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Epicardial spindle orientation controls cell entry into the myocardium

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Summary

During heart morphogenesis, epicardial cells undergo an epithelial to mesenchymal transition (EMT) and migrate into the subepicardium. The cellular signals controlling this process are poorly understood. Here, we show that epicardial cells exhibit two distinct mitotic spindle orientations, directed either parallel or perpendicular to the basement membrane. Cells undergoing perpendicular cell division subsequently enter the myocardium. We found that loss of β-catenin led to a disruption in adherens junctions and a randomization of mitotic spindle orientation. Loss of adherens junctions also disrupted Numb localization within epicardial cells and disruption of Numb and Numblike expression in the epicardium led to randomized mitotic spindle orientations. Taken together, these data suggest that directed mitotic spindle orientation contributes to epicardial EMT and implicate a junctional complex of β-catenin and Numb in the regulation of spindle orientation.

Keywords

epicardium; β-catenin; Numb

Introduction

The establishment of cell polarity is important for many biological processes including asymmetric cell division and directed cell migration. Par6, aPKC and Par3 are part of the polarity complex that establishes apical-basal polarity (Kemphues et al., 1988; Muller and Wieschaus, 1996), directs asymmetric cell division (Cheng et al., 1995; Kemphues et al., 1988), and positions adherens junctions during epithelial development (Harris and Peifer, 2004). As adherens junctions are the primary sites of epithelial cell attachment, they are essential for maintaining epithelial sheet integrity and apical-basal polarity, and one necessary component of the adherens junction is β-catenin (Yap et al., 1997).

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The epicardium is a single layer of mesothelial cells that attaches to and spreads over the myocardium beginning at E9.5 in the mouse. During heart morphogenesis, a subset of these cells migrates into the subepicardial space via the process of epithelial to mesenchymal transition (EMT) and subsequently differentiates into fibroblasts and vascular smooth muscle cells (VSMC) (Gittenberger-de Groot et al., 1998; Mikawa and Fischman, 1992; Poelmann et al., 1993; Wessels and Perez-Pomares, 2004). Previously, it has been demonstrated that β-catenin is essential for normal epicardial cell EMT (Zamora et al., 2007), but the cellular mechanisms behind this requirement remain unclear.

Here, we show that epicardial cells divide predominantly along two distinct axes, either parallel or perpendicular to the basement membrane. Cell divisions perpendicular to the basement membrane result in one cell remaining in the epicardium while the other cell exits the mesothelial monolayer. This process is reminiscent of an asymmetric cell division, a mechanism for cell differentiation in several epithelial cell populations (Lechler and Fuchs, 2005). The loss of β-catenin in the epicardium led to disruption of adherens junctions and randomized epicardial cell division patterns.

Furthermore, we found that Numb, an intracellular adaptor protein, associates with β-catenin in epicardial cells. Numb was originally identified in *Drosophila* as a cell fate determinant, where it is asymmetrically distributed in two daughter cells during neural progenitor division (Frise et al., 1996; Petersen et al., 2002; Spana and Doe, 1996; Uemura et al., 1989; Wirtz-Peitz et al., 2008). In mammals, two homologs, *Numb* and *Numblike*, exist (Verdi et al., 1996; Zhong et al., 1996), and they appear to function redundantly during development (Li et al., 2003; Petersen et al., 2004). Although the best known function of Numb is its role in asymmetric cell division (Knoblich et al., 1995; Rhyu et al., 1994; Wu et al., 2007), it also has been suggested to function in migration (Nishimura and Kaibuchi, 2007), cell death (Orgogozo et al., 2002), cell adhesion, and cell polarity (Rasin et al., 2007). We found that Numb and β-catenin co-localized to adherens junctions, and that epicardial cells with *Numb* and *Numblike* deletion displayed abnormal spindle orientation and failed to enter into the myocardium similar to epicardial cells lacking β-catenin.

Results

Epicardial cell proliferation is required for entry into myocardium

Epicardial cells arise from a small outcropping of cells called the proepicardium (Komiyama et al., 1987; Viragh and Challice, 1981). To determine how this limited number of cells expands to encompass the entire heart, we examined epicardial cell proliferation using BrdU incorporation. Because epicardial cells are a single layer of mesothelial cells surrounding the myocardium, we were able to use collagen IV staining to delineate the epicardial cell population (Fig. 1A). From the point of epicardial spreading until a few days after birth, we observed the highest rate of proliferation at E11.5 with a consistent decrease in the proliferation rate each subsequent day (Fig. 1B–C).

