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JTV1 co-activates FBP to induce USP29 transcription and stabilize p53 in response to oxidative stress

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***c-myc* and p53 networks control proliferation, differentiation, and apoptosis and are responsive to, and cross-regulate a variety of stresses and metabolic and biosynthetic processes. At *c-myc*, the far upstream element binding protein (FBP) and FBP-interacting repressor (FIR) program transcription by looping to RNA polymerase II complexes engaged at the promoter. Another FBP partner, JTV1/AIMP2, a structural subunit of a multi-aminoacyl-tRNA synthetase (ARS) complex, has also been reported to stabilize p53 via an apparently independent mechanism. Here, we show that in response to oxidative stress, JTV1 dissociates from the ARS complex, translocates to the nucleus, associates with FBP and co-activates the transcription of a new FBP target, ubiquitin-specific peptidase 29 (USP29). A previously uncharacterized deubiquitinating enzyme, USP29 binds to, cleaves poly-ubiquitin chains from, and stabilizes p53. The accumulated p53 quickly induces apoptosis. Thus, FBP and JTV1 help to coordinate the molecular and cellular response to oxidative stress.**

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Introduction

The far upstream element (FUSE) binding protein (FBP) and the FBP-interacting repressor (FIR) interact with the transcription factor TFIIH to close a topological loop that controls promoter escape by RNA polymerase II. This loop is critical both to maintain *c-myc* steady-state levels and to program the pulse of expression induced by serum (Liu *et al*, 2001, 2006). Disruption of this loop by TFIIH mutation or by knockdown of FBP and FIR delays post-induction *c-myc* shutoff (Liu *et al*, 2006) and increases cell-to-cell variation of c-Myc levels (Weber *et al*, 2005). Although FBP has been suggested to be an important regulator of apoptosis (Jang *et al*, 2009), how FBP, its interacting factors and their targets respond to cellular stresses and stimuli remains largely unexplored.

One FBP partner, JTV1 (p38/AIMP2), has been shown to mediate the ubiquitination and degradation of FBP. Knockout of JTV1 increases the levels of FBP and c-Myc in fetal lung and intestines (Kim *et al*, 2003b). JTV1 is also a non-catalytic subunit of the cytoplasmic multi-aminoacyl-tRNA synthetase (ARS) complex (Kim *et al*, 2002). Besides being important for efficient protein synthesis, additional physiological roles for ARS complex components have been discovered (Lee *et al*, 2004; Park *et al*, 2005). In response to signals, individual subunits of the complex may be released to participate in a variety of cellular processes, including transcription (Kim *et al*, 2003b), translational silencing (Sampath *et al*, 2004), angiogenesis (Park *et al*, 2002) and apoptosis (Park *et al*, 2005; Han *et al*, 2008). For example, following DNA damage, JTV1 is liberated from the ARS complex, phosphorylated in a JNK2-dependent pathway and translocated into the nucleus where it has been suggested to bind and sequester p53 from Mdm2-dependent ubiquitination (Han *et al*, 2008). JTV1 has also been shown to be a substrate of E3 ligase Parkin (Corti *et al*, 2003). Accumulation of JTV1 as a result of Parkin mutation has been speculated to contribute to the characteristic dopaminergic cell death observed in Parkinson patients (Ko *et al*, 2005).

Although evidence indicates that abnormal levels or subcellular localization of JTV1 correlates with apoptosis, how JTV1 modulates apoptosis has been incompletely described. Here, we report that JTV1 co-activates the transcription of a previously uncharacterized target, ubiquitin-specific peptidase 29 (USP29), via FBP bound tightly at an upstream site. USP29 realizes much of JTV1's proapoptotic potential by binding with, deconjugating ubiquitin from, and stabilizing p53. We also show that in response to oxidative stress, endogenous JTV1 migrates into the nucleus and associates with nuclear FBP to activate USP29 transcription. Regulating *c-myc* and p21 expression (Rabenhorst *et al*, 2009), as well as p53 protein levels via USP29, the FBP–JTV1 system is poised to shift the balance between cell proliferation, survival and death under physiological and pathological conditions.

Results

JTV1 activates USP29 transcription through an FBP-binding site on the USP29 promoter

Both JTV1 and FBP have been suggested to regulate apoptosis, the former through p53 and the latter via Myc. Because

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JTV1 and FBP interact, cross-talk between their respective apoptotic pathways seemed likely. Although FBP target genes have been documented (Chung *et al*, 2006), a role for JTV1 in the transcriptional regulation of human genes is unknown. To interrogate the mechanisms through which JTV1 and FBP collaborate to regulate apoptosis, a series of expression microarray experiments were performed to identify candidate JTV1 target genes. Besides the full-length JTV1, a naturally existing, alternative splice form of JTV1 (JTV1-Alt, JA (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?exdb=AceView&db=36a&term=jtv1&submit=Go)) was also used in these microarray assays due to its higher affinity for FBP observed in yeast two-hybrid assays (data not shown). JA lacks the second exon but retains the amino-acid sequences required to interact with FBP and the ubiquitin E3-ligase Parkin (Corti *et al*, 2003; Kim *et al*, 2003b). Immunoprecipitation results indicated that JA interacted with endogenous FBP similar to full-length JTV1 (Figure 1A). JA and JTV1 were also ubiquitinated to similar extents (Figure 1B). These data indicated that both JTV1 and JA preserved the molecular features needed to interact with FBP and to modify its function.

To identify candidate JTV1 target genes, GFP-JTV1 or GFP-JA were transiently expressed in HeLa cells. Transfected cells were harvested and sorted by green fluorescence for extraction of total RNA and protein. The mRNA profiles in GFP-JTV1 or GFP-JA transfected cells were compared with GFP-transfected cells using microarrays. Among the 142 genes changing over two-fold with JTV1 or JA expression, 96 were upregulated and 46 were downregulated (Supplementary Table I). Qualitatively, an increased fraction of dead cells were noted in the cells transiently transfected with GFP-JTV1 or -JA. Mindful of a connection between JTV1 and apoptosis, especially that JTV1 affects p53 stability (Ko *et al*, 2005; Han *et al*, 2008), ubiquitin-specific peptidase 29 (USP29) was an especially inviting candidate to study further since deubiquitination is a major mechanism that stabilizes p53 and induces apoptosis. USP29 is a type-2 ubiquitin carboxyl-terminal hydrolase that contains the two conserved domains found in ubiquitin-specific processing proteases. Other members of this USP family modify the stability of transcription factors including p53 (Li *et al*, 2002; Cummins and Vogelstein, 2004; Stevenson *et al*, 2007; Yuan *et al*, 2010), FOXO4 (van der Horst *et al*, 2006) and c-Myc (Popov *et al*, 2007). Consistent with reports that USP29 is paternally imprinted and is either not expressed or is expressed at extremely low levels in adult tissues except testis (Kim *et al*, 2000, 2003a), the USP29 levels from the GFP controls were barely above background, making quantification unreliable. The upregulation of USP29 by JTV1/JA in these RNA samples was confirmed using qPCR (Figure 1C). Utilization of an optimized primer-probe set detected little if any USP29 mRNA under normal growth conditions; however, 48 h post-transfection, JTV or JA-driven USP29 mRNA levels were comparable to those of endogenous *c-myc* (Supplementary Figure S1), indicating that JTV1 expression overcame whatever the mechanisms preventing USP29 transcription (Kim *et al*, 2003a; Huang and Kim, 2009).

The sequence of JTV1 reveals no known nucleic acid binding domain. Moreover, the inability of Gal4-DBD-JTV1 to activate a Gal4-UAS-driven reporter (data not shown) also indicated that JTV1 is not an autonomous transcription activator. To activate USP29 transcription, JTV1 would need to cooperate with a DNA-binding activator such as FBP. Inspection of the USP29 promoter revealed a 54-bp segment 2.5 kb upstream of the transcription start site that included a better match with the optimal FBP-binding element, than found at the *c-myc* FUSE (mFUSE). To test if JTV1 activated USP29 transcription, a 2.9-kb promoter fragment, as well as derivatives deleting 400 bp (dF) or 63 bp (Δ63) encompassing the USP29 FUSE (uFUSE), were cloned upstream of a luciferase reporter, and assayed for luciferase following co-transfection with either JTV1 or JA expression plasmids. To dissociate any effects that JTV1 or JA might have on reporter activity from its putative influence on p53 (Han *et al*, 2008), p53-null H1299 cells were used for these assays. JTV1 and JA induced luciferase activity from the 2.9-kb promoter by 1.9- and 3.7-fold, respectively (Figure 1D). Neither JTV1 nor JA activated the dF or Δ63 reporters, indicating that the JTV1/JA responsiveness mapped to the uFUSE.

FBP and/or JTV1 binding with uFUSE were tested using electophoretic mobility shift assays (EMSA). As predicted, FBP bound tightly with the uFUSE probe (Figure 1E, lane 2). JTV1 alone formed no DNA complex (lane 6), but with FBP yielded a complex migrating more slowly than FBP-uFUSE (lanes 3 and 5); this complex was further supershifted by antibodies to either protein (lanes 8 and 9). As expected, the FBP-uFUSE and FBP–JTV1–uFUSE complexes were reduced by competition with FUSE-bearing oligonucleotides (lane 4) but not by irrelevant sequences (lane 5).

