

RESEARCH ARTICLE

Cell division and cadherin-mediated adhesion regulate lens epithelial cell movement in zebrafish

Toshiaki Mochizuki, Yi-Jyun Luo, Hsieh-Fu Tsai, Akane Hagiwara and Ichiro Masai*

ABSTRACT

In vertebrates, lens epithelial cells cover the anterior half of the lens fiber core. During development, lens epithelial cells proliferate, move posteriorly and differentiate into lens fiber cells after passing through the equator. To elucidate the mechanisms underlying lens epithelial cell movement, we conducted time-lapse imaging of zebrafish lens epithelium. Lens epithelial cells do not intermingle but maintain their relative positions during development. Cell division induces epithelial rearrangement, which subsequently promotes cell movement towards the equator. These data suggest that cell division is the major driving force for cell movement. In zebrafish, E-cadherin is expressed in lens epithelium, whereas N-cadherin is required for lens fiber growth. E-cadherin reduced lens epithelial cell movement, whereas N-cadherin enhanced it. Laser ablation experiments revealed that lens epithelium is governed by pulling tension, which is modulated by these cadherins. Thus, cell division and cadherin-mediated adhesion regulate lens epithelial cell movement via modulation of epithelial tension.

KEY WORDS: Cadherin, Cell movement, Epithelium, Lens, Tension, Zebrafish

INTRODUCTION

Cell proliferation is a key regulator of tissue morphogenesis (Gillies and Cabernard, 2011). In the *Drosophila* wing disc, high proliferation in the central region increases anisotropic mechanical tension in the peripheral region, which subsequently orients cell division and tissue growth (LeGoff et al., 2013; Mao et al., 2013). Tension-oriented cell division reduces tissue tension and facilitates tissue spreading during zebrafish gastrulation (Caminho et al., 2013). These examples indicate that cell proliferation and epithelial tension cooperatively shape tissue growth. However, mechanisms that coordinate cell proliferation and epithelial tension for tissue shaping remain to be elucidated.

In the vertebrate lens, lens epithelium covers the anterior half of the lens fiber core. During development, lens epithelial cells proliferate and move towards the periphery of the lens epithelium, called the equator (Hanna and O'Brien, 1961). At the equator, lens epithelial cells start to differentiate into lens fiber cells (McAvoy, 1978). Thus, cell proliferation and movement towards the equator are important for lens fiber growth. However, several interesting issues have not been addressed. For example, to keep a spheroid

shape during lens growth, spatial and temporal frequencies at which lens epithelial cells pass through the equator must be equal around the equatorial circumference. However, it is unknown whether cell division density is radially uniform in the lens epithelium and, if not, how the same rate of cell movement at the equator is ensured. Quantitative and theoretical analyses on lens epithelial cell proliferation in mice was applied to understand the mechanism that regulates the size and shape of the lens during its growth (Shi et al., 2015; Šikić et al., 2015). However, cellular dynamics in the lens epithelium are largely unknown.

Classic studies using chick and mammalian lenses revealed that cell proliferation is low in the anterior region and high in the peripheral region of lens epithelium. This highly proliferative peripheral region is called the germinative zone (McAvoy, 1978; Modak et al., 1968; Zhou et al., 2006). We have established a zebrafish transgenic line that visualizes cell-cycle phases, and found a similar, highly proliferative zone in peripheral lens epithelium of zebrafish (Mochizuki et al., 2014). Furthermore, cell division orientation is biased longitudinally in the anterior region and circumferentially in the peripheral region of lens epithelium, suggesting a spatial pattern of cell division orientation. Although the FGF-Ras-MAPK pathway is required for active cell proliferation in the germinative zone in mice (Upadhyay et al., 2013), it is unclear how cell proliferation and cell division orientation are spatially regulated and how these spatial patterns influence cell movement.

Here, we have conducted time-lapse imaging of lens epithelium using a zebrafish transgenic line that visualizes cell-cycle phases. Lens epithelial cells do not intermingle but maintain their relative positions during development. Furthermore, cell division triggers epithelial rearrangement, which subsequently promotes cell movement towards the equator. Thus, cell division is a major force for cell movement. In vertebrates, E-cadherin is expressed in lens epithelium, whereas N-cadherin is required for lens fiber growth (Mochizuki et al., 2014; Pontoriero et al., 2009; Xu et al., 2002). E-cadherin reduced cell movement, whereas N-cadherin enhanced it. A laser ablation experiment revealed that lens epithelium is governed by pulling tension, which is modulated by these cadherins. Thus, cell division and cadherin-mediated cell adhesion regulate lens epithelial cell movement by modulating epithelial tension.

RESULTS

Cell division promotes cell movement in lens epithelium

We conducted time-lapse imaging of lens epithelium from 33–45 h post-fertilization (hpf), using a zebrafish transgenic line *Tg(h2afva:GFP; EF1α:mCherry-zGem)*, which visualizes cell nuclei and cell-cycle phases (Mochizuki et al., 2014) (Fig. 1A). First, we focused on the anterior lens epithelium, which covers the region from the anterior pole to the anterior border of the germinative zone (60 cells at 33 hpf, Fig. 1B and Movie 1). During the scanned period, lens epithelial cells did not intermingle, but maintained their relative

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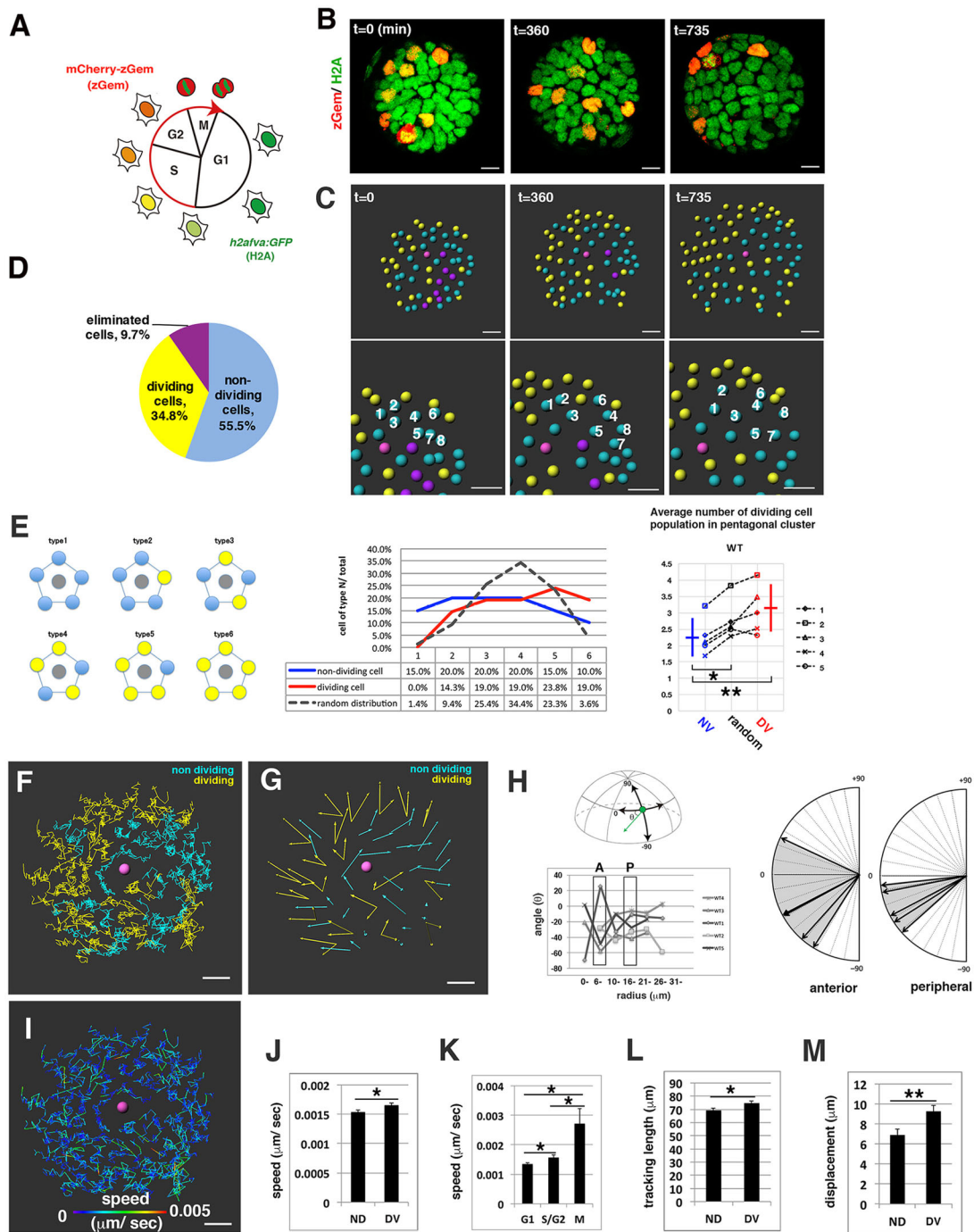


