Ion channel TRPV1-dependent activation of PTP1B suppresses EGFR-associated intestinal tumorigenesis

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Introduction

The mammalian intestinal epithelium reveals a complex interplay among intestinal stem cell (ISC) self-renewal in the crypts of Lieberkühn, progenitor cell proliferation, differentiation, and, ultimately, apoptosis (1). The high rate of intestinal epithelial cell turnover within a genotoxic microenvironment needs to be tightly regulated to minimize the risk of neoplasia development from ISCs or dedifferentiated progenitor cells (2). The main drivers of IEC proliferation are the Wnt, Notch, and epidermal growth factor receptor (EGFR) pathways (3). Somatic mutations that result in gain of function of these and associated oncogenic potential (4, 5). Similarly, whereas Toll-like receptor signaling in IECs provides essential proliferative signals (6), these microbiota-induced epithelial tumorigenesis (7). Microenvironmental cues that signal IECs to shift gears from a progenitor to a differentiated mode at the crypt-villus junction are instrumental in the regulation of crypt homeostasis (8). This includes the release of BMP and Hedgehog proteins along a spatial gradient. Interruption of this mesenchymal-epithelial crosstalk results in distortion of the crypt-villus architecture (9–11) and predisposes the epithelium to neoplasia (12, 13).

The regulatory role of physiological signals transduced by sensory ion channels in crypt homeostasis, and their implications for epithelial tumorigenesis, have not been fully studied. One exception is the reported homeostatic role of the stretch-activated receptor Piezo1. Overcrowding within the epithelial sheet is detected by the Piezo1 cation channel, which leads to live cell extrusion through Rho kinase activation, thereby keeping cell numbers in check. Interestingly, colonic polyps show increased cell densities in crypt sides compared with healthy epithelium, which suggests that aberrant cell extrusion may be an early oncogenic event in the intestines (14).

In the search for sensory receptors in epithelial tissues, the comprehensive family of TRP ion channels holds particular interest, as these sense and integrate a broad range of thermal, mechanical, and chemical environmental stimuli (15). The mammalian TRP family consists of multiple subfamilies, including canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP), and mucolipin (TRPML), which form multimeric proteins that function as cation channels, display diverse gating mechanisms, and are ubiquitously expressed in various excitable and nonexcitable cell types (16). TRP channels are well represented in the complex milieu of the digestive tract (17). Moreover, various TRP channels are expressed in epithelial tissues (18, 19), and some data suggest that they promote cell proliferation...
and may play a role in cellular transformation (20). Mechanistically, this has been shown to involve potentiation of EGFR signaling in other epithelial tissues, such as TRPV3 in keratinocytes (21) and TRPC1 in lung carcinoma cell lines (22). However, the physiological relevance of TRP-EGFR interactions and their associated signaling pathways in the intestinal epithelium are not known.

Here we demonstrate an unconventional role for the TRPV1 channel in intestinal crypt homeostasis and tumorigenesis. We found that TRPV1 was intrinsically activated by EGFR. Our molecular dissection showed that TRPV1 triggering subsequently initiated a molecular cascade — involving Ca\(^{2+}\), calpain, and finally protein tyrosine phosphatase 1B (PTP1B) — that served as a negative feedback loop on EGFR activity. Deficiency of Trpv1 resulted in IEC hyperproliferation in vivo and in intestinal organoid cultures in vitro, as well as increased formation of intestinal neoplasia. Collectively, our data suggest that TRPV1 is a nonredundant, intrinsic negative regulator of cell proliferation and intestinal tumorigenesis. Thus, rather than transducing physical signals, TRPV1 senses and regulates growth factor signaling in IECs in order to suppress intestinal tumorigenesis.

Results

TRPV1 is a functional Ca\(^{2+}\) channel in IECs and organoids. To evaluate the expression of TRPV1 family members in IECs, quantitative real-time RT-PCR (Q-PCR) screening for the abundance of Trpv1–Trpv6 transcripts in both primary IEC isolates and intestinal organoids was performed. The latter is an autonomous IEC culture system that consists of self-organizing “miniguts” that recapitulate intestinal crypts, free of any potentially contaminating mesenchymal, neuronal, or bone marrow–derived cells (23). We found that Trpv1, Trpv4, Trpv5, and Trpv6 were expressed in the intestinal epithelium, with similar expression patterns in freshly isolated IECs and intestinal organoid cultures (Figure 1, A and B, and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI72340DS1). While the molecular effects of TRPV4 and TRPV5/6 in IECs have been reported previously (24, 25), the physiological role of TRPV1 in IEC biology is unknown. We validated expression of Trpv1 mRNA in IECs using Trpv1\(^+/−\) mice (26) for both organoid cultures and freshly isolated crypts (Supplemental Figure 1C). We also confirmed TRPV1 mRNA expression in human and rodent IEC lines (Supplemental Figure 1D). To show TRPV1 protein expression, we first validated the specificity of the anti-TRPV1 Ab by using CHO cells that stably overexpress TRPV1 (Supplemental Figure 1, E and F). We confirmed TRPV1 expression in IECs using flow cytometry (Figure 1C). TRPV1 expression by primary IECs was demonstrated by immunofluorescent staining of intestinal organoids (Figure 1D). Staining with secondary antibody only (Alexa Fluor 488–conjugated anti-Rb) did not produce a positive signal (data not shown). We next evaluated TRPV1 ion channel functionality in IECs. As TRPV1 is primarily a Ca\(^{2+}\) channel (27), we first validated our experimental setup to measure cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) measurements in cells loaded with Fura-2 acetoxymethyl ester (Fura-2 AM) (28, 29). We used CHO\(^{Trpv1}\) cells after stimulation with the TRPV1-specific agonist capsaicin (Supplemental Figure 1G). More importantly, addition of capsaicin induced [Ca\(^{2+}\)]\(_{cyt}\) elevations in the IEC line HCT116 that were dose-dependent and abrogated by TRPV1 knockdown (Figure 1E). Pretreatment with the TRPV1 antagonist BCTC also inhibited capsaicin-induced Ca\(^{2+}\) influxes in HCT116 cells (Supplemental Figure 1H). Similar results were generated by applying heat as a TRPV1 activator (Figure 1F). To confirm the functionality of TRPV1 channels in primary IECs, we used intestinal organoids from WT and Trpv1\(^−/−\) mice in the same experimental setup. Organoid-derived crypts were then stimulated with the TRPV1 agonist capsaicin, which resulted in marked [Ca\(^{2+}\)]\(_{cyt}\) increases in WT, but not Trpv1\(^−/−\), crypts (Figure 1, G–I). Collectively, these data demonstrated that TRPV1 is a functional Ca\(^{2+}\) channel in IECs.