To show that JTV1 and FBP bound with the endogenous USP29 promoter, cells transfected with GFP, GFP-JTV1 or GFP-JA were assayed by chromatin immunoprecipitation (ChIP). In GFP-control cells, neither FBP nor JTV1 were found at the endogenous uFUSE (Figure 1Fa), consistent with the observed low or absent USP29 expression. FBP was bound at the *c-myc* FUSE (mFUSE) in these cells (Figure 1Fd, lane 3), as expected (Liu *et al*, 2006). In contrast, transfected GFP-JTV1 or GFP-JA were each co-recruited with endogenous FBP to uFUSE (Figure 1Fa and c, lanes 2 and 4), paralleling USP29 induction. Concurrently, GFP-JTV1 or GFP-JA was also recruited to *c-myc* mFUSE (Figure 1Fb and c versus e and f). ChIP using anti-GFP rather than anti-JTV1, more likely reveals the relative binding of GFP-JTV1 versus GFP-JA because of the markedly reduced immunoreactivity of anti-JTV1 with exon 2-lacking JA (Figure 1Fc and f, lane 4; Supplementary Figure S2). One explanation for the absence of JTV1 at *c-myc* in GFP-control cells (Figure 1Fd, lane 4) would be limited nuclear availability of endogenous JTV1 (see below). The recruitment of JTV1 and FBP to their target sequences supports a transcriptional role for these proteins.

JTV1 cooperates with FBP to activate USP29 transcription

If JTV1 induces USP29 transcription via FBP, then FBP should also participate in the USP29 induction. Indeed, transfected FBP upregulated the USP29 promoter (Figure 2A) in a dose-dependent manner (data not shown). As with *c-myc*, USP29 was even more strongly activated by FBP3, the strongest transactivator of the FBP family (Chung *et al*, 2006). Thus, uFUSE and mFUSE have similar properties.

To test if endogenous FBP and JTV1 at physiological levels contribute to USP29 promoter activity, H1299 cells were transfected with siRNAs to FBP or JTV1 and incubated for

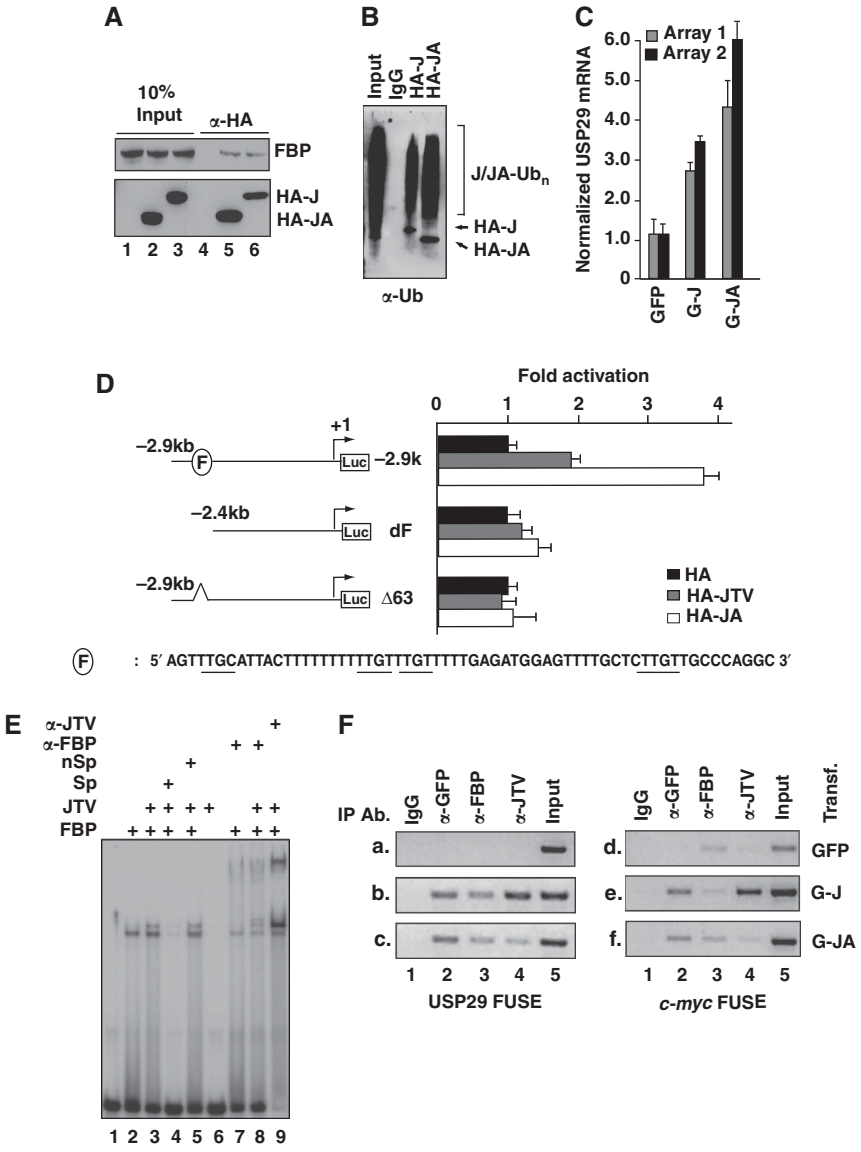

Figure 1 JTV1/JA activates USP29 transcription through a FBP-binding site on the USP29 promoter. (**A**) The alternatively spliced JTV1 form (JA) includes sufficient sequence to interact with FBP. Whole-cell extracts from HeLa cells transfected with HA-JTV1 or HA-JA were immunoprecipitated monoclonal α-HA, washed and eluted. The precipitates and 10% of input were blotted and probed with either polyclonal α-HA or α-FBP. (**B**) JA is ubiquitinated to a similar extent as JTV1. HeLa cells transfected with HA-JTV1 or HA-JA were treated with 10 μM MG-132 for 4 h and harvested. Gels of cell lysates immunoprecipitated with polyclonal α-HA were blotted and probed with α-ubiquitin. (**C**) JTV1 and JA activate endogenous USP29 transcription. HeLa cells were transfected with GFP, GFP-JTV1 or GFP-JA. After 20 h of incubation, cells were sorted by flow cytometer according to green fluorescence. Total RNA and whole-cell extracts from sorted cells were analysed with microarray, real-time PCR, and western blot. USP29 mRNA levels from the same samples used for the two microarrays were analysed by real-time RT–PCR and normalized to γ-tubulin. qPCR was performed in triplicate with the RNA used for the two independent microarrays. Fluorescence from GFP transfected cells was defined as 1. (**D**) Activation of USP29 requires FUSE. −2.9 kb to +87 bp of USP29 promoter was cloned upstream of luciferase. Deletion derivatives lacking the putative FUSE were also cloned as shown on the left. The candidate USP29 FUSE (uFUSE) sequence is shown at the bottom. In all, 25 ng of reporter was co-transfected with 300 ng of HA, HA-JTV1 or HA-JA into H1299 cells. Cells were harvested 16 h post-transfection and assayed for luciferase activity. Luciferase activities were normalized to the activity of cells co-transfected with luciferase and empty expression vector. (**E**) JTV1 and FBP form a complex on uFUSE. An end-labelled 54 nt oligonucleotide containing the putative USP29 FUSE was incubated with highly purified FBP (5 ng) or JTV1 (25 ng). Specific (lane 4), non-specific competitors (5), as well as antibody to FBP (7, 8) or JTV1 (9) were also added to determine binding specificity. Bands were resolved on 6% PAGE. (**F**) FBP and JTV1 are recruited to the endogenous USP29 promoter. MCF7 cells transfected with GFP, GFP-JTV1 or GFP-JA were fixed and sonicated. Chromatin solutions were immunoprecipitated with normal rabbit IgG, anti-GFP, anti-FBP or anti-JTV1. Enrichment of the USP29 or *c-myc* FUSE regions was detected by PCR using primer sets flanking either site.

48 h prior to transfection of the USP29 reporter. Knockdown of FBP or JTV1 reduced USP29 promoter activity by three- and two-fold, respectively (Figure 2B, graph). Basal JTV1 may support the low level of USP29 reporter activity through two non-exclusive mechanisms: ARS-augmented translation (Kim *et al*, 2002) or JTV1-mediated transcription via very low-level leakage of JTV1 into the nucleus. Knockdown of either FBP or JTV1 had little influence on the level of the

