The ENTH domain protein Clint1 is required for epidermal homeostasis in zebrafish

M. Ernest Dodd1, Julia Hatzold2, Jonathan R. Mathias1, Kevin B. Walters3, David A. Bennin5, Jennifer Rhodes4, John P. Kanki4, A. Thomas Look3, Matthias Hammerschmidt2 and Anna Huttenlocher1,3,5,*

Epidermal hyperproliferation and inflammation are hallmarks of the human condition psoriasis. Here, we report that a zebrafish line with a mutation in the cargo adaptor protein Clint1 exhibits psoriasis-like phenotypes including epithelial hyperproliferation and leukocyte infiltration. Clint1 is an ENTH domain-containing protein that binds SNARE proteins and functions in vesicle trafficking; however, its in vivo function in animal models has not been reported to date. The clint1 mutants exhibit chronic inflammation characterized by increased Interleukin 1β expression, leukocyte infiltration, bidirectional trafficking and phagocytosis of cellular debris. The defects in clint1 mutants can be rescued by expression of zebrafish clint1 and can be phenocopied with clint1-specific morpholinos, supporting an essential role for Clint1 in epidermal development. Interaction studies suggest that Clint1 and lethal giant larvae 2 function synergistically to regulate epidermal homeostasis. Accordingly, clint1 mutants show impaired hemidesmosome formation, loss of cell-cell contacts and increased motility suggestive of epithelial to mesenchymal transition. Taken together, our findings describe a novel function for the ENTH domain protein Clint1 in epidermal development and inflammation and suggest that its deficiency in zebrafish generates a phenotype that resembles the human condition psoriasis.

KEY WORDS: Epidermis, Zebrafish, Inflammation, Hemidesmosome

INTRODUCTION

The epidermis is a stratified epithelium that provides a crucial barrier against pathogens and other environmental insults. An essential function of the epidermis is to respond to physical insults with repair mechanisms that include wound healing characterized by regulated epithelial proliferation and leukocyte infiltration. A hallmark of epithelial disorders, including psoriasis and some epidermal cancers, is uncontrolled epithelial proliferation and inflammation. Although a genetic predisposition contributes to the development of psoriasis and other epidermal conditions, the precise molecular mechanisms that lead to the development of these heterogeneous disorders remain unknown and are likely to be multifactorial. Therefore it is essential to develop model systems to dissect pathways that regulate epidermal homeostasis and inflammation.

Recent studies indicate that the zebrafish represents a powerful model system with which to study mechanisms of epidermal development. The identification in zebrafish of homologs of human genes that are involved in epidermal tissues, such as p63 (also known as tp63 – ZFIN) (Bakkers et al., 2002; Lee and Kimelman, 2002), rbp4 (Tingaud-Sequeira et al., 2006) and keratin isoforms (Imboden et al., 1997; Chua and Lim, 2000; Martorana et al., 2001; Thissen and Thissen, 2004), suggests that genetic conservation exists between zebrafish and mammals. Furthermore, the identification of mutants such as hai1 (spint1) (Carney et al., 2007; Mathias et al., 2007), penner (lgl2) (Sonawane et al., 2005), lama5 (Webb et al., 2007) and psoriasis (Webb et al., 2008) is providing more clues as to the genetic program of epidermal development in zebrafish.

The epidermis in developing zebrafish embryos contains two layers: a basal layer and a superficial layer of keratinocytes (Le Guèvrec et al., 2004). Adhesive structures that anchor the basal layer to the underlying basement membrane or mediate cell-cell contacts are essential for the integrity of epithelial tissues. A recent study reported that zebrafish lethal giant larvae 2 (Lgl2; Lgl2) functions in the formation and maintenance of hemidesmosomes, and deficiency of Lgl2 results in loss of epidermal integrity in zebrafish (Sonawane et al., 2005). Additionally, the recently reported mutants hai1 and lama5 demonstrate abnormal cell-cell contacts and impaired epidermal integrity (Carney et al., 2007; Webb et al., 2007).

The zebrafish also provides a powerful model with which to study inflammation and leukocyte trafficking. The innate immune system is highly conserved in zebrafish (de Jong and Zon, 2005; Carradice and Lieschke, 2008), and they are used to study host-pathogen interactions (Davis et al., 2002; van der Sar et al., 2003; Pressley et al., 2005). Models of acute inflammation have been described using transgenic zebrafish that allow high-resolution observation of leukocyte recruitment (Mathias et al., 2006; Renshaw et al., 2006; Hall et al., 2007; Meijer et al., 2008). These reports demonstrate that acute inflammation in zebrafish involves the active recruitment of neutrophils into tissues, which can be resolved by reverse chemotaxis (Mathias et al., 2006).

To identify genes involved in inflammation, we screened a collection of zebrafish mutants for abnormal infiltration of neutrophils into the fin. Here we describe the hit1520 line that was identified in this screen and carries an insertion in the clathrin interactor 1 (clint1, also known as enthoptin and epsinR) locus. Clint1 is an adaptor molecule in clathrin-mediated vesicular transport that binds membrane and clathrin coat components as well as cargo proteins, such as the vesicle-associated SNARE proteins.