To test whether epicardial cell division was required for epicardial cell entry into the myocardium, we arrested the cell cycle using a specific inhibitor of DNA polymerase, aphidicolin. E12.5 hearts were treated with aphidicolin or vehicle and then labeled with BrdU. As shown by BrdU incorporation, aphidicolin treatment led to an arrest in the cell cycle (Fig. S1A). We next determined if aphidicolin treatment resulted in a failure of epicardial cell entry into the myocardium. Epicardial cells were traced by using a *PDGFRα*$^{GFP/+}$ knock-in line (Hamilton et al., 2003). In this line, H2b–eGFP is under the control of the *PDGFRα* promoter. In heterozygous *PDGFRα*$^{GFP/+}$ mice, GFP expression faithfully recapitulates PDGFRα expression and at E12.5 is restricted to a single layer of epicardial cells surrounding the heart (Fig. S1B). A secondary means of identifying

epicardial and epicardial derived cells (EPDC) was the expression of Wilm's tumor antigen 1 (WT1) (Moore et al., 1999;Zhou et al., 2008). At time zero in the control, $WT1^+$ and GFP^+ (Fig. 1D–E) cells resided solely within the epicardial layer, but after 48 hours ex vivo culture, control hearts contained labeled cells within the subepicardium. In contrast, there were significantly fewer $WT1^+$ or GFP^+ cells in this region in aphidicolin treated hearts (Fig. 1D–F). The inability of aphidicolin treated epicardial cells to enter the subepicardial space was not permanent, because epicardial cells entered the subepicardial space after aphidicolin was removed (Fig. 1D–F). The reduced number of GFP^+ cells in myocardium was not because of suppression of *PDGFR*α transcription by aphidicolin, as *PDGFR*α mRNA expression and GFP intensity was not significantly altered (Fig. S1 C – D). We observed very similar results when a second cell cycle inhibitor, doxorubicin, was used (Fig. S1E). Conversely, when we overexpressed cyclin D1 using adenoviral transduction (Ad-cyclin D1) to drive cell cycle progression, we observed an increase in the number of epicardial cells that had entered into the myocardium (Fig. 1G–H). Fig. S1F illustrates that there was a high efficiency of epicardial transduction. These results indicate that cell division is an important component of epicardial cell entry into the myocardium.

Epicardial mitotic spindles occur in two orientations

During development a subset of epicardial cells must exit the mesothelial monolayer and become mesenchymal. To determine if orientation of cell division impacts these events, we investigated spindle orientation of dividing epicardial cells identified by phosphorylated Histone H3 (p-Histone H3) and podoplanin (Mahtab et al., 2008). We observed that epicardial mitotic spindles occurred predominantly in two orientations, either perpendicular or parallel to the basement membrane. We quantified these patterns using α, β, or γ-tubulin staining. If the angle of the spindle was less than 30° from the basement membrane it was defined as a parallel division. If the angle of the mitotic spindle was 60–90° from the basement membrane it was defined as a perpendicular division (Fig. 2A–B and Fig. S2A).

To specifically identify dividing epicardial cells, we stained for WT1 and α-Tubulin. We found WT1 expressing cells undergoing perpendicular and parallel divisions (Fig. S2B). To demonstrate that the perpendicular division results in a viable cell within the subepicardium, we used murine stem cell virus (MSCV) transduction to preferentially target dividing cells. Whole hearts were transduced with MSCV-GFP and after 48 hours were sectioned and imaged. As expected we found pairs of cells in two configurations. The first configuration was one cell in the epicardium and the other abutting it in the subepicardium; the second configuration resulted in both cells residing in the epicardium (Fig. S2C). Quantification of these events showed that 27% ± 2.5% of the pairs at E12.5 and 74% ±3.2% at E13.5 resulted from a perpendicular cell division (n=172 pairs from 6 E12.5 hearts and n=136 pairs from 3 E13.5 hearts). These results are consistent with the division ratio demonstrated by tubulin staining at E12.5 and E13.5 (Fig. 2C–E). At E12.5 parallel divisions were more prominent, while at E13.5 perpendicular divisions occurred more often.

