Matriptase generates a tissue damage response via promoting Gq signalling, leading to RSK and DUOX activation

Running Title: Matriptase activation of a tissue damage response

Jiajia MA¹, Claire A. SCOTT²-³, Ying Na HO¹, Harsha MAHABALESHWAR¹, Katherine S. MARSAY²,⁴, Changqing ZHANG¹, Christopher K. J. TEOW¹, Ser Sue NG², Weibin ZHANG², Vinay TERGAONKAR², Lynda J. PARTRIDGE⁴, Sudipto ROY²,⁵,⁶, Enrique AMAYA³, Tom J. CARNEY¹,²,*

¹Lee Kong Chian School of Medicine, Experimental Medicine Building, Yunnan Garden Campus, 59 Nanyang Drive, Nanyang Technological University, 636921, Singapore
²Institute of Molecular and Cell Biology (IMCB), A*STAR (Agency for Science, Technology and Research), 61 Biopolis Drive, Proteos, 138673, Singapore
³Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, M13 9PT, UK
⁴Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, S10 2TN, UK.
⁵Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543
⁶Department of Pediatrics, Yong Loo Ling School of Medicine, National University of Singapore, 1E Kent Ridge Road, Singapore 119288

*Author for correspondence: tcarney@ntu.edu.sg

Abstract Tissues respond to damage by increasing inflammation and epithelial cell motility. How damage detection and responses are orchestrated is unclear. Overexpression of the membrane bound protease, Matriptase, or mutation of its inhibitor, Hai1, results in inflamed epithelia, in which cells have increased motility and are prone to carcinoma. How Matriptase leads to these cellular outcomes is unknown. We demonstrate that zebrafish hai1a mutants show increased H₂O₂, NfκB signalling, and IP₃R-mediated calcium flashes, and that these promote inflammation, but do not generate epithelial cell motility. In contrast, inhibition of the Gq subunit rescues both the hai1a inflammation and epithelial phenotypes, with the latter recapitulated by the DAG analogue, PMA. We demonstrate that hai1a has elevated pERK, inhibition of which rescues the epidermal defects. Finally, we identify RSK kinases as pERK targets disrupting adherens junctions in hai1a mutants. Our work maps novel signalling cascades mediating the potent effects of Matriptase on epithelia, with implications for tissue damage response and carcinoma progression.

Keywords: Epithelia, RSK, Par2, Matriptase, St14, zebrafish, epidermis, inflammation, hydrogen peroxide, Hai1, Gq
Introduction

Cells express diverse systems on their cell surface that ensure they can detect and respond to local tissue damage. Dysregulation of these systems are known to promote tumour development, and tumours have been proposed to represent non-healing wounds. Type II transmembrane serine proteases have been implicated in many diverse physiological processes and pathologies, including iron homeostasis, epithelial barrier formation, hypertension, cancer, and viral infection (Szabo & Bugge, 2008). Of these, Matriptase, encoded by the ST14 gene, has been extensively studied due to its broad expression in most epithelia, its consistent dysregulation in human carcinomas and its ability to promote malignancy when overexpressed in mice (List et al., 2005; Martin & List, 2019). Matriptase is essential for epidermal cornification and desquamation, with ST14 mutations found in patients with various forms of ichthyosis (Basel-Vanagaite et al., 2007). The oncogenic potency of Matriptase is mitigated by a Kunitz type transmembrane serine protease inhibitor, Hai1, encoded by the SPINT1 gene. Indeed, loss of mouse Hai1 creates epidermal and intestinal barrier defects, and failure of placental labyrinth formation, which is due unrestricted Matriptase activity (Kawaguchi et al., 2011; Nagaike et al., 2008; Szabo, Molinolo, List, & Bugge, 2007).

Mutation of the zebrafish orthologue Hai1a (encoded by spint1a gene) also results in epidermal defects, including removal of E-cadherin from the cell membrane leading to aberrant mesenchymal behaviour of keratinocytes, entosis, cell extrusion and sterile inflammation, which can all be ameliorated by reduction of Matriptase levels (Armistead, Hatzold, van Roye, Fahle, & Hammerschmidt, 2020; Carney et al., 2007; Mathias et al., 2007; Schepis et al., 2018). Similarly, overexpression of Matriptase in the mouse epidermis leads to epidermal papillomas, ulcerative and invasive carcinomas, and inflammation, suggesting there is a conserved response to unregulated Matriptase activity in keratinocytes of vertebrates (List et al., 2005). Clinically, an increase in the Matriptase:HAI-1 ratio has been found in a range of tumours, and is predictive of poor outcome (Martin & List, 2019).

Due to the clinical implications of its dysregulation, the targets of Matriptase, and the signalling pathways activated pathologically, are increasingly investigated. In transgenic mouse models and breast cancer cell lines, maturation of HGF by Matriptase has been implicated in activating a cMET-Gab1-PI3K-Akt-mTor pathway, resulting in carcinoma progression (Szabo et al., 2011; Zoratti et al., 2015). In contrast, cMet is not required for the effects of uninhibited Matriptase in zebrafish (Carney et al., 2007). In addition to HGF, elegant genetic analysis using mouse and zebrafish have established that the G-protein coupled receptor, Proteinase-activated receptor-2 (Par2; encoded by the F2rl1 gene) is essential for the oncogenic and also the inflammatory effects of uninhibited Matriptase (Sales et al., 2015; Schepis et al., 2018). Like HGF, Par2 is a direct proteolytic target of Matriptase, which activates Par2 by cleaving within the extracellular N-terminal domain, removing an inhibitory peptide, and revealing a tethered ligand. Ligand bound Par2 then activates number of Gα protein subunits. It displays biased agonism, with different proteases/cleavage sites within the N-terminal domain activating distinct Gα subunits (Pawar, Buzza, & Antalis, 2019). Early studies in keratinocytes linked Par2 activation with intracellular Ca²⁺ mobilization via phospholipase C, thus implicating Gq subunit as the canonical target (Schechter, Brass, Lavker, & Jensen, 1998). Alternate Gα subunits, including Gi. Gs and G12/13 are now known to also be activated by Par2, thus placing cAMP and Rho pathways downstream of Par2 (Zhao, Metcalf, & Bunnett, 2014). The logic of the pathway utilised depends on cell context and the activating protease. In vitro analyses implicated both Par2 and Gi in Matriptase mediated Nfkb pathway activation. This work however excluded involvement of other Gα subunits in vitro (Sales et al., 2015). Whilst this explains the inflammatory phenotype of uninhibited Matriptase, it fails to explain how Par2 suppresses the carcinoma phenotypes in the epidermis. It is also unclear how Par2 and HGF both contribute to the pro-oncogenic effects of Matriptase. In addition to Gα subunits, Par2 can transactivate EGFR through an unknown mechanism, and inhibition of EGFR alleviates certain basal keratinocyte phenotypes of zebrafish hai1a mutants (Schepis et al., 2018). Thus, the identity, contribution, and interactions of the pathways downstream of Matriptase remain unclear.