1Department of Medical Microbiology and Immunology, University of Wisconsin–Madison, Madison, WI 53706, USA. 2Institute for Developmental Biology, Center for Molecular Medicine Cologne, and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, University of Cologne, Cologne, Germany. 3Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Madison, WI 53706, USA. 4Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA. 5Department of Pediatrics, University of Wisconsin-Madison, Madison, WI 53706, USA.

*Author for correspondence (e-mail: huttenlocher@wisc.edu)

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(v-SNAREs) involved in the vesicular fusion machinery (Kalthoff et al., 2002; Wasiak et al., 2002; Mills et al., 2003; Miller et al., 2007). However, previous studies have not addressed the function of Clint1 during the development of multicellular organisms.

Here we describe a novel function for Clint1 in epidermal development in zebrafish. clint1 mutants exhibit epidermal aggregation, hyperproliferation and inflammation. Leukocytes display phagocytosis of cellular debris and robust bidirectional trafficking between epidermal tissues and the vasculature. Rescue and knockdown experiments confirm the contribution of clint1 to these phenotypes. Furthermore, interaction studies suggest that Clint1 functions synergistically with Lgl2 to regulate epidermal homeostasis, and electron micrographs of clint1 mutants demonstrate defects in hemidesmosome formation. Time-lapse microscopy demonstrates both a loss of cell-cell contact and increased motility of clint1-deficient epidermal cells, suggestive of epithelial to mesenchymal transition (EMT). Taken together, our findings describe a novel function for the ENTH domain protein Clint1 in epidermal development and suggest that its deficiency generates a phenotype in zebrafish that resembles the human condition psoriasis.

**MATERIALS AND METHODS**

**Zebrafish maintenance**

All zebrafish protocols were approved by the University of Wisconsin-Madison Research Animal Resources Center. Adult AB zebrafish and embryos were maintained (Nueslein-Volhard and Dahm, 2002) and staged (Kimmel et al., 1995) as established. Wounding was performed as described (Mathias et al., 2006). The clint1 allele hi1520 was isolated in an insertional mutagenesis screen (Amsterdam et al., 1999) and is maintained in heterozygous adults. hi1520 adults were crossed to Tg(ef1a:GFP)ex11032 (Mathias et al., 2006) adults to yield clint1::GFP embryos. The Tg(β-actin·hras·GFP) allele vu118 line was described previously (Cooper et al., 2005).

**RNA isolation and RT-PCR**

Total RNA was isolated from single or pooled embryos using STAT-60 (Tel-Test). mRNA transcripts were detected using a one-step RT-PCR Kit (Qiagen). Primers for detection of levels and alternative splicing of clint1 mRNAs were labeled by whole-mount in situ hybridization (WISH) as described (Hammerschmidt et al., 1996; Bennett et al., 2006). Antibodies and dilutions used were: anti-p63 (1:300, Novus Biologicals), anti-BrdU (1:100,Sigma), anti-β-actin (1:200, Santa Cruz SC-216), FITC donkey anti-rabbit IgG (1:300, Jackson Immunoresearch), Rhodamine Red X anti-mouse IgG (1:500, Molecular Probes), Rhodamine Red X goat anti-mouse Fab fragment (1:300, Jackson Immunoresearch), Cy3 anti-mouse IgG (1:200, Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (1:200, Invitrogen). DAPI staining was performed with a 1:1000 dilution of DAPI (1 mg/ml) in PBS. BrdU incorporation (Sonawane et al., 2005), Mpo/L-plastin double-immunolabeling and Acridine Orange (AO) staining (Walters et al., 2009) were performed as described. Co-immunolabeling of BrdU and p63 was performed as described (Lee and Kimelman, 2002), except that a Rhodamine Red X goat anti-mouse Fab fragment secondary antibody was used. Quantifications of BrdU, AO and Plastin were performed for representative experiments with at least 19 embryos per group. A box was duplicated onto each image in MetaMorph to ensure equivalency of the areas sampled. BrdU and AO signals were scored within the caudal fin fold within the boxed region. Displaced Mpo and L-plastin included all signals outside of the caudal hematopoietic tissue. Statistical analysis was performed in Prism (GraphPad) using paired two-tailed Student’s t-tests with a 95% confidence interval and significance at P<0.05.

**Zebrafish embryo sectioning**

Unstained embryos or WISH-labeled embryos were fixed in 4% PFA, washed in PBS, suffused in 30% sucrose, embedded in paraffin and sectioned. Hematoxylin and Eosin staining was as described (Hsu et al., 2004). Immunolabeled embryos were post-fixed in 4% PFA, dehydrated through an ethanol series to 100% ethanol, embedded in Durcupan (Sigma) and sectioned.

**Neutrophil tracking**

Neutrophils from clint1::mpo::GFP embryos were tracked and analyzed as described (Mathias et al., 2006). Percentages of time stopped and directionality index (D/T) were calculated as described (Mathias et al., 2007). Data were collected from movies of three mutant embryos before (clint1 mutant) and after (clint1 post-wound) wounding of the caudal fin. For clint1 post-wound cell tracks (n=22 from three fish), data include points from the onset of migration to the arrival at the wound (average duration of 27 minutes). For clint1 mutant cell tracks (n=39 from three fish), data for percentages of time stopped include all points, and directionality data are derived from the first 27 minutes of each time-lapse. Data are presented as averages of three separate experiments.

