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# Cell-mediated reduction of human $\beta$ -defensin 1: a major role for mucosal thioredoxin

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Human  $\beta$ -defensin 1 (hBD-1) is an antimicrobial peptide expressed by epithelia and hematopoietic cells. We demonstrated recently that hBD-1 shows activity against enteric commensals and *Candida* species only after its disulfide bonds have been reduced by thioredoxin (TRX) or a reducing environment. Here we show that besides TRX, glutaredoxin (GRX) is also able to reduce hBD-1, although with far less efficacy. Moreover, living intestinal and lymphoid cells can effectively catalyze reduction of extracellular hBD-1. By chemical inhibition of the TRX system or specific knockdown of TRX, we demonstrate that cell-mediated reduction is largely dependent on TRX. Quantitative PCR in intestinal tissues of healthy controls and inflammatory bowel disease patients revealed altered expression of some, although not all, redox enzymes, especially in ulcerative colitis. Reduced hBD-1 and TRX localize to extracellular colonic mucus, suggesting that secreted or membrane-bound TRX converts hBD-1 to a potent antimicrobial peptide *in vivo*.

## INTRODUCTION

Defensins are small cationic antimicrobial peptides that protect human surfaces against infection by bacteria, fungi, and some viruses.<sup>1,2</sup> Human  $\beta$ -defensin 1 (hBD-1) is one member of the defensin family that exhibits microbicidal activity predominantly against Gram-negative bacteria.<sup>3,4</sup> It is produced constitutively by epithelial surfaces including urinary tract, kidney, pancreas, airways, skin, and intestine.<sup>5–7</sup> In addition, several hematopoietic cells like monocytes, dendritic cells<sup>8</sup> and platelets<sup>9</sup> have also been reported to express hBD-1. Different single-nucleotide polymorphisms in the hBD-1 encoding gene *DEFB1* have been associated with colonic Crohn's disease (CD), infections in cystic fibrosis, atopic dermatitis and periodontitis.<sup>10,11</sup> However, under standard laboratory conditions, hBD-1 showed only weak antimicrobial killing compared with other human antimicrobial peptides. Yet, the conditions used did not necessarily reflect the environment in the human body. For instance, the colon is heavily colonized with microorganisms, reaching densities of up to  $10^{11}$ – $10^{12}$  bacteria per g of luminal content.<sup>12</sup> The majority are anaerobic commensal bacteria, including Bifidobacteria and Lactobacilli, whose metabolism leads to a low redox potential of  $-200$  to  $-300$  mV with a very low oxygen partial pressure.<sup>12,13</sup> In consequence, intestinal

epithelial cells are adapted to a state of “physiologic hypoxia”<sup>14</sup> and a reducing environment. We recently reported that hBD-1 shows dramatically enhanced activity against enteric commensals after its three characteristic disulfide bonds have been reduced.<sup>15</sup> Specifically, antimicrobial activity against Bifidobacteria and Lactobacilli could only be detected when hBD-1 was reduced to a linearized peptide (redhBD-1) whereas the oxidized hBD-1 peptide (oxhBD-1) was inactive against these microbes. Moreover, only reduced hBD-1 exhibits activity toward several strains of the opportunistic fungus *Candida albicans*.<sup>15</sup> Enhanced antimicrobial effects of the reduced form of hBD-1 seem to be strain specific to a subset of bacteria or fungi, as reduction of disulfides in hBD-1 did not increase activity against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis*.<sup>16</sup>

Intramolecular disulfide bonds have long been viewed only as static motifs, stabilizing the structure of mature proteins. Over the past few years, it became apparent that the disulfide bonds of extracellular proteins can also be cleaved to modify protein functions.<sup>17–20</sup>

One important mediator of cleavage of disulfide bridges is the oxidoreductase thioredoxin-1 (TRX, gene name *TRX*). In addition to its localization in the cytoplasm, membrane-

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bound TRX has been demonstrated in a variety of cells,<sup>21</sup> it can be secreted<sup>22–24</sup> and hence detected at significant levels, for example, in human blood.<sup>25</sup> TRX is an enzyme engaged in antioxidative defense and is responsible for preserving intra- and extracellular redox equilibria.<sup>23,26,27</sup> Along with the low-molecular-weight antioxidant glutathione (GSH), TRX protects against reactive oxygen species (e.g., superoxide, hydrogen peroxide, or hydroxyl radicals). For example, human regulatory T cells were shown to express high levels of TRX and exhibit greater tolerance toward oxidative stress than conventional CD4<sup>+</sup> T cells.<sup>28,29</sup> In addition, TRX plays a decisive role in innate immune functioning.<sup>30,31</sup> In line with these observations, we could show that hBD-1 can be reduced by the TRX system *in vitro*, thereby increasing its antimicrobial activity.<sup>15</sup>

The dithiol-sequence motive of TRX responsible for the activation of hBD-1 is not unique to TRX. Similar redox active amino-acid sequences can be found in a variety of proteins expressed in mucosal epithelia, for example, protein disulfide isomerase 1 (PDI, gene name *PDI*), glutaredoxin-1 (GRX, gene name *GRX*), or macrophage migration inhibitory factor (MIF). Protein disulfide isomerases are a large family of proteins primarily located to the endoplasmic reticulum, where they guide proper folding of immature proteins. On cell surfaces, PDI functions in the reduction of extracellular proteins.<sup>32,33</sup> GRX is a protein with antioxidant properties that can be detected in the cytoplasm of mucosal epithelium, serum, and sputum of healthy individuals.<sup>34,35</sup> GRX is regenerated by GSH to its active dithiol form and regulates redox states of intra- and extracellular proteins.<sup>34</sup> The pro-inflammatory cytokine MIF is constitutively expressed in intraepithelial lymphocytes and epithelial cells of normal intestinal mucosa<sup>36</sup> and is involved in the response to endotoxin and Gram-negative bacteria.<sup>37</sup> Its oxidoreductase activity has been demonstrated,<sup>38</sup> but the precise role for this function *in vivo* has yet to be determined.

We have shown earlier that hBD-1 can be reduced by the TRX system in an assay of purified enzymes.<sup>15</sup> In this study we aimed to investigate if reduction of hBD-1 can also be performed by additional oxidoreductases. Furthermore, we inquired if the extracellular surface of living cells can reduce hBD-1, and if its reduction is also mediated by the TRX system. As we have shown that TRX expression is diminished in colonic inflammatory bowel disease (IBD),<sup>15</sup> we now conducted a systematic analysis of TRX mRNA expression in sigmoid colon and ileum and characterized expression levels of additional redox enzymes in healthy controls and inflamed/noninflamed tissue in IBD. Finally, we employ immunofluorescence microscopy to reveal extracellular localization of TRX and redhBD-1 *in vivo*.

## RESULTS

### Reduction of hBD-1 can be mediated by TRX and GRX

We recently identified the TRX system as an efficient activator of hBD-1 *in vitro*.<sup>15</sup> To investigate if this effect is limited to TRX, we compared different redox enzymes in their capacity to catalyze conversion of oxidized hBD-1 into the reduced

peptide. We used reversed-phase, high-performance liquid chromatography (HPLC) analysis to differentiate between the oxidized and the reduced peptide.<sup>15</sup> Comparable to our previous results, 3  $\mu$ M TRX in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase-1 (TXNRD) completely reduced oxhBD-1, shown in **Figure 1a**. In contrast, the same concentration of PDI did not effectively reduce hBD-1.

