NITRIC OXIDE-MEDIATED TRANSCRIPTIONAL REGULATION

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ABBREVIATIONS

AFC	7-amino-4-trifluoromethyl coumarin
AFX	acute-lymphocytic-leukemia-1 fused gene from Chr X
AIF	apoptosis- inducing factor
AP-1	activator protein -1
ATF	activating transcription factor
ATP	adenosine tri phosphate
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
Caspase	cysteine dependent aspartate specific proteinase
Cdk	cyclin dependent kinase
cDNA	complementary deoxy ribonucleic acid
cGMP	cyclic guanosine mono phosphate
CHAPS	3-cholamidopropyl-dimethylammonio-1-propanesulfonate
CHOP	C/EBP homology protein
CNS	central nervous system
CRE	cAMP responsive element
CREB	CRE-binding protein
DEVD	aminomethylcoumarin
DMSO	dimethyl sulfoxide
dNTP	deoxy nucleotide tri-phosphate
DOC	downstream of CHOP
DTT	dithiothreitol
DYRK1a	dual-specificity tyrosine regulated kinase 1A
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EMSA	electro-mobility shift assay
ER	endoplasmic reticulum
ERSE	endoplasmic reticulum stress element
FAD	flavine adenine dinucleotide
FKHR	forkhead in rhabdomyosarcoma
FKHRL1	FKHR-like 1
FMN	flavin mononucleotide
FOXO	Forkhead box class O
GADD	growth arrest DNA damage
GTP	guanosine tri phosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horse radish peroxidase
IAP	inhibitor of apoptosis
IFN-gamma	interferon gamma

IKK	IκB kinase
IL	interlukin
IRS	insulin response element
JNK	Jun N-terminal kinase
LDH	lactate dehydrogenase
MAPK	mitogen activated protein kinase
Mdm	murine double minute
MPT	mitochondrial permeability transition
NADH	nicotinamide adenine dinucleotide
NES	nuclear export signal
NFkB	nuclear factor k B
NGF	nerve growth factor
NLS	nuclear localization signal
NO	nitric oxide
NOS	nitric oxide synthase
orf	open reading frame
PAGE	poly acrylamide gel electrophoresis
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK	3'-phosphoinositide dependent kinase-1
PI3K	Phosphoinositide 3-kinase
PKB	protein kinase B
PLK	polo like kinase
PMSF	phenyl methyl sulphonyl fluoride
RE	restriction enzyme
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
RT-PCR	reverse transcription – polymerase chain reaction
SDS	sodium dodecyl sulphate
sGC	soluble guanylyl cyclase
SGK	serum- and glucocorticoid-inducible kinase
SNP	sodium nitroprusside
SOD	super oxide dismutase
STAT	signal transducers and activators of transcription
Tet	tetracycline
TF	transcription factor
TNF	tumor necrosis factor

LIST OF PUBLICATION(S)

- Protein/DNA array based identification of Nitric oxide regulated transcription factor activities governing cell death and cell survival in neuroblastoma cells. *Dhakshinamoorthy S, Sridharan S, Ng PH, Boxer LM and Porter AG.* Manuscript submitted to Cell Death and Differentiation.

SUMMARY

Nitric oxide (NO) is a gas that potently effects changes in cells, including gene expression, cell cycle arrest, apoptosis and differentiation. NO has a role to prevent malignancy and help fight bacterial and viral infections, as well as cause CNS injury, angiogenesis, ischemia, and autoimmune diseases. All the physiological roles of NO are dependent on the concentration of NO and the nature of the cell type involved, indicating the importance of the macro and micro environment in determining whether NO infusion ultimately results in cell death or survival. The mechanism of all these different effects, however, is still not clear. In this study, I aimed to understand NO-mediated regulation of gene transcription leading to cell death or survival in neuroblastoma cell lines by examining the roles of two transcription factors, AFX (acute-lymphocytic-leukemia-1 fused gene from Chr X) and CHOP (C/EBP homology protein).

Forkheads are a family of transcription factors with a classical "winged helix" and are known to be regulators of longetivity and stress-resistance. Here, I found evidence for a novel mechanism of NO affecting the AFX protein. NO appeared to increase the affinity of the FOXO factors to bind their consensus cis-elements and induced Ser-197 phosphorylation of AFX, without affecting the localization of the protein. Reporter assays showed a decrease in the capacity of phosphorylated AFX to mediate transcriptional activation following NO treatment. Inducible over-expression of AFX killed cells independently of NO, indicating that AFX may play a role in regulating viability of neuroblastoma cells. Construction of serine to alanine and serine to aspartate mutants and the study of their effect on NO-mediated FOXO DNA-binding, AFX transactivational capacity and cell survival will help us further understand the significance of NO-mediated AFX Ser-197 phosphorylation.

CHOP (GADD153), an important player of the Endoplasmic Reticulum (ER) stress response pathway, is believed to be involved in a possible alternative pathway for NO-mediated apoptosis. CHOP mRNA and protein levels were found to be induced by NO in SH-Sy5y cells. Tet-inducible CHOP- sense and -anti-sense cell lines were used to further investigate the role that CHOP might play in neuroblastoma cells. Cell death assays showed CHOP sense stable cells were sensitized to NO-induced apoptosis and the anti-sense stable cells were protected from NO-induced apoptosis. These tools developed could be utilized to identify downstream targets of CHOP by differential microarray analysis, thereby helping us further understand the role of the ER stress pathway acting as an apoptotic trigger through the transcriptional activity of CHOP.

A screening project was undertaken to determine the panel of transcription factors and their targets that are activated by NO. Evidence for NO-mediated transcriptional up-regulation of Bcl-2 (B-cell leukemia- 2) was found. As part of the follow-up of the study, I developed Bcl-2 over-expressing SH-Sy5y cells. This study appears to suggest that the transcriptional up-regulation of Bcl-2 delayed cytochrome c release and reduced caspase-3 activity, thereby protecting neuroblastoma cells from NO-induced apoptosis.

CHAPTER 1 - INTRODUCTION

1.1 Apoptosis

Apoptosis or programmed cell death is an evolutionally conserved mechanism for multi-cellular organisms to eliminate unwanted cells during development and homeostasis. It is an active and highly orderly process as evident by the characteristic morphological changes including nuclear condensation and fragmentation, cytoplasmic shrinkage, plasma membrane blebbing, and exposure of phosphatidylserine. The dead cells eventually fragment into membrane-bound apoptotic bodies, which are phagocytosed by macrophages and surrounding cells without inducing an inflammatory response (Yuan *et al*., 2003).

With few exceptions, the terminal apoptotic programme of mammalian cells depends on the activation of intracellular cysteine endopeptidases called caspases and modification of protein substrates within the nucleus and cytoplasm. Two overlapping pathways are known to be upstream of these effecter events. The first is the activation of the receptor-mediated death-signaling pathways that ultimately trigger caspase-8. The second originates from the mitochondria, which are central targets for intracellular oxidative stress. Stressed mitochondria release cytochrome c and Apaf-1 (apoptotic peptidase activating factor 1), which contributes to a sub-organellar molecular cluster, called the apoptosome, which in turn activates caspase-9 (Rich *et al*., 2000). Both these pathways can be profoundly influenced by both pro- and anti-apoptotic members of the Bcl-2 family, which, in turn, are regulated by various transcription factors and signaling pathways. The mitochondrion is the reservoir of many other death agonists like AIF (an NADH oxidase, a mitochondrial flavoprotein), and endonuclease G, which may contribute to apoptosis by a caspase-independent nuclear degradation process (Punj and Chakrabarty, 2003).

1.1.1 Apoptosis in neurons

Barring the recent discovery of neuronal stem cells indicating the possibility of neurogenesis in the adult brain, most neurons live as long as we do, making them among the most long-lived cell types. But, neurons are not invulnerable to stress and homestatic signals. Neuronal cell death helps sculpt the nervous system by removing excess neurons to ensure proper and precise pre- and postsynaptic connections. Neurons may also die prematurely in adult brains, when subjected to acute or chronic neurotoxic conditions caused by accidental or genetic factors (Yuan *et al.*, 2003). In fact, apart from some cases of autophagy and necrosis, it has now been established that approximately half of all neurons in the neuroaxis and >99.9% of the total number of cells generated during the course of a human life time go on to die through the process of apoptosis (Datta *et al.*, 1999).

The balance of death and survival signals tightly controls cellular apoptotic machinery. In the case of neurons, NGF (nerve growth factor) is a prototypic example for a survival pathway. NGF binding to its receptor tyrosine kinase, TrkA, activates a host of pro-survival proteins like PI-3 kinase, Akt and MAPK (mitogen activated protein kinase), which in turn regulate apoptosis by inhibiting pro-apoptotic proteins such as Bad and Forkhead factors, and by activating pro-survival proteins such as CREB (CRE-binding protein) and NF-κB (nuclear factor kB), which directly or indirectly regulate the apoptotic machinery. These events are reversed in the absence of NGF (Yuan *et al.*, 2003). Nitric Oxide (NO) is another such signaling molecule known to have pleotropic effects on the cell, including maintenance of the delicate balance between death and survival in adverse conditions (Holmqvist et al., 2004).

1.2 Nitric oxide

1.2.1 Endogenous NO

The synthesis of NO is catalyzed by NO synthases (NOS), a group of evolutionarily conserved cytosolic or membrane-bound isozymes that convert the amino acid L-arginine to citrulline and NO in mammalian and non-mammalian animals (including protozoa and insects) as well as in plants. All NOS isoforms are homodimeric enzymes that require the same co-substrates (molecular oxygen, NADPH) and cofactors (FMN, FAD, tetrahydrobiopterin, heme, Ca^{2+}/ calmodulin, and possibly, also Zn^{2+} ions) (Stuehr, 1999). To date, no nucleated cell has been described that does not express at least one of the three major isoforms (named after the cell type from which they were first isolated and cloned - neuronal NOS or NOS1 or nNOS, inducible NOS or NOS2 or iNOS and endothelial NOS or eNOS or NOS3). These NOS isoforms differ with respect to their main mode of regulation, their key functions, the average concentration of NO produced and their tissue expression pattern. nNOS and eNOS are regulated by Ca^{2+} fluxes and reversible calmodulin-binding, while iNOS is Ca^{2+} independent. iNOS, when induced in cells activated by inflammatory stimuli (interleukin 1, tumour necrosis factor α, interferon-γ or bacterial products such as lipopolysaccharide), is capable of sustained synthesis of high amounts of NO that is typical in inflammation. Induction of iNOS involves a delay of 6-8 hours before the onset of NO production but, once induced the enzyme is active for hours to days and produces up to 1000-fold larger quantities of NO than the constitutive enzymes eNOS and nNOS (Pfeilschifter *et al.*, 2001).

1.2.2 Chemistry

Most biological functions of NO can be attributed to Reactive Nitrogen Intermediates (RNI), rather than NO itself. The term RNI refers to oxidation states and adducts of the product of NOS, ranging from nitric oxide ($°NO$) to nitrate (NO_3), analogous to reactive oxygen intermediates (ROI) that encompass intermediate products when oxygen is reduced to water. In the presence of oxygen, $°NO$ is oxidized to $°NO_2$ and the reaction between $°NO$ and $°NO_2$ gives N_2O_3. As a free radical, NO can quickly react with superoxide ($°O_2^-$), which leads to the formation of peroxynitrite ($ONOO^-$). Peroxynitrite is a strong oxidant, which can irreversibly oxidize thiol residues to sulfenic and sulfonic acids and nitrate peptides and proteins at the phenyl side chain of tyrosine residues. It can also lead to the formation of $°NO_2$ and $°OH$ (Bogdan, 2001).

$°NO_2$ can give rise to nitrite (NO_2^-) and/ or nitrate (NO_3^-), depending on the oxygen concentration. Synthesis of SNO (S-nitrosyl) is facilitated by electron acceptors or metals that mediate the formation of thiol-reactive nitrosonium (NO^+) ions or N_2O_3. S-nitrosylation is among the more studied NO modifications, because it fulfills the criterion of reversibility, essential for many signaling events. S-nitrosylation of cysteines include those that are part of intermolecular disulphide bonds (-S-S-), mixed disulphide bonds (-S-S-R) or cysteine sulphenic acids (RS-OH), all of which affect the (di)sulphide bridges that are crucial for tertiary structure and function.

$°NO$ can also interact with transition metals such as Fe, Zn or Cu. The formation of metal-nitrosyl complexes underlies the regulatory effect of NO on a range of proteins including soluble guanylyl cyclase (sGC) where NO disrupts the bond between the ferrous iron and histidine-105 in the heme moiety, Zn-finger

proteins such as Sp1, EGR-1, vitamin D3 receptor, retinoid X receptor and mRNA binding proteins such as the iron-regulatory protein 1 (Bogdan, 2001).

The reversible reactions have regulatory functions in biological systems, including cytoprotection, while, on the contrary, irreversible modifications are presumably associated with loss of protein function and cytotoxicity (Nicotera *et al*., 1999).

1.2.3 Physiological roles

The biological attributes of NO are numerous and complex. NO is known to play a fundamental role at a very early stage of development in mammals, when the NOS activity in male gametocytes is required for the activation of eggs immediately after insemination (Bogdan, 2001). Various studies have indicated spatio-temporal actions of NO during embryogenesis in the formation of the central and peripheral nervous system, with possible involvement in processes such as neurogenesis, organogenesis and early physiology (Holmqvist *et al*., 2004).

Nitric oxide is implicated in a broad range of physiological and patho-physiological functions. They include cell division, tissue differentiation, response to pathogens, and disease. On a positive note, they help fight malignancy and bacterial or viral infections, while on the other hand, they might also be involved in causing CNS injury, angiogenesis, ischemia, or auto immune diseases. All physiological roles are dependent on the concentration of NO and the type of cell involved – indicating the importance of the macro and micro-environment in determining whether NO ultimately results in cell death or survival. The molecular mechanism of all these different effects, however, is still not clear.

A classic example for the signaling effects of NO is the relaxation of vascular smooth muscle cells when they are exposed to low levels of NO by adjacent

endothelial cells. This effect, which was the first function of NO to be discovered, is due to the activation of soluble heme enzyme sGC, resulting in the formation of cyclic GMP (cGMP), an important messenger molecule which subsequently activates cGMP-dependent ion channels, phosphodiesterases and kinases, ultimately mediating vascular homeostasis (Bogdan, 2001).

When in low levels, NO is a key signaling molecule that almost exclusively mediates physiological functions and counteracts patho-physiological processes in blood vessels, brain, kidney, heart, and many other organs. However, at higher concentrations, produced mainly by iNOS and eventually also by nNOS, in the setting of appropriate micro-environmental conditions, it interacts with thiol groups or transition metal-containing proteins and can alter protein function or initiate gene expression to trigger cell adaptation. There is a continuous shift towards cell damage or apoptosis at increasing concentrations, causing tissue damage.

Mouse knockouts of the NOS isoforms surprisingly show only limited phenotypes. Though the iNOS knock out mice show an increased susceptibility to bacterial and viral pathogens, the other abnormalities are restricted to elevated systemic and pulmonary pressure and resistance to ischemic and certain inflammatory injury, implicating the possibility of NO and NOS's performing possibly some redundant physiological roles (Mashimo and Goyal, 1999).

1.2.4   NO in neurons

Although NO was first discovered as an endothelium-derived relaxing factor, it is now clear that the brain is a primary source for its production in the body. It is an important neurotransmission regulator and can inhibit the uptake of dopamine, noradrenaline, and serotonin (5-HT), and is able to enhance the release of acetylcholine from forebrain basal neurons (Contestabile et al., 2003). It is interesting

to note that unlike other neurotransmitters, NO is not stored in synaptic vesicles and its action is not mediated by any specific membrane receptors, but rather through the cGMP channel of signal transduction. Recent evidence also indicates that NO is an important player in the programme of brain development, by influencing the switch from the proliferative status to the differentiated state of neural progenitor cells (Contestabile *et al*., 2003).

With respect to the neuropathological role of NO, NOS inhibitors have been shown to protect striatal neurons from MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) neurotoxicity in the mouse models for Parkinson's disease (Contestabile *et al*., 2003). A decrease in nNOS expression and activity in patients with Huntington's disease as well as an indication of glutamate-induced neurotoxicity in Amyotrophic Lateral Sclerosis (ALS) and a similar co-operation between β-amyloid and glutamate, in vitro, are indicative of the role of NO in various neuro-pathological conditions. Regionally variable decreases in catalytic activity and/or NOS expression/ localization in aging mice, have also indicated a role for NO in brain aging (Contestabile *et al*., 2003).

1.2.5   Nitric oxide and apoptosis

NO-induced cell death is important in the context of NO killing of pathogens, tumor cells and non-pathogenic host cells; and such cell death has been implicated in a wide range of inflammatory, infectious, ischemic, and neurodegenerative conditions. NO can cause cell death by two general pathways - necrosis and apoptosis. The former often results from ATP depletion, causing failure of sodium and calcium pumps – with energy depletion occurring largely due to inhibition of mitochondrial respiration, inhibition or uncoupling of glycolysis and activation of PARP. The causes of NO-induced apoptosis are, however, less clear and probably more diverse.

The threshold for an apoptosis triggering effect of NO varies considerably from one cell to another, but there is sufficient evidence to conclude that NO mediated apoptosis is dependent on the mitochondria. Inhibition of respiration, along with inhibition of mitochondrial enzymes including aconitase and cytochrome c oxidase, is known to contribute to the pro-apoptotic effect of NO *via* membrane potential reduction, transition pore opening and release of cytochrome c. In a cell-free system containing mitochondria and nuclei, NO induced mitochondrial permeability transition (MPT) and promoted apoptosis which was inhibited by permeability transition inhibitors (Brune, 2003).

Caspase-3, -7, -2 and -9 have been determined to be central components in a cascade triggered by NO in certain cell systems. Processing of these caspases has been observed, implicating their involvement in NO mediated cell death. Besides, blocking of mitochondrial permeability attenuated caspase activation, further confirming the sequence of NO-mediated pro-death events in the cell (Bogdan, 2001).

NO, generated either via iNOS or the breakdown of NO donors, evoked p53 accumulation in murine macrophages (Zhou *et al*., 2004). However, accumulation of p53 cannot be taken as proof that DNA damage is involved because NO does not induce significant DNA damage at the cellular level. However, p53-null mice or mutant p53 human cells showed increased resistance to NO-induced apoptosis, underscoring the role of p53 in transmitting a pro-death NO response (Brune, 2003). p53 activity has been shown to be required for the up-regulation of cell cycle regulators such as p21or pro-apoptotic proteins such as, Bax, PUMA and NOXA. Akt attenuated p53-dependent transcriptional activation and suppressed NO-elicited cell death. NO-mediated Ser-15 phosphorylation (increases p53 stability/ activity), down-regulation of Mdm-2 and attenuated nuclear export, p38 and/or JNK activation have

all been implicated in the accumulation and activation of p53 (Brune, 2003). However, the p53 response is not operational in all cells, and alternate pathways are known to exist. One of them is the ER stress pathway, induced through the unfolded protein response, and involves the activation of the transcription factor CHOP. Perhaps, mitochondrial and downstream cascades modulated by Bcl-2 and IAP family members are possible intersections of p53-dependent and p53-independent apoptosis (Li *et al*., 2004a).

Bcl-2 and Bcl-X_L (B-cell leukemia extra long) over-expression studies have shown the role played by Bcl-2 family members downstream of p53, and upstream of cytochrome c relocation and caspase activation. NO-evoked decreased expression of anti-apoptotic Bcl-2 family members was also shown in monocytes (Brune *et al*., 1999; Yabuki *et al*., 1997).

Lipid peroxidation (a chain reaction that results in the oxidation of phospholipids to lipid hydroperoxides and in the formation of secondary diffusible products, such as aldehydes) is another trigger for MPT (mitochondrial permeability transition) and apoptosis. NO can both promote and inhibit the process – in the presence of superoxide, peroxynitrite generation could rapidly cause lipid peroxidation; while, on the other hand, NO scavenging of the hydroxyl radical (OH°) is also a potent inhibitor of the lipid chain reaction (Contestabile *et al*., 2003).

The NO-caspase connection has been an important component of several review articles addressing the anti-apoptotic role of NO. The active sites of almost all caspase family members are known to become S-nitrosylated, resulting in loss of enzyme function. Interference by NO has also been observed in the pro-apoptotic signaling cascade by not allowing caspase processing rather than inhibition of enzyme activity per se (Brune, 2003). But the relevance, in the light of parallel caspase

activation events documented in so many instances in association with the pro-apoptotic role of NO, is still not clear.

Other anti-apoptotic roles of NO involve the transcriptional up-regulation of cytoprotective genes such as Cox-2, CuZn superoxide dismutase, heme-oxygenase-1, heat shock protein 70 and manganese superoxide dismutase (Brune *et al*., 1999). Most of these are downstream of various different transcription factor-coupled pathways, and their regulation downstream of NO is associated with attenuation of NO toxicity.

1.3 NO and gene regulation

The notion of a linear sequence of signaling events – extracellular ligands binding to cell surface receptors, activation of specific linear cassettes of signal transduction pathways enabling a cell to constantly adapt and coordinate its activities with changes in the microenvironment, is seriously challenged by the ligand NO, which freely diffuses through cell membranes to reach its targets at different intracellular locations and alters signaling networks by redox-sensitive modifications (Pfeilschifter *et al*., 2001). The early and rapid mechanisms of NO signaling depend primarily on post-translational modifications of pre-existing cellular proteins; however, the late phases that are required to accommodate the changing micro environment are mediated by changes in gene expression (Pfeilschifter *et al*., 2001). Table 1 shows a brief summary of the transcription factors known to be modulated by NO in various cell types (Bogdan, 2001).

The adaptive long-term regulation occurs mainly at the level of transcription and is controlled by transcription factors. To date, there is no evidence for the existence of DNA elements in the promoters of genes which directly respond to NO. In a coordinated fashion, various repressors and activators (as a direct and an indirect effect

of NO) that selectively bind their cognate sites in the regulatory elements of NO-responsive genes come together and activate/ repress gene transcription. Sensitivity of various cellular components to the cellular redox status primarily enables NO modulation of transcription factors, of the translation or stability of mRNA, or of the processing of the primary gene product. Some of the known effects of NO on transcription factors are discussed below.

