Identification and Characterization of Apoptosis-associated Genes in Hematopoietic Cells using Gene trapping and Microarray Technologies

Dissertation

A thesis submitted in fulfilment of the

requirements for the degree of

Doctor of Philosophy

Vorgelegt beim Fachbereich Biologie
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
Ji Yeon Yang
aus Seoul, Republic of Korea

Frankfurt am Main, 2003

SUPERVISED BY

Professor Harald von Melchner, M.D., Ph.D.
Department of Molecular Hematology
Internal Medicine III
Johann Wolfgang Goethe University

Frank Wempe, Ph.D.
Department of Molecular Hematology
Internal Medicine III
Johann Wolfgang Goethe University

REVIEWED BY

Professor Anna Starzinski-Powitz, Ph.D.
Department of Biology of Human Genetics
Johann Wolfgang Goethe University

OFFICIAL OPPONENT

_____

_____

Nature first issue, Nov. 4, 1869

NATURE: APHORISMS BY GOETHE

NATURE! We are surrounded and embraced by her: powerless to separate ourselves from her, and powerless to penetrate beyond her. Without asking, or warning, she snatches us up into her circling dance, and whirls us on until we are tired, and drop from her arms. She is ever shaping new forms: what is, has never yet been; what has been, comes not again. Everything is new, and yet nought but the old. We live in her midst and know her not. She is incessantly speaking to us, but betrays not her secret. We constantly act upon her, and yet have no power over her. The one thing she seems to aim at is Individuality; yet she cares nothing for individuals. She is always building up and destroying; but her workshop is inaccessible. Her life is in her children; but where is the mother? She is the only artist; working-up the most uniform material into utter opposites; arriving, without a trace of effort, at perfection, at the most exact precision, though always veiled under a certain softness. Each of her works has an essence of its own; each of her phenomena a special characterisation: and yet their diversity is in unity. She performs a play; we know not whether she sees it herself, and yet she acts for us, the lookers-on. Incessant life, development, and movement are in her, but she advances not. She changes for ever and ever, and rests not a moment. Quietude is inconceivable to her, and she has laid her curse upon rest. She is firm. Her steps are measured, her exceptions rare, her laws unchangeable. She has always thought and always thinks; though not as a man, but as Nature. She broods over an all-comprehending idea, which no searching can find out. rejoices the more they win. With many, her moves are so hidden, that the game is over before they know it. That which is most unnatural is still Nature; the stupidest philistinism has a touch of her genius. Whoso cannot see her everywhere, sees her nowhere rightly. She loves herself, and her innumberable eyes and affections are fixed upon herself. She has divided herself that she may be her own delight. She causes an endless succession of new capacities for enjoyment to spring up, that her insatiable sympathy may be assuaged. She rejoices in illusion. Whoso destroys it in himself and others, him she punishes with the sternest tyranny. Whoso follows her in faith, him she takes as a child to her bosom. Her children are numberless. To none is she altogether miserly; but she has her favourites, on whom she squanders much, and for whom she makes great sacrifices. Over greatness she spreads her shield. She tosses her creatures out of nothingness, and tells them not whence they came, nor whither they go. It is their business to run, she knows the road. Her mechanism has few spring, but they never wear out, are always active and manifold.

The spectacle of Nature is always new, for she is always renewing the spectators. Life is her most exquisite invention; and death is her expert contrivance to get plenty of life. She wraps man in darkness, and makes him forever long for light. She creates him dependent upon the earth, dull and heavy; and yet is always shaking him until he attempts to soar above it. She creates needs because she loves action. Wondrous! That she produces all this action so easily. Every need is a benefit, swiftly satisfied, swiftly renewed. —Every fresh want is a new source of pleasure, but she soon reaches an equilibrium. Every instant she commences an immense journey, and every instant she has reached her goal. She is vanity of vanities; but not to us, to whom she has made herself of the greatest importance. She allows every child to play tricks with her; every fool to have judgment upon her; thousands to walk stupidly over her and see nothing; and takes her pleasure and finds her account in them all. We obey her laws even when we rebel against them; we work with her even when we desire to work against her. She makes every gift a benefit by causing us to want it. She delays, that we may desire her; she hastens, that we may not weary of her. She has neither language nor discourse; but she creates tongues and hearts, by which she feels and speaks. Her crown is love. Through love alone dare we come near her. She separates all existences, and all tend to intermingle. She has isolated all things in order that all may approach one another. She holds a couple of draughts from the cup of love to be fair payment for the pains of a lifetime. She is all things. She rewards herself and punishes herself; is her own joy and her own misery. She is rough and tender, lovely and hateful, powerless and omnipotent. She is an eternal present. Past and future are unknown to her. The present is her eternity. She is beneficent. I praise her and all her works. She is silent and wise. No explanation is wrung from her; no present won from her, which she does not give freely. She is cunning, but for good ends; and it is best not to notice her tricks. She is complete, but never finished. As she works now, so can she always work. Everyone sees her in his own fashion. She hides under a thousand names and phrases, and is always the same. She has brought me here and will also lead me away. I trust her. She may scold me, but she will not hate her work. It was not I who spoke of her. No! What is false and what is true, she has spoken it all. The fault, the merit, is all hers.

Translated by T. H. HUXLEY (Original script is in Appendix IX)

Molekulare Hematologie, Haus 23B Labor EI45, Klinikum J.W. Goethe-Universität, Theodor-Stern-Kai7, 60590 Frankfurt.

Declaration

The work described in this thesis is original and has not previously been submitted for a degree or diploma in any other University or College, and to the best of my knowledge, does not contain material previously published or presented by another person, except where due reference is made in the text.

Ji Yeon Yang

Acknowledgments

The present thesis was carried out at the laboratory of Prof. Harald von Melchner, M.D., Ph.D. during the years 1999-2002 in Molecular Hematology, Internal Medicine III, Johann Wolfgang Goethe University. This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG) and Schering AG, Berlin.

The most important person for my career development is Prof. Dr. Anna Starzinski-Powitz in the Department of Biology of Human Genetics, Johann Wolfgang Goethe University. I would like to thank her for providing me the opportunity to study as Ph.D. student. My warm thanks go to my supervisor Prof. H. von Melchner, who kindly took me in as part of his group and always provided me with excellent research facilities to do scientific work. Especially, his pinpointing advice was always so important for the progress of my works. My grateful thanks go to Dr Frank Wempe whose supervision all through the years of this study was invaluable. I would like to thank him again for providing constant guidance, contribution, encouragement, and for promoting tremendous professional growth during my graduate studies. It is hard for me to find proper and enough words for all his guidance and for making this journey of mine a pleasurable and an unforgettable one.

Without the altruistic help of a wonderful band of my lab colleagues, I could not have accomplish this study. I am genuinely grateful to these people. Especially I want to extend my gratitude to Doris-Maria Viel with various matters. I deeply appreciate Dr Shenchu Jin and Dr Joachim Altschmied for their help of correcting the manuscript.

Finally, I would like to thank my parents, Chang Se Yang and late mother Kyung Ae Seo who passed away last year on Jun. 30 after a hard struggle with cancer. They have always been behind of me and have inspired me to pursue my studies. I owe my most deepest and loving thanks to my lovely wife So-Hyun, my two daughters Shin-Myung and Su-Jin. Without their continuous love and warm support, the making of this thesis would have been much more difficult. I know that I am privileged when having the chance to share my life and so many unforgettable moments with them. They have given me enormous energy to deal with both daily routine and pleasure. I am sorry for those numerous lonely evenings and weekends I had to leave them alone because of my intense work. It is my great pleasure to dedicate this thesis to my family.

Ji-Yeon Yang

Abbreviations

aa:	amino acid
A_{260}; A_{280}:	absorbance at the wavelength 260 or 280 nm
BLAST:	Basic Local Alignment Research Tool
bp:	base pairs
BSA:	bovine serum albumin
°C:	degrees Celsius
cDNA:	complementary DNA obtained by reverse transcription of RNA
Cre:	causes recombination
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytidine triphosphate
DEPC:	diethylpyrocarbonate
DMEM:	Dulbecco's modified Eagle's medium with phenol red
DMSO:	dimethylsulphoxide
DNA:	deoxyribonucleic acid
DNase:	deoxyribonuclease
dNTP:	deoxynucleoside 5'-triphosphate
dsDNA:	double stranded DNA
DTT:	dithiothreitol
E. coli:	*Escherichia coli*
EDTA:	ethylenediamine tetra-acetic acid
EMBL:	European Molecular Biology Laboratory
ERK:	extracellular regulated kinase
ES:	embryonic stem (cells)
EST:	expressed sequence tag
EtBr:	ethidium bromide
EtOH:	ethanol
FCS:	fetal calf serum
FDCP:	factor dependent cell-Paterson (Paterson Insitute, UK)
FPLC:	fast protein liquid chromatography
g:	gravity (force)
GAPDH:	glyceraldehyde phosphate dehydrogenase
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GTSTs:	gene trap sequence tags
h:	hour
HEPES:	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
IL-3:	Interleukin-3
IMDM:	Iscove's modified Dulbecco's medium
IPTG:	isopropyl β-D-thiogalacto pyranoside

J. W. Goethe:	Johann Wolfgang Goethe
kb:	kilobase pairs
kDa:	kilodaltons
LB:	Luria-Bertani medium
*lox*P:	locus of *x*-ing over *P*1
LMW:	low molecular weight
LTR:	long terminal repeat
MAPK:	mitogen activated protein kinase
µCi:	microcurie
MEK:	mitogen/extracellularly regulated kinase kinase
min:	minutes
mol. wt.:	molecular weight
MoMuLV:	Moloney murine leukemia virus (also: MMLV)
MOPS:	3-(N-morpholino) propanesulphonic acid
mRNA:	messenger ribonucleic acid
NF-κB:	nuclear factor kappa beta
nt:	DNA nucleotide(s)
oligo(dT):	oligodeoxythymidylic acid
ORF:	open reading frame
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PGK:	phosphoglycerate kinase
pgk:	phosphoglycerate kinase promoter
poly(A)$^+$-RNA:	polyadenylated RNA (mRNA)
RACE:	rapid amplification of cDNA ends
RNA:	ribonucleic acid
RNase:	ribonuclease
RPMI 1640:	Roswell Park Memorial Institute medium 1640
RT:	reverse transcription (or transcriptase)
RT-PCR:	reverse transcription-polymerase chain reaction
SDS:	sodium dodecyl sulfate
ssDNA:	single stranded DNA
TAE:	Tris/acetate/EDTA (buffer)
Taq:	*Thermus aquaticus* DNA polymerase
TE:	Tris/EDTA buffer
T_m:	melting (or midpoint) temperature; thermal denaturation
Tris:	tris-(hydroxymethyl)-aminomethane
UV:	ultraviolet
w/v:	weight/volume
X-gal:	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Abstract

A gene trap strategy was used to identify genes induced in hematopoietic cells undergoing apoptosis by growth factor withdrawal. IL-3 dependent survival of hematopoietic cells relies on a delicate balance between proliferation and apoptosis that is controlled by the availability of cytokines (Thompson, 1995; Iijima *et al.*, 2002). From our previous results of gene trap assay, we postulated that transcriptionally activated antagonistic genes against apoptosis might actually block or delay cell death (Wempe *et al.*, 2001) causing cells to have carcinogenic behavior. The analysis attempted to better understand the outcome of a death program following IL-3 deprivation and to identify those survival genes whose expression is affected by time dependent manner.

As described in the chapter 4, there would be two major conclusions evident from the three separate experiments (Genetrap, Atlas cDNA array and Affymetrix chips): Firstly 56% of trapped genes, that are up-regulated by IL-3 withdrawal (28 of 50), are directly related to cell death or survival. Secondly, unlike most array technologies, gene trapping only selects for the transiently induced genes that is independent of pre-existing steady state mRNA levels. In regarding correlations of the genes with potential carcinogenesis, the pre-existing mRNA makes difficult to describe the unique characteristics of deregulated tumor tissue genes.

For a joint project with Schering (Schering AG, Berlin), the genes of our GTSTs were examined. The first screen with custom array was used to look for whether the survival genes of our GTSTs are involved in various cancer cell lines, whilst the second screen with Matched Tumor/Normal Array was used to characterize if the selected seven genes (ERK3, Plekha2, KIAA1140, PI4P5Kα/γ, KIAA0740, KIAA1036 and PEST domains) are transformation-related genes or not in different tumor tissues.

Twenty-six genes were identified as either induced or repressed in one or more cell lines. Genetic information is expressed in complex and ever changing patterns throughout a life span of cells. A description of these patterns and how they relate to the

tissue specific cancer is crucial for our understanding of the network of genetic interactions that underlie the processes of normal development, disease and evolution. The development of cancer and its progression is clearly a multiplex phenotype, as a function of time, involving dozens of primary genes and hundreds of secondary modifier genes.

There would be a major conclusion evident from the three separate experiments (Genetrap, Affymetrix mouse chip and Matched Tumor/Normal Array): ERK3 could play a significant role in breast, stomach and uterus carcinogenesis with tissue specific regulations. It is clear that ERK3 is obvious putative survival gene in these tumor tissues. Especially, in breast tumors, seven times up-regulation was considerable and the activation of ERK3 could be a feature of breast tumors. My results imply that the unique deregulation of ERK3 is perhaps the major consequence of possible transformation of normal cells into malignant cancer cells, even though further analysis remains to be determined whether an alterated activity of associated survival genes is primarily responsible for a carcinogenesis. However unlike all the other known MAP Kinases, no stimuli and no nuclear substrates of ERK3 is reported. Therefore, it will be necessary first to determine the spectrum of substrates and to identify the proximal effectors for the ERK3 in breast carcinoma cells.

Publications

Wempe F, **Yang JY**, Hammann J, von Melchner H. (2001) Gene trapping identifies transiently induced survival genes during programmed cell death. *Genome Biol.* **2**(7): 0023.1-0023.10.

Hamdan S, Bulloch EM, Thompson PR, Beck JL, **Yang JY**, Crowther JA, Lilley PE, Carr PD, Ollis DL, Brown SE, Dixon NE (2002) On the Mechanism of Hydrolysis of the 5'-4-Nitrophenyl Ester of TMP by the Proofreading Exonuclease (ε) Subunit of *Escherichia coli* DNA Polymerase III. *Biochemistry* **41**(16): 5266-5275.

Keniry MA, Berthon HA, **Yang JY**, Miles CS and Dixon NE (2000) NMR solution structure of the θ subunit of DNA polymerase III from *Escherichia coli. Protein Science* **9**:721–733.

Hamdan S, Brown SE, Thompson PR, **Yang JY**, Carr PD, Ollis DL, Otting G, Dixon NE (2000) Preliminary X-Ray crystallographic and NMR studies on the exonuclease domain of the epsilon subunit of *Escherichia coli* DNA polymerase III. *J. Struct. Biol.* **131**(2): 164-169.

Contents

FIGURES AND TABLES

List of Figures

List of Tables

CHAPTER 1

CHAPTER 1

INTRODUCTION

1.1 Background

Recently, the nucleotide sequencing of the entire human genome was completed (McPherson *et al*., 2001; Venter *et al.*, 2001; Claverie, 2001). The total genomic sequences of other organisms (such as *E. coli* and many other bacteria, yeast, *Arabidopsis, Caenorhabditis, Drosophila,* zebrafish and mouse) are determined. However, this achievement is just the beginning of the functional understanding of the genome of humans and other organisms (Smaglik, 2000; Roest Crollius *et al*., 2000; Sachidanandam *et al.*, 2001). Adequate expression or functional information is available for only ~15% of them (Stanford *et al*., 2001). The coding sequences of unknown genes can be identified by computational methods such as alignment of Expressed Sequence Tags (ESTs) with other genomes. Following sequencing, the next step must be to assign functions to the identified genes.

Gene-trap mutagenesis is a technique that randomly generates loss-of-function mutations and reports the expression of many mouse genes. Gene-trapping methodology (Section 1.4) is a powerful strategy for cloning and identifying functional genes, as it marks a gene with a tag and simultaneously generates a corresponding genetic variation at that particular locus.

For this reason, gene trapping has become a favorable standard tool for functional genomics, establishing a correlation between the physical and genetic maps of the genome (Thompson *et al.,* 1995; Russ *et al*., 1996b; Evans *et al*., 1997; Thorey *et al*., 1998; Stanford *et al*., 2001). Gene trapping studies using interleukin-3 (IL-3) dependent cell lines have begun to shed light on the transcriptional regulation of apoptosis. A mouse bone marrow-derived hematopoietic cell line, FDCP-1, (Sections 1.3 and 2.1.2) was employed as our model system.

1.2 Apoptosis

Programmed cell death (PCD) is a tightly controlled process of cellular self-destruction at predictable time and places during development (Steller *et al*., 1995). The decision to die cannot be taken lightly, and the activities of many genes influence a cell's likelihood of activating its self-destruction program. Once the decision is taken, proper execution of the apoptotic program requires the coordinated activation and execution of multiple subprograms.

Here I review the basic components of the death machinery, describe how they interact to regulate apoptosis in a coordinated manner, and discuss the main pathways that are used to activate cell death. Apoptosis is a kind of death that is best described as cellular suicide and it is important for normal tissue homeostasis. The term apoptosis was coined by Kerr (Kerr *et al*., 1972; Table 1.1) to describe a morphologically distinguished form of cell death compared to necrosis. In necrosis, uncontrolled cell death leads to lysis of cells, inflammatory responses and potentially to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why it is often referred to as cell suicide, Figure 1.1).

Three major pathways have been elucidated so far (Gross *et al*., 1999; Yin, 2000) which all result in the activation of caspase-3, a cysteine proteinase that cleaves substrates after aspartic acid residues (Figure 1.2). One is the Type I (mitochondria-independent, Bcl-2-insensitive) pathway. The pathway involves the binding of Fas/TNF-R1 death receptor signals to cell surface receptors, which are members of the tumor necrosis factor receptor (TNF-R) superfamily that activate different cellular processes depending on cell type and environmental context.

Stimulation of apoptosis via proteolytic cleavage results in a cascade of caspase activation within the cell. The caspases comprise the key effectors of the apoptotic pathway and are expressed in all living cells in inactive forms. Following interactions with caspase activator proteins, they become active and cell death quickly

ensues. Finally, granzyme B (a cytolytic T-cell product) directly cleaves and activates several caspases, resulting in apoptosis.

Table 1.1: Features of apoptosis vs necrosis (Kerr *et al*., 1972).

Apoptosis	Necrosis
Chromatin condensation	Nuclear swelling
Cell shrinkage	Cell swelling
Preservation of organelles and cell membranes	Disruption of organelles
Rapid engulfment by neighboring cells	Rupture of cell and release of cellular contents
DNA fragmentation	Inflammatory response

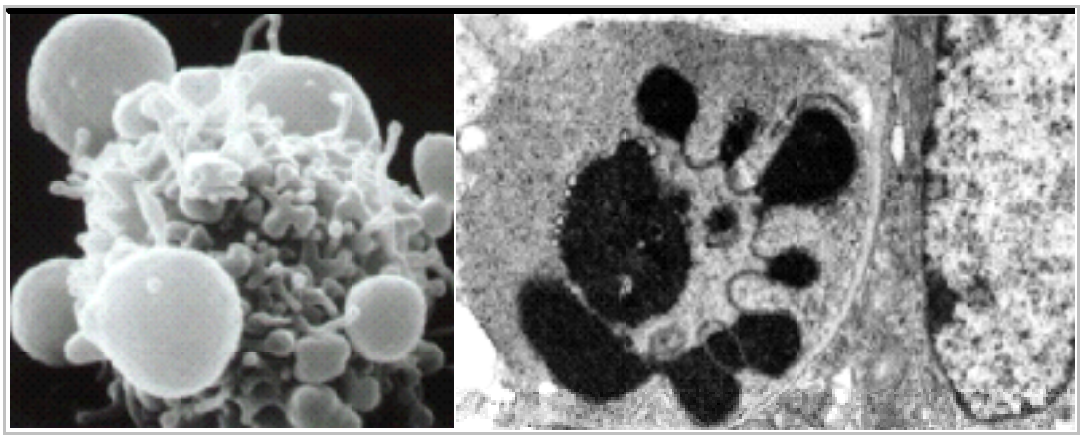


Figure 1.1: Cells dying by apoptosis. The electron micrograph shows morphological changes of nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum and membrane blebbing in an apoptotic mammary gland cell. (Courtesy of Dr David Ferguson)

The other is the mitochondrial pathway (mitochondria-dependent, Bcl-2-sensitive) pathway, largely mediated through Bcl-2 family members, which results in release of cytochrome *c* (cyt *c*), activation of apoptotic protease activating factor-1 (Apaf-1), and caspase-9 form a complex known as the apoptosome (Figure 1.2). This results in the formation of a wheel-like structure that contains 7 molecules each of Apaf-1, cyt *c* and ATP. This wheel-like structure, the apoptosome, permits the recruitment of 7 molecules of procaspase-9 to the complex (Figure 1.5). Recently, however, mitochondria was also suggested as an integrator in tumorigenesis

(Augenlicht and Heerdt, 2001). The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

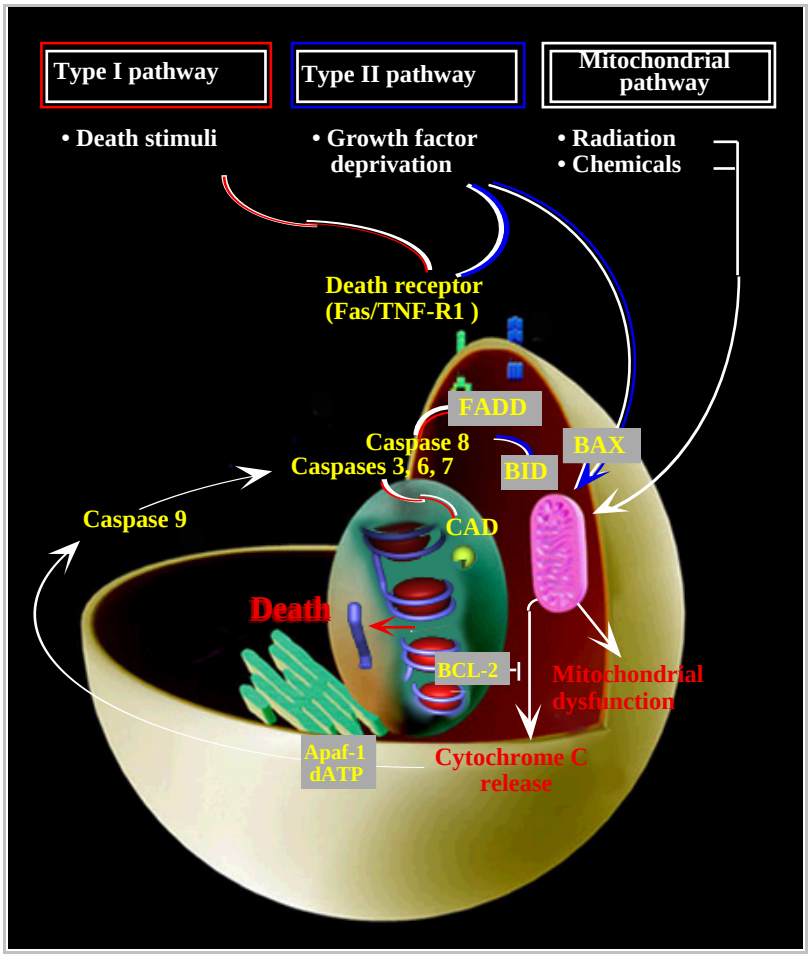


Figure 1.2: Three major pathways of apoptosis. Type I (mitochondria-independent, Bcl-2-insensitive) pathway, mitochondria-dependent (Bcl-2-sensitive) pathway and Type II (which shares Type I and mitochondria-dependent pathways).

The result of these biochemical changes is appearance of morphological changes in the cell, such as nuclear chromatin condensation (Wyllie *et al*., 1984), cytoplasmic shrinking, dilated endoplasmic reticulum and membrane blebbing (Figure 1.1). Several protein families (Sections 1.2.2 and 1.2.3) have been identified whose members are the products of constitutively expressed genes that stimulate apoptosis (Oltvai *et al*., 1994; White *et al*., 1996). Apoptotic cell death can be triggered by a wide variety of stimuli. One of the well documented stimuli is DNA damage (Decker and Muller, 2002), caused by irradiation (Zhan *et al*., 2002) or by

drugs used for cancer chemotherapy, which leads to apoptotic death via a p53-dependent pathway (Zhu *et al*., 1994). However, in many cases, apoptosis involves p53-independent as well as p53-dependent pathways (Pan and Griep, 1995; Blandino *et al.*, 1995; JacksonGrusby *et al*., 2001). The p53-dependent pathway may involve activation of caspases 1, 11, and 12, whereas the p53-independent pathway may involve activation of members of the Bcl-2 and death receptor families (Choi *et al*., 2001). There are a number of mechanisms through which apoptosis can be induced in cells (McKinstry *et al.,* 1997). The sensitivity of cells to any of these stimuli can vary depending on several of factors such as the expression of pro- and anti-apoptotic proteins, the severity of the stimulus and the stage of the cell cycle. Some of the major stimuli that can induce apoptosis are described in the following Sections.

1.2.1 *Death receptors*

The death receptor pathway is initiated at the cell surface through the Fas/TNF-R1 (tumor necrosis factor receptor-1) family proteins. Fas (also CD95 or APO-1, Ashkenazi and Dixit, 1998) induces apoptosis upon stimulation with Fas ligand (FasL, also CD95L or APO-1L). Fas is a type I transmembrane protein that has multiple cysteine-rich repeats in the extracellular domain and an intracellular motif termed a death domain (DD). They play an important role in very rapid apoptosis and can activate a caspase cascade within seconds of ligand binding (Ashkenazi and Dixit, 1998; Dhein *et al*., 1994; Miyake *et al*., 2003).

Binding of FasL to the Fas extracellular domain induces trimerization of Fas, resulting in the recruitment of the Fas-associated death domain (FADD), which then recruits pro-caspase 8 (Chinnaiyan *et al.*, 1996). FADD has a carboxy-terminal DD and an amino-terminal death effector domain (DED). FLICE (FADD-like interleukine-1β-converting enzyme) is a key molecule in Fas-induced apoptosis that recruits the DED-containing apoptosis-initiating proteases caspase-8 (Muzio *et al.*, 1996) and caspase-10 (Kischkel *et al.*, 2001; Wang *et al.*, 2001) to the Fas receptor to assemble a DISC (Kischkel *et al.*, 1995). Activation of pro-caspase 8 through self-

cleavage leads to a series of downstream events, including activation of pro-caspase 3 (Thornberry *et al.*, 1997), which subsequently cleaves its substrates such as DNA fragmentation factor 45 (DFF45, Liu *et al.*, 1997), to execute programmed cell death.

In a similar fashion, binding of TNF to TNF-R1 results in the trimerization of the receptor and recruitment of TRADD, which is followed by a similar reaction seen in the activation of Fas receptors (Figure 1.3). The known difference is that TRADD also recruits other molecules, such as TRAF2, which actually activates a protective pathway through the transcription factors NF-κB. Thus in many types of cells, TNF induces cell death only in the presence of transcriptional inhibitors. The best characterised of the death receptor is TNF-R1 (Figure 1.3).

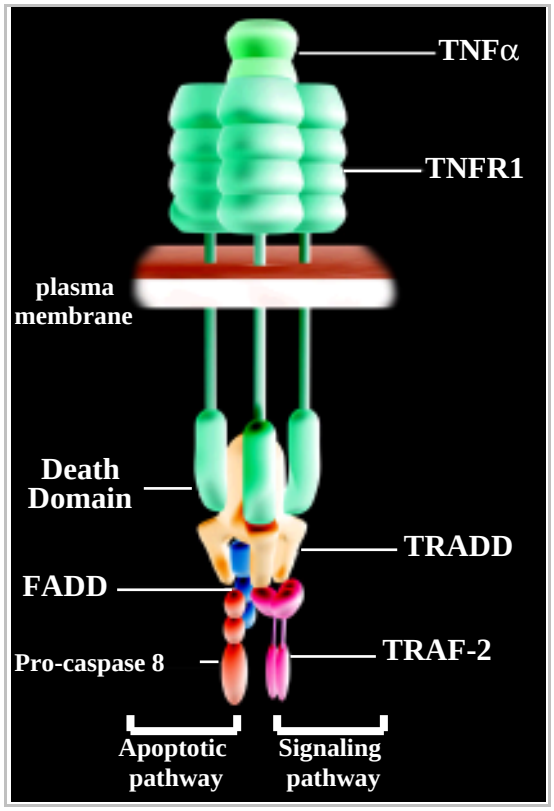


Figure 1.3: TNF receptor signaling. Binding of TNF-α to TNF-R1 results in receptor trimerisation and clustering of intracellular death domains. This allows binding of an intracellular adapter molecule called TRADD (TNF-R-associated death domain) via interactions between death domains. TRADD has the ability to recruit a number of different proteins to the activated receptor. Recruitment of TRAF2 (TNF-associated factor 2) leads to activation of NF-κB and the JNK/Apaf-1 pathway.

1.2.2 *Bcl-2 protein family*

The Bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as Bcl-2, Bcl-X_L and Mcl-1) are anti-apoptotic, while others (such as Bad, Bax and Bak) are pro-apoptotic (Gross *et al.*, 1999, Figure 1.4).

The BH1 and BH2 domains of Bcl-2 are required to heterodimerize with Bax and to repress cell death; conversely, the BH3 domain (Wang *et al*., 1996) of Bax is required to heterodimerize with Bcl-2 and to promote cell death. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis; when there is an excess of anti-apoptotic proteins the cells will tend to be less sensitive. The pro-apoptotic Bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located.

This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins and can lead to the formation of pores in the mitochondrial membrane and the release of cyt *c* and other pro-apoptotic molecules from the intermembrane space (Figure 1.5).

1.2.3 *Caspase protein family*

Caspases are a family of proteins that contains some of the main effectors of apoptosis (Salvesen and Dixit, 1997). They are a group of cysteine proteases that exist within the cell as inactive pro-forms or zymogens (Donepudi and Grutter, 2002). These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10, which can then activate other caspases in a cascade (Donepudi and Grutter, 2002).

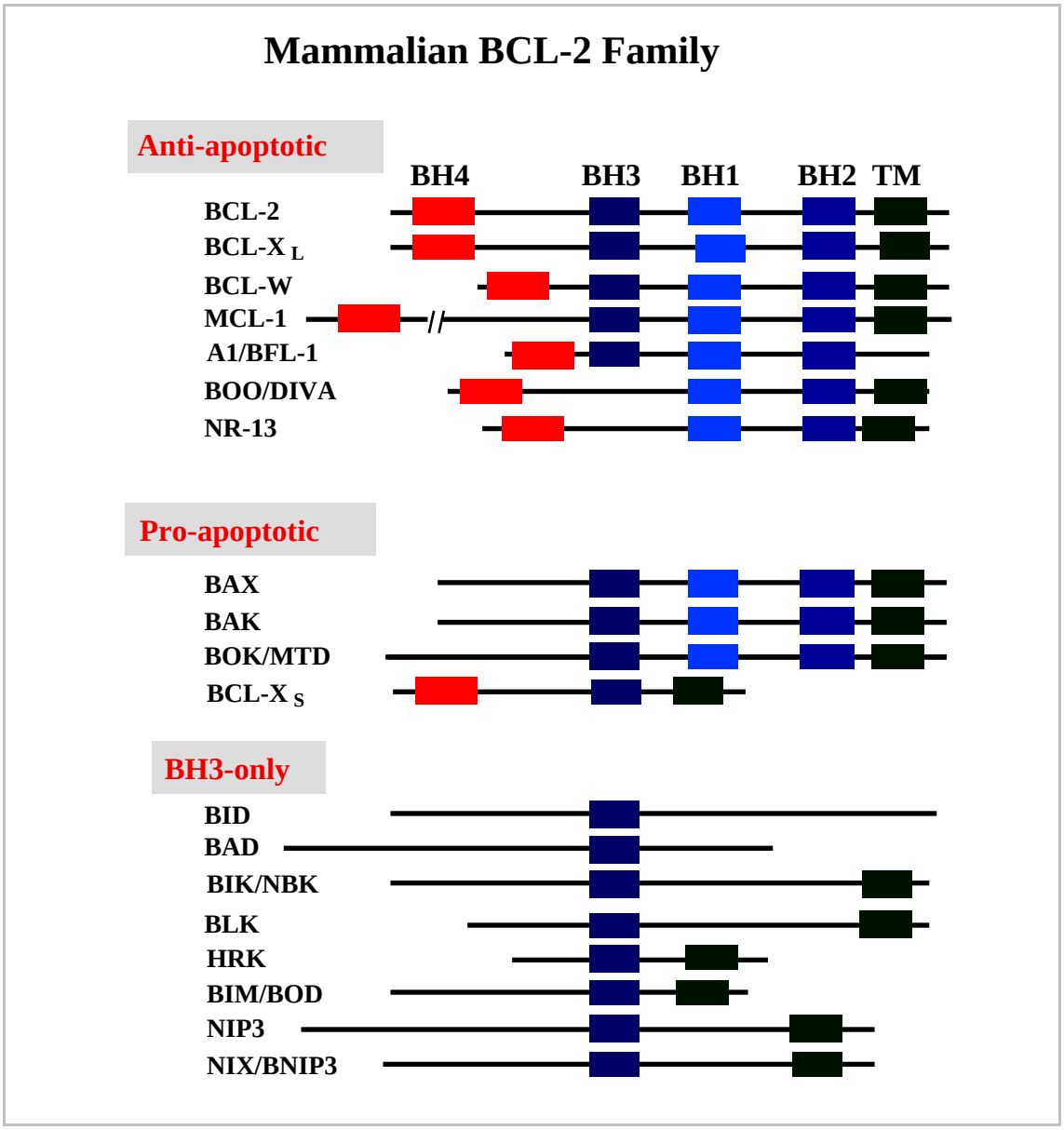


Figure 1.4: Bcl-2 family proteins are key regulators of apoptosis (Gross *et al*., 1999). According to their Structure and function, they can be classified into 3 groups: anti-apoptotic, pro-apoptotic. The proteins with only BH3 domain are required for enhancing cell death interaction with pro-apoptotic proteins.

During apoptosis, cyt *c* is nitrosylated in mitochondria and then rapidly released into the cytoplasm in the absence of Bcl-2 or Bcl-X_L overexpression (Schonhoff *et al*., 2003). Interestingly, arginine antimetabolite L-canavanine induces apoptotic cell death in human Jurkat T cells via caspase-3 activation regulated by Bcl-2 or Bcl-xL (Jang *et al*., 2002). In vitro nitrosylation of cyt *c* increases caspase-3 activation in cell lysates. Inhibition of intracellular cyt *c* nitrosylation is associated with a decrease in apoptosis, suggesting that cyt *c* nitrosylation is a pro-apoptotic

modification (Schonhoff *et al.*, 2003). This eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These are in turn responsible for the cleavage of key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis.

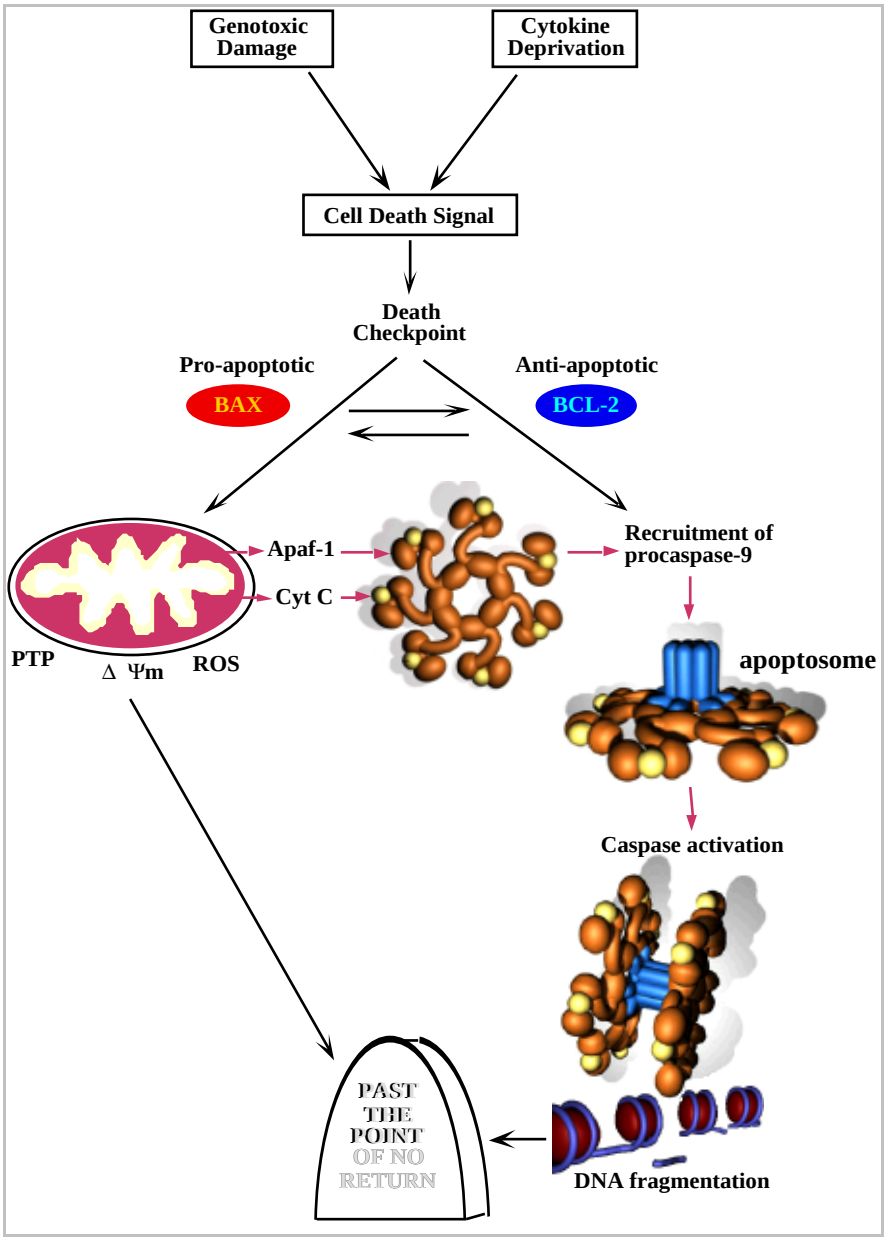


Figure 1.5: Apoptosis is initiated by growth factor deprivation or genotoxic demage. In one case the Apaf-1, cyt *c* and procaspase-9 complex can act as a stage to activate cytosolic procaspase-9 as it is recruited to the apoptosome (a multi-protein complex consisting of cyt *c*, Apaf-1, pro-caspase 9 and ATP) and the activation of the caspase cascade. ΔΨm: mitochondrial membrane potential, ROS: Reactive oxygen species, PTP: Protein tyrosine phosphatase.

Interestingly, formation of noncanonical high molecular weight caspase-3 and -6 complexes and activation of caspase-12 during serum starvation induced apoptosis in AKR-2B mouse fibroblasts (Kilic *et al*., 2002).

In some cases, the induction of cell death by cytotoxic T-lymphocytes (CTL) or natural killer (NK) cells is one of the main ways by which higher organisms protect themselves from infected cells with a virus or posing a risk of cancer (Shi *et al*., 1997; Sutton *et al*., 2000; Pinkoski *et al*., 2001).

The granule exocytosis mechanism plays a vital role in defense against virus infection (granzyme mediated cell death; Sutton *et al*., 2003). Cytolytic lymphocytes (CL) utilize two independently initiated pathways involving either ligation of death receptors or perforin mediated trafficking of granzyme B to the target cell cytosol to activate a family of death proteases (caspases 3, 7, 8 and 10) in the target cell.

However, if caspases are not activated, CL can utilize a caspase-independent pathway to ensure the death of the target cell (Sutton *et al.*, 2000; Pinkoski *et al*., 2001). Bcl-2 was found to block granzyme B mediated apoptosis and maintained the survival of some cells, indicating that mitochondrial disruption is central to the granzyme B apoptotic pathway (Sutton *et al*., 1997).

In other cases, apoptosis is initiated by intrinsic cellular stress signals. Cellular stress may occur from exposure to radiation, chemicals or to viral infection. It might also be a consequence of growth factor (e.g., IL-3) deprivation or genotoxic demage (Figure 1.5). Protection of hematopoietic FDCP cells from genotoxin-induced death, by Pim-1 kinase, was observed (Pircher *et al*., 2000; see Section 4.1.1.1).

These intrinsic signals initiate apoptosis via the involvement of the mitochondria. The mitochondria are the key regulators of the caspase cascade and apoptosis. Release of cyt *c* from mitochondria can lead to the activation of caspase 9, and then of caspase 3. This effect is mediated through the formation of an apoptosome (Figure 1.5).

1.3 The murine myeloid cell line (FDCP-1) responsive to IL-3

During hematopoesis, differentiation of leukocytes are regulated by hematopoietic growth factors and cytokines (Vaux *et al*., 1988; Rodriquez-Tarduchy *et al*., 1990). In a mouse model, a leukocyte cell population was generated from the established factor-dependent (FDCP-Mix) hemopoietic cell lines (Spooncer and Dexter, 1997). These cells can be differentiated into monocytes or granulocytes by the addition of specific growth factors. FDCP-1 is a cell line which was generated from FDCP-Mix by long-term mouse marrow cultures with blocking of clonal differentiation (Dexter *et al*., 1980, See Section 2.1.2). FDCP-1 cells, are non-transformed and dependent on pleiotropic growth factors such as IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) for proliferation (Miyajima *et al.*, 1993) and rapidly undergo programmed cell death within 1-2 days of IL-3 deprivation, are suitable for investigation of collective mRNA expression from candidate genes that antagonize or protagonize apoptosis. The apoptotic response in FDCP-1 cell lines are relatively well characterized (Williams *et al*., 1990; Antoku *et al.*, 1998; Cho *et al*., 1999; Bittorf *et al*., 2000a) because FDCP-1 cells are closer to primary cells than any other such cell lines (e.g., BA/F3, Palacios *et al*., 1984; 32D, Migliaccio *et al.*, 1989; TF-1, Drexler *et al.*, 1997). However, the precise role of IL-3 in regulating massive numbers of pro- and anti-apoptosis genes, at transcriptional level, is still intricate (Robb *et al.*, 1995; Nicola *et al*., 1996; Nishinakamura *et al.*, 1995; 1996). For the reason described above, FDCP-1 was used as model cell in our laboratory to isolate genes that are transcriptionally activated during programmed cell death by IL-3 withdrawal (Russ *et al*., 1996b; Rayner *et al*., 1994).

1.3.1 *Induction of apoptosis following IL-3 withdrawal and regulation of survival signaling*

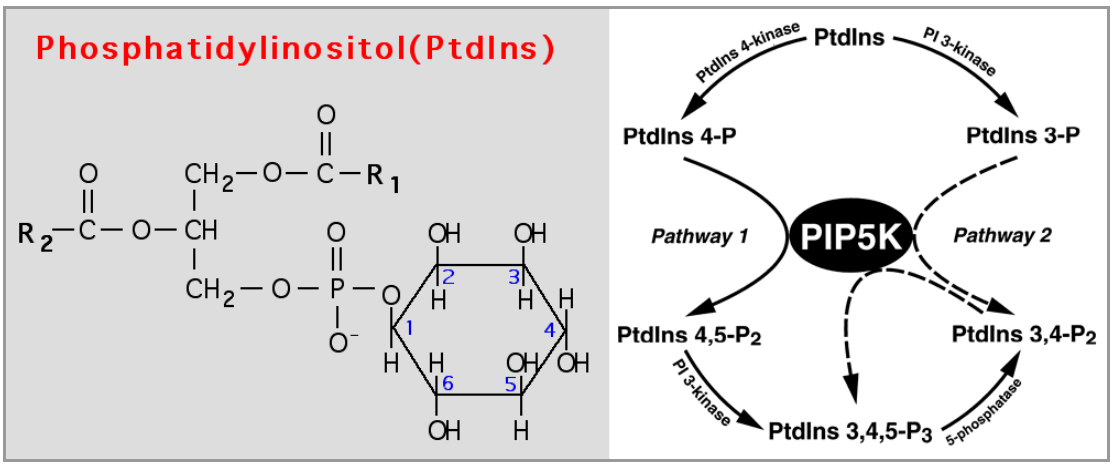
Eventually, the withdrawal of IL-3 from FDCP-1 cells leads to the activation of caspase proteases and a commitment to cell death (Bojes et al., 1999). However in the early course of apoptosis, IL-3 deprivation also stimulates the activation of

survival cellular kinases, including phophatidylinositol 3'-OH-kinase (PI-3-K) and Akt kinase (Section 1.3.1.1).

The signal transduction pathways, activated by IL-3 and GM-CSF in FDCP-1 cells, was further correlated with various biological effects such as (i) cell proliferation, (ii) inhibition of apoptosis (Blalock *et al.*, 1999; Reddy *et al.*, 2002). Activation of each of the Jak-Stat (Section 1.3.1.2), MAPK (Section 1.3.1.3) or PI-3K-Akt alone is not sufficient either to stimulate cell proliferation or inhibit apoptosis, suggesting that these processes are regulated by orchestrated activation of multiple signalling cascades (Ratajczak *et al*., 2001). Although cell proliferation was related to simultaneous activation of Jak-Stat and MAPK, the effect on cell survival is more correlated with activation of PI-3K-Akt proteins (Section 1.3.1.1).

1.3.1.1 PI-3-K and Akt survival signaling

Survival of hemopoietic progenitors (e.g., FDCP-1) is maintained by at least two different intracellular signaling pathways, one requiring PI-3-K and one that does not (Minshall *et al.*, 1996). PI-3-K is a lipid kinase that catalyzes the transfer of phosphate from ATP to the D3 position of phosphoinositide (PtdIns, Figure 1.6) generating phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3, Wymann and Pirola, 1998) and these stimulates the activation of protein kinase-dependent cell survival signaling pathways (Pirola *et al*., 2001; Storz and Toker, 2002). The most relevant survival factor among the downstream effectors of PI-3-K is Akt/PKB (see Figure 1.7; Nolte *et al.*, 1996, Wymann and Pirola, 1998). Akt was originally identified as the oncogene transduced by the acute trans-forming retrovirus (Akt-8) that was isolated from an AKR thymoma (Staal *et al*., 1977; Staal *et al*., 1987; Bellacosa *et al*., 1991). The serine-threonine kinase Akt exerts its anti-apoptotic effects through several downstream targets. Akt was recognized to phosphorylate the pro-apoptotic Bcl-2 family member, BAD, on Ser136 (Datta *et al*., 1997; del Peso *et al.*, 1997). Expression of activated Akt inhibits apoptosis and G1 arrest induced by growth factor withdrawal in hematopoietic cells.



(Zhang *et al.*, 1997)

Figure 1.6: The multiple reactions that PIP5Ks catalyze phosphoinositide pathways leading to production of PtdIns-3,4,5-P_3. Phosphorylation of D3-phosphate-containing phosphatidylinositols by PIP5Ks provides routes for PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 production.

The biological effects of Akt were linked to the induction of c-Myc oncogene product and Bcl-2 (Ahmed *et al.*, 1997). When BAD is phosphorylated, it gains affinity for the cytosolic protein 14-3-3 and forms an inactive complex with this protein. Nonphosphorylated BAD can heterodimerize with the anti-apoptotic Bcl-2 family member Bcl-X_L. Upon phosphorylation of BAD, its binding to 14-3-3 decrease formation of the BAD/Bcl-X_L heterodimer, thus permitting free Bcl-X_L to protect the cell from apoptosis (Zha *et al*., 1997).

The anti-apoptotic effects of both PI-3-K and Akt suggested that these two genes might be involved in human cancer (Coffer *et al*., 1998). The supporting evidence of Akt involvement in human malignancies has been well documented (Shayesteh *et al*., 1999; Cantley and Neel, 1999). Frequently, overexpression of Akt occurs in more aggressive tumors. Akt inhibits a conformational change in the pro-apoptotic Bax protein, thus preventing the disruption of the mitochondrial inner membrane potential, caspase-3 activation, and apoptosis in pre-B hematopoietic cells following IL-3 withdrawal (Yamaguchi and Wang, 2001).

1.3.1.2 Jaks and Stats signaling

Jaks (Janus kinase) and Stats (Signal Transducer and Activator of Transcription) are essential components of cytokine receptor signaling for regulating growth, survival, differentiation and pathogen resistance (Heim *et al.*, 1999; Ihle, 2001). Cytokine binding induces receptor dimerization, activating the associated Jaks, which phosphorylate the receptor itself (Figure 1.7). Receptor-bound Stats are phosphorylated by Jaks, dissociate from the receptor, dimerize and translocate into the nucleus. Stat dimers bind specific enhancers, regulating the transcription of target genes (Heinrich *et al.*, 1998; Nelms *et al.*, 1999). Interleukin-3 signals through multiple isoforms of Stat5 (Azam *et al.*, 1995). In the analysis of myeloid FDCP-1 cells, that stably expressing mutant erythropoietin receptor proteins (EPOR), show that receptors having a high potential to mediate anti-apoptotic signals also effectively activate Stat5, whereas receptors lacking Stat5 docking sites are diminished (Bittorf *et al.*, 2000b). The suppressor of cytokine signaling (SOCS) family of proteins dampen receptor signaling via homologous or heterologous feedback regulation (Starr and Hilton, 1999; Figure 1.7).

Anti-apoptotic signaling with Stat5 has been documented (Bittorf *et al.*, 2000a). When Stats are activated, Jak kinases phosphorylate other signaling-adaptor proteins, linking Jak signaling (Quelle *et al.*, 1994) to other pathways such as the MAP kinases. The ligand-dependent activation of the Jak/Stat pathway has been implicated in the explanation of cytokine-specific regulation of gene expression (Bittorf *et al.*, 2000b).