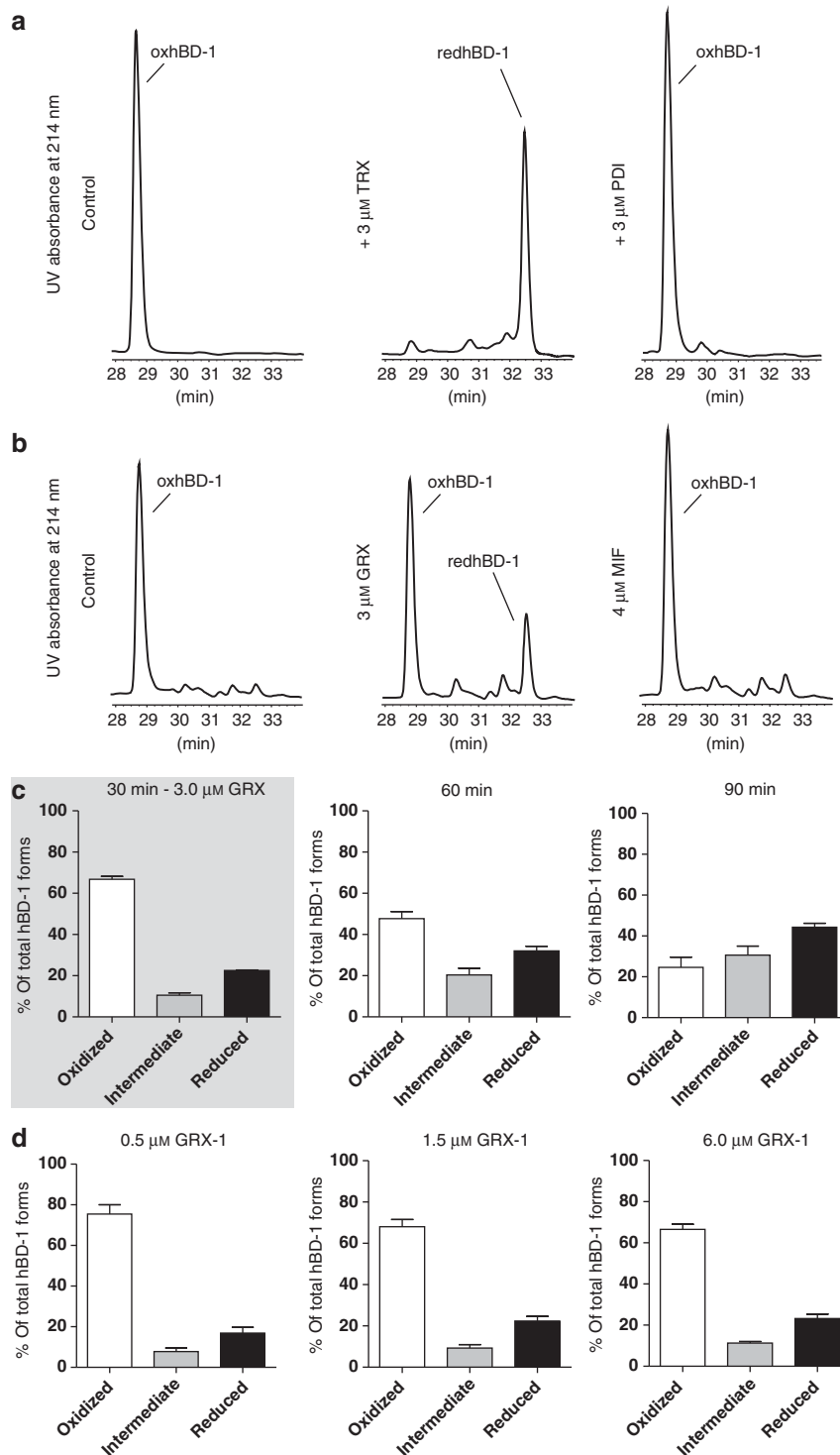
For GRX and MIF, reduction assays were performed with 5 mM reduced GSH. Although 5 mM GSH alone was insufficient (**Figure 1b**, left panel), oxhBD-1 was reduced in part when 3  $\mu$ M GRX was added (**Figure 1b**, middle panel). Extending the incubation time with GRX from 30 to 90 min increased the amounts of reduced hBD-1 and intermediate forms (**Figure 1c**), but increasing the concentration of GRX to up to 6.0  $\mu$ M did not significantly increase the amount of reduced hBD-1 (**Figure 1d**). For MIF, a concentration of 4  $\mu$ M did not reduce oxhBD-1 (**Figure 1b**, right panel). Based on our data, PDI and MIF are not able to reduce oxhBD-1 to a significant extent, whereas GRX was identified as an additional factor that mediates cleavage of the disulfide bonds of oxhBD-1 *in vitro*. However, the efficacy of the reaction is considerably lower and slower than observed in the TRX system. Hence, we hypothesize that the TRX system is the major physiological system for hBD-1 reduction and that GRX only plays an ancillary role.

### Reduction of oxhBD-1 is mediated by surfaces of epithelial and inflammatory cells

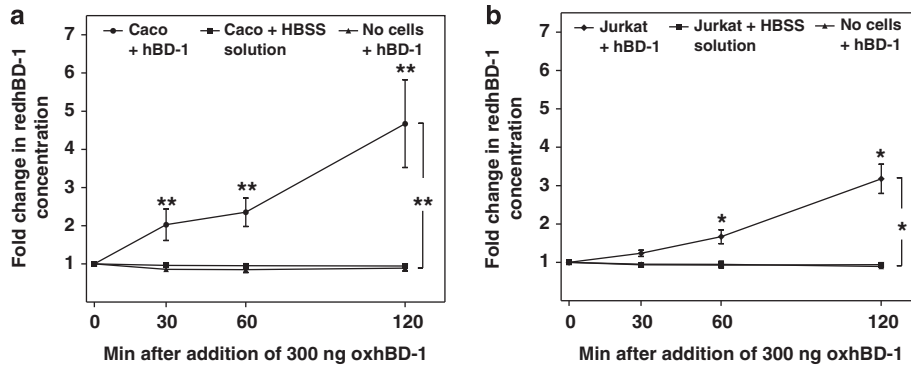
hBD-1 can be secreted into lumen and plasma by epithelia and other cells, presumably as an oxidized peptide. To investigate if oxidized, extracellular hBD-1 can be reduced and thus activated by the extracellular surface of living cells, we conducted cell culture experiments with colonic and lymphoid cell lines. The oxhBD-1 in a balanced salt solution was added to the washed cells and conversion of oxhBD-1 to redhBD-1 was followed by enzyme-linked immunosorbent assay. At 30 min after adding oxhBD-1, supernatants of Caco-2/TC7 cells showed a twofold higher concentration of redhBD-1, whereas after 120 min 4.7 times higher values of redhBD-1 were measured ( $P < 0.01$ , **Figure 2a**). With Jurkat cells, redhBD-1 concentration in the supernatant was also significantly increased after 60 min, and after 120 min, a 3.2-fold increase compared with baseline was noted ( $P < 0.05$ , **Figure 2b**). To exclude any cell toxic effects of hBD-1, we performed a flow cytometric assay using tetramethylrhodamine methyl ester (TMRM +), but addition of hBD-1 did not alter the percentage of viable cells significantly (data not shown). In summary, reduction of hBD-1 cannot only be catalyzed by purified enzymes but also mediated by viable cells, strengthening the hypothesis that secreted hBD-1 can be activated against microorganisms by the extracellular surface of intestinal epithelia or immune cells.

### Cell surface-mediated conversion of hBD-1 depends on the TRX system

We have shown previously that the TRX system can catalyze reduction of hBD-1 in an enzymatic assay with



**Figure 1** Reduction of hBD-1 by different redox enzymes. **(a)** A total of 10 μM oxhBD-1 was incubated with rat thioredoxin reductase and NADPH. Conversion of oxidized into reduced hBD-1 was monitored by RP-HPLC analysis. Left panel: control; middle panel: addition of 3 μM TRX; right panel: addition of 3 μM PDI. **(b)** oxhBD-1 was incubated with 5 mM glutathione (left panel); middle panel: addition of 3 μM GRX; right panel: addition of 4 μM MIF. **(c)** oxhBD-1 was incubated with 3 μM GRX as described for **b** and incubated for 30, 60, or 90 min. Quantification of hBD-1 forms in **c** and **d** was performed by integration of areas under the respective peaks from at least two independent experiments; data are presented as means ± s.e.m. GRX, glutaredoxin-1; MIF, macrophage migration inhibitory factor; NADPH, nicotinamide adenine dinucleotide phosphate; oxhBD-1, oxidized human β-defensin 1; PDI, protein disulfide isomerase A1; redhBD-1, reduced human β-defensin 1; RP-HPLC, reversed-phase, high-performance liquid chromatography; TRX, thioredoxin-1. Gray shadow in **c** indicates that this experiment is quantification of **b**, middle panel.

