

**THE ROLE OF P38 MAPK IN CELL CYCLE
CHECKPOINT CONTROL FOLLOWING DNA DAMAGE**

Mark Phong Siew Peng

(B.Sc. Eng(Hons), B.A.(Hons) , University of Pennsylvania, Philadelphia PA, USA)

A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
DEPARTMENT OF PHARMACOLOGY
YONG LOO LIN SCHOOL OF MEDICINE
NATIONAL UNIVERSITY OF SINGAPORE

2009

ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks and gratitude to my thesis supervisors Dr. Xiang Ye (Oncology Division, Lilly Research Labs, Eli Lilly & Co.) and Prof. Uttam Surana (Institute of Molecular and Cellular Biology, A*STAR & Dept of Pharmacology NUS) for their continuous help, advice and guidance throughout my candidature. I would like to thank Dr Greg Tucker-Kellogg (LSCDD) for his ideas, support and for the discussions that helped me move my thesis forward. I would like to thank Dr Robert Morris Campbell (LSCDD) for his help in providing ideas and suggestions for my thesis, for advice in navigating the maze of Lilly corporate structure and for pointing me towards Dr Xiang Ye as my supervisor. I would like to thank Danny Van Horn and Li Fan, both of whom work in Dr Xiang Ye's lab for training me in the basics of molecular biology. I would like to thank Dr Song Qing Na (CI&CS DHT, LRL Eli Lilly & Co.) for the generous gift of a stable-line MK2^{-/-} Hela cell construct. I would like to acknowledge and thank my former academic supervisor Dr. Guna Rajagapol (New Jersey Cancer Institute) and Dr. Michael Schroter (LSCDD) for their roles in setting up my thesis and for their help in settling the complex legal framework to facilitate this work I also would like to thank my friends, colleagues and former colleagues at LSCDD for their help, support and encouragement during my candidature, especially Marie Wong, Jaga Virayah, Anindyo Chakravarty, Dr. Christopher Taylor, Dr. Ketan Patel, Dr. Vinisha K. Kanjilal, Dr Kok Long Ang, Dr Horst Flotow, Dr Asja Preator, Connie Er, Rajween Kaur, and Chris Sim. I would like to thank the management of Lilly Singapore Center for Drug Discovery and Eli Lilly & Co. for their sponsorship and support to enable this work.

Lastly, I would like to thank my wife Agnes, my parents Bart and Janet and my sister Maria for their unconditional love, support and understanding during the 5 ½ years of my candidature. Completing this candidature would not have been possible without their love and support.

Sincerely,

Mark Phong

FOR MY LOVING FAMILY

TABLE OF CONTENTS

Summary	6
List of Tables	8
List of Figures	9
Chapter 1: Introduction	15
1.1 Role of Signal Transduction in response to Extra-Cellular Stimuli	15
1.2 Types of External Stimuli	16
1.3 Signal Transduction Receptors	18
1.4 Intracellular Signaling Cascades	20
1.5 The p38 MAPK	28
1.6 Physiological response to Growth Signals	32
1.7 Physiological Response to Stress	47
1.8 Effectors of Cell Cycle Arrest	59
1.9 The Induction of Apoptosis in response to DNA damage	70
Chapter 2: Materials and Methods	80
2.1 Materials	80
2.1.9 Computational Programs and Tools	89
2.2 Methods in Mammalian Cell Culture	90
2.3 Protein Analytical Techniques	93
2.4 Statistical Analysis of Microarray Data	99
Chapter 3: The role of p38 MAPK in regulation of DNA damage G2 cell cycle checkpoint control	102
3.1 Background	102
3.2 p38 MAPK is activated during DNA damage at all stages of the cell cycle ..	102
3.3 LY479754 and SB203580 are effective inhibitors of p38 pathway	107
3.4 Adriamycin dose titration to find optimal dose for cell-cycle experiments....	109
3.5 Biochemical inhibition of p38 MAPK cannot abrogate Adriamycin induced G2 checkpoint arrest in HeLa Cells	111
3.6 Transient and stable knock-out of p38 or its down-stream substrate MK2 has no effect on Adriamycin induced G2 DNA damage checkpoint.	123
3.7 Biochemical Inhibition of p38 cannot abrogate UV induced DNA damage G2 checkpoint response in HeLa cells	129
3.8 Activation of p38 at G2 without DNA damage does not inhibit entry into mitosis 137	
3.13 Summary	140
Chapter 4: Effect of p38 inhibition on TNF- α induced inflammatory response and apoptosis	142
4.1 Background	142
4.2 TNF α induces p38 MAPK activity in Calu6 Cells	144
4.2.1 LY479754 effectively inhibits TNF- α induced p38 activity	145
4.3 Gene-Chip Experimental Design	146
4.4 TNF- α induces inflammatory response genes in a time dependent manner ...	146
4.5 Early transcriptome effects of TNF- α treatment on Calu6 cells	148
4.6 Effect of TNF- α and p38 inhibition at the mid time point (2hrs)	163

4.7	Effect of TNF- α treatment and p38 inhibition at the late time points (4hrs & 7hrs)	184
4.8	<i>In-Vitro</i> Validation.....	199
4.9	Summary.....	201
Chapter 5: Alternative roles for p38 in response to DNA Damage outside G2 cell cycle checkpoint response.....		204
5.1	Background.....	204
5.2	A role for p38 MAPK activity during mitotic progression.....	204
5.2.1	Inhibition of p38 during regular mitosis has no impact on completion of mitosis	206
5.3	Role of p38 in recovery from Adriamycin damage.....	215
5.4	Biochemical Inhibition of p38 leads to Apoptosis in conjunction with genotoxic agents	218
5.5	Summary.....	227
Chapter 6: Discussion and Conclusions.....		229
6.1	Inhibition of Chk1 but not p38 is critical to the maintenance of the G2 DNA damage checkpoint.....	230
6.2	Inhibition of p38 degrades anti-apoptosis response to TNF- α in Calu6 cells.	236
6.2	p38 MAPK activates cell survival pathways in response to DNA Damage...	238
6.4	A tentative new model for p38's role in DNA Damage Response.....	241
6.5	Role of p38 in Recovery from DNA damage.....	242
6.6	Conclusion.....	242
Chapter 7: Future Direction.....		244
7.1	What is the mechanism of the pathway attenuation of p38 in G2 cell cycle checkpoint signaling?.....	244
7.2	Where p38 signaling impinge upon apoptosis signaling?.....	246
7.3	Exploring p38 and p53 interactions, especially at the G1/S cell cycle checkpoint transition.....	246
Publications.....		249

Summary

The response of mammalian cells to DNA damage has been an area of great interest, as loss of genomic integrity is often implicated in tumorigenic and oncogenic events. Critical to the ability of healthy cells in maintaining genomic integrity are the cell cycle checkpoints that act as a brake against inappropriate cell division in the presence of DNA damage. Recent publications have implicated the p38 MAPK as a critical kinase for the establishment and maintenance of a DNA damage-induced cell cycle arrest in G2. The ability of cancer cells to establish a cell cycle arrest in response to genotoxic agents is one of the reasons for their resistance to chemotherapy. Cancer cells with the ability of under-going a reversible cell cycle arrest in response to genotoxic agents such as Adriamycin have the ability to survive chemotherapy and continue proliferation post therapy, leading to poor patient outcome.

In this study, we investigated whether inhibition of p38 with a potent and selective p38 inhibitor (LY479754) could act as a chemo-sensitizer in response to genotoxic agents such as Adriamycin and to environmental stress such as UV irradiation. To lend physiological context to p38's role at G2 DNA damage checkpoint arrest, we also examined the role of Chk1, a canonical member of the ATM/ATR pathway, in DNA damage-induced G2 checkpoint control.

While examining the role of p38 in the G2 checkpoint pathway, we found that inhibition of p38 by biochemical or siRNA was unable to affect G2 cell cycle arrest induced by Adriamycin, UV or MMS. Inhibition of Chk1, on the other hand, led to the abrogation of DNA damage-induced G2 arrest in p53 functionally null cancer cells.

We also discovered a strong link between p38 activity and the increase in cell survival signaling in response to both DNA damage and TNF- α stress. Investigation of the link between p38 and the regulation of apoptosis revealed that p38 plays a significant role in the early induction of anti-apoptotic signaling in response to DNA damage and TNF- α stress. Inhibition of p38 led to the strong down-regulation of BCL2 and BCL-xl, members of the BCL2 anti-apoptotic protein family and up-regulation of pro-apoptotic proteins such as FADD and TRADD.

These results imply that, while p38 activation is associated with DNA damage G2 arrest, its activity is not required for the execution or maintenance of the checkpoint. Instead, p38 activation in response to DNA damage and to TNF- α stress is linked to the strong induction of anti-apoptotic signaling in immediate response to stress. Inhibition of Chk1 kinase activity serves as an appropriate counter point to p38 inhibition, as loss of Chk1 activity in a p53 functionally null cancer cell prevents the establishment or maintenance of an effective checkpoint-induced G2 arrest.

The data suggests that both inhibition of p38 and Chk1 may be useful therapeutic strategies for oncology treatment in combination with chemotherapeutic agents. It also suggests that while both kinases are activated in a similar manner to DNA damage, the downstream effect of each protein's activation is fundamentally different. Understanding the functional role of both proteins in response to DNA damage may aid in the development of successful and relevant therapeutic strategies for cancer.

List of Tables

S/No.	Table ID	Description	Page Number
1	2.1	Table 2.1: Table of Laboratory chemicals and biochemicals	80
2	2.2	Table 2.2: List of Commercial assay kits, buffers and systems	81
3	2.3	Table 2.3: Table of primary antibodies	82
4	2.4	Table 2.4: Table of secondary antibodies and reagents	83
5	2.5	Table 2.5: Table of biochemical inhibitors used in this study	84
6	2.6	Table 2.6: Cell culture reagents	84
7	2.7	Table 2.7: Cell-Line Models used in this study	85
8	2.8	Table 2.8: Table of siRNA duplex reagents used in this study	86
9	2.9	Table 2.9: Table of Analytical Instruments and Systems used in this study	89
10	2.10	Table 2.10: Cell seeding density for assay plates	92
11	4.1	Table 4.1: Gene table of early response genes induced by TNF- α and modulated by LY479754 treatment	152
12	4.2	Table 4.2: Anti-Apoptotic Genes induced by TNF- α in early time points, all genes FDR<0.1	157
13	4.3	Table 4.3: Genes induced early by TNF- α associated with cell proliferation.	162
14	4.4	Table 4.4: Top functional pathways for TNF- α +LY479754 at 2hour time point	164
15	4.5	Table 4.5: Genes functionally related to Apoptosis, induced by TNF- α and modulated by p38i (LY479754), all genes FDR<0.1.	171
16	4.6	Table 4.6: Apoptosis related genes induced strongly by TNF- α , but not modulated significantly by p38i (LY479754) at 2hr time point, all genes FDR<0.1	175
17	4.7	Table 4.7: NFkB related genes directly modulated by TNF- α treatment at 2hrs, all genes FDR<0.1	182
18	4.8	Table 4.8: Top networks for genes modulated by TNF- α at the late time points.	185
19	4.9	Table 4.9: Top 40 Apoptosis Genes modulated by TNF- α in the late time points	191
20	4.10.	Table 4.10: Inflammatory genes induced by TNF- α at the late time points	194

List of Figures

S/No.	Figure ID	Description	Page Number
1	1.1	Figure 1.1: Overview of Signal Transduction in mammalian cells	16
2	1.2	Figure 1.2: Canonical overview of the MAPK signaling cascade	21
3	1.3	Figure 1.3: Canonical p38 MAPK signaling pathway: Receptors and signaling cascades leading to ERK, JNK & p38 MAPK activation	23
4	1.4	Figure 1.4: Overview of the Mammalian Cell Cycle: Key cyclins and CDKs required for transition through the cell cycle	34
5	1.5	Figure 1.5: Canonical representation of Chk1 & Chk2 activation in response to DNA damage leading to deactivation of CDK1/CyclinB1 complex leading to G2 arrest	65
6	1.6	Figure 1.6: Putative new role for p38 at G2 checkpoint, acting through direct regulation of CDC25B phosphatases	68
7	1.7	Figure 1.7: Canonical Apoptosis Pathway: Activation of apoptosis from both extrinsic and intrinsic apoptosis pathways	72
8	3.1	Figure 3.1: p38 MAPK is activated by various DNA damaging stresses.	104
9	3.2	Figure 3.2: p38 MAPK is activated at all stages of the cell cycle	106
10	3.3	Figure 3.3: In-vitro kinase assay for LY479754 in HeLa cells	107
11	3.4	Figure 3.4: In-vitro kinase assay for SB203580 in HeLa cells	108
12	3.5	Figure 3.5: Adriamycin Dose Response in HeLa cells at 20hrs	110
13	3.6	Figure 3.6: Inhibition of Chk1 but not p38 abrogates Adriamycin induced G2 Arrest	113
14	3.7	Figure 3.7: Dose titration of Adriamycin and p38-inhibitor (LY479754) in thymidine synchronized HeLa cells.	115
15	3.8	Figure 3.8: Confocal Microscopy images of thymidine synchronized HeLa cells damaged with 160nM Adriamycin and dosed with either 320nM p38 inhibitor or 2uM Chk1-inhibitor	118
16	3.9	Figure 3.9: Biochemical inhibition of p38 is unable to abrogate Adriamycin induced G2 arrest in Calu6 cells	120

17	3.10.	Figure 3.10: Biochemical inhibition of p38 and Chk1 was unable to abrogate Adriamycin induced G2 arrest in A549 & U2OS cells.	122
18	3.11	Figure 3.11: Effect of siRNA KD of p38, MK2 and Chk1 transcript on establishment of Adriamycin induced G2 DNA damage checkpoint	125
19	3.12	Figure 3.12: Effect of Adriamycin damage on HeLa ^{MK2-/-} cells.	127
20	3.13	Figure 3.13: Effect of biochemical inhibition of p38, MK2 and Chk1 on UV damage in thymidine synchronized HeLa cells	131
21	3.14	Figure 3.14: Effect of siRNA KD of p38 and Chk1 on UV damage induced G2 checkpoint arrest	132
22	3.15	Figure 3.15: Effect of siRNA KD of MK2 with UV-C irradiation in U2OS cells. (A) FACS scatter plot of phospho-Histone H3 and DNA content of siMK2 or siGFP transfected cells +/- 20J/m ² UV-C irradiation and 165nM nocodazole. (B) Western blot assay of siMK2 or siGFP transfected cells +/- 20J/m ² UV-C irradiation and 165nM nocodazole.	134
23	3.16	Figure 3.16: Effect of biochemical inhibition of p38, and Chk1 on UV damage in thymidine synchronized A549 cells, mitotic index plot (ph-Histone H3)	136
24	3.17	Figure 3.17: Effect of non-genotoxic stimulation of p38 on ability of cancer cells to enter mitosis.	139
25	4.1	Figure 4.1: MAPK pathway is strongly induced by TNF- α treatment in Calu6 cells	144
26	4.2	Figure 4.2: Phospho-MAPKAPK2 levels as marker of p38 MAPK activity, post TNF- α treatment. 320nM LY479754 effectively inhibits p38 activity in response to TNF- α	145
27	4.3	Figure 4.3: Experimental design for Gene-Chip experiment involving p38 inhibitor (LY479754) and TNF- α in Calu6 Cells	146
28	4.4	Figure 4.4: Count of number of significant probesets at each timepoint for TNF vs DMSO comparison.	147

29	4.5	Figure 4.5: Overlap of significant genes (probesets) at 30mins and 60mins TNF- α treatment	149
30	4.6	Figure 4.6: Compacted Heatmap of genes significantly modulated by TNF- α at 60mins, with FC(log2)>1.5 filter & FDR<0.1	149
31	4.7	Figure 4.7: Boxplot of selected immediate early response genes	152
32	4.8	Figure 4.8: Genes belonging to death receptor and programmed cell death pathway, induced by TNF- α treatment in the early timepoints.	153
33	4.9	Figure 4.9: Anti-apoptosis genes induced by TNF- α and modulated by p38-inhibitor (LY479754) at 60mins	155
34	4.10.	Figure 4.10: Boxplots of log2 normalized MAS5 signal: Inhibition of p38 with TNF- α modulates BCL2 anti-apoptosis proteins BCL2 & BCL-xl	156
35	4.11	Figure 4.11: Boxplots of log2 normalized MAS5 signal: Members of FAS signaling pathway are modulated by p38 inhibition in the early time points.	158
36	4.12	Figure 4.12: Genes associated with increased proliferation, induced by TNF- α and modulated by p38i treatment	160
37	4.13	Figure 4.13: Boxplot of selected cell proliferation genes induced strongly by TNF- α and modulated by p38 inhibition	161
38	4.14	Figure 4.14: Heatmap of genes classified as developmental genes, significantly changed by TNF- α . A large sub cluster of genes are also modulated by p38i (LY479754) treatment.	165
39	4.15	Figure 4.15: Boxplot of selected development and cell differentiation genes, induced by TNF- α and modulated by p38-inhibition.	166
40	4.16	Figure 4.16: IPA network analysis of cellular development genes modulated by p38i (LY479754) at 2hours	167
41	4.17	Figure 4.17: Venn Diagram of TNF- α and TNF- α +LY479754 modulated apoptosis genes at 2hrs time point	168
42	4.18	Figure 4.18: Heatmap of 22 genes related to apoptosis, modulated by p38 inhibitor in conjunction with TNF- α treatment at 2hrs.	170
43	4.19	Figure 4.19: A pathway/network diagram of the top network of genes significantly regulated by p38 inhibitor LY479754 and TNF- α at the 2hrs timepoint	172

44	4.20.	Figure 4.20: Heatmap of 57 genes (probesets) functionally classified as apoptosis related, strongly induced by TNF- α but unaffected by p38 inhibition (LY479754).	174
45	4.21	Figure 4.21: Cell cycle genes are modulated by TNF- α , but relatively unaffected by p38-inhibitor	178
46	4.22	Figure 4.22: Boxplots of selected Cell cycle related genes, modulated by TNF- α at 2hrs time point.	179
47	4.23	Figure 4.23: Heatmap of NFkB genes induced by TNF- α treatment at 2hrs time point, all genes FDR<0.1	180
48	4.24	Figure 4.24: Heatmap of Cell Death related genes induced by TNF- α at the late time points (4hrs & 7hrs), all genes FDR<0.1	186
49	4.25	Figure 4.25: Ingenuity pathway analysis network diagram of apoptosis related genes strongly induced by TNF- α at late time points.	187
50	4.26	Figure 4.26: IAP and other pro-cell survival genes are strongly expressed across time in response to TNF- α treatment	188
51	4.27	Figure 4.27: Boxplot of apoptosis related genes, modulated by p38 inhibition at the late time points (4hrs & 7hrs)	189
52	4.28	Figure 4.28: Heatmap of Genes associated with inflammatory response at late time points (7hrs), all genes FDR<0.1	192
53	4.29	Figure 4.29: Heatmap of Genes associated with Cell cycle progression and regulation at late time points (7hrs), all genes FDR<0.1	195
54	4.30.	Figure 4.30: Boxplot of select cell cycle regulator genes, induced by TNF- α but unaffected by p38 inhibition at late time points (7hrs), all genes filtered by FDR< 0.1	196
55	4.31	Figure 4.31: Line plots of select cell cycle regulator genes, induced by TNF- α but unaffected by p38 inhibition across time, all genes filtered by FDR< 0.1	197
56	4.32	Figure 4.32: Western blot of TNF- α and TNF- α +LY479754 treated Calu6 cells over a 48hour time series.	200
57	5.1	Figure 5.1: p38 MAPK was activated during normal mitosis, without any DNA Damage	205
58	5.2	Figure 5.2: Effect of inhibition of p38, and Chk1 on mitotic progression in Hela cells	207
59	5.3	Figure 5.3: Effect of Adriamycin on cells in Mitosis	210

60	5.4	Figure 5.4: MMS damage in mitosis leads to disruption of mitosis	213
61	5.5	Figure 5.5: Recover from Adriamycin Damage at G2	217
62	5.6	Figure 5.6: Apoptosis Induction in Hela cells in response to Adriamycin and varying doses of p38 inhibitor	220
63	5.7	Figure 5.7: Inhibition of p38 induces apoptosis in A549 cells	221
64	5.8	Figure 5.8: Apoptosis induction by MMS and LY479754 in HeLa & A549	223
65	5.9	Figure 5.9: Effect of Adriamycin and siRNAs	226
66	6.1	Figure 6.1: A new model of p38's role in DNA damage response	241

LIST OF SYMBOLS & ABBREVIATIONS

S/No.	Symbol	Description
1	Dox	Doxyrubicin HCL (Adriamycin)
2	MMS	Methyl Methanesulfonate
3	UV	Ultra-Violet Radiation
4	p38i	p38 inhibitor: LY479754
5	MK2i	MAPKAPK2 Inhibitor: LY2441693
6	Chk1i	Chk1 Inhibitor: LY2494516 SmithKline Beecham p38 Inhibitor:
7	SB	SB203580
8	FDR	False Discovery Rate p-value

Chapter 1: Introduction

1.1 Role of Signal Transduction in response to Extra-Cellular Stimuli

Mammalian cells do not live in isolation, making it necessary for them to respond to and coordinate a wide degree of extracellular stimuli from their external environment. Cells respond to changes in their external environment by activating a complex series of intracellular signaling pathways. This allows cells to change physiological processes in response to external stimuli (12).

While there are many types of external stimuli, the two largest groups of stimuli can generally be classified as growth stimuli, and stress stimuli (376). The signals from these major categories stimulate a rapid transmission of signal from the exterior of the cell to the interior (68). There are many components that make up the cellular machinery responsible for efficient signal transduction. As the stimuli originate external to the cell, cell surface receptors play a critical part in the detection of the stimuli. Once the stimuli is detected by the cell surface receptors, rapid conformational changes in the receptor recruit both extracellular and intracellular binding partners that are responsible for the transduction of the signal (184). The transduction of the signal from the cell surface to the internal compartments of the cell requires a series of complex post-translational modifications or translocations of intracellular signaling proteins (66,117). The end result of the rapid induction of signal transduction pathways depends on the nature of the external stimuli, with most resulting in significant physiological effects including transcriptional activation of specific genes, or activation of specific protein networks that may result in cell division or cell death (68).

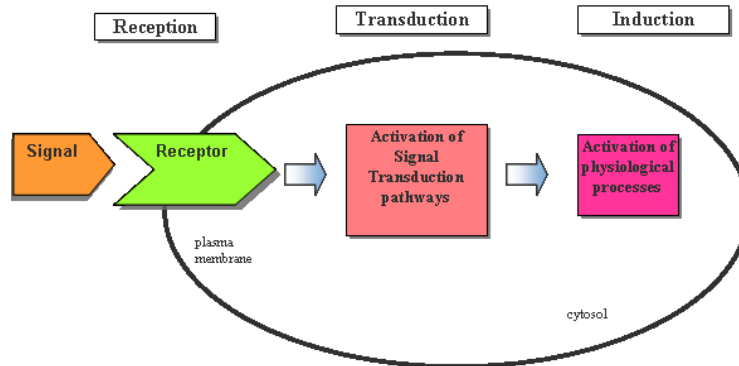


Figure 1.1: *A broad scheme for Signal Transduction in mammalian cells*

1.2 Types of External Stimuli

Signal transduction involves the reception and internalization of external cell stimuli. While there are many different types of external signaling moieties, the two of greatest interest to this research are factors that stimulate cell growth and factors that initiate stress response.

1.2.1 Growth Signals

A growth factor is broad classification of proteins whose expression results in the induction of growth and proliferation (105,471). Another term often associated with growth factors is the term cytokine. A cytokine was originally used to classify secreted factors that influenced hematopoietic and immune system cells (225,484). As research in this area progressed, however, it became clear that many cytokines also influenced the function of other cell types as well. Cytokines do not always induce cell growth, for instance, FasL, a common cytokine, induces apoptosis. The term cytokine today is used

in a neutral context, as a cytokine may have either a growth inducing or non-growth inducing role (1,394)

A large number of secreted factors are classified as growth factors. Included in this group are proteins such as EGF (epidermal growth factor), PDGF (platelet derived growth factor), FGF (fibroblast growth factor), VEGF (vascular endothelial growth factor) and the TGF (Transforming growth factor) family of proteins (39,89,358). A general effect of growth signals is the induction of cellular proliferation pathways that eventually act to stimulate progression through the cell cycle. Induction of proliferation signals is usually accompanied by strong transcriptional activation and secretion of additional growth factors, leading to positive feedback loops for increased cellular proliferation (160,336,386). Growth factors can also elicit other cellular responses such as new blood vessel formation, wound healing and others.. The dysregulation of growth factor production is associated with the onset of diseases, specifically cancer. The establishment of the tumor microenvironment and the onset of angiogenesis is highly associated with dysregulated production of cytokines and growth factors (101,339,457).

1.2.2 Stress Signals

Another major class of external stimuli that are sensed by cells is stress signals. Cells can be exposed to a large number of stresses on a regular basis, and have developed complex signaling networks to respond appropriately to each type of stress. (225,438) The types of stress that can be experienced by a cell can range from mechanic stress such as shear stress, foreign organism invasion such as bacterial and viral infection, to chemical and environmental damage such as ultra-violet (UV) radiation, osmotic stress or

genotoxic agents (21,22,82,87,135,335). While the specific cellular response to different stresses is inherently different, the overall response to stress exhibits a general pattern. The cellular surveillance mechanism assesses the degree of severity of a stress, this response then triggers a halt to the cell cycle in proliferating cells and induces an appropriate cellular repair pathway. However, if the stress or damage is too great, the apoptotic pathway is activated (55,217,247,310,352).

The cellular response to stress is an area of great interest, as incorrect or inappropriate response to stress leads to the onset of many diseases. The hallmarks of cancer as defined by Weinberg et al (138), depict that cancer cells have acquired the ability to escape anti-proliferative and pro-death signals while maintaining endless replicative potential.

1.3 Signal Transduction Receptors

Signal transduction receptors play a major role in the transmission of external stimuli to the inside of the cell. A large number of signal transduction receptors are found on the surface of the cell and are termed cell surface receptors. Cell surface receptors are responsible for the detection of external stimuli, and for the activation of intracellular signaling pathways. (145,192,233,333) Cell surface receptors can comprise of simple ion channels that respond to changes in extracellular ion concentrations to the more complex protein structures activated by ligand binding relationships (83).

Signal transduction receptors can be grouped broadly into three general classes. These classes are:

- i. The first class of receptors penetrates the plasma membrane and has intrinsic enzymatic activity. Examples of this type of receptors include the receptor tyrosine kinases (RTK), the serine/threonine kinase receptors, the tyrosine phosphatases and the guanylate cyclases. Epidermal growth factor receptor (EGFR), the platelet derived growth factor receptor (PDGF) and the insulin growth factor receptor (IGFR) are examples of RTK (145,147,153,286,319,404). Similarly, transforming growth factor beta receptor (TGF- β receptor) belongs to the serine/threonine kinase receptors class, CD45 to tyrosine phosphatase receptors class and natriuretic peptide receptors to guanylate cyclases (100,418,463). Receptors with intrinsic tyrosine kinase activity have the capability to auto-phosphorylate themselves as well as their down-stream substrates.
- ii. Receptors belonging to the second class are coupled intracellularly to GTP-binding and hydrolyzing G-proteins. The G-protein coupled receptors (GPCRs) have a characteristic 7 transmembrane spanning domain and are sometimes referred to as serpentine receptors (153,196,255,447). Adrenergic receptors, odorant receptors and certain hormone receptors (angiotensin, vasopressin and bradykinin) are examples of GPCRs.
- iii. The 3rd general class of receptors is found intracellularly and upon ligand binding migrates to the nucleus where the ligand-receptor complexes directly modulate gene transcription (287,359). These receptors are known as nuclear receptors, and generally have both a ligand binding domain and a DNA

binding domain. Examples of this class of receptors include the large steroid and thyroid hormone receptors (e.g. Estrogen receptor) (142,276,301).

Having introduced the major classes of cell surface receptors involved in receiving exogenous signals, we will now discuss the intracellular mechanism involved in the transmission of the external signal.

1.4 Intracellular Signaling Cascades

The cytoplasmic receptors activate a cascade of intracellular signaling pathways to perpetuate the signal away from the site of ligand/receptor binding, into the cell proper. These intracellular signaling cascades are critical for the efficient and fast response to extra-cellular stimuli. Many of the intracellular signaling cascades that respond to extracellular growth or stress signals are not direct substrates of receptors with kinase activity such as the RTKs or serine/threonine kinase receptors (141,325). Instead intracellular adaptor molecules and other signaling kinases link receptor activation with the down-stream effector molecules (49). As this thesis is focused on the role of p38 MAPK, we will focus on reviewing the intracellular signaling cascades responsible for p38 activation, with some brief overview of other parallel signaling pathways.

1.4.1 Mitogen Activated Protein Kinase Activation Cascade (MAPK Cascade)

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes and have been implicated in cell migration, invasion, proliferation, angiogenesis, cell differentiation and cell survival (5). MAPKs are serine/threonine protein kinases mediating the response of cells to extracellular stimuli to critical

regulatory targets within the cell (297,338). At least four distinctly regulated groups of MAPKs are expressed in mammals, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 $\alpha/\beta/\gamma/\delta$) and ERK5 (59). A major function of MAPK pathways is the control of gene expression by either direct phosphorylation of transcription factors, but they can also target coactivators and corepressors (109). All MAPKs are activated through a dual phosphorylation on an exposed surface loop, normally referred to as the phosphorylation loop. All MAPKs are activated by a dual phosphorylation on a threonine and tyrosine residue following a Thr-Xxx-Tyr dual phosphorylation motif (130). The basic structure of the MAPK cascade is well conserved in all eukaryotic cells and it consists of a 3-layer activation cascade consisting of a MAPKKK activating a MAPKK, which in turn activates a MAPK (348). The MAPK cascade is detailed in Figure 1.2.

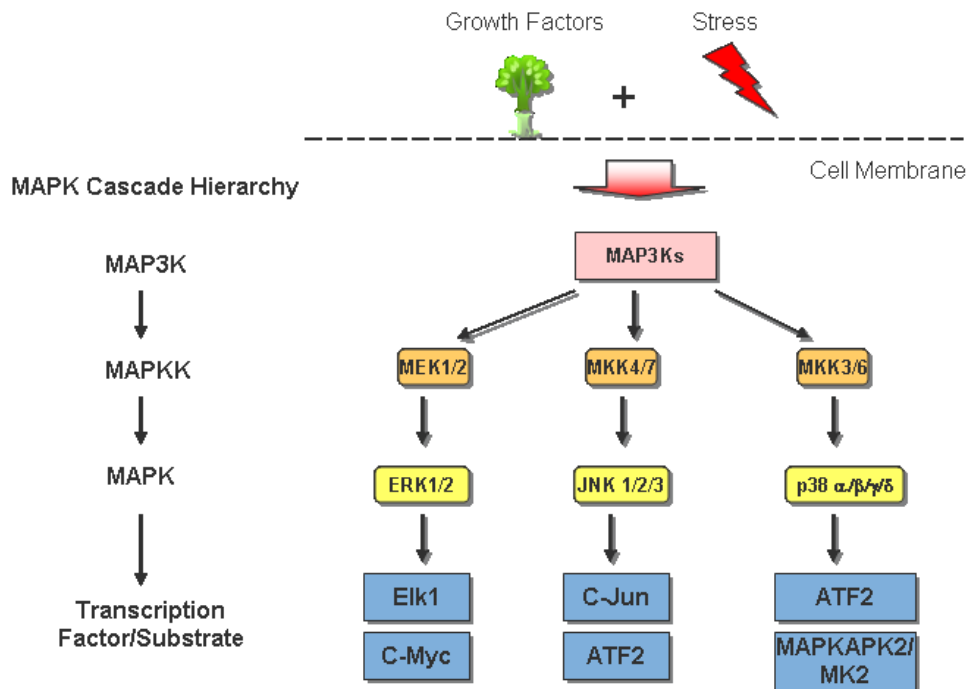


Figure 1.2: *Canonical overview of the MAPK signaling cascade*

The most prominently studied MAPK cascade is the pathway leading to the activation of ERK1/2 by RTKs (54,461). Stimulation of RTKs leads to the recruitment of the adaptor protein Grb2 and association and activation of the RAS-GEF Sos, which subsequently activates membrane-associated Ras. Ras in turn induces the serine/threonine kinase activity of the MAPK kinase kinase (MAPKKK) Raf-1 which phosphorylates and activates the MAPK kinases 1/2 (MAPKK, MEK 1/2). Finally, MEK1/2 activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory TEY-motif (23,65). Thereafter, ERK1/2 either translocate into the nucleus to regulate gene expression or effect cytoplasmic or membrane bound effectors, such as influencing transmembrane protein processing by phosphorylation of the intracellular domain of the metalloprotease ADAM17 (393).

The JNK-family MAPKs are also known as stress-activated kinases as their activation result from response to environmental stress and radiation and growth factors (419,427,439).

With the focus of this thesis being the role of p38 in DNA damage response, the basic structure of the stress induced p38 MAPK cascade will be discussed, starting with LPS stimulation of the Toll receptors (TLRs).

1.4.2 Upstream activation of p38 MAPK

The TLRs are activated in response to the presence of LPS in a cell's external environment. The p38 MAPK was first discovered as a 38-kDa protein that was phosphorylated in response to the presence of LPS (225,262). We begin my exploration

of the mechanics of p38 activation by examining the intracellular signaling arising from LPS stimulation. Besides LPS stimulation, p38 has been shown to be strongly activated by many other secreted factors including TNF- α , IL1 and certain growth factors and hormones. The canonical signaling pathways leading to the activation of the MAPK cascades and p38 specifically are depicted in Figure 1.3.

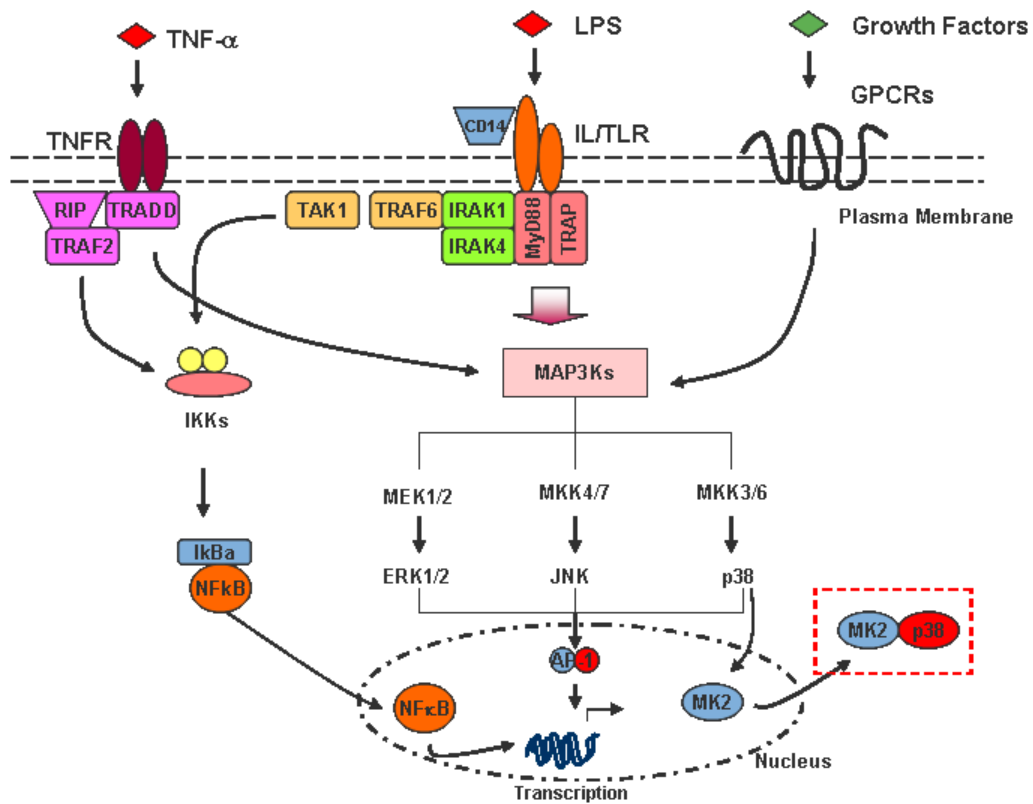


Figure 1.3: *Canonical p38 MAPK signaling pathway: Receptors and signaling cascades leading to ERK, JNK & p38 MAPK activation*

1.4.3 Cytoplasmic Adaptor Proteins

Connecting the receptors to intracellular signaling networks are the intracellular adaptor proteins. These proteins are often recruited to the site of receptor activation by

conformational changes in the intracellular domain of the receptor, which facilitates recruitment and binding (84).

The key intracellular adaptor proteins for the TNF receptor family are the RIP protein and the TRADD protein (251,329). Together they are responsible for the activation of the downstream signaling cascade that includes recruitment and activation of TRAF2 and eventually the activation of IKKs and NF κ B. The TNFR signaling pathway is tightly associated with extrinsic induction of apoptosis and the production of inflammatory cytokines (252).

For the TLR, the key intracellular domain responsible for recruitment of adaptor proteins is known as the Toll/Interleukin receptor (TIR) domain. The TIR domain is responsible for the recruitment of adaptor molecules to the cytoplasmic face of the receptor as well as to facilitate homo or hetero-dimerization of the receptors (294). The common adaptor molecule shared by all the different TLR is called Myeloid differentiation response gene 88 (MyD88). MyD88 is an adaptor molecule that is recruited to the cytoplasmic domain of TLR through homophilic interactions of TIR homology domains between TLR and MyD88 (6). MyD88 functions to recruit the interleukin receptor associated kinase 1 and 4 (IRAK1 & 4) and is a key adaptor molecule for the TLR pathways because it contains both a TIR domain as well as a death domain. However The TIR domain has been most commonly associated with host defense in plant and mammalian cells, while the death domain is normally associated with induction of apoptotic stimuli (85). It has also been shown through MyD88 deficient macrophages that LPS can induce an inflammatory response both in the presence and absence of MyD88 (10). The kinetics of the activation of inflammation in MyD88

deficient cells is significantly slower than wild type cells. This suggests that LPS can signal through both a MyD88 dependent and independent pathway (10).

IRAK1,4 a serine/threonine kinase, is a key adaptor molecule mediating the LPS and IL-1 signaling cascade (345). Upon activation, IRAK1/4 dissociates from the receptor complex in order to associate and activate their downstream substrates, which include TRAF6. (302) IRAK1/4 has been shown through mouse knockout studies to be vital for the induction of the NF κ B, JNK and p38 MAPK stress induced pathways (188,211). IRAK1/4 has also been shown to be responsible for the translocation of the key adaptor molecule TAB2 from the membrane space to the cytoplasmic space (322).

The complexities of cell signaling pathways at this level are tremendous, however much of this complexity could be due to the way scientists have probed the various components of cell signaling pathways. Over-expression studies have been shown to induce associations and correlations in-vitro when no such associations are observed in-vivo. There are numerous possible binding partners that could be recruited to the TLR4/MyD88/IRAK1/4 cytoplasmic complex, the most important protein involved in p38 MAPK induction from the TLR pathway however is TRAF6 (260,322). IRAK1/4 signaling has also been shown to mediate the translocation of TAB2 from the plasma membrane to the cytosol (322). TAB2, as discussed later is a key component of transforming growth factor β associated kinase 1 (TAK1) activation.

Tumor necrosis factor associated factor 6 (TRAF6) was identified through yeast two-hybrid assays as a key binding partner to members of the TLR adaptor complex (395). Gene knockout and dominant negative over-expression of TRAF6 in mouse models, has shown the importance of TRAF6 for response to inflammatory stress and the

activation of NF κ B, p38 and JNK pathways (290). TRAF6 is cited as a key upstream molecule that interacts and facilitates the phosphorylation of various Mitogen Activated Protein Kinase Kinase Kinases (Alias: MAPKKK, MAP3K, MEKK). Upon activation, TRAF6 forms a complex with 2 ubiquitin molecules Ubc13 and Uev1A (170). Collectively these molecules form a ubiquitin conjugating enzyme (E2), where TRAF6 serves as the ubiquitin ligase. TRAF6 is a key adaptor/scaffold protein that helps link upstream activating kinases like IRAK1/4 to its downstream substrates such as the MAP3K family of proteins (170).

1.4.4 MAP3K family

The highest level of the hierarchical MAPK activation cascade is the MAP3K (MAPKKK) family of protein kinases. In humans there are 21 proteins that currently identified to play a role as a MAP3K (71). Based on protein homology modeling, studies have identified up to 4 separate groups of MAP3Ks, suggesting distinct yet overlapping down-stream signaling pathways (71). Some of the best studied MAP3Ks are the RAF family of proto-oncogenes and the TAK1 family of proteins. As TAK1 is shown to activate the MAPK cascade leading to p38 activation, its role in signaling is studied in greater details below.

TAK1, a member of the MAP3K family of kinases is activated by various stimuli, including stress, growth factors and cytokines (88). Activated TAK1 dissociates from its inhibitory associated proteins and leads to the activation of the p38 and JNK MAPK pathways as well as the NF κ B pathway, as shown in mouse knock-out studies (166).

Immuno-precipitation and yeast two hybrid studies of activated TAK1 indicate that it normally associates with 2 binding proteins, TAB1 and TAB2. As explained earlier in this review, TAB2 translocates from the membrane to the cytosol during LPS and IL-1 stimulation, and is linked with the transient association with TRAF6 (369).

TAB1 was originally identified as a binding partner of TAK1 and is associated with the activation of TAK1. TAB1 however, has also been shown through immuno-precipitation and mouse knock-out studies to be able to activate p38, independent of TAK1 (402). This suggests a more extended role for TAB1 beyond being a binding partner of TAK1, leading to TAK1's activation (236).

TAK1 is just one of many MAPKKKs that have been implicated *in-vitro* and *in-vivo* to activate p38 MAPK. TAK1 activates a number of MAPKK like MKK6, MKK3 and MKK4, which lead to the activation of p38 (98).

1.4.5 The MKK6, MKK3, MKK4 kinases

The kinases that directly phosphorylate the MAPKs are referred to as MAP kinase kinases, and are variously abbreviated as MAPKKs, MEKs, or MKKs (477). MKK3 and MKK6 have been implicated as the major upstream activators of p38 MAPK *in vitro* and *in vivo* (265). MKK6 appears to phosphorylate all 4 p38 MAPK isoforms, whereas MKK3 phosphorylates only p38 α , p38 γ , and p38 δ (98). In addition to MKK3 & 6, a recent gene-targeting study revealed that MKK4^{-/-} fibroblast cells exhibited defects in both JNK and p38 MAPK phosphorylation in response to TNF, anisomycin or hyperosmotic stress, suggesting a possible *in vivo* pathway from MKK4 to p38 MAPK (437).

1.5 The p38 MAPK

The p38 MAP kinases are a family of serine/threonine protein kinases that play a critical role in cellular responses to external stresses (64). They belong to a set of core intra-cellular signaling pathways that are critical for the internalization of a host of external signals. The p38 MAPK family of proteins was first discovered in response to LPS stimulation in murine cells (225). Since its discovery in relationship to cytokine production, the p38 MAPK proteins have been implicated in response to biological processes such as inflammation, immune response, pulmonary disease, osmotic stress and hypoxia (186,200). The 4 isoforms of p38 MAPK are p38 α , p38 β , p38 γ and p38 δ . The best-characterized isoforms of p38 are the α and β isoforms (98). The p38 α and p38 β proteins are ubiquitously expressed in all cell types, but p38 γ and p38 δ are expressed in a more restricted fashion and only in limited cell types (476).

To be fully activated, p38 needs to be dual phosphorylated on the threonine 180 residue (Thr180) and the tyrosine 182 residue (Tyr182) by its upstream activating kinases (476).

This study will focus on the p38 α and β isoforms of p38, as they are the best characterized and understood isoforms of p38. This study does not discount isoform specific effects of p38 in response to stress and DNA damage, however as the reagents to closely study the γ and δ isoforms are not easily available, we have decided to set aside the isoform specific differences for now and focus on the main isoforms of p38 for now, namely the α and β isoforms. When we refer to p38 from hence forth, we will be referring to the α and β isoforms.

1.5.1 Sub-cellular location of Activated p38

Cytoplasmic p38 has also been shown to translocate to the nucleus upon activation (347). Activated p38 has also been shown to be exported from the nucleus while associated with one of its downstream substrates MAPKAPK2. This suggests a dual location for activated p38 (347).

1.5.2 Downstream Targets of substrates of p38 MAPK

Like all members of the MAPK family, p38 MAPK is a proline-directed kinase, phosphorylating its substrates on serine/threonine-proline motifs. The structural basis for this motif selection is unknown, since a portion of the activation loop, for example, occludes the Ser+1 pocket in the unphosphorylated p38 structure (97).

The targets of p38 can be divided into 3 categories: Cytosolic proteins, Transcription Factors that are directly phosphorylated or activated by p38 and other kinases that are activated by p38. These 3 classes of molecules describe the majority of p38's activity in a host of different cell lines and cell types. This list does not currently differentiate abundance of these substrates in different cell types.

1. Cytoskeletal and Cytosolic Proteins:

The study of p38 MAPK's effect on cytoskeletal proteins was first studied using p38 inhibitors. The inhibitor was found to have profound effect on cell migration in epithelial, smooth muscle and endothelial cells. SB203580 was found to block cell migration substantially in these cells (481). The exact p38 substrates responsible for this effect on

cell migration still have yet to be elucidated. The direct cytosolic substrates of p38 that have been elucidated are: (116,227,431)

- a. Microtubule associate protein (Tau)
- b. Cytosolic phospholipase A2 (in Platelets)
- c. Angiotensin II mediated regulation of NHE1 in vascular smooth muscle cells
- d. F-Actin (Endothelial Cells)
- e. HSP27

Upon activation and association with these substrates, p38 serves to phosphorylate and activate these molecules. The direct effect of p38 phosphorylation on most of these molecules is not well characterized with the exception of Hsp27 and Tau.

Under non-stress conditions, HSP27 forms large oligomers that, together with hsp70, act as molecular chaperones to stabilize and refold various proteins (11,223).

2. Nuclear Substrates: p38 phosphorylates and activates many transcription factors directly and through intermediate nuclear kinases, controls the expression of a large number of genes. p38 also has significant influence on transcriptional control elements such ATF 1/2 and CHOP/GADD 153 and multiple CREBs (cyclic AMP response element binding proteins) (30,137,441,479). Through its role as an activating and regulating kinase of a large number of transcriptional control elements, the p38 MAPK pathway is an integral part of many signal

transduction pathways. These pathways include most of the stress response and many of the growth response pathways.

The nuclear transcription factors targeted by p38 include:

- a. ATF-1/2 (30,409)
 - b. Myocyte enhancer factor 2A& 2C (MEF2A/2C) (479)
 - c. ELK-1/4 (SAP-1) (152)
 - d. NF κ B (97)
 - e. CHOP/GADD153 (441)
 - f. Ets-1 (400)
 - g. MAX (Myc binding partner) (303)
 - h. p53 (163,356)
3. The p38 MAPK controls an important *cis* element, the AP-1B binding site and through AP-1 regulates the expression of many genes. Transcriptional control regions containing elements for both CREB and ATF1 are strongly influenced by p38 since the p38 pathway phosphorylates both elements. There are at least three known CREB kinases downstream of p38: MK2, MSK1, and RSK-B.
- a. Downstream Protein Kinases:
 - i. MAPKAPK-2 (MK2) & MAPKAPK3 (MK3) (462)
 - ii. MNK-1 (40)
 - iii. MSK-1 (Alias: RSK-B, RLPK) (79)
 - iv. PRAK (209)
 - b. Transcriptional control elements controlled by p38 include:

- i. *c-jun* (309,335)
- ii. *c-fos* (70)
- iii. ATF1,2 (398)
- iv. Cyclic AMP response element binding protein (CREB) (79)

Having described the major components of the p38 signaling network, we now switch to describing the physiological responses to growth signals, and stress signals that are associated with p38 activation.

1.6 Physiological response to Growth Signals

Having detailed the intracellular signaling components that make up the p38 MAPK pathway, we shift focus to explore the physiological effects of growth signals and of stress signals.

The general response of increased growth signals is the induction of the mammalian cell cycle (376). The cell cycle is the process which cells use to undergo cell-division, where one cell divides into two identical daughter cells. Regulation of the cell cycle is necessary to control the rate of proliferation as well as the accuracy of duplication.

1.6.1 The Mammalian Cell Cycle

The mammalian cell cycle consists of a set of tightly regulated and ordered events that occur in sequence, culminating eventually in cell growth and cell division into two daughter cells (346,376). The molecular pathways and systems that govern this tightly regulated system have been the subject of intense study, as dysfunction in the cell cycle

control has been implicated in human diseases, especially in tumorigenesis. Uncontrolled proliferation is one of the key hallmarks of cancer as defined by Weinberg et al (138), in their landmark paper describing the various properties underpinning the occurrence and growth of cancer. Loss of cell cycle checkpoint control and regulation is one of the main mechanisms employed by cancers to gain limitless potential to proliferate (49,263,425). Before we explore the role of p38 MAPK in the control of DNA damage cell cycle checkpoint control, it is necessary to first layout concisely the current understanding of the mammalian cell cycle. In this chapter, we will describe the basic molecular signaling components that govern cell cycle progression. We will also discuss the various checkpoints that are positioned at critical junctures throughout the cell cycle to prevent premature advancement through the cell cycle. These checkpoints play a critical role in halting the cell cycle and function to ensure the integrity of the genomic code. A pictorial scheme of the mammalian cell cycle is provided in figure 1.4 below.

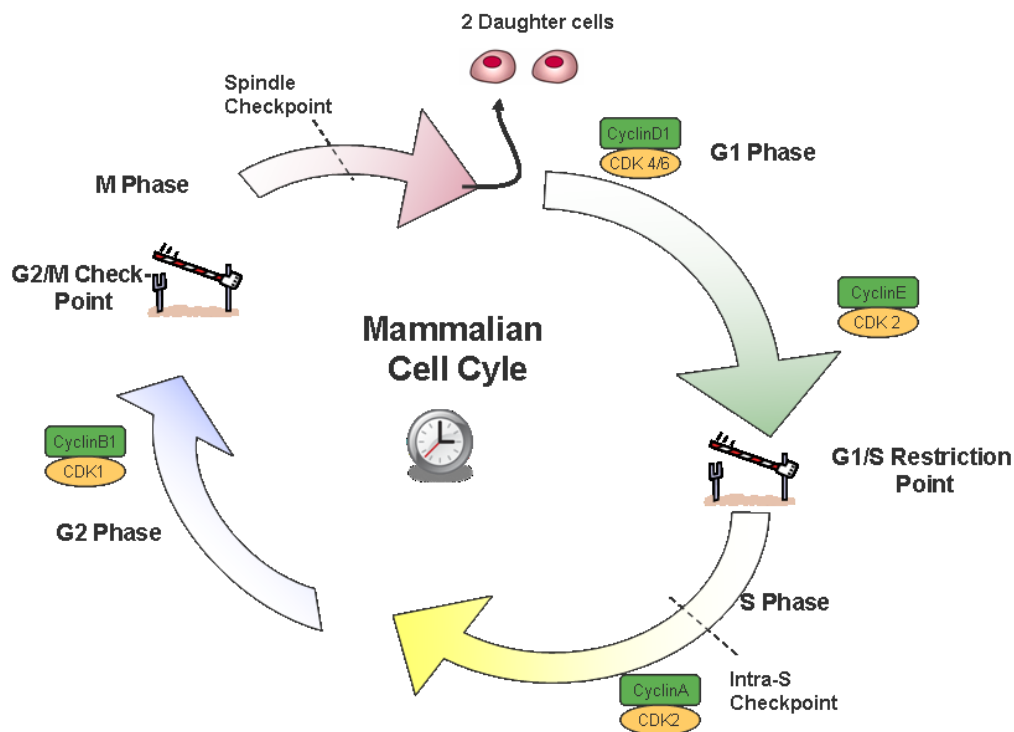


Figure 1.4: *Overview of the Mammalian Cell Cycle: Key cyclins and CDKs required for transition through the cell cycle*

The mammalian cell cycle is conveniently divided into 4 ordered phases, namely the G1, S, G2 and M phase. Besides these 4 phases that comprise the normal cell cycle for growing cells, an additional state called the G0 or quiescence phase is also available for cells that are no longer proliferating and are outside the regular cell cycle (9,346,425). Normal progression through the cell cycle requires that a cell completes each phase in order starting from G1. Each phase of the cell cycle is governed by a number of regulatory proteins called cyclins and their interacting family of kinases called cyclin dependent kinases (CDKs). The cyclins are a family of proteins that are centrally involved in cell cycle regulation which share structurally conserved “cyclin box” regions (346). As the name suggests, CDKs require cyclin to properly function, and until recently it was suggested that each phase of the cell cycle had its own unique pair of

cyclin/cdk interactions that drove the proliferating cell from one stage of the cell cycle to another (284). Recent data now suggests that for some of the critical cyclin/cdk interactions have built in redundancies and cyclin/cdk complex may play a role at more than 1 component of the cell cycle (341). In the next few sections of this review, we will explore the transitions between each phase of the cell cycle. Understanding the key regulatory mechanism that governs these transitions will highlight the impact of defects on the ability of cells to proliferate in an uncontrolled manner, often leading to tumorigenesis.

1.6.2 The G1 to S transition

The G1 phase is the longest phase of the normal mammalian cell cycle. For cells that have a regular doubling time of 24 hours, the G1 phase can span for 10-12hrs or more, usually half or more of the total doubling time (266). Much of what is currently know about the mammalian cell cycle was originally derived through studies in experimental systems such as yeasts, fly and *Xenopus* oocytes (76,113,261). Yeast genetic studies revealed many of the key regulators of the cell cycle, including Cdc2/cyclinB complex, now known CDK1 (100). The main goal of cells in the G1 phase of the cell cycle is to prepare for DNA synthesis in the ensuing S phase. The entire genome is replicated once and once only per cell cycle (456). Genetic studies in yeast have yielded insight into the molecular activity in the pre-replication stage of the cell cycle. During G1/S transition, complexes called pre-replicative complexes or pre-RCs are formed at thousands of sites across the genome. The pre-RCs are formed by the interaction of CDC6, minichromosome maintenance proteins and the origin-recognition

complex (ORC). The presence of pre-RCs on the chromatin renders the DNA replication competent and facilitates the signal transduction pathway that pushes the cell through the G1 restriction point, or start in yeast, into S phase (183,224,228,243,330).

There are numerous regulatory proteins that govern the exit from the G1 phase. The principal cyclin active during the G1 to S transition of mammalian cells is cyclin D, and it has been found to associate with two corresponding CDKs, which are CDK4 and CDK6. The best studied regulatory substrates of the cyclin D and CDK4/6 complex is the Retinoblastoma protein (Rb).

Rb is a critical protein whose activity controls progression through the G1/S transition point. Rb has been found to actively interact with many other critical proteins during G1/S transition, these proteins include E2F family of transcription factors, and the p53 tumor suppressor protein (421). Rb was first predicted to exist through studies of families with Retinoblastoma in 1971 by Knudson et al (204). Cytogenetic studies performed later found a large number of Retinoblastoma patients with lost heterozygosity at the 13q14 locus, the genetic locus of the Rb protein (205). Rb's role as a tumor suppressor protein was solidified by examination of patients with small cell lung cancer (SCLC). It was found that approximately 20% of patients with SCLC had a resulting loss of Rb at the Rb locus (203). Rb tumor suppressing activity stems primarily from its role in governing the transition of the cell cycle forward into S-phase (132). In normal growing cells in early G1, the Rb protein is found to be active in a hypo-phosphorylated form. In this form, it interacts with and suppresses E2F1 transcriptional activity, which is responsible for activating the transcription of many pro-proliferation genes, such as cyclinE (433). The Cyclin D/CDK4/6 complex phosphorylation of Rb at multiple sites,

leads to its inactivation and the decoupling of Rb from the E2F1 transcription factor family (112). Rb not only regulates the E2F1 transcription factor, but also plays a role in the regulation of the activity of the RNA polymerase pol1 and pol2 (257,299,433,465). The combination in the regulation of the transcription factors and the RNA polymerase together makes Rb a critical regulator in G1 to S transition. The phosphorylation of Rb by the cyclin D/CDK4/6 complex is an essential step in the forward progression of cells through the Rb mediated G1/S restriction point (20). Loss of Rb, as in the case of patients suffering from cancers such as retinoblastoma and SCLC, inactivates a critical anti-proliferation checkpoint leading to the ability of these cells to escape anti-proliferative signals and become cancerous (465,466).

1.6.3 Transition through S-Phase

The primary activity during the S-phase of the mammalian cell cycle is DNA replication (159). As discussed in section 1.6.2, cells in G1 have prepared for S phase by increasing cell mass to prepare for DNA synthesis and subsequent cell division and have assembled the pre-RC complexes on their chromatin (13). The subsequent transition of pre-RC formation to the initiation of DNA synthesis provides another level by which CDKs exert regulatory control on progression through S-phase. During G1 phase the loading of the pre-RC complex signals the chromatin ready for DNA replication. Once cells move past G1 into S phase, however, the pre-RC must be reconfigured to form the pre-initiation complex or pre-IC (91). The pre-IC in yeast is composed of the CDC45 and the MCM complex (228). This protein complex is required for the initiation of DNA

replication. In animal cells, loading of the MCM complex to chromatin is governed by the activity of cyclin E/CDK2 (228,272,357).

The key cyclins active during S-phase of the mammalian cell cycle are Cyclin E and Cyclin A, both of which are found to interact with CDK2 (162). Through genetic studies in mice, it has been shown that cyclin E/CDK2 is required primarily for the passage through the G1/S restriction point. Studies have shown the cyclin E/CDK2 complex also phosphorylates Rb, however, the dynamics of cyclin E/CDK2 phosphorylation is believed to be subsequent to the initial phosphorylation event by cyclin D/CDK4/6 (214). This suggests that cyclin D/CDK4/6 phosphorylation begins the process of Rb inactivation. Then, additional phosphorylation of Rb by cyclin E/CDK2 promotes complete inhibition of Rb at the G1/S transition point (46).

While CDK2 is a key kinase required for normal progression into S-phase, homologous knock-outs of CDK2 in mice have shown that loss of CDK2 does not prevent cell cycle progression, suggesting that there are sufficient compensatory pathways in mammalian cells through other CDKs for S-phase progression. Loss of CDK2 is not without effect however, CDK2^{-/-} mice are sterile, suggesting an irreplaceable role of CDK2 during germ cell development (250). Another observation noted for CDK2^{-/-} mice was their delayed kinetics for entry and completion of S-phase. So while some of the essential kinase functions of CDK2 can be compensated, loss of a critical S-phase CDK is not without effect and consequence on an organism(264). Studies from genetic knock-outs of CDK2 have suggested that the CDK2 function is required for germline cell proliferation, but can be largely compensated by other CDKs in somatic cells.

The cyclin A/CDK2 complex regulates initiation of the DNA replication and restricts the initiation of replication as a once only event within a cell cycle (273). Cyclin A/CDK2 is activated in early S-phase and remains active until early M phase. While cyclin E plays an important role during G1/S transition as discussed earlier, there are reports suggesting that cyclin A can substitute for cyclin E function at the G1/S restriction point (280). However loss of cyclin A2 cannot be substituted for by other cyclins, as loss of cyclin A2 results in embryonic lethality (291). Cyclin A2 has been found to be at both the G1/S transition as well as the G2 transition. Cyclin A2 activity is also required for initiation of DNA replication (origin firing) as well as chromosome replication (171,187,342).

1.6.4 The G2 to M transition

After completion of DNA replication, mammalian cells undergo a 2nd gap phase in the cell cycle prior to mitosis and division (110). As discussed in section 1.6.3, cyclin A/CDK2 activity is important for progression through S-phase and completion of DNA replication. The cyclin A/CDK2 complex remains active during G2 phase (221,273,456). By the end of S-phase, DNA is replicated and repackaged into adjoining chromosomes. At G2 phase, cells hence have 2 copies of each chromosomes or measured as a 4N DNA content.

The G2 phase is thought to be a final staging phase prior to the act of cell division. The main cyclin/CDK complex that governs the transition from G2 to M is the cyclin B/CDK1 (*cdc2/cdc28* in yeasts) complex (151,221). This is believed to be the last major cellular checkpoint prior to the entry into mitosis which is demarcated by the

formation of polar spindles and a metaphase plate. The importance of accurate and error free cell-division is paramount to survival of an organism. The cyclin B/CDK1 complex governs the last checkpoint prior to entry into mitosis, sensing incomplete DNA replication and DNA damage resulting in both single and double strand breaks.(344,349,397) The signaling cascades that signal into the G2 checkpoint have been an area of intense research, as defects in G2 checkpoint have been exploited by cancers as a mechanism to evade growth inhibitory signals. The key intracellular kinases that suppress the activity of the cyclin B1/CDK1 complex are identified as Myt1 and Wee1 kinase. These kinases restrain CDK1 activation by maintaining inhibitory phosphorylation at Thr14 & Tyr15 (387). As cells progress through G2 phase, the CDC25 family of phosphatases are activated to remove the inhibitory phosphorylation on CDK1 (397). Simultaneously, the Wee1 & Myt1 kinases are inactivated through ubiquitin-mediated proteolysis. The consequence of down-regulation of Wee1 & Myt1 and the up-regulation of the CDC25 phosphatases culminates in the rapid activation of CDK1 (378). Network analysis of CDK1 activation dynamics has suggested a closely regulated positive feedback loop aiding in its rapid activation (242). CDK1 has been shown to phosphorylate its inhibitory kinases Myt1 and Wee1, leading to their deactivation and eventual degradation by poly-ubiquitination (143). Once CDK1 activity progresses beyond its activation threshold, this positive feedback mechanism drives down the activity of its inhibitory kinases leading to maximal activation of CDK1. There are numerous kinase signaling pathways including Plk1 and the p38 pathway that have been implicated in the activation dynamics of CDK1 and the entry into mitosis.

The active cyclin B1/CDK1 complex triggers initiation of mitosis, promoting, nuclear envelope breakdown, DNA condensation and formation of polar spindles (174). A key marker used in this study and others to mark the transition from G2 into mitosis is the phosphorylation of Histone-H3 on its ser10 residue (403). We use this mitotic marker as a critical biochemical marker to study the potential role of p38 on the G2 DNA damage checkpoint. Another key kinase that plays a regulatory role at the G2 transition is the polo-like kinase, Plk1. Plk1 has been shown to play a significant role in G2, especially in preparation of centrosomes for separation during mitosis (222,305). Plk1 is involved in the recruitment of γ -tubulin to the centrosome, a required component of the mitotic spindle. Plk1 has also been shown to regulate several centrosome associated proteins including Nlp (ninein-like protein), Katanin and the microtubule stabilizing protein TCTP (47,296). Besides being involved in the active recruitment of key components of the microtubule machinery to the centrosome, Plk1 is also implicated as a key component of the G2 DNA damage checkpoint (172,415).

There are other signaling pathways besides the Plk1 pathway that are believed to regulate the activation and maintenance of the DNA damage G2 checkpoint. One of the pathways recently implicated in the regulation of G2 to M transition is the p38 MAPK pathway. Elucidation of the putative role of p38 MAPK in the regulation of the G2 DNA damage checkpoint is a key area of my graduate research. we will, therefore dedicate an entire section of this literature review to highlight the key signaling and mechanistic components of the G2 checkpoint.

1.6.5 Transition through Mitosis

The final stage of the cell-cycle is the act of cell division. In terms of total time, the act of cell-division is a very rapid and spectacular event (122). All the inter-dependent events during inter-phase, culminate in a series of complex processes during mitosis to segregate DNA equally and to complete cell division into two daughter cells. The key kinase that regulates the entry into mitosis have been identified as cyclin B1/Cdc2 complex, now commonly known as CDK1 (241,340,412). As reviewed in the preceding section, a delicate balance of inhibitory kinases and activating phosphatases keeps CDK1 in check until cells are ready to undergo mitosis. Upon reaching the threshold of activity, CDK1 triggers cells into mitosis (366). CDK1 undergoes a nuclear translocation at the beginning of mitosis. The translocation of CDK1 from the cytoplasm to the nucleus correlates with chromosome condensation and nuclear envelope breakdown (318). Besides CDK1, early mitosis is also driven by the activity of the S-phase cyclin-A . As mentioned in previous sections, cyclin-A plays a critical role during S-phase, driving successful DNA and centrosome duplication. Cyclin A levels however stay high throughout G2 and into early mitosis, also correlating with chromosome condensation and nuclear envelope breakdown (148). As cyclin A is degraded post nuclear envelope breakdown, cyclin A, like the mitotic cyclin B, also forms a complex with Cdc2 drives some of the early events during mitosis, including chromosome condensation and nuclear envelope breakdown (129,215,273,274).

The next major features noticeable during mitosis after nuclear envelope breakdown is the formation of polar mitotic spindles and the formation of the metaphase plate (485). Active CDK1 is found localized to the centrosomes at the end of G2. The

localization of activated CDK1 at the centrosome is an indication that high CDK1 activity is critical for the initiation of centrosome separation (72,212,242). Centrosomes duplicated during S-phase now move quickly to opposing ends to form the bipolar mitotic spindle. Sister chromatids are moved and oriented in a bi-directional fashion and attached to the mitotic spindle (133). The correct orientation and attachment of all chromosomes to the mitotic spindle is the single most important event during metaphase (108). Incomplete or inaccurate attachment of sister chromatids to the mitotic spindle often leads to aneuploidy, as frequently associated in cancer cells (311). The spindle checkpoint is the key mitotic checkpoint that ensures that all sister chromatids are appropriately aligned and attached prior to separation (78). Some of the key regulatory proteins responsible for the spindle assembly checkpoint include Mad2 and BubR1. Mad2 is found active at the sites of unattached kinetochore proteins (478). The kinetochore is a centromere protein complex that microtubules associate with for sister chromatid separation by the mitotic spindle. Mad2 and BubR1 actively signal to inhibit the activation of APC/cyclosome activity (107,154). These inhibitory proteins ensure that all chromosomes are attached to the mitotic spindle before activation of the anaphase promoting complex (APC/cyclosome complex). While the exact mechanism governing Mad2 and BubR1 activity are not yet fully discovered, active Mad2 is believed to bind and inhibit cdc20, a key co-activator of the APC/cyclosome complex (122,277). Another co-activator Cdh1, is believed to be responsible for progression through late anaphase and onto to the start of cytokinesis (389). Some of the key signaling proteins that feed into the spindle checkpoint include the polo-like kinases Plk1 and Plk2, and the aurora kinases (401,455). These signaling kinases are believed to be part of complex feedback or

feed forward signaling networks that contribute to the regulation of APC/cyclosome activity to ensure that all chromosomes are attached to the mitotic spindle before chromosome separation can occur, and is commonly referred to as the spindle assembly checkpoint (115,234,446,459,464).

The anaphase promoting complex is an ubiquitin ligase that is the key driver for the completion of mitosis. One of the main roles of the APC/cyclosome complex in mitosis is the destruction of cyclin B1 and inactivation of CDK1 (167). Destruction of cyclin B1 generally marks the end of metaphase and the beginning of cytokinesis (244). Inhibition of the APC/cyclosome complex by members of the polo-like kinase family facilitates the maintenance of high levels of active CDK1 complex for the period of time necessary for the correct attachment of chromosomes to the spindle (120,140). Inactivation of the spindle checkpoint occurs only when all chromosomes are oriented and attached in an appropriate fashion to kinetochores (485). Upon attachment of chromosomes, the inhibitory signaling by Mad2 at each respective kinetochore is silenced. Inactivation of Mad2 releases cdc20 from inhibition, and facilitates the activation of APC/cyclosome complex (107). The other major co-activator of the APC/cyclosome complex is the Cdh1 protein. Cdh1 is found to complex with APC/cyclosome and believed to aid in substrate specificity to APC mediated proteosomal degradation. Cdh1 is negatively regulated by CDK1 complex through phosphorylation (73,74). As already mentioned, multiple signaling pathways cooperate to ensure that APC is not activated pre-maturely. The combination of the spindle associated proteins such as Mad2 and BubR1 and the activity of CDK1 act in concert to inhibit activators of APC until the appropriate moment (244).

Inactivation of the spindle assembly checkpoint, releases the APC/C complex from inhibitory control leading to the rapid degradation of cyclin B1 and inactivation of CDK1. The APC/C complex has multiple binding partners even during mitosis. The interaction between APC/C and cdc20 in early mitosis is dependent on high CDK1 activity (237,368). APC/C, cdc20 complex is activated by phosphorylation by Plk1 and CDK1, facilitating a positive feedback loop driving the destruction of cyclin B1 to initiate the rapid DNA segregation process during anaphase. Cdh1 is, however, prevented from associating with the APC/C complex by inhibitory phosphorylation by CDK1. This highlights the importance of delicate balance and control in the progression of mitosis. Cdh1 is unable to bind to APC/C until CDK1 activity is inhibited by the destruction of cyclin B1. This carefully choreographed sequence of events highlights the importance of signaling pathways in the control of APC/C activity (8,368,475).

Upon activation, APC/C with its co-activator cdc20 target cyclin B1 for proteosomal degradation (365). Degradation of cyclin B1, leads to inactivation of CDK1, and marks the transition of cells from metaphase to anaphase. Activated APC/C targets the protein securin, located on kinetochores for proteosomal degradation. Degradation of securin releases the protein separase from inhibition (124,292). Activated separase cleaves Scc1 (Sister chromatid cohesion 1), a member of the cohesin complex, which results in the loss of sister chromatid cohesion. This facilitates the separation of sister chromatids from each other, leading to two independent sets of chromosomes.

Upon completion of chromosome separation, cells have moved beyond metaphase into anaphase and telophase, which is marked by the events of spindle elongation, spindle disassembly and cytokinesis (424). The APC/C^{cdc20} complex that has been the main

driver responsible for the events leading to separation of sister chromatids attached at the mitotic spindle, disassociates and APC/C forms a new complex with Cdh1. Cdh1 is inhibited from binding to APC/C by CDK1 activity prior to chromosome segregation (73). Destruction of cyclin B1 by the APC/C^{cdc20} complex leads to the inhibition of CDK1 activity, when coupled with the activity of the phosphatase cdc14, leads to the activation of the APC/C^{Cdh1} complex, the complex responsible for driving the signaling network responsible for mitotic exit (219). The APC/C^{Cdh1} complex drives the completion of mitosis through proteolytic degradation of key mitotic proteins. Following chromosome separation APC/C^{Cdh1} targets cdc20 and securin for degradation (134). Unlike cdc20, the Cdh1 complex with APC/C stays active through the subsequent G1 phase and is only then inactivated at the beginning of S-phase to prevent re-activation of mitotic activities in G1. The APC/C^{Cdh1} complex is believed to be essential for the formation of pre-RCs during G1 phase and for the suppression of mitotic cyclins and kinases during G1 phase (19,161,370,445). The APC/C^{Cdh1} complex is one of the key components of the mitotic exit network, a network of signaling and associated motor proteins responsible for the division of a cell into two identical daughter cells.

The key features of mitotic exit also include ordered dephosphorylation of CDK1 substrates by mitotic phosphatases. Failure to dephosphorylate CDK1 substrates has led to prolonged and failed mitotic division (114,453).

The microtubule motor proteins belonging to the kinesin and dynein family of motor proteins are responsible for bipolar mitotic spindle formation, DNA segregation and cytokinesis (450). The Plk1 signaling pathway has been implicated as being a key signaling kinase in late mitosis, and is believed to be responsible for driving events

leading to cytokinesis (115,140,401,459). Recent knock-out studies have highlighted the importance of Plk1 in late mitosis. Loss of Plk1 often leads to arrest in early mitosis, however, impairment between interactions of Plk1 and the mitotic kinesin Pavarotti has been shown to lead to failed cytokinesis. Plk1 has also been shown to interact with Mklp2 and NudC proteins, the two key motor proteins responsible for cytokinesis (240).

As this thesis focuses on the role of p38 in DNA damage response, specifically at the G2 to M transition point, we will next review the signaling networks that comprise the cellular response to DNA damage, with a focus on the G2 checkpoint governing the entry into mitosis.

1.7 Physiological Response to Stress

Cells are faced with many different forms of stress throughout their life, making an appropriate response to different forms of stress critical for long term viability and survival. While cells may respond differently to various forms of stress, it is possible to generalize the response of cells to most common types of stress. Stresses ranging from mechanical, chemical or environmental damage cause physical damage to the cell and elicit strong activation of similar stress response pathways in a cell (22,288,458). Cellular response to changes in external environment such as immune system activation and the presence of inflammatory cytokines largely depend on similar signaling networks (60,63). This suggests a high degree of overlap between stress pathways responsible for sensing changes in the external environment and internal damage.

In the following sections we will briefly detail the role of p38 MAPK in response to inflammatory cytokine stimulation and its role in cytokine production. The focus will be on the pathways responsible for sensing internal damage to the cell.

1.7.1 Response to Inflammatory Cytokines

One of the phenotypic effects mediated by TNF- α through p38 MAPK signaling is the production and secretion of inflammatory cytokines in numerous macrophage and lymphocyte cell lines (449).

Studies have shown that TNF- α or LPS induced inflammatory cytokine production is mediated through a p38 MAPK mediated response. Mouse mutagenesis studies have identified MAPKAPK-2 as one of the principal p38 substrates that is responsible for the production and secretion of inflammatory cytokines (407). Mouse knockout studies have shown that mice lacking MAPKAPK-2 are resistant to LPS induced stress response and a decrease of ~90% TNF- α and IL6 production (210). The effects of MAPKAPK-2 are thought to be mediated via a mechanism involving cytokine mRNA turnover and its effect on protein translation (388).

1.7.2 Effect of p38 MAPK on ARE binding proteins

The p38 MAPK's effect on cytokine production involves ARE binding proteins bound to AU-rich elements (ARE) in the 3' untranslated region of an inducible cytokine like IL-1, TNF- α and IL-6. The AREs are known to target mRNAs for rapid deadenylation and degradation and may enhance decapping (45). The p38 MAPK pathway stabilizes many otherwise unstable ARE-containing mRNAs encoding proteins involved in inflammation(452). It is postulated that some ARE binding proteins facilitate the degradation and turnover of these cytokine mRNA. Whereas other ARE binding proteins are known to stabilize the mRNA through their association with the ARE. The activation of the p38 MAPK pathway facilitates the phosphorylation of these ARE

binding proteins and hence leads to cytokine mRNA stability either through disassociation or association.

The effects of ARE binding proteins is unique to certain cytokines. TNF- α mRNA instability for example is associated with the presence of heterogeneous nuclear ribonucleoproteins (hnRNPs) bound to their ARE regions(57). The activation of the p38 MAPK pathway leads to MAPKAPK-2 dependent phosphorylation of these hnRNPs and their subsequent disassociation. The disassociation of the hnRNPs confers stability to the mRNA and allows their translation (25).

The effects of ARE binding proteins on IL6 and IL1 secretion are opposite to what has been seen with TNF- α secretion. The binding of hnRNPs is seen to confer a degree of stability to the mRNAs that allow their subsequent translation. The p38 MAPK pathway through MAPKAPK-2 mediates the binding of these hnRNPs. The evidence for this was a 10-fold decrease in IL6 mRNA stability in MAPKAPK-2 deficient mice (448). There is evidence also, however, that MAPKAPK-2 is not the only kinase downstream of p38 that regulates post-transcriptional activity of inducible cytokine mRNA. MAPKAPK-3 and MAPKAPK-5, two other downstream targets of p38, are also capable of phosphorylating hnRNPs in the absence of MAPKAPK-2(434).

Bollig et al has identified 18 macrophage ARE binding proteins that are involved in cytokine mRNA instability (25). The phosphorylation of some of these ARE binding proteins is attributed to MAPKAPK-2, a direct kinase substrate of p38 MAPK. Kontoyiannis et al, showed that IL10's inhibitory effect on TNF- α production was mediated principally through a p38/MAPKAPK-2 pathway, further confirming MAPKAPK-2 involvement in cytokine mRNA stability (208).

The p38 MAPK working through its downstream kinase substrates MAPKAPK-2, 3&5 is a key mediator of inducible cytokine stability and expression. The mechanisms through which it controls TNF- α , IL1, and IL6 mRNA expression is through the phosphorylation of ARE binding proteins (374). The activation of the p38 MAPK pathway also increases the transcription of inflammatory cytokine mRNA through its action with the AP-1 binding site and its activation of the ATF-2 transcription factor (454).

We intend to investigate p38 MAPK's role in inflammation response as a secondary question in this thesis. We will explore p38's role in inflammatory stress response to give a physiological context to p38's putative role in DNA damage response. As p38 is a critical kinase in stress response, it is conceivable that p38 activation during DNA damage may be a general, protective stress response and may not necessarily be specific for the DNA damage response, per se. we will explore this topic in detail in chapter 4 of this thesis, where we analyze whole genome gene expression data to gain a mechanistic understanding of p38 in inflammatory stress response through perturbation of p38 activity with a selective kinase inhibitor.

