CHAPTER 1

INTRODUCTION
1.1 Transcriptional Control of Gene Expression - An Overview

Eukaryotic cells carry a tremendous amount of genetic information just to encode the 6000 to 100,000 proteins necessary to perpetuate life from yeast to animals (Lemon and Tjian, 2000). In metazoans, thousands of protein coding genes must be differentially expressed in specific cell types, during development, and in response to a wide variety of extracellular signals (Smale, 2001). The question of how gene expression is regulated in complex eukaryotic genomes has focussed on the molecular machines that mediate transcriptional control. Transcriptional regulation is all about getting RNA polymerase to the right place on the gene at the right time and making sure it is competent enough to conduct transcription (Tansey, 2001). Biochemical and genetic analysis of various model organisms has identified an astounding number of protein factors responsible for transcriptional control. Large assortments of gene-specific DNA-binding regulators and a huge number of co-regulators (co-activators and co-repressors) have imposed multiple layers of control on the transcriptional machinery.

1.1.1 Regulation of Transcription Factor Activity and Function

Different genes in the genome are discriminated by defining unique sets of cis-regulatory sequences that are bound by sequence-specific transactivating factors. A typical transcription factor contains a specific DNA-binding domain, a multimerisation domain that allows for the formation of homo- or heteromultimers, and a transcriptional activation domain, which can directly interact with components of the basal transcription apparatus. The levels and activity of these proteins and the resulting gene expression therefore, is regulated by diverse mechanisms responsive to a variety of signals.

(a) Stability: Transcription factors tend to be some of the most unstable proteins in the cell. Mammalian transcription factors such as E2F-1, Jun, Fos, p53, Stat-1 and Myc, for example, are all short-lived proteins that are destroyed by Ubiquitin-mediated proteolysis (Muratani and Tansey, 2003). In these cases, protein turnover rates dictate functional levels of the transcription factor in the cell and hence influence transcription of genes controlled by that transcription factor. There is also compelling evidence to indicate functional relationships between the transcription activation domains (TADs) and degradation signals (degrons). In almost all unstable
transcription factors that have been characterized, there is an overlap between TAD and degron sequences (Salghetti et al., 2000). Simply put, efficient recruitment of activator-target protein complexes to the promoter means that they are subjected to rapid degradation by proteasomes. Proteasome-mediated control of the intracellular levels of transcriptional activators is therefore, an important regulator of gene expression (Molinari et al., 1999).

(b) Influence of co-regulators: The vast diversity of molecular signals that must be interpreted by the transcriptional machinery makes it untenable to visualize a unique target for each transcription factor. It is now evident that a given transcription factor can partner and function with multiple types of co-activators and co-repressors. These co-regulators may influence their targets at different levels in the transcription machinery. Some of the properties of these co-regulators include chromatin remodelling activities (SWI/SNF), ability to interact with and modulate proteins of the core promoter machinery (tightly associated factors of the TFIID complex, the TAFs), acetyltransferase or deacetylase activities with multiple substrates (Lemon and Tjian, 2000). These transcriptional co-regulators may have tissue-restricted expression (Spiegelman and Heinrich, 2004) or sequestered in specific nuclear compartments to further specify the target spectrum of a transcription factor (Stein et al., 2003).

(c) Localisation: Some transcription factors are sequestered in the cytoplasm or otherwise inactivated and require signal transduction to instigate transcription, for example the NF-κB family. The five members of the mammalian NF-κB family, p65 (RelA), Rel-B, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), exist in unstimulated cells as homo- or heterodimers bound to the IκB family proteins. Binding to IκB prevents the NF-κB:IκB complex from translocating to the nucleus, thereby maintaining NF-κB in an inactive state. NF-κB activation, for example during stimulation with tumour necrosis factor α (TNFα) occurs by triggering a cascade leading to the phosphorylation of IκB. Phosphorylated IκB proteins are recognized by the ubiquitin ligase machinery leading to their poly-ubiquitination and subsequent degradation. The freed NF-κB dimers then translocate to the nucleus, where they bind specific sequences in the promoter or enhancer regions of various target genes (Hayden and Ghosh, 2004).
(d) **Post-translation modifications:** Modification of the transcription machinery by acetylation, phosphorylation, ubiquitinylation can have gain of function or loss of function effects. Acetylation of lysine residues within histones and transcription factors has emerged as a major mechanism used by the cell to overcome repressed chromatin states. Several transcription factors have been identified as substrates of histone acetyltransferases (HATs) particularly for the HATs; CREB binding protein (CBP), p300 and p300/CBP-associated factor (pCAF). Acetylation of transcription factors can alter their ability to bind DNA (in the case of p53, E2Fs, GATA1), to interact with other proteins (as with c-Jun) or to remain in the nucleus in some cases (Polevoda and Sherman, 2002).

Protein phosphorylation and dephosphorylation can directly regulate distinct aspects of transcription factor function, including cellular localisation, stability, DNA binding and transactivation (Whitmarsh and Davis, 2000). The STAT (signal transducers and activators of transcription) family of transcription factors is probably the best example to illustrate this. Upon exposure to cytokines, the STATs are tyrosine phosphorylated by Janus kinases (JAKs) and dimerize via interactions between these phosphorylated tyrosine residues. This change in structure allows for nuclear import and an increased DNA binding affinity, the STAT dimers are trapped in the nucleus till they are dephosphorylated (Vinkemeier, 2004).

Ubiquitin, the 76-amino acid peptide 'tag' that targets eukaryotic proteins for degradation by the proteasome, has also been implicated in transcriptional activation. Indeed, ubiquitination does not always presage destruction; there is evidence to support a mechanism by which ubiquitination of activators might facilitate transcriptional elongation (Herrera and Triezenberg, 2004).

SUMO, an ubiquitin like molecule has also been described to affect transcription factor localization, stability and DNA binding. In many cases that have been described to date, attachment of SUMO seems to repress the activity of transcriptional activators. SUMO acceptor sites in the transcription factors such as GR (glucocorticoid receptor), Myb, Sp3, C/EBP have been mapped to previously mapped inhibitory regions and mutation of these acceptor sites dramatically increases the transcriptional activity of these proteins (Gill, 2003).
1.1.2 Combinatorial Gene Regulation: A Symphony of Transcription factors and their Regulators

Combinatorial gene regulation strategies are required to generate a diverse array of expression patterns by a genome of fixed size. Most genes are regulated by mixing and matching different types of activators and repressors in a coordinated fashion (Lemon and Tjian, 2000). What are mechanisms that allow for creating and modulating such combinations? A part of the answer lies in the modular nature of the transcription factors, enhancers and promoters (Tjian and Maniatis, 1994; Dynan, 1989). In molecular terms, specificity in gene transcription via the combinatorial theory of gene expression would arise from the assembly of unique enhancer-transcription factor complexes, termed “enhanceosomes”. Given the unlimited possibilities for mixing and matching enhancer and protein modules, we could imagine that there might be an endless number of unique gene expression programs hard-wired into genomes of relatively limited size (Merika and Thanos, 2001).

Mammalian core promoters by themselves appear diverse; there is a variable occurrence of consensus or near-consensus TATA, Initiator (Inr) and downstream promoter elements (DPE). Enhancers may exhibit core promoter specificities, which then could make an important contribution to combinatorial gene regulation (Smale, 2001).

The virus-inducible enhancer of the interferon-β (IFN-β) gene provides one of the best-understood examples of how combinatorial interactions between transcription factors can lead to a highly specific gene expression program. The gene is normally silent but is highly inducible upon viral infection. The enhancer region responsible for this inducibility contains binding sites for NF-κB, IRF-1 and ATF-2/c-Jun transcription factors. None of these sites function on their own, but two or more copies of each of them can act as a virus-inducible enhancer. However, these synthetic enhancers are less inducible and not as specific as the intact enhancer; the natural intact enhancer is thus distinct in its specificity and activity. Put simply, the specific function of the enhancer is not a collection of individual enhancers (a single transcription factor-enhancer DNA interaction) functions but appears to emerge from a network of synergistic interactions between the elements and their transcription factors. The high mobility group protein, HMG I (Y) is the main player in creating and maintaining a stable IFN-β enhanceosome (higher order three-
1.2 Apoptosis - A Brief Overview

Apoptosis or programmed cell death (PCD) is a genetically regulated cellular suicide mechanism that plays a crucial role in development and in the defence of homeostasis (Cryns and Yuan, 1998). This highly regulated mechanism enables metazoans to control cell number in tissues and to eliminate individual cells that threaten the animal’s survival. Morphologically, cells undergoing apoptosis demonstrate nuclear/cytoplasmic condensation and membrane protrusions. These initial changes are followed by fragmentation of the nuclear contents and subsequent encapsulation of these fragments into “apoptotic bodies” that are quickly and unobtrusively consumed by phagocytic cells (Kerr et al., 1972; Ravichandran, 2003).

