Loss of vacuolar type H\textsuperscript{+}-ATPase induces caspase-independent necrosis-like death of hair cells in zebrafish neuromast

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Key words: Vacuolar type H\textsuperscript{+}-ATPase (V-ATPase), zebrafish, neuromast hair cell, necrosis-like cell death, mitochondrial membrane potential

Summary statement:
V-ATPase mutations cause necrosis-like death of mechanosensory hair cells in zebrafish. This work identifies new cell type-specific V-ATPase functions and may help understand causes of sensorineural deafness.

ABSTRACT

The vacuolar type H\textsuperscript{+}-ATPase (V-ATPase) is a ubiquitous membrane-bound, multi-subunit proton pump that regulates pH of cellular compartments. V-ATPase activity is known to modulate several cellular processes, but cell type-specific V-ATPase functions remain poorly understood. Patients with mutations in specific V-ATPase subunits can develop sensorineural deafness, but underlying mechanisms are unclear. Here, we show that V-ATPase mutations disrupt formation of zebrafish neuromasts, which serve as a model system to investigate the underpinnings of hearing loss. Neuromasts consist of support cells surrounding mechanosensory hair cells that function similarly to hair cells in the mammalian inner ear. In V-ATPase mutant zebrafish embryos, neuromasts are small, malformed, and contain pyknotic nuclei that denote dying cells. Using molecular markers and live imaging, we find that loss of V-ATPase induces hair cells, but not neighboring support cells, to undergo caspase-independent necrosis-like cell death. This is the first demonstration that loss of V-ATPase can lead to necrosis-like cell death in a specific cell type in vivo. Mechanistically, loss of V-ATPase reduces mitochondrial membrane potential in hair cells, which has
previously been associated with necrotic cell death. Modulating the mitochondrial permeability transition pore, which regulates mitochondrial membrane potential, improves hair cell survival. These results have implications for understanding causes of sensorineural deafness, and more broadly, reveal functions for V-ATPase in regulating mitochondrial function and promoting survival of a specific cell type in vivo.

INTRODUCTION

The vacuolar type H+-ATPase (V-ATPase) protein complex localizes to membranes of organelles and vesicles and translocates protons (H+) into their lumens by hydrolyzing ATP (Nishi and Forgac 2002, Marshansky and Futai 2008, Holliday 2014). Tightly regulated V-ATPase activity maintains proper pH in these compartments, which is critical for multiple cellular functions that include vesicle trafficking, protein degradation, and ion homeostasis (Kane 2007, Vasanthakumar and Rubinstein 2020). In some cell types, V-ATPase also localizes to the plasma membrane to regulate extracellular pH. Inhibition of V-ATPase activity can impact cell proliferation, migration, or survival (Cotter, Stransky et al. 2015). The V-ATPase holoenzyme is composed of a transmembrane V₀ domain and a cytosolic V₁ domain, and each of these domains is comprised of multiple core subunits (Marshansky, Rubinstein et al. 2014, Cotter, Stransky et al. 2015) (Fig 1A). Some of these subunits have multiple isoforms, which can show tissue-specific expression and/or function. There are accessory proteins that associate with the V-ATPase in some contexts. Although complete loss of V-ATPase function is embryonic lethal in animal models (Davies, Goodwin et al. 1996, Inoue, Noumi et al. 1999, Sun-Wada, Murata et al. 2000), recessive loss-of-function mutations in specific human subunits can cause disorders that affect distinct tissue types, which include distal renal tubular acidosis (kidney) (Karet, Finberg et al. 1999, Stover, Borthwick et al. 2002), osteopetrosis (bone) (Kornak, Schulz et al. 2000), and cutis laxa (skin) (Kornak, Reynders et al. 2008). These findings reveal V-ATPase has cell-type specific functions in different organs, but these functions are only beginning to be understood.

The zebrafish embryo provides a useful system to investigate in vivo V-ATPase functions because embryos develop externally, and V-ATPase subunits are maternally supplied (Adams, Robinson et al. 2006, Nuckels, Ng et al. 2009, Gokey, Dasgupta et al. 2015). This allows analysis of cellular processes in zygotic V-ATPase mutants, as well as acute loss-of-function studies using gene knockdown or pharmacological approaches. In previous work, we used zebrafish to analyze V-ATPase functions in cells that give rise to the left-right organizer, an embryonic structure that establishes the left-right body axis in vertebrate embryos (Gokey, Dasgupta et al. 2015). We found
that small molecule inhibition of V-ATPase activity, or gene knockdown of the \textit{atp6ap1b} gene that encodes a V-ATPase accessory protein, reduced the size of the left-right organizer, and caused organ laterality defects. Compromised V-ATPase activity was found to reduce proliferation of cells that give rise to the left-right organizer. Analysis of a loss-of-function mutation in \textit{atp6ap1b} also revealed defects in neuromasts along the zebrafish lateral line. Neuromast size was reduced in \textit{atp6ap1b} mutants, but the mechanisms by which V-ATPase impacts neuromast development were not pursued (Gokey, Dasgupta et al. 2015).

Here, we focused on the zebrafish neuromast to further investigate cell-type specific V-ATPase functions. Neuromasts are sensory organs in the lateral line system on the surface of aquatic vertebrates that detect directional water movement (Lush and Piotrowski 2014, Pickett and Raible 2019). Each neuromast is comprised of non-sensory support cells that surround a central cluster of mechanosensory hair cells (Fig. 1B). Hair cells extend a single long microtubule-based kinocilium and a staircase-like bundle of several shorter actin-based stereocilia from its apical surface. Mechanical bending of stereocilia opens cation channels to generate electrical impulses that are carried by neurons to the brain (Gillespie and Muller 2009). Hair cells in the zebrafish neuromast are structurally and functionally similar to hair cells in other vertebrates (Whitfield 2002, Nicolson 2005). Hair cells in human inner ear convert sound vibrations into electrical signals to provide the basis for hearing. Some patients with distal renal tubular acidosis that have mutations in \textit{ATP6V1B1} or \textit{ATP6V0A4}, which encode the V-ATPase V\textsubscript{1}B1 and V\textsubscript{o}a4 subunits, respectively, develop sensorineural hearing loss, which is typically caused by damage to the sensory hair cells and/or nerve fibers of the inner ear (Karet, Finberg et al. 1999, Stover, Borthwick et al. 2002, Vargas-Poussou, Houillier et al. 2006, Subasioglu Uzak, Cakar et al. 2013). How these mutations impact hearing is not completely understood. \textit{Atp6v1b1} and \textit{Atp6v0a4} knockout mice can show severe hearing loss and enlarged endolymphatic compartments in the inner ear, but hair cells appear normal (Norgett, Golder et al. 2012, Lorente-Canovas, Ingham et al. 2013, Tian, Gagnon et al. 2017). However, \textit{Atp6v1b1} null mutations in a different strain of mice have no effect on hearing (Dou, Finberg et al. 2003), indicating genetic background has a significant impact. Since these mutations affect subunits with multiple isoforms, there may be redundancy and/or compensation mechanisms to support V-ATPase functions in the inner ear. In zebrafish, a mutation in \textit{rabconnectin 3a}, which encodes a V-ATPase assembly factor, alters acidification of synaptic vesicles and reduces synaptic transmission from neuromast hair cells (Einhorn, Trapani et al. 2012), which suggests specific functions for V-ATPase in hair cells. However, loss of \textit{rabconnectin 3a} only alters pH regulation of synaptic vesicles. Thus, in both mouse and zebrafish models, the impact of complete loss of V-ATPase function on hair cells remains unknown.
Using mutations in genes encoding core V-ATPase subunits—*atp6v1f* or *atp6v1h*—we show that loss of V-ATPase activity alters zebrafish neuromast formation. We find that defects in neuromast size and architecture in V-ATPase mutants are due to reduced survival of hair cells. Additional analyses indicate mutant hair cells, but not surrounding support cells, undergo necrosis-like cell death that is independent of caspase activity. As loss of V-ATPase has typically been associated with apoptotic cell death, this is the first example of necrosis-like cell death *in vivo*. At the molecular level, loss of V-ATPase activity results in depolarization of the mitochondrial membrane in hair cells, which has previously been linked to mitochondrial dysfunction and ultimately cell death. Directly inhibiting opening of the mitochondrial permeability transition pore (mPTP), which regulates mitochondrial membrane polarization, reduced hair cell death. In addition, blocking the mitochondrial calcium uniporter that controls calcium ion (Ca$^{2+}$) influx into mitochondria, which also regulates mPTP opening, improved hair cell survival. Taken together, these results uncover novel *in vivo* cell-type specific functions for V-ATPase that regulate mitochondrial health in hair cells that contributes to cell survival. This protective function for V-ATPase in hair cells may contribute to our understanding of causes of sensorineural deafness.