1.7.3 The sensing of DNA Damage: Role of ATR and ATM

The key members of the DNA damage checkpoint pathways have been identified to be members of the PIKK (or phosphatidylinositol 3' kinase like kinase) family of proteins. The most prominent members of this family are the ATM (Ataxia-Telangiectasia Mutated) and ATR (Ataxia Telangiectasia mutated and Rad3 related) proteins (361). Traditionally it was convenient to divide the response to the different classes of DNA damage between these two key sensors that share sequence homology. Early studies attributed response to double strand chromosomal breakage primarily to ATM while response to single strand breaks and other DNA lesions was assigned to ATR (392). More recent studies, however, have shown that such a simplistic view of the DNA damage response pathway was inaccurate. ATR has also been found to play a key role in the activation of cell cycle checkpoint arrest and DNA repair and/or apoptosis induction signaling in response to DSB events. While ATM has also been shown to be active to damage that does not ordinarily cause DSB (2,443). This section of the review will detail some of the key components responsible for detection of DNA damage.

ATM was identified as the gene mutated in the inherited cancer predisposition syndrome ataxia telangiectasia and is a key player in the response to double strand chromosomal breaks (DSB) (256). ATR was identified to be the sensor of a much wider variety of DNA damage including detection of DNA lesions induced by UV irradiation and stalled replication forks induced by chemical genotoxics such as hydroxyurea (HU) and topo-isomerase II inhibitors such as Adriamycin (Doxorubicin HCL) (43,92,104). Both ATM and ATR have been found to localize to areas of DNA damage. This localization has been shown to be the result of other adaptor and co-binding proteins. The

initiation of ATM mediated signaling has been shown to require the protein RPA. Extensive studies by various groups have shown that the PIKK family of signaling proteins works in concert with these co-initiators in a multi-protein complex. It has also been shown that the loss of certain key members of these multi-protein complexes greatly inhibits the ability of cells to respond effectively to DNA damage, often leading to disease phenotypes such as tumorigenesis.

ATM is a key sensor of DSB in eukaryotic cells. Studies utilizing ionizing radiation (IR) have shown that cells exhibiting defects or mutations in ATM are hypersensitive to IR (270). IR causes primarily DNA double strand breaks, implicating ATM as one of the primary sensors of DNA DSB events. Extensive studies conducted in yeast and other simple multi-cellular organisms have identified numerous adaptor and co-factor proteins necessary for full ATM function. The Mre11-NBS1-Rad50 (MNR) complex is essential for activation of ATM (99). Studies have shown that loss of components of the MNR complex lead to inefficient ATM activation including its inability to auto-phosphorylate itself. It is interesting to note that while Nbs1 is part of the MNR complex, which is required for ATM activation, Nbs1 itself has been found to be a target of ATM. Phosphorylation of Nbs1 by ATM is suggested to have an important role in efficient transduction of DNA damage signal to downstream effector molecules including Chk2, one of the primary substrates of ATM (178).

In mammalian cells, the ATR protein is essential for cell viability even in the absence of exogenous DNA damage (444). This role in a normal cell cycle has been attributed to the tremendous rigors involved in replicating larger genomes found in mammalian cells. Such replication often requires an ATR dependent response to ssDNA

formed in almost every cell cycle (90). Conventional thought had originally associated ATR activation and function in response to lesions formed on DNA, as well as to stalled replication forks. DNA lesions could be grouped according to the chemical nature of the base modification. DNA lesions include dimers, mismatches, base modifications such as oxidation, methylation and depurination, bulk lesions, intra and inter-strand cross-linking such as that caused by UV damage (131,384). Responding to stalled replication forks such as caused by hydroxyurea (HU) was one of the primary roles first identified for the ATR protein. Early studies that showed that ATR was preferentially activated by stalled replication forks and that ATM was preferentially activated by DSB, led to the belief that these 2 key sensors of DNA damage had independent and separate roles in activating a cellular response to DNA damage (361).

Recent studies in yeast and other model organisms, however have painted a slightly more complicated picture. Studies have shown that there is close inter-play between ATM and ATR for the detection of DSB events in genome. There is much evidence to indicate that the activation of an ATR dependent response to a chromosomal DSB is triggered by the recognition of ssDNA moieties (2,392). The formation of ssDNA is a common product, found at locations of base excision repair and stalled replication forks. DSBs are often processed by 5' to 3' exo-nucleases also resulting in the formation of ssDNA. This makes ssDNA a common and useful checkpoint initiation signal for the DNA damage checkpoint pathway. A key adaptor protein called RPA has been shown to be vital for ATR activation of downstream signaling components in the event of DNA damage. The recruitment of ATR to sites of double strand breakage in the genome is assisted by adaptor proteins, Ddc2 and the ATR interacting protein ATRIP (199).

In yeast, Mec1 (ATR) and Ddc2 form a complex in cells independent of DNA damage and genetic studies with Mec1 Δ and Ddc2 Δ cells show the same defects, namely the complete loss of checkpoint arrest and a failure to activate the downstream signaling components of the DNA damage pathway, such as Rad9, Rad53, Chk1 or Pds1 (Securin) (96,281,306). Studies on Mec1 have suggested that its kinase activity is not tightly regulated by DNA damage. However Ddc2's role in checkpoint activation is thought to be the recruitment of Mec1 to damaged DNA. This suggests that Mec1 can only interact with its substrates while bound to damaged DNA, which may help prevent spurious checkpoint activation in undamaged cells (75).

Through its role in processing ssDNA generated by exonucleases in the event of DSB, the ATR protein has been shown to be a key integrator of numerous exogenous DNA damaging agents including UV, MMS and Adriamycin (383). While the end result of these different DNA damaging agents range from inter and intra strand fusions and thymidine dimers formed by UV damage to nucleotide base alkylation caused by MMS, the by product of most of these agents was the production of single strand DNA (ssDNA) and a stalled replication fork. The activated ATR complex phosphorylates a number of key downstream signaling substrates. These substrates include the Chk1 kinase, Brca1 and the p53 tumor suppressor protein (331). Translation of these findings into the context of human cancer cells, could provide the next step in deeper understanding of the molecular pathways

Having identified the main players in the sensing of DNA damage, we now focus on identifying the main players in the transduction of the DNA damage signal to the downstream effectors of cell cycle checkpoint arrest.

1.7.4 Signal Transduction of DNA Damage Signal

After DNA damage is detected by upstream sensors such as the ATM/ATR complexes, a kinase signaling cascade then amplifies and relays the signal to checkpoint targets leading to cell-cycle arrest, DNA repair and/or apoptosis induction (259). The task of relaying and amplifying a damage signal is crucial in the ability of cells to maintain genomic stability. The tiered nature of the signal transduction pathways allows the damage signal to be rapidly transmitted, with built in feedback mechanism allowing authentic signals to be amplified while spurious signals are more likely to be damped out and ignored. A number of prominent signaling pathways have been identified as substrates to ATM/ATR signaling. These include the Chk1/Chk2 pathways which have been implicated as one of the primary signaling transduction pathways activated in response to DNA damage leading to cell cycle arrest, repair and apoptosis (308). Besides the Chk1/2 pathways, other key players identified in response to DNA damage are the Brca1 SMC1 and p53 pathways (429,470). Both Brca1 and p53 play prominent roles in maintenance of cellular genomic integrity and are classified as tumor-suppressors. While most of the work in the past has been focused on understanding this handful of signal transduction and integrators of DNA damage signals, recently a new crop of possible players in DNA damage response have also been identified. A study by Mu et al, using advanced proteomics techniques has recently identified up to 20 new phospho-protein substrates of ATR signaling (285). Additional studies have also started focusing on the MAPKs, specifically the p38 MAPK as another possible key signaling pathway that plays a role in the transduction of DNA damage signal. This review will briefly cover the

current state of knowledge on these signaling pathways and how activation of these pathways leads to effective cell cycle checkpoint arrest.

1.7.5 Chk1 and Chk2 are substrates of ATM/ATR signaling

Some of the signal transduction pathways responsible for transmitting the DNA damage response signals are highly conserved evolutionarily from yeast to humans. Much of the early work in unraveling the complex signaling networks involved in DNA damage response was conducted in budding yeast *Saccharomyces cerevisiae* or fission yeast, *Schizosaccharomyces pombe* and with *Xenopus* egg extracts (258,411). The primary signaling cascade following activation of Mec1 (ATM/ATR checkpoints) in yeast is the Chk2 family of kinases Rad53 whose activation also requires the adaptor protein Rad9 (96,343). Studies in yeast have shown that without Mec1, Rad53 is not functional. Hence activation of the PIKK (ATM) mediated phosphorylation of Rad53 serve several functions, namely the activation of the Rad53 (Chk2) kinase activity, the promotion of oligomerization, trans-autophosphorylation and the creation of an interface for the Rad53-Dun1 interaction (314,390). Activated Rad53 also interacts with numerous nuclear import factors. Nuclear import and localization has been identified as a necessary part of Rad53 activation and the transduction of DNA damage signaling (314).

Similar to Rad53, activation of Chk1 also requires Rad9 for its activation and function (144). Structural analysis of the Rad9 protein shows that the activation region of the Rad9 protein for Rad53 is different from Chk1 (391). Chk1 does not have an FHA domain like Rad53 and it can be activated by an allele of Rad9 without the SQ/TQ cluster required for Rad53 activation. Conversely, an N-terminal truncated mutant of Rad9 is

unable to activate Chk1 but is able to activate Rad53 in response to a single DSB in *S. Cerevisiae*. Structural modification studies on Chk1 have suggested that similar to Rad53, Chk1 requires oligomerization and probably autophosphorylation for full activity (218). The Chk1 kinase is one of the primary substrates of ATR following DNA damage response. Understanding the role of Chk1 activation in DNA damage response has helped advance the understanding behind the underlying mechanism of cell cycle checkpoint arrest following DNA damage (131).

1.7.6 Other known targets of ATM/ATR signaling

The Chk1 and Chk2 (Rad53) pathways are the best characterized members of the canonical DNA damage response pathway. Recently, additional signaling proteins have been implicated as key players in the transmission of DNA damage response. Protein targets such as BRCA1, SMC1 and p53 have been implicated as targets of ATM/ATR signaling and are known to play a crucial role in checkpoint arrest and DNA repair (86). The role of p53 has been well studied for its role in G1-S DNA damage checkpoint control. Briefly, p53 is stabilized by phosphorylation which allows it to separate from its negative inhibitor MDM2. Stabilization of p53 activates the p53 damage response transcriptional pathways, leading ultimately to the activation of the Rb protein (42,61,216). This section will not focus on these specific mediators of p53 dependent DNA damage response, but will instead point readers to other recent excellent reviews on this topic (430,486).

Recently a lot of interest has focused on the possibility of members of the MAPK family of proteins especially p38 MAPK and its downstream substrates in playing a

major role in initiation and maintenance of cell cycle checkpoint response to DNA damage (35,48,313). The p38 MAPK plays a significant role in response to endotoxins, and in inflammatory response. The p38 MAPK has also been implicated in active immune response and antigen presentation to T-lymphocytes. Beyond its well established role in inflammatory response, a new role for p38 has been suggested in DNA damage response. A more detailed review of p38 and its possible roles in DNA damage checkpoint control is included as a separate section below.

1.8 Effectors of Cell Cycle Arrest

Having introduced the key signal transduction pathways activated in response to stress and DNA damage, we now will discuss some of the common physiological consequences resulting from the activation of DNA damage response pathways.

The DNA damage response (DDR) signal transduction pathways transmit and amplify the initial signal from the sensors of DNA damage to the end effector proteins or pathways that initiate and maintain the checkpoint arrest. In eukaryotic cells there are 3 major checkpoints during interphase and they are termed the G1-S, intra-S and G2-M checkpoints (4,308,382). Each of these specific checkpoints has a specific set of effector molecules responsible for their activities which will be reviewed briefly in the following sections. In addition, as the focus of this thesis is centered on the role of p38 MAPK, a separate section will focus on examining the literature evidence for its role in G2 cell cycle checkpoint arrest.

1.8.1 The G1/S Restriction Point

Entry into S-phase from G1 is critical to cells, marking a point of no return to a new round of cell cycle (377). Thus, not surprisingly mammalian cells have developed elaborate regulatory mechanisms governing G1/S transition. While cyclin D1/CDK4/6 complexes are key activators of the G1/S transition, there are two equally important counter-balances to the CDK family of proteins, namely the CDK inhibitors (363). The two major CDK inhibitors in the G1/S transition consist of the CDKN1 (p21^{CIP/WAF1}) and CDKN2 (p16^{INK} & p14^{ARF}) family of proteins (24). Both p21 and p16 inhibit CDK4/6 activity to keep Rb in a hypo-phosphorylated form, in response to DNA damage (213). The p14^{ARF} protein product of the CDKN2A locus does not interact with the cyclin D/CDK4/6 complex but instead has been found to interact with MDM2, leading to p53 activation (146,246). These two key proteins as well as PI3KCA (PTEN), guard against damage to the genome and help to maintain genomic integrity (33,139,307,371,377). As this family of CDK inhibitors is critical to the healthy maintenance of the genome, it is no surprise that the gene locus for CDKN1A, CDKN2A and PI3KCA are some of the most frequently mutated and copy loss areas of the human genome (189). A majority of human tumors studied were found to have a loss or mutation of either one or all of these gene loci, rendering the G1/S restriction point deficient.

A second key tumor suppressor that governs the G1/S transition along with Rb pathway is the p53 tumor suppressor protein, a protein most often implicated in tumorigenesis and the response to DNA damage (232,327). The goal of this thesis and review is not to exhaustively review the role and function of the p53 protein in the cell cycle. Instead we will point readers to a number of in-depth reviews on p53

(34,202,289,373). Here we will highlight some of the functions of p53 in relation to DNA damage and cell cycle control relevant to my research.

The p53 protein is a tightly regulated protein that requires a series of phosphorylation events in order to be activated. In its inactive state, p53 is found tightly bound to the MDM2 protein, which suppresses its transcriptional activity (53,155). The p53 protein has been implicated as a principal mediator of stress response to a host of environmental and other damages to DNA. The p53 response, functions through the trans-activation of a number of key downstream genes as a transcription factor (351,380,432). Some of the best understood response genes to p53 activation include the p21^{CIP/Waf1}, a protein responsible for the activation of G1/S as well as G2 checkpoint arrest (150,206,344). Other genes that are direct targets of p53 regulation in response to DNA damage are the GADD45 protein, a growth arrest & DNA damage inducible gene, and the primary negative regulator of p53 function, MDM2 itself (180,360). The central nature of p53 in DNA damage checkpoint response has resulted in it being one of the most highly mutated genes found in cancers (193,372). Some studies have shown that functional inactivation of p53 is found in more than half of all human tumors. Such a high propensity of functional inactivating mutations highlights the importance of p53 as a critical protein in the maintenance of genomic stability (42,216,279,321,355).

There are a number of reports to suggest that p38 may play an active role in the regulation of the G1/S transition in response to DNA damage. The current opinion of the field is that p38 may act to modulate the G1/S checkpoint through interaction and regulation of p53 activity (201,269,410). Inhibition of p38 has been shown to affect the levels of p21 mediated regulation of Rb, contributing to the failure of checkpoint control

in response to DNA damage. More published papers show that p38 may play a role at the G1/S transition point (69,81,282,283,410). While we are mindful of the important role of p38 at the G1/S restriction point, the point of this thesis was not to study p38 at the G1/S restriction point.

1.8.2 The Intra-S Checkpoint

In response to single or double strand breaks during S-phase, the intra-S checkpoint activates to halt cell cycle progression during DNA replication. The cyclin A/CDK2 complex is a critical for S-phase progression (273). It is shown that CDK2 inhibition leads to a significant halt in cell cycle progression (215). In response to environmental, chemical or physical damage, the ATM/ATR pathway activates a number of checkpoint signaling proteins that lead to the inhibition of the cyclin E or A/CDK2 complex (162,482). These signaling pathways include the well studied Chk1 and Chk2 as well as activation of p53 via ser15 and ser33 phosphorylation. Activation of the Chk1, Chk2 pathway regulates CDK2 activity through phosphorylation of the CDC25 phosphatase protein family (121). The CDC25 phosphatases are key regulatory proteins that remove inhibitory phosphorylation on key checkpoint proteins such as Rb and CDK1. The negative regulation of the CDC25 family of phosphatases during S phase inhibits CDK2 activity through inhibition of cyclin E/A binding and complex disruption (18,334,350). Besides the Chk1 & Chk2 signaling pathways, activation of the p53 tumor suppressor pathway is another key factor during intra-S phase arrest. Increased p21^{waf1} activity has been linked to decreased CDC25A levels (187). Increased levels of p53 activity have also been implicated in decreased cyclin A/CDK2 complex activity. The

mechanism of p53 mediated cyclin A/CDK2 inhibition is still under investigation, the complexity of the feedback mechanisms governing p53 activity during G1 & S phase leads to difficulty in fine determination of cause and effect of activation of this pathway (7,344). In response to UV damage during the G1/S phase, increased p53 activity is associated with cell cycle arrest in S-phase. Increased levels of ATM/ATR signaling in response to DNA damage leads to strong checkpoint activation. From canonical pathway activation, this implies a top down activator/effector relationship between signaling kinases and cell cycle effectors like CDK2. However, studies to establish the importance of CDK2 in G1/S transition have shown that p53 and ATM/ATR pathways are activated in response to direct CDK2 inhibition in the absence of external stress (482). This study highlights the importance of understanding the cause and effect relationship of complex signaling pathways in checkpoint activation. Additional in-depth review of the activation mechanism of ATM/ATR and the p53 pathway are covered in the section on DNA damage checkpoint activation. The activation mechanism of p53 in G1/S phase is an area of complex work, which has made it an ideal target for complex mathematical modeling.

Similar to a putative role of p38 at G1/S transition, p38 has also been implicated in the regulation of the intra-S checkpoint. As p38 has been reported to have the ability to phosphorylate and stabilize p53 activity, p38 can be considered to have a putative role in the activation of the intra-S checkpoint. The activity of p38 has been shown to increase in conjunction with stalled replication forks. This increase in activity in correlation with intra-S checkpoint activation is consistent with a role for p38 at the intra-S checkpoint (182,279,480).

1.8.3 The G2-M Checkpoint

The G2 checkpoint is the final checkpoint during interphase, and regulates the entry into mitosis. It is imperative that cells halt the cell-cycle and not enter mitosis with DNA damage. Mitotic spindle separation with damaged DNA often results in catastrophe, resulting in imbalanced chromosomal segregation and the perpetuation of genomic DNA errors (194). The key players in the G2 to M transition are the cyclin dependent kinase CDK1 and its regulatory subunit cyclin-B. For CDK1/Cyclin-B complex to be active, an inhibitory phosphorylation on tyrosine 15 must be removed by the CDC25 class of dual-specificity phosphatases from CDK1 (Cdc2) (241). In mammalian cells, there are 3 isoforms of CDC25, CDC25A,B & C (28). The 3 isoforms of this family of phosphatases have been shown to have both over-lapping and independent functions in the control of cell cycle progression. The main CDC25 phosphatase isoforms that regulate entry into mitosis are the CDC25B and C isoforms with CDC25A primarily involved in the regulation of CDK2/CyclinE, governing the G1-S transition (241,350). The canonical representation of a G2 checkpoint arrest caused by DNA damage is activation of ATM/ATR checkpoint as depicted in figure 1.5 below.

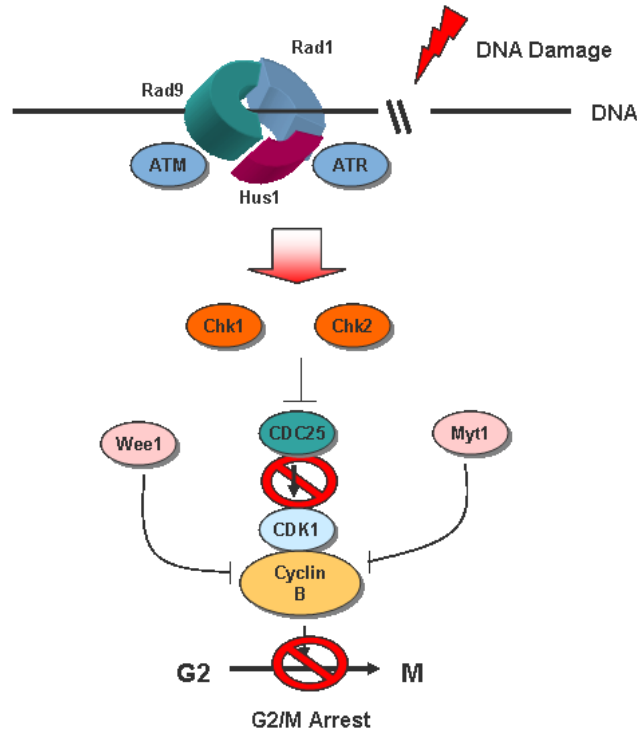


Figure 1.5: *Canonical representation of Chk1 & Chk2 activation in response to DNA damage leading to deactivation of CDK1/CyclinB1 complex leading to G2 checkpoint arrest*

In response to DNA damage, both Chk1 and Chk2 phosphorylate CDC25B and C on multiple residues on their non-catalytic N-terminal domain (190). This phosphorylation at the N-terminal on CDC25 provides a binding site for 14-3-3 proteins, sequesters the CDC25 phosphatase away from the nucleus and leads to eventual ubiquitination. In human cells Chk1 and Chk2 phosphorylate CDC25C at Ser216, leading to cytoplasmic localization and blocking the interaction with CDK1 (17,241,397). This sequestration of CDC25C prevents the removal of the inhibitory phosphorylation by Wee1 and Myt1 on CDK1, leading to a G2-M checkpoint arrest. The active CDK1/CyclinB1 complex is responsible for numerous vital events during mitosis such as nuclear envelope breakdown, centrosome separation, spindle assembly, chromosome condensation and golgi fragmentation (245,304,318,366,412). Hence the

dephosphorylation event of CDK1 by CDC25B/C is a critical event prior to entry into mitosis. The other protein strongly linked to CDC25 and CDK1 activity at the G2-M transition is the Polo-like kinase 1 (PLK1).

Plk1 is found localized to the centrosomes during G2/M phase of the cell cycle, and is activated in concert with CDK1/CyclinB1 during transition from G2 to mitosis (455). This tight correlation has led to conclusions that Plk1 is linked with the CDK1/CyclinB1 complex in driving cells into mitosis. Studies with Topo-II inhibitors have shown that an ATR dependent inhibition of Plk1 kinase activity leads to G2 checkpoint arrest. Furthermore, Plk1 kinase activity has been shown to decrease with ATR and Chk1 dependent activation following DNA damage. These observations serve to associate Plk1 kinase activity with the CDK1/CyclinB1 complex as a cooperative complex whose activation leads to cells entering mitosis (102,455).

1.8.5 Role of p53 in G2-M Transition

The p53 tumor suppressor protein plays a pivotal role in DNA damage checkpoint response, especially at the G1/S transition checkpoint. The role of p53 at the G2 to M transition point however has not been well studied. There is growing evidence that p53 may play a significant role in the control of G2 to M transition. Studies have shown that ATR and Chk1 can both stabilize and activate p53 through phosphorylation of its N-terminal domain. Activated p53 induces transcription of p21, Wee1 and Myt1, all direct or indirect inhibitors of CDK1. The activation of p53 also leads to decreased transcription of CDC25C, the phosphatase required for activation of the CDK1/CyclinB1 complex.

Taken together, activation of p53 transcriptional pathways lead directly to the inhibition of CDK1, leading to the activation of the G2-M checkpoint arrest (14,312,344).

In various publication, in-vitro kinase and phenotypic assays were employed to highlight that p38 could interact with p53 and phosphorylate it on a number of serine sites. These serine sites include ser15, ser33, ser44 and ser389. As phosphorylation at these sites are known to induce p53 protein stability and loss of inhibitory binding of p53 to MDM2, these studies suggest that p38 is an important upstream kinase capable of activating p53 in response to DNA damage (37,51,56,313).

1.8.4 Role of p38 in G2-M Transition

The p38 MAPK is one of the key responders to environmental stress, its role in inflammatory response and response to bacterial invasion have already been discussed in this review. Seeing how p38 plays a critical role in general stress response, there has been increasing interest in understanding if p38 may also play a role in response to DNA damage and perhaps play an important role in cell cycle control (3,27,230).

A number of key publications in the last 8 year implicate p38 as a key component of various DNA damage checkpoints. Initial reports began to implicate p38 in response to environmental damage including UV damage and ionizing radiation (38,163,320,364,396). The initial basis of this, was due to the fact that p38 activity could be induced rapidly in response to environmental and chemical damage to mammalian cells. Further to the rapid activation, strong chemical inhibition of p38 has resulted in significant biochemical and phenotypic changes. Taken together, these observations

strongly implicated p38 as a key responder to environmental and chemical induced DNA damage.

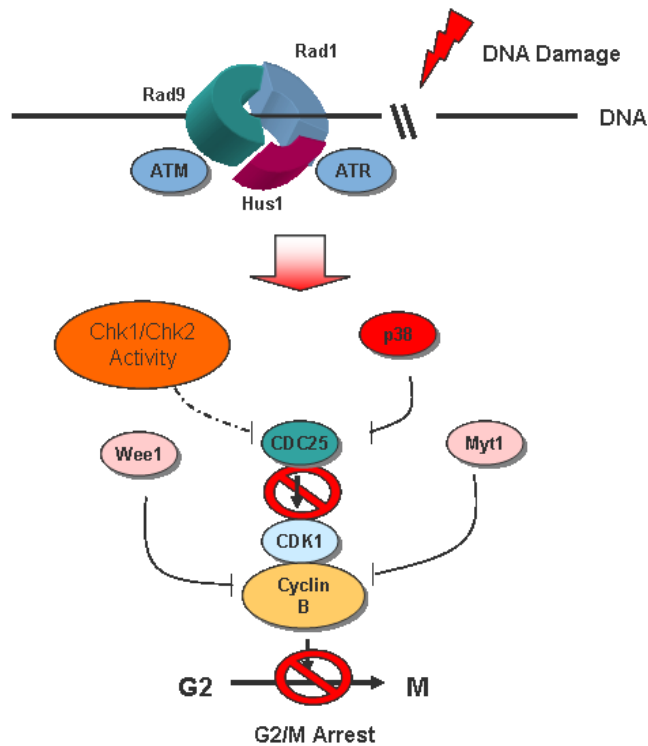


Figure 1.6: *Putative new role for p38 at G2 checkpoint, acting through direct regulation of CDC25B phosphatases*

The two major pathways that are implicated as being impinged by p38 signaling are the p53 tumor suppressor pathway at the G1/S restriction point and the CDC25 phosphatases at G2 checkpoint. Both p53 and members of CDC25 family of protein phosphatases are believed to be regulated through direct interaction with p38 or indirect phosphorylation through its direct downstream substrate MK2 (38,163,191,356,483). At the G2 to M transition checkpoint, a complex network of positive and negative regulators of CDK1 controls the entry into mitosis. Recent work by numerous authors has suggested that the CDC25B and CDC25C isoforms are phosphorylated and activated by p38 or its downstream kinase MK2. Activation of CDC25 family of phosphatases facilitates the

release of inhibitory phosphorylation of CDK1 at tyr14 and tyr15. As mentioned in a previous section, the loss of phosphorylation at these two sites enables CDK1 to bind to cyclinB1 to form the active cyclinB/CDK1 complex. The line of evidence linking p38 to the CDC25 family of phosphatases consists of both biochemical and structural data. Immunoprecipitation pull-down of p38 on DNA damage treated samples were found to be positive for CDC25B (230,254). Furthermore, chemical inhibition of p38 with high concentrations of SB203580 (10 μ M and above) have shown phenotypic effects including increased ph-Histone H3 levels, suggesting that under those conditions, unsynchronized cells enter mitosis with the inhibition of p38 (36,51,220,254,313). It has also been reported that p38 is activated in unperturbed entry into mitosis (48).

While the volume of articles implicating p38 in G2 DNA damage checkpoint control is growing, there are still aspects of p38's role at G2 cell cycle checkpoint that warrant a more in-depth analysis. A recent publication by Mikhailov et al (267), has reported that the p38 MAPK pathway has no function at the G2 cell cycle checkpoint in transformed cancer cells. This publication and other aspects of the prior body of literature warrant a revisit of p38's role in the establishment and control of the G2 cell cycle checkpoint in response to DNA damage. This thesis will focus on exploring p38's role in response to DNA damage at G2 in a cell-cycle centric manner, employing a combination of chemical biology and standard molecular biology techniques.

1.85 Transgenic Mouse Models of p38

In addition to traditional *in-vitro* human and mouse model systems, there have been a number of studies that have utilized transgenic p38 knock-in or conditional knock-out

systems to study *in-vivo* the role of p38 in G2 checkpoint arrest and in general stress response (168).

Studies by Bulavin et al in transgenic mouse models have shown a role for p38 MAPK pathway activity in the regulation of G2 checkpoint in response to a number of stresses including DNA damage (80).

1.9 The Induction of Apoptosis in response to DNA damage

The cellular response to DNA damage consists of at least three distinct responses, the first response is to halt the cell cycle to prevent the perpetuation of any damage to the DNA. Concurrent to the halt of the cell cycle, the DNA damage repair pathway assesses the damage to the genome and attempts to repair the damage. DNA damage repair consists principally of two major mechanisms, the first being nucleotide excision repair or base excision repair. The second major mechanism of DNA damage repair consists of non-homologous end re-joining. As the molecular mechanism of DNA damage repair is not the focus of this thesis, we will not be reviewing the underlying signaling pathways leading to DNA damage repair. Instead we point readers to a number of recent reviews that describe in detail the pathways and components responsible for the repair of DNA damage.

1.9.1 The Canonical Apoptosis Pathway

If damage to the cell is too severe, the 3rd major response to DNA damage is triggered. If the damage to the cell is too severe to be repaired or tolerated, the cellular mechanism for programmed cell death is triggered. It is commonly believed that this cellular self-destruct mechanism is a physiologically necessary response in order to

preserve the overall integrity of a multi-cellular organism. Apoptosis or programmed cell death is an elaborate biological mechanism that helps maintain cellular homeostasis in mammalian organisms (158). It is a biological process that functions to ensure correct developmental and growth in embryonic and early development. It is also used by the immune system to maintain cellular systems in an orderly fashion, by targeting cells with abnormal phenotypes for cell death. Escape from apoptosis is considered a hallmark of cancer by Weinberg et al, cancer cells develop mechanisms to escape extrinsic induction of cell death by the immune system (138).

The programmed cell death or apoptosis pathway has two canonical pathways of activation. There is the extrinsic apoptosis pathway that relays signals from cell surface receptors such as Fas-receptor to the intracellular caspase protein network, which is responsible for execution of the apoptosis signal (94). The extrinsic apoptosis pathway relies on the activation of a number of cell surface receptors, including the TNF-receptor superfamily, and the Fas/death domain receptor family of receptors. These cell surface receptors trigger the canonical extrinsic apoptosis pathway. The extrinsic apoptosis pathway is often used by the immune system in mammalian organisms to stimulate unhealthy cells to die through programmed cell death (158).

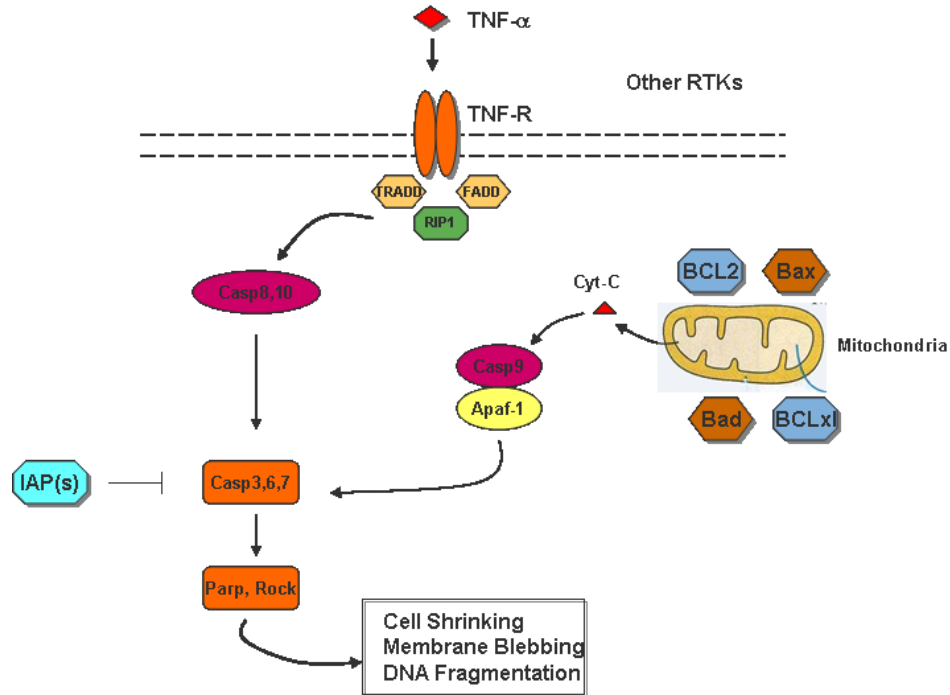


Figure 1.7: Canonical Apoptosis Pathway: Activation of apoptosis from both extrinsic and intrinsic apoptosis pathways

The central molecular players in the induction of cell death are the caspase proteins. The caspase proteins are divided into two functional classes, the initiator caspases and the executioner caspases. The caspase family of proteins is a number of closely conserved cysteine peptidases, many of which share a common CARD or caspase caspase recruitment domain motif (118). Caspases in normal unperturbed cells are found in an inactive pro-form. All caspases undergo proteolytic cleavage for activation. For the extrinsic apoptosis pathway, the initiator caspases, casp8 and casp10 are recruited to the intracellular domain of the death receptors such as Fas-Receptor. This forms the death inducing signaling complex (DISC) which comprises FADD and other adaptor molecules that lead to the sequential catalytic activation of casp8 and casp10. Initiation of casp8 or casp10 leads to the activation of the down-stream intracellular caspase pathway, which ultimately leads to the catalytic activation of the executioner caspases, casp3, casp6 and

casp7. One of the immediate downstream substrates of activated casp3 or casp7 is parp, a pro-survival protein. Catalytic cleavage by casp3 or casp7 leads to the deactivation of parp's pro-survival function and ultimately leads to cell death. Parp-cleavage is a commonly used biochemical marker of apoptosis induction and is often referred to in this thesis as we study p38's potential role in the regulation of apoptosis induction (118,367,430).

The second major activation pathway for apoptosis is commonly referred to as the intrinsic or mitochondria apoptosis pathway and does not normally rely on external signals for initiation of apoptosis. The main cellular organelle responsible for intrinsic apoptosis signaling is the mitochondria. Central to the activation of mitochondria dependent apoptosis is the permeabilization of the mitochondrial membrane that allows the escape of several pro-apoptosis factors including cytochrome c. Cytochrome C is the critical trigger factor for the conformational activation of apoptosis inducing factor (APAF-1). APAF-1 subsequently binds to and oligomerizes pro-caspase9, which undergoes proteolytic cleavage to activate. The formation of a cytochrome c, APAF-1 and cleaved-caspase9 complex results in the formation of the mitochondrial apoptosis initiation complex known as the apoptosome (169).

The mitochondrial sensitivity to apoptosis is regulated closely by the BCL2 (B-Cell leukemia/lymphoma 2) family of pro and anti-apoptotic proteins. These proteins share common protein homology, in conserved BCL2 homology (BH) domains. The anti-apoptotic members of the BCL2 family include the BCL2 and the BCL-XL proteins. These two proteins show high degree of conservation across all 4 BH domains. The pro-apoptotic members of the BCL2 family include the Bax protein (BCL2 associated X-

protein) and the Bak protein (BCL2 antagonistic killer). These proteins show conservation in the BH1 to BH3 domains. The anti-apoptotic members of the BCL2 family act to restrain the pro-apoptotic members through direct inhibitory binding. Bax and Bak must disassociate from the inhibitory binding of BCL2 prior to their activation and subsequent permeabilization of the mitochondrial membrane. Subsequent permeabilization of the mitochondrial membrane facilitates the assembly of the apoptosome, culminating in the activation of the executioner caspases, caspase3 and caspase7 (177,317).

Caspases are regulated through a negative feedback mechanism through direct inhibitory proteins known as inhibitors of apoptosis proteins or IAPs. These IAPs represent an integral checkpoint in the activation of programmed cell death. IAPs regulate the execution of the apoptosis signal through direct binding and suppression of active caspase activity. IAPs hence have the ability to halt an active apoptosis pathway, and the signaling pathways leading to the deactivation of IAP regulation is an area of intense study. IAP pathways are exploited by cancer cells to mitigate the effect of apoptosis signaling. Over-expression and significant copy number gains of IAPs such as Survivin and XIAP have been reported in a large number of solid tumors. Abnormal inhibition of pro-apoptotic BCL2 members has also been attributed to selective pressure associated with tumor formation (15,381).

The importance of p53 in the proper functioning of mitochondrial apoptosis has been revealed through numerous knock-out and tumorigenic model studies (50,173). The p53 protein is considered one of the essential mediators of DNA damage induced apoptosis induction. The p53 protein functions primarily as a transcription activator, upon

separation from its primary inhibitory protein MDM2, p53 acts to transactivate the transcription of numerous pro-apoptotic protein transcripts. The central role of p53 as a tumor suppressor has resulted in the tumor selection pressure to inactivate p53 by a combination of genetic (truncating nonsense) mutations, copy number deletion and over expression of its direct regulator MDM2 (HDM2 in human cells). As a result, p53 is genetically inactivated in over 50% of tumors and is often functionally inactivated through over-expression of its negative regulators in the remainder of tumors.

1.9.2 The role of p38 in Regulation of Apoptosis

The role of p38 MAPK in the regulation of apoptosis induction has been an area of interest for many years. The p38 MAPK signaling pathway is one of the principal responders to external stress inducing elements including TNF- α and LPS. The connection between activation of p38 and inflammatory pathway signaling, led to the studies implicating p38 as a key responder to TNF- α and Fas-L induced cell death signaling (126,128,238,295,300).

The Mammalian immune system acts as a surveillance mechanism against abnormal growth phenotypes, and the principal means to deal with cells exhibiting such properties is the activation of the extrinsic apoptosis pathway via the death receptor signaling pathway. The primary ligands used for this purpose include TNF- α and Fas-L. The key signaling pathways instigated by TNF- α treatment include the NF κ B pathway and the 3 major MAPK pathways including p38. Studies involving p38 gene knock-out and siRNA gene knock-down have revealed that in these physiological conditions, p38 is essential for the effective activation of caspase3 and apoptosis (26,126,435).

Furthermore, p38's role in activation of AP1 family of transcription factors leads to a positive feedback loop between p38 activity and increased TNF- α production. In this sense, p38 is a central positive activator and amplifier of TNF- α induced death signaling. Studies have also found an association between TNF α and Fas-L induced apoptosis and p38 mediated modulation of BCL2 anti-apoptotic proteins. In response to TNF α stimulation, studies have shown that inhibition of p38 leads to a decrease in BCL-xl protein levels and increased expression of Bax protein (185,195,293). These studies suggest that p38 activity is directly connected to extrinsic apoptosis pathway stimulation due to suppression of anti-apoptotic factors and increased expression of pro-apoptotic factors. As death receptor mediated apoptosis is tightly connected to the immune system and to the production of inflammatory cytokine production, p38 MAPK's association with these phenomena is in-line with p38's role in inflammation response.

Numerous studies have also reported that p38 plays an active role in the induction of apoptosis in mitochondrial dependent apoptosis. Studies have shown that p38 either directly or indirectly is able phosphorylate p53 on a number of key serine residues including ser33 and ser46. Phosphorylation on these residues is associated with stabilization and activation of p53 in response to stress and cell damage. Through its regulation of p53 activity, p38 is implicated as an activating kinase of mitochondrial dependent apoptosis in response to hypoxia, nitric oxide and DNA damage (31,62,179,198,469). Many of these studies have focused on p38 activity being necessary in a p53 wild-type environment, where p38 activates a p53 dependent apoptosis pathway (31,62,77,226,435). In addition to its role in regulation of p53 dependent apoptosis, studies have highlighted p38's interaction with the BCL2 family of pro and anti-apoptotic

proteins to induce apoptosis in conjunction with hypoxia, UV and other environmental stresses (111,123,293,375,413). These studies implicate p38 in apoptosis induction through a p53 independent, but BCL2 dependent mechanism. These studies suggest that in line with p38's role as a central responder to external shocks and stress, p38 activity is tied directly to apoptosis induction in both normal and transformed cells. The majority of the evidence for p38's role in positive induction of apoptosis has been derived from normal or untransformed cells, where the activation of death domain receptors is a primary mechanism for p38 induced cell death. Studies in tumor cells have focused on the loss of sensitivity of cells to death inducing stimuli such as FasL upon inhibition of p38.

The role of p38 in regulation of apoptosis does not stop as a simple mediator of death domain receptor induced apoptosis. While high activity of p38 in response to extrinsic apoptotic signaling via the death receptors is associated with significantly increased cell death, there are an increasing number of studies that have implicated p38 activity as cytoprotective, especially in tumor cells (44,93,136,235,239,278,328,405). Numerous studies have found that in certain cellular contexts, inhibition of p38 significantly increases apoptotic potential in tumor cells. This suggests that loss of p38 activity drives cells towards an apoptotic phenotype. It has been suggested that the transactivation potential of p38 in response to inflammatory cytokine induction has been co-opted by tumor cells to promote increased cell survival and to perpetuate an inflamed micro-environment, suitable for increased angiogenesis and tumor development (414). It is in this context that many p38 inhibitors have been developed with the hopes of reversing angiogenic factors sustaining a tumor. A second context that many researchers

have found inhibition of p38 as a potential useful therapeutic strategy is in combination with genotoxic agents. In numerous cancer cell models, researchers have found that inhibition of p38 with SB203580, synergizes well with numerous chemotherapeutic agents. These observations lead to the conclusion that in certain physiological contexts in tumors, p38 activity does not behave as a pro-apoptotic factor but as a pro-survival factor. The studies that suggest p38 acts as in a cyto-protective role, were often performed in conjunction with DNA damaging alkylating, methylating agents or microtubule/spindle poisons (248,323,474). It is possible that these genotoxic agents do not impinge on the death domain receptor signaling pathway and instead evoke a cellular survival response where p38 switches from a death promoting kinase to a survival promoting kinase. Some evidence that p38 may impact pro-survival pathways include its ability to phosphorylate and activate ph-CREB (79,420). CREB signaling is often implicated in pro-survival settings, and there have been numerous reports of tumors using CREB related pro-survival pathways to escape genotoxic agents such as temozolomide in glioblastoma. (315)

This conflicting evidence of p38's role in the regulation of apoptosis may at first seem contradictory. There are however many examples of proteins having dual and at times opposing functions in biology (e.g. TGF β and NF κ B), where the cellular and physiological context of a protein's activity may need to be considered when determining its function.

1.10 Aims of this Study

The primary aim of this study is to investigate the role of p38 MAPK in DNA damage response. Specifically we aim to understand if p38 activity is required for the establishment and maintenance of a G2 cell cycle checkpoint arrest in response to DNA damage in cancer cells.

In addition to DNA damage induced G2 checkpoint regulation, we will also explore if p38 activity plays a role in other physiological processes in response to DNA damage in cancer cells. Lastly we seek to understand if there are mechanistic similarities between p38's role in DNA damage response and its principal role in inflammatory stress response.

To answer these questions, we will employ a cell-cycle centric approach using a specific p38 inhibitor (LY479754) as well as RNAi technology (siRNAs & shRNAs) to specifically test the effect of p38 pathway inhibition on the ability of cancer cells to respond to DNA damage and TNF- α inflammatory stress.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Laboratory Chemicals and Biochemicals

S/No.	Chemical	Catalog Number	Company
1	Adriamycin/Doxyrubicin	D1515-10MG	Sigma Aldrich
2	Anisomycin	10522-25MG	Sigma Aldrich
3	Aphidicolin	A0781	Sigma Aldrich
4	Aprotinin	A6103	Sigma Aldrich
5	Benzamidine	12072	Sigma Aldrich
6	Beta Gly PO ₄	G6251	Sigma Aldrich
7	Bovine Albumin Fraction V Solution (7.5%)	15260-037	Invitrogen/Gibco
8	Caffeine	C8960	Sigma Aldrich
9	Complete, Mini, EDTA Free	11836170001	Sigma Aldrich
10	DMSO (Dimethyl sulfoxide)	D8418	Sigma Aldrich
11	DTT (Dithiothreitol)	646563	Sigma Aldrich
12	EDTA (Ethylenediaminetetraacetic acid)	E6758	Sigma Aldrich
13	HEPES ((N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	15630-130	Invitrogen/Gibco
14	Leupeptin	L2884	Sigma Aldrich
15	L-Glutamine	21051-024	Invitrogen/Gibco
16	Lipofectamine™ 2000	11668-019	Invitrogen/Gibco
17	Lipofectamine™ RNAiMAX	13778-150	Invitrogen/Gibco
18	MMS (methyl methanesulfonate)	129925	Sigma Aldrich
19	Nocodazole	M1404	Sigma Aldrich
20	Okadaic Acid	O7885	Sigma Aldrich
21	PMSF (Phenylmethanesulfonyl fluoride)	P7626	Sigma Aldrich
22	PNPP (4-Nitrophenyl phosphate di(tris) salt)	N3254	Sigma Aldrich
23	Ponceau S	P3504	Sigma Aldrich
24	Sodium azide	08591	Sigma Aldrich
25	Sodium Chloride (NaCl)	S7653	Sigma Aldrich
26	Sodium Fluoride	S7920	Sigma Aldrich
27	Sodium metavanadate (NaVO ₃)	289361	Sigma Aldrich
28	Sodium persulfate	3690	Sigma Aldrich
29	Sodium Pyrophosphate Dibasic	71501	Sigma Aldrich
30	Sodium pyrophosphate dibasic (Na ₂ H ₂ P ₂ O ₇)	71501	Sigma Aldrich
31	TAME (N α -p-Tosyl-L-arginine methyl ester hydrochloride)	T4626	Sigma Aldrich
32	Thymidine	T1895-5G	Sigma Aldrich
33	TNF-a	T0157-10UG	Sigma Aldrich
34	TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone)	T4376	Sigma Aldrich
35	Tris pH7.5	42029	Sigma Aldrich
36	Tris, ph7.5 (Trizma)	T1503	Sigma Aldrich
37	Triton-X100	234729	Sigma Aldrich
38	Trypsin-chymotrypsin inhibitor	T9777	Sigma Aldrich

Table 2.1: Table of Laboratory chemicals and biochemicals

2.1.2 Kits and commercially available buffers and systems

we used the following commercially available products for assays run during this study. We used the Invitrogen™ X-Lock mini-gel system as the primary western blotting module. We used the Novex pre-cast gel system as the primary gel system for protein electrophoresis.

S/No.	Name/Description	Catalog Number	Company
1	10X PBS Stock, pH 7.4	75889	USB
2	20X TBS Stock, pH 7.4	75892	USB
3	20X TBST Stock, pH 7.4	77500	USB
4	BioRad RC DC Protein Assay	500-0120	BioRad
5	Caspase-Glo® 3/7 Assay	G8090	Promega
6	Complete, Mini, EDTA Free	11836170001	Roche
7	ECL Advance Western Blotting Detection Kit	RPN2135	Amersham
8	ECL Plus Western Blotting Detection Kit	RPN2132	Amersham
9	Halt phosphatase inhibitor Cocktail	78420	Pierce
10	Laemmli Sample Buffer	161-0737	BioRad
11	Lipofectamine™ RNAiMAX	13778-075	Invitrogen
12	MagicMark XP Protein Ladder	LC5602	Invitrogen
13	Novex® 4-20% Tris-Glycine Gel 1.5 mm, 15 well	EC60285BOX	Invitrogen
14	Novex® Tris-Glycine SDS Running Buffer (10X denaturing)	LC2675-4	Invitrogen
15	Novex® Tris-Glycine Transfer Buffer (25X)	LC3675	Invitrogen
16	NuPAGE® 4-12% Bis Tris Gel 1.5 mm, 15 well	NP0336BOX	Invitrogen
17	NuPAGE® Antioxidant	NP0005	Invitrogen
18	NuPAGE® LDS 4X LDS Sample Buffer	NP0008	Invitrogen
19	NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X)	NP0001	Invitrogen
20	NuPAGE® Novex 12% Bis-Tris Gel 1.0 mm, 10 well	NP0341BOX	Invitrogen
21	NuPAGE® Sample Reducing Agent (10X)	NP0004	Invitrogen
22	NuPAGE® Transfer Buffer (20X)	NP0006-1	Invitrogen
23	NuPAGE® Transfer Buffer (20X)	NP0006-1	Invitrogen
24	One-Step RT-PCR Kit	78350	USB
25	PrepEase® RNA Spin Kit	78766	USB
26	QIAquick PCR purification Kit	28104	Qiagen
27	RT-PCR Master Mix (2X)	78370	USB
28	Seeblue Plus2 Protein Ladder	LC5925	Invitrogen
29	XCell SureLock® Mini-Cell and XCell II™ Blot Module Kit CE Mark	EI0002	Invitrogen
30	ZOOM® Dual Power Supply	ZP10002	Invitrogen

Table 2.2: List of Commercial assay kits, buffers and systems

2.1.3 Primary Antibodies

S/No	Antibody Name	Description	Catalog Number	Company/Source
1	ph-p38,t180&y182	Rabbit Polyclonal Antibody targeting p38 phosphorylated on Thr180 & Tyr182	9216	Cell-Signaling Technology (CST)
2	p38 MAPK(5F11),mAB	Mouse monoclonal antibody targeting total p38	9217	Cell-Signaling Technology (CST)
3	p-MK2,T334	Rabbit Polyclonal Antibody targeting MAPKAPK2, phosphorylated at Thr334	3041	Cell-Signaling Technology (CST)
4	p-p53,S37	Rabbit Polyclonal Antibody targeting p53 phosphorylated at ser37	558369	BD
5	p-p53,S15 (16G8), mAB	Mouse Monoclonal (16G8) Antibody targeting p53 phosphorylated at ser15	9286	Cell-Signaling Technology (CST)
6	p-p53,S33	Rabbit Polyclonal Antibody targeting p53 phosphorylated at ser33	2526	Cell-Signaling Technology (CST)
7	HSP27 (G31) Mouse mAb	Mouse Monoclonal (G31) Antibody targeting HSP27 protein	2402	Cell-Signaling Technology (CST)
8	p-HSP27, ser15	A Rabbit Polyclonal Antibody targeting HSP27 phosphorylated at ser15	2404	Cell-Signaling Technology (CST)
9	p16	p16 INK4A Antibody detects endogenous levels of p16 INK4A protein.	4824	Cell-Signaling Technology (CST)
10	Cyclin-B1, mAB	Mouse monoclonal Antibody recognising endogenous levels of Cyclin-B1	554176	Becton Dickinson Biosciences (BD)
11	Cyclin-D1,2,3 mAB	Mouse monoclonal Antibody recognising endogenous levels of Cyclin-D1,2,&3	554203	Becton Dickinson Biosciences (BD)
12	cdc25b	cdc25B Antibody recognizes endogenous levels of total cdc25B protein.	9525	Cell-Signaling Technology (CST)
13	ph-chk-1,ser345	Phospho-Chk1 (Ser345) Antibody detects endogenous levels of Chk1 only when phosphorylated at serine 345.	2341	Cell-Signaling Technology (CST)
14	ph-chk2,thr68	Phospho-Chk2 (Thr68) Antibody detects endogenous levels of Chk2 only when phosphorylated at threonine 68	2661	Cell-Signaling Technology (CST)
15	ph-Chk1,ser 317	Phospho-Chk1 (Ser317) Antibody detects endogenous levels of Chk1 only when phosphorylated at serine 317	A300-163A	Bethyl Labs
16	Cleaved-PARP	Cleaved PARP (Asp214) (19F4) Mouse mAb (Human Specific) detects endogenous levels of the large fragment (89 kDa) of human PARP1 resulting from cleavage at aspartic acid 214	9546L	Cell-Signaling Technology (CST)
17	Cleaved-caspase3	Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175	9661L	Cell-Signaling Technology (CST)
18	b-Actin	β -Actin Antibody detects endogenous levels of β -actin. This antibody may cross-react with the γ -actin (cytoplasmic) isoform. It does not cross-react with α -skeletal, α -cardiac, α -vascular smooth, or γ -enteric smooth muscle isoforms	4967	Cell-Signaling Technology (CST)
19	a/b Tubulin	The α/β -Tubulin Antibody detects endogenous levels of both α - and β -tubulin total protein, and does cross-react with both recombinant α - and β -tubulin	2148	Cell-Signaling Technology (CST)
20	Pericentrin	Rabbit Polyclonal Antibody that detects endogenous levels of peri-centrin protein	ab4448	Abcam
21	Pericentrin	Mouse monoclonal Antibody that detects endogenous levels of peri-centrin protein	ab28144	Abcam
22	Gamma tubulin	Rabbit Polyclonal Antibody that detects endogenous levels of gamma-tubulin protein	ab11317	Abcam
23	p-Histone H3,s10	Rabbit Polyclonal Antibody that detects Histone H3 phosphorylated at ser10	06-570	Millipore (Upstate)
24	p-MPM2	The MPM2 monoclonal antibody binds to a phospho amino acid-containing epitope (peptides containing LTPLK and FTPLQ domains) present on more than 50 proteins of M-phase eukaryotic cells	05-368	Millipore (Upstate)
25	c-Casp7, Asp198	Cleaved Caspase-7 (Asp198) Antibody detects endogenous levels of the large fragment of caspase-7 resulting from cleavage at aspartic acid 198	9491	Cell-Signaling Technology (CST)
26	c-Casp9, Asp330	Cleaved Caspase-9 (Asp330) Antibody (Human Specific) detects endogenous levels of the large fragment (37 kDa with prodomain/17 kDa) of caspase-9 following cleavage at aspartic acid 330	9501	Cell-Signaling Technology (CST)
27	FADD	FADD Antibody (Human Specific) detects endogenous levels of human FADD protein	2782	Cell-Signaling Technology (CST)
28	ph-FADD,ser192	Phospho-FADD (Ser194) Antibody (Human Specific) detects endogenous levels of human FADD protein only when phosphorylated at serine 194	2781	Cell-Signaling Technology (CST)

Table 2.3: Table of primary antibodies

The primary antibodies listed in table 2.4 were used in all primary protein analytical assays including western blotting, acumen high content imaging, FACS (flow cytometry analysis) and immuno-fluorescence and confocal imaging.

Antibody Dilution:

- All antibodies were diluted to manufacturers recommended levels for specific application
- All CST antibodies for western blotting were diluted at 1:1000
- Antibodies were diluted to 1:500 for Acumen High Content Imaging applications unless otherwise specified.

Secondary Antibodies and DNA stains

S/No.	Antibody/Reagent Name	Description	Catalog Number	Company/Source
1	Anti-Rabbit HRP Linked	ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey)	NA934-1ML	Amersham (GE)
2	Anti-Mouse HRP Linked	ECL Mouse IgG, HRP-Linked Whole Ab (from sheep)	NA931-1ML	Amersham (GE)
3	Anti-Rabbit Alexa 488 Linked	Alexa Fluor® 488 goat anti-rabbit IgG (H+L)	A-11008	Invitrogen
4	Anti-Mouse Alexa 488 Linked	Alexa Fluor® 488 goat anti-mouse IgG (H+L) *2 mg/mL*	A-11001	Invitrogen
5	Anti-Rabbit Alexa 568 Linked	Alexa Fluor 568 donkey anti - rabbit IgG	A10042	Invitrogen
6	Anti-Mouse Alexa 568 Linked	Alexa Fluor 568 goat anti - Mouse IgG	A-11004	Invitrogen
7	Anti-Rabbit Alexa 680 Linked	Alexa Fluor 680 donkey anti - rabbit IgG	A10043	Invitrogen
8	Propidium Iodide	propidium iodide - 1.0 mg/mL solution in water	P3566	Invitrogen
9	Hoescht stain	Hoechst 33342, trihydrochloride, trihydrate - 10 mg/mL solution in water	H3570	Invitrogen
10	ProLong Anti-Fade	ProLong® Gold antifade reagent with DAPI	P-36931	Invitrogen

Table 2.4: Table of secondary antibodies and reagents

Antibody Dilution:

- Secondary antibodies for western blotting were diluted at 1:10,000
- Secondary antibodies for Immuno-fluorescence were diluted at 1:1000
- Propidium Iodide and Hoescht stain were diluted at 1:500

HRP linked antibodies were used for western blotting applications as previously described, Alexa488 linked antibodies were used for acumen HCA assays and for FACS applications. Combinations of Alexa488, Alexa568 and/or Alexa680 were used for immuno-fluorescence imaging applications.

DNA staining with propidium iodide was used in both acumen HCA and FACS assays for accurate cell count and determination of DNA content (cell cycle state). DNA counter staining with Hoescht was used in immuno-fluorescence applications for staining of nuclei.

2.1.4 Biochemical Inhibitors

S/No.	Chemical	Description	Company/Source
1	LY479754	Lilly p38 α / β MAPK Inhibitor	Eli Lilly & Co.
2	LY2441693	Lilly MAPKAPK2 Inhibitor	Eli Lilly & Co.
3	LY2494516	Lilly Synthesized copy of Published Pfizer Chk1 Inhibitor (PF-00477736)	Eli Lilly & Co.
4	SB203580	SmithKline Beecham p38 MAPK Inhibitor	Calbiochem
5	RO-3306	Roche CDK1 Inhibitor	Calbiochem

Table 2.5: Table of biochemical inhibitors used in this study

2.1.5 Cell Culture Media and reagents

All cell culture media and additives were purchased from Gibco (Invitrogen).

I. Cell Culture Reagents

S/No.	Name	Description	Catalog Number	Company/Source
1	DMEM Media	Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (4.5 g/L D-glucose)	21063-029	Invitrogen
2	MEM Media	Minimum Essential Medium (MEM) (1X), liquid	11090-073	Invitrogen
3	McCoy's 5A Media	McCoy's 5A Medium (modified) (1X), liquid	12330-031	Invitrogen
4	RPMI-1640 Media	RPMI Medium 1640 (1X), liquid	11879-020	Invitrogen
5	Opti-Mem Media	Opti-MEM [®] I Reduced-Serum Medium (1X), liquid	11058-021	Invitrogen
6	FBS	Fetal Bovine Serum, Certified (USA)	16000-044	Invitrogen
7	FBS (Heat Inactivated)	Fetal Bovine Serum, Certified, Heat-Inactivated (USA)	10082-147	Invitrogen
8	MEM Non-Essential Amino Acids	MEM Non-Essential Amino Acids Solution 10 mM (100X), liquid	11140-050	Invitrogen
9	L-Glutamine	L-Glutamine 200 mM (100X), liquid	25030-024	Invitrogen
10	Sodium Pyruvate	MEM Sodium Pyruvate Solution 100 mM (100X), liquid	11360-070	Invitrogen
11	Penicillin-Streptomycin	Penicillin-Streptomycin, liquid	15070-063	Invitrogen
12	Puromycin	Puromycin is used to select for cell lines that have been transformed by vectors that express puromycin-N-acetyl-transferase (pac),	631305	Clontech

Table 2.6: Cell culture reagents

II. Cell-Lines

S/No.	Name	Description	Catalog Number	Company/Source
1	HeLa	Human Cervical Adenocarcinoma Cell-Line	CCL-2	ATCC
2	A549	Human Lung Carinoma Cell-Line	CCL-185	ATCC
3	Calu6	Human Non-Small Cell Lung Carinoma Cell-Line	HTB-56	ATCC
4	U2OS	Human Osteosarcoma Cell-Line	HTB-96	ATCC
5	HeLa, MK2 ^{-/-}	Stable Transfected sh-MK2(Lentiviral) in HeLa Cells,	NA	LRL (Indianapolis IN)
6	HeLa, GFP ^{-/-}	Stable Transfected sh-GFP (Lentiviral) in HeLa Cells,	NA	LRL (Indianapolis IN)

Table 2.7: Cell-Line Models used in this study

III. Growth media conditions for Cell-Lines:

1. HeLa:

- DMEM Media
- 10% FBS

2. A549:

- RPMI-1640 Media
- 10% FBS
- 1% Sodium Pyruvate

3. Calu6:

- MEM Media
- 10% FBS
- 1% Sodium Pyruvate
- 1% MEM non-essential Amino-Acids
- 1% L-Glutamine

4. U2OS:

- McCoy-5A Media

- 10% FBS
5. HeLa shMK2^{-/-} or shGFP^{-/-}:
- DMEM Media
 - 10% FBS
 - 1% Sodium Pyruvate
 - 1% Puromycin

2.1.6 siRNA Oligonucleotides

Small interfering 21-nucleotide RNA duplexes (siRNAs) were purchased from Qiagen sciences. The siRNA duplexes used in this study are listed in the table below. For every endogenous gene target, a minimum of 2 independent siRNA duplexes was used to confirm gene target knock-down.

S/No.	Target Gene	Figure Legend Name	Reagent Description	Catalog Number	Company/Source
1	p38 MAPK (MAPK14)	p38-1	Hs_MAPK14_5 HP Validated siRNA (NM_001315, NM_139012, NM_139013, NM_139014)	SI00300769	Qiagen Sciences
2	p38 MAPK (MAPK14)	p38-2	Hs_MAPK14_6 HP Validated siRNA (NM_001315, NM_139012, NM_139013, NM_139014)	SI00605157	Qiagen Sciences
3	MK2 (MAPKAPK2)	MK2-1	Hs_MAPKAPK2_5 HP Validated siRNA (NM_004759, NM_032960)	SI02223697	Qiagen Sciences
4	MK2 (MAPKAPK2)	MK2-2	Hs_MAPKAPK2_6 HP Validated siRNA (NM_004759, NM_032960)	SI00288246	Qiagen Sciences
5	Chk1 (Chek-1)	Chk1-1	Hs_CHEK1_13 HP Validated siRNA (NM_001114121, NM_001114122, NM_001274)	SI02660007	Qiagen Sciences
6	Chk1 (Chek-1)	Chk1-2	Hs_CHEK1_7 HP Validated siRNA (NM_001114121, NM_001114122, NM_001274)	SI00299859	Qiagen Sciences
7	Chk2 (Chek2)	Chk2-1	Hs_CHEK2_10 HP Validated siRNA (NM_001005735, NM_007194)	SI02224271	Qiagen Sciences
8	Chk2 (Chek2)	Chk2-2	Hs_CHEK2_14 HP Validated siRNA (NM_001005735, NM_007194, NM_145862)	SI02655422	Qiagen Sciences
9	Rb1	Rb-1	Hs_RB1_5 HP Validated siRNA (NM_000321)	SI00301651	Qiagen Sciences
10	Rb1	Rb-2	Hs_RB1_7 HP Validated siRNA (NM_000321)	SI02653819	Qiagen Sciences

Table 2.8: Table of siRNA duplex reagents used in this study

2.1.7 Buffers

1. Modified RIPA Buffer for cell lysis
 - RIPA Buffer, (Santa Cruz Biotechnology cat: sc-24948)
 - 1X Complete-EDTA protease inhibitor cocktail (Roche, cat:)
 - 1X Halt Phosphatase Inhibitor Cocktail
 - 10mM Sodium pyrophosphate

- 2mM Sodium orthovanadate
 - 10mM Sodium Fluoride
 - 1mM phenylmethylsulfonyl fluoride
 - 0.5 ug/ml Okadaic acid
2. Loading Buffer (Western Blotting)
 - 4X LDS Loading Buffer
 - 1X Sample Reducing Buffer (DTT)
 - Final concentration of 1ug/ml protein lysate, dilution in modified RIPA Buffer
 3. 2X Prefer Fixative Buffer
 - Prefer fixative diluted from 16X Prefer Stock (Anatech Ltd, MI USA) to 2X final
 - 100% ethanol diluted to 2X ethanol (40%) final
 - 1X PBS
 4. 1X Prefer Fixative Buffer
 - Prefer fixative diluted from 16X Prefer Stock (Anatech Ltd, MI USA) to 1X final
 - 100% ethanol diluted to 1X ethanol final
 - 1X PBS
 5. 4% Paraformaldehyde (PFM) Fixative Buffer
 - 16% PFM stock diluted to 4% PFM in PBS
 6. 1X Wash Buffer (Western Blotting)
 - 1X TBS-Tween
 7. 1X Blocking Buffer (Western Blotting)
 - 2% ECL blocking reagent in TBS

8. 1X Primary Antibody Buffer (Western Blotting)
 - 5% BSA in 1X TBS-Tween
 - 0.01% Sodium Azide
9. 1X Secondary antibody Buffer (Western Blotting)
 - 2% ECL Blocking reagent in 1X TBS-Tween
 - Or 5% low-fat milk in 1X TBS-Tween
10. 1X Stripping Buffer (Western Blotting)
 - 1X Restore Plus Strip Buffer (Pierce, Cat No. 21059)
11. 1X Wash Buffer (Acumen or FACS)
 - 1X PBS
12. 0.1% Triton-X 100 Buffer (Permeabilization buffer, Acumen)
 - Triton-X 100 diluted to 0.1% in PBS
13. 1X Antibody/Blocking Buffer (Acumen or FACS)
 - 1% BSA in 1X PBS-Tween
 - 0.01% Sodium Azide
14. 1X Antibody/Blocking Buffer (Confocal)
 - 2% BSA in 1X PBS-Tween

2.1.8 Analytical Instruments and Systems

The following analytical instruments and systems were used during the course of this study.

S/No.	Name	Description	Company
1	Acumen Explorer	TTP Labtech Acumen Explorer Microplate Cytometer	TTP Labtech Ltd, Cambridge UK
2	BD LSR II System FACS	The BD LSR II can be configured with up to four fixed alignment lasers and the ability to detect up to 18 colors, utilizing a revolutionary optical design. The BD LSR II can be configured with a 488 nm, 405 nm, 633 nm, and 355nm UV laser.	BD Biosciences, NJ USA
3	Bio-Rad MRC600	Bio-Rad MRC 600 Laser Scanning Confocal Microscope	Bio-Rad Ltd, Hercules CA USA
4	ImageQuant RT-ECL	ImageQuant™ RT ECL™ system is designed as a general-purpose, fully featured imager, but with particular focus on high-sensitivity chemiluminescence imaging.	GE Health Care Life Sciences
5	Safire 2 Plate Reader	Tecan Safire Fluorescence, Absorbance and Luminescence Reader	Tecan Trading AG, Switzerland
6	SpectraMax M5	SpectraMax M5 is a dual monochromatic, multi-detection microplate reader with a triple mode curvette port and a 6-384 microplate reading capability	Molecular Devices Ltd

Table 2.9: Table of Analytical Instruments and Systems used in this study

2.1.9 Computational Programs and Tools

All statistical modeling and analysis used in this study were carried out using the R-Statistical analysis tool (www.R-Project.org) with associated Bioconductor modules (www.bioconductor.org) or author generated custom code and scripts. Spreadsheet and tables were generated using the Microsoft Excel program.

Genomic and gene annotation was derived from Ensembl's BioMart database based on Ensembl build 38 reference data. Ensembl build 38 uses the current NCBIv36 human genome build. Access to Ensembl's BioMart was based on pre-written R and perl based APIs.

Functional pathway annotation was accomplished using the web-based NIH DAVID tool (<http://david.abcc.ncifcrf.gov/>) and canonical pathway and gene network analysis was done using the Ingenuity Pathway Analysis (IPA) software program. All

author created images were produced using Microsoft Powerpoint, the freeware software tool GIMP[®] or Paint.Net.

Dynamic modeling and simulation was carried out using the Matlab[®] software suite, with user written simulation code. Kinetic constants for pathway simulation were derived from Literature survey or from public kinetic pathway databases.

II. Methods in Molecular Biology

2.2 Methods in Mammalian Cell Culture

2.2.1 General Cell Culture

Cell lines were grown in a humidified 95% air, and 5% CO₂ environment in a cell culture incubator. Cells were grown in Falcon T75 or T150 flasks and passaged at regular intervals when cells reached approximately 80% confluence. The cell growth media conditions for the cell-lines were described in section 2.1.5 of this chapter.

Cells to be passaged upon reaching 80% confluence, were first washed once with 10ml 1X warm PBS. This was followed by 1ml trypsin-EDTA solution which was allowed to sit for 1 minute prior to being aspirated out. Cells were then placed back in the incubator for 5mins to allow cells to detach from the surface of the flask. Cells were then washed with warm growth media and counted using a nucleo-counter. New flasks were seeded with approximately 0.5×10^6 cells and placed back in the incubator to grow.

2.2.2 Thymidine Block/Release Synchronization

Cells were synchronized using a double thymidine block/release cycle technique. The result of a successful double thymidine block/release cycle was the homogenous synchronization of cells at the G1/S boundary upon the 2nd release from thymidine block.

The protocols for synchronization of cells with excess amounts of thymidine are well established and understood (231,451). The general protocol used is as follows.

1. Cells were treated with 3mM Thymidine for 12 hours
2. Wash out thymidine 2x with warm growth media
3. Trypsinize and replate at 30% cell density, incubate for 16hours
4. Replace growth media with 3mM Thymidine for 12-14 hours.
5. Wash out thymidine 2x with warm growth media.
6. Check that cells are at G1/S boundary.

2.2.3 CDK1 Block/Release Synchronization

Cells were synchronized using a CDK1 inhibitor block/release technique. Cells synchronized with a CDK1 inhibitor arrest at the G2 to M boundary and upon release transition into mitosis. The protocol used was similar to what was previously described (423), using the commercially available Roche CDK1 inhibitor RO-3306. The essential steps for this method are as follows:

1. Cells grown in 6-well dishes are incubated with 9uM RO-3306 for 21 hours
2. CDK1 inhibitor (RO-3306) is washed out 3x with warm growth media.
3. Cells are incubated with growth media and watched for entry into mitosis

2.2.4 RNA Interference

Transfection of 21-nucleotide siRNA duplexes (Qiagen Sciences, Germantown MD,USA) for targeting endogenous genes was carried out using Lipofectamine RNAimax (Invitrogen, Carlsbad CA, USA) and 5ug siRNA duplex per well as previously described in low serum (Opti-mem) media (467).

Transfection occurs for 48hours following which transfection efficiency is assayed using RT-PCR or western blotting assays. High efficiency of gene silencing was achieved using Qiagen validated siRNA duplexes, where experimental validation for gene silencing is performed by manufacturer. For every gene knock-down in this study, 2 independent siRNA duplexes was used to confirm gene knock-down effect.

2.2.5 Biochemical compounds

The use of biochemical inhibitors and chemical genotoxic compounds in this study was performed as previously described (467). Biochemical inhibitors and compounds were diluted to final concentrations from 100uM working stock in 100% DMSO. Working stock was derived from 10mM master stock solution in 100% DMSO. All biochemical inhibitors and compounds were stored at -20°C.

Chemical inhibitors and genotoxic compounds were diluted to final concentration in whole growth media just prior to addition to 6-well or 96 well dishes for assay. DMSO was matched by volume in the control samples as appropriate DMSO control in all assays.

2.2.6 Cell Seeding Density for various assay plates

The cell seeding density and plate type for various assays is described in the table below.

S/No.	Name	Description	Cell Seeding Density	Company/Source	Assays
1	6-Well Plate	Multi-well cell culture plates with CellBIND® surface, 6 well	0.5x10 ⁶ cells/ml, 2ml per well	Corning	Western Blotting, RT-PCR, FACS
2	96-Well Plate (No-Poly-lysine)	BD Optilux 96-well Microplates, tissue-culture treated, flat-bottom, with lid	3x10 ⁴ cells/ml, 100ul per well	BD Falcon	Acumen HCA
3	96-Well Plate (Coated w/Poly-lysine)	BD Optilux 96-well Microplates, black/clear, tissue-culture treated polystyrene, flat bottom, with lid	3x10 ⁴ cells/ml, 100ul per well	BD Falcon	Acumen HCA

Table 2.10: Cell seeding density for assay plates

2.3 Protein Analytical Techniques

2.3.1 Lysis of cells with custom/modified RIPA buffer

Prior to lysis, cells were grown to 80% confluence and were treated with biochemical inhibitors, chemical agonists, or UV irradiation as per figure legend. Cells were washed with ice cold PBS and then lysed for 10mins on ice with custom/modified RIPA buffer. Lysates were precleared by centrifugation at 13000 rpm for 10mins at 4°C.

2.3.2 Determination of protein concentrations in cell lysates

Protein concentration in cell lysates was determined using the BioRad RC DC colorimetric protein assay in 96-well micro-titer format. Protein levels were read using a spectramax spectrophotometer plate reader.

2.3.3 SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as previously described (422) using the Invitrogen X-Lock system. Invitrogen NuPage Bis-Tris or Tris-Glycine gels were used for all SDS-PAGE applications. All SDS-PAGE gels were run as per manufacturer's instruction. Two protein ladders were incorporated for all gels:

1. MagicMark XP (HRP ladder)
2. SeeBlue Plus 2 (Visible Ladder)

2.3.4 Transfer of proteins onto nitrocellulose membranes

For immunoblot (western blotting) analysis, proteins were transferred onto nitrocellulose membranes as previously described (467). Transfer was performed using a

“semi-dry” X-Lock transfer device by Invitrogen. Transfer of denatured proteins, was performed using manufacturers recommended transfer buffer containing 20% methanol at recommended voltage and current for 1 hour. Following transfer of protein onto nitrocellulose membrane, membranes were stained with Ponceau S reversible protein stain to ascertain efficient protein transfer.

2.3.5 Immunblot Detection

After electroblotting, the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked with either 5% low-fat milk or 2% ECL advance blocking reagent (Amersham) for at least 1 hour at room temperature. The membrane was then probed with a primary antibody diluted to a final concentration of 1:500 to 1:1000 in antibody buffer and incubated overnight at 4°C. The membrane was washed 3x 10mins in 1X wash buffer on a rocker and then incubated with secondary HRP-linked antibody diluted to 1:10,000 in secondary antibody buffer for 1hour at room temperature. Following secondary antibody incubation, membranes were washed in 1X wash buffer (Western Blotting) on a rocker for 3x 30mins at room temperature.

Luminescent substrate detection was performed using the ECL Advance or ECL plus chemi-luminescent kit (Amersham). Chemi-luminescent signal was detected using a high resolution GE Gel-blot imager.

Membranes were stripped of bound antibodies by incubating for 1hour in 1X stripping buffer at 50°C. Stripped membranes were re-blocked in 1X blocking buffer and re-probed with a different primary antibody.

2.3.6 Total Protein Loading Control for Immunoblotting

For biochemical inhibitor studies, total p38 α was used for loading control as total p38 α protein levels are stable and unaffected by the different biochemical inhibitors used in this study.

For RNAi studies, the β -actin house keeping gene was used for loading control, as RNAi leads to protein ablation of target proteins including p38 α .

2.3.7 Acumen Multi-Parametric Cytometry High Content Analysis (HCA)

Prior to Acumen HCA, cells were grown in black 96-well BD Falcon opti-lux micro-titer plates. Cells were strained using a 40-micron cell strainer prior to seeding to aid in dis-aggregation of cell clumps. Cells were seeded at approximately 30,000 cell/ml density in 100ul volumes per well.

Following treatment with chemical inhibitors, chemical agonists and/or UV irradiation, cells were fixed without aspiration of media with 2X prefer fixative buffer (Anatech Ltd, MI USA) for 30mins at room temperature (for study of mitotic index) or with 1X prefer fixative for 30mins with removal of cell media for cell studies. Black micro-titer plates were then washed once with 1X wash buffer (PBS), using an automated BioTek micro-titer plate washer.

Following fixing of cells to the surface of micro-titer plate, cells were permeabilized using 0.1% Triton-X 100 permeabilization buffer for 10mins at room temperature. Triton-X 100 buffer is washed out once with 1X wash buffer using an automated BioTek micro-titer plate washer.

Fixed and permeabilized cells are now accessible to immuno-detection reagents, and to minimize non-specific immuno-detection, cells are blocked in 1X blocking

solution for at least 1 hour at room temperature on an orbital shaker. Following blocking step, cells are incubated with primary antibody diluted to a range of 1:200 to 1:1000 in 1X antibody buffer overnight at 4°C. After incubation with primary antibody overnight, plates are washed 3X in 1X wash buffer using an automated BioTek washer. Incubation of Alexa488 linked secondary antibody diluted to a final concentration of 1:1000 in antibody buffer occurred for 1 hour, at room temperature in the dark. Secondary antibody was washed out with 3x wash in 1X wash buffer.

DNA staining for DNA content was achieved with 500nM propidium iodide buffer with 200ug/ml DNase free RNase-A. Stained 96 well plates were scanned with Acumen Explorer (TTP, UK), a laser scanning cytometer with 488 nm laser excitation and 4 PMTs as detectors. We used 7x7 mm whole wellscan for 96 well plates at 1µm(X) x 2 µm(Y) resolution. Emission signals were collected with channel-1 (500nm-530nm) mean fluorescence intensity for cyclin B1, channel 3 (575 nm-640nm) peak fluorescence for DNA condensation and channel 4(655 nm-705 nm) total fluorescence for DNA content. Single cells were identified with gating DNA content covering 2N to 4N range. Total single cells, 2N cells, 4N cells and cells between 2-4N (S phase cells), cells with DNA condensation, and channel 1 (Alexa488) positive cells were individually identified. Percentage of each subpopulation was calculated based on total cell populations.

