

# Def defines a conserved nucleolar pathway that leads p53 to proteasome-independent degradation

Ting Tao<sup>1,\*</sup>, Hui Shi<sup>1,\*</sup>, Yihong Guan<sup>1</sup>, Delai Huang<sup>1</sup>, Ye Chen<sup>1</sup>, David P Lane<sup>3</sup>, Jun Chen<sup>2</sup>, Jinrong Peng<sup>1</sup>

<sup>1</sup>Key Laboratory for Molecular Animal Nutrition, College of Animal Sciences, Ministry of Education, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou, Zhejiang 310058, China; <sup>2</sup>College of Life Sciences, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou, Zhejiang 310058, China; <sup>3</sup>p53 Laboratory, Agency for Science and Technology Research, 8A Biomedical Grove, #6-06, Immunos, Singapore 138648

**p53 protein turnover through the ubiquitination pathway is a vital mechanism in the regulation of its transcriptional activity; however, little is known about p53 turnover through proteasome-independent pathway(s). The digestive organ expansion factor (Def) protein is essential for the development of digestive organs. In zebrafish, loss of function of *def* selectively upregulates the expression of p53 response genes, which raises a question as to what is the relationship between Def and p53. We report here that Def is a nucleolar protein and that loss of function of *def* leads to the upregulation of p53 protein, which surprisingly accumulates in the nucleoli. Our extensive studies have demonstrated that Def can mediate the degradation of p53 protein and that this process is independent of the proteasome pathway, but dependent on the activity of Calpain3, a cysteine protease. Our findings define a novel nucleolar pathway that regulates the turnover function of p53, which will advance our understanding of p53's role in organogenesis and tumorigenesis.**

**Keywords:** Def; p53;  $\Delta 133p53/\Delta 113p53$ ; ubiquitination; Calpain; digestive organ development; nucleolus  
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## Introduction

p53 is under exquisitely fine regulation and acts as a transcription factor that regulates the expression of thousands of genes that control apoptosis, cell cycle arrest, senescence, metabolism, fertility, aging and autophagy [1]. Regulation of p53 turnover is essential to keep p53 activity under control in a normal cell. Multiple E3 ligases that mediate p53 degradation through the ubiquitination-26S proteasome pathway have been identified, including the key E3 ligase Mdm2 and others such as Pirh2, COP1, Topors, Arf-BP1, Synoviolin, CARP1/2, E6-AP and Trim24 (reviewed in [2]). p53 can also be degraded through the MG132-inhibitable but ubiquiti-

nation-independent pathway [3]. However, very little is known to date about p53 turnover through proteasome-independent pathway(s), although some reports have shown that certain cytoplasmically abundant calpains might contribute to the degradation of p53 [4–7].

The nucleolus is also involved in regulating the function of p53. Disruption of the nucleolus in a cell in response to either internal or external stress normally activates the p53 pathway. In most cases, the activation of p53 is due to the binding of Mdm2 by factors such as ARF [8], PML [9], RPL5 [10] or RPL11 [11], which results in physical separation of Mdm2 from p53, thus preventing p53 from degradation by the ubiquitination pathway [12].

Def was first identified as a pan-endodermal-enriched factor that is essential for the growth of digestive organs in zebrafish [13]. Def belongs to a novel protein family that is evolutionally conserved from yeasts to humans and is a component of the ribosomal small subunit (SSU) processome in the nucleolus [14–16]. Human  $\Delta 133p53$  and its zebrafish  $\Delta 113p53$  counterpart are N-terminally truncated p53 isoforms whose transcriptional expression

\*These two authors contributed equally to this work.

Correspondence: Jinrong Peng<sup>a</sup>, Jun Chen<sup>b</sup>, David P Lane<sup>c</sup>

<sup>a</sup>E-mail: pengjr@zju.edu.cn

<sup>b</sup>E-mail: chenjun2009@zju.edu.cn

<sup>c</sup>E-mail: dplane@p53Lab.a-star.edu.sg

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is initiated by an alternative p53 promoter that is totally p53 dependent [13, 17-19]. Both  $\Delta 133p53$  and  $\Delta 113p53$  have been found to modulate p53 activity [17, 19] and  $\Delta 133p53$  is upregulated in certain human cancers [18, 20, 21]. In the zebrafish *def* null mutant (*def*<sup>hi429</sup>), the transcriptional expression of p53 response genes, including *113p53*, *mdm2* and *p21*, is selectively upregulated. However, the level of full-length *p53* transcripts was not obviously altered in the *def*<sup>hi429</sup> mutant [13], which raises the question of whether p53 protein is stabilized or over-activated to upregulate the expression of *113p53* in the *def*<sup>hi429</sup> mutant.

In this report, we analyzed the effect of Def on p53 in both zebrafish and human cells and found that Def triggers the degradation of p53 and its isoform  $\Delta 133p53/\Delta 113p53$ . More importantly, Def-mediated degradation of p53 is dependent on the activity of a specific cysteine protease, Calpain 3 (CAPN3), rather than acting through the 26S proteasome pathway. Our results demonstrated that both zebrafish and humans share a conserved common nucleolar pathway that mediates p53 degradation.

## Results

### *Both zebrafish and human Def are localized in the nucleolus*

Def homologues in yeast (Upt25p) [14, 15] and *Arabidopsis* (NOF1) [16] are nucleolar proteins. Zebrafish Def contains a putative nucleolar localization signal (NoLS) [22] (Supplementary information, Figure S1A). Co-immunostaining of Def and the nucleolar marker, Fibrillarlin (Fib) [23] showed that Def was colocalized with Fib in the nucleoli in the intestinal epithelia of the wild-type fish at 3.5 days post-fertilization (dpf) but not in those of the *def*<sup>hi429</sup> mutant (Supplementary information, Figure S1B and S1C). The human *def* gene (*hu-def*) is located in 1q32.2 and is called *c1orf107*. We found that hu-Def was also localized in the nucleolus in MCF-7 cells (Supplementary information, Figure S1D and S1E).

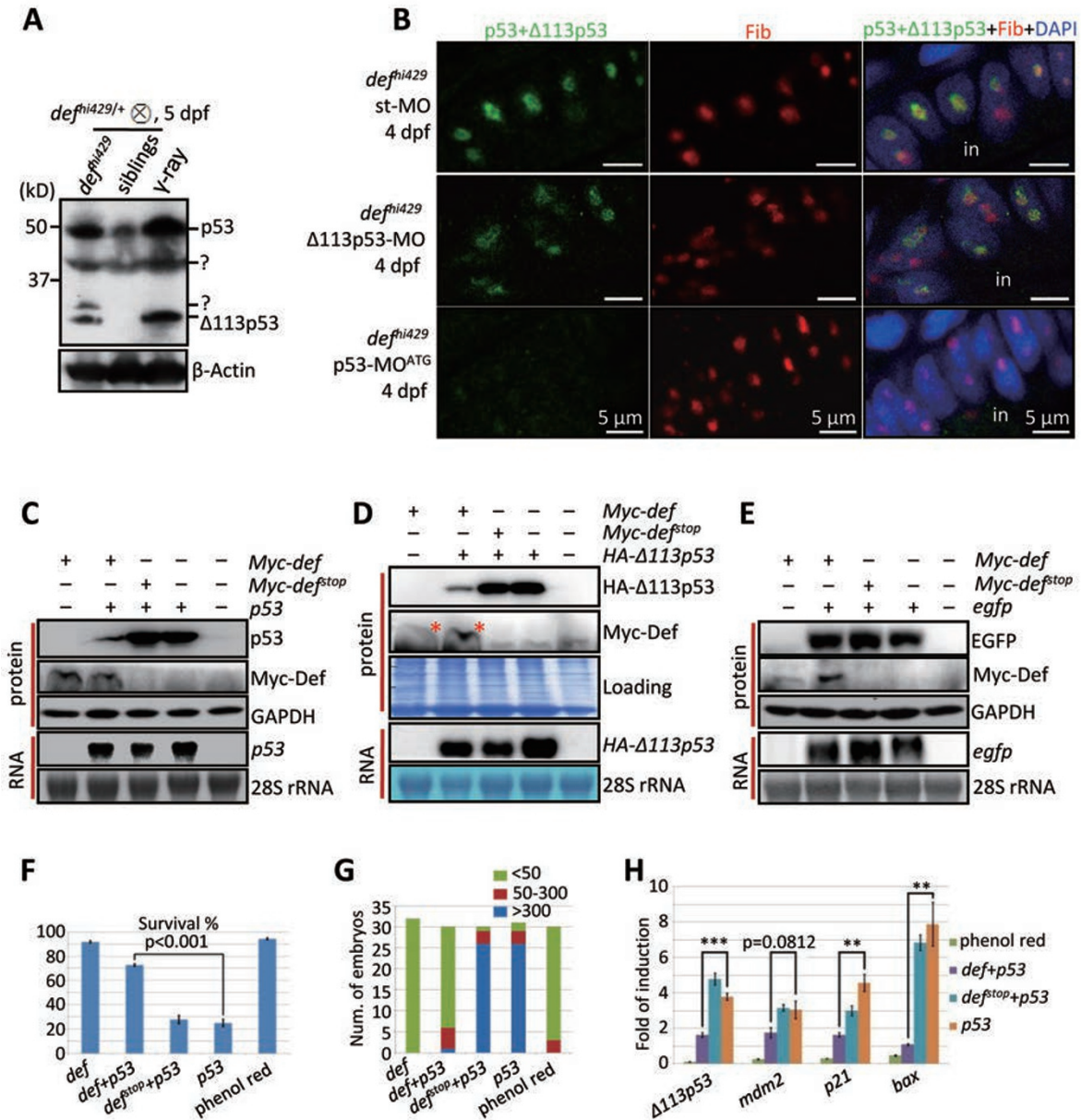
### *p53 is upregulated and specifically accumulated in the nucleolus in the *def*<sup>hi429</sup> mutant*

