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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, 14-3-3σ) 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 unappreciated 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; Espinosa, 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).

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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 transactivation 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
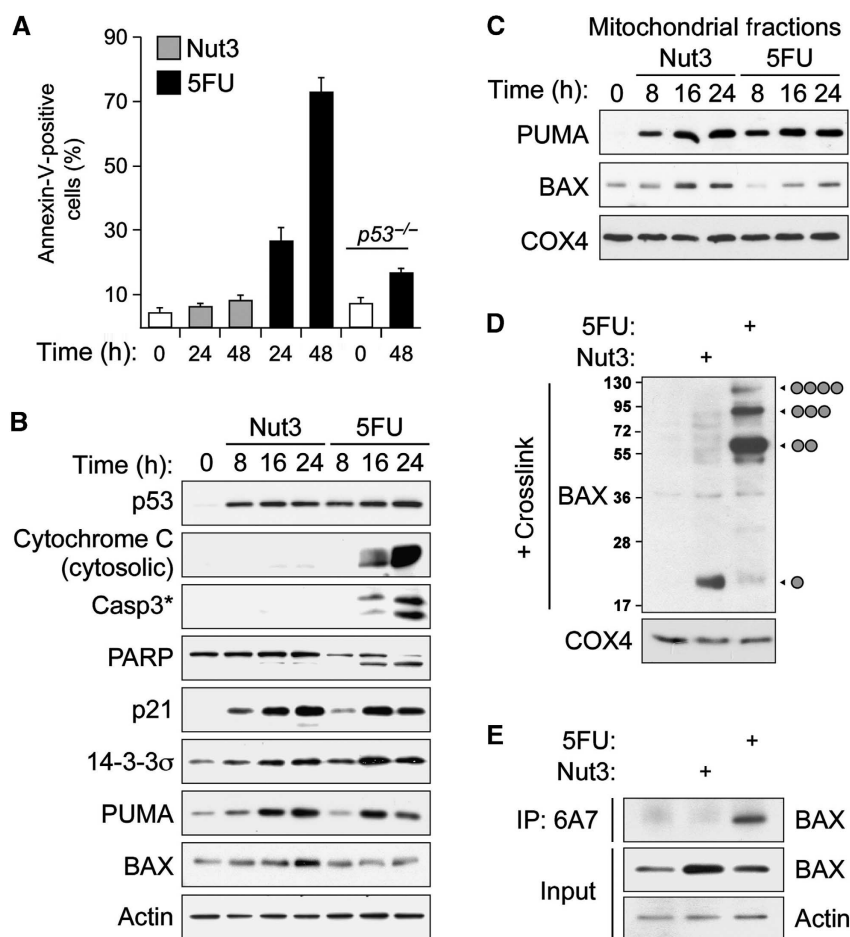
Figure 1 Stimulus-specific responses to p53 activation are defined by differential BAX oligomerization. (**A**) HCT116 cells undergo p53-dependent apoptosis in response to 5FU, but not Nut3. Cells were treated with Nut3 or 5FU for the indicated times, stained with Annexin-V-FITC and analysed by flow cytometry. Isogenic p53-null HCT116 cells are denoted as $p53^{-/-}$. Data shown are the mean ± s.d. of at least three independent experiments. (**B**) Immunoblot analysis reveals that several genes mediating cell-cycle arrest and apoptosis are expressed equally in response to Nut3 or 5FU treatment. Casp3* denotes the p19/17 doublet indicative of caspase 3 activation. (**C**) Nut3-treated cells accumulate as much mitochondrial PUMA and BAX as do 5FU-treated cells. COX4 is a mitochondrial protein serving as a loading control. HCT116 cells were treated as in (**B**) before preparation of mitochondrial fractions (see Supplementary Figure S1 for fractionation controls). (**D**) BAX oligomerization is observed only in 5FU-treated cells. Filled circles on the right represent the predicted migration of BAX mono-, di-, tri-, and tetramers. (**E**) Stimulus-specific activation of the BAX protein was detected by immunoprecipitation with the conformer-specific 6A7 antibody followed by western blot. Figure source data can be found in Supplementary data.

