HIGHLIGHTS

- The zebrafish hatching glands contain high levels of free Zn^{2+}
- Zinc content of hatching glands is regulated by two opposingly acting zinc transporters, Zip10 (import) and Znt1a (export)
- Zip10 and free Zn^{2+} is required for terminal differentiation of hatching gland cells, their developmental apoptosis, and embryo hatching
- The involvement of Zn^{2+} in regulation of hatching enzyme appears to be evolutionary conserved as the human hatching enzyme homologue, ovastacin, cleaves the zona pellucida protein, ZP2, under the influence of zinc

Hatching gland development and hatching in zebrafish embryos: a role for zinc and its transporters Zip10 and Znt1a

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ABSTRACT

Zinc transporters of the Zip (Slc39, importers) and ZnT (Slc30, exporters) protein families have evolutionary conserved roles in biology. The aim of the present study was to explore the role of zinc, and zinc transporters Zip10 and Znt1a in zebrafish hatching gland development and larval hatching. In the study, knockdown of genes for Zip10 and Znt1a in zebrafish embryos was achieved using morpholino-modified oligonucleotides. A partial loss-of-function Znt1a mutant ($Znt1a^{sa17}$) allowed comparison with the Znt1a morphant. Free Zn^{2+} in embryos and apoptosis were investigated using fluorescent dyes whereas gene expression was investigated by whole-mount in situ hybridization. The results showed high levels of free Zn^{2+} in the hatching gland cells (HGC) along with abundant expression of *zip10* and *znt1a* in normal embryo. Knockdown of *zip10* reduced free Zn^{2+} in HGC, ceased their normal developmental apoptosis, and resulted in displacement and later disappearance of hatching glands and hatching enzymes *he1a* and *catL1b*, and inability to hatch. Conversely, knockdown of *znt1a* or the $Znt1a^{sa17}$ mutation accelerated hatching and coincided with high expression of hatching enzymes and free Zn^{2+} in the HGC. Thus, Zip10 and free Zn^{2+} in the HGC are required both for their development and function. This study also demonstrated the opposite functions of the two zinc transporters, ZIP10 and ZnT1 as well as shedding light on the role of Zn^{2+} in regulation of the human hatching enzyme homologue, ovastacin, which is activated by zinc and cleaves the zona pellucida protein, ZP2, to prevent polyspermy.

Key words: Zebrafish, Slc30a1, Slc39a10, apoptosis, Zn, he1a, catL1b

INTRODUCTION

Hatching is a critical process in oviparous animals, and in the zebrafish hatching occurs between 48-72 hours post fertilisation (hpf) releasing the larvae into the water [1]. In viviparous species a simiar process occurs, for example the human embryo hatches out of its *zona pellucida* encasement at the blastula stage about five days post fertilisation in a process termed blastocyst hatching [2, 3]. Blastocyst hatching allows implantation in the endometrium to occur and provides the embryo space for further growth.

Zinc is an essential element required for virtually all biological proccesses, including embryonic development [4]. For example, 'zinc sparks' released by mammalian oocytes from cortical granules during fertilisation prevent polyspermy by transiently blocking sperm penetration of the zona matrix. This is followed by cleavage of zona pellucida glycoproteins (ZP2) by ovastacin, a zinc metalloprotease that is also released from cortical granules [5, 6]. Ovastacin belongs to the astacin family of zinc metalloproteases [7] and is homologous to the hatching enzymes of fish and crustacean [8-10]. In fact, the astacin protein family is named after the *Astacus* genus of crayfish. The hatching enzymes in fish are secreted by hatching gland cells (HGC), which have their final location in different areas of the embryo depending on fish species [9, 11, 12]. The hatching gland is derived from the polster, which is an accumulation of cells found anterior, and below, the forebrain of the embryo at the tail bud stage. The polster, in turn, is derived from the anterior mesendoderm. Differentiation of the polster and, later, the hatching gland cells, is controlled by specific gene regulation, a process programmed early in embryogenesis [13, 14]. In zebrafish, the hatching enzymes is known as hatching enzyme 1 (he1) and is highly expressed in the HGC [9], which also contains an abundance of free Zn^{2+} [15, 16]. At the time of hatching, the exocrine HGC release their contents of zinc and enzymes into the perivitelline space. Zinc is an inhibitor of caspases [17] and regulates the apoptotic process initiating the breakdown of the zona pellucida, an event that can be observed as early as 24hpf [18]. However, exposure of zebrafish and other teleost fish embryos to excess waterborne zinc paradoxically causes delayed hatching with low hatchability and developmental abnormalities [19-22].

The numerous zinc-dependent processes in the vertebrate body are tightly homeostatically controlled. In this regard, two families of transmembrane proteins play critical roles in transporting zinc in and out of cells. These are the Cation Diffusion Facilitator (CDF/ZnT/SLC30) family, which traffics zinc out of the cytosol, and the Zinc/Iron regulated

transport (Zrt, Irt)-like protein (ZIP/SLC39) family, which moves zinc into the cytosol [23-25]. Zip10 (Slc39a10) is highly expressed in the HGC of zebrafish [26] and was therefore considered a candidate zinc importer in these cells. Tissue-specific mRNA expression of *zip10* is notable at pre-polster mesendoderm, polster and HGC and persists there until 48hpf before the expression gradually disappears [26]. This pattern of expression indicates that *zip10* is an early marker of mesendoderm with a role in early embryonic development. Additionally, we previously observed that *znt1a* (*slc30a1a*) is also expressed in the HGC of embryos [16]. Thus, in the present study, we followed the expression of hatching gland development and its dependence on Zn^{2+} ions and zinc transporters Zip10 and Znt1a, using four HGC expression markers: *zip10*, *znt1a*, *catL1b* and *he1a*.

