Polarity and morphogenesis of the eye epithelium requires the adhesion junction associated adaptor protein Traf4

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ABSTRACT
During development, neuroepithelial progenitors acquire apico-basal polarity and adhere to one another via apically located tight and adherens junction complexes. This polarized neuroepithelium must continue to integrate cells arising through cell divisions and intercalation, and allow for cell movements, at the same time as undergoing morphogenesis. Cell proliferation, migration and intercalation all occur in the morphing embryonic eye. To understand how eye development might depend on dynamic epithelial adhesion, we investigated the function of a known regulator of junctional plasticity, Tumour necrosis factor receptor-associated factor 4 (Traf4). 

traf4a mRNA is expressed in the developing eye vesicle over the period of optic cup morphogenesis, and Traf4a loss leads to disrupted evagination and elongation of the eye vesicles, and aberrant organization and apico-basal polarity of the eye epithelium. We propose a model whereby Traf4a regulates apical junction plasticity in nascent eye epithelium, allowing for its polarization and morphogenesis.

Symbols and Abbreviations: AB: apico-basal; aPKC: atypical protein kinase-C; CRISPR: clustered regularly-interspaced short palindromic repeats; GFP: green fluorescent protein; hpf: hours post-fertilization; MO: antisense morpholino oligonucleotide; pHH3: phospho histone H3; ss: somite stage; Traf4: Tumour necrosis factor receptor-associated factor 4; ZO-1: zona occludens-1

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Introduction
Epithelial remodelling is a feature of tissues undergoing morphogenesis. Tissue morphogenesis is driven in part by the migration and intercalation of cells. How these dynamic behaviours occur within the context of a nascent epithelium of adherent cells is poorly understood. Presumably, molecular mechanisms exist that would allow for individual cells to change position without disrupting the integrity of the epithelium. 

The eye undergoes extensive morphogenesis to achieve its final form. First, the specified eye field splits into two eye vesicles that evaginate from the anterior neural plate and elongate. Subsequently, the vesicles invaginate around the lens to form the optic cup, and lastly the two poles of the ventral optic cup meet and fuse [1,2]. Cell behaviours underlying these events have been studied in fish, where it is possible to track individual cells in the live embryo [3–7]. Formation of the eye vesicles appears to be driven both by individual cell migration and cell intercalation. In medaka, it is reported that migration of eye progenitors drives both the evagination of bilateral eye vesicles from a single eye field, and their subsequent elongation [7]. Recent work in zebrafish argues that eye vesicle evagination and elongation are driven predominantly by the intercalation of mesenchymal-like progenitors from the core of the eye field into the epithelium forming at its lateral edges [4]. Additional cell movements include the migration of progenitors from the telencephalon into the elongating eye vesicles [5], and movement of the epithelium of the dorsal and ventral portions of the inner leaflet of the eye vesicle around the distal rim of the invaginating optic cup [3,6].

Cells within an epithelium exhibit apico-basal (AB) polarity – with neighbouring cells adhering to one another at their apical surface through tight junctions and cadherin-dependent adherens junctions. As cells rearrange, these junctions must maintain adhesion to not compromise epithelial integrity, but at the same time allow cells to move. One potential mechanism is
the shrinkage of the junctional contacts between neighbouring cells [8]. Thus, apical adhesive junctions that are plastic with regard their extent and stability may be an important feature of an embryonic epithelium. The molecules that control such epithelial plasticity, however, are poorly understood.

Tumour necrosis factor receptor-associated factor 4 (TRAF4) belongs to the TRAF family of proteins, but unlike other TRAFs, TRAF4 has no defined role in the immune system. TRAF4 encodes a well-conserved adaptor protein with cytoplasmic, nuclear and membrane localization [9–11]. Moreover, TRAF4 is associated with both tight and adherens junctions [12,13], and can control the subcellular location of proteins [13,14]. In Drosophila, Traf4 regulates the association of Armadillo (β-Catenin) in the adherens junctions of constricting mesoderm cells [13], and is required for proper formation of the imaginal eye discs and a correct photosensory neuronal array in the brain [15]. While TRAF4 is required in mice for neural tube closure and neural crest cell development [16,17], a role for Traf4 in the control of cell-cell adhesion in vertebrate systems has not been explored.

Here we employ loss of Traf4a in zebrafish to understand the need for dynamic regulation of adhesive interactions between progenitors of the eye vesicle for morphogenesis. We find that traf4a mRNA is expressed in the developing eye field and vesicles. Loss or knockdown of Traf4a protein in the early embryo results in disrupted symmetric emergence and elongation of the bilateral eye vesicles, as well as failure of some eye progenitors within the core of the eye field to integrate into the eye epithelium. Additionally, the organization of the eye epithelium is disrupted, with cells of the evaginating vesicles exhibiting aberrant AB polarity, morphology, and radial orientation of their nuclei. We propose a model whereby Traf4a controls apical junction distribution and stability to allow for plasticity of adhesion between epithelial cells that accommodates eye morphogenesis, while maintaining epithelial integrity.

