Published in final edited form as:

Cell Signal. 2014 October ; 26(10): 2086–2095. doi:10.1016/j.cellsig.2014.06.006.

Prohibitin 1 modulates mitochondrial function of Stat3

Jie Han^a, Chunhua Yu^b, Rhonda F. Souza^b, and Arianne L. Theiss^{a,c}

^aDepartment of Internal Medicine, Division of Gastroenterology, Baylor Research Institute, Baylor University Medical Center, Dallas, TX

^bDepartment of Medicine, Veterans Affairs North Texas Health Care System, University of Texas Southwestern Medical Center, Dallas, TX

Abstract

Mitochondrial dysfunction in intestinal epithelial cells (IEC) is thought to precede the onset of inflammatory bowel diseases (IBD). Expression of Prohibitin 1 (PHB), a mitochondrial protein required for optimal electron transport chain (ETC) activity, is decreased in mucosal biopsies during active and inactive IBD. In addition to its activities as a transcription factor, Signal Transducer and Activator of Transcription 3 (Stat3) resides in the mitochondria of cells where phosphorylation at S727 is required for optimal ETC activity and protects against stress-induced mitochondrial dysfunction. Here, we show that PHB overexpression protects against mitochondrial stress and apoptosis of cultured IECs induced by TNF α , which is a pro-inflammatory cytokine involved in IBD pathogenesis. Expression of pS727-Stat3 dominant negative eliminates protection by PHB against TNF α -induced mitochondrial stress and apoptosis. PHB interacts with pS727-Stat3 in the mitochondria of cultured IECs and in colonic epithelium from wild-type mice. Our data suggest a protective role of PHB that is dependent on pS727-Stat3 to prevent mitochondrial dysfunction in IECs. Reduced levels of PHB during IBD may be an underlying factor promoting mitochondrial dysfunction of the intestinal epithelium.

Keywords

mitochondria; Prohibitin; Stat3; intestinal epithelium; electron transport chain; oxidative stress; TNF α

© 2014 Elsevier Inc. All rights reserved.

^cAddress correspondence to: Arianne L. Theiss, Division of Gastroenterology, 1053 Wadley Tower, 3600 Gaston Avenue, Baylor Research Institute, Baylor University Medical Center, Dallas, Texas 75246, Tel: 214-818-3150, Fax: 214-818-9292, arianne.theiss@baylorhealth.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures: The authors have nothing to disclose. No conflicts of interest exist.

Author contributions: Study concept and design: ALT, RFS. Acquisition of data: JH, CY, ALT. Analysis and interpretation of data: RFS, ALT. Drafting the manuscript: JH, ALT. Critical revision of the manuscript for important intellectual content: RFS.

1. Introduction

Disturbed intestinal epithelial cell (IEC) homeostasis is a key feature of inflammatory bowel diseases (IBD), the most common forms being Crohn's disease and ulcerative colitis. Structurally, an intact epithelium barrier that prevents the translocation of intraluminal contents and subsequent immune cell activation is the basis of mucosal healing and an initial event in suppressing inflammation deeper in the bowel wall [1]. Multiple studies have reported mitochondrial dysfunction in epithelial cells during IBD and in animal models of colitis [2-7]. Mitochondrial dysfunction impairs energy production and cell homeostasis. Mitochondrial structural abnormalities in IECs of patients with IBD are thought to occur early in disease progression since they are evident prior to other ultrastructural alterations or the onset of mucosal inflammation [8-10]. Exogenous reactive oxygen species (ROS) and the pro-inflammatory cytokine tumor necrosis factor α (TNF α), both of which are increased during IBD, promote cellular injury via mitochondrial ROS production from respiratory chain dysfunction and mitochondrial damage [11, 12]. Recent studies suggest that mitochondria are integrators of cellular danger signaling and that mitochondrial stress participates in the pathology of IBD [13, 14].

Prohibitin 1 (PHB) is a highly conserved protein with diverse functions including regulation of cell cycle progression, apoptosis, and transcription depending on its subcellular localization [15]. In IECs, PHB is predominantly localized in the mitochondria [16]. PHB is the major component protein of the mitochondrial inner membrane where it maintains the structure and function of mitochondria [17]. PHB interacts with and is required for optimal activity of complexes I and IV of the electron transport chain (ETC) [9, 18, 19]. Expression of PHB is decreased in uninvolved and inflamed epithelium in IBD patients compared to healthy control patients [9, 16]. Multiple animal models of colitis also exhibit decreased PHB expression [16, 20, 21]. Transgenic mice overexpressing PHB in IECs are protected from experimental colitis and exhibit less oxidative stress in the colon [20, 22]. This is in agreement with emerging data that suggest a role of PHB in protection against oxidative stress-induced injury in multiple cells types [23-27]. Gene silencing of PHB in cultured IECs induces mitochondrial membrane depolarization and cellular stress pathways including intracellular ROS generation, autophagy, and apoptosis [28]. Furthermore, cultured IECs overexpressing PHB exhibit less intracellular ROS, autophagy, and apoptosis [28], suggesting that relative levels of PHB modulate epithelial cell homeostasis.

Signal Transducer and Activator of Transcription 3 (Stat3), a transcription factor known to control cell growth and host responses to inflammation and cellular stress, is increased in the mucosa of IBD patients and in animal models of intestinal inflammation [29]. Intestinal epithelial Stat3 has been shown to promote mucosal healing and regulation of intestinal homeostasis in response to injury during colitis [30, 31]. In addition to its activities as a transcription factor, a pool of Stat3 resides in the mitochondria of cells [32]. Although it is well-known that mitochondrial Stat3 regulates metabolic function in the mitochondria of Ras-transformed cells [33], additional data suggest that mitochondrial Stat3 regulates cellular respiration and is required for optimal ETC activity in non-transformed cells via its interaction with complexes I and II [32, 34]. Furthermore, phosphorylation of mitochondrial Stat3 at serine 727 (S727), but not tyrosine 705 (Y705), is crucial for optimal activity of

complexes I and II of the ETC and protects against stress-induced generation of ROS by the ETC [32, 35]. These results suggest a protective role of mitochondrial Stat3 during cellular injury. Here, our studies suggest that the interaction of PHB and Stat3 prevents epithelial cell mitochondrial dysfunction characteristic of IBD.

2. Materials and Methods

2.1. Cell culture

The Caco2-BBE human colonic adenocarcinoma epithelial cell line and IEC-6 rat small intestinal epithelial cell line were used as in vitro models of polarized intestinal epithelium. Both cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Caco2-BBE) supplemented with penicillin (40 mg/l), streptomycin (90 mg/l), and 10% fetal bovine serum. Caco2-BBE cells and IEC-6 cells were plated on permeable supports to allow the cells to reach confluency and polarize (pore size, 0.4µm; transwell-clear polyester membranes; Costas life science, Acton, MA). All experiments performed on Caco2-BBE cells were between passages 32 and 45 and IEC-6 were between passages 10 and 18. All cells were treated with 10 ng/ml recombinant human TNFα added to the basolateral chamber.