**Rescue studies**

Zebrafish clint1 cDNA was subcloned into pcDNA3.1 (Invitrogen), and clint1 mRNA was synthesized using mMessage Machine (Ambion). Embryos obtained from hi1520 adults were injected at the 1- to 2-cell stage with 0.5 pl clint1 mRNA (150 or 250 ng/μl) or nuclease-free H2O (control). Statistical analysis was performed in Prism (GraphPad) using one-way ANOVA with Tukey’s multiple comparison post-hoc test with significance at P<0.05.

**Morpholino oligonucleotide microinjection**

Morpholino oligonucleotides (MOs) (GeneTools) were resuspended in 1× Danieau Buffer at a stock concentration of 1 mM. Morpholino oligonucleotide microinjection (MO) volumes and concentrations used were (5′-3′): standard control MO, 0.5 nl of 500 μM (GeneTools); puf1 MO, 1 nl of 1 mM (Rhodes et al., 2005); clint1-atg MO, 0.5 nl of 600 μM (CGCAGCTTCTTCA-CATATCCATAC); clint1-ex1 MO, 0.5 nl of 400 μM (ACATC-CAAAATACATACGCTT); vitil-atg, 0.5 nl of 700 μM (CCGACCATTTCGAGTCAAACTAC); vitil-ex1, 0.5 nl of 250 μM (ATACGAGGTAAATACCTCCTCG). MOs for interaction studies were: 1.0-1.5 nl of both clint1-ex1 (22 μM) and lgil (22 μM); GCCG-CATGCGGGCAATATGCAG. For injection into 8- to 32-cell embryos (see Fig. 5G,H), single cells were injected with 60 pl of cell tracer dye, 10 mg/ml tetramethyl Rhodamine-conjugated dextran (TMRD, Molecular Probes) or 500 μM clint1-atg MO in TMRD.
Expression analysis of clint1 and vti1b

A fusions construct with mCherry (5') obtained from Dr R. Tsien (University of California, San Diego, CA, USA), and clint1 cDNA (3') was cloned into a Tol2 construct (Walters et al., 2009). Tol2/CMV:mCherry-clint1 DNA (25 ng) and transposase mRNA (45 ng) were co-injected at the 1-cell stage in a total volume of 1 nl, and in vivo confocal imaging was performed at 2 days post-fertilization (dfp) with a 60X water immersion objective and a 4.8X digital zoom. The vti1b cDNA clone (accession no. BC055131, ATCC) was subcloned into the egfp-C1 vector (Clontech). Co-expression and imaging of mCherry-clint1 and vti1b-GFP in HEK293 cells were performed as described (Doan and Huttenlocher, 2007).

Cell transplants

One to four cells from Tg(β-actin:kras-eGFP) donors were transplanted into non-transgenic hosts to obtain clusters of GFP-labeled basal keratinocytes as described (Carney et al., 2007). Transplants (wild-type donor into wild-type recipient; clint1 morphant donor into wild-type recipient) were used to assay the autonomy of the clint1 mutant proliferation phenotype, and GFP-positive cells were counted at 2 and 3 dpf. Homotypic transplants (wild-type donor into wild-type recipient; clint1 morphant donor into clint1 morphant recipient) were used to image the behavior of keratinocytes in wild-type or clint1 mutant environments. Recipients were allowed to develop to 36 hours post-fertilization (hpf), mounted and analyzed by time-lapse confocal microscopy as described (Carney et al., 2007). GFP-labeled keratinocytes in wild-type or clint1 mutant environments were tracked using ImageJ (v1.41) and the MTrackJ plug-in. Statistical analysis was performed in Prism (GraphPad) using paired two-tailed Student’s t-tests with significance at P<0.05.

Transmission electron microscopy

Tissues were fixed in 2.5% glutaraldehyde/2% PFA in PBS (0.1 M, pH 7.4) overnight at 4°C, or in 4% PFA overnight at 4°C followed by incubation in 2% glutaraldehyde in PBS for 2 hours at 4°C. Samples were post-fixed in 1% osmium tetroxide in PBS for 2 hours, dehydrated through an ethanol series, further dehydrated in propylene oxide, embedded in Durcupan, sectioned with an ultramicrotome (Leica) and contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol. Ultrathin sections were observed with a Philips CM120 electron microscope, and images were captured with a MegaView III side-mounted digital camera.

