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Essential role for epithelial HIF-mediated xenophagy in control of *Salmonella* infection and dissemination

Alexander S. Dowdell[1,2], **Ian M. Cartwright**[1,2], **David A. Kitzenberg**[1], **Rachael E. Kostelecky**[1], **Omemh Mahjoob**[1], **Bejan J. Saeedi**[1], **Nichole Welch**[1], **Louise E. Glover**[1,3], **Sean P. Colgan**[1,2,4,*]

[1]Mucosal Inflammation Program and Division of Gastroenterology and Hepatology, Department of Medicine, University of Colorado School of Medicine, Aurora, CO, USA

[2]Rocky Mountain Veterans Hospital, Aurora, CO, USA

[3]School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland

[4]Lead contact

SUMMARY

The intestinal mucosa exists in a state of "physiologic hypoxia," where oxygen tensions are markedly lower than those in other tissues. Intestinal epithelial cells (IECs) have evolved to maintain homeostasis in this austere environment through oxygen-sensitive transcription factors, including hypoxia-inducible factors (HIFs). Using an unbiased chromatin immunoprecipitation (ChIP) screen for HIF-1 targets, we identify autophagy as a major pathway induced by hypoxia in IECs. One important function of autophagy is to defend against intracellular pathogens, termed "xenophagy." Analysis reveals that HIF is a central regulator of autophagy and that *in vitro* infection of IECs with *Salmonella* Typhimurium results in induction of HIF transcriptional activity that tracks with the clearance of intracellular *Salmonella*. Work *in vivo* demonstrates that IEC-specific deletion of HIF compromises xenophagy and exacerbates bacterial dissemination. These results reveal that the interaction between hypoxia, HIF, and xenophagy is an essential innate immune component for the control of intracellular pathogens.

Graphical abstract

*Correspondence: sean.colgan@cuanschutz.edu.

AUTHOR CONTRIBUTIONS

A.S.D., L.E.G., and S.P.C. were responsible for project conceptualization, methodology, and formal analysis. A.S.D., D.A.K., O.M., B.J.S., N.W., and L.E.G. were responsible for investigation. I.M.C. and R.E.K. provided experimental resources. A.S.D. and S.P.C. wrote the original manuscript draft and subsequent revisions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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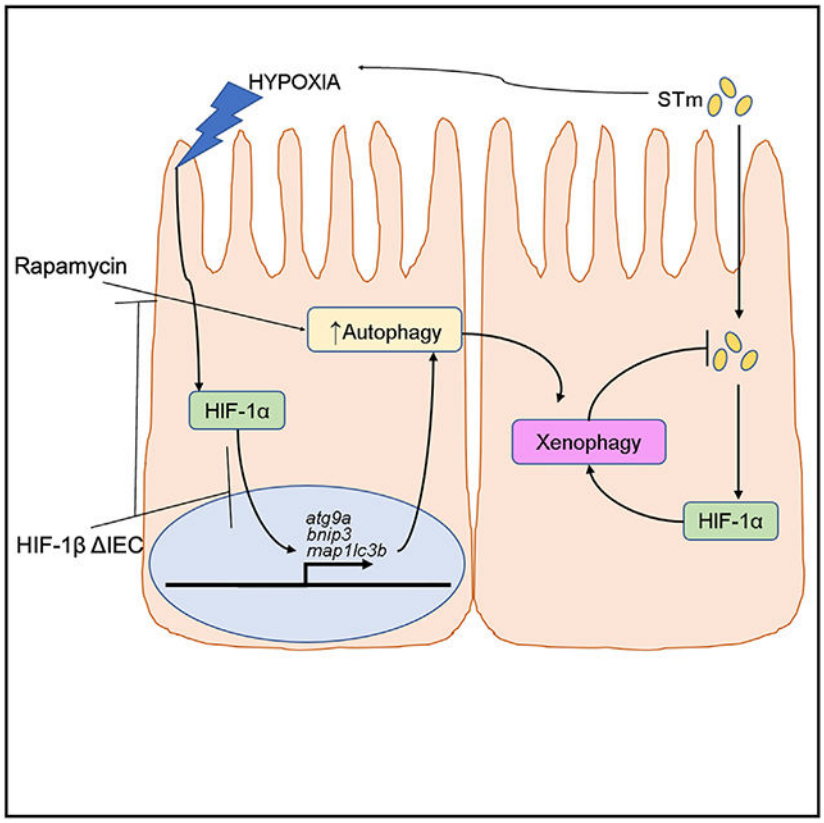

In brief

Dowdell et al. show that hypoxia, through stabilization of HIF-1α, activates autophagy in intestinal epithelial cells (IECs). Further, the model invasive bacterium *Salmonella* Typhimurium stabilizes HIF in IECs to trigger anti-bacterial autophagy (xenophagy). This mechanism demonstrates an essential mucosal innate immune response for control of invasive pathogens.

INTRODUCTION

Inflammatory bowel disease is a family of disorders characterized by chronic, relapsing inflammation of the gastrointestinal tract (Jairath and Feagan, 2020). The prevalence of inflammatory bowel disease (IBD) varies worldwide and corresponds roughly with the degree of industrialization; as a result, developed countries show the highest rates of IBD—greater than 1 in 200 in the USA and Europe—but with accelerating prevalence rates in the developing world due to burgeoning industrialization (Molodecky et al., 2012). IBD is currently subdivided into two major classes, Crohn's disease (CD) and ulcerative colitis (UC), and although each demonstrates differing presentations and susceptibility factors, all forms of IBD are lifelong conditions with no cure that imparts a reduced quality of life (Cleynen et al., 2016). Although environmental factors (Ananthakrishnan et al., 2018) and genetics (Cleynen et al., 2016) have been implicated, the driving forces for IBD remain unclear, and the precise molecular mechanisms of IBD pathophysiology have yet to be elucidated.

One of the first genetic polymorphisms identified to confer susceptibility to IBD was in the gene *nod2/card15*, which encodes a cytoplasmic sensor to muramyl dipeptide (MDP; a peptidoglycan component) (Hugot et al., 2001; Ogura et al., 2001). These polymorphisms are most commonly found in the MDP-sensing domain of NOD2, resulting in a nonfunctional protein unable to detect intracellular bacteria and a corresponding aberrant inflammatory response (Girardin et al., 2003; Gutierrez et al., 2002; Yamamoto and Ma, 2009). Notably, NOD2 is expressed in distinct intestinal epithelial lineages, including Paneth cells—a cell type important for host-microbe interactions in the intestine (Ogura et al., 2003). Consequently, lack of functional NOD2 results in elevated levels of commensal bacteria and a compromised ability to kill intestinal pathogens such as *Salmonella* Typhimurium (STm) and *Listera monocytogenes* (Petnicki-Ocwieja et al., 2009). The mechanism of increased IBD susceptibility imparted by *nod2* mutations was later found to be dependent on (macro)autophagy, a highly conserved eukaryotic system in which cytoplasmic constituents are targeted to and degraded in the lysosome (Yang and Klionsky, 2010). Autophagy acts on numerous intracellular substrates, such as misfolded proteins and organelles, but can also target intracellular pathogens in a process termed “xenophagy” (Dikic and Elazar, 2018). Defects in NOD2 impair the ability for autophagy proteins, notably ATG16L1, to localize to the plasma membrane at the site of bacterial entry, preventing an adequate xenophagic response and permitting intracellular bacterial survival (Travassos et al., 2010). ATG16L1 is an essential autophagy protein that, like NOD2, has been found to be mutated at a higher frequency in patients with IBD patients (Hampe et al., 2007; Mizushima et al., 2003; Rioux et al., 2007). The lack of functional ATG16L1, in turn, compromises autophagy and the xenophagic defense against intracellular bacteria(Homer et al., 2010; Kuballa et al., 2008). The integration of NOD2 and ATG16L1 as an intracellular xenophagic pathway was further confirmed by the discovery that the protein IRGM, itself also an IBD risk factor, orchestrates the interaction between NOD2 and ATG16L1 to promote anti-bacterial xenophagy (Brest et al., 2011; Chauhan et al., 2015; Parkes et al., 2007). Although it is currently understood that xenophagy plays an essential role in intestinal homeostasis and IBD pathogenesis, the regulation of the autophagic factors necessary for efficient bacterial clearance is not as clearly defined and the mechanisms of xenophagy regulation *in vivo* are unclear.

The intestinal tract is noteworthy in that it exists in a state of “physiologic hypoxia”—that is, it maintains homeostasis at oxygen tensions far below that of ambient air (Taylor and Colgan, 2017). Adaptation to this austere environment is mediated through the transcription factor family hypoxia-inducible factor (HIF), which is composed of three oxygen-labile subunits (HIF-1α, −2α, and −3α) and an oxygen-insensitive nuclear binding partner (HIF-1β/ARNT) (Colgan et al., 2020). These factors, members of the Per-ARNT-Sim family of basic helix-loop-helix transcription factors, modulate gene expression of a large number of direct targets including those responsible for a metabolic shift toward glycolysis and proteins involved in maintenance of epithelial integrity and regulation of innate immune function (Colgan et al., 2016; Dengler et al., 2014; Glover et al., 2016). Recent work has also shed light on the importance of HIF signaling in the regulation of autophagy. HIF-1α has been shown to regulate hypoxia-induced autophagy in mouse embryonic fibroblasts through BNIP3 and BNIP3L/NIX; likewise, the essential autophagy protein

ATG9A, necessary for intestinal barrier/tight junction biogenesis, was found to regulated by HIF/hypoxia in intestinal epithelial cells (IECs) (Bellot et al., 2009; Dowdell et al., 2020; Saitoh et al., 2009).

