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A novel GRAIL E3 ubiquitin ligase promotes environmental salinity tolerance in euryhaline tilapia

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ABSTRACT

Background: Tilapia (*Oreochromis mossambicus*) are euryhaline fishes capable of tolerating large salinity changes. In a previous study aimed to identify genes involved in osmotolerance, we isolated an mRNA sequence with similarity to GRAIL (Gene Related to Anergy In Lymphocytes), which is a critical regulator of adaptive immunity and development. Tilapia GRAIL contains a PA (protease associated) domain and a C3H2C3 RING finger domain indicative of E3 ubiquitin ligase activity.
Scope of review: Western blots analysis was used to assess GRAIL expression pattern and responses to hyperosmotic stress. Immunohistochemistry was used to reveal the cellular localization of GRAIL in gill epithelium. Overexpression in HEK293 T-Rex cells was used to functionally characterize tilapia GRAIL. Salinity stress causes strong up-regulation of both mRNA and protein levels of tilapia GRAIL in gill epithelium. Tissue distribution of GRAIL protein is mainly confined to gill epithelium, which is the primary tissue responsible for osmoregulation of teleost fishes. Overexpression of tilapia GRAIL in HEK293 cells increases cell survival (cell viability) while decreases apoptosis during salinity challenge.
Major conclusions: Our data indicate that tilapia GRAIL is a novel E3 ubiquitin ligase involved in osmotic stress signaling, which promotes environmental salinity tolerance by supporting gill cell function during hyperosmotic stress.
General significance: Involvement of tilapia GRAIL in the osmotic stress response suggests that GRAIL E3 ubiquitin ligases play a broader role in environmental stress responses, beyond their documented functions in adaptive immunity and development.

1. Introduction

The global mean sea level of the oceans has been rising and is projected to continue to rise at an accelerated rate due to global climate change, which will induce salinization of coastal freshwater habitats, in particular in low-lying river deltas, e.g., in Southeast Asia [1,2]. In addition, increased evaporation and droughts resulting from global warming are projected to cause salinity increases in inland water systems [2]. Because coastal areas are rich in life, both from a species diversity and a biomass perspective, salinization of those areas will significantly impact ecosystem structure. Understanding how organisms respond to salinity stress and how evolution has equipped some organisms (euryhaline species) with the ability to tolerate large increases in salinity sheds light on biological processes and molecular functions that will be subject to increased selection pressure in coastal ecosystems in the future.

Tilapia (*Oreochromis mossambicus*) is a euryhaline species of bony fish (teleost) that is native to the Great East African rift lakes but has invaded many marine and limnic ecosystems world-wide. This fish represents an excellent model for studying salinity tolerance because it utilizes the underlying mechanisms very effectively to gain an extreme salinity tolerance range of 0 (fresh water, FW) to 120 ppt (>3× seawater, SW) [3]. Tilapia and other euryhaline fishes are capable of switching the direction of active ion transport across their gill epithelium from salt absorption mode (in plasma–hyposmotic environments such as FW) to salt secretion mode (in plasma–hyperosmotic environments such as SW). Such switching entails many molecular and morphological adjustments that lead to reorganization of the gill epithelium and is controlled by an osmotic stress signaling network [4]. While characterizing this network, we have recently identified a gene that is highly and rapidly induced during salinity stress in tilapia gill epithelium and shows a high degree of similarity to Gene Related to Anergy In Lymphocytes (GRAIL) E3 ubiquitin ligases [5].

The original GRAIL prototype was discovered in mice based on its induction paralleling the occurrence of an anergic phenotype in CD4(+) CD25(−) T cells [6]. A dominant negative mutant of GRAIL suppressed

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the anergic phenotype suggesting that GRAIL is required for T cell anergy, which is a state of immune unresponsiveness resulting from stimulation of T cell antigen receptor [7]. GRAIL is a transmembrane E3 ubiquitin ligase with homology to RING zinc-finger proteins [8]. An alternative name for GRAIL that has been used for *Drosophila*, ascidian, human, and rat GRAIL orthologs is Goliath [9–12]. *Drosophila* Goliath was discovered prior to mouse GRAIL and has a role in development [10]. GREUL1 (Goliath Related E3 Ubiquitin Ligase 1) has been identified in *Xenopus*, where it is also involved in development [13].