RESULTS

An insertion in the zebrafish clint1 locus causes epidermal defects and inflammation

To identify genes that are involved in regulating inflammation, a collection of zebrafish insertional mutants (Amsterdam et al., 1999) was screened at 2.5 dpf by WISH for expression of the neutrophil-specific myeloperoxidase (mpo, also known as mpx) gene (Bennett et al., 2001; Lieschke et al., 2001). Three of 276 lines screened displayed abnormal tissue distributions of neutrophils. Two of these lines, hi2217 and hi1019, have been characterized (Carney et al., 2007; Mathias et al., 2007; Walters et al., 2009), and here we describe the third line, hi1520, with infiltration of neutrophils into the fin (Fig. 1F,F'). The retroviral insertion in the hi1520 line was mapped to the intron between the first two coding exons of the clint1 locus (Fig. 1A) (Amsterdam et al., 2004), and embryos with the hi1520 insertion have alternatively spliced clint1 transcripts due to an exon trap, as previously discussed (Amsterdam and Hopkins, 2004). Embryos homozygous for this insertion (clint1 mutants) have reduced full-length clint1 transcripts and display inflammatory phenotypes with increased expression of the inflammatory cytokine il1b (Fig. 1B). In addition, developmental defects of clint1 mutants include embryonic lethality, smaller body size, pericardial edema, unconsumed yolk and impaired development of the liver and gut (Amsterdam et al., 2004).

The tissue distribution of clint1 was determined by WISH for clint1 mRNA and revealed predominant expression at 36 hpf within the epidermis (Fig. 1C-E), including the fin fold (Fig. 1C,E) and the lateral epidermis covering the trunk (Fig. 1C,D). clint1 expression...
was detected in both p63-positive and p63-negative cells of the epidermis (Fig. 1D,E). Consistent with the epidermal expression of clint1, clint1-deficient mutants displayed an abnormal fin morphology, with epithelial aggregates at 48 hpf (Fig. 1G,G’), allowing the earliest reliable sorting of mutants from wild-type siblings. Further examination of embryos at 48 hpf by transverse paraffin sectioning showed that epidermal aggregates of clint1 mutants protruded from the epidermal sheet, especially in the fin fold (Fig. 1H’J’) and the lateral epidermis covering the trunk and yolk sac extension (Fig. 1H’I’), as compared with control embryos (Fig. 1H-J). Thus, our findings suggest a novel role for Clint1 in regulating epidermal morphology.

**clint1 mutants exhibit epidermal hyperproliferation, cell death and inflammation**

To determine whether epithelial aggregates are associated with cell proliferation, BrdU pulse-labeling was combined with anti-p63 staining to identify basal keratinocytes in control and mutant embryos. Mutants exhibited significantly increased BrdU labeling within both p63-positive and p63-negative cells of the epidermis at 48 hpf (Fig. 2A,A’,D), indicating epithelial hyperproliferation. The p63 staining highlighted morphological abnormalities of mutant cells along the edges of the caudal fin fold; mutants showed flattened, aggregated cells in comparison to the rounded and ordered appearance of these cells in wild-type siblings (Fig. 2A,A’). Proliferating cells were found dispersed throughout the fin fold and within the epidermal aggregates (Fig. 2A’). Similarly, AO staining was increased throughout the mutant fin fold at 48 hpf (Fig. 2B,B’,E), suggesting an increase in epidermal cell death. A time course showed significant elevations in epidermal proliferation and cell death in clint1 mutants as early as 36 hpf (see Fig. S1A-F in the supplementary material); however, leukocyte infiltration was not observed in clint1 mutants until 48 hpf (Fig. 2C’). Quantification of neutrophils outside the caudal hematopoietic tissue (CHT) revealed that clint1 mutants have statistically significant neutrophil displacement as compared with wild-type siblings (Fig. 2F). Together, the findings demonstrate that clint1 mutants display enhanced proliferation, cell death and leukocyte infiltration.

**clint1 mutants display persistent inflammation in epidermal tissues**

The clint1 mutant was identified by a chronic inflammation phenotype characterized by leukocyte infiltration in the fin (Fig. 1F’) and increased il1b expression (Fig. 1B). To further characterize the inflammation in clint1 mutants, embryos were co-immunolabeled with antibodies to Mpo and L-plastin (Lcp1), a general leukocyte marker (Meijer et al., 2008). In clint1 mutants at 48 hpf, two populations of cells – L-plastin-positive/Mpo-positive (neutrophils) and L-plastin-positive/Mpo-negative (macrophages and other leukocytes) – were observed throughout the epidermis, including the fin fold (Fig. 3A’,B’), the retinal pigment epithelium (data not shown) and the epidermis covering the trunk (Fig. 3B’). In wild-type siblings, leukocytes were excluded from these areas (Fig. 3A,B). Additionally, inflammation was not affected by raising the embryos in sterile E3 versus E3 containing *Escherichia coli* (see Fig. S2F in the supplementary material), suggesting that the inflammation phenotype was not affected by changes in bacterial load. Together, these findings indicate that clint1 mutants have chronic inflammation associated with impaired epidermal homeostasis.

