ABSTRACT

SIMMONS, ALICIA NICOLE. The Role of TGFß-Activated Kinase 1 Signaling in Reactive Oxygen Species and Intestinal Homeostasis. (Under the direction of Dr. Jun Ninomiya-Tsuji).

Proper function of intestinal epithelial cells is essential for overall homeostasis of the host in that if intestinal epithelial cells are unable to carry out their functions, structural disruptions may occur leading to leaky barriers, invasion of bacteria, and cell death. In this study we investigated the role of TGF-β activated kinase 1 (TAK1) in intestinal homeostasis. TAK is a member of the mitogen activated protein kinase (MAPK) kinase kinase (MAPKKK) family and is activated by various chemical and physical stressors and inflammatory cytokines to promote cell survival through activation of MAPKs and a transcription factor, NF-κB. We used a mouse model harboring an inducible intestinal epithelial specific deletion of the *Tak1* gene.

We initially found that when *Tak1* is deleted, cell death, upregulation of reactive oxygen species (ROS), and loss of Paneth cells, which play a major role in gut microbe regulation, are induced in the intestine. We hypothesize that TAK1 regulation of the antioxidant pathway prevents microbial-induced ROS and is integral for Paneth cell integrity. To test the involvement of bacteria, we depleted commensal bacteria using an antibiotic cocktail. We found that bacteria depletion greatly reduced ROS in *Tak1*-deficient intestinal epithelium. Furthermore, intestinal epithelial-specific deletion of Myd88, a common adaptor protein of several Toll-like receptors, also reduced the accumulation of ROS. These demonstrate that commensal bacteria induce ROS accumulation through TLR-MyD88 pathway in the *Tak1*-deficient intestinal epithelium. However, unexpectedly, we found that depletion of Paneth cells was not rescued by either commensal bacteria depletion or Myd88

deletion. These results suggest that Paneth cell depletion is independent of ROS accumulation. We later found that deletion of receptor interacting kinase 3 (*Ripk3*), which is a mediator of one type of cell death, necroptosis, prevented Paneth cell loss. These results demonstrate that TAK1 regulates ROS and Paneth cells, both of which are critical for intestinal homeostasis. Chronic intestinal inflammatory diseases such as Crohn's disease are associated with oxidative stress and loss of Paneth cells. Our results provide a better understanding of the regulatory mechanisms for ROS and Paneth cells, which could lead to new approaches to control these diseases.

In a subsequent study we analyzed the role of TAK1 in the regulation of the antioxidant transcription factor Nrf2. Nrf2 is a leucine-zipper transcription factor that regulates a number of antioxidant enzymes thereby playing a major role in the cellular antioxidant system. We found that a protein kinase, TAK1 physically interacts with Nrf2 through Keap1 and upregulates Nrf2 protein stability, suggesting that TAK1 regulates ROS through Nrf2.

Collectively, these studies revealed previously uncharacterized roles of TAK1 in regulating Paneth cell integrity, as well as modulating Nrf2-Keap1 signaling. Our results indicate that TAK1 pathways are a potentially effective target to control ROS and Paneth cells integrity in the intestine.

The Role of TGFß-Activated Kinase 1 Signaling in Reactive Oxygen Species and Intestinal Homeostasis

by
Alicia Nicole Simmons

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APPROVED BY:

_____	_____
Jun Ninomiya-Tsuji Committee Chair	James Bonner
_____	_____
Robert Smart	Yoshiaki Tsuji

DEDICATION

I would like to dedicate my dissertation to my parents Mr. & Mrs. Lucius Simmons Jr. for their continued love and support of my dreams and aspirations for as long as I can remember. I want to especially thank them for always being present in my life through not only the pursuit of me obtaining my PhD but for being there whenever I needed them for anything. You both never pressured me and always told me that I could stop or take a break from school whenever I felt like it. I will never forget the love and support that I received from you throughout this journey. I don't know what I would do without you. It is because of you that I am the woman I am today. I love you mom and dad! This is for you!!!

BIOGRAPHY

Alicia Nicole Simmons was born on October 26, 1985 to Lucius and Christine Simmons Jr, in Winston-Salem, NC. She is the oldest of two and has a younger brother, Lucius Simmons, III. Alicia attended elementary school at Forest Park Elementary followed by middle school at Atkins Middle, both which are located in Winston-Salem, NC. In 2004, Alicia graduated high school form East Forsyth High School and was admitted to and enrolled at Johnson C. Smith University in Charlotte, North Carolina. She later graduated Cum Laude from Johnson C. Smith University in May 2008 with a Bachelor of Science degree in Biology.

After graduating from JCSU, Alicia moved to Chapel Hill, NC and worked as a post baccalaureate student for two years at the University of North Carolina at Chapel Hill (UNC). While there Alicia worked under the mentorship of Dr. Shawn Ahmed, PhD in the department of Genetics where she studied germ cell immortality using the nematode roundworm *Caenorhabditis elegans* (*C. elegans*). Additionally, Alicia learned and accomplished a lot while at UNC and attended and presented at her first International Conference at UCLA in the summer of 2009.

In the summer of 2010 Alicia left UNC and began working as a Research Assistant in the laboratory of Dr. Jun Ninomiya-Tsuji at North Carolina State University. During this time, Alicia took some graduate level courses and conducted research, using mice as a model system. She received good preliminary data and applied to and was accepted into the Cellular and Molecular Toxicology PhD program at NC State the following summer and continued with Dr. Ninomiya-Tsuji's lab as her Principal Investigator. Alicia's project was to determine the role of TAK1 signaling in intestinal homeostasis. In the summer of 2014 Alicia passed

her written preliminary examination and successfully defended her oral preliminary examination of October of the same year. In the summer of 2015 Alicia plans to finish up her work towards her PhD and then begin her postdoctoral fellowship position at the NIEHS in the Signal Transduction Research group of Dr. Xiaoling Li, PhD.

"Discipline is the bridge between goals and accomplishment." – Jim Rohn

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have always been supportive of anything and everything that I have wanted to do. I knew that when I embarked on this journey it would be a long and tough road but you always reiterated that I could do this and that it was destiny. Thank you for making me take study breaks, cooking me food whenever I just worked so hard that I would only eat junk food because it was easier and quicker. You took me on walks, told me jokes, and will forever be the best vacation partner a girl could ask for. I appreciate you and love you! You are definitely one of a kind!

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GENERAL INTRODUCTION

Chronic inflammation is associated with a diverse set of human diseases not limited to the well known inflammatory diseases but also many cancers, and metabolic diseases. Therefore, better understanding of the mechanisms leading to chronic inflammation is much anticipated. Among the many diseases associated with inflammatory conditions, I focus on inflammatory bowel disease (IBD), which is known to be associated with oxidative stress induced tissue injury. I fortunately found a very effective mouse model system to investigate intestinal chronic inflammation, which was previously developed by our research group. These mice harbor gene deletion of a protein kinase, *Tak1* that exhibits profound IBD-like oxidative injury in the intestine. In this dissertation project, I explore the mechanism by which chronic oxidative injury is developed. This introduction serves to provide background information regarding intestine physiology, IBD, oxidative stress, and TAK1, which will support my hypothesis for this project described at the end of the introduction.

The Intestine

The intestine consists of a small and large intestine. The small intestine contains the duodenum, jejunum, and the ileum, which is where most of the nutrient and water absorption occurs whereas the large intestine also known as the colon or large bowel is the last part of the digestive system where waste is stored as feces before defecation. A layer of microvilli, which contain enzymes needed for digestion as well as nutrient transporters, covers the intestinal epithelial cells. Two major regions of the intestinal epithelium are the villus and the crypt of Lieberkuhn. The villus lining contains a single layer of epithelial cells that is renewed every 3-5 days by the proliferative progenitor cells that are organized and supplied

from the crypt stem cell. The stem cells continuously proliferate to support the constant turnover of all of the differentiated epithelial cells. These differentiated cells include the absorptive enterocytes, goblet cells, and enteroendocrine cells. Additionally, in the small intestine, the specialized epithelial Paneth cells are also generated from the stem cells where they reside at the crypt base, and their role is to secrete antimicrobial peptides, defensins, and lysozymes to aid in intestinal homeostasis (Ayabe et al., 2000). We note that Paneth cells are not present in the large intestine.

Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a chronic disease of the colon and small intestine that primarily consists of ulcerative colitis (UC) and Crohn's disease (CD) and is one of the major health problems in the US. This chronic disease involves interaction of immune cells, cytokines, chemokines, and intestinal flora until that induce a net imbalance of the redox equilibrium (Biasi et al., 2013). The peak disease incidence is in individuals between the ages of 15-35 years old although there are some instances where late onset IBD may occur (~ 40 years old). IBD is mostly prevalent in the United States and Northern Europe however studies have shown that the incidence of IBD is increasing in other regions of Europe as well as Asia (Biasi et al., 2013). Symptoms of IBD include bloating, abdominal pain, diarrhea, bleeding, and fatigue (Olendzki et al., 2014). These are often difficult to diagnose without extensive testing because many individuals have experienced these symptoms at some point in life and do not have IBD. Because IBD encompasses both UC and CD and they are two distinct conditions, histological features differ between the two. Histological features that are seen in UC include structurally an irregular surface, crypt distortion, and an increased crypt

density in the large intestine (Geboes, 2008). Whereas, features that favor CD include inflammation, tissue damage of the ileum (ileitis), discontinuous crypts, mucin preservation, discontinuous inflammation, Paneth cells loss, and epitheloid granulomas (Geboes, 2008). The presence or absence of these features is dependent upon duration and activity of the disease as well as medical treatment (Kleer and Appelman, 1998).

Although the pathogenesis of IBD has been extensively studied there is still a large gap in our understanding at the molecular level of how phenotypes associated with IBD as well as how genetics, and exogenous factors impact our ability to handle the circumstances of IBD. My goal of the study is to provide a molecular mechanism of development of IBD-like disorders that will potentially lead to a new approach to combat the major problems associated with IBD including Paneth cell loss by investigating our mouse model that resembles IBD pathologies.

Pathogenesis of IBD

Both genetic and environmental factors have been associated with the pathogenesis of IBD. These factors can lead to the destruction of the intestinal barrier, translocation of microbial products, and chronic inflammation.

It is widely accepted that the pathogenesis of IBD results when there are overly aggressive acquired immune responses towards commensal bacteria in susceptible individuals/hosts; and the environment precipitates the onset or reactivation of the disease. Patients with IBD have shown that mucosal immune cells in response to environmental factors i.e. commensal bacteria overproduce both pro and anti-inflammatory cytokines. For example, macrophages and $CD4^+$ Tcells secrete increased IL-6, a pro-inflammatory cytokine,

in patients with IBD as well as experimental colitis mouse models (Atreya et al., 2000; Kai et al., 2005). Additionally, in experimental ulcerative colitis mouse models (Sugimoto et al., 2008), dendritic cells and neutrophils produce increased levels of IL-22, which is a member of the anti-inflammatory IL-10 cytokine family (Dumoutier et al., 2000a; Dumoutier et al., 2000b), that induces the production of antibacterial proteins and chemokines.

Several genetic mutations and polymorphisms are closely associated with IBD pathogenesis. Some include mutated genes associated with autophagy (*ATG16*) (Hampe et al., 2007), with the anti-inflammatory cytokine IL-10 (Franke et al., 2008), and polymorphisms in *NOD2* leading to increased risk for Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). *ATG16L1* (T100A) gene mutation is associated with Paneth cell granule abnormalities (Rioux et al., 2007). Autophagy is a cellular homeostasis process by which a double-membraned structure, the autophagosome, surrounds the cytoplasm to degrade captured proteins and cytoplasmic organelles (Cadwell et al., 2008). In patients with healthy ileums, Paneth cells in the crypts of the ileum have granules and secrete peptides such as antimicrobial peptides and lysozyme to control the gut microbiota. However, loss of autophagy caused by the *ATG16L1* mutation in patients with Crohn's disease or through a decrease in the gene product in $Atg16L1^{HM}$ mice results in fewer granules or diffused granule contents in the cells (Cadwell et al., 2008; Deuring et al., 2014). *NOD2* is a intracellular pattern recognition receptor that plays a role in immune response by recognizing bacteria moiety, muramyl dipeptide, and activating NF-κB. About 50 different variations in the NOD2 gene is associated with an increase risk of CD (Fritz et al., 2011). NOD2 mutation is known to increase NF-κB activation thereby potentiating inflammation (Maeda et al., 2005). Moreover, mutations in the NOD2 gene is known to be associated with decreased defensin

production in Paneth cells (Wehkamp et al., 2004). Thus, the combination of alterations in gut environment such as changes in gut bacteria or nonpathogenic/pathogenic virus infection with autophagy genes or NOD2 mutations may promote CD pathogenesis as suggested by a previous study conducted by (Cadwell et al., 2010).

Oxidative Stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS), including superoxides and peroxides, and the availability of antioxidant defenses (Du et al., 2009). ROS are generated and produced naturally as a product of cellular metabolism. During oxidative phosphorylation electrons are passed to electron carriers (cytochrome c and ubiquinone). The final electron acceptor is an oxygen molecule and typically this reaction will combine with hydrogen to form water. However, small amounts of peroxide are also produced, which is one of the most common reactive oxygen species. Intracellular ROS that can cause damage to lipids, proteins, and DNA, which could disrupt plasma and organelle membrane integrity and protein function and/or inactivating some enzymes by oxidation of cofactors (Cederbaum et al., 2009). The cells in the body can naturally produce antioxidants and enzymes to reduce ROS. There are three main pathways that are involved in the removal of ROS that involve reduced glutathione, thioredoxin, and catalase. One of the most common antioxidants is glutathione, which provides electrons to the oxidants so that ROS are reduced (Du et al., 2009). Both glutathione and thioredoxin pathways rely heavily on NADPH production for sustaining their activity, whereas catalase acts independently of NADPH (Gorrini et al., 2013).

Oxidative stress has been linked to several diseases and pathologies such as increased inflammation, cancers, and atherosclerosis (Le Lay et al., 2014). Additionally, oxidative damage in the epithelium is causally associated with chronic inflammatory diseases such as inflammatory bowel disease (IBD) as discussed below.

Oxidative Stress & IBD

It is widely accepted that under normal homeostatic conditions, the primary role of ROS is to defend against invading microorganisms. In the intestine, as generally discussed above, dietary antioxidants such as vitamin E, or vitamin C as well as glutathione, thioredoxin, and enzymatic antioxidants like superoxide dismutase and catalase protect tissues from the damaging effects of ROS (Puertollano et al., 2011; Gough and Cotter, 2011). However, an excess amount of ROS and impairment in production or maintenance of the antioxidants has been shown to lead to intestinal oxidative damage. Such aberrant ROS accumulation can be induced through several mechanisms. One prominent cause of ROS in the intestine is leukocyte infiltration, where these cells produce large amounts of ROS and reactive nitrogen species (RNS) (Espey, 2013). However, ROS are generated not only by immune cells but also in intestinal epithelial cells, which will be described in a later section. Nonetheless, ROS accumulation is closely associated with pathology of IBD. Indeed, it has been well documented that intestinal samples from IBD patients possess an increased activity of ROS production and a higher level of superoxide compared to healthy control samples (Bouzid et al., 2013). Furthermore, the levels of the lipid and protein oxidation are highly increased in the intestine samples from IBD patients (Bouzid et al., 2013).

Gut microbiota Involvement

Bacterial *phyla Firmicutes* and *Bacteroidetes* account for over 90% of the intestinal microbiota (Eckburg et al., 2005). Several studies have documented that there are changes in the gut microbiota (dysbiosis) in patients with IBD and dysbiosis is emerging as the cause of IBD (Dalal and Chang, 2014). Dysbiosis may be at least in part due to the increasing consumption of the western type diets (high fat, low fiber), which are high in processed sugars, protein, and overall calories. For example, many people who consume a high fat, low fiber diet generally have an increase in Bacteroidetes and Actinobacteria, while the bacteria Firmicutes and Proteobacteria are associated with low fat, high fiber diets (Wu et al., 2011). The mechanism by which dysbiosis in IBD is induced is not yet clear. Recently, it was reported that bacteria short chain fatty acid (SCFA) production is decreased in IBD patients, which is necessary for intestinal mucosal homeostasis. Therefore, this could lead to an exacerbated pro-inflammatory phenotype in these hosts (Smith et al., 2013). Dysbiosis is also clearly associated with increased oxidative stress (Henao-Mejia et al., 2012).

Paneth cells & IBD

As previously mentioned, Paneth cells reside at the base of the small intestinal crypts where they produce defensins, antimicrobial peptides, and enzymes such as lysozyme which are stored in secretory vesicles and secreted constantly into the lumen upon bacteria stimulation such as bacteria moieties, lipopolysaccharides (LPS) and (muramyl dipeptide) MDP (Ayabe et al., 2000). Paneth cells are derived from intestinal stem cells in which their differentiation is controlled by the Wnt signaling pathway (van Es et al., 2005). In patients with IBD, their Paneth cells usually show some abnormalities. It has been shown histologically that many IBD patients do not have any Paneth cells (Gunther et al., 2011). A decrease in expression of

defensins, lysozyme and other antimicrobial peptides is usually indicative of IBD pathogenesis where microbes are able to invade the mucosa and cause inflammation. Specifically, CD patients display a reduction in defensins (Wehkamp et al., 2005). In contrast, UC exhibits mainly colon abnormalities and goblet cells have a defective and/or reduced ability to secrete mucin, which disturbs the mucosal barrier leading to colonic inflammation and UC (McCormick et al., 1990; Pullan et al., 1994), and this is consistent with the fact that Paneth cells are present only in the small intestine.

TAK1

Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is activated by various cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), and ligands of Toll-like receptors and NOD-like receptors, which recognize bacterial and viral moieties (Kim et al., 2008; Sato et al., 2005; Takaesu et al., 2001; Ninomiya-Tsuji et al., 1999). In response to these stimuli, TAK1 is activated through interaction with TAK1 binding proteins: TAK1 binding protein 1 (TAB1), TAK1 binding protein 2 (TAB2), and TAK1 binding protein 3 (TAB3) (Mihaly et al., 2014a; Scholz et al., 2010; Inagaki et al., 2008; Kishimoto et al., 2000; Shibuya et al., 1996). It is known that TAK1 regulates mitogen-activated protein kinase (MAPK) pathways leading to activation of c-Jun N-terminal kinase (JNK) and p38, and also IKK signaling pathways to activate NF-κB (Ikeda et al., 2014; Omori et al., 2012; Ishitani et al., 2003; Takaesu et al., 2000). Although MAPK and NF-κB are extensively studied, TAK1 can activate several other downstream targets including Ski-related novel protein N (SnoN) (Kajino et al., 2007) and Nemo-like kinase (NLK) (Kanei-Ishii et al.,

2004), which could include other unidentified targets.