To observe these division patterns in real time, we used time-lapse confocal microscopy to image whole hearts. Using confocal microscopy and hearts from $PDGFR\alpha^{GFP/+}$, we traced the division patterns of epicardial cells. Consistent with our above results, epicardial cells displayed two distinct division patterns. Furthermore, the orientation of cell division appeared to influence the fate of the epicardial cell. When perpendicular epicardial cell division occurred, we observed one GFP^+ cell in the epicardium and one cell in the subepicardium (Fig. 2F). When a parallel division occurred the two GFP^+ cells remained within the plane of the epicardium (Fig. 2G). Using GFP to track epicardial cell divisions via time-lapse imaging, we found that 33% of the epicardial cells divided perpendicularly and 67% divided in the parallel orientation (109 cell divisions from 18 E12.5 hearts). In total, we observed 38 cells entering the myocardium, and 36 cells entered via perpendicular division,

while two appeared to migrate in without any apparent cell division. Time-lapse movies of a vertical and perpendicular cell division are provided in supplementary data (Movie S1a and S1b).

It has been proposed that epicardial cells undergo EMT in a stereotypic fashion where the dorsal cells at the atrio-ventricular junction undergo EMT earlier than ventral cells (Komiyama et al., 1987). Our data suggested that preferential perpendicular divisions coincide with the induction of EMT. To document this, we imaged E12.5 and E13.5 whole hearts for epicardial cell spindle orientation. The epicardial cells were identified by podoplanin staining (Mahtab et al., 2008) and spindle orientation was determined by p-Histone H3 and α-tubulin. The dominant spindle orientation of E12.5 epicardial cells was parallel, while for E13.5 epicardial cells, it was perpendicular (Fig. 3A–B and Table SI). Taken together these data suggest that two distinct orientations of cell division occur within the epicardium and that perpendicular divisions follow the pattern of EMT in the developing epicardium.

β-catenin is required to establish adherens junctions and cell polarity

Previous work has shown that epicardial deletion of *β-catenin* (βcat^{EKO}) results in failure of epicardial cell EMT *in vivo*, although *β-catenin* null epicardial cells still undergo EMT in vitro (Zamora et al., 2007). As β-catenin is an essential component of the adherens junction complex, we determined if adherens junctions were disrupted in βcat^{EKO} epicardial cells. In vertebrates, adherens junction components include the transmembrane protein cadherins, α-catenin and β-catenin (Hartsock and Nelson, 2008; Tepass, 2002), and we investigated the localization of N-cadherin and α-catenin in epicardial cells. We found that the adherens junctions proteins, N-cadherin and α-catenin, localized to the basal lateral domains of epicardial cells in control but not in βcat^{EKO} hearts (Fig. 4A–B), while the tight junction marker ZO-1 displayed normal localization (Fig. 4A–B) in both genotypes. Adherens junctions are also required to establish cell polarity and asymmetric cell division (Kaplan et al., 2009; Lu et al., 2001a; Marthiens and ffrench-Constant, 2009), and this notion has been supported by reports that β-catenin localization establishes cell polarity in some tissues (Cox et al., 1996; Fu et al., 2006; Morel and Arias, 2004). We then assessed whether β-catenin deficient epicardium displayed disrupted epicardial cell polarity. We first determined the distribution of cell polarity machinery proteins in wild type epicardial cells. Par3, Par6, and aPKC (PKCζ) are important components of the epithelial cell polarity complex (Knoblich, 2008). We found that all three of these proteins were localized to the apical surface of epicardial cells (Fig. 4C–E). These data demonstrate that the epicardium is a polarized epithelium. We also examined the polarity of these proteins in βcat^{EKO}. Although we did not see abnormal localization of these polarity proteins in interphase cells (data not shown), in 33 out of 40 dividing βcat^{EKO} cells, Par3 was no longer localized to the apical cortex (Fig. 4F–G).

Loss of β-catenin leads to randomized spindle orientation

Previous reports have suggested that adherens junctions are important for mitotic spindle orientation (den Elzen et al., 2009; Kraut et al., 1996; Lechler and Fuchs, 2005; Lu et al., 2001a). Because we observed a disruption in epicardial cell adherens junctions, we examined the spindle orientation in βcat^{EKO} epicardial cells. βcat^{EKO} epicardial cells at E12.5 contained a lower percentage of perpendicular divisions than the controls. Similar results were obtained in E13.5 epicardial cells (Fig. 5A–B). In the absence of β-catenin, epicardial cell division occurred randomly, which correlated with fewer cells entering the myocardium (Zamora et al., 2007). These results suggest that β-catenin may be important for directing epicardial spindle orientation.