1.3.1.3 Mitogen Activated Protein (MAP) kinase signaling

Ras mediates the activation of Raf (with other necessary factors for maximal activation of its kinase activity; Figure 1.7). Phosphorylation of both serine/threonine and tyrosine residues is believed to have a role in the full activation of Raf (Morrison and Cutler, 1997). The activated Raf can phosphorylate MEK (mitogen/extracellular-signal regulated kinase kinase), leading to its activation. KSR (kinase suppressor of

Ras) has been proposed as a molecular scaffold regulating the Raf/MEK/ERK kinase cascade (Brennan *et al*., 2002). Suppression of c-Myc-induced apoptosis, by Ras signalling through PI-3-K and PKB, was described by Kauffmann-Zeh (Kauffmann-Zeh *et al*., 1997).

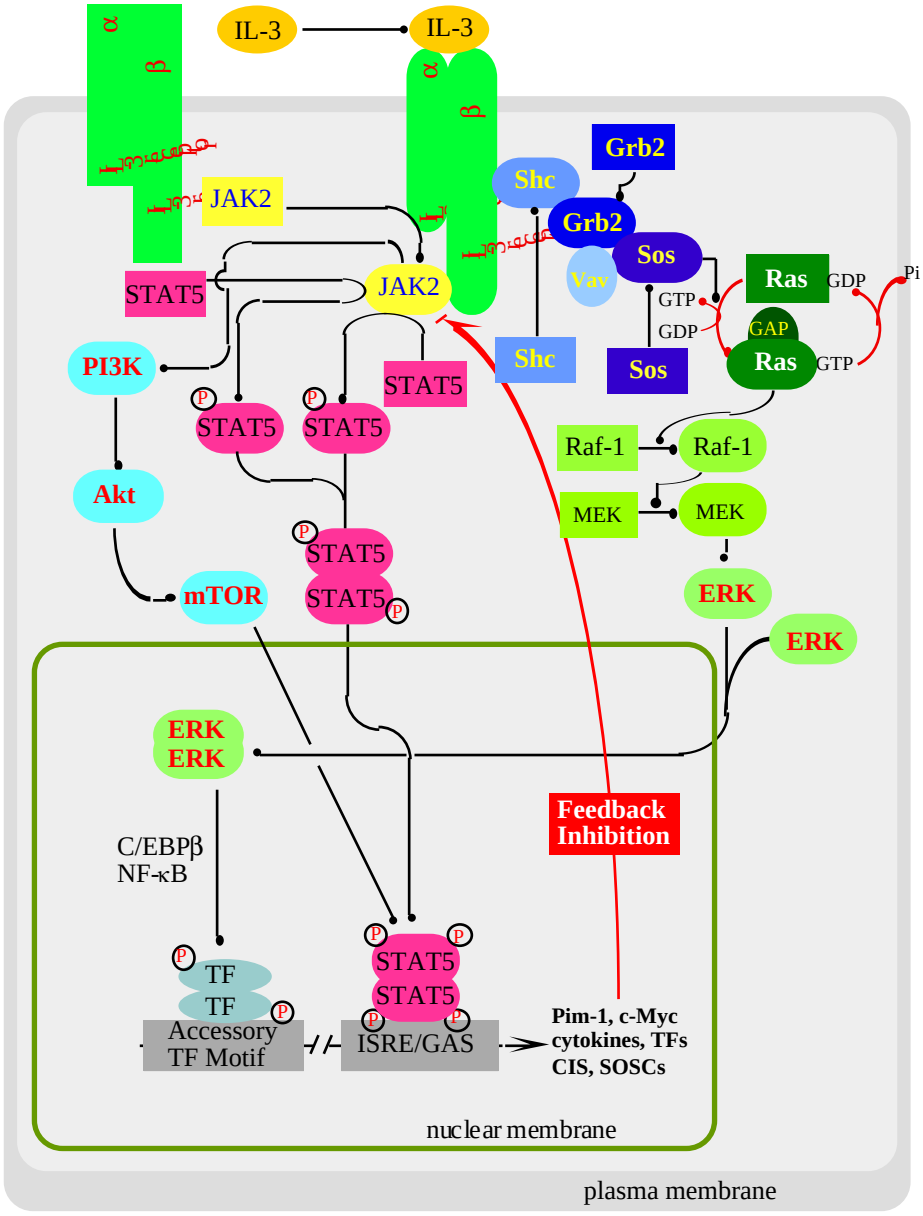


Figure 1.7: Jak/Stat, MAPK and PI-3-K/Akt signaling pathways in IL-3 dependent cells. Cytokine binding induces receptor dimerization that signaling activation of Jaks and Stats. Then, dimerized Stats are translocated into the nucleus and bind specific enhancers, regulating the transcription of target genes. In the Raf/MEK/ERK kinase cascade pathway, Ras mediates the activation of Raf and the activated Raf phosphorylate MEK leading to its activation (see Section 4.3.1 for ERK). The most relevant survival factor among the downstream effectors of PI-3-K is Akt/PKB.

1.3.2 *Cell death by default*

Apoptosis is a fundamental aspect of the maintenance of the balance between cell growth and cell death. If the balance is tilted (e.g., by unregulated cell growth), the result could be cancer. If tipped the other direction and the result can again be cancer. As described in Section 1.2 and Figure 1.2, however, nature provides a way of cell death by default (the type I pathway that ensues quick apoptosis). The concept of cell death by default, has forced a shift in the paradigm of cancer development for more than three decades (Parkin *et al*., 2001). However, the likelihood that radiation and chemotherapy, both major modalities of cancer therapy, could not benefit from strategies that modulate programmed cell death by default. The apoptosis process is the result of differential expression of a number of genes, which are described in Section 4.2.2.

1.4 Identification of transcriptionally induced genes after IL-3 withdrawal

Gene trapping has become a standard approach of functional genomics (Stanford *et al.*, 2001) because of its efficiency for simultaneously characterizing gene function, sequence and expression. Current gene-trapping principle is based on the three different types of trap vectors: the enhancer-, promoter- and gene-trap vectors (Stanford *et al.*, 2001). The gene trap technique combined with Cre recombinase (Cre: causes recombination) and locus of *x*-ing over *P*1 (loxP) recombination system (Figure 1.8) were previously developed in our laboratory (Russ *et al.*, 1996b). This technique was used to isolate genes from cells undergoing apoptosis by growth factor withdrawal and to identify transcriptional mechanisms that interfere with cell death and survival. As illustrated in the Figure 1.8, the reporter cell line FLOXIL3 (an IL-3-dependent hematopoietic cell line) expresses a fused reporter gene tk*neo* that codes for thymidine kinase and neomycin phosphotransferase (Russ *et al.*, 1996a). FDCP-1 cells expressing this reporter gene survive out of selection procedure with G418 (neomycin) and IL-3. The FLOXIL3 cells were infected again in suspension cultures of U3Cre gene trap virus with a multiplicity of

infection (MOI) ≅ 0.5 Activation of Cre (Cre-recombinase) expression from the transduced genes delete the tk*neo* gene by placing the IL-3 gene just downstream of the *pgk* promoter (Figure 1.8) that resulted in a permanent switching of the FLOXIL3 cells to IL-3 independence but losing neomycin resistance (Dexter *et al.*, 1980; Sauer and Henderson, 1988; Vaux *et al*., 1988; Gu *et al.*, 1993; Russ *et al.*, 1996b). This rescues IL-3 dependent FDCP-1 (FLOXIL3) cells (Vaux *et al.*, 1988; Dexter *et al.*, 1980) from apoptotic cell death induced by IL-3 deprivation.

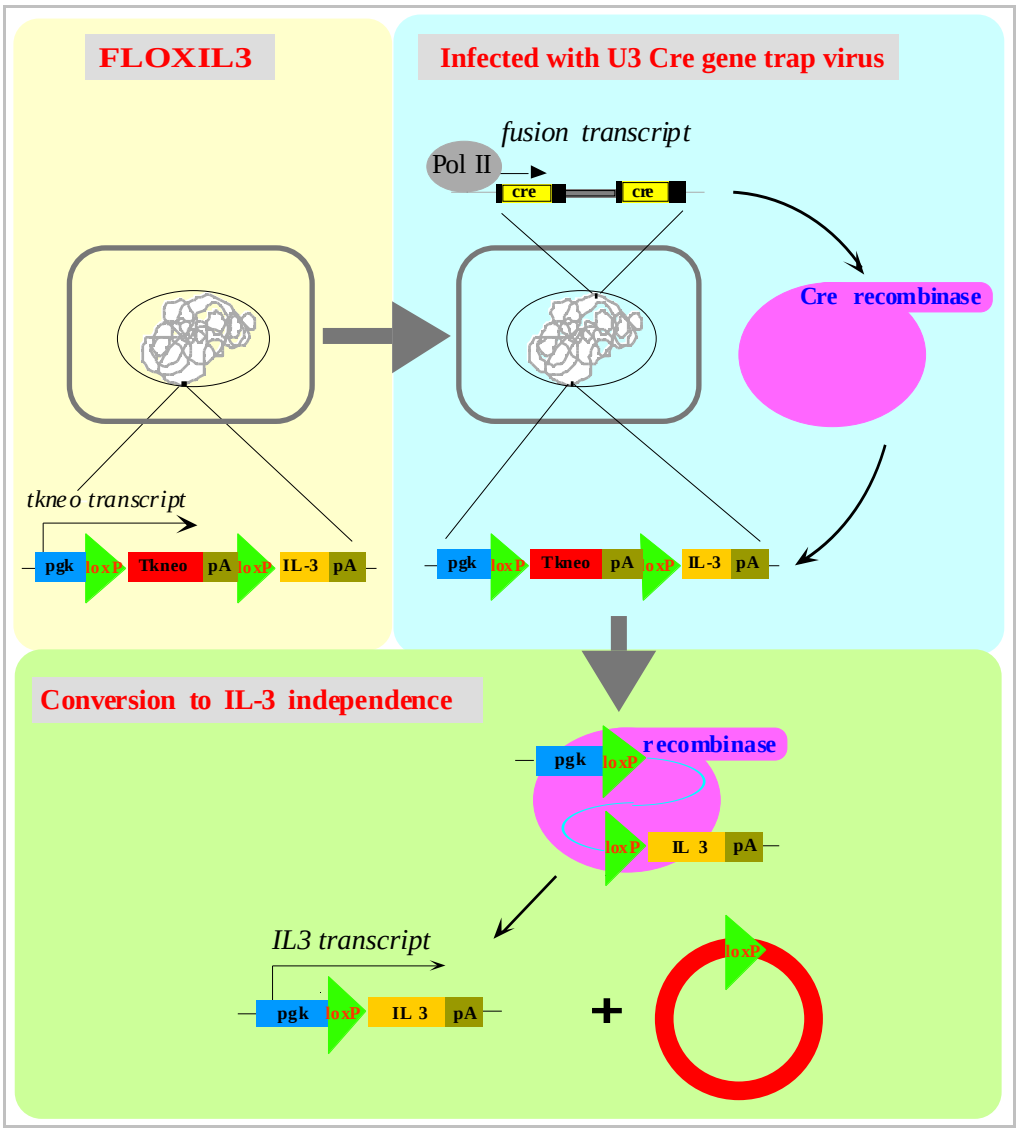


Figure 1.8: Site specific recombination with Cre/loxP exon trap system, applied to transiently induced gene trapping. Activated Cre-recombinase delete the tk*neo* gene by placing the IL-3 gene that resulted in a permanent switching of the FLOXIL3 cells to IL-3 independence from apoptotic cell death induced by IL-3 deprivation. The phenomenon allowed selection for gene-trap integrations of activated survival genes upon factor deprivation.

Two PCR-based methods were applied for amplification of subtracted cDNA: Rapid Amplification of cDNA Ends (RACE) and inverse PCR (Figure 1.9). This approach allowed to identify several functional classes of genes (Section 3.1.1). The gene trap sequence tags (GTSTs) library served as a reference (described in Chapter 3) for the survival genes that prevent growth factor withdrawal-mediated apoptosis in the FDCP-1.

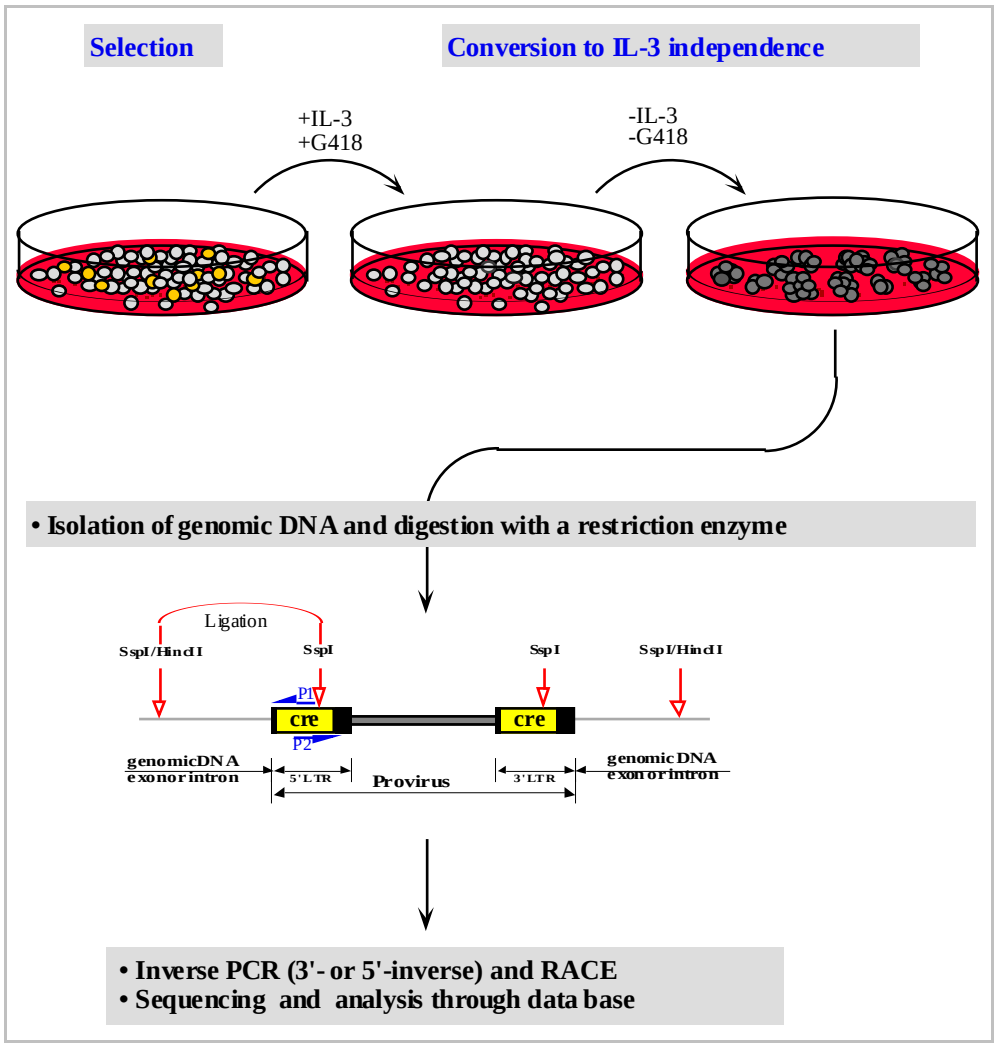


Figure 1.9: Isolation and analysis of transiently induced genes. Positive selection: FLOXIL3 expresses thymidine kinase and neomycin phosphotransferase that endows to FDCP-1 cells survive out of selection procedure with G418 (neomycin) and IL-3. Negative selection: when FLOXIL3 cells were infected with U3Cre gene trap virus, FLOXIL3 cells become independence to IL-3 but losing neomycin resistance. The subtracted cDNAs were amplified by the RACE and inverse PCR methods for the generation of GTSTs library.

1.5 Aims

In hematopoietic systems, apoptosis is highly regulated as it can function to restrict cell number, eliminate autoreactive immune cells, and control differentiation. Followings are the well defined facts on basis of the accumulated knowledge that published since more than a decade (Williams *et al*., 1990).

(a) Hematopoietic colony stimulating factors, such as IL-3, promote cell survival by suppressing apoptosis.

(b) Withdrawal of essential cytokines has been shown to initiate the apoptotic cascade in variety of myeloid lineages and cells.

However, our previous results and observations (unpublished data, Wempe, F. and von Melchner, H.) strongly suggested that IL-3 does not singularly control survival of the hematopoietic cell line FDCP-1, because some of survival genes (as well as apoptotic genes) are transiently expressed after 2-4 h of IL-3 withdrawal (Wempe *et al*., 2001). This phenomenon suggested a possibility that IL-3 might activate some apoptosis genes in the different pathways. In this particular hematopoietic system, our interest were the balance of mechanisms by which IL-3 equally mediates its biological effects on both survival and death.

Major aims:

I. To validate the gene trapping strategy for the recovery of transcriptionally regulated survival genes.

II. To assess the efficiency of the gene trapping strategy in recovering apoptosis related genes in the context of related Microarray and GeneChip analysis.

III. To identify cancer related genes among GTSTs recovered from FDCP-1 cells undergoing apoptosis.

CHAPTER 2

CHAPTER 2

MATERIALS AND METHODS

2.1 Routine preparations

2.1.1 *Chemicals, reagents, enzymes and instruments*

All chemicals and reagents used in this research were analytical grade and were obtained from commercial suppliers (see Appendix II). Routine procedures, especially when they originate from current standard protocols described elsewhere, are mentioned in this Chapter with or without modifications.

2.1.2 *Eukaryotic cell lines and cell culture*

Eukaryotic cells were grown in culture plates or flasks at 37 °C, 5% CO_2 with 100% humidity. Cells were maintained in culture by refreshing the culture medium every 2 to 3 days. After decantation of the culture medium, adherent cells were incubated for 5 min at 37 °C with 3-5 ml of commercially available PBS buffer containing 0.25% (w/v) trypsin. The reaction was stopped by addition of 10 ml of culture medium. The cells were collected by centrifugation at 300 x *g* for 5 min, resuspended in 10 ml of the appropriate medium and propagation was continued.

Except for the cell line FDCP-1 (factor dependent cell from Paterson Institute, Dexter *et al*., 1980; Spooncer *et al*., 1997), all cells used for RNA purification were of human origin and were obtained from the American Type Culture Collection (ATCC). FDCP-1 cell lines were generously provided by the †German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig).

†German Collection of Microorganisms and Cell Cultures:
URL: http://www.dsmz.de/
E-mail: dsmzmutz@gbf-braunschweig.de

Table 2.1: Cell lines used as source of RNA for array assays (see Section 3.4.2 and Table 3.6).

Cell lines	Cell type	Medium*	Accession number
FDCP-1	bone marrow culture of mice	1	ACC 368 †
U937	histiocytic lymphoma	2	CRL-1593.2
K562	chronic myeloid leukemia	2	CCL-243
HEL	erythroleukemia	2	TIB-180
HL-60	acute myeloid leukemia	3	CCL-240
Raji	Burkitt's lymphoma	2	CCL-86

* Media used for cell culture were:

1. Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL), supplemented with 10% (v/v) fetal calf serum (FCS), 5 ng/ml recombinant mouse IL-3 and 100U/gentamycin
2. RPMI 1640 medium (Roswell Park Memorial Institute, Moore *et al.*, 1993) supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 100 U of /penicillin and streptomycin
3. IMDM (Iscove's modified Dulbecco's medium, Uittenbogaart *et al.*, 1983) supplemented with 20% FCS, 1.5 g/l sodium bicarbonate, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin

2.1.3 *IL-3 withdrawal from FDCP-1 cell culture medium*

The FDCP-1 cells (6 x 35 ml) were propagated to a concentration of 1–2 x 10^6 cells/ml in DMEM. The cells were pooled and separated into 6 x 50 ml Falcon tubes. Then, they were collected by centrifugation (300 x *g*) at 4 °C for 4 min and resuspended in fresh DMEM lacking IL-3. This step was repeated one more time. Finally, five of the six pellets were resuspended in DMEM (supplemented only with 10% (v/v) FCS, 100 U/ml gentamycin) and portioned into 5 x 50 ml plates (density: ~1.2 x 10^6).

One pellet was harvested directly and used as control. For study of the time course of induction, the cells were cultured in the absence of IL-3 for 1, 2, 4, 6, and 8 h. The cells treated under each condition were collected by centrifugation, and then the pellet was directly frozen in liquid nitrogen and kept at –80 °C until used for RNA preparation.

2.1.4 *Cell storage*

For storage, 1 x 10^7 cell was harvested by centrifugation at 300 x *g* for 10 minutes and washed with PBS. They were then resuspended in 0.5 ml of RPMI containing 20% (v/v) FCS, and then mixed with 0.5 ml of RPMI containing 20% (v/v) DMSO. The cell suspensions were immediately transferred into cryovials and placed into a freezing container (Mr. Frosty, Nalgene), then stored at –80 °C freezer overnight, before being transferred into a liquid nitrogen storage freezer.

To recover the stored cells, cryopreserved cells were thawed rapidly in a 37 °C water bath and subsequently resuspended in culture medium. Following one washing step with medium (Section 2.1.2) to remove residual DMSO, the cells were placed into culture flasks.

2.1.5 *Media for bacterial growth*

Strains of *E. coli* were grown in LB medium (Luria and Burrous, 1957) at 37 °C supplemented with ampicillin (50 μg/ml). Stock ampicillin (50 mg/ml in H_2O) kept at –20 °C until use.

2.1.6 *Agar plates for color screening*

For blue white color screening, LB agar plates were treated (30 min prior to plating the transformants) with 100 μl of 10 mM IPTG (isopropyl β-D-thiogalacto pyranoside) and 100 μl of 2% X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) by spreading over the plates.

2.1.7 *Preparation of plasmid DNA*

The method for small-scale plasmid DNA extraction by alkaline lysis, for isolating plasmids from bacterial culture, was adapted from Silhavy's method (Silhavy *et al.*, 1984). However, for the best yield of plasmid DNA with efficiency, the preparations were performed with commercially available kits and protocols (Qiagen, Inc.). After purification, the plasmid DNAs were resuspended in TE buffer (10 mM Tris.HCl pH 7.6, 1 mM EDTA) and stored at –20 °C.

2.1.8 *DNA precipitation with ethanol*

DNA was concentrated by precipitation with ethanol at low temperature in the presence of moderate concentrations of sodium acetate. One-tenth volume of 3 M sodium acetate, pH 5.2 and 2 volumes of ice cold ethanol was added to an aqueous DNA sample. The precipitation was performed by treatment at –20 °C overnight and by centrifugation at 12000 x *g* for 15 minutes at 4 °C. The DNA pellet was rinsed with ice cold 80% (v/v) ethanol. The DNA pellet was dried and dissolved in TE buffer (see Appendix III).

2.1.9 *DNA extraction from agarose gels*

QIAquick™ Spin (Qiagen) kits were used to extract and purify DNA fragments (in the size range between 70 bp and 3 kb) from gels in Tris/acetate/EDTA electrophoresis buffer (TAE, Appendix III). The optimal elution efficiency was achieved with 10 mM Tris-HCl (pH 7.0-8.5) and the eluted DNA was stored at –20 °C.

2.1.10 *Determination of nucleic acid concentrations*

The nucleic acid solution was concentrated or diluted to give an optimal range of concentration for photometric measurement. A quartz cuvette (Ultraspec®, Pharmacia Biotech) was filled with dilution buffer and the base line was recorded. The absorbance of the sample was measured at 260 nm.

The measured A_{260} was translated into the concentration of the nucleic acids by the spectrophotometric conversions shown in Table 2.2. The ratio between the absorbance readings at 260 and 280 nm gives an estimate of nucleic acid purity. The A_{260}/A_{280} ratio of pure DNA and RNA are 1.8 and 2.0, respectively.

Table 2.2: Spectrophotometric conversion of A_{260} to concentrations of nucleic acids.

1 A_{260} unit of double-stranded DNA equals 50 μg/ml
1 A_{260} unit of single-stranded DNA equals 33 μg/ml
1 A_{260} unit of single-stranded RNA equals 40 μg/ml

2.1.11 *Restriction-endonuclease digestion of DNA*

Target DNAs were digested with commercially available restriction endonucleases in suitable buffers (Appendix II). Digestions were usually performed at 37 °C for 0.5-2 h (if not recommended otherwise*) and terminated by addition of 0.25-0.5 volume of restriction-endonuclease stop mix (50 mM EDTA, 17% (v/v) glycerol, 0.07% bromophenol blue, pH 8.5). For partial digestion, a small-scale reaction was carried out first for optimization of the reaction. Restriction enzymes were diluted serially and made in equivalent volumes of reaction mixtures containing identical concentrations of plasmid DNA. Electrophoresis on agarose gels (with 1xTAE buffer) was routinely used to see the results of digests.

* One unit of enzyme: the minimal amount of enzyme required to digest all of 1 μg DNA in 1 hour.

2.1.12 *Agarose gel electrophoresis*

Agarose gels, from 0.7 to 2% (w/v) in 1 x TAE buffer (Appendix III) containing 0.2 μg/ml ethidium bromide, were used for the electrophoretic separation of DNA molecules for the following purposes.

(a) To check the progression of a restriction enzyme digestion (Section 2.1.11).

(b) To quickly determine the yield and purity of PCR products (Figure 3.9).

(c) To purify DNA fragments (Section 2.1.9).

Agarose gel electrophoresis, for DNA separation, was carried out in a horizontal configuration (Davis system; Sambrook *et al.*, 1989).

Agarose and buffer mix was heated in a microwave oven until the agarose was completely melted. The agarose solution was cooled to about 50 °C and supplemented with ethidium bromide to a final concentration of 0.2 µg/ml. The warm agarose solution was poured into a gel tray. The gel was completely submerged by about 1 mm with the electrophoresis buffer. Samples of DNA were mixed with 1/4 volume of a loading buffer (2% bromophenol blue, 50% glycerol).

After samples were loaded onto a gel, electrophoresis was carried out at 50 V until the DNA had entered the gel matrix. Electrophoresis was continued at 100 V until the desired resolution of DNA fragments was achieved.

2.1.13 *5´-Dephosphorylation of linear plasmid DNA*

The terminal 5' phosphates were removed using calf intestinal alkaline phosphatase (CIP) to prevent self-ligation of linearised vector DNA (Sambrook *et al.*, 1989). Generally 1 µg of DNA was mixed with 1 Unit of CIP and 5 µl of 10 x CIP buffer (500 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 10 mM $ZnCl_2$) in a 50 µl volume. The mixture was incubated at 37 °C for 20 min before a second aliquot of CIP was added, then further incubated for 20 min. The enzyme was inactivated by heating the sample at 65 °C for 20 min.

2.1.14 *Ligation of DNA fragments*

Fragments of DNA with sticky ends were ligated with T4 DNA ligase in the ligation buffers described by Sambrook *et al.* (1989).

Ligations were carried out in a 10 µl reaction mixture containing linearized vector, a threefold molar excess of the insert, 1 Unit of T4-DNA ligase and 1 µl of 10 x ligation buffer (250 mM Tris-HCl pH7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol, 5 mM ATP). The mixtures were incubated overnight at 16 °C.

2.1.15 *Preparation of competent bacteria*

The competent bacteria (XL1-Blue; Hanahan, 1983) were prepared using the standard $CaCl_2$ method (Morrison, 1979), dispensed into chilled sterile microcentrifuge tubes and frozen immediately by immersing the tube in liquid nitrogen. The aliquots (50 µl) were stored at -80 °C. However, when it was necessary for increased transformation efficiency and blue-white color screening, commercially available XL10-Gold ultracompetent cells were used (Table 2.3, Stratagene).

Table 2.3: Genotypes of the competent bacteria. The genes present on the F´ episome represent the wild type bacterial alleles

Host strain	Genotype
XL10-Gold	Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB* lac^qZΔ*M15* Tn *10* (Tet^R) Amy Cam^R]
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZΔM15* Tn*10* (Tet^R)]

2.1.16 *Cloning in pGEM-T vector*

Double stranded PCR products made with Taq DNA polymerase (the resulting A-tailed fragments) were cloned using pGEM®-T Vector Systems (Promega, Figure 2.1). The greatly improved efficiency of ligation is based on the fact that Taq DNA polymerase preferentially adds a single 3' A-deoxynucleotide to double-stranded DNA fragments by a non-template-dependent extension reaction (Clark, 1988).

"Blue-white" color screening methods are based on the ability of β-galactosidase (IPTG induced *LacZ* gene product) to hydrolyse 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), resulting in the blue staining of a colony. As shown in Figure 2.1, T7 and SP6 RNA polymerase promoters flank a multiple cloning region within the peptide coding region for the β-galactosidase α-peptide. Insertional inactivation of β-galactosidase allows recombinant clones to be directly identified by color screening on indicator plates (Section 2.1.6).

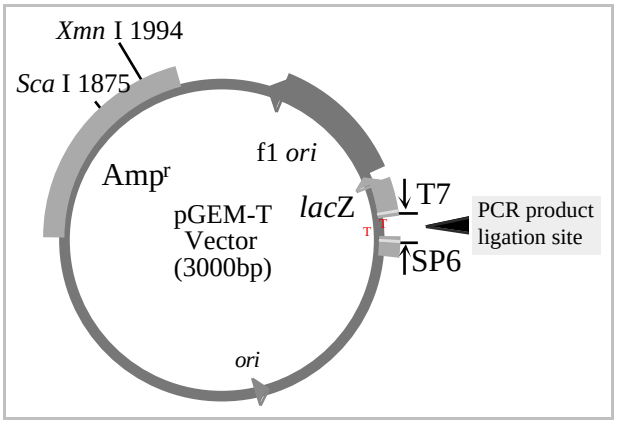


Figure 2.1: Genetic map of the pGEM-T vector. (Promega)

2.1.17 *Transformation of bacteria*

An aliquot of competent *E .coli* bacteria was taken out of a -80 °C freezer, and after 10 min resting on ice, 10-100 ng of plasmid (or ligation mix) were added. Bacteria were left on ice for the next 30 min and then exposed to a 42 °C heat shock for 2 min. 500 µl of LB (without antibiotic) were added, and samples were incubated for 1 h in a shaking incubator. Afterwards, bacteria were transferred onto LB-ampicillin (50 µg/ml, Section 2.1.5) agar plates. Following overnight incubation on 37 °C, individual single colonies were initially selected on the basis of ampicillin resistance and "blue-white" color screening. The described orientation of insert was then confirmed by PCR (Section 2.2) and sequencing of the gene (Section 2.1.18).

2.1.18 *Sequencing of DNA*

Sequencing reactions with double stranded plasmid DNA templates were performed using ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems). The underlying principle is the use of a set of four different dye-labeled ddNTPs as terminators in a primer extension reaction. In this reaction, DNA polymerase, dNTP mix and four fluorescence-labeled ddNTPs were used to produce DNA ladders for the individual bases in one reaction tube. The method is based on non-isotopic fluorescent detection of the labeled nucleic acids.

In a standard protocol, DNA (plasmid DNA: 3 µg, PCR fragment: 100-300 ng) was mixed with 10 pmol of a primer and 4 µl of the Terminator Ready Reaction

Mix (0.05 Units/µl AmpliTaq® DNA polymerase, 400 µM each dNTP mix, 30 mM Tris-HCl pH 8.05, 5 mM $MgCl_2$, 100 mM KCl and labeled ddNTPs) in a final volume of 20 µl. This mixture was denatured for 5 min at 94 °C and PCR was performed with 25 cycles, each consisting of 30 sec denaturation at 94 °C, 30 sec annealing at 50 °C and 4 min elongation at 60 °C.

The DNA product was purified using Centri-Sept® (Princeton Separation, USA). Following the purification 4 µl of DNA solution was mixed with 12 µl of Template Suppression Reagent (Applied Biosystems) and denatured for 3 min at 94 °C. Separation and detection of fragments was carried out using an ABI PRISM 310 Genetic Analyser (Pekin Elmer Biosystems).

2.2 Polymerase chain reaction (PCR)

PCR techniques for amplifying segments of DNA (that lie between two regions of known sequences or primers) consist of three basic steps: Repeated cycles of (i) denaturation, (ii) annealing and (iii) DNA extension. The annealing temperature and extension time were decided by G-C contents in primers and product considerations. The automated thermal cycler (Robo Cycler Gradient 96, Stratagene) was programmed according to the manufacturers' instructions. The target DNA for amplification was denatured for 1 min at 94 °C and annealed for 1 min at 50-60 °C (if GC content was ≤50%, annealed at 54 °C; if >50%, annealed at 60 °C), then extended during 1-3 min at 72 °C (if product length was ≤500 nucleotides, extended 1 min; if >500 nucleotides, extended 3 min). Usually, the program was run for 20-30 cycles. However, in the case of cDNA amplification (with minimal amount of genomic DNA template, Figure 2.7), 45 cycles were carried out. Specific applications of PCR are described in the individual Sections.

2.2.1 *Oligonucleotides*

To identify nucleotide sequences that carrying our target DNAs, the GenBank accession numbers (see Appendix I) were used as a query to search nucleotide

databases. Specific PCR primers (Appendix IV, V and VI) were designed (with MacVector and DNA Strider, Appendix II) from the acquired sequence information (Clontech data bank or NCBI: http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi). All oligonucleotides were provided by Interactiva Biotechnology and were purified by HPLC. Usually, 10 pmol/µl (in TE buffer, pH 7.5; Appendix III) working solution was used for PCR.

2.2.2 *Quick analysis of recombinant plasmid by PCR*

PCR is frequently used because it is the most sensitive assay for determining the orientation and size of an insert in recombinant DNA. After blue-white screening of transformants (Section 2.1.16) on the plates, single colonies were picked up with sterilized toothpicks and cells were vigorously vortexed in 50 µl of TE buffer.

After they were centrifuged for 20 min at 30,000 *g*, 1 µl of the supernatant, containing recombinant DNA, was used as template. Sense and antisense primers were designed for the screening by PCR.

2.3 Reverse transcription-PCR for generation of cDNA fragments

RT-PCR was adapted to detect transcripts, and to amplify their sequences to permit cloning and/or nucleotide sequencing.

Especially, for Northern blot and matched tumor/normal expression array assays, part of the corresponding cDNA was amplified from the original cDNA fragment that was generated from the total RNA of related cells by reverse transcription (Superscript II RT; Gibco-BRL). Then, the cDNA was ligated into the pGEM®-T vector (Section 2.1.16). Sequencing of the double stranded cDNA was done as described in Section 2.1.18.

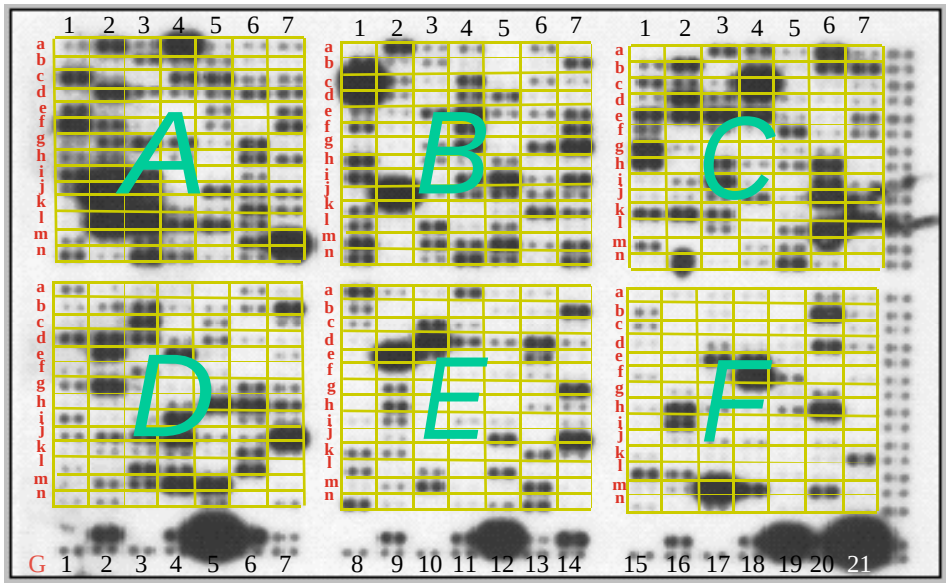
Reverse transcription was used for another reason to produce α-^{32}P-labeled first strand cDNA probes that were synthesized from total RNA (Custom array assay; Section 2.6.5). In this case, I used gene specific primers (Section 2.2.1; see Appendix VI) and SuperScript ™ II RNaseH reverse transcriptase (Gibco-BRL).

2.3.1 *RT-PCR for generating cDNA templates for matched tumor/normal array*

RT-PCR was used to produce cDNA from total RNA of cancer cells. The total 30 µl PCR reaction mixtures in diethylpyrocarbonate (DEPC) H_2O, contained 2 µl of SuperScript ™ II RNaseH reverse transcriptase in 1 x reaction buffer (5 x reaction buffer: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 1 x dNTP mix (10 x dNTP: each of 5 mM dNTPs) with 1 µl of random hexamer primers (1 µg/µl), 3 µl of dithiothreitol (DTT, 100 mM) and 2.5 µg of total RNA of cancer cells. After the initial denaturation of RNA at 65 °C for 10 min, the PCR reactions followed by switching temperatures to 30 °C for 10 min, 42 °C for 90 min and final incubation at 65 °C for 15 min. 1 µl of this cDNA template was used for amplification of target genes (Section 2.5.2).

2.4 cDNA array analysis

I used a commercially available cDNA microarray to examine the modulation of mRNA expression following IL-3 withdrawal (Section 2.1.3). The Atlas™ Mouse cDNA Expression Array (CLONTECH) includes 588 genes involved in key processes (as shown in Figure 2.2) to facilitate differential expression profiling. Each cDNA fragment contained 10 ng of cDNA, and was immobilized (on a nylon membrane) in duplicate to assure the quality of hybridization. As shown in the Figure 2.2, the genes in the membrane were subdivided into six groups according to their function. **A**: Oncogenes/tumor suppressors/cell cycle regulators; **B**: Stress response/ion channels and transport; **C**: Apoptosis/DNA synthesis, repair and recombination; **D**: Transcription factors/DNA-binding proteins; **E**: Receptors for growth factors (interleukin, interferon), hormones and neurotransmitters; **F**: Cell-cycle communication/cytoskeleton and mobility; **G**: 5, 6, 7, 12, 13, 14, 19, 20 and 21 are housekeeping genes that are commonly defined as genes that are ubiquitously expressed at stable levels in different biological contexts. They have been used to standardize quantitative expression studies. The methods that underlie use of the Atlas™ Mouse cDNA Expression Array are described in Figure 2.3.



* The list of genes is available at Clontech's web site (http://www.Clontech.com).

Figure 2.2: The Atlas™ Mouse cDNA Expression Array.

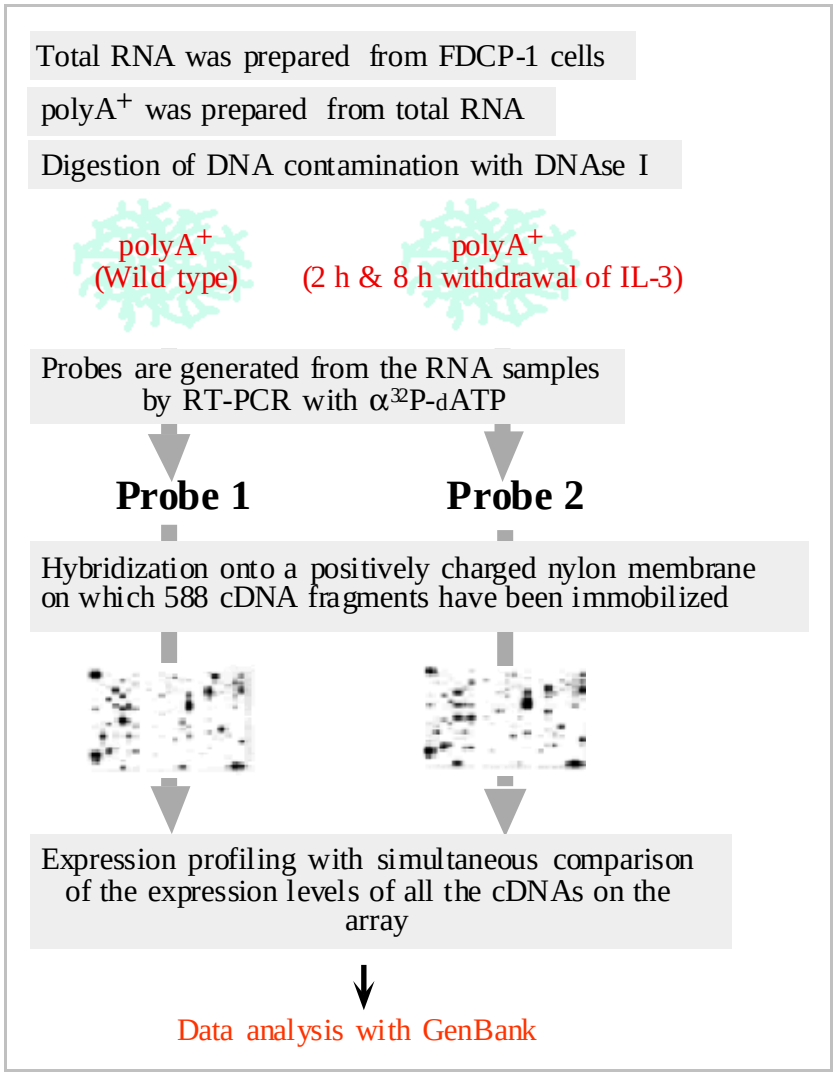


Figure 2.3: A scheme for mouse cDNA expression array assay.

2.4.1 *Isolation of total RNA from FDCP-1 cells*

All of the solutions used in dealing with RNA were prepared with DEPC treated H_2O. DEPC (0.2 ml) was added to 100 ml of H_2O and autoclaved. FDCP-1 cells were grown either in the presence (+IL-3) or the absence of IL-3 (–IL-3) for periods between 1 and 8 h (Section 2.1.3). Total RNAs were prepared by the following methods. The cell pellet (~1.2 x 10^8 cells) was dissolved in 2 ml of "RNAzol™ B-RNA" solution that contains 4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (w/v) sodium N-lauroyl sarcosinate, 0.1 M 2-mercaptoethanol, 0.2 M sodium acetate (pH 4.0) and phenol (Chomczynski *et al.*, 1987). Guanidinium isothiocyanate (with phenol) is a strong agent that inactivates RNases. To extract RNA (by phase separation), 0.1 volume of chloroform was added, mixed by shaking and the phases were separated by centrifugation at 5000 x *g* for 10 min at room temperature. The cellular DNA was precipitated at the interface of the two phases. The aqueous phase containing RNA was transferred to a fresh tube and extracted with 800 µl of chloroform to remove the residual phenol. After centrifugation at 12000 x *g* (10 min, 4 °C), the supernatant was transferred to a fresh tube. The RNA was precipitated by mixing with 0.7 volume of isopropanol and by incubation for 30 min in ice. The RNA was collected by centrifugation at 12000 x *g* (10 min, 4 °C). The RNA pellet was rinsed with 70% (v/v) ethanol and centrifuged at 12000 x *g* for 10 min. The ethanol was removed and the RNA was dissolved in DEPC-H_2O and stored at –80 °C.

2.4.2 *Isolation of polyA^+-RNA from total RNA*

There is a lower limit to the sensitivity of Northern blotting, so that only moderately abundant mRNAs can be detected. One way to increase the sensitivity of Northern blotting is to enrich the RNA preparation for mRNA. When RNA is isolated from cell lines or tissue sources, all RNA species (total RNAs: ribosomal and transfer RNA as well as mRNA) are isolated. As noted in Figure 2.4, most mRNAs destined for the cytoplasm and translation are modified by the addition of a 3' poly(A) tract.

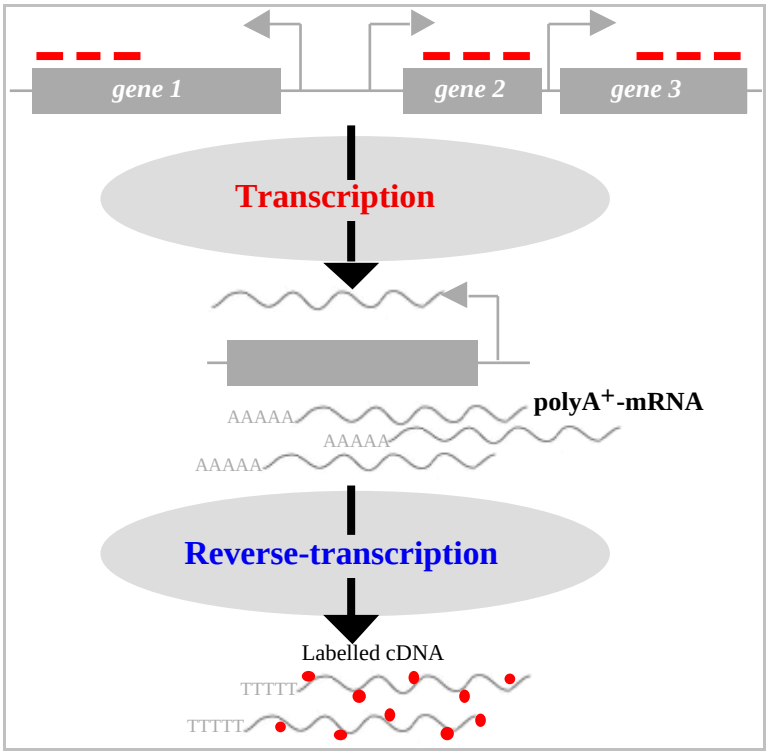


Figure 2.4: Probe synthesis from polyA$^+$-RNA (see Section 2.3.1).

An RNA preparation could be greatly enriched for mRNA species by removing all RNA molecules that lack the 3' poly(A) tail. Usually, mRNA makes up less than 10% of the total RNA content of a cell or tissue. In this work, ~500 µg of total RNA was prepared using the methods described in Section 2.4.1, and it was used to obtain 10-20 µg of poly(A)$^+$-RNA. The method was followed as described in Klaus *et al*. (1990). Total RNA (500 µg) was resuspended with 45 ml of lysis buffer (in a 50 ml Falcon tube); 2.5 ml of sodium dodecyl sulfate (SDS, 20%) and 0.3 mg/ml proteinase K were added and the mixture was incubated for 3 h at 37 °C. After addition of 2 ml of oligo (dT)-cellulose (Pharmacia) suspension to the Falcon tube, it was incubated for 18 h at room temperature on a rolling incubator. The supernatant was discarded and nonpoly(A)-containing RNA was washed away three times with 45 ml of 1 x binding buffer (Appendix III). The RNA/ oligo (dT)-cellulose solution was then placed into a Poly-Prep® chromatography column, (Bio-Rad), which was washed with 30 ml of 2 x wash buffer (Appendix III). The poly(A)$^+$-RNA was eluted with 10 ml of elution buffer (Appendix III) at room temperature. Fractions (500 µl each) were collected and 5 µl of each was loaded on a 1% ethidium bromide-stained agarose dry gel (without

running the sample by electrophoresis) to quickly check which fractions contained poly(A)$^+$-RNA samples. Fractions containing RNA were visible on the gel. Then, the fractions were pooled and precipitated by mixing with 0.7 volume of isopropanol and by incubation for 30 min on ice. The RNA was collected by centrifugation at 12000 x *g* (10 min, 4 °C). The RNA pellet was rinsed with 70% (v/v) ethanol and centrifuged at 12000 x *g* for 10 min. The ethanol was removed and the RNA was dissolved in DEPC-H_2O. To avoid genomic DNA contamination, the polyA$^+$-RNA samples were treated with 0.5 Units of RNase-free DNase I for 2 h at 37 °C and purified with CHROMA SPIN-200 column (Clontech). Then, a possible genomic DNA contamination of the sample was checked by PCR (Section 2.2) with GAPDH primers (Appendix IV) and analyzed on 2% agarose gel (Figure 3.3).

2.4.3 *Probe synthesis from polyA$^+$-RNA for Atlas Array assay*

1 µg of polyA$^+$-RNA was converted into α-^{32}P-labeled first strand cDNA. A master mix (Table 2.4) was prepared for all labeling reactions. First, the polyA$^+$-RNA (1 µg) was mixed with 2 µl of CDS Primer Mix (Clontech) in a 0.5 ml microcentrifuge tube. Then, the tube was incubated in a preheated PCR thermal cycler at 70 °C for 2 min. The temperature of the thermal cycler was reduced to 50 °C and the sample was incubated for 2 min. 8 µl of the master mix (Table 2.4) was added to each reaction tube without removing the RNA samples from the thermal cycler, then it was incubated at 50 °C for 25 min. The reaction was stopped by adding 1 µl of 10 x termination mix (Appendix III) and column chromatography followed (CHROMA SPIN-200, Clontech).

Table 2.4: Composition of the master mix for generating labeled cDNA probes

	1 x
5 x Reaction buffer	2 µl
10 x dNTP mix (for dATP label)	1 µl
α-^{32}P-dATP (3,000 Ci/mmol, 10 mCi/ml)	3.5 µl
DTT (100 mM)	0.5 µl
MMLV reverse transcriptase (50 units/ml)	1 µl
Total volume	8 µl

2.4.4 *Hybridization of cDNA probes to the Atlas Array*

First, the ExpressHyb™ solution (Clontech) and sheared salmon testes DNA (Sigma) were prepared as follows. ExpressHyb (50ml) was prewarmed at 68 °C. Sheared salmon testes DNA (500 μg) was heated at 95–100 °C for 5 min, then chilled quickly on ice. Then, the heat-denatured sheared salmon testes DNA was mixed with the prewarmed ExpressHyb and maintained in a hybridization oven (Hybridiser HB-1D, Appendix II) at 68 °C until used.

Second, a hybridization container (Biometra) was filled with deionized H_2O. The Atlas Array membrane was placed into the container. All the water was poured from the container. The hybridization solution (5ml), prepared in the first step, was added into the bottle. This step was performed as quickly as possible to prevent the array membrane from drying. It was then prehybridized for 30 min with continuous agitation at 68 °C.

