GADD34 Function in Protein Trafficking Promotes Adaptation to Hyperosmotic Stress in Human Corneal Cells

Graphical Abstract

Highlights

- GADD34, a stress-inducible subunit of the PP1 phosphatase, promotes osmoadaptation
- The functions of GADD34 in osmoadaptation are independent of its substrate, eIF2α-P
- GADD34/PP1 facilitates cis- to trans-Golgi SNAT2 protein trafficking
- Pharmacologic and genetic inhibition of GADD34/PP1 induces Golgi fragmentation

Authors

Dawid Krokowski, Bo-Jhih Guan, Jing Wu, ..., Eric Pearlman, Anna Blumental-Perry, Maria Hatzoglou

Correspondence
dmk102@case.edu (D.K.), axb811@case.edu (A.B.-P.), mxh8@case.edu (M.H.)

In Brief

Here, Krokowski et al. show that GADD34/PP1 protects the microtubule network, prevents Golgi fragmentation, and preserves protein trafficking independent of its action in the integrated stress response (ISR). In osmoadaptation, GADD34 facilitates trans-Golgi-mediated processing of the endoplasmic reticulum (ER)-synthesized amino acid transporter SNAT2, which in turn increases amino acid uptake.

Krokowski et al., 2017, Cell Reports 21, 2895–2910
December 5, 2017 © 2017 The Author(s).
https://doi.org/10.1016/j.celrep.2017.11.027
GADD34 Function in Protein Trafficking Promotes Adaptation to Hyperosmotic Stress in Human Corneal Cells

Dawid Krokowski,1,* Bo-Jhih Guan,1 Jing Wu,1 Yuke Zheng,1 Padmanabhan P. Pattabiraman,2 Raul Jobava,1 Xing-Huang Gao,1 Xiao-Jing Di,3 Martin D. Snider,1 Ting-Wei Mu,1 Shijie Liu,5 Brian Storrie,1 Eric Pearlman,6 Anna Blumental-Perry,7,* and Maria Hatzoglou1,8,*

1Department of Genetics and Genome Sciences
2Department of Ophthalmology and Visual Science
3Department of Physiology and Biophysics
4Department of Biochemistry
Case Western Reserve University, Cleveland, OH 44106, USA
5Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA
6Institute for Immunology, University of California, Irvine, CA 92697, USA
7Department of Surgery, Case Western Reserve University, Cleveland, OH 44106, USA
8Lead Contact
*Correspondence: dmk102@case.edu (D.K.), axb811@case.edu (A.B.-P.), mxh8@case.edu (M.H.)
https://doi.org/10.1016/j.celrep.2017.11.027

SUMMARY

GADD34, a stress-induced regulatory subunit of the phosphatase PP1, is known to function in hyperosmotic stress through its well-known role in the integrated stress response (ISR) pathway. Adaptation to hyperosmotic stress is important for the health of corneal epithelial cells exposed to changes in extracellular osmolarity, with maladaptation leading to dry eye syndrome. This adaptation includes induction of SNAT2, an endoplasmic reticulum (ER)-Golgi-processed protein, which helps to reverse the stress-induced loss of cell volume and promote homeostasis through amino acid uptake. Here, we show that GADD34 promotes the processing of proteins synthesized on the ER during hyperosmotic stress independent of its action in the ISR. We show that GADD34/PP1 phosphatase activity reverses hyperosmotic-stress-induced Golgi fragmentation and is important for cis- to trans-Golgi trafficking of SNAT2, thereby promoting SNAT2 plasma membrane localization and function. These results suggest that GADD34 is a protective molecule for ocular diseases such as dry eye syndrome.

INTRODUCTION

Homeostasis in tissues exposed to significant changes in osmolarity (Brocker et al., 2012) is maintained through an adaptive cellular response. Failure of adaptation is implicated in the development of various pathophysiological conditions. Among the most prominent is dry eye syndrome (Bhavsar et al., 2011), where hyperosmolarity of the tear film has been linked to both inflammation and corneal cell loss due to apoptosis (Lam et al., 2009; Lin et al., 2011; Luo et al., 2007; Yeh et al., 2003). Understanding the mechanisms of adaptation to hyperosmotic stress is a key to the development of therapeutic approaches to the treatment of dry eye syndrome and related diseases (Brocker et al., 2012). Initial stress-induced cell shrinkage activates Na”/H” exchangers of the Slc9 transporters family; Na*, K*, and 2Cl-/Ca2+ cotransporters of the Slc12 family (Eveloff and Warnock, 1987; Pedersen et al., 2007); and possibly increased uptake of extracellular Ca2+, all of which contribute to regulatory volume increase (RVI) (Burg et al., 2007; Lang et al., 1998; Sánchez and Wilkins, 2004). This initial RVI response does not require de novo protein synthesis. A second phase replaces these inorganic ions by increasing the pool of intracellular organic osmolytes (Burg et al., 2007; Franchi-Gazzola et al., 2006). This second phase requires the synthesis of proteins mediating the uptake and biosynthesis of osmolytes (Dall’Asta et al., 1994; Franchi-Gazzola et al., 2006). Among these is the amino acid transporter SNAT2, which mediates the concentrative uptake of small neutral amino acids, which act as osmolytes to maintain RVI (Bröder, 2014).

Osmoadaptation has been studied extensively at the level of transcriptional control (Burg et al., 2007) and, to a lesser extent, at the level of regulation of protein synthesis. A signaling pathway that regulates protein synthesis in diverse stress conditions is the phosphorylation of the translation initiation factor eIF2α, which is known as the integrated stress response (ISR). Phosphorylation of eIF2α causes a decrease in global protein synthesis and at the same time induces translational reprogramming that leads to adaptation to stress conditions. An important component of this response is the induction of GADD34, a regulatory subunit of the protein phosphatase PP1 that dephosphorylates eIF2α-P (Novoa et al., 2001). This dephosphorylation restores protein synthesis and allows execution of the stress-induced transcription programs. The cellular response to hyperosmotic stress also involves eIF2α-P/GADD34 signaling (Bevilacqua et al., 2010; Krokowski et al., 2015). However, inhibition of protein synthesis
Adaptation to mild stress, but not to severe stress (Bevilacqua et al., 2012). We tested the levels of SNAT2 in cells exposed to mild (500 mOsm) or severe (600 mOsm) stress. These two stress conditions were chosen based on our previous reports that cells adapt to mild stress, but not to severe stress (Bevilacqua et al., 2010; Krokowski et al., 2015). SNAT2 mRNA levels were gradually induced by mild stress similar to MEFs (Krokowski et al., 2015), but induction was absent in severe stress (Figure 1A). Increased SNAT2 mRNA was reflected in a higher uptake of its substrate, methylaminoisobutyric acid (MeAIB; Figure 1B), and accumulation of transporter protein (Figure 1C) during mild, but not severe, stress. We have shown previously that SNAT2 expression in MEFs is coordinated with the induction of GADD34 (Krokowski et al., 2015); a similar regulation was observed in corneal epithelial cells exposed to 500 mOsm, but not 600 mOsm, media (Figures 1A and 1C).