**RESULTS**

**Mutations in core V-ATPase subunits cause neuromast defects**

Previous work in zebrafish revealed that loss of the V-ATPase accessory protein Atp6ap1b reduced neuromast size in the lateral line (Gokey, Dasgupta et al. 2015). Recent work indicates ATP6AP1 in humans (Wang, Wu et al. 2020) and its homolog Voa1 in yeast (Jansen, Timal et al. 2016) are central subunits involved in V-ATPase assembly. However, in zebrafish two genes—*atp6ap1a* and *atp6ap1b*—encode ATP6AP1-like proteins, and the function(s) of each of these subunits remain unknown. To further investigate the function of V-ATPase activity in neuromasts, we chose to analyze two previously described loss-of-function mutations that disrupt expression of the V1F (*atp6v1f*hi1988Tg allele) or V1H (*atp6v1h*hi923Tg allele) subunits (Nuckels, Ng et al. 2009), because each is an essential subunit encoded by a single gene in zebrafish. Work in yeast has demonstrated that both V1F and V1H are required for V-ATPase activity (Ho, Hirata et al. 1993, Nelson, Mandiyan et al. 1994). Zebrafish homozygous zygotic *atp6v1f*hi1988Tg and *atp6v1h*hi923Tg mutant (referred to here as *atp6v1f*hi1988Tg and *atp6v1h*hi923Tg) are indistinguishable from wild-type siblings during early development but become easily identifiable at 2 days post-fertilization (dpf) due to hypopigmentation (Nuckels, Ng et al. 2009) (Fig. 1C, Fig. S1A-B). Zygotic *atp6v1f*hi1988Tg and *atp6v1h*hi923Tg mutants continue to develop for several days, likely supported by maternal supply of subunit protein, but both mutations are ultimately
lethal, with mutant larvae dying after 5 dpf. To analyze lateral line neuromast development in V-ATPase mutants, we used the Tg(scm1:GFP) transgene that expresses GFP in support cells (Behra, Bradsher et al. 2009) and the Tg(cldnb:lynEGFP) transgene that expresses EGFP in all neuromast cells (Haas and Gilmour 2006) as markers. The number and pattern of neuromasts in the head (anterior lateral line or aLL) and trunk (posterior lateral line) at 4 dpf was similar among V-ATPase mutants and wild-type siblings (Fig. 1C, Fig. S1C). To analyze individual neuromasts, we focused on the caudal-cranial region of the aLL, specifically the Otic O1, O2 and Middle MI1 neuromasts (Van Trump and McHenry 2008), and used acetylated tubulin antibodies to mark hair cells and DAPI to identify nuclei. At 4 dpf, neuromast area was smaller in atp6v1f/f− and atp6v1h/h− mutants, compared to wild-type siblings (Fig. 1D-F). To more closely examine neuromast architecture, we performed scanning electron microscopy (SEM) on atp6v1f/f−, atp6v1h/h−, and wild-type embryos at 4 dpf. V-ATPase mutant neuromasts appeared smaller and malformed as compared to wild-type (Fig. 1G). SEM revealed that both kinocilia and stereocilia were present on V-ATPase mutant hair cells, but the number of these structures appeared to be reduced (Fig. 1G). These results indicate loss of an essential V-ATPase subunit results in neuromast phenotypes, implicating V-ATPase activity in neuromast development.

V-ATPase subunit expression in atp6v1f/f− and atp6v1h/h− mutants

The atp6v1f/h988Tg and atp6v1h/h923Tg mutant alleles, identified in a large-scale insertion mutagenesis screen (Amsterdam, Nissen et al. 2004), have a retroviral insertion in the first exon (atp6v1f) or first intron (atp6v1h) that cause a reduction of zygotic transcription of atpv1f or atp6v1h, respectively, when analyzed at 5 dpf (Nuckels, Ng et al. 2009). To assess expression at earlier time points in atp6v1f/f− and atp6v1h/h− mutants, we collected total RNA from embryos at 2, 3 and 4 dpf for non-quantitative reverse transcriptase (RT)-PCR. Expression of atp6v1f mRNA was undetected at each of the time points in atp6v1f/f− mutants, whereas other representative V-ATPase subunit transcripts were detected (Fig. 2A, Fig. S2). In atp6v1h/h− mutants however, atp6v1h mRNA was detected at all three timepoints (Fig. 2A). Similar to atp6v1f/f− mutants, other subunit mRNAs were detected in atp6v1h/h− mutants (Fig. 2A, Fig. S2). Based on these results, we focused primarily on using atp6v1f/f− mutants for subsequent experiments.

To determine the impact of atp6v1f/f− mutation on V-ATPase subunit protein expression in neuromasts, we used a previously described antibody against the V1A subunit (Atp6v1a) (Einhorn, Trapani et al. 2012, Gokey, Dasgupta et al. 2015) in whole-embryo immunofluorescence experiments. Interestingly, we found V-ATPase staining enriched in the hair cells relative to the surrounding support cells in wild-type neuromasts marked by Tg(cldnb:lynEGFP) transgene (Fig. 2B).
Using confocal optical sections to focus on hair cells, we detected V$_1$A staining throughout wild-type cells, with a notable accumulation in the basal region (Fig. 2C) that has been previously reported (Einhorn, Trapani et al. 2012). In *atp6v1f*<sup>-/-</sup> mutants, the basal accumulation of V$_1$A protein was reduced in hair cells (Fig. 2C). These results suggest that loss of Atp6v1f alters localization and/or assembly of the V-ATPase holoenzyme in hair cells, which would be predicted to compromise V-ATPase function.

**Defects in organelle acidification and autophagy indicate V-ATPase activity is compromised in *atp6v1f*<sup>-/-</sup> mutant hair cells**

V-ATPase activity is critical for acidifying the lumens of vesicles and organelles. Therefore, to directly assess V-ATPase activity in hair cells we used the vital dye Lysotracker that label lysosomes and other acidic cellular compartments. In wild-type neuromasts marked by *Tg(cldnb:lynEGFP)* transgene expression, we observed intense Lysotracker staining in the basal region of hair cells (Fig. 3A), which is reminiscent of previously reported V$_1$A subunit accumulation (Fig. 2C) (Einhorn, Trapani et al. 2012). Also, similar to V$_1$A immunostaining, Lysotracker intensity was higher in hair cells than in surrounding support cells. In contrast to wild-type, lysotracker staining was significantly reduced in *atp6v1f*<sup>-/-</sup> mutant hair cells at 4 dpf (Fig. 3A) indicating a defect in acidification.

As a functional test for loss of V-ATPase activity in *atp6v1f*<sup>-/-</sup> mutants, we assessed autophagy. Autophagy—the process of degrading, recycling, and reusing cellular components—depends on fusion of autophagosomes with acidified and functional lysosomes that contain hydrolyzing enzymes (Parzych and Klionsky 2014, Saha, Panigrahi et al. 2018). In several cellular contexts, V-ATPase mutations result in an accumulation of autophagosomes, which denotes a block in autophagic flux (Nakamura, Matsuura et al. 1997, Mangieri, Mader et al. 2014, Mauvezin, Nagy et al. 2015, Xia, Liu et al. 2019). Since hair cells are known to upregulate autophagy as a survival mechanism in response to stress (He, Guo et al. 2017), we analyzed autophagy in *atp6v1f*<sup>-/-</sup> mutant hair cells. To assess autophagic flux, we used transgenic *Tg(CMV:EGFP-map1lc3b)* embryos that express GFP fused with the LC3b protein (LC3b-GFP) marking autophagosomes in living embryos (He, Bartholomew et al. 2009). At 3 dpf and 4 dpf, *atp6v1f*<sup>-/-</sup> mutant neuromasts show an accumulation of LC3b-GFP aggregates that are not present in wild-type (Fig. 3B-C). Live imaging of LC3b-GFP in *Tg(myo6b:tdtomato)* embryos—in which hair cells express red fluorescent tdTomato specifically in hair cells (Toro, Trapani et al. 2015) revealed LC3b-GFP aggregates were present in hair cells and not in surrounding support cells (Fig. S3). Next, immunostaining with lysosomal marker Lamp1 revealed Lamp1 puncta localized with LC3b-GFP aggregates in V-ATPase mutant hair cells (Fig. 3D). These results suggest that autophagosomes can fuse with lysosomes in V-ATPase mutant hair
cells, and the aggregation of autophagosomes is due to defective lysosomes that are unable to degrade the contents. Taken together these results indicate V-ATPase activity is compromised in \textit{atp6v1f}\textsuperscript{-/-} mutant neuromasts.

