Type I Interferons Control Proliferation and Function of the Intestinal Epithelium

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Wnt pathway-driven proliferation and renewal of the intestinal epithelium must be tightly controlled to prevent development of cancer and barrier dysfunction. Although type I interferons (IFN) produced in the gut under the influence of microbiota are known for their antiproliferative effects, the role of these cytokines in regulating intestinal epithelial cell renewal is largely unknown. Here we report a novel role for IFN in the context of intestinal knockout of casein kinase 1α (CK1α), which controls the ubiquitination and degradation of both β-catenin and the IFNAR1 chain of the IFN receptor. Ablation of CK1α leads to the activation of both β-catenin and IFN pathways and prevents the unlimited proliferation of intestinal epithelial cells despite constitutive β-catenin activity. IFN signaling contributes to the activation of the p53 pathway and the appearance of apoptotic and senescence markers in the CK1α-deficient gut. Concurrent genetic ablation of CK1α and IFNAR1 leads to intestinal hyperplasia, robust attenuation of apoptosis, and rapid and lethal loss of barrier function. These data indicate that IFN play an important role in controlling the proliferation and function of the intestinal epithelium in the context of β-catenin activation.

Indeed, high levels of cell surface IFNAR1 are specifically required for the antiproliferative effects of IFN as opposed to their ability to elicit an antiviral state (14, 19). These levels of IFNAR1 are tightly regulated by its phosphorylation-dependent ubiquitination and subsequent endocytosis and lysosomal degradation (20–22). The rate-limiting event in these processes is the phosphorylation of serine residues within the IFNAR1 degron that enables the recruitment of beta-transducin repeat containing protein (βTrcp) E3 ubiquitin ligase and IFNAR1 ubiquitination (21, 23). Importantly, while this phosphorylation can be induced by IFN via the activation of protein kinase D2 (24, 25), there is also a ligand-independent pathway that removes IFNAR1 from the surface of cells that are yet to encounter IFN (23, 26). This pathway was shown to be activated by inflammatory cytokines (27) and may contribute to the lack of phenotypic differences between wild-type and Ifnar1 knockout mice under inflammatory conditions (28). We previously purified and characterized casein kinase 1α (CK1α) as a major ligand-independent kinase that is capable of phosphorylating IFNAR1 in vitro (29).

Importantly, CK1α is also a critical mediator of β-catenin ubiquitination and degradation (2). Priming phosphorylation of β-catenin by CK1α greatly increases the phosphorylation of the β-catenin degron by glycogen synthase kinase 3β (GSK3β) (30,
that is required for its recognition by the βTrcp E3 ubiquitin ligases and subsequent ubiquitination and proteasomal degradation (32–36). Our recent studies demonstrated that gut-specific knockout of the Csk1α1 gene that encodes CK1α leads to robust stabilization of β-catenin and activation of Wnt target genes (37, 38). Intriguingly, ablation of Csk1α1 alone did not lead to either epithelial cell hyperproliferation or tumorigenesis. Instead, inactivation of CK1α induced the DNA damage response (DDR) and p53/p21-dependent senescence. These events appear to prevent tumorigenesis driven by hyperactive β-catenin because the concurrent ablation of CK1α with either p53 or p21 resulted in hyperproliferation and a rapid development of aggressive and invasive intestinal tumors (37, 38).

Here we determined the role of CK1α in the regulation of IFNAR1 ubiquitination and levels in vivo. We found that despite the accumulation of IFNAR1 protein (but not mRNA), the ubiquitination of IFNAR1 was decreased in CK1α-deficient intestinal tissues. In addition, the expression of IFN-stimulated genes was increased in the gut upon Csk1α1 ablation. As the lack of CK1α stabilized both β-catenin and IFNAR1, the phenotype associated with the loss of Csk1α1 highlighted the contribution of IFN signaling to control IEC proliferation and function. Intriguingly, IFN signaling was required for the effective activation of p53 and p21 and induction of senescence and apoptosis in the CK1α-deficient intestinal epithelium. Furthermore, the concurrent ablation of CK1α with Ifnar1 led to unrestricted IEC proliferation to an extent that caused profound aberrations of gut barrier function and rapid animal death. These results demonstrate that IFN play an important role in restricting intestinal epithelial cell proliferation elicited by the activated β-catenin pathway.

MATERIALS AND METHODS

Animals. All experiments with animals were carried out under protocol 803995 approved by the IACUC of the University of Pennsylvania. All mice were on the C57BL/6 background, had water ad libitum, and were fed regular chow. Ifnar1−/− mice (a kind gift of Dong-er Zhang, UCSF) were crossed with Vil1-Cre-ERT2 (37), to generate Ifnar1−/−/Vil1Cre-ERT2 mice, which bear floxed Csk1α1 and Ifnar1. Genotyping was performed with the following PCR primers (37–39).

