Matriptase activation of Gq drives epithelial disruption and inflammation via RSK and DUOX

Jiajia Ma¹, Claire A Scott²,³, Ying Na Ho¹, Harsha Mahabaleshwar¹, Katherine S Marsay²,³, Changqing Zhang¹, Christopher KJ 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, Singapore, Singapore; ²Institute of Molecular and Cell Biology (IMCB), A*STAR (Agency for Science, Technology and Research), Singapore, Singapore; ³Division of Cell Matrix Biology and Regenerative Medicine, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom; ⁴Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom; ⁵Department of Biological Sciences, National University of Singapore, Singapore, Singapore; ⁶Department of Pediatrics, Yong Loo Ling School of Medicine, National University of Singapore, Singapore, Singapore

Abstract

Epithelial tissues are primed to respond to insults by activating epithelial cell motility and rapid inflammation. Such responses are also elicited upon overexpression of the membrane-bound protease, Matriptase, or mutation of its inhibitor, Hai1. Unrestricted Matriptase activity also predisposes 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 in hai1a mutants rescues both the inflammation and epithelial phenotypes, with the latter recapitulated by the DAG analogue, PMA. We demonstrate that hai1a has elevated MAPK pathway activity, inhibition of which rescues the epidermal defects. Finally, we identify RSK kinases as MAPK 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.

Introduction

The transmembrane serine protease, Matriptase, encoded by the ST14 gene, has potent oncogenic properties and is consistently dysregulated in human carcinomas. Overexpression of Matriptase in the mouse epidermis leads to epidermal papillomas, ulcerative and invasive carcinomas, and inflammation (List et al., 2005; Martin and List, 2019). These effects of Matriptase are mitigated by a cognate serine protease inhibitor, HAI-1. Clinically, an increase in the Matriptase:HAI-1 ratio has been found in a range of tumours and is predictive of poor outcome (Martin and List, 2019). Loss of mouse Hai1 leads to epidermal and intestinal barrier defects, epithelial inflammation, and failure of placental labyrinth formation, which are all due to unrestricted Matriptase activity (Kawaguchi et al., 2011; Nagaike et al., 2008; Szabo et al., 2007). The response of epithelia to
unregulated Matriptase activity appears conserved across vertebrates. Mutation of the zebrafish orthologue, Hai1a, also results in epidermal defects, including loss of membrane E-cadherin, aberrant mesenchymal behaviour of keratinocytes, which form cell aggregations over the body and loss of fin fold integrity. The epidermis also displays sterile inflammation and is invaded by highly active neutrophils. Genetic ablation of the myeloid lineage demonstrated that the keratinocyte phenotypes are not a consequence of the inflammation (Carney et al., 2007). The strong hai1afr26 allele is embryonic lethal, dying within the first week, whilst the more mild allele, hai1ah2217, is semi-viable, with epithelial defects resolved through sphingosine-1-phosphate-mediated entosis and cell extrusion (Armistead et al., 2020). All hai1a mutant phenotypes can be ameliorated by reduction of Matriptase levels (Carney et al., 2007; Mathias et al., 2007).

Due to the clinical implications of its dysregulation, the signalling pathways activated pathologically by Matriptase are of interest. The G-protein-coupled receptor, proteinase-activated receptor-2 (Par2), is essential for the oncogenic and inflammatory effects of uninhibited Matriptase in zebrafish and mouse (Sales et al., 2015; Schepis et al., 2018). Par2 is directly activated by Matriptase proteolysis and signals through a number of heterotrimeric Gα protein subunits. Early studies in keratinocytes linked Par2 activation with intracellular Ca2+ mobilisation via phospholipase C, thus implicating Gq subunit as the canonical target (Schechter et al., 1998). Alternate Gα subunits, including Gi, Gs, and G12/13, are now known to also be activated by Par2 (Zhao et al., 2014). Par2 displays biased agonism, and the logic of the pathway utilised depends on cell context and the activating protease. In vitro experiments using HEK293 cells implicated both Par2 and Gi in Matriptase-mediated Nfxb pathway activation (Sales et al., 2015). Whilst this explains the inflammatory phenotype of uninhibited Matriptase, it does not address whether Par2 promotes carcinoma phenotypes directly in keratinocytes in vivo. In zebrafish, as the keratinocyte defects are not dependent on inflammation, but are dependent on Par2, it is likely that there is a direct effect of Par2 on promoting keratinocyte motility.
Figure 1. The epidermis of hai1a mutants displays elevated hydrogen peroxide. (A, B)Projected confocal images showing neutrophils populate the tail of hai1a<sup>h2217</sup> mutants (B) but just the vasculature of WT (A) at 4dpf labelled by the Tg(mpx:EGFP)<sup>114</sup> line. Fin extremity outlined in white. (C) Neutrophils move significantly faster in hai1a<sup>h2217</sup> than WT. n = 15; t-test; ***p<0.001. (D, E) Tracks of neutrophil migration taken from Video 1 in WT (D) and hai1a<sup>h2217</sup> (E). (F) Schematic of the Hai1a protein with protein domains given, signal peptide as purple line and Figure 1 continued on next page
Par2 can also 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 and Par2 remain unclear. Here through use of the zebrafish hai1a mutant, we comprehensively map the essential pathways downstream of zebrafish Matriptase and Par2, leading to inflammation and epithelial disruption.

Results

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

Neutrophils in hai1a embryos invade the epidermis, are highly motile, but move randomly (Carney et al., 2007; Mathias et al., 2007; Figure 1A–E, Video 1). To establish the nature of their stimulus, we tested if neutrophils in hai1a altered their behaviour in the presence of a large fin wound. In wild-type larvae, neutrophils were recruited from a great distance and tracked to the wound with high directionality. However, neutrophils in the hai1a mutant appeared largely apathetic to the wound and remained migrating randomly. There was a mild increase in neutrophil speed in hai1a larvae following wounding, indicating that they retain capacity to respond to additive stimuli (Figure 1—figure supplement 1A–D, Video 2). Co-labelling of basal keratinocyte nuclei (using TP63 immunostaining), neutrophils (Tg(fli1:EGFP)transgenic), and TUNEL labelling of apoptotic cells highlighted that whilst the epidermis of hai1a mutants, unlike WT, had regions of apoptosis at 24hpf (arrowhead, Figure 1—figure supplement 1E, F), 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, or more, potent as that of wounds.

To identify the neutrophil activator in hai1a, 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 has many phenotypic similarities to the strong hai1a 2s26 allele (van Eeden et al., 1996). Crosses between ddf 2s251 or ddf 419 and hai1a 2h2217 failed to complement, and sequencing of hai1a CDNA from both ddf alleles identified a nonsense mutation in the ddf 419 allele (c.771T>G; p.Tyr257Ter) and a missense mutation of a highly conserved amino acid in the ddf 2s251 allele (c.749G>A; p.Gly250Asp) (Figure 1F, Video 1). Neutrophils in WT and hai1a 2h2217 4dpf larva. Projected confocal timelapses of eGFP-positive neutrophils in the tail region of 4dpf Tg(mpx:eGFP)114 (left) and hai1a 2h2217, Tg(mpx:eGFP)114 (right) larvae taken every 45 s for 45 min. Scale bar: 50 μm. https://elifesciences.org/articles/66596#video1
To demonstrate that, as with other phenotypes, the H$_2$O$_2$ increase in hai1a was due to unrestrained activity of Matriptase1a, we used a matriptase1a mutant allele, st14a$^{sq10}$, which prematurely terminates the protein at 156 amino acids (Figure 2A, Figure 2—figure supplement 1A–C; Lin et al., 2019). Zygotic st14a mutants showed no overt phenotype; however, maternal zygotic mutants lacked ear otoliths (Figure 2B, C). As expected, when crossed into the hai1a background, embryos lacking otoliths (st14a$^{sq10}$; hai1a$^{hi2217}$ double mutants) never displayed the hai1a epidermal and neutrophil phenotypes (Figure 2D–F; Table 1). Double mutants also had significantly reduced H$_2$O$_2$ levels (Figure 2F, Figure 2—figure supplement 1D). To determine if reduced H$_2$O$_2$ could account for the rescue of hai1a phenotypes by st14a mutation, 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 (Figure 2, Figure 2—figure supplement 1D) and neutrophil inflammation in hai1a mutants but did not rescue the epithelial defects (Figure 2F, G). Treatment with a known Duox inhibitor, diphenyleneiodonium (DPI), also resulted in amelioration of neutrophil inflammation, but not epithelial aggregates, in hai1a mutants (Figure 2G, Figure 2—figure supplement 1E). We conclude that Matriptase1 activity leads to excess H$_2$O$_2$ in hai1a mutants, which partially accounts for the neutrophil inflammation, but not epithelial 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 et al., 2013). We injected hai1a$^{hi2217}$ with RNA encoding the calcium reporter GCaMP6s. Timelapse imaging at 24hpf indicated that hai1a mutants had significantly more calcium flashes in both the trunk and tail (Figure 3A, B, E, Figure 3—figure supplement 1A, B, Video 3). Increased intracellular calcium dynamics was observable as early as 16hpf, concomitant with increased H$_2$O$_2$, but prior to onset of hai1a phenotypes (Figure 3G, H, Video 4). 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 are reduced by treatment with the IP$_3$R inhibitor, 2-APB compared to control (Figure 3C, D, F, Figure 3—figure supplement 1C, D, Video 5). Reducing calcium flashes in hai1a mutant embryos with 2-APB also significantly reduced H$_2$O$_2$ levels (Figure 3I, J, Figure 3—figure supplement 1E) and partially reduced inflammation; however, the epidermal defects were not noticeably rescued (imaged by DIC (Differential Interference Contrast) or labelled with the TP63 antibody) (Figure 3I–K). 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 (Figure 3K, Figure 3—figure supplement 1F, G). This suggests, in hai1a mutants, that 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 et al., 1991). We crossed the hai1a fr26 allele to the NfkB sensor transgenic line Tg(6xHsa.NFKB:EGFP)nc1. In WT embryos, NfkB signalling was