We demonstrated previously that the transcriptional expression of *113p53* is totally p53 dependent and that the transcript level of *113p53* was greatly elevated in the *def*<sup>hi429</sup> mutant [13, 17]. Interestingly, the transcript level of p53 was not obviously affected in the *def*<sup>hi429</sup> mutant, which prompted us to speculate that p53 protein might be stabilized or become more active in the mutant to activate the expression of *113p53*. Western blot using a monoclonal antibody that detects both p53 and  $\Delta 113p53$  of zebrafish showed that both proteins were upregulated in the *def*<sup>hi429</sup> mutant at 5 dpf (Figure 1A). The

*def*-specific morpholino (*def*-MO) could knock down Def protein expression in zebrafish by targeting the *def* gene at its splicing junction of exon 2 and intron 2 [13]. Western blot showed that p53 and  $\Delta 113p53$  were upregulated in the *def*-MO morphants at 3 dpf (Supplementary information, Figure S1F and S1G). Immunostaining using a polyclonal antibody that recognizes both p53 and  $\Delta 113p53$  of zebrafish revealed a clear and strong positive signal colocalized with Fib-labeled nucleoli in the cells of the intestinal epithelium of 4 dpf *def*<sup>hi429</sup> mutants but not in those of wild-type zebrafish (Figure 1B; Supplementary information, Figure S2A and S2B). Knockdown of  $\Delta 113p53$  by its specific morpholino *113p53*-MO [17] had no obvious effect, while knockdown of p53 by its specific morpholino, *p53*-MO<sup>ATG</sup>, almost abolished the nucleolar staining pattern of p53 in *def*<sup>hi429</sup> mutants (Figure 1B). Therefore, the loss of function of *def* upregulated p53 protein expression, and p53 protein accumulated in the nucleoli in the *def*<sup>hi429</sup> mutant cells. As  $\Delta 113p53$  can form a complex with p53 [17], we speculated that the upregulated  $\Delta 113p53$  protein probably accumulates, together with p53, in the nucleoli of the mutant cells, although further concrete evidence is needed to prove this hypothesis.

### *Def selectively triggers the degradation of p53 and $\Delta 113p53$ protein*

The above results suggested that Def regulates the stability of p53. Indeed, we found that co-injection of *Myc-def* but not of *Myc-def*<sup>stop</sup> (a *def* mutant that harbors a premature stop codon at codon 55 created by site-directed mutagenesis) [13] mRNA drastically reduced the level of p53 protein (Figure 1C, protein panels) but not that of *p53* mRNA (Figure 1C, RNA panels) at 6 h post-injection (hpi). In fact, Def reduced the level of p53 as early as 1 hpi (Supplementary information, Figure S2C). To our surprise, overexpression of Def also reduced the level of HA- $\Delta 113p53$  protein (Figure 1D, protein panels) but not that of mRNA (Figure 1D, RNA panels) at 6 hpi. To determine whether Def reduced the level of p53 selectively, we replaced *p53* mRNA with *egfp* (enhanced green fluorescent protein) or *rcl1* (encoding a nucleolar protein) mRNA and found that *Myc-def* did not affect the protein levels of EGFP (Figure 1E) or Rcl1 [24] (Supplementary information, Figure S2D). Interestingly, Def failed to reduce the protein levels of two p53 mutants, R143H and R250W (Figure 3D). As R250W and R143H differ from p53 only by an A<sup>748</sup> to T<sup>748</sup> single nucleotide change and by an R<sup>143</sup> codon AGA to H<sup>143</sup> codon CAC change in the p53 coding region, respectively, which are relatively distant from the translation start codon ATG, we therefore concluded that Def overexpression selec-



**Figure 1** Def selectively induced the degradation of p53 and  $\Delta 113p53$  proteins. **(A)** Western blot of p53 and  $\Delta 113p53$  using the A7-C10 monoclonal antibody to detect both proteins in *def<sup>hi429</sup>* homozygotes and non-homozygous siblings at 5 dpf and in  $\gamma$ -ray-treated wild-type embryos. ?, uncharacterized p53 isoforms;  $\beta$ -actin, loading control. **(B)** Coimmunostaining of Fib and p53/ $\Delta 113p53$  in a *def<sup>hi429</sup>* mutant embryo injected with st-MO (upper panel),  $\Delta 113p53$ -MO (middle panel) or *p53*-MO<sup>ATG</sup> (bottom panel) morpholinos at 4 dpf. Nuclei were stained with DAPI. st-MO: standard control morpholino. in: intestine. **(C)** Western blot (top three panels) of p53 protein and northern blot (bottom two panels) of *p53* mRNA in embryos injected with different mRNA mixes at 6 hpi as shown. 28S rRNA: RNA loading control. GAPDH, protein loading control. **(D)** Same as in **(C)**, but analysis of  $\Delta 113p53$ . **(E)** Same as in **(C)**, but analysis of EGFP. **(F)** *tp53<sup>M214K</sup>* mutant embryos were injected with different mRNA mixes or phenol red dye. The survival rate of embryos in each treatment group at 12 hpi was analyzed. The values plotted represent mean  $\pm$  SEM (three repeats of  $n = 100$ -200 embryos each), with test *P*-values indicated. **(G)** Analysis of apoptosis in embryos described in **(F)** at 10 hpi. Embryos were categorized based on their number of apoptotic cells. Category 1, < 50 apoptotic cells per embryo; category 2, between 50-300 apoptotic cells per embryo; category 3, > than 300 apoptotic cells per embryo. Graphics shows the number of embryos in each category in each case. **(H)** qPCR analysis of p53 target genes in embryos described in **(F)**. The qPCR values were normalized against *elf1a* and expressed as fold change in expression. The values plotted represent mean  $\pm$  SEM. The *P*-value was obtained by performing the two-tailed unpaired *t*-test. \*\*\**P* < 0.001; \*\**P* < 0.01.



tively promotes the degradation of p53 and  $\Delta 113p53$  protein without affecting their mRNA stability or protein translation in zebrafish.

Eight *def* deletion constructs (Supplementary information, Figure S3A) were generated and each *in vitro* synthesized mRNA was co-injected with *HA-p53* mRNA. The level of HA-p53 was then analyzed at 6 hpi. Deletion of the first 189 (construct *D1*) or 377 (construct *D2*) amino acids (AAs) from the N-terminus, which contains the NoLS, almost totally abolished the ability of Def to promote HA-p53 degradation. In contrast, deletion of the last 376 AAs (constructs *D3*, *D4* and *D5*) from the C-terminus did not affect Def-mediated p53 degradation (Supplementary information, Figure S3B). Surprisingly, removal of the last 64 AAs (construct *D6*) inactivated the Def protein, whereas deletion of AAs 566-627 (construct *D7*) caused a moderate loss of Def activity. We also deleted the internal region between AAs 628 and 689 (construct *D8*) and found that the resultant protein was more effective in promoting HA-p53 degradation than the full-length Def (Supplementary information, Figure S3C). Thus, we concluded that the NoLS is essential for the activity of Def on p53. Meanwhile, we identified two promoting regions (AAs 1-189 and 566-627) and one repressing region (AAs 628-689) in the Def protein function, suggesting a highly regulated and complex mechanism of action in Def-mediated p53 degradation.

#### *Def* protects the survival of embryos by antagonizing p53 apoptotic activity

The zebrafish mutant *tp53*<sup>M214K</sup> carries a M<sup>214</sup> to K<sup>214</sup> substitution in the p53 DNA-binding domain that creates a loss-of-function allele of *p53* [25]. Injection of the normal *p53* mRNA alone caused high embryo mortality (~70%) at 12 hpi in the *tp53*<sup>M214K</sup> mutant background. Co-injection of *def* and *p53* mRNAs greatly reduced the embryo mortality seen following injection of *p53* mRNA alone (Figure 1F; Supplementary information, Figure S2E and Table S1). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showed that injection of the *p53* mRNA alone caused massive cell apoptosis, and that injection of *def* but not of *def*<sup>stop</sup> mRNA strongly inhibited p53-induced apoptosis in the co-injected embryos at 10 hpi (Figures 1G; Supplementary information, Figure S2F and Table S2). Concomitantly, compared with their expression in embryos injected with the *p53* mRNA alone, the expression of all p53 target genes, including  *$\Delta 113p53$* , *mdm2*, *p21* and *bax*, was downregulated by the injection of *def* mRNA in the co-injected embryos at 6 hpi (Figure 1H and Supplementary information, Table S3).