We have utilized the zebrafish hai1a mutant as an in vivo platform to determine mechanisms and pathways activated by Matriptase. Informed by an unbiased proteomics approach, we demonstrate that Gq is essential for all phenotypes, with IP₃,R dependent Ca²⁺ release predominantly accounting for DUOX and Nfkb mediated sterile inflammation, whilst PKC and pERK drive the epithelial defects. We demonstrate that inhibition of RSK kinases, known pERK targets, also rescues the epithelial defects and restore E-cadherin to the membrane of basal keratinocytes in hai1a mutants. This has allowed us to construct a framework of the pathways downstream of zebrafish Matriptase.
Results

Increased hydrogen peroxide and calcium flashes contribute to inflammation in hai1a mutants

The hai1a zebrafish mutant combines epidermal defects and inflammation through loss of Matriptase1a inhibition, and subsequent activation of the GPCR, Par2b. Using the Tg(mpx:eGFP)i114 transgenic line to label neutrophils, we examined the inflammation phenotype to determine the mechanistic basis. As previously shown, neutrophils in hai1a occupy the epidermis, are highly active and motile, but move randomly (Mathias et al., 2007) (Fig. 1A-E; Video 1). Neutrophils in hai1a are successfully recruited locally to a small needle wound of the larval fin but were not recruited from further away or from the vasculature as in wild-type (Mathias et al., 2007). To better compare the signals recruiting neutrophils to a wound and those activating neutrophils in hai1a we performed a larger fin wound in wild-type and hai1a mutants by scalpel cut of the tail fin at 4dpf. We noted that again, whilst there was brief local recruitment in the mutant, more distant neutrophils appeared apathetic to the wound and remained migrating randomly, unlike in wild-type where neutrophils were recruited from a great distance and tracked to the wound with high directionality (Fig. 1F, G; Video 2). We noted, however, that although hai1a mutant neutrophils lacked directionality following wounding, they did increase their speed during their random movements, suggesting other damage response components present (Fig. S1A). We further tested if the early recruitment of neutrophils to the epidermis in hai1a mutants was dependent on apoptosis by performing a triple staining for neutrophils labelled by Tg(fli1:EGFP) with an immunofluorescent antibody labelling against the basal keratinocyte transcription factor TP63, and TUNEL labelling of apoptotic cells. Whilst the epidermis of hai1a mutants, unlike WT, had regions of apoptosis at 24hpf (arrowhead, Fig. 1H, I), neutrophils were not associated, but rather found at nascent TUNEL negative aggregates of basal keratinocytes (arrow). We conclude that epidermal cell death does not directly contribute to inflammation and that the effector stimulating neutrophils in hai1a mutants is as, or more, potent as that of wounds.

To identify the neutrophil activator in hai1a and visualize proteins cleaved by Matriptase1a, we employed an unbiased approach using 2D-gel proteomics to compare the wild-type proteome with that of strong hai1a alleles. The dandruff (ddf) mutant, isolated from an ENU screen, had many phenotypic similarities to the strong hai1a^{260} allele (van Eeden et al., 1996). Crosses between ddf^{t419} or ddf^{ti257} and hai1a^{t4217} failed to compliment. Sequencing of hai1a cDNA from both ddf alleles identified a missense mutation (c.749G>A; p.Gly250Asp) in ddf^{t419} and a nonsense mutation (c.771T>G; p.Tyr257Ter) in ddf^{ti257} (Fig. 1J; Fig. S1B, C). The amino acid substituted in ddf^{t419} is broadly conserved in Hai1 proteins of vertebrates suggesting its mutation is deleterious to function (Fig. S1D). We used both alleles for comparative 2D protein gel analysis at 24hpf and 48hpf. Rather than finding proteins with clear altered molecular weight, Peroxiredoxin4 (Prdx4) was identified as having a higher pI in both hai1a^{t419} and hai1a^{t4217} mutants at 24hpf and 48hpf, indicative of a change in oxidation state (Fig. 1F, Fig. S1E). Peroxiredoxins are hydrogen peroxide scavengers, and its altered oxidation state suggests that hai1a has higher H$_2$O$_2$ levels, a known activator of inflammation in larval zebrafish (Niethammer, Grabher, Look, & Mitchison, 2009). Using the stain pentafluorobenzenesulfonyl fluorescein (PFBSF) (Maeda et al., 2004), we observed significantly higher levels of H$_2$O$_2$ in hai1a^{t4217} mutants was dependent on apoptosis by performing a triple staining for neutrophils labelled by Tg(fli1:EGFP) with an immunofluorescent antibody labelling against the basal keratinocyte transcription factor TP63, and TUNEL labelling of apoptotic cells. Whilst the epidermis of hai1a mutants, unlike WT, had regions of apoptosis at 24hpf (arrowhead, Fig. 1H, I), neutrophils were not associated, but rather found at nascent TUNEL negative aggregates of basal keratinocytes (arrow). We conclude that epidermal cell death does not directly contribute to inflammation and that the effector stimulating neutrophils in hai1a mutants is as, or more, potent as that of wounds.

To demonstrate that, as with other phenotypes, this H$_2$O$_2$ increase in hai1a was due to unrestrained activity of Matriptase1a, we generated a st14a (matriptase1a) mutant by zinc finger mediated mutagenesis, which introduced 5bp into exon 6, altering the reading frame, and introducing a premature termination at 156 amino acids (st14a^{t465}; c.465_466insTCACA; p.Ala156SerfsTer8) (Fig. 2A, Fig. S2A-C). Homozygous zygotic st14a mutants showed no overt phenotype, however maternal zygotic mutants lacked ear otoliths (Fig. 2B-C). When crossed into the hai1a background, embryos lacking otoliths never displayed the hai1a epidermal phenotype (Table S1). Further, all st14a^{t465}, hai1a^{t4217} double mutants lacked the epidermal and neutrophil phenotypes of hai1a^{t4217} single mutants as expected (Fig. 2D-F). Double mutants also had significantly reduced H$_2$O$_2$ levels (Fig. 2F; Fig. S2D). To determine if this could account for the rescue of hai1a phenotypes, we used genetic and pharmacological inhibition of the main enzyme responsible for generating H$_2$O$_2$ in zebrafish, Duox. A morpholino directed against duox successfully reduced H$_2$O$_2$ levels (Fig. 2F; Fig. S2D) and neutrophil inflammation in hai1a mutants but did not rescue the epithelial defects (Fig. 2F-G). Treatment with a known Duox inhibitor, diphenyleneiodonium (DPI), also resulted in amelioration of neutrophil inflammation, but not epithelial aggregates in hai1a mutants (Fig. 2G, Fig. S2E). We conclude that excess H$_2$O$_2$ in hai1a mutants partially accounts for the neutrophil inflammation, but not epidermal defects.
Duox is regulated by calcium through its EF-Hand domains, and calcium flashes are known to generate H$_2$O$_2$ in epidermal wounds in Drosophila (Razzell, Evans, Martin, & Wood, 2013). We injected hai1a$^{fr26}$ with RNA encoding the calcium reporter GCaMP6s. Timelapse imaging at 24hpf indicated hai1a mutants had significantly more calcium flashes in both the trunk and tail (Fig. 3A, B, E; Video 3). Release of calcium from intracellular stores is regulated by IP$_3$ receptors located on the endoplasmic reticulum. The frequency and number of calcium flashes in the trunk and tail of hai1a mutants is reduced by treatment with the IP$_3$R inhibitor 2-APB compared to control (Fig. 3C, D, F; Video 4). Reducing calcium flashes in hai1a mutant embryos with 2-APB, also significantly reduced H$_2$O$_2$ levels (Fig. 3G; Fig. S3A), and partially reduced inflammation, however the epidermal defects were not noticeably rescued (imaged by DIC or labelled with the TP63 antibody) (Fig. 3G-I). We observed similar reduction in neutrophil inflammation, but not rescue of epidermal defects, in hai1a mutants treated with Thapsigargin, which inhibits the replenishment of ER calcium stores by SERCA (Fig. 3I; Fig. S3B-C). Thus, in hai1a mutants, IP$_3$R dependent calcium flashes activate Duox, flooding the epidermis with H$_2$O$_2$ and leading to inflammation.

Hydrogen peroxide elevates NfkB signalling in hai1a mutants

Increased Matriptase, Par2 activity or hydrogen peroxide levels are known to activate NfkB signalling (Kanke et al., 2001; Sales et al., 2015; Schreck, Rieber, & Baeuerle, 1991). We crossed the hai1a$^{fr26}$ allele to the NfkB sensor transgenic line Tg(6xHsa.NFKB:EGFP)$_{nc1}$. In WT embryos, NfkB signalling was mostly restricted to neuromasts at 48hpf, whilst in hai1a mutants we observed an increase in fluorescence at 48hpf.
and a strong increase at 48hpf. Fluorescence at both timepoints was noted in epidermal aggregates and fin folds, locations of strong inflammation (Fig. 4A, B; Fig. S4A, B). This increase in signalling in 48hpf hai1a mutant embryos was confirmed by qRT-PCR of the NfkB target gene, nfkbiaa (Fig. 4C). To determine the extent that NfkB signalling accounts for the hai1a phenotypes, we generated a mutant in the ikbkg (\(=\) ikg or nemo) gene by TALEN mediated mutagenesis, which encodes a scaffold protein required for activating the NfkB pathway (Rothwarf, Zandi, Natoli, & Karin, 1998) (ikbkg sq304 Gly80ValfsTer11; Fig. S4C). Crossing this mutant to hai1a hi2217 on the Tg(mpx:eGFP) i114 background realised a very strong rescue of neutrophil inflammation, but no improvement of hai1a epidermal defects (Fig. 4D-I). To demonstrate that this increase in NfkB signalling was dependent on H\(_2\)O\(_2\), we injected hai1a hi2217; Tg(6xHsa.NFKB:EGFP) nc1 embryos with duox MO. We noted a strong reduction in NfkB pathway activation compared to uninjected hai1a mutant controls (Fig. 4J, K). Conversely, genetic ablation of NfkB signalling, through use of the ikg mutant, did not reduce H\(_2\)O\(_2\) levels in hai1a mutants (Fig. S4D-E). Similarly, we tested if reduction of calcium flashes could also reduce NfkB signalling in hai1a mutants using 2-APB, but noticed only a slight reduction (Fig. S4F-G). We conclude that upon loss of Hai1a, IP\(_3\)R mediated release of calcium activates Duox to increase H\(_2\)O\(_2\). This acts upstream of NfkB pathway activation, which is necessary for the inflammation phenotype.