E3 ubiquitin ligases are key players in the process of protein ubiquitination, which represents a posttranslational modification that alters many proteins either by mono- or polyubiquitination. Ubiquitination is carried out by the sequential action of several proteins including E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligases) enzymes. The specificity of the reaction relies on the action of the E3 ubiquitin ligases, many hundreds of which are encoded by animal genomes. Each E3 enzyme has typically a narrow group of targets (substrate proteins). Functional consequences of ubiquitination are diverse and depend on ubiquitin chain length and topology. They include proteasome-mediated degradation, protein sorting, protein kinase activation and inactivation, DNA repair, cell cycle regulation, and apoptosis [14].

The present study analyzes the structure and functions of a new E3 ubiquitin ligase—tilapia GRAIL, its localization, and its regulation during exposure of tilapia to environmental salinity stress. Our data indicate that tilapia GRAIL participates in osmotic stress signaling and promotes environmental salinity tolerance by supporting gill cell function and survival and by suppressing apoptosis during hyperosmotic stress.

2. Materials and methods

2.1. Animals

Tilapia (*O. mossambicus*) were maintained in large (4-ft. diameter) tanks supplied with flow-through heated (26 °C) well water (=FW, Na^+=28 mg l^{-1}, K^+<5 mg l^{-1}, Ca^{2+}=33 mg l^{-1}, Mg^{2+}= 36 mg l^{-1}, pH 8.0) at the Center for Aquatic Biology and Aquaculture (CABA) of the University of California, Davis. SW acclimated fish were maintained at least for 4 weeks in SW tanks. SW was collected at Bodega Bay, California (1000 mOsm/kg, ~32 ppt) or prepared with Instant Ocean sea salt to the same concentration. Fish were sampled, gills were perfused, and gill epithelium was collected by scraping off from the cartilage of individual gill arches as described in previous work [15]. In addition, muscle (posterior to the dorsal fin), and the whole intestine, kidneys, heart, brain, liver, and testis or ovaries were collected. All different tissues were kept at −80 °C and subsequently utilized for RNA or protein isolation. All procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee (IACUC, Protocol #15013).

2.2. Cloning of full-length tilapia GRAIL

Full-length sequence for tilapia *GRAIL* gene was cloned using SMART RACE cDNA Amplification kit (Clontech, USA). Tilapia *GRAIL* clone SSH#30 was used as a starting point and extended with 3′ RACE and 5′ RACE until completion of the full-length cDNA. Sequence was submitted to Genbank (DQ465380).

2.3. Protein extraction and Western blot analysis

For protein extraction, cells were lysed in a buffer that contained 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 tablet of minicomplete protease inhibitor mixture (Roche) per 10 ml, 1 mM activated Na_3VO_4, and 1 mM NaF. Protein concentrations were determined by BCA protein assay according to the manufacturer's instructions (Pierce). Protein separation by SDS-PAGE (25 μg/lane) and Western blots were carried out as described previously [16]. A custom-made anti-tilapia GRAIL antibody at 1:500 dilution was used (raised in rabbits by Sigma-Genosys using as antigen the peptide DDRKESFSAESPPD, amino acid 303 to 316). Blots were developed with SuperSignal Femto (Pierce) and imaged with a ChemiImager (Alpha Innotech).

2.4. Immunohistochemistry

Branchial tissues were fixed and embedded in paraffin using a Tissue Tek vacuum infiltration processor (Sakura Finetek, Torrance, CA, USA). Paraffin blocks were constructed with a Tissue Tek tissue embedding center (Sakura Finetek, Torrance, CA, USA). Blocks were sectioned at 5-μm thickness using a Bromma 2218 Historange microtome (LKB, Uppsala, Sweden) and sections were floated on to a poly-lysine-coated glass microscope slide; slides were dried overnight at 44 °C. Once dry, slides were deparaffinized in xylene for 5 min (3×), 100% EtOH (2×), 95% EtOH (2×), and 80% EtOH (1×). For detection, slides were incubated in blocking solution (PBS containing 2% BSA (Roche Applied Science)) for 1 h at room temperature. After blocking, slides were incubated with anti-tilapia GRAIL antibody; 1:80 in blocking solution for 1 h at room temperature. Slides were washed three times for 5 min each in PBS and then incubated with Alexa Fluor® goat anti-rabbit IgG antibody (A-11070, Molecular Probes, Eugene, OR, USA) diluted 1:100 in PBS containing 2% BSA for 1 h at room temperature. After five washes in PBS, slides were counterstained in propidium iodide (PI) (Sigma; 10 μg/ml) for 5 min at room temperature. After three washes for 5 min in PBS, slides were dried, 20 μl of ProLong® Gold antifade reagent (P36930, Invitrogen) was applied, and coverslips were mounted and sealed with clear nail polish. Slides were examined with a laser scanning cytometer (Compucyte, Cambridge, MA, USA) using a 40× objective (UPlanFL 40×/0.75/∞/0.17, Olympus, Melville, NY, USA) in combination with UV and Argon lasers (400, 488 nm). Images were acquired using Wincyte software (Compucyte). Controls using preimmune rabbit IgG instead of primary antibody were processed in parallel and were negative.

