A DR4:tBID axis drives the p53 apoptotic response by promoting oligomerization of poised BAX

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The cellular response to p53 activation varies greatly in a stimulus- and cell-type-specific manner. Dissecting the molecular mechanisms defining these cell fate choices will assist the development of effective p53-based cancer therapies and also illuminate fundamental processes by which gene networks control cellular behaviour. Using an experimental system wherein stimulus-specific p53 responses are elicited by non-genotoxic versus genotoxic agents, we discovered a novel mechanism that determines whether cells undergo proliferation arrest or cell death. Strikingly, we observe that key mediators of cell-cycle arrest (p21, 14-3-3ε) and apoptosis (PUMA, BAX) are equally activated regardless of outcome. In fact, arresting cells display strong translocation of PUMA and BAX to the mitochondria, yet fail to release cytochrome C or activate caspases. Surprisingly, the key differential events in apoptotic cells are p53-dependent activation of the DR4 death receptor pathway, caspase-8-mediated cleavage of BID, and BID-dependent activation of poised BAX at the mitochondria. These results reveal a previously unrecognized role for DR4 and the extrinsic apoptotic pathway in cell fate choice following p53 activation.

Introduction

The p53 network is the most commonly deregulated gene circuitry in human cancer. More than half of tumours carry mutations in the TP53 gene while in the remaining fraction p53 is likely attenuated by hyperactivation of repressors such as MDM2 and MDM4 (Brown et al, 2009). The p53 protein acts mostly as a transcription factor, but cytoplasmic functions of p53 have also been documented (Laptenko and Prives, 2006; Green and Kroemer, 2009). p53 behaves as a signalling node that is activated by a plethora of stress signals and it in turn participates in the orchestration of various cellular responses including, but not restricted to, cell-cycle arrest, senescence, apoptosis, and autophagy (Vousden and Prives, 2009). As observed for other master transcriptional regulators of cell behaviour, the cellular response to p53 activation varies greatly with the context. Stimulus- and cell type-specific p53 responses have been extensively documented (Vousden and Lu, 2002). The same cell type may undergo strikingly different p53-dependent responses upon exposure to distinct stress signals, whereas the same p53-activating agent can trigger dissimilar responses across various cell types. Despite many research efforts in this arena, we still lack a thorough understanding of how alternative p53 responses are defined. The biomedical importance of this problem cannot be overstated. Elegant studies in animal models have clearly established that reactivation of p53 in tumours is a valid therapeutic strategy, as increased p53 activity leads to tumour clearance via senescence or cell death (Ventura et al, 2007; Xue et al, 2007). However, any efforts to pharmacologically reactivate mutant p53 or block the repressive effects of MDM2/MDM4 on wild-type p53 will be hampered by the fact that p53 is highly pleiotropic. This pleiotropy has become evident with the advent of inhibitors of the p53–MDM2 interaction such as Nutlin-3 (Nut3), a non-genotoxic small molecule that binds to MDM2 and prevents its association with p53 (Vassilev et al, 2004). Remarkably, most cancer cell types fail to undergo p53-dependent apoptosis following Nut3 treatment, instead adopting a reversible cell-cycle arrest phenotype (Tovar et al, 2006; Paris et al, 2008). However, as shown in this report, these ‘Nutlin-resistant’ cell types effectively undergo p53-dependent apoptosis in response to a genotoxic stimulus. Thus, understanding the molecular mechanisms driving stimulus-specific p53 responses is a prerequisite for the successful design of p53-based therapies.

Pleiotropy and context dependence are the hallmarks of biological systems. The overall impact of a given gene product on the function of a cell, tissue or organism is ultimately defined by myriad interactions with other gene products. Context-dependent variations in this web of interactions define the biological function of a gene in different scenarios. The p53 network provides an excellent paradigm to investigate how gene networks orchestrate alternative cell fates. Several models have been proposed to explain how different p53-dependent responses are established (Vousden and Lu, 2002; Espinoza, 2008). p53-centric models ascribe the cell fate choice to molecular events affecting the p53 molecule itself. For example, p53 post-translational modifications and p53-binding proteins have been shown to modulate its transcriptional competence in a gene-specific manner (Samuels-Lev et al, 2001; Sykes et al, 2006; Tang et al, 2006; Das et al, 2007). Alternative models focus instead on the fact that p53 target genes themselves are subject to multiple regulatory influences acting at both the transcriptional and post-transcriptional levels (Espinosa et al, 2003; Gomes et al, 2006; Donner et al, 2007b; Tanaka et al, 2007; Paris et al, 2008; Beckerman et al, 2009; Morachis et al, 2010).
According to the latter view, the transcriptional competence of p53 is largely invariant in different contexts, and the cell fate choice is instead defined by variations in p53-autonomous mechanisms affecting its target genes. Despite their differences, both models converge on the assumption that cell fate choice results from an imbalance in the activity of p53 target genes acting in different pathways. For example, it is well established that p53-dependent cell-cycle arrest is mostly mediated by cell-cycle regulators such as CDKN1A (p21) and SFN (14-3-3σ) (el-Deiry et al., 1993; Hermeking et al., 1997), while p53-dependent apoptosis is mediated by pro-apoptotic factors such as the mitochondrial pore protein BAX and the BH3-only protein BBC3 (PUMA) (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Yu et al., 2003). Thus, the prevailing views support the idea that differential transcriptional output between the p21 and 14-3-3σ loci versus the BAX and PUMA loci is a key determinant of cell fate choice (Sykes et al., 2006; Tang et al., 2006; Das et al., 2007). However, a formal demonstration of this model is lacking. In fact, we show here that stimulus-specific responses to p53 activation can be delivered without any difference in the transcriptional output of these key genes.