**Both inflammation and epidermal aggregates of hai1a mutants are resolved by Gq inhibition**

IP\(_3\) is generated from cleavage of PIP\(_2\) by Phospholipase C. The sensitivity of the hai1a mutants to 2-APB
implies that IP₃ levels are increased and therefore there may be an increase in Phospholipase C activity. Numerous attempts to inhibit PLC in hai1a mutants failed, and we were unable to find a dosage window that rescued without gross embryo deformity. Hence, we tested rescue of hai1a mutants with YM-254890, an inhibitor of the heterotrimeric G protein alpha subunit, Gq, which directly activates PLC isoforms. We found that not only did this significantly reduce neutrophil inflammation (Fig. 5D, F), but surprisingly, it also significantly rescued the epidermal defects in hai1a mutants (Fig. 5A-E). There was a visible reduction in epidermal aggregates in the trunk and improved tail fin fold integrity at 48hpf, either imaged by DIC or through TP63 immunostaining of basal keratinocytes (Fig. 5A-D).

PMA treatment phenocopies the hai1a mutant

As IP₃ inhibition only blocks inflammation in hai1a mutants, but an inhibitor of a PLC activator (Gq) additionally reduces the epidermal defects as the second product of PIP₂ cleavage (along with IP₃). Indeed, treating zebrafish embryos with phorbol 12-myristate 13-acetate (PMA), a DAG analogue, has been described to generate global embryological phenotypes (Hrubik et al., 2016). Treating WT embryos from 15hpf to 24hpf with 125ng/ml PMA resulted in embryos with striking similarities to strong hai1a mutants, including a thin or absent yolk sac extension, lack of head straightening, lack of lifting the head off the yolk, and multiple epidermal aggregates on the skin (Fig. 6A-C). These aggregates were due, at least partially, to displacement of basal keratinocytes as shown by TP63 staining where the basal keratinocyte nuclei lost their uniform distribution (Fig. 6D-E). Treatment of WT embryos from 24hpf to 48hpf with 125ng/ml PMA led to a fin defect similar to the dysmorphic hai1a mutant fin (Fig. 6F-G). It has been shown that the basal keratinocytes in hai1a lose their epithelial nature and adopt a partially migratory phenotype (Carney et al., 2007) (Video Figure 3: Calcium dynamics in hai1a mutants regulate H₂O₂ and inflammation  

A-D: Projected confocal images of eGFP in the trunk (top row) or tail (bottom row) of WT (A), or hai1a<sup>fr26</sup>; (B-D) injected with GCaMP6s RNA, imaged at 24hpf indicating calcium dynamics. Embryos are either untreated (A-B), treated with DMSO (C), or with 2.5µM 2-APB (D). Images are temporal projections of timelapse movies taken at maximum speed intervals (2 minutes for tail movies, 3 minutes for trunk) and projected by time. E-F: Graphs comparing eGFP intensities from GCaMP6s RNA timelapses in trunk and tail between 24hpf WT and hai1a<sup>fr26</sup> (E) and between hai1a<sup>fr26</sup> treated with DMSO and 2.5µM 2-APB (F). n= 10; t-test; * = p<0.05, *** = p<0.001. G-H: Projected confocal images of PFBSF staining at 24hpf (left column), TP63 (magenta) and eGFP (green) antibody staining at 48hpf (middle column) with DIC imaging (right column) for hai1a<sup>fr2217/fr237</sup> mutants (G), or hai1a<sup>fr2217/fr247</sup> mutants treated with 2.5µM 2-APB (H). Individuals for middle and right columns were hemizygous for the Tg(mpxeGFP)<sup>114</sup> transgene. I: Counts of eGFP positive neutrophils in the fins at 48hpf of Tg(mpxeGFP)<sup>114</sup>, or hai1a<sup>fr2217</sup>; Tg(mpxeGFP)<sup>114</sup> treated with 0.5% DMSO, 2.5µM 2-APB or 6.5µM Thapsigargin. n=20; t-test; *** = p<0.001. Scale bars A-D = 50µm; G-H = 100µm.
We treated Tg(krtt1c19e:lyn-tdtomato) sq16 larvae (Lee, Asharani, & Carney, 2014) with 37.5 ng/ml PMA for 12 hours and imaged the basal epidermis at 3 dpf by light-sheet timelapse. Whilst the DMSO treated transgenic larvae had very stable keratinocyte membranes and shape, PMA treatment led to a less stable cell membrane topology and dynamic cell shape, similar to hai1a mutants (Fig. 6H; Videos 5, 6). Kymographs taken from Video 6 highlighted both the more labile and weaker cell membrane staining following PMA treatment (Fig. 6I). The potency of PMA was dependant on region and reduced with age.

Most PMA treated Tg(mpx.eGFP)i114 larvae at 48 hpf also had more neutrophils in the epidermis than untreated controls, which were highly migratory (Fig. 6F-G, J-K'; Video 7). We determined H$_2$O$_2$ levels in PMA treated embryos using PFBSF staining, and found it was significantly increased in both trunk and tail at 24 hpf (Fig. 6L-O, R). In contrast, we treated GcaMP6s RNA injected embryos with PMA, we failed to see an increase in calcium flashes, as seen in hai1a mutants (Fig. 6P, Q, S). To see if the heightened H$_2$O$_2$ and inflammation was also correlated with increased NfkB signalling we treated Tg(6xHsa.NFKB:EGFP)$^{nc1}$ embryos reporting NfkB signalling levels at 32 hpf of hai1a$^{hi2217}$ uninjected (J) or injected with duox MO (K). Loss of H$_2$O$_2$ reduces NfkB signalin levels in hai1a mutants. Scale bars A, K = 200µm; D' , I = 50µm.

The phenocopy and the rescue of hai1a by PMA and Gq inhibition respectively implies DAG is elevated in hai1a mutants. If this is true, then inhibition of its target, Protein Kinase C, should also ameliorate the hai1a mutant phenotype. We treated hai1a$^{hi2217}$ embryos with the PKC inhibitor, GFX109203, and found this was able to reduce the epidermal aggregates and disruption of fin morphology as imaged by DIC or immunostaining for TP63 (Fig. 7A-F). Neutrophil inflammation in the epidermis was somewhat reduced, but not to a significant degree (Fig. 7C-G). Thus, the epithelial defects of hai1a are due to PKC activation.