**Leukocytes exhibit phagocytosis of cellular debris and bidirectional trafficking in clint1 mutants**

To explore the dynamic properties of inflammation in vivo, the clint1 mutant line was crossed with the mpo:GFP transgenic line (Mathias et al., 2006). Time-lapse images of clint1;mpo:GFP mutant larvae at 3 dpf revealed extensive infiltration of highly motile leukocytes within the epidermis. The leukocytes also displayed phagocytosis of cellular debris (Fig. 3C,D; see Movie 1 in the supplementary material). Time-lapse imaging and cell tracking of leukocyte motility in the clint1 mutants at 3 dpf showed that the spontaneous leukocyte migration in the fin was characterized by periods of random motility alternating with frequent pauses (moving <0.75 μm/minute an average of 28% of the time; see Fig. S2A in the supplementary material). Neutrophils also displayed robust bidirectional trafficking between the epidermal tissues and the vasculature in the clint1 mutant, suggesting that reverse migration and resolution were not impaired (see Fig. S2D and Movie 2 in the supplementary material). Neutrophil motility in mutants showed reduced cell velocity (5.7 μm/minute) and low directional persistence (0.4) as compared with the directed neutrophil response to wounding (8.3 μm/minute, average D/T=0.67; see Fig. S2B,C,E and Movie 3 in the supplementary material). Our findings indicate that neutrophils in clint1 mutants retain the ability to respond to tissue wounding. Taken together, these findings demonstrate that...
leukocytes in clint1 mutants display robust phagocytosis of cellular debris and bidirectional trafficking between the epidermal tissues and vasculature.

**Clint1 mediates the hi1520 mutant phenotype**

To directly implicate Clint1 in the hi1520 mutant phenotype, we determined whether ectopic expression of wild-type clint1 mRNA could rescue the epidermal phenotype of clint1 mutants. Control-injected embryos yielded 28% with wild-type clint1 mutant phenotypes (Fig. 4A-C), close to the predicted Mendelian ratio of 25%, whereas clutches injected with two different concentrations of clint1 mRNA (150 or 250 ng/l) yielded increased numbers of embryos with wild-type morphology (87% or 94%, respectively), few with clint1 mutant phenotypes (3% or 0%) and some with a partial rescue phenotype (10% or 6%) (Fig. 4A,D,E). There appeared to be a dosage-dependent effect of clint1 mRNA expression on the morphological phenotype. Ectopic expression of clint1 mRNA in the mutant background also significantly reduced inflammation and hyperproliferation (Fig. 4E-G).

To further implicate clint1 in the epidermal phenotypes of hi1520 mutants, morpholinos (MOS) were used to deplete wild-type embryos of Clint1. clint1-specific MOS targeted to the translational start site (clint1-atg MO) or the splice donor site of intron 1 (clint1-ex1 MO) (Fig. 5E) recapitulated the hi1520 mutant phenotypes (Fig. 5A’,B’,C’,D; see Fig. S3 in the supplementary material), whereas a standard control MO (con MO) had no effect (Fig. 5A-D; see Fig. S3 in the supplementary material). RT-PCR analysis with primers targeted to exons 1 and 4-5 (Fig. 5E) revealed that injection of clint1-ex1 MO reduced the amount of full-length clint1 mRNA to below the detection threshold (Fig. 5F). Both clint1-atg and clint1-ex1 MOS induced epidermal hyperproliferation and aggregation of p63-positive cells by 36 hpf (Fig. 5A’,B), similar to clint1 mutants. However, in contrast to clint1 mutants, neutrophilic inflammation was evident in clint1 morphants by 36 hpf (Fig. 5C’,D). We were unable to determine whether cell death was also an early feature of the clint1 morphant phenotype at 36 hpf because control morphants exhibited significant increases in AO staining compared with uninjected wild-type embryos (data not shown). These findings demonstrate that the clint1 morphant phenotype is similar to that of clint1 mutants and support the genetic contribution of clint1 to these phenotypes.

**Clint1 localizes to intracellular vesicles**

Recent work has shown that Clint1 interacts with SNAREs, such as Vti1b and syntaxins, to regulate clathrin-mediated vesicular trafficking (Wasiak et al., 2002; Chidambaram et al., 2008). Ectopically expressed zebrafish Clint1 was observed in intracellular vesicles in zebrafish epidermal cells (see Fig. S4A and Movie 4 in the supplementary material), and the zebrafish Clint1 and Vti1b isoforms were found to co-localize at intracellular vesicles in HEK293 cells (see Fig. S4B-D in the supplementary material). These data suggest a conserved function between the zebrafish and human Clint1 and Vti1b pathways; however, vti1b morphants...
showed no epidermal defects (see Fig. S4E,H-L in the supplementary material). Taken together, our findings implicate Clint1-mediated vesicular trafficking in the regulation of epidermal homeostasis.

**Inflammation and proliferation are secondary phenotypes in clint1 mutants**

The timing of inflammation in clint1 mutants suggests that it is a secondary phenotype. To determine whether inflammation causes the epithelial defects, clint1 mutants were injected with MOs specific for pu.1 (spi1) to inhibit myeloid lineage development (Rhodes et al., 2005) (see Fig. S5 in the supplementary material). The pu.1 MO blocked the development of macrophages and neutrophils in clint1 mutants without preventing epithelial aggregation (see Fig. S5C in the supplementary material) or hyperproliferation (see Fig. SSD in the supplementary material). Limited efficacy of the pu.1 MO after 2 dpf prevented an assessment of the role of leukocytes in the progression of the fin phenotype at later stages. These findings suggest that the induction of the epithelial phenotypes in the clint1 mutants is independent of leukocyte infiltration.

