Chapter 5

REGULATION OF THE P53 RESPONSE BY CELLULAR GROWTH AND SURVIVAL FACTORS

Lauren Brown and Samuel Benchimol
Ontario Cancer Institute / Princess Margaret Hospital and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

INTRODUCTION

In response to abnormal proliferative signals and many forms of cellular stress including DNA damage and ribonucleotide depletion, p53 induces cells to undergo a transient arrest in G1 that is believed to allow time for repair of damaged DNA before the initiation of S phase. Failure to arrest in G1 can lead to chromosome aberrations and genomic instability. Activated p53 can also eliminate cells from the proliferative population through mechanisms that involve prolonged arrest in G1 (as seen during telomere-initiated replicative senescence and stress/DNA damage-induced premature senescence) and apoptosis (Levine, 1997; Oren, 2003; Vogelstein et al., 2000). The elimination of damaged, stressed or abnormally proliferating cells by p53 is considered to be the principal means by which p53 mediates tumour suppression (Symonds et al., 1994; Schmitt et al., 2002). Inappropriate or prolonged activation of p53 in normal tissues, however, can lead to tissue damage and has been associated with multiple sclerosis (Wosik et al., 2003), neurodegenerative disorders and exacerbation of ischemic damage from stroke or cardiac arrest (Mattson et al., 2001; Komarova and Gudkov, 2001). Accordingly, the regulation of p53 function is important for the maintenance of tissue homeostasis.

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p53-mediated apoptosis is dependent on the Apaf-1/caspase-9 pathway (Soengas et al., 1999) and involves mitochondrial cytochrome c release (Schuler et al., 2000). How p53 elicits the release of cytochrome c to promote caspase activation remains elusive. p53-mediated apoptosis involves transcriptional regulation of target genes (Chao et al., 2000; Jimenez et al., 2000) as well as transcription-independent functions of p53, possibly reflecting distinct mechanisms of p53 action in different cell types (Oren, 2003; Vousden, 2000; Benchimol, 2001). A number of p53-regulated genes have been identified and some of these promote apoptosis when overexpressed. A subset has been shown, additionally, to attenuate apoptosis when disrupted through antisense RNA, siRNA or gene deletion methods including: *Bax* (Miyashita and Reed, 1995), *Noxa* (Oda E. et al., 2000; Shibue et al., 2003; Villunger et al., 2003), *Puma* (Villunger et al., 2003; Nakano and Vousden, 2001; Yu et al., 2001, 2003; Jeffers et al., 2003), *PERP* (Ihrie et al., 2003), *p53AIP1* (Oda K. et al., 2000), *Pidd/Lrdd* (Lin et al., 2000), *p53DINP1* (Okamura et al., 2001), *PAC1* (Yin et al., 2003), *UNC5H2* (Tanikawa et al., 2003), and *TSAP6* (Passer et al., 2003). Bax, Noxa, Puma and p53AIP1 proteins are localized at the mitochondria and each has been shown to associate with Bcl-2. So far, however, no single molecule can be considered to be the principal mediator of p53-dependent apoptosis.

It remains unclear why certain cells undergo apoptosis in response to p53 activation while other cells undergo p53-dependent cell cycle arrest. Differences in the cellular response to p53 activation have been attributed to extracellular survival factors and to intrinsic factors that might reflect differences in DNA repair, p53 expression and activation, intracellular death/survival pathways, oncogene activation, or selective transactivation/ repression of p53-target genes in different cell types. For example, normal fibroblasts undergo p53-dependent G1 arrest in response to DNA damage whereas hyperproliferative fibroblasts such as those expressing ectopic E1A, c-myc or E2F-1 undergo p53-dependent apoptosis (Levine, 1997); cells expressing ectopic Bcl-2 or Bcl-X_L are protected from p53-dependent apoptosis (Chiou et al., 1994; Schott et al., 1995; Wang et al., 1993) and constitutively active PI3K and PKB delay the onset of p53-mediated apoptosis (Lin et al., 2002; Sabbatini and McCormick., 1999). Promoter selectivity by p53 may also contribute to cellular outcome (Oren, 2003). This could reflect differences in the affinity of various promoters for p53, such that some are responsive only to high levels of p53 or to certain modified forms of p53 (Resnick-Silverman et al., 1998). Beside covalent modification of p53, promoter selectivity leading to cell cycle arrest or apoptosis can be regulated by the interaction of p53 with other proteins including ASPP, JMY, WT1, BRCA1, p63 and p73 (Oren, 2003; Flores et

al., 2002; Vousden and Lu, 2002). Here we describe how anti-apoptotic Bcl-2 family members and the MAPK and PI3K/PKB signalling pathways regulate the cellular response to p53 activation.

ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS

The cellular decision to undergo apoptosis is governed by the integration of death and survival signals. The mitochondrial death pathway is triggered by a variety of stress-induced signals, including genotoxic agents, metabolic inhibitors and inadequate growth factor stimulation. These signals act initially on proapoptotic members of the BH3-only subset of the Bcl-2 family of proteins (e.g. Bid, Bim, Bmf, Bik, Noxa, Puma), which associate with anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-X_L, Mcl-1) residing in the outer mitochondrial membrane and neutralize their ability to maintain membrane integrity. This, combined with the oligomerization of other pro-apoptotic family members (e.g. Bax and Bak), results in mitochondrial damage and release of mitochondrial proteins including cytochrome c and other apoptogenic factors that lead to caspase activation and apoptosis (Cory and Adams, 2002) . The ratio of anti- to pro-apoptotic Bcl-2 family members is thought to determine the susceptibility of a cell to undergo apoptosis. Survival and death signals influence the concentration and activity of anti- and pro-apoptotic Bcl-2 family members, tipping the balance in favour of cell survival or cell death. Overexpression of Bcl-2 and other anti-apoptotic family members in cancer attests to the importance of this family of oncoproteins in suppressing apoptosis and prolonging malignant cell survival (Cory et al., 2003). The expression of anti-apoptotic Bcl-2 proteins correlates with the survival of numerous hematopoietic cell lines in the presence of their lineage-specific cytokines (Lotem and Sachs, 1999).