Third, the labeled probe (entire pool; 0.5–20 x 10^6 cpm) was mixed with 1/10 volume of 10 x denaturing solution (1 M NaOH, 10 mM EDTA; Appendix III). Then, it was incubated at 68 °C for 20 min. Human Cot-1 DNA (5 μl) and 1/2 volume of 2 x neutralizing solution (1 M NaH_2PO_4/Na_2HPO_4, pH 7.0; Appendix III) was added to the denatured probe. Incubation was continued at 68 °C for 10 min. After the addition of the probe, the membrane was hybridized overnight with continuous agitation in a hybridization oven (Hybridiser HB-1D, Appendix II) at 68 °C. On the following day, the wash solutions 1 and 2 (Appendix III) were prewarmed at 68 °C. After removal of the hybridization solution, the Atlas Array membrane was washed (by the wash solution 1; 200 ml) with continuous agitation for 30 min at 68 °C. This step was repeated three more times. One 30 min wash with 200 ml of prewarmed wash solution 2 was then carried out with continuous agitation at 68 °C.

Finally, it was washed in 200 ml of HPLC H_2O for 15 min without agitation at room temperature. After the washes, the membrane was sealed immediately in a plastic wrap to prevent drying and exposed at –80 °C to Kodak BioMax MS film with

an intensifying screen. Several exposures were tried for varying lengths of time (3–6 h, overnight, and 3 days). Alternatively, the membrane was exposed to a phosphor imaging plate (Molecular Dynamics) for two to six days; the plate was scanned in a phosphorimage analyzer (Phosphorimager^{TM} SI, Molecular Dynamics).

2.4.5 *Stripping cDNA probes*

To re-use the Atlas Array after exposure to X-ray film or phosphorimaging, the cDNA probes were routinely removed by stripping. First, the stripping solution (500 ml DEPC H_2O with 0.5% SDS) was heated to boiling. Then, the plastic wrap was removed from the Atlas Array and the membrane was immediately placed into the boiling solution. Boiling was continued for 5–10 min. The solution was removed from heat and allowed to cool down for 10 min. The Array was removed from the solution and the damp membrane was immediately wrapped in plastic wrap. The efficiency of stripping was checked by exposure to X-ray film overnight. When radioactivity was detected, the stripping procedure was repeated. The Array was sealed and stored in plastic wrap at –20 °C until needed.

2.4.6 *General procedures and computer analysis*

The hybridization signals were analyzed by using Atlas Image TM 1.0 software (Clontech). The two images to be compared were normalized for the calculations by selecting and utilizing 7 out of the 9 housekeeping genes included on the membrane. In addition, the membrane contained three negative control DNAs in triplicate. The negative control spots had to be free of radioactive signals. The results were visually confirmed. Selective analyses were repeated twice to validate reliability and reproducibility of the method.

To create gene expression profiles for FDCP-1 cells, the expression levels of the 588 known genes (immobilized on the membrane) were analyzed first, then I focused later on the genes of particular interest (related with apoptosis, DNA synthesis, repair and recombination). The genes, which showed an adjusted intensity

ratio above 1 or less than 1 in samples were considered to be differentially expressed (see Section 3.1.2 and Table 3.3).

2.5 Northern hybridization

2.5.1 *Isolation of polyA$^+$-RNA from FDCP-1 cells*

The poly(A)$^+$-RNA from FDCP-1 cells was isolated (see Section 2.4.1 and 2.4.2) as described in Klaus *et al*. (1990). Samples (2.5 μg) from each of FDCP-1 cells that were grown in different conditions (Section 2.1.3) were used for electrophoresis.

2.5.2 *Formaldehyde gels and Northern blot membrane*

The composition of a formaldehyde agarose gel differs from that of a standard agarose gel used for electrophoresis of DNA. However, the same general principles of gel preparation were followed. Formaldehyde denatures RNA by reacting covalently with the amine groups of adenine, guanine, and cytosine and so prevents the reformation of G-C and A-T base pairs. Usually, 0.5 μg of poly A$^+$-RNA was used (with RNA size markers, 0.24-9.5 kb, Gibco-BRL) for the analysis of RNA quality. A sample (2.5 μg) of poly A$^+$-RNA was electrophoresed in 1% formaldehyde agarose gels, transferred to a nylon membrane (Hybond-N, Amersham Life Science), immobilized by UV light exposure (UV crosslinker® 1800, Stratagene). Following are more details of the methods of preparations.

First, 1 g of agarose was completely dissolved in 80 ml of DEPC-water by heating. The solution was allowed to cool to 60 °C and 10 ml of 10 x MOPS buffer and 5.4 ml of 12.3 M formaldehyde were added to the solution. Then, DEPC-water was added up to 100 ml final volume and poured into an electrophoresis chamber.

Second, RNA samples were prepared by mixing 2.5 μg of poly A$^+$-RNA (in 3 μl of DEPC-water) with 1.5 μl of 10 x MOPS buffer, 3 μl of 37% formaldehyde and 7.5 μl of deionized formamide. The RNA samples were denatured by treatment at 65 °C for 10 min and chilled immediately on ice.

Third, 2 µl of loading buffer was added to the sample and it was loaded on the formaldehyde gel. The RNA was separated at 5 V/cm using 1 x MOPS buffer. After electrophoresis, the RNA was visualized (by staining in DEPC-water with 0.5 µg/ml ethidium bromide and extensive washing to remove traces of ethidium bromide) followed by UV transillumination (see Figure 3.2).

2.5.3 *Labeling of DNA probes for Northern hybridization and Matched Tumor/Normal expression array assay*

Random-primed labeling was devised by Feinberg and Vogelstein (1983, 1984). The labeling is catalyzed by Klenow fragment, the large fragment of *E. coli* polymerase I obtained by subtilisin cleavage. Klenow fragment lacks the 5'⇒3' exonuclease activity of the intactenzyme but still contains the 5'⇒3' polymerase activity as well as the 3'⇒5' proofreading exonuclease activity.

The modified random-primed labeling protocol and commercially available Rediprime™ II® reaction kit (Amersham Pharmacia Biotech) were used to obtain uniform labeling. First, the DNA sample (PCR product, see also Appendix IV and V) was diluted to a concentration of 5-25 ng in 45 µl of TE buffer, denatured by heating to 95 °C for 5 min in a boiling water bath, and cooled immediately on ice. Then, the denatured DNA was added to a Rediprime™ II® reaction tube containing dATP, dGTP, dTTP, Klenow enzyme and random primers. The reaction solution was mixed by pipetting in 5 µl of α-^{32}P-dCTP (3000 Ci/mol; 50 µCi) and then incubated at 37 °C for 60 min. Unincorporated nucleotides were separated using a Sephadex S-200 micro spin column (Pharmacia). Finally, α-^{32}P-dCTP labeled probe was prepared with the specific activity of ~10^8 cpm/µg.

2.5.4 *Hybridization*

The membrane (carrying the immobilized RNA, Section 2.5.2) was wetted in 6 x SSC and placed in a hybridization bottle. Prewarmed ExpressHyb solution (20 ml) was added to it and the bottle was placed in a hybridization oven and incubated with rotation for 30 min at 65 °C. The probe (prepared as in Section 2.5.3) was

denatured by heating in a hot block for 5 min at 100 °C, then transferred to ice. The probe was added into the hybridization bottle and incubation was continued for 1.5 h. The membrane was then washed using prewarmed washing solutions (65 °C, 2 x SSC, 0.1% SDS) for 15 min and autoradiography was performed as described in Section 2.8.

2.5.5 *Stripping cDNA probes*

The membranes were reused up to 4 times. After exposure, they were stripped to remove the cDNA probe by boiling in 0.5% SDS solution for 10–15 min. The efficiency of stripping was checked by exposure to Kodak BioMax MS film overnight.

2.6 Self-made array assay (custom made array)

Based on the knowledge gained from the gene trap assay (Section 3.1 and Table 3.1), I designed and developed my own array system (Figure 2.5) containing seventy cDNA spots on a nylon membrane (low density array, Figure 3.10) to verify whether our target genes are biologically relevant and differentially expressed in tumor vs. normal tissue cell lines.

The gene specific primers (Appendix VI) were designed with MacVector software (International Biotechnologies) employing a sequence data base, and PCR products were generated (Figure 3.9). Spotting on nylon membranes (Figure 3.10 and Appendix VIII) was kindly performed by Drs Bernhard Korn and Matthias Schick from Deutsche Krebsforschungszentrum (DKFZ).

2.6.1 *Preparation of templates for cDNA amplification*

Templates were prepared for amplification of target genes (from GTSTs libraries, Table 3.1) for spotting on nylon membranes. A cDNA template was generated from polyA$^+$-RNA or genomic DNA of FDCP-1 cells (Figure 2.6) that were serially diluted to give an optimal concentration for PCR (Figure 2.7).

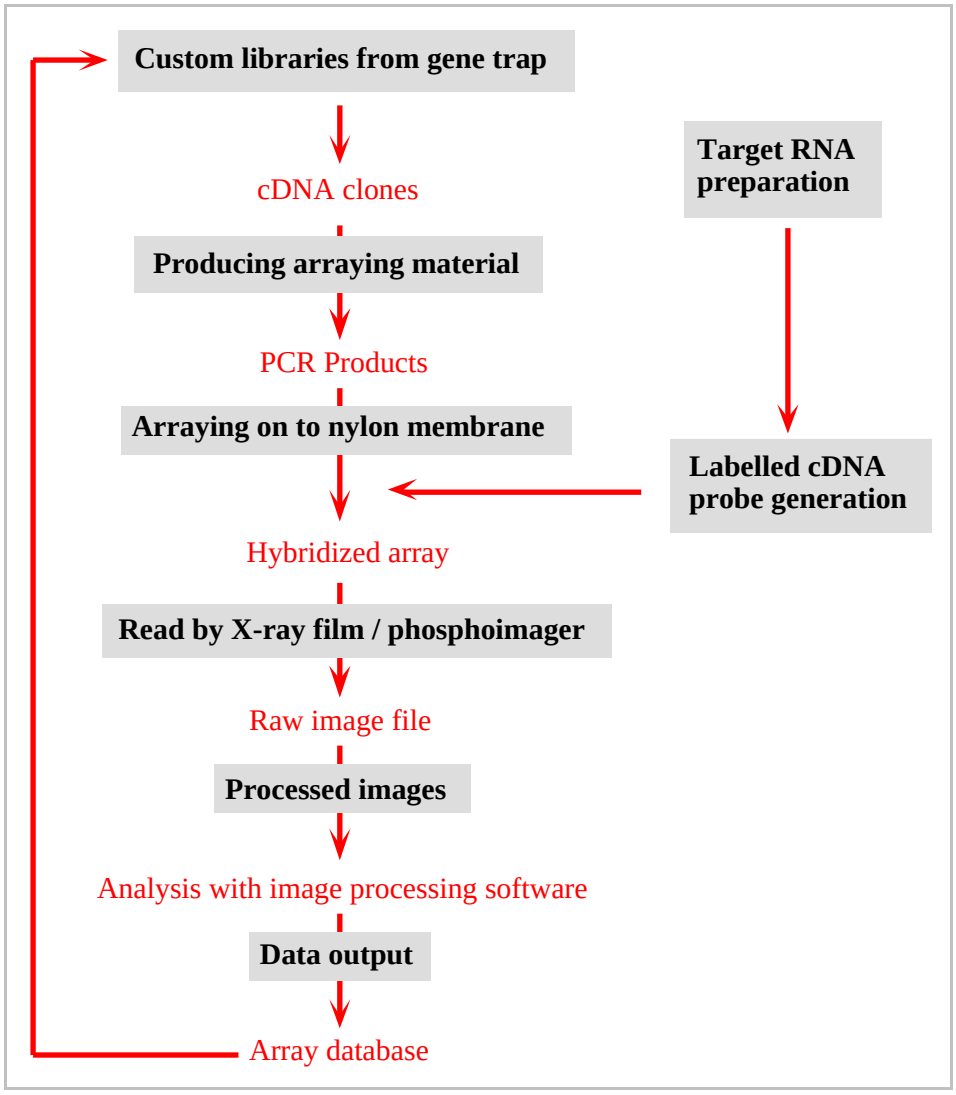


Figure 2.5: A scheme for use of self made array (custom made arrays) assay.

2.6.2 *Cycling conditions and PCR optimization*

Two different templates (cDNA and genomic DNA) and two different annealing temperatures (54 or 61 °C) were compared to determine the optimal settings of PCR (Figure 2.6). For PCR amplification, 1 x PCR buffer, each of the dNTPs at 0.2 mM, 1.5 mM $MgCl_2$, each of primers at 0.5 µM (Appendix V), 1 µl of cDNA template (Section 2.3) and 5 units of Taq DNA polymerase were used (total volume: 25 µl). After the PCR, 5 µl from each sample was loaded on to 1~2% agarose gels. A cycling condition was considered optimized when a single clean band of the expected size was obtained (Section 3.1.4.1, Figure 3.9).

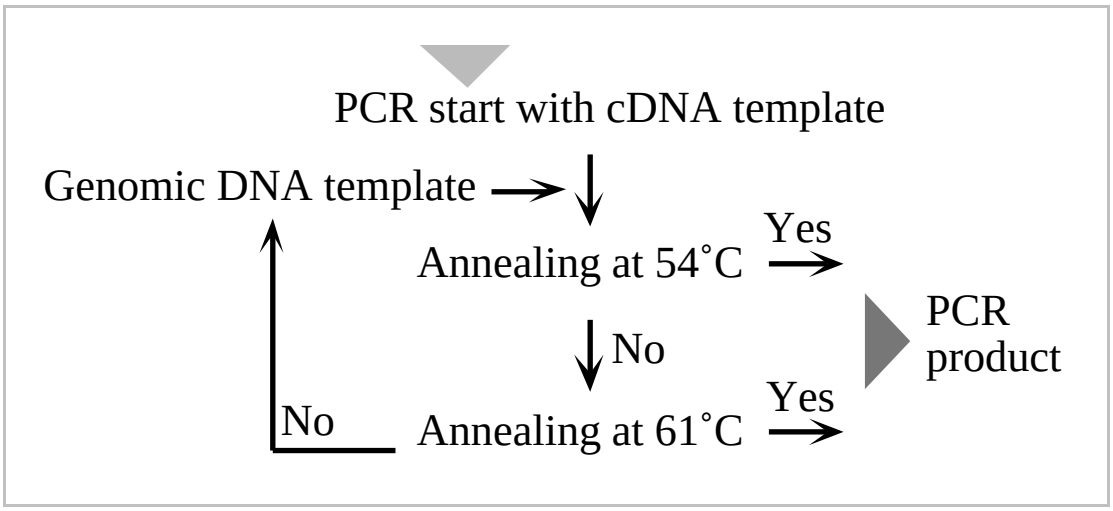


Figure 2.6: Cycling conditions for PCR optimization.

For optimization of PCR with a genomic DNA template, I started with 200 ng of mammalian genomic DNA template(1:1) and serial dilution of it ($1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$), and 55 cycles at 54 °C for amplification of a sample DNA (26-32-3, Appendix VI) as shown in Figure 2.7A.

PCR was run again for 45 cyles with the diluted genomic DNA template (from 100 to 0.01 pg) and the target DNA primer pairs (26-32-3 in Appendix VI; Figure 2.7B). After PCR, the products were displayed on 1-2% agarose gels and examined for yield, specific size and specificity. The number of cycles used depended on both the efficiency of the reaction and the amount of template DNA in the reaction.

As shown in Figure 2.7B, 10% of the reaction produced a band that is readily visible on an ethidium bromide-stained gel as a single predominant band. With more templates, fewer cycles were sufficient. In this case, 45 cycles was optimal for our target DNA amplification.

I confirmed seventy PCR products were effectively amplified and their sizes were correct (Figure 3.9, see the Table in Appendix VI for the conditions of PCR). 100 pmol of each were spotted onto nylon membranes as described in Figure 3.10.

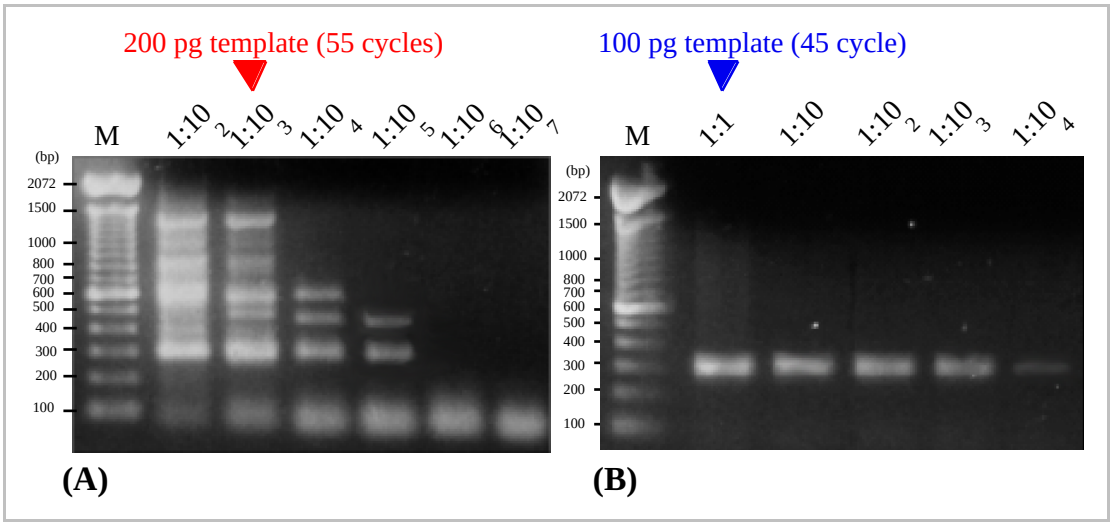


Figure 2.7: Optimization of PCR condition with a mouse genomic DNA template. **A**: Templates (consisting of 2 000, 200, 20, 2, 0.2 and 0.02 pg) used for 55 cycles at 54 °C for amplification of a sample DNA. **B**: Serial dilutions consisting of 100 (1:1), 10, 1, 0.1 and 0.01 pg were used as templates for 45 cycles at 54 °C.

2.6.3 *Purification of RNA*

The methods of purification of total RNA and polyA$^+$-RNA from the cultured cells (Table 2.1) were the same as described in Sections 2.3.1 and 2.3.2. For a second purification, RNeasy kit (QIAGEN) was used. However, most of the total RNAs (for cancer profiling assay) were kindly provided by AstaMedica (See Table 3.6).

2.6.4 *cDNA labeling primers for self made array*

Gene-specific primer cocktails were used for cDNA labeling (α-^{32}P-labelled first single-strand cDNA). The Gene-specific primer cocktails were the mixtures of reverse primers (antisense) for all sequences represented in Appendix VI. The mixture of primers (100 pmol each) were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and finally redissolved in 70 µl of DEPC-H_2O to give ~1 µg/µl.

2.6.5 *Probe synthesis from total RNA for custom array assay*

Total RNA was converted into α-^{32}P-labeled first strand cDNA (Figure 2.5). First, the total RNA (5 µg) was precipitated with 1/5 volume of 3 M sodium acetate

(in DEPC-H_2O, Appendix III) and 2.5 volume of 95% ethanol. It was kept at –20 °C for 1 h, then centrifuged for 10 min in 13,000 rpm in a microfuge. The pellet was washed with 80% ethanol and dried for 10 min, then resuspended with 3 µl of gene specific primer solution (Section 2.6.4) in a 0.5 ml microcentrifuge tube and incubated in a preheated PCR thermal cycler at 70 °C for 2 min. After the temperature of the thermal cycler was reduced to 45 °C, the reagents mixture (Table 2.5) was added to the tube that was incubated at 45 °C for 25 min.

The reaction was stopped by adding 1 µl of 10 x termination mix (Appendix III). The α-^{32}P-labeled first strand cDNA was purified from the unincorporated ^{32}P and small cDNA fragments (< 0.1 kb) with a CHROMA SPIN-200 column.

Table 2.5: Reagents mixture for generating labeled first strand cDNA probes

	1 x
5 x Reaction Buffer	2 µl
10 x dNTP Mix (for dATP label)	1 µl
α-^{32}P-dATP (3,000 Ci/mmol, 10 mCi/ml)	4 µl
DTT (100 mM)	0.5 µl
Superscript II RT (Gibco-BRL)	1.5 µl
Total volume	9 µl

2.6.6 *Hybridization of cDNA probes to the custom array*

Following are the methods for hybridization of 300 µl of cDNA probe (entire pool; ~9 x 10^7 cpm) to the custom array. ExpressHyb (20 ml) was prewarmed at 55 °C. Human Cot-1 DNA (8 µl, Gibco-BRL) and 200 µg of sheared salmon testes DNA were heated at 95–100 °C for 5 min, then chilled quickly on ice. The heat-denatured sheared salmon testes DNA (200 µg) was mixed with prewarmed ExpressHyb (20 ml) and added into the hybridization bottle. The membrane was prehybridized for 30 min with continuous agitation at 55 °C. The labeled probe was mixed together with 1/10 volume 10 x denaturing solution (1 M NaOH, 10 mM EDTA; Appendix III). Then, it was incubated at 55 °C for 20 min. Human Cot-1 DNA (10 µl) and 1/2 volume of 2 x neutralizing solution (1 M sodium phosphate, pH 7.0; Appendix III)

were added to the denatured probe. Incubation was continued at 55 °C for 10 min. After the addition of the probe to the membrane and hybridization solution, it was hybridized overnight with continuous agitation at 55 °C.

2.6.7 *Washing steps and documentation*

After hybridization, the membrane was quickly washed twice with 50 ml of preheated (at 50 °C), washing solution 1 (Appendix III). Two 30 min washes were performed in 100 ml of prewarmed wash solution 1 in a shaking water bath (KötterMann, Labor Technik). Two additional 20 min washes were applied in 100 ml preheated washing solution 2. Finally, a 20 min wash in HPLC H_2O was done at room temperature without agitation. The membrane was sealed in a plastic wrap and exposed to X-ray film at –80 °C for 18 h, 36 h, and 2 days (see Section 2.8). Then, the membrane was exposed to a phosphor imaging plate (Molecular Dynamics) for three to five days.

2.6.8 *Analysis of data*

The steps required to determine the expression ratios from microarray experiments are spot finding, spot quantification, background subtraction and normalization. Prior to analysis of matched tumor/normal array data, ImageQuant version 1.2 (Molecular Dynamics) was used as a visualization tool for the phosphorimage file (see Appendix VII) or the TIFF file from scanned X-ray film (Figure 3.11). Spots below a threshold intensity were eliminated and the expression ratio data were median-centered, then the data were replaced with colors by Excel.

2.7 Matched tumor/normal expression arrays

A major limitation of gene expression profiling using microarrays has been the substantial amount of RNA required for standard probe labeling techniques. The cDNAs were generated (from 68 human tumors and corresponding normal tissues of the same individual) with CLONTECH's PCR-based SMART technology (switch mechanism at the 5'-end of RNA templates that allows accurate cDNA amplification

from nanogram quantities of total RNA; Vernon *et al*., 2000). This amount of RNA ensures that SMART amplification yields a pool of cDNA, which reflects the sample's original complexity, and relative abundance of the original RNA sample.

The array is a nylon membrane on which cDNAs have been immobilized in separate dots as shown in Figure 2.8. The array contains (a) six negative controls (included on the right of the membrane in Figure 2.8) that were used to estimate levels of nonspecific background (yeast total RNA, yeast tRNA, *E. coli* DNA, Poly(A), Cot-1 DNA, human genomic DNA); and (b) a positive control (UBI: cDNA probe for ubiquitin, on the right top and bottom corners of the membrane).

Total cDNAs from the cancer cell lines (1: HeLa, 2: Daudi, 3: K562, 4: HL60, 5: G361, 6: A549, 7: Molt4, 8: SW480 and 9: Raji) were spotted at the bottom from left to right.

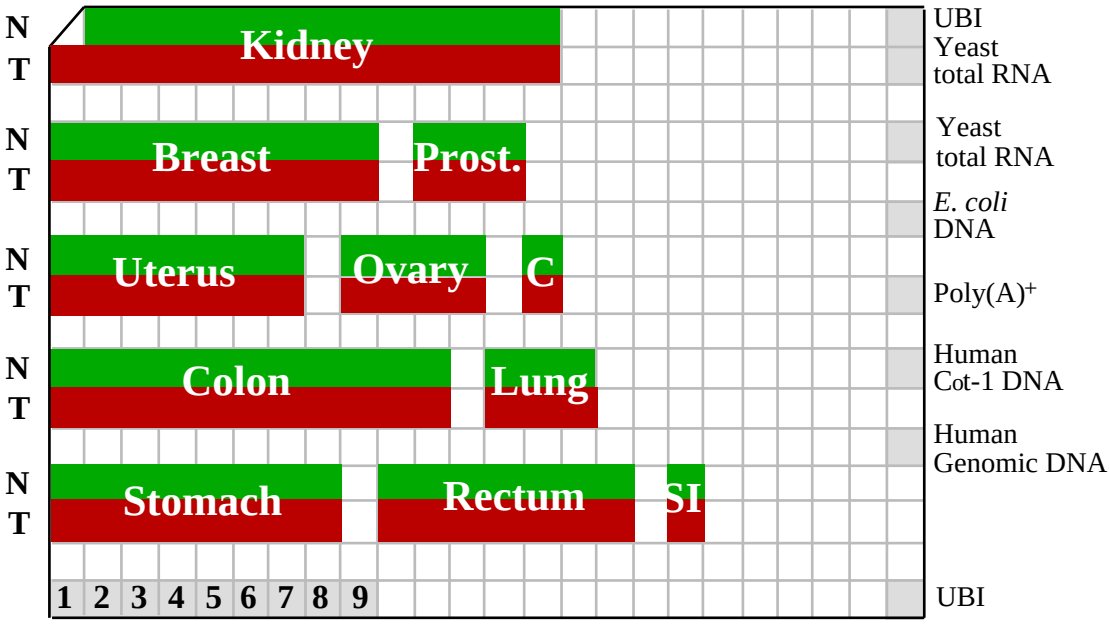


Figure 2.8: Matched tumor/normal expression array. N: cDNA from normal tissue. T: cDNA from tumor tissue.

2.7.1 *Target cDNA probe preparation*

For the amplification of gene specific probes by PCR, templates were prepared from 1 µg each of total RNAs of tumor cell lines from breast (BT-474), ovary (OVCAR-4), pancreas (BXPC-3), lung (NCI-H-460) and colon (COLO-205) as

described in Figure 2.9. The templates were mixed and 1 μl of the mixture was used for generating the target cDNAs (Section 2.3.1). The primers for amplification of cDNAs are described in Appendix V.

The amplified cDNA fragments were ligated into pGEM-T vector (Section 2.1.16). The competent cells (XL10-Gold) were transformed with the plasmids (Section 2.1.15) and single colonies containing required plasmids were selected (Section 2.1.17). The cDNAs were amplified from the plasmids, then purified and sequenced. The cDNA fragment was then labeled with α-^{32}P-dCTP (Section 2.5.3) and used as a probe (Figure 2.9). The hybridization temperatures of each probe are described in Appendix V.

2.7.1.1. Preparation of ExpressHyb solution and sheared salmon testis DNA.

ExpressHyb solution (20 ml) was prewarmed at 50–60 °C. Sheared salmon testis DNA (1.5 mg) was denatured at 95–100 °C for 5 min, and then put on ice. Then, the salmon testis DNA was mixed with the ExpressHyb solution.

2.7.2 *Hybridization*

On the basis of confirmation of expressed target genes (Figure 2.9) by RT-PCR, PCR and sequencing, the DNA probes (Section 2.7.1 and Appendix V) were labeled as described in Section 2.5.3 and hybridized on the matched tumor/normal array membrane (Figure 2.8 and Appendix V).

The methods of hybridization were similar to those described in Section 2.4.4 and 2.5.4 except for the following. The labeled cDNA probe (Section 2.7.1) was mixed with 30 μg of Cot-1 DNA, 150 μg of sheared salmon testis DNA, and 50 μl of 20 x SSC in a total volume of 200 μl. The mixture was heated at 95–100 °C for 5 min and kept at 68 °C for 30 min, then was added to the prehybridized matched tumor/normal array membrane in a hybridization container (Biometra). Hybridization was carried out overnight at 60-65 °C. After removal of the hybridization solution, the array membrane was washed as described in Section 2.5.5.

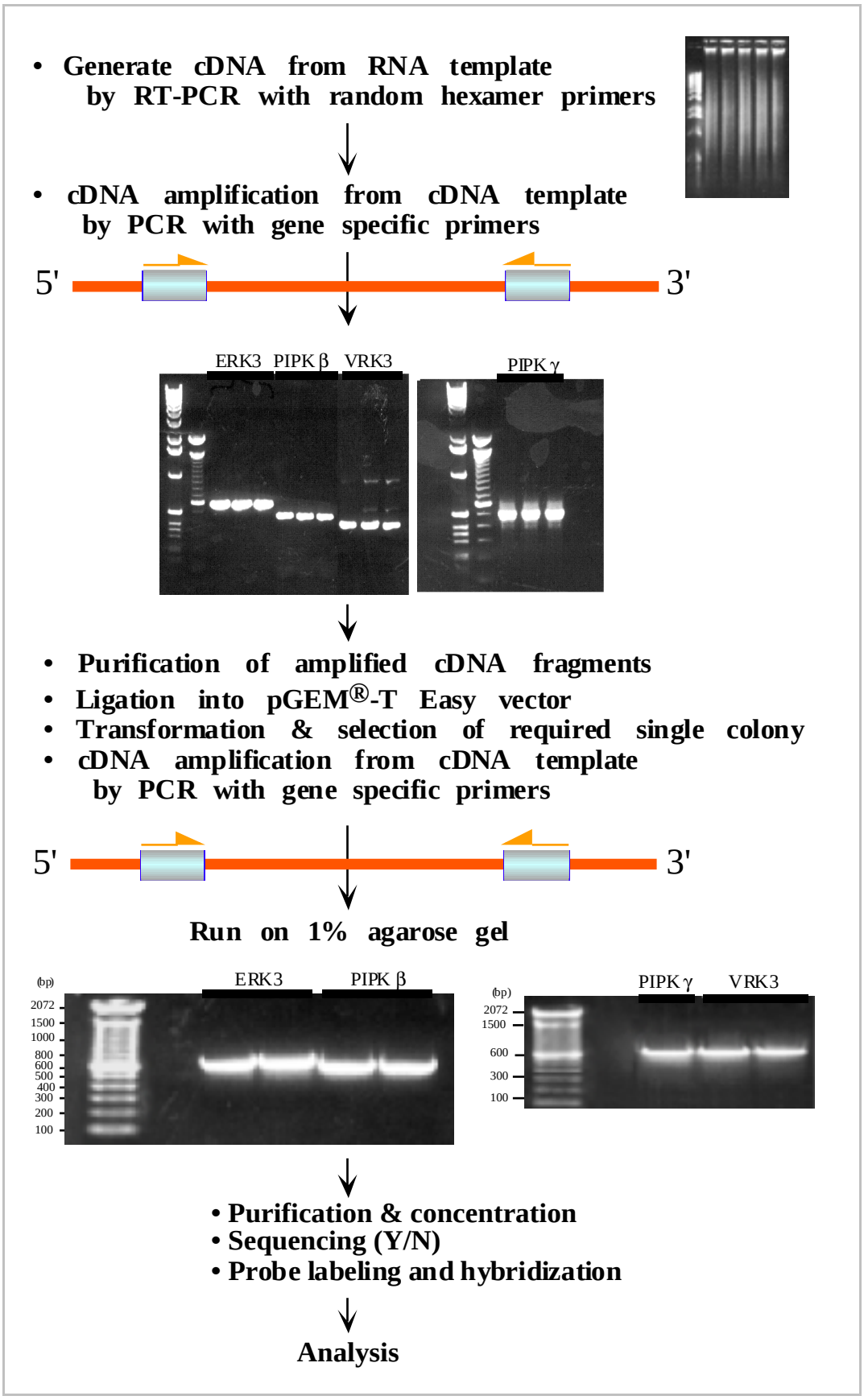


Figure 2.9: A scheme describing the matched tumor/normal expression array assay

2.7.3 *Normalization of hybridization signals*

Prior to an analysis of differential gene expression using the matched tumor/normal expression array, ubiquitin (housekeeping gene, 23-kDa basic protein) cDNAs was employed to normalize the results of hybridization. The array was re-hybridized with a radiolabeled ubiquitin probe and hybridization signals were detected by phosphorimaging for analysis (Figure 2.10).

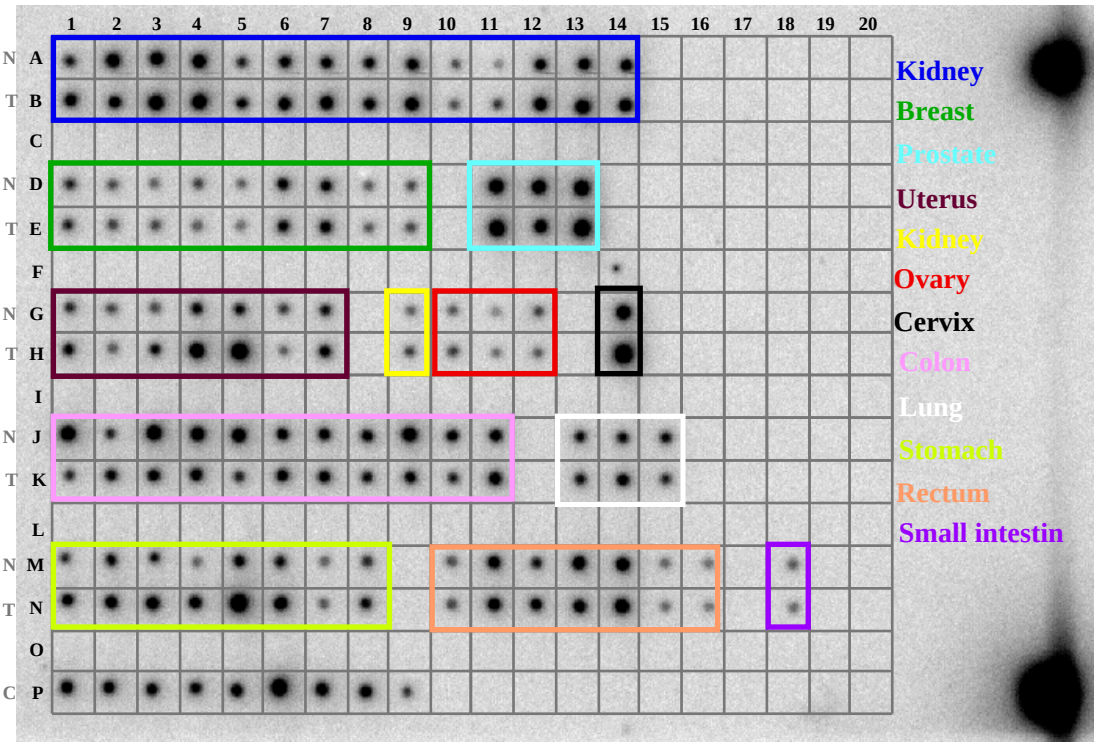


Figure 2.10: Normalization with human ubiquitin cDNA control probe. Matched colors designate tissue types on the array membrane. Two spots (top and down corners from right) indicate ubiquitin cDNA. N, normal; T, tumor; C, cancer cell line cDNAs.

2.7.4 *Analysis*

The hybridized array was exposed to a phosphorimage plate for 2-5 days. After the visualized image was acquired (by scanning the plate), spot finding and spot quantification were done with ImageQuant version 1.2 software (Figure 2.11). The mean value of the background was generated from three randomly selected spots (yellow colored B1, B2 and B3 in Figure 2.11). For background subtraction and normalization, the Excel (Microsoft) program was used. Normalization eliminated

values below a threshold intensity. The final expression ratio data of tumor cells were the values compared with normal cells (when the normal cell expression level is set to 1). An example of the data processing procedure of ERK3 is illustrated in Figure 3.12 and Tables 3.8 and 3.9.

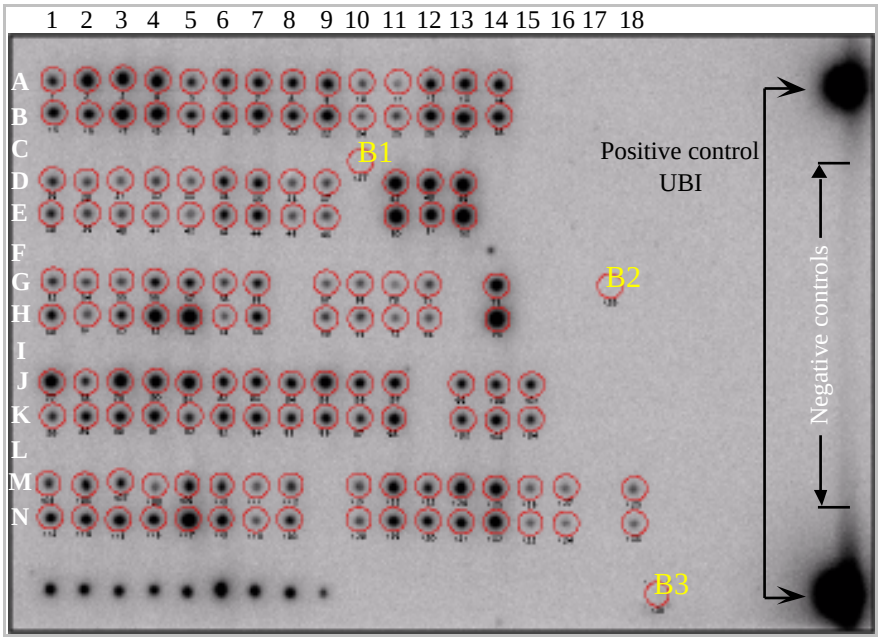


Figure 2.11: Spot finding, spot quantitation and background subtraction with ImageQuant.

2.8 Documentation

For routine laboratory documentation, photographs were taken using a Polaroid camera or digital camera with computer and software (Herolab, Appendix II). However, for documentation of hybridization analysis, autoradiography and phosphorimaging (Phosphorimager™ SI, Molecular Dynamics) were used. After the washing steps (Section 2.6.7), the membranes were exposed to X-ray film (Kodak BioMax MS film with the corresponding intensifying screen) at –80 °C or exposed to a phosphorimaging screen at room temperature. The optimal exposure time was decided after overnight exposure. Figures presented in this Thesis were processed by scanning photographs with a scanner (SNAPscan 600, AGFA) or phosphor images were used. For drawing illustrations, Adobe®Photoshop® 4.0 and Canvas™ 3.5 were employed.

CHAPTER 3

CHAPTER 3

RESULTS

3.1 Gene trapping identifies transiently induced survival genes during programmed cell death

As described in Section 1.6, gene trap mutagenesis and site specific recombination (Cre/loxP) were employed to identify genes that are transiently induced in FDCP-1 cells undergoing apoptosis by growth factor (IL-3) withdrawal (Wempe *et al.*, 2001). From a FDCP1 (FLOXIL3) gene trap integration library corresponding to approximately 2 x 10^6 proviral insertions, we isolated 125 clones that converted to factor independence upon IL-3 deprivation.

Cellular sequences adjacent to gene trap (U3Cre) integration sites (gene trap sequence tags; GTSTs) were obtained from 102 clones by inverse PCR or 5'RACE using Cre-specific primers. Eighty-six out of 102 GTSTs (>30 nt in length) were informative of which 73 belonged to known genes and 13 corresponded to expressed sequence tags (ESTs) within the GenBank/ENSEMBL databases (Figure 3.1).

This clearly reflects the progress of the international genome sequencing projects, as only 2 years ago the majority of the GTSTs had no match in the public databases (Wempe *et al*, 2001). The recovered genes are listed in Table 3.1. Among fifty genes having well defined functions, twenty one (42%) are directly or indirectly related to cellular survival functions (Figure 3.1, Table 3.1). The results indicate that the gene trap strategy effectively selected for this class of genes. Only five genes (marked with green) demonstrate cell death function.

Table 3.1: Trapped genes induced by IL-3 withdrawal*.

Clone	Trapped gene	Genbank ID	Clone	Trapped gene	Genbank ID
26-11-1	TREP-132	NM_172622	30-32-6	KIAA0740/Rhobtb1	XM_125637
26-11-2	Muf1	BC022976	30-32-11	NADPH Cyto' p450 reductase	BC031463
26-11-3	Tif II e β	NM_026584	30-32-12	α-1,2-mannosidase IC	XM_285851
26-11-4	NEF-sp	AK084640	30-32-14	FHOS	XM_134529
26-11-5	1190005B03Rik	NM_144536	30-32-16	YB-1	X57621
26-11-6	MEG3	NM_144513	30-32-18	Eferin/ mKIAA0665	AK083658
26-11-7	Annexin A6	NM_013472	30-41-4	ICAM4	NM_023892
26-12-2	AF15q14 homolog	AK033132	30-42-1	cappucino	AY186603
26-12-3	SBBI54 homolog	BC018244	30-42-4	PSCDBP	BC007144
26-12-4	Vav-1	BC020487	30-42-7	Syntaxin 3A	D29797
26-21-3	Rad 50	NM_009012	30-42-10	Tel oncogene	BC052163
26-21-4	KIAA0376	BC037464	AW16	VMP1	NM_029478
26-22-1	ATF1	NM_007497	AW17	Tmp21-I	BC006774
26-22-4	1200014P05Rik	NM_028765	AW19	STK-19	NM_019442
26-22-7	Collybistin II	AK038840	AW21	same as AW16	
26-22-8	AlkB	XM_127049	AW22	Syntaxin 7	NM_016797
26-31-1	13E, HSP281fis	AK008409	AW24	Jade-1	NM_172303
26-31-3	Same as 26-31-1		AW30	Ect2	BC023881
26-31-7	Same as 26-22-8		AW32	GNA15	NM_010304
26-32-1	Plekha2	NM_031257	AW33	ADH2	AJ245750
26-32-2	KIAA1036	XM_138099	AW34	same as AW22	
26-32-4	14-3-3 zeta	NM_011740	AW36	same as 30-42-7	
26-32-5	Net1	AJ010045	AW37	IP3 receptor isoform 1	NM_010585
26-32-9	Vezf1	NM_016686	AW65	PI transfer protein	AB077281
26-41-1	PI4P5K type I γ	NM_008844	AW66	Glucocorticoide-induced gene1	AF292939
26-41-2	L34	BC052086	AW69	TNFRSF19L	NM_177073
26-41-3	PEST cont. nuclear prot.	XM_132579	AW75	Smurf1	BC029097
26-41-6	ZnF407	XM_140518	AW602	ASC-1 subunit P50	AK007519
30-11-3	Myosin IB	NM_010863	AW604	Pde4d	NM_011056
30-11-4	GSA7	NM_028835	AW606	TOX	NM_145711
30-11-5	**ERK3 (?)**	NM_015806	AW607	nin283	AF378525
30-11-6	VMAT2	AJ555564	AW608	Formin related protein	AF215666
30-12-1	5730501N20Rik	XM_127032	AW611	PHAPI	NM_009672
30-12-2	Hexokinase I	NM_010438	AW613	Angiopoetin-1	NM_009640
30-12-4	PIP5K type I β	BC003763	AW615	LOC226651	AK031319
30-21-2	EDEM	NM_138677	AW618	MC1-R	NM_008559
30-21-3	Nitrilase 1	NM_012049	AW619	NOPAR2/TRALPUSH	NM_177855
30-21-4	LPG22P homolog	AK088457	AW623	SatB1	NM_009122
30-22-1	Rec8	BC052155	AW625	2900052L18Rik	NM_175248
30-22-2	NYD-sp12	NM_029150	NEUA	Siat8d	NM_009183
30-22-4	Ureb1	BC017642	NEUC	Vars2	BC053703
30-31-5	PI145P-5Ptase	XM_133934	NEUD	ALS2	AB053307
30-31-6	4632413C14Rik	NM_029857	NEUE	BTEB1	NM_010638
30-32-1	SMARRC2	BC040363	Un1g	KIAA1140	BC025613
30-32-2	L28	NM_009081	Un3g	same as 26-32-1	
30-32-3	Annexin A11	BC012875	Un11g	2610305M23Rik	BC019973

* Red: genes with survival functions. Green: pro-apoptotic genes. Light blue: genes with pro- and anti-apoptotic functions. KIAs, Riks and fis are full-length cDNAs with unknown functions.

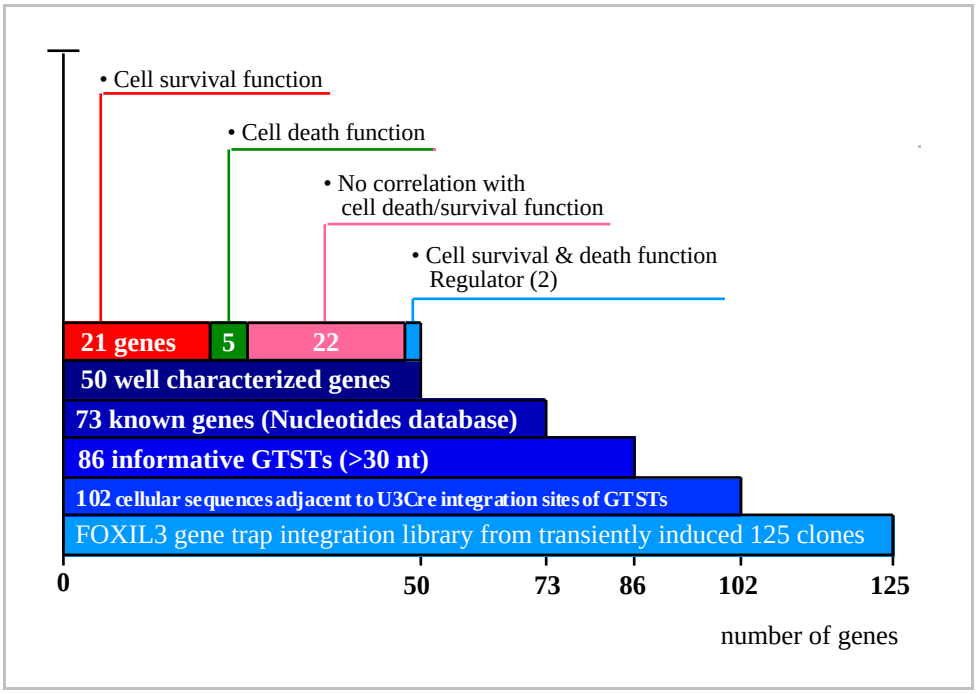


Figure 3.1: GTSTs recovered from the FLOXIL3 gene trap integration library following IL-3 withdrawal.

Table 3.2: Functional classification of the recovered survival genes.

Transcription factors		**Ubiquitin system**	
26-22-1	ATF1	AW75	Smurf1
26-32-9	Vezf1	**DNA repair**	
30-32-16	YB-1	26-21-3	Rad 50
30-42-10	Tel oncogene	26-22-8	AlkB
NEUE	BTEB1	30-22-1	Rec8
AW602	ASC-1 subunit P50	**Small GTPases/Effectors**	
AW623	SatB1	26-22-7	Collybistin II
Metabolic enzyme		26-32-5	Net1
30-32-11	Cyto' p450 reductase	**Other functions**	
Kinases & phosphorylase		26-32-4	14-3-3 zeta
26-41-1	PI4P5K type I γ	30-11-6	VMAT2
30-12-4	PIP45K type I β	30-21-2	EDEM
30-11-5	ERK3	30-42-10	Tel oncogene
30-12-2	Hexokinase I	AW30	Ect2
AW19	STK-19	AW608	Formin related protein
Cytoskeletal binding		AW618	MC1-R
26-11-7	Annexin A6		

3.2 Mouse cDNA expression arrays reveal differential expression of cell death and survival genes following IL-3 withdrawal

To further study transcriptional regulation during growth factor starvation, we analyzed the expression patterns of a large number of previously characterized genes using Atlas™ mouse cDNA arrays (Clontech).

Atlas™ arrays contain cDNA probes for 588 genes involved in development, oncogenesis, DNA repair, tumor suppression and apoptosis. PolyA$^+$-RNA was prepared from FDCP-1 cells at 0 and 2 hours after IL-3 withdrawal.

After verifying mRNA quality on 1% formaldehyde-agarose gels (Figure 3.2) and excluding contamination with genomic DNA (Figure 3.3) the mRNA was reverse transcribed in the presence of ^{32}P-dATP using array gene specific primers (see section 2.4.3 for details). Figure 3.4 shows that of the 588 genes displayed on the arrays, only 13 genes were differentially expressed after factor deprivation (Table 3.3).

Differential expression of these genes was reproducible in independent array experiments and was validated by Northern hybridization. For Northern blots, polyA$^+$-RNA was recovered from FDCP1 cells after 0, 2, 4, 6 and 8 hours of factor deprivation using oligo (dT) beads (see section 2.4.2).

This procedure increases the mRNA detection sensitivity on Northern blots and was chosen because several of the regulated genes were not very highly expressed on the arrays. Moreover, attention was paid to mRNA quantification to insure even gel-loading (Figure 3.5).

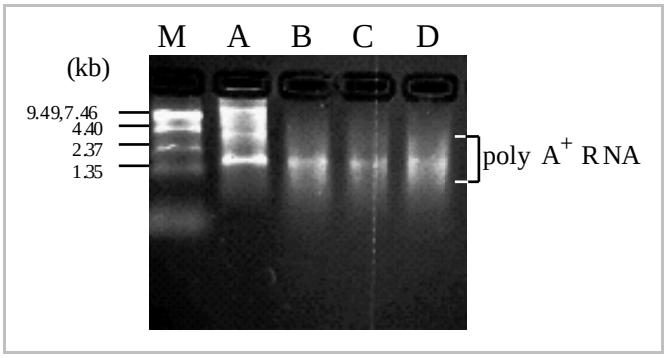


Figure 3.2: Quality assessment of FDCP-1 mRNA on a 1% formaldehyde-agarose gel. **M**: RNA ladder (0.24-9.5 kb, GIBCO-BRL), **A**: Total RNA; **B**: polyA$^+$-RNA from non-deprived cells; **C**: polyA$^+$-RNA from cells deprived of IL-3 for 2 hours; **D**: polyA$^+$-RNA from cells deprived of IL-3 for 8 hours.