Biochemically, apoptotic cells are characterized by:
(a) Reduction in mitochondrial membrane potential (Zamzami et al., 1995, Green and Kroemer, 2004)
(b) Intracellular acidification (Gottlieb et al., 1996)
(c) Externalisation of phosphatidyl serine (Martin et al., 1995)
(d) Selective proteolysis of a subset of cellular proteins (Lazebnik et al., 1994) mediated by proteases called caspases (Nicholson, 1999)
(e) Degradation of DNA into inter-nucleosomal fragments (Wyllie et al., 1984)

1.2.1 Demolition Machinery of the Cell - Caspases

Caspases are a conserved family of cysteine proteases that cleave after certain aspartate residues in their substrates (Nicholson, 1999). The first mammalian member of the caspase family to be identified was the Interleukin 1β-converting enzyme (ICE) or caspase-1 on the basis of sequence similarity to the ced-3 gene of Caenorhabditis elegans (Yuan et al., 1993). Caspases are expressed in most cell types, but to preclude unwarranted cell death, each is maintained as a zymogen.
The table 1.1 summarizes the general properties of the known mammalian caspases.

<table>
<thead>
<tr>
<th>CASPASE</th>
<th>PRO-DOMAIN</th>
<th>ACTIVATORS</th>
<th>ESTABLISHED OR PROPOSED FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1 Initiator</td>
<td>CARD</td>
<td>Inflammasome, Ipaf, RIP2, ASC</td>
<td>IL-1β/IL-18 maturation, apoptosis</td>
</tr>
<tr>
<td>Caspase-2 Initiator</td>
<td>CARD</td>
<td>PIDDosome</td>
<td>Premitochondrial stress</td>
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<tr>
<td>Caspase-3 Executioner</td>
<td>short</td>
<td>Apoptosome, caspase-8</td>
<td>Cleavage of most apoptotic substrates</td>
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<tr>
<td>Caspase-4 Initiator</td>
<td>CARD</td>
<td>-</td>
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<tr>
<td>Caspase-5 Initiator</td>
<td>CARD</td>
<td>Inflammasome</td>
<td>IL-1β/IL-18 maturation, LPS stress</td>
</tr>
<tr>
<td>Caspase-6 Executioner</td>
<td>short</td>
<td>Caspase-3 and 7</td>
<td>Lamin cleavage</td>
</tr>
<tr>
<td>Caspase-7 Executioner</td>
<td>short</td>
<td>Apoptosome, caspase-8</td>
<td>Cleavage of most apoptotic substrates</td>
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<tr>
<td>Caspase-8 Initiator</td>
<td>DED</td>
<td>DISC</td>
<td>Death receptor signalling</td>
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<tr>
<td>Caspase-9 Initiator</td>
<td>CARD</td>
<td>Apaf-1 and cytochrome c</td>
<td>Caspase-3/7 activation after mitochondrial stress</td>
</tr>
<tr>
<td>Caspase-10 Initiator</td>
<td>DED</td>
<td>DISC</td>
<td>Death receptor signalling</td>
</tr>
<tr>
<td>Caspase-12 Initiator</td>
<td>CARD</td>
<td>TRAF-2, calpain</td>
<td>ER stress</td>
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<tr>
<td>Caspase-14</td>
<td>-</td>
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CARD- Caspase Recruitment Domain; DED- Death Effector Domain, DISC- Death Inducing Signalling Complex.

Table 1.1. Mammalian Initiator and Executioner Caspases (adapted from Adams, 2003).

Before the "executioner" caspases can attack their cellular substrates; their zymogens must be proteolytically cleaved, typically by the "initiator" caspases (Shi, 2002; Slee et al., 1999). While the executioner caspases have very short pro-
domains, the initiator caspase has a long prodomain and activation is triggered by dimerization of the zymogen on a dedicated adaptor or scaffold protein (Shi, 2004).

A major factor that has been recognized to control caspase activity is the inhibitors of apoptosis (IAP) family of proteins. IAPs are the only known endogenous proteins that regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7). The BIR (Baculoviral IAP Repeat) domains of the IAPs can bind and inhibit caspases, for e.g. BIR3 domains of XIAP (X-chromosome linked IAP), cIAP1 and cIAP2 (Cellular IAP) have been demonstrated to bind and inhibit caspase-9 (Deveraux and Reed, 1999; Liston et al., 2003).

1.2.2 Extrinsic and Intrinsic Pathways of Apoptosis

The extrinsic apoptotic pathway reflects a kind of "instructive" apoptosis, wherein specialised cell surface receptors transmit apoptotic signals initiated by specific "death ligands" enabling the cell to self-destruct (Ashkenazi and Dixit, 1998). A death ligand such as FasL (Fas Ligand) binds to its death receptor, Fas, leading to the recruitment of a cytosolic adaptor, FADD (Fas Associated Death Domain-containing protein) and caspase-8, forming an oligomeric death-inducing signalling complex (DISC). This leads to caspase-8 activation, which then cleaves and activates the effector caspase, caspase-3 (Schulze-Osthoff et al., 1998; Riedl and Shi, 2004).

The intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth factor withdrawal, hypoxia, DNA damage and oncogene induction. Signals transduced in response to these stresses converge mainly on the mitochondria, and several proteins are released from the inter membrane space of the mitochondria into the cytoplasm (Wang, 2001).

Some of the well-characterized proteins released from the mitochondria include:
(a) Cytochrome c: It binds and activates Apaf-1 in the cytoplasm (Li, 1997; Cecconi, 1999). The binding of cytochrome c to Apaf-1 induces a conformational change that allows Apaf-1 to bind dATP and form the "apoptosome". The apoptosome mediates caspase-9 activation thereby triggering a cascade of caspase activation (Srinivasula et al., 1998; Zou et al., 1999).
(b) **SMAC (Second Mitochondria-Derived Activator of Caspase) / DIABLO (Direct Inhibitor of Apoptosis (IAP)-Binding Protein with Low pI)**: Smac/Diablo binds to the BIR (Baculoviral IAP Repeat) domain of IAPs (Chai *et al.*, 2000). IAPs are a family of intracellular proteins that contain one or multiple BIR domains, which inhibit active caspases (Deveraux and Reed, 1999). Smac/Diablo binding of the IAPs disrupts the latter's inhibition of caspases.

(c) **OMI/HTRA2 (High-Temperature Requirement Protein A2)**: Omi is a serine protease that is released from mitochondria and antagonises IAPs (Suzuki *et al.*, 2001). But unlike Smac/DIABLO, Omi/HtrA2 can cause catalytic cleavage of IAPs and this is a key mechanism for it to irreversibly inactivate IAPs and promote apoptosis (Yang *et al.*, 2003). A catalytically inactive mutant of HtrA2 does not induce cell death supporting a role for this serine-protease activity in inducing apoptosis. However, when over expressed extra-mitochondrially, HtrA2 induces atypical cell death, which is neither accompanied by a significant increase in caspase activity nor inhibited by caspase inhibitors, including XIAP (Suzuki *et al.*, 2001).

(d) **AIF (Apoptosis-Inducing Factor)**: When released, AIF translocates to the nucleus and causes chromatin condensation and large scale DNA fragmentation independent of caspases. AIF has no measurable DNase activity; it may work with other protein(s) to mediate the effect (Susin *et al.*, 1999).

(e) **EndoG (Endonuclease G)**: It is a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis. EndoG cleaves chromatin into nucleosomal fragments independently of caspases (Li *et al.*, 2001).

