Grxcr1 Promotes Hair Bundle Development by Destabilizing the Physical Interaction between Harmonin and Sans Usher Syndrome Proteins

Highlight

- Grxcr1, a deglutathionylating enzyme, promotes hair cell mechanoreceptor development
- grxcr1, ush1c, and ush1ga zebrafish mutants have similar hair bundle defects
- Glutathionylation of Ush1c promotes the interaction between Ush1c and Ush1ga
- Grxcr1 prevents physical interaction between Ush1c and Ush1ga

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In Brief

Deafness and vestibular areflexia in Usher syndrome (USH) are due to defective assembly of USH proteins into macromolecular complexes. Blanco-Sánchez et al. show that Grxcr1 negatively regulates the assembly of Ush1c and Ush1ga into complexes and that its activity is essential for correct mechanoreceptor morphology.

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Grxcr1 Promotes Hair Bundle Development by Destabilizing the Physical Interaction between Harmonin and Sans Usher Syndrome Proteins

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SUMMARY

Morphogenesis and mechanoelectrical transduction of the hair cell mechanoreceptor depend on the correct assembly of Usher syndrome (USH) proteins into highly organized macromolecular complexes. Defects in these proteins lead to deafness and vestibular areflexia in USH patients. Mutations in a non-USH protein, glutaredoxin domain-containing cysteine-rich 1 (GRXCR1), cause non-syndromic sensorineural deafness. To understand the deglutathionylating enzyme function of GRXCR1 in deafness, we generated two grxcr1 zebrafish mutant alleles. We found that hair bundles are thinner in homozygous grxcr1 mutants, similar to the USH1 mutants ush1c (Harmonin) and ush1ga (Sans). In vitro assays showed that glutathionylation promotes the interaction between Ush1c and Ush1gα and that Grxcr1 regulates mechanoreceptor development by preventing physical interaction between these proteins without affecting the assembly of another USH1 protein complex, the Ush1c-Cadherin23-Myosin7aa tripartite complex. By elucidating the molecular mechanism through which Grxcr1 functions, we also identify a mechanism that dynamically regulates the formation of Usher protein complexes.

INTRODUCTION

The hair cells of the inner ear are the sensory cells that mediate hearing and balance. They sense mechanical stimuli induced by sound waves or movement via their mechanoreceptor, an apical organelle bathed in the endolymph. This specialized organelle is composed of a single kinocilium and interconnected actin-rich stereocilia arranged in a staircase pattern that, together, form the hair bundle (Tilney and DeRosier, 1986; Tilney et al., 1986). The mechanoreceptor acts as an antenna, converting mechanical forces into ionic currents that result in signaling to the brain. This process is known as mechanoelectrical transduction (MET). Defects in the morphology or polarity of the mechanoreceptor can affect hair cell function. Thus, the morphogenesis, development, and structural integrity of the mechanoreceptor are tightly regulated. Numerous proteins, including actin-bundling proteins and adhesion molecules, participate in these processes (Barr-Gillespie, 2015; Dror and Avraham, 2009; Lu and Sipe, 2016; McGrath et al., 2017; Petit and Richardson, 2009), although the fine regulation of each process at the molecular level is still understudied.

Glutathionylation is a reversible posttranslational modification of cysteine residues that can alter enzyme activity, protein stability, DNA binding, actin polymerization, and protein distribution. Defects in glutathionylation are linked to many diseases, including cancer (Allocati et al., 2018; Board and Menon, 2016; Johnson et al., 2015; Matsui et al., 2017; Pastore and Pielmonte, 2012). Glutaredoxin domain-containing cysteine-rich 1 (GRXCR1) is an enzyme that removes glutathione groups from proteins. Mutations in GRXCR1 are associated with nonsyndromic hearing loss DFNB25 (Odeh et al., 2010; Schraders et al., 2010). Patients with mutations in GRXCR1 present with phenotypic variation, from congenital severe or profound deafness to early-onset mild to moderate hearing loss (Mori et al., 2015; Odeh et al., 2010; Schraders et al., 2010). Some patients report vestibular dysfunction or dizziness. Mice homozygous mutant for Grxcr1 have profound deafness and abnormal vestibular function (Beyer et al., 2000; Erven et al., 2002). Ultrastructural analysis of the cochlear and vestibular mechanoreceptors showed that Grxcr1 mutant mice have morphological defects in their stereocilia. In the Grxcr1 mutant organ of Corti and vestibular system, the staircase arrangement of stereocilia is present at birth but becomes progressively disorganized during subsequent development (Beyer et al., 2000; Erven et al., 2002). These phenotypes are indicative of defects in stereocilium growth, a three-step process characterized by stereocilium elongation, elongation arrest and widening, followed by reinitiation of elongation (Cotanche, 1987). Grxcr1 localizes in the stereocilia of inner ear hair cells (Odeh et al., 2010), but its role in hair bundle development is unknown.
Stereocilium growth depends on the proper assembly of the tip link, a complex of Usher syndrome type 1 (USH1) proteins. Disorganization of stereocilia in Grxcr1 mutant mice is reminiscent of that observed in USH1 mutant mice and zebrafish (Blanco-Sánchez et al., 2014; Ernest et al., 2000; Johnson et al., 2003; Lefèvre et al., 2008; Phillips et al., 2011; Seiler et al., 2005; Sollner et al., 2004). In humans, mutations in USH1 genes prevent tip link assembly and lead to non-syndromic deafness or Usher syndrome (USH), a condition characterized by congenital deafness, vestibular areflexia, and progressive retinal degeneration (Mathur and Yang, 2015). Harmonin, encoded by USH1C, and Sans, encoded by USH1G, are two USH1 scaffold proteins that form complexes with other USher proteins (Mathur and Yang, 2015). Because glutathionylation can affect protein binding (Wang et al., 2018), we hypothesized that Grxcr1 regulates the assembly of USH1 protein complexes. To test this, we created two zebrafish grxcr1 mutants and analyzed USH1 protein interactions. We found that Ush1c (Harmonin) and Ush1ga (one of the two zebrafish Sans proteins) are glutathionylated or bound to a glutathionylated protein and that removal of the glutathione by Grxcr1 promotes interaction of Ush1c with Ush1ga.