Nuclear factor κB (NFκB), best known for its stimulatory functions in B cells and macrophages, is a hetero dimeric transcription factor, which exists in an inactive state in the cytosol, where it is bound to members of the IκB (inhibitor of NFκB)family. Activating signals like cytokines or oxygen radicals, lead to IκB kinase (IKK) mediated phosphorylation, ubiquitination and proteosomal degradation of IκB, allowing the free NFκB to translocate to the nucleus. NO is known to affect NFκB activity in at least four different ways. Firstly, NO is known to activate signals upstream of IκB such as $p21^{ras}$ or IKK-α activity in T lymphocytes and endothelial cells, respectively. NO might scavenge $°O_2^-$, thereby reducing the formation of H_2O_2 and impeding the action of NFκB, as seen in the NFκB-mediated transcriptional down-regulation of monocyte chemo attractant protein-1 and macrophage colony stimulation factor-1. Thirdly, NO upregulates and stabilizes the expression of IκB-α mRNA and protein and promotes the translocation of IκB-α to the nucleus where it prevents NFκB interacting with its DNA- binding sites (which mainly accounts for the down-regulation of vascular cellular adhesion molecule-1 expression, seen in primary endothelial cells) (Bogdan, 2001). Lastly, in cell-free systems, NO inhibits the DNA-binding activity of NFκB through the S-nitrosylation of a crucial cysteine residue within the p50 subunit. Taken together, depending on the cell type investigated, the

activating stimulus applied, and other factors, NO can activate or inhibit NFκB gene

transcription by interacting with multiple intracellular targets (Bogdan, 2001).

Table 1. Selected examples for the regulatory effect of NO on mammalian transcription factors (Bogdan, 2001).

Transcription factor	Experimental setting; source of NO	Effect of NO	Refs
NF-κB	Recombinant p50/p50, p65/p65 or p50/p65 (cell-free); SNP SNAP, acidified NaNO$_2$	DNA binding ↓ via S-nitrosylation of p50 subunit (Cys62)	46,55
	Human peripheral blood mononuclear cells, human T-cell line (Jurkat); NO gas (in H$_2$O), SNAP, SNP	NF-κB activation ↑ due to activation of p21^{ras} (GDP–GTP nucleotide exchange ↑)	1,43
	Primary human endothelial cells (resting or TNF-stimulated); endogenous NO (eNOS), DETA-NO, SNOG	NF-κB activation ↓; IκB mRNA/protein ↑; M-CSF and VCAM-1 expression ↓	45,56
	Mouse endothelial cell line (TC10) (stimulated with TNF); GTN	NF-κB activation ↑ (100–500 μm GTN) or ↓ (>500 μm GTN); IKKα-activity ↑ (500 μm GTN)	44
	Human vascular smooth muscle cells (IL 1α- or IFN-γ-stimulated); SNP, SIN-1, SNOG	NF-κB activation ↓; VCAM-1 gene transcription ↓	57
	Astrocytes; endogenous NO (nNOS), NOR-3 donor	Constitutive or PDTC-induced activation of NF-κB ↓	58
	Mouse macrophages (ANA-1) (LPS-activated); endogenous NO (NOS2), SNAC	NF-κB p50 activation ↓ presumably owing to S-nitrosylation of NF-κB p50; NOS2 and M-CSF transcription ↓	55
	Liver or lung from mice resuscitated from hemorrhagic shock; endogenous NO (NOS2)	NF-κB activation ↑	47
AP-1 (c-Fos/Jun)	Recombinant c-Jun homodimers or c-Fos/c-Jun heterodimers; NO gas (in H$_2$O)	DNA-binding ↓ presumably through S-nitrosylation of Cys272 (c-Jun) and Cys154 (c-Fos)	59
	Recombinant c-Jun homodimers; DETA-NO (in the presence of sulfhydrylglutathione)	DNA-binding ↓ through S-glutathionylation (of Cys269 in the DNA-binding domain)	60
	Primary mouse cerebellar granule cells (stimulated by NMDA); SNP	AP-1 activation ↓; c-Fos mRNA expression ↓	61
	Pheochromocytoma PC12 cells; SNP	AP-1 activation ↑; c-Fos/JunB mRNA expression	48
	Stably or transiently trans-fected rat thyroid follicu-lar cells or fibroblasts; SNP, SIN-1, SNOG	AP-1 activation/TRE-binding ↑; c-Fos- and JunB-transcription ↑ (cGMP-dependent)	49
	Primary embryonic rat stri-atal neurons; SIN-1, SNAP, SNOG	c-Fos mRNA expression ↑	62
Sp1	Recombinant Sp1; SNOC	DNA-binding ↓	29
	Human monocytic cell line (U937), differentiated with PMA; SNAP, SNOG	Sp1 activation ↓ (inhibition of PKA-mediated phospho-rylation or disruption of the zinc–cysteine interac-tions?); TNF production ↑	51
	Mouse lymphoma cells (EL4), stimulated with IL 1β; SNOC, PAPA/NO	Sp1 activation ↓; IL 2 mRNA expression ↓	29
Egr-1	Recombinant Egr-1; SNOC	DNA-binding ↓	29
	Rat mesangial cells; SNOG	Egr 1 activation ↓; Egr 1 protein ↓; cell proliferation ↓	52
VDR/RXR	Recombinant VDR/RXR heterodimers; SNOC, MAMA/NO	Binding to VDREs ↓	30
	COS-7 cells (transfected with VDRE/luciferase reporter construct and VDR/RXR expression vector); DETA/NO	Reporter gene expression ↓	30
HIF-1	Human cell lines: hepa-tocellular carcinoma cells (Hep3B), embryonic kid-ney cells (293), glioblas-toma cells (A-172)	Under *normoxia*: HIF-1 DNA binding ↑; HIF-1α protein ↑; VEGF promotor activity ↑	5,54

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Activating protein (AP)-1, a heterodimeric transcription factor that belongs to the basic leucine zipper family, consists in its most active form of two proto-oncogenic products, c-Fos and c-Jun. It binds to phorbol-ester response elements (TREs), present in the regulatory region of many genes. NO stimulation of sGC and cGMP, through G-kinase, is known to increase *c-fos* and *jun-B* gene expression. Modulation of JNK2 (Jun N-terminal kinase- 2) and JNK2 kinases, protein kinase cascades upstream of AP-1, has also been observed. S-glutathionylation of c-Jun at cys-269, has been reported to inhibit DNA-binding (Pfeilschifter *et al.*, 2001). Basal c-Jun/AP-1 activity has also been found to be important for counteracting apoptosis induced by low levels of NO through regulation of the expression of *sgII* (gene encoding secretogranin II) (L. Li, per. comm.) and NCAM140 (neural cell adhesion molecule 140) (Feng *et al.*, 2002). But at the same time, at toxic concentrations of NO, c-Jun is phosphorylated exclusively on Ser-63 in a JNK-dependent manner, which enhances c-Jun/AP-1 transcriptional activity, counteracts the protective function of basal AP-1, and eventually leads to apoptosis in SH-Sy5y cells (Li *et al.*, 2004b).

NO has also been implicated in the translocation of Nrf2 (nuclear factor-erythroid 2 related factor) into the nucleus and its enhanced binding (along with small Maf proteins) to the antioxidant response element (ARE), found in the promoter of NAD(P)H: quinine oxidoreductase1 (Nqo1) and other detoxifying enzymes. This early response to NO, resulting in the transcriptional upregulation of cytoprotective genes, is known to counteract NO-induced apoptosis (Dhakshinamoorthy and Porter, 2004).

NO destroys ZnS clusters and ejects Zn^{2+} from various proteins including transcription factors that contain Zn-coordinated DNA-binding motifs ('zinc fingers'). NO-mediated S-nitrosylation of critical cysteine thiol groups and subsequent oxidation

causes the loss of in vitro DNA binding capacity, as demonstrated for p53, Sp1, Egr-1, the glucocorticoid receptor, Vit D receptor, and retinoid X receptor (Pfeilschifter *et al.*, 2001).

In addition, a novel pathway for gene silencing based on the activation of the DNA methyltransferase by S-nitrosylation of critical cysteine residues and the subsequent modification of the CpG island methylation has been reported (FMR1 gene, in mouse macrophages) - methylated CpG-dinucleotides favor a repressive chromatin structure that impede the promoter binding of transcription activators (Pfeilschifter *et al.*, 2001).

NO has also been implicated in affecting mRNA stability and hence, the gene expression status of the cell. For example, NO-mediated stabilization of the transferrin receptor, hemoxygenase-1 and iNOS mRNA itself by a positive feed back loop, have been observed (Turpaev and Litvinov, 2004).

Temporal patterns of NO-mediated cascades of gene expression has been observed with both protective and anti-apoptotic implications, or at times both, depending on the intensity of the signal (Hemish *et al.*, 2003). Though much work has been put into analyzing the transcription machinery reacting to NO in mammalian cells, there is still no clear picture; in fact contradictory results for even the same transcription factors have been observed. This could be a result of the use of different sources of NO (specifically, this could include the use of chemical compounds that preferentially release one of the many RNIs; along with common donors like SNP that also release trace amounts of ferricyanide), or different concentrations of NO, the use of cell free versus cell-containing systems, or of different cell types, or even variations in the redox status within the same cells that have been cultured differently.

Regulation of apoptosis is known to occur at multiple levels in three-dimensional signal transduction pathways and many different incoming signals including NO initiate a varied broad spectrum of responses, regulating the initiation of apoptosis or its inhibition. There is a huge scope to decipher and better understand the details of the transmission of pro- and anti-apoptotic signals by NO that ultimately determine the fate of the cell.

1.4 FOXOs

1.4.1   The forkhead family

Initial investigations leading to identification of Forkhead transcription factors as key effectors of PI3K-PKB signaling began in the nematode worm *Caenorhabditis elegans*. When environmental conditions are adverse, these worms can enter a so-called Dauer or "non-aging" larval stage where they can survive for months. This state of developmental arrest is associated with reduced metabolic activity and increased resistance to oxidative stress. The process is regulated by a neuroendocrine pathway that has been extensively studied by genetic analysis (Birkenkamp and Coffer, 2003a). Regulation of a *C. elegans* PI3K (age-1) signal transduction pathway was found to be essential in modulation of dauer entry. Critically, mutations in the Forkhead transcription factor DAF-16 were found to completely suppress the dauer arrest, metabolic shift and longevity observed in age-1 mutants (Lee *et al.*, 2003). DAF-16 was found to be negatively regulated by DAF-2, an insulin receptor-like protein, and further epistatic analysis implicated PI-3K, PTEN, PDK-1 and PKB/ Akt in the DAF-2 regulation of DAF-16. Several consensus sequences for phosphorylation by PKB/ Akt were later identified in the forkhead protein (Burgering and Medema, 2003).

The forkhead family of transcription factors comprises more than 100 members in species ranging from yeast to humans. About 40 different forkheads have been identified till date in mammalian cells. Mammalian homologues of the Forkhead superfamily are characterized by the presence of a conserved 110 amino acid DNA binding domain, that was shown to form a typical butterfly-shaped structure made up of three tightly packed N-terminal -helices, three ß-sheets, and two loop regions located at the C-terminal end that shape the wings of the structure called the "forkhead" domain or "winged helix" domain (Burgering and Medema, 2003).

Recently, a new nomenclature for these factors has been adopted, and they are now denoted Forkhead box (FOX) factors. This superfamily has been classified into 17 sub-families ranging from FOXA to FOXQ, based on phylogenetic analysis. The Forkhead box class O (FOXO) factors are a subclass within the superfamily. This sub-family of proteins is the only one that is known to be regulated by the PKB/Akt pathway and also share the most homology to the *C.elegans*' DAF-16 protein. Three mammalian proteins belong to this subfamily: forkhead in rhabdomyosarcoma (FKHR), FKHR-like 1 (FKHRL1), and acute-lymphocytic-leukemia-1 fused gene from chromosome X (AFX). FKHR is now known as FOXO1, FKHRL1 is known as FOXO3a, and AFX is known as FOXO4 (Burgering and Medema, 2003). FOXO4 is studied in my thesis.

These three FOXO proteins interact preferentially with a core consensus recognition motif 5'-TTGTTTAC-3', although the residues flanking this core contribute to some DNA binding specificity. Interestingly, the FOXO factors were initially identified at chromosomal breakpoints in several human tumors. For instance, the t(X;11)(q13;q23) chromosomal translocation found in acute lymphoblastic

leukemia gives rise to a fusion between the coding region of the mixed-lineage leukemia (MLL) gene, a thritorax-related transcription factor and FOXO4 (Borkhardt *et al.*, 1997). These genetic alterations of FOXOs in human cancers strongly suggested that they may play a role in the regulation of proliferation, survival or differentiation in higher organisms.

The tissue expression pattern of the FOXO proteins is similar. Given the role of FOXOs in apoptosis and stress responses, most cell types express one or more of the FOXO isoforms. FKHR expression is enriched in adipose tissue, liver and thymocytes, while, FKHRL1 and AFX are observed to be expressed at higher levels in post-mitotic neurons in the brain and muscle tissues, respectively (Tran *et al.*, 2003).

The physiological functions of FOXO proteins, as deduced from loss/ gain of function studies in transgenic and knock out mice, are complex. Mice lacking the three FOXO iso-forms display remarkably different phenotypes. But most transgenics do share a defective insulin signaling pathway as a common aberration– FOXO ablation in mice can rescue diabetes caused due to pancreatic β cell failure while, on the other hand, FOXO over-expression sensitizes mice to diabetes. Insulin signaling, being key to regulation of glucose uptake and metabolism, also affects the life span of these animals (Accili and Arden, 2004).

1.4.2   Forkhead regulation

The FOXO factors are phosphorylated in vivo on multiple threonine and serine residues. In the absence of PKB activity, the FOXO members are predominantly nuclear and thus presumed to be active; however, PKB-mediated phosphorylation induces their re-localization from the nucleus to the cytoplasm (Datta *et al.*, 1999).

Phosphoinositide 3-kinases (PI3Ks) are activated by several proteins including G-proteins and tyrosine kinases, downstream of various growth factor and other signals. Recruitment of PI3K to membrane receptors relocates PI3K to its lipid substrates on the plasma membrane where it phosphorylates the 3'-OH group of the inositol ring of phosphatidylinositol to generate the phosphoinositide phosphates PIP2 and PIP3. These two signalling molecules trigger downstream serine/ threonine kinases including 3'-phosphoinositide dependent kinase-1 (PDK-1) and protein kinase B (PKB). The latter is also known as Akt, a member of the AGC family (cAMP-dependent protein kinase/protein kinase G/protein kinase C extended family) of protein kinases that is catalytically activated through recruitment and phosphorylation by PDK-1. Activated PKB detaches from the plasma membrane and translocates to the cytosol and nucleus where it phosphorylates serine or threonine residues within a PKB phosphorylation motif in target proteins (Van Der Heide *et al*., 2004).
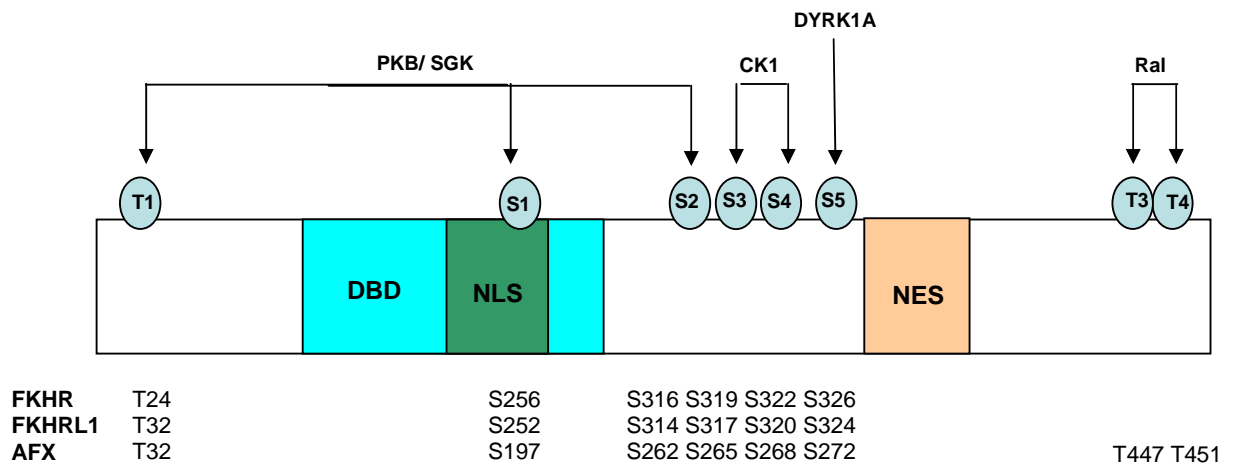
Figure 1. A schematic representation of FOXO transcription factors and their potential sites of phosphorylation.

One such candidate is the FOXO family of proteins. FOXOs are known to be phosphorylated on multiple sites (the implicated serines and threonines are denoted as

Ss and Ts on Fig. 1). Three of these, an N terminal threonine and two serines (T1, S1, and S2) lie within a consensus motif for phosphorylation by PKB. PKB-mediated phosphorylation therein, induces the binding of 14-3-3 protein (which harbours a NES - nuclear export signal) to the FOXO proteins in the nucleus, which results in the release of the FOXO protein from the DNA and subsequent translocation from the nucleus to the cytoplasm (Zheng *et al*., 2002). Nuclear export of FOXO members is sensitive to leptomycin B treatment, indicating that export depends on Crm1 (chromosomal region maintenance-1) too (Kops *et al*., 1999). All three FOXO members also harbour a putative NES. Deletion of the putative NES clearly affects the nuclear export of FOXO4, yet surprisingly does not affect Crm1 binding (Brownawell *et al*., 2001). In addition, phosphorylation does not significantly modulate the binding between FOXO proteins and Crm1. FOXO proteins also contain a sequence that conforms to a non-classical NLS. This NLS consists of three arginine residues present in the forkhead domain C- terminus, and three lysine residues located 19 residues further downstream. The S1 phosphorylation site is present within this motif and the basic nature of the NLS is perturbed by the negative charge introduced by the phosphorylation of S1. The phosphorylated serine thus hinders the NLS function sterically.

As indicated in Fig. 1, not all phosphorylation of FOXO members occurs as a result of PI3K–PKB signaling. For example, it was reported that serum and glucocorticoid-inducible kinase (SGK) can also phosphorylate T1 and S2 of FOXO3a (Figure 1). Like PKB, SGK is a member of the AGC-kinase family, and PDK1 (3'-phosphoinositide dependent kinase-1) appears to be responsible for T-loop phosphorylation and activation of SGK, just as it phosphorylates PKB. Thus, in PI3K-dependent phosphorylation of T1, S1 and S2, SGK can act either redundantly or

independently of PKB to increase the phosphorylation of these sites (Van Der Heide *et al*., 2004).

The S5 site can be phosphorylated by dual-specificity tyrosine-phosphorylated and -regulated kinase 1A (DYRK1a) (Fig. 1). Although phosphorylation at this residue in FOXO1 affects nuclear localization, DYRK1a itself appears to be constitutively active, so the relevance of this phosphorylation in regulated shuttling remains unclear. Casein kinase 1 has been shown to phosphorylate S3 and S4, again the implications are unclear (Kops *et al*., 1999).

Finally, a role for signaling by the small GTPase Ras has also been suggested in the regulation of FOXO4. Like PI3K, Ras is activated following treatment of cells with insulin. Activated Ras can stimulate different downstream signalling cascades, one of which proceeds when active Ras binds the exchange factor (RalGEF) for another small GTPase, Ral. In this way, RalGEF binding to Ras results in Ral activation. The phosphorylation site of AFX regulated by Ras–Ral signalling has been identified and consists of two adjacent threonines (T3 and T4; Fig. 1). Mutational analysis shows that phosphorylation of these residues is essential for AFX activity (Burgering and Kops, 2002).

PKB and other kinase mediated phosphorylation of FOXO can regulate its transcriptional activity in several ways other than merely regulating their nuclear import or export. First, the binding of the 14-3-3 protein to FOXO proteins (which is induced by PKB phosphorylation) has been suggested to inhibit their binding to DNA (Daly *et al*., 2004). It is to be noted here that the S1 phosphorylation site is also part of the DNA binding region of the FOXO proteins and any phosphorylation event therein could affect the affinity of the proteins to bind DNA (Zhang *et al*., 2002).

Secondly, phosphorylation might affect the stability of the FOXO proteins, though FOXO4 is known to be relatively stable, with a low turnover. Lastly, phosphorylation might disturb FOXO interaction with various cofactors that are essential to activate transcription. A handful of proteins are known to interact with FOXOs and help mediate gene expression profile changes as well as determine which target genes are regulated after specific signals. A recent study described the histone acetylase p300/CREB-binding protein (CBP) as a co-activator for FOXO1 (Kops *et al*., 1999). Furthermore, FOXO1 was shown to interact with both steroid and non-steroid nuclear receptors (Li *et al*., 2003). FOXO1 differentially regulated the transactivation mediated by these different nuclear receptors, either as a co-activator or co-repressor, depending on the receptor and cell type (Zhao *et al*., 2001). Roles for FOXOs in modulating HNF-4 (hepatocyte nuclear factor- 3) and STAT3-mediated transcription have been reported recently (Hirota *et al*., 2003; Kortylewski *et al*., 2003). Interestingly, FOXO1 increased STAT3- but not STAT5-mediated transcription, demonstrating specificity for the STAT transcription factor iso-forms. However, it remains to be determined whether (and how) phosphorylation modulates the interaction between FOXO members and all its other interacting partners.

1.4.3   Physiological roles of FOXOs

FOXO mediated transcriptional control of key metabolic enzymes such as IGF-binding proteins, glucose-6-phosphatase and phospho-enol-pyruvate carboxy kinase (PEPCK) is well documented (Nakae *et al*., 2001). The other transcriptional targets that mediate the direct effects of FOXO activity on cell survival, cell cycle control, and DNA damage repair are just beginning to be identified (Fig. 2).

Regulation of Apoptosis:

The death agonist Fas ligand (FasL) has been identified as a mediator of FOXO-induced cell death (Brunet *et al*., 1999). Up-regulation of the pro-apoptotic Bcl-2 family member Bim plays a role in FOXO-dependent cell death upon cytokine withdrawal in a number of hematopoietic cell types (Sunters *et al*., 2003; Tran *et al*., 2003). TRAIL, a pro-apoptotic member of the TNF family, has also been shown to be under the transcriptional contol of FOXOs (Ghaffari *et al*., 2003; Modur *et al*., 2002).

Cell Cycle Regulation:

FOXO activity mediates G_1 cell cycle arrest at least in part by inducing expression of the cell cycle inhibitor p27kip1. p27kip1 acts to block the activity of the cyclin E – CDK2 (cyclin-dependent kinase 2) complex, thereby preventing entry into S phase (Coffer, 2003; Zheng *et al*., 2002). The suppression of the D-type cyclins has also been implicated in FOXO-mediated growth arrest. As cells progress through G_1, D-type cyclins are synthesized and form a complex with their corresponding catalytic partners CDK4 and CDK6. These cyclinD-CDK complexes function to inactivate the retinoblastoma tumor suppressor protein, permitting progression into the S phase (Schmidt *et al*., 2002; Tran *et al*., 2003).