Epithelial TRPV1 signaling inhibits EGFR activity in vivo. Next we used Trpv1\(^−/−\) mice to evaluate the physiological function of TRPV1 signaling in IECs. TRPV1 deficiency did not significantly affect gross intestinal crypt-villus morphology or IEC differentiation (Supplemental Figure 2A). Consistent with these in vivo findings, we observed similar numbers of goblet and Paneth cells per crypt in intestinal organoids generated from WT and Trpv1\(^−/−\) mice (Supplemental Figure 2B). In contrast, we observed a substantial increase in IEC proliferation in Trpv1\(^−/−\) mice by immunostaining for Ki67, a marker of transient amplifying (TA) cells (Figure 2A). These data suggest that TRPV1 signaling negatively regulates epithelial cell proliferation. Since EGFR is a physiological inducer of IEC proliferation and has previously been associated with other TRP channels (21, 22), we hypothesized that the TRPV1 and EGFR signaling pathways may functionally interact. Constitutive phosphorylation of EGFR\(^\text{Tyr1068}\), indicative of EGFR kinase activity, was increased in colonic crypts in Trpv1\(^−/−\) mice (Figure 2, B–D). Moreover, Trpv1\(^−/−\) crypts displayed increased constitutive phosphorylation of ERK1/2, a MAPK that relays downstream signaling of EGFR. This effect was reversed by treatment with the EGFR kinase inhibitor gefitinib (Figure 2B). Gefitinib also reversed the increased IEC proliferation in Trpv1\(^−/−\) mice (Figure 2, E and F), which suggests that enhanced constitutive EGFR activation was responsible for the epithelial hyperproliferation observed in these mice. We then tested the effects of TRPV1 triggering on EGFR signaling in IECs in vivo. Expression levels of immediate early response genes reflect the transcriptional activity of EGFR signaling (30). A single oral administration of capsaicin (3 mg/kg) decreased expression levels of c-Fos, Fosl2 (also known as Fra2), and c-Jun in WT mice, but not Trpv1\(^−/−\) mice (Figure 2, G and H). Importantly, cotreatment with gefitinib (50 mg/kg) did not enhance the suppressive effect of capsaicin, which suggests that both agents optimally targeted the same pathway (i.e., EGFR). Oral administration of capsaicin also inhibited ligand–induced EGFR activation in IECs and suppressed epithelial cell proliferation (Supplemental Figure 2, C–E). Notably, oral capsaicin treatment did not significantly affect goblet or Paneth cell numbers (Supplemental Figure 2, F–H). Together, these results are suggestive of negative regulation of IEC proliferation and epithelial EGFR signaling by TRPV1.

In order to unambiguously address whether epithelial TRPV1 directly modulates EGFR kinase activity, we generated mice that overexpress TRPV1 (31) in IECs on the C57BL/6J background (referred to herein as TRPV1\(^\text{IEC}\) mice). Excision of a stop sequence by Cre under the control of the Villin promoter resulted in conditional TRPV1 overexpression in IECs (Figure 2I). TRPV1\(^\text{IEC}\) mice showed increased TRPV1 protein and mRNA expression in IECs,
but not in spinal cord homogenates (Supplemental Figure 3, A and B). Consistent with our results from *Trpv1<sup>−/−</sup>* mice, TRPV1<sup>IEC</sup> animals showed reduced constitutive p-EGFR<sup>Y1068</sup> levels in colon crypts, whereas total EGFR levels were unaffected (Figure 2J). Furthermore, IEC-specific TRPV1 overexpression suppressed EGF-induced EGFR activity in freshly isolated IECs, inhibited epithelial cell proliferation in vivo, and suppressed cell proliferation and expression of EGFR target genes in intestinal organoid cultures in vitro as well as the intensity of p-EGFR<sup>Y1068</sup> staining (Figure 2K and Supplemental Figure 3, C–G). Collectively, these findings suggest that epithelial TRPV1 inhibits IEC proliferation by antagonizing EGFR signaling.
resulted in enhanced ligand-induced EGFR\textsuperscript{Y1068} phosphorylation (Figure 3B). This effect coincided with an increased rate of cell proliferation, which was reversed by gefitinib treatment (Figure 3C). These data further support the functional and intrinsic association between the TRPV1 and EGFR signaling pathways in IECs.

To compare the effects of the TRPV1 channel with those of other TRPV family members that we found to be expressed by IECs, we repeated our in vitro assays for the thermosensors TRPV3 and TRPV4, as well as the Ca\textsuperscript{2+} transporter TRPV5. Overexpression of TRPV3 potentiated ligand-induced EGFR\textsuperscript{Y1068} phosphorylation in HCT116 cells (consistent with previous findings in keratinocytes; ref. 21), whereas TRPV4 overexpression had no significant effects (Supplemental Figure 4C). Interestingly, like TRPV1, TRPV5 significantly suppressed ligand-induced EGFR\textsuperscript{Y1068} phosphorylation (Supplemental Figure 4, D and E). These data suggest a more gen-

TRPV1 intrinsically inhibits EGFR signaling and epithelial cell proliferation. We then further explored the molecular interactions between TRPV1 and EGFR in vitro. Similar to the data derived from TRPV1\textsuperscript{IEC} transgenic mice, overexpression of TRPV1 in HCT116 cells inhibited ligand-induced EGFR\textsuperscript{Y1068} phosphorylation compared with mock-transfected cells (Figure 3A). This was recapitulated in CHO cells with reconstituted expression of WT-EGFR, with or without TRPV1 (Supplemental Figure 4A). Exploration of other EGFR phosphorylation sites showed that TRPV1 suppressed phosphorylation of Y992 and Y1045, in addition to Y1068, but not S1046/S1047 (Supplemental Figure 4B). Since the Y1068 phosphorylation site was a more robust readout, and is associated with multiple proproliferative signaling pathways, we focused on phospho-EGFR\textsuperscript{Y1068} levels in subsequent assays. Stable knockdown of TRPV1 in HCT116 cells by shRNA (validated in Figure 1, E and F)
eral role for TRP channel–mediated Ca\(^{2+}\) influx in the regulation of EGFR signaling in IECs. However, as these effects might occur by different mechanisms, we focused in subsequent experiments on dissecting the TRPV1-mediated effects on EGFR signaling.