Given the physiologic state of hypoxia in the intestine and considering the demonstrated regulation of autophagy by HIF/hypoxia, it follows that HIF-driven autophagy/xenophagy may regulate host-microbe interactions at the intestinal mucosa. In this study, we demonstrate an essential role for HIF in the regulation of autophagy in the intestinal epithelium. Further, we show that HIF/hypoxia regulates anti-bacterial autophagy (xenophagy) in IECs both *in vitro* and *in vivo* and, conversely, that intestinal bacteria regulate HIF and HIF target gene expression in the intestinal epithelium. Finally, we show that HIF is protective during infection of IECs with the model invasive pathogen STm using both *in vitro* and *in vivo* models. The results presented herein shed light on the role of HIF in regulating host-microbe interactions at the intestinal mucosa and provide insight into the xenophagic mechanisms dysregulated in IBD, informing the development of the next generation of IBD therapeutics.

RESULTS

HIF drives autophagy gene expression in IECs

Previous work suggests that HIF drives autophagy in a variety of cell types, including epithelial cell lineages (Bellot et al., 2009; Dowdell et al., 2020). Using our previously reported chromatin immunoprecipitation (ChIP)-chip screen (Glover et al., 2013) in which Caco-2 IECs were subjected to hypoxia, immunoprecipitated DNA was obtained using either anti-HIF-1α or anti-HIF-2α antibodies following specific fixation protocols. ChIP-enriched DNA was then hybridized to a custom microarray comprising a genome-wide set of predicted transcription start site flanking sequences at 50 bp resolution. From this dataset, we asked whether autophagy targets would be enriched in HIF-precipitated samples. Principal-component analysis (PCA) demonstrated that autophagy genes grouped well together with known HIF target genes, including *pdk1* and *hmox1* (Figure 1A). Further, we observed that autophagy and HIF-regulated genes clustered together separately from housekeeping genes such as *tuba1* and *rps27l*, suggesting a distinct HIF-directed autophagic transcriptional program induced by hypoxia. Autophagy and HIF target genes showed distinct enrichment in ChIP-chip analysis as visualized by heatmap analysis (Figure 1B), whereas no such enrichment was observed for a variety of housekeeping genes. Notably, several autophagy genes (e.g., *atg9a*, *atg5*) demonstrated increased enrichment in HIF-1α-precipitated samples versus HIF-2α counterparts (Figure 1B), suggesting a distinct transcriptional response guided by a specific HIF isoform. We then sought to validate our ChIP-chip analysis through qPCR analysis of hypoxia-treated Caco-2 cells. Analysis of this cell line indicated up-regulation of a variety of targets genes implicated in both autophagy and xenophagy, including *irgm*, *map1lc3a*, and *bnip3* (Figure 1C), in agreement with our ChIP-chip observations. Finally, we sought to confirm our observations using nontransformed cells, as previous observations have indicated that differences in autophagy can exist between normal and cancer IECs (Groulx et al., 2012). Validation via hypoxia treatment of murine colonoids from wild-type C57BL/6J mice resulted in the significant

induction of a subset cohort of autophagy genes compared with normoxia-treated controls (Figure 1D), indicating that our initial observations were not due to a cancer cell phenotype and suggesting that expression of autophagy genes are regulated by hypoxia and, more specifically, by HIF.

Autophagic flux is enhanced by hypoxia in IECs

Given the finding that autophagy genes appear to be HIF targets in IECs, we next sought to examine whether autophagic flux itself is regulated by hypoxia in IECs. Autophagic flux describes "the entire process of autophagy over a period of time" and more accurately reflects the biological process of autophagy in living systems than autophagic gene expression alone (Klionsky et al., 2021). To do so, we first exposed Caco-2 IECs plated on glass coverslips to hypoxia (1% O_2, 24 h), then fixed and stained them against the autophagosome-associated proteins LC3 and p62/SQSTM1. Observation of stained cells by indirect immunofluorescence (IF) indicated an increased degree of LC3 and p62 puncta formation and co-localization in hypoxia-treated cells versus normoxia-treated controls (Figure 2A), a finding suggestive of increased autophagic flux. We then assessed LC3 and p62 abundance in IECs by western blot (WB) with and without hypoxia treatment for varying lengths of time. We observed a time-dependent increase in the band intensity of LC3-I (upper band) to LC3-II (lower band) conversion in hypoxia-treated cells, as well as a corresponding decrease in p62 band intensity, compared with normoxia-treated controls (Figure 2B). These results indicate that molecular markers of autophagic flux, namely LC3 lipidation and p62 degradation (Jiang and Mizushima, 2015), are both enhanced by hypoxia and suggest that autophagic flux is enhanced by hypoxia. We investigated this further by treating tandem-fluorescent LC3 (tf-LC3)-expressing Caco-2 cells with hypoxia and imaging by IF. As previously reported, tf-LC3 is an mRFP-GFP-LC3 fusion protein that demonstrates both green and red fluorescence in the cytoplasm but, due to acidic quenching of GFP, demonstrates only red fluorescence after autophagosome-lysosome fusion (Kimura et al., 2007). In addition, tf-LC3 shows increased puncta formation during heightened autophagic flux (similar to wild-type [WT] LC3), resulting in an increase in yellow puncta (autophagosomes) and red puncta (autolysosomes) during certain stresses, such as starvation (Kuma et al., 2017). To this end, we observed an increase in both yellow and red puncta in hypoxia-treated, tf-LC3-expressing Caco-2 cells versus normoxic controls (Figure 2C), indicating an active increase in autophagic flux in these cells during hypoxia treatment. Finally, treatment of murine C57BL/6J colonoids with hypoxia resulted in stabilization of HIF-1α and induction of BNIP3 (Figure 2D), a protein previously shown to be important for hypoxia-related autophagy (Bellot et al., 2009), further demonstrating that hypoxia induces autophagy through HIF in IECs and that this induction is not limited to cancer cell lines.

Infection of IECs by STm stabilizes HIF and activates HIF signaling

Previous findings indicate that bacteria can regulate HIF signaling both *in vitro* and *in vivo* in a variety of cell types (Hartmann et al., 2008; Peyssonnaux et al., 2005). As HIF plays an indispensable role in autophagy, and given that regulation of autophagy appears to be governed in an HIF-1α-dependent fashion, we sought to examine the role of bacterial regulation of HIF-1α-driven autophagy *in vitro*. We first examined this by infecting Caco-2 cells with STm, a model invasive bacterium used extensively in the study of xenophagy

(Birmingham et al., 2006; Mimouna et al., 2014; Tattoli et al., 2012), in a dose-response manner by treating at varying multiplicities of infection (MOIs) and in a time course fashion at a set MOI. 300 μM $CoCl_2$ was used as a positive control for HIF stabilization, as previously described (Epstein et al., 2001). We observed that HIF-1α was stabilized by infection with STm in a time- and dose-dependent manner (Figure 3A), indicating bacterial regulation of HIF as reported previously and confirming that STm stabilizes HIF in our model system. We further examined expression of HIF target genes by qPCR to assess whether stabilized HIF-1α was transcriptionally active. We found that expression of HIF target genes involved in autophagy was induced by STm (Figure 3B), suggesting regulation of the HIF transcriptional repertoire through bacterial infection.

HIF stabilization by STm through oxygen consumption

We next sought to examine the mechanism by which STm stabilizes HIF in IECs *in vitro*. Previous reports have documented HIF stabilization in a variety of cell lines by pathogenic bacteria, though the mechanism(s) of this stabilization are unclear (Cane et al., 2010; Hartmann et al., 2008; Legendre et al., 2011; Mimouna et al., 2014; Peyssonnaux et al., 2005, 2008; Sharma et al., 2011; Werth et al., 2010). While one study reported the secretion of siderophores as one possible mechanism for bacterial-mediated HIF stabilization (Hartmann et al., 2008), we suspected alternative mechanisms and asked whether oxygen consumption was important for HIF stabilization due to STm. Using an oxygen-sensing microplate system previously utilized by our group to measure *in vitro* oxygen tensions and consumption (Campbell et al., 2014; Kelly et al., 2015), we found that treatment of IECs with STm resulted in a dramatic decrease in oxygen levels to well below the threshold usually considered to stabilize and activate HIF after an initial O_2 equilibration/stabilization period (Figure 4A) (Koh and Powis, 2012). In contrast, uninfected IECs stabilized at a nonhypoxic oxygen tension. Crucially, hypoxia was not observed in control wells containing buffer alone or buffer + STm. To corroborate these results, HeLa cells were transiently transfected with a plasmid containing luciferase driven by 5× serial hypoxia-response elements (HREs) derived from *vegf* (HRE-Luc); this construct has been shown to demonstrate luciferase induction during HIF stabilization (Walton et al., 2018). HeLa cells were utilized, as they are an excellent model cell line for *Salmonella* infection, including in studies of autophagy, and are highly transferable (Steele-Mortimer, 2008; Tattoli et al., 2012). Infection of HeLa cells containing HRE-Luc with STm resulted in a significant increase in luciferase activity versus uninfected cells (Figure 4B), indicating that STm drives activity from HREs during infection of epithelial cells through stabilization of HIF.