Using a model system where the two major p53 responses, cell-cycle arrest and apoptosis, are triggered alternatively by simply using different p53-activating agents, we investigated the contribution of ~20 components of the p53 network to cell fate choice. In our system, cancer cells are driven into p53-dependent cell-cycle arrest with Nut3 versus p53-dependent apoptosis with the anti-metabolite 5-fluorouracil (5FU). Strikingly, gene expression analysis at the RNA and protein levels reveals that cells undergoing p53-dependent cell-cycle arrest nevertheless display effective transactivation of PUMA and several other pro-apoptotic target genes, concurrent with translocation of BAX to the mitochondria. However, these cells fail to release cytochrome C into the cytosol, activate caspases or trigger apoptosis. Conversely, cells undergoing p53-dependent apoptosis show strong transcription of p21, 14-3-3σ and other genes involved in cell-cycle arrest. However, these cells fail to arrest and show instead p53-dependent activation of caspases. Careful genetic dissection of different components of the apoptotic apparatus led to the unexpected observation that the key stimulus-specific molecular events defining cell fate choice are (1) p53-dependent activation of caspase 8, (2) caspase 8-dependent activation of the BH3-only protein BID, and (3) BID-dependent activation of BAX. Interestingly, the p53 target gene DR4 is required for all of these events to occur. DR4 expression is strongly induced only in 5FU-treated cells via a combination of p53-dependent transactivation and p53-independent mRNA stabilization. Thus, p53 action is complemented by other stress-induced events to tip the balance towards the apoptotic response. These results demonstrate a critical role of the extrinsic apoptotic pathway in cell fate choice and also illuminate key molecular events defining the shortcomings of novel p53-based therapies.

Results

Cell fate choice to p53 activation is defined by the stimulus-specific activation of poised BAX at the mitochondria

In order to uncover novel mechanisms contributing to p53-dependent cell fate choices, we established a tissue culture system in which p53 activation results in different cellular fates depending on the p53-activating agent utilized. Our group and others have previously demonstrated that HCT116 cells undergo reversible p53-dependent cell-cycle arrest upon treatment with Nut3 (Tovar et al., 2006; Paris et al., 2008). In contrast, these same cells undergo robust p53-dependent apoptosis upon treatment with 5FU (Figure 1A) (Bunz et al., 1999). Interestingly, while both Nut3 and 5FU induce equivalent p53 protein accumulation, only 5FU causes release of cytochrome C into the cytosol, caspase 3 activation and PARP cleavage, demonstrating that differences in p53 protein levels do not account for the stimulus-specific responses seen in our paradigm (Figure 1B).

Work from several laboratories has put forth a model wherein differential transcriptional activation of key p53 target genes mediating cell-cycle arrest, such as p21 and 14-3-3σ, versus genes mediating apoptosis, such as PUMA and BAX, can determine the cellular outcome to p53 activation (Samuels-Lev et al., 2001; Sykes et al., 2006; Tang et al., 2006; Das et al., 2007). Accordingly, we hypothesized that stimulus-specific regulation of one or more of these genes could steer the p53 response towards a specific outcome in our system. Interestingly, both Nut3 and 5FU treatment lead to identical accumulation of p21 and 14-3-3σ protein (Figure 1B). Thus, although it is well established that these two cell-cycle regulators are required for p53-dependent cell-cycle arrest (el-Deiry et al., 1993; Hermeking et al., 1997) and that they cooperate to exert protective effects against 5FU-induced apoptosis (Chan et al., 2000), they are clearly not the determinants of cell fate choice in our system. PUMA and BAX are direct transcriptional targets of p53 required for 5FU-induced apoptosis (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Yu et al., 2001). Remarkably, Nut3 treatment induces the accumulation of PUMA and BAX protein to levels equal to or higher than those observed upon 5FU treatment (Figure 1B). Furthermore, mitochondrial fractionation experiments demonstrate that Nut3 treatment leads to effective translocation of PUMA and BAX to the mitochondria (Figure 1C, fractionation controls are shown in Supplementary Figure S1). These observations suggest that physiological induction and mitochondrial accumulation of these key apoptotic factors is not sufficient to trigger p53-dependent cell death.

The lack of cytochrome C release into the cytosol of Nut3-treated cells suggests that mitochondrial outer membrane permeabilization (MOMP) is impaired under these conditions. One explanation for this could be the failure of BAX to oligomerize and form pores in the mitochondrial outer membrane. To test this, we evaluated BAX oligomerization using chemical crosslinking of mitochondrial fractions, which revealed the existence of BAX oligomers in 5FU-treated cells but not in cells treated with Nut3 (Figure 1D). This suggests that in Nut3-treated cells, BAX fails to undergo the requisite conformational change prior to oligomerization (Kim et al., 2009). We investigated this possibility by immunoprecipitating BAX with the conformation-specific antibody 6A7, which specifically detects the activated conformer of BAX (Hsu and Youle, 1997). Confirming our hypothesis, BAX activation is detectable in 5FU-treated cells, but not in cells treated with Nut3 (Figure 1E). Taken together, these data demonstrate that although Nut3 treatment leads to effective accumulation of PUMA and BAX at
the mitochondria, it fails to promote BAX oligomerization, MOMP and apoptosis.

**BID is required for stimulus-specific BAX activation and apoptosis**

It is well established that MOMP is regulated by the balance between pro- and anti-apoptotic members of the BCL2 protein family, many of which are transcriptionally regulated by p53 in some cell types, such as BAX, PUMA, NOXA, BID and BCL2 (Miyashita et al, 1994; Miyashita and Reed, 1995; Nakano and Vousden, 2001; Wu et al, 2001; Yu et al, 2001; Sax et al, 2002; Schuler et al, 2003). Having ruled out differential expression of PUMA and BAX as responsible for stimulus-specific MOMP in our system, we next examined the expression of the pro-apoptotic BH3-only proteins NOXA, BID, the truncated active form of BID (tBID), as well as the anti-apoptotic proteins BCL2 and BCL-XL. Interestingly, we observed both stimulus-specific induction of NOXA protein and proteolytic activation of BID upon 5FU treatment (Figure 2A). Stimulus-specific activation of NOXA and tBID is also reflected in their mitochondrial levels (Figure 2B). In contrast, the total and mitochondrial levels of BCL2 and BCL-XL remain unchanged by either treatment (Figure 2A and B). Differential induction of NOXA is also observed at the mRNA level, and in agreement with a previous report (Yu et al, 2001), we found that NOXA upregulation upon 5FU treatment is p53-independent (Supplementary Figure S2). Although BID has been described as a direct transcriptional target of p53 in other settings (Sax et al, 2002), BID mRNA expression is not induced by Nut3 or 5FU in our system (Supplementary Figure S3). To test if differential activation of NOXA and tBID contributes to cell fate choice in our paradigm, we established stable knockdown cell lines for each factor and measured the apoptotic response following 5FU treatment. Interestingly, reducing NOXA expression well below basal levels has no significant effect on 5FU-induced apoptosis (Figure 2C and D). In contrast, BID depletion significantly reduces 5FU-induced apoptosis, blocking cell death as effectively as a BAX knockout (Figure 2E). Importantly, BID knockdown severely impairs
BAX oligomerization in 5FU-treated cells (Figure 2F). Taken together, these data demonstrate that BID is an important factor mediating apoptosis in response to 5FU, and suggests that the failure of Nut3-activated p53 to induce BID cleavage determines cell fate choice in our experimental model.