**Physiological effects of epithelial TRPV1 signaling in intestinal organoids.** To substantiate our findings on TRPV1 in a more physiological setting, we studied the growth kinetics of intestinal organoids derived from WT and Trpv1–/– mice. Mature Trpv1–/– organoids produced larger crypts compared with WT controls under similar culture conditions (Figure 4, A and B). Q-PCR analysis of Mkt1 transcripts showed significantly increased expression in Trpv1–/– organoids, which was confirmed by immunostaining (Figure 4, C-E, and Supplemental Figure 5, A-C). Moreover, this increased proliferation coincided with increased expression of the EGFR target gene c-Fos in Trpv1–/– organoids (Figure 4F). We then hypothesized that Trpv1–/– organoids would have a reduced requirement for exogenous EGF, as recently reported for another negative regulator of EGFR signaling, leucine-rich and immunoglobulin-like domain protein 1 (Lrig1; ref. 32). Indeed, WT organoids showed an almost complete loss of proliferation in the absence of exogenous EGF, whereas Trpv1–/– organoids maintained active TA cells under the same conditions (Figure 4, G and H, and Supplemental Figure 5D). Consistent with our in vivo findings, these data suggest that TRPV1 is an intrinsic regulator of EGFR signaling in IECs, acting to confine the proliferating cell compartment in the intestinal crypt.

TRPV1 regulates EGFR through Ca\(^{2+}\)/calcineurin and PTP1B. To identify the molecular mechanism by which TRPV1, a nonsellective Ca\(^{2+}\) channel, regulates EGFR activity, we explored the involvement of Ca\(^{2+}\)-dependent effectors, including the calmodulin, calcineurin, and calpain pathways (20). Calmodulin-dependent kinase II-mediated (CaMKII-mediated) Ser phosphorylation of the intracellular tail of EGFR can inhibit EGFR kinase activity (33), which we indeed found to be enhanced in TRPV1-overexpressing cells (Supplemental Figure 4B). However, even though the CaM inhibitor W-7 efficaciously blocked ionomycin-induced CaMKII activation, pretreatment with W-7 did not reverse the inhibitory effects of TRPV1 overexpression on EGFR\(^{Y1068}\) phosphorylation (Supplemental Figure 6, A and B). Similarly, and despite the reported inhibitory role of Ca\(^{2+}\)/calcineurin activity on EGFR signaling (34), calcineurin inhibition by FK506 failed to reverse the TRPV1-mediated effects on EGFR\(^{Y1068}\) phosphorylation (Supplemental Figure 6, C and D).

We then focused on the Ca\(^{2+}\)/calcineurin pathway. Regulation of EGFR\(^{Y1068}\) phosphorylation by TRPV1 was prevented by pretreatment with the cell-permeable calpain inhibitor ALLM (Figure 5A), suggestive of direct or indirect inhibitory effects of calpain on EGFR kinase activity. Dephosphorylation of EGFR by protein tyrosine phosphatases (PTPs) is an important early regulatory loop that controls receptor tyrosine kinase signaling (35). Since there is no evidence in the literature for direct posttranslational modulation of the EGFR by calpain, we hypothesized that calpain might control the activity of a PTP, which in turn regulates the phosphorylation status of EGFR. Indeed, pretreatment with the nonspecific PTP inhibitor sodium orthovanadate (Na\(_3\)VO\(_4\)) reversed the negative regulatory effect of TRPV1 on EGFR\(^{Y1068}\) phosphorylation (Figure 5B). PTPs that are known to regulate EGFR kinase activity include LAR (encoded by Ptp1f), PTP-\(\sigma\) (Ptpsr), PTP1B (Ptprb), TCPTP (Ptpn2), and SHP-1 (Ptnp6) (36). We found all of these PTPs to be expressed by IECs (Figure 5C), although their expression levels were similar in WT and Trpv1–/– mice (Supplemental Figure 6E). Of these PTP candidates, only PTP1B is known to be functionally associated with Ca\(^{2+}\)/calpain (37). Posttranslational cleavage of PTP1B by calpain increases its phosphatase activity (38). Accordingly, pretreatment with the cell-permeable PTP1B inhibitor Compound 3 recapitulated the effect of Na\(_3\)VO\(_4\) in reversing TRPV1-mediated dephosphorylation of EGFR (Figure 5B). The crucial role of this PTP in the TRPV1-mediated effects on EGFR was confirmed by siRNA-mediated knockdown of PTP1B (Figure 5D). Another target of PTP1B upon EGF stimulation is the early endosomal protein hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) (39). TRPV1-mediated Ca\(^{2+}\) influx and subsequent calpain activation should therefore also affect Hrs\(^{334}\) phosphorylation, the major phosphorylated tyrosine residue in response to EGF (40). Indeed, we found that overexpression of TRPV1 resulted in suppression of Hrs\(^{334}\) phosphorylation upon EGF stimulation (Supplemental Figure 6F). Finally, to confirm...
EGFR and TRPV1 are part of a homeostatic molecular circuit. The activity of certain TRP channels can be potentiated by phospholipase C–coupled (PLC-coupled) receptor tyrosine kinases (15). One of the proposed models of TRP activation is the release from its tonic inhibition by membrane lipids, such as phosphatidylinositol-4,5-bisphosphate (PIP2). Hydrolysis of PIP2 in the plasma membrane (44), we hypothesized that EGFR activation might therefore facilitate TRPV1 gating. To test this hypothesis, we transfected cells with EGFR and/or TRPV1 and measured intracellular [Ca2+] after EGF stimulation. We found that expression of EGFR or TRPV1 alone did not result in EGF-induced [Ca2+] increases in CHO or HEK293 cells (Figure 6, A and B). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D). This functional coupling between EGFR and TRPV1 was abrogated by addition of exogenous PIP2, or by PLCG1 knockdown (Figure 6, B and D).
TRPV1 signaling inhibits the development of intestinal neoplasia. EGFR signaling accelerates the development of neoplasia in mice genetically susceptible to intestinal adenoma formation (i.e., ApcMin/+ mice; ref. 45). Given these tumor-promoting properties of EGFR signaling and the data presented above, we hypothesized that genetic deletion of Trpv1 would promote the development of intestinal neoplasia. As predicted, ApcMin/+ Trpv1–/– mice developed significantly more tumors in the intestinal tract, in accordance with recent data (46), an effect that was prevented by gefitinib treatment (Figure 7, A and B). This was associated with increased morbidity in Apc Min/+ Trpv1–/– versus Apc Min/+ mice, as shown by decreased levels of blood hemoglobin (HgB) as well as reduced lifespan (Figure 7, C and D). We observed increased constitutive EGFR Y1068 phosphorylation and elevated expression of the proliferation marker PCNA in IECs from ApcMin/+ Trpv1–/– mice, which was reversed by gefitinib treatment (Figure 7E). The epithelial hyperproliferation in ApcMin/+ Trpv1–/– versus ApcMin/+ mice was confirmed by Ki67 staining (Figure 7F). Furthermore, deletion of Trpv1 resulted in significantly increased expression levels of the EGFR-regulated oncoproteins c-Fos and c-Myc on the ApcMin/+ background, without affecting the Wnt target Axin2 (Figure 7G and Supplemental Figure 8, A and B). These findings support the concept that increased constitutive EGFR signaling in ApcMin/+ Trpv1–/– mice predisposed this strain to develop more intestinal neoplasia.