Results

Traf4a mRNA is expressed by eye progenitors

Two traf4 orthologs of human TRAF4 are present in zebrafish, traf4a and traf4b [10]. In the embryo, traf4b mRNA is weakly and ubiquitously expressed, whereas traf4a mRNA is expressed in a highly specific manner. While traf4a expression in the eye of the 3–10 somite stage (ss) zebrafish embryo was reported previously [10], spatial and temporal aspects of the expression in the eye vesicle between the 10 ss and 24 hours post fertilization (hpf) were not described. Thus, we investigated the expression of traf4a by wholemount in situ hybridization from the 6 ss to 18 hpf. The zebrafish eye field is specified during gastrulation and separates into bilateral eye vesicles that start to evaginate at the 5 ss, and elongate over the next 3 hours [1,5]. traf4a mRNA was expressed throughout the 6–12 ss eye vesicle (Figure 1A–C,E), and eye expression continued through to at least 18 hpf (Figure 1F). Of note, Traf4a protein is reported to localize to the apical surface of epithelia [12,13], labeled at the 12 ss by the tight junction associated protein ZO-1 [18] (Figure 1D), and the adherens junction protein E-cadherin [19] (data not shown).

Traf4a is required for eye development

Given the embryonic expression of traf4a, we tested its involvement in eye development by reducing traf4a mRNA using an antisense morpholino oligonucleotide (MO) that successfully targeted the splice junction between exon 1 and intron 1 (e1i1) (Figure 2H). The MO was injected into one cell Tg(rx3:GFP) embryos, in which the rx3 promoter drives GFP expression in eye and hypothalamic progenitors [7]. The bilateral eye vesicles of controls had elongated at the 12 ss and were of comparable size (Figure 2A). While the eye fields of traf4a morphants also separated into bilateral eye vesicles that evaginated, one or both vesicles were significantly smaller than control (Figure 2B), with vesicles asymmetric in size in over 40% of embryos (3/30 control vs. 18/41 morphants present with

![Figure 1](image-url). traf4a is expressed in the developing eye vesicle. A–C, E: Wholemount in situ hybridization viewed laterally (A,B) and in anterior (C) and posterior (E) transverse sections with antisense riboprobe for traf4a mRNA. D: Immunolabeling of transverse sections through an eye vesicle and forebrain of a 12ss embryo with an antibody against ZO-1 to identify apical surface of eye vesicle. F: traf4a mRNA is still expressed in the 18 ss eye vesicle. Orientation bar in C applies to panels C–E. a, apical; b, basal; D, dorsal; e, eye vesicle; tel, telencephalon; V, ventral.
Figure 2. Traf4a knockdown results in small and asymmetric eye vesicles. A-G: Dorsal views of 12 ss (A-D) and 18 ss (E-G) Tg(rx3:GFP) control embryos (A,E), and embryos injected with either e1i1 MO (B,F), e3i3 MO (G), wild-type zebrafish traf4a mRNA (C), or e1i1 MO along with wild-type zebrafish traf4a mRNA (D). In G, the left eye vesicle outline is superimposed on the right eye. Morphant eye vesicles (outlined) are misshaped, and can be smaller in anterior-posterior length. e, eye vesicle; hy, hypothalamus. H: RT-PCR showing knockdown or missplicing of the traf4a transcript with the e1i1 and the e3i3 antisense MOs, respectively, with ef1α as a loading control. I,K,M: Eye size (µm) as measured by the anterior-posterior length of the shorter eye vesicle for 18 ss control, and e1i1 (I) and e3i3 (K) traf4a morphant embryos; 12 ss control embryos, and embryos injected with e1i1 with or without wild-type zebrafish traf4a mRNA (M). J,L: Mean percentage difference between the length of e1i1 (J) or e3i3 (L) embryos’ two eye vesicles. Error bars are standard deviation, and numbers above bars indicate number of embryos. Statistics ( * p < 0.05, **, p < 0.01) represent: two-tailed, unpaired Student’s t-tests for I,K (N = 3) and J,L (N = 4); One Way ANOVA, Dunnett’s post-hoc test (M, N = 4).

≥ 10% size difference). Three hours later at the 18 ss, when the eye vesicles invaginate around the lens to form the optic cups, the size disparity had not resolved (Figure 2E,F,I,J; 2/25 control and 11/33 morphant embryos).

