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Usp28 counteracts Fbw7 in intestinal homeostasis and cancer

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Abstract

The stability of several oncoproteins, including c-Myc, is regulated by ubiquitin-dependent degradation mediated by the SCF(Fbw7) ubiquitin ligase. This activity is antagonized by the deubiquitinase Usp28, which is highly expressed in murine and human intestinal cancers. Usp28 was previously shown to interact with its substrates via a "piggyback" interaction with Fbw7, which suggested that Fbw7 is required for Usp28 activity. Unexpectedly, we found that genetic deletion of Usp28 rescued the lethality of Fbw7-deficient primary fibroblasts. Moreover, Usp28 inactivation in the intestine (*Usp28*$^{\Delta IEC}$) ameliorated the hyperproliferation and the impaired goblet and Paneth cell differentiation observed in *Fbw7*$^{\Delta IEC}$ mice. The aggressive intestinal tumor formation of *APC*$^{Min/+}$; *Fbw7*$^{\Delta IEC}$ mice was restrained when Usp28 was inactivated concomitantly. In both fibroblasts and intestinal cells, Usp28 deficiency corrected the accumulation of SCF(Fbw7) substrate proteins, including NICD1, c-Jun and c-Myc. These findings suggested that Usp28 function does not depend on the presence of Fbw7, but instead independently recognizes and deubiquitylates the same substrates as SCF(Fbw7). Fbw7 binds to a phosphorylated motif termed the phosphodegron and we found that Usp28 also interacted with this same motif, but only when it is unphosphorylated, offering a mechanistic explanation for identical substrate selection by Fbw7 and Usp28. Our results indicate an unusually direct antagonism between an E3 ligase and a deubiquitinase, Fbw7 and Usp28, in modulating intestinal homeostasis and cancer.

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Keywords

Ubiquitination; Usp28; Fbw7; c-Myc; colorectal cancer

Introduction

Fbw7 (also known as Fbxw7, CDC4, Ago and hSel10) is the substrate recognition component of an evolutionarily conserved SCF (Skp1, Cul1 and F-box protein)-type E3 ubiquitin ligase complex. SCF(Fbw7)-mediated ubiquitination targets several proteins that function in proliferation and differentiation for degradation, including c-Myc, Cyclin E1, NICD1 and c-Jun (1). In accordance with its destabilizing effect on these oncoproteins, *Fbw7* is a haploinsufficient tumor suppressor gene for several cancer types in the mouse, including intestinal cancer (2). In humans, *FBW7* loss-of-function mutations occur in a variety of tumors, and *FBW7* copy number loss is particularly frequent in cancers of the colon (3). Low expression of *FBW7* correlates with poor prognosis in colorectal cancer patients (3).

The control of c-Myc is particularly important in intestinal tumorigenesis: c-Myc is required for the altered proliferation and differentiation induced by APC inactivation (4, 5), and conditional inactivation of c-Myc impairs intestinal tumor formation in the $APC^{min/+}$ tumor model (5). Genomic data from human cancers suggests that most colorectal cancer mutations converge on c-Myc misregulation (6).

c-Myc is a highly labile protein, and its stability is regulated by a balance between ubiquitination (by Fbw7 and at least one other E3 ubiquitin ligase, Skp2), and deubiquitination, by the ubiquitin-specific protease Usp28 (7-11). Fbw7 recognises c-Myc by its phosphodegron motif, which contains phospho-threonine 58 (11). Usp28 binds to Fbw7, and Usp28 was shown to be recruited to c-Myc protein indirectly through Fbw7 (the “piggyback” model) (7).

Usp28 was originally described as part of the DNA damage response, since it binds the double-strand break repair protein 53BP1 and is phosphorylated following ionizing radiation in an ATM-dependent manner (12). Although Usp28 is recruited to damage sites by 53BP1, it is not crucial for double-strand break repair, and Usp28 germline deficient mice show normal lifespan, immunological development and radiation responses (13). However, since Usp28 also counteracts the ubiquitin-mediated degradation of several Fbw7 substrates, including c-Myc, c-Jun, Notch-1 and cyclin E, it antagonizes Fbw7’s tumor suppressive effect, placing Usp28 as a tumor-promoting factor (7, 14). In particular, we recently found that deleting Usp28 in established tumors slows their progression and extends lifespan in the $APC^{min/+}$ colorectal cancer mouse model (14).

