

## RESEARCH ARTICLE

# Tight junctions negatively regulate mechanical forces applied to adherens junctions in vertebrate epithelial tissue

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## ABSTRACT

Epithelia are layers of polarised cells tightly bound to each other by adhesive contacts. Epithelia act as barriers between an organism and its external environment. Understanding how epithelia maintain their essential integrity while remaining sufficiently plastic to allow events such as cytokinesis to take place is a key biological problem. In vertebrates, the remodelling and reinforcement of adherens junctions maintains epithelial integrity during cytokinesis. The involvement of tight junctions in cell division, however, has remained unexplored. Here, we examine the role of tight junctions during cytokinesis in the epithelium of the *Xenopus laevis* embryo. Depletion of the tight junction-associated proteins ZO-1 and GEF-H1 leads to altered cytokinesis duration and contractile ring geometry. Using a tension biosensor, we show that cytokinesis defects originate from misregulation of tensile forces applied to adherens junctions. Our results reveal that tight junctions regulate mechanical tension applied to adherens junctions, which in turn impacts cytokinesis.

This article has an associated First Person interview with the first author of the paper.

**KEY WORDS:** Epithelium, Tight junctions, Adherens junctions, ZO-1, GEF-H1, Mechanotransduction, *Xenopus laevis*, Cytokinesis

## INTRODUCTION

Epithelia are found in all metazoans and represent a basic type of cell organisation that is required to ensure the physical cohesion necessary to form tissues and organs. By acting as mechanical and chemical barriers, epithelia constitute vital fences between different tissue compartments, allowing polarised absorption and secretion. Epithelia comprise polarised cells, which maintain strong cell–cell contacts to guarantee the integrity necessary for barrier functions. At their apical side, epithelial cells develop a junctional complex, formed by adherens and tight junctions, which seals cell–cell contacts. Adherens junctions provide mechanical attachments between neighbouring cells (Lecuit and Yap, 2015). These junctions are composed of cadherin, a transmembrane protein whose extracellular domain creates homophilic interactions with cadherin of neighbouring cells. Cadherin indirectly links the actin cytoskeleton, via association of  $\alpha$ - and  $\beta$ -catenins to its intracytoplasmic tail (Leckband and de Rooij, 2014). In vertebrates, tight junctions (zonula occludens, ZO) are the most apical

junctions. They act as regulators of transepithelial permeability, intramembrane barriers and signalling platforms (Citi et al., 2014). Tight junctions are composed of a variety of transmembrane proteins, such as claudins and occludin, which are connected intracytoplasmically to the actin cytoskeleton via ZO proteins.


Although epithelia maintain strong cell–cell contacts, which guarantee the integrity indispensable for barrier functions, they also preserve plasticity, which is crucial during development and morphogenesis. Morphogenesis relies on diverse cellular events, including division, intercalation and extrusion – all of which depend on the contractility of the actomyosin cytoskeleton. During tissue remodelling, which occurs during development and morphogenesis, cell–cell contacts are maintained, thus preserving tissue integrity. This requires that adherens junctions and mechanical forces produced by the actomyosin cytoskeleton are intimately linked (Herszberg et al., 2013). Maintaining epithelial plasticity and integrity is also vital throughout adult life, during which epithelia are constantly remodelled or regenerated by cell proliferation. How epithelia remain plastic while simultaneously maintaining their integrity is a key question. To decipher this problem, it is important to investigate the regulation of mechanical forces produced by the actomyosin cytoskeleton at adherens junctions.

Cell division in epithelia provides a physiological model for studying the antagonistic constraints between the maintenance and plasticity of cell–cell contacts. Indeed, during cytokinesis, cell–cell contacts are challenged by drastic shape changes, both of the dividing cell and of its neighbours, which resist division (Founounou et al., 2013). During cell division, a contractile actomyosin ring causes ingression of the plasma membrane and the associated cytoskeleton cortex, thus allowing separation of the two daughter cells. The contractile actomyosin ring triggers the reinforcement of adherens junctions, explaining how these junctions counteract the cytokinetic ring constriction and contribute to maintenance of epithelial integrity (Higashi et al., 2016). However, the main question of how the cytokinetic actomyosin ring overcomes the resistance of neighbouring cells, ultimately allowing cytokinesis progression, remains unanswered.

In *Drosophila*, the contractile ring is anchored at the adherens junctions (Guillot and Lecuit, 2013). This led to the hypothesis that constriction of the cytokinetic ring would induce variation of tensile forces applied on adherens junctions. Recently, we used a cadherin tension biosensor to investigate how mechanical forces acting at adherens junctions are regulated during cell division in the epithelium of *Xenopus* embryos (Herbomel et al., 2017). Using this approach, we have shown that the epithelium is under stable tension. In addition, mechanical tension across cadherin is similar in dividing and non-dividing epithelial cells, indicating either that variations of tensile force applied on cadherin are modest, or that adherens junctions are not the sites where mechanical forces vary. This suggests that other factors that remain to be identified are involved in the regulation of mechanical tension applied at the adherens junctions.

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Because adherens junctions are main sites where mechanical forces apply, their role during cell division is a key focus of previous studies, particularly in *Drosophila* (Founounou et al., 2013; Guillot and Lecuit, 2013; Herszterg et al., 2013; Morais-de-Sá and Sunkel, 2013). In contrast, the involvement of tight junctions has remained unexplored. Here, we investigate the involvement of two tight junction-associated proteins, ZO-1 and GEF-H1, in the regulation of mechanical forces applied on adherens junctions and their involvement in cytokinesis in the epithelium of the *Xenopus* embryo. Our results provide insight into the role of tight junctions in the negative regulation of mechanical forces applied to adherens junctions necessary for cytokinesis to occur.

## RESULTS

### Tight junctions are essential for cytokinesis

To investigate the possible role of tight junctions during epithelial cell cytokinesis, we knocked down the two tight junction-associated proteins ZO-1 and GEF-H1. ZO-1 (Stevenson et al., 1986) is a core tight junction protein, which acts as a scaffold linking tight junction transmembrane proteins, such as occludin (Furuse et al., 1994), with the actin cytoskeleton (Fanning et al., 1998). GEF-H1 (guanine nucleotide exchange factor H1) is a component of tight junctions (Benais-Pont et al., 2003) and exhibits RhoA small GTPase activity (Ajijaz et al., 2005); it regulates tight junctions (Benais-Pont et al., 2003) and is involved in apical constriction, which contributes to morphogenetic movements, leading to neural tube closure (Itoh et al., 2014).

To knockdown ZO-1 and GEF-H1, we used two antisense morpholino (Mo) oligonucleotides, which decrease ZO-1 and GEF-H1 levels in *Xenopus* embryo (Fig. S1 and Itoh et al., 2014). At the gastrula stage, these two proteins were depleted to about half ( $54.6 \pm 0.6\%$  in Mo ZO-1-treated cells and  $47.7 \pm 2.7\%$  in Mo GEF-H1-treated cells) compared with untreated cells (Fig. 1A). We observed cell division failures in ZO-1- and GEF-H1-depleted cells of living embryos, (Fig. 1B,C). This was correlated to the presence of multinucleated cells (Fig. 1D). Taken together, our results suggested that ZO-1 and GEF-H1 depletions induce cytokinesis defects. Although most embryos injected with ZO-1 antisense morpholinos were affected (proportion of embryos exhibiting multinucleated cells: control, 4.5% of embryos analysed; Mo ZO-1, 79.6%; Mo ZO-1+ZO-1-GFP, 17.4%), numerous cells exhibited one nucleus, indicating that they had divided correctly (Fig. 1D). Moreover, these depletions did not detectably alter the overall structure of the epithelium (Fig. 1A), allowing further analysis.