The role of* TAK1 *in vivo

The role of TAK1 during development is not completely elucidated but it is known that mice deficient in *Tak1* die in utero at around E10.5 (Shim et al., 2005b; Jadrich et al., 2006). These mice have morphological defects such as neural tube defects and reduced vessel architecture. Because the early lethality prevents detailed analysis of the roles of TAK1, our group has been using tissue specific and/or inducible gene knock out mice in order to fully understand the role of TAK1 *in vivo*. We used the LoxP-Cre system where exon 2 of the *Tak1* gene is flanked by two loxP sites so that when Cre recombinase is activated, a truncated form of TAK1, TAK1Δ, lacking the ATP binding domain is expressed (Sato et al., 2005). TAK1Δ is unfunctional and often unstable in cells. The phenotypes of our *Tak1* exon 2 deletion mice are identical to total deletion of *Tak1* gene expression (Sato et al., 2005; Shim et al., 2005a; Jadrich et al., 2006). For the intestinal epithelial specific deletion, we used the intestinal epithelial specific promoter (villin promoter) driven inducible Cre system that contains a fusion protein of Cre recombinase and a mutant estrogen receptor (CREERT2). The CREERT2 can be activated by treatment of the synthetic estrogen tamoxifen but not endogenous estrogen (Indra et al., 1999). Generating tissue specific TAK1 inducible knock out mice show the importance of TAK1 in tissue integrity. For example, endothelial-specific *Tak1* deletion causes defects in vascular formation, as well as increased cell death and vessel regression (Morioka et al., 2012). Hematopoietic specific *Tak1* deletion causes loss of hematopoietic stem cells (Takaesu et al., 2012). Macrophages die upon *Tak1* deletion (Mihaly et al., 2014a) and epidermal specific *Tak1* deletion causes severe inflammation of the skin and increased keratinocyte death (Omori et al., 2006). These results indicate that

TAK1 plays a diverse set of roles in different tissues, but that TAK1 is in general important for cell survival.

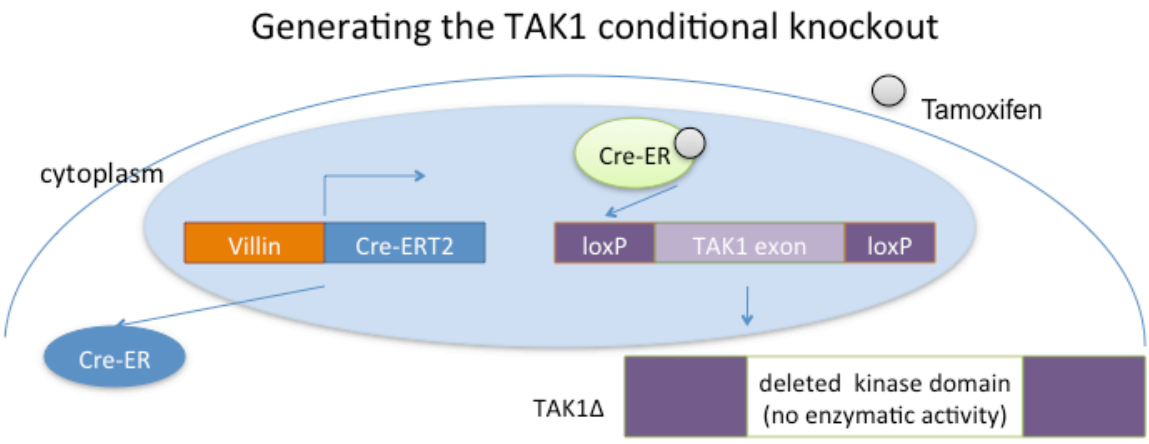

Figure 1: Generating a TAK1 intestinal epithelium conditional knockout mouse

The mechanism of cell death

Apoptosis or programmed cell death is characterized by the ability of multicellular organisms to physiologically remove unwanted cells as well as damaged cells (Kerr et al., 1972). Some key features of apoptosis include: cell shrinkage, membrane blebbing, nuclear condensation & fragmentation into small pieces (Kerr et al., 1972; Wyllie et al., 1980). At the molecular level, apoptosis is characterized by activation of caspases (Los et al., 2002) through two major pathways: the intrinsic and extrinsic pathways (Danial and Korsmeyer, 2004). The extrinsic pathway is activated by death receptor families including TNF, FAS, and TNF-related apoptosis-inducing ligand (TRAIL) (Walczak and Krammer, 2000; LeBlanc and Ashkenazi, 2003). In the TNF signaling pathway, adaptor molecules including TNF receptor type-1 associated death domain protein (TRADD), TNF receptor-associated factor 2 and 5

(TRAF2 and TRAF5), cellular inhibitor of apoptosis 1 and 2 (cIAP1/2), and receptor interacting protein kinase 1 (RIPK1) are recruited to the receptor complex (TNFR1 Complex I). Under some circumstances, the TNF bound receptor complex dissociates from TNFR1 leading to molecular reorganization and formation of a cytosolic death-inducing signaling complex (DISC) including TRADD, FAS-associated protein with a death domain (FADD), RIPK1 and caspase 8, known as Complex IIa (Micheau and Tschopp, 2003b). In this complex caspase-8 dimerizes, self-cleaves, and is activated to further activate executioner caspases such as caspase-3, which leads to apoptotic cell death.

In contrast, the intrinsic pathway is mainly activated by non-receptor signaling associated with Bcl-2 family proteins (Ledgerwood and Morison, 2009). For example, DNA damage activates p53, which transcriptionally activates several pro-apoptotic Bcl-2 family proteins (i.e. Puma, Noxa). Bcl-2 regulates mitochondrial integrity, and activation of pro-apoptotic Bcl-2 causes mitochondrial membrane permeabilization (Vander Heiden et al., 1997). Cyctochrome c and other cell death factors are released by MMP, which induces the activation of caspase-9 which in turn cleaves and activates downstream caspases such as caspase-3, -6, and -7 (Zamzami et al., 1998; Boldin et al., 1996; Muzio et al., 1996; Srinivasula et al., 1996).

There are several mechanisms to prevent apoptosis. One is the NF-κB, which is known to upregulate antiapoptotic proteins such as cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) and inhibitor of apoptosis (IAP) to inhibit the activation of caspases (Chang et al., 2006; Wang et al., 1998).

Unlike apoptosis, necrosis is not a programmed form of cell death as it is caused by cell injury that results in the uncontrolled, premature death of cells (Proskuryakov et al.,

2003). Characteristics of necrosis include: cell swelling, membrane rupture, inflammation, and release on intracellular contents such as nucleotides and lipids into the extracellular space. Necrotic cells trigger inflammation, which is caused by the release of such cellular contents so-called, danger associated molecular patterns (DAMPs) or also referred to as alarmins that stimulate pattern recognition receptors on macrophages, dendritic cells, and natural killer cells (Oppenheim and Yang, 2005; Chen et al., 2007). This stimulation enables the cells the ability to activate T cells and elicit immune responses (Trinchieri and Sher, 2007).

Several new forms of cell death have been recently reported, which are considered programmed but different from apoptosis. One of such types of cell death that I want to describe here is necroptosis. Necroptosis is induced by death receptors, toll-like receptors, interferons, intracellular RNA & DNA sensors, as well as other unidentified mediators (Pasparakis and Vandenabeele, 2015). Most information regarding necroptosis comes from studies regarding TNF signaling which is a potent inducer of cell death (Vandenabeele et al., 2010). As discussed above, TNF recruits several proteins to form Complex 1, which leads to activation of NF-κB and MAPKs. Deubiquination of RIPK1 cIAP proteins promotes the formation of Complex II leading to apoptosis. However, when cells have high expression of one protein kinase, receptor interacting protein kinase 3 (RIPK3), which is a structurally related protein kinase of RIPK1, or when caspases are inhibited, RIPK1 forms a complex with RIPK3 to initiate necroptosis (Vandenabeele et al., 2010; Micheau and Tschopp, 2003a; Wang et al., 2008). As mentioned above, *Tak1* deletion leads to ROS accumulation in cultured cells and epithelial cells *in vivo.* Additionally, this ROS has been shown to be associated with apoptosis however, necroptosis is also involved which I will later discuss.

Cell death in Tak1 deficient cells and tissues

Tak1 deficient cultured cells including fibroblasts and keratinocytes grow normally and do not exhibit any increase in cell death compared to wild type cells (Omori et al., 2006; Morioka et al., 2009). However, following treatment with TNF, these cells die, indicating that TNF is the cause of cell death in *Tak1* deficient cells. This cell death is associated with caspase activation and also increased ROS. TNF induced early cell death (apoptosis) is blocked by inhibition of caspases (Omori et al., 2008), but *Tak1* deficient cells still die with long-term incubation of TNF (Morioka et al., 2014b). Several recent reports indicate that *Tak1* deletion cause necroptotic cell death following TNF treatment (Lamothe et al., 2013; Arslan and Scheidereit, 2011; Dondelinger et al., 2013). Thus, *Tak1* deficiency induces apoptosis and necroptotic cell death upon TNF stimulation. In contrast to the *in vitro* observations, *in vivo* epithelial cells die without any exogenous stimulation. This led us to question of what is the endogenous activator of cell death in *Tak1* deficient tissues? One prominent possibility is endogenous TNF, which is known to be constantly expressed at certain levels in many tissues and provide basal immunity to prevent invasion of microorganisms (Pasparakis et al., 1996). Indeed, ablation of TNF signaling in the *Tak1* deficient epidermis, cell death is largely reduced (Omori et al., 2006). Cell death in the *Tak1* deficient endothelium is also largely rescued by compound deletion of the TNF receptor (Morioka et al., 2012). In the intestinal epithelium, TNF deletion rescued of cell death and inflammatory conditions in neonatal intestinal epithelial specific *Tak1* deficient mice (Kajino-Sakamoto et al., 2010). However, it does not result in a rescue of cell death, ROS, or inflammatory conditions in adult intestinal epithelial specific *Tak1* deficient mice (Kajino-Sakamoto et al., 2010). Mice having compound intestinal epithelial specific *Tak1* deletion

and germline deletion of TNF receptor 1 (hereafter referred to as Tak1^{IEKO} Tnfr1$^{-/-}$) which is the major receptor of TNF induced cell death, develop normally until two weeks of age; however, intestinal epithelial cell death and inflammation are observed in the adults.

Pathogenic phenotype of Tak1 deficient intestinal epithelium

Our recent studies in the Tak1^{IEKO} Tnfr1$^{-/-}$ intestinal epithelium have revealed several interesting features. For example, mice with Tak1^{IEKO} Tnfr1$^{-/-}$ intestinal epithelium have a profound accumulation of ROS positive cells, which is correlated with caspase activation leading to cell death (Kajino-Sakamoto et al., 2010). These mice also have a loss of barrier function (Kajino-Sakamoto et al., 2010) as shown by disrupted intestinal morphology and leaky tight junctions. Most interestingly, Paneth cells are depleted in these adult Tak1^{IEKO} Tnfr1$^{-/-}$ mice. These characteristics are very similar and resemble IBD pathology.

Thus, Tak1^{IEKO} Tnfr1$^{-/-}$ mice are potentially a useful model for IBD, and we asked by what mechanism are Paneth cells depleted and ROS are accumulated in the *Tak1* deficient intestinal epithelium?

TAK1 ***Regulation of Oxidative Stress***

How does TAK1 regulate the level of ROS? One prominent possibility is through the regulation of the NF-κB pathway, which is downstream of TAK1 and regulates antioxidant systems. However, our group previously demonstrated that loss of NF-κB does not increase ROS as high as the level in *Tak1* deficient cells (Omori et al., 2008). There may be a possibility that TAK1 regulates cellular antioxidant systems through unidentified mechanisms.

Keap1/Nrf2 Signaling

An important mechanism in cellular defense against oxidative stress is activation of an anti-oxidant transcription factor NF-E2 related factor-2 (Nrf2). Nrf2 is a leucine zipper DNA binding protein that, under normal conditions, is sequestered in the cytoplasm by its binding partner Keap1. Keap1 is an adaptor protein that is responsible for the degradation of Nrf2 by recruiting the ubiquitination complex, E1, E2 and E3 Cullin 3 (Kobayashi et al., 2004). Under oxidative stress or electrophilic stress, the Keap1- Cullin 3 ubiquinator system is disrupted and Nrf2 cannot be degraded. This results in accumulation of Nrf2 in the cytoplasm and Nrf2 is then translocated into the nucleus leading to transcriptional activation of target anti-oxidant genes. In the nucleus Nrf2 binds to antioxidant response element (ARE) to regulate a number of antioxidant enzymes thereby playing a major role in the cellular antioxidant system. Ablation of Nrf2 is known to be associated with increased sensitivity to oxidative stress and with inflammatory diseases, cancers, and cardiovascular disease (Mitsuishi et al., 2012; Wu et al., 2014).

Nrf2 and Inflammatory disorders

It has been suggested that Nrf2 may contribute to maintaining intestinal homeostasis by preventing excess ROS production in the intestinal epithelium (Mariani et al., 2014). It was shown that deletion of *Nrf2* causes some of the IBD pathology seen in mouse models (Khor et al., 2008). *Nrf2* knockout mice show a decreased expression of antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase, quinone 1 (NQO-1), and glutathione s transferase (GST) and are highly susceptible to colitis mouse models. Moreover, these mice also showed severe inflammation, decreased colon length, and loss of

crypts with infiltration of inflammatory cells (Khor et al., 2008; Khor et al., 2006). Collectively, these prompt us to investigate the possibility that TAK1 regulates ROS through modulating Nrf2.

Hypotheses (see Fig. 2 Model)

Hypothesis 1: Commensal bacteria induce ROS in the *Tak1* deficient intestinal epithelium and TAK1 regulates Paneth cell maintenance directly or through ROS.

Hypothesis 2: TAK1 regulates ROS through Nrf2.

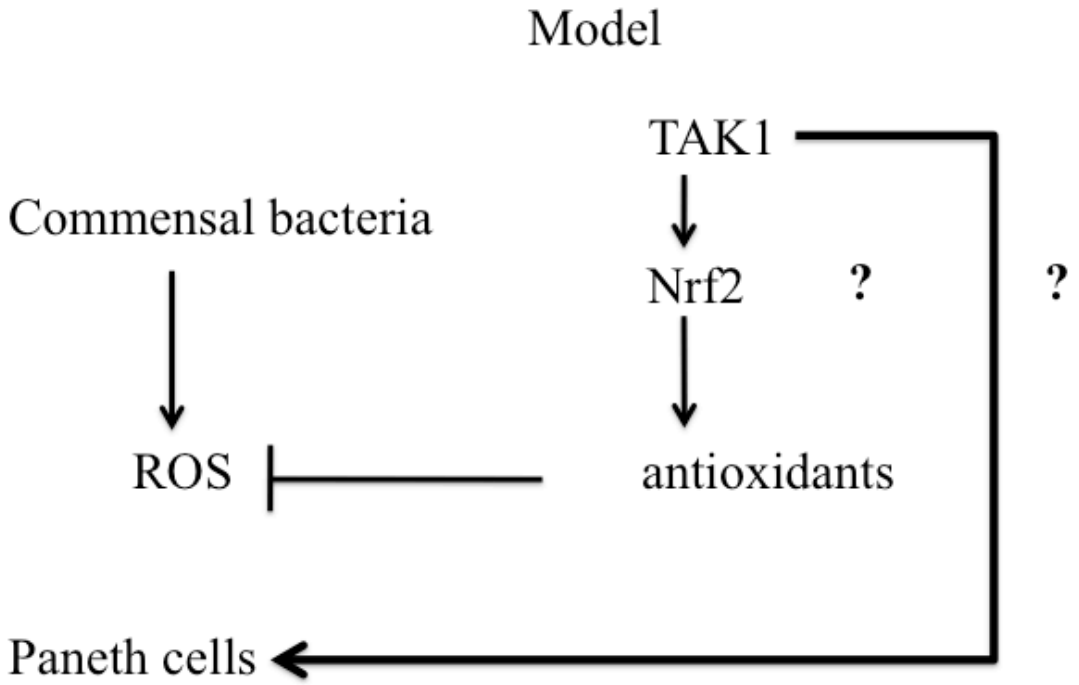

Figure 2: Model

MANUSCRIPT I

TAK1-dependent Paneth cell maintenance is required for prevention of intestinal oxidative stress

Alicia N. Simmons[1], Rie Kajino-Sakamoto[1,2], and Jun Ninomiya-Tsuji[1,3]

[1]Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695-7633 USA

[2]Current address: Division of Molecular Pathology, Aichi Cancer Center Research Institute, Nagoya, Aichi 464-8681 JAPAN

[3]To whom correspondence should be addressed:

Jun Ninomiya-Tsuji

E-mail: Jun_Tsuji@ncsu.edu

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ABSTRACT

Paneth cells reside at the base of crypts of the small intestine and secrete antimicrobial factors to control gut microbiota. Paneth cell loss is often observed in the chronically inflamed intestine; however, the relationship between Paneth cell loss and inflammatory tissue injury is not yet clear. Intestinal epithelial-specific deletion of a protein kinase *Tak1* (*Map3k7*) depletes Paneth cells and highly upregulates reactive oxygen species (ROS) in the mouse model. We found that depletion of gut bacteria or *Myd88*, a mediator of bacteria-derived cell signaling, reduced ROS but did not block Paneth cell loss, suggesting that gut bacteria are the cause of ROS accumulation but bacteria-induced ROS are not the cause of Paneth cell loss. In contrast, deletion of the cell death signaling intermediate, *Ripk3,* partially blocked Paneth cell loss and reduced ROS. These results demonstrate that *Tak1* deficiency-induced Paneth cell loss is the cause but not the result of oxidative stress in the intestine, which illustrates importance of Paneth cells in prevention of intestinal oxidative stress.

INTRODUCTION

TAK1 (MAP3K7) is a member of mitogen-activated protein kinase kinase kinase (MAP3K), and an indispensable signaling intermediate of proinflammatory cytokine and TLR/NLR signaling pathways leading to activation of transcription factors, NF-κB and AP-1 (reviewed in (Mihaly et al., 2014)). NF-κB and AP-1 induce expression of a number of proinflammatory and cell survival genes including several antioxidant genes (Sen and Packer, 1996). TAK1 was also found to regulate another redox transcription factor, Nrf2 (Kajino-Sakamoto et al., 2010). Through these transcription factors, TAK1 participates in the maintenance of the cellular antioxidant system. Deletion of *Tak1* impairs the cellular redox balance resulting in ROS accumulation in cultured cells (Morioka et al., 2009; Omori et al., 2010; Omori et al., 2008). *Tak1* deficiency causes cell death primarily through apoptosis (Morioka et al., 2014) but also induces a regulated type of necrosis so-called necroptosis (Arslan and Scheidereit, 2011; Lamothe et al., 2013; Vanlangenakker et al., 2011; Vucur et al., 2013). Increased ROS are causally associated with apoptosis in *Tak1*-deficient cells (Morioka et al., 2009; Omori et al., 2011; Omori et al., 2008), whereas the mechanism by which *Tak1* deficiency induces necroptosis is not yet clear.