Table 1. Prevalence of otolith and epithelial phenotypes in hai1a and st14a double mutants: p<0.0001 (Chi-squared test).

| Observed (expected) | WT epidermis | hai1a epidermis | Total |
|---------------------|--------------|----------------|-------|
| Wild-type otoliths  | 72 (65)      | 60 (65)        | 132 (130) |
| No otoliths         | 128 (65)     | 0 (65)         | 128 (130) |
| Total               | 200 (130)    | 60 (130)       | 260    |
Figure 3. Calcium dynamics in hai1a mutants regulate H$_2$O$_2$ and inflammation. (A–D) Projected confocal images of eGFP in the tail of WT (A) or hai1a$^{fr26}$ (B–D) injected with GCaMP6s RNA, imaged at 24hpf, indicating calcium dynamics. Embryos are either untreated (A, B), treated with DMSO (C), or with 2.5 µM 2-APB (D). Images are temporal projections of timelapse movies taken at maximum speed intervals (2 min) and projected by time. (E, F) Graphs comparing eGFP intensities from GCaMP6s RNA timelapses in trunk and tail between 24hpf WT and hai1a$^{fr26}$ (E) and between hai1a$^{fr26}$ treated with DMSO and 2.5 µM 2-APB (F). n = 10; t-test; *p<0.05, ***p<0.001. (G, H) Projected light-sheet images of Tg(actb2:GCaMP6s, myl7:mCherry)$^{fr26}$ embryos indicating calcium dynamics at 16hpf of sibling (G) or hai1a$^{hi2217}$ (H). Images are temporal projections of timelapse movies taken at 45 s intervals. (I–K) Analysis of neutrophil number in fins of hai1a$^{fr26}$ embryos treated with DMSO, 2.5 µM 2-APB, or 6.5 µM Thapsigargin. 

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mostly restricted to neureomasts at 48hpf, whilst in hai1a mutants we observed an increase in fluorescence at 24hpf and a strong increase at 48hpf. Fluorescence at both timepoints was noted in epidermal aggregates and fin folds, locations of strong inflammation (Figure 4A, B, Figure 4—figure supplement 1A, B). This increase in signalling in 48hpf hai1a mutant embryos was confirmed by qRT-PCR of the NfkB target gene, nfkbiaa (Figure 4C). Unlike calcium and H$_2$O$_2$, NfkB signalling is not present at early stages prior to phenotype (Figure 4—figure supplement 1C, D). To determine the extent that NfkB signalling accounts for the hai1a phenotypes, we generated a mutant in the ikbkg (=ikkg or nemo) gene, which encodes a scaffold protein required for activating the NfkB pathway (Rothwarf et al., 1998) (ikbkg$^{Gly80ValfsTer11}$; Figure 4—figure supplement 1E). Crossing this mutant to hai1a$^{hi2217}$ on the Tg(mpx:eGFP)$^{T14}$ background realised a very strong rescue of neutrophil inflammation at 48hpf, but no improvement of hai1a epidermal defects (Figure 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)$^{+1}$ embryos with duoxMO. We noted a strong reduction in NfkB pathway activation compared to uninjected hai1a$^{hi2217}$ mutant controls (Figure 4J, K). Conversely, genetic ablation of NfkB signalling, through use of the ikbkg mutant, did not reduce H$_2$O$_2$ levels in hai1a mutants (Figure 4—figure supplement 1F, G). 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 (Figure 4—figure supplement 1H, I). We propose 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 occurs at later stages, and 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 IP$_3$ 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 (Figure 5D, F), but surprisingly, it also significantly rescued the epidermal defects in hai1a mutants, with a significant reduction in TP63-positive epidermal aggregates in the trunk and improved tail fin fold integrity at 48hpf (Figure 5A–E).
PMA treatment phenocopies the hai1a mutant

As IP₃R inhibition only blocks inflammation in hai1a mutants, but an inhibitor of a PLC activator (Gq) additionally reduces the epidermal defects, we considered that diacyl glycerol (DAG) might contribute to the epidermal defects as the second product of PIP₂ cleavage (along with IP₃). Indeed, treating WT embryos from 15hpf to 24hpf with 125 ng/ml phorbol 12-myristate 13-acetate (PMA), a DAG analogue, 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 (Figure 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 (Figure 6D, E). Treatment from 24hpf to 48hpf with 125 ng/ml PMA led to a fin defect similar to the dysmorphic hai1a mutant fin (Figure 6F, G).

Most PMA-treated Tg(mpx.eGFP)¹⁷⁴ larvae at 48hpf also had more neutrophils in the epidermis than untreated controls, which were highly migratory (Figure 6F–G, J–K, Video 8). We determined H₂O₂ levels in PMA-treated embryos using PFBSF staining and found that it was significantly increased in both trunk and tail at 24hpf (Figure 6L–O, R). In contrast, when we treated GCaMP6s RNA-injected embryos with PMA, we failed to see an increase in calcium flashes, as seen in hai1a (Figure 6P, Q, S). To see if the heightened H₂O₂ and inflammation was also correlated with increased NfkB signalling, we treated Tg(6xHsa.NFKB:EGFP)¹⁴⁷ embryos with 125 ng/ml PMA. There...
was a robust increase in fluorescence, indicating that PMA activates the NfkB pathway (Figure 6T, U).

The phenocopy and the rescue of hai1a by PMA and Gq inhibition respectively imply that DAG is elevated in hai1a mutants. Elevated cellular DAG leads to relocalisation of Protein Kinase C isoforms to the plasma and nuclear lipid membranes where they bind DAG and become activated. Using a

Figure 4. NfkB signalling is elevated in hai1a mutants and is necessary for neutrophil inflammation. (A, B) Lateral confocal projections of Tg(6xHsa.NFKB:EGFP)embryos reporting NfkB signalling levels at 48hpf for WT (A) and hai1a626 (B). (C) qPCR of cDNA levels of Nfk target gene nfkbiaa in hai1a626 vs. sibs at 48hpf. n = 3, 200 embryos pooled in each, t-test *p<0.05. (D–E') Projected confocal images of the tail fins of 48hpf Tg(avgpx:eGFP)i114; hai1aht217 embryos, immunostained for TP63 (magenta) and eGFP (green) (D, E) with corresponding DIC image (D', E'). Embryos were either mutant for ikbkg (ikbkg7899, E–E') or heterozygous (ikbkg7899; D–D'). (F) Counts of eGFP-positive neutrophils in the fins at 48hpf of hai1aht217; ikbkg7899 and hai1aht217; ikbkg7899. Embryos were hemizygous for Tg(avgpx:eGFP)i114, n = 9; t-test; ***p<0.001. (G–I) Lateral DIC images of the trunk of hai1aht217, ikbkg7899 and hai1aht217; ikbkg7899. Loss of IKBKG does not rescue epidermal defects of hai1a mutants (arrowheads). (J, K) Lateral confocal projections of Tg(6xHsa.NFKB:EGFP)embryos reporting NfkB signalling levels at 32hpf of hai1aht217 uninjected (J) or injected with duox MO (K). Loss of H2O2 reduces NfkB signalling levels in hai1a mutants. Scale bars: (A, K) = 200 μm; (D', I) = 50 μm.