**Elevated MAPK signalling generates epithelial defects in hai1a**

We next sought to determine which pathways downstream of PKC are responsible for the epidermal defects. The MAPK pathway is a major target pathway of multiple PKC isoforms, and activation of this pathway in zebrafish epidermis has previously been shown to induce papilloma formation which have very similar attributes to hai1a mutant aggregates (Chou et al., 2015). Although whole embryo western analysis of hai1a mutants failed to show an overall increase in pERK (Armistead et al., 2020), we performed wholemount immunofluorescent analysis in case there was only a localized effect. Indeed, we observed a
significant and localised increase in cytoplasmic pERK immunoreactivity (phospho-p44/42 MAPK (Erk1/2) \((\text{Thr}^{202}/\text{Tyr}^{204})\)) in the regions of epidermal aggregate formation in \(\text{hai}1\alpha\) mutants and in PMA treated embryos, including under the yolk at 24hpf and in the fins at 48hpf (Fig. 8A-H, M; Fig. S5A-F). There was no increase in total ERK levels in the mutant (Fig. 8K-L). Increased pERK was seen in the both the cytoplasm and nucleus of TP63 positive cells but was only increased in the nucleus of periderm cells (Fig. 8F; Fig. S5D). To establish that this is an early marker of aggregate formation, and not a sequela, we stained \(\text{hai}1\alpha\) mutant embryos at earlier timepoints. We found that even nascent aggregates at 16hpf have pERK staining (Fig. 8I-J), which increases in number over time (Fig. S5G-J).

To determine if elevated pERK is causative of epidermal defects, we attempted to rescue using pERK inhibitors. Initially we used PD0325901, however this appeared to give fin fold deformities, even in WT embryos (Anastasaki, Rauen, & Patton, 2012), precluding ability to assess rescue in \(\text{hai}1\alpha\), although there was a noticeable reduction in epidermal aggregates forming under the yolk-sac extension (data not shown). Instead we tried U0126 and CI-1040, other well-known pERK inhibitors (Allen, Sebolt-Leopold, & Meyer, 2003; Favata et al., 1998). Both inhibitors showed a significant reduction in \(\text{hai}1\alpha\) mutant epidermal aggregates under the yolk, and restoration of the overall and tail epithelial morphology, with embryos showing a \(\text{hai}1\alpha\) phenotype class significantly reduced (Fig. 9A-G; Fig. S6A-F). Similarly, the epidermal defects of the trunk, yolk and tail following PMA treatment were also ameliorated by concomitant U0126 treatment (Fig. 9H-I; Fig. S6G-H). Rescue of aggregates and tail morphology following PMA treatment or in \(\text{hai}1\alpha\) mutants could be visualized by immunolabelling TP63 in basal keratinocyte nuclei (Fig. 9J-O; Fig. S6-J). Treatment with U0126 did not significantly reduce neutrophil inflammation of \(\text{hai}1\alpha\) mutants or PMA treatment (Fig. 9L-P). It has been shown that the epidermal defects in \(\text{hai}1\alpha\) are associated with loss of E-Cadherin from adherens junctions (Carney et al., 2007). As there was a rescue of the epithelial phenotype following pERK inhibition, we looked at the status of the adherens junction marker \(\beta\)catenin. Whilst the WT basal epidermal cells of the 48hpf tail showed strong staining at the membrane, \(\text{hai}1\alpha\) mutants and PMA treated embryos showed a significant loss of \(\beta\)catenin at the membrane and increase in the cytoplasm (Fig. 9Q-V, Y, Z). Treatment of \(\text{hai}1\alpha\) mutants with U0126 restored the membrane localisation of \(\beta\)catenin (Fig. 9W, X, AA).
Phosphorylation of cytoplasmic RSK by pERK, leads to loss of E-cadherin at the hai1a keratinocyte membrane

As increased pERK appeared to contribute strongly to loss of adherens junctions, and removal of E-Cadherin/Catenin from the membrane, we sought to determine how pERK signalling might affect adherens junctions. We predicted that this would occur through a cytoplasmic target of pERK, as we have previ-
ously shown that there is no transcriptional downregulation of E-cadherin levels in hai1a, making a nuclear transcription factor target less likely to be relevant. The p90RSK family of kinases represent cytoplasmic direct targets of Erk1/2 phosphorylation which regulate cell motility, and thus were good candidates for mediators disrupting cell-cell adhesion (Caslavsky, Klimova, & Vomastek, 2013; Tanimura & Takeda, 2017). We determined that at least RSK2a (=p90RSK2a, encoded by rps6ka3a) is expressed in the basal keratinocytes at 24hpf (Fig. 10A, B), and other RSK family members may also be expressed there. To gauge if there was an alteration in phosphorylation of RSK family members in the epidermis of hai1a mutants, we used an anti-body which detects a phosphorylated site of mouse p90RSK (Phospho-Thr348). This site is phosphorylated in an ERK1/2 dependant manner (Romeo, Zhang, & Roux, 2012). We noticed a substantial increase in cytoplasmic signal in both hai1a mutants and PMA treated embryos. Where p90RSK-pT348 signal was largely nuclear in both basal and periderm cells in WT, it was more broadly observed in hai1a mutant fins, with an increase in the cytoplasm leading to a more uniform staining (Fig. 10C-D’). This cytoplasmic signal was lost upon U0126 treatment showing it was pERK dependant (Fig. 10E-E’). Similarly, increased cytoplasmic p90RSK-pT348 was observed following PMA treatment that was reduced by co-treatment with U0126 (Fig. 10F-H’). The increase in cytoplasmic p90RSK-pT348 signal, and its reduction by U0126, was significant in both hai1a mutants and PMA treated embryos (Fig. 10I-J).

If phosphorylation of an RSK protein is required for mediating the pERK epidermal defects in hai1a mutants, then inhibition of RSK should rescue the epidermal defects. As morpholino targeted inhibition of rps6ka3a was unsuccessful, we employed established pan-RSK inhibitors BI-D1870 and Dimethyl Fumarate (Andersen et al., 2018; Sapkota et al., 2007). We noted that both inhibitors were able to reduce epidermal aggregates in hai1a mutants and restore fin morphology, when visualised by DIC or TP63 immunofluorescence (Fig. 10K-N; Fig.S7 A-B, D-E). Reduction of mutant phenotype classes was significant at both 24hpf and 48hpf (Fig. S7C). We then assayed if RSK inhibition can reduce the aberrant cytoplasmic E-Cadherin staining in hai1a mutant basal keratinocytes and observed that Dimethyl fumarate treatment restored membrane localisation of E-Cadherin in the mutants (Fig. 10O-Q’). Thus, phosphorylation of RSK proteins is altered in hai1a mutants and their inhibition can restore E-Cadherin to the membrane and reduce epidermal aggregate formation.

Figure 7: Inhibition of PKC rescues epidermal defects of hai1a
A-B: Lateral brightfield images of 48hpf hai1a[i2217] larvae treated with 0.5% DMSO (A) or 85µM GFX109203 (B). Epidermal aggregates and fin deterioration are rescued by the PKC inhibitor (arrowheads).
C-F: DIC (C, E) and projected confocal images (C’, E’, D, F) of hai1a[i2217]; Tg(mpox:eGFP)i114 trunk at 24hpf (C-C’, E-E’) and tail at 48hpf (D, F), either treated with 0.5% DMSO (C-D) or 85µM GFX109203 (E-F). Embryos are immunostained for TP63 (magenta) and eGFP (green), highlighting rescue of epidermal phenotype and partial rescue of neutrophils by GFX109203.
G: Counts of eGFP positive neutrophils in the fins at 48hpf of Tg(mpox:eGFP)i114, or hai1a[i2217]; Tg(mpox:eGFP)i114 treated with 0.5% DMSO, or 85µM GFX109203. n= 8; ANOVA, Dunn’s Multiple comparisons; ** = p<0.01. Scale bars B = 200µm; F = 100µm.
Figure 8: Elevation of pERK levels in PMA treated and hai1a mutant epidermis

A-L: Lateral projected confocal images of trunks (A, B, E, F, K, L), and tails (C, D, G, H) of embryos immunostained for TP63 (A-L; magenta) and pERK (green; A-J) or total ERK (tERK; green K, L) at 24hpf (A-F, K, L), 48hpf (G, H) and 16hpf (I, J). Both hai1afr26 (B, D, F, J) and 125ng/ml PMA treated (H) embryos show increased epidermal pERK levels compared to untreated WT (A, C, E, G, I). There was no increase in tERK in hai1afr26 (L) compared to WT (K). Elevation of epidermal pERK in hai1afr26 mutants and PMA treated embryos is seen in the trunk (B, F) and tail (D, H) as well as in nascent aggregates (arrowhead, J).