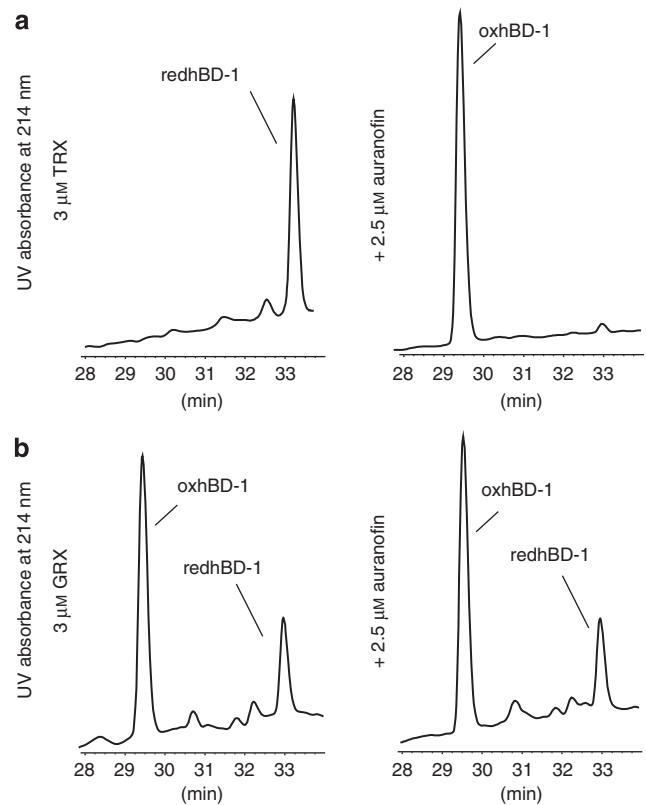


**Figure 2** Reduction of oxidized hBD-1 by living cells. Oxidized hBD-1 was incubated with (a) Caco-2/TC7 cells or (b) Jurkat cells for up to 120 min. For defined time points (0, 30, 60, and 120 min), redhBD-1 concentration in the supernatants was measured with ELISA and normalized to the concentration at 0 min after addition of oxidized hBD-1. Results represent the means  $\pm$  s.e.m. of at least four individual experiments. \* $P < 0.05$  and \*\* $P < 0.01$  by Mann–Whitney  $U$ -test. ELISA, enzyme-linked immunosorbent assay; hBD-1, human  $\beta$ -defensin 1; HBSS, Hank's balanced salt solution; oxhBD-1, oxidized human  $\beta$ -defensin 1; redhBD-1, reduced human  $\beta$ -defensin 1.

purified enzymes.<sup>15</sup> To investigate if reduction of hBD-1 by living cells is also dependent on the TRX system, we aimed to specifically block TXNRD with the gold compound auranofin.<sup>39,40</sup> In a first approach, we found that 2.5  $\mu$ M auranofin blocked reduction of oxhBD-1 by the TRX system (Figure 3a) but not by the GRX–GSH system (Figure 3b), confirming specificity of the inhibitor. To specifically analyze the role of the TRX system in Caco-2/TC7-mediated reduction of hBD-1, we used auranofin to block TXNRD in cell culture experiments. Addition of auranofin suppressed the reduction of extracellular oxhBD-1 in a dose-dependent manner, as higher concentrations of auranofin led to decreased levels of reduced hBD-1 after 120 min (Figure 4a). The cytotoxic effects of auranofin were not observed at these levels and were only detectable above the concentration range employed in the final experiments (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Figure 4b). To verify results obtained by chemically blocking the TRX system, we performed small interfering RNA (siRNA) knockdown of TRX in Caco-2/TC7 cells and analyzed reduction of extracellular oxhBD-1. As shown in Figure 4c an approximate 90% knockdown of TRX (Figure 4d) decisively diminished reduction of oxhBD-1, indicating a dominant role of TRX in reduction of hBD-1 in intestinal epithelia. Combining our results from chemical inhibition and knockdown experiments, the TRX system seems to be critical for reduction of hBD-1 in living cells. Inhibiting TXNRD or TRX activity both led to strongly decreased amounts of reduced hBD-1 in cell culture supernatant. The residual concentration of redhBD-1 observed in spite of auranofin or TRX knockdown could be because of the activity of secreted GRX or not yet identified redox-active proteins.

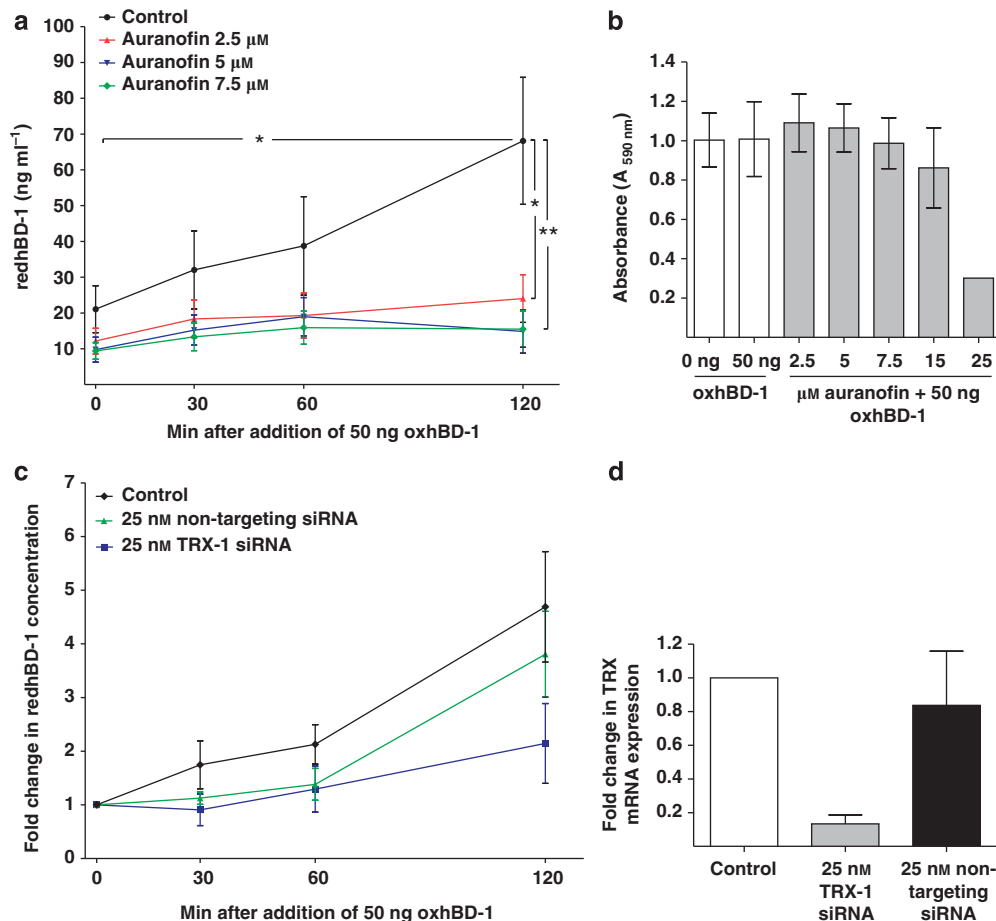
### Expression levels of additional redox enzymes in the intestines

As we could demonstrate earlier that activation of hBD-1 is mediated by the oxidoreductase TRX and that colonic TRX mRNA expression is reduced in IBD patients,<sup>15</sup> we were interested if there is a general defect in expression of redox



**Figure 3** Blockade of the TRX system by auranofin. (a) Oxidized hBD-1 was incubated with human TRX, rat thioredoxin reductase, and NADPH while 0  $\mu$ M (left) or 2.5  $\mu$ M auranofin (right) was added. (b) Oxidized hBD-1 was incubated with 5 mM glutathione and 3  $\mu$ M GRX without (left) and with 2.5  $\mu$ M auranofin (right). Reaction mixtures were analyzed by RP-HPLC as in Figure 1. GRX, glutaredoxin-1; NADPH, nicotinamide adenine dinucleotide phosphate; oxhBD-1, oxidized human  $\beta$ -defensin 1; redhBD-1, reduced human  $\beta$ -defensin 1; RP-HPLC, reversed-phase, high-performance liquid chromatography; TRX, thioredoxin-1.

enzymes during active IBD. Therefore, we investigated the expression levels of redox enzymes in intestinal biopsies of a cohort of healthy and diseased humans. Figure 5a shows a comparison between expression levels of TRX, TXNRD, GRX,