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Figure supplement 1. NfkB signalling is elevated in hai1a mutants, and mutation of ikbkg rescues neutrophil inflammation.
GFP-tagged PKC\(\delta\) fusion protein (Sivak et al., 2005), we showed that in the WT embryo there was largely diffuse cytoplasmic PKC\(\delta\)-GFP signal, however, it translocated to plasma and nuclear membranes in \(hai1a\) mutants, indicating increased levels of DAG (Figure 7A, B, Figure 7—figure supplement 1A, B). This is indeed relevant to the epidermal defects, as treatment of \(hai1a^{h2217}\) embryos with the PKC inhibitor, GFX109203, reduced the epidermal aggregates and disruption of fin morphology as imaged by DIC or immunostaining for TP63 (Figure 7C–H). Neutrophil inflammation in the epidermis was somewhat reduced, but not to a significant degree (Figure 7E–I). Thus, these experiments strongly suggest that epithelial defects of \(hai1a\) are due to DAG generation and 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.,...
Figure 6. Phorbol 12-myristate 13-acetate (PMA) induces epidermal aggregates, motility, H$_2$O$_2$, NfkB, and inflammation. (A, B) Lateral micrographs of embryos treated with DMSO (A) or 125 ng/ml PMA (B, C) showing generation of epidermal aggregates (arrowheads). (D, E) Projected confocal images of the trunk of 24hpf WT embryos treated with 0.1% DMSO (D) or 125 ng/ml PMA (E) and immunostained for TP63 (magenta), showing aggregation of TP63-positive cells. (F, G) Projected confocal images superimposed on DIC image of the tail of 48hpf Tg(mpx:eGFP)$_{i114}$ embryos treated with 0.1% DMSO (F) or 125 ng/ml PMA (G) showing fin defect and Figure 6 continued on next page.
2020), we performed whole-mount immunofluorescent analysis in case there was only a localised effect. Indeed, we observed a significant and localised increase in cytoplasmic pERK immunoreactivity (phospho-p44/42 MAPK (Erk1/2) (Thy202/Tyr204)) in the regions of epidermal aggregate formation in hai1a mutants and in PMA-treated embryos, including under the yolk at 24hpf and in the fins at 24hpf and 48hpf (Figure 8A–K, Figure 8—figure supplement 1A–F). There was no increase in total ERK levels in the mutant (Figure 8—figure supplement 1M, N). Increased pERK was seen in both the cytoplasm and nucleus of TP63-positive cells but was only increased in the nucleus of periderm cells (Figure 8E–F, Figure 8—figure supplement 1D). To establish that this is an early marker of aggregate formation, and not a sequela, we stained hai1a mutant embryos at earlier timepoints. We found that at 16hpf regions of the epidermis have pERK staining before overt aggregation formation (Figure 8G–H), whilst nascent aggregates also contain pERK staining which increases in number over time (Figure 8—figure supplement 1G–L).

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 et al., 2012), precluding ability to assess rescue in hai1a, 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 et al., 2003; Favata et al., 1998). Both inhibitors showed a significant reduction in hai1a mutant epidermal aggregates under the yolk, and restoration of the overall and tail epithelial morphology, with embryos showing a hai1a phenotype class significantly reduced (Figure 9A–G, Figure 9—figure supplement 1A–F). Similarly, the epidermal defects of the trunk, yolk, and tail following PMA treatment were also ameliorated by concomitant U0126 treatment (Figure 9H, I, Figure 9—figure supplement 1G, H). Rescue of aggregates and tail morphology following PMA treatment or in hai1a mutants could be visualised by immunolabelling TP63 in basal keratinocyte nuclei (Figure 9J–O, Video 6).
Initiating U0126 treatment later at 26hpf led to only a partial rescue, indicating that the epidermal phenotypes were likely due to sustained pERK activation (Figure 9—figure supplement 1K–M).

Treatment with U0126 did not significantly reduce neutrophil inflammation of hai1a mutants or PMA treatment (Figure 9L–P). This suggests that the inflammation phenotype is not simply a consequence of the epidermal defects. Furthermore, dye penetration assays showed that the epithelial barrier was not globally and overtly compromised in hai1a, underscoring that inflammation is not simply a consequence of epithelial defects (Figure 9—figure supplement 2A–H). It has been shown that the epidermal defects in hai1a 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 β-catenin. Whilst the WT basal epidermal cells of the 48hpf tail showed strong staining at the membrane, hai1a mutants and PMA-treated embryos showed a significant loss of β-catenin at the membrane and increase in the cytoplasm (Figure 9Q–V, Y, Z). Treatment of hai1a mutants with U0126 restored the membrane localisation of β-catenin (Figure 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/β-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 previously shown that there is no transcriptional downregulation of E-cadherin levels in hai1a, making a nuclear transcription factor target less likely to be relevant (Carney et al., 2007). The p90RSK family of kinases represents direct cytoplasmic targets of Erk1/2 phosphorylation which regulate cell motility, and thus were good candidates for mediators disrupting cell-cell adhesion (Časlavský et al., 2013; Tanimura and Takeda, 2017). We determined that at least RSK2a (=p90RSK2a, encoded by rps6ka3a) is expressed in basal keratinocytes at 24hpf (Figure 10A, B). To gauge if there was an alteration in phosphorylation of RSK family members in the epidermis of hai1a mutants, we used an antibody which detects a phosphorylated site of mouse p90RSK (Phospho-Thr348). This site is phosphorylated in an ERK1/2-dependent manner (Romeo et al., 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 (Figure 10C–D). This increase in cytoplasmic levels of p90RSK-pT348 was observable at 17hpf prior to epithelial defects (Figure 10—figure supplement 1A–C). p90RSK cytoplasmic signal...
Figure 7. Inhibition of PKC rescues epidermal defects of hai1a. (A, B) Confocal images of the ventral fin of 22hpf sibling (A) or hai1a hi2217 (B) embryos injected with RNA encoding PKCδ-GFP. Mostly cytoplasmic distribution in sibling was relocated to cell and nuclear membranes in hai1a mutants. (C, D) Lateral brightfield images of 48hpf hai1a hi2217 larvae treated with 0.5% DMSO (C) or 85 μM GFX109203 (D). Epidermal aggregates and fin deterioration are rescued by the PKC inhibitor (arrowheads). (E–H) DIC (E, G) and projected confocal images (E', G', F, H) of hai1a hi2217; Tg(mpx:eGFP)i114 trunk at 24hpf (E–E', G–G') and tail at 48hpf (F, H), either treated with 0.5% DMSO (E–F) or 85 μM GFX109203 (G–H). Embryos are immunostained for TP63 (magenta) and eGFP (green), highlighting rescue of epidermal phenotype and partial rescue of neutrophils by GFX109203. (I) Counts of eGFP-positive neutrophils in the fins at 48hpf of Tg(mpx:eGFP)i114, or hai1a hi2217; Tg(mpx:eGFP)i114 treated with 0.5% DMSO or 85 μM GFX109203. n = 8; ANOVA, Dunn's multiple comparisons; **p<0.01. Scale bars: (A) = 10 μm; (D) = 200 μm; (H) = 100 μm.

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was lost upon U0126 and GFX109203 treatments, showing that it was pERK and PKC dependant (Figure 10E, E, Figure 10—figure supplement 1D, E). Similarly, increased cytoplasmic p90RSK-pT^{348} was observed following PMA treatment which was reduced by co-treatment with U0126 (Figure 10F–H). The increase in cytoplasmic p90RSK-pT^{348} signal, and its reduction by U0126, was significant in both hai1a mutants and PMA-treated embryos (Figure 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). Dimethyl fumarate treatment reduced the extent of cytoplasmic p90RSK-pT^{348} in hai1a (Figure 10—figure supplement 1F, G). 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 (Figure 10K–N, Figure 10—figure supplement 1H, I, K, L). Reduction of mutant phenotype classes was significant at both 24hpf and 48hpf (Figure 10—figure supplement 1J). 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 (Figure 10O–Q). Thus, phosphorylation of RSK proteins is altered in hai1a mutants, and their inhibition appears to restore E-cadherin to the membrane and reduce epidermal aggregate formation.

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, suggesting conservation of downstream pathways. What the conserved ancestral role of the Matriptase-Hai1 might have been is unclear. Matriptase dysregulation in the mouse is associated with cancer progression (Martin and List, 2019). Tumours have long been considered to represent non-healing wounds, and the cellular- and tissue-level phenotypes of hai1a have similarities to tumours. Epidermal cells in zebrafish transformed by MAPK activation both promote and respond to inflammation through similar mechanisms to wound responses (Feng et al., 2010; Schäfer and Werner, 2008). Further, tissue damage of the zebrafish epidermis perturbs osmolarity and releases nucleotides, leading to inflammation and epithelial cell motility, with the resulting phenotypes strikingly similar to hai1a mutants (de Oliveira et al., 2014; Enyedi and Niethammer, 2015; Gault et al., 2014; Hatzold et al., 2016). Indeed, the tissue responses initiated by loss of zebrafish Hai1a have been previously suggested to represent an early injury response (Schepis et al., 2018), whilst PAR2 synergises with P2Y purinergic and EGF receptors to promote cell migration in scratch assays (Shi et al., 2013). Thus our analysis supports the previous hypothesis of the Hai1-Matriptase system as a component of tissue injury responses (Schepis et al., 2018), which, if inappropriately activated, promotes carcinoma.