Fig. 1. Cell movement of wild-type anterior lens epithelium. (A) Fluorescence pattern of *Tg(h2afva:GFP; EF1α:mCherry-zGem)*. Adapted, with permission, from Mochizuki et al. (2014). (B) Anterior views of lens epithelium of *Tg(h2afva:GFP; EF1α:mCherry-zGem)*. Time indicates the time elapsed after 33 hpf. This lens is designated as WT1. (C) (Upper) Schematics of images shown in B. Yellow, blue and purple indicate dividing, non-dividing and eliminated cell populations, respectively. (Lower) Enlarged views of upper panels. The numbers indicate individual cells in the non-dividing cell cluster. (D) Average percentages of non-dividing, dividing and eliminated cell populations in five wild-type lenses at 33 hpf. (E) (Left) Six pentagonal patterns consisting of non-dividing (blue) and dividing (yellow) cell populations. (Middle) Distribution of pentagonal patterns of WT1 at 45 hpf. The broken gray line indicates the estimated profile of pentagonal patterns when non-dividing and dividing cell populations are randomly selected. Blue and red lines indicate the profiles of pentagonal patterns, in which non-dividing and dividing cell populations are in pentagonal centers. Non-dividing and dividing cell populations tend to assemble with the same cell type. (Right) Average number of dividing cell population in non-dividing cell population-centered (blue), dividing cell population-centered (red) pentagonal clusters and random distribution model (black). Values of five wild-type lenses are plotted. Averages and s.d. are indicated by horizontal and vertical bars, respectively. (F,G) Trajectory (F) and displacement (G) in WT1. (H) Cell movement direction along the AP axis for five wild-type lenses. (Left) AP position is defined by the lens sphere radius: $r=0$ (anterior) to $31\ \mu\text{m}$ (equator). Cell movement direction is defined by the angle (θ) between the displacement vector and the circumferential axis: $\theta=-90^\circ$ (longitudinal) to 0° (circumferential). (Right) Cell movement direction in anterior and peripheral regions, which correspond to squares marked A and P. The range of cell movement becomes more narrow in peripheral region than in the anterior region. (I) Speed of cell movement in WT1. (J,L,M) Average speed (J), tracking length (L) and displacement (M) of non-dividing (ND) and dividing (DV) cell populations in WT1. (K) Average speed of G1, S/G2 and M phase cells in WT1. Data in J-M are mean \pm s.e.m. Student's *t*-test: * $P<0.05$, ** $P<0.01$. Scale bars: $10\ \mu\text{m}$.

positions, pushing each other back and forth. Cell division mainly induces surrounding cells to move.

Lens epithelial cells are classified into three groups: during the 12 h scanned period, the first group did not undergo cell division (Fig. 1C, blue). The second group underwent one round of cell division (Fig. 1C, yellow), and the third group disappeared due to cell death or movement into lens fiber area (Fig. 1C, purple). Hereafter, these three groups are designated as the non-dividing cell population, the dividing cell population and the eliminated cell population. The average fractions of non-dividing, dividing, and eliminated cell populations in five wild-type lenses, which are designated WT1–WT5, at 33 hpf were 55.5, 34.8 and 9.7%, respectively (Fig. 1D, Fig. S1A–E); thus, more than 50% of anterior lens epithelial cells did not divide during the 12 h scan. In the eliminated cell population, 84.6% were associated with the collapse of chromatin, whereas 15.4% moved into lens fiber area, suggesting that cell death is a major cause of cell elimination (Fig. S2A).

Interestingly, non-dividing and dividing cell populations were spatially segregated (Fig. 1C, upper panels). Using another zebrafish transgenic line, *Tg(h2afva:GFP; EF1a:mCherry-CAAX)*, which visualizes cell nuclei and plasma membranes, we observed that cell division often occurred sequentially within neighboring cells, which led to clustering of their daughter cells (Fig. S2B; Movie 2). Furthermore, non-dividing cell clusters maintained their associations and moved as cell assemblies (Fig. 1C, lower panels). To examine whether clustering of dividing and non-dividing cell populations is significant, we selected five adjacent cells surrounding each lens epithelial cell and examined the profile of six pentagonal cluster types, which consist of different combinations of dividing and non-dividing cell populations (Fig. 1E, left). We compared distributional profiles of pentagonal clusters, in which dividing and non-dividing cell populations were in the center, with the estimated profile under a random distribution model (see supplementary Materials and Methods). In wild-type lenses, both dividing and non-dividing cell population-centered pentagonal clusters tend to contain the same cell type (Fig. 1E, middle). Average number of dividing cell population in dividing cell population-centered clusters was significantly higher than that of non-dividing cell population-centered clusters (Fig. 1E, right), supporting the observation that both dividing and non-dividing cell populations tend to cluster with the same cell type. We also examined pentagonal patterns of cells pre-designated as dividing or non-dividing cell population at the initial ($t=0$ min) and middle point ($t=360$ min) of the scanned period (Fig. S3A–B). The pentagonal distribution pattern of both populations was less segregated from that of the random distribution model at $t=0$ and 360 than at $t=735$ (Fig. S3C), suggesting that dividing and non-dividing cell populations became segregated during development, probably through clustered cell divisions.

Next, we examined the trajectory of cell movement (Fig. 1F) and cell displacement (Fig. 1G). The spatial movement pattern was variable in the anterior region among individual lenses; however, the movement direction was circumferentially biased in the peripheral region in wild-type lenses (Fig. 1H). The circumferential shift of cell movement in the peripheral region correlates with the spatial pattern of cell division orientation (Mochizuki et al., 2014), suggesting that cell division promotes cell movement.

We further examined three parameters of cell movement: speed (Fig. 1I), total tracking length and displacement. We compared the speed of the dividing cell population with that of the non-dividing cell population in the lens shown in Fig. 1C, namely WT1 (as a wild-type representative lens). On average, the dividing cell

population moved faster than the non-dividing cell population (Fig. 1J). We also compared cell movement speed in different cell cycle phases of all lens epithelial cells. Cells in M phase moved markedly faster than cells in G1 or S/G2 phase (Fig. 1K). Tracking length (Fig. 1L) and displacement (Fig. 1M) were longer in the dividing cell population than in the non-dividing cell population. Displacement was roughly 10% of tracking length (Fig. 1M), supporting the observation that cells pushed each other back and forth without forward movement. We confirm a similar tendency with regard to speed, tracking, and displacement in five wild-type lenses, including WT1 (Fig. 2A–C), although the difference of tracking length was less significant between dividing and non-dividing cell population ($P=0.074$, Fig. 2B). Thus, cell division promotes cell movement in lens epithelium.

E-cadherin suppresses cell movement in the anterior lens epithelium

E-cadherin (Cdh1) is expressed in lens epithelium. We previously examined a hypomorphic allele of zebrafish E-cadherin mutant, *half baked*^{rk3} (*hab*^{rk3}), and reported that the spatial pattern of cell division orientation was affected, although lens epithelial cell proliferation and lens fiber differentiation are generally normal (Mochizuki et al., 2014). In mouse E-cadherin mutants, epithelial-to-mesenchymal transition (EMT) occurs in lens epithelium after postnatal stages (Pontoriero et al., 2009). However, such EMT was not observed in the *hab*^{rk3} mutant until 96 hpf (data not shown).