Induction of SNAT2 Is an Important Element of the Adaptation Program in Corneal Epithelial Cells

We first examined the role of SNAT2 in preventing hyperosmotic-stress-induced apoptosis. Severe hyperosmotic stress in MEFs decreases cell viability through induction of apoptosis via an intrinsic pathway (Bevilacqua et al., 2010; Saikia et al., 2014). Similarly, in corneal epithelial cells, apoptosis was induced by severe, but not mild, stress, as demonstrated by increases in the proteolytic cleavage of PARP and caspase-3 and the activity of caspase-3 (Figures S1A and S1B). We tested the importance of SNAT2 in protecting cells from apoptosis during mild stress by depleting SNAT2 mRNA through the use of SNAT2-directed short hairpin RNA (shRNA). SNAT2 depletion inhibited the induction of both SNAT2-mediated transport and SNAT2 protein levels during mild stress (Figures S1C and S1D). This reduction in stress-induced SNAT2 expression resulted in an increase in caspase-3 cleavage and activity (Figures S1D and S1E). We also found that absence of SNAT2 expression induces more caspase-3 cleavage and higher caspase-3 activity at earlier time points during the treatment of corneal epithelial cells with severe stress (Figures S1F and S1G). Under severe stress conditions, although SNAT2 mRNA is not induced (Figure 1A), its sustained basal levels offer protection from the early induction of apoptosis. Our data support the importance of SNAT2 in corneal epithelial cell osmoadaptation.

Maturation of the SNAT2 Protein Requires Active GADD34/PP1

The uptake of neutral amino acids by SNAT2 is crucial for adaptation to hyperosmolar conditions in corneal epithelial cells (Figure S1) and MEFs (Bevilacqua et al., 2005; Franchi-Gazzola et al., 2006). We showed previously that GADD34 expression had no impact on the levels of SNAT2 mRNA or its association with translating ribosomes, suggesting that translation of this mRNA is insensitive to the stress-induced inhibition of protein synthesis (Krokowski et al., 2015). Conceivably, the well-characterized ability of GADD34/PP1 to dephosphorylate eIF2a-P and reactivate translation during different stress conditions (Novoa et al., 2001) may not be important for SNAT2 expression. To test this directly, we used GADD34/PP1 inhibitors. Sal003, a salubrinal (Sal) derivative that inhibits the phosphatase activity of GADD34/PP1 (Boyce et al., 2005), caused an increase in eIF2a-P (Figure S2A). Sal003 also inhibited the increase in SNAT2 transport activity caused by hyperosmotic stress in a dose-dependent manner (Figure S2B). Sal003 inhibits dephosphorylation of eIF2a-P by targeting the interaction of PP1 with either CReP or
Figure 1. Adaptation of Human Corneal Epithelial Cells to Hyperosmotic Stress Involves SNAT2 and GADD34 Induction

(A) qRT-PCR analysis of mRNAs from cells treated with 500 or 600 mOsm media for the indicated times. mRNA levels were normalized to the levels of GAPDH mRNA and are presented as a fold of induction over control.

(B) SNAT2 activity as measured by 14C-MeAIB uptake in cells exposed to hyperosmotic media for the indicated times.

(C) Immunodetection of SNAT2 and GADD34 proteins in extracts from cells treated with 500 or 600 mOsm media for the indicated times. A nonspecific band on the immunoblot is indicated (#). Positions of protein size markers are indicated.

(D) Western blot analysis of the membrane fraction from cells treated with 500 mOsm media for 5 hr in the presence of Sal003 (30 μM). Positions of protein size markers are indicated.

(E) Immunodetection of SNAT2 in plasma membrane fractions. Biotinylated surface proteins and cell extract (10 μg) were analyzed by western blotting. Positions of protein size markers are indicated.

(F) Quantification of mature SNAT2 from (E) by densitometry. Signal intensities of mature SNAT2 were normalized to α-1 ATPase.

(G) Cellular distribution of SNAT2 (green channel) was visualized by confocal fluorescence microscopy. Nuclei are stained with Hoechst. Black and white insets show magnifications of boxed areas with green channel signal to demonstrate SNAT2 on the plasma membrane (white arrowheads). Scale bars represent 10 μm (main image) and 2 μm (insert).

Data in (A), (B), and (F) are presented as mean of 3 independent experiments ± SD.
GADD34 regulatory subunits (Hetz et al., 2013). To determine which subunit is important for SNAT2 expression, we tested the effects of guanabenz (Guan) and sephin 1 (Seph), which target GADD34/PP1, but not CReP/PP1 (Carrara et al., 2017; Das et al., 2015; Hetz et al., 2015; Tsaytler et al., 2011). These inhibitors have similar structures; however, guanabenz is also a potent agonist of the α2-adrenergic receptor (Das et al., 2015; Tsaytler et al., 2011). Because this receptor has been reported in corneal epithelial cells (Grueb et al., 2008), we studied both compounds to exclude any contribution of adrenergic signaling. GADD34/PP1 inhibitors showed dose-dependent inhibition of hyperosmolarity-induced SNAT2 transport activity (Figure S2C). None of these inhibitors significantly affected SNAT2 mRNA induction (Figure S2D).

We next tested whether SNAT2 PM localization in corneal epithelial cells requires GADD34/PP1 activity. Sal003 treatment decreased the accumulation of mature SNAT2 protein at the PM in corneal epithelial cells, as evidenced by analysis of total membrane fractions (Figure 1D) and fractions of proteins biotinylated on the cell surface (Figures 1E and 1F). In agreement with our biochemical data, hyperosmotic stress led to an increase in the SNAT2 signal, with a significant fraction visible on the cell surface (Figures 1G and S2E). Inhibition of GADD34/PP1 function by Sal003 treatment resulted in a dramatic decrease in SNAT2 delivery to the cell surface (Figures 1G and S2E) and a decrease in SNAT2-mediated amino acid uptake (Figure S2B) and levels of mature SNAT2 protein (Figures 1D and 1E).

To confirm that the effects of Sal003 are specific to GADD34/PP1, we depleted GADD34 protein using shRNA. Introduction of GADD34 shRNA into cells resulted in a decrease in stress-induced expression of GADD34 mRNA and protein (Figures S3A and S3C) but had no effect on the levels of SNAT2 mRNA, indicating that GADD34 is not involved in the regulation of SNAT2 gene expression (Figure S3A). Even though GADD34 shRNA did not affect SNAT2 mRNA levels, there was inhibition of the stress-induced increase in transport activity (Figure S3B). A decrease in protein levels was observed when western blots of whole-cell lysates and membrane fractions were analyzed (Figures S3C–S3E). Finally, downregulation of GADD34 expression led to an increase in apoptosis, as evidenced by increased caspase-3 cleavage and caspase-3 activity (Figures S3C and S3F). These data emphasize the adaptive functions of GADD34 during hyperosmotic stress.