To address the relationship between epithelial aggregation and inflammation, we knocked down Clint1 in a subset of embryonic cells and monitored the distribution of neutrophils. Tracer dye, TMRD, or clint1-atg MO with TMRD, was injected into single cells of embryos at the 8- to 32-cell stage to facilitate asymmetric distribution of the clint1 MO. Embryos injected with the clint1-atg MO plus TMRD exhibited localized epithelial aggregates in areas that contained TMRD (Fig. 5H), suggesting that a localized deficiency in Clint1 can cause localized cell aggregation. These aggregates were associated with neutrophil infiltration (Fig. 5H), suggesting that neutrophil recruitment is secondary to the localized epithelial defects.

To determine whether the epidermal proliferation in clint1 morphants is a primary or secondary effect of the epithelial defects, a controlled number (2–4) of presumptive epidermal cells from clint1 morphant or wild-type Tg(β-actin:hras-eGFP) embryos were transplanted into unlabeled wild-type recipients. Following transplantation at the shield stage, GFP-positive cells were counted at 2 and 3 dpf and expressed as average cell number per transplanted (see Fig. S6C in the supplementary material). Although clint1 morphant cells often displayed rounder shapes than transplanted wild-type control cells, the average clone size was similar (see Fig. S6B,B′ in the supplementary material), suggesting that the epithelial hyperproliferation in non-mosaic clint1 morphants and mutants is due to a non-cell-autonomous effect of the environment, such as a secondary consequence of the loss of epithelial homeostasis.

**Clint1 and Lgl2 function synergistically to regulate epithelial homeostasis**

To investigate potential defects in epithelial cell-cell adhesion, embryos were co-labeled with p63 and pan-cadherin antibodies. In clint1 mutants, the epithelial organization of keratinocytes in areas of cell aggregation was disrupted, with overlapping cells (Fig. 6A′) and cell shedding (see Fig. 7). In epithelial aggregates, cadherin appeared to be localized to the membrane (Fig. 6A,A′), suggesting that cell-cell borders, although disorganized, were maintained.

Previous studies of penner (lgl2) mutant larvae have reported that mutant basal epidermal cells exhibit migratory behavior. Specifically, basal cells labeled with a pan-cytokeratin antibody are restricted to the dorsal and lateral epidermis in wild-type larvae but are found in the fin folds of penner mutants (Sonawane et al., 2005). To investigate this possibility in clint1 mutants, larvae were immunolabeled with the pan-cytokeratin antibody. In wild-type larvae at 3 dpf, keratin expression appeared normal in the lateral epidermis (Fig. 6B) and included weak expression within the fin fold (Fig. 6C). We observed keratinocyte aggregation (Fig. 6B′–D′), with increased keratin staining in the fin folds of clint1 mutants at 72 and 120 hpf (Fig. 6C′,D′). These findings indicate that the epithelial cells in clint1 mutants exhibit defects similar to those reported for penner mutants.

To determine whether Clint1 and Lgl2 function synergistically to regulate epithelial homeostasis, low doses of MOs targeting both gene products were co-injected into wild-type embryos. Co-injection of clint1-ex1 and lgl2 MOs produced severe epithelial defects more frequently (32%) than clint1-ex1 and control MO (<5%) or lgl2 and control MO (<1%) co-injected embryos (Fig. 6F-I). This synergistic enhancement points to an interaction between clint1 and lgl2 in the regulation of epithelial homeostasis and morphology.

**clint1 mutants have defects in hemidesmosome formation**

As the primary defect of penner mutants is in hemidesmosome formation (Sonawane et al., 2005), we analyzed hemidesmosome formation in clint1 mutants at 4 dpf by transmission electron
microscopy. Numerous electron-dense mature and immature hemidesmosomes were observed along the basal lamina of wild-type embryos, but few of these structures were observed in clint1 mutant siblings (Fig. 7A,B,A’,B’). Other adhesive structures, such as desmosomes (Fig. 7C,C’) and tight junctions (Fig. 7D,D’), were unaffected in clint1 mutants. Cellular fragmentation (Fig. 7A’,F) and shedding (Fig. 7C’) and nuclear condensation (Fig. 7E) were observed in epidermal cells of clint1 mutants, which are consistent with the AO and BrdU labeling results documented above (Fig. 2; see Fig. S1 in the supplementary material). Furthermore, previous studies of penner mutants determined that Lgl2 regulates hemidesmosome formation through a Pkcζ-independent mechanism (Sonawane et al., 2009), and no differences were observed in transverse sections (see Fig. S6D,E in the supplementary material) or lateral views (data not shown) of the wild type and clint1 mutants immunolabeled for Pkcζ. Thus, these data suggest that Clint1 and Lgl2 function synergistically to regulate hemidesmosome formation in the zebrafish epidermis using a Pkcζ-independent mechanism.

clint1 morphants display mesenchymal-like properties

For penner mutants, the aberrant keratin pattern suggested basal cell displacement. To study mesenchymal and migratory properties in clint1 morphant cells, cell morphology and motility was assessed using cell transplants. Time-lapse confocal imaging was performed of basal keratinocytes in wild-type (wild-type recipient) or lateral epidermis (data not shown) or for keratin (red) (B-D,B’-D’). Arrows (A’-D’) identify keratinocyte aggregation. Arrowheads (C,D’) identify keratin expression within fin folds. (E-H)p63 expression highlights epidermal morphology in un.injected (E), clint1-ex1 plus control MO (F), lgl2 plus control MO (G), or clint1-ex1 plus lgl2 MO (H) co-injected wild-type embryos. (I) Quantification of morphological phenotypes observed in E-H. Scale bar: 50 μm.