To determine the role of E-cadherin in cell movement, we conducted time-lapse scanning of lens epithelium of the same allele of E-cadherin mutant, *hab*^{rk3}, combined with the transgenic line *Tg(h2afva:GFP; EF1a:mCherry-zGem)* from 33 to 45 hpf (Fig. 3A; Movie 3). The average fraction of non-dividing, dividing, and eliminated cell populations in three *hab*^{rk3} mutant lenses, which are designated E-cad1 to E-cad3 (Fig. S1F), at 33 hpf were 64.9, 22.6, and 12.4%, respectively (Fig. S1A). In the *hab*^{rk3} mutant, the non-dividing and dividing cell fractions were higher and lower than in wild type, respectively (Fig. S1B,C), although their difference was less significant. The eliminated cell fraction was not different (Fig. S1D). However, in the *hab*^{rk3} mutant, the eliminated cell population that moved into the lens fiber area was 47.8%, higher than in wild type (Fig. S2A), suggesting that E-cadherin knockdown increases the fraction of cells that moved into lens fiber area. As in the wild type, non-dividing cells assembled and moved together during the scanned period (Fig. 3B; Movie 3). However, both dividing and non-dividing cell population-centered pentagonal clusters showed a distribution pattern similar to that of the random distribution model in two of three *hab*^{rk3} mutant lenses, namely E-cad1 and E-cad2, during scanned period (Fig. 3C and Fig. S3A,B). In the remaining *hab*^{rk3} mutant lens, namely E-cad3, the dividing cell population was spatially localized in a one-sided peripheral area (Fig. S3F), so pre-designated dividing and non-dividing cell populations were already segregated at 33 hpf (Fig. S3A,B). Even in this situation, the average number of dividing cell population in pentagonal clusters was not significantly different between dividing and non-dividing cell population-centered clusters in *hab*^{rk3} mutant lenses (Fig. S3C), suggesting that dividing and non-dividing cell populations fail to be segregated.

Next, we examined the trajectory of cell movement (Fig. 3D) and cell displacement (Fig. 3E) in the *hab*^{rk3} mutant. The displacement pattern was less coordinated in the *hab*^{rk3} mutant than in the wild type (Fig. 3E). Cell movement direction was significantly less circumferential in the peripheral region of *hab*^{rk3} mutant lenses (Figs 2D and 3F). Next, we examined speed, tracking length and

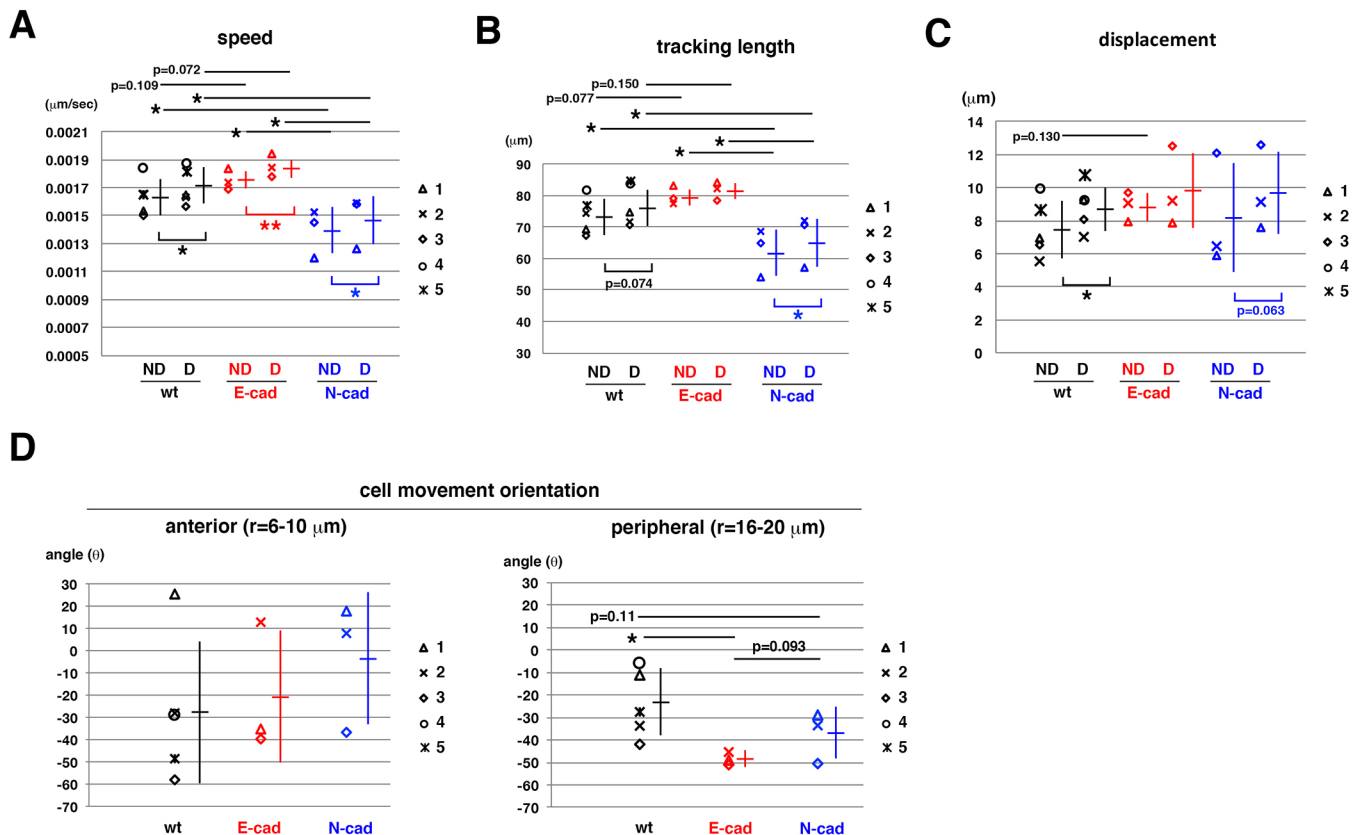


Fig. 2. Cell movement parameters of wild-type, E-cadherin mutant and N-cadherin morphant lenses. (A–D) Speed (A), tracking length (B), displacement length (C), cell movement direction (D) of anterior lens epithelial cells. Values of five wild-type, three E-cadherin mutant and three N-cadherin morphant lenses are plotted. ND and DV in A–C indicate non-dividing and dividing cell populations, respectively. Cell movement angle in the anterior zone ($r=6\text{--}10\text{ }\mu\text{m}$) and the peripheral zone ($r=16\text{--}20\text{ }\mu\text{m}$) are indicated in the left and right panels. Averages and s.d. are indicated by horizontal and vertical bars, respectively. Probabilities were calculated using Student's *t*-test: * $P<0.05$. *P*-values less than 0.15 are also indicated. Images of wild-type, E-cadherin mutant and N-cadherin morphant lenses numbered with a 1 (WT1, E-cad1 and N-cad1) are shown in Figs 1B, 3A and 4A, respectively.

displacement of a *hab^{rk3}* mutant lens shown in Fig. 3A, namely E-cad1 (as an E-cadherin mutant representative lens), and compared with those of the wild-type representative lens, WT1. The average speed of all lens epithelial cells was faster in E-cad1 than in WT1 (Fig. 3G,H), and both dividing and non-dividing cell populations contributed to this increased speed (Fig. 3I). Movement of G1 phase cells was significantly faster in E-cad1 than in WT1, whereas the speed of S/G2 and M phase cells did not differ between E-cad1 and WT1 (Fig. 3J), suggesting that movement is enhanced mainly in G1 phase in the *hab^{rk3}* mutant. Average tracking length of all lens epithelial cells was longer in E-cad1 than in WT1 (Fig. 3K). Longer tracking length was observed in both dividing and non-dividing cell populations in E-cad1 (Fig. 3L). By contrast, average displacement of all lens epithelial cells was similar between WT1 and E-cad1 (Fig. 3M). Displacement of non-dividing and dividing cell populations was also similar between WT1 and E-cad1 (Fig. 3N). These data suggest that E-cadherin suppresses cell movement in the anterior lens epithelium.

We compared five wild-type and three *hab^{rk3}* mutant lenses, including WT1 and E-cad1. Average speed and tracking length were slightly higher in the *hab^{rk3}* mutant than in wild type for both dividing and non-dividing cell population, although their differences were less significant ($0.05<P<0.15$, Fig. 2A,B). Average displacement of non-dividing cell population in the *hab^{rk3}* mutant was slightly higher than in wild type ($P=0.13$), but displacement of dividing cells was not different from that of wild

type (Fig. 2C). Thus, cell movement speed is faster in the E-cadherin mutant than in wild type, but faster speed does not correlate with longer displacement, especially for a dividing cell population.

Cell movement in the equatorial region

In wild type, cell movement direction is circumferentially biased in peripheral lens epithelium (Fig. 1H). At the equator, cells start to differentiate into lens fibers, which elongate along the anteroposterior (AP) axis of the lens sphere. We conducted time-lapse imaging of the equatorial region of wild-type lenses from 33 to 45 hpf, using *Tg(h2afva:GFP; EF1α:mCherry-zGem)* (Fig. S4A, upper panels; Movie 4). We focused on seven cell rows covering the equatorial region along the AP axis (Fig. S4A, lower panels), and examined their trajectories and displacements (Fig. S4B). Cells located anterior to the equator moved circumferentially, whereas cells in or posterior to the equator moved posteriorly. Thus, cell movement direction changes at the equator (Fig. S4E).