In a mouse model, intestinal epithelial-specific *Tak1* deletion causes cell death, severe inflammatory conditions and perinatal animal lethality (Kajino-Sakamoto et al., 2008). Ablation of a proinflammatory cytokine TNF signaling by *Tnfr1* gene deletion effectively alleviates severe inflammation. However, the *Tak1*-deficient intestine still exhibits increased apoptosis in the crypt of the ileum and milder inflammatory conditions, which are similar to human ileitis (Kajino-Sakamoto et al., 2010). ROS are highly increased in the *Tak1*-deficient intestinal epithelium even on a *Tnfr1*$^{-/-}$ background (Kajino-Sakamoto et al., 2010).

Furthermore, we found that one specific cell type of the intestinal epithelium, Paneth cells, was depleted in the *Tak1*-deficient small intestine (shown in the current study). Paneth cells reside at the base of the crypts in the small intestine and are specialized to secret antimicrobial enzymes and peptides such as lysozyme C and α-defensins, which control commensal microbiota (Bevins and Salzman, 2011). Paneth cells are a unique cell type among the specialized intestinal epithelial cells, which have a very long life span of around 6-8 weeks, while other cells are constantly renewed about every 3-6 days in the mouse intestinal epithelium (Ireland et al., 2005; Sancho et al., 2003). Inflammatory bowel disease (IBD) is a group of chronic inflammatory diseases in the intestine, which is characterized by oxidative damage in the intestinal epithelium and are sometime associated with degradation of Paneth cells (Kaser et al., 2010; Xavier and Podolsky, 2007). One type of IBD, Crohn's disease is specifically characterized by ileitis and dysfunction of Paneth cells, which resemble the *Tak1*-deficient intestinal epithelium. In the current study, we sought to determine the mechanism by which *Tak1* deficiency causes IBD-like pathology i.e. increased ROS and loss of Paneth cells. We postulated two scenarios: one is that *Tak1* deficiency causes oxidative stress due to an impaired cellular redox system, which is the cause of Paneth cell loss: the other is that *Tak1* deficiency causes Paneth cell death, which results in disruption of normal gut microbiota leading to increased ROS. Better understanding of the relationship between two major IBD disorders: oxidative stress and Paneth cell loss could shed new insights into IBD pathogenesis, which is still largely undetermined.

RESULTS

Intestinal epithelial-specific deletion of *Tak1* depletes Paneth cells

To determine the mechanism by which *Tak1* deletion causes IBD-like intestinal injury, we initially re-evaluated the intestinal morphology in the *Tak1*-deficient intestinal epithelium. We used mice having intestinal epithelium-specific *Tak1* deletion on a *Tnfr1* null background (Tak1$^{IE\text{-}KO}$ Tnfr1$^{-/-}$). *Tnfr1* null background was used to block uncontrollable inflammatory conditions in Tak1$^{IE\text{-}KO}$, which does not reduce the number of cell death or the level of ROS in the *Tak1*-deficient intestinal epithelium (Kajino-Sakamoto et al., 2010). The intestine in Tak1$^{IE\text{-}KO}$ Tnfr1$^{-/-}$ mice was morphologically intact at postnatal day 17 (Fig. 1A and also see ref. (Kajino-Sakamoto et al., 2008)). The total number of intestinal epithelial cells per crypt did not decrease but rather increase in Tak1$^{IE\text{-}KO}$ Tnfr1$^{-/-}$ mice (Fig. 1A. and also see ref. (Kajino-Sakamoto et al., 2008)). Goblet and enteroendocrine cells are developed around birth and the number of those cells are not altered by *Tak1* deficiency (Kajino-Sakamoto et al., 2008). Paneth cells become detectable around 2-3 weeks of age concomitantly with the establishment of commensal microbiota (Bry et al., 1994). Paneth cells were also normally developed in Tak1$^{IE\text{-}KO}$ Tnfr1$^{-/-}$ at postnatal day 17 (P17) (Fig. 1A, bottom panels, and also see ref. (Kajino-Sakamoto et al., 2008)). These indicate that *Tak1* deficiency does not impair intestinal epithelial stem cells or their ability to differentiate toward specialized intestinal epithelial cells including Paneth cells. However, we found that Paneth cells were completely depleted in the adult Tak1$^{IE\text{-}KO}$ Tnfr1$^{-/-}$ mice (Fig. 1B). These results indicate that *Tak1*-deficient Paneth cells can complete their differentiation processes but they are not maintained. To further investigate Paneth cell loss in the *Tak1*-deficient intestinal epithelium, we used mice carrying an inducible intestinal epithelial-specific *Tak1* gene deletion system on a *Tnfr1*$^{-/-}$ background, *villin.CreER*^{T2} *Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$ (Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$). In this system, TAK1 is intact without an inducer of gene deletion, tamoxifen, and, upon

intraperitoneal injection of tamoxifen for 3 consecutive days (day 3), intestinal epithelium TAK1 protein was diminished and *Tak1* deletion was afterward maintained without additional tamoxifen treatment (Kajino-Sakamoto et al., 2010) (supplementary Fig. S1A). We found that the number of Paneth cells (granulated cells in the base of crypts) was gradually decreased starting at day 4 and depleted around day 7 (Fig. 1C). We note here that heterozygous deletion of *Tak1*, *Villin.CreER*^{T2} *Tak1*$^{flox/+}$ *Tnfr1*$^{-/-}$, did not exhibit any abnormality with tamoxifen treatment (supplementary Fig. S1B), demonstrating that the observed phenotypes are dependent on *Tak1* deletion but not on artifacts from inducible Cre expression. While the small intestine exhibited Paneth cell loss, the colon was found to be relatively intact in Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ mice even after 2 months (supplementary Fig. S2). Paneth cell loss was observed around day 7, which is much shorter than the lifespan of Paneth cells. Thus, the cause of Paneth cell depletion should not be due to impairment in the renewal processes but should be due to premature removal (cell death) of pre-existing Paneth cells. Indeed, we observed morphologically disrupted Paneth cells in Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ crypt at day 4 (Fig. 1C, top right panel, arrows). These results suggest that *Tak1* deletion induces Paneth cell depletion, which is likely to be caused by Paneth cell death.

Gut bacteria are the cause of accumulation of ROS in the *Tak1*-deficient intestinal epithelium.

Intestinal epithelial-specific *Tak1* deficiency induces ROS accumulation and apoptosis (Kajino-Sakamoto et al., 2010), which might be the cause of Paneth cell loss. To test this, we first attempted to reduce ROS in the Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ intestinal epithelium. The level of ROS in the intestine was monitored by CM-H_2DCFDH. Cellular peroxides convert CM-

H_2DCFDH to a fluorescent product that is trapped inside of the cells (Halliwell and Whiteman, 2004). Earlier studies have shown CM-H_2DCFDH staining using tissue sections in the intestine (Kajino-Sakamoto et al., 2010) and in the endothelium (Liu et al., 2013). We used unfixed freshly frozen sections and ROS positive signals were validated by treatment of a ROS scavenger, butylated hydroxyanisole (BHA) as shown previously (Kajino-Sakamoto et al., 2010). Bacterial moieties are major inducers of ROS in the intestinal epithelium (reviewed by (Lambeth and Neish, 2014)). Thus, we postulated that depletion of gut bacteria could reduce ROS in the *Tak1*-deficient intestinal epithelium. We treated mice with an antibiotic cocktail, ampicillin (1 g/l), vancomycin (1 g/l), neomycin sulfate (1 g/l), and metronidazole (1 g/l), which is commonly used for depletion of commensal bacteria (Rakoff-Nahoum et al., 2004), for 4 weeks and subsequently treated with tamoxifen to delete *Tak1*. In the absence of antibiotic treatment, ROS were highly increased by *Tak1* gene deletion and ROS accumulation was predominantly observed in the lower part of the crypts at day 7-10 (Fig. 2A), which is consistent with our previous results at day 3 (Kajino-Sakamoto et al., 2010). The level of ROS was greatly reduced with the pre-treatment of antibiotics (Fig. 2A and 2B). Apoptotic cells were also observed in the lower part of crypts (Fig. 2C), and antibiotic treatment greatly reduced the number of apoptotic cells in the $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ intestinal epithelium (Fig. 2C and 2D). These results suggest that commensal bacteria are the cause of ROS accumulation and apoptotic cell death in the *Tak1*-deficient intestinal epithelium.

TLR-MyD88 pathway mediates ROS accumulation.

Bacterial moieties are known to induce the production of ROS in host cells through TLR pathway (Lambeth and Neish, 2014). TLRs activate NADPH oxidases and also upregulate

mitochondrial ROS production (Laroux et al., 2005; Lipinski et al., 2009; West et al., 2011). We asked whether TLR signaling is responsible for ROS accumulation in the *Tak1*-deficient intestinal epithelium. TLR signaling pathways are mediated through two key adaptor proteins i.e. MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Kawai and Akira, 2010). Among them, TLR-MyD88 pathway is implicated in activation of ROS production (Laroux et al., 2005; Lipinski et al., 2009). To test the involvement of TLR-MyD88 signaling in *Tak1* deficiency-induced ROS, we utilized the inducible *Myd88*-deficient system (Hou et al., 2008). We generated mice harboring compound inducible deletion of *Tak1* and *Myd88* on a background of $Tnfr1^{-/-}$ ($Tak1^{IE\text{-}IKO}$, $Myd88^{IE\text{-}IKO}$ $Tnfr1^{-/-}$). We examined ROS levels in $Tak1^{IE\text{-}IKO}$, $Myd88^{IE\text{-}KO}$ $Tnfr1^{-/-}$ and *Myd88* heterozygous inducible deletion littermate ($Tak1^{IE\text{-}IKO}$, $Myd88^{Het}$ $Tnfr1^{-/-}$) mice. *Myd88* heterozygous intestinal epithelium exhibited increased ROS similar to *Tak1* single-deficient intestinal epithelium (Fig. 3B), but homozygous deletion of Myd88 alleviated accumulation of ROS (Fig. 3A and 3B). Apoptotic cells were also decreased in $Tak1^{IE\text{-}IKO}$, $Myd88^{IE\text{-}KO}$ $Tnfr1^{-/-}$ mice (Fig. 3C and 3D). Thus, *Myd88* deletion resembles the antibiotic treatment, suggesting that commensal bacteria-induced TLR-MyD88 signaling is the primary pathway to induce excess ROS accumulation in the *Tak1*-deficient intestinal epithelium. We note here that this partial prevention of ROS accumulation by the antibiotic treatment or *Myd88* deletion slightly improved intestinal injury (supplementary Fig. S3).

Paneth cell loss is independent on gut bacteria or MyD88

If ROS are the cause of Paneth cell loss, antibiotic treatment or *Myd88* deletion should block loss of Paneth cells in the *Tak1*-deficint intestinal epithelium. However, hematoxylin and

eosin (H&E) staining revealed that granulated cells in the base of crypt were still not observed in the antibiotic treated Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ intestinal epithelium (Fig. 4A), and only a few crypt base cells was detected as positive with a Paneth cell marker, lysozyme (Fig. 4B and 4C). Similarly, Paneth cells were not increased in Tak1$^{IE\text{-}IKO}$ Myd88$^{IE\text{-}KO}$ Tnfr1$^{-/-}$ intestinal crypts compared to control mice (Fig. 4D-F). Thus, ROS are not the cause of loss of Paneth cells in the *Tak1*-deficient intestinal epithelium. These results suggest that commensal bacteria are causally involved in increased ROS in the *Tak1*-deficient intestinal epithelium, whereas Paneth cells are depleted through a bacteria-ROS-independent mechanism.

RIPK3-dependent cell death is involved in Paneth cell loss and is the cause of ROS accumulation.

Our results above demonstrate that Paneth cell depletion is not due to bacteria-ROS-induced apoptosis. However, Paneth cells were depleted within a period shorter than their life span, and *Tak1* deletion structurally disrupts Paneth cells (see Fig. 1C). Thus, the cause of Paneth cell loss is still likely due to cell death. Ablation of *Tak1* is known to primarily induce apoptotic cell death (Morioka et al., 2014); however, it is also implicated in induction of necroptosis (Arslan and Scheidereit, 2011; Lamothe et al., 2013; Vanlangenakker et al., 2011; Vucur et al., 2013). Intestinal epithelial-specific deletion of *Tak1* could potentially induce apoptosis or/and necroptosis in Paneth cells. Interestingly, it was reported that intestinal epithelial-specific deletion of necroptosis inhibitors such as caspase 8 and its activator Fas-associated protein with death domain (FADD) induces Paneth cells loss (Gunther et al., 2011; Welz et al., 2011). This might suggest that Paneth cells are sensitive to

necroptosis. Necroptosis is morphologically indistinguishable from necrosis but characterized by a specific feature, dependency on a protein kinase, receptor interacted protein kinase 3 (RIPK3) (Vandenabeele et al., 2010). To determine whether Paneth cell loss in the *Tak1*-deficient intestinal epithelium is caused by necroptosis, we generated intestinal epithelial-specific deletion of *Tak1* on a background of $Ripk3^{-/-}$ mice ($Tak1^{IE\text{-}IKO}$ $Ripk3^{-/-}$). Intestinal injury was still somewhat observed in $Tak1^{IE\text{-}IKO}$ $Ripk3^{-/-}$ mice (Fig. 5A). However, we found that Paneth cell loss was partially blocked by *Ripk3* deletion (Fig. 5B and C). These suggest that Paneth cells in the *Tak1*-deficient intestinal epithelium were depleted at least partially due to necroptosis. Paneth cells are key intestinal epithelial cells to control commensal microbiota, and their dysfunction induces dysbiosis (Clevers and Bevins, 2013). Dysbiosis is known to be associated with increased production of ROS (Morgan et al., 2012). This raises the possibility that loss of Paneth cells may be the cause of excess accumulation of ROS in the *Tak1*-deficient intestinal epithelium. If this is the case, *Ripk3* deletion should alleviate accumulation of ROS in the *Tak1*-deficient intestinal epithelium. Indeed, we found that the level of ROS was moderately decreased by deletion of *Ripk3* compared to the *Tak1*-deficient intestinal epithelium on a $Tnfr1^{-/-}$ background (Fig. 5D, 5E and also see Fig. 2A). Collectively, these results suggest that loss of Paneth cells is the cause but not the result of bacteria-induced ROS in the *Tak1*-deficient intestinal epithelium.

DISCUSSION

Paneth cells are unique epithelial cells in the small intestine, which are raised from intestinal epithelial stem cells as are other intestinal epithelial cell types but migrate downward while all other cell types migrate upwards. Paneth cells are specialized to secret anti-microbial peptides and enzymes to control microbiota in the small intestinal crypts. Paneth cells are also visually unique in histological analysis, in which eosinophilic large granules occupy most of the cytoplasm. Destruction of Paneth cells is often histologically observed in human ileitis such as one type of IBD, Crohn's disease (Kaser et al., 2010; Xavier and Podolsky, 2007). Given their importance in gut microbiota homeostasis, disrupted Paneth cells are likely to be causally associated with ileitis. Indeed, Paneth cell loss has recently been implicated in the initiation of intestinal inflammation (Adolph et al., 2013). Thus, determination of the mechanism of how Paneth cells are maintained is important for better understanding of IBD pathology and treatment. Paneth cell loss has been reported in several genetically engineered mouse models. Most intriguingly, intestinal epithelium-specific deletion of *caspase 8* or its activator, *Fadd*, which are inhibitors of necroptosis, depletes Paneth cells (Gunther et al., 2011; Welz et al., 2011). This loss of Paneth cells is rescued by deletion of necroptosis mediator, *Ripk3*. Furthermore, RIPK3 is found to be increased in the intestine in IBD patient samples (Gunther et al., 2011). Thus, activation of necroptosis is likely to be one of the causes of pathological Paneth cell loss. However, the pathway of how necroptosis is activated in the intestinal epithelium is not clear. Our current study reveals that TAK1 is required for prevention of Paneth cell death. Since this cell death is prevented by deletion of a necroptosis mediator *Ripk3*, *Tak1*-deletion causes Paneth cell death through necroptosis. TAK1 is a protein kinase mediating inflammatory intracellular signaling

pathways leading to NF-κB and AP-1, which is activated by a variety of inflammatory stimuli including TNF, IL-1 and Toll-like receptor ligands. In these signaling pathways, another protein kinase RIPK1 is ubiquitilyated, which serves as a scaffold of signaling molecules including TAK1 (Ea et al., 2006). Both RIPK1 and TAK1 are essential molecules in these inflammatory signal transduction pathways. Recently, intestinal epithelial specific deletion of *Ripk1* is reported to deplete Paneth cells (Dannappel et al., 2014; Takahashi et al., 2014). Thus, deletion of either *Tak1* or *Ripk1* results in Paneth cell loss. This raises the possibility that impairment of inflammatory signaling causes Paneth cell loss. Given the fact that intestinal epithelium is constantly exposed to gut bacteria and immune cell-derived cytokines, it may not be surprising that proper inflammatory signaling from bacteria and cytokines is involved in the maintenance of Paneth cells. Homeostatic intestinal inflammatory signaling may be one of the key factors to maintain Paneth cells through preventing RIPK3-dependent necroptosis.

In the *Tak1*-deficient intestinal epithelium, ROS are highly accumulated and the intestinal epithelium is severely damaged. Our results demonstrate that gut bacteria cause ROS accumulation in the *Tak1*-deficient intestinal epithelium. Because inhibition of Paneth cell loss reduces ROS accumulation in the *Tak1*-deficient intestinal epithelium, Paneth cell loss-induced dysbiosis but not normal commensal bacteria is the predominant cause of ROS accumulation. However, oxidative injury in the *Tak1*-deficient intestinal epithelium seems to be much more pronounced compared to other genetically engineered mouse models harboring Paneth cell depletion. For example, *caspase 8* or *Fadd* deletion gradually induces ileitis in a non-inducible version of intestinal epithelium specific gene deletion system (Gunther et al., 2011; Welz et al., 2011), whereas the same deletion system causes severe

tissue damage and neonatal lethality when *Tak1* is deleted (Kajino-Sakamoto et al., 2008). This suggests that additional mechanisms are involved in the oxidative damage by *Tak1* deletion. *Tak1* deletion has been shown to reduce the capacity of cellular antioxidant system through downregulation of antioxidant transcription factors such as NF-κB, AP-1 and Nrf2 (Kajino-Sakamoto et al., 2010; Omori et al., 2008). Thus, the impaired antioxidant system may contribute to the high accumulation of ROS in the *Tak1*-deficient intestinal epithelium. These collectively suggest that homeostatic inflammatory signaling, which activates TAK1 in the intestine, is important not only for Paneth cell maintenance but also for integrity of cellular antioxidant system. Our results reveal the potential importance of homeostatic inflammatory signaling in the intestinal epithelium.

Our results clearly demonstrate that removal of bacterial-induced ROS did not block Paneth cell loss, whereas blockade of Paneth cell loss reduces ROS accumulation. Thus, Paneth cell necroptosis is not the result of excess ROS accumulation but rather the cause of dysbiosis-induced ROS. Paneth cells are emerging as the critical modulation system of intestinal homeostasis (Adolph et al., 2013; Clevers and Bevins, 2013). Our current study further illustrates the importance of Paneth cells in the regulation of oxidative stress.