Given the potential role of TRPV1-expressing sensory neurons in the regulation of intestinal tumorigenesis, we also evaluated the phenotype of ApcMin/+ mice that underwent systemic ablation of TRPV1+ neurons. Apc Min/+ mice were treated with the ultrapotent TRPV1 agonist resiniferatoxin (RTX) during the neonatal period (47). Ocular challenge with capsaicin (eye wipe test) confirmed systemic TRPV1+ sensory neuron ablation. In contrast to mice with global deletion of Trpv1, the RTX-treated mice showed a slight survival benefit, whereas intestinal tumor counts and HgB levels were comparable to those of ApcMin/+ mice (Supplemental Figure 8, C and D). Thus, these results indirectly substantiate the tumor-suppressive role of epithelial TRPV1 in the intestines.

Finally, administration of the dietary TRPV1 agonist capsaicin, mixed at 0.01% (w/w) with chow, significantly increased the sur-
activation (42, 43). This proposed PLCγ-PIP2-TRPV1 signaling axis was initially inferred from the observation that activation of TrkA sensitizes TRPV1 to physical or chemical stimuli (53). More recently, others showed that activation of another receptor tyrosine kinase, c-Met, evoked TRPV1 channel activity in hepatocytes (54). Our present findings are consistent with a model in which receptor tyrosine kinases functionally interact with TRPV1. Moreover, our data suggest that the physiological role of TRPV1 in the intestinal epithelium is to restrain EGFR signaling. This negative feedback downstream of TRPV1 is dependent on the intracellular actions of Ca2+/calpain and a phosphatase (PTP1B), regulates the proliferation rate of IECs, and suppresses intestinal tumorigenesis. The proposed molecular pathway and its in vivo relevance are summarized in Figure 8.

Incidentally, we also found the TRPV5 Ca2+ channel to be capable of suppressing ligand-induced EGFR activation (Supplemental Figure 4E). TRPV5 is potentiated by PIP2, in contrast to TRPV1 (55), suggestive of a distinct mechanistic link between TRPV5 and EGFR that remains to be elucidated (Supplemental Figure 9). The highly selective TRPV5 Ca2+ channel has been described to be involved in transcellular absorption of Ca2+ in the intestines, osteoclastic Ca2+ resorption of bone tissue, as well as active Ca2+ reabsorption in the kidneys (56). Thus, TRPV5 also exerts many extraintestinal effects in the regulation of Ca2+ homeostasis. A functional relationship with the EGFR pathway, such as that previously described for the Mg2+ channel TRPM6 in distal convoluted tubule cells of the kidney (56, 57), has not been reported for

**Discussion**

Here we propose that TRPV1 plays an indispensable role in the intestinal epithelium as an intrinsic regulator of growth factor receptor signaling, cell proliferation, and tumorigenesis. In this context, epithelial TRPV1 may be activated independently from its classical agonists: heat, capsaicin, lipid mediators, oxidative agents, and low pH (50–52). Our results suggest a different mode of activation of TRPV1, by PLCγ-mediated depletion of PIP2, a tonic negative regulator of TRPV1, after receptor tyrosine kinase activation (42, 43). This proposed PLCγ-PIP2-TRPV1 signaling axis was initially inferred from the observation that activation of TrkA sensitizes TRPV1 to physical or chemical stimuli (53). More recently, others showed that activation of another receptor tyrosine kinase, c-Met, evoked TRPV1 channel activity in hepatocytes (54). Our present findings are consistent with a model in which receptor tyrosine kinases functionally interact with TRPV1. Moreover, our data suggest that the physiological role of TRPV1 in the intestinal epithelium is to restrain EGFR signaling. This negative feedback downstream of TRPV1 is dependent on the intracellular actions of Ca2+/calpain and a phosphatase (PTP1B), regulates the proliferation rate of IECs, and suppresses intestinal tumorigenesis. The proposed molecular pathway and its in vivo relevance are summarized in Figure 8.