- Forward primers: 
  - 5’-GCCAACTTCATTTG
  - 5’-GCCACGCTG

- Reverse primers: 
  - 5’-TCCACACGGT
  - 5’-TCTTCACGCT

Here we used previously described primers (37).

- For antibiotic treatment, Csk1α1Cre-lox mice were intraperitoneally (120 mg/kg of body weight) on two consecutive days. On day 5 after the last injection, mice were euthanized. The jejunum, the ileum, and the entire large intestine were flushed with ice-cold phosphate-buffered saline (PBS); cut open longitudinally; and subjected to fixation in 4% formaldehyde and paraffin embedding. Small pieces of the jejunum were embedded in Tissue-Tek OCT compound (Sakura) and frozen at −80°C. IECs were isolated from the middle part of the small intestine as described previously (39) but with the following slight modifications: intestinal cells were separated into single cells and Hanks’ balanced salt solution containing 5 mM EDTA at 4°C for 30 min. For antibiotic treatment, Csk1α1Cre-lox; Ifnar1−/− mice were gavaged with 100 mg streptomycin (Sigma), and the drinking water was immediately replaced with filter-sterilized water containing ampicillin (1 g/liter; American Bioanalytical), vancomycin (0.5 g/liter; MP Biomedical), neomycin (1 g/liter; Sigma), metronidazole (1 g/liter; Sigma), and 1% sucrose (Fisher). Antibiotic-containing water was replaced at least once a week during the course of the experiment. For all experimental groups, either mice were housed or their feces were swapped daily between cages to minimize potential differences in the gut microbiota.

Histology and immunotechniques. Sections (5 μm) were cut for hematoxylin and eosin (H&E) staining and immunohistochemistry analysis. For immunohistochemistry, sections were incubated with antibodies to detect CK1α (C-19 [1:1,000]; Santa Cruz Biotechnology), β-catenin (1:200; Cell Signaling), cyclin D1 (SP4 [1:100]; Thermo Scientific), Ki67 (2.5 mg/ml; BioLegend), cleaved caspase-3 (1:100; Cell Signaling Technology), and p53 (1:500; NovoCasta). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-goat, and anti-rat antibodies (Millipore, Cell Signaling Technology). 3’,5’-Diaminobenzidine (DAB) chromogen (Lab Vision) was used for detection. For whole-tissue immunofluorescence, pieces of small intestine harvested from mice were frozen in Tissue-Tek OCT compound, cryosectioned by using Leica CM3050 S cryostat, fixed in acetone, washed, and blocked with PBS containing 5% goat serum. The sections were incubated for 1 h with primary antibodies to detect IFNAR1 (2 μg/ml; Sino Biological), γH2AX (1:100; Millipore), E-cadherin (1:500; Millipore), or TJP1/ZO-1 (1:100; Thermo Fisher). The sections were then washed, incubated with the corresponding secondary antibodies labeled with Alexa Fluor 488 or 594 (Invitrogen) for 1 h, washed again, and mounted onto coverslips by using mounting solution with 4,6-diamino-2-phenylindole (DAPI) (Prolong Gold). For se-nescence-associated β-galactosidase (SA-βGal) staining (described in detail in reference 40), 10-μm sections were cut from OCT-embedded frozen tissue and allowed to adhere to coated slides at 25°C for 1 min before fixation for 15 min. Staining was performed according to the in-struction provided with the senescence β-galactosidase staining kit (cat-log number 9860S; Cell Signaling). After staining, sections were counter-stained with nuclear fast red, dehydrated, and mounted. Periodic acid-Schiff (PAS) staining for goblet cell determination was performed according to standard protocols. Numbers of γH2AX-, IFNAR1-, and Ki67-positive foci per crypt/villus axis were determined by counting foci in 40 or 20 low-power fields (magnification of ×200 or ×400). The number of positive cells per crypt (Ki67, cleaved caspase-3, and SA-βGal) was determined by counting foci in 20 low-power fields (magnification of ×200).