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-X$_L$. 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-X$_L$ 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
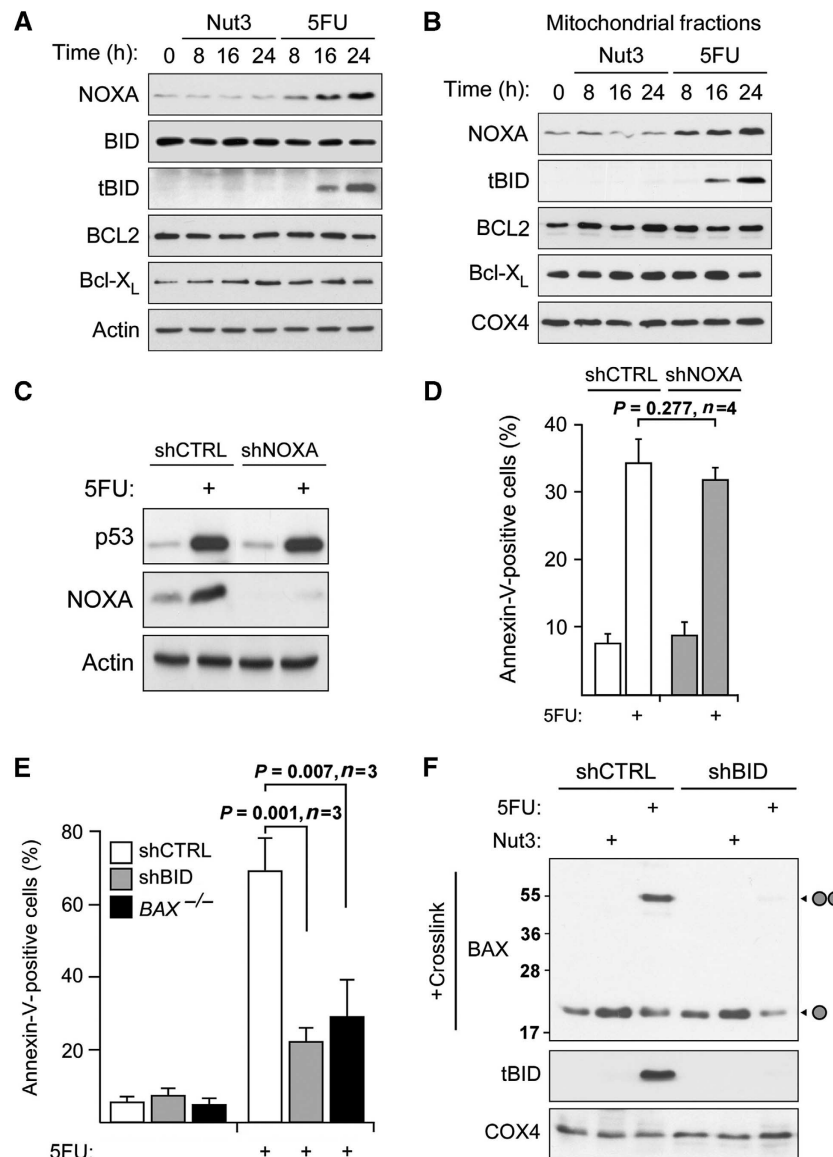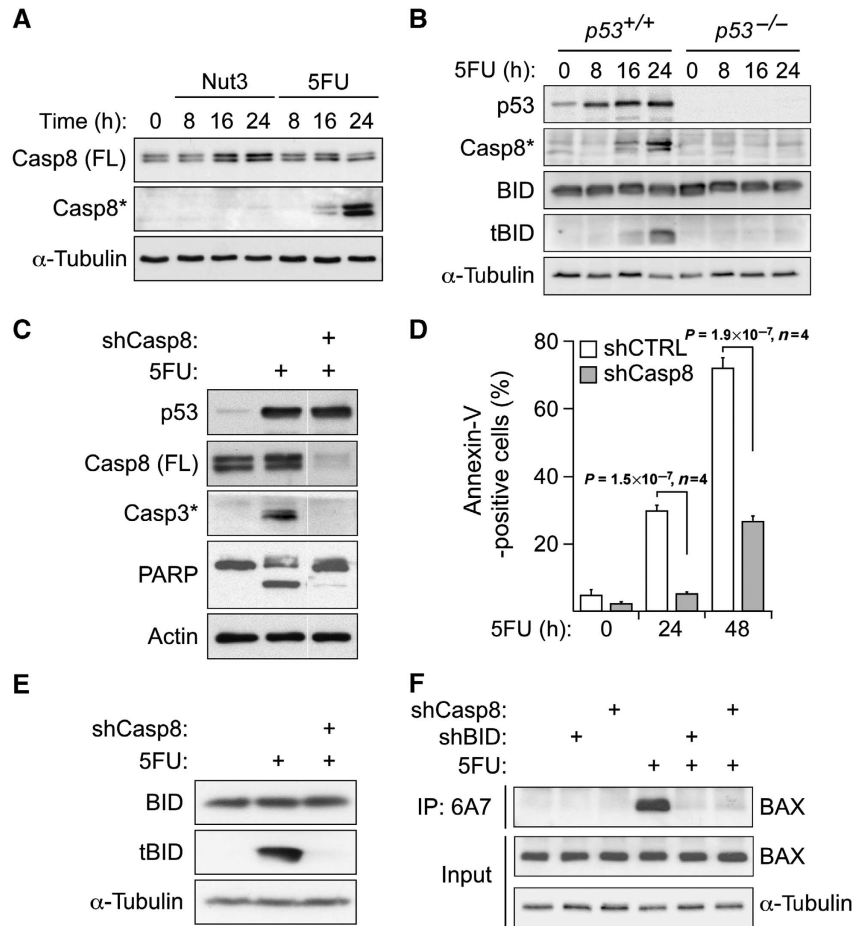
Figure 2 BID cleavage is stimulus-specific and required for BAX oligomerization and apoptosis. (**A**) NOXA and tBID proteins accumulate in 5FU-treated, but not in Nut3-treated cells, whereas BCL2 and BCL-X_L levels are similar between treatments. (**B**) Levels of mitochondria-localized NOXA and tBID increase following 5FU, but not Nut3, whereas the levels of BCL2 and BCL-X_L remain similar between treatments. The COX4 panel is the same as in Figure 1C. (**C**) Immunoblot assessment of NOXA knockdown. (**D**) Apoptotic index assays show no significant effect of NOXA knockdown on 5FU-induced apoptosis. Data shown are the mean ± s.d. (**E**) Apoptotic index assays indicate that BID knockdown abrogates 5FU-induced apoptosis as effectively as *BAX* knockout. Data shown are the mean ± s.d. (**F**) Western blot analysis of BAX dimerization in Nut3 and 5FU-treated cells expressing shCTRL or shBID. 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.