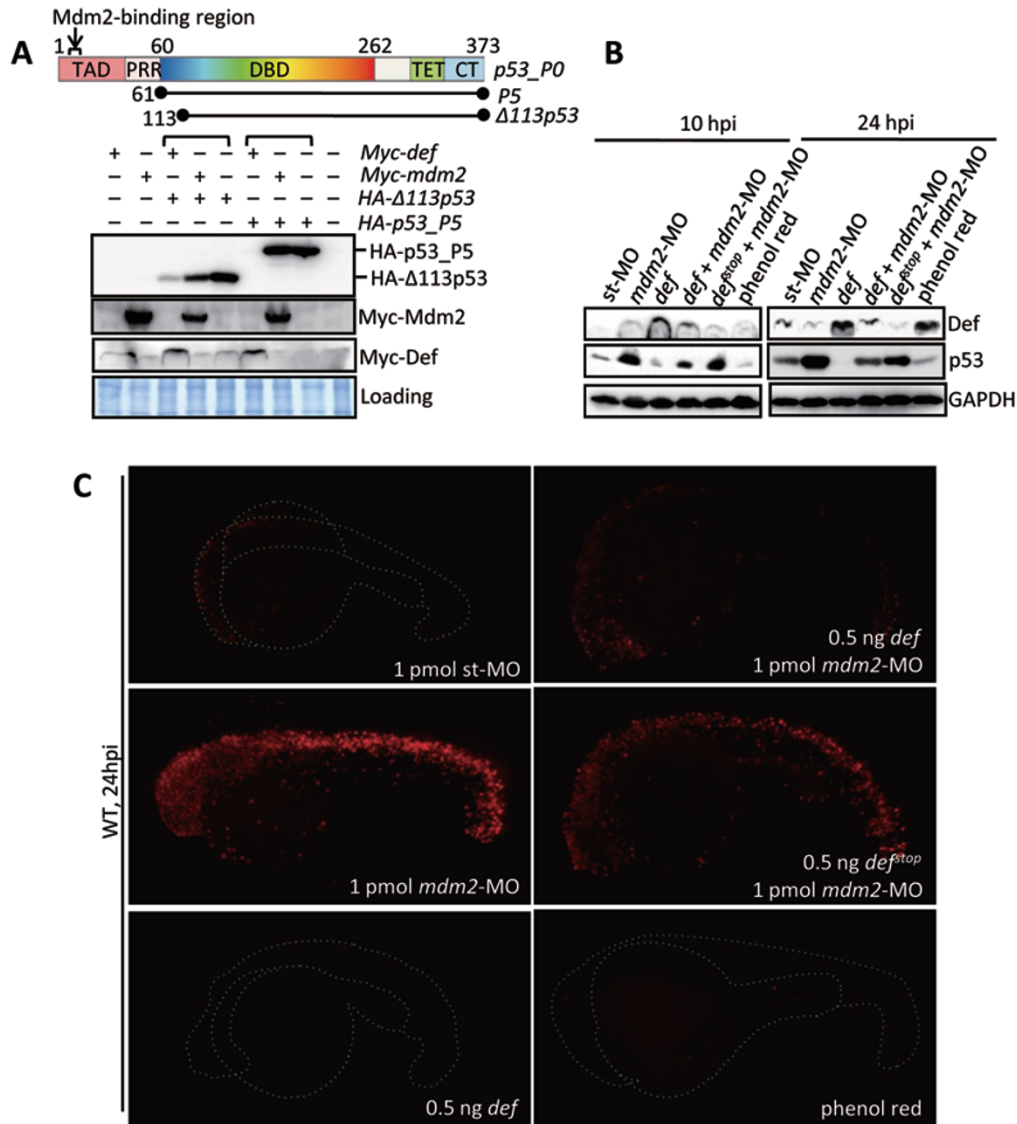
#### *Def*-mediated degradation of p53 and $\Delta 113p53$ is independent of *Mdm2*

$\Delta 113p53$  lacks the motif essential for Mdm2 binding [26]. Mdm2 binds to the N-terminus of p53 and targets the C-terminus of p53 for ubiquitination, a prerequisite for p53 degradation through the 26S proteasome [2]. We therefore predicted that  $\Delta 113p53$  would be resistant to Mdm2-mediated protein degradation. We co-injected *Myc-def* or *Myc-mdm2* mRNA with *HA- $\Delta 113p53$*  or *HA-p53\_P5* mRNA (similarly to  $\Delta 113p53$ , P5 lacks the Mdm2-binding motif) and found that Def but not Mdm2 promoted the degradation of both  $\Delta 113p53$  and *p53\_P5* at 6 hpi (Figure 2A). In addition, knockdown of Mdm2 by an *mdm2*-specific morpholino (*mdm2*-MO) did not prevent the degradation of p53 by Def in the injected embryos, and *vice versa* (Supplementary information, Figure S4). Mdm2 is the key E3 ligase that mediates the degradation of p53 through the 26S proteasome pathway, and its depletion causes embryo lethality in zebrafish [27]. We used *mdm2*-MO to knock down Mdm2 protein expression and found that the level of endogenous p53 protein was greatly elevated in the *mdm2*-MO morphants (Figure 2B). We co-injected *def* mRNA and *mdm2*-MO and found that Def reduced the level of p53 protein in the *mdm2*-MO morphants at both 10 and 24 hpi (Figure 2B), concomitant with a reduction in their apoptosis at 24 hpi (Figure 2C). These results demonstrated that Def promotes the degradation of both p53 and  $\Delta 113p53$  in an Mdm2-independent manner.

#### *Def* targets the p53 DNA-binding domain for degradation

Six *p53* deletion constructs (*P1-P6*) (Figure 3A) were generated for the identification of the Def-targeting domain(s). Def only promoted the degradation of those deletion products that contained the p53 DNA-binding domain (*P2*, *P4* and *P5*) at 6 hpi (Figure 3B). Noteworthy, Mdm2 triggered the degradation of the *P6* product only, but had no or only a weak effect on the products that were truncated from the N- (*P2*, *P3*, and *P5*) or C-terminus (*P2* and *P4*) of p53 (Figure 3C). This is consistent with earlier studies that showed that both the N-terminal Mdm2-binding domain and the C-terminal ubiquitin target sites of p53 are requisite for the Mdm2-driven degradation of p53 [26].

Three zebrafish p53 mutants were created, each with a point mutation that alters a crucial functional residue, including R143H, R250W and R313C that are counterparts of human hu-p53-R175H, -R282W and -R337C, respectively. hu-p53-R175H and -R282W are known to lose DNA-binding capacity [28] whereas hu-p53-R337C displayed the highest DNA-binding activity [29]. We

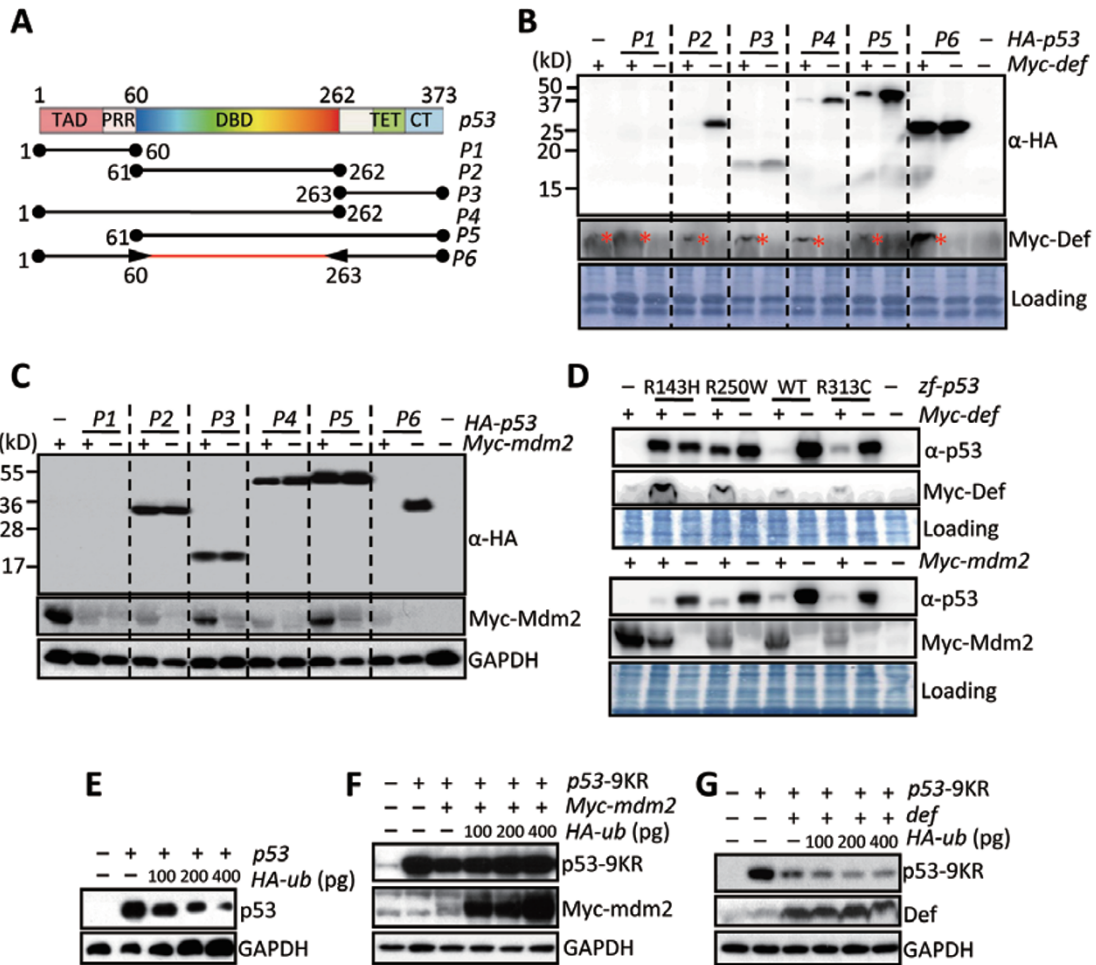


**Figure 2** Def-mediated p53 degradation was independent of Mdm2. **(A)** Diagram on the top shows the Mdm2-binding domain and other key functional domains of the p53 protein, and shows the truncated sites for  $\Delta 113p53$  and p53-P5. TAD, transactivation domain; PRR, proline-rich region; DBD, DNA-binding domain; TET, tetramerization domain; CT, C-terminus regulation domain. Numerical numbers denote the amino acid position from Met. Western blot analysis of p53, Def and Mdm2 examined the effects of Def and Mdm2 on the stability of  $\Delta 113p53$  and p53-P5 proteins at 6 hpi. Coomassie Blue staining, protein loading control. **(B, C)** Western blot of p53 and Def for analyzing the effect of Def on endogenous p53 induced by *mdm2*-MO at 10 hpi and 24 hpi, respectively **(B)**. TUNEL assay for analysis of apoptosis in embryos injected with *mdm2*-MO alone or *mdm2*-MO+*def* mRNA mix at 24 hpi **(C)**. st-MO morpholino, *def*<sup>stop</sup> mRNA and phenol red dye injections were used as the controls.

found that although Mdm2 triggered the degradation of all these three p53 mutant proteins, Def was unable to promote the degradation of zebrafish zf-p53-R143H and -R250W but promoted zf-p53-R313C degradation at 6 hpi (Figure 3D). Therefore, Def-induced p53 degradation is dependent on the molecular status of the p53 protein that requires a wild-type DNA-binding domain.