2.2. Isolation of colonic epithelial cells (IECs) from mice

Colonic IEC isolation from 8-week old wild-type C57Bl/6 mice was performed as previously described [36]. All mice were grouped-housed in standard cages under a controlled temperature (25°C) and photoperiod (12-hour light/dark cycle) and were allowed standard chow and tap water *ad libitum*. All experiments were approved by the Baylor Research Institute Institutional Animal Care and Use Committee.

2.3. PHB overexpression

Generation of stably-transfected Caco2-BBE cells expressing pEGFPN1-PHB or empty pEGFPN1 vector was previously described [28]. Stable cell lines were selected under 0.12% geneticin (Sigma-Aldrich, St. Louis, MO). IEC-6 cells were transiently transfected with either pEGFPN1 expression vector or pEGFPN1-PHB for 72 hours using Amaxa® Cell Line Optimization Nucleofector® Kit T (Lonza, Basel, Switzerland).

2.4. Generation of mitochondrial-targeted S727A-Stat3 dominant negative construct

A single PCR product corresponding to mitochondrial localization signal (MLS; 80 bp) of cytochrome c oxidase subunit VIIIa was generated by PCR using DNA from Caco-2BBE cells and the following primers: sense 5'-CGCCGAATTCAAGCTTGTGACCATGTCCGTCCTGACGCCGCTGCTGCTGCGGGG-3', EcoRI site is underlined; antisense 5'-CGCCGATATCCAACGAATGGATCTTGGC-3', EcoRV site is underlined. The PCR product was ligated into pcDNA His Max C vector (Invitrogen Life Technologies, Grand Island, NY) at the EcoRV and EcoRI restriction enzyme sites using the Quick Ligation Kit (New England Biolabs, Ipswich, MA) and sequenced. S727A-Stat3 pRc/CMV was purchased from Addgene (Cambridge, MA). S727A-Stat3 was subcloned into pcDNA His Max C containing the MLS at the NotI and

ApaI restriction enzyme sites and sequenced. Using this cloning strategy the MLS is linked to a His tag and is fused to the N terminus of Stat3. Caco2-BBE or IEC-6 cells were transiently transfected with MLS-S727A-Stat3 or MLS-vector using Amaxa electroporation.

2.5. Immunoprecipitation and western blot analysis

Mitochondrial and cytosolic extracts from Caco2-BBE cells, IEC-6 cells, or freshly isolated mouse colonic IECs were isolated as previously described [16]. 1 µg of PHB (Thermo Fisher), Stat3 (Santa Cruz Biotechnology), pS727-Stat3 (Cell Signaling Technology, Danvers, MA), pY705-Stat3 (Cell Signaling Technology), or His tag (Santa Cruz Biotechnology) antibody was incubated with mitochondrial or cytosolic extracts for immunoprecipitation experiments followed by incubation with 30 µl 50% protein A or G sepharose beads (GE Healthcare, Piscataway NJ). An IgG control antibody (Cell Signaling) was used as a negative control during the immunoprecipitation. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Laemmli's 2x SDS sample buffer and AnyKD™ gradient polyacrylamide gels (Bio-Rad, Hercules, CA) followed by electrotransfer to nitrocellulose membranes (Bio-Rad). As a positive control, whole cell extracts (WCE) were separated by SDS-PAGE but were not immunoprecipitated. Membranes were incubated with primary antibodies at 4°C overnight and subsequently incubated with corresponding peroxidase-conjugated secondary antibodies. Membranes were washed and immunoreactive proteins were detected using Amersham ECL Plus™ reagent (GE Healthcare, Piscataway, NJ). Western blots were reprobed with β-tubulin or β-actin (Sigma-Aldrich) antibody and COX IV (Cell Signaling) antibody to ensure purity of cytosolic and mitochondrial extracts, respectively. Mouse monoclonal GFP antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.6. Measuring mitochondrial ROS

Cells were treated with 10 ng/ml TNFα for 4 hr. Cells treated with 300 µM peroxynitrite were used as a positive control. Cells were then incubated with Hank's balanced salt solution (HBSS) with 5 µM MitoSOX Red Mitochondrial Superoxide Indicator dye (Invitrogen) for 10 min at 37°C. Cells were washed twice with warm HBSS and fluorescent intensity was measured at 510 nm excitation/580 nm emission.

2.7. Measuring ATP concentration

The concentration of adenosine triphosphate (ATP) in Caco2-BBE cells treated with 10 ng/ml TNFα for 4 hr was determined using the ENLIGHTEN ATP Assay Bioluminescence Detection kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.8. Detection of mitochondrial complex I and IV activities

The activities of complexes I and IV were measured in whole cell lysates of Caco2-BBE cells treated with 10 ng/ml TNFα for 4 hr using Complex I Enzyme Activity Microplate Assay Kit (Abcam) and Complex IV Human Enzyme Activity Microplate Assay Kit (Abcam), respectively, according to the manufacturer's instructions using 20 µg of protein.

2.9. Cytotoxicity test

Lactate dehydrogenase (LDH) cytotoxicity detection kit (Clontech, Mountain View, CA) was used to measure cell viability. Caco2-BBE cells or IEC-6 cells were treated with 10 ng/ml TNF α for 16 hr prior to collection. An aliquot of 100 μ l of culture media was added to 100 μ l of LDH reagent and % cytotoxicity and % viable cells were measured according to the manufacturer's protocol.

2.10. Measuring cell apoptosis

Fold increase in apoptotic Caco2-BBE cells after treatment with 10 ng/ml TNF α for 16 hr was measured using the Cell Death Detection Elisa Plus kit (Roche, Indianapolis, IN) as described by the manufacturer.

2.11. Statistical analysis

Values are expressed as mean $\pm$ s.e.m. Comparisons between PHB overexpression, S727-Stat3 dominant negative, or TNF α treatment were analyzed by two-way analysis of variance and subsequent pair-wise comparisons used Bonferroni post-hoc tests to test for significant differences between two particular groups. $p < 0.05$ was considered statistically significant in all analyses.

3. Results

3.1. PHB interacts with pS727-Stat3 in the mitochondria of Caco2-BBE and IEC-6 cell lines and colonic IECs *in vivo*

It is well established that PHB interacts with multiple mitochondrial proteins involved in mitochondrial function and structural stability [17]. Recent studies have identified a pool of Stat3 residing in mitochondria with functions distinct to those in the nucleus not involving transcriptional regulation [37]. In this current study, we initially examined whether PHB and Stat3 interact in the mitochondria of IECs. To determine the subcellular localization of PHB and Stat3, mitochondrial and cytosolic extracts were isolated from polarized Caco2-BBE cells, a model intestinal epithelial cell line. Western blotting revealed that a portion of phospho-S727-Stat3 (pS727-Stat3), but not pY705-Stat3, localized to the mitochondrial fraction (Fig. 1A and 1B). PHB was exclusively expressed in the mitochondrial fraction (Fig. 1A and 1B).