HIF and hypoxia are protective during *in vitro* infection of IECs with STm

Given that STm stabilizes HIF and drives expression of HIF-regulated genes, including autophagy genes, and as autophagy plays an essential role in the defense against intracellular pathogens (xenophagy) (Huang and Brumell, 2014), we asked whether HIF-regulated autophagy was protective during infection of IECs with STm. To investigate this, we generated stable HIF knockdown (KD) IEC lines through delivery of short hairpin RNA (shRNA) via lentiviral transduction. These lines were validated by incubating with or without the HIF stabilizing agent $CoCl_2$ and assessing HIF isoforms through WB; results

indicate successful depletion of the targeted HIF isoforms in the various cell lines relative to cells expressing nontargeting shRNA (Figure 5A). Next, we infected these cell lines with STm and measured bacterial replication with or without hypoxia treatment, a metric that has been previously used to show increased susceptibility to *Salmonella* in autophagy-deficient cells (Birmingham et al., 2006). Bacterial replication was quantified by infecting IECs with STm, then treating them with the membrane-impermeable antibiotic gentamicin to restrict extracellular growth of bacteria. This results in only intracellular bacteria remaining viable, with differences in bacterial replication reflected in changes in viable colony-forming units (CFUs) following collection of cell lysates and plating of serial dilutions (Steele-Mortimer, 2008). Quantification of intracellular CFUs demonstrated that hypoxia resulted in a decrease in intracellular growth of STm in control cells, a phenotype that was abolished in HIF KD cells and suggestive of HIF-driven xenophagy (Figure 5B). No differences in cell survival were observed between experimental groups, and, as each group was infected at an equal MOI, these results indicate that the observed differences in intracellular CFUs were likely due to variations in xenophagy. Interestingly, HIF-1α KD cells demonstrated significantly elevated levels of bacterial replication compared with control IECs; however, all HIF KD cell lines showed no benefit from hypoxia incubation, demonstrating that the protective effects of hypoxia with regards to restricting intracellular growth of STm were mediated by HIF. We then confirmed these observations by infecting WT IECs with STm in the presence of compounds previously shown to enhance (rapamycin [RAP]; through inhibition of mTOR) or inhibit (3-methyladenine) autophagy. As expected, an increase in autophagic flux via treatment with RAP was protective by reducing the intracellular burden of STm, which was phenocopied by treatment with hypoxia (Figure 5C). Further, inhibition of autophagy by 3-methyladenine was detrimental and enhanced intracellular STm levels. These results recapitulate previous findings in the nonintestinal HeLa cell line HeLa and in peritoneal macrophages, validating our observations that autophagy positively regulates STm infection *in vitro* (Owen et al., 2014; Tattoli et al., 2012). Taken together, these results indicate that xenophagy is a critical mechanism by which intracellular STm replication is controlled in IECs and that hypoxia (through HIF) is protective through regulating anti-STm xenophagy.

Regulation of autophagy by HIF and activation of HIF by STm *in vivo*

Next, we sought to address whether our observations *in vitro* held true in animal models. To do so, we first utilized mice expressing a luciferase construct fused to the oxygen-dependent degradation domain (ODD) from HIF-1α (ODD-Luc). These mice show luciferase activity only under conditions that permit HIF stabilization; as such, the ODD-Luc fusion protein acts as a faithful reporter for hypoxia/HIF stabilization and has been used *in vivo* by our group for this purpose (Campbell et al., 2014). Following infection of ODD-Luc mice with STm, we observed enhanced luciferase activity in the terminal ileum, cecum, and proximal colon compared with uninfected ODD-Luc controls, indicating increased HIF stabilization (Figure 6A). These results recapitulate our *in vitro* results demonstrating induction of HIF signaling by STm infection (Figures 3B and 4B). Next, we asked what role HIF signaling plays in the xenophagic defense against STm *in vivo*. To investigate this, we first generated mice selectively lacking Hif-1β in the intestinal epithelium (Hif-1β ΔIEC) by crossing mice with a floxed *Hif-1β* allele to mice expressing Cre recombinase

under the *Villin* promoter, which shows high specificity for expression in the intestinal epithelium (Maunoury et al., 1992). Hif-1β ΔIEC mice were used as opposed to Hif-1α ΔIEC mice due to the potential redundancy and/or compensation by Hif-2α, as has been noted before (Koh and Powis, 2012). The strategy behind the generation, and the subsequent genotype validation, of Hif-1β ΔIEC mice has been described elsewhere by our group, and, notably, these mice show no defects in gross intestinal development (Glover et al., 2013). Examination of autophagy in epithelial-enriched intestinal scrapings of Hif-1β ΔIEC mice reveals a striking defect in autophagic flux, evidenced by the diminished conversion of LC3-I to LC3-II and increased levels of p62, compared with littermate controls expressing Hif-1β (i.e., Cre^- littermates) (Figure 6B). These findings indicate an *in vivo* dependence of autophagy on HIF signaling and indicate that its absence impairs normal autophagic flux. Next, we sought to examine expression of representative target genes in the colonic epithelium of Hif-1β-expressing and Hif-1β ΔIEC mice. As expected, Hif-1β ΔIEC mice show significantly diminished colonic *Hif-1β* expression in the colonic epithelium relative to littermate controls, as well as diminished expression of the prototypic HIF targets *Pgk1* and *Ckb* (Figure 6C) (Glover et al., 2013). However, Hif-1β ΔIEC mice also demonstrated significantly lower expression of *Atg9* in the colonic epithelium; as Atg9 is an essential autophagy gene (Saitoh et al., 2009), this indicates that normal levels of autophagy are compromised at the transcriptional level by the loss of Hif-1β and that HIF signaling is an inherently important component for autophagy in the intestinal epithelium. Given these observations, we then asked whether HIF-regulated autophagy would be protective *in vivo* during STm infection (i.e., through xenophagy) as it was observed to be *in vitro*. Hif-1β ΔIEC mice and littermate controls were infected with STm through established protocols (Bellot et al., 2009; Conway et al., 2013), after which mice were euthanized and samples collected. Although no difference was observed between HIF-1β ΔIEC mice and controls with regards to extracellular colonization by STm (i.e., fecal samples) (Figure 6D), HIF-1β ΔIEC mice demonstrated a significantly higher dissemination of STm to extraintestinal sites, including liver and spleen, as well as a reduced colon length—a metric that is directly correlated with the severity of intestinal inflammation (Figure 6E) (Garcia-Hernandez et al., 2021). These results demonstrate that HIF regulates autophagy in the normal intestinal epithelium *in vivo*, similar to what is observed *in vitro*, and that HIF-driven autophagy is protective during STm infection by limiting extraintestinal spread of pathogens and limiting inflammation.

HIF-xenophagy axis regulates susceptibility to *Salmonella* infection *in vivo*

Finally, we asked whether pharmacologic modulation of the HIF-xenophagy axis in HIF-1β ΔIEC mice could rescue the phenotypes of defective xenophagy and susceptibility to STm infection (Figure 6). To do so, we treated HIF-1β ΔIEC mice and littermate controls either with the mTOR inhibitor (autophagy activator) RAP or vehicle daily beginning 3 days prior to infection using a formulation and dosage scheme previously demonstrated to achieve robust induction of autophagy (Johnson et al., 2013). We chose to use RAP in order to clarify the underlying mechanism by which HIF-dependent autophagy regulates host-microbial interactions. Preliminary experiments in WT C57BL/6J mice indicated robust activation of autophagy in colonic IECs using this treatment regimen (Figure S1). With this approach, we then infected mice with STm as done before and assessed the extent

of disease severity. RAP treatment did not influence colonization of mice (Figure S2); however, we did observe significantly diminished extracellular dissemination of STm to the liver in Hif-1β$^{+/+}$ mice treated with RAP compared with vehicle-treated controls (Figure 7A), suggesting that activation of autophagy via RAP was protective through limiting systemic spread of STm. Further, we saw no such protection in RAP-treated HIF-1β ΔIEC mice, suggesting that the observed protection by RAP in Hif-1β$^{+/+}$ mice was due to activation of autophagy specifically in the intestinal epithelium. In agreement with these data, we observed significantly lower levels of pro-inflammatory tissue cytokines in RAP-treated Hif-1β$^{+/+}$ mice (Figure 7B), indicating attenuated intestinal inflammation during RAP administration. In contrast, HIF-1β ΔIEC mice showed elevated levels of tissue cytokines versus vehicle-treated controls, demonstrating a more severe state of intestinal inflammation in the HIF-1β ΔIEC+ RAP cohort. Finally, blinded histopathological analysis (Figure 7C) of cecal tissue from RAP-treated Hif-1β$^{+/+}$ mice showed a decrease in the "epithelial integrity" subscore relative to vehicle-treated controls, indicative of a more intact epithelium with less severe erosion and ulceration (Figure 7D, top) (Barthel et al., 2003). By contrast, RAP-treated HIF-1β ΔIEC mice showed a significantly higher epithelial integrity histopathology score versus vehicle controls, which is indicative of more severe epithelial deterioration (Figure 7D, bottom). These data demonstrate that the protective effect of RAP was lost in the absence of epithelial Hif-1β, resulting in more severe damage to the intestinal epithelium during infection. Taken together, these results show that HIF is protective during STm infection through regulating autophagy/xenophagy in IECs and that the protective pharmacological activation of autophagy is lost in the absence of epithelial HIF signaling.