We demonstrate that Zip10 and Znt1a have opposing effects on time to hatch, with deficiency of ZnT1a expression or activity accelerating the process and loss of Zip10 causing delayed or no hatching. We propose that this relates to the free Zn^{2+} concentration in the HGC whereby Zip10 knockdown results in less free Zn^{2+} in the HGC while Znt1a deficiency causes more HGC free $Zn^{2+}$and accelerated hatching. Our study identifies Zip10 as the major importer and Znt1a as the major exporter and demonstrates that free Zn^{2+} in the HGC is necessary for the terminal differentiation, migration and function of HGC in zebrafish. Based on these results we propose that the amount of free Zn^{2+} present in the HGC determines the time for hatching. We further provide evidence that the effect of waterborne zinc on hatching time is unrelated to HGC function and might be explained by the action of zinc on the chorion.

MATERIALS AND METHODS

Animal model

Zebrafish embryos were obtained from adult fish of King's Wild-type 2 (KWT2) strain immediately after spawning and were used for antisense morpholino-mediated gene knockdown analysis as well as for zinc exposure/depletion. All experiments were performed in accordance with licences held under the UK Animals (Scientific Procedures) Act 1986 and later amendments and conforming to ARRIVE and all relevant guidelines and regulations.

To study the effects of zinc exposure and depletion, embryos were incubated in embryo water (composed of reverse osmosis deionized water supplemented with 60 mg/l of sea salt and 200µMCaCl2) in a Petri dish either supplemented with zinc by 100µM of $ZnSO_4$ or depleted of zinc by 5µM TPEN (N,N,N',N'-Tetrakis(2-pyridymethyl) ethylene diamine and incubated at 28.5^{o}C for 5 days observation period with no appreciable effect of treatment on survival of the embryos. The control embryos were incubated in standard fish tank water at the same temperature for the same period of time. The number of hatched larvae at various time intervals were observed and recorded for different treatments.

For studies on Znt1a homozygous mutant embryo (Znt1a^{sa17}), embryos derived from zebrafish with a heterozygous mutation in *slc30a1a* (*znt1a*; strain sa17) generated through TILLING by the Sanger Centre [27] were used in the present study. The mutation caused a transition of nucleotide ''A'' to ''T'' at position 1568 in the coding sequence leading to premature termination of the Znt1a protein and resulting in a truncated protein, short of the last 40 amino acids of the C-terminus as previously described [16]. Mutant embryos were transferred to King's College London where they were reared to adults and genotyped. Heterozygous adults were cross-bred to obtain homozygous mutants which were further reared to adults and mated to obtain homozygous mutant embryos [16].

Gene knockdown

Anti-sense morpholino-modified oligonucleotides (MO) for *zip10* and *znt1a* genes were designed (Table 1) and then procured from GeneTools LLC (Philomath, USA). Wild-type zebrafish embryos at 1-4 cell stage of development were either micro-injected with 2-4 ng of translation blocking MO for *zip10* or *znt1a* gene knockdown as previously described [16, 28]. Injected embryos were then incubated at 28.5^{o}C and monitored through developmental stages until the time of hatching. Another set of embryos at the same developmental stage were also injected with a scrambled or mismatched MO in a similar way while other embryos, also at the same stage of development, were left un-injected to serve as injected and un-injected controls respectively. Because of the off-target effect in some MOs [29], a splice-site blocking MO for each gene (*zip10* and *znt1a*) was injected and the stages of embryogenesis were compared with those of translation blocking MO-injected embryos. In other experiments, a *p53* translation blocking MO was either injected alone or co-injected along

with each of the two types of MOs for *zip10* and *znt1a* in the ratio of 1.0:1.5 respectively. This control treatment is based on the observation that most of the off-target effects of some MOs are a result of *p53* gene activation causing a non-specific neuronal cell death [30, 31]. Sequences for all MO used (translational and splice blockers) are shown in Table 1.

Imaging of free Zn^{2+} and apoptotic cells in embryos

Abundance of free zinc (Zn^{2+}) ions was imaged *in vivo* in 24hpf dechorionated embryos using a synthetic small molecule ratiometric zinc-specific probe termed "ZTRS" which was a kind gift from Dr. Zhaochao Xu of the University of Cambridge [15]. Embryos were incubated in standard fish tank water with 10µM of the ZTRS probe for 10 min and the Zn^{2+} ion fluorescence intensity of the hatching gland cells was observed with an epi-fluorescence microscope (Nikon eclipse 400) at 360/530nm excitation/emission wavelength as previously described [16]. For visualisation of apoptotic cells, embryos were incubated in 5-10µg/ml of acridine orange dye for 10 min and observed under a fluorescence microscope at 360/630nm excitation/emission wavelength.

Gene expression analysis

Digoxygenin-labelled anti-sense RNA probes were produced against two zinc transporter genes (*zip10 & znt1a*) as well as against two hatching gland marker genes (*catL1b & he1a*) according to standard molecular gene cloning techniques followed by *in vitro* transcription [32]. These probes were used for gene expression analysis on the embryos at various developmental stages using the whole mount *In Situ* Hybridization (ISH) technique [33]. The primer sequences used for amplification of each gene are shown in Table 2.