The piggyback model indicates that Fbw7 is required for Usp28 substrate recognition, suggesting that Usp28 would only be able to promote tumorigenesis in the presence of functional Fbw7. Here we test this hypothesis, and examine the effect of *Usp28* deletion in the absence of functional Fbw7. As well as shedding light on the substrate recognition capabilities of Usp28, this work clarifies the role of Usp28 activity in a mutational

background common in human colorectal cancer, underlining its importance as an oncogene and putative drug target.

Materials and Methods

Mice

Mouse lines have been previously described: *Usp28*$^{F/F}$ (14); *Fbw7*$^{F/F}$ (2); *Villin-Cre* (15) and the intestinal tumor model *APC*$^{min/+}$ (16) (see Supplementary Methods). All experimental mice were in the C57BL/6 genetic background. Experiments were carried out with the approval of the London Research Institute's Ethical Review Committee according to the Animals (Scientific Procedures) Act 1986.

Isolation of MEFs

Mouse embryos were sacrificed at E10.5. Dissected limb tissue was dissociated in DMEM (10% FCS/1% penicillin-streptomycin). MEFs were maintained at 37°C/3% O_2/5% CO_2/95% humidity for a minimum of 3 days before reseeding and infection 2 days later with Adeno-CMV-Cre virus. Recombination was confirmed by genotyping PCR.

Histology

Mice were injected intraperitoneally (i.p.) with 100 mg/kg BrdU (Sigma) 2.5hr prior to sacrifice. Intestinal sections were cut at 4 μm for staining; 100 full crypts or villi were scored from at least 3 mice of each genotype.

Western blotting

Immunoblots were carried out as previously described (2). See Supplementary information for details of antibodies.

q-RT-PCR

Total mRNA was isolated from dissected ileum as previously described (2). Primer sequences are given in ref. (14).

Computational analysis

Human *USP28* and *FBW7* expression data from Skrzypczak Colorectal 2 (20 normal and 10 tumor samples) were downloaded from GEO (ID GSE20916).

Cell lines

HCT116 cells were from Cancer Research UK Cell Services and HCT116ΔFBW7 cells from B. Vogelstein (17). Both were authenticated by short tandem repeat profiling and FBW7 loss was verified by western blotting. *KP* and *KP Fbw7*$^{\Delta/\Delta}$ cell lines were generated from primary murine adult epithelial cells harboring a conditional allele for *p53* (*p53*$^{F/F}$), the lox-stop-lox mutant *KRas*^{G12D} allele and in the latter case, a conditional allele for *Fbw7* (*Fbw7*$^{F/F}$). Following in vitro recombination by infection with adenovirus encoding Cre recombinase (Vectorcore, University of Iowa), deletion of *p53* and *Fbw7* was confirmed by

PCR. Cell lines were cultured in DMEM (10% FBS/1% penicillin-streptomycin) at 37°C/5% CO_2/95% humidity.

Immunoprecipitation

For co-immunoprecipitation, cells were lysed 48h after transfection and cleared lysates incubated with anti-HA conjugated agarose or Flag-conjugated sepharose for 2h rotating at 4°C. For peptide pulldown, biotinylated c-MYC$^{46\text{-}74}$ peptides bound to M280 Streptavidin Dynabeads (Invitrogen) were incubated with cell lysates from *KP* and *KP Fbw7*$^{\Delta/\Delta}$ cell lines for 2h at 4°C. Washed immunoprecipitates were analyzed by western blotting. See Supplementary information for details of antibodies, buffer compositions, and peptide sequences.

Ubiquitin pulldown

24hr after transfection with the indicated constructs, cells were treated with proteasome inhibitor (MG132, Merck Biosciences) for 3hr, washed with cold PBS and lysed as above. His–Ubiquitin was affinity purified with nickel-NTA-agarose beads, and ubiquitin complexes analyzed by western blotting.

Statistics

Histogram bars indicate the mean average of results from 3 mice of the same genotype and error bars indicate SEM. Figure 2B histograms indicate mean and SEM of 3 independent means (from mice each with 30+ crypts/villi quantified). Statistical evaluation was performed by Student's unpaired t test or Chi-squared test (Fig.3D). $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Usp28 loss rescues cell proliferation and substrate protein levels in Fbw7-null cells