To delineate the precise mechanism of action of GADD34 on SNAT2 protein maturation in corneal epithelial cells, we analyzed the presence of different forms of SNAT2 resolved in SDS-PAGE gels from cells in which GADD34/PP1 activity was inhibited. The addition of Sal003 resulted in a decrease in mature SNAT2 protein and an increase in a faster-migrating species (Figure 2A). The two forms of SNAT2 differ in their N-linked glycosylation status: the faster-migrating form corresponds to the ER-processed SNAT2 with endo-H-sensitive glycans (Freeze and Kranz, 2010) (immature SNAT2), whereas the slower-migrating form corresponds to the Golgi-localized SNAT2 with further processed endo-H-resistant glycans (Freeze and Kranz, 2010) (mature SNAT2) (Figure 2A). To confirm the identity of the rapidly migrating form, we used inhibitors of protein glycosylation and intracellular trafficking. The addition of tunicamycin (Tu), which blocks the assembly of N-linked glycans in the ER, caused accumulation of the fastest migrating form of SNAT2, consistent with the inhibition of N-linked glycosylation (Figure 2A, unglycosylated). The addition of inhibitors of COPI vesicle formation (brefeldin A [BFA] and golgicide A [GCA]) caused the pronounced accumulation of ER-glycosylated SNAT2 species relative to that observed with Sal003 treatment (Figure 2A). In brief, all of these inhibitors had an effect on intracellular SNAT2 trafficking to the trans-Golgi-located glycosyltransferases, which would modify the protein to form mature SNAT2 (Figure 2A).

Next, we examined how inhibition of GADD34/PP1 affects the SNAT2 distribution within the secretory pathway. The addition of Sal003 during hyperosmotic treatment increased the levels of immature protein (Figures 2A–2C), suggesting a retention of SNAT2 in compartments proximal to trans-Golgi, where terminal glycosylation occurs. Retention of the protein in the ER would make it a substrate for endoplasmic-ribosome-associated protein degradation (ERAD; Ruggiano et al., 2014). To evaluate the potential contribution of ERAD to clearance of SNAT2 from the ER compartment, we used the proteasome inhibitor MG132, which blocks the final degradation steps. MG132 caused an accumulation of unglycosylated SNAT2 and the expected increase in levels of ubiquitinated proteins. The accumulation of unglycosylated SNAT2 was only observed in MG132-treated cells, suggesting that any unglycosylated intermediate is normally cleared rapidly by the proteasome. Nevertheless, MG132 did not affect the level of immature ER-glycosylated SNAT2 in stressed cells with or without GADD34/PP1 inhibition (Figures 2B and 2C), suggesting that this form is not degraded by ERAD.

These data are consistent with the idea that Sal003 treatment does not induce accumulation of the immature form in the ER.

Figure 2. Hyperosmotic-Stress-Induced GADD34/PP1 Activity Promotes Post-ER SNAT2 Protein Processing in Corneal Epithelial Cells
(A) Western blot analysis of extracts from cells treated with 500 mOsm media alone for 5 hr or supplemented with Sal003 (30 μM), tunicamycin (Tu; 500 nM), brefeldin A (BFA; 20 μM), or golgicide A (GCA; 20 μM). Positions of protein size markers are indicated.
(B) Western blot analysis of total cell extracts from cells treated with 500 mOsm media for 5 hr with or without Sal003 (30 μM) supplementation. MG132 (100 μM) was added for the last 1 hr of treatment. Cell extracts from cells treated with 500 mOsm media and tunicamycin (Tu) or brefeldin A (BFA) were analyzed by loading one-third the amount of the other samples.
(C) Quantification of immature (left) and unglycosylated (right) SNAT2 levels from cells treated as in (B). Signal intensities were normalized to α-tubulin.
(D) Subcellular distribution of SNAT2 (green channel) and ER-resident proteins (red channel, visualized by anti-KDEL antibody staining) in cells grown in control or 500 mOsm media for 5 hr with or without Sal003 (30 μM) addition. Scale bars, 10 μm.
(E) Boxed image areas from (D). White arrowheads indicate SNAT2 protein, and dotted arrows point to KDEL-positive structures. Scale bar, 1 μm.
(F) Quantification of SNAT2 co-localization with KDEL reporter in cells exposed to 500 mOsm media with or without Sal003. Masks of SNAT2 and KDEL signals were created and overlap between areas was calculated in 4 separate planes. 9 and 11 cells were analyzed, respectively.
Data are presented as mean ± SD.
Figure 3. Osmoprotective Functions of GADD34/PP1 in Corneal Epithelial Cells Are Mediated through Reversion of Hyperosmotic-Stress-Induced Golgi Apparatus Fragmentation

(A) Immunofluorescent staining of GM130 (green channel) in cells incubated in control or hyperosmotic (600 mOsm) media for 3 hr. Scale bar, 10 μm.

(B) Quantification of Golgi fragmentation in cells incubated in the indicated conditions for 3 hr.

(C) Quantification of Golgi fragmentation in cells exposed to 500 mOsm media for the indicated times.

(D) Quantification of Golgi fragmentation in cells expressing shRNA against GADD34 incubated in 500 mOsm media.

(E) Quantification of Golgi fragmentation in cells treated with the indicated concentrations of GADD34/PP1 inhibitors in control media for 2 hr.

(legend continued on next page)
compartment. To further confirm this notion, we examined the effects of hyperosmotic stress and Sal003 on SNAT2 distribution using immunofluorescence microscopy. Cells were stained for SNAT2 (green) and the ER marker KDEL (red). KDEL staining showed the characteristic pattern for ER-reticular staining with perinuclear concentration. None of the treatments used significantly affected KDEL distribution. SNAT2 staining was weak in untreated cells and control cells treated with Sal003 (Figure 2D). It consisted of punctate structures with little to no co-localization to KDEL staining (Figures 2D and 2E). Hyperosmotic stress resulted in significant increase in SNAT2 staining intensity and redistribution of SNAT2 to vesicular structures, which do not co-localize with KDEL and possibly correspond to SNAT2 post-Golgi trafficking and PM localization (Figure 2E, white arrowheads). The addition of Sal003 to cells experiencing hyperosmotic stress neither affected the increase in SNAT2 intensity nor induced its significant retention in the ER, but it did block SNAT2 trafficking to the PM (Figures 2D–2F). Therefore, ER retention of SNAT2 in cells lacking functional GADD34/PP1 cannot account for the accumulation of immature transporter during hyperosmotic stress. The majority of SNAT2 in stress conditions with or without Sal003 is localized outside of the ER in Golgi-like structures.