The various molecular pathways known to be activated by Matriptase have not been fully delineated or integrated. Par2 has previously been shown to be required for the hai1a phenotype in zebrafish and contributes to the phenotypes of Matriptase overexpression in the mouse. Exactly which heterotrimeric G-protein Par2 is activating in vivo and how this links to phenotypes has not been identified. Our analyses allow us to propose a pathway downstream of Par2 which accounts for both the inflammatory and the epidermal phenotypes (Figure 11). Firstly, inhibition of Gq rescued both the inflammation and epithelial defects. PAR2 activation of Gq has been documented to occur in many cell types including keratinocytes, where inhibition of Gq and PKC reduces PAR2-mediated Nfkb signalling (Böhm et al., 1996; Goon Goh et al., 2008; Macfarlane et al., 2005). Although we were unable to rescue hai1a phenotypes with a PLC inhibitor due to toxicity, genetic sensors demonstrated increased levels of Ca^{++} and DAG in hai1a epidermis. Our analysis demonstrated that the different products of PIP2 hydrolysis appear to invoke the two main hai1a phenotypes to different
Figure 8. Elevation of pERK levels in phorbol 12-myristate 13-acetate (PMA)-treated and hai1a mutant epidermis. (A–L) Lateral projected confocal images of trunks (A, A', B, E, E', F), yolk surface (G, G', H) and tails (C, C', D, I, I', J) of embryos immunostained for TP63 (A, B, C, D, E, F, G', H, I, J, magenta) and pERK (A, J, green) at 24hpf (A–F), 16hpf (G–H), and 48hpf (I–J). Both hai1a<sup>f26</sup> (A, A', C, C', E, E', G, G') and 125 ng/ml PMA-treated (I, I') embryos show increased epidermal pERK levels compared to untreated WT (B, D, F, H, J). Elevation of epidermal pERK in hai1a<sup>f26</sup> mutants and PMA- treated embryos is seen in the trunk (A, E) and tail (C, I) as well as in epidermis over the yolk prior to overt phenotype manifestation (G). (K) Quantification of pERK immunofluorescent intensity in the tail of 24hpf hai1a<sup>f26</sup> larvae compared to siblings. n = 5; Mann–Whitney test; **p<0.01. Scale bars: (B) = 100 μm; (D, H, J) = 50 μm; (F) = 20 μm.

The online version of this article includes the following figure supplement(s) for figure 8:

Figure 8 continued on next page
extents. IP₃R-dependent calcium release in hai1a epidermis was required for Duox activity, high hydrogen peroxide levels, and, later, increased NFkB signalling. Reduction of these attenuated the inflammatory, but not epithelial, defects. Conversely, inhibiting the DAG receptor, PKC, rescued the epithelial phenotypes, and the inflammation slightly. The DAG analogue, PMA, phenocopied the epidermal defects of hai1a mutants but also increased H₂O₂, NFkB, and neutrophil inflammation, indicating that PKC activation may be sufficient, but not necessary, for inflammation. This is in line with known activation of Duox and IKK by PKC (Rigutto et al., 2009; Turvey et al., 2014). In addition, expression of activated Ras in zebrafish keratinocytes has been shown to lead to H₂O₂ release and neutrophil attraction (Feng et al., 2010). Thus, there is likely to be dual contribution to the inflammatory phenotype from IP3 and DAG. It is important to stress however that the inflammation is not simply a result of epithelial defects or an overt loss of barrier. Firstly, we see increase in Ca²⁺ and H₂O₂ very early in the epidermis prior to skin defects. Secondly, barrier assays failed to conclusively show a broad increase in permeability. Finally, rescue of epithelial defects by PKC and pERK inhibition did not fully rescue the inflammation. We conclude in our model that DAG contributes to both aspects of the phenotype, but IP₃ promotes only the inflammation.

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. That we can rescue the epithelial defects using a MEK inhibitor indicated that this increase in epidermal pERK is likely critical to the phenotype. The MAPK pathway is known to regulate cell motility (Tanizawa and Takeda, 2017). In the zebrafish epidermis, misexpression of activated MEK2 generated papillomas with remarkable resemblance to the epidermal aggregates in hai1a mutants (Chou et al., 2015), and which are not overtly proliferative. In astrocytes and oesophageal or breast tumour cell lines, PAR2 stimulates migration and invasiveness through MAPK/ERK, activation of which required Gq and PIP2 hydrolysis (Jiang et al., 2004; McCoy et al., 2010; Morris et al., 2006; Sheng et al., 2019).

One of the main molecular defects defined for zebrafish hai1a is the removal of adherens junction proteins from the membrane (Carney et al., 2007). MAPK signalling has been shown to reduce E-cadherin expression at adherens junctions and promote cytoplasmic accumulation through phosphorylation of the effector, RSK (Časlavský et al., 2013). Like Matriptase, activation of RSK2 is associated with tumour progression, 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 activated RSK1, which promotes invasion of melanoma clinically as well as in vitro and zebrafish melanoma models (Salhi et al., 2015). Intriguingly, proximity protein labelling has identified p120-catenin as a target of RSK phosphorylation. This catenin promotes cell-cell adhesion by stabilising cadherins at junctions, a function inhibited by RSK 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; Shi et al., 2018). Thus, we propose that pERK signalling, through RSK members, significantly contributes to dissolution of adherens junctions and the hai1a epidermal phenotype. We observed increased pERK in the cytoplasm and also the nucleus of keratinocytes, with comparatively more nuclear levels in periderm cells. Thus, whilst RSKs are phosphorylated by pERK, it is also likely that other cytoplasmic and also nuclear targets, such as cFos and Ets transcription factors, may also be activated, and that there are underlying transcriptional changes in hai1a mutants. It is not clear why pERK shows slightly different subcellular localisation patterns between the two different epidermal layers, but the two layers do respond differently to ErbB2 inhibition (Scherpis et al., 2018), whilst calcium is recently described to alter nuclear shuttling of pERK (Chuderland et al., 2020).

Our model for how Matriptase invokes cellular responses is highly likely to be incomplete. Indeed, others have indicated MMPs, HB-EGF, EGFR, and AKT and are downstream of Matriptase and PAR2.
function (List et al., 2005; Schepis et al., 2018; Darmoul et al., 2004; Chung et al., 2013; Rattenholl et al., 2007). Furthermore, Matriptase promotes HGF–cMet signalling in mouse (Szabo et al., 2011). We do not think that 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 et al., 2008; van der Merwe et al., 2009). Furthermore, there is evidence that PKC activates both MEK/ERK and EGFR independently following PAR2 stimulation, and that PI3K is activated by PAR2 via Gq (Wang and DeFea, 2006; Al-Ani et al., 2010). Cell identity, subcellular 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

#### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Gene (Danio rerio) | hai1a | GenBank | NM_213152 | =spint1a |
| Gene (Danio rerio) | matriptase1a | GenBank | NM_001040351 | =st14a |
| Gene (Danio rerio) | duox | GenBank | XM_017354273 | =dual oxidase |
| Gene (Danio rerio) | ikbkg | GenBank | NM_001014344 | =ikky =nemo |
| Gene (Danio rerio) | nfkbiaa | GenBank | NM_213184 | =ikbaa |
| Gene (Danio rerio) | rps6ka3a | GenBank | NM_212786 | =RSK2a =p90RSK2a |
| Gene (Danio rerio) | tp63 | GenBank | NM_152986 | =delta Np63 |
| Strain, strain background (Escherichia coli) | Top10 | Invitrogen | C404010 | Chemical competent cells |
| Strain, strain background (Danio rerio) | AB | ZIRC | | Wild-type strain |
| Strain, strain background (Danio rerio) | TL | ZIRC | | Wild-type strain |
| Genetic reagent (Danio rerio) | Tg(mpox:EGFP)p114 | Uni of Sheffield PMID:16926288 | ZFIN ID: ZDB-ALT-070118-2 |
| Genetic reagent (Danio rerio) | Tg(fli1:EGFP)p1 | ZIRC PMID:16671106 | ZFIN ID: ZDB-ALT-011017-8 |
| Genetic reagent (Danio rerio) | hai1a[T266] | Hammerschmidt lab; Max Planck Freiburg PMID:31819976 | ZFIN ID: ZDB-ALT-200618-2 =spint1a[T266] |
| Genetic reagent (Danio rerio) | hai1a[a2217] | Nancy Hopkins lab; Massachusetts Institute of Technology PMID:17728346 | ZFIN ID: ZDB-ALT-040924-4 =spint1a[a2217] |
| Genetic reagent (Danio rerio) | ddf[T251] | Nuesslein-Volhard lab; Max Planck Tuebingen PMID:9007245 | ZFIN ID: ZDB-ALT-980203-1462 =dandruff spint1a[T251] =hai1a[T251] |
| Genetic reagent (Danio rerio) | ddf[y19] | Nuesslein-Volhard lab; Max Planck Tuebingen PMID:9007245 | ZFIN ID: ZDB-ALT-120409-6 =dandruff spint1a[y19] =hai1a[y19] |
| Genetic reagent (Danio rerio) | st14a[y10] | Our lab PMID:31645615 | ZFIN ID: ZDB-ALT-200219-5 |
| Genetic reagent (Danio rerio) | Tg(6xNFkB:EGFP)y1 | Rawls lab PMID:21439961 | ZFIN ID: ZDB-ALT-120409-6 |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Genetic reagent (Danio rerio)     | Tg(krtt1c19e:LY-Tomato)\(^{16}\) | Our lab. Lee et al: PMID:24400120 | ZFIN ID: ZDB-ALT-140424-2 | |
| Genetic reagent (Danio rerio)     | Tg(actb2:GCaMP6s, myl7:mCherry)\(^{2}\) | This paper | | Plasmid from Solnica-Krezel Lab. Injected with Tol2 RNA to make line |
| Antibody                         | Chicken anti-eGFP antibody | Abcam | ab13970, RRID:AB_300798 | 1:500 |
| Antibody                         | Rabbit anti-eGFP | Torrey Pines Biolabs | Tp401, RRID:AB_10013661 | 1:500 |
| Antibody                         | Rabbit anti-FITC | Thermo Fisher Scientific | 71-1900, RRID:AB_2533978 | 1:200 |
| Antibody                         | Rabbit anti-p90RSK (Phospho-Thr\(^{348}\)) | GenScript | A00487 | 1:100 |
| Antibody                         | Rabbit anti-beta catenin | Abcam | ab6302, RRID:AB_305407 | 1:200 |
| Antibody                         | Mouse anti-E-cadherin | BD Biosciences | 610181, RRID:AB_397580 | 1:200 |
| Antibody                         | Mouse anti-Tp63 | Biocare Medical | CM163, RRID:AB_10582730 | 1:200 |
| Antibody                         | Rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr\(^{202}/\text{Tyr}\(^{204}\)) | Cell Signaling Technology | Cat# 4370, RRID:AB_2315112 | 1:100 |
| Antibody                         | Rabbit anti-p44/42 MAPK (Erk1/2) | Cell Signaling Technology | Cat# 9102, RRID:AB_330744 | 1:100 |
| Antibody                         | Alexa Fluor-488 Donkey anti-rabbit | Life Technologies | A21206, RRID:AB_2535792 | 1:700 |
| Antibody                         | Alexa Fluor-647 Donkey anti-rabbit | Life Technologies | A31573, RRID:AB_253618 | 1:700 |
| Antibody                         | Alexa Fluor-546 Donkey anti-mouse | Life Technologies | A10036, RRID:AB_2534012 | 1:700 |
| Antibody                         | Alexa Fluor-488 Goat anti-chicken | Life Technologies | A11039, RRID:AB_2534096 | 1:700 |
| Recombinant DNA reagent          | pCS2+-PKC\(^{\text{d}}\)-GFP | Amaya Lab, Uni of Manchester | PMID:15866160 | For making PKCd-GFP RNA |
| Recombinant DNA reagent          | pT3Ts-Tol2 | Ekker Lab, Mayo Clinic | PMID:17096595 | Addgene Plasmid #31831, RRID:Addgene_31831 |
| Recombinant DNA reagent          | pCS2+-GCaMP6s | Solnica-Krezel Lab, Washington University School of Medicine, St. Louis, MO | | For making GCaMP6s RNA |
| Recombinant DNA reagent          | p(actb2:GCaMP6s, myl7:mCherry) | Solnica-Krezel Lab, Washington University School of Medicine, St. Louis, MO. PMID:28322738 | | For making stable transgenic line |
| Sequence-based reagent           | duox morpholino | GeneTools | PMID:19494811 | 5’-AGTGAATTAGAGAAA TGACCTTTT 3’ (0.4 mM) |
| Sequence-based reagent           | p53 morpholino | GeneTools | PMID:19494811 | 5’-GGGCCATTGCTTTGCA AGAATTG 3’ (0.2 mM) |
| Sequence-based reagent           | Oligo(dT)12–18 Primer | Invitrogen | PMID:18418012 | |
| Sequence-based reagent           | nfkbiaa | This paper | PCR primers | F-5’-AGACGCACAAAGGAGC AGTGTAG 3’ R- 5’-TGTTGTCTGCAGCA AGGTC 3’ |

*Continued on next page*
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Sequence-based reagent            | ee1a111     | This paper          | PCR primers | F'-5' CTGGAGGCCAGC TCAAAACAT 3' R'-5' ATCAAGAAGAGTAGT ACGGTACGATTAC 3' |
| Sequence-based reagent            | rps6ka3a    | This paper          | PCR primers for cloning probe | F'-5' ATACTCCAGTCCC ACGGA 3' R'- 5'TGGTGATGATGTT AGACTCGC 3' |
| Peptide, recombinant protein      | Proteinase K | Thermo Scientific   | EO0491      | 0.5 µg/µl              |
| Commercial assay or kit           | SuperScript III | Invitrogen         | 18080093    |                        |
| Commercial assay or kit           | TRizol Reagent | Invitrogen         | 15596026    |                        |
| Commercial assay or kit           | GoTaq G2 Green Master Mix | Promega         | M7823       | Functions used: TrackMate Reslice Average Intensity |
| Commercial assay or kit           | iTaq Universal SYBR Green Supermix | Bio-Rad      | 1725121    | Functions used: Spot   |
| Commercial assay or kit           | mMESSAGE mMACHINE SP6 Transcription Kit | Invitrogen  | AM1340      | Tests: Student's t-test, Chi-squared test, Mann–Whitney test, ANOVA with Bonferroni or Dunn’s post-tests |
| Commercial assay or kit           | mMESSAGE mMACHINE T3 Transcription Kit | Invitrogen  | AM1348      |                        |
| Commercial assay or kit           | MEGAShortscript T7 Transcription Kit | Invitrogen  | AM1350      |                        |
| Commercial assay or kit           | pGEM-T Easy | Promega             | A137A       |                        |
| Commercial assay or kit           | pCR 2.1-TOPO TA vector | Invitrogen      | K450040     |                        |
| Commercial assay or kit           | QIAquick PCR Purification Kit  | Qiagen        | 28104       |                        |
| Commercial assay or kit           | DIG RNA Labeling Kit   | Roche          | 11175025910 |                        |
| Commercial assay or kit           | SP6 RNA Polymerase     | Roche          | 10 810 274 001 |                        |
| Commercial assay or kit           | NBT/BCIP Stock Solution | Roche         | 11681451001 |                        |
| Chemical compound, drug           | Diphenyleneiodonium chloride | Sigma-Aldrich  | D2926       | 40 µM                  |
| Chemical compound, drug           | Thapsigargin         | Sigma-Aldrich   | T9033       | 6.25 µM                |
| Chemical compound, drug           | Bisindolylmaleimide I (GF109203X) | Selleckchem   | S7208       | 85 µM                  |
| Chemical compound, drug           | YM-254890          | FocusBiomolecules | 10-1590-0100 | 32 µM            |
| Chemical compound, drug           | 2-Aminoethyl diphenylborinate | Sigma-Aldrich  | D9754       | 2.5 µM                |
| Chemical compound, drug           | BI-D1870           | Axon Medchem    | Axon-1528   | 1.2 µM                |

Continued on next page
| Reagent type (species) or resource | Designation                          | Source or reference         | Identifiers | Additional information |
|-----------------------------------|--------------------------------------|-----------------------------|-------------|------------------------|
| Chemical compound, drug          | Dimethyl fumarate                    | Sigma-Aldrich               | 242926      | 9 µM                   |
| Chemical compound, drug          | Phorbol 12-myristate 13-acetate       | Sigma-Aldrich               | P8139       | 37.5 or 125 ng/ml      |
| Chemical compound, drug          | U0126                                | Cell Signaling Technology   | 9903        | 100 µM                 |
| Chemical compound, drug          | PD184352 (CI-1040)                   | Selleckchem                 | S1020       | 1.3 µM                 |
| Chemical compound, drug          | DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) | Invitrogen                  | D1306       | 5 µg/ml                |
| Chemical compound, drug          | Penta-fluorobenzenesulfonyl fluorescein | Cayman Chemicals           | 10005983    | 12.5 µM                |
| Chemical compound, drug          | Fluorescein isothiocyanate–dextran   | Sigma-Aldrich               | FD4         | 2.5 mg/ml              |
| Software, algorithm              | Fiji (ImageJ 1.52p)                  | NIH                         |             | Functions used: TrackMate Reslice Average Intensity |
| Software, algorithm              | Imaris 9.6.0                         | Oxford Instruments         |             | Functions used: Spot   |
| Software, algorithm              | Prism 9.1.1                          | GraphPad                    |             | Tests: Student’s t-test, Chi-squared test, Mann–Whitney test, ANOVA with Bonferroni or Dunn’s post-tests |
| Software, algorithm              | Photoshop 22.1.1 release             | Adobe                       |             |                        |