To distinguish between the adherens junction function of β-catenin and its role in transcription, we rescued *βcat*^{EKO} hearts with either full-length or truncated, constitutively active β-catenin (Barth et al., 1999; Reya et al., 2003). The truncated β-catenin lacks the transactivation domain (Ad-trβcat) (Hsu et al., 1998; Vleminckx et al., 1999). Expression of both the truncated β-catenin and full length β-catenin promoted epicardial cell entrance into the myocardium (Fig. 5C and Fig. S3A). In contrast, Ad-GFP or a dominant negative TCF4 (Ad-dnTCF4) (Kolligs et al., 1999) failed to rescue epicardial cell EMT (Fig. 5C). We also found that Ad-TCF4 and Ad-dnTCF4 did not alter wild type epicardial cell entry into the myocardium (Fig. 5D). We verified that the Ad-trβcat and Ad-dnTCF4 inhibited Wnt3a induced gene transcription in primary cultured epicardial cells (Fig. S3B). In primary cultured *βcat*^{EKO} epicardial cells, Ad-βcat induced Wnt target gene transcription, while Ad-trβcat did not (Fig.S3C), confirming the transactivation incompetence of the truncated β–catenin.

To further demonstrate that the transactivation activity of β–catenin is not required for epicardial cell entry into the myocardium, we treated hearts with Inhibitor of Wnt Response (IWR) ENDO, which abrogates Axin degradation (Chen et al., 2009). We found that activation of β-catenin signaling was reduced by IWR ENDO (Fig. S3D–F). However, this reduction did not affect epicardial cell entry into the myocardium (Fig. S3G–H). In summary, these results suggest that β-catenin transcriptional activity was not required for epicardial cell entry into the myocardium.

Numb and β-catenin associate with each other

The polarization of epicardial cells caused us to further investigate proteins that are asymmetrically localized. Because Numb associates with β-catenin (Rasin et al., 2007) and has been shown to localize asymmetrically in progenitor cell populations (Zhong et al., 1996), we examined its expression in the epicardium. Numb was expressed by both cardiomyocytes and epicardial cells but in the latter localized to adherens junctions (Fig. 6A, Fig. S4A–B and Fig. S5A). We then observed that Numb localization changed in dividing cells. In late metaphase, regardless of the orientation of cell division, Numb weakly localized to the basal lateral surface in roughly 70% of the dividing epicardial cells (n=114 mitotic events in E12.5 10 hearts) (Fig. S4B and data not shown). Consistent with reports that localization of Numb and apical proteins are reciprocal (Smith et al., 2007), Par3 and Numb localized to the apical and basal domain of cells respectively, regardless of their division pattern (Fig. 4F, Fig. 6A, Fig. S4, Fig. S5A and data not shown).

The disrupted cell polarization and adherens junctions of *βcat*^{EKO} epicardial cells prompted us to examine Numb localization in these hearts. In wild type epicardium, Numb was localized to adherens junctions and co-localized with β-catenin. However, in *βcat*^{EKO} epicardial cells, Numb localization in adherens junctions and at the basal surface did not occur (Fig. 6A). We then determined whether Numb, β-catenin and N-cadherin existed in a complex using primary epicardial cell lysates. We found that β-catenin and N-cadherin precipitates, but not Par3 precipitates, contained Numb. Similarly, Numb and N-cadherin, precipitates contained β-catenin (Fig. 6B). These data show that β-catenin and Numb exist in a complex similar to what had been suggested previously (Rasin et al., 2007). To determine if the interaction between Numb and N-cadherin was β-catenin dependent, we precipitated N-cadherin from *βcat*^{EKO} and control epicardial cells lysates. We found that the N-cadherin and Numb interaction occurred in the absence of β-catenin (Fig. 6C). These results suggested that N-cadherin, β-catenin and Numb may exist as a complex.