Western blotting. Proteins were extracted from intestinal epithelial cell pellets in protein lysis buffer containing protease and phosphatase inhibitors according to whole-cell extract protocols. IFNAR1 was immuno-noprecipitated from whole-cell lysates by using MAR1-5A3 (Leinco Technolo-gies, Inc.), as previously described (28). Membranes were incubated with antibodies to detect IFNAR1 (2 μg/ml; Sino Biological), ubiquitin (Ub) (P0D1 [1:1,000]; Santa Cruz Biotechnology), Csk1α (C-19 [1:1,000]; Santa Cruz Biotechnology), p21CIP1/WAF1 (ab7960 [1:1,000]; Abcam), interferon regulatory factor 7 (IRF7) (ab62805 [1:1,000]; Abcam), ZO-1 (1:100; Thermo Fisher), and β-actin (AC-74 [1:5,000]; Sigma). Secondary antibodies conjugated to horseradish peroxidase were purchased from Millipore Bioscience Research Reagents. Blots were processed as previously described (23) and developed by using ECL (GE Healthcare).

RNA analysis. Total RNA was extracted from cell pellets by using TRizol reagent and phenol-chloroform methods. RNA (1 μg) was subjected to reverse transcription using a first-strand cDNA synthesis kit (Thermo Scientific), and mRNA expression levels were measured by quantitative real-time PCR using an Applied Biosystems 7500 Fast real-time PCR system. Relative quantities of gene transcripts were normalized to β-actin transcript levels. Sequences of PCR primers are as follows: 5’-TAGGCCGAATGAAGTGGAC (forward primer for Axin2), 5’-CTGG TACCCCAAACAGGAGT (reverse primer for Axin2), 5’-CAGTACTCTCCGGAGTACGG (forward primer for Cdh4), 5’-GCAACTTTAGTGGTCCAT (reverse primer for Cdh4), 5’-GGTGCGGGAAGGACATG (reverse primer for Csk1α1), 5’-TGTAACCAGACTCCTCGCC (reverse primer for Csk1α1), 5’-GGTGCGGGAAGGACATG (forward primer for Csk1α1), 5’-TTCCAAGTCCCTCGCC (reverse primer for Csk1α1), 5’-TTGACTGTCGGAAGTCTGTTG (reverse primer for cyclin D1), 5’-CACCTAGTGAGGTGGCAAGCA (reverse primer for cyclin D1), 5’-CTGGCTGGCTGGCTTATAG (forward primer for Ifitm3), 5’-GCTGCTGGTGGCTGATA (reverse primer for Ifitm3), 5’-TTCCAAGGATGATGATGAGCA (forward primer for Cdkn1a), 5’-AGA CAACGGACACTTCTG (reverse primer for Cdkn1a), 5’-GGAGCTC
AGCAAGACTCTGG (forward primer for Sox9), 5'-TGTAACTGGGAGGTCTCTTCT (reverse primer for Sox9), 5'-GCCCTAATCGTCGAGAAGAAAG (forward primer for Ascl2), 5’-CACCTAGTTGGAAATCCTAAG (reverse primer for Ascl2), 5’-CCCTGTGAGGAAATGAGCTTA (forward primer for Oas2), 5’-CCCTGTGAGGAAATGAGCTTA (reverse primer for Oas2), 5’-CTTGTTGGAAGACCCTACGTTACA (reverse primer for Ifnb1), 5’-ACAGCATCTGCTGGTTGAAG (reverse primer for Ifnb1), 5’-GACCATGATGGTGGAAATCCTAAG (forward primer for Ifnb1), 5’-GAGGAAGATGATGGTGGAAATCCTAAG (reverse primer for Ifnb1).

Ifnar1

- TCAAA (reverse primer for Ifnar1), 5’-ATCTACCATTACCAAGAACAGAAG (forward primer for Ifnar1), 5’-ACCCCAACAAAGGACAAGGAAATCCTAAG (forward primer for Tp1), 5’-ATATACGCGCCGCTAGAC (reverse primer for Tp1), 5’-GGCACAACACCAAGAATGCTCTGCT (forward primer for Tp53), 5’-GGCACAACACCAAGAATGCTCTGCT (reverse primer for Tp53), 5’-ATATACGCGCCGCTAGAC (forward primer for Bak1), 5’-AGGCCATCTTGTTGGAAG (reverse primer for Bak1), 5’-ATGCTCTGGATCTGGGCTGCTCCT (forward primer for Fas), 5’-GACAATTTGCTGCTGGTTGAAG (reverse primer for Fas).

Ifnar1

- CCTCAAGTTTTGCCCTTTA (forward primer for Fas), 5’-GACAATTTGCTGCTGGTTGAAG (reverse primer for Fas), 5’-CCTCAAGTTTTGCCCTTTA (forward primer for Casp1), 5’-CTCTCTTAATGGTTCTTGTCCATG (reverse primer for Casp1), 5’-AGAGGAAATCCTAGGGTCGCAG (forward primer for β-actin), and 5’-CTCTCTTAATGGTTCTTGTCCATG (reverse primer for β-actin).