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Figure 6. EGFR and TRPV1 are part of a homeostatic circuit. (A) EGFR triggering induced TRPV1-mediated Ca2+ influx. CHO cells did not show Ca2+ influxes in response to EGF (100 ng/ml; green bars) when only EGFR or TRPV1 was expressed (n = 43 and 42, respectively). ATP (10 μM; red bars) served as a positive control. (B) CHO cells expressing both EGFR and TRPV1 showed [Ca2+]cyt increases in response to EGF stimulations, which was prevented by addition of exogenous PIP2 (n = 50 and 52, respectively). (C) HEK293 cells did not show EGF-induced Ca2+ increases in response to only EGFR or TRPV1 (n = 50 and 52, respectively). (D) HEK293 cells showed [Ca2+]cyt increases in response to EGF when the EGFR and TRPV1 were coexpressed, which was prevented by PLCγ1 knockdown (n = 50 and 52, respectively). HEK293 cells were transfected with control or PLCγ1 siRNA, together with EGFR and TRPV1 expression plasmids. Ca2+ imaging was performed at day 3 after transfection. Mean ± SEM from individual [Ca2+]cyt measurements of Fura-2 AM–loaded cells, representative of 2 (B and D) or 3 (A and C) independent experiments.
that promotes cellular growth and the development of colorectal neoplasia (58). To prevent sporadic tumorigenesis, EGFR activation is coupled to the prompt initiation of cell-intrinsic inhibitory mechanisms, including dephosphorylation by PTP, receptor endocytosis, and the transcriptional upregulation of a variety of inhibitors (59). An example of the latter is the pan-ErbB inhibitor Lrig1, which promotes EGFR degradation (60). Importantly, Lrig1 was recently shown to be a physiological regulator of EGFR signaling in the intestinal crypt (32). Genetic deletion of Lrig1 not only leads to crypt expansion, but also unleashes the oncogenic potential of TRPV5. Needless to say, the association between TRPV5 and the EGFR needs to be further addressed. Furthermore, given the high expression levels of TRPV5 in IECs, the potential physiological role of TRPV5 with regard to EGFR signaling should be directly compared with that of TRPV1 to evaluate whether mechanistic differences exist between these TRP channels.

The dynamic events within the intestinal crypt include proliferative signals from the crypt base (i.e., Wnt and Notch) that are counteracted by negative regulatory signals at the crypt-villus junction (8). EGFR signaling represents a third pathway that promotes cellular growth and the development of colorectal neoplasia (58). To prevent sporadic tumorigenesis, EGFR activation is coupled to the prompt initiation of cell-intrinsic inhibitory mechanisms, including dephosphorylation by PTP, receptor endocytosis, and the transcriptional upregulation of a variety of inhibitors (59). An example of the latter is the pan-ErbB inhibitor Lrig1, which promotes EGFR degradation (60). Importantly, Lrig1 was recently shown to be a physiological regulator of EGFR signaling in the intestinal crypt (32). Genetic deletion of Lrig1 not only leads to crypt expansion, but also unleashes the oncogenic potential of TRPV5. Needless to say, the association between TRPV5 and the EGFR needs to be further addressed. Furthermore, given the high expression levels of TRPV5 in IECs, the potential physiological role of TRPV5 with regard to EGFR signaling should be directly compared with that of TRPV1 to evaluate whether mechanistic differences exist between these TRP channels.

**Figure 7.** TRPV1 signaling inhibits intestinal neoplasia development. (A) Increased tumor burden in Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice. Shown are representative examples of small intestinal polyps (asterisks) in untreated and gefitinib-treated Apc<sup>min/+</sup> and Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice. Scale bar: 4 mm. (B) 10-week-old Apc<sup>min/+</sup> and Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice were treated with gefitinib (50 mg/kg/d) until 20 weeks, and polyps (>1 mm) were counted. n = 12 (untreated Apc<sup>min/+</sup>), 9 (gefitinib-treated Apc<sup>min/+</sup> and untreated Apc<sup>min/+ Trpv1<sup>−/−</sup></sup>), 7 (gefitinib-treated Apc<sup>min/+ Trpv1<sup>−/−</sup></sup>). (C) Hgb levels at 20 weeks. (D) Survival curves of Apc<sup>min/+</sup> and Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice, treated with normal chow (n = 16 and 24, respectively) or capsaicin (Cap; n = 11 and 5, respectively). ***P < 0.0004, Apc<sup>min/+</sup> capsaicin vs. Apc<sup>min/+ Trpv1<sup>−/−</sup></sup>; ****P < 0.0003, Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> vs. Apc<sup>min/+</sup>; ***P < 0.004, Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> capsaicin vs. Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> capsaicin; *P < 0.019, Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> capsaicin vs. Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> capsaicin, log-rank test. (E) Increased p-EGFR<sup>Y1068</sup> and PCNA levels in Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> colon crypts was reversed by gefitinib treatment, as in Figure 2B. (F) Increased IEC proliferation in Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice. Scale bar: 100 μm. (G) Expression of EGFR- and Wnt-regulated genes in IEC lysates from 15-week-old Apc<sup>min/+</sup> and Apc<sup>min/+ Trpv1<sup>−/−</sup></sup> mice (n = 9 and 5, respectively). Mean ± SEM, expressed relative to Apc<sup>min/+</sup>. *P < 0.05, t test. (H) Apc<sup>min/+</sup> mice were treated with regular chow (n = 16; different cohort from D), chow mixed with 300 ppm celecoxib (n = 9), or celecoxib plus capsaicin (n = 12). **P < 0.001, celecoxib vs. control; ***P < 0.0001, celecoxib+capsaicin vs. control; ****P = 0.0002, celecoxib+capsaicin vs. celecoxib, log-rank test. (I) Polyp counts and (J) Hgb levels at 20 weeks. *P < 0.05, **P < 0.001, ***P < 0.0001 vs. control or as indicated, ANOVA (B, C, I, and J).
TRPV1 signaling regulates the EGFR pathway and its associated regulatory systems appear to act in a nonredundant fashion in the intestinal epithelium to suppress neoplasia development. Our dissection of the intracellular components that negatively regulate EGFR activation downstream of TRPV1 outlined a requirement for the Ca\(^{2+}\)/calpain signaling axis and identified PTP1B as a key target of this pathway. PTP1B is a prototypical, nonreceptor PTP that directly interacts with the EGFR and dephosphorylates Tyr residues upon receptor activation. Furthermore, Lrig1 expression is restricted to the intestinal stem cell population, whereas our data suggested a more diffuse expression pattern of TRPV1 in intestinal crypts. Thus, these 2 regulatory systems appear to act in a nonredundant fashion in the intestinal epithelium to suppress neoplasia development.