### Depletion of ZO-1 and GEF-H1 alters cytokinesis duration and contractile ring geometry

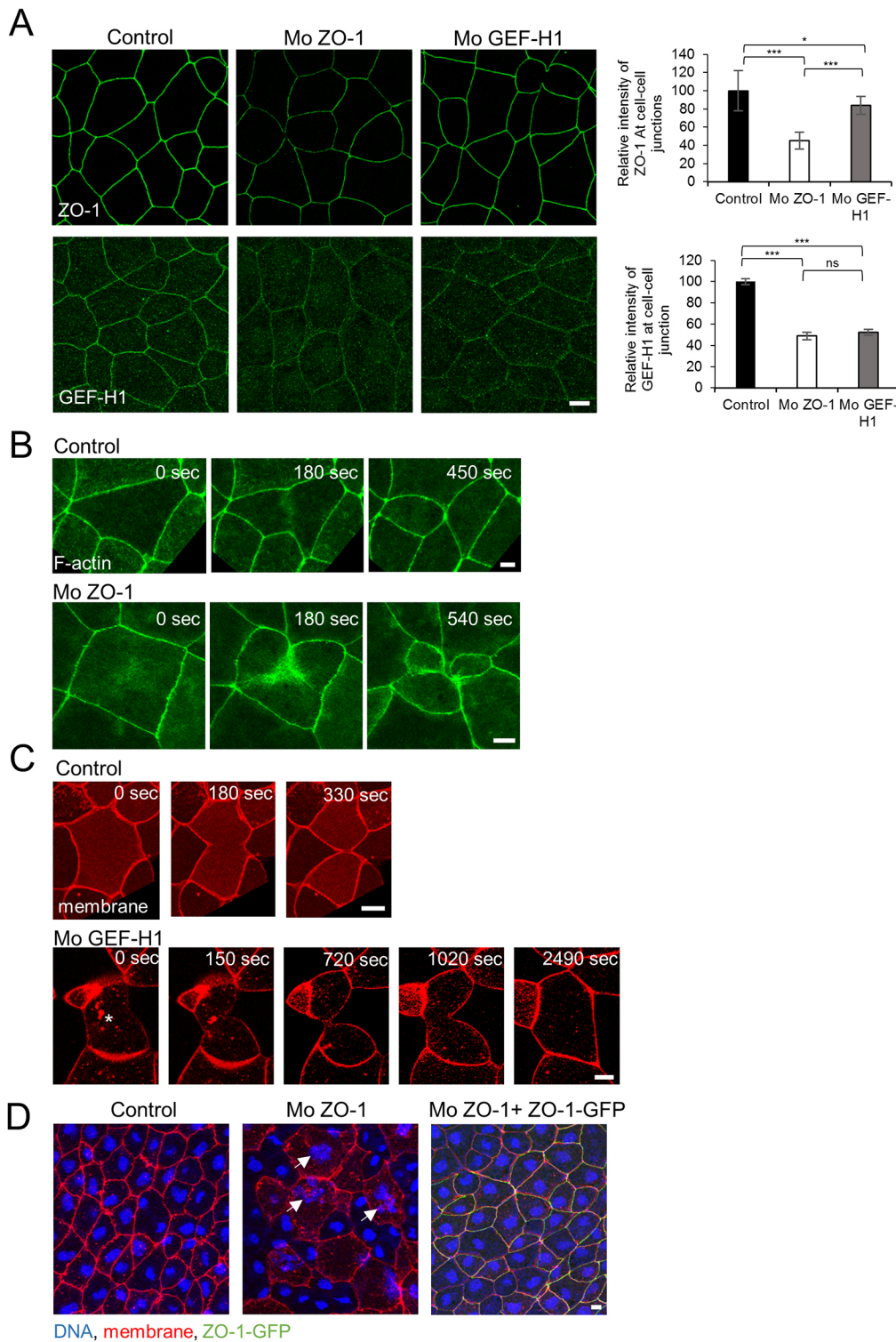
To analyse the effect of ZO-1 and GEF-H1 depletion during cytokinesis, constriction of the actomyosin contractile ring was followed *in vivo* in *Xenopus* gastrulae using actin fluorescent probes. At this developmental stage, epithelial cells divide by asymmetric furrowing from the basal side towards the apical pole (Le Page et al., 2011). Apical cell-cell contacts were deformed at the division site (Fig. 2A, arrowheads in control 180 s). As constriction advanced, the contractile ring adopted an ovoid shape (Fig. 2A, side view, 360 s). In ZO-1- and GEF-H1-depleted cells, the basal-to-apical asymmetric furrowing was maintained (Fig. 2A, Mo ZO-1 and Mo GEF-H1). However, the contractile ring was flattened (Fig. 2A, side views, 300 s) with a ring shape index (RSI, the ratio between ring length and height, where  $RSI=1$  is a circle) significantly higher in cells treated with Mo ZO-1 [ $RSI=4.7 \pm 0.2$  (mean $\pm$ s.e.m.)] and Mo GEF-H1 ( $3.8 \pm 0.2$ ) compared with controls

( $2.1 \pm 0.1$ ) (Fig. 2B). Moreover, apical constriction of the ring was significantly slower in depleted cells (Mo ZO-1,  $648 \pm 45.1$  s, 21/21 recorded cells; Mo GEF-H1,  $538.3 \pm 27.6$  s, 13/22 cells) than in controls ( $377.5 \pm 11.4$  s, 46/46 cells) (Fig. 2C). Expression of ZO-1-GFP in Mo ZO-1 or GEF-H1 in Mo GEF-H1 knockdown embryos restored both the cytokinesis duration and the ring shape (Fig. 2A–C).

Altogether, our results indicate that ZO-1 and GEF-H1 are required for epithelial cell cytokinesis. Moreover, defects induced by ZO-1 and GEF-H1 depletion evoked increased cell–cell junction resistance to cell division, which might result from increased mechanical forces applied to cell–cell junctions (Founounou et al., 2013; Guillot and Lecuit, 2013).

### Tight junctions regulate tension applied to adherens junctions

Because adherens junctions that link neighbouring cell membranes and their internal actin cytoskeletons are the main sites affected when mechanical forces are applied, we focused on these junctions. Although levels of C-cadherin (also termed EP-cadherin), the main adherens junction molecule of the *Xenopus* gastrula (Choi et al., 1990; Ginsberg et al., 1991), and  $\beta$ -catenin, which links cadherin with  $\alpha$ -catenin, were reduced in ZO-1- and GEF-H1-depleted cells, the perijunctional actin filaments (F-actin) were significantly increased in GEF-H1-depleted cells compared with control cells (Fig. 3A,  $192.5 \pm 11.6\%$ ) and slightly increased in ZO-1-depleted cells (Fig. 3A,  $130.6 \pm 12.2\%$ ). As shown previously (Nandadasa et al., 2012), depletion of  $\alpha$ -catenin, which indirectly links C-cadherin to the actomyosin cytoskeleton also led to a significant decrease of C-cadherin (Fig. 4A). However, in contrast to ZO-1 and GEF-H1 depletion, depletion of  $\alpha$ -catenin induced a significant reduction of perijunctional F-actin without altering the duration of cytokinesis and the contractile ring shape (Fig. 4A–C). These results suggested that the flattened shape of the contractile ring and the increased cytokinesis duration in ZO-1- and GEF-H1-depleted cells could result from the increase of F-actin perijunctional cytoskeleton. We hypothesized that reinforcement of the perijunctional F-actin might induce an increase of the tensile force applied to adherens junctions. To test this assumption, we used CcadTSMo, a FRET tension-sensing probe. It consists of a tensile force-sensitive module (TSMo) integrated into the cytosolic domain of C-cadherin (Herbomel et al., 2017) (Fig. 3B). We have previously shown that this tension probe detects tensile force variations on adherens junctions in the *Xenopus* embryo. Whereas in control cells the CcadTSMo mean fluorescence lifetime measured on entire fields of acquisition (10 cells per field) was  $2596.9 \pm 11.6$  ps, it significantly increased to  $2662.9 \pm 16.5$  ps and  $2660.3 \pm 14.4$  ps in ZO-1- and GEF-H1-depleted cells, respectively (Fig. 3C). As a control, we used a construct without the cytosolic domain (CcadTL) which is not linked to the actomyosin cytoskeleton. Therefore, it is not subjected to mechanical forces and exhibits the minimum fluorescence lifetime (Borghi et al., 2012). CcadTL fluorescence lifetime was similar in control, ZO-1- and GEF-H1-depleted cells (respectively  $2483.8 \pm 7.6$  ps,  $2480.7 \pm 6.1$  ps and  $2484.1 \pm 4.2$  ps, Fig. 3C). Correlated with the reduction of perijunctional F-actin (Fig. 4A), the CcadTSMo fluorescence lifetime was significantly decreased in  $\alpha$ -catenin-depleted cells compared with controls ( $2544.2 \pm 5.0$  ps versus  $2604.8 \pm 6.9$  ps) (Fig. 4C). These results indicated an increase in global tensile forces applied to adherens junctions induced by depletion of ZO-1 and GEF-H1. This led us to hypothesize that the increase in tensile forces may cause cytokinesis defects induced by ZO-1 and GEF-H1 depletion.