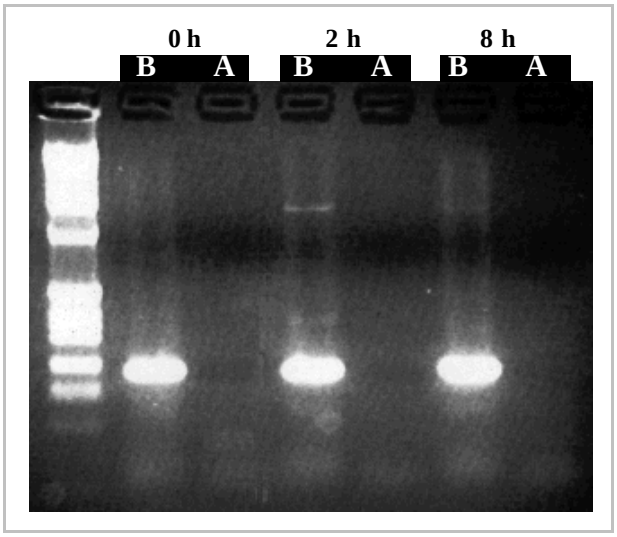


Figure 3.3: Estimating genomic DNA contamination of mRNA preparations. PolyA$^+$ RNAs were prepared from FDCP-1 cells at 0, 2 and 8 hours after IL-3 withdrawal. Aliquots were treated for 2 hours at 37 °C with 0.5 U of DNase I and then subjected to PCR using intron specific GAPDH primers. Amplification products were resolved on a 2% agarose gel. Lanes **A** = after DNase treatment; Lanes **B** = before DNase treatment.

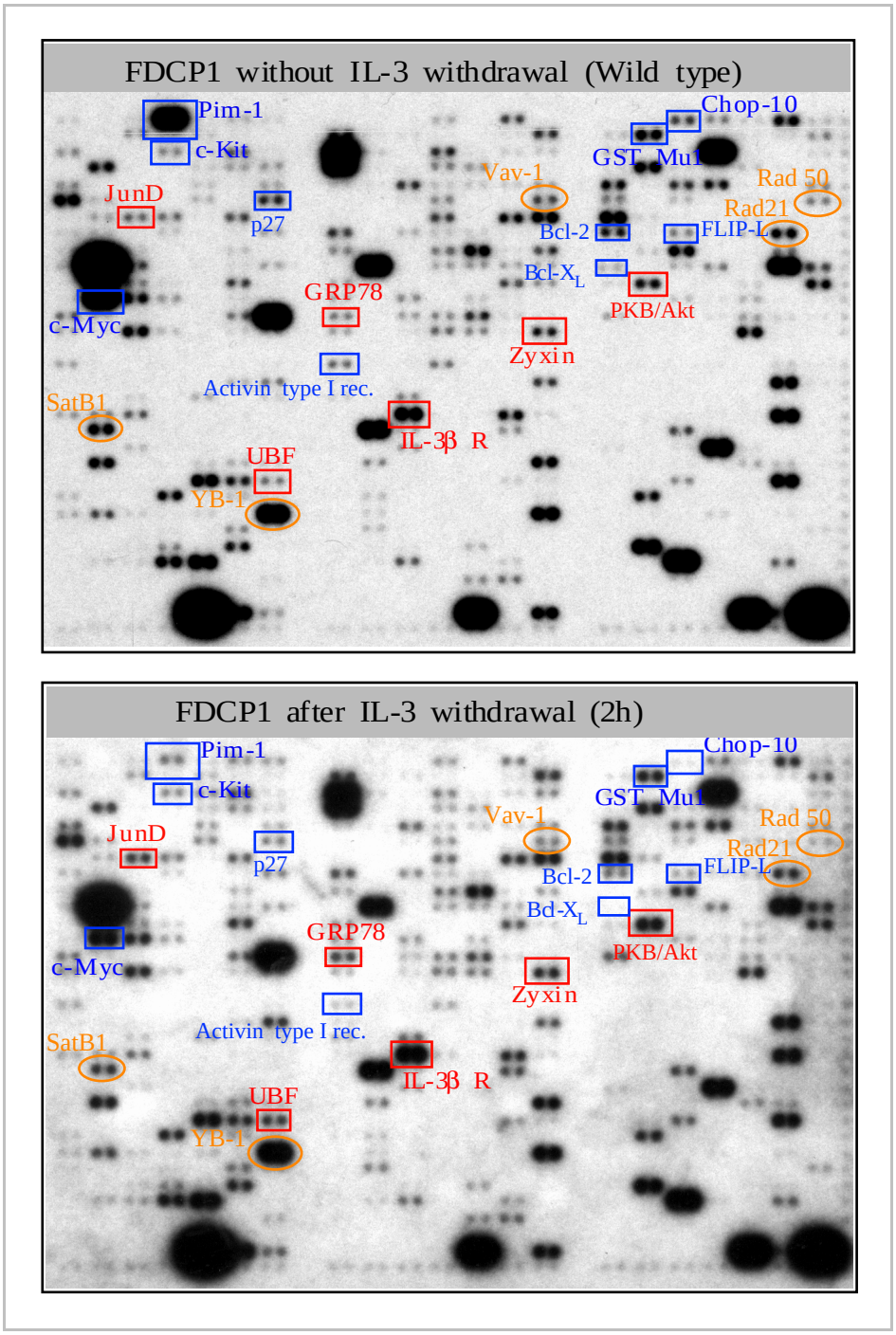


Figure 3.4: Differential gene expressions in FDCP-1 cells after IL-3 withdrawal. The Atlas arrays were hybridized to polyA$^+$ from control and factor deprived (2 hours) cells The membranes were exposed for 16 hours to Kodak BioMax Ms X-ray film at –80 °C. Red and blue rectangles correspond to induced and repressed genes, respectively. Orange circles: genes that are not differentially expressed on the Atlas arrays but were recovered by the gene trap approach.

Table 3.3: Summary of the results obtained with the Atlas arrays.*

Gene/protein	Regulation ratio	Coordinate
c-Myc (proto-oncogene; Selten *et al.*, 1984)	0.6	A2l
Pim-1 (proto-oncogene; Selten *et al.*, 1986)	0.1	A4a
c-Kit (proto-oncogene; Qiu *et al.*, 1988)	1.6 (8 h)	A4c
Chop-10 (murine Gadd153; Ron *et al.*, 1992)	0.1	C3a
GST (glutathione-S-transferase; Huang *et al.*, 2003)	1.2 (8 h)	C2b
Zyxin (α-actinin binding protein; Macalma *et al.*, 1996)	1.6	B7n
IL-3 receptor (Itoh *et al.*, 1990)	1.6	E3d
Akt (proto-oncogene, protein kinase B; Section 1.3.1.1)	2.4	C2k
JunD (transcription factor; Li *et al.*, 1990)	1.7	A3g
Bcl-2 (apoptosis inhibitor; Section 1.2.2)	0.7	C1h
Bcl-X_L (apoptosis regulator Bcl-X long form; Section 1.2.2)	0.1	C1j
FLIP-L (apoptosis inhibitor; Irmler *et al.*, 1997)	0.5	C3h
Activin type I receptor (Verschuern *et al.*, 1995)	0.1	E1a

* Gene expression was quantified using the Atlas Image 1.01a (Clontech) software. To obtain the regulation ratio, signal intensity value produced by each gene on the membrane after hybridizing to mRNA from FDCP-1 cells deprived of IL-3 for 2 hours (8 hours for c-kit and GST) was divided by the value obtained for the same gene with mRNA from control cells (0 h). Hence, regulation ratios below or above 1 reflect gene repression or induction, respectively.

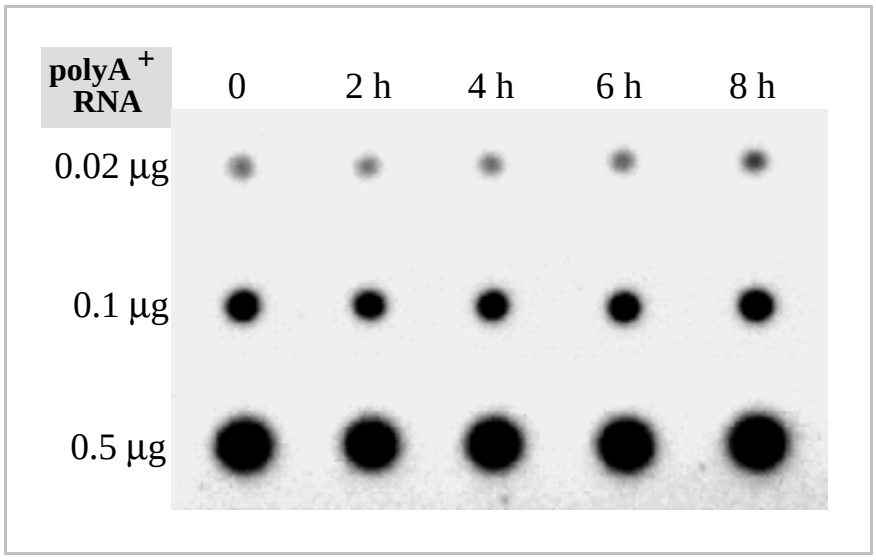


Figure 3.5: Quantification of polyA$^+$-RNA by dot-blot analysis. Serial dilutions of polyA$^+$-RNA isolated from FDCP1-cells (0.5, 0.1 and 0.02 µg from each of sample corresponding to 0, 2, 4, 6 and 8 h after IL-3 withdrawal were spotted onto a nylon membrane using a microsyringe. α-^{32}P-labeled GAPDH cDNA probes were used for quantification.

Similar to the gene trapping results, several genes with survival functions were up-regulated in the absence of IL-3. Among these were the survival kinase Akt, the c-kit receptor tyrosine kinase, zyxin, a protein closely related to proteins implicated tumorigenesis and steroid receptor binding, and the IL-3 receptor itself (Figures 3.6 and 3.7).

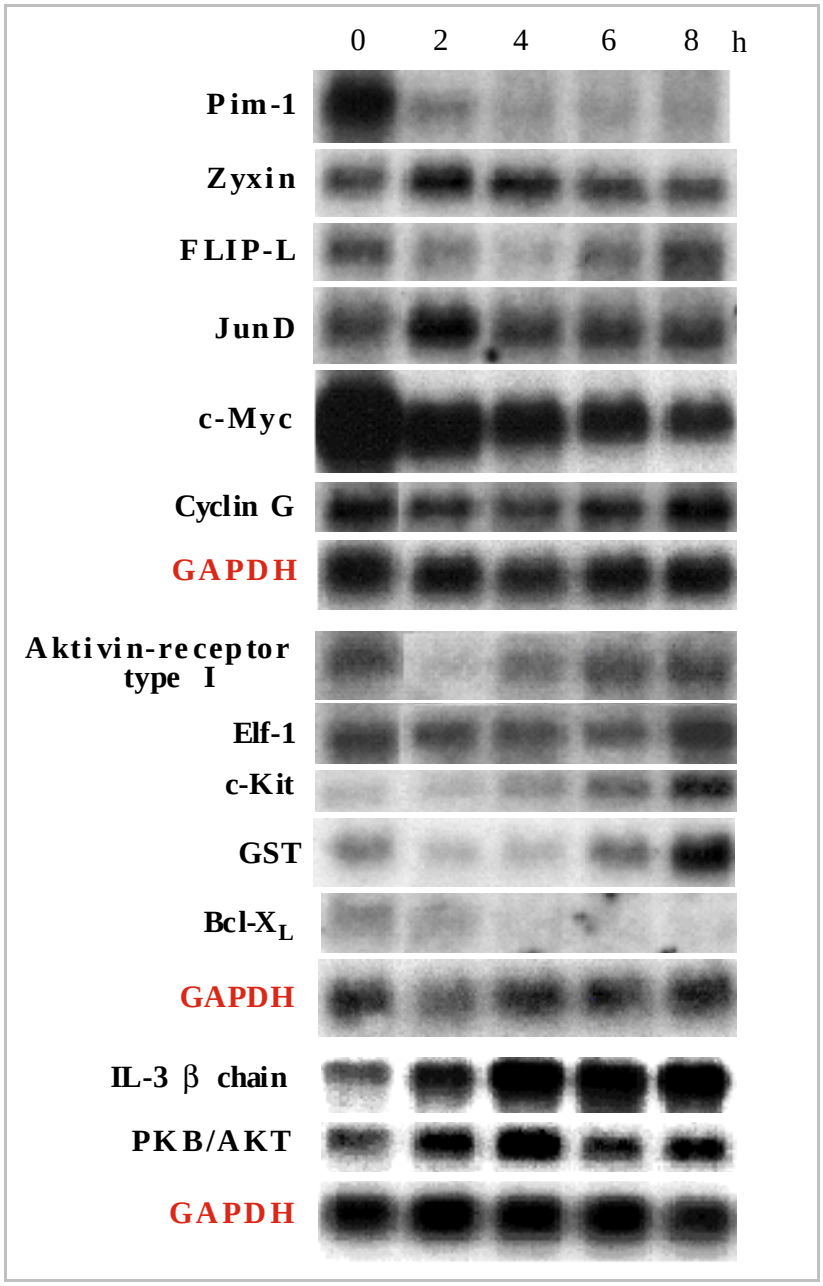


Figure 3.6: Northern blot analysis of differentially regulated genes. PolyA$^+$ RNAs derived from FDCP-1 cells at various times after IL-3 withdrawal were resolved on 1% formaldehyde-agarose gels, blotted onto nylon filters and hybridized to ^{32}P-labeled gene specific probes. Hybridizing bands were visualized by autoradiography using Kodak BioMax MS film with the corresponding intensifying screen.

Moreover, survival functions seemed strengthened by the transient down-regulation of the pro-apoptotic activin receptor (Figure 3.8). In confirmation of previous results, however, the expression of several survival genes appeared IL-3 dependent. Thus, transcripts for anti-apoptotic Pim-1, c-Myc, Bcl-X_L and potent apoptosis inhibitor FLIP-L proteins were repressed in absence of IL-3. Finally, only JunD with arguably pro-apoptotic functions was transiently up-regulated (Figure 3.7) in these experiments.

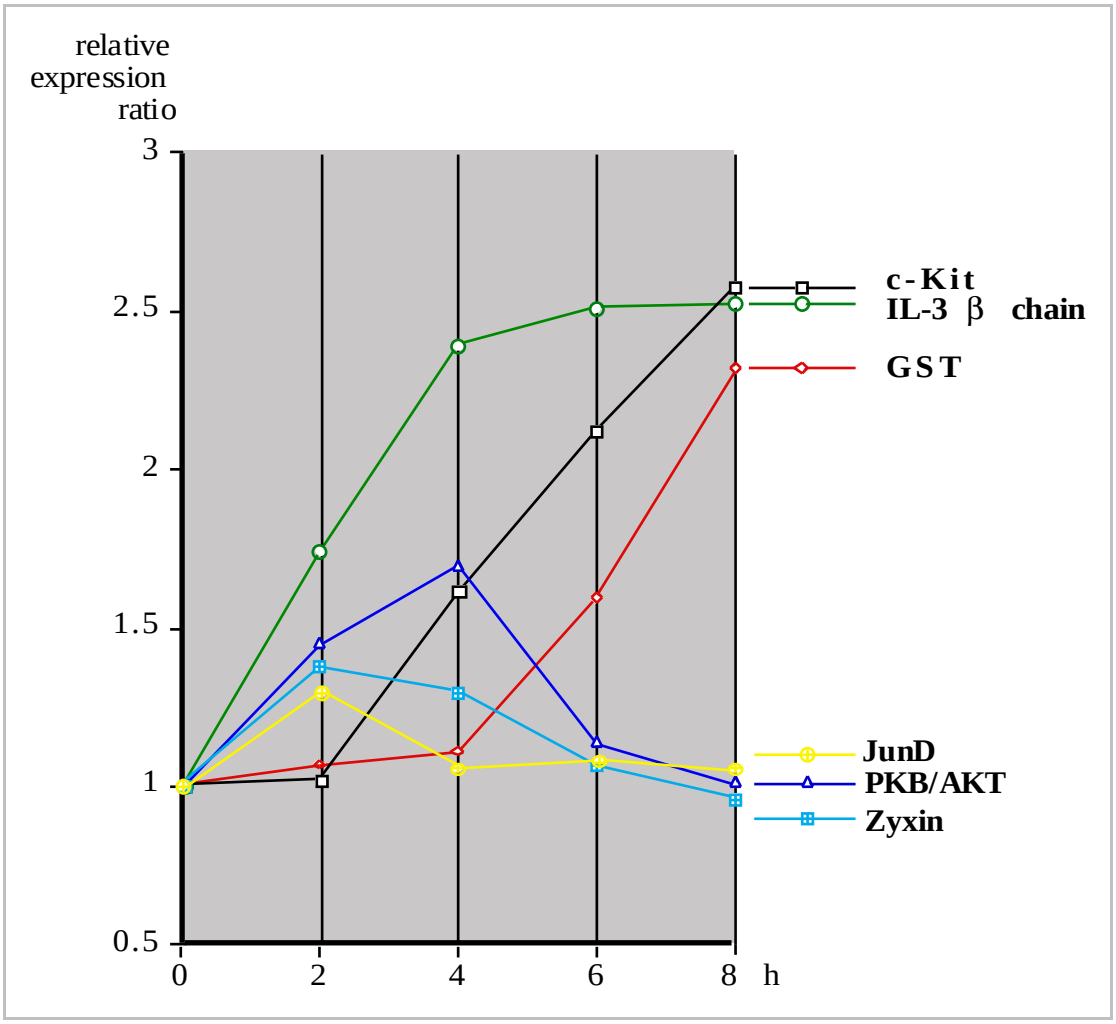


Figure 3.7: Kinetics of gene induction after IL-3 withdrawal. Relative expression ratios were calculated by dividing the signal intensity values obtained with mRNA from FDCP-1 cells after 2, 4, 6, and 8 days of factor withdrawal to the value obtained with mRNA from non-deprived FDCP-1 cells. Signal intensities of Phosphoimager scans were quantified with the ImageQuant software.

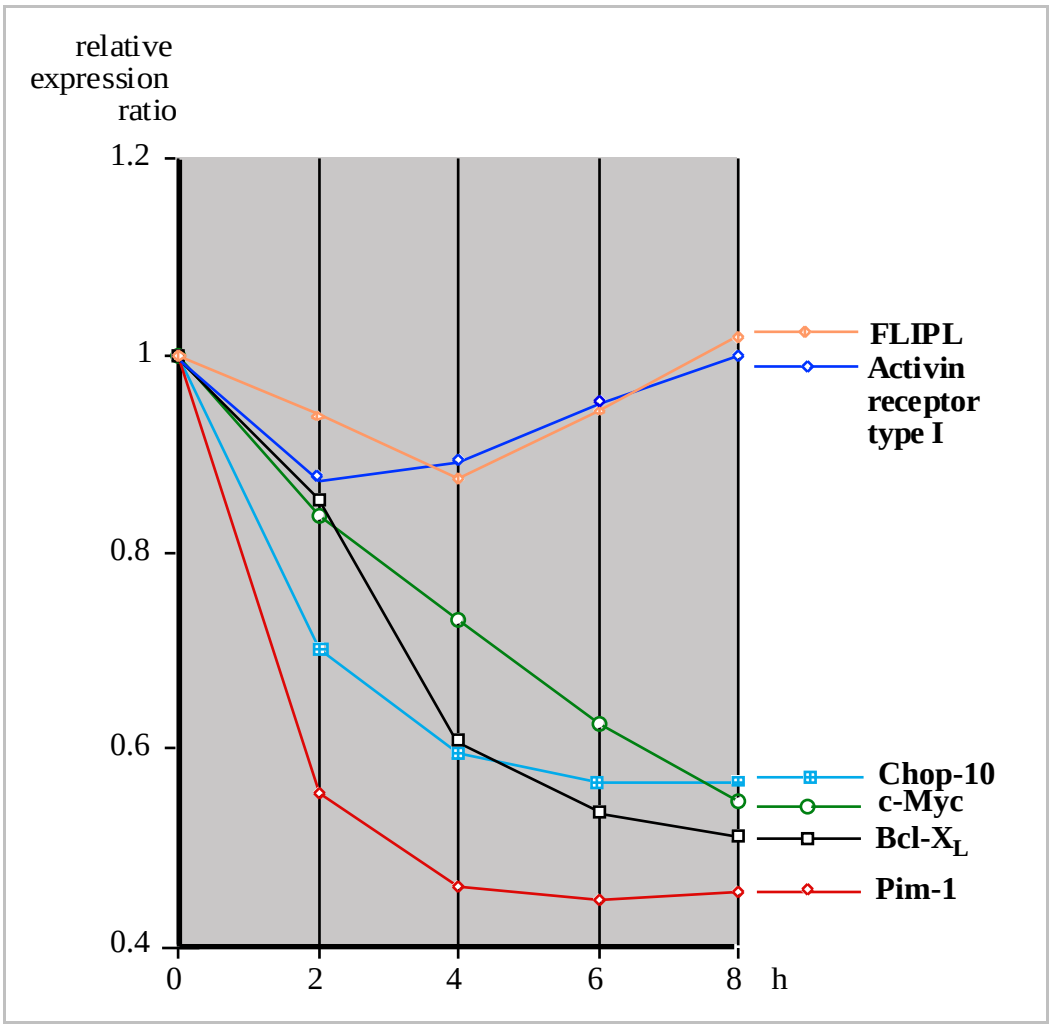


Figure 3.8: Kinetics of gene repression after IL-3 withdrawal. For details see legend to Figure 3.7.

3.3 Affymetrix Genechip arrays detect a large number of genes induced by growth factor deprivation

Since the FDCP-1 integration library for recovering genes induced in cells undergoing apoptosis was created at a time when neither the genome sequence nor reliable high density microarrays were available, it was of interest to compare the efficiency of the gene trap methodology with high throughput expression profiling at a time when both genome sequence and high density microarrays are available. To this end, an Affymetrix chip analysis was performed in collaboration with Schering AG (Berlin). PolyA$^+$ RNAs from factor deprived FDCP-1 cells were hybridized to Affymetrix Mouse A chips containing oligonucleotide probes for 5000 known genes. We found that about 25% of all genes that were comparatively induced at some time

point after IL-3 withdrawal. For practical reason, more than three times up or three times down regulated genes were selected from 5000 known genes (see Table 3.4). They are also the genes that corresponded to literature database (PubMed) for their defined functions.

Of these 35 genes, 24 genes (69%) were related to cell death and survival. As described in Table 3.4, the 21 genes of survival functions are named with red and the 3 genes having cell death functions named with green.

Similarly, 125 genes were obtained from an estimated screen of 100,000 genes in the gene trap approach. Of these 50 genes with defined functions, 28 genes (56%) were related to cell death and survival (see Figure 3.1 and Table 3.1).

This high process specificity together with the significantly lower yield of genes induced by factor deprivation suggests that the gene trap approach detects less process-unrelated genes than the Gene chip arrays. Moreover, the gene trap strategy selects for real gene inductions and, unlike the arrays and Northern blots, is independent of pre-existing steady state mRNA levels subjected to post-transcriptional regulation.

Taken together, these considerations suggest that for the functional analysis of the mammalian genome, gene trapping effectively complements microarray based strategies, which are unable to distinguish between transcriptional and/or post-transcriptional changes of gene expression.

Table 3.5 shows the differential expression of several genes recovered by gene trapping on Affymetrix chips. While over half of these genes were also induced on the chips, a substantial number was unexpectedly repressed.

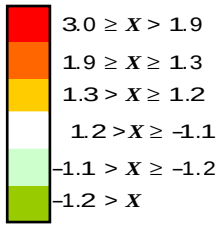
Interestingly, most of these repressed genes were recovered from antisense gene trap insertions (Table 3.5), suggesting that IL-3 deprivation induces gene specific inhibitory (antisense) transcripts. Such potentially inhibitory transcripts are readily detected by gene trapping.

Table 3.4: Regulated genes in FDCP-1 cells by IL-3 withdrawal from Affymetrix Mouse A chips assay*

* The 21 genes with survival functions are named with red; the 3 genes with cell death functions named with green. The numbers in the legends represent fold of regulation in comparison with control (0 h). The positive and negative values indicate up- and down-regulation, respectively. The extent of the regulations is schematically visualized in seven different colors.

Table 3.5: Differentially expressed genes that recovered by gene trapping on Affymetrix Mouse A chips*.

	2h	4h	6h	8h
26-12-4 Vav1 (inv?)				
26-21-3 Rad 50 (inv)				
26-22-1 ATF-1				
26-22-4 ACOX (inv.)				
26-32-4 14-3-3 zeta				
30-11-5 ERK3				
30-12-2 Hexokinase I				
30-21-3 Prefoldin2(inv.)				
30-32-3 Annexin A11	undef			
30-32-16 YB-1				
30-42-7 Syntaxin 3A				
NeuA Siat8d				
Un1g KIAA1140 (inv)				
AW17 Tmp21-I				
AW30 ect2				
AW32 GNA15 (inv)				
AW34 Syntaxin-7 (inv)				
AW607 nin283				
AW608 FORMIN RELATED PROTEIN (inv)				
AW611 PHAPI				
Angiopoetin-1 (inv)				
AW618 MC1-R (inv.)				
AW623 SatB1				



* Blue cells mark genes with sense gene trap insertions. Yellow cells mark genes with antisense gene trap insertions. The numbers in the legends represent fold of regulation in comparison with control (0 h). The positive and negative values indicate up- and down-regulation, respectively. The extent of the regulations are schematically visualized in six colors

3. 4 GTST custom arrays for the identification of cancer-relevant genes

Based on the assumption that some of the cell death and survival genes assembled in the GTST library might be relevant to cancer, the cDNAs from GTSTs were spotted onto nylon membranes to collectively evaluate their differential

expressions in various types of human cancers (Table 3.6). This was intended to identify interesting genetic targets for anti-cancer drug development.

3.4.1 *Custom array production*

In a first step, we attempted to generate probes from all GTSTs. At that time, 72% of the GTSTs were still classified as "novel" (Wempe *et al*, 2001). Therefore the following strategies were used: (i) RT-PCR using primers against the coding regions of known genes, (ii) RT-PCR using primers against GTSTs belonging to unknown genes and (iii) genomic PCR using primers against GTSTs belonging to unknown genes for which RT-PCR failed to produce an amplification product.

To circumvent hybridization kinetics variability with different sized probes, primers were designed to amplify fragments similar in size (although this was not always possible). The sizes of the majority of fragments are listed in Appendix VI. Clean amplification products were obtained for 77 GTSTs (Figure 3.9).

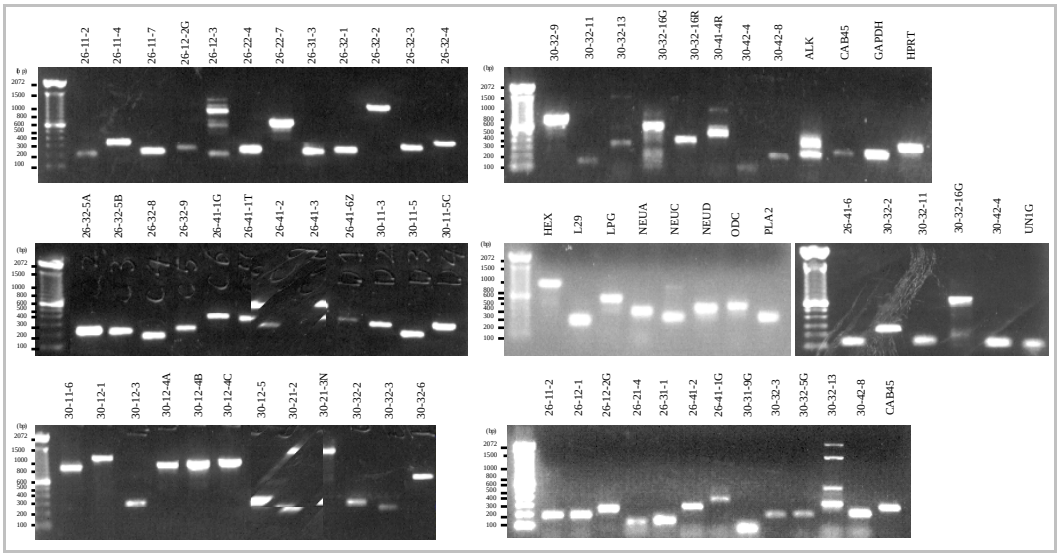


Figure 3.9: GTST probes obtained by PCR. Ethidium bromide stained agarose gels (1~2%) showing the size of the amplification products.

After the amplification, products were verified by sequencing. 100 pmol of each were spotted onto nylon membranes on collaboration with Drs Bernhard Korn and Matthias Schick (Deutsche Krebsforschungszentrum; DKFZ Heidelberg). Figure

3.10 shows probes for each individual gene were spotted in duplicates to eliminate potential non-specific single spot hybridization. Genes and their positions on the self made array are listed in Appendix VIII.

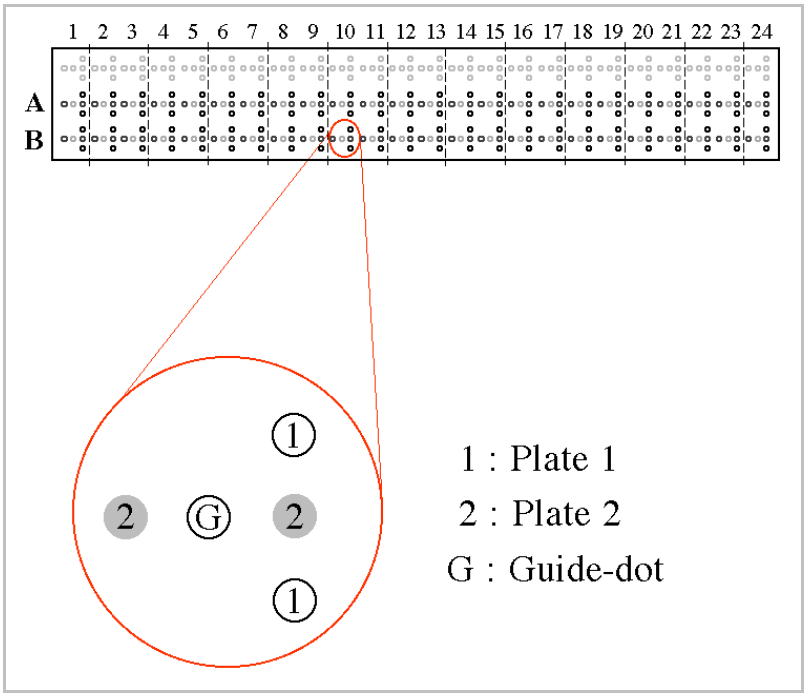


Figure 3.10: Typical self-made array with seventy cDNA probes spotted in duplicates around the guide dot G, 1 and 2 represent different probes.

3.4.2 *Array hybridization of probes from human tumor cells*

We first used a trial and error approach to optimize the detection sensitivity of human transcripts on mouse arrays. Random hexamer and oligo-d(T)$_{18}$ based labeling and array probe-specific primer cocktails (see Section 2.6.4 and Appendix VI for details) were used for labeling the mRNA for array hybridization. Gene specific primers were clearly superior to oligo-d(T)$_{18}$ hexamers as they offered increased sensitivity and produced a lower background.

To check reproducibility, four randomly selected RNA samples from Hel 92.1.7, KM12, SW-620 and HL60 cells (see Table 3.6) were hybridized to the membranes in 2 separate experiments . While some variability was noticed with different RNA preparations, repeated hybridization with the same mRNA gave similar results. Most of tumor cell RNA samples, which were used for hybridization,

were donated from an RNA bank assembled by AstaMedica (Table 3.6). Differential expression of the various genes on cancer cell lines was estimated by comparing spot intensity generated by cancer cells to that generated by normal tissue controls (Table 3.6). Results of the hybridizations are summarized in Table 3.7.

Table 3.6: List of human tumor cell lines and controls used for expression profiling.

Sample Name	Type of cancer	Sample Name	Type of cancer	Sample Name	Type of cancer
BA/F3	Blood	SCC-15	Head&Neck	HS766t	Pancreas
EOL-1	Blood	SCC-25	Head&Neck	PA-TU-8902	Pancreas
BT-20	Breast	HOP-62	Lung	PA-TU-8988T	Pancreas
BT-474	Breast	NCI-H-226	Lung	SU8686	Pancreas
MDA-MB-157	Breast	NCI-H-460	Lung	HUP-T4	Pancreas
MDA-MB-436	Breast	H322M	Lung	YAPC	Pancreas
MDA-MB-453	Breast	BEN	Lung	BPH-1	Prostate
MX-1	Breast	LcLc97M1	Lung		
SK-BR-3	Breast	ScLc214	Lung		
SISO	Cervix	H82	Lung	Control	Source of Sample
CaCo-2	Colon	VM-CuB1	Lung	U937	Eukaryotic cell lines
COLO-205	Colon	SK-Mel1	Melanoma	K562	Eukaryotic cell lines
CX-1	Colon	SK-Mel3	Melanoma	Hel	Eukaryotic cell lines
HCC-2998	Colon	IGROV1	Ovary	DOHH	Eukaryotic cell lines
HCT-15	Colon	OVCAR-3	Ovary	Hel 92.1.7	Eukaryotic cell lines
KM12	Colon	OVCAR-4√	Ovary	Laz509	Eukaryotic cell lines
LOVO	Colon	OVCAR-5√	Ovary	HL60	Eukaryotic cell lines
SW-480	Colon	OVCAR-8	Ovary	Raji	Eukaryotic cell lines
SW-620	Colon	SK-OV-3	Ovary	Tumor	Human lung tissue
CX-14	Colon	SW-626	Ovary	Normal	Human lung tissue
Hec-1a	Endometrium	AS-PC-1	Pancreas	Normal	Human Brain tissue
JShikawa	Endometrium	BXPC-3	Pancreas	Normal	Human colon tissue
SCC-27	Head&Neck	Capan-2	Pancreas	Normal	Human Prostate tissue
SCC-4	Head&Neck	CFPAC-1	Pancreas	Frank	Monocyte (PMNL I)
SCC-9	Head&Neck	DAN-G	Pancreas	Jiyeon	Monocyte (PMNL II)

About 50% of the cDNAs showed hybridization signals from the various mRNA sample. Intensities of individual spots were quantified with the ImageQuant software after exposing the membranes to a Phosphoimager screen, or alternatively, to a Kodak-BioMax X-ray film (Figure 3.11).

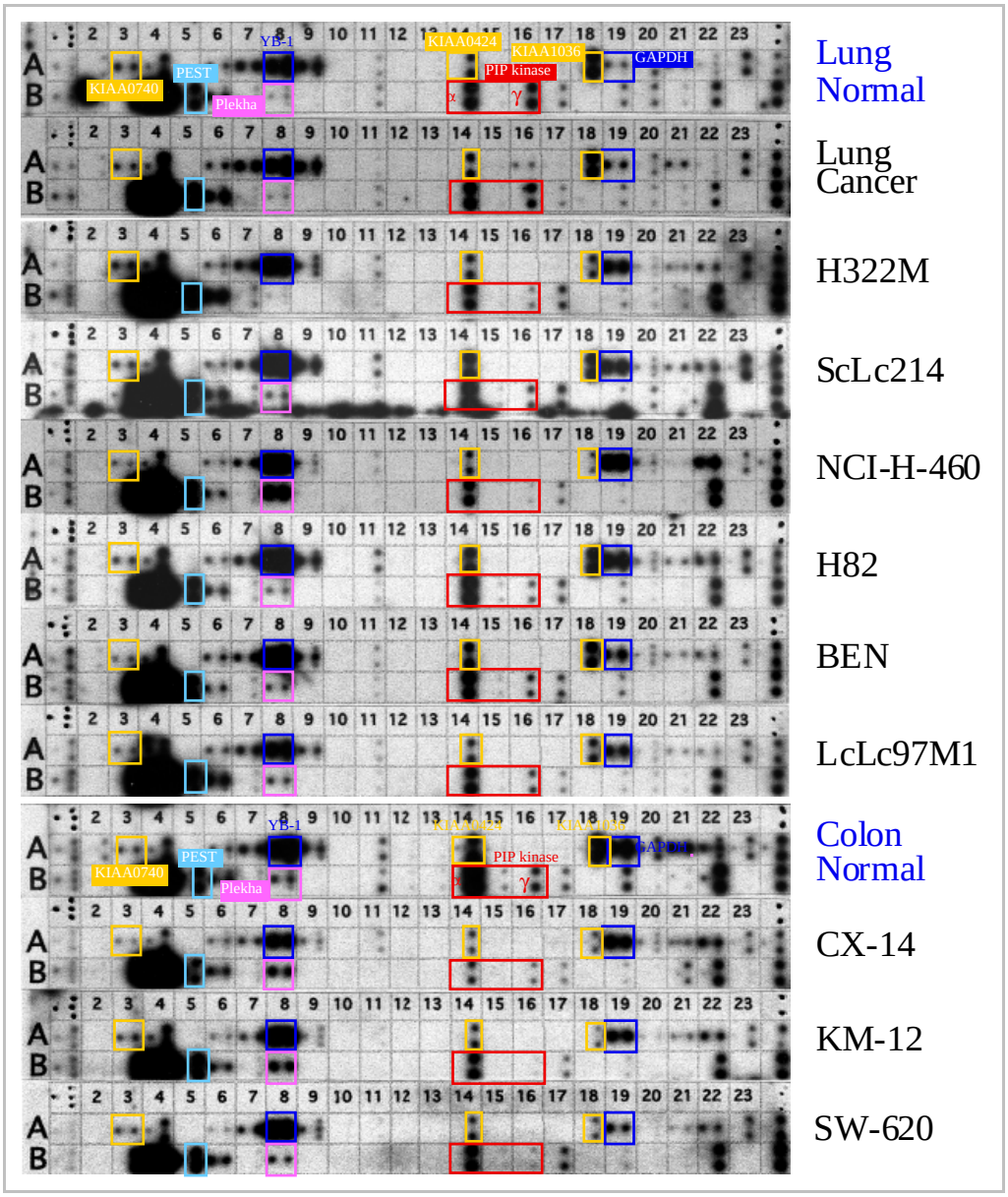
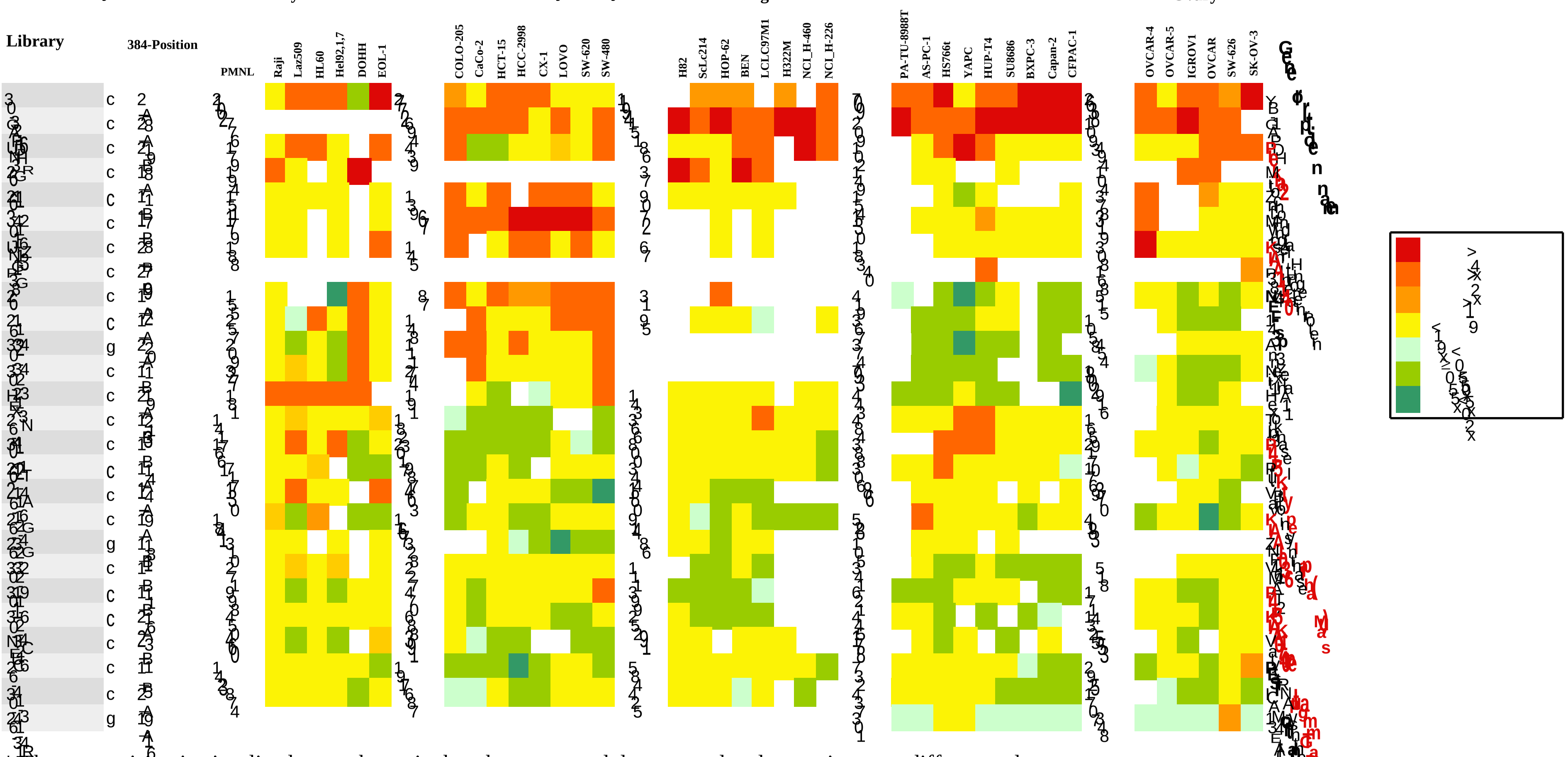


Figure 3.11: Some examples of differential gene expressions of our selected GTSTs in various cell lines. These arrays are chosen from others (see Appendix VII) on the basis of intensity of hybridization signals that are in good contrast to background. These genes (KIAA, PEST, Plekha) are mostly unknown or not well defined in data bases. Lung normal, mRNA prepared from normal lung tissues of 28 year old male (Invitrogen); lung cancer, mRNA from tumor lung tissues of 66 year old female who got moderately differentiated alveous cell carcinoma. Colon normal, pooled mRNA prepared from normal whole colon (with mucosal lining) tissues of two male (ages 35 and 50) who got sudden death (Clontech).

Table 3.7

: Hybridization data obtained with the self made array assay.*



* The comparision is visualized as a schematic that shows up- and down- regulated genes in seven different colors. Red squares, most up-regulated; dark green squares, most down-regulated; yellow, not regulated.

C: cDNA template; G: genomic DNA template (see Figure 2.6)

Of the most highly regulated genes, the following were selected for further analysis: Plekha2, KIAA0740, KIAA1036, KIAA1140, PI4P5K type I α, PI4P5K type I γ and PEST containing nuclear protein.

Since the project was performed on collaboration with Schering AG (Berlin) the selection containing of genes for further analysis included a potential drugability of the targets. ERK3 was additionally selected, since our collaboration partners were especially interested in targets that could be inhibited by kinase antagonists.

3.4.3 *Matched tumor/normal expression array assay*

Commercially available matched tumor arrays (containing sample pairs from 68 patients of different tumor types) were hybridized to probes derived from Plekha2, KIAA0740, KIAA1036, KIAA1140, PI4P5K type I α, PI4P5K type I γ, PEST containing nuclear protein and ERK3. Probes were prepared by RT-PCR using an RNA pool from 5 cancer cell lines and gene specific primers (see section 2.7.1 and Figure 2.9). Hybridization patterns obtained on the matched tumor arrays with the selected probes were as follows.

(I) ERK3 is downregulated in 10 out of 11 colon cancers and in most of the kidney and rectum cancers (Table 3.9). In contrast, ERK3 is significantly up-regulated in breast cancers and to a lesser extend in stomach and uterus cancers.

(II) PIPK β (corresponds to murine PIPK α) was slightly up-regulated in colon cancers.

(III) KIAA0740 was slightly up-regulated in uterus cancer.

(IV) KIAA1140 was slightly up-regulated in stomach cancer, but showed no significant change in the others.

Tumors consist of different cell populations and clones, and usually different parts of a tumor are not separated for the analysis. The aberrations detected in a tumor represent a “pool” of changes. This holds true for the specific expression patterns in various carcinoma tissues. Using matched tumor/normal expression array (Figure 2.8)

analysis we investigated differential gene expressions of our seven target genes in eleven specific tumor types (including patient age and gender, tumor stage, extent of local invasion and sites of metastasis). As described in the following Table 3.16, gains and losses of expression levels of the target genes in the specific tumor types turned out to be genetically extremely complex.

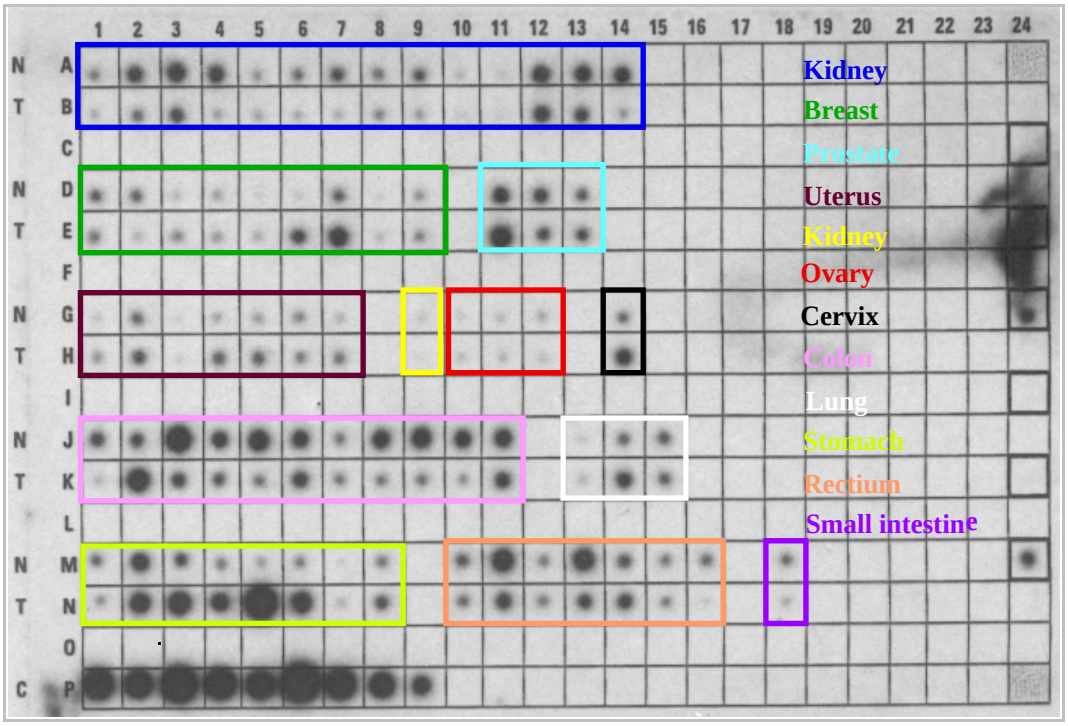


Figure 3.12: Tissue-specific differential expression patterns of ERK3
The colored name indicates tissue types on the membrane:

blue:	kidney	green:	breast
azure:	prostate	dark brown:	uterus
red:	ovary	black:	cervix
pink:	colon	white:	lung
lime:	stomach	terracotta:	rectum
violet:	small intestine		
N:	normal	T:	tumor.

Table 3.8: Generation of differential tumor gene expression ratio against normal tissue gene (data for ERK3 from Figure 3.12)*

Kidney	Nornal	Kidney	Tumor	Expression
A1	0.45	B1	0.1	**0.2**
A2	0.64	B2	0.34	**0.5**
A3	0.84	B3	0.44	**0.5**
A4	0.68	B4	0.13	**0.2**
A5	0.37	B5	0.19	**0.5**
A6	0.4	B6	0.12	**0.3**
A7	0.64	B7	0.1	**0.2**
A8	0.46	B8	0.28	**0.6**
A9	0.51	B9	0.16	**0.3**
A10	0.34	B10	0.15	**0.4**
A11	0.5	B11	0.15	**0.3**
A12	0.95	B12	0.73	**0.8**
A13	0.87	B13	0.5	**0.6**
A14	0.9	B14	0.21	**0.2**
Breast	**Normal**	**Breast**	**Tumor**	**Expression**
D1	0.61	E1	0.48	**0.8**
D2	0.73	E2	0.12	**0.2**
D3	0.13	E3	0.44	**3.2**
D4	0.25	E4	0.36	**1.4**
D5	0.05	E5	0.29	**5.7**
D6	0.05	E6	0.77	**14.6**
D7	0.53	E7	1.16	**2.2**
D8	0.04	E8	0.21	**5.5**
D9	0.42	E9	0.55	**1.3**
Prostate	**Normal**	**Prostate**	**Tumor**	**Expression**
D11	0.61	E11	0.82	**1.3**
D12	0.52	E12	0.46	**0.9**
D13	0.29	E13	0.33	**1.1**
Uterus	**Normal**	**Uterus**	**Tumor**	**Expression**
G1	-0.01	H1	0.18	**0**
G2	0.66	H2	1	**1.5**
G3	-0.05	H3	-0.05	**0**
G4	0.1	H4	0.31	**3**
G5	0.18	H5	0.27	**1.5**
G6	0.45	H6	0.63	**1.4**
G7	0.12	H7	0.37	**3.2**
Ovary	**Normal**	**Ovary**	**Tumor**	**Expression**
G10	0.14	H10	0.1	**0.7**
G11	0.26	H11	0.25	**1**
G12	0.21	H12	0.14	**0.6**

Cervix	Normal	Cervix	Tumor	Expression
G14	0.25	H14	0.46	**1.9**
Colon	**Normal**	**Colon**	**Tumor**	**Expression**
J1	0.44	K1	0.11	**0.3**
J2	0.94	K2	1.5	**1.6**
J3	1.03	K3	0.65	**0.6**
J4	0.66	K4	0.45	**0.7**
J5	0.92	K5	0.48	**0.5**
J6	0.97	K6	0.78	**0.8**
J7	0.36	K7	0.32	**0.9**
J8	1.11	K8	0.4	**0.4**
J9	0.87	K9	0.38	**0.4**
J10	1.06	K10	0.31	**0.3**
J11	1.01	K11	0.72	**0.7**
Lung	**Normal**	**Lung**	**Turmor**	**Expression**
J13	0.08	K13	0.08	**1**
J14	0.48	K14	0.8	**1.7**
J15	0.59	K15	0.65	**1.1**
Stomach	**Normal**	**Stomach**	**Tumor**	**Expression**
M1	0.58	N1	0.2	**0.3**
M2	1.17	N2	1.09	**0.9**
M3	0.79	N3	1.13	**1.4**
M4	0.63	N4	1	**1.6**
M5	0.19	N5	0.87	**4.6**
M6	0.32	N6	0.98	**3**
M7	-0.04	N7	0.21	**0**
M8	0.46	N8	0.7	**1.5**
Rectium	**Normal**	**Rectium**	**Tumor**	**Expression**
M10	1.02	N10	0.73	**0.7**
M11	1.2	N11	0.73	**0.6**
M12	0.43	N12	0.34	**0.8**
M13	1.03	N13	0.63	**0.6**
M14	0.46	N14	0.55	**1.2**
M15	0.72	N15	0.65	**0.9**
M16	1.03	N16	0.08	**0.1**
SI	**Normal**	**SI**	**Tumor**	**Expression**
M18	0.81	N18	0.27	**0.3**

* The hybridization signals were detected from the obtained phosphorimage. Values shown in second and forth columns are those after normalization. The final value of ERK3 expression ratio (in fifth column) was converted into a schematically visualized table (as shown in Table 3.16).