Several lines of evidence support the specificity of the eye phenotype for traf4a knockdown. First, a similar phenotype was observed with a second MO targeting the splice junction between exon 3 and intron 3 (e3i3) (Figure 2G,K,L), which caused missplicing of the traf4a mRNA (Figure 2H): morphants exhibited small eyes, sometimes with the two eyes of an embryo being of different sizes (1/27 control and 8/38 MO embryos). The e1i1 MO was used for all subsequent experiments. Second, the phenotype did not arise from inappropriate p53 upregulation, a documented off-target response of antisense MOs in fish [20], in that simultaneous knockdown of both traf4a and p53 failed to rescue the eye phenotype seen with Traf4a knockdown (Supp. Figure 1). Finally, the traf4a morphant eye phenotype was rescued by co-injection of the traf4a MO along with full-length zebrafish traf4a mRNA (not targeted by the e1i1 splice MO) (Figure 2C,D, M). Interestingly, Traf4a overexpression had no significant effect on eye vesicle size or morphogenesis (Figure 2C,M). Finally, because TRAF4 is upregulated in several cancers [21], and controls the asymmetric cell division of Drosophila neuroblasts [22,23], we also investigated cell proliferation. An antibody against phospho-Histone H3 (pHH3), which labels cells actively undergoing mitosis, reveals similar numbers of pHH3 positive cells in
both traf4a morphant and control 12 ss embryos (Supp Figure 2).

Anterior neural tube patterned normally with Traf4a knockdown

Aberrant development of the eye vesicles could result from defects in the patterning of the early anterior neural tube from which it derives, and where traf4a is expressed (Figure 1E). To address this possibility, we examined by in situ hybridization the mRNA expression of several transcription factors key in the development of anterior neural structures, including: rx3, critical for eye morphogenesis and proliferation [24,25] (Figure 3A-B); six3, key for retinal specification [26] (Figure 3C-D); pax6, known to function in forebrain and eye development [27,28] (Figure 3E-F); vax1, an optic stalk marker [29] (Figure 3G-H), and; vax2, a marker of the early anterior eye vesicle [30] (Figure 3I-J). In addition, we visualized fibroblast growth factor 8a (fgf8a) mRNA (Figure 3K-L), a morphogen that patterns the forebrain and midbrain [31]. Despite obvious defects in eye vesicle elongation, the general patterns of expression of all of these genes were similar in controls and traf4a morphants.

Apical junction complexes disrupted in Traf4a morphants

In Drosophila, Traf4 is associated with adherens junctions and is required for apical constriction during ventral furrow formation [13]. Epithelial adherens junctions are comprised of the cell adhesion molecule E-cadherin, which associates intracellularly with β-catenin and the actin cytoskeleton [19]. We investigated the localization of all three proteins. In control, a β-catenin antibody (Figure 4A-D; N = 2, 11/11) and rhodamine-phalloidin that marks F-actin (Figure 4I-L; N = 3, 5/5) labeled the membranes of the cells that spanned the width of the brain and eye epithelium, with particularly bright apical and basal signal (Figure 4B,D). In contrast, the epithelium of morphant eyes was disorganized, with disrupted β-Catenin labeling of the basal epithelium (Figure 4E-H; N = 2, 16/16; compare white arrowheads in Figure 4B,D,F,H), and bright accumulations of β-catenin at the apical surface (Figure 4F; white arrows). F-actin label was also discontinuous basally (Figure 4M-P; N = 3, 26/26; white arrowheads N,P), and disorganized within the epithelium (Figure 4P). Finally, E-cadherin immunoreactivity in control accumulated at the apical surface of the eye vesicle epithelium (Figure 4Q-S arrowheads; N = 4, 15/17), whereas in morphants E-Cadherin failed to collect at the apical surface (Figure 4U-W, arrows; N = 4, 17/18). Disrupted localization of apical adherens junction proteins, along with physical gaps between the two normally adherent leaflets of the eye vesicle (Figure 4F,G,U), as well as progenitors with no obvious apical attachment (Figure 6H), suggest that adhesive junctions are disrupted in the traf4a morphants. Interestingly, bulges off the eye vesicle (Figure 4G, white arrow) were sometimes observed, as well as clusters of GFP+ cells within the ventricles of the eye vesicles and forebrain (Figure 4E,F,N,T,X). These cells expressed the polarity protein atypical Protein Kinase C (aPKC) (Figure 4X).

Eye phenotype present in Traf4a-/ CRISPR genetic mutant

Because of potential concerns about the specificity of phenotypes arising through the use of antisense MOs [32], we generated a traf4a genetic mutant by using CRISPR gene silencing technology [33]. A guide RNA complementary to exon1 of the traf4a gene was injected along with Cas-9 nuclease into the one cell embryo. A single F0 female founder was bred to homozygosity in the F2 generation: sequencing identified a 10 bp deletion that produced a frameshift introducing a premature stop codon (Figure 5A), as seen by a smaller RT-PCR product amplified with traf4a specific primers from mRNA of mutants than wildtype (data not shown). traf4a homozygous mutants were viable and fertile, and embryogenesis was grossly normal (Figure 5C). The eyes of 12 ss F3 embryos showed a comparable phenotype to the traf4a

Figure 3. Gross patterning of the anterior neural keel is not disrupted with Traf4a knockdown. Dorsal views of 12 ss control (A,C,E,G,I,K) and traf4a morphant (B,D,F,H,J,L) embryos processed with antisense riboprobes for rx3 (A-B; N = 2), six3 (C-D; N = 2), pax6 (E-F; N = 2), vax1 (G-H; N = 1), vax2 (I-J; N = 1), and fgf8a (K-L; N = 1). Eye vesicles outlined in white dots. br, brain; e, eye vesicle; hy, hypothalamus; mhb, midbrain/hindbrain border; os, optic stalk.
In the majority of embryos (Figure 5E,F,G,J), one or both eyes were smaller than observed in wild-type or traf4a± embryos (Figure 5B,D). In many cases an asymmetry in the size of the two eyes was present (Figure 5E,F,K; 0/33 wildtype and 24/43 traf4a-/− embryos), though in some embryos both eyes were equally small in size (Figure 5G). The generally smaller size of the mutant eyes remained at 48 hpf (Figure 5H,I).