M: Quantification of pERK immunofluorescent intensity in the tail of 24hpf hai1afr26 larvae compared to siblings. n= 5; Mann-Whitney test; ** = p<0.01. Scale bars B, J = 100µm; D, H, L = 50µm; F = 20µm.
Discussion

There are a number of similarities between loss of Hai1a in zebrafish and overexpression of Matriptase in the mouse epidermis, including inflammation, hyperproliferation and enhanced keratinocyte motility. This implies that there is an ancient function for this protease/protease inhibitor system. In mouse and human, the loss of Matriptase causes stratum corneum defects including ichthyosis, loss of filaggrin processing and defects in desquamation. As there is no stratum corneum in zebrafish, this cannot be the ancestral role. Since the only overt phenotype in zebrafish Matriptase1a maternal zygotic mutants was transient loss of ear otoliths, and hai1a; st14a double mutants appear normal, it is unclear why the Hai1-Matriptase system evolved in fish at all. Our previous analysis of the epithelial aggregates in zebrafish hai1a mutants led us to conclude that nascent aggregates are not forming due to increased proliferation, but...
which occurs at later timepoints, but due to increased keratinocyte motility. Further, although early and prominent, we have shown that the inflammation in hai1a mutants is not required for epithelial defects but occurs in parallel to it. These two primary phenotypes, sterile inflammation and epithelial cell motility,
are the hallmarks of the damage response during early wound detection. Wounds are detected through a combination of osmotic surveillance, release of DAMPs from cell lysis and mechanotransduction at the wound edge (Enyedi & Niethammer, 2015). Indeed, disrupted osmolarity differences and nucleotide release in the zebrafish epidermis generate inflammation and epithelial cell motility as part of a damage response (de Oliveira et al., 2014; Enyedi & Niethammer, 2015; Gault, Enyedi, & Niethammer, 2014; Hatzold et al., 2016), with the resulting phenotypes strikingly similar to hai1a mutants. Further, PAR2 promotes cell migration in scratch assays in conjunction with P2Y purinergic and EGF receptors (K. Shi, Queiroz, Stap, Richel, & Spek, 2013). It is, therefore, tempting to propose that the Hai1–Matriptase system as part of the early wound detection process. How it might act as a sensor is not clear; however, Matriptase activity is highly sensitive to reduced pH and has been linked to response to acidosis (Tseng et al., 2010). Altered pH has recently been proposed to regulate wound progression (Stolwijk et al., 2020). In the mammalian epidermis, Matriptase becomes active at the transition to the stratum corneum where the matrix becomes acidic (Miller & List, 2013), and the acid sensing ion channel, ASIC1, is also a target of Matriptase further supporting a link between Matriptase and environmental pH (Clark, Jovov, Rooj, Fuller, & Benos, 2010). Matriptase activation is also increased by redox state and the oxidative effects of heavy metals such as cadmium and cobalt, and loss of Matriptase makes cells more sensitive to heavy metal toxicity (J. K. Wang et al., 2014). We postulate that the Hai1–Matriptase system evolved in the epidermis as a surveillance system of altered external pH, osmolarity, redox state or metal ions, which may arise due to wounding of the epithelial barrier. Such a surveillance system would likely act redundantly with other pathways of the damage response, and promote responses through PAR2 signalling as also suggested by Schepis et al. (2018).

There are strong links between Matriptase dysregulation and cancer progression (Martin & List, 2019), and the cellular and tissue level phenotypes of hai1a have similarities to tumours. Tumours have long been considered to represent non-healing wounds and transformed cells in zebrafish both promote and respond to inflammation through similar mechanisms to wound responses (Feng, Santoriello, Mione, Hurlstone, & Martin, 2010; Schafer & Werner, 2008). We have shown that the inflammation in hai1a has similar molecular signatures to that following wounding, or other damage sensors such as ATP release and osmotic stress. Indeed, we show that increased IP₃, R₃-dependent calcium release in hai1a epidermis activates Duox, leading to high hydrogen peroxide levels, which in turn leads to increased NFκB signalling. This pathway has also been shown to act downstream of ATP via purinergic GPCRs, to recruit neutrophils in zebrafish (de Oliveira et al., 2014). We were unable to definitively rescue hai1a phenotypes with a PLC inhibitor due to toxicity, however an inhibitor of Gq very effectively rescued the inflammation, but surprisingly also the epithelial defects. We showed that the DAG analogue, PMA can phenocopy the epidermal defects of hai1a mutants. Conversely, inhibiting the DAG receptor, PKC, rescued the epithelial phenotypes. Furthermore, PMA treatment increased H₂O₂, NFκB and neutrophil inflammation, which is in line with known activation of Duox and IKK by PKC (Rigutto et al., 2009; Turvey et al., 2014). Seminal experiments in transgenic mice overexpressing Matriptase in the epidermis and treated with a DMBA/PMA regime, concluded that Matriptase and PMA activate functionally similar carcinoma promoting pathways (List et al., 2005). Our subsequent analysis suggests that this would include the MAPK pathway, as we see increased phosphorylated-ERK in the epidermis of both hai1a mutants and also PMA treated embryos. This was surprising as previous reports did not detect this by western analysis of whole embryos, although we observed pERK increased only in the epidermis, which may account for a limited global proportion. That we can rescue the epithelial defects using a MEK inhibitor indicated that this increase in epidermal pERK is critical to the phenotype. The MAPK pathway, in addition to well-defined roles in cell proliferation, translation, differentiation and survival, is known to regulate cell motility (Tanimura & Takeda, 2017). Indeed, misexpression of activated MEK2 in zebrafish epidermis generated papillomas with remarkable resemblance to the epidermal aggregates in hai1a mutants (Chou et al., 2015), and which are not overtly proliferative. Additionally, PAR2 activates motility of an oesophageal tumour line partially via MAPK/ERK pathway (Sheng et al., 2019). One major effector phosphorylated by pERK is the kinase RSK2 (= p90RSK2), which is encoded by the RPS6KA3 gene. Like Matriptase, activation of RSK2 is associated with tumour progression (Kang & Chen, 2011), particularly promoting invasiveness and metastasis of glioblastomas and head and neck squamous cell carcinomas (Kang et al., 2010; Sulzmaier et al., 2016). Promotion of invasiveness has also been noted for other members of the p90RSK family such as RSK1, activation of which promotes invasion of melanoma clinically as well as in vitro and zebrafish melanoma models (Salhi et al., 2015). MAPK signalling has also been shown to reduce E-Cadherin expression at adherens junctions and promoting its cytoplasmic accumulation in an RSK dependant manner, without reducing E-Cadherin protein levels (Caslavska et al., 2013). We previously showed an identical effect on E-Cadherin relocation in zebrafish hai1a mutants (Carney et al., 2007). Intriguingly, recent proximity protein labelling has identified p120-catenin being phosphorylated by p90RSK activity. This catenin promotes cell-cell adhesion by...
regulating Cadherin stability at the membrane, a function inhibited by p90RSK phosphorylation (Meant et al., 2020). More broadly, RSK2 activity promotes cell motility through other mechanisms, including inactivation of Integrins and activation of the RhoGEF, LARG (Gawecka et al., 2012; G. X. Shi, Yang, Jin, Matter, & Ramos, 2018). Thus, we propose pERK signalling, through p90RSK members, significantly contributes to the hai1a epidermal phenotype.