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 initiator 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 cleavage upon 5FU treatment also requires p53 (Figure 3B). Importantly, BAX activation is significantly blocked in cells depleted of caspase 8 or BID as seen by immunoprecipitation with 6A7 antibody (Figure 3F). Stimulus-specific activation of caspase 8 was also observed in RKO colorectal cancer cells, which also undergo cell-cycle arrest upon Nut3 treatment

Figure 3 Caspase 8 activation is stimulus-specific, p53-dependent and required for BID cleavage and BAX activation. (**A**) Caspase 8 activation is stimulus-specific (FL) indicates full-length caspase 8 (55–57 kDa), Casp8* indicates the 43–41 kDa cleavage products. (**B**) 5FU-induced caspase 8 activation and BID cleavage in HCT116 cells are p53-dependent events. (**C**) Caspase 8 knockdown prevents caspase 3 activation and PARP cleavage upon 5FU treatment. White line indicates cropping of intervening lanes from the same blot. (**D**) Apoptotic index assays show that caspase 8 is required for 5FU-induced apoptosis. Data shown are the mean ± s.d. *P*-values were calculated by Student's *t*-test (unpaired, two-tailed). (**E**) BID cleavage in 5FU-treated cells requires caspase 8. (**F**) Immunoblot analysis of 6A7 immunoprecipitates indicates that BAX activation following 5FU treatment is both caspase 8- and BID-dependent. Figure source data can be found in Supplementary data.