Table 3.9: Tissue-specific differential expression of ERK3 in eleven organs (tumor tissue against the matching normal tissue).

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean ± SE	0.5±0.08	7.4±2.4*	1.1±0.1	2.3±0.3	0.85±0.09	1.9

	Colon	Lung	Stomach	Rectum	SI
Mean ± SE	0.95±0.2	1.35±0.2	2.45±0.8	0.65±0.2	0.3

* In the breast, the differential expressions of ERK3 was significant, although the standard error value (SE) was high because of relatively strong variations among individual patients.

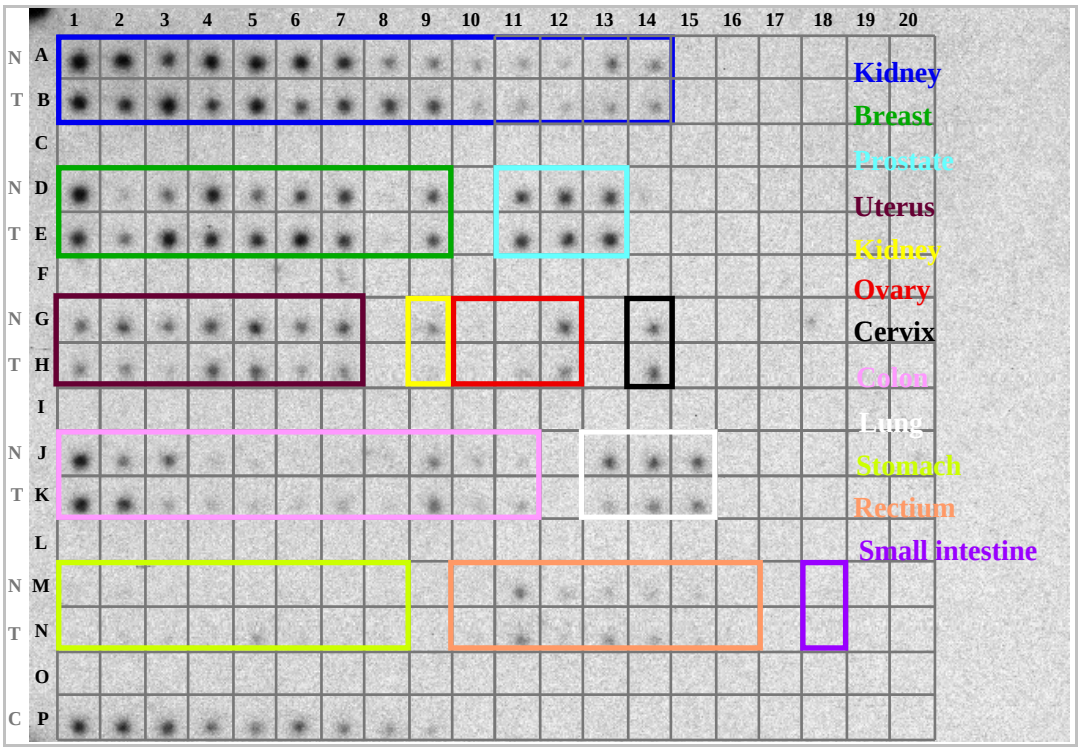


Figure 3.13: Tissue-specific differential expression patterns of PIPK β between normal and tumor tissues. As shown in 4E, PIPK β expression has been shown to be not regulated in a patient who got medullary carcinoma but with no metastasis.

Table 3.10: Tissue-specific differential expression of PIPK β in eleven organs* (tumor tissue against the matching normal tissue)

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	0.95±0.12	1.1±0.1	1.0	0.6±0.08	1.15±0.3	0.8

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	1.6±0.2	0.7±0.18	0.85±0.16	1.2±0.15	1.0

* The expressions of PIPK β were slightly down-regulated or not changed at all from 7 out of 11 specific tumor types. Probably, differential expression of PIPK β is not a prime feature of these cancers.

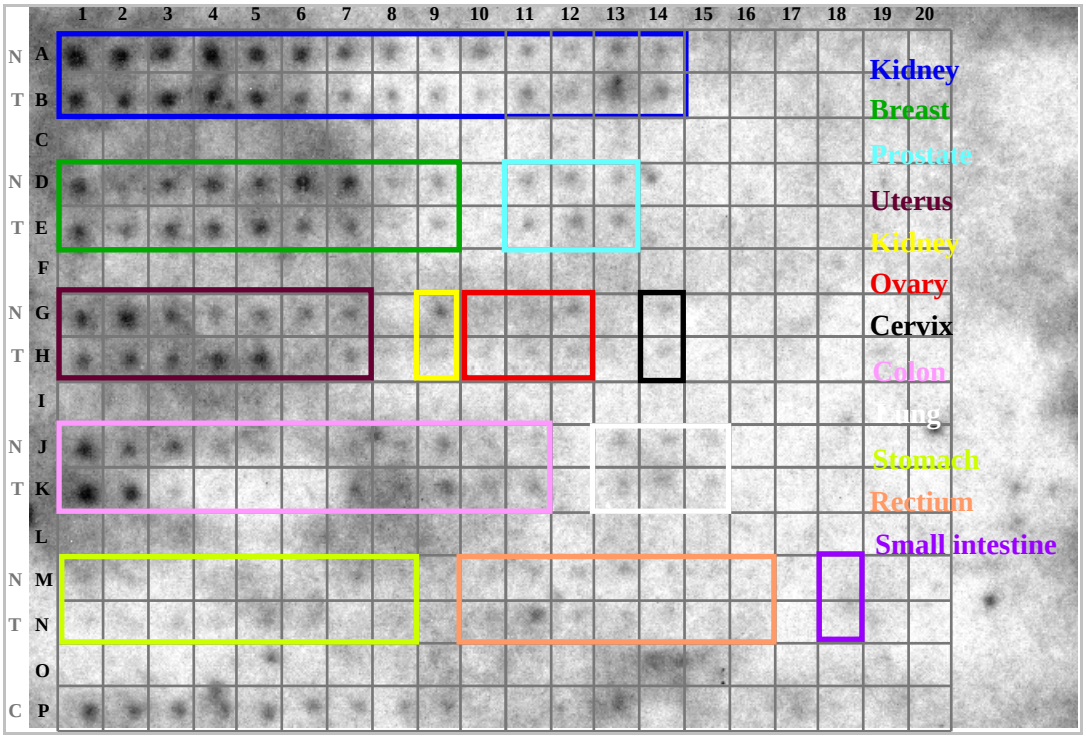


Figure 3.14: Tissue-specific differential expression patterns of PIPK γ between normal and tumor tissues. The phosphorimage was obtained following five days exposure to the imaging plate. *This array and Figure 3.16 have partially darker background that results in variation in gene expression analysis. Therefore, some values (Table 3.11) of differential expressions of PIPK γ couldn't be regarded as genuine differences.

Table 3.11: Tissue-specific differential expression of PIPK γ in eleven organs (tumor tissue against the matching normal tissue).

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	0.7±0.1	0.8±0.07	1.05±0.15	0.9±0.12	0.5±0.06	0.8

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	1.5±0.4	1.3±0.1	0.55±0.16	1.3±0.4	1.0

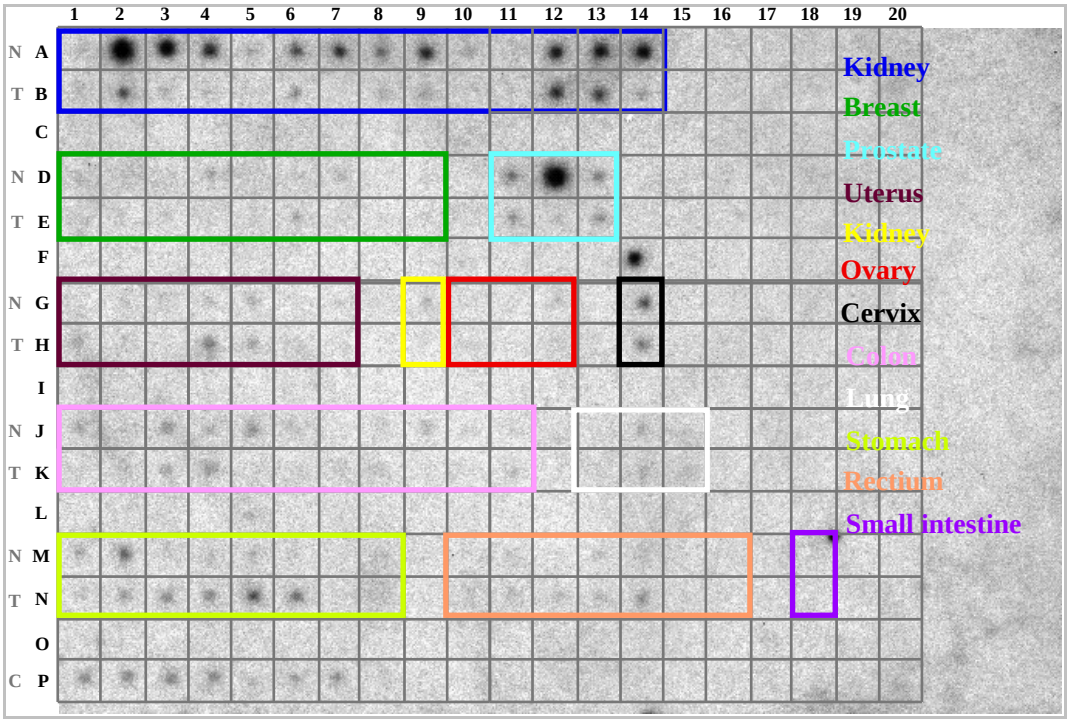


Figure 3.15: Tissue-specific differential expression patterns of KIAA0740 between normal and tumor tissues. The phosphorimage was obtained following three days exposure.

Table 3.12: Tissue-specific differential expression of KIAA0740 in eleven organs (tumor tissue against the matching normal tissue)

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	0.5±0.08	0.85±0.15	0.4±0.18	3.05±1.0*	1.1	0.6

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	1.25±0.04	1.13±0.2	1.4±0.3	1.2±0.3	0.9

***** In the uterus, the differential expressions of KIAA0740 was significant, although the standard error value (SE) was high because of relatively strong variations among individual patients

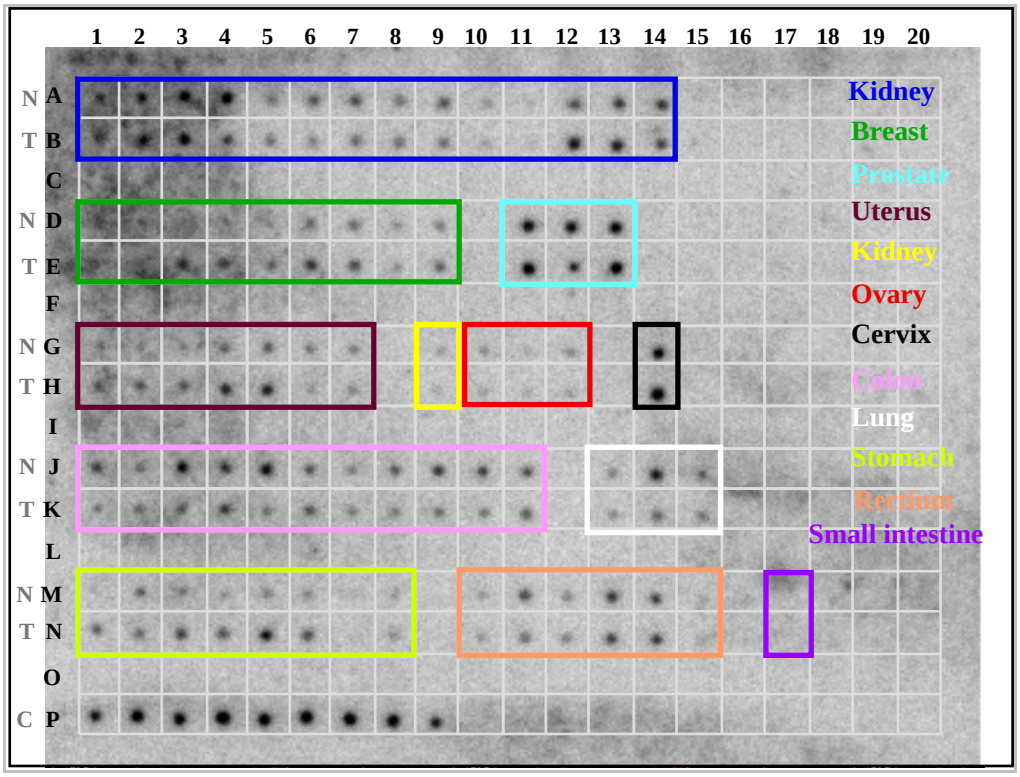


Figure 3.16: Tissue-specific differential expression patterns of KIAA1036 (after five days exposure). As explained in Figure 3.14, the partially darker background that results in variation in gene expression analysis.

Table 3.13: Tissue-specific differential expression of KIAA1036 in eleven organs (tumor tissue against the matching normal tissue)

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	1.0±0.1	1.35±0.15	1.0±0.3	1.4±0.2	0.9±0.2	1.4

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	0.83±0.2	0.6±0.35	1.53±0.09	0.95±0.1	1.1

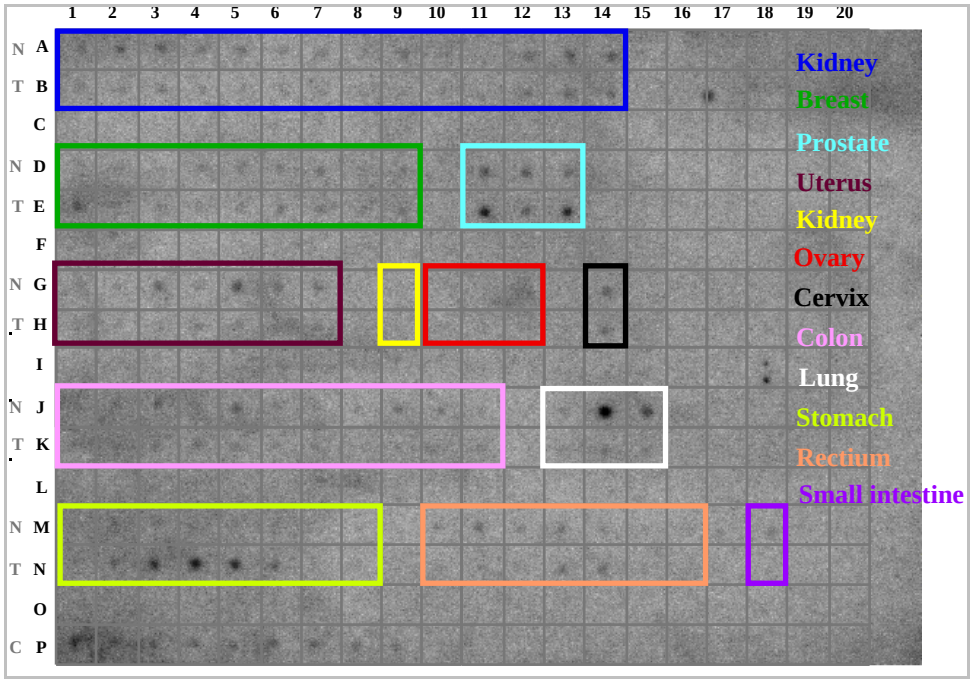


Figure 3.17: Tissue-specific differential expression patterns of KIAA1140 (five days exposure). This array and Figure 3.18 are uniformly darker than the other arrays and that has an effect on the apparent variation in expression levels of the genes for a comparison analysis with the other results .

Table 3.14: Tissue-specific differential expression of KIAA1140 in eleven organs (tumor tissue against the matching normal tissue)

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	0.85±0.07	1.1±0.3	1.25±0.09	0.7±0.2	0.8	0.7

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	0.9±0.2	0.6±0.17	2.3±0.07	1±0.08	0.7

.

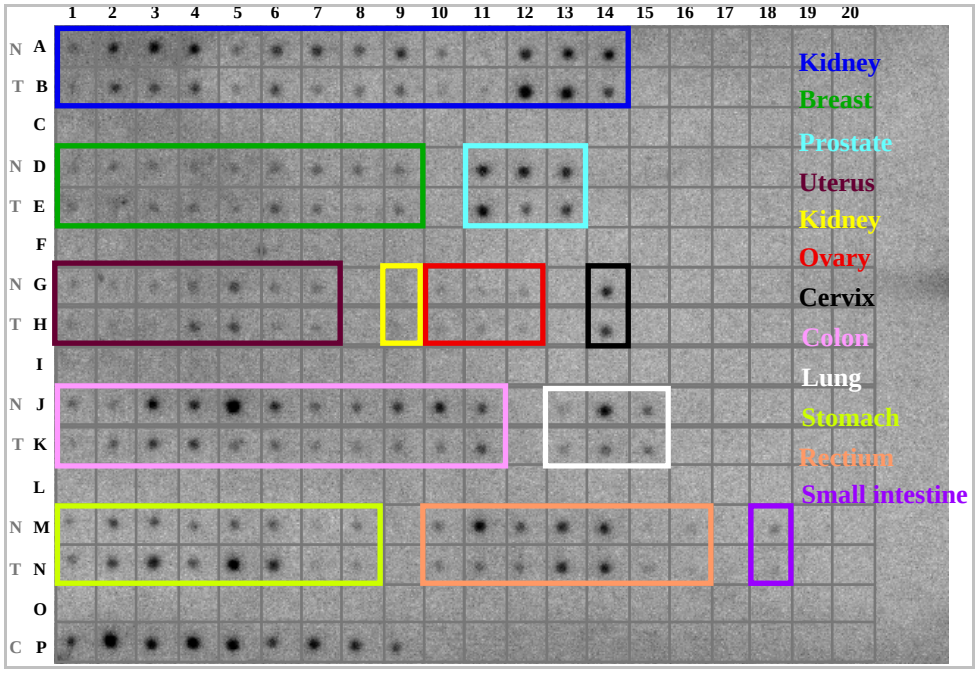


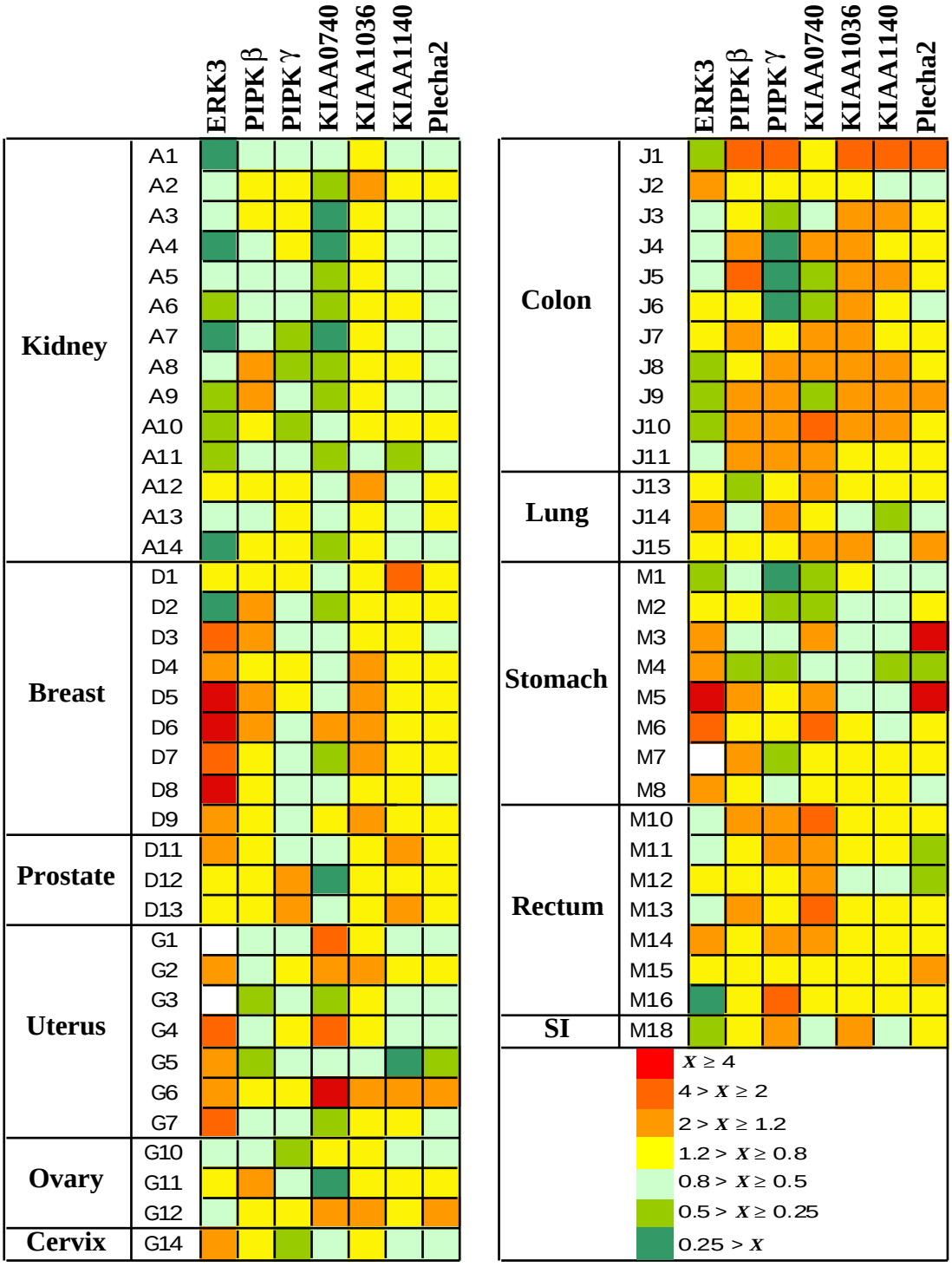
Figure 3.18: Tissue-specific differential expression patterns of Plekha2 .

Table 3.15: Tissue-specific differential expression of Plekha2 in eleven organs (tumor tissue against the matching normal tissue).

	Kidney	Breast	Prostate	Uterus	Ovary	Cervix
Mean±SE	0.8±0.08	0.9±0.1	0.85±0.03	0.9±0.15	0.85±0.03	1.0

	Colon	Lung	Stomach	Rectum	SI
Mean±SE	0.75±0.3	0.95±0.2	1.7±0.1	0.95±0.2	0.9

Table 3.16: Seven target genes with relevance to various human tumor tissues*



* The numbers in legends indicate the fold of regulation in cancer against normal tissues. Values greater than 1.2 are defined as up-regulation and those smaller than 0.8 are down-regulation. The magnitude of regulation is visualized in different color.

CHAPTER 4

CHAPTER 4

DISCUSSION

Apoptosis is the counterpoint to proliferation in the regulation of cell growth. The operational activities of cells are based on an awareness of their current state, coupled to a programmed response to internal and external cues in a context-dependent manner. The essence of the balance between life and death is the ability to express only certain portions of the genome in particular cells at particular times (Dunham, 2000; Levy and Darnell, 2002; Kavurma and Khachigian, 2003).

Apoptotic genes are activated or inactivated in a series of events that underlie the multistage development of life and death. Therefore, these incidents possibly reflect the differential and coordinate expression of specific genes (Gillen *et al*., 1998) in the maintenance of the balance between cell growth and cell death (Thompson *et al*., 1995).

After the removal of the survival factors, the cytokine-dependent hematopoietic cell lines undergo apoptosis at a greatly accelerated pace (Askew *et al*., 1991; Evan *et al*., 1992; Askew *et al.*, 1993; Juin *et al*., 1999; Juin *et al*., 2002). Our previous studies have shown that the stimulation of survival (anti-apoptotic) mechanisms during the early stage of apoptosis induced by IL-3 deprivation results in reduced apoptosis and improved survival of cells. Part of our findings is supported by the studies of others that established the relevancies between some regulators (e.g., c-Myc, Bcl-2, Bcl-X_L and Pim-1) in anti- and pro-apoptotic pathways.

Since the constitutive up-regulation of survival genes and/or the inactivation of pro-apoptotic genes are hallmarks of cancer, the susceptibility of tumor cells to apoptosis induced by antitumor drugs could depend on the balance between pro-apoptotic and survival (anti-apoptotic) gene regulation (Tsuruo *et al*., 2003).

The goal of this thesis was to identify genes that are differentially expressed during genes apoptosis and tumorigenesis.

To achieve this, a gene trap strategy was employed to recover transcriptionally regulated genes in FDCP-1 cells undergoing apoptosis after growth factor (IL-3) withdrawal. Furthermore, the efficiency of recovering such genes by gene trapping was compared to the efficiency of recovering similar genes on Atlas arrays and Affymetrix chips. To identify cancer related genes, custom arrays containing 70 gene trap sequence tags (GTSTs) isolated from FDCP1 cells undergoing apoptosis were hybridized to mRNAs derived from a large collection of cancer cell lines. Finally, to define potential targets for anti-cancer drug development, several candidate genes identified on the custom arrays were used as probes on matched tumor/normal-arrays from patients with various types of cancer. Of all identified targets, the most promising was the ERK-3 serine/threonine kinase because it is highly and specifically upregulated in breast and uterine cancers and susceptible to synthetic antagonists (= drugable).

4.1 Assessment of the technologies

Gene trap technology is a powerful tool for identifying genes, whose expression is induced by certain biological stimuli (Wempe *et al*., 2001). In this effort, cells with gene trap insertions in loci transcriptionally induced by IL-3 withdrawal were selected from a random integration library in FDCP-1 cells. From 2 x 10^6 unique gene trap insertions, we recovered a total of 102 useful gene trap sequence tags (GTSTs). A significant proportion of the trapped genes with well-defined functions (28 out of 50 = 56%) were either directly or indirectly involved in cell survival and/or cell death. A third of these genes (9 out of 28) were directly related to the IL-3 - and small Rho-GTPase survival pathways (Table 3.1).

To compare the gene trap approach with other strategies used to identify differentially expressed genes, we first hybridized commercially available Atlas cDNA arrays (Clontech) to mRNAs from FDCP1 cells undergoing apoptosis. Out of

588 genes, 47 appeared differentially expressed. Out of these, only 13 could be confirmed as genuinely induced by IL-3 withdrawal.

Similar experiments were performed using Affymetrix chips containing oligonucleotide probes from 5000 known mouse genes. At low stringeny about 25% of all genes seemed inducible by IL-3 withdrawal. However, at high stringency (over 3 fold up- or downregulated), only 34 genes appeared differentially expressed. Of these, 24 genes (69%) were directly related to cell death and survival.

Although the number of relevant genes recovered by the two different technologies (gene trapping vs. arrays) is quite similar (56% and 69%, respectively), the type of genes is different. First, unlike most array technologies, gene trapping has no bias for highly expressed genes and readily identifies subtle changes in gene expression. Second, gene trapping selects for real gene inductions and, unlike arrays or Northern blots, is independent of pre-existing steady state of mRNA levels subjected to posttranscriptional regulation. Taken together, the results suggest that gene trapping effectively complements microarray based strategies unable to distinguish between transcriptional and post-ranscriptional gene regulation.

Interestingly, several genes recovered by gene trapping that were also represented on the Affymetrix arrays were regulated in the opposite direction on the chips. With one exception, the genes down-regulated on the chips corresponded to GTSTs obtained from anti-sense gene trap insertions. As repression correlated with anti-sense gene trap insertions, it is likely that the anti-sense transcripts induced by IL-3 deprivation have an inhibitory function. Thus, unlike array technologies, gene trapping can detect potential inhbitors of specific gene expression. The presence of overlapping anti-sense transcripts has been demonstrated for several genes (e.g., cyclin E2 and p53BP1, Yelin *et al.*, 2003) and it has been speculated that they might have regulatory functions (Bains *et al.*, 1997; Carmichael, 2003)

Fifty percent of all gene trap insertions recovered in these expreriments were activated from non-coding strands. Compared to other gene trap screens performed in our laboratory, the frequency of antisense gene trap insertions is relatively high and may reflect the high sensitivity of the Cre-recombinase reporter gene used for creating the FDCP1 integration library. Alternatively, as most antisense transcripts are non-coding, lack of upstream ATGs may favor the selection of the Cre ATG as the translation initiation codon in the fusion transcript. It is predicted that over 8% of human genes have antisense transcripts (Yelin *et al*., 2003), although their significance still remains to be established. In many cases sense-antisense overlaps could have regulatory functions as has been shown repeatedly for short interfering RNAs (RNAi) (Hannon 2002).

In summary, gene trapping appears to provide an effective and sensitive tool for the functional annotation of the mammalian genome.

4.2 Expression profiling of the trapped genes

4.2.1 *Genes recovered by gene trapping*

A total of 28 genes involved in cell death and survival were recovered from a U3Cre gene trap integration library in FDCP1 cells. These genes can be classified into 4 functional groups:

I. Apoptosis or survival-related proteins

Formin/diaphanous-related protein (FHOS), is a member of the formin homology (FH) protein family which is implicated especiall that interacts with Rac-1, a small Rho-GTPase and activates transcription from the serum response element (Westendorf, 2001). Similarly, another member of this family, the **Formin-related protein (FRL)** also binds Rac-1 and when expressed as a dominant negative deletion mutant reduces attachment which initiates apoptosis (anoikis) in cell culture (Yayoshi-Yamamoto *et al*., 2000). Further examples are several guanine nucleotide exchange factors (GEFs) such as **NET1**, **Ect2**, **Vav-1** and **Collibistin II** (Table 3.2).

The GEFs belong to the Rho-subfamily of GTPases, which regulate cell growth and can transform cells in culture (Grosskreutz *et al.*, 2001; Schmidt and Hall, 2002; Bassermann *et al.*, 2002; Piccolella *et al*., 2003). This transformation is dependent on the highly conserved DB-1 homology (DH) domain and in most cases requires N-terminal truncation of the protein. Interestingly, a novel splice variant of NET1, NET1A, was found to encode an N-terminally truncated protein that possesses transforming activity (Alberts and Treisman, 1998). In addition to the DH domain, GEFs have a pleckstrin homology domain that connects the Rho signal transduction pathway to the phosphatidyl-3-phosphate kinase (PI3K) signal transduction pathway (Bellanger *et al*., 2000; Blomquist *et al*., 2000). The latter is responsible for mediating survival signals, particularly in hematopoietic cells, and is directly activated by IL-3 (Guthridge *et al*., 2000). Surprisingly, two members of this pathway, the phosphatidylinositol 4,5-**phosphate kinases (PIP5K) type I**β (which corresponds to human PIP5K type Iα) and **type I**γ, were induced by IL-3 withdrawal. PIP5K converts the lipid phosphatidylinositol 4-phosphate (PI4-P) to phosphatidylinositol 4,5-bisphosphate (PI4,5-P_2), which enhances cell survival by at least three mechanisms. First, PI4,5P_2 directly inhibits apoptosis by inactivating caspases 8, 9 and 3. Moreover, PIP5K type Iα overexpression in cultured cells prevents apoptosis induced by caspase 9 or TNF-α (Secchiero *et al*., 2003). Second, PI3K phosphorylates PI4,5-P_2 to phosphatidylinositol 3,4,5-triphosphate (PIP_3), which activates the survival kinase **Akt**. Akt prevents cell death by phosphorylating and inactivating the pro-apoptotic proteins BAD, caspase 9 and the forkhead family transcription factor (FKHRL1). FKHRL1 induces the expression of Fas-ligand (Suhara *et al*., 2002). Phosphorylated BAD is sequestrated and thereby inactivated by **14-3-3** ζ isoform (Shinoda *et al.*, 2003). Interestingly, 14-3-3 ζ was also trapped in these experiments. Third, PIP5K activates ATP-dependent potassium channels thereby helping to maintain the polarity of the cell membrane (Shyng *et al*., 2000). As has been shown previously, apoptotic signals can cause membrane depolarization that leads to cell death by directly inhibiting potassium channels (Pei *et al*., 2003).

II. Transcription factors

The transcription factor **YB-1** is directly involved in cell survival by repressing Fas gene expression (Lasham *et al*., 2000). Moreover, YB-1 activates the expression of the multi-drug resistance gene (MDR1) whose product, the P-glycoprotein, is an efflux pump that eliminates a variety of toxins from the cell, including pro-apoptotic anticancer drugs (Saji *et al*., 2003).

ATF1, which binds the same DNA motive like the Hypoxia induced factor-1 (HIF-1), activates a signal transduction pathway leading to increased expression of genes known to compensate for hypoxic or oxidative stress (Zaman *et al*., 1999).

Vezf1/DB1 is described as an endothelial transcription factor that regulates endothelin-1 expression, which has been shown to be a survival factor during c-Myc induced apoptosis (Aitsebaomo *et al*., 2001; Louro *et al*., 2002).

Tel, a member of the Ets family of transcription factors, is a fusion partner of AML1 or ABL in leukemic translocations (Hannemann *et al*., 1998). Overexpression of Tel leads to reduced cell growth, even in cells transformed by activated Ras (Sakurai *et al*., 2003).

BTEB1, a ZNF-containing transcription factor, promotes cell growth by inducing several cell-cycle regulating genes (Zhang *et al*., 2002).

ASC-1 was originally isolated as a transcriptional coactivator of nuclear receptors. However, it also appears to play an essential role in transactivation through AP-1, SRF, and NF-κB (Jung *et al*., 2002) and could thus potentially modulate survival responses of cells.

SatB1 is a nuclear protein that recruits chromatin remodeling proteins to AT-rich DNA. SatB1 is cleaved by caspase 6 in thymocytes, which leads to its detachment from the DNA (Galande *et al*., 2001). SatB1 seems to be involved in the apoptotic clonal deletion during thymocyte maturation (Yasui *et al*., 2002).

III. Proteins for DNA repair

The mouse homologs of the bacterial **AlkB,** the yeast **rec8** and **rad50** genes were recovered as DNA-repair genes. While AlkB protects DNA from damage by

alkylating agents (Wei *et al*., 1996), rec8 and rad50 are proteins responsible for double DNA strand break reapir (Parisi *et al*., 1999; Dasika *et al*., 1999; Luo *et al*., 1999). The double strand breaks develop as a result of any apoptotic process.

IV. Proteins with diverse functions

VMAT2 (vesicular monoamine transporter 2) is a cytoprotective protein. It moves cytoplasmic monoamine transmitters into secretory vesicles and prevents cell death by sequestering pro-apoptotic molecules such as 1-methyl-4-phenylpyridinium (MPP^+) and histamine (Moriyama and Yamada, 1996; Grundemann *et al*., 1999).

NADPH-cytochrome P450 oxidoreductase transfers electrons from NADPH to cytochrome P450. By catalyzing the one-electron reduction of many drugs and xenobiotic compounds (Smith *et al.*, 1994), this oxidoreductase is part of the cell's detoxifying machinery and thus promotes survival.

Hexokinase I is a common metabolic enzyme of glycolysis. However, it has been demonstrated that increased hexokinase activity protects renal epithelial cells against acute oxidant-induced cell death (Bryson *et al*., 2002) and that mitochondrial hexokinase association promotes both mitochondrial integrity and cell survival following growth factor withdrawal (Gottlob *et al*., 2001)

ERK3 (extracellular signal-regulated kinase 3) is a recently characterized member of the MAPK (mitogen-activated protein kinases) signal transduction protein family. It is involved in the ubiquitin-proteasome pathway of protein degradation and assumed to regulate cellular differentiation (Coulombe *et al*., 2003). Since ERK-3 is likely an interesting genetic target for anti-cancer drug development it will be discussed in more detail in Section 4.4.

Annexin V: There are numerous reports that overexpression of annexins (annexin A6 and annexin A11 were trapped) leads to subsequent apoptosis (Canaider *et al*., 2000; Kirsch *et al*., 2000; Solito *et al*., 2001). Previous findings show that annexin-induced apoptosis is connected to annexin dependent Ca^{2+} influx into the cells (Hawkins *et al*., 2002; Wang *et al*., 2003).

Smurf1, an E3 ubiquitin ligase for bone morphogenetic protein-specific Smads, is recruited by Smad-7 to the TGF-β (transforming growth factor-beta) type I receptor, resulting in proteasome-dependent degradation of the receptor (Ebisawa *et al.*, 2001). As TGF-β is known to prevent epithelial cell proliferation and to induce apoptosis in lymphocytes, liver and prostate (Schuster and Krieglstein, 2002), Smurf1 could play an important modulatory role in these processes by regulating receptor availability.

EDEM (endoplasmic reticulum-degradation-enhancing α-mannosidase–like protein) is a quality control receptor responsible for sorting terminally malfolded glycoproteins that are destined to destruction (Wang and Hebert, 2003)

MC1-R (melanocyte-stimulating hormone receptor) stimulates proliferation and survival in melanocytes (Kadekaro *et al.*, 2003).

UREB1 (upstream regulatory element binding protein 1) is a nuclear phospho-protein that inhibits p53-stimulated transcription (Gu *et al.*, 1995). Since p53 is a critical regulator of growth arrest and apoptosis, UREB1 promotes cell survival by inhibiting p53 function.

PHAPI (putative HLA-DR-associated protein I), initially identified as an MHC class I binding protein proteins, activates caspase 9 within the apoptosome (Jiang *et al.*, 2003) and thus suppresses tumor growth.

In conclusion, a large fraction of genes recovered by gene trapping encode survival functions. Their induction after IL-3 withdrawal seems to reflect a short lived cellular response to prevent cell death induced by an accidental exposure to an apoptotic stimulus such as temporary factor deprivation. In line with this temporary activation of a survival program is the phenomenon of apoptotic preconditioning where a cell epxosed transiently to an apoptotic stimulus becomes more resistant to a subsequent permanent apoptotic stimulus (Wempe *et al.*, 2001).

However, several genes with proapoptotic functions were also induced by factor deprivation, suggesting that the balance between pro- and antiapoptotic

functions is crucial for the final outcome. During growth factor withdrawal apoptosis, this balance is tilted initially towards survival (Wempe *et al*., 2001).

4.2.2 *Genes recovered by Atlas arrays*

Twelve out of the 588 genes spotted onto the Atlas arrays (2%) were differentially expressed in IL-3 deprived cells, as the observed regulation could be confirmed by Northern blotting. All these twelve genes are linked to cell death and survival functions.

4.2.2.1 Genes downregulated by IL-3 withdrawal

The **c-Myc** proto-oncogene (Selten *et al*., 1984; Pelengaris *et al*., 2002) is an important positive regulator of cell growth and proliferation. However, c-Myc is also a potent inducer of apoptosis when expressed in the absence of serum or growth factors (Wagner *et al*., 1993; Evan and Littlewood, 1998; You *et al*., 2002). This implies that Myc-induced apoptosis can limit the growth of tumors which at certain volumes experience shortage of growth factors. (Evan and Littlewood, 1993; Koskinen and Alitalo, 1993; Pelengaris *et al*., 2002). This protective mechanism is counteracted by Bcl-2 (Section 1.5.2) (Fanidi *et al.*, 1992, Bissonnette *et al*., 1992, Wagner *et al*., 1993). Consistent with this inhibition of c-Myc mediated apoptosis Bcl-2 cooperates with c-Myc in malignant transformation of lymphoid cells in transgenic mice (Strasser *et al*., 1990).

Pim-1 (pathogenesis of virally-induced mouse lymphoma gene). was originally identified as a genetic locus frequently activated by proviral insertion in murine leukemia virus induced T cell lymphomas (Cuypers *et al.*, 1984, Selten *et al.*, 1985, Selten *et al.*, 1986,). Although this serine/threonine kinase is primarily a survival factor in FDCP-1 cells (Lilly and Kraft, 1997; Lilly *et al.*, 1999), Pim-1 functions in concert with c-Myc in inducing apoptosis in the absence of growth factors (Mochizuki *et al*., 1997). This cooperation is also in effect in oncogenesis (Verbeek *et al*., 1991) suggesting that Pim-1 modulates Myc-triggered intracellular pathways common to transformation and apoptosis. One substrate and effector for

Pim-1 kinase is Cdc25A (Mochizuki *et al.*, 1999), a direct transcriptional target of c-Myc indicatingthat the signals of Pim-1 and c-Myc might converge at this molecule.

Bcl-2 and **Bcl-X_L.** Overexpression of Bcl-2 protects IL-3 dependent cells from apoptosis induced upon IL-3 withdrawal (Lin *et al*., 1996; Ito *et al*., 1997). It has also been shown that Bcl-X_L expression is upregulated by hemopoietins in both immortal and primary myeloid progenitors. Accordingly, Bcl-X_L overexpression in hematopoietic cells is highly protective against apoptosis induced by IL-3 withdrawal. (Boise *et al*., 1993). It was suggested that the survival genes Bcl-X_L and Bcl-2 inhibit c-Myc mediated apoptosis by inducing the cyclin mediated kinase inhibitor p27 (Moser *et al*., 1997; Greider *et al*., 2002).

4.2.2.2 Genes upregulated by IL-3 withdrawal

c-Kit. Activation of the receptor tyrosine kinase c-Kit by its ligand SCF (stem cell factor) eventually leads to phosphorylation of the antiapoptotic protein Stat1 (Deberry *et al*., 1997). In addition, activation of c-Kit is a likely determinant of the unrestrained growth characteristic for malignant cells (Moses *et al*., 2002; Yamazaki *et al*., 2003).

The IL-3 receptor βc chain. Binding of IL-3βc turns the low affinity complex of IL-3 and the α-receptor chain into a high affinity receptor, which stimulates cell proliferation and survival.

GST (Glutathione S-transferases) catalyzes the glutathione-dependent detoxification of several chemotherapeutic drugs (conjugation of electrophilic substrates with glutathione, Hayes and Pulford, 1995; Dirven *et al.*, 1996). Mitochondrial damage and the associated release of free radicals that are antagonized by GST are major features of the apoptotic process (Khaled *et al*., 2001; Facchinetti and Leon, 2002; Ding and Nam, 2003, Matsuyama *et al*., 2000; Figure 1.5). It has recently been suggested that Glutathione S-transferase is implicated in protection against apoptosis.(Huang *et al*., 2003). Thus, the activation of GST is likely reflecting

an antiapoptotic mechanism against the oxidative stress developing after IL-3 withdrawal (Dickinson *et al*., 2003).

4.2.2.3 Transiently up- or downregulated genes

Activin receptor type I. Two types of activin receptors (I and II) have been characterized as transmembrane serine/threonine receptor kinases (Willis *et al*., 1996; Harrison *et al*., 2003). Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-β (TGF-β) superfamily. They are involved in the control of cell proliferation by initiating signaling through a heteromeric complex of activin receptors. Binding of activin to its recptors (Schulte *et al.*, 2001) triggers association of the rasGAP-binding protein (Dok-1) with Smad3 (Yamakawa *et al.*, 2002), which inhibits growth of certain types of cells (e.g., colonic epithelial cells).

PKB/Akt is one of the most important cellular survival kinases, which already serves a a target for several anticancer drugs (Tsuruo *et al.*, 2003). It operates within the phosphatidylinositol-3 kinase (PI-3-K) signal transduction pathway which has been shown to stimulate survival of many cells in culture (Stein 2001). Whereas repression of PKB/Akt leads to an irreversible commitment to apoptotic cell death, its effective recruitment by appropriate survival signals results in suppression of p53-dependent apoptosis (Fu *et al*., 2002).

FLIP-L (FLICE-like apoptosis inhibitory protein long form) is structurally similar to procaspase 8 as it contains two death effector domains and a caspase-like domain (Irmler *et al*., 1997). However, the caspase domain lacks the critical site for catalytic activation. The protein is recruited to the CD95 DISC (death induced signalling complex) in a stimulation-dependent fashion, where it then prevents activation of procaspase 8 (Scaffidi *et al*., 1999).

Zyxin (α-actinin binding protein; Rottner *et al.*, 2001) is a LIM domain protein, which displays the architectural features of an intracellular signal transducer (Beckerle, 1997). Its expression is significantly upregulated in melanoma cells when

compared to melanocytes, where upregulation can be induced by mitogens, indicating that zyxin is an important growth regulator (van der Gaag *et al*., 2002)

JunD (c-jun-related transcription factor, member of the jun proto-oncogene family; Li *et al.*, 1990, Weitzman *et al.*, 2000) is a growth factor-inducible transcription factor, It mediates survival signaling by the JNK signal transduction pathway through collaboration with NF-κB to increase antiapoptotic gene expression. (Lamb *et al*., 2003)

The specific regulation of several pro-and anti-apoptotic genes detected with the arrays suggests induction of not only survival but also pro-apoptotic functions in factor deprived FDCP1 cells, the balance of which will finally determine, whether the cell will initiate apoptosis. Early after IL-3 deprivation the survival functions seem to be dominant, as the cells can still be rescued by readdition of the cytokine. However, during prolonged factor withdrawal this balance will shift towards apoptosis.

4.3 Identifying candidate genes for anti-cancer drug development

Since the up-regulation of survival genes and/or the inactivation of pro-apoptotic genes are common in cancer, the relevance of the recovered GTSTs to various cancers was investigated by first using GTST custom arrays and mRNAs from established tumor cell lines. Of the 70 different cancer cell line mRNAs hybridized to the GTST arrays, 26 were differentially expressed in tumor cells. Seven genes were subjected to a final validation screen using matched tumor/normal arrays. They were selected based on their enzymatic activity as protein kinases, which can easily be targeted with small molecule inhibitors, or because they represent so far uncharacterized proteins, which might be of interest in the future.

There are no previous reports of regulation of **KIAA0740**, **KIAA1036** and **KIAA1140** in both carcinoma or normal cell lines. Correlations between these three genes and their clinical behavior have not been demonstrated so far, because they are still hypothetical proteins with unknown biological properties. KIAA0740 is a

putative new GTPase from the Rho-Family; which was moderately up-regulated only in uterine tumors. KIAA1036, a completely unknown protein, was differentially expressed in electronic Northern experiments performed in cooperation with Schering AG and showed up-regulation in stomach and breast tumor tissues. KIAA1140 encodes a protein with 8 tetratricopeptide repeats (TPR), a motif which could serve as an interface for interaction with activated Ras (Marty *et al*., 2003). The RNA for this protein was upregulated in stomach tumors.

Plekha2 contains 2 PH-domains that implicate a role in signal transduction. However, in most tumor tissues, Plekha2 expression was down-regulated or not changed suggesting Plekha2 is possibly not a major sign of these cancers. Conversely, in the analysis with the custom made array Plekha2 was up-regulated in four different types of cancer cell lines. Probably, these discrepancies are due to differences of cultured cancer cell lines and real tumor tissue.

However, before these proteins might be useful in target directed drug development approaches, they have to be characterized in more detail. Currently, one of the most interesting classes of target molecules for drug discovery are protein kinases, as such enzymes can effectively be inhibited with small molecule effectors.

PI4P5Kα and γ. Although many cancer related functions, such as proliferation (Klippel *et al*., 1998; Darmoul *et al*., 2003), cell adhesion (Thamilselvan *et al*., 2003), apoptosis (Kennedy *et al*., 1997; Ehemann *et al*., 2003) and Ras signaling (Downward, 1998; Bakin *et al*., 2003) have been suggested to be mediated by the PI3K-signal transduction pathway, and PI4P5K type I β and γ were upregulated in a variety of cancer cell lines, they appeared either repressed or not-regulated on the matched tumor/normal arrays (Tables 3.10 and 3.11).

The most interesting candidate gene identified in this analysis was the serine/threonine kinase **ERK3**. It is consistently upregulated in breast cancer and provides a promising target for high throughput screening of small molecule libraries

for specific kinase-inhibitory drugs as it has different properties than the other known proteins of this family.

4.4 ERK3 as putative target genes for drug development

ERK3 (=MAPK6) is the most recently described member of the MAPK family of proteins and has been characterized in humans, rat and mouse (Boulton *et al.*, 1991, Meloche *et al*., 1996, Turgeon *et al*., 2000). The ERK3 gene subfamily of consists of the two functional genes, MAPK6 and MAPK4 and several processed pseudogenes (Turgeon *et al*., 2002).