**Stimulus-specific activation of caspase 8 is required for BID cleavage and BAX activation**

Caspase 8 is a major initiate caspase in the death receptor-dependent (extrinsic) apoptotic pathway and a known mediator of BID cleavage in response to genotoxic stress (Li *et al.*, 1998). Interestingly, we found that only 5FU treatment leads to caspase 8 activation (Figure 3A) and that this event requires p53 (Figure 3B). Furthermore, caspase 8 knockdown strongly inhibits activation of executioner caspase 3, and cleavage of the caspase 3 substrate, PARP (Figure 3C). In fact, caspase 8-deficient cells are significantly impaired in their ability to undergo apoptosis following 5FU treatment (Figure 3D). Next, we asked whether caspase 8 is required for BID cleavage in our system and found that indeed, proteolytic processing of BID is greatly reduced in 5FU-treated cells lacking caspase 8 (Figure 3E). Expectedly, BID depletion prevents BAX dimerization in response to 5FU treatment. P-values shown in (D, E) were calculated by Student’s t-test (unpaired, two-tailed). Figure source data can be found in Supplementary data.
As observed for HCT116 cells, caspase 8 knockdown impairs BID cleavage, caspase 3 activation, PARP cleavage, and apoptosis in this cell line (Supplementary Figure S4). In sum, these data indicate that stimulus-specific activation of the extrinsic apoptotic pathway involving p53, caspase 8, and tBID is required for BAX activation and commitment to apoptosis in our paradigm.

**Differential activation of the extrinsic pathway requires FADD, not TRADD**

In an effort to understand in greater detail how Nut3 and 5FU differ in their ability to trigger p53-dependent apoptosis, we investigated the signalling pathway responsible for caspase 8 activation upon 5FU treatment. Of note, caspase 8 can be directly activated downstream of death receptors, but also by a death receptor-independent mechanism involving caspase 3 (von Haefen et al., 2003). To discriminate between these possibilities, we established stable knockdown cell lines for FADD and TRADD (shFADD and shTRADD), two important death domain-containing adaptor proteins that transmit death ligand signals from death receptors to caspase 8 (Jin and El-Deiry, 2005). If 5FU-induced caspase 8 activation requires either of these adaptor proteins, this would suggest that caspase 8 is activated via death receptors in our experimental setting. Indeed, we found that FADD depletion abrogates both caspase 3 activation and Annexin-V positivity, whereas depletion of TRADD does not significantly affect either event (Figure 4A and B). Furthermore, shFADD cells are impaired in their ability to activate BAX (Figure 4C). Together, these results indicate that 5FU treatment causes apoptosis through a FADD-dependent, TRADD-independent pathway. Additionally, we observed that BID knockdown impairs caspase 3 activation more than it does caspase 8 activation, providing additional evidence that caspase 8 activation occurs upstream of caspase 3 activation in 5FU-treated HCT116 cells (Supplementary Figure S5). Thus, we conclude that the key molecular event defining cell fate choice in our model system likely resides within the death receptor pathway.

**DR4 is required for BAX activation and apoptosis**

We hypothesized that one or more death receptors acting upstream of FADD may be differentially expressed upon p53...
activation by Nut3 versus 5FU. To test this, we examined the expression and cell surface localization of DR4 (TNFRSF10A/APO2/TRAIL-R1), DR5 (TNFRSF10B/TRAIL-R2/KILLER) and FAS (TNFRSF6/APO-1/CD95), all of which require FADD to mediate pro-apoptotic signalling and have also been previously described as p53 target genes (Wu et al., 1997; Muller et al., 1998; Liu et al., 2004; Jin and El-Deiry, 2005). Western blot analysis of the three death receptors revealed markedly different, stimulus-specific patterns of expression for DR4, DR5, and FAS (Figure 5A). At an early time point (8 h), Nut3 induces DR4 to the same extent as 5FU; however, in Nut3-treated cells, DR4 protein returns to basal levels at later time points (16 and 24 h) but remains elevated in 5FU-treated cells. In contrast, DR5 expression is induced equally by Nut3 and 5FU at 8 h, but Nut3-treated cells maintain steady DR5 expression, whereas DR5 protein levels decrease slightly in 5FU-treated cells at later time points. Lastly, both Nut3 and 5FU treatments lead to sustained FAS induction over the course of the experiment, although 5FU does induce FAS to a greater extent than Nut3. Next, we investigated the cell surface expression of DR4, DR5, and FAS by flow cytometry, which is a more accurate measurement of the active pool of these receptors. In close agreement with total protein levels, 5FU treatment leads to higher cell surface levels of DR4 than does Nut3 treatment, with mean fluorescence indices (MFIs) of 1.34 ± 0.04 and 1.04 ± 0.03, respectively (Figure 5B). In contrast, despite differences in the total protein levels seen by western blot, cell surface levels of FAS and DR5 are nearly identical in cells treated with Nut3 or 5FU (MFI\textsubscript{FAS} 2.10 ± 0.04 and 2.07 ± 0.05; MFI\textsubscript{DR5} 1.47 ± 0.08 and 1.40 ± 0.12). Based on these results, we focused our efforts on testing the possible role of DR4 in 5FU-induced apoptosis. Interestingly, whereas FAS knockdown has no significant effect on cell death, knocking down DR4 expression significantly attenuates 5FU-induced apoptosis (Figure 5C). Next, we tested if DR4 is required for 5FU-induced BAX activation. Indeed, immunoprecipitation of activated BAX with the 6A7 antibody revealed that DR4 ablation blocks BAX activation upon 5FU treatment (Figure 5D). Importantly, DR5 has been previously shown to contribute to 5FU-induced apoptosis (Wang and El-Deiry, 2004), and we confirmed these results in our system (Supplementary Figure S6). Thus, although both DR4 and DR5 mediate the apoptotic effects of this genotoxic agent, only DR4 is differentially activated in 5FU-treated cells as compared with Nut3-treated cells, and thus is more likely to define the cell fate choice.