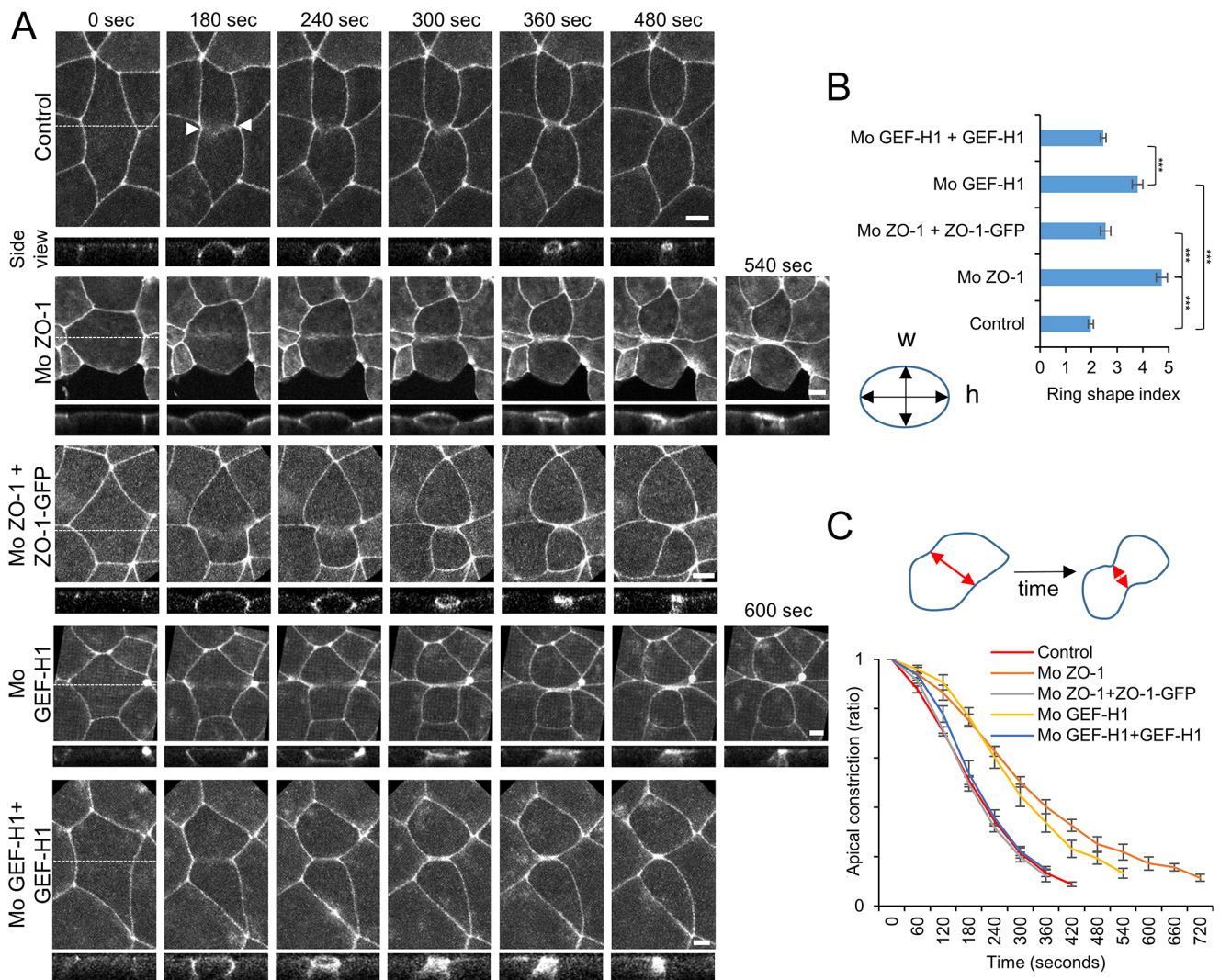
### Fig. 1. Depletion of ZO-1 and GEF-H1 leads to cytokinesis defects.

(A) Endogenous ZO-1 and GEF-H1 proteins in untreated (control), ZO-1-depleted (Mo ZO-1) and GEF-H1-depleted (Mo GEF-H1) embryos. Images are maximal projections. Relative intensities of endogenous ZO-1 and GEF-H1 proteins at cell-cell junctions in control, ZO-1-depleted and GEF-H1-depleted cells are presented as histograms on the right. (B) Still images extracted from time lapse of control and ZO-1-depleted cells expressing a fluorescent F-actin probe. The ZO-1-depleted cell exhibits multiple cytokinetic furrows. (C) Still images extracted from time lapse of control and GEF-H1-depleted cells traced with RFP-membrane. Cell marked with an asterisk failed to divide. (D) ZO-1 depletion in *Xenopus* gastrulae. Injected cells were traced with membrane marker (red) and DNA was stained with Topro3 (blue). Mouse ZO-1-GFP, resistant to Mo ZO-1 (because of the divergence of its nucleotide sequence from the *Xenopus* sequence), expressed in ZO-1-depleted cells traced with membrane marker (Mo ZO-1+ZO-1-GFP). Arrows indicate multinucleated cells. Proportion of embryos exhibiting multinucleated cells: control, 4.5%,  $n=22$  embryos analysed, Mo ZO-1, 79.6%,  $n=23$ ; Mo ZO-1+ZO-1-GFP, 17.4%,  $n=23$ . Data represent means  $\pm$  s.e.m. ns, not significant, \* $P<0.05$ , \*\*\* $P<0.0001$ . Scale bars: 10  $\mu$ m.

### Increased tensile forces applied on adherens junctions alters contractile ring geometry and cytokinesis

To test a potential relationship between increased forces and the cytokinetic defects induced by ZO-1 and GEF-H1 depletions, we increased tensile forces applied to adherens junctions by incubating embryos in 2'-deoxyadenosine 5-triphosphate (dATP), which stimulates contractility (Regnier et al., 2000). Under these conditions, tensions applied to adherens junctions, perijunctional

F-actin recruitment and RSI were significantly increased compared with levels in control cells (Fig. 5A–D), thus mimicking depletion of ZO-1 and GEF-H1. Thus, increased tensile forces applied to adherens junctions correlated with a ring geometry defect. To further challenge a causal relationship between increased forces on adherens junctions and cytokinetic defects, we took advantage of a recently developed vinculin deletion mutant (VinD1) (Higashi et al., 2016), which decreases vinculin recruitment. Vinculin is an