**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 crossings and staged as per Kimmel et al., 1995 and raised in 0.5 x E2 medium (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM MgSO4, 75 µM KH2PO4, 25 µM Na2HPO4, 0.5 M CaCl2, 0.35 mM NaHCO3). Anaesthesia was administered in E2 medium (embryos) or fish tank water (adults) using 0.02% pH 7.0 buffered Tricaine MS-222 (Sigma). The hai1a/ddf alleles used were hai1a<sup>h2217</sup>, hai1a<sup>h26</sup>, ddf<sup>p251</sup>, and ddf<sup>p419</sup>. The st14a<sup>qt10</sup> allele was generated previously (Lin et al., 2019). For imaging neutrophils and keratinocytes, the transgenic lines Tg (mpx:EGFP)<sup>ip114</sup> (Renshaw et al., 2006) and Tg(krtt1c19e:lyn-tomato)<sup>ip16</sup> (Lee et al., 2014) were used, whilst early leukocytes were imaged with Tg(fli1:EGFP)<sup>y1</sup> (Redd et al., 2006). To image NfkB pathway activity, the Tg(6xHsa.NFKB:EGFP)<sup>nc1</sup> sensor line was used (Kanther et al., 2011). Calcium imaging was performed by injection of GCaMP6s RNA (see below) or using a Tg(actb2:GCaMP6s, myl7::Cherry)<sup>kc2</sup> stable transgenic line, generated via plasmid (Chen et al., 2017) and Tol2 RNA co-injection.

**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 4 hr in Lysis buffer (10 mM Tris pH 8.3, 50 mM KCI, 0.3% Tween20, 0.3% Nonidet P-40, 0.5 µg/µl Proteinase K). PCRs were performed using GoTaq (Promega) on a Veriti thermal cycler (Applied Biosystems) and purified with a PCR purification kit (Qiagen). TRizol (Invitrogen) was used for RNA extraction following provided protocol, and cDNA generated from 1 µg total RNA.
Figure 9. 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), 1.3 μM CI-1040 (B), or 100 μM U0126 (D, F) showing rescue of general morphology (B), trunk (D), and tail (F) epidermal phenotypes compared to DMSO-treated hai1a<sup>h2217</sup>. Epidermal aggregates under the yolk are reduced in the treated mutants (A–D; arrowheads). (G) Proportion of 48hpf larvae derived from hai1a<sup>+/h2217</sup> incross showing mild or severe hai1a epidermal phenotype following DMSO (upper) or U0126 (lower) treatment (Chi-squared test). (H, I) Lateral DIC images of 24hpf embryo treated with 125 ng/ml phorbol 12-myristate 13-acetate (PMA) (H) or PMA and U0126 (I). Yolk-associated epidermal aggregates are reduced. (J–M) Lateral projected confocal images of hai1a<sup>h2217</sup>; Tg(mp1: eGFP)<sup>i114</sup> trunk at 24hpf (J, K) and tail at 48hpf (L, M), either treated with 0.5% DMSO (J, L) or U0126 (K, M). Embryos are immunostained for TP63 (magenta) and eGFP (green), highlighting rescue of epidermal phenotype but no reduction of neutrophils. (N, O) Lateral projected confocal images of Tg(mp1:eGFP)<sup>i114</sup> treated with PMA alone (N) or PMA with U0126 (O) and immunostained for TP63 (magenta) and eGFP (green). Fin morphology is Figure 9 continued on next page
using SuperScript III Reverse Transcriptase (Invitrogen) with Oligo(dT)12-18 primer. 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 \( \text{nfkbiaa} \) mRNA by qPCR, the following primers (5\(^{\prime}\) to 3\(^{\prime}\)) were used to amplify a region encoded on exons 4 and 5: F-AGACGCAAAGGAGCAGTGTAG, R-TGTGTGTCTGCCGAAGGTC. Reference gene was \( \text{eef1a1l1} \) and the primers used amplified between exon 3 to 4: F-CTGGAGGCCAGCTCAAACAT, R- ATCAAGAAGAGTAGTACCGCTAGCA TTAC.

RNA synthesis

RNAs for GCaMP6s and \( \text{PKC}^{\beta}-\text{GFP} \) were synthesised from pCS2-based plasmids containing the respective coding sequences (Sivak et al., 2005; Chen et al., 2017). These were linearised with NotI (NEB), and RNA in vitro transcribed with mMESSAGE mMACHINE SP6 Transcription Kit (Ambion). RNA for Tol2 was generated from the pT3Ts-Tol2 plasmid, linearised with SmaI (NEB), and transcribed with the mMESSAGE mMACHINE T3 Transcription Kit (Ambion). RNA for injection was purified by lithium chloride precipitation.

Embryo injection and morpholino

Embryos were aligned on an agarose plate and injected at the one-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 (0.5 mm inner diameter, Sutter) on a Sutter P-97 micropipette puller. The Duox morpholino (AGTGAATTAGAGAAATGCACCTTTT) was purchased from GeneTools and injected at 0.4 mM with 0.2 mM of the tp53 morpholino (GCGCCA TTGCTTTGCAAGAATTG).

TALEN mutagenesis

To generate the \( \text{ikbkg} \) mutant, TALEN vectors targeting the sequence ATGGAGGGCTGG in second exon were designed and constructed by ToolGen (http://toolgen.com). TALEN vectors were linearised with PvuII (NEB) and purified using a PCR purification kit (Qiagen), and then used for in vitro transcription with the MEGAshortscript T7 kit (Ambion). About 170–300 pg of supplied ZFN RNAs or purified TALEN RNAs were then injected into one-cell stage WT zebrafish embryos, which were raised to 24 hr, 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 0.5× E2 Embryo Medium and embryos treated by immersion. The compounds, and concentrations used, with catalogue numbers were diphenyleneiodonium chloride (DPI), 40 \( \mu \text{M} \) (D2926, Sigma); thapsigargin, 6.25 \( \mu \text{M} \) (T9033, Sigma); bisindolylmaleimide I (GF109203X), 85 \( \mu \text{M} \) (S7208, Selleckchem); YM-254890, 32 \( \mu \text{M} \) (S2866, Selleckchem).
Figure 10. Altered RSK status in hai1a hi2217 accounts for epidermal defects. (A, B) In situ hybridisation of rps6ka3a at 24hpf under low- (A) and high-power (B) magnification showing expression in basal keratinocytes. Open arrowheads in (B) indicate borders of EVL cells bisecting nuclei of underlying rps6ka3a-positive cells. (C–H) Lateral projected confocal images of the tails of embryos immunostained for p90RSK (Phospho-Thr348) (C–H) and TP63 (C–H). In both the hai1a hi2217 (D, D') and 125 ng/ml phorbol 12-myristate 13-acetate (PMA)-treated (G, G') embryos, there is an increase in cytoplasmic levels of p90RSK (Phospho-Thr348) signal above the nuclear only signal seen in WT (C, C') or DMSO (F, F'). Treatment with the pERK inhibitor U0126 reduced cytoplasmic levels but did not affect nuclear signal (E, E'; H, H'), (I, J) Quantification of immunofluorescent intensity of cytoplasmic levels of p90RSK (Phospho-Thr348) in basal keratinocytes of tails of 48hpf WT and hai1a hi2217, treated with DMSO or U0126 (I), and PMA or PMA plus U0126 (J). Nucleus signal was excluded by masking from the DAPI channel. n = 5; t-test; ***p<0.001, **p<0.01, *p<0.05. (K–N) Lateral DIC images of hai1a hi2217 embryos at 24hpf (K, L) and 48hpf (M, N) untreated (K, M) or treated with 1.2 μM BI-D1870 (L) or 9 μM dimethyl fumarate (DMF). Locations of epidermal aggregates and loss of tail fin morphology in hai1a mutants, and their rescue by RSK inhibitor treatment are indicated by arrowheads. (O–Q) Lateral projected confocal images of the tails of embryos immunostained with antibodies against E-cadherin and TP63 in WT (O, P) and hai1a hi2217 treated with DMF (Q). (O'–Q') Profile plots of fluorescence distribution across cells of WT (O), hai1a hi2217 (P'), and hai1a hi2217 treated with DMF (Q'). X-axis represents width of the cell. β-Catenin immunofluorescence intensity (Y-axis) shows majority at cell edge (E-cadherin domain demarcated in light purple) and centre of cell (nucleus demarcated in light green) in WT and rescued hai1a mutants, but there is no clear membrane signal in the untreated hai1a mutants. Two cells per five larvae were analysed. Scale bars: (A, K, N) = 100 μM; (B, H) p=20 μM.