Next, we examined equatorial cell movement in the *hab^{rk3}* mutant (Fig. S4C,D; Movie 5). As in the wild type, cell movement direction changes at the equator in the *hab^{rk3}* mutant (Fig. S4E). We also examined speed, tracking length and displacement in the equatorial region. In wild-type lenses, speed and tracking length gradually increased along the AP axis (Fig. S4F,G), and displacement increased markedly after cells pass through the equator (Fig. S4H). In the *hab^{rk3}* mutant, speed and tracking length corresponded to the highest part of the wild-type range

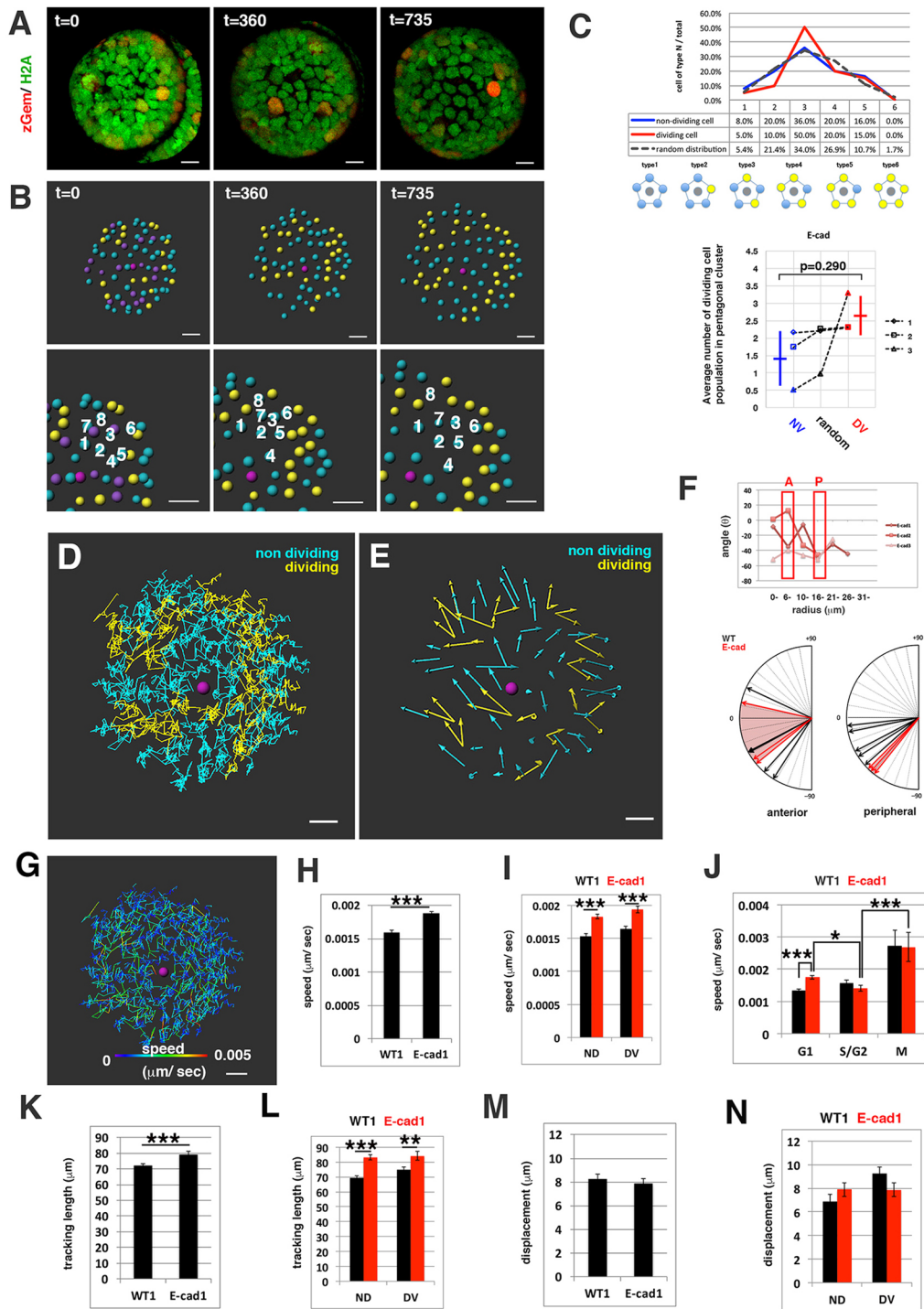


Fig. 3. Cell movement in anterior lens epithelium of the E-cadherin mutant. (A) *hab^{rk3}* mutant lens epithelium combined with *Tg(h2afva:GFP; EF1α:mCherry-zGem)*. This lens is designated as E-cad1. (B) (Upper) Schematics of images shown in A. Yellow, blue and purple indicate dividing, non-dividing and eliminated cell populations, respectively. (Lower) Enlarged view of upper panels. Numbers indicate individual cells in the non-dividing cell cluster. (C) (Upper) Distribution of pentagonal patterns of E-cad1 at 45 hpf. Peaks of non-dividing and dividing cell populations are the same as that of the random distribution model, suggesting that dividing and non-dividing cell populations fail to segregate. (Bottom) Average number of dividing cell population in non-dividing cell population-centered (blue), dividing cell population-centered (red) pentagonal clusters and random distribution model (black). Values of three *hab^{rk3}* mutant lenses are plotted. E-cad1 is indicated by the number 1. Averages and s.d. are indicated by horizontal and vertical bars, respectively. (D,E) Trajectory (D) and displacement (E) of cell movement in E-cad1. (F) (Upper) Cell movement direction in three *hab^{rk3}* mutant lenses. A and P indicate anterior and peripheral regions, respectively. (Bottom) Cell movement direction in anterior and peripheral regions, which correspond to squares marked A and P, in wild-type (black arrows, shown in Fig. 1H) and *hab^{rk3}* mutant lenses (red arrows). The range of cell movement direction of the *hab^{rk3}* mutant is indicated in red and is less circumferential than that of the wild type in the peripheral region. (G) Speed of cell movement on trajectory of E-cad1. (H,K,M) Cell movement speed (H), tracking length (K) and displacement (M) of all lens epithelial cells in WT1 and E-cad1. (I,L,N) Cell movement speed (I), tracking length (L) and displacement (N) of non-dividing (ND) and dividing (DV) cell populations in E-cad1 (red) and WT1 (black). (J) Cell movement speed of G1, S/G2 and M phase cells in E-cad1 (red) and WT1 (black). The error bars in H–N indicate s.e.m. Student's *t*-test: **P*<0.05, ***P*<0.01, ****P*<0.005. Scale bars: 10 μm.

(Fig. S4F,G), whereas displacement was highly variable (Fig. S4H). Thus, E-cadherin mutant cells move faster than wild-type cells in the equatorial region, but higher speed did not correlate with longer displacement.

N-cadherin promotes cell movement in the anterior lens epithelium

In chick, N-cadherin (Cdh2) promotes lens fiber differentiation, by complexing with α -catenin and actin filaments (Leonard et al., 2011). We also reported that N-cadherin is required for lens fiber elongation in zebrafish (Masai et al., 2003). Consistently, zebrafish N-cadherin was expressed in lens epithelium, but more intensely in elongating lens fiber cells (Fig. S5A). Here, we injected a morpholino antisense oligo against N-cadherin (MO-Ncad) at low concentrations. This induced typical N-cadherin mutant defects, such as failure of neural tube closure (Fig. S5B), but did not grossly affect lens epithelial cell proliferation and lens fiber differentiation (Fig. S5C,D). In the N-cadherin morphant, the lens fiber core was smaller than in wild-type lenses, but the number of lens epithelial cells was not altered (Fig. S5E). Thus, low doses of MO-Ncad reduces only the size of the lens fiber core.