2.5. Overexpression of tilapia GRAIL in HEK293 T-Rex cells

Tilapia GRAIL ORF was amplified with primers ATGGGTGAGAAGACACAACCAC and TCATCCCCTTGCAGCCTCTGAG. Amplified product was confirmed by double-pass sequencing. Construct pcDNA5/GRAIL was created by inserting PCR product into pcDNA5/FRT/TO TOPO TA expression vector (Invitrogen). The construct was then propagated in *Escherichia coli* strain DH5α (Invitrogen). Endotoxin-free plasmid Megapreps were performed using a kit as described by the manufacturer (Qiagen GmbH, Hilden, Germany). Stable cell lines were established by transfecting HEK293 T-Rex cells (Invitrogen) with 2 μg of a 1:9 mix of pcDNA/GRAIL plasmid DNA:pOG44 plasmid DNA and 4 μl of LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours after transfection, cells were exposed to selection in medium containing 0.15 μg/ml hygromycin (Invitrogen). After 2 weeks, individual colonies were picked, expanded, and tested for expression of tilapia GRAIL and qPCR analysis. In parallel, HEK293 T-Rex cells were transfected with empty pcDNA5/FRT/TO TOPO TA expression vector in order to obtain the Empty cell line used as control in the experiments.

2.6. Cell viability and apoptosis assays

HEK293 cells were cultured in white-walled 96-well plates and exposed to isoosmotic (300 mOsm/kg) or variable hypersaline (500, 550, 575, 600, 625, 650, 675, 725, 757, and 830 mOsm/kg) media. Hypersaline media were prepared by addition of an appropriate amount of NaCl to isosmotic medium and osmolality confirmed after thorough mixing using a freezing point osmometer (μOsmette, Advanced Instruments,

Norwood, MA USA). The viability of cells exposed to 550 mOsm/kg was analyzed at 12, 24, 48, and 72 h. Cells exposed to other salinities were analyzed at 72 h (viability assay) or at 24 h (apoptosis assay). Viability was analyzed with ViaLight cytotoxicity assay (Pierce) following the manufacturer's indications. Similar results were obtained when viable cells were counted Neubauer hemocytometer chambers using 0.2% methylene blue as indicator of dead cells. Apoptosis was assessed using Caspase-Glo 3/7 Assay (Promega).

2.7. Bioinformatics and statistical analysis

Multiple sequence alignments were performed with ClustalX2 software [17]. Phylogenetic tree was generated using the neighbor-joining program ClustalX package [17] and visualized using TreeView version 1.6.0 [18]. Bootstrap resampling (1000 trials) was used to estimate the degree of confidence in the branching order. Differences between pairs of data were analyzed by *t*-test using Sigma Stat version 3.5 (Systat). Significance threshold was set at $P<0.05$. Values are presented as mean ± standard error of mean (SEM).

3. Results

3.1. Cloning, primary structure, and phylogenetic analysis of tilapia GRAIL

A partial mRNA sequence of tilapia GRAIL was initially cloned using the Suppressive Subtractive Hybridization (SSH) technique and named SSH#30 based on its induction during exposure of tilapia to environmental salinity stress [5]. Here we cloned the full-length tilapia GRAIL transcript using a PCR-RACE approach (Fig. 1A). The ORF of tilapia GRAIL encodes a polypeptide of approximately 40 kDa molecular mass. Identification of SSH#30 as a GRAIL E3 ubiquitin ligase was achieved as a result of multiple sequence alignment, homology analysis, and phylogenetic analysis. Two conserved protein domains were identified: a protease associated domain (PA) and a C3H2C3 RING finger domain, which are both characteristics of the RING Finger protein (Rnf) families (Fig. 1B). Additional conserved features that were identified in the tilapia GRAIL transcript were a signal peptide and a transmembrane region (Fig. 1B).