Since phosphorylation of Stat3 at serine 727 (S727) has been shown to be necessary for mitochondrial Stat3 function [32], we determined whether phosphorylation of Stat3 is required for interaction with PHB in the mitochondria of IECs. PHB was immunoprecipitated from mitochondria and cytosolic extracts from Caco2-BBE cells, separated by SDS-PAGE, and membranes were immunoblotted for pY705-Stat3 and pS727-Stat3 expression. PHB co-immunoprecipitated with pS727-Stat3, but not with pY705-Stat3, in the mitochondrial fractions (Fig. 1C). Immunoprecipitation using a Stat3 antibody followed by immunoblotting with a PHB antibody further demonstrated that Stat3 and PHB interact in the mitochondria but not the cytosol of Caco2-BBE cells (Fig. 1D). These results were corroborated when PHB expression was detected in mitochondrial extracts that were

immunoprecipitated with a pS727-Stat3 antibody (Fig. 1E) but not with a pY705-Stat3 antibody (Fig. 1F).

Although Caco2-BBE cells are commonly used as a model of intestinal epithelium due to many morphological and biochemical characteristics of small intestinal enterocytes including their ability to form polarized monolayers and secrete brush border enzymes, these cells originated from a colonic adenocarcinoma [38]. We therefore utilized the IEC-6 non-transformed intestinal epithelial cell line. Similar to Caco2-BBE cells, PHB localized exclusively to the mitochondrial fraction of IEC-6 cells (Fig. 2A). A portion of pS727-Stat3, but not pY705-Stat3, was present in the mitochondrial fraction of IEC-6 cells (Fig. 2A). To determine whether PHB interacts with mitochondrial Stat3, protein extracts from IEC-6 cells were subjected to co-immunoprecipitation experiments using PHB, pY705-Stat3, or pS727-Stat3 antibodies. PHB co-immunoprecipitated with pS727-Stat3 in mitochondrial extracts but not with pY705-Stat3 in IEC-6 cells (Fig. 2B-D). These results were corroborated *in vivo* using freshly isolated colonic epithelium from wild-type mice in which PHB and pS727-Stat3 localized to the mitochondrial fraction and mitochondrial pS727-Stat3 co-immunoprecipitated with PHB (Fig. 3). Collectively, these results suggest that PHB interacts with pS727-Stat3 in the mitochondria of IECs.

3.2. S727 of Stat3 is necessary for PHB/Stat3 interaction

Stat3 harboring a mutation of serine 727 to an alanine (S727A) functions as a pS727 dominant negative [32]. To target S727A-Stat3 to the mitochondria, a His-tagged mitochondrial targeting sequence of cytochrome c oxidase subunit VIII was fused to the N terminus of S727A-Stat3 (MLS-S727-Stat3dn). Cell fractionation of Caco2-BBE cells expressing MLS-S727-Stat3dn indicated that the dominant negative construct specifically localized to the mitochondria (Fig. 4A, B). MLS-S727-Stat3dn had a slightly higher molecular weight compared to endogenous Stat3 due to the addition of the mitochondrial targeting sequence and His tag (Fig. 4A). Cells expressing MLS-S727-Stat3dn exhibited reduced levels of pS727-Stat3 in the mitochondria but not cytosol, while pY705-Stat3 and total Stat3 protein expression remained unaffected, indicating specificity of the dominant negative construct to inhibit mitochondrial pS727-Stat3 (Fig. 4B). In addition, PHB protein expression in cells expressing MLS-S727-Stat3dn was comparable to cells transfected with empty MLS-vector, suggesting that mitochondrial pS727-Stat3 does not modulate expression levels of PHB protein (Fig. 4B). Cells expressing MLS-S727-Stat3dn exhibited a decrease in the amount of Stat3 that co-immunoprecipitated with PHB, suggesting that S727 of Stat3 is necessary for PHB/Stat3 interaction (Fig. 4C and 4D).

3.3. TNF α decreases endogenous PHB expression and interaction of pS727-Stat3 with PHB

Since previous studies have shown that TNF α promotes cellular injury via mitochondrial ROS production from respiratory chain dysfunction [11, 12], we utilized TNF α treatment as an inducer of mitochondrial stress in Caco2-BBE cells. Specifically, TNF α decreases the activity of ETC complex I, decreases ATP production, and increases mitochondrial-derived ROS [39]. TNF α treatment (10 ng/ml for 4 hr) decreased PHB protein expression in the mitochondria but did not affect levels of pS727-Stat3 (Fig. 5A). Co-immunoprecipitation

experiments revealed that TNF α decreased interaction of pS727-Stat3 with PHB, which could be at least partially due to decreased expression of PHB by TNF α (Fig. 5B and 5C).

To determine whether PHB overexpression could sustain PHB interaction with pS727-Stat3 during TNF α treatment, Caco2-BBE cells were transfected with a GFP-tagged PHB construct or empty GFP vector and treated with TNF α . Western blotting of mitochondrial and cytosolic extracts revealed that exogenous GFP-tagged PHB localized to the mitochondria (Fig. 6A). Co-immunoprecipitation experiments revealed that TNF α decreased interaction of pS727-Stat3 with endogenous PHB in both vector- and GFP-PHB-transfected cells (Fig. 6B). However, in cells overexpressing PHB, interaction between exogenous GFP-PHB and pS727-Stat3 was maintained during TNF α treatment (Fig. 6B).

3.4. Mitochondrial pS727-Stat3 mediates the protective effect of PHB overexpression on mitochondrial function

TNF α caused an increase in mitochondrial superoxide generation, as indicated by MitoSox Red fluorescence, in Caco2-BBE cells stably overexpressing empty vector, whereas in cells overexpressing PHB MitoSOX Red fluorescence was similar to untreated cells (Figs. 7A, 8A). Expression of MLS-S727-Stat3dn in PHB overexpressing cells eliminated the protective effect of PHB to prevent mitochondrial ROS production induced by TNF α treatment (Figs. 7A, 8A). Furthermore, TNF α decreased ATP concentration in vector control cells but did not affect ATP concentration in PHB overexpressing cells (Fig. 7B). Inhibition of pS727-Stat3 with expression of the dominant negative construct diminished the effect of PHB overexpression on maintaining ATP concentration during TNF α treatment (Fig. 7B). These results suggest that PHB overexpression protects against TNF α -stimulated mitochondrial ROS generation and decreased ATP production and that this protection by PHB is dependent on pS727-Stat3.

Blockade of forward electron flow via inhibition of ETC complexes leads to electrons accumulating at upstream complexes, ROS generation, and decreased ATP production [34]. Since both Stat3 and PHB have been shown to interact with and are required for optimal activity of complex I of the ETC [18, 32, 35], and complex I is a primary site of ROS generation in the mitochondria [39], we next assessed whether activity of complex I was altered by PHB overexpression, and subsequently, the involvement of pS727-Stat3. Although complex I activity was increased in cells overexpressing PHB compared to vector, this increase did not reach statistical significance (Fig. 7C). TNF α treatment decreased complex I activity in vector-transfected cells as shown previously [39], but not in PHB overexpressing cells which exhibited increased complex I activity compared to untreated cells. Expression of MLS-S727-Stat3dn decreased basal complex I activity and abolished the TNF α -induced increase in complex I activity in PHB overexpressing cells (Fig. 7C). In addition to complex I, PHB has been shown to modulate activity of complex IV of the ETC [19]. TNF α treatment decreased complex IV activity in vector-transfected cells compared to untreated cells and increased complex IV activity in PHB overexpressing cells (Fig. 7D). Similar to the effect on complex I activity, expression of MLS-S727-Stat3dn abolished the TNF α -induced increase in complex IV activity in PHB overexpressing cells (Fig. 7D).