Next, we conducted time-lapse imaging of lens epithelium of the N-cadherin morphant, combined with *Tg(h2afva:GFP; EF1a:mCherry-zGem)* from 33 to 45 hpf (Fig. 4A; Movie 6). The average fractions of non-dividing, dividing and eliminated cell populations in three N-cadherin morphant lenses [which are designated N-cad1 to N-cad3 (Fig. S1G)] at 33 hpf were 49.8, 40.4 and 9.8%, respectively (Fig. S1A), values that are not significantly different from wild type (Fig. S1B–D). In contrast to the *hab^{rk3}* mutant, 94.7% of eliminated cells were associated with cell death in the N-cadherin morphant, whereas only 5.3% of eliminated cells moved into the lens fiber area (Fig. S2A). Non-dividing and dividing cell populations became spatially segregated to form clusters in the N-cadherin morphant during development (Fig. 4B, upper panels). Furthermore, non-dividing cell clusters moved as cell assemblies during the scanned period (Fig. 4B, lower panels). Interestingly, non-dividing and dividing cell population clusters were more segregated in the N-cadherin morphant than in wild type at 45 hpf (Fig. 4C). We examined the formation of non-dividing and dividing cell population clusters in the N-cadherin morphant during development (Fig. S3A, B). The average number of dividing cell population was significantly higher in dividing cell population-centered clusters and lower in non-dividing cell population-centered clusters than in the random distribution model after $t=360$ min (Fig. S3C), suggesting that segregation of dividing and non-dividing cell populations is enhanced in the N-cadherin morphant.

Next, we examined trajectories of cell movement (Fig. 4D) and cell displacement (Fig. 4E) in N-cadherin morphant lenses. Cell movement direction was less biased circumferentially in the peripheral region, although the difference between wild-type and N-cadherin morphant was less significant ($P=0.11$) (Figs 2D and 4F). We examined speed (Fig. 4G), tracking length and displacement in the N-cadherin morphant. We compared a N-cadherin morphant lens shown in Fig. 4A, namely N-cad1 and the representative wild-type lens WT1. The average speed of all lens epithelial cells was slower in N-cad1 than in WT1 (Fig. 4H), and the slower speed was observed in both dividing and non-dividing cell populations (Fig. 4I). Speeds of G1 and S/G2 cells were significantly lower in N-cad1 than in WT1, whereas the speed of M-phase cells was not significantly different (Fig. 4J). Average tracking length of all lens epithelial cells was shorter in N-cad1 than in WT1 (Fig. 4K), and the shorter tracking length was observed in

both non-dividing and dividing cell populations (Fig. 4L). Average displacement of all lens epithelial cells was shorter in N-cad1 than in WT1 (Fig. 4M). However, the shorter displacement was significant only in dividing cell populations (Fig. 4N). These data suggest that N-cadherin promotes cell movement in the anterior lens epithelium.

We compared five wild-type lenses with three N-cadherin morphant lenses, including WT1 and N-cad1. In the N-cadherin morphant, average speed and tracking length were significantly lower than those of wild type for dividing and non-dividing cell populations (Fig. 2A,B). However, displacement was not different between wild type and the N-cadherin morphant (Fig. 2C). This is partly because one of three N-cadherin morphant lenses, N-cad3, showed exceptionally high displacement (Fig. 2C). We examined the spatial pattern of displacement (Fig. S6). In WT1 and N-cad1, cells with high displacements (>15 μm) were few and were located in the peripheral region. By contrast, many peripheral cells showed high displacement (>15 μm) in N-cad3. These peripheral cells are the primary dividing cell population that moved *en masse* toward the equator (Fig. S6). Such large clustered cell divisions near the equator may increase displacement length, regardless of lower speed. Thus, speed is slower, but slower speed does not correlate with shorter displacement in the N-cadherin morphant.

N-cadherin suppresses cell movement toward the equator

We conducted time-lapse scanning of the equatorial region in N-cadherin morphant lenses combined with *Tg(h2afva:GFP; EF1a:mCherry-zGem)* (Fig. S7A, upper panels; Movie 7). We focused on seven cell rows covering the equatorial region (Fig. S7A, lower panels). Cell trajectories (Fig. S7B) and the spatial profile of cell movement direction (Fig. S7C) indicate that all rows of cells moved posteriorly in the N-cadherin morphant. Speed, tracking length and displacement increased in the N-cadherin morphant compared with wild type (Fig. S7D–F). In contrast to the anterior lens epithelium, N-cadherin prevents cell movement in the peripheral region.

Epithelial tension is reduced in E- and N-cadherin knockdown lenses

In general, cadherin-mediated cell adhesion counterbalances epithelial tension. To clarify lens epithelial tension, we used a laser to ablate single cells in the anterior lens epithelium of the transgenic line *Tg(h2afva:GFP; EF1a:mCherry-CAAX)*, and performed time-lapse imaging to examine the temporal change of the ablated area from 15 s before ablation to 255 s post-ablation. In 50 hpf wild-type lenses, the ablated area initially increased to a maximum at 30 s post-ablation, and progressively decreased thereafter (Fig. 5A,B). Initial expansion of the ablated area was isotropic (data not shown). We examined actomyosin contraction, which represents healing processes associated with epithelial damage (Abreu-Blanco et al., 2011, 2012), using a zebrafish transgenic line *Tg(actb1:myl12.1-eGFP; EF1a:mCherry-CAAX)*, which visualizes myosin 2 and cell membranes (Behrndt et al., 2012). In wild type, myosin 2 accumulated beneath cell membranes adjacent to the ablated area by 99 s post-ablation (Fig. S8; Movie 8), suggesting that initial expansion and later shrinkage of the ablated area depend on physical tension and on actomyosin-mediated healing, respectively. Initial expansion of the ablated area suggests that wild-type lens epithelial cells pull each other.

In *hab^{rk3}* mutant lenses, the ablated area did not increase after ablation (Fig. 5A,C,E), suggesting that epithelial tension is low. Furthermore, the ablated area failed to shrink after 30 s post-ablation

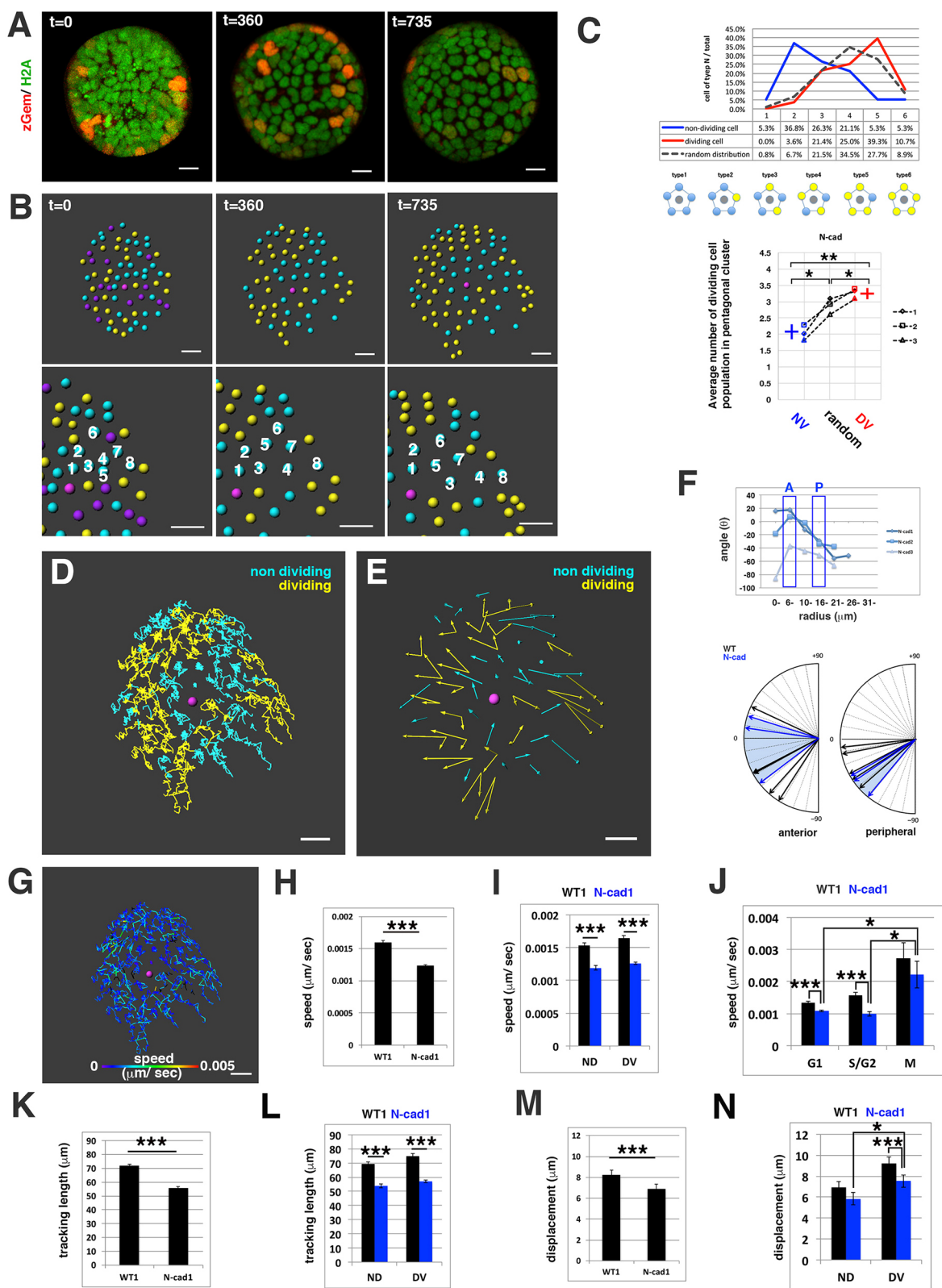


Fig. 4. See next page for legend.