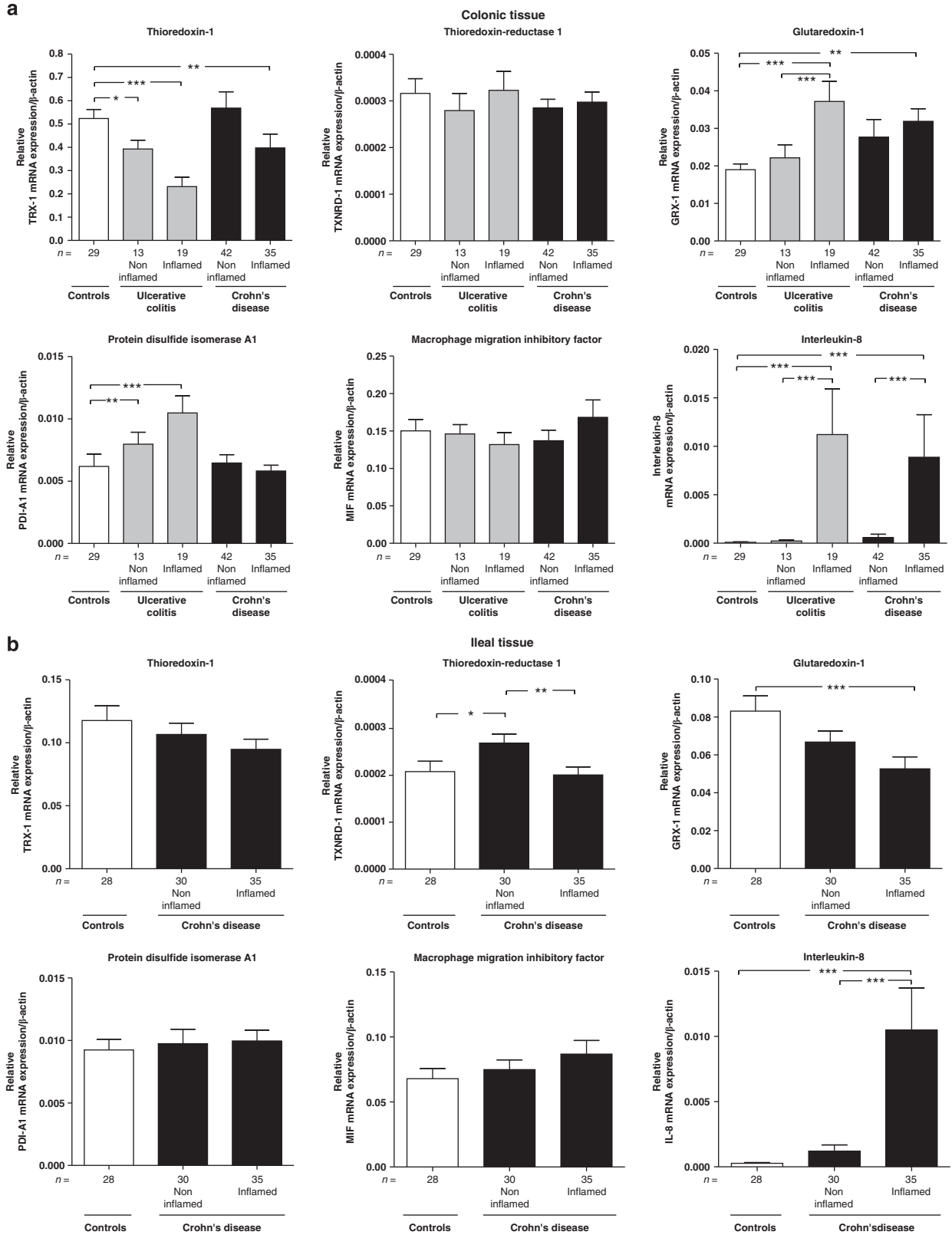


**Figure 4** Cell-mediated reduction of hBD-1 is dependent on the TRX system. (a) Oxidized hBD-1 was incubated with Caco-2/TC7 cells after preincubation with 0, 2.5, 5, and 7.5  $\mu$ M auranofin. At defined time points, redhBD-1 in the supernatants was detected by ELISA. Six individual experiments were carried out in triplicates; values represent means  $\pm$  s.e.m. (b) Viability test (MTT assay) of untreated/treated cells to check cytotoxicity of auranofin and oxidized hBD-1. Values represent means  $\pm$  s.e.m.; experiments were carried out in triplicates. (c) Knockdown of TRX with siRNA in Caco-2/TC7 cells. At 48 h after transfection, oxidized hBD-1 was added to the washed cells as described for **Figure 2a**. (d) After the experiment, TRX knockdown was confirmed by quantification of mRNA levels, shown as values normalized to the noninhibited control. ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide; oxhBD-1, oxidized human  $\beta$ -defensin 1; redhBD-1, reduced human  $\beta$ -defensin 1; siRNA, small interfering RNA; TRX, thioredoxin-1. \* $P < 0.05$  and \*\* $P < 0.01$  by Mann-Whitney  $U$ -test.

*PDI*, and *MIF* in colonic biopsies from unaffected controls or patients with ulcerative colitis (UC) or CD. For UC and CD patients, we also differentiated for inflammation status, which is also visualized by the expression of the proinflammatory cytokine interleukin 8 (IL-8). As shown previously, *TRX* expression is reduced in UC patients, whereas in CD patients *TRX* expression is only reduced in inflamed samples.<sup>15</sup> In addition, as we found now, mucosal *TXNRD* is expressed at a low level and its expression is unchanged in patients with IBD, independent of inflammation. *GRX* expression levels are only  $\sim 1/30$  of *TRX* ( $\sim 900,000$  copies), underlining the abundance of *TRX* in intestinal redox processes. Conversely to the inflammation-dependent reduction of *TRX* in inflamed IBD, *GRX* is significantly increased in both inflamed UC and CD compared with healthy controls. Also, expression levels of *PDI* show increased expression in UC patients, which is even more pronounced in inflamed samples. For CD patients, no difference in comparison with healthy controls was observed.

Colonic mRNA levels of *MIF* did not show statistically significant changes with inflammation status.

In contrast to colonic tissue, *TRX* mRNA expression in ileal biopsies of CD patients showed no significant differences between the examined cohorts, as during inflammation, ileal *TRX* expression remains stable (**Figure 5b**). Remarkably, expression levels in the small intestine are roughly 1/4 of those found in the colon as in healthy controls  $\sim 246,000$  compared with  $\sim 900,000$  copies ( $P < 0.0001$ ) were measured (data not shown). Ileal expression levels for *TXNRD*, *PDI*, and *MIF* also showed no significant differences between controls or CD patients. Only *GRX* expression was significantly reduced in inflamed ileal CD, whereas noninflamed ileal CD did not statistically differ from unaffected controls. In summary, expression levels of redox enzymes in intestinal biopsies show a wide range, and *TRX* is quantitatively by far the most important. Although colonic *TRX* expression is decreased, especially in biopsies of UC patients,



**Figure 5** mRNA expression of redox enzymes in human intestinal biopsies. mRNA transcript levels per 10 ng total mRNA are normalized to the expression level of  $\beta$ -actin in the corresponding biopsies. Colonic biopsies (**a**) are from the sigmoid colon and ileal tissue (**b**) is from terminal ileum. Data are presented as means  $\pm$  s.e.m. Values were considered to be statistically significant with \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  using Mann–Whitney test. CD, Crohn’s disease; GRX, glutaredoxin-1; MIF, macrophage migration inhibitory factor; PDI, protein disulfide isomerase A1; TRX, thioredoxin-1; TXNRD, thioredoxin-reductase 1; UC, ulcerative colitis.



GRX and PDI expression is increased in exactly these subgroups.

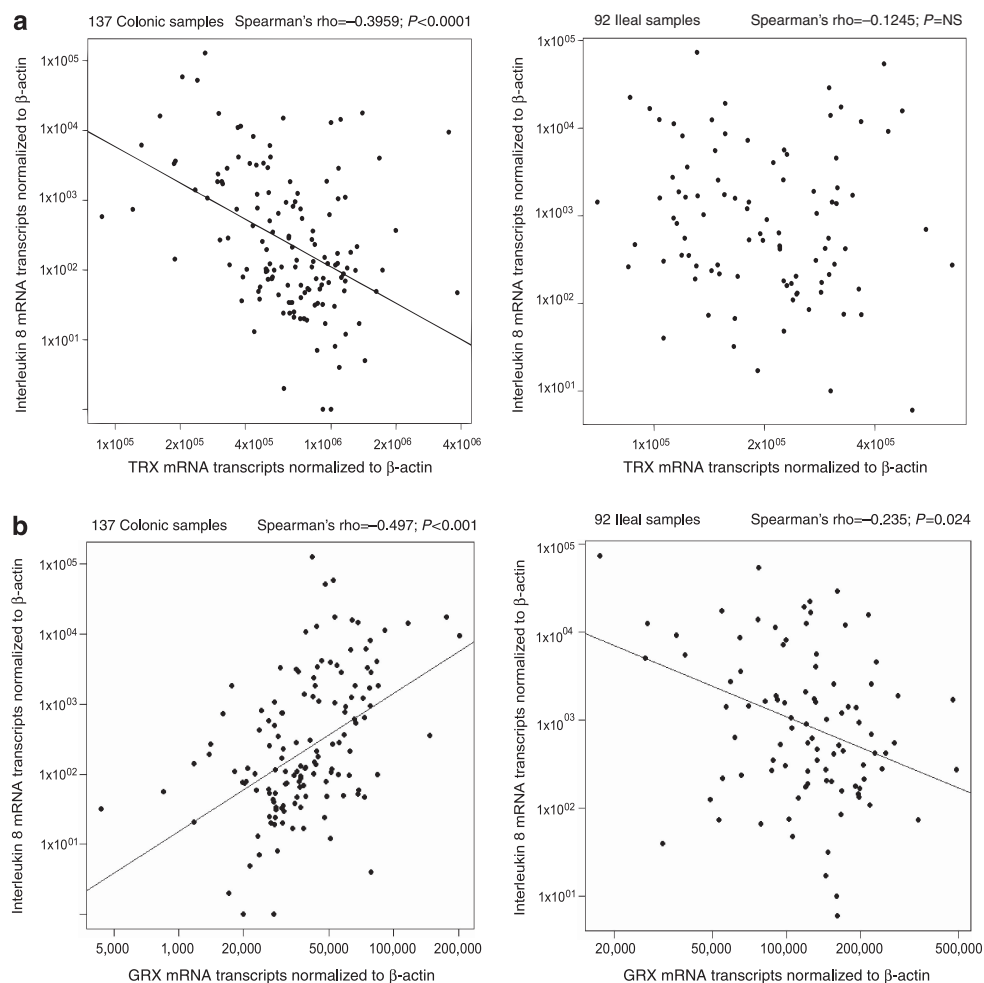
### TRX and GRX behave differently during active colonic inflammation