Like the other ERK proteins, ERK3 encodes a protein serine/threonine kinase, which is activated by phosphorylation. However, despite a 50% identity between ERK3 and ERK1 and ERK2 in their catalytic domains key properties of ERK3 are different from the two classic MAPKs (Robinson *et al.*, 2002), Structurally ERK3is unique among the MAP kinases as the highly conserved TXY sequence in the activation loop is replaced in ERK3 by the amino acids SEG. This distinction might explain the activation by different upstream regulators (Robinson *et al*., 2002).

The intracellular localization of ERK3 is still a matter of discussion, however, there seems to be a shuttling between nucleus and cytoplasm, which is independent of mitogenic or stress stimuli and the activation status of the protein, but has an influence on the effects of ERK3 on cell cycle progression (Julien *et al*., 2003).

It has been shown that ERK3 expression is upregulated upon proteasome inhibition and that this upregulation is not dependent on p53, Bcl-2 and caspase 3 but is apparently linked to the p38 signal transduction pathway. That indicates that ERK3 expression is a consequence of p38 pathway activation and most probably represents an intracellular defense or rescue mechanism against cell stress and damage induced by proteasome inhibition(Zimmermann *et al.*, 2001). Recent microarray studies revealed that ERK3 is also significantly upregulated by actived H-Ras (Teramoto *et al.*, 2003), suggestingthat it may participate in H-Ras mediated oncogenesis. In contrast, other oncogenes such as RhoA, Rac1 and Cdc42 seem to have little effect on

ERK3 activation. In a study of colorectal cancers, ERK3 kinase activity was found increased in about half the cases (Wang *et al*., 2000). Collectively, these studies and our findings suggest that ERK3 may be involved in carcinogenesis as part of a yet unidentified MAPK pathway.

Three ways to antagonize ERK3 function in the future could be envisioned:

I. Targeting the SEG motif with small molecules derived from a substance library after screening for specific ERK3 phosphorylation inhibitors.

II. Targeting the ERK3 transcript by RNAi and, finally

III. Manipulating ERK3 degradation by interfering with proteasome function. Unlike all the other MAP kinases, ERK3 is an unstable protein that is constitutively degraded by the proteasome following ubiquitinylation in proliferating cells, hence its half life is short (30 min) (Coulombe *et al.,* 2003). Unlike other protein kinases, the catalytic activity of ERK3 is not responsible for its short half-life (Coulombe *et al.,* 2003). Thus, by targeting the two destabilization regions (NDR-1 and -2) in the N-terminal lobe of ERK3, molecules may be obtained that promote increased ERK3 degradation whithout affecting the other MAPKs.

CHAPTER 5

CHAPTER 5

SUMMARY AND CONCLUSIONS

The cellular homeostasis of a multicellular organism depends on a fine balance between proliferation and apoptosis. Disturbances of this balance can lead to degenerative or hyperproliferative diseases. Therefore, the knowledge about the mechanisms involved in the regulation of proliferation and apoptosis is of prime interest for the definition of molecules, which can be targeted by pharmacological intervention to treat diseases like cancer.

Proliferation and differentiation within the hematopoeitic system is controlled by a large number of growth factors and cytokines and the lack of such factors often leads to apoptosis. One model system to study the processes involved in apoptosis induction is the murine cell line FDCP-1, a myeloic precursor of the monocyte-granulocyte lineage. This cell line is dependent on interleukin-3 (IL-3) and withdrawal of the cytokine leads to induction of apoptosis.

Besides the well characterized posttranslational processes involved in the initiation and execution of apoptosis, transcriptional regulation also seems to one of the early events. In an attempt to characterize genes upregulated upon apoptosis induction a gene trap approach, which allows the tagging of transiently induced genes, was used to identify loci upregulated by IL-3 withdrawal in FDCP-1 cells.

From an integration library of 2×10^6 gene trap insertions, we recovered a total of 102 useful gene trap sequence tags (GTSTs). More than half of the trapped genes with defined functions, were either directly or indirectly involved in cell survival and/or cell death and about one third of these are directly related to the IL-3 and small Rho-GTPase pathways.

A comparison of the gene trap approach with other strategies used to identify differentially expressed genes showed that the number of relevant genes recovered by the different technologies is quite similar, although the types of genes are different. Whereas array based searches look at steady state levels of RNA, the gene trap used selects for true transcriptional induction and can also detect genes expressed at extremely low levels. However genes induced from an already basic expression level will be missed due to the selection strategy employed. Taken together, the results suggest that gene trapping effectively complements microarray-based strategies unable to distinguish between transcriptional and post-transcriptional gene regulation.

The analysis of the genes recovered by the different approaches revealed that not only pro-apoptotic genes, but also a large number of survival genes are transcriptionally induced upon IL-3 withdrawal. This suggests that the primary response of the cells is the prevention of apoptosis. However, the upregulation of pro-apoptotic genes indicates that it is the balance between the two responses that determines the fate of the cell.

To identify potential target molecules for targeted drug discovery several candidate genes were analyzed for their relevance in human tumors by using custom arrays and matched tumor/normal arrays. This screen revealed that ERK3 might be a suitable candidate for further evaluation due its consistent upregulation in breast tumors and its unique properties setting it apart from other proteins of this family.

REFERENCES

Ahmed, N. N., Grimes, H. L., Bellacosa, A. Chan, T. O. and Tsichlis, P. N. (1997) Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc. Natl. Acad. Sci. USA* **94**: 3627-3632.

Aitsebaomo, J., Kingsley-Kallesen, M. L., Wu, Y., Quertermous, T. and Patterson, C. (2001) Vezf1/DB1 is an endothelial cell-specific transcription factor that regulates expression of the endothelin-1 promoter. *J. Biol. Chem.* **276**: 39197-39205.

Alberts, A. S. and Treisman, R. (1998) Activation of RhoA and SAPK/JNK signalling pathways by the RhoA-specific exchange factor mNET1. *EMBO. J.* **17**: 4075-4085.

Antoku, K., Liu, Z. and Johnson, D. E. (1998) IL-3 withdrawal activates a CrmA-insensitive poly (ADP-ribose) polymerase cleavage enzyme in factor-dependent myeloid progenitor cells. *Leukemia* **12**: 682-689.

Ashkenazi, A. and Dixit, V. M. (1998) Death receptors: signaling and modulation. *Science* **281**: 1305-1308.

Askew, D. S., Ashmun, R. A., Simmons, B. C., Cleveland, J. L. (1991) Constitutive c-myc expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and induces apoptosis. *Oncogene* **6**: 1915-1922.

Askew, D. S., Ihle, J. N. and Cleveland, J. L. (1993) Activation of apoptosis associated with enforced myc expression in myeloid progenitor cells is dominant to the suppression of apoptosis by interleukin-3 or erythropoietin. *Blood* **82**: 2079-2087.

Augenlicht, L. H. and Heerdt, B. G., (2001) Mitochondria: integrators in tumorigenesis. *Nat. Genet.* **28**: 104-105.

Azam, M., Erdjument-Bromage, H., Kreider, B. L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J. N. and Schindler, C. (1995) Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J.* **14**: 1402-1411.

Bains, M. A., Giles, I. and Wright, D. H. (1997) Distribution and configuration of c-myc RNA during transcriptional attenuation in differentiating cells in-situ. *Histochem. Cell. Biol.* **107**: 259-263.

Bakin, R. E., Gioeli, D., Sikes, R. A., Bissonette, E. A. and Weber, M. J. (2003) Constitutive Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway Promotes Androgen Hypersensitivity in LNCaP Prostate Cancer Cells. *Cancer Res.* **63**: 1981-1989.

Bassermann, F., Jahn, T., Miething, C., Seipel, P., Bai, R. Y., Coutinho, S., Tybulewicz, V. L., Peschel, C., Duyster, J. (2002) Association of Bcr-Abl with the proto-oncogene Vav is implicated in activation of the Rac-1 pathway. *J. Biol. Chem.* **277**: 12437-12445.

Beckerle, M. C. (1997) Zyxin: zinc fingers at sites of cell adhesion. *Bioessays.* **19**: 949-957.

Bellacosa, A., Testa, J. R., Staal, S. P. and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254**: 274-277.

Bellanger, J. M., Astier,C., Sardet, C., Ohta, Y., Stossel, T. P. and Debant, A. (2000) The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nat. Cell. Biol.* **12**: 888-892.

Bissonnette, R. P., Echeverri, F., Mahboubi, A. and Green, D. R. (1992) Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. *Nature* **359**: 552-554.

Bittorf, T., Sasse, T., Wright, M., Jaster, R., Otte, L., Schneider-Mergener, J. and Brock, J. (2000a) cDNA cloning and functional analysis of a truncated STAT5 a protein from autonomously growing FDCP-1 cells. *Cell Signal.* **12**: 721-730.

Bittorf, T., Seiler, J., Ludtke, B., Buchse, T., Jaster, R. and Brock, J. (2000b) Activation of STAT5 during EPO-directed suppression of apoptosis. *Cell Signal* **12**: 23-30.

Blalock, W. L., Weinstein-Oppenheimer C., Chang, F., Hoyle, P. E., Wang, X.Y., Algate, P. A., Franklin, R. A., Oberhaus, S. M., Steelman, L. S., McCubrey, J. A. (1999) Signal transduction, cell cycle regulatory, and anti-apoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. *Leukemia* **13**: 1109-1166.

Blandino, G., Scardigli, R., Rizzo, M. G., Crescenzi, M., Soddu, S. and Sacchi, A. (1995) Wild-type p53 modulates apoptosis of normal, IL-3 deprived, hematopoietic cells. *Oncogene* **10**: 731-737.

Blomquist, A., Schworer, G., Schablowski, H., Psoma, A., Lehnen, M., Jakobs, K. H. and Rumenapp, U. (2000) Identification and characterization of a novel Rho-specific guanine nucleotide exchange factor. *Biochem. J.* **352**: 319-325.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**: 597-608.

Bojes, H. K., Feng, X., Kehrer, J. P. and Cohen, G. M. (1999) Apoptosis in hematopoietic cells (FL5.12) caused by interleukin-3 withdrawal: relationship to caspase activity and the loss of glutathione. *Cell Death Differ.* **6**: 61-70.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**: 663–675.

Brennan, J. A., Volle, D. J., Chaika, O. V. and Lewis, R. E. (2002) Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of Ras. *J. Biol. Chem.* **277**: 5369-5377.

Bryson, J. M., Coy, P. E., Gottlob, K., Hay, N. and Robey, R. B. (2002) Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death. *J. Biol. Chem.* **277**: 11392-11400.

Canaider, S., Solito, E., de Coupade, C., Flower, R. J., Russo-Marie, F., Goulding, N. J. and Perretti, M. (2000) Increased apoptosis in U937 cells over-expressing lipocortin 1 (annexin I). *Life Sci.* **66**: 265-270.

Cantley, L. C. and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. USA* **96**: 4240-4245.

Carmichael, G. (2003) Antisense starts making more sense. *Nature Biotech.* **21**: 371-372.

Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt,S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.* **271**: 4961-4965.

Cho, R. L. and Johnson, D. E. (1999) Characterization of caspase proteases in cytokine-dependent myeloid progenitor cells using enzyme affinity labeling. *J. Cell. Biochem.* **73**: 79-89.

Choi, Y. J., Mendoza, L., Rha, S. J., Sheikh-Hamad, D., Baranowska-Daca, E., Nguyen, V., Smith, C. W., Nassar, G., Suki, W. N. and Truong, L. D., (2001) Role of p53-dependent activation of caspases in chronic obstructive uropathy: evidence from p53 null mutant mice. *J. Am. Soc. Nephrol.* **12**: 983-992.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. **162**: 156-159.

Clark, J. M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* **16**: 9677-9686.

Claverie, J. M. (2001) Gene number. What if there are only 30 000 human genes? *Science* **291**: 1255-1257.

Coffer, P. J., Schweizer, R. C., Dubois, G. R., Maikoe, T., Lammers, J. W. and Koenderman, L. (1998) Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. *Blood* **91**: 2547-2557.

Coulombe, P., Rodier, G., Pelletier, S., Pellerin, J. and Meloche, S. (2003) Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. *Mol. Cell. Biol.* **23**: 4542-4558.

Cuypers, H. T., Selten, G., Quint, W., Zijlstra, M., Maandag, E. R., Boelens, W., van Wezenbeek, P., Melief, C. and Berns, A. (1984) Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell.* **37**:141-150.

Darmoul, D., Gratio, V., Devaud, H., Lehy, T. and Laburthe, M. (2003) Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. *Am. J. Pathol.* **162**: 1503-1513.

Dasika, G. K., Lin, S. C., Zhao, S., Sung, P., Tomkinson, A. and Lee, E. Y. (1999) DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. *Oncogene* **18**: 7883-7899.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**: 231-241.

d'Auriol, L., Mattei, M. G., Andre, C. and Galibert, F. (1988) Localization of the human c-kit protooncogene on the q11-q12 region of chromosome 4. *Hum. Genet.* **78**: 374-376.

Deberry, C., Mou, S. and Linnekin, D. (1997) Stat1 associates with c-kit and is activated in response to stem cell factor. *Biochem. J.* **327**: 73-80.

Decker, P. and Muller, S. (2002) Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Curr. Pharm. Biotechnol.* **3**: 275-283.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**: 687-689.

Dexter, T. M, Garland, J., Scott, D., Scolnick, E. and Metcalf, D. (1980) Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* **152**: 1036-1047.

Dickinson, D. A., Iles, K. E., Zhang, H., Blank, V. and Forman, H. J. (2003) Curcumin alters EpRE and AP-1 binding complexes and elevatesglutamate-cysteine ligase gene expression. *FASEB J.* **17**: 473-475.

Ding, W. X., and Nam, Ong. C. (2003) Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett.* **220**: 1-7.

Dirven, H. A., van Ommen, B., Van Bladeren, P. J. (1996) Glutathione conjugationof alkylating cytostatic drugs with a nitrogen mustard group and the role of glutathione S -transferases. *Chem. Res. Toxicol.* **9**: 351-360.

Donepudi, M. and Grutter, M. G. (2002) Structure and zymogen activation of caspases. *Biophys. Chem.* **101**: 145-153.

Downward, J. (1998) Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**: 49-54.

Drexler, H. G., Zaborski, M. and Quentmeier, H. (1997) Cytokine response profiles of human myeloid factor-dependent leukemia cell lines. *Leukemia* **11**: 701-708.

Dunham, I. (2000) The gene guessing game. *Yeast* **17**: 218-224.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T. and Miyazono, K. (2001) Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J. Biol. Chem.* **276**: 12477-12480.

Ehemann, V., Sykora, J., Vera-Delgado, J., Lange, A. and Otto, H. F. (2003) Flow cytometric detection of spontaneous apoptosis in human breast cancer using the TUNEL-technique. *Cancer Lett.* **194**: 125-131.

Evan, G. I. and Littlewood, T. D. (1993) The role of c-myc in cell growth. *Curr. Opin. Genet. Dev.* **3**: 44-49.

Evan, G. I. and Littlewood, T. D. (1998) A matter of life and cell death. *Science* **281**: 1317-1322.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood,T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. and Hancock, D. C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**: 119-128.

Evans, M. J., Carlton, M. B. and Russ, A. P. (1997) Gene trapping and functional genomics. *Trends Genet.* **13**: 370-374.

Facchinetti, F. S. and Leon A. (2002) H_2O_2 induces upregulation of Fas and Fas ligand expression in NGF-differentiated PC12 cells: Modulation by cAMP. *J. Neurosci. Res.* **69**: 178-188.

Fanidi, A., Harrington, E. A. and Evan, G. I. (1992) Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* **359**: 554-556.

Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**: 6-13.

Feinberg, A. P. and Vogelstein, B. (1984) "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity". *Addendum. Anal. Biochem.* **137**: 266-267.

Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**: 41-50.

Fukada, T. and Tonks, N. K. (2003) Identification of YB-1 as a regulator of PTP1B expression: implications for regulation of insulin and cytokine signaling. *EMBO. J.* **22**: 479-493.

Galande, S., Dickinson, L. A., Mian, I. S., Sikorska, M. and Kohwi-Shigematsu, T. (2001) SATB1 cleavage by caspase 6 disrupts PDZ domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Mol. Cell.Biol.* **21**: 5591-5604.

Gillen, C., Jander, S. and Stoll, G. (1998) Sequential expression of mRNA for proinflammatory cytokines and interleukin-10 in the rat peripheral nervous system: comparison between immune-mediated demyelination and Wallerian degeneration. *J. Neurosci. Res.* **51**: 489-496.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B. and Hay, N. (2001) Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* **15**:1406-1418.

Greider, C., Chattopadhyay, A., Parkhurst, C. and Yang, E. (2002) BCL-x(L) and BCL2 delay Myc-induced cell cycle entry through elevation of p27 and inhibition of G1 cyclin-dependent kinases. *Oncogene* **21**: 7765-7775.

Gross, A., Yin, X. M., Wang, K., Wie, M. C., Jockel, J., Milliman, C., Erdjument-Bromage , H., Tempst, P. and Korsmeyer, S. J. (1999) Caspase Cleaved BID Targets Mitochondria and Is Required for Cytochrome c Release,

while BCL-XL Prevents This Release but Not Tumor Necrosis Factor-R1/Fas Death. *J. Biol. Chem.* **274**:1156-1163.

Gross A., McDonnell, J. M., and Korsmeyer, S. J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes & Development* **13**: 1899-1911.

Grosskreutz, Y., Hermann, A., Kins, S., Fuhrmann, J. C., Betz, H. and Kneussel, M. (2001) Identification of a gephyrin-binding motif in the GDP/GTP exchange factor collybistin. *Biol. Chem.* **382**: 1455-1462.

Grundemann, D., Liebich, G., Kiefer, N., Koster, S and Schomig, E. (1999) Selective substrates for non-neuronal monoamine transporters. *Mol. Pharmacol.* **56**: 1-10.

Gu, H., Zou, Y. R. and Rajewsky, K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73**: 1155-1164.

Gu, J., Dubner, R., Fornace, A. J. Jr and Iadarola, M. J. (1995) UREB1, a tyrosine phosphorylated nuclear protein, inhibits p53 transactivation. *Oncogene.* **11**: 2175-2178.

Guthridge, M. A., Stomski, F. C., Barry, E. F., Winnall, W. Woodcock, J. M., McClure, B. J., Dottore, M., Berndt, M. C., and Lopez, A. F. (2000) Site-specific serine phosphorylation of the IL-3 receptor is required for hemopoietic cell survival. *Mol. Cell* **6**: 99-108.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557-580.

Hannemann, J.R., McManus, D. M., Kabarowski, J. H. and Wiedemann, L. M. (1998) Haemopoietic transformation by the TEL/ABL oncogene. *Br. J. Haematol.* **102**: 475-485.

Hannon, G. J. (2002) RNA interference. *Nature* **418**: 244-251.

Harrison, C. A., Gray, P. C., Koerber, S. C., Fischer, W. and Vale, W. (2003) Identification of a functional binding site for activin on the type I receptor ALK4. *J. Biol. Chem.* **278**: 21129-21135.

Hawkins, T. E., Das, D., Young, B. and Moss, S. E. (2002) DT40 cells lacking the Ca2+-binding protein annexin 5 are resistant to Ca2+-dependent apoptosis. *Proc. Natl. Acad. Sci. USA.* **99**: 8054-8059.

Hayes, J. D., and Pulford, D. J., (1995) The glutathione S -transferase supergene family:regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**: 445 -600.

Heim, M. H. (1999) The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J. Recept. Signal. Transduct. Res.* **19**: 75-120.

Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F. and Graeve, L. (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**: 297-314.

Huang, J., Tan, P. H., Thiyagarajan, J. and Bay, B. H. (2003) Prognostic significance of glutathione S-transferase-pi in invasive breast cancer. *Mod. Pathol.* **16**: 558-565.

Ihle, J. N. (2001) The Stat family in cytokine signaling. *Curr. Opin. Cell. Biol.* **13**: 211-217.

Iijima, Y., Okuda, K., Tojo, A., Tri, N. K., Setoyama, M., Sakaki, Y., Asano, S., Tokunaga, K., Kruh, G. D. and Sato, Y. (2002) Transformation of Ba/F3 cells and Rat-1 cells by ETV6/ARG. *Oncogene* **21**: 4374-4383.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroeter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E. and Tschopp, J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* **388**: 190-195.

Ito, T., Deng, X., Carr, B. and May, W. S. (1997) Bcl-2 phosphorylation required for anti-apoptosis function. *J. Biol. Chem.* **272**: 11671-11673.

Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Arai, K. and Miyajima, A. (1990) Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. *Science* **247**: 324-327.

JacksonGrusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E. and Jaenisch, R. (2001) Loss of genomic methylation causes p53dependent apoptosis and epigenetic deregulation. *Nat. Genet.* **27**: 31-39.

Jang, M. H., Jun, do. Y., Rue, S. W., Han, K., Park, W. and Kim, Y. H. (2002) Arginine antimetabolite L-canavanine induces apoptotic cell death in human Jurkat T cells via caspase-3 activation regulated by Bcl-2 or Bcl-xL. *Biochem. Biophys. Res. Commun.* **295**: 283-288.

Jiang, X., Kim, H. E., Shu, H., Zhao, Y., Zhang, H., Kofron, J., Donnelly, J., Burns, D., Ng, S. C., Rosenberg, S. and Wang, X. (2003) Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. *Science.* **299**: 223-226.

Juin, P., Hueber, A. O., Littlewood, T. and Evan, G. (1999) c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. *Genes Dev.* **13**:1367-1381.

Juin, P., Hunt, A., Littlewood ,T., Griffiths, B., Swigart, L. B., Korsmeyer, S. and Evan, G. (2002) c-Myc functionally cooperates with Bax to induce apoptosis. *Mol. Cell. Biol.* **22**: 6158-6169.

Julien, C., Coulombe, P. and Meloche, S. (2003) Nuclear export of ERK3 by a CRM1-dependent mechanism regulates its inhibitory action on cell cycle progression. *J. Biol. Chem.* Aug 12 [Epub ahead of print].

Jung, D. J., Sung, H. S., Goo, Y. W., Lee, H. M., Park, O. K., Jung, S. Y., Lim, J., Kim, H. J., Lee, S. K., Kim, T. S., Lee, J. W. and Lee, Y.C. (2002) Novel transcription coactivator complex containing activating signal cointegrator 1. *Mol. Cell. Biol.* **22**: 5203-5211.

Kadekaro, A. L., Kanto, H., Kavanagh, R. and Abdel-Malek, Z. A. (2003) Significance of the melanocortin 1 receptor in regulating human melanocyte pigmentation, proliferation, and survival. *Ann. NY. Acad. Sci.* **994**: 359-365.

Kauffmann-Zeh, A., Rodriquez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Suppression of c-Myc-induced

apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**: 544-548.

Kavurma, M. M. and Khachigian, L. M. (2003) Signaling and transcriptional control of Fas ligand gene expression. *Cell Death and Differentiation* **10**: 36-44.

Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N. and Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.* **11**: 701-713.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239-257.

Khaled, A. R., Moor, A. N., Li, A., Kim, K., Ferris, D. K., Muegge, K., Fisher, R. J., Fliegel, L. and Durum, S. K. (2001) Trophic factor withdrawal: p38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalinization. *Mol. Cell. Biol.* **22**: 7545-7557.

Kilic, M., Schafer, R., Hoppe, J. and Kagerhuber, U. (2002) Formation of noncanonical high molecular weight caspase-3 and -6 complexes and activation of caspase-12 during serum starvation induced apoptosis in AKR-2B mouse fibroblasts. *Cell Death Differ.* **9**: 125-137.

Kirsch, T., Swoboda, B. and Nah, H. (2000) Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage. *Osteoarthritis Cartilage.* **8**: 294-302.

Kischkel, F. C., Hellbardt, S., Berhmann, I., Germer, M., Pawlita, M., Krammer, P. H. and Peter, M. E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO. J.* **14**: 5579-5588.

Kischkel, F. C., Lawrence, D. A., Tinel, A., LeBlanc, H., Wirmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D. and Ashkenazi A. (2001) Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J. Biol. Chem.* **276**: 46639-46646.

Klaus, G. G. and Harnett, M. M. (1990) Cross-talk between B cell surface immunoglobulin and interleukin 4 receptors: the role of protein kinase C and Ca2(+)-mediated signals. *Eur. J. Immunol.* **20**: 2301-2307.

Klippel, A., Escobedo, M. A., Wachowicz, M. S., Apell, G., Brown, T. W., Giedlin, M. A., Kavanaugh, W. M. and Williams, L. T. (1998) Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. *Mol. Cell. Biol.* **18**: 5699-5711.

Koskinen, P. J. and Alitalo, K. (1993) Role of myc amplification and overexpression in cell growth, differentiation and death. *Semin. Cancer Biol.* **4**: 3-12.

Lamb, J. A., Ventura, J. J., Hess, P., Flavell, R. A. and Davis, R. J. (2003) JunD mediates survival signaling by the JNK signal transduction pathway. *Mol. Cell.* **11**:1479-1489.

Lasham, A., Lindridge, E., Rudert, F., Onrust, R. and Watson, J. (2000) Regulation of the human fas promoter by YB-1, Puralpha and AP-1 transcription factors. *Gene.* **252**: 1-13.

Levy, D. E. and Darnell, J. E. (2002) Signalling: Stats: transcriptional control and biological impact. *Nature Reviews Molecular Cell Biology* **3**: 651-662.

Li, L., Hu, J. S. and Olson, E. N. (1990) Different members of the jun proto-oncogene family exhibit distinct patterns of expression in response to type beta transforming growth factor. *J. Biol. Chem.* **265**: 1556-1562.

Lin, E. Y., Orlofsky, A., Wang, H. G., Reed, J. C. and Prystowsky, M. B. (1996) A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. *Blood* **87**: 983-992.

Lilly, M. and Kraft, A. (1997) Enforced expression of the Mr 33,000 Pim-1 kinase enhances factor-independent survival and inhibits apoptosis in murine myeloid cells. *Cancer Res.* **57**: 5348-5355.

Lilly, M., Sandholm, J., Cooper, J. J., Koskinen, P. J. and Kraft, A. (1999) The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. *Oncogene* **18**: 4022-4031.

Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89**: 175-184.

Louro, I. D., Bailey, E. C., Li, X., South, L. S., McKie-Bell, P. R., Yoder, B. K., Huang, C. C., Johnson, M. R., Hill, A. E., Johnson, R. L. and Ruppert, J. M. (2002) Comparative gene expression profile analysis of GLI and c-MYC in an epithelial model of malignant transformation. *Cancer Res*. **62**: 5867-5873.

Luo, G., Yao, M. S., Bender, C. F., Mills, M., Bladl, A. R., Bradley, A. and Petrini, J. H. (1999) Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. Proc. Natl. Acad. Sci. USA **96**: 7376-7381.

Luria, S. E., and Burrous, J. W. (1957) Hybridization between *Escherichia coli* and *Shigella. J. Bacteriol*. **74**: 461-476.

Macalma, T., Otte, J., Hensler, M. E., Bockholt, S. M., Louis, H. A., Kalff-Suske, M., Grzeschik, K. H., von der Ahe, D. and Beckerle, M. C. (1996) Molecular characterization of human zyxin. *J. Biol. Chem.* 271: 31470-31478.

Marty, C., Browning, D. D. and Ye, R. D. (2003) Identification of tetratricopeptide repeat 1 as an adaptor protein that interacts with heterotrimeric G proteins and the small GTPase Ras. *Mol. Cell. Biol.* **23**: 3847-3858.

Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R.Y. and Reed, J. C. (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell Biol.* **2**: 318-325.

McKinstry, W. J., Li, C. L., Rasko, J., Nicola, N. A., Johnson, G. R. and Metcalf, D. (1997) Cytokine Receptor Expression on Hematopoietic Stem and Progenitor Cells. *Blood* **89**: 65-71.

McPherson, J., Marra, M., Hillier, L., Waterston, R., Chinwalla, A., Wallis, J., Sekhon, M., Wylie, K., Mardis, E. R., Wilson, R. K., Fulton, R., Kucaba, T. A., Yada, T., Park, H. S., Shimizu, Shuichi (2001) A physical map of the human genome. *Nature* **409**: 934-941.

Meloche, S., Beatty, B. G. and Pellerin, J. (1996) Primary structure, expression and chromosomal locus of a human homolog of rat ERK3. *Oncogene.* **13**: 1575-1579.

Migliaccio, G., Migliaccio, A. R., Kreider, B. L., Rovera, G. and Adamson, J. W. (1989) Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32D. *J. Cell. Biol.* **109**: 833-841.

Minshall, C., Arkins, S., Freund, G. G. and Kelley, K. W. (1996) Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J. Immunol.* **156**: 939-947.

Miyajima, A., Mui, A. L., Ogorochi, T. and Sakamaki, K. (1993) Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3 and interleukin-5. *Blood* **82**: 1960-1974.

Miyake, Y., Kakeya, H., Kataoka, T. and Osada, H. (2003) Epoxycyclohexenone inhibits Fas-mediated apoptosis by blocking activation of pro-caspase-8 in death-inducing signaling complex. *J. Biol. Chem.* **278**: 11213-11220.

Mochizuki, T., Kitanaka, C., Noguchi, K., Muramatsu, T., Asai, A. and Kuchino, Y. (1999) Physical and functional interactions between Pim-1 kinase and Cdc25A phosphatase. Implications for the Pim-1-mediated activation of the c-Myc signaling pathway. *J. Biol. Chem.* **274**: 18659-18666.

Mochizuki, T., Kitanaka, C., Noguchi, K., Sugiyama, A., Kagaya, S., Chi, S., Asai, A. and Kuchino, Y. (1997) Pim-1 kinase stimulates c-Myc-mediated death signaling upstream of caspase-3 (CPP32)-like protease activation. *Oncogene*. **15**: 1471-1480.

Moore, G. E. and Hood, D. B. (1993) Modified RPMI 1640 culture medium. *In Vitro Cell Dev. Biol. Anim.* **29**: 268.

Moriyama, Y. and Yamada, H. (1996) Vesicular monoamine transporter in microvesicles from bovine posterior pituitaries is immunologically similar to but distinct from the chromaffin granule counterpart in its sensitivities to 1-methyl-4-phenylpyridinium and histamine. *Biochem. Biophys. Res. Commun.* **22**: 790-794.

Morrison, D. A. (1979) Transformation and preservation of competent bacterial cells by freezing. *Methods Enzymol.* **68**: 326-331.

Morrison, D. K. and Cutler, R. E. (1997) The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**: 174-179.

Moser, M., Pscherer, A., Roth, C., Becker, J., Mucher, G., Zerres, K., Dixkens, C., Wies, J., Guay-Woodford, L., Buettner, R. and Fassler, R. (1997) Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2beta. *Genes Dev.* **11**: 1938-1948.

Moses, A. V., Jarvis, M. A., Raggo, C., Bell, Y. C., Ruhl, R., Luukkonen, B. G., Griffith, D. J., Wait, C. L., Druker, B. J., Heinrich, M. C., Nelson, J. A. and Fruh, K. (2002) Kaposi's sarcoma-associated herpesvirus-induced upregulation of the c-kit proto-oncogene, as identified by gene expression profiling, is essential for the transformation of endothelial cells. *J. Virol.* **76**: 8383-8399.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E. and Dixit, V. M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85**: 817-827.

Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J. and Paul, W. E. (1999) The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* **17**: 701-738.

Nicola, N.A., Robb, L., Metcalf, D., Cary, D., Drinkwater, C.C., Begley, C.G. (1996) Functional inactivation in mice of the gene for the interleukin-3 (IL-3)-specific receptor beta-chain: Implication for IL-3 function and the mechanism of receptor transmodulation in hematopoietic cells. *Blood* **87**: 2665-2674.

Nishinakamura, R., Nakayama, N., Hirabayashi, Y., Inoue, T., Aud, D., McNeil, T., Azuma, S., Yoshida, S., Toyoda, Y., Arai, K. (1995) Mice deficient for the IL-3/GM-CSF/IL-5 beta c receptor exhibit lung pathology and impaired immune response, while beta IL-3 receptor-deficient mice are normal. *Immunity* **2**: 211-222.

Nishinakamura, R., Miyajima, A., Mee, P.J., Tybulewicz, V.L.J., Murray, R. (1996) Hematopoiesis in mice lacking the entire granulocyte macrophage-colony stimulating factor/interleukin-3/interleukin-5 functions. *Blood* **88**: 2458-2464.

Nolte, R. T., Eck, M. J., Schlessinger, J., Shoelson, S. E., Harrison, S. C. (1996) Crystal structure of the PI 3-kinase p85 amino-terminal SH2 domain and ist phosphopeptide complexes. *Nat. Struct. Biol.* **3**: 364-374.

Oltvai, Z. N. and Korsmeyer, S. J. (1994) Checkpoints of dueling dimers foil death wishes. *Cell* **79**: 189-192.

Palacios, R., Henson, G., Steinmetz, M. and McKearn, J. P. (1984) Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. *Nature* **309**: 126-131.

Pan, H. and Griep, A. E. (1995) Temporally distinct patterns of p53-dependent and p53-independent apoptosis during mouse lens development. *Genes & Dev.* **9**: 2157-2169.

Parisi, S., McKay, M. J., Molnar, M., Thompson, M. A., van der Spek, P. J., van Drunen-Schoenmaker, E., Kanaar, R., Lehmann, E., Hoeijmakers, J.H. and Kohli, J. (1999) Rec8p, a meiotic recombination and sister chromatid cohesion phosphoprotein of the Rad21p family conserved from fission yeast to humans. *Mol. Cell. Biol.* **19**: 3515-3528.

Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2001) Estimating the world cancer burden: GLOBOCAN2000. *Int. J. Cancer* **94**: 153-156.

Pei, L., Wiser, O., Slavin, A., Mu, D., Powers, S., Jan, L. Y. and Hoey, T. (2003) Oncogenic potential of TASK3 (Kcnk9) depends on K+ channel function. *Proc. Natl. Acad. Sci. USA.* **100**: 7803-7807.

Pelengaris, S., Khan, M. and Evan, G. (2002) c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer* **10**: 764-776.

Piccolella, E., Spadaro, F., Ramoni, C., Marinari, B., Costanzo, A., Levrero, M., Thomson, L., Abraham, R. T. and Tuosto, L. (2003) Vav-1 and the IKK alpha subunit of I kappa B kinase functionally associate to induce NF-kappa B activation in response to CD28 engagement. *J. Immunol.* **170**: 2895-2903.

Pinkoski, M. J., Waterhouse, N. J. and Heibein, J. A. (2001) Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2 inhibitable mitochondrial pathway. *J. Bio. Chem.* **276**: 12060-12067.

Pircher, T. J., Zhao, S., Geiger, J. N., Joneja, B. and Wojchowski, D. M. (2000) Pim-1 kinase protects hematopoietic FDC cells from genotoxin-induced death. *Oncogene* **19**: 3684-3692.

Pirola, L., Zvelebil, M. J., Bulgarelli-Leva, G., Van Obberghen, E., Waterfield, M. D. and Wymann, M. P. (2001) Activation loop sequences confer substrate specificity to phosphoinositide 3-kinase alpha (PI3Kalpha). Functions of lipid kinase-deficient PI3Kalpha in signaling. *J. Biol. Chem.* **276**: 21544-21554.

Qiu, F. H., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H. and Besmer, P. (1988) Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family, oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *EMBO. J.* **7**: 1003-1011.

Quelle, F. W., Sato, N., Witthuhn, B. A., Inhorn, R. C., Eder, M., Miyajima, A., Griffin, J. D. and Ihle, J. N. (1994) JAK2 associates with the betac chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol. Cell. Biol.* **14**: 4335-4341.

Ratajczak, J., Majka, M., Kijowski, J., Baj, M., Pan, Z. K., Marquez,L. A., Janowska-Wieczorek, A. and Ratajczak, M. Z. (2001) Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br. J. Haematol.* **115**: 195-204.

Rayner, J. R. and Gonda, T. J. (1994) A simple and efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector. *Mol. Cell. Biol.* **14**: 880-887.

Reddy, G. P., McAuliffe, C. I., Pang, L., Quesenberry, P. J. and Bertoncello, I. (2002) Cytokine receptor repertoire and cytokine responsiveness of Ho(dull)/Rh(dull) stem cells with differing potentials for G1/S phase progression. *Exp. Hematol.* **30**: 792-800.

Robb, L., Drinkwater, C. C., Metcalf, D., Li, R., Kontgen, F., Nicola, N. A. and Begley, C. G. (1995) Hematopoietic lung abnormalities in mice with a null mutation of the common beta subunit of the receptors for granulocyte-macrophage colony-stimulating factor and interleukin 3 and 5. *Proc. Natl. Acad. Sci. USA* **92**: 9565-9569.

Robinson, M. J., Xu, B. E., Stippec, S. and Cobb, M. H. (2002) Different domains of the Mitogen-activated protein kinases ERK3 and ERK2 direct subcellular localization and upstream specificity in vivo. *J. Biol. Chem.* **277**: 5094-5100.

Rodriquez-Tarduchy, G., Collins, M. K. L., Lopez-Rivas, A. (1990) Regulation of apoptosis in interleukin-3-dependent hemopoietic cells by interleukin-3 and calcium ionophores. *EMBO. J.* **9**: 2997-3002.

Roest Crollius, H., Jaillon, O., Bernot, A., Dasilva, C., Bouneau, L., Fischer, C., Fizames, C., Wincker, P., Brottier, P., Quetier, F., Saurin, W. and Weissenbach, J. (2000) Estimate of human gene number provided by genome-wide analysis using Tetraodon nigroviridis DNA sequence. *Nat. Genet.* **25**: 235-238.

Ron, D. and Habener, J. F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6**: 439-453.

Rottner, K., Krause, M., Gimona, M, Small, J. V. and Wehland, J. (2001) Zyxin is not colocalized with vasodilator-stimulated phosphoprotein(VASP) at lamellipodial tips and exhibits different dynamics tovinculin, paxillin, and VASP in focal adhesions. *Mol. Biol. Cell* **12**: 3103-3113.

Russ, A. P., Friedel, C., Grez, M. and von Melchner, H. (1996a) Self-deleting retrovirus vectors for gene therapy. *J. Virol.* **70**: 4927-4932.

Russ, A. P., Friedel, C., Ballas, K., Kalina, U., Zahn, D., Strebhardt, K. and von Melchner, H. (1996b) Identification of genes induced by factor deprivation in hematopoietic cells undergoing apoptosis using gene-trap mutagenesis and site-specific recombination. *Proc. Natl. Acad. Sci. USA* **93**: 15279-15284.

Sachidanandam, R., Weissman, D., Schmidt, S. C., Kakol, J. M., Stein, L. D., Marth, G., Sherry, S., Mullikin, J. C., Mortimore, B. J., Willey, D. L., Hunt, S. E., Cole, C. G., Coggill, P. C., Rice, C. M., Ning, Z., Rogers, J., Bentley, D. R., Kwok, P. Y., Mardis, E. R., Yeh, R. T., Schultz, B., Cook, L., Davenport, R., Dante, M., Fulton, L., Hillier, L., Waterston, R. H., McPherson, J. D., Gilman, B., Schaffner, S., Van Etten, W. J., Reich, D., Higgins, J., Daly, M. J., Blumenstiel, B., Baldwin, J., Stange-Thomann, N., Zody, M. C., Linton, L., Lander, E. S. and Altshuler, D. (2001) A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* **409**: 928-933.

Saji, H., Toi, M., Saji, S., Koike, M., Kohno, K. and Kuwano, M. (2003) Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human breast carcinoma. *Cancer Lett.* **190**: 191-197.

Sakurai, T., Yamada, T., Kihara-Negishi, F., Teramoto, S., Sato, Y., Izawa, T. and Oikawa, T. (2003) Effects of overexpression of the Ets family transcription factor TEL on cell growth and differentiation of K562 cells. *Int. J. Oncol.* **22**: 1327-1333.

Salvesen, G. S. and Dixit, V. M. (1997) Caspases: Intracellular signaling by proteolysis. *Cell* **91**: 443-446.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2 nd ed., Cold Spring Harbor laboratory, Cold Spring Harpor, NY.

Sauer, B. and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85**: 5166-5170.

Scaffidi, C., Schmitz, I., Krammer, P. H. and Peter, M. E. (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* **274**: 1541-1548.

Schmidt, A. and Hall, A. (2002) The Rho exchange factor Net1 is regulated by nuclear sequestration. *J. Biol. Chem.* **277**: 14581-14588.

Schonhoff, C. M., Gaston, B. and Mannick, J.B. (2003) Nitrosylation of cytochrome c during apoptosis. *J. Biol. Chem.* **278**: 18265-18270.

Schulte, K. M., Jonas, C., Krebs, R. and Roher, H. D. (2001) Activin A and activin receptors in thyroid cancer. *Thyroid* **11**: 3-14.

Schuster, N. and Krieglstein, K. (2002) Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res.* **307**: 1-14.

Secchiero, P., Milani, D., Gonelli, A., Melloni, E., Campioni, D., Gibellini, D., Capitani, S. and Zauli, G. (2003) Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF-alpha promote the NF-kappaB-dependent maturation of normal and leukemic myeloid cells. *J. Leukoc. Biol.* **74**: 223-232.

Selten, G., Cuypers, H. T. and Berns, A. (1985) Proviral activation of the putative oncogene Pim-1 in MuLV induced T-cell lymphomas. *EMBO. J.* **4**:1793-1798.

Selten, G., Cuypers, H. T., Boelens, W., Robanus-Maandag, E., Verbeek, J., Domen, J., van Beveren,C. P. and Berns, A. (1986) The primary structure of the putative oncogene pim-1 shows extensive homology with protein kinases. *Cell* **46**: 603-611.

Selten, G., Cuypers, H. T., Zijlstra, M., Melief, C. and Berns, A. (1984) Involvement of c-Myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. *EMBO. J.* **3**: 3215-3222.

Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B. and Gray, J. W. (1999) PIK3CA is implicated as an oncogene in ovarian cancer. *Nat. Genet.* **21**: 99-102.

Shi, L., Mai, S., Israels, S., Browne, K., Trapani, J. A., Greenberg, A. H. and Granzyme, B. (1997) (GraB) auton omously crosses the cell membrane and per forin initiates apoptosis and GraB nuclear localization. *J. Exp. Med.* **185**: 855-866.

Shinoda, S., Schindler, C. K., Quan-Lan, J., Saugstad, J. A., Taki, W., Simon, R. P. and Henshall, D. C. (2003) Interaction of 14-3-3 with Bid during seizure-induced neuronal death. *J. Neurochem.* 86: 460-469.

Shyng, S. L., Barbieri, A., Gumusboga, A., Cukras, C., Pike, L., Davis, J. N., Stahl, P. D. and Nichols, C. G. (2000) Modulation of nucleotide sensitivity of ATP-sensitive potassium channels by phosphatidylinositol-4-phosphate 5-kinase. *Proc. Natl. Acad. Sci. USA.* **97**: 937-941.

Silhavy, T. J., Berman, M. L. and Enquist, L. W. (1984) *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y.

Smaglik, P. (2000) Researchers take a gamble on the human genome. *Nature* **405**: 264.

Smith, G. C., Tew, D. G., Wolf, C. R. (1994) Dissection of NADPH-cytochrome P450 oxidoreductase into distinct functional domains. *Proc. Natl. Acad. Sci. USA.* **91**: 8710-8714.

Solito, E., de Coupade, C., Canaider, S., Goulding, N. J. and Perretti, M. (2001) Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation. *Br. J. Pharmacol.* **133**: 217-228.

Spooncer, E. and Dexter, T. M. (1997) Culturing primitive hemopoietic cells. Long-term mouse marrow cultures and the establishment of factor-dependent (FDCP-Mix) hemopoietic cell lines. *Methods Mol. Biol.* **75**: 209-219.

Staal, S. P., Hartley, J. W. and Rowe, W. P. (1977) Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. *Proc. Natl. Acad. Sci. USA* **74**: 3065-3067.

Staal, S. P. (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc. Natl. Acad. Sci. USA* **84**: 5034-5037.

Stanford, W. L., Cohn, J. B. and Cordes, S. P. (2001) Gene-trap mutagenesis: past, present and beyond. *Nat. Rev. Genet.* **2**: 756-768.

Starr, R. and Hilton, D. J. (1999) Negative regulation of the JAK/STAT pathway. *Bioessays* **21**: 47-52.

Stein, R. C. (2001) Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocr Relat Cancer*. **8**: 237-248.

Steller, H. (1995) Mechanisms and genes of cellular suicide. *Science* **267**: 1445-1449.

Storz, P. and Toker, A. (2002) 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. *Front Biosci.* **7**: 886-902.

Strasser, A., Harris, A. W., Bath, M. L. and Cory, S. (1990) Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl*-2. *Nature* **348**: 331-333.

Suhara, T., Kim, H. S., Kirshenbaum, L. A. and Walsh, K. (2002) Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. *Mol. Cell. Biol.* **22**: 680-691.

Sutton, V. R., Davis, J. E. and Cancilla, M. (2000) Initiation of apoptosis by granzyme B requires di- rect cleavage of bid, but not direct granzyme B-mediated caspase activation. *J. Exp. Med.* **192**: 1403-1414.

Sutton, V. R., Vaux, D. L. and Trapani, J. A. (1997) Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. *J. Immunol.* **158**: 5783-5790.

Sutton, V. R., Wowk, M.E., Cancilla, M., and Trapani, J. A. (2003) Caspase activation by granzyme B is indirect, and caspase autoprocessing

requires the release of proapoptotic mitochondrial factors. *Immunity* **18**: 319-329.

Teramoto, H., Malek, R. L., Behbahani, B., Castellone, M. D., Lee, N. H. and Gutkind J. S. (2003) Identification of HRas,RhoA,Rac1 and Cdc42 responsive genes. *Oncogene* **22**: 2689-2697.

Thamilselvan, V., Fomby, M., Walsh, M. and Basson, M. D. (2003) Divalent cations modulate human colon cancer cell adhesion. *J. Surg. Res.* **110**: 255-265.

Thompson, C. (1995) Apoptosis in the pathogenesis and Treatment of disease. *Science* **267**: 1456-1462.

Thorey, I. S., Muth, K., Russ, A. P., Otte, J., Reffelmann, A. and von Melchner H. (1998) Selective disruption of genes transiently induced in differentiating mouse embryonic stem cells by using gene trap mutagenesis and site-specific recombination. *Mol. Cell. Biol.* **18**: 3081-3088.

Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo,M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T. and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **272**: 17907-17911.

Tsuruo, T., Naito, M., Tomida, A., Fujita, N., Mashima, T., Sakamoto, H. and Haga, N. (2003) Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci.* **94**: 15-21.

Turgeon, B., Lang, B. F. and Meloche, S. (2002) The protein kinase ERK3 is encoded by a single functional gene: genomic analysis of the ERK3 gene family. *Genomics* **80**: 673-680.

Turgeon, B., Saba-El-Leil, M. K. and Meloche, S. (2000) Cloning and characterization of mouse extracellular-signal-regulated protein kinase 3 as a unique gene product of 100 kDa. . *Biochem. J.* **346**:169-175.

Uittenbogaart, C. H., Cantor, Y. and Fahey, J. L. (1983) Growth of human malignant lymphoid cell lines in serum-free medium. *In Vitro* **19**: 67-72.

van der Gaag, E. J., Leccia, M. T., Dekker, S. K., Jalbert, N. L., Amodeo, D. M., Byers and H. R. (2002) Role of zyxin in differential cell spreading and proliferation of melanoma cells and melanocytes. *J. Invest. Dermatol.* **118**: 246-254.

Vaux, D. L., Cory, S., and Adams, J. M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**: 440-442.

Venter, C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R. Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder,S., Clark, A. G., Nadeau, J., McKusick,V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon,M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan,M., Florea, L., Halpern,

A.,Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick,K., Bonazzi,V., Brandon,R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di, V., Dunn, F.P., Eilbeck,K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji,R., Ke, Z., Ketchum,K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam, B., Nusskern,D., Rusch, D.B., Salzberg, S., Shao,W., Shue, B., Sun, J., Wang,Z.Y., Wang, A., Wang, X., Wang, J., Wei, M-H., Wides,R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong,F., Zhong, W., Zhu, S.C., Zhao, S., Gilbert, D., Baumhueter, S., Spier,G., Carter, C. (2001) The Sequence of the Human Genome. *Science* **291**: 1304-1351.

Verbeek, S., van Lohuizen, M., van der Valk, M., Domen, J., Kraal, G. and Berns, A. (1991) Mice bearing the E mu-myc and E mu-pim-1 transgenes develop pre-B-cell leukemia prenatally. *Mol. Cell. Biol.* **11**: 1176-1179.

Vernon, S. D., Unger, E. R., Rajeevan, M., Dimulescu, I. M., Nisenbaum, R. and Campbell, C. E. (2000) Reproducibility of alternative probe synthesis approaches for gene expression profiling with arrays. *J. Mol. Diagn.* **2**: 124-127.

Verschuern, K., Dewulf, N., Goumans, M. J., Lonnoy, O., Freijen, A., Grimsby, S., Vande Spiegle, K., ten Dijke, P., Moren, A., Vanscheeuwijck, P., Heldin, C. H., Miyazono, K., Mummery,C., Van Den Eijnden-Van Raaij, J. and Huylebroeck, D. (1995) Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. *Mech. Dev.* **52**: 109-123.

Wagner, A. J., Small, M., B. and Hay, N. (1993) Myc-mediated apoptosis is blocked by ectopic expression of Bcl-2. *Mol. Cell. Biol.* **4**: 2432-2440.

Wang, J., Chun, H. J., Wong, W., Spencer, D. M. and Lenardo, M. J. (2001) Caspase-10 is an initiator caspase in death receptor signaling. *Proc. Natl. Acad. Sci. USA.* **98**: 13884-13888.

Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L. and Korsmeyer, S. J. (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev.* **10**: 2859-2869.

Wang, Q., Ding, Q., Dong, Z., Ehlers, R. A. and Evers, B. M. (2000) Downregulation of mitogen-activated protein kinases in human colon cancers. *Anticancer Res.* **20**: 75-83.