The extrinsic and intrinsic pathways are connected by a BH3 (Bcl-2 Homology)-only protein, Bid. Bid can be cleaved by caspase-8 in many cell types (e.g. hepatocytes); cleaved bid can promote mitochondrial permeabilization and downstream caspase-9 activation (Yin *et al.*, 1999). BH3 only molecules trigger apoptosis in response to insufficient trophic support (Bim), intracellular damage (Puma, Noxa) and also developmental cues (*c.elegans* Egl-1) (Adams, 2003). The "multi-BH" domain proteins Bax, Bak and Bok are also pro-apoptotic; they act further
Figure 1.1. Extrinsic (Death Receptor) and Intrinsic (Stress) pathways of apoptosis. This flow chart depicts the two-apoptotic pathways that can be engaged by a cell. Cleavage of Bid by caspase-8 to form truncated Bid (t-bid) in some cell-types forms the only link between these otherwise disparate apoptotic pathways in the cell.
downstream to promote caspase activation by causing mitochondrial outer membrane permeabilisation or MOMP (Cory and Adams, 2002; Green and Kroemer, 2004). The pro-survival Bcl-2 family proteins, Bcl-2, Bcl-xL and Bcl-w potently inhibit apoptosis in response to many cytosolic insults and also prevent the release of many pro-apoptotic molecules from the mitochondria. Bcl-2 is targeted to the endoplasmic reticulum and mitochondrial membranes in normal cells. To prevent apoptosis, Bcl-2 has been proposed to interact with Bax and Bak and inhibit the membrane perturbing properties of the latter (Cory et al., 2003).

BH3 only molecules can bind Bcl-2 neutralizing their pro-survival function by altering the topology of Bcl-2 in membranes. A peptide corresponding to the BH3 domain of Bim triggers a conformational change in Bcl-2, supporting such a hypothesis (Kim et al., 2004). The intrinsic cell death machinery described above is highly conserved from worms to man and reflects the evolutionary conservation of the cell death program (Willis et al., 2003).

1.2.3 Transcriptional Control of the Cell Death Machinery

Although the pre-existing cellular machinery mediates much of the control of caspase activation, transcriptional regulation has also started to emerge as an important means of controlling the core cell death machinery. Most cells constitutively contain both prosurvival and proapoptotic components of the apoptotic machinery; an alteration in the balance of the pro and antideath molecules can be achieved by transcription of molecules in response to relevant stimuli (Kumar and Cakouros, 2004).

Transcriptional control of programmed cell death (PCD) during development

Most of our knowledge on developmental PCD is from non-mammalian model organisms, C.elegans and Drosophila. One of the best examples to illustrate the importance of a transcriptional program in developmental PCD comes from the study of stage-specific apoptosis initiated by the hormone, ecdysone in the Drosophila salivary glands during metamorphosis. Ecdysone suppresses the expression of diap2 (Drosophila IAP) by switching on expression of E75A and E75B genes that repress diap2 and switching off βFTZ-F1 that activates diap2. Ecdysone-receptor complex can also transactivate the Drosophila cell death inducers, rpr (reaper) and hid (head involution defective) leading to apoptosis (Jiang et al., 2000).
Transcriptional regulation of the intrinsic apoptotic pathway

Several key players in the intrinsic apoptotic pathway are regulated by a transcriptional program in response to stresses like growth factor withdrawal and DNA damage. Bim (pro-apoptotic BH3-only family protein) expression is upregulated by the forkhead family transcription factor, FOXO3, on withdrawal of the growth factor IL-2 in T-cells. In the presence of IL-2, FOXO3 is phosphorylated in a PI3-kinase dependent manner and retained in the cytoplasm, thereby keeping it transcriptionally inactive. On IL-2 withdrawal, phosphorylation of FOXO3 ceases and it becomes free to transactivate Bim expression that starts off the apoptotic cascade (Stahl et al., 2002).

p53 and the E2F family of transcription factors are key regulators of DNA damage induced apoptosis. The role of p53 in mediating an apoptotic response in response to several cellular stresses like genotoxic drugs, UV and γ- radiation, hypoxia, oncogene induction is discussed in a separate section (section 1.3) in this thesis. E2F-1 can induce apoptosis in several ways. Although E2F-1 partly mediates apoptosis through p53, in p53 null cells, E2F-1 can transactivate p73 (a p53 family protein) to mediate apoptosis (Irwin et al., 2000; Stiewe and Putzer, 2000). E2F-1 also binds the promoter of Apaf-1 and switches on Apaf-1 gene expression in response to DNA damage stimuli (Moroni et al., 2001).

The general paradigm for the role of NF-κB family of transcription factors is in cell survival. NF-κB induces the expression of several anti-apoptotic genes like Bcl-2, Bcl-xL, c-IAP1 and c-IAP2 that highlight the strong link between NF-κB and cell survival (Burstein and Duckett, 2003). Decreased NF-κB baseline activity also decreases Mdm2 expression and enhances p53 stabilization in response to chemotherapeutic drugs, indicating the protective role of NF-κB against p53-mediated apoptosis (Tergaonkar et al., 2002).

Transcriptional control of the death receptor pathway of apoptosis

The TNF family member, FasL (CD95L) acts through its receptor Fas (CD95) to induce apoptosis, this interaction is crucial in maintaining homeostasis of immune cells. The promoter of the gene encoding CD95L is highly complex and is directly or indirectly regulated by a large number of transcription factors, such as NF-AT, NF-κB, AP1, c-Myc, IRFs and Sp1 (Li-Weber and Krammer, 2003). Sp1 and Ets-1
transcriptionally regulate caspase-8, the initiator caspase in the death receptor pathway and caspase-8 promoter is also induced by p53 (Liedtke et al., 2003).

1.3 Tumour Suppressor p53 – Guardian of the Genome

The path to discovery of the p53 tumour suppressor was filled with more than a few twists and turns (Sherr, 2004). Linzer and Levine (1979) and Lane and Crawford (1979) first detected p53 in complexes with the SV40 T-antigen. Earlier thought as an immortalizing oncogene (Eliyahu et al., 1984; Parada et al., 1984), it was only 10 years after its discovery that p53 was demonstrated to act as a suppressor of cell transformation (Finlay et al., 1989), classifying it as a bonafide tumour suppressor gene. Mutations of the p53 gene have been documented in around 50% of all human tumours (Hollstein et al., 1996) and were also revealed to be a causative genetic factor in patients with the familial Li-Fraumeni syndrome (Malkin et al., 1990).

p53 is a protein with many talents, including triggering apoptosis, cell cycle arrest, senescence and differentiation (Vousden, 2000). The apoptosis inducing property of p53 is largely recognized to be most important for its ability to function as a tumour suppressor (Schmitt et al., 2002). Levels of p53 within the cell increase dramatically following cellular stresses such as ionizing radiation, UV-radiation, hypoxia, heat shock, growth factor withdrawal, oncogene activation and the application of cytotoxic drugs (Vogelstein et al., 2000; Vousden and Lu, 2002). p53 is a transcription factor and can therefore both initiate and suppress gene expression following these stresses to mediate its responses. The functions of the p53 target genes are diverse, this may be the foundation by which p53 acts as a multifunctional protein and may also explain why loss of p53 activity has been found to result in human tumours that are associated with a wide range of phenotypes such as lack of differentiation, increased levels of angiogenesis and metastasis (Slee et al., 2004).

1.3.1 Domains of the p53 Protein

p53 is a DNA-binding protein of 393 aminoacids that binds to specific sequences in the promoter or enhancer elements of its target genes to activate or repress transcription. The various domains and their functions are listed below-

(a) N-terminal acidic transcriptional activation (TA) domain-The TA domain is the region to which Mdm2, a negative regulator and TAF (TATA-binding protein (TBP) associated factor) bind. The TA domain is also the site of many
regulatory phosphorylations that affect the stability and activity of the p53 protein (Giaccia and Kastan, 1998).

(b) A proline rich domain (PRD) that binds the corepressor complex, mSin3a protecting p53 from proteasome mediated degradation (Zilfou et al., 2001).

(c) A central DNA binding domain (DBD) which binds to at least two repeats of the consensus sequence, 5' PuPuPuC(A/T)(T/A)GPyPyPy 3' (El-Deiry et al., 1992; Funk et al., 1992). DBD is the site of the vast majority of point mutations; these may either disrupt protein-DNA interactions or alter the overall conformation of the DNA-binding domain (Cho et al., 1994).

(d) The C-terminal domain (CTD) is the region of major allosteric regulation of p53 function and contains the sequences necessary for dimerization and tetramerization. It is also the site for regulatory acetylations and phosphorylations that modulate p53 activity (Giaccia and Kastan, 1998).

Figure 1.2. Domains of the p53 protein. The various domains and the region of Mdm2 binding, phosphorylations (p) and acetylations (Ac) are indicated. Some of the mutational “hot-spots” clustered in the DNA binding domain are also indicated.

p53 exists as a dimer of dimers, the two monomers within a p53 dimer bind to a half-site (two consecutive quarter-sites, → ← → ←) but not to separated (alternating) quarter-sites. Tetramers bind similarly, with the two dimers within each tetramer binding to pairs of half-sites. In a random sequence of DNA, single quarter-sites
frequently would be encountered, but relatively stable binding would occur only if a half-site was encountered. Since tetramers bound to a half-site would still have a short half-life, they would be able to dissociate and sample many more sequences of DNA. When a rare consensus site is finally encountered, both dimers within the p53 tetramer would bind, thereby enhancing the affinity of tetramer binding (McLure and Lee, 1998; Nicholls et al., 2002).