Cytokine suppression of p53 apoptosis by up-regulation of anti-apoptotic Bcl-2 proteins

Cytokines have a well-documented role in apoptosis suppression, illustrated by the requirement of colony stimulating factors (G-CSF, M-CSF and GM-CSF), interleukin-3 (IL-3) and erythropoietin (EPO) to maintain the viability of hematopoietic cells in culture (Lotem et al., 1991; Williams et al., 1990; Koury and Bondurant, 1990). In addition to apoptosis induced by growth factor withdrawal, hematopoietic cells undergo apoptosis upon exposure to γ-irradiation, treatment with chemotherapeutic agents as well as forced expression of wild-type p53 (Yonish-Rouash et al., 1991; Canman et

al., 1995; Abrahamson et al., 1995; Quelle et al., 1998; Lin and Benchimol, 1995). In some cases, apoptosis that is dependent upon p53 can be suppressed when cells are cultured in the presence of their lineage-specific cytokines. Cells that are rescued from apoptosis remain in a viable, growth arrested state. The common ability of certain cytokines to suppress p53-induced apoptosis is striking and may reflect a mechanism by which tumours that retain wild-type p53 gain resistance to apoptosis-inducing anti-cancer agents (Lotem and Sachs, 1999).

EPO and IL-3 bind to type I cytokine receptors, causing receptor dimerization. Lacking intrinsic kinase activity, type I cytokine receptors recruit members of the Janus kinase (JAK) tyrosine kinase family to mediate phosphorylation of tyrosine residues located within the intracellular portion of the receptor dimer (Wojchowski et al., 1999). An immediate downstream target of JAK2 after EPO activation is Signal Transducer and Activator of Transcription 5 (STAT5) and it has been proposed that STAT5-dependent transcriptional up-regulation of Bcl-X_L mediates survival downstream of EPO (Socolovsky et al., 1999, 2001). In contrast, EPO has been shown to up-regulate Bcl-2 and Bcl-X_L transcripts in cells expressing EPO-R mutants incapable of activating STAT5 (Quelle et al., 1998). Using an erythroleukemia cell line expressing a temperature sensitive p53 mutant (p53ts) that can be induced to undergo p53 dependent apoptosis at 32^{o}C, we have shown that EPO promotes survival and suppresses p53-dependent apoptosis through a mechanism that is dependent on JAK2 but independent of STAT5. Moreover, we observed that EPO stimulation resulted in an increase in Bcl-X_L expression that was regulated primarily through a posttranscriptional mechanism involving Bcl-X_L protein modification (Lin et al., 2002). Although the mechanism regulating Bcl-X_L expression in response to EPO is controversial (Socolovsky et al., 1999; Teglund et al., 1998), the importance of Bcl-X_L as a mediator of EPO-dependent erythroid survival is well established by animal studies. Bcl-X_L deficient mice have severe hematopoietic defects resulting from massive cell death of erythroid progenitors and JAK2 deficient mice die in utero from a block in definitive eythropoiesis, a maturation program during embryogenesis when red blood cell production switches from the yolk sac to the fetal liver (Motoyama et al., 1995; Parganas et al., 1998). The phenotype of JAK2 deficient mice bears a striking resemblance to that of EPO and EPO-R deficient mice (Wu et al., 1995). Ectopic Bcl-X_L expression alone has been shown to substitute for EPO during differentiation of primary mouse erythroblasts in culture. Hence, the primary role of EPO during erythropoiesis appears to be apoptosis protection through the up regulation of Bcl-X_L protein expression, and terminal erythroid differentiation of the surviving cells is thought to depend on an intrinsic default differentiation program (Dolznig et al., 2002).

How do cytokines rescue cells from p53-dependent apoptosis and regulate Bcl-X_L and/or Bcl-2 expression? The dependency of this survival signal upon JAK2 is established, however, the signalling components that connect JAK2 activation and the activation of anti-apoptotic Bcl-2 proteins is not fully understood (Lin et al., 2002; Quelle et al., 1998). Pro-survival cytokines activate STAT5, MAPK and PI3K signalling pathways, and the relative importance of these pathways in providing protection against p53-induced apoptosis, is an area of intense investigation. We have observed that EPO-suppression of p53-dependent apoptosis is independent of PI3K (Lin et al., 2002) and the three MAPK pathways (unpublished data). These experiments also revealed that chemical inhibition of PI3K markedly increased p53-dependent apoptosis suggesting that intrinsic levels of activated PI3K/PKB, commonly present in transformed cells, limit the ability of p53 to induce cell death (Lin et al., 2002). This could be problematic for gene therapy approaches that attempt to reconstitute p53 expression in p53 null tumours with the expectation of inducing apoptosis. The observation that survival pathways impinge on p53-dependent cell death is widespread across many cell types. The following sections discuss mechanisms by which the MAPK and PI3K/PKB pathways interact with p53 and regulate the cellular response to p53 activation.