#### *Def-mediated p53 degradation is independent of the ubiquitination pathway*

In addition to Mdm2, many other E3 ligases have been found to trigger the degradation of p53 through the ubiquitination pathway [2]. We injected one-cell stage zebrafish embryos with *HA-ubiquitin* mRNA and found that this promoted degradation of p53 in a dosage-dependent manner at 6 hpi (Figure 3E). Multiple lysine residues

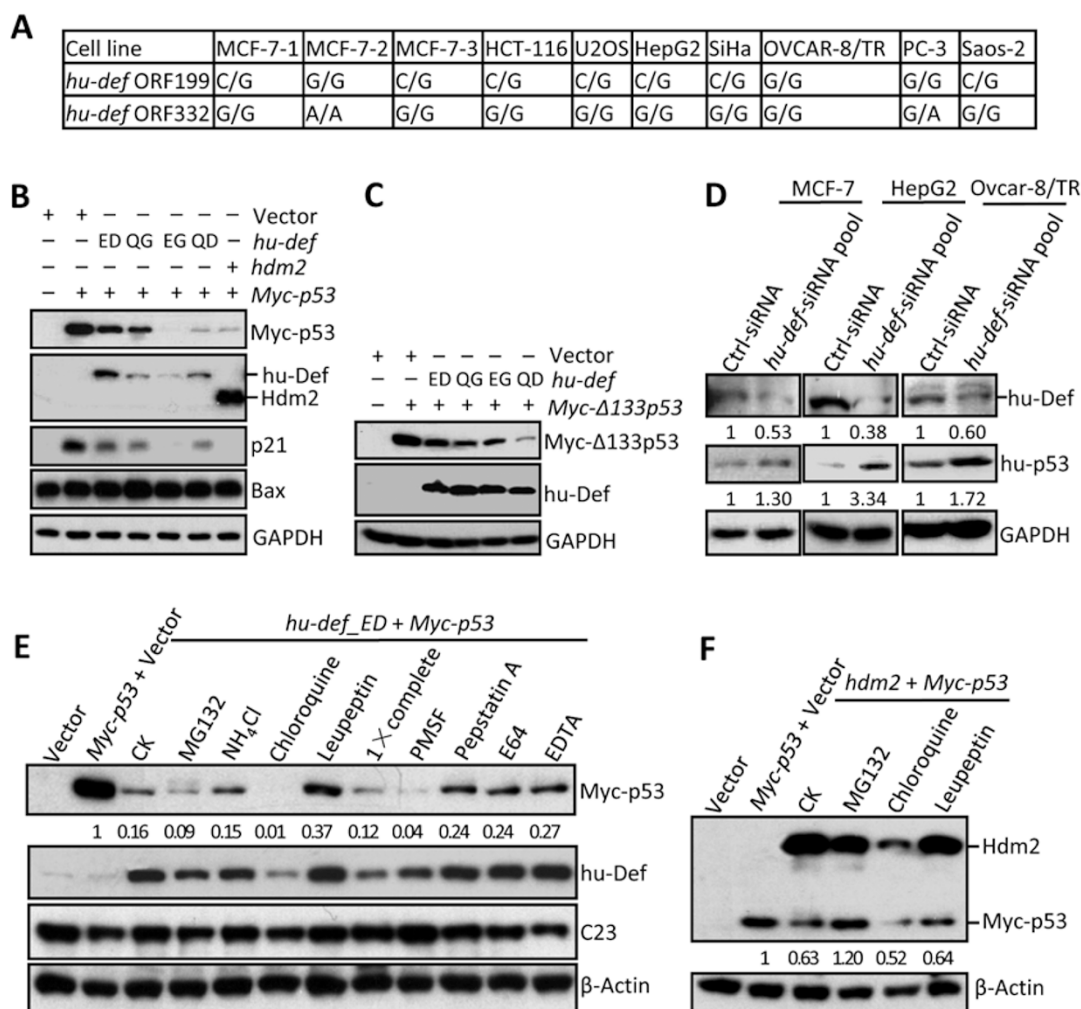


**Figure 3** Def-mediated p53 degradation is independent of the ubiquitination pathway. **(A)** Diagram shows the structure of p53 protein (refer to Figure 2A) and six p53 deletion mutant constructs. **(B)** Western blot of p53, six p53 deletion mutant products and Def for analyzing the effect of Def on their stability in the co-injected embryos at 6 hpi. **(C)** Same as in **(B)** but analyzing the effect of Mdm2. **(D)** Western blot of p53, Def and Mdm2 for comparing the effect of Def and Mdm2 on the stability of p53 mutant proteins R143H, R250W and R313C in the injected embryos at 6 hpi. **(E)** Western blot of p53 for comparing the effect of different dosages of ubiquitin on the stability of p53 in the injected embryos at 6 hpi. **(F)** Western blot of p53 and Mdm2 for analyzing the effect of Mdm2 plus ubiquitin on the stability of p53 mutant protein p53-9KR in the injected embryos at 6 hpi. **(G)** Same as in **(F)** but analyzing the effect of Def.

in the p53 protein are known to serve as the sites for ubiquitination [26]. We selected nine lysine (K) residues (K<sup>292</sup>, K<sup>293</sup>, K<sup>295</sup>, K<sup>345</sup>, K<sup>347</sup>, K<sup>348</sup>, K<sup>360</sup>, K<sup>361</sup>, and K<sup>365</sup>) and mutated them to arginine (R) (construct *zf-p53-9KR*). We co-injected *zf-p53-9KR* with *Myc-mdm2* or *def* mRNA together with *HA-ubiquitin* mRNA. The results showed that *zf-p53-9KR* was totally stable after the co-injection of Mdm2 with 400 pg *HA-ubiquitin* mRNA (Figure 3F) but was degraded by Def with or without HA-ubiquitin at 6 hpi (Figure 3G). Thus, Def-mediated p53 degradation occurs via an ubiquitination-independent pathway.

### Two naturally occurring single-nucleotide polymorphisms (SNPs) in *hu-def* alter *hu-Def* activity on p53

Before studying *hu-Def*, we sequenced *hu-Def* cDNA cloned from different human cell lines including MCF7, HCT116, U2OS, HepG2, SiHa, Ovarc-8/TR, PC-3 and Saos-2 and searched the dbSNP database (<http://www.ncbi.nlm.nih.gov/snp/>). We identified two SNPs (G/C at position ORF<sup>199</sup>, converting E<sup>67</sup> to Q<sup>67</sup>, and A/G at position ORF<sup>332</sup>, converting D<sup>111</sup> to G<sup>111</sup>) in its coding region (Figure 4A). We found that overexpression of the products encoded by all four combinations of these two SNPs in MCF7 cells downregulated the levels of *hu-p53* (Figure 4B) and  $\Delta 133p53$  (Figure 4C) at 24 h post-transfection



**Figure 4** Def-mediated p53 degradation was inhibited by the cysteine protease inhibitor. **(A)** Sequencing genomic DNA from different human cells identified two SNPs (G/C at codon position ORF<sup>199</sup>, A/G at codon position ORF<sup>332</sup>) in the *hu-Def* coding region. **(B)** Western blot of p53, Def, Hdm2, p21 and Bax for comparing the effect of *hu-Def* encoded by four combinations of (G/C)<sup>199</sup> and (A/G)<sup>332</sup> SNPs on the stability of p53 in MCF-7 cells co-transfected with MYC-tagged p53 plasmid, and the expression of p53 target genes p21 and Bax at 24 hpt. **(C)** Same as in **(B)** but analysis of  $\Delta 133p53$  in co-transfected cells. **(D)** Western blot of p53 and Def in MCF-7, HepG2 or Ovar-8/TR cells treated with *def* specific siRNA pool (*hu-def*-siRNA pool) at 24 hpt. **(E)** Western blot of p53 and Def for comparing the effect of various inhibitors on *hu-Def*-mediated p53 degradation. **(F)** Same as in **(E)** but analysis of the effect of selected inhibitors on Hdm2-mediated p53 degradation. In **(D-F)**, fold changes in expression for p53 or *hu-Def* against their respective controls (set as 1) are shown under their corresponding panels. GAPDH or  $\beta$ -actin was used as the normalization control.