The number of hair cells is reduced in V-ATPase mutant neuromasts

To understand why neuromasts are smaller in V-ATPase mutants, we used molecular markers to determine which neuromast cell type(s) are affected by loss of V-ATPase activity. In immunostaining experiments, we used acetylated tubulin as a marker to count the number of hair cells (Harris, Cheng et al. 2003, Sarrazin, Villablanc et al. 2006) in all neuromasts. At 2 dpf, \textit{atp6v1f}\textsuperscript{-/-}, \textit{atp6v1h}\textsuperscript{-/-} mutants and wild-type have similar hair cell numbers (Fig. 4A-C). However, at 4 dpf the number of hair cells was significantly reduced in \textit{atp6v1f}\textsuperscript{-/-} and \textit{atp6v1h}\textsuperscript{-/-} mutant neuromasts (Fig. 4A-C). We next used antibodies against Parvalbumin as a marker for mature hair cells (Lopez-Schier and Hudspeth 2005). Similar to acetylated tubulin staining results, the number of Parvalbumin positive hair cells at 4 dpf was reduced in mutants compared to wild-type siblings (Fig. S4). The reduction in hair cells in V-ATPase mutant neuromasts is consistent with the reduction of apical hair cell structures—kinocilia and stereocilia—observed using SEM (Fig. 1G). Using Sox2 antibodies to label neuromast support cells (Hernandez, Olivari et al. 2007, Froehlicher, Liedtke et al. 2009, Montalbano, Capillo et al. 2018) revealed largely similar numbers of support cells between wild-type and V-ATPase mutants (Fig. 4D). We detected a statistically significant difference in support cell number at 2 dpf in \textit{atp6v1f}\textsuperscript{-/-} mutants, but there was no difference at 4 dpf when neuromast size is reduced (Fig. 4E). In \textit{atp6v1h}\textsuperscript{-/-} mutants, there was no difference in the number of support cells at 2 dpf or 4 dpf (Fig. 4F). Together, these findings indicate that the smaller neuromast size at 4 dpf in V-ATPase mutants is due to a reduced number of hair cells.

V-ATPase mutant hair cells undergo caspase 3-independent necrosis-like cell death

We next wanted to understand the cellular mechanism(s) that underlie the reduced number of hair cells in V-ATPase mutant neuromasts. Fewer hair cells could be due to defects in cell proliferation and/or cell survival. We first used bromodeoxyuridine (BrdU) incorporation or phospho-Histone H3 (pHH3) immunostaining to detect proliferating cells in neuromasts. Between 2 and 4 dpf, cell proliferation rates are low in neuromasts, and we did not detect differences between wild-type and \textit{atp6v1f}\textsuperscript{-/-} mutants (Fig. 5A-B, Fig. S5). However, while investigating cell division DAPI staining of DNA revealed the presence of pyknotic nuclei in V-ATPase mutant neuromasts (arrowheads in Fig. 5A). Pyknosis, the irreversible condensation of chromatin during cell death, can be a result of either apoptotic or necrotic cell death (Hou, Liu et al. 2016). There was a significant increase in the number of pyknotic nuclei between 2 and 4 dpf in \textit{atp6v1f}\textsuperscript{-/-} neuromasts, whereas wild-type siblings had few or
no pyknotic nuclei (Fig. 5C). A similar increase in pyknotic nuclei was observed in atp6v1h-/- neuromasts (Fig. 5D). We next used the fluorescent vital dye Ethidium Homodimer III (EthD III) as a cell death marker in live imaging experiments. In mammalian cell cultures EthD III is impermeant to living cells but binds DNA in necrotic cells and late apoptotic cells that have lost membrane integrity. Interestingly, we found that EthD III accumulates in the cytoplasm of what appear to be healthy, intact hair cells in wild-type neuromasts at 4 dpf (Fig. S6A). EthD III was also found to co-localize with Hoechst staining of DNA in pyknotic nuclei in atp6v1f-/- neuromasts (Fig. S6B). Fortuitously, Hoechst stained the nuclei in hair cells. In positive control experiments, EthD III labeled pyknotic nuclei in wild-type neuromasts treated with the aminoglycoside antibiotic neomycin that is known to induce hair cell death (Fig. S6C)(Owens, Cunningham et al. 2007). These results suggested loss of V-ATPase does not alter support cell proliferation but reduces cell survival in neuromasts.

The increase in the number of pyknotic nuclei between 2 and 4 dpf (Fig. 5C-D) coincides with the reduction of hair cells in V-ATPase mutants (Fig. 4B-C), which suggests hair cells are dying in mutant neuromasts. To directly visualize the cell type(s) dying, we followed neuromast development in live transgenic embryos with fluorescently label support cells (marked by Tg(scm1:GFP) expression) and hair cells (marked by Tg(myo6b:tdtomato) expression). Live imaging captured the swelling and bursting of hair cells in atp6v1f-/- mutant neuromasts (Fig. 5E), whereas support cells remained healthy over time (Movies 1-2). No dying cells were observed in wild-type siblings imaged using the same conditions (Fig. 5E, Movies 3-4). The specific death of hair cells, and not neighboring support cells, identifies hair cells as being highly dependent on V-ATPase activity for survival. Taken together, these results indicate hair cell death is the cellular mechanism that leads to fewer hair cells and smaller neuromast size in V-ATPase mutants.

Next, we sought to identify the mode of cell death of V-ATPase mutant hair cells. Hair cells facing cellular stresses, such as the presence of neomycin or other aminoglycosides, upregulate autophagy as survival mechanism (Fujimoto, Iwasaki et al. 2017, He, Guo et al. 2017), but then typically undergo apoptosis (Dinh, Goncalves et al. 2015, Pang, Xiong et al. 2018). However, hair cells have also been found to die by necrosis (Owens, Cunningham et al. 2007, Dinh, Goncalves et al. 2015). Previous work in zebrafish V-ATPase mutant embryos found that cells in the retina aberrantly undergo apoptosis, which is readily detected by immunostaining for active caspase 3 (Nuckels, Ng et al. 2009). To test whether atp6v1f-/- mutant hair cells also undergo apoptosis, we first used anti-cleaved caspase 3 antibodies to detect active caspase 3. At 2 or 4 dpf, we observed little or no active caspase 3 in wild-type or mutant neuromasts (Fig. 6A). However, retina in the same 4 dpf mutant embryos contained caspase 3 positive cells (Fig. 6A) as described (Nuckels, Ng et al. 2009).
In additional positive control experiments, caspase 3 positive cells were detected in wild-type neuromasts treated with 400 µM neomycin, which activates caspase-dependent apoptosis of hair cells (Fig. 6A) (Harris, Cheng et al. 2003, Uribe, Kawas et al. 2015, Wiedenhoft, Hayashi et al. 2017). The absence of active caspase 3 in atp6v1f\(^{-}\) neuromasts suggested that the hair cell death is independent of caspase 3 pathway.

To further test the role of caspase activity in hair cell death in V-ATPase mutants, we used the pan-caspase inhibitor ZVAD-FMK that blocks apoptotic cell death. Previous work in zebrafish neuromasts has shown that ZVAD-FMK can prevent hair cell death induced by low doses of neomycin (Williams and Holder 2000, Matsui, Ogilvie et al. 2002). However, ZVAD-FMK treatments did not block hair cell death in atp6v1f\(^{-}\) mutants (Fig. 6B-C), which provides additional evidence that V-ATPase mutant hair cells die independent of caspase activity. Next, live imaging of atp6v1f\(^{-}\) mutants expressing the Tg(myo6b:tdtomato) transgene revealed that dying hair cells do not undergo typical morphological changes associated with apoptosis, which include plasma membrane blebbing, cell shrinkage, and formation of apoptotic bodies (Chen, Kang et al. 2018, Nirmala and Lopus 2020). In contrast, dying mutant hair cells swell and rupture, which are changes associated with necrosis (Golstein and Kroemer 2007, Nirmala and Lopus 2020) (Fig. 6D, Movie 5). These morphological changes in mutant hair cells were similar to changes in wild-type hair cells treated with 10 µM CuSO\(_4\) (Movie 6), which is known to induce necrosis in neuromast hair cells (Olivari, Hernandez et al. 2008, Kasica-Jarosz, Podlasz et al. 2018). Together, these results indicate hair cells undergo necrosis-like cell death that is independent of caspase activity.