In comparison to wild-type eye vesicles (Figure 6A,D; N = 2, 10/10), the epithelium of the mutant (Figure 6B,C,E; N = 3, 16/16) eye vesicles were disorganized: nuclei often failed to orient perpendicular to the apical surface of the eye vesicle epithelium (Figure 6B-E,M), and F-actin on both basal and apical surfaces of the epithelium was disrupted and unevenly distributed (compare Fig. 6A,D with B,C,E'). Similar to the traf4a morphants, mutants sometimes exhibited ectopic GFP positive cell clusters in the ventricles of the eye epithelium (Figure 6C',C'' arrows), and aberrant bulges from the eye vesicle epithelium (Fig. 6B',B'',C'',C''''). Moreover, with Traf4a loss, membrane associated red fluorescent protein (mRFP)-expressing eye cells (from a cDNA plasmid injected at the one-cell stage) were present that did not span the epithelium, and had no obvious apically directed process with an attachment to the apical surface, similar to what is seen in traf4a morphants (Figure 6F-H). Finally, comparable to what was observed in traf4a morphants (Figure 4U-W), E-Cadherin failed to accumulate at the apical surface of the eye epithelium in 14 ss traf4a-/− mutants (compare Figure 6J-L).

TRAF4 can act as either an anti- or pro-apoptotic factor (Rousseau et al., 2011). As such, we examined cell death in our traf4a mutant and morphant eye vesicles by immunohistochemistry for activated Caspase 3, at different time points between the 12 ss and 18 ss. traf4a mutant 12–18 ss eye vesicles exhibited
similar numbers of apoptotic cells as compared to control (Figure 6I). More apoptotic cells were present in the eye vesicles of $traf4a$ morphants at the 18ss, though numbers were relatively small, and importantly eye vesicle defects were seen in morphant embryos at embryonic stages (12 and 14 ss) when apoptosis was minimal. Thus, apoptosis does not appear to be a significant contributor towards the eye vesicle phenotypes observed at the 12–14 ss with the loss of $Traf4a$.

Importantly, for the validity of the morphant phenotypes we described, we saw no exacerbation of the mutant phenotype (Figure 6N-Q) when we injected $traf4a$ MO into mutant embryos, either in terms of the length of the smallest eye vesicle (Figure 6R) or the size asymmetry of the two eyes (Figure 6S).

**Epithelial polarity disrupted by $Traf4a$ loss**

Given that Traf4 regulates adhesion junctions [12,13], and apical E-Cadherin is disrupted with Traf4a loss, we next asked if the polarity of the eye epithelium was also affected. We first performed immunolabeling for aPKC, which localizes to the apical surface of epithelia [34]. In control, aPKC (Figure 7A-C,J) was found at the apical midline of the forebrain and eye vesicle epithelia (N = 4, 18/18 embryos). In $traf4a$ morphants (Figure 7D-F,K; N = 4, 15/17 embryos) and mutants (Figure 7G-I,L; 10/10 embryos), while this polarized localization was somewhat preserved, aPKC was unevenly distributed, with mosaic patches of little or intense apical immunoreactivity, and ectopic non-apical expression. Further, a
Figure 6. Eye epithelium disrupted in traf4a CRISPR mutant. A-E: Transverse sections through the eye vesicles (e) of wild-type (A,D) and traf4a/- 12 ss Tg(rx3:GFP) (B,C,E) embryos revealing DAPI-stained nuclei (A-E) and rhodamine-phalloidin stained F-actin (A’-E’), with merge in A’’-E’’. In B’ arrowhead points to a bulge off the eye vesicle (shown at higher magnification in C’’), and in C’,’ arrow point to cell cluster in ventricle (ve). Scale bar in A is 100 µm for A-B, 75 µm for C,’C’’ and 25 µm for C’’,C’’’,D,E-J-L. Higher magnification view (D,E) reveals disruption of organization of DAPI nuclei (E) and non continuous F-actin (E’, arrows) in mutant. F-H: mRFP expressing progenitors in the eyes of wildtype (F), traf4a/- (G) and traf4a morphant (H) 14 ss embryos. I: Graph of the mean number of activated Caspase-3 positive cells in the whole eye of embryos at the indicated somite stages. Error bars are standard error of the mean (s.e.m.) and numbers above the bars indicate the numbers of embryos assessed (data pooled from two independent experiments). **, p < 0.01 One Way ANOVA, Bonferroni correction. J-L: E-Cadherin labeling of 14 ss wildtype (J) and traf4a/- (K) rx3:GFP embryo (high power in L). Arrowheads show accumulation of E-Cadherin label at the apical surface in wildtype. M: Schematic of DAPI-labeled nuclei in boxed areas in A and B, with the orientation of selected nuclei indicated by red arrows. N-Q: Dorsal brightfield images of traf4a+/+ (N), e1ii MO+ traf4a+/+ (O), traf4a/- (P), and e1ii MO+ traf4a/- (Q) embryos with the eye vesicle and brain outlined. R-S: Quantitation of size of smallest 12 ss eye (R) and the % difference between the sizes of the two eyes (S). Number of embryos analyzed is shown. Error bars are standard deviation. *p < 0.05, Kruskal Wallis One Way ANOVA, followed by Dunn’s method for multiple comparisons. a, apical; b, basal.
marker of the basal surface of the epithelium, Laminin, appeared downregulated and discontinuous on the dor-
sal surface of the eye epithelium in the 
traf4a
morphants
(Figure 7P-R; N = 2, 11/13 embryos) and mutants
(Figure 7S-U; 10/10 embryos) as compared to controls
(Figure 7M-O; N = 2, 0/20 embryos).

