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Redox biology of the intestine

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Abstract

The intestinal tract, known for its capability for self-renew, represents the first barrier of defense between the organism and its luminal environment. The thiol/disulfide redox systems comprising the glutathione/glutathione disulfide (GSH/GSSG), cysteine/cystine (Cys/CySS) and reduced and oxidized thioredoxin (Trx/TrxSS) redox couples play important roles in preserving tissue redox homeostasis, metabolic functions, and cellular integrity. Control of the thiol-disulfide status at the luminal surface is essential for maintaining mucus fluidity and absorption of nutrients, and protection against chemical-induced oxidant injury. Within intestinal cells, these redox couples preserve an environment that supports physiological processes and orchestrates networks of enzymatic reactions against oxidative stress. In this review, we focus on the intestinal redox and antioxidant systems, their subcellular compartmentation, redox signaling and epithelial turnover, and contribution of luminal microbiota, key aspects that are relevant to understanding redox-dependent processes in gut biology with implications for degenerative digestive disorders, such as inflammation and cancer.

Keywords

Intestinal redox status and control of redox balance; extracellular cysteine/cystine (Cys/CySS) redox state; cellular glutathione/glutathione disulfide (GSH/GSSG) redox state; redox control of intestinal cell phenotypic transitions; mucosal GSH and GSH-dependent enzymes; GSH and intestinal oxidative stress; intestinal disorders and tissue redox state; intestinal microbiota; intestinal NFκB redox signaling

Introduction: Overview of structural and functional organization of the intestinal epithelium

The mammalian gastrointestinal tract is lined with a single layer of epithelial cells that is capable of self-renewal every 4–5 days, and is among the highest proliferative tissue in the organism [1]. The gut is comprised of the small intestine and the colon; the small intestinal epithelium is extensively folded to maximize the absorptive surface area, resulting in distinct villus and crypt (of Lieberkühn) regions (Figure 1A). Within the crypt, stem cells proliferate, and actively dividing progenitors cells differentiate into secretory (Paneth, mucin-secreted goblet, and enteroendocrine cells) or absorptive (enterocyte) lineages. Current paradigm is consistent with the self-renewing and multi-potent intestinal crypt stem cells (ISC) being the source of the four major differentiated epithelial cell types. Cell proliferation begins at the bottom of intestinal crypts where one to six ISC transform into a transient population of rapidly dividing progenitor cells (Figure 1B). During migration

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towards the villus tip, progenitor cells differentiate into goblet, enteroendocrine cells or enterocytes. Three days after their functional differentiation, intestinal cells undergo apoptosis and are shed into the lumen. Paneth cells differentiate and reside within the crypts and undergo phagocytosis three weeks post differentiation [2]. The surrounding stromal/mesenchymal cells, together with signals generated by stem cells create a complex microenvironment, known as the stem cell niche which regulates stem cell behavior [3]. Unlike the small intestine, the colonic epithelium is devoid of villi and Paneth cells [4], and proliferative and differentiated cells are localized to the bottom of crypt invaginations and to surface epithelium, respectively.

The absorptive enterocytes account for over 80% of the intestinal cells [5], and are highly polarized, consisting of an apical surface facing the lumen and a basolateral surface adjacent to the stroma. Adjacent epithelial cells are connected along the cell length from the luminal to lamina propria surfaces via tight junctions, adherens junctions, and desmosomes. These junctional structures provide architectural integrity, maintain an apical-to-basolateral polarity, and regulate paracellular water and electrolyte flux [6,7]. A viscous glycoprotein layer covering the enterocyte brush border membrane functions as a physical barrier that limits the access of luminal proteins and microorganisms. An underlying dense microvilli layer serves as a second barrier that impede intestinal access of most macromolecules, viruses, and bacteria [8]. Goblet cells, representing ~4 to 16% in the duodenum and colon [9] secrete mucins, forming a protective barrier against shear stress and chemical insult. Enteroendocrine cells, which comprise less than 1% of the epithelial cells, release gastrointestinal hormones (serotonin, substance P, secretin), and Paneth cells produces lysosyme, defensin, or antimicrobials that control the luminal flora.

The mucosal epithelium is supported by the lamina propria which consists of the extracellular matrix, blood and lymphatic vessels, neuronal and smooth muscle cells, and various immune cells (lymphocytes, macrophages), the latter participating in the mucosal immune response [10]. The gut-associated lymphoid tissue (GALT) is one of the largest lymphoid organs in the body and is formed by isolated and aggregated lymphoid follicles known as Peyer's patches [11] (Figure 1). Peyer's patches are surrounded by the follicle-associated epithelium (FAE), comprised of specialized, highly invaginated enterocytes named M (for microfold) cells. In contrast to the villus epithelium, the FAE is characterized by poorly organized brush border membrane with short, irregular microvilli, weak mucus production, and a thin glycocalyx that exhibits different glycosylation pattern, a feature that allows for easy access to antigens from the intestinal lumen [12]. Specific luminal antigens (e.g. pathogens, toxins, or commensal bacteria) are sampled by the M cells, phagocytosed, and transported to the underlying immune cells which trigger the immune response [13]. M cell basolateral membranes contain a unique intraepithelial invagination ("pocket") where antigen-presenting dendritic cells, B-and T-lymphocytes, and macrophages reside [14]. This pocket reduces the distance for the trans-epithelial transport of antigens and enables a faster immune response. Since antigens do not undergo major alterations during transcytosis, M cells are exploited by different pathogens like *S. typhimurium, Yersinia enterocolitica, L. monocytogenes* and *V. cholera* as entry sites to the underlying host tissues [15,16]. Oral tolerance against commensal bacteria and proteins is acquired via generation of antigen-specific T lymphocytes that suppress inflammatory immune responses, and thus protects the intestinal mucosa.

General consideration of intracellular redox, antioxidant defense systems and subcellular compartmentation

Concept of redox state and redox environment

The "redox state" of a specific redox couple such as glutathione (GSH), thioredoxin (Trx) or cysteine (Cys) refers to the ratio of their inter-convertible reduced and oxidized forms, i.e., GSH/GSSG, Trx/TrxSS, or Cys/CySS. The term, "redox environment" represents the sum total of the product of the reducing potential and reducing capacity of these linked redox couples [17]. Given the difficulty in quantifying the cellular status of all linked redox couples, and the large cellular GSH pool size, the status of GSH/GSSG is generally considered a good estimate of the intracellular redox environment. The tendency of GSH to accept or donate electrons is determined by its redox potential (electromotive force, E_h), mathematically represented by the Nernst equation: $E_h = E_0 + (RT/nF)\ln([GSSG/GSH]^2)$ [18], where R is the gas constant, T is the absolute temperature, F is Faraday's constant and n is the number of electrons transferred. E_0 represents the standard potential for the redox couple and is calculated at equilibrium conditions relative to a standard hydrogen electrode. Under physiological conditions, E_h for the GSH/GSSG couple is between −260 to −200mV [18].

In biologic systems, the GSH/GSSG, Trx/TrxSS, and Cys/CySS redox couples are found to be far from equilibrium [18], and each redox couple reportedly functions as unique rheostat on/off switches in the redox regulation of cellular proteins [18]. The prevailing argument is that the existence of such distinctive "redox control nodes or circuitry" could afford an elegant mechanism for redox control in optimizing protein function based on the subcellular concentrations and fluxes of the redox couples, thus permitting a mode for independent regulation of the redox status of individual and/or specific protein sets [19]. Accordingly, the disruption of these redox control nodes would dramatically affect activities of various enzymes with redox sensitive catalytic site cysteines or methionines [19,20]. The notion that on/off "sulfur switches" could represent a generalized redox signaling mechanism in the control of redox-sensitive biological processes in normal cell physiology [21] allows redefining cellular oxidative stress as the disruption of physiological redox signaling events [22], beyond the classical definition of oxidative stress as an imbalance between pro- and anti-oxidant systems [23]. This notwithstanding, there remains key unanswered questions; for instance, the degree of cross-talk among redox couples, such as GSH/Trx, within and between compartments, e.g., cytosol, nucleus, or mitochondria, [20], and how changes in the redox state of one or more redox couples in single or multi compartments influence the overall cellular redox environment, cell signaling and, ultimately, cell metabolism or cell fate. Several recent reviews provide excellent discussion of the integration of redox compartments and implications for redox processes in biologic systems [18,24].

Cellular redox systems and their compartmentation

Glutathione/glutathione disulfide (GSH/GSSG) system—Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is present in millimolar concentrations (2–10mM) within cells and is a primary determinant of the cellular redox environment [17]. Intracellular GSH exists mainly as the biologically active reduced-thiol form; the oxidation of GSH to GSSG and subsequent decrease in the GSH-to-GSSG ratio is often associated with oxidative stress. Thus, the GSH-to-GSSG ratio is a simple and useful indicator of cellular oxidative stress [17]. Central to the maintenance of cellular redox homeostasis is *de novo* GSH synthesis, GSSG reduction, and exogenous GSH uptake. *De novo* GSH synthesis in the cytosol is catalyzed by γ-glutamylcysteine ligase and GSH synthetase [25], while the regeneration of GSH from GSSG is catalyzed by NADPH-dependent GSSG reductase, and GSH transport occurs via plasma membrane carriers [26]. Extracellular GSH uptake has been shown in

transport epithelial cells such as enterocytes [27] and proximal tubular cells [28]. Figure 2 illustrates the relationships among these pathways of cellular GSH homeostasis (synthesis, reduction, transport), and GSH consumption in detoxication reactions by GSH-dependent enzymes, such as glutathione reductase (GR), glutaredoxin (Grx), and the family of glutathione peroxidases (GPxs) and glutathione-S-transferases (GSTs). Redox modulation of enzyme function is an important regulatory mechanism in the removal of reactive oxygen or nitrogen species (ROS/RNS), carcinogens, drugs, and xenobiotics.