**Loss of V-ATPase alters mitochondria in hair cells**

We next wanted to begin to understand mechanistically how loss of V-ATPase activity leads to necrosis-like death of hair cells. V-ATPase is known to mediate several different cellular functions, therefore we predicted that instead of one specific defect that triggers cell death, there may be several underlying problems that contribute to hair cells dying. Since loss of V-ATPase has previously been shown to increase reactive oxygen species (ROS) (Milgrom, Diab et al. 2007, Yokomakura, Hong et al. 2012), and elevated ROS levels can induce hair cell death (Esterberg, Linbo et al. 2016), we first tested whether ROS levels are elevated in V-ATPase mutant hair cells. Surprisingly, using the fluorescent probe CellROX in live neuromasts as described (Esterberg, Linbo et al. 2016, Razaghi, Steele et al. 2018), we did not detect an increase in ROS in atp6v1f\(^{-}\) mutant hair cells. On the contrary, quantification of ROS probe fluorescence indicates hair cell ROS is decreased in mutants (Fig. S7). This suggests loss of V-ATPase does not trigger an increase in ROS that contributes to hair cell death.
Since mitochondria are the primary source of cellular ROS (Kausar, Wang et al. 2018), the reduction of ROS in \textit{atp6v1f}/ mutant hair cells suggested a mitochondrial defect. Mitochondria are key players in cell death, and previous work has highlighted links between V-ATPase activity, lysosome function and mitochondrial health (Hughes and Gottschling 2012, Bartel, Pein et al. 2019, Yambire, Rostosky et al. 2019). In mammalian cancer cell lines, pharmacological suppression of V-ATPase activity can result in depolarization of the mitochondrial membrane and apoptosis (Hong, Nakano et al. 2006, De Miltolo, lessi et al. 2007, McHenry, Wang et al. 2010). In other contexts, loss of mitochondrial membrane potential (\(\Delta \Psi_m\)), which leads to mitochondrial dysfunction and loss of ATP production, is associated with necrosis (Navarro and Boveris 2004, Karch and Molkentin 2015, Claudia Jara 2019). To analyze mitochondria in V-ATPase mutant hair cells, we co-stained living embryos with the vital dye MitoTracker to label the mitochondria and tetramethylrhodamine ester (TMRE) to visualize \(\Delta \Psi_m\) (Fig. 7A), as previously described in zebrafish neuromasts (Owens, Cunningham et al. 2007, Esterberg, Hailey et al. 2014, Alassaf, Daykin et al. 2019). At 4 dpf, we measured a decrease in overall MitoTracker fluorescence intensity between wild-type and \textit{atp6v1f}/ hair cell clusters (Fig. 7C), indicating reduced mitochondrial mass in mutants. Additionally, we found a more pronounced decrease in TMRE labeling in \textit{atp6v1f}/ hair cells as compared to wild-type (Fig. 7B), which indicates lower \(\Delta \Psi_m\). The ratio of TMRE/MitoTracker (Fig. 7D) indicates reduced TMRE staining in the mutants is not only due to loss of mitochondria. Consistent with reduced ROS levels, these results indicate that V-ATPase loss leads to structural and functional defects in hair cell mitochondria.

\textbf{Modulating the mitochondrial permeability transition pore improves survival of V-ATPase mutant hair cells}

We next focused on whether mitochondrial membrane depolarization (reduced \(\Delta \Psi_m\)) contributes to V-ATPase mutant hair cell death. A decrease in \(\Delta \Psi_m\) can be due to prolonged opening of the mitochondrial permeability transition pore (mPTP). Inhibiting mPTP opening has been shown to prevent necrotic cell death of hepatocytes and cardiomyocytes (Kinnally, Peixoto et al. 2011, Kwong and Molkentin 2015). To modulate mPTP, we treated embryos with the mPTP inhibitor cyclosporin A (CsA). CsA interacts with Cyclophilin D (CypD) protein that regulates mPTP and has been successfully used in zebrafish hair cells to inhibit mPTP opening and increase \(\Delta \Psi_m\) (Esterberg, Hailey et al. 2014). Immunostaining experiments revealed that CsA treatments increased the number of hair cells (Fig. 7E-F) and reduced the number of pyknotic nuclei (Fig. S8A) in \textit{atp6v1f}/ mutant embryos. This increase in hair cell survival was modest, but robust: a statistically significant increase
in hair cell number was observed in mutant neuromasts in three independent experiments. These results suggest that inhibiting mPTP opening, and thereby modulating mitochondrial membrane potential, can moderately improve hair cell survival in V-ATPase mutants.

As a second approach to modulate mPTP, we sought to test another regulator of mPTP opening. The primary stimulators that open the mPTP are thought to be ROS and calcium ions (Ca²⁺) (Bonora and Pinton 2014). Since ROS levels are not elevated in atp6v1f⁻/⁻ mutant hair cells (Fig. S7), we asked whether loss of V-ATPase activity alters Ca²⁺ handling in hair cells, which may impact mPTP function. Loss of V-ATPase has previously been associated with defects in Ca²⁺ homeostasis in diverse cell types (Forster and Kane 2000, Christensen, Myers et al. 2002, Lopez, Camello-Almaraz et al. 2005), including mammalian macrophages and platelets in which loss of V-ATPase increases cytoplasmic Ca²⁺ levels. We hypothesized that loss of V-ATPase may lead to a cytoplasmic Ca²⁺ overload that contributes to mPTP opening, mitochondrial defects, and cell death. To functionally test this hypothesis, we inhibited the mitochondrial calcium uniporter (MCU) with the Ruthenium Red derivative RU360 (Esterberg, Hailey et al. 2014, Esterberg, Linbo et al. 2016). The MCU is a Ca²⁺-activated Ca²⁺ channel that controls cytoplasmic Ca²⁺ entry into mitochondria. Prolonged elevation of Ca²⁺ levels in mitochondria result in mPTP opening and cell death (Bonora and Pinton 2014). RU360 treatments from 2 to 4 dpf were detrimental to wild-type neuromasts and led to reduced hair cell number (Fig. 7G). In contrast, RU360 increased hair cell number in atp6v1f⁻/⁻ mutants (Fig. 7G). This suggests Ca²⁺ handling is quite different between wild-type and mutant hair cells. The increase in mutant hair cell survival with RU360 treatments was comparable to CsA treatments that inhibit mPTP opening (Fig. 7F). The number of pyknotic nuclei was also reduced in atp6v1f⁻/⁻ embryos treated with RU360 but did not reach statistical significance (Fig. S8B). Taken together, these results support a model in which loss of V-ATPase alters Ca²⁺ homeostasis and mPTP regulation, which leads to mitochondrial depolarization and dysfunction that is a contributing factor to necrosis-like death of hair cells.

DISCUSSION

In this study we identify new cell-type specific functions for V-ATPase in vivo. We show V-ATPase activity is critical for survival of mechanosensory hair cells, but not neighboring support cells, in zebrafish neuromasts. This is the first analysis of complete loss of V-ATPase activity in hair cells in any vertebrate. Multiple previous reports indicate V-ATPase associated cell death occurs via apoptosis (De Milito, Iessi et al. 2007, Nuckels, Ng et al. 2009, You, Jin et al. 2009). However, loss of V-ATPase has also been found to cause caspase-independent cell death in cultured cell lines
Our work provides the first in vivo evidence that loss of V-ATPase activity leads to caspase-independent necrosis-like death of neuromast hair cells. In addition, our results provide a new in vivo case study that supports a growing body of evidence that links V-ATPase activity with mitochondrial function (De Milito, Iessi et al. 2007, Graham, Thompson et al. 2014). Our results suggest loss of V-ATPase alters Ca\(^{2+}\) flow via the mitochondrial calcium uniporter, impacts regulation of the mPTP, and disrupts mitochondrial membrane potential in hair cells. Together, these findings suggest mitochondrial dysfunction contributes to caspase-independent necrosis-like death of hair cells. We predict there is a vast scope of functions for V-ATPase activity in specific cell types that we are only beginning to uncover. Identifying these V-ATPase functions may help understand underlying causes of disease.

Elucidating V-ATPase functions in specific cell types

Human gene mutations and studies using animal models have implicated V-ATPase activity in diverse functions in specific cells. Global knockout of the essential Atp6v0c subunit in mice results in severe developmental defects shortly after implantation (E 5.5-6.5) that lead to embryonic lethality (Inoue, Noumi et al. 1999), but conditional knockouts have shed light on functions of V-ATPase subunits in specific cell types. In addition, the zebrafish embryo has emerged as a useful model to understand V-ATPase functions. Zebrafish zygotic V-ATPase loss-of-function mutants, which complete embryogenesis and develop for several days due to maternal supply of subunit mRNA and/or protein, provide a platform for broad-based phenotyping and in vivo mechanistic studies. In addition to altered pigmentation, which likely reflects altered pH in melanosomes (Dooley, Schwarz et al. 2013), phenotypes in several other cell types have been analyzed in zebrafish V-ATPase mutants. Analysis of microphthalmia (small eyes) in mutants uncovered roles for V-ATPase activity regulating cell cycle exit of retinoblasts, proliferation of retinal stem cells, and survival of developing neurons (Nuckels, Ng et al. 2009). In the developing zebrafish gastrointestinal tract, analysis of membrane protein trafficking in mutant intestinal epithelial cells revealed roles for V-ATPase-mediated trans-golgi network luminal acidification in apical sorting and transport of membrane proteins (Levic, Ryan et al. 2020). In a separate study, loss of V-ATPase was found to alter intrahepatic biliary duct formation, which was proposed to result from faulty protein sorting (EauClaire, Cui et al. 2012). Finally, CRISPR-mediated knockout of atp6v1h reduced bone formation, likely via upregulation of matrix metalloproteinases in osteoclast cells (Zhang, Huang et al. 2017). This study also identified a family of patients with decreased bone density that have a deleterious mutation in ATP6V1H, which highlights the use of zebrafish to model human disease. It becomes clear from these examples that V-ATPase has a broad spectrum of cell-type specific functions.
Inhibiting V-ATPase activity during early zebrafish development altered positioning of the heart and gastrointestinal tract along the left-right body axis (Gokey, Dasgupta et al. 2015). V-ATPase activity has been linked to left-right axis determination for many years (Adams, Robinson et al. 2006), and more recently, a copy number variants screen in patients with laterality defects (Cowan, Tariq et al. 2016) identified a duplication of the ATP6V1G1 suggesting a role for V-ATPase in human laterality. In zebrafish, V-ATPase gene knockdowns or small molecule inhibitors reduced proliferation—but not survival—of precursor cells that formed a left-right organizer that was reduced in size (Gokey, Dasgupta et al. 2015). Similarly, neuromast size was reduced in the V-ATPase accessory gene atp6ap1b mutant embryos (Gokey, Dasgupta et al. 2015). We predicted the reduced size of neuromasts would be a consequence of reduced cell proliferation, as we observed in the left-right organizer. However, we found that loss of V-ATPase reduces the survival of mechanosensory hair cells in developing neuromasts. Moreover, we found that hair cells do not die via caspase-dependent apoptosis as described in the zebrafish retina (Nuckels, Ng et al. 2009), but rather undergo caspase-independent necrosis-like cell death. These novel findings highlight context-specific functions for V-ATPase activity during embryo development.