**Isolation and culture of intestinal crypts.** Cultured crypts were performed as previously described (41). After intestinal crypt isolation, a total of 500 crypts were mixed with 50 μl of Matrigel (BD Biosciences) and plated into 24-well plates. After polymerization of Matrigel, 500 crypts were mixed with 50 μl of Matrigel (BD Biosciences) and plated into 24-well plates. After polymerization of Matrigel, 500 crypts were mixed with 50 μl of Matrigel (BD Biosciences) and plated into 24-well plates.

**Microarray.** Microarray analyses were performed with an Illumina whole-genome array. Total RNA was isolated from intestinal epithelial cells of Csnk1a1+/−, Ifnar1+/− (single knockout [SKO]) and Csnk1a1−/−, Ifnar1−/− (double knockout [DKO]) mouse cells at day 5 after the last tamoxifen treatment by using a miRNAeasy minikit (Qiagen). The RNA was labeled with Cy3 using a Target-Amp-Nano labeling kit (Epigen) as recommended by the manufacturer. Thereafter, 75 μg of RNA was hybridized to Illumina Sentrix Mouse-6 v1 BeadChips, which were scanned with an Illumina BeadStation 500 instrument (both from Applied Biosystems-Life Technologies, Inc.). Data were collected with Illumina BeadStudio 3.1.1.0 software, and statistical analyses were conducted with IlluminaGUI-R-package 15.70.

**Intestinal permeability assay.** Barrier function was evaluated by measuring in vivo paracellular permeability to fluorescein-labeled dextran. Mice were fasted for 4.5 h and then gavage fed fluorescein isothiocyanate (FITC)-labeled 4.4-kDa dextran (FD4, Sigma). Plasma was obtained 5 h after gavage administration by terminal cardiac puncture after CO2 anesthesia. The plasma FD4 concentration was calculated by comparing samples with serial dilutions of known standards by using a Varispec 900 Fluorimeter (Thermo Scientific) with excitation at 485 nm and emission at 530 nm.

**Statistical analyses.** Data are presented as averages ± standard errors of the means (SEM). Statistical analysis was performed by using SigmaStat (Systat Software). Statistical significance was calculated by using a two-tailed Student t test. A P value of <0.05 was considered significant.

**Microarray data accession number.** Raw data were deposited in the GEO database under accession number GSE76512.

**RESULTS**

**Activation of IFN signaling in response to CK1α ablation.** To determine the role of CK1α in the regulation of IFNAR1 levels and signaling in the intestinal epithelium, we crossed Csnk1a1−/− mice with animals expressing Vill-Cre-ER22 (37). Both strains harbored a double wild-type allele for Ifnar1 (Ifnar1+/−). Treatment of the progeny from these crosses with tamoxifen resulted in mice lacking CK1α exclusively in the intestinal epithelium (Csnk1a1−/−, Ifnar1+/− [SKO]). The decrease in CK1α expression in the intestinal epithelium was monitored by assessing the levels of Csnk1α mRNA (Fig. 1A) and protein (Fig. 1B and C). We next crossed these mice to mice lacking Ifnar1 and observed a similar extent of CK1α ablation in these DKO mice (Fig. 1A to C).

CK1α can phosphorylate IFNAR1 within its degron in vitro (29). Phosphorylation of this degron enables ubiquitination of IFNAR1 (21, 22). Here we aimed to determine the role of CK1α in the regulation of IFNAR1 ubiquitination and levels in the intestinal tissues. We detected IFNAR1 protein and its ubiquitination in intestinal epithelial tissue lysates from Csnk1a1−/−, Ifnar1−/− mice (Fig. 1C). Importantly, deletion of Csnk1a1 resulted in decreased IFNAR1 ubiquitination concurrent with a dramatic increase in the overall level of IFNAR1 protein (Fig. 1C and D). Given that IFnar1 mRNA levels were not affected by the ablation of Csnk1α (Fig. 1A), these data are consistent with the hypothesis that CK1α is a major regulator of IFNAR1 ubiquitination and proteolytic degradation in vivo.

Importantly, the IFN-inducible IRF7 protein was upregulated in CK1α-deficient mice (Fig. 1C). Furthermore, CK1α ablation induced the mRNA levels of Ifib1 along with a number of other IFN-stimulated genes (Fig. 1E and F). Induction of mRNA of the ligand IFN-β and stimulation of the transcription factor IFnar1 by IFN-β resulted in increased IFNAR1 expression (44). Thus, we sought to investigate the role of IFN signaling in the DDR by comparing the consequences of CK1α ablation in Ifnar1+/− and Ifnar1−/− mice.