Traf4a is reported to associate with apically loca-
lized tight junctions \[35\]. As such, we also investigated
the expression of the tight junction protein ZO-1 \[18\].

In control, ZO-1 immunoreactivity was associated
with the apical surface of the neuroepithelium of the eye
vesicle (Figure 7V; N = 3, 20/20). With Traf4a knock-
down (Figure 7W; N = 3, 19/19) and loss (Figure 7X;
14/14 embryos), ZO-1 immunoreactivity was still asso-
ciated with the apical surface but in a disorganized
fashion, and like aPKC could be mis-localized away
from the apical surface of the eye vesicle.

**Improper eye vesicle evagination and elongation with Traf4a loss**

To better understand the defects of early eye develop-
ment observed with loss of Traf4a, we followed
the morphogenesis of control and 
traf4a
loss Tg(rx3:GFP)
eye vesicles by confocal time-lapse microscopy, over the
period that eye vesicles evaginate and elongate (6–18 ss)
(Figure 8). In a dorsal view, in the control embryo the
eye field separated and the eye vesicles evaginated in a
symmetrical manner from the diencephalon (Figure 8a,
Supp. Movie 1). The control eye vesicles then elongated
gradually over time and separated fully at the midline
(Figure 8A,C). In the 
traf4a-/-
embryo, however, the
eye field at the 6 ss was not symmetric in shape,
exhibiting an aberrant bulge (Figure 8A, arrow; Supp.
Movie 2), and elongation of the two eye vesicles was
impaired (Figure 8C). Nonetheless, the eye vesicles did
separate completely. In other 
traf4a-/-
mutant embryos,
one eye vesicle appeared to evaginate and elongate
normally, while the other failed to elongate
(Figure 8B, Supp. Movie 3). A similar failure of one
or both eye vesicles to elongate was observed in the

**Discussion**

Our data support an important role for the apical
junction-associated adaptor protein Traf4a in the
embryonic zebrafish eye in allowing proper formation of
a polarized pseudostratified eye epithelium – a feature
that appears critical for morphogenesis. 

\[51x147\]
expressed by early eye progenitors. Both knockdown and loss of Traf4a protein result in a disorganized embryonic eye epithelium with disrupted AB polarity and apical progenitor attachments, and improper evagination and elongation of the eye vesicles. Our data support the idea [4] that development of an eye epithelium with appropriately localized apical adhesive junctions and basal proteins is required to allow for the cell movements that drive early eye vesicle morphogenesis.

Traf4a loss or knockdown causes several distinct defects in eye epithelial organization. Importantly, comparable defects are present in both the traf4a morphants and CRISPR genetic mutant. As such, the disruptions in epithelial organization we described are highly likely to be specific to low or absent Traf4a. These defects include many eye progenitors showing disrupted AB polarity, a loss of the elongated, radial orientation of nuclei, a failure of eye vesicles to evaginate and elongate.
epithelium, and an abnormally distributed actin cytoskeleton. Also present are clusters of adherent GFP+ eye progenitors in the ventricle of the eye vesicle, which could reflect core eye field progenitors that failed to intercalate into the eye epithelium [4], or the delamination and extrusion of progenitors [6]. Loss of Traf4a presents with extra evaginations at random locations off the eye vesicle. Interestingly, time-lapse imaging suggests that these protrusions are transient and resorb into the eye vesicle proper (data not shown). Finally, the antero-posterior extent of one or both of the eye vesicles is often smaller than in control. The time lapse microscopy suggests a few possible explanations. First, with Traf4a loss the eye field can split in a non-symmetric fashion, resulting in eye vesicles of different sizes. Second, eye vesicles do not always elongate appropriately. Elongation of the eye vesicles in zebrafish is thought to be driven by the intercalation of mesenchymal-like eye progenitors from the core of the eye field into the lateral eye vesicle epithelium [4], and so potentially the loss of Traf4a alters the adhesiveness of the epithelium and blocks progenitor intercalation. Indeed, we do observe GFP+ eye progenitors within the ventricle of the forebrain and eye vesicles with Traf4a loss. Survival of progenitors within the eye vesicles did not appear to be impacted by Traf4a loss, and so likely did not contribute directly to a smaller eye vesicle size.