MAPK PATHWAYS

Ras/RAF/MEK/ERK

The Ras/Raf/MEK/ERK mitogenic activated protein kinase signalling pathway (Ras/ERK) has a well-documented role in suppressing apoptosis downstream of survival-promoting growth factors in cell types ranging from cultured murine fibroblasts and rat neurons to the developing Drosophila eye and nervous system (Bergmann et al., 1998, 2002; Xia et al., 1995; Gardner and Johnson, 1996; Parrizas et al., 1997; Kurada and White, 1998) . Upon growth factor binding, Receptor Tyrosine Kinases (RTKs) dimerize and activate Ras through the interaction of adaptor proteins that recognize phosphorylated tyrosines residues within the cytoplasmic domain and recruit GDP-bound Ras to the membrane. SOS, a guanine nucleotide exchange factor, then catalyzes the exchange of GDP for GTP, generating activated GTP-bound Ras, which in turn activates downstream kinases in the signalling cascade (Figure 1). The Ras/ERK signalling pathway promotes survival through transcriptional and post-transcriptional processes.

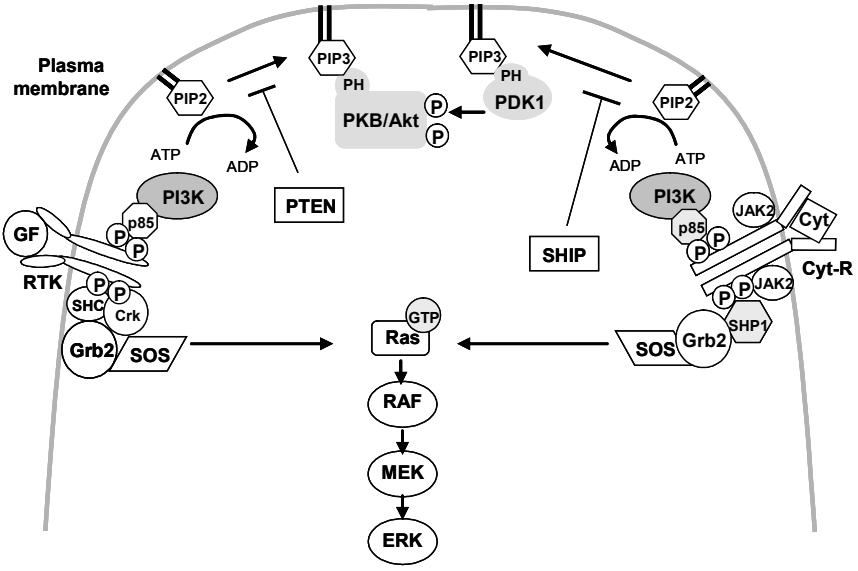

Figure 1. Activation of the Ras/MAPK and PI3K signalling pathways by growth factor (GF) binding to growth factor receptor tyrosine kinases (RTK) and pro-survival cytokine (Cyt) binding to cytokine receptors (Cyt-R).

ERK1/2 activate pp90 ribosomal S6 kinase (pp90rsk) which in turn phosphorylates and inactivates the pro-apoptotic Bcl-2 family member BAD on Serine residue 112 (Shimamura et al., 2000; Bonni et al., 1999). Phosphorylated BAD is bound by 14-3-3 proteins and sequestered in the cytoplasm, rendering it incapable of inhibiting the action of anti-apoptotic Bcl-2 family members at the mitochondrial membrane (Zha et al., 1996). In neurons, BDNGF-mediated survival is dependent on Ras/ERK-mediated phosphorylation and activation of the CREB transcription factor (Bonni et al., 1999). In hematopoietic cells treated with GM-CSF and thrombopoietin, cell survival involves pp90rsk-mediated phosphorylation of CREB on Ser 133 (Kwon et al., 2000; Zauli et al., 1998). Transcriptional targets of CREBl that may play a direct role in apoptosis suppression downstream of the Ras/ERK pathway include the anti-apoptotic genes *bcl-2*, and *bag-l* (Riccio et al., 1999; Wilson et al., 1996; Perkins et al., 2003). pp90rsk activation requires phosphorylation by both ERK and phospho-inositide-dependent kinase 1 (PDK1), activated by phospholipid second messengers generated by PI3K (Richards et al., 1999; Jensen et al., 1999). Thus, Ras/ERK signalling represents one of two pathways that contribute to cell survival through pp90rsk .

Ras/ERK signalling and p53

A number of studies have investigated the connection between the Ras/ERK signalling pathway and p53 activation (Figure 2). A complex and incomplete picture has emerged in which the Ras/ERK pathway converges upon p53 and has opposing effects on p53 function. The outcome of these opposing effects is likely determined by cell type or growth conditions (Ries et al., 2000). Some studies place p53 and Ras/ERK signalling components within the same linear pathway with p53 acting upstream or downstream of Ras/ERK. Other studies propose that Ras/ERK signalling operates in a parallel pathway to facilitate/oppose p53 functions.

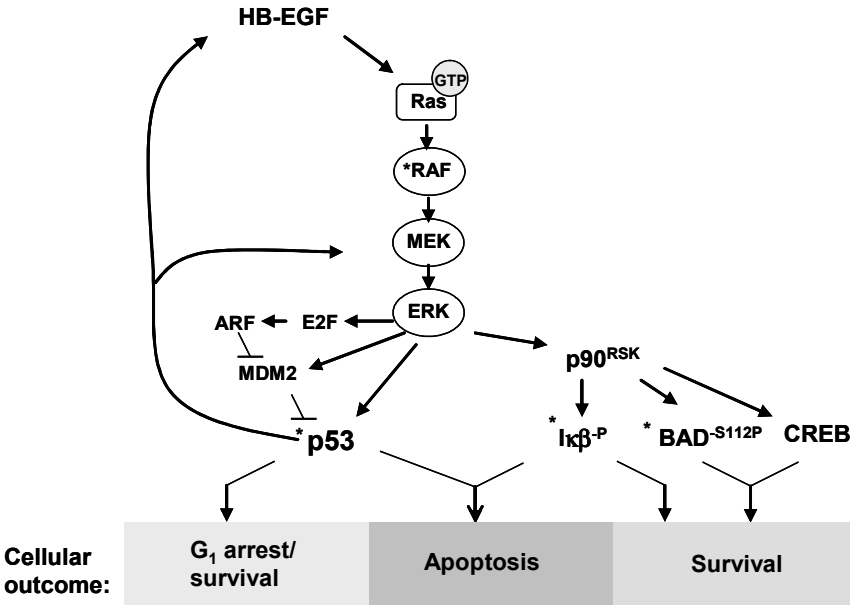

Figure 2. Apoptotic and Survival pathways induced downstream of the Ras/MAPK signalling pathway. * Denotes proteins regulated by multiple signaling pathways.