2.3.8 Immuno-Fluorescence Microscopy and Confocal Microscopy

Cell culture and sample preparations for immunofluorescence microscopy were done as previously described (467). Briefly, cells were grown on cover slips or in LabTek 4-well chamber slides. After cell treatment with chemical inhibitors, chemical agonist or UV irradiation, cells were fixed with 4% PFM buffer for 30mins. Cells were

permeabilized with 0.2% triton-X 100 buffer for 10mins, followed by blocking in 2% BSA blocking solution for at least 1 hour. Primary antibody mixtures were incubated overnight at 4°C at a final concentration of 1:50 to 1:1000 depending on antibody optimization step or as per manufacturer's recommendation. Alexa488 linked and Alex568 linked secondary antibody were incubated for 1 hour at a final concentration of 1:1000 for 1 hour at room temperature. DNA counterstaining was achieved with 500nM Hoescht stain in DI water.

Cell imaging was acquired with a Bio-Rad MRC600 confocal microscope at 40X (air or oil) or 60X (oil) magnification.

2.3.9 FACS analysis

Cell culture and sample preparation for FACS analysis were done as previously described (467). Briefly, cells were grown on 10cm diameter dishes or 6-well plates to 80% confluence, and treated with chemical inhibitors, chemical agonist or UV irradiation as per figure legend. After chemical treatment, cells were detached from plate bottom with trypsin-EDTA, collected into a Falcon spin tube and were fixed with 1X prefer fixative buffer or 4% PFM buffer for 30mins. Fixed cells were pelleted via centrifuge at 1200RPM for 5mins and washed with 1X PBS wash buffer. Cells were permeabilized with 0.2% Triton-X 100 for 10mins, and cell pellet was collected via centrifuge at 1200 RPM for 5mins and washed with 1X PBS wash buffer. Cells were blocked in 5% low-fat milk for at least 1 hour and then incubated with primary antibody at final dilution of 1:200 to 1:1000 depending on optimization step, overnight at 4°C. Following washing 3x in 1X PBS wash buffer, Alexa488 and/or Alexa568 linked secondary antibodies are added in sequence for 1 hour at room temperature. RNase digestion was carried out with

200ug/ml DNase free RNase-A for 30mins. DNA counter staining is achieved with 1mM propodium iodide for DNA content analysis.

Multi-parametric FACS analysis was carried out on the BD LSR-II Flow-Cytometry system. A minimum of 10,000 independent events was required for a successful data capture run. In this study we gated using a band-pass filter for 530/30 for the Alexa488 emission, a band-pass filter of 575/26 for the PI stain and a 488/10 filter for the side scatter filter. We used the ratio of forward scatter vs side scatter to determine the population of interest.

2.3.10 cDNA microarray hybridization (Affymetrix Arrays)

Total RNA from Calu6 cells was isolated with RNA STAT-60 (Tel-Test) according to the manufacturer's protocol. 5 micrograms of total RNA were labeled and hybridized to Affymetrix U133plus2 arrays according to the Affymetrix protocol. Each sample was hybridized to a single microarray. All samples were assessed for RNA quality such as microarray scaling factors, background levels, percent present calls, β -actin and GAPDH 3'/5' ratios, etc. Samples with 3'/5' ratios above a threshold 3 were excluded from further analysis.

Signal intensities representing gene expression values were obtained from Microarray Suite version 5.0 (MAS5) using the default settings, except 2% trimmed mean was set to 1500.

III. Mathematical & Statistical Methods in Computational Biology

2.4 Statistical Analysis of Microarray Data

To apply statistical analysis to the experiment, 2 different statistical algorithms were used depending on the nature of the statistical problem. For a two-sided problem, we employed the SAM (Significance Analysis of Microarray) algorithm to identify genes differentially expressed between two groups of genes. (Tibshirani et al, PNAS 2001) Multiple testing correction has been built into the SAM algorithm based on the false discovery rate (FDR) and Q-value algorithm (Storey et al, 2002). The FDR rate for every gene list derived in this analysis was standardized to $FDR \leq 0.1$.

For a multi-parametric time series analysis we employed a mixed-effects analysis of variance model (ANOVA). The random effect in this analysis was classified as the technical replicates for each treatment group and time point. The nested linear mixed-effect ANOVA model follows this format.

$$y(x) = \gamma_0 + \gamma_1 \cdot Type/Treatment + \gamma_2 \cdot Time + \epsilon_{Error}(Rep)$$

The p values of the ANOVA model were adjusted for multiple testing using the false discovery rate (326). The adjusted P values, or the false discovery rates (FDR), are designated as Q values, where $Q = P \cdot n/i$ (n = total number of probe sets on the microarrays, i = sorted rank of P values). The fold change was calculated as the ratio of the two group means based on the observed signal values from MAS5 and the gene expression signal change was calculated as the difference of the two group means. Differential gene lists were generated for time series analysis using a FDR $FDR \leq 0.1$ for all factors.

2.4.1 Hierarchical Clustering, Correlation Analysis and Principal Component Analysis (PCA)

In addition to generating gene lists using SAM or ANOVA, we also applied a number of unbiased, unsupervised clustering techniques to assess natural groupings found within the data. For hierarchical clustering we used complete linkage with a Euclidean distance, similarity metric to measure distance between groups of genes. For my correlation analysis, we used a boot-strapped adjusted (permutation based) pearson correlation metric to find genes that clustered across and within each timepoint. We also plotted the 1st, 2nd and 3rd principal components of all expressed genes to understand if treatment conditions naturally separated in dimensionally reduced projections.

2.4.2 Pathway Analysis and Functional Annotation Clustering

For all gene lists generated in the microarray experiment, we performed functional annotation clustering and canonical pathway analysis using two computational tools. We used the NIH DAVID tool (Lempicki et al, *Genome Biology* 2003) for functional clustering of gene lists. We used the Ingenuity pathway analysis tool (www.ingenuity.com) for canonical pathway over-lay and for canonical pathway network construction. Functional analysis and pathway analysis gives context to gene lists produced from statistical analysis of microarray data.

Chapter 3: The role of p38 MAPK in regulation of DNA damage G2 cell cycle checkpoint control

3.1 Background

The p38 MAPK is one of the main stress induced kinases that is critical for response to many types of environmental stress including bacterial infection, osmotic stress and UV damage (41). The response of p38 to different types of stress has been detailed concisely in chapter 1. This chapter will focus on my studies addressing p38's role in the control of the cell cycle specifically at the G2 to M transition point and in other aspects of p38's role in DNA damage response. Our interest with p38 stems primarily from reports that p38 was activated in response to DNA damage and suggestions that inhibition of p38 signaling would provide an ideal new mechanism to effectively target cancer cells. Prior publications as detailed in chapter 1 have suggested a link between p38 activity and DNA damage checkpoint response at the G2 to M phase of the cell cycle. As many of these published studies were conducted in mouse cells and in untransformed cells, we were interested in definitively exploring p38's role in the control of cell cycle progression specifically in the response to DNA damage and in the context of cancer cells. Our goal was to the gain a deeper mechanistic understanding of p38 function at the G2 checkpoint, and to test that inhibition of p38 was an effective anti-cancer strategy that should be developed further for clinical testing.

3.2 p38 MAPK is activated during DNA damage at all stages of the cell cycle

Since p38 is known to be activated in response to DNA damage, we asked if the extent of p38 activation is correlated with the sources of DNA damage and the underlying cell cycle states. Prior publications have shown that p38 MAPK is activated during events

leading to DNA damage (181,316,332). These events include genotoxic damage caused by Topo-isomerase II inhibitors such as Doxyrubicin-HCL (Adriamycin), and radiation damage by UV and IR radiation. To test the effect of different sources of DNA damage on p38 pathway and cell cycle checkpoint response, unsynchronized and synchronized HeLa cells were treated with 160nM Adriamycin (Figure 3.1A), increasing doses of UV irradiation (Figure 3.1B), and 0.01% MMS (Figure 3.1C). Based on multi-parametric Acumen & FACS cytometry data, treatment with Adriamycin, UV and MMS damage all led to a G2 checkpoint arrest in p53 functionally null HeLa cells (Top panels of figure 3.1A, B&C). Western blot assays of p38 and DNA damage response pathways to the different sources of DNA damage also showed strong induction of p38 pathways, corresponding to the G2 checkpoint arrest observed in multi-parametric cytometry data (Bottom panel Figure 3.1A, B&C).

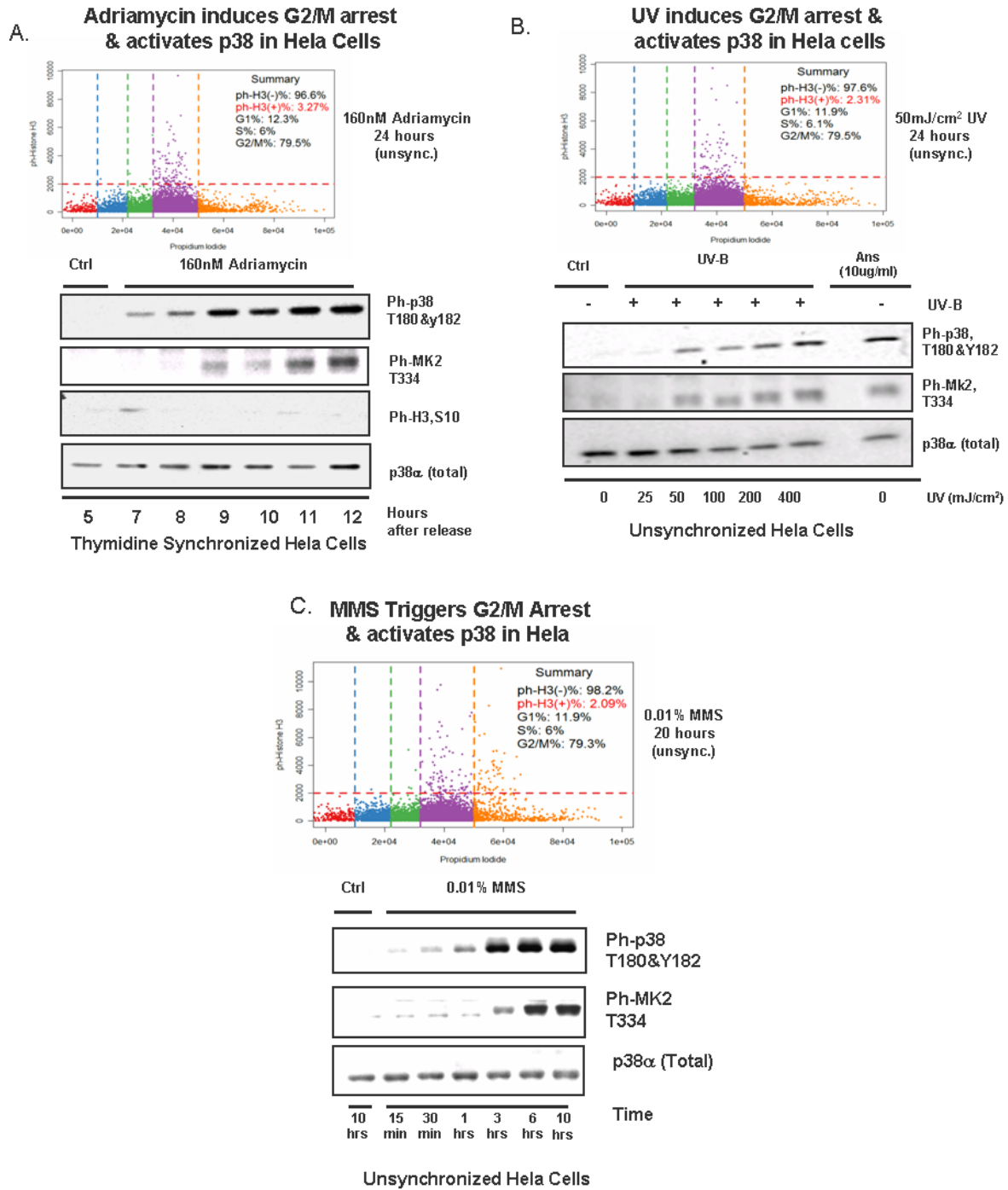


Figure 3.1: *p38 MAPK is activated by various DNA damaging stresses. (A) Activation of p38 by Topo-II inhibitors. (B) p38 MAPK is activated by UV-B irradiation. (C) p38 pathway activated by 0.01% MMS damage*

Having demonstrated that p38 is activated strongly by different genotoxic agents and by environmental UV damage. We next asked if p38 could be activated equally well at different stages of the cell cycle. For this, HeLa cells were synchronized in G1 by 48hrs serum-starvation (Figure 3.2A) or in early S phase by double thymidine block/release (Figure 3.2B) or in G2 by CDK1 inhibitor (RO-3306) block for 21hrs (Figure 3.2C) and then released in fresh growth medium containing 0.01% MMS.

DNA damage following the different cell synchronization techniques all showed rapid induction of p38 and Chk1 pathway activity (Figure 3.2).

This shows that p38 is activated by DNA damage at all stages of the cell cycle.

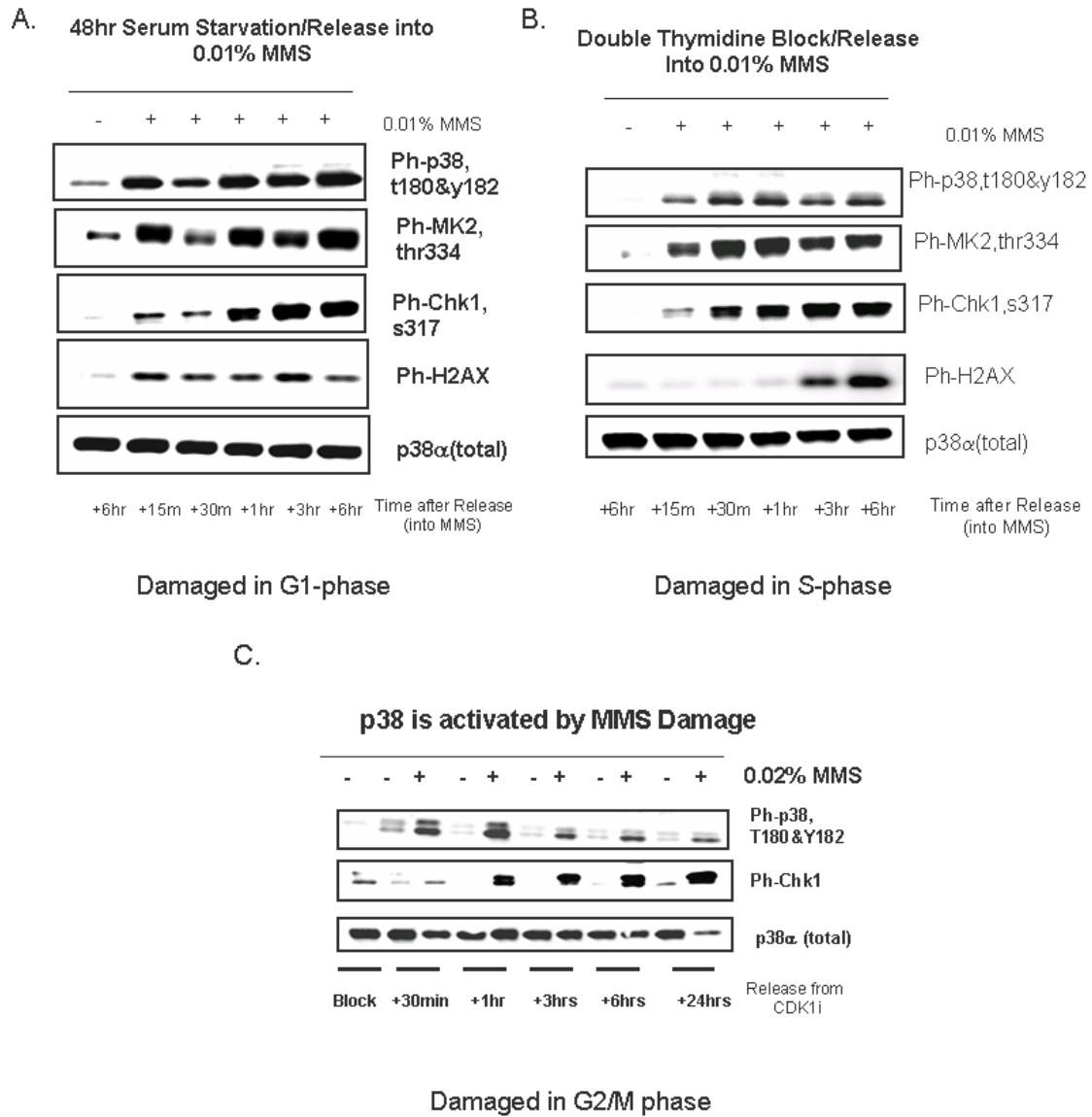


Figure 3.2: *p38 MAPK is activated at all stages of the cell cycle. (A) p38 is activated by MMS damage after 48hours serum starvation, cells in G1 phase. (B) p38 is activated by MMS damage after double thymidine block/release protocol, cells are in S phase. (C) p38 is activated by MMS following release from CDK1i (RO-3306), cells in G2.*

3.3 LY479754 and SB203580 are effective inhibitors of p38 pathway

To probe the effect of p38 in the DNA damage response pathway, we used multiple *in-vitro* biochemical tools including a potent p38 inhibitor LY479754 (referred to subsequently as p38 inhibitor) (106). We showed using anisomycin, a potent anti-microbial agent, that a dose in the range of 80nM to 160nM of LY479754 can effectively suppress MAPKAPK2 (MK2) phosphorylation in HeLa cells (Figure 3.3). Concurrent analysis of phospho-Chk1 and phospho-JNK status showed that doses of up to 1 μ M of LY479754 had no effect on the expression of these proteins.

LY479754 is an effective and specific inhibitor of p38 activity

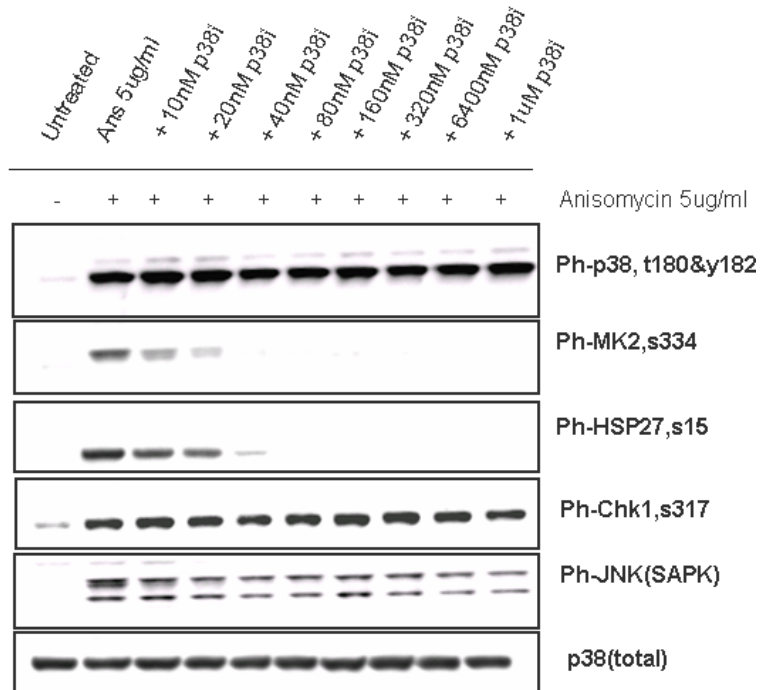


Figure 3.3: *In-vitro* kinase assay for LY479754 in HeLa cells. HeLa cells were treated for 1hr with 5 μ g/ml of anisomycin and co-incubated with stated concentration of p38 inhibitor (LY479754). MAPKAPK2 phosphorylation at serine 334 was used as a marker of p38 pathway activity.

As the majority of prior publications have used SB203580 to biochemically inhibit p38 pathway. We conducted a dose response experiment to determine the effectiveness of the inhibitor.

SB203580 is an inhibitor of p38 activity

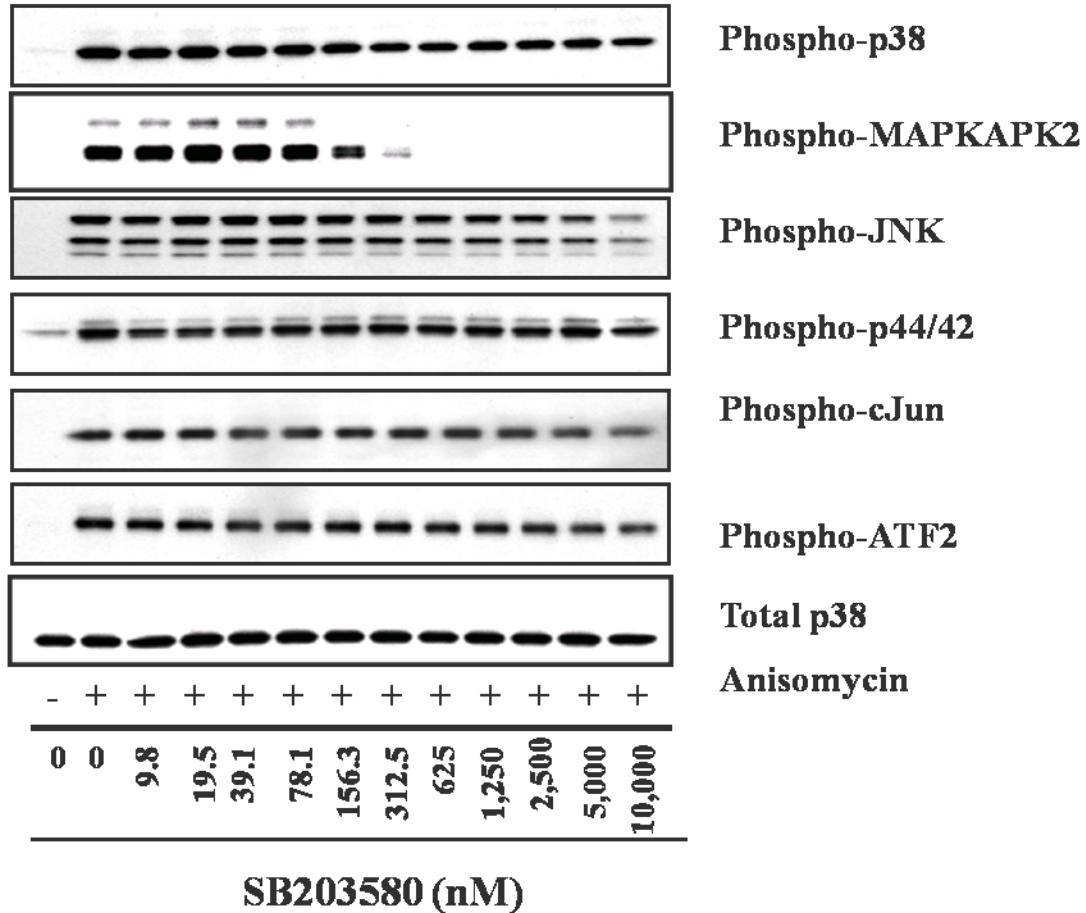


Figure 3.4: *In-vitro* kinase assay for SB203580 in HeLa cells. HeLa cells were treated for 1hr with 5 μ g of anisomycin and co-incubated with stated concentration of p38 inhibitor (SB203580). MAPKAPK2 phosphorylation at serine 334 was used as a marker of p38 pathway activity.

SB203580 is an effective inhibitor of p38 pathway activity. In the dose response study conducted in HeLa cells, SB203580 dramatically inhibits p38 pathway activity in

the range of 312nM, with complete suppression of phospho-MAPKAPK2 observed at 625nM (Figure 3.4). It should be noted that most prior publications use dose concentrations of SB203580 of around 10 μ M to 40 μ M to suppress p38 activity, many times over what we observe to be the effective dose for SB203580.

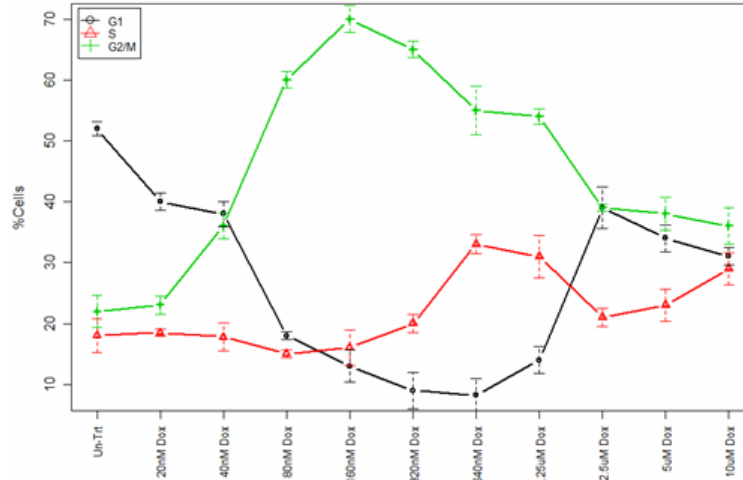
3.4 Adriamycin dose titration to find optimal dose for cell-cycle experiments

The reports of the p38 pathway acting as a key regulator of G2 checkpoint activity and fidelity have stirred considerable interest in the area of oncology treatment. Activation of DNA damage checkpoint at the G2 to M transition in response to chemotherapeutic treatment has been implicated as a mechanism that allows some cancer cells to escape apoptosis and cell death. We began this exercise with the hypothesis that the combination of a potent p38 inhibitor with a genotoxic chemotherapeutic agent would increase the efficacy of treatment by abrogating the DNA damage G2 checkpoint, forcing cells pre-maturely into mitosis. Forcing cells pre-maturely into mitosis has been known to lead to a phenomenon known as mitotic catastrophe, leading to increased cell death.

To begin this experimental series, Adriamycin, a Topo-isomerase II inhibitor (Doxurubicin HCL) was selected as a genotoxic agent to explore the role of p38 activity in G2 DNA damage checkpoint function. Before undertaking a study of p38 function in G2 checkpoint function, an optimal dose of Adriamycin that induced a strong G2 checkpoint without excessive cytotoxicity had to be determined. To address this, a dose titration experiment of Adriamycin in HeLa cells was conducted using a multi-parametric Acumen HCA assay (Figure 3.5). In this assay, the 3 primary parameters under consideration were the DNA content (propidium iodide), mitotic index (phospho-Histone H3) and cell viability (cell count). From these 3 parameters, the ideal dose of Adriamycin

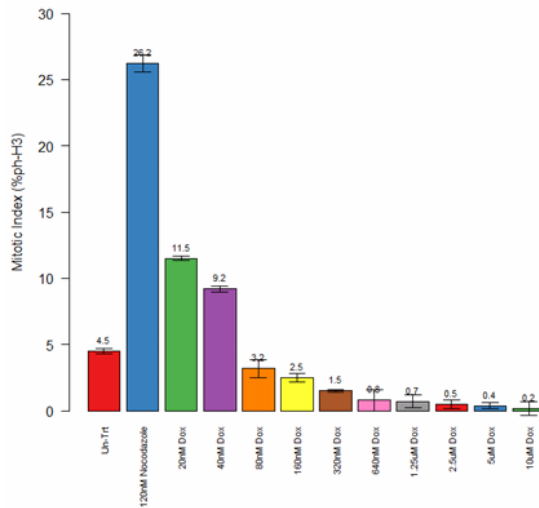
would have a majority 4N DNA content, low phospho-Histone H3 levels and high cell viability.

A. Adriamycin Drug Dose Response (Cell Cycle State)



B.

Adriamycin DDR, Mitotic Index



C.

Adriamycin DDR, Viable Cell Count

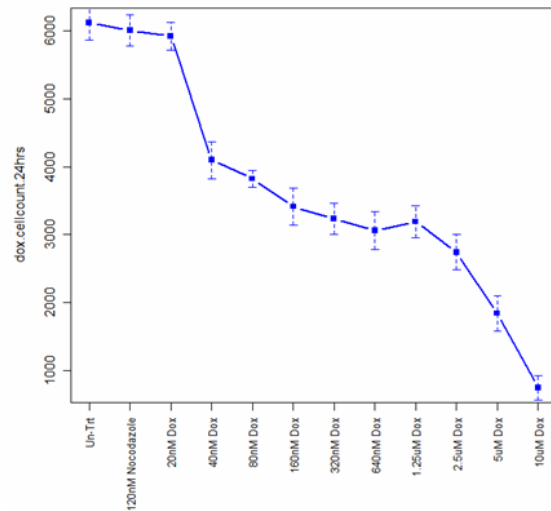
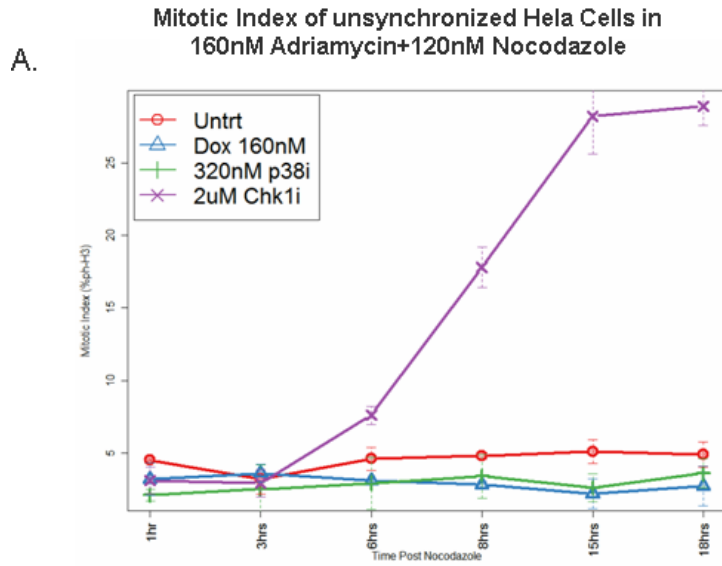


Figure 3.5: *Adriamycin Dose Response in HeLa cells at 20hrs. (A) Distribution of Cell Cycle States at 20hrs post Adriamycin, in an Adriamycin Dose response experiment (B) Mitotic Index in HeLa cells in a dose response to Adriamycin. (C) Cell viability assay: Cells were stained with PI and counted.*

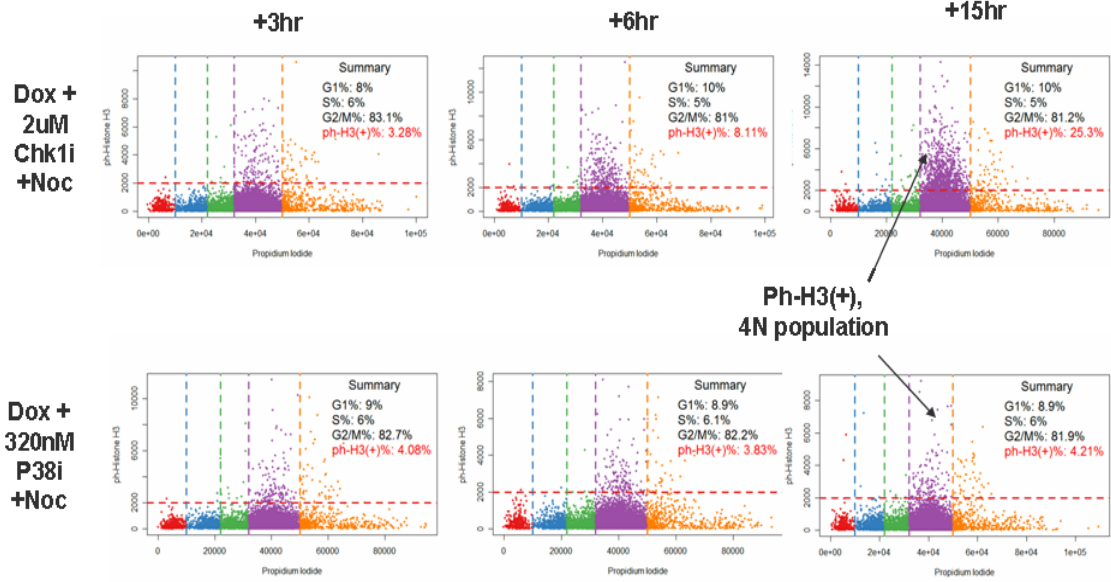
Cells with the highest 4N DNA content population could be achieved in the dose range of 80nM to 320nM (Figure 3.5A). At doses higher than 1.2 μ M, cells tend to arrest in S-phase instead of G2. A dose of Adriamycin below 80nM led to leaky G2 checkpoint arrest with significant positive phospho-Histone H3 signal (Figure 3.5B). Lastly, high doses of Adriamycin led to significant cell death, with doses of Adriamycin above 320nM leading to significant loss of cells (>50% loss) (Figure 3.5C). The results of the dose response studies with Adriamycin showed that a dose of around 160nM was optimal, as a strong G2 cell cycle arrest was induced with acceptable levels of cellular toxicity based on cell viability (Figure 3.5).

3.5 Biochemical inhibition of p38 MAPK cannot abrogate Adriamycin induced G2 checkpoint arrest in HeLa Cells.

As p38 is strongly activated in response to DNA damage resulting in a G2 checkpoint arrest, its activity is implicated as critical for proper checkpoint establishment and function. If so, then inhibition of p38 activity during G2 would be expected to abrogate the checkpoint -mediated arrest in response to DNA damage. To test this, unsynchronized HeLa cells were dosed with 160nM of Adriamycin for 20hrs to allow for the establishment of a robust G2 checkpoint arrest. Following 20hrs, original Adriamycin only media was replaced with growth media containing 320nM of LY479754 or 1.5 μ M of the reference Chk1 inhibitor and 160nM Adriamycin. An additional nocodazole trap consisting of 120nM of nocodazole was added to trap in mitosis, any cells that escape the G2 checkpoint arrest. Cells were fixed and analyzed for mitotic index (phospho-Histone H3) and for cell cycle state (propidium iodide) using multi-parametric Acumen high content cytometry or by multi-parametric FACS (Figure 3.6A &B).



B. Multi-Parametric Cytometry Analysis of unsynchronized HeLa Cells



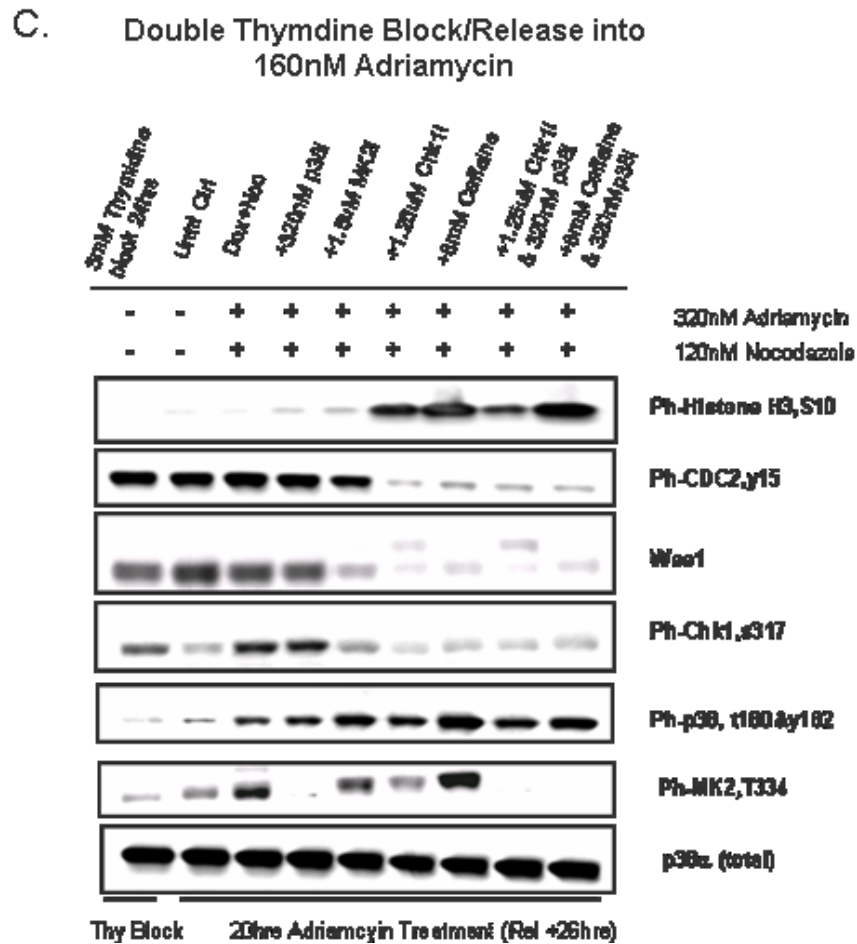


Figure 3.6: Inhibition of Chk1 but not p38 abrogates Adriamycin induced G2 checkpoint Arrest: (A) Summary line graph of mitotic index across time for different treatment conditions. (B) Representative multi-parametric plots of mitotic index (ph-Histone H3) and DNA content (PI) at 15hrs post p38 inhibitor+nocodazole or Chk1 inhibitor+Nocodazole addition. (C) Western Blots of synchronized HeLa cells (thymidine block/release) treated with Adriamycin and chemical inhibitors (160nM p38i, 2uM MK2i, 1.5uM Chk1i)

HeLa cells treated with 160nM of Adriamycin arrest with a majority 4N population after 20 hours of treatment. Our expectation that p38 activity is necessary for proper G2 checkpoint activation implies that inhibition of p38 activity should release cells from G2 checkpoint arrest. HeLa cells treated with 320nM of LY479754 do not exhibit any significant increase in mitotic index in the 20 hours after inclusion of p38

inhibitor with a nocodazole mitotic trap (Figure 3.6A). This result contrasts strongly with the effect of a Chk1 inhibitor (LY2494516), where we saw a dramatic increase in mitotic index in the same time period with a nocodazole mitotic trap (Figure 3.6A &B).

Multi-parametric Acumen cytometry data was further supported by data produced from synchronized HeLa cells in a western blot assay. In this experimental regime, HeLa cells were synchronized using 3mM thymidine block/release protocol. In this experimental regime with synchronized HeLa cells, Adriamycin induced a strong G2 checkpoint arrest, which was relieved by specific inhibition of Chk1 with a Chk1 inhibitor or by inhibition of the ATM/ATR response with 6mM caffeine, but not by inhibition of p38 or its downstream substrate MK2 by measuring levels of phospho-Histone H3. (Figure 3.6C) To better understand the effect of biochemical inhibition on the G2 DNA damage checkpoint, we probed for additional cell-cycle related checkpoint kinases that were known to play a role at this checkpoint. These included phosphorylated CDC2 (CDK1) at tyr15, and the levels of Wee1 kinase. Co-analysis of phospho-CDC2 and Wee1 levels show that inhibition of the ATM/ATR pathway with either a specific Chk1 inhibitor (1.5uM) or the generic ATM/ATR inhibitor caffeine (6mM) led to dramatic decreases in expression of both these proteins, corroborating with an increase in phospho-Histone H3, indicating the effective abrogation of the G2 checkpoint (Figure 3.6C). Inhibition of p38 with LY479754 or a specific MK2 inhibitor (LY2441693) had no such effect on these mitotic entry markers. (Figure 3.6C) Additionally, we noticed that abrogation of G2 checkpoint with either the Chk1 inhibitor or caffeine still occurs with high levels of phospho-p38 and phospho-MK2 still present.

Taken together this data suggests that inhibition of the ATM/ATR pathway with either a specific Chk1 inhibitor or with caffeine was able to abrogate the G2 DNA

damage checkpoint induced by Adriamycin whereas inhibition of p38 was unable to abrogate the G2 DNA damage checkpoint.

Additional experiments involving various doses of p38 inhibitor (5nM to 2.5µM) and multiple concentrations of Adriamycin (9nM to 160nM) demonstrate that these results were not specific for a particular dose range of LY479754 or Adriamycin (Figure 3.7).

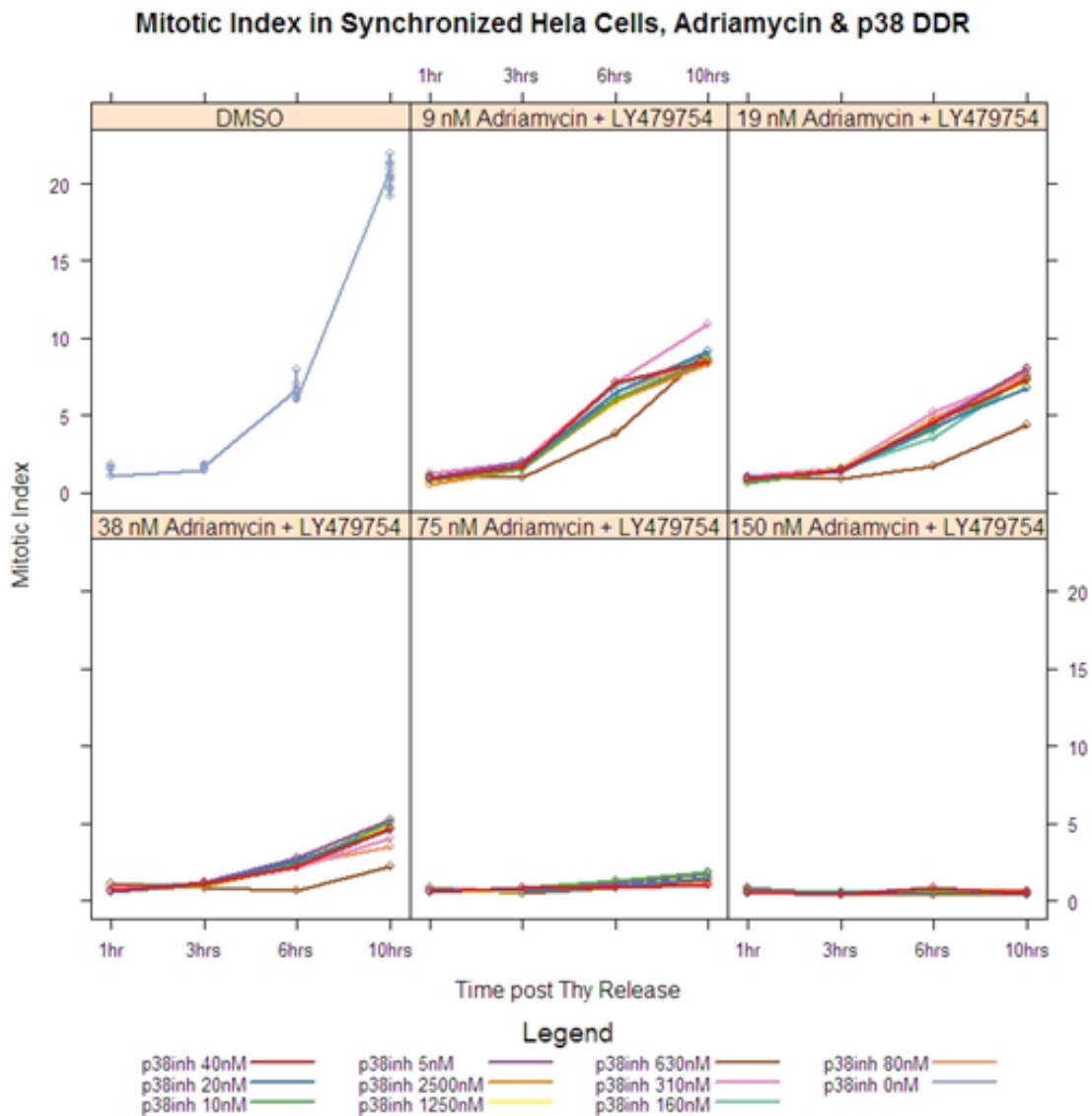


Figure 3.7: *Dose titration of Adriamycin and p38-inhibitor (LY479754) in thymidine synchronized HeLa cells.*

Confocal Immunofluorescence Microscopy

To further substantiate the results in HeLa cells beyond biochemical assays, a series of confocal microscopy experiments were performed to image cells damaged by Adriamycin and treated with either the p38-inhibitor or the Chk1-inhibitor. HeLa cells were synchronized with double thymidine block and upon release subsequently damaged with 160nM Adriamycin in combination with either 320nM p38-inhibitor or 2uM Chk1-inhibitor. Cells were fixed 10 hours after treatment with Adriamycin and inhibitors and imaged according to the confocal microscopy methodology described in the methods and materials section (Figure 3.8).

In concordance with the results of other assays, the confocal microscopy imaging shows that Adriamycin induced a strong G2 checkpoint arrest in synchronized HeLa cells. 10 hours post inhibitor treatment, cells treated with Adriamycin showed a “spread-out” and flat morphology characteristic of cells in interphase. Hoerscht stained DNA revealed un-condensed DNA and no Histone-H3 phosphorylation (ser10) was detectable (Figure 3.8 left panel). Combining the lack of ph-Histone-H3 staining with CyclinB1 primary co-localization within the cytoplasm and the lack of condensed DNA, imaging data presents the case from multiple markers that Adriamycin induces a strong G2 checkpoint arrest. Imaging of HeLa cells treated with 320nM p38-inhibitor and 160nM Adriamycin showed very little differences in terms of morphology or immunofluorescent staining patterns with cells treated with only Adriamycin. As similar patterns of no phospho-Histone-H3, primarily cytoplasmic CyclinB1, the lack of cells with a mitotic spindles and uncondensed DNA were seen in cells treated with the p38 inhibitor (Figure 3.8, middle panel).

Significant differences however, were observed in HeLa cells treated with 2μM Chk1-inhibitor and adriamycin. Treatment with a Chk1 inhibitor led to cells with distinct

bipolar mitotic spindles, condensed DNA, nuclear cyclin B1 and strong phospho-Histone H3 levels (Figure 3.8 right panel). Taken together this imaging data shows that inhibition of Chk1 but not p38 was able to induce cells to bypass an Adriamycin induced G2 checkpoint arrest.

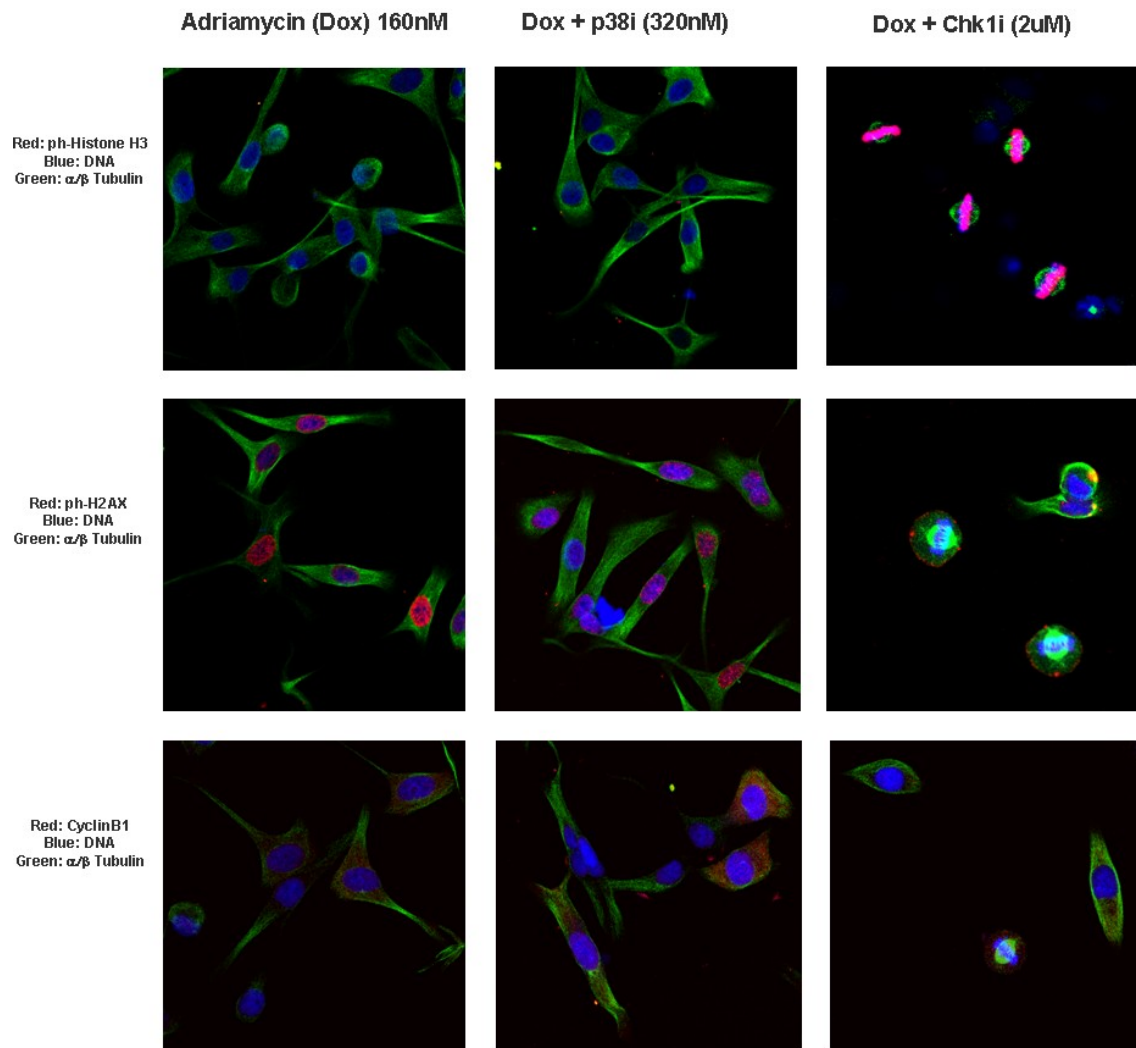


Figure 3.8: *Confocal Microscopy images of thymidine synchronized HeLa cells damaged with 160nM Adriamycin (Left) and dosed with either 320nM p38 inhibitor (Middle) or 2uM Chk1-inhibitor (Right). Red Dye: Alexa 555, Green Dye: Alexa 488, Blue Dye: Hoerscht Stain.*

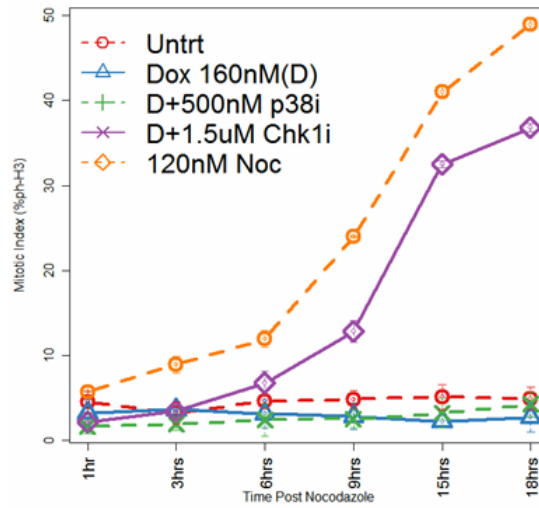
3.5.1 Biochemical inhibition of p38 cannot abrogate Adriamycin induced G2 checkpoint arrest in other cancer cell-lines

While a large part of this study was conducted using HeLa cells as the primary cell-line model, we were interested to investigate if these results could be reproduced in other commonly used cancer cell-line models. To address this issue, we studied the effect of p38 inhibition in combination with Adriamycin stress in Calu6 (Lung Adenocarcinoma), A549 cells (Lung non-small cell carcinoma) and in U2OS cells (Osteosarcoma cells).

There were some complications involved in switching cell-line models between HeLa cells and other cancer cell-line models. The vastly varying genetic background between the different cancer cell-lines resulted in varying degrees of sensitivity to both Adriamycin damage and to the p38 inhibitor LY479754. For each cell-line model, we conducted a small pilot experiment to determine optimal cell treatment conditions. We found that a dose range between 160nM and 320nM of Adriamycin produced a very strong G2 checkpoint arrest in all three cell-lines. For the p38 inhibitor we found that a dose in the range of between 320nM-500nM and above effectively inhibited phospho-MK2,T334 expression in all cell-lines.

In Calu6, lung adenocarcinoma cell-lines, we were able to reproduce the results observed in HeLa cells well. We found that strong biochemical inhibition of p38 kinase activity had no effect on the ability of Calu6 cells to mount a G2 checkpoint arrest in response to adriamycin damage. Similar to our observations in HeLa cells, inhibition of Chk1 via a biochemical inhibitor led to a dramatic increase in mitotic index within a 20 hour timeframe, while cells were trapped with a nocodazole metaphase trap (Figure 3.9).

A. Unsynchronized Calu6 Cells into 160nM Adriamycin (Dox)+Nocodazole



B.

Adriamycin Damage in Calu6 Cells

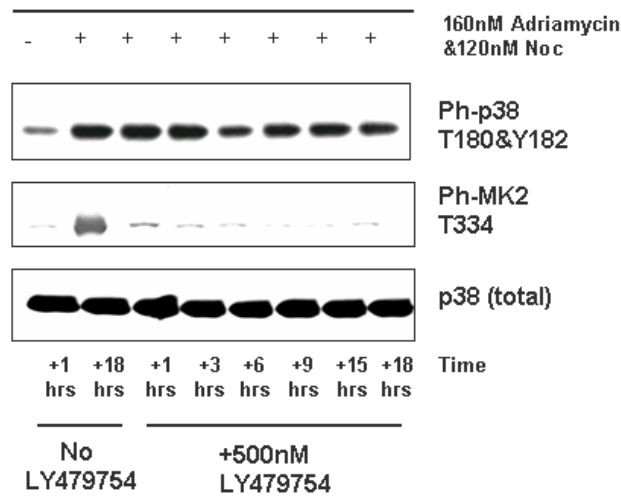
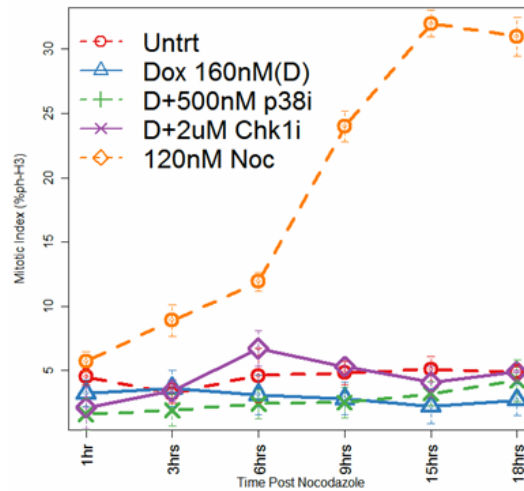


Figure 3.9: Biochemical inhibition of p38 is unable to abrogate Adriamycin induced G2 checkpoint arrest in Calu6 cells. (A) Mitotic index plot (ph-Histone H3,s10) based on an Acumen Explorer HCA scan across time of Calu6 cells treated with 320nM of Adriamycin and p38 or Chk1 inhibitors. (B) Western blot assay of Adriamycin treated Calu6 cells with p38 inhibitor (LY479754)

In addition to Calu6 cell-lines, the effect of p38 inhibition on Adriamycin induced G2 checkpoint arrest was tested on A549 and U2OS cancer cell lines. Inhibition of p38 in A549 and U2OS cells was again unable to abrogate a G2 checkpoint arrest mounted in response to Adriamycin damage (Figure 3.10).

A. **Unsynchronized A549 Cells into 160nM Adriamycin (Dox)+ Nocodazole**



B. **Unsynchronized U2OS Cells into 320nM Adriamycin (Dox)+ Nocodazole**

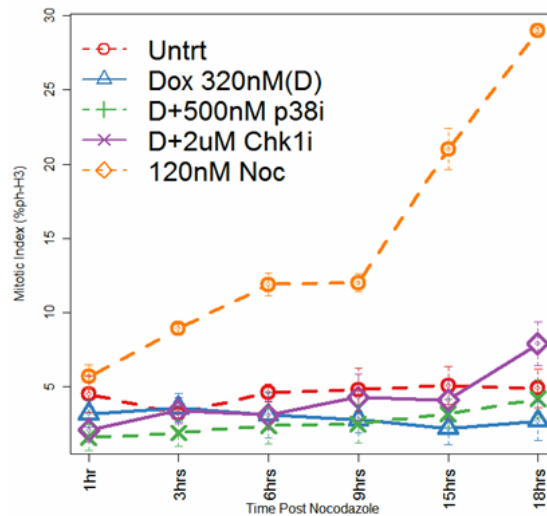


Figure 3.10: Biochemical inhibition of p38 and Chk1 was unable to abrogate Adriamycin induced G2 checkpoint arrest in A549 & U2OS cells. (A) Mitotic index plot (ph-Histone H3,s10) based on an Acumen Explorer HCA scan across time of A549 cells treated with 160nM of Adriamycin and 120nM nocodazole with p38 or Chk1 inhibitors. (B) Mitotic index plot (ph-Histone H3,s10) based on an Acumen Explorer HCA scan across time of U2OS cells treated with 320nM of Adriamycin and 120nM Nocodazole with p38 or Chk1 inhibitors

However in both A549 and U2OS cells, inhibition of Chk1 did not lead to a significant increase in mitotic index within a 24 hour time frame. This result in A549 and U2OS cells differed significantly from the results observed in HeLa and Calu6 cells. One possible explanation in this discrepancy could be the p53 status in these cells-lines. Both HeLa and Calu6 have a non-functional p53 pathway, whereas A549 and U2OS are regarded to have an intact p53 signaling pathway. A search of literature revealed prior publications that suggested that inhibition of Chk1 alone in a p53 functional environment was not sufficient to overcome a DNA damage G2 checkpoint arrest (59,217,232).

As Chk1 kinase was not the primary target of this study, we did not pursue further investigations into this phenomena, however future studies may delve into dissecting the Chk1, p53 pathway interaction in response to Adriamycin induced cell cycle arrest.

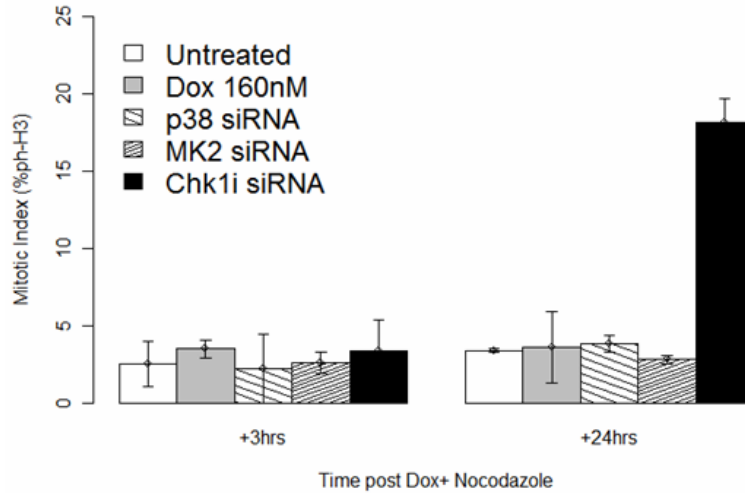
3.6 Transient and stable knock-out of p38 or its down-stream substrate MK2 has no effect on Adriamycin induced G2 DNA damage checkpoint.

As chemical inhibitors are often reported to have off-target or dose dependent effects, we wanted to test the effect of p38 pathway suppression through a separate mechanism. To address this problem, we tested the effect of both transient siRNA knock-down and stable-line shRNA knock down of p38. For the transient knock-down experiments we used validated QiagenTM oligonucleotides designed against the p38alpha, MK2 and Chk1 transcripts. In this experimental regime, unsynchronized HeLa cells were transfected with siRNA oligonucleotides using Lipofectamine-RNAiMax (Invitrogen) for 48hrs in low serum media for 48 hours. Following 48 hours, siRNA transfection media was replaced with fresh growth media containing 160nM Adriamycin and 120nM nocodazole acting as a mitotic trap.

In HeLa cells, transient knock-down of p38 after 48hrs treatment with Qiagen siRNAs was unable to abrogate the G2 DNA damage checkpoint induced by Adriamycin (Figure 3.11A). Over a 24 hour time period, cells transfected with siRNA oligonucleotides targeting p38 showed no significant increase in phospho-Histone H3 levels, while in a nocodazole mitotic trap. Cells transfected with siRNA oligonucleotides targeting Chk1, in contrast registered a significant increase in phospho-Histone H3 levels over a 24 hour period, indicating abrogation of the G2 checkpoint and mitotic entry (Figure 3.11A).

Additional experiments using western blots showed that despite effective siRNA knock-down of p38 and MK2 protein levels, no corresponding increase in phospho-histone H3 levels were detected. (Figure 3.11B) These assays showed that effective siRNA knock-down of Chk1 protein, led to an increase in phospho-Histone H3 and a concordant decrease in phospho-CDC2 (CDK1), Y15 levels. (Figure 3.11B)

A. Effect of siRNA KD in Adriamycin Treated HeLa Cells



B. Effect of siRNA KD on Adriamycin G2/M Checkpoint In HeLa

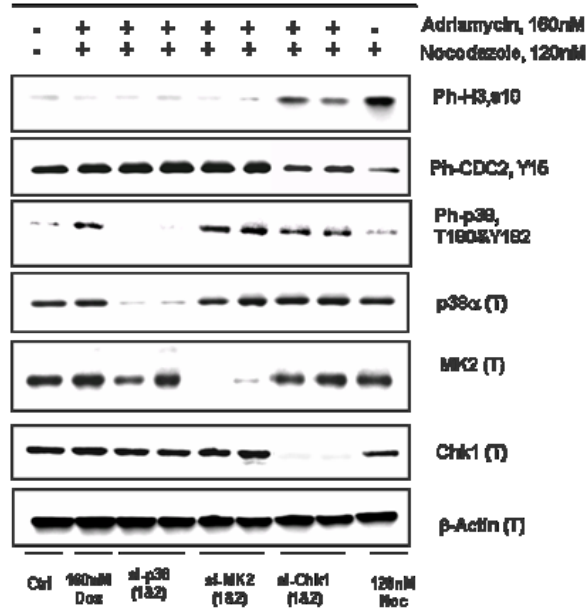


Figure 3.11: Effect of siRNA KD of p38, MK2 and Chk1 transcript on establishment of Adriamycin induced G2 DNA damage checkpoint. (A) Mitotic index derived from Acumen HCA assay in HeLa cells following reverse transfection of siRNA vectors with Lipofectamine RNAi-Max and 24hrs 160nM Adriamycin treatment. (B) Western Blot assay of effect of siRNA KD of p38, MK2 and Chk1 on Adriamycin induced G2 checkpoint arrest, 24hrs post Adriamycin & nocodazole.

Together this data shows that in a p53 functional null system that knock-down of Chk1 can effectively abrogate the G2 checkpoint induced by Adriamycin. In addition to p38 knock-down, siRNA knock-down of MK2 was explored as a secondary hypothesis within this experimental context. While siRNA knock-down of MK2 could be achieved successfully (Figure 3.11B), it had no impact on the ability of cancer cells to mount a G2 checkpoint arrest in response to Adriamycin (Figure 3.11A). These results show that siRNA knock-down of either p38 or MK2 had no significant impact on the proper function of the DNA damage G2 cell cycle checkpoint.

As a follow-up to studies involving transient siRNA knock-down of p38 and MK2, stable-lines incorporating a lentiviral vector against MK2 were made in HeLa cells. As MK2 has been implicated as both a downstream component of p38 pathway signaling as well as a direct substrate of CDC25B binding, we wanted to further investigate if stable-line knock-down of MK2 would produce any difference in the ability of cells to mount a successful G2 checkpoint in response to Adriamycin DNA damage. HeLa^{MK2^{-/-}} and their respective control cells HeLa^{Vect} (empty vector) were synchronized with double thymidine block/release and treated with 160nM Adriamycin.

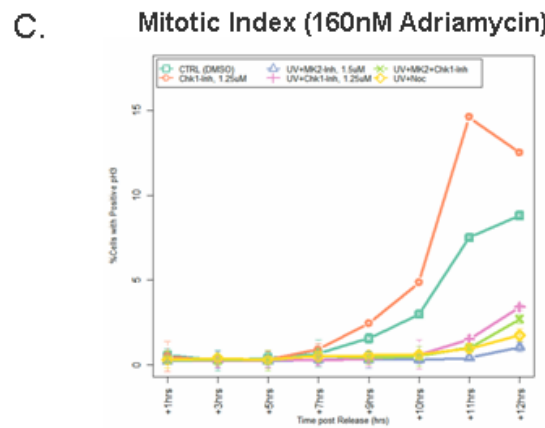
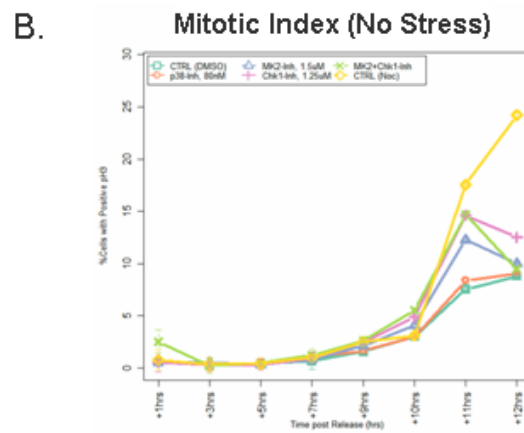
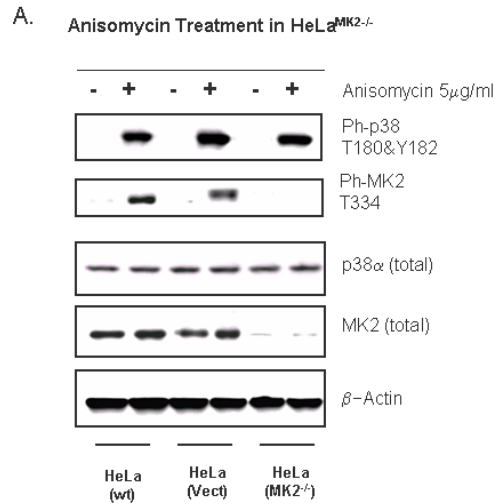


Figure 3.12: Effect of Adriamycin damage on HeLa^{MK2-/-} cells. *A. Western blots of HeLa cells incorporating an shMK2 vector or an empty vector control treated with anisomycin (1hour) and Adriamycin (24hrs). B. Mitotic index of synchronized HeLa MK2-null cells and synchronized MK2-null cells treated with Adriamycin.*

HeLa cells transfected with a lentiviral shRNA vector targeting MK2, and grown under constant selection pressure using puromycin results in a stably incorporated cell-line with MK2 knocked down. Using anisomycin to test the effect of MK2 knock-down, showed that the MK2 pathway in HeLa^{MK2^{-/-}} cells was effectively silenced (Figure 3.12A). Having shown that the HeLa^{MK2^{-/-}} cells do not have a functioning MK2 pathway, these cells and their respective empty vector controls were synchronized with double thymidine block release with 3mM thymidine in growth media. Following release from thymidine block, these cells were treated with 160nM of Adriamycin and with biochemical inhibitors against p38 and Chk1 kinase as additional controls.

To show that these cells do not possess unusual cell cycle properties, HeLa^{MK2^{-/-}} cells were demonstrated to be able to enter and exit mitosis as normal in an unstressed environment (Figure 3.12B). Following treatment with Adriamycin, HeLa^{MK2^{-/-}} cells were able to mount a successful G2 checkpoint arrest, as measured by low levels of phospho-Histone H3 (Figure 3.12C). The addition of LY479754 to these cells treated with Adriamycin was unable to abrogate the checkpoint arrest, whereas HeLa^{MK2^{-/-}} and the corresponding empty-vector incorporated HeLa cells that were treated with 1.5uM of Chk1i were found to enter mitosis in a slight delayed fashion versus un-perturbed cells (Figure 3.12C).

This data shows that despite stable knock-down of MK2 and treatment with a potent biochemical inhibitor of p38, loss of p38/MK2 pathway activity did not impact the establishment of a G2 checkpoint arrest in response to Adriamycin.

3.7 Biochemical Inhibition of p38 cannot abrogate UV induced DNA damage G2 checkpoint response in HeLa cells.

Previous reports implicating p38 as a critical kinase in G2 checkpoint function had utilized UV irradiation as a source of DNA damage. Earlier reports by Bulavin et al (38) and others had suggested that inhibition of p38 sensitizes cells to UV damage through abrogation of the G2 DNA damage checkpoint. Since p38 activity does not appear to be necessary for G2 checkpoint arrest in Adriamycin induced G2 checkpoint arrest, we wanted to investigate the role of p38 activity in response to UV damage.

UV damage is known to induce single strand breaks as well as bulky adjuncts to DNA through dimer cross-linking (67). Such damage to DNA is known to halt the cell cycle through induction of single strand DNA response signals as detailed in preceding chapters. In this series of experiments we seek to understand the role of p38 MAPK activity on cellular response to UV induced DNA damage. We believe that if p38 plays a key role in G2 DNA damage in response to UV, both biochemical inhibition of p38 activity as well as RNAi knock-out of p38 should have phenotypic differences.

We first investigated if inhibition of p38 affected the ability of cancer cells to enter mitosis following UV damage. Similar to the experiments involving Adriamycin as a DNA damaging agent, a selective Chk1-inhibitor was used as a suitable counter-point to selective p38 pathway inhibition

To test the effect of p38 inhibition on response to UV damage, unsynchronized HeLa cells were treated with 50mJ/cm² of UV irradiation, followed by the immediate addition of fresh media containing 320nM p38 inhibitor or 2μM Chk1 inhibitor. In addition, 120nM of nocodazole was added to the media to trap in mitosis, cells that may escape the G2 checkpoint arrest. Measuring phospho-Histone H3 as a marker for mitotic entry, HeLa cells treated with a Chk1 inhibitor had a dramatic increase in mitotic index,

while cells treated with a p38 inhibitor had no significant increase in mitotic index over time (Figure 3.13A).

In addition, the effect of UV irradiation and p38 inhibition was tested on synchronized HeLa cells. HeLa cells were synchronized by double thymidine block and upon release, dosed with 100mJ/cm² UV irradiation. Following irradiation cells were incubated with fresh media containing p38 inhibitor or MK2 inhibitor or Chk1 inhibitor. Cells were harvested 10 hours after release from thymidine block, as by this time the majority of control (un-irradiated) cells were observed to be in mitosis (Figure 3.13B lane1). Effective inhibition of p38 and MK2, as measured by decreased levels of phospho-MK2 and phospho-HSP27 had no measurable increase in phospho-Histone H3 levels, contrasting with the effect of Chk1 inhibition which had a significant increase in the mitotic marker (Figure 3.13B).

This data showed that despite effective inhibition of p38 pathway activity, HeLa cells were still able to mount an effective checkpoint arrest to UV damage. Inhibition of Chk1 activity however, led to an abrogation of the UV damage induced G2 checkpoint arrest.

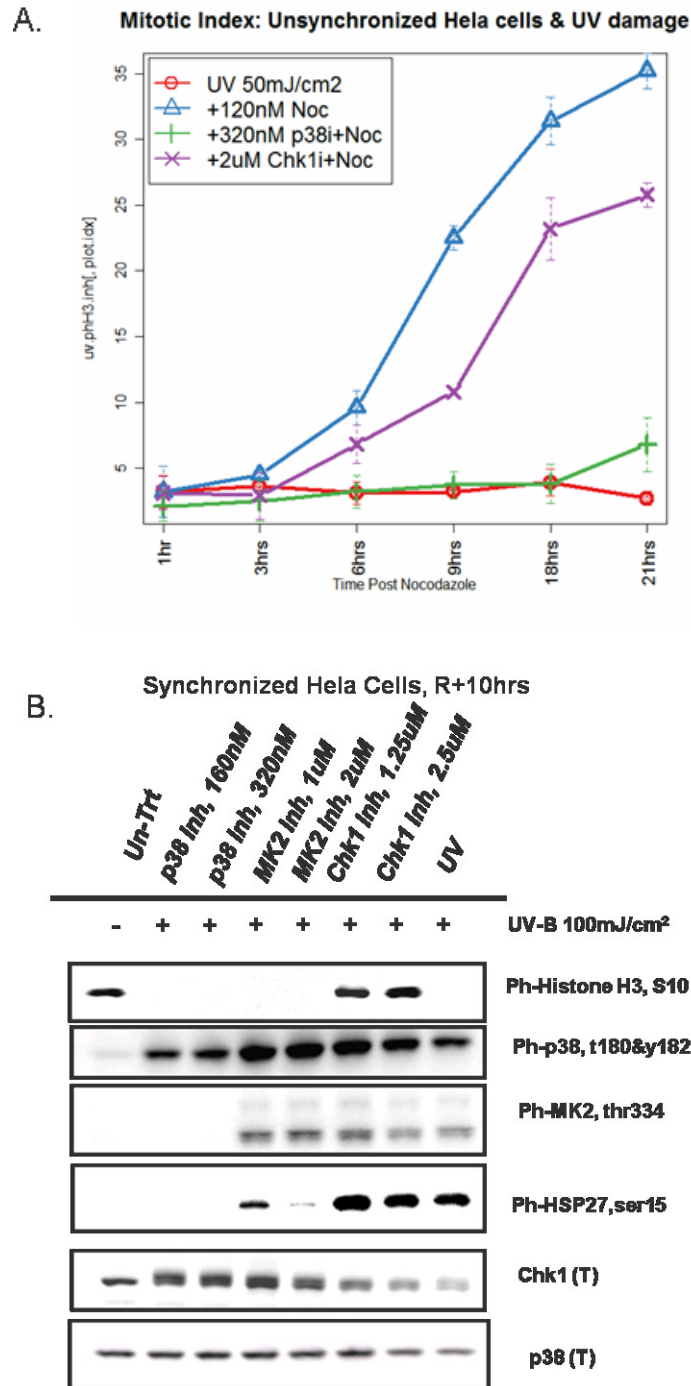


Figure 3.13: Effect of biochemical inhibition of p38, MK2 and Chk1 on UV damage in thymidine synchronized HeLa cells. (A) Plot of mitotic index (%ph-Histone H3) at select time points of unsynchronized HeLa cells treated with either 320nM p38 inhibitor or 2 μ M Chk1 inhibitor and 120nM Nocodazole. (B) Western blot assay of 3mM thymidine synchronized HeLa cells, 10hrs post release from UV and treated with respective levels of biochemical inhibitors.

3.7.1 Transient KD of p38 was unable to abrogate UV DNA damage checkpoint in HeLa or U2OS cells

Similar to experiments involving Adriamycin, we were interested in examining if the effects observed with the use of biochemical inhibitors could be reproduced using an alternate mechanism of silencing p38 pathway such as RNAi. For this experimental series, commercially validated Qiagen siRNA oligonucleotides were used to transiently silence the p38 and Chk1 pathways. HeLa cells were transfected with siRNA oligonucleotides targeting p38 and Chk1 for 48 hours. Following 48hour transfection, cells were exposed to 50mJ/cm² of UV irradiation, and incubated in fresh growth media with 120nM nocodazole which will trap in mitosis any cells that escape the G2 checkpoint arrest (Figure 3.14).

Effect of siRNA KD on UV Damage in Hela

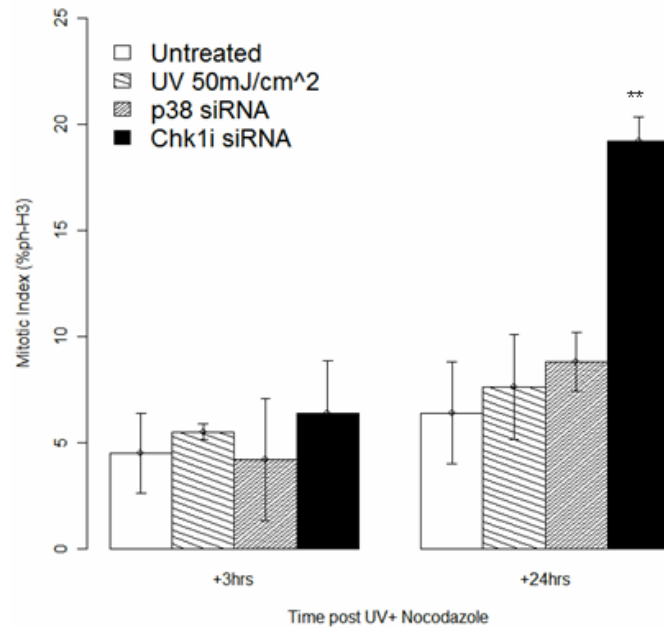
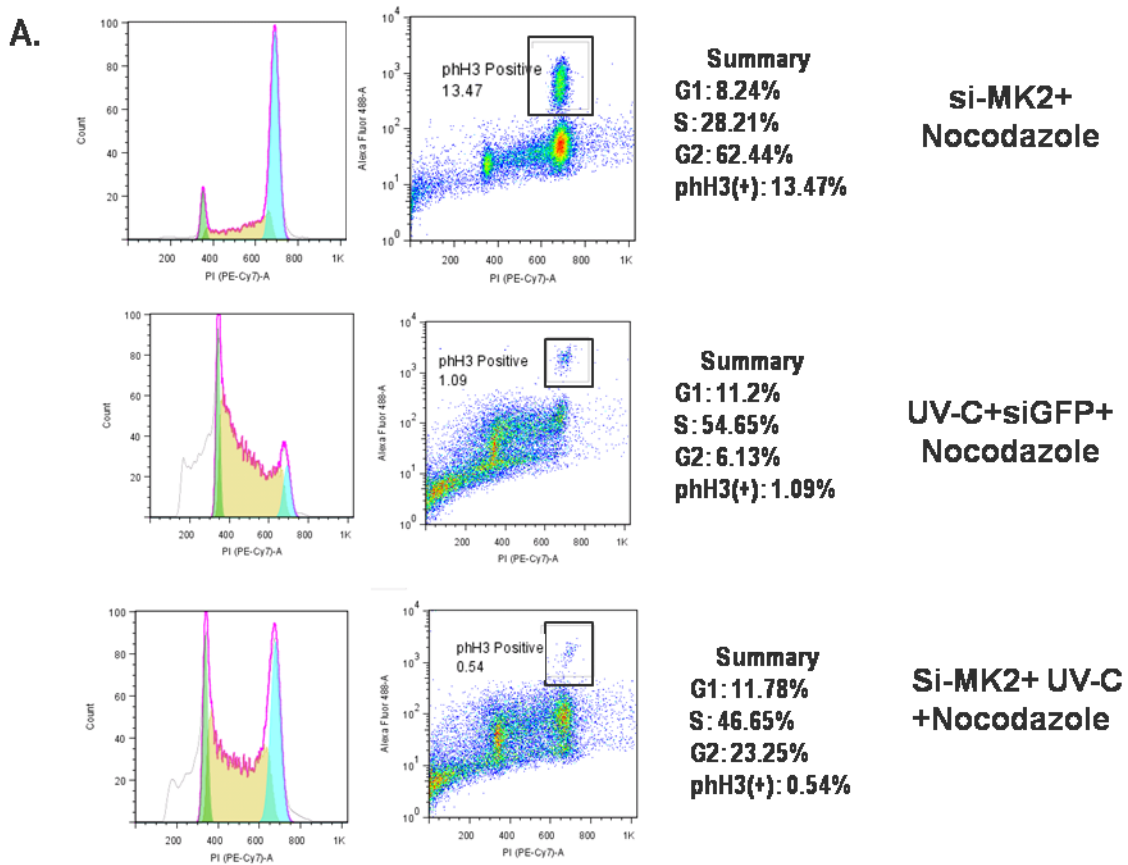


Figure 3.14: Effect of siRNA KD of p38 and Chk1 on UV damage induced G2 checkpoint arrest

Transient siRNA knock-down of p38 in HeLa cells had no significant effect on the mitotic index, measured by phospho-Histone H3 levels over time. SiRNA knock-down of Chk1 in contrast, led to an increase in phospho-Histone H3 levels, similar to what was observed by biochemical inhibition (Figure 3.14). Previous western blot data as shown in figure 3.11B, demonstrated that the siRNA oligonucleotides targeting both p38 and Chk1 effectively reduced their protein expression. This data supports the observations made with LY479754, that inhibition or knock-down of p38 is unable to abrogate a UV DNA damage G2 checkpoint in HeLa cells.