FOXO activity has also been observed to induce cells to exit the cell cycle and enter a non-dividing, quiescent state (G_0). This switch is characterized by reduced protein synthesis and increased abundance of the retinoblastoma family protein p130 which itself appears to represent a direct target of FOXO. Although the FOXO-induced increase in p130 does not affect cyclin E-dependent kinase activity and is therefore not believed to have a role in the FOXO-induced G_1 arrest, p130 does appear

to be required for the FOXO-induced decrease in protein synthesis that has been correlated with cellular quiescence. FOXO thus acts to block cell cycle progression prior to DNA synthesis and to induce cellular quiescence via a variety of target genes (Kops *et al.*, 2002b).

Human cyclin B and polo-like kinase (PLK), two genes whose products have critical roles in G_2-M phase progression, have also been found to be regulated by FOXOs at the transcriptional level (Birkenkamp and Coffer, 2003b). Cyclin B levels rise in G_2 phase and allow for entry into and progression through M phase, whereas exit from mitosis is promoted by cyclin B ubiquitination and degradation. PLK controls this ubiquitination process by regulating the anaphase-promoting complex, a ubiquitin ligase that causes cyclin B degradation and M phase completion. These results further emphasize the key role that FOXO family members play in controlling mitosis by regulating the expression of a set of genes that act as crucial mediators of M phase progression.

Thus, in addition to their roles as mediators of apoptosis, FOXO factors regulate cell proliferation at multiple phases of the cell cycle. Surprisingly, FOXO activity appears to exert opposing effects at different points in the cell cycle; under normal physiological conditions, FOXO family members regulate the expression of genes such as cyclin B and PLK that promote progression through M phase, whereas ectopic FOXO expression can act to arrest cells in G_1 and G_2. The G_1-S and G_2-M transitions represent important sites of checkpoint regulation by the cell to ensure replicative fidelity, raising the possibility that at these critical checkpoints, FOXO factors may sense and act to coordinate cellular responses to DNA damage or other stress stimuli.

Stress Response:

Foxos have been implicated in a variety of stress responses ranging from antioxidant defense to DNA repair. They have been shown to control the expression of antioxidant enzymes manganese superoxide (MnSOD) (Kops *et al*., 2002a) and catalase. MnSOD can convert $°O_2^-$ into H_2O_2, and catalase can convert H_2O_2 into H_2O and O_2, thereby catalyzing a simple linear chain of events that ultimately protects cells from superoxide. These events in particular are associated with forkhead mediated induction of quiescence in cells and reflect in the increased resistance shown by cells to glucose starvation, especially in the absence of active PKB/ Akt.

Gadd45-α (growth arrest and DNA damage 45-α) has also been discovered as a FOXO target that functions in G_2 arrest. Gadd45-α is up-regulated under conditions of stress or growth factor deprivation and it contributes to G_2 arrest by preventing the interaction of the cyclin-dependent kinase cdc2 with cyclin B (Burgering and Medema, 2003; Furukawa-Hibi *et al*., 2002).
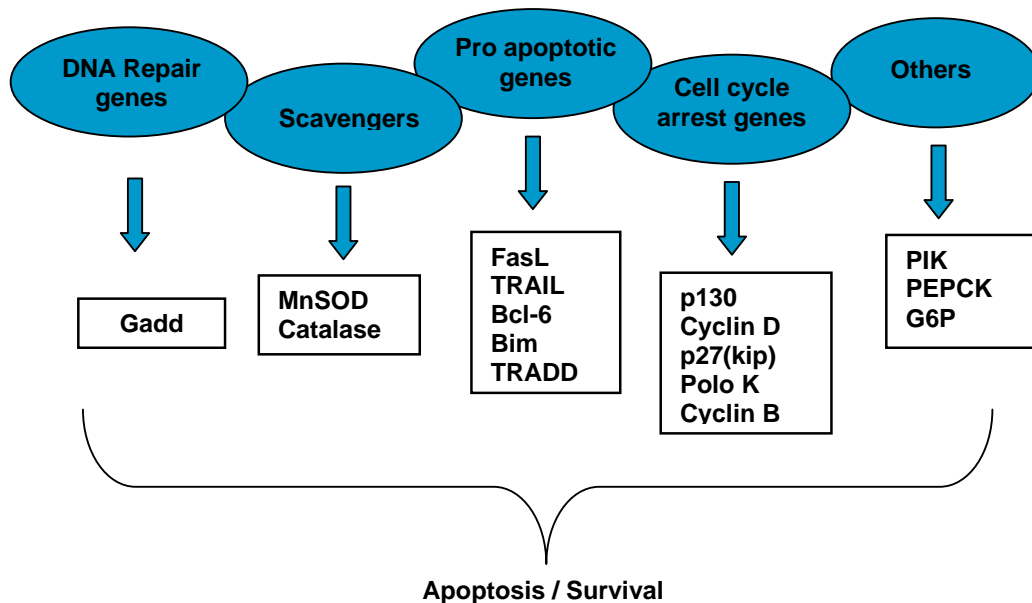
Figure. 2 A summary of known transcriptional targets of FOXOs, classified according to functionality.

1.4.4 Rationale

Apoptotic stimuli are known to activate both pro-death and pro-survival molecules. The seemingly contradictory implication of the up-regulation of both Bim and MnSOD, by FKHRL1 in GM-701 fibroblast cells, after growth factor deprivation, is a relevant example in this case (Liu *et al*., 2005). Other studies have implicated both up and down regulation of Cu/ ZnSOD, Bcl-2, Bcl-X$_L$, cytochrome c, at least partially due to direct FOXO regulation (confirmed by the absence of such regulation in FOXO dominant-negative constructs or primary cells from knockout animals). In cells of hematopoietic origin, mere activation of a FOXO factor is sufficient to activate a variety of pro-apoptotic genes and to trigger apoptosis. In contrast, in most other cell types, activation of FOXOs blocks cellular proliferation and drives cells into a quiescent state. In such cell types, FOXO factors also provide the protective mechanisms that are required to adapt to the altered metabolic state of quiescent cells. Thus, FOXO factors can determine the fate of a cell, can initiate long-term survival in a quiescent state or programmed cell death (Burgering and Medema, 2003). It is in this context that it will be interesting to study the relationships of FOXOs and NO, another important molecule that is known to regulate both death and survival.

1.5 C/EBP HOMOLOGY PROTEIN (CHOP)

1.5.1 Endoplasmic Reticulum (ER) stress

Several experiments have suggested the existence of p53-independent signaling pathways operating during NO-mediated apoptosis. Islet cells lacking poly ADP-ribose polymerase (PARP) and p53-deficient microglial cells, still undergo apoptosis even though they are more resistant to NO (Kawahara *et al*., 2001). The

endoplasmic reticulum (ER) stress pathway is one of the contenders for a p53-independent signaling pathway.

The ER plays several important roles in the folding, export, and processing of newly synthesized proteins. Exposure of cells to stress results in protein unfolding and impairment of cellular processes. When such stress results in the transcriptional activation of genes harboring ER stress response elements within their promoters that help alleviate the pressure on the ER, pre-emptive apoptosis occurs in cells. The collective response is referred to as the ER stress response pathway (schematically represented in Fig. 3).

A variety of triggers including agents such as tunicamycin, which blocks protein glycosylation, brefeldin A, which blocks ER-Golgi transport, and general inhibitors like dithiothreitol that block formation of disulphide bonds and affect protein folding can result in the accumulation of unfolded proteins in the ER lumen (Rao *et al.*, 2004). These then bind to Bip/ Grp78 and competitively disrupt the interaction between Bip/ Grp78 and Ire-α (inositol- requiring). The free Ire-α cleaves 28S rRNA and inhibits translation in the cell. ER stress also results in the cleavage of ATF-6 (activating transcription factor-6). Its cytosolic domain translocates to the nucleus, where it functions as a basic leucine zipper transcription factor of the ATF/CREB family, leading to the activation of genes possessing an ER stress element (ERSE) in the promoter region (Gotoh *et al.*, 2002). Such genes include chaperones such as Bip/ Grp78 and calreticulin, other mitochondrial stress proteins like mtDnaJ and ClpP (caseinolytic protease P) and transcription factors such as CHOP (Ferri and Kroemer, 2001).
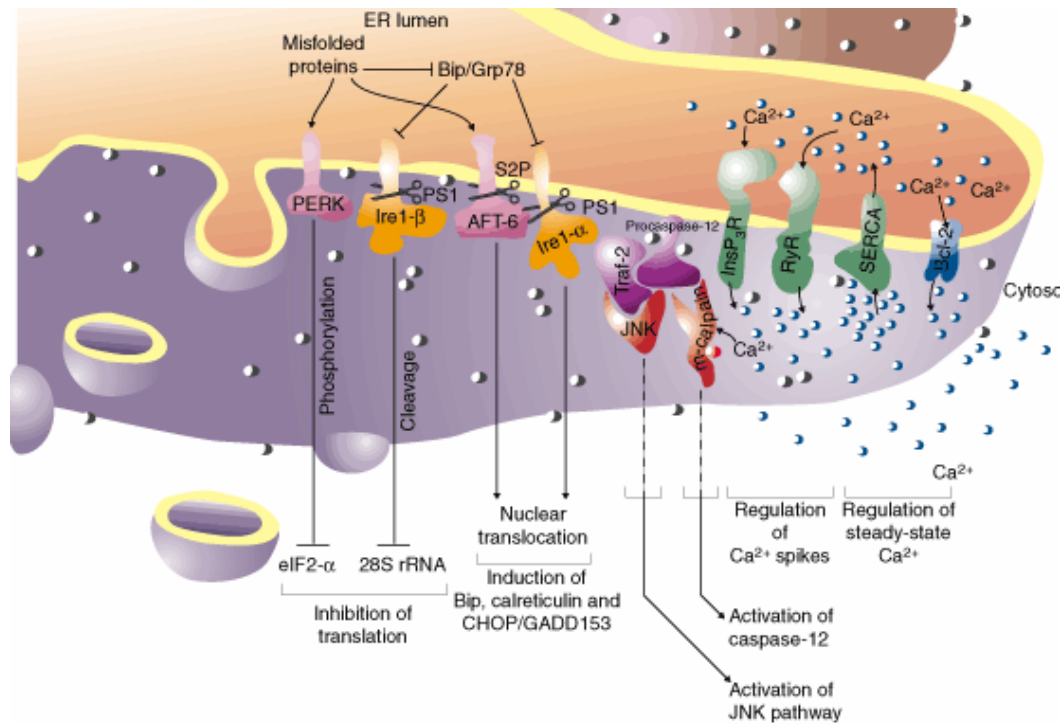
Figure 3. Schematic representation of the ER stress response in mammalian cells (Ferri and Kroemer, 2001).

1.5.2 CHOP in ER stress

CHOP (also called GADD153 - growth arrest DNA damage 153) is a bZIP transcription factor and is a member of the C/EBP family. The GADD genes are a group of genes that are induced by genotoxic stress and growth arrest signals. There are three distinct GADD genes, GADD34, GADD45 and GADD153, but there is no similarity among them. C/EBPs form a family of transcription factors that regulate a variety of genes involved in a broad range of physiological processes, including immune functions as well as cell differentiation and proliferation (Oh-Hashi *et al.*, 2001). To date, six distinct members of the C/EBP family have been identified. CHOP protein is composed of two known functional domains, an N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper (bZIP) domain consisting of a basic amino-acid-rich DNA-binding region followed by a leucine zipper dimerization

motif (Oyadomari and Mori, 2004). CHOP negatively modulates the C/EBP (CCAAT/enhancer-binding protein) transcription factors by heterodimerizing with members of the family. These stable heterodimers do not bind to the classical C/EBP sites and thus inhibit the expression of genes that contain the consensus element in their promoter regions (Igase *et al*., 2001). There is no evidence for CHOP homodimers. However, some novel specific DNA sequences have also been identified as possible recognition elements for the heterodimers as well as monomeric CHOP (Ubeda *et al*., 1996).

CHOP is known to be up-regulated by a variety of stress responses including hypoxia, growth arrest, DNA damage, amino acid and glucose deprivation, apart from exposure to known direct ER stressors. A consensus ERSE has been identified in CHOP and is known to be involved in p50ATF6-mediated expression of CHOP mRNA (Ubeda and Habener, 2000).

Further proof that CHOP is induced by ER stress and plays a role in growth arrest and cell death comes from the fact that the unfolded protein response and the induction of CHOP can be attenuated by the over-expression of an ER chaperone, BiP/Grp78 (Wang *et al*., 1996). CHOP is also induced by activation of Ire1α or Ire1β, ATF6, and PERK, all of which are upstream mediators of the ER stress pathway (Fig. 3). Targeted disruption of the CHOP gene in mice, protected cells against ER stress-induced apoptosis, as was seen in the case of protection against tunicamycin-induced apoptosis in proximal renal tubular epithelia (Oyadomari *et al*., 2001).

1.5.3   NO, ER stress and CHOP

What are the possible mediators of the cell death pathway between NO and ER stress initiation? NO is known to make oxidative/ nitrosylative, nitrosative changes to

numerous proteins and affect ZnS clusters and disulphide bonding - all of which perturb the tertiary structure of proteins, possibly initiating the unfolded protein response. Oyadomari et al (2001) also reported that ER Ca^{2+} stores could be a previously unrecognized target of NO. NO was shown to deplete ER Ca^{2+}, cause ER stress and apoptosis through the ER stress pathway in pancreatic-β cells. Over-expression of calreticulin increased the Ca^{2+} content of the ER and protected cells against NO-mediated apoptosis. Molecular components of ER Ca^{2+} homeostasis including pumps for Ca^{2+} uptake (SERCAs), Ca^{2+}-binding proteins, and channels for Ca^{2+} release, inositol 1,4,5-triphosphate receptors and ryanodine receptors are the possible target molecules of NO in NO-induced depletion of ER Ca^{2+} (Oyadomari *et al*., 2001).

CHOP also undergoes stress-inducible phosphorylation by a p38-type MAP kinase and this phosphorylation event is known to further enhance the transcriptional activation potential of the protein (Oh-Hashi *et al*., 2001).

Target genes of CHOP, designated as DOCs (downstream of CHOP) are just beginning to be identified. Wang et al (1998) found candidate target genes of the CHOP protein using representational difference analysis. However none of these have been shown to be directly involved in the process of programmed cell death. A novel form of carbonic anhydrase V1, identified as one of the DOCs, may promote apoptosis indirectly by increasing intracellular proton concentrations (Wang *et al*., 1998). Recently CHOP expression has also been implicated in down-regulation of Bcl-2 expression, depletion of cellular glutathione, and exaggerated production of reactive oxygen species (Kawahara *et al*., 2001). Bcl-2 antagonizes CHOP-induced apoptosis and sensitization to ER stress, further confirming its important role in effecting CHOP mediated cell death (Ferri and Kroemer, 2001). CHOP-mediated down-regulation of

Bcl-2 can be expected to facilitate MMP (mitochondrial membrane potential) induction as ER stress has been observed to finally trigger the induction (Reimertz *et al*., 2003). However, the precise apoptosis cascade downstream of CHOP remains to be identified.

1.5.4   Rationale

The role of CHOP as an ER stress response element is well established, though details of how it mediates cell death through its transcriptional activation capability is still not clear. NO, on the other hand, has also been implicated in causing apoptosis through multiple pathways, with ER stress (through the unfolded protein response pathway) being one of them. But any precise role of CHOP in NO-mediated cell death, specifically, is not well documented. Here I intend to begin my investigation with the observation of (any) induction of CHOP after NO treatment of neuroblastoma cells. I also intend to follow up on its physiological relevance, with reference to sensitivity to NO-mediated cell death, preferably, solely transmitted through CHOP and its transcriptional activity. The elucidation of mechanisms of how CHOP regulates cell death and identification of its transcriptional targets relevant to apoptosis will help precisely chart out the role of CHOP in NO-mediated cell death.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

2.1.1 Common reagents

A list of some of the common reagents used in this study and their source:

Description	Source
Media preparation components	Difco laboratories
Laboratory chemicals	Sigma
Deta-NO	Sigma
SNP	Sigma
Okadaic acid	Roche
Insulin	Sigma
Hygromycin	Clontech Laboratories
Geneticin (Neomycin)	Invitrogen
Metaphor agarose	Cambrex Bioscience
Culture dishes	Nunc
1 Kb DNA ladder	Promega
10 bp DNA ladder	Invitrogen
Broad range protein ladder	Invitrogen
pcDNA3.1/ V5-His TOPO TA kit	Invitrogen
pcDNA4/ TO vector	Invitrogen
pGL3 (with SV-40 promoter)	Promega
pRl-Tk (Renilla) vector	Promega
Blasticidin	Invitrogen
Zeocin	Invitrogen
Stripping buffer	Pierce

2.1.2 Antibodies

Details of the antibodies used for immunoflouroscence and western- blotting in this study:

Antibody	Source
AFX	Cell Signalling
FKHR	Cell Signalling
FKHRL1	Santa Cruz Biotechnology
Phospho- AFX (Ser 197)	Cell Signalling
Phospho- FKHR (Ser256)	Cell Signalling
Phospho-FKHRL1(Thr32)	Santa Cruz Biotechnology
CHOP	Santa Cruz Biotechnology
Bcl-2	Santa Cruz Biotechnology
V5	Invitrogen
Myc	Invitrogen
Caspase-3	Transduction Laboratories
PARP	BD Pharmingen
Anti- rabbit, HRP conjugated	Chemicon International
Anti- mouse, HRP conjugated	Chemicon International
Anti- goat, HRP conjugated	Chemicon International
Fluorescent secondary antibodies	Alexa Chemicals

2.1.3 Primers

Oligonucleotides (synthesized by Research Biolabs, Singapore) used in this study, are listed below (F stands for forward and R for reverse, primers):

Oligonucleotide	Sequence (5' to 3')
AFX orf F	CCA CGT ATG GAT CCG GGG AAT
AFX orf R	GGG ATC TGG CTC AAA GTT GAA
AFX GFP F	CCC AAG CTT CCA CGT ATG GAT CCG GGG AAT
AFX GFP R	CGG GGT ACC GGG ATC TGG CTC AAA GTT GAA
AFX orf p4 F	CCC AAG CTT CCA CGT ATG GAT CCG GGG AAT
AFX orf p4 R	CCG CTC GAG TCA GGG ATC TGG CTC AAA GTT
AFX S197A F	CCG GGC CGC CGC CAT GGA TAG CA
AFX S197A R	TGC TAT CCA TGG CGG CGG CCC GG
Gadd 153 F	ACT GCA GAG ATG GCA GCT GAG TCA T
Gadd 153 R	TGC TTG GTG CAG ATT CAC CAT TCG

IRS 3X XmaI F	CCG GGC AAA ACA AAC TTA TTT TGA AGC AAA ACA ACT TAT TTT GAA GCA AAA CAA ACT TAT TTT GAA
IRS 3X XhoI R	TCG ATT CAA AAT AAG TTT GTT TTG CTT CAA AAT AAG TTT GTT TTG CTT CAA AAT AAG TTT GTT TTG C
Mut IRS 3X XmaI F	CCG GGC AAA A<u>G</u>A AAC TT<u>C</u> TTT TGA AGC AAA A<u>G</u>A AAC TT<u>C</u> TTT TGA AGC AAA A<u>G</u>A AAC TT<u>C</u> TTT TGA A
Mut IRS 3X XhoI R	TCG ATT CAA AA<u>G</u> AAG TTT <u>C</u>TT TTG CTT CAA AA<u>G</u> AAG TTT <u>C</u>TT TTG CTT CAA AA<u>G</u> AAG TTT <u>C</u>TT TTG C
FKHRL1 orf F	GGC GGC GAA GAT GGC AGA GGC ACC G
FKHRL1 orf R	GCC TGG CAC CCA GCT CTG AGA TGA G
FKHR N F	CAC AGC AAG TTC ATT CGT GTG CAG A
FKHR N R	GGA GAG TCA GAA GTC AGC AAC TCC T
BimEL F	TGA TGT AAG TTC TGA GTG TG
BimEL R	GTG TCA AAA GAG AAA TAC CC
CyclinD1 F	CGA GGA GCT GCT GCA AAT GG
CyclinD1 R	GGT ATC AAA ATG CTC CGG AGA GG
Trail S F	TGC TGG CAA GTC AAG TGG CA
Trail S R	GCC AAC TAA AAA GGC CCC GA
CyclinD2 F	AGC AGC GGG AGA AGC TGT CTC TGA TCC
CyclinD2 R	ATG GAC GCG TCT CTC TCT TTC GGC C
p27 F	CGG GAG AAA GAT GTC AAA CGT GCG A
p27 R	CGT TTG ACG TCT TCT GAG GCC AGG C
p130 F	GGG GTG CGC TAT GCC GTC GGG AGG T
p130 R	TCC CAG GTT CTA GAA ACC AGT GA G
Trail F	TGA CAG GAT CAT GGC TAT GAT GGA G
Trail R	GCC AAC TAA AAA GGC CCC GAA AAA A
Bcl6 F	TGA AGA CAA AAT GGC CTC GCC GGC T
Bcl6 R	CAG GCA TCC GGA CAT CCC GAA ACT C
B actin F	ATG GAT GAT GAT ATC GCC GCG CTC G
B actin R	GAA GCA TTT GCG GTG GAC GAT GGA G

The underlined nucleotides indicate the introduction of a base pair change, as compared to the native (genomic) sequence.

2.1.4   Stable cell lines

List of SH-Sy5y cell lines selected for stable expression of the constructs mentioned alongwith:

Name	Description
p3.1-AFX	AFX- pcDNA3.1 transfected SH-Sy5y cells; Neomycin resistant.
p3.1	pcDNA3.1 transfected SH-Sy5y cells (vector control); Neomycin resistant.
Tet-12	pcDNA6 (expressing tetracyclin repressor) transfected SH-Sy5y cells; Blasticidin resistant; kindly provided by Dr. Chris Redfern, University of Newcastle, UK
p4- TO	pcDNA4- TO transfected Tet-12 cells (vector control); Blasticidin and Zeocin resistant.
p4- AFX	AFX-pcDNA4 transfected Tet-12 cells; Blasticidin and Zeocin resistant.
AFX- EGFP	AFX-EGFP transfected SH-Sy5y cells; Hygromycin resistant.
EGFP	EGFP (N2) transfected SH-Sy5y cells (vector control); Hygromycin resistant.
p4- S CHOP	CHOP-pcDNA4 transfected Tet-12 cells; Blasticidin and Zeocin resistant.
p4- AS CHOP	Anti- sense CHOP-pcDNA4 transfected Tet-12 cells; Blasticidin and Zeocin resistant.