Germline inactivation of *Usp28* is well tolerated in vivo (13), while germline *Fbw7* deletion is embryonic lethal, due to a failure of cardiovascular development (18). In mouse embryonic fibroblasts (MEFs), deletion of *Fbw7* results in cell cycle arrest (19). To determine the effect of *Usp28* deletion in this system, we isolated MEFs from *Usp28*$^{F/F}$, *Fbw7*$^{F/F}$ and double mutant mice and infected them with a Cre recombinase-expressing adenovirus (Ad5-Cre-GFP). Efficient recombination was achieved in all three cell lines, resulting in loss of *Usp28* and/or *Fbw7* expression according to genotype (Fig. 1A). *Usp28*$^{\Delta/\Delta}$ MEFs proliferated normally in culture, while *Fbw7*$^{\Delta/\Delta}$ MEFs arrested proliferation after the first passage, consistent with previous data (20). However, *Fbw7*$^{\Delta/\Delta}$*; Usp28*$^{\Delta/\Delta}$ MEFs were able to proliferate (Fig. 1B and C). Cell cycle-arrested *Fbw7*$^{\Delta/\Delta}$ MEFs showed elevated levels of Notch intracellular domain 1 (NICD1), as previously described. In *Fbw7*$^{\Delta/\Delta}$*; Usp28*$^{\Delta/\Delta}$ MEFs, NICD1 levels were reduced, consistent with the alleviation of arrest (20)(Fig. 1D). In addition, *Usp28* deletion reduced the levels of other substrates elevated in *Fbw7*$^{\Delta/\Delta}$ cells, namely c-Myc and c-Jun (Fig. 1D). These data indicate that loss of Usp28 can partially rescue the *Fbw7*$^{\Delta/\Delta}$ phenotype.

Usp28 deficiency partially reverts hyperproliferation and impaired differentiation in *Fbw7$^{\Delta IEC}$* gut

To test whether Usp28 antagonizes Fbw7 function *in vivo*, we generated *Usp28$^{F/F}$; Fbw7$^{F/F}$; Villin-Cre (Usp28$^{\Delta IEC}$; Fbw7$^{\Delta IEC}$)* double mutant mice. As expected, *Usp28$^{\Delta IEC}$; Fbw7$^{\Delta IEC}$* animals showed negligible expression of Fbw7 and Usp28 in the intestinal epithelial cells (Supplementary Fig. S1A). Consistent with our previous observations, *Fbw7$^{\Delta IEC}$* animals showed significantly reduced numbers of differentiated goblet and Paneth cells present in intestinal crypts and villi (2). Importantly, concomitant deletion of Usp28 partially rescued this phenotype, significantly restoring goblet and Paneth cell numbers (Fig. 2A and B). Loss of Usp28 also partially rescued the hyperproliferation of *Fbw7$^{\Delta IEC}$* cells, reducing BrdU incorporation as well as the number of transit-amplifying cells marked by Sox9 in *Usp28$^{\Delta IEC}$; Fbw7$^{\Delta IEC}$* compared with *Fbw7$^{\Delta IEC}$* crypts (Fig. 2A and B). Consistent with reduced proliferation and increased differentiation, expression of many differentiation markers was increased and progenitor markers decreased in double mutant compared with *Fbw7$^{\Delta IEC}$* intestine (Supplementary Fig. S1B). The increased levels of p-c-Myc, c-Jun, Cyclin E1 and NICD1 were reduced to levels comparable to wild-type in *Usp28$^{\Delta IEC}$; Fbw7$^{\Delta IEC}$* double-mutant mice (Fig. 2C). Moreover, the altered expression of Notch-1 target genes, most notably *Hes1*, in *Fbw7$^{\Delta IEC}$* intestine was rescued (Fig. 2D). Thus, loss of Usp28 reverts the phenotypes observed in Fbw7-deficient intestine.

Loss of Usp28 ameliorates tumorigenesis in *APC$^{min/+}$; Fbw7$^{\Delta IEC}$* mice

Analysis of publically available expression data from human colorectal samples shows that expression of *USP28* is increased in adenomas compared with normal tissue, while *FBW7* expression is mildly decreased (Fig. 3A). As *FBW7* is also frequently mutated in colorectal cancer (2), it is not clear whether Usp28 requires Fbw7 function to promote tumorigenesis. We therefore asked whether loss of *Usp28* was able to suppress tumor growth in *Fbw7*-deficient mice. *APC$^{min/+}$; Fbw7$^{\Delta IEC}$* offspring were born at sub-Mendelian frequency, but concomitant deletion of *Usp28* rescued this synthetic lethality phenotype (Fig. 3B).