Phylogenetic analysis unambiguously classified clone SSH#30 as a GRAIL E3 ubiquitin ligase belonging to the Rnf128 protein family. On phylogenetic trees tilapia GRAIL clustered with salmon Rnf128s (Genbank: ACN11723 and Genbank: ACI33627) and zebrafish Rnf128 (Genbank: CAQ13285) (Fig. 2). Interestingly, while two Rnf128s have been reported in Salmon, only one Rnf128 could be detected by Blast analysis surveying five fish species for which sequenced genomes or draft genomes are available (*Oryzia latipes*, *Tetraodon nigroviridis*, *Danio rerio*, *Takifugu rubripes*, and *Gasterosteus aculeatus*) (not shown). The conserved sequence features identified in tilapia GRAIL are also present in these other GRAIL orthologs and, except for the signal peptide sequence, represent the regions with the highest sequence identity (Fig. 3).

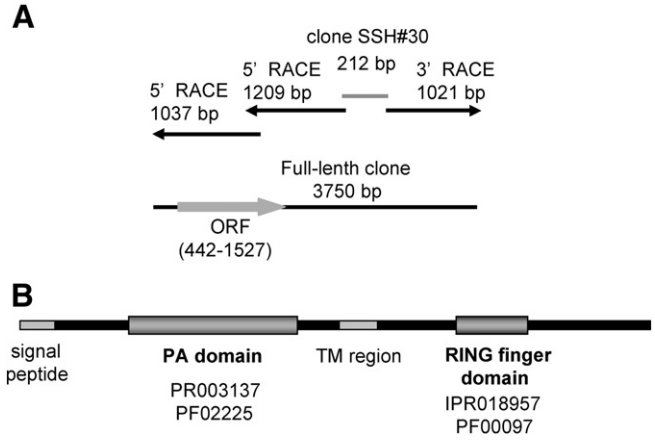

Fig. 1. Cloning of tilapia GRAIL cDNA. (A) Clone SSH #30 was extended to full-length using RACE-PCR-based methods. (B) Schematic of tilapia GRAIL transcript product. Conserved domain IDs for Pfam (http://pfam.sanger.ac.uk/) and InterPro (http://www.ebi.ac.uk/interpro/index.html) domain databases are indicated. ORF: open reading frame, PA: protease associated domain, TM: transmembrane region.

3.2. Osmotic regulation and localization of GRAIL protein in tilapia

Analysis of the level of protein expression of tilapia GRAIL in different tissues of FW-acclimated tilapia shows that this protein is primarily and almost exclusively expressed in the gills, with the only other tissues showing a noticeable (although much smaller) level of expression being liver and kidney (Fig. 4). GRAIL protein was reported to be glycosylated in cells and thus the observation of multiple bands in the Western blot might be attributable to differences in the extent of glycosylation [8]. To reveal the cellular localization of GRAIL in gill epithelium, we used immunohistochemistry. With this method tilapia GRAIL was localized to epithelial cells covering the secondary lamellae with the highest level of abundance at the tips of the lamellae. This localization pattern did not change when tilapia were subjected to increased environmental salinity (Fig. 5). However, the abundance of GRAIL protein increased significantly when tilapia were acclimated from FW to SW (Fig. 6). These data match well with our previously reported data on GRAIL mRNA expression, which show that salinity stress causes a rapid transient increase followed by a sustained augmented expression of GRAIL transcript [5].

3.3. Effects of tilapia GRAIL overexpression during salinity stress

Stably transfected cell lines expressing tilapia GRAIL were established in HEK293 T-Rex cells to functionally characterize tilapia GRAIL. Expression of GRAIL was confirmed by RT-PCR (data not shown) and immunohistochemistry (Fig. 7).

Given the increase in GRAIL abundance in tilapia gill epithelium following salinity stress, we hypothesized that this enzyme may be beneficial to the cells during hyperosmotic stress. Therefore, we tested the response of GRAIL overexpressing cells to hyperosmotic media. A control cell line was prepared by transfection of the empty pcDNA5 vector instead of the pcDNA5 vector containing tilapia GRAIL.