1.3.2 Functions of p53

p53 is involved in mediating many cellular functions particularly in response to DNA damage.

(a) Cell Cycle Arrest

p53 can control cell cycle check points by mediating the gene expression of certain key players involved in progression through the G1 and G2 phases of the cell cycle. p53-dependent G1 arrest results mainly from the transactivation of p21/WAF1 (Wild type p53 Activated Factor) (El-Deiry et al., 1993). p21 interferes with cell cycle progression and prevents S-phase entry by blocking the activity of Cdns (Cyclin Dependent Kinase). Inhibition of G1-phase specific kinase activity maintains Rb in a hypophosphorylated state, which represses E2F-specific transcription of genes required for entry into the S-phase (Weinberg, 1995).

\[ \text{14-3-3} \sigma \uparrow \rightarrow \text{14-3-3} \sigma/ \text{Cdc25C} \rightarrow \text{Inactive cdc2} \rightarrow \text{G2 block} \]

\[ \text{p53} \uparrow \rightarrow \text{p21} \uparrow \rightarrow \text{p21/Cdns} \rightarrow \text{Inactive cdks} \rightarrow \text{G1 block} \]

**Figure 1.3.** The combination of p21 and 14-3-3 \( \sigma \) proteins mediate a significant part of the cell cycle effects of p53 following DNA damage.

14-3-3 \( \sigma \), a protein capable of causing arrest in the G2 phase of the cell cycle is also transcriptionally upregulated by p53. DNA damage for e.g. caused by
irradiation, triggers the activation of Chk1 (Checkpoint kinase1), which subsequently phosphorylates the Cdc25C phosphatase. The 14-3-3 group of proteins sequesters the phosphorylated Cdc25C preventing it from dephosphorylating Cdc2, which is essential for entry into mitosis (Hermeking et al., 1997).

(b) Apoptosis

p53 knockout mice form spontaneous tumours after birth (Donehower et al., 1992) and this has largely been attributed to its ability to induce apoptosis. Thymocytes from p53\(^{-/-}\) mice are resistant to apoptosis induced by \(\gamma\)-irradiation (which increases p53 protein levels in wild-type cells) but not that induced by agents like PMA (phorbol 12-myristate 13-acetate)/calcium ionophore, dexamethasone or glucocorticoids (these do not increase p53 protein levels in wild-type cells). This clearly demonstrated the requirement of functional p53 protein for \(\gamma\)-radiation induced apoptosis in thymocytes (Lowe et al., 1993; Clarke et al., 1993). Subsequent work for over a decade has clearly established apoptosis as the most important cellular function of p53.

Contribution of apoptosis to p53-mediated tumour suppression

The importance of apoptosis for p53-mediated tumour suppression has been suggested from numerous studies linking p53 loss to apoptotic defects in many mouse models of tumour progression (Attardi and Jacks, 1999). Anti-apoptotic activities can accelerate tumourigenesis in transgenic mouse models of cancer (Eischen et al., 2001; Strasser et al., 1990; Jiang and Milner, 2003). Furthermore, certain p53 wild-type tumours harbour mutations that can suppress apoptosis downstream of p53, for e.g. mutations in the Apaf-1 gene in melanomas (Soengas et al., 2001). Inactivation of apoptosis downstream of p53 alleviates pressure to mutate p53 during Myc-induced lymphomagenesis, implying that apoptosis is the only p53 effector function that limits tumour development in this setting (Schmitt et al., 2002). Also, some tumour-derived p53 mutants are defective at inducing apoptosis but not cell-cycle arrest (Aurelio et al., 2000). Furthermore, unlike p53 knockout mice, mice lacking p21 do not develop tumours (Deng et al., 1995).

All these observations imply that it is the ability of p53 to induce apoptosis rather than growth arrest that is central to its role as a tumour suppressor.
Dependence of p53-mediated apoptosis on transcription

Mouse models expressing transactivation-deficient mutant of p53 have provided evidence for the role of transactivation function of p53 in p53-tumour suppression. Murine embryonic stem (ES) cells that express p53 with Gln and Ser in place of Leu25 and Trp26, (termed p53 GS, here residues essential for transcriptional activation and Mdm2 binding are mutated) did not undergo DNA damage-induced apoptosis (Chao et al., 2000). Transgenic mice expressing this mutant p53 allele showed defects in cell cycle regulation and apoptosis. Thymocytes and MEFs derived from these mice were resistant to apoptosis. These mice were tumour prone like p53 knockout mice (Jimenez et al., 2000). But the interpretation from this study had complications because of an additional temperature sensitive mutation in the p53 allele used in this study. More recently, studies on knock-in p53 GS mice (without additional mutations) pointed to an important role for the transactivation function of p53. MEFs from these mice were severely compromised for the induction of several p53 target genes in response to DNA damage (Johnson et al., 2005). Although apoptosis induced by DNA damaging agents like doxorubicin was compromised in these mice, apoptosis in response to hypoxia was not. Transcriptional repression by p53 is likely to play an important role in mediating apoptosis to hypoxic stress (Johnson et al., 2005).

Whether through activation or repression of its target genes, the ability of p53 to modulate transcription of several genes is key to its ability to induce apoptosis and tumour suppression. p53 is able to elevate the expression of many genes that have pro-apoptotic roles. These include genes contributing to both the death receptor and the stress-induced pathways of apoptosis.

Targets in the death receptor pathway-

The death receptors, Killer/DR5 (Wu et al., 1997), Fas/CD95 (Owen-Schaub et al., 1995, Zalcenstien et al., 2003) and PIDD (p53-induced protein with a death domain; Lin et al., 2000) have been reported as transcriptional targets of p53. Caspase-8 promoter has also shown to be inducible by p53 through a site downstream of its transcription start site (Liedtke et al., 2003).

Targets in the Bcl-2 family-

The pro-apoptotic Bcl-2 family members, Bax (Miyashita and Reed, 1995; Thomborrow et al., 2002), PUMA (Nakano and Vousden, 2001; Yu et al., 2001),
NOXA (Oda et al., 2000a) and Bid (Sax et al., 2002) are transcriptionally induced by p53 and contribute to p53-mediated apoptosis.

**Targets in the apoptotic machinery**

Apaf-1 (Moroni et al., 2001; Kannan et al., 2001; Robles et al., 2001), the caspase-9 adaptor is transcriptionally upregulated by p53 and plays a pivotal role in the regulation of apoptosis after neuronal injury (Fortin et al., 2001). Human caspase-1 has been shown as a direct transcriptional target of p53, demonstrating that the executioners of apoptosis themselves can be regulated at the level of transcription (Gupta et al., 2001). Other members of the caspase family including caspase-6, 8 and 10 were subsequently also shown as transcriptional targets of p53 (MacLachlan and El-Deiry, 2002; Liedtke et al., 2003; Rikhof et al., 2003).

**Targets involved in redox regulation**

p53 can upregulate genes that affect redox metabolism and ROS (Reactive oxygen species) production such as REDD1/HIF-1 (Ellielsen et al., 2002), ferredoxin reductase and p53-inducible genes or PIGs (Hwang et al., 2001; Polyak et al., 1997).

**Other targets**

Several other proteins capable of inducing apoptosis have been shown to be p53-inducible. Scotin (Bourdon et al., 2002), mtCLIC/CLIC4 (Fernandez-Salas et al., 2002), PERP (Attardi et al., 2000), p53AIP1 (Oda et al., 2000b) are all p53-inducible genes capable of inducing apoptosis. ASC, an adaptor for Bax that promotes its translocation to the mitochondria to initiate apoptosis is also transcriptionally induced by p53 (Ohtsuka et al., 2004).

PML, a positive regulator of p53 transcription that targets p53 to the nuclear bodies, is itself induced by p53 in response to oncogenes and DNA damaging therapeutics thereby constituting a positive feedback loop (de Stanchina et al., 2004). A comprehensive list of all the transcriptional targets of the p53 family can be found in the reference, Harms et al; 2004.