Regardless of the status of Ifnar1, we observed that CK1α deletion leads to a robust accumulation of β-catenin (Fig. 2A). Consistent with this result, similar levels of induction of cyclin D1 protein was observed in Csnk1a1−/−; Ifnar1−/− and Csnk1a1−/−; Ifnar1−/− mice (Fig. 2B). Furthermore, animals of both genotypes exhibited comparable increases in the levels of several Wnt target genes, including Sox9, Cnd1, Axin2, Ascl2, and Cdh4 (Fig. 2C). These results indicate that IFN signaling is dispensable for β-catenin stabilization and transcriptional activation in the absence of CK1α.

Intriguingly, the concurrent ablation of CK1α and Ifnar1 noticeably decreased the extent of DDR signaling as seen from the number of phosphorylated histone H2AX foci in CK1α-deficient tissues (Fig. 3A and B). Our previously reported results showed that IFN-β-induced response to DNA damage can further augment the extent of this damage. Furthermore, DDR-stimulated IFN was shown to play an important role in the induction of the p53 tumor suppressor protein transcriptional target gene Cdkn1a, which encodes the p21Cip1/Waf1 protein (43). The accumulation
of p53 protein seen upon the deletion of Csnk1a1 was not affected by the status of Ifnar1 (Fig. 3C). However, inactivation of Ifnar1 led to noticeable decreases in the levels of p21 CIP1/WAF1 protein (Fig. 1C) and mRNA (Fig. 3D) in CK1α-deficient tissues. Given that Ifnar1 ablation also attenuated the induction of other p53-dependent genes (Fig. 3D and E), these results strongly suggest an important role for IFN signaling in the activation of DDR and p53 activities in CK1α-deficient intestinal tissues.

Endogenous IFN restrict proliferation of intestinal epithelial cells and elicit their apoptosis. We previously postulated that Csnk1a1 ablation simultaneously elicits the proproliferative activity of canonical Wnt pathway target genes through β-catenin stabilization along with another yet-to-be-understood antiproliferative pathway that activates p53-p21, restricts IEC proliferation, and prevents tumorigenesis in spite of constitutive β-catenin activity (37). We therefore investigated the role of IFN in inhibiting...
epithelial cell proliferation. Intriguingly, immunofluorescence staining demonstrates the mutual exclusion of the IFNAR1 protein with the marker of cell replication Ki67 in normal intestine (Fig. 4A and B). Whereas Ki67 staining in Ifnar1−/− mice was elevated upon Csklk1a ablation, a further significant increase was seen in DKO mice (Fig. 4C and D). Consistent with this finding, the number of senescence-associated β-galactosidase (SA-β-gal)-positive cells was also dramatically lower in intestinal tissues of Csklk1a−/−, Ifnar1−/− double-knockout mice (Fig. 4E and F). Finally, gene set enrichment analysis (GSEA) revealed a significant increase in the expression of genes associated with cell proliferation in Csklk1a−/−, Ifnar1−/− double-knockout mice versus mice with the Csklk1a−/− knockouot alone (Fig. 4G). Collectively, these results indicate that IFN signaling plays an important role in restricting IEC proliferation within the context of β-catenin stabilization caused by the inactivation of CK1δ.

Persistent activation of the Wnt pathway by stabilized β-catenin was shown to increase the number of apoptotic cells in the intestine (37, 45). Importantly, the concurrent deletion of Ifnar1 and Csklk1a decreased the enrichment of the apoptosis-associated gene signature (Fig. 4H) and decreased the number of cleaved caspase-3-positive cells (Fig. 4I and J). Taken together with the lower level of induction of p53-dependent proapoptotic genes such as Casp1, Fas, and Bak1 (Fig. 3D), these results implicate IFN signaling in apoptosis that occurs upon constitutive β-catenin stabilization.