Control *APC$^{min/+}$* mice had an average life span of 144 days before succumbing to intestinal tumors. In agreement with previous work (2), *APC$^{min/+}$; Fbw7$^{\Delta IEC}$* mice showed accelerated tumor formation and survived on average for only 69 days (Fig. 3C and Supplementary Fig. S2A). Strikingly, Usp28 loss in *APC$^{min/+}$; Fbw7$^{\Delta IEC}$* mice resulted in an increase in survival to an average of 122 days, although the tumor burden at death was similar (Fig. 3C and Supplementary Fig. S2A and B). Histological analysis showed that deletion of *Usp28* resulted in loss of Usp28 protein throughout the tumor. Moreover, tumor cell proliferation was reduced, and goblet cell differentiation within the tumor was restored in *APC$^{min/+}$; Fbw7$^{\Delta IEC}$* compared with *Fbw7$^{\Delta IEC}$* tumors, as illustrated by Ki67 staining and AB/PAS staining for goblet cells (Fig. 3D). Thus, Usp28 loss reverted the accelerated tumorigenesis induced by deletion of *Fbw7*, suggesting that Usp28 contributes to tumorigenesis in Fbw7-deficient animals.

Usp28 can interact with and deubiquitinate its substrates in the absence of Fbw7

Previous work has shown that Usp28 interacts with its substrates via binding to Fbw7 (7). To determine how the Usp28–substrate interaction is affected in the absence of Fbw7, we

used a previously described HCT116 cell line, in which the *FBW7* gene has been deleted (HCT116ΔFBW7) (17). In agreement with the previous study (7), the interaction of c-MYC with USP28 was reduced in the absence of FBW7. However, USP28 was still able to co-immunoprecipitate c-MYC from HCT116ΔFBW7 cells (Fig. 4A). We confirmed this observation by coimmunoprecipitation of c-Myc with endogenous Usp28 in murine *Fbw7*-deficent cancer cells (*KP*ΔFbw7) (Supplementary Fig. S3). Overexpression of USP28 in FBW7-null cells also induced c-MYC deubiquitination (Fig. 4B). These data confirm previous observations that Fbw7 facilitates Usp28 recruitment to substrates, but also show that Usp28 is capable of recognizing and deubiquitinating common substrates independently of Fbw7 (Supplementary Fig. S4A).

To determine why Usp28 can recognize the same substrates as SCF(Fbw7) in Fbw7-deficient cells, we tested whether Usp28 recognizes the same motif as Fbw7. We generated four short peptides spanning the c-MYC phosphodegron (amino acids 46-74), either unphosphorylated or phosphorylated at Thr58 and/or Ser62 (Fig. 4C). Fbw7 preferentially bound peptides phosphorylated at Thr58, its known recognition site on c-Myc (11)(Fig. 4C). In the presence of Fbw7, Usp28 was able to bind all four peptides (7). Interestingly, in Fbw7-null cells, Usp28 strongly bound to the unphosphorylated peptide, but not to the modified peptides (Fig. 4C). These data suggest that Usp28 binding to c-Myc can occur via the degron motif in an Fbw7-independent fashion. Moreover, addition of unphosphorylated degron peptide strongly reduced the interaction between USP28 and c-MYC, presumably by titration of Usp28 protein, while the p-Thr58/p-Ser62 peptide did not affect the interaction (Fig. 4D). These data suggest that the unphosphorylated degron motif can mediate Usp28 binding in the absence of Fbw7 (Supplementary Fig. S4A).