2.2 Mammalian cell culture

2.2.1   Cell lines, passaging and treatments

Human neuroblastoma cell line SH-Sy5y and mouse fibroblast cell line NIH-3T3 were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, USA; 4.5g/l glucose), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Hyclone Laboratories, USA), 100u/ml penicillin and 0.1mg/ml streptomycin (both from Sigma Chemicals, St. Louis, MO). All cells were incubated in a humidified

37°C incubator, under partial CO_2 pressure of 5%. 0.125% Trypsin- Versene and PBS were used during passages and washes. Cells were passaged (1 flask into 4) every 4-5 days.

2', 2'(hydroxynitrosohydrazono) bis-ethanamine (Deta-NO) and sodium nitroprusside (SNP) were the two extrinsic NO donors that were used in this study. Deta-NO is a relatively pure NO donor releasing only NO and the free amine, diethylenetriamine (Borutaite and Brown, 2003). While NO delivery is almost instantaneous at acidic pH, the release of NO follows much slower kinetics at physiological pH. Deta-NO is also easily soluble in water and is stable (with a half life around 30 hours). SNP, on the other hand, requires enzymatic bio-activation or reaction with thiols. Its NO release is accompanied by the release of H_2O_2, cyanide, ferrocyanide and ferricyanide (Kimura et al., 2002).

For NO treatment of cells, 1.5 and 2 mM concentrations of Deta-NO and SNP, respectively, were found to cause apoptosis starting 12 hours (Feng et al., 2002), in SH-Sy5y cells and hence, were the preferred concentrations for treatments facilitating the observation of the effects of inhibitory levels of NO in mammalian cells.

NIH-3T3 cells were used in parallel as positive controls, by exploiting the AFX S-197 phosphorylation that is known to occur post insulin treatment, through the activation of PI-3 kinase and PKB (Guo et al., 1999). Cells were serum starved for 24 hours and subsequently treated with 1ug/ ml of insulin for 30 minutes. Immunoflourescense or protein analysis by western blotting was done to show the phosphorylation of AFX and its subsequent nuclear export.

2.2.2 Freezing and thawing of cells

70-80% confluent cells were trypsinised, centrifuged at 1000 rpm, for 5 min, at 4°C, washed with PBS and resuspended in freezing media – 10% (v/v) DMSO in heat-inactivated FCS. In order to prevent the formation of intra cellular crystals, the aliquots were frozen in ethanol, and subsequently transferred to liquid nitrogen for long-term storage. Recovery involved rapid thawing of the vials and immediate re-suspension in flasks holding pre-warmed culture media.

2.2.3 Transient transfection

1ug of plasmid DNA was transfected in 6-well plates of 60-70% confluent cells using Lipofectin (Life Technologies Inc, USA). Up to 5ul of the lipofectin reagent was mixed with the plasmid DNA in 200ul of Opti-MEM reduced serum medium (Invitrogen, Carlsbad, CA), and left at room temperature for up to 30 minutes to allow DNA-liposome complexes to form. Cells were washed once with Opti-MEM media before being over-laid with 2 ml of the media containing the DNA- lipofectin mixture.

The liposome- DNA mix was incubated with cells for 12-24 hours before the cells were treated and analyzed using appropriate protocols for reporter assays, western blots, etc.

2.2.4 Generation of stable cell lines

Transfection was carried out as above. 12 hours post- transfection, neomycin (Invitrogen, Carlsbad, CA) or hygromycin (Sigma Chemicals, St. Louis, MO) containing media was added to the cells, at concentrations of 600 and 100ug/ml, respectively. The media was refreshed at 48 hour intervals, for up to 10 days, when colonies of resistant cells begin to be seen on the plate. These are then isolated by trypsinisation and plated in 96-well plates, at a dilution that will allow the isolation of

single cells in separate wells. These were then progressively cultured into bigger flasks, and then analyzed for expression of the desired protein, through western blotting. Confirmed clones were maintained at 200 ug/ml and 50 ug/ ml neomycin or hygromycin concentrations, respectively.

Stable clones with pcDNA4 vectors expressing the protein of interest (AFX and CHOP), were made by transfection of the plasmid DNA in Tet-12 background cell lines, which in turn, is a stable cell line selected for expression of the Tetracyclin repressor protein (by stable integration of the pcDNA6- TetR construct). Tet-12 cells were maintained in 5 ug/ml Blasticidin (Invitrogen, Carlsbad, CA) containing culture media. Subsequent selection for pcDNA4- AFX/ CHOP expressing constructs was done by selection for growth in media containing an additional antibiotic - 250 ug/ml Zeocin (Invitrogen, Carlsbad, CA). 1 ug/ml of tet (Sigma Chemicals, St. Louis, MO) was used to induce the expression of the protein of interest, over a 4-24 hour period. The expression levels were checked using western blots before further experiments involving the AFX/ CHOP over-expressing cell lines were set up.

2.2.5 Preparation of whole cell extracts

Cells were washed with cold PBS and trypsinised. Pelleted cells were then lysed in lysis buffer [50mM HEPES, 150 mM NaCl, 1mM EDTA and 1% NP-40 with 1 protease tablet (Complete, Roche) dissolved in 30 ml of the buffer] by placing on ice, for up to 30 minutes. The mixture was then centrifuged, the supernatant separated and the protein content quantitated by Bradford's assay before subsequent use.

2.2.6 Preparation of nuclear extracts

The nuclear extraction kit from Panomics Inc. (Redwood City, CA) was used to isolate the nuclear fractions of mammalian cells. Briefly, pelleted mammalian cells were lysed in the presence of a protease inhibitor cocktail to release the nuclei. The

nuclei were separated by centrifugation and the nuclear proteins were released by vigorous shaking during an overnight incubation in the nuclear lysis buffer (provided as part of the kit). The debris was pelleted and removed, and the nuclear proteins quantitated before use.

2.2.7 RNA extraction

80 cm^2 flasks containing 70-80% confluent cells were homogenized in 1ml of Tri reagent (Molecular Research Centre, USA). The lysate was passed several times through a pipette and stored at room temperature for 5 min, to completely dissociate nucleoprotein complexes. 200ul of chloroform was then added and the mixture thoroughly shaken, before centrifugation at 12000g for 15 minutes. The upper aqueous phase, holding the RNA exclusively, was separated with care from the phenol-chloroform phase and the interphase, holding the protein- cell debris and DNA, respectively. 500ul of isopropanol was added to the precipitate and stored at room temperature for 5 minutes and centrifuged at 12000g for 8 minutes, to precipitate the RNA. The white-pellet like RNA precipitate was then washed with 75% ethanol, air-dried and dissolved in RNase-free water, and quantitated before use.

2.3 DNA methodology

2.3.1 Plasmid constructs

List of plasmids, constructed and used in this study:

Construct	Description
AFX- pcDNA3.1	Full length AFX cDNA cloned into pcDNA3.1 vector by TA cloning.
AFX- pcDNA4	Full length AFX cDNA cloned into pcDNA4 vector as a HindIII- XhoI fragment.
S197A AFX- pcDNA3	Mutant full length AFX cDNA cloned in pcDNA3 vector.

AFX- EGFP	Full length AFX cDNA cloned into EGFP N3 vector as a HindIII- KpnI fragment.
3X IRS- pGL3	3 consecutive copies of the forkhead consensus sequence from the IRS promoter cloned into pGL3 vector as an NheI- XhoI fragment.
Mutant 3X IRS- pGL3	The above sequence cloned into pGL3 vector as an NheI- XhoI fragment with a key base pair mutated in every copy.

2.3.2 PCR

The PCR reactions were performed using the Pfu polymerase kit (Promega, Madison, WI). The following recipe was used -

Component	Volume	Final concentration
Pfu polymerase (3units/ul)	1 ul	3 units
10X buffer	5 ul	1X
dNTP (10mM)	2 ul	0.4 mM
Forward primer (10uM)	1 ul	20 pm
Reverse primer (10 uM)	1 ul	20 pm
Template DNA (50 ng/ul)	1 ul	50 ng
Distilled water	39 ul	-

Appropriate primers, as mentioned in 2.1.3, were used in the reactions, to generate PCR fragments that encoded the full-length cDNA. PCR conditions were as follows:

Condition	Temperature	Duration
1. Initial denaturation	94°C	5 minutes
2. Denaturation	94°C	30 seconds
3. Annealing *	55- 68°C	1 minute
4. Extension ^	72°C	1 minute per kb
5. Final Extension	72°C	10 minutes

*Approximately 5°C below the Tm of primers
^The reaction is repeated up to 30 times, from step 2 to 4.

PCR products were separated using gel electrophoresis (see 2.3.4) and visualized using a UV trans-illuminator. PCR fragments of the right size were excised and eluted from the gel (see 2.3.5) before restriction enzyme digestion of the ends and ligation with the vector.

2.3.3 Site-directed mutagenesis

In order to further understand the role played by the NO-mediated phosphorylation event at S-197 of AFX, I constructed dominant negative cell lines expressing the protein with a site directed mutation, converting the serine to an alanine, which would prevent recognition and phosphorylation of the site. Forward and reverse primers containing the mutated residue were designed, and the high fidelity Expand kit (Roche, Basel, Switzerland) was used for the PCR. The pre-constructed pcDNA3-AFX construct was used as a template, and the primers were used to re-synthesize the entire plasmid, but with the insert containing the mutated residue this time. The PCR reaction was set up as follows –

Component	Volume	Final concentration
Taq high fidelity polymerase	0.75 ul	3 units
10X buffer	5 ul	1X
dNTP (10mM)	2 ul	0.4 mM
Forward primer (10uM)	1 ul	20 pm
Reverse primer (10uM)	1 ul	20 pm
Template DNA (100 ng/ul)	2 ul	200 ng
Distilled water	38 ul	-

The PCR conditions were as follows:

Condition	Temperature	Duration
1. Initial denaturation	94°C	2 minutes
2. Denaturation	94°C	15 seconds
3. Annealing *	55°C	30 seconds
4. Extension ^	68°C	8 min (for 7 Kb)
5. Final Extension	68°C	7 minutes

* Approximately 5°C below the Tm of the AFX S197A primers.
^ The reaction is repeated up to 14 times, from step 2 to 4.

The PCR products were then digested with DpnI for 2 hours at 37°C. This effectively removed the entire methylated template DNA (the original plasmid was from a dam positive, DH5-α transformant, leaving the GATC's in its sequence methylated) leaving behind, only the mutagenised plasmid. The DNA is purified from the salt solution by ethanol precipitation and transformed into nick- repair capable Xl-1 blue competent cells, by heat- shock. The plasmids were subsequently sequence verified.

2.3.4 Agarose gel electrophoresis

1% (w/v) agarose gel was prepared by melting 1 gram agarose in 100 ml of TBE buffer (89 mM Tris, 89 mM Boric Acid, 2.5 mM EDTA- pH 8). 0.5 ug/ml ethidium bromide was added before allowing the gel to solidify on a gel casting mould. The gel was subsequently immersed in an electrophoresis tank with 1X TBE buffer. 6X loading dye (0.1% bromophenol blue, 0.1% xylene cyanol, 40% glycerol) was added to the plasmid DNA/ PCR products to a final concentration of 1X, and loaded in wells, along with 0.5 ug of a suitable DNA ladder. Electrophoresis was carried out at 100V. The separated DNA bands were visualized using a UV trans-illuminator.

2.3.5 Elution of DNA from agarose gel

DNA fragments of interest were excised from the agarose gel under UV trans-illumination, using scalpels. The QIAquick gel extraction kit was used to elute the DNA. Briefly, the excised gel fragment was weighed and a 3X volume of buffer QG was added. The mixture was left at 52°C, with occasional agitation, to dissolve the gel thoroughly. 1X volume of isopropanol was then added and the mixture loaded onto the mini-columns provided with the kit. The columns were centrifuged at 12000 rpm on a table-top microfuge for 1 minute. The column (whose resin binds and retains the DNA from the mixture) is then washed with buffer PE. The wash buffer is completely removed by centrifugation at high speed. The DNA is then eluted in a suitable volume of buffer TE, by centrifuging at 12000 rpm for 1 minute.

2.3.6 RE digestion

The following reaction mixes were set up for restriction enzyme digestion of the plasmids –

Component	Volume	Final concentration
Plasmid DNA	5 ul	1 ug
RE enzyme(s)	0.5 ul	10 units
10X RE buffer	4 ul	1X
Distilled water	30.5 ul	-

The mixture was incubated at 37°C for at least 2 hours. The digestion of the plasmids were confirmed by gel electrophoresis and subsequently eluted from the gel, for ligation.

2.3.7 Ligation

Restriction enzyme digested, sticky-ended plasmid and PCR fragments were ligated by setting up the following mixture –

Component	Volume	Final concentration
T4 DNA ligase (400 units/ ul)	1 ul	400
10 X buffer	1 ul	1X
Vector DNA: insert DNA	upto 500 ng	1:3 ratio
Distilled water	top up to 10 ul	-

The reaction mix was incubated at 16°C for 16 hours before transformation into competent DH5-α cells.

In the case of annealing inserts into the pcDNA3.1 vector, the T overhangs within the TOPO-conjugated vector were utilized. The PCR fragments of the insert, with A overhangs added by Taq polymerase, was directly ligated onto the linearised vector with the T overhangs, and then subsequently transformed.

2.3.8   Preparation of competent cells

E.coli (DH5-α or Xl-1 blue) was cultured in 200 ml liquid broth. Growth was monitored to collect cells in the logarithmic phase, at an A_{600} of 0.3-0.6. Cells were chilled on ice for 15-30 minutes before pelleting by spinning at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended in half the volume of cold 100 mM $CaCl_2$ and incubated on ice for 20 minutes. The cells were pelleted again and resuspended in 1/10 th volume of cold 100 mM $CaCl_2$ and incubated on ice for 1 hour. Sterile glycerol was added to a final concentration of 15%, and the cells were aliquoted in 0.5 ml micro-centrifuge tubes, and stored at -80° C.

2.3.9   Transformation by heat shock

The ligation mixture (up to 2 ul, refer to 2.3.7) was carefully mixed with 50 ul of competent cells and left on ice, for up to 30 minutes. The vial was placed in a 42°C water bath for 30 seconds and then put on ice, immediately. After recovery on ice for 10 minutes, liquid broth (up to 800 ul) was added to the cells-DNA mixture and

incubated at 37°C for 1 hour. The cells were then plated on to agar plates, containing appropriate concentrations of the selective antibiotics (ampicillin – 100 ug/ ml; kanamycin – 50 ug/ ml) and left to incubate at 37°C overnight. Colonies, if any, were picked, grown in liquid broth over night, the plasmid isolated (refer to 2.3.10) and checked for transformed cells containing the required construct.

2.3.10 Isolation of plasmid DNA

A mini prep kit from Qiagen (Valencia, CA) was used to isolate plasmid DNA from transformed cells. Briefly, logarithmically growing bacterial cell cultures were pelleted, and resuspended in ice-cold buffer P1 (250 ul of resuspension buffer for 1 ml of cell culture, OD~0.5). An equal volume of the lysis buffer, P2, was gently mixed with the cells. 350 ul of buffer P3 was added to the same, to neutralize the solution. The mixture was then spun down at 13000 rpm, for 10 min and the supernatant loaded onto the resin spin columns provided with the kit, and centrifuged. The columns were then washed with buffer PE and the DNA eluted from the resin with 50 ul of buffer EB.

2.3.11 Plasmid sequencing for construct verification

The inserts in all the plasmid constructs made were sequence verified by using appropriate primers designed upstream and downstream of the insert, encoded on the vector itself, in its multiple cloning region. A PCR mix was set up as follows –

Component	Volume	Final concentration
Template DNA (100 ng/ul)	2 ul	200 ng
Sequencing primer (10 uM)	2 ul	20 pm
Big Dye*	8 ul	*
Distilled water	8 ul	-

*dNTP mixture along with fluorescent labeled ddNTPs; kindly provided by DNA sequencing facility, IMCB.

The PCR conditions were as follows -

Condition	Temperature	Duration
1. Initial denaturation	94°C	2 minutes
2. Denaturation	94°C	30 seconds
3. Annealing	50°C	15 seconds
4. Extension ^	60°C	4 minutes

^The reaction is repeated up to 25 times, from step 2 to 4.

DyeEx columns (Qiagen, Valencia, CA) were used to purify the PCR products, following manufacturer's instructions.

2.3.12 Sub-cloning of short oligonucleotides

Cloning of the 3X FOXO cis-element into pGL3 involved the insertion of a 60 base-pair oligonucleotide, which was achieved by annealing sticky-ended full length oligonucleotides, than by conventional 'PCR with primers followed by restriction enzyme digestion of the ends' technique. Single stranded full-length oligonucleotides were used with relevant restriction enzyme recognition sites at the 5' ends (in this case, NheI and XhoI). These were annealed by transient heating at 70°C, followed by a gradual cooling to room temperature. T4 polynucleotide kinase was then used to phosphorylate the 5' ends. The pGL3 vector, cut with the same two restriction enzymes, was ligated with the oligonucleotide and transformation performed as in 2.3.9.

The small size of the insert also necessitates the use of a special higher percentage agarose gel – in this case a 4% Metaphor gel was cast to visualize the insert bands after digesting the plasmid from the transformants with suitable restriction enzymes.

2.3.13 Semi- quantitative RT-PCR

Various downstream targets of forkheads as well as the mRNA levels of the transcription factors themselves were checked using RT-PCR, with primers as listed in 2.1.3. In every case, a parallel RT-PCR with β-actin primers was done to normalize for the quality and quantity of the template RNA in the reaction mixes. The one-step RT-PCR kit (Promega, Madison, WI) was used to set up RT-PCR reactions; up to 2 ug of total RNA (see 2.2.7), isolated from mammalian cells was used as a template.

The reaction was set up as below –

Component	Volume	Final concentration
Enzyme mix*	1 ul	3 units
10X buffer	5 ul	1X
dNTP (10mM)	2 ul	0.4 mM
Forward primer (10uM)	1 ul	20 pm
Reverse primer (10uM)	1 ul	20 pm
Total RNA (1 ug/ul)	2 ul	200 ng
Distilled water	38 ul	-

* DNA polymerase and reverse transcriptase mix

The RT-PCR conditions were as follows:

Condition	Temperature	Duration
1. Reverse transcription	50°C	30 minutes
2. Initial denaturation	94°C	5 minutes
3. Denaturation	94°C	30 seconds
4. Annealing *	55- 68°C	1 minute
5. Extension ^	72°C	1 minute per kb
6. Final Extension	72°C	10 minutes

*Approximately 5°C below the Tm of primers.
^The reaction is repeated upto 30 times, from step 3 to 5.

2.4 Protein methodology

2.4.1 Protein concentration determination by Bradford's assay

Bradford's solution (Bio-Rad, Hercules, CA) was used to quantify whole cell and nuclear protein extracts. 1 ml of the Bradford's solution was mixed with 1-2 ul of the protein extract and left to stand at room temperature for 10 minutes before absorbance at 595 nm was measured. A standard curve was plotted every time using known (serial dilutions from 0 to 2 ug per ml) quantities of BSA and the concentration of the protein samples, based on their corresponding absorbance values, was deduced from the same.

2.4.2 SDS-PAGE and Western blot analysis

SDS-PAGE was carried out as described in standard protocols (Molecular Cloning by Maniatis, *et al.*) using Mini- PROTEAN II electrophoresis system (Bio-Rad, Hercules, CA) in Tris- glycine buffer (25mM Tris, 250mM glycine, 01.% SDS). A 10% polyacrylamide gel was set up and 10 ug each of the protein samples were denatured in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 0.1% bromophenol blue, and 5% β- mercaptoethanol) and heated at 95°C for 5 minutes before loading. The resolved proteins were then transferred onto a Hybond C-plus nitrocellulose membrane (Amersham, UK) using the Bio-Rad wet transfer system in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). All subsequent immunoblotting was carried out in PBS with 0.1% Tween 20 and 5% skimmed milk (henceforth referred to as the 'blocking buffer'). The membrane was blocked for one hour at room temperature. This was followed by over-night incubation of the membrane in appropriate dilution of the primary antibody in the blocking buffer at 4°C. The membrane was washed extensively (3 times, each lasting 10 minutes) using PBS containing 0.1% Tween 20. The membrane was then incubated

in blocking buffer containing the appropriate horse radish peroxidase conjugated secondary anti-body for 2 hours at room temperature. The membrane was washed extensively as before. Antigen-antibody complexes were visualized using luminal chemilumniscent substrate (LumiGLO reagent, Cell Signalling, Beverly, MA).

2.4.3 Protein/DNA array analysis

SH-SY5Y cells were treated with NO donors for the times indicated, and nuclear proteins were prepared. The protein/DNA array was performed following manufacturer's instructions (Panomics, Redwood city, CA). Briefly, the nuclear extracts were incubated with biotin-labeled DNA probe mix in binding buffer for 30 min at 15°C. Protein/DNA complexes were resolved in a 2% agarose gel and excised. DNA probes were recovered from the protein/DNA complexes and hybridized to the transignal Protein/DNA array membrane. Hybridization signals were visualized using horseradish peroxidase-mediated chemiluminescence. The resulting autoradiograph was scanned by densitometry, and the mean values were represented as optical density (OD) along with the standard deviation (SD).

2.4.4 Protein- DNA interaction –electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides were generated by annealing the forward and reverse 3X IRS oligonucleotides (as in 2.1.3; encoding 3 successive copies of the FOXO cis-element) in annealing buffer by heating at 70°C followed by slow cooling. Up to 100 ng of these oligonucleotides were then end-labeled with [γ-P^{32}] ATP and T4 polynucleotide kinase, by incubation at 37°C for 45 minutes. Mutant 3X oligonucleotides with one of the 8 conserved base pairs mutated, was used as a negative control in parallel. Excess unused nucleotides were removed using Biospin 30 (Bio-Rad, Hercules, CA) columns. The specific activities of the probes were then checked using a scintillation counter. A master EMSA mix was made as follows-

Component	Volume	Final concentration
0.5 M Hepes (K+) pH 7.8	25	10 mM
0.5 M MgCl2	12	5 mM
25 mM DTT	20	2.5mM
Glycerol	100	10%
2.5% NP-40	10	0.05%
2.5 M KCl	20	50 mM

10 ul of the above mix was gently added to 10 ug of nuclear proteins (see 2.2.6) and 2 ug of dI-dC was added to the same, as the source of non-specific competitor DNA. An appropriate volume of the probe (up to 100, 000 counts per reaction) was added and the mix left to stand at room temperature for 20 minutes, for the DNA- protein complexes to form. In order to prove the specificity of the DNA-protein interaction, cold, wild type or mutant oligonucleotides were added to the mix, at increasing concentrations, to show the interaction being competed out in the former, while in the latter, the DNA-protein complex was unaffected.

The sample was subsequently mixed gently with 6X DNA loading dye and loaded onto a 5% native polyacrylamide gel. Electrophoresis was carried out in 0.25X TBE buffer at a constant current of 25 mA for 3 hours at 4°C. The gel was then peeled off the glass plates and dried and exposed to a BioMax MR (Kodak, Rochester, NY) X-ray film to detect the bands.