DISCUSSION

Previous studies have highlighted the varied mechanisms by which HIF influences intestinal homeostasis; these include regulation of creatine energetics, expression of pro-barrier genes/factors, and regulating the cellular response to "beneficial" microbiota-derived metabolites (e.g., SFCAs) (Glover et al., 2013; Karhausen et al., 2004; Kelly et al., 2015). Similarly, *in vivo* activation of HIF signaling has been demonstrated to be protective in murine models of colitis (Cummins et al., 2008; Robinson et al., 2008), and as such, pharmacological activation of HIF is a promising strategy for the development of IBD therapeutics (Manresa and Taylor, 2017). However, the mechanism by which HIF signaling promotes intestinal homeostasis and resilience are not well understood; indeed, HIF has been shown to directly regulate dozens of target genes (Dengler et al., 2014) and possibly hundreds more indirectly, resulting in many possible complementary pathways through which HIF is protective during intestinal inflammation. The studies presented here provide insight into an underexplored area of HIF-assisted innate immunity that adds to the arsenal of the mucosa in defense of potentially invasive microbes.

One biochemical pathway shown to be associated with HIF is (macro)autophagy, a highly conserved eukaryotic pathway in which cytoplasmic organelles, proteins, etc., are enveloped in characteristic double-membraned autophagosomes, then targeted to the lysosome for degradation (Mehrpour et al., 2010). Autophagy occurs in every cell in the body, and, accordingly, defects in autophagy underlie the molecular pathology of a diverse collection of diseases such as neurodegenerative disorders (e.g., Alzheimer's disease) (Wong and

Cuervo, 2010) and cardiomyopathies (e.g., Danon disease) (Gottlieb and Mentzer, 2013) and the development and treatment of various cancers (Mathew et al., 2007). IBD is no different, and it is theorized that defects in anti-bacterial autophagy, or xenophagy, underlie the pathogenesis of IBD based on previously characterized IBD susceptibility genes (Lassen and Xavier, 2017). Although anecdotal evidence exists for the use of autophagy activating compounds in the treatment of IBD (Dumortier et al., 2008; Massey et al., 2008), no FDA-approved therapy currently exists that leverages autophagy to treat IBD.

The present study presents evidence that autophagy is regulated by HIF in the intestinal epithelium and that this regulation, through activation of xenophagy, is protective both *in vitro* and *in vivo* against the prototypic invasive pathogen STm. Further, we present evidence that exogenous stimulation of autophagy via pharmacological intervention is beneficial during STm infection through stimulating xenophagy in an HIF-dependent manner. These findings, collectively considered, demonstrate an underappreciated role for HIF signaling in regulating xenophagy in the intestinal epithelium. They are in accord with previous studies that show an essential role for autophagy in the defense against invasive pathogens and the maintenance of commensal microorganisms (Birmingham et al., 2006; Conway et al., 2013; Petnicki-Ocwieja et al., 2009). Further, these results expand upon the already well-established role of HIF in maintaining intestinal epithelial homeostasis, indicating a unique function for HIF *in vivo* through the regulation of protective xenophagy (Glover et al., 2013; Karhausen et al., 2004). Finally, results presented herein suggest that modulation of the HIF-autophagy axis may be beneficial in the context of intestinal inflammation through regulating host-microbe interactions at the intestinal epithelium. Interestingly, we observed that the beneficial effect of RAP was lost in Hif-1β ΔIEC mice, suggesting a bi-directional mode of communication between the HIF and autophagy pathways. Although our data do support a conclusion that HIF drives autophagy in the intestinal epithelium, one possibility is that the loss of baseline autophagy resulting from HIF-1β knockout (Figures 6B and 6C) causes the intestine to become less sensitive to autophagy induction mediated by RAP. This would suggest that a dependency exists between autophagy and HIF signaling for regulation of host-microbe interactions in the gut.

Although the work presented here demonstrates a distinct role for the regulation of xenophagy by HIF, observations regarding the HIF-xenophagy axis have been described before in more limited contexts. One study previously suggested a role for HIF-1α in the xenophagic response to adherent-invasive *Escherichia coli* (AIEC) in IECs (Mimouna et al., 2014). However, that mechanism was found to be dependent on the specific AIEC receptor CEACAM6 (Barnich et al., 2007), and, as such, it is unclear how broadly applicable these findings are to intestinal host-microbe interactions. In particular, STm appears to recognize a diverse array of cell-surface receptors including GP2, MUC1, and CD209 (Hase et al., 2009; Li et al., 2019; Ye et al., 2019). This suggests that the observations made in this study are more broadly applicable toward a range of enteric microorganisms, and current studies in our group are focused on defining the role of HIF-driven autophagy/xenophagy in response to a wider variety of commensal, opportunistic (pathobionts), and pathogenic microbes. Additionally, a recent study found that HIF-1α appears not to contribute toward the host defense against STm *in vivo* (Robrahn et al., 2022). Although the authors of this study did observe STm-induced HIF-1α stabilization in the intestinal epithelium, epithelial-

specific loss of HIF-1α did not appear to worsen outcomes during STm typhlitis/colitis. One explanation for the discrepancy between this study and the results published herein is the potential that HIF-2α can compensate for the loss of HIF-1α in the setting of HIF-driven xenophagy. HIF-2α has been previously shown to be induced by the loss of HIF-1α in a compensatory manner (Carroll and Ashcroft, 2006), which could obscure any phenotype resulting from the loss of HIF-1α alone. It is also notable that, in addition to the xenophagic response described here, HIF-1-mediated signaling influences other epithelial antimicrobial defenses, including the transcriptional regulation of defensins (Kelly et al., 2013). Our studies done *in vivo* rely on the loss of HIF-1β, abolishing overall HIF signaling and allowing for interrogation of the role of HIF in general to intestinal epithelial xenophagy.

Although intact HIF-driven xenophagy is essential for the control of invasive bacteria, STm, as well as several other intracellular bacteria, have evolved strategies in which they can evade host xenophagic responses to establish productive infections (Huang and Brumell, 2014). In STm, these strategies include reactivation of mTOR following infection by targeting LKB1, SIRT1, and AMPK to the lysosome for degradation, suppressing autophagy, and promoting intracellular bacterial survival (Ganesan et al., 2017; Tattoli et al., 2012). In addition, *Shigella flexneri* evades xenophagy through the secretion of IcsB, a virulence factor that masks the bacterial autophagy target IcsA/VirG on the bacterium's surface and thereby prevents recognition by host ATG proteins (Ogawa et al., 2005). Expression of ActA by *Listeria monocytogenes* similarly "hides" the bacteria from host xenophagy by recruiting host cytoskeletal proteins and camouflaging itself as an organelle (Yoshikawa et al., 2009). Further, *Mycobacterium tuberculosis* can suppress the induction of autophagy altogether in macrophages through secretion of the factor Eis, which induces interleukin-10 (IL-10) expression and activates PI3K/Akt/mTOR signaling (Duan et al., 2016). These findings underscore the observation that pathogenic bacteria and their host cells exist in a sort of molecular "tug of war"—mechanisms that the eukaryotic host use to control infection are simultaneously antagonized by their targeted microorganisms.

Our results suggest that pharmacological intervention targeting the HIF-autophagy axis is a viable strategy for attenuation of intestinal inflammation, particularly that stemming from an infectious source. This observation has particular importance in the current "post-antibiotics" era, in which antimicrobial resistance (AMR) is a major health crisis and is responsible for an estimated 4.95 million deaths in 2019 alone (Antimicrobial Resistance, 2022). Further, one review of medical data from 2012 to 2017 found that >20% of USA hospitalizations were due to multi-drug-resistant (MDR) bacteria, representing over 41.6 million hospitalizations in that 6 year span and highlighting the severe burden that MDR microbes place on the USA healthcare system (Jernigan et al., 2020). The crisis of AMR is complicated by a dearth of therapeutics currently in development as well as wide-spread improper use of existing antimicrobials, which further contributes toward the prevalence of AMR (Ardal et al., 2020; Kardas et al., 2005). By mobilizing the epithelium's innate defenses against invasive pathogens, activation of the HIF-autophagy axis would promote clearance of pathogens without encouraging consequent AMR. Such an approach has already been demonstrated to be beneficial *in vitro* against STm (Tattoli et al., 2012) and *M. tuberculosis* (Gutierrez et al., 2004), and our findings suggest that such an approach is applicable toward *in vivo* systems. In addition, *in vivo* stabilization of HIF has been

demonstrated to be protective during chemical models of colitis (Cummins et al., 2008; Robinson et al., 2008), and, as these are highly dependent on the presence and constitution of the gut microbiota (Gkouskou et al., 2014), it is possible that one mode by which HIF stabilizer confers protection is by activating HIF-regulated autophagy/xenophagy to maintain epithelial homeostasis. We are currently exploring the role for pharmacological agents in regulating the HIF-autophagy axis as a means of regulating host-microbial interactions, including those involving MDR bacteria and other clinically relevant microbes.

Taken together, we have found that HIF controls autophagy in the intestinal epithelium and that this interaction promotes xenophagy in a protective fashion during infection by invasive bacteria. These findings reveal a distinctive homeostatic innate immune mechanism for HIF in the intestine. Likewise, this work lays the ground-work for future studies seeking to modulate host-microbial interactions through clinical intervention via the HIF-autophagy axis.

Limitations of the study

Although our group has strived to be comprehensive and discerning with regards to experimental design and interpretation, we do acknowledge some limitations of the current study. Specifically, our group utilizes STm as a model intracellular organism, and it is unclear as to the broad applicability of these findings toward other enteric microbes. The universality of HIF activation by prokaryotes is an area under active investigation by our group. Further, we acknowledge that our mouse model is deficient in both Hif-1α and Hif-2α signaling through deletion of Hif-1β. Although current evidence suggests that HIF-1α is the primary factor through which HIF-driven xenophagy is activated, we cannot fully exclude a role for HIF-2α in the bacterial activation of autophagy/xenophagy. Further studies will seek to elucidate the specific roles of HIF isoforms in governing the HIF-regulated autophagic response to intestinal bacteria. Lastly, the murine model of *Salmonella* used relies on ablation of the intestinal microbiota through streptomycin pretreatment to diminish colonization resistance. The role of the commensal microbiota in regulating HIF-driven autophagy, therefore, could not be interrogated, and its role in regulating intestinal autophagy is an area of active investigation by our group.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sean Colgan (sean.colgan@cuanschutz.edu).