RESULTS

The zinc concentration in the water is inversely related to time of hatching

The hatching rates of wildtype untreated zebrafish embryos at 48, 51 and 72hpf were 18%, 28% and 85%, respectively. Exposure of embryos to waterborne zinc (100µM) markedly delayed and reduced hatching (Fig. 1). In contrast, treating the embryos with 5µM of the zinc chelator, TPEN, accelerated hatching whereby about 75% hatched at 48hpf and 100% at 51hpf (Fig. 1). Embryos incubated with 100µM Zn and 5µM TPEN in combination showed no difference in hatching frequency compared to the un-exposed embryos (Fig. 1).

Zinc importer Zip10 and zinc exporter Znt1a are highly expressed in HGC

There was distinct expression of *zip10* and *znt1a* in HGC of 24hpf wild-type embryos, which remained following injection with mis-matched MO control (Fig. 2). However, the expression disappeared in embryos injected with the respective MO to *zip10* and *znt1a* genes (Fig. 2). Embryos homozygous for a loss-of-function mutation in *znt1a* (Znt1a^{sa17}) showed diffuse expression of and *znt1a* and little or no expression of *znt1a* in the HGC (Fig. 2).

Zinc importer Zip10 and zinc exporter Znt1a have opposing effects on hatching

Both translation- and splice-blocking morpholino-modified oligonucleotides (MO) were used to silence expression of *zip10* or *znt1a* in zebrafish embryos. The effects of the *zip10* or *znt1a* morpholinos appeared to be specific and persisted when either of these was co-injected with *p53* MO, indicating no off-target p53 activation. Knockdown of *zip10* by MO drastically delayed hatching and reduced the percentage of hatched embryos at 72hpf (Fig. 3) without appreciable delay in the stages of development as compared to wild-type control. Only 38% of the *zip10* MO embryos later hatched and were alive by 7dpf (168hpf). In contrast, *znt1a* MO knockdown accelerated the hatching process whereby an increased number of embryos hatched at 51hpf compared to the wild-type control. A hatching effect similar to that of *znt1a* MO knockdown was observed in homozygous embryos with a loss-of-function mutation in the *znt1a* gene (Znt1a^{sa17}). Embryos injected with scrambled MO did not differ from the un-injected wild-type control (Fig. 3). These results indicate that hatching is modulated by processes that either supply (importer) or remove (exporter) intracellular zinc in HGC.

Zip10 and Znt1a have opposing effects on Zn^{2+} accumulation and apoptosis of HGC

There was an abundance of free Zn^{2+} in the HGC of wild-type, *znt1a* MO-injected morphant, and *znt1a^{sa17}* homozygous loss-of-function mutant embryos at 24hpf. In contrast, free Zn^{2+} was not detected in the HGC of *zip10* MO-injected embryos (Fig 4A). Apoptotic cells were observed in the HGC of wild-type and *znt1a* MO-injected embryos, but not in *zip10* MO-injected embryos (Fig. 4B). The developmental apoptosis of HGC was not due to p53 activation, as evidenced by embryos injected with *p53* MO (Fig 4C). Apotosis was abolished in embryos treated with the caspase-3 inhibitor, fluoromethylketone (Z-DVD-FMK). Neither caspase-3 inhibition nor *p53* knockdown visibly influenced ZTRS fluorescence for free Zn^{2+} in the HGC (Fig. 4C). Thus, free Zn^{2+} in the HGC provided by Zip10 is necessary to effect HGC developmental apoptosis.

Zip10 is essential for hatching gland development

The zebrafish hatching enzyme (*he1a*) and cathepsin L (*catL1b*), markers for the HGC, were expressed in a distinct and well-defined pattern in control wild-type and control MO-injected embryos at all developmental stages investigated. In *zip10* MO-injected embryos, expression of *catL1b* and *he1a* was normal in the polster (10hpf) and hatching gland tissue continued to develop up to 24hpf. However, already at 24hpf, the shape of the hatching gland tissue was irregular and isolated groups of HGC began to appear elsewhere on the yolk. At 33hpf, staining for the HGC markers declined and was found at a variety of punctate locations, disappearing completely at 48hpf (Fig. 5). Since *catL1b* and *he1a* are both expressed in the polster and in the HGC at least up to 24hpf it appears that Zip10 is not critical for the specification of HGC from their precursors but is required for their migration and terminal differentiation.

The *znt1a* MO-injected and Znt1a^{sa17} homozygous mutant embryos showed abundant expression of the hatching gland markers *catL1b* and *he1a* at all developmental stages and, if anything, had more prominent hatching gland patterning than the un-injected control (Fig. 6).

DISCUSSION

Our results show how the control of embryonic zinc is involved in the process of zebrafish embryo hatching. We found opposing roles of two zinc transporters and describe Zip10 as the main importer responsible for the presence of zinc in HGC and Znt1a as the main zinc exporter that reduces zinc in HGC. Knockdown of *zip10* or *znt1a*, or reduced Znt1a expression and function in HGC of the the Znt1a^{sa17} mutant [16] influences the time to hatch with opposing effects. Through the use of a fluorescent Zn^{2+} probe we were able to show *in-vivo* that high levels of free Zn^{2+} are present in the HGC and that this is necessary for successful hatching.