These results suggest that PHB modulation of pS727-Stat3 prevents TNF α -induced inhibition of complexes I and IV of the ETC.

Since severe mitochondrial damage can lead to cell death by necrosis or apoptosis, we measured cell viability in cells overexpressing PHB during TNF α -induced mitochondrial stress. PHB overexpressing cells were protected from TNF α -induced cell cytotoxicity and this protective effect of PHB was eliminated by expression of MLS-S727-Stat3dn (Figs. 7E, 8B). Cell death induced by TNF α was at least in part mediated by apoptosis in vector overexpressing cells (Fig. 7F). Cells overexpressing PHB did not exhibit TNF α -induced apoptosis unless pS727-Stat3 was inhibited by expression of MLS-S727-Stat3dn (Fig. 7F).

4. Discussion

Our data demonstrate a potential mechanistic link between PHB and mitochondrial Stat3 in the intestinal epithelium in preventing mitochondrial dysfunction, which is thought to participate in the pathogenesis of IBD [14]. We show that PHB interacts with pS727-Stat3 in the mitochondria of cultured intestinal epithelial cell lines and *in vivo* in mouse colonic epithelium. PHB/pS727-Stat3 interaction is decreased during mitochondrial stress induced by TNF α , due at least in part to decreased PHB expression. PHB overexpression maintains PHB/pS727-Stat3 interaction during TNF α treatment and protects against TNF α -induced mitochondrial dysfunction and apoptosis of IECs via mitochondrial pS727-Stat3. Our study supports the hypothesis that relative levels of PHB in the intestinal epithelium modulate mitochondrial function dependent on pS727-Stat3.

It is well-established that IEC mitochondrial dysfunction is associated with IBD, as demonstrated by reduced ATP levels, activated mitochondrial unfolded-protein response, reduced activities of ETC complexes, and increased mitochondrial-derived ROS [5, 13, 40-42]. Ultrastructural abnormalities of epithelial cell mitochondria including swelling and irregular and/or dissolved cristae indicative of impaired function are thought to be an early event in the onset of mucosal inflammation since they are evident prior to endoscopic or histologic inflammation [8, 9]. Recent studies suggest mitochondrial stress is not just a consequence of intestinal inflammation but participates in disease pathogenesis [13, 43]. Furthermore, polymorphisms in the genes encoding SLC22A5, IRGM, and UCP2, which are crucial for mitochondrial ATP production, are susceptibility loci in IBD [44-46]. PHB resides predominantly in the mitochondria of IECs and has been shown to regulate mitochondrial function, including optimal ETC complex I and IV assembly and function in multiple tissues [15]. Expression of PHB is decreased in uninvolved and inflamed epithelium from IBD patients compared to healthy control patients, suggesting that down-regulation of PHB may be an early event rather than a consequence during the development of disease [9, 16]. Decreased PHB expression was specific to IBD since expression was not altered in nonspecific, infectious colitis [9]. Multiple animal models of colitis also exhibit decreased PHB expression [16, 20, 21]. Transgenic mice overexpressing PHB in IECs are protected from experimental colitis and exhibit less oxidative stress in the colon [20, 22]. This is in agreement with emerging data that suggest a role of PHB in protection against oxidative stress-induced injury in multiple cells types [23-27].

TNF α , a pro-inflammatory cytokine that plays a central role in IBD pathogenesis, promotes cellular injury via mitochondrial ROS production from respiratory chain dysfunction [11, 12, 47]. We show here that TNF α decreased expression of endogenous PHB, thereby decreasing the amount of PHB interacting with pS727-Stat3. We speculate that phosphorylation status of Stat3 at S727 could be directly controlled by PHB or could be a secondary effect of changes in mitochondrial environment brought about by alterations in PHB protein expression. We utilized TNF α treatment to induce mitochondrial stress in IECs as demonstrated by increased mitochondrial-derived ROS, decreased ATP production, decreased activity of complexes I and IV of the ETC, and apoptosis. Mitochondrial stress induced by TNF α was at least in part mediated by loss of PHB as evidenced by the ability of PHB overexpression to prevent TNF α -induced mitochondrial dysfunction in IECs. Protection against TNF α -induced mitochondrial stress by PHB overexpression was eliminated by expression of pS727-Stat3 dominant negative, suggesting that pS727-Stat3 is essential for PHB modulation of mitochondrial function.

Recent data suggest that mitochondrial Stat3, representing 5-10% of total Stat3, regulates cellular respiration and is required for optimal ETC activity via its interaction with complexes I and II [32, 34, 48]. Phosphorylation of mitochondrial Stat3 at S727, but not Y705, is crucial for optimal activity of complexes I and II of the ETC and protects against stress-induced generation of ROS by the ETC [32, 35]. However, the exact mechanism of Stat3 interaction with complexes I and II is unknown [34]. Relative to complexes I/II, mitochondrial Stat3 is 10⁵-fold less abundant, which suggests that Stat3 may not be a structural component of complexes I or II but likely modifies mitochondrial function indirectly via signal transduction pathways or post-translational modifications [49].

The action of Stat3 in IBD is cell-type dependent. Stat3 in the colonic epithelium is thought to be protective against intestinal inflammation. Loss of Stat3 in the intestinal epithelium promotes pro-inflammatory cytokine and chemokine expression, delays wound healing, and leads to more severe chronic inflammation following acute dextran sodium sulfate (DSS)-induced colitis [31, 50]. It is not known the specific effect of loss of mitochondrial Stat3 in the intestinal epithelium as these studies focused on loss-of-function of Stat3 from all cellular locations. Interleukin-6 (IL-6), a well-known activator of Stat3, is protective against colitis, cardiac inflammation, and oxidative stress in the liver and lung [51-55]. A recent study suggests that IL-6 provides protection against colitis via a mechanism dependent on Stat3 activation in the intestinal epithelium [56]. We and others have shown that IL-6 increases expression of PHB through Stat3 transcriptional activation to promote survival of IECs, cardiomyocytes, and hepatocytes [53-55, 57]. IL-6 protective effects in cardiomyocytes are dependent upon IL-6 induction of PHB [58] and phosphorylation of mitochondrial S727-Stat3 [59]. Future studies will assess whether IL-6 mediates protection against mitochondrial dysfunction in IECs through modulation of PHB levels and phosphorylation of Stat3 at S727.