RESULTS

Grxcr1 Is Required for Stereocilium Elongation

To understand the role of Grxcr1 in hair cells, we first verified that grxcr1 is expressed in developing hair cells. RT-PCR analysis showed that grxcr1 is maternally deposited and then expressed until at least early larval stages, when hair cells become functional in zebrafish (Figure S1A). Using an RNA probe spanning the four coding exons of grxcr1, we found, by in situ hybridization, that this gene is expressed specifically in the brain, eyes, and hair cells of the inner ear and neuromasts 5 days postfertilization (dpf; Figures S1B and S1C).

To study Grxcr1 function, we generated two independent grxcr1 mutant alleles that delete the glutaredoxin domain. Mutations in the grxcr1b1259 and grxcr1b1260 alleles delete 19 bp in exon 1 and 8 bp in exon 2, respectively (Figures S2 A and S2B). In each allele, deletions lead to a shift of the open reading frame that introduces either 64 amino acids (grxcr1b1259) or 1 (grxcr1b1260) amino acid, followed by an early stop codon. The mutant Grxcr1b1259 protein lacks the entire glutaredoxin domain, whereas Grxcr1b1260 lacks about a third of the enzymatic domain (Figure S2C).

We first analyzed the structure of the anterior macula at 5 dpf. We found that the number of hair bundles and hair cells was similar in grxcr1 mutant and wild-type (WT) sibling larvae (Figures 1A–1D and S3A). Consistent with this, we found no change in cell death, as indicated by terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate (dUTP) nick end labeling (TUNEL; Figure 1E).

Figure 1. Grxcr1 Is Required for Stereocilium Widening in the Anterior Macula

(A–C) Phalloidin staining of the whole anterior macula in grxcr1+/+ (A), grxcr1b1259/b1259 (B), and grxcr1b1260/b1260 mutant larvae (C).

(D) Average number of hair bundles in grxcr1+/+ (n = 41 larvae), grxcr1b1259/b1259 (n = 35 larvae), and grxcr1b1260/b1260 mutant larvae (n = 32 larvae).

(E) Number of TUNEL-positive cells in grxcr1+/+ (n = 18 larvae) and grxcr1−/− mutant larvae (n = 28 larvae).

(F) Average hair bundle width at half their height in grxcr1+/+ (n = 40 larvae), grxcr1b1259/b1259 (n = 34 larvae), and grxcr1b1260/b1260 mutant larvae (n = 36 larvae).

Data are represented as mean ± SEM. *p < 0.05, **p < 0.01; 5 dpf.

Stereocilium growth depends on the proper assembly of the tip link, a complex of Usher syndrome type 1 (USH1) proteins. Disorganization of stereocilia in Grxcr1 mutant mice is reminiscent of that observed in USH1 mutant mice and zebrafish (Blanco-Sánchez et al., 2014; Ernest et al., 2000; Johnson et al., 2003; Lefèvre et al., 2008; Phillips et al., 2011; Seiler et al., 2005; Sollner et al., 2004). In humans, mutations in USH1 genes prevent tip link assembly and lead to non-syndromic deafness or Usher syndrome (USH), a condition characterized by congenital deafness, vestibular areflexia, and progressive retinal degeneration (Mathur and Yang, 2015). Harmonin, encoded by USH1C, and Sans, encoded by USH1G, are two USH1 scaffold proteins that form complexes with other USher proteins (Mathur and Yang, 2015). Because glutathionylation can affect protein binding (Wang et al., 2018), we hypothesized that Grxcr1 regulates the assembly of USH1 protein complexes. To test this, we created two zebrafish grxcr1 mutants and analyzed USH1 protein interactions. We found that Ush1c (Harmonin) and Ush1ga (one of the two zebrafish Sans proteins) are glutathionylated or bound to a glutathionylated protein and that removal of the glutathione by Grxcr1 promotes interaction of Ush1c with Ush1ga.
observed hair bundle defects (Figures S3D–S3H), demonstrating the thinner and longer hair bundle phenotype is specifically due to loss of Grxcr1 function.

**grxcr1 Mutants Have Decreased Locomotor Activity**

Hair cell defects in USH1 and some other heritable diseases of deafness result in vestibular as well as hearing defects. To characterize the functional consequences of grxcr1 mutation on balance, we analyzed the behavior of grxcr1+/− mutants and their siblings at 5 dpf. We first measured the total distance traveled by the larvae over a period of 5 min (Figure 2A) as well as the time spent in motion during that period (Figure 2B). The two groups of grxcr1 sibling larvae swam an average of 580 and 614 mm and were in motion 32% and 37% of the time, respectively. In contrast, grxcr1b1259/b1259 mutants swam about 297 mm, 51% of the distance traveled by their siblings, and were in motion 22% of the time, a third less than their siblings. These results indicate a significant decrease in locomotion in grxcr1 mutants. In contrast, we found that grxcr1b1260/b1260 mutants spend more time close to the surface of the water than their siblings. We then examined the routes followed by each larva to assay spatial exploratory activity. We found that, unlike their siblings, grxcr1b1259/b1259 mutants spend more time exploring their environment, similar to their siblings (Figures 2C and 2D). The differences in the hair cell phenotypes between the grxcr1b1259/b1259 and grxcr1b1260/b1260 mutants (Figure 1) thus correlate with differences in locomotion defects (Figure 2). The significant decreases in locomotor activity in the more severely affected grxcr1b1259/b1259 mutants could correlate with the vestibular dysfunction and dizziness reported by some patients (Mori et al., 2015; Schraders et al., 2010).

We also assayed other aspects of hair cell function and found that grxcr1−/− mutants performed normally in the startle response test at 5 dpf (Figure S4A). In addition, incorporation of FM1-43 into the hair bundles of hair cells from the anterior macula and the neuromasts was normal (Figures S4B–S4E). These results suggested that, unlike in USH1 mutants, there were no mechanosensory defects in grxcr1−/− mutants at this stage of development. Homozygous grxcr1 mutants are viable. We thus tested behavior in adults using the potentiated


**Figure 3. ush1c–/– and ush1ga–/– Mutants Have Dysmorphic Hair Bundles**

(A and B) Phalloidin staining of the anterior macula in ush1c siblings (A) and ush1c<sup>h293/h293</sup> mutant larvae (B). The red arrowhead points to splayed hair bundles.