(Fig. 5C,F), suggesting that healing requires E-cadherin. Interestingly, in the E-cadherin morphant, myosin 2 accumulation was slower than in wild type, and proceeded irregularly in patches (Fig. S8; Movie 9).

In N-cadherin morphant lenses, the ablated area did not expand after ablation (Fig. 5A,D,E), suggesting that epithelial tension is low. Later shrinkage of the ablated space normally proceeded after 30 s post-ablation (Fig. 5D,F), suggesting that healing does not

Fig. 4. Cell movement in anterior lens epithelium of the N-cadherin morphant. (A) N-cadherin morphant lens epithelium combined with *Tg(h2afva::GFP; EF1a::mCherry-zGem)*. This lens is designated as N-cad1. (B) (Upper) Schematics of images shown in A. Yellow, blue and purple indicate dividing, non-dividing and eliminated cell populations, respectively. (Lower) Enlarged view of upper panels. Numbers indicate individual cells in the non-dividing cell cluster. (C) (Upper) Distribution of pentagonal patterns of N-cad1 at 45 hpf. A peak shift is observed in both non-dividing and dividing cell populations. Furthermore, the segregation level is more prominent in the N-cadherin morphant. (Bottom) Average number of dividing cell population in non-dividing cell population-centered (blue), dividing cell population-centered (red) pentagonal clusters and random distribution model (black). Values of three N-cadherin morphant lenses are plotted. N-cad1 is indicated by the number 1. Averages and s.d. are indicated by horizontal and vertical bars, respectively. (D,E) Trajectory (D) and displacement (E) of cell movement in N-cad1. (F) (Upper) Cell movement direction in three N-cadherin morphant lenses. A and P indicate the anterior and peripheral regions, respectively. (Bottom) Cell movement direction in anterior and peripheral regions, corresponding to squares marked A and P, in wild-type (black arrows, shown in Fig. 1H) and N-cadherin morphant lenses (blue arrows). The range of cell movement direction of N-cadherin morphants is indicated in blue and is less circumferential than that of the wild type in the peripheral region. (G) Speed of cell movement on trajectory of N-cad1. (H,K,M) Cell movement speed (H), tracking length (K) and displacement (M) of all lens epithelial cells in WT1 and N-cad1. (I,L,N) Cell movement speed (I), tracking length (L) and displacement (N) of non-dividing (ND) and dividing (DV) cell populations in N-cad1 (blue) and WT1 (black). (J) Cell movement speed of G1, S/G2 and M phase cells in N-cad1 (blue) and WT1 (black). The error bars in H–N indicate the s.e.m. Student's *t*-test: **P*<0.05, ***P*<0.01, ****P*<0.005. Scale bars: 10 μ m.

require N-cadherin. In the N-cadherin morphant, myosin 2 accumulation was initiated with normal timing (Fig. S8; Movie 10), but proceeded irregularly in patches, as with the E-cadherin morphant.

Apical cell size, and perhaps nuclear volume, are influenced by the balance between epithelial tension and cell adhesion. Previously, we reported that the perimeter length of lens epithelial cells is longer in the E-cadherin mutant than in wild type (Mochizuki et al., 2014). We found that the apical size of anterior lens epithelial cells was larger in the *hab^{rk3}* mutant and smaller in the N-cadherin morphant at 50 hpf (Fig. 5G). We also measured nuclear volumes of lens epithelial cells. Nuclear volume was more variable in the *hab^{rk3}* mutant than in wild type, whereas it was significantly lower in the N-cadherin morphant than in wild type (Fig. S9).

Cell division triggers cell intercalation, which subsequently promotes cell movement

Cell division is likely to relax epithelial tension, enabling surrounding cells to move towards the equator. To clarify the relationship between cell division, epithelial rearrangement and cell movement, we examined their temporal profiles in lens epithelium. Using time-lapse imaging of anterior lens epithelium in wild-type transgenic line *Tg(h2afva::GFP; EF1a::mCherry-CAAX)* (Fig. S2B), we focused on a particular area consisting of 10–15 lens epithelial cells. In wild type, cell division often occurred sequentially in short time windows, followed by cell intercalation (Fig. 6A, Fig. S10A,B). Thus, the period of cell division was segregated from that of cell intercalation. We further calculated the rate of area expansion per scanned frame and sorted it into three periods: cell division, cell intercalation and the rest period when no cell division or cell intercalation occurred (Fig. 6C, Fig. S10E–F). The average rate of area expansion of three wild-type lenses (Fig. 6E) was 0.090 (% of original area size/min) for the cell-division period, but 0.234 (%/min) for the cell-intercalation period, which is twice higher than that of the cell-division period. Interestingly, the expansion rate was -0.015 (%/min) during the rest period. These data suggest that cell

division itself does not promptly increase the area, but later cell intercalation increases the area accompanied with cell movement.

Next, we examined temporal profiles of cell division, cell intercalation and lens epithelial area size in the *hab^{rk3}* mutant. In contrast to wild type, cell intercalation often occurred during the cell division period (Fig. 6B; Fig. S10C,D). We calculated the rate of area expansion per scanned frame (Fig. 6D; Fig. S10G,H). The average area expansion rate of three *hab^{rk3}* mutant lenses (Fig. 6E) was 0.181, 0.154 and 0.179 (%/min) for periods of cell division, cell intercalation and rest, respectively. These data suggest that expansion rate increases during the cell division period in the *hab^{rk3}* mutant, although the *hab^{rk3}* mutant showed more individual variation. Furthermore, continuous enlargement occurs during the rest period in the *hab^{rk3}* mutant. Overlapping of cell division and cell intercalation periods indicate that the reduction of cell adhesion allows surrounding cells to move immediately after cell division. Thus, E-cadherin suppresses intercalation of surrounding cells neighbor to cell division, resulting in temporal segregation of cell division and cell intercalation periods. Furthermore, E-cadherin inhibits expansion of the apical areas of lens epithelial cells during the rest period.

DISCUSSION

In this study, we conducted time-lapse imaging of lens epithelium using zebrafish, and found several interesting traits of lens epithelial cells. First, lens epithelial cells are classified into dividing and non-dividing cell populations. These two populations are spatially segregated during development. Second, in non-dividing cell clusters, cells do not intermingle, but move as cell assemblies. Third, cell movement direction is biased circumferentially in the peripheral lens epithelium, similar to cell division orientation, indicating a correlation between cell division and cell movement. Finally, cell division triggers cell intercalation, which subsequently promotes cell movement. Thus, cell division is a major driving force for cell movement in lens epithelium.

Cell division triggers cell intercalation, which promotes lens epithelial cell movement

As both cell movement direction and cell division orientation are similarly biased to the circumferential direction in the peripheral lens epithelium in zebrafish, we initially assumed that cell division simply generates directional cell movement, as previously reported for zebrafish gastrulation (Gong et al., 2004) and *Drosophila* germband extension (da Silva and Vincent, 2007). However, the case of zebrafish lens is more complex. Cell division often occurred sequentially within neighboring cells, resulting in clustering of dividing cells. Furthermore, the non-dividing cell population did not intermingle, but moved as a cell assembly. Thus, it is likely that expansion of dividing cell clusters promotes collective movement of neighboring non-dividing cells. Intriguingly, clustered cell divisions do not overlap temporally with cell intercalation, and the expansion rate of lens epithelial area is higher during the cell interaction period than during cell division period. These observations suggest that cell division does not drive prompt movement of surrounding cells, but induces cell intercalation, which subsequently promotes cell movement.