Stat3 is required for survival of IECs through its protection against apoptosis [60]. However, this function of Stat3 can be a double-edged sword in that proliferation and survival of tumor-initiating IECs plays a key role in colitis-associated tumorigenesis. In fact, IEC-specific ablation of Stat3 inhibited mutagen-induced colitis-associated cancer induction and

growth [60, 61]. Mitochondrial Stat3 has been shown to be involved in the regulation of glycolytic and oxidative phosphorylation activities characteristic of the Warburg effect in cancer cells and facilitates Ras transformation [33]. Mitochondrial dysfunction is a common characteristic of cancer cells believed to be the consequence of ROS and mitochondrial chaperone proteins, including PHB [15]. A recent study found that mitochondrial dysfunction and loss of PHB expression preceded the development of dysplasia during ulcerative colitis and that mitochondrial function and PHB expression were restored in cancer cells [62]. Although PHB expression is increased in many transformed cells and tumors [15], we and others previously showed that colonic mucosal PHB protein expression was reduced after experimental colitis-associated cancer in mice using the azoxymethane-DSS model [63, 64] and that mice overexpressing PHB in the intestinal epithelium exhibited fewer tumors than wild-type mice [63]. We speculate that PHB may prevent tumor formation via mitochondrial stabilization. We show here that PHB/pS727-Stat3 interaction occurs in both transformed and non-transformed intestinal epithelial cell lines and *in vivo* in normal colonic epithelium. Also, we show that PHB protection from mitochondrial ROS generation and cell death induced by TNF α treatment was dependent on pS727-Stat3 in both Caco2-BBE and non-transformed IECs, suggesting a role of PHB modulation of mitochondrial Stat3 in normal cell mitochondrial function, not just during transformation. These results support the concept that mitochondrial dysfunction is not a cause of cancer metabolism but a consequence of oncogene-directed metabolic reprogramming required to meet the increased anabolic requirements of tumor growth [65].

Our data suggest a protective role of PHB modulation of mitochondrial Stat3 during IEC injury to prevent mitochondrial dysfunction characteristic of IBD. The non-transcriptional function of mitochondrial Stat3 in maintaining respiratory activity is only beginning to emerge. Less is known regarding the regulation of mitochondrial Stat3. We show here that the mitochondrial inner membrane protein PHB, regulates Stat3 to maintain mitochondrial homeostasis during pro-inflammatory conditions. By identifying Stat3 as a mediator of PHB-regulated mitochondrial function, we have identified a potential target to restore mitochondrial function and IEC homeostasis during colitis when PHB expression is deficient.

5. Conclusions

The onset of IBD is preceded by intestinal epithelial mitochondrial dysfunction associated with decreased expression of PHB. Here, we show that restoring PHB levels via exogenous PHB expression constructs protects against mitochondrial stress and apoptosis of IECs induced by TNF α . This protective effect of PHB on mitochondrial function is mediated by mitochondrial Stat3 and requires interaction of PHB with pS727-Stat3. Reduced levels of PHB during chronic intestinal inflammation may be an underlying factor promoting mitochondrial dysfunction and apoptosis of the intestinal epithelium. Restoration of PHB expression in IECs represents a potential therapeutic approach to maintain cell homeostasis during chronic mucosal inflammatory diseases such as IBD.

Acknowledgments

The authors thank the late Dr. Shanthi V. Sitaraman from Emory University, Atlanta, GA for her scientific guidance. We thank Mackenzie A. Butcher (Baylor Research Institute) for technical assistance. This work was supported by National Institutes of Health grants K01-DK085222 (A.L.T.) and R03-DK098229 (A.L.T.) and funds from the Baylor Research Institute.

Abbreviations

ETC	electron transport chain
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
PHB	prohibitin 1
ROS	reactive oxygen species
Stat	signal transducer and activator of transcription
TNF	tumor necrosis factor

References

- [1]. Neurath MF, Travis SP. Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut*. 2012; 61:1619–1635. [PubMed: 22842618]
- [2]. Du G, Mouithys-Mickalad A, Sluse FE. Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro. *Free Radic Biol Med*. 1998; 25:1066–1074. [PubMed: 9870560]
- [3]. O'Morain C, Smethurst P, Levi J, Peters TJ. Subcellular fractionation of rectal biopsy homogenates from patients with inflammatory bowel disease. *Scand J Gastroenterol*. 1985; 20:209–214. [PubMed: 3992179]
- [4]. Restivo NL, Srivastava MD, Schafer IA, Hoppel CL. Mitochondrial dysfunction in a patient with crohn disease: possible role in pathogenesis. *J Pediatr Gastroenterol Nutr*. 2004; 38:534–538. [PubMed: 15097444]
- [5]. Sifroni KG, Damiani CR, Stoffel C, Cardoso MR, Ferreira GK, Jeremias IC, Rezin GT, Scaini G, Schuck PF, Dal-Pizzol F, Streck EL. Mitochondrial respiratory chain in the colonic mucosal of patients with ulcerative colitis. *Mol Cell Biochem*. 2010; 342:111–115. [PubMed: 20440543]
- [6]. Tirosh O, Levy E, Reifen R. High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. *Nutrition*. 2007; 23:878–886. [PubMed: 17936198]
- [7]. Vanderborght M, Nassogne MC, Hermans D, Moniotte S, Seneca S, Van Coster R, Buts JP, Sokal EM. Intractable ulcerative colitis of infancy in a child with mitochondrial respiratory chain disorder. *J Pediatr Gastroenterol Nutr*. 2004; 38:355–357. [PubMed: 15076640]
- [8]. Delpre G, Avidor I, Steinherz R, Kadish U, Ben-Bassat M. Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis. *Am J Gastroenterol*. 1989; 84:1038–1046. [PubMed: 2773897]
- [9]. Hsieh SY, Shih TC, Yeh CY, Lin CJ, Chou YY, Lee YS. Comparative proteomic studies on the pathogenesis of human ulcerative colitis. *Proteomics*. 2006; 6:5322–5331. [PubMed: 16947118]
- [10]. Nazli A, Yang PC, Jury J, Howe K, Watson JL, Soderholm JD, Sherman PM, Perdue MH, McKay DM. Epithelia under metabolic stress perceive commensal bacteria as a threat. *Am J Pathol*. 2004; 164:947–957. [PubMed: 14982848]
- [11]. Goossens V, Grooten J, De Vos K, Fiers W. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc Natl Acad Sci U S A*. 1995; 92:8115–8119. [PubMed: 7667254]