**V-ATPase and cell death**

Functions for V-ATPase in cell survival and death is complex and context dependent. Since V-ATPase is involved in essential cellular processes, it makes sense that loss of V-ATPase would be lethal to cells. In many cell types, including aforementioned zebrafish retinal neurons (Nuckels, Ng et al. 2009), murine osteoclasts (Okahashi, Nakamura et al. 1997), human cortical neurons (Hirose, Cabrera-Socorro et al. 2019), and several human cancer cells such as leukemic (Zhang, Schneider et al. 2015) and breast cancer cells (von Schwarzenberg, Wiedmann et al. 2013), absence or prolonged inhibition of V-ATPase activity leads to cell death via the apoptotic pathway. Indeed, inhibiting V-ATPase has been identified as a promising therapeutic target to kill cancer cells (Stransky, Cotter et al. 2016). Several studies suggest cancer cells are more susceptible to V-ATPase inhibition than non-cancer cells, which indicates V-ATPase activity promotes cancer cell survival. On the other hand, V-ATPase activity is required for necrosis-like death of yeast cells and C. elegans neurons induced by stress (Syntichaki, Samara et al. 2005, Kim, Kim et al. 2012) and killing human cancer cell lines with CDK4/6 inhibitors (Hino, Iriyama et al. 2020), likely by creating an acidic environment that mediates specific cell death pathways. In these cases, V-ATPase loss or inhibition increases cell survival. These results reveal different functions for V-ATPase in different scenarios that promote cell survival or cell death.
Several studies indicate that loss of V-ATPase triggers apoptosis by increasing ROS. High levels of ROS result in mitochondrial membrane depolarization, release of cytochrome C, and activation of apoptotic machinery that includes caspases (Lin and Beal 2006, Du, Li et al. 2015, Wang, Wang et al. 2017). However, we observed a decrease in CellROX signals in the atp6v1f<sup>-/-</sup> mutant hair cells, indicating elevated levels of ROS is not a cause of hair cell death in these mutants. Work in hepatocellular carcinoma cell lines indicates V-ATPase inhibition that causes mitochondrial impairment can hamper ROS generation (Bartel, Pein et al. 2019). Similarly, mitochondrial defects in V-ATPase mutant hair cells likely explain reduced ROS levels. In some in vitro studies, loss of V-ATPase has been associated with caspase-independent cell death. Treating cultured leukemia cell lines (Yuan, Song et al. 2015) or hepatocellular carcinoma cell lines (Yan, Jiang et al. 2016) with the V-ATPase inhibitor Bafilomycin A1 reduced proliferation and induced death in these cell types. In both studies, the mode of cell death was determined to be caspase-independent because caspase 3 activation was not observed and the pan-caspase inhibitor ZVAD-FMK had no effect. Additional experiments in leukemia cells uncovered mitochondrial membrane depolarization and release of apoptosis-inducing factor to the nucleus (Yuan, Song et al. 2015). In contrast, death in hepatocellular carcinoma cell lines was proposed to involve autophagy and p38-MAPK pathways (Yan, Jiang et al. 2016). It is important to note that these studies only tested the effect of Bafilomycin A1 on cancer cell death, so it is possible that cell death results from an off-target effect that is independent of V-ATPase. Using gene mutations, our results provide clear evidence that loss of V-ATPase activity can indeed lead to caspase-independent death of a specific cell type in vivo. However, the morphology of dying hair cells is consistent with necrotic-like cell death rather than apoptosis, and, in preliminary experiments, pharmacological inhibitors of autophagy or p38-MAPK did not change hair cell death in V-ATPase mutant zebrafish neuromasts. Instead, we found evidence that mitochondrial dysfunction contributes to hair cell death in V-ATPase mutants. Mitochondrial membrane depolarization is known to cause mitochondrial dysfunction, loss of ATP production and ultimately cell death. However, other consequences of mitochondrial depolarization, such as the potential for release of pro-death signals, may contribute to hair cell death. Since modulating mitochondrial membrane potential only partially rescued hair cell survival in mutant neuromasts, we hypothesize that loss of V-ATPase induces additional defects that contribute to hair cell death. Future work is needed to test this hypothesis and potentially identify other mechanisms by which V-ATPase promotes hair cell survival.

**V-ATPase in hair cells**

Hair cells are intriguing cell types that transduce mechanical stimuli into chemical signals; this includes detecting water flows by zebrafish neuromast and sounds in the mammalian inner ear. We report here that loss of V-ATPase leads to hair cell death in zebrafish neuromasts. Previous work has
implicated V-ATPase in signaling pathways—including Notch and Wnt—that are known to be active in neuromasts. This made us wonder whether alterations in these pathways influenced the neuromast defects in V-ATPase mutants. First, loss of V-ATPase activity has been reported to reduce Notch signaling in diverse cell types, including drosophila follicle cells and imaginal disc cells (Yan, Denef et al. 2009), rat retina cells (Valapala, Hose et al. 2013), and mouse neural precursors (Lange, Prenninger et al. 2011). V-ATPase-mediated pH regulation is thought to be critical for endocytosis, protease activation, and protein degradation during Notch signal transduction (Sun-Wada and Wada 2015). In mouse (Kiernan, Cordes et al. 2005) and zebrafish (Itoh and Chitnis 2001), blocking Notch signaling results in an increased number of hair cells at the expense of support cells. In contrast, we do not observe an increase in hair cells or a decrease in support cells in V-ATPase mutants. Second, blocking V-ATPase activity in turn blocks endosomal processing and transmission of canonical Wnt signals (Buechling, Bartscherer et al. 2010, Cruciat, Ohkawara et al. 2010, Tuttle, Hoffman et al. 2014). Wnt signaling is known to promote cell proliferation in neuromasts, which is restricted by the Wnt antagonist Dickkopf (Dkk) proteins (Valdivia, Young et al. 2011, Head, Gacioch et al. 2013, Wada, Ghysen et al. 2013). Inhibiting Wnt by over-expressing Dkk reduces neuromast size and hair cell number, which is similar to V-ATPase mutants. However, in contrast to V-ATPase mutants, blocking Wnt signaling was found to reduce cell proliferation in neuromasts, with no effect on cell survival (Wada, Ghysen et al. 2013). Although we do not rule out the possibility that subtle changes in Notch and/or Wnt signaling could contribute to V-ATPase mutant neuromast phenotypes, it is clear that the reduced survival of hair cells cannot be explained exclusively by alterations in one of these pathways.