**Stimulus-specific DR4 expression is achieved via stabilization of the DR4 mRNA**

Next, we investigated the mechanism driving stimulus-specific activation of DR4. Interestingly, Q-RT–PCR analysis revealed stimulus-specific induction of the DR4 mRNA over time (Figure 6A). Importantly, full induction of DR4 mRNA requires p53; however, the mRNA still accumulates ~2-fold in p53-null cells, suggesting the combined action of p53-dependent and p53-independent events. In contrast, the p21 mRNA is equally induced by both drugs in a p53-dependent manner. Since the DR4 locus is a known direct transcriptional target of p53 (Liu et al., 2004), we hypothesized that stimulus-specific transcription of DR4 could explain differential DR4 expression. To test this, we measured initiating and elongating forms of RNA polymerase II (Ser5-phospho-RNAPII and Ser2-phospho-RNAPII, respectively) at the DR4 locus by chromatin immunoprecipitation (ChIP) assays. Activation of p53 with Nut3 or 5FU for 12 h results in increased SSP-RNAPII occupancy at the DR4 promoter region as well as within the gene body, indicating increased transcription initiation with both stimuli (Figure 6B). Likewise, S2P-RNAPII also increases throughout the DR4 gene body after both treatments, surprisingly demonstrating equivalent increases in transcriptional elongation. We employed the p21 locus for comparison purposes, which displays strong RNAPII activation by both p53-activating agents. Taken together, these results suggest that the stimulus-specific accumulation of DR4 mRNA seen in our system cannot be adequately explained by stimulus-specific differences in DR4 transcription. Therefore, we asked whether Nut3 and 5FU differentially affect DR4 mRNA stability. Indeed, mRNA half-life assays demonstrate that 5FU treatment has a significant effect on DR4 mRNA stability, nearly doubling its half-life relative to DMSO- or Nut3-treated cells (Figure 6C; Supplementary Figure S7). Interestingly, the DR4 mRNA was also stabilized in p53\textsuperscript{−/−} cells. Taken together, these data suggest that Nut3 and 5FU treatments lead to an equal but modest induction of DR4 transcription, but that stimulus-specific accumulation of DR4 following 5FU treatment results from increased stabilization of the DR4 mRNA. Of note, the NOXA mRNA, which is also induced in a p53-independent manner upon 5FU treatment (Supplementary Figure S2), is also strongly stabilized by 5FU regardless of p53 status. However, mRNA stabilization upon 5FU is not a universal phenomenon, as it is not observed for the p53 target gene MDM2 (Figure 6C). Thus, we conclude that 5FU induces mRNA stabilization of a subset of
Conserved action of the DR4/FADD/caspase 8/tBID axis

In order to test whether the mechanism of cell fate choice identified in HCT116 colorectal cancer cells is conserved in cancer cells of different tissue origin, we employed H460 cells (non-small cell lung cancer), which also undergo cell-cycle arrest upon Nut3 treatment (Tovar et al., 2006). Indeed, we found that although both Nut3 and 5FU activate p53 in this cell line, only 5FU triggers caspase 8 activation, BID cleavage and caspase 3 activation (Figure 7A). As observed in HCT116 cells, differential induction of apoptosis correlates with higher expression of DR4 protein and mRNA at late time points of 5FU treatment (Figure 7A). As observed in HCT116 cells, differential induction of apoptosis correlates with higher expression of DR4 protein and mRNA at late time points of 5FU treatment (Figure 7A). Importantly, knockdown of BID, caspase 8, DR4, or FADD decreases BID cleavage and caspase 3 activation (Figure 7C and D). Thus, we conclude that the DR4/FADD/caspase 8/tBID axis contributes to cell fate choice across cancer cells of different origins.

Finally, we investigated whether this same pathway is activated by other genotoxic stimuli. To test this, we performed a side-by-side comparison of the effects of Nut3, 5FU, doxorubicin, camptothecin, etoposide, and Ultra Violet Light C (UVC) on caspase activation and apoptosis. Although all of these drugs lead to p53 activation, we and others have previously shown that only 5FU and UVC lead to rapid apoptosis in HCT116 cells, whereas the topoisomerase inhibitors lead to cell-cycle arrest mostly in G2/M, and to a lesser extent, in G1 (Donner et al., 2007a; Bunz et al., 1998, 1999; Gomes and Espinosa, 2010). Interestingly, only 5FU and UVC lead to activation of caspase 8 (Supplementary Figure S9). In order to define if 5FU and UVC induce apoptosis by identical mechanisms, we tested the impact of shRNAs against BID, caspase 8, DR4, and FADD, as well as p53 knockout, on UVC-induced apoptosis. Expectedly, p53 knockout significantly reduces caspase 3 activation upon UVC treatment. Importantly, we found that whereas knockdown of BID, caspase 8, or FADD impairs activation of caspase 3 following UVC treatment, the effects of DR4 knockdown are negligible. Thus, we conclude that both 5FU and UVC require a functional FADD/caspase 8/BID axis to induce apoptosis, but that only 5FU requires DR4, suggesting that UVC utilizes an alternative pathway for caspase 8 activation.

Discussion

Given the high prevalence of cancer and the fact that p53 is the most commonly mutated tumour suppressor gene, anomalies in the p53 network cause an unacceptable amount of disease and death. Despite thousands of publications on this important tumour suppressor, there is still much that we do not know. In particular, a key question remains unanswered: what determines the cellular response to p53 activation? Given the intrinsically p53-centric nature of the field, most efforts in this area have focused on regulatory events affecting the p53 molecule itself. These efforts have produced several models where the cellular outcome upon p53 activation is defined by p53 post-translational modifications and/or p53-binding proteins (Samuels-Lev et al., 2001; Sykes et al., 2006; Tang et al., 2006; Das et al., 2007). In this report, we took an alternative approach and investigated this problem from a gene network perspective, where the p53 molecule is embedded in a gene circuit composed of its many target genes and their interactors (Figure 8). Our efforts identified key regulatory events affecting cell fate choice that seem to
reside away from the p53 molecule itself. Our results cannot be explained by any of the previous models proposing that cell fate choice is determined by differential transcriptional regulation of cell-cycle arrest genes, such as p21 and 14-3-3s, versus the apoptotic genes PUMA and BAX (Figure 1). Thus, we turned our attention towards other gene modules within the network that could behave as molecular switches governing cell fate choice. This led to the unexpected discovery that the key differential event between arresting and apoptotic cells was activation of the extrinsic apoptotic pathway downstream of DR4 and DR5. As discussed below, these observations contribute to our understanding of several molecular processes including, but not restricted to, regulation of p53 DNA-binding and transactivation activities, regulation of the intrinsic and extrinsic apoptotic pathways by p53, and modulation of BAX activity by BH3-only proteins.