(RKO, Supplementary Figure S4) (Tovar *et al*, 2006). 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.
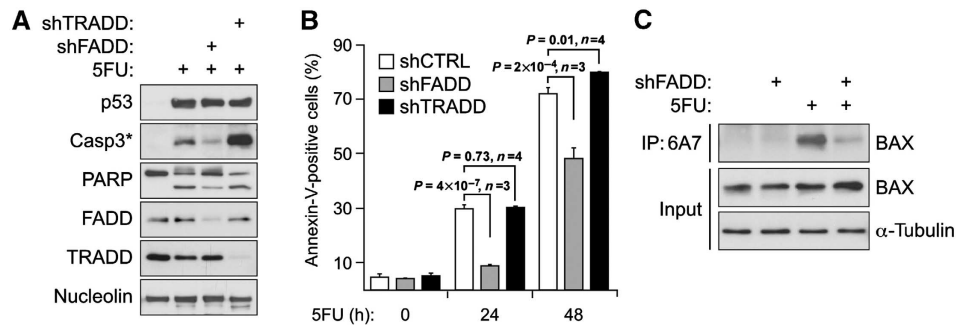
Differential activation of the extrinsic pathway requires FADD, not TRADD

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
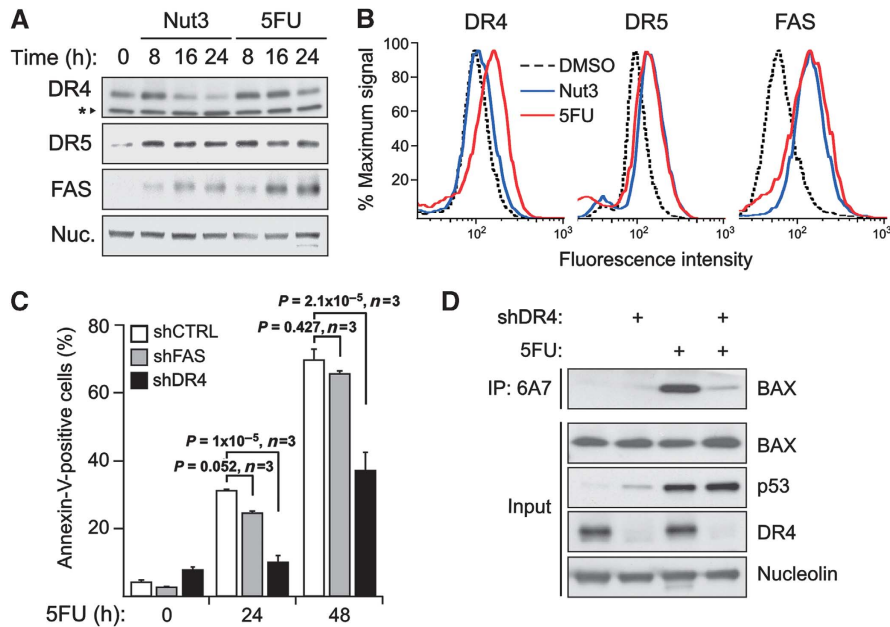
Figure 4 Stimulus-specific activation of the extrinsic apoptotic pathway is mediated by FADD. (**A**) Caspase 3 activation requires the adaptor protein FADD, not TRADD. (**B**) Apoptotic index assays demonstrate that FADD, not TRADD, is required for full induction of apoptosis upon 5FU treatment. Data shown are the mean ± s.d. *P*-values were calculated by Student's *t*-test (unpaired, two-tailed). (**C**) Immunoblot analysis of 6A7 immunoprecipitates indicates that BAX activation following 5FU treatment is FADD-dependent. Figure source data can be found in Supplementary data.