2.4.5   Immunoflourescent staining

Mammalian cells, grown in chamber slides, were fixed with 3.7% para-formaldehyde solution, for 20 minutes at room temperature. Cells were then washed with PBS and blocked with 1% normal goat serum, diluted in PBS containing 0.1% Triton X-100, for 2 hours at room temperature. The primary antibody (1 in 100 dilution) was added to fresh blocking solution and the cells incubated at 4°C over-

night. They were washed 3 times, for 5 minutes each, with PBS containing 0.1% Triton X-100, before incubation with appropriate fluorescent labeled secondary antibody (1:200-1:500), at room temperature for 2 hours. All incubations with antibodies were carried out in darkened humidified chambers. Cells were again washed with PBS containing 0.1% Triton X-100 before being mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA). TOPRO-3 (Molecular Probes, Carlsbad, CA) was diluted 1 in 5000 in PBS, and was used to stain the nuclei before mounting. Cells were observed and photographed using a Bio-Rad MRC 2000 confocal microscope (Zeiss, Germany) connected to a Photometrics Sensys CCD camera.

2.4.6 Localisation studies using GFP tagged proteins

The sub-cellular localization of GFP-tagged proteins in live cells, after appropriate treatments, was observed using an epifluorescence microscope (Zeiss, Germany) with an attached Nikon Coolpix CCD camera.

2.4.7 Luciferase reporter assay

The dual luciferase reporter assay kit (Promega, Madison, WI) was used to assay for luciferase activity in transfected cells, according to the manufacturer's instructions. Cells were transfected with the appropriate pGL3 vector (up to 1 ug per dish, on a 6-well plate) and the control Renilla construct (50 ng per dish). 24 hours post-transfection, appropriate reagents (NO donors or insulin or tet, as required) were added. At the end of the time courses, cells were washed three times with 2ml PBS. The media and the PBS were removed thoroughly as residual NO/ culture media is known to interfere with the luminescence readings. Up to 500 ul 1X passive lysis

buffer (Promega, Madison, WI) was added to the wells, and the cells were lysed at room temperature by shaking gently for 15- 20 minutes.

The lysate was diluted 10 times by mixing 10 ul the lysate with 90 ul 1X lysis buffer. To 95 ul of the first substrate, 10 ul of the above was added, mixed well and the luminescence (emitted by the action of the firefly luciferase, encoded by the pGL3 construct) noted immediately in a TD-20e luminometer. 95ul of the stop and glo substrate was then added, mixed well and the luminometer reading noted again - this substrate quenched the activity of the firefly luciferase and at the same time acted as a substrate for the Renilla luciferase, encoded by the control Renilla vector. Assuming the stop and glo reading as the equivalent of a 1000 units of luciferase activity, the relative luciferase activity of the different constructs of the pGL3 plasmid and the treatments were then deduced.

2.5 Cell viability and death assays

2.5.1 Crystal violet staining and quantitation

Cells were seeded in 6-well plates for approx. 70% confluency and treated as required. The media was aspirated and 0.5% crystal violet in 30% methanol was carefully overlaid on the cells and left at room temperature for 10 minutes, to fix and stain the cells. The excess stain was completely removed by washing with distilled water many times. The plates were then dried. The dye taken up by the cells was eluted in 50% methanol, 1% acetic acid solution. Absorbance was measured at 540 nm in an immunoabsorbent assay reader. Crystal violet binds proteins and since most dead SH-Sy5y cells float away, there is a good correlation between the total viable cell number and the absorbance at 540 nm after staining. Cell death was expressed as a percentage difference in dye-uptake between treated and non-treated cells.

2.5.2 Lactate dehydrogenase (LDH) release assay

Release of LDH from the intracellular compartment into the surrounding medium was measured as an indicator of cell death. Cells were seeded in 96-well plates and appropriate treatments were carried out. The supernatant of the treated and un-treated control cells were collected and the cells lysed in an equal volume of lysis buffer (Dulbecco's modified Eagle's medium with 0.1% Triton X-100), by gentle shaking at room temperature for 30 minutes.

The assay is based on a coupled enzymatic reaction involving the conversion of a tetrazolium salt (INT) into a formazan product. The reaction is catalyzed by LDH released from cells and diaphorase present in the assay substrate mixture.

Lactate + NAD(+) --> Pyruvate + NADH

NADH + INT --> NAD(+) + Formazan

Final formazan concentrations, which act as an indirect indicator of the LDH enzyme activity in the assay mixture, were determined by measuring optical absorbance at 492 nm in a 96 well format. The percentage LDH release is calculated by dividing the level of LDH in the medium by the total amount of LDH in the medium and the cell lysate together.

2.5.3 Sytox Hoescht staining

The membrane permeable DNA dye Hoechst-33342 and the membrane impermeable DNA dye Sytox was used to observe cell death morphologically and distinguish between apoptosis and necrosis. 50 ul of a 250 uM Sytox stock (in DMSO) and 10 ul of a 10mg/ ml Hoechst stock (both from Molecular Probes, Carlsbad, CA), were mixed in 190 ul of DMSO to make a 500X working solution (a final concentration of 100 nM- 200 ng/ml Sytox- Hoescht dye mixture was found to be optimal for SH-Sy5y cells). The dye mixture was added to the culture media of

treated cells and the plates examined under a fluorescence microscope after incubation

for 15 minutes at 37°C.

The nuclei of living cells are stained evenly by membrane permeable Hoechst.

The cells with bright, condensed chromatin and fragmented nuclei under Hoechst dye

staining were identified as apoptotic cells. The cells with normal-shaped nuclei and

stained by Sytox Green were counted as necrotic cells; in my experiments, most of

these were a result of secondary necrosis, caused by long treatments with NO donors.

Cells in five random fields under a magnitude of 20X were counted using an

epifluorescence microscope (Zeiss, Germany) with an attached Nikon Coolpix digital

camera, to gauge cell death patterns.

2.5.4   Caspase 3 cleavage assay

The enzymatic activity of caspases was measured by monitoring the cleavage

of exogenous fluorogenic peptide substrates, yielding 7-amino-4-trifluoromethyl

coumarin (AFC) as the fluorescent signal. Cells were seeded in 24- well plates and

treated for appropriate times with NO donors. The supernatant was gently removed

and discarded while the cells were lysed with lysis buffer (1% Triton X-100,115 mM

NaCl, 1 mM KH_2PO4, 4 mM KCl, 1 mM dithiothreitol, 25 mM Hepes, pH 7.4, 1 mM

benzamidine, 1 mM PMSF, 10 ug/ml phenanthroline, 10 ug/ml aprotinin, 10 ug/ml

leupeptin and 10 ug/ml pepstatin) on ice for 10 minutes. The lysates (20 ul from a

total of 250 ul) were diluted to a final volume of 100 ul with substrate buffer (60 mM

fluorogenic substrate DEVD-afc in 50mM HEPES pH 7.4, 1% sucrose, 0.1% CHAPS,

10mM DTT) for detection of caspase-3-like activity. The generation of free AFC at

37°C was kinetically determined over a period of 30 minutes by fluorescence

measurement (excitation: 385 nm; emission: 505 nm) using the Spectrafluor Plus plate

reader (Tecan, Crailsheim, Germany). The specific activities (pmol afc/min*mg protein) were calculated using the protein concentrations of lysates as determined by the BCA protein assay (Pierce, IL).

CHAPTER 3 - RESULTS

3.1 NO mediated regulation of FOXOs

3.1.1 Cis-element array shows NO increases binding of FKHR consensus cis-elements

In an attempt to obtain an overview of all the different transcriptional changes effected by NO in cells, we chose to treat SH-Sy5y cells with cytotoxic concentrations of SNP for 2 to 12 hours, and isolated the nuclear extract. A TranSignal protein/DNA array (Panomics, Redwood city, CA) spotted with 150 different consensus transcription factor-binding sequences, with each recognition sequence corresponding to a specific transcription factor, was used. Biotin-labeled oligonucleotides (TranSignal Probe Mix) were pre-incubated with the nuclear extracts to allow the formation of protein/DNA (or TF/DNA) complexes. The protein/DNA complexes were then separated from the free probes, and the probes in the complexes were then subsequently extracted and hybridized to the TranSignal Array. Signals were detected using chemiluminescence imaging.

The array picked up changes in the levels of a few of the transcription factors binding to their respective cis-elements upon NO treatment - changes that were subsequently confirmed by conventional EMSA (Dhakshinamoorthy *et al*., unpublished).

Various known and novel changes in DNA-binding affinity were observed, including AP1, AP2, Brn3, Cre, EGR, PPAR, E2F and FKHR. Beginning from 4 hours after treatment with SNP, there was a significant increase in the binding to both the mouse and human FKHR consensus elements as shown in Fig. 4. I decided to focus on the significance of this perceived change in FKHR-binding activity from the array.
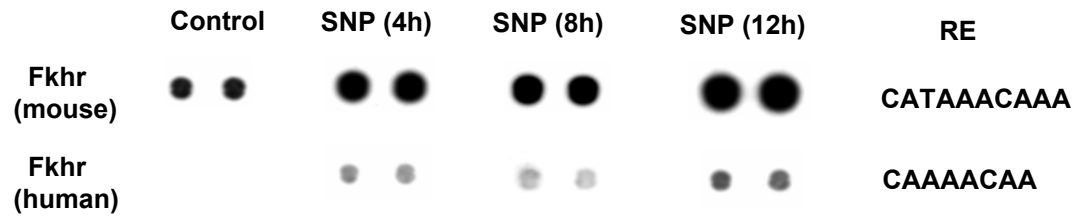
Figure. 4 FOXO cis-elements on TranSignal protein-DNA array. Increased binding affinity of NO-treated SH-Sy5y nuclear proteins to mouse and human consensus FKHR recognition elements as seen on the TranSignal array.

3.1.2 Increased binding of FKHR consensus cis-elements confirmed by EMSA

The human IRS promoter which responds to FOXO transcriptional control via the conserved FKHR elements in its promoter, was chosen as the basis for designing oligonucleotides to perform an EMSA (Guo *et al*., 1999). A 35 base-pair sequence containing the core CAACAAAA FKHR recognition element (Fkhr RE) was end-labeled with [γ-P^{32}] ATP. 100,000 cpm of the labeled FKHR RE was then incubated with 10 ug of NO-treated SH-Sy5y nuclear extracts and analyzed in a 5% native polyacrylamide gel. The shifted FKHR-nuclear protein complex is *arrowed* in Fig. 5A. In a similar experiment, in order to prove the specificity of the interaction between the nuclear proteins to the FKHR RE, competition assays were set up using cold FKHR oligonucleotides or oligonucleotides with the key recognition site mutated (Fig. 5B). The former increasingly competed out the appearance of the labeled oligonucleotide-protein complex with increasing concentrations, whereas the mutant oligonucleotide showed no such competing effect; the same is re-emphasised in the graph in Fig. 5C, which shows a quantitation of the shifts observed – nuclear extracts of cells treated with Deta-NO for 4 hours were used in the above experiment. Panel D on Fig. 5 shows the sequence of the FKHR-specific oligonucleotide used. The EMSA clearly confirmed the increase in FOXO DNA binding seen in the array after NO treatment of SH-Sy5y cells – the inherent differences in the nature of the two NO donors used, however, do show up as minor variations in the pattern of the band-shift observed.
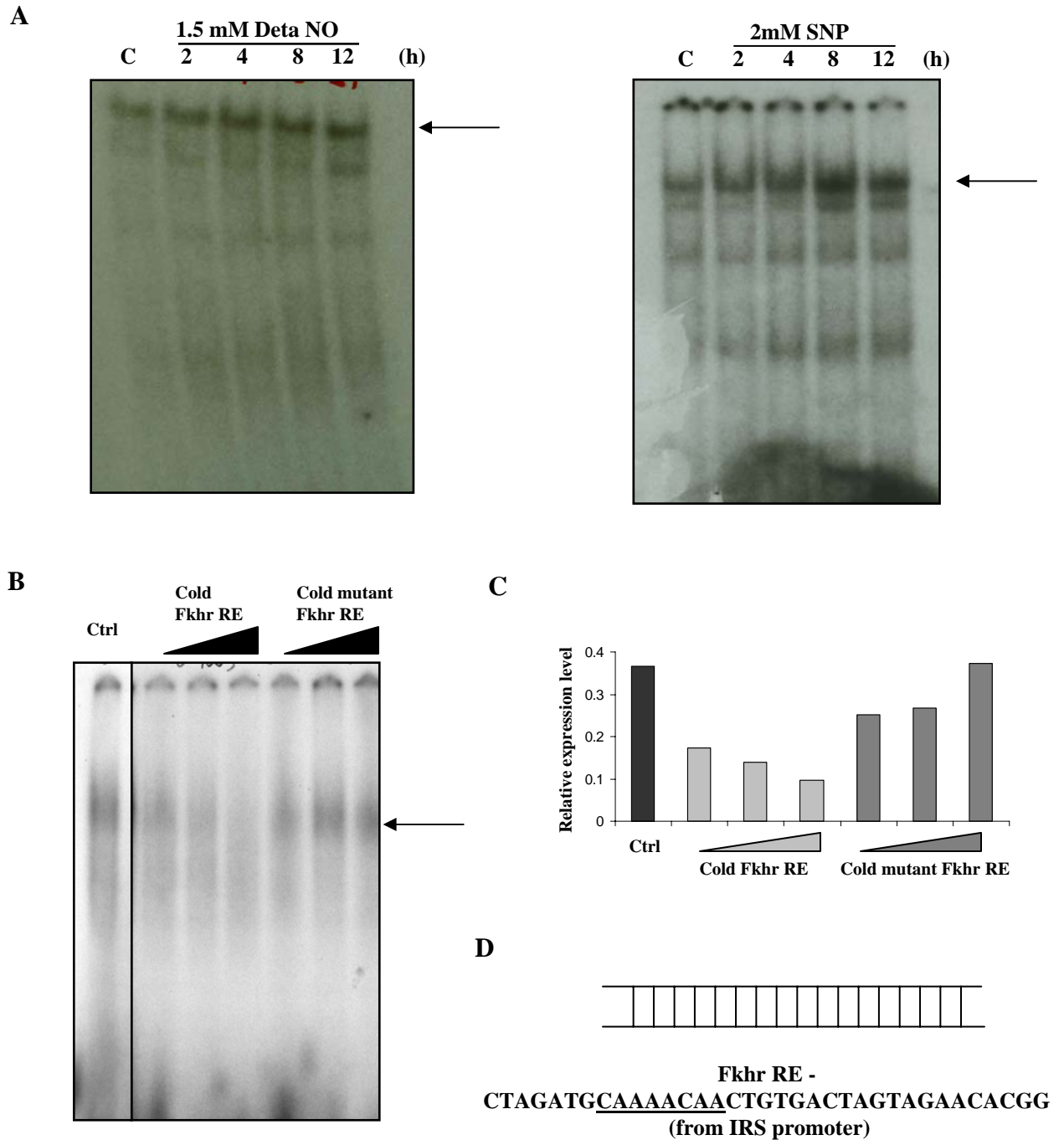
A

B

C

D

Fkhr RE -
CTAGATGC<u>CAAAACAA</u>CTGTGACTAGTAGAACACGG
(from IRS promoter)

Figure 5. EMSA with Fkhr RE (A) NO enhances the binding of nuclear proteins to the Fkhr cis-element. EMSA with labelled FKHR RE - panel on the left shows interaction with nuclear proteins after 1.5 mM Deta-NO treatment of SH-Sy5y cells while the panel on the right shows the same, but with 2 mM SNP-treated cells. (B) Competition assay. Unlabelled Fkhr and mutant Fkhr cis-elements were incubated with the labeled oligonucleotides in increasing concentration, with 4 hour Deta-NO treated nuclear extracts, to prove the specificity of the observed shift. (C) Quantitation of the above proving the specificity of the observed shift. (D) The Fkhr recognition element sequence used in the above gel-shift assays.

3.1.3   NO-mediated down-regulation of FOXO transcriptional activity

To investigate whether the increase in DNA binding also affected the transcriptional activity of the FOXO proteins, I decided to look at the functionality of the FKHR recognition elements in mediating transcription after NO treatment. The pGL3 luciferase reporter system was used for this purpose.

The over-expression of AFX in an increasing concentration, resulted in a significant up-regulation of the pGL3-3X IRS-dependent luciferase gene expression (pGL3-3X IRS contains 3 successive copies of the FKHR RE from the FOXO responsive IRS gene promoter) (Fig 6A).   Treatment with 2mM SNP, however, suppressed the basal activity by up to 30%, beginning around 8 hours post-NO treatment (Fig. 6B). Parallel experiments with another NO donor, Deta-NO, also show a 30% reduction from basal activity, starting 8 hours post treatment (Fig. 6C). But the difference in the mechanism and concentration of NO (and other by-products, especially peroxynitrite, a by-product of the breakdown of SNP) released by the 2 donors, brings up subtle differences in the early transcriptional activation kinetics. It is worth mentioning here that, with the similarity in the transcriptional down-regulation and DNA binding profile, seen with both Deta-NO and SNP, at 8 and 12 hours post- treatment, and the induction of S197 phosphorylation around 8 hours (see Fig. 7B), will mean a similar effect of the 2 donors, on FOXO regulation and activity-hence, at least in the responses starting 8 hours from NO treatment. Hence, both the donors were treated without any prejudice in the study. Attempts were also made to duplicate most experiments with both donors and through out the study, no major differences were observed in any of the NO mediated effects on FOXO. The insulin – PI3 Kinase – PKB pathway, where insulin is known to cause phosphorylation of FOXO proteins and prime their export into the cytosol, was used as a positive control.

Treatment of serum-starved NIH-3T3 cells with 1ug/ml insulin/ serum caused a fall in luciferase activity as previously reported (Kops *et al*., 1999) (Fig. 6D).

In order to rule out any generic fall in luciferase activity/ FOXO transactivation with time, the above experiments were performed in parallel in medium without NO donors. Luciferase assays at appropriate time points did not show any change in the levels of enzyme activity without NO (data not shown).

3.1.4 AFX mRNA and protein status in NO-treated cells

In order to ascertain if the NO-induced changes in the DNA-binding and transcriptional activation were due to expression level changes of the FOXO transcription factors, I checked the mRNA and protein levels of the FOXO proteins by RT-PCR and western blotting, respectively. SH-Sy5y cells were treated for 2, 4, 8 and 12 hours with 2 mM SNP and the cells were harvested for subsequent total RNA and protein extraction. RT-PCR analysis was carried out with suitable primers. Representative results for AFX, along with β-actin controls, are shown in Fig. 7A. Nuclear extracts were made and the expression levels of the proteins were determined using suitable antibodies by western blot analysis (left panels on Fig.7B, C). No changes were observed in both mRNA and protein expression levels after NO treatment.
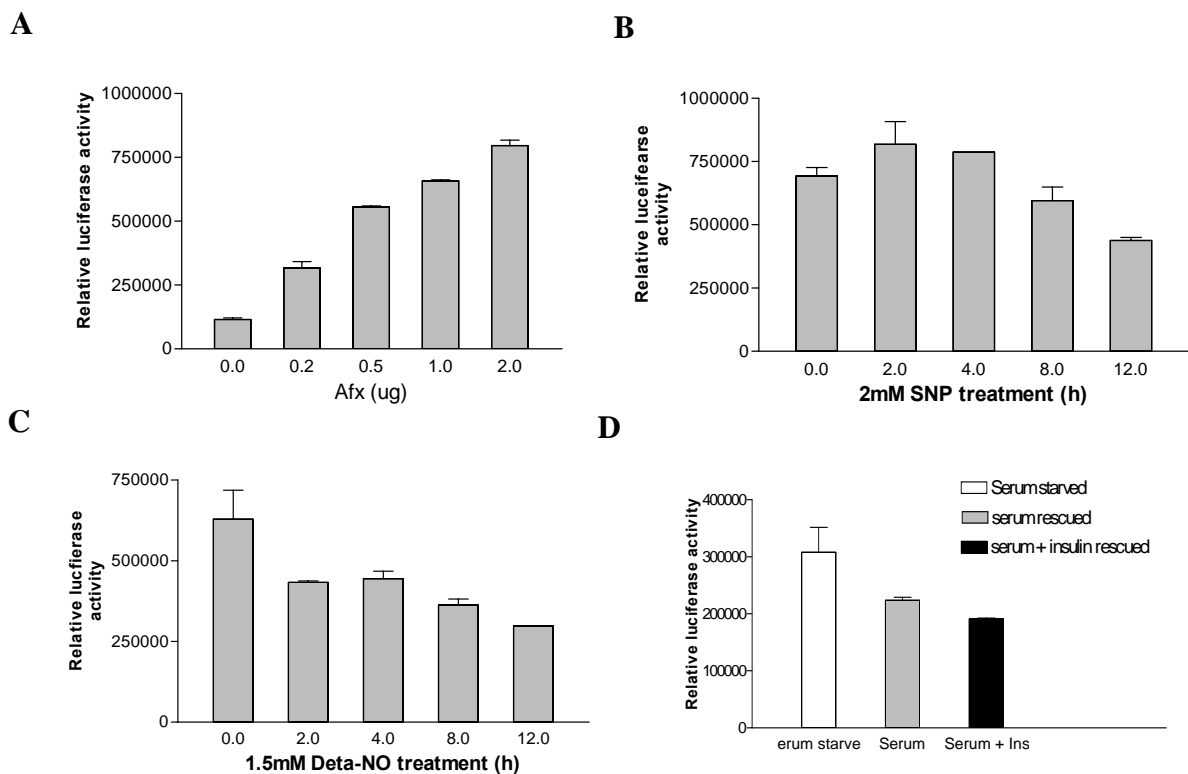
Figure. 6 Induction of 3X IRS-mediated luciferase activity by co-expression of AFX and its down-regulation by insulin and NO treatment. (A) SH-Sy5y cells were transfected with 1ug of the pGL3-3XIRS plasmid and the AFX expression plasmid (in increasing concentrations) as shown; and the cells analyzed for luciferase activity. (B) SH-Sy5y cells were transfected with 1 ug of pGL3-3XIRS and 24 hours post transfection, 2 mM SNP containing media was added to the cells for 2, 4, 8 and 12 hours before harvesting and analysis for luciferase activity. (C) As in B, but 1.5 mM Deta-NO was used as NO donor. (D) NIH-3T3 cells were transfected with 1 ug of the pGL3-3XIRS plasmid. 24 hours post transfection, the cells were grown in serum-starved conditions before induction with serum and serum containing 1 ug/ml insulin for 30 minutes, before harvesting and analysis for luciferase activity. 0.1 ug of plasmid pRL-Tk, encoding Renilla luciferase, was used as a control for normalizing the efficiency of transfection in every case. The results obtained are representative of multiple independent experiments.