Figure 8. Proposed model for EGFR-TRPV1 crosstalk in IECs. TRPV1 and PTP1B are part of a homeostatic signaling circuit that restrains EGFR-induced epithelial cell proliferation. EGFR kinase activity mediates proproliferative and thus protumorigenic effects in IECs (dashed red arrows). TRPV1 and PTP1B are predominantly expressed in the crypt compartment that contains TA cells (dark blue cells) with active EGFR signaling. Ligand-induced autophosphorylation of the EGFR results in PLC activation, which cleaves PIP\(_2\), a tonic inhibitor of TRPV1, into diacylglycerol (DAG) and inositol triphosphate (IP\(_3\); green arrows). This results in TRPV1 triggering and Ca\(^{2+}\) influx, which activates calpain and subsequently PTP1B. PTP1B then dephosphorylates EGFR (blue lines). This coupling between EGFR and TRPV1 exerts negative feedback on growth factor receptor signaling, inhibits crypt progenitor cell (dark blue cells) turnover, and hence reduces the risk of intestinal neoplasia development (red cells). +4 ISC, ISC at +4 position; CBCC, crypt-based columnar stem cell.
In conclusion, our data suggest that TRPV1 sensors and regulates cell proliferation in the intestinal epithelium. Mechanistically, we identified a novel pathway downstream of TRPV1 through Ca\(^{2+}\)/calpain and PTP1B that regulates cell growth signaling and affects IEC homeostasis and tumorigenesis. Our data also suggest that TRPV1 triggering by dietary administration of capsaicin suppressed intestinal tumorigenesis. Based on these results, we propose that the administration of TRPV1 agonists in combination with a COX-2 inhibitor may prevent the adenoma-to-carcinoma sequence in humans.

### Methods

**Reagents.** Recombinant mEGF for in vitro and in vivo stimulations was purchased from PeproTech. DMEM, DTT, ATP, capsaicin (≥95% purity, from *Capsicum* sp.), and Na\(_2\)VO\(_4\) were purchased from Sigma-Aldrich. Gefitinib, resiniferatoxin, and celecoxib were obtained from LC Labs. EDTA and G418 (Geneticin) were obtained from Invitrogen. Puromycin was obtained from InvivoGen. PIP, was purchased from Echelon (catalog no. P-4508). BCTC, W-7, and FK506 were obtained from Tocris. ALLM and the selective PTIPB inhibitor Compound 3 (3-(5,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonic-acid-(4-[(thiazol-2-yl)sulfanyl]-phenyl)-amide; ref. 75) were obtained from Calbiochem. Alexa Fluor 488–conjugated wheat germ agglutinin, Alexa Fluor 546–conjugated phalloidin, and Alexa Fluor 488–conjugated secondary antibodies for immunostainings were obtained from Molecular Probes.

**Antibodies.** Anti-phospho-EGFR (Y992, Y1045, S1046/S1047, and Y1068), anti-EGFR, anti-phospho-ERK1/2 (T202/Y204), anti-ERK1/2, anti-PCNA, anti-MMP7, anti-PTIPB (for Western blotting), anti-synaptophysin, and anti-PLC\(\gamma\) antibodies were from Cell Signaling Technologies; anti-β-actin antibody was from Sigma-Aldrich; anti-Ki67 was from Abcam; and anti-occludin was from Neomarkers. Anti-PTP1B antibody for immunohistochemistry was obtained from Santa Cruz Biotechnology Inc. Anti-TRPV1 antibody for immunofluorescent stainings was obtained from Alomone Labs (catalog no. ACC-030). Anti-phospho-Hrs (Y334) antibody was provided by S. Urbé (University of Liverpool, Liverpool, United Kingdom; ref. 40).