GADD34/PP1 Is Involved in Maintaining Golgi Integrity

To further understand the mechanism of action of GADD34/PP1 on SNAT2 post-ER processing during hyperosmotic stress, we examined Golgi morphology and SNAT2 distribution in cells with or without active GADD34/PP1. Our analysis of SNAT2 protein during adaptation to hyperosmotic stress emphasized the importance of its intracellular transport and glycosylation. Because the Golgi complex is vital for trafficking of SNAT2 to the PM, we examined the effect of hyperosmotic stress on Golgi morphology using GM130 as a Golgi marker. Hyperosmotic stress induced Golgi fragmentation (Figure 3A), and the percentage of cells with fragmented Golgi complexes increased with stress intensity (Figure 3B). The fragmentation during mild stress was transient; after the initial increase in fragmentation at 1 hr, the Golgi complex partially recovered its morphology (Figure 3C). This recovery corresponded with the timing of SNAT2 and GADD34 induction (Figures 3C and 1A–1C). Therefore, we conclude that during mild stress, the initial fragmentation of the Golgi is followed by a recovery that correlates with the expression of the osmostable membrane protein SNAT2.

Because GADD34/PP1 is required for the stress-induced expression and maturation of SNAT2 (Figures 1D–1G, S2, and S3), we studied its effects on Golgi fragmentation. Silencing of GADD34 expression by shRNA increased the number of cells with fragmented Golgi in both control and hyperosmotic media (Figures 3D and S3G). The effect of GADD34 silencing was most evident after prolonged stress, when physiological GADD34 levels are induced to support the adaptation to stress. In agreement with a role of GADD34/PP1 in maintaining Golgi morphology, the addition of GADD34/PP1 inhibitors (Sal003 and sephin 1) caused a dose-dependent increase in Golgi fragmentation in control cells (Figure 3E), with Sal003 having a more prominent effect. Sephin 1 is a very selective GADD34/PP1 inhibitor (Carrara et al., 2017; Das et al., 2015); in contrast, Sal003 has an additional inhibitory effect on PP1 when it contains the constitutively expressed regulatory subunit CRP instead of GADD34 (Choy et al., 2015). We next tested whether the effects of GADD34/PP1 on Golgi fragmentation involve the well-known eIF2α-P-mediated responses in the ISR. It is well established that during the ISR, GADD34 promotes dephosphorylation of eIF2α-P with the subsequent reversal of eIF2α-P-mediated stress-induced reprogramming of cells, including reversal of the induction of the transcription factor ATF4, the master regulator of the stress response (Novoa et al., 2001; Sidrauski et al., 2015). The integrated stress response inhibitor (ISRIB) has been shown to reverse eIF2α-P-mediated stress-induced effects independently of the actions of GADD34/PP1 (Di Prisco et al., 2014; Sidrauski et al., 2015). If the effects of GADD34/PP1 on Golgi fragmentation during hyperosmotic stress are independent of its target, eIF2α-P, then treatment of cells with ISRIB should not reverse sephin-1-induced Golgi fragmentation. Treatment of cells with ISRIB combined with sephin 1 did not prevent the induction of fragmented Golgi (Figure 3F). As expected (Sidrauski et al., 2015), ISRIB treatment of corneal cells during thapsigargin (Tg)-induced ER stress reversed the induction of ATF4, but not the non ISR-mediated splicing of XBP1 mRNA (Figure S3H). Taken together, these findings show that inhibition of GADD34/PP1 induces Golgi fragmentation via mechanisms that do not involve eIF2α-P-mediated effects in the ISR.

Both microtubules and actin filaments undergo dynamic changes in response to cell shrinkage induced by hyperosmolarity (Nunes et al., 2013; Yamamoto et al., 2006). Fitness of the Golgi apparatus, including proper cellular distribution of Golgi cisterns and transport events that couple microtubules and motor proteins that drive motility, rely on the proper assembly of microtubules (Allan et al., 2002). We therefore investigated changes in the microtubule and actin networks in corneal cells exposed to hyperosmotic stress. In untreated cells, actin exhibited peripheral accumulation in cortical rims and actin stress fibers (Figure 3G). The microtubules were distributed across the cytoplasm and extended to the PM (Figure 3H). Shortly after exposure to hyperosmotic stress, we observed an increase in actin stress fibers and a decrease in microfilaments interacting with the PM. These cytoskeletal network changes were reversed during longer stress treatment (Figure 3G), and this reversal was inhibited by treatment of cells during stress with the GADD34/PP1 inhibitor sephin 1 (Figures 3G and 3H). These data suggest an involvement of GADD34 in the regulation of tubulin polymerization.
which can also explain its functions on maintaining Golgi integrity.

Our results point to the importance of GADD34/PP1 in promoting Golgi integrity under basal conditions and during adaptation to stress. It is possible that the action of GADD34/PP1 via unknown targets is necessary to reverse the initially high Golgi fragmentation as part of the mechanism that promotes SNAT2 maturation and PM-localized activity.

Induction of Golgi Fragmentation Results in Decreased SNAT2 Expression and Processing during Hyperosmotic Stress

The regulated fragmentation of the Golgi during mild hyperosmotic stress reveals a mechanism of osmoadaptation via the coordinated actions of GADD34/PP1 and SNAT2. Golgi fragmentation has been shown to have a negative impact on the trafficking of some proteins but a stimulatory effect on the trafficking of others (Xiang et al., 2013). It was previously shown that the GADD34 interactome includes cytoskeletal elements, as well as KIF3A, a motor protein driving intracellular, microtubule-based anterograde transport (Chambers et al., 2015; Hasegawa et al., 2000). First, we tested whether this association is maintained in hyperosmolar conditions.

We used cornea epithelial cells transiently expressing a FLAG-tagged GADD34 protein and attempted to co-immunoprecipitate α-tubulin and KIF3A, as well as the known interactors eIF2α and the PP1γ subunit of PP1 phosphatase. All four proteins were found to interact with GADD34 in control and stress conditions when cytoplasmic protein GAPDH was absent in the immunoprecipitations (Figure S4A). At the same time, inhibition of GADD34/PP1 by sephin 1 did not change the interaction of GADD34 with α-tubulin, KIF3A, and eIF2α (Figure S4A).

We next tested the effect of nocodazole (Noc), a microtubule polymerization inhibitor, on the subcellular distribution of SNAT2 during hyperosmotic stress. In the presence of Noc throughout the entire treatment with hyperosmotic media (5 hr), we did not observe SNAT2 localization to the PM but instead observed an intracellular punctate pattern (Figure S4B).