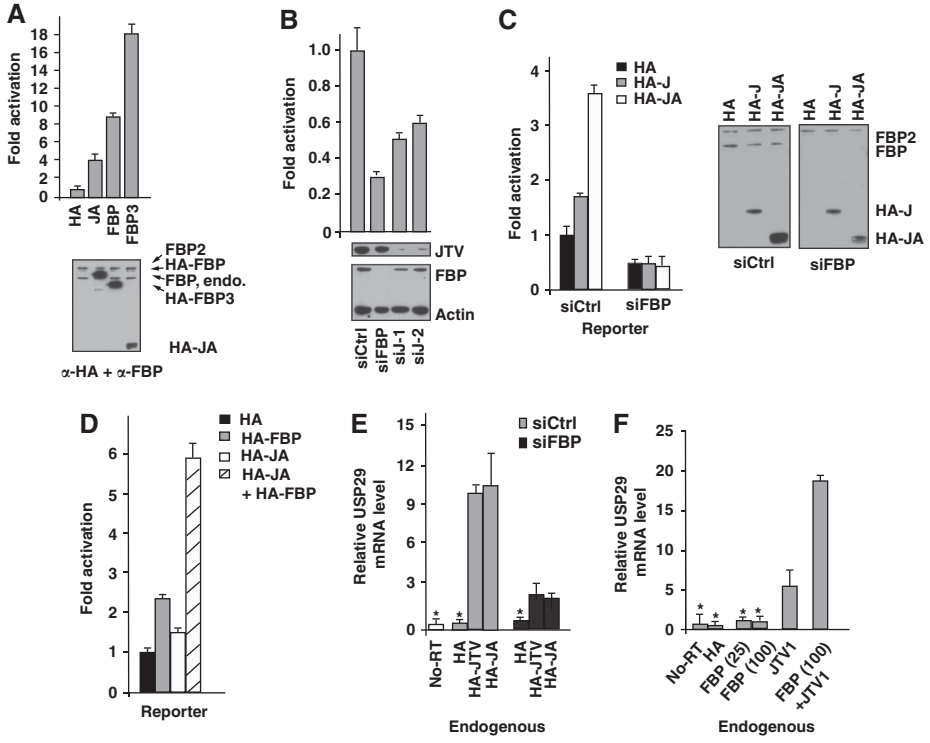

Figure 2 Activation of USP29 by JTV1 requires FBP. (**A**) FBP family members activated the USP29 promoter. FBP (100 ng), FBP3 (125 ng) or JA (300 ng) expression plasmids were co-transfected with 25 ng of reporter into H1299 cells. Luciferase activity (graph) and levels of recombinant protein expression (western blot) are shown. Because this anti-FBP also recognizes FBP2, the FBP2 band was used as a loading control. (**B**) Endogenous FBP and JTV affect USP29 promoter activity. In all, 20 nM of control siRNA (siCtrl), siRNA to FBP (siFBP) or JTV1 (siJ-1 and siJ-2) were transfected to H1299 cells and incubated for 48 h. Reporter plasmid (−2.9 kb) was then transfected. Cells were harvested 16 h later for luciferase assay (graph) and western blot analysis. (**C**) Activation of USP29 reporter by JTV1 requires FBP. Transfections and reporter assays were performed in H1299 cells exactly as described in (**B**). We have previously shown that short-term knockdown of FBP has no effect on cellular FBP2 level and thus FBP2 was used as a loading control for the western blot. (**D**) JTV1 cooperates with FBP to activate USP29 promoter. FBP (10 ng) and JA (100 ng) were transfected either separately or together to H1299 cells. Luciferase activity was measured as above. (**E**) Activation of endogenous USP29 by JTV1 requires FBP. siRNA and 300 ng of JTV1 or 100 ng of FBP expression plasmids were transfected to HeLa cells as described in (**C**). After DNase I treatment, 1 μg of total RNA from these cells were reverse transcribed and 4% of the reaction was used for qPCR. Relative mRNA level was calculated based on standard curve and normalized to γ-tubulin (see Supplementary Figure S2 for detail). *The value is out of the range of the standard curve and is extrapolated. (**F**) JTV1 (100 ng) and FBP (25 or 100 ng) were transfected to HeLa cells individually or together as indicated. USP29 RNA levels were measured as in (**E**).

other (Figure 2B, blot), indicating that in cultured cells under normal growth conditions, FBP and JTV1 were independently maintained as expected since FBP and JTV1 were segregated in separate subcellular compartments. Knockdown of FBP blunted activation by JTV1 (Figure 2C), demonstrating that JTV1 acts through FBP to upregulate USP29 transcription. JTV1 co-activated FBP since their co-expression boosted USP29 promoter activity beyond additive levels (Figure 2D).

Although knockdown of FBP dramatically attenuated JTV1 or JA activation of endogenous USP29 transcription in HeLa cells (Figure 2E), increasing FBP levels alone by transfection did not stimulate endogenous USP29 transcription based on qPCR (Figure 2F, HA-FBP) unless JTV1 levels were also increased (Figure 2F), indicating that nuclear JTV1 is limiting relative to endogenous FBP for the activation of the native USP29 gene. Whereas JA was a more potent activator of the USP29 reporter than JTV1, they were equally effective activators of endogenous USP29 expression, suggesting that other parameters modulate their action in native chromatin. The data indicate that JTV1 helps to overcome the repressive epigenetic modifications that render the USP29 promoter inaccessible to FBP.

Nuclear uptake of JTV1 defines its role as an FBP co-activator

In transfection assays, JA was a more potent activator of the USP29 reporter though both were expressed equivalently and bound to FBP with similar affinity. So some other feature(s) might distinguish the functional interaction between FBP and JTV1 or JA, such as subcellular localization. Immunostaining using anti-JTV1 antibody, revealed JTV1 to be almost exclusively cytoplasmic in HeLa cells under normal growth conditions (Figure 3Aa) consistent with its essential role in the maintenance of the cytoplasmic eukaryotic ARS complex (Kim *et al*, 2002). At low levels of expression, transfected GFP-JTV1 localized in the cytoplasm similar to endogenous JTV1 (Figure 3Ab and e). Cytoplasmic GFP-JTV1 inclusion bodies suspected to cause cell death (Corti *et al*, 2003; Ko *et al*, 2005) were only observed in cells expressing extremely high levels of the recombinant protein (Figure 3Ad). In contrast, previous studies clearly showed that both endogenous FBP and a variety of fluorescent protein-FBP fusions are exclusively nuclear (He *et al*, 2000; Chung *et al*, 2006) (Figure 3Ae and f). If JTV1 is to bind with, and modulate FBP stability as reported, JTV1 must be redeployed to the nucleus. The subcellular localiza-