Wang, T. and Hebert, D. N. (2003) EDEM an ER quality control receptor. *Nat. Struct. Biol.* **10**: 319-321.

Wang, W., Xu, J. and Kirsch, T. (2003) Annexin-mediated Ca2+ influx regulates growth plate chondrocyte maturation and apoptosis. *J. Biol. Chem.* **278**: 3762-3769.

Wei, Y. F., Carter, K. C., Wang, R. P. and Shell, B. K. (1996) Molecular cloning and functional analysis of a human cDNA encoding an Escherichia coli AlkB homolog, a protein involved in DNA alkylation damage repair. *Nucleic Acids Res.* **24**: 931-937.

Weitzman, J. B., Fiette, L., Matsuo, K. and Yaniv, M. (2000) JunD protects cells from p53-dependent senescence and apoptosis. *Mol. Cell.* **6**: 1109-1119.

Wempe, F., Yang, J. Y., Hammann, J. and von Melchner, H. (2001) Gene trapping identifies transiently induced survival genes during programmed cell death. *Genome Biology* **2**: 0023.1-0023.10.

Westendorf, J. J. (2001) The formin/diaphanous-related protein, FHOS, interacts with Rac1 and activates transcription from the serum response element. *J. Biol. Chem.* **276**: 46453-46459.

White, E. (1996) Life, death, and the pursuit of apoptosis. *Genes Dev.* **10**: 1-15.

Williams, G., Smith, C., Spooncer, E., Dexter, T. and Taylor, D. (1990) Haematopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**: 76-79.

Willis, S. A., Zimmerman, C. M., Li, L. I. and Mathews, L. S. (1996) Formation and activation by phosphorylation of activin receptor complexes. *Mol. Endocrinol.* **10**: 367-379.

Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* **142**: 67-77.

Wymann, M. P. and Pirola, L. (1998) Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta.* **1436**: 127-150.

Yamaguchi, H. and Wang, H. G. (2001) The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* **20**: 7779-7786.

Yamakawa, N., Tsuchida, K. and Sugino, H. (2002) The rasGAP-binding protein, Dok-1, mediates activin signaling via serine/threonine kinase receptors. *EMBO. J.* **21**:1684-1694.

Yamazaki, K., Sakamoto, M., Ohta, T., Kanai, Y., Ohki, M. and Hirohashi, S. (2003) Overexpression of KIT in chromophobe renal cell carcinoma. *Oncogene* **22**: 847-852.

Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P. and Kohwi-Shigematsu, T. (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* 419: 641-645.

Yayoshi-Yamamoto, S., Taniuchi, I. and Watanabe, T. (2000) FRL, a novel formin-related protein, binds to Rac and regulates cell motility and survival of macrophages. *Mol. Cell. Biol.* **20**: 6872-6881.

Yelin, R., Dahary, D., Sorek, R., Levanon, E. Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., Nemzer, S., Pinner, E., Walach, S., Bernstein, J., Savitsky, K. and Rotman, G. (2003) Widespread occurrence of antisense transcription in the human genome. *Nature Biotechnology* **21**: 379-386.

Yin, X. M. (2000) Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. *Cell Research* **10**: 161-167.

You, Z., Madrid, L. V., Saims, D., Sedivy, J. and Wang, C. Y. (2002) c-Myc sensitizes cells to tumor necrosis factor-mediated apoptosis by inhibiting nuclear factor kappa B transactivation. *J. Biol. Chem.* **277**: 36671-36677.

Zaman, K., Ryu, H., Hall, D., O'Donovan, K., Lin K, I., Miller, M. P., Marquis, J. C., Baraban, J. M., Semenza, G. L. and Ratan, R. R. (1999) Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. *J. Neurosci.* **19**: 9821-9830.

Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G. and Korsmeyer, S. J. (1997) BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. *J. Biol. Chem.* **272**: 24101-24104.

Zhan, Q., Jin, S., Ng, B., Plisket, J., Shangary, S., Rathi, A., Brown, K. D. and Baskaran, R. (2002) Caspase-3 mediated cleavage of BRCA1 during UV-induced apoptosis. *Oncogene* **21**: 5335-5345.

Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W. and Anderson, R. A. (1997) Phosphatidylinositol-4-phosphate 5-Kinase Isozymes Catalyze the Synthesis of 3-Phosphate-containing Phosphatidylinositol Signaling Molecules. *The American Society for Biochemistry and Molecular Biology* **272**: 17756-17761.

Zhang, X. L., Simmen, F. A., Michel, F. J. and Simmen, R. C. (2002) Increased expression of the Zn-finger transcription factor BTEB1 in human endometrial cells is correlated with distinct cell phenotype, gene expression patterns, and proliferative responsiveness to serum and TGF-beta1. *Mol. Cell. Endocrinol.* **181**: 81-96.

Zhu, Y.M. , Bradbury, D. A. and Russell, N. H. (1994) Wild-type p53 is required for apoptosis induced by growth factor deprivation in factor-dependent leukaemic cells. *Br. J. Cancer* **69**: 468-472.

Zimmermann, J., Lamerant, N., Grossenbacher, R. and Fürst P. (2001) Proteasome- and p38-dependent Regulation of ERK3 Expression. *J. Biol. Chem.* **276**: 10759-10766.

APPENDIX I

Target genes and their accession numbers

Protein name:	FLIP-L; apoptosis inhibitor; FLICE-like inhibitory protein long form
GenBank Accession:	U97076
Protein ID:	AAC53281.1
Data base reference:	GI: 2253683
Date base source:	locus U97076, accession U97076.1

Protein name:	Bcl-XL apoptosis regulator (bcl-x long); Bcl-2 family member
GenBank Accession:	L35049
Protein ID:	AAA51039.1
Data base reference:	GI: 561741
Date base source:	locus MUSBCLX, accession L35049.1

Protein name:	Jun-D; c-jun-related transcription factor
GenBank Accession:	J05205
Protein ID:	AAA39345.1
Data base reference:	GI: 293681
Date base source:	locus MUSJUNDR, accession J05205.1

Protein name:	c-Kit proto-oncogene (mast/stem cell growth factor receptor tyrosine kinase)
GenBank Accession:	Y00864
Protein ID:	CAA68772.1
Data base reference:	GI: 50424, SWISS-PROT: P05532
Date base source:	locus 50423

Protein name:	Zyxin; LIM domain protein; alpha-actinin binding protein
GenBank Accession:	X99063
Protein ID:	CAA67510.1
Data base reference:	GI: 1430883, SWISS-PROT: Q62523
Date base source:	locus MMZYX

Protein name:	Chop10; murine homologue of Gadd153 (growth arrest and DNA-damage-inducible protein)
GenBank Accession:	X67083
Protein ID:	CAA47465.1
Data base reference:	GI: 50407, SWISS-PROT: P35639
Date base source:	embl locus MMCHOP10, accession X67083.1

Protein name:	Pim-1 protein kinase
GenBank Accession:	M13945
Protein ID:	AAA39930.1
Data base reference:	GI: 387510
Date base source:	locus MUSPIM1, accession M13945.2

Protein name: Cyclin G (G2/M-specific)
GenBank Accession: Z37110
Protein ID: CAA85474.1
Data base reference: GI: 558381, SWISS-PROT: P51945
Date base source: embl locus MMCYCLGMR, accession Z37110.1

Protein name: Activin type I receptor
GenBank Accession: Z31663
Protein ID: CAA83483.1
Data base reference: GI: 840813, SPTREMBL: Q61271
Date base source: embl locus MMACT1B, accession Z31663.1

Protein name: Elf-1 Ets family transcription factor
GenBank Accession: U19617
Protein ID: AAB17097.1
Data base reference: GI: 972940
Date base source: locus MMU19617, accession U19617.1

Protein name: Mouse interleukin-3 receptor
GenBank Accession: M29855
Protein ID: AAA39295.1
Data base reference: GI: 309406
Date base source: locus MUSIL3R

Protein name: Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
GenBank Accession: M32599
Protein ID: AAA37659.1
Data base reference: GI: 309243
Date base source: locus MUSGAPDH, accession M32599.1

Protein name: c-myc protein
GenBank Accession: X01023
Protein ID: CAA25508.1
Data base reference: GI: 50468, SWISS-PROT: P01108
Date base source: locus CAA25508

Protein name: interleukin-3 [Mus musculus]
GenBank Accession: BAA09848
Protein ID: g4433276
Data base reference: GI: 4433276
Date base source: locus D63766.1

APPENDIX II (Commercial Suppliers)

Chemicals	**Suppliers**
Acetic acid	Merck, Darmstadt
Acetone	Riedl-de Heaen, Seelze
Agarose (for cell culture)	Gibco-BRL (Life Technologies)
Agarose (for gel analysis)	Roth GmbH, Karlsruhe
Alcohol (methyl-, ethyl-)	J.T Baker, Holland
Alcohol (propyl-, butyl-)	Sigma, Steinheim
Alkaline phosphatase and buffer	Boehringer-Mannheim
Ammonium sulfate	Gibco-BRL (Life Technologies)
Ampicillin	Boehringer-Mannheim
Bradford reagent	Bio-Rad
Bromophenolblue	Sigma, Deisenhofen
BSA	Sigma, Steinheim
Calcium chloride	BDH
Chloroform	Merck, Darmstadt
Coomassie brilliant blue	Aldrich
DEPC	ICN Biomedical, Ohio, USA
DMEM, RPMI 1640 (cell culture)	Gibco-BRL (Life Technologies)
DMSO	Merck, Darmstadt
DNA ladder (1 kb, 100 bp)	Gibco-BRL (Life Technologies)
DNA purification kit (Mini,Midi,Maxi &PCR)	QIAGEN
dNTPs (dGTP/dATP/dCTP/dTTP)	Gibco-BRL (Life Technologies)
DTT	Gibco-BRL (Life Technologies)
EDTA	Appli Chem., Darmstadt
Enhancer PCR kit	Gibco-BRL (Life Technologies)
Ethidium bromide	Roth GmbH, Karlsruhe
ExpressHyb™	Clontech
Fetal bovine serum (FCS, cell culture)	Gibco-BRL (Life Technologies)
Ficoll	Sigma, Deisenhofen
Formamide	Fluka, Seelze
Formaldehyde 37%	Riedl-de Heaen, Seelze
Glucose	Sigma, Deisenhofen
(L) Glutamine	Gibco-BRL (Life Technologies)
Glycerol	Appli Chem., Darmstadt
Glycogen	Boehringer, Mannheim
HEPES	Sigma, Steinheim
HPLC-H_2O	J.T Baker, Holland
Human Cot-1 DNA®	Gibco-BRL (Life Technologies)
Hydrochloride, 25%	Roth GmbH, Karlsruhe
IPTG	Sigma, Steinheim
Kanamycin	Sigma, Steinheim
Magnesium chroride-Hexahydrate	Merck, Darmstadt
Manganese chloride tetrahydrate	Sigma, Steinheim
β-Mercaptoethanol	Merck, Darmstadt
Murine interleukin-3 (IL-3, cell culture)	TEBU

Oligo (dT) cellulose	Pharmacia
Oligonucleotids	Interactiva
^{32}P-dATP & ^{32}P-dCTP	Amersham Pharmacia
Penicillin/Streptomycin	Gibco-BRL (Life Technologies)
pGEM®-T & pGEM®-T EASY vector	Promega (Madison, USA)
Phenol	Appli Chem., Darmstadt
Potassium chloride	Merck, Darmstadt
RNA ladder (0.24-9.5 kb)	Gibco-BRL (Life Technologies)
RNasin® Ribonuclease inhibitor	Promega
RNAzol™ B (for RNA purification)	Cinna Scientific, Inc.
Salmon testes DNA	Sigma
SDS	Appli Chem., Darmstadt
Sephadex G-25	Pharmacia
Sodium acetate trihydrate	Gibco-BRL (Life Technologies)
Sodium chloride	Sigma, Steinheim
Sodium hydroxide	Roth GmbH, Karlsruhe
Tris-Base	Sigma, Steinheim
Trypan-blue	Gibco-BRL (Life Technologies)
XL10-Gold ultracompetent cells	Stratagene

Enzymes	**Suppliers**
Alkaline phosphatase	New England Biolabs,Taunus
DNase I (RNase-free)	Boehringer, Mannheim
Klenow enzyme	Boehringer, Mannheim
Lysozyme	Sigma
Proteinase K	Sigma, Deisenhofen
T4 DNA ligase	New England Biolabs, Taunus
Taq-DNA-Plolymerase (Platinium & HiFi)	Gibco-BRL (Life Technologies)
Rediprime™ II (for random prime labelling)	Amersham Pharmacia
Restriction endonuclease	New England Biolabs, Taunus
Reverse transcriptase (Superscript II RT)	Gibco-BRL (Life Technologies)
RNase A	Boehringer, Mannheim
RNase H	Gibco-BRL (Life Technologies)
RNeasy (for 2nd RNA purification)	QIAGEN
Sequencing Kit	Amersham, Braunschweig

Instruments	**Suppliers**
ABI PRISM$^{TM\ 310}$ Genetic Analyser	Perkin Elmer, Norwalk USA
ABI PRISM Sequencing Kits	Applied Biosystems
Atlas Image 1.01a (Analysis program)	Clontech
Cell culture flasks	Nunk, Roskilde, Denmark
Cell culture plates	Nunk, Roskilde, Denmark
Centricon (micro filter)	Amicon
Centrifuge (Sorvall RC5B)	Heraeus (Dupont), Hanau
Centrifuge (Eppendorf)	Hamburg
Chroma spin -200 column	Clontech
Cuvettes (quartz), Ultraspec®	Pharmacia Biotech, Freiburg
Cuvettes (plastic, 1 ml)	Sarstedt
DMEM, RPMI 1640	Gibco-BRL (Life Technologies)
Eppendorf tube	Sarstedt, Nümbrecht

Eppendorf tube (Screw cap)	Sorenson
Film BioMax	Kodak, Rochester, USA
Gel electrophoresis (XCell II™)	NOVEX
Glass bottle for hybridization	Biometra
Hybond-N (and N^+)	Amersham
Hybridiser HB-1D	Techne
Latex gloves	Ansell
Laboratory scales	Sartorious
MEGAscript™ T7	Ambion
Micro Centrifuge 5415D	Eppendorf, Hamburg
NuPAGE™ 10% Bis-Tris Gel	Invitrogen
Parafilm™	American National Can Co.
PCR machine, DNA Themal Cycler 480	Perkin Elmer, Weiterstade
PCR machine, Robo Cycler Gradient 96	Stratagene
PCR tubes (200 µl)	Molecular Bioproducts
Peristaltic pump (Minipuls II)	Gilson
Petri dish (plastic)	Biolab Scientific Pty
$_p$H-meter CG825	Scott
Phosphorimager™ SI	Molecular dynamics
Pipettes (10, 20, 200, 1000 µl)	Gilson
Polaroid camera (Model:	E.A.S.Y RH) Herolab
Power supplies	Consort, Belgium
Rotors (Sorvall GS3, SE12, SS34)	Heraeus (Dupont), Hanau
Scanner (SNAPSCAN600)	Agfa
Scintillation Analyzer, TRI-CARB 1500	Packard
Spectrohotometer (Gene Quant II)	Pharmacia Biotech, Freiburg
UV crosslinker® 1800	Stratagene, Hamburg
UV Spectrophotometer , computer & softwere	Herolab (Easy Image Plus)
Vortex (REAX 2000)	Heidolph
Water purification (milliQ system)	Millipore
(shaking) water bath	KötterMann, Labor Technik

Sequence analysis softwares	**Suppliers**
DNA Strider	Christian Marck Service de Biochimie-Bat 142 Centre d'Etudes Nuclaires de Saclay 91191 Gif-sur-Yvette Cedex France
MacVector	International Biotechnologies P.O. Box 9558 New Haven, CT 06535
Plasmid Artist	Clontech Laboratory 4030 Fabian Way Palo Alto, CA 94303

MicroArray analysis software	**Suppliers**
Atlas Image 1.01a (Analysis program)	Clontech
ImageQuant version 1.2	Molecular Dynamics

APPENDIX III (commonly used stock solutions)

Followings are commonly used buffers and stock solutions in the manipulation of nucleic acids and proteins. Always deionized, distilled water and reagents of the highest grade available were used for preparing stock solutions. Sterilization was done by filtration through a 0.22-0.45 µm filters or by standard autoclaving.

Ammonium acetate, 10 M

Dissolve 385.4 g ammonium acetate in 150 ml H_2O

Add H_2O to 500 ml

$CaCl_2$, 1 M

147 g $CaCl_2 \cdot 2H_2O$

H_2O to 1 liter

Dithiothreitol (DTT), 1 M

Dissolve 15.45 g DTT in 100 ml H_2O

Store at -20°C

EDTA (ethylenediamine tetraacetic acid), 0.5 M (pH 8.0)

Dissolve 186.1 g $Na_2EDTA \cdot 2H_2O$ in 700 ml H_2O

Adjust pH to 8.0 with 10 M NaOH (~50 ml)

Add H_2O to 1 liter

Ethidium bromide, 10 mg/ml

Dissolve 0.2 g ethidium bromide in 20 ml H_2O

Mix well and store at 4°C in dark

HCl, 1 M

Mix in the following order:

913.8 ml H_2O

86.2 ml concentrated HCl

KCl, 1 M

74.6 g KCl

H_2O to 1 liter

$MgCl_2$, 1 M

20.3 g $MgCl_2 \cdot 6H_2O$

H_2O to 100 ml

$MgSO_4$, 1 M

24.6 g $MgSO_4 \cdot 7H_2O$

H_2O to 100 ml

MOPS buffer

0.2 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0

0.5 M sodium acetate

0.01 M EDTA

NaCl, 5 M (292 g NaCl and H_2O up to 1 liter)

NaOH, 10 M

Dissolve 400 g NaOH in 450 ml H_2O

Add H_2O to 1 liter

PBS (phosphate-buffered saline)

*10**x** stock solution, 1 liter:*

80 g NaCl

2 g KCl

11.5 g $Na_2HPO_4 \cdot 7H_2O$

2 g KH_2PO_4

Working solution, pH ~7.3:

137 mM NaCl

2.7 mM KCl

4.3 mM $Na_2HPO_4 \cdot 7H_2O$

1.4 mM KH_2PO_4

Sodium acetate, 3 M

Dissolve 408 g sodium acetate$\cdot 3H_2O$ in 800 ml H_2O (or DEPC-H_2O)

Add H_2O to 1 liter

Adjust pH to 4.8 or 5.2 (as desired) with 3 M acetic acid

SSC (sodium chloride/sodium citrate), 20x

3 M NaCl (175 g/liter)

0.3 M Na_3citrate$\cdot 2H_2O$ (88 g/liter)

Adjust pH to 7.0 with 1 M HCl

TAE (Tris/acetate/EDTA) electrophoresis buffer

50x stock solution:

242 g Tris base

57.1 ml glacial acetic acid

37.2 g $Na_2EDTA \cdot 2H_2O$

H_2O to 1 liter

Working solution, pH ~8.5:

40 mM Tris·acetate

2 mM $Na_2EDTA \cdot 2H_2O$

TE (Tris/EDTA) buffer

10 mM Tris·Cl, pH 7.4, 7.5, or 8.0

1 mM EDTA, pH 8.0

Tris·Cl [tris(hydroxymethyl)aminomethane], 1 M

Dissolve 121 g Tris base in 800 ml H_2O

Adjust to desired pH with concentrated HCl
Mix and add H_2O to 1 liter

Approximately 70 ml of HCl is needed to achieve a pH 7.4 solution, and approximately 42 ml for a solution that is pH 8.0.

1 x Binding buffer (poly(A)$^+$-RNA isolation)

0.5 M NaCl; 10 mM Tris, pH 7.2; 0.1 mM EDTA; 0.2 % SDS

Wash buffer (poly(A)$^+$-RNA isolation)

0.1 M NaCl; 10 mM Tris, pH 7.2; 0.1 mM EDTA; 0.2 % SDS

Elution buffer (poly(A)$^+$-RNA isolation)

10 mM Tris, pH 7.2; 0.1 mM EDTA; 0.2 % SDS

10X DNase I Buffer (Atlas array)

400 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM $MgCl_2$

Phenol:chloroform:isoamyl alcohol (poly(A)$^+$-RNA isolation)

25:24:1; equilibrated with 0.1 M sodium citrate, pH 4.5, 1 mM EDTA

10X Termination Mix (Atlas array)

0.1 M EDTA, pH 8.0, 1 mg/ml glycogen

10X denaturing solution (Atlas array)

1 M NaOH, 10 mM EDTA

2X neutralizing solution (Atlas array)

1 M NaH_2 PO_4 ,pH 7.0

Wash Solution 1 (Atlas array)

2X SSC, 1% SDS

Wash Solution 2 (Atlas array)

0.1X SSC, 0.5% SDS

APPENDIX IV

Primers for generation of probes for Northern Hybridization

Target genes		Primer Sequences
Flip-L	Sense Antisense	5'- GCCAGCTCTCTTTTGCTACCTTG -3' 5'- GCTTGCTCTGCATCCTGTAAGTCTC -3'
Bcl-X_L	Sense Antisense	5'- AGAGAGGCAGGCGATGAGTTTG -3' 5'- GGCTCTAGGTGGTCATTCAGATAGG -3'
JunD	Sense Antisense	5'- TCGACATGGACACGCAAGAACG -3' 5'- GTTGACGTGGCTGAGGACTTTC -3'
zyxin	Sense Antisense	5'- GGAGATTGACTCTCTGTCCTCACTG -3' 5'- GGCACCATTTTTTGATTGGCTG -3'
GST	Sense Antisense	5'- ACCTTGCCCGAAAGCACAAC -3' 5'- TTGCCCAGAAACTCAGAGTAGAGC -3'
Chop-10	Sense Antisense	5'- TGAGTCCCTGCCTTTCACCTTG -3' 5'- ACGCAGGGTCAAGAGTAGTGAAGG -3'
Pim-1	Sense Antisense	5'- ATGGAAGTGGTCCTGTTGAAGAAG -3' 5'- TGGCTCACCATCAAAGTCCG -3'
Aktivin type I rec'	Sense Antisense	5'- CTACCATAACCGCCAGAGGTTG -3' 5'- CAAGTCTCGATGAGCAATTCCC -3'
c-myc	Sense Antisense	5'- TCTGTGGAGAAGAGGCAAACCC -3' 5'- GCTTGCTCTGCATCCTGTAAGTCTC -3'
Elf-1	Sense Antisense	5'- CAAACCTGACATGAACTACGAGACC-3' 5'- CCCTCCTTTAATTCCTGGACTTG-3'
GAPDH	Sense Antisense	5'- CCAAACGGGTCATCATCTC -3' 5'- TGGATGCAGGGATGATGTT -3'

APPENDIX V

Primers for generation of probes for matched tumor/normal array assay

Target genes		Primer Sequences	Hybridization Temperature
ERK3	Sense Antisense	5'- ACG ATG TGG GCT CTC TTA CGG AAC -3' 5'- CCA GGA AAT CCA GTG CTT CTC G -3'	68°C
PIPKβ	Sense Antisense	5'- ATG TGC CTG ATG CTA AGC GGA C -3' 5'- GGC TGC TAA AAC TTT GTC CTT CAG TC -3'	65°C
PIPKγ	Sense Antisense	5'- GCT GTG AAA CCG CTG GGG -3' 5'- CGC CCT CCT CGT CTG AGG C -3'	65°C
KIAA 0740	Sense Antisense	5'- CGGATGGAGTAAGGGGTTCATTG -3' 5'- TCGGGAGATACACCTCACTGTTGG -3'	65°C
KIAA 1036	Sense Antisense	5'- GAAACTGCTGGTTCTGCTGGTG -3' 5'- CCATTCTGGGTCCTCCGCC -3'	63°C
KIAA 1140	Sense Antisense	5'- GAATGCCGCAGCCATCTATG -3' 5'- CATCTTGCTTGGACTTCAGGGTG -3'	68°C
Plecha2	Sense Antisense	5'- GCGGCAAGTTTCTGCGG -3' 5'- GGCTGATGGGTGTGTGGACC -3'	68°C

APPENDIX VI

Primers for generation of array materials for self-made arrays
(see APPENDIX VII for the Arrangement)

Seq ID		Primer Sequences	T_a	Template	cDNA (bp)
26-11-1	Sense Antisense	5'-AGGGCTTTGGGCAGGAG -3' 5'-CAAACAGGAAGTTTATGAGAAA -3'	61°C	Genomic DNA	95
26-11-2	Sense Antisense	5'-AGGGGGGGGGAGAATGA -3' 5'-GAATGTGAAAGTGTAACTGGAG -3'	54°C	cDNA	797
26-11-3	Sense Antisense	5'-ATATTGTATATAAATCCAATGTTTTC -3' 5'-AGGACAGCATTTCATTGGG -3'	54°C	Genomic DNA	587
26-11-4	Sense Antisense	5'-GTCAGGATGGGAACTCAAACA -3' 5'-GGGAAGGCTTTGCATCTAA -3'	61°C	cDNA	314
26-11-5	Sense Antisense	5'-AGAAATGTCCAGCAGCCCA -3' 5'-GCAACCTTATAATGGAGAAACAA -3'	54°C	cDNA	667
26-12-1	Sense Antisense	5'-GGAGGTGACTGGGGTGAG -3' 5'-CATCTGATCCTGAGCCACTT -3'	54°C	Genomic DNA	173
26-12-3	Sense Antisense	5'-GTGTGTCTGTGTGCCACC -3' 5'-CTCCAGTTGAATTCTTAATACTT -3'	54°C	cDNA	254
26-21-4	Sense Antisense	5'-CCCCCTTTCGCTCCTG -3' 5'-GAAGCCAGCCTTAACCAG -3'	54°C	Genomic DNA	115
26-22-4	Sense Antisense	5'-GTTTTCTCTGCAAACAAACCC -3' 5'-TATGTGCTTTTTAGATACCCAA -3'	54°C	Genomic DNA	240
26-22-7	Sense Antisense	5'-TCAAGGACATTTGCGAGGG -3' 5'-GTAGATCCAGGCCATCTCTC -3'	61°C	cDNA	622
26-31-1	Sense Antisense	5'-GGAAGTGAATGTATGTTTCGT -3' 5'-AAAACCTATAATCGTGTTCCC -3'	54°C	Genomic DNA	130
26-31-3	Sense Antisense	5'-CAGTGTCTCGTAAGTGGTC -3' 5'-TCTTAAATTTCATAAACTTTGGTCT -3'	54°C	Genomic DNA	300
26-31-7	Sense Antisense	5'-AGACAGAGGCAGGCAAAC -3' 5'-TCTCGGCATCACGCTCA -3'	61°C	cDNA	177
26-32-1	Sense Antisense	5'-GAGGTGTGGGGGGGGTG -3' 5'-ACTTCCTTAACCTGGCATTC -3'	61°C	cDNA	780
26-32-2	Sense Antisense	5'-GTCCCACCCCCGTTGAG -3' 5'-TACCTTATACTTCAGTTGTAGA -3'	54°C	cDNA	1383
26-32-3	Sense Antisense	5'-CTTCATAAAGTTAAGAAAGACCC -3' 5'-CAGGCGCATAAGTCAGTT -3'	54°C	Genomic DNA	301
26-32-4	Sense Antisense	5'-CAAAGGCGCACATTTAAAGA -3' 5'-GATCGTAGAAGCCTGACG -3'	61°C	cDNA	362
26-32-5	Sense Antisense	5'-AGCCAAGCAATAAGAGAGT -3' 5'-TGAGAGAGAGACCCAAACC -3'	61°C	Genomic DNA	1653
26-32-5A	Sense Antisense	5'-GCAGAAGCAGAGCTCCA -3' 5'-AACATCTAAGACTCGGATCGT -3'	61°C	cDNA	223
26-32-5B	Sense Antisense	5'-AGCCGGCGGCTCAGAA -3' 5'-GCATCATCATCTTTCTCTCTT -3'	61°C	cDNA	216
26-32-8	Sense Antisense	5'-AGTTCAATTTCCATCACCCAC -3' 5'-GAGAGCCTTCTGAGTGTTG -3'	54°C	cDNA	175
26-41-2	Sense Antisense	5'-AAATAAACATGCAGCTTTAAGT -3' 5'-GTCTTTATGCTGTTTTCTTTTTG -3'	54°C	Genomic DNA	276
26-41-3	Sense Antisense	5'-GAAAGGGTGGCAAGTACAC -3' 5'-CTTTTCGTTCCTCCTACCC -3'	54°C	cDNA	588
26-41-6	Sense Antisense	5'-TGGTCTGTGATTTATTCAATGG -3' 5'-ATGGACCTAGAATTTTCCTACAC -3'	54°C	cDNA	1565
30-11-3	Sense Antisense	5'-GAGTGACTGGAAGGTTATGA -3' 5'-AATGTCCGGCTGTACACA -3'	54°C	cDNA	326

Seq ID		Primer Sequences	T_a	Template	cDNA (bp)
30-11-5	Sense Antisense	5'-GGAACAGTAATACTGAAGCG -3' 5'-GCTTGAGAGTTTCTAGAAATG -3'	54°C	Genomic DNA	245
30-11-6	Sense Antisense	5'-CTGCTCACCGTCGTAGTT -3' 5'-GGCTCCAGCATGGCTATC -3'	61°C	cDNA	841
30-12-1	Sense Antisense	5'-GACGGGTTCAGCAATGGG -3' 5'-CATACAACATCAGTCATCAGG -3'	61°C	cDNA	1100
30-12-2	Sense Antisense	5'-GAACTGCCAGCGAGGAC -3' 5'-GGAAGTGGGACAGGGTTT -3'	54°C	cDNA	1504
30-12-3	Sense Antisense	5'-GCCTTATAAGATTTGAATATTAAC -3' 5'-ATACCACCTATAATGTCTGTGT -3'	54°C	Genomic DNA	290
30-12-4A	Sense Antisense	5'-GCAAGACCAAGACGAAGAA -3' 5'-TGCTGTCACTCTCTCCTTC -3'	54°C	cDNA	878
30-12-4B	Sense Antisense	5'-CCTGCTGGTACCGCAG -3' 5'-ACATCAACAGGCTGTAGTCC -3'	54°C	cDNA	876
30-12-4C	Sense Antisense	5'-GCCGTGACGGCGGAA -3' 5'-CAGCAGCAGGCTGTAGT -3'	54°C	cDNA	917
30-12-5	Sense Antisense	5'-TCTTCCCTAGCCCTCTCA -3' 5'-AGTCTCTCCAGATGTGGCA -3'	54°C	Genomic DNA	249
30-21-2	Sense Antisense	5'-CCCAGACTGCTTAGCTTTC -3' 5'-AGAGACAATTACTGACACAAG -3'	54°C	Genomic DNA	180
30-21-3N	Sense Antisense	5'-CAGCTCTAGAGATCCGAC -3' 5'-AGCCCTCAGCATAACTTCA -3'	61°C	cDNA	1266
30-21-3P	Sense Antisense	5'-ACAGAAGATCATTGAGACACT -3' 5'-TTGGCTGCTGGCTTCTC -3'	54°C	cDNA	116
30-21-4	Sense Antisense	5'-GCACGAGGAAAGGTGCA -3' 5'-AAACACTGCTGGAAAGGAC -3'	54°C	Genomic DNA	945
30-22-1	Sense Antisense	5'-ACACACACACACACAATGC -3' 5'-CCACAGAAGACATCCAGCAT -3'	54°C	Genomic DNA	148
30-22-4	Sense Antisense	5'-GTGAGCAGAGACAGGAAA -3' 5'-CTCATGCAGGTACCCTCC -3'	54°C	Genomic DNA	90
30-31-3	Sense Antisense	5'-TCTCCAGCTCGCCAGT -3' 5'-CACTTCACAGGCTGCACAC -3'	61°C	cDNA	32?
30-32-1	Sense Antisense	5'-ACCTGCCTGCCTCTGTC -3' 5'-GACGGAGGTAACATTTAACAA -3'	54°C	cDNA	281
30-32-11	Sense Antisense	5'-CCAGGCGTGGAATTCAG -3' 5'-CTGAGACTCCATCATAAACT -3'	54°C	Genomic DNA	187
30-32-13	Sense Antisense	5'-AGTGTCCTGTGTTTGTAGTG -3' 5'-GGGCTGCCTGCAGAACA -3'	54°C	cDNA	318
30-32-14	Sense Antisense	5'-AATCTCATCTCCTGGAAATTCT -3' 5'-CGAAAGAGTGGGTTTCAGG -3'	54°C	cDNA	72
30-32-2	Sense Antisense	5'-CCAGTGCTCTGTGCAGT -3' 5'-CGGTGTGGCTGACTTG -3'	54°C	Genomic DNA	247
30-32-3	Sense Antisense	5'-TGCCAACCATTGCTCATCA -3' 5'-AGAACTTCCTTCCCTCTCC -3'	54°C	cDNA	192
30-32-6	Sense Antisense	5'-GGCTCAGGAGATGGTCAG -3' 5'-GCTTTCCTCTCTTTTTTTTCC -3'	54°C	cDNA	608
30-32-9	Sense Antisense	5'-AGGGACAGGGAGGAGAAA -3' 5'-TAGATTGGAAATGCTGAGACA -3'	54°C	Genomic DNA	729
30-41-7	Sense Antisense	5'-TTTATCACATTTCAAGATACTGG -3' 5'-TGGTAGAGCAATTAACCTAATAT -3'	54°C	Genomic DNA	192
30-42-10	Sense Antisense	5'-GAGCCCGTCAAGCTGTAAT -3' 5'-GGCGTGGGAACACTGGA -3'	54°C	Genomic DNA	96

Seq ID		Primer Sequences	T_a	Template	cDNA (bp)
30-42-4	Sense Antisense	5'-ATAAAGTCGTCAGAATTAATGA -3' 5'-CAACAGTAAGGAGTCATGCT -3'	54°C	cDNA	81
30-42-8	Sense Antisense	5'-ATTTGAATGTCTTCTGTGAAATG -3' 5'-TGCAACCTTATAATGGAGAAAC -3'	54°C	Genomic DNA	218
30-42-10	Sense Antisense	5'-TGGTTCATCACCGTAGCAAAGG-3' 5'-GGCGTGGGAACACTGGA-3'	61°C	cDNA	96
30-42-10E	Sense Antisense	5'-TTTTCTGGAGACTTCTGGATGCC-3' 5'-TCCTCTGTACCTCACTTGGAGTCA-3'	54°C	cDNA	138
CAB45	Sense Antisense	5'-TGGATGGACACCTCAACAA-3' 5'-CTTTATATTCATCCCACGAAACA-3'	54°C	cDNA	285
GAPDH	Sense Antisense	5'-CCAAACGGGTCATCATCTC-3' 5'-TGGATGCAGGGATGATGTT-3'	54°C	cDNA	300
HPRT	Sense Antisense	5'-GCCGAGGATTTGGAAAAAGTG-3' 5'-TGCAACCTTAACCATTTTGGGG-3'	54°C	cDNA	399
L29	Sense Antisense	5'-AAGATGGGTCACCAGCAGCTCTAC-3' 5'-AAAGACTAGCATGATCGGTTCCAC-3'	54°C	cDNA	232
NEUA	Sense Antisense	5'-TAGACTCTCCATGCTGAATGA-3' 5'-GCGTTGGAAAAGTATCTATATTT-3'	54°C	cDNA	345
NEUC	Sense Antisense	5'-TACCAGCACTTAAAGGGAAA-3' 5'-AGTGCCGCCAGCACAG-3'	54°C	cDNA	266
NEUD	Sense Antisense	5'-TGGAGAGTTTGGAGTACATTC-3' 5'-GGAAGCAGTAAGCCAGAA-3'	54°C	cDNA	397
NEUF	Sense Antisense	5'-GCAAGTCCCAAGTCTGATG-3' 5'-GTATACAGGCTCTCCATTACA-3'	54°C	cDNA	139
NEUG	Sense Antisense	5'-GAGTAAGCCTAAGTAAGAAAAGA-3' 5'-TGCTGCTGGTGGGGGT-3'	54°C	Genomic DNA	107
ODC	Sense Antisense	5'-AGTCGCCAGAGCACATCC-3' 5'-CTGATGCGACATAGTATCTG-3'	61°C	cDNA	425
PLA2	Sense Antisense	5'-GCAGATGGCTCGAGAATA-3' 5'-TGAGGGCCAGACCCAGT-3'	61°C	cDNA	290
UN1G	Sense Antisense	5'-CTAAGAGTCCTCCTGCTCATATCC-3' 5'-TGATGAAGCTGGCTCAGT-3'	61°C	cDNA	73
UN3G	Sense Antisense	5'-AAGAAGAGGCCACGGTGTT-3' 5'-CCCTTCAGTGGGCTCTCC-3'	61°C	cDNA	64

APPENDIX VII

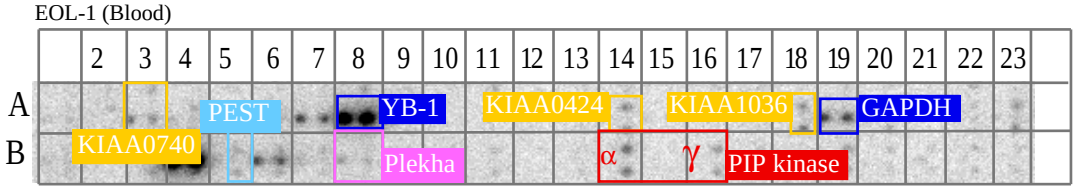
Gene positions on the self-made arrays

Gene No.	Position	Gene No.	Position	Gene No.	Position
26-11-2	1_A1	26-41-6	1_B6	30-42-1G	2_A11
26-11-4	1_A2	26-41-6Z	1_B7	30-42-4	2_A12
26-11-5	1_A3	30-11-3	1_B8	30-42-5G	2_A13
26-11-6G	1_A4	30-11-5	1_B9	30-42-8	2_A14
26-11-7	1_A5	30-11-5C	1_B10	30-42-10	2_A15
26-12-1	1_A6	30-11-6	1_B11	30-42-10E	2_A16
26-12-2G	1_A7	30-12-1	1_B12	ALK	2_A17
26-12-3	1_A8	30-12-3	1_B13	CAB45	2_A18
26-12-4G	1_A9	30-12-4A	1_B14	GAPDH	2_A19
26-21-2G	1_A10	30-12-4B	1_B15	HPRT	2_A20
26-21-3	1_A11	30-12-4C	1_B16	HEX	2_A21
26-21-4	1_A12	30-12-5	1_B17	L29	2_A22
26-22-4	1_A13	30-21-2	1_B18	LPG	2_A23
26-22-7	1_A14	30-21-3N	1_B19	NEUA	2_A24
26-31-1	1_A15	30-22-1	1_B20	NEUC	2_B1
26-31-3	1_A16	30-22-4	1_B21	NEUD	2_B2
26-32-1	1_A17	30-31-3	1_B22	NEUF	2_B3
26-32-2	1_A18	30-31-9G	1_B23	NEUG	2_B4
26-32-3	1_A19	30-32-2	1_B24	ODC	2_B5
26-32-4	1_A20	30-32-3	2_A1	PLA2	2_B6
26-32-5	1_A21	30-32-5G	2_A2	UN1G	2_B7
26-32-5A	1_A22	30-32-6	2_A3	UN3G	2_B8
26-32-5B	1_A23	30-32-9	2_A4	P38	2_B9
26-32-8	1_A24	30-32-11	2_A5		
26-32-9	1_B1	30-32-13	2_A6		
26-41-1G	1_B2	30-32-16G	2_A7		
26-41-1T	1_B3	30-32-16R	2_A8		
26-41-2	1_B4	30-41-4R	2_A9		
26-41-3	1_B5	30-41-7	2_A10		

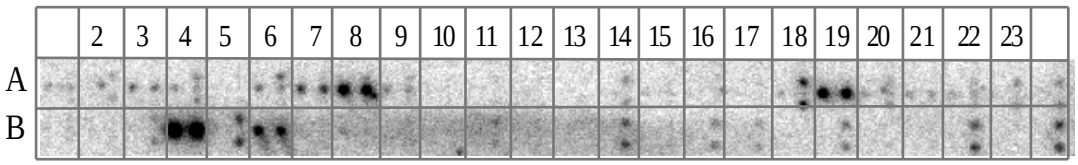
APPENDIX VIII

Phosphorimage files of Cancer Profiling

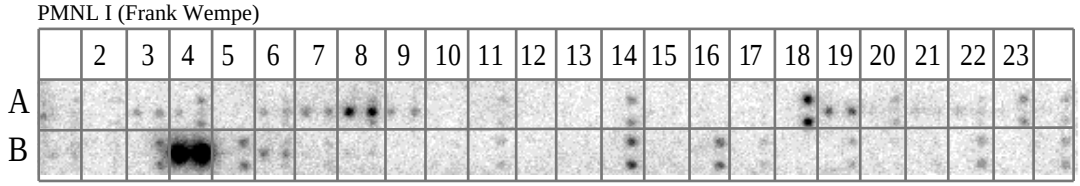
EOL-1 (Blood)



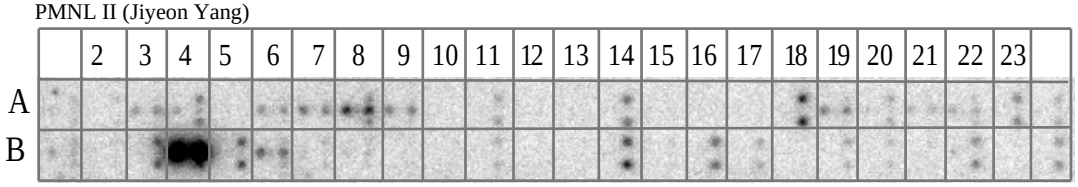
Ba/F3 (Blood)



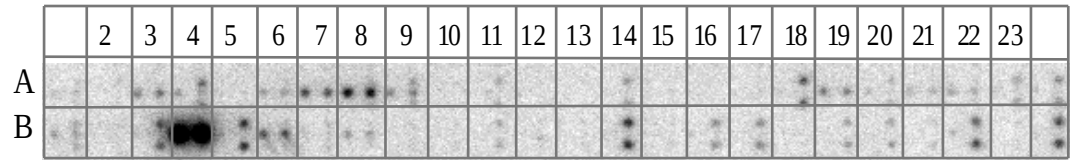
PMNL I (Frank Wempe)



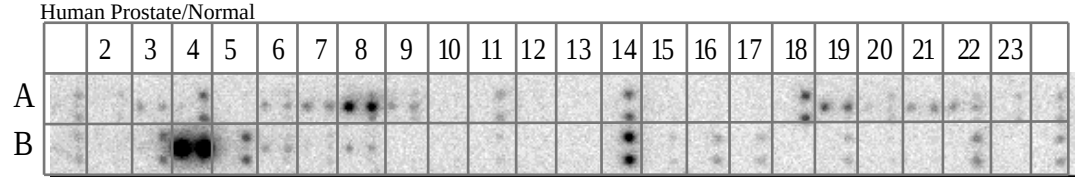
PMNL II (Jiyeon Yang)



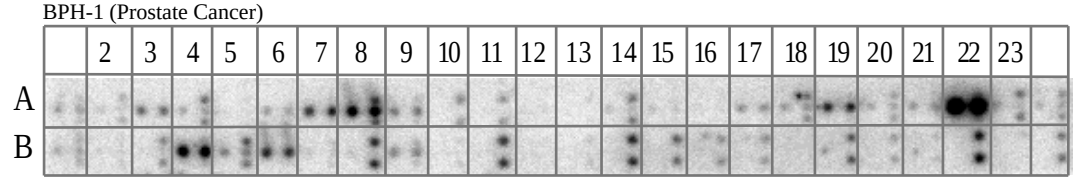
Human Brain/Normal



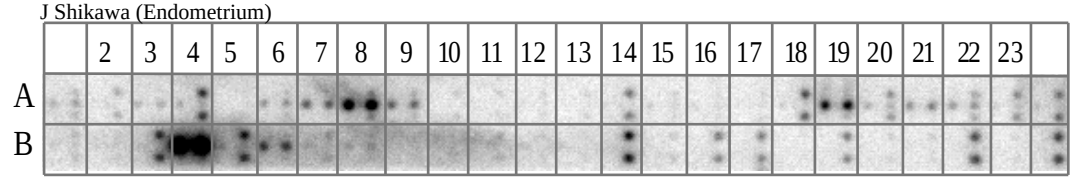
Human Prostate/Normal



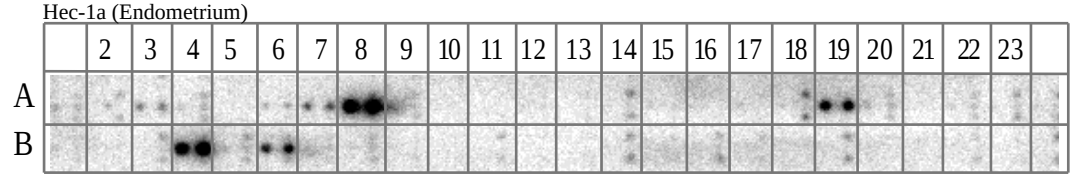
BPH-1 (Prostate Cancer)

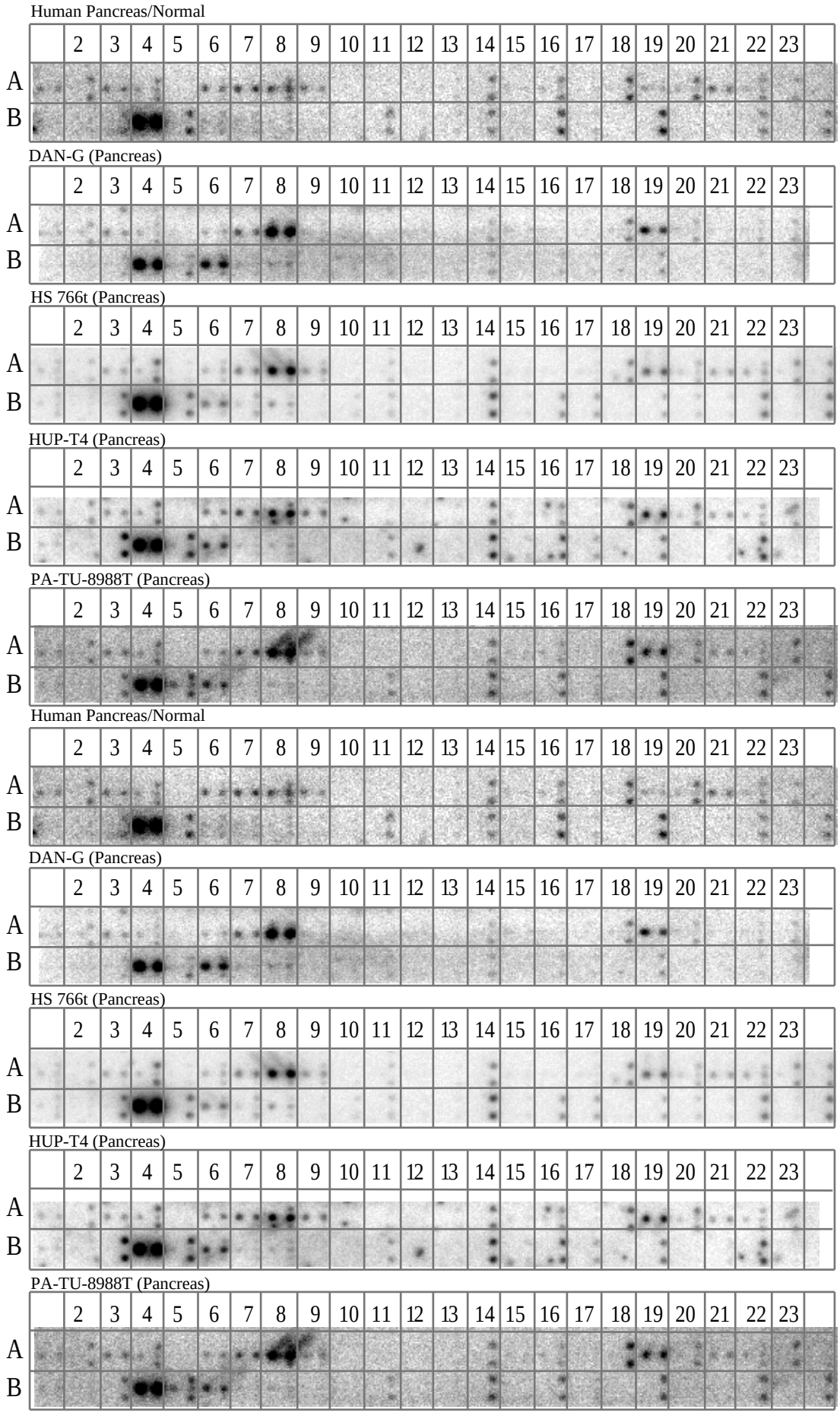


J Shikawa (Endometrium)

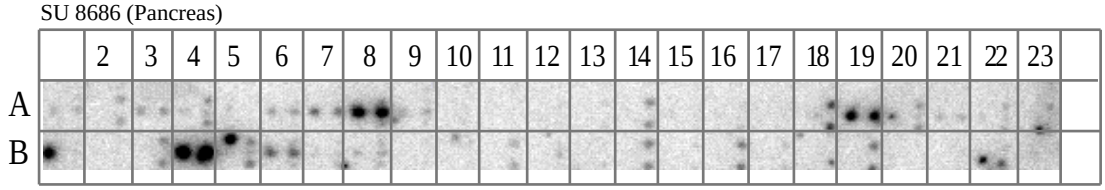


Hec-1a (Endometrium)