In addition to experiments conducted in HeLa cells, work was done to reproduce the Manke et al, publication on UV-C damage in U2OS cells.



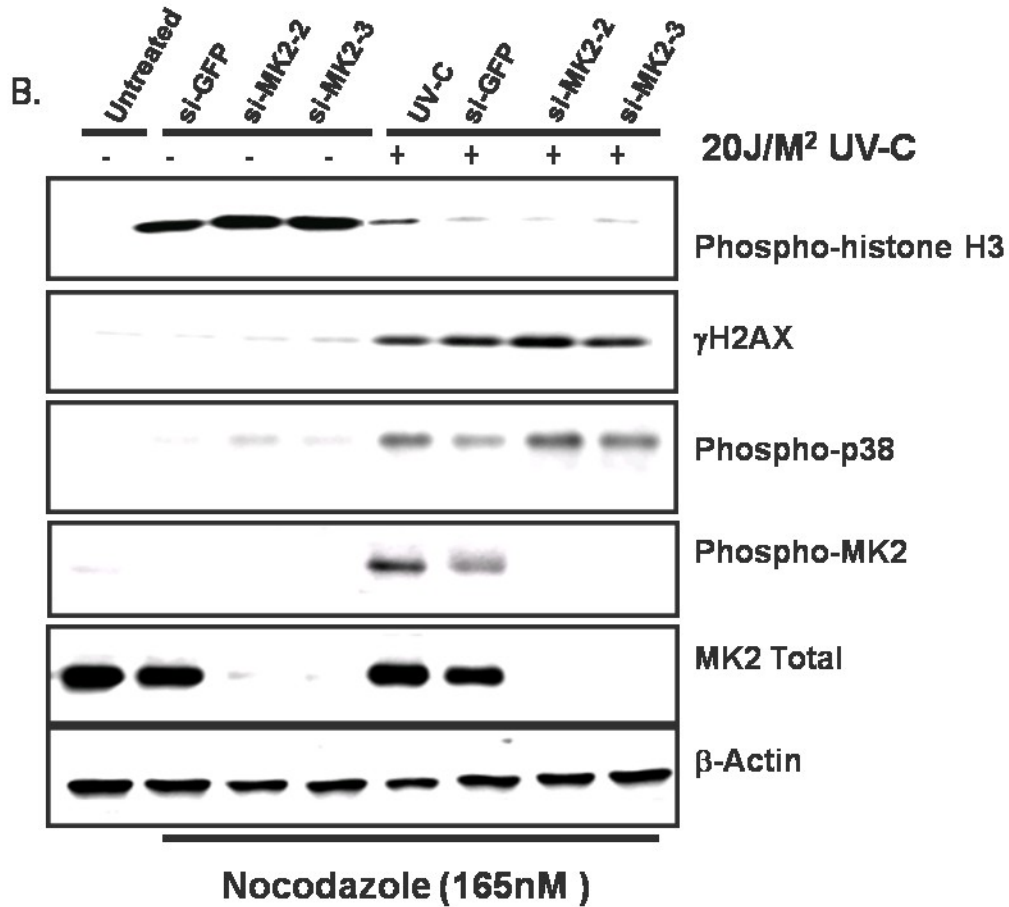


Figure 3.15: Effect of siRNA KD of MK2 with UV-C irradiation in U2OS cells. (A) FACS scatter plot of phospho-Histone H3 and DNA content of siMK2 or siGFP transfected cells +/- 20J/m² UV-C irradiation and 165nM nocodazole. (B) Western blot assay of siMK2 or siGFP transfected cells +/- 20J/m² UV-C irradiation and 165nM nocodazole.

Despite repeated attempts to reproduce the Manke et al results with siRNA knock-down of MK2 and response to UV-C irradiation, we found that siRNA knock-down of MK2 did not compromise the ability of U2OS cells to mount an effective G2 DNA damage checkpoint arrest.

3.7.2 Biochemical Inhibition of p38 and Chk1 have no effect on UV induced G2 checkpoint Arrest in A549 cells

Similar to the experimental regime for the study of Adriamycin induced G2 checkpoint arrest, for UV damage we were interested to test if the the results were specific to the HeLa cell-line model system. To address this, A549 lung cancer cell-lines were synchronized by double thymidine block and upon release irradiated with 50mJ/cm² of UV irradiation. Immediately following UV irradiation, fresh growth media containing 500nM p38 inhibitor or 1.5μM Chk1 inhibitor were added. Cells were tracked for a 24 hour time period post irradiation.

In A549 lung NSCLC cell-lines, biochemical inhibition of p38 did not have any significant effects on the mitotic index of thymidine synchronized A549 cells (Figure 3.16). We noted that control A549 cells released from thymidine block begin to enter mitosis around 12 hours after release from 2nd thymidine block, and that nocodazole treated cells show steady accumulation in mitosis across a 24hour period. Within the same time period, biochemical inhibition of p38 and Chk1 kinase in a p53 functional background, were unable to abrogate the G2 checkpoint in response to UV damage (Figure 3.16). The results of A549 cells in response to UV damage are similar to results previously reported for Adriamycin DNA damage. The data presented in this section also supports the hypothesis that Chk1 kinase inhibition is not sufficient to over-come a G2 DNA damage arrest in p53 wild-type cells.

UV Damage in Thymidine Synchronized A549 Cells

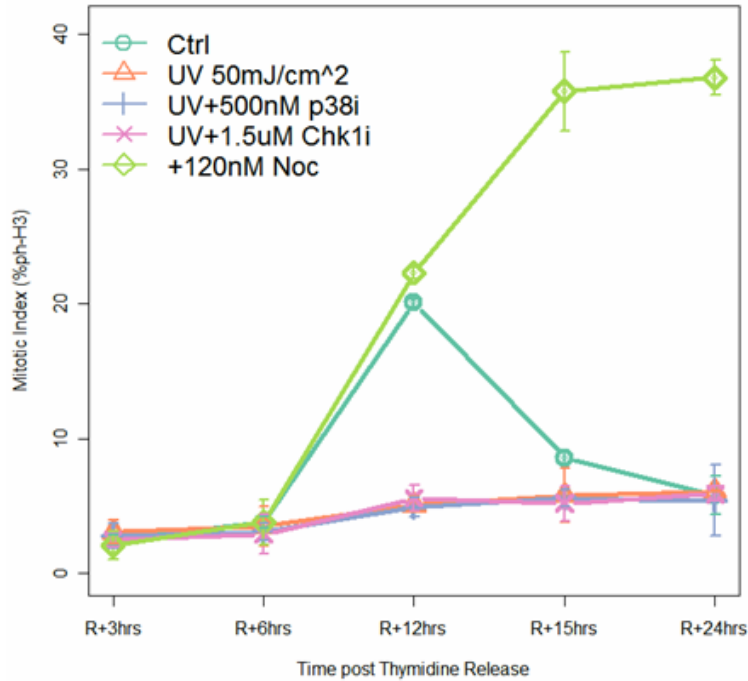


Figure 3.16: *Effect of biochemical inhibition of p38, and Chk1 on UV damage in thymidine synchronized A549 cells, mitotic index plot (ph-Histone H3)*

3.8 Activation of p38 at G2 without DNA damage does not inhibit entry into mitosis

If p38 activity is indeed important in execution and maintenance of DNA damage checkpoint induced G2 arrest, activation of p38 outside of the checkpoint context may be expected to impede progression into mitosis by untimely activation of the G2 checkpoint. Therefore, we investigated the effect of non-genotoxic activation of p38 by anisomycin, a potent antimicrobial agent, on the onset of mitosis.

In HeLa cells, we first synchronized cells using the CDK1-inhibitor (RO-3306) block/release protocol as discussed previously. As CDK1 inhibitor treatment effectively blocks cells at the G2 to M boundary, CDK1 inhibition was an appropriate synchronization agent to use for this experiment. Anisomycin is an antimicrobial agent that functions through inhibition of protein synthesis. Short term exposure of anisomycin has been shown to strongly induce p38 in cancer cells, without inducing DNA damage. We performed a series of experiments in HeLa cells to understand if strong stimulation of p38, with an anti-microbial agent was able to prevent or impede cells from entering mitosis following release from CDK1 block. Briefly, HeLa cells were synchronized with 9uM CDK1 inhibitor (RO-3306) for 21 hours, and 1 hour prior to release from CDK1 inhibitor block 2ug/ml anisomycin was added. Cells were subsequently released from CDK1 block by 3x fresh media wash out and tracked across time for entry into mitosis.

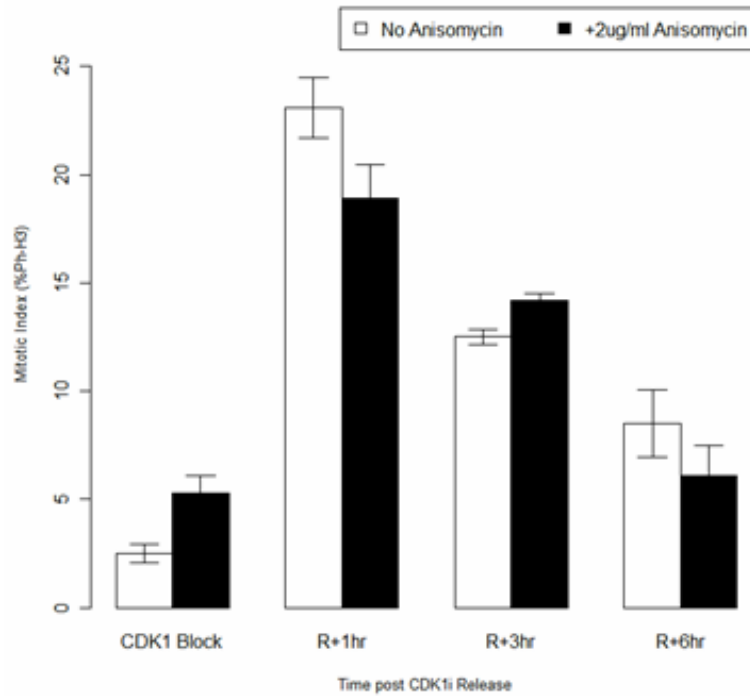
HeLa cells released from CDK1 inhibitor block, entered mitosis within 1hour of release marked by high levels of phospho-Histone H3, as previously described (423). HeLa cells treated with anisomycin also entered mitosis in the same time frame as untreated control cells, despite anisomycin treatment inducing strong activation of p38 (Figure 3.17).

Strong activation of p38 by anisomycin was unable to block cells from progressing into mitosis. Based on an Acumen multi-parametric high content imaging assay, HeLa cells treated with anisomycin were not impeded in their entry into mitosis following release from CDK1 inhibition (Figure 3.17A). Protein assays conducted at matching time points to identically treated HeLa cells show a strong activation of p38 by anisomycin prior to CDK1 release and subsequently post release (Figure 3.17 lower panel). In addition, both untreated control and anisomycin treated cells displayed a steady decrease in ph-Histone H3 levels as time progressed, suggesting that cells were not being trapped in mitosis. This data suggests that despite high levels of p38 activity, cells were able to enter and exit mitosis normally. This implies that p38 activity alone was not sufficient to activate a cell cycle arrest at the G2 to M transition point.

Taken together with data produced with the p38 inhibitor and siRNA experiments, it suggests that p38 does not have a role in the regulation of cells transiting through the G2 checkpoint into mitosis.

Non-Genotoxic Activation of p38 does not inhibit mitotic entry

A.



B.

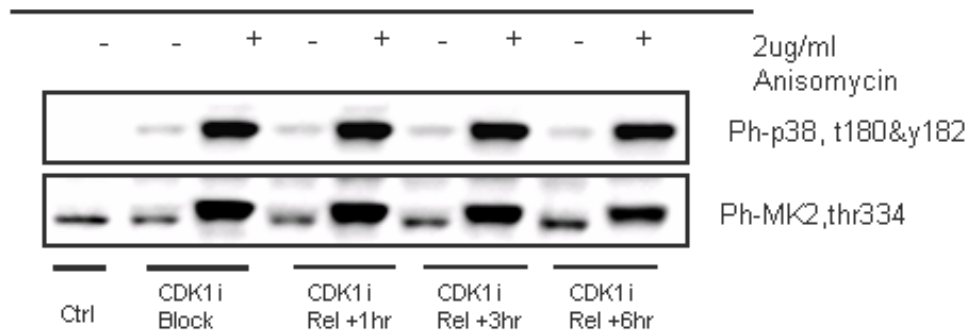


Figure 3.17: Effect of non-genotoxic stimulation of p38 on ability of cancer cells to enter mitosis. (A) Acumen High content assay of mitotic index (ph-Histone H3) of HeLa cells first synchronized with 9uM RO-3306 (CDK1 inhibitor) and treated with 2ug/ml anisomycin, a 6 hour time series. (B) Western Blots of HeLa cells synchronized with 9uM RO-3006 and subsequently treated with 2ug/ml anisomycin.

3.13 Summary

In this study we explored p38's role in the control of the G2 DNA damage checkpoint. Using a combination of chemical biology as well as RNAi technology, we explored if there was a strong causal link between p38 activity and the establishment and maintenance of the DNA damage G2 cell cycle checkpoint. To our surprise and contrary to some earlier publications, we found that in various cancer cell line models there was no strong link between p38 activity, and the establishment or maintenance of the G2 DNA damage checkpoint. This was shown via biochemical inhibition of p38 with a potent and specific inhibitor to p38 having no effect on the establishment and maintenance of the G2 DNA damage checkpoint in multiple tumor cell-line models in response to Adriamycin, UV and MMS damage. In addition, effective siRNA knock-down of p38 was also unable to relieve the G2 checkpoint in response to Adriamycin and UV in these cancer cell line models. We also investigated the effect of non-genotoxic activation of p38 through the use of the anti-bacterial agent anisomycin, and found that untimely activation of p38 was unable to prevent cells from entering mitosis.

Taken together this shows that inhibition of p38 through both biochemical and siRNA approaches as well as non-genotoxic activation of p38 had no effect on the G2 DNA damage checkpoint function in cancer cell-lines. Instead inhibition of Chk1 in a p53 functionally null environment led to deactivation of the DNA damage G2 cell cycle checkpoint. Our data corroborates previously published data suggesting that Chk1 kinase activity is critical for the proper function of the G2 checkpoint in a p53 functionally null environment (440). As the majority of cancers have dysfunctional p53 pathways, this data suggests that inhibition of Chk1 may be a promising therapeutic strategy for increasing sensitivity to chemotherapeutic agents.

In summary, Chk1 activity but not p38 activity is required for the proper activation and maintenance of the DNA damage G2 cell cycle checkpoint in p53 null, transformed cancer cell lines.

Chapter 4: Effect of p38 inhibition on TNF- α induced inflammatory response and apoptosis

4.1 Background

As p38 is activated in response to DNA damage, but its activity is not associated with the execution or maintenance of the G2 checkpoint (Chapter 3), it is possible that its activity is related to another critical cellular function that may be beneficial to cells exposed to physiological stress. To explore this possibility, we decided to return to the original context of p38 activation: response to inflammatory cytokines. The p38 MAPK was originally discovered as a 38 kDa protein that underwent rapid tyrosine phosphorylation in response to lipopolysacchride (LPS) stress (225). The role of p38 in inflammatory response is well studied and its involvement in the initiation of angiogenesis (399,472), contributing to the establishment of the tumor microenvironment has made it an attractive target for therapeutic intervention.

The tumor microenvironment consists of the cells surrounding and supporting the ability of a tumor mass to proliferate, grow and escape death. The tumor microenvironment or stroma is believed to be a key factor relating to the ability of tumor cells to initiate, grow and ultimately metastasize to secondary locations. The stroma consists of a mixture of extracellular matrix components, cells and extracellular matrix molecules such as growth factors and cytokines (229,298).

Activities associated with the establishment of a tumor microenvironment include angiogenesis, or the ability to stimulate and grow blood vessels to promote tumor growth have been linked to the plethora of growth factors and cytokines that are produced by the stroma tissue and from the tumor itself. (354) Over-expression and dysregulation of cytokines such as TNF- α and FGF-1 are believed to play key roles in the establishment of

a persistent inflammatory response that contributes to the growth of a tumor. The interaction between the stroma and the tumor cell is believed to be dynamic and changes throughout the different stages of tumor growth (417). In the initial stages of tumor development the surrounding stroma sets the ground for successful tumor establishment by increased production of cytokines, neutrophils and growth factors. The increased production of cytokines and growth factors leads to sustained inflammation at the site of tumor growth (353,428).

The link between the need to sustain an inflammatory microenvironment and tumor progression has led to research on the molecular pathways involved in inflammatory response. TNF- α is one of the cytokines that is believed to be highly dysregulated during tumor formation. While TNF- α has a role in normal immune response including the induction of extrinsic apoptosis pathway, high levels of TNF- α have been associated with increased tumorigenesis. (275,329,436). It is reported that high TNF- α levels contributes to the establishment and maintenance of the tumor microenvironment as well as aids in up-regulation of cell survival pathways. A key pathway in the response to mitogenic growth signals and a key player in sustained response to inflammation is the p38 MAPK pathway.

In this experiment, we explored the molecular pathways regulated by TNF- α induction in Calu6 cells through whole genome transcriptome analysis. As the p38 MAPK pathway plays a critical role in the response to inflammatory cytokine induction, we wanted to explore the effect of p38 inhibition on the response to TNF- α treatment. The use of DNA micro-arrays (gene chip) creates a new unbiased paradigm in understanding gene regulation induced by TNF- α in Calu6 lung carcinoma cells. In the upcoming analysis, we investigated the global function of p38 MAPK in response to

TNF- α induction, through DNA gene chip technology. The study design for this experiment is detailed further in section 4.3 of this chapter.

4.2 TNF α induces p38 MAPK activity in Calu6 Cells

While p38 is reported to be strongly activated by TNF- α treatment in both untransformed and transformed cells, the aberrant genetic nature of cancer cells requires that we check the degree of p38 induction in Calu6 cells prior to beginning a gene expression study. To test the effect of TNF- α , Calu6 cells are treated with 25ng/ml of TNF- α for a 7 hour time series (Figure 4.1).

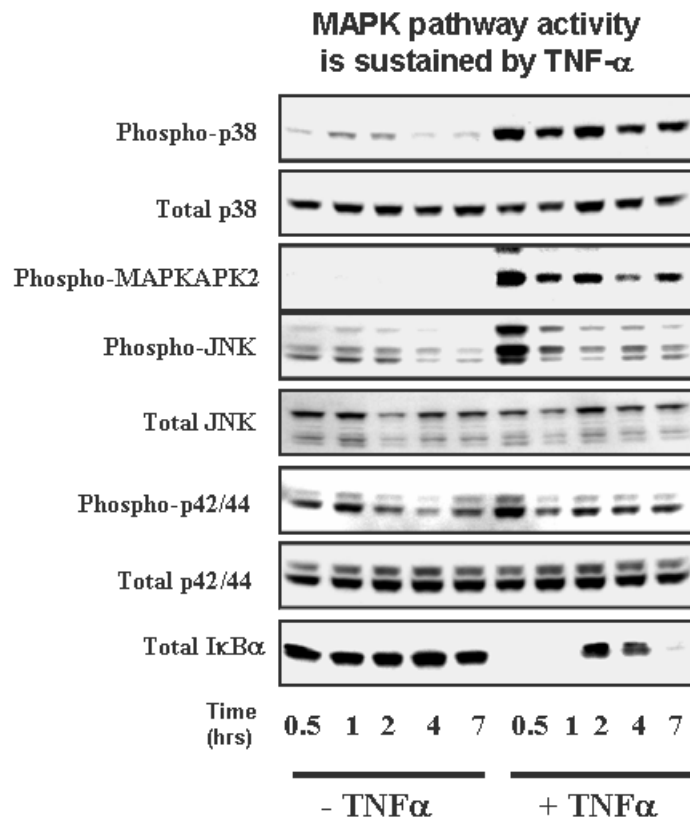


Figure 4.1: *MAPK pathway is strongly induced by TNF- α treatment in Calu6 cells*

Treatment with 25ng/ml TNF- α strongly and rapidly activates p38 MAPK in Calu6 lung carcinomas (Figure 4.1). TNF- α treatment also strongly activates other members of the MAPK family, as p44/42 (ERK1/2) and JNK (SAPK) also show concordant activation in the same timescale as p38 MAPK.

The rapid and sustained activation of p38 by TNF- α makes this an ideal stress for the study of p38 MAPK activity in response to inflammatory cytokines.

4.2.1 LY479754 effectively inhibits TNF- α induced p38 activity

Similar to experimental assays discussed in chapter 3 of this thesis, we use LY479754 as a biochemical inhibitor of p38 MAPK activity. We show that in response to TNF- α induced activity, LY479754 effectively suppresses p38 MAPK activity.

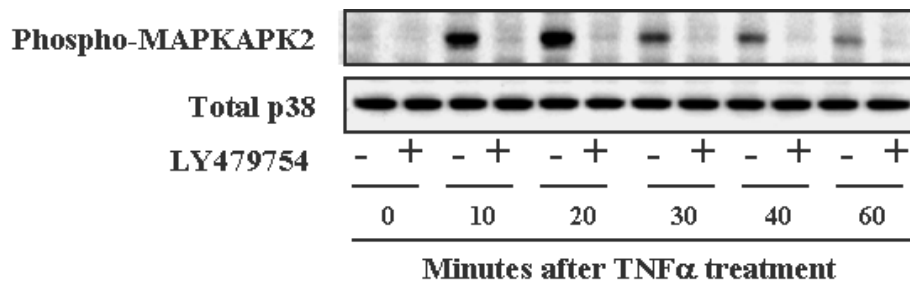


Figure 4.2: *Phospho-MAPKAPK2 levels as marker of p38 MAPK activity, post TNF- α treatment. 320nM LY479754 effectively inhibits p38 activity in response to TNF- α*

As shown in figure 4.2, LY479754 effectively suppresses ph-MK2 levels post TNF- α treatment. LY479754 is thus an effective inhibitor of p38 MAPK activity induced by TNF- α treatment.

4.3 Gene-Chip Experimental Design

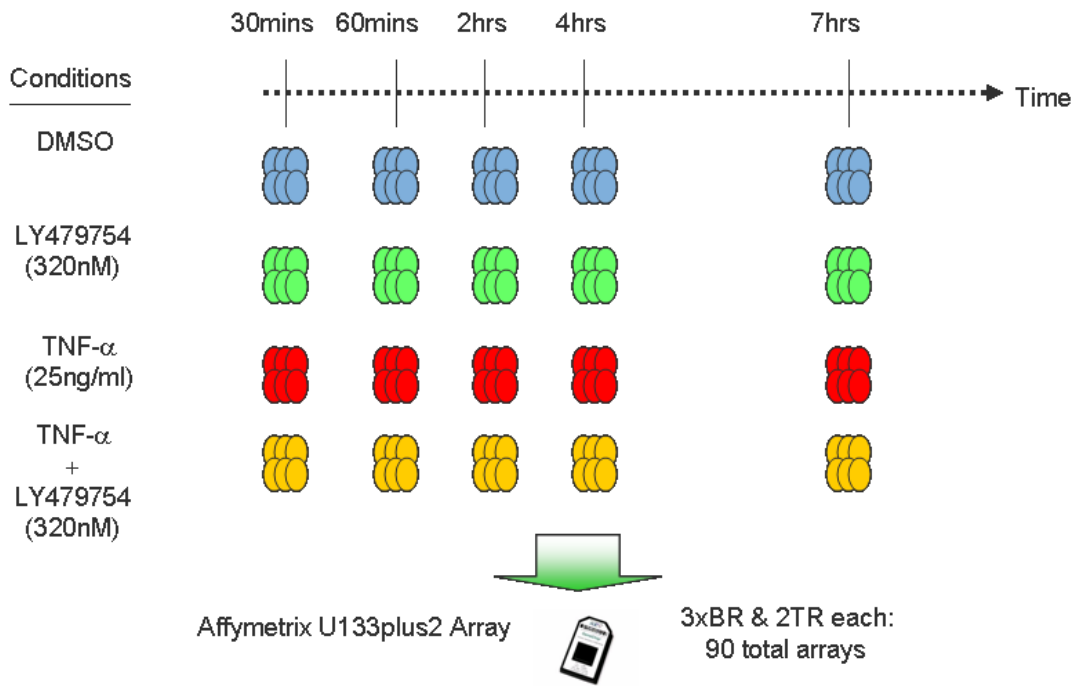


Figure 4.3: *Experimental design for Gene-Chip experiment involving p38 inhibitor (LY479754) and TNF- α in Calu6 Cells*

We tested 4 treatment conditions during this experiment: 1. DMSO only, as the control condition, 2. LY479754 only, as a control for single agent effects of the p38 inhibitor, 3. TNF- α (25ng/ml) only, to understand the effect of TNF- α , and 4. TNF- α +LY479754 to understand the combinatorial effect of p38 inhibition with TNF- α treatment.

All conditions were run in 3 independent biological replicates and 2 technical replicates each for a total of 6 affymetrix U133plus2 chips per condition.

4.4 TNF- α induces inflammatory response genes in a time dependent manner

We first analyzed the gene expression data generated on the Affymetrix HG-U133A chip, by examining the effect of TNF- α on Calu6 cells. As described in the

methods and materials chapter, significant genes were identified using the *Significance Analysis of Microarray (SAM)* algorithm developed by Tibshirani et al. (385,416) we used a local false discovery rate (FDR) of 0.1 and a pair-wise fold change of 1.5, as the cut-off for significance across each condition.

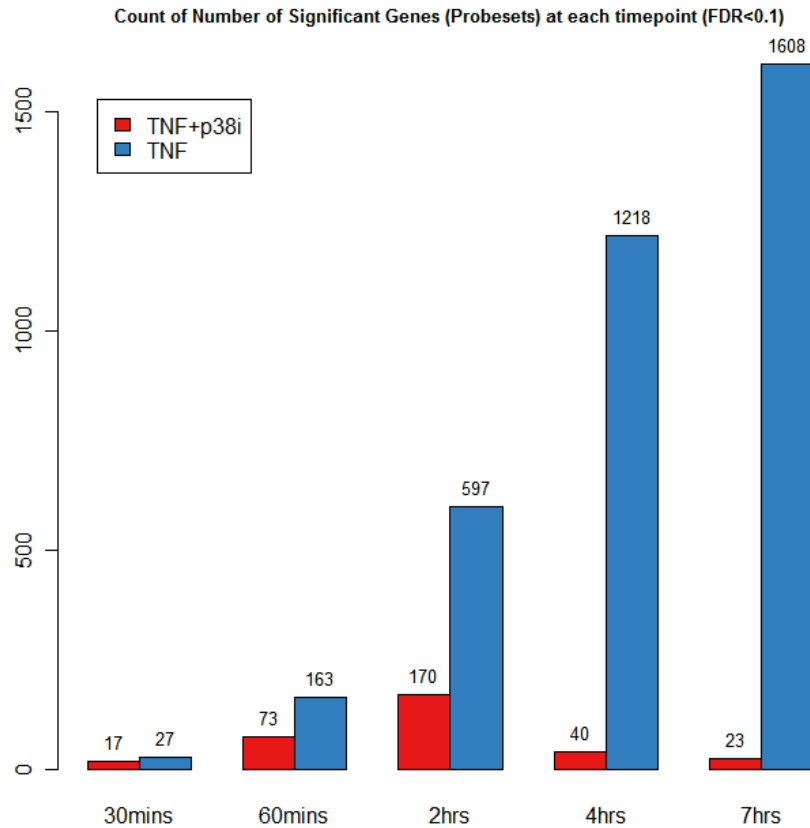


Figure 4.4: *Count of number of significant probesets at each timepoint for TNF vs DMSO comparison.*

As can be observed in figure 4.3, upon TNF- α treatment, the number of significant genes (probesets) increases steadily across time, suggesting that TNF- α induces a strong transcriptional response with positive feedback loops leading to more significantly expressed genes at later time points. The number of genes induced by TNF-

α and significantly modulated by a p38 inhibitor (LY479754), shows very strong activity in the early time points, but significantly reduced activity at the later time points. This suggests that inhibition of p38 may have strong effects on the early transcriptional response to TNF- α , but may not play as significant a role in the later time points.

The analysis of TNF- α effects on the Calu6 cells will be divided into three major time categories, mainly early, mid and late. The early timepoints will cover the 30min and 60min time point. The mid time period will cover the 2hour time point and the period of late will cover the 4 hour and 7 hour timepoint.

4.5 Early transcriptome effects of TNF- α treatment on Calu6 cells

The early transcriptional response to TNF- α mainly consists of the activation of immediate early response genes. The immediate response of cancer cells to TNF- α induction is indicative of the key pathways that are induced to sustain the inflammatory response, a key factor to maintaining a suitable microenvironment for tumor growth. As seen in figure 4.4, we observed that 24 genes and 133 genes were significantly modulated by TNF- α at the 30min and 60min time points respectively. An overlap of the genes modulated at the early time points by TNF- α showed that 90% of the genes modulated significantly at 30mins were also modulated at 60mins (Figure 4.5). Subsequent downstream analysis of genes for early transcriptional response to TNF- α , will be based on the 60min gene list.

Overlap of Significant genes at 30mins & 60mins
For TNF- α Treated Cells

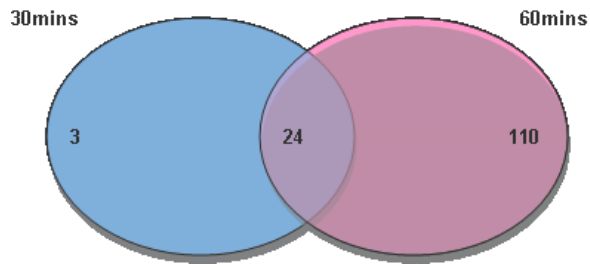


Figure 4.5: *Overlap of significant genes (probesets) at 30mins and 60mins TNF- α treatment*

Genes modulated by TNF, 60min Time point
in Calu-6

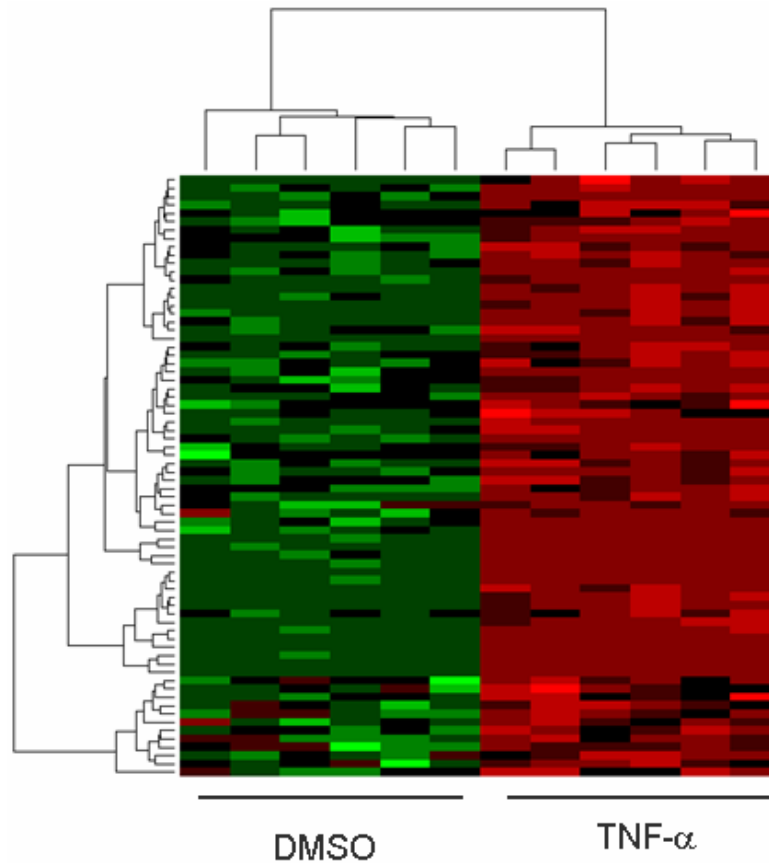


Figure 4.6: *Compacted Heatmap of genes significantly modulated by TNF- α at 60mins, with $FC(\log_2) > 1.5$ filter & $FDR < 0.1$*

As can be observed in figure 4.6, a significant number of genes are strongly modulated by TNF at the 60min time point. Genes strongly modulated by TNF- α at 30min were also significantly modulated at 60min, representing a strong overlap in signaling pathways. The genes that are regulated by TNF- α include early response genes such as EGR2, IER3 and the cytokines IL8, CXCL3, the Jun kinase and members of the NF κ B family. These genes have been characterized to play a role in stress response, mitogen response and inflammation

Due to the high dimensionality of gene expression data, we will highlight functional pathways of significance that are modulated by TNF- α treatment. Functional pathway analysis for the following sub-sections was conducted using two pathway analysis tools. The first is the DAVID tool published by the NIH for functional annotation and clustering of gene expression data (164,165). The second tool used in this analysis is the Ingenuity Pathway Analysis Tool (324,337). The DAVID tool relies primarily on Gene Ontology, protein domain (e.g. Interpro & PdB), and public pathway and interaction databases such as KEGG and Bind to functionally annotate gene lists. The Ingenuity pathway analysis tool relies on manual curation of literature, and a combination of public and proprietary protein-protein, protein-DNA databases to find networks and associations between expressed genes.

In the following analysis we will also integrate the effects of the p38 inhibitor, as the primary purpose of this study to gain a mechanistic understanding of the effects of inhibition of p38 in response to TNF- α stress. This will allow the highlighting of genes and pathways that are modulated by TNF- α and are also the target of a specific p38 MAPK inhibitor.

I. *TNF- α induces Immediate Early Response Genes that is blocked by p38 inhibitor*

Among the groups of genes that were strongly modulated by TNF- α in the early time points, immediate early response genes such as IER2& IER3 as well as the EGR1 genes prominently stood out. In addition to the genes which are named to correspond with their function in early response to stress, the Jun transcription factor is also induced strongly, early in response to TNF- α treatment. These genes are associated with immediate early response to stress, and upon TNF- α treatment are strongly induced.

To illustrate that p38 MAPK plays a key role in the propagation of early stress signals, we showed that addition of 320nM of p38 MAPK inhibitor LY479754 is capable of modulating the gene expression response of some of these early response genes. The normalized gene expression signals for this class of genes are illustrated in figure 4.7. The boxplots (plotted in log₂ scale), illustrate a significant up-regulation of expression in the immediate aftermath of treatment with 25ng/ml of TNF- α . These plots show that p38 plays a role in early response to stress by observing a statistically significant decrease in expression upon p38 inhibitor treatment (Figure 4.7).

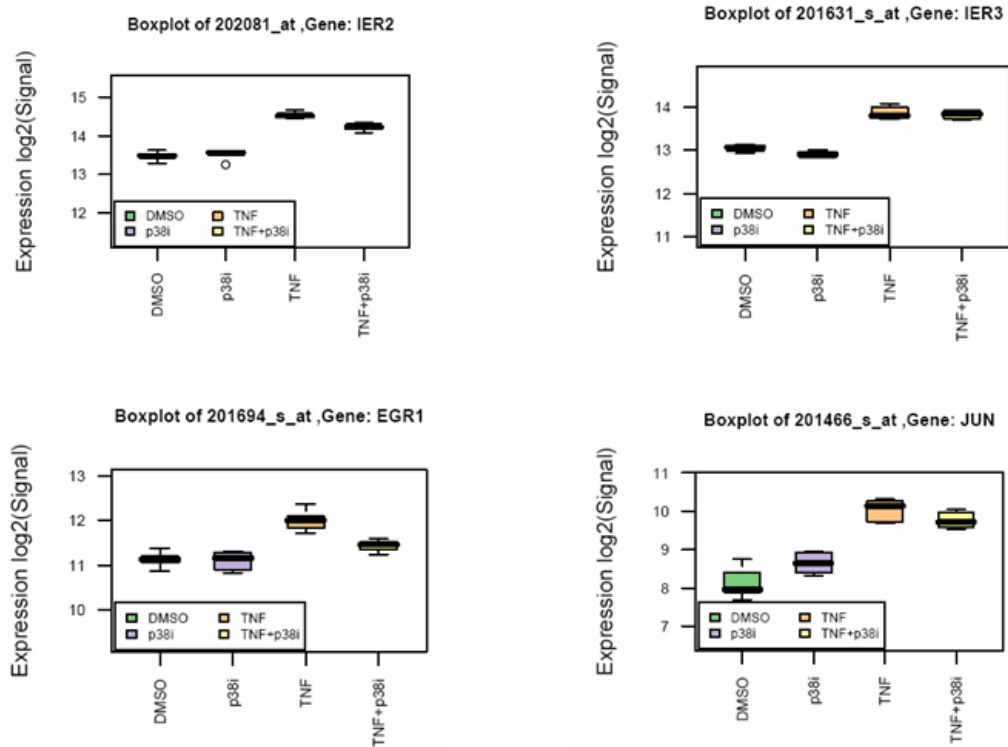


Figure 4.7: *Boxplot of selected immediate early response genes. Boxplot plot order(L-R): 1.DMSO control, 2. p38i (LY479754), 3. TNF- α , 4. TNF- α +p38i (LY479754)*

Affy ID	Gene Name	TNF/DMSO log2(FC) (30min)	TNF+p38i/ TNF log2(FC) (30min)	Entrez Description
201694_s_at	EGR1	1.31170662	-0.856174	Early growth response protein 1 (EGR-1) (Krox-24 protein) (Transcription factor Zif268) (Nerve growth factor-induced protein A) (NGFI-A) (Transcription factor ETR103) (Zinc finger protein 225) (AT225). [Source:Uniprot/SWISSPROT;Acc:P18146]
205249_at	EGR2	1.50800623	-0.750126	Early growth response protein 2 (EGR-2) (Krox-20 protein) (AT591). [Source:Uniprot/SWISSPROT;Acc:P11161]
201473_at	JUNB	1.73309883	-0.727426	Transcription factor jun-B. [Source:Uniprot/SWISSPROT;Acc:P17275]
202081_at	IER2	1.06134708	-0.311251	Immediate early response gene 2 protein (Protein ETR101). [Source:Uniprot/SWISSPROT;Acc:Q9BTL4]

Table 4.1: *Gene table of early response genes induced by TNF- α and modulated by LY479754 treatment*

II. *TNF- α treatment activates death receptor signaling pathways, and anti-apoptotic genes*

In normal physiological contexts, TNF- α treatment is known to induce cell death through the induction of the programmed cell death pathway. In the immediate aftermath of TNF- α treatment, genes and pathways related to the programmed cell death pathway as determined by the DAVID bioinformatics tool, were strongly induced by TNF- α treatment (Figure 4.8). The genes related to programmed cell death induced early by TNF- α treatment are plotted as a heatmap below.

TNF- α induces Early Apoptosis pathway Signaling

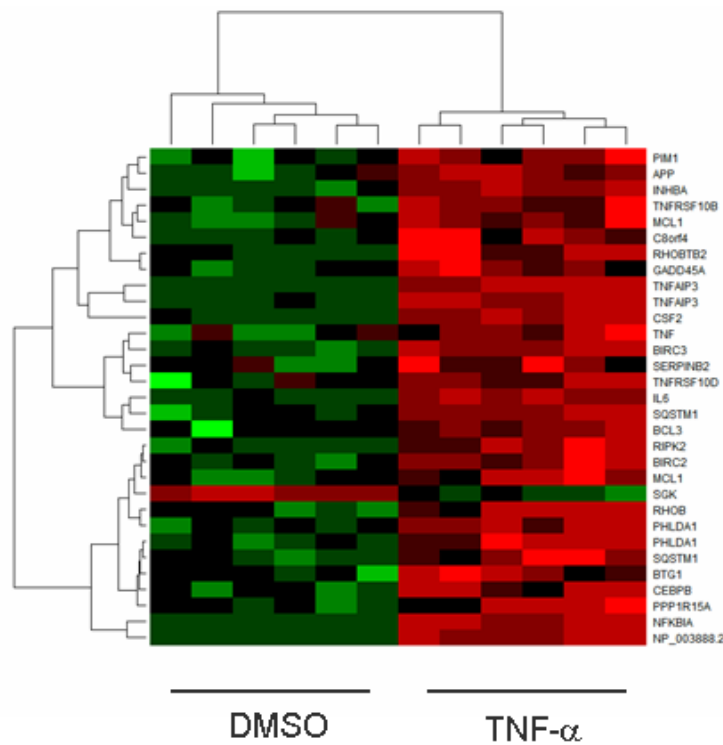


Figure 4.8: *Genes belonging to death receptor and programmed cell death pathway, induced by TNF- α treatment in the early timepoints.*

In cancer cells, normal extra-cellular signaling networks for cytokine and chemokine production have been subverted for the promotion and establishment of a tumor micro-environment (175). As we explored the transcriptional response of cancer cells to TNF- α treatment, we focused on the effect of TNF- α on the activation of the extrinsic apoptosis pathway. A key hallmark of cancer highlighted by Weinberg et al (138), is the ability of cancer cells to evade apoptosis inducing signals. Among the many genes significantly modulated by TNF- α treatment, a number of them belonged to the negative regulation of apoptosis sub-cluster (as defined by functional cluster by DAVID tool). Specifically members of the IAP family of proteins (BIRC2 & BIRC3) as well as the BCL2 family of anti-apoptotic proteins were strongly induced in the early response to TNF- α (Figure 4.9).

Anti-Apoptosis Genes Induced by TNF- α & modulated by p38i

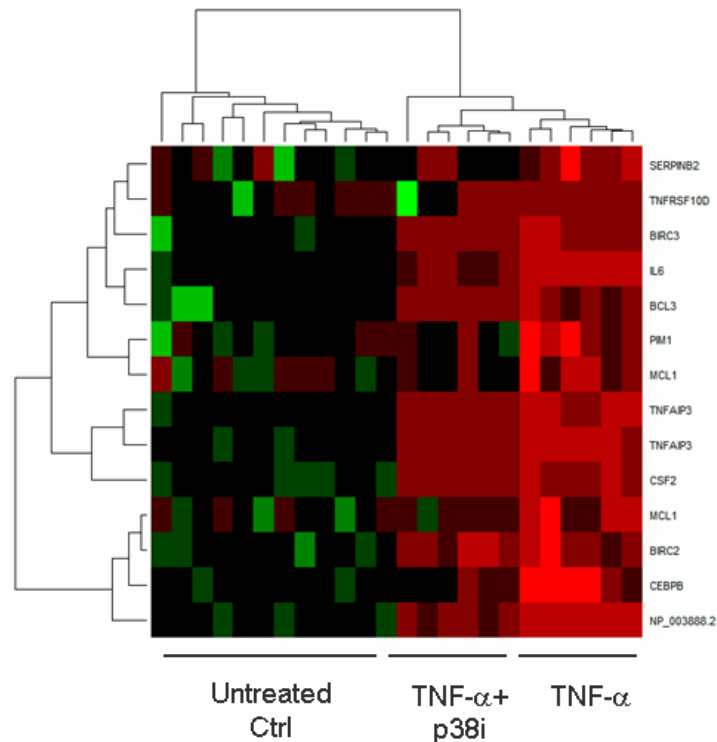


Figure 4.9: *Anti-apoptosis genes induced by TNF- α and modulated by p38-inhibitor (LY479754) at 60mins*

From the heatmap plotted as figure 4.9, it is clear that treatment with a potent p38 inhibitor is able to alter the transcriptional profile of a number of anti-apoptotic genes induced by TNF- α treatment. In the immediate aftermath of TNF- α treatment, cancer cells respond to pro-apoptotic stimulus by inducing strong anti-apoptotic signals to counteract the effect of TNF- α . The p38 MAPK is implicated as a central responder to stress and in this context may play a cyto-protective role.

In addition to genes modulated by both TNF- α and a p38 inhibitor we find that some members of the BCL2 family of genes are strongly modulated by the p38 inhibitor. These genes include the anti-apoptosis gene BCL2L1 (BCL-XL) and BCL2. We plot these two genes as separate box plots below.

Inhibition of p38, modulates BCL2 family proteins at 60mins

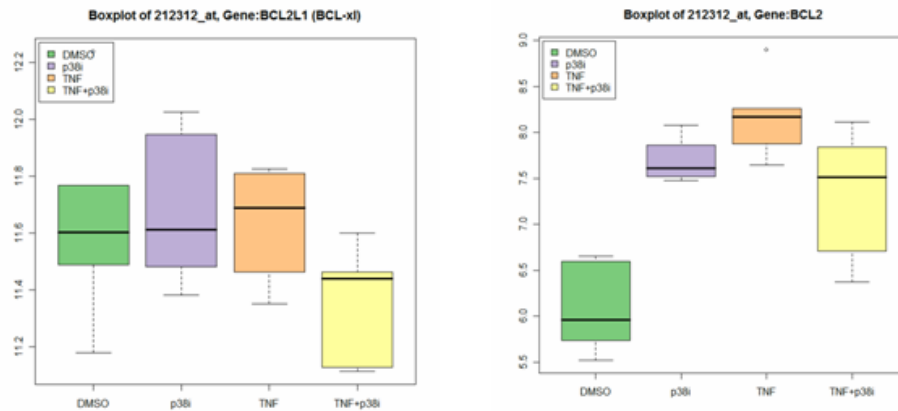


Figure 4.10: *Boxplots of log₂ normalized MAS5 signal: Inhibition of p38 with TNF- α modulates BCL2 anti-apoptosis proteins BCL2 & BCL-xl*

This data suggests that in an early response to stress and to extrinsic pro-apoptotic stimuli, cancer cells activate strongly an anti-apoptotic mechanism. This is evident in the strong induction of inhibitor of apoptosis proteins (BIRC family) as well as the anti-apoptotic members of the BCL2 family of protein (BCL2& BCL-xl). The p38 MAPK may play a role in the transduction of this cell survival signaling pathway as inhibition of p38 with a potent p38 inhibitor significantly impinges on the ability of Calu6 cells from mounting a fast anti-apoptotic response to TNF- α . This microarray data provides some potential mechanistic insight into the multi-faceted role of p38 MAPK in stress response. It may suggest that p38 plays a pro-cell survival role in the early response to stress or extrinsic pro-apoptotic stimuli. A table of genes functionally annotated to have a role in negative regulation of apoptosis (by NIH DAVID tool) is given below as table 4.2.

Affy ID	gene	FC.TNF. CTRL.60 min	FC.p38i. TNF.60 min	Description
205207_at	IL6	2.21193	-0.9002	Interleukin-6 precursor (IL-6) (B-cell stimulatory factor 2) (BSF-2) (Interferon beta-2) (Hybridoma growth factor) (CTL differentiation factor) (CDF). [Source:Uniprot/SWISSPROT;Acc:P05231]
202643_s_at	TNFAIP3	3.42122	-0.8223	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT;Acc:P21580]
202644_s_at	TNFAIP3	4.2892	-0.7124	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT;Acc:P21580]
210538_s_at	BIRC3	2.55434	-0.1245	Baculoviral IAP repeat-containing protein 3 (Inhibitor of apoptosis protein 1) (HIAP1) (HIAP-1) (C-IAP2) (TNFR2-TRAF-signaling complex protein 1) (IAP homolog C) (Apoptosis inhibitor 2) (API2) (RING finger protein 49). [Source:Uniprot/SWISSPROT;Acc:Q13489]
209193_at	PIM1	0.37277	-0.3099	Proto-oncogene serine/threonine-protein kinase Pim-1 (EC 2.7.11.1). [Source:Uniprot/SWISSPROT;Acc:P11309]
204614_at	SERPINB2	0.76686	-0.5927	Plasminogen activator inhibitor 2 precursor (PAI-2) (Placental plasminogen activator inhibitor) (Monocyte Arg-serpin) (Urokinase inhibitor). [Source:Uniprot/SWISSPROT;Acc:P05120]
202076_at	BIRC2	0.48623	-0.0049	Baculoviral IAP repeat-containing protein 2 (Inhibitor of apoptosis protein 2) (HIAP2) (HIAP-2) (C-IAP1) (TNFR2-TRAF-signaling complex protein 2) (IAP homolog B) (RING finger protein 48). [Source:Uniprot/SWISSPROT;Acc:Q13490]
201631_s_at	NP_003888.2	1.78313	-0.6381	immediate early response 3 [Source:RefSeq_peptide;Acc:NP_003888]
200796_s_at	MCL1	0.94105	-0.5507	Induced myeloid leukemia cell differentiation protein Mcl-1 (Bcl-2- related protein EAT/mcl1) (mcl1/EAT). [Source:Uniprot/SWISSPROT;Acc:Q07820]
200798_x_at	MCL1	0.50899	-0.3271	Induced myeloid leukemia cell differentiation protein Mcl-1 (Bcl-2- related protein EAT/mcl1) (mcl1/EAT). [Source:Uniprot/SWISSPROT;Acc:Q07820]
212501_at	CEBPB	0.45254	-0.2935	CCAAT/enhancer-binding protein beta (C/EBP beta) (Nuclear factor NF-IL6) (Transcription factor 5). [Source:Uniprot/SWISSPROT;Acc:P17676]
210229_s_at	CSF2	5.5859	-0.3533	Granulocyte-macrophage colony-stimulating factor precursor (GM-CSF) (Colony-stimulating factor) (CSF) (Sargramostim) (Molgramostim). [Source:Uniprot/SWISSPROT;Acc:P04141]
210654_at	TNFRSF10D	0.91016	-0.6811	Tumor necrosis factor receptor superfamily member 10D precursor (Decoy receptor 2) (DcR2) (TNF-related apoptosis-inducing ligand receptor 4) (TRAIL receptor 4) (TRAIL-R4) (TRAIL receptor with a truncated death domain) (CD264 antigen). [Source:Uniprot/SWISSPROT;Acc:Q9UBN6]
204908_s_at	BCL3	1.81007	-0.0576	B-cell lymphoma 3-encoded protein (Protein Bcl-3). [Source:Uniprot/SWISSPROT;Acc:P20749]
212312_at	BCL2L1	-0.00875	-0.2739	Apoptosis regulator Bcl-X (Bcl-2-like 1 protein). [Source:Uniprot/SWISSPROT;Acc:Q07817]
207005_s_at	BCL2	1.10862	-0.2654	Apoptosis regulator Bcl-2. [Source:Uniprot/SWISSPROT;Acc:P10415]

Table 4.2: Anti-Apoptotic Genes induced by TNF- α in early time points, all genes FDR<0.1

Inhibition of p38 restores FAS death domain signaling in Calu6 cells

In addition to the fast up-regulation of anti-apoptotic pathway components, we observed that Calu6 lung carcinoma cells also attempted to escape apoptosis through direct down-regulation of key intracellular components of the FAS, or death domain receptor signaling pathway. These components include FADD, the key intra-cellular signaling component of the FAS signaling pathway. In addition to FADD a number of

other signaling kinases connected to the death receptor pathway were down-regulated upon TNF- α treatment.

FAS pathway signaling modulated by p38 inhibition at 60mins

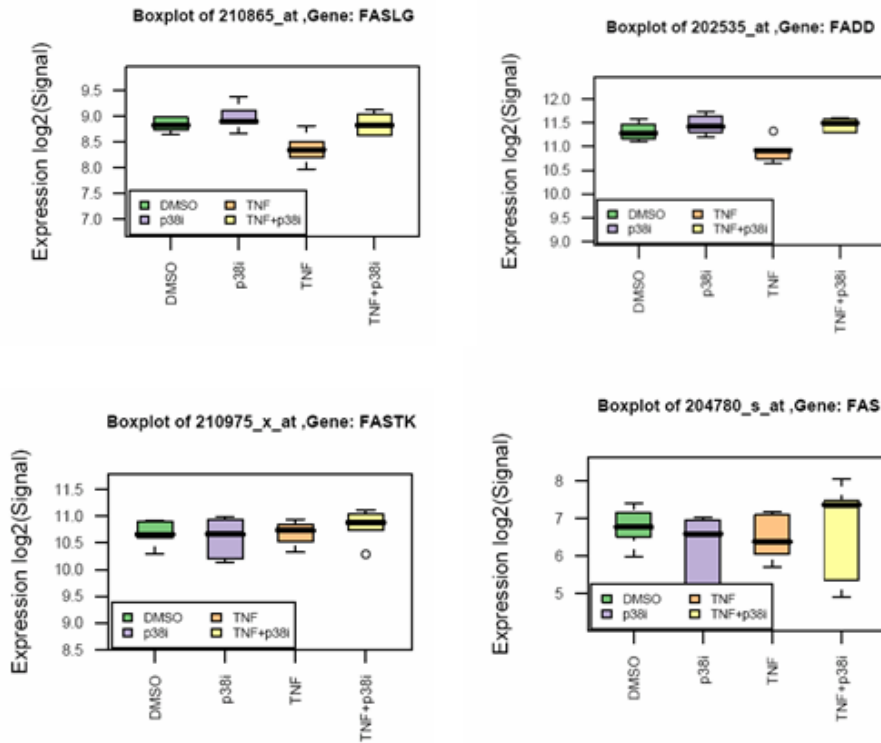


Figure 4.11: *Boxplots of log₂ normalized MAS5 signal: Members of FAS signaling pathway are modulated by p38 inhibition in the early time points.*

Similar to observations that p38 inhibition affected certain components of the inhibitor of apoptosis pathway, inhibition of p38 led to the recovery or increased the expression of these FAS pathway components. This data again suggests that the p38 MAPK pathway is linked to the pro-cell survival response to TNF- α induced stress.

In summary, we observed that genes associated with anti-apoptotic function are induced strongly in early response to TNF- α . These genes include members of the IAP family and the BCL2 family of anti-apoptotic proteins. Inhibition of p38 with a potent

p38 inhibitor shows some effects on modulating some members of this gene list associated with the negative regulation of apoptosis. In addition we found that members of the FASR signaling pathway were down-regulated in response to TNF- α treatment, p38 inhibition reversed this down-regulation. Combining the observations from this gene expression study with the work discussed in chapter 3 of this thesis, we there is mechanistic evidence that p38 may play a role in regulating a cellular response to apoptosis. Specifically p38 MAPK may in a stress context play a role in the early induction of cell survival pathways to prevent the early onset of apoptosis.

III. Inhibition of p38 modulates TNF- α induced cell proliferation

TNF- α induction has been shown to increase cytokine and growth factor production in both cancer and untransformed cells (406). The over-expression of cytokines including TNF- α has been linked to the increased proliferation potential of tumor cells and the establishment of a suitable tumor microenvironment for tumor growth. We find that in the early time points post treatment with TNF- α , genes linked to increased cell-proliferation are strongly induced by TNF- α treatment.

Proliferation Genes induced by TNF- α & modulated by p38i

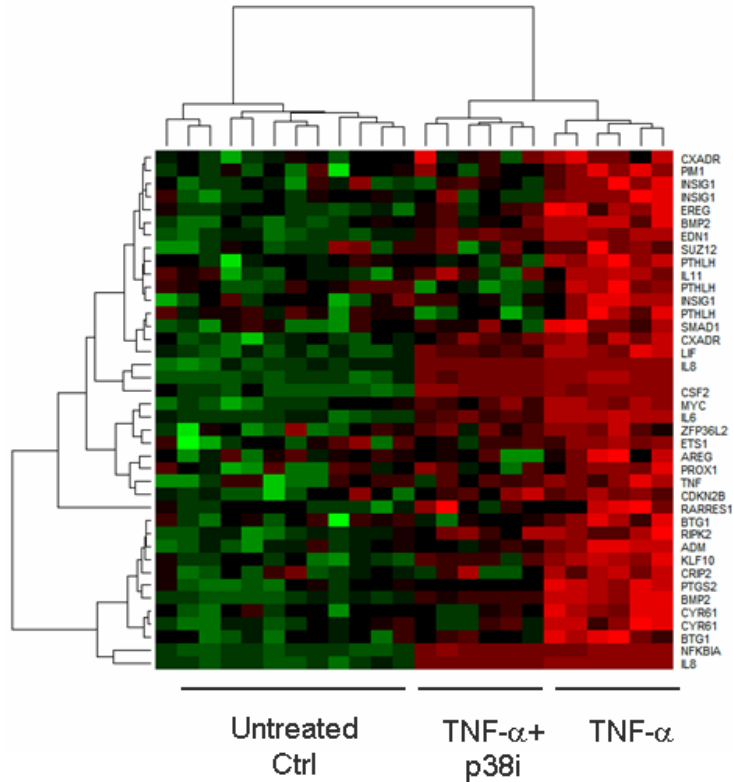


Figure 4.12: Genes associated with increased proliferation, induced by TNF- α and modulated by p38i treatment

In total, 31 genes associated with cell proliferation were strongly induced by TNF- α treatment at 60mins. Among the genes induced strongly in the early time points include pro-inflammatory cytokines such as IL6 & IL8 as well as TNF- α . As can be observed in the heatmap plotted as figure 4.12, treatment with a potent p38 inhibitor led to a significant decrease in a large number of genes associated with cell proliferation. A more detailed plot of some of these genes is given as boxplots (Figure 4.13).

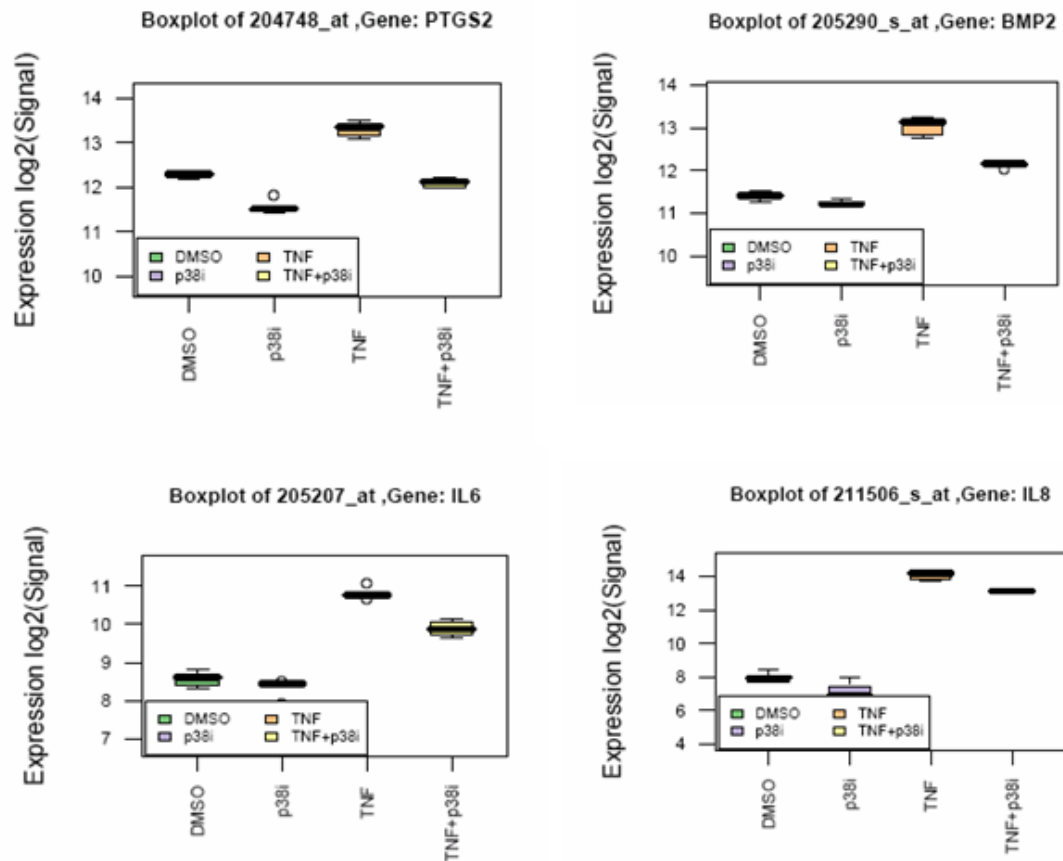


Figure 4.13: Boxplot of selected cell proliferation genes induced strongly by TNF- α and modulated by p38 inhibition

Genes such as PTGS2 (alias: Cox-2) and IL6 and IL8 are strongly associated with induction of pro-inflammatory response and are observed to increase dramatically almost immediately upon treatment with TNF- α . BMP2 is a morphogenic protein strongly associated with bone and osteoclast formation. BMP2 has primarily been linked to TGF- β signaling, but is also observed to increase in expression levels upon cytokine stimulation. BMP2 is strongly induced by TNF- α treatment and is also observed to be inhibited significantly by p38 inhibition. The significant inhibition of cell proliferation signaling through inhibition of p38, confirms in this study the link between p38 signaling and cell proliferation. Inhibition of p38 strongly modulated a significant number of genes

associated with proliferation in the early time points, where 19/41 (45%) of genes (probesets) associated with cell proliferation were significantly modulated by p38 inhibition. The gene table associated with early TNF- α induced cell-proliferation is displayed as table 4.3.

Affy ID	Gene	FC.TNF.C TRL.60min	FC.p38i.T NF.60min	Description
211506_s_at	IL8	6.462	-0.943	Interleukin-8 precursor (IL-8) (CXCL8) (Monocyte-derived neutrophil chemotactic factor) (MDNCF) (T-cell chemotactic factor) (Neutrophil-activating protein 1) (NAP-1) (Protein 3-10C) (Granulocyte chemotactic protein 1) (GCP-1) (Monocyte-derived neutrophil [Source:Uniprot/SWISSPROT,Acc:P10145]
210229_s_at	CSF2	5.586	-0.353	Granulocyte-macrophage colony-stimulating factor precursor (GM-CSF) (Colony-stimulating factor) (CSF) (Sargramostim) (Molgramostin). [Source:Uniprot/SWISSPROT,Acc:P04141]
201502_s_at	NFKBIA	3.540	-0.270	NF-kappa-B inhibitor alpha (Major histocompatibility complex enhancer-binding protein MAD3) (I-kappa-B-alpha) (IkappaBalpha) (IKB-alpha). [Source:Uniprot/SWISSPROT,Acc:P25963]
205207_at	IL6	2.212	-0.900	Interleukin-6 precursor (IL-6) (B-cell stimulatory factor 2) (BSF-2) (Interferon beta-2) (Hybridoma growth factor) (CTL differentiation factor) (CDF). [Source:Uniprot/SWISSPROT,Acc:P05231]
205266_at	LIF	1.800	-0.622	Leukemia inhibitory factor precursor (LIF) (Differentiation-stimulating factor) (D factor) (Melanoma-derived LPL inhibitor) (MLPL) (Emflemmin). [Source:Uniprot/SWISSPROT,Acc:P15018]
218995_s_at	EDN1	1.655	-0.442	Endothelin-1 precursor (Preproendothelin-1) (PPE1) [Contains: Endothelin-1 (ET-1); Big endothelin-1]. [Source:Uniprot/SWISSPROT,Acc:P05305]
205290_s_at	BMP2	1.647	-0.911	Bone morphogenetic protein 2 precursor (BMP-2) (BMP-2A). [Source:Uniprot/SWISSPROT,Acc:P12643]
207113_s_at	TNF	1.629	-0.861	Tumor necrosis factor precursor (TNF-alpha) (Tumor necrosis factor ligand superfamily member 2) (TNF-a) (Cachectin) [Contains: Tumor necrosis factor, membrane form; Tumor necrosis factor, soluble form]. [Source:Uniprot/SWISSPROT,Acc:P01375]
206392_s_at	RARRES1	1.510	-0.528	Retinoic acid receptor responder protein 1 (Tazarotene-induced gene 1 protein) (RAR-responsive protein TIG1). [Source:Uniprot/SWISSPROT,Acc:P49788]
205289_at	BMP2	1.361	-0.854	Bone morphogenetic protein 2 precursor (BMP-2) (BMP-2A). [Source:Uniprot/SWISSPROT,Acc:P12643]
202431_s_at	MYC	1.307	-0.574	Myc proto-oncogene protein (c-Myc) (Transcription factor p64). [Source:Uniprot/SWISSPROT,Acc:P01106]
205767_at	EREG	1.233	-0.782	Epiregulin precursor (EPR). [Source:Uniprot/SWISSPROT,Acc:O14944]
204748_at	PTGS2	1.034	-1.214	Prostaglandin G/H synthase 2 precursor (EC 1.14.99.1) (Cyclooxygenase-2) (COX-2) (Prostaglandin-endoperoxide synthase 2) (Prostaglandin H2 synthase 2) (PGH synthase 2) (PGHS-2) (PHS II). [Source:Uniprot/SWISSPROT,Acc:P35354]
207530_s_at	CDKN2B	0.955	-0.298	Cyclin-dependent kinase 4 inhibitor B (p14-INK4b) (p15-INK4b) (p15INK4B) (Multiple tumor suppressor 2) (MTS2). [Source:Uniprot/SWISSPROT,Acc:P42772]
202393_s_at	KLF10	0.856	-0.482	Kruppel-like factor 10 (Transforming growth factor-beta-inducible early growth response protein 1) (TGFB-inducible early growth response protein 1) (TIEG-1) (EGR-alpha). [Source:Uniprot/SWISSPROT,Acc:Q13118]
207401_at	PROX1	0.719	-0.474	Homeobox prospero-like protein PROX1 (PROX 1). [Source:Uniprot/SWISSPROT,Acc:Q92786]
201626_at	INSIG1	0.683	-0.626	Insulin-induced gene 1 protein (INSIG-1). [Source:Uniprot/SWISSPROT,Acc:O15503]
202912_at	ADM	0.673	-0.539	ADM precursor [Contains: Adrenomedullin (AM); Proadrenomedullin N-20 terminal peptide (ProAM-N20) (ProAM N-terminal 20 peptide) (PAMP)]. [Source:Uniprot/SWISSPROT,Acc:P35318]
210355_at	PTH1H	0.666	-0.789	Parathyroid hormone-related protein precursor (PTH-rP) (PTHrP) [Contains: PTHrP[1-36]; PTHrP[38-94]; Osteostatin (PTHrP[107-139])]. [Source:Uniprot/SWISSPROT,Acc:P12272]
201289_at	CYR61	0.615	-0.578	Protein CYR61 precursor (Cysteine-rich, angiogenic inducer, 61) (Insulin-like growth factor-binding protein 10) (Protein GIG1). [Source:Uniprot/SWISSPROT,Acc:O00622]
213134_x_at	CXADR	0.612	-0.221	Coxsackievirus and adenovirus receptor precursor (Coxsackievirus B-adenovirus receptor) (hCAR) (CVB3-binding protein) (HCVADR). [Source:Uniprot/SWISSPROT,Acc:P78310]
210764_s_at	CYR61	0.608	-0.588	Protein CYR61 precursor (Cysteine-rich, angiogenic inducer, 61) (Insulin-like growth factor-binding protein 10) (Protein GIG1). [Source:Uniprot/SWISSPROT,Acc:O00622]
206924_at	IL11	0.555	-0.334	Interleukin-11 precursor (IL-11) (Adipogenesis inhibitory factor) (AGIF) (Oprelvekin). [Source:Uniprot/SWISSPROT,Acc:P20809]
201627_s_at	INSIG1	0.550	-0.610	Insulin-induced gene 1 protein (INSIG-1). [Source:Uniprot/SWISSPROT,Acc:O15503]
201369_s_at	ZFP36L2	0.550	-0.286	Butyrate response factor 2 (Protein TIS11D) (EGF-response factor 2) (ERF-2). [Source:Uniprot/SWISSPROT,Acc:P47974]
205548_s_at	CXADR	0.544	-0.317	Coxsackievirus and adenovirus receptor precursor (Coxsackievirus B-adenovirus receptor) (hCAR) (CVB3-binding protein) (HCVADR). [Source:Uniprot/SWISSPROT,Acc:P78310]
213971_s_at	SUZ12	0.505	-0.359	Polycomb protein SUZ12 (Suppressor of zeste 12 protein homolog) (Joined to JAZF1 protein) (Chromatin precipitated E2F target 9 protein) (ChET 9 protein). [Source:Uniprot/SWISSPROT,Acc:Q15022]
205239_at	AREG	0.499	-0.701	Amphiregulin precursor (AR) (Colorectum cell-derived growth factor) (CRDGF). [Source:Uniprot/SWISSPROT,Acc:P15514]
200920_s_at	BTG1	0.490	-0.395	Protein BTG1 (B-cell translocation gene 1 protein). [Source:Uniprot/SWISSPROT,Acc:P62324]
214447_at	ETS1	0.461	-0.409	Protein C-ets-1 (p54). [Source:Uniprot/SWISSPROT,Acc:P14921]
209545_s_at	RIPK2	0.420	-0.163	Receptor-interacting serine/threonine-protein kinase 2 (EC 2.7.11.1) (RIP-like-interacting CLARP kinase) (Receptor-interacting protein 2) (RIP-2) (CARD-containing interleukin-1 beta-converting enzyme-associated kinase) (CARD-containing IL-1 beta ICE-kina [Source:Uniprot/SWISSPROT,Acc:O43353]
201625_s_at	INSIG1	0.390	-0.309	Insulin-induced gene 1 protein (INSIG-1). [Source:Uniprot/SWISSPROT,Acc:O15503]
209193_at	PIM1	0.373	-0.310	Proto-oncogene serine/threonine-protein kinase Pim-1 (EC 2.7.11.1). [Source:Uniprot/SWISSPROT,Acc:P11309]
210993_s_at	SMAD1	0.364	-0.216	Mothers against decapentaplegic homolog 1 (SMAD 1) (Mothers against DPP homolog 1) (Mad-related protein 1) (Transforming growth factor-beta-signaling protein 1) (BSP-1) (hSMAD1) (JV4-1). [Source:Uniprot/SWISSPROT,Acc:Q15797]
208978_at	CRIP2	0.232	-0.237	Cysteine-rich protein 2 (CRP2) (Protein ESP1). [Source:Uniprot/SWISSPROT,Acc:P52943]
211756_at	PTH1H	0.178	-0.474	Parathyroid hormone-related protein precursor (PTH-rP) (PTHrP) [Contains: PTHrP[1-36]; PTHrP[38-94]; Osteostatin (PTHrP[107-139])]. [Source:Uniprot/SWISSPROT,Acc:P12272]

Table 4.3: Genes induced early by TNF- α associated with cell proliferation. A significant portion of these genes are strongly modulated by p38 inhibition (LY479754), all genes FDR<0.1

Summary of Early Time Points (30mins & 60mins)

In the immediate aftermath of TNF- α treatment, a strong induction of a number of physiological pathways and functional clusters including the immediate early response stress pathway, the regulation of apoptosis pathway and the cell proliferation pathway. Within these functional clusters, genes and pathways associated with pro-inflammatory signaling were strongly induced by TNF- α , and were attenuated by a p38 inhibitor. In addition to inflammation, a significant number of genes related to apoptosis and proliferation pathways were significantly modulated by inhibition of p38. Modulation of these pathways provides some mechanistic insight into the potential roles of p38 in the immediate response to stress, and to pro-apoptotic extrinsic death induction by TNF- α .

4.6 Effect of TNF- α and p38 inhibition at the mid time point (2hrs)

As can be observed in the summary plot of the number of genes modulated by TNF- α and the p38 inhibitor in figure 4.4, the mid time point of this study has an increasing number of genes induced by TNF- α treatment and has the largest number of genes also modulated by p38 inhibition. The 2 hour time point in this study could be the “cross-over” point, where the effect of TNF- α triggers a host of secondary signaling pathways and the effect of the p38 inhibitor has potential maximum transcriptional effect.

A summary of the top 10 functional pathways/networks as determined by the Ingenuity Pathway Analysis (IPA) tool is provided below (Table 4.4).

Top Functions	Score	Focus Molecules
Protein Synthesis, Cellular Compromise, Cellular Growth and Proliferation	52	31
Cancer, Hematological Disease, Cellular Development	47	29
Gene Expression, Cellular Development, Nervous System Development and Function	44	28
Cancer, Cell Death, Connective Tissue Disorders	42	27
Gene Expression, Carbohydrate Metabolism, Small Molecule Biochemistry	35	24
Cellular Development, Organ Morphology, Organismal Functions	34	24
Embryonic Development, Tissue Development, Cellular Development	29	21
Infection Mechanism, Cellular Development, Gene Expression	27	20
Cancer, Hematological Disease, Ophthalmic Disease	25	19
Cellular Assembly and Organization, Developmental Disorder, Cellular Growth and Proliferation	23	18

Table 4.4: Top functional pathways for TNF- α +LY479754 at 2hour time point

I. TNF- α induces cell developmental and differentiation pathways

We found that after 2hours of TNF- α treatment, approximately 600 genes (probesets) were significantly modulated by TNF- α . Of these genes, approximately 30% were modulated by a p38 inhibitor. The largest functional category according to both DAVID and Ingenuity pathway analysis tools was related to cell development and differentiation pathways.

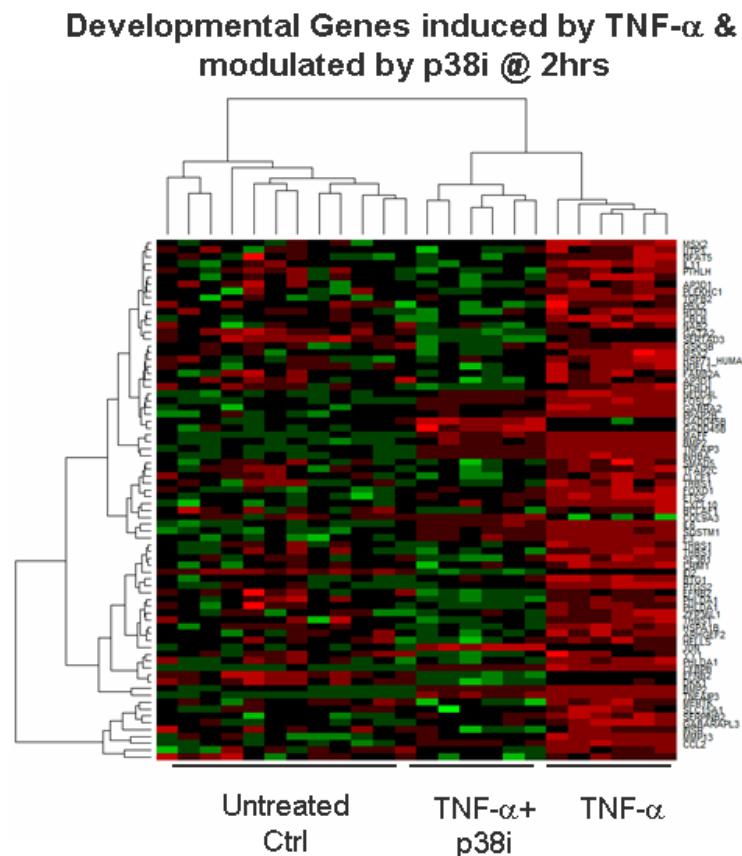


Figure 4.14: *Heatmap of genes classified as developmental genes, significantly changed by TNF- α . A large sub cluster of genes are also modulated by p38i (LY479754) treatment.*

Within the functional cluster of genes related to growth, development and cell differentiation, we found members of a number of key signaling pathways whose deregulation leads to oncogenesis. These pathways include the TGF- β signaling pathway, the MMP pathway and the Hedgehog/Notch signaling pathway. The components of these pathways are plotted as boxplots below. Besides the genes plotted for illustration in figure 4.15 below, other components of the TGF- β and the Hedgehog/Notch signaling pathway can be observed being modulated by TNF- α in this mid-time point.