To further explore the action of Noc on SNAT2 maturation, we treated cells with this inhibitor for the last 2 hr of a 5-hr stress treatment. This strategy allows for both accumulation of SNAT2
Figure 5. Inhibition of GADD34/PP1 Results in the SNAT2 Retention in cis-Golgi in Corneal Epithelial Cells Exposed to Hyperosmotic Stress

(A) Intra-Golgi localization of SNAT2 in cells treated with 500 mOsm media with or without Sal003 (30 μM) for 5 hr, where the cis-Golgi marker is GM130 and the trans-Golgi marker is TGN46. Arrows point to the areas enlarged in (B). Note Golgi fragmentation and separation of the trans-Golgi blue color from SNAT2 and GM130 in Sal003-treated cells. Scale bar, 10 μm.

(B) Line-scan analysis of SNAT2 intra-Golgi localization, with representative examples of line scans and plots of three channel intensities through regions with separated cis- and trans-Golgi. Scale bar, 1 μm.

(C) Representative Golgi morphology and overlap between cis- and trans-Golgi staining in cells treated with 500 mOsm media with or without Sal003.

(D) Graph represents quantitation of analysis of 14 line scans from 6 cells treated with 500 mOsm media and 45 line scans taken from 14 cells treated with 500 mOsm media in the presence of Sal003 (30 μM), respectively. Data are presented as mean ± SD.

(legend continued on next page)
protein and observation of its continuing maturation in the presence of the inhibitor. Inhibition of microtubule polymerization resulted in accumulation of the immature form of SNAT2 (Figure S4C) and subsequent decrease of transporter activity (Figure S4D). These data suggested decreased maturation and delivery of SNAT2 to the PM after disruption of the microtubule network. Noc (last 2 hr of treatment with stress) induced disassembly of microtubules and diffused staining of monomeric α-tubulin (Figure S4E). At the same time, the Golgi apparatus was fragmented, as shown by the distribution of the cis-Golgi marker GM130 (Figure S4E).

We also used a genetic model of Golgi fragmentation by depletion of the Golgi reassembly stacking protein 2 (GORASP2) using shRNA. GORASP2 establishes the stacked morphology of the Golgi, and its depletion was previously shown to induce Golgi fragmentation (Xiang et al., 2013). GORASP2 silencing resulted in lower levels of SNAT2 protein both in the basal condition and upon exposure to mild hyperosmotic stress (Figure 4A). These data suggest that Golgi fragmentation has a negative effect on SNAT2 maturation. Next, we tested whether increased Golgi fragmentation due to stress intensity would have similar effects on SNAT2 maturation. Cells exposed to mild stress (500 mOsm) followed by a switch to 600 mOsm media showed an increase in the percentage of cells with fragmented Golgi (Figure 4B). We then used this experimental paradigm to further examine the maturation of SNAT2. Cells were exposed to mild hyperosmotic stress for 3 hr, which caused the accumulation of both mature and immature SNAT2 (Figure 4C, immature SNAT2). Cells were subsequently incubated in 500 or 600 mOsm media for an additional 2 hr in the presence of cycloheximide (CX) to prevent de novo SNAT2 protein synthesis during this time frame. In 500 mOsm media, the immature form diminished after 30 min. In contrast, a shift to 600 mOsm media delayed the anterograde trafficking of the immature form, and the levels of mature SNAT2 did not change (Figures 4C, 4D, and S4F). These data strongly support the conclusion that maturation and delivery of SNAT2 to the PM requires Golgi integrity and are negatively correlated with the degree of Golgi fragmentation and the intensity of stress.

To demonstrate the contribution of GADD34/PP1 to SNAT2 maturation during mild stress, we hypothesized that GADD34/PP1 inhibition after 3 hr of exposure to cells to mild stress would result in immature transporter accumulation. In this condition, inhibition of GADD34/PP1 by Sal003 induced eIF2α phosphorylation and accumulation of immature SNAT2 (Figures 4E and 4F). We then incubated cells with hyperosmotic media in the presence of CX with or without Sal003 and studied the trafficking of immature SNAT2 protein (Figures 4G and 4H). The existing immature SNAT2 declined during subsequent incubation in the absence of Sal003, consistent with its export from the ER and processing in the Golgi. Significantly, the inhibition of GADD34/PP1 by Sal003 delayed the loss of immature SNAT2, consistent with decreased conversion to the mature form by glycosylation in the Golgi.

**GADD34/PP1 Inhibition Delays Trafficking of SNAT2 from cis- to trans-Golgi**

Immunofluorescent staining of SNAT2 clearly demonstrated a very strong cisternae-like pattern of SNAT2 subcellular distribution, which suggests that a significant amount of the protein in the cell is localized to the Golgi complex at any given time (Figure 1G), with or without GADD34.

To study the movement of SNAT2 through the Golgi and the contribution of GADD34 to this process, cells were exposed to mild hyperosmotic stress for 5 hr with or without the addition of Sal003, and SNAT2 intra-Golgi distribution was analyzed. GM130 was used as a cis-Golgi marker (labeled red), TGN46 was used as a trans-Golgi marker (labeled blue), and SNAT2 was labeled using green color (Figure 5A). We adopted line-scan methodology for intra-Golgi distribution of proteins, which was originally developed to analyze the localization of resident Golgi proteins (Dejgaard et al., 2007). This approach is based on visual separation between cis- and trans-Golgi ribbons in order to assign the protein of interest to either compartment. In cells experiencing hyperosmotic stress, the peak SNAT2 signal spreads between peaks of cis- and trans- Golgi (GM130 and TGN46) (Figure 5B), which correspond to the trafficking of “cargo” protein through different cis- and trans-Golgi compartments in order to assign the protein of interest to either compartment.

During the analysis, we noted that in corneal epithelial cells experiencing hyperosmotic stress for 5 hr, the Golgi structure is very compact, which is shown as the almost complete overlap of red and blue signal (Figure 5C). In the presence of Sal003, the separation of cis- and trans-Golgi markers was clearly visible (Figure 5C, right), and a significant amount of TGN46 (blue signal) was not being masked by GM130 (red). The same changes from compacted Golgi structure to visibly separated cis- and trans-Golgi compartments were obtained using a different trans-Golgi marker protein, the UDP-galactose transporter SLC35A2 (Song, 2013). This effect of Sal003 is very similar to the well-described action of Noc, which increases separation between cis- and trans-Golgi compartments in HeLa cells (Dejgaard et al., 2007) due to the effects of Noc on microtubules. Indeed, Noc addition resulted in an increased distance between cis- and trans-Golgi markers in corneal epithelial cells (Figure S5A). Importantly, not only the addition of Sal003 but also expression of shRNA targeting GADD34 led to separation between cis- (GM130) and trans-Golgi (SLC35A2) markers (Figures S5A–S5C). Therefore, inactivation of GADD34/PP1 by Sal003 or shRNA resulted in Golgi

---

(E) Subcellular localization of SNAT2 and the cis-Golgi marker GM130 in cells treated with 500 mOsm media for 5 hr with or without Sal003 (30 μM). Scale bar, 10 μm.