Numb family proteins (NFP) are required for epicardial cell development

To study the functions of NFP in epicardial cells, we generated animals lacking Numb and Numblike in epicardial cells (NFP^{EKO}). Fig. S5A–B demonstrates efficient loss of expression Numb in epicardial cells. Real-time PCR demonstrated a 76%±3 and 56%± 7 reduction in Numb and Numblike expression levels in primary epicardial cell cultures, respectively (data not shown). Loss of NFP in the epicardium resulted in embryonic lethality between E12.5 and E14.5. Because our data suggested an interaction with β-catenin, we examined the EMT process in these mutant hearts. We found that fewer $WT1^+$ cells entered the myocardium in NFP^{EKO} hearts at both E13.5 and E14.5 (Fig. 6D and Fig. S5C). We also used Ad-GFP transduction (Mellgren et al., 2008) to trace epicardial cell migration ex vivo and found that NFP^{EKO} hearts had significantly fewer GFP^+ cells in the myocardium after stimulation (Fig. 6E). Finally, we used $GATA5Cre^{Tg}$:$ROSA26R^{YFP}$ lineage tracing to follow the epicardially derived cells and found fewer cells within the subepicardial space (Fig. S5D–E).

As Numb is a component of adherens junctions (Kuo et al., 2006; Wang et al., 2009), we investigated whether NFP^{EKO} epicardial cells displayed adherens junction and cell polarity defects similar to βcat^{EKO} hearts. Similar to βcat^{EKO}, adherens junction proteins were mislocalized (Fig. 6F). Par3, aPKC and Par6 were also mis-localized in NFP^{EKO} epicardial cells (Fig. 7A–C). By contrast, ZO-1 localization was not disrupted (Fig. 6F).

We then investigated spindle orientation in NFP^{EKO} epicardial cells. Compared to controls, NFP^{EKO} epicardial cells had fewer cells undergoing perpendicular divisions, and the spindle orientation appeared to occur more in the parallel orientation (Fig. 7D–E). Taken together, these results suggest that β-catenin and NFP expression are important for directing the orientation of epicardial cell division and that this may have some impact on the ability of epicardial cells to undergo EMT.

Discussion

The development of the epicardium is a highly dynamic process that plays a critical role in producing coronary VSMC and cardiac fibroblasts. Little is known about the processes that direct a subpopulation of these cells to exit the mesothelial epicardium. A better understanding of these events may allow the manipulation of adult epicardial cells to facilitate myocardial regrowth after cardiac damage has occurred.

During EMT, epithelial cells undergo morphological changes and become mobile mesenchymal cells (Boyer and Thiery, 1993; Hay, 2005; Liebner et al., 2004). EMT is a highly regulated, yet heterogeneous, multiple-step process (Thiery and Sleeman, 2006). EMT starts with the disassembly of cell-cell junctions, loss of apical-basal polarity, and the transition to a purely mesenchymal cell type (Hay, 2005; Thiery and Sleeman, 2006). However, these cellular events are not obligatory for each EMT process. EMT is a species and cell context specific process, and each EMT uses unique initiation cues, transcription factors, and signal transduction cascades. During mouse gastrulation (an EMT dependent process), TGFβ, Wnt, and FGF pathways are important for mesoderm cell formation (Nakaya and Sheng, 2008), while TGFβ, BMP, and notch signaling are important for endocardial cushion EMT (Boyer et al., 1999; Camenisch et al., 2002; Ma et al., 2005; Timmerman et al., 2004). During neural crest EMT, cells from the dorsal neural tube undergo EMT and dissociate from the neural epithelium and migrate to their destined regions in a context dependent manner (Duband et al., 1995; Duband and Thiery, 1987; Nichols, 1981; Sauka-Spengler and Bronner-Fraser, 2008; Tucker et al., 1988). Recently, it has been demonstrated that neural crest cells leave the neural tube by diverse morphological

processes (Ahlstrom and Erickson, 2009). This suggests that even within a given cell population, EMT may not be a canonical process.

Recent studies demonstrate that WT1 is an essential component of the epicardial EMT process by controlling Snail and E-cadherin expression (Martinez-Estrada et al., 2009), yet it is apparently expressed in all mesothelial epicardial cells and transiently expressed in EPDCs. Because WT1 shuttles between the nucleus and cytoplasm, it could be a candidate factor that distributes asymmetrically during epicardial EMT. However, we were unable to observe any differences in WT1 intensity or localization when we compared WT1 expression in the epicardium and in cells entering the subepicardial space (data not shown). Therefore, WT1 may be involved in an EMT function that is downstream of oriented cell division.