Our analyses allow us to propose a pathway which accounts for both the inflammatory and the epidermal phenotypes (Fig. 11). Whilst the inflammatory phenotype is strongly reliant on IP3 and calcium release, there is likely a partial contribution from PKC activation, as PKC also promotes inflammation and PKC inhibition partially reduces inflammation. In contrast, only inhibition of Gq, PKC, pERK and p90RSK rescued the epidermal phenotype. This model invokes activation of Gq and Phospholipase C to generate Ca++ and DAG. This has been well documented to occur downstream of PAR2 in many cell types including keratinocytes, where inhibition of Gq and PKC reduces PAR2 mediated Nfκb signalling (Bohm et al., 1996; Goon Goh et al., 2008; Macfarlane et al., 2005). PAR2 agonists have also been shown to activate the ERK1/2 MAPK pathway, although the mechanisms proposed are varied. In breast cancer cell lines, ERK is activated by PAR2 and promotes invasiveness and correlates with PAR2 mediated PIP2 hydrolysis (Jiang et al., 2004; Morris et al., 2006). Similarly, PAR2 stimulation of astrocyte migration required Gq, but not Gi/o, stimulation of pERK (McCoy, Traynelis, & Hepler, 2010). However, a number of publications have implicated EGFR transactivation in signalling downstream of PAR2, including stimulation of the MAPK pathway (Darmoul, Gratio, Devaud, & Laburthe, 2004). Other work has indicated that PAR2, through PI3K and AKT, lead to release of MMPs, which in turn lead to maturation of HB-EGF (Chung, Ramachandran, Hollenberg, & Muruve, 2013; Rattenholl et al., 2007), although an MMP-independent mechanism through Src has also been proposed (van der Merwe, Hollenberg, & MacNaughton, 2008). Our model for how Matriptase invokes cellular responses is highly likely to be incomplete. Indeed, others have indicated EGFR and AKT are downstream of Matriptase function (List et al., 2005; Schepis et al., 2018). We do not think these conflict with our model but will interface with it. A number of reports have demonstrated that PI3K/AKT and MEK/ERK function in parallel downstream of PAR2 (Sheng et al., 2019; Tanaka, Sekiguchi, Hong, & Kawabata, 2008; van der Merwe, Moreau, & MacNaughton, 2009). Furthermore, there is evidence that PKC activates both MEK/ERK and EGFR independently following PAR2 stimulation (Al-Ani et al., 2010), and that PI3K is activated by PAR2 via Gq (P. Wang & DeFea, 2006). Cell identity, sub-cellular localisation, β-arrestin scaffolding and biased agonism/antagonism are known to generate alternative downstream outputs from PAR2 (Zhao et al., 2014). To understand fully the roles of Matriptase and PAR2 in epithelial homeostasis and carcinoma, it will be critical to map how, when, and where they activate different downstream pathways.

**Materials and Methods**

**Zebrafish husbandry and lines**

Fish were housed at the IMCB and the NTU zebrafish facilities under IACUC numbers #140924 and #A18002 respectively, and according to the guidelines of the National Advisory Committee for Laboratory Animal Research. Embryos were derived by natural crosses and staged as per Kimmel et al (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995) and raised in 0.5x E2 medium (7.5mM NaCl, 0.25mM KCl, 0.5mM MgSO4, 75μM KH2PO4, 25μM Na2HPO4, 0.5M CaCl2, 0.35mM NaHCO3). Anaesthesia was administered in E2 medium (embryos) or fish tank water (adults) using 0.02% pH7.0 buffered Tricaine MS-222 (Sigma). The hai1a/ddf alleles used were hai1a2217, hai1a2226, ddf257, and ddf419. For imaging neutrophils and keratino-

![Figure 11: Model of pathway activated downstream of Hai1 and Matriptase](image-url)
cytes, the transgenic lines Tg(mpx:EGFP)i114 (Renshaw et al., 2006) and Tg(krt1c19e:lyn-tdtomato)sq16 (Lee et al., 2014) were used, whilst early leukocytes were imaged with Tg(fli1:EGFP)y1 (Redd, Kelly, Dunn, Way, & Martin, 2006). To image NfkB pathway activity, the Tg(6xHsa.NFKB:EGFP)ect sensor line was used (Kanther et al., 2011).

Genomic DNA and RNA extraction, Reverse transcription, and PCR
Adult fin clips or embryos were isolated following anaesthesia, and genomic DNA extracted by incubation at 55°C for 4hrs in Lysis buffer (10mM Tris pH8.3, 50mM KCl, 0.3% Tween20, 0.3% Nonidet P-40, 0.5µg/µl Proteinase K). PCRs were performed using GoTaq (Promega) on a Veriti thermal cycler (Applied Biosystems), and purified with a PCR purification kit (Zymo Research). TRIzol™ (Invitrogen) was used for RNA extraction following provided protocol, and cDNA generated from 1µg total RNA using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamer primers. For qPCR, iTaq SYBR green (Bio-Rad) was used to amplify, with reaction dynamics measured on a Bio-rad CFX96 Real-Time PCR Detection System. For measuring nfkbiaa mRNA by qPCR, the following primers (5’ to 3’) were used to amplify a region encoded on exons 4 and 5: F-AGACGCAAAGGAGCAGTGTAG, R- TGTGTGTCTGCAGAGGTC. Reference gene was eef1a1l1 and the primers used amplified between exon 3 to 4: F-CTGGAGGCCAGCTCAAACAT, R- AT-CAAGAAGAGTAGTACCGCTAGCATTAC.

RNA synthesis
RNA for GCaMP6s was synthesised from a pCS2 plasmid containing the GCaMP6s coding sequence (gift of the Solnica-Krezel Lab; (Chen, Xia, Bruchas, & Solnica-Krezel, 2017)). This was linearised with NotI (NEB), RNA in vitro transcribed with mMESSAGE mMACHINE SP6 Transcription Kit (Ambion) and purified by lithium chloride precipitation.

Embryo injection and Morpholino
Embryos were aligned on an agarose plate and injected at the 1-cell stage with RNA or Morpholino diluted in Phenol Red and Danieau’s buffer using a PLI-100 microinjector (Harvard Apparatus). Injection needles were pulled from borosilicate glass capillaries (0.5mm inner diameter, Sutter) on a Sutter P-97 micropipette puller. The Duox morpholino (AGTGAATTAGAGAAATGCACCTTTT) was purchased from GeneTools and injected at 0.4mM with 0.2mM of the tp53 morpholino (GCGCCATTGCTTTGCAAGAATTG).

Zinc Finger and TALEN Mutagenesis
To generate the st14a (=matriptase1a) mutant, CompoZr Knockout Zinc Finger Nucleases were designed and manufactured by Sigma-Aldrich, targeting the following sequence in Exon 6: CAGTTCCAGCAGCA-CACGaagcaGCAGTGGATCAGGCTGTG, where the forward and reverse strand ZFN binding sites are in uppercase and the cut site is in lowercase. To generate the ikbkg mutant, TALEN vectors targeting the sequence ATGGAGGGCTGG in second exon were designed and constructed by ToolGen (http://toolgen.com). TALEN vectors were linearized with PvuII (NEB) and purified using a PCR purification kit (Zymo Research), and then used for in vitro transcription with the MEGAshortscript T7 kit (Ambion). About 170-300pg of supplied ZFN RNAs or purified TALEN RNAs were then injected into one-cell stage WT zebrafish embryos, which were raised to 24hrs, then genomic DNA extracted.

For detection of fish with edited loci, PCR was performed on genomic DNA of injected fish with primers flanking the target site, cloned by TA cloning into pGEMT-Easy (Promega) or pCR2.1-TOPO-TA (Invitrogen) and individual clones sequenced to establish efficiency. Other embryos were raised to adulthood and their offspring were similarly genotyped to identify founder mutants.

Small molecule treatment
All compounds for treating embryos were dissolved in DMSO, diluted in 0.5x E2 Embryo Medium and embryos treated by immersion. The compounds, and concentrations used, with catalog numbers were: Diphenyleneiodonium Chloride (DPI), 40µM (D2926, Sigma); Thapsigargin, 6.25µM (T9033, Sigma); Bisindolylmaleimide I (GF109203X), 85µM (S7208, Selleckchem); YM-254890, 32µM (10-1590-0100, Focus Biomolecule); Bisindolylmaleimide I (GF109203X), 85µM (S7208, Selleckchem); YM-254890, 32µM (10-1590-0100, Focus Biomolecule); 2-Aminoethyl diphenylborinate (2-APB), 2.5µM (D9754, Sigma), BI-D1870, 1.2µM (Axon-1528, Axon Medchem); Diphenyl fumarate, 9µM (242926, Sigma); Phorbol 12-myristate 13-acetate (PMA), 9µM (242926, Sigma); Phorbol 12-myristate 13-acetate (PMA), 37.5 or 125ng/ml (P8139, Sigma); U0126, 100µM (9903, Cell Signaling Technology); PD184352 (CI-1040), 1.3µM (S1020, Selleckchem). Unless otherwise stated, controls for all experiments were exposed to 0.5% DMSO carrier in 0.5x E2 Embryo Medium.
Proteomic Analysis

Batches of 100 WT, \textit{ddf}^{419} and \textit{ddf}^{B257} embryos were collected at 24hrs and 48hrs, dechorionated, deyolked and protein extracted as per (Alli Shaik et al., 2014). Protein was precipitated in 100\% methanol at 4°C, then resuspended in 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4\% CHAPS). 2-D DIGE and Mass spectrometry protein identification was performed by Applied Biomics (Hayward, CA). Protein samples were labelled with either Cy2, Cy3 or Cy5, mixed and then subjected to 2-D DIGE to separate individual proteins. Gels were scanned using Typhoon TRIO (Amersham BioSciences) and analysed by Image QuantTL and DeCyder (ver. 6.5) software (GE-Healthcare). Spots with more than 1.5 fold change were picked, in-gel trypsin digested and protein identification performed by MALDI-TOF mass spectrometry and MASCOT search engine in the GPS Explorer software (Matrix science).