- [12]. Hansen JM, Zhang H, Jones DP. Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor- α -induced reactive oxygen species generation, NF- κ B activation, and apoptosis. *Toxicol Sci.* 2006; 91:643–650. [PubMed: 16574777]
- [13]. Rath E, Berger E, Messlik A, Nunes T, Liu B, Kim SC, Hoogenraad N, Sans M, Sartor RB, Haller D. Induction of dsRNA-activated protein kinase links mitochondrial unfolded protein response to the pathogenesis of intestinal inflammation. *Gut.* 2012; 61:1269–1278. [PubMed: 21997551]
- [14]. Rath E, Haller D. Mitochondria at the interface between danger signaling and metabolism: Role of unfolded protein responses in chronic inflammation. *Inflamm Bowel Dis.* 2012; 18:1364–1377. [PubMed: 22183876]
- [15]. Theiss AL, Sitaraman SV. The role and therapeutic potential of prohibitin in disease. *Biochim Biophys Acta.* 2011; 1813:1137–1143. [PubMed: 21296110]
- [16]. Theiss AL, Idell RD, Srinivasan S, Klapproth JM, Jones DP, Merlin D, Sitaraman SV. Prohibitin protects against oxidative stress in intestinal epithelial cells. *Faseb J.* 2007; 21:197–206. [PubMed: 17135366]
- [17]. Thuaud F, Ribeiro N, Nebigil CG, Desaubry L. Prohibitin ligands in cell death and survival: mode of action and therapeutic potential. *Chem Biol.* 2013; 20:316–331. [PubMed: 23521790]
- [18]. Bourges I, Ramus C, Mousson de Camaret B, Beugnot R, Remacle C, Cardol P, Hofhaus G, Issartel JP. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. *Biochem J.* 2004; 383:491–499. [PubMed: 15250827]
- [19]. Tsutsumi T, Matsuda M, Aizaki H, Moriya K, Miyoshi H, Fujie H, Shintani Y, Yotsuyanagi H, Miyamura T, Suzuki T, Koike K. Proteomics analysis of mitochondrial proteins reveals overexpression of a mitochondrial protein chaperon, prohibitin, in cells expressing hepatitis C virus core protein. *Hepatology.* 2009; 50:378–386. [PubMed: 19591124]
- [20]. Kathiria AS, Butcher MA, Hansen JM, Theiss AL. Nrf2 is not required for epithelial prohibitin-dependent attenuation of experimental colitis. *Am J Physiol Gastrointest Liver Physiol.* 2013; 304:G885–896. [PubMed: 23494124]
- [21]. Yuan C, Chen WX, Zhu JS, Chen NW, Lu YM, Ou YX, Chen HQ. IL-10 treatment is associated with prohibitin expression in the Crohn's disease intestinal fibrosis mouse model. *Mediators Inflamm.* 2013; 2013:617145. [PubMed: 23690666]
- [22]. Theiss AL, Vijay-Kumar M, Obertone TS, Jones DP, Hansen JM, Gewirtz AT, Merlin D, Sitaraman SV. Prohibitin is a novel regulator of antioxidant response that attenuates colonic inflammation in mice. *Gastroenterology.* 2009; 137:199–208. 208, e191–196. [PubMed: 19327358]
- [23]. Lee JH, Nguyen KH, Mishra S, Nyomba BL. Prohibitin is expressed in pancreatic beta-cells and protects against oxidative and proapoptotic effects of ethanol. *Febs J.* 2010; 277:488–500. [PubMed: 20030709]
- [24]. Henschke P, Vorum H, Honore B, Rice GE. Protein profiling the effects of in vitro hyperoxic exposure on fetal rabbit lung. *Proteomics.* 2006; 6:1957–1962. [PubMed: 16447161]
- [25]. Ko KS, Tomasi ML, Iglesias-Ara A, French BA, French SW, Ramani K, Lozano JJ, Oh P, He L, Stiles BL, Li TW, Yang H, Martinez-Chantar ML, Mato JM, Lu SC. Liver-specific deletion of prohibitin 1 results in spontaneous liver injury, fibrosis, and hepatocellular carcinoma in mice. *Hepatology.* 2010; 52:2096–2108. [PubMed: 20890892]
- [26]. Liu X, Ren Z, Zhan R, Wang X, Wang X, Zhang Z, Leng X, Yang Z, Qian L. Prohibitin protects against oxidative stress-induced cell injury in cultured neonatal cardiomyocyte. *Cell Stress Chaperones.* 2009; 14:311–319. [PubMed: 18958584]
- [27]. Schleicher M, Shepherd BR, Suarez Y, Fernandez-Hernando C, Yu J, Pan Y, Acevedo LM, Shadel GS, Sessa WC. Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. *J Cell Biol.* 2008; 180:101–112. [PubMed: 18195103]
- [28]. Kathiria AS, Butcher LD, Feagins LA, Souza RF, Boland CR, Theiss AL. Prohibitin 1 modulates mitochondrial stress-related autophagy in human colonic epithelial cells. *PLoS One.* 2012; 7:e31231. [PubMed: 22363587]

- [29]. Mudter J, Weigmann B, Bartsch B, Kiesslich R, Strand D, Galle PR, Lehr HA, Schmidt J, Neurath MF. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol*. 2005; 100:64–72. [PubMed: 15654782]
- [30]. Neufert C, Pickert G, Zheng Y, Wittkopf N, Warntjen M, Nikolaev A, Ouyang W, Neurath MF, Becker C. Activation of epithelial STAT3 regulates intestinal homeostasis. *Cell Cycle*. 2010; 9:652–655. [PubMed: 20160497]
- [31]. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr HA, Hirth S, Weigmann B, Wirtz S, Ouyang W, Neurath MF, Becker C. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med*. 2009; 206:1465–1472. [PubMed: 19564350]
- [32]. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC. Function of mitochondrial Stat3 in cellular respiration. *Science*. 2009; 323:793–797. [PubMed: 19131594]
- [33]. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science*. 2009; 324:1713–1716. [PubMed: 19556508]
- [34]. Szczepanek K, Chen Q, Larner AC, Lesnefsky EJ. Cytoprotection by the modulation of mitochondrial electron transport chain: the emerging role of mitochondrial STAT3. *Mitochondrion*. 2012; 12:180–189. [PubMed: 21930250]
- [35]. Szczepanek K, Chen Q, Derecka M, Salloum FN, Zhang Q, Szelag M, Cichy J, Kukreja RC, Dulak J, Lesnefsky EJ, Larner AC. Mitochondrial-targeted Signal transducer and activator of transcription 3 (STAT3) protects against ischemia-induced changes in the electron transport chain and the generation of reactive oxygen species. *J Biol Chem*. 2011; 286:29610–29620. [PubMed: 21715323]
- [36]. Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*. 2007; 446:557–561. [PubMed: 17361131]
- [37]. Szczepanek K, Lesnefsky EJ, Larner AC. Multi-tasking: nuclear transcription factors with novel roles in the mitochondria. *Trends Cell Biol*. 2012; 22:429–437. [PubMed: 22705015]
- [38]. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stamatii A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol*. 2005; 21:1–26. [PubMed: 15868485]
- [39]. Lopez-Armada MJ, Riveiro-Naveira RR, Vaamonde-Garcia C, Valcarcel-Ares MN. Mitochondrial dysfunction and the inflammatory response. *Mitochondrion*. 2013; 13:106–118. [PubMed: 23333405]
- [40]. Santhanam S, Rajamanickam S, Motamarri A, Ramakrishna BS, Amirtharaj JG, Ramachandran A, Pulimood A, Venkatraman A. Mitochondrial electron transport chain complex dysfunction in the colonic mucosa in ulcerative colitis. *Inflamm Bowel Dis*. 2012; 18:2158–2168. [PubMed: 22374887]
- [41]. Kameyama J, Narui H, Inui M, Sato T. Energy level in large intestinal mucosa in patients with ulcerative colitis. *Tohoku J Exp Med*. 1984; 143:253–254. [PubMed: 6474455]
- [42]. Santhanam S, Venkatraman A, Ramakrishna BS. Impairment of mitochondrial acetoacetyl CoA thiolase activity in the colonic mucosa of patients with ulcerative colitis. *Gut*. 2007; 56:1543–1549. [PubMed: 17483192]
- [43]. Bar F, Bochmann W, Widok A, von Medem K, Pagel R, Hirose M, Yu X, Kalies K, Konig P, Bohm R, Herdegen T, Reinicke AT, Buning J, Lehnert H, Fellermann K, Ibrahim S, Sina C. Mitochondrial gene polymorphisms that protect mice from colitis. *Gastroenterology*. 2013; 145:1055–1063. e1053. [PubMed: 23872498]
- [44]. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M,

Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008; 40:955–962. [PubMed: 18587394]

- [45]. Singh SB, Ornatowski W, Vergne I, Naylor J, Delgado M, Roberts E, Ponpuak M, Master S, Pilli M, White E, Komatsu M, Deretic V. Human IRGM regulates autophagy and cell-autonomous immunity functions through mitochondria. *Nat Cell Biol.* 2010; 12:1154–1165. [PubMed: 21102437]
- [46]. Yu X, Wieczorek S, Franke A, Yin H, Pierer M, Sina C, Karlsen TH, Boberg KM, Bergquist A, Kunz M, Witte T, Gross WL, Epplen JT, Alarcon-Riquelme ME, Schreiber S, Ibrahim SM. Association of UCP2 -866 G/A polymorphism with chronic inflammatory diseases. *Genes Immun.* 2009; 10:601–605. [PubMed: 19387457]
- [47]. Theiss AL, Jenkins AK, Okoro NI, Klapproth JM, Merlin D, Sitaraman SV. Prohibitin inhibits tumor necrosis factor alpha-induced nuclear factor-kappa B nuclear translocation via the novel mechanism of decreasing importin alpha3 expression. *Mol Biol Cell.* 2009; 20:4412–4423. [PubMed: 19710421]
- [48]. Elschami M, Scherr M, Philippens B, Gerardy-Schahn R. Reduction of STAT3 expression induces mitochondrial dysfunction and autophagy in cardiac HL-1 cells. *Eur J Cell Biol.* 2013; 92:21–29. [PubMed: 23102833]
- [49]. Phillips D, Reilley MJ, Aponte AM, Wang G, Boja E, Gucek M, Balaban RS. Stoichiometry of STAT3 and mitochondrial proteins: Implications for the regulation of oxidative phosphorylation by protein-protein interactions. *J Biol Chem.* 2010; 285:23532–23536. [PubMed: 20558729]
- [50]. Willson TA, Jurickova I, Collins M, Denson LA. Deletion of intestinal epithelial cell STAT3 promotes T-lymphocyte STAT3 activation and chronic colitis following acute dextran sodium sulfate injury in mice. *Inflamm Bowel Dis.* 2013; 19:512–525. [PubMed: 23429443]
- [51]. Kida H, Yoshida M, Hoshino S, Inoue K, Yano Y, Yanagita M, Kumagai T, Osaki T, Tachibana I, Saeki Y, Kawase I. Protective effect of IL-6 on alveolar epithelial cell death induced by hydrogen peroxide. *Am J Physiol Lung Cell Mol Physiol.* 2005; 288:L342–349. [PubMed: 15475383]
- [52]. Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med.* 2002; 8:1089–1097. [PubMed: 12219085]
- [53]. Jia Y, Zhou F, Deng P, Fan Q, Li C, Liu Y, Fu X, Zhou Y, Xu X, Sun X. Interleukin 6 protects H(2)O(2)-induced cardiomyocytes injury through upregulation of prohibitin via STAT3 phosphorylation. *Cell Biochem Funct.* 2012; 30:426–431. [PubMed: 22431190]
- [54]. Matsumoto T, O'Malley K, Efron PA, Burger C, McAuliffe PF, Scumpia PO, Uchida T, Tschoeke SK, Fujita S, Moldawer LL, Hemming AW, Foley DP. Interleukin-6 and STAT3 protect the liver from hepatic ischemia and reperfusion injury during ischemic preconditioning. *Surgery.* 2006; 140:793–802. [PubMed: 17084723]
- [55]. Teoh N, Field J, Farrell G. Interleukin-6 is a key mediator of the hepatoprotective and proliferative effects of ischaemic preconditioning in mice. *J Hepatol.* 2006; 45:20–27. [PubMed: 16600417]
- [56]. Spehlmann ME, Manthey CF, Dann SM, Hanson E, Sandhu SS, Liu LY, Abdelmalak FK, Diamanti MA, Retzlaff K, Scheller J, Rose-John S, Greten FR, Wang JY, Eckmann L. Trp53 deficiency protects against acute intestinal inflammation. *J Immunol.* 2013; 191:837–847. [PubMed: 23772033]
- [57]. Theiss AL, Obertone TS, Merlin D, Sitaraman SV. Interleukin-6 transcriptionally regulates prohibitin expression in intestinal epithelial cells. *J Biol Chem.* 2007; 282:12804–12812. [PubMed: 17324931]
- [58]. Gratia S, Kay L, Michelland S, Seve M, Schlattner U, Tokarska-Schlattner M. Cardiac phosphoproteome reveals cell signaling events involved in doxorubicin cardiotoxicity. *J Proteomics.* 2012; 75:4705–4716. [PubMed: 22348821]

- [59]. Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res Cardiol.* 2010; 105:771–785. [PubMed: 20960209]
- [60]. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell.* 2009; 15:103–113. [PubMed: 19185845]
- [61]. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell.* 2009; 15:91–102. [PubMed: 19185844]
- [62]. Ussakli CH, Ebaee A, Binkley J, Brentnall TA, Emond MJ, Rabinovitch PS, Risques RA. Mitochondria and tumor progression in ulcerative colitis. *J Natl Cancer Inst.* 2013; 105:1239–1248. [PubMed: 23852949]
- [63]. Kathiria AS, Neumann WL, Rhees J, Hotchkiss E, Cheng Y, Genta RM, Meltzer SJ, Souza RF, Theiss AL. Prohibitin attenuates colitis-associated tumorigenesis in mice by modulating p53 and STAT3 apoptotic responses. *Cancer Res.* 2012; 72:5778–5789. [PubMed: 22869582]
- [64]. Rahman MM, Seo YR. Discovery of potential targets of selenomethionine-mediated chemoprevention in colorectal carcinoma mouse model using proteomics analysis. *Carcinogenesis.* 2013; 34:1575–1584. [PubMed: 23504501]
- [65]. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell.* 2012; 21:297–308. [PubMed: 22439925]

Highlights

- IBD is associated with intestinal epithelial mitochondrial dysfunction.
- The mitochondrial protein PHB is decreased during IBD.
- PHB overexpression protects against intestinal epithelial mitochondrial stress.
- PHB protection is dependent on mitochondrial Stat3.
- Reduced levels of prohibitin during IBD may underlie mitochondrial dysfunction.