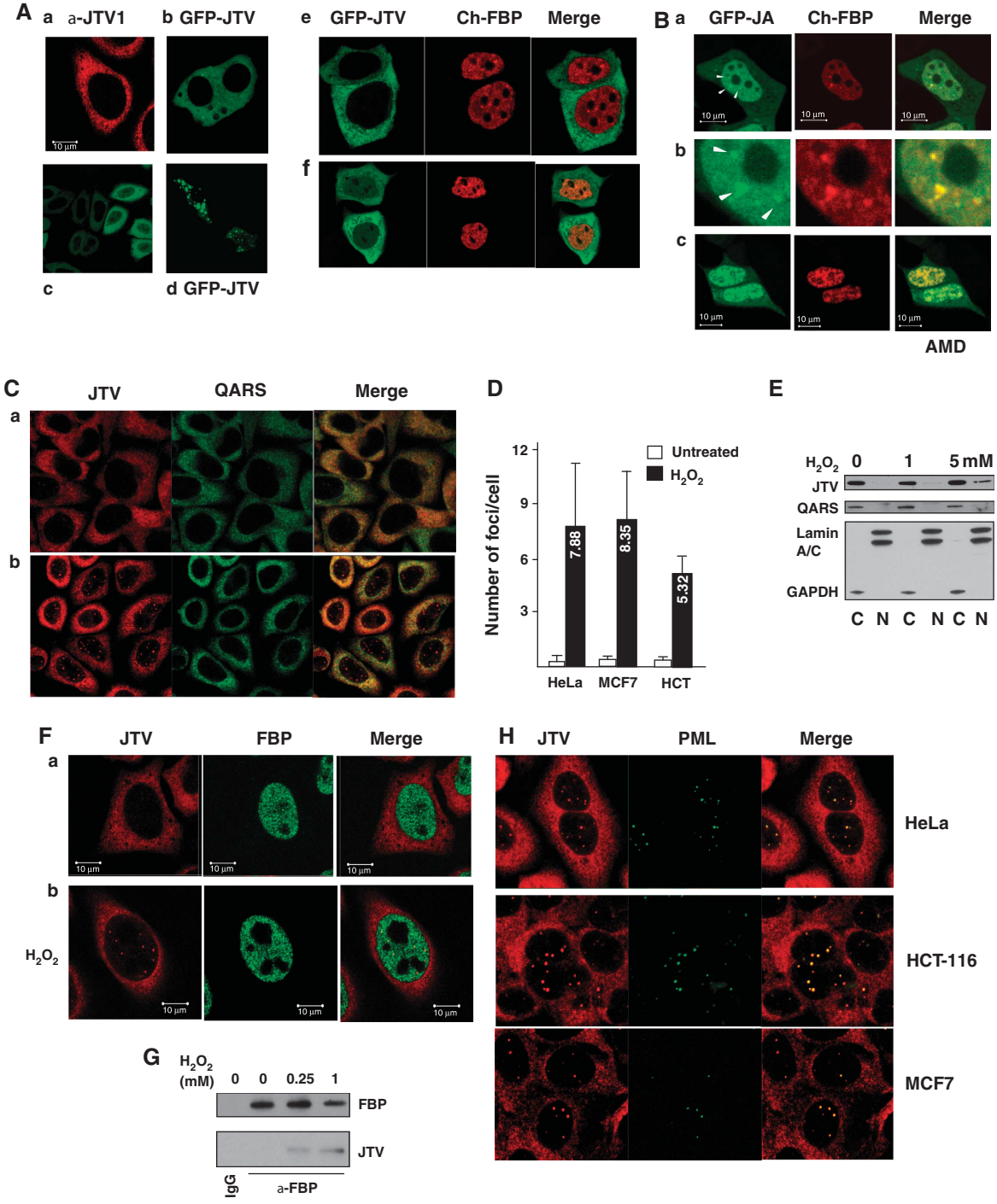

Figure 3 Oxidative stress induces nuclear uptake of JTV1 and the subsequent JTV1–FBP interaction. (**A**) Subcellular localization of JTV1 and FBP. (a) JTV1 in HeLa cells was stained with anti-JTV1 and visualized by anti-rabbit IgG-Alexa594. (b, c, d) Representative confocal image showing subcellular localization of transfected GFP-JTV1 in HeLa cells. The images were taken from the same slide. (e, f) Representative live cell confocal image showing the subcellular localization of transfected GFP-JTV1 and Cherry-FBP in HeLa cells. (**B**) Focal co-enrichment of GFP-JA and FBP in the nucleus. (a) Representative live cell confocal image of transfected GFP-JA and Cherry-FBP in HeLa cells. (b) Zoomed view of the nuclear GFP-JA and Cherry-FBP foci observed in (a). (c) Representative live confocal image showing subcellular localization of GFP-JA and Cherry-FBP after 2 h of incubation with 1.25 μg/ml actinomycin D. (**C**) Oxidative stress induces nuclear uptake of JTV1 but not other components of the ARS complex. Subcellular localization of HeLa cell endogenous JTV1 and QARS prior to (a) or after 30 min treatment of 1 mM H_2O_2 (b) were analysed by confocal imaging. (**D**) Numbers of JTV1 nuclear foci prior to and after H_2O_2 treatment. Confocal images of HeLa, HCT-116 or MCF7 cells were randomly chosen and JTV1 nuclear foci were counted ($n = 40$ per cell line per treatment). Because these numbers were obtained from confocal images rather than *z*-stacks, the differences between samples are relative and not absolute and account for the standard error indicated. Representative images for HCT-116 and MCF7 cells are shown in Supplementary Figure S6. (**E**) HeLa cells were treated with indicated concentrations of H_2O_2 for 30 min. The cells were washed and the cytoplasmic and nuclear extracts were prepared using Cytobuster. The lysates were blotted with indicated antibodies. (**F**) Oxidative stress induced JTV1 nuclear foci partially overlapped with FBP. Representative confocal image showing localization of endogenous JTV1 and FBP prior to (a) or after (b) 30 min treatment of 1 mM H_2O_2. (**G**) Oxidative stress induced JTV1–FBP interaction. Nuclear extract from HeLa cells treated with H_2O_2 were subjected to immunoprecipitation with α-FBP antibody. The precipitates were blotted with either α-FBP or α-JTV1. Only a small portion of nuclear JTV1 co-purified with FBP. (**H**) Oxidative stress induced JTV1 nuclear foci completely co-localized with PML nuclear body. Representative confocal image showing localization of endogenous JTV1 and PML after 30 min treatment of 1 mM H_2O_2.

tions of FBP and JTV1 might be a likely parameter governing their functions.

To test whether augmenting the expression of JTV1 relative to other ARS complex components would drive it into the nucleus, HeLa cells were co-transfected with GFP-JTV1 and Cherry-FBP, and the subcellular localization of JTV1 and FBP were analysed by live cell confocal imaging. The results showed that although GFP-JTV1 was always predominantly cytoplasmic; cells with increased GFP-JTV1 expression exhibited an increase in nuclear fluorescence that partly co-localized with nuclear FBP (Figure 3Ae versus f and 3Ac).

In contrast, JA, lacking the majority of the amino acids required for incorporation into the ARS complex (Kim *et al*, 2002), was predominantly nuclear regardless of expression levels (Figure 3Ba; Supplementary Figure S3). Live cell confocal imaging showed that GFP-JA co-localized with FBP at multiple intranuclear foci (Figure 3Ba and b (zoomed view of panel a)). Dissolution of these foci following a 2-h actinomycin D treatment suggested that JTV1/FBP complexes are maintained by ongoing transcription (Figure 3Bc). Similar nuclear foci were also observed with different fluorescent protein tags (Supplementary Figure S4). Because FBP is recruited to active genes, it seemed that JTV1 might partner with FBP (and conceivably with other transcription factors; note that nuclear GFP-JA and Cherry-FBP did not entirely co-localize) to modify the spectrum of target gene expression.

Oxidative stress induces nuclear uptake of endogenous JTV1

Our previous data indicate the FBP-TFIIH system has a critical role in programming a serum-activated pulse of transcription (Liu *et al*, 2006). If FBP and JTV1 were to co-regulate such a pulse of USP29, then some stimulus or stress must drive JTV1 into the nucleus. Some studies have indicated that oxidative stress associated with dopamine metabolism or with the direct application of H_2O_2, elevates JTV1 levels concomitant with neuronal cell death (Ko *et al*, 2005). Because p53 also contributes to oxidative-stress-induced apoptosis, and has been reported to associate with JTV1, JTV1 localization was examined following this p53-elevating stress. HeLa cells were treated with hydrogen peroxide and stained with antibodies, as indicated. Confocal imaging showed that under normal growth conditions, JTV1 resided exclusively in the cytoplasm, and co-localized with QARS, another subunit of the ARS complex (Figure 3Ca). After oxidative stress, nuclei became diffusely tinted with JTV1 that also concentrated at several bright, large intranuclear foci, similar to the foci observed with transfected GFP-JA. In contrast, QARS remained cytoplasmic (Figure 3Cb). Fractionation of HeLa cells confirmed the presence of JTV1, but not QARS, in nuclear extracts from oxidatively stressed cells (Figure 3E). The data indicated that oxidative stress may liberate JTV1 from the ARS complex to enable its nuclear translocation. JTV1 relocalization to the nucleus was also observed with MCF7 and HCT-116 cells (Figure 3D; Supplementary Figure S6), indicating that nuclear uptake of JTV1 in response to oxidative stress is a general phenomenon. FBP localization was unaffected by oxidative stress, remaining diffusely nuclear with some punctile JTV1 co-localization, suggesting that oxidative stress might license limited intranuclear interaction between FBP and JTV1 (Figure 3F). The physical association between endogenous FBP and JTV1 after oxidative stress was confirmed by co-immunoprecipitation (Figure 3G). Consistent with microscopy, only a subpopulation of nuclear JTV1 co-immunoprecipitated with FBP, indicating that this oxidative-stress-dependent interaction may be transient. A transient JTV1–FBP interaction might help to temporally delimit FBP-programmed pulses of transcription (Liu *et al*, 2006).

Because FBP and JTV1 interact, but FBP is not found at intranuclear JTV1 foci, either JTV1 is independently recruited to different nuclear compartments lacking FBP or else it traffics between compartments for function, processing or degradation. Because the JTV1 foci resembled PML bodies in size, number and morphology (Dellaire *et al*, 2006; Bernardi and Pandolfi, 2007; Weidtkamp-Peters *et al*, 2008), oxidatively stressed cells were stained for JTV1 and PML to confirm their co-localization (Figure 3H). Recent evidence shows that after oxidative stress, some proteins traffic to PML bodies for degradation (Jeanne *et al*, 2010). One possibility would be that once nuclear, JTV1 interacts with and modifies the transcriptional response of FBP prior to disposal.

JTV1 induces USP29 transcription to stabilize p53

Ubiquitin-specific proteases (USPs) deconjugate ubiquitin moieties from their substrates, often stabilizing proteins otherwise slated for degradation (D'Andrea and Pellman, 1998; Nijman *et al*, 2005). Different USPs discriminate among target proteins such as p53, Hdm2 (Li *et al*, 2002, 2004; Cummins and Vogelstein, 2004; Stevenson *et al*, 2007; Yuan *et al*, 2010), FOXO4 (van der Horst *et al*, 2006) and c-Myc (Popov *et al*, 2007). Because JTV1/JA both upregulated USP29 and increased apoptosis in transfected cells (vide infra), p53 levels were examined in the same JTV1/JA transfected HeLa cells analysed by microarray. GFP-JTV1 and -JA each countered the E6AP directed destabilization of p53 (Scheffner *et al*, 1993, 1994), increasing p53 protein (4- and 9-fold, respectively) compared with GFP controls (Figure 4A). JTV1/JA transfection also increased p53 protein in MCF7 (Figure 4B, lanes 1–3), indicating that this response is general. It seemed likely that JTV1/JA increased p53 post-transcriptionally because p53 mRNA levels changed only slightly if at all in the transfected HeLa cells (1.5- and 1.3-fold for JTV1 and JA on microarray, 1- and 0.8-fold by qPCR). If JTV1/JA increased p53 via USP29, then transfected USP29 would support even bigger increases, just as observed (Figure 4B, lane 4).