(C) Average width of hair bundles at half their height in ush1c<sup>h293/h293</sup> siblings (n = 15 larvae) and ush1c<sup>h293/h293</sup> (n = 14 larvae).

(D) Number of hair bundles splayed in ush1c<sup>h293/h293</sup> siblings (n = 15 larvae) and ush1c<sup>h293/h293</sup> (n = 14 larvae).

(E-H) Phalloidin staining of the anterior macula at in ush1ga siblings (E and G), ush1ga<sup>b1342/b1342</sup> (F), and ush1ga<sup>b1240/b1240</sup> mutant larvae (H).

(I) Average hair bundle width at half their height in ush1ga<sup>b1342/b1342</sup> siblings (n = 10 larvae), ush1ga<sup>b1342/b1342</sup> (n = 11 larvae), ush1ga<sup>b1240/b1240</sup> siblings (n = 28 larvae), and ush1ga<sup>b1240/b1240</sup> mutant larvae (n = 9 larvae).

(J) Number of hair bundles splayed in ush1ga<sup>b1342/b1342</sup> siblings (n = 15 larvae), ush1ga<sup>b1342/b1342</sup> (n = 13 larvae), ush1ga<sup>b1240/b1240</sup> siblings (n = 35 larvae), and ush1ga<sup>b1240/b1240</sup> mutant larvae (n = 14 larvae).

Data are represented as mean ± SEM. *p < 0.01; 5 dpf.

Dorsal light reflex (Nicolson et al., 1996) and found a normal response. Together, our results indicate that the tip links are functional and that MET does not depend upon Grxcr1 activity.

**The Hair Bundle Morphology of grxcr1 Mutants Is Reminiscent of ush1c and ush1ga Mutants**

In zebrafish, mutations in the USH1 genes cadherin 23 (cdh23), protocadherin 15a, and myosin 7aa (myo7aa) lead to severe structural defects of the hair bundles with splayed stereocilia (Blanco-Sánchez et al., 2014; Ernst et al., 2000; Seiler et al., 2005; Söllner et al., 2004). However, we previously showed that ush1c mutants have a milder hair bundle phenotype (Blanco-Sánchez et al., 2014; Phillips et al., 2011), and zebrafish ush1g mutants have not been described.

As in grxcr1 mutants, we measured the hair bundle height and the width at half height in ush1c<sup>h293/h293</sup> mutants and siblings. We found that hair bundles were significantly longer (9%), thinner (21%), and more frequently splayed (4 times) in ush1c mutants than in their siblings (Figures 3A–3D and S3).

Zebrafish have two USH1G orthologs, ush1ga and ush1gb. To compare grxcr1 and ush1g mutant phenotypes, we generated two independent mutant alleles of ush1ga. The ush1ga<sup>b1342</sup> allele contains a 10-bp deletion in exon 2, and the ush1ga<sup>b1240</sup> allele has a 13-bp deletion in exon 1 (Figures S5A and S5B). These deletions lead to frameshifts that introduce 6 (ush1ga<sup>b1342</sup>) or 5 (ush1ga<sup>b1240</sup>) amino acids, followed by early stop codons. The mutant Ush1ga<sup>b1240</sup> protein lacks the sterile alpha motif (SAM) domain, whereas Ush1ga<sup>b1240</sup> lacks both the ankyrin repeat (ANK) and SAM domains (Figure S5C).

Examination of mechanoreceptors of the anterior macula in ush1g mutants revealed defects in hair bundle morphology. We found that hair bundles were 10% longer and 8% narrower at half height in ush1ga<sup>b1342/b1342</sup> and 12% longer and 18% narrower at half height in ush1ga<sup>b1240/b1240</sup> mutants (Figures 3E–3I and S3J). In addition, there were about 3 times more splayed hair bundles in the ush1ga<sup>b1342/b1342</sup> mutants and 4 times more in ush1ga<sup>b1240/b1240</sup> than in WT siblings (Figure 3J). Analysis of the anterior macula also revealed significant decreases in the number of hair cells and hair bundles in ush1ga<sup>b1342/b1342</sup> but no significant differences in ush1ga<sup>b1240/b1240</sup> (Figures S3K and S3L). This latter result could be due to the presence of a second transcript variant of ush1ga. Supporting this, Ensembl (Ensembl Release 92) predicts a second transcript with a start codon in exon 2 (Zerbino et al., 2018), downstream of the ush1ga<sup>b1240</sup> frameshift in exon 1.

Together, our data show that grxcr1, ush1c, and ush1ga zebrafish mutants have very similar phenotypes with significantly thinner, fewer hair bundles and splayed stereocilia. These phenotypes differ from those found in the USH1 cdh23, pcdh15a, and myo7aa mutants and suggest that Grxcr1, Ush1c, and Ush1ga might act together to regulate hair bundle morphogenesis.

**Ush1c Is Glutathionylated**

Because of the deglutathionylation activity of Grxcr1 and the overlap in phenotypes of the three types of mutants, we
examined whether Ush1c and Ush1ga are glutathionylated. Each contains cysteine residues, one in Ush1c and seven in Ush1ga, that are potential sites for glutathionylation. We transfected Madin-Darby canine kidney (MDCK) cells with a vector encoding hemagglutinin (HA)-tagged Ush1c or HA-tagged Ush1ga. Cell lysates were immunoprecipitated using an antibody against glutathione (GSH) and then analyzed by immunoblot with Ush1c or HA antibodies. Under nonreducing conditions, neither Ush1c nor Ush1ga were detected in the input, unbound, or bound fractions (Figure 4A, lanes 7–9; Figure 4B, lanes 5 and 6), indicating that there was no background expression of the untagged proteins.