The disrupted AB polarity of eye progenitors is reminiscent of that reported for zebrafish missing Laminin function [4,36]. Laminin-1 is a component of the basal lamina and is required for many eye progenitors to maintain proper AB polarity and an elongated radial morphology. In the absence of Laminin-1, apical proteins are present both apically and ectopically on the basal surfaces of the eye epithelium, progenitors lose their apical attachments, and eye evagination is consequently disrupted [4,36]. We find the basal expression of Laminin is patchy with Traf4a loss. Thus, the defects in eye evagination and elongation that occur when Traf4a is absent might result similarly from disruptions of normal AB cellular polarity and epithelial organization. Interestingly, despite the disorganized epithelium, eye vesicles usually do evaginate and elongate to some extent, arguing that early eye morphogenesis is a robust phenomenon. Subsequent events of eye morphogenesis, including eye vesicle invagination around the lens and choroid fissure fusion, appear to not require Traf4a, while Laminin-alphal is necessary for both early eye vesicle elongation and for subsequent optic cup morphogenesis [36].

Interestingly, the defects in AB polarity of the eye progenitors with Traf4a loss are mosaic in nature. Disruptions in polarity protein, and actin and β-Catenin localization are patchy in nature and vary in location and severity from eye vesicle to eye vesicle. The mosaicism could be explained by redundant mechanisms permitting proper AB polarity to be achieved in some regions of the epithelium. In support, a Traf4-independent mechanism must carry out similar functions to Traf4a in organizing the location of proteins of the Xenopus eye epithelium, where traf4 is not obviously expressed [16]. The fact that only 30% of Traf4a-/− mice exhibit embryonic lethality, with the remainder showing a number of less penetrant defects [17], and that we find that some zebrafish embryos exhibit apparently normal eye vesicle development with Traf4a loss, also argues for redundant mechanisms. Mosaic penetrance of the defects within the eye epithelium could explain the differences in phenotype observed between the two eye vesicles of a single embryo. Presumably, morphogenetic defects arise only if large enough patches of the epithelium are disorganized and contain cells with defective AB polarity and morphology. One possibility is that if the associations between eye progenitors are sufficiently disrupted at the eye field stage to impact the extent and/or direction of evagination of the tissue from the diencephalon, eye elongation is impacted. If not, eye elongation occurs normally.

How might Traf4a control the organization and polarity of a nascent epithelium? Traf4 is known to regulate the subcellular localization of proteins, to control cell apoptosis and proliferation, and to associate with both tight and adherens junctions. Traf4a is unlikely to be controlling epithelial homeostasis as it does for cultured mammary epithelial cells [12], in that Traf4a is absent from the nuclei of eye progenitors and Traf4a loss causes no dramatic proliferation or apoptosis defects over the early stages of eye development. Instead, based on the known roles for Traf4 in the literature, our data suggest a model (Figure 8G) whereby Traf4a controls the localization and function of apical adhesive junctions to control associations between eye progenitors and maintain the organization of the pseudostratified eye epithelium, and allow for proper eye evagination and elongation. In support, Traf4a protein is enriched at the apical surface of the eye epithelium, along with tight junction and adherens junction associated proteins. Moreover, we find the apical localization of both the E-Cadherin and β-Catenin adherens junction proteins, and the ZO-1 tight junction protein, along with the apical attachments of progenitors, are disrupted with Traf4a loss.

In some systems, Traf4 promotes the plasticity of junctional complexes by negatively regulating junction stability. For instance, in breast cancer cells Traf4 is upregulated and destabilizes tight junctions [35], and indirectly regulates
adherens junctions by promoting TGFβR1 signaling-dependent down regulation of E-cadherin [37]. Such plasticity is important to allow epithelial cells to retain contacts while undergoing morphogenesis [38]. While Traf4 can regulate tight junctions [35], a critical role for tight junctions in epithelial remodelling is unclear [39], and we find ZO-1 is only mildly impacted by Traf4a loss. In contrast, the regulation of adherens junctions is key for epithelial remodelling [40], and Traf4 associates with and fine tunes the assembly of adherens junction complexes in invaginating Drosophila mesodermal cells [13]. Thus, our data revealing a loss of the accumulation of E-Cadherin at the apical surface of the eye epithelium, and non-uniform apical localization of β-Catenin and αPKC, support the idea that the eye phenotype observed with Traf4a loss is best explained by Traf4a regulation of adherens junctions. Our data, however, argue against Traf4a de-stabilizing apical junctions between eye epithelial cells, as with Traf4a loss E-Cadherin fails to accumulate at the apical surface. Whether normally Traf4a controls localization of E-Cadherin or β-Catenin to the apical surface of eye progenitors is unclear, though data suggests Traf4a can interact directly with β-Catenin [13]. Nonetheless, the two proteins critical for adherens junction function become mislocalized with Traf4a loss, with apical β-Catenin released from E-Cadherin interactions clumping, and E-Cadherin failing to collect at the apical surface, respectively. The apparent result is that adhesive interactions in the eye vesicle are impacted, with progenitors losing their apical attachments and AB polarity, and the two leaflets of the eye vesicles failing to adhere to one another.