If JTV1/JA increases p53 via USP29, then USP29 knockdown should block this increase. Therefore, MCF7 cells were transfected with siRNA to USP29, incubated for 48 h, then transfected with various expression plasmids. After another 18 h, p53 and transfected protein levels were analysed. siUSP29 not only blocked HA-USP29 expression (Figure 4C, lane 4 versus lane 8) as expected, it also prevented p53 accumulation by JTV1, JA or USP29, whereas control siRNA did not (Figure 4C, lanes 2–4 versus lanes 6–8). These data revealed that USP29 was the agent through which JTV1/JA increased p53 protein levels. Furthermore, USP29 was able to override the destabilization of p53 mediated by either Hdm2 or E6AP. Importantly, under normal growth conditions, knockdown of USP29 did not alter cellular p53 levels (Figure 4C, lane 1 versus lane 5), indicating that USP29 may only regulate p53 under stress conditions. This is

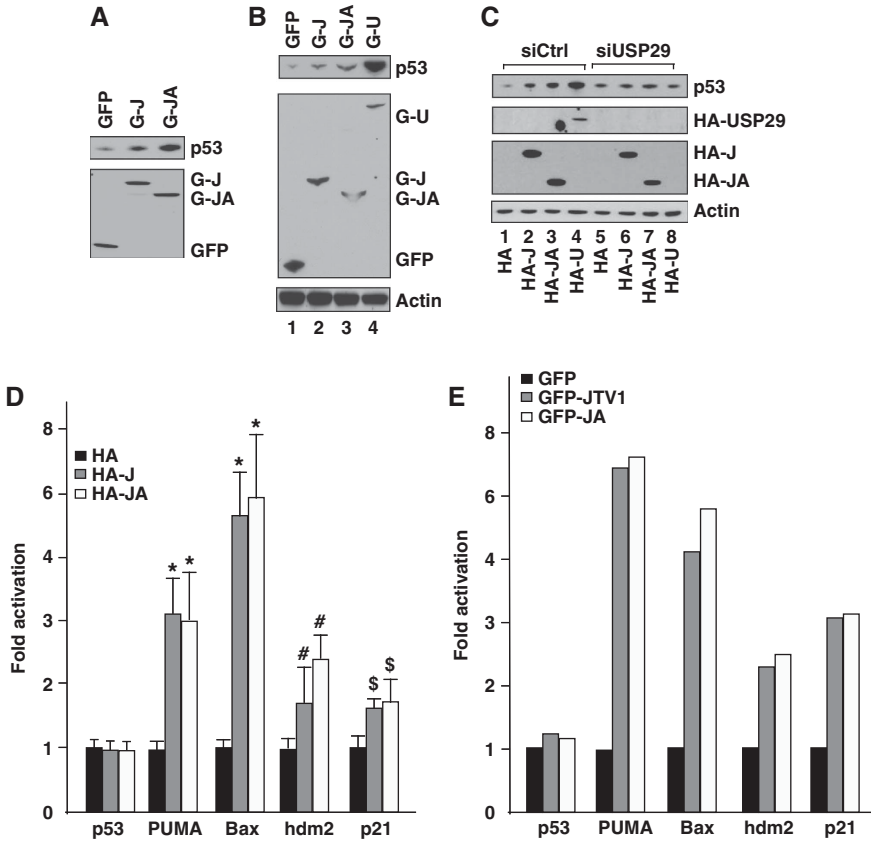

Figure 4 USP29 directly stabilizes p53. (**A**) Overexpression of JTV1/JA increases p53 protein levels. Whole-cell extracts from the same HeLa cells used for microarray were immunoblotted with α-p53 or α-GFP. Lysates from ~5000 cells were loaded per lane and the densities of the p53 bands were determined by densitometry. (**B**) Western blots of MCF7 cells transfected with 200 ng of GFP, GFP-JTV1 (G-J), GFP-JA (G-JA) or GFP-USP29 (G-U). Cells were harvested 20 h post-transfection and blotted. (**C**) USP29 mediates stabilization of p53 by JTV1. MCF7 cells were transfected with siRNA against USP29 (siUSP29) or control siRNA (siCtrl). After 48 h, expression vectors were transfected, and cells were harvested for western blotted. (**D**) Stabilization of p53 induces the transcription of p53 targets. HeLa cells were transfected with HA, HA-JTV1 of HA-JA expression vectors. Cells were harvested 36 h post-transfection and RNA was extracted. mRNA levels of indicated p53 targets were measures by RT–qPCR and normalized to γ-tubulin. The data represent two independent transfections. qPCR were performed in triplicates. $^{*}P<0.001$; $^{\#}P<0.01$; $^{\$}P<0.05$. (**E**) The same assay as in (**D**) was repeated in HCT-116 cells, except the transfected cells were first sorted by flow cytometer due to much lower transfection efficiency in HCT-116 cells.

consistent with the report that USP29 is expressed at very low levels in the absence of stress or signalling (Kim *et al*, 2000).

Transient cycles of p53 induction occur in normal cells without upregulating its targets unless accompanied by p53 post-translational modifications (Loewer *et al*, 2010). To confirm that JTV1/JA-driven increases of p53 levels are functional, the mRNA levels of several known p53 targets in HeLa or HCT-116 cells transfected with JTV1 or JA were monitored by qPCR (Figure 4D and F). In the absence of any observable changes of p53 mRNA levels, PUMA and Bax levels were clearly induced. Moderate and statistically significant increases of Hdm2 and p21 were also observed. The data reveal that JTV1 is capable of initiating apoptosis by increasing the level of active p53.

USP29 deubiquitinates p53 through direct interaction

USPs must recognize and bind their substrates to deconjugate ubiquitin (Li *et al*, 2002, 2004; Cummins and Vogelstein, 2004; van der Horst *et al*, 2006; Popov *et al*, 2007; Stevenson *et al*, 2007; Yuan *et al*, 2010). To test if USP29 binds p53, lysates of MCF7 cells expressing GFP, GFP-JTV1, GFP-JA or GFP-USP29 were immunoprecipitated with anti-GFP and blotted with either anti-p53 or anti-GFP. p53 co-precipitated only with GFP-USP29 (Figure 5A). JTV1 and p53 did not co-immunoprecipitate in our assays, and so we were unable to confirm a prior report that JTV1 bound to p53 sterically, preventing poly-ubiquitination by Hdm2 (Han *et al*, 2008). In principle, enzymatic removal of ubiquitin by USPs (D'Andrea and Pellman, 1998) would stabilize p53 more efficiently than stoichiometric complex formation. To test if USP29 opposes the Hdm2 directed, ubiquitin-dependent degradation of p53, similar to other USPs (Li *et al*, 2002, 2004; Cummins and Vogelstein, 2004; Stevenson *et al*, 2007; Yuan *et al*, 2010), H1299 cells were transfected with his-tagged ubiquitin, p53 and Hdm2 along with GFP or GFP-USP29. After 20 h of incubation, 10 mM MG-132 was added and the cells were incubated for another 8 h. Cells were harvested and ubiquitinated proteins were purified by Ni-beads and blotted. As expected, Hdm2 promoted p53-ubiquitinylation (Figure 5B, lane 1). Co-expression of USP29 completely removed poly-ubiquitin from p53 (Figure 5B, lanes 2 and 3) and increased the level of p53 monomer (Figure 5B, lanes 5 and 6). Unlike HAUSP, which targets both p53 and Hdm2 (Vogelstein *et al*, 2000; Brooks and Gu, 2004, 2006; Poyurovsky and Prives, 2006), USP29 removed ubiquitin much more efficiently from the former. The effect of USP29