To learn whether Ush1c is glutathionylated directly, we transfected MDCK cells with a vector encoding HA-tagged Ush1c. Cell lysates were immunoprecipitated using an antibody against Ush1c and then analyzed by immunoblot with Ush1c and GSH antibodies. The band recognized by the Ush1c antibody was also detected by the GSH antibody (Figure 4C, lanes 1 and 2). No bands were observed when the vector encoding HA-tagged Ush1c was not transfected (Figure 4C, lanes 3 and 4). This result indicates that Ush1c is glutathionylated.

**USH Complex Proteins Are Substrates of Grxcr1**

To test whether Grxcr1 deglutathionylates Ush1c, we transfected MDCK cells with a vector encoding HA-tagged Ush1c or with vectors encoding both HA-tagged Ush1c and HA-tagged Grxcr1. After immunoprecipitation with an antibody against GSH and immunoblot analysis with an antibody against Ush1c, Ush1c was detected in the bound fraction only when cells were transfected with HA-tagged Ush1c (Figure 4D, lane 3) but not when cells were co-transfected with both HA-tagged Ush1c and HA-tagged Grxcr1 (Figure 4D, lane 6). Detection of Ush1c and Grxcr1 in the input and unbound fractions (Figure 4D, lanes 4 and 5, respectively) showed that co-transfected cells expressed both proteins and that failure to detect Ush1c in the bound fraction is due to deglutathionylation by Grxcr1.

Similar experiments were done using GFP-tagged Ush1ga. After immunoprecipitation with an antibody against GSH and...
immunoblot analysis with an antibody against GFP, Ush1ga was detected in the bound fraction only when cells were transfected with GFP-tagged Ush1ga (Figure 4E, lane 3) but not when cells were co-transfected with both GFP-tagged Ush1ga and HA-tagged Grxcr1 (Figure 4E, lane 6).

Together, these data indicate that glutathionylated Ush1c is a substrate of Grxcr1 and that glutathionylated Ush1ga or a glutathionylated protein to which Ush1ga binds (possibly Ush1c) are substrates of Grxcr1.

**Grxcr1 Activity May Affect Protein Trafficking**

To understand where Grxcr1 acts in hair cells, we used an antibody specific to Grxcr1 (Figures S6A–S6D). We found Grxcr1 associated with both the stereocilia and kinocilium of the mechanoreceptor (Figure 5A, red and yellow arrows, respectively).

Because Grxcr1 localizes to the cis-Golgi (Figure 5B), we used GM130 to analyze the Golgi structure. We found that the integrity of the Golgi was abnormal in grxcr1/C0/C0 mutants (Figures 6A–6C). GM130 labeling was 19% greater in mutants than in the WT. Within the Golgi apparatus, proteins are modified and packed into vesicles before they are transported to their final destinations. The abnormal appearance of the Golgi suggested that Grxcr1 functions in the Golgi apparatus and that loss of Grxcr1 activity may affect protein trafficking.

**Grxcr1 Prevents the Physical Interaction between Ush1c and Ush1g**

To examine whether Grxcr1 may affect protein trafficking, we investigated the subcellular localization of Ush1c and Ush1ga in the cell bodies of anterior macula hair cells. We found that
the intensity of the Ush1c signal was increased by 35% in
grxcr1<sup>−/−</sup> mutants (Figures 6D and 6F), suggesting an accumu-
lation of Ush1c in the cell body. Using an antibody that
recognizes zebrafish Ush1ga but not zebrafish Ush1gb
(Figures S6F–S6H), we found that the level of Ush1ga signal
was reduced by 26% in grxcr1<sup>−/−</sup> mutants compared with
grxcr1<sup>+/+</sup> (Figures 6G–6I). Because the epitope recognized by
the Ush1ga antibody is located in the SAM domain, known
to interact with Ush1c (Figure S6F; Yan et al., 2010), we exam-
ined whether the decrease in the Ush1ga signal was due to the
epitope not being accessible. For this, we used a commercially
available antibody against human USH1G that targets an
epitope in exon 2 (Figure S6F) and recognizes Ush1ga but not Ush1gb (Figures S6I and S6J). We found that the level of Ush1g signal was unchanged in the mutants (Figures 6J–6L), indicating that the decrease in Ush1ga signal is due to inaccessibility of the epitope when Ush1ga binds to proteins via its SAM domain.

We then analyzed the physical proximity of Ush1c and Ush1g using an in situ proximity ligation assay (PLA) that we previously adapted to quantify USH1 protein-protein interactions in hair cells (Blanco-Sánchez et al., 2014). In WT animals, we found that, when both Ush1c and Ush1g antibodies (experimental condition) or only the anti-Ush1g antibody (control condition) were used in the assay, the PLA signals between Ush1c and Ush1g were not statistically different (Figures 7A and 7C). This suggested that, under normal conditions, Ush1c and Ush1g are not in close proximity in the cell body of hair cells and/or that they form fewer complexes than can be detected with this assay. In contrast, the Ush1c-Ush1g PLA signal was increased by 35% in the grxcr1−/− mutants (Figures 7A–7C). We also examined Ush1c and Ush1g interactions by immunoprecipitation. For this, MDCK cells were co-transfected with vectors encoding HA-tagged Ush1c and GFP-tagged Ush1ga with or without a vector encoding HA-tagged Grxcr1. Cell lysates were immunoprecipitated using an anti-GFP antibody and analyzed by immunoblot with Ush1c, GFP, and HA antibodies (Figure 7D). We observed an increase of 65% in the amount of Ush1c pulled down with Ush1ga when Grxcr1 was absent compared with when Grxcr1 was present (Figure 7E). This suggests that Grxcr1 activity reduces the number of Ush1c-Ush1ga complexes that form, consistent with results from the PLA (Figures 7A–7C).

Together, these observations indicate that Grxcr1 promotes widening of the stereocilia by preventing physical interaction between Ush1c and Ush1g.

**Figure 7. Grxcr1 Destabilizes Ush1c-Ush1ga Interactions**

(A and B) Proximity ligation assay for Ush1c and Ush1g in the anterior maculae of grxcr1+/+ (A) and grxcr1−/− larvae (B) at 5 dpf.