MATERIALS AND METHODS

Mice

Mice carrying a floxed Map3k7 allele (*Tak1*$^{fl/fl}$) (Sato et al., 2005) were backcrossed to C57BL/6 mice for at least seven generations. *Tnfr1*-deficient (*Tnfr1*$^{-/-}$) (Pfeffer et al., 1993), Myd88-floxed (*Myd88*$^{fl/fl}$) (Hou et al., 2008) and an intestinal epithelium-specific deleter (*villin.Cre*) (Madison et al., 2002) mice with a C57BL/6 background were from The Jackson

Laboratory. The inducible intestinal epithelium-specific deleter (*villin.CreER^{T2}*) (a gift from Dr. Robine) (el Marjou et al., 2004) and *Ripk3*-deficient (*Ripk3$^{-/-}$*) (a gift from Dr. Dixit) (Newton et al., 2004) were also used. We generated constitutive and inducible versions of intestinal epithelium-specific *Tak1* (*villin.Cre Tak1$^{fl/fl}$,* TAK1$^{IE\text{-}KO}$; and *villin.CreER^{T2} Tak1$^{fl/fl}$,* TAK1$^{IE\text{-}IKO}$; respectively) on a *Tnfr1$^{-/-}$* or *Ripk3$^{-/-}$* background. Intestinal epithelium-specific compound deletion of *Tak1* and *Myd88* (*villin.CreER^{T2} Tak1$^{fl/fl}$ Myd88$^{fl/fl}$,* TAK1 Myd88$^{IE\text{-}IKO}$) mice were generated on a *Tnfr1$^{-/-}$* background. Littermate control mice (no-Cre *Tak1$^{fl/fl}$* or *villin.CreER*^{T2} *Tak1$^{fl/+}$* on a *Tnfr1$^{-/-}$* or *Ripk3$^{-/-}$* background or no-Cre *Tak1$^{fl/fl}$ Myd88$^{fl/fl}$* on a *Tnfr1$^{-/-}$* background) were included in all experiments, but some age matched control mice were also used. All control (*Tak1* wild type or heterozygous deletion) mice exhibited no ROS accumulation and 4-6 Paneth cells were observed in each crypt. To induce gene deletion, 6 to 12 weeks-old mice were given intraperitoneal injections of tamoxifen (1 mg per mouse, approximately 20 g body weight, per day) for three to five consecutive days. The first day of tamoxifen injection is herein referred to as day 1. For antibiotic treatment, the antibiotic cocktail consisting of ampicillin (1 g/l), vancomycin (1 g/l), neomycin sulfate (1 g/l), and metronidazole (1 g/l) (Rakoff-Nahoum et al., 2004) was added to the drinking water of 6-8 week old mice for 4 weeks prior to the tamoxifen injected. The antibiotic treatment was continued during and after the tamoxifen injection until the end of experiments. Mice were maintained in ventilated cages at the specific pathogen free animal facility and fed regular chow diet. All animal experiments were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering.

Histology, ROS and immunofluorescent staining

For hematoxylin and eosin (H&E) staining, a part of ileum was fixed in 4% paraformaldehyde and embedded into paraffin, and cross sections were stained by H&E. Sections are scored in a blinded fashion on the scale described previously (Kajino-Sakamoto et al., 2008). Remaining ileums were embedded into optimum cutting temperature (OCT) compound and frozen immediately. Cryosections (8 μm) were incubated with the ROS staining dye (CM-H_2DCFDA, Life Technologies) for 30 min at room temperature. To detect apoptotic cells, cryosections were fixed with 4% paraformaldehyde and cleaved caspase 3 was detected using a polyclonal antibody against cleaved caspase 3 (1:200, Cell Signaling). Bound antibodies were visualized by the Alexa Fluor 488 fluorescence dye-conjugated secondary antibody (1:1000, Life Technologies). Nuclei were counterstained with DAPI. Images were visualized using a fluorescent microscope (BX41; Olympus) controlled by the CellSens imaging software (Olympus). Random portions of the intestine were selected and images were visualized and photographed using the same exposure times. More than 100 crypts per mouse were counted and representative pictures are shown.

Analysis of Paneth Cells

4% paraformaldehyde fixed paraffin sections were rehydrated, heat induced antigen retrieval was performed in a citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), and the sections were stained using the muramidase (lysozyme) primary antibody (1:200, NovoCasta) overnight at 4^{o}C. Bound antibodies were visualized by the Alexa Fluor 488 fluorescence dye-conjugated secondary antibody. Random portions of the slides were

selected and photographed using the same exposure times. More than 100 crypts per mouse were counted.

Immunoblot analysis of intestinal epithelial cells

The small intestine was harvested and flushed with phosphate buffer saline (PBS). One end of the intestine was tied off, filled with Hanks' Balanced Salt Solution (HBSS, Sigma) containing 10 mM EDTA and incubated in a PBS bath at 37^{o}C for 5-10 min. The contents (intestinal epithelial cells) were collected and lysed in a cell extraction buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA, 10mM NaF, 2 mM DTT, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 μM aprotinin, and 0.5% Triton X-100. Proteins were electrophoresed on SDS-PAGE and transferred to Hypond-P membranes (GE Healthcare). The membranes were immunoblotted with anti-TAK1 (Ninomiya-Tsuji et al., 1999) and β-actin (AC-15, Sigma), the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Statistical Analysis

All experiments were conducted using at least three mice and the results are confirmed by at least three separately performed experiments. The box plots show medians (line), lower and upper quartiles (boxes), 10th and 90th percentiles (whiskers) and outliers. The column graphs represent the mean ± the standard deviation. Differences between experimental groups were assessed for significance by using the one-way ANOVA with Tukey's multiple comparisons test, or the unpaired Students t test (two-tailed) with equal distributions.

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Author contributions statement

AS and RK performed experiments. AS, RK and JNT designed the experiments, analyzed data and wrote the manuscript.

Conflict-of-interest disclosure

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1

Tak1 deletion depletes Paneth cells.

(A) H&E staining (upper panels) and immunofluorescent staining of Paneth cell marker, lysozyme (red) (lower panels) of control and the non-inducible version of intestinal epithelial-specific *Tak1*-deficient ileum on a *Tnfr1*$^{-/-}$ background at postnatal day 17. Scale bars, 20 μm.

(B) H&E staining of the crypts of ileum (3 months old). Scale bars; upper panels, 20 μm; lower panels (high magnifications), 10 μm.

(C) H&E staining of control and the inducible version of intestinal epithelial-specific *Tak1*-deficient crypts of ileum on a *Tnfr1*$^{-/-}$ background. Tamoxifen was treated for 3 consecutive days and analyzed at 4, 7 or 2 months after the initial tamoxifen treatment. Black arrows indicate structurally disrupted Paneth cells. Black scale bars, 20 μm: red scale bars, 10 μm.

Figure 2

Antibiotic treatment reduces ROS and apoptosis.

(A) ROS were determined by ROS sensitive dye (CM-H_2DCFDA) staining using fresh unfixed cryosections at day 7-10.

(B) ROS puncta in each crypt of samples in (A) was counted, and the data shown are from 4-6 mice of the average of ROS puncta in more than 100 crypts per mouse. Control Tnfr1$^{-/-}$ mice; without (n = 5) and with (n =4) antibiotics (Ab). Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ mice; without (n = 4) and with (n = 6) antibiotics. The box plots show medians (line), lower and upper quartiles (boxes), 10th and 90th percentiles (whiskers). **, $p < 0.01$ (one-way ANOVA).

(C) Immunofluorescent staining of cleaved caspase 3. Scale bars, 50 μm.

(D) Cells with cleaved caspase 3-positive staining were counted (more than 100 crypts per mouse). 3 mice per treatment group were analyzed. The plots show medians (line) and 10th and 90th percentiles (whiskers). **, $p < 0.01$ (two-tailed unpaired Student's t test).

Figure 3

Myd88 deletion reduces ROS and apoptosis.

(A) ROS sensitive dye staining of *Myd88* heterozygous or *Myd88* homozygous deletion $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ ileum crypts at day 10-12. Scale bars, 50 μm.

(B) ROS puncta in each crypt of samples in (A) was counted (more than 100 crypts per mouse). *Myd88* heterozygous deletion $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ mice; n = 7; *Myd88* homozygous deletion $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ mice; n = 8. The box plots show medians (line), lower and upper quartiles (boxes), 10th and 90th percentiles (whiskers) and an outlier. **, $p < 0.01$ (two-tailed unpaired Student's t test).

(C) Immunofluorescent staining of cleaved caspase 3. Scale bars, 50 μm.

(D) Quantification of cleaved caspase 3-positive staining (more than 100 crypts per mouse). *Myd88* heterozygous deletion $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ mice; n = 3; *Myd88* homozygous deletion $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ mice; n = 4. The box plots show medians (line), lower and upper quartiles (boxes), and 10th and 90th percentiles (whiskers). **, $p < 0.01$ (two-tailed unpaired Student's t test).

Figure 4

Antibiotic treatment or *Myd88* deletion does not block Paneth cell loss.

(A) H&E staining and (B) immunofluorescent staining of lysozyme in the crypts of ileum with and without antibiotic treatment at day 7-10. Scale bars, (A), 20 μm; (B), 40 μm.

(C) Quantification of lysozyme staining-positive cells. More than 100 crypts per mouse were counted. Control Tnfr1$^{-/-}$ mice; without (n = 5) and with (n =5) antibiotics. Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ mice; without (n = 4) and with (n = 7) antibiotics. The box plots show medians (line), lower and upper quartiles (boxes), and 10th and 90th percentiles (whiskers). NS, not significant (one-way ANOVA).

(D) H&E staining and (E) immunofluorescent staining of lysozyme of *Myd88* heterozygous or *Myd88* homozygous deletion Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ ileum crypts at day 10-12. Scale bars, (D), 20 μm; (E), 50 μm.

(F) Quantification of (E). *Myd88* heterozygous deletion Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ mice; n = 6; *Myd88* homozygous deletion Tak1$^{IE\text{-}IKO}$ Tnfr1$^{-/-}$ mice; n = 7. The box plots show medians (line), lower and upper quartiles (boxes), and 10th and 90th percentiles (whiskers). NS, not significant (one-way ANOVA).

Figure 5

Ripk3 deletion partially rescues Paneth cells loss and ROS accumulation.

(A) H&E staining and (B) immunofluorescent staining of lysozyme of the indicated mouse genotype ileum crypts at day 14. Scale bars, (A), 20 μm; (B), 50 μm.

(C) Quantification of (B). Ripk3$^{-/-}$, n = 5; Tak1$^{IE\text{-}IKO}$ Ripk3$^{+/-}$; n = 3; Tak1$^{IE\text{-}IKO}$ Ripk3$^{-/-}$; n = 5. The box plots show medians (line), lower and upper quartiles (boxes), and 10th and 90th percentiles (whiskers). *, $p < 0.05$ (one-way ANOVA).

(D) ROS sensitive dye staining of Ripk3$^{-/-}$ and Tak1$^{IE\text{-}IKO}$ Ripk3$^{-/-}$ ileum crypts at day 14.

(E) Quantification of (D). $Ripk3^{-/-}$, n = 4; $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$; n = 3; $Tak1^{IE\text{-}IKO}$ $Ripk3^{-/-}$; n = 8. The box plots show medians (line), lower and upper quartiles (boxes), and 10th and 90th percentiles (whiskers). **, $p < 0.01$ (one-way ANOVA).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1

(A) Intestinal epithelial cells were collected at day 12 and TAK1 protein was analyzed by immunoblotting. β-actin is shown as a loading control. Asterisk indicates a non-specific band. In this *Tak1*-floxed system (Sato et al., 2005), a truncated version of TAK1 (TAK1Δ) lacking the ATP binding site was produced by the gene deletion but it was expressed at a lower level compared to intake TAK1 presumably due to instability of a mutant protein.

(B) Heterozygous deletion of *Tak1* and inducible Cre expression does not cause Paneth cell loss. H&E staining of the ileum crypts at day 8. Scale bars, 20 μm.

Figure S2

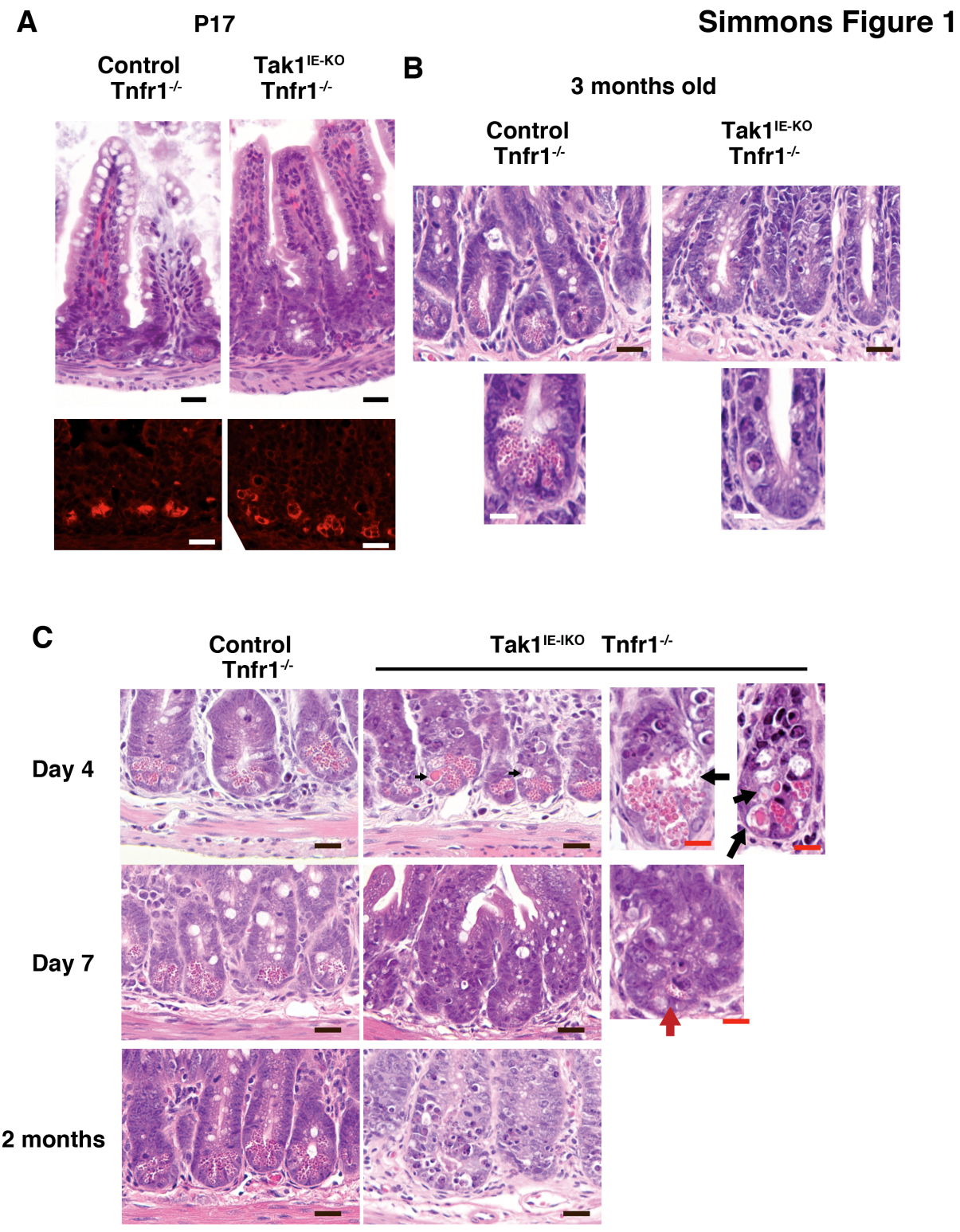
Tak1-deficient colonic crypts are relatively intact. H&E staining of the colonic crypts at day 4, day 7 and 2 months after the initiation of *Tak1* gene deletion. Arrows indicate cells morphologically showing apoptotic features. Scale bars, 20 μm.

Figure S3

Intestinal injury is slightly improved by antibiotic treatment or *Myd88* deletion in *Tak1*-deficient intestinal epithelium.

Histology scores at day 7-12 are shown. No-Cre $Tnfr1^{-/-}$ without (n = 5) and with (n = 6) antibiotic treatment (Abx); $Tak1^{IE\text{-}IKO}$ $Tnfr1^{-/-}$ without (n = 7) and with (n = 7) antibiotic treatment; $Tak1^{IE\text{-}IKO}$ $Myd88^{Het}$ $Tnfr1^{-/-}$ and $Tak1^{IE\text{-}IKO}$ $Myd88^{IE\text{-}IKO}$ $Tnfr1^{-/-}$, n = 5. Means ± SD. *, $p < 0.05$.