To assess if the expression of TRX and GRX correlates with inflammatory status, we measured the expression of the proinflammatory chemokine IL-8 (Figure 5 and Figure 6). Macroscopic evaluation during endoscopy showed a very high conformity with the expression of IL-8 in ileal and colonic tissue (data not shown). A moderate negative correlation between TRX and IL-8 expression could be found in colonic tissue, showing diminished TRX levels in biopsies with stronger inflammation (Spearman's  $r = -0.3959$ ,  $P < 0.0001$ , shown in Figure 6a, left panel), although whereas in the ileum, no significant correlation could be detected (Figure 6a, right panel). This indicates a reduced expression of TRX during inflammatory conditions that is specific for colonic IBD. Interestingly, GRX positively correlates with IL-8 expression in the colonic tissue. A good correlation between stronger inflammation and increasing GRX transcript numbers is

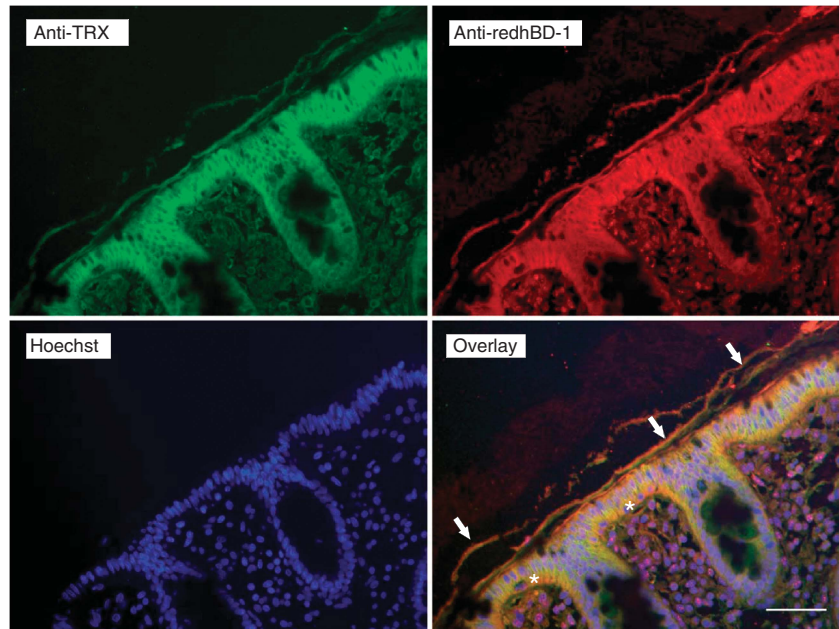
evident (Spearman's  $r = 0.497$ ,  $P < 0.001$ , shown in Figure 6b, left panel). In contrast, in ileal tissue, a mild, but negative correlation was detected (Spearman's  $r = -0.235$ ,  $P = 0.024$ , Figure 6b, right panel). Upregulation of GRX during colonic inflammation could thus be an attempt to compensate for the lost oxidoreductase activity due to lowered TRX.

### TRX and redhBD-1 localize to extracellular mucus *in vivo*

We have detected redhBD-1 and TRX in epithelial cells of colonic tissue samples previously.<sup>15</sup> After demonstrating now that oxidized hBD-1 can be reduced by the extracellular surface of epithelial and lymphatic cells, we sought to investigate TRX and redhBD-1 localization *in vivo* on the luminal side of intestinal mucosa. We used Carnoy-fixed (a method that preserves the mucus layer) colonic resection specimens from macroscopically and microscopically noninflamed areas. By using immunofluorescence staining, we confirmed epithelial presence of red hBD-1 and TRX in the cytoplasm of epithelial cells (Figure 7). The cytoplasm of the enterocytes is homogeneously colored and inflammatory cells of the lamina propria are also stained, which is in line with previous observations and



**Figure 6** Correlation between mRNA expression levels of interleukin-8 and (a) TRX or (b) GRX. Colonic biopsies ( $n = 137$ ) are shown in the left panels and ileal biopsies ( $n = 92$ ) in the right panels. Data are presented as means  $\pm$  s.e.m. mRNA transcript levels per 10 ng total mRNA are normalized with respect to the expression level of  $\beta$ -actin in the corresponding ileal or colonic biopsies. GRX, glutaredoxin-1; TRX, thioredoxin-1.



**Figure 7** TRX and redhBD-1 localize to extracellular mucus *in vivo*. Colonic mucosa tissue from a noninflamed control person was stained for TRX (upper left panel; green) and redhBD-1 (upper right panel; red). The lower left panel shows nuclear staining with the DNA-selective Hoechst dye (blue). Yellow areas in the lower right overlay panel indicate identical localization of redhBD-1 and TRX protein. Asterisks indicate epithelial presence of both proteins whereas arrows indicate reduced hBD-1 and thioredoxin secreted into the mucus. Magnification bar = 50  $\mu\text{m}$ . redhBD-1, reduced human  $\beta$ -defensin 1; TRX, thioredoxin-1.

our own results from conventional immunohistochemistry with redhBD-1 (data not shown). In addition, and for the first time, we could also detect intensive staining for redhBD-1 and TRX in intestinal mucus overlaying the colonic epithelia (Figure 7). With this observation we suggest that extracellular reduction of hBD-1 seems to be a physiological mechanism activating hBD-1 in the lumen to protect the intestinal epithelium against pathogenic and commensal microbes.

## DISCUSSION

Recently, we could show that the intramolecular disulfide bridges of hBD-1 can be cleaved either by a reducing environment or enzymatically by secreted or surface-associated molecules like TRX.<sup>15</sup> In this study we demonstrate that the antimicrobial peptide hBD-1 can be reduced and thereby activated by the surface of epithelial and lymphoid cell lines, supporting a physiological role of this activation mechanism. The cellular reduction of hBD-1 is largely dependent on TRX, as a blockade of the system by auranofin or siRNA knockdown of TRX impairs activation of hBD-1 by Caco-2/TC7 cells. As we have shown previously that colonic TRX expression was diminished in active colonic IBD,<sup>15</sup> we now analyzed TRX mRNA expression in the ileum and also evaluated intestinal TXNRD, GRX, PDI, and MIF expression. For these molecules, huge differences in baseline expression levels are evident, but alteration in active IBD can only be observed in colonic tissue for TRX, GRX, and PDI. TRX is by far the most abundantly transcribed redox enzyme in intestinal tissue, facilitating its role in the activation of hBD-1. Combining the functional biochemical approach with our mRNA data from IBD patients,

it can be speculated that reduced expression of TRX in inflamed UC would lead to diminished activation of hBD-1 at the intestinal mucosa. However, in this work we could show that GRX is also able to catalyze the conversion of hBD-1 *in vitro*, although at lower efficacy. GRX is expressed at higher levels in inflamed UC patients; thus, it is possible that in this case GRX functions as a back-up enzyme, compensating for decreased TRX function, which was suggested recently for this enzyme.<sup>41</sup>

As evidenced by immunofluorescence staining, redhBD-1 and TRX can be detected in the epithelium and extracellular mucus of the large intestine. In this setting, the TRX system could convert hBD-1 to a potent antimicrobial peptide toward enteric commensals.

Interestingly, activation of extracellular transglutaminase 2 (TG2), an intestinal enzyme involved in the pathogenesis of celiac disease, crucially depends on the TRX system as well.<sup>42</sup> TG2 is inactive in its oxidized state and Jin *et al.*<sup>42</sup> have elegantly demonstrated that the reduction of disulfide bonds in TG2 is mediated by TRX in an assay using cryosections of human small intestinal biopsies. IL-4 is another protein whose function can be altered by the TRX system. IL-4 has three intramolecular disulfide bridges and these can be reduced by surfaces of HeLa cells. Reduced IL-4 is no longer able to bind to the IL-4R. In this experimental setting, preincubation of cell with auranofin, a blocker of TXNRD, suppressed reduction of the disulfides in IL-4. This suggests a regulatory function on IL-4 signaling mediated by the TRX system.<sup>39</sup> The TRX system might thus facilitate the activation and deactivation of various extracellular molecules other than hBD-1.