(C) Quantification of Ush1c-Ush1g interactions in grxcr1+/+ (n = 44 larvae), grxcr1−/− larvae (n = 45 larvae), and grxcr1−/− larvae that were incubated with only one primary antibody (control, n = 11 larvae). Data were normalized to grxcr1+/+. Individual hair cells are outlined in white. Nuclei are outlined in blue.

(D) MDCK cells were transfected with ush1c-HA, ush1ga-GFP, and grxcr1-HA (+) or not (−). Extracted proteins were immunoprecipitated with an anti-GFP antibody and blotted using anti-Ush1c, anti-GFP, and anti-HA antibodies.

(E) Quantification of the Ush1c level in bound fractions in the presence or absence of Grxcr1 (n = 4 experiments). Data were normalized to input and are presented as a ratio amount of normalized Ush1c in the presence of Grxcr1 over the amount of normalized Ush1c in the absence of Grxcr1. Data are represented as mean ± SEM. *p < 0.05.

**DISCUSSION**

The proper development, morphogenesis, and structural integrity of the hair cell mechanoreceptor rely on interactions among USH1 proteins assembled into complexes (Cosgrove and Zalocchi, 2014; El-Amraoui and Petit, 2005; Pan and Zhang, 2012). Ush1c and Ush1g are scaffold proteins that serve as organizational hubs for these complexes. Previous studies showed that Ush1c and Ush1g can interact together and with other USH1 proteins via their PDZ and SAM domains (Adato et al., 2005; Boëda et al., 2002; Siemens et al., 2002; Weil et al., 2003; Wu et al., 2011; Yan et al., 2010). However, because each scaffold protein contains limited numbers of
protein-protein interaction domains, it is unlikely that all USH1 proteins interact with Ush1c and Ush1g at the same time. Thus, assembly of USH1 proteins into different complexes is most probably dynamically regulated. Here we show that deglutathionylation by Grxcr1 regulates the formation of USH1 protein complexes by destabilizing the interactions between Ush1c and Ush1g and that failure to deglutathionylate leads to defects in hair bundle morphology.

Widening of the hair bundle during normal development occurs by increasing the size of the stereocilia with greater numbers of actin filaments and by addition of new stereocilia (Tilney et al., 1992; Tilney and DeRosier, 1986). Elongation relies initially on extension of the stereocilia surrounding the kinocilium and then stereocilia further away (Tilney and DeRosier, 1986). This wave of extension gives the hair bundle its staircase shape. Previous work revealed a role for the MET machinery in actin polymerization and, thus, in hair bundle development and maintenance (Caberlotto et al., 2011a, 2011b). In Ush1g mutants, the amplitude of the MET current is decreased, and loss of tip links prevents F-actin renewal, leading to regression of tants, the amplitude of the MET current is decreased, and loss of tip links prevents F-actin renewal, leading to regression of.

Thus, assembly of USH1 proteins into different complexes is most probably dynamically regulated. Here we show that deglutathionylation by Grxcr1 regulates the formation of USH1 protein complexes by destabilizing the interactions between Ush1c and Ush1g and that failure to deglutathionylate leads to defects in hair bundle morphology.

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Glutathionylation is a reversible posttranslational modification by addition of glutathione to a protein cysteine via a disulfide bond. In zebrafish, the transcript variant A of Ush1c is the most abundant isoform (Phillips et al., 2011), and the encoded protein has a single cysteine located in the first PDZ domain (PDZ1). Structural analysis of the interaction between Ush1c PDZ1 and the SAM-PDZ binding motif (PBM) domain of USH1G revealed that this conserved cysteine localizes in the hydrophobic pocket of PDZ1, where the PBM domain of Ush1g binds (Yan et al., 2010). Colocalization of Ush1c and Ush1g is decreased when interactions between the USH1G PBM and Ush1c PDZ1 are weakened by mutations in these domains (Yan et al., 2010). Our finding that Ush1c and, possibly, Ush1g are glutathionylated is consistent with this interaction model and further shows that glutathionylation dynamically regulates the stability of Ush1c and Ush1g interaction.

The roles of posttranslational modifications in the development of the mechanoreceptor remain understudied. Bauß et al. (2014) showed previously that Ush1g phosphorylation regulates Magi2-mediated endocytosis and that these two proteins can form a complex in the periciliary region of photoreceptors in the murine retina (Bauß et al., 2014). In Drosophila, the E3 ubiquitin ligase Ubr3 regulates development of the Johnston’s auditory organ by negatively regulating MyoVIIa interaction with MyoII (Li et al., 2016). In humans, the Clrn1 USH protein is involved in regulation of the actin cytoskeleton (Tian et al., 2009), and a common missense mutation that substitutes a lysine for an asparagine leads to defective N-linked glycosylation of Clrn1, misfolding, and degradation by the proteasome (Tian et al., 2009). Our studies add to the importance of posttranslational modifications of USH proteins in hair cell development and function.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.10.005.