FIG 3 Inhibition of IFN signaling suppresses DNA damage responses in CK1δ-deficient mouse intestines. (A) Immunofluorescence analysis of γH2AX expression in intestinal tissues from mice of the indicated genotypes. Bar, 100 μm. (B) Quantitation of the number of γH2AX-positive foci per basal crypt in small intestines of three mice (30 to 50 crypts/villi were analyzed for each mouse). Data are shown as averages ± SEM; asterisks above Csklk1aSKO (SKO) indicate a significant difference between wild-type (WT) and SKO mice, and asterisks above Csklk1aSKO, Ifnar1−/− (DKO) indicate a significant difference between SKO and DKO mice. *, P < 0.05; ***, P < 0.001. (C) Immunohistochemical analysis of p53 levels in intestinal tissues from mice of the indicated genotypes. Magnification, ×10. (D) Relative mRNA levels of the indicated p53 target genes in IECs from mice of the indicated genotypes as assessed by quantitative PCR (levels in untreated Csklk1aSKO, Ifnar1−/− mice are taken as 1.0). (E) GSEA of the transcriptome profiles showing a significant enrichment of p53 target gene signatures in Csklk1aSKO (SKO) versus Csklk1aSKO, Ifnar1−/− (DKO) IECs at day 5 after CK1δ ablation. NES, normalized enrichment score; FDR, false discovery rate.
FIG 4 Endogenous IFN restrict proliferation of intestinal epithelial cells and elicit their apoptosis. (A) Double-immunofluorescence analysis of IFNAR1 and Ki67 expression in intestinal tissues from mice of the indicated genotypes. Bar, 100 μm. (B) Quantitation of the number of IFNAR1-positive, Ki67-positive, or double-positive cells per crypt/villus axis in the small intestines of three mice (30 to 50 crypts/villi were analyzed for each mouse). Data are shown as averages ± SEM; asterisks above Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (SKO) indicate a significant difference between wild-type (WT) and SKO mice, and asterisks above Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (DKO) indicate a significant difference between SKO and DKO mice. ***, P < 0.001. (C) Immunohistochemistry analysis of Ki67 levels in intestinal tissues from mice of the indicated genotypes. Bars, 100 μm. (D) Quantitation of the number of Ki67-positive cells per basal crypt in the small intestines of three mice (30 to 50 crypts were analyzed for each mouse). ***, P < 0.001 for differences between SKO and WT and between SKO and DKO mice. (E) Analysis of SA-βGal-positive cells in intestinal tissues (counterstained with nuclear fast red) from mice of the indicated genotypes. (F) Quantitation of the number of SA-βGal-positive cells per basal crypt in the small intestines of three mice (30 to 50 crypts were analyzed for each mouse). (G) GSEA of transcriptome profiles showing a significant enrichment of proliferation-associated gene signatures in Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (DKO) versus Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (SKO) intestinal epithelial cells at day 5 after CK1α ablation. NES, normalized enrichment score; FDR, false discovery rate. (H) GSEA of transcriptome profiles showing a significant enrichment of apoptosis-associated gene signatures in Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (DKO) versus Csk1a1<sup>+/+</sup>; Ifnar1<sup>−/−</sup> (DKO) IECs at day 5 after CK1α ablation. (I) Immunohistochemistry analysis of cleaved caspase-3 levels in intestinal tissues from mice of the indicated genotypes. (J) Quantitation of the numbers of cleaved caspase-3-positive cells per basal crypt in the small intestines of three mice (30 to 50 crypts were analyzed for each mouse).
Altering CK1α and IFNAR1 levels can affect intestinal cell growth and survival directly or/and indirectly. To assess the direct contribution of these regulators, we assessed the proliferation and viability of IECs grown in wild-type and Ifnar1−/− intestinal organoids treated with 10 μM the CK1α inhibitor D4476 at day 4. (B) Quantitation of live organoids in wild-type and Ifnar1−/− cultures compared with their vehicle-treated counterparts at day 4 (at least 10 random fields were analyzed for each culture). Data are shown as averages ± SEM. *, P < 0.05; **, P < 0.001. (C) Representative bright-field images of WT organoids treated with 10 μM the CK1α inhibitor D4476 in the presence or absence of 10 μg/ml IFN-β neutralizing antibodies at day 4. (D) Quantitation of live organoids in WT cultures compared with vehicle- and IgG-treated counterparts at day 4 (at least 10 random fields were analyzed for each culture).

Furthermore, Csnka1α KO; Ifnar1−/− mice rapidly became moribund within a week after tamoxifen treatment was completed (Fig. 7A). Their life span could be extended by intraperitoneal injections of buffered saline solution, suggesting that an imbalance of water and electrolytes due to a differentiation block and potential loss of barrier function contributed to this early lethality. To test barrier function, we administered fluorescent dextran by gavage into the gastrointestinal tract of Csnka1α/Ifnar1−/− double-knockout mice exhibited markedly increased translocation of dextran into the bloodstream, consistent with compromised barrier function (Fig. 7B). While the levels of E-cadherin in Csnka1α KO; Ifnar1−/− mice were not affected by the status of Ifnar1, the double-knockout mice displayed decreased levels of the ZO-1 tight junction protein (Fig. 1C and 7C) and its mRNA (Fig. 7D). These results implicate the IFN pathway in regulating the barrier function of the gut.