Cytosolic GSH is highly reduced (E_h for GSH/GSSG of −260mV) [18], and is the source of distinct GSH redox pools within the cellular compartments of mitochondria, endoplasmic reticulum (ER) and nucleus. Mitochondrial GSH, at concentrations similar to those in the cytosol, can account for 15% to 30% of total GSH [29,30]. The inner mitochondrial membrane GSH transporters, dicarboxylate (DIC) and 2-oxoglutarate (OGC) carriers [31] maintain the matrix GSH/GSSG E_h at −300mV [32]. Interestingly, the GSH/GSSG redox state of the intermembrane space (IMS) is more oxidized despite free access of cytosolic GSH through porin channels [33]. A redox potential of E_h −255mV in the IMS is believed to be an ideal redox environment for disulfide bond formation of imported cytosolic proteins [34]. Similarly, the highly oxidized GSH redox milieu of the ER matrix (GSH:GSSG E_h between −170mV and −185mV [35]) ensures proper folding of nascent proteins, and maintains the redox buffering capacity in this compartment [36]. Cytosol-to-nuclear GSH distribution occurs through passive diffusion via nuclear pores [37]. While the size of the nuclear GSH pool is unclear, it is reportedly higher than that of the cytosol, suggesting that the two pools are independently maintained [19]. Additionally, a higher ratio of reduced- to-glutathionylated nuclear proteins [38] is consistent with a more negative GSH/GSSG redox potential in the nucleus [21]. The interaction of nuclear and cytosolic GSH is dynamic; during cell cycle, cytosolic GSH is distributed to the nucleus [38] (4 times higher) while confluent cells exhibited equal nuclear-to-cytosol GSH distribution [38].

Thioredoxin (Trx) and Trx-dependent redox system—Reduced and oxidized Trx (Trx-S/Trx-SS) maintain intracellular redox homeostasis in conjunction with GSH/GSSG [20]. Trx are small ubiquitous proteins that catalyze the reversible reduction of disulfide bonds resulting from oxidation of active site cysteines, Cys-XX-Cys (CGPC) in proteins. Reduction of the CGPC motif is mediated by NADPH-dependent Trx reductase (TrxR). The mammalian Trx1 and Trx2 are distinct cytosolic/nuclear and mitochondrial isoforms, respectively that exhibit redox potential of −280mV and −300mV, and −330mV [18]. Trx1/2 are regulated independently and are differentially sensitive to various stimuli [20,39]. *TrxR* isoenzymes are ubiquitous selenoproteins that catalyze the NADPH-dependent reduction of oxidized Trx. TrxR1 and TrxR2 are respectively cytosolic and mitochondrial enzymes while TGR is exclusively found in the testis [40]. The C-terminal motif contains a selenium-Cys (Sec) residue that is essential for catalytic activity; the loss of Sec is associated with enzyme inactivation. Deficiency inTrxR1 or TrxR2 genes results in embryonic lethality [41].

Peroxiredoxins (Prxs) are non-seleno thiol-specific peroxidases that catalyze the reduction of H_2O_2 and organic hydroperoxides wherein the peroxidatic Cys is oxidized to sulfenic acid (Cys-SOH) that forms a disulfide bond with a C-terminal Cys residue. Regeneration of active cysteines is catalyzed by Trx and TrxR. To date, six Prx isoforms (Prx 1–6) are described and divided into typical 2-Cys Prxs, atypical 2-Cys Prxs, and 1-Cys Prx based on the number of active cysteines [40]. Among these, Prx 1–4 are typical 2-Cys Prxs with different subcellular localizations: cytosol, Prx 1 and 2; extracellular space, Prx 4; and mitochondria, Prx3. Prx 5 is the only atypical 2-Cys enzyme widely distributed in the mitochondria, cytosol, nucleus, and peroxisomes [42] while Prx 6 is a cytosolic 1-Cys Prx [43]. High Prx expression (up to 1% of the cellular proteins) and catalytic rates

($\sim 10^7 M^{-1}s^{-1}$) suggests that Prxs may be responsible for the bulk of intracellular H_2O_2 reduction under physiological conditions [44]. However, it remains to be resolved as to whether Prxs are more significant than GPxs in the quantitative elimination of H_2O_2 generated within the various subcellular organelles. Localized H_2O_2 accumulation and the inactivation of Prx via peroxidatic Cys-to-sulfinic acid ($-SO_2H$) oxidation, a process that was reversed by sulfiredoxins [45], was implicated in cell signaling [46]. More recent evidence show that plasma membrane signaling was linked to specific inactivation of membrane-associated Prx1 through receptor engagement and tyr^{194} phosphorylation, a novel mechanism that allowed for H_2O_2 accumulation and signal propagation initiated at the receptor site [47].

Cysteine/cystine (Cys/CySS) redox system—Cysteine (Cys) and cystine (CySS) constitute the most abundant low-molecular thiols in extracellular fluids with concentrations reaching 40 μM and 8–10 μM, respectively. The status of Cys and CySS contributes to maintaining the extracellular redox environment [48]. The measured Cys/CySS redox potential, E_h, in cell culture media or in plasma of healthy human subjects is around −80mV, a value that is more oxidized than that of GSH/GSSG and Trx/TrxSS redox systems [18]. Plasma Cys/CySS homeostasis is highly regulated by dietary Cys/CySS [49], hydrolysis of exported GSH [50], thiol-disulfide exchange between CySS and plasma GSH or homocysteine [51], and intracellular/extracellular Cys/CySS shuttle [52]. An oxidized Cys/CySS redox state has been associated with age-related diseases like diabetes, cardiovascular disease, and atherosclerosis [53]. Since the plasma Cys/CySS as well as GSH/GSSG redox states serve as quantitative measures of oxidative stress, a broader question is whether the redox states of these couples in the plasma could function as predictive markers of health and disease, a notion championed by Jones [48].

Redox balance in the intestine: physiological and pathological significance

Intestinal redox systems and related enzymes

Mucosal GSH and GSH-dependent enzymes—Like most tissues, the intestinal epithelium contain millimolar concentrations of GSH [54–56] that is maintained by *de novo* synthesis [57], regeneration from GSSG [58], and GSH uptake at the apical membrane [55,59]. Induction of GSH deficiency by inhibition of synthesis caused severe epithelial degeneration *in vivo* [60] while oral GSH administration reversed jejunal and colonic degeneration in accordance with increased tissue GSH [27], consistent with a role of GSH in intestinal integrity. Uptake of intact GSH across the brush-border membrane is stimulated by cations (Na^+, K^+, Li^+) [59,61] that was not linked to GSH synthesis [57], indicating that GSH transport and intracellular GSH synthesis are independently regulated [62].

Exogenously administered GSH is efficiently taken up and excreted across the intestinal basal membrane, thereby increasing plasma GSH levels and GSH bioavailability [63]; however, uptake of precursor amino acids did not alter plasma GSH, consistent with local consumption of newly synthesized GSH within intestinal cells [63]. In vascularly perfused rat small intestine, milimolar concentrations of luminal GSH was absorbed intact across intestinal apical membranes; ~70–80% of [^{3}H]GSH (1mM) was transported as intact GSH into blood with half-maximal transport at 1mM and saturation at 3mM [59]. Interestingly, at 0.3mM GSH ([^{35}S]-labeled), abluminal recovery of GSH consisted of intact GSH (60%) and cysteine/cystine products (30 and 10%). At 0.1mM, the percent of intact GSH absorbed (45%) equaled that of cysteine and cystine (45% and 10%), indicating that substantial hydrolysis of luminal GSH occurred at submillimolar levels [59]. Intestinal GSH uptake and GSH hydrolysis are thus independent processes that occur concurrently, and the final metabolic fate of GSH is a function of its luminal concentration. In animal studies, dietary intake and biliary output are major contributors to luminal GSH homeostasis [54,64].

Additionally, intestinal GSH export into the lumen can occur during periods of fasting [65]. Liver is the major source of biliary GSH, and duodenal luminal concentrations can account for 50% of the hepatic GSH pool [54,64]. GSH contents in the human diet is highly variable; GSH-rich sources include fresh fruits, vegetables, and lean meat, while processed foods, grains, or dairy products generally have low GSH [66,67]. Therefore, dietary habit would contribute to the variability of sulfur amino acid and GSH levels and varied degree of oxidative stress in the lumen and plasma [68].

The luminal GSH pool is important in intestinal absorptive and detoxication functions and in protecting the mucus layer [65]. In this regard luminal GSH participates in dietary disulfide reduction and uptake [69] and in maintenance of mucus fluidity [70]. Mucins, which comprised the matrix/mucus gel layer, are glycosylated glycoproteins that assemble into homo-oligomeric structures via disulfide bonds formation between cysteine-rich domains. Thus, mucus viscosity can be modulated by GSH [71,72] and NAC [73] through reduction of disulfide bonds and disassembly of mucin oligomers. In addition, the hydration of the mucus layer can indirectly be influenced by transepithelial GSH transport via CFTR (cystic fibrosis transmembrane conductance regulator) channel and altered electrolyte (chloride and bicarbonate) fluxes [74,75]. Mucus-associated GST [76] catalyzed the conjugation-detoxication of luminal reactive electrophiles, carcinogens, drugs or food preservatives and prevent damage to the intestinal epithelium [77]; luminal GSH levels (250 μM) can stimulate GST-dependent conjugation reactions by 300% [76]. However, GSH can bind divalent metals like Cu, Se, Cr or Zn, and non-enzymatic oxidative damage to the intestinal epithelium can occur as a consequence of metal-mediated ROS production [78]. Of significance, therapeutic use of iron complexes could result in increased availability of free iron in the intestinal lumen [79]. Since ascorbic acid is typically administered in combination to increase iron bioavailability from storage ferritin [80], the potential for H_2O_2 generation is enhanced through Fe-catalyzed two-electron oxidation of ascorbate [81]. During iron/ascorbate supplementation, an elevated consumption of the luminal GSH pool for ascorbate reduction and ROS scavenging could impose a significant stress on the redox buffering capacity of the intestinal lumen.