Over the past decade, a number of groups have shown that primary human cells exposed to DNA damage or oncogenic stimulation undergo a prolonged p53-dependent and Rb-dependent arrest in G1, and exhibit a senescence-like state that is commonly referred to as "premature senescence" (Di Leonardo et al., 1994; Serrano et al., 1997; Wright and Shay, 2002). Ras-induced growth arrest is dependent upon Raf-1 and MEK1 kinases, and is associated with an increase in ERK kinase activity (Serrano et al., 1997; Lin et al., 1998; Zhu et al., 1998). Oncogenic Ras, as well as

constitutive activation of the Ras/ERK signalling cascade is associated with increased expression of p53, p16INK4a and p19ARF (Ries et al., 2000; Serrano et al., 1997; Agarwal et al., 2001). Ras/ERK signalling is essential for activation of cyclin D transcription, resulting in the generation of cyclin D/cdk4 activity that leads to Rb phosphorylation and E2F1 activation. E2F1 induces p19ARF expression, likely through direct transcriptional activation via E2F sites in the ARF promoter. p19ARF binds to Mdm2 and blocks its interaction with p53 resulting in p53 stabilization (Pomerantz et al., 1998; Kamijo et al., 1998). Mdm2 acts as a negative regulator of p53 through a direct interaction that targets p53 for ubiquitin-mediated degradation. Activation of the Ras/ERK pathway also results in elevated levels of Mdm2 (Ries et al., 2000). Thus, p53 protein levels are determined by a balance between these opposing effects of the Ras/ERK pathway.

ERK activation was also shown to increase the level of p53 mRNA and this effect could be blocked by treatment with the MEK inhibitor U0126 (Agarwal et al., 2001). Two studies reported that ERK could phosphorylate p53 on Ser15, a modification that disrupts the MDM2-p53 interaction resulting in p53 protein accumulation (Persons et al., 2000; Wang et al., 2001). Two recent studies indicate that p53 can activate the Ras/ERK pathway. Using a p53-inducible cell model, Ryan et al. (Ryan et al., 2000) reported that p53 expression resulted in NF-kB activation involving the Ras/ERK pathway and activation of pp90rsk. NF-kB activation and apoptosis in response to inducible p53 expression were blocked by treatment with a MEK1 inhibitor (Ryan et al., 2000). This study provides a rare instance in which NF-kB is associated with pro-apoptotic activity rather than survival. Aaronson and colleagues have identified HB-EGF (heparin-binding EGF-like growth factor) as the product of a p53-responsive gene. HB-EGF is secreted and through its interaction with the EGF receptor is capable of activating the Ras/ERK pathway. p53-induced HB-EGF protects cells from death in response to oxidative stress and DNA damage through ERK activation and might facilitate cell cycle rentry after DNA repair is complete (Fang et al., 2001; Lee et al., 2000).

It is pertinent to consider potential differences between oncogenic mutant Ras and normal Ras proteins in initiating the Ras/ERK signalling cascade and how this might impact on cell survival or cell death. Physiological activation of this pathway by normal Ras proteins might produce a transient and less intense signal compared with oncogenic mutant Ras proteins that produce an intense and prolonged signal (Sewing et al., 1997; Woods et al., 1997). The cellular response to these two types of signals may be profoundly different; the former leading to proliferation and survival and the latter leading to p53 activation, and cell cycle arrest or apoptosis in an effort to suppress neoplasia and eliminate oncogene-expressing cells. Sustained

ERK activation in response to oncogenic Ras may lead to inappropriate accumulation of phosphorylated substrates and activation of transcription factors that would otherwise not occur in response to a transient signal from normal Ras (Marshall, 1995).

MEKK1/MKK(4 and 7)/JNK

Of the three known JNK family members, JNK1 and 2 are ubiquitously expressed whereas JNK 3 is expressed primarily in the brain, heart and testis (Gupta et al., 1996; Ip et al., 1998). Each is able to activate the c-jun transcription factor by phosphorylating Ser residues 63 and 73, located within the N-terminal transactivation domain (Hibi et al., 1993; Pulverer et al., 1991; Adler et al., 1992). As with ERK, JNKs are activated by sequential phosphorylation of protein kinases involved in an archetypical MAPK cascade. Based on their initial identification as stress-activated kinases, early research focused on the role of JNKs in apoptosis. Indeed, when activated by stress stimuli such as UV irradiation and growth factor withdrawal JNK has an apoptotic role (Xia et al., 1995; Tournier et al., 2000); emerging evidence, however, suggests that JNK additionally functions to promote cell survival.

In neurons, JNK1/2 play a critical role in stress-induced apoptosis in response to nerve-growth factor (NGF) withdrawal. PC12 neuronal cells deprived of NGF undergo rapid cell death, blocked by the expression of a dominant-interfering JNK mutant. Conversely, PC12 cells expressing constitutively activated MEKK1, the upstream kinase activator of JNK, undergo apoptosis (Xia et al., 1995). Overexpression of c-jun in cultured sympathetic neurons induces apoptosis, and expression of a dominant-interfering c-jun mutant protects against apoptosis due to NGF-withdrawal, implicating it as one of the downstream targets of JNK in this type of neuronal cell death (Ham et al., 1995). In PC12 cells, death from NGF withdrawal is associated with an increase in Fas ligand and cognate death receptor activation (Le-Niculescu et al., 1999).