**Cell culture, in vitro assays, and transfections.** HCT116, HEK293, IEC-6, and CMT-93 cells were cultured in high-glucose DMEM (Mediatech); HCA-7 cells were cultured in low-glucose DMEM. Culture medium was supplemented with 4 mM glucose, 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. The rTRPV1 (pcDNA3) and mTRPV3 (pcDNA5) plasmids, CHO\(^{k1}\) cell line, and stable CHO\(^{TRPV}\) transgenic cell line were provided by A. Patapoutian (Scripps Research Institute, La Jolla, California, USA). CHO\(^{TRPV}\) cells were maintained in G418-containing selection medium. The WT-EGFR (pJWERNL) plasmid was provided by F. Furnari (UCSD, La Jolla, California, USA), the mTRPV4-FLAG (pcDNA3) plasmid was provided by G. Walz (University Hospital Freiburg, Freiburg, Germany), and the rBTPV5 (pEGFP-N3) plasmid was provided by C.-L. Huang (UT Southwestern, Dallas, Texas, USA). Plasmids containing HA-tagged human full-length PTIPB (pJ3H-PPTP435) and C-terminal truncation mutants pJ3H-PPTP370 (aa 1-370) and pJ3H-PPTP377 (aa 1-377) were donated by J. Chernoff (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA). CHO and HCT116 cells were transfected with WT-EGFR and/or TRPV1, TRPV3, TRPV4, TRPV5, or PTIPB plasmids (1–3 μg DNA per 10\(^6\) cells) with Nucleofector (Amaxa), as previously described (76). Cells were serum-starved 2–4 hours before EGFR stimulation (1–10 ng/ml, as indicated). In some conditions, cells were pre-treated for 1 hour with W-7 (10 μM), FK506 (1–10 μM), Na\(_2\)VO\(_4\) (10 μM), Compound 3 (10 μM), ALLM (10 μM), or DMSO. MTX assay (Sigma-Aldrich) was performed according to the manufacturer’s instructions. Knockdown in HCT116 and HEK293 cells was performed with Nucleofector. TRPV1 shRNA consisted of a pool of 3 independent, target-specific shRNAs: sc-36826-SHA (GATCCGAAGACCTGCTT-GCTGAAATTCAAGAGATTTCAGACAGGTCTTCTTTT) and corresponding siRNA sequences (sc-36826A) (sense, GAAACCCUGUCUGGAAATGT; antisense, UUCAGAGAAGAGAGACGUGUGT), sc-36826-SHB (GATCCGCACTTCTACTCTCCAATTTCTCAAGAGTTTGAAGTAGAAGATCGGGTTTTT) and corresponding siRNA sequences (sc-36826C) (sense, CCAGACUUCUUCUUCUCCAGT; antisense, AGUUAGAGAACAGACGUGT), TRPV1 siRNA consisted of a pool of 3 different siRNA duplexes: sc-36826A, sc-36826B, and sc-36826C. PTIPB siRNA consisted of a pool of 3 different siRNA duplexes: sc-36328A (sense, UCUCGUGUAGUCAAGAAT; antisense, UUCUGAUCAUCAAGGAAATG), sc-36328B (sense, CGAGACUGAUAUAUAUAAT; antisense, UGAUAAGUACAGGUGGTT), and sc-36328C (sense, UCUCGCAAUUAAAGCAACAT; antisense, UGUGACUAGAAGGAGGAGT). PLC\(\gamma\) siRNA consisted of a pool of 4 different siRNA duplexes: sc-29452A (sense, CAAACCUAUGCCCAUUUUT; antisense, AAAGUUGGCAUGGGUUGTT), sc-29452B (sense, GCAAGAAUGUCCUGUAGTT; antisense, UAGUGAAAGACUUGUUGGTT), sc-29452C (sense, ACAGAGCAUUAAUGUUGGT; antisense, UACUGAAGAACCUUGUUGGTT), and sc-29452D (sense, CAACUCAUUGCCACAUAGT; antisense, AAUGUGGCCAGAAGUUGGTT). shRNA and siRNA sequences were purchased from Santa Cruz. Nontargeting control shRNA lentiviral particles (copGFP Control Lentiviral Particles; Santa Cruz) or control siRNA (nontargeting siRNA #2; Dharmacon) were used as controls. Knockdown with shRNA was followed by puromycin selection and functional validation.

**Mice and in vivo intervention studies.** To conditionally overexpress TRPV1 in IECs in mice, the *ROSA-stop\(_{\text{loxP}}\)-TRPV1-ires-ECFP* strain (31) was crossed to Villin-Cre mice. *Apc\(^{Min}\)/Trpv1\(^{-/-}\) mice were generated by crossing *Apc\(^{Min}\)/mice with *Trpv1\(^{-/-}\) mice (26). Follow-up and health monitoring of *Apc\(^{Min}\)/mice was performed as previously published (7). All mouse strains were on the C57BL/6J background and obtained from The Jackson Laboratory. Gefitinib, reconstituted in DMSO, was delivered by gavage in 0.05% (hydroxypropyl)methylcellulose and 0.02% Tween 80 vehicle at 50 mg/kg according to the indicated dosing schedules. For dietary studies, C57BL/6J mice were fed standard laboratory chow or chow mixed with 0.01% (w/w) capsaicin ad libitum (77) for the indicated time periods. *Apc\(^{Min}\)/ and *Apc\(^{Min}\)/Trpv1\(^{-/-}\) mice received normal chow (control) or chow plus 0.01% (w/w) capsaicin ad libitum starting at 8 weeks of age and were followed for survival studies or until they reached 20 weeks of age. Other cohorts of *Apc\(^{Min}\)/mice received low-dose celecoxib (300 ppm) mixed with chow (78), with or without 0.01% (w/w) capsaicin ad libitum. Chow was prepared

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freshly every week. For neonatal TRPV1– sensory neuron ablation, Apcmin/+ mice were treated with resiniferatoxin (50 μg/kg s.c.) on days 1, 2, and 7 after birth. Validation of systemic TRPV1– neuronal ablation was performed with the eye wipe test by ocular challenge with 0.01% (w/v) capsaicin in saline (47).

**Intestinal organoid culture and immunofluorescent staining.** Mouse intestinal organoids were generated from small intestines, following published protocols (23). Organoids were maintained in ENR (EGF, Noggin, RspoI) medium, which consisted of Advanced DMEM/F12 Reduced Serum Medium (Invitrogen) supplemented with 2 mM GlutaMAX, 10 mM HEPES, 100 μM penicillin and 100 μg/ml streptomycin, and B27 and N2 supplements (Invitrogen) with 50 ng/ml mEGF (Invitrogen), 100 ng/ml mNoggin (PeproTech), and 10% (v/v) RspoI conditioned medium. RspoI conditioned medium was generated using the 293T-HA-RspoI-Fc cell line (provided by C. Kuo, Stanford University, Stanford, California, USA). Matrigel (GFR) was obtained from BD Biosciences. WT and Trpv1+ organoids at similar developmental stages in parallel cultures were compared in the analyses. For EGF starvation experiments, EGF was removed from the culture medium 2 days after passing, for a period of 48 hours before analysis. For immunofluorescent stainings, organoids were grown in Matrigel and stained in 8-well chamber slides (Nunc). Immunofluorescent stainings were performed using anti-TRPV1 (Alomone Labs), anti-MMIP7 (Cell Signaling), anti-Ki67 (Abcam), or anti-PTPIB (Epitomics) primary Abs. Briefly, fixed organoids in Matrigel were stained with primary Abs (1:100 dilution) and detected with Alexa Fluor 488–conjugated anti-Rb secondary antibody (1:500 dilution). After counterstaining with Alexa Fluor 546–conjugated phallodin and DAPI, organoids were analyzed by confocal fluorescence microscopy. Staining with Alexa Fluor 488–conjugated wheat germ agglutinin and Alexa Fluor 546–conjugated phallodin were performed according to the manufacturer’s instructions. For Q-PCR analysis, organoids were extracted from Matrigel using BD Cell Recovery Solution following the manufacturer’s instructions.