In addition to modulating the Notch and Wnt pathways, V-ATPase regulates mTOR signaling. V-ATPase recruits the mTOR complex 1 (mTORC1) to the lysosomal membrane, in response to amino acid stimulation or nutrient changes (Zoncu, Bar-Peled et al. 2011). Activation of mTORC1 at the lysosome promotes cellular growth. mTOR has not been implicated in hair cell development, but mTORC1 and mTORC2 are expressed in the mature mammalian cochlea. Reports of inhibiting mTOR with the small molecule Rapamycin provide conflicting results on hair cell survival. In vitro treatments of cultured rat cochlear explants with Rapamycin reduced hair cell number (Leitmeyer, Glutz et al. 2015), whereas in vivo injections of Rapamycin into rats protected hair cells from dying when exposed to the ototoxic drug cisplatin (Fang and Xiao 2014). In our hands, inhibiting mTOR with Rapamycin in zebrafish decreased hair cell number in both wild-type and atp6v1f /− mutant neuromasts, but we did not observe an increase in the number of pyknotic nuclei in wild-type or mutants. This suggests mTOR signaling may promote hair cell formation (potentially via proliferation control) but does not impact hair cell survival during zebrafish neuromast development.
Our analysis of *atp6v1f*-/mutants indicates V-ATPase activity is required to maintain low pH in acidic cellular compartments in hair cells, which include endosomes, synaptic vesicles, and lysosomes. The accumulation of autophagolysosomes in V-ATPase mutant hair cells indicates lysosomal function is indeed impaired in these cells. Previous work has indicated lysosomal function is linked to mitochondrial function. In yeast, genetic or pharmacological inhibition of V-ATPase reduced acidification of the vacuole (analogous to the lysosome) and led to an increase in cytosolic acidity, dissipation of $\Delta\Psi m$, and mitochondrial degradation (Hughes and Gottschling 2012). More recently, inhibition of lysosomal acidification using V-ATPase inhibitors in mouse fibroblasts was shown to cause a reversible iron ($Fe^{2+}$) deficiency that was linked to mitochondrial dysfunction and caspase-independent cell death (Yambire, Rostosky et al. 2019). However, in preliminary trials, iron supplementation did not prevent hair cell death in zebrafish V-ATPase mutants. In addition to $Fe^{2+}$, intracellular $Ca^{2+}$ levels, which are known to regulate mitochondria, are also modulated by lysosomes and V-ATPase activity (Lawrence and Zoncu 2019). We found that using the drug RU360 to inhibit the mitochondrial calcium uniporter, which regulates $Ca^{2+}$ flow into mitochondria, protects some hair cells in V-ATPase mutants. This is intriguing since RU360 treatments were harmful to wild-type neuromasts and reduced the number of hair cells. These results suggest a working model in which gradual loss of maternal V-ATPase activity in mutant neuromasts changes $Ca^{2+}$ homeostasis, such that mitochondrial $Ca^{2+}$ overload leads to mitochondrial depolarization and dysfunction that contributes to caspase-independent necrosis-like death of hair cells.

Our findings identify zebrafish hair cells to be highly enriched for V-ATPase expression, and highly vulnerable to V-ATPase loss. This may be relevant on two biomedical fronts. First, although knockout mice can recapitulate sensorineural hearing loss found in patients with *ATP6V1B1* or *ATP6V0A4* mutations, exactly how V-ATPase functions in hearing is not fully understood. It is proposed that V-ATPase activity regulates pH and ionic composition of the endolymphatic fluid in contact with hair cells in the inner ear and when the homeostasis is disrupted leads to loss of endocochlear potential, and enlarged endolymph compartment and vestibular aqueduct (Lorente-Canovas, Ingham et al. 2013, Tian, Gagnon et al. 2017). Interestingly, hair cells appeared largely normal in deaf knockout mice at the stages analyzed (Dou, Finberg et al. 2003, Hennings, Picard et al. 2012, Lorente-Canovas, Ingham et al. 2013). Our results indicate hair cells depend on V-ATPase activity for survival, which raises the possibility that compromised V-ATPase activity in patients may sensitize hair cells to damage or stress that leads to an accumulation of hair cell death over time and ultimately results in hearing loss. A second consideration is the potential use of V-ATPase inhibitors as anti-cancer treatments. Some cancer cells are highly dependent on V-ATPase for survival and are
more sensitive to V-ATPase inhibition than non-cancerous cells (Stransky, Cotter et al. 2016), which makes V-ATPase inhibitors attractive candidates for chemotherapy. Similar to cancer cells, we found that neuromast hair cells are highly sensitive to loss of V-ATPase activity. This suggests hair cell death may be a potential side effect of V-ATPase inhibitor treatments, similar to currently used platinum-based cancer drugs (cisplatin) and aminoglycoside antibiotics (Schacht, Talaska et al. 2012). Future work is needed to test how V-ATPase inhibitor doses that kill cancer cells impact hair cells.

From the work presented here we conclude loss of V-ATPase activity induces caspase-independent necrosis-like death of hair cells in zebrafish neuromasts. Our work indicates V-ATPase activity is necessary to maintain mitochondrial membrane polarization and mitochondrial health in hair cells, which are highly dependent on V-ATPase for survival relative to neighboring support cells in the neuromast. These results advance our understanding of cell-type specific functions for V-ATPase activity, and provide insight into underlying causes of sensorineural hearing loss and potentially other V-ATPase-associated diseases.

METHODS

Zebrafish strains

Zebrafish (*D. rerio*) were maintained using standard protocols. Zebrafish embryos were collected from natural matings and staged according to (Kimmel, Ballard et al. 1995). Mutant strains used in this study include *atp6v1f*\(^{hi1988Tg}\) and *atp6v1h*\(^{hi923Tg}\) that were obtained from the Zebrafish International Resource Center. Transgenic strains include *Tg(cldnb:lynGFP)* (Haas and Gilmour 2006), *Tg(CMV:EGFP-map1lc3b)* (He, Bartholomew et al. 2009), *Tg(myo6b:tdTomato)*, and *Tg(scm1:GFP)* (Behra, Bradsher et al. 2009). All experiments were approved by SUNY Upstate Medical University’s Institutional Animal Care and Use Committee.
Immunostaining

For fluorescent immunostaining experiments, embryos were fixed with 4% Paraformaldehyde (Alfa Aesar) in phosphate buffered saline + 1% Tween20 (PBST) overnight at 4°C. Next day, fix was removed, and the embryos were washed in PBST once for 15 minutes. This was followed by incubating the embryos in acetone for 8 minutes at -20°C, and another wash with PBST for 15 minutes. The embryos were then blocked with PBS + 10% BSA for an hour at room temperature. Primary antibodies diluted in PBS + 10% BSA were incubated with embryos overnight at 4°C. The embryos were then washed in PBST eight times for 15 minutes each. The embryos were again blocked and incubated with secondary antibodies in PBS + 10% BSA overnight at 4°C, and washed eight times in PBST. Primary antibodies: mouse anti-acetylated tubulin, 1:200 (Sigma-Aldrich, T7451), mouse anti-parvalbumin, 1:200 (Sigma-Aldrich, MAB1572), rabbit anti-SOX2, 1:200 (Abcam, ab97959), chicken anti-GFP, 1:200 (GeneTex, GTX13970), rabbit anti-ATP6V1A, 1:200 (Proteintech, 17115-1-AP), rabbit anti-LAMP1, 1:200 (abcam, ab24170), mouse anti-BrdU, 1:200 (Santa Cruz Biotechnology, sc-32323), rabbit anti-pHH3, 1:200 (Cell Signaling Technology, 9701S), and rabbit anti-cleaved Caspase3, 1:200 (Abcam, ab13847). Secondary antibodies: goat anti-mouse AlexaFluor 568, 1:200 (Thermo Fisher Scientific, A-11004), goat anti-rabbit AlexaFluor 488, 1:200 (Abcam, ab150077), goat anti-chicken AlexaFluor 488, 1:200 (Invitrogen, A-11039). DAPI, 1:500 (Thermo Fisher Scientific, 62248) was used to stain nuclei.

Spinning disc confocal microscopy

Immunostained or live zebrafish embryos were placed on their side on a 35 mm petri dish with a cover glass bottom (MatTek) and immobilized in 2% low melting agarose. For live imaging, embryos were anesthetized using 0.4% Tricaine (Tokyo chemical industry, T0941). Images were captured using a Perkin-Elmer Ultra VIEW Vox spinning disc confocal microscope with a 40x objective lens and a Hamamatsu C9100-50 camera. Laser power and exposure times were kept the same between control and test groups for fluorescence intensity measurement experiments.
Image processing and analysis

Confocal images were analyzed using Fiji (NIH) software. Neuromast area was measured by drawing a circle around the neuromast using DAPI as a marker. Volume of the aggregates of Lc3b-GFP were measured by using 3D object counter. Counting the number of hair cells and support cells in a neuromast was done by going through each Z slice of a stack of images through one neuromast. Representative images of neuromast hair cells and support cells were made by taking the maximum intensity projection of a few z-slices. Number of pyknotic nuclei was quantified by using DAPI as a marker of nuclei using cell counter in Fiji. Both pHH3 and BrdU positive cells were counted using these markers and DAPI to identify nucleus using cell counter. TMRE and Mitotracker fluorescence mean intensity was measured by manually drawing region of interest (ROI) around cluster of hair cell of individual neuromast in one channel and then copy-and-pasting the ROI in the other channel in Fiji.

Scanning Electron Microscopy

Embryos were fixed with 2.5% Glutaraldehyde and 2 mM CaCl$_2$ in 0.1 M Cacodylate buffer for 1.5 hours. The embryos were then washed three times for 5 minutes each with Cacodylate buffer. These embryos were then post fixed with 1% Osmium tetroxide and 4 mM CaCl$_2$ in 80 mM Cacodylate buffer for 10 minutes on ice. Followed by three washes for 5 minutes each with milliQ water. Embryos were then dehydrated using 50 to 100% graded ethanol (Kindt, Finch et al. 2012). Critical point drying was achieved using Tousimis samdri-PVT-3b, sputter coated with Palladium using Edwards sputter and coater. Images were collected using a JEOL JSM-IT100LA scanning electron microscope.