**p53-mediated trans-repression**

p53 does not activate canonical target genes during hypoxia-induced apoptosis, suggesting that transactivation may not have a key role in this context. It
has been proposed that transcriptional repression might be crucial for the ability of p53 to respond to hypoxic stress (Koumenis et al., 2001; Johnson et al., 2005). Many gene array analysis studies have shown that p53 specifically curbs the expression of anti-apoptotic genes such as Bcl-2, MAP4 and survivin. Although the promoters of genes like survivin contain a p53-binding site, many genes that are repressed by p53 do not. It remains unclear how p53 specifically blocks the expression of these genes. Forming complexes with general repressor proteins like mSin3a may provide a potential explanation of how p53 may repress gene expression (Murphy et al., 1999; Zilfou et al., 2001).

Figure 1.4. p53 can trigger components of both the Extrinsic (Death Receptor) and Intrinsic apoptotic pathways. In addition, p53 directly localises to the mitochondria to mediate apoptosis.
Because of the large number of p53 target genes that have been identified and proposed to mediate apoptosis it has been unclear whether any single target is critical. The BH3-only molecules Puma and Noxa have merited particular attention in this regard. Loss of Puma protected lymphocytes from DNA-damage-induced apoptosis. Puma deficiency also protected cells against diverse p53-independent cytotoxic insults, including cytokine deprivation and exposure to glucocorticoids, staurosporine or PMA (Villunger et al., 2003). Mouse embryonic fibroblasts (MEFs) from Noxa-deficient mice showed increased resistance to DNA damage induced apoptosis, which was further increased by introducing an additional nullizygosity for Bax (Shibue et al., 2003). *The expression of p53-induced genes reveals striking tissue specificity with distinct regulation in different cells and tissue compartments, as well as variations in dependence on p53 for basal expression* (Fei et al., 2002). There are also marked differences in the affinities and damage specific recruitment of transcription initiation components to the p53-response elements in the different target genes (Espinosa et al., 2003). These observations may explain the reason why none of the p53 targets fully recapitulate the effect of the p53 knockout.

**Transcription-independent pro-apoptotic roles for p53**

There is growing evidence to suggest that p53 can induce apoptosis independently of its ability to bind DNA and activate transcription. A mutant p53 protein with substitutions of two residues within the TA domain (L22Q, W23S) failed to upregulate expression of particular target genes but retained the ability to induce apoptosis (Haupt et al., 1995). *A molecular basis for observations supporting a transcription-independent role came from the study showing evidence that p53 translocation to the mitochondria occurs in vivo in irradiated thymocytes. p53 can directly induce MOMP by forming complexes with Bcl-xL and Bcl-2, resulting in cytochrome c release. The interaction with Bcl-xL is through the DNA-binding domain of p53; tumour-derived p53 mutants (R175H, R273H, L194F and R280 K) are compromised in their ability to bind Bcl-xL. These tumour derived p53 mutations may therefore represent 'double hits' by abrogating the transcriptional and mitochondrial apoptotic activity of p53 (Mihara et al., 2003; Manfredi, 2003; Erster et al., 2004). p53 can also activate Bax in a transcription-independent manner and*
thereby mediate mitochondrial membrane permeabilization and apoptosis (Chipuk et al., 2004).

However, the relevance of p53-dependent apoptosis proceeding through transcription-independent mechanisms, in vivo has not yet been determined. Multiple lines of evidence support a role for the ability of p53 to function as a transcription factor as crucial for mediating cell cycle arrest and apoptosis.

(a) The mutant p53<sup>GS</sup> knock-in mice are severely compromised in the transactivation of p53 target genes like p21, Puma, Noxa, PERP and consequently are also compromised for apoptosis induced by DNA damaging agents like doxorubicin (Johnson et al., 2005).

(b) Puma, Noxa knockout mice show defects in apoptosis induction in response to DNA damage stimuli (Villunger et al., 2003). In general, a compromise in the activity of many well-characterised p53 target genes manifests as apoptotic or cell cycle arrest defects.

(c) In cases where transcriptional activation of target genes is dispensable, like in hypoxia-induced apoptosis, p53-mediated transcriptional repression appears to have a major role in inducing apoptosis (Johnson et al., 2005).

Therefore, transactivation/repression of its target genes by p53 and their consequent roles in apoptosis is crucial to its function as a tumour suppressor.

(c) Cellular Senescence

Senescence is a safeguard program that limits the growth potential, but not necessarily the viability of a dividing cell as a consequence of progressive shortening of telomeres. Regulators of cell-cycle checkpoints and cellular integrity such as p53 and p16<sup>INK4A</sup> are induced by various senescent stimuli like UV-radiation and anti-cancer agents (Kahlem et al., 2004). In an in vivo model of drug-senescence mouse lymphomas, repeated anti-cancer therapy eventually selected against the senescence controlling genes like p53 and p16<sup>INK4A</sup>, thereby producing relapse tumours that resumed growth in an aggressive manner. These studies implicate cellular senescence mechanisms mediated by p53 and other proteins as an important factor in tumourigenesis and anti-cancer therapy in vivo (Schmitt et al., 2002).
(d) DNA Repair

There is evidence for the participation of p53 in repair processes, and for a p53-dependent activation of DNA repair. The GADD45 gene, which has been linked to DNA repair, is a transcriptional target of p53 (Kastan et al., 1992). p53R2, a p53 target gene encodes a ribonucleotide reductase that is directly involved in the p53 checkpoint for repair of damaged DNA (Tanaka et al., 2000; Nakano et al., 2000). The identification of these genes as p53 targets supports a direct role for p53 in DNA repair, in addition to inhibition of growth of damaged cells. p53 interacts with components of DNA repair pathways and binds single-stranded DNA in a non-specific manner; it also binds to the ends of double stranded breaks, holliday junctions and DNA mismatches (Albrechtsen et al., 1999). These activities might be important for p53's ability to bind damaged DNA.

1.3.3 General Themes for Modulating p53 Activity

p53 can be thought of as a critical "node" of the cellular circuitry (Vogelstein et al., 2000). It is therefore not surprising that multiple mechanisms have evolved to regulate its activity (Brooks and Gu, 2003). The myriad of modulatory mechanisms probably exist both to tightly and rapidly control the activities of p53 and to provide alternative regulatory mechanisms for different cell types and different physiologic stimuli (Giaccia and Kastan, 1998).

(a) Protein stability: The p53-Mdm2 circuit

p53 is a short-lived nuclear protein with a half-life of 5-20 minutes in most cell types studied. Mdm2 (Mouse double minute2) protein functions as an E3-Ubiquitin ligase for p53 and targets p53 for rapid proteasomal degradation (Honda et al., 1997). Activation of p53 by either DNA damage or by oncogenes via p19 ARF are both associated with decreased binding of Mdm2 to p53 (Shieh et al., 1997; Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998). Mdm2 can also promote NEDD8 (a ubiquitin-like molecule) conjugation to itself and to p53; NEDDylation of p53 subsequently inhibits its transcriptional activity (Xirodimas et al., 2004).
1.3 Tumour Suppressor p53 – Guardian of the Genome

Ras, Myc oncogenic stress

Figure 1.5. The p53-Mdm2 Circuit.

(b) Binding proteins that modulate p53 activity

Many cellular and viral proteins interact with p53 and work by different mechanisms to influence p53 function. Apoptosis stimulating protein of p53 (ASPP) enhances DNA binding and transactivation function of p53 on the promoters of apoptotic genes in vivo. ASPP specifically enhances p53-induced apoptosis but not cell cycle arrest, and expression of ASPP is frequently down regulated in human breast carcinomas expressing wild type (wt) p53 but not mutant p53. ASPP can therefore regulate the tumour suppression function of p53 in vivo (Samuels-Lev et al., 2001).

Herpesvirus-associated ubiquitin specific protease (HAUSP) specifically deubiquitinates p53 both in vitro and in vivo, thereby stabilizing p53. HAUSP strongly stabilizes p53 even in the presence of excess Mdm2, and induces p53-dependent cell cycle arrest and apoptosis (Li et al., 2002). Glycogen synthase kinase-3β (GSK-3β), a central figure in Wnt signalling directly binds p53 and contributes to p53-mediated increase in p21 levels and caspase-3 activity (Watcharasit et al., 2002). Hypoxia (lack of oxygen in body tissues) is perhaps the most physiological inducer of the wild type p53 protein. Hypoxia increases p53 protein levels via induction of HIF-1α (Hypoxia Inducible factor), which can bind to and stabilize p53 (An et al., 1998).