FIGURES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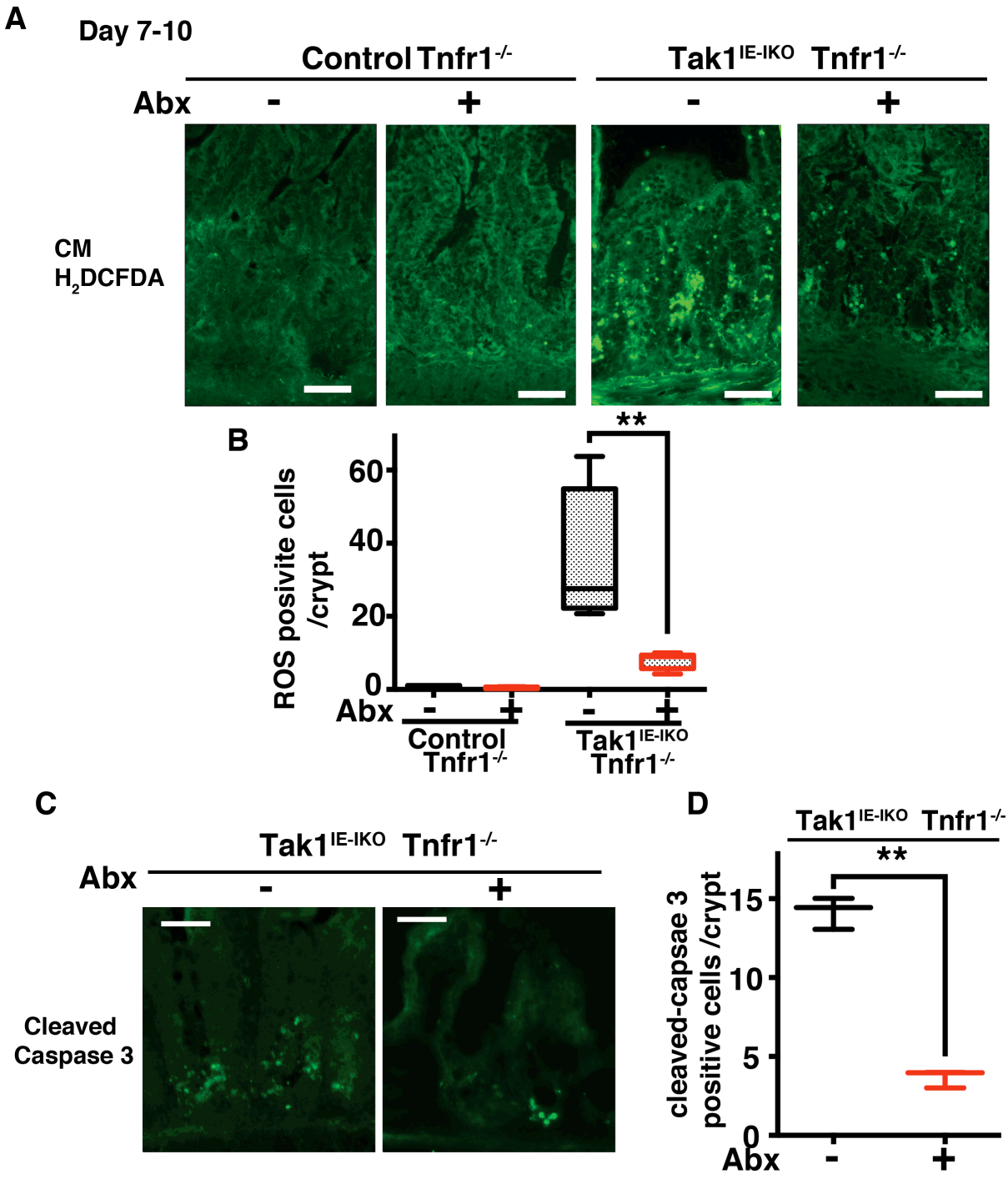
P17
Simmons Figure 1
Control
Tnfr1-/-
Tak1IE-KO
Tnfr1-/-
B
3 months old
Control
Tnfr1-/-
Tak1IE-KO
Tnfr1-/-
C
Control
Tnfr1-/-
Tak1IE-IKO Tnfr1-/-
Day 4
Day 7
2 months

Simmons Figure 2



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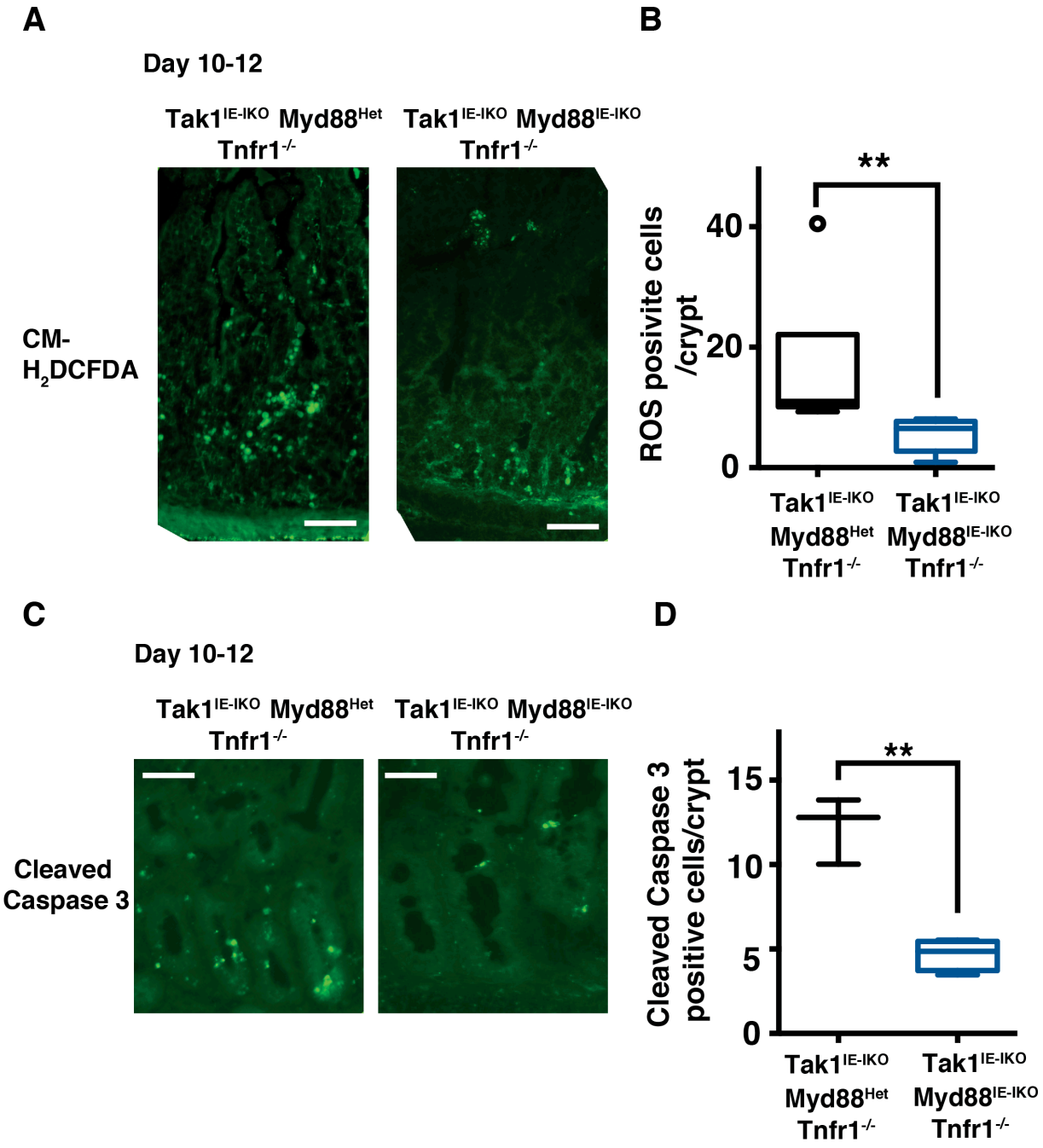

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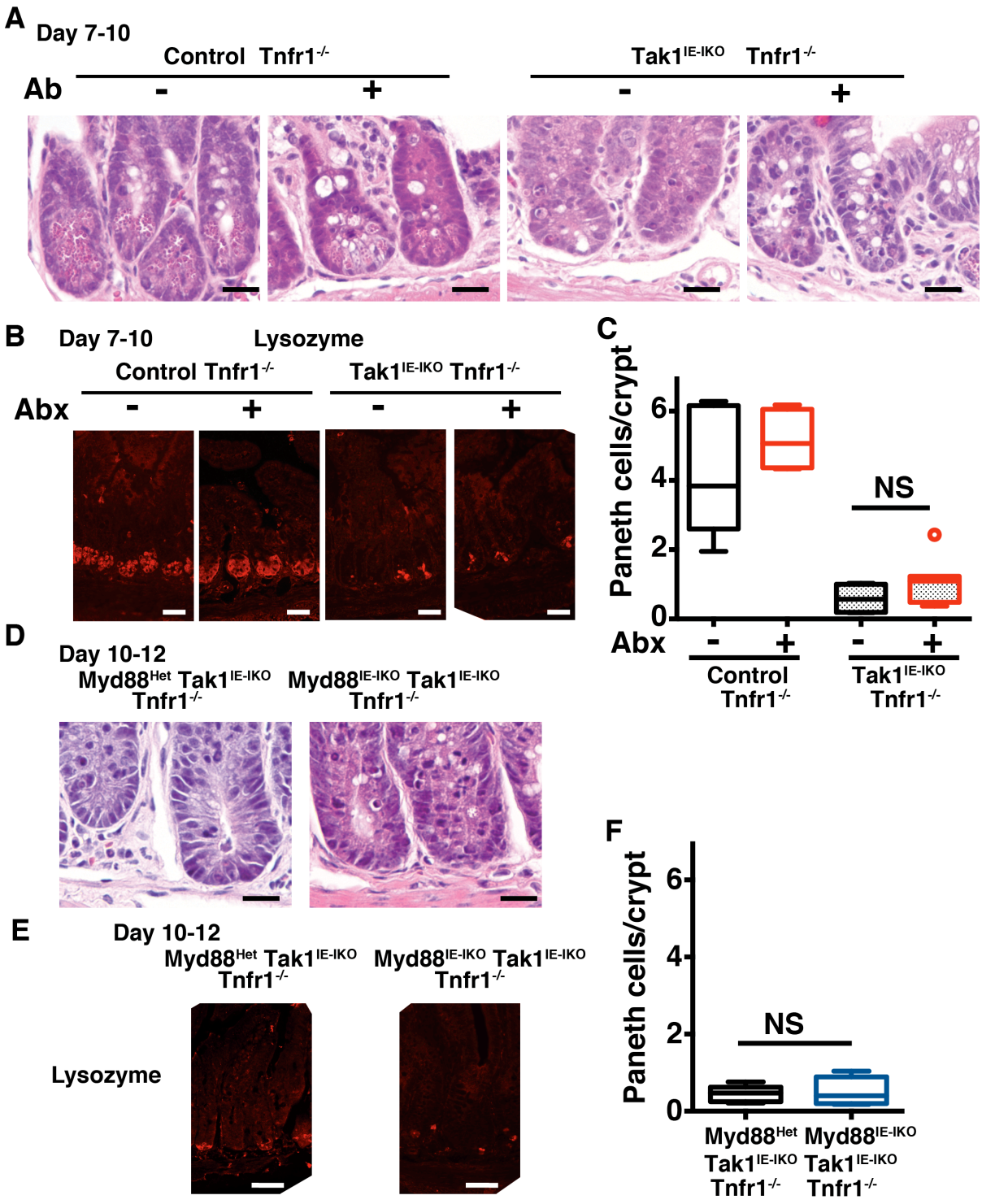
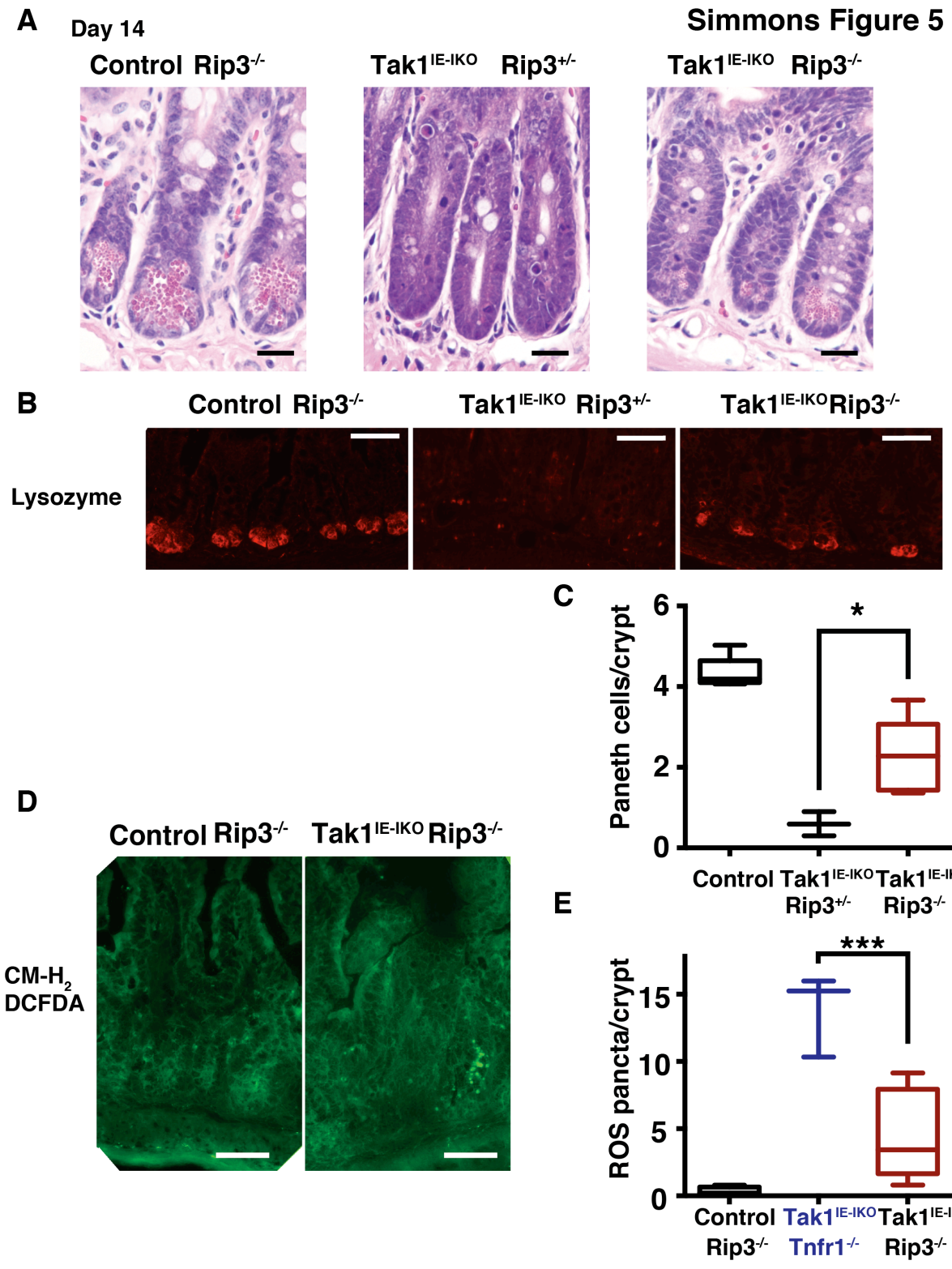
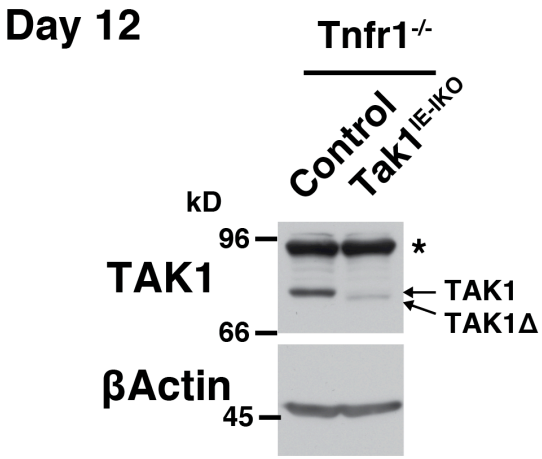


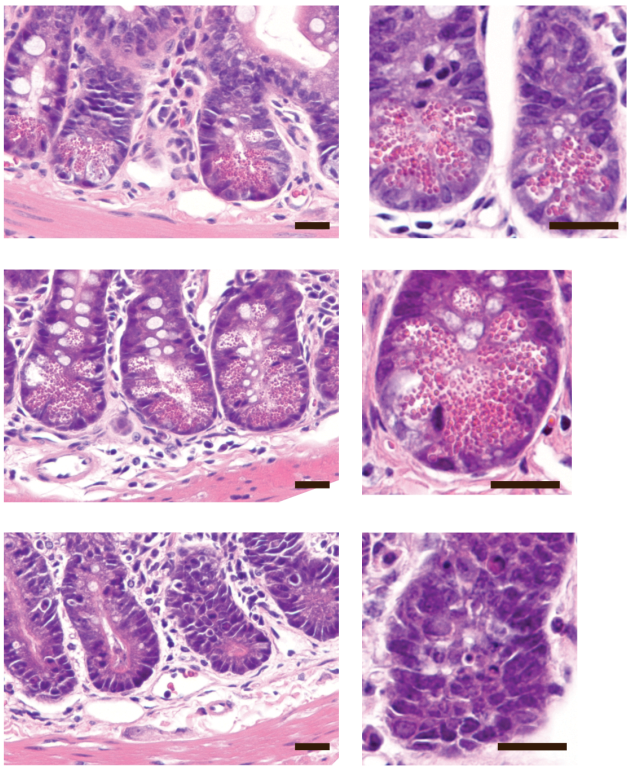

A
Day 14
Simmons Figure 5
Control Rip3-/-
Tak1IE-IKO Rip3+/-
Tak1IE-IKO Rip3-/-
B
Control Rip3-/-
Tak1IE-IKO Rip3+/-
Tak1IE-IKO Rip3-/-
Lysozyme
C
Paneth cells/crypt
6
4
2
0
*
Control
Tak1IE-IKO Rip3+/-
Tak1IE-IKO Rip3-/-
D
Control Rip3-/-
Tak1IE-IKO Rip3-/-
CM-H2 DCFDA
E
ROS puncta/crypt
15
10
5
0
***
Control Rip3-/-
Tak1IE-IKO Tnfr1-/-
Tak1IE-IKO Rip3-/-

Simmons Supplementary Figure S1

A



B Day 8



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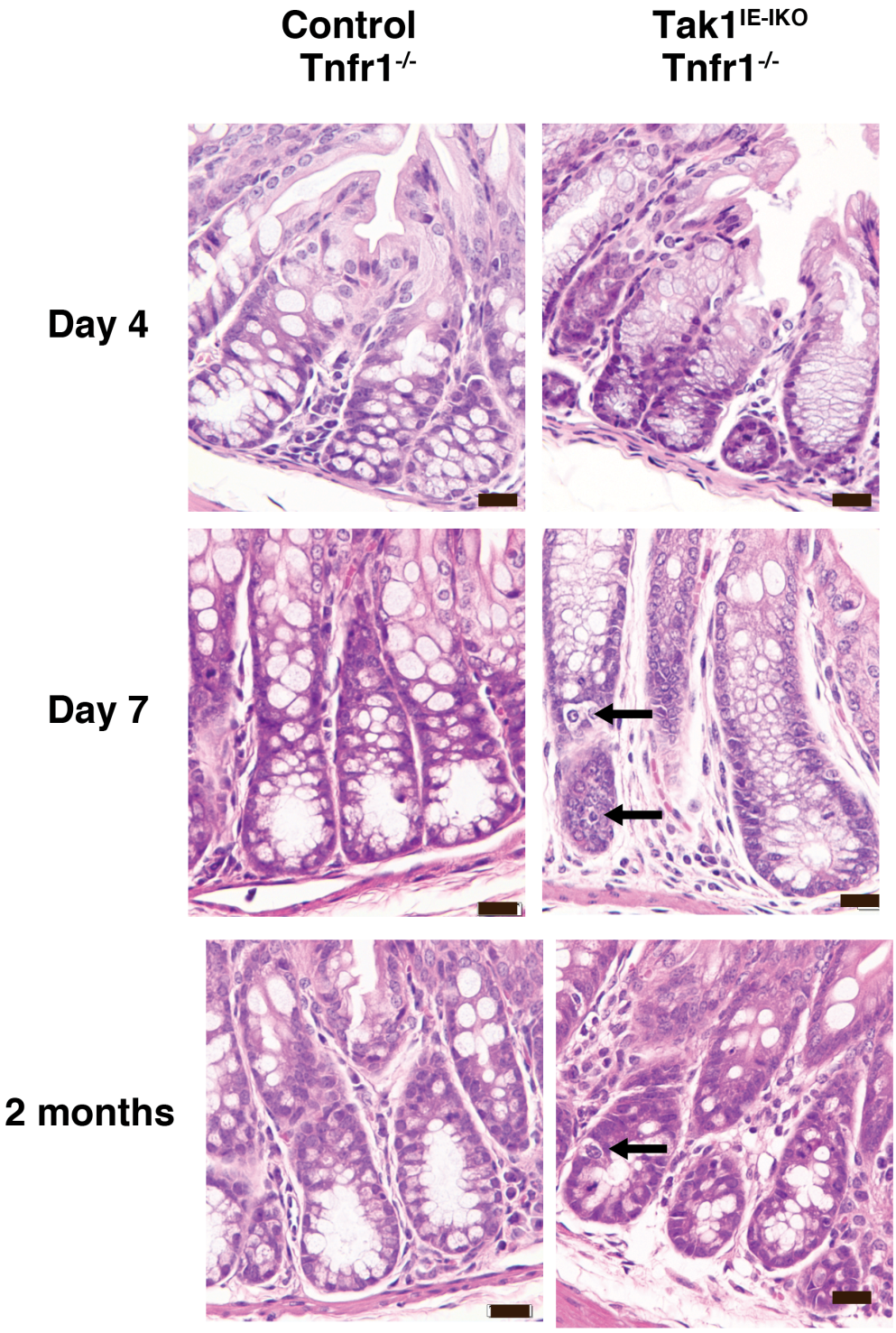