3.1.5 NO induces AFX phosphorylation at Ser 197

Since phosphorylation is a major means of FOXO transcriptional activity regulation, I followed up the protein analysis with antibodies available against specific phospho-FOXO proteins. Various commercial antibodies against the known serine and threonine phosphorylation sites on the FOXOs (see Fig. 1) were used. The AFX phospho-Ser197 antibody was found to detect increasing amounts of phosphorylated proteins in the nuclear extracts of SH-Sy5y cells exposed to NO donors for 8 hours or more (right panel on Fig. 7B). Parallel positive controls were set up by exploiting the known phosphorylation of FOXO proteins in NIH-3T3 cells upon insulin treatment. Cells were serum starved for 24 hours and then recovered in medium containing serum and 1ug/ml insulin. Insulin is known to cause phosphorylation of AFX at Ser197, among other sites, and the western blot clearly showed the known insulin-mediated phosphorylation of AFX at Ser197 (right panel on Fig. 7B).

As seen in Fig. 1, Ser 256 is the equivalent analogous serine, comparable to the serine at 197 in AFX, implicated in the insulin- PI3Kinase- PKB pathway of phosphorylation of FOXOs. Interestingly, NO-mediated phosphorylation of FKHR was observed with a phospho-Ser 256 antibody after 8 hours of treatment with NO donors (right panel on Fig. 7C). The high degree of homology between these two proteins along with the published literature evidence that usually club them together on most occasions in growth factor and stress mediated pathways, further strengthens the possibility of NO modulating FOXO functions and thereby probably affecting the survival status of the cell.
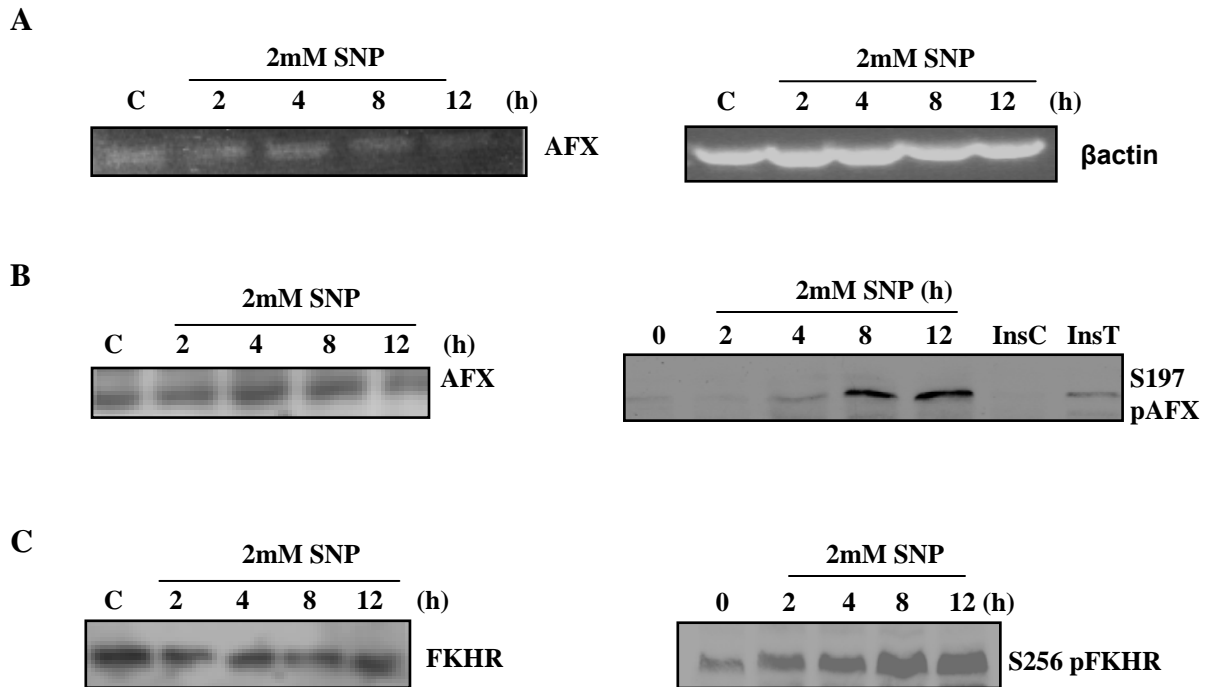
Figure 7. AFX mRNA and protein levels in cells after NO treatment. (A) SH-Sy5y cells were treated with 2 mM SNP for various times and the RNA isolated was used for RT-PCR analysis of the AFX mRNA. β-actin is shown as a control.on the right panel. (B) 2 mM SNP-treated SH-Sy5y nuclear extracts (10 ug each) were analysed for AFX protein expression by Western blotting. Panel on the right shows the same nuclear extracts, but probed with antibody against AFX phospho-Ser197. Serum starved cells (denoted as InsC on the panel) and serum-starved cells recovered with serum and 1 ug/ ml insulin for 30 minutes (denoted as InsT on the panel) served as a control for the observed phosphorylation. (C) Panel on the left shows 2 mM SNP-treated SH-Sy5y nuclear extracts probed with antibody against FKHR; panel on the right shows the same, but probed with antibody against FKHR phospho-Ser256.

3.1.6   NO-mediated phosphorylation at Ser197 does not cause nuclear export of AFX

Insulin-dependent PI-3 Kinase- and PKB-mediated phosphorylation of Ser-197 is known to cause the translocation of AFX from the nucleus into the cytosol. I wanted to check if NO-mediated phosphorylation at this site also prompted the nuclear export of the protein. Fluorescent antibodies were used to stain NO-treated and untreated SH-Sy5y cells to track any translocation of the protein. Immunofluorescence revealed that under normal conditions, AFX (red) was localized in the nucleus (Fig. 8A). NO did not cause any translocation of the protein from the nucleus (nucleus stained blue with TO-PRO 3) under conditions in which Ser-197 of AFX is phosphorylated. Insulin treatment for 30 minutes, on the other hand, caused sequestration of the protein in the cytosol (Fig. 8B).

Live tracking of the protein was also attempted such that any transient translocation of the protein outside the discrete time points used during immuno fluorescense is not missed. For this purpose, a GFP-tagged AFX construct was made, and transiently transfected SH-Sy5y cells were constantly monitored for the localization of the protein after NO treatment; but AFX appeared to remain sequestered in the nucleus (data not shown).

A

Untreated

AFX

TO-PRO3

2mM SNP (8h)

B

Untreated   1ug/ml insulin (30')

AFX
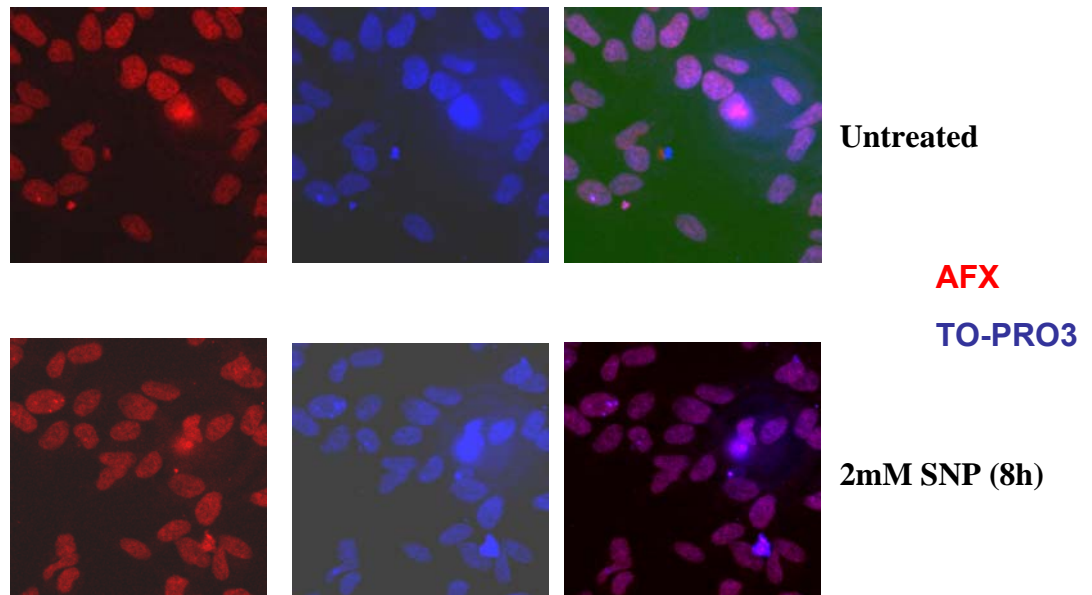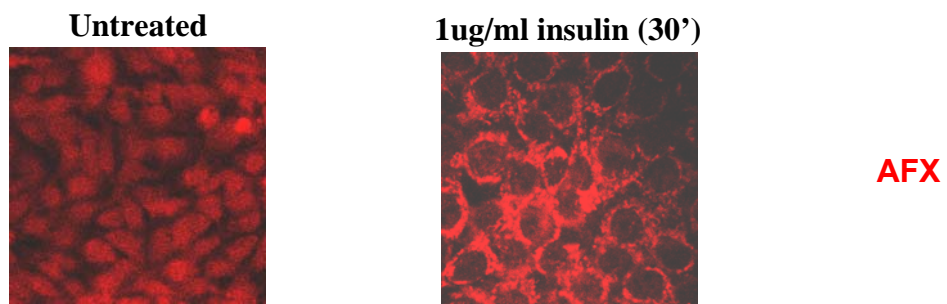
Figure 8. Immunofluourescense to check localisation status. (A) SH-Sy5y cells were grown in chamber slides and treated for 8 hours with 2 mM SNP, as shown. After treatment, the cells were fixed, washed and stained with AFX antibody, appropriate flourescent secondary antibody and TO-PRO 3, as in 2.4.4. AFX staining is shown in red, TOPRO3 staining is shown in blue. The purple merge panel shows the localization of AFX in the nucleus. (B) NIH-3T3 cells show AFX translocation after 30 minutes of treatment of serum starved cells with 1 ug/ml insulin.

3.1.7   S197A mutants

To further understand the significance of the NO-induced phosphorylation at serine 197 of AFX, the serine was modified to an alanine, such that the site can no longer be phosphorylated. The mutant pcDNA3 construct is denoted as AFX S197A.

Luciferase assays were set up in order to investigate the effect of the mutation on the transcriptional activity of AFX. Transient transfection of pGL3-3XIRS and the control Renilla vector were done. The pcDNA3 constructs with the full length native AFX or the mutant AFX were co-transfected into the cells. Treatment with NO donors 24 hours post transfection was followed by analysis of luciferase activity. As seen in Fig. 9A, the 30% fall in luciferase activity seen with AFX co-expression was not observed in the case of the AFX S197A co-expression, post-NO treatment. This implicates the phosphorylation at S197 as a probable mediator of the decrease in FOXO transcriptional activity observed upon NO treatment. But the fall in AFX transactivation upon introduction of the S197A mutant in untreated cells raises the question of the effect of the expression of the dominant-negative construct *per se* in mediating the transcriptional effect of AFX. It is to be noted that the FKHR recognition sequence used in these experiments is generic to the FOXO family and some interference of the other FOXO proteins could be expected. The significance of the phosphorylation at S197 is still inconclusive – further experiments with whole promoters of appropriate down stream targets and/ or promoter fragments that respond to AFX solely will help complete the picture.
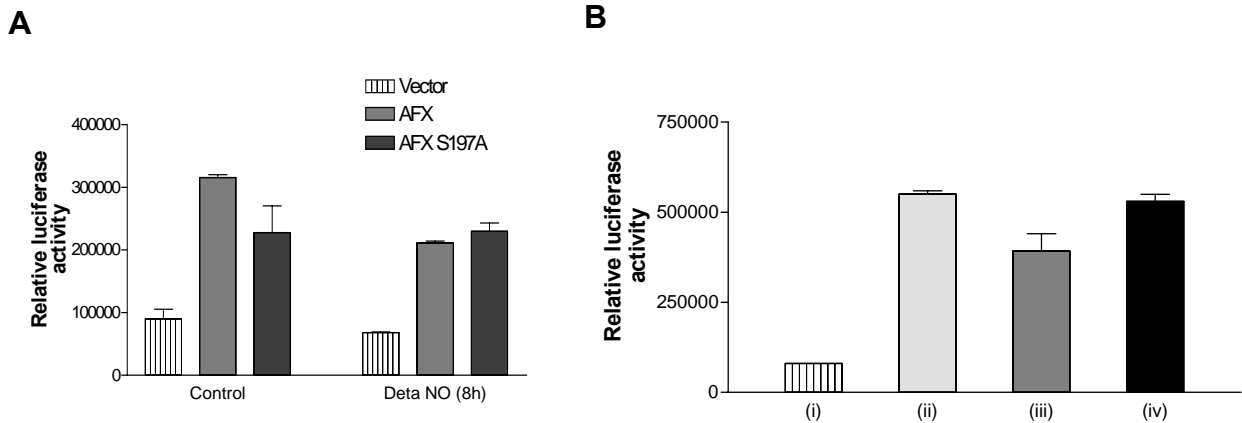
Figure 9. Luciferase assays with S197A AFX and tet-inducible AFX. (A) pGL3-3X IRS was co-transfected into SH-Sy5y cells with 0.5 ug of pcDNA3 expression vectors with native AFX or S197A AFX inserts. 24 hours post transfection, the cells were treated with 1.5 mM Deta-NO for 8 hours. Luciferase assay results are as shown. Cells not treated with NO show similar luciferase values 24 and 32 hours post transfection. (B) Stable tet-inducible AFX cells were transfected with pGL3-3X IRS. Bar (i) shows luciferase activity of the cells 40 hours post transfection. Bar (ii) shows luciferase activity after 12 hours of 1 ug/ml tet induction, 24 hours post transfection. Bar (iii) represents the fall in activity when the tet-induced cells are further treated with NO for 8 hours. Bar (iv) represents the luciferase activity of the same condition, but without NO donors in the medium – it shows enzyme activity levels similar to that seen after only tet induction (bar ii). pRL-Tk, encoding Renilla luciferase, was used as a control for normalizing the efficiency of transfection in every case. The results obtained are representative of multiple independent experiments.

3.1.8 AFX over-expression studies

In order to further understand the role of AFX-mediated transcriptional changes in the SH-Sy5y cell response to cytotoxic concentrations of NO, AFX over-expressing cell lines were developed. Since FOXO proteins are known to be death mediators, the tet-inducible pcDNA4 system was chosen as the preferred vector construct for over-expression of the protein.

AFX cDNA was cloned into the pcDNA4 vector and the construct transfected into tet repressor-expressing Tet-12 cells, in order to generate SH-Sy5y cells producing tet-inducible AFX. Various parameters (concentration, time of induction, etc.) of tet induction were tested. An 8 hour induction protocol with 1 ug/ml tet was found to be suitable to induce AFX in SH-Sy5y cells (Fig. 10A).

The 30% fall in FOXO transcriptional activity upon NO treatment was reconfirmed in stable cells expressing tet-inducible AFX. The p4-AFX cells were seeded in 6 well plates and the pGL3-3X IRS constructs along with the control Renilla construct were transiently transfected. Tet induction increased the luciferase activity by at least 6-fold. 8 hours of treatment with NO donors reduced this activity by 30%. Parallel treatment with medium without NO donors was without effect, confirming the NO-specific effect on the transcriptional capacity of FOXO consensus elements (Fig. 9B). However, the NO mediated decrease in the transcriptional activity of AFX should be seen in perspective, along with the massive apoptosis triggered by AFX over-expression in SH-Sy5y cells (see below; Fig. 10 B and 10C) – a decreasing pattern in endogenous AFX transcriptional activity is seen to begin from 8 hours post-NO treatment (Fig. 6B and 6C) and we decided not to look beyond the early 8 hour response of the transcriptional activity of Tet induced cells, for the above reason (Fig. 9A).

AFX is known to be an important modulator of cell death/ survival signals in cells. The fact that NO, another molecule that can determine cell viability, modulates FOXO function, immediately suggests that NO-modulated AFX might regulate apoptosis in mammalian cells. The p4-AFX cell lines were used to examine the effect of over-expression of AFX on cell death in SH-Sy5y cells. As seen in Fig. 10B, mere tet induction was enough to induce apoptosis. Further addition of NO donors did not further increase cell death. The above result was confirmed by using both NO donors and measuring cell death using various methods, including Sytox-Hoechst staining and LDH release assays (Fig. 10B, C).

The cell death sensitization might be modulated towards either pro- or anti-apoptosis by the S197 phosphorylation. Parallel experiments with S197A and S197D stable cell lines will help further understand the role of the S197 phosphorylation in modifying cell death after NO treatment in neuroblastomas. These will also need to be correlated with the identification of appropriate direct or indirect transcriptional targets of AFX that transmit the signals further downstream. The transcriptional profiles of these protein(s) and promoter analyses using EMSA and functional reporter assays, should match the NO-mediated changes we observed with AFX and FOXO elements.
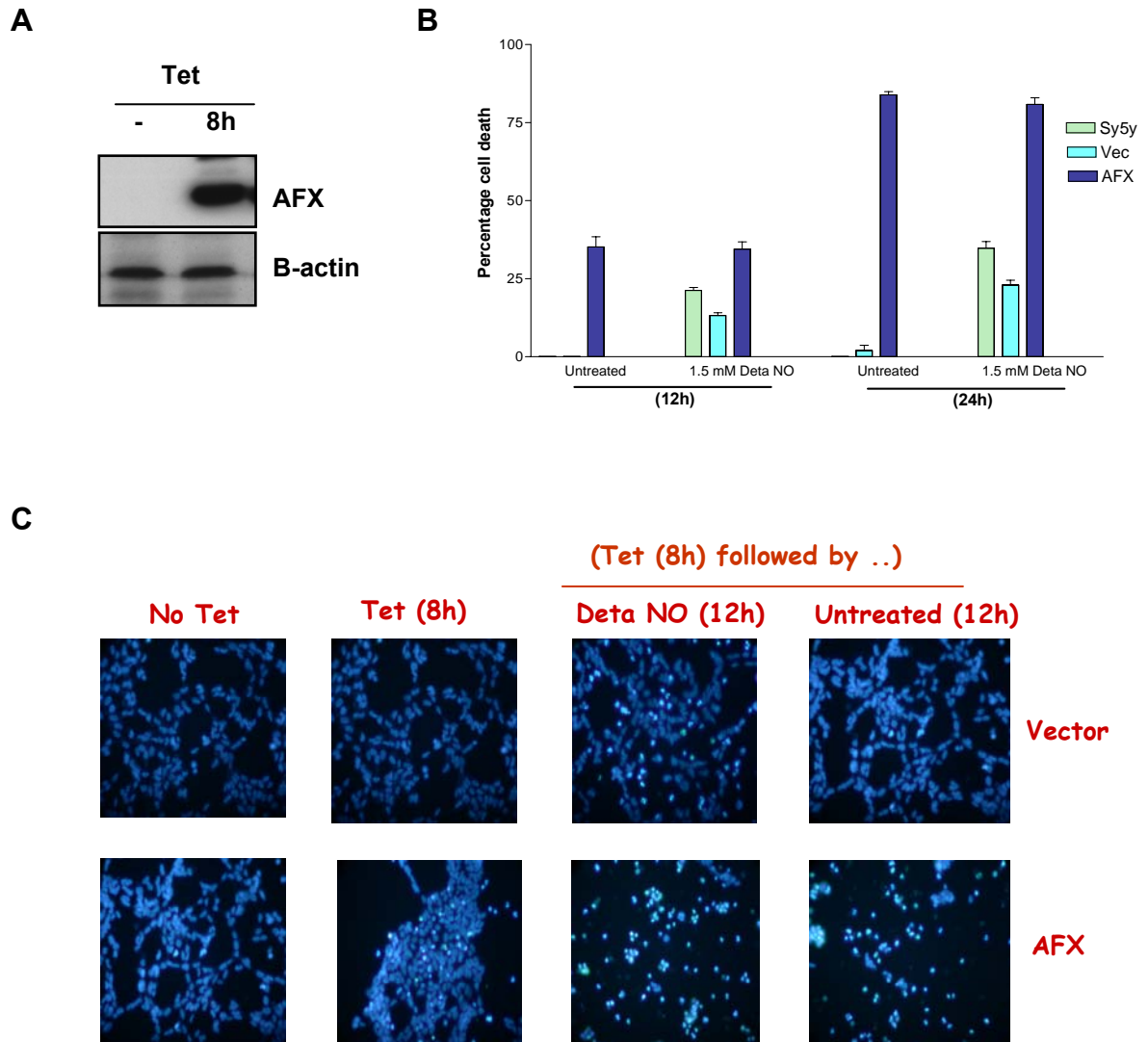
A

Tet

-	8h

AFX

B-actin

B

C

(Tet (8h) followed by ..)

No Tet	Tet (8h)	Deta NO (12h)	Untreated (12h)

Vector

AFX

Figure 10. Tet-induced over-expression of AFX in SH-Sy5y cells sensitizes cells to apoptosis. (A) Western blot analysis of SH-Sy5y cells that stably over-express AFX after 8 hours of induction with 1 ug/ml tet; bottom panel shows corresponding Western blot for the β-actin control. (B) LDH release. SH-Sy5y, vector control and AFX stable cells were induced with 1 ug/ml tet for 8 hours; cells were then either recovered in normal media ("untreated") or treated with 1.5 mM Deta-NO, for 12 and 24 hours. The cell supernatants and the lysates were then subjected to the LDH assay and the percentage cell death calculated. (C) Sytox- Hoechst staining. Cells were treated as in (B) and stained with Sytox-Hoechst to observe any differences in cell death in the AFX over-expressing stable cells.

3.1.9 NO regulation of known FOXO target genes

FOXO is known to play a part in the transcriptional regulation of a number of genes. Most of these are involved in the regulation of cell death, cell cycle control and cyto-protection. NO-mediated changes in the mRNA expression profiles of genes known to be downstream of forkheads should predict which NO-mediated changes in protein levels might occur as a result. We can subsequently correlate these changes with any change in sensitivity to cell death after NO treatment, and after AFX over-expression. With this purpose in mind, semi- quantitative RT-PCRs were set up.

Gadd45α, p130, Bcl-6, cyclin D1, catalase, MnSOD and BimEL were chosen, being known FOXO targets potentially involved in apoptosis initiation or cytoprotection. SH-Sy5y cells were treated with NO donors and total RNA was extracted from cells. Semi-quantitative RT-PCR was set up for each of the different genes, and expression patterns after 2, 4, 8 and 12 hours of NO treatment were noted (Fig. 11).