In terms of disease pathogenesis, TRX has already been implicated in colonic IBD independently. Tamaki *et al.*<sup>43</sup> have shown that in mice overexpressing human TRX, dextran sodium sulfate-induced colitis takes a milder course and that intraperitoneal administration of recombinant TRX leads to attenuation of dextran sodium sulfate colitis when given as a prophylactic or as treatment. The authors demonstrate that TRX downregulates the proinflammatory cytokines tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and MIF in cultured murine colonic tissue. These findings complement the established and well-characterized role of TRX in the scavenging of reactive oxygen species; furthermore, reactive oxygen species themselves can induce expression of TRX.<sup>44</sup> TRX or other components of the TRX system might thus be increased in inflamed tissues, which is in fact the case in rheumatoid arthritis. There, significantly elevated TRX and TXNRD levels have been detected in synovial fluid and tissues.<sup>45</sup> An entirely different observation was evident from our analysis of intestinal tissue, where TXNRD is uniformly expressed on mRNA level throughout all groups, and TRX expression is significantly diminished in UC and the subgroup of inflamed colonic IBD. The question remains if altered TRX expression has a functional effect as long as TXNRD expression remains stable. However, our TRX knock-down experiments provide evidence that, independently of TXNRD expression, decreasing TRX expression has indeed a functional consequence. Along these lines, Watson *et al.*<sup>46</sup> found that siRNA knockdown of TXNRD expression did not lead to altered redox function of TRX in HeLa cells, whereas chemical inhibition of TXNRD with monomethylarsonous acid led to extensive oxidation of TRX.

In accordance with these observations, we demonstrate a significant, dose-dependent decrease in conversion of oxhBD-1 to redhBD-1 by Caco-2/TC7 cells when preincubating cells with the chemical inhibitor auranofin (Figure 4a). Together with our immunofluorescence images documenting localization of redhBD-1 and TRX in intestinal epithelia and mucus, we speculate that *in vivo*, hBD-1 is activated extracellularly by intestinal epithelia in a TRX-dependent fashion.

A wealth of redox enzymes other than TRX is expressed in intestinal tissues. We therefore analyzed the oxidoreductases PDI, GRX, and MIF with respect to their ability to reduce hBD-1. We show that GRX can catalyze reduction of hBD-1 *in vitro*, although the *in vivo* relevance remains unclear because of the relatively low efficiency compared with TRX. GRX could nonetheless be responsible for the small amount of redhBD-1 detected at the end of cell culture experiments in which the TRX system has been blocked by auranofin or TRX expression has been diminished by siRNA knockdown. A relatively high GRX expression is found in the healthy intestine and transcript numbers were significantly increased in active colonic IBD. Upregulation of GRX during inflammation has been observed previously, for example, during oxidative stress,<sup>47</sup> and GRX transcript numbers positively correlate with the proinflammatory cytokine IL-8 in the colon (Figure 6b). GRX can be upregulated by tumor necrosis factor- $\alpha$ ,<sup>48</sup> and hBD-1 can be upregulated by interferon- $\gamma$  or lipopolysaccharide.<sup>49</sup> A supporting role of GRX

in the activation of antimicrobial peptides during states of inflammation is therefore a plausible alternative.

This study demonstrates that hBD-1 can be biologically activated by colonic and lymphocytic cells, and this activation seems to be mediated in great part by the reducing capabilities of TRX. The high expression in intestinal tissues, its localization to cell surfaces, and detection in extracellular mucus make TRX the most likely candidate to mediate reduction of hBD-1 *in vivo*. This concept is supported by the localization of these two molecules in epithelium and mucus of human colonic tissue. Thus, the TRX-mediated activation of hBD-1 by cellular surfaces described in this study extends the understanding of innate immunity and allows speculation if defects in this mechanism might be relevant in IBD.

## METHODS

***In vitro* reduction assays.** hBD-1 reduction assays were performed as described previously.<sup>15</sup> A reaction mixture of 10  $\mu$ M oxhBD-1 (Peptide Institute, Osaka, Japan), 0.8 mM NADPH (Biomol, Hamburg, Germany), and 100 nM rat thioredoxin reductase (TXNRD, purchased from IMCO, Stockholm, Sweden) in a 0.1 M potassium phosphate buffer with 2 mM EDTA at pH 7.0 was used. Then, 3  $\mu$ M human thioredoxin-1 (Sigma-Aldrich, Steinheim, Germany), 3  $\mu$ M bovine protein disulfideisomerase (Sigma-Aldrich), or equivalent volume of buffer was added to the assay. In the inhibition experiment, 2.5  $\mu$ M of the TXNRD inhibitor auranofin was employed. Incubation lasted for 30 min at 37°C. For the oxidoreductases GRX and MIF, a GSH-based system was used. A total of 5 mM reduced GSH (Roth, Karlsruhe, Germany) in a 100 mM sodium phosphate buffer with 2 mM EDTA was incubated for 30 min (37°C) with 10  $\mu$ M oxhBD-1 and 0–6  $\mu$ M active GRX (Abcam, UK) or 4  $\mu$ M active MIF (Abcam, Cambridge, UK). For GRX, incubation times of 60 and 90 min were used. After incubation, all reaction mixtures were acidified with 0.1% trifluoroacetic acid and analyzed with HPLC as described below. Conversion from oxhBD-1 to redhBD-1 peptide was followed by retention time.

**HPLC analysis.** HPLC analyses were carried out with an Agilent 1200 series system (Agilent, Santa Clara, CA) and a Vydac 218TP-C18 column (250  $\times$  4.6 mm, 5 mm; Grace, Hesperia, CA). Gradient increased from 2% B to 35% B in 33 min (solvent A, water + 0.18% (v/v) trifluoroacetic acid; solvent B, acetonitrile + 0.15% (v/v) trifluoroacetic acid) at 25°C and 0.8 ml min<sup>-1</sup>. In case of GRX assays, quantification was performed by integration of areas under the respective peaks.

**Cell culture experiments.** The colon adenocarcinoma cell line Caco-2/TC7<sup>50</sup> was kindly provided by U. Meyer (University of Basel, Basel, Switzerland) and cultivated in Dulbecco's modified Eagle's medium (Gibco Life Technologies, New York, NY), buffered with 25 mM HEPES. Medium was completed with 10% fetal calf serum (PAA, Pasching, Austria), 1% non-essential amino acids, 1% penicillin/streptomycin, and 1% sodium pyruvate (Gibco Life Technologies). Jurkat cells were cultivated in Dulbecco's modified Eagle's medium as described for Caco-2/TC7, but without addition of non-essential amino acids.

Caco-2/TC7 cells were seeded at a density of 150,000 per well (24-well culture plates) or 28,500 cells per well (96-well culture plates), and grown to ~80–90% confluency. Jurkat suspension cells were seeded at 400,000 cells in 960  $\mu$ l Hank's balanced salt solution with CaCl<sub>2</sub> and MgCl<sub>2</sub> (HBSS, Gibco Life Technologies), and used for experiments after 1 h.

After decanting the growth medium, the cells were washed with HBSS and 40  $\mu$ l 25  $\times$  proteinase inhibitor cocktail (Roche, Mannheim, Germany) and 300 ng oxhBD-1 (Peptide Institute) were added. At 0, 30, 60, and 120 min, medium was decanted and centrifuged for

5 min at 16,000 *g*. Subsequently, open thiol groups of proteins were irreversibly alkylated with 20 mM iodoacetamide in 10 mM ammonium-hydrogencarbonate buffer. Samples were snap frozen in liquid nitrogen and stored at  $-20^{\circ}\text{C}$ . At least four independent experiments were performed on different days.

For inhibition experiments, the thioredoxin reductase inhibitor auranofin (Sigma) was solved in stock solution of 25 mM in dimethyl sulfoxide. Final dimethyl sulfoxide concentrations did not exceed 1%. Cells were incubated for 60 min with 0.5–25  $\mu\text{M}$  auranofin, whereas a range from 2.5 to 7.5  $\mu\text{M}$  was used in our assays. After incubation with auranofin, cells were washed with HBSS, and 200  $\mu\text{l}$  HBSS with 50 ng oxhBD-1 were added. At defined time points, supernatants were pipetted off the cells and alkylated with iodoacetamide as described above. Six independent experiments were carried out as triplicates.

**Cell viability.** A colorimetric MTT assay was used to determine cell viability at the end of the experiments with auranofin.