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^{FAS} 2.10 ± 0.04 and 2.07 ± 0.05; MFI^{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 S5P-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$^{-/-}$ 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

Figure 5 DR4 is required for stimulus-specific BAX activation and apoptosis. (**A**) Immunoblots show that total protein levels of the DR4 and FAS death receptors, but not DR5, are higher in 5FU-treated cells than in those treated with Nut3. (*) Indicates a non-specific band. (**B**) Nut3 fails to induce DR4 cell surface expression as measured by flow cytometry. Nut3 and 5FU induce DR5 and FAS cell surface localization equally. Data shown are representative of a least three independent experiments. (**C**) Apoptotic index assays show that DR4, but not FAS, is required for 5FU-induced apoptosis (see Supplementary Figure S6 for western blot analysis of FAS knockdown). Data shown are the mean ± s.d. *P*-values were calculated by Student's *t*-test (unpaired, two-tailed). (**D**) Immunoblot analysis of 6A7 immunoprecipitates indicates that BAX activation following 5FU treatment is DR4-dependent. Figure source data can be found in Supplementary data.

genes in a p53-independent manner, and in the case of DR4, this event contributes to cell fate choice upon p53 activation.

### 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 and B). Importantly, knockdown of BID, caspase 8, DR4, or FADD decreases BID cleavage, activation of caspase 3 and Annexin-V staining in this cell line (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
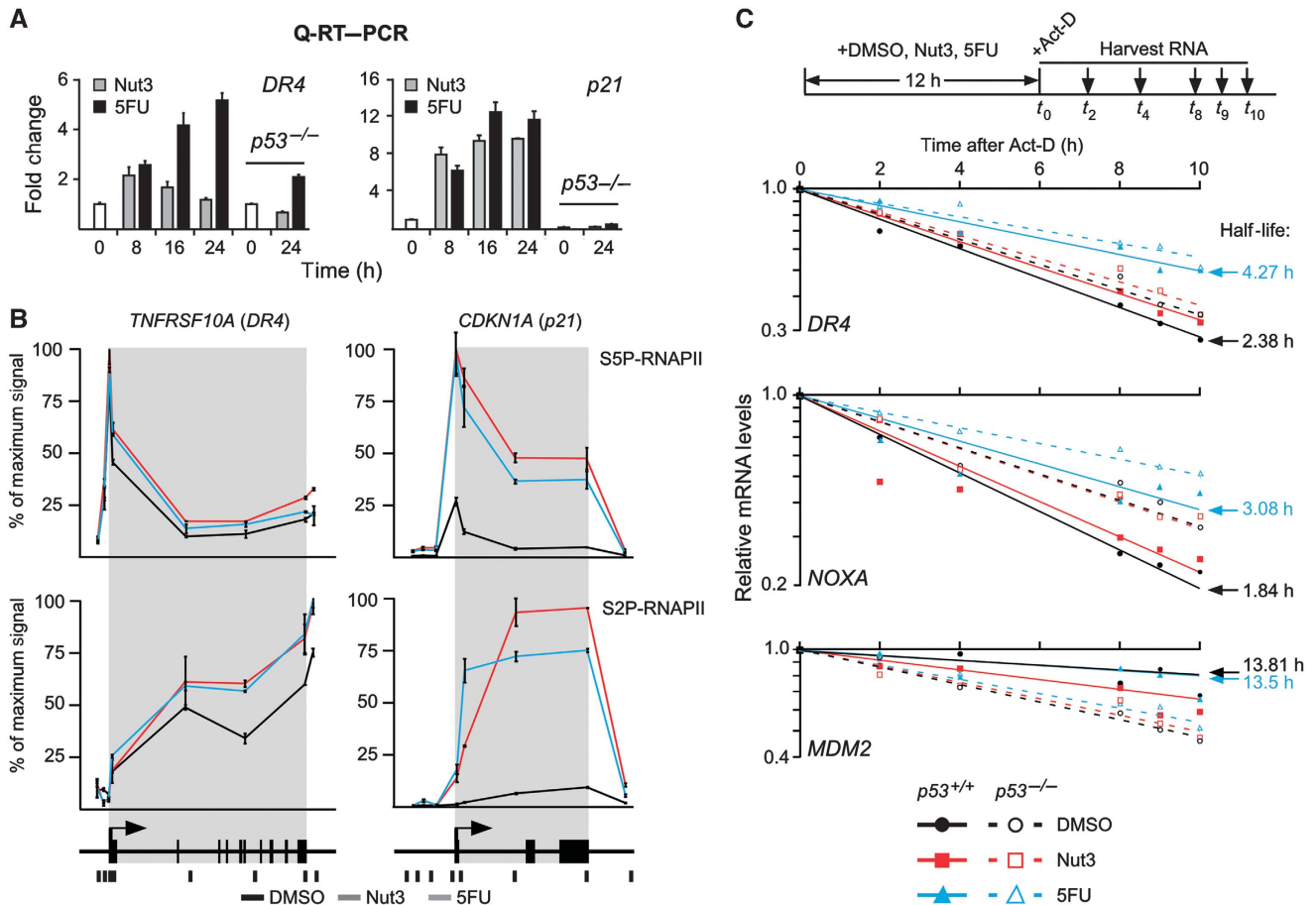
Figure 6 Stimulus-specific stabilization of the *DR4* mRNA. (**A**) Q-RT–PCR analysis reveals that the *DR4* mRNA, but not the *p21* mRNA, accumulates more strongly in 5FU-treated cells. (**B**) ChIP analysis of the *DR4* and *p21* loci show increased levels of initiating (S5P-RNAPII) and elongating (S2P-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 12 h of DMSO, Nut3 or 5FU treatment of HCT116 cells of different p53 status, RNA synthesis was halted by ActD 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.