Cells were exposed to hyperosmotic media and analyzed at 12, 24, 48, and 72 h for survival. Cells exposed to salinity stress (550 mOsm/kg) for a period of 24 h or shorter showed no differences in survival relative to controls (Fig. 8A). However, cells that overexpressed tilapia GRAIL showed significantly higher survival rates than control cells at 48 and 72 h (Fig. 8A). Strikingly, when cells were exposed to higher salinities for 72 h, then cells that overexpressed GRAIL showed survival rates that exceeded those of control cells by 30%. A large fraction of GRAIL overexpressing cells survived prolonged exposure to 625 and 650 mOsm/kg, osmolalities that were lethal for control cells (Fig. 8B). When responses were assessed using a sublethal assay such as caspase 3/7 activity assay for apoptosis, significant differences between GRAIL overexpressing cells and controls could be observed at earlier time points such as 24 h (Fig. 8C).

4. Discussion

In this work, we present the characterization of tilapia GRAIL, a novel E3 ubiquitin ligase that was originally identified using a suppression subtractive hybridization approach [5]. Tilapia GRAIL is predominantly expressed in gills that is the primary tissue responsible for osmoregulation of teleost fishes. However, no GRAIL expression was observed in intestine, which is also subject to major epithelial remodeling upon FW to SW transfer of fish. In addition, GRAIL protein

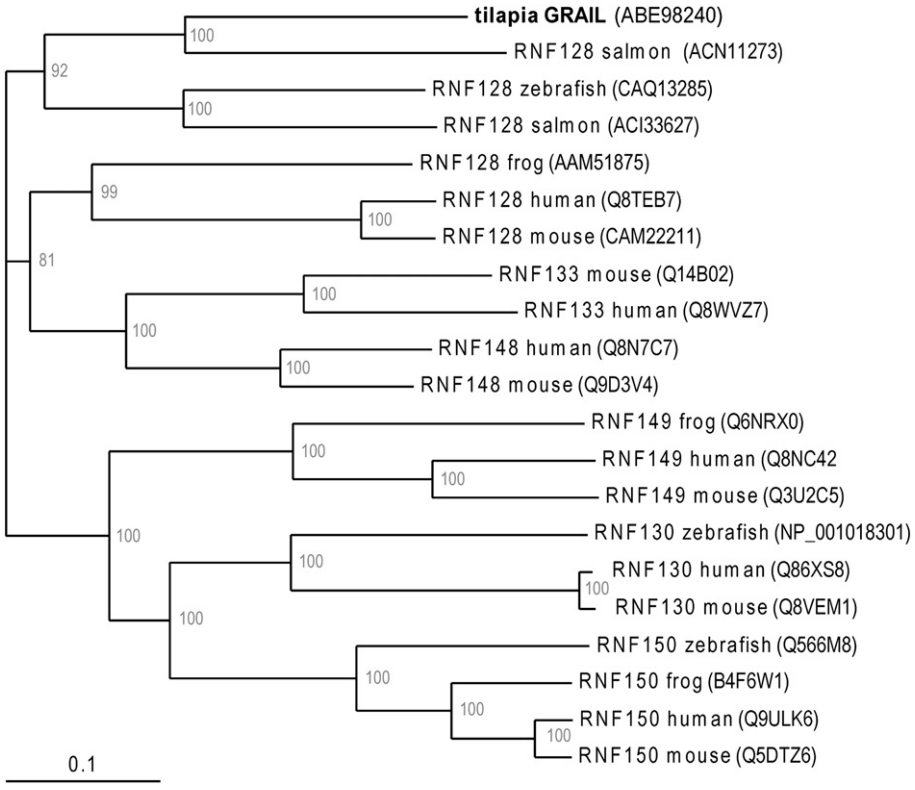

Fig. 2. Phylogenetic analysis of tilapia GRAIL. Tilapia GRAIL was identified as a member of the Ring Finger Protein 128 (Rnf128) family. Phylogenetic tree was generated using the neighbor-joining program ClustalX package. Numbers at each node indicate the percentage of trees representing the particular node out of 1000 bootstrap replicates. The database accession ID for each sequence is indicated. Scale bar represents the numbers of substitutions per site.