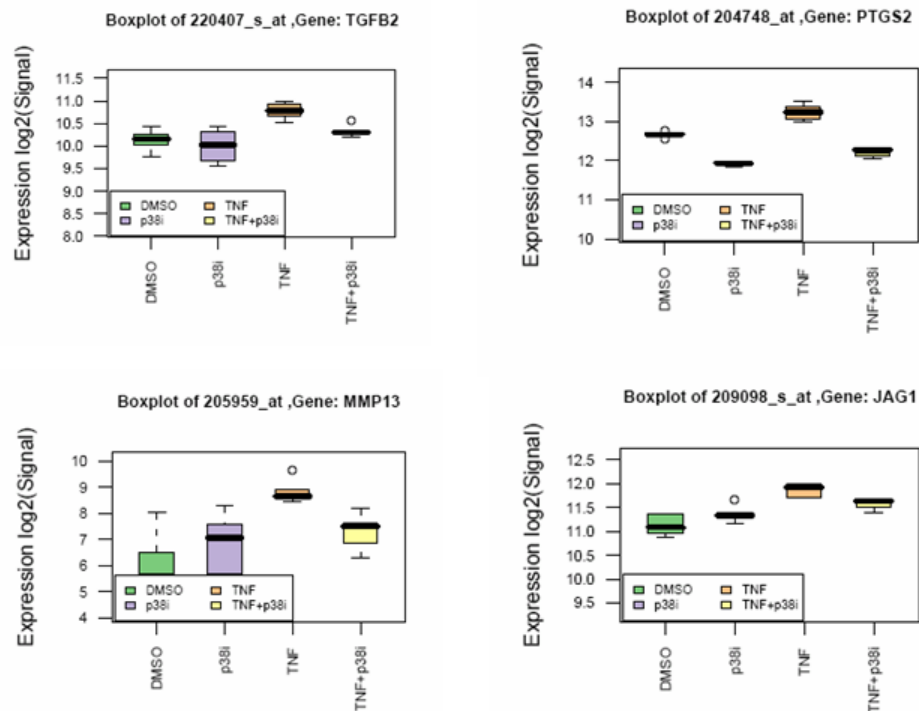


Figure 4.15: Boxplot of selected development and cell differentiation genes, induced by TNF- α and modulated by p38-inhibition.

To give some context to the regulation of cellular development genes by p38 inhibition, genes modulated in this section were analyzed through network analysis in IPA. The nearest neighbor linkage analysis in IPA produces a network of putative relationships among genes in a gene list and is presented as an acyclical directed graph. The sub-network for developmental genes modulated by p38 inhibition is presented in figure 4.16.

Network 6: Observation 2: TNF_sig_genes_2hrs bt: Observation 2

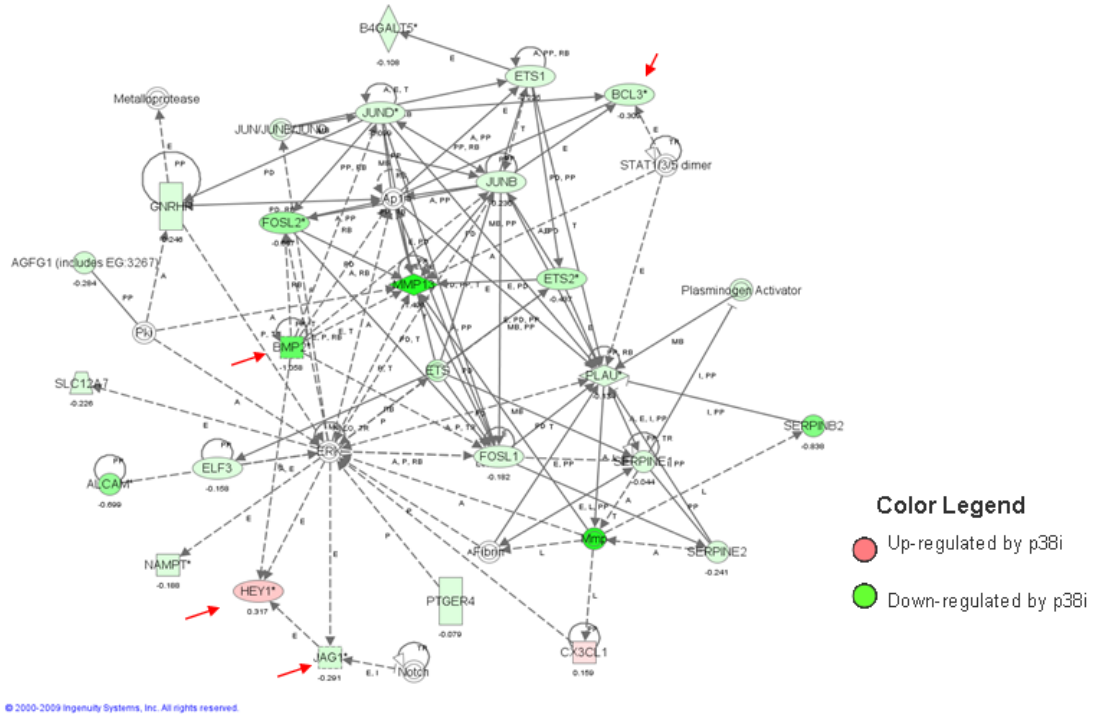


Figure 4.16: IPA network analysis of cellular development genes modulated by p38i (LY479754) at 2 hours

A potent p38 inhibitor is able to influence the transcriptional response of a number of key developmental signaling pathways including components of the TGF- β and Hedgehog/Notch signaling pathway (Figure 4.16). While TNF- α has been studied extensively as one of the key components of immune system stimulated death receptor signaling, its dysregulation and over-expression could promote sustained inflammatory signaling leading to induction of a host of growth and proliferation pathways. Mechanistically we have identified that p38 MAPK signaling may be critical to the early induction of a number of these pathways, suggesting that a p38 MAPK inhibitor may be a useful therapeutic agent targeting the formation and establishment of a successful tumor microenvironment.

II. TNF- α induces both pro and anti-apoptotic signaling pathways that are marginally counteracted by inhibition of p38.

Following 2 hours of TNF- α treatment, the immediate early response to the stress has activated a host of secondary signaling pathways that in turn activate their own respective down-stream signaling pathways. In the early time points, we observed a strong up-regulation of some pro and anti-apoptotic factors, many of which were also modulated partly by p38 inhibition. At this time point, many more components of the apoptosis signaling machinery have been activated. Some components of this machinery show some dependence on p38 signaling, other components behave in a p38 independent manner. At this time point we also observed within this functional cluster, a group of genes that were down-regulated by TNF- α treatment, but whose expression recovers upon p38 inhibition. We will separately dissect each of these groups of genes in order to gain a better mechanistic understanding of p38 activity in response to TNF- α stimulation in Calu6 lung cancer cells.

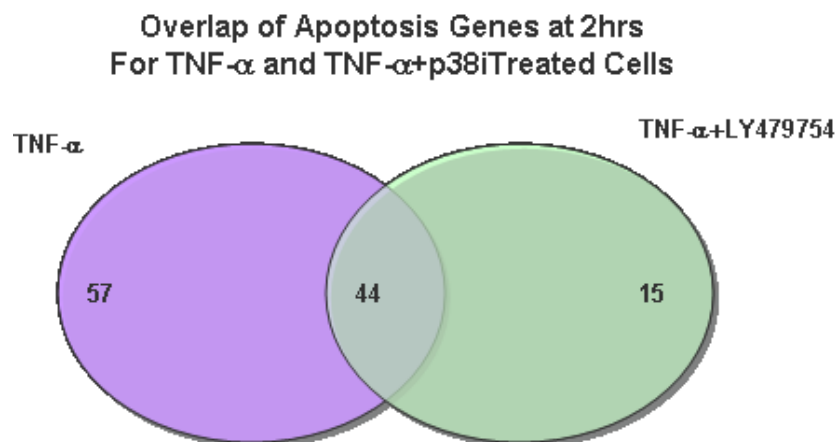


Figure 4.17: *Venn Diagram of TNF- α and TNF- α +LY479754 modulated apoptosis genes at 2hrs time point*

A large number of genes functionally related to apoptosis are affected by TNF- α , 2 hours post treatment. A small but significant number of these genes are directly modulated by a p38 inhibitor in the same time-frame. Functional analysis of expressed genes suggests that there are two classes of genes modulated within this functional cluster, one that depends on p38 signaling and another class that does not. We will deal with each class of genes separately in the following sections.

A heatmap plot of genes involved in apoptosis and modulated by the p38 inhibitor is plotted in figure 4.18.

Apoptosis Genes modulated by both TNF- α & p38i @ 2hrs

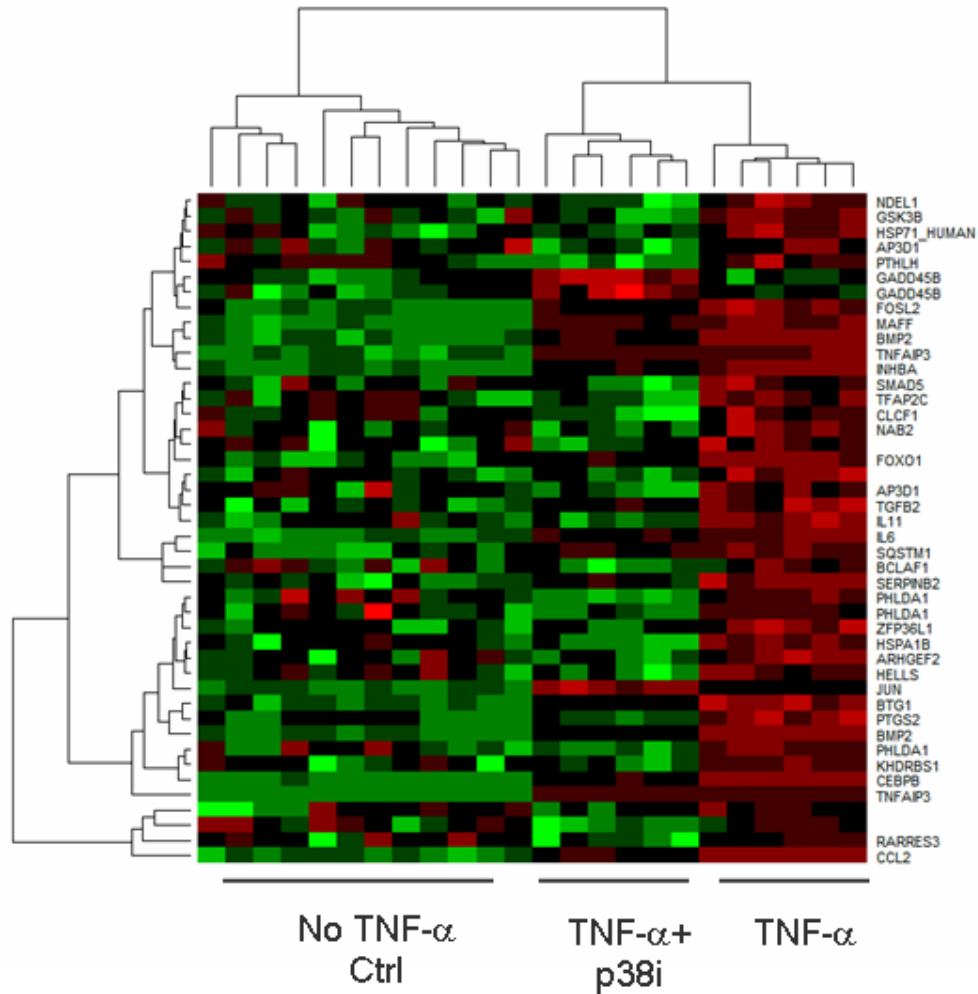


Figure 4.18: Heatmap of 22 genes related to apoptosis, modulated by p38 inhibitor in conjunction with TNF- α treatment at 2hrs.

A number of the genes classified as being involved in apoptosis and modulated by the p38 inhibitor belong to cytokine, chemokine and growth factor families. These include IL6 & IL8 as well as TGF- β . The transcription factor FOXO1A is also among the genes strongly induced by TNF- α and down-regulated by a p38 inhibitor. A table of genes classified by DAVID related to apoptosis and significantly modulated by TNF- α and the p38 inhibitor is presented in table 4.5.

Affy ID	gene	FC.TNF.CTRL. 60min	FC.p38i.TNF.60m in	Description
216598_s_at	CCL2	3.978725469	-1.40426449	Small inducible cytokine A2 precursor (CCL2) (Monocyte chemoattractant protein 1) (MCP-1) (Monocyte chemoattractant protein 1) (Monocyte chemoattractant and activating factor) (MCAF) (Monocyte secretory protein JE) (HC11). [Source:Uniprot/SWISSPROT;Acc:P13500]
204070_at	RARRES3	0.413760882	-1.124299872	Retinoic acid receptor responder protein 3 (Tazarotene-induced gene 3 protein) (RAR-responsive protein TIG3) (Retinoid-inducible gene 1 protein). [Source:Uniprot/SWISSPROT;Acc:Q9JUL19]
205290_s_at	BMP2	2.520670866	-1.058372452	Bone morphogenetic protein 2 precursor (BMP-2) (BMP-2A). [Source:Uniprot/SWISSPROT;Acc:P12643]
206924_at	IL11	0.63024089	-1.031756387	Interleukin-11 precursor (IL-11) (Adipogenesis inhibitory factor) (AGIF) (Oprevekin). [Source:Uniprot/SWISSPROT;Acc:P20809]
204748_at	PTGS2	0.579058866	-1.009579251	Prostaglandin G/H synthase 2 precursor (EC 1.14.99.1) (Cyclooxygenase-2) (COX-2) (Prostaglandin-endoperoxide synthase 2) (Prostaglandin H2 synthase 2) (PGH synthase 2) (PGHS-2) (PHS II). [Source:Uniprot/SWISSPROT;Acc:P35354]
219500_at	CLCF1	0.125030976	-0.939026333	Cardiotrophin-like cytokine factor 1 precursor (B cell-stimulating factor 3) (BSF-3) (Novel neurotrophin-1) (NNT-1). [Source:Uniprot/SWISSPROT;Acc:Q9UBD9]
210511_s_at	INHBA	2.533084698	-0.855228996	Inhibin beta A chain precursor (Activin beta-A chain) (Erythroid differentiation protein) (EDF). [Source:Uniprot/SWISSPROT;Acc:P08476]
204614_at	SERPINB2	1.596214756	-0.837508363	Plasminogen activator inhibitor 2 precursor (PAI-2) (Placental plasminogen activator inhibitor) (Monocyte Arg-serpin) (Urokinase inhibitor). [Source:Uniprot/SWISSPROT;Acc:P05120]
211756_at	PTH1H	0.004894029	-0.826188994	Parathyroid hormone-related protein precursor (PTHrP) (PTHrP) [Contains: PTHrP[1-36], PTHrP[38-94], Osteostatin (PTHrP[107-139])]. [Source:Uniprot/SWISSPROT;Acc:P12272]
205289_at	BMP2	2.343790349	-0.790532805	Bone morphogenetic protein 2 precursor (BMP-2) (BMP-2A). [Source:Uniprot/SWISSPROT;Acc:P12643]
202724_s_at	FOXO1	1.294088968	-0.701289796	Forkhead box protein O1A (Forkhead in rhabdomyosarcoma). [Source:Uniprot/SWISSPROT;Acc:Q12778]
213112_s_at	SQSTM1	2.739736191	-0.596780932	Sequestosome-1 (Phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa) (Ubiquitin-binding protein p62) (EB1-associated protein of 60 kDa) (p60) (EBIAP). [Source:Uniprot/SWISSPROT;Acc:Q13501]
218000_s_at	PHLDA1	0.089180759	-0.588012308	Pleckstrin homology-like domain family A member 1 (T-cell death-associated gene 51 protein) (Apoptosis-associated nuclear protein) (Proline- and histidine-rich protein) (Proline- and glutamine-rich protein) (PQ-rich protein). [Source:Uniprot/SWISSPROT;Acc:Q8WV24]
205287_s_at	TFAP2C	0.191801274	-0.565013887	Transcription factor AP-2 gamma (AP2-gamma) (Activating enhancer-binding protein 2 gamma) (Transcription factor ERF-1). [Source:Uniprot/SWISSPROT;Acc:Q92754]
217997_at	PHLDA1	0.008769307	-0.543758632	Pleckstrin homology-like domain family A member 1 (T-cell death-associated gene 51 protein) (Apoptosis-associated nuclear protein) (Proline- and histidine-rich protein) (Proline- and glutamine-rich protein) (PQ-rich protein). [Source:Uniprot/SWISSPROT;Acc:Q8WV24]
205188_s_at	SMAD5	0.245773962	-0.542182847	Mothers against decapentaplegic homolog 5 (SMAD 5) (Mothers against DPP homolog 5) (Smad5) (hSmad5) (JVS-1). [Source:Uniprot/SWISSPROT;Acc:Q99717]
201083_s_at	BCLAF1	0.344616302	-0.527244652	Bcl-2-associated transcription factor 1 (Btf). [Source:Uniprot/SWISSPROT;Acc:Q9NYF8]
202643_s_at	TNFAIP3	3.331777004	-0.502423526	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT;Acc:P21580]
217996_at	PHLDA1	0.101872887	-0.498056531	Pleckstrin homology-like domain family A member 1 (T-cell death-associated gene 51 protein) (Apoptosis-associated nuclear protein) (Proline- and histidine-rich protein) (Proline- and glutamine-rich protein) (PQ-rich protein). [Source:Uniprot/SWISSPROT;Acc:Q8WV24]
212803_at	NAB2	0.396684749	-0.487507493	NGFI-A-binding protein 2 (EGR-1-binding protein 2) (Melanoma-associated delayed early response protein) (Protein MADER). [Source:Uniprot/SWISSPROT;Acc:Q15742]
205207_at	IL6	2.23056109	-0.483706561	Interleukin-6 precursor (IL-6) (B-cell stimulatory factor 2) (BSF-2) (Interferon beta-2) (Hybridoma growth factor) (CTL differentiation factor) (CDF). [Source:Uniprot/SWISSPROT;Acc:P05231]
200920_s_at	BTG1	0.769166693	-0.469781029	Protein BTG1 (B-cell translocation gene 1 protein). [Source:Uniprot/SWISSPROT;Acc:P62324]
220407_s_at	TGF2	0.651501991	-0.452603171	Transforming growth factor beta-2 precursor (TGF-beta-2) (Glioblastoma-derived T-cell suppressor factor) (G-TSF) (BSC-1 cell growth inhibitor) (Polygerin) (Cetermin). [Source:Uniprot/SWISSPROT;Acc:P61812]
202581_at	HSPA1B	0.145045683	-0.449927573	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70.1/HSP70.2). [Source:Uniprot/SWISSPROT;Acc:P08107]
208710_s_at	AP3D1	0.148470806	-0.436981455	AP-3 complex subunit delta-1 (Adapter-related protein complex 3 subunit delta-1) (Delta-adaptin 3) (AP-3 complex subunit delta) (Delta-adaptin). [Source:Uniprot/SWISSPROT;Acc:Q14617]
220085_at	HELLS	0.21416943	-0.418289833	Lymphoid-specific helix-loop-helix (EC 3.6.1.-) (SWI/SNF2-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5) (Proliferation-associated SNF2-like protein). [Source:Uniprot/SWISSPROT;Acc:Q9NRZ9]
211962_s_at	ZFP36L1	0.390864709	-0.410385401	Butyrate response factor 1 (Protein TIS11B) (EGF-response factor 1) (ERF-1). [Source:Uniprot/SWISSPROT;Acc:Q07352]
218880_at	FOSL2	0.900234223	-0.408904656	Fos-related antigen 2. [Source:Uniprot/SWISSPROT;Acc:P15408]
208093_s_at	NDEL1	0.201032375	-0.406478243	Nuclear distribution protein nudE-like 1 (Protein Nudel) (Mitosis-associated protein 1). [Source:Uniprot/SWISSPROT;Acc:Q9GZM8]
36711_at	MAFF	2.339103588	-0.401336449	Transcription factor MafF (V-maf musculoaponeurotic fibrosarcoma oncogene homolog F) (U-Maf). [Source:Uniprot/SWISSPROT;Acc:Q9ULX9]
200799_at	HSP71_H UMAN	0.437020685	-0.395013397	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70.1/HSP70.2). [Source:Uniprot/SWISSPROT;Acc:P08107]
209945_s_at	GSK3B	0.289660532	-0.371796596	Glycogen synthase kinase-3 beta (EC 2.7.11.26) (GSK-3 beta). [Source:Uniprot/SWISSPROT;Acc:P49841]
202644_s_at	TNFAIP3	4.441217388	-0.356360388	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT;Acc:P21580]
212501_at	CEBPB	1.086785628	-0.35590921	CCAAT/enhancer-binding protein beta (C/EBP beta) (Nuclear factor NF-IL6) (Transcription factor 5). [Source:Uniprot/SWISSPROT;Acc:P17676]
210974_s_at	AP3D1	0.146754243	-0.342760339	AP-3 complex subunit delta-1 (Adapter-related protein complex 3 subunit delta-1) (Delta-adaptin 3) (AP-3 complex subunit delta) (Delta-adaptin). [Source:Uniprot/SWISSPROT;Acc:Q14617]
209435_s_at	ARHGEF2	0.257443753	-0.30739874	Rho/Rac guanine nucleotide exchange factor 2 (GEF-H1 protein) (Proliferating cell nuclear antigen p40). [Source:Uniprot/SWISSPROT;Acc:Q92974]
201488_x_at	KHDRBS1	0.226939165	-0.279571376	KH domain-containing, RNA-binding, signal transduction-associated protein 1 (p21 Ras GTPase-activating protein-associated p62) (GAP-associated tyrosine phosphoprotein p62) (Src-associated in mitosis 68 kDa protein) (Sam68) (p68). [Source:Uniprot/SWISSPROT;Acc:Q07666]
209304_x_at	GADD45B	0.171306819	0.592541814	Growth arrest and DNA-damage-inducible protein GADD45 beta (Negative growth-regulatory protein MyD118) (Myeloid differentiation primary response protein MyD118). [Source:Uniprot/SWISSPROT;Acc:Q75293]
207574_s_at	GADD45B	0.081866814	0.617291061	Growth arrest and DNA-damage-inducible protein GADD45 beta (Negative growth-regulatory protein MyD118) (Myeloid differentiation primary response protein MyD118). [Source:Uniprot/SWISSPROT;Acc:Q75293]
201464_x_at	JUN	0.74734261	0.634279604	Transcription factor AP-1 (Activator protein 1) (AP1) (Proto-oncogene c-jun) (V-jun avian sarcoma virus 17 oncogene homolog) (p39). [Source:Uniprot/SWISSPROT;Acc:P05412]

Table 4.5: Genes functionally related to Apoptosis, induced by TNF- α and modulated by p38i (LY479754), all genes FDR<0.1.

A further network analysis of these genes puts into pathway context some of the potential relationships between the genes in the list (Figure 4.19).

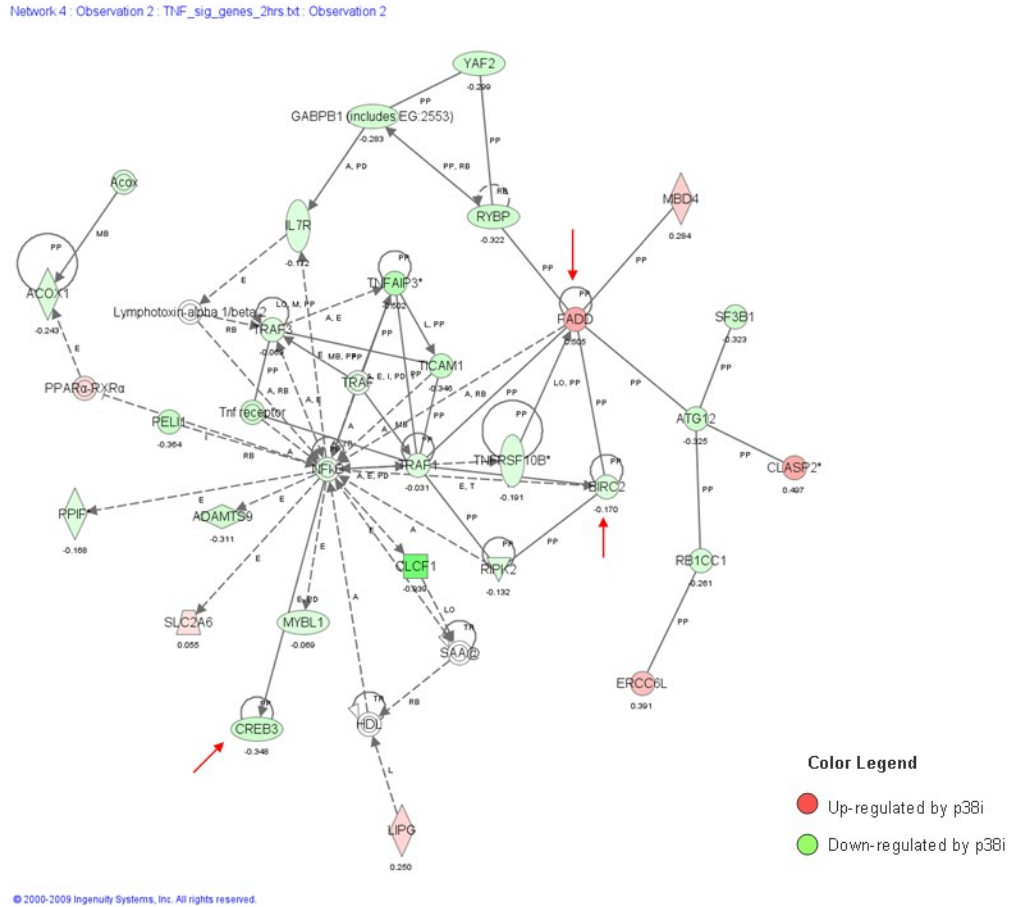


Figure 4.19: A pathway/network diagram of the top network of genes significantly regulated by p38 inhibitor LY479754 and TNF- α at the 2hrs timepoint

As can be observed in the pathway/network diagram (Figure 4.18), p38 inhibition modulates the expression of many anti-apoptotic and pro-cell survival genes, including CREB3 and BIRC2. It also increases the expression of FAS, death domain associated protein (FADD), suggesting that inhibition of p38 leads to the decrease in certain anti-apoptosis genes and up-regulates other pro-apoptosis genes.

The second category of genes functionally related to apoptosis in the mid-time point is strongly modulated by TNF- α but are generally not significantly affected by a p38 inhibitor. These genes are part of a transcriptional response that diverges away from direct p38 pathway control, beginning at this time point.

We note that in the unsupervised clustering shown in Figure 4.20, the TNF- α condition and the TNF- α +LY479754 conditions are intermingled and undistinguishable from each other. These are genes whose transcriptional response at 2hrs are generally unaffected by inhibition of p38 signaling.

Apoptosis related genes, unaffected by p38i 2hrs Time point

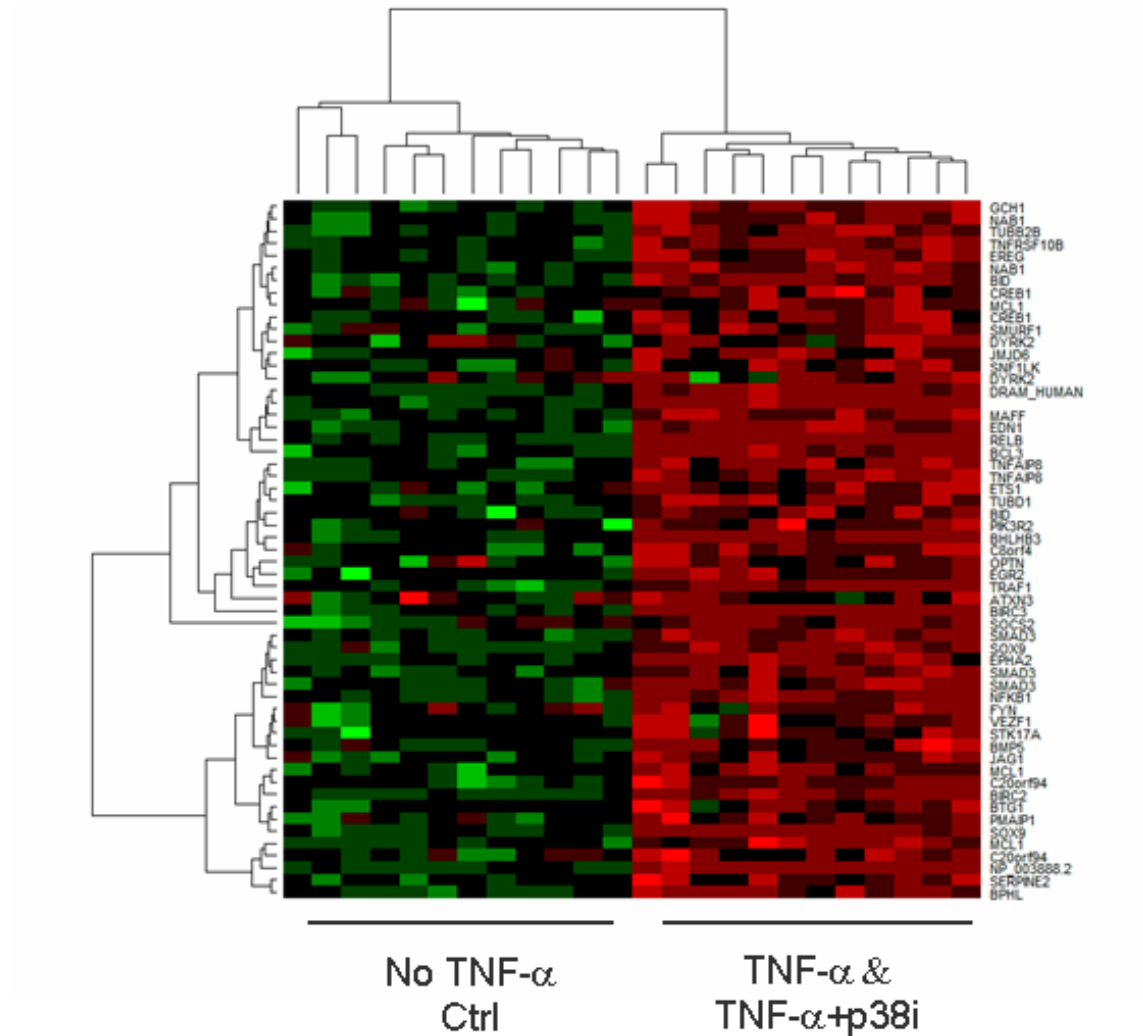


Figure 4.20: Heatmap of 57 genes (probesets) functionally classified as apoptosis related, strongly induced by TNF- α but unaffected by p38 inhibition (LY479754).

Affy ID	gene	TNFvsCTRL,2 hr log ₂ (FC)	TNFvsp38i, 2hr log ₂ (FC)	Description
210538_s_at	BIRC3	5.005456642	-0.01356175	Baculoviral IAP repeat-containing protein 3 (Inhibitor of apoptosis protein 1) (HIAP-1) (C-IAP2) (TNFR2-TRAF-signaling complex protein 1) (IAP homolog C) (Apoptosis inhibitor 2) (API2) (RING finger protein 49). [Source:Uniprot/SWISSPROT,Acc:Q13489]
205205_at	RELB	2.295949076	0.062447601	Transcription factor RelB (I-Rel). [Source:Uniprot/SWISSPROT,Acc:Q01201]
205599_at	TRAF1	2.155463027	-0.03056745	TNF receptor-associated factor 1 (Epstein-Barr virus-induced protein 6). [Source:Uniprot/SWISSPROT,Acc:Q13077]
204908_s_at	BCL3	1.894032853	-0.22451406	B-cell lymphoma 3-encoded protein (Protein Bcl-3). [Source:Uniprot/SWISSPROT,Acc:P20749]
218995_s_at	EDN1	1.537804435	0.265125651	Endothelin-1 precursor (Preproendothelin-1) (PPET1) [Contains: Endothelin-1 (ET-1); Big endothelin-1]. [Source:Uniprot/SWISSPROT,Acc:P05305]
203372_s_at	SOCS2	1.436154969	0.136393416	Suppressor of cytokine signaling 2 (SOCS-2) (Cytokine-inducible SH2 protein 2) (CIS-2) (STAT-induced STAT inhibitor 2) (SSI-2). [Source:Uniprot/SWISSPROT,Acc:O14508]
202076_at	BIRC2	1.336640492	-0.17009575	Baculoviral IAP repeat-containing protein 2 (Inhibitor of apoptosis protein 2) (HIAP2) (HIAP-2) (C-IAP1) (TNFR2-TRAF-signaling complex protein 2) (IAP homolog B) (RING finger protein 48). [Source:Uniprot/SWISSPROT,Acc:Q13490]
221530_s_at	BHLHB3	1.332251418	0.057516718	Class B basic helix-loop-helix protein 3 (bHLHB3) (Differentially expressed in chondrocytes protein 2) (hDEC2) (Enhancer-of-split and hairy-related protein 1) (SHARP-1). [Source:Uniprot/SWISSPROT,Acc:Q9C0J9]
201631_s_at	NP_003888.2	1.292304678	-0.06255506	immediate early response 3 [Source:RefSeq_peptide,Acc:NP_003888]
205249_at	EGR2	1.285660536	-0.10559882	Early growth response protein 2 (EGR-2) (Krox-20 protein) (AT591). [Source:Uniprot/SWISSPROT,Acc:P11161]
208296_x_at	TNFAIP8	1.221947394	-0.25069771	Tumor necrosis factor, alpha-induced protein 8 (Head and neck tumor and metastasis-related protein) (TNF-induced protein GG2-1) (SCC-S2) (NF-kappa B-inducible DED-containing protein) (NDED) (MDC-3.13). [Source:Uniprot/SWISSPROT,Acc:O95379]
209239_at	NFKB1	1.08984003	-0.23777011	Nuclear factor NF-kappa-B p105 subunit (DNA-binding factor KBF1) (EBP-1) [Contains: Nuclear factor NF-kappa-B p50 subunit]. [Source:Uniprot/SWISSPROT,Acc:P19838]
205767_at	EREG	1.073429857	-0.25891527	Epiregulin precursor (EPR). [Source:Uniprot/SWISSPROT,Acc:O14944]
218627_at	DRAM_HUMAN	0.966020753	0.038106703	Damage-regulated autophagy modulator. [Source:Uniprot/SWISSPROT,Acc:Q8N682]
210405_x_at	TNFRSF10B	0.922604174	-0.19131416	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor 2) (TRAIL-R2) (CD262 antigen). [Source:Uniprot/SWISSPROT,Acc:O14763]
204224_s_at	GCH1	0.913934136	-0.16032137	GTP cyclohydrolase I (EC 3.5.4.16) (GTP-CH4). [Source:Uniprot/SWISSPROT,Acc:P30793]
209272_at	NAB1	0.854460608	-0.14097838	NGFI-A-binding protein 1 (EGR-1-binding protein 1) (Transcriptional regulatory protein p54). [Source:Uniprot/SWISSPROT,Acc:Q13506]
203499_at	EPHA2	0.815165538	-0.05892298	Ephrin type-A receptor 2 precursor (EC 2.7.10.1) (Tyrosine-protein kinase receptor ECK) (Epithelial cell kinase). [Source:Uniprot/SWISSPROT,Acc:P29317]
214023_x_at	TUBB2B	0.812098366	0.100972391	Tubulin beta-2B chain. [Source:Uniprot/SWISSPROT,Acc:Q9BVA1]
210389_x_at	TUBD1	0.713428535	-0.04186773	Tubulin delta chain (Delta tubulin). [Source:Uniprot/SWISSPROT,Acc:Q9JUT1]
202935_s_at	SOX9	0.70431792	0.06313959	Transcription factor SOX-9. [Source:Uniprot/SWISSPROT,Acc:P48436]
204493_at	BID	0.651178055	-0.07895956	BH3-interacting domain death agonist (BID) (p22 BID) [Contains: BH3-interacting domain death agonist p15 (p15 BID); BH3-interacting domain death agonist p11 (p11 BID)]. [Source:Uniprot/SWISSPROT,Acc:P55957]
214447_at	ETS1	0.598326625	-0.22471705	Protein C-ets-1 (p54). [Source:Uniprot/SWISSPROT,Acc:P14921]
205397_x_at	SMAD3	0.577586783	-0.09846844	Mothers against decapentaplegic homolog 3 (SMAD3) (Mothers against DPP homolog 3) (Mad3) (hMAD-3) (JV15-2) (hSMAD3). [Source:Uniprot/SWISSPROT,Acc:P84022]
205398_s_at	SMAD3	0.573164318	-0.19006719	Mothers against decapentaplegic homolog 3 (SMAD3) (Mothers against DPP homolog 3) (Mad3) (hMAD-3) (JV15-2) (hSMAD3). [Source:Uniprot/SWISSPROT,Acc:P84022]
202971_s_at	DYRK2	0.569688717	-0.27253507	Dual specificity tyrosine-phosphorylation-regulated kinase 2 (EC 2.7.12.1). [Source:Uniprot/SWISSPROT,Acc:Q92630]
204285_s_at	PMAIP1	0.567176484	-0.2144124	Phorbol-12-myristate-13-acetate-induced protein 1 (PMA-induced protein 1) (Immediate-early-response protein APR). [Source:Uniprot/SWISSPROT,Acc:Q13794]
200921_s_at	BTG1	0.544740826	-0.29288674	Protein BTG1 (B-cell translocation gene 1 protein). [Source:Uniprot/SWISSPROT,Acc:P62324]
204141_at	BPHL	0.518344153	-0.12670196	Valacyclovir hydrolase precursor (EC 3.1.-.-) (VACVase) (Biphenyl hydrolase-like protein) (Biphenyl hydrolase-related protein) (Bph-rp) (Breast epithelial mucin-associated antigen) (MCNAA). [Source:Uniprot/SWISSPROT,Acc:Q86WA6]
204313_s_at	CREB1	0.512733797	-0.05641874	cAMP response element-binding protein (CREB). [Source:Uniprot/SWISSPROT,Acc:P16220]
205431_s_at	BMP5	0.475781758	-0.28059907	Bone morphogenetic protein 5 precursor (BMP-5). [Source:Uniprot/SWISSPROT,Acc:P22003]
212722_s_at	JMJD6	0.44763762	-0.17220512	JmjC domain-containing protein 6 (Jumonji domain-containing protein 6) (Protein PTDSR) (Phosphatidylserine receptor). [Source:Uniprot/SWISSPROT,Acc:Q6NYC1]
212666_at	SMURF1	0.441012146	-0.18040362	E3 ubiquitin-protein ligase SMURF1 (EC 6.3.2.-) (Smad ubiquitination regulatory factor 1) (Smad-specific E3 ubiquitin ligase 1) (hSMURF1). [Source:Uniprot/SWISSPROT,Acc:Q9HCE7]
208078_s_at	SNF1LK	0.428132157	-0.19040319	Serine/threonine-protein kinase SNF1-like kinase 1 (EC 2.7.11.1) [Serine/threonine-protein kinase SNF1LK) (SIK1). [Source:Uniprot/SWISSPROT,Acc:P57059]
205416_s_at	ATXN3	0.41722863	-0.22951688	ataxin 3 isoform 2 [Source:RefSeq_peptide,Acc:NP_109376]
200796_s_at	MCL1	0.401166284	-0.04267441	Induced myeloid leukemia cell differentiation protein Mcl-1 (Bcl-2-related protein EAT/mcl1) (mcl1/EAT). [Source:Uniprot/SWISSPROT,Acc:Q07820]
212190_at	SERPINE2	0.398258846	-0.24117354	Glia-derived nexin precursor (GDN) (Protease nexin I) (PN-1) (Protease inhibitor 7). [Source:Uniprot/SWISSPROT,Acc:P07093]
202693_s_at	STK17A	0.38641042	-0.21327638	Serine/threonine-protein kinase 17A (EC 2.7.11.1) (DAP kinase-related apoptosis-inducing protein kinase 1). [Source:Uniprot/SWISSPROT,Acc:Q9UEE5]
202171_at	VEZF1	0.314382022	-0.17769504	Vascular endothelial zinc finger 1 (Zinc finger protein 161) (Putative transcription factor DB1). [Source:Uniprot/SWISSPROT,Acc:Q14119]
207105_s_at	PIK3R2	0.295880622	-0.09623386	Phosphatidylinositol 3-kinase regulatory subunit beta (PI3-kinase p85-subunit beta) (PtdIns-3-kinase p85-beta). [Source:Uniprot/SWISSPROT,Acc:O00459]
210105_s_at	FYN	0.242814425	-0.13240405	Proto-oncogene tyrosine-protein kinase Fyn (EC 2.7.10.2) (p59-Fyn) (Protooncogene Syn) (SLK). [Source:Uniprot/SWISSPROT,Acc:P06241]

Table 4.6: Apoptosis related genes induced strongly by TNF- α , but not modulated significantly by p38i (LY479754) at 2hr time point, all genes FDR<0.1

At the two hour time point, it is observed that genes and pathways strongly

regulated by TNF- α , are relatively unaffected by p38 inhibition. These genes and

pathways are activated and behave independently of p38 regulation. Some key genes that stick out strongly at this time point include anti-apoptotic genes relating to the IAP proteins BIRC2 and BIRC3. We had observed a slight down-regulation of both of these genes at earlier time points, with p38 inhibition, but this cyto-protective pathway seems to increase in intensity as time progresses on through this experiment. This data suggests that Calu6 cancer cells may escape death inducing TNF- α extrinsic apoptosis pathway activation by strong up-regulation of IAP proteins. We note that at earlier time points, p38 inhibition impinges upon this pathway, however at later time points, alternative signaling pathways seem to be able to over-come blockade of p38 signaling to enable these anti-apoptotic pathways to activate. In addition to anti-apoptosis pathways behaving independently of p38 activity, we observe that a number of transcription factors related to cell growth and proliferation are also activated at this time point. These transcription factors include the Creb1, RELB and Maff, which relate to diverse pathways such as NFkB (RELB), cell survival (Creb1) and transcription regulation (Maff). The strong induction of Creb1, may be suggestive of additional trans-activation of pro-survival genes to help in evasion of TNF- α induced apoptosis.

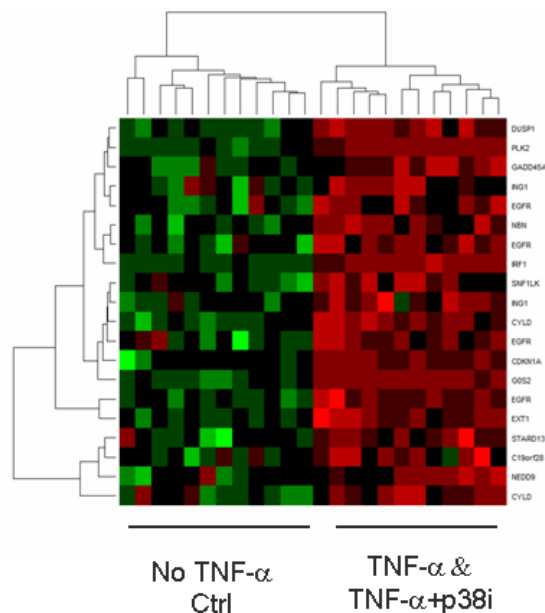
II. TNF- α induces genes that regulate the Cell Cycle

Another functional cluster identified in the genes significantly modulated by TNF- α at the 2hrs time point was the cell cycle related cluster. These genes have been functionally documented to play a role either in regulation of cell cycle or play a role in the progression of the mammalian cell cycle. As previous chapters of this thesis have focused extensively on gaining a mechanistic understanding of p38 MAPK's role in cell

cycle progression and control, this cluster of genes is of particular interest to this study.

The genes identified to belong to this cluster are plotted below.

A. Cell-Cycle related genes, Induced by TNF- α & unaffected by p38i, 2hrs Time point



B. Cell-Cycle related genes, reduced by TNF- α & unaffected by p38i, 2hrs Time point

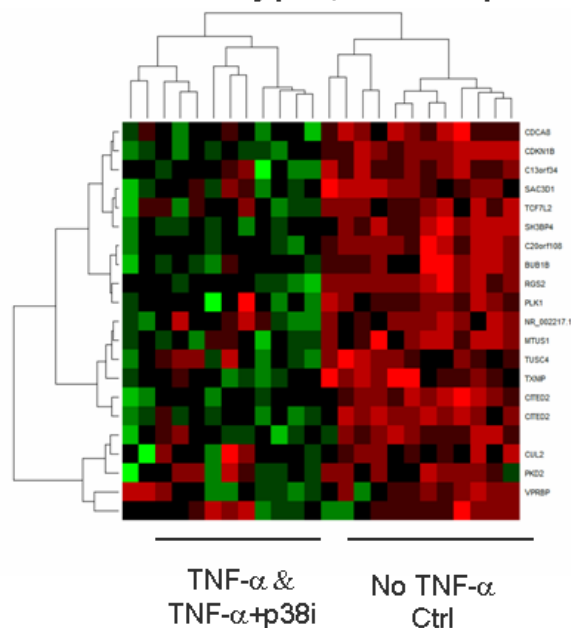


Figure 4.21: Cell cycle genes are modulated by TNF- α , but relatively unaffected by p38-inhibitor: (A) Heatmap of 24 genes induced by TNF- α , related to cell cycle as determined by SP_PIR database (protein super-family database), all genes $FDR < 0.1$ (B) 21 Cell cycle related genes down-regulated by TNF- α and unaffected by p38i (LY479754), all genes $FDR < 0.1$

We observed that a significant number of genes related to the progression or regulation of the cell cycle were induced or repressed by TNF- α treatment at the 2hrs time point. It is very interesting to note that in this unsupervised clustering, the TNF- α conditions and the TNF- α +LY479754 conditions cluster together. This shows that these genes related to the progression or regulation of the cell cycle are relatively unaffected by inhibition of p38 at this time point. Certain genes worth highlighting from this functional cluster include PLK1 (down), PLK2 (up) and CDKN1A (up) genes. The exception to this general observation was the gene GADD45A, a gene related to DNA damage arrest response. However we find that inhibition of p38 does not down-regulate GADD45A, instead inhibition of p38 leads to even higher expression of GADD45A, when compared to both DMSO control and to TNF- α treatment alone. The normalized expression patterns of these highlighted genes are illustrated as boxplots below.

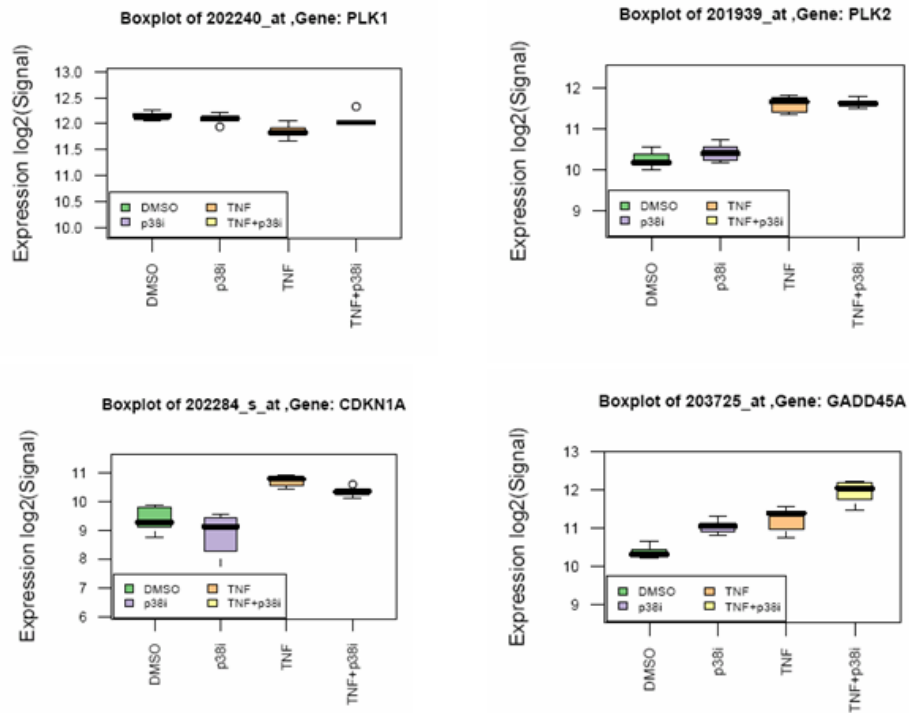


Figure 4.22: Boxplots of selected Cell cycle related genes, modulated by TNF- α at 2hrs time point.

IV. TNF- α induces NF κ B signaling that is not significantly modulated by p38 inhibition at 2hours

The NF κ B pathway is a central pathway involved in stress response, cytokine production and induction of apoptosis. It is one of the main pathways believed to be induced by extrinsic cytokine signaling, and is reported to be responsive to TNF- α induction. Among the genes found significantly modulated by TNF- α in the 2hrs time point, we found a significant functional cluster associated directly with NF κ B signaling. The normalized gene expression of these genes is plotted below.

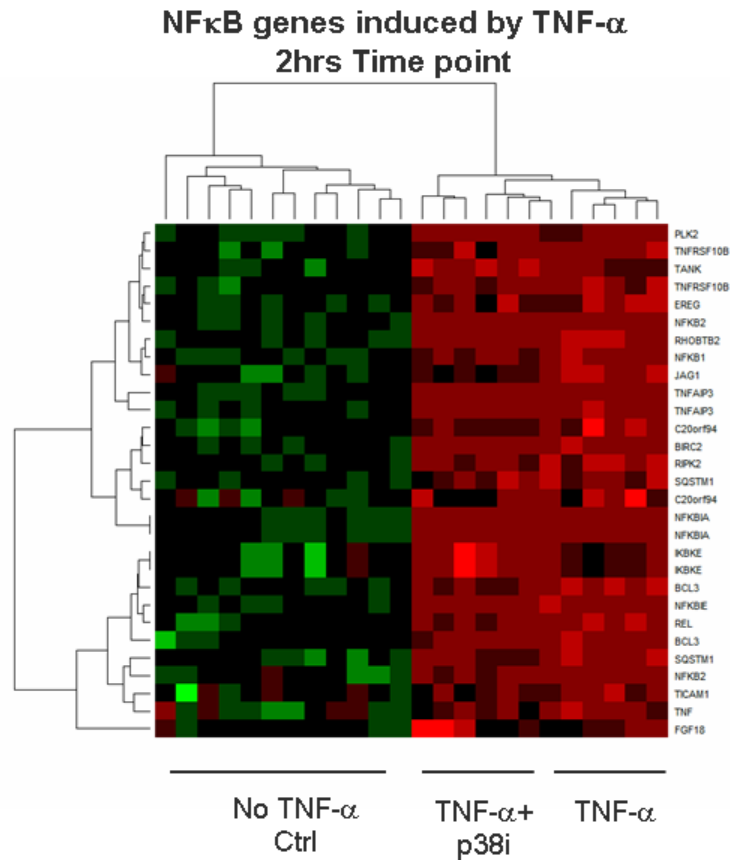


Figure 4.23: Heatmap of NF κ B genes induced by TNF- α treatment at 2hrs time point, all genes FDR<0.1

Genes and pathways associated directly or down-stream of NF κ B signaling are induced by TNF- α treatment. In figure 4.23, we plotted genes that have the associated GO term: GO:0007249~I-kappaB kinase/NF-kappaB cascade. We find that the majority of these genes are strongly induced by TNF- α treatment, in concordance with previously reported studies linking the NF κ B pathway with TNF- α response. We also find that the majority of the genes linked to the NF κ B pathway do not have strong transcriptional response to a p38 inhibitor. While the genes can be segregated into two distinct clusters based on unsupervised clustering, the degree of modulation by the p38 inhibitor is very slight, with most being statistically insignificant (FDR, p-val >0.1) and fold-changes <0.5.

This observation leads to the conclusion that at the mid-time point of this study, the p38 pathway and the NF κ B pathway do not experience significant cross-talk in their signaling networks. High activity of the NF κ B pathway at this time point, moving forward would suggest an alternative or parallel pathway from the p38 MAPK pathway for cancer cells to maintain a high expression of cytokine and chemokines. In addition, the induction and regulation of apoptosis pathways are closely tied to NF κ B signaling, which may suggest a plausible explanation as to the lack of effect of a p38 inhibitor on modulating a large number of apoptosis related genes in this time point. From a signaling pathway standpoint, it is likely that the cell has transitioned from an immediate early response pathway like the p38 MAPK pathway to a more long term responder of stress in the NF κ B pathway. This does not necessarily negate the role of p38 in the long term response to stress, it just illustrates that a cancer cells has multiple parallel pathways in which to respond to external stress or mitogenic signals. Genes directly related to the NF κ B pathway are tabulated in table 4.7 below.

Affy ID	gene	TNFvsCTRL, 2hr log ₂ (FC)	TNFvsp38i, 2hr log ₂ (FC)	Description
202644_s_at	TNFAIP3	4.441217388	-0.35636039	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT,Acc:P21580]
201502_s_at	NFKBIA	3.882233151	0.077807488	NF-kappa-B inhibitor alpha (Major histocompatibility complex enhancer-binding protein MAD3) (I-kappa-B-alpha) (IkappaBalpha) (IKB-alpha). [Source:Uniprot/SWISSPROT,Acc:P25963]
209636_at	NFKB2	2.934868799	-0.22506084	Nuclear factor NF-kappa-B p100 subunit (DNA-binding factor KBF2) (H2TF1) (Lymphocyte translocation chromosome 10) (Oncogene Lyt-10) (Lyt10) [Contains: Nuclear factor NF-kappa-B p52 subunit]. [Source:Uniprot/SWISSPROT,Acc:Q00653]
213112_s_at	SQSTM1	2.739736191	-0.59678093	Sequestosome-1 (Phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa) (Ubiquitin-binding protein p62) (EBI3-associated protein of 60 kDa) (p60) (EBIAP). [Source:Uniprot/SWISSPROT,Acc:Q13501]
207113_s_at	TNF	2.046688092	-0.5809232	Tumor necrosis factor precursor (TNF-alpha) (Tumor necrosis factor ligand superfamily member 2) (TNF-a) (Cachectin) [Contains: Tumor necrosis factor, membrane form; Tumor necrosis factor, soluble form]. [Source:Uniprot/SWISSPROT,Acc:P01375]
204908_s_at	BCL3	1.894032853	-0.22451406	B-cell lymphoma 3-encoded protein (Protein Bcl-3). [Source:Uniprot/SWISSPROT,Acc:P20749]
203927_at	NFKBIE	1.667812366	-0.105588	NF-kappa-B inhibitor epsilon (NF-kappa-BIE) (I-kappa-B-epsilon) (IkappaBepsilon) (IKB-epsilon) (IKBE). [Source:Uniprot/SWISSPROT,Acc:O00221]
206036_s_at	REL	1.480042714	-0.16417491	C-Rel proto-oncogene protein (C-Rel protein). [Source:Uniprot/SWISSPROT,Acc:Q04864]
209295_at	RHOBTB2	1.403643072	-0.14655622	Rho-related BTB domain-containing protein 2 (Deleted in breast cancer 2 gene protein) (p83). [Source:Uniprot/SWISSPROT,Acc:Q9BYZ6]
201939_at	PLK2	1.376822332	0.023503744	Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2) (Serine/threonine-protein kinase SNK) (Serum-inducible kinase). [Source:Uniprot/SWISSPROT,Acc:Q9NYY3]
202076_at	BIRC2	1.336640492	-0.17009575	Baculoviral IAP repeat-containing protein 2 (Inhibitor of apoptosis protein 2) (HIAP2) (HIAP-2) (C-IAP1) (TNFR2-TRAF-signaling complex protein 2) (IAP homolog B) (RING finger protein 48). [Source:Uniprot/SWISSPROT,Acc:Q13490]
211029_x_at	FGF18	1.170717228	0.522656352	Fibroblast growth factor 18 precursor (FGF-18) (zFGF5). [Source:Uniprot/SWISSPROT,Acc:O76093]
209239_at	NFKB1	1.08984003	-0.23777011	Nuclear factor NF-kappa-B p105 subunit (DNA-binding factor KBF1) (EBP- 1) [Contains: Nuclear factor NF-kappa-B p50 subunit]. [Source:Uniprot/SWISSPROT,Acc:P19838]
205767_at	EREG	1.073429857	-0.25891527	Epiregulin precursor (EPR). [Source:Uniprot/SWISSPROT,Acc:O14944]
209294_x_at	TNFRSF10B	1.063571345	-0.1225767	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor 2) (TRAIL-R2) (CD262 antigen). [Source:Uniprot/SWISSPROT,Acc:O14763]
209545_s_at	RIPK2	0.894255841	-0.13174171	Receptor-interacting serine/threonine-protein kinase 2 (EC 2.7.11.1) (RIP-like-interacting CLARP kinase) (Receptor-interacting protein 2) (RIP-2) (CARD-containing interleukin-1 beta-converting enzyme-associated kinase) (CARD-containing IL-1 beta ICE-kinase) [Source:Uniprot/SWISSPROT,Acc:O43353]
213191_at	TICAM1	0.768758719	-0.34551643	toll-like receptor adaptor molecule 1 isoform 2 [Source:RefSeq_peptide,Acc:NP_891549]
209098_s_at	JAG1	0.757700224	-0.29145389	Jagged-1 precursor (Jagged1) (hJ1) (CD339 antigen). [Source:Uniprot/SWISSPROT,Acc:P78504]
207616_s_at	TANK	0.602998512	0.229765436	TRAF family member-associated NF-kappa-B activator (TRAF-interacting protein) (I-TRAF). [Source:Uniprot/SWISSPROT,Acc:Q92844]
214398_s_at	IKBKE	0.545187563	0.265926607	Inhibitor of nuclear factor kappa-B kinase subunit epsilon (EC 2.7.11.10) (I kappa-B kinase epsilon) (IKBKE) (IKK-epsilon) (IKK-E) (Inducible I kappa-B kinase) (IKK-i). [Source:Uniprot/SWISSPROT,Acc:Q14164]

Table 4.7: NFkB related genes directly modulated by TNF- α treatment at 2hrs, all genes FDR<0.1

Summary of Mid-Time point (2hrs)

In this time point we observed a very large increase in the number of genes positively and negatively regulated by TNF- α treatment. We also observed the largest number of genes that were modulated by a p38 inhibitor (LY479754) versus the TNF- α treatment condition. In this section we highlighted 4 major functional clusters that were modulated by TNF- α , of which 2 of these functional clusters were observed to not be sensitive to p38 inhibition. The two functional clusters that were observed to be sensitive to p38 inhibition were the cell proliferation, development and differentiation cluster and

the regulation of apoptosis cluster. In the cell proliferation/development/differentiation cluster, we found that a large number of induced genes including cytokines such as IL6, IL8 and TNF- α were inhibited by a p38 inhibitor. The transcriptional repression of this class of genes, suggests that p38 MAPK is required for these genes to effectively respond to TNF- α stimulation. In the apoptosis functional cluster we identified 2 groups of genes, the first group whose expression was observed to be sensitive to a p38 inhibitor, and a second group whose expression was insensitive to p38 inhibition. This data suggests that similar to the effects observed in the early time points, p38 plays a role in the regulation of apoptosis, however there is clear evidence that there are parallel signaling pathways that regulate apoptosis in addition to p38 signaling. One such pathway that has come up prominently in this time point is the NF κ B pathway. NF κ B response to TNF- α is well documented so it is no surprise to observe strong induction of NF κ B signaling in response to TNF- α . We observed that the transcriptional response of the majority of genes belonging to the NF κ B pathway were not responsive to a p38 inhibitor, suggesting that at this time point, we observe little cross talk between the p38 and NF κ B pathway. Lastly, as a large proportion of this thesis is focused on gaining a mechanistic understanding of p38 MAPK in the context of control of the cell cycle. We observe that TNF- α , a non-genotoxic stress induces a fairly large transcriptional response in genes related to cell cycle progression or cell cycle regulation. We observe that inhibition of p38 in the most part has little effect on genes functionally related to the cell cycle. This lends some support to the observation that p38 activity at the G2 cell cycle arrest checkpoint may be associated with a stress response and not a cell cycle arrest phenotype. Cell cycle genes induced strongly by TNF- α and are unaffected by p38 inhibition include CDKN1A (alias: p21^{Waf1}).

4.7 Effect of TNF- α treatment and p38 inhibition at the late time points (4hrs & 7hrs)

In the early (30min & 60mins) and mid (2hrs) time points, we observed significant induction of gene expression in response to TNF- α treatment. Many of the genes induced in the early and mid time points in this study belonged to early response signaling pathways. In the later time points, however we find that a lot of the signaling and response networks are likely due to sustained activation or longer term response pathways in response to stress. As observed in figure 4.3, the number of genes (probsets) significantly modulated by TNF- α at these two later time points is very large. We present the significant genes at this time point as a collection of functionally related pathways. We will highlight a number of key pathways that are of interest to this study. In table 4.8 below, we present the top 15 (neighborhood) gene networks of relevance derived from the Ingenuity pathway analysis tool, for genes modulated by TNF- α at the late time points.

S/No.	Top Functions	Score	Focus Molecules
1	Infectious Disease, Cancer, Cell Death	43	25
2	Cell Death, Reproductive System Disease, Hematological Disease	33	22
3	Cell-To-Cell Signaling and Interaction, Antigen Presentation, Antimicrobial Response	32	21
4	Cellular Development, Cancer, Cellular Movement	31	20
5	Lipid Metabolism, Small Molecule Biochemistry, Cellular Compromise	31	20
6	Nervous System Development and Function, Cell Signaling, Cancer	29	19
7	Cancer, Cell Cycle, Neurological Disease	29	19
8	Cancer, Cell Death, Immunological Disease	28	20
9	Cancer, Cell-mediated Immune Response, Hematological System Development and Function	24	17
10	Infection Mechanism, Carbohydrate Metabolism, Molecular Transport	20	15
11	Cellular Compromise, Hypersensitivity Response, Cancer	17	13
12	Amino Acid Metabolism, Cancer, Cell Morphology	17	13
13	Cell-mediated Immune Response, Cellular Development, Hematological System Development and Function	16	12
14	Genetic Disorder, Skeletal and Muscular Disorders, Cancer	14	11
15	Carbohydrate Metabolism, Small Molecule Biochemistry, Lipid Metabolism	7	7

Table 4.8: Top networks for genes modulated by TNF- α at the late time points.

As detailed in table 4.8 above, TNF- α induces a host of physiological pathways 7 hours post treatment. The functional pathways with the highest clustering score include the cell death, cell to cell signaling, cellular development and inflammatory response. As many of these functional pathways have been observed in the earlier time points, within each section we will also examine the pattern of gene expression across time for the group of genes that are highly expressed across time.

I. TNF- α induces sustained pro and anti-apoptotic signaling

As observed in other time points during this study, TNF- α treatment induces a strong apoptotic response in Calu6 cells. Genes related to the induction and the regulation of

apoptosis are among the top functionally related clusters in the large group of genes modulated by TNF- α treatment. We plot these genes as a heatmap below.

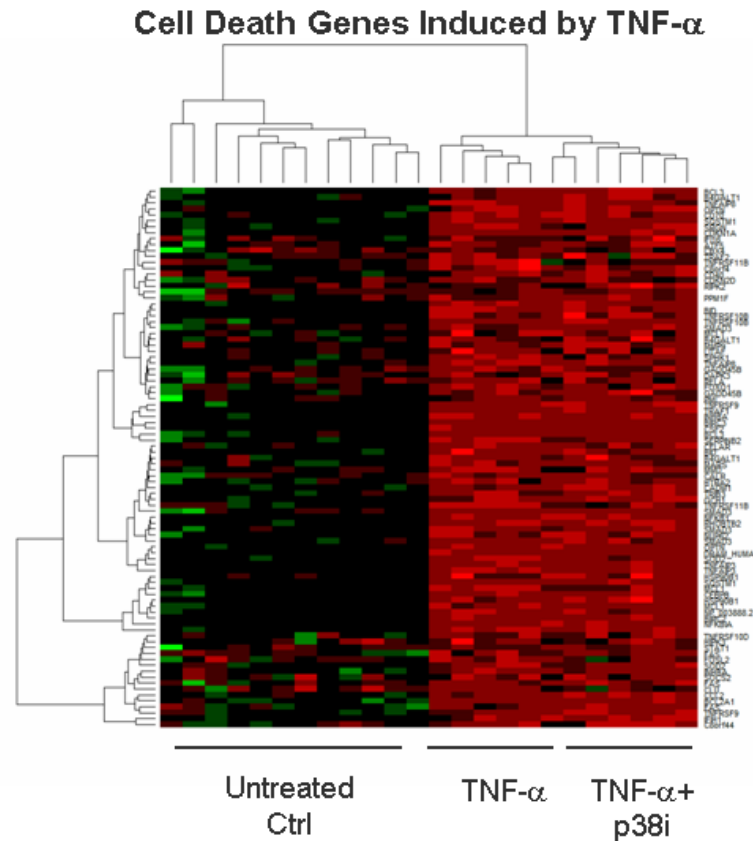


Figure 4.24: Heatmap of Cell Death related genes induced by TNF- α at the late time points (4hrs & 7hrs), all genes FDR<0.1

A large number of the cell death and apoptosis related genes induced strongly by TNF- α are not strongly modulated by p38 inhibition at this time point (Figure 4.24). This is likely due to the large number of feedback and parallel signaling pathways already induced by this time. There are a number of genes however whose transcriptional response is degraded by p38 inhibition even at this later time point. These genes whose expression is still affected by p38 inhibition, allow us to dissect the apoptosis and cell

death pathways to understand what genes and pathways still depend on p38 kinase activity.

Genes specific to induction of apoptosis are plotted as a sub-network to illustrate relationships among genes.

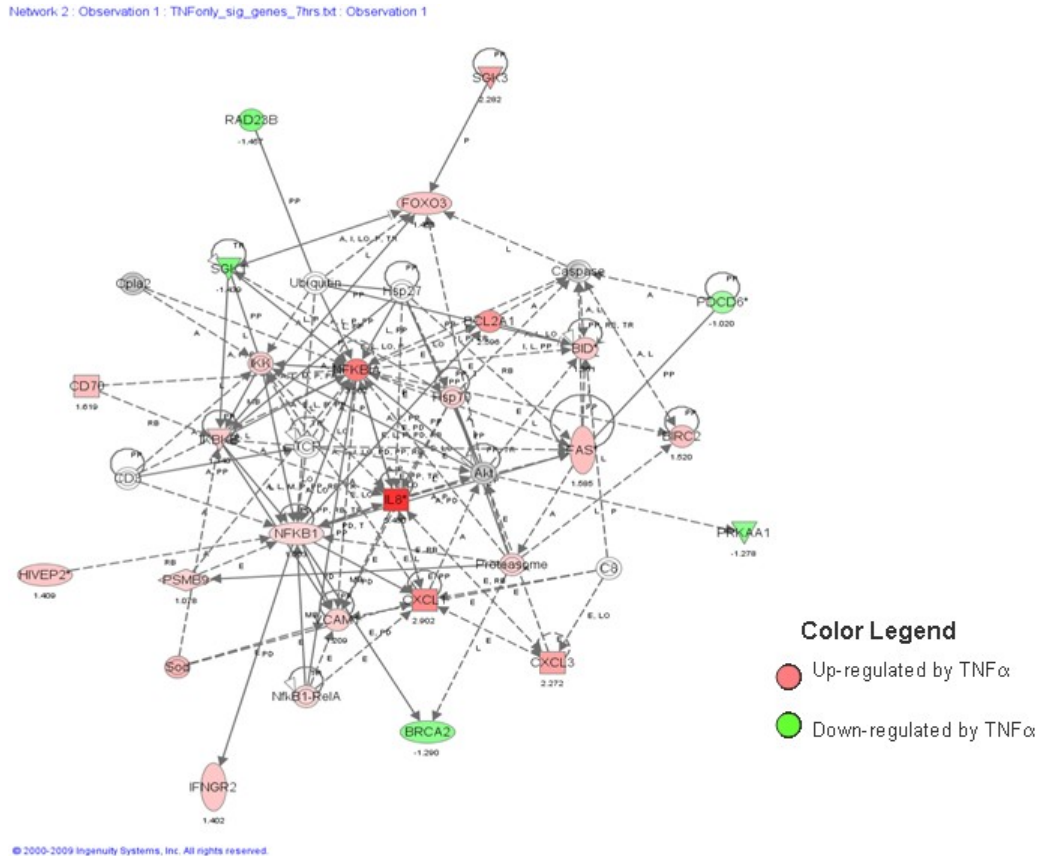


Figure 4.25: Ingenuity pathway analysis network diagram of apoptosis related genes strongly induced by TNF- α at late time points.

The network diagram (Figure 4.25) shows a strong interconnected relationship between genes strongly induced by TNF- α and the NF κ B pathway, suggesting that by the late time points, early response signaling through the p38 pathway has been replaced by signaling through the NF κ B pathway.

In addition to pro-apoptotic genes induced at the late time points, a few genes observed to be highly expressed in the late time points have been observed to be

significantly expressed in the earlier time points. Among the genes, whose activity is consistently up-regulated by TNF- α treatment across time is a small family of anti-apoptosis genes, including the IAP (Inhibitor of Apoptosis) proteins. As the IAP proteins are strongly expressed across time in response to TNF- α treatment, we conclude that they play a major role in protecting Calu6 lung cancer cells from TNF- α induced apoptosis (Figure 4.26).

IAP & Pro-Survival proteins strongly induced across time

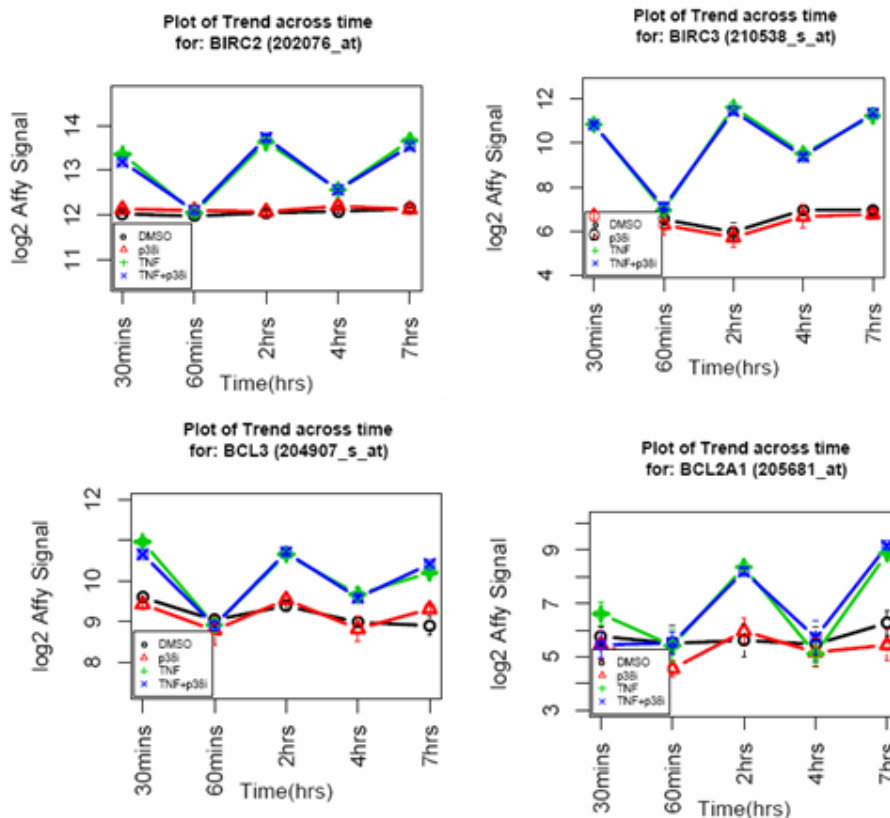


Figure 4.26: IAP and other pro-cell survival genes are strongly expressed across time in response to TNF- α treatment

We find that while the number of genes modulated strongly by p38 inhibition at this time point is small, they still consist of a number of highly relevant genes for the

control of apoptosis. The gene CLU (Clusterin) has been implicated to interact and inactivate the pro-apoptosis BCL2 protein Bax. The gene TRADD is a central component of the TNF receptor death domain complex. The FOSL2 is one of 4 members of the FOS family that is known to play a role in cell proliferation and survival. Finally Casp6 is central component of the central caspase activation pathway. These genes and others all show a similar pattern in that TNF- α decreases apoptosis potential while inhibition of p38 restores the expression to levels consistent with a more pro-apoptotic setting. A subset of these genes is plotted as boxplots below (Figure 4.27).

Apoptosis Related Genes, modulated by p38 inhibition At Late time points (4 & 7hrs)

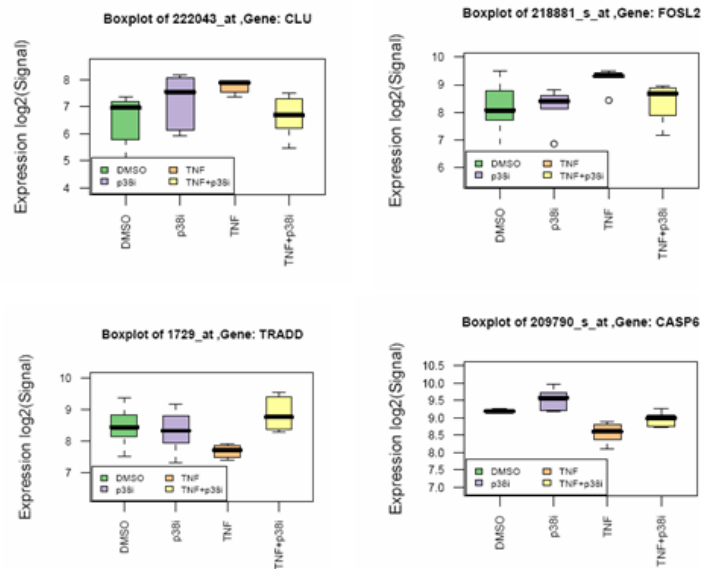


Figure 4.27: *Boxplot of apoptosis related genes, modulated by p38 inhibition at the late time points (4hrs & 7hrs)*

Calu6 cells respond to TNF- α stress by strong activation of anti-apoptotic proteins or reduction of key components of the pro-apoptotic signaling cascade at late time points. At early and mid time points, p38 activity was found to play a role in supporting

a pro-survival response to TNF- α . The complication as time progresses in this study is the host of parallel signaling pathways that have been activated by this time point. Many of these signaling pathways (i.e. NF κ B) play a strong role in the control of apoptosis and genes that may have depended on p38 activity in the earlier time points may now be induced by parallel signaling pathways.

A table annotating the genes discussed in this section is displayed below as table 4.9.