Mucosal antioxidant defense are mediated by GSH-dependent enzymes including GR, Gpx, Grx, and GST that are ubiquitously present along the intestinal tract. Among these, GR and GRx (thiol-disulfide oxidoreductases) reduce GSSG or GSH-mixed disulfides in targeted proteins through thiol-disulfide exchanges [82]. Mammalian Grx isoenzymes exhibit different catalytic properties and are localized to various subcellular compartments of cytosol (Grx1) and mitochondrion (Grx2(a)); however, two Grx2 variants, Grx2b and Grx2c, were identified in the cytosol and nucleus. The monothiol Grxs (cytosolic Grx3, mitochondrial Grx5) lack thiol-disulfide oxidoreductase activities; Grx3, also called PICOT or TXNL-2, contains two $[2Fe\text{-}2S]^{2+}$ clusters and reportedly functions as redox sensors for ROS/RNS signals rather than participate in redox reactions [83]. All Grx isoforms have been detected in mouse intestine with high expression of Grx2 in duodenal enterocytes and preferred localization of Grx5 and Grx3 to apical and apical/lateral surfaces, respectively [84]. Similarly, Grx2 is highly expressed in jejunal enterocytes, Paneth and certain lamina propria cells [84]. The location of nuclear Grx5 in enterocytes, and its presence in Paneth cells and colonic epithelium [84] suggest a role for this isoform in intestinal/colonic redox reactions. The pathophysiolgical or clinical significance of the existence of defined intestinal Grx isoenzymes and their subcellular distribution remains to be investigated.

Of the eight characterized mammalian Gpxs (Gpx1–8), most are selenoproteins (Gpx1, Gpx2, Gpx3, Gpx4, and Gpx6) with different subcellular locations [85]. Gpx-catalyzed removal of H_2O_2 is kinetically fast (~ 5×10^7 M^{-1} s^{-1}), thus rendering the elimination of H_2O_2 an efficient process even at high hydroperoxide concentrations. Gpx1 and Gpx2 are

the major H_2O_2 reducing enzymes in the intestinal epithelium [86]. Although these isoenzymes have similar substrate specificity and cytosolic location, Gpx1 is uniformly distributed along the crypt-villus axis, while Gpx2 predominates in the crypt [86,87]. In addition, Gpx2, previously known as GSHPx-GI [88], is an intestinal specific GSH peroxidase that is highly expressed in the ileum and cecum [89]. A notable distinction between Gpx1 and Gpx2 is their sensitivity to selenium (Se) deprivation. Unlike Gpx2, Gpx1 protein and mRNA expression are highly vulnerable to Se deficiency and is rapidly degraded; the relative resistance of Gpx2 to Se variation [90] suggests the importance of this isoenzyme in intestinal cell survival. Gpx3, an extracellular Gpx isoform secreted by intestinal cells into the lumen, reportedly participates in protecting the intestinal mucosa against oxidant injury [91]. Gpx4, the only Se-Gpx protein known to reduce phospholipid hydroperoxides, is present in the cytoplasmic and nuclear compartments of human small intestine and colon [92]; interestingly, Se deficiency has no effect on Gpx4 message level or its stability [90]. Pprotein expression and enzymatic activity of Gpx4 are notably increased in differentiated colonocytes in association with enhanced antioxidant protection, especially in the nuclear compartment [92].

GST proteins are abundant in the gastrointestinal tract. However, GST distribution varies considerably along the digestive tract and their activity and expression is influenced by diet, drug exposure, and clinical conditions [93]. Human GST multigenic family of isoenzymes is divided into the Alpha, Mu, Pi, Omega, Theta, Zeta, Sigma, Kappa, and microsomal GSTs [94]. Typically, GST activity and the expression of GST isoforms, such as Pi and Mu are lower in the colon as compared to the small intestine [93], a fact that may explain the rare incidence of neoplasia in the small intestine given that GST catalyzes GSH-dependent detoxication of luminal electrophiles and carcinogens. In human colon, the highest expressed isoform is GST Pi [95]; GST Mu is also abundant in primary colonocytes [96] while GST alpha expression is low. The decrease in GST activity from proximal to distal colon [93] is consistent with decreased colonic xenobiotic detoxication and increased cancer risk.

Luminal Cys/CySS redox status—Aside from GSH, the Cys/CySS pool plays an important role in regulating the luminal thiol-disulfide redox state [65] and preserving the redox status of extracellular proteins [97]. In rat intestine, GSH hydrolysis, catalyzed by γ-glutamyl transpeptidase and dipeptidase (Figure 2) accounted for ~40% of luminal Cys. Luminal Cys exhibit dual functions in facilitating the absorption of diverse redox sensitive nutrients [98], and in maintaining the fluidity and integrity of the intestinal mucus layer [70]. Redox control of extracellular surface proteins by luminal Cys/CySS is believed to regulate signaling processes at the apical plasma membrane [99]. Cys/CySS homeostasis is maintained by dietary sources, the Cys/CySS shuttle, and the degradation of luminal GSH (Figure 3). A recent study with minipigs found that ~60% of dietary cysteine was captured and sequestered by the small intestine, and only a small portion was released into the blood [100]. Dietary methionine, another sulfur amino acid, can be taken up by enterocytes at the brush border membrane, and through intracellular trans-sulfuration, yields Cys that supports the synthesis of mucosal GSH and extracellular mucin secreted by goblet cells [23]. It is suggested that the requirements for these processes govern the rate of methionine trans-sulfuration [101,102].

Cys was found to be released at a high rate into the lumen of the vascularly perfused rat small intestine *in situ* [65] where it reduced GSSG to GSH with resultant formation of CySS. A more reduced luminal E_h for Cys/CySS than that for GSH/GSSG is consistent with the function of extracellular Cys in luminal GSSG reduction [69]. CySS uptake across the brush border membrane [103] was reduced intracellularly by GSH and Cys is released back into the lumen, thus constituting a Cys/CySS shuttle (Figure 3), a pivotal mechanism in

controlling the luminal thiol-disulfide redox status [69]. The transport of Cys and CySS at the apical and basal membranes [104,105] is facilitated by sodium-dependent and independent carrier systems. These include the apical amino acid transporters: $b^{0,+}$, X^{-}_{AG}, ASC, respectively, neutral and cationic amino acid transporter, aspartate/glutamate anionic amino acid transporter, and alanine/serine/cysteine carrier system, as well as basal amino acid transporters: L, y^{+}L, ASC, respectively, leucine and large hydrophobic neutral amino acid carrier system, cationic amino acid transporter and alanine/serine/cysteine carrier system [104,105]. Characterization of the apical and basal thiol-disulfide redox control in polarized Caco-2 epithelial cells revealed that the regulation of E_h for Cys/CySS at the basal and apical surfaces occurred at different rates [105]. Moreover, the regulation of the extracellular Cys/CySS redox status on the basal surface was mediated by the y^{+}L and x^{-}_{c} system [105]. What this means is that basal surface proteins, such as receptors or transporters could exhibit greater redox sensitivity than proteins situated at the apical surface and that redox signaling events at these opposite polar membrane surfaces would be independently controlled.

Previous studies have shown that the redox couples of Cys/CySS and GSH/GSSG in the plasma are considerably displaced from equilibrium and their E_h are tightly regulated to the values of −80mV and −140mV, respectively [19,53]. Cys in plasma predominantly exists as CySS which undergoes thiol-disulfide exchange with GSH released from the liver, a process that is important for maintaining the extracellular/plasma E_h for the Cys/CySS redox couple [106]. γ-glutamyl transferase- and dipeptidase- catalyzed hydrolysis of CySSG [107] and intestinal methionine uptake from the arterial blood [108] have been described as mechanisms that contribute to the homeostasis of plasma and intestinal Cys/CySS redox status (Figure 3).

Intestinal Trx redox system—The presence of a full complement of Trx proteins suggests their function in antioxidant defense and redox regulation in the intestinal tract [84]. A notable role for mucosal Trx is in gut immune response. Trx expression is particularly high in the intestinal mucosa [109], and is reportedly involved in redox regulation of human β-defensin 1 (hBD-1) which possesses antimicrobial activity [110]. Trx-catalyzed reduction of disulfide bridges in hBD-1 enhances hBD-1 killing activity against commensal bacteria and opportunistic fungi, consistent with Trx function in innate immunity [110]. Unstimulated lamina propria T lymphocytes (LP-T) similarly exhibit high expression of Trx which contributed to the regulation of intracellular redox homeostasis in these cells [111]. In stimulated LP-T cells, constitutive as well as inducible Trx function as amplifiers of the inflammatory response by promoting and sustaining T-cell cytokine production [111]. Potential Trx/TrxSS involvement in the etiology of intestinal disorders is a relevant topic in the redox pathobiology of the intestine that warrants further study.

Luminal commensal bacteria and intestinal redox state

A vast consortium of bacteria comprising 500–1,000 species and an estimated 10^{8} to $10^{11–12}$ bacteria per ml of luminal content in the distal small intestine and the colon, forms a complex luminal microenvironment within the human intestinal tract [112,113]. The microflora of facultative bacteria (*Enterobacteriacae, Enteroccocus, and Streptococcus)* expands during infancy and creates a reducing environment that supports population of the gut by anaerobic strains (*Bifidobacterium, Bacteroides,* and *Clostridium*). By year 1, the gut microflora assumes the adult pattern of 100–1000 times greater anaerobes to aerobes [114,115]. The microbiota prevents colonization by exogenous pathogens, provides nutrients for the intestinal cells from undigested food [113] or regulates the mucosal immune system [116]. A “dysbiotic” flora characterized by an abundance of pathogenic species leads to aberrant mucosal immune responses that contribute to chronic inflammatory intestinal

disorders [116]. The diverse microbiota metabolic profiles under various nutrient conditions directly affect functions of the host intestinal tract such as lipid, carbohydrate, and amino acid metabolism [117] at different anatomical locations [113]. For instance, elevated threonine, a mucin constituent is cytoprotective against gastric acid injury in the duodenum while elevated phosphocholines, GSH, taurine and betaine support bile acid and lipid metabolism in the ileum [118]. Jejunal GSH levels of germ-free mice inoculated with human infant microbiota, were similar to those of conventional mice despite decreased constituent amino acid levels, suggesting that microbiota can modulate jejunal GSH synthesis [118].