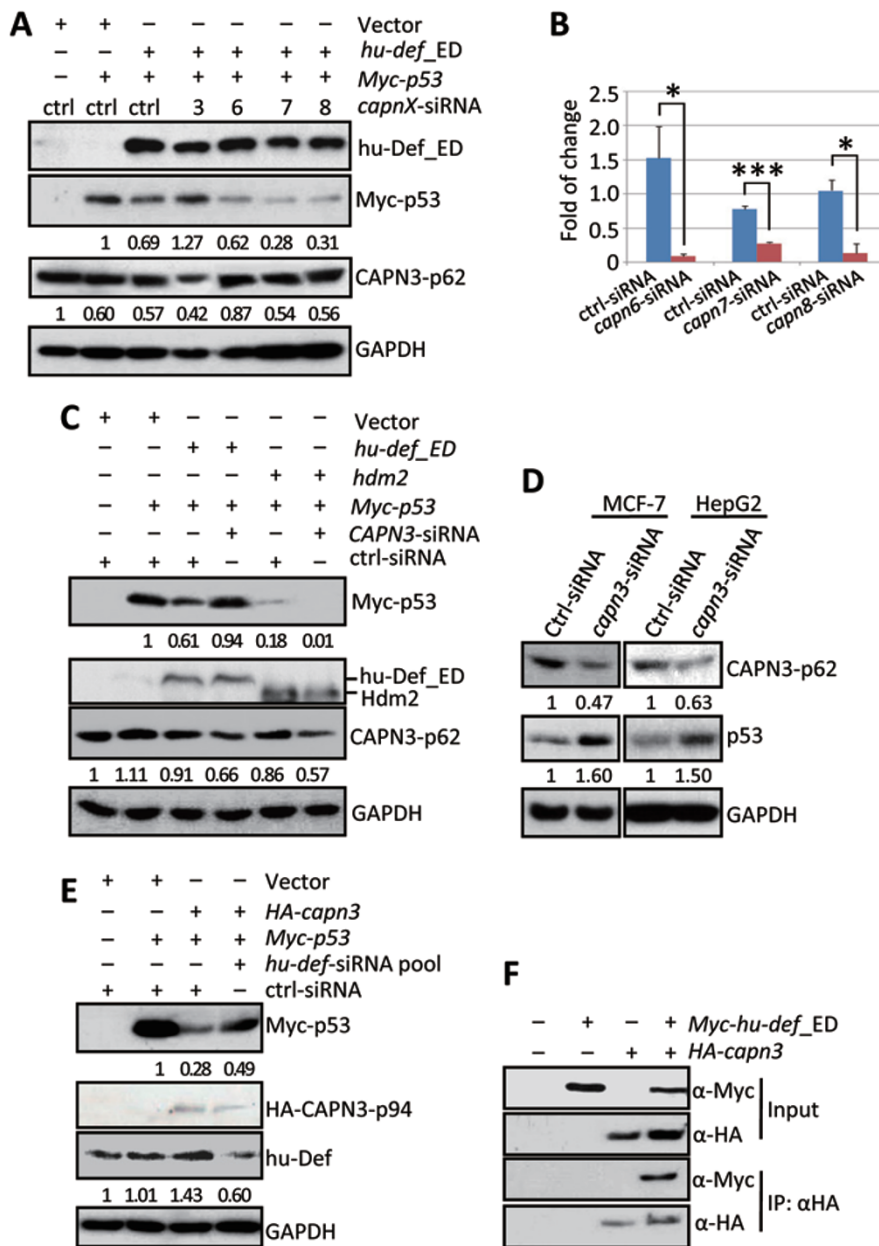
(hpt). Interestingly, the products of the combination of G<sup>199</sup>G<sup>332</sup> (*hu-Def*\_EG) and C<sup>199</sup>A<sup>332</sup> (*hu-Def*\_QD) exhibited much higher activity than those of G<sup>199</sup>A<sup>332</sup> (*hu-Def*\_ED) and C<sup>199</sup>G<sup>332</sup> (*hu-Def*\_QG) in the degradation of p53 (Figure 4B) and  $\Delta 133p53$  (Figure 4C). Examination of the p53 target genes showed that p21 but not Bax was downregulated in the *hu-def* and *p53* co-transfected cells (Figure 4B). In contrast, we found that the knockdown of *hu-Def* by its specific small interfering RNA (siRNA)

upregulated the level of endogenous p53 protein in MCF-7, HepG2 and Ovar-8/TR cells (both MCF-7 and HepG2 cells carry wild-type p53 whereas Ovar-8/TR has a mutated p53 with an in-frame deletion of 126-132 AAs) at 24 hpt (Figure 4D).

#### *Def*-mediated p53 degradation is dependent on Calpain 3

We then tested the effects of various protein degradation inhibitors on *Def*-mediated p53 degradation. Only





**Figure 5** Def-mediated p53 degradation depends on cysteine protease, CAPN3. **(A)** Western blot of p53 and Def for comparing the effect of knockdown of CAPN3, -6, -7 and -8 by their specific siRNAs on hu-Def-mediated p53 degradation. **(B)** qPCR showing the efficiency of knockdown of *capn6*, *capn7* and *capn8* by their respective siRNAs. The relative expression level of the genes was shown in fold change as normalized against human *elf1a*. **(C)** Western blot of p53, Def, Hdm2 and CAPN3 in MCF-7 cells treated with CAPN3-siRNA. **(D)** Western blot of p53 and CAPN3 in CAPN3-siRNA treated MCF-7 and HepG2 cells at 24 hpt. **(E)** Western blot of p53, CAPN3 and Def in cells transfected with CAPN3, p53 overexpression plasmids and hu-Def siRNA. **(F)** Co-IP analysis of interaction between hu-Def and CAPN3 in H1299 cells. Cells were transfected with plasmids as indicated. In **(A, and C-E)**, fold changes in expression for p53, hu-Def or CAPN3 against their respective controls (set as 1) are shown under their corresponding panels. GAPDH was used as the normalization control.

cysteine protease inhibitors (leupeptin, pepstatin A and E64) and EDTA, but not inhibitors of the 26S proteasome (MG132), lysosomes (NH<sub>4</sub>Cl and chloroquine) or autophagy (3-methyladenine, data not shown), inhibited Def-mediated p53 degradation (Figure 4E). In contrast, although MG132 showed a strong inhibitory effect, leupeptin showed only a negligible effect on Hdm2-mediated p53 degradation (Figure 4F). This result also ruled out the possibility of the involvement of free 20S proteasome in Def-mediated degradation of p53 because MG132 was also an effective inhibitor of this process. Interestingly, hu-p53-R175H, which is poorly sensitive to Calpain (a

type of cysteine proteases) [4], was more resistant to Def-mediated degradation (Supplementary information, Figure S5).

The above results suggested that Def and Calpain might act together to mediate p53 degradation. Humans have 15 known *calpain* (*capn*) genes [30]. We designed specific siRNAs against these 15 human *capn* genes (Supplementary information, Table S4) and used them to knockdown the expression of their corresponding CAPNs. We then tested the effect of hu-Def on p53 in cells treated with each siRNA. We found that only the knockdown of CAPN3 expression, but not

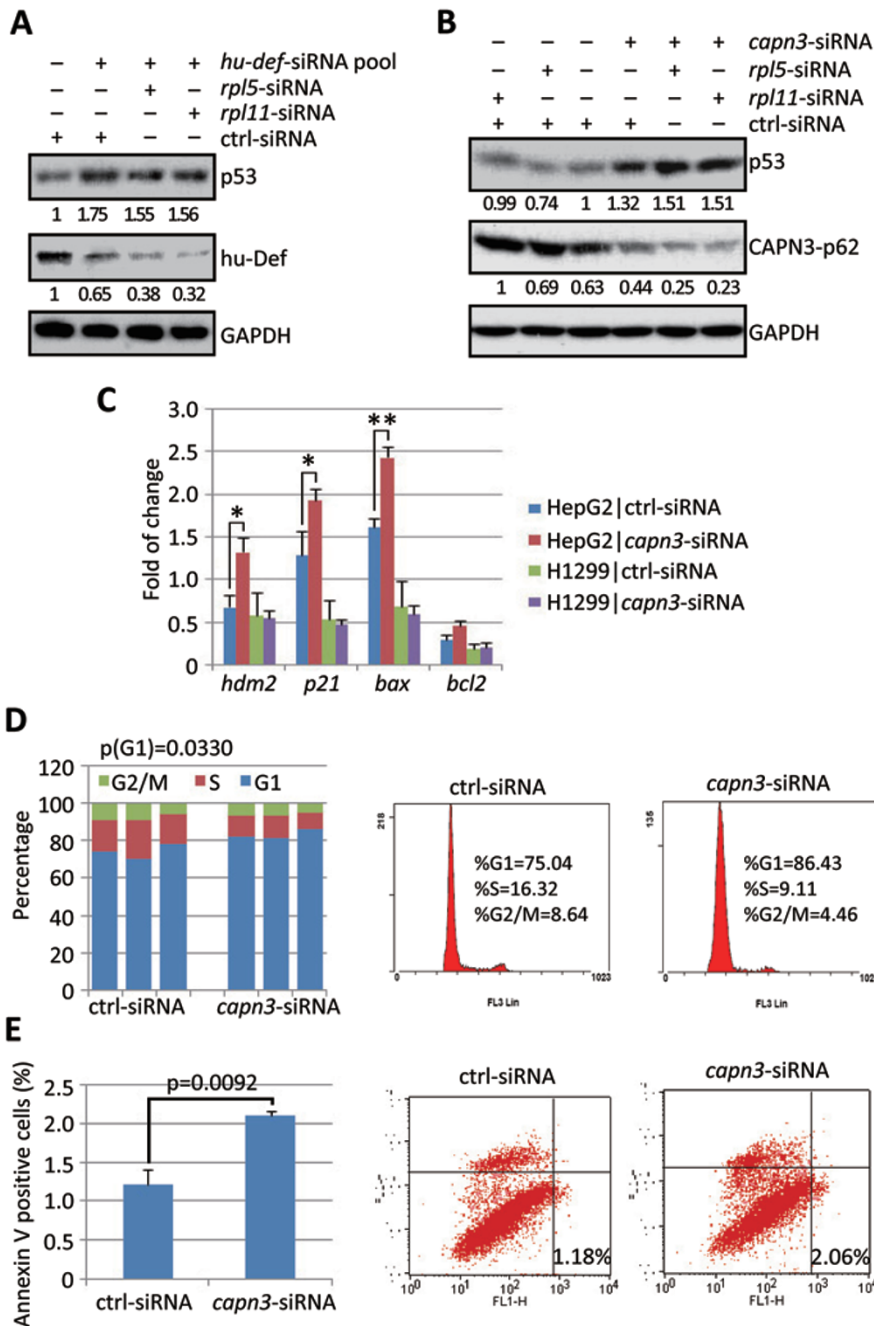


that of CAPN6, -7 or -8 (Figure 5A and 5B) or others (Supplementary information, Figure S6A), blocked Def-mediated p53 degradation. In contrast, the knockdown of CAPN3 expression did not block Hdm2-mediated p53 degradation (Figure 5C). We then determined the effect of the knockdown of CAPN3 on p53 and found that it significantly increased the level of endogenous p53 protein at 24 hpt in MCF7 and HepG2 cells (Figure 5D). Therefore, Def-mediated p53 degradation was CAPN3 dependent. More importantly, because CAPN3

is the only CAPNs known to date to be localized in the nucleolus [31], the above results strongly suggested that the Def- and CAPN3-mediated p53 degradation pathway operates in the nucleolus.