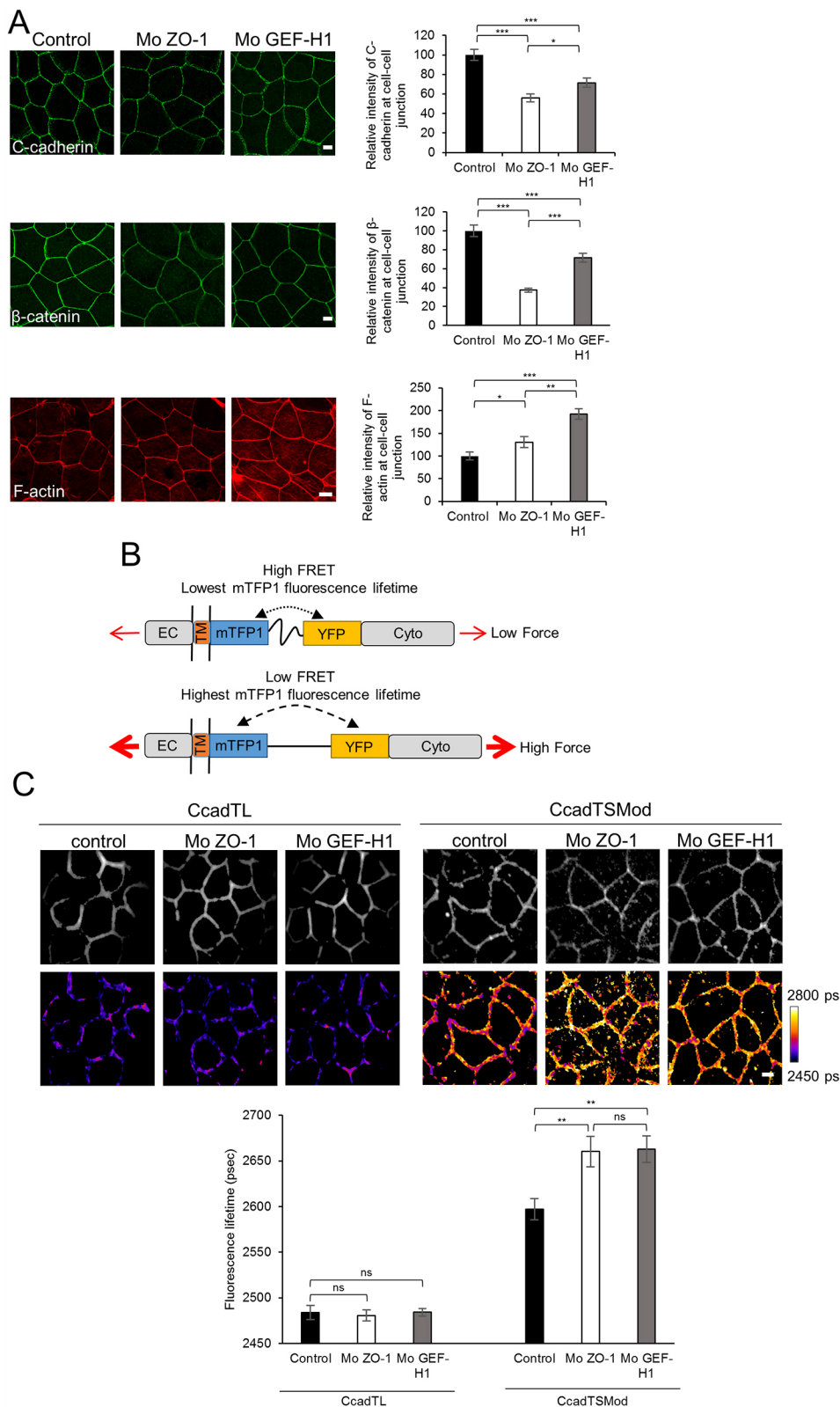
**Fig. 2. Tight junctions are essential during cytokinesis.** (A) Still images extracted from time lapse of dividing cells expressing fluorescent F-actin probes alone (control) or in ZO-1-depleted embryos (Mo ZO-1), ZO-1-depleted embryos expressing ZO-1-GFP (Mo ZO-1+ZO-1 GFP), GEF-H1-depleted embryos (Mo GEF-H1) and GEF-H1-depleted embryos expressing GEF-H1 (Mo GEF-H1+GEF-H1). Maximal projections of en face views and side views parallel to the cleavage plane through the contractile ring (dashed line in  $x-y$  views) are shown. Cells shown are representative of 46/46 recorded cells for the control (100%), 21/21 for Mo ZO-1 (100%), 10/13 for Mo ZO-1+ZO-1-GFP (77%), 13/22 for Mo GEF-H1 (59.1%) and 16/26 for Mo GEF-H1+GEF-H1 (61.5%). (B) Contractile ring shape index (RSI) in conditions shown in A. The oval represents the contractile ring; w, width; h, height. Control,  $2.1 \pm 0.1$  (mean  $\pm$  s.e.m.),  $n=46$  cells; Mo ZO-1,  $4.7 \pm 0.2$ ,  $n=21$ ; Mo ZO-1+ZO-1-GFP,  $2.5 \pm 0.1$ ,  $n=10$ ; Mo GEF-H1,  $3.8 \pm 0.2$ ,  $n=13$ ; Mo GEF-H1+GEF-H1,  $2.4 \pm 0.1$ ,  $n=16$ . (C) Apical constriction at the cleavage site in conditions shown in A. The red arrow represents the distance separating plasma membranes at the division site measured over time. Control,  $377.5 \pm 11.4$  s; Mo ZO-1,  $648 \pm 45.1$  s; Mo ZO-1+ZO-1-GFP,  $370 \pm 10$  s; Mo GEF-H1,  $538 \pm 27.6$  s; Mo GEF-H1+GEF-H1,  $384 \pm 6$  s. Data represent means  $\pm$  s.e.m. \*\*\* $P < 0.0001$ . Scale bars: 10  $\mu$ m.

F-actin binding protein recruited at the adherens junction by a tension-induced conformational change of  $\alpha$ -catenin (Yonemura et al., 2010) leading to adherens junction reinforcement. VinD1 expression significantly decreased the duration of cytokinesis, as reported previously (Higashi et al., 2016), modestly increased the RSI and did not significantly affect the CcadTSMOD fluorescence lifetime (Fig. 5F–H). The absence of any effect on the tensile force could be explained by the fact that in *Xenopus* epithelium tensile forces applied on cadherin at the adherens junctions are at equilibrium; they are  $\sim 3$  pN (Herbomel et al., 2017), which is below the 5 pN required to unfold  $\alpha$ -catenin and recruit vinculin (Yao et al., 2014). Expression of VinD1 suppressed the ring shape defect (Fig. 5E,F, RSI= $4.6 \pm 0.3$  for Mo ZO-1 and  $2.3 \pm 0.1$  for Mo ZO-1+VinD1 compared with  $2.0 \pm 0.1$  in controls), cytokinesis duration defect (Fig. 5G,  $741 \pm 55$  s for Mo ZO-1 and  $378 \pm 18$  s for

Mo ZO-1+VinD1 compared to  $393 \pm 17$  s in controls) and increased tensile forces (mean fluorescence lifetime, Fig. 5H) induced by ZO-1 depletion. Taken together, these results indicate that in ZO-1- and GEF-H1-depleted cells, a global increase of tensile forces applied to cadherin enhance the resistance of apical junctions to cytokinesis, which ultimately affects the contractile ring geometry and the cytokinesis duration. They also indicate that tight junction-associated proteins ZO-1 and GEF-H1 are implicated in the negative regulation of global tensile force applied to adherens junctions.

#### GEF-H1 could act by regulating membrane trafficking via the exocyst complex

Next, we investigated how GEF-H1 regulates tensile forces applied on adherens junctions. Recently, GEF-H1 has been shown to regulate assembly and localization of the exocyst (Pathak et al.,