The tumor suppressor p53 protein also binds to the products of several viral oncoproteins, including SV40 large T antigen, human papillomavirus (HPV) E6 and adenoviral E1B 55K. p53 was in fact initially isolated as a protein bound to the SV40 T antigen (Linzer and Levine, 1979; Lane and Crawford, 1979). These viral proteins target different regions of p53. SV-40 T antigen binds to p53’s core DNA-binding domain (Ruppert and Stillman, 1993; Li and Fields, 1993), whereas adenoviral E1B
55K binds to, and silences, p53’s N-terminal transactivation domain (Yew and Berk, 1992). HPV E6 interacts with p53’s core DNA-binding domain and C terminus and targets p53 for ubiquitin-dependent proteolysis (Scheffner et al., 1990; Werness et al., 1990).

(c) **Phosphorylation**

Several residues at both the amino terminus and the carboxyl terminus of p53 are phosphorylated or dephosphorylated in response to genotoxic stress. Phosphorylation events contribute to increased p53 half-life largely by decreasing the ability of Mdm2 to bind to p53 (Shieh et al., 1997). The ATM (Ataxia Telangiectasia Mutated) kinase has been demonstrated to phosphorylate Ser15 in response to ionizing radiation (IR). Both p53 Ser15 phosphorylation and total p53 levels are reduced in ATM null cells following IR (Kastan et al., 1992). IR also causes Ser376 dephosphorylation in the carboxyl terminal of p53 in an ATM-dependent manner enhancing the DNA binding ability of p53, although the phosphatase responsible for this has not yet been identified (Waterman et al., 1998). ATM is also required for the phosphorylation of p53 at Ser20 through the activation of Chk2 (Checkpoint kinase2) protein (Hirao et al., 2000).

Homeodomain-Interacting protein kinase-2 (HIPK2), a nuclear Ser/Thr kinase binds to and activates p53 by directly phosphorylating it on Ser46. In response to ultraviolet (UV) radiation, HIPK2 is activated and selectively phosphorylates p53. It also colocalizes with p53 and PML-3 in the nuclear bodies and promotes p53-dependent target gene expression (Hofmann et al., 2002; D’Orazi et al., 2002). p53DINP1 (p53-dependent damage inducible nuclear protein 1), a p53 target gene also regulates the phosphorylation of p53 at Ser46 in response to genotoxic stress to upregulate p53AIP-1 expression that subsequently causes apoptosis (Oda et al., 2000b; Okamura et al., 2001).

(d) **Acetylation**

p53 can be acetylated by the HATs, p300/CBP and pCAF at many residues in the carboxyl terminus. p53 is acetylated in vivo in response to a variety of cellular stress signals, at K372, K373, K381 and K382 by p300/CBP and at K320 by pCAF (Prives and Manley, 2001). Acetylation of p53 at these sites can stimulate the ability of p53 to bind DNA (Gu and Roeder, 1997; Sakaguchi et al., 1998). But this
increased ability to bind DNA is restricted to short oligonucleotides and does not seem to affect binding to sites within in the p21 promoter where they are present in a much longer DNA molecule (Espinosa and Emerson, 2001).

Acetylation of p53 might also be a mechanism to recruit deacetylases to eventually dampen expression. p53 can associate with mSin3a, a co-repressor to repress transcription of its target genes by recruiting HDACs (Murphy et al., 1999). Acetylation of p53 is stimulated by its positive regulator (phosphorylation) and abrogated by its negative regulator (Mdm2). This supports the idea that, however it works, acetylation of p53 is likely to be critical for it to function as a tumour suppressor (Prives and Manley, 2001).

(e) Methylation

In addition to acetylation and phosphorylation, a novel mechanism of p53 regulation through lysine methylation by the Set9 methyltransferase has recently been reported. Set9 specifically methylated p53 at K372 in its carboxyl-terminus regulatory region. Methylation increased the stability of p53 in response to DNA damage and methylated p53 was restricted to the nucleus. The expression of many p53 regulated genes like p21, Bax and Mdm2 in response to DNA damage was augmented by overexpression of the Set9 methyltransferase. Methylation may therefore represent an important DNA-damage-induced modification mark required for p53 function in vivo (Chuikov et al., 2004)

1.3.4 Inactivation of p53 in Human Tumours

The most commonly occurring loss of a pro-apoptotic regulator through mutation involves the p53 gene. While the p53 gene is mutated directly in about half of all human tumours (Hollstein et al., 1996), it is also becoming clear that in the vast majority of the remaining 50% of the tumours that retain wild type p53 expression, the function of the protein is severely compromised (Michael and Oren, 2002). Loss of p53 function endows cells with the ability to evade apoptosis, one of the key hallmarks of cancer (Hanahan and Weinberg, 2000; Green and Evan, 2002).

Mutations in the p53 gene

Unlike many other tumour suppressor genes, more than 85% of p53 mutations result in single amino-acid substitutions rather that deletions or
frameshifts. A comprehensive list of mutations in the p53 gene can be found in the p53 mutation database at http://p53.curie.fr. The vast majority of these mutations are within the central region of p53 (residues 102-292). DNA-binding defective p53 mutants are either contact mutants, wherein amino-acid residues that are essential for contacting DNA are mutated (R273, R248) or structural mutants, wherein key residues required for maintenance of p53 structure are mutated (R175, G245)(Slee et al., 2004).

Although considerable evidence supports the ability of p53 mutant alleles to inhibit wild type p53 function by exerting a 'dominant-negative' effect, the effects can be quite complex. For example, some mutant p53 alleles can inhibit the ability of wild type p53 to transactivate target genes involved in apoptosis but not those involved in cell cycle arrest (Aurelio et al., 2000). Point mutant alleles of p53 expressed under physiological control have been shown to have an enhanced oncogenic potential beyond the simple loss of p53 function (Olive et al., 2004). The pro-tumourigenic 'gain-of-function' effects of mutant p53 maybe attributed to its ability to inhibit other members of the p53 family, p63 and p73 (Lang et al., 2004). A role for p63 and p73 in p53-dependent apoptosis and tumour suppression has been an area of debate with experimental data that support as well as negate a tumour suppressive function for p63 and p73 (Flores et al., 2002; Senoo et al., 2004).

Abnormal expression of cellular regulators of p53

Affecting the levels and activity of the regulators of p53 can also compromise the apoptotic signalling cascade triggered by wild type p53. Augmented expression of Mdm2, the negative regulator of p53, can overcome tumour suppression by p53. Amplification of Hdm2 (human Mdm2) is found in many tumour types including soft-tissue sarcomas, osteosarcomas, lymphoblastic leukaemia and bronchogenic carcinoma (Oliner et al., 1992; Freedman et al., 1999). Overexpression of iASPP (inhibitor of ASPP) has also been found in many breast carcinomas that retain wild type p53 expression (Bergamaschi et al., 2003).

p53 function can also be nullified by inactivating mutations that encode positive regulators or effectors of p53 function. One such example is p14ARF (p19ARF in mouse) that signals oncogene activation in the cell and stabilizes p53 by blocking Hdm2 activity. Oncogenes like ras and β-catenin signal p53 stabilization through
1.4 Caspase-1 - An Inflammatory cum Apoptotic Protease

ARF, perhaps explaining the selective pressure for abrogation of ARF expression in colorectal and other cancers (Michael and Oren, 2002). Expression of ASPP proteins is also frequently down regulated in human breast cancers expressing wild type p53 (Samuels-Lev et al., 2001). Downstream of p53 activation, the p53 responsive gene Apaf-1 has been shown to be inactivated in chemo-resistant malignant lymphomas, a tumor type that rarely exhibits p53 mutation (Soengas et al., 2001).

Taken together, down-regulation of proteins that cooperate with p53 to induce cell death or the increased expression of p53 inhibitors are both mechanisms that can disrupt wild type p53 function in many human tumours.

1.4 Caspase-1 - An Inflammatory cum Apoptotic Protease

Caspase-1 or \( \text{IL-1}\beta \)-converting enzyme (ICE) was originally identified as the enzyme responsible for processing a 33kDa precursor pro-IL-1\( \beta \) to its 17kDa mature form and also as an enzyme bearing significant homology to the \textit{C.elegans} cell death gene, \textit{ced-3} (Cerretti et al., 1992; Yuan et al., 1993). IL-1\( \beta \) is a potent inflammatory mediator of the activation of endothelial and mononuclear cells, assigning a key role for caspase-1 in inflammatory pathways. Caspase-1 knockout mice are resistant to lipopolysaccharide (LPS) induced endotoxic shock ascertaining its role as an inflammatory caspase (Li et al., 1995; Kuida et al., 1995). Although caspase-1 deficient mice have no apparent defects in developmental programmed cell death, recent evidence has indicated that caspase-1 could be important for some cases of pathological cell death where cell stress occurs (Zhang et al., 2003). It is now realized that several caspases can play a role in both inflammatory and apoptotic cascades, including caspase -1, -11 and -12 (Zhang et al., 2003; Wang et al., 1998; Nakagawa et al., 2000).