Fig. 3. Alignment of Rnf128 family proteins. Selected Rnf128 family members were aligned by ClustalX. Asterisks show key cysteine and histidine residues in the RING domain. Represented sequences are *O. mossambicus* (GenBank: ABE983240), *S. salar* (GenBank: ACI33627 and GenBank: ACN11273), *D. rerio* (GenBank: CAQ13285), *O. latipes* (Ensembl: ENSORLP00000003451), *T. nigroviridis* (GenBank: CAF99842), *Xenopus laevis* (GenBank: AAM5187), *Homo sapiens* (SwissProt: Q8TEB7), *Mus musculus* (GenBank: CAM22211), and *Drosophila melanogaster* (GenBank: ACZ94572).

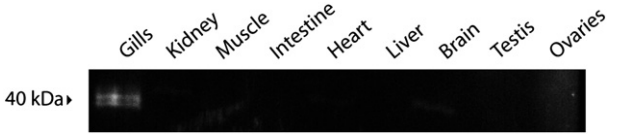

Fig. 4. Tilapia GRAIL expression pattern. GRAIL protein expression was detected by Western blot in different organs of FW acclimated tilapia. A representative image is presented, 25 μg of protein extracts was loaded in each lane.

expression in specific cell types is suggested, given its enriched localization in the tips of secondary lamellae. Up-regulation at the mRNA [5] and protein levels in response to hyperosmotic stress suggests its participation in the osmotic stress response.

A role of the ubiquitin pathway in the physiological response to osmotic stress has been observed before in mammalian cells regarding the regulation of the ion channel ENaC and aquaporin-1 [19,20]. ENaC is ubiquitinated by the E3 ubiquitin ligase Nedd4, causing an increase in turnover during hyperosmotic conditions [19]. Conversely, aquaporin-1 stability is increased by a reduction in ubiquitination during hyperosmotic stress [20]. In addition, ubiquitination plays a key role in the regulation of the transcription factor c-jun during osmotic stress [21].

Another E3 ubiquitin ligase from fish, Shop21 (Salmon hyperosmotic protein 21), was previously reported as responsive to osmotic stress [22]. Shop21 is markedly induced at 24 h during moderate hyperosmotic stress in salmon branchial lamellae (550 mOsm/kg) [22]. However, no induction of Shop21 was recorded at salinities greater than or equal to 800 mOsm/kg. In contrast, tilapia GRAIL is most highly induced at osmolalities exceeding 600 mOsm/kg. In addition, no homology exists between Shop21 and tilapia GRAIL (data not shown).

Although this is the first report concerning tilapia GRAIL, its mammalian ortholog has been studied in T-lymphocytes [23], even though it is also expressed in various other cell types and organs [8]. In T-lymphocytes GRAIL plays an important role in the regulation of anergy [7,24,25], participating in the ubiquitination and degradation of the tetraspanins CD40 [24], CD83 [26] as well as CD81 and CD151 [27]. Another GRAIL target protein previously identified by the same research group is the cellular Rho family inhibitor RhoGDI [28]. Of interest, RhoGDI GRAIL-mediated ubiquitination did not result in proteosomal degradation suggesting that this modification has a signaling function [28].

The original ubiquitination model for GRAIL proposes that a single transmembrane E3 ligase subunit binds target proteins through the PA domain in the extracellular/luminal compartment while the RING finger domain catalyzes the ubiquitination process in the cytosol [27]. The high degree of conservation between tilapia and mammalian GRAIL, including the presence of all conserved features (PA domain, TM region, and RING domain), suggests that tilapia GRAIL might be a functional E3 ligase. The ability of tilapia GRAIL to promote an increase

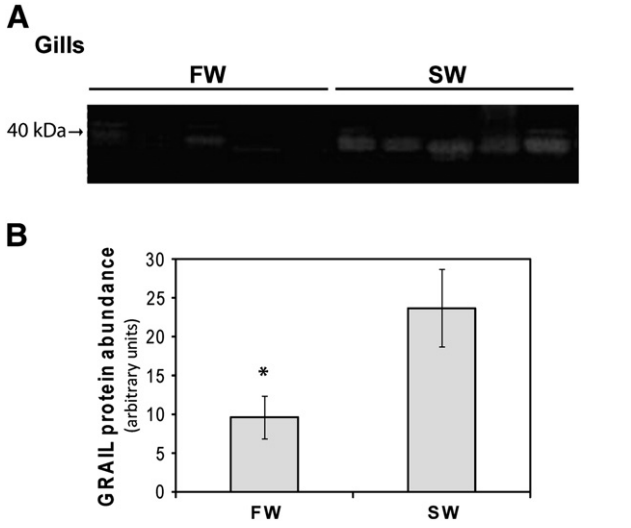

Fig. 6. Tilapia GRAIL protein expression in gill epithelial cells of FW and SW acclimated fish. (A) Western blot, (B) densitometry quantification. Twenty-five micrograms of protein extracted from individual fish was loaded in each line. An asterisk denotes a significant difference ($P<0.5$).

in osmotolerance in HEK293 cells also supports GRAIL functionality as an efficient heterologous E3 ligase in this cell line model and suggests the existence of conserved ubiquitination pathways between fish and mammals.