An interesting question is how are dividing cell populations clustered? In wild-type lens, dividing and non-dividing cell populations are gradually segregated during development, suggesting that cell division promotes clustering of these two cell populations. Furthermore, these two cell populations failed to segregate in the E-cadherin mutant. As the fraction of dividing cells is reduced in the E-cadherin mutant, decreased cell division may delay cluster formation. The second possibility is that segregation of

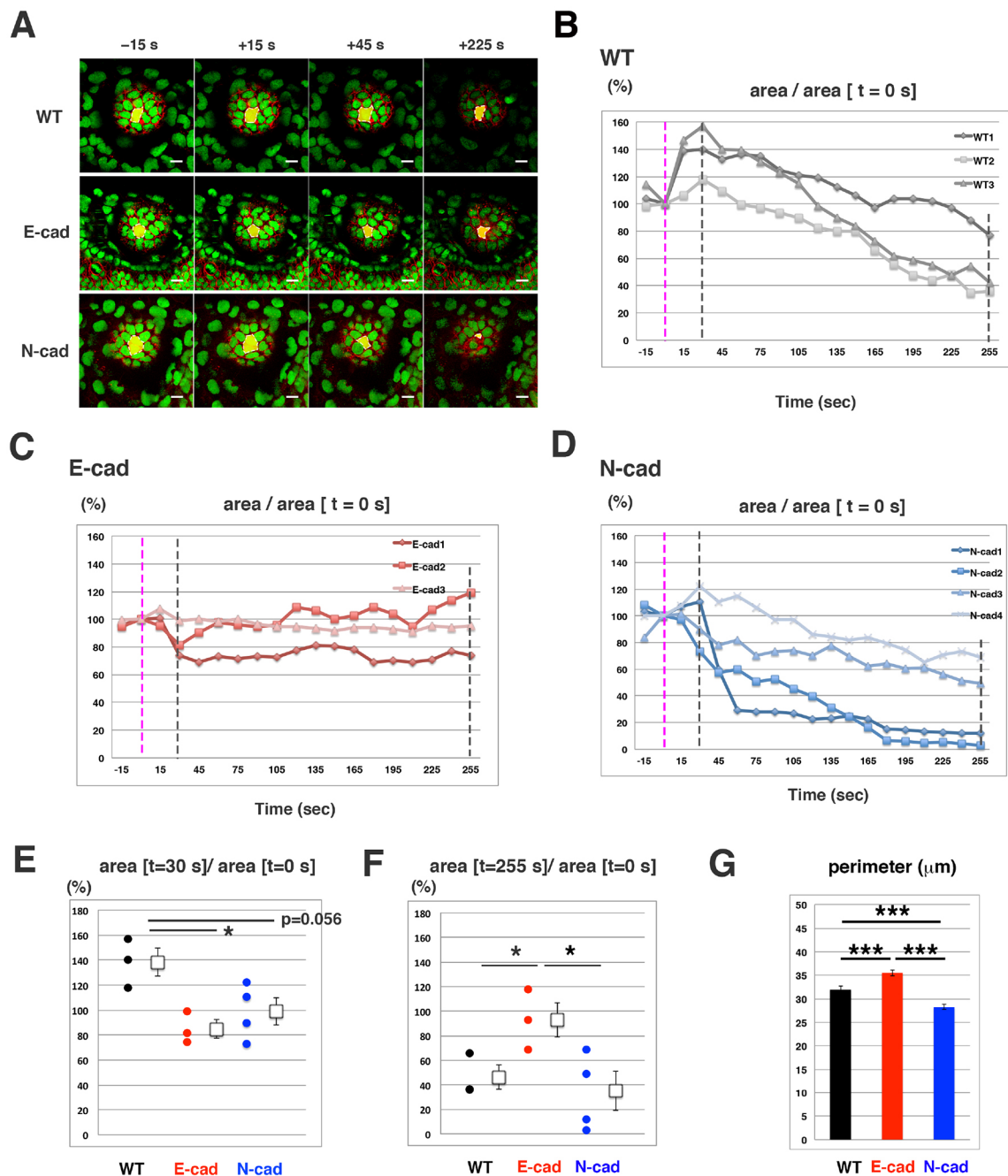


Fig. 5. Laser ablation of lens epithelial cells. (A) Time lapse scanning of 50 hpf wild-type, E-cadherin mutant and N-cadherin morphant lenses. Time indicates the time elapsed after laser ablation. Ablated cells are indicated in green. (B-D) Temporal profile of the ablated area at 50 hpf wild-type (B), E-cadherin mutant (C) and N-cadherin morphant (D) lenses. Ablated areas are normalized by the initial area at t=0 (pink dotted line). Black dotted lines indicate the time when the ablated area reaches its peak in the wild type (t=30) as well as the time at the end of scanning (t=255). (E,F) The ablated area at t=30 (E) and t=255 (F) relative to the initial area at t=0. (G) Perimeter length of wild-type, E-cadherin mutant and N-cadherin morphant lenses at 50 hpf. The error bars indicate the s.e.m. Student's *t*-test: **P*<0.05, ****P*<0.005. Scale bars: 10 μm.

dividing and non-dividing cell populations is not maintained in the E-cadherin mutant. In general, cell intercalation is regulated by remodeling of adherens junctions, which requires dynamic, but stable, coupling with E-cadherin and myosin 2 accumulation (Bardet et al., 2013; Levayer and Lecuit, 2013). As cell intercalation occurs abnormally during the cell division period in the E-cadherin mutant, loss of adherens junctions in the E-cadherin mutant may lead to improper epithelial remodeling, which disrupts clustering of non-dividing cells.

It was reported that rat kidney epithelial cells show stochastically pulsed MAPK activation *in vitro*, the frequency of which correlates with cell proliferation rate (Aoki et al., 2013). Interestingly, this pulsed MAPK activity is propagated to neighbor cells through EGF/Ras/MAPK signaling pathways. A similar propagation mechanism of cell proliferation may underlie clustering of cell division in zebrafish lens epithelium. Given that the frequency of MAPK activity pulses depends on cell density (Aoki et al., 2013), it would be interesting to investigate whether the level of cell proliferation