(F) Magnifications of cell area as indicated in (E). Note SNAT2 areas that do not overlap with GM130 staining in 500-mOsm-treated cells and their disappearance upon Sal003 addition. Scale bar, 1 μm.

(G) Quantification of a SNAT2 area overlapping with GM130. Data are presented as the mean ± SD of 3 experiments.
fragmentation, which was accompanied by a high degree of separation between cis- and trans-Golgi regions.

Analysis of SNAT2 intra-Golgi distribution (Figure 5D) suggested that the absence of GADD34 leads to decreased SNAT2 trafficking from cis- to trans-Golgi. The percentage of the area stained by SNAT2 (green color) and GM130 overlapping within single planes was calculated. In stressed cells, the steady-state distribution of SNAT2 was wider than that of GM130. There was only partial co-localization between these two proteins, and a significant portion of the SNAT2 signal was localized in close proximity (but without exclusive overlap) with GM130 (Figures 5E–5G). This is explained by SNAT2 transitioning through cis- and trans-Golgi. The addition of Sal003 resulted in a significant increase in co-localization of SNAT2 with GM130 (Figures 5E–5G). We additionally confirmed these results in corneal cells expressing shRNA against GADD34. In cells with decreased levels of GADD34 exposed to hyperosmotic media, co-localization of SNAT2 with the cis-Golgi marker was higher as compared to shCon (Figures 5G and S5D). Therefore, inactivation of GADD34 genetically or pharmacologically leads to retention of SNAT2 in the cis-Golgi compartment.

In conclusion, adaptation to mild hyperosmotic stress is characterized by fast processing and trafficking of SNAT2 through the Golgi complex (from cis- to trans-Golgi). The activity of GADD34/PP1 is required to maintain Golgi integrity during stress, which promotes maturation of SNAT2 during processing and trafficking to the PM. In the absence of functional GADD34/PP1, Golgi morphology does not recover from stress-induced fragmentation, resulting in increased separation of cis-trans-compartments and trapping of SNAT2 in the cis-Golgi, with significant inhibition of its membrane localization.

Function of GADD34 in SNAT2 Maturation Is Independent of eIF2α Phosphorylation Status

A well-studied function of the GADD34/PP1 phosphatase is the dephosphorylation of eIF2α and the subsequent regulation of protein synthesis rates (Novoa et al., 2001). Our studies with ISRIB (Figure 3F) suggested that the effects of GADD34/PP1 on maturation and trafficking of SNAT2 during hyperosmotic stress are independent of its target, eIF2α-P. We further tested this idea in MEFs with a homozygous mutation of the eIF2α phosphorylation site (S51A). We previously reported that hyperosmotic stress induces SNAT2 and GADD34 in MEFs (Krokowski et al., 2015). Similar to wild-type (WT) cells, GADD34 and SNAT2 were induced in S51A MEFs (Figure 6A and S6A). SNAT2 protein localized in membranes (Figures 6B and S6B) and was functional, as demonstrated by the increased uptake of its substrate, MeAIB (Figure 6C). Similarly to corneal epithelial cells and WT MEFs (Krokowski et al., 2015), induction of transporter activity was attenuated when GADD34/PP1 was inhibited without a decrease in SNAT2 mRNA levels (Figures 6C and 6D). To demonstrate that the effects of GADD34/PP1 activity on SNAT2-mediated transport is not due to changes in SNAT2 protein levels, we compared the levels of an N-terminal fragment of the SNAT2 protein in cell extracts treated with o-iodoxybenzoic acid (IBX), which breaks polypeptide chains on tryptophan residues. This approach releases a ~20 kDa (1~190 aa) fragment at the N terminus of SNAT2, which does not contain glycosylated asparagine residues (Figure S6C). This fragment migrates as a single band in SDS-PAGE and is recognized by the antibody directed against SNAT2, thus allowing a direct comparison of SNAT2 protein levels between different samples. As expected, the N-terminal SNAT2 protein fragment was induced by hyperosmotic stress (Figure S6D). Neither Sal003 nor sephin 1 decreased this induction (Figure S6D). These data support the conclusion that the action of GADD34/PP1 on SNAT2 maturation is independent of SNAT2 gene transcription or SNAT2 mRNA translation (Krokowski et al., 2015).

Inhibition of GADD34/PP1 in S51A MEFs resulted in the accumulation of the immature SNAT2 protein corresponding to the ER glycosylated form, similar to the observations in corneal epithelial cells (Figure 6E). Furthermore, genetic depletion of GADD34 decreased levels of mature SNAT2 protein (Figure 6F). We next confirmed that similar to the observations in corneal epithelial cells, both hyperosmotic stress and inhibition of GADD34/PP1 induced Golgi fragmentation (Figures 6G and S6E). Furthermore, the effect of GADD34 inhibitors is specific to their target protein, as GADD34/PP1-deficient MEFs...

Figure 6. GADD34 Promotes SNAT2 Maturation Indepentently of eIF2α Phosphorylation Status in Mouse Embryonic Fibroblasts

Experiments were conducted in mouse embryonic fibroblasts expressing mutated eIF2α protein (S51A MEFs).

(A) Immunodetection of SNAT2 protein in extracts from cells exposed to 500 mOsm media for the indicated times.

(B) Immunodetection of SNAT2 in membrane fractions from cells treated as in (A).

(C) 14C-MeAIB uptake in cells exposed to 500 mOsm media for 5 hr with or without Sal003 (30 μM) sephin 1 (Seph; 100 μM) or guanabenz (Guan; 100 μM).

(D) qRT-PCR analysis of SNAT2 mRNA levels from cells treated with 500 mOsm media for 5 hr in the presence of GADD34/PP1 inhibitors. Values were normalized to GAPDH mRNA and are plotted as a fold of induction over control.

(E) Western blot analysis of extracts from cells treated with 500 mOsm media for 5 hr in the presence of Sal003 (30 μM), tunicamycin (Tu; 500 nM) or golgicide A (GCA; 20 μM). A nonspecific band is indicated (#). Positions of protein size markers are marked.

(F) Western blot analysis of extracts from cells expressing shRNA against GADD34 and treated with 500 mOsm media.

(G) Quantification of Golgi fragmentation in cells incubated in the indicated stress conditions for 3 hr or treated with the GADD34/PP1 inhibitors Sal003 (30 μM) or sephin 1 (Seph; 50 μM) in control media for 2 hr.

(H) Immunofluorescent staining of F-actin (green channel) and α-tubulin (red channel) in control cells and after exposure to 500 mOsm media with or without sephin 1 (Seph; 50 μM). Scale bar, 20 μm (top). The 3 lower panels represent magnifications of boxed areas from the top panel (scale bar, 5 μm).

(I) Immunodetection of SNAT2 protein in extracts from cells incubated in 500 mOsm for 5 hr with the indicated concentrations of nocodazole during the last 2 hr of treatment.