DISCUSSION

Clint1 is a novel regulator of epidermal homeostasis and hemidesmosome formation

Previous studies have reported an important role for Clint1 in vesicle transport; however, an in vivo function has not been reported to date. Here, we identify Clint1 as a novel regulator of epidermal homeostasis that is required for embryonic survival in the zebrafish. Clint1 is expressed in keratinocytes and localizes to intracellular vesicles. The epidermis of clint1 mutants displays hyperproliferation and cell death by 36 hpf and inflammation by 48 hpf. The defects in clint1 mutants can be rescued by injection of clint1 mRNA and are phenocopied by lgl2 MOs. Electron micrographs show that tight junctions and desmosomes are intact, but hemidesmosome formation is impaired in clint1 mutants. The lateral epidermis appears disorganized, with protruding epidermal cells, and keratinized cells appear in the fin fold, suggestive of epidermal displacement related to EMT. Time-lapse movies of clint1 morphants show enhanced protrusive activity and motility of epidermal cells. Taken together, our findings suggest that Clint1 is indispensable for epidermal homeostasis. Furthermore, the findings indicate that a deficiency of Clint1 induces a phenotype that resembles the human condition psoriasis.

Comparison of clint1 mutants with other psoriasis-like mutants

Recent progress has been made in defining pathways that regulate epidermal integrity and function. The identification of mutants with psoriasis-like phenotypes, such as penner (Sonawane et al., 2005), psoriasis (Webb et al., 2008) and hail (Carney et al., 2007; Mathias et al., 2007), has provided clues as to the genetic program of epidermal development in zebrafish. A crucial component of these epidermal phenotypes is keratinocyte hyperproliferation and the development of epidermal aggregates. Comparison of these phenotypes with those observed in clint1 mutants suggests potential relationships between these signaling pathways.

Previous work has suggested a link between epithelial hyperproliferation and the regulation of epithelial polarity. An example of this connection is the association between mutations in the polarity gene lgl2 and the development of hyperproliferation of epithelial cells in Drosophila (Bilder, 2004) and zebrafish (Sonawane et al., 2005; Vasioukhin, 2006). Previous studies have suggested a possible role for Clint1 in the regulation of cell polarity through a proposed interaction between the ENTH domain of Clint1 and the polarity gene lgl2.
and the GTPase-activating proteins (GAPs) for Cdc42 (Aguilar et al., 2006; Ritter and McPherson, 2006). Cytokeratin is an early polarity marker in zebrafish that displays basal localization of keratin filaments at 3 dpf (Sonawane et al., 2005). In penner mutants, cytokeratin is not restricted to the basal cortex, suggesting a loss of polarity, and ectopic expression of cytokeratin is suggestive of EMT. Although cytokeratin staining did not indicate polarity defects (data not shown), a similar pattern of ectopic keratin expression was observed within the fin fold of clint1 mutants at 5 dpf, suggesting EMT. Finally, defects in hemidesmosome formation were evident in electron micrographs of both penner (Sonawane et al., 2005) and clint1 mutants. A recent report on the penner mutant suggests that Lgl2 functions antagonistically with E-cadherin to regulate hemidesmosome formation through a Pkcζ-independent mechanism (Sonawane et al., 2009). Although the relationship between Clint1 and E-cadherin remains unclear, Pkcζ staining in clint1 mutants also suggests that Clint1 regulates hemidesmosome formation through a Pkcζ-independent mechanism. Our findings support an interaction between lgl2 and clint1 in the regulation of epidermal homeostasis and hemidesmosome formation.

The mechanism by which these two pathways interact remains unknown; however, both Clint1 (Hirst et al., 2004; Miller et al., 2007; Chidambaram et al., 2008) and Lgl2 (Musch et al., 2002) interact with SNARE proteins, such as syntaxins, providing a potential link between the two pathways. Based on recent reports, Vti1b was an obvious candidate to regulate epidermal homeostasis. Zebrafish Vti1b and Clint1 were found to co-localize at intracellular vesicles. However, no epidermal phenotypes were observed in vti1b-deficient embryos, and a functional interaction was not observed between Clint1 and Vti1b (data not shown). Taken together, our findings suggest that Clint1 regulates epithelial homeostasis through a vesicle transport pathway that functions synergistically with Lgl2.