An increase in Gadd45α mRNA after NO treatment was immediately obvious. p130, Bcl-6, cyclin D1, catalase showed a gradual decrease in mRNA transcript levels, post-NO treatment. p130 is particularly interesting as its mRNA abruptly decreases after 8 hours of NO treatment. This incidentally coincided with the time frame of the increased DNA-binding and the decreased luciferase activity of the FOXO cis-element and the AFX phosphorylation observed in my study (Fig 5A, 6B and 7B, respectively).

Further involvement of these genes in mediating the pro-death effects of AFX after NO treatment will require an analysis of their promoter regions. EMSA showing AFX and phospho-AFX binding to the promoters, and NO modulating this binding, along with reporter assays that show the functional significance of the NO effect on the promoter through AFX for p130 or other targets, will help in better understanding

this novel transcriptional effect of NO. The tet-inducible AFX system will also help
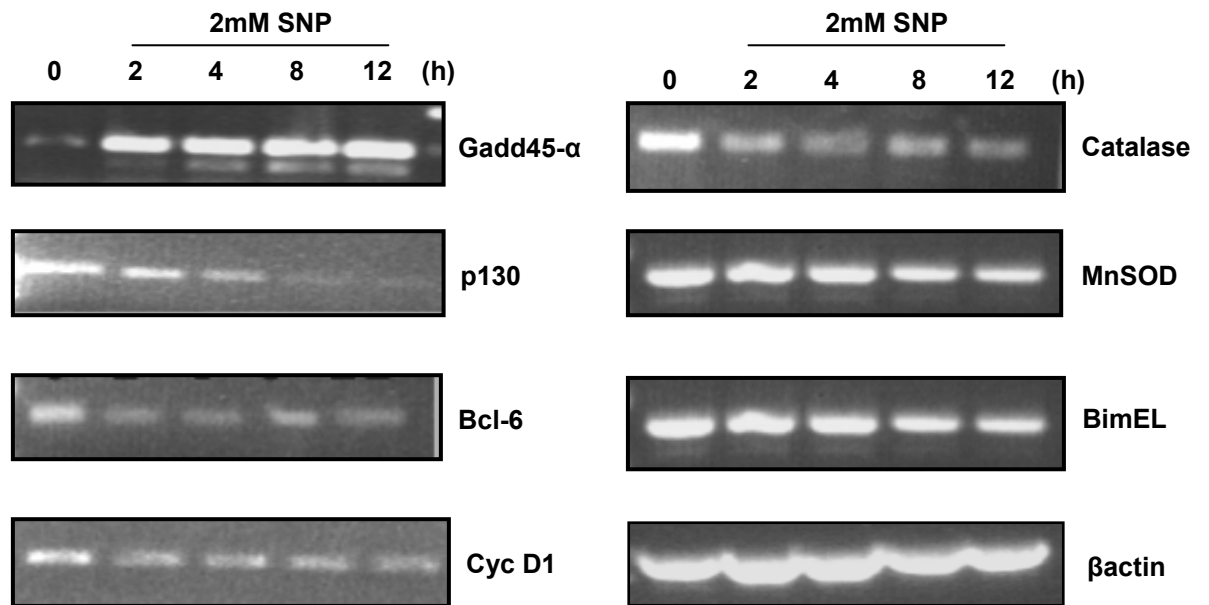
offer interesting insights into the above experiments.

Figure 11. RT-PCR analysis of genes known to be downstream of FOXO factors. SH-Sy5y cells were treated with 2 mM SNP; cells were subsequently lysed. and the total RNA extracted was used for semi-quantitative RT-PCR analysis of Gadd45-α, p130, Bcl-6, cyclin D1, catalase, MnSOD and Bim El. β-actin is shown as a control.

3.1.10 Discussion

Various transcription factors have been identified to be modulated by NO in various cell types including neurons. Most factors directly respond to the change in the redox status of the cell, or are oxidized or nitrosylated by NO or RNIs, while a few discrete signaling pathways are also known to be activated upstream of transcription factors, resulting in kinase cascades and subsequent effects on transcription factors and gene targets further downstream (see section 1.3).

The Panomics protein/ DNA array showed a collection of cis-elements that respond to NO treatment of SH-Sy5y cells. These implicated some known transcriptional factors such as AP-1, AP-2, CREB, Sp-1, EGR, STAT and ARE along with some novel factors or elements including Brn3, PPAR, CRE, E2F and FKHR. Various other factors including SRE, CEBP, ERE, Ets, ISRE, IRF, NF-1, HNF, GRE and GATA were found to be unaffected (Dhakshinamoorthy *et al.*, unpublished).

In particular, FOXOs, known to play important roles in initiating apoptosis, regulating cell cycle progression and the initiation of quiescence in various cell types, have been identified as novel targets of NO in neuroblastoma cells in my study.

NO-mediated increase in DNA-binding was accompanied by a decrease in its transcriptional activation capacity. This indicates a scenario where the particular FOXO transcription factor acts as a repressor. FOXO proteins do have a precedence for acting as repressors of gene transcription – downregulation of cyclin D1 and survivin promoters has been observed (O'Connor *et al.*, 2000; Tran *et al.*, 2003). In this case, limited NO-mediated down-regulation of cyclin D1 mRNA levels is observed – the phenomenon subscribes to the pattern of increased DNA-binding and the decreased functional activity of the promoter element, as observed in earlier

studies; but further analysis of the NO and AFX effects on the cyclin D1 promoter needs to be done before any conclusive results can be drawn.

Further examination of FOXO expression levels showed there was no NO-mediated change in the mRNA or protein levels of the proteins. However, 8 hours of NO treatment produced a sharp increase in Ser197 phosphorylation of AFX, which coincided with increased cis-element binding and a decrease in reporter activity. AFX phosphorylation at Ser197 has been shown to be the primer for the subsequent phosphorylation of T32 and S262 in the insulin-PI3Kinase-PKB pathway; though phosphorylation at this site has been proved to be vital for Akt-mediated nuclear export of AFX, with or without the phosphorylation at the other two sites, in some instances (Zhang *et al*., 2002).

Interestingly, NO does not appear to cause any change in the localization of this protein in my study. The absence of commercial phospho-specific antibodies for the other sites precluded analysis of their phosphorylation status. But the continued retention of phospho-AFX only further supports my observations of the increased DNA-binding affinity and the decreased reporter activity of the FOXO elements. In any event, nuclear exclusion will eliminate any possibility of AFX-mediated effects on promoter elements. It is interesting to note that the recently identified FOXO homologue, FOXO6, lacks the 14-3-3 binding motif, and is completely nuclear; but its transcriptional activity is still effectively regulated by growth factors to the same extent as the other FOXOs (Van Der Heide *et al*., 2004). FOXO translocation, hence, cannot be the only means to regulate FOXO transcriptional activity in cells.
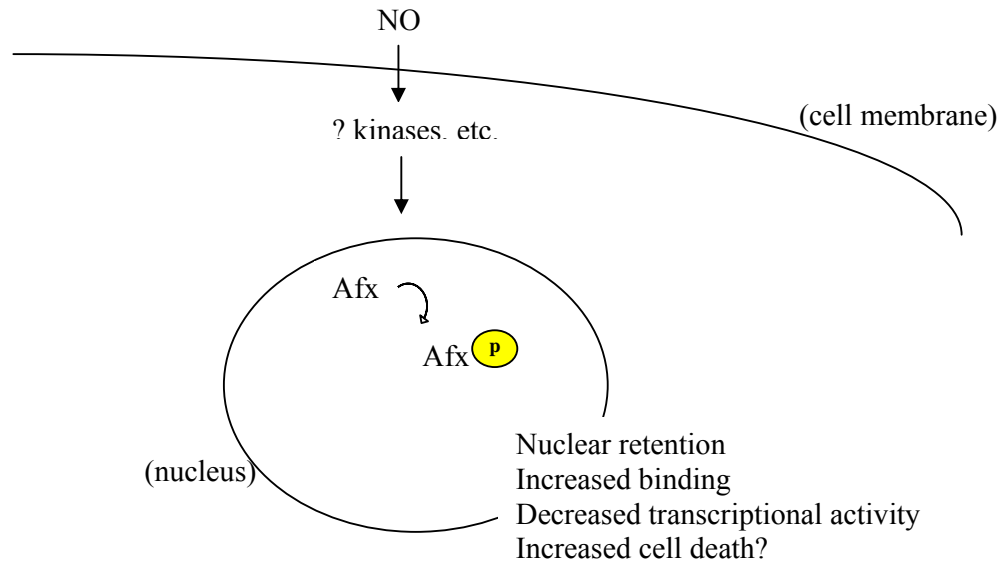
Figure. 17. Schematic representation of NO regulation of AFX.

The S197 site is also part of the DNA-binding domain of the AFX protein. Earlier reports have shown that phosphorylation at this site can affect the affinity of the protein to bind negatively-charged DNA. The decreased functionality of the FOXO cis-element, seen with luciferase assays (Fig. 6B and 6C), appears to suggest that NO could indeed negatively regulate FOXO activity by this phosphorylation event, though the increase in DNA-binding I observed (Fig. 5A) appears to be contradictory. The identification of particular downstream targets that respond to NO-mediated AFX transcriptional regulation and further analysis of its promoter for AFX binding motifs, along with DNA binding studies and reporter assays of the whole promoter (native and with the FOXO site mutated), are crucial before we can conclusively understand the implication of the S197 phosphorylation.

Since all the transcriptional changes observed were the responses to cytotoxic concentrations of NO donors, and given that members of the FOXO family are

involved in regulating the cell cycle and apoptosis, AFX over-expressing stable cell lines were developed to further examine the role played by AFX in the neuroblastoma response to NO. Tet-inducible over-expression of the protein greatly increased the susceptibility of SH-Sy5y cells to apoptosis, in the absence of NO. In fact, the susceptibility was so massively enhanced that no additional sensitivity to NO could possibly be obtained when compared to SH-Sy5y cells with endogenous AFX (see Fig. 10B and 10C).

NO appears to affect the transcriptional profile of a subset of genes that can influence the survival of the cell – the increase in the mRNA levels of Gadd45-α and the decrease in the level of catalase, cyclin D1 and MnSOD may tilt the cells towards cell death (*via* growth arrest, loss of free radicle scavenging potential, etc.) but there could be a counterbalancing force *via* the decrease in Bcl-6, p130 and BimEL mRNA transcripts. The sum effect of these (and other) gene expression profiles will ultimately decide the fate of the cell. But only a closer examination of the effect of NO on the respective promoters of these genes will shed light on the particular role of AFX in NO-induced apoptosis in neuroblastomas (it should be noted that all the above-mentioned genes are known to have FOXO cis- elements in their promoters, and have been shown to be under FOXO regulation in other signaling pathways or during growth factor deprivation).

The development of site directed mutants of the Ser197 site will also be helpful in understanding the role of AFX. Conversion of serine to alanine in AFX-expression constructs inhibited the 30% fall in reporter activity seen with the native AFX constructs following NO-treatment. Similar studies with serine to aspartate mutants, which will mimic phosphorylation at the site, should complement the above observation. Though the AFX over-expression (seen in Fig. 10A) appears to be

phosphorylated, post- NO treatment, the massive over-expression of protein in the system, prevents us from drawing any conclusions from the observation. Cell death assays with stable over-expression of the above mentioned site directed mutant constructs will give a clearer picture of any definitive role played by AFX, and phospho- AFX in particular, in NO-mediated cell death in neuroblastomas.

A handful of kinases have been implicated in the phosphorylation of FOXO proteins in cells (see Fig. 1). But most of these are part of signaling cascades responding to insulin or other growth factor deprivation. The S197 site in AFX that is observed to undergo NO-mediated phosphorylation is part of a PKB-recognition motif and is known to be phosphorylated by PKB and SGK. However, the question of which kinases are involved in NO-mediated phosphorylation of AFX S197 in neuroblastoma cells requires additional experiments involving the use of specific kinase inhibitors and S197A knock in.

3.2   NO mediated regulation of CHOP

3.2.1   NO-mediated CHOP induction in SH-Sy5y cells

The role of CHOP (GADD153) in NO-mediated cell death in cells with wild type p53 is not well documented. Following up on the Gotoh *et al.* study in RAW 264.7 macrophages in which CHOP was shown to be induced downstream of bacterial lipopolysacharides and interferon- γ treatment (mediated *via* NO) (Gotoh *et al.*, 2002), I decided to examine the role of CHOP in mediating cell death in SH-Sy5y cells after NO treatment.

To investigate a role for CHOP in NO-mediated cell death, the mRNA and protein levels of CHOP were analyzed following NO treatment. I treated SH-Sy5y cells with 2 mM SNP and 1.5 mM Deta NO and isolated total mRNA and whole cell

lysates. The former was used as template for semi-quantitative RT-PCR. NO treatment was shown to steadily increase the CHOP mRNA transcript levels in SH-Sy5y cells (Fig. 12A). Western blotting with CHOP antibody using cell lysates from NO-treated cells showed that the increase in mRNA was reflected in a comparable NO-mediated increase in CHOP protein (Fig. 12B). It is interesting to note that the durations after NO treatment at which I observed increases in CHOP mRNA and protein correlate with the onset of cell death (Feng *et al*., 2002) as well as the onset of AFX phosphorylation (Fig. 7B).

3.2.2   Inducible CHOP cell lines

Since the cellular response of neuroblastoma cells to NO involves an increase in CHOP, cell lines over-expressing CHOP in parallel with CHOP knock downs will be essential to further investigate the possible role played by CHOP in NO-mediated cell death in neuroblastoma cells. As part of a research collaboration, I obtained the tet-inducible CHOP sense- and anti-sense-expressing stable SH-Sy5y cell lines developed by C. Redfern, University of Newcastle, UK (Corazzari *et al*., 2003).

The CHOP expression levels were checked in these cell lines after tet induction. To begin with, it was observed that CHOP levels in the vector control cells were elevated when compared to the wild type SH-Sy5y cells. But among themselves, the vector control, sense and anti-sense CHO stable cells showed similar levels of CHOP expression (data not shown). The elevated levels could be explained by differences that could be inherent in the parent SH-Sy5y line used in the collaborators' lab, which were used to generate the stable cells. It is also possible that the process of selection with the two antibiotics could have increased general stress levels in the cells, which might be reflected as elevated CHOP protein levels. Nevertheless, 24 hours of induction with 1 ug/ml tet showed at least a three-fold increase in CHOP

A

2mM SNP

0   2   4   8   12   (h)

CHOP

B

2mM SNP

0   2   4   8   12   (h)

CHOP

Figure 12. NO induces CHOP mRNA and protein expression. SH-Sy5y cells were treated with 2 mM SNP and total RNA and whole cell extracts prepared. (A) RT-PCR showing increasing levels of CHOP mRNA with NO treatment. (B) Western blot analysis showing the increase in CHOP protein.

A

Control

Sy5y   V   S   AS

1.5 mM Deta NO (12h)

Sy5y   V   S   AS

CHOP

B
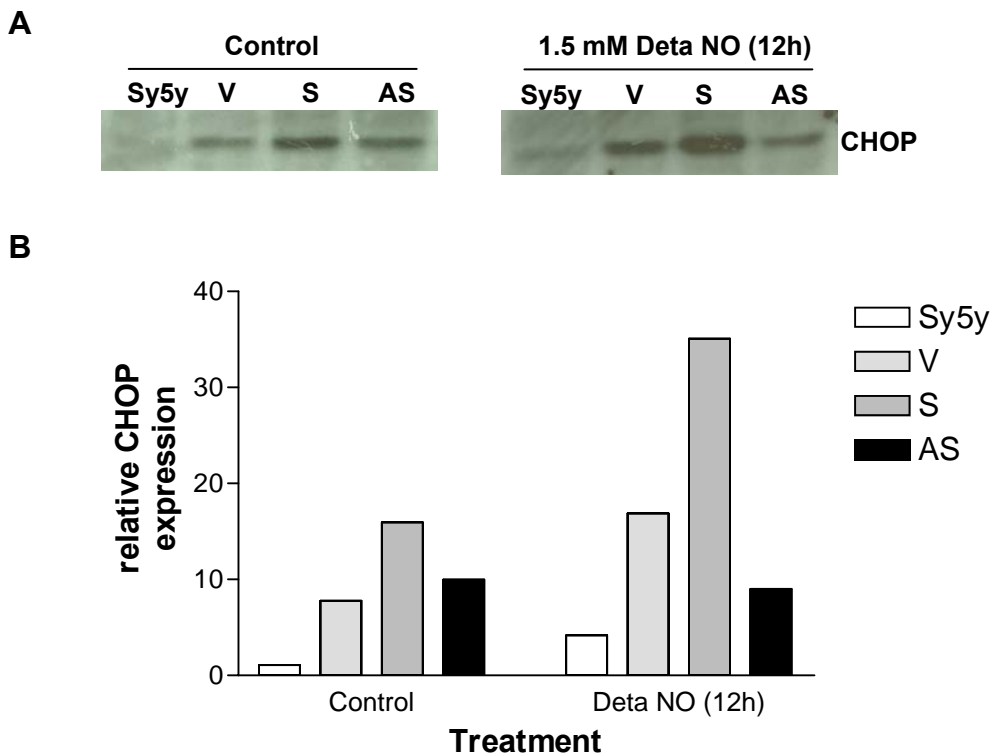
Figure 13. Tet-inducible stable cell lines expressing sense- and anti-sense CHOP. (A) Western blot analysis of SH-Sy5y, vector control (V), sense-CHOP (S) and anti-sense CHOP (AS) cells after 1 ug/ml tet induction for 24 hours. The panel on the right shows tet-induced cells further treated with 1.5 mM Deta NO for 12 hours prior to Western blot analysis of CHOP expression. (B) Quantitation of the above blot, showing relative levels of CHOP expression as fold changes.

protein yield in the sense-CHOP stable cells. The anti-sense stable cell line, on the other hand, did not show an appreciable decrease in CHOP upon tet induction (Fig. 13A, left panel). Deta-NO treatment in addition to tet induction resulted in a further 2-fold induction of CHOP expression with the sense CHOP construct, whereas only a slight reduction in CHOP expression occurred with the anti-sense CHOP stable cell lines (panel on the right in Fig. 13A; also quantified in Fig. 13B).

3.2.3   Cell death

The tet-inducible CHOP sense and anti-sense cell lines were then used to study the effect of CHOP on NO-mediated cell death. The cells were seeded in 24 well plates, along with vector control and wild-type SH-Sy5y cells, then tet-induced followed by NO treatment. Various means of measuring cell death including crystal violet uptake, LDH release assays and Sytox-Hoechst staining/ counting were performed (Fig. 14).

Over-expression of CHOP sensitized SH-Sy5y cells to NO-mediated cell death by a modest 7%, whereas cells with lower levels of CHOP due to the induction of the anti-sense construct showed up to 15% protection from cell death, when compared to vector control cells.
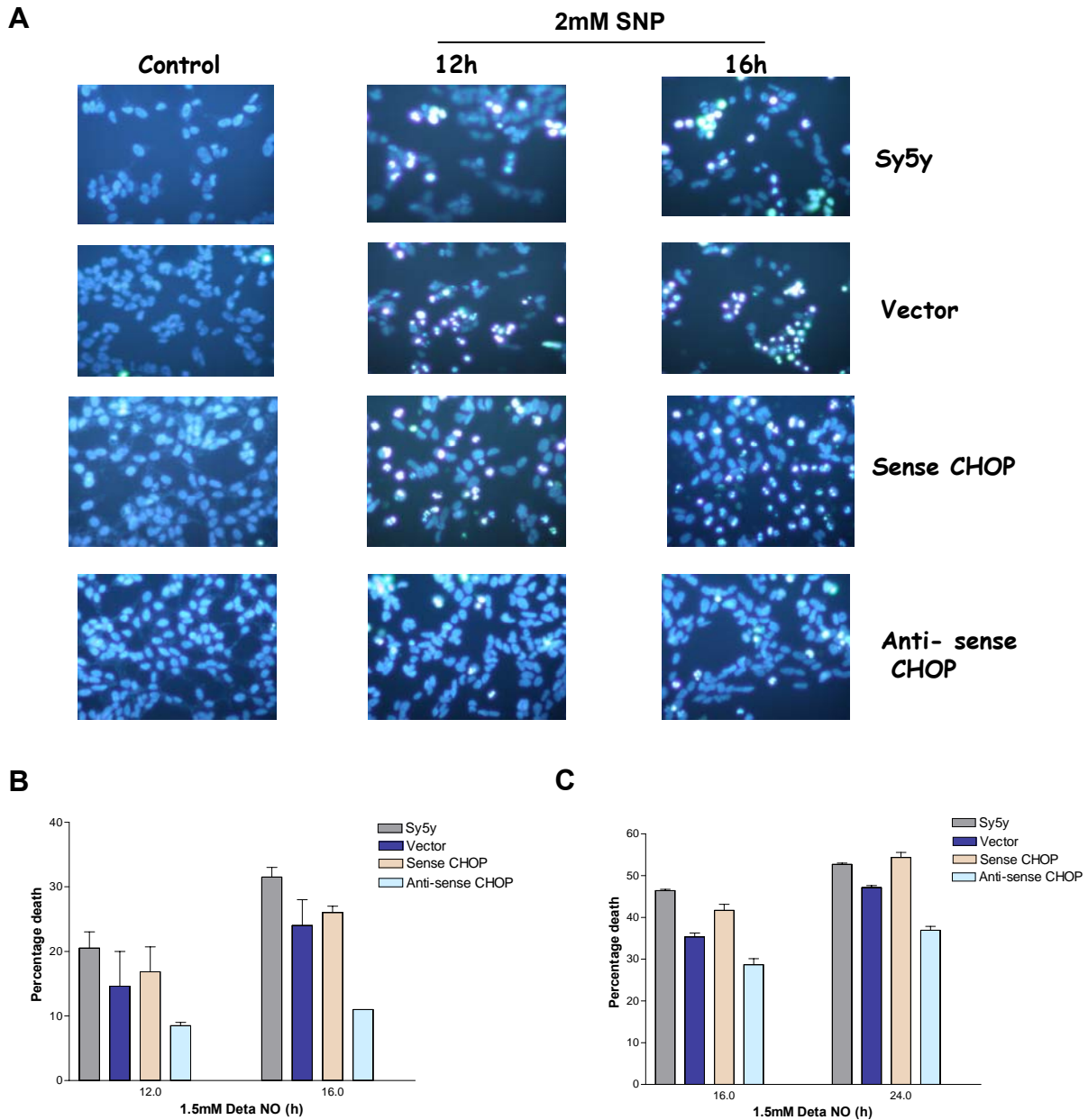
Figure 14. CHOP over-expression sensitizes SH-Sy5y cells to NO-mediated cell death; CHOP knock down protects. (A) Sytox-Hoechst staining of stable cell lines along with the vector control and wild type SH-Sy5y cells – tet induction for 24 hours was followed by treatment with 2 mM SNP for 12 and 16 hours before staining for visualization of cell death. (B) Multiple fields were chosen and live and apoptotic cells counted after Sytox-Hoechst staining, to deduce percentage death. (C) Crystal violet staining was carried out under similar conditions and the percentage of cell death calculated as in 2.5.1.