The online version of this article includes the following figure supplement(s) for figure 10:

Figure supplement 1. RSK inhibitors rescue the hai1a phenotype.
Proteomic analysis

Batches of 100 WT, ddf^{419} and ddf^{251} embryos were collected at 24 hr and 48 hr, 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 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 and 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), permeabilised in –20°C acetone for 7 min, washed in PBT,
blocked for 3 hr 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, Thermo Fisher), Rabbit anti-beta catenin, 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), and Rabbit anti-p90RSK (Phospho-Thr348), 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 (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 60 min at room temperature with 12.5 μM PFBSF (#10005983, Cayman Chemicals), then rinsed in Embryo Medium, anaesthetised, 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. Epidermal permeability assays were conducted by immersing 36hpf embryos in 2.5 mg/ml fluorescein isothiocyanate-dextran 3–5 kDa (Sigma) or 0.075% methylene blue for 30 min and then destained in E2 medium.

**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 1 mm inner diameter capillary for light-sheet time-lapse. When imaging was performed on live embryos, the embryo media were supplemented with buffered 0.02% Tricaine and imaging conducted at 25°C. Image processing was done using Zen 3.1 software (Zeiss), Fiji (ImageJ, ver. 1.52p), or Imaris (Bitplane) and compiled using Photoshop 2020 (Adobe). Neutrophils were tracked with TrackMate in Fiji or using the Spot function in Imaris. Kymographs were generated using the Reslice function in Fiji following generation of a line of interest across image. Fluorescence intensities were calculated using the Average Intensity function in Fiji following generation of a Region of Interest and masking of the DAPI channel to exclude the nucleus when required. In statistical analyses, n = number of embryos or cells measured, and as defined in the figure legend. GraphPad Prism was used for statistical analyses and graph generation. In all statistical tests, *p<0.05, **p<0.01, ***p<0.001. Tests used are indicated in the associated figure legend and were Student’s t-test, Chi-squared test, Mann–Whitney test, or ANOVA with Bonferroni or Dunn’s post-tests.

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Author ORCIDs
Tom J Carney  https://orcid.org/0000-0003-2371-1924

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Animal experimentation: 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. Approval was provided by the Institutional Animal Care and Use Committees of the Biological Resource Centre (IMCB) and NTU according to Agri-Food and Veterinary Authority (AVA) Rules and the National Advisory Committee for Laboratory Animal Research (NAACLAR) requisirments.

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Additional files
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References
Al-Ani B, Hewett PW, Cudmore MJ, Fujisawa T, Saifeddine M, Williams H, Ramma W, Sissaoui S, Jayaraman PS, Ohba M, Ahmad S, Hollenberg MD, Ahmed A. 2010. Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells. *Hypertension* **55**:689–697. DOI: https://doi.org/10.1161/HYPERTENSIONAHA.109.136333, PMID: 20124108

Allen LF, Sebott-Leopold J, Meyer MB. 2003. CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). *Seminars in Oncology* **30**:105–116. DOI: https://doi.org/10.1053/j.seminoncol.2003.08.012, PMID: 14613031

Alli Shaik A, Wee S, Li RH, Li Z, Carney TJ, Mathavan S, Gunaratne J. 2014. Functional mapping of the zebrafish early embryo proteome and transcriptome. *Journal of Proteome Research* **13**:5536–5550. DOI: https://doi.org/10.1021/pr5005136, PMID: 25230361

Anastasaki C, Rauen KA, Patton EE. 2012. Continual low-level MEK inhibition ameliorates cardio-facio-cutaneous phenotypes in zebrafish. *Disease Models & Mechanisms* **5**:546–552. DOI: https://doi.org/10.1242/dmm.008672, PMID: 22301711

Andersen JL, Gesser B, Funder ED, Nielsen CJF, Gottfred-Rasmussen H, Rasmussen MK, Toth R, Gothelf KV, Arthur JSC, Iversen L, Nissen P. 2018. Dimethyl fumarate is an allosteric covalent inhibitor of the p90 ribosomal S6 kinases. *Nature Communications* **9**:4344. DOI: https://doi.org/10.1038/s41467-018-06877-w, PMID: 30341347

Armistead J, Hatzold J, van Roye A, Fahle E, Hammerschmidt M. 2020. Entosis and apical cell extrusion constitute a tumor-suppressive mechanism downstream of matrilapse. *Journal of Cell Biology* **219**:e201905190. DOI: https://doi.org/10.1083/jcb.201905190

Böhm SK, Khitin LM, Grady EF, Aponte G, Payan DG, Bunnell NW. 1996. Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *Journal of Biological Chemistry* **271**:22003–22016. DOI: https://doi.org/10.1074/jbc.271.36.22003, PMID: 8703006
Carney TJ, van der Hardt S, Sonntag C, Amsterdam A, Topczewski J, Hopkins N, Hammerschmidt M. 2007. Inactivation of serine protease Matriptase1a by its inhibitor Hai1 is required for epithelial integrity of the zebrafish epidermis. Development 134:3461–3471. DOI: https://doi.org/10.1242.dev.004556, PMID: 17728346

Čáslavský J, Klimová Z, Vomastek T. 2013. ERK and RSK regulate distinct steps of a cellular program that induces transition from multicellular epithelium to single cell phenotype. Cellul. Signalling 25:2743–2751. DOI: https://doi.org/10.1016/j.cellsig.2013.08.024, PMID: 24012955

Chen J, Xia L, Bruchas MR, Solnica-Krezel L. 2017. Imaging early embryonic calcium activity with GCaMP6s transgenic zebrafish. Developmental Biology 430:385–396. DOI: https://doi.org/10.1016/j.ydbio.2017.03.010, PMID: 28322738

Chou CM, Chen YC, Su S, Chen GD, Huang KY, Lien HW, Huang CJ, Cheng CH. 2015. Activation of MEK2 is sufficient to induce skin papilloma formation in transgenic zebrafish. Journal of Biomedical Science 22:102. DOI: https://doi.org/10.1186/s12929-015-0207-2, PMID: 26572230

Chuderland D, Marmor G, Shainskaya A, Seger R. 2020. Calcium-Mediated interactions regulate the subcellular localization of extracellular Signal-Regulated kinases (ERKs). Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Pathology, Biochemistry, and Pharmacology 54:474–492. DOI: https://doi.org/10.33594/000000231, PMID: 32392404

Chung H, Ramachandran R, Hollenberg MD, Muruve DA. 2013. Proteinase-activated receptor-2 transactivation of epidermal growth factor receptor and transforming growth factor-β receptor signaling pathways contributes to renal fibrosis. Journal of Biological Chemistry 288:37319–37331. DOI: https://doi.org/10.1074/jbc.M113.492793, PMID: 24253040

Darmoul D, Gratio V, Devaud H, Laburthe M. 2004. Protease-activated receptor 2 in Colon cancer: trypsin-induced MAPK phosphorylation and cell proliferation are mediated by epidermal growth factor receptor transactivation. The Journal of Biological Chemistry 279:20927–20934. DOI: https://doi.org/10.1074/jbc.M401430200, PMID: 15010475

de Oliveira S, López-Muñoz A, Candel S, Pelegrin P, Calado Â, Mulero V. 2014. ATP modulates acute inflammation in vivo through dual oxidase 1-derived H2O2 production and NF-kB activation. Journal of Immunology 192:5710–5719. DOI: https://doi.org/10.4049/jimmunol.1302902, PMID: 24842759

Enyedi B, Niethammer P. 2015. Mechanisms of epithelial wound detection. Trends in Cell Biology 25:398–407. DOI: https://doi.org/10.1016/j.tcb.2015.02.007, PMID: 25813429

Favata MF, Horuchi KY, Manos EJ, Dauerlo AJ, Stradley DA, Feerer WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. Journal of Biological Chemistry 273:18623–18632. DOI: https://doi.org/10.1074/jbc.273.29.18623, PMID: 9660836

Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. 2010. Live imaging of innate immune cell sensing of transformed cells in zebrafish larva: parallels between tumor initiation and wound inflammation. PLOS Biology 8:e1000562. DOI: https://doi.org/10.1371/journal.pbio.1000562, PMID: 21179501

Gault WJ, Enyedi B, Niethammer P. 2014. Osmotic surveillance mediates rapid wound closure through nucleotide release. Journal of Cell Biology 207:767–782. DOI: https://doi.org/10.1083/jcb.201408049, PMID: 25533845

Gawecka JE, Young-Robbins SS, Sulzmaier FJ, Caliva MJ, Heikillé MM, Matter ML, Ramos JW. 2012. RSK2 protein suppresses integrin activation and fibronectin matrix assembly and promotes cell migration. Journal of Biological Chemistry 287:43424–43437. DOI: https://doi.org/10.1074/jbc.M112.423046, PMID: 23118220

Goon Goh F, Sloss CM, Cunningham MR, Nilsson M, Cadalbert L, Plevin R. 2008. G-protein-dependent and -independent pathways regulate proteinase-activated receptor-2 mediated p65 NF kappa B activation. Journal of Biological Chemistry 283:18623–18632. DOI: https://doi.org/10.1074/jbc.273.29.18623, PMID: 18320872