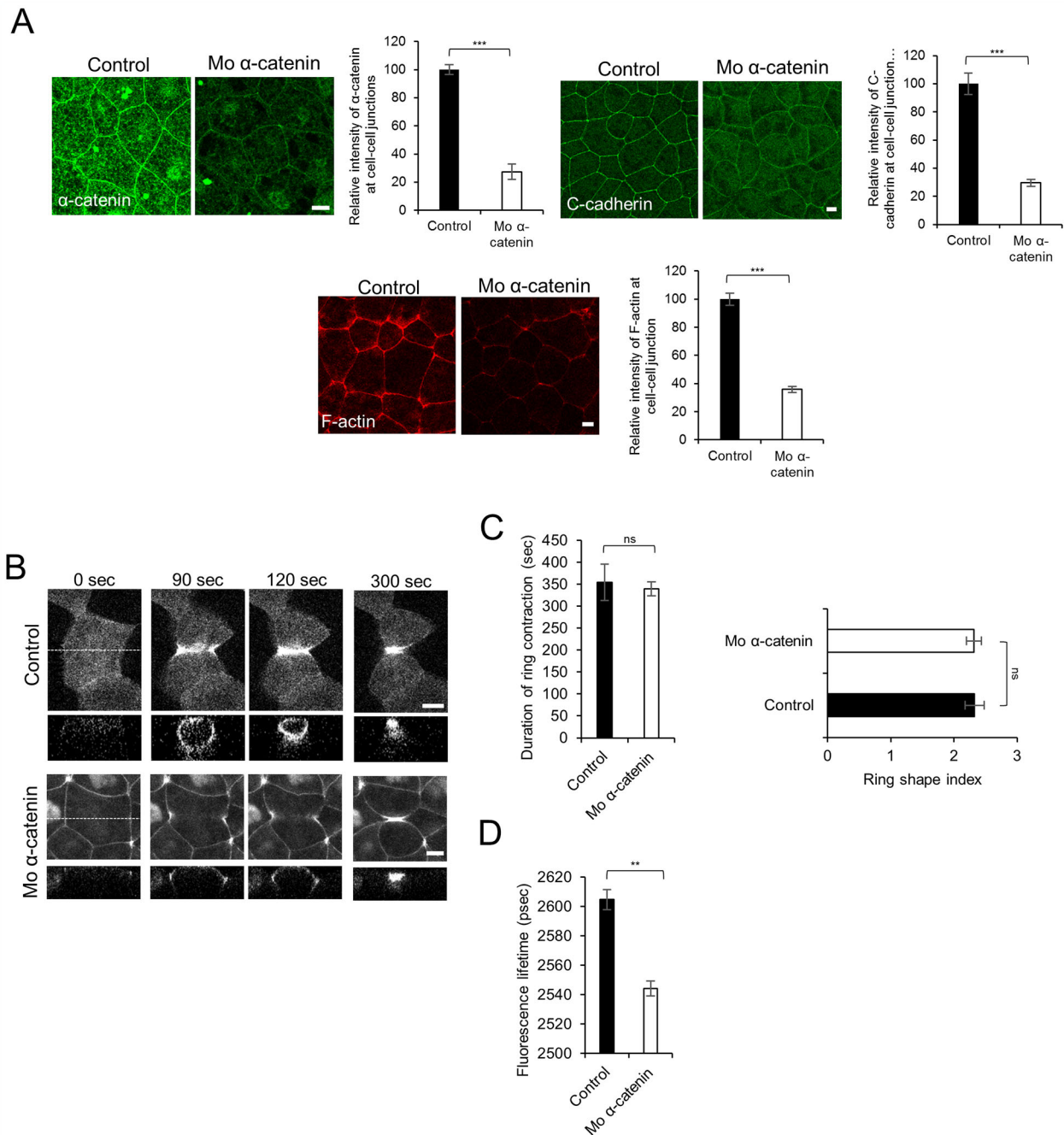


**Fig. 3. ZO-1 and GEF-H1 regulate tension applied to adherens junctions.**

(A) Endogenous C-cadherin, β-catenin and F-actin in control, ZO-1-depleted (Mo ZO-1) and GEF-H1-depleted (Mo GEF-H1) cells. Maximal projections of confocal sections are shown. Graphs: relative intensity of each protein at cell-cell junctions in control and depleted cells. C-cadherin, 55.7±4.1% in Mo ZO-1 and 71.7±4.7% in Mo GEF-H1; β-catenin, 37.3±2.1% in Mo ZO-1 and 71.4±4.7% in Mo GEF-H1; F-actin, 130.6±12.2% in Mo ZO-1 cells and 192.5±11.6% in Mo GEF-H1. (B) Schematic representation of the CcadTSMOD biosensor. The relationship between the FRET and donor fluorescence lifetime is inverse and in the absence of tension [top, the tension sensor module (TSMOD, black line) is not elongated], FRET is high and fluorescence lifetime is low. Conversely, in the presence of tension (bottom, the TSMOD is elongated), FRET decreases and the donor fluorescence lifetime increases. EC, Cyto and TM: respectively extracellular, cytoplasmic and transmembrane domains of C-cadherin. (C) Tensile forces applied to adherens junctions in control, ZO-1-depleted (Mo ZO-1) and GEF-H1-depleted (Mo GEF-H1) cells expressing CcadTL or the CcadTSMOD biosensor. Top panel shows representative images of mTFP1 fluorescence (first row) and the corresponding fluorescence lifetimes (second row) are shown. The pseudocolor scale represents pixel-by-pixel fluorescence lifetime expressed in ps. Graph of mean fluorescence lifetime of CcadTL and CcadTSMOD tension probe at the cell-cell contacts in control ( $n=25$  and  $29$  fields, respectively), ZO-1-depleted ( $n=29$  and  $19$  fields) and GEF-H1-depleted ( $n=41$  and  $22$  fields) cells. Data are representative of 3 independent experiments. Data represent means±s.e.m. \* $P<0.05$ , \*\* $P<0.001$ , \*\*\* $P<0.0001$ ; ns, not significant. Scale bars:  $10\ \mu\text{m}$ .

2012). The exocyst is a multiprotein complex which plays a crucial role in tethering and fusion of vesicles at the plasma membrane (Wu and Guo, 2015). This regulation requires GEF-H1 GTP exchange activity and interaction of GEF-H1 with the Sec5 subunit of the exocyst complex (Pathak et al., 2012). To test whether exocyst regulation by GEF-H1 could be involved in tensile forces regulation

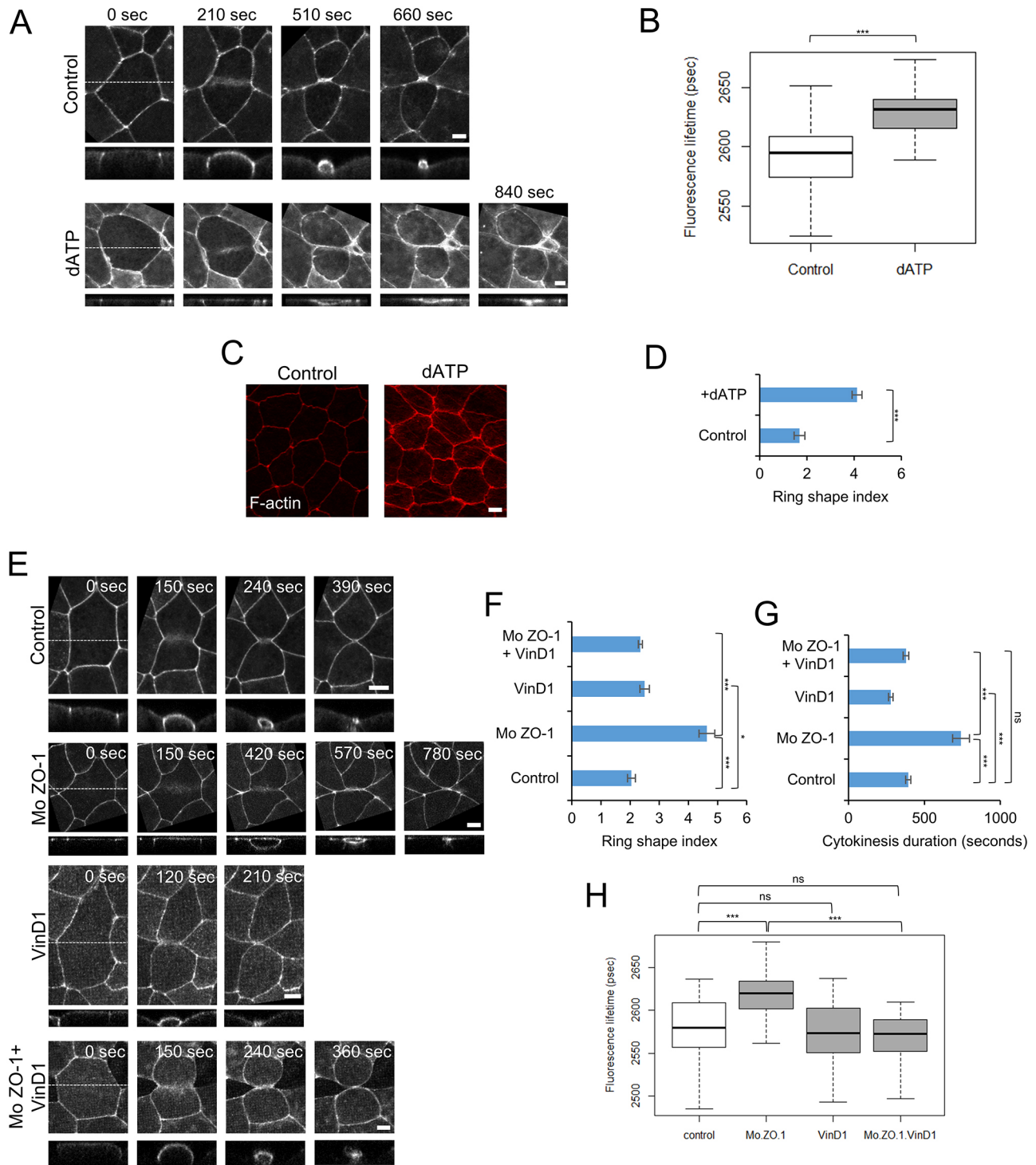
applied on adherens junctions, we expressed the inactive GEF-H1<sup>Y384A</sup> mutant (Itoh et al., 2014; Krendel et al., 2002) or GEF-H1<sup>(aa 108-226)</sup>, which links Sec5 and acts as a competing peptide (Pathak et al., 2012). The fluorescence lifetime of CcadTSMOD increased in cells expressing GEF-H1<sup>Y384A</sup> and GEF-H1<sup>(aa 108-226)</sup> mutants compared with levels in controls (Fig. 6A, respectively



**Fig. 4. Cytokinesis is not altered in  $\alpha$ -catenin-depleted cells.** (A) Endogenous  $\alpha$ -catenin, C-cadherin and F-actin in control and embryos treated with  $\alpha$ -catenin morpholino (Nandadasa et al., 2012). Histograms show relative fluorescence intensities for each protein at cell-cell contacts.  $\alpha$ -catenin,  $27.4 \pm 5.5\%$  of controls; C-cadherin,  $29.6 \pm 2.5\%$ ; F-actin,  $35.8 \pm 2.2$ . (B) Still images from time-lapse movies of dividing control and  $\alpha$ -catenin-depleted (Mo  $\alpha$ -catenin) cells expressing fluorescent Lifeact F-actin probe. Maximal projections and side views parallel to the cleavage plane (along dashed lines) are presented. Control, 17 out of 17 recorded cells (100%); Mo  $\alpha$ -catenin, 17 out of 19 recorded cells (89.5%). (C) Cytokinesis duration in control and  $\alpha$ -catenin depleted cells. Control,  $354 \pm 41.6$  s; Mo  $\alpha$ -catenin;  $339 \pm 16.1$  s (left). Ring shape index in control and  $\alpha$ -catenin depleted cells. Control,  $2.1 \pm 0.1$ ; Mo  $\alpha$ -catenin,  $2.3 \pm 0.1$  (right). (D) Variation of fluorescence lifetimes of the CcadTSM tension probe in control cells or  $\alpha$ -catenin-depleted cells. Control,  $n=8$  fields; Mo  $\alpha$ -catenin,  $n=32$  fields. Data represent means  $\pm$  s.e.m. ns, not significant; \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . Scale bars: 10  $\mu$ m.