Mice deficient for either JNK1 or 2 show no obvious phenotype, with the exception of immunodeficiency due to a defect in T-cell function (Constant et al., 2000; Sabapathy et al., 1999a). In response to UV irradiation, only Jnk1-/- single knockout MEFs display impaired apoptosis compared to their wild-type or Jnk2-/- counterparts, yet still undergo some cell death (Tournier et al., 2000). The lack of resistance to UV stress in the single knockout studies is believed to result from the ability of JNK1 and 2 to function in a compensatory manner, supported by the fact that JNK1/2 double knockout (Jnk1/2-/-) MEFs are completely resistant to death from UV irradiation. Jnk1/2-/- mice are embryonic lethal and show exencephaly of the hindbrain

at E9.25 due to a reduction in hindbrain apoptosis. Also evident is an increase in apoptosis in the forebrain and hindbrain post neural tube closure at approximately E10.5 (Sabapathy et al., 1999b; Kuan et al., 1999). This points to a role for JNK1/2 in both apoptosis and survival at different times during fetal mouse brain development. Evidence from tumour cell models suggests that JNK acts as a potent survival factor. Several transformed cell lines express constitutive activated JNK, and expression of a c-jun S63/73A mutant, lacking JNK phosphorylation sites, suppresses the transforming ability of several oncogenes (Ip et al., 1998; Behrens et al., 2000). In addition, JNK suppresses apoptosis via inhibitory phosphorylation of the proapoptotic Bcl-2 family protein BAD on Thr201 (Yu et al., 2004).

JNK signalling and p53

In response to stress stimuli, p53 undergoes a complex series of post-translation modifications including phosphorylation and acetylation that lead to protein stabilization, accumulation and transcriptional activation (Prives and Hall, 1999). JNK along with other kinases can phosphorylate and activate p53 (Milne et al., 1995; Hu et al., 1997; Fuchs et al., 1998b; She et al., 2002), however, the role of p53 in JNK-induced apoptosis/survival and the specific phosphorylation events that mediate these responses have yet to be determined. In addition, JNK can bind p53 and target it for ubiquitin-mediated proteosomal degradation (Fuchs et al., 1998a). These opposing effects of JNK on p53 depend in part on cell type, the stimulus used to activate JNK signalling, and cellular growth conditions.

In addition to p53 and c-jun, JNK also activates JunB and ATF-2 by phosphorylation (Davis 2000; Lin, 2003) and targets these transcriptions factors for ubiquitin-mediated degradation, but only when they are in their unphosphorylated state (Fuchs et al., 1996, 1997; Musti et al., 1997). In non-stressed, proliferating cells an estimated 30 % of p53 is found in complex with JNK. Binding is associated with p53 ubiquitination and decreased p53 protein levels suggesting that JNK and/or associated factors target p53 for ubiquitin-mediated proteosomal degradation (Fuchs et al., 1998a), (Figure 3). In cells exposed to UV-irradiation (a known activator of JNK), or expressing constitutively activated MEKK1, p53 is phosphorylated, no longer ubiquitinated, accumulates and becomes transcriptionally active (Fuchs et al., 1998b). The current view is that in unstressed cells, JNK binds p53 and other targets to promote ubiquitin-dependent degradation. In response to certain cellular stresses, in particular UV-irradiation, activated JNK phosphorylates bound targets resulting in their dissociation from JNK and associated factors that mediate degradation (Fuchs et al., 1996, 1997, 1998b; Musti et al., 1997). Thus, in UV-irradiated cells, JNK switches from

an ubiquitin-targeting enzyme to a pro-apoptotic kinase that phosphorylates p53 and protects it from degradation. This model is consistent with other observations including our own that suggest that basal JNK activity in proliferating cells under non-stressed conditions plays a critical role in cell survival (see below).

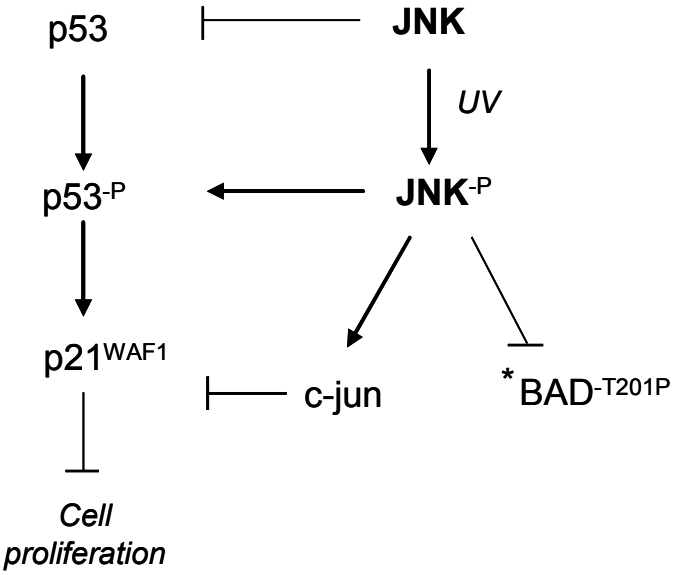

Figure 3. Mechanisms of cell survival and proliferation mediated by JNK under stressed and non-stressed conditions.

JNK-mediated degradation of p53 occurs independently of Mdm2. This is supported by the observation that mutant p53, unable to bind Mdm2, is still degraded by JNK and mutant p53 unable to bind JNK is degraded by Mdm2. In synchronously growing cells JNK/p53 complexes are observed as cells enter G1, whereas Mdm2/p53 complexes are observed as cells enter the G2/M phase of the cell cycle (Fuchs et al., 1998a). These studies suggest that p53 stability is affected by JNK independently of Mdm2 in a cell cycle-dependent manner. One intriguing possibility suggested by these findings is that JNK may normally be involved in regulating the level of latent p53 protein in unstressed cells whereas Mdm2, which is induced by stress in a p53-dependent manner, may serve to down-regulate activated p53 and to terminate the p53-dependent stress response.