Materials availability—This study did not generate unique reagents.

Data and code availability

Section 1: Data

This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.

Section 2: Code

This paper does not report original code.

Section 3:

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mammalian and bacterial cell culture—C2BBe1 is a colorectal adenocarcinoma cell line described previously by our group (Alexeev et al., 2021; Dowdell et al., 2020; Glover et al., 2013). HeLa is a cervical adenocarcinoma cell line with high transfection efficiency. Caco-2 and HeLa cells were maintained in IMDM supplemented with 1× GlutaMAX, 1× Penicillin/Streptomycin, and 10% heat-inactivated bovine calf serum. Murine colonic organoids ("colonoids") were prepared from healthy, wild-type C57BL/6J mice and maintained using standard protocols (Miyoshi and Stappenbeck, 2013). When necessary, antibiotics were omitted from cell culture media to prevent killing during bacterial treatments. Mammalian cell lines were maintained at 37°C, 5% CO_2 in humidified incubators decontaminated regularly. Hypoxia incubations were performed in a humidified controlled atmosphere incubation chamber (Coy Laboratory Products, Inc.) fed with a feed gas of 94% N_2, 5% CO_2, and 1% O_2 and kept at 37°C. All cell lines used were tested for mycoplasma at least once a month using established protocols (Young et al., 2010). Stable knockdown of HIF isoforms were performed via lentiviral transduction according to previously established protocols (Cartwright et al., 2020; Wang et al., 2020) using the TRCN accession numbers given in the Key Resources Table. Plasmids containing shRNA for lentiviral packaging were purchased from the Functional Genomics Core Facility (CU Anschutz, Aurora, CO).

Salmonella enterica subsp. *enterica* serovar Typhimurium strain SL1344 (STm) was maintained as glycerol stocks (15% (v/v) glycerol) at −80°C and struck weekly onto fresh plates for preparation of liquid cultures. STm was regularly propagated in LB-Miller broth or on LB-Miller agar, each containing 100 μg/mL streptomycin. Subcultures of STm were prepared by inoculating 20 mL of LB-Miller broth (without antibiotics) in a 250 mL baffled shaker flask with 600 μL of an overnight (16–18 h at 37°C, 250 RPM) culture and incubating for approximately 3.5 h at 37°C, 250 RPM. 1 mL of sub-culture was then pelleted at 8,000 × *g* for 2 min, at which point 900 μL was removed and replaced with 900 μL of sterile PBS, pH 7.4. The bacterial pellet was then gently resuspended and used immediately.

Mouse lines and* Salmonella *infections—All animal work was performed according to protocols reviewed and approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC). All mice used were between 8 – 12 weeks old and consisted of a mix of male and female littermates. C57BL/6J "wild-type" mice and mice with IEC-specific deletion of *Hif1b* have been described previously (Glover et al., 2013). Specifically, mice containing a floxed-*Hif1b* allele were crossed with mice expressing *Villin*-driven Cre recombinase, resulting in selective loss of *Hif1b* expression

in the intestinal epithelium (Glover et al., 2013). Villin-cre mice used were of the B6.Tg (Vil1-cre)997Gum/J background from Jackson Laboratories (Strain# 004586) with specific transgene expression in the gastrointestinal tract (Madison et al., 2002). Experiments using the streptomycin pretreatment model of STm *in vivo* infection were performed as described elsewhere (Conway et al., 2013). Briefly, mice were gavaged with 20 mg/mouse streptomycin sulfate in order to abolish colonization resistance, then 24 h later infected orally with STm SL1344. Mice were euthanized 48 h post-infection for collection of tissues. Where indicated, mice were treated daily by intraperitoneal injection with either 8 mg/kg RAP or vehicle control prepared with an equivalent volume of DMSO (Johnson et al., 2013). Histopathological scoring was performed by a trained pathologist in a blinded fashion using established metrics for scoring *Salmonella*-induced typhlitis/colitis (Barthel et al., 2003).

ODD-luciferase ("ODD-Luc") mice express luciferase fused C-terminally to the oxygen-dependent degradation domain (ODD) from HIF-1α, resulting in degradation of luciferase under normoxia and, conversely, stabilization under hypoxic conditions. Use of ODD-Luc in intestinal inflammation assays, including *in situ* imaging of tissue luciferase activity, has been previously described by our group (Campbell et al., 2014).

METHOD DETAILS

Chromatin immunoprecipitation-microarray (ChIP-chip) and analysis—ChIP-chip data used in this paper has been reported elsewhere and is accessible in the Gene Expression Omnibus (GSE43108). Principal component analysis (PCA) of ChIP-chip data sets was performed using the freely available webtool ClustVis (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015).

RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)—Total RNA was prepared from *in vitro* cell culture samples by lysis and purification using TRIzol reagent. Total RNA was prepared from murine intestinal tissue using an EZ-10 DNAaway RNA Miniprep Kit. Both approaches were performed according to the manufacturers' instructions, with the resulting RNA quantified using a NanoDrop One instrument (Thermo Scientific). Complementary DNA (cDNA) was synthesized from purified total RNA using iScript Supermix reagent. qPCR was performed using 2× Power SYBR Green master mix on either an Applied Biosystems 7300 (96-well) or 7900HT (384-well) instrument. Primers used for qPCR are given in Table S1. Analysis of qPCR data was performed using LinRegPCR (Untergasser et al., 2021).

Immunoblotting—Samples were first collected by lysing in either RIPA buffer (50 mM Tris-Cl, pH 8.0; 150 mM sodium chloride; 1% (v/v) Triton X-100; 0.5% (w/v) sodium deoxycholate; 0.1% (w/v) sodium dodecyl sulfate (SDS)) or 1× SDS-PAGE sample buffer. In either case, HALT protease inhibitor cocktail and EDTA were included at 1× and 0.5 mM final concentrations, respectively (Thermo# 78438). *In vivo* samples were collected as done before by scraping intestinal tissue with a clean scalpel and transferring the liberated epithelium to a fresh microcentrifuge tube (Kao et al., 2017; Zheng et al., 2017). Samples were sonicated as needed to reduce viscosity. Samples were analyzed by SDS-PAGE using standard techniques (Brunelle and Green, 2014) and transferred to PVDF for blotting. Blots

were blocked in 5% (w/v) nonfat dry milk and probed with antibodies listed in the Key Resources Table. Blots were developed using Clarity Max ECL reagent and imaged using a Bio-Rad ChemiDoc MP instrument.

In vitro infection of IECs—For the observation of HIF stabilization and induction of HIF target genes, epithelial cells were infected by direct addition of bacterial subcultures at a given multiplicity of infection (MOI) based on previously described protocols (Hartmann et al., 2008). For the enumeration of intracellular colony forming units (CFUs) as a metric of intracellular replication, cells were infected briefly with STm then treated with the membrane-impermeable antibiotic gentamicin to kill extracellular bacteria (while intracellular bacteria remain protected) as described elsewhere (Wrande et al., 2016).

Oxodish measurement of in vitro oxygen concentrations—Use of Oxodish sensor plates for real-time *in vitro* oxygen consumption has been previously described (Kelly et al., 2015). Briefly, Caco-2 cells were grown to confluence on Transwell semi-permeable inserts (0.4μm, Corning# 3470). Cells were then washed and placed into Hanks' Balanced Salt Solution containing Ca^{2+}/Mg^{2+} and supplemented with 10mM HEPES ($HBSS^{+}$) (Cartwright et al., 2021). Cells were then incubated on an Oxodish sensor plate with or without apical STm, and the percent O_2 of the surrounding buffer was measured over time. Note that due to the high altitude (>5000 ft.) of the laboratory where experiments were performed, fully oxygenated air is less than the ~21% observed at sea level. Control wells included $HBSS^{+}$ only, as well as wells containing $HBSS^{+}$ + bacteria without IECs.

In vitro measurement of HIF-driven luciferase—HeLa cells were transiently transfected with pGL4.22-VEGF-HRE:dLUC (Walton et al., 2018) using FuGENE HD transfection reagent according to the manufacturer's instructions. 24 h after transfection, cells were infected with STm for six hours then lysed using a Dual Luciferase Assay Kit and analyzed on 96-well plates in triplicate using a Promega GloMax 96-well luminometer.