Reverse transcriptase-PCR

For qualitative analysis of V-ATPase subunit mRNA expression, total mRNA was isolated from $atp6v1f^{+/+}$, $apt6v1h^{+/+}$, and wild-type sibling embryos using Trizol (Invitrogen, 15596018). cDNA was synthesized using the reverse transcriptase iScript kit (Bio-Rad), and PCR was used to amplify
cDNAs of selected V-ATPase subunits and β-actin (primer sequences available upon request). PCR amplicons were scored as present or absent via agarose gel electrophoresis.

**Vital dyes**

Live embryos were incubated in vital dyes diluted in embryo water at 28.5°C. After the incubation, live embryos were imaged using spinning disc confocal microscopy. Vital dyes used in this study: Lysotracker (Invitrogen, L7528) 100 nM for 20 minutes, CellROX (Invitrogen, C10444) 2 µM for 20 minutes, TMRE (Thermo Fisher Scientific, T669) 20 nM for 20 minutes, Mitotracker (Thermo Fisher Scientific, M7514) 100 nM for 10 minutes, Ethidium homodimer III (Biotium, 30065) diluted according to the manufacturer for 30 minutes, and Hoechst 33342 (NucBlue; Invitrogen, R37605) 8 µM for 30 minutes.

**BrdU assay**

Embryos were incubated with 15% BrdU in embryo water for one hour at 28.5°C. Embryos were then fixed with 4% PFA overnight at 4°C, washed next day with PBST (1% Tween20), followed by incubation in 2N HCl for 30 minutes at 37°C (Cai, Lin et al. 2016). The immunostaining protocol was followed as mentioned above for anti-BrdU immunostaining and confocal imaging. The number of BrdU positive cells per neuromast was determined by analyzing Z-stacks.

**Pharmacological treatments**

To induce hair cell death, embryos were incubated in 10, 200 or 400 µM Neomycin sulfate (Sigma-Aldrich, 1458009) freshly prepared in embryo media for 1 hour at 28.5°C as described (Ma, Rubel et al. 2008, Cruz, Kappedal et al. 2015, Uribe, Kawas et al. 2015), or 10 µM Copper (ii) sulfate pentahydrate (Sigma-Aldrich, C8027) for 40 minutes at 28.5°C as described (Olivari, Hernandez et al. 2008). For all other drug treatments, embryos were incubated in embryo media containing the drug or
DMSO (vehicle control) from 2 to 4 dpf at 28.5°C. The media was refreshed daily. Drugs used include: 300µM pan-caspase inhibitor ZVAD-FMK (Enzo Life Sciences, ALX-260-020-M001) as described (Williams and Holder 2000, McNeill, Paulsen et al. 2007, Coffin, Williamson et al. 2013), 1 µM or 5 µM cyclosporin A (CsA) (Millipore Sigma, C3662) (Alassaf, Daykin et al. 2019) and 1µM Ru360 (Millipore Sigma, 557440).

**Statistical analysis**

All statistical analyses were done using GraphPad Prism 9. Graphs show ‘cleaned data’ wherever applicable and is devoid of outliers as determined by Graphpad Prism following the ROUT method with Q=1%. P values were calculated using unpaired t-test with Welch’s correction when comparing means of two unpaired groups of data without assuming that they have identical standard deviation or Two-way ANOVA when comparing means of multiple groups of data involving more than one variable followed by post-hoc Bonferroni- Šidák multiple comparisons since we compare selected sets of means, where p value needs to be less than alpha (0.05) to be considered statistically significant. All data are pooled from at least three independent experiments and represented as mean ± s.d. (standard deviation).

**Acknowledgements**

We thank Alexis Whellan, Victoria Berlandi-Short, Ellyn Prusinowski and Hannah Tarolli for technical help. We also thank Dr. Susan Anagnost and Ben Zink for assistance with scanning electron microscopy. We are grateful to Dr. Patricia Kane for providing feedback on this manuscript and thank Dr. Cameron MacQuarrie for helpful discussions. We thank Dr. Jeffrey Gross, Dr. Jason Myers, Dr. Katie Kindt and Dr. Michel Bagnat for sharing the zebrafish strains. This work was supported by NIH (NICHD) grant R03HD097543.
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Figure 1. V-ATPase mutant embryos show neuromast defects. **A.** Diagram of the V-ATPase holoenzyme complex. Adapted from (Collins and Forgac 2018, Abbas, Wu et al. 2020). **B.** Diagram of a lateral view of a zebrafish neuromast with centrally organized hair cells (orange) surrounded by support cells (green). Each hair cell has a single kinocilium (pink), a stair-case of stereocilia (red), and a nucleus (blue). **C.** Wild-type and *atp6v1f<sup>−/−</sup>* embryos at 4 dpf expressing the *Tg(scm1:GFP)* transgene that labels lateral line neuromasts (white arrowheads). **D.** Top-down view of neuromasts in wild-type, *atp6v1f<sup>−/−</sup>* and *atp6v1h<sup>−/−</sup>* embryos labeled with acetylated tubulin to mark hair cells (magenta) and DAPI to stain nuclei (blue). Dashed circle indicates the approximate neuromast boundary. **E-F.** Quantification of neuromast area in *atp6v1f<sup>−/−</sup>* (E) and *atp6v1h<sup>−/−</sup>* (F) embryos at 4 dpf relative to wild-type (WT) siblings. n=number of embryos examined. ***p=0.0002, ****p<0.0001 by unpaired t-test with Welch’s correction. **G.** Pseudo-colored scanning electron micrographs of wild-type, *atp6v1f<sup>−/−</sup>* and *atp6v1h<sup>−/−</sup>* neuromasts at 4 dpf with pink representing kinocilia and red representing stereocilia.
Figure 2. Expression of V-ATPase subunits in wild-type and V-ATPase mutant zebrafish. A. Reverse transcriptase (RT)-PCR analysis of total mRNA from wild-type and V-ATPase mutant embryos at 2, 3 and 4 dpf. *atp6v1f* mRNA is not detected in *atp6v1f*”;胚胎s, whereas *atp6v1h* mRNA is detected in the *atp6v1h*”;胚胎s. β-actin mRNA was amplified as a positive control, and reactions without reverse transcriptase (no RT) were negative controls. B. Antibodies against the V-ATPase V1A subunit (magenta) show enriched staining in centrally localized hair cells in a wild-type neuromast labeled by *Tg(cldnb:lynEGFP)* expression (green). C. Optical sections of hair cells reveal V1A subunit localizes throughout wild-type hair cells with an accumulation in the basal region. This basal localization is disrupted in *atp6v1f*”; mutant hair cells. Approximate boundaries of individual hair cells are outlined, and asterisks mark hair cell nuclei. Arrowheads indicate basal accumulation of V1A in wild-type hair cells, and lack thereof in *atp6v1f*”; hair cells.
Figure 3. V-ATPase loss of function alters pH and induces autophagy defects in neuromasts.