JNK activation results in the induction of c-jun following UV-irradiation. c-jun has been shown to inhibit the association of p53 with p21 promoter DNA in UV-irradiated cells thereby suppressing p53-mediated activation of

p21WAF1 expression (Shaulian et al., 2000). As a result, c-jun has been implicated in promoting cell cycle re-entry following p53-dependent G1 arrest, presumably once damaged DNA has been repaired. In the absence of c-jun, UV-activated p53 results in a prolonged growth arrest that is associated with protection from apoptosis. In cells that express c-jun constitutively, p21WAF1 induction is blocked and the predominant cellular response to activated p53 is apoptosis (Shaulian et al., 2000). Potapova et al. (2000) reported that inhibition of JNK in p53-null cells caused growth suppression due to apoptosis. In p53 intact cells, JNK inhibition resulted in p53-dependent increase in p21WAF1 expression and survival of growth arrested cells (Potapova et al., 2000). This agrees with the model in which basal JNK in nonstressed cells suppresses p53 by targeting it for ubiquitin-mediated degradation. Therefore, in nonstressed cells JNK promotes p53 degradation, whereas in stressed cells JNK activates p53 and c-jun by phosphorylation and c-jun attenuates p53-dependent activation of p21WAF1 mRNA expression (Figure 3). We have observed that basal levels of JNK protect cells from p53-dependent cell death. Murine erythroleukemia cells expressing a p53ts allele show enhanced p53-dependent apoptosis upon treatment with the chemical inhibitor SP600125 or following expression of a dominant interfering JNK mutant. Neither treatment alone induces apoptosis of parental cells or p53ts-expressing cells grown at the non-permissive temperature (unpublished observations).

MKK(3 and 6)/p38

The p38 MAPKs exists in 4 isoforms, α, β, γ and δ, with the α and β isoforms having the widest range of mammalian tissue expression (Martin-Blanco, 2000). Like JNK, initial identification of p38 as a kinase activated by cellular stress and inflammatory cytokines linked it with an apoptotic cellular response. p38 is now also implicated in cell proliferation and survival (Kyriakis and Avruch, 2001). p38 has been shown to play a role in apoptosis in response to stress due to growth factor withdrawal. PC12 neuronal cells undergo apoptosis upon NGF withdrawal and this can be blocked with a p38 chemical inhibitor (PD169316) or with a dominant-interfering p38 mutant kinase. Rat-1 cells showed a similar p38-dependent apoptotic response upon serum-depletion, and in both cell lines, factor withdrawal was associated with an increase in p38 kinase activity (Xia et al., 1995; Kummer et al., 1997). Notably, dominant-interfering kinases of both p38 and JNK were able to block apoptosis induced by NGF withdrawal, suggesting that they may act in concert in mediating this type of neuronal cell death (Xia et al., 1995). Treatment of normal human diploid fibroblasts with the non-steroidal anti-inflammatory drug Sodium Salicylate (NSAID)

activates p38 and leads to apoptosis that can be blocked with the p38 chemical inhibitor, SB203580. p38 activation might represent a mechanism by which NSAIDs exert their anti-neoplastic effect (Schwenger et al., 1997). Excitatory amino acids such as glutamate induce p38-dependent apoptosis of rat cerebellar granule neurons (Kawasaki, et al., 1997). *Jnk3-/-* mice are resistant to apoptosis induced in hippocampal neurons with kainite, an acid agonist of glutamate, suggesting that JNK3 also plays a role in excitatory-induced neuron apoptosis (Yang et al., 1997).

In hematopoietic cells, treatment with EPO and IL-3 have been shown to activate p38 MAPK activity and promote survival and differentiation (Nagata et al., 1997, 1998). Blocking expression of either JNK1/2 or p38 with antisense oligonucleotides inhibited erythroid differentiation (Nagata, et al., 1998). The phenotype of p38 deficient mice further illustrates that p38 has a critical role in EPO-mediated survival, at least during embryogenesis when red blood cell production switches from the yolk sac to the fetal liver (Klingmuller, 1997). Viable *p38-/-* mice are severely anemic due to a defect in definitive erythropoiesis, however, this failure of erythropoiesis is attributed to diminished EPO gene expression, placing EPO downstream of p38 in this process (Tamura et al., 2000). We have observed that EPO-mediated rescue of p53-dependent apoptosis in erythroid cells occurs independently of p38 (unpublished data). Intriguingly, the p53ts erythroid cell line used in these investigations has basal p38 kinase activity that effectively limits p53-dependent death (unpublished observations). This suggests that, like JNK, basal p38 plays a role in cell survival.

p38 signalling and p53

In response to UV-irradiation, p38 MAPK phosphorylates p53 on Ser residues 33, 46 and 389 (Huang et al., 1999; Keller et al., 1999, Bulavin et al., 1999; Takekawa et al., 2000). Although the physiological relevance of p53 Ser389 phosphorylation is controversial, p38-mediated phosphorylation of p53 on Ser33 and 46 is important for transcriptional activation and for the ability of p53 to induce arrest and/or apoptosis in response to UV (Bulavin et al., 1999). Takekawa et al. (2000) identified Wip1/PPM1D as a serine/threonine protein phosphatase that dephosphorylates and inactivates p38 thereby attenuating the cellular response to UV-irradiation. Overexpression of Wip1/PPM1D reduced p38-dependent p53 phosphorylation at Ser 33 and 46. Wip1/PPM1D, was originally identified as a p53 regulated gene (Fiscella et al., 1997). Moreover, Wip1/PPM1D expression was shown to be dependent upon p38, as treatment with SB203580 prevented its induction with UV. p38, p53 and Wip1/PPM1D, therefore, function in a negative regulatory loop in response to UV-