(J) Immunofluorescent staining of α-tubulin (red channel) and the Golgi marker GM130 (green channel) in cells treated with 500 mOsm stress with or without nocodazole (Noc; 2 μM). Scale bar, 20 μm.

Data in (C) and (G) are presented as the mean ± SD of 3 experiments.
(GADD34ΔC/ΔC) did not induce Golgi fragmentation when treated with sephin 1 (Figure S7A). GADD34ΔC/ΔC MEFs express a truncated GADD34 protein that is unable to interact with the PP1 holoenzyme (Novoa et al., 2003). As expected, Sal003 caused some increase in Golgi fragmentation, most likely due to its inhibitory effect on CReP (Figure S7A). We next showed that hyperosmotic stress induced significant changes in the microtubule network in S51A MEFs. Short exposure to mild stress (500 mOsm, 30 min) resulted in the detachment of the microtubule structures from the PM, which was shown by the actin-positive peripheral zones without α-tubulin signal (Figure 6H). As adaptation progressed (5 hr of stress), the microtubule network was restored and the cytoplasm was uniformly labeled with both F-actin- and α-tubulin-positive structures (Figure 6H). The presence of sephin 1 in control or hyperosmotic media resulted in the retraction of microtubules from the PM, with minor changes in the actin network (Figure 6H). These data further support the conclusion that changes in the dynamics of tubulin polymerization can be the cause of Golgi fragmentation in response to GADD34/PP1 inhibition. To further support this hypothesis, we treated stressed cells with Noc (Minin, 1997). Noc had effects similar to the GADD34/PP1 inhibitor, indicated by the accumulation of immature SNAT2 (Figure 6I). Immuno- fluorescence staining revealed that the expected disassembly of microtubules was also accompanied by Golgi fragmentation (Figure 6J).

In summary, inhibition of GADD34/PP1 induced changes in the microtubule network and increased Golgi fragmentation in a manner independent of its functions on eIF2α-P. Furthermore, the decreased SNAT2 activity upon GADD34/PP1 inhibition is the result of inhibited SNAT2 protein processing and maturation, resulting in accumulation of the immature ER-glycosylated form. This function of GADD34/PP1 is independent of the well-established actions of phosphatase in the regulation of protein synthesis during stress.

Genetic Depletion of Active GADD34/PP1 Attenuates GPI-EGFP Trafficking from the ER to the PM during Hyperosmotic Stress

To test whether the effects of GADD34 on Golgi integrity and protein trafficking are extended to other than SNAT2 cargo proteins during hyperosmotic stress, we used RUSH technology. Two fusion proteins were introduced into the cells: the ER anchor KDEL fused to streptavidin and the cargo reporter GPI-EGFP fused to the streptavidin-binding peptide (Boncompain et al., 2012). GPI protein was used as a cargo reporter because it undergoes posttranslational modifications in the Golgi apparatus (Knoshita et al., 2013). The KDEL-streptavidin anchor ensures ER localization of GPI-EGFP. The addition of biotin disrupts the anchor-cargo interaction and induces a synchronous release of GPI-EGFP from the ER. GPI-EGFP release was tested in WT and GADD34ΔC/ΔC MEFs subjected to hyperosmotic stress. Prior to the addition of biotin at time point 0 in MEFs transfected with the plasmid expressing both proteins, GPI-EGFP showed uniform distribution, with characteristics of an ER-reticular pattern (Figure 7A). Upon the addition of biotin, GPI-EGFP was released from the ER and trafficked to the Golgi apparatus (center-located structure), from where it subsequently reached the PM (Figure 7A).

We observed that, after 40 min of biotin treatment of WT MEFs, the majority of GPI-EGFP localizes to Golgi-like structures in over 90% of the cells, whereas, after 60 min, GPI-EGFP clears from the Golgi and reaches the PM in over 75% of the cells (Figure S7B). Therefore, 40 min is an appropriate time point to analyze trafficking of GPI-EGFP to the Golgi, and 60 min is a proper time point to analyze trafficking from the Golgi to the PM.

Next, the effect of hyperosmolar stress on trafficking of GPI-EGFP was compared in WT and GADD34ΔC/ΔC MEFs. 40 min after the addition of biotin, GPI-EGFP reached the central Golgi-like structure as well as some vesicular intracellular compartments in the majority of cells. No significant differences were found between WT and GADD34ΔC/ΔC cells (Figure S7B; compare the total number of cells with GPI-EGFP in the Golgi). Therefore, trafficking of GPI-EGFP to the Golgi does not depend on functional GADD34. 60 min after the addition of biotin, as
expected from the time course in unstimulated cells, GPI-EGFP cleared from the Golgi and reached the PM in 75% of WT MEF cells (Figure 7B). During hyperosmotic stress, clearance of GPI-EGFP from the Golgi-like structures and delivery to the PM (cells as in Figure 7A, PM, no visible central Golgi-like structure) was significantly slower in GADD34 trans/GADD34 cis MEFs (only 49% of the cells cleared GPI-EGFP from Golgi) (Figures 7 and S7B). Moreover, Golgi trapping and slow PM delivery can be seen in GADD34 trans/GADD34 cis MEFs in both control and hyperosmotic stress conditions, but the effect is more prominent in cells exposed to stress (Figures 7B and S7B). GADD34 regulates trafficking and maturation of proteins modified through the Golgi apparatus, especially in cells exposed to stress.

**DISCUSSION**

We report here the response of cultured human corneal epithelial cells to increased osmolarity generated by sodium chloride, a physiologically relevant solute of the tear film (Stahl et al., 2012). Mild stress (500 mOsm) triggered an osmoadaptive program that involved the actions of GADD34/PP1 phosphatase and the amino acid transporter SNAT2, which facilitates the uptake of compatible osmolytes. This osmoadaptation mechanism was shown to consist of (1) mild-stress-induced gene expression of both SNAT2 and the regulatory subunit of the PP1 phosphatase GADD34 and (2) post-translational processing of SNAT2 via the Golgi apparatus and subsequent localization to the PM via the positive actions of GADD34/PP1 on attenuating stress-induced Golgi fragmentation. In contrast, the lack of SNAT2 and GADD34 induction during severe hyperosmotic stress (600 mOsm) abolished osmoadaptation.

Adaptation to increased extracellular osmolarity has been extensively studied at the level of transcriptional control (Burg et al., 2007). We show here that GADD34/PP1 is required for the adaptive recovery of Golgi integrity in response to mild hyperosmotic stress. In agreement with the adaptive role of GADD34 in protein trafficking, we have also shown that GADD34/PP1 inhibition does not attenuate increased transcription (Krokowski et al., 2015) or stress-insensitive translation of SNAT2 mRNA (Gaccioli et al., 2006). Instead, it inhibits SNAT2 protein maturation in the Golgi. SNAT2 as a membrane protein undergoes numerous maturation steps on its way from the ER through the Golgi to the cell surface. The glycosylation of two asparagine residues of the SNAT2 protein in the ER is followed by extensive modification in the Golgi (Bröer, 2014). Inactivation of GADD34 caused accumulation of the ER-glycosylated form in cis-Golgi and a decrease in the levels of the mature transporter protein on the PM.