First and foremost, p53 is a transcription factor. Naturally, many efforts aimed at understanding context-dependent p53 responses have focused on the regulation of its DNA-binding and transactivation activities. Several studies have indicated that these biochemical steps can be regulated by stress-induced p53 post-translational modifications and p53-binding proteins. Repeatedly, the p53 target genes reported to be affected by these modifications and cofactors are p21, 14-3-3s, PUMA, and BAX. For example, the HZF protein was shown to associate with p53 to allow binding and transactivation of the p21 and 14-3-3s promoters while blocking binding to the BAX locus (Das et al., 2007). Conversely, the p53-binding protein ASPP2 was shown to increase binding to and transactivation of BAX, but not p21 (Samuels-Lev et al., 2001). Similarly, acetylation of lysine 120 in the p53 DNA-binding domain was shown to stimulate transactivation of PUMA, but not p21 (Sykes et al., 2006; Tang et al., 2006).

However, none of these gene-specific effects seem to play a role in our paradigm, as p21, 14-3-3s, PUMA, and BAX are equally induced by p53 in Nut3- and 5FU-treated cells. Of note, prior studies have shown equal p21 upregulation in cells that undergo cell-cycle arrest versus those that undergo apoptosis upon p53 activation (el-Deiry et al., 1994). In contrast to the prevalent models, our research revealed an unprecedented role for the death receptor DR4 in p53-dependent cell fate choice. DR4 is activated in a stimulus-specific manner and is required to overcome BAX poising at the mitochondria, which also requires both caspase 8 and caspase 9.

Figure 6 Stimulus-specific stabilization of the DR4 mRNA. (A) Q-RT–PCR analysis reveals that the DR4 mRNA, but not the p21 mRNA, accumulates more strongly in 5FU-treated cells. (B) ChIP analysis of the DR4 and p21 loci show increased levels of initiating (SSP-RNAPII) and elongating (SSP-RNAPII) forms of RNAPII in response to p53 activation by both Nut3 and 5FU. Grey regions represent the transcribed region of each locus, arrows indicate transcription start sites, black boxes represent exons, and black dashes indicate the position of PCR amplicons used for analysis of ChIP-enriched DNA. (C) Schematic of experimental procedure for DR4 mRNA half-life determination (top). Following 12h of DMSO, Nut3 or 5FU treatment of HCT116 cells of different p53 status, RNA synthesis was halted by Act-D and the levels of DR4, NOXA and MDM2 mRNA levels were measured over the time, revealing that 5FU treatment leads to stabilization of the DR4 and NOXA mRNAs in a p53-independent fashion. The numbers to the right indicate the mRNA half-lives for the corresponding mRNAs in HCT116 p53+/− cells treated with DMSO (black) or 5FU (blue). A table with all half-life measurements can be found in Supplementary Figure S7.
One possible explanation for the failure of Nut3 to induce apoptosis in a majority of cancer cell lines is that additional, p53-independent events may be required to overcome oncogenic pro-survival signalling. Since 5FU has pleiotropic effects on cellular metabolism whereas Nut3 activates p53 very specifically, it is likely that 5FU treatment provides both p53-dependent and -independent signals to efficiently induce apoptosis. Consistent with this idea, increased expression of DR4 mRNA after 5FU treatment is not completely abrogated in HCT116 p53−/− cells (Figure 6A), suggesting that full DR4 induction requires both p53 and additional, unidentified factors. In fact, our research revealed that stimulus-specific induction of DR4 mRNA is not due to a differential ability of Nut3 and 5FU to transactivate the DR4 locus, but rather to p53-independent mRNA stabilization upon 5FU treatment. Thus, the coordinated action of p53 and other complementary stress-activated pathways tips the balance towards the apoptotic response in 5FU-treated cells. Unfortunately, almost nothing is known about the regulatory factors governing DR4 mRNA turnover, although one study has shown that DR4 mRNA levels are increased in cells that lack the RNA-binding protein HuR (Ghosh et al., 2009). Interestingly, we found that the NOXA mRNA was also stabilized in a p53-independent manner upon 5FU treatment, yet the MDM2 mRNA was not affected in this way. These observations point to the action of one or more gene-specific regulators of mRNA stability whose activities are likely regulated by genotoxic stress. Future studies will be required to identify and characterize these regulators.

It is important to note that although we focused our efforts on the role of DR4 in cell fate choice, it has been amply demonstrated that DR5 also contributes to the p53 apoptotic response in cells treated with 5FU and other genotoxic agents. Elegant work by the El-Deiry group showed that knockout
of DR5 reduces 5FU-induced cell death of HCT116 cells and reduces tumour growth in vivo (Wang and El-Deiry, 2004). In mice, where there is a single pro-apoptotic TRAIL receptor that is transactivated by p53 (likely a hybrid of DR4 and DR5), gene knockout experiments showed that this pathway contributes to p53-dependent, ionizing radiation-induced apoptosis in various tissues (Finnberg et al., 2005). Since DR4 and DR5 bind the same ligand, it is not clear if and how they have specialized to mediate activation of the extrinsic apoptotic pathway in different contexts. In this regard, our work shows that while both receptors are induced upon 5FU treatment and required for 5FU-induced apoptosis, DR4 is not activated upon non-genotoxic activation of p53 with Nut3, thus revealing important regulatory differences between these two genes in human cells. Interestingly, in response to ionizing radiation, p53 activation in mice leads to DR4/DR5 induction in tissues that undergo apoptosis but not in those that undergo cell-cycle arrest (Burns et al., 2001; Fei et al., 2002; Finnberg et al., 2005). Thus, differential activation of the extrinsic pathway downstream of DR4 and/or DR5 could be a better predictor of the tissue-specific responses elicited by p53 activation in human patients, as compared with activation of p21 or some mitochondrial apoptotic factors, which seem to be activated regardless of outcome in various scenarios.