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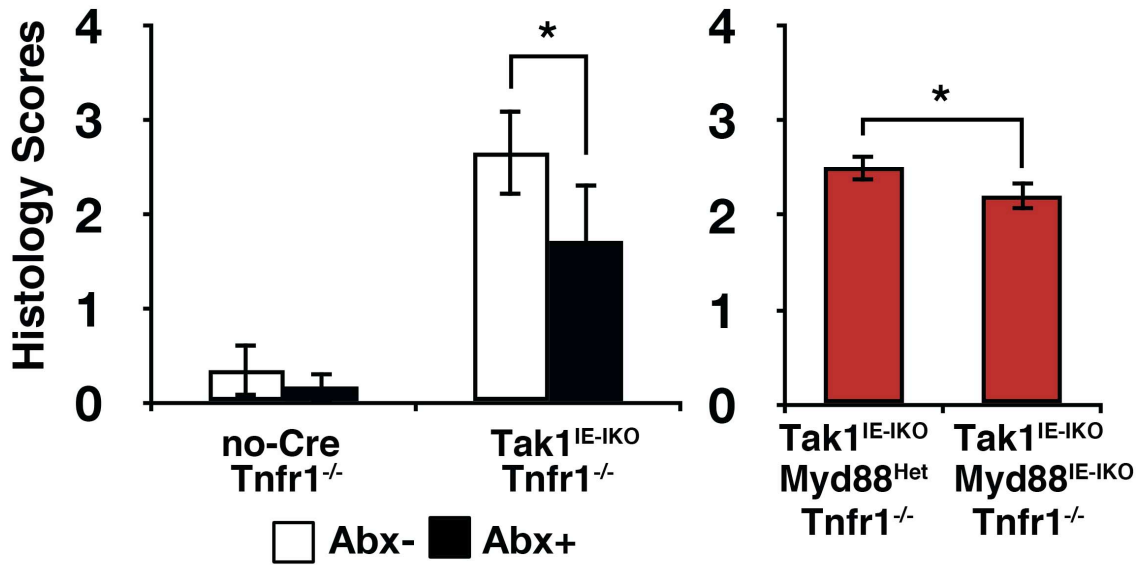

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MANUSCRIPT II:

TAK1-dependent and redox-independent regulation of Nrf2-Keap1 is required for prevention of oxidative tissue injury

Alicia N. Simmons[1], Kazunori Hashimoto[1], Rie Kajino-Sakamoto[1,2], Yoshiaki Tsuji[1] and Jun Ninomiya-Tsuji[1,3]

[1]Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695-7633 USA

[2]Current address: Division of Molecular Pathology, Aichi Cancer Center Research Institute, Nagoya, Aichi 464-8681 JAPAN

[3]To whom correspondence should be addressed:

Jun Ninomiya-Tsuji

E-mail: Jun_Tsuji@ncsu.edu

Key words: intestine, TAK1, Nrf2, Keap1

Running title: TAK1 regulates Nrf2

ABSTRACT

Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) and its binding partner Kelch-like ECH associated protein 1 (Keap1) are the master regulators of cellular redox homeostasis, which control antioxidant gene expression by sensing the cellular redox levels. Here we report that a protein kinase TGF-β activated kinase 1 (TAK1) is a previously uncharacterized modulator of Nrf2-Keap1. We found that TAK1 physically interacts with Nrf2 through Keap1 and upregulates Nrf2 protein stability. Ablation of TAK1 upregulated Keap1 and reduced Nrf2 levels under steady-state conditions. *Tak1* deficiency did not prevent redox-dependent increase of Nrf2. In the *in vivo* setting, intestinal-epithelial specific *Tak1* deletion downregulated Nrf2 and profoundly increased reactive oxygen species (ROS) without any exogenous oxidative stressors, while enforced activation of Nrf2 through the redox pathway restored the level of Nrf2 and reduced accumulation of ROS in the intestine. These results demonstrate that TAK1 regulation of Keap1-Nrf2 is required for a steady-state antioxidant protection, which is critical to prevent unattended increase of ROS in the *in vivo* environment.

INTRODUCTION

Nrf2 is a member of the Cap'n' Collar/basic leucine zipper (CNC)/(bZIP) families of proteins and transcriptionally upregulates a number of antioxidant genes (Jaramillo and Zhang, 2013; Kensler et al., 2007; Kobayashi et al., 2006). Cytoplasmic Nrf2 is constantly associated with Kelch-like ECH associated protein 1 (Keap1) (Itoh et al., 2010). Keap1 acts as an adaptor molecule for the Cul3 E3 ubiquitin ligase, which targets Nrf2 for degradation via the proteasome pathway (Kang et al., 2004). Conformational changes by covalent modifications on Keap1 block Cul3 ubiquitylation of Nrf2. Consequently, Keap1 is occupied by Nrf2 and newly synthesized Nrf2 stays as a Keap1-free form, which translocates into the nucleus (Baird et al., 2013; Itoh et al., 2003). Nuclear Nrf2 binds to a specific DNA element so-called antioxidant responsive element (ARE) and upregulates antioxidant genes including glutathione S-transferase (GST), NADPH quinone oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO1) (Taguchi et al., 2011).

Nrf2-Keap1 complex is primarily regulated by the cellular redox status through modification of specific cysteine residues including Cys151, Cys273 and Cys288 in mouse Keap1 (Dinkova-Kostova et al., 2002; Eggler et al., 2007; Yamamoto et al., 2008; Zhang and Hannink, 2003). Increased cellular ROS oxidize thiols to disulfides of these cysteine residues, or electrophilic oxidative stressors; sulforaphane and quinone compounds such as tert-butylhydroquinone (tBHQ), directly modify these cysteine residues, which cause conformational changes of Keap1 and stabilize Nrf2 (Abiko et al., 2011; Li et al., 2005; Takaya et al., 2012). In addition, Nrf2-Keap1 is modulated during tumorigenesis and metabolic changes. Somatic mutations in Nrf2 and Keap1 genes are reported to be associated

with tumorigenesis (Taguchi et al., 2011). Keap1 expression level is also regulated epigenetically through promoter methylation (Wang et al., 2008). These alterations downregulate Keap1 and upregulate Nrf2, which facilitates tumorigenesis by increasing antioxidants (Jaramillo and Zhang, 2013). Tumor suppressor Trp53 stabilizes Nrf2 through sequestration of Keap1 by its target gene products, p21 and sestrins (Bae et al., 2013; Chen et al., 2009). Nrf2 stability is also modulated by changes in a cellular catabolic process, autophagy, in which a scaffold protein p62/SQSTM1 binds to and recruits Keap1 into autophagosomes (Copple et al., 2010; Jain et al., 2010; Komatsu et al., 2010b; Lau et al., 2010). Accordingly, the level of Keap1 is downregulated and Nrf2 is upregulated by accumulation of p62/SQSTM1, which occurs when the autophagic catabolic process is disrupted and is generally associated with increased cell proliferation (Bjorkoy et al., 2009). Collectively, Nrf2 is regulated by acute oxidative stress through direct modifications on Keap1, which provides effective means to reduce excessive ROS: and Nrf2 is also chronically upregulated by elevated anabolism (inhibition of catabolic process) in the highly proliferative cells, which helps cell proliferation by removing hazardous ROS.

TGF-β activated kinase 1 (TAK1) is an intermediate of inflammatory signal transduction pathways, which is commonly activated by a diverse set of inflammatory stimuli including proinflammatory cytokines, microorganism moieties and chemical and physical stressors. TAK1 transmits signals from receptor or non-receptor activated signaling complexes to downstream targets including but not limited to two types of protein kinase cascades: mitogen activated protein kinase (MAPK) cascade and IκB kinase (IKK)-NF-κB cascade (Mihaly et al., 2014; Ninomiya-Tsuji et al., 1999). In this context, TAK1 is activated by autophosphorylation mediated by binding with the upstream protein complexes through

its binding proteins TAK1 binding protein 1 (TAB1) and TAK1 binding protein 2 (TAB2), and TAK1 phosphorylates and activates downstream protein kinases (Mihaly et al., 2014). Genetic ablation of TAK1 in mouse models have revealed that TAK1 is required for prevention of oxidative tissue injury in the epidermis, intestinal epithelium, and liver as well as in the skin tumors (Inokuchi et al., 2010; Kajino-Sakamoto et al., 2010; Omori et al., 2010; Omori et al., 2008). *Tak1* deficiency impairs activation of MAPKs and IKK-NF-κB, and one of MAPKs, JNK and its target c-Jun are shown to be partially involved in TAK1 regulation of reactive oxygen species (ROS) (Omori et al., 2008). However, IKK-NF-κB pathway is totally dispensable for TAK1 regulation of ROS (Omori et al., 2008). Furthermore, *Tak1* deletion is found to reduce the protein level of Nrf2 in the intestinal epithelium (Kajino-Sakamoto et al., 2010), suggesting that TAK1 modulates ROS also through the Nrf2-Keap1 pathway. In the current study, we attempted to define the role of TAK1 in the regulation of Nrf2-Keap1.

RESULTS AND DISCUSSION

TAK1 physically interacts with Nrf2 through Keap1.

Intestinal epithelial-specific *Tak1* deletion reduces the protein amount of Nrf2 in the intestine, however, the Nrf2 mRNA level is not altered (Kajino-Sakamoto et al., 2010). These suggest that TAK1 regulates Nrf2 at the protein level but not transcription of Nrf2 gene. We first examined whether activation of TAK1 upregulates Nrf2 protein levels independent of transcription. Overexpression of active form of TAK1 (co-expression of TAK1 and TAB1) increased the amount of Nrf2 in human embryonic kidney 293 (HEK293) cells, even when Nrf2 is expressed driven by an ectopic constitutive promoter (Fig. 1A). This confirms that TAK1 post-transcriptionally modulates Nrf2. A kinase-dead form of TAK1 was incapable of upregulating Nrf2 (Fig. 1B), indicating that this regulation requires TAK1 protein kinase activity. Post-transcriptional regulations may involve a physical protein-protein interaction. We then examined whether TAK1 binds to Nrf2 and Keap1. TAK1 was not co-precipitated with Nrf2 (Fig. 1C), whereas Keap1 was co-precipitated with TAK1 (Fig. 1D). Interestingly, Nrf2 is co-precipitated with TAK1 when co-expressed together with Keap1 (Fig. 1E), suggesting that TAK1 interacts with Nrf2 through Keap1. These raise the possibility that TAK1 modulates Nrf2 through Keap1.

TAK1 stabilizes Nrf2 through Keap1.

The level of Nrf2 protein is primarily regulated by modulation of Nrf2 protein stability (Itoh et al., 2010). We next examined whether TAK1 stabilizes Nrf2. An active form of TAK1 (co-expression of TAK1 and TAB1), Nrf2 with and without Keap1 were expressed in HEK293 cells and Nrf2 protein stability was assessed by treatment with cycloheximide. Nrf2 was

degraded quickly and the half-life was less than one hour (Fig. 2A and 2B), which is consistent with a number of earlier studies (McMahon et al., 2003; Nguyen et al., 2003). Activated TAK1 greatly reduced degradation of Nrf2 (Fig. 2A, third panel, and 2B). Interestingly, TAK1 blocked Nrf2 degradation more effectively when Keap1 was co-expressed compared to the efficiency without Keap1 co-expression (Fig. 2A, third and bottom panel, and 2B). Nrf2 is known to be degraded through both Keap1-dependent and –independent mechanisms (Itoh et al., 2003). When Nrf2 but not Keap1 is exogenously overexpressed, only a fraction Nrf2 can form a complex with endogenous Keap1, which increases Keap1-free Nrf2 molecules. Such Nrf2 may not be regulated by TAK1, since TAK1 interacts with Nrf2 through Keap1. Thus, the results in Fig. 2 suggest that TAK1 blocks Nrf2 degradation through modulating Keap1.

TAK1 modulates Keap1 independently of electrophilic modification.

We next asked how TAK1 modulates Keap1. We found that Keap1 was upregulated in *Tak1*-deficient mouse embryonic fibroblasts (Fig. 3A), and was also increased by *Tak1* knockdown in HEK293 (Fig. 3B). These suggest that TAK1 may limit the Keap1 protein levels, which could block Nrf2 degradation. Keap1 is known to be regulated through several binding proteins such as p21, sestrins and p62/SQSTM1 (Bae et al., 2013; Chen et al., 2009; Copple et al., 2010; Jain et al., 2010; Komatsu et al., 2010b; Lau et al., 2010). Among them, p62/SQSTM1 binds to and targets Keap1 for autophagic degradation (Komatsu et al., 2010a). Intriguingly, deletion of *Tak1* is known to upregulate p62/SQSTM1 (Inokuchi-Shimizu et al., 2014). Thus, TAK1 may modulate Keap1 protein level through p62/SQSTM1. Furthermore, p62/SQSTM1 can physically interact with the TAK1 signaling complex containing TNF

receptor associated factor 6 (TRAF6) (Sanz et al., 2000), which raises an additional possibility that TAK1 signaling blocks Keap1 activity through sequestering Keap1 into the TAK1 inflammatory signaling complex. If TAK1 modulates Keap1 protein levels through p62/SQSTM1, this regulation should be independent of the canonical oxidative stress regulation of Keap1. We asked whether *Tak1* deletion alters electrophilic modulation of Nrf2-Keap1 by using tBHQ, which is one of the most extensively studied electrophilic compounds and covalently modifies Keap1 at Cys151 (Abiko et al., 2011; Takaya et al., 2012). We used wild type and *Tak1*-deficient keratinocytes, which expressed a detectable level of Nrf2 without any oxidative stresses, and the steady-state level of Nrf2 is lower in *Tak1*-deficient cells compared to wild type with tBHQ treatment (Fig. 4A and 4B), which is consistent with the results in intestinal epithelium (Kajino-Sakamoto et al., 2010). The Nrf2 levels could be highly upregulated by blockade of proteasome using MG132 in both wild type and *Tak1*-deficient keratinocytes (Fig. 4A). The protein level of Nrf2 was highly upregulated upon tBHQ treatment in both wild type and *Tak1*-deficient cells (Fig. 4A and 4B), although the accumulation of Nrf2 was slower in *Tak1*-deficint cells (Fig. 4B). The fold-increase of Nrf2 was higher in *Tak1*-deficent cells compared to wild type. These suggest that TAK1 regulation of Nrf2-Keap1 is independent of the redox regulation, and that TAK1 determines the steady-state level of Nrf2.

Electrophilic activation of Nrf2 prevents ROS in *Tak1*-deficient intestinal epithelium

We previously reported that intestinal epithelial-specific inducible *Tak1* deletion ($Tak1^{IE\text{-}IKO}$) mice develop inflammatory bowel disease-like tissue injury upon *Tak1* gene deletion, which is associated with profound increase of ROS in the intestine (Kajino-Sakamoto et al., 2010).

Severe tissue injury can be blocked by additional gene deletion of TNF receptor 1, which is due to reduced inflammation. However, increased ROS are still observed in Tak1$^{IE\text{-}IKO}$ on the background of TNF receptor 1 deletion (*Tnfr1-/-*) (Kajino-Sakamoto et al., 2010). We further demonstrated that treatment of butylated hydroxyanisole (BHA), which is metabolized to tBHQ in the liver, could prevent the increase of ROS (Kajino-Sakamoto et al., 2010). However, the involvement of Nrf2 was not explored in this *in vivo* setting. Our current study revealed that TAK1 regulation of Nrf2 is independent of tBHQ-induced Nrf2 activation. This raises the possibility that BHA treatment may upregulate Nrf2 in the *Tak1*-deficient intestinal epithelium. Control (*Tnfr1-/-*) and Tak1$^{IE\text{-}IKO}$ *Tnfr1-/-* mice were fed with BHA containing food starting from one week prior to the induction of gene deletion, and *Tak1* gene deletion was induced by an inducer tamoxifen injection once per day for 3 consecutive days. Proteins from control and *Tak1*-deficient intestinal epithelium were analyzed by immunoblotting (Fig. 5A). Nrf2 protein was highly upregulated by BHA treatment in Tak1$^{IE\text{-}IKO}$ *Tnfr1-/-*intestinal epithelium, which was correlated with the reduction of ROS (Fig. 5B). These results demonstrate that enforced upregulation of Nrf2 reduces intestinal ROS in *Tak1*-deficient intestinal epithelium. Collectively, TAK1 regulates steady-state Nrf2, and disruption of this regulation is causally associated with increased ROS *in vivo*. Gene deletion of *Nrf2* in mouse models does not cause any abnormalities in tissues including the intestine (Chan et al., 1996). Thus, it is clear that reduced Nrf2 in *Tak1*-deficient intestinal epithelium is not solely the cause of ROS accumulation. Accordingly, TAK1 is likely to modulate multiple biological processes including Nrf2, which are associated with the regulation of ROS in the intestine, and *Tak1* deletion impairs these multiple processes resulting in ROS accumulation. If this is correct, enforced Nrf2 activation would reduce ROS, but it should not restore other impaired

processes. Indeed, in a separate study, we found that intestinal epithelial-specific *Tak1* deletion causes abnormality in one specific cell type, named Paneth cell, among intestinal epithelial cells, which was not restored by BHA treatment (unpublished results). Thus, TAK1 regulation of Nrf2 is not the sole but one of the critical processes in the TAK1 regulation of tissue homeostasis.

In summary, our results demonstrate that TAK1 is a previously uncharacterized regulator of Nrf2-Keap1, and this regulation is likely through physical interaction between TAK1 and Keap1. TAK1 seems to limit steady-state Keap1 protein levels potentially through modulating p62/SQSTM1. Our *in vivo* results further suggest that TAK1 regulation of Keap1 is critical for normal tissue homeostasis by participating in prevention of ROS accumulation.