In summary, our results demonstrate an unusually direct antagonism between an E3 ligase/deubiquitinase pair, Fbw7 and Usp28. Our finding that Usp28 can recognize and promote deubiquitination of c-Myc in the absence of Fbw7 might explain why Usp28 is most strongly expressed in intestinal crypt stem cells, where levels of Fbw7 are low (2, 14). We speculate that overlapping gradients of Fbw7 and Usp28 activity function to control the levels of key proliferation and differentiation determinants, such as c-Jun (Supplementary Fig. S4B) and thereby delineate the boundary between the stem cell and transit-amplifying compartments in the intestinal crypt. Thus our findings indicate that substrate stabilization by Usp28, with or without functional Fbw7, is crucial for both intestinal homeostasis and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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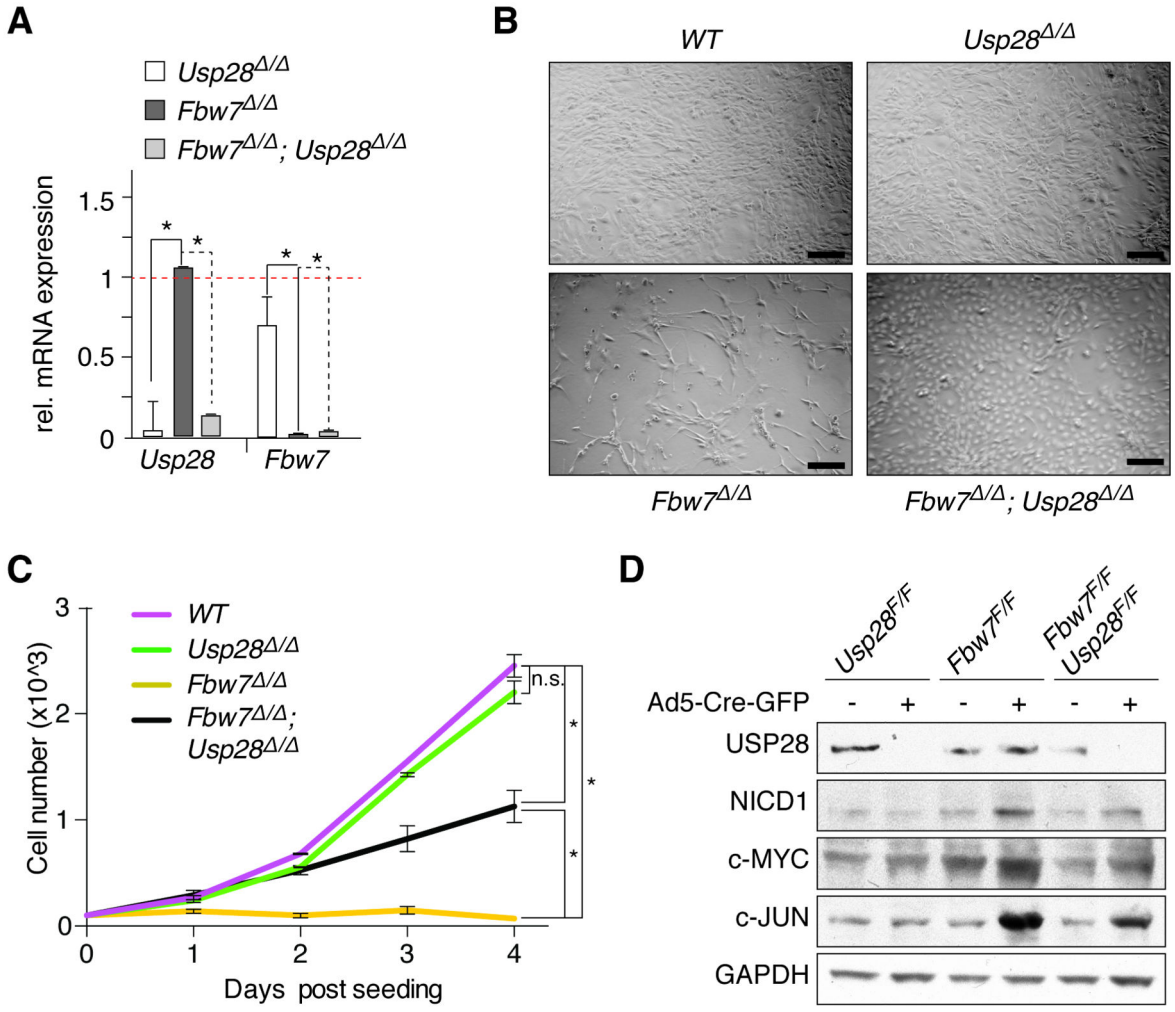

Figure 1. Usp28 loss rescues cell proliferation and substrate protein levels in Fbw7-null cells

(A) qRT-PCR analysis of *Usp28* and *Fbw7* expression in $Usp28^{\Delta/\Delta}$, $Fbw7^{\Delta/\Delta}$, and $Fbw7^{\Delta/\Delta}$; $Usp28^{\Delta/\Delta}$ MEFs. (B-C) Phase-contrast micrographs (B) and growth curves (C) showing proliferation of MEFs of the above genotypes. *p<0.05; n.s., not significant. Scale bar represents 200μm. Error bars indicate SEM. (D) Western blots showing Usp28 substrate levels in MEFs of the indicated genotypes. Data are representative of results from 3 independent MEF isolations.