Regulation of BAX activity is a critical event in apoptosis. BAX mediates permeabilization of the outer mitochondrial membrane for release of pro-apoptotic molecules such as cytochrome C and SMAC/DIABLO (Jin and El-Deiry, 2005). Members of the BCL2 family of pro-survival factors bind to BAX and prevent its activation (Jin and El-Deiry, 2005). Members of the BH3-only family of apoptotic proteins promote BAX activity by: (1) binding to BCL2 family members and preventing their interaction with BAX (‘derepression/sensitiser’ mechanism) and/or (2) binding to and allosterically activating BAX (‘direct activation’ mechanism) (Jin and El-Deiry, 2005). The precise contribution of different BH3-only proteins to the ‘derepression’ versus ‘direct activation’ mechanisms is currently the subject of intense debate and is under investigation by several groups. This debate is clearly illustrated by PUMA, which has been proposed to mediate apoptosis by engaging multiple BCL2 family members without binding BAX or BAK (Willis et al., 2007), but also shown to directly activate BAX by triggering both its mitochondrial translocation and oligomerization (Kim et al., 2009). These models are not necessarily mutually exclusive and it is possible that PUMA contributes differentially to each mechanism in a context-dependent fashion. In this regard, our results illuminate a novel facet of PUMA in p53-dependent apoptosis. While there is no doubt that PUMA contributes to p53-dependent apoptosis in response to 5FU and other genotoxic agents in HCT116 and other cell types (Nakano and Vousden, 2001; Yu et al., 2001; Chipuk et al., 2005; Gomes and Espinosa, 2010), our analyses clearly indicate that transactivation of endogenous PUMA by p53 is not sufficient to induce apoptosis. In our system, total and mitochondrial levels of PUMA protein are identical between arresting and apoptotic cells, revealing the requirement for additional signals to induce cell death. Of note, we and others have shown that ectopic PUMA overexpression is sufficient to induce apoptosis in HCT116 cells and many other cell types (Nakano and Vousden, 2001; Yu et al., 2001; Gomes and Espinosa, 2010), which indicates that these cells can be driven into apoptosis by the mass action of BH3-only proteins. Thus, overexpression experiments must be interpreted carefully. One possibility is that cells are protected from the killing effects of endogenous PUMA by the action of survival factors, some of which may be induced by Nut3 itself. Indeed, our previous work showed that HCT116 cells are protected from the killing effects of Nut3 by the fact that p53 induces p21, 14-3-3ζ, and miR-34a, three factors mediating cell-cycle arrest downstream of p53 and thus partially protecting from apoptosis (Paris et al., 2008). In fact, we found that HCT116 p21−/− 14-3-3ζ−/− cells where miR-34a is inactivated with ‘antagomirs’ fail to properly arrest and show signs of apoptosis upon prolonged Nut3 treatment. Additionally, we reported that cell lines where Nut3 induces rapid apoptosis show impaired induction of one or more of these cell-cycle inhibitors. In the extreme case of the BV173 chronic myelogenous leukaemia cell line, which undergoes rapid apoptosis within 24 h of Nut3 treatment, we found that the p21 mRNA is rapidly degraded and the p21 protein never expressed, that the 14-3-3ζ promoter is silenced by DNA methylation and that the primary transcript for miR-34a is not processed into the mature microRNA. In addition to the effect of these cell-cycle inhibitors, HCT116 cells are also protected from the killing effects of PUMA by members of the BCL2 family. In fact, we and others have found that knockdown of BCL2 sensitizes cells to Nut3-induced apoptosis and that the BH3 mimetic ABT-737 synergizes with Nut3 to induce cell death (Sullivan and Espinosa, unpublished results; Wade et al., 2008).

One key observation from our present work is that arresting cells are primed for apoptosis by having translocated BAX to the mitochondria, but this form of BAX is not fully activated and remains poised in a monomeric state. Importantly, it has been shown that BAX is activated in a stepwise fashion, where two distinct biochemical steps mediate membrane translocation and oligomerization (Kim et al., 2009). In the simplest interpretation, PUMA induction suffices only to induce the first biochemical step in our system. Our results indicate that tBID then completes BAX activation at the mitochondria by inducing its oligomerization. Although both PUMA and tBID have been considered equally competent to induce both steps of BAX activation in certain cell-free assays (Kim et al., 2009), our results are more consistent with a model where PUMA and tBID cooperate to induce BAX activation. Functional collaboration between PUMA and tBID has been observed in other cell-free assays, where pre-treatment of mitochondria with PUMA BH3 domain peptides sensitized them to tBID-induced permeabilization by ~100–200-fold (Chipuk and Green, 2009). Furthermore, overexpression of PUMA is significantly less efficient at inducing apoptosis in bid−/− MEFs as compared with wild-type cells (Chipuk and Green, 2009). Our results reinforce the importance of this cooperation in cell fate choice and identify a key biochemical event that could be modulated to drive cells into alternative p53 responses. It remains to be determined to what degree this cooperation between the intrinsic and extrinsic pathways is conserved across diverse cell types. Our finding that the DR4/FADD/caspase 8/tBID pathway defines cell fate choice in both colorectal cancer and lung cancer cells suggests that it is not a rare event. Interestingly, the El-Deiry group has recently shown that the intrinsic pathway may rely on extrinsic signals in more...
scenarios than previously expected (Kuribayashi et al., 2011). In fact, they found that dr4/5−/− and puma−/− animals do not show additive protection from radiation-induced apoptosis in any of the investigated tissues.

In the post-genome era, the advent of functional genomics and proteomics has accelerated the cataloguing of genetic interactions and created a view of gene networks where degeneracy, redundancy, and context dependency are the norm. It is in this scenario that efforts to dissect how a finite number of gene modules adopt stimulus- and cell type-specific configurations to generate a plethora of biological responses become critical. Given the biomedical relevance of the p53 network and the validity of p53 as a bona-fide therapeutic target in cancer, advances such as those reported here will bring us closer to the development of effective p53-based therapies for the selective elimination of cancer cells.