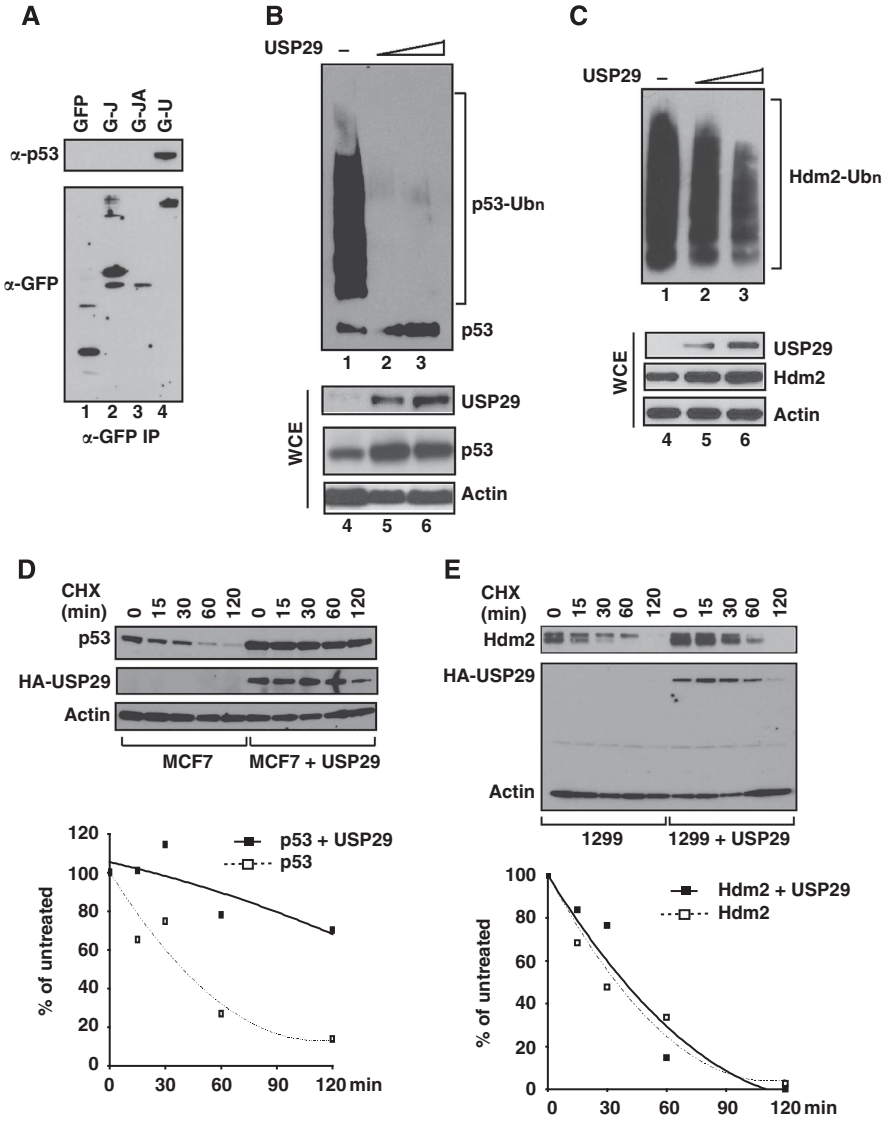

Figure 5 USP29 deubiquitinates p53 through direct interaction. (**A**) USP29 interacts with p53. MCF7 cells were transfected as indicated. Cell lysates were immunoprecipitated with polyclonal α-GFP and analysed with either α-p53 or monoclonal α-GFP. (**B**) USP29 deubiquitinates p53. H1299 cells grown on six-well plates were transfected with expression plasmids as follows: 100 ng of p53, 500 ng of his-ubiquitin, 500 ng of HA-Hdm2, and 125 or 500 ng of GFP-USP29. After 20 h of incubation, MG-132 was added to the media to 10 μM and cells were incubated for an additional 8 h. Cells were harvested and 10% of the cells were lysed with SDS loading buffer, lightly sonicated and subject to western blot (lanes 4–6). Poly-ubiquitinated proteins from the rest of cells were purified by Ni-beads and eluted for western blot (lanes 1–3). (**C**) Hdm2 is not a high affinity USP29 substrate. H1299 cells were transfected and analysed the same as in (**B**) except p53 was omitted. (**D**) Expression of USP29 extended p53 half-life. MCF7 cells were transfected with either HA or HA-USP29 expression vector. At 12 h post-transfection, cycloheximide were added to a final concentration of 50 μg/ml. Cells were harvested for western blot analysis at indicated times after addition of cycloheximide. Intensity of p53 bands were normalized to these of actin and plotted (bottom). (**E**) USP29 did not affect Hdm2 half-life. H1299 cells were transfected and cellular Hdm2 levels were analysed as in (**D**).

on Hdm2 poly-ubiquitination was quite modest at the same doses of expression vector that removed essentially all of the ubiquitin from p53 (Figure 5C, lanes 1–3). In the absence of p53, USP29 yielded only a minor increase of Hdm2 (Figure 5C, lanes 4–6). If USP29 is indeed an antagonist of p53 degradation, then it would be expected to prolong the half-life of p53 protein and to oppose Hdm2-mediated degradation. A vector directing the expression of USP29 or empty vector was expressed in MCF7 cells for 12 h and then protein synthesis was blocked with cycloheximide and the decay of p53 was monitored over time. Whereas without USP29, the p53 half-life was very short (~35 min) as expected (Reich *et al*, 1983), in the presence of USP29 the p53 half-life was too long to be estimated as its degradation was almost undetectable (Figure 5D). Consistent with our data that USP29 only marginally reduced poly-ubiquitinated Hdm2 form, USP29 had no impact on Hdm2 half-life (Figure 5E). Consistent with its dramatic capacity to intercept Hdm2, GFP-USP29 was, like the former, an entirely nuclear protein (Supplementary Figure S5) with ready access to p53 when expressed.

Although the most straightforward interpretation of all of these data is that USP29 binds and catalytically cleaves ubiquitin moieties from p53, a definitive proof of this concept awaits further biochemical characterization of the enzyme,

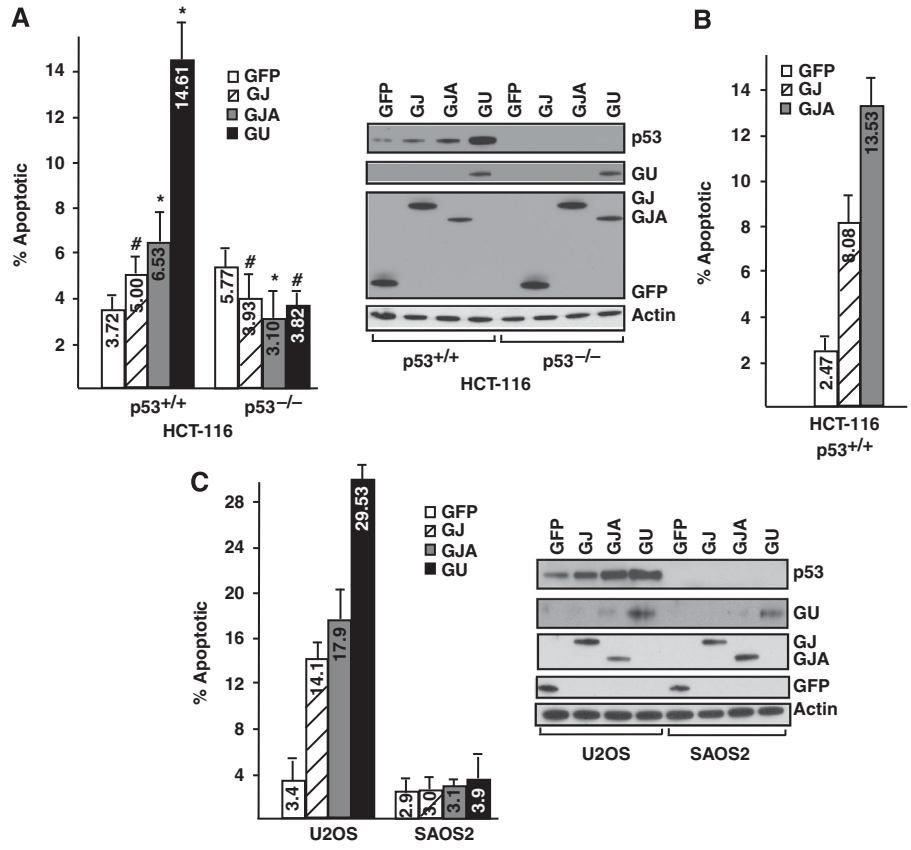

Figure 6 Stabilization of p53 by JTV1 or USP29 induces apoptosis. (**A**) HCT-116 $p53^{+/+}$ or $p53^{-/-}$ cells were transfected with GFP, GFP-JTV1 (GJ), GFP-JA (GJA) or GFP-USP29 (GU) using Lipofectamine Plus. After 24 h of incubation, cells were stained with Annexin V-PE. Green and red fluorescence signals were analysed by flow cytometry. After compensation, cells were gated according to green and red fluorescence. The percentage of Annexin V-PE-positive cells in the untransfected population was subtracted from the transfected cell population to correct for background staining and the differences were graphed. Mean and s.d. from three independent assays with duplicates were shown. $^{*}P<0.001$; $^{\#}P<0.01$. The levels of the relevant proteins of corresponding cells were analysed by western blot (right). (**B**) HCT-116 $p53^{+/+}$ cells were transfected as in (**A**) and apoptosis was measured 48 h post-transfection. (**C**) The same analyses as in (**A**) were repeated with U2OS and SAOS2 48 h post-transfection. The mean and s.d. are from two independent transfections in duplicates.

its substrates and its kinetics using purified components. Nevertheless, the dramatic increase of p53 seen upon transfection of USP29 even in the absence of JTV1 suggests that catalytic deubiquitinylation by the former rather than stoichiometric binding by the latter is likely to be the prevailing mechanism that stabilizes the tumour suppressor (Han *et al*, 2008).

Stabilization of p53 by JTV1/JA induces apoptosis

Cellular attempts to proliferate through accumulating wild-type p53 drive apoptosis (Vogelstein *et al*, 2000; Poyurovsky and Prives, 2006). So p53 stabilization following JTV1/FBP-driven USP29 expression should elicit apoptosis of the transfected cells. To test this, p53 wild-type or null HCT-116 cells were transfected with GFP, GFP-JTV1, GFP-JA or GFP-USP29, harvested 24 h post-transfection, stained with Annexin V-PE and analysed by flow cytometry. After background correction, JTV1 and JA weakly but reproducibly induced apoptosis. USP29 elicited more apoptosis despite lower levels of expression, reflecting dramatically increased p53 in these cells (Figure 6A). In p53-null cells, none of these proteins were proapoptotic, proving that the JTV1/JA/USP29 system required p53 to kill cells. More apoptosis occurred 48 h post-transfection of JTV1 or JA than at 24 h (Figure 6B), indicating that these factors must accumulate to trigger apoptosis in the absence of genotoxic stress. To confirm JTV1 or USP29-induced apoptosis is not limited to HCT-116 cells or HeLa cells (Supplementary Figure S7), another commonly used cell pair was tested. At 48 h post-transfection, JTV1, JA or USP29 all drastically increased apoptotic population in U2OS cells but not in the p53-null SAOS2 cells (Figure 6C).