The concentration of zinc in the water is inversely related to the time of hatching (Fig. 1), an observation that corrobroates previous findings [20, 21]. This effect appears counterintutive because Zn^{2+} in the HGC is required for hatching (Fig. 4). However, the embryos of oviparous vertebrates are protected by the chorion from fluctuating zinc levels in the water [34], which do not necessarily reflect the internal free Zn^{2+}. We observed no change in Zn^{2+} accumulation of the HGC or in the expression of hatching gland gene markers in zinc-

exposed or zinc-depleted embryos compared to the untreated control. Salvaggio et al., [22] reported that water concentrations between 760 and 2,300μM did not increase the zinc accumulation of zebrafish embryos compared to un-exposed controls before hatching. Since in the present study we investigated embryos exposed to 100μM waterborne zinc or 5μM TPEN up to 72hpf only, the observed effects of waterborne zinc exposure and depletion are probably not caused by changes in zinc contents of the embryonic tissues, but potentially attributed to the consequences of zinc deposition on, or removal from, the chorion, making it either harder or softer for the embryo to break through during the time of hatching.

The loss of expression of hatching gland markers (*catL1b* and *he1a*) at 48 hpf in *zip10* knockdown embryos was associated with delayed or complete lack of hatching in these embryos. Gardiner et al., [35] observed a similar effect in zebrafish with Krüppel-like factor 4 (*klf4*) morpholino knockdown showing lack of expression of hatching gland marker *catL1b* and subsequent failure to hatch. Noteworthy, in the mouse intestine expression of *Klf4*, which codes for a zinc finger transcription factor, is increased during zinc restriction and reportedly induces expression of the *Zip10* paralogue, *Zip4* [36]. Whilst it is interesting in this context that *Klf4* appears to be a zinc sensor there is no emperical evidence suggesting that it is regulating *zip10* transcription in zebrafish.

The location of irregular and isolated groups of HGC elsewhere on the yolk at 24hpf in $Zip10^{MO}$ embryos resembles that of abnormal morphology and positioning of HGC in E-cadherin mutant and morphant zebrafish resulting from defective migratory activity of the cells [37, 38]. In the present study, the loss of HGC was a gradual process brought about by displacement and disappearance of the gland tissue between 24 and 48hpf (Fig. 3B). This effect may be a result of an insufficient concentration of Zn^{2+} in the HGC, leading to only transient expression of hatching gland markers.

Whilst He1a is a zinc-dependent metalloprotease, which in $Zip10^{MO}$ embryos may have lost activity to digest the chorion, zinc is an inhibitor of CatL1b, the other proteolytic enzyme required for hatching in zebrafish]10, 39]. Human CATL1 is inhibited by nanomolar concentrationsof Zn^{2+} [40] and it is therefore possible that the lack of Zn^{2+} in Zip10 deficient embryos resulted in premature activation of CatL1a in the HGC with consequential loss of the tissue [40-43]. Hence, the failure of $Zip10^{MO}$ embryos to hatch and accelerated hatching in embryos with Znt1a morphant or mutant can be explained by the opposing functions of

both transporters, whereby Zip10 and Znt1a imports and exports zinc in and out of the HGC, respectively.

Apoptosis is one of the processes in the terminal phase of hatching gland development [18]. At low levels zinc inhibits apoptosis [44], but high concentrations of zinc can induce apoptosis through other pathways [45]. In untreated 24hpf wild-type embryos HGC stained intensely for Zn^{2+} and apoptosis whereas in Zip10 morphants there was no staining for either (Fig. 4), although the HGC at this point in development were still present (Fig. 5). Thus, zinc in the HGC could have a role in inducing developmental apoptosis in zebrafish at 18hpf [45]. Similar to observations in $Zip10^{MO}$ knockdown embryos, inhibition of caspase-3 activity also inhibited developmental apoptosis of HGC [18] as confirmed in our present study. Morpholino knockdown of *p53* did not interfere with the apoptotic process, suggesting that the developmental apoptosis of the HGC is not through the mitochondrial p53-dependant apoptosis pathway but through other pathway(s) that converge on caspase-3. The results suggest that free Zn^{2+} in the HGC is required for developmental apoptosis by activating caspase-3 through an p53-independent pathway. Interestingly, knockdown of *zip6* (which is a relative of *zip10*) also showed a decrease in intracellular Zn levels as well as increase caspase-related cell apoptosis in both zebrafish cells and human T cells [46].

The process of hatching in oviparous vertebrates is recapitulated at two events in mammalian embryogenesis. The first of these is during sperm penetration of the egg whereby the mammalian hatching enzyme homologue, ovastacin, cleaves the zona pellucida protein, ZP2, under the influence of zinc to prevent polyspermy [3, 5, 6, 47]. Both ovastacin and zinc are released from the cortical granule of the oocyte [5, 6] and, intruigingly, ZIP10 along with ZIP6 is instrumental in delivering the zinc that is incorporated into the cortical granules of the mammalian oocyte during the oocyte-to-egg transition [48]. A Zip6:Zip10 heterodimer also regulates epithelial-to-mesenchymal transition during zebrafish gastrulation [49, 50]. The second evolutionary recapitulated event is the hatching *per se*, which happens at the blastula stage in mammals [2, 3]. Embryo-derived cathepsin accelerates hatching in mammals [2]. This process mirrors the process of lysis of the zona pellucida (ZP) of the chorion (choriolysis) in the zebrafish embryo [51].