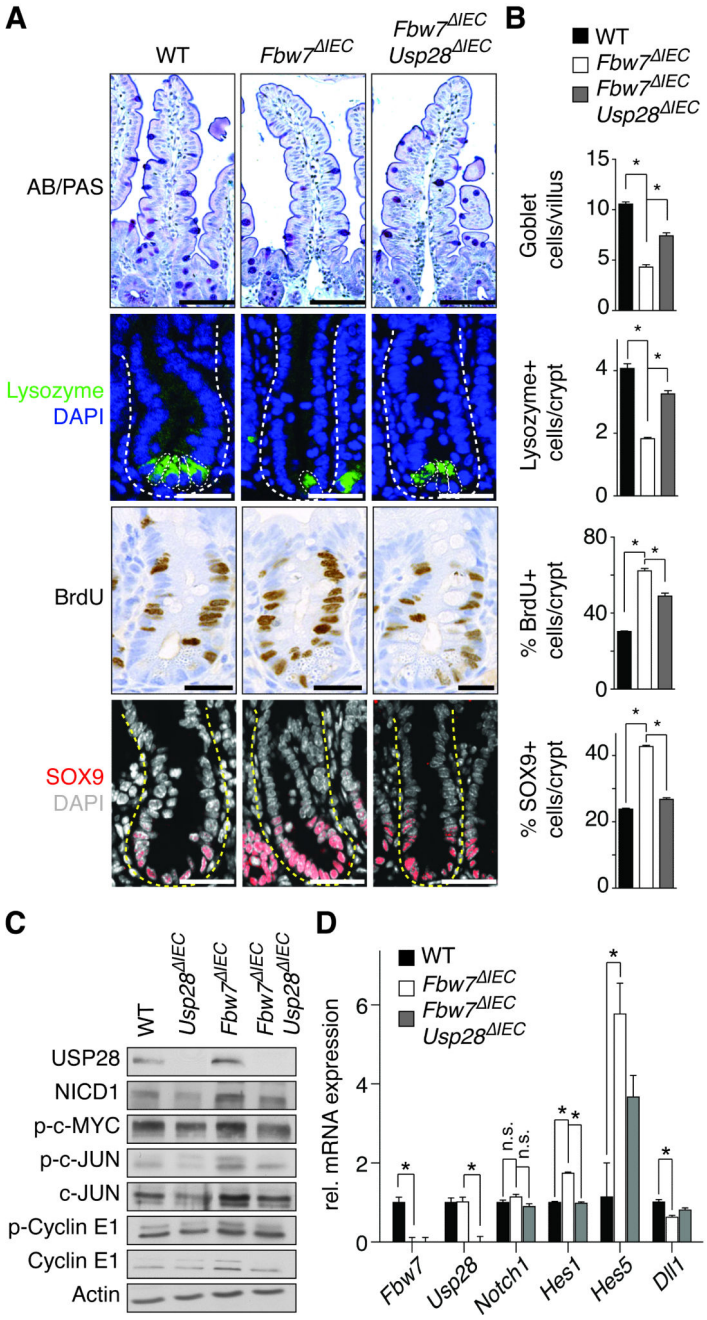

Figure 2. Usp28 deficiency partially reverts hyperproliferation and impaired differentiation in *Fbw7*$^{\Delta IEC}$ gut

(A-B) Representative images (A) of murine wild-type (*Usp28*$^{F/F}$), *Fbw7*$^{\Delta IEC}$ and *Fbw7*$^{\Delta IEC}$*; Usp28*$^{\Delta IEC}$ intestines, stained with AB/PAS (goblet cells), Lysozyme (Paneth cells, outlined), BrdU, and SOX9 (stem/progenitor cells). Scale bars represent 100μm (AB/PAS) or 25μm (lower panels). Stained cells are quantified (B). n = 3 animals/genotype (31 villi or 32 crypts counted per mouse). (C) Western blots analyzing isolated crypt cells from mice of the indicated genotypes. (D) qRT-PCR analysis of *Notch-1*, *Hes1*, *Hes5* and *Dll1* mRNA

isolated from crypt cells of 3 animals/genotype. *$p < 0.05$; n.s., not significant. Error bars in (B) and (D) indicate SEM.