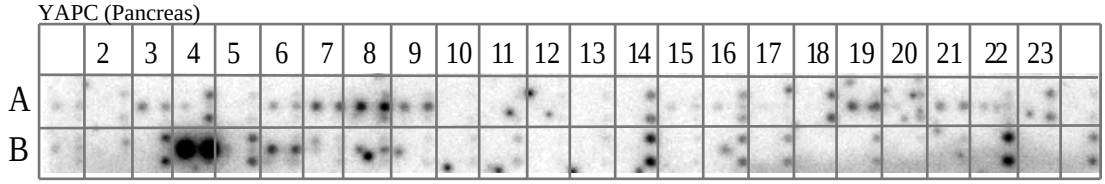



Human Pancreas/Normal
DAN-G (Pancreas)
HS 766t (Pancreas)
HUP-T4 (Pancreas)
PA-TU-8988T (Pancreas)
Human Pancreas/Normal
DAN-G (Pancreas)
HS 766t (Pancreas)
HUP-T4 (Pancreas)
PA-TU-8988T (Pancreas)
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
B

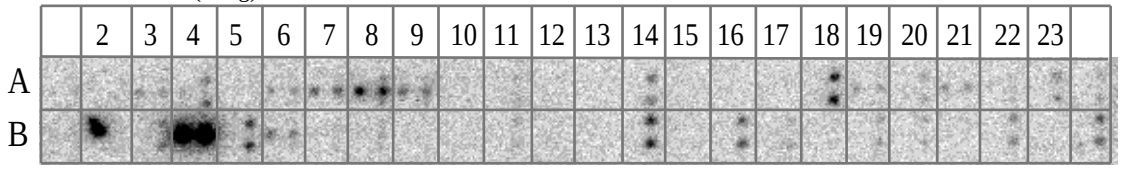
SU 8686 (Pancreas)



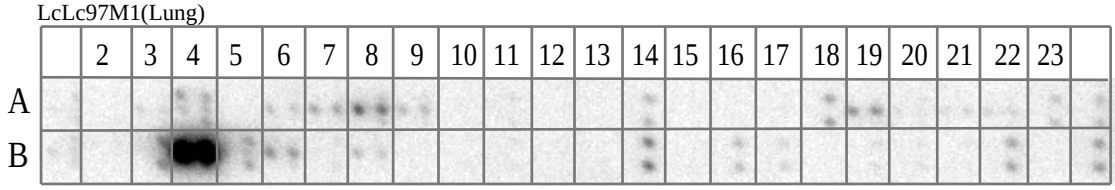
YAPC (Pancreas)



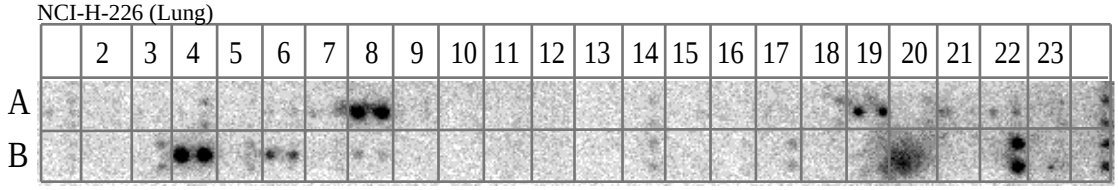
Human /Normal (Lung)



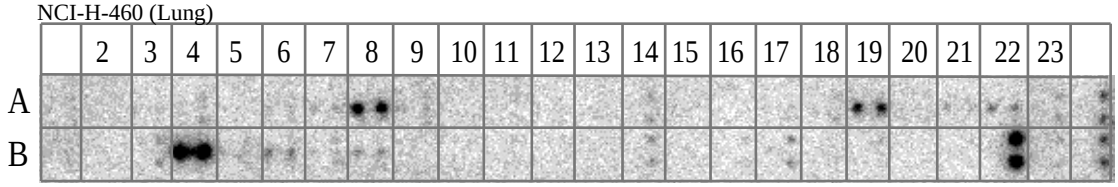
LcLc97M1(Lung)



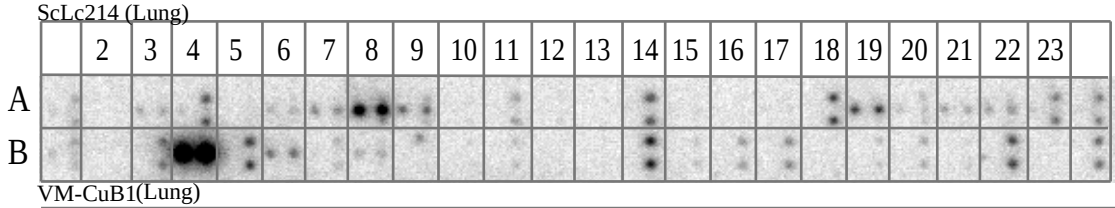
NCI-H-226 (Lung)



NCI-H-460 (Lung)

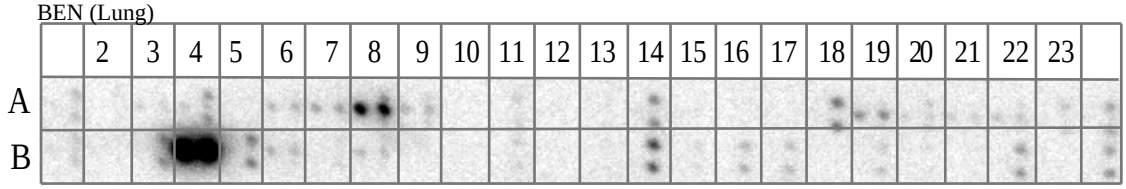


ScLc214 (Lung)

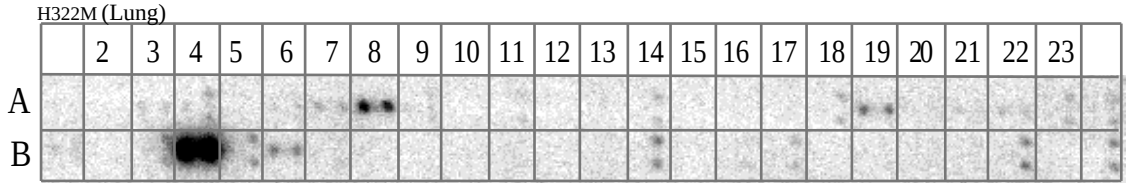


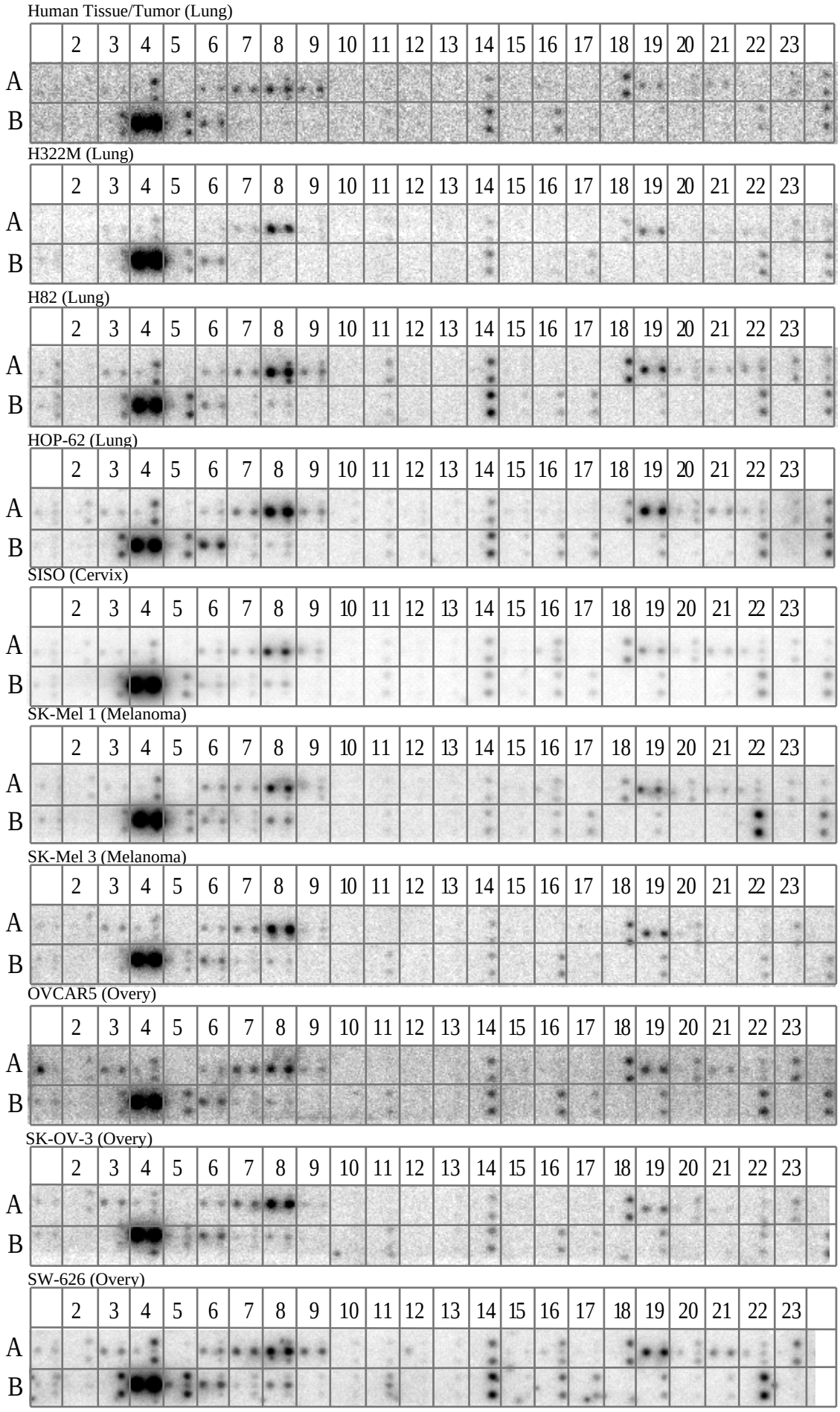
VM-CuB1(Lung)

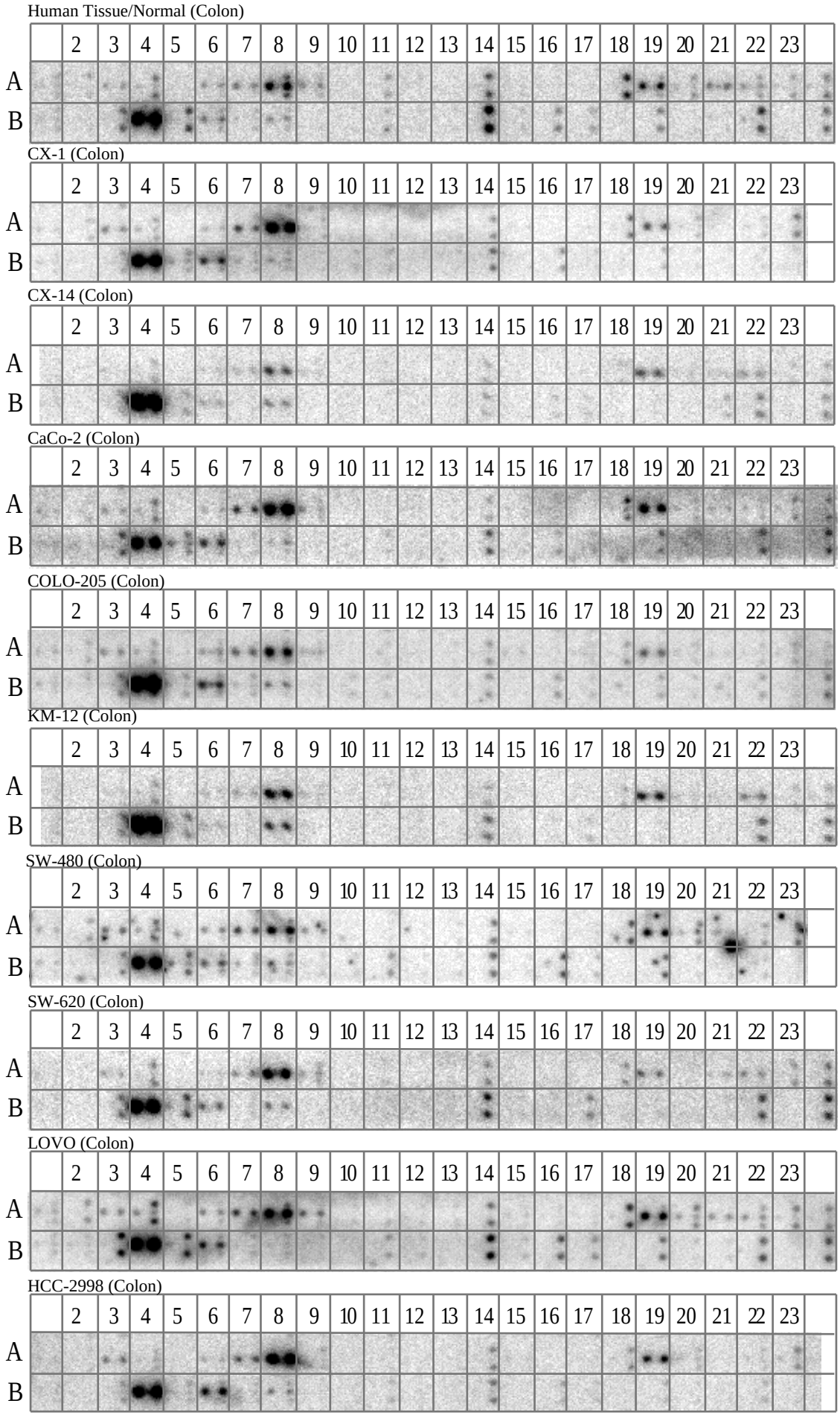
BEN (Lung)

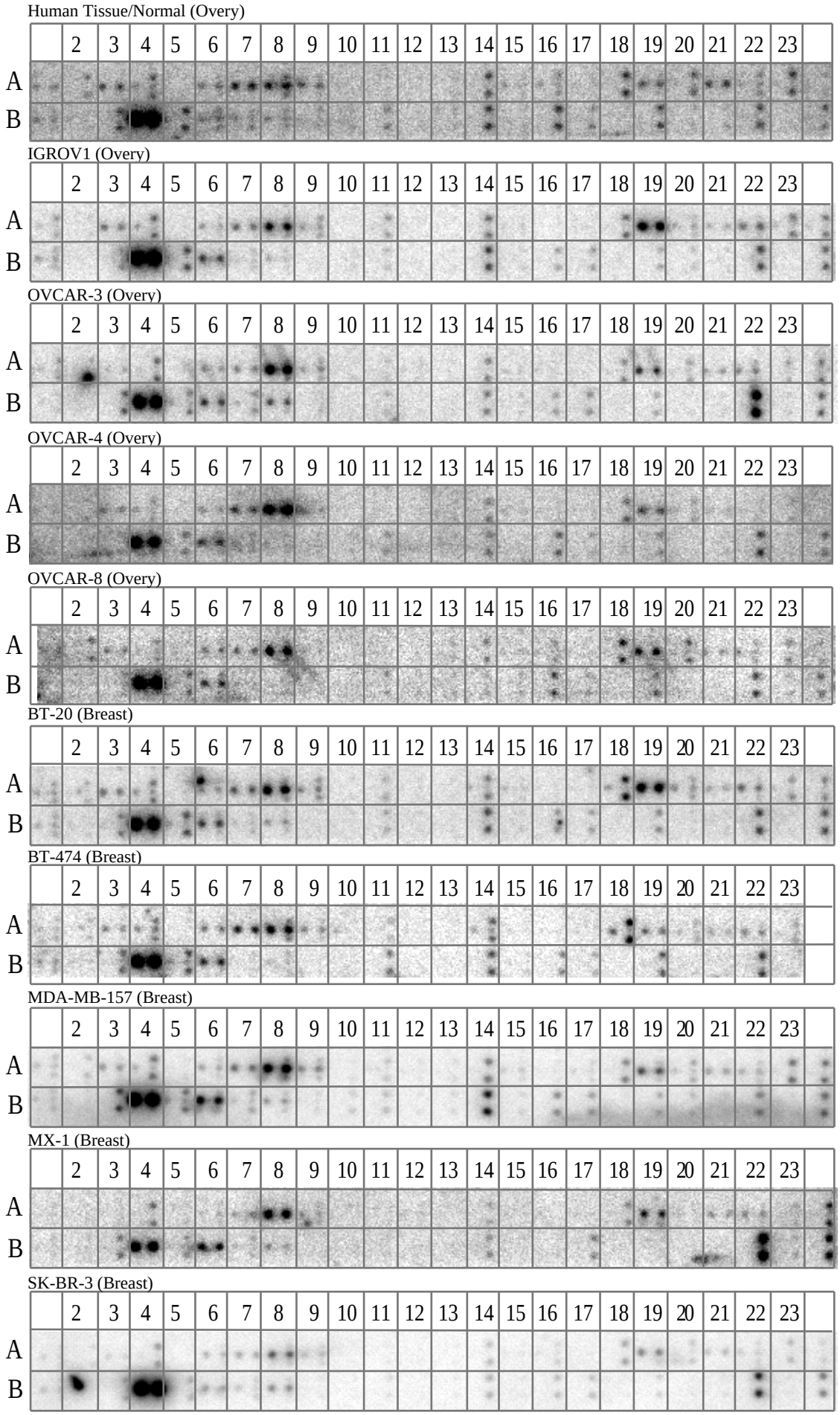


H322M (Lung)

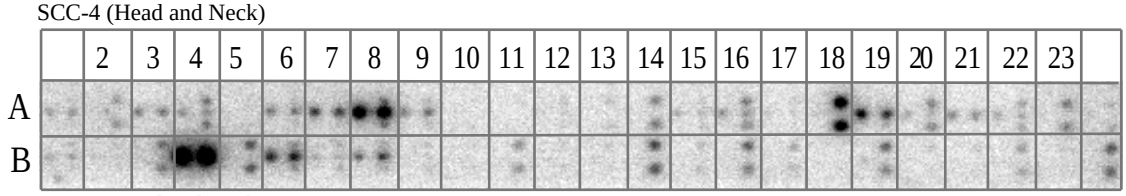



Human Tissue/Tumor (Lung)
H322M (Lung)
H82 (Lung)
HOP-62 (Lung)
SISO (Cervix)
SK-Mel 1 (Melanoma)
SK-Mel 3 (Melanoma)
OVCAR5 (Overy)
SK-OV-3 (Overy)
SW-626 (Overy)
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
B

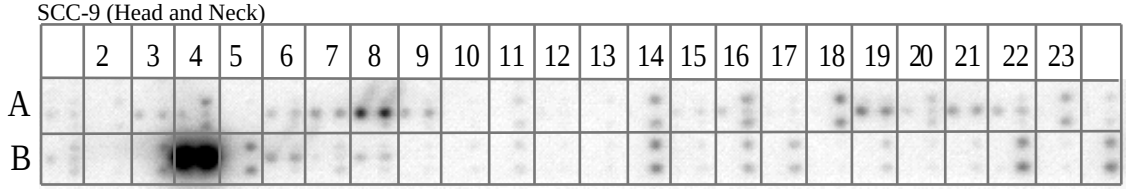

Human Tissue/Normal (Colon)
CX-1 (Colon)
CX-14 (Colon)
CaCo-2 (Colon)
COLO-205 (Colon)
KM-12 (Colon)
SW-480 (Colon)
SW-620 (Colon)
LOVO (Colon)
HCC-2998 (Colon)
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
B


Human Tissue/Normal (Overy)
IGROV1 (Overy)
OVCAR-3 (Overy)
OVCAR-4 (Overy)
OVCAR-8 (Overy)
BT-20 (Breast)
BT-474 (Breast)
MDA-MB-157 (Breast)
MX-1 (Breast)
SK-BR-3 (Breast)
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
B

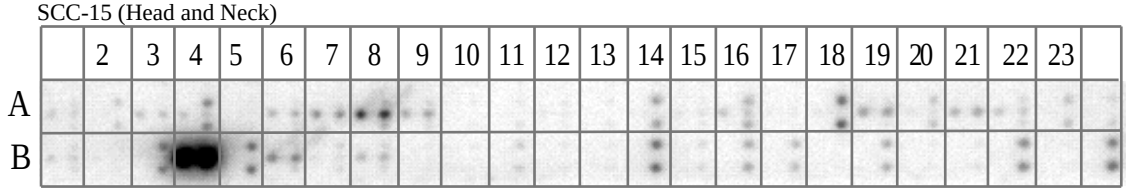
SCC-4 (Head and Neck)



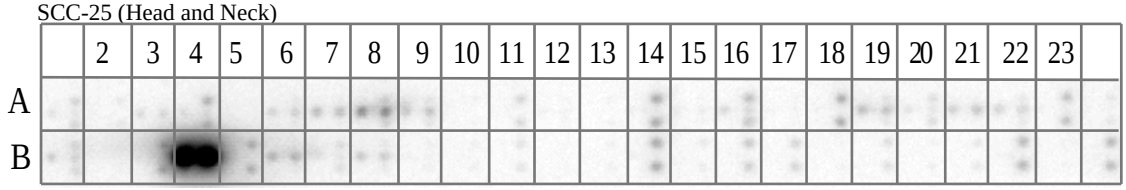
SCC-9 (Head and Neck)



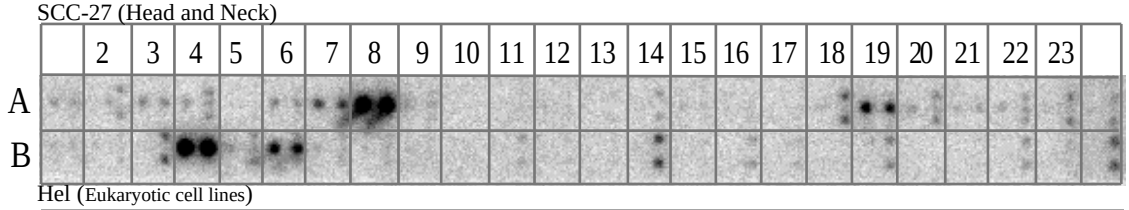
SCC-15 (Head and Neck)



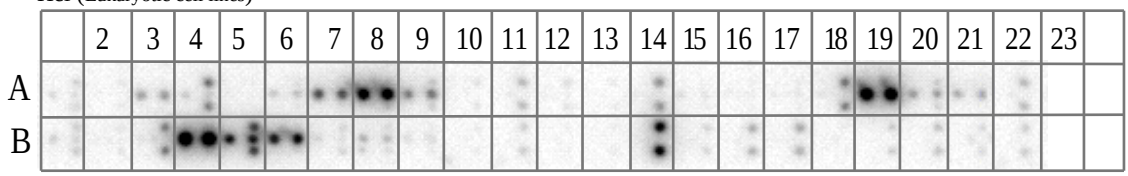
SCC-25 (Head and Neck)



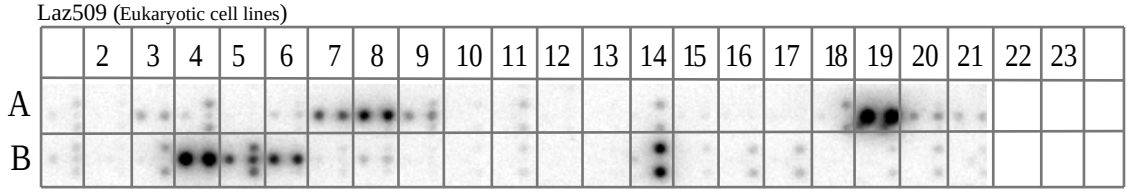
SCC-27 (Head and Neck)



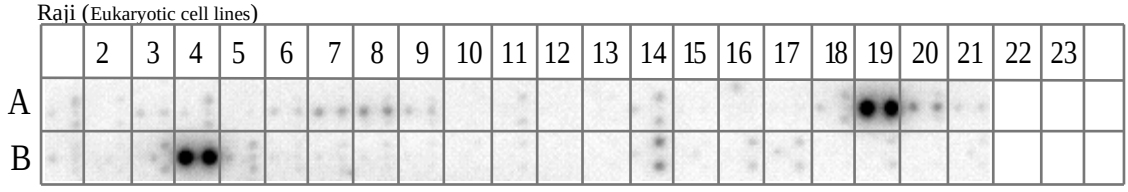
Hel (Eukaryotic cell lines)



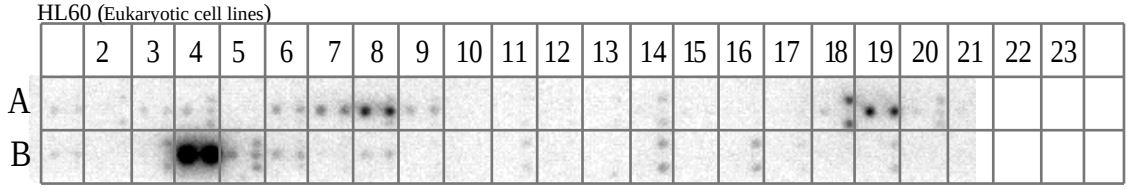
Laz509 (Eukaryotic cell lines)



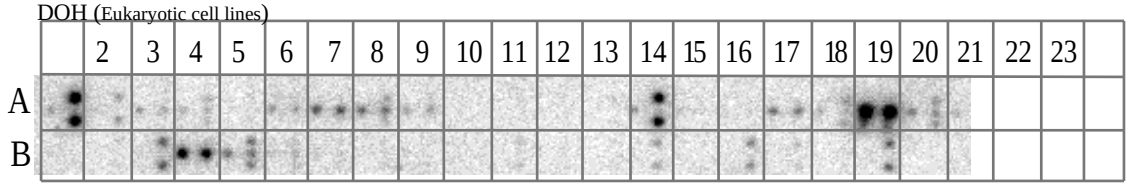
Raji (Eukaryotic cell lines)



HL60 (Eukaryotic cell lines)



DOH (Eukaryotic cell lines)



APPENDIX IX

DIE NATUR. Aphoristisch
Fragment [*verfaßt von Georg Christoph Tobler*]
[*32. Stück des Journal von Tiefurt, privatim; Ende 1782 oder Anfang 1783. Pfälzisches Museum 1783. I, 5*]

Natur! Wir sind von ihr umgeben und umschlungen - unvermögend aus ihr herauszutreten, und unvermögend tiefer in sie hineinzukommen. Ungebeten und ungewarnt nimmt sie uns in den Kreislauf ihres Tanzes auf und treibt sich mit uns fort, bis wir ermüdet sind und ihrem Arme entfallen.

Sie schafft ewig neue Gestalten; was da ist war noch nie, was war kommt nicht wieder- alles ist neu, und doch immer das Alte.

Wir leben mitten in ihr, und sind ihr fremde. Sie spricht unaufhörlich mit uns, und verrät uns ihr Geheimnis nicht.

Wir wirken beständig auf sie, und haben doch keine Gewalt über sie.

Sie scheint alles auf Individualität angelegt zu haben, und macht sich nichts aus den Individuen. Sie baut immer und zerstört immer, und ihre Werkstätte ist unzugänglich. Sie lebt in lauter Kindern, und die Mutter, wo ist sie?- Sie ist die einzige Künstlerin: aus dem simpelsten Stoff zu den größten Kontrasten; ohne Schein der Anstrengung zu der größten Vollendung-zur genausten Bestimmtheit, immer mit etwas Weichem überzogen. Jedes ihrer Werke hat ein eigenes Wesen, jede ihrer Erscheinungen den isoliertesten Begriff, und doch macht alles Eins aus.

Sie spielt ein Schauspiel: ob sie es selbst sieht wissen wir nicht, und doch spielt sie’s für uns die wir in der Ecke stehen.

Es ist ein ewiges Leben, Werden und Bewegen in ihr, und doch rückt sie nicht weiter. Sie verwandelt sich ewig, und ist kein Moment Stillestehen in ihr. Fürs Bleiben hat sie keinen Begriff, und ihren Fluch hat sie ans Stillestehen gehängt. Sie ist fest. Ihr Tritt ist gemessen, ihre Ausnahmen selten, ihre Gesetze unwandelbar. Gedacht hat sie und sinnt beständig; aber nicht als ein Mensch, sondern als Natur. Sie hat sich einen eigenen allumfassenden Sinn vorbehalten, den ihr niemand abmerken kann.

Die Menschen sind alle in ihr und sie in allen. Mit allen treibt sie ein freundliches Spiel, und freut sich je mehr man ihr abgewinnt. Sie treibt’s mit vielen so im verborgenen, daß sie’s zu Ende spielt ehe sie’s merken.

Auch das Unnatürlichste ist Natur, auch die plumpste Philisterei hat etwas von ihrem Genie. Wer sie nicht allenthalben sieht, sieht sie nirgendwo recht.

Sie liebt sich selber und haftet ewig mit Augen und Herzen ohne Zahl an sich selbst. Sie hat sich auseinandergesetzt um sich selbst zu genießen. Immer läßt sie neue Genießer erwachsen, unersättlich sich mitzuteilen.

Sie freut sich an der Illusion. Wer diese in sich und andern zerstört, den straft sie als der strengste Tyrann. Wer ihr zutraulich folgt, den drückt sie wie ein Kind an ihr Herz.

Ihre Kinder sind ohne Zahl. Keinem ist sie überall karg, aber sie hat Lieblinge an die sie viel verschwendet und denen sie viel aufopfert. Ans Große hat sie ihren

Schutz geknüpft. Sie spritzt ihre Geschöpfe aus dem Nichts hevor, und sagt ihnen nicht woher sie kommen und wohin sie gehen. Sie sollen nur laufen; die Bahn kennt sie. Sie hat wenige Triebfedern, aber nie abgenutzte, immer wirksam, immer mannigfaltig. Ihr Schauspiel ist immer neu, weil sie immer neue Zuschauer schafft. Leben ist ihre schönste Erfindung, und der Tod ist ihr Kunstgriff viel Leben zu haben. Sie hüllt den Menschen in Dumpfheit ein, und spornt ihn ewig zum Lichte. Sie macht ihn abhängig zur Erde, träg und schwer, und schüttelt ihn immer wieder auf.

Sie gibt Bedürfnisse, weil sie Bewegung liebt. Wunder, daß sie alle diese Bewegung mit so wenigem erreicht. Jedes Bedürfnis ist Wohltat; schnell befriedigt, schnell wieder erwachsend. Gibt sie eins mehr, so ist's ein neuer Quell der Lust; aber sie kommt bald ins Gleichgewicht. Sie setzt alle Augenblicke zum längsten Lauf an, und ist alle Augenblicke am Ziele.

Sie ist die Eitelkeit selbst, aber nicht für uns denen sie sich zur größen Wichtigkeit gemacht hat. Sie läßt jedes Kind an sich künsteln, jeden Toren über sich richten, Tausende stumpf über sich hingehen und nichts sehen, und hat an allen ihre Freude und findet bei allen ihre Rechnung.

Man gehorcht ihren Gesetzen, auch wenn man ihnen wiederstrebt; man wirkt mit ihr, auch wenn man gegen sie wirken will.

Sie macht alles was sie gibt zur Wohltat, denn sie macht es erst unentbehrlich. Sie säumet, daß man sie verlange; sie eilet, daß man sie nicht satt werde.

Sie hat keine Sprache noch Rede, aber sie schafft Zungen und Herzen durch die sie fühlt und spricht.

Ihre Krone ist die Liebe. Nur durch sie kommt man ihr nahe. Sie macht Klüfte zwischen allen Wesen, und alles will sich verschlingen. Sie hat alles isoliert,, um alles zusammenzuziehen. Durch ein paar Züge aus dem Becher der Liebe hält sie für ein Leben voll Mühe schadlos.

Sie ist alles. Sie belohnt sich selbst und bestraft sich selbst, erfreut und quält sich selbst. Sie ist rauh und gelinde, lieblich und schrecklich, kraftlos und allgewaltig. Alles ist immer da in ihr. Vergangenheit und Zukunft kennt sie nicht.Gegenwart ist ihr Ewigkeit. Sie ist gütig. Ich preise sie mit allen ihren Werken. Sie ist weise und still. Man reißt ihr keine´Erklärung vom Leibe, trutzt ihr kein Geschenk ab, das sie nicht freiwillig gibt. Sie ist listig, aber zu gutem Ziele, und am besten ist's ihre List nicht zu merken.

Sie ist ganz, und doch immer unvollendet. So wie sie's treibt, kann sie's immer treiben. Jedem erscheint sie in einer eignen Gestalt. Sie verbirgt sich in tausend Namen und Termen, und ist immer dieselbe. Sie hat mich hereingestellt, sie wird mich auch herausführen. Ich vertraue mich ihr. Sie mag mit mir schalten. Sie wird ihr Werk nicht hassen. Ich sprach nicht von ihr. Nein, was wahr ist und was falsch ist alles hat sie gesprochen. Alles ist ihre Schuld, alles ist ihr Verdienst.

Johann Wolfgang Goethe

ZUSAMMENFASSUNG

ZUSAMMENFASSUNG

Apoptose ist ein zelluläres Suizidprogramm, das in jeder Zelle eines mehrzelligen Lebewesens stattfinden kann. Apoptose ist im Gegensatz zur Nekrose ein physiologischer Prozess, dessen Ziel die Eliminierung von Zellen ist, die vom Gesamtorganismus entweder nicht mehr benötigt werden oder diesem schaden können. Hämatopoetische Zellen sind abhängig von einer Vielzahl verschiedener Zytokine, die deren Überleben und Differenzierung gewährleisten. Entzieht man hämatopoetischen Zellen ihre entsprechenden Wachstumsfaktoren, gehen die Zellen in kurzer Zeit in Apoptose.

FDCP1-Zellen sind murine Leukozyten, die myeloische Vorläuferzellen der Monozyten-Granulozyten-Linie darstellen. FDCP-1 Zellen können in Zellkultur mit GM-CSF oder dem pleiopotenten Wachstumsfaktor IL-3 gehalten werden (in unserem Falle IL-3). Nach Entzug von IL-3 folgt eine prä-apoptotische Phase von etwa 8 Stunden, in denen die Apoptose durch Wiederzugabe von IL-3 vollständig rückgängig gemacht werden kann. Bei Wiederzugabe von IL-3 erst nach etwa 12 Stunden sterben fast alle Zellen, was bedeutet, dass in diesen Zellen zwischen Stunde 8 und 12 die unwiederbringliche Entscheidung zur Einleitung der Apoptose gefällt wird.

Wie viele andere biologische Prozesse ist die Regulation der Apoptose hierarchisch strukturiert. Die Aktivierung einer exekutiven Ebene (Caspasen, DNasen) führt zur Zerstörung wichtiger Zellfunktionen und zum unwiederbringlichen Zelltod. Über dieser exekutiven Ebene steht eine regulative Ebene (Bcl2-homologe Proteine, regulative Caspasen und weitere Faktoren). Die Proteine der exekutiven Ebene sind meist konstitutiv in den Zellen vorhanden und werden bei einem Überschreiten eines Schwellenwertes auf einer regulativen Ebene nach dem "Schneeballprinzip" aktiviert. Auf regulativer Ebene findet neben posttranslationaler Regulation (Bcl2-homologe Proteine und regulative Caspasen) eine transkriptionelle Aktivierung statt. Diese transkriptionell aktivierten Faktoren (z.B. p53 Kinase oder NF-κB regulierte Gene) können direkt oder indirekt Apoptose positiv oder negativ regulieren.

Der zeitlich streng geordnete Ablauf der Apoptose in FDCP1-Zellen sowie die Tatsache, dass bis zu einem bestimmten Zeitpunkt die Zellen durch Wiederzugabe von IL-3 zu retten sind, spricht dafür, dass viele regulative Gene während der

Apoptose nur transient aktiviert werden.

Bereits vor Beginn der praktischen Arbeit dieser Dissertation wurde in unserem Laboratorium ein Genfallensystem entwickelt, um transkriptionell induzierte regulative Gene während der Apoptose zu identifizieren. Mit Hilfe eines molekularen Schalters, der auch durch kurzfristige Expression der Cre-Rekombinase (hier das Reportergen) von der Expression des "gefloxten" Genes A (TKneo) auf die Expression des Genes B (IL-3) umschaltet, sollten Gene identifiziert worden, die während der Apoptose nach IL-3 Entzug in FDCP1-Zellen transkriptionell induziert werden. Das Prinzip der Genfalle besteht darin, ein promotorloses Reportergen in natürliche Gene einzuführen. Das Reportergen soll bei der Integration ins Zielgenom unter die Kontrolle des natürlichen Promotors gelangen und wie das getroffene natürliche Gen exprimiert werden. Entscheidend für die Nutzbarkeit dieses Systems ist die möglichst unbeeinflusste zufällige Integration ins Genom. Retrovirale Vektoren eignen sich besonders gut als Genfalle, da sie neben der Zufälligkeit des Integrationsortes eine genau definierte Struktur als Provirus im Zielgenom aufweisen. Die Position des Reportergens im Provirus wird so planbar und kann wie in unserem Projekt so gewählt werden, dass der Beginn des Reportergens möglichst weit am 5'-Ende des Provirus liegt.

Für 102 „Genetrap specific tags“ (GTSTs) konnten Provirus-flankierende DNA-Sequenzen generiert werden. 86 waren lang genug um den Integrationsort im Mausgenom zu bestimmen. von diesen 86 Klonen konnten 73 Virusintegrationen spezifischen Genen zugeordnet werden. Bei den übrigen kann es sich um bisher nicht identifizierter kodierende oder nichtkodierende Gene handeln.

Bei der Überprüfung der Virusintegrationen stellte sich heraus, dass bei etwa 50% der Klone die Virusintegration in inverser (antisene) Orientierung zu dem getroffenen Gen vorlag. Es muss in diesen Fällen davon ausgegangen werden, dass die Aktivierung des Cre-Rekombinase Reportergenes durch eine Integration in eine antisense RNA stattgefunden hat. Nach Schätzungen haben vermutlich etwa 8% der Gene antisense RNAs. Die ungewöhnliche Häufung der antisense Integrationen konnte auch in weiteren Projekten mit Cre-Rekombinase als Reportersystem festgestellt werden (andere gebräuchliche Reportergene in Genfallen zeigen diese Eigenschaft nicht). Da bereits eine sehr geringe Konzentration der Cre-Rekombinase reicht aus, um eine Rekombination hervorzurufen, ist zu vermuten, dass auch sehr

schwach exprimierte mRNAs mit dem Cre-Reportersystem identifiziert werden können. Zudem könnte das Fehlen einer kodierenden Region in den meisten antisense RNAs dazu führen, dass die Integration zu besonders günstigen Voraussetzungen für den Translationsstart des Cre-Reportergenes führt. Bei der Analyse der Expression der Gene mit antisense Integrationen konnte festgestellt werden, das diese in fast allen Fällen nach IL-3 Entzug herunterreguliert werden, was die Vermutung nahe legt, dass den antisense Transkripten eine regulatorische Rolle zukommt.

Von den 50 identifizierten Genen mit wohldefinierter Funktion konnten 28 Genen Überlebens- oder Apoptose-Funktionen zugewiesen werden. Der große Anteil von Genen mit Überlebensfunktionen ist bemerkenswert, da Apoptose die Konsequenz des IL-3-Entzugs ist und eigentlich erwartet wurde, dass hauptsächlich pro-aopoptotische Gene hochreguliert würden. Da, wie bereits beschrieben, einige der integrierten Gene auch herunterreguliert werden (Integrationen in antisense Orientierung), war unklar ob die jeweilige Regulation pro- oder anti-apoptotische Folgen hat.

Bei der Betrachtung der Regulation dieser Genen, fällt auf, dass pro- wie anti-apoptotische Gene herunter- wie heraufreguliert werden. Es ergibt sich eine durchaus widersprüchliche, ambivalente Antwort auf den Apoptosestimulus. Wie bereits zuvor gezeigt werden konnte, erzeugt ein zeitlich limitierter IL-3-Entzug in FDCP1-Zellen eine reduzierte nachfolgende Apoptose nach einem weiteren IL-3-Entzug. Die Induktion von Überlebens-Genen so wie Herunterregulation von Apoptose-Genen scheint also in einer zeitlich befristeten ersten Phase nach IL-3-Entzug von großer Bedeutung zu sein.

Mit der Absicht, diese Hypothese mit weiteren Genexpressionsmustern zu erweitern, wurde eine Hybridisierung mit Atlas Arrays von Clontech durchgeführt. Bei den Hybridisierungen wurde ein Vergleich der Transkriptmengen der auf dem Filter vorhandenen 588 Gene vor IL-3-Entzug mit den Mengen 4 und 8 Stunden nachher durchgeführt. Für etwa 50% der gebundenen Gene konnte ein Hybridisierungssignal detektiert werden. Nur 12 Gene zeigten signifikante Regulation nach IL-3 Entzug. Bis auf einen Fall konnten die Ergebnisse des Atlas Arrays in Northern Blot Experimenten verifiziert und um weitere Messzeitpunkte erweitert werden. Von den 12 identifizierten Genen konnten 9 Apoptose oder Überlebensfunktionen zugeordnet werden. Die starke Herunterregulation von c-Myc und Pim-1 spiegelte zudem bereits

aus der Literatur bekannte Daten wieder.

Zusammenfassend stellen die durch den Atlas-Array erhaltenen Daten eine Bestätigung der Hypothese einer ambivalenten Antwort der Zelle auf den Apoptosestimulus dar.

Da diese Arbeit Teil einer Kooperation mit der Schering AG (Berlin) war, hatten wir die Möglichkeit, die Genexpression nach IL-3-Entzug in FDCP1-Zellen auch mittels Hybridisierungen mit Affymetrix Chip zu untersuchen. Die Hybridisierungen wurden bei Schering in Berlin durchgeführt. Für die Analyse wurden mRNA Proben von 0, 2, 4, 6 und 8 Stunden miteinander verglichen. Ausgehend von der Fülle der Rohdaten wurden Gene ausgewählt, die im Zeitraum von 0 bis 8 Stunden mindestens zu einem Zeitpunkt 3-fach hoch- oder herunterrguliert waren. Auch in diesem Fall konnte ein Anteil von 70% der Gene der Apoptose- oder Überlebensfunktionen zugeordnet werden.

Erstaunlicherweise konnten trotz der sehr unterschiedlichen Methoden zur Identifizierung regulierter Gene etwa ein gleicher Anteil von Genen identifiziert werden, denen man Apoptose- oder Überlebensfunktionen zuordnen kann. Dieser hohe Anteil spricht für eine sehr spezifische Antwort der FDCP1-Zellen bei Wachstumsfaktorentzug. Die Regulation der Apoptose nach IL-3-Entzug lässt sich nach diesen Daten nicht auf die Regulation weniger Faktoren zurückzuführen, sondern stellt eine sehr komplexe Antwort dar, die je nach Situation der jeweiligen Zelle zu einem schnelleren oder auch verzögerten Verlauf der Apoptose führt.

Arrays beruhen auf der Vorwahl der gebundenen Gene. Der Atlas Array liegt nach heutigen Maßstäben mit seiner sehr limitierten Zahl von 588 Genen (etwa 1-2% aller Gene) weit hinter dem Affymetrix Chip mit etwa 5000 Genen (etwa 20-30% aller Gene). Mit 3-4 solcher Affymetrix Chips können alle Gene eines Säugertranskriptoms abgedeckt werden. Es steht außer Frage, dass diese Methode an Effektivität schwer zu überbieten ist. Ein Nachteil wird jedoch immer die Vorauswahl der Gene bleiben. Hohe Anforderungen stellt bei der Analyse von Affymetrix Chip-Hybridisierung der große Umfang der ermittelten Ergebnisse dar.

Genfallensysteme beruhen auf völlig anderen Bedingungen. Sie sind nicht von einer Vorauswahl von Genen als experimenteller Randbedingung abhängig, so dass der Anteil bekannter Gene etwa dem momentanen Wissenstand zu den Funktionen aller Gene entspricht. Da das Cre-Rekombinase- Reportersystem besonders sensitiv zu sein

scheint, könnte ein höherer Anteil an unbekannten bzw. niedrig exprimierten Genen nachgewiesen werden. Vergleicht man die Genfallenergebnisse mit den Affymetrixdaten, konnten nur etwa 23,5% der durch Genfallen identifizierten Gene auf dem Affymetrix Array wieder gefunden werden. Dieser geringe Anteil spricht für das Genfallensystem als System zur Identifizierung noch unbekannter Gene.

Die von uns verwendete Methode zur Herstellung der Genfallen-Integrationsbank beginnt mit der Negativselektion zur Eliminierung konstitutiv aktiver Loci. Da viele Gene, wenngleich hochreguliert, auch im nichtinduzierten Zustand eine basale Expression zeigen, werden Zellklone mit Genfallenintegrationen in diesen Loci aus dem Klonpool eliminiert. Dadurch sinkt die Zahl der möglichen identifizierbaren Gene. Solche relativ hoch exprimierten Gene sind nur dann zu isolieren, wenn durch eine sehr ungünstige Provirusintegration die Translation des Cre-Reportergenes stark reduziert ist.

Während der Tumorigenese kommt es in vielen Fällen zu Mutationen, Überexpressionen oder Inaktivierungen von Apoptose- oder Überlebensgenen. Verstärkte Proliferation ergibt sich nicht nur aus einer Aktivierung des Zellzyklus, sondern kann auch durch eine verminderte Apoptose verursacht werden..

Es war nun naheliegend zu untersuchen, ob aus unserer GTST-Integrationsbank weitere, vielleicht weniger bekannte Gene eine Tumor-relevante Funktion haben. Basierend auf unseren Erfahrungen mit der Arrayhybridisierung wurden 70 GTST-spezifische DNA-Proben generiert und auf eine Nitrozellulosemembran aufgebracht (sog. „custom array“). Diese Arrays wurden dann mit den cDNAs aus Tumorzellen bzw. aus normalen Geweben hybridisiert. Der Vergleich der Hybridisierungssignale ermöglicht die Eingrenzung von GTSTs mit differenzieller Expression in Tumorzellen gegenüber den jeweiligen normalen Geweben. Eine Gruppe von 7 Genen, deren Genprodukte möglicherweise Zielstrukturen für die Entwicklung von Therapeutika sein könnten, wurden ausgewählt und mit Hilfe von Matched Tumor Arrays auf ihre differenzielle Expression in Patientenproben untersucht. Dabei wurde festgestellt, dass in den Tumorproben nur ERK3 und mehrere Gene für bisher unbekannte Proteine differenziell exprimiert wurden.

Von diesen scheint ERK3 als mögliches Zielprotein für eine Tumortherapie von besonderer Bedeutung zu sein: 1) Die mit den Matched Tumor Arrays gewonnenen Ergebnisse zeigen, dass ERK3 am stärksten in Brustkrebs (7-fach), Magenkrebs (2,5-

fach) und Gebärmutterkrebs (2,3-fach) hochreguliert wird. Diese differenzielle Expression von ERK3 mit hoher Organspezifität erlaubt möglicherweise eine sehr spezifische Hemmung. 2) Es wurde bereits gezeigt, dass die Expression von ERK3 durch spezifische Inhibitoren der p38 Kinase und Proteasomen stark unterdrückt wird. Bei den Inhibitoren handelt es sich um Ableitungen von Peptiden oder kleine chemische Moleküle, was für die Entwicklung von Medikamenten von großem Vorteil ist. 3) ERK3 besitzt eine völlig unterschiedliche Phosphorylierungsstelle als die anderen ERKs. Daher sollte es möglich sein, bei der Hemmung der Aktivierung von ERK3 eine hohe Selektivität gegenüber anderen ERKs zu erreichen. 4) Es wurde nachgewiesen, dass ERK3 ein relativ kurzlebiges Protein ist ($t_{1/2}$ = ca. 30 min.), was ein *Targeting* auch auf der transkriptionalen Ebene, z.B. durch RNA Interferenz, ermöglicht

Trotz all dieser Vorteile setzt die Beurteilung von ERK3 als ein gutes Zielprotein letztendlich voraus, das die Unterdrückung der Expression oder Aktivität von ERK3 tatsächlich zur verringerten Proliferation oder verstärkten Apoptose in Tumorzellen führen wird. Da über die nachfolgenden Ereignisse der ERK3-Aktivierung bislang wenig bekannt ist, sind weitere Untersuchungen nötig, um die anti-apoptotische oder pro-proliferative Wirkung von ERK3 aufzuklären bzw. zu bestätigen.

Lebenslauf

Persönliche Daten

Name	Yang
Vorname	**Ji Yeon**
Geburtsdatum	17.11.1961
Geburtsort	Seoul, Südkorea
Staatsangehörigkeit	Südkorea
Familienstand	**verheiratet, 2 Kinder**

Schulbildung

03/1968 – 02/1974	**Grundschule in Nam Won, Südkorea**
03/1974 – 02/1977	**Mittelschule in Yong Seong, Südkorea**
03/1977 – 02/1980	**Gymnasium in Shin-Hung, Südkorea**
1981 – 1983	**Zivildienst**
1984 – 1985	**Vorbereitungskurs für Aufnahmeprüfung zum Studium in Südkorea (Privatschule)**

Hochschulausbildung

03/1985 – 01/1989	**Grundstudium (Bakkalaureus der Naturwissen schaften) in Biochemie/Diplom an der Hanyang Universität in Seoul, Korea**
02/1989 – 08/1991	**Technischer Assistent an der Bond Universität in Gold Coast, Australien**
10/1991 – 12/1992	**Diplomarbeit unter der Leitung von Dr. Jill E. Gready an der Universität von Sydney zum Thema: „Ligand Binding Studies to Dihydrofolate Reductase by Fluorescence Spectroscopy“**
01/1993 – 12/1994	**Wissenschaftlicher Mitarbeiter an der Research School of Chemistry in Canberra, Australien**
01/1995 – 12/1997	**Magisterarbeit in der Research School of Chemistry in Canberra, Nationale Universität, Australien zum Thema: „Aspects of Core Subunits of DNA Polymerase III Holoenzyme in *E. coli*“**
01/1998 – 11/1998	**Technischer Assistent im Biozentrum der J. W. Goethe Universität, Frankfurt a. M.**
Seit 03/1999	**Anfertigung der Doktorarbeit in der Abteilung Molekulare Hämatologie des Klinikums der J. W. Goethe Universität unter der Leitung von Prof. Dr. H. von Melchner sowie Dr. F. Wempe. Thema: „Identifizierung und Charakterisierung Apoptose-assoziierter Gene in Hämatopoetischen Zellen unter besonderer Verwendung der Array-Technologie“**

Frankfurt a. M., den