CONCLUSION

Our study reveals three remarkable findings, that (1) the zebrafish hatching gland contains high levels of free Zn^{2+}, (2) the Zn^{2+} in the HGC is provided by a single zinc transporter, Zip10, and that (3) Zip10 is required for terminal differentiation of HGC, their developmental apoptosis, and embryo hatching. Interestingly, deficiency in expression or function of the principal zinc exporter, Znt1a, in HGC accelerated hatching, indicating that the zinc content of HGC influences time to hatch.

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IM conducted all the experiments and drafted the manuscript; CH lead and supervised the project while NRB and WM assisted in supervision. IM, CH, NRB, and WM contributed to editing of the manuscript.

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FIGURE LEGENDS

Figure1: Effects of zinc and TPEN treatment on zebrafish hatching. Wild-type embryos were treated with either waterborne zinc (100μM) or with the cell-permeable zinc chelator, TPEN (5μM) or both and the rate of hatching was compared to untreated wild-type control at 48, 51 and 72hpf. Data are expressed as percentage of hatched embryos and presented as the mean ± SEM with significance accepted at $p \leq 0.05$ using 1-way ANOVA followed by Dunnett's post-hoc test. n=15 with 10 embryos per n in each group. **Key;** *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$.

Figure 2: Effect of *zip10* or *znt1a* morpholino knockdown or *znt1a* loss-of-function mutation on expression of *zip10* or *znt1a*. Expression of *zip10* and *znt1a* mRNA in 24 hpf embryos of wild-type, morphants ($Zip10^{MO}$, $Znt1a^{MO}$ and $control^{MO}$) and Znt1a homozygote mutant ($Znt1a^{sa17}$). Note the staining of mRNA for *Zip10* and *Znt1a* in HGC and absence of

staining in the HGC on the respective morphants and mutant. N=3 experiments per treatment group with 10 embryos in each group with consistent observations.

Figure 3: Effect of zinc transporter knockdown or mutation on time to hatch. Wild-type embryos were micro-injected with either mis-matched control MO, or MO against Zip10 ($Zip10^{MO}$) or Znt1a ($Znt1a^{MO}$) and hatching was compared to wild-type un-injected control at 51 and 72 hpf. Hatching of embryos with Znt1a mutation the sa17 ($Znt1^{sa17}$) was also compared with the wild-type un-injected control. Data are expressed as percentage of hatched embryos and presented as the mean ± SEM with significance accepted at $p \leq 0.05$ using 1-way ANOVA followed by Dunnett's post-hoc test. n=15 with 10 embryos per n in each group. **Key;** significant *at $p \leq 0.05$, ** at $p \leq 0.01$, *** at $p \leq 0.001$.

Figure 4: Effect of Zip10 knockdown or Znt1a knockdown/mutation on free Zn^{2+}levels and developmental apoptosis of Hatching Gland Cells (HGC) at 24 hpf. Free Zn^{2+} in zebrafish embryos was monitored by the fluorescent Zn^{2+} probe, ZTRS, and apoptotic cells visualised using acridine orange. (**A**) Fluorescence of ZTRS Zn^{2+} was detected in the hatching glands of 24hpf control wild-type (WT), Znt1a homozygote ($Znt1a^{sa17}$) and Znt1a morphant ($Znt1a^{MO}$) indicating the presence of Zn^{2+} but was absent in the Zip10 morphant ($Zip10^{MO}$). (**B**) Fluorescence of acridine orange dye in the HGC of wild-type and Znt1 morphant embryos ($Znt1^{MO}$) as indicator of apoptosis which was absent in Zip10 morphant embryos ($Zip10^{MO}$). (**C**) Effect of *p53* knockdown ($p53^{MO}$) or caspase 3 inhibition (fluoromethylketone) on fluorescence of the free Zn^{2+} indicator, ZTRS, and acridine orange staining of apoptotic cells of the hatching gland as observed in untreated wild-type control. All experiments were carried out in five repeats (n=5) with consistent observations.

Figure 5: Effect of *zip10* morpholino knockdown on expression of HGC markers in embryos at different stages of development. Expression of mRNA for hatching gland markers (*catL1b* and *he1*a) at 10, 24, 33 & 48 hpf in wild-type embryos ($Control^{WT}$), control morphants ($Control^{MO}$) and Zip10 morphants ($Zip10^{MO}$). Note the gradual displacement of gland tissue from its normal position on the yolk at 24hpf (white arrow) and33hpf (red arrows), and eventual disappearance at 48hpf. N=3 experiments per treatment group with 10 embryos in each groupwith consistent observations.

Fig 6: Effect of *znt1a* morpholino knockdown or loss-of-function mutation on expression of HGC markers in embryos at different stages of development. Expression of mRNA for

hatching gland markers (*catL1b* and*he1a*) at 24 and 48 hpf in wild-type, *znt1a* morphant ($Znt1a^{MO}$) and *znt1a* homozygote mutant embryos ($Znt1a^{sa17}$). N=3 experiments per treatment group with 10 embryos in each group with consistent observations.

Tables

Table 1. Sequences of MO used for gene knockdown experiment. TB = translation blocker, SB = Splice junction blocker.