The relationship between bacteria-host interaction and intestinal redox biology is not completely understood and is of considerable research interest. Much attention is focused on hydrogen sulfide (H_2S), a product of methionine and cysteine transsulfuration [119]. While bacteria production of H_2S are in millimolar range [119], H_2S is largely catabolized within the lumen [120,121]. H_2S at high concentrations inhibits mitochondrial cytochrome oxidase [122], a process that would trigger ROS production and intracellular redox changes. However, in HT-29 cells, mitochondrial sulfide quinone reductase (SQR) catalyzed the oxidation of H_2S to sulfide, a substrate of similar effectiveness as succinate in stimulating mitochondrial respiratory rate [123]. Moreover, unique to colonic cells, reverse electron flow between SQR and complex I yields NADH [124]. Thus, the removal of H_2S/sulfide is a priority for colonic cells to prevent mitochondrial inhibition. As yet to be demonstrated in intestinal cells, H_2S was shown to scavenge ROS in neuronal cells [125], promote intraneuronal GSH production through stimulation of cystine/cysteine transport, and protect against H_2O_2-induced oxidative stress via redistribution of newly synthesized GSH to the mitochondria [126]. The question of how the microbiota controls intestinal redox signaling is unresolved; emerging evidence implicates a role for bacteria-induced mucosal ROS generation (see section on NF-κB signaling).

Redox modulation of intestinal cell fate: proliferation, differentiation, and apoptosis

Cellular redox potential and normal phenotypic cell transition—The intestinal epithelium turns over continuously and the canonical Wnt/β-catenin and Notch signaling pathways have been identified as major determinants of intestinal stem cell commitment to one of the four specialized intestinal epithelial cell types (for review [4,127]). Major functions of the canonical Wnt/β-catenin pathway include maintenance of crypt stem/progenitors proliferation via cell cycle control and inhibition of differentiation, migration of epithelial cells along the crypt-villus axis, and development of secretory lineage and terminal differentiation of Paneth cells in the crypt. Thus, an active Wnt pathway is consistent with intestinal stem cell proliferation, a pre-requisite for cell differentiation and migration toward the villus tip. Furthermore, the Notch signaling cascade controls cell fate evolution to absorptive or secretory lineages. In addition, cell proliferation and the determination of cell fate are also controlled by bone morphogenic protein (BMP) and PI3K/Akt signaling [128,129]. The mechanism of how these signaling pathways interact to maintain intestinal homeostasis and the implication of dysfunctional signaling for intestinal disorders is not completely understood. Recent evidence suggest that ROS and redox mechanisms could coordinate the interplay of Wnt and Notch signaling in controlling the balance between goblet and absorptive cell phenotypes and the proliferative responses in the colon [130]. Direct relationships among NADPH oxidase 1 (NOX-1)-dependent ROS generation, differential activation of Wnt/β-catenin or Notch signaling pathways, and intestinal proliferation and lineage commitment were demonstrated [130].

Quantitative changes in GSH/GSSG and Cys/CySS redox potentials have been correlated with normal intestinal phenotypic cell transitions; a reducing redox environment favors proliferation, whereas an oxidized milieu favors growth arrest and differentiation. A highly

oxidized redox environment associates with cells undergoing apoptosis or necrosis. Specifically, E_h for GSH/GSSG varies from −260mV to −240mV at proliferation, switches towards more oxidizing conditions (−220mV to −200mV) at differentiation and growth arrest, and is typically −170mV or more oxidized (−150mV) at apoptosis or necrosis (Figure 4A) [131,132]. Similar changes in E_h of extracellular Cys/CySS redox couple from −80mV to −50mV are associated with the transition of cells from proliferation to a growth-arrested, non-dividing or differentiated state [99] (Figure 4A). In contrast, intestinal cell transition is associated with little or no change in the E_h of the Trx/TrxS redox couple [133]. While the progression of Caco-2 cells from a proliferative to a differentiated state was accompanied by 40mV and 28mV oxidation of intracellular GSH/GSSG and extracellular Cys/CySS redox status, respectively, the E_h of the Trx/TrxSS redox system remained essentially unchanged [133], at a constant reduced potential of −300mV (Figure 4A) [18,21,133].

Our laboratory has established that the cellular GSH/GSSG redox status governs cell transitions from quiescence to that of a proliferative, growth arrested, differentiated or apoptotic states in intestinal and other cell types [131,134] (Figure 4B). Fully differentiated epithelial cells are typically arrested in the quiescent state, but mitotic-competent enterocytes maintain a genetic program that signals proliferation in response to an altered redox stimulus. For instance, an increase in GSH oxidation or lower GSH/GSSG ratio allows entry into cell cycle likely through overcoming regulatory checkpoints (G0/G1 transition) and mitotic block; depending on the severity of the redox shift, proliferation, growth arrest, differentiation, or apoptosis may predominate (Figure 4B). Similarly, cell transitions are modulated by the extracellular Cys/CySS redox status [99,131,134,135]. Culturing Caco-2 cells in media with variable E_h for Cys/CySS (0 to −150mV), elicited proliferation rates (measured by DNA synthesis) that were 2-fold higher in reduced (−150mV) as compared to oxidized conditions (0mV) [99]. The interaction between the GSH/GSSG and Cys/CySS redox systems in the redox control of the intestinal cell's phenotypic fate is poorly understood; importantly, these two redox systems are displaced from equilibrium and each functions independently in redox regulation [99,136,137]. Interestingly, we demonstrated that exogenous cysteine promoted CaCo-2 cell proliferation only under conditions of GSH depletion, but not GSH repletion, yet the proliferative response is independent of intracellular GSH concentration or of cysteine-mediated *de novo* GSH synthesis [136]. Moreover, oxidation of the extracellular Cys/CySS pool, without a change in the cellular GSH/GSSG redox status, was sufficient to elicit CaCo-2 proliferation via redox activation of growth receptors at the plasma membrane [99]. Although the E_h of Trx/TrxSS remained essentially unchanged through cell proliferation and differentiation [133] (Figure 4A), oxidation of Trx1 (by ROS/RNS) resulted in activation of MAP kinase signaling and colonic cell apoptosis [138].

Manipulation of intracellular and extracellular redox environment induces intestinal cell transition—An imposed shift in the redox environment, such as occurs during oxidative stress, can induce the exit of cells from normal quiescence into cell cycle or cell apoptosis [131,134]. In early studies, we validated the notion that for a given oxidant, such as lipid hydroperoxide (LOOH), a different phenotypic outcome can result from titrating the oxidant (LOOH) load and the associated extent of GSH/GSSG redox disruption [139]. At low levels of LOOH (1–5 μM) an early loss of GSH-to-GSSG redox balance triggered a proliferative response in Caco-2 cells as evidenced by increases in ornithine decarboxylase activity, DNA synthesis, and expression of cyclin D1/cyclin-dependent kinase 4, consistent with transition from G_0/G_1 to S phase [139]. Higher LOOH doses (10–50 μM) resulted in severe GSH redox imbalance that mediated Caco-2 cell apoptosis as evidenced by mitochondrial dysfunction, caspase 3 activation, and DNA fragmentation [140]. Interestingly, induction of sustained GSH/GSSG redox imbalance (24h) induced Caco-2 growth arrest, specifically the blockade of cells at the G_1-to-S transition and G_2/M

phases of the cell cycle [139,141]. Parallel studies in rat intestine *in vivo* supported our hypothesis that intestinal epithelial cell transition is modulated by mucosal GSH/GSSG redox status. The proliferative and apoptotic phases of intestinal epithelial cells follow a circadian rhythm under physiological conditions that was correlated with the feeding and post-prandial periods [142,143] wherein feeding stimulated ornithine decarboxylase activity and epithelial cell growth [142] while apoptosis was initiated post-prandially upon cessation of feeding [143]. Chronic administration of a peroxidized lipid diet for 2–8 weeks significantly disrupted mucosal GSH-to-GSSG redox status, and induced a cytostatic state in the intestine, indicating that impairment of normal epithelial turnover occurred in conjunction with the loss of basal mucosal GSH/GSSG balance [144,145]. LOOH-mediated disruption of normal intestinal turnover activity was abrogated by restoration of mucosal GSH/GSSG redox status through exogenous GSH supplementation [145]. Collectively, our studies underscore the centrality of mucosal GSH/GSSG redox status in intestinal cell responses; the final phenotypic outcome is a function of the magnitude and the duration of GSH redox disruption.

The addition of exogenous growth factors like insulin-like growth factor-I, epidermal growth factor (EGF), or the amino acid glutamine contributed to a more reduced extracellular Cys/CySS E_h which stimulated CaCo-2 cell growth [99,135]. This finding suggests that changes in extracellular redox environment induced by the Cys/CySS redox couple was connected with activation of redox signaling cascade at the plasma membrane [99,135]. Indeed, the most reduced E_h for Cys/CySS couple (−150mV) has been shown to increase EGFR phosphorylation which triggered p44/42 MAPK signaling and cell growth [146]. We similarly demonstrated a potentiation of ornithine decarboxylase activity by EGF administration in rat small intestine and colon previously challenged by chronic lipid hydroperoxide exposure [99,135]. The suggestion that mild oxidative stress, through modulating the extracellular Cys/Cyss redox state, could prime the intestine for EGF-induced EGFR phosphorylation and hyperproliferation is an intriguing notion that requires experimental validation.

Under certain conditions, such as massive small bowel resection, a more oxidized colonic GSH/GSSG and Cys/CySS redox status has been shown to promote mucosal growth in rat colon [147]. Moreover, BSO-induced decreases in colonic GSH and Cys significantly stimulated colonic mucosal growth as evidenced by crypt depth, cell/crypt and increased DNA synthesis without an effect on cell apoptosis [147]. Despite massive depletion of colonic tissue GSH and Cys and an oxidized E_h of plasma GSH/GSSG, these rats exhibited increased plasma Cys and CySS levels, suggesting that elevated plasma Cys/CySS redox status may be responsible for the stimulation of mucosal proliferation [147]. This finding raises the interesting, heretofore untested notion that the initiation of redox signaling of proliferative cascades originates from the basal polar of the intestinal epithelium, possibly by altering the plasma Cys/CySS redox status (see Figure 3). Subsequent studies revealed that local GSH redox status did not influence the adaptive growth of the remnant intestine following small bowel resection [148], and that the stimulation of ileal mucosal growth in rats supplemented with dietary sulfur amino acid was through reduction of the mucosal thiol/disulfide redox state [149].