*hu-Def and CAPN3 form a complex*

As shown above, CAPN3 knockdown blocked hu-Def-mediated p53 degradation (Figure 5A-5C). To determine whether hu-Def knockdown could block CAPN3-mediated p53 degradation, MCF7 cells were cotransfected with



**Figure 6** CAPN3 knockdown activated p53 response. (A, B) Western blot analysis of the effect of RPL5 or RPL11 knockdown on the stabilized p53 induced by Def (A) or CAPN3 (B) knockdown. Fold changes in expression for p53, hu-Def or CAPN3 against their respective controls (set as 1) are shown under their corresponding panels. GAPDH was used as the normalization control. (C) qPCR analysis of expression of p53 response genes in HepG2 or H1299 cells treated with control siRNA (ctrl-siRNA) or *capn3*-siRNA. The relative expression level of the genes is shown in fold change as normalized against human *elf1a*. (D) FACS analysis of the ratio of cells at G2/M, S and G1 phases (histogram on the left) after cells were treated with ctrl-siRNA (middle graph) or *capn3*-siRNA (graph on the right). (E) Analysis of apoptotic cells (histogram on the left) after Annexin V staining on cells treated with ctrl-siRNA (middle graph) or *capn3*-siRNA (graph on the right).

CAPN3 overexpression plasmid and hu-Def siRNA, and p53 protein was analyzed. Western blot results showed that hu-Def knockdown clearly partially blocked the effect of CAPN3 on p53 (Figure 5E). Apparently, Def and CAPN3 are mutually dependent for the maximization of their effect on p53. We next overexpressed Myc-tagged hu-Def and HA-tagged CAPN3 together in H1299 (*p53*-null) and MCF7 (*p53* wild type) cells and performed coimmunoprecipitation (Co-IP) using the anti-HA antibody. Western blot of Co-IP products using the anti-Myc antibody showed that hu-Def was successfully coprecipitated with CAPN3 (Figure 5F; Supplementary information, Figure S6B). This result demonstrated that Def and CAPN3 can form a complex even in the absence of p53.

#### *The Def-CAPN3 pathway is independent of the RPL5/RPL11 pathway*

Previous studies have shown that the ribosomal proteins, RPL5 and RPL11, stabilize and activate p53 by sequestering MDM2 to the nucleolus under conditions of nucleolar stress [10, 11]. Knockdown of RPL5 or RPL11 would leave MDM2 free in the nucleoplasm, which would trigger the degradation of p53 even under nucleolar stress [12]. Our extensive data shown above demonstrated that Def-mediated p53 degradation is independent of the Mdm2 pathway. To further verify this conclusion, we tested the effect of the combined knockdown of Def and RPL5, Def and RPL11, CAPN3 and RPL5, and CAPN3 and RPL11 by their respective siRNAs. Clearly, the elevated levels of p53 induced by Def or CAPN3 knockdown were not affected by the concomitant knockdown of either RPL5 or RPL11 (Figure 6A and 6B).

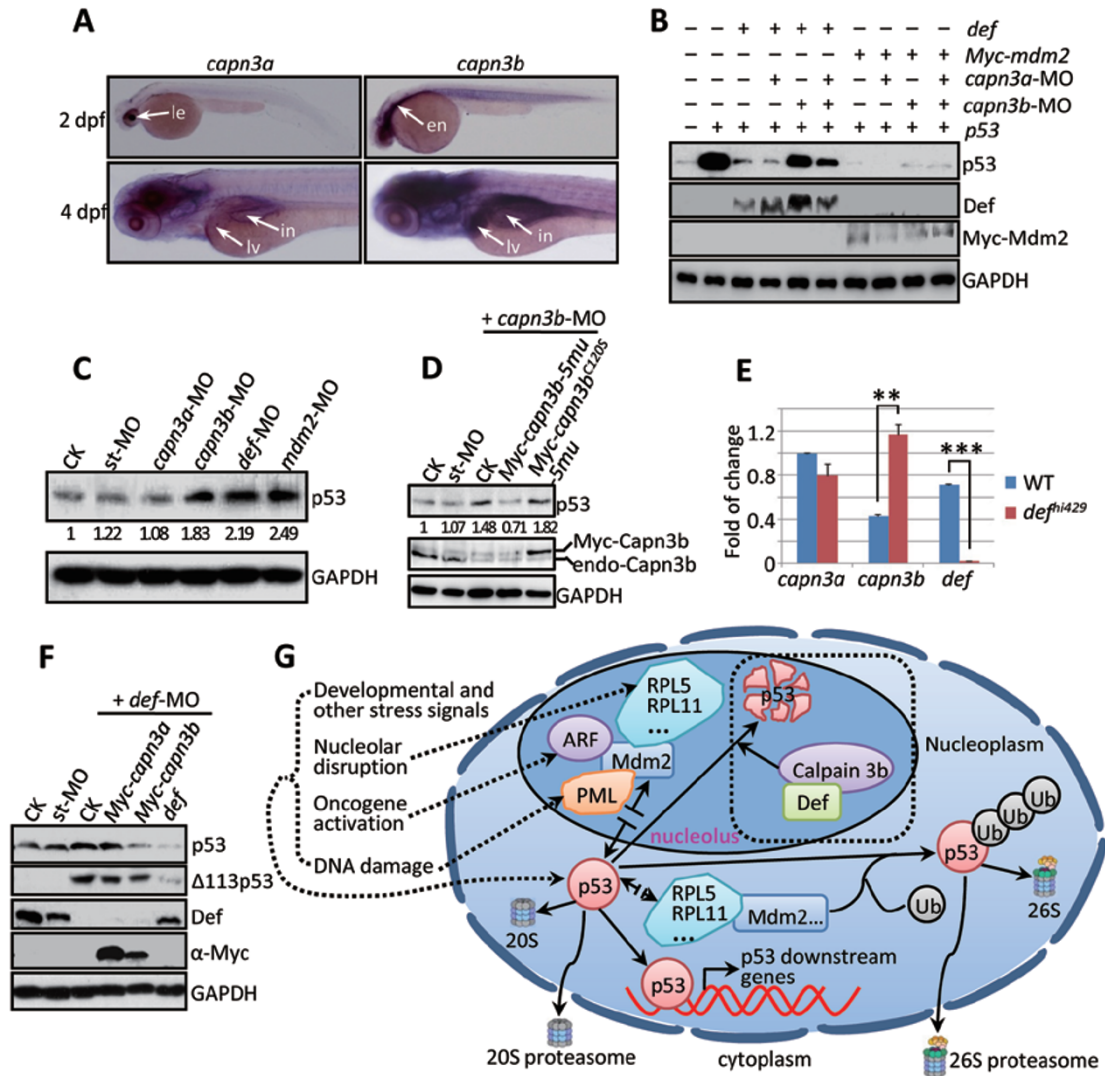
#### *Knockdown of CAPN3 arrests cells at the G1 phase and promotes cell apoptosis*

We then performed qPCR to examine the expression of p53 response genes, including *hdm2*, *p21*, *bax* and *bcl2* in HepG2 (*p53* wild type) and H1299 cells after treatment with CAPN3 siRNA at 24 h. We found that *hdm2* and *bax* were significantly upregulated in HepG2 cells but not in H1299 cells after CAPN3 knockdown (Figure 6C). We also analyzed the ratio of cells in the G1, S and G2/M phases after CAPN3 siRNA treatment. The flow cytometry analysis (FACS) result showed that CAPN3 knockdown in HepG2 cells arrested cells at the G1 phase (an average of 74.7% G1 cells in control siRNA samples versus 83.5% in CAPN3 siRNA samples) (Figure 6D). FACS analysis of apoptotic cells after Annexin V staining showed that CAPN3 siRNA-treated HepG2 cells exhibited a much higher level of apoptosis than those treated with control siRNA (Figure 6E).

#### *Capn3b acts together with Def to mediate p53 degradation in zebrafish*

A database search in the zebrafish genome assembly Zv9 identified two *capn3* genes, located on chromosomes 17 (named *capn3a*) and 20 (named *capn3b*). Amino acid sequence alignment revealed that human CAPN3 shares 62% and 57% identity with zebrafish CAPN3a and CAPN3b, respectively (Supplementary information, Figure S7). However, only CAPN3b contains the presumed NoLS KKKxKP (Supplementary information, Figure S8A). We first examined the expression patterns of *capn3a* and *capn3b* in zebrafish embryos at 2 and 4 dpf, respectively. Whole-mount *in situ* hybridization (WISH) results showed that *capn3a* is mainly expressed in the lens at 2 dpf as recorded in the database (database for Gene Expression, <http://zfin.org>), and is then enriched in the brain region between the eyes (Figure 7A). In contrast, *capn3b* expression is enriched in the digestive organs at both 2 and 4 dpf and in the head region at 4 dpf (Figure 7A), and displays a pattern resembling that observed for *def* [13]. To determine whether Def-mediated p53 degradation in zebrafish is also CAPN3 dependent, we co-injected *def* and *p53* mRNA with a *capn3a*-specific (*capn3a*-MO) or *capn3b*-specific (*capn3b*-MO) morpholino (Supplementary information, Figure S8B and S8C) and examined p53 protein levels in the injected embryos. The results showed that *capn3b*-MO but not *capn3a*-MO effectively blocked Def-mediated p53 degradation at 6 hpi (Figure 7B). Furthermore, we found that *capn3b*-MO but not *capn3a*-MO also increased the level of the endogenous p53 protein as *def*-MO and *mdm2*-MO at 3 dpi (Figure 7C). The elevated endogenous p53 protein level could be downregulated by co-injection with *Myc-capn3b-5mu* mRNA that lacks the target sequence for *capn3b*-MO (Figure 7D). In contrast, mutating the active site Cys<sup>120</sup> (corresponding to Cys<sup>129</sup> at the active site in human Capn3) [32] to a serine (Capn3b-C120S) abolished the ability of the mutant Capn3 to downregulate the p53 level induced by the injection of *capn3b*-MO (Figure 7D). This result suggested that Capn3b catalytic activity is essential for Def-mediated p53 degradation and that Capn3b itself does not simply provide a platform for p53 degradation by an alternative mechanism. In humans, CAPN3-C129S was found to be more stable than the wild-type CAPN3 [32]. We found that, as expected, the overexpressed zebrafish Capn3b-C120S is more stable than the wild-type Capn3b (Figure 7D).