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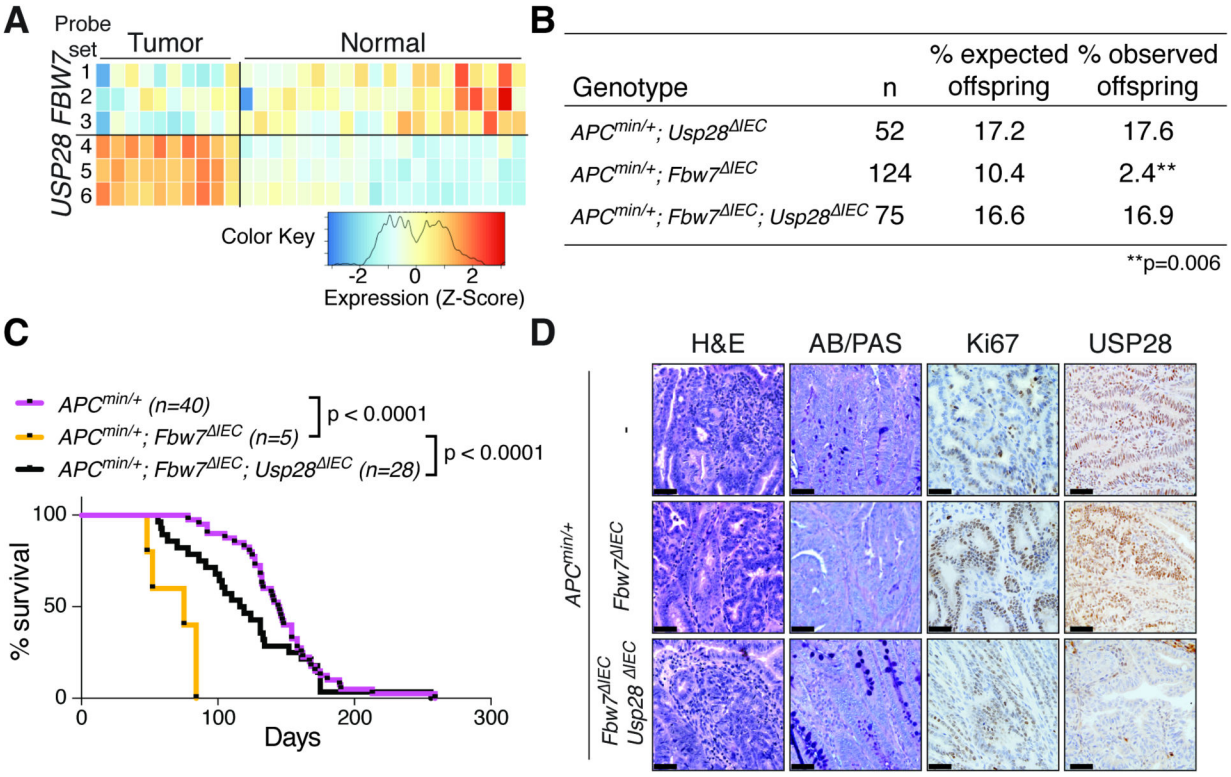

Genotype	n	% expected offspring	% observed offspring
$APC^{min/+}$; $Usp28^{\Delta IEC}$	52	17.2	17.6
$APC^{min/+}$; $Fbw7^{\Delta IEC}$	124	10.4	2.4**
$APC^{min/+}$; $Fbw7^{\Delta IEC}$; $Usp28^{\Delta IEC}$	75	16.6	16.9

**p=0.006

Figure 3. Loss of Usp28 ameliorates tumorigenesis in $APC^{min/+}$; $Fbw7^{\Delta IEC}$ mice

(A) Heat map showing *USP28* and *FBW7* expression in human intestinal samples from tumors and the corresponding normal tissue. Data from GEO. (B) Loss of Usp28 restores Mendelian distribution of $APC^{min/+}$; $Fbw7^{\Delta IEC}$ animals. **indicates significant deviation from expected value (Chi-squared test with Yates' correction). (C) Kaplan-Meier curves showing survival of $APC^{min/+}$, $APC^{min/+}$; $Fbw7^{\Delta IEC}$ and $APC^{min/+}$; $Fbw7^{\Delta IEC}$; $Usp28^{\Delta IEC}$ animals. (D) Immunohistological analysis of intestinal tumors from mice of the indicated genotypes. Scale bar represents 50μm. Data are representative of at least 5 animals/ genotype.