Intestinal oxidative challenge and role of GSH

The intestinal epithelial barrier is constantly challenged by diet- and endogenous-derived oxidants that can accumulate and disrupt mucosal redox control with deleterious consequences. Examples of luminal oxidants include products of lipid peroxidation and organic hydroperoxides [150], toxic metals like aluminium [151], and redox cycling compounds like menadione [152]. Enterocytes are replete with GSH- and Trx- dependent systems in all segments of the intestinal tract that function to preserve cellular redox

homeostasis and intestinal cell integrity [84,93]. Using the conscious, lymph and bile fistula rat model, we demonstrated that mucosal GSH is crucial in the intestinal elimination of luminal peroxidized lipids and the prevention of lymphatic peroxide transport [55,56]. Enhanced accumulation of lipid peroxides [56] in enterocytes and in intestinal lymph was associated with decreased mucosal GSH concentrations, consistent with attenuated Gpx-catalyzed peroxide catabolism [56,131]. Elevated luminal GSH, either through exogenous GSH supplementation or from biliary GSH output, promoted luminal lipid peroxide uptake and reduced lymphatic peroxide transport, the result of increased intracellular GSH-dependent peroxide metabolism [56,131]. Studies in freshly isolated rat enterocytes further revealed that intestinal metabolism of organic hydroperoxides subscribes to regulation by glucose availability for the production of NADPH that is pivotal for GR-catalyzed reduction of GSSG [153]. Significantly, NADPH generation from glucose flux through the pentose phosphate pathway was rate limiting in hydroperoxide catabolism, consistent with reductant supply as a major determinant of peroxide detoxication. Interestingly, the activities of enterocyte Gpx and GR did not appear to be limiting factors under these conditions [153] and in other pathophysiological conditions of diabetes [153], chronic hypoxia [153], and dietary peroxide intake [153]. Collectively, these findings underscore the centrality of GSH availability and/or the capacity for its regeneration, i.e., NADPH production, in intestinal hydroperoxide detoxication.

In colonic CaCo-2 or HT-29 cell lines, significant loss of cellular GSH either through oxidative or non-oxidative processes was associated with colonic cell apoptosis, and mitochondrial GSH (mtGSH) is a major player in colonic cell survival. A consistent observation was that an early spike in GSSG after peroxide (*tert*-butyl hydroperoxide, *t*BH) treatment was linked to the activation of the mitochondrial apoptotic cascade [154,155]. Importantly, apoptotic initiation occurred within a narrow window of GSH/GSSG redox shift (15–30 min post-oxidant exposure) that preceded cell death at 24h; subsequent restoration of GSH redox homeostasis did not prevent the apoptotic outcome [140,155,156]. The observation that an early loss of GSH/GSSG redox balance can trigger apoptotic signaling also characterizes *t*BH-induced apoptosis in other cell types, such as neuronal-like PC12 cells [157,158], which suggests that GSH oxidation is likely a common early event in oxidative stress mediated apoptosis in different cell types. Interestingly, induction of GSH loss by non-oxidants, such as staurosporine (STP), a broad spectrum inhibitor of protein kinases [156] can elicit colonic HT-29 cell apoptosis [156]. STP-induced GSH efflux and cell apoptosis was linked to caspase 3 activation, but independently of caspase 8 or 9 involvement [156], thus suggesting participation of a non classical pathway of apoptosis. Moreover, STP-induced GSH export was driven by γ-glutamyl transferase-mediated extracellular GSH hydrolysis without changes in the cellular GSH/GSSG ratio [156]. Precisely how GSH exit is coupled to the initiation of apoptotic signaling at the plasma membrane is an unresolved question that needs to be addressed.

Mitochondrial GSH control and intestinal integrity—Mitochondria involvement in oxidant-mediated cell apoptosis is well established [24,159], and early studies demonstrated that mitochondrial GSH (mtGSH) depletion sensitizes cells to oxidant-induced cell injury [160]. Mechanistically, the loss of mtGSH causes mitochondrial transition pore opening [161], inhibition of respiratory complexes [162], decreased ATP [155], and increased ROS production [163], that collectively lead to cell apoptosis [155,164]. Matrix GSH homeostasis is acquired through active GSH transport from the cytosolic compartment [31]. The DIC and OGC carriers are the main contributors [31,162,165], but a tricarboxylate carrier also accounted for mtGSH uptake in brain mitochondria [166]. Mitochondrial membrane dynamic plays an important role in the proper function of OGC and DIC carriers [165]; factors that impair membrane fluidity (e.g. cholesterol deposition, ceramide production, changes in phospholipid content) decreased membranal transport and induced mtGSH

depletion [167,168]. This mechanism of selective mtGSH loss was implicated in acetaminophen- and TNF-α-induced apoptosis in hepatocytes [169,170] and amyloid β peptide-induced neuronal inflammation and toxicity [171]. In contrast, overexpression of DIC or OGC transporters, such as in rat renal proximal tubular NRK-52E cells, protected against *tert*-butylhydroperoxide- or S-(1,2-dichlorevinyl)-L-cysteine-induced cell apoptosis [172,173], thus underscoring the role of mtGSH in preserving cell survival.

Interestingly, OGC and DIC-mediated mtGSH transport in liver and kidney mitochondria is promoted by high mitochondrial ATP and energetic status [31,174], consistent with the role of these carriers in the transport of both mtGSH and respiratory substrates. Indeed, dicarboxylates such as malate, malonate, butylmalonate and phenylsuccinate are effective inhibitors of mtGSH uptake [31,175]. A close link between the matrix status of GSH and respiratory substrates is evidenced by a parallel increase in mtGSH levels and high mitochondrial flux of intermediary metabolites in the hypermetabolic remnant kidney following nephrectomy of the hypertrophied kidney; enhanced DIC and OGC activity was suggested to be a major cause of this high mtGSH uptake [176]. Mitochondrial respiratory substrates additionally regulate mitochondrial redox status through supply of reducing equivalents for NADPH-dependent reductive processes [177]. In isolated rat brain mitochondria, substrates such as succinate, malate and/or glutamate were shown to elevate mtGSH levels and NAD(P)H-dependent reduction of mitochondrial GSSG and S-glutathiolated proteins [177]. How these mechanisms contribute to preserving redox homeostasis and integrity of an oxidant-prone mitochondrial compartment [155] in intestinal cells remains unresolved.

In recent studies, we established the importance of mtGSH in preserving intestinal mitochondrial genomic [154] and functional integrity [155] in colonic NCM460 and HT29 cells against oxidative challenge by the redox cycling quinone, menadione (MQ). Enhancement of matrix GSH levels through promoting OGC and DIC expression and cytosolic GSH synthesis prevented against MQ-induced mitochondrial dysfunction, oxidative mitochondrial DNA damage and apoptosis [154,155]. Mitochondrial demise was accentuated by blockade of mtGSH transport and inhibition of cytosolic GSH production [155]. Interestingly, specific inhibition of DIC and OGC transport *per se* resulted in an increase in baseline mtGSH, suggesting that these carriers may function in mitochondria-to-cytosol GSH efflux [155], an intriguing notion that warrant further investigation. Significantly, the loss of mitochondrial GSH/GSSG redox balance was associated with a disrupted cellular pyridine nucleotides (NAD^+/NADH, $NADP^+$/NADPH) redox status that compromised cellular ATP, mitochondrial respiratory activity, and NADPH-dependent reducing capacity in colonic HT-29 cells [178]. Such redox and metabolic disruptions were corrected in part by the glucose-sparing mitochondrial substrates, succinate and glutamate [178], which underscores the quantitative importance of glucose in reductant (NADPH) supply.

Altered tissue redox status in intestinal inflammation and associated pathology

Intestinal GSH/GSSG redox state in tissue inflammation and pathology

An altered intestinal redox status is a recognized risk factor in the development of gut pathologies. Inflammatory bowel diseases, intestinal cancers, or oxidative conditions like diabetes are often associated with attenuated intraepithelial GSH status that contributes to disease progression and exacerbation of the pathological states. Chronic inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic inflammation of the intestinal tract [179,180]. As such, excessive

ROS generation by infiltrated inflammatory cells (macrophages and neutrophils) in the inflamed gut of IBD patients leads to oxidative stress, reportedly a pivotal factor in the onset and development of chronic gut inflammation. Elevated tissue GSSG have been correlated with the severity of mucosal inflammation [181,182], and decreased GSH synthetic enzymes and lower precursor Cys levels contributed to diminished mucosal GSH concentrations in IBD patients [183]. Significantly lower levels of GSH were similarly found in inflamed ileal mucosa from patients with CD [181], and in colonic mucosa of active UC disease state [184].

The mechanism by which an impaired epithelial redox environment signals inflammatory disease initiation and/or progression is unclear. It is generally thought that intestinal oxidative stress is secondary to the inflammatory process; however, we previously demonstrated that the loss of mucosal GSH/GSSG redox balance preceded the onset of colonic inflammation and manifestations of clinical colitis in SCID mice reconstituted with $CD^{4+}CD45RB^{high}$ T-lymphocytes, an immune-based mouse model of experimental UC [185]. This finding suggests the involvement of redox dependent mechanisms in UC development. Supporting evidence comes from the finding that antioxidant therapy that counteracted ROS production and restored tissue redox homeostasis attenuated disease manifestations [185,186]. Unexpectedly, antioxidant therapy (vitamin C, vitamin E, GSH) in HLA-B27 transgenic rats, a model of IBD, was without beneficial effect despite low colonic mucosal GSH levels and high neutrophil infiltration [187]. One explanation could be that there was little evidence of tissue oxidative stress due to compromised neutrophilic respiratory burst in these mutant mice. Overall, these results support a link between oxidative stress, mucosal GSH redox status, and intestinal inflammation; however, establishment of causality between intestinal GSH/GSSG redox disruption and IBD development requires further study.