As shown in Figure 1A, the level of p53 protein was increased in the zebrafish *def*<sup>hi429</sup> mutant. To determine whether *capn3a* and *capn3b* were expressed normally in the *def*<sup>hi429</sup> mutant, we measured their expression levels in zebrafish. The qPCR results showed that *capn3b* but



**Figure 7** Def and CAPN3 duet in zebrafish. **(A)** WISH analysis of the expression patterns of *capn3a* and *capn3b* in embryos at 2 and 4 dpf, respectively, using *capn3a* or *capn3b* probes. en, endoderm tube; in, intestine; le, lens; lv, liver. **(B)** Western blot of p53, Def and Mdm2 for comparing the effect of knockdown of zebrafish Capn3a or Capn3b with their specific morpholinos *capn3a-MO* or *capn3b-MO* on Def-mediated or Mdm2-mediated p53 degradation in the injected embryos at 6 hpi. **(C)** Western blot of the endogenous p53 in *capn3a-MO*, *capn3b-MO* and *def-MO* morphants at 3 dpi. **(D)** Western blot of p53 for examining the effect of *capn3b* mRNA lacking the *capn3b-MO* target sequence (*Myc-capn3b-5mu*) or the mutant mRNA carrying a mutation changing the codon for the active site Cys<sup>120</sup> to Ser<sup>120</sup> (*Myc-capn3b<sup>C120S</sup>-5mu*) on elevated endogenous p53 induced by *capn3b-MO*. Capn3b was detected using a polyclonal antibody against zebrafish Capn3b. Myc-Capn3b, Myc-tagged Capn3b; endo-Capn3b, endogenous Capn3b. **(E)** qPCR analysis of *capn3a* and *capn3b* transcripts in *def<sup>hi429</sup>* mutant embryos and wild-type controls (WT). The relative expression level of the genes was shown in fold change as normalized against zebrafish *elf1*. **(F)** Western blot analysis of p53, Δ113p53, Def and CAPN3 (α-Myc) in the embryos co-injected with *def-MO* morpholino plus *capn3a* mRNA or *def-MO* morpholino plus *capn3b* mRNA at 3 dpi. Wild-type embryos (CK) and st-MO-injected embryos were used as the controls. **(G)** Graphics summarizes the roles of the nucleolus in regulation of p53 homeostasis. In response to stress conditions such as nucleolar disruption, oncogene activation, DNA damage or developmental defect, p53 is stabilized or activated. Nucleolar factors including Arf, PML, RPL5 and RPL11 can associate with Mdm2 to prevent p53 ubiquitination and degradation through the 26S proteasome in the nucleoplasm and cytoplasm. In contrast, the role of the Def-CAPN3 pathway is to prevent the accumulation of p53 in the nucleolus by triggering *in situ* p53 degradation.

not *capn3a* was expressed at a higher level in the mutant than in the wild-type control (Figure 7E), demonstrating

that Capn3b-mediated p53 degradation is dependent on Def. As shown in Supplementary information, Figure



S1F, the level of p53 protein was elevated in the *def*-MO morphants. We co-injected *def*-MO with *capn3a* or *capn3b* mRNA and found that the overexpression of CAPN3b but not that of CAPN3a partially downregulated the p53 protein level (Figure 7F). It should be noted that the extent of p53 degradation induced by *capn3b* mRNA was less than that induced by *def* mRNA, demonstrating that Capn3b-mediated p53 degradation is dependent on Def, as has been observed in human cells. Therefore, the Def- and Capn3b-mediated p53 degradation pathway is also functional in zebrafish.

## Discussion

In this study, we demonstrated that Def, a novel nucleolar factor, can induce degradation of the p53 protein in both human and zebrafish, and that this process is independent of the proteasome pathway but is dependent on a specific nucleolus localized cysteine protease, CAPN3. Hence, we concluded that Def and CAPN3 together define a conserved nucleolar protein degradation pathway that negatively regulates the function of p53 by mediating its turnover (Figure 7G).

The regulation of p53 turnover is essential to maintain a low level of p53 in normal cells. Many E3 ligases have been found to mediate p53 degradation through the ubiquitination pathway [2]. The role of the nucleolus in regulating the p53 function is through the binding of Mdm2 by some nucleolar factors, such as p14ARF, RPL5 and RPL11, so that Mdm2 is physically separated from the nucleoplasmic p53. In this way, p53 degradation through the ubiquitination pathway is blocked [12, 23]. We found (1) that the upregulated p53 and possibly  $\Delta 113p53$  clearly accumulated in the nucleolus in the *def*<sup>hi429</sup> mutant, and (2) that RPL5 or RPL11 knockdown failed to compromise the elevated level of p53 induced by CAPN3 knockdown. These results demonstrated that the Def-CAPN3-p53 pathway differs from the p14ARF-, PML- or RPL11-mediated nucleolar pathways [8, 12, 23] (Figure 7G).

Calpains belong to the family of Ca<sup>2+</sup>-dependent cysteine proteases. Although CAPN1 and CAPN2 are considered to be ubiquitous, CAPN3 is mainly expressed in the muscle [33]. A study of a *Calpain3*-null knockout mouse revealed that this model exhibited a phenotype that resembles the human limb girdle muscular dystrophy type 2A (LGMD2A) due to aberrant myofibrillogenesis. CAPN3 exists in many alternatively spliced isoforms. The main isoform in muscle is p94, whereas the main isoforms in human melanoma cells are p62, hMp78 and hMp84 (it is proposed that p62 is derived from hMp78 and hMp84). Surprisingly, in human melanoma cells,

p62 and hMp78 were located in both the cytoplasm and nucleoli [31].

It has been reported that Calpains can regulate p53 stability, however, this was attributed to cytoplasmic abundant calpains [4-7]. The nature of the nucleolar localization of CAPN3 explains why Def-mediated p53 degradation depends on the activity of CAPN3 but not that of other Calpains. Importantly, the catalytic activity of Capn3b is indispensable for p53 degradation. Furthermore, we found that Def and CAPN3 can form a complex, which is consistent with the finding that Def and CAPN3 are mutually dependent for the mediation of p53 degradation in the nucleolus. We tried different means to study the potential protein-protein interaction between Def and p53, however, we did not observe any interaction between them. It would be interesting in the future to determine whether the Def-CAPN3-p53 pathway contributes to LGMD2A in the *Calpain3*-null mouse model.

It appeared that Def knockdown was more effective than that of Capn3b in stabilizing p53. We speculated that an additional nucleolar factor(s) might be required for the formation of a fully functional Def-Capn3 complex. Def might serve as a scaffold protein to recruit the other factor(s). In this scenario, Def knockdown would be more effective in disrupting the complex, whereas the complex could still form after Capn3b knockdown and the residual Capn3b could still induce p53 degradation. This hypothesis might explain why p53 and  $\Delta 113p53$  are highly stabilized despite that *capn3* is highly expressed in the *def*<sup>hi429</sup> mutant or overexpressed in the *def*-MO morphants. Certainly, the possibility of the existence of other Def effector(s) cannot be excluded. The creation of a *capn3b* knockout mutant by the transcription activation-like effector nuclease (TALEN) method will help to resolve this query in the future.

Previous studies showed  $\Delta 113p53$  and  $\Delta 133p53$  are p53 target genes and function to antagonize p53 apoptotic activity [17, 19]. Our study also demonstrated that  $\Delta 113p53/\Delta 133p53$  was degraded by the Def-CAPN3 pathway when overexpressed. This is the first pathway that was found to regulate the turnover of  $\Delta 113p53/\Delta 133p53$ , although further efforts are needed to prove that this pathway also operates *in vivo*. The fact that Def acts on the wild-type p53, but not on point mutants p53-R143H and -R250W, and also acts on  $\Delta 113p53/\Delta 133p53$  implies that  $\Delta 113p53/\Delta 133p53$  protein adopts much of the native structure in its truncated DNA-binding domain. Our findings might provide an explanation of why tumorigenesis occurs in certain cells/tissues even when they harbor wild-type p53. Therefore, the downregulation of hu-Def or CAPN3 in such cancer cells might be a new approach for cancer therapy.

Clearly, the Def-Capn3-p53 pathway has an important role in protecting embryos from the p53-induced reduction in survival. We hypothesized that during early embryogenesis, the Def-Capn3b-p53- $\Delta$ 113p53 pathway may serve as a check point to monitor environmental changes that would allow the organism to adjust its physiological response to certain cellular stresses and thus to safeguard the proceeding of normal development. However, many questions remain unanswered. For example, how does the Def-CAPN3 pathway control the genesis of the digestive organs? Are other proteins targeted by the Def-CAPN3 pathway? Are the two SNPs in *hu-def* related to any disease(s)? The resolution of these questions will undoubtedly help to further our understanding of the function of the nucleolus.