$2653.4 \pm 7.8$  ps and  $2635 \pm 4.7$  ps; control,  $2599.4 \pm 6.9$  ps). Moreover, cells expressing GEF-H1<sup>Y384A</sup> or GEF-H1<sup>(aa108-226)</sup> also exhibited the contractile ring apical constriction defect (Fig. 6B,C). Similar to GEF-H1 depletion, expression of GEF-H1<sup>Y384A</sup> or GEF-H1<sup>(aa108-226)</sup> led to decreased C-cadherin (Fig. 6D) and  $\beta$ -catenin (Fig. S3) levels. Taken together, these results suggest that GEF-H1-dependent regulation of the exocyst may participate in

the negative regulation of tensile force applied on the adherens junctions. As ZO-1 depletion affects GEF-H1 levels (Fig. 1A), it could be possible that the effect on the force applied on adherens junctions could occur via GEF-H1-dependent regulation of the exocyst. Further studies will be necessary to explore the involvement of GEF-H1-dependent exocyst regulation in the control of forces applied on adherens junctions.



**Fig. 5. Increased tensile forces applied on adherens junctions induce cytokinesis defects.** (A) Still images from time-lapse movies of dividing cells expressing fluorescent Lifeact F-actin probe in embryos treated with dATP or not (control). (B) Boxplot of mean fluorescence lifetimes (in ps) of the CcadTSMoD tension probe at cell–cell contacts in controls ( $2590.3 \pm 6.6$  ps,  $n=23$  fields) and dATP-treated ( $2627.1 \pm 4.7$  ps,  $n=24$  fields) cells. (C) Endogenous F-actin in control and dATP-treated embryos. (D) Ring shape index and cytokinesis duration in control and dATP-treated cells. Control: RSI,  $1.7 \pm 0.2$ ,  $n=12$  cells; +dATP: RSI,  $4.1 \pm 0.2$ ,  $n=32$  cells. (E) Still images extracted from time lapse of dividing cells expressing fluorescent Lifeact F-actin probe alone (control) or in ZO-1-depleted (Mo ZO-1), VinD1-expressing (VinD1) and ZO-1-depleted and -expressing VinD1 (Mo ZO-1+VinD1) embryos. For each condition, maximal projections of en face views (top rows) and side views (below, along dashed line) parallel to the cleavage plane through the contractile ring at are shown. Controls, 26/26 of recorded cells (100%); Mo ZO-1, 21/23 (91.3%); VinD1, 8/10 (80%); Mo ZO-1+VinD1, 26/39 (66.7%). (F) Ring shape index and (G) cytokinesis duration in control, ZO-1-depleted (Mo ZO-1), VinD1-expressing (VinD1) and ZO-1-depleted and -expressing VinD1 (Mo ZO-1+VinD1) cells. Controls,  $n=21$  cells; Mo ZO-1,  $n=10$  cells; VinD1,  $n=16$  cells; Mo ZO-1+VinD1,  $n=21$  cells. (H) Tensile forces applied to adherens junctions in control, ZO-1-depleted, and ZO-1-depleted and -expressing VinD1 (Mo ZO-1+VinD1) cells. Boxplot of mean fluorescence lifetimes of the CcadTSMoD tension probe at cell–cell contacts in control ( $2579 \pm 5.1$  ps,  $n=47$  fields), ZO-1-depleted (Mo ZO-1,  $2618.3 \pm 5.6$  ps,  $n=29$  fields), VinD1-expressing (VinD1,  $2576.6 \pm 6.8$  ps,  $n=26$  fields) cells and ZO-1-depleted and expressing VinD1 (Mo ZO-1+VinD1,  $2566.0 \pm 8.4$  ps,  $n=16$  fields). Data represent means  $\pm$  s.e.m. ns, not significant; \* $P < 0.05$ , \*\*\* $P < 0.0001$ . Scale bars: 10  $\mu$ m.

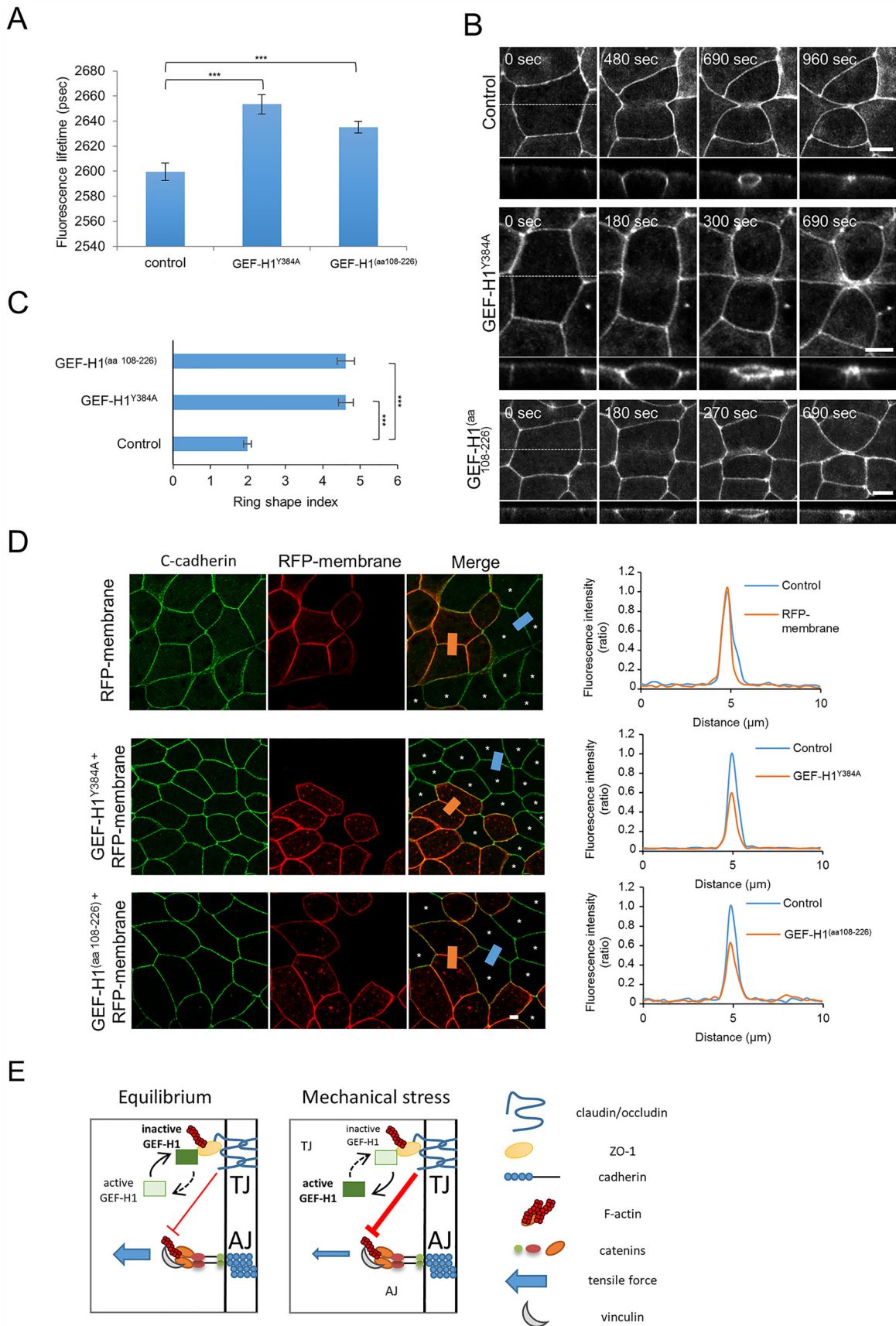


Fig. 6. See next page for legend.