Precisely how altered redox signaling contributes to the onset of immunological response associated with chronic inflammation is not completely understood. Increasing evidence suggests that the redox milieu in the lamina propria is an important determinant of LP-T proliferation and of their responsiveness to luminal bacteria [188]. In normal gut, the hyporeactivity of LP-T against luminal flora is evidenced by their low proliferative capacity [189] due to a low intracellular GSH [190]. LP-T activation and proliferation rely on a reducing extracellular environment that is provided by antigen presenting cells [191]; resident lamina propria macrophages are unable to secrete cysteine, and are therefore unable to stimulate LP-T [192]. Furthermore, ROS-mediated NF-κB-dependent activation of an pro-inflammatory response was attenuated by a constitutively higher Trx1 redox status in LP-T cells as compared to peripheral blood T cells [111]. Oxidative damage to the epithelia caused the recruitment of cysteine-secreting blood-borne macrophages which increased LP-T GSH levels and induced LP-T proliferation [190]. The subsequent transition of T cells from a bacteria-tolerant to a reactive state creates and perpetuates a sustained inflammatory phenotype in the lamina propria [193]. Consistent with this scenario, IBD patients exhibit increased $CD14^{+}$, cysteine positive macrophages as well as $CD3^{+}$ LP-T cells with elevated intracellular GSH [190].

NF-kB signaling and intestinal inflammation

IBD is associated with activation of the NFκB pathway in intestinal mucosa [194,195] and lamina propria macrophages [196], as well as the overproduction of NF-κB-dependent pro-inflammatory cytokines [196,197]. In quiescent cells, members of the NF-κB family (RELA, RELB, REL, p50 and p52) exist as inactive dimers in association with the inhibitor of NF-κB protein (IκB). Inflammatory mediator induced NF-κB activation is achieved through IκB phosphorylation by the IκB kinase (IKK) complex (consists of IKKα, IKKβ catalytic subunits and NEMO regulatory subunit), dissociation of NF-κB-IκB interaction, nuclear

translocation of NF-κB, and transcription of proinflammatory and/or anti-apoptotic genes. The relationship between NF-κB signaling and intestinal inflammation is highly complex and not fully resolved; the reader is referred to several excellent recent reviews on the subject [196–198]. As an example, while the inhibition of IKKβ-dependent NF-κB activation attenuated chronic inflammation, acute inflammation was enhanced [199], suggesting that duration of signaling is likely a pivotal factor in disease outcome. Other interesting observations support an opposing notion to a generally accepted paradigm for a pro-inflammatory function of NF-κB. For instance, NF-κB dysregulation in myeloid cells mediates inflammatory cell influx into the intestinal epithelia and elicits a pro-inflammatory response [199]. Mice deficient in intestinal epithelial cell (IEC) NEMO protein ($NEMO^{IEC\text{-}KO}$) were characterized by excessive epithelial cell apoptosis, despite presenting with widespread colonic inflammation, innate immune cell infiltrate and spontaneous colitis early after birth [200]. Thus it appears that normal NF-κB signaling in these animals would be toward upregulation of anti-apoptotic genes and epithelial survival.

The redox mechanisms of NF-κB activation in intestinal epithelium are poorly characterized. ROS mediated oxidation or S-glutathiolation of redox-sensitive cysteines of NF-κB subunits can activate or inhibit signaling (see reviews [201,202]). Moreover, NF-κB-nuclear DNA binding depends on the redox status of Cys-62 of the p50 subunit; oxidation of this cysteine significantly decreased transcription of NF-κB-dependent genes [203] while its reduction by nuclear Trx1/Ref-1 system restored NF-κB binding affinity [204,205].

Emerging evidence implicates a role for ROS in the cross-talk between the microbiota and NF-κB redox signaling in intestinal epithelia [206]. Mitochondria-derived ROS caused by butyrate, a bacterial fermentation product of polysaccharides, was shown to mediate transient alteration of the redox status of cytosolic and mitochondrial Trx that promoted NF-κB inhibition [207]. Similarly, bacteria-intestinal epithelial cell co-culture resulted in oxidation of intestinal Trx and GSH by NADPH oxidase-derived ROS that modulated ubiquitin-like conjugating enzymes (cullin-1 and Ubc12) and NF-κB inactivation [206]. Specifically, H_2O_2-mediated oxidation of Ubc12 redox-active cysteine prevented TNFα–induced nuclear translocation of the p65 subunit and blocked the inflammatory response that was reversed by NAC treatment [206]. This means that commensal bacteria modulation of the ubiquitin-proteasome system through mucosal ROS could influence the epithelial outcome of NF-κB signaling. Furthermore, bacteria-induced intestinal ROS generation was shown to inactivate low molecular weight protein tyrosine phospatase (LMW-PTP) and SHP-2 and promoted epithelial restitution and recovery from dextran sulfate-mediated injury in mice [208]. The *Lactobacillus* strain exhibits a higher capacity for ROS generation, and their luminal abundance can be deleterious for the epithelial layer due to altered NF-κB protective function, and exacerbation of inflammatory processes secondary to invasion of luminal bacteria into the mucosa. Microbiota composition can alter tissue susceptibility to intestinal pathogens. For instance, an enriched Bacteroides microbiota from C57BL/6 resistant mice protected against *Citrobacter rodentium*-induced colitis in C3H/HeOuJ susceptible mice through GSH/GSSG-mediated changes in inflammatory cytokines and systemic pathogen load [209]. It is unclear what caused the change in epithelial GSH redox, but an oxidative stress response was important for microbiota to modulate inflammation-associated subset of genes that enhanced pathogen clearance. It is notable that greater pathogenic bacteria strains comprised the intestinal microbiota of IBD patients [210]; in the TNBS-induced injury rat model of colitis, the administration of probiotic bacteria can significantly enhance epithelial GSH and attenuate mucosal inflammation [211,212]. Loss of commensal bacteria induces epithelial apoptosis that was exacerbated by oxidative stress (ischemia/reperfusion) mediated by NF-κB-dependent transcription of pro-inflammatory cytokines [213]. The collective evidence underscore a role for intestinal GSH/GSSG and

redox signaling through NF-κB in preserving the fragile balance between the luminal microenvironment and epithelial survival.

The delay in colitis onset in IL-10-deficient mice due to loss of IKKβ in macrophages/neutrophils but not in epithelial cells suggests that infiltrated immune cells mediate chronic inflammation [199], sustained by NF-κB-dependent inflammatory cytokine production [214]. Binding of the bacterial endotoxin, lipopolysaccharide (LPS) to toll-like receptors on immune cell surface triggers NADPH oxidase-catalyzed ROS generation and NF-κB-dependent pro-inflammatory cytokine production, indicating that redox signaling also governs NF-κB activation of immune cells. LPS-induced intestinal inflammation was exacerbated in Srx1 [215], Prx3 [216], or Prx2 [217]-deficient mice and those whose macrophages that are deficient in nuclear factor-E2-related factor 2 (Nrf2), master regulator of antioxidant responsive genes [218]. These results suggest that excessive immune cell generation of ROS and inflammatory cytokines contribute to mucosal damage [219], and that NF-κB redox signaling is central to immune cell-mediated intestinal inflammation.

Mucosal redox status and other inflammation associated intestinal disorders

Chronic gut inflammation increases cancer incidence, and cancer development is attributed to a persistent state of oxidative stress and diminished ROS elimination. Low GSH content and increased levels of 4-hydroxy-2-nonenal, malonyldialdehyde, and 8-hydroxy-2′-deoxy guanosine are associated with tissue malignancy [220–222]. The modulation of GSH- and/or Trx-dependent enzyme levels and activities was reported to enhance cancer cell proliferation, migration and metastasis while evading apoptosis [222,223], underscoring a role for redox involvement. Compelling evidence indicates that altered states of redox couples and/or redox-dependent systems are common in cancers of the intestinal tract although contribution of individual/combined redox systems at cancer stages has not been defined. Up-regulation of Se-insensitive Gpx2 has been correlated with increased cancer cell proliferation and rapid growth of intestinal tumors [86]. Interestingly, increases in Gpx2 protein occurred only in the early and not the advanced stages of human colorectal cancer, suggesting differential functional roles for Gpx2 during cancer progression [224]. Aberrant and reduced expression of proteins that are sensitive to Se deprivation, such as Gpx1, Gpx3, and selenoprotein P (SePP) in colon cancers is consistent with the sensitivity of Se-proteins to dietary Se and the susceptibility of the colonic tissue to oxidative stress [225].

Genetically engineered mice deficient in Gpx genes have provided useful experimental models to evaluate the role of these Se-proteins in the pathogenesis of intestinal inflammation, colitis and cancer. Homozygous mice lacking the wild-type (WT) *Gpx1* or *Gpx2* genes exhibit no overt phenotype for pathological symptoms in the gastrointestinal tract [226]. However, mice that express one wild-type allele of *Gpx1* ($Gpx1^{+/-}\,Gpx2^{-/-}$) presented with low incidence of IBD symptoms that were exacerbated by Se-deficiency [88]. Regardless of Se content, $Gpx1^{-/-}\,Gpx2^{+/-}$ mice seldom exhibit IBD pathology, indicating that Gpx2 afforded protection of the intestinal mucosa against inflammatory reactions [226]. Interestingly, the Gpx2 null mouse exhibited a compensatory increase in Gpx1 throughout the intestine, predominantly in colon and ileum which may explain why Gpx2 deficient animals do not develop IBD pathologies [227]. When Gpx2 deficient mice were fed Se-deficient diet, increased apoptosis was observed in colonic crypts [227], a region that normally supports cell proliferation [86] (see Figure 1) and expresses high levels of Gpx2 [224]. The double knockout mice (Gpx1/2-DKO) develop early ileocolitis and severe inflammation in the distal ileum at 6–9 month; ~25% of these mice exhibit microflora-associated cancer in the ileum and colon [226,228]. Taken together, these studies highlight a physiological role for Se-Gpx proteins in intestinal integrity and are therefore feasible target candidates in anti-cancer therapy.

