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−/−) ameliorated the hyperproliferation and the impaired goblet and Paneth cell differentiation observed in Fbw7+/−/IEC mice. The aggressive intestinal tumor formation of APCmin/+; Fbw7−/−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. Cancer Res; 75(7); 1181–6. ©2015 AACR.

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 APCmin/+ tumor model (5). Genomic data from human cancers suggest 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 recognizes 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; ref. 7). Usp28 was originally described as part of the DNA damage response, as 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, immunologic development, and radiation responses (13). However, as 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 \( \text{APC}^{\text{min}/-} \) 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: \( \text{Usp}28^{\text{F/F}} \) (14); \( \text{Fbw}7^{\text{F/F}} \) (2); Villin-Cre (15), and the intestinal tumor model \( \text{APC}^{\text{min}/-} \) (16; see Supplementary Materials and 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% \( \text{O}_2/5\% \text{CO}_2 \) / 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 BrdUrd (Sigma) 2.5 hours before sacrifice. Intestinal sections were cut at 4 \( \mu \text{m} \) 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.

**qRT-PCR**

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

**Computational analysis**

Human \( \text{USP28} \) and \( \text{FBW7} \) 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 HCT116/ΔFBW7 cells from B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD; ref. 17). Both were authenticated by short tandem repeat profiling and FBW7 loss was verified by Western blotting. KP and KP \( \text{Fbw}7^{\text{D/D}} \) cell lines were generated from primary murine adult epithelial cells harboring a conditional allele for \( \text{p53}^{\text{F/F}} \), the lox-stop-lox mutant \( \text{KRas}^{G12D} \) allele and in the latter case, a conditional allele for \( \text{Fbw}7 \) (\( \text{Fbw}7^{\text{D/D}} \)). After in vitro recombination by infection with adenovirus encoding Cre recombinase (Vectorcore, University of
Iowa, Iowa City, IA), deletion of p53 and Fbw7 was confirmed by PCR. Cell lines were cultured in DMEM (10% FBS/1% penicillin-streptomycin) at 37°C/5% CO2/95% humidity.

Immunoprecipitation

For immunoprecipitation, cells were lysed 48 hours after transfection and cleared lysates incubated with anti-HA conjugated agrose or Flag-conjugated sepharose for 2 hours rotating at 4°C. For peptide pulldown, biotinylated c-MYC peptides bound to M280 Streptavidin Dynabeads (Invitrogen) were incubated with cell lysates from K Poll and KP Fbw7Δ/Δ cell lines for 2 hours at 4°C. Washed immunoprecipitates were analyzed by Western blotting. See Supplementary Information for details of antibodies, buffer compositions, and peptide sequences.

Ubiquitin pulldown

Twenty-four hours after transfection with the indicated constructs, cells were treated with proteasome inhibitor (MG132, Merck) 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). Usp28Δ/Δ MEFs proliferated normally in culture, whereas Fbw7Δ/Δ MEFs arrested proliferation after the first passage, consistent with previous data (20). However, Fbw7Δ/Δ; Usp28Δ/Δ MEFs were able to proliferate (Fig. 1B and C). Cell cycle–arrested Fbw7Δ/Δ MEFs showed elevated levels of Notch intracellular domain 1 (NICD1), as previously described. In Fbw7Δ/Δ; Usp28Δ/Δ 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 Fbw7Δ/Δ cells, namely c-Myc and c-Jun (Fig. 1D). These data indicate that loss of Usp28 can partially rescue the Fbw7Δ/Δ phenotype.

Usp28 deficiency partially rescues hyperproliferation and impaired differentiation in Fbw7Δ/Δ gut

To test whether Usp28 antagonizes Fbw7 function in vivo, we generated Usp28Δ/Δ; Fbw7Δ/Δ; Villin-Cre (Usp28ΔMEC; Fbw7ΔMEC) double mutant mice. As expected, Usp28ΔMEC; Fbw7ΔMEC animals showed negligible expression of Fbw7 and Usp28 in the intestinal epithelial cells (Supplementary Fig. S1A). Consistent with our previous observations, Fbw7ΔMEC 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 Fbw7ΔMEC cells, reducing BrdUrd incorporation as well as the number of transit-amplifying cells marked by Sox9 in Usp28ΔMEC; Fbw7ΔMEC...
most notably Histologic analysis showed that deletion of Fbw7 was similar (Fig. 3C and Supplementary Fig. S2A and S2B). Strikingly, Usp28 loss in APC\textsuperscript{min/-}; Fbw7\textsuperscript{MIEC} double-mutant mice resulted in an increase in survival to 122 days, although the tumor burden at death was reduced to levels comparable to those observed in Fbw7 null mice (Supplementary Fig. S3). Overexpression of USP28 in FBW7-null cells also rescued this synthetic lethality phenotype (Fig. 3B). Thus, loss of Usp28 reverts the phenotypes observed in Fbw7-deficient intestine.

Loss of Usp28 ameliorates tumorigenesis in APC\textsuperscript{min/-}; Fbw7\textsuperscript{MIEC} mice

Analysis of publicly available expression data from human colorectal cancer samples shows that expression of USP28 is increased in adenomas compared with Fbw7\textsuperscript{MIEC} intestine (Supplementary Fig. S1B). The increased levels of p-c-Myc, c-Jun, Cyclin E1, and NICD1 were reduced to levels comparable with wild-type in Usp28\textsuperscript{IEC}; Fbw7\textsuperscript{MIEC} double-mutant mice (Fig. 2C). Moreover, the altered expression of Notch-1 target genes, most notably Hes1, in Fbw7\textsuperscript{MIEC} intestine was rescued (Fig. 2D). Thus, loss of Usp28 reverts the phenotypes observed in Fbw7-deficient intestine.

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 (HCT116\textsuperscript{ΔFbw7}; ref. 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 coimmunoprecipitate c-MYC from FBW7-null cells, we tested whether Usp28 bound to the unphosphorylated c-Myc peptide. The interaction of c-MYC with USP28 in FBW7-null cells was reduced compared with wild-type cells (Fig. 4A). We confirmed this observation by coimmunoprecipitation of c-Myc with endogenous Usp28 from mutant Fbw7-null cells (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 (Fig. 4C; ref. 11). 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, whereas
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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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