Owing to its long N-terminal pro-domain, caspase-1 belongs to the group of initiator caspases and is therefore expected to act proximally in a caspase activation cascade (Creagh et al., 2003). Structural characterisation of caspase-1 revealed a homo-dimeric structure (Wilson et al., 1994); active caspase-1 enzyme is a hetero-tetramer of two p20 and two p10 subunits (Gu et al., 1995). The catalytic site is formed by amino acids from both the p20 and p10 subunits, with the active site cysteine located within the p20 subunit. The caspase-1 pro-domain contains a CARD (Caspase Recruitment Domain). By analogy with established caspase-activation
paradigms, the caspase-1 prodomain is likely to function as a protein-protein interaction motif, enabling recruitment of the latter to an activation complex (Bouchier-Hayes and Martin, 2002).

![Diagram of caspase-1](image)

**Figure 1.6.** Schematic representation of caspase-1 showing the sites of cleavage required for activation (shown in block arrows) to form the large (p20) and small (p10) subunits. The light arrow represents an additional cleavage site.

### 1.4.1 Caspase-1 Activation Pathways

Although data on the mechanism of caspase-1 activation are still far from complete, recent studies have proposed several possible routes to caspase-1 processing. To date, a number of candidate caspase-1 adapters have been identified.

(a) **CARDIAK/RICK/RIP2**

The first molecule to be proposed as a caspase-1 activating adapter was the CARD-containing IL-1β-converting enzyme associated kinase (CARDIAK) also called RICK or RIP2 (Receptor Interacting Protein2). RIP2 is capable of interacting with caspase-1 through its C-terminal CARD and inducing the processing of full-length caspase-1 on over expression (Thome *et al.*, 1998). However, RIP-2 deficient mice do not show defects in IL-1β processing. RIP2 has also been implicated in NF-κB activation and signalling events in innate and adaptive immune responses (Kobayashi *et al.*, 2002; Chin *et al.*, 2002). RIP2 is induced by hypoxia/ischaemia in neuronal cells and activates caspase-1 processing in response to these stresses (Zhang *et al.*, 2003).
(b) Ipaf/CLAN/CARD12

Ipaf, an Apaf-1 like adapter and a CED-4 homologue can recruit and promote caspase-1 processing (Poyet et al., 2001; Damiano et al., 2001; Geddes et al., 2001). Ipaf contains a N-terminal CARD, a central NBD (Nucleotide Binding Domain) and multiple leucine rich repeats (LRRs) at its carboxyl terminus. Ipaf interacts with caspase-1 through a mutual CARD-CARD interaction and removal of the LRRs induces caspase-1 dependent apoptosis in cells (Poyet et al., 2001). Macrophages from Ipaf null mice are resistant to cell death induced by Salmonella typhimurium infection, suggesting a role for Ipaf in apoptosis induced by intracellular pathogens (Mariathasan et al., 2004). If a scaffold similar to Apaf-1 (adapter for caspase-9) is required, the best current candidate for caspase-1 activation during apoptosis seems to be Ipaf (Adams, 2003).

(c) ASC/PyCARD

Asc (Apoptosis-associated speck like protein with CARD) is a bipartite molecule containing a CARD and a pyrin motif (both these motifs are members of the death domain super family), which has also been shown to be an activating adapter for caspase-1 (Srinivasula et al., 2002). The PyPAF (Pyrin-containing Apaf-1 like proteins) family of proteins, specifically PyPAF -1, -5 and -7 assemble with Asc and result in NF-κB activation and in some cases, caspase-1 activation (Manji et al., 2002; Wang et al., 2002; Grenier et al., 2002). Asc-deficient macrophages exhibited defective maturation of IL-1β and Asc-null mice were resistant to LPS induced endotoxic shock (like caspase-1 knockout mice). Activation of caspase-1 in response to Salmonella typhimurium was also severely abrogated in Asc-null macrophages (Mariathasan et al., 2004). This evidence is concurrent with the role of Asc as a physiological inducer of caspase-1 processing and is emphasized by the presence of Asc in the caspase-1 activating complex, the inflammasome.

The Inflammasome: A macromolecular complex for caspase-1 activation

A novel molecular complex containing active caspase-1 has been described to link pro-IL-1β to its upstream activators like LPS. This complex is composed of Asc, NALP1 (a pyrin and CARD containing protein), caspase-1 and caspase-5. NALP1 promotes formation of the multi-protein caspase-1/caspase-5 activating
complex by recruiting Asc (Martinon et al., 2002). The stimulus downstream of LPS interaction with TLR4 (Toll like receptor 4), which prompts the assembly of the inflammasome, is yet to be elucidated.

Figure 1.7. The Inflammasome. A macro-molecular complex that triggers caspase-1 activation and subsequent IL-1β production in response to pro-inflammatory stimuli like lipopolysaccharide (LPS) (taken from Creagh et al., 2003).

Proteins homologous to NALP1 namely, NALP2 and NALP3/cryopyrin also associate with Asc, the CARD-containing protein Cardinal, and caspase-1 (but not caspase-5), forming an inflammasome with high pro-IL-1β-processing activity (Agostini et al., 2004). The NALP3/cryopyrin inflammasome induces caspase-1 activation in response to muramyl dipeptide, a component of the bacterial
peptidoglycans, independent of the Toll like receptors or TLRs (Martinon et al., 2004).

**Negative Regulation of Caspase-1**

In addition to the positive regulators of caspase-1 activation discussed above, proteins capable of negatively regulating caspase-1 processing and activation have also been identified. Three CARD-only proteins, PseudoiCE/COP (CARD only protein), ICEBERG and CARD8 have been identified that bind the CARD of caspase-1 and negatively regulate its activity (Humke et al., 2000; Druilhe et al., 2001; Razmara et al., 2002). These proteins are thought to act in a dominant-negative manner by competing with the caspase-1 activating adapters for binding the caspase-1 CARD.

**1.4.2 Pro-apoptotic Roles for Caspase-1**

Evidence for caspase-1 activation has been demonstrated in a variety of *in vitro* and *in vivo* cell death paradigms.

**Role of caspase-1 in *Salmonella*, *Shigella*-induced macrophage cell death**

The bacterial pathogen, *Shigella* induces macrophage apoptosis by directly activating caspase-1 through its invasion plasmid antigen B (IpaB). Caspase-1 is activated and processed after *Shigella* infection, macrophages from caspase-1 knockout mice are also resistant to *Shigella*-induced apoptosis (Hilbi et al., 1998). Another pathogenic bacterium, *Salmonella* also induces cell death in macrophages through the *Salmonella* invasive protein B (SipB) that interacts with caspase-1 and causes its activation (Hersh et al., 1999). Macrophages from caspase-1 deficient mice, as well as those from mice deficient in any one of the caspase-1 activators, Asc or Ipaf, are resistant to *Salmonella* induced cell death (Mariathasan et al., 2004). Caspase-1 is therefore responsible for macrophage cell death and acute inflammation characteristic of pathogenic infections of *Shigella* or *Salmonella*.

**Role of caspase-1 in ischaemia and hypoxia induced cell death in the nervous system**

Both caspase-1 and caspase-3 are activated in neurons following spinal cord injury and ischaemia in the mouse brain (Li et al., 2000a; Hara et al., 1997a).
Inhibition of caspase activity by using the broad-spectrum inhibitor, ZVAD-fmk can reduce the neuronal damage caused by cerebral ischaemia (Rabuffetti et al., 2000; Hara et al., 1997a). Ischaemic brain injury is reduced in caspase-1-deficient mice suggesting that caspase-1 contributes to the pathology of ischaemia (Schielke et al., 1998). Furthermore, a dominant negative caspase-1 mutant (C285G) could prevent neuronal cell death induced by trophic factor withdrawal and ischaemic brain injury (Friedlander et al., 1997; Hara et al., 1997b).

Functional roles for caspase-1 in the pathology of neurodegenerative diseases like the Huntington's disease (HD) and familial amyotrophic lateral sclerosis (ALS) have also been demonstrated (Ona et al., 1999; Li et al., 2000b). Dominant negative mutant of caspase-1 extends survival and delays the pathology in a transgenic mouse model of HD (Ona et al., 1999). Minocycline, a tetracycline derivative, delays mortality in the same mouse model because of its ability to inhibit caspase-1 and caspase-3 expression (Chen et al., 2000). Intranuclear huntingtin (polyglutamine repeats in huntingtin protein is responsible for HD) can increase expression of caspase-1 and increased levels of caspase-1 contribute to HD-associated cell death (Li et al., 2000c).