The over-expression of Grx and Trx is a characteristic feature of colon cancer [229], and elevated Trx1 in primary colorectal cancer cells was associated with aggressive tumor growth and poor prognosis for survival [230]. In colon carcinoma, the expression of Grx3, an intestinal epithelial specific Grx isoform, was increased ~50-fold with no alteration in the other Grx proteins levels [231], consistent with Grx3 function in tumor growth and survival [232]. Indeed, the knock down of Grx3 in breast cancer cells promoted cellular ROS production and GSH oxidation; the resultant oxidized redox environment destabilized p65 and relB subunits and attenuated NF-κB survival signaling that inhibited tumor growth and migration [233]. Thus, targeting NF-κB signaling, a major promoter of tumorigenesis, tumor growth and metastasis as well as Grx3 expression in Grx3 overexpressing colon cancer cells, [233] would be a viable strategic approach in colon cancer therapy.

Specific intestinal pathologies that are directly attributed to the oxidative condition of the diabetic state have yet to be defined. However, diabetes associated hyperglycemia, oxidative and carbonyl stress can augment the susceptibility of the gastrointestinal system to oxidant- or ischemia/reperfusion-induced damage [234,235]. Significantly, diabetic rats exhibit impaired antioxidant defense capacity, evidenced by lower mucosal SOD activity and depressed GSH levels [234,236]. In accordance with an increased capacity to scavenge ROS, the administration of SOD or GSH was able to reverse oxidant-mediated gastrointestinal injury [234] while GSH depletion via starvation induced gastric lesions in streptozotocin-treated diabetic rats [237]. Importantly, the intestinal capacity for ROS elimination are majorly influenced by factors that modulate mucosal GSH regeneration, including glucose availability, cysteine supply, and NADPH production [237], rather than the activities of “core” redox enzymes such as Gpx, GR, and glucose-6-phosphate Surprisingly, glycemic control by short-term insulin treatment decreased mucosal GSH and suppressed activities of these core enzymes which were reversed by 7-day insulin treatment [235], suggesting a heightened vulnerability of the diabetic intestine to acute glycemic fluctuation. Significantly, the capacity of the diabetic intestine for peroxide (oxidant) detoxication is directly impacted by the GSH redox cycle function, regulated by cysteine supply for GSH synthesis and NADPH supply for GSH regeneration.

Concluding remarks

Intracellular and extracellular thiol-disulfide redox homeostasis is central to intestinal function and integrity. Maintenance of the intestinal epithelial redox environment is essential for the activities of key physiological processes that include digestion and absorption, cell proliferation and apoptosis, and immune response. Much is known of the GSH/GSSG and Cys/CySS redox systems at the apical epithelial surface from studies in animal models and *in vitro* intestinal cell culture systems. These two redox couples ensure a redox environment that supports the gut microflora, facilitates nutrient absorption, counteracts oxidant-induced epithelial injury, and regulates intestinal cell transformation and apoptosis. The fine tuning of the extracellular redox environment is also crucial in the intestinal stem cell niche that signals intestinal cell genesis. However, little is known of the redox mechanisms governing extracellular/membrane signaling events that mediate intracellular immunological responses that control pathogen-host interactions or inflammation and the contribution of the microbiota to these processes. Within intestinal cells, Trx/TrxSS and GSH/GSSG redox status as well as Trx- and GSH-dependent enzyme systems preserve a redox environment that supports redox signaling and regulation of cell metabolism, orchestrates antioxidant defense and initiates cell death-associated processes during oxidative stress. The distinctness of subcellular compartmentation of individual redox systems provides a unique level of independence and elegance in specific redox control of biological processes. Indeed, subcellular redox compartmentation could be a generalized underlying determinant of cell function. In the past decade, there is growing interest in redox

involvement in the pathobiology of the intestine. Given the conceptual advances that shaped our current understanding of cellular redox regulation and signaling, the challenge for future research is to define the mechanistic relationship between disrupted regulation and communication of compartmental redox systems and luminal microbiota with altered intestinal processes such as enhanced inflammatory responses or aberrant cell proliferation, processes that fundamentally underlie intestinal disorders like cancer or IBD.

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Abbreviations

γ-GT	γ-glutamyl transferase
Akt	serine/threonine protein kinase Akt
BSO	L-buthionine-S,R-sulfoximine
CD	Crohn's disease
Cys	cysteine
CySS	cystine
CyS-SG	cysteine-glutathione disulfide
DIC	dicarboxylate transporter
DP	dipeptidase
ER	endoplasmic reticulum
FAD	flavin adenine nucleotide
FAE	follicle-associated epithelium
GALT	gut-associated lymphoid tissue
GCL	glutamate-cysteine ligase
Gpx	glutathione peroxidase
GR	glutathione reductase
Grx	glutaredoxin
GS	glutathione synthase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferases
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
hBD-1	human β-defensin 1
IBD	inflammatory bowel diseases
IκB	inhibitor of NF-κB
IKK	IκB kinase

IMS	mitochondrial intermembrane space
ISC	intestinal crypt stem cells
LP-T	lamina propria T lymphocytes
LMW-PTP	low molecular weight protein tyrosine phosphatase
Met	methionine, MQ, menadione
mtGSH	mitochondrial GSH
NF-κB	nuclear factor-κB
NOX-1	NADPH oxidase 1
NNT	nicotinamide nucleotide transhydrogenase
OGC	2-oxoglutarate transporter
PPs	Payer's patches
PPP	pentose phosphate pathway
PI3K	phosphatidylinositol-3′-kinase
Pr-SSG	protein disulfides
Prx	peroxiredoxin
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCFA	short chain fatty acids
SQR	sulfide quinone reductase
STP	staurosporine
***t*BH**	*tert*-butyl hydroperoxide
Trx	reduced thioredoxin
TrxR	Trx reductase
TrxSS	oxidized thioredoxin
TNBS	trinitrobenzensulfonic acid
UC	ulcerative colitis

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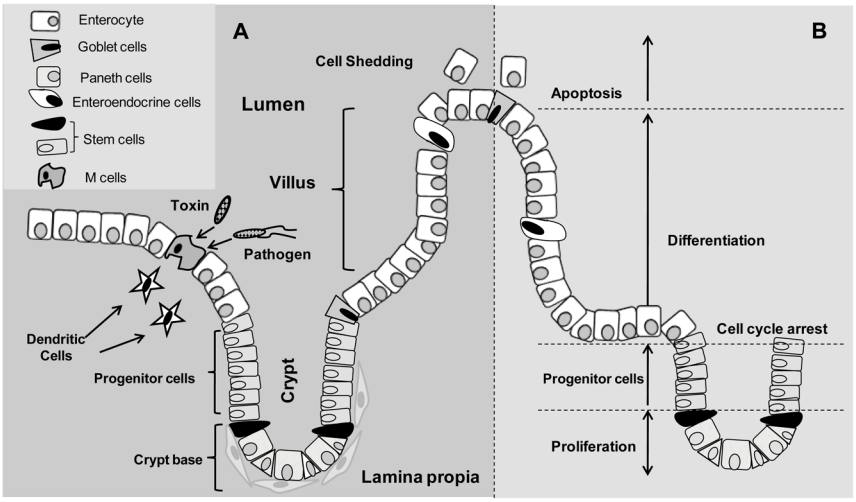

Figure 1. Organization of the small intestinal epithelium

A. The intestinal epithelium, consisting of a single layer of cells, is extensively folded that results in distinct crypt and villus regions. Four types of cells including enterocytes, Paneth, goblet, and enteroendocrine cells reside in the epithelium and are involved in digestive and immunological functions of the intestine. Particulate antigens (pathogens and toxins) are sampled by the M cells present in the gut-associated lymphoid tissues at the luminal site and presented to dendritic cells or other antigen-presenting cells at their basolateral surface. At the basolateral membrane, cells of the intestinal epithelium associate with and are supported by lamina propria. **B**. The intestinal epithelium is a highly proliferative tissue. Cell proliferation originates at the base of the crypt where intestinal stem cells reside. Progenitors of the stem cells proliferate and migrate bi-directionally. The precursors that migrate toward the tip of the villus differentiate into one of the following cell types: enterocyte, goblet cell, or enteroendocrine cell; the progenitors that migrate toward the bottom of the crypt differentiate into Paneth cells. At 4–5 days post differentiation, villus tip cells die by apoptosis are shedded into the lumen while Paneth cells remain in the crypt for about 23 days and thereafter are phagocytozed.