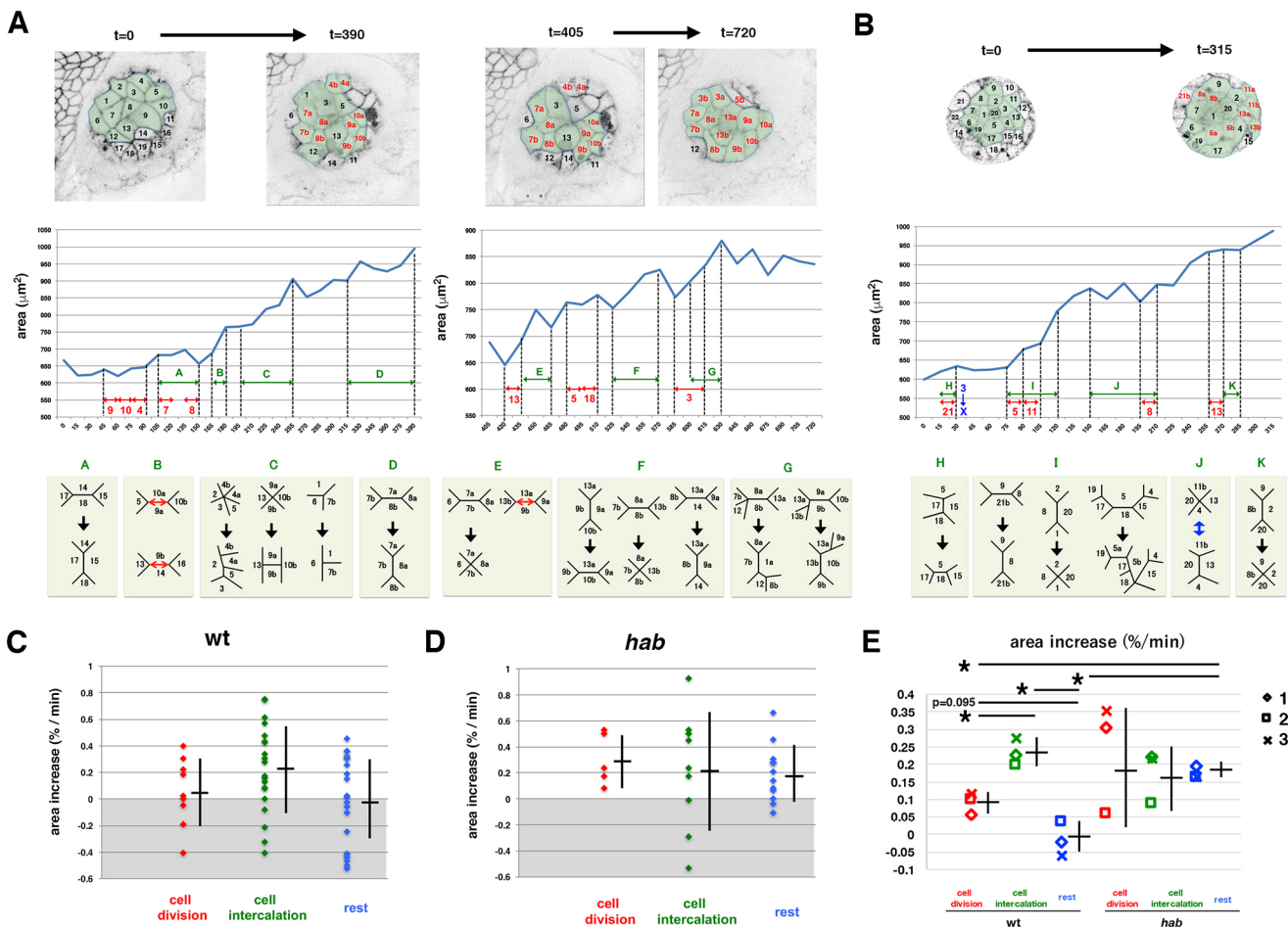


Fig. 6. Temporal profile of lens epithelial expansion of the wild type and the E-cadherin mutant. (A) Wild-type lens epithelial area, consisting of 12 cells during the first 390 min (left panels) and 10 cells during the last 315 min (right panels). The top panels indicate the composition of individual lens epithelial cells. Divided daughter cells are in red. Middle panels display temporal profiles of the area. Red and green arrows indicate cell division events and periods when cell intercalation occurred, respectively. Bottom panels indicate cell intercalation that occurred in the corresponding period of middle panels. (B) Temporal change of *hab^{rk3}* mutant lens epithelial area consisting of 14 cells during the first 315 min. (C,D) Expansion rate per time frame of lens epithelial area of wild-type (C) and *hab^{rk3}* mutant (D) lens shown in A and B, respectively, during periods of cell division (red), cell intercalation (green) and the rest period (blue). Averages and s.d. are indicated by horizontal and vertical bars, respectively. The average value of the cell intercalation period is higher than others in wild type, whereas average values are similar during all three periods in *hab^{rk3}* mutant. (E) The average rate of lens epithelial expansion in three wild-type and *hab^{rk3}* mutant lenses. Averages and s.d. are indicated by horizontal and vertical bars, respectively. Although values of cell division period in the *hab^{rk3}* mutant are variable, profiles were similar to those shown in C and D. Student's *t*-test: **P*<0.05.

depends on epithelial tension. Epithelial tension is transmitted through adherens junctions via E-cadherin (Borghi et al., 2012; Yonemura et al., 2010). A smaller dividing cell population in the E-cadherin mutant may indicate that higher tension promotes cell proliferation in zebrafish lens epithelium.

Cadherins regulate cell movement through epithelial tension

Speed of lens epithelial cell movement is increased in E-cadherin mutant, but decreased in N-cadherin morphant. Our laser ablation experiments show that lens epithelial cells pull each other, and that such pulling tension is reduced in both E-cadherin mutant and N-cadherin morphant. As N-cadherin is required for lens fiber elongation (Masai et al., 2003), defects in this process may affect the interface structure between the lens epithelium and lens fiber core, which physically reduces lens epithelial cell movement in the N-cadherin morphant. Alternatively, lower epithelial tension may cause slower cell movement in the N-cadherin morphant. Because lens epithelium covers the anterior half of the lens fiber core, the growth of the lens fiber core promoted by N-cadherin may increase

tensile force on the lens epithelium, which E-cadherin-dependent cell adhesion counterbalances to preserve as elastic energy (Fig. S11A). As E-cadherin is required for actomyosin accumulation underneath plasma membranes (Levayer and Lecuit, 2013), irregular and patchy accumulation of myosin 2 in response to laser ablation suggests that E-cadherin-mediated cell adhesion, which balances with epithelial tension in equilibrium, becomes lower in the N-cadherin morphant. In this case, epithelial tension generated by lens fiber growth is decreased in the N-cadherin morphant, resulting in slower cell movement and smaller apical cell and nuclear size (Fig. S11B).

However, the situation in the E-cadherin mutant is complex. Although N-cadherin-generated tensile force may be intact in the E-cadherin mutant, E-cadherin mutant epithelium could not preserve epithelial tension in the lens epithelium because of the loss of adherens junctions. Furthermore, E-cadherin mutant cells are likely to have increased fluidity of cell movement. By sudden removal of single cells using laser ablation in the E-cadherin mutant, surrounding cells could not sense tension from neighboring cells

but just filled the ablated space because of increased cell fluidity (Fig. S11B). The fraction of cell division is decreased in E-cadherin mutant lens epithelium, which may reduce epithelial tension by the delay of lens fiber growth. Reduction of lens epithelial cell number and higher flexibility of cell shape may explain increased apical cell size in E-cadherin mutant. Loss of adherens junction may compromise force transmission from plasma membranes to nuclear membranes, which would contribute to variable nuclear size. Further investigation is required to fully address molecular mechanisms that induce these E-cadherin mutant phenotypes.

In summary, our findings suggest that E-cadherin and N-cadherin cooperatively regulate lens epithelial cell movement by modulating epithelial tension. Epithelial tension is a key mediator that links cell division and cell movement in zebrafish lens epithelium.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish (*Danio rerio*) were maintained as described previously (Westerfield, 1993). OIST wild type, *oki*, zebrafish *e-cadherin/cdh1* mutant, *half baked*^{rk3} (Shimizu et al., 2005) and the transgenic lines *Tg(EF1α:mCherry-zGem)*^{oki011} (Mochizuki et al., 2014), *Tg(h2afva:GFP)*^{kca6/kca66} (Pauls et al., 2001), *Tg(EF1α:mCherry-CAAX)*^{oki049} and *Tg(actb1:myl12.1-eGFP)* (Behrndt et al., 2012) were used. Animal care and experimental protocols were approved by the OIST Institutional Animal Care and Use Committee.

Tg(EF1α:mCherry-CAAX) transgenic line

The CAAX domain of zebrafish *hrasa* (NM_001017263) fused to mCherry was subcloned into a Tol2 transposon-based expression vector, pT2AL200R150G (Urasaki et al., 2006). This DNA construct was injected into fertilized eggs with Tol2 transposase mRNA, to establish a transgenic line, namely *Tg(EF1α:mCherry-CAAX)*.

Morpholino

Morpholino antisense oligos against *n-cadherin/cdh2* (MO-Ncad) and *e-cadherin/cdh1* (MO-Ecad) were designed as described previously [see ncad-MO1 in Lele et al. (2002) and MO3-cdh1 in Babb and Marrs (2004)]. MO-Ncad and MO-Ecad were injected into fertilized eggs at 50 and 20 μM, respectively.

Time-lapse imaging and image analyses

Time lapse images of zebrafish lens epithelium with the transgene *Tg(h2afva:GFP; EF1α:mCherry-zGem)* or *Tg(h2afva:GFP; EF1α:mCherry-CAAX)* were obtained using the confocal laser scanning microscope and analyzed using Imaris software (ver. 7.6.5, Bitplane). Detailed procedures are described in the supplementary Materials and Methods.

Laser ablation experiments

Single cells were ablated using a UV laser pulse and epithelial cells were scanned in time-lapse at 11–15 s intervals. Using Image-J software, the ablated area was analyzed. Detailed procedures are described in the supplementary Materials and Methods.

Determination of cell movement direction

Cell movement direction in the lens epithelium was calculated using time-lapse 3D images from an anterior view. Detailed procedures are described in the supplementary Materials and Methods.

Evaluation of clustering

Using images of *Tg(h2afva:GFP; EF1α:mCherry-zGem)*, we selected the five cells closest to the cell of interest, forming a virtual pentagonal of surrounding cells. Combinations of dividing and non-dividing cell numbers in pentagonal cell clusters were determined. Distribution profiles of pentagonal cluster patterns with a centered dividing cell population and a centered non-dividing cell population, and of the random distribution model were compared. Detailed procedures are described in the supplementary Materials and Methods.

Histology

Immunostaining was performed as described previously (Imai et al., 2010). Antibodies against Pax6 (Covance PRB-278P, 1:100), PCNA (clone PC10; Sigma, 1:100), Prox1 (GTX128354; Gene Tex, 1:500) and N-cadherin (GTX125885; Gene Tex, 1:100–200) were used.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.M. and I.M. designed the experiments, analyzed the data and prepared the manuscript. T.M. and Y.-J.L. performed time-lapse imaging experiments. T.M., H.-F.T. and A.H. performed laser ablation experiments.

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Supplementary information

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