Importantly, Csnka1α/Ifnar1−/− mice eventually became moribund despite the administration of buffered saline solution (Fig. 7A), suggesting additional mechanisms by which the IFN pathway contributes to intestinal homeostasis. A number of studies suggested an important role of microbiota in IFN induction and effects of IFN on innate immunity responses to commensal bacteria (reviewed in reference 47). Consistent with this role of IFN, the stromal elements of the gut in Csnka1α KO; Ifnar1−/− mice displayed signs of injury and leukocyte infiltration and contained enlarged and inflamed lymph nodes. A robust inflammatory response was seen near the basement membrane, including leukocyte infiltration, combined with mucosal injury and increased epithelium permeability (Fig. 7E). Observed alterations were indicative of an increased microbiota-induced ability to penetrate the barrier and induce inflammation. Indeed, treatment of Csnka1α/Ifnar1−/− mice with antibiotics efficiently prevented their death (Fig. 7A). These data collectively suggest that IFN signaling plays an important role in maintaining intestinal barrier functions and homeostasis of the host interaction with the microbiota.

DISCUSSION

Our data presented here demonstrate that induction of IFN signaling appears to contribute to the activation of the DNA damage responses and apoptotic pathways as well as the suppression of intestinal epithelial proliferation that occurs upon the inactivation of CK1α. The latter event stabilizes both β-catenin and IFNAR1, thereby highlighting the conditions that determine the role of IFN signaling in restricting IEC proliferation. In addition, IFN contributes to the vitally important function of maintenance of intestinal barrier function.

CK1α is capable of phosphorylating numerous proteins and affecting a multitude of signaling pathways and transcriptional activities toward specific genes (reviewed in reference 48). Whereas CK1α was capable of phosphorylating the IFNAR1 degron in vitro (29), the role of other kinases in stimulating the recruitment of βTrcp to IFNAR1 and promoting its ubiquitination, endocytosis, and degradation was also demonstrated (24, 25). Our current data clearly characterize CK1α as a major regulator of IFNAR1 ubiquitination and stability in vivo (Fig. 1). Furthermore, these results implicate this kinase in the negative regulation of the IFN pathway in intestinal tissues and underscore the importance of CK1α function for the proliferation of the intestinal epithelium and its permeability.
Furthermore, our data suggest that the role of CK1 in regulating intestinal homeostasis is at least in part mediated by its effects on the stability and levels of IFNAR1 and the ensuing alterations in IFN signaling. Although microbiota-supported constitutive tonic IFN signaling has been described in the gut (11, 12), the role of this signaling in regulating gut renewal and function was a challenge to evaluate due to the potential phenotypic similarity of mice lacking the Ifnar1 gene and wild-type mice exhibiting rapid IFNAR1 degradation under inflammatory conditions (28). The concurrent stabilization of IFNAR1 and $\beta$-catenin upon CK1$\alpha$ inactivation enabled us to determine that IFN plays an important role in restricting the proliferation and viability of the intestinal epithelium (Fig. 4 and 5) and contributes to the maintenance of the equilibrium of the host-microbiota interaction and barrier function (Fig. 7).

Our data further indicate that the IFN pathway contributes to the expression of p53-driven genes (including proapoptotic genes and $Cdkn1a$) stimulated in the absence of CK1$\alpha$ (Fig. 3). These data are consistent with our recently reported data demonstrating that IFN can amplify DNA damage responses (43) as well as with data from previous reports that exogenous IFN can trigger an increase in p53 activities (44, 49). Importantly, these effects of IFN may provide an additional mechanism for the activation of the p53 pathway in the CK1$\alpha$-deficient gut in addition to the previously reported downregulation of MDMX and the ensuing stabilization of the p53 protein (37).

Intriguingly, activation of the p53 pathway was not observed in the Apc-deficient gut (37, 46). Given that the ablation of either CK1$\alpha$ or APC results in the stabilization of $\beta$-catenin and stimulation of the Wnt pathway, the difference between these pheno-
types and underlying gene profile signatures can be explained by at least two diverse reasons. First, APC possesses important functions that do not depend on the timely degradation of β-catenin. For example, APC can bind RNA and regulate the microtubule scaffold (50). Furthermore, the loss of Apc leads to the upregulation of Musashi proteins, which are pleiotropic translational regulators that affect numerous important signaling cascades, including the mTorc1 pathway (51,52). Second, characteristic for the deletion of Cnsk1a (but not Apc) is the induction of the DNA damage response, activation of the p53-driven genes, and cell senescence (46) and is characterized by a similar gene expression signature (Fig. 6).

The restriction of proliferation of Csk1al-deficient IECs could be lifted by the concurrent inactivation of either p53, p21Cip1/Waf1 (37, 38), or, to a lesser extent, IFNAR1 (this work). Importantly, ablation of either p53 or p21Cip1/Waf1 in CK1α-deficient animals led to the development of malignant tumors (37, 38). However, we did not observe these tumors in any groups of CK1α/IFNAR1-deficient mice that developed a lethal disruption of intestinal barrier function (Fig. 7), suggesting a contribution of IFN-independent pathways to p53 function as a tumor suppressor in CK1α-null intestines.