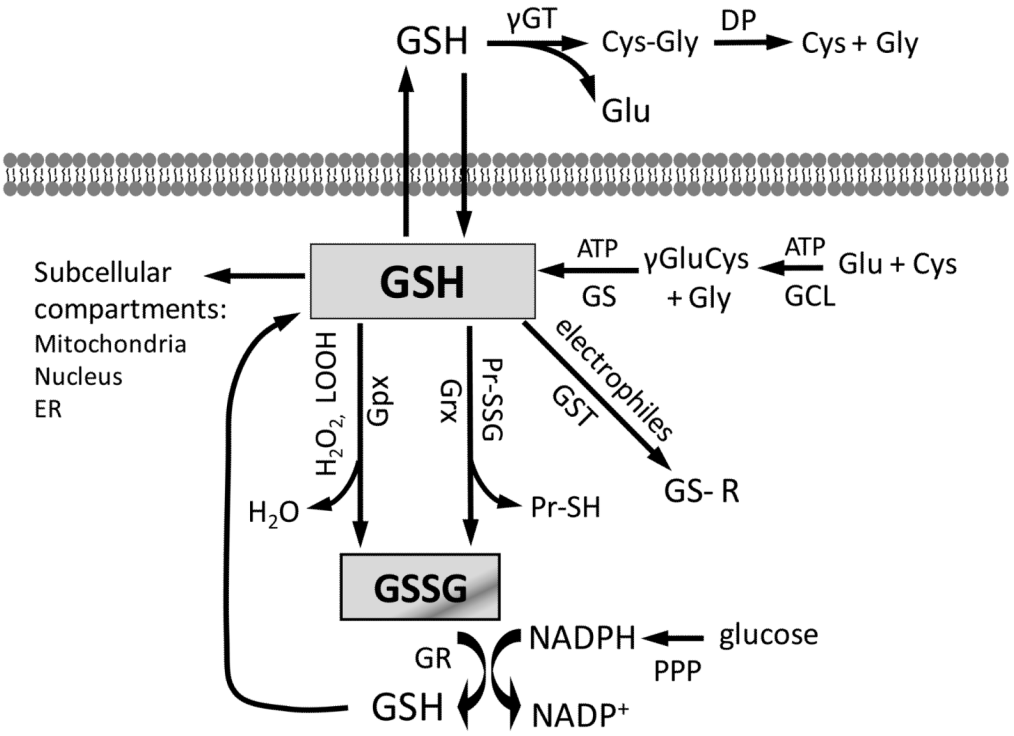

Figure 2. Cellular GSH homeostasis and GSH-dependent reactions

Intracellular GSH balance is maintained by *de novo* synthesis, regeneration from GSSG, and extracellular GSH uptake. In transport epithelial cells, such as enterocytes, γ-glutamyl transferase (γ-GT) and dipeptidase (DP) catalyzed the hydrolysis of extracellular GSH to its constituent amino acids, glutamate, cysteine and glycine. Additionally, intestinal epithelial cells can import intact GSH from the lumen via specific plasma membrane transporters. Cytosolic synthesis of GSH takes place in two ATP-dependent reactions catalyzed by glutamate-cysteine ligase (GCL) and glutathione synthase (GS). The intracellular GSH pool, present in millimolar concentrations, is involved in various GSH-dependent reactions. Compartmentation of GSH within the mitochondria, nucleus, or endoplasmic reticulum creates distinct and independently regulated subcellular redox pools. As part of the antioxidant defense system, GSH participates in conjugation reactions catalyzed by glutathione-S-transferases (GSTs), in the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH) catalyzed by glutathione peroxidases (Gpxs), and the reduction of protein-disulfides (PrSSG) catalyzed by glutaredoxins (Grxs). The reduction of glutathione disulfide (GSSG) by glutathione reductase (GR) in the GSH redox cycle regenerates GSH. GSSG reduction occurs at the expense of NADPH, produced from the pentose phosphate pathway (PPP) from glucose oxidation.

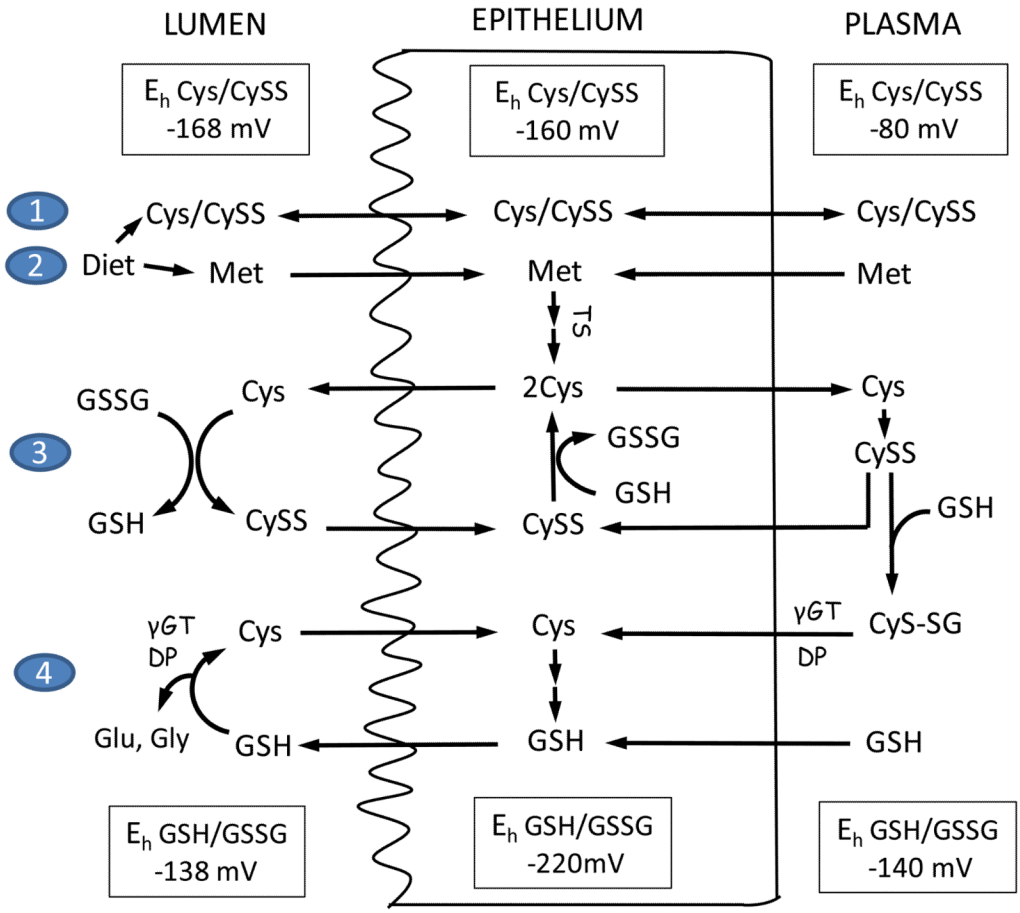

Figure 3. Homeostatic control of Cys/CySS redox status in intestinal lumen, intestinal epithelium, and plasma

At the brush-border membrane, uptake of dietary Cys and methionine (**1 & 2**); Cys/CySS shuttle (**3**), and γ-glutamyl transferase (γGT) and dipeptidase (DP)-catalyzed hydrolysis of extracellular GSH (**4**) participate in Cys homeostasis. The Cys/CySS shuttle and luminal GSH hydrolysis maintains the E_h for luminal Cys/CySS at −168mV and that of GSH/GSSG at −138mV. Within the intestinal epithelium, CySS is reduced by GSH, the resultant Cys is exported or utilized in GSH synthesis. Additionally, intracellular Cys is increased through the trans-sulfuration (TS) of imported dietary or circulatory methionine (Met). Plasma Cys exists mainly as CySS, and the redox state of plasma Cys is controlled by thiol/disulfide exchange with liver-derived GSH. The hydrolysis of Cys-GSH mixed disulfide (CyS-SG) releases Cys which is taken into enterocytes by basolateral membrane associated transporters. The E_h for Cys/CySS and GSH/GSSG redox couples are tightly regulated at values of −80mV and −140mV, respectively. Luminal values of E_h for Cys/CySS and GSH/GSSG redox couples were taken from [69] and the plasma values were from [53,106].

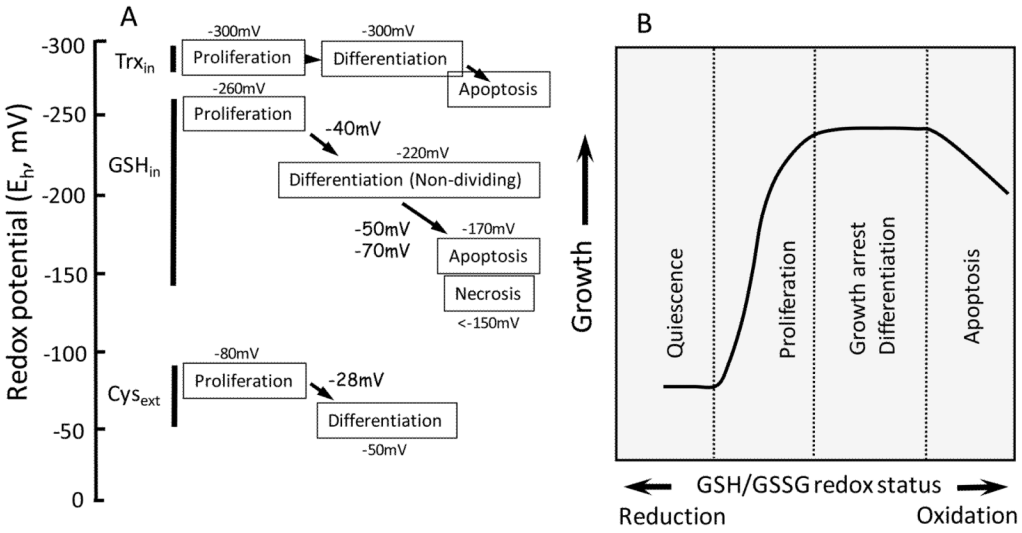

Figure 4. Redox potential (E_h) of thiol redox couples during normal phenotypic transition of intestinal cell (A) and cellular responses to changes in the cellular GSH/GSSG redox status (B)

A. Normal intestinal cell transitions from proliferation to differentiation or growth arrest, and apoptosis are associated with increased oxidation of the redox potentials (E_h) of the intracellular GSH/GSSG (GSH_{in}) or extracellular Cys/CySS (Cys_{ext}) redox couples. A 40mV oxidation (from −260mV to −220mV) in E_h for cellular GSH/GSSG is associated with cell transition from proliferation to differentiation. An additional 50mV or 70mV oxidation characterizes apoptotic or necrotic cells, respectively [18]. Similarly, an increase in 28mV oxidation of E_h for extracellular Cys/CySS (from −80mV to −50mV) accompanies intestinal cell progression from proliferation to differentiation [99]. In contrast, the E_h for intracellular Trx/TrxSS (Trx_{in}) remains unchanged at −300mV through cell proliferation and differentiation [99], but significant Trx 1 oxidation results in cell apoptosis. **B.** Fully differentiated intestinal cells typically exhibit a biological constraint to proliferate due to a mitotic block and are arrested in a quiescent state. An imposed change in the status of the cellular GSH/GSSG redox couple during oxidative challenge can initiate entry into cell cycle, differentiation/growth arrest, or apoptosis, depending on the duration and severity of the redox shift.