Immunofluorescence—Caco-2 cells were plated onto sterile glass coverslips in 24-well plates and treated with/without hypoxia. In some cases, Caco-2 cells expressed tf-LC3 (Kimura et al., 2007). Cells were fixed using 4% (w/v) paraformaldehyde, then permeabilized using either 0.1% saponin or 0.1% Triton X-100 and stained with primary and secondary antibodies described in Key Reagents Table. Cells were then protected using ProLong Anti-Fade reagent and imaged using a Zeiss Axio Imager A1 microscope. Visible puncta were manually quantified in a blinded fashion and, afterwards, experimental groups were compared.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed using either Microsoft Excel or GraphPad Prism. Details on statistical analyses, including tests performed, can be found in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Autophagy genes are HIF targets in intestinal epithelial cells
- S. Typhimurium induces HIF stabilization and transcriptional activity
- HIF signaling is protective against *Salmonella* both *in vitro* and *in vivo*
- Xenophagy response to *Salmonella* infection is dependent on IEC HIF activity

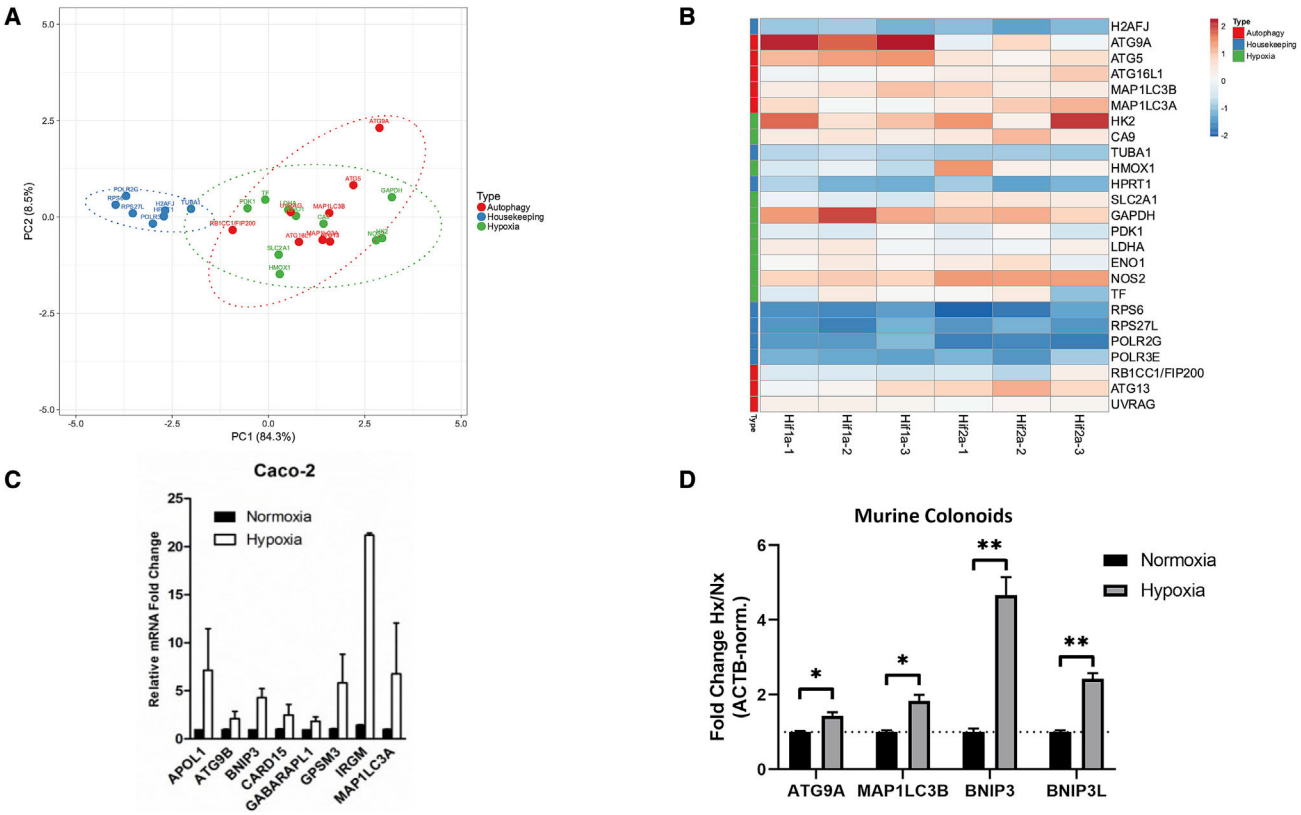

Figure 1. Autophagy genes are HIF targets

(A) Principal-component analysis (PCA) of Caco-2 IECs analyzed by ChIP-chip demonstrates association of autophagy genes with hypoxia genes, in contrast with housekeeping genes.

(B) Heatmap of genes plotted in (A) shows conserved patterns between autophagy and hypoxia targets. Sample names at bottom of columns represent biological replicates.

(C) Validation of ChIP-chip results by qPCR in Caco-2 IECs demonstrates induction of autophagy genes under hypoxic conditions.

(D) Analysis of gene expression in primary murine colon-derived organoids by qPCR shows significant increase in autophagy gene expression by hypoxia. *$p < 0.05$, **$p < 0.01$ by t test. ChIP-chip results reflect three biological replicates per sample group. qPCR results reflect at least three biological replicates.

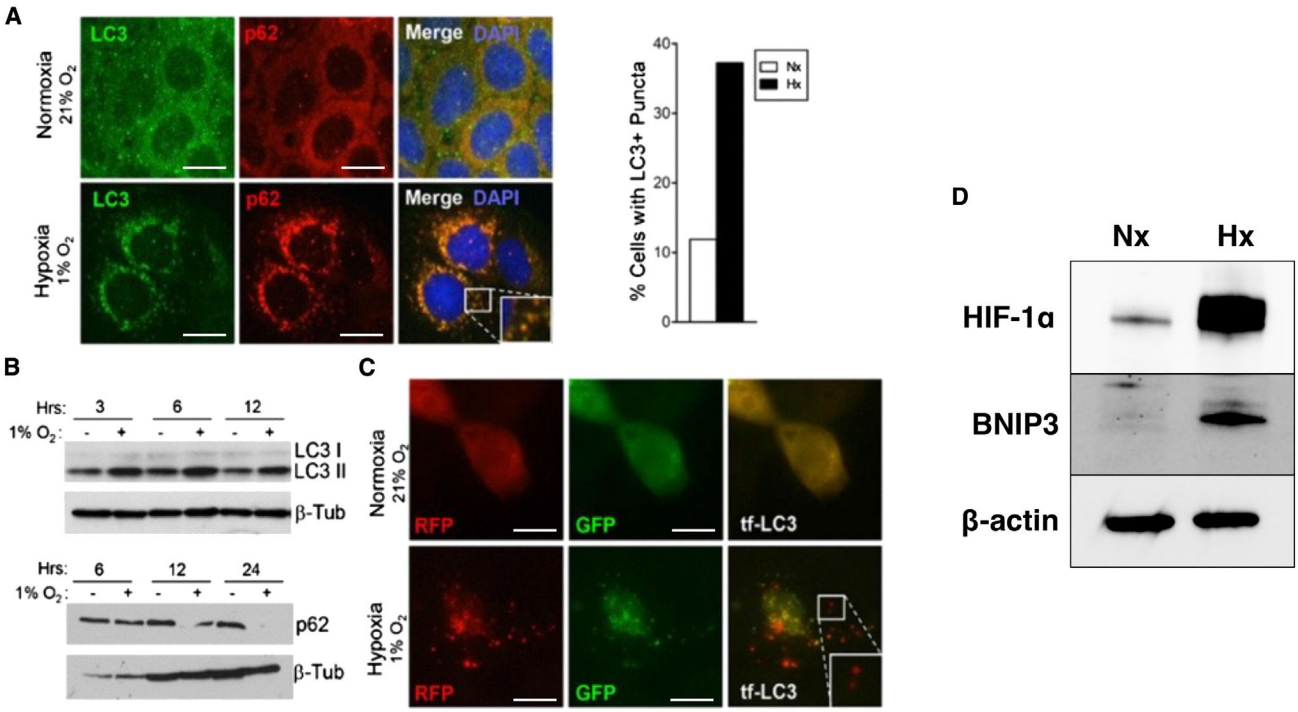

Figure 2. Hypoxia induces autophagy in IECs

(A) Incubation of Caco-2 IECs in either normoxia or hypoxia (1%O_2, 24 h), followed by immunofluorescence staining, reveals increased LC3 and SQSTM1/p62 puncta in hypoxia-treated cells.

(B) Measurement of LC3 and p62 by western blot in Caco-2 IECs during time course incubation at normoxia or hypoxia.

(C) Caco-2 IECs transfected with tf-LC3 followed by incubation at normoxia or hypoxia. The increase in red puncta (bottom panels) demonstrates increased autophagic flux in hypoxia-treated cells.

(D) Caco-2 IECs incubated at hypoxia (Hx) show increased expression of HIF-1α and BNIP3 compared with normoxic (Nx) controls. Results are representative of at least two separate experiments. Microscopy images at 1,000×; scale bar: 10 μm.

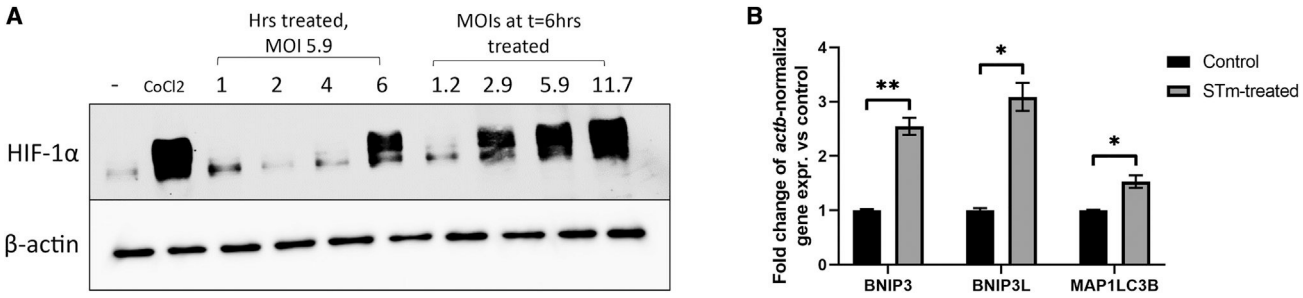

Figure 3. Infection of IECs by *Salmonella* Typhimurium stabilizes HIF and induces HIF signaling

(A) Caco-2 cells were infected with *Salmonella* Typhimurium (STm) at a set MOI for the given length of time or for a constant time with variable MOIs, then whole-cell lysates were prepared and analyzed for HIF by western blot. Uninfected cells and $CoCl_2$-treated cells were used as negative and positive controls for HIF stabilization, respectively.