**Transfection with siRNA.** Caco-2/TC7 cells were plated at a density of 5,000 cells per well (in 96-well cell culture plates) and grown for 24 h according to the manufacturer's protocol (Dharmacon, Thermo-Scientific, Lafayette, CO). Cells were transfected with 25 nM siGENOME SMARTpool siRNA (targets four different regions specific to TRX-1) or 25 nM siCONTROL non-targeting Pool #1 (designed to target no known human genes). Controls were treated with DharmaFECT 1 transfection reagent but no siRNA was added. After incubation for an additional 48 h, cells were washed with phosphate-buffered saline, and 50 ng oxhBD-1 in HBSS was added to all experimental groups. As above, triplicates for each time point (0, 30, 60, and 120 min) were analyzed for protein levels of redhBD-1 by enzyme-linked immunosorbent assay. Four independent experiments were conducted on different days. At the end of each experiment, cells were homogenized with the QiaShredder Kit Nr. 79656 (Qiagen, Hilden, Germany), total RNA was isolated using RNeasy Mini Kit (Qiagen), and mRNA levels of TRX were determined as described in the section "Quantitative real-time PCR."

**Enzyme-linked immunosorbent assay for reduced hBD-1.** The 96-well plates were coated with 10  $\mu\text{g ml}^{-1}$  of the rabbit polyclonal antibody specifically directed against reduced hBD-1 (ref. 15) in a 50 mM sodium carbonate buffer at  $4^{\circ}\text{C}$  overnight. Wells were blocked with 1% bovine serum albumin in phosphate-buffered saline. Reduced-alkylated hBD-1 as standards, negative controls, and samples were incubated for 45 min at  $37^{\circ}\text{C}$ . To detect bound defensin, 1 ng  $\mu\text{l}^{-1}$  biotinylated redhBD-1 antibody was used. As detection system, streptavidin-peroxidase conjugate (1:10,000, Roche) and the liquid chromogen ABTS (Sigma) was used. Absorption was read at 405 nm (reference wave length 492 nm). Results were calculated from optical density values after blank reduction and standard curves were created with the four-parameter fit.

**Patients.** All biopsy samples were collected at the Robert Bosch Hospital (Stuttgart, Germany) between 2002 and 2009. The diagnosis of IBD was based on combined clinical, radiological, and endoscopic data (Table 1). All patients gave their written informed consent and the study was approved by the ethical committee of the University of Tübingen (Germany). Endoscopic biopsy specimens from a total of 173 patients were collected for this study and immediately snap-frozen in liquid nitrogen. For real-time PCR analysis, 93 ileal and 139 sigmoid biopsies were used.

**RNA isolation and reverse transcription.** Frozen biopsies were mechanically disrupted and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Ribosomal ratio (18S/28S rRNA) and the algorithm-based RIN (RNA Integrity Number) was calculated to check RNA quality. Total RNA (1  $\mu\text{g}$ ) was reverse transcribed to complementary DNA using oligo (dT) primers and AMV reverse transcriptase according to the supplier's protocol (Promega, Mannheim, Germany).

**Table 1 mRNA analysis—patient characteristics**

	Controls	CD	UC
Number of patients	34	95	32
Mean age in years (range)	50 (16–80)	38 (16–70)	39 (18–67)
Sex			
Male	13	44	20
Female	21	51	12
Age at diagnosis			
< 16 years		9	0
16–40 years		64	26
> 40 years		11	6
Localization (Montreal)			
L1 (ileal)		31	
L2 (colonic)		24	
L3 (ileocolonic)		40	
L4 (upper GI)		3	
E1 (proctitis)			7
E2 (left-sided colitis)			12
E3 (pancolitis)			13
Behavior			
Nonstricturing/penetrating		30	
Stricturing		27	
Penetrating		38	
Perianal disease		10	
Medical treatment for IBD			
Oral/topical 5-ASA		25/1	12/6
Systemic/topical steroids		41/1	10/1
Oral budesonide		16	—
Azathioprine/6-Mercaptopurine		22	5
Methotrexate		4	—
Tacrolimus		—	1
Anti-TNF- $\alpha$		3	0
Antibiotics		8	1
Surgery, total		55	1 <sup>a</sup>
Ileocecal resection		24	
Segmental resection			
Ileal segments		9	
Colonic segments		7	
Ileostomy		1	
Appendectomy		5	
Perianal fistulas		9	
Mean activity index <sup>b</sup>			
CDAI		199	
CAI			8.7

5-ASA, 5-aminosalicylic acid; CAI, Clinical Activity Index; CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; GI, gastrointestinal; IBD, inflammatory bowel disease; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; UC, ulcerative colitis.

<sup>a</sup>Surgery was performed after biopsies were taken.

<sup>b</sup>Owing to missing data, activity index was available for 48 CD patients and 25 UC patients only.

**Table 2 Primers used in real-time PCR**

Target	Forward primer	Reverse primer	Product size
β-Actin	5'-GCCAACCGCGAGAAGATGA-3'	5'-CATCACGATGCCAGTGGTA-3'	120
TRX	5'-TGGTGTGGGCCTTGCAAAATGA-3'	5'-TTCACCCACCTTTTGTCCCTTC-3'	174
TXNRD	5'-CAGACGGGGAGGCTTTTC-3'	5'-CGAGAGCGTTCCTCCAG-3'	118
GRX	5'-ATCAAACAAGGGCTTCTGGA-3'	5'-CACTGCATCCGCTATACAA-3'	142
MIF	5'-TGACACAGCATCGGCAAGATCGG-3'	5'-TGATGTAGACCCTGTCGGGGCTGA-3'	108
PDI (P4HB)	5'-CTGCTGGTGGAGTCTATGCCCT-3'	5'-ATGGTGGGATAGCCGCGCAC-3'	176
IL-8	5'-ATGACTTCCAAGCTGGCCGTGGC-3'	5'-TCTCAGCCCTTTCAAAACTTC-3'	292

GRX, glutaredoxin-1; IL-8, interleukin 8; MIF, macrophage migration inhibitory factor; PDI, protein disulfide isomerase A1; TRX, thioredoxin-1; TXNRD, thioredoxin-reductase 1.

**Quantitative real-time PCR.** For mRNA quantification, real-time PCR was carried out in a fluorescence temperature cycler (LightCycler 480; Roche Diagnostics). Single-stranded complementary DNA or gene-specific plasmids as controls served as a template with specific oligonucleotide primer pairs (Table 2). Plasmids for each product were generated with the TOPO TA Cloning Kit (Invitrogen) according to the supplier's protocol. The sequence of the PCR-amplified DNA fragments was confirmed and internal standard curves were produced. The mRNA transcript numbers were normalized to the respective β-actin mRNA transcript number in a given colonic or ileal biopsy. The mRNA data are presented in bars with s.e.m.

**Immunofluorescence microscopy.** Samples were obtained from patients who underwent colonic resection because of colonic cancer. Tissue areas of disease-free resection margins were fixated with Carnoy's solution containing 60% ethanol, 10% glacial acetic acid, and 30% chloroform and embedded in paraffin. Paraffin sections (5 μm) of tissue samples were deparaffinized and rehydrated before heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). Slides were blocked with 10% bovine serum albumin in Tris-buffered saline, pH 7.4, before staining. Staining was performed at 4°C overnight using 5 μg ml<sup>-1</sup> of an affinity-purified polyclonal rabbit antibody against redhBD-1 (from ref. 15) and subsequently a goat TRX antibody (R&D Systems, Minneapolis, MN, 1:500). After washing, slides were incubated with a mixture of secondary antibodies (chicken anti-goat Alexa Fluor 488 and a donkey anti-rabbit Alexa Fluor 546; Invitrogen) for 1 h at room temperature. Sections were counterstained with the DNA-selective bisbenzimidazole dye (blue; Hoechst 33258). To exclude artificial autofluorescence secondary to the preparation of the sections, control sections were stained without primary antibodies and no unspecific labeling was observed following incubation with secondary antibodies (data not shown).

**Statistics.** Statistical analyses were performed and all graphs were generated using GraphPad Prism (GraphPad Software, La Jolla, CA, version 5.04). Mann-Whitney test was used in mRNA and cell culture experiments. *P*-values of <0.05 were considered statistically significant.

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#### DISCLOSURE

B.O.S., E.F.S., and J.W. filed a patent application for therapeutic use of reduced hBD-1.