The idea that disrupted adherens junction function could explain some of the epithelial phenotypes we observe is supported by the literature. For instance, increases in the levels of proteins that promote apical localization of adherens junctions cause ectopic folds of the dorsal epithelium during Drosophila gastrulation [41]. Thus, one possibility is that in the absence of Traf4a, mis-localized adherens junction proteins cause ectopic folds in the eye epithelium, and account for the ectopic evaginations we observe. Traf4a may also, via the regulation of adherens junctions, control the intercalation of progenitors of the eye field core [4], in that a role for adherens junctions in cell intercalation is well established [42–44].

We also find Traf4a concentrated at the basal surface of the eye vesicle epithelium, where we observe disruptions in the localization of Laminin, F-actin and β-Catenin expression. The function of Traf4 that is best supported by the literature is as a regulator of the localization of apically targeted proteins [12,13], and certainly we see disruptions in the localization of apical proteins with Traf4a loss. Thus, the defects in basally located proteins may arise secondary to apical defects. Nonetheless, we cannot rule out a direct role of Traf4a at the basal surface of eye epithelial cells. Certainly, Laminin is known for its role in polarizing epithelia [45]. Moreover, in platelets, Traf4 is in a complex with a glycoprotein collagen receptor, upstream of focal adhesion kinase [46], suggesting it may play roles in extracellular matrix protein signaling. Future experiments will need to resolve the exact role Traf4 plays in controlling adhesion in the eye epithelium.

**traf4a** expression exhibits dynamic spatial changes over embryonic development, and Traf4a in zebrafish is expressed in tissues other than the eye, such as the somites, which also undergo morphogenesis. In cancers where Traf4 is overexpressed [47,48], cells fail to adhere to their neighbours, epithelial integrity is compromised and migration is promoted. Thus, Traf4a may be a general regulator of the plasticity of adhesion between cells within epithelia that are experiencing cell movement and morphogenesis.

### Materials and methods

**Zebrafish husbandry**

Zebrafish embryos were developmentally staged as per [49]. Dr. Wittbrodt (U. Heidelberg) provided Tg(rx3:GFP) fish. The University of Calgary Animal Care Committee approved all procedures.

#### In situ hybridization

RNA in situ hybridization was performed as described previously [50]. Antisense riboprobe was made for traf4a by using linearized Image Clone 6970326 with the UTR removed (MGC:77418 Accession BC0659691, Open Biosystems). rx3, vax1, and six3 riboprobes were provided by Dr. D. Kurusch (U. Calgary), and the vax2 riboprobe from an Image clone (#9038293). fgf8a and pax6 riboprobes were synthesized from PCR fragments amplified from embryonic zebrafish cDNA using the primers: fgf8a: forward primer; GATGAGACTCATACCTTCAC, and reverse primer+ SP6: ATTTAGGTGACACTATAGA TCAACGCTCTCCTGAGTAG. pax6: forward primer: CTGACGTTTTTGACGAGAA, reverse+ T7: GAAA TTAATACGACTCATATAGAG, reverse primer+ SP6: ATTTAGGTGACACTATAGA TCAACGCTCTCCTGAGTAG.

### RNA isolation and RT-PCR

Total RNA from 50–75 embryos at the 21 ss was prepared by using Trizol/chloroform (Invitrogen). First strand
cDNA was made using the Superscript II RT-PCR protocol (Invitrogen), and amplified by PCR using HiFi polymerase (Thermo Scientific) and the following primers: \textit{traf4a} e1i1 MO; forward: TTCTCGTCTCTCGTCC, reverse: TAATCCAATGGGACCTGTC. \textit{traf4a} e3i3 MO; forward: GACCAGCTCCCATTGAGATTA, reverse: GACAGTCGTGTTGCAGGTGT. \textit{ef1α} forward: CGGACAGTCTCCAACACCCAA; reverse: ACCAGTCTCCAACACCCAA.