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**AUTHOR CONTRIBUTIONS**

B.B.-S. and A.C. conceptualized the project and designed the experiments. B.B.-S. and A.C. performed cell culture and immunoprecipitation experiments with support from P.J.W. S.S. performed behavior experiments with support from P.J.W. B.B.-S., A.C., J.B.P., J.W., and J.L.P. generated and maintained and performed cell culture and immunoprecipitation experiments with support from P.J.W. B.B.-S. and A.C. performed immunohistochemistry and western blot experiments, image analysis and quantification, and statistical analysis. J.F. Jr. performed cell culture and immunoprecipitation experiments with support from P.J.W. B.B.-S., A.C., J.B.P., J.W., and J.L.P. generated and maintained and performed initial phenotypical characterization of the zebrafish mutant lines. J.M.P. performed immunohistochemistry. B.B.-S., A.C., and M.W. wrote the manuscript. All authors reviewed and edited the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit polyclonal anti-GRXCR1 | Novus Biologicals | Cat# NBP1-90591; RRID: AB_11028153 |
| Mouse monoclonal anti-GM130 | BD Biosciences | Cat# 610822; RRID: AB_398141 |
| Mouse monoclonal anti-KDEL | Millipore | Cat# 420400-50UG; RRID: AB_212090 |
| Rabbit polyclonal anti-USH1C | Novus Biologicals | Cat# 20920002; RRID: AB_11017833 |
| Guinea pig polyclonal anti-USH1GA | This paper | N/A |
| Rabbit polyclonal anti-USH1G | Novus Biologicals | Cat# NBP1-89076; RRID: AB_11036031 |
| Mouse monoclonal anti-USH1G | Santa Cruz Biotechnology | Cat# sc-514418 |
| Goat polyclonal anti-USH1G | Santa Cruz Biotechnology | Cat# sc-69070 |
| Rabbit polyclonal anti-TRAPPC3 | Sigma Aldrich | Cat# SAB4501193; RRID: AB_10745271 |
| Rabbit polyclonal anti-CDH23 | Sigma Aldrich | Cat# HPA07232; RRID: AB_1845903 |
| Goat polyclonal anti-cadherin-23 | Santa Cruz Biotechnology | Cat# sc-26338; RRID: AB_634513 |
| Mouse monoclonal anti-Myosin VIIa | Santa Cruz Biotechnology | Cat# sc-74516; RRID: AB_2148626 |
| Chicken polyclonal anti-Myo7aa | Blanco-Sánchez et al., 2014 | N/A |
| Rabbit polyclonal anti-GFP | Torrey Pines Biolabs | Cat# TP401 071519; RRID: AB_10013661 |
| Mouse monoclonal anti-HA | Covance | Cat# MMS-101R |
| Mouse monoclonal anti-Glutathione | ViroGen | Cat# 101-A-100 |
| Goat anti-mouse IgG, biotinylated | Vector Laboratories | Cat# BA-9200; RRID: AB_2336171 |
| Goat anti-rabbit IgG, biotinylated | Vector Laboratories | Cat# BA-1100; RRID: AB_2336201 |
| Horse anti-goat IgG, biotinylated | Vector Laboratories | Cat# BA-9500; RRID: AB_2336123 |
| Goat anti-guinea pig IgG, biotinylated | Vector Laboratories | Cat# BA-7000; RRID: AB_2336132 |
| Goat anti-chicken IgG, biotinylated | Vector Laboratories | Cat# BA-9010; RRID: AB_2336114 |
| Goat anti-mouse, Alexa Fluor-568-conjugated | Molecular Probes | Cat# A-11004; RRID: AB_141371 |
| Goat anti-rabbit, Alexa Fluor-488-conjugated | Molecular Probes | Cat# A-11008; RRID: AB_143165 |
| Goat anti-guinea pig, Alexa Fluor-546-conjugated | Thermo Fisher Scientific | Cat# A-11074; RRID: AB_2534118 |
| Cy3 AffiniPure Donkey Anti-Goat IgG | Jackson ImmunoResearch Labs | Cat# 705-165-147; RRID: AB_2307351 |
| Sheep Anti-Digoxigenin Fab fragments Antibody, AP Conjugated | Roche | Cat# 11093274910; RRID: AB_514497 |
| Alexa Fluor 488 Phalloidin | Thermo Fisher Scientific | Cat# A12379; RRID: AB_2315147 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| MS-222 (tricaine-S) | Syndel USA | N/A |
| Vectashield mounting medium | Vector Laboratories | Cat# H-1000 |
| nProtein A Sepharose 4 fast flow | GE Healthcare | Cat# 17-5280-01 |
| FM1-43fx | Molecular probes | Cat# F3555 |
| Critical Commercial Assays |        |            |
| Lipofectamine 2000 Transfection Reagent | Thermo Fisher Scientific | Cat# 11668027 |
| VECTASTAIN Elite ABC-HRP Kit | Vector Laboratories | Cat# PK-6100 |
| Duolink In Situ Detection reagents green | Sigma Aldrich | Cat# DUO92014 |
| ApopTag Peroxidase In Situ Apoptosis Detection kit | Millipore | Cat# 57100 |
| Experimental Models: Cell Lines |        |            |
| Canine: MDCK | ATCC | N/A |
| Experimental Models: Organisms/Strains |        |            |
| Zebrafish: grxcr1<sup>1259</sup> | This paper | N/A |
| Zebrafish: grxcr1<sup>1260</sup> | This paper | N/A |

(Continued on next page)
**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bernardo Blanco Sanchez (berde@uoneuro.uoregon.edu).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Zebrafish**

Homozygous wild-types (WT), *grxcr1^{+/+}*; homozygous mutants, *grxcr1^{b1259/b1259}* and *grxcr1^{b1260/b1260}*; heterozygous mutants, *grxcr1^{b1259/+}* (p.G67fsX132), *grxcr1^{b1260/+}* (p.C201fsX203), *ush1ga^{b293/+}* (p.L162X), *ush1ga^{b1240/+}* (p.D15fsX21) and *ush1ga^{b1342/+}* (p.144fsX151); *Tg[myo6b:kdel-crimson]^{b1319}*; and WT ABCxTu adult zebrafish were maintained as previously described (Westerfield, 2007). Embryos and larvae were staged according to the standard staging series (Kimmel et al., 1995). Siblings are defined as a mix of homozygous WT and heterozygous mutants generated by incrosses of heterozygous mutant adults. All experimental procedures were approved by the local IACUC.

**Cell lines**

MDCK cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin (40 U/ml)/streptomycin (40 μg/ml) at 37°C under 5% CO₂. Cells were transfected with 1 μg of plasmid of interest using Lipofectamine 2000. For cells that were treated with the reducing agent dithiotreitol (DTT), 30 mM DTT was added to the cell medium for 1 hour at 37°C.