A few overlapping and non-mutually exclusive mechanisms by

FIG 7 CK1α and IFN pathways regulate intestinal barrier function. (A) Kaplan-Meier survival analysis of mice of the indicated genotypes after induction of intestinal CK1α ablation. Csk1al+/–; Ifnar1–/– mice received intraperitoneal injections of 1 ml saline every day starting on day 1 after intestinal CK1α ablation (DKO+Saline) or were administered antibiotics (Ab) in drinking water. ***, P < 0.001. Asterisks next to the DKO line indicate comparisons between SKO and DKO mice, asterisks next to the DKO+Saline indicate comparisons between DKO and DKO+Saline mice, and asterisks next to the DKO+Ab line indicate comparisons between DKO and DKO+Ab mice. Neither wild-type nor Ifnar1–/– mice died during the course of the experiment. (B) Levels of FITC-dextran (FD4) in plasma samples from mice of the indicated genotypes 5 h after FD4 gavage (n = 3 for each group). Data are shown as averages ± SEM; an asterisk above Csk1al+/– (SKO) indicates a significant difference between wild-type (WT) and SKO mice, and an asterisk above Csk1al+/–; Ifnar1–/– (DKO) indicates a significant difference between SKO and DKO mice. *, P < 0.05. (C) Immunofluorescence analysis of E-cadherin (top) and tight junction protein 1 (TJP1) (zona occludens 1 [ZO-1]) (bottom) expression in intestinal tissues from mice of the indicated genotypes. Bar, 100 μm. (D) Relative mRNA levels of Tjp1 in intestinal epithelial cells from mice of the indicated genotypes as assessed by quantitative PCR (levels in untreated Csk1al+/–; Ifnar1–/– mice are taken as 1.0). (E) H&E staining of intestinal tissues from mice of the indicated genotypes at day 30 after tamoxifen treatment (DKO mice were treated with saline). Bar, 200 μm.
which IFN contributes to the maintenance of the barrier function of the gut can be proposed. First, IFN-mediated restriction of the rate of cell proliferation should enable better differentiation, maturation, and establishment of the cohesive sheet of enteroctyes and colonocytes. Second, IFN can contribute to immune defenses against conditionally pathogenic microbiota and intestinal inflammation. Katataka and coauthors previously reported a greater sensitivity of Ifnar1-null mice to intestinal inflammation caused by dextran sodium sulfate (53). Those authors also mentioned a positive effect of IFN on the protection of barrier properties of the epithelial cell sheet in vitro. Importantly, our results implicate IFN in regulating the expression of the ZO-1 protein involved in the formation of tight junctions that separate the basolateral epithelial space from the microbiota. Potential immune-related effects of IFN on the ability to withstand the pathogenic effects of microbiota are illustrated by the rescue of CskΔ11/Ifnar1-deficient mice upon administration of antibiotics (Fig. 7).

Genetic alterations in the IFNAR1 gene in humans were linked with the susceptibility locus for inflammatory bowel disease (54), and IFN-based pharmaceutical formulations have been used to treat patients with these disorders albeit with variable results (reviewed in reference 55). As evident from the literature and our current results, the effects of IFN on intestinal homeostasis are pleiotropic. Roles of IFN in antigen recognition and immune function, cell differentiation and proliferation, and gut barrier function are likely to contribute to the complexity of patient responses to IFN. Although some of the long-term IFN effects, such as suppression of tissue-regenerative abilities in the gastrointestinal tract, could be genuinely detrimental (17, 28), it is also plausible that rapid degradation of IFNAR1 may constitute an additional challenge for the efficacy of IFN-based therapies (reviewed in reference 13). Thus, future studies may determine the potential application of poorly bioavailable gut-restricted CK1α inhibitors to stabilize IFNAR1 and improve intestinal barrier function.

ACKNOWLEDGMENTS

We declare no conflict of interest.

This work was supported by NIH/NCI grants RO1 CA165997 and PO1 CA165997 (to S.Y.F.), including help from the Scientific Cell/Tissue Morphology Core and its principal investigator, Qian-Chun Yu.

We thank D. E. Zhang for reagents and A. Ortiz for help with manuscript preparation.

FUNDING INFORMATION

Office of Extramural Research, National Institutes of Health (OER) provided funding to Serge Y. Fuchs under grant number CA165997. Office of Extramural Research, National Institutes of Health (OER) provided funding to Serge Y. Fuchs under grant number CA092900.

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