**Embryo injections**

Antisense morpholino oligonucleotides (Gene Tools LLC), mRNA (450 pg mRNA), or morpholino/mRNA combinations were injected at the 1-cell stage. mRNA for embryo injection was made by using cDNA plasmids encoding either a zebrafish \textit{traf4a}, or membrane-associated red fluorescent protein (mRFP; provided by Dr. P. Huang) and the mMessage Machine T7 Kit (Ambion) as per kit instructions. Morpholino concentrations and sequences are as follows; \textit{traf4a} e1i1, 4–8 ng (GGCCAAACGTTGCTCTTACCTGAGA), and \textit{traf4a} e3i3 12 ng (GAGATGAAAAGCGTGATTACCTGTA). The p53 MO is Danio rerio p53 from Gene Tools, LLC. For rescue experiments, \textit{traf4a} e1i1 MO was injected on its own, or with mRNA encoding full-length zebrafish \textit{traf4a}. Injected embryos that were delayed in development (> 1 hr behind control) and those with severe convergent extension defects where embryo staging was not possible were not included in our analysis.

**CRISPR/Cas9 targeted mutation of \textit{traf4a}**

The single guide RNA (sgRNA) target sequence for \textit{traf4a} (AGCGTCTCTGCCTGGCTCTCC) was identified by using MIT Optimized CRISPR Design software (Zhang lab), and made using the Maxiscript T7 In Vitro Transcription Kit (Ambion) [51]. 1nL of a mixture of \textit{traf4a} sgRNA (82ng/µL) and Cas9 Nuclease (80ng/µL, New England Biolabs) were injected into single cell Tupfel Long fin embryos. RNA was quantified by using a Nanodrop spectrophotometer. Injected embryos were pooled at 24 hpf, genomic DNA was extracted and the targeted region was amplified by PCR to detect mutations via a T7 Endonuclease assay (F: CGACTCTAGCCTGCATTGA, R: TTCCTGCAGGAGGATTACCTGTA). The p53 MO is Danio rerio p53 from Gene Tools, LLC. For rescue experiments, \textit{traf4a} e1i1 MO was injected on its own, or with mRNA encoding full-length zebrafish \textit{traf4a}. Injected embryos that were delayed in development (> 1 hr behind control) and those with severe convergent extension defects where embryo staging was not possible were not included in our analysis.

**Confocal time-lapse imaging**

\textit{Tg(rx3:GFP)} transgenic embryos were live imaged from the 6–12 ss or the 10–18 ss with a 10X or 20X objective. Embryos were embedded in 0.8% low melt agarose on glass bottom dishes (Mat-Tek). For transverse optical sections, embryos were positioned at a slight angle with their yolk sac touching the bottom of the dish and caudal end closer to the bottom than the head. For both transverse and dorsal orientations, optical sections of 3 µm step size through the eye vesicle were taken on a Zeiss LSM 700 inverted microscope every 5 minutes over a 3.5-hour window. Stacks were processed in Zen Lite and presented as maximal projections of 4–5 sections, or for the movies as weighted averages of 30 sections.

**Immunohistochemistry**

Embryos were fixed in 4% paraformaldehyde, infiltrated in 25% followed by 35% sucrose (EM Science), and embedded in Optimal Cutting Temperature (OCT; Tissue Tek). Twelve µm sections were cut on a Microm HM 500 OM cryostat, and immunostaining performed as described previously [52]. The following antibodies were used; activated Caspase-3 1:500 (Promega, G748A lot#47473), Laminin 1:200 (Sigma, L9393, Batch 103M4779), pH3 1:500 (Millipore, 06–570, lot#2202541), ZO-1 1:150 (Invitrogen, 393100, lot#QA210455), E-Cadherin 1:250 (GeneTex, GTX125890, lot#41388), β-Catenin 1:500 (Santa Cruz), aPKC 1:500 (Santa Cruz, SC-216, lot#J2604), anti-mouse or anti-rabbit Alexa Fluor 546 secondary 1:1000 (Invitrogen). Hoechst dye (1:1000, Invitrogen) and AlexaFluor 546 phalloidin (Invitrogen) were used to label nuclei and F-actin, respectively. Images were taken on a Zeiss compound microscope using an Axiovision MRc camera (Zeiss). Images were processed in Adobe Photoshop for brightness and contrast. pH3 and activated Caspase-3 immunostaining were performed in 12 ss wholemount embryos. pH3 labeled embryos were embedded in JB4 media, 7 µm transverse sections cut on a Leica microtome, and pH3+ cells labeled cells counted within the GFP + eye where it evaginates from the brain. For activated Caspase-3+, cells with the GFP+ eye vesicle were counted in wholemount. In both experiments, the experimenter was blinded to the treatment.
Eye size measurements

Photomicrographs of the dorsal view of the bilateral eye vesicles were taken of embryos with a dissecting stereo-compound epifluorescent microscope with an Axiovision HRc camera (Zeiss). The antero-posterior lengths of the right and left eye vesicles (µm) were measured.

Statistics

Statistical analyzes were performed by using SigmaStat 3.0. Error bars represent the standard deviation of the average of data from independent replicates (N). Statistical tests, numbers of independent replicates, and the numbers of embryos analyzed, are presented in the figures and their corresponding legends.

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Author Contributions

CH did all the experiments except for the time-lapse imaging done by RH, and both CH and RH provided experimental guidance and edited the manuscript. SM designed the experiments, collected data, and wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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