**METHOD DETAILS**

**Generation of indel mutant lines using CRISPR and TALEN**

For the mutant lines generated with CRISPR, two single guide RNAs (sgRNA) were designed that contained targeting sequences in exon 1 (5\'-GACACCTGTGGAGACTCTCT-3\') and exon 2 (5\'-GGAGACTCGCTGCAAACGAG-3\') of *grxcr1*, and one sgRNA was designed that contained a targeting sequence in exon 2 (5\'-GGCCTTCCGGGATGCCGAA-3\') of *ush1ga*. sgRNA and cas9 RNAs were co-injected at the 1-cell stage.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Zebrafish: *ush1c^{b293}* | Phillips et al., 2011 | N/A |
| Zebrafish: *ush1ga^{b1240}* | This paper | N/A |
| Zebrafish: *ush1ga^{b1342}* | This paper | N/A |
| Zebrafish: *Tg[myo6b:kdel-crimson]^{b1319}* | This paper | N/A |
| Oligonucleotides | | |
| *grxcr1*-sgRNA1: GACACCTGTGGAGACTCTCT | This paper | N/A |
| *grxcr1*-gF1: GTCAACGCAatgaggtgc | This paper | N/A |
| *grxcr1*-gR1: CCAACAATCTACATATGGACC | This paper | N/A |
| *grxcr1*-sgRNA2: GGAGACTCGCTGCAAACGAG | This paper | N/A |
| *grxcr1*-gF2: aattgactcttggtttgccc | This paper | N/A |
| *grxcr1*-gR2: CAAATCAATAGGCTTGGACC | This paper | N/A |
| *ush1ga*-sgRNA: GGGGTTCCGGGATGCCGAA | This paper | N/A |
| *ush1ga*-gF2: GTCAACGCAatgaggtgc | This paper | N/A |
| *ush1ga*-gR2: CCAACAATCTACATATGGACC | This paper | N/A |
| Recombinant DNA | | |
| *grxcr1*-HA | This paper | N/A |
| *ush1c*-HA | Blanco-Sanchez et al., 2014 | N/A |
| *ush1ga*-HA | This paper | N/A |
| *ush1ga*-GFP | This paper | N/A |
| *ush1gb*-HA | This paper | N/A |
| Software and Algorithms | | |
| ImageJ 1.44o | NIH | RRID: SCR_003070 |
| Prism 6 | GraphPad | RRID: SCR_002798 |
For the mutant lines generated with TALEN, a TALEN target site was designed in exon 1 (‘-GGGATGGTACCTCAGGACGTCTGAA’, spacer is indicated in bracket) of ush1g. TALEN mRNA was injected at the 1-cell stage. Small mutations were identified by sequencing: Targeted sequence in exon 1 of grxcr1: forward 5’-GCAACGCAATGGAGGAGTCGCA-3’ and reverse: 5’-CCCTACTATAGTAAATTGCC-3’; targeted sequence in exon 2 of grxcr1: forward: 5’-AATTGAGTCTTTGATATGACC-3’ and reverse: 5’-CACCAATTAGGGCTGACC-3’; targeted sequence in exon 1 of ush1g: forward 5’-CTCCAAAACTCTATGCTAAG-C-3’ and reverse: 5’-CTGGGTCTTACCCCTAGGCTGG-3’; targeted sequence in exon 2 of ush1g: forward 5’-GTCTTCTCTGCTGTGTCCTTTGG-3’ and reverse: 5’-CTGGGAGTAGCAGACATTAGTG-3’.

Immunolabeling and phalloidin staining

Wholemount larvae labeling was performed following our published protocol (Blanco-Sánchez et al., 2014) with minor modifications. 5 dpf larvae were anesthetized with MS-222 (tricaine-S; Syndel USA) at 4 g/l, fixed in BT fix overnight and permeabilized in 2.5% tween-20 for 18 hours. For colocalization with ER markers, 15 min acetone crack was performed. For single and double immunolabeling, following a 2-hours blocking step (PBS + 0.01% Tween-20 + 5% bovine serum albumin + 5% goat serum), the larvae were incubated overnight at 4°C with primary antibodies. Primary antibodies were rabbit anti-Grxcr1 (Novus Biologicals, 1:500), mouse anti-Gm130 (BD Transduction Laboratories, 1:500), mouse anti-KDEL (Calbiochem, 1:500), rabbit anti-Grxcr1 (Novus Biologicals, 1:500), guinea pig anti-Ush1ga (custom made against C-terminal region, 1:500), rabbit anti-Ush1g (Novus Biologicals, 1:500), mouse anti-Ush1g (Santa Cruz Biotechnolgy, 1:500), goat anti-Ush1g (Santa Cruz Biotechnology, 1:500), rabbit anti-Trappc3 (Sigma, 1:500), rabbit anti-Cdh23 (Sigma, 1:500), chicken anti-Myo7aa (custom made against N-terminal region, 1:500). Larvae were then washed with four washes in PBST to remove unbound antibodies, blocked for two hours and incubated overnight at 4°C with secondary antibodies. Secondary antibodies were goat biotinylated horse anti-mouse, goat biotinylated horse anti-rabbit (Vector Laboratories, 1:500), biotinylated horse anti-guinea pig, biotinylated horse anti-goat, biotinylated horse anti-chicken (Vector Laboratories, 1:250). Detection was done using the Vectastain ABC Elite kit (Vector laboratories) and stopped in PBST. For double immunolabeling, larvae were post-fixed for 20 minutes in BT fix and washed in PBST. The horse radish peroxidase (HRP) was quenched using 0.1 M HCl. After 2-hours block, the samples were incubated overnight at 4°C with secondary antibodies. Detection was done as described above.

Phalloidin staining procedure followed similar steps as the wholemount larvae labeling described here, but phalloidin was added instead of primary antibodies.

For cell-culture labeling, cells were fixed in BT fix for 10 minutes and permeabilized with 0.25% Triton X-100 in PBS. Detection was performed using rabbit anti-Grxcr1 (Novus Biologicals, 1:250), mouse anti-Grxcr1 (Novus Biologicals, 1:250), mouse anti-Ush1ga (custom made against C-terminal region, 1:250), rabbit anti-Ush1g (Novus Biologicals, 1:500), mouse anti-Ush1g (Santa Cruz Biotechnology, 1:500), goat anti-Ush1g (Santa Cruz Biotechnology, 1:500), rabbit anti-Cdh23 (Sigma, 1:500), goat anti-Cdh23 (Sigma, 1:500), chicken anti-Myo7aa (custom made against N-terminal region, 1:500). The samples were equilibrated 20 minutes in BT fix and incubated in acetone at −20°C. The samples were equilibrated for 1 hour at room temperature in equilibration buffer, incubated for 90 minutes at 37°C in reaction solution (16 µL TdT enzyme + 38 µL reaction buffer). The reaction was stopped by addition of 200 µL stop buffer overnight at 37°C. The samples were fixed for 2 hours and incubated overnight at 4°C with an anti-Dig coupled to HRP. After several washes in PBST, the reaction product was detected with DAB as a substrate.