Materials and methods

Cell culture

Cells were grown in McCoy’s 5A (HCT116), DMEM (RKO), or RPMI1640 (H460) medium supplemented with 10% FBS (Sigma-Aldrich) and antibiotic/antimycotic mix (Invitrogen) at 37°C/5.0% CO2. Unless otherwise noted, DMSO vehicle control (Sigma-Aldrich), Nutlin-3R (Nut3; Cayman Chemical), and 5FU (Sigma-Aldrich) were used at 0.1%, 10.0, and 375.0 μM, respectively. Doxorubicin (Sigma-Aldrich), Camptothecin (Sigma-Aldrich), and Etoposide (Sigma-Aldrich) were used at 0.5, 10.0, and 375.0 μM, respectively. For UVC irradiation of HCT116 cells, media was aspirated from the plate prior to treatment with UVC (50 J/m²) in a UV Stratalinker® 2400 (Stratagene). Media was then replaced and the cells incubated at 37°C/5.0% CO2 for 24 h prior to harvesting for western blot analysis.

Western blots and antibodies

Protein preparation, quantification, and immunoblot analyses were performed as previously described (Gomes et al., 2006). Antibody information is supplied in Supplementary Table SI.

Q-RT–PCR

RNA extractions, cDNA synthesis, and Q-RT–PCR were performed as previously described (Gomes et al., 2006). Briefly, total RNA was isolated using an RNeasy kit (Qiagen) and first-strand cDNA was synthesized using the qScript kit (Quanta Biosciences) according to the manufacturer’s instructions. cDNA was analysed by Q-RT–PCR Absolute Quantification method (SYBR green, ABI) on an ABI 7900HT instrument. Primer sequences are listed in Supplementary Table SII.

shRNA-mediated knockdown

shRNAs were designed using the PSICOLIGOMAKER 1.5 software, freely available from the Jacks Lab website (http://web.mit.edu/jacks-lab/protocols/psico.html), and cloned into the pLL3.7 expression vector. Commercially available shRNAs pre-cloned into the pLKO.1-Puro vector (shCTRL and shDR4) were purchased from Sigma-Aldrich. Oligonucleotide sequences for all shRNAs used in this study are listed in Supplementary Table SIII. Lentiviral particles were produced in HEK293FT packaging cells. HCT116 and RKO cells were transduced with 0.45 μM-filtered viral supernatants. Selection of stably transduced cells was carried out for 2–7 days with either 100.0 μg/ml G418 or 10.0 μg/ml puromycin (Sigma-Aldrich).

ChIP and Q-PCR

ChIP assays were performed as described in Gomes et al. (2006). Briefly, cells were treated for 12 h with DMSO, Nut3, or 5FU, fixed with 1.0% formaldehyde and harvested for whole-cell lysate preparation in RIPA buffer. In all, 1.0 mg of protein lysate was used per ChIP with the indicated antibodies (Supplementary Table SI) and Protein-G sepharose beads (GE Healthcare). ChIP-enriched DNA was analysed by Q-PCR as described in Gomes et al. (2006). See Supplementary Table SIV for Q-PCR primer sequences.

DR4 mRNA half-life determination

Cells were treated with DMSO, Nut3, or 5FU for 12 h, followed by the addition of 10.0 μg/ml Actinomycin D (ActD; Sigma-Aldrich) to the culture medium. Total RNA was isolated at several time points after the addition of ActD, reverse-transcribed and subjected to Q-RT–PCR analysis as described above.

Flow cytometry

Aptoptic index assays were performed as described in Gomes et al. (2006). Data were collected on a Cyan-ADP flow cytometer (Dako) and analysed using Summit 5.3 software (Beckman-Coulter).

Quantification of cell surface death receptor levels

HCT116 cells were treated for 24 h, trypsinized, and resuspended in 1.0% BSA (Fraction V) in PBS. In all, 1 × 10⁵ cells were labelled with the appropriate antibody or isotype control for 1.0 h at 25°C, washed with 1% BSA/PBS, and stained with secondary antibody for 1.0 h at 25°C in the dark. Flow cytometry was carried out as described above. MFIs were calculated as the ratio between the mean fluorescence value of the specific antibody and that of the appropriate isotype control. Histograms were created using the FlowJo software package (Tree Star, Inc.). Antibodies used for detection are listed in Supplementary Table SI.

Mitochondrial purification and crosslinking

Subcellular fractionation was performed as described in Pallotti and Lenaz (2007). Briefly, cells were washed with cold PBS, resuspended in swelling buffer (10.0 mM Tris–HCl pH 7.5, 10.0 mM NaCl, 1.5 mM MgCl₂), and incubated on ice for 5 min. Cell suspensions were adjusted to 250.0 mM sucrose and dounce homogenized. Undisrupted cells and large cellular fragments were pelleted by centrifugation at 10 000 g and loaded onto a 1.0–1.7 M discontinuous sucrose gradient. Mitochondria in the supernatant were separated from the cytosolic fraction by centrifugation at 10 000 g and loaded onto a 1.0–1.7 M discontinuous sucrose gradient. Mitochondria were then recovered from the interphase. Isolated mitochondria were either directly lysed in SDS buffer (1.0% SDS, 10.0% glycerol, 100.0 mM Tris, pH 7.4) or first crosslinked with bismaleimidohexane (Pierce) using the manufacturer’s protocol prior to lysis. Samples were then analysed by western blot.

BAX immunoprecipitation

Immunoprecipitation of activated BAX protein was performed as described (Kim et al., 2009) with minor modifications. Cells were treated with DMSO, Nut3, or 5FU for 24 h, washed once in PBS, resuspended in 1.0% CHAPS Buffer (1.0% (w/v) CHAPS, 142.5 mM KCl, 2.0 mM CaCl₂, 20.0 mM Tris–Cl, pH 7.4) and dounce homogenized. Lysates were cleared by centrifugation (21 000 g for 20 min at 4°C). In all, 1.0 mg of protein extract was pre-cleaned with Protein-G sepharose beads (GE Healthcare) for 1.5 h at 4°C and incubated with 5.0 μg of mouse 6A7 antibody ( Trevigen) overnight at 4°C. IPs were washed with 1.0% CHAPS buffer, eluted by boiling in 1× SDS–PAGE loading dye, and subjected to immunoblot analysis.

Supplementary data

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Author contributions: REH designed and performed most of the experiments, interpreted the data, and co-wrote the manuscript with JME. ZA, RP, and MDG contributed to study design and performed the experiments in collaboration with REH.

Conflict of interest

The authors declare that they have no conflict of interest.
References

Beckerman R, Donner AJ, Mattia M, Peart MJ, Manley JL, Espinosa JM, Prives C (2009) A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. *Genes Dev* 23: 3643–3646.

Brown CJ, Lain S, Verma CS, Fersht AR, Lane DP (2009) Awakening guardian angels: drugging the p53 pathway. *Nat Rev Cancer* 9: 862–873.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 282: 1497–1501.

Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Chan TA, Hwang PM, Hermeking H, Kinzler KW, Vogelstein B (2007a) Stimulus-specific positive coregulator of p53 target genes. *Science* 310: 624–637.

Burns TF, Bernhard EJ, El-Deiry WS (2003) Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis *in vivo*. *Oncogene* 20: 4601–4612.

Chan TA, Hwang PM, Hermekeing H, Kinzler KW, Vogelstein B (2000) Cooperative effects of genes controlling the G(2)/M checkpoint. *Genes Dev* 14: 1584–1588.

Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR (2007) Modulating p53 transactivation through stress- and promoter-specific recruitment of transcription factors. *Cell Cycle* 6: 2594–2598.

Donner AJ, Hoover JM, Zsostek SA, Espinosa JM (2007a) Stimulus-specific transcriptional regulation within the p53 network. *Cell Cycle* 6: 2594–2598.

Donner AJ, Zsostek S, Hoover JM, Espinosa JM (2007b) CDK8 is a stimulus-specific positive coregulator of p53 target genes. *Mol Cell* 27: 121–133.

el-Deiry WS, Harper JW, O'Connor PM, Veculcescu VE, Canman CE, Jackman K, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wilman KG, Mercer KG, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, Vogelstein B (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 54: 1169–1174.

el-Deiry WS, Tokino T, Veculcescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817–825.

Espinosa JM (2008) Mechanisms of regulatory diversity within the p53 transcriptional network. *Oncogene* 27: 4013–4023.

Espat JA, Xu X, Gerber JS, Lee RE, Espinosa JM (2003) p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. *Mol Cell* 12: 1051–1027.

Fei P, Bernhard EJ, el-Deiry WS (2002) Tissue-specific induction of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis *in vivo*. *Oncogene* 20: 4601–4612.

Fei P, Bernhard EJ, el-Deiry WS (2002) Tissue-specific induction of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis *in vivo*. *Oncogene* 20: 4601–4612.

Gomes NP, Bjerke G, Llorente B, Szostek SA, Emerson BM, Espinosa JM (2009) Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell* 36: 487–499.

Kuribayashi K, Finnegn B, Jeffers JR, Zambetti GP, Hsieh JJ, Chiang EH (2009) Stimulus-specific activation of BAX and BAK by BID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell* 36: 487–499.

Laptenko O, Prives C (2006) Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ* 13: 951–961.

Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 489–501.

Liu X, Yue P, Khuri FR, Sun SY (2004) p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res* 64: 5078–5083.

Miyashita T, Krajewska S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene* 9: 1799–1805.

Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80: 293–299.

Morachis JM, Murawsky CM, Emerson BM (2010) Regulation of the p53 transcriptional response by structurally diverse core promoters. *Genes Dev* 24: 135–147.

Muller M, Wilder S, Bannasch N, Israel D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH (1998) p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* 188: 2033–2045.

Nakano K, Voussden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 7: 683–694.

Pallotti F, Lenaz G (2007) Isolation and subfractionation of mitochondrial organelles from animal cells and tissue culture lines. *Methods Cell Biol* 80: 3–44.

Paris R, Henry RE, Stephens JS, McBrady M, Espinosa JM (2008) Multiple p53-independent gene silencing mechanisms define the cellular response to p53 activation. *Cell Cycle* 7: 2427–2433.

Samuels-Lev Y, O’Connor DJ, Bergamaschi D, Triguante G, Hsieh JK, Zhong S, Campagne I, Naumovski L, Crook T, Lu X (2001) ASPF proteins specifically stimulate the apoptotic function of p53. *Mol Cell* 8: 781–794.

Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS (2002) Cell Cycle dependence of p53 contributes to chemosensitivity. *Nat Cell Biol* 4: 842–849.

Schuler M, Maurer U, Goldstein JC, Breitenbucher F, Hoffarth S, Waterhouse NJ, Green DR (2003) p53 triggers apoptosis in oncogene-expressing fibroblasts by the induction of Noxa and mitochondrial Bax translocation. *Cell Death Differ* 10: 451–460.

Sykes SM, Melleri HS, Holbert MA, Li K, Marmorstein R, Lane WS, McMahon SB (2006) Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* 24: 841–851.

Tanaka T, Ohkubo S, Tatsuno I, Prives C (2007) hCAS/CSEIL associates with chromatin and regulates expression of select p53 target genes. *Cell* 130: 638–650.

Tang Y, Luo J, Zhang W, Gu W (2006) Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell* 24: 827–839.

Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, Zhao X, Yu BT, Qing W, Packman K, Myklebost O, Heimbrock DC, Vassiliev LT (2006) Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci USA* 103: 1888–1893.

Vassiliev LT, Yu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303: 844–848.

Ventura A, Kirsch DG, McLoughlin ME, Tuveson DA, Grimm J, Lintanit L, Newman J, Reczek EE, Weissleder R, Jacks T (2007) Restoration of p53 function leads to tumour regression in vivo. *Nature* 449: 661–665.

von Haefen C, Wieder T, Essmann F, Schulze-Osthoff K, Dorken B, Daniel PT (2003) Paclitaxel-induced apoptosis in BJAB cells
proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene* 22: 2236–2247
Vousden KH, Lu X (2002) Live or let die: the cell’s response to p53. *Nat Rev Cancer* 2: 594–604
Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. *Cell* 137: 413–431
Wade M, Rodewald LW, Espinosa JM, Wahl GM (2008) BH3 activation blocks Hdmx suppression of apoptosis and cooperates with Nutlin to induce cell death. *Cell Cycle* 7: 1973–1982
Wang S, El-Deiry WS (2004) Inducible silencing of KILLER/DR5 in vivo promotes bioluminescent colon tumor xenograft growth and confers resistance to chemotherapeutic agent 5-fluorouracil. *Cancer Res* 64: 6666–6672
Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* 315: 856–859
Wu GS, Burns TF, McDonald III ER, Jiang W, Meng R, Krantz ID, Kao G, Can DD, Zhou JY, Muschel R, Hamilton SR, Spinner NB, Markowitz S, Wu G, el-Deiry WS (1997) KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 17: 141–143
Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM (2001) Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene* 20: 240–251
Xue W, Zender L, Miething C, Dickens RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW (2007) Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445: 656–660
Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci USA* 100: 1931–1936
Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 7: 673–682