Putative targets for tilapia GRAIL and their participation in the osmotic stress response can be anticipated based on validated target proteins that have been identified for mammalian GRAIL. Several reports implied the tetraspanin GRAIL targets in the osmotic regulation of ion pumps and channels. For instance, the tetraspanin protein CD63 enhances the internalization of the H,K-ATPase β-subunit [29] and also increases the inhibition of the apical K^+ channel ROMK, which is induced by protein-tyrosine kinase [30]. In the heterologous expression system employed in our work, tilapia GRAIL could mediate the degradation of a conserved regulatory tetraspanin, which in turn would promote an osmoregulatory increase in K^+ excretion. Participation of tetraspanins in kidney function has been reported previously in a CD63 knockout mice study; CD63 knockout mice showed increased water intake, increased urinary flow, and reduced urine osmolality [31].

On the other hand, RhoGDI proteins, another candidate GRAIL target, regulate the cytosol-membrane shuttling of Rho GTPases [32]. RhoGDI binds and extracts Rho from the membrane, blocking its accessibility to guanine nucleotide exchange factors and GTPase-activating proteins, thereby inhibiting nucleotide exchange and GTP-hydrolyzing activities [32]. Interestingly, it was reported that RhoGDI protects cancer cells against drug-induced apoptosis [33]. The

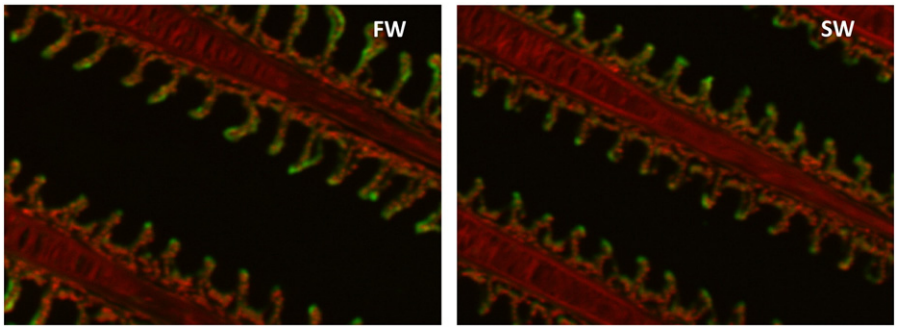

Fig. 5. Immunohistochemical detection of GRAIL protein in FW and SW tilapia gills. Green staining: GRAIL immunoreactivity; red staining: nuclear DNA stained with propidium iodide.

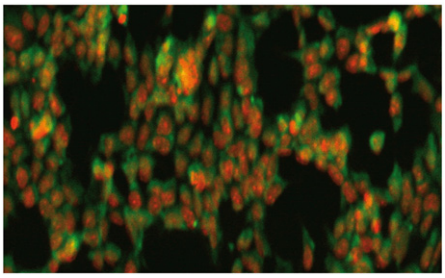

Fig. 7. Immunohistochemical detection of tilapia GRAIL protein in transfected HEK293 cells. Full-length GRAIL ORF was cloned into the expression vector pcDNA5/TO and expressed in HEK 293T-Rex cells. Green staining: GRAIL immunoreactivity; red staining: nuclear DNA stained with propidium iodide.