irradiation (Figure 4); p38, activated in response to UV, phosphorylates p53 on Ser33 and 46, and activated p53 induces transcription of Wip1/PPM1D which terminates the UV-response by dephosphorylating p38 (Takekawa et al., 2000). The proposed function of this loop is to downregulate the p38-p53 response to UV-irradiation, allowing cells to re-enter the cell cycle once genetic lesions are repaired. In the event of irreparable DNA damage, sustained p38 MAPK activity overcomes the action of Wip1/PPM1D and p53-dependent apoptosis ensues. According to this model, Wip1/PPM1D acts as a key regulator of the p53 decision to induce cellular arrest or apoptosis in response to UV-irradiation.

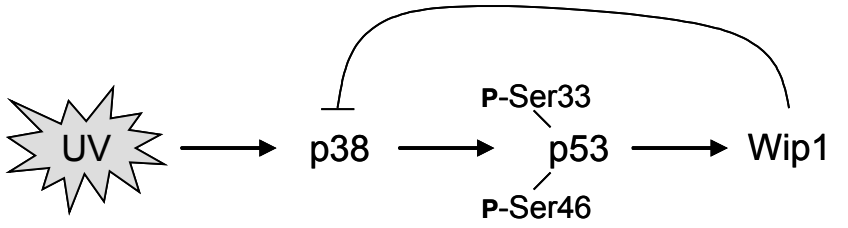

Figure 4. UV-irradiation induces p53-dependent Wip1 expression which functions in a negative regulatory loop to suppress p38 activity.

Nitric oxide (NO)-induced death of cultured chondrocytes has also been linked to p38 and p53 (Kim et al., 2002a). In this model, p38 activates p53 by at least two mechanisms: p38 activation of NF-kB which regulates p53 transcription, and direct p38 phosphorylation of p53 on Ser15, which disrupts the p53-Mdm2 interaction and leads to p53 stabilization (Kim et al., 2002b). Chemotherapeutic agents, such as cisplatin and doxorubicin, induce p38-dependent phosphorylation of p53 on Ser 33, illustrating a putative mechanism utilized by these agents to induce apoptosis during cancer therapy (Sanchez-Prieto et al, 2000). Overall, p38 regulation of p53 occurs through multiple mechanisms that are stimulus-dependent.

PI3K/PKB

A major pathway of cell survival upon activation of RTKs and cytokine receptors is through the activation of PI3K/PKB. Phospho-tyrosine residues within the cytoplasmic domains of these receptors are recognized by the p85 regulatory subunit of PI3K, which recruits the p110 catalytic subunit to the plasma membrane where it catalyzes the addition of a phosphate group to the D3 position of membrane-bound phosphatidylinositol-4,5-bisphosphate, generating phosphatidylinositol-3,4,5-triphosphate (PIP3) (Klingmuller,

1997; Cantley, 2002). PI3K-generated PIP3 acts as a second messenger to activate a number of downstream pathways involved in cell growth, migration and survival (Cantley, 2002). Key to the survival response is the recognition of PIP3 by PKB/Akt and PDK1 through their lipid binding Pleckstrin Homology (PH) domains (Scheid and Woodgett, 2003). Once localized to the membrane, PDK1 phosphorylates PKB within its catalytic domain activation loop (Thr308) to allow substrate binding (Alessi et al., 1996). To become fully active, PKB also requires phosphorylation within a hydrophobic carboxy-proximal region (Ser473), thought to occur through auto-phosphorylation or phosphorylation by an as yet unidentified kinase (Scheid and Woodgett, 2001). Disruption of PDK1 by gene targeting or anti-sense inhibition renders cells unresponsive to PKB activation in response to growth factor stimulation, evidence that PDK1 is the major kinase responsible for PKB phosphorylation and activation (Flynn et al., 2000; Williams et al., 2000).

In addition to being activated by a number of growth factors to prevent apoptosis of factor dependent cells, PKB activation is known to protect cells from apoptosis in response to a number of death-inducing stimuli, such as UV irradiation, treatment with sorbitol, cyclohexamide and TNF-α (Sabattini and McCormick, 1999; Kulik et al., 1997; Ulrich et al., 1998; Ahmed et al., 1997; Stambolic et al., 1998). Activated PKB phosphorylates a number of downstream targets involved in cell survival such as glycogen synthase kinase (GSK), Forkhead transcription factors FKHR1 and AFX, pro-apoptotic BAD and IkB kinase; in all cases, phosphorylation inhibits the function of these proteins (Datta et al., 1997; del Peso et al., 1997; Liang et al., 2003; Brunet et al., 1999; Ozes et al., 1999; Romashkova et al., 1999). Src-homology 2 (SH2)-containing phosphatase (SHIP) and Phosphatase and tensin homologue deleted from chromosome ten (PTEN), serve as negative regulators of PI3K/PKB signalling through their ability to dephosphorylate PIP3 to phosphatidylinosotol-3,4-bisphosphate and phosphatidylinositol-4,5-biphosphate, respectively (Maehama and Dixon, 1999; Liu et al., 1999; Aman et al., 1998).