**Fig. 6. Involvement of GEF-H1 activity and potential interaction with the exocyst complex in the regulation of tensile forces applied on adherens junctions.** (A) Variation of fluorescence lifetimes of the CcadTSMod tension probe in cells expressing mutants as shown in B: GEF-H1<sup>Y384A</sup> ( $n=28$  fields) and GEF-H1<sup>(aa 108-226)</sup> ( $n=35$ ). Data are representative of three independent experiments. (B) Still images extracted from time-lapse movies of cells expressing a fluorescent F-actin probe only (control, 100% of recorded cells,  $n=38$ ) or coexpressing GEF-H1<sup>Y384A</sup> (100%,  $n=12$  cells), GEF-H1<sup>(aa 108-226)</sup> (69%,  $n=42$ ). (C) Ring shape index in control ( $2.0\pm 0.1$ ,  $n=38$  cells), and GEF-H1<sup>Y384A</sup> ( $4.6\pm 0.2$ ,  $n=12$ )- and GEF-H1<sup>(aa 108-226)</sup> ( $4.6\pm 0.2$ ,  $n=29$ )-expressing cells. (D) Endogenous C-cadherin, in control, and in GEF-H1<sup>Y384A</sup>- and GEF-H1<sup>(aa 108-226)</sup>-expressing cells. Co-expression of RFP-membrane was used as a tracer. For each condition, an image of a field containing cells expressing and not expressing RFP-membrane is shown. Cells that do not express RFP-membrane (asterisks) are used as internal control for fluorescence quantification. Red and blue bars on merged images indicate cell–cell contacts where fluorescence was quantified. Line scans on the right show C-cadherin intensity in internal control (blue) and cells expressing the RFP-membrane (red) in each condition. See also Fig. S3. (E) Model of the regulation by tight junctions (TJ) of tensile forces applied to adherens junctions (AJ). The two tight junction-associated proteins ZO-1 and GEF-H1 are involved in the regulation of mechanical forces applied to adherens junctions. When tensile forces are at the equilibrium, GEF-H1 is inhibited by junctional recruitment (Aijaz et al., 2005) and the inhibition (exerted by tight junctions) of mechanical forces applied on adherens junctions is low. Under mechanical stress, such as cytokinesis, GEF-H1 will be released from junctional inhibition. Active GEF-H1 will trigger, at the tight junction level, a response leading to increased inhibition of the mechanical forces applied on adherens junctions. Data represent means $\pm$ s.e.m. \*\*\* $P<0.0001$ . Scale bars: 10  $\mu$ m.

## DISCUSSION

Our results reveal that the tight junction-associated proteins ZO-1 and GEF-H1 are involved in negative regulation of mechanical forces applied to adherens junctions necessary for cytokinesis completion in the epithelium of *Xenopus* embryos.

Constriction of the cytokinetic actomyosin ring triggers reinforcement of adherens and tight junctions (Higashi et al., 2016). This reinforcement was proposed to participate in the maintenance of the epithelium integrity during cell division. However, it remained unclear how the contractile ring ultimately wins the ‘tug of war’ against apical junctions, allowing cytokinesis progression. In the present study, measurement of tensile forces applied to C-cadherin molecules showed that tight junction-associated proteins ZO-1 and GEF-H1 are involved in the regulation of mechanical forces applied to adherens junctions. Our results are in agreement with those obtained in cultured epithelial cells, which show that ZO-1 depletion increases intercellular tension (Bazellières et al., 2015), and that double depletion of ZO-1 and ZO-2 increases contractility of the perijunctional actomyosin associated with the adherens junctions (Choi et al., 2016; Fanning et al., 2012). In contrast, in endothelial cells cultured *in vitro*, ZO-1 depletion decreases the tensile forces applied to VE-cadherin (Tornavaca et al., 2015). This discrepancy might lie in the cell types studied, epithelial versus endothelial, which display different apical junction organization (Dejana et al., 2009). Nevertheless, our results support a model in which ZO-1 and GEF-H1 regulate apical cell–cell contact resistance to the contractile ring constriction. Owing to cell–cell contacts and the mechanical coupling between epithelial cells (Twiss and de Rooij, 2013), the shape modifications of the cell that divides deform neighbouring cells, thereby producing a mechanical stress (Bosveld et al., 2016; Founounou et al., 2013; Herszterg et al., 2013). GEF-H1 was previously shown to be involved in mechanotransduction (Guilluy et al., 2011). Upon mechanical stimuli, GEF-H1 activates Rho GTPase and promotes cellular stiffness. In epithelial cells, GEF-H1

is inhibited by junctional recruitment (Aijaz et al., 2005), suggesting a potential signalling role of this protein. We speculate that during cytokinesis, GEF-H1 inhibition might be suppressed by mechanical stress produced by the dividing cell, allowing the cell–cell contact adaptation necessary for cell division (Fig. 6E). In our model, active GEF-H1 released from junctional inhibition would, via RhoA GTPase, activate actomyosin contraction at the level of tight junctions. This would lead to an increase in tight junction inhibition of forces applied on adherens junctions. Further studies will be necessary to understand how tight junctions sense and regulate mechanical forces applied to adherens junctions.

In this study, we showed that depletion of ZO-1 or GEF-H1 has a different impact on C-cadherin,  $\alpha$ - and  $\beta$ -catenin levels. However, the CcadTSMod mean fluorescence lifetimes are similar (Fig. 3C), suggesting that tensions reached a maximum. We hypothesise that ZO-1 depletion could induce a stronger and transient effect on tensile forces than GEF-H1 depletion does. Increased tensile forces induced by ZO-1 depletion could reach a threshold value harmful for epithelium integrity and function. We propose that a compensatory mechanism activated by increased tensile forces induced by ZO-1 depletion would reduce the amount of C-cadherin, and  $\alpha$ - and  $\beta$ -catenins at the adherens junctions, which would limit the increase of tensile forces induced by ZO-1 depletion. Because tensile forces in GEF-H1-depleted cells did not reach the threshold value, the compensatory mechanism would not be activated. A compensatory mechanism acting at the apical junctional complex has already been described for ZO-depleted cells treated with a ROCK inhibitor (Choi et al., 2016). Such a hypothetical mechanism would protect the epithelium against oversized forces, which might alter the epithelium integrity and function when tight junctions are compromised. Further investigations will be required to understand the details of this mechanism.

It was previously shown that cytokinesis defects in cells mutated for the tumour suppressor gene p53 could lead to genomic instability and cancer (Fujiwara et al., 2005). Our results showing that alterations in tight junctions leading to misregulation of mechanical tension on adherens junctions represents a previously unreported origin of cell division defects which might ultimately lead to cancer formation and progression.

## MATERIALS AND METHODS

### DNA constructs and morpholinos

pT7T-ZO-1-GFP (mouse) and pT7T-GEF-H1a<sup>Y384A</sup> (*Xenopus*) were PCR amplified from pCAGGS-ZO-1-GFP (Matsuda, 2004) and pCS2-myc-GEF-H1-Y444A (a kind gift from Sergei Y. Sokol, Icahn School of Medicine at Mount Sinai, New York, USA), respectively (Itoh et al., 2014). pT7T-GEF-H1 WT and pT7T-GEF-H1<sup>(aa 108-226)</sup> were generated by PCR amplifying the corresponding sequences from *Xenopus* GEF-H1a obtained from Source Bioscience (clone image: 4173243). pT7T-VinD1 was obtained by amplification of the first 258 amino acids of chicken vinculin (Grashoff et al., 2010). All PCR fragments were subcloned into the pT7T vector. pCS2-GFP-GPI (GFP-membrane) and pCS2-mRFP-GPI (RFP-membrane) plasmids were kindly provided by Michael Brand (Dresden University of Technology, Germany), pCS2-mCherry-Utr-CH was a gift from William M. Bement (Burkel et al., 2007), pCS2-Life-act-GFP was a gift from Ulrike Engel (Marx et al., 2013). For tension measurements, pT7T-CadherinTSMod (Herbomel et al., 2017) was used. The CadherinTSMod contains the entire FRET tension module created by Grashoff et al. (2010) composed of an elastic linker interspaced between the fluorescence proteins mTFP1 and YFP integrated into the cytosolic cadherin domain. We have previously shown that CcadTSMod and EcadTSMod (Borghini et al., 2012) tension sensors exhibit similar fluorescence lifetimes in the blastula embryo (Herbomel et al., 2017). This is also the case in gastrula embryos that we used in the present study (Fig. S2). As a control, a construct without the

cytosolic domain (CcadTL) was used. Because CcadTL is not linked to the actomyosin cytoskeleton, it is not submitted to mechanical forces and therefore exhibits the minimum fluorescence lifetime (Borghi et al., 2012). All plasmids were linearised and mRNAs were generated by *in vitro* transcription using the T7 or SP6 mMessage mMachine kit (Ambion).