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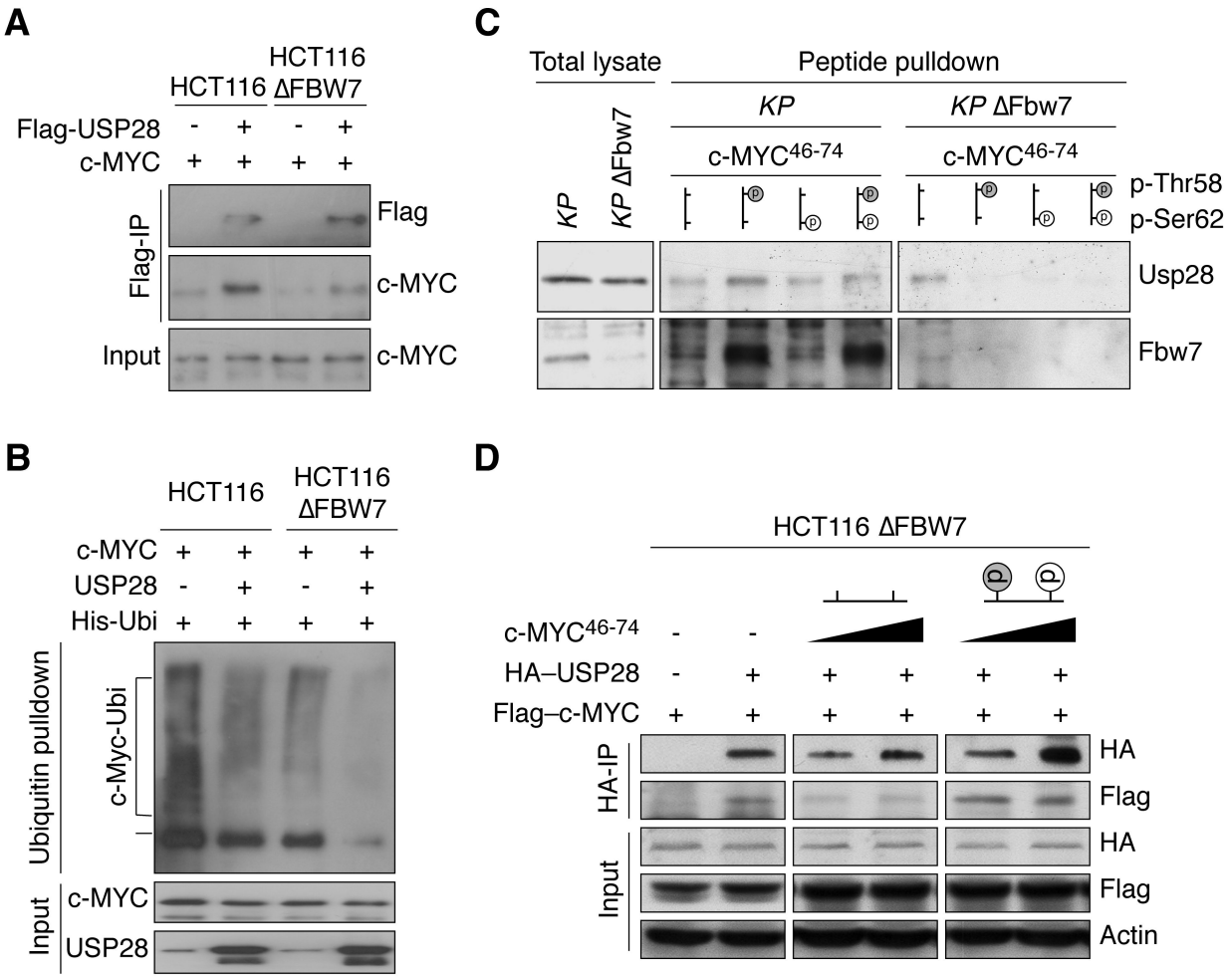

Figure 4. Usp28 binds and deubiquitinates c-Myc in the absence of Fbw7

(A) Co-immunoprecipitation of c-MYC and Flag-USP28 from HCT116 or HCT116ΔFBW7 cells transfected as indicated. (B) Ubiquitin pulldown from HCT116 or HCT116ΔFBW7 cells transfected with c-MYC and His-tagged ubiquitin (His-Ubi) in the absence or presence of USP28. Cells were treated with MG132 for 3hr before lysis. Ubiquitinated complexes were immunoprecipitated using Ni-NTA beads and analyzed by western blotting. (C) Peptide pulldown of Usp28 and Fbw7 from *KRas*^{G12D}*; p53*$^{\Delta/\Delta}$ (*KP*) or *KP Fbw7*$^{\Delta/\Delta}$ cell lines. (D) Co-immunoprecipitation of Flag–c-Myc and HA–Usp28 from HCT116 in the presence of unphosphorylated or p-Thr58/p-Ser62 c-MYC$^{46-74}$ peptide. Experiments were replicated at least 3 times with similar results.