(B) Expression of HIF-regulated autophagy genes in HeLa cells infected with STm were measured by qPCR. *$p < 0.05$, **$p < 0.01$ by t test. Western blot results are representative of at least two separate experiments. qPCR results reflect at least three biological replicates per group.

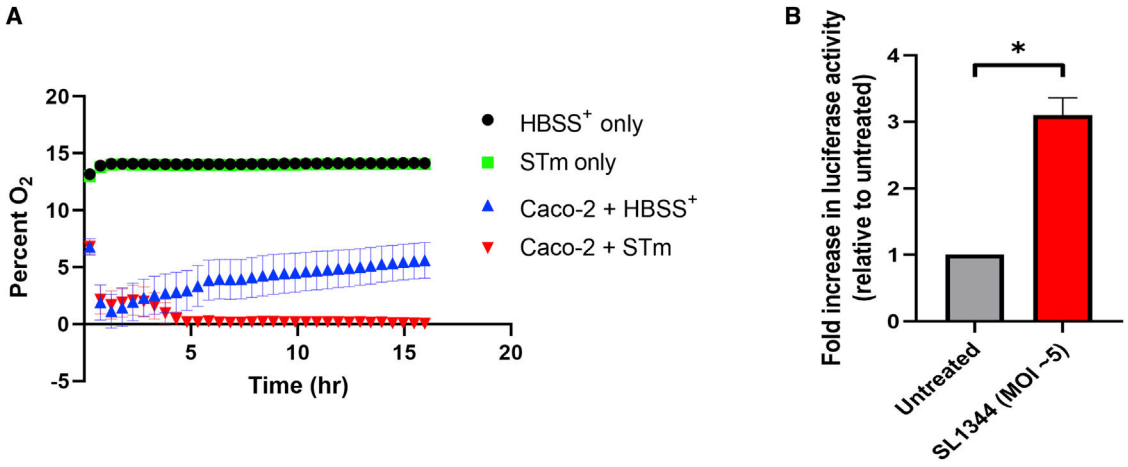

Figure 4. STm induces cellular Hx *in vitro*

(A) Caco-2 IECs, STm, or a combination of both were incubated at 37°C on Transwell inserts in an Oxodish oxygen measurement system. HBSS+ was used as the assay buffer and as the negative control.

(B) HeLa cells transfected with a plasmid containing luciferase under Hx-response element (HRE) control were infected with STm, then luciferase activity was measured. Both Oxodish and luciferase experiments utilized at least six biological replicates per group.

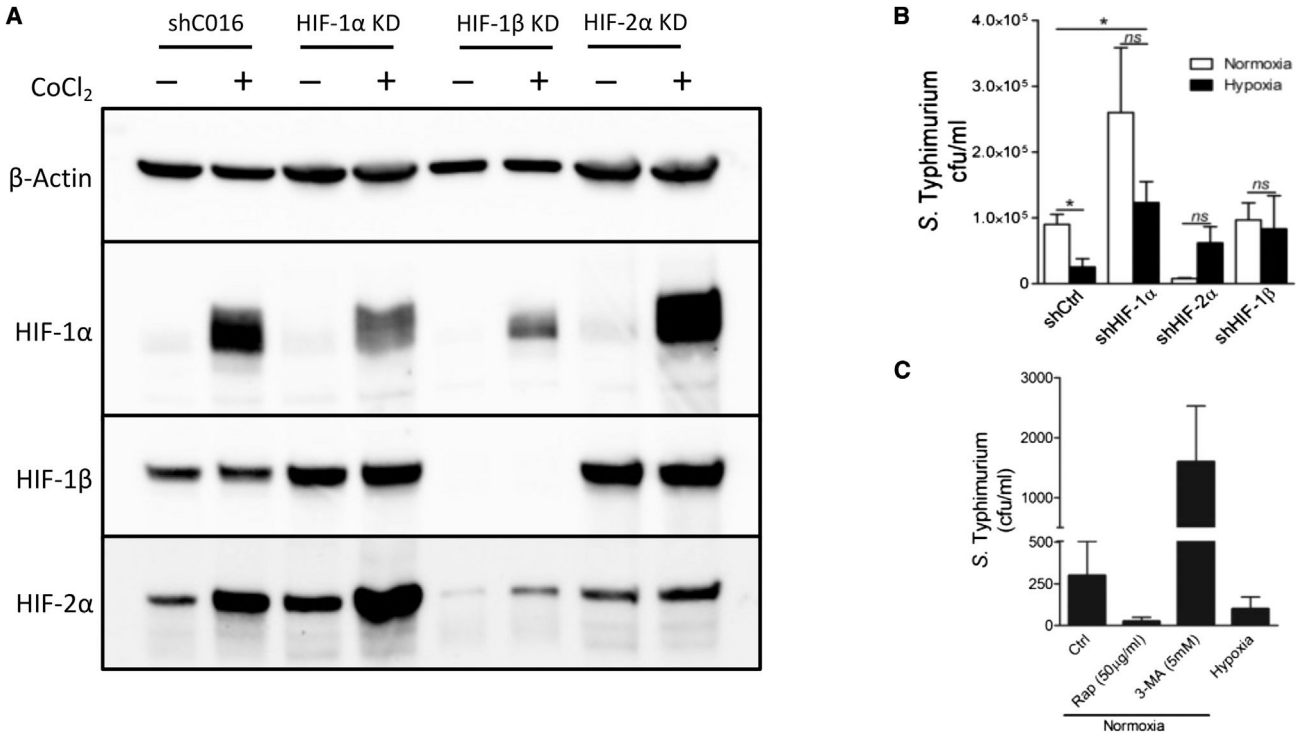

Figure 5. HIF/Hx promotes clearance of intracellular STm by xenophagy

(A) HIF isoforms were knocked down in IECs using lentiviral-delivered shRNA. $CoCl_2$ treatment was used to stabilize HIF for analysis of knockdown efficiency.

(B) Knockdown of HIF isoforms impairs Hx-mediated reduction of intracellular STm. *$p < 0.05$ by t test.

(C) Intracellular burden of STm in IECs is reduced by either incubation at Hx or treatment with the autophagy activator RAP. Conversely, inhibition of autophagy with 3-methyladenine (3-MA) increases intracellular proliferation of STm. MOI = 10. Results are representative of at least two separate experiments; in the case of STm infection, at least three biological replicates were used per experimental group.

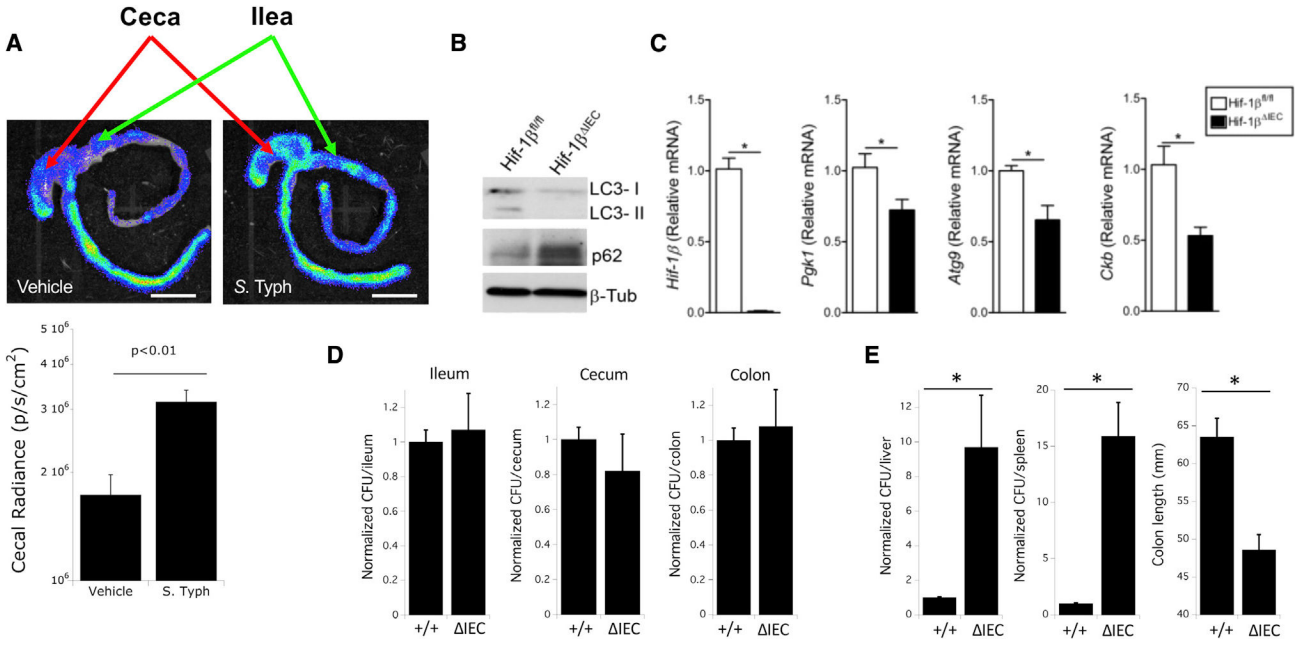

Figure 6. HIF signaling is essential for *in vivo* control of STm by xenophagy

(A) Intragastric infection of ODD-Luc mice with STm increases HIF stabilization (luciferase activity) in the cecum. Quantification of cecal radiance shown in plot below images (n = 4 per group, $p < 0.01$). Scale bar: 1 cm.