Oxidative stress induces USP29 transcription to stabilize p53

If the induction of USP29 by transfected GFP-JTV1/JA reflects a physiological process, then the nuclear influx of endogenous JTV1 in response to H_2O_2 treatment should induce endogenous USP29 expression. Indeed, USP29 RNA was induced shortly after H_2O_2 treatment (Figure 7A). Concurrently, both JTV1 and FBP were recruited to the FUSE element on the USP29 promoter (Figure 7B). To test whether blocking the USP29 pathway may increase tolerance of cells to oxidative stress, siRNA to JTV1, USP29 or p53 was first transfected to HeLa cells. At 48 h post-transfection, the cells were treated with increasing concentrations of H_2O_2 for 12 h and apoptosis was analysed by flow cytometry. siUSP29 and si-p53 were equally effective at blocking H_2O_2-induced apoptosis (Figure 7C, graph). Both siJTV1

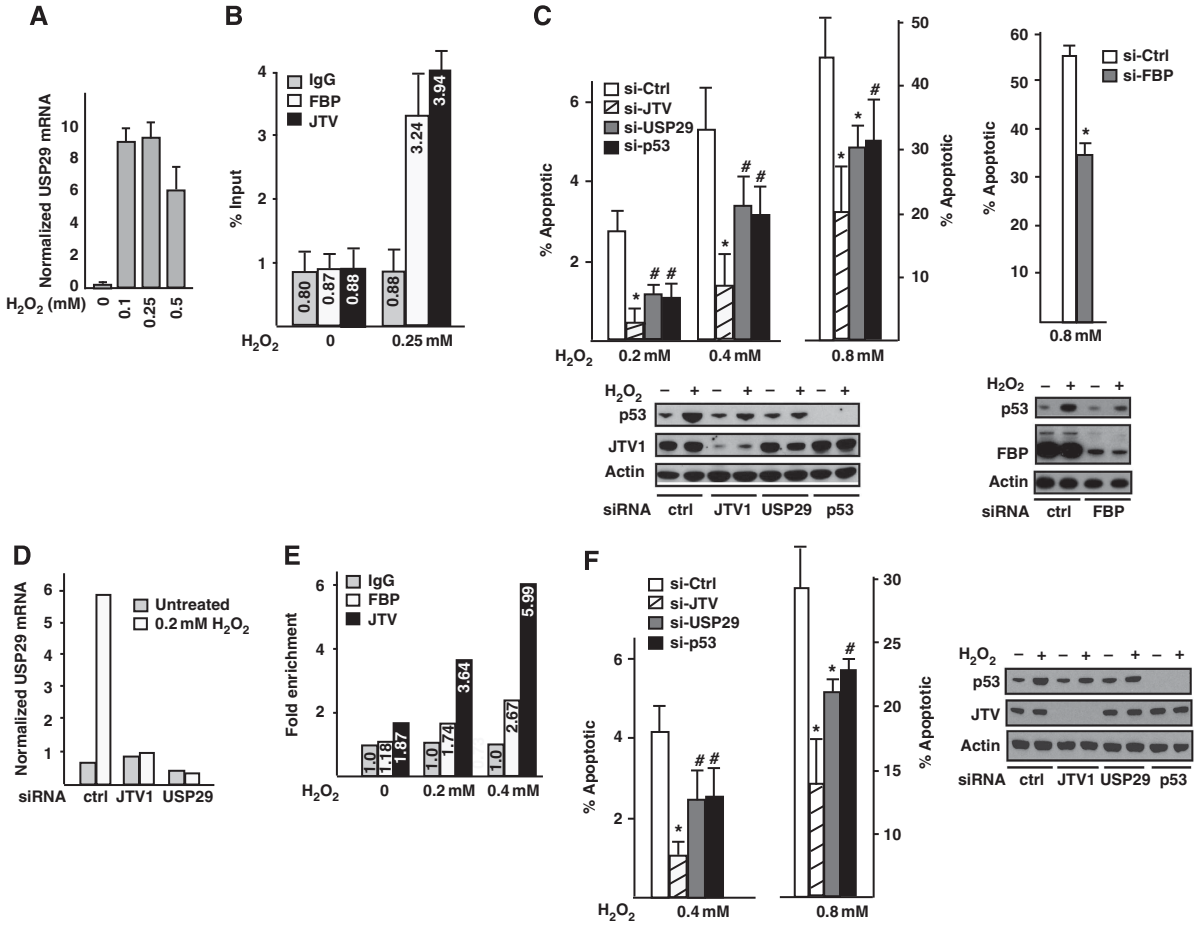

Figure 7 Oxidative stress induces USP29 transcription to stabilize p53. (**A**) Oxidative stress induces USP29 transcription. HeLa cells were treated with indicated concentrations of H_2O_2 for 30 min and incubated in fresh media for 1 h. USP29 mRNA levels were measured by qPCR as described. (**B**) JTV1 and FBP are recruited to the USP29 promoter after oxidative stress. HeLa cells were treated with 0.25 mM H_2O_2 for 30 min, fixed and sonicated. Chromatin solutions were immunoprecipitated with IgG, α-JTV1 or α-FBP. The precipitated DNAs were measured by qPCR and normalized to input. (**C**) Knockdown of JTV1 or USP29 blocks oxidative stress induced p53 accumulation and subsequent apoptosis. HeLa cells were transfected with siRNA to JTV1, USP29 or p53. After 48 h of incubation, these cells were treated with indicated concentrations of H_2O_2 for 12 h. Apoptosis was measured by flow cytometry using Annexin V-PE. Since knockdown of JTV1, USP29 or p53 had no impact on apoptosis in the absence of oxidative stress (Supplementary Figure S10), the percentage of apoptotic cells without oxidative stress was subtracted and only the increase of apoptosis was presented. Mean and s.d. were calculated from three independent experiments in duplicates. $^{*}P<0.001$; $^{\#}P<0.01$. For western blots, cells were treated with 0.2 mM H_2O_2 for 1 h and harvested. (**D**–**F**) JTV1 mediates USP29 induction and apoptosis in response to H_2O_2 in HCT-116 cells. Similar experiments to those described in (**A**–**C**) were performed in HCT-116 cells. The mean and s.d. were calculated from two independent experiments, each in duplicate.

and siUSP29 prevented the massive p53 build-up upon H_2O_2 treatment (Figure 7C, blot). All of these data reveal USP29 to be an important regulator of p53 levels. To confirm that the above phenomenon is not peculiar to HeLa cells that lack Hdm2 regulation of p53 levels, these experiments were repeated in HCT-116 cells (Figure 7D–F). In HCT-116 cells, while low levels of oxidative stress (H_2O_2 up to 0.1 mM) caused G2/M arrest, concentrations higher than 0.2 mM, progressively augmented the population of apoptotic cells (Supplementary Figure S8). To investigate effects of JTV1 on oxidative-stress-induced apoptosis, experiments were performed using 0.4 and 0.8 mM H_2O_2. Knockdown of JTV1 entirely abrogated USP29 induction by H_2O_2 (Figure 7D) and knockdown of either JTV1 or USP29 further boosted resistance to oxidative stress, as expected. Because knockdown of JTV1 inhibits apoptosis more effectively than knockdown of p53, we surmise that JTV1 may co-activate other proapoptotic targets as well. This is consistent with pervious data that p53 is only partly responsible for oxidative induced cell death.

The induction of JTV1 nuclear uptake and subsequent activation of USP29 transcription seemed to be specific to oxidative stress as JTV1 nuclear foci were not observed following camptothecin or UV irradiation; JTV1 knockdown had no effect on p53 levels after CPT treatment (Supplementary Figure S9). Taken together, these data suggest that JTV1 nuclear uptake with the subsequent activation of USP29 transcription and p53 stabilization may be a major cellular response to oxidative stress.

Discussion

The increasing morphological complexity from lower to higher life forms is seemingly at odds with the modest, even minimal expansion in the number of protein coding genes. One pathway to yield increased complexity is to layer

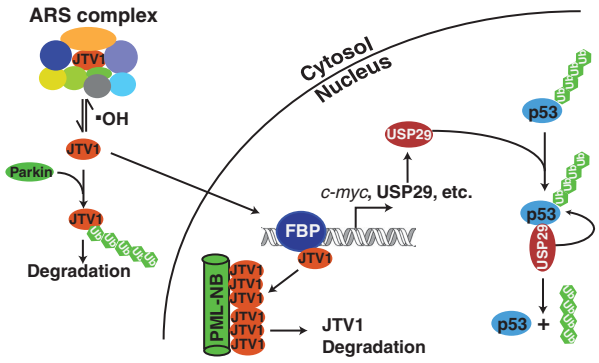

Figure 8 Proposed scheme for JTV1 as an FBP co-activator. Under physiological conditions, JTV1 is an integral component of the cytoplasmic ARS complex and is restrained from entering the nucleus. JTV1 not incorporated into the ARS complex is ubiquitinated by Parkin and quickly degraded. In response to physiological signalling or stresses, or under conditions where Parkin activity is compromised, monomeric JTV1 translocates into the nucleus. Nuclear JTV1 interacts with FBP and activates transcription of FBP target genes such as USP29. USP29 in turn, post-translationally upregulates p53. Following the initial stress, nuclear JTV1 is quickly sequestered to the PML nuclear body and kept away from FBP. The final fate of nuclear JTV1 is unknown, although further degradation of JTV1 mediated by PML nuclear body is likely.

additional functions onto existing mono-functional molecules. Indeed many proteins, from enzymes of intermediary metabolism to ribosomal proteins, possess secondary activities not obviously related to the primary function (Zhang and Lu, 2009). Two of JTV1's reported activities, ARS complex nucleation and FBP binding, are they functionally coherent or do they reflect molecular opportunism, the evolutionary overlaying of a second, unrelated function onto an existing protein (Doolittle, 1988)? Maintenance of the ARS complex promotes efficient protein synthesis; release of JTV1 from the ARS complex and its consequent trafficking to nucleus, is provoked by oxidative stress, a condition associated with growth arrest, reduced macromolecular biosynthesis and/or apoptosis. Therefore, toggling JTV1 between its cytoplasmic and intranuclear partners makes sense. Besides stabilizing the ARS complex and interacting with FBP, JTV1 has most recently been reported to possess a third function, p53 binding, and it has been proposed that this interaction sterically and stoichiometrically prevents Hdm2-mediated poly-ubiquitination and subsequent degradation of p53. The results presented here suggest that JTV1 regulates p53 indirectly via FBP activation of USP29 expression (Figure 8). USP29 enzymatically reverses Hdm2-directed p53 ubiquitination to protect p53 from degradation. This enzymatic stabilization of p53 is in principle, more efficient than an alternative mechanism in which a JTV1–p53 complex sterically interferes with Hdm2–p53 interaction (Han *et al*, 2008).