The process of epicardial EMT has been studied extensively in avian proepicardial explants (Dettman et al., 1998; Lu et al., 2001b), but until recently very few experiments have been performed in mice. Interestingly, we found epicardial EMT may not be a canonical EMT process. The epicardium is a mesothelial layer rather than a true epithelial layer. For example, vimentin is expressed uniformly in the mouse epicardium (Mellgren et al., 2008), which suggests that the mouse epicardium may be more mesothelial than epithelial in nature. In addition, unlike many other developmental EMT events, cell division appears to be required for epicardial cell EMT. Finally, in many EMT processes, β-catenin nuclear localization is the signal that initiates the mesenchymal transcription profiles (Hay, 2005), yet, here we show that β-catenin localization in the adherens junctions may be a critical component of epicardial EMT.

β-catenin is well known for its function as a transcriptional activator in cell proliferation and cell fate determination in response to Wnt signaling (Cadigan and Nusse, 1997), and our results do not exclude a transcriptional role later in epicardial cell differentiation. Another activity for β-catenin is connecting adherens junctions with the cytoskeleton (Huber and Weis, 2001; Nelson and Nusse, 2004; Tepass, 2002). Others have suggested a role for β-catenin in directing spindle orientation (Le Borgne et al., 2002; Walston et al., 2004), centrosome separation (Bahmanyar et al., 2008), and asymmetric cell division (Mizumoto and Sawa, 2007; Schneider and Bowerman, 2007). Thus, β-catenin function is probably context dependent and has to be examined in a cell type specific manner.

Centrosomes, part of the microtubule organizing center (MTOC), are important for directing spindle orientation and asymmetric cell division (Rusan and Peifer, 2007; Yamashita and Fuller, 2008; Yamashita et al., 2007). In epicardial cells, we observe that the centrosome localizes to the apical domain during interphase. During mitosis, centrosomes localize to the lateral domains in a parallel division and to the apical-basal domains in a perpendicular division. In the *Drosophila* male germline, the inheritance of the mother centrosome determines if the cell remains a stem cell (Yamashita et al., 2007). Future work will determine if a similar scenario exists in the mouse epicardium. Recently, it has been reported that Connexin 43, a gap junction protein, is required for efficient epicardial cell directed migration after EPDC formation (Rhee et al., 2009). In this model, gap junctional signaling helps organize actin stress fibers and permits directed migration. *In vitro*, examination of MTOC demonstrated *connexin 43* mutants exhibited a random location of the MTOC compared to the leading edge organization observed in control epicardial cells. Thus, the MTOC could be involved in entry into the myocardium after an epicardial cell perpendicular division *in vivo*.

In summary, we show that loss of *β-catenin* leads to a disruption of Numb localization to adherens junctions, consistent with results reported for the retina (Fu et al., 2006). Our

results suggest that Numb, β-catenin and N-cadherin exist as a complex and that loss of NFP leads to disrupted adherens junctions and cell polarity: a phenotype resembling the loss of *β-catenin* phenotype. Finally, we show that β-catenin and NFP were involved in mitotic spindle orientation of epicardial cells. These results are similar to the discovery that α-catenin and cadherins are involved in the control of mitotic spindle orientation *in vivo* (Lechler and Fuchs, 2005) and in vitro (den Elzen et al., 2009). We suggest a junctional function for β-catenin and NFP in the regulation of epicardial cell polarity and spindle orientation and that loss of these proteins leads to a failure in epicardial EMT.

Materials and methods

Experimental animals

Mouse strains used were: $PDGFR\alpha^{GFP/+}$ (Hamilton et al., 2003), $\beta\text{-}catenin^{fl/fl}$ (Brault et al., 2001), $Numb^{fl/fl}$ and $Numblike^{fl/fl}$ (Wilson et al., 2007; Zilian et al., 2001) and $Gata5Cre^{Tg/0}$ (Merki et al., 2005) mice. Embryos whose genotype were $\beta\text{-}catenin^{fl/fl}$;$Gata5Cre^{Tg/0}$ were designated as βcat^{EKO}, while embryos whose genotype were $Numb^{fl/fl}$; $Numblike^{fl/fl}$; $Gata5Cre^{Tg/0}$ were designated as NFP^{EKO} . All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center and performed according to the Guide for the Care and Use of Laboratory Animals published by the NIH.