Figure 1. PHB interacts with pS727-Stat3 in the mitochondria of Caco2-BBE cells

(A) Western blots of mitochondrial (mito) and cytosolic extracts, performed in triplicate as indicated by numbers 1-3, from polarized Caco2-BBE cells compared to whole-cell extracts (WCE). β -tubulin and COX IV were used to assess purity of cytosolic and mitochondrial fractions, respectively. (B) The same protein extracts as in A were probed for Y705-Stat3 by western blot. (C) PHB was immunoprecipitated (IP) from mitochondrial or cytosolic cell extracts, performed in triplicate. Immunoprecipitates were separated by SDS-PAGE and membranes were immunoblotted (IB) for pY705-Stat3, pS727-Stat3, or PHB expression. An IgG control antibody was used for the immunoprecipitation as a negative control. Whole cell extracts (WCE) were separated by SDS-PAGE but did not undergo the immunoprecipitation

step. Extracts from Caco2-BBE cells immunoprecipitated for total Stat3 (D), pS727-Stat3 (E), or pY705-Stat3 (F) were immunoblotted for PHB or Stat3 as described in C.

Figure 2. PHB interacts with mitochondrial pS727-Stat3 in non-transformed IEC-6 cells
 (A) Western blots of Stat3 and PHB expression in mitochondria (mito), cytosolic, and whole-cell extracts (WCE), performed in duplicate as indicated by numbers 1-2, from IEC-6 cells. β -tubulin and COX IV were used to assess purity of cytosolic and mitochondrial fractions, respectively. (B) PHB was immunoprecipitated (IP) from mitochondrial or cytosolic extracts from IEC-6 cells. Immunoprecipitates were separated by SDS-PAGE and membranes were immunoblotted (IB) for pY705-Stat3, pS727-Stat3, total Stat3, or PHB expression. An IgG control antibody was used for the immunoprecipitation as a negative control. Whole cell extracts (WCE) were separated by SDS-PAGE but did not undergo the immunoprecipitation step. IEC-6 protein extracts immunoprecipitated for pS727-Stat3 (C) or pY705-Stat3 (D) were immunoblotted for PHB or Stat3.

Figure 3. PHB and pS727-Stat3 interact in mitochondria of *in vivo* colonic intestinal epithelium

(A) Western blots of Stat3 and PHB expression in mitochondria (mito), cytosolic, and whole-cell extracts (WCE), performed in duplicate as indicated by numbers 1-2, from freshly isolated wild-type mouse colonic intestinal epithelial cells. β -tubulin and COX IV were used to assess purity of cytosolic and mitochondrial fractions, respectively. (B) PHB was immunoprecipitated from mitochondrial or cytosolic extracts from isolated colonic epithelium from wild-type mice. Immunoprecipitates were immunoblotted for pY705-Stat3, pS727-Stat3, total Stat3, or PHB expression. (C) Colonic epithelium extracts immunoprecipitated for total Stat3 were immunoblotted for PHB or Stat3. A non-specific band is evident when immunoprecipitating tissue extracts and blotting with the PHB antibody (denoted with *).

Figure 4. MLS-S727A-Stat3 dominant negative construct localizes to the mitochondria and PHB/Stat3 interaction is dependent on S727-Stat3

(A) Mitochondrial (mito) or cytosolic extracts were isolated from Caco2-BBE cells transfected with His-tagged MLS-S727A-Stat3 dominant negative (MLS-S727 Stat3dn). Immunoprecipitation (IP) using a His antibody followed by immunoblotting (IB) for total Stat3 or His expression indicate that His-tagged MLS-S727A-Stat3 localizes to the mitochondria. An IgG control antibody was used for the immunoprecipitation as a negative control. Whole cell extracts (WCE) were separated by SDS-PAGE but did not undergo the immunoprecipitation step. (B) Representative western blots of pS727-Stat3, pY705-Stat3, and total Stat3 protein expression in mitochondrial, cytosolic, or whole cell extracts from

Caco2-BBE cells transfected with MLS-S727 Stat3dn construct or MLS-vector. MLS-S727 Stat3dn band is denoted with a * in western blots probed for total Stat3. Blots were subsequently probed for PHB, Cox IV and β -actin to ensure purity of subcellular fractions. (C-D) Caco2-BBE cells were transfected with MLS-S727 Stat3dn or MLS-vector and interaction of Stat3 with PHB was assessed by co-immunoprecipitation experiments.

Figure 5. TNF α decreases endogenous PHB expression and interaction of pS727-Stat3 with PHB
 (A) Mitochondrial (mito) and cytosolic extracts from Caco2-BBE cells treated with 10 ng/ml TNF α for 4 hr were assessed for PHB and Stat3 protein expression by western blot. β -tubulin and COX IV were used to assess purity of cytosolic and mitochondrial fractions, respectively. (B-C) Caco2-BBE cells were treated with 10 ng/ml TNF α for 4 hr and the interaction of PHB with pS727-Stat3 was assessed by co-immunoprecipitation experiments.

Figure 6. TNF α decreases interaction of pS727-Stat3 with endogenous PHB but not with exogenous GFP-tagged PHB

(A) Western blots of mitochondria (mito) and cytosolic extracts from Caco2-BBE cells stably overexpressing pEGFPN1 vector or pEGFPN1-PHB (GFP-PHB) were probed with an anti-GFP antibody to determine whether exogenous GFP-tagged PHB localizes to mitochondria. COX IV and β -tubulin were used to assess purity of mitochondrial and cytosolic fractions, respectively. (B) Caco2-BBE cells overexpressing pEGFPN1-vector or GFP-PHB were treated with 10 ng/ml TNF α for 4 hr. The interaction of pS727-Stat3 with exogenous GFP-tagged PHB with was assessed by co-immunoprecipitation experiments.

Figure 7. Mitochondrial pS727-Stat3 mediates the protective effect of PHB overexpression on mitochondrial function

Caco2-BBE cells stably transfected with pEGFPN1-vector (V) or pEGFPN1-PHB (PHB) were transfected with MLS-S727A-Stat3 dominant negative construct (MLS-S727-Stat3dn) or MLS-vector for 24 hr. Cells were treated with 10 ng/ml TNF α for 4 hr and assessed for mitochondrial ROS levels using MitoSOX dye (A), ATP concentration (B), complex I activity (C), or complex IV activity (D). Peroxynitrite treatment was used as a positive control for mitoSOX measurement. Cells were treated with 10 ng/ml TNF α for 16 hr and

assessed for viability by LDH test (E) and apoptosis by ELISA (F). n = 3 per treatment across 2 separate experiments. $**P < 0.01$, $*P < 0.05$, ns, not significant.

Figure 8. Mitochondrial pS727-Stat3 mediates the protective effect of PHB overexpression on mitochondrial function in IEC-6 cells

IEC-6 cells were co-transfected with pEGFPN1-vector (V) or pEGFPN1-PHB (PHB) and MLS-S727A-Stat3 dominant negative construct (MLS-S727-Stat3dn) or MLS-vector for 24 hr. (A) Cells were treated with 10 ng/ml TNF α for 4 hr and assessed for mitochondrial ROS levels using MitoSOX dye. Peroxynitrite treatment was used as a positive control. (B) Cells were treated with 10 ng/ml TNF α for 16 hr and assessed for viability by LDH test. $n = 3$ per treatment across 2 separate experiments. $**P < 0.01$, $*P < 0.05$.