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 *14-3-3σ*, 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*, *14-3-3σ*, *PUMA*, and *BAX*. For example, the HZF protein was shown to associate with p53 to allow binding and transactivation of the *p21* and *14-3-3σ* 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, 14-3-3σ, 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
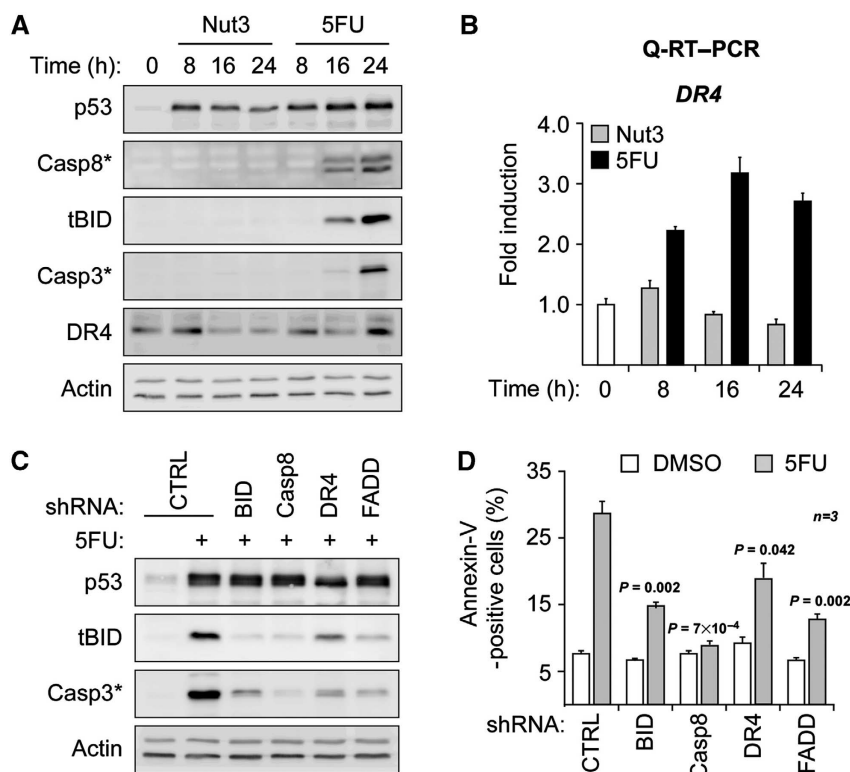
Figure 7 The DR4/FADD/caspase 8/tBID axis also defines cell fate choice in the H460 lung cancer cell line. (**A**) Western blot analysis of H460 cells shows that both Nut3 and 5FU activate p53 effectively, yet only 5FU leads to caspase 8 activation, tBID formation and caspase 3 activation. Differential cell fate choice correlates with higher levels of DR4 at late time points after 5FU treatment. (**B**) Q-RT–PCR analysis shows that the *DR4* mRNA accumulates only in 5FU-treated H460 cells. (**C**) Knockdown of BID, caspase 8, DR4, or FADD impairs both tBID formation and activation of the executioner caspase 3. Immunoblot assessment of knockdown efficiency in H460 cells can be found in Supplementary Figure S8. (**D**) Apoptotic index assays reveal that BID, caspase 8, DR4, and FADD are all required for efficient induction of apoptosis upon 5FU treatment. Data shown are the mean ± s.d. The indicated *P*-values represent the statistical significance of the 5FU-treated value for the respective knockdown line relative to the CTRL shRNA value and were calculated by Student's *t*-test (unpaired, two-tailed). Figure source data can be found in Supplementary data.
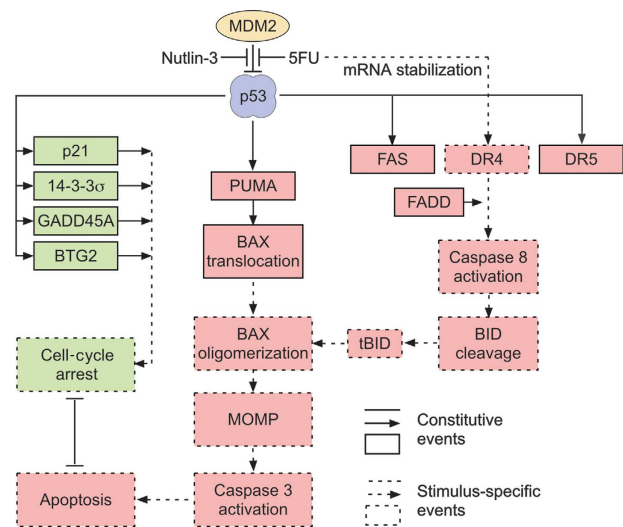
Figure 8 Model of stimulus-specific configurations of the p53 network defining cell fate choice in response to Nutlin-3 and 5FU. See Discussion for details.