For knockdown experiments, antisense morpholinos were produced by Gene Tools. For ZO-1 depletion, an antisense morpholino (5'-GCCGGTGTCAGTATGAGTCCCCAGA-3') has been designed to target the 5'UTR of the two genes encoding ZO-1. Previously described morpholinos targeting the 5'UTR of GEF-H1a (5'-AGGTGCGGTCAATGCCGGACATTGA-3') (Itoh et al., 2014) and the initiation codon for  $\alpha$ -catenin (5'-ATGTTTCTGTATTGAGAGTCATGC-3') (Nandadasa et al., 2012) were used. A standard morpholino (5'-CCTCTTACTCAGTTACAATTTATA-3', Gene Tools) was used as a control.

### Preparation of *Xenopus* embryos and micro-injection

*Xenopus laevis* albinos were obtained from Biological Resources Centre (CRB, Rennes, France). All animal experiments were performed in accordance with the approved protocols and guidelines at Rennes 1 University by the Comité Rennais d'Éthique en Matière d'Expérimentation Animale (C2EA-07) and the French Ministry for Education and Research (3523813). Eggs were fertilised *in vitro* and embryos were collected as described previously (Hatte et al., 2014). After de-jelling, embryos were placed in 5% Ficoll in F1 buffer (10 mM HEPES pH 7.6, 31.2 mM NaCl, 1.75 mM KCl, 59  $\mu$ M MgCl<sub>2</sub>, 2 mM NaHCO<sub>3</sub>, and 0.25 mM CaCl<sub>2</sub>) for microinjection. mRNAs were microinjected into one blastomere of 4- or 8-cell-stage embryos. For GEF-H1 knockdown experiments, 40 ng morpholino in 13.8 nl was microinjected into one blastomere at the 4-cell stage, and for Mo ZO-1 and Mo  $\alpha$ -catenin, 40 ng and 20 ng, respectively, in 9.2 nl was microinjected into one blastomere at the 8-cell stage. mRNA quantities injected were: GFP-membrane, 0.5 ng; RFP-membrane, 0.5 ng; life-act-GFP, 0.5 ng; mCherry-utr-ch, 0.5 ng; ZO-1-GFP, 2 ng; GEF-H1 WT, 0.25 ng; CcadTSMoD, 2 ng; CcadTL, 2 ng; VinD1, 1 ng; GEF-H1<sup>Y384A</sup>, 8 ng; and GEF-H1<sup>(aa 108-226)</sup>, 8 ng. Embryos were then placed at 16°C overnight and analyzed at gastrula stage (stage 11; Nieuwkoop and Faber, 1967).

### Indirect immunofluorescence

Albino embryos were injected with membrane marker (GFP- or RFP-membrane) used as lineage tracers and with or without Mo GEF-H1, Mo ZO-1 or Mo  $\alpha$ -catenin. Indirect immunofluorescence was performed as described previously (Hatte et al., 2014). Briefly, embryos were fixed with 2% trichloroacetic acid (TCA) in 1× F1 buffer. After embryos were devitellinated, cells were permeabilised in PBS+1% Triton X-100 for 20 min at room temperature, incubated in PBS+0.1% Triton X-100 (PBST 0.1%) for 10 min and then blocked for 1 h in 2% BSA in PBST 0.1% (BSA-PBST). Embryos were incubated overnight in BSA-PBST at 14°C with anti-GFP (Roche, clone 7.1 and 13.1, 11914460001, 1:100), anti-mCherry (Abcam, Ab167453, 1:200), anti-ZO-1 (Zymed, 33-9100, 1:200), anti-GEF-H1 (Exalpha, Sheep lfc X1089P, 1:100), anti-C-cadherin (DHSB, 6B6, 1:200), anti- $\beta$ -catenin (Santa Cruz, H102, sc-7199, 1:100), anti- $\alpha$ -catenin (ThermoFisher Scientific, PA1-25081, 1:100) and anti-phospho S20 myosin light chain (S19 in *Xenopus*) (Abcam, ab2480, 1:100). After washing with 0.1% PBST, embryos were incubated with Alexa Fluor-488 or -555 anti-mouse, anti-rabbit or anti-sheep (ThermoFisher Scientific) and TO-PRO-3 to stain DNA (Invitrogen, 0.5  $\mu$ g/ml) in BSA-PBST for 1 h at room temperature. After washing with 0.1% PBST, embryos were mounted in Vectashield (Vector). For actin staining, embryos were fixed in 3.7% formaldehyde for 2 h at room temperature, permeabilised and blocked as previously described. After blocking, embryos were incubated with Alexa Fluor 555-conjugated Phalloidin (Life Technologies, 1:200, 1 U/ml) and TO-PRO3 for 2 h, and mounted in Vectashield.

### Western blot analysis

Proteins were extracted as previously described (Reyes et al., 2014). Protein blots were incubated with anti-ZO-1 (Zymed, 33-9100, 1:500) and anti- $\beta$ -tubulin (TUB 2.1, Sigma, 1:1000) antibodies. Secondary anti-mouse peroxidase-coupled immunoglobulins were from Jackson.

### Treatment with dATP

Embryos at the 4-cell stage were microinjected with mRNA encoding GFP-GPI (GFP-membrane) as described above. When they reached the gastrula stage, embryos were treated with 1 mM dATP (Promega, U120D) in 1× F1 buffer for 20 min prior to imaging.

### Confocal microscopy with living and fixed embryos

Living embryos were mounted as previously described (Hatte et al., 2014). Fixed and live imaging was performed using a Leica SP5 confocal microscope with 40×/1.25–0.75 HC PL APO (WD 0.1 mm) or 63×/1.4–0.6 HCX PL APO (WD 0.14 mm) oil-immersion objective lens or a Leica SP8 confocal microscope with 40×/1.30 HC PL APO or 63×/1.4 HC PL APO oil-immersion objective lens (Microscopy Rennes Imaging Center, MRiC). For FLIM measurements, a FastFLIM microscope previously described (Leray et al., 2013) was used, a time lapse of 5 consecutive time frames was acquired for all the conditions.

### Image processing and quantification

Images were processed using ImageJ software (<http://rsb.info.nih.gov/ij>) and figures were prepared using ImageJ and Photoshop. Graphs were drawn using R software and Microsoft Excel. In live experiments, only cells with one furrow showing no cytokinesis failure were analysed.

### Quantification of immunolabelling at adherent and tight junctions

The mean fluorescence pixel intensity at cell–cell junctions was measured along a linear ROI placed at the maximum fluorescence plane. The mean fluorescence pixel intensity in proximity to cell–cell junctions was measured along a linear ROI with identical line size to the ROI used for quantification at cell–cell junctions and subtracted from the pixel intensity at the junction.

### Cytokinesis timing and apical constriction measurement

The cytokinesis duration is the time for which the contractile ring contracts. To standardise measurements, the beginning and end of cytokinesis were respectively standardised as the time point just before F-actin started accumulating at the cleavage site and when the actomyosin contractile ring fully ingressed and concentrates at the apical pole of the cell.

### Contractile ring shape

The ring shape index (RSI) was calculated as the ratio between the length (parallel to the epithelium plane) and height (orthogonal to the plane of the epithelium) of the contractile ring. To avoid bias due to differences in the cytokinesis duration which could distort the ring shape index, measurements of the ring length were standardised: for all conditions they were done when the ring height reached 4  $\mu$ m.

### FLIM measurements

All analyses were done as previously described (Herbomel et al., 2017). Briefly, the percentage of photobleaching was determined in a small area in the fluorescence image, if the decrease was <10% between the first and the last time, the field was not analysed. Then, a mask corresponding to the FRET sensor or controls localised at cell junctions was created on fluorescence images and subsequently used to retrieve the fluorescence lifetime of this protein pool. One value represents the mean of the whole field values (10 cells per field), results are expressed in ps. Fire LUT was applied to fluorescence lifetime images.

### Statistical analysis

No statistical method was used to predetermine sample size. The experiments were not randomised and investigators were not blinded to allocation during experiments and outcome assessment. Experiments were repeated at least twice. Sample sizes are specified for each experiment in figure legends. For each representative image, sample sizes (n) are indicated in the figure legend. A Shapiro–Wilk test was done for each group to determine the distribution normalities. Where appropriate, a two-tailed unpaired Student's *t*-test was applied to determine statistical significance. In cases where variances were different, the unequal variance *t*-test was used. For groups not displaying a normal distribution, a Wilcoxon signed-rank test was done as indicated in figure legends. Statistical analysis was performed in R software.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: J.T.; Validation: G.H.; Formal analysis: G.H.; Investigation: G.H., J.T.; Writing - original draft: G.H., J.T.; Writing - review & editing: C.P., J.T.; Visualization: G.H., J.T.; Supervision: J.T.; Funding acquisition: C.P., J.T.

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**Supplementary information**

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.208736.supplemental>

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