Paraffin and frozen section immunohistochemistry (IHC)

IHC was performed as previously published (Lechler and Fuchs, 2005; Mellgren et al., 2008). The following primary antibodies were used: Numb (1:300, Abcam, ab14140); Numb (1:100, Abcam, ab4147); β-catenin (1:500, BD Pharmingen, 61053); Phosphorylated Y489 β-catenin (1:50, DSHB, University of Iowa); α-catenin (1:100, Abcam ab51032); N-Cadherin (1:250, BD Transduction, 610920); pan-Cadherin (1:200, Sigma C3678); Par3 (1:100, Upstate, 07–330); Par6 (1:100, Abcam, ab6022); aPKC (1:100, Santa Cruz Biotechnology, sc-216); BrdU(1:50, Becton Dickinson, 347583); Ki67(1:100, Novocastra Laboratories, NCL-L-Ki67-MM1); α-Tubulin (1:1000, Sigma, T6199), Collagen IV (1:1000, Chemicon, AB756P), β-tubulin (1:1000, BD Pharmingen, 556321), γ-Tubulin (1:1000, Sigma, T5326), acetylated α-Tubulin (1:1000, Sigma, T6793) and WT1 (1:100, DAKO, M3561 or 1:50, Santa Cruz Biotechnology, sc-19).

Epicardial cell proliferation

Pregnant females or postnatal pups were injected with 100 μg BrdU (Sigma) per gram of body weight for four hours before embryo or heart isolation. Hearts were fixed in 4% PFA for at least 1 hours, then frozen embedded, and sectioned. Antigen retrieval was carried out by microwave antigen retrieval as previously described (Mellgren et al., 2008). Sections were then blocked 45 minutes in 3% BSA in 0.1% TWEEN-20, and stained for 2 hours or overnight with a 1:50 dilution of anti-BrdU antibody (Becton Dickinson) and a 1:1000 dilution of anti-Collagen IV (Chemicon). Epicardial cells were designated as the cells adjacent to the basement membrane on the exterior of the heart. Proliferation index was calculated by dividing the number of BrdU positive cells in the epicardium by the number of DAPI stained epicardial nuclei and multiplying by 100. A minimum of six sections per heart from a total of at least three hearts was quantified at each stage.

Imaging

The following equipment was used: confocal imaging (LSM510META) (grant NIH 1-S10-RR019406-01), fluorescent imaging (Zeiss Axiovert 200 with a Hamamatsu ORCA-ER

camera), time-lapse confocal imaging (Perkin Elmer Ultraview ERS spinning confocal microscope) and color imaging (Zeis Axiovert 200 with an Olympus DP71 camera).

MSCV virus

VSVG pseudotyped virus was produced, and viral transduction was carried out as described (Duncan et al., 2005) with some modifications. Briefly, concentrated MSCV-IRES-GFP viral solutions were incubated with E12.5 or E13.5 hearts for 20 hours. Hearts were washed and incubated in fresh medium for another 24 hours. Hearts were fixed and frozen-sectioned. Divisions where two daughter cells both localized to epicardium were counted as parallel division, while divisions where one daughter was in the subepicardium and the other daughter was in the epicardium were counted as perpendicular division.

Epicardial cell entry into myocardium

Wild type or *PDGFRα*$^{GFP/+}$ E12.5 whole hearts were cultured as previously described (Mellgren et al., 2008) and treated with aphidicolin (Calbiochem) at a concentration of 2μg/ml or vehicle for the indicated time. Some hearts were stained with WT1. WT1 or GFP^{+} cells that were present within the myocardium were quantified. At least 6 entire midcoronal sections per heart were quantified. Neural crest cells were excluded. In one group, aphidicolin treated hearts were treated for 24 hours, and then aphidicolin was washed out. After an additional 24 hours, hearts were processed. A minimum of 3 hearts for each treatment was quantified.

Epicardial cell viral transduction

Full length cyclin D1 cDNA was cloned using SuperScript III reverse transcriptase (Invitrogen). The full-length cDNA was then cloned into pAd/CMV/V5-DEST adenovirus vector (Invitrogen, V493-20) via the Gateway LR system. Cyclin D1 was overexpressed in epicardial cells of E12.5 *PDGFRα*$^{GFP/+}$ hearts for 48 hours via adenovirus-mediated transduction (Mellgren et al., 2008). GFP^{+} cells that were present within the myocardium were quantified. At least 6 entire midcoronal sections per heart and at least three hearts for each treatment were quantified as described above. Adenoviral transduction of the epicardium proved to be very efficient with near 100% of the epicardial cells expressing the adenoviral construct. See Fig. S1