Caspase-1 has also been demonstrated to be an apical caspase in neuronal cell death pathways induced by hypoxia and ischaemia. The upstream activator of caspase-1, RIP2 is inducible by hypoxia/ischaemia and regulates caspase-1 activation in the neurons. Bid cleavage, release of mitochondrial apoptogenic factors (cytochrome c, smac/Diablo and AIF) and activation of caspase-9 and 3 are all compromised in neurons from caspase-1-deficient mice (Zhang et al., 2003).

Role of caspase-1 in triggering cell death in other systems

Studies on caspase-1 knockout mice have revealed the role of caspase-1 in mediating apoptosis in different tissue types, in response to diverse stress stimuli.

- Caspase-1 knockout mice show impaired Fas-mediated killing of lymphocytes implicating caspase-1 function in Fas-mediated apoptosis (Enari et al., 1996; Kuida et al., 1995).
- Endothelial cells from caspase-1 knockout mice are resistant to apoptosis induced by serum withdrawal showing a direct role for caspase-1 in mediating this cell death pathway (King et al., 2003).
1.4 Caspase-1: An Inflammatory and Apoptotic Protease

- A function for caspase-1 in modulating apoptosis in neutrophils has also been described. Caspase-1 deficient peripheral blood neutrophils show delayed constitutive apoptosis when cultured in vitro as compared to wild-type neutrophils (Rowe et al., 2002).

- Caspase-1 contributes to the pathogenesis of cisplatin-induced acute renal failure. In this model of cisplatin injury, caspase-1 has been shown to activate caspase-3 to trigger apoptosis (Faubel et al., 2004); caspase-1 is also known to process caspase-3 in vitro (Van de Craen et al., 1999).

Apaf-1 and caspase-9 (apoptosome) deficient lymphocytes also undergo apoptosis in response to cytokine-withdrawal, γ-radiation, etoposide and dexamethasone; this cell death is compromised on Bcl-2 over expression. Processed caspase-1 was detected in these dying cells raising the possibility that caspase-1 may act as an initiator caspase and trigger an apoptotic cascade independent of the apoptosome (Marsden et al., 2002).

The tumour-suppressor p53 can directly upregulate human caspase-1 mRNA levels and a dominant negative mutant of caspase-1 could inhibit apoptosis induced by p53 over expression or doxorubicin (genotoxic agent) treatment in MCF-7 cells. This suggests that caspase-1 may be a mediator of p53-dependent apoptosis in human cells (Gupta et al., 2001; Gupta et al., 2002).

1.4.3 Transcriptional Regulation of Caspase-1

Caspases are produced as zymogens that are cleaved on the receipt of relevant stimuli to ensure activation only when needed. Regulation of caspases at the level of transcription also offers a handle to modulate apoptotic or inflammatory responses in response to specific stimuli.

The cytokine interferon-gamma (IFN-γ) is a potent inducer of caspase-1 transcription. This is largely mediated by the activation of interferon regulatory factors (IRFs) and STATs. A functional IRF-1 binding site in the caspase-1 promoter allows for transcriptional upregulation of caspase-1 mRNA (Iwase et al., 1999). IRF-1 is also involved in mediating DNA-damage induced apoptosis in lymphocytes and IRF-1 mediated caspase-1 gene expression may contribute to apoptosis in these cells (Tamura et al., 1995; Tamura et al., 1997). Tamoxifen-induced apoptosis in human mammary epithelial cells also proceeds through IRF-dependent signalling.
events that result in caspase-1 expression and a subsequent activation of caspase-3 (Bowie et al; 2004). IFN-γ induced apoptosis in pancreatic islet cells and ovarian carcinoma cells is also dependent on IRF-1 induced caspase-1 gene expression (Karlsen et al., 2000; Kim et al., 2002).

IFN-γ induced STAT-1 as well as over expressed STAT-1 can activate transcription from the caspase-1 promoter, although a STAT-1 responsive site has not yet been identified in the caspase-1 promoter (Chin et al., 1997; Stephanou et al., 2000). STAT-1 appears to be required for the constitutive expression of caspase-1 (Kumar et al., 1997).

LPS is a strong inducer of caspase-1 expression and consequent IL-1β generation (Tingsborg et al., 1996). Induction of caspase-1 mRNA in different tissues in response to LPS has been described but the cis-regulatory sequences responsible for LPS-responsiveness are not known. The hormone estrogen plays a critical role in protection from apoptosis. In several cell types, withdrawal of estrogen leads to increased apoptosis in tissues such as brain, endothelium, testes and uterus. Caspase-1 transcription is rapidly stimulated after estrogen withdrawal and probably contributes to apoptosis in these tissues (Monroe et al., 2002).

High levels of visible light induce apoptosis of photoreceptors, a process dependent on the activation of the transcription factor, AP1. Expression of caspase-1 increased after light exposure in the retinas of wild type but not c-fos-deficient mice, which are protected from light induced apoptosis in the retina. This suggests the contribution of an AP1 dependent caspase-1 expression in light induced degeneration of the retina (Grimm et al., 2000). Caspase-1 mRNA is also upregulated in many pathological conditions including pneumococcal meningitis (Von Mering et al., 2001), ALS (Ando et al., 2003), ischaemia and Huntington's disease (Friedlander et al., 2003). The increased caspase-1 levels are believed to contribute to the pathology of these diseases.

1.5 Background and Objectives of the Current Study
p53 is a transcription factor that mediates its biological effects largely by the transcriptional activation of its target genes. The target genes identified till date for p53 are predominantly involved in mediating cell cycle arrest (like p21) or in triggering apoptosis (like Bax, Puma, caspase-1).
Human Caspase-1 is a direct transcriptional target of p53. A p53-binding site in the minimal promoter region of the human caspase-1 gene allows for its p53-dependent induction (Gupta et al., 2001). Caspase-1 is also one of the mediators of p53-dependent apoptosis in human cells because blocking caspase-1 function either by a chemical inhibitor or by mutant caspase-1 (a dominant-negative) inhibits p53-induced as well as doxorubicin-induced apoptosis. However, overexpression of caspase-1 by itself induces much less apoptosis than p53 (Gupta et al., 2002). Therefore it is likely that an activator of caspase-1 may also be induced by p53 to mediate its apoptotic program through a caspase-1 dependent pathway.

Caspase-1, like all other caspases, is produced as an inactive zymogen and is subsequently processed to its active form. Many activators that can cause pro-caspase-1 processing have been identified. These include Asc (Apoptosis-associated speck like protein with CARD), Ipaf (ICE protease activating factor) and RIP2 (Receptor interacting protein 2). In this study we have focussed on the gene regulation of the caspase-1 activator, Ipaf, by p53 and its role in apoptosis mediated by p53.

The main objectives of this work have been-

a) To study the regulation of Ipaf gene expression by p53 and DNA damage including an analysis of the mechanism by which p53 activates transcription of the Ipaf gene.

b) To analyse the contribution of Ipaf to p53-dependent apoptosis.

c) To study the role of p53 family members, p73α and p73β in regulating Ipaf gene expression.

Chapter 2 provides the list of reagents used and the detailed description of methodologies used in this study.

Chapter 3 describes results showing the transcriptional induction of Ipaf mRNA levels by exogenous as well as endogenous p53. Over-expression of p53 as well as p73 (another p53 family member) increased the levels of Ipaf gene expression. Genotoxic stresses that increase endogenous p53 levels in the cell, like treatment with doxorubicin, also induced the expression of Ipaf. The Ipaf promoter region was subsequently cloned and shown to be p53-responsive in promoter-reporter assays.
The results presented in this chapter conclusively show that the caspase-1 activator, lpaf is a p53-inducible gene.

Chapter 4 describes results showing the involvement of lpaf in p53-mediated apoptosis. Two independent strategies to inhibit lpaf function were used. A dominant negative mutant was made which is expected to inhibit the function of lpaf protein. The RNA interference strategy was used to specifically down regulate p53-induced lpaf gene expression. Blocking lpaf function by both these strategies compromised p53 and doxorubicin mediated apoptosis in human tumour cell lines. These results establish a role for lpaf in p53-mediated apoptosis. In addition these results suggest the recruitment of an lpaf-caspase-1 pathway in mediating p53-dependent apoptosis.