As a proapoptotic stabilizer of p53, USP29 expression must be restricted in most tissues and cells, possibly through DNA methylation or repressive chromatin compaction (Kim *et al*, 2000, 2003a; Huang and Kim, 2009). If uFUSE behaves like *c-myc* FUSE, low-level USP29 transcription must precede FBP/JTV1 binding (Liu *et al*, 2006). The fact that overexpressing JTV1 alone, but not FBP triggers USP29 suggests that intranuclear JTV1 is limiting for the JTV1–FBP interaction; it is tempting to speculate that JTV1 may cooperate with other factors to surmount genetic or epigenetic barriers to the expression of other targets.

Our data suggest that FBP and JTV1 are recruited neither synchronously nor in fixed ratio at all common binding sites. FBP residency at the *c-myc* FUSE is independent of nuclear JTV1. In contrast, the USP29 uFUSE is devoid of FBP unless JTV1 accumulates in the nucleus. While the rules and parameters governing JTV1 binding to chromatin remain largely unknown, its binding with FBP conceptually links JTV1 action with single-stranded DNA formation induced by supercoiling (highly context-specific properties and so easily customized) or with other B-DNA-destabilizing processes. Therefore, some other stimulus or factor must preactivate the USP29 promoter sufficiently to melt uFUSE for FBP to bind. Potential candidates to initiate USP29 transcription include p53 itself, as binding of p53 protein to the USP29 promoter *in vivo* has been demonstrated using ChIP with paired-end di-tag sequencing (PET); another candidate is c-Myc, which has also been noted to bind to the USP29 promoter using ChIP-PET (http://www.genome.ucsc.edu/cgi-bin/hgc?hgsid=120964399&o=62325726&t=62325803&g=encodeGisChipPet&i=12487-3&c=chr19&l=62323320&r=62335106&db=hg17&pix=800). Following melting and FBP binding at uFUSE, JTV1 recruitment would promote two opposing FBP-related functions, first, transcriptional co-activation of USP29, then ubiquitin-dependent degradation of FBP. The net effect would be a pulse of expression ending upon the removal of FBP as likely occurs at other FBP targets such as *c-myc* (Liu *et al*, 2006) and p21 (Rabenhorst *et al*, 2009).

USPs are the major subclass of deubiquitinating enzymes (DUBs). Correlation of increasing numbers of USPs with increasing numbers of E3 ligases indicates a co-evolutionary expansion of these two antagonistic processes (Nijman *et al*, 2005). The physiological outcome of ubiquitination is defined by the expression kinetics and profiles of the various E3 ligases in response to particular inducing signals, by the binding specificities of the E3 and by the availability of their substrates and co-factors. Likewise, USP target specificities will also be dictated by the protein–protein interaction motifs of USPs and their substrates. It is possible, and perhaps expected that USP29 stabilizes other targets besides p53. Elevated levels of FBP and JTV1 in afflicted brain regions of Parkinson's victims (Corti *et al*, 2003; Dawson and Dawson, 2003; Cookson, 2005; Ko *et al*, 2005) may indicate that disturbances in the levels of Myc or USP29 (and consequently their downstream targets) occur in this disease.

Materials and methods

Tissue culture, constructs, transfections and reporter assays

HeLa, MCF7, U2OS, SAOS2, NCI-H1299 (ATCC) and HCT-116 (a kind gift from Dr B Vogelstein) cells were cultured in DMEM with 10% FBS. USP29 coding region (the entire open reading frame is within Exon 4 of the gene) and promoter were PCR amplified from HeLa genomic DNA. JA was cloned from IMAGE 5111063. Hdm2 and p53 expression vectors were from Dr X Wang. All reporter assays were performed in H1299 cells using Lipofectamine 2000 (Invitrogen) and cells were harvested 16 h post-reporter transfection. Stealth siRNA (Invitrogen) was transfected with Lipofectamine 2000 at 20 nM and cells were incubated for 48 h before secondary plasmid transfections.

Microarray, real-time PCR and ChIP

For microarray, HeLa cells were transfected with GFP, GFP-JTV1 or GFP-JA vectors. Cells were harvested 20–24 h post-transfection and sorted by flow cytometer. Green fluorescent cells were collected for total RNA and whole-cell extract preparation. Microarray was performed as described previously (Chung *et al*, 2006). Quantitative PCR was performed using UPL (Roche) with matching primers. Due to the lack of an appropriate antibody to detect endogenous USP29, expression of USP29 was only measured by qPCR. γ-Tubulin served as control. ChIP was performed exactly as described previously.

Immunoprecipitation

For protein–protein interactions, whole cell or nuclear extract in BC-250 (10 mM Tris, pH 7.9, 250 mM NaCl, 1 mM EDTA, 0.05% NP-40, 10% glycerol with protease inhibitors and 10 μM MG-132 (Calbiochem)) were incubated with the indicated antibodies for 6 h at 4°C, washed four times with BC-300, eluted with 1% SDS and blotted. For ubiquitination assays, cells were treated with 10 μM MG-132 in growth media for 4 h and lysed in RIPA buffer (25 mM Tris–HCl, pH 7.6, 300 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with 10 μM MG-132 and protease inhibitors. Cell lysates were immunoprecipitated for 4 h at 4°C, washed four times with RIPA buffer, eluted with 1% SDS and analysed.

Fluorescent imaging

Cells growing on cover slips were transfected with Lipofectamine Plus (Invitrogen), incubated overnight, washed with PBS and fixed with 4% paraformaldehyde for 12 min before imaging. Endogenous proteins were stained with affinity purified anti-JTV1 (1:1000), anti-PML (sc-966, 1:50) or anti-QARS (ab55644, 1:200) for 1.5 h at 37°C, washed and visualized with anti-Rabbit IgG Alexa-594 or anti-mouse IgG Alexa-488 (Invitrogen). Live cell confocal microscopy was performed exactly as described (Chung *et al*, 2006). Cells expressing high levels of recombinant protein were disregarded.

Apoptosis assay

Cells were transfected with Lipofectamine Plus. At 24 or 48 h after transfection, cells were harvested, washed, stained with Annexin V-PE (Invitrogen) and analysed by flow cytometry.

Electrophoresis mobility shift assay

SF-9 cell expressed FBP was extensively purified. Inclusion bodies containing bacterially expressed his-tagged JTV1 were denatured with 6 M guanidine, bound to Ni-NTA column, washed, renatured and eluted. End-labelled USP29 FUSE probe was incubated with protein(s) and antibodies as indicated in binding buffer (10 mM Tris, pH 7.9, 20 mM NaCl, 0.5 mM DTT, 5% glycerol) for 15 min at room temperature and analysed on 6% PAGE using 0.5 × TBE.

Ubiquitination assay

H1299 cells in six-well plates were transfected with 0.5 μg his-Ubiquitin, 100 ng p53, 0.5 μg HA-Hdm2 together with 125 or 500 ng GFP-USP29. At 20 h post-transfection, the media was replaced with fresh media containing 10 mM MG-132. After another 8 h, cells were harvested. Ten percent of the cells were lysed in SDS buffer, lightly sonicated and used for western analysis. The rest of the cells were resuspended in lysis buffer (6 M Guanidine, 10 mM β-mecaptoethanol, 0.1 M Na-phosphate, 10 mM Tris, pH 8.0) and mixed with Ni-beads for 4 h. The beads were washed once each with lysis buffer and urea buffer (8 M urea, 10 mM β-mecaptoethanol, 0.1 M Na-phosphate, 10 mM Tris, pH 8.0 and pH 6.3). Bound proteins were eluted with elution buffer (150 mM Tris, pH 6.8, 200 mM imidazole, 10% glycerol, 0.72 M β-mecaptoethanol and 1% SDS).

Supplementary data

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

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Author Contributions: DL, JL and HJC designed the study, analysed all the data and wrote the manuscript. JL and HJC performed the majority of the experiments. MD and DM performed the initial confocal imaging studies and provided valuable assistance for the processing and analysis of confocal images. LH performed the initial FACS studies and assisted with other FACS experiments. MV performed some of ChIP assays. YJ designed and performed part of the ubiquitination assays.

Conflict of interest

The authors declare that they have no conflict of interest.

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