A. Lysotracker staining in a wild-type neuromast at 4 dpf was reduced in \textit{atp6v1f}−/− mutants. Neuromast cells are marked by \textit{Tg(cldnb:lynEGFP)} transgene expression. Arrowheads point out basal accumulation of Lysotracker in wild-type in hair cells. B. Wild-type and \textit{atp6v1f}−/− mutant neuromasts at 4 dpf stained for Lc3b-GFP (green) and nuclei using DAPI (blue). Arrowheads point out Lc3b-GFP aggregates. C. Quantification of volume of individual Lc3b-GFP aggregates in neuromasts from wild-type and \textit{atp6v1f}−/− embryos. The number of data points reflect the finding that each embryo can have multiple neuromast aggregates. n=number of embryos. ***p=0.0005 and ****p<0.0001 by unpaired t-test with Welch’s correction. D. Lamp1 antibody staining shows co-localization with Lc3b-GFP aggregates in \textit{atp6v1f}−/− mutant neuromast. Yellow dashed circle indicates hair cell cluster within a neuromast.
Figure 4. V-ATPase loss reduces the number of hair cells in neuromasts. A. Acetylated tubulin staining detects hair cells in wild-type, \textit{atp6v1f}^{-/-} and \textit{atp6v1h}^{-/-} neuromasts at 2 and 4 dpf. Arrowheads point to individual hair cells. B-C. The number of hair cells per neuromast at 2 and 4 dpf in \textit{atp6v1f}^{-/-} embryos and wild-type (WT) siblings (B), and \textit{atp6v1h}^{-/-} and wild-type siblings (C). D. Sox2 staining labels neuromast support cells at 2 and 4 dpf. E-F. The number of support cells per neuromast at 2 and 4 dpf in \textit{atp6v1f}^{-/-} embryos and wild-type siblings (E), and \textit{atp6v1h}^{-/-} and wild-type siblings (F). \(n\)=number of embryos. ***\(p=0.0009\) and ****\(p<0.0001\) by unpaired t-test with Welch’s correction. ns=not significant.
Figure 5. Loss of V-ATPase does not alter proliferation but induces hair cell death in mutant neuromasts. A-B. A one-hour pulse with BrdU results in a similar number of BrdU positive cells in wild-type and atp6v1f-/- neuromasts at 2 and 3 dpf. Representative images show BrdU labeled cells (orange) and neuromast nuclei stained with DAPI (blue). B. Quantification of the number of BrdU labeled cells per neuromast at 2 and 3 dpf. C-D. The number of pyknotic nuclei increases from 2 dpf through 4 dpf in both atp6v1f-/- (C) and atp6v1h-/- (D) neuromasts. Arrowheads denote pyknotic nuclei in panel A. E. Snapshots from live time-lapse imaging of wild-type and atp6v1f-/- hair cells marked by Tg(myo6b:tdTomato) expression. White arrowhead follows a single hair cell in the atp6v1f-/- neuromast over time as it dies. Fluorescent tdTomato protein accumulates into aggregates that are reminiscent of LC3b-GFP aggregates (see Fig. 3). Wild-type hair cells remained healthy. n=number of embryos. *** p=0.0009 and ****p<0.0001 by unpaired t-test with Welch’s correction. ns=not significant.
Figure 6. V-ATPase mutant hair cell death is independent of Caspase 3 and morphologically resembles necrosis. A. Cleaved Caspase 3 staining was absent in wild-type and \textit{atp6v1f}^{-/-} mutant neuromasts at 4 dpf, but was detected in the retina of \textit{atp6v1f}^{-/-} embryos, and in wild-type hair cells treated with neomycin (Neo) that induces caspase 3 activation (yellow arrowheads). Nuclei were detected using DAPI. White arrowheads indicate pyknotic nuclei. B. DAPI staining of neuromast nuclei at 4 dpf in wild-type and \textit{atp6v1f}^{-/-} embryos treated with either DMSO (control) or 300\textmu M ZVAD-FMK from 2 to 4 dpf. White arrowheads indicate pyknotic nuclei. C. Quantification of pyknotic nuclei per neuromast in control and 300\textmu M ZVAD-FMK treated embryos. \textit{n}=number of embryos. \textit{ns}=not significant by unpaired t-test with Welch’s correction. D. 3D rendering of time-lapse snapshots of \textit{Tg(myo6b:tdtomato); atp6v1f}^{-/-} hair cells undergoing necrosis-like morphological changes. The white arrowhead follows one hair cell swelling and then bursting.
Figure 7. Reduced mitochondrial membrane potential contributes to hair cell death in V-ATPase mutant neuromasts. A. The vital dyes Mitotracker and TMRE were used to assess mitochondrial mass and mitochondrial transmembrane potential, respectively in live wild-type and atp6v1f<sup>−/−</sup> embryos at 4 dpf. B-C. Quantification of mean fluorescence intensity measurements per neuromast of Mitotracker (B) and TMRE (C) in wild-type and atp6v1f<sup>−/−</sup> embryos at 4 dpf. D. Ratio of TMRE fluorescence intensity to Mitotracker. n=number of embryos. ****p<0.0001 by unpaired t-test with Welch’s correction.

E. Representative images of acetylated tubulin immunostaining of hair cells in wild-type and atp6v1f<sup>−/−</sup> embryos at 4 dpf after treatment with DMSO (vehicle control), CsA or RU360 from 2 dpf to 4 dpf. F-G. The number of hair cells per neuromast in control embryos and embryos treated with CsA (F) or RU360 (G). n=number of embryos. *p<0.04 by Two-way ANOVA with Bonferroni-Šidák post hoc test.
Fig. S1. A-B. Representative images of wild-type, \( atp6v1f^- \) and \( atp6v1h^- \) embryos at 2, 3 and 4 dpf. V-ATPase mutants are characterized by smaller size than wild-type, hypopigmentation, microphthalmia, facial malformations, and lack of swim bladder. C. Wild-type and \( atp6v1h^- \) embryos at 4 dpf expressing the \( Tg(cldnb:lynGFP) \) transgene that labels lateral line neuromasts.
**Fig. S2.** Qualitative RT-PCR analysis of the representative V-ATPase subunits atp6v1aa, atp6v0b and atp6v0ca in wildtype siblings and *atp6v1f<sup>−/−</sup>* and *atp6v1h<sup>−/−</sup>* mutants at 2, 3 and 4 dpf. mRNA was extracted from whole embryo lysates. Amplification of β-actin was used as a positive control, and reactions lacking reverse transcriptase (no RT) served as negative controls.
Fig. S3. Live images of wild-type and atp6v1f⁻/⁻ embryos expressing the Tg(myo6b:tdtomato) and Tg(CMV:EGFP-map1lc3b) transgenes that label hair cells (purple) and autophagosomes (white). Lc3b-GFP was found to accumulate in atp6v1f⁻/⁻ hair cells.
**Fig. S4.** A. Parvalbumin immunostaining labels hair cells (magenta) in wild-type and *atp6v1f<sup>-/-</sup>* neuromasts. DAPI stains nuclei (blue). B. Quantification of parvalbumin positive hair cell per neuromast in wild-type WT and *atp6v1f<sup>-/-</sup>*. The number of parvalbumin positive hair cells is reduced in *atp6v1f<sup>-/-</sup>* mutant neuromasts. Each data point represents an individual neuromast measured in n=number of embryos. ****p<0.0001 by unpaired t-test with Welch’s correction.
**Fig. S5.** A. Representative images of phospho-histone H3 (pHH3) positive cells (orange) in wild-type and atp6v1f/- neuromasts. DAPI stains nuclei (blue). B. Quantification of pHH3-positive cells in the neuromasts at both 2 and 4 dpf. n=number of embryos. ns=not significant by unpaired t-test with Welch’s correction.
Fig. S6. A-B. 4 day old wild-type or \textit{atp6v1f}^-^- embryos were incubated with EthD III and Hoechst DNA stain for 30 min, and then neuromasts were imaged. Only hair cell nuclei stained with Hoechst in neuromasts. EthD III staining was found to accumulate in the cytoplasm of wild-type hair cells (A) and co-localize with Hoechst staining of pyknotic nuclei in \textit{atp6v1f}^-^- mutants (B). (C) As a positive control experiment, wild-type embryos were treated with 200 \textmu M neomycin to induce hair cell death. EthD III staining co-localized with Hoechst staining of pyknotic nuclei in dying hair cells. Arrows point out pyknotic nuclei. \textit{n}=number of neuromasts analyzed.
**Fig. S7.** A. The vital dye CellROX was used to visualize ROS at 4 dpf in live embryos. A heatmap representation of fluorescence intensity indicates CellROX staining is reduced in *atp6v1f*-/- neuromasts relative to wild-type siblings. As a positive control, wild-type embryos were treated with 400µM neomycin (Neo), which is known to increase ROS in hair cells. B. CellROX staining is reduced in *atp6v1f*-/- hair cells, which are marked by Tg(*myo6b:tdtomato*) transgene expression. C. Quantification of CellROX staining in neuromasts at 4 dpf. n= number of embryos. ****p<0.0001 by unpaired t-test with Welch’s correction.
Fig. S8. A-B. The number of pyknotic nuclei per neuromast in wild-type and $atp6v1f^{-/-}$ embryos treated with or without CsA (A) or RU360 (B) from 2 dpf to 4 dpf. n=number of embryos. **p=0.0096 by Two-way ANOVA with Bonferroni-Šidák post hoc test.
**Movie 1.** Live imaging of *atp6v1f−/−* mutant neuromast hair cells expressing *Tg(myo6b:tdtomato)* at 4 dpf. Arrowhead follows a single hair cell that swells over time. Note that fluorescent tdTomato protein accumulates into aggregates that are reminiscent of LC3b-GFP aggregates that mark autophagosomes (see Fig. 3). Time stamp=hours:minutes

**Movie 2.** Live imaging of *atp6v1f−/−* mutant neuromast cells expressing *Tg(scm1:GFP)* at 4 dpf. Time stamp=hours:minutes
**Movie 3.** Live imaging of wild-type neuromast hair cells expressing *Tg(myo6b:tdtomato)* at 4 dpf. Time stamp=hours:minutes

**Movie 4.** Live imaging of wild-type neuromast support cells expressing *Tg(scm1:GFP)* at 4 dpf. Time stamp=hours:minutes
**Movie 5.** Live imaging of \textit{atp6v1f/-} mutant neuromast hair cells expressing \textit{Tg(myo6b:tdtomato)} at 4 dpf. The hair cell marked by asterisk detaches from the hair cell cluster, then swells and bursts. Note that fluorescent tdTomato protein accumulates into aggregates that are reminiscent of LC3b-GFP aggregates that mark autophagosomes (see Fig. 3).

**Movie 6.** Live imaging of wild-type neuromast hair cells expressing \textit{Tg(myo6b:tdtomato)} at 4 dpf treated with 10 µM CuSO$_4$ for 20 minutes at 28°C and then immediately imaged. The asterisk follows a hair cell undergoing CuSO$_4$ induced necrosis, where the hair cell disengages from the hair cell cluster, swells, and bursts.