Hatzold J, Beleggia F, Herzig H, Altmüller J, Nürnberg P, Bloch W, Wollnik B, Hammerschmidt M. 2016. Tumor suppression in basal keratinocytes via dual non-cell-autonomous functions of a Na,K-ATPase beta subunit. eLife 5:e4277. DOI: https://doi.org/10.7554/eLife.4277, PMID: 27240166

Jiang X, Bailly MA, Panetti TS, Cappello M, Konigsberg WH, Bromberg ME. 2004. Formation of tissue factor-VIIa-factor xa complex promotes cellular signaling and migration of human breast Cancer cells. Journal of Thrombosis and Haemostasis 2:93–101. DOI: https://doi.org/10.1111/j.1538-7836.2004.00545.x, PMID: 14717972

Kang S, Elf S, Lythgoe K, Hitosugi T, Taunton J, Zhou W, Xiong L, Wang D, Muller S, Fan S, Sun SY, Gu TL, Chen ZG, Shin DM, Hurlstone A, Hitosugi T, Taunton J, Zhou W, Xiong L, Wang D, Muller S, Fan S, Sun SY, Gu TL, Chen ZG, Shin DM, Chen J. 2010. p90 ribosomal S6 kinase 2 promotes invasion and metastasis of human renal fibrosis. Journal of Clinical Investigation 120:1165–1177. DOI: https://doi.org/10.1172/JCI45082, PMID: 20234090

Kanke T, Macfarlane SR, Seattter MJ, Davenport E, Paul A, McKenzie RC, Plevin R. 2001. Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory kappa B kinases in NCTC 2544 keratinocytes. Journal of Biological Chemistry 276:31657–31666. DOI: https://doi.org/10.1074/jbc.M103077200, PMID: 11413129

Kanther M, Sun X, Mühlbauer M, Mackey LC, Flynn EJ, Bagnat M, Jobin C, Rawls JF. 2011. Microbial colonization induces dynamic temporal and spatial patterns of NF-κB activation in the zebrafish digestive tract. Gastroenterology 141:197–207. DOI: https://doi.org/10.1053/j.gastro.2011.03.042, PMID: 21439961

Kawaguchi M, Takeda N, Hoshiko S, Yorita K, Baba T, Sawaguchi A, Nezu Y, Yoshikawa T, Fukushima T, Kataoka H. 2011. Membrane-bound serine protease inhibitor HA1-1 is required for maintenance of intestinal epithelial
integral. The American Journal of Pathology 179:1815–1826. DOI: https://doi.org/10.1016/j.ajpath.2011.06.038, PMID: 21840293

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Developmental Dynamics 203:253–310. DOI: https://doi.org/10.1002/aja.1002030302, PMID: 8589427

Lee RT, Asharani PV, Carney TJ. 2014. Basal keratinocytes contribute to all strata of the adult zebrafish epidermis. PLOS ONE 9:e84858. DOI: https://doi.org/10.1371/journal.pone.0084858, PMID: 24400120

Lin Q., Low LWL, Lau A., Chua EWL, Matsuoka Y., Lian Y., Monteiro A., Tate S., Gunaratne J., Carney TJ. 2019. Tracking genome-editing and associated molecular perturbations by SWATH mass spectrometry. Scientific Reports 9:15240. DOI: https://doi.org/10.1038/s41598-019-51612-z, PMID: 31645615

List K., Szabo R., Molinolo A., Suriarapong V., Redey V., Murdock T., Burke B., Nielsen BS., Gutkind JS., Bugge TH. 2005. Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. Genes & Development 19:1934–1950. DOI: https://doi.org/10.1101/gad.1300705, PMID: 16103220

Macfarlane SR, Sloss CM, Cameron P., Kanke T., McKenzie RC, Pleavin R. 2005. The role of intracellular Ca2+ in the regulation of proteinase-activated receptor-2 mediated nuclear factor kappa B signalling in keratinocytes. British Journal of Pharmacology 145:535–544. DOI: https://doi.org/10.1038/sj.bjp.0706204, PMID: 15821758

Maeda H., Fukuysu Y., Yoshida S., Fukuda M., Saeki K., Matsuho N., Yamauchi Y., Yoshida K., Hirata K., Miyamoto K. 2004. Fluorescent probes for hydrogen peroxide based on a non-oxidative mechanism. Angewandte Chemie International Edition 43:2389–2391. DOI: https://doi.org/10.1002/anie.200405238, PMID: 15114569

Méant A., Gao B., Lavoie G., Nourredrine S., Jung F., Aubert L., Tcherkezian J., Gingras AC, Roux PP. 2020. Proteomic analysis reveals a role for RSK in p120-catenin phosphorylation and melanoma Cell-Cell adhesion. Molecular & Cellular Proteomics 19:50–64. DOI: https://doi.org/10.1074/mcp.RA119.001811, PMID: 31678930

Morris DR, Ding Y., Ricks TK, Guillapalli A., Wolfe BL, Trejo J. 2006. Protease-Activated Receptor-2 is essential for factor VIIa and Xa-Induced Signaling, Migration, and Invasion of Breast Cancer Cells. Cancer Research 66:307–314. DOI: https://doi.org/10.1158/0008-5472.CAN-05-1735

Nagaike K., Kawaguchi M., Takeda N., Fukushima T., Sawaguchi A., Kohama K., Setoyama M., Kataoka H. 2008. Defect of hepatocyte growth factor activator inhibitor type 1/serine protease inhibitor, Kunitz type 1 (Hai-1/ Spint1) leads to ichthyosis-like condition and abnormal hair development in mice. The American Journal of Pathology 173:1464–1475. DOI: https://doi.org/10.2335/ajpath.2008.071142, PMID: 18832587

Niethammer P., Grabber C., Look AT, Mitchison TJ. 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature 459:996–999. DOI: https://doi.org/10.1038/nature08119, PMID: 19494811

Rattenholl A., Seelig L., Buddenkotte J., Schön M., Schön MP, Ständner S., Vergnolle N., Steinhoff M. 2007. Proteinase-activated receptor-2 (PAR2): a tumor suppressor in skin carcinogenesis. Journal of Investigative Dermatology 127:2245–2252. DOI: https://doi.org/10.1038/sj.jid.5700847, PMID: 17476297

Razzell W., Evans IR, Martin P., Wood W. 2013. Calcium flashes orchestrate the wound inflammatory response through DUOX activation and hydrogen peroxide release. Current Biology 23:424–429. DOI: https://doi.org/10.1016/j.cub.2013.01.058, PMID: 23948344

Redd MJ, Kelly G., Dunn G., Way M., Martin P. 2006. Imaging macrophage chemotaxis in vivo: studies of microtubule function in zebrafish wound inflammation. Cell Motility and the Cytoskeleton 63:415–422. DOI: https://doi.org/10.1002/cm.20133, PMID: 16671106

Renshaw SA, Lounes CA, Truswell DM, Elworthy S., Ingham PW, Whyte MK. 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108:3976–3978. DOI: https://doi.org/10.1182/blood-2006-05-024075, PMID: 16926288

Rigutto S., Hoste C., Grasberger H., Milenkovic M., Communi D., Dumont JE, Corvillain B., Miot F., Dep D. 2009. Activation of dual oxidases Duox1 and Duox2: differential regulation mediated by camp-dependent protein kinase and protein kinase C-dependent phosphorylation. The Journal of Biological Chemistry 284:6725–6734. DOI: https://doi.org/10.1074/jbc.M806893200, PMID: 19144650

Romeo Y., Zhang X., Roux PP. 2012. Regulation and function of the RSK family of protein kinases. Biochemical Journal 441:553–569. DOI: https://doi.org/10.1042/BJ20110289, PMID: 22187936

Rothwarf DM, Zandi E., Natoli G., Karin M. 1998. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature 395:297–300. DOI: https://doi.org/10.1038/26261, PMID: 9751060

Sales KU, Friis S, Konkel JE, Godilksen S, Hatakeyama M., Hansen KK, Rogatto SR, Szabo R, Vogel LK, Chen W., Gutkind JS, Bugge TH. 2015. Non-hematopoietic PAR-2 is essential for matriptase-driven pre-malignant progression and potentiation of ras-mediated squamous cell carcinogenesis. Oncogene 34:346–356. DOI: https://doi.org/10.1038/onc.2013.563, PMID: 24469043

Salhi A., Farhadian JA, Giles KM, Vega-Saenz de Miera E., Silva IP, Bourque C., Yeh K., Chhangawala S., Wang J., Ye F., Zhang DY, Hernando-Monge E., Houwras Y., Osman I. 2015. RSK1 activation promotes invasion in nodular
Ma et al. eLife 2021;10:e66596. DOI: https://doi.org/10.7554/eLife.66596