Proximity ligation assay

The larvae were prepared as described in the previous section. Primary antibodies were rabbit anti-Harmonin (Ush1c, Novus Biologicals), rabbit anti-Ush1g (Novus Biologicals, 1:500), mouse anti-Ush1g (Santa Cruz Biotechnology), Rabbit anti-Cdh23 (Sigma), goat anti-Cdh23 (Santa Cruz Biotechnology), mouse anti-Myo7a (Santa Cruz Biotechnology). All primary antibodies were used at 1:500. After washing off the primary antibodies in PBST, the larvae were incubated 1 hour at 37°C in PBST, incubated for 4 hours at 37°C with the Duo-Link ligation solution (Olink Bioscience) and then overnight in Duo-Link amplification solution (Olink Bioscience).

Images of immunolabeled larvae and cells were acquired using a Zeiss LSM 5 confocal microscope.

TUNEL labeling

Apoplast plus Peroxidase In situ Apoptosis Detection Kit was used. 5 dpf larvae were anesthetized with MS-222, fixed in BT fix overnight and stored overnight in 100% Methanol. After rehydration, the larvae were permeabilized for 1 hour in Proteinase K (1 µg/ml), post-fixed for 20 minutes in BT fix and incubated in acetic acid/ethanol mix (1:2) for 15 minutes at −20°C. The samples were equilibrated for 1 hour at room temperature in equilibration buffer, incubated for 90 minutes at 37°C in reaction solution (16 µL TdT enzyme + 38 µL reaction buffer). The reaction was stopped by addition of 200 µL stop buffer overnight at 37°C. The samples were blocked for 2 hours and incubated overnight at 4°C with an anti-Dig coupled to HRP. After several washes in PBST, the reaction product was detected with DAB as a substrate.

Behavior assay

5 dpf larvae were placed individually into polystyrene tissue culture flasks containing 50 mL embryo medium and recorded at 6 Hz for 5 min using a Mightex BCN-B050-U camera at a resolution of 1296x972 pixels. Images were background subtracted and the particle analysis feature in ImageJ was used to obtain the position of the larvae in the flask at each time point. 20 zebrafish from each genotype (grxcr1<sup>−/−</sup>, grxcr1<sup>b1259+/−</sup>, grxcr1<sup>b1259+/+</sup>, grxcr1<sup>b1260+/−</sup>, and grxcr1<sup>b1260+/+</sup>) were tested. Larvae that were immobile for greater
than 90% of the test period were excluded from statistical analysis (5 grxcr1<sup>b1259</sup>/+, 6 grxcr1<sup>b1259</sup>/−, 2 grxcr1<sup>b1260</sup>/+, and 3 grxcr1<sup>b1260</sup>/− mutant larvae).

Co-immunoprecipitation and western blotting

Cells were lysed in 125 μl RIPA buffer for 20 minutes at 4°C. Proteins were extracted as previously described (Blanco-Sánchez et al., 2014). After centrifugation at 5000 rpm for 5 minutes, the supernatant was incubated overnight at 4°C with either mouse anti-GSH (Virogen), rabbit anti-Ush1c (Novus Biologicals) or rabbit anti-GFP (Torrey Pines Biolabs). Proteins were precipitated with protein A-Sepharose beads, washed in lysis buffer and eluted in lysis buffer and non-reducing sample buffer (1:1). The eluted proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Detection was performed using rabbit anti-Harmonin (Ush1c, Novus Biologicals, 1:600), mouse anti-HA (Covance, 1:500), rabbit anti-GFP (Torrey Pines Biolabs, 1:250), and mouse anti-GSH (Virogen, 1:500) antibodies and the Odyssey Western Blotting Kit I LT (LI-COR) following the manufacturer recommendations.

RT-PCR

RNA was extracted from 0.75 and 3 hpf and 1, 2, 3, 4 and 5 dpf. PCR was performed after retro-transcription using the SuperScript® III First-Strand Synthesis System. Primers used were: grxcr1 forward: 5'-AATTGACTCTTGAGTTTGGCCG-3', grxcr1 reverse: 5'-TTAGTGAGAACAGCTGGAGCA-3', ef1rt forward: 5'-GCCCTGCCAATGTAACCAC-3', ef1rt reverse: 5'-TGCCAGGACCATCTCAACAA-3'.

in situ hybridization and histology

The procedure followed previously published protocol (Thisse and Thisse, 2004). Larvae were hybridized with a digoxigenin labeled RNA probe spanning the whole coding sequence of grxcr1. Stained larvae were embedded in 1% agarose, 0.5% agar, 5% sucrose medium and 16 μm cryosections were cut. Images of larvae stained by in situ hybridization were acquired using a Zeiss Axioplan2 compound microscope.

Startle response test

The startle test was performed at 5 dpf by taping on the Petri dish containing the tested larvae (Phillips et al., 2011). Larvae swimming away from the tap were scored as wild-types. Larvae that did not respond, circled or reacted to the tap but did not swim away were scored as larvae with balance defects.

FM1-43 incorporation

Larvae were incubated for 3.5 minutes (inner ear hair cells) or 30 seconds (neuromast hair cells) in 3 mM FM1-43fx (Molecular probes). They were then rinsed in embryo medium, fixed in BT fix overnight and stored in vectashield.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis and quantification

Images were all analyzed using ImageJ. Quantification of hair bundles and hair cells, hair bundle length and width measurements and signal intensity quantification were all performed using ImageJ.

The intensity of the immunoblot signals was measured using ImageJ and normalized.

Statistical analysis

Each experiment was repeated at least three times. The number of larvae, cells or experiments used are reported in the figures.

For all statistical analysis, a Student’s t test was performed. Standard error of the mean (SEM) and significance are indicated in the figures.