BID. 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 knockdown

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, 14-3-3σ, 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$^{-/-}$ 14-3-3σ$^{-/-}$ 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 14-3-3σ 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 $37^\circ C/5.0\%$ CO_2. 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 $375.0\,\mu M$, respectively. Doxorubicin (Sigma-Aldrich), Camptothecin (Sigma-Aldrich), and Etoposide (Sigma-Aldrich) were used at $0.5\,\mu M$, $2.0\,\mu M$, and $1.0\,\mu g/$ml, respectively. For UVC irradiation of HCT116 cells, media was aspirated from the plate prior to treatment with UVC ($50\,J/m^2$) in a UV Stratalinker® 2400 (Stratagene). Media was then replaced and the cells incubated at $37^\circ C/5.0\%$ CO_2 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 $100.0\,\mu g/ml$ G418 or $10.0\,\mu g/ml$ 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 $10.0\,\mu g/ml$ 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
Apoptotic 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, 1×10^5 cells were labelled with the appropriate antibody or isotype control for 1.0 h at $25^\circ C$, washed with 1% BSA/PBS, and stained with secondary antibody for 1.0 h at $25^\circ C$ 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 $MgCl_2$), 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 $1000\,g$. 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 $CaCl_2$, 20.0 mM Tris–Cl, pH 7.4) and dounce homogenized. Lysates were cleared by centrifugation ($21\,000\,g$ for 20 min at $4^\circ C$). In all, 1.0 mg of protein extract was pre-cleared with Protein-G sepharose beads (GE Healthcare) for 1.5 h at $4^\circ C$ and incubated with $5.0\,\mu g$ of mouse 6A7 antibody (Trevigen) overnight at $4^\circ C$. IPs were washed with 1.0% CHAPS buffer, eluted by boiling in $1\times$ SDS–PAGE loading dye, and subjected to immunoblot analysis.

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

Conflict of interest

The authors declare that they have no conflict of interest.

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