MATERIALS & METHODS

Tissue culture and mouse models

Human embryonic kidney (HEK) 293 and fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone) and 50 I.U./ml penicillin-streptomycin at 37 °C in 5% CO_2. Keratinocytes (immortalized) were originally isolated from epidermal-specific *Tak1*-deficient and control mice (Omori et al., 2006), and cultured in Ca^{2+}-free Eagle's minimal essential medium (BioWhittaker) supplemented with 4% Chelex-treated bovine growth serum, 10 ng/ml of human epidermal growth factor (Invitrogen), 0.05 mM calcium chloride, and 50 I.U./ml penicillin-streptomycin at 33°C in 8% CO_2. Some cells were treated with tert-butylhydroquinone (tBHQ) (Sigma) and/or MG132 (Millipore). Mice carrying a floxed *Map3k7* allele ($Tak1^{fl/fl}$) (Sato et al., 2005) were backcrossed to C57BL/6 mice for at least seven generations. *Tnfr1*-deficient ($Tnfr1^{-/-}$) (Pfeffer et al., 1993) (Jackson lab) and an intestinal epithelium-specific inducible deleter ($villin.CreER^{T2}$) (el Marjou et al., 2004) mice with a C57BL/6 background were used. We generated inducible intestinal epithelium-specific *Tak1* ($villin.CreER^{T2}$ $Tak1^{flox/floxl}$, $TAK1^{IE\text{-}IKO}$) on a *Tnfr1-/-* background. To induce gene deletion, 6 to 12 weeks-old mice were given intraperitoneal injections of tamoxifen (1 mg per mouse, approximately 20 g body weight, per day) for three consecutive days. The first day of tamoxifen injection is herein referred to as day 1. Some mice were fed with food containing 0.7% butylated hydroxyanisole (BHA) from 1 week prior to the tamoxifen treatment, and mice were continuously given BHA food for the entire period of the experiments. All animal experiments were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering.

Transfection, immunoprecipitation and immunoblotting

Expression vectors for HA-tagged TAK1, TAB1 (Ninomiya-Tsuji et al., 1999), Nrf2 (Huang et al., 2013), and T7-tagged Keap1 (Furukawa and Xiong, 2005) were used. The standard calcium phosphate transfection method was used. Cell lysates were prepared at 48 h post transfection using an extraction buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μM aprotinin, and 0.5% Triton X-100. In some experiments for detecting endogenous Nrf2, nuclear extracts were prepared using Nuclear Extraction kit (Active Motif). Cell extracts were immunoprecipitated withanti-TAK1 (Ninomiya-Tsuji et al., 1999), anti-HA (16B12) (Biolegend), anti-T7 (Millipore) or non-immunized control IgG with Protein G Sepharose 4 fast flow (GE Healthcare). The beads were washed three times with a wash buffer (20 mM HEPES pH 7.4, 10 mM MgCl2, 0.5 M NaCl) and once with a kinase buffer (10 mM HEPES pH 7.4, 1 mM DTT, 5 mM MgCl2). Immunoprecipitates and whole cell lysates were resolved on SDS-PAGE and transferred to Hypond-P membranes (GE Healthcare). The membranes were immunoblotted with anti-TAK1, anti-HA, anti-T7, anti-Keap1 (clone 144, Millipore) and anti-Nrf2 (C2, Santa Cruz), and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Protein stability Assay

HEK293 cells were transfected with expression vectors for TAK1+TAB1 (active TAK1), Nrf2, and T7-Keap1. Cells were treated with 100 μg/ml cycloheximide (CHX) at 48 h after transfection and harvested at 0, 1 and 5 h post CHX treatment. The Nrf2 protein amounts were determined by immunoblotting and semi-quantified by Image J densitometry software.

Tak1 knockdown

siRNAs *TAK1* and non-targeting control siRNAs were obtained from Sigma (*TAK1* siRNA, 5'-GAGAUCGACUACAAGGAGA-3' and Non-targeting siRNA, 5'-UUCUCCGAACGUGUCACGU-3'). HEK293 cells were transfected siRNAs by a standard electroporation method using Gene Pulser Xcell™ Electroporation System (Bio-Rad).

ROS staining

Ileums were embedded into optimum cutting temperature (OCT) compound and frozen immediately. Cryosections (8 μm) were incubated with the ROS staining dye (CM-H_2DCFDA, Life Technologies) for 30 min at room temperature. Images were visualized using a fluorescent microscope (BX41; Olympus) controlled by the CellSens imaging software (Olympus). Random portions of the intestine were selected and images were visualized and photographed using the same exposure times.

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FIGURE LEGENDS

Figure 1

TAK1 upregulates Nrf2, and binds to Nrf2 through Keap1.

(A) HEK293 were transfected with expression vectors (CMV promoter) for increasing amounts of HA-TAK1 (0, 1, 10, 100, 500 ng), and the same amount of TAB1 (100 ng) and Nrf2 (500 ng). Nrf2 and TAK1 amounts were analyzed by immunoblotting. Lamin B and tubulin are shown as loading controls.

(B) HEK293 cells were transfected with expression vectors for HA-TAK1 or HA-tagged kinase-dead form of TAK1 [HA-TAK1(K63W)] together with TAB1 and Nrf2. HA-TAK1(K63W) was migrated faster than wild type HA-TAK1 (3rd panel), which is known to be due to lack of autophosphorylation (Kishimoto et al., 2000).

(C) HEK293 cells were transfected with expression vectors for TAK1 and Nrf2, and cell lysates were immunoprecipitated with Protein G beads (no-antibody), non-immunized IgG, or anti-Nrf2. The precipitates were analyzed by immunoblotting. Inputs (1/100 volume used for immunoprecipitation) are also shown.

(D, E) HEK293 cells were transfected with expression vectors for HA-TAK1, T7-Keap1, Nrf2 as indicated, and cell lysates were immunoprecipitated with anti-HA (HA), anti-T7 (T7) or control IgG (C).

Figure 2

TAK1 stabilizes Nrf2.

HEK293 cells were transfected with expression vectors for Nrf2, TAK1, TAB1 and Keap1, and were treated with 100 μg/ml cycloheximide (CHX) at 48 h post transfection. Cell lysates

were prepared at 0, 1 and 5 h after CHX treatment, and analyzed by immunoblotting with anti Nrf2. The Nrf2 band intensities were quantified and the relative values (percent) to that of 0 time are shown (right graphs). The results shown are a representative from similar three independent experiments.

Figure 3

Ablation of TAK1 upregulates Keap1.

(A) Wild type and *Tak1*-deficient fibroblasts were analyzed by immunoblotting. GAPDH and b-actin are shown as loading controls.

(B) HEK293 cells were transfected with non-targeted (control) or targeted against *Tak1* siRNA, and cell lysates at 48 h post transfection were analyzed by immunoblotting.

Figure 4

Electrophilic activation of Nrf2 is intact in *Tak1*-defieicnt cells.

(A) Wild type and *Tak1*-deficient (*Tak1* Δ/Δ) keratinocytes were treated with 100 μM tBHQ for 6 hand cell lysates were analyzed by immunoblottings. Some cells were pretreated with MG132 for 2 h prior to tBHQ treatment to block proteasome-dependent protein degradation. Lamin B is shown as a loading control.

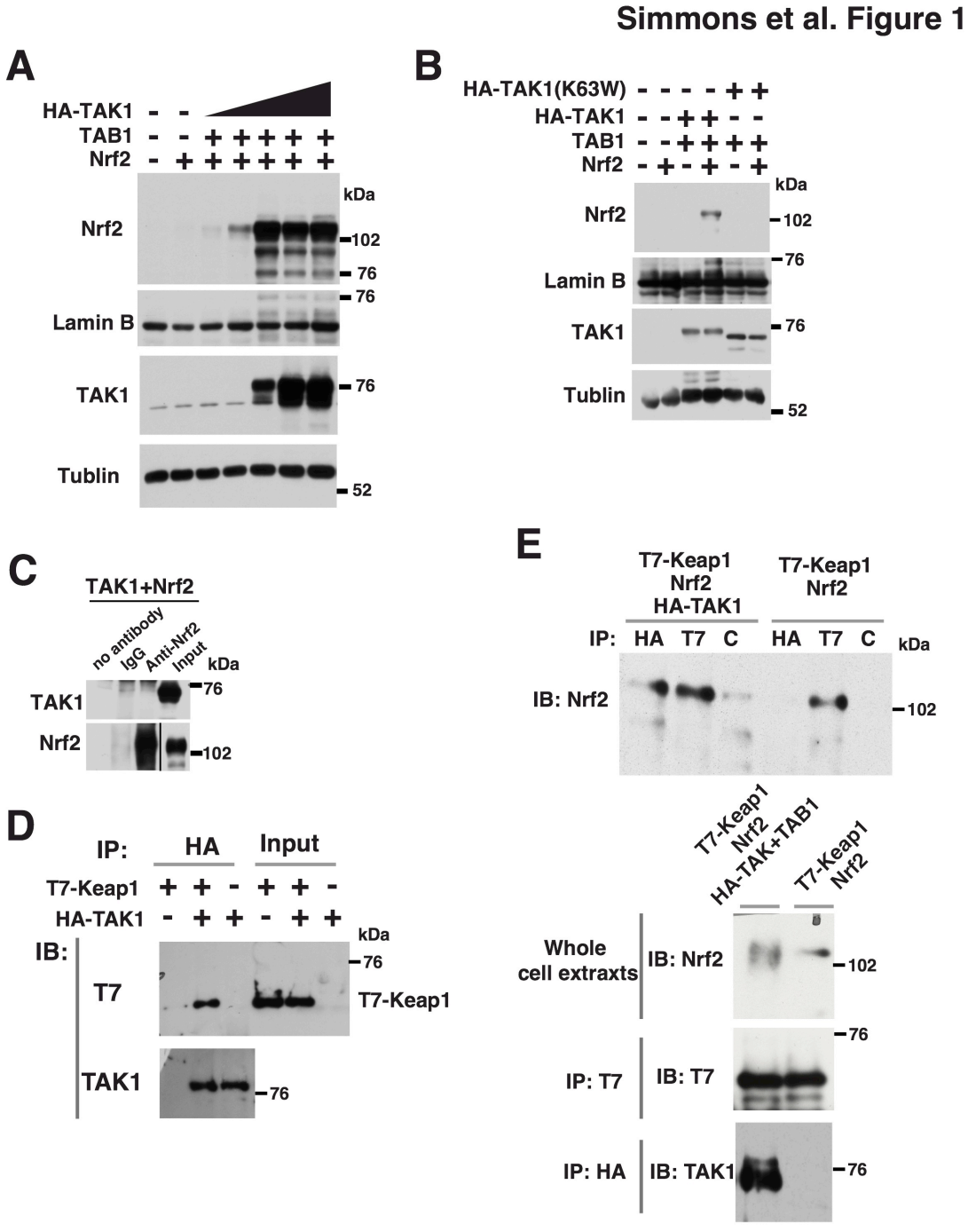
(B) Time course of Nrf2 accumulation with 100 μM tBHQ treatment.

Figure 5

Electrophilic activation of Nrf2 blocks ROS accumulation in *Tak1*-deficient intestinal epithelium.

Control (*Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$) and TAK1$^{IE\text{-}IKO}$ *Tnfr1*$^{-/-}$ mice were fed with BHA contain food from one week prior to injection of tamoxifen for 3 consecutive days, and the small intestine was harvested at day 3 (B) or 7 (A). Protein extracts from the intestinal epithelium were analyzed with immunoblotting (A). Fresh frozen sections without any fixation were stained by CM-H_2DCFDA. Scale bars, 50 μm.

FIGURES


Simmons et al. Figure 1
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Tublin
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TAK1+Nrf2
no antibody
IgG
Anti-Nrf2
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TAK1
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Nrf2
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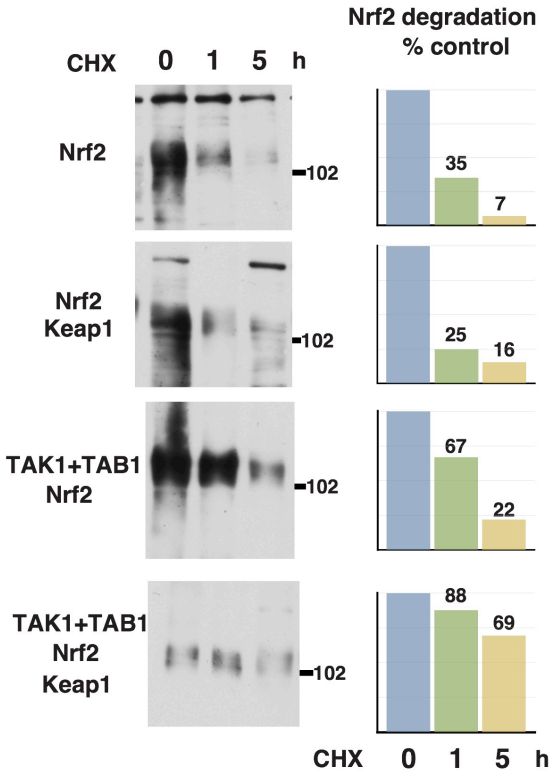
Simmons et al. Figure 2



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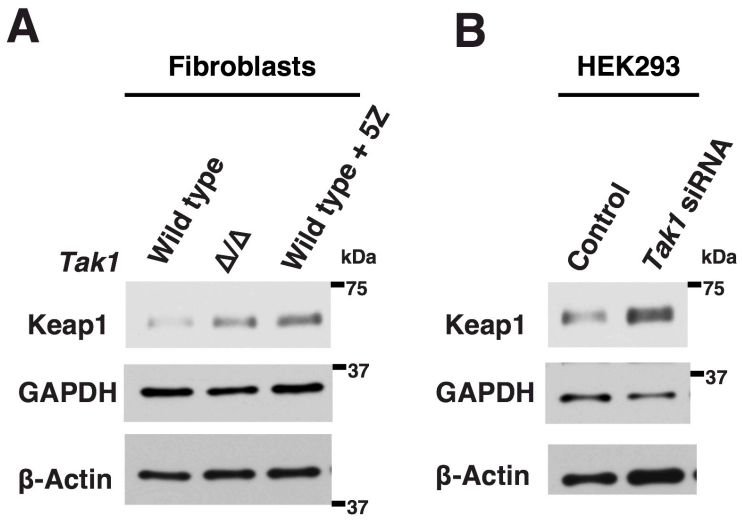

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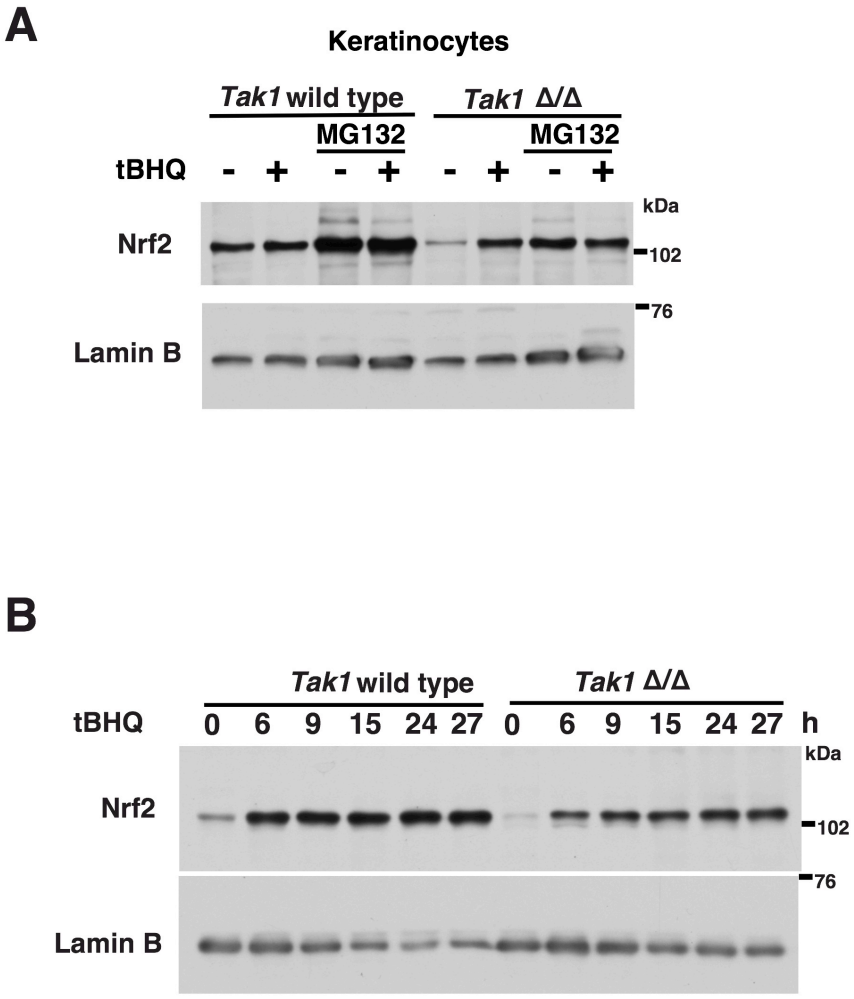

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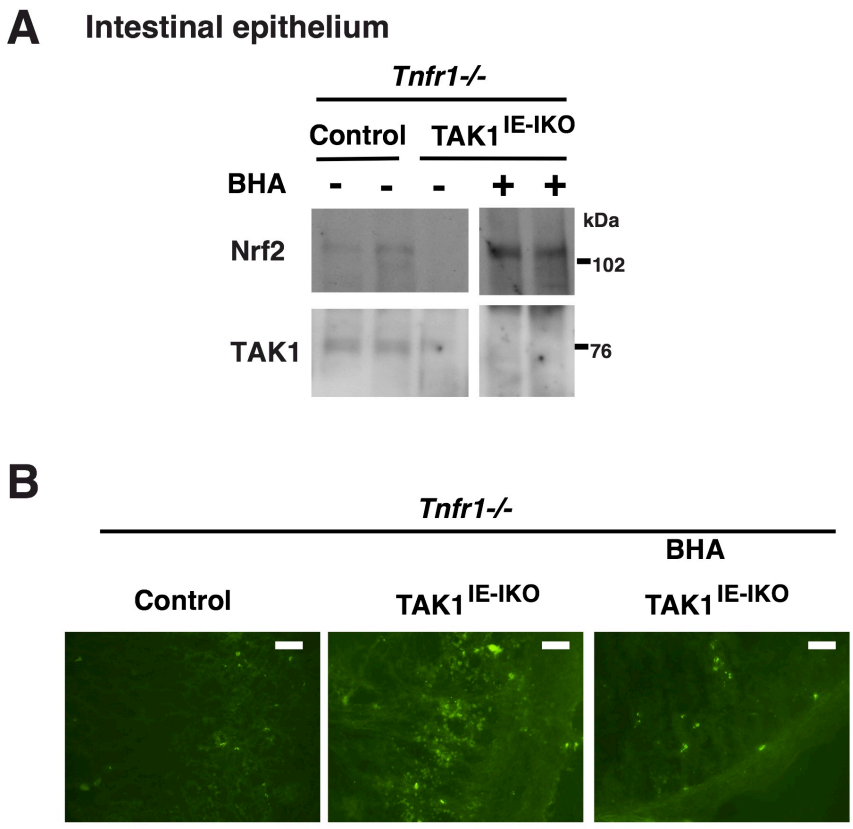

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GENERAL DISCUSSION

Role of Paneth cells in Host Defense and Intestinal Integrity

It is well understood that dysfunction of the intestinal epithelium as a result of increased invasion of commensal and pathogenic bacteria into the lamina propria, causes inflammatory conditions further leading to the pathology seen in inflammatory bowel disease (IBD) (Duchmann et al., 1995). In healthy individuals, the intestinal epithelium provides a tight physical barrier that protects against toxic chemicals, pathogens, and microorganisms (Podolsky, 1999). Additionally, the intestine normally acts as a selective filter that allows the absorption of essential nutrients and water, which also requires a functional physical barrier. To maintain intestinal barrier function, epithelial cells are constantly renewed and commensal microorganisms are properly regulated. One of the reasons the intestine can regulate commensal microorganisms is because of several specialized epithelial cells such as the Paneth cells, enteroendocrine cells, and goblet cells. Enteroendocrine cells produce and secrete gastrointestinal hormones and peptides into the bloodstream in response to various stimuli (Furness et al., 2013). Goblet cells are responsible for secreting high molecular weight glycoproteins known as mucins (Johansson et al., 2013). Lastly, Paneth cells produce and secrete alpha defensins, phospholipase A2, and lysozyme to control microbiota in the intestinal (Figure 1). Paneth cells are also known to help sustain and modulate the intestinal stem cells by acting as niche cells (Sato et al., 2011). Paneth cells can promote stem cell derived crypt organoid formation (Sato et al., 2011). Through controlling microbiota and supporting intestinal stem cells, Paneth cells have shown to be critical cell types in overall host protection, in that loss of Paneth cells can result in loss of barrier function of the

intestine leading to pathogenic bacteria invasion and virus infection (Cadwell et al., 2010; Adolph et al., 2013).