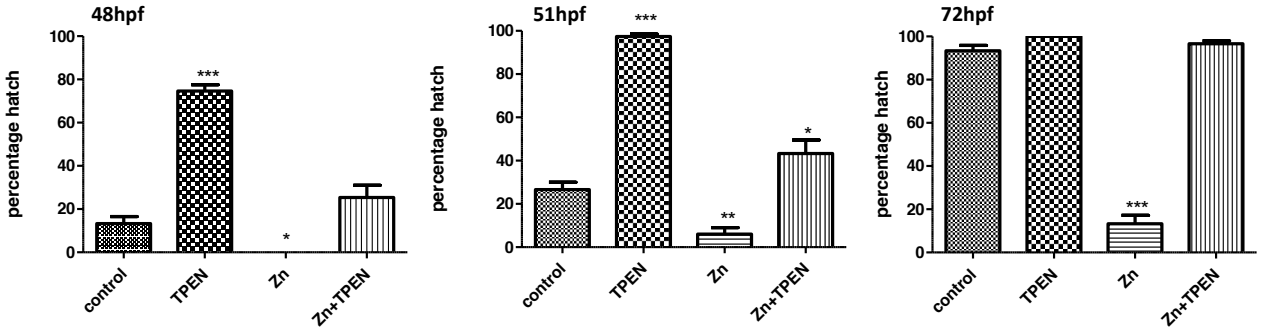
MO type	Sequences (5'-3')
zip10 TB	TGGTATGTGTGTGAACTCTCATCAT
zip10 SB	ATCACAGCACTGAGACTCACCTCTT
znt1a TB	GCGGAGCACAGACAGAAACAAAAGCT
znt1a SB	AGAAAACAAACCCCATTTACCGGCA
p53 TB	GCGCCATTGCTTTGCAAGAATTG
Random-control-MASO (mismatch) 5'A 3'	

Table 2. Primer sequences used for amplification of genes for WISH probe. Genes of interest with their primer sequences, product lengths and accession numbers

Gene	Primer sequences (5'-3')	Amplicon size (bp)
Slc30a1a (*znt1a*)	F: AGACCCAGTCCACCAACAAG R: AGGACATGCAGGAAAACACC	538
slc39a10 (*zip10*)	F: TCAGAAATGTCCTGCAATGG R: TGGGCTTTGACCTTAGATGG	823
he1a	F: CCCTCTCCATTCTGCTTCTG R: GCCGTTTTTCCATAGTGCAT	652
catL1b	F: AGCCAGCATGGAAAAAGCTA R: CAGAGAAGGCTCCAGTGACC	935

Figure

Figure 1



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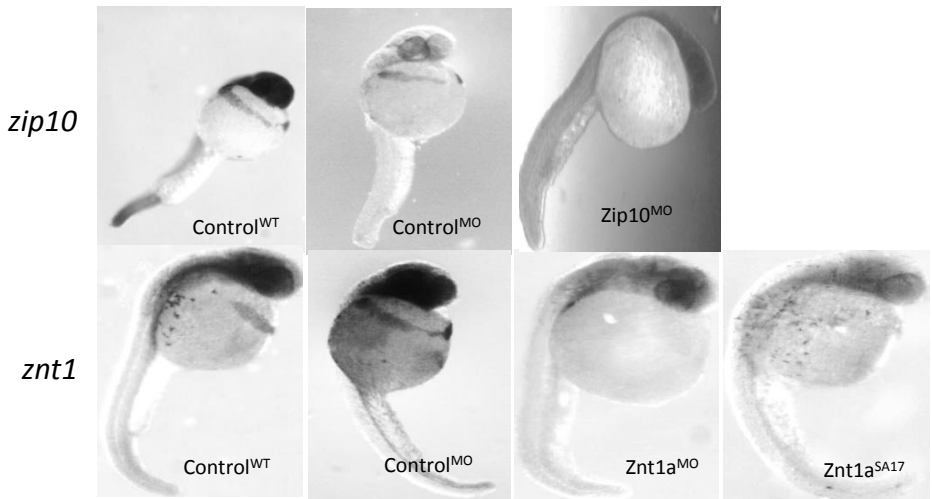
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Zip10^MO
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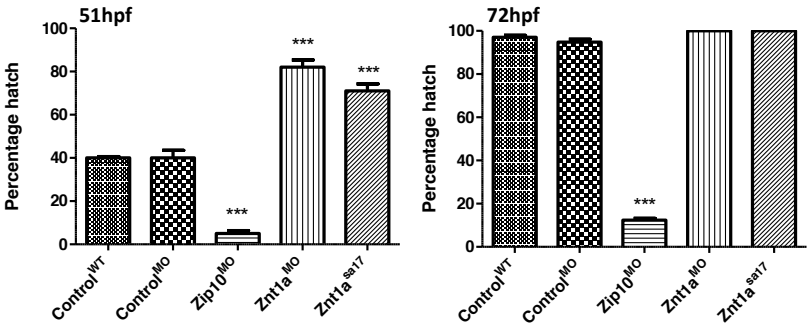
Znt1a^SA17