3.2.4   Discussion

NO-induced apoptosis is generally considered to be mediated by DNA damage or mitochondrial damage (see 1.2.5); however, the cascade leading to cell death has not been fully clarified, and variations in the response to death stimuli definitely exist, dependent on the cell type and nature of the death signal. The ease with which NO can affect the integrity of proteins in the cell through the various oxidative, nitrosative/ nitrosylative reactions and its effect on the redox status of the cell, would suggest the ER stress pathway being a likely candidate for mediation of apoptosis.

Experiments with RAW264.7 macrophages showed NO-dependent up-regulation of Bip/Grp 78 and the activation of ATF6 as well as other markers of ER stress. p50, the cleaved product of ATF6 post-ER stress, has been shown to induce the expression of CHOP through the ERSE element in its promoter (Gotoh *et al.*, 2002). NO-dependent, ER stress-mediated CHOP up-regulation has also been shown in B cell islets. The latter are known to have a particularly weak p53 response to NO. Though the involvement of CHOP and ER stress in NO-mediated apoptosis seems likely, the mechanism of action of CHOP remains unclear.

NO-mediated CHOP up-regulation in neuroblastoma cells was shown for the first time in my study (as in Fig. 12). The mRNA and protein up-regulation was marked only after 8 hours of treatment with cytotoxic concentrations of NO donors, which correlates with the onset of NO-induced apoptosis(Feng *et al.*, 2002), anticipating a role in cell death.

In order to further analyze the role of CHOP in neuroblastoma cell death, stable cell lines with tet-inducible CHOP over-expression and tet-inducible anti-sense CHOP expression were employed. The amount of cell death with NO following CHOP induction with tet was slightly enhanced when compared with NO without tet,

whereas protection was slightly but significantly noticeable with the antisense construct. One possible explanation for the relatively minor effects of sense or anti-sense CHOP on NO-induced apoptosis might be that NO-induced *endogenous* CHOP mRNA is already saturating and maximizes cell death (to its best possible extent); and consequently, tet-induced *exogenous* CHOP cannot mediate much additional killing. On the other hand, the anti-sense CHOP only marginally reduces CHOP mRNA, and so the NO-induced *endogenous sense* CHOP mRNA would presumably hybridize to what little anti-sense is expressed, and counteract its effect. Another possible disadvantage could be the inherent 'leakiness' of the tet induced expression systems.

It is established that there are several NO-induced cell death pathways in neuroblastoma cells (Brune *et al*., 1999), and over-expression of CHOP alone would not in any event be expected to overwhelmingly affect the outcome of NO treatment. Thus, the results also imply the relative importance of the ER stress pathway in mediating apoptosis in neuroblastoma cells, acknowledging the existence of robustly functioning p53 and the MPT pathways and their obvious effects on NO-mediated cell death. This is further confirmed by the study in beta cell islets where the p53 pathway is known to be rather weak – this accentuated its susceptibility and up to 50% increased sensitivity was found when CHOP was over-expressed in these cells.

Though a CHOP responsive cis-element and a few downstream targets have been identified, the precise role of CHOP in gene regulation and physiology is not clear. The sense CHOP cell line after NO treatment shows a massive increase in CHOP expression, when compared to basal CHOP expression in SH-Sy5y cells (see Fig. 13B). This should be exploited in micro-array analysis to positively identify CHOP-regulated transcriptional targets specific for apoptosis. A differential micro array exploring NO-mediated gene transcription *via* CHOP, over all other gene targets

responding to over-expressed CHOP alone, could also help identify (any) NO-specific regulation of the transcriptional activities of CHOP, apart from the initial NO mediated up-regulation of the CHOP protein itself.

3.3 Bcl-2 is a downstream mediator of NO induced apoptosis

3.3.1 Bcl-2 over-expression in SH-Sy5y cells

In an attempt to characterize the 'global' transcriptional response to NO, a screening project was undertaken as mentioned previously. Through the use of a transcription factor array (Panomics), various transcription factors were identified and shown to bind with increasing/ decreasing affinity to their respective consensus elements (S. Dhakshinamoorthy, unpublished). In parallel, the expression profiles of a few candidate NO-induced apoptosis-regulating genes were examined, some of which might be up- or down-regulated *via* the identified NO-activated *cis*-elements. Bcl-2 mRNA and protein were found to be strongly up-regulated starting from 2 hours of NO treatment of SH-Sy5y cells (S. Dhakshinamoorthy, unpublished).

Analysis of the *bcl-2* promoter showed the importance of an upstream CRE element in mediating the transcriptional up-regulation of the gene after NO treatment. Deletion analysis of the *bcl-2* promoter showed the presence of at least three regulatory elements -- two SP-1 sites and a CRE (S. Dhakshinamoorthy *et al.*, unpublished). The CRE element has been implicated in neuronal cell responses to hypoxia *via* Bcl-2 protein induction (Freeland *et al.*, 2001). A similar correlation between NO-mediated up regulation of Bcl-2 mRNA and subsequent cyto- protection was observed here. Luciferase assays using the *bcl-2* heterologous promoter and the isolated CRE showed NO inducibility starting from two hours of treatment with NO donors. Mutations introduced in the core sequence of the CRE (but not the SP-1 sites)

in the *bcl-2* promoter totally abolished this response (S. Dhakshinamoorthy, unpublished).

I developed Bcl-2 over-expressing stable neuroblastoma cell lines to determine if Bcl-2 plays any role in protecting cells from NO-induced apoptosis. This mimics the NO-dependent upregulation of Bcl-2 mRNA and protein and would implicate Bcl-2 in protecting SH-Sy5y cells from NO-mediated apoptosis (especially given the abundant evidence supporting Bcl-2 as an anti-apoptotic protein in cells).

Stable over-expression of Myc-tagged Bcl-2 was confirmed in three selected independent clones of SH-Sy5y cells by Western blotting (Fig. 15A). Cell death assays were then set up to examine the effect of the over-expression of the protein. Sytox-Hoechst staining of the cells showed up to 30% protection from NO-mediated cell death at 24 hours after NO treatment (Fig. 15C). Similar results were obtained with LDH release assays performed in parallel for the Bcl-2 clones, vector control and wild-type SH-Sy5y cells (data not shown).

Caspase-3 cleavage was examined next. The Bcl-2 clones showed a near complete absence of caspase-3-like cleavage activity even 16 hours after treatment with NO (Fig. 15B). This was also reflected in the absence of the 17-kDa cleaved caspase-3 products in Western blots performed with protein extracts of Bcl-2-overexpressing clones (prepared after 16 hours of NO treatment) when compared to the vector control cells (Fig. 16A). Protection from apoptosis was also indicated by the fact that the 89-kDa cleaved PARP band was virtually absent in the Bcl-2 stable cells, even after 16 hours of NO treatment (Fig. 16B). Next, cytochrome c release was examined by immunoflourescense. Bcl-2 over-expression appeared to delay cytochrome c release into the cytosol when compared to wild type/ vector control cells
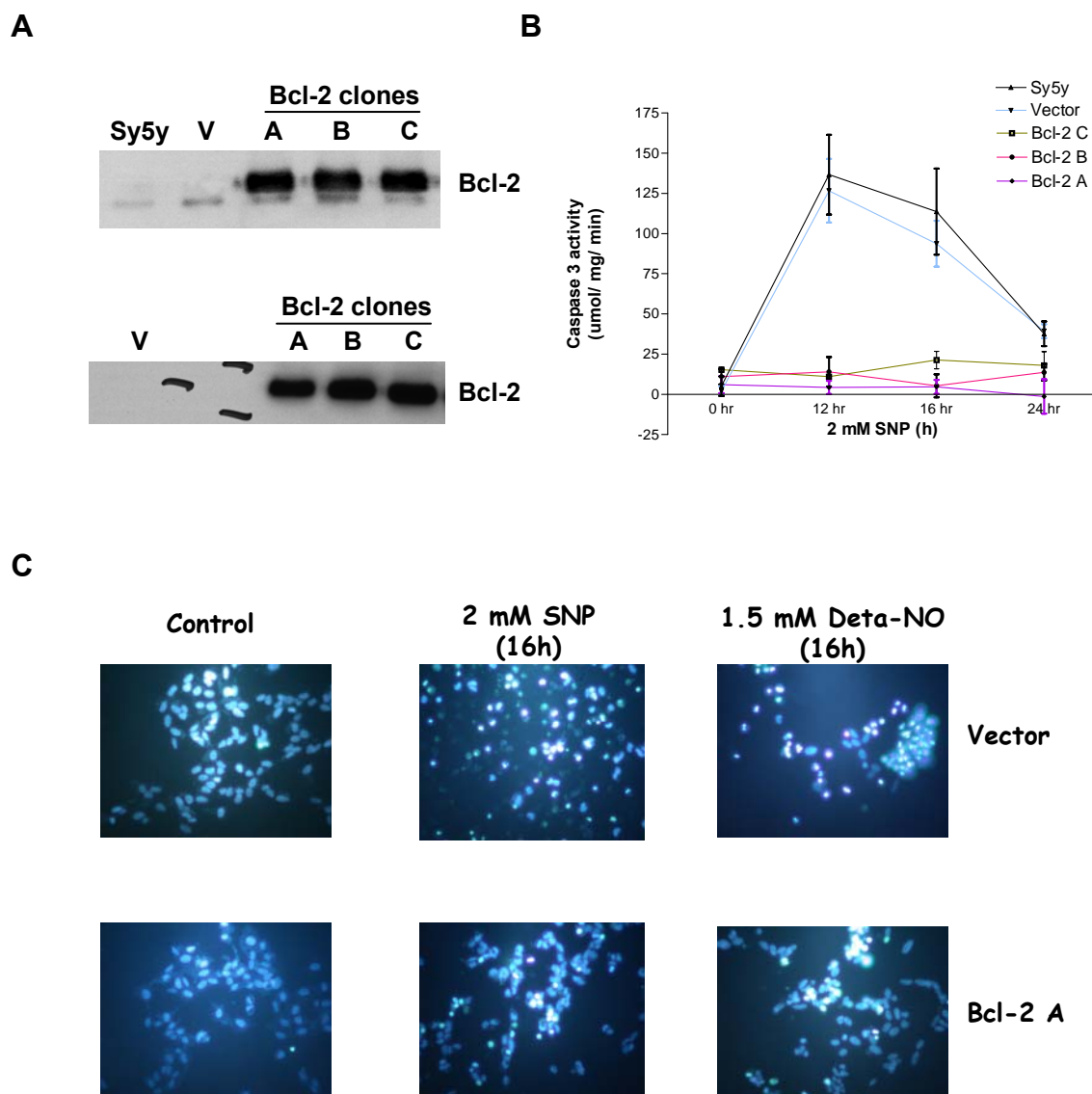
A

B

C

Figure 15. Stable Bcl-2 over-expression in SH-Sy5y cells. (A) Western blot analysis of SH-Sy5y, vector control and three selected clones over-expressing Myc-tagged Bcl-2. The top panel, probed with Bcl-2 antibody, shows tagged bcl-2 migrating 4 kDa above the native protein. Western blotting with the Myc antibody reveals only the tagged proteins in the stable clones (the panel below). (B) SH-Sy5y, vector and stable Bcl-2 cells were treated with 2 mM SNP for 12, 16 and 24 hours, before assay for caspase-3 cleavage. (C) Sytox-Hoechst staining of vector and Bcl-2 clone A cells after 16 hours of treatment with 2 mM SNP and 1.5 mM Deta NO.
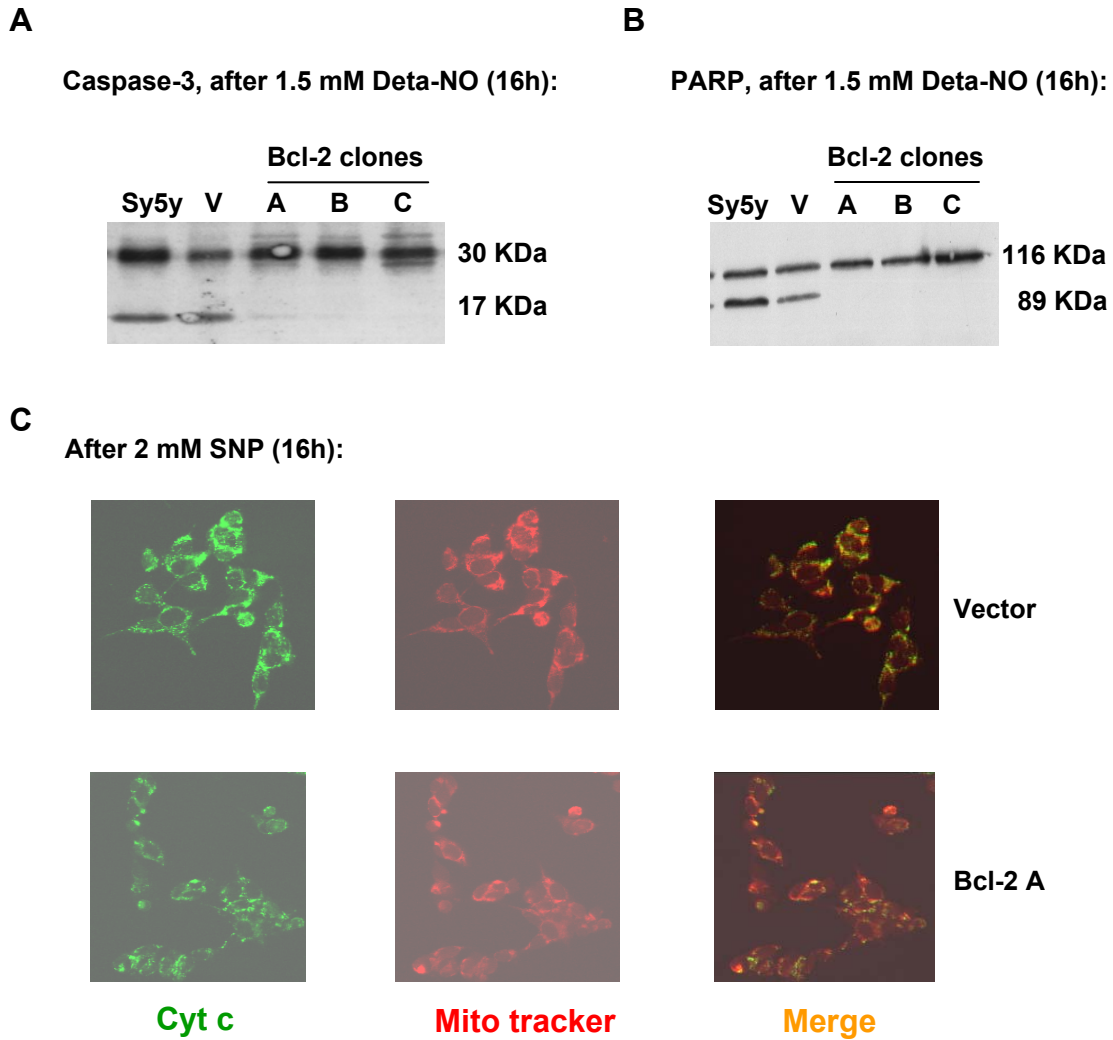
A

Caspase-3, after 1.5 mM Deta-NO (16h):

B

PARP, after 1.5 mM Deta-NO (16h):

C

After 2 mM SNP (16h):

Cyt c **Mito tracker** **Merge**

Figure 16. Bcl-2 over-expression protects SH-Sy5y cells from NO-mediated cell death. (A) Western blot analysis of SH-Sy5y, vector control (V) and Bcl-2 over-expressing clones, A, B and C, showing caspase-3 cleavage in cells treated with 1.5 mM Deta-NO for 16 hours. The un-cleaved 30 kDa and the cleaved 17-kDa bands are seen. (B) Western blot analysis, as above, but showing PARP cleavage. The un-cleaved 116 kDa and the cleaved 89 kDa bands are seen. (C), cytochrome c release. Vector control and Bcl-2 over-expressing cells treated with 2 mM SNP for 16 hours and stained with FITC-cyt c antibody and a red-colored mito-tracker. The third panel shows the merged pictures – co-localization in mitochondria is seen as yellow coloured stains; the cytochrome c release into the cytosol looks more apparent in the 'vector' cells than in the Bcl-2 clones, where the red mitotracker appears to co-localize mostly with the green cyt c, in the mitochondria, even after NO treatment.

with endogenous levels of Bcl-2, further confirming its overwhelming cytoprotective role of Bcl-2 (Fig. 16C).

3.3.2 Discussion

Overexpression of the Bcl-2 protein protects a wide variety of cell types from many death-inducing stimuli including growth factor withdrawal, treatment with calcium ionophores, glucose withdrawal, membrane peroxidation, glucocorticoid treatment, chemotherapeutic agents, and virus infection, implying that Bcl-2 functions at the point of convergence of many different signals. Bcl-2 is known to bind and inhibit pro-apoptotic family members; regulate ion flux across the mitochondria and stabilize the membrane potential and thus regulate cytochrome c release. It is also known to inhibit ROS production. Bcl-2 has also been implicated in regulation of VDAC (voltage-dependent anion channel) and thus the ATP/ ADP ratio of the cell; all of which make it among the most prolific of all the cyto-protective proteins of the cell (Cory *et al*., 2003).

Our study showed for the first time the protective role played by Bcl-2 in NO-induced apoptosis of neuroblastoma cells. The evidence for such protection came from transcriptional up regulation of Bcl-2 mRNA and protein (S. Dhakshinamoorthy, unpublished), and from my further experiments confirming the strong protection afforded by over-expressed Bcl-2 in the presence of NO. Bcl-2 up-regulation was observed as early as two hours after NO treatment, well before the onset of cell death, which further indicates its importance in counteracting NO-induced apoptosis. These results altogether emphasize the importance of death signal-mediated regulation of apoptosis through mRNA transcription.

CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

The study of transcriptional regulation of apoptosis and particularly NO-mediated apoptosis are relatively new fields. There is little information on specific details of the cascades of kinases or other modulators affecting specific transcription factors and appropriate downstream targets that modulate apoptosis. Variation in NO treatment regimens, the type of donors used, the duration of treatment, the redox status of the cells, hinders the development of uniform models of transcriptional cascades in mammalian cells downstream of NO. Thus, the available literature often shows contradictory roles for even the same transcription factor, as seen in the case of AP-1. The large family of AP-1 dimers is known to counteract cell death induced by DNA damage as well as protect macrophages from excess NO (via c-jun). In undifferentiated neuroblastoma cells, AP-1 affords protection from NO-induced apoptosis, in part through the expression of neuroprotective NCAM-140 (Feng *et al*., 2002); but in contrast, toxic concentrations of NO lead to c-Jun phosphorylation on Ser-63 by JNK that triggers apoptosis in the same cells, via unknown c-Jun targets (Li *et al*., 2004b).

In this study, I have shown evidence for NO-mediated phosphorylation of AFX without nuclear exclusion (see Figs. 7B and 8A). These effects appear to be concurrent with modification of the DNA-binding and transactivation potential of FOXO factors in reporter assays (see Figs. 5A, 6B). Interestingly, over-expression of AFX alone is toxic to cells, suggesting AFX is pro-apoptotic in neuroblastomas, but it remains to be determined whether this killing depends on S197 phosphorylation. It will be important to identify the actual downstream targets of phosphor-AFX that might regulate the cell sensitivity to apoptosis. Furthermore, the role of other FOXO factors such as FKHR or FKHRL1, if any, could also be investigated. To this end, I have identified a number

of mRNAs that are either up- or down-regulated by NO treatment, such as GADD45-α, catalase, Bcl-6, p130, and cyclin D1 (see Fig. 11). It is interesting that the regulation of these genes by NO occurs early after NO treatment and well before the onset of cell death, allowing the possibility these genes can govern cell viability under NO stress.

Cell death assays with SH-Sy5y stable cell lines that express S197A site-directed mutants of AFX will also help us better understand the significance of this particular serine phosphorylation. If this is not important, other serines in AFX that are known to be phosphorylated in other systems should be investigated. Identification of the upstream kinases that phosphorylate AFX downstream of NO will also be useful.

We have considered ER stress as being a significant contender for mediation of NO-mediated apoptosis by examining the status of CHOP in neuroblastoma cells. Up-regulation of endogenous CHOP by NO coincides with the onset of apoptosis, implying a role for CHOP in the demise of the cell by apoptosis. The small sensitization and protection (from cell death) seen with CHOP over-expression and knockdown, respectively, although disappointing, may implicate the unfolded protein response-ER stress pathway, but the precise role of CHOP as a transcription factor contributing to these effects is still not clear. The CHOP sense stable cell lines will be useful to identify CHOP-regulated genes (by micro-array) that contribute to NO-mediated apoptosis in mammalian cells.

The cyto-protection shown by the transcriptional up-regulation of Bcl-2 (see Fig. 15C) is further evidence for the existence of transcriptional control in cell survival. A better under standing of the transcriptional profiles in response to NO, the identification of all the relevant transcription factors and their possible NO-mediated modification and modulation, along with the appropriate gene targets that protect or

sensitize cells to apoptosis, is necessary before suitable applications can be considered to counter various NO-mediated pathologies in neurons and other mammalian cells.

The role of endogenously produced NO, the molecular identity of the active nitrogen species, its primary target molecules, the chemical modification(s) induced, and the mechanism of action have not yet been defined *in vivo*. To this end, replication of experiments in this study and other studies done on transformed cell lines with chemical NO donors will need to be done in primary cell lines or in *in-vivo* conditions in order to have better understanding of the relevance of NO in physiological situations. The latter will be particularly significant when the attempt is to understand the 'global' response to NO in mammalian cells.

The relevance of low levels/ endogenous levels of NO to cell signaling is relatively well studied. Only recently has the 'signaling' possibilities of cytotoxic levels of NO been studied. The published literature as well as studies in our own laboratory emphasizes the complexity of this task. NO, with its wide ranging pleiotropic effects on kinases, phosphatases and transcription factors, along with other crucial modulators, appears to be capable of drastically modulating gene transcription and thereby influencing the physiological and patho-physiological status of the cell. Cytotoxic levels of NO have been found to induce time-dependent cascades of transcriptional events, where the early events involve the induction of metabolic and cyto-protective genes and the later events involve the activation of pro-death genes (Hemish *et al.*, 2003). The relevance of transcription itself, however, with reference to the preparation of the cells for, and the induction of apoptosis, is still controversial. Further analysis of transcriptional events dictated by cytotoxic levels of NO is a valid approach to achieve a better understanding of the molecular events in neurodegeneration, stroke and trauma in which NO is a key player.

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