Affy ID	gene	TNFvsCTRL, 7hr log2(FC)	TNFvsp38i, 7hr log2(FC)	Description
210229_s_at	CSF2	4.941218187	-0.2652719	Granulocyte-macrophage colony-stimulating factor precursor (GM-CSF) (Colony-stimulating factor) (CSF) (Sargramostim) (Molgramostin). [Source:Uniprot/SWISSPROT,Acc:P04141]
210538_s_at	BIRC3	4.27390536	0.09933606	Baculoviral IAP repeat-containing protein 3 (Inhibitor of apoptosis protein 1) (HIAP-1) (C-IAP2) (TNFR2-TRAF-signaling complex protein 1) (IAP homolog C) (Apoptosis inhibitor 2) (API2) (RING finger protein 49). [Source:Uniprot/SWISSPROT,Acc:Q13489]
210511_s_at	INHBA	3.729153655	-0.8011857	Inhibin beta A chain precursor (Activin beta-A chain) (Erythroid differentiation protein) (EDF). [Source:Uniprot/SWISSPROT,Acc:P08476]
202644_s_at	TNFAIP3	3.622028592	-0.3444714	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT,Acc:P21580]
216598_s_at	CCL2	3.409838295	0.20436378	Small inducible cytokine A2 precursor (CCL2) (Monocyte chemoattractant protein 1) (MCP-1) (Monocyte chemoattractant protein 1) (Monocyte chemoattracting and activating factor) (MCAF) (Monocyte secretory protein JE) (HC11). [Source:Uniprot/SWISSPROT,Acc:P13500]
201502_s_at	NFKBIA	3.301642698	0.13442105	NF-kappa-B inhibitor alpha (Major histocompatibility complex enhancer-binding protein MAD3) (I-kappa-B-alpha) (IkappaBalpha) (IKB-alpha). [Source:Uniprot/SWISSPROT,Acc:P25963]
204908_s_at	BCL3	3.163416956	0.12543009	B-cell lymphoma 3-encoded protein (Protein Bcl-3). [Source:Uniprot/SWISSPROT,Acc:P20749]
204614_at	SERPINB2	3.066751227	-0.9992378	Plasminogen activator inhibitor 2 precursor (PAI-2) (Placental plasminogen activator inhibitor) (Monocyte Arg-serpin) (Urokinase inhibitor). [Source:Uniprot/SWISSPROT,Acc:P05120]
202643_s_at	TNFAIP3	2.664326322	-0.2830518	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT,Acc:P21580]
205681_at	BCL2A1	2.596495096	0.28612983	Bcl-2-related protein A1 (Protein BFL-1) (Hemopoietic-specific early response protein) (Protein GRS). [Source:Uniprot/SWISSPROT,Acc:Q16548]
207536_s_at	TNFRSF9	2.570207665	0.64205657	Tumor necrosis factor receptor superfamily member 9 precursor (4-1BB ligand receptor) (T-cell antigen 4-1BB homolog) (T-cell antigen ILA) (CD137 antigen) (CDw137). [Source:Uniprot/SWISSPROT,Acc:Q07011]
205599_at	TRAF1	2.532916039	0.14763185	TNF receptor-associated factor 1 (Epstein-Barr virus-induced protein 6). [Source:Uniprot/SWISSPROT,Acc:Q13077]
211786_at	TNFRSF9	2.413171465	0.85655463	Tumor necrosis factor receptor superfamily member 9 precursor (4-1BB ligand receptor) (T-cell antigen 4-1BB homolog) (T-cell antigen ILA) (CD137 antigen) (CDw137). [Source:Uniprot/SWISSPROT,Acc:Q07011]
219209_at	IFIH1	2.360926264	1.00740951	Interferon-induced helicase C domain-containing protein 1 (EC 3.6.1.-) (Interferon-induced with helicase C domain protein 1) (Helicase with 2 CARD domains) (Helicard) (Melanoma differentiation-associated protein 5) (MDA-5) (RNA helicase-DEAD box protein 1). [Source:Uniprot/SWISSPROT,Acc:Q9BYX4]
205397_x_at	SMAD3	2.07289337	0.09810407	Mothers against decapentaplegic homolog 3 (SMAD3) (Mothers against DPP homolog 3) (Mad3) (hMAD-3) (JV15-2) (hSMAD3). [Source:Uniprot/SWISSPROT,Acc:P84022]
216841_s_at	SOD2	1.703815447	-0.4764707	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1). [Source:Uniprot/SWISSPROT,Acc:P04179]
210260_s_at	TNFAIP8	1.607161198	-0.3436462	Tumor necrosis factor, alpha-induced protein 8 (Head and neck tumor and metastasis-related protein) (TNF-induced protein GG2-1) (SCC-S2) (NF-kappa B-inducible DED-containing protein) (NDED) (MDC-3 13). [Source:Uniprot/SWISSPROT,Acc:Q95379]
204780_s_at	FAS	1.584566872	0.45011613	Tumor necrosis factor receptor superfamily member 6 precursor (FASLG receptor) (Apoptosis-mediating surface antigen FAS) (Apo-1 antigen) (CD95 antigen). [Source:Uniprot/SWISSPROT,Acc:P25445]
212501_at	CEBPB	1.559301688	-0.2221893	CCAAT/enhancer-binding protein beta (C/EBP beta) (Nuclear factor NF-IL6) (Transcription factor 5). [Source:Uniprot/SWISSPROT,Acc:P17676]
201859_at	SRGN	1.53456041	-0.3359442	Serglycin precursor (Secretory granule proteoglycan core protein) (Platelet proteoglycan core protein) (P.PG) (Hematopoietic proteoglycan core protein). [Source:Uniprot/SWISSPROT,Acc:P10124]
203063_at	PPM1F	1.371246042	0.09826989	Protein phosphatase 1F (EC 3.1.3.16) (Ca ²⁺ /calmodulin-dependent protein kinase phosphatase) (CaM-kinase phosphatase) (CaMKPase) (Partner of PI3 2) (hFEM-2). [Source:Uniprot/SWISSPROT,Acc:P49593]
204907_s_at	BCL3	1.29754631	0.21254527	B-cell lymphoma 3-encoded protein (Protein Bcl-3). [Source:Uniprot/SWISSPROT,Acc:P20749]
222043_at	CLU	1.279018008	-1.1211298	Clusterin precursor (Complement-associated protein SP-40.40) (Complement cytolytic inhibitor) (CLI) (NA1/NA2) (Apolipoprotein J) (Apo-J) (Testosterone-repressed prostate message 2) (TRPM-2) [Contains: Clusterin beta chain (ApoJalpha) (Complement cytolytic inhibitor)]. [Source:Uniprot/SWISSPROT,Acc:P10909]
202073_at	OPTN	1.197171039	0.29566698	Optineurin (Optic neuropathy-inducing protein) (E3-14.7K-interacting protein) (FIP-2) (Huntingtin-interacting protein HYPL) (NEMO-related protein) (Transcription factor IIIA-interacting protein) (TFIIIA-IntP). [Source:Uniprot/SWISSPROT,Acc:Q96CV9]
218881_s_at	FOSL2	1.129130092	-0.8409005	Fos-related antigen 2. [Source:Uniprot/SWISSPROT,Acc:P15408]
209031_at	CADM1	0.998247971	0.21298599	Cell adhesion molecule 1 precursor (Immunoglobulin superfamily member 4) (Nectin-like protein 2) (NECL-2) (Tumor suppressor in lung cancer 1) (TSLC-1) (Synaptic cell adhesion molecule) (Spermatogenic immunoglobulin superfamily) (SglGSF). [Source:Uniprot/SWISSPROT,Acc:Q9BY67]
209294_x_at	TNFRSF10B	0.973310169	0.14785516	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor 2) (TRAIL-R2) (CD262 antigen). [Source:Uniprot/SWISSPROT,Acc:Q14763]
204413_at	TRAF2	0.930495397	-0.2264981	TNF receptor-associated factor 2 (Tumor necrosis factor type 2 receptor-associated protein 3). [Source:Uniprot/SWISSPROT,Acc:Q12933]
216627_s_at	B4GALT1	0.860010421	0.17256231	Beta-1,4-galactosyltransferase 1 (EC 2.4.1.-) (Beta-1,4-GalTase 1) (Beta4Gal-T1) (b4Gal-T1) (UDP-galactose beta-N-acetylglucosamine beta-1,4-galactosyltransferase 1) (UDP-Gal:beta-GlcNAc beta-1,4-galactosyltransferase 1) [Includes: Lactose synthase A pr]. [Source:Uniprot/SWISSPROT,Acc:P15291]
209878_s_at	RELA	0.856882634	-0.2409945	Transcription factor p65 (Nuclear factor NF-kappa-B p65 subunit). [Source:Uniprot/SWISSPROT,Acc:Q04206]
211012_s_at	PML	0.819903269	0.10060996	Probable transcription factor PML (Tripartite motif-containing protein 19) (RING finger protein 71). [Source:Uniprot/SWISSPROT,Acc:P29590]
203414_at	MMD	0.759211451	-0.2407004	Monocyte to macrophage differentiation protein (Progesterin and adipoQ receptor family member XI). [Source:Uniprot/SWISSPROT,Acc:Q15546]
200797_s_at	MCL1	0.734573028	-0.2851063	Induced myeloid leukemia cell differentiation protein Mcl-1 (Bcl-2-related protein EAT/mcl1) (mcl1/EAT). [Source:Uniprot/SWISSPROT,Acc:Q07820]
204933_s_at	TNFRSF11B	0.728407811	-0.5945603	Tumor necrosis factor receptor superfamily member 11B precursor (Osteoprotegerin) (Osteoclastogenesis inhibitory factor). [Source:Uniprot/SWISSPROT,Acc:Q00300]
210148_at	HIPK3	0.708304296	-0.3826146	Homeodomain-interacting protein kinase 3 (EC 2.7.11.1) (Homolog of protein kinase YAK1) (Fas-interacting serine/threonine-protein kinase) (FIST) (Androgen receptor-interacting nuclear protein kinase) (ANPK). [Source:Uniprot/SWISSPROT,Acc:Q9H422]
209544_at	RIPK2	0.687281079	0.15052039	Receptor-interacting serine/threonine-protein kinase 2 (EC 2.7.11.1) (RIP-like-interacting CLARP kinase) (Receptor-interacting protein 2) (RIP-2) (CARD-containing interleukin-1 beta-converting enzyme-associated kinase) (CARD-containing IL-1 beta ICE-kinase). [Source:Uniprot/SWISSPROT,Acc:Q43353]
1729_at	TRADD	-0.78128711	1.17749078	Tumor necrosis factor receptor type 1-associated DEATH domain protein (TNFR1-associated DEATH domain protein) (TNFRSF1A-associated via death domain). [Source:Uniprot/SWISSPROT,Acc:Q15628]

Table 4.9: Top 40 Apoptosis Genes modulated by TNF- α in the late time points

II. TNF- α induces sustained Inflammatory response

Among the many top functional clusters identified among the genes induced by TNF- α treatment at the late time points is the cluster for inflammatory response. As TNF- α is known to play a key role in inflammatory cytokine production, this observation should

not be surprising. We observed throughout the different time points in this study, the onset, and continuous induction of pro-inflammatory pathways. We find that TNF- α triggers a strong transcriptional response to sustain an inflamed phenotype. This contributes to the ability of cancers cells to maintain an inflamed tumor microenvironment, which is ideal for increased cell proliferation and tumor growth. In this section we highlight the key signaling and pathway components that contribute to this sustained inflammation response.

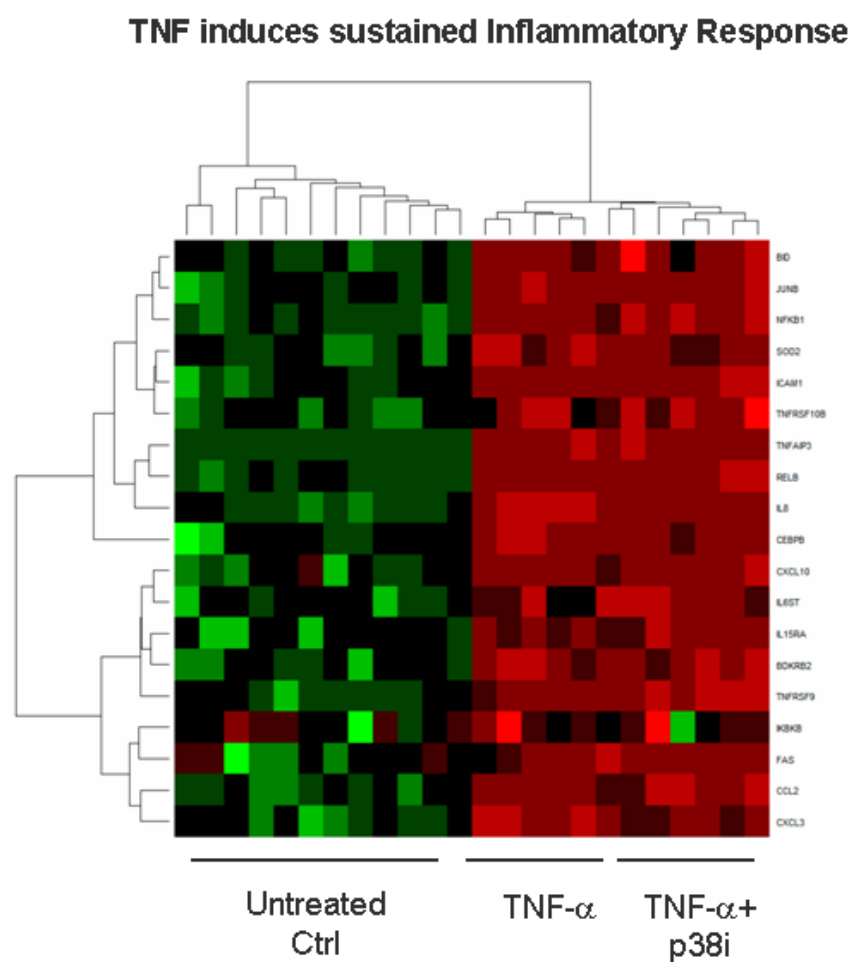


Figure 4.28: *Heatmap of Genes associated with inflammatory response at late time points (7hrs), all genes FDR<0.1*

Among the genes related to inflammation strongly induced at the late time point, include a number of inducible cytokines including IL8, IL6 and CXCL3 (Figure 4.28). In addition to evidence of positive feedback loops inducing additional cytokine production, we observed that TNF- α induced multiple components of the NF κ B pathway strongly. The expression of these genes shows that Calu6 cells increase pro-inflammatory molecules in response to TNF- α induction. When taken into context with increased anti-apoptotic gene induction as described in preceding sections, it shows that TNF- α treatment in a cancer setting leads to an increased inflammatory response with a marked decrease in associated apoptosis induction.

This data shows again that NF κ B plays a role in the induction of cytokine production in response to TNF- α treatment. As the p38 pathway was responsible for early induction of cytokine production, in the late time points, this role has been taken over by the NF κ B pathway, leading to sustained cytokine production. This switch from a p38 dependent (early response) to an NF κ B dependent signaling network is a possible explanation as to why inhibition of p38 no longer significantly affects the transcriptional response rate of inflammatory cytokines at the later time points. This suggests that at the later time points, the NF κ B pathway plays a strong role in the induction of both cytokine production pathways as well as signaling pathways that regulate the induction of apoptosis.

This provides a potential mechanistic understanding of how cancer cells are able to hijack dual functioning cytokines such as TNF- α to promote the rapid establishment of a tumor microenvironment without undergoing sustained cell death through apoptosis induction. In addition we find that by this time in the study, inhibition of p38 has only marginal effects on the components of the inflammation response. A plausible

explanation as stated earlier is the induction of a host of parallel or redundant signaling pathways that lead to the induction of inflammatory pathways despite p38 inhibition. A table describing the gene expression of the genes discussed in this section is presented below (Table 4.10).

Affy Id	gene	FC.TNF.CTR L.60min	FC.p38i.TN F.60min	Description
202859_x_at	IL8	5.430017511	-0.79777671	Interleukin-8 precursor (IL-8) (CXCL8) (Monocyte-derived neutrophil chemotactic factor) (MDNCF) (T-cell chemotactic factor) (Neutrophil activating protein 1) (NAP-1) (Protein 3-10C) (Granulocyte chemotactic protein 1) (GCP-1) (Monocyte-derived neutrophil [Source:Uniprot/SWISSPROT,Acc:P10145])
202644_s_at	TNFAIP3	3.622028592	-0.3444714	Tumor necrosis factor, alpha-induced protein 3 (EC 3.-.-.) (Putative DNA-binding protein A20) (Zinc finger protein A20). [Source:Uniprot/SWISSPROT,Acc:P21580]
216598_s_at	CCL2	3.409838295	0.20436378	Small inducible cytokine A2 precursor (CCL2) (Monocyte chemotactic protein 1) (MCP-1) (Monocyte chemoattractant protein 1) (Monocyte chemotactic and activating factor) (MCAF) (Monocyte secretory protein JE) (HC11). [Source:Uniprot/SWISSPROT,Acc:P13500]
205205_at	RELB	2.955355782	0.22562461	Transcription factor RelB (I-Rel). [Source:Uniprot/SWISSPROT,Acc:Q01201]
207536_s_at	TNFRSF9	2.570207665	0.64205657	Tumor necrosis factor receptor superfamily member 9 precursor (4-1BB ligand receptor) (T-cell antigen 4-1BB homolog) (T-cell antigen ILA) (CD137 antigen) (CDw137). [Source:Uniprot/SWISSPROT,Acc:Q07011]
207850_at	CXCL3	2.271800362	-0.3310059	Macrophage inflammatory protein 2-beta precursor (MIP2-beta) (CXCL3) (Growth-regulated protein gamma) (GRO-gamma) (GRO-gamma(1-73)) [Contains: GRO-gamma(5-73)]. [Source:Uniprot/SWISSPROT,Acc:P19876]
215485_s_at	ICAM1	2.140413059	0.24039421	Intercellular adhesion molecule 1 precursor (ICAM-1) (Major group rhinovirus receptor) (CD54 antigen). [Source:Uniprot/SWISSPROT,Acc:P05362]
207375_s_at	IL15RA	1.820131838	0.15264607	Interleukin-15 receptor alpha chain precursor (IL-15R-alpha) (IL-15RA). [Source:Uniprot/SWISSPROT,Acc:Q13261]
205870_at	BDKRB2	1.799985479	-0.0817761	B2 bradykinin receptor (BK-2 receptor) (B2R). [Source:Uniprot/SWISSPROT,Acc:P30411]
204780_s_at	FAS	1.584566872	0.45011613	Tumor necrosis factor receptor superfamily member 6 precursor (FASLG receptor) (Apoptosis-mediating surface antigen FAS) (Apo-1 antigen) (CD95 antigen). [Source:Uniprot/SWISSPROT,Acc:P25445]
212501_at	CEBPB	1.559301688	-0.2221893	CCAAT/enhancer-binding protein beta (C/EBP beta) (Nuclear factor NF-IL6) (Transcription factor 5). [Source:Uniprot/SWISSPROT,Acc:P17676]
215223_s_at	SOD2	1.505747401	-0.2184291	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1). [Source:Uniprot/SWISSPROT,Acc:P04179]
201473_at	JUNB	1.354134424	-0.0286354	Transcription factor jun-B. [Source:Uniprot/SWISSPROT,Acc:P17275]
204533_at	CXCL10	1.24690463	-0.0154067	Small inducible cytokine B10 precursor (CXCL10) (10 kDa interferon-gamma-induced protein) (Gamma-IP10) (IP-10) [Contains: CXCL10(1-73)]. [Source:Uniprot/SWISSPROT,Acc:P02778]
209239_at	NFKB1	1.003189623	-0.0302694	Nuclear factor NF-kappa-B p105 subunit (DNA-binding factor KBF1) (EBP-1) [Contains: Nuclear factor NF-kappa-B p50 subunit]. [Source:Uniprot/SWISSPROT,Acc:P19838]
210405_x_at	TNFRSF10B	0.97005959	0.06642402	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (TNF-related apoptosis-inducing ligand receptor 2) (TRAIL receptor 2) (TRAIL-R2) (CD262 antigen). [Source:Uniprot/SWISSPROT,Acc:O14763]
211000_s_at	IL6ST	0.957947275	0.310149	Interleukin-6 receptor subunit beta precursor (IL-6R-beta) (Interleukin-6 signal transducer) (Membrane glycoprotein 130) (gp130) (Oncostatin-M receptor alpha subunit) (CD130 antigen) (CDw130). [Source:Uniprot/SWISSPROT,Acc:P40189]
211725_s_at	BID	0.728377507	-0.0605463	BH3-interacting domain death agonist (BID) (p22 BID) [Contains: BH3-interacting domain death agonist p15 (p15 BID), BH3-interacting domain death agonist p13 (p13 BID), BH3-interacting domain death agonist p11 (p11 BID)]. [Source:Uniprot/SWISSPROT,Acc:P55957]
209342_s_at	IKKBK	0.21172991	-0.2829886	Inhibitor of nuclear factor kappa-B kinase subunit beta (EC 2.7.11.10) (I-kappa-B kinase beta) (IKK(B) (IKK-beta) (IKK- β) (I-kappa-B kinase 2) (IKK2) (Nuclear factor NF-kappa-B inhibitor kinase beta) (NFKBIKB). [Source:Uniprot/SWISSPROT,Acc:O14920]
207851_s_at	INSR	0.171083134	0.23012072	Insulin receptor precursor (EC 2.7.10.1) (IR) (CD220 antigen) [Contains: Insulin receptor subunit alpha, Insulin receptor subunit beta]. [Source:Uniprot/SWISSPROT,Acc:P06213]
214272_at	CYLD	0.027457053	0.62223429	Probable ubiquitin carboxyl-terminal hydrolase CYLD (EC 3.1.2.15) (Ubiquitin thioesterase CYLD) (Ubiquitin-specific-processing protease CYLD) (Deubiquitinating enzyme CYLD). [Source:Uniprot/SWISSPROT,Acc:Q9NQC7]

Table 4.10: Inflammatory genes induced by TNF- α at the late time points

III. TNF- α induces genes linked to progression of cell cycle

The last major category of genes that we explored in-depth in this analysis are the genes related to the progression of the cell cycle. Similar to observations at the mid-time point, we find a sizeable functional cluster of genes induced by TNF- α treatment related to the progression and control of the cell cycle. In general, sustained exposure to TNF- α seems to increase genes related to progression of the cell cycle and cellular proliferation.

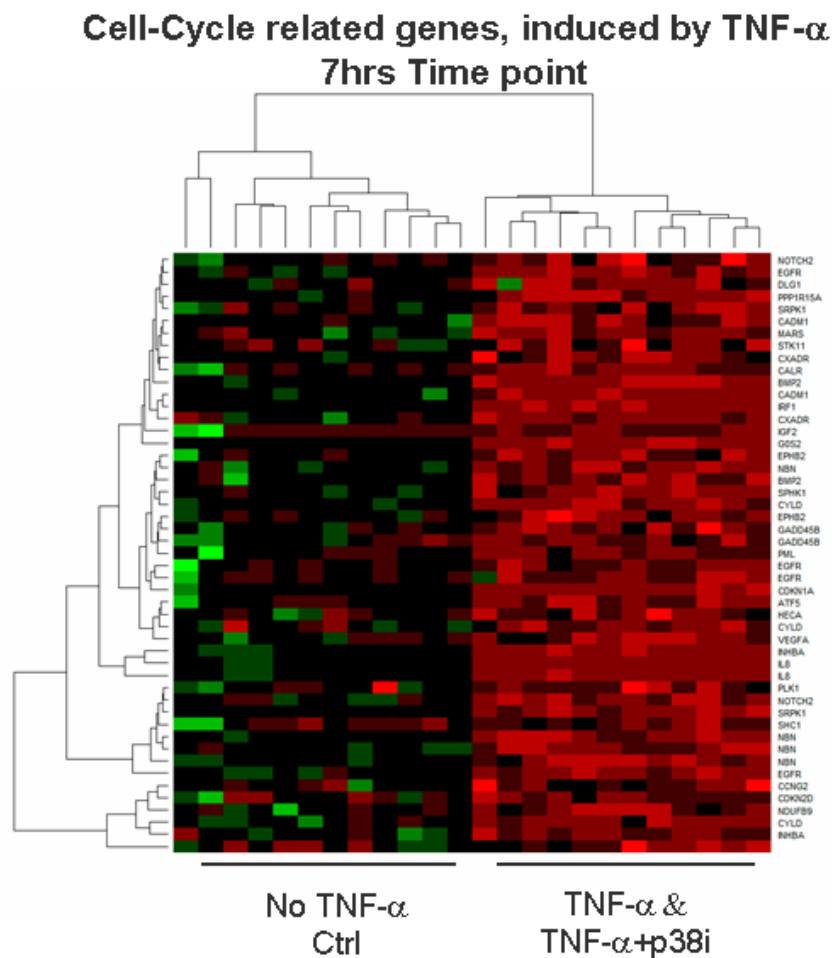


Figure 4.29: Heatmap of Genes associated with Cell cycle progression and regulation at late time points (7hrs), all genes $FDR < 0.1$

Similar to observations made at the 2hrs time point, TNF- α induces strong expression of genes linked to cell cycle progression or increased cell proliferation. For the majority of these genes, we find that inhibition of p38 had little impact on their transcriptional response. Genes within this differential list, linked specifically to progression of the cell cycle, included CDKN1A (p21^{waf1}), CDKN2D (p19^{INK4}), PLK1, NBN and CCNG2 (cyclin G2). Detailed boxplots of these genes are provided below.

Inhibition of p38 has little impact on TNF- α induced cell cycle regulators

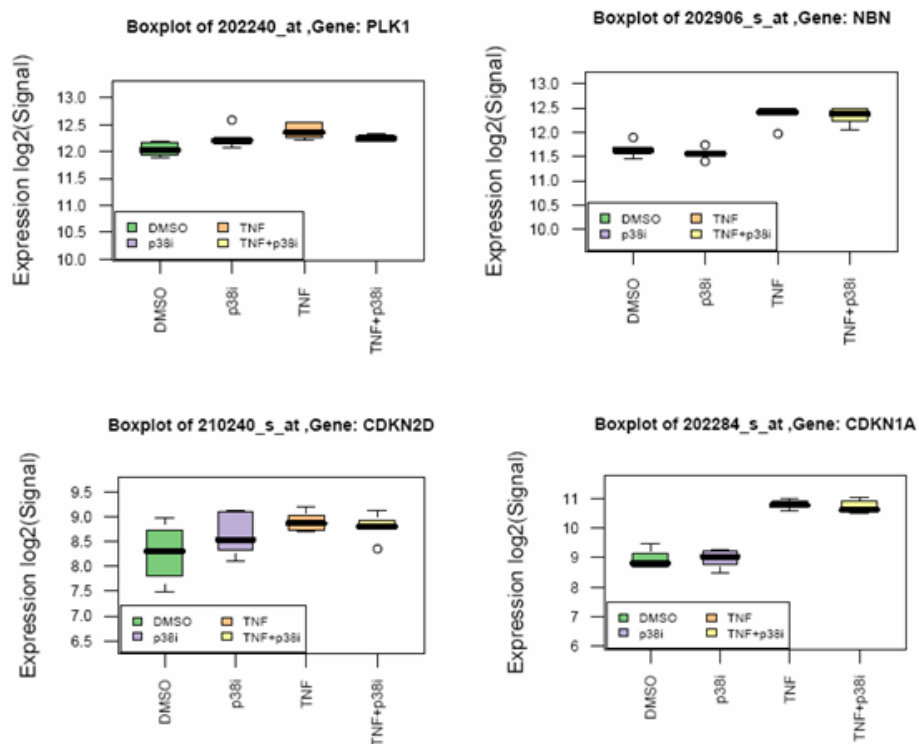


Figure 4.30: *Boxplot of select cell cycle regulator genes, induced by TNF- α but unaffected by p38 inhibition at late time points (7hrs), all genes filtered by $FDR < 0.1$*

Many of the cell cycle regulatory proteins induced by TNF- α at the late time points were also observed to be induced at other time points. We observed that a large number of the genes linked to cell cycle regulation or progression were not significantly impacted by p38 inhibition. We illustrate this by plotting some of these cell cycle genes in line graphs across time. (Figure 4.27) we note that some of these genes have cyclical expression cycles. It is not clear if the cyclical nature of their gene expression is due to the nature of their activation pathway or if this is an aberration of the microarray platform.

Cell Cycle Regulators Induced by TNF- α across time

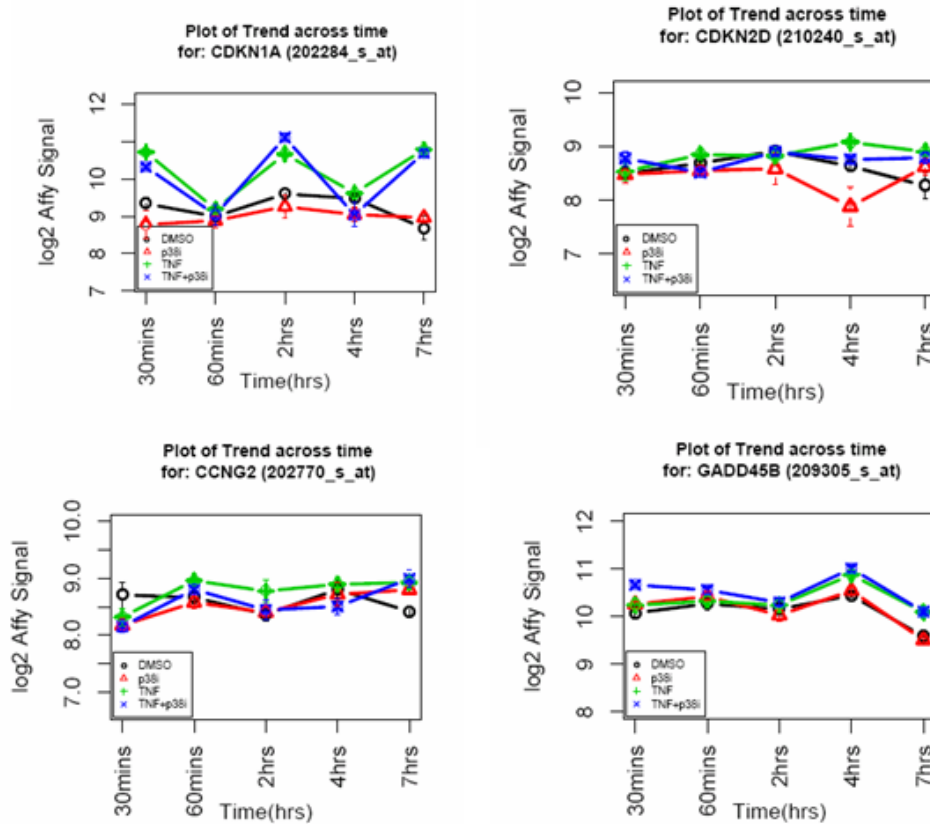


Figure 4.31: Line plots of select cell cycle regulator genes, induced by TNF- α but unaffected by p38 inhibition across time, all genes filtered by FDR < 0.1

TNF- α in the context of Calu6 lung carcinoma cells, strongly induces proliferation through up-regulation of many genes associated with progression of the cell cycle as well as cellular proliferation. Similar to what was observed at the 2hrs time point, inhibition of p38 MAPK seemed to have little effect on the transcriptional expression of the majority of the genes associated with cell cycle progression. This suggests that at a transcriptional level, inhibition of p38 MAPK does not have any dramatic impact on genes related to the cell cycle, and hence may not have much impact on the ability of cells to progress through the cell cycle during a stress event.

Summary of Late Time Points (4hrs & 7hrs)

The 4hrs and 7hrs time points are the last time points for this microarray study on the effects of TNF- α and concurrent p38 MAPK inhibition of the Calu6 lung carcinoma cell line. As time progresses we find that the number of genes significantly induced or modulated by TNF- α increases dramatically. Post 7 hours of TNF- α treatment, we find a dramatic increase in gene expression versus the earlier time points. Integrating the statistics of genes modulated by p38 inhibition, we find that the over-all count of genes strongly modulated by p38 inhibition drops off considerably by 7hours post TNF- α +LY479754 treatment.

In the late time points, we observed the largest functional cluster of genes relating to the induction of apoptosis/cell death and cellular development/differentiation. Other large functional clusters identified within the list of statistically significant genes included clusters for inflammation response and for cell cycle control. We found in detailed analysis of the apoptosis/cell death cluster that there was a consistent transcriptional pattern of up-regulation of anti-apoptosis components such as IAP proteins and anti-apoptotic members of the BCL2 family. In addition to direct up-regulation of anti-apoptosis gene pathways, we also found a concerted effort in Calu6 lung cancer cells to down-regulate key components of the death receptor signal transduction pathway. Among these genes included down-regulation of TRADD, a critical complex member of the TNF- α death receptor pathway and to a lesser extent FADD, a critical component of the FAS signaling pathway. Inhibition of p38 MAPK seems to be able to reverse this pro-survival trend through recovery in expression of pro-apoptotic genes as well as down-regulation of pro-survival genes like BCL-xl.

We note that at a transcriptional level, inhibition of p38 does not seem to have much impact on genes related to the regulation or progression of the cell cycle. Sustained treatment of TNF- α does seem to have sustained impact on cell cycle and proliferation genes. Sustained exposure to TNF- α seems to induce strong cellular proliferation signaling.

4.8 *In-Vitro* Validation

To confirm some of the observations and hypothesis generated from the analysis of the microarray data, a small series of *in-vitro* validation was carried out in Calu6 cells.

Within the context of the original microarray experiment, p38's role in immediate early response was validated through RT-PCR assays at LRL.

However, we were interested to test the hypothesis that inhibition of p38 led to a degradation of anti-apoptosis response to TNF- α treatment. To test this hypothesis, unsynchronized Calu6 cells were treated in paired fashion with TNF- α or TNF- α +LY479754 and tracked over a 48 hour period at the times indicated (Figure 4.32).

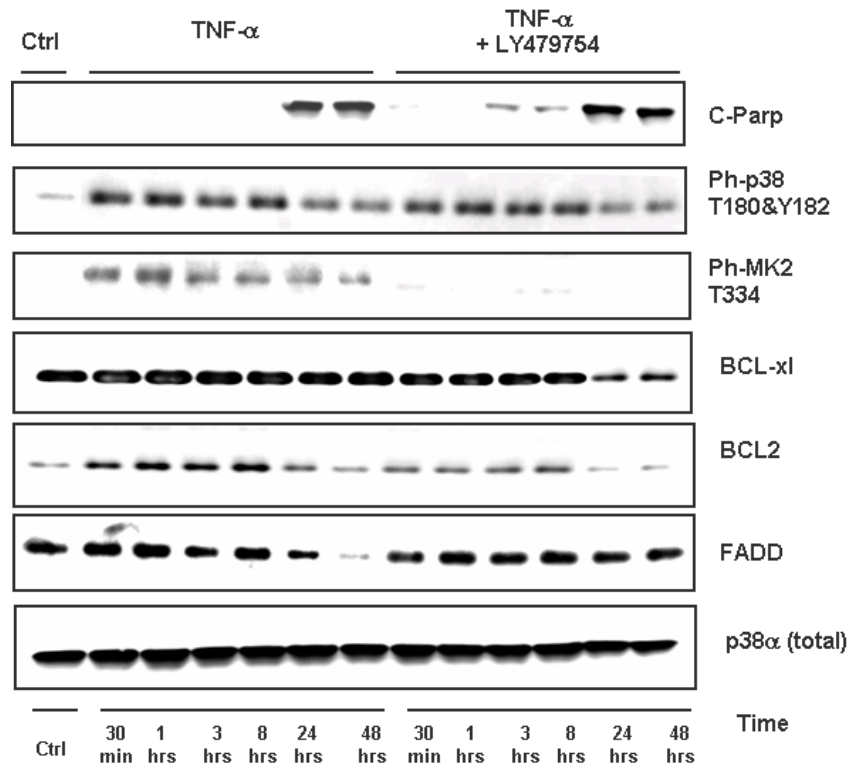


Figure 4.32: *Western blots of TNF- α and TNF- α +LY479754 treated Calu6 cells over a 48hour time series.*

In Calu6 cells, LY479754 effectively inhibits p38 pathway activity, in response to TNF- α treatment (Figure 4.32, phospho-MK2 blot). TNF- α induces apoptosis in Calu6 cells over the 48 hour time periods with or without the p38 inhibitor. However, Calu6 cells treated with a p38 inhibitor seem to have low levels of parp-cleavage much earlier than cells treated only with TNF- α (Figure 4.32). Levels of BCL2 and BCL-xl are also decreased at the later time points in cells treated with the p38 inhibitor. Lastly, expression of the FAS receptor death domain (FADD) decreases dramatically with TNF- α treatment, in line with what was observed in the transcriptional data. FADD protein levels remain strongly expressed in Calu6 cells treated with the p38 inhibitor.

This data confirms some aspects of the hypothesis generated from the microarray data. We observed that in response to TNF- α , components of the BCL2 anti-apoptosis

family are strongly induced, while elements of the extrinsic apoptosis pathway are down-regulated. The expression of these proteins is reversed with p38 inhibition, leading to the conclusion that p38 activity plays a role in cell survival signaling in response to TNF- α treatment.

4.9 Summary

In this chapter we explored the potential role of p38 MAPK in response to TNF- α treatment in the Calu6 lung carcinoma cell line. DNA microarrays allow the simultaneous measurement of tens of thousands of gene transcripts, giving an unbiased view of the effect of stress and damage response on the transcriptome. In this study we utilized DNA microarrays to gain a mechanistic insight into the role of p38 MAPK in the response of cells to TNF- α treatment. TNF- α is a secreted cytokine responsible for the induction of extrinsic apoptosis pathway as well as for inflammatory response. As solid tumors require the establishment of an appropriate tumor microenvironment for successful growth and proliferation, the dysregulation of inflammation pathways is considered one of the first steps in tumor formation. Our interest was to gain a mechanistic understanding of p38 MAPK in the context of inflammatory and non-genotoxic stress response.

We found that p38 MAPK played a significant role in a number of key physiological pathways in response to TNF- α induced inflammatory stress. In the early time points p38 activity was needed for the activation of immediate/early response pathways. We also observed that at multiple time points throughout the study, p38 activity was needed for pro-survival signaling. As TNF- α elicits strong responses from multiple signaling pathways, cancer cells need to inactivate pro-apoptosis pathways. To counteract pro-apoptotic stimuli, Calu6 activate strongly the IAP pathway, and the anti-

apoptotic members of the BCL2 family. BIRC2 and BIC3 were found to be activated by as much as 10-fold across the different time points in this study, while components of the BCL2 family were activated by a smaller extent, topping out at around 4-6 fold. Inhibition of p38 changed the transcriptional response of many of the anti-apoptosis proteins significantly. This hypothesis generated from the microarray data was validated in a series of in-vitro experiments that showed that p38 inhibition led to the modulation of anti-apoptotic pathway components such as FADD, BCL-xl and BCL2 (Figure 4.32).

The most dramatic changes in transcriptional response due to p38 inhibition were observed at the earlier time points (30mins, 60mins and 2hrs). Early modulation of transcriptional events has been linked with significant changes in protein levels at later time a point, hence observing significant changes to transcriptional response at the early time points is believed to lead to significant physiological differences.

Inhibition of p38 also dramatically affected the regulation of proliferation and inflammation response genes especially at the early time points. These genes include known TNF- α targets including TGF- β , BMP2 pathway and components of the NF κ B. Inflammation response modulated strongly by TNF- α treatment included cytokines such as IL6, IL8 and PTGS. We found that biochemical inhibition of p38 led to dramatic decrease in many up-regulated genes involved in proliferation and inflammatory response.

The gene expression data strongly supports the prior observations that p38 MAPK activity may be linked to cyto-protection in response to stress. This extends the work presented in chapter 3 of this thesis to support the idea that p38's activity in response to DNA damage is related to protection from stress induced apoptosis pathways. This suggests that targeted inhibition of p38 may have therapeutic benefits in conjunction with

chemotherapeutic treatments. Inhibition of cell survival pathways with a potent p38 inhibitor will have the potential to greatly enhance the cell killing potential of current genotoxic agents, increasing the efficacy of current treatment regimes.

Chapter 5: Alternative roles for p38 in response to DNA Damage outside G2 cell cycle checkpoint response

5.1 Background

The p38 MAPK is activated strongly in response to stress including DNA damage. While the traditional role for p38 in stress response has been in the realm of response to inflammatory cytokines, numerous roles for p38 in other types of stress response have been suggested. In chapter 3, we explored the role of p38 activity in DNA damage induced G2 cell cycle checkpoint arrest. Our results showed that while p38 is strongly activated in response to DNA damage, its activity is not required for the activation or maintenance of the G2 cell cycle checkpoint arrest. As p38 activity is not required for G2 cell cycle checkpoint function, we reasoned that its activity may be associated with other physiological processes related to DNA damage. In this chapter, we explore a number of alternative hypotheses that connect p38 activity in response to DNA damage to non G2 cell-cycle checkpoint function. The goal is to attempt to elucidate other possible roles for p38 activity beyond the original hypothesized role in G2 cell cycle checkpoint function.

5.2 A role for p38 MAPK activity during mitotic progression

Beyond the role of p38 in G2 DNA damage cell cycle checkpoint function, numerous publications have suggested some alternative roles for p38 activity in cell cycle progression. A publication by Fornace et al (48), showed that p38 was activated during normal transition from G2 to M phase of the cell cycle in the absence of stress, and remained activated and localized to the centrosomes during metaphase. This suggests a potential non-DNA damage role for p38 activation during mitotic transition.

To confirm the observations made in the paper, a part of the results from this publication was reproduced. HeLa cells were synchronized by double thymidine block and released into fresh growth media. Cells were tracked for 12 hours after release for thymidine block and harvested according to the time points presented in Figure 5.1.

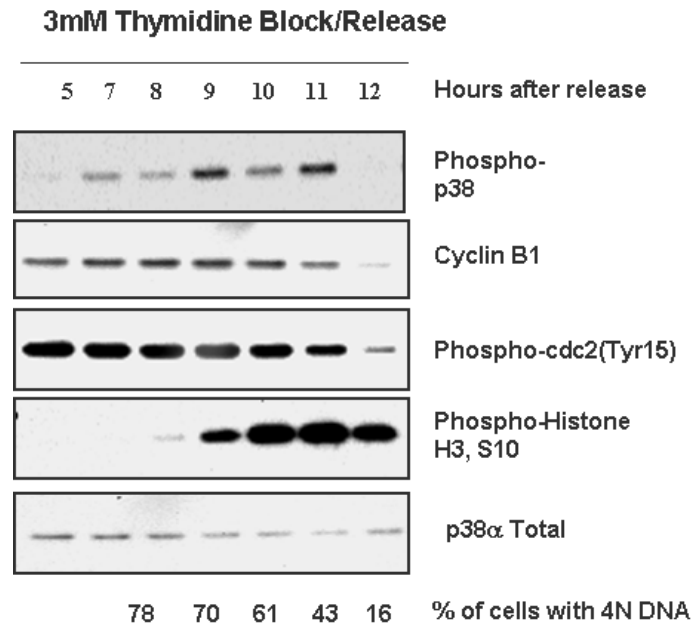


Figure 5.1: *p38 MAPK was activated during normal mitosis, without any DNA Damage. Western blot of hela cells, synchronized with double thymidine block/release technique. % cells with 4N DNA determined by flow cytometry.*

As can be observed in figure 5.1, phosphorylation of p38 can be observed starting at 7 hours post release from thymidine block. Based on levels of ph-CDC2,tyr15 and phospho-Histone H3 at the same time points, it was clear that p38 was activated just prior to entry into mitosis. Based on other mitotic markers at the same time points, p38 seems to be active in early mitosis when cyclin B1 was still present. Loss of p38 activity seems to correlate with loss of cyclin B1, suggesting that p38's activity decays at the end of mitosis or as cells exit mitosis. This data reproduces the observations made in the

Fornace et al paper, showing that p38 activation occurs during mitotic progression without exogenous stress.

5.2.1 Inhibition of p38 during regular mitosis has no impact on completion of mitosis

As high p38 activity is correlated with the onset of mitosis in unperturbed cells, it was natural to assume that p38 plays a critical role in some context within mitosis. To understand if p38 kinase activity plays a critical role in mitosis, we first tried to inhibit p38 activity without any exogenous stress to the system. As p38 activity was high in the early phases of mitosis, and was known to co-localize to the centrosomes, inhibition of p38 may have some impact on the kinetics of mitosis. To test this hypothesis, we synchronized HeLa cells with a CDK1 inhibitor (RO-3306) as described previously. Upon release from CDK1 inhibitor, cells were dosed with 500nM p38 inhibitor (LY479754) or 1 μ M Chk1 inhibitor or 120nM Nocodazole and tracked for 6hours post release (Figure 5.2A&B).

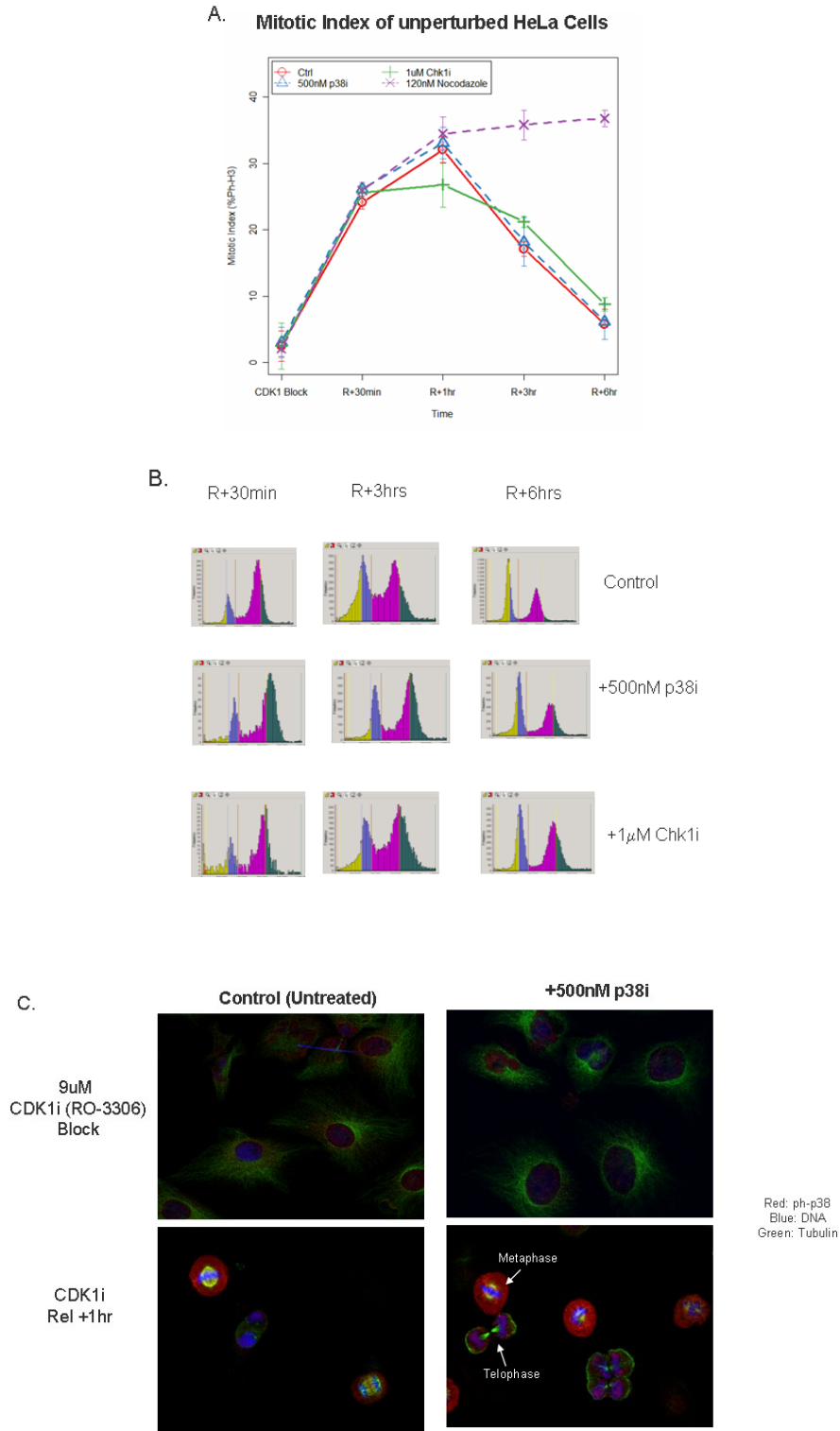


Figure 5.2: *Effect of inhibition of p38, and Chk1 on mitotic progression in HeLa cells. (A) HeLa cells synchronized with 9uM RO-3306 were released into 160nM p38i or 1.5uM Chk1i (B) Cell cycle profile (3mM PI) of cells treated with p38i or Chk1i or control cells (C) Confocal Microscopy of HeLa cells synchronized with CDK1 inhibitor*

Block/Release, with or without 500nM p38-inhibitor. Color Code: Red: ph-p38 (thr180/tyr182), Green: α/β Tubulin, Blue: DNA

CDK1-inhibitor (RO-3306) block/release treatment was able to synchronize hela cells at the G2 to M boundary after dosing for 21 hours. Using both the mitotic index and the cell cycle profile as readouts for the state of cells, we see that untreated cells enter mitosis within 1 hour of release and begin exiting mitosis within 3 hours of release from CDK1 inhibition (Figure 5.2A & B). Treatment with a potent p38 inhibitor and a Chk1 inhibitor did not seem to have a significant effect on the ability of cells to progress through mitosis, whereas treatment with 120nM nocodazole traps cells in mitosis (Figure 5.2A).

Additional confocal immunofluorescence microscopy shows that CDK1 inhibitor block effectively synchronizes cells at the G2 to M boundary, with cells rapidly entering mitosis 1hour post release (Figure 5.2C, left panel). Treatment of CDK1 inhibitor synchronized HeLa cells with 500nM LY479754 also does not seem to impede cells progressing through mitosis, as cells treated with p38 inhibitor are captured under going metaphase and telophase (Figure 5.2C, right panel).

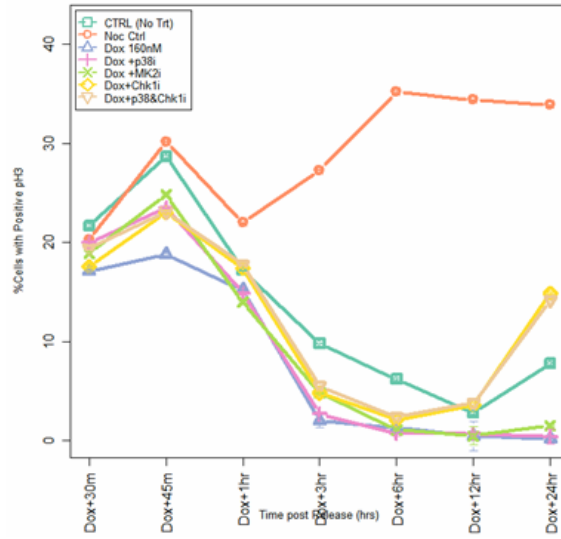
These results imply that while p38 was active in unperturbed cells during mitosis, inhibition of p38's kinase activity does not have any discernable effect at the phenotypic level. Cells seem to transition through mitosis in the same kinetics as untreated and there was no discernable increase in polyploidy cells or cells with other discernable defects in mitosis.

5.2.2 Adriamycin damage has no effect on mitotic progression

Having tested the effect of inhibition of p38 on the ability of cells to complete mitosis in the absence of genotoxic stress, the next question was what effect DNA damage during mitosis may have. We sought to understand if genotoxic agents that induce cell cycle arrest during interphase had an effect in mitosis, the final stage of the cell cycle. As p38 was induced in mitosis, we asked whether p38 activity plays a role in any response to DNA damage during mitosis.

To test this, cells were synchronized using the CDK1-inhibitor block/release protocol described previously. Post release from CDK1i block cells were tracked for mitotic entry visually by looking for a “round-up” phenotype in a standard light microscope. Unstressed cells released from CDK1 inhibitor block can be observed entering mitosis with a large percentage of cells achieving this “round-up” phenotype within 30mins of release. At this point, mitotic cells were harvested using a “tap-off” protocol and seeded onto 96well micro-titer plates in fresh growth media containing 160nM Adriamycin and p38 inhibitor or Chk1 inhibitor. A subset of the mitotic cells were treated with 120nM nocodazole to trap them in mitosis while another subset were left untreated to act as untreated control. Cells were tracked for 24 hours post damage with Adriamycin (Figure 5.3).

A. Mitotic Index of CDK1 B/R Synchronized Hela Cells with Adriamycin Treatment



B. γ -H2AX Levels in CDK1 B/R Synchronized Hela cells with Adriamycin Treatment

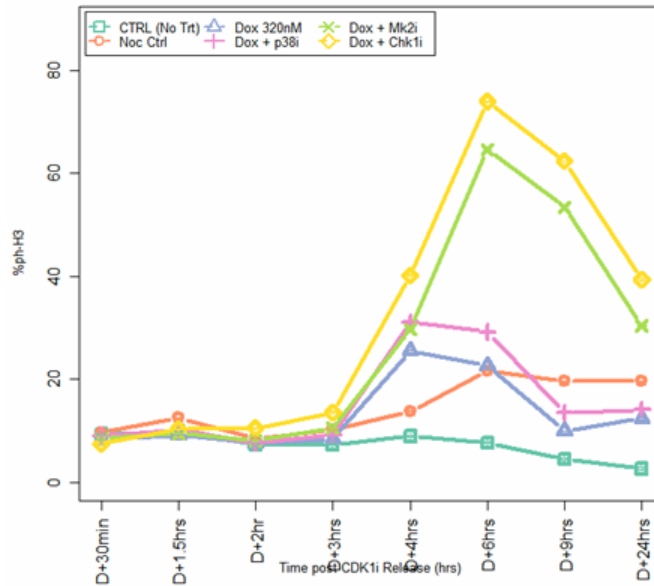


Figure 5.3: Effect of Adriamycin on cells in Mitosis: (A) Mitotic index of hela cells synchronized by CDK1i block/release treated with Adriamycin during mitosis. (B) DNA damage index (γ -H2AX) of cells of hela cells synchronized with CDK1i block/release.

Adriamycin treatment does not seem to have any effect on the ability of cells already in mitosis from completing mitosis based on mitotic index (Figure 5.3). The 24 hour time course Acumen multi-parametric cytometry data shows that Adriamycin only seems to have an effect on cells during the interphase component of the next subsequent cell cycle and does not exhibit any effect on mitotic cells. Considering that Adriamycin is a Topo-II inhibitor, this result is not surprising. As DNA was fully condensed during mitosis, it was likely that the topo-isomerase enzymes do not have a role during mitosis, hence a topo-isomerase inhibitor like Adriamycin only has the opportunity to impinge on the cell cycle at the next subsequent cell cycle, through disruption of DNA replication.

Addition of a p38 inhibitor to Adriamycin damage also did not appear to have any effect on cells progressing through mitosis. As we have shown previously that inhibition of p38 in undamaged or unstressed cells has no discernable effect, it may not be surprising that a p38 inhibitor does not alter the kinetics of mitosis with Adriamycin in HeLa cells.

5.2.3 MMS damage during mitosis interrupts mitotic progression

As we were interested to investigate if p38 kinase activity had a role in response to DNA damage in mitosis, we explored if MMS, a strong alkylating agent was able to affect cells completing mitosis. HeLa cells were synchronized with CDK1 inhibitor (RO-3306) block/release cycle as describe previously. Upon release, mitotic cells were collected using a mitotic “tap-off” procedure. Mitotic cells harvested in this manner were seeded onto 96 well plates in fresh growth media containing 0.02% MMS and a series of increasing doses of p38 inhibitor (80nM to 500nM). Cells were tracked for a 24 hours time series.

MMS damage of hela cells synchronized with CDK1 inhibitor block/release protocol while in mitosis lead to some interesting observations. We observed that the dynamics of p38 activation in response to MMS damage was very rapid, within 30mins of MMS treatment (Figure 5.4D). This contrasts with the onset of the markers of DNA damage ph-H2AX and that of ph-Chk1 (ser317), which was observed at 1hrs post MMS treatment respectively. This suggests that the dynamics of p38 activation precedes that of Chk1 and the classical marker of DNA damage (γ H2AX).

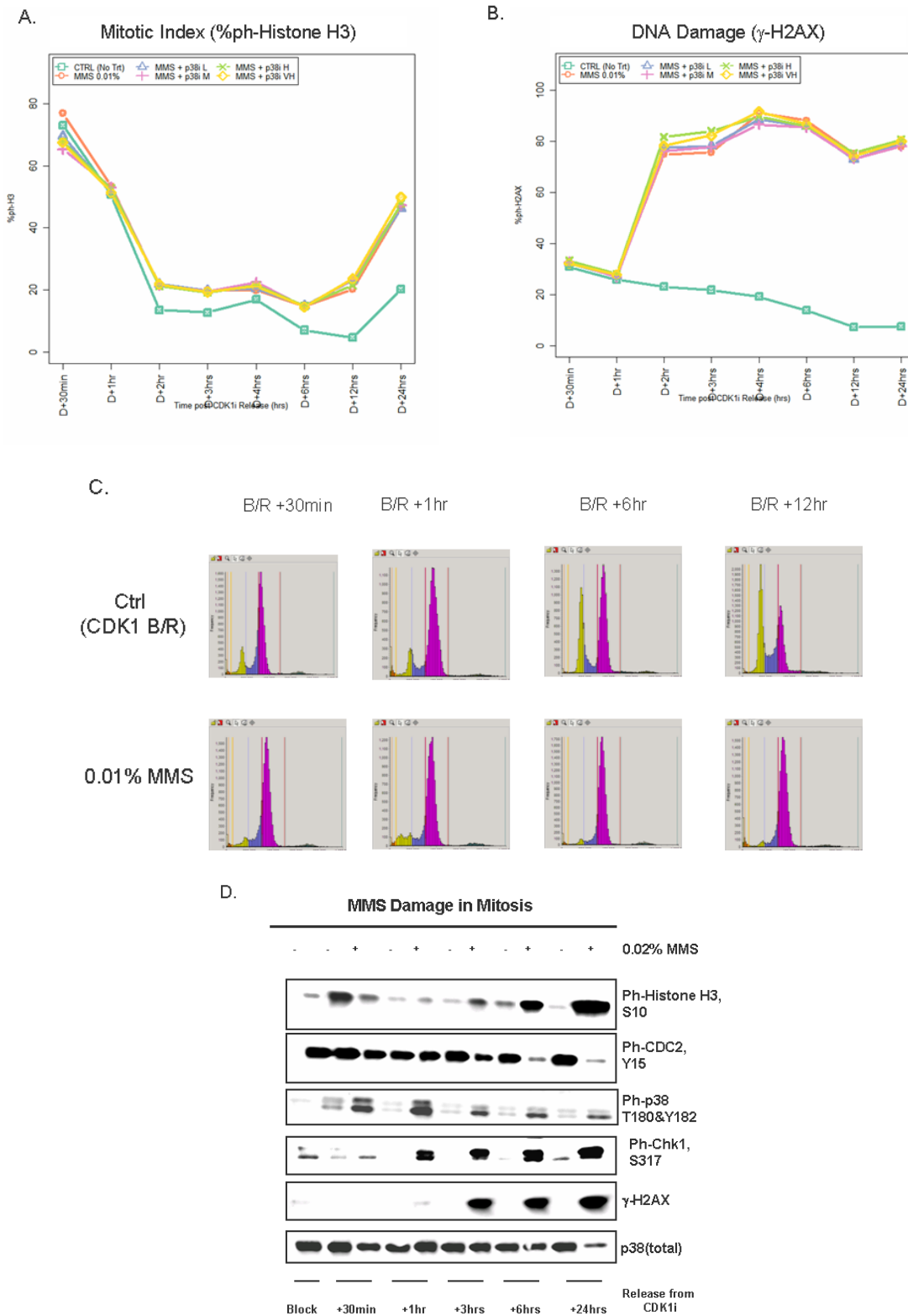


Figure 5.4: *MMS damage in mitosis leads to disruption of mitosis. (A) Mitotic index plot of HeLa cells synchronized by CDK1i block/release and treated with 0.02% MMS in mitosis. (B) DNA damage (γ -H2AX) in HeLa cells treated with 0.02% MMS in mitosis.*

(C) Select cell cycle states (DNA content: Propidium Iodide) of CDK1 block/release cells and CDK1 block released cells + 0.01% MMS. (D) Corresponding western blots of HeLa cells synchronized by CDK1i Block/Release and treated with 0.02% MMS in mitosis.

In terms of an effect on mitotic progression, we observed that unlike Adriamycin, MMS damage seemed to have a profound impact on cell cycle progression. MMS damage appeared to alter the ability of cells to complete mitosis. This observation stems primarily from the observation that a 2N (G1) peak was not observed within the 24 hour time series for this set of experiments (Figure 5.4C). We do however observe a temporary decrease in histone H3 phosphorylation within the first 8 hours of MMS damage, this decrease however was reversed by 12 hours of MMS damage, and we observe that cells again have very high levels of ph-Histone H3, suggesting that cells were in mitosis again. However as we do not observe a return of the 2N peak within the same 24hr period, it may suggest that cells never leave mitosis. The oscillation of ph-histone H3 levels were observed in a careful time series experiment in both an acumen high content assay as well in traditional western blots.

Despite this observation of oscillations in the levels of histone H3, in a number of limited experiments we did not find that the biochemical inhibition of p38 having any effect on any of the markers under observation (ph-histone H3, & DNA content). It was observed that prolonged treatment of cells with MMS leads to dramatic induction of apoptosis. A possible explanation for these phenomena was that continuous exposure to MMS led to dramatic damage to the DNA, preventing cells from progressing through mitosis. The last canonical checkpoint during mitosis is the spindle assembly checkpoint, which guards against improper and incomplete attachment of sister chromatids to the

mitotic spindle (485). While MMS acts as an alkylating agent, its activity may prevent the proper attachment of chromosomes to the spindle, halting progression in mitosis. As further exploration of mitotic progression was not the primary focus of this thesis, an underlying mechanism to explain this observation is still not understood. Additional ideas and comments on this observation are provided in Chapter 7.

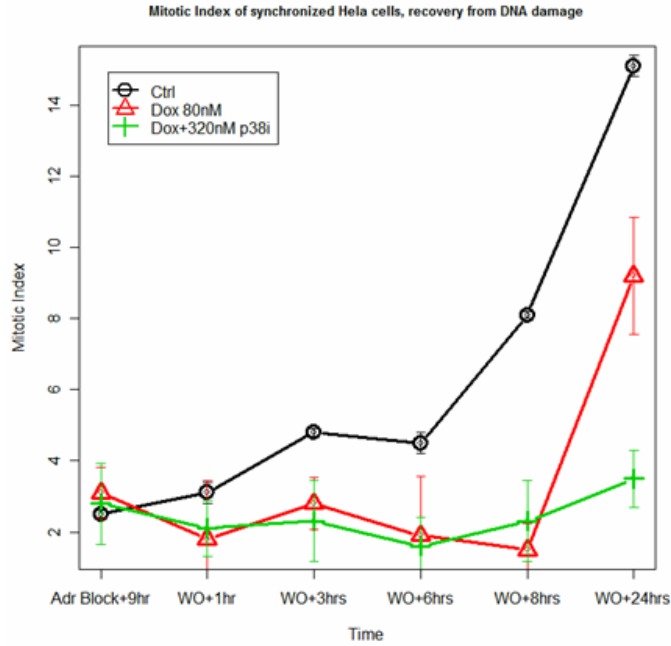
5.3 Role of p38 in recovery from Adriamycin damage

The three usual outcomes of DNA damage are generally considered to be successful repair of damage and subsequent re-initiation of the cell cycle or unsuccessful repair, adaptation and re-initiation of the cell cycle or apoptosis. As cells have the ability to respond to DNA damage and either repair the damage or adapt to the damage, a signaling pathway to prepare cells for recovery and re-initiation of cell cycle is necessary. As p38 MAPK is strongly activated in conjunction to DNA damage in association with a G2 cell cycle arrest, it may play a role in recovery from DNA damage. The majority of research on DNA damage response has focused on identifying the key signaling regulators for the initiation of the cell cycle checkpoint arrest. Less research has been done on the components responsible for the release and recovery from DNA damage induced checkpoint. As our data (Chapter 3) has shown that despite p38 being activated in response to DNA damage, its activity was not linked to the function of the G2 checkpoint, we explored if p38 activity may be tied to recovery from DNA damage.

To explore this, we used the Hela cell system, and studied the kinetics of repair and release from low levels of Adriamycin damage. As Adriamycin is a potent Topo-II inhibitor, the dose range selection for DNA damage had to be chosen carefully as too low a dose of Adriamycin led to a leaky arrest and too high a dose led to unrecoverable DNA

damage arrest (for at least 48hrs). In addition to the dose, the duration of Adriamycin treatment was also an important factor that needed to be controlled. Too long an incubation even at lower doses, led to permanent checkpoint arrest within the 24 hour observation window. The optimal dose range for this recovery experiment was determined to be 80nM of Adriamycin for 9 hours in HeLa cells. In this experimental regime, HeLa cells were synchronized with double thymidine block and upon release were subsequently treated for 9hours at 80nM Adriamycin with or without 320nM p38 inhibitor, following which Adriamycin was washed out with fresh growth media (3x), samples pre-treated with p38 inhibitor were re-incubated with 320nM p38 inhibitor. The recovery from Adriamycin was tracked over a 24 hour time series where the mitotic index (phospho-Histone H3) and the cell cycle state (DNA content) was tracked using a multi-parametric Acumen Explorer cytometry assay (Figure 5.5).

A.



Cell-Cycle Plots

B.

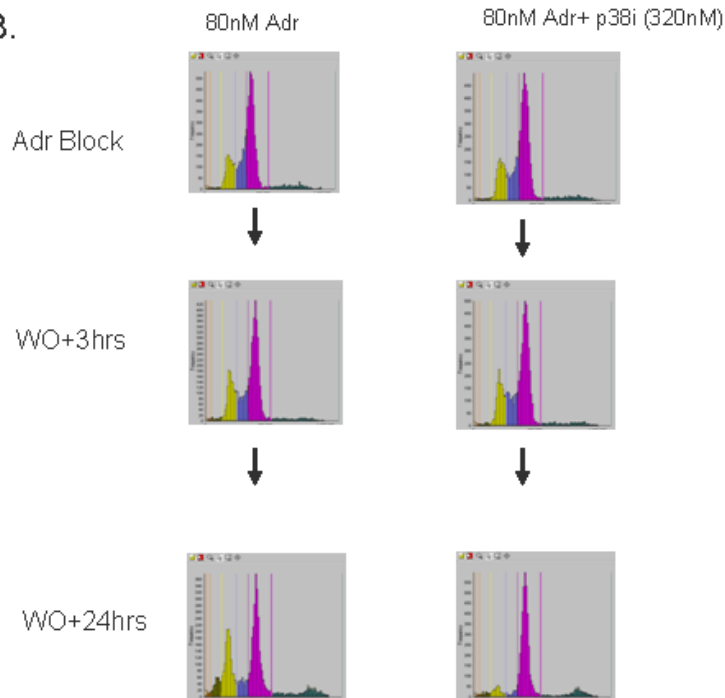


Figure 5.5: Recovery from Adriamycin Damage at G2. (A) Mitotic Index (*ph*-H3) of *hela* cells treated with 80nM Adriamycin or 80nM Adriamycin and 320nM LY479754 (*p38i*). (B) Select cell cycle profiles of cells treated with 80nM Adriamycin or 80nM Adriamycin+ 320nM *p38i*.

In thymidine synchronized Hela cells, 80nM of Adriamycin induces a sufficient G2 checkpoint arrest by the majority 4N peak (>75%) and low levels of ph-Histone H3 (Figure 5.5). It was observed that washout of Adriamycin post 9hrs incubation, allowed cells to recover from Adriamycin induced G2 checkpoint arrest within 24hours of washout. It was also observed that inhibition of p38 with a potent biochemical inhibitor was able to inhibit recovery from Adriamycin. As can be observed from the cell-cycle profiles, and the mitotic index, cells treated with 320nM of LY479754 never recover a G1 (2N) peak nor do they exhibit high levels of ph-Histone H3 24hrs post washout.

This initial data suggests that p38 activity in response to DNA damage from genotoxics may also be linked to the ability of cells to recover from DNA damage. While we have made an observation that inhibition of p38 by a potent biochemical inhibitor has the ability to delay or prevent recover from DNA damage, we have not yet begun to explore any underlying mechanism that may explain this observation. Additional thoughts and comments on this topic were addressed in Chapter 7 of this thesis.

5.4 Biochemical Inhibition of p38 leads to Apoptosis in conjunction with genotoxic agents

Analysis of TNF- α treated Calu6 cells in Chapter 4 strongly suggested that inhibition of p38 interfered with the ability of cancer cells to mount an immediate early response to apoptosis signals. Inhibition of p38 strongly decreased the expression of anti-apoptotic BCL2 family proteins and also reversed inhibition of components of the pro-apoptotic signaling pathway (Chapter 4). Given these observations, there was a strong interest to extend these findings to p38's response to DNA damage. We reasoned that if p38 MAPK activity played a significant role in the regulation of apoptosis, inhibition of

p38 should have dramatic impact on the induction of apoptosis in response to genotoxic agents. To explore if there was any evidence that p38 activity was a critical regulator of apoptosis induction, unsynchronized HeLa cells were treated with increasing doses of p38 inhibitor (LY479754), with or without 160nM Adriamycin and fixed after 48 hours (Figure 5.6A). The degree of apoptosis induction was measured by levels of cleaved-caspase3 in a multi-parametric Acumen cytometry assay. In this experimental regime, apoptosis induction was significantly induced with increasing doses of p38 inhibitor in combination with 160nM of Adriamycin. Yet cells treated with either the p38 inhibitor alone or with Adriamycin alone did not exhibit strong levels of cleaved-caspase3 signal. This data shows that inhibition of p38 in combination with a genotoxic agent leads to strong induction of apoptosis (Figure 5.6A)

Follow up experiments in unsynchronized HeLa cells, showed in a western blot assay format, that inhibition of p38 kinase activity led to significant induction of apoptosis pathway in combination with Adriamycin (Figure 5.6B). Similar to results obtained from Acumen multi-parametric assays, western blot data showed that strong induction of cleaved-parp and cleaved caspase-3 in combination with Adriamycin was associated with inhibition of phospho-MK2, T334 levels (Figure 5.6B).

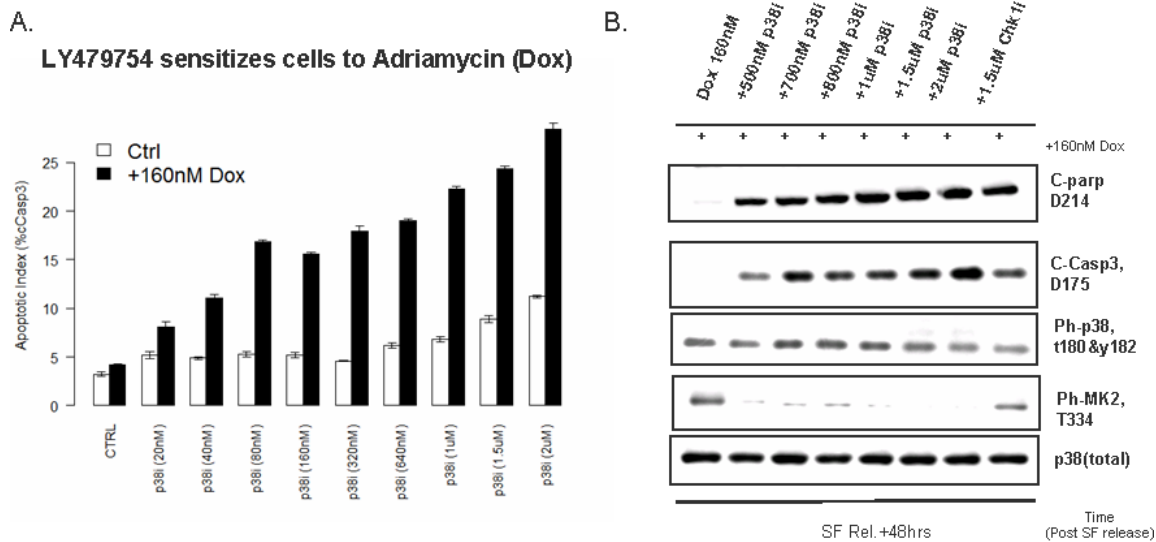


Figure 5.6: Apoptosis Induction in HeLa cells in response to Adriamycin and varying doses of p38 inhibitor. (A) Apoptosis Index (c-casp3) of HeLa cells at 48hrs post treatment with Adriamycin and varying doses (20nM to 2µM) of LY479754 (p38i). (B) Western blots of HeLa cells treated with Adriamycin and varying doses (500nM to 2µM) of p38 inhibitor.

5.4.1 Inhibition of p38 modulates BCL2 anti-apoptotic proteins

As our initial results in HeLa cells showed a strong induction of apoptosis with p38 inhibition in combination with Adriamycin, we were interested to check if this observation could be extended beyond the HeLa cell model and Adriamycin damage. To test this, A549 NSCLC cells synchronized with 48 hours serum starvation were treated with 160nM of Adriamycin and 1µM p38 inhibitor (LY479754) for 48 hours (Figure 5.7A). The combination of p38 inhibitor and Adriamycin leads to strong induction of cleaved parp and cleaved-caspase7 in A549 cells (Figure 5.7), whereas individual treatment of p38 inhibitor or Adriamycin did not produce a strong apoptotic response.

This result is similar to phenotype observed in HeLa cells, suggesting that this phenomena is not unique to HeLa cells, but may instead be linked to a role for p38 in the regulation of apoptosis induction in cancer cells.

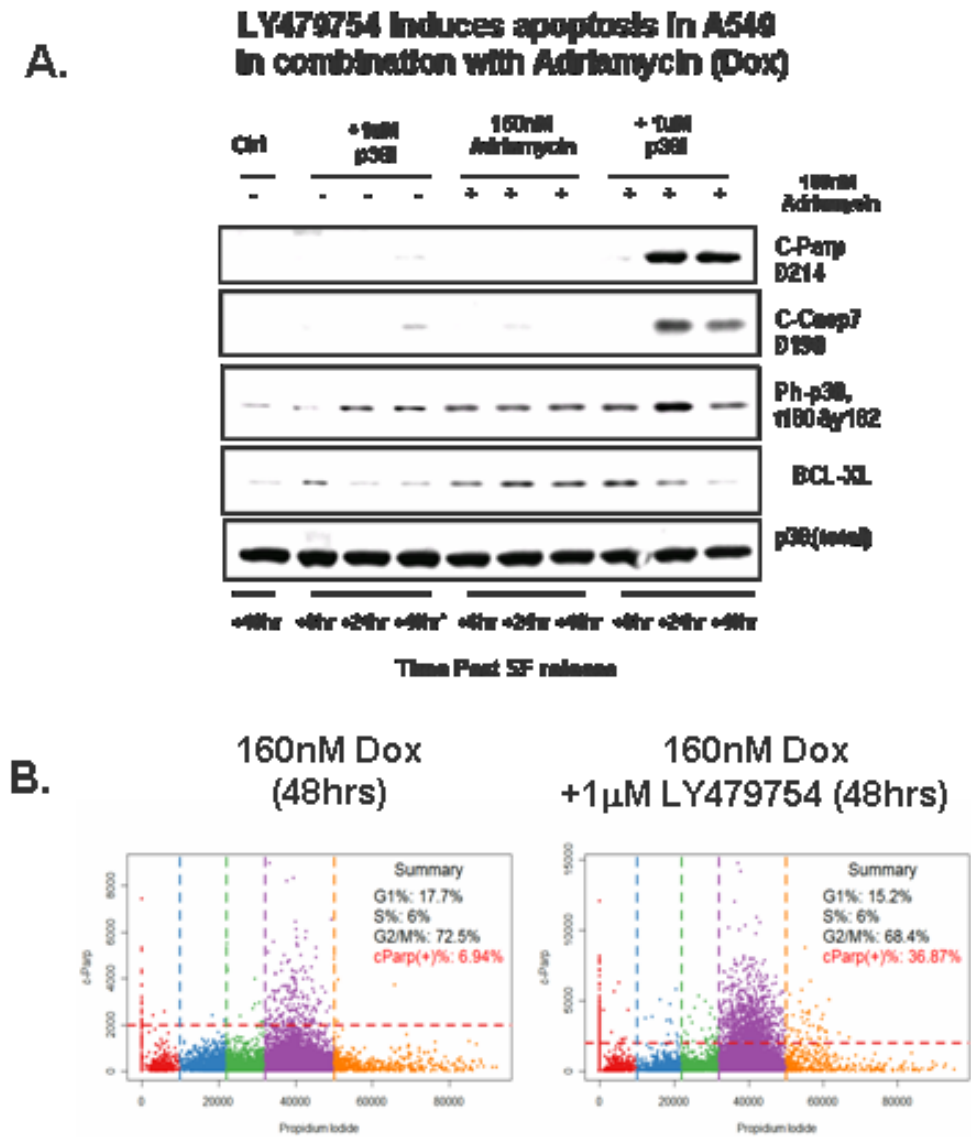


Figure 5.7: Inhibition of p38 induces apoptosis in A549 cells: (A) A549 cells synchronized with 48hrs serum starvation treated with Adriamycin and or Adriamycin+LY479754. (B) Multi-parametric Acumen Cytometry data, measuring cleaved-parp and PI (DNA content) of A549 cells treated with 160nM Adriamycin and 160nM Adriamycin+1µM LY479754, 48hours.

Having observed an increase in apoptotic induction with the inhibition of p38 in response to Adriamycin, we asked if the apoptosis induction was specific to Adriamycin stress. To test this hypothesis, unsynchronized HeLa cells were treated with 0.01% MMS, a strong DNA alkylating agent and treated with 1 μ M p38 inhibitor. As p38 inhibition of TNF- α cells led to strong transcriptional repression of anti-apoptotic response in the early and mid-time points and MMS strongly induces apoptosis post 24hours, a short time series of experiment in HeLa cells was carried out (Figure 5.8A).

Inhibition of p38 in combination with MMS in HeLa cells leads to strong early induction of apoptosis as seen by levels of parp cleavage (Figure 5.8A). Cleaved-parp is only strongly detectable in MMS only treated samples at the 10hour time point, versus the 3 hour time point in samples with 1 μ M p38 inhibitor added. In addition, inhibition of p38 again leads to strong inhibition of BCL2 and BCL-xl protein expression, suggesting inhibition of anti-apoptotic BCL2 protein expression as a mechanism of action. A follow-up study conducted in A549 confirms a strong loss of BCL2 as a consequence of p38 inhibition in combination with MMS damage (Figure 5.8B).

Together this data suggests that biochemical inhibition of p38 may suppress some anti-apoptotic members of the BCL2 family, leading to loss of cell survival capacity in the presence of genotoxic agents.

5.4.2 siRNA KD of p38 sensitizes cells to Adriamycin DNA damage

To test that the chemo-sensitization data derived from the p38 inhibitor was not due to an off target effect, we conducted a series of siRNA ablation experiments to knock down the p38 pathway. We reasoned that if p38 plays a critical role in cell survival in response to DNA damaging agents, loss of p38 protein would be detrimental to the survival capabilities of cancer cells in response to DNA damaging agents such as Adriamycin.

Using Hela cells again as our experimental model, we knock-downed p38, MK2 and Chk1 protein using validated Qiagen siRNAs oligonucleotides against the respective targets. As a control for transfection, we used random scramble siRNAs (NS-AS). Following 48hrs treatment with siRNA reagents (as per manufacturers instructions), we

dosed cells with in a paired fashion with 160nM of Adriamycin for 48hrs or DMSO control.