Figure 3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Percentage hatch
100
80
60
40
20
0
***
***
***
Control WT
Control MO
Zip10 MO
Znt1a MO
Znt1a sa17
72hpf
Percentage hatch
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60
40
20
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***
Control WT
Control MO
Zip10 MO
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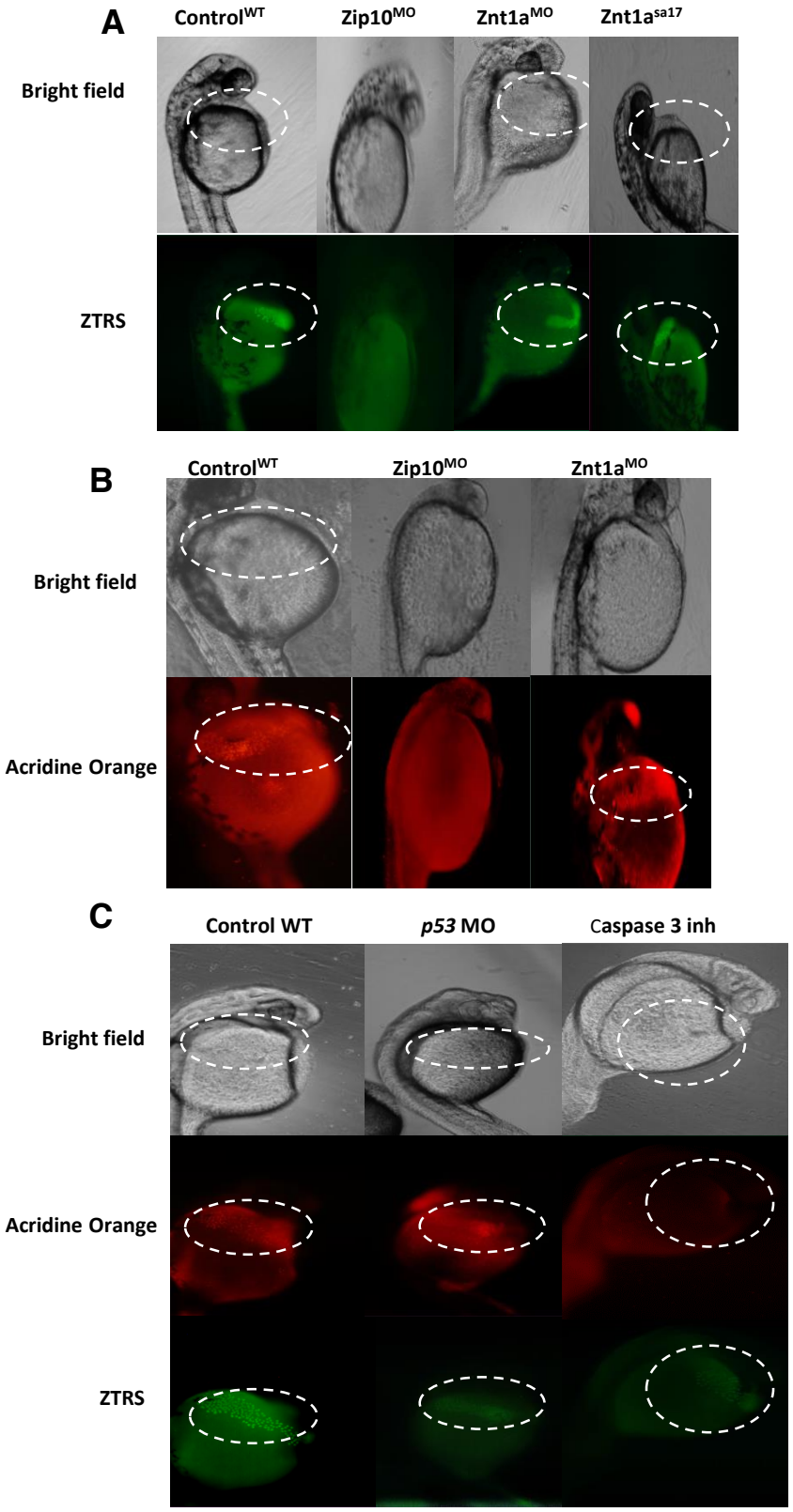
Znt1a sa17