Cadherins are transmembrane linker proteins that mediate cell-cell contacts. In clint1 mutants, cell-cell contacts were disrupted in areas of epithelial aggregation, but cadherin surface expression at cell-cell contacts appeared normal, similar to reports for the psoriasis mutant (Webb et al., 2008). Similarly, apical cell-cell junctions also appeared to be intact in penner mutants by β-catenin staining at lateral-apical cell-cell junctions (Sonawane et al., 2005). Other similarities between clint1 and psoriasis mutants include persistent hyperproliferation that continues beyond 120 hpf and the non-cell-autonomous nature of the epidermal proliferation defects. However, in contrast to penner and clint1 mutants, the psoriasis mutant displays impaired epidermal development related to the differential expression of keratin genes, suggesting a potentially different mode of action. Future studies will be needed to identify the causal mutation of psoriasis mutants and to investigate potential interactions with Clint1 in regulating epidermal homeostasis.

Finally, comparison of clint1 and hai1 mutants also highlights similarities. Epithelial cells in clint1 mutants displayed transient losses of cell-cell contacts and exhibited mesenchymal-like characteristics with single-cell motility, as reported with hai1 mutants (Carney et al., 2007). Further studies are needed to address possible differences in mesenchymal behaviors in the different zebrafish mutants. It appears that partial or complete EMT may be a common feature of psoriasis-like mutants in zebrafish and might contribute to the epithelial aggregation observed in these mutants.

**Inflammation in clint1 mutants**

The human condition psoriasis is characterized by epithelial proliferation and inflammation of the skin (Bowcock and Krueger, 2005; Lowes et al., 2007). Similar to clint1 mutants, zebrafish hai1 mutants exhibited hyperproliferation and inflammation involving neutrophils and macrophages (Mathias et al., 2007). Leukocytes were observed in similar locations in hai1 and clint1 mutants. The persistent inflammation in hai1 mutants was characterized by leukocyte recruitment into the fin in the absence of reverse migration back to the vasculature, suggesting that the persistent inflammation might, in part, be due to impaired resolution. By contrast, leukocyte
trafficking in the clint1 mutants showed robust bidirectional trafficking between the fin folds and the vasculature, suggesting that reverse migration remains intact. In both clint1 and hai1 mutants, individual neutrophil migration was characterized by periods of motility alternating with frequent pauses, although pausing was more frequent in clint1 mutants, a likely reflection of differences in phenotypic severity.

The recruitment signals responsible for persistent leukocyte infiltration remain unknown. Candidate factors include the inflammatory cytokine il1b, expression of which is increased in clint1 mutants. However, il1b alone did not appear to be sufficient to induce the inflammatory response because an il1b-specific MO did not block inflammatory cell recruitment in the clint1 mutants (data not shown). Alternatively, inflammation can be induced by dying cells in order to allow for clearance of cellular debris. Although caspase inhibitors did not affect the leukocyte infiltration (data not shown), we cannot rule out the possibility that tissue damage signals contribute to leukocyte recruitment in the clint1 mutant. In support of this possibility, removal of tissue debris by phagocytic cells was observed in the clint1 mutants. Another potential target is Toll-like receptor (TLR) signaling, as Tlr3 was recently implicated as an endogenous sensor of tissue necrosis during acute inflammation (Cavassani et al., 2008). It is also possible that one of these factors alone is not sufficient for leukocyte infiltration in clint1 mutants, and that inhibition of multiple pathways might be necessary to limit the inflammatory response.

EMT and leukocyte infiltration

A deficiency of either Clint1 or Hai1 leads to a phenotype characterized by epithelial proliferation, EMT and leukocyte infiltration, similar to a wound response. Although leukocyte infiltration has not been assessed in all zebrafish epidermal mutants, evidence indicates that EMT and/or a loss of epithelial integrity would be accompanied by inflammation. In accordance with this possibility, a recent study reported that loss of epidermal Caspase 8 induces a wound healing response characterized by epidermal hyperproliferation, apoptosis and inflammation (Lee et al., 2009), similar to the phenotype of the clint1 mutant. Further studies are needed to examine the relationship between inflammation and EMT in zebrafish models, and to investigate the ability of leukocyte infiltration to exacerbate the EMT phenotype.

In summary, clint1 is an essential gene for the regulation of epidermal homeostasis in zebrafish. Importantly, this is the first reported in vivo function for Clint1 in an animal model. The findings suggest that Clint1 deficiency results in a phenotype that resembles the human condition psoriasis. It is of substantial importance to identify model systems that are amenable to drug screening and that can provide insight into the factors that contribute to inflammation in response to damaged tissues, or in the context of proliferative epidermal diseases. The clint1 mutant will provide a powerful tool with which to dissect the pathways that regulate epidermal homeostasis and contribute to the onset and resolution of inflammatory responses.

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Competing interests statement

The authors declare no competing interests.

Supplementary material

Supplementary material for this article is available at http://dx.biologists.org/cgi/content/full/115/2591/DC1

References


Chua, K. L. and Lim, T. M. (2000). Type I and type II cytokeratin cDNAs from the zebrafish (Danio rerio) and expression patterns during early development. Dev. Dyn. 216, 359-368.