The exact mechanism by which GADD34/PP1 activity assists SNAT2 protein maturation is not known. However, our data suggest that during hyperosmotic stress, the induction of GADD34 is associated with the rescue from stress-induced changes in tubulin polymerization and Golgi fragmentation. Recovery from these changes promotes SNAT2 maturation. Here, we observed that the hyperosmotic-stress-induced initial microtubule detachment from the PM was followed by restoration of the microtubule network. This reversal correlated with accumulation of GADD34 protein levels. Inhibition of GADD34/PP1 showed (1) a retraction of the microtubule network from the cell periphery, (2) fragmentation and a spatial separation between the cis- and trans-cisternae of the Golgi apparatus, (3) decreased accumulation on the PM of Golgi-processed SNAT2, and (4) decreased presence of microtubule threads extending to the cell periphery. Moreover, fragmentation of Golgi and separation of cis-cisternae upon GADD34/PP1 inhibition or depletion are strikingly similar to the effects of Noc, an inhibitor of microtubule polymerization (Dejgaard et al., 2007; Sandovai et al., 1984). Hyperosmotic stress has a pronounced effect on the microtubule network, with transient depolymerization upon exposure to stress and reassembly during adaptation (Nunes et al., 2013). Hyperosmolarity has been shown to induce a series of phosphorylation events, including modification of α-tubulin and stathmin, proteins involved in cytoskeletal organization (Ban et al., 2013; Yip et al., 2014). It is therefore possible that GADD34/PP1 modulates phosphorylation events on unknown targets that are involved in microtubule organization during hyperosmotic stress. In support of this idea, numerous cytoskeletal elements, including α- and β-tubulins (Chambers et al., 2015), as well as a microtubule-based anterograde intracellular transport motor protein of the kinesin family (Hasegawa et al., 2000), were found to be part of the GADD34 interactome. Future studies will determine how GADD34/PP1 regulates cytoskeleton dynamics during hyperosmotic stress and thereby promotes Golgi integrity and insures the successful onset of osmoadaptation.

Our finding that GADD34/PP1 promotes SNAT2 cis- to trans-Golgi trafficking may also imply a direct action of the phosphatase on the Golgi apparatus that is independent of the microtubule network. Golgi fragmentation is a regulated process. For example, the Golgi undergoes extensive transient fragmentation at the onset of mitosis. Several protein kinases that are activated by hyperosmotic stress are also involved in the regulation of Golgi integrity during cell division. Because phosphorylation is involved and global inhibition of phosphatases has been shown to induce Golgi fragmentation (Lucqc et al., 1991), it is possible that GADD34 is required to reverse stress-induced changes in the Golgi apparatus. This would involve unknown targets of the GADD34/PP1 phosphatase. The subcellular localization of GADD34 supports this hypothesis. In stressed cells, GADD34 is recruited to intracellular membrane compartments via its N-terminal domain (Brush et al., 2003), which can assist the proximity of the phosphatase complex with proteins involved in Golgi integrity.

The uptake of amino acids during hyperosmotic stress is known to be an important part of the RVI. Of the ten most abundant amino acids in tears, five are substrates for the uptake by SNAT2 (Nakatsukasa et al., 2011). In addition, amino acids (including the SNAT2 substrates Gly and Pro) have been used in teardrops to treat dysfunctional tear production (Aragona et al., 2013). Our research provides a rationale for using amino acids as a component of tear substitutes and offers an interesting opportunity to use amino acids and non-metabolized amino acid analogs as a potential pharmacological intervention to increase adaptation as a treatment for dry eye syndrome. In addition to the benefits of our studies for ocular diseases, our finding that GADD34/PP1 functions in Golgi...
integrity reveals an unrecognized molecular mechanism that can control the sensitivity of cells to stress-induced Golgi fragmentation and therefore influence the development of diseases in a cell-type-specific manner.

**EXPERIMENTAL PROCEDURES**

**Cells**
Immortalized human corneal epithelial cells (10,014 pRSV-T) were grown in Keratinocyte-SFM (Thermo Fisher Scientific) with 5 ng/mL epidermal growth factor, 0.05 mg/mL bovine pituitary extract, 0.005 mg/mL insulin, and 500 ng/mL hydrocortisone on dishes coated with fibronectin, type I bovine collagen, and BSA. MEFs were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept in a humidified atmosphere of 5% CO2 at 37°C.

**Viral Particles and Chemicals**
Propagation of lentiviral particles expressing shRNA against human SNAT2 and GORASP2 and human and mouse GADD34 (TRCN0000020239, TRCN0000278406, TRCN0000003041, and TRCN0000353349, respectively; Sigma-Aldrich) or empty vector (pLKO.1) was performed in HEK293T cells. The osmolarity of media was increased by addition of NaCl for corneal epithelial cells or sorbitol for MEFs. Sal003, Noc, ISRIB, breffelin A, and golcicide A were purchased from Tocris, and CX, MG132, guanabenz, and tunicamycin were purchased from Sigma-Aldrich. Sephin 1 was a generous gift from Anne Bertolotti (University of Cambridge, UK).

**SNAT2 Amino Acid Transporter Activity**
SNAT2-mediated amino acid uptake (system A activity) was measured as previously described (Krokowski et al., 2015). Cells were grown in 24-well plates, and the uptake of 14C-MeAIB was measured in Earle’s balanced salt solution in the presence of sodium ions for 3 min at 37°C. Cells were washed twice with ice-cold PBS to remove MeAIB, and the intracellular amino acid pool was extracted with absolute ethanol. Radioactivity was measured using a scintillation counter and normalized to protein content.

**Statistical Methods**
To evaluate statistical significance, a one-way or two-way ANOVA with post hoc Bonferroni test was used. Analysis was conducted using Origin software. A p value of less than 0.05 was considered statistically significant (*p < 0.05, **p < 0.01, and ***p < 0.001).

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.027.

**AUTHOR CONTRIBUTIONS**

**ACKNOWLEDGMENTS**
The authors thank Kenneth Farabaugh for editing the manuscript. This work was supported by the NIH (grants R37-DK60596 and R01-DK53307 to M.H. and grants R01GM092960 and U54GM105814 to B.S.), the VSRC (core grant P30-EY11373), and the American Diabetes Association (postdoctoral fellowship 1-17-PDF-129 to X.-H.G.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Received: March 9, 2017
Revised: September 1, 2017
Accepted: November 6, 2017
Published: December 5, 2017

**REFERENCES**