PI3K/PKB signalling and p53

The expression of PKB alone has been demonstrated to overcome p53-dependent apoptosis, an effect associated with a decrease in p53 DNA-binding and transcriptional activation of pro-apoptotic targets like Bax (Sabbatini and McCormick, 1999; Yamaguchi et al., 2001). These observations lead to the idea that some opposing regulation between p53 and PKB exists (Oren et al., 2002). One link between these two pathways involves Mdm2. PKB, whether activated by IL-3, IGF-1 or an oncogenic

RTK, binds and phosphorylates Mdm2 at two serine residues (Ser166 and Ser186). PKB-mediated phosphorylation of Mdm2 results in its translocation to the nucleus where it binds p53 and targets it for ubiquitin-mediated proteosomal degradation (Zhou et al., 2001; Mayo and Donner, 2001; Gottlieb et al., 2002). Earlier work suggested that the binding of nuclear Mdm2 to p53 is facilitated by p300, which participates in the formation of a ternary complex that stabilizes the Mdm2-p53 interaction (Grossman et al., 1998). This leads to a decrease in p53 protein and transcriptional activity and is consistent with the view that the E3-ligase activity and nuclear import and export signals of Mdm2, encompassing Ser166 and 186, are important for Mdm2-dependent p53 degradation (Zhou et al., 2001; Mayo and Donner, 2001; Woods and Vousden, 2001). p19ARF also binds Mdm2 and inhibits its ability to promote p53 degradation (Pomerantz et al., 1998; Kamijo et al., 1998). Zhou et al. (2001) have proposed that in the presence of survival factors, PKB-dependent phosphorylation of Mdm2 leads to ternary complex formation with p300 and p53 in the nucleus and p53 degradation; unphosphorylated Mdm2 (e.g. in the absence of activated of PKB) is bound by p19ARF and is incapable of targeting p53 for degradation (Figure 5).

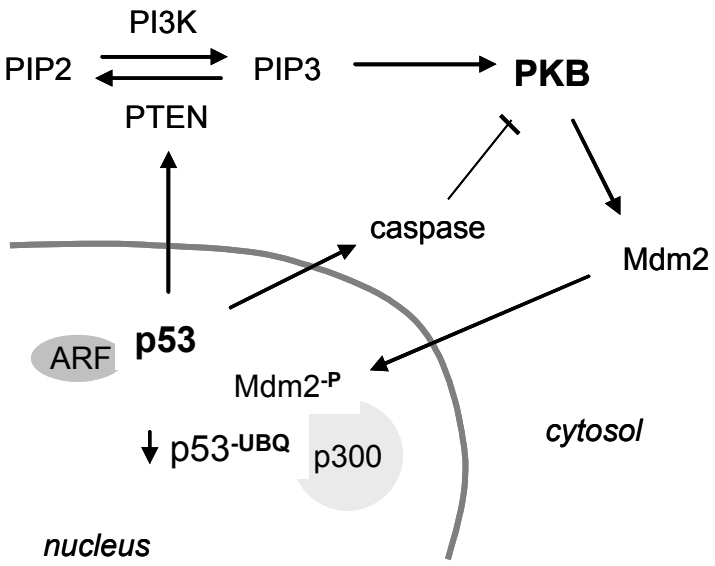

Figure 5. Opposing regulation of PI3K/PKB and p53.

The finding that PTEN is a transcriptional target of p53 adds an intriguing link between the p53 apoptotic program and PKB survival

pathway. There are 2 half sites within the PTEN promoter identical to the p53 consensus binding site, with the exception that the PTEN spacer region does not conform to the typical spacer region being 14 bp as opposed to 10-13 bp. Nevertheless, p53 binds this region in a sequence-specific manner to activate PTEN transcription; both promoter binding and transcriptional activation are inhibited by mutation within the p53-consensus binding site of PTEN (Stambolic et al., 2001). PTEN-/- cells are impaired in their apoptotic response to death-inducing stimuli such as UV-irradiation and TNF-α treatment. In addition, PTEN-/- MEFs are resistant to apoptosis induced by forced expression of p53 (Stambolic et al., 1998). These observations suggest that p53-dependent regulation of PTEN expression is important for p53 induced cell death (Stambolic et al., 1998, 2001). This is consistent with our own observations that chemical inhibition of PI3K markedly potentiates p53-dependent apoptosis in cells with a constitutively activated PI3K/PKB pathway (Lin et al., 2002). Thus, in order to effect maximal killing, p53 must not only induce effectors of apoptosis such as Bax, Noxa, Puma and PIDD (Benchimol, 2001), it must also down-regulate intrinsic survival pathways such as PI3K/PKB. Restoring PTEN function in tumour cells that lack PTEN or that overexpress Mdm2 restores their sensitivity to apoptosis-inducing chemotherapeutic agents such as etoposide and doxorubicin, respectively, further supporting a role for PKB in suppressing p53-dependent apoptosis (Zhou et al., 2003; Mayo et al., 2002). The opposing effects of p53 and PKB on death and survival are depicted in the model shown in Figure 5. In cells primed to undergo apoptosis (e.g. from growth-factor deprivation), p53 signals prevail and PKB activation is decreased either through caspase-mediated degradation of PKB protein (Gottlieb et al., 2002), or through PTEN-mediated dephosphorylation of PIP3 (Stambolic et al., 1998, 2001). Under conditions that favour survival, PKB phosphorylates and activates Mdm2 leading to p53 degradation. PKB has many other targets that promote survival independently of any direct effect upon p53.

SUMMARY

Death and life decisions within a cell are regulated through a complex and integrated network that we are still trying to understand. Protooncogenes like c-myc and tumour suppressor genes like p53 encode proteins that can promote survival under certain conditions and death under other conditions. How these decisions are determined remains elusive and under investigations in numerous laboratories. In a similar vein, the three arms of the MAPK pathway, once thought to regulate proliferation/survival (ERK) or apoptosis (JNK, p38) are now known to act in a far more complex fashion

promoting death or survival in a context-dependent manner. We have focussed on intrinsic and extrinsic factors that govern death/survival pathways (Bcl-2 family, MAPK pathways, and PI3K/PKB pathway) that ultimately converge on p53 either directly or indirectly to determine the final cellular outcome. .

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