Figure 4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Zip10^MO
Znt1a^MO
Znt1a^sa17
Bright field
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B
Control^WT
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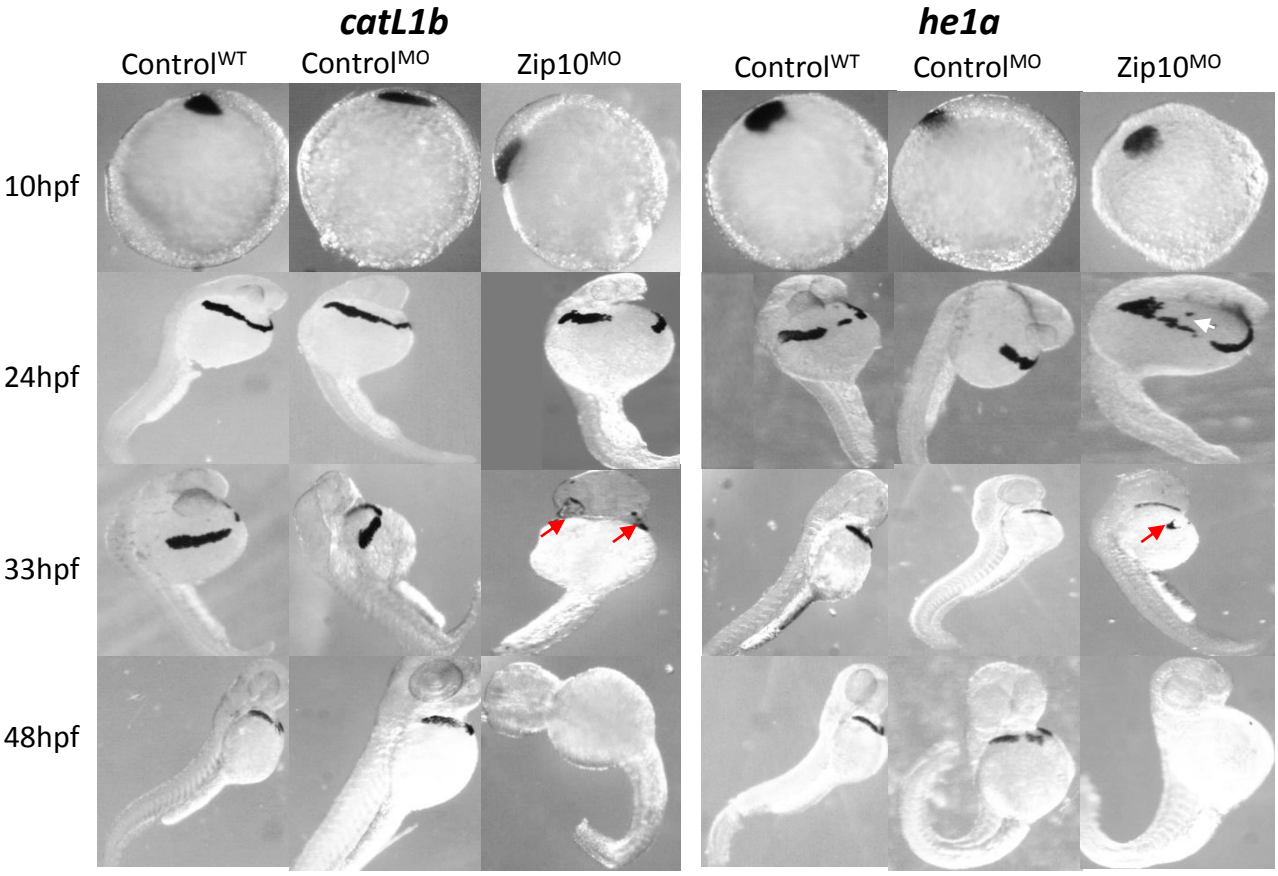
p53 MO
Caspase 3 inh
Bright field
Acridine Orange
ZTRS

Figure 5


catL1b
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Zip10^MO
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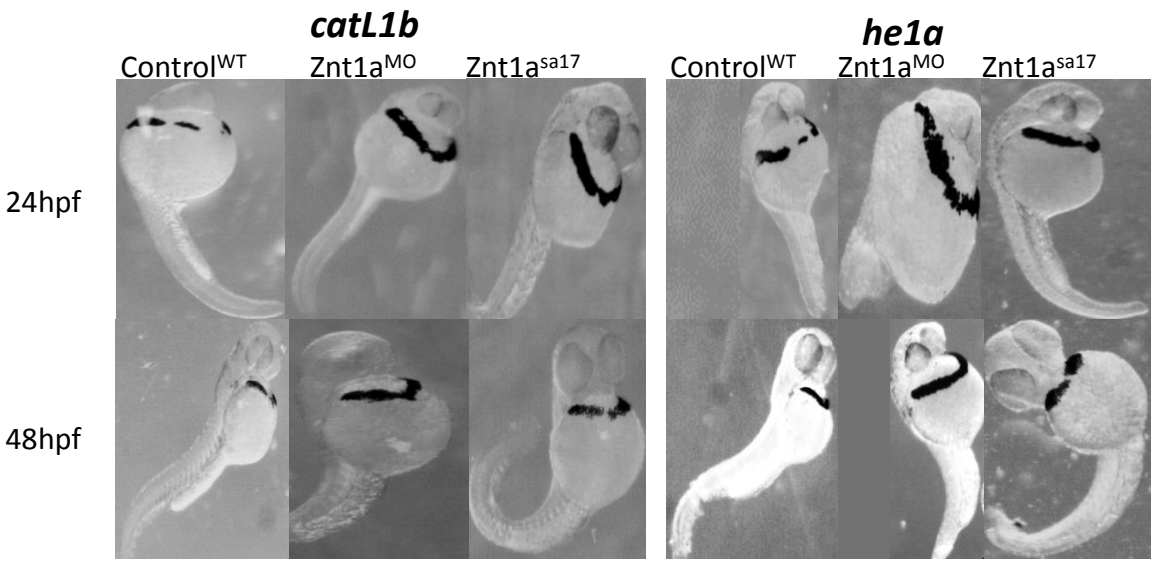
Zip10^MO
10hpf
24hpf
33hpf
48hpf

Figure 6


catL1b
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24hpf
48hpf

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KING'S College LONDON Founded 1829

University of London

Thursday, 07 May 2020

RE: DECLARATION OF INTEREST

Dear Editor,
We would greatly appreciate if you considered our manuscript "Hatching gland development and hatching in zebrafish embryos: a role for zinc and its transporters Zip10 and Znt1a" for publication in *Biochemical and Biophysical Research Communications*.

Meanwhile, the authors hereby have no conflict of interest to declare.

Best wishes,

Christer Hogstrand