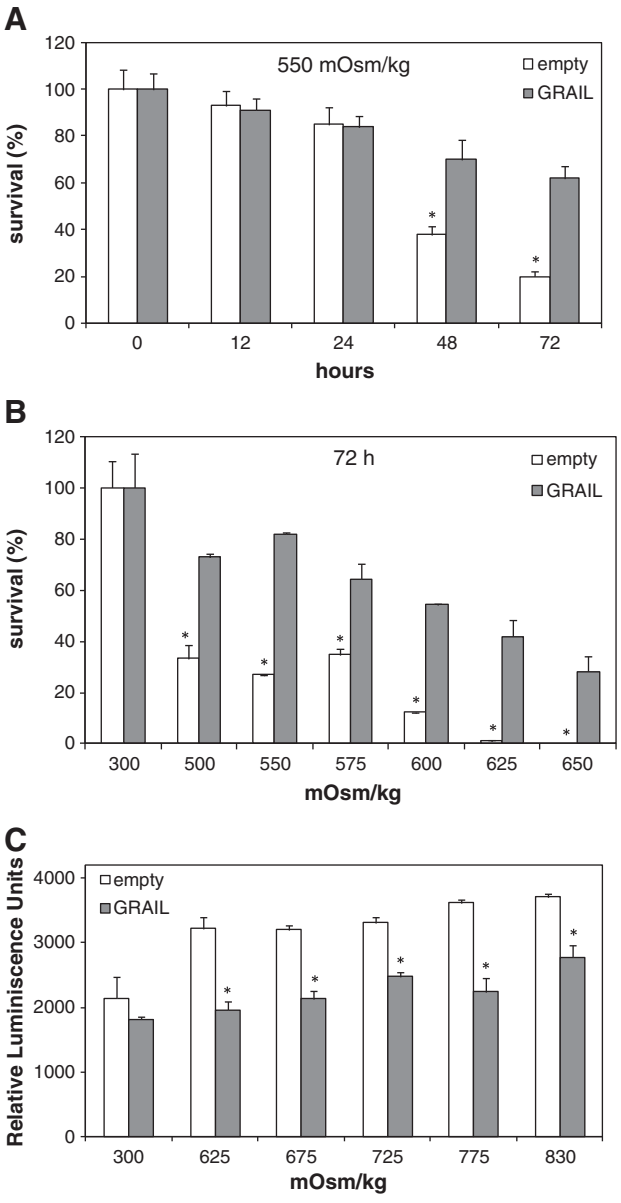

Fig. 8. Tilapia GRAIL overexpression in HEK293 cells. Full-length GRAIL ORF was cloned into the expression vector pcDNA5/TO and expressed in HEK 293T-Rex cells. Control cells were transfected with the empty vector pcDNA5/TO. (A) Cells were exposed to 550 mOsm/kg and survival was analyzed at different time points. (B) Cells were exposed to different salinities and survival was analyzed at 72 h. (C) Cells were exposed to different salinities for 24 h and apoptosis was assessed quantifying caspase 3/7 activity using a fluorescent assay. An asterisk denotes a significant difference ($P<0.5$).

increase in osmotolerance observed in GRAIL overexpressing HEK293 cells, accompanied with a decrease in the number of cells undergoing apoptosis, may then be explained by a regulatory modification carried out by the E3 ligase on RhoGDI proteins. Interestingly, Rho regulated pathways have also been related to hyperosmotic stress responses. For example, hyperosmotic stress induces a rapid and substantial increase in the level of active Rho in kidney tubule cells [34]. Conversely, it was reported that expression of GRAIL in T cells resulted in specific inhibition of RhoA GTPase activation [28]. This apparent contradiction may indicate that the physiological role of GRAIL is context-dependent and illustrates the need for more in-depth analysis of GRAIL, in particular in systems other than T cells.

Taken together, these results suggest that tilapia GRAIL has a role in targeting selected proteins (e.g., tetraspanin regulators of ionocyte structure, or RhoGDI proteins involved in the regulation of specific Rho activated pathways) that promote adaptation of tilapia to SW. Once ubiquitinated by GRAIL target proteins may be either removed via the proteasome pathway (e.g., tetraspanins) or translocated to a different cellular compartment (e.g., RhoGDI). The reduced caspase 3/7 activity in salinity-stressed cells overexpressing tilapia GRAIL suggests that this protein promotes cell survival during hyperosmotic stress via inhibition of programmed cell death. Future work aimed at more specific localization of tilapia GRAIL and its targets in different types of mitochondria-rich cells and pavement cells will contribute to the elucidation of the mechanisms underlying tolerance to salinity increases in tilapia and other vertebrates.

Our data indicate that tilapia GRAIL is a novel E3 ubiquitin ligase involved in osmotic stress signaling, which promotes environmental salinity tolerance by supporting gill cell function and survival by suppressing apoptosis during hyperosmotic stress. We conclude that GRAIL E3 ubiquitin ligases play a broader role in environmental stress responses, beyond their documented functions in adaptive immunity and development.

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