Similar to what we observed using biochemical inhibitors, we observed significant apoptosis induction with the siRNA knock-down of p38 and its downstream kinase MK2 in response to Adriamycin damage (Figure 5.9). We observed strong induction of cleaved-parp as well as cleaved-caspase7 in the p38 and MK2 knock-down conditions but did not observe such an effect in Adriamycin alone or non-specific scramble (NS-AS) plus Adriamycin conditions. As a positive control, we observed that loss of Chk1 kinase also led to strong induction of apoptosis in conjunction with Adriamycin damage. Recent reports have highlighted the inhibition of Chk1 kinase as a new mechanism to enhance chemo-sensitization. Our findings in this study were in line with such reports, showing that inhibition and knock-out of Chk1 strongly induces apoptosis in cancer cells. In addition we also showed that knock-down of p38 also led to induction of apoptosis in combination with Adriamycin. While knock-down of Chk1 induced apoptosis is likely linked to a defective G2 checkpoint, we have seen that reduction of p38 does not affect the ability of cancer cells to effectively mount a checkpoint response. We hypothesize that p38 activity may be an integral part of a general stress response, including stress induced by DNA damaging agents. This could suggest that p38's activity in response to DNA damage is correlated to protecting cells from pre-mature apoptosis induction in response to stress.

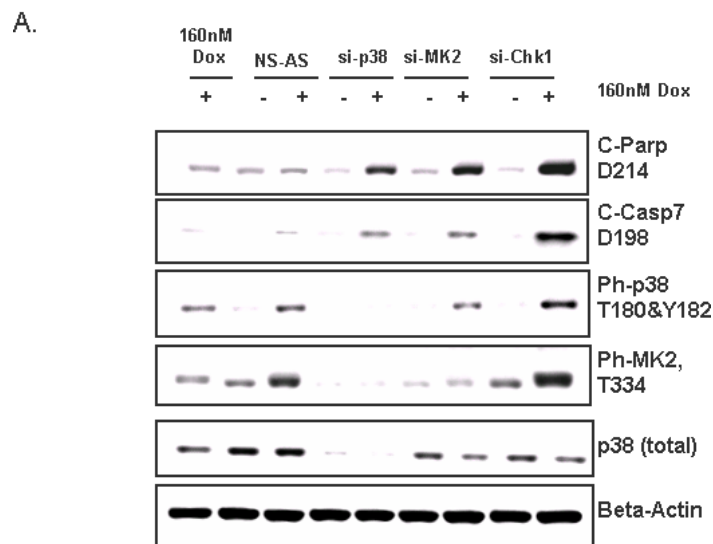


Figure 5.9: Effect of Adriamycin and siRNAs. (A) Western blot analysis of 48hrs siRNA knock-down of p38, MK2 and Chk1 followed by 160nM Adriamycin

This data in combination with the biochemical inhibitor data establishes a link between p38 activity in response to DNA damage and increased cell survival. Testing a hypothesis derived from gene expression analysis of TNF- α treated Calu6 cells, we show that inhibition of p38 in the context of DNA damage degrades a cancer cells' ability to induce anti-apoptotic pathways, leading to induction of apoptosis. This data suggests an alternative role for p38 in response to DNA damage. We hypothesize that rapid activation of p38 in response to DNA damage in cancer cells activates a strong cell survival signal that prevents early induction of apoptosis. This anti-apoptotic response may allow cancer cells to escape pro-apoptotic induction during treatment with genotoxic agents. This pro-survival mechanism possibly acts in concert with cell cycle checkpoint response, allowing cancer cells to activate the necessary stress and DNA repair pathways that allow the continued perpetuation of proliferation. Inhibition of p38 limits a cancer cells' ability to escape apoptosis in combination with a genotoxic agent, leading to increased cell

death. This suggests that p38 may be an attractive target for combination therapy in treatment of cancers.

5.5 Summary

In this chapter we examined alternative roles for p38 MAPK activity in response to DNA damage. Our results, presented in Chapter 3 showed that while p38 is strongly induced in response to DNA damage, its activity has no role in the establishment and maintenance of the G2 cell cycle checkpoint. While cell cycle checkpoint regulation is a critical aspect of DNA damage response, there are a number of other physiological processes that p38 activity may impact in response to DNA damage.

We found that despite being activated in unstressed mitotic progression, inhibition of p38 had no impact on unstressed cells progressing through and completing mitosis. This data suggests that while p38 is activated during mitosis, inhibition of its kinase function does not have an discernable impact on mitotic progression.

In exploring p38's role in the recovery from DNA damage, we found that inhibition of p38 impacted a cell's ability to recover from Adriamycin damage. Inhibition of p38 prevented cells from re-entering the cell cycle after low levels of Adriamycin damage. In a series of limited studies, we showed that inhibition of p38 prevented cells from recovering from low levels of Adriamycin damage. While inhibition of p38 in conjunction with Adriamycin prevented cells from recovering a 2N DNA state, and from entering mitosis, we had also found that inhibition of p38 in undamaged cells did not have an effect on cell cycle progression through mitosis. This suggests that the inability of cells to recover from DNA damage in the presence of p38 inhibitor may not necessarily be related to cell cycle progression and that the inability to recover from DNA

damage may be linked instead to disruption of DNA repair. Unfortunately there was insufficient time in this thesis to explore further the underlying mechanism underpinning this observation. We review our thoughts on additional future work on this topic in chapter 7 of this thesis.

Lastly, our studies found a strong positive link between p38 activity in response to the DNA damage and increased cell survival. Biochemical inhibition of p38 and siRNA knock-down of p38 led to significantly increased levels of caspase activation and parp cleavage. We also showed that p38 inhibition led to modulation of the BCL2 family of anti-apoptotic proteins. We found that inhibition of p38 in conjunction with a genotoxic agent led to a decrease in BCL-XL and BCL2 expression. This suggests that p38 activity confers its cell survival protection on cancer cells through modulation of the BCL2 family of apoptosis regulatory proteins.

We conclude from these studies that p38 may be an attractive target for cancer therapeutics especially in combination with genotoxic agents for the induction of apoptosis. This data highlights that p38 does not function as a principal controller of the G2 DNA damage checkpoint, hence inhibition of p38 is unlikely to induce apoptosis through a mitotic catastrophe type mechanism. Instead, this data shows that chemosensitization effects of a potent p38 inhibitor are instead conferred through disruption of pro-survival signaling.

Chapter 6: Discussion and Conclusions

The role of p38 MAPK in DNA damage response has been an area of growing interest in recent times. The idea that p38, a key responder to general stress also plays a role in the control of cell cycle progression is a strong and attractive idea for new and innovative treatments for many diseases especially cancer. Numerous publications in the past few years have implicated p38 as a potential new regulator of cell cycle progression, especially at the G2 checkpoint.

A recent observation that p38 MAPK pathway is attenuated in transformed cancer cells (267), however, has called into question the role of the p38 MAPK pathway in the G2 checkpoint in cancer cells. Furthermore, both p38 and Chk1 are reportedly activated under similar circumstances in cancer cells and are both believed to directly inactivate the same CDC25 family of protein phosphatases in response to DNA damage at G2, keeping CDC2 in its inactive phosphorylated state, preventing mitotic entry (212,366). Prior work has focused primarily on either p38 or on Chk1, and has made arguments that in response to certain DNA damaging agents, inhibition of either p38 or Chk1 is sufficient to overcome the G2 checkpoint (149,254,268,313). We aimed to gain a better understanding of the underlying mechanisms governing the function of the G2 DNA damage checkpoint by investigating the specific roles of p38 and Chk1 activity in cancer cells. A better understanding of the functioning of these proteins at the G2 cell cycle checkpoint could ultimately lead to superior therapeutic treatments for oncology in the future.

6.1 Inhibition of Chk1 but not p38 is critical to the maintenance of the G2 DNA damage checkpoint

In this study, we revisited the role of p38 activity in the context of the G2 DNA damage checkpoint. Specifically we were interested to understand if p38 activity was required for the activation and the maintenance of a G2 checkpoint arrest in response to DNA damage. To address these issues, a series of in-depth, cell-cycle focused experiments were conducted in different cancer cell-lines to examine the role of p38 in DNA damage-induced G2 checkpoint. In these studies, cells were synchronized at different stages of the cell cycle using various techniques including 48hour serum starvation (G1 phase), double thymidine block/release (G1/S phase), and the recently published CDK1 inhibitor block/release technique (G2 phase). The use of cell synchronization techniques and careful tracking of cell progression through the cell cycle allowed a relatively homogenous cell population prior to the addition of DNA damaging agents. As the context of cellular response to DNA damage is often framed in the context of the cell cycle state, a more homogenous cell population would be expected to exhibit a more uniform response.

In a series of studies, designed to understand p38's and Chk1's function at the G2 DNA damage checkpoint, the checkpoint was activated with a low doses of Adriamycin, and effects of the inhibition of p38 and Chk1 on the cells' ability to maintain checkpoint-induced G2 arrest was ascertained. A key aspect of the experimental design was the use of the lowest effective dose of Adriamycin that would trigger largely uniform G2 checkpoint arrest. This allowed for controlled experiments on the G2 checkpoint without additional confounding effects of high doses of genotoxic agents. Indeed, it was observed that high concentrations of Adriamycin (>500nM) did not induce a G2 arrest,

instead damaged cells arrested in S-phase (Figure 3.4A). Hence the decision to use 160nM of Adriamycin, instead of 5 to 10uM as previous publications had used (327).

Contrary to previous published results, we found that the G2 DNA damage checkpoint induced in HeLa cells by treatment with Adriamycin or UV could not be effectively abrogated through the inhibition of p38. In Chapter 3, by using a potent and highly selective inhibitor, p38 kinase activity could be inhibited without significantly increasing the number of cells entering mitosis over a 24 hour period after initiation of an Adriamycin G2 checkpoint arrest. Furthermore, treatment of thymidine synchronized cancer cells with a p38 inhibitor showed that inhibition of p38 activity before cells even enter G2 phase, had no effect on the establishment of G2 checkpoint arrest. In contrast to the results of p38 inhibition, inhibition of Chk1, a key member of the ATM/ATR DNA damage checkpoint, with a potent and selective Chk1 inhibitor allowed cells to enter mitosis despite the continuous presence of DNA damage. In addition to the Chk1 inhibitor, suppression of ATM/ATR response through the use of caffeine also achieved similar results. These results confirmed prior publications that showed that Chk1 is responsible for G2 checkpoint activation and maintenance in a p53 null environment (58,207,232).

The inability of cells treated with a p38 inhibitor to escape a DNA damage G2 cell cycle arrest shows that p38 activity is not required for the maintenance of the DNA damage G2 checkpoint. Furthermore, by successfully abrogating the G2 checkpoint with a selective Chk1 inhibitor and with caffeine, we were able to demonstrate that cells were able to enter mitosis despite high levels of phospho-p38 and phospho-MK2 activity, showing that high p38 activity does not prevent entry into mitosis. The results described showing inhibition of p38 having no effect on cancer cells mounting a DNA damage G2

checkpoint arrest were reproduced in Calu6 cells, a lung carcinoma cell-line, as well as A549 NSCLC cells and U2OS osteosarcoma cell-lines.

The results in different cancer cell-lines diverged in terms of the effect of Chk1 inhibition. In HeLa and Calu6 cells, inhibition of Chk1 led to dramatic abrogation of a DNA damage G2 checkpoint. In A549 and U2OS cells however, inhibition of Chk1 did not yield the same physiological outcome, in that no significant abrogation of G2 checkpoint arrest was observed. While this result was surprising initially, a search of literature revealed that the underlying p53 functional status strongly influences the effect of Chk1 activity on the function of G2 checkpoint (217,249,408,426). Inhibition of Chk1 in a p53 functional cell-line was not sufficient to over-come the G2 checkpoint, which was in-line with previous publications (217,249,408,426). The data from Calu6, A549 and U2OS cells however were in agreement that the inhibition of p38 was insufficient to abrogate DNA damage induced G2 checkpoint arrest, corroborating the main body of results from Hela cells.

We also briefly examined the hypothesis that p38 behaves as a checkpoint kinase through its interaction with CDC25B. It has been reported earlier (36) that in a p53 null environment, p38 binds to and phosphorylates CDC25B on its ser309 residue. This phosphorylation leads to the binding of CDC25B to the 14-3-3 proteins which leads to its sequestration in the cytoplasm away from its nuclear target CDC2. While we did not directly study the phosphorylation state of CDC25B, due to a lack of good quality commercial antibodies, biochemical suppression of p38 was unable to dramatically affect CDC2, tyr15 phosphorylation in Adriamycin treated Hela cells (Figure 3.5C). While it is possible that p38 does phosphorylate and interact with CDC25B, it is also well studied that the CDC25 phosphatases are the primary targets of Chk1 mediated ATM/ATR DNA

damage response pathway. Inhibition of Chk1 in HeLa cells, led to a decrease in CDC2, tyr15 phosphorylation, and decrease in the abundance of Wee1 kinase, a phenotype not observed in p38 inhibited cells in response to Adriamycin damage (Figure 3.5C).

In addition to Adriamycin, we also tested UV damage and a DNA alkylating agent MMS to examine if the effects observed were unique to Adriamycin damage or whether similar effects could be observed from other common sources of DNA damage. Inhibition of p38 in response to UV or to MMS in HeLa cells had no statistically significant effect on the mitotic index within the observed 24hour time period.

To further corroborate the results from the p38 inhibitor, siRNA oligonucleotides were used to knock-down p38, its down-stream kinase MK2 and Chk1 followed by DNA damage with Adriamycin and UV. SiRNA knock-down of p38 and MK2 in conjunction with Adriamycin and UV damage did not have any dramatic effect on the ability of cells to mount an effective G2 checkpoint in HeLa cells. This contrasted with the siRNA knock-down of Chk1 which again produced a dramatic phenotype in terms of abrogation of Adriamycin induced G2 checkpoint in HeLa cells.

Taken together with biochemical inhibitor data, these results shows that in a p53^{-/-} tumor environment, inhibition of Chk1, but not p38 in response to DNA damage leads to cells bypassing the G2 cell cycle checkpoint.

Given the recent increase in work implicating the p38 MAPK as a critical kinase in the response to DNA damage, this set of results may seem surprising. However, the findings of this study that p38 does not play a critical role in the establishment of G2 checkpoint arrest is supported by work recently published by Mikhailov et al (267), who found that the p38 G2 arrest pathway is attenuated in cancer cells, but active in untransformed cells. HeLa cells, one of our primary *in-vitro* models has been documented

to be both p53 and Retinoblastoma (Rb) negative (non-functional) (29,156,253). It is proposed that in this p53 and Rb functionally negative context, Chk1 kinase, but not p38 is required for the establishment and maintenance of the DNA damage induced G2 checkpoint arrest. Numerous prior publications studying the role of p38 in DNA damage G2 checkpoint have used untransformed human fibroblasts, and untransformed mouse embryonic fibroblast cells. Some of the discrepancies in our data and results previously published could be explained by the underlying differences between untransformed mouse and human fibroblast cells and transformed human endothelial cancer cells.

The loss of heterozygosity of both the p53 and Rb gene is a common phenotype registered in numerous types of primary tumors, including cervical cancer.(119,125,197) Hence the use of selective Chk1 kinase inhibitors as a potential chemo-sensitizer and in combination therapy with chemotherapeutics may prove to be more effective in the context of bypassing G2 checkpoint and reducing an avenue that cancers cells use to escape cell death after chemotherapy treatment.

6.1.1 Comparison of results for p38's role at G2 checkpoint arrest with prior publications

We showed that p38 activation was strongly induced by DNA damage and was correlated with G2 checkpoint arrest. Contrary to some prior publications (36,220,254), however, our data shows that p38 pathway activity is not necessary for the G2 checkpoint arrest in response to DNA damage

We were interested in the exciting possibility of using potent and selective p38 kinase inhibitors as chemo-sensitizers to enhance anticancer efficacy of chemotherapies.

Thus, lack of G2 checkpoint abrogation by a highly selective and potent p38 kinase inhibitor comes as a big surprise to us. A closer examination of earlier publications, however, reveals a certain degree of discrepancies concerning the role of p38 in G2 checkpoint control in response to different types of DNA damage and the function of p53 (36,254,327). In addition, earlier studies used older generation of p38 kinase inhibitors at very high concentrations (36,327). At such high concentrations these p38 kinase inhibitors would be most likely to have off-target activities as shown recently (16,103). Our data are consistent with a more recent report that demonstrates using the RNAi approach that only Chk1 but not Chk2 or MK2 is responsible for DNA damage-induced G2 checkpoint arrest in cancer cells (460). Furthermore, it is also recently shown that the p38 pathway response at the G2 checkpoint is strongly attenuated in transformed cells (267). Earlier studies that implicated p38 activity in DNA damage induced G2 checkpoint were performed in untransformed human cells and mouse embryonic fibroblasts (36,313,327). However, untransformed mammalian cells have intact p53 and Chk1 functions. Thus, it is inconceivable that normal, untransformed mammalian cells would depend on p38 for G2 DNA damage checkpoint function with functioning p53 and Chk1, but not cancer cells. Furthermore, if p38 MAPK plays an important role in the G2 checkpoint control only in untransformed normal cells but not in transformed cancer cells, that would then rule out the feasibility of developing a p38 inhibitor as a chemosensitizer to enhance efficacy of chemotherapies. Future follow-up on the observations that p38 may play a role in G2 checkpoint pathway in untransformed cells should be done.

6.1.2 Requirement of Chk1 for G2 DNA damage checkpoint in p53 null cells

Inhibition of Chk1 through both biochemical and RNAi techniques led to the abrogation of the G2 checkpoint only in p53 functionally null cancer cells. While p53 was not a central focus of this thesis, this observation warrants further investigation. As a p53 function is a central prosecutor of DNA damage response, it is feasible that the downstream pathways activated by p53 are sufficient to activate the G2 checkpoint, making the activity of Chk1 redundant.

Some possible mechanistic pathways downstream of p53 include the transcriptional activation of p21^{waf1}, a known inhibitor of CDK1 function (52). In this context up-regulation of p21^{waf1} led to sequestering of CDC25b and prevented the dephosphorylation of CDK1 at Tyr15. In cancer cells that retain p53 functional activity, it is likely that this pathway works in conjunction with the ATM/ATR mediated activation of Chk1 to induce G2 arrest. When p53 is functionally inactivated as in >50% of tumors, ATM/ATR mediated Chk1 activity may be the last resort to impose the G2 arrest. This suggests that in cancer cells with functionally inactive p53, a combination of Chk1 inhibitor with a chemotherapeutic agent could be a successful new therapeutic strategy.

6.2 Inhibition of p38 degrades anti-apoptosis response to TNF- α in Calu6 cells

If p38 is not necessary for the execution or the maintenance of the G2 checkpoint in transformed cells, what is its role in DNA damage response? To understand the role for p38 activity in response to DNA damage, we returned to the original context of p38 activation and investigated the nature of its involvement in response to inflammatory cytokines (TNF- α). TNF- α is a cytokine that is associated with the induction of inflammatory response, apoptosis and proliferation. An in-depth analysis of the

transcriptional pathways differentially modulated by p38 inhibition revealed p38's role in many pathways and gene networks. P38 activity was critical for the rapid induction of genes and networks associated with immediate early stress response, cellular proliferation, production of inflammatory cytokines and the regulation of apoptosis and cell death. Of particular interest in this study was p38's role in the rapid activation of the anti-apoptosis pathway in response to TNF- α treatment. The results highlighted in chapter 4 showed that TNF- α treatment resulted in a rapid up-regulation of an anti-apoptosis response, including the rapid induction of inhibitor of apoptosis proteins (IAPs) and anti-apoptotic members of the BCL2 family and a concordant decrease in pro-apoptotic components such as TRADD and FADD. Inhibition of p38 significantly degraded the anti-apoptosis transcriptional response, especially at the early time points.

Analysis of TNF- α and p38 inhibitor transcriptional data revealed the importance of p38 kinase activity in many key regulatory pathways including the regulation of apoptosis. Our data and analysis of p38's role in TNF- α response provides a possible link between p38 activity and the induction of pro-survival signaling.

Given p38's well documented role in promoting FAS-ligand, death domain receptor mediated apoptosis (157,435,442), it was surprising to find that inhibition of p38 led to increased cell death in the context of DNA damage response. In the context of induction of extrinsic apoptosis pathway, increased p38 activity is associated with increased cell death in many untransformed cells including fibroblasts and macrophages. It is possible that p38's response to FAS and other extrinsic stimuli depends on different cellular and environmental contexts.

The nature of three-tiered p38 MAPK activation system makes it an efficient transducer of cell death signals from external sources. In transformed cancer cells

however, the p38 MAPK pathway may be subverted by other oncogenic proteins such as Ras to switch its signaling from pro-apoptotic to anti-apoptotic (293). In the context of DNA damage in cancer cells, the speed and intensity of p38 activation may also make it a suitable candidate for holding apoptosis induction in check, until other factors have determined that the damage is too severe and cell death is necessary. Our observations that p38 may play a cyto-protective role in cells is supported by other studies which have also found that p38 may be tied to the BCL2 family and to anti-apoptotic pathways (127,128,235,239,271,473).

6.2 p38 MAPK activates cell survival pathways in response to DNA Damage

The traditional response to DNA damage is comprised of 3 main areas: cell cycle arrest, DNA damage repair and apoptosis. The classical response to DNA damage is believed to comprise of a series of decision tree like actions, where in response to DNA damage, cells immediately arrest, assess the damage, attempt to repair the damage and finally undergo apoptosis if the damage is too severe. It is well documented that p38 is rapidly activated in response to a plethora of exogenous stress signals. It has also been proposed that p38 MAPK may act as a central integrator of stress transduction signals, hence the topology of its 3 tier activation cascade. As shown in chapter 3, while p38 is strongly activated in response to DNA damage, its activity is not required for the proper execution of the G2 cell cycle checkpoint.

Transcriptional analysis of TNF- α treated Calu6 cells revealed that cancer cells induce strong anti-apoptosis signaling in response to TNF- α treatment and inhibition of p38 in this context leads to strong degradation of anti-apoptosis signaling. This suggested that p38 activity is an important component of the anti-apoptotic signaling pathway,

activated in response to TNF- α stress. To extend these observations to the context of DNA damage response, we explored the role of p38 activity in the regulation of apoptosis induction.

We observed that inhibition of p38 in combination with a genotoxic agent such as Adriamycin led to a dramatic increase in apoptotic markers in both p53 functionally null as well as p53 wild-type cancer cell-lines. A dose titration exercise with a p38 inhibitor revealed that chemo-potential could be observed at doses as low as 80 to 160nM. In conjunction with apoptosis induction with a p38 inhibitor, we also showed that inhibition of Chk1 eventually leads to dramatic increase in apoptosis. The time dynamics of Chk1 mediated apoptosis induction suggests that bypassing the G2 checkpoint and pre-mature entry into mitosis may be a necessary step for cells treated with a Chk1 inhibitor prior to the initiation of apoptosis. This would be in line with the hypothesis that inhibition of ATM/ATR signaling in conjunction with DNA damage would lead eventually to mitotic catastrophe.

Considering that inhibition of p38 in cancer cell-line models was unable to dramatically increase the percentage of cells bypassing the G2 cell cycle checkpoint mounted in response to DNA damage, cells undergoing apoptosis in combination with a genotoxic agent are mostly still stuck in G2 with 4N DNA content. This contrasts dramatically with the mitotic catastrophe phenotype observed with inhibition of ATM/ATR signaling, where premature entry into mitosis is believed to be the main trigger of apoptosis. As we found that cancer cells do not enter mitosis despite strong p38 inhibition, apoptosis induction observed with a combination of a DNA damaging genotoxic agent and p38 inhibition must be triggered by a separate mechanism.

A possible mechanism for how p38 activity plays a role in regulation of apoptotic response is through modulation of the anti-apoptotic BCL2 family proteins. The decreased expression of BCL2 and BCL-xl was observed in association with p38 inhibition and genotoxic agents like Adriamycin and MMS as seen in Chapter 5. This suggests that p38 activity in response to DNA damage plays an important role in the activation of pro-survival signaling, preventing the early onset of apoptosis. A possible biological explanation for this could be that p38's cyto-protective role in the early response to DNA damage is to protect cells from pre-mature apoptosis while DNA damage repair pathways assess the extent of the damage. As increased cell-killing can be observed in both p53 functionally null (Hela) and in p53 wild-type cells (A549), we concluded that p38's impact on apoptosis was not dependent on p53 status. Our suggestions that p38 plays a cyto-protective role in connection with DNA damage also implies that p38 functions through a stress induced but cell cycle independent mechanism. While we observed that p38 inhibition in conjunction with genotoxic agents modulates certain members of the BCL2 family of proteins. We do not discount other mechanisms including increased synergy with H2AX, leading to increased cell death (217).

6.4 A tentative new model for p38's role in DNA Damage Response

Based on the results of Our study of p38's role in DNA damage response, we have developed a tentative new model for p38's role in DNA damage response.

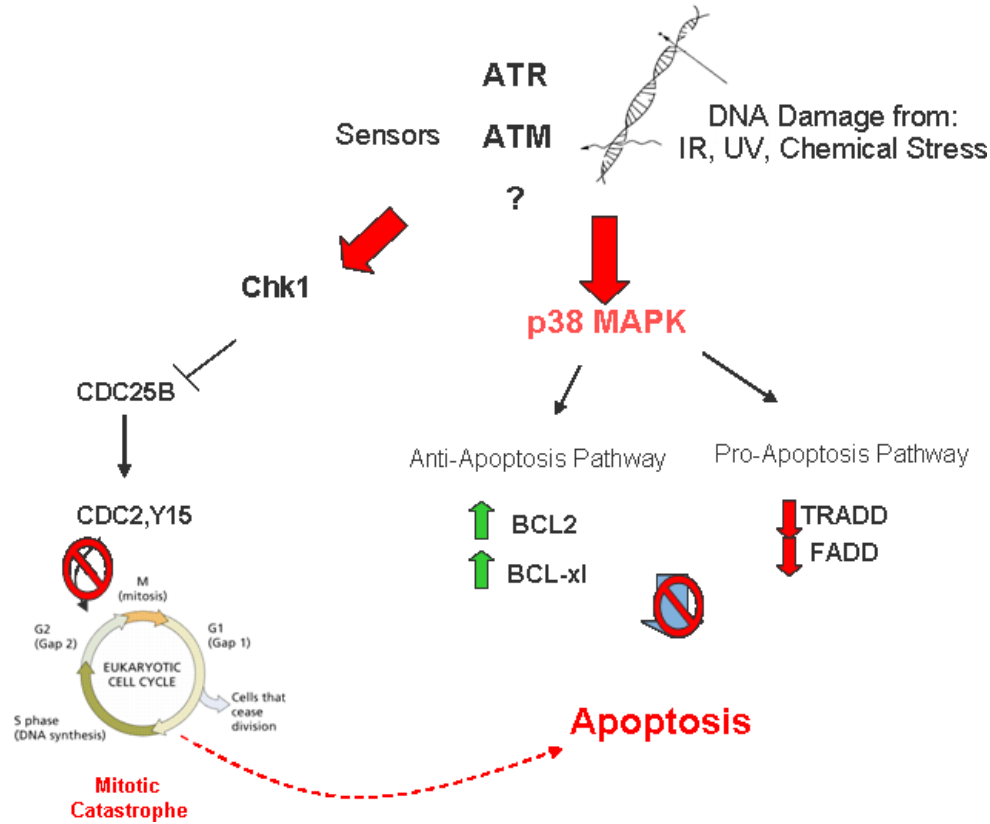


Figure 6.1: A new model of p38's role in DNA damage response

While p38 activity is highly associated with the onset of DNA damage, our results suggest that its activity is not required for the functioning of the pathway. Instead, the link between inhibition of p38 activity and increased apoptosis in conjunction with DNA damage and with TNF- α stress suggests a role for p38 in cell survival signaling following stress. The results documented in Chapter 5 show that in cancer cells, p38 activity is most

likely linked with the up-regulation of cell survival pathways and that p38 activity prevents early onset of apoptosis.

6.5 Role of p38 in Recovery from DNA damage

We explored a few other possible roles for p38 activity in response to DNA damage. As DNA damage cell cycle arrest is often reversible upon repair of the damage, we explored if p38 activity was necessary for cells to recover efficiently from DNA damage. Our initial data suggested that there may be a link between inhibition of p38 and irreversible cell cycle arrest with low levels of Adriamycin. Recovery from DNA damage is a relatively unexplored research area, and there are suggestions that p38 activity may be a necessary component of the recovery pathway.

6.6 Conclusion

In summary, we conclude that while p38 is highly associated with the onset of checkpoint arrest in G2, our findings show that p38 activity is not required for the successful mounting and maintenance of this checkpoint. We find that in a p53 functionally null environment, ATM/ATR signaling culminating in Chk1 activity is required for a properly functioning G2 DNA damage checkpoint. We do, however, find a possible role for p38 in the protection of cells from apoptosis in G2, while cells are undergoing cell cycle arrest, and DNA damage repair. We find that p38 may play this cyto-protective role through regulation of the pro/anti apoptotic BCL2 family proteins. These findings suggest that p38's early activity in G2 checkpoint activation is related to protection from apoptosis and not related to the establishment of cell cycle checkpoint arrest. The p38 MAPK, acting as a central integrator of external stress signals serves as a

central node in damage assessment and may ultimately help perpetuate cell growth and cell death signals in response to stress.

The findings of this study suggest that biochemical inhibition of Chk1 in combination with a chemotherapeutic agent may have a strong therapeutic value in p53 defective cancer cells. As the majority of cancer cells are p53 functionally deficient while untransformed cells have an intact p53 signaling pathway, synergy with a Chk1 inhibitor may provide an appropriate efficacy window for combination therapy with lower toxic side effects.

Our findings also imply that a p38 inhibitor is a useful therapeutic agent in synergy with chemotherapeutic agents. The reversal of anti-apoptotic signaling response with a p38 inhibitor could significantly increase the efficacy of chemotherapeutic agents. Our results suggest that the efficacy of the p38 inhibitor in combination with a genotoxic agent is not dependent on the cell cycle state of the cell, possibly allowing a greater degree of flexibility in future combination therapy strategies.

Chapter 7: Future Direction

As is often the case for extensive bodies of work such as this, this study contains some unresolved issues and raises numerous interesting questions to be tackled in the future. The multiple upstream regulators and down-stream substrates of p38 signaling, make it an extremely complicated pathway to study, especially in the context of cell cycle regulation and control of apoptosis. While this study addressed a number of fundamental issues involving the p38 pathway, the results generated from this study also produced a number of new/on-going questions especially in the area of underlying mechanism.

In the next few sections we will address a number of these issues and raise them as problems for future investigation.

7.1 What is the mechanism of the pathway attenuation of p38 in G2 cell cycle checkpoint signaling?

While p38 is strongly activated in response to DNA damage in cancer cells, its activity is not required for the activation of a G2 DNA damage checkpoint arrest, as shown in this study. The fact that p38 signaling into the G2 cell cycle checkpoint is attenuated in transformed cancer cells was first reported by Mikhailov et al (267). In this paper, they attributed the loss of p38 pathway significance in transformed cancer cells to G2 checkpoint function, to increased Ras/Raf signaling and loss of p53/Rb status. Yet the underlying mechanism of how Ras/Raf signaling feeds into the CDC25/CDC2 checkpoint control is not understood. They and others report that in untransformed cells, activation of p38 significantly impedes entry into mitosis. Hence the process of transformation from a normal cell to a cancer cell, somehow mitigates the effect of the p38 pathway on the G2 cell cycle checkpoint.

An immediate follow-up to this thesis would be to investigate if the p38 pathway is required for G2 checkpoint activation in untransformed human fibroblast cells. Using the same strategies employed in cancer cell models, a careful cell cycle based investigation of the G2 checkpoint would validate the hypothesis that p38 pathway activity is only necessary in untransformed human cells, but not in transformed cancer cells.

Gaining a better mechanistic understanding of how the p38 pathway is attenuated in relation to the cell cycle checkpoint, could have great therapeutic value for treatment of cancer. If the process of attenuation of p38 effect at G2 checkpoint could be restored, a new mechanism for halting uncontrolled cellular proliferation could be achieved. This would be an interesting question to address as a follow-up to results from this study.

7.2 Transcriptional Analysis of Adriamycin Treated HeLa Cells

The microarray dataset used during this thesis explored the effect of p38 inhibition on TNF α mediated stress in Calu6 cells. As this thesis was focused on DNA damage response, an improved microarray experiment exploring the effect of p38 inhibition on the expression profile on cells arrested in G2 by adriamycin treatment should be conducted.

It will be interesting to see if p38 plays a role in modulating the immediate early response proteins and in transcriptional regulation of anti-apoptotic proteins such as BCL2 cells arrested in G2 by adriamycin treatment. The transcriptional network analysis suggested that the p38 signaling plays a role primarily at the early time points and may

play a role in prevention of early or pre-mature apoptosis. It will be important to see if these same transcriptional networks are also strongly induced by adriamycin treatment.

7.2 Where does p38 signaling impinge upon apoptosis signaling?

In this study, inhibition of p38 in conjunction with genotoxic agents leads to dramatic increase in apoptotic induction. One possible mechanism for loss of pro-survival signaling was linked with p38 activity being required for induction of BCL2 and BCL-xl expression. However, inhibition of Chk1 in conjunction with genotoxic agents also leads to apoptosis induction, via pre-mature entry into mitosis (mitotic catastrophe). Induction of apoptosis through the inhibition of Chk1 pathway does not require inhibition of p38.

Furthermore, p38 MAPK signaling has been widely reported to play a role in pro-apoptotic signaling in the context of death receptor signaling and DNA damage (176,375,413,468). Yet results from this study and others have shown that inhibition of p38 also leads to apoptosis. A possible explanation for this could be that the signaling pathways in transformed versus untransformed cells are fundamentally different.

Another possible explanation could be that p38 MAPK plays a dual role and depending on environmental cofactors, switches between a pro-survival and pro-apoptotic phenotype. Clearly the underlying mechanism of how p38 behaves in different contexts for the regulation of apoptosis is not well understood.

Further exploration of the role of p38 signaling in the regulation of apoptosis would be an interesting question to pursue in the future.

7.3 Exploring p38 and p53 interactions, especially at the G1/S cell cycle checkpoint transition

One question that was not addressed in this study due to insufficient time was the relationship between p38 and p53. Numerous publications have found a link between p38

activity and increased p53 activity, suggesting that p38 is an upstream mediator of p53 signaling (31,163,180,327,356,362,379). It has been shown in these publications that p38 can directly or via MK2, phosphorylate p53 on numerous N-terminal residues. This relationship between p38 and p53 may be especially relevant at the G1/S transition, which depends on a functional p53 and Rb pathway. In addition to regulating the G1/S transition point, p38 may have a role in p53 induced apoptosis following DNA damage.

The majority of the experiments for the cell cycle component of this study utilized HeLa and Calu6 cells, which are both p53 functionally null. However some very preliminary data in A549 cells suggests that p38 may modulate p53 signaling and thus impact the function of the G1/S restriction point in response to DNA damage.

The complexity of the p38 and p53 signaling pathways makes this a difficult study. The large number of potential up-stream regulators of p53 makes identifying a p38 specific interaction with p53 difficult. It is likely that high degree of redundancy in the protein signaling networks leading to p53 activation would make deciphering of the pathway challenging. Yet gaining a greater understanding of p38's role in G1/S transition, in a p53 wild-type environment would have potential therapeutic value.

7.4 Understanding how p38 signaling affects recovery from DNA damage

A relatively unstudied field in DNA damage response is the process of how cells recover from DNA damage and reinitiate the cell cycle. As shown in section 5.3, HeLa cells can recover from low levels of adriamycin damage (Figure 5.5). Inhibition of p38 in this context leads to cells not recovering from DNA damage. Due to time constraints we were unable to further delve into the mechanism of how p38 activity influences the process of recovery and reinitiation of cell cycle.

One possibility is that p38 may link the DNA damage response to the DNA repair pathway (149). Inhibition of p38 may impede the proper functioning of the DNA repair pathway, leading to cells not being able to sufficiently repair the damage and return to the cell cycle. Further investigation of the underlying mechanism of p38's role in DNA repair and recovery would be an interesting topic for future research.

Publications

1. Phong M¹, Li S³, Tucker-Kellogg G¹, Surana U⁴, Ye X². **The p38 MAPK promotes cell survival in response to DNA damage but is not required for the execution of cell cycle arrest in G2 in cancer cells.** (Revision submitted to Journal of Molecular and Cellular Biology, December 2009)

Reference List

1. **Abreu, M. T., E. T. Arnold, L. S. Thomas, R. Gonsky, Y. Zhou, B. Hu, and M. Arditi.** 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem.* **277**:20431-20437.
2. **Adams, K. E., A. L. Medhurst, D. A. Dart, and N. D. Lakin.** 2006. Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex. *Oncogene.* **25**:3894-3904.
3. **Adler, H. S., S. Kubsch, E. Graulich, S. Ludwig, J. Knop, and K. Steinbrink.** 2007. Activation of MAP kinase p38 is critical for the cell-cycle-controlled suppressor function of regulatory T cells. *Blood.* **109**:4351-4359.
4. **Agarwal, M. L., A. Agarwal, W. R. Taylor, and G. R. Stark.** 1995. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **92**:8493-8497.
5. **Aggeli, I. K., C. Gaitanaki, A. Lazou, and I. Beis.** 2001. Activation of multiple MAPK pathways (ERKs, JNKs, p38-MAPK) by diverse stimuli in the amphibian heart. *Mol. Cell Biochem.* **221**:63-69.
6. **Akira, S., K. Hoshino, and T. Kaisho.** 2000. The role of Toll-like receptors and MyD88 in innate immune responses. *J Endotoxin. Res* **6**:383-387.
7. **Akiyama, T., T. Yoshida, T. Tsujita, M. Shimizu, T. Mizukami, M. Okabe, and S. Akinaga.** 1997. G1 phase accumulation induced by UCN-01 is associated with dephosphorylation of Rb and CDK2 proteins as well as induction of CDK inhibitor p21/Cip1/WAF1/Sdi1 in p53-mutated human epidermoid carcinoma A431 cells. *Cancer Res.* **57**:1495-1501.
8. **Amador, V., S. Ge, P. G. Santamaria, D. Guardavaccaro, and M. Pagano.** 2007. APC/C(Cdc20) controls the ubiquitin-mediated degradation of p21 in prometaphase. *Mol. Cell.* **27**:462-473.
9. **Ammit, A. J. and R. A. Panettieri, Jr.** 2001. Invited review: the circle of life: cell cycle regulation in airway smooth muscle. *J Appl. Physiol.* **91**:1431-1437.
10. **Andreakos, E., S. M. Sacre, C. Smith, A. Lundberg, S. Kiriakidis, T. Stonehouse, C. Monaco, M. Feldmann, and B. M. Foxwell.** 2004. Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood* **103**:2229-2237.
11. **Armstrong, S. C., M. Delacey, and C. E. Ganote.** 1999. Phosphorylation state of hsp27 and p38 MAPK during preconditioning and protein phosphatase inhibitor protection of rabbit cardiomyocytes. *J. Mol. Cell Cardiol.* **31**:555-567.

12. **Arteaga, C. L., F. Khuri, G. Krystal, and S. Sebti.** 2002. Overview of rationale and clinical trials with signal transduction inhibitors in lung cancer. *Semin. Oncol.* **29**:15-26.
13. **Austin, C. A. and L. M. Fisher.** 1990. DNA topoisomerases: enzymes that change the shape of DNA. *Sci. Prog.* **74**:147-161.
14. **Badie, C., J. E. Itzhaki, M. J. Sullivan, A. J. Carpenter, and A. C. Porter.** 2000. Repression of CDK1 and other genes with CDE and CHR promoter elements during DNA damage-induced G(2)/M arrest in human cells. *Mol. Cell Biol.* **20**:2358-2366.
15. **Bai, J., J. Sui, A. Demirjian, C. M. Vollmer, Jr., W. Marasco, and M. P. Callery.** 2005. Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro. *Cancer Res.* **65**:2344-2352.
16. **Bain, J., L. Plater, M. Elliott, N. Shpiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alessi, and P. Cohen.** 2007. The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* **408**:297-315.
17. **Baldin, V., C. Cans, M. Knibiehler, and B. Ducommun.** 1997. Phosphorylation of human CDC25B phosphatase by CDK1-cyclin A triggers its proteasome-dependent degradation. *J Biol. Chem.* **272**:32731-32734.
18. **Bartek, J., C. Lukas, and J. Lukas.** 2004. Checking on DNA damage in S phase. *Nat. Rev. Mol. Cell Biol.* **5**:792-804.
19. **Bashir, T. and M. Pagano.** 2004. Don't skip the G1 phase: how APC/CCdh1 keeps SCFSKP2 in check. *Cell Cycle.* **3**:850-852.
20. **Bastians, H., L. M. Topper, G. L. Gorbsky, and J. V. Ruderman.** 1999. Cell cycle-regulated proteolysis of mitotic target proteins. *Mol. Biol. Cell.* **10**:3927-3941.
21. **Beck, F. X., R. Grunbein, K. Lugmayr, and W. Neuhofer.** 2000. Heat shock proteins and the cellular response to osmotic stress. *Cell Physiol Biochem.* **10**:303-306.
22. **Bhattacharya, S.** 1998. Mechanisms of signal transduction in the stress response of hepatocytes. *Int. Rev. Cytol.* **184**:109-56.:109-156.
23. **Bian, Z. M., S. G. Elner, A. Yoshida, S. L. Kunkel, J. Su, and V. M. Elner.** 2001. Activation of p38, ERK1/2 and NIK pathways is required for IL-1beta and TNF-alpha-induced chemokine expression in human retinal pigment epithelial cells. *Exp. Eye Res.* **73**:111-121.
24. **Bockstaele, L., H. Kooken, F. Libert, S. Paternot, J. E. Dumont, L. Y. de, P. P. Roger, and K. Coulonval.** 2006. Regulated activating Thr172 phosphorylation

- of cyclin-dependent kinase 4(CDK4): its relationship with cyclins and CDK "inhibitors". *Mol. Cell Biol.* **26**:5070-5085.
25. **Bollig, F., R. Winzen, M. Gaestel, S. Kostka, K. Resch, and H. Holtmann.** 2003. Affinity purification of ARE-binding proteins identifies polyA-binding protein 1 as a potential substrate in MK2-induced mRNA stabilization. *Biochem. Biophys. Res. Commun.* **301**:665-670.
 26. **Bouaouina, M., E. Blouin, L. Halbwachs-Mecarelli, P. Lesavre, and P. Rieu.** 2004. TNF-induced beta2 integrin activation involves Src kinases and a redox-regulated activation of p38 MAPK. *J. Immunol.* **173**:1313-1320.
 27. **Bradham, C. and D. R. McClay.** 2006. p38 MAPK in development and cancer. *Cell Cycle.* **5**:824-828.
 28. **Brault, L. and D. Bagrel.** 2008. Activity of novel Cdc25 inhibitors and preliminary evaluation of their potentiation of chemotherapeutic drugs in human breast cancer cells. *Life Sci.* **82**:315-323.
 29. **Braun, K., V. Ehemann, W. Waldeck, R. Pipkorn, H. Corban-Wilhelm, J. Jenne, L. Gissmann, and J. Debus.** 2004. HPV18 E6 and E7 genes affect cell cycle, pRB and p53 of cervical tumor cells and represent prominent candidates for intervention by use peptide nucleic acids (PNAs). *Cancer Lett.* **209**:37-49.
 30. **Brinkman, B. M., J. B. Telliez, A. R. Schievella, L. L. Lin, and A. E. Goldfeld.** 1999. Engagement of tumor necrosis factor (TNF) receptor 1 leads to ATF-2- and p38 mitogen-activated protein kinase-dependent TNF-alpha gene expression. *J Biol. Chem.* **274**:30882-30886.
 31. **Brown, L. and S. Benchimol.** 2006. The involvement of MAPK signaling pathways in determining the cellular response to p53 activation: cell cycle arrest or apoptosis. *J. Biol. Chem.* **281**:3832-3840.
 32. **Bucher, N. and C. D. Britten.** 2008. G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. *Br. J Cancer.* **98**:523-528.
 33. **Buchynska, L. G. and I. P. Nesina.** 2006. Expression of the cell cycle regulators p53, p21(WAF1/CIP1) and p16(INK4a) in human endometrial adenocarcinoma. *Exp. Oncol.* **28**:152-155.
 34. **Bueter, M., M. Gasser, T. Lebedeva, G. Benichou, and A. M. Waaga-Gasser.** 2006. Influence of p53 on anti-tumor immunity (review). *Int. J Oncol.* **28**:519-525.
 35. **Bulavin, D. V., S. A. Amundson, and A. J. Fornace.** 2002. p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. *Curr. Opin. Genet. Dev.* **12**:92-97.

36. **Bulavin, D. V., Y. Higashimoto, I. J. Popoff, W. A. Gaarde, V. Basrur, O. Potapova, E. Appella, and A. J. Fornace, Jr.** 2001. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature*. **411**:102-107.
37. **Bulavin, D. V., O. Kovalsky, M. C. Hollander, and A. J. Fornace, Jr.** 2003. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of Gadd45a. *Mol. Cell Biol.* **23**:3859-3871.
38. **Bulavin, D. V., S. Saito, M. C. Hollander, K. Sakaguchi, C. W. Anderson, E. Appella, and A. J. Fornace, Jr.** 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J.* **18**:6845-6854.
39. **Burmeister, L. A. and C. N. Mariash.** 1990. Oncogenes, growth factors, and signal transduction. *N. Engl. J Med.* **322**:1322.
40. **Buxade, M., J. L. Parra, S. Rousseau, N. Shpiro, R. Marquez, N. Morrice, J. Bain, E. Espel, and C. G. Proud.** 2005. The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. *Immunity.* **23**:177-189.
41. **Canesi, L., M. Betti, C. Ciacci, A. Scarpato, B. Citterio, C. Pruzzo, and G. Gallo.** 2002. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAP kinases. *Dev. Comp Immunol.* **26**:325-334.
42. **Canman, C. E., C. Y. Chen, M. H. Lee, and M. B. Kastan.** 1994. DNA damage responses: p53 induction, cell cycle perturbations, and apoptosis. *Cold Spring Harb. Symp. Quant. Biol.* **59**:277-86.:277-286.
43. **Caporali, S., S. Falcinelli, G. Starace, M. T. Russo, E. Bonmassar, J. Jiricny, and S. D'Atri.** 2004. DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. *Mol. Pharmacol.* **66**:478-491.
44. **Cappellini, A., P. L. Tazzari, I. Mantovani, A. M. Billi, C. Tassi, F. Ricci, R. Conte, and A. M. Martelli.** 2005. Antiapoptotic role of p38 mitogen activated protein kinase in Jurkat T cells and normal human T lymphocytes treated with 8-methoxypsoralen and ultraviolet-A radiation. *Apoptosis.* **10**:141-152.
45. **Carter, A. B., M. M. Monick, and G. W. Hunninghake.** 1999. Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell Mol. Biol.* **20**:751-758.
46. **Casagrande, F. and J. M. Darbon.** 2001. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem. Pharmacol.* **61**:1205-1215.
47. **Casenghi, M., F. A. Barr, and E. A. Nigg.** 2005. Phosphorylation of Nlp by Plk1 negatively regulates its dynein-dynactin-dependent targeting to the centrosome. *J Cell Sci.* **118**:5101-5108.

48. **Cha, H., X. Wang, H. Li, and A. J. Fornace, Jr.** 2007. A functional role for p38 MAPK in modulating mitotic transit in the absence of stress. *J Biol. Chem.* **282**:22984-22992.
49. **Chang, F., L. S. Steelman, J. G. Shelton, J. T. Lee, P. M. Navolanic, W. L. Blalock, R. Franklin, and J. A. McCubrey.** 2003. Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). *Int. J Oncol.* **22**:469-480.
50. **Chang, N. S.** 2002. A potential role of p53 and WOX1 in mitochondrial apoptosis (review). *Int. J Mol. Med.* **9**:19-24.
51. **Chao, J. I. and P. C. Kuo.** 2004. Role of p53 and p38 MAP kinase in nitric oxide-induced G2/M arrest and apoptosis in the human lung carcinoma cells. *Carcinogenesis.* **25**:645.
52. **Charrier-Savournin, F. B., M. T. Chateau, V. Gire, J. Sedivy, J. Piette, and V. Dulic.** 2004. p21-Mediated nuclear retention of cyclin B1-Cdk1 in response to genotoxic stress. *Mol. Biol. Cell.* **15**:3965-3976.
53. **Chen, C. Y., J. D. Oliner, Q. Zhan, A. J. Fornace, Jr., B. Vogelstein, and M. B. Kastan.** 1994. Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc. Natl. Acad. Sci. U. S. A.* **91**:2684-2688.
54. **Chen, L., H. Y. He, H. M. Li, J. Zheng, W. J. Heng, J. F. You, and W. G. Fang.** 2004. ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. *Cancer Lett.* **215**:239-247.
55. **Chen, Q. M., J. Liu, and J. B. Merrett.** 2000. Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. *Biochem. J.* **347**:543-551.
56. **Chen, Y., Z. H. Miao, W. M. Zhao, and J. Ding.** 2005. The p53 pathway is synergized by p38 MAPK signaling to mediate 11,11'-dideoxyverticillin-induced G2/M arrest. *FEBS Lett.* **579**:3683-3690.
57. **Chen, Y. L., Y. L. Huang, N. Y. Lin, H. C. Chen, W. C. Chiu, and C. J. Chang.** 2006. Differential regulation of ARE-mediated TNFalpha and IL-1beta mRNA stability by lipopolysaccharide in RAW264.7 cells. *Biochem. Biophys. Res. Commun.* **346**:160-168.
58. **Chen, Z., Z. Xiao, J. Chen, S. C. Ng, T. Sowin, H. Sham, S. Rosenberg, S. Fesik, and H. Zhang.** 2003. Human Chk1 expression is dispensable for somatic cell death and critical for sustaining G2 DNA damage checkpoint. *Mol. Cancer Ther.* **2**:543-548.
59. **Chen, Z., Z. Xiao, W. Z. Gu, J. Xue, M. H. Bui, P. Kovar, G. Li, G. Wang, Z. F. Tao, Y. Tong, N. H. Lin, H. L. Sham, J. Y. Wang, T. J. Sowin, S. H. Rosenberg, and H. Zhang.** 2006. Selective Chk1 inhibitors differentially

- sensitize p53-deficient cancer cells to cancer therapeutics. *Int. J Cancer*. **119**:2784-2794.
60. **Chi, D. S., S. M. Fitzgerald, S. Pitts, K. Cantor, E. King, S. A. Lee, S. K. Huang, and G. Krishnaswamy.** 2004. MAPK-dependent regulation of IL-1- and beta-adrenoreceptor-induced inflammatory cytokine production from mast cells: implications for the stress response. *BMC. Immunol.* **5**:22.:22.
 61. **Chiarugi, V., L. Magnelli, and M. Cinelli.** 1997. Complex interplay among apoptosis factors: RB, p53, E2F, TGF-beta, cell cycle inhibitors and the bcl2 gene family. *Pharmacol. Res.* **35**:257-261.
 62. **Chowdhury, R., S. Chowdhury, P. Roychoudhury, C. Mandal, and K. Chaudhuri.** 2009. Arsenic induced apoptosis in malignant melanoma cells is enhanced by menadione through ROS generation, p38 signaling and p53 activation. *Apoptosis.* **14**:108-123.
 63. **Chung, E. Y., S. J. Kim, and X. J. Ma.** 2006. Regulation of cytokine production during phagocytosis of apoptotic cells. *Cell Res.* **16**:154-161.
 64. **Clerk, A., S. J. Fuller, A. Michael, and P. H. Sugden.** 1998. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J Biol. Chem.* **273**:7228-7234.
 65. **Clerk, A., J. G. Harrison, C. S. Long, and P. H. Sugden.** 1999. Pro-inflammatory cytokines stimulate mitogen-activated protein kinase subfamilies, increase phosphorylation of c-Jun and ATF2 and upregulate c-Jun protein in neonatal rat ventricular myocytes. *J. Mol. Cell Cardiol.* **31**:2087-2099.
 66. **Cogoli-Greuter, M., P. Lovis, and S. Vadrucchi.** 2004. Signal transduction in T cells: an overview. *J Gravit. Physiol.* **11**:53-56.
 67. **Cooke, M. S., M. T. Finnegan, K. E. Herbert, and J. Lunec.** 1995. Cell cycle and dose-dependence of DNA damage and p53 expression following UVA irradiation. *Biochem. Soc. Trans.* **23**:481S.
 68. **Cornell, T. T. and T. P. Shanley.** 2005. Signal transduction overview. *Crit Care Med.* **33**:S410-S413.
 69. **Correze, C., J. P. Blondeau, and M. Pomerance.** 2005. p38 mitogen-activated protein kinase contributes to cell cycle regulation by cAMP in FRTL-5 thyroid cells. *Eur. J Endocrinol.* **153**:123-133.
 70. **Coss, D., C. M. Hand, K. K. Yaphockun, H. A. Ely, and P. L. Mellon.** 2007. p38 mitogen-activated protein kinase is critical for synergistic induction of the FSH(beta) gene by gonadotropin-releasing hormone and activin through augmentation of c-Fos induction and Smad phosphorylation. *Mol. Endocrinol.* **21**:3071-3086.

71. **Craig, E. A., M. V. Stevens, R. R. Vaillancourt, and T. D. Camenisch.** 2008. MAP3Ks as central regulators of cell fate during development. *Dev. Dyn.* **237**:3102-3114.
72. **Crasta, K., P. Huang, G. Morgan, M. Winey, and U. Surana.** 2006. Cdk1 regulates centrosome separation by restraining proteolysis of microtubule-associated proteins. *EMBO J.* **25**:2551-2563.
73. **Crasta, K., H. H. Lim, T. H. Giddings, Jr., M. Winey, and U. Surana.** 2008. Inactivation of Cdh1 by synergistic action of Cdk1 and polo kinase is necessary for proper assembly of the mitotic spindle. *Nat. Cell Biol.* **10**:665-675.
74. **Crasta, K. and U. Surana.** 2006. Disjunction of conjoined twins: Cdk1, Cdh1 and separation of centrosomes. *Cell Div.* **1**:12.:12.
75. **Craven, R. J., P. W. Greenwell, M. Dominska, and T. D. Petes.** 2002. Regulation of genome stability by TEL1 and MEC1, yeast homologs of the mammalian ATM and ATR genes. *Genetics.* **161**:493-507.
76. **Cross, F. R.** 2003. Two redundant oscillatory mechanisms in the yeast cell cycle. *Dev. Cell.* **4**:741-752.
77. **Cuadrado, A., V. Lafarga, P. C. Cheung, I. Dolado, S. Llanos, P. Cohen, and A. R. Nebreda.** 2007. A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. *EMBO J.* **26**:2115-2126.
78. **D'Angiolella, V., C. Mari, D. Nocera, L. Rametti, and D. Grieco.** 2003. The spindle checkpoint requires cyclin-dependent kinase activity. *Genes Dev.* **17**:2520-2525.
79. **Delghandi, M. P., M. Johannessen, and U. Moens.** 2005. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal.* **17**:1343-1351.
80. **Demidov, O. N., C. Kek, S. Shreeram, O. Timofeev, A. J. Fornace, E. Appella, and D. V. Bulavin.** 2007. The role of the MKK6/p38 MAPK pathway in Wip1-dependent regulation of ErbB2-driven mammary gland tumorigenesis. *Oncogene.* **26**:2502-2506.
81. **Densham, R. M., D. E. Todd, K. Balmanno, and S. J. Cook.** 2008. ERK1/2 and p38 cooperate to delay progression through G1 by promoting cyclin D1 protein turnover. *Cell Signal.* **20**:1986-1994.
82. **Detmers, P. A., D. Zhou, E. Polizzi, R. Thieringer, W. A. Hanlon, S. Vaidya, and V. Bansal.** 1998. Role of stress-activated mitogen-activated protein kinase (p38) in beta 2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. *J. Immunol.* **161**:1921-1929.
83. **Deupi, X. and B. Kobilka.** 2007. Activation of G protein-coupled receptors. *Adv. Protein Chem.* **74**:137-66.:137-166.

84. **Dhanasekaran, D. N., K. Kashef, C. M. Lee, H. Xu, and E. P. Reddy.** 2007. Scaffold proteins of MAP-kinase modules. *Oncogene*. **26**:3185-3202.
85. **dib-Conquy, M. and J. M. Cavaillon.** 2002. Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to MyD88 and not by modulating TLR4 expression. *J Biol Chem*. **277**:27927-27934.
86. **Ding, S. L., L. F. Sheu, J. C. Yu, T. L. Yang, B. F. Chen, F. J. Leu, and C. Y. Shen.** 2004. Abnormality of the DNA double-strand-break checkpoint/repair genes, ATM, BRCA1 and TP53, in breast cancer is related to tumour grade. *Br. J Cancer*. **90**:1995-2001.
87. **Dmitrieva, N. I., D. V. Bulavin, A. J. Fornace, Jr., and M. B. Burg.** 2002. Rapid activation of G2/M checkpoint after hypertonic stress in renal inner medullary epithelial (IME) cells is protective and requires p38 kinase. *Proc. Natl. Acad. Sci U. S. A.* **99**:184-189.
88. **Dong, W., Y. Liu, J. Peng, L. Chen, T. Zou, H. Xiao, Z. Liu, W. Li, Y. Bu, and Y. Qi.** 2006. The IRAK-1-BCL10-MALT1-TRAF6-TAK1 cascade mediates signaling to NF-kappaB from Toll-like receptor 4. *J Biol. Chem*. **281**:26029-26040.
89. **Druker, B. J., H. J. Mamon, and T. M. Roberts.** 1989. Oncogenes, growth factors, and signal transduction. *N. Engl. J Med*. **321**:1383-1391.
90. **Dubrana, K., A. H. van, F. Hediger, and S. M. Gasser.** 2007. The processing of double-strand breaks and binding of single-strand-binding proteins RPA and Rad51 modulate the formation of ATR-kinase foci in yeast. *J Cell Sci*. **120**:4209-4220.
91. **Duncker, B. P., P. Pasero, D. Braguglia, P. Heun, M. Weinreich, and S. M. Gasser.** 1999. Cyclin B-cdk1 kinase stimulates ORC- and Cdc6-independent steps of semiconservative plasmid replication in yeast nuclear extracts. *Mol. Cell Biol*. **19**:1226-1241.
92. **Durocher, D. and S. P. Jackson.** 2001. DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr. Opin. Cell Biol*. **13**:225-231.
93. **Duval, D., M. Malaise, B. Reinhardt, C. Kedinger, and H. Boeuf.** 2004. A p38 inhibitor allows to dissociate differentiation and apoptotic processes triggered upon LIF withdrawal in mouse embryonic stem cells. *Cell Death. Differ*. **11**:331-341.
94. **Elmore, S.** 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol*. **35**:495-516.
95. **Enders, G. H.** 2008. Expanded roles for Chk1 in genome maintenance. *J Biol. Chem*. **283**:17749-17752.

96. **Enomoto, S., L. Glowczewski, and J. Berman.** 2002. MEC3, MEC1, and DDC2 are essential components of a telomere checkpoint pathway required for cell cycle arrest during senescence in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* **13**:2626-2638.
97. **Enslin, H., D. M. Brancho, and R. J. Davis.** 2000. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *EMBO J.* **19**:1301-1311.
98. **Enslin, H., J. Raingeaud, and R. J. Davis.** 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.* **273**:1741-1748.
99. **Ewald, B., D. Sampath, and W. Plunkett.** 2008. ATM and the Mre11-Rad50-Nbs1 complex respond to nucleoside analogue-induced stalled replication forks and contribute to drug resistance. *Cancer Res.* **68**:7947-7955.
100. **Ewen, M. E., C. J. Oliver, H. K. Sluss, S. J. Miller, and D. S. Peeper.** 1995. p53-dependent repression of CDK4 translation in TGF-beta-induced G1 cell-cycle arrest. *Genes Dev.* **9**:204-217.
101. **Eyler, C. E. and J. N. Rich.** 2008. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin. Oncol.* **26**:2839-2845.
102. **Fabbro, M., B. B. Zhou, M. Takahashi, B. Sarcevic, P. Lal, M. E. Graham, B. G. Gabrielli, P. J. Robinson, E. A. Nigg, Y. Ono, and K. K. Khanna.** 2005. Cdk1/Erk2- and Plk1-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. *Dev. Cell.* **9**:477-488.
103. **Fabian, M. A., W. H. Biggs, III, D. K. Treiber, C. E. Atteridge, M. D. Azimioara, M. G. Benedetti, T. A. Carter, P. Ciceri, P. T. Edeen, M. Floyd, J. M. Ford, M. Galvin, J. L. Gerlach, R. M. Grotzfeld, S. Herrgard, D. E. Insko, M. A. Insko, A. G. Lai, J. M. Lelias, S. A. Mehta, Z. V. Milanov, A. M. Velasco, L. M. Wodicka, H. K. Patel, P. P. Zarrinkar, and D. J. Lockhart.** 2005. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* **23**:329-336.
104. **Falck, J., J. Coates, and S. P. Jackson.** 2005. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature.* **434**:605-611.
105. **Falco, J. P., W. G. Taylor, P. P. Di Fiore, B. E. Weissman, and S. A. Aaronson.** 1988. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of Balb/MK keratinocytes. *Oncogene.* **2**:573-578.
106. **Fan, L., X. Yang, J. Du, M. Marshall, K. Blanchard, and X. Ye.** 2005. A novel role of p38 alpha MAPK in mitotic progression independent of its kinase activity. *Cell Cycle.* **4**:1616-1624.

107. **Fang, G., H. Yu, and M. W. Kirschner.** 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* **12**:1871-1883.
108. **Fang, G., H. Yu, and M. W. Kirschner.** 1999. Control of mitotic transitions by the anaphase-promoting complex. *Philos. Trans. R. Soc. Lond B Biol. Sci.* **354**:1583-1590.
109. **Fessler, M. B., K. C. Malcolm, M. W. Duncan, and G. S. Worthen.** 2002. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. *J. Biol. Chem.* **277**:31291-31302.
110. **Fishman, D. D., S. Segal, and E. Livneh.** 1998. The role of protein kinase C in G1 and G2/M phases of the cell cycle (review). *Int. J. Oncol.* **12**:181-186.
111. **Flacke, J. P., S. Kumar, S. Kostin, H. P. Reusch, and Y. Ladilov.** 2009. Acidic preconditioning protects endothelial cells against apoptosis through p38- and Akt-dependent Bcl-xL overexpression. *Apoptosis.* **14**:90-96.
112. **Fogal, V., J. K. Hsieh, C. Royer, S. Zhong, and X. Lu.** 2005. Cell cycle-dependent nuclear retention of p53 by E2F1 requires phosphorylation of p53 at Ser315. *EMBO J.* **24**:2768-2782.
113. **Forbes, K. C., T. Humphrey, and T. Enoch.** 1998. Suppressors of cdc25p overexpression identify two pathways that influence the G2/M checkpoint in fission yeast. *Genetics.* **150**:1361-1375.
114. **Forester, C. M., J. Maddox, J. V. Louis, J. Goris, and D. M. Virshup.** 2007. Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proc. Natl. Acad. Sci U. S. A.* **104**:19867-19872.
115. **Fu, Z., L. Malureanu, J. Huang, W. Wang, H. Li, J. M. van Deursen, D. J. Tindall, and J. Chen.** 2008. Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression. *Nat. Cell Biol.*
116. **Garcia, J. G., P. Wang, K. L. Schaphorst, P. M. Becker, T. Borbiev, F. Liu, A. Birukova, K. Jacobs, N. Bogatcheva, and A. D. Verin.** 2002. Critical involvement of p38 MAP kinase in pertussis toxin-induced cytoskeletal reorganization and lung permeability. *FASEB J.* **16**:1064-1076.
117. **Gelowitz, D. L. and S. P. Berger.** 2001. Signal transduction mechanisms and behavioral sensitization to stimulant drugs: an overview of cAMP and PLA2. *J. Addict. Dis.* **20**:33-42.
118. **Giannetti, L., U. Consolo, C. Magnoni, and M. L. Lo.** 2004. Apoptosis: escaping strategies in human skin cancer (Review). *Oncol. Rep.* **11**:401-405.
119. **Gleich, L. L., Y. Q. Li, P. W. Biddinger, P. S. Gartside, P. J. Stambrook, Z. P. Pavelic, and J. L. Gluckman.** 1996. The loss of heterozygosity in

- retinoblastoma and p53 suppressor genes as a prognostic indicator for head and neck cancer. *Laryngoscope*. **106**:1378-1381.
120. **Golan, A., Y. Yudkovsky, and A. Hershko.** 2002. The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. *J Biol. Chem.* **277**:15552-15557.
121. **Goldstone, S., S. Pavey, A. Forrest, J. Sinnamon, and B. Gabrielli.** 2001. Cdc25-dependent activation of cyclin A/cdk2 is blocked in G2 phase arrested cells independently of ATM/ATR. *Oncogene*. **20**:921-932.
122. **Golias, C. H., A. Charalabopoulos, and K. Charalabopoulos.** 2004. Cell proliferation and cell cycle control: a mini review. *Int. J Clin. Pract.* **58**:1134-1141.
123. **Gomez-Lazaro, M., M. F. Galindo, C. G. Concannon, M. F. Segura, F. J. Fernandez-Gomez, N. Llecha, J. X. Comella, J. H. Prehn, and J. Jordan.** 2008. 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. *J Neurochem.* **104**:1599-1612.
124. **Gorr, I. H., D. Boos, and O. Stemmann.** 2005. Mutual inhibition of separase and Cdk1 by two-step complex formation. *Mol. Cell.* **19**:135-141.
125. **Goto, A., H. Kanda, Y. Ishikawa, S. Matsumoto, N. Kawaguchi, R. Machinami, Y. Kato, and T. Kitagawa.** 1998. Association of loss of heterozygosity at the p53 locus with chemoresistance in osteosarcomas. *Jpn. J. Cancer Res.* **89**:539-547.
126. **Grethe, S., M. P. Ares, T. Andersson, and M. I. Porn-Ares.** 2004. p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). *Exp. Cell Res.* **298**:632-642.
127. **Grethe, S., N. Coltella, M. F. Di Renzo, and M. I. Porn-Ares.** 2006. p38 MAPK downregulates phosphorylation of Bad in doxorubicin-induced endothelial apoptosis. *Biochem. Biophys. Res. Commun.* **347**:781-790.
128. **Grethe, S. and M. I. Porn-Ares.** 2006. p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-alpha induced endothelial apoptosis. *Cell Signal.* **18**:531-540.
129. **Guadagno, T. M. and J. W. Newport.** 1996. Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. *Cell.* **84**:73-82.
130. **Gum, R. J. and P. R. Young.** 1999. Identification of two distinct regions of p38 MAPK required for substrate binding and phosphorylation. *Biochem. Biophys. Res. Commun.* **266**:284-289.

131. **Guo, Z., A. Kumagai, S. X. Wang, and W. G. Dunphy.** 2000. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts. *Genes Dev.* **14**:2745-2756.
132. **Haapajarvi, T., L. Kivinen, K. Pitkanen, and M. Laiho.** 1995. Cell cycle dependent effects of u.v.-radiation on p53 expression and retinoblastoma protein phosphorylation. *Oncogene.* **11**:151-159.
133. **Hagan, I. M.** 2008. The spindle pole body plays a key role in controlling mitotic commitment in the fission yeast *Schizosaccharomyces pombe*. *Biochem. Soc. Trans.* **36**:1097-1101.
134. **Hagting, A., E. N. Den, H. C. Vodermaier, I. C. Waizenegger, J. M. Peters, and J. Pines.** 2002. Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J Cell Biol.* **157**:1125-1137.
135. **Haimovitz-Friedman, A.** 1998. Radiation-induced signal transduction and stress response. *Radiat. Res.* **150**:S102-S108.
136. **Hamanoue, M., K. Sato, and K. Takamatsu.** 2007. Inhibition of p38 mitogen-activated protein kinase-induced apoptosis in cultured mature oligodendrocytes using SB202190 and SB203580. *Neurochem. Int.* **51**:16-24.
137. **Han, J., Y. Jiang, Z. Li, V. V. Kravchenko, and R. J. Ulevitch.** 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature.* **386**:296-299.
138. **Hanahan, D. and R. A. Weinberg.** 2000. The hallmarks of cancer. *Cell.* **100**:57-70.
139. **Hangaishi, A., S. Ogawa, N. Imamura, S. Miyawaki, Y. Miura, N. Uike, C. Shimazaki, N. Emi, K. Takeyama, S. Hirosawa, N. Kamada, Y. Kobayashi, Y. Takemoto, T. Kitani, K. Toyama, S. Ohtake, Y. Yazaki, R. Ueda, and H. Hirai.** 1996. Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4A/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies. *Blood.* **87**:4949-4958.
140. **Hansen, D. V., A. V. Loktev, K. H. Ban, and P. K. Jackson.** 2004. Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFbetaTrCP-dependent destruction of the APC Inhibitor Emi1. *Mol. Biol. Cell.* **15**:5623-5634.
141. **Hao, D. and E. K. Rowinsky.** 2002. Inhibiting signal transduction: recent advances in the development of receptor tyrosine kinase and Ras inhibitors. *Cancer Invest.* **20**:387-404.
142. **Harari, D. and Y. Yarden.** 2000. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene.* **19**:6102-6114.

143. **Harvey, S. L., A. Charlet, W. Haas, S. P. Gygi, and D. R. Kellogg.** 2005. Cdk1-dependent regulation of the mitotic inhibitor Wee1. *Cell*. **122**:407-420.
144. **Hayashi, K., H. Kuniyasu, N. Oue, H. Shigeishi, K. Kuraoka, H. Nakayama, and W. Yasui.** 2002. Induction of hRAD9 is required for G2/M checkpoint signal transduction in gastric cancer cells. *Pathobiology*. **70**:40-46.
145. **Heldin, C. H.** 1995. Dimerization of cell surface receptors in signal transduction. *Cell*. **80**:213-223.
146. **Herrington, C. S.** 2006. p16, p14, p53, and Cyclin D1 expression and HPV analysis in small cell carcinomas of the uterine cervix by Horn et al. *Int. J Gynecol. Pathol.* **25**:408.
147. **Hibi, M. and T. Hirano.** 1998. Signal transduction through cytokine receptors. *Int. Rev. Immunol.* **17**:75-102.
148. **Hinchcliffe, E. H., E. A. Thompson, F. J. Miller, J. Yang, and G. Sluder.** 1999. Nucleo-cytoplasmic interactions that control nuclear envelope breakdown and entry into mitosis in the sea urchin zygote. *J Cell Sci.* **112**:1139-1148.
149. **Hirose, Y., M. Katayama, D. Stokoe, D. A. Haas-Kogan, M. S. Berger, and R. O. Pieper.** 2003. The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. *Mol. Cell Biol.* **23**:8306-8315.
150. **Ho, J. S., W. Ma, D. Y. Mao, and S. Benchimol.** 2005. p53-Dependent transcriptional repression of c-myc is required for G1 cell cycle arrest. *Mol. Cell Biol.* **25**:7423-7431.
151. **Hohegger, H., D. Dejsuphong, E. Sonoda, A. Saberi, E. Rajendra, J. Kirk, T. Hunt, and S. Takeda.** 2007. An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells. *J Cell Biol.* **178**:257-268.
152. **Hodge, C., J. Liao, M. Stofega, K. Guan, C. Carter-Su, and J. Schwartz.** 1998. Growth hormone stimulates phosphorylation and activation of elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-regulated kinases 1 and 2. *J. Biol. Chem.* **273**:31327-31336.
153. **Hollenberg, M. D.** 1995. Tyrosine kinase-mediated signal transduction pathways and the actions of polypeptide growth factors and G-protein-coupled agonists in smooth muscle. *Mol. Cell Biochem.* **149-150**:77-85.:77-85.
154. **Homer, H. A., A. McDougall, M. Levasseur, A. P. Murdoch, and M. Herbert.** 2005. Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes. *Reproduction.* **130**:829-843.
155. **Hoppe-Seyler, F. and K. Butz.** 1993. Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human

- papillomavirus type 16 E6, human mdm-2, and mutant p53. *J. Virol.* **67**:3111-3117.
156. **Horner, S. M., R. A. DeFilippis, L. Manuelidis, and D. DiMaio.** 2004. Repression of the human papillomavirus E6 gene initiates p53-dependent, telomerase-independent senescence and apoptosis in HeLa cervical carcinoma cells. *J. Virol.* **78**:4063-4073.
157. **Hou, S. T., X. Xie, A. Baggley, D. S. Park, G. Chen, and T. Walker.** 2002. Activation of the Rb/E2F1 pathway by the nonproliferative p38 MAPK during Fas (APO1/CD95)-mediated neuronal apoptosis. *J. Biol. Chem.* **277**:48764-48770.
158. **Houtgraaf, J. H., J. Versmissen, and W. J. van der Giessen.** 2006. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc. Revasc. Med.* **7**:165-172.
159. **Hozak, P. and P. R. Cook.** 1994. Replication factories. *Trends Cell Biol.* **4**:48-52.
160. **Hrzenjak, M. and S. A. Shain.** 1997. Fibroblast growth factor-2 and TPA enhance prostate-cancer-cell proliferation and activate members of the Ras and PKC signal transduction pathways. *Recept. Signal. Transduct.* **7**:207-219.
161. **Hsu, J. Y., J. D. Reimann, C. S. Sorensen, J. Lukas, and P. K. Jackson.** 2002. E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nat. Cell Biol.* **4**:358-366.
162. **Hu, B., J. Mitra, H. S. van den, and G. H. Enders.** 2001. S and G2 phase roles for Cdk2 revealed by inducible expression of a dominant-negative mutant in human cells. *Mol. Cell Biol.* **21**:2755-2766.
163. **Huang, C., W. Y. Ma, A. Maxiner, Y. Sun, and Z. Dong.** 1999. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J. Biol. Chem.* **274**:12229-12235.
164. **Huang, d. W., B. T. Sherman, and R. A. Lempicki.** 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**:44-57.
165. **Huang, d. W., B. T. Sherman, Q. Tan, J. Kir, D. Liu, D. Bryant, Y. Guo, R. Stephens, M. W. Baseler, H. C. Lane, and R. A. Lempicki.** 2007. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res.* **35**:W169-W175.
166. **Huang, H., J. Ryu, J. Ha, E. J. Chang, H. J. Kim, H. M. Kim, T. Kitamura, Z. H. Lee, and H. H. Kim.** 2006. Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-kappaB transactivation by RANKL. *Cell Death. Differ.* **13**:1879-1891.

167. **Huang, J. Y., G. Morley, D. Li, and M. Whitaker.** 2007. Cdk1 phosphorylation sites on Cdc27 are required for correct chromosomal localisation and APC/C function in syncytial *Drosophila* embryos. *J Cell Sci.* **120**:1990-1997.
168. **Hui, L., L. Bakiri, E. Stepniak, and E. F. Wagner.** 2007. p38alpha: a suppressor of cell proliferation and tumorigenesis. *Cell Cycle.* **6**:2429-2433.
169. **Humlova, Z.** 2002. Protooncogene bcl-2 in process of apoptosis. Review article. *Sb Lek.* **103**:419-425.
170. **Ichijo, H., E. Nishida, K. Irie, D. P. ten, M. Saitoh, T. Moriguchi, M. Takagi, K. Matsumoto, K. Miyazono, and Y. Gotoh.** 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science.* **275**:90-94.
171. **Ioachim, E., M. Michael, N. E. Stavropoulos, E. Kitsiou, K. Hastazeris, M. Salmas, S. Stefanaki, and N. J. Agnantis.** 2004. Expression patterns of cyclins D1, E and cyclin-dependent kinase inhibitors p21(Waf1/Cip1) and p27(Kip1) in urothelial carcinoma: correlation with other cell-cycle-related proteins (Rb, p53, Ki-67 and PCNA) and clinicopathological features. *Urol. Int.* **73**:65-73.
172. **Ismail, I. A., K. S. Kang, H. A. Lee, J. W. Kim, and Y. K. Sohn.** 2007. Genistein-induced neuronal apoptosis and G2/M cell cycle arrest is associated with MDC1 up-regulation and PLK1 down-regulation. *Eur. J Pharmacol.* **575**:12-20.
173. **Ito, K., T. Nakazato, Y. Miyakawa, K. Yamato, Y. Ikeda, and M. Kizaki.** 2003. Caffeine induces G2/M arrest and apoptosis via a novel p53-dependent pathway in NB4 promyelocytic leukemia cells. *J Cell Physiol.* **196**:276-283.
174. **Jackman, M., C. Lindon, E. A. Nigg, and J. Pines.** 2003. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat. Cell Biol.* **5**:143-148.
175. **Jackson, J. R., B. Bolognese, L. Hillegass, S. Kassis, J. Adams, D. E. Griswold, and J. D. Winkler.** 1998. Pharmacological effects of SB 220025, a selective inhibitor of P38 mitogen-activated protein kinase, in angiogenesis and chronic inflammatory disease models. *J. Pharmacol. Exp. Ther.* **284**:687-692.
176. **Jameel, N. M., C. Thirunavukkarasu, T. Wu, S. C. Watkins, S. L. Friedman, and C. R. Gandhi.** 2009. p38-MAPK- and caspase-3-mediated superoxide-induced apoptosis of rat hepatic stellate cells: reversal by retinoic acid. *J Cell Physiol.* **218**:157-166.
177. **Jansen, B., V. Wacheck, E. Heere-Ress, H. Schlagbauer-Wadl, C. Hoeller, T. Lucas, M. Hoermann, U. Hollenstein, K. Wolff, and H. Pehamberger.** 2000. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet.* **356**:1728-1733.