In situ Hybridisation

A probe corresponding to the final 1078 bp of \textit{rps6ka3a} (RSK2a; NM\_212786.1) was generated by cloning a PCR-derived cDNA fragment into pGEMT-Easy (Promega), linearising with Apal (NEB) and transcribing a DIG probe with SP6 RNA polymerase (Roche). Whole mount in-situ hybridisation developed with NBT/BCIP (Roche) was performed as described (Thisse & Thisse, 2008).

Immunofluorescent, Dye staining and TUNEL

For antibody staining, embryos were fixed in 4\% paraformaldehyde overnight at 4°C and then washed in PBT (0.1\% Triton in PBS), permeabilized in -20°C Acetone for 7 minutes, washed in PBT, blocked for 3 hours in Block solution (PBT supplemented with 4\% BSA and 1\% DMSO), then incubated overnight at 4°C with primary antibody diluted in Block solution, washed extensively in PBT, re-blocked in Block solution then incubated overnight at 4°C with fluorescent secondary antibody diluted in Block solution. Following extensive PBT washing, embryos were cleared in 80\% glycerol/PBS before imaging. Primary antibodies used and their dilutions are as follows: Chicken anti-eGFP antibody, 1:500 (ab13970, Abcam), Rabbit anti-eGFP, 1:500 (Tp401, Torrey Pines Biolabs), Rabbit anti-FITC, 1:200 (#71-1900, ThermoFisher), Rabbit anti-beta Cat-enin, 1:200 (ab6302, Abcam), Mouse anti-E-Cadherin, 1:200 (#610181, BD Biosciences), Mouse anti-Tp63, 1:200 (CM163, Biocare Medical), Rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), 1:100 (#4370, Cell Signaling Technology), Rabbit anti-p44/42 MAPK (Erk1/2), 1:100 (#9102, Cell Signaling Technology), Rabbit anti-p90RSK (Phospho-Thr358), 1:100 (A00487, Genscript). All secondary antibodies were purchased from Invitrogen and used at 1:700 and were Alexa Fluor-488 Donkey anti-rabbit (A21206), Alexa Fluor-647 Donkey anti-rabbit (A21206), Alexa Fluor-487 Donkey anti-rabbit (A31573), Alexa Fluor-546 Donkey anti-mouse (A10036) and Alexa Fluor-488 Goat anti-chicken (A-11039). Nuclei were counterstained using 5\µg/ml of DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; D1306, Invitrogen) added during secondary antibody incubation.

To stain hydrogen peroxide, embryos were incubated for 60min at room temperature with 12.5\µM penta-fluorobenzenesulfonyl fluorescein (PFBSF) (#10005983, Cayman Chemicals), then rinsed in Embryo Medium, anaesthetized and imaged.

Fluorescent TUNEL staining was performed using the Fluorescein In Situ Cell Death Detection Kit (11684795910, Roche), with the fluorescein detected by antibody staining using rabbit anti-FITC, and co-immunostained for TP63 and eGFP.

Microscopy and Statistical analysis

Still and timelapse imaging was performed on upright Zeiss AxioImager M2, Zeiss Light-sheet Z.1, upright Zeiss LSM800 Confocal Microscope or Zeiss AxioZoom V16 microscopes. Embryos were mounted in 1.2\% Low Melting Point Agarose (Mo Bio Laboratories) in 0.5x E2 medium in 35 mm glass-bottom imaging dishes (MatTek) or in a 1mm inner diameter capillary for Light-sheet timelapse. When imaging was performed on live embryos, the embryo media were supplemented with 0.02\% Tricaine. Image processing was done using Zen 3.1 software (Zeiss), ImageJ (ver. 1.52p) or Imaris (Bitplane) and compiled using Photoshop CS6 (Adobe). Graphpad Prism was used for statistical analyses and graph generation. In all statistical tests, * = p<0.05, ** = p<0.01, *** = p<0.001.
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Supplementary Figures

Supplementary Figure 1: The epidermis of hai1a mutants displays elevated hydrogen peroxide

A: Graph of neutrophil speeds before and after wounding in WT and hai1a<sup>h2217</sup>. Neutrophils in wounded WT move as fast as unwounded hai1a mutants. Wounding hai1a mutants accelerates neutrophils.

B-C: Sequence chromatograms of genomic DNA from WT (upper panels) and dandruff alleles ti257 (B) and t419 (C) (lower panels). Altered codon is underlined in red with altered base indicated by arrow.

D: ClustalW protein alignment of the amino acid substitution in the ti257 allele showing broad conservation of across vertebrates.

E-F: Selected regions of 2D gel of protein extracted from 48hpf embryos for hai1a<sup>t419</sup> (E) or hai1a<sup>t257</sup> (F) in red, superimposed over WT protein samples (green in both). The shift in pi of Peroxiredoxin4 in both alleles is indicated with an arrow.

G: Box and whiskers plot of PFBSF fluorescent staining intensity of WT and hai1a<sup>fr26</sup> mutants at 24hpf in trunk and tail. n=9; t-test *** p<0.001.

H: Lateral projected confocal views of PFBSF staining of WT (upper row) and hai1a<sup>fr26</sup> (lower row) trunks and tails at 24hpf and 48hpf. Scale Bar H = 50µm.
Supplementary Figure 2: Generation of st14a mutant, and Duox inhibition reduces hydrogen peroxide and neutrophils in hai1a mutants

A-B: Sequence chromatograms of part of exon 6 of the zebrafish st14a gene from WT (A) and the st14a sq43 allele showing the 5bp insertion highlighted by red bar.

C: Effect of the 5bp insertion (red) in the sq43 allele leads to alteration of reading frame, 8 aberrant amino acids followed by a termination codon (red lettering). The codon altered to nonsense codon is underlined in both WT and mutant sequences.

D: Box and whiskers plot of PFBSF fluorescent staining intensity of 24hpf WT, hai1a h12217 mutants, hai1a h12217 st14a sq43 double mutants and hai1a h12217 injected with duox MO. n=8; ANOVA with Bonferroni post-test *** p<0.001, ** = p<0.01.

E: TP63 (magenta) and eGFP (green) antibody staining (left column) with DIC imaging (right column) for 48hpf hai1a h12217; Tg(mpx:eGFP) i114 mutants, either untreated (top row), or treated with 40µM DPI (bottom row). Scale bar D = 100µm.
Supplementary Figure 3: Thapsigargin and 2-APB reduces neutrophil inflammation but not epidermal defects in hai1a mutants

A: Plot of PFBSF fluorescent staining intensity of hai1afr26 mutants at 24hpf in trunk and tail, either untreated or treated with 2-APB. n=14; ANOVA with Bonferroni post-test *** = p<0.001 ** = p<0.01.

B-C: Projected confocal images of tail fins of 48hpf larvae immunostained with TP63 (magenta) and eGFP (green) (left column) with DIC imaging (right column). Larvae were both hemizygous for Tg(mpx:eGFP)i114 transgene and were treated with 6.5µM Thapsigargin. Genotypes are hai1a+/hi2217 sibling (A), and hai1a hi2217 mutant (B). Rescue of neutrophil inflammation, but not epidermal defect is apparent in treated hai1a mutant. Scale bar B = 100µm.
Supplementary Figure 4: NfkB signalling is elevated in hai1a mutants and mutation of ikbkg rescues neutrophil inflammation

A-B: Lateral confocal projections of Tg(6xHsa.NFKB:EGFP)nc1 embryos reporting NfkB signalling levels of hai1afr26 at 24hpf (B), and WT (A).