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#### REFERENCES

- Harder, J., Glaser, R. & Schroder, J.M. Human antimicrobial proteins effectors of innate immunity. *J. Endotoxin. Res.* **13**, 317–338 (2007).
- Huttner, K.M. & Bevins, C.L. Antimicrobial peptides as mediators of epithelial host defense. *Pediatr. Res.* **45**, 785–794 (1999).
- Singh, P.K. *et al.* Production of beta-defensins by human airway epithelia. *Proc. Natl. Acad. Sci. USA* **95**, 14961–14966 (1998).
- Goldman, M.J., Anderson, G.M., Stolzenberg, E.D., Kari, U.P., Zasloff, M. & Wilson, J.M. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**, 553–560 (1997).
- Zhao, C., Wang, I. & Lehrer, R.I. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett.* **396**, 319–322 (1996).
- McCray, P.B.J. & Bentley, L. Human airway epithelia express a beta-defensin. *Am. J. Respir. Cell. Mol. Biol.* **16**, 343–349 (1997).
- Tollin, M., Bergman, P., Svenberg, T., Jornvall, H., Gudmundsson, G.H. & Agerberth, B. Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides* **24**, 523–530 (2003).
- Presicce, P., Giannelli, S., Taddeo, A., Villa, M.L. & Della, B.S. Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. *J. Leukoc. Biol.* **86**, 941–948 (2009).
- Kraemer, B.F. *et al.* Novel Anti-bacterial Activities of beta-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. *PLoS Pathog.* **7**, e1002355 (2011).
- Prado-Montes de Oca, E. Human β-defensin 1: a restless warrior against allergies, infections and cancer. *Int. J. Biochem. Cell. Biol.* **42**, 800–804 (2010).
- Kocsis, A.K. *et al.* Association of beta-defensin 1 single nucleotide polymorphisms with Crohn's disease. *Scand. J. Gastroenterol.* **43**, 299–307 (2008).
- Berg, R.D. The indigenous gastrointestinal microflora. *Trends Microbiol.* **4**, 430–435 (1996).
- Wilson, M. The gastrointestinal tract and its indigenous microbiota. In *Microbial Inhabitants of Humans*, 251–317; ISBN-13:9780521841580 (Cambridge University Press, Cambridge, 2005).
- Glover, L.E. & Colgan, S.P. Hypoxia and metabolic factors that influence inflammatory bowel disease pathogenesis. *Gastroenterology* **140**, 1748–1755 (2011).



15. Schroeder, B.O. *et al.* Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1. *Nature* **469**, 419–423 (2011).
16. Scudiero, O. *et al.* Novel synthetic, salt-resistant analogs of human beta-defensins 1 and 3 endowed with enhanced antimicrobial activity. *Antimicrob. Agents Chemother.* **54**, 2312–2322 (2010).
17. Hogg, P.J. Disulfide bonds as switches for protein function. *Trends Biochem. Sci.* **28**, 210–214 (2003).
18. Xie, L., Chesterman, C.N. & Hogg, P.J. Control of von Willebrand factor multimer size by thrombospondin-1. *J. Exp. Med.* **193**, 1341–1349 (2001).
19. Azimi, I., Matthias, L.J., Center, R.J., Wong, J.W. & Hogg, P.J. Disulfide bond that constrains the HIV-1 gp120 V3 domain is cleaved by thioredoxin. *J. Biol. Chem.* **285**, 40072–40080 (2010).
20. Matthias, L.J. *et al.* Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1. *Nat. Immunol.* **3**, 727–732 (2002).
21. Wollman, E.E., Kahan, A. & Fradelizi, D. Detection of membrane associated thioredoxin on human cell lines. *Biochem. Biophys. Res. Commun.* **230**, 602–606 (1997).
22. Rubartelli, A., Bonifaci, N. & Sitia, R. High rates of thioredoxin secretion correlate with growth arrest in hepatoma cells. *Cancer Res.* **55**, 675–680 (1995).
23. Angelini, G. *et al.* Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. *Proc. Natl. Acad. Sci. USA* **99**, 1491–1496 (2002).
24. Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. & Sitia, R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267**, 24161–24164 (1992).
25. Nakamura, H. *et al.* Chronic elevation of plasma thioredoxin: inhibition of chemotaxis and curtailment of life expectancy in AIDS. *Proc. Natl. Acad. Sci. USA* **98**, 2688–2693 (2001).
26. Gromer, S., Urig, S. & Becker, K. The thioredoxin system—from science to clinic. *Med. Res. Rev.* **24**, 40–89 (2004).
27. Arner, E.S. & Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267**, 6102–6109 (2000).
28. Mouggiakakos, D., Johansson, C.C., Jitschin, R., Bottcher, M. & Kiessling, R. Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress. *Blood* **117**, 857–861 (2011).
29. Mouggiakakos, D., Johansson, C.C. & Kiessling, R. Naturally occurring regulatory T cells show reduced sensitivity toward oxidative stress-induced cell death. *Blood* **113**, 3542–3545 (2009).
30. Takeuchi, J. *et al.* Thioredoxin inhibits tumor necrosis factor- or interleukin-1-induced NF-kappaB activation at a level upstream of NF-kappaB-inducing kinase. *Antioxid. Redox. Signal.* **2**, 83–92 (2000).
31. Hirota, K. *et al.* Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J. Biol. Chem.* **274**, 27891–27897 (1999).
32. Turano, C., Coppari, S., Altieri, F. & Ferraro, A. Proteins of the PDI family: unpredicted non-ER locations and functions. *J. Cell. Physiol.* **193**, 154–163 (2002).
33. Gallina, A. *et al.* Inhibitors of protein-disulfide isomerase prevent cleavage of disulfide bonds in receptor-bound glycoprotein 120 and prevent HIV-1 entry. *J. Biol. Chem.* **277**, 50579–50588 (2002).
34. Lundberg, M., Fernandes, A.P., Kumar, S. & Holmgren, A. Cellular and plasma levels of human glutaredoxin 1 and 2 detected by sensitive ELISA systems. *Biochem. Biophys. Res. Commun.* **319**, 801–809 (2004).
35. Peltoniemi, M.J. *et al.* Modulation of glutaredoxin in the lung and sputum of cigarette smokers and chronic obstructive pulmonary disease. *Respir. Res.* **7**, 133 (2006).
36. O’Keeffe, J. *et al.* Flow cytometric measurement of intracellular migration inhibition factor and tumour necrosis factor alpha in the mucosa of patients with coeliac disease. *Clin. Exp. Immunol.* **125**, 376–382 (2001).
37. Roger, T., David, J., Glauser, M.P. & Calandra, T. MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature* **414**, 920–924 (2001).
38. Kleemann, R. *et al.* Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase. *J. Mol. Biol.* **280**, 85–102 (1998).
39. Curbo, S. *et al.* Regulation of interleukin-4 signaling by extracellular reduction of intramolecular disulfides. *Biochem. Biophys. Res. Commun.* **390**, 1272–1277 (2009).
40. Peters-Golden, M. & Shelly, C. The oral gold compound auranofin triggers arachidonate release and cyclooxygenase metabolism in the alveolar macrophage. *Prostaglandins* **36**, 773–786 (1988).
41. Du, Y., Zhang, H., Lu, J. & Holmgren, A. Glutathione and glutaredoxin act as backup of human thioredoxin reductase 1 to reduce thioredoxin 1 preventing cell death by aurothioglucose. *J. Biol. Chem.* **287**, 38210–38219 (2012).
42. Jin, X. *et al.* Activation of extracellular transglutaminase 2 by thioredoxin. *J. Biol. Chem.* **286**, 37866–37873 (2011).
43. Tamaki, H. *et al.* Human thioredoxin-1 ameliorates experimental murine colitis in association with suppressed macrophage inhibitory factor production. *Gastroenterology* **131**, 1110–1121 (2006).
44. Taniguchi, Y., Taniguchi-Ueda, Y., Mori, K. & Yodoi, J. A novel promoter sequence is involved in the oxidative stress-induced expression of the adult T-cell leukemia-derived factor (ADF)/human thioredoxin (Trx) gene. *Nucleic Acids Res.* **24**, 2746–2752 (1996).
45. Maurice, M.M. *et al.* Expression of the thioredoxin-thioredoxin reductase system in the inflamed joints of patients with rheumatoid arthritis. *Arthritis Rheum.* **42**, 2430–2439 (1999).
46. Watson, W., Heilman, J., Hughes, L. & Spielberger, J. Thioredoxin reductase-1 knock down does not result in thioredoxin-1 oxidation. *Biochem. Biophys. Res. Commun.* **368**, 832–836 (2008).
47. Song, J.J., Rhee, J.G., Suntharalingam, M., Walsh, S.A., Spitz, D.R. & Lee, Y.J. Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H<sub>2</sub>O<sub>2</sub>. *J. Biol. Chem.* **277**, 46566–46575 (2002).
48. Pan, S. & Berk, B.C. Glutathiolation regulates tumor necrosis factor-alpha-induced caspase-3 cleavage and apoptosis: key role for glutaredoxin in the death pathway. *Circ. Res.* **100**, 213–219 (2007).
49. Pazgier, M., Hoover, D.M., Yang, D., Lu, W. & Lubkowski, J. Human beta-defensins. *Cell. Mol. Life Sci.* **63**, 1294–1313 (2006).
50. Chantret, I. *et al.* Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. *J. Cell. Sci.* **107** (Pt 1), 213–225 (1994).



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