## Materials and Methods

### Zebrafish lines and maintenance

Zebrafish were raised and maintained according to the standard procedure. The *def*<sup>hi429</sup> mutant line was provided by Prof Nancy Hopkins. Two pairs of primers derived from *lacZ* and *def* were used to genotype the *def*<sup>hi429</sup> mutant [13]. The *p53*<sup>-/-</sup> mutant allele *tp53*<sup>M214K</sup> line [25] was provided by Professor Thomas Look.

### Cell lines

MCF-7-1, MCF-7-3, HepG2 and SiHa cells were maintained in the DMEM medium (high glucose, GIBCO), MCF-7-2 in DMEM/F12 (1:1) medium (GIBCO), HCT-116, U2OS and Saos-2 in McCoy's 5A (modified) medium, H-1299 and OVCAR-8/TR in RPMI medium 1640 (GIBCO), and PC-3 in Ham's F12. All media were supplemented with 10% newborn calf serum (NBCS, GIBCO) with the exception of 15% NBCS for the Saos-2. Plasmids were transfected into the cell by FuGENE HD (Roche) and siRNAs with Lipofectamine 2000 (GIBCO) transfection reagent according to the manufacturer's recommendations.

### Phylogenetic analysis

Human and zebrafish CAPN3 amino acid sequences were retrieved from the GenBank and Ensembl Genome Browser and aligned using the Clustal W program. The phylogenetic tree was built using the MEGA 4 program [34].

### Plasmid construction

Each *def* and *p53* deletion construct was derived from pCS2<sup>+</sup>-*def*-D0 and pCS2<sup>+</sup>-*p53*-P0, respectively. The corresponding primer pairs used to generate all relevant plasmids are listed in Supplementary information, Table S5. The method to generate the internal deletion constructs *MYC-def\_D7*, *MYC-def\_D8*, *def*<sup>stop</sup>-pCS2<sup>+</sup> and the internal deletion construct *HA-p53\_P6*-pCS2<sup>+</sup> has been described previously [17]. Zebrafish *mdm2* was cloned by PCR from cDNA of 1 dpf embryos, *capn3a* and *capn3b* from cDNA of 5 dpf embryos, using the primer pairs listed in Supplementary information, Table S5. *Capn3* and *hu-def\_ED* cDNAs were obtained from MCF7 and *hu-def\_QG* cDNA from RKO cells, and all cDNAs were cloned into the pcDNA3.1<sup>+</sup> vector. The  $\Delta$ 113p53 construct was subcloned from *hu-p53* plasmid. The *hdm2* and *ubiquitin* con-

structs were provided by Dr Shuyong Lin and Dr Huizhe Huang, respectively. *zf-p53* 9KR, R143H, R250W, R313C, *hu-def\_EG*, *hu-def\_QD*, *hu-p53* R175H, *Myc-capn3b-5mu-pCS2*<sup>+</sup> and *Myc-capn3b*<sup>C120S</sup>-*5mu-pCS2*<sup>+</sup> were constructed through site-directed mutagenesis using the primer pairs listed in Supplementary information, Table S5. The 5'-untranslated regions (5' UTR) of *capn3a* and *capn3b* were cloned upstream of the *egfp* reporter gene to create *cmv:capn3a\_5'UTR-egfp* and *cmv:capn3b\_5'UTR-egfp* constructs in pCS2<sup>+</sup>, respectively. HA or MYC tags were designed into the primers to generate the tagged constructs. For mRNA injection, mRNAs were synthesized *in vitro* using the mMACHINE Kit (Ambion) according to the manufacturer's instructions. 1 nl mRNA or morpholinos or plasmid containing phenol red dye was injected into zebrafish embryos at the one-cell stage.

### DNA, RNA and protein analysis

The primer pair 5'-AACCTGTGGCCTAATGCCTGGGA-3' and 5'-CTGGCAGTGTCAACCACTCGCC-3' was used to amplify the genomic DNA fragment harboring *hu-def* from different human cells and the DNA fragment was sequenced with the primer 5'-TTAAAAGTGGGGCAGTAGCAGT-3'.

Total RNA was extracted from different samples using TRIzol reagent (Invitrogen). Probes were labeled with Digoxigenin (DIG) and northern blot hybridization was performed according to the manufacturer's instructions (Roche Diagnostics). The *p53*-P1 probe and *egfp* probe have been described previously [13]. The qPCR was performed with a CFX96 Real-Time System (Bio-Rad) using SsoFast EvaGreen Supermix (Bio-Rad). The primer pairs and detailed PCR conditions for amplifying zebrafish  $\Delta$ 113p53, *mdm2*, *p21* and *bax* have been described previously [13]. The primer pairs for amplifying zebrafish *capn3a*, *capn3b*, *def* and human *hdm2*, *p21*, *bax* and *bcl2* are listed in Supplementary information, Table S6.

The methods used for protein extraction from zebrafish embryos and from cultured cells have been described previously [17, 18]. Protein electrophoresis and western blot were performed according to the instructions provided by the manufacturers.

### Protease inhibitors

Protease inhibitors MG132, chloroquine and pepstatin A were purchased from Sigma, Complete Protease Inhibitor Cocktail Tablet (EDTA-free) from Roche, and NH<sub>4</sub>Cl, leupeptin, PMSF (phenylmethanesulfonyl fluoride), E64 and EDTA from Sangon (Shanghai, China).

### Morpholinos and siRNAs

Morpholinos were purchased from Gene Tools (Philomath, USA). *def*-MO,  $\Delta$ 113p53-MO, *p53*-MO<sup>ATG</sup>, *mdm2*-MO, and the human  $\beta$ -globin antisense morpholino (st-MO) were used as described previously [13, 17, 27], *capn3a*-MO (5'-AACCCAGACGGCGTATAGGGCATCT-3') and *capn3b*-MO (5'-CTGAAACTTCTGTTCTTCTGCCAT-3') were designed to target the start codon ATG of the corresponding genes. All morpholinos (0.25 pmol) were injected into one-cell stage embryos.

Stealth *C1orf107* (*hu-def*) siRNAs and Negative Control Duplex (control siRNA) were obtained from Invitrogen (Carlsbad, USA). The *hu-def* siRNA pool containing three duplex oligonucleotides (*hu-def*-siRNA-1, *hu-def*-siRNA-2 and *hu-def*-siRNA-3),

CAPN1 siRNA [35], CAPN2 siRNA [36], CAPN3 siRNA, CAPN5 to CAPN16 siRNAs, RPL5 and RPL11 siRNAs [12], and the negative control duplex (control siRNA) were obtained from GenePharma (Shanghai, China). All information on the siRNA sequences is listed in Supplementary information, Table S4.

### Antibodies

The zf-p53 monoclonal antibody A7-C10 and polyclonal antibody against the 113-373 aa region of p53 were raised in mouse and rabbit, respectively. Polyclonal antibody against the 34-174 aa region of hu-Def was raised in rabbit. The zf-Def rabbit polyclonal antibody has been described previously [13]. Anti-Myc mouse monoclonal (9E10) antibody was purchased from Clontech (#631206), anti-HA mouse monoclonal (HA-7) antibody from Sigma (H3663), mouse monoclonal (38F3) antibody against human Fibrillarin (ab4566) and rabbit polyclonal antibodies against GFP (ab6556) and CAPN3 (ab38963) from Abcam. Rabbit monoclonal antibody (EPR1977Y) against GAPDH was from Epitomics (#2251-1), and rabbit polyclonal antibody against  $\beta$ -actin (#4967) was from Cell Signaling Technology. Mouse monoclonal antibodies against human p53 (DO-1, sc-126), Hdm2 (SMP14, sc-965), p21 (F-5, sc-6246), C23 (H-6, sc-55486) and rabbit polyclonal antibody against human Bax (N-20, sc-493) were from Santa Cruz Biotechnology. Polyclonal antibody against the 1-89 aa region of Capn3b was raised in rabbit by HuaAn Biotechnology (Hangzhou, China).

### WISH, cryo-sectioning and immunostaining

Probe labeling and WISH were performed as described previously [13]. The 3'-UTRs of *capn3a* and *capn3b* were cloned (Supplementary information, Table S5) and used as probes.

Cryosectioning and immunofluorescence staining were performed as described previously [17]. For immunofluorescence staining of the cultured cells, cells were plated on coverslips in a six-well plate. Primary antibodies were used at a 1:100 dilution. Immunofluorescence was detected after staining with Alexa Fluor conjugated secondary antibody (Invitrogen) (1:400 dilution). DAPI was used to stain the nuclei. All immunofluorescence staining images were taken under a Leica TCS SP5 confocal microscope.

### Coimmunoprecipitation (Co-IP)

Human cell lines transfected with various plasmids were harvested with Tris-HCl protein lysis buffer for total protein extraction. Co-IP was performed using an HA Tag IP/Co-IP Kit (Pierce) according to the manufacturer's instructions. The input and precipitated protein samples were subjected to western blot analysis with corresponding antibodies as stated.

### TUNEL assay and embryo viability counting

The TUNEL assay was performed using the *in situ* Cell Death Detection Kit, TMR red (Roche) as described previously [13]. For viability counting, dead embryos were characterized by the presence of large, dark debris in the embryos.

### Flow cytometry analysis (FACS)

Human cells were digested with trypsin and precipitated at  $500\times g$ , 4 °C. After washing in PBS, the cells were stained with PI (Beyotime) or costained with Annexin V-FITC and PI according to the manufacturer's instructions, and subjected to FACS analysis

of the cell cycle and apoptosis using a Beckman Coulter FC 500 MCL and a BD FACSCalibur flow cytometer, respectively.

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