C: Nature of the ikbkgsq304 mutant allele showing TALEN site location within the intron-exon structure of the gene (coding and non-coding exons depicted as filled and open boxes respectively). Sequence of part of exon 3 shown below with the TALEN binding site in red. The 14bp deletion in the ikbkgsq304 allele indicated under the WT sequence as dashes. This leads to a frameshift changing codon 80 from GGC (Gly) to GGT (Val), then 9 aberrant amino acids followed by a stop codon.

D-E: Lateral widefield fluorescent images of hai1ahi2217; ikbkg+/+ (D) and hai1ahi2217; ikbkgsq304 (E) embryos at 24hpf stained with PFBSF showing no loss of H2O2 in hai1a mutants upon mutation of ikbkg.

F-G: Lateral confocal projections of 48hpf hai1afr26; Tg(6xHsa.NFKB:EGFP)nc1 embryos, treated with DMSO (F) or with 2.5µM 2-APB (G). Scale bars A, F = 200µm, D = 50µm.
Supplementary Figure 5: Elevation of pERK levels in PMA treated and hai1a mutant epidermis

A-J: Lateral projected confocal images of ventral trunks (A-C), tails (D-F) and whole embryos (G-J) immunostained for TP63 (magenta) and pERK (green) at 24hpf (A-D, I, J), 48hpf (E, F) and 20hpf (G, H). Both hai1a\textsuperscript{fr26} (C, D, F, H, J) and 125ng/ml PMA treated (B) embryos show increased epidermal pERK levels compared to untreated WT (A, E, G, I). Elevation of epidermal pERK is seen in hai1a\textsuperscript{fr26} mutants and PMA treated embryos as well as in nascent aggregates (arrowheads, H). Scale bars: C, F = 50µm; D = 20µm; G, I, J = 100µm.
Supplementary Figure 6: Rescue of the hai1a epidermal phenotype by pERK inhibitors

A-F: Lateral DIC images of 24hpf (A-B) or 48hpf (C-F) hai1a<sup>h2217</sup> embryos treated with either DMSO (A, C, E), U0126 (B) or CI-1040 (D, F) showing rescue of general morphology (B), trunk (D) and tail (F) epidermal phenotypes compared to DMSO treated hai1a<sup>h2217</sup>. G-H: Lateral DIC images of tails of 48hpf WT treated with 125ng/ml PMA alone (G) or PMA and U0126 (H). I-J: Lateral projected confocal images of trunks of 48hpf hai1a<sup>h2217</sup> embryos treated with DMSO (I) or U0126 (J) and then fluorescently immunostained for TP63. Scale bars: B = 200µm; C, H, I = 100µm.
Supplementary Figure 7: RSK inhibitors rescue the hai1a phenotype

A-B: Lateral DIC images of hai1a hi2217 embryos at 24hpf either untreated (A) or treated with 9µM Dimethyl Fumarate (B). Locations of epidermal aggregates and loss of tail fin morphology in hai1a mutants, and their rescue by RSK inhibitor treatment are indicated by arrowheads. C: Proportions of epidermal phenotypes from hai1a hi2217 x hai1a +/hi2217 determined at 24hpf and 48hpf following treatments with DMSO, BI-D1870 or Dimethyl Fumarate (DMF), n=100; Chi-squared test; *** = p<0.001. D-E: Lateral projected confocal images of trunks of 48hpf hai1a hi2217 embryos, untreated (D) or treated with 9µM Dimethyl Fumarate (E), and then fluorescently immunostained for TP63. Scale bars: A=200µm; D = 100µm.
### Supplementary Table

#### Supplementary Table 1: Prevalence of otolith and epithelial phenotypes in hai1a and st14a double mutants:

| hai1a<sup>+/hi2217</sup>; st14a<sup>+/sq43</sup> ♂ x hai1a<sup>hi2217/hi2217</sup>; st14a<sup>sq43/sq43</sup> ♀ |
|-----------------------------------------------|
| **Observed (Expected)** | WT Epidermis | hai1a epidermis | Total |
|-------------------------|--------------|----------------|-------|
| **Wildtype Otoliths**   | 72 (65)      | 60 (65)        | 132 (130) |
| **No otoliths**         | 128 (65)     | 0 (65)         | 128 (130) |
| **Total**               | 200 (130)    | 60 (130)       | 260   |

p<0.0001 (Chi-squared test)
Supplementary Videos

**Supplementary Video 1: Neutrophils in WT and hai1ahi2217 4dpf larva**
Projected confocal timelapses of eGFP positive neutrophils in the tail region of 4dpf Tg(mpx:eGFP)i114 (left) and hai1ahi2217; Tg(mpx:eGFP)i114 (right) larvae taken every 45 seconds for 45 minutes. Scale bar 50µm.

**Supplementary Video 2: Neutrophils in WT and hai1ahi2217 4dpf larva before and after fin wound**
Projected confocal timelapses of eGFP positive neutrophils in the tail region of 4dpf Tg(mpx:eGFP)i114 (left) and hai1ahi2217; Tg(mpx:eGFP)i114 (right) larvae taken every 50 seconds for 250 minutes with the tail fin cut at 50 minutes. GFP is overlaid on DIC channel. Scale bar 50µm.

**Supplementary Video 3: Calcium dynamics in WT and hai1afz26 embryos at 24hpf**
Projected confocal timelapses of eGFP in the trunks (left side) and tails (right side) of a 24hpf WT (top row) and hai1afz26 (bottom row) embryos injected with GCaMP6s RNA, indicating calcium dynamics. Scale bar 50µm.

**Supplementary Video 4: Calcium dynamics in DMSO and 2-APB treated hai1afz26 embryos at 24hpf**
Projected confocal timelapses of eGFP signal in the trunks (left side) and tails (right side) of 24hpf hai1afz26 embryos injected with GCaMP6s RNA and treated with 0.03% DMSO (top row) and 2.5µM 2-APB (bottom row), indicating reduced calcium dynamics following 2-APB treatment. Scale bar 50µm.

**Supplementary Video 5: Basal keratinocyte membrane and neutrophil dynamics in 3dpf wild-type and hai1ahi2217 larvae carrying the Tg(krt1c19e:lyn-tdtomato)q16 and Tg(mpx:eGFP)i114 transgenes**
Projected light-sheet timelapses of the trunk of 3dpf WT (left) and hai1ahi2217 (right) larvae with neutrophils and basal keratinocyte membranes labelled by eGFP and Lyn-tdTomato respectively. Both larvae carried the Tg(krt1c19e:lyn-tdtomato)q16; Tg(mpx:eGFP)i114 transgenes. The hai1a mutants have highly dynamic neutrophils and keratinocyte membrane dynamics. Scale bar 20µm.

**Supplementary Video 6: Basal keratinocyte membranes in DMSO and PMA treated 3dpf Tg(krtt1c19e:lyn-tdtomato)q16 larvae**
Zoomed projected light-sheet timelapses of basal keratinocyte membranes labelled by Lyn-tdTomato in the trunk of 3dpf Tg(krt1c19e:lyn-tdtomato)q16 larvae treated with 0.1% DMSO (left) and 37.5ng/ml PMA (middle and right) for 18hrs. Membranes are stable in DMSO treated larvae but were dynamic in PMA treated larvae. Images were captured every 20 seconds. Scale bar 10µm.

**Supplementary Video 7: Neutrophils and basal keratinocyte membranes in DMSO and PMA treated 3dpf Tg(krt1c19e:lyn-tdtomato)q16; Tg(mpx:eGFP)i114 larvae**
Lateral projection of light-sheet timelapse of neutrophils labelled by eGFP and basal keratinocyte cell membranes labelled by lyn-tdTomato in the trunks of 3dpf Tg(krt1c19e:lyn-tdtomato)q16 larva treated with 0.1% DMSO (left) and 37.5ng/ml PMA (right) for 18hrs. PMA treatment leads to slightly dynamic cell membranes and motile neutrophils. Images were captured every 20 seconds for 30 minutes. Scale bar 50µm.