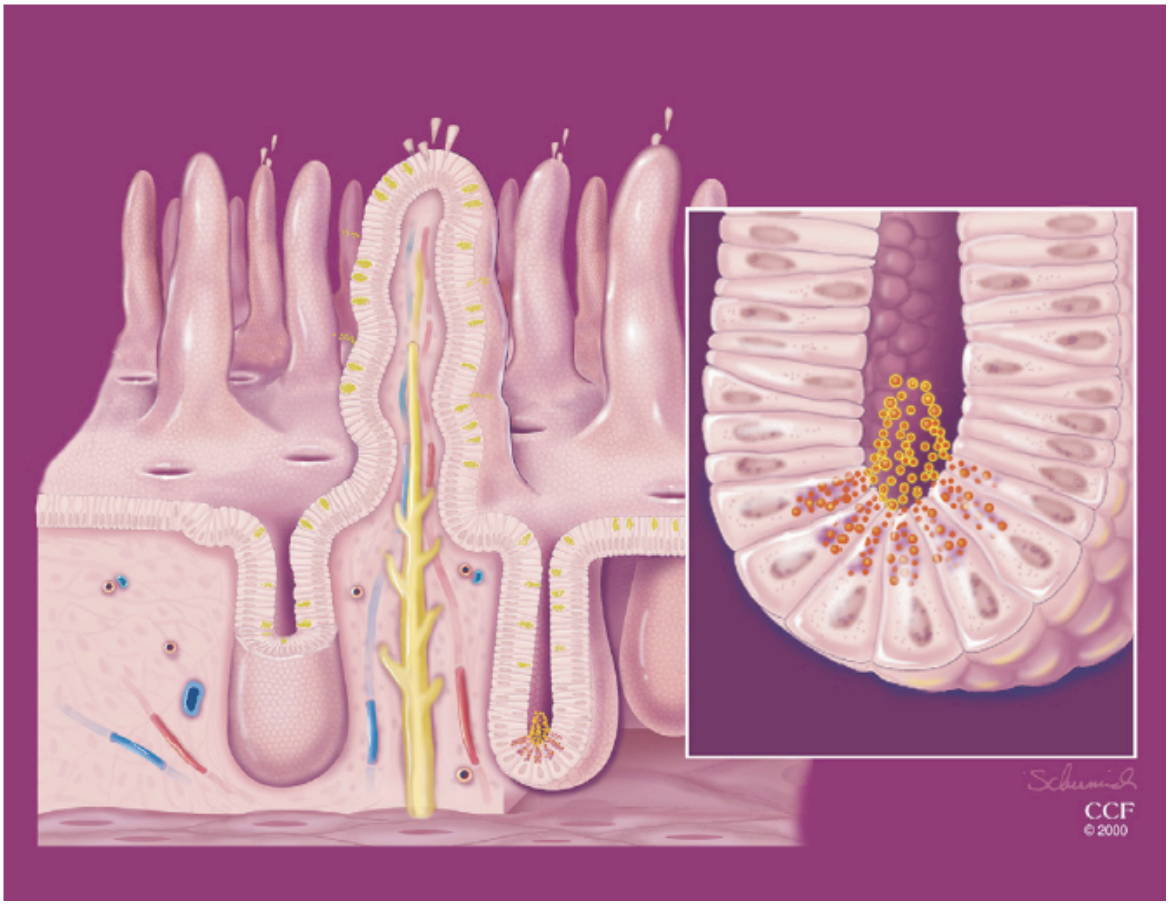

Figure 1: adapted from (Salzman et al., 2007) Paneth cells at the base of the crypts discharging antimicrobial peptides (AMPs)

Paneth cells are regulated by Necroptosis

As mentioned in the introduction, intestinal epithelial cells (goblet, enteroendocrine, and absorptive cells) in the small intestine live from 3 to 5 days, migrating up the villi and are thought to shed off via apoptosis (Barker et al., 2007). However, Paneth cells migrate downward near the crypt bottom and can live up to about 6-8 weeks. Recently it has been shown that Paneth cells do not die via apoptosis but by necrosis in one type of IBD, Crohn's

disease patient (Gunther et al., 2011). In the mouse model, two separate studies demonstrated that Paneth cells are sensitive to necroptotic cell death. One study has been reported that intestinal epithelium specific deletion of an apoptotic signaling molecule, Fas-Associated protein with a Death Domain (FADD) results in epithelial cell necrosis, loss of Paneth cells, and severe inflammation (Welz et al., 2011). Because FADD is an adaptor protein essential for death receptor induced apoptosis (Muppidi and Siegel, 2004), it is reasonable to assume that ablation of FADD would block apoptotic cell death but in fact, the cells die by necrosis, as shown by electron microscopy analysis (Welz et al., 2011). This result was confirmed by additional deletion of receptor interacting protein kinase 3 (*Ripk3*), which is the critical mediator of necroptosis. Paneth cell loss in the intestinal epithelium specific deletion of FADD is rescued by *Ripk3* deletion. These mice developed normally, with no signs of inflammation or Paneth cell loss (Welz et al., 2011). Another group reported a similar observation where they showed that mice deficient in caspase 8 in the intestinal epithelium spontaneously developed severe inflammation, increased necrotic cells, and loss of Paneth cells. In this study, they used a pharmacological inhibitor, Necrostatin-1 (Nec-1), which is known to block necroptosis by inhibiting another necroptosis mediating protein kinase, RIPK1. Treatment with Nec-1 reduced animal lethality, intestinal tissue damage, and Paneth cell loss in the caspase 8 intestinal epithelium deficient mice (Gunther et al., 2011). Intestinal epithelial-specific *Fadd* or *caspase* 8 deficient mice are viable although they exhibit IBD-like pathology, and therefore the intestinal epithelium is still functional but Paneth cells are not present. Thus, Paneth cells seem to be highly sensitive to necroptosis compared to other intestinal cells.

TAK1 is a critical regulator of Paneth cells

In our previous and current studies, we observed that in the *Tak1* intestinal epithelium inducible knockout mouse, reactive oxygen species are highly upregulated (Kajino-Sakamoto et al., 2010) and Paneth cell granules are disappeared (this study, Chapter I). Moreover, immunohistochemistry staining with the Paneth cell marker lysozyme, revealed that Paneth cells are not detectable in the *Tak1* deficient intestinal epithelium (this study, Chapter I). ROS upregulation in the gut is known to be associated with gut dysbiosis (Morgan et al., 2012) so we explored the possibility of the involvement of gut microbiota in ROS accumulation and Paneth cell loss in the *Tak1* deficient intestinal epithelium. Depletion of gut bacteria resulted in reduced ROS but not Paneth cell restoration. Similarly, intestinal epithelium deletion of the bacteria derived adaptor molecule, *Myd88* also reduced ROS but did not restore Paneth cells. Thus, gut bacteria are associated with ROS in our mouse model but that gut bacteria induced ROS is not the cause of Paneth cell loss.

Role of TAK1/RIPK3 in Paneth cell Survival

Ablation of TAK1 is known to be associated with inducing apoptosis (Morioka et al., 2014a; Mihaly et al., 2014b); however, it is also involved in the induction of necroptosis in some cells such as macrophages (Lamothe et al., 2013). RIPK3 is a cell death signaling intermediate that is a critical regulator of programmed necrosis (necroptosis) (He et al., 2009a). As discussed above, Paneth cells are sensitive to necroptosis. Therefore, we tested whether our mouse models' Paneth cells died via necroptosis. Generation of intestinal epithelium deletion of *Tak1* on *Rip3-/-* background revealed a partial rescue of Paneth cells, which suggests that Paneth cells were deleted at least in part due to necroptosis (this study,

Chapter I). Also, *Ripk3* deletion resulted in decreased ROS accumulation, which suggests that loss of Paneth cells is causally related to the accumulation of ROS in the *Tak1* deficient intestinal epithelium (this study, Chapter I).

Why are Paneth cells sensitive to necroptosis?

Paneth cells function as a barrier to bacterial invasion by secreting antimicrobial peptide containing granules (Vaishnava et al., 2008; Kaser et al., 2008). Thus, Paneth cells constantly generate secretion vesicles, which are important for their function. The Paneth cell secretion vesicles are generated by using the intracellular membrane fusion process, and the process is shared by a cell catabolic process, so called autophagy (Klionsky et al., 2008).

Autophagy is a term that describes the sequestration of cytoplasmic contents into the double membrane vacuole (autophagosome). The outer autophagosomal membrane then fuses with the lysosome leading to the intracellular degradation of cytoplasmic components (Mizushima et al., 2008). There are number of genes associated with autophagy, ATG5 is a protein involved in the early stages of autophagosome formation (Mizushima et al., 1998), while ATG8 promotes autophagosome maturation (Klionsky et al., 2008). Interestingly, one of the ATG genes ATG16, mutations is associated with IBD, which causes Paneth cell loss. Because Paneth cells are one of the cell types actively generating secretion vesicles in the intestinal epithelium, ATG16 mutations may specifically disrupt Paneth cells. Thus, Paneth cells may be specifically sensitive to any impairment associated with membrane integrity.

Necroptosis is a form of cell death that is described as a form regulated necrosis that depends on the serine/threonine kinase activity of RIP1 and (Kroemer et al., 2009) and its related kinase RIP3 (Cho et al., 2009; He et al., 2009b). Some of the characteristics

associated with necroptosis involve calcium influx, increased ROS, ATP depletion, and plasma membrane rupture (Cai et al., 2014). Recently, RIPK3 was reported to be oligomerized and also promotes oligomerization of the downstream protein, mixed lineage kinase domain-like protein (MLKL), which drives membrane rupture (Wang et al., 2014). Thus, necroptosis seems to be predominantly associated with disruption of membrane. This raise the possibility Paneth cells are highly sensitive to necroptosis due to their requirement of intact membrane structure. It has been shown that the Paneth cells and goblet cells of mice with intestinal epithelial specific deletion of *Ripk1* are sensitive to necroptosis (Dannappel et al., 2014). Like Paneth cells, goblet cells are also secretory cells however, they provide a protective function against physical and chemical injury by the secretion of high molecular weight glycoproteins called mucins (Artis, 2008). Collectively, high demand of membrane integrity may be associated with sensitivity to necroptosis.

How does *Tak1* deletion cause necroptosis?

We have observed that although other *Tak1* deficient intestinal epithelial cells are dying via apoptosis (Kajino-Sakamoto et al., 2010), these dying cells do not seem to be near the base of the crypts where Paneth cells reside, but near the region where proliferating cells reside. As discussed earlier, *Tak1* deletion normally causes apoptosis. However, when apoptosis is inhibited, necroptosis can occur. It is known that inhibition of apoptosis induces necroptosis (Pasparakis and Vandenabeele, 2015). Since *Tak1* gene is deleted in all intestinal epithelial cells in our mouse model, we assume that the cell death pathway is equally activated in all epithelial cells. Proliferating cells are prone to accumulate ROS due to their active mitochondrial respiration, which promotes apoptosis in the absence of *Tak1*. In contrast,

Paneth cells may be lacking signaling components of apoptosis, leading to a shift to necroptotic cell death.

Role of TAK1 in cellular antioxidant system

Our results described in Chapter I revealed that TAK1 regulation of Paneth cells is critical in intestinal homeostasis and prevention of ROS accumulation. However, blockade of Paneth cell loss not completely rescue ROS accumulation in *Tak1* deficient intestinal epithelium. TAK1 may have additional roles to participate in intestinal integrity. We show here that TAK1 is a potential regulator of Nrf2 through Keap1 as described in Chapter II. *Tak1* knockdown in cultured cells and *Tak1* deficient intestinal epithelium decreased the protein expression of Nrf2, which reduces cellular antioxidant levels.

Thus, it is likely that TAK1 regulation of Nrf2 is likely to also participate in the maintenance of intestinal integrity in addition to TAK1 regulation of Paneth cells. Nrf2 germline deletion mice are grossly normal and do not develop any intestinal or other tissue abnormalities (Chan et al., 1996). However, Nrf2-/- mice are sensitive to intestinal injury such as one of the IBD model injury, dextran sodium sulfate (DSS) treatment (Khor et al., 2006). This suggests that Nrf2 deficiency alone is not sufficient to induce intestinal abnormalities but Nrf2 is critical to prevent exogenous stress-induced intestinal injury. *Tak1* deletion causes Paneth cell loss, which causes dysbiosis. Under such conditions, reduced Nrf2 may be required for prevention of ROS accumulation. Our current study has not yet determined the precise molecular mechanism by which TAK1 regulates Nrf2-Keap1 system, which should be one of future studies in the TAK1 research field.

Current studies (Chapter I and II) revealed two critical roles for TAK1 in the regulation of intestinal homeostasis. One is maintenance of Paneth cells by preventing necroptosis and the other is maintenance of basal antioxidant levels by modulating the Nrf2-Keap1 system (Fig. 2)

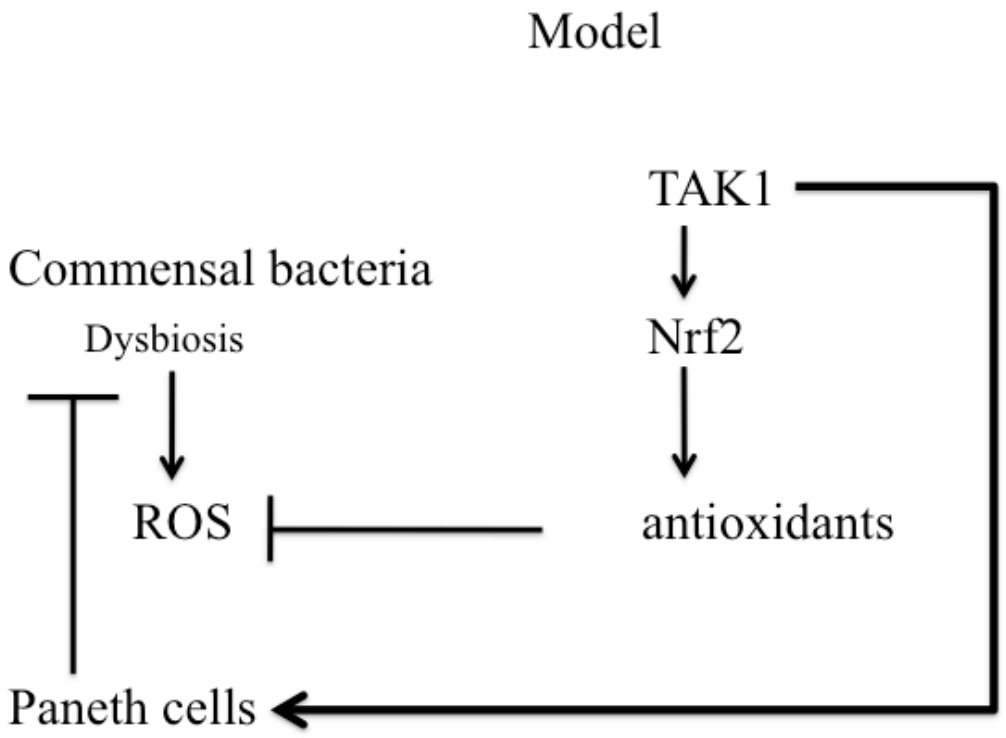

Figure 2: Model

Human Health Implications

IBD is a condition that is increasingly becoming more and more of a global concern and health problem. This is because there a steady increase worldwide in the incidence number per year (Molodecky et al., 2012). It is important to develop new treatments and therapies for this disease as this chronic condition is without a cure and usually requires a lifetime of care. Current treatments of IBD include therapeutic treatments to improve the patient quality of life by inducing and maintaining remission, predicting, preventing and treating complications, and restoring nutritional deficits (Triantafillidis et al., 2011). Antibiotics and anti-inflammatory drugs are also commonly used to help reduce pain and symptoms but they cannot be used for long term due to the increased risk of liver disease/failure.

Although our work is in mice, we see very similar intestinal phenotypes that are present in humans (i.e. cell death, ROS, dysbiosis, inflammation etc.). The current IBD mouse models include supplementing the drinking water with dextran sulfate sodium (DSS) to induce a model similar to that of human ulcerative colitis. These mice show characteristics of bloody stool, diarrhea, ulcerations, and granulocyte infiltration (Okayasu et al., 1990; Mahler et al., 1998). Other mouse models such the IL-10 knockout mice that are more closely related to human Crohn's disease (Rennick et al., 1997). These mice develop chronic intestinal inflammation, epithelial hyperplasia, and focal ulcers. However, as these mice began to reach the age of 6 months, colitis and carcinomas can develop (Rennick et al., 1997). Human Crohn's disease is characterized by inflammation of a specific region of the small intestine, the ileum. However, a model system resembling ileitis has not been developed. Our results raise the possibility intestinal epithelial specific *Tak1* deficient mice are an alternative animal model to IBD, specifically ileitis. In addition, our results also raise

the possibility that activation of TAK1 mat be beneficial for promoting Paneth cell integrity and reduction of ROS. However, sustained or excessive activation of TAK1 could upregulate inflammation through its downstream MAPKs and NF-κB and disrupt intestinal integrity by destroying the homeostatic balance. Nonetheless, this work demonstrates that in the future TAK1 may be interesting to study as a therapeutic target in individuals with IBD or IBD like symptoms.

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