(B) Hif1bΔIEC mice demonstrate defects in autophagy in the intestinal epithelium by LC3 and p62 by western blot analysis of intestinal scrapings.

(C) Hif1bΔIEC mice show reduced intestinal levels of the HIF targets Pgk1 and Ckb, as well as the HIF-driven autophagy protein Atg9, by qPCR analysis. *$p < 0.05$ by t test.

(D) Deletion of intestinal epithelial HIF-1β (Hif1bΔIEC) *in vivo* does not change levels of colonization following infection with STm in the ileum, cecum, or colon. Results are normalized to wild-type (+/+) controls. N = 5 mice per group.

(E) Deletion of intestinal HIF-1β (Hif1bΔIEC) *in vivo* increases dissemination of STm to liver and spleen following intragastric infection and provokes increased intestinal inflammation as measured by shortened colon length. Results are normalized to wild-type (+/+) controls. N = 5 mice per group, where *$p < 0.025$ by t test.

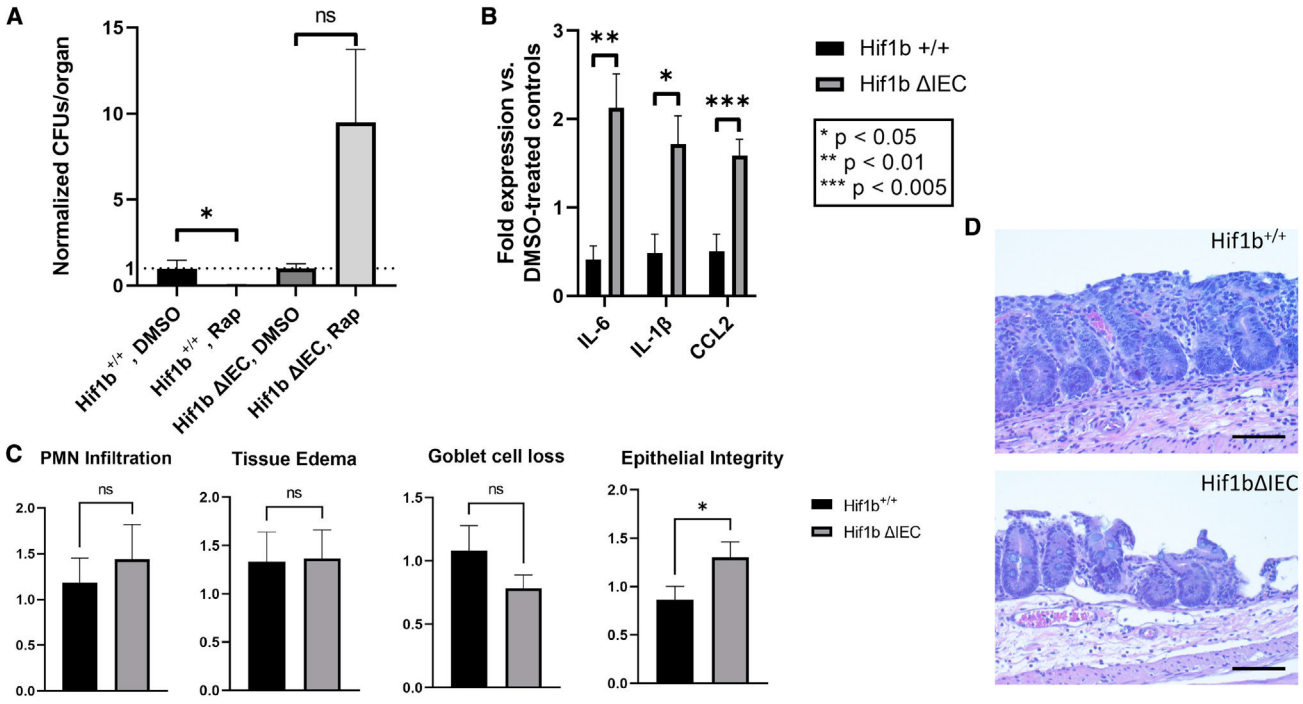

Figure 7. Treatment of mice with autophagy agonist RAP ameliorates STm-induced inflammation in a HIF-1β-dependent manner

(A) Measurement of liver STm CFUs in mice treated either with DMSO or RAP. Data presented as CFUs per organ, normalized to DMSO-treated controls.

(B) Expression of pro-inflammatory cytokines in terminal ilea of mice infected with STm and treated with either DMSO or RAP. Data represented as fold expression of RAP-treated mice versus DMSO-treated controls.

(C) Histopathology of *Salmonella*-infected, RAP-treated Hif1b$^{+/+}$ or Hif1bΔIEC mice was evaluated by H&E staining and microscopy. Data presented as total final score for indicated parameters.

(D) Representative histopathologic images of cecal tissue from Hif1b$^{fl/fl}$ (top) or Hif1bΔIEC (bottom) mice administered RAP in combination with STm. Note loss of epithelial integrity in Hif1bΔIEC. For all experiments, n = 9–10 mice per experimental group.

All statistics calculated by one-way ANOVA (A) or unpaired t test (B and C). *p < 0.05, **p < 0.01, ***p < 0.005 in all panels. Microscopy images at 400×; scale bar: 50 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-ACTB	Abcam	Cat#ab8227
anti-LC3B	Sigma-Aldrich	Cat#L7543
anti-p62/SQSTM1	Abcam	Cat#ab56416
anti-HIF1A	BD	Cat#610959
anti-HIF1B/ARNT	BD	Cat#611079
anti-HIF2A	Novus Biologicals	Cat#NB100-122
anti-BNIP3	Abcam	Cat#ab109362
anti-TUBB	Abcam	Cat#ab6046
anti-Mouse IgG, HRP-conjugated	MP Biomedical	Cat#0855550
anti-Rabbit IgG, HRP-conjugated	MP Biomedical	Cat#0855676
anti-p62	MBL	Cat#M162
anti-LC3	Novus Biological	Cat#NB100-2220
anti-LC3	Cell Signaling Technology	Cat#2775S
Bacterial and virus strains		
Salmonella enterica serovar Typhimurium strain SL1344	Lab stock	DSM 24522
Chemicals, peptides, and recombinant proteins		
Rapamycin	Thermo-Fisher	Cat#J62473
Streptomycin sulfate	Sigma-Aldrich	Cat#S6501
Phoshate buffered saline (PBS)	Thermo-Fisher	Cat#10010023
LB-Miller broth	BD	Cat#244610
Agar	BD	Cat#214010
Glycerol	Sigma-Aldrich	Cat#G5516-500ML
IMDM	Corning	Cat#10-016-CV
GlutaMAX	Thermo-Fisher	Cat#35050061
Penicillin/streptomycin	Thermo-Fisher	Cat#15140122
Bovine calf serum	Cytiva	Cat#SH30072.03
TRIzol reagent	Thermo-Fisher	Cat#15596026
iScript Supermix reagent	Bio-Rad	Cat#1708841
2× Power SYBR Green	Thermo-Fisher	Cat#4367659
SDS-PAGE sample buffer	Bio-Rad	Cat#1610747
HALT Protease Inhibitor	Thermo-Fisher	Cat#78438
Clarity Max ECL reagent	Bio-Rad	Cat#1705062
ProLong Gold Anti-Fade reagent	Thermo-Fisher	Cat#P36930
Critical commercial assays		
Oxodish oxygen sensor plates	Presens	Cat#OD24
EZ-10 DNAaway RNA Miniprep Kit	Bio Basic	Cat#BS88136
Dual Luciferase Assay kit	Promega	Cat#E1960
FuGENE HD	Promega	Cat#E2311

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
HIF-1α and HIF-2α ChIP-chip profiling in intestinal epithelial cells	NCBI GEO	Accession#GSE43108
Experimental models: Cell lines		
HeLa	Lab stock	RRID:CVCL_0030
C2BBe1	Lab stock	RRID:CVCL_1096
Colonoids	Healthy C57BL/6 mice	Generated in-house according to established protocols
Experimental models: Organisms/strains		
Hif1b-flox/villin-cre	Lab colony	Previously generated (PMID# 24248342)
C57BL/6J wild-type	Lab colony	RRID:IMSR_JAX:000664
ODD-luciferase	Lab colony	Previously described (PMID# 24412613)
Oligonucleotides		
Primers	This publication	See Table S1 for sequences
anti-HIF1A shRNA	Functional Genomics Facility (Aurora, CO)	Accession#TRCN0000003811
anti-HIF1B shRNA	Functional Genomics Facility (Aurora, CO)	Accession#TRCN0000003819
anti-HIF2A shRNA	Functional Genomics Facility (Aurora, CO)	Accession#TRCN0000003806
Recombinant DNA		
pGL4.22-VEGF-HRE::dLUC	Chi Van Dang, via Addgene	RRID:Addgene_128096
tf-LC3	Tamotsu Yoshimori, via Addgene	RRID:Addgene_21074
Software and algorithms		
LinRegPCR	Untergasser et al. (PMID# 34433408)	n/a
ClustVis	Metsalu and Vilo (PMID# 25969447)	n/a