178. **Jazayeri, A., A. Balestrini, E. Garner, J. E. Haber, and V. Costanzo.** 2008. Mre11-Rad50-Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. *EMBO J.* **27**:1953-1962.
179. **Jeon, H. K., S. U. Choi, and N. P. Jung.** 2005. Association of the ERK1/2 and p38 kinase pathways with nitric oxide-induced apoptosis and cell cycle arrest in colon cancer cells. *Cell Biol. Toxicol.* **21**:115-125.
180. **Jin, S., T. Tong, W. Fan, F. Fan, M. J. Antinore, X. Zhu, L. Mazzacurati, X. Li, K. L. Petrik, B. Rajasekaran, M. Wu, and Q. Zhan.** 2002. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. *Oncogene.* **21**:8696-8704.
181. **Jinlian, L., Z. Yingbin, and W. Chunbo.** 2007. p38 MAPK in regulating cellular responses to ultraviolet radiation. *J Biomed. Sci.* **14**:303-312.
182. **Jirmanova, L., D. V. Bulavin, and A. J. Fornace, Jr.** 2005. Inhibition of the ATR/Chk1 pathway induces a p38-dependent S-phase delay in mouse embryonic stem cells. *Cell Cycle.* **4**:1428-1434.
183. **Johnson, A. and M. O'Donnell.** 2005. Cellular DNA replicases: components and dynamics at the replication fork. *Annu. Rev. Biochem.* **74**:283-315.:283-315.
184. **Jordan, M. S., A. L. Singer, and G. A. Koretzky.** 2003. Adaptors as central mediators of signal transduction in immune cells. *Nat. Immunol.* **4**:110-116.
185. **Jung, M. S., D. H. Jin, H. D. Chae, S. Kang, S. C. Kim, Y. J. Bang, T. S. Choi, K. S. Choi, and D. Y. Shin.** 2004. Bcl-xL and E1B-19K proteins inhibit p53-induced irreversible growth arrest and senescence by preventing reactive oxygen species-dependent p38 activation. *J. Biol. Chem.* **279**:17765-17771.
186. **Kacimi, R., J. Chentoufi, N. Honbo, C. S. Long, and J. S. Karliner.** 2000. Hypoxia differentially regulates stress proteins in cultured cardiomyocytes: role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. *Cardiovasc. Res.* **46**:139-150.
187. **Kan, Q., S. Jinno, H. Yamamoto, and H. Okayama.** 2007. Chemical DNA damage activates p21 WAF1/CIP1-dependent intra-S checkpoint. *FEBS Lett.* **581**:5879-5884.
188. **Kanakaraj, P., K. Ngo, Y. Wu, A. Angulo, P. Ghazal, C. A. Harris, J. J. Siekierka, P. A. Peterson, and W. P. Fung-Leung.** 1999. Defective interleukin (IL)-18-mediated natural killer and T helper cell type 1 responses in IL-1 receptor-associated kinase (IRAK)-deficient mice. *J Exp. Med.* **189**:1129-1138.
189. **Kanellou, P., A. Zaravinos, M. Zioga, A. Stratigos, S. Baritaki, G. Soufla, O. Zoras, and D. A. Spandidos.** 2008. Genomic instability, mutations and expression analysis of the tumour suppressor genes p14(ARF), p15(INK4b), p16(INK4a) and p53 in actinic keratosis. *Cancer Lett.* **264**:145-161.

190. **Karlsson-Rosenthal, C. and J. B. Millar.** 2006. Cdc25: mechanisms of checkpoint inhibition and recovery. *Trends Cell Biol.* **16**:285-292.
191. **Keller, D., X. Zeng, X. Li, M. Kapoor, M. S. Iordanov, Y. Taya, G. Lozano, B. Magun, and H. Lu.** 1999. The p38MAPK inhibitor SB203580 alleviates ultraviolet-induced phosphorylation at serine 389 but not serine 15 and activation of p53. *Biochem. Biophys. Res. Commun.* **261**:464-471.
192. **Kent, U. M., S. Y. Mao, C. Wofsy, B. Goldstein, S. Ross, and H. Metzger.** 1994. Dynamics of signal transduction after aggregation of cell-surface receptors: studies on the type I receptor for IgE. *Proc. Natl. Acad. Sci U. S. A.* **91**:3087-3091.
193. **Khan, S. A., H. C. Thomas, M. B. Toledano, I. J. Cox, and S. D. Taylor-Robinson.** 2005. p53 Mutations in human cholangiocarcinoma: a review. *Liver Int.* **25**:704-716.
194. **Kim, E. M. and D. J. Burke.** 2008. DNA damage activates the SAC in an ATM/ATR-dependent manner, independently of the kinetochore. *PLoS. Genet.* **4**:e1000015.
195. **Kim, H. J., J. E. Oh, S. W. Kim, Y. J. Chun, and M. Y. Kim.** 2008. Ceramide induces p38 MAPK-dependent apoptosis and Bax translocation via inhibition of Akt in HL-60 cells. *Cancer Lett.* **260**:88-95.
196. **Kim, J., H. Y. Yang, and Y. S. Jang.** 2006. A G protein-associated ERK pathway is involved in LPS-induced proliferation and a PTK-associated p38 MAPK pathway is involved in LPS-induced differentiation in resting B cells. *Mol. Immunol.* **43**:1232-1242.
197. **Kim, J. W., C. G. Lee, S. M. Han, K. S. Kim, J. O. Kim, J. M. Lee, I. K. Kim, and S. E. Namkoong.** 1997. Loss of heterozygosity of the retinoblastoma and p53 genes in primary cervical carcinomas with human papillomavirus infection. *Gynecol. Oncol.* **67**:215-221.
198. **Kim, S. J., S. G. Hwang, D. Y. Shin, S. S. Kang, and J. S. Chun.** 2002. p38 kinase regulates nitric oxide-induced apoptosis of articular chondrocytes by accumulating p53 via NFkappa B-dependent transcription and stabilization by serine 15 phosphorylation. *J Biol. Chem.* **277**:33501-33508.
199. **Kim, S. M., A. Kumagai, J. Lee, and W. G. Dunphy.** 2005. Phosphorylation of Chk1 by ATM- and Rad3-related (ATR) in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP. *J Biol. Chem.* **280**:38355-38364.
200. **Kim, Y. J., M. Song, and J. C. Ryu.** 2009. Inflammation in methotrexate-induced pulmonary toxicity occurs via the p38 MAPK pathway. *Toxicology.* **256**:183-190.

201. **Kishi, H., K. Nakagawa, M. Matsumoto, M. Suga, M. Ando, Y. Taya, and M. Yamaizumi.** 2001. Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20. *J. Biol. Chem.* **276**:39115-39122.
202. **Kmet, L. M., L. S. Cook, and A. M. Magliocco.** 2003. A review of p53 expression and mutation in human benign, low malignant potential, and invasive epithelial ovarian tumors. *Cancer.* **97**:389-404.
203. **Knudson, A.** 2005. Retinoblastoma: teacher of cancer biology and medicine. *PLoS. Med.* **2**:e349.
204. **Knudson, A. G., Jr.** 1971. Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci U. S. A.* **68**:820-823.
205. **Knudson, A. G., Jr., A. T. Meadows, W. W. Nichols, and R. Hill.** 1976. Chromosomal deletion and retinoblastoma. *N. Engl. J Med.* **295**:1120-1123.
206. **Koljonen, V., E. Tukiainen, C. Haglund, and T. Bohling.** 2006. Cell cycle control by p21, p27 and p53 in Merkel cell carcinoma. *Anticancer Res.* **26**:2209-2212.
207. **Koniaras, K., A. R. Cuddihy, H. Christopoulos, A. Hogg, and M. J. O'Connell.** 2001. Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells. *Oncogene.* **20**:7453-7463.
208. **Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias.** 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity.* **10**:387-398.
209. **Koprak, S., M. J. Staruch, and F. J. Dumont.** 1999. A specific inhibitor of the p38 mitogen activated protein kinase affects differentially the production of various cytokines by activated human T cells: dependence on CD28 signaling and preferential inhibition of IL-10 production. *Cell Immunol.* **192**:87-95.
210. **Kotlyarov, A., A. Neininger, C. Schubert, R. Eckert, C. Birchmeier, H. D. Volk, and M. Gaestel.** 1999. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat. Cell Biol.* **1**:94-97.
211. **Koziczak-Holbro, M., C. Joyce, A. Gluck, B. Kinzel, M. Muller, C. Tschopp, J. C. Mathison, C. N. Davis, and H. Gram.** 2007. IRAK-4 kinase activity is required for interleukin-1 (IL-1) receptor- and toll-like receptor 7-mediated signaling and gene expression. *J Biol. Chem.* **282**:13552-13560.
212. **Kramer, A., N. Mailand, C. Lukas, R. G. Syljuasen, C. J. Wilkinson, E. A. Nigg, J. Bartek, and J. Lukas.** 2004. Centrosome-associated Chk1 prevents premature activation of cyclin-B-Cdk1 kinase. *Nat. Cell Biol.* **6**:884-891.

213. **Kramer, D. L., S. Vujcic, P. Diegelman, J. Alderfer, J. T. Miller, J. D. Black, R. J. Bergeron, and C. W. Porter.** 1999. Polyamine analogue induction of the p53-p21WAF1/CIP1-Rb pathway and G1 arrest in human melanoma cells. *Cancer Res.* **59**:1278-1286.
214. **Krasinska, L., E. Besnard, E. Cot, C. Dohet, M. Mechali, J. M. Lemaitre, and D. Fisher.** 2008. Cdk1 and Cdk2 activity levels determine the efficiency of replication origin firing in *Xenopus*. *EMBO J.* **27**:758-769.
215. **Krasinska, L., E. Cot, and D. Fisher.** 2008. Selective chemical inhibition as a tool to study Cdk1 and Cdk2 functions in the cell cycle. *Cell Cycle.* **7**:1702-1708.
216. **Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan.** 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. U. S. A.* **89**:7491-7495.
217. **Kulkarni, A. and K. C. Das.** 2008. Differential roles of ATR and ATM in p53, Chk1, and histone H2AX phosphorylation in response to hyperoxia: ATR-dependent ATM activation. *Am. J Physiol Lung Cell Mol. Physiol.* **294**:L998-L1006.
218. **Kumagai, A., S. M. Kim, and W. G. Dunphy.** 2004. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. *J Biol. Chem.* **279**:49599-49608.
219. **Kurland, J. F. and W. P. Tansey.** 2004. Crashing waves of destruction: the cell cycle and APC(Cdh1) regulation of SCF(Skp2). *Cancer Cell.* **5**:305-306.
220. **Kurosu, T., Y. Takahashi, T. Fukuda, T. Koyama, T. Miki, and O. Miura.** 2005. p38 MAP kinase plays a role in G2 checkpoint activation and inhibits apoptosis of human B cell lymphoma cells treated with etoposide. *Apoptosis.* **10**:1111-1120.
221. **L'Italien, L., M. Tanudji, L. Russell, and X. M. Schebye.** 2006. Unmasking the redundancy between Cdk1 and Cdk2 at G2 phase in human cancer cell lines. *Cell Cycle.* **5**:984-993.
222. **Lane, H. A. and E. A. Nigg.** 1996. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol.* **135**:1701-1713.
223. **Leal, R. B., F. M. Cordova, L. Herd, L. Bobrovskaya, and P. R. Dunkley.** 2002. Lead-stimulated p38MAPK-dependent Hsp27 phosphorylation. *Toxicol. Appl. Pharmacol.* **178**:44-51.
224. **Lebofsky, R. and A. Bensimon.** 2005. DNA replication origin plasticity and perturbed fork progression in human inverted repeats. *Mol. Cell Biol.* **25**:6789-6797.

225. **Lee, J. C. and P. R. Young.** 1996. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J Leukoc. Biol.* **59**:152-157.
226. **Lee, Y. J., H. C. Kuo, C. Y. Chu, C. J. Wang, W. C. Lin, and T. H. Tseng.** 2003. Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. *Biochem. Pharmacol.* **66**:2281-2289.
227. **Lee, Y. M., J. O. Lee, J. H. Jung, J. H. Kim, S. H. Park, J. M. Park, E. K. Kim, P. G. Suh, and H. S. Kim.** 2008. Retinoic acid leads to cytoskeletal rearrangement through AMPK-Rac1 and stimulates glucose uptake through AMPK-p38 MAPK in skeletal muscle cells. *J Biol. Chem.* **283**:33969-33974.
228. **Lei, M.** 2005. The MCM complex: its role in DNA replication and implications for cancer therapy. *Curr. Cancer Drug Targets.* **5**:365-380.
229. **Lejeune, F. J., D. Lienard, M. Matter, and C. Ruegg.** 2006. Efficiency of recombinant human TNF in human cancer therapy. *Cancer Immun.* **6**:6:6.
230. **Lemaire, M., C. Froment, R. Boutros, O. Mondesert, A. R. Nebreda, B. Monsarrat, and B. Ducommun.** 2006. CDC25B phosphorylation by p38 and MK-2. *Cell Cycle.* **5**:1649-1653.
231. **Lepikhin, A. F., O. V. Malinovskii, N. N. Peshin, and T. A. Sheikina.** 1973. [Generation cycle of HeLa Zh-63 cells in synchronized culture. 3. Effect of preliminary synchronization with hydroxyurea and excess of thymidine]. *Tsitologiya.* **15**:226-229.
232. **Levesque, A. A., A. A. Fanous, A. Poh, and A. Eastman.** 2008. Defective p53 signaling in p53 wild-type tumors attenuates p21waf1 induction and cyclin B repression rendering them sensitive to Chk1 inhibitors that abrogate DNA damage-induced S and G2 arrest. *Mol. Cancer Ther.* **7**:252-262.
233. **Li, C., B. Guo, S. Ding, C. Rius, C. Langa, P. Kumar, C. Bernabeu, and S. Kumar.** 2003. TNF alpha down-regulates CD105 expression in vascular endothelial cells: a comparative study with TGF beta 1. *Anticancer Res.* **23**:1189-1196.
234. **Li, H., Y. Wang, and X. Liu.** 2008. Plk1-dependent phosphorylation regulates functions of DNA topoisomerase IIalpha in cell cycle progression. *J Biol. Chem.* **283**:6209-6221.
235. **Li, J., J. A. Deane, N. V. Campanale, J. F. Bertram, and S. D. Ricardo.** 2006. Blockade of p38 mitogen-activated protein kinase and TGF-beta1/Smad signaling pathways rescues bone marrow-derived peritubular capillary endothelial cells in adriamycin-induced nephrosis. *J. Am. Soc. Nephrol.* **17**:2799-2811.
236. **Li, J., E. J. Miller, J. Ninomiya-Tsuji, R. R. Russell, III, and L. H. Young.** 2005. AMP-activated protein kinase activates p38 mitogen-activated protein

- kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. *Circ. Res.* **97**:872-879.
237. **Li, M., J. P. York, and P. Zhang.** 2007. Loss of Cdc20 causes a securin-dependent metaphase arrest in two-cell mouse embryos. *Mol. Cell Biol.* **27**:3481-3488.
238. **Li, Y. P., Y. Chen, J. John, J. Moylan, B. Jin, D. L. Mann, and M. B. Reid.** 2005. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J.* **19**:362-370.
239. **Lim, S. J., Y. J. Lee, and E. Lee.** 2006. p38MAPK inhibitor SB203580 sensitizes human SNU-C4 colon cancer cells to exisulind-induced apoptosis. *Oncol. Rep.* **16**:1131-1135.
240. **Lindon, C. and J. Pines.** 2004. Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. *J Cell Biol.* **19**;164:233-241.
241. **Lindqvist, A., H. Kallstrom, A. Lundgren, E. Barsoum, and C. K. Rosenthal.** 2005. Cdc25B cooperates with Cdc25A to induce mitosis but has a unique role in activating cyclin B1-Cdk1 at the centrosome. *J Cell Biol.* **171**:35-45.
242. **Lindqvist, A., Z. W. van, R. C. Karlsson, and R. M. Wolthuis.** 2007. Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression. *PLoS. Biol.* **5**:e123.
243. **Lioni, M., K. Noma, A. Snyder, A. Klein-Szanto, J. A. Diehl, A. K. Rustgi, M. Herlyn, and K. S. Smalley.** 2008. Bortezomib induces apoptosis in esophageal squamous cell carcinoma cells through activation of the p38 mitogen-activated protein kinase pathway. *Mol. Cancer Ther.* **7**:2866-2875.
244. **Listovsky, T., A. Zor, A. Laronne, and M. Brandeis.** 2000. Cdk1 is essential for mammalian cyclosome/APC regulation. *Exp. Cell Res.* **255**:184-191.
245. **Litvak, V., R. Argov, N. Dahan, S. Ramachandran, R. Amarilio, A. Shainskaya, and S. Lev.** 2004. Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. *Mol. Cell.* **14**:319-330.
246. **Liu, H., Y. R. Han, Y. Zhan, Y. F. Chen, Y. H. Zhang, H. Yu, T. J. Zhao, and Y. Z. Zhuo.** 2007. A dynamical model on the network of p53-Mdm2 feedback loop regulated by p14/19ARF. *Conf. Proc. IEEE Eng Med. Biol. Soc.* **2007**:4235-8.:4235-4238.
247. **Liu, S., H. Mizu, and H. Yamauchi.** 2007. Molecular response to phototoxic stress of UVB-irradiated ketoprofen through arresting cell cycle in G2/M phase and inducing apoptosis. *Biochem. Biophys. Res. Commun.* **364**:650-655.

248. **Lu, M., L. Xiao, and Z. Li.** 2007. The relationship between p38MAPK and apoptosis during paclitaxel resistance of ovarian cancer cells. *J Huazhong. Univ Sci Technolog. Med. Sci.* **27**:725-728.
249. **Lu, X., B. Nannenga, and L. A. Donehower.** 2005. PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes Dev.* **19**:1162-1174.
250. **Macias, E., P. L. Miliani de Marval, S. A. De, C. J. Conti, A. M. Senderowicz, and M. L. Rodriguez-Puebla.** 2008. CDK2 activation in mouse epidermis induces keratinocyte proliferation but does not affect skin tumor development. *Am. J Pathol.* **173**:526-535.
251. **Madge, L. A., M. R. Sierra-Honigmann, and J. S. Pober.** 1999. Apoptosis-inducing agents cause rapid shedding of tumor necrosis factor receptor 1 (TNFR1). A nonpharmacological explanation for inhibition of TNF-mediated activation. *J Biol. Chem.* **274**:13643-13649.
252. **Madge, L. A., M. R. Sierra-Honigmann, and J. S. Pober.** 1999. Apoptosis-inducing agents cause rapid shedding of tumor necrosis factor receptor 1 (TNFR1). A nonpharmacological explanation for inhibition of TNF-mediated activation. *J. Biol. Chem.* **274**:13643-13649.
253. **Maehara, K., K. Yamakoshi, N. Ohtani, Y. Kubo, A. Takahashi, S. Arase, N. Jones, and E. Hara.** 2005. Reduction of total E2F/DP activity induces senescence-like cell cycle arrest in cancer cells lacking functional pRB and p53. *J. Cell Biol.* **168**:553-560.
254. **Manke, I. A., A. Nguyen, D. Lim, M. Q. Stewart, A. E. Elia, and M. B. Yaffe.** 2005. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol. Cell.* **17**:37-48.
255. **Markou, T. and A. Lazou.** 2002. Phosphorylation and activation of mitogen- and stress-activated protein kinase-1 in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires both extracellular-signal-regulated kinase and p38 mitogen-activated protein kinase. *Biochem. J.* **365**:757-763.
256. **Matei, I. R., C. J. Guidos, and J. S. Danska.** 2006. ATM-dependent DNA damage surveillance in T-cell development and leukemogenesis: the DSB connection. *Immunol. Rev.* **209**:142-58.:142-158.
257. **Mateo, M. S., M. Sanchez-Beato, J. C. Martinez, A. Orfao, J. L. Orradre, and M. A. Piris.** 1995. p53, Rb and bcl-2 expression during the cell cycle: a study in phytohaemagglutinin stimulated lymphocytes and microwave irradiated lymphoid tissue sections. *J. Clin. Pathol.* **48**:151-159.
258. **Matsuda, N., H. Tanaka, S. Yamazaki, J. I. Suzuki, K. Tanaka, T. Yamada, and M. Masuda.** 2006. HIV-1 Vpr induces G2 cell cycle arrest in fission yeast

- associated with Rad24/14-3-3-dependent, Chk1/Cds1-independent Wee1 upregulation. *Microbes. Infect.* ..
259. **Matsuoka, S., B. A. Ballif, A. Smogorzewska, E. R. McDonald, III, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi, and S. J. Elledge.** 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science*. **316**:1160-1166.
260. **McDermott, E. P. and L. A. O'Neill.** 2002. Ras participates in the activation of p38 MAPK by interleukin-1 by associating with IRAK, IRAK2, TRAF6, and TAK-1. *J Biol. Chem.* **277**:7808-7815.
261. **Meister, P., A. Taddei, A. Ponti, G. Baldacci, and S. M. Gasser.** 2007. Replication foci dynamics: replication patterns are modulated by S-phase checkpoint kinases in fission yeast. *EMBO J.* **26**:1315-1326.
262. **Meja, K. K., P. M. Seldon, Y. Nasuhara, K. Ito, P. J. Barnes, M. A. Lindsay, and M. A. Giembycz.** 2000. p38 MAP kinase and MKK-1 co-operate in the generation of GM-CSF from LPS-stimulated human monocytes by an NF-kappa B-independent mechanism. *Br. J Pharmacol.* **131**:1143-1153.
263. **Mercer, W. E.** 1992. Cell cycle regulation and the p53 tumor suppressor protein. *Crit Rev. Eukaryot. Gene Expr.* **2**:251-263.
264. **Merrick, K. A., S. Larochelle, C. Zhang, J. J. Allen, K. M. Shokat, and R. P. Fisher.** 2008. Distinct activation pathways confer cyclin-binding specificity on cdk1 and cdk2 in human cells. *Mol. Cell.* **32**:662-672.
265. **Merritt, C., H. Enslin, N. Diehl, D. Conze, R. J. Davis, and M. Rincon.** 2000. Activation of p38 mitogen-activated protein kinase in vivo selectively induces apoptosis of CD8(+) but not CD4(+) T cells. *Mol. Cell Biol.* **20**:936-946.
266. **Michalides, R. J., B. M. van de, and F. Balm.** 2002. Defects in G1-S cell cycle control in head and neck cancer: a review. *Head Neck.* **24**:694-704.
267. **Mikhailov, A., D. Patel, D. J. McCance, and C. L. Rieder.** 2007. The G2 p38-mediated stress-activated checkpoint pathway becomes attenuated in transformed cells. *Curr. Biol.* **17**:2162-2168.
268. **Mikhailov, A., M. Shinohara, and C. L. Rieder.** 2004. Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway. *J Cell Biol.* **166**:517-526.
269. **Mikule, K., B. Delaval, P. Kaldis, A. Jurczyk, P. Hergert, and S. Doxsey.** 2007. Loss of centrosome integrity induces p38-p53-p21-dependent G1-S arrest. *Nat. Cell Biol.* **9**:160-170.
270. **Mirzayans, R., D. Severin, and D. Murray.** 2006. Relationship between DNA double-strand break rejoining and cell survival after exposure to ionizing radiation

- in human fibroblast strains with differing ATM/p53 status: implications for evaluation of clinical radiosensitivity. *Int. J Radiat. Oncol. Biol. Phys.* **66**:1498-1505.
271. **Mishima, K., A. Baba, M. Matsuo, Y. Itoh, and R. Oishi.** 2006. Protective effect of cyclic AMP against cisplatin-induced nephrotoxicity. *Free Radic. Biol. Med.* **40**:1564-1577.
272. **Mitkova, A. V., E. E. Biswas-Fiss, and S. B. Biswas.** 2005. Modulation of DNA synthesis in *Saccharomyces cerevisiae* nuclear extract by DNA polymerases and the origin recognition complex. *J. Biol. Chem.* **280**:6285-6292.
273. **Mitra, J. and G. H. Enders.** 2004. Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. *Oncogene.* **23**:3361-3367.
274. **Mitra, J., G. H. Enders, J. zizkhan-Clifford, and K. L. Lengel.** 2006. Dual regulation of the anaphase promoting complex in human cells by cyclin A-Cdk2 and cyclin A-Cdk1 complexes. *Cell Cycle.* **5**:661-666.
275. **Mocellin, S. and D. Nitti.** 2008. TNF and cancer: the two sides of the coin. *Front Biosci.* **13**:2774-83.:2774-2783.
276. **Mohamood, A. S., P. Gyles, K. V. Balan, V. W. Hollis, W. R. Eckberg, A. Asseffa, Z. Han, J. H. Wyche, and W. A. Anderson.** 1997. Estrogen receptor, growth factor receptor and protooncogene protein activities and possible signal transduction crosstalk in estrogen dependent and independent breast cancer cell lines. *J Submicrosc. Cytol. Pathol.* **29**:1-17.
277. **Moir, R. D., T. P. Spann, R. I. Lopez-Soler, M. Yoon, A. E. Goldman, S. Khuon, and R. D. Goldman.** 2000. Review: the dynamics of the nuclear lamins during the cell cycle-- relationship between structure and function. *J Struct. Biol.* **129**:324-334.
278. **Moolman, J. A., S. Hartley, W. J. Van, E. Marais, and A. Lochner.** 2006. Inhibition of myocardial apoptosis by ischaemic and beta-adrenergic preconditioning is dependent on p38 MAPK. *Cardiovasc. Drugs Ther.* **20**:13-25.
279. **Moon, S. K., S. Y. Jung, and C. H. Kim.** 2004. Transcription factor Sp1 mediates p38MAPK-dependent activation of the p21WAF1 gene promoter in vascular smooth muscle cells by pyrrolidine dithiocarbamate. *Biochem. Biophys. Res. Commun.* **316**:605-611.
280. **Moore, G. D., T. Ayabe, G. S. Kopf, and R. M. Schultz.** 1996. Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. *Mol. Reprod. Dev.* **45**:264-275.
281. **Mordes, D. A., E. A. Nam, and D. Cortez.** 2008. Dpb11 activates the Mec1-Ddc2 complex. *Proc. Natl. Acad. Sci U. S. A.* **105**:18730-18734.

282. **Moro, T., T. Ogasawara, H. Chikuda, T. Ikeda, N. Ogata, Z. Maruyama, T. Komori, K. Hoshi, U. I. Chung, K. Nakamura, H. Okayama, and H. Kawaguchi.** 2005. Inhibition of Cdk6 expression through p38 MAP kinase is involved in differentiation of mouse prechondrocyte ATDC5. *J. Cell Physiol.* **204**:927-933.
283. **Moro, T., T. Ogasawara, H. Chikuda, T. Ikeda, N. Ogata, Z. Maruyama, T. Komori, K. Hoshi, U. I. Chung, K. Nakamura, H. Okayama, and H. Kawaguchi.** 2005. Inhibition of Cdk6 expression through p38 MAP kinase is involved in differentiation of mouse prechondrocyte ATDC5. *J Cell Physiol.* **204**:927-933.
284. **Morris, M. C. and G. Divita.** 1999. Characterization of the interactions between human cdc25C, cdks, cyclins and cdk-cyclin complexes. *J Mol. Biol.* **286**:475-487.
285. **Mu, J. J., Y. Wang, H. Luo, M. Leng, J. Zhang, T. Yang, D. Besusso, S. Y. Jung, and J. Qin.** 2007. A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. *J Biol. Chem.* **282**:17330-17334.
286. **Mueller, H., P. Loop, R. Liu, K. Wosikowski, W. Kueng, and U. Eppenberger.** 1994. Differential signal transduction of epidermal-growth-factor receptors in hormone-dependent and hormone-independent human breast cancer cells. *Eur. J Biochem.* **221**:631-637.
287. **Mulholland, D. J., J. T. Read, P. S. Rennie, M. E. Cox, and C. C. Nelson.** 2003. Functional localization and competition between the androgen receptor and T-cell factor for nuclear beta-catenin: a means for inhibition of the Tcf signaling axis. *Oncogene.* **22**:5602-5613.
288. **Muller, C., M. Sanguinetti, E. Riboulet, L. Hebert, B. Posteraro, G. Fadda, Y. Auffray, and A. Rince.** 2008. Characterization of two signal transduction systems involved in intracellular macrophage survival and environmental stress response in *Enterococcus faecalis*. *J Mol. Microbiol. Biotechnol.* **14**:59-66.
289. **Munro, A. J., S. Lain, and D. P. Lane.** 2005. P53 abnormalities and outcomes in colorectal cancer: a systematic review. *Br. J Cancer.* **92**:434-444.
290. **Muroi, M. and K. Tanamoto.** 2008. TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-kappaB. *J Leukoc. Biol.* **83**:702-707.
291. **Murphy, M.** 1999. Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. *Nat. Genet.* **23**:481.
292. **Nagao, K. and M. Yanagida.** 2006. Securin can have a separate cleavage site by substitution mutations in the domain required for stabilization and inhibition of separase. *Genes Cells.* **11**:247-260.

293. **Nelyudova, A., N. Aksenov, V. Pospelov, and T. Pospelova.** 2007. By blocking apoptosis, Bcl-2 in p38-dependent manner promotes cell cycle arrest and accelerated senescence after DNA damage and serum withdrawal. *Cell Cycle*. **6**:2171-2177.
294. **Neumann, D., C. Kollwe, K. Resch, and M. U. Martin.** 2007. The death domain of IRAK-1: an oligomerization domain mediating interactions with MyD88, Tollip, IRAK-1, and IRAK-4. *Biochem. Biophys. Res. Commun.* **354**:1089-1094.
295. **Nguyen, J., J. Gogusev, P. Knapnougel, and B. Bauvois.** 2006. Protein tyrosine kinase and p38 MAP kinase pathways are involved in stimulation of matrix metalloproteinase-9 by TNF-alpha in human monocytes. *Immunol. Lett.* **106**:34-41.
296. **Nishino, M., Y. Kurasawa, R. Evans, S. H. Lin, B. R. Brinkley, and L. Y. Yu-Lee.** 2006. NudC is required for Plk1 targeting to the kinetochore and chromosome congression. *Curr. Biol.* **16**:1414-1421.
297. **Niwa, K., O. Inanami, T. Ohta, S. Ito, T. Karino, and M. Kuwabara.** 2001. p38 MAPK and Ca²⁺ contribute to hydrogen peroxide-induced increase of permeability in vascular endothelial cells but ERK does not. *Free Radic. Res.* **35**:519-527.
298. **Norrby, K.** 1996. TNF-alpha and de novo mammalian angiogenesis. *Microvasc. Res.* **52**:79-83.
299. **O'Connor, D. J., E. W. Lam, S. Griffin, S. Zhong, L. C. Leighton, S. A. Burbidge, and X. Lu.** 1995. Physical and functional interactions between p53 and cell cycle co-operating transcription factors, E2F1 and DP1. *EMBO J.* **14**:6184-6192.
300. **O'Sullivan, A. W., J. H. Wang, and H. P. Redmond.** 2009. The role of P38 MAPK and PKC in BLP induced TNF-alpha release, apoptosis, and NFkappaB activation in THP-1 monocyte cells. *J Surg. Res.* **151**:138-144.
301. **Obrero, M., D. V. Yu, and D. J. Shapiro.** 2002. Estrogen receptor-dependent and estrogen receptor-independent pathways for tamoxifen and 4-hydroxytamoxifen-induced programmed cell death. *J. Biol. Chem.* **277**:45695-45703.
302. **Ogasa, M., Y. Miyazaki, S. Hiraoka, S. Kitamura, Y. Nagasawa, O. Kishida, T. Miyazaki, T. Kiyohara, Y. Shinomura, and Y. Matsuzawa.** 2003. Gastrin activates nuclear factor kappaB (NFkappaB) through a protein kinase C dependent pathway involving NFkappaB inducing kinase, inhibitor kappaB (IkappaB) kinase, and tumour necrosis factor receptor associated factor 6 (TRAF6) in MKN-28 cells transfected with gastrin receptor. *Gut.* **52**:813-819.

303. **Okajima, E. and U. P. Thorgeirsson.** 2000. Different regulation of vascular endothelial growth factor expression by the ERK and p38 kinase pathways in v-ras, v-raf, and v-myc transformed cells. *Biochem. Biophys. Res. Commun.* **270**:108-111.
304. **Onischenko, E. A., N. V. Gubanova, E. V. Kiseleva, and E. Hallberg.** 2005. Cdk1 and okadaic acid-sensitive phosphatases control assembly of nuclear pore complexes in *Drosophila* embryos. *Mol. Biol. Cell.* **16**:5152-5162.
305. **Oshimori, N., M. Ohsugi, and T. Yamamoto.** 2006. The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nat. Cell Biol.* **8**:1095-1101.
306. **Paciotti, V., M. Clerici, G. Lucchini, and M. P. Longhese.** 2000. The checkpoint protein Ddc2, functionally related to *S. pombe* Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast. *Genes Dev.* **14**:2046-2059.
307. **Paik, J. H., Y. K. Jeon, S. S. Park, Y. A. Kim, J. E. Kim, J. Huh, S. S. Lee, W. H. Kim, and C. W. Kim.** 2005. Expression and prognostic implications of cell cycle regulatory molecules, p16, p21, p27, p14 and p53 in germinal centre and non-germinal centre B-like diffuse large B-cell lymphomas. *Histopathology.* **47**:281-291.
308. **Palermo, C. and N. C. Walworth.** 2005. Assaying cell cycle checkpoints: activity of the protein kinase Chk1. *Methods Mol. Biol.* **296**:345-54.:345-354.
309. **Park, J. H., M. Y. Lee, J. S. Heo, and H. J. Han.** 2008. A potential role of connexin 43 in epidermal growth factor-induced proliferation of mouse embryonic stem cells: involvement of Ca²⁺/PKC, p44/42 and p38 MAPKs pathways. *Cell Prolif.* **41**:786-802.
310. **Park, J. I., C. M. Grant, P. V. Attfield, and I. W. Dawes.** 1997. The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal transduction pathway. *Appl. Environ. Microbiol.* **63**:3818-3824.
311. **Pathak, S. and A. S. Multani.** 2006. Aneuploidy, stem cells and cancer. *EXS*.49-64.
312. **Paulsen, M. T., A. M. Starks, F. A. Derheimer, S. Hanasoge, L. Li, J. E. Dixon, and M. Ljungman.** 2006. The p53-targeting human phosphatase hCdc14A interacts with the Cdk1/cyclin B complex and is differentially expressed in human cancers. *Mol. Cancer.* **19**;5:25.:25.
313. **Pedraza-Alva, G., M. Koulis, C. Charland, T. Thornton, J. L. Clements, M. S. Schlissel, and M. Rincon.** 2006. Activation of p38 MAP kinase by DNA double-strand breaks in V(D)J recombination induces a G2/M cell cycle checkpoint. *EMBO J.* **25**:763-773.

314. **Pelliccioli, A., C. Lucca, G. Liberi, F. Marini, M. Lopes, P. Plevani, A. Romano, P. P. Di Fiore, and M. Foiani.** 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. *EMBO J.* **18**:6561-6572.
315. **Perry, C., E. H. Sklan, and H. Soreq.** 2004. CREB regulates AChE-R-induced proliferation of human glioblastoma cells. *Neoplasia.* **6**:279-286.
316. **Pillaire, M. J., A. R. Nebreda, and J. M. Darbon.** 2000. Cisplatin and UV radiation induce activation of the stress-activated protein kinase p38gamma in human melanoma cells. *Biochem. Biophys. Res. Commun.* **278**:724-728.
317. **Poliseno, L., L. Mariani, P. Collecchi, A. Piras, L. Zaccaro, and G. Rainaldi.** 2002. Bcl2-negative MCF7 cells overexpress p53: implications for the cell cycle and sensitivity to cytotoxic drugs. *Cancer Chemother. Pharmacol.* **50**:127-130.
318. **Porter, L. A. and D. J. Donoghue.** 2003. Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog. Cell Cycle Res.* **5**:335-47.:335-347.
319. **Premack, B. A. and P. Gardner.** 1992. Signal transduction by T-cell receptors: mobilization of Ca and regulation of Ca-dependent effector molecules. *Am. J Physiol.* **263**:C1119-C1140.
320. **Price, M. A., F. H. Cruzalegui, and R. Treisman.** 1996. The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light. *EMBO J.* **15**:6552-6563.
321. **Pruneri, G., N. Carboni, L. Baldini, D. Intini, M. Colombi, F. Bertolini, S. Valentini, P. Maisonneuve, G. Viale, and A. Neri.** 2003. Cell cycle regulators in multiple myeloma: prognostic implications of p53 nuclear accumulation. *Hum. Pathol.* **34**:41-47.
322. **Qian, Y., M. Commane, J. Ninomiya-Tsuji, K. Matsumoto, and X. Li.** 2001. IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. *J Biol. Chem.* **276**:41661-41667.
323. **Quan, H., Y. Xu, and L. Lou.** 2008. p38 MAPK, but not ERK1/2, is critically involved in the cytotoxicity of the novel vascular disrupting agent combretastatin A4. *Int. J Cancer.* **122**:1730-1737.
324. **Raponi, M., R. T. Belly, J. E. Karp, J. E. Lancet, D. Atkins, and Y. Wang.** 2004. Microarray analysis reveals genetic pathways modulated by tipifarnib in acute myeloid leukemia. *BMC. Cancer.* **4**:56.:56.
325. **Rebay, I.** 2002. Keeping the receptor tyrosine kinase signaling pathway in check: lessons from *Drosophila*. *Dev. Biol.* **251**:1-17.
326. **Reiner, A., D. Yekutieli, and Y. Benjamini.** 2003. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics.* **19**:368-375.

327. **Reinhardt, H. C., A. S. Aslanian, J. A. Lees, and M. B. Yaffe.** 2007. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell.* **11**:175-189.
328. **Ricote, M., I. Garcia-Tunon, B. Fraile, C. Fernandez, P. Aller, R. Paniagua, and M. Royuela.** 2006. P38 MAPK protects against TNF-alpha-provoked apoptosis in LNCaP prostatic cancer cells. *Apoptosis.* **11**:1969-1975.
329. **Rivas, M. A., R. P. Carnevale, C. J. Proietti, C. Rosembli, W. Beguelin, M. Salatino, E. H. Charreau, I. Frahm, S. Sapia, P. Brouckaert, P. V. Elizalde, and R. Schillaci.** 2008. TNF alpha acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, Akt and NF-kappa B-dependent pathways. *Exp. Cell Res.* **314**:509-529.
330. **Robinson, N. P. and S. D. Bell.** 2005. Origins of DNA replication in the three domains of life. *FEBS J.* **272**:3757-3766.
331. **Rocha, S., M. D. Garrett, K. J. Campbell, K. Schumm, and N. D. Perkins.** 2005. Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *EMBO J.* **24**:1157-1169.
332. **Rolli-Derkinderen, M. and M. Gaestel.** 2000. p38/SAPK2-dependent gene expression in Jurkat T cells. *Biol. Chem.* **381**:193-198.
333. **Rosales, C., V. O'Brien, L. Kornberg, and R. Juliano.** 1995. Signal transduction by cell adhesion receptors. *Biochim. Biophys. Acta.* **1242**:77-98.
334. **Rother, K., R. Kirschner, K. Sanger, L. Bohlig, J. Mossner, and K. Engeland.** 2006. p53 downregulates expression of the G(1)/S cell cycle phosphatase Cdc25A. *Oncogene.* ..
335. **Roulston, A., C. Reinhard, P. Amiri, and L. T. Williams.** 1998. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J. Biol. Chem.* **273**:10232-10239.
336. **Rozengurt, E.** 1999. Autocrine loops, signal transduction, and cell cycle abnormalities in the molecular biology of lung cancer. *Curr. Opin. Oncol.* **11**:116-122.
337. **Rybaczyk, L., A. Rozmiarek, K. Circle, I. Grants, B. Needleman, J. E. Wunderlich, K. Huang, and F. L. Christofi.** 2009. New bioinformatics approach to analyze gene expressions and signaling pathways reveals unique purine gene dysregulation profiles that distinguish between CD and UC. *Inflamm. Bowel. Dis.*
338. **Ryter, S. W., S. Xi, C. L. Hartsfield, and A. M. Choi.** 2002. Mitogen activated protein kinase (MAPK) pathway regulates heme oxygenase-1 gene expression by hypoxia in vascular cells. *Antioxid. Redox. Signal.* **4**:587-592.

339. **Sakariassen, P. O., L. Prestegarden, J. Wang, K. O. Skaftnesmo, R. Mahesparan, C. Molthoff, P. Sminia, E. Sundlisaeter, A. Misra, B. B. Tynes, M. Chekenya, H. Peters, G. Lende, K. H. Kalland, A. M. Oyan, K. Petersen, I. Jonassen, K. A. van der, B. G. Feuerstein, A. J. Terzis, R. Bjerkvig, and P. O. Enger.** 2006. Angiogenesis-independent tumor growth mediated by stem-like cancer cells. *Proc. Natl. Acad. Sci U. S. A.* **103**:16466-16471.
340. **Salaun, P., Y. Rannou, and C. Prigent.** 2008. Cdk1, Plks, Auroras, and Neks: the mitotic bodyguards. *Adv. Exp. Med. Biol.* **617:41-56**:41-56.
341. **Sanchez, I. and B. D. Dynlacht.** 2005. New insights into cyclins, CDKs, and cell cycle control. *Semin. Cell Dev. Biol.* **16**:311-321.
342. **Santamaria, D. and S. Ortega.** 2006. Cyclins and CDKS in development and cancer: lessons from genetically modified mice. *Front Biosci.* **11:1164-88**:1164-1188.
343. **Santocanale, C. and J. F. Diffley.** 1998. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature.* **395**:615-618.
344. **Satyanarayana, A., M. B. Hilton, and P. Kaldis.** 2008. p21 Inhibits Cdk1 in the absence of Cdk2 to maintain the G1/S phase DNA damage checkpoint. *Mol. Biol. Cell.* **19**:65-77.
345. **Sayama, K., Y. Hanakawa, Y. Shirakata, K. Yamasaki, Y. Sawada, L. Sun, K. Yamanishi, H. Ichijo, and K. Hashimoto.** 2001. Apoptosis signal-regulating kinase 1 (ASK1) is an intracellular inducer of keratinocyte differentiation. *J. Biol. Chem.* **276**:999-1004.
346. **Schafer, K. A.** 1998. The cell cycle: a review. *Vet. Pathol.* **35**:461-478.
347. **Schett, G., M. Tohidast-Akrad, J. S. Smolen, B. J. Schmid, C. W. Steiner, P. Bitzan, P. Zenz, K. Redlich, Q. Xu, and G. Steiner.** 2000. Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. *Arthritis Rheum.* **43**:2501-2512.
348. **Schett, G., J. Zwerina, and G. Firestein.** 2008. The p38 mitogen-activated protein kinase (MAPK) pathway in rheumatoid arthritis. *Ann. Rheum. Dis.* **67**:909-916.
349. **Schmitt, E., M. Beauchemin, and R. Bertrand.** 2007. Nuclear colocalization and interaction between bcl-xL and cdk1(cdc2) during G2/M cell-cycle checkpoint. *Oncogene.* **26**:5851-5865.
350. **Schmitt, E., R. Boutros, C. Froment, B. Monsarrat, B. Ducommun, and C. Dozier.** 2006. CHK1 phosphorylates CDC25B during the cell cycle in the absence of DNA damage. *J. Cell Sci.* **119**:4269-4275.

351. **Schreiber, M., A. Kolbus, F. Piu, A. Szabowski, U. Mohle-Steinlein, J. Tian, M. Karin, P. Angel, and E. F. Wagner.** 1999. Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev.* **13**:607-619.
352. **Schwartz, D. and V. Rotter.** 1998. p53-dependent cell cycle control: response to genotoxic stress. *Semin. Cancer Biol.* **8**:325-336.
353. **Sethi, G., B. Sung, and B. B. Aggarwal.** 2008. TNF: a master switch for inflammation to cancer. *Front Biosci.* **13**:5094-107.:5094-5107.
354. **Seynhaeve, A. L., A. M. Eggermont, and T. L. ten Hagen.** 2008. TNF and manipulation of the tumor cell-stromal interface: "ways to make chemotherapy effective". *Front Biosci.* **13**:3034-45.:3034-3045.
355. **Shaulsky, G., A. Ben-Ze'ev, and V. Rotter.** 1990. Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. *Oncogene.* **5**:1707-1711.
356. **She, Q. B., N. Chen, and Z. Dong.** 2000. ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. *J. Biol. Chem.* **275**:20444-20449.
357. **Shechter, D., C. Y. Ying, and J. Gautier.** 2004. DNA unwinding is an Mcm complex-dependent and ATP hydrolysis-dependent process. *J. Biol. Chem.* **279**:45586-45593.
358. **Shen, J. T. and V. Falanga.** 2003. Growth factors, signal transduction, and cellular responses. *J Dermatol.* **30**:5-16.
359. **Shen, Q. and P. H. Brown.** 2003. Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways. *J Mammary. Gland. Biol. Neoplasia.* **8**:45-73.
360. **Shih, R. S., S. H. Wong, N. W. Schoene, and K. Y. Lei.** 2008. Suppression of Gadd45 alleviates the G2/M blockage and the enhanced phosphorylation of p53 and p38 in zinc supplemented normal human bronchial epithelial cells. *Exp. Biol. Med. (Maywood.).* **233**:317-327.
361. **Shiloh, Y.** 2001. ATM and ATR: networking cellular responses to DNA damage. *Curr. Opin. Genet. Dev.* **11**:71-77.
362. **Shimada, K., M. Nakamura, E. Ishida, M. Kishi, and N. Konishi.** 2003. Roles of p38- and c-jun NH2-terminal kinase-mediated pathways in 2-methoxyestradiol-induced p53 induction and apoptosis. *Carcinogenesis.* **24**:1067-1075.
363. **Shimamura, T., J. Shibata, H. Kurihara, T. Mita, S. Otsuki, T. Sagara, H. Hirai, and Y. Iwasawa.** 2006. Identification of potent 5-pyrimidinyl-2-aminothiazole CDK4, 6 inhibitors with significant selectivity over CDK1, 2, 5, 7, and 9. *Bioorg. Med. Chem. Lett.* **16**:3751-3754.

364. **Shimizu, H., Y. Banno, N. Sumi, T. Naganawa, Y. Kitajima, and Y. Nozawa.** 1999. Activation of p38 mitogen-activated protein kinase and caspases in UVB-induced apoptosis of human keratinocyte HaCaT cells. *J. Invest Dermatol.* **112**:769-774.
365. **Shirayama, M., A. Toth, M. Galova, and K. Nasmyth.** 1999. APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature.* **402**:203-207.
366. **Shiromizu, T., H. Goto, Y. Tomono, J. Bartek, G. Totsukawa, A. Inoko, M. Nakanishi, F. Matsumura, and M. Inagaki.** 2006. Regulation of mitotic function of Chk1 through phosphorylation at novel sites by cyclin-dependent kinase 1 (Cdk1). *Genes Cells.* **11**:477-485.
367. **Shivapurkar, N., J. Reddy, P. M. Chaudhary, and A. F. Gazdar.** 2003. Apoptosis and lung cancer: a review. *J Cell Biochem.* **88**:885-898.
368. **Shteinberg, M., Y. Protopopov, T. Listovsky, M. Brandeis, and A. Hershko.** 1999. Phosphorylation of the cyclosome is required for its stimulation by Fizzy/cdc20. *Biochem. Biophys. Res. Commun.* **260**:193-198.
369. **Singhirunnusorn, P., S. Suzuki, N. Kawasaki, I. Saiki, and H. Sakurai.** 2005. Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. *J. Biol. Chem.* **280**:7359-7368.
370. **Skaar, J. R. and M. Pagano.** 2008. Cdh1: a master G0/G1 regulator. *Nat. Cell Biol.* **10**:755-757.
371. **Smeds, J., P. Berggren, X. Ma, Z. Xu, K. Hemminki, and R. Kumar.** 2002. Genetic status of cell cycle regulators in squamous cell carcinoma of the oesophagus: the CDKN2A (p16(INK4a) and p14(ARF)) and p53 genes are major targets for inactivation. *Carcinogenesis.* **23**:645-655.
372. **Smith, M. L. and A. J. Fornace, Jr.** 1995. Genomic instability and the role of p53 mutations in cancer cells. *Curr. Opin. Oncol.* **7**:69-75.
373. **Smith, N. D., J. N. Rubenstein, S. E. Eggener, and J. M. Kozlowski.** 2003. The p53 tumor suppressor gene and nuclear protein: basic science review and relevance in the management of bladder cancer. *J Urol.* **169**:1219-1228.
374. **Smith, S. J., P. S. Fenwick, A. G. Nicholson, F. Kirschenbaum, T. K. Finney-Hayward, L. S. Higgins, M. A. Giembycz, P. J. Barnes, and L. E. Donnelly.** 2006. Inhibitory effect of p38 mitogen-activated protein kinase inhibitors on cytokine release from human macrophages. *Br. J Pharmacol.* **149**:393-404.
375. **So, K. S., J. E. Oh, J. H. Han, H. K. Jung, Y. S. Lee, S. H. Kim, Y. J. Chun, and M. Y. Kim.** 2008. Induction of apoptosis by a stilbene analog involves Bax translocation regulated by p38 MAPK and Akt. *Arch. Pharm. Res.* **31**:438-444.

376. **Sorrentino, V.** 1989. Growth factors, growth inhibitors and cell cycle control (review). *Anticancer Res.* **9**:1925-1935.
377. **Soto Martinez, J. L., C. M. Cabrera Morales, O. S. Serrano, and M. A. Lopez-Nevot.** 2005. Mutation and homozygous deletion analyses of genes that control the G1/S transition of the cell cycle in skin melanoma: p53, p21, p16 and p15. *Clin. Transl. Oncol.* **7**:156-164.
378. **Squire, C. J., J. M. Dickson, I. Ivanovic, and E. N. Baker.** 2005. Structure and inhibition of the human cell cycle checkpoint kinase, Wee1A kinase: an atypical tyrosine kinase with a key role in CDK1 regulation. *Structure.* **13**:541-550.
379. **Srsen, V., N. Gnadt, A. Dammermann, and A. Merdes.** 2006. Inhibition of centrosome protein assembly leads to p53-dependent exit from the cell cycle. *J. Cell Biol.* **174**:625-630.
380. **Steeenga, W. T., L. T. van, A. Shvarts, C. Terleth, A. J. van der Eb, and A. G. Jochemsen.** 1995. Distinct modulation of p53 activity in transcription and cell-cycle regulation by the large (54 kDa) and small (21 kDa) adenovirus E1B proteins. *Virology.* **212**:543-554.
381. **Steinman, R. A. and D. E. Johnson.** 2000. p21WAF1 prevents down-modulation of the apoptotic inhibitor protein c-IAP1 and inhibits leukemic apoptosis. *Mol. Med.* **6**:736-749.
382. **Stewart, Z. A. and J. A. Pietenpol.** 2001. p53 Signaling and cell cycle checkpoints. *Chem. Res. Toxicol.* **14**:243-263.
383. **Stiff, T., S. A. Walker, K. Cerosaletti, A. A. Goodarzi, E. Petermann, P. Concannon, M. O'Driscoll, and P. A. Jeggo.** 2006. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J.* **25**:5775-5782.
384. **Stokes, M. P., J. Rush, J. Macneill, J. M. Ren, K. Sprott, J. Nardone, V. Yang, S. A. Beausoleil, S. P. Gygi, M. Livingstone, H. Zhang, R. D. Polakiewicz, and M. J. Comb.** 2007. Profiling of UV-induced ATM/ATR signaling pathways. *Proc. Natl. Acad. Sci U. S. A.* **104**:19855-19860.
385. **Storey, J. D. and R. Tibshirani.** 2003. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci U. S. A.* **100**:9440-9445.
386. **Strobl, J. S., W. F. Wonderlin, and D. C. Flynn.** 1995. Mitogenic signal transduction in human breast cancer cells. *Gen. Pharmacol.* **26**:1643-1649.
387. **Stumpff, J., T. Duncan, E. Homola, S. D. Campbell, and T. T. Su.** 2004. Drosophila Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis. *Curr. Biol.* **14**:2143-2148.

388. **Subbaramaiah, K., T. P. Marmo, D. A. Dixon, and A. J. Dannenberg.** 2003. Regulation of cyclooxygenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. *J Biol. Chem.* **278**:37637-37647.
389. **Sudo, T., N. T. Ueno, and H. Saya.** 2004. Functional analysis of APC-Cdh1. *Methods Mol. Biol.* **281:189-98**:189-198.
390. **Sun, M. and M. Fasullo.** 2007. Activation of the budding yeast securin Pds1 but not Rad53 correlates with double-strand break-associated G2/M cell cycle arrest in a *mec1* hypomorphic mutant. *Cell Cycle.* **6**:1896-1902.
391. **Sweeney, F. D., F. Yang, A. Chi, J. Shabanowitz, D. F. Hunt, and D. Durocher.** 2005. *Saccharomyces cerevisiae* Rad9 acts as a Mec1 adaptor to allow Rad53 activation. *Curr. Biol.* **15**:1364-1375.
392. **Syljuasen, R. G., C. S. Sorensen, L. T. Hansen, K. Fugger, C. Lundin, F. Johansson, T. Helleday, M. Sehested, J. Lukas, and J. Bartek.** 2005. Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. *Mol. Cell Biol.* **25**:3553-3562.
393. **Sylvester, J., A. Liacini, W. Q. Li, and M. Zafarullah.** 2004. Interleukin-17 signal transduction pathways implicated in inducing matrix metalloproteinase-3, -13 and aggrecanase-1 genes in articular chondrocytes. *Cell Signal.* **16**:469-476.
394. **Takahashi, T., F. Hato, T. Yamane, H. Fukumasu, K. Suzuki, S. Ogita, Y. Nishizawa, and S. Kitagawa.** 2001. Activation of human neutrophil by cytokine-activated endothelial cells. *Circ. Res.* **88**:422-429.
395. **Takatsuna, H., H. Kato, J. Gohda, T. Akiyama, A. Moriya, Y. Okamoto, Y. Yamagata, M. Otsuka, K. Umezawa, K. Semba, and J. Inoue.** 2003. Identification of TIFA as an adapter protein that links tumor necrosis factor receptor-associated factor 6 (TRAF6) to interleukin-1 (IL-1) receptor-associated kinase-1 (IRAK-1) in IL-1 receptor signaling. *J Biol. Chem.* **278**:12144-12150.
396. **Takekawa, M., M. Adachi, A. Nakahata, I. Nakayama, F. Itoh, H. Tsukuda, Y. Taya, and K. Imai.** 2000. p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. *EMBO J.* **19**:6517-6526.
397. **Takizawa, C. G. and D. O. Morgan.** 2000. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr. Opin. Cell Biol.* **12**:658-665.
398. **Tan, Y., J. Rouse, A. Zhang, S. Cariati, P. Cohen, and M. J. Comb.** 1996. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.* **15**:4629-4642.
399. **Tanaka, K., M. Abe, and Y. Sato.** 1999. Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of

- basic fibroblast growth factor in endothelial cells during angiogenesis. *Jpn. J. Cancer Res.* **90**:647-654.
400. **Tanaka, K., N. Oda, C. Iwasaka, M. Abe, and Y. Sato.** 1998. Induction of Ets-1 in endothelial cells during reendothelialization after denuding injury. *J. Cell Physiol.* **176**:235-244.
401. **Tang, J., X. Yang, and X. Liu.** 2008. Phosphorylation of Plk1 at Ser326 regulates its functions during mitotic progression. *Oncogene.* **27**:6635-6645.
402. **Tanno, M., R. Bassi, D. A. Gorog, A. T. Saurin, J. Jiang, R. J. Heads, J. L. Martin, R. J. Davis, R. A. Flavell, and M. S. Marber.** 2003. Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. *Circ. Res.* **93**:254-261.
403. **Taylor, W. R.** 2004. FACS-based detection of phosphorylated histone H3 for the quantitation of mitotic cells. *Methods Mol. Biol.* **281:293-9**.:293-299.
404. **Thibonnier, M.** 1992. Signal transduction of V1-vascular vasopressin receptors. *Regul. Pept.* **38**:1-11.
405. **Thiefes, A., S. Wolter, J. F. Mushinski, E. Hoffmann, O. ttrich-Breiholz, N. Graue, A. Dorrie, H. Schneider, D. Wirth, B. Luckow, K. Resch, and M. Kracht.** 2005. Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. *J Biol. Chem.* **280**:27728-27741.
406. **Thirunavukkarasu, C., S. C. Watkins, and C. R. Gandhi.** 2006. Mechanisms of endotoxin-induced NO, IL-6, and TNF-alpha production in activated rat hepatic stellate cells: role of p38 MAPK. *Hepatology.* **44**:389-398.
407. **Thuraisingam, T., Y. Z. Xu, J. Moisan, C. Lachance, J. Garnon, M. S. Di, M. Gaestel, and D. Radzioch.** 2007. Distinct role of MAPKAPK-2 in the regulation of TNF gene expression by Toll-like receptor 7 and 9 ligands. *Mol. Immunol.* **44**:3482-3491.
408. **Tian, H., A. T. Faje, S. L. Lee, and T. J. Jorgensen.** 2002. Radiation-induced phosphorylation of Chk1 at S345 is associated with p53-dependent cell cycle arrest pathways. *Neoplasia.* **4**:171-180.
409. **Tindberg, N., M. Porsmyr-Palmertz, and A. Simi.** 2000. Contribution of MAP kinase pathways to the activation of ATF-2 in human neuroblastoma cells. *Neurochem. Res.* **25**:527-531.
410. **Todd, D. E., R. M. Densham, S. A. Molton, K. Balmanno, C. Newson, C. R. Weston, A. P. Garner, L. Scott, and S. J. Cook.** 2004. ERK1/2 and p38 cooperate to induce a p21CIP1-dependent G1 cell cycle arrest. *Oncogene.* **23**:3284-3295.

411. **Tournier, S., D. Leroy, F. Goubin, B. Ducommun, and J. S. Hyams.** 1996. Heterologous expression of the human cyclin-dependent kinase inhibitor p21Cip1 in the fission yeast, *Schizosaccharomyces pombe* reveals a role for PCNA in the chk1+ cell cycle checkpoint pathway. *Mol. Biol. Cell.* **7**:651-662.
412. **Tsichlis, P. N., M. Hatziapostolou, and P. W. Hinds.** 2007. Timing is everything: regulation of Cdk1 and aneuploidy. *Dev. Cell.* **12**:477-479.
413. **Tsuchiya, T., N. H. Tsuno, M. Asakage, J. Yamada, S. Yoneyama, Y. Okaji, S. Sasaki, J. Kitayama, T. Osada, K. Takahashi, and H. Nagawa.** 2008. Apoptosis induction by p38 MAPK inhibitor in human colon cancer cells. *Hepatogastroenterology.* **55**:930-935.
414. **Tsukahara, K., A. Nakao, M. Hiraguri, S. Miike, M. Mamura, Y. Saito, and I. Iwamoto.** 1999. Tumor necrosis factor-alpha mediates antiapoptotic signals partially via p38 MAP kinase activation in human eosinophils. *Int. Arch. Allergy Immunol.* **120 Suppl 1**:54-9.:54-59.
415. **Tsvetkov, L. and D. F. Stern.** 2005. Phosphorylation of Plk1 at S137 and T210 is inhibited in response to DNA damage. *Cell Cycle.* **4**:166-171.
416. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci U. S. A.* **98**:5116-5121.
417. **Tzukerman, M. and K. L. Skorecki.** 2006. A novel experimental platform for investigating cancer growth and anti-cancer therapy in a human tissue microenvironment derived from human embryonic stem cells. *Methods Mol. Biol.* **331**:329-46.:329-346.
418. **Uchiyama-Tanaka, Y., H. Matsubara, Y. Mori, A. Kosaki, N. Kishimoto, K. Amano, S. Higashiyama, and T. Iwasaka.** 2002. Involvement of HB-EGF and EGF receptor transactivation in TGF-beta-mediated fibronectin expression in mesangial cells. *Kidney Int.* **62**:799-808.
419. **Ura, S., N. Masuyama, J. D. Graves, and Y. Gotoh.** 2001. MST1-JNK promotes apoptosis via caspase-dependent and independent pathways. *Genes Cells.* **6**:519-530.
420. **Vaishnav, D., P. Jambal, J. E. Reusch, and S. Pugazhenti.** 2003. SP600125, an inhibitor of c-jun N-terminal kinase, activates CREB by a p38 MAPK-mediated pathway. *Biochem. Biophys. Res. Commun.* **307**:855-860.
421. **van den, H. S. and N. J. Dyson.** 2008. Conserved functions of the pRB and E2F families. *Nat. Rev. Mol. Cell Biol.* **9**:713-724.
422. **van der Wolf, J. M., B. van, Jr., P. M. de Vries, and J. W. van Vuurde.** 1994. Verification of ELISA results by immunomagnetic isolation of antigens from extracts and analysis with SDS-PAGE and western blotting, demonstrated for *Erwinia* spp. in potatoes. *J Appl. Bacteriol.* **77**:160-168.

423. **Vassilev, L. T.** 2006. Cell cycle synchronization at the G2/M phase border by reversible inhibition of CDK1. *Cell Cycle*. **5**:2555-2556.
424. **Vassilev, L. T., C. Tovar, S. Chen, D. Knezevic, X. Zhao, H. Sun, D. C. Heimbrosk, and L. Chen.** 2006. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc. Natl. Acad. Sci U. S. A.* **103**:10660-10665.
425. **Vermeulen, K., D. R. Van Bockstaele, and Z. N. Berneman.** 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* **36**:131-149.
426. **Vitale, I., L. Galluzzi, S. Vivet, L. Nanty, P. Dessen, L. Senovilla, K. A. Olaussen, V. Lazar, M. Prudhomme, R. M. Golsteyn, M. Castedo, and G. Kroemer.** 2007. Inhibition of Chk1 kills tetraploid tumor cells through a p53-dependent pathway. *PLoS. ONE*. **2**:e1337.
427. **Wada, T., E. Stepniak, L. Hui, A. Leibbrandt, T. Katada, H. Nishina, E. F. Wagner, and J. M. Penninger.** 2008. Antagonistic control of cell fates by JNK and p38-MAPK signaling. *Cell Death. Differ.* **15**:89-93.
428. **Wajant, H.** 2009. The Role of TNF in Cancer. *Results Probl. Cell Differ.*
429. **Wang, B., S. Matsuoka, P. B. Carpenter, and S. J. Elledge.** 2002. 53BP1, a mediator of the DNA damage checkpoint. *Science*. **298**:1435-1438.
430. **Wang, G., E. Reed, and Q. Q. Li.** 2004. Apoptosis in prostate cancer: progressive and therapeutic implications (Review). *Int. J Mol. Med.* **14**:23-34.
431. **Wang, Q. and C. M. Doerschuk.** 2001. The p38 mitogen-activated protein kinase mediates cytoskeletal remodeling in pulmonary microvascular endothelial cells upon intracellular adhesion molecule-1 ligation. *J Immunol.* **166**:6877-6884.
432. **Wang, S. and W. S. El-Deiry.** 2006. p73 or p53 directly regulates human p53 transcription to maintain cell cycle checkpoints. *Cancer Res.* **66**:6982-6989.
433. **Wang, S., N. Nath, A. Minden, and S. Chellappan.** 1999. Regulation of Rb and E2F by signal transduction cascades: divergent effects of JNK1 and p38 kinases. *EMBO J.* **18**:1559-1570.
434. **Wang, S. W., J. Pawlowski, S. T. Wathen, S. D. Kinney, H. S. Lichenstein, and C. L. Manthey.** 1999. Cytokine mRNA decay is accelerated by an inhibitor of p38-mitogen-activated protein kinase. *Inflamm. Res.* **48**:533-538.
435. **Wang, W. H., G. Gregori, R. L. Hullinger, and O. M. Andrisani.** 2004. Sustained activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathways by hepatitis B virus X protein mediates apoptosis via induction of Fas/FasL and tumor necrosis factor (TNF) receptor 1/TNF-alpha expression. *Mol. Cell Biol.* **24**:10352-10365.

436. **Wang, X., W. Chen, and Y. Lin.** 2007. Sensitization of TNF-induced cytotoxicity in lung cancer cells by concurrent suppression of the NF-kappaB and Akt pathways. *Biochem. Biophys. Res. Commun.* **355**:807-812.
437. **Wang, X., A. Destrument, and C. Tournier.** 2007. Physiological roles of MKK4 and MKK7: insights from animal models. *Biochim. Biophys. Acta.* **1773**:1349-1357.
438. **Wang, X., J. L. Martindale, Y. Liu, and N. J. Holbrook.** 1998. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem. J.* **333**:291-300.
439. **Wang, X., H. Tokuda, K. Hirade, and O. Kozawa.** 2002. Stress-activated protein kinase/c-Jun N-terminal kinase (JNK) plays a part in endothelin-1-induced vascular endothelial growth factor synthesis in osteoblasts. *J. Cell Biochem.* **87**:417-423.
440. **Wang, X. Q., E. J. Stanbridge, X. Lao, Q. Cai, S. T. Fan, and J. L. Redpath.** 2007. p53-dependent Chk1 phosphorylation is required for maintenance of prolonged G2 Arrest. *Radiat. Res.* **168**:706-715.
441. **Wang, X. Z. and D. Ron.** 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science.* **272**:1347-1349.
442. **Wang, Y., L. Sun, C. Xia, L. Ye, and B. Wang.** 2008. P38MAPK regulates caspase-3 by binding to caspase-3 in nucleus of human hepatoma Bel-7402 cells during anti-Fas antibody- and actinomycin D-induced apoptosis. *Biomed. Pharmacother.*
443. **Wang, Z., Z. J. Zhou, D. P. Liu, and J. D. Huang.** 2008. Double-stranded break can be repaired by single-stranded oligonucleotides via the ATM/ATR pathway in mammalian cells. *Oligonucleotides.* **18**:21-32.
444. **Ward, I. M., K. Minn, and J. Chen.** 2004. UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. *J Biol. Chem.* **279**:9677-9680.
445. **Wasch, R. and F. R. Cross.** 2002. APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. *Nature.* **418**:556-562.
446. **Watanabe, N., T. Sekine, M. Takagi, J. I. Iwasaki, N. Imamoto, H. Kawasaki, and H. Osada.** 2008. Deficiency in chromosome congression by the inhibition of PLK1 polo box domain-dependent recognition. *J Biol. Chem.*
447. **Waters, C., S. Pyne, and N. J. Pyne.** 2004. The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction. *Semin. Cell Dev. Biol.* **15**:309-323.

448. **Westermann, D., S. Rutschow, L. S. Van, A. Linderer, C. Bucker-Gartner, M. Sobirey, A. Riad, M. Pauschinger, H. P. Schultheiss, and C. Tschope.** 2006. Inhibition of p38 mitogen-activated protein kinase attenuates left ventricular dysfunction by mediating pro-inflammatory cardiac cytokine levels in a mouse model of diabetes mellitus. *Diabetologia*. **49**:2507-2513.
449. **Westra, J., M. B. Doornbos-van der, B. P. de, M. A. van Leeuwen, M. H. van Rijswijk, and P. C. Limburg.** 2004. Strong inhibition of TNF-alpha production and inhibition of IL-8 and COX-2 mRNA expression in monocyte-derived macrophages by RWJ 67657, a p38 mitogen-activated protein kinase (MAPK) inhibitor. *Arthritis Res. Ther.* **6**:R384-R392.
450. **Wheatley, S. P., E. H. Hinchcliffe, M. Glotzer, A. A. Hyman, G. Sluder, and Y. Wang.** 1997. CDK1 inactivation regulates anaphase spindle dynamics and cytokinesis in vivo. *J Cell Biol.* **138**:385-393.
451. **Whitmore, G. F. and S. Gulyas.** 1966. Synchronization of mammalian cells with tritiated thymidine. *Science*. **151**:691-694.
452. **Winzen, R., G. Gowrishankar, F. Bollig, N. Redich, K. Resch, and H. Holtmann.** 2004. Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. *Mol. Cell Biol.* **24**:4835-4847.
453. **Wolf, F., R. Sigl, and S. Geley.** 2007. '... The end of the beginning': cdk1 thresholds and exit from mitosis. *Cell Cycle*. **6**:1408-1411.
454. **Won, J. S., Y. B. Im, A. K. Singh, and I. Singh.** 2004. Dual role of cAMP in iNOS expression in glial cells and macrophages is mediated by differential regulation of p38-MAPK/ATF-2 activation and iNOS stability. *Free Radic. Biol. Med.* **37**:1834-1844.
455. **Wong, O. K. and G. Fang.** 2007. Cdk1 phosphorylation of BubR1 controls spindle checkpoint arrest and Plk1-mediated formation of the 3F3/2 epitope. *J Cell Biol.* **179**:611-617.
456. **Woo, R. A. and R. Y. Poon.** 2003. Cyclin-dependent kinases and S phase control in mammalian cells. *Cell Cycle*. **2**:316-324.
457. **Wu, G., J. Luo, J. S. Rana, R. Laham, F. W. Sellke, and J. Li.** 2006. Involvement of COX-2 in VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial cells. *Cardiovasc. Res.* **69**:512-519.
458. **Wu, J. J. and A. M. Bennett.** 2005. Essential role for mitogen-activated protein (MAP) kinase phosphatase-1 in stress-responsive MAP kinase and cell survival signaling. *J. Biol. Chem.* **280**:16461-16466.
459. **Wu, Z. Q., X. Yang, G. Weber, and X. Liu.** 2008. Plk1 phosphorylation of TRF1 is essential for its binding to telomeres. *J Biol. Chem.* **283**:25503-25513.

460. **Xiao, Z., J. Xue, T. J. Sowin, and H. Zhang.** 2006. Differential roles of checkpoint kinase 1, checkpoint kinase 2, and mitogen-activated protein kinase-activated protein kinase 2 in mediating DNA damage-induced cell cycle arrest: implications for cancer therapy. *Mol. Cancer Ther.* **5**:1935-1943.
461. **Xie, J., J. Qian, J. Yang, S. Wang, M. E. Freeman, III, and Q. Yi.** 2005. Critical roles of Raf/MEK/ERK and PI3K/AKT signaling and inactivation of p38 MAP kinase in the differentiation and survival of monocyte-derived immature dendritic cells. *Exp. Hematol.* **33**:564-572.
462. **Xu, L., S. Chen, and R. C. Bergan.** 2006. MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. *Oncogene.* **25**:2987-2998.
463. **Yakymovych, I., U. Engstrom, S. Grimsby, C. H. Heldin, and S. Souchelnytskyi.** 2002. Inhibition of transforming growth factor-beta signaling by low molecular weight compounds interfering with ATP- or substrate-binding sites of the TGF beta type I receptor kinase. *Biochemistry.* **41**:11000-11007.
464. **Yamaguchi, T., H. Goto, T. Yokoyama, H. Sillje, A. Hanisch, A. Uldschmid, Y. Takai, T. Oguri, E. A. Nigg, and M. Inagaki.** 2005. Phosphorylation by Cdk1 induces Plk1-mediated vimentin phosphorylation during mitosis. *J Cell Biol.* **171**:431-436.
465. **Yamamoto, M., M. Yoshida, K. Ono, T. Fujita, N. Ohtani-Fujita, T. Sakai, and T. Nikaido.** 1994. Effect of tumor suppressors on cell cycle-regulatory genes: RB suppresses p34cdc2 expression and normal p53 suppresses cyclin A expression. *Exp. Cell Res.* **210**:94-101.
466. **Yamamoto, Y., E. Shimizu, N. Masuda, M. Takada, and S. Sone.** 1998. RB protein status and chemosensitivity in non-small cell lung cancers. *Oncol. Rep.* **5**:447-451.
467. **Yang, H., T. Burke, J. Dempsey, B. Diaz, E. Collins, J. Toth, R. Beckmann, and X. Ye.** 2005. Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. *FEBS Lett.* **579**:3385-3391.
468. **Yang, J., L. J. Wu, S. Tashiro, S. Onodera, and T. Ikejima.** 2008. Nitric oxide activated by p38 and NF-kappaB facilitates apoptosis and cell cycle arrest under oxidative stress in evodiamine-treated human melanoma A375-S2 cells. *Free Radic. Res.* **42**:1-11.
469. **Yang, S. R., S. D. Cho, N. S. Ahn, J. W. Jung, J. S. Park, E. H. Jo, J. W. Hwang, S. H. Kim, B. H. Lee, K. S. Kang, and Y. S. Lee.** 2005. The role of p38 MAP kinase and c-Jun N-terminal protein kinase signaling in the differentiation and apoptosis of immortalized neural stem cells. *Mutat. Res.* **579**:47-57.

470. **Yarden, R. I., S. Pardo-Reoyo, M. Sgagias, K. H. Cowan, and L. C. Brody.** 2002. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat. Genet.* **30**:285-289.
471. **Yarden, Y. and A. Ullrich.** 1988. Molecular analysis of signal transduction by growth factors. *Biochemistry.* **27**:3113-3119.
472. **Ye, J. and L. Yuan.** 2007. Inhibition of p38 MAPK reduces tumor conditioned medium-induced angiogenesis in co-cultured human umbilical vein endothelial cells and fibroblasts. *Biosci. Biotechnol. Biochem.* **71**:1162-1169.
473. **Yeste-Velasco, M., J. Folch, M. Pallas, and A. Camins.** 2008. The p38(MAPK) signaling pathway regulates neuronal apoptosis through the phosphorylation of the retinoblastoma protein. *Neurochem. Int.*
474. **Yu, C., S. Wang, P. Dent, and S. Grant.** 2001. Sequence-dependent potentiation of paclitaxel-mediated apoptosis in human leukemia cells by inhibitors of the mitogen-activated protein kinase kinase/mitogen-activated protein kinase pathway. *Mol. Pharmacol.* **60**:143-154.
475. **Yudkovsky, Y., M. Shteinberg, T. Listovsky, M. Brandeis, and A. Hershko.** 2000. Phosphorylation of Cdc20/fizzy negatively regulates the mammalian cyclosome/APC in the mitotic checkpoint. *Biochem. Biophys. Res. Commun.* **271**:299-304.
476. **Zarubin, T. and J. Han.** 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* **15**:11-18.
477. **Zechner, D., R. Craig, D. S. Hanford, P. M. McDonough, R. A. Sabbadini, and C. C. Glembotski.** 1998. MKK6 activates myocardial cell NF-kappaB and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. *J Biol. Chem.* **273**:8232-8239.
478. **Zhang, D., W. Ma, Y. H. Li, Y. Hou, S. W. Li, X. Q. Meng, X. F. Sun, Q. Y. Sun, and W. H. Wang.** 2004. Intra-oocyte localization of MAD2 and its relationship with kinetochores, microtubules, and chromosomes in rat oocytes during meiosis. *Biol. Reprod.* **71**:740-748.
479. **Zhao, M., L. New, V. V. Kravchenko, Y. Kato, H. Gram, P. F. Di, E. N. Olson, R. J. Ulevitch, and J. Han.** 1999. Regulation of the MEF2 family of transcription factors by p38. *Mol. Cell Biol.* **19**:21-30.
480. **Zhu, K. Q. and S. J. Zhang.** 2003. Involvement of ATM/ATR-p38 MAPK cascade in MNG induced G1-S arrest. *World J. Gastroenterol.* **9**:2073-2077.
481. **Zhu, T. and P. E. Lobie.** 2000. Janus kinase 2-dependent activation of p38 mitogen-activated protein kinase by growth hormone. Resultant transcriptional activation of ATF-2 and CHOP, cytoskeletal re-organization and mitogenesis. *J Biol. Chem.* **275**:2103-2114.

482. **Zhu, Y., C. Alvarez, R. Doll, H. Kurata, X. M. Schebye, D. Parry, and E. Lees.** 2004. Intra-S-phase checkpoint activation by direct CDK2 inhibition. *Mol. Cell Biol.* **24**:6268-6277.
483. **Zhu, Y., X. O. Mao, Y. Sun, Z. Xia, and D. A. Greenberg.** 2002. p38 Mitogen-activated protein kinase mediates hypoxic regulation of Mdm2 and p53 in neurons. *J. Biol. Chem.* **277**:22909-22914.
484. **Zier, K., B. Gansbacher, and S. Salvadori.** 1996. Preventing abnormalities in signal transduction of T cells in cancer: the promise of cytokine gene therapy. *Immunol. Today.* **17**:39-45.
485. **Zimmerman, S., R. R. Daga, and F. Chang.** 2004. Intra-nuclear microtubules and a mitotic spindle orientation checkpoint. *Nat. Cell Biol.* **6**:1245-1246.
486. **Zusman, I., P. Gurevich, E. Gurevich, and H. Ben-Hur.** 2001. The immune system, apoptosis and apoptosis-related proteins in human ovarian tumors (a review). *Int. J Oncol.* **18**:965-972.