**Ca2+ imaging.** Ca2+ imaging was performed as described previously (28, 29). Briefly, for imaging with cell lines (CHO, HEK293, or HCT116), cells were grown on coverslips and loaded with 5 μM Fura-2 AM (Invitrogen) dissolved in 0.01% Pluronic F-127 (Invitrogen) plus 0.1% DMSO in physiological salt solution (140 mM Na+, 5 mM K+, 2 mM Ca2+, 147 mM Cl−, 10 mM HEPES, and 10 mM glucose, pH 7.4) at room temperature (20°C) for 1 hour. Cells were then washed for 0.5 hours, mounted in a perfusion chamber on a Nikon microscope stage, and perfused with PSS. After obtaining baseline measurements at room temperature, cells were then stimulated with capsaicin, heated PSS (up to 40°C), or EGF (100 ng/ml). ATP (10 μM) was used as a positive control for [Ca2+]i measurements. In some conditions, cells were pretreated with either the TRPV1 antagonist BCTC (1–10 μM) or PIP2 (30 μM) for 30 minutes. For Ca2+ imaging with intestinal organoids, organoids generated from WT and Trpv1+ mice were passed and seeded 1 day before imaging. On the day of analysis, organoids were recovered from Matrigel, stained with 5 μM Fura-2 AM (1 hour), washed, attached on BD Cell-Tek–coated coverslips, and stimulated with capsaicin (30 μM). Data are shown as mean ± SEM from individual [Ca2+]i measurements based on recordings of F420/380 nm ratios. 

**IEC isolation and ex vivo stimulation.** Colons or small intestines were opened longitudinally, and feces were removed without scraping. Intestines were then washed in PBS (1 mM DTT) on ice. Intestines were minced into 3- to 5-mm pieces and incubated in HBSS (5 mM EDTA, 10 mM HEPES, and 0.5 mM DTT) in conical 50-ml tubes for 1 hour at 37°C. Intestinal pieces were then vigorously shaken (15 seconds) to detach crypts. The supernatants containing IECs were separated from larger pieces of nonepithelial tissue and spun. Pellets were washed in PBS, resuspended in lysis buffer, and stored at −80°C until protein or RNA analysis. IEC fractionation was performed following published protocols (79). Briefly, small intestines from WT mice were fractionated in 2 villus fractions (from distal to proximal along the crypt-villus axis) and 1 crypt fraction. For ex vivo stimulation of IECs, colons were cut open longitudinally and washed in PBS on ice. They were cut in halves and stimulated with or without EGF (50 ng/ml) for 15 minutes (37°C). Immediately after EGF stimulation, IEC isolation was performed (all steps on ice), and crypts were lysed in total cell lysis buffer for Western blot analysis.

**Flow cytometry.** TRPV1 antibodies were preincubated with 10-fold excess of blocking peptide or PBS. Cells were then stained with anti-TRPV1 (diluted 1:100) or goat IgG for 1 hour at 4°C. Primary goat anti-TRPV1 Abs were detected with rabbit anti-goat secondary Abs (Alexa Fluor 488 conjugate). Cells were run on a BD FACS Calibur flow cytometer or Accuri C6 (BD Biosciences).

**Immunohistochemistry.** Immunohistochemistry was performed as described previously (7), with minor modifications. Briefly, 4- to 6-μm paraffin sections from murine ileums or colons were incubated with anti-Ki67, anti-MMIP, anti-phospho-EGFR, or anti-PTPIB antibodies overnight. Abs were detected with biotinylated secondary Abs, HRP-streptavidin conjugates (Jackson), and visualized with DAB (Vector Laboratories). Counterstaining was performed with Hematoxylin 560 (Sur-gipath). The Aican Blue-P.A.S. Stain kit was obtained from American MasterTech, and stainings were performed according to the manufacturer’s instructions. The NanoZoomer 2.0-HT (Hamamatsu) slide scanner and NanoZoomer Digital Pathology software was used for histological analyses. Image analysis was performed with ImageJ (version 1.47) with the Colour Deconvolution or ImmunRatio plugin (version 1.0c).

**Q-PCR and gel electrophoresis.** RNA isolation was performed with the RNeasy Mini Kit or Micro Kit (Qiagen), and cDNA synthesis was performed with the gScript cDNA superMix kit (Quanta Biosciences) or the RT– First Strand Kit (Qiagen). Q-PCR was performed on the AB7300 Real-Time PCR System (Applied Biosystems) using PerfeCta SYBR Green FastMix (Quanta Biosciences) or a Roche Lightcycler 480 using SYBR Green qPCR Master Mix (SABiosciences/Qiagen). The custom-designed oligonucleotide sequences (IDT Technologies) used for Q-PCR are summarized in Supplemental Tables 1 and 2. Ptpn1 (NM_012101), Ptpn2 (NM_001211), Ptpn6 (NM_00177705, NM_013545), Ptpn7 (NM_011213), and Ptpn8 (NM_001252453, NM_001252455, NM_001252456, NM_011218) primer sets were obtained from SABiosciences/Qiagen. PCR products were run on 2% agarose gels and visualized with SYBR Safe DNA (Invitrogen).

**Statistics.** Data are presented as mean ± SD or mean ± SEM, as indicated. Unpaired, 2-tailed Student’s t test was used for statistical analyses to compare 2 data sets with normal distribution, with the exception of Figure 4, C and F (1-tailed Student’s t test); Mann-Whitney U test was used for nonparametric data; ANOVA was used to compare multiple data sets; and log-rank analysis was applied for survival curves (Graph-Pad Prism 5.0). A P value less than 0.05 was considered significant.

**Study approval.** All experimental procedures involving mice were reviewed and approved by the UCSD Institutional Animal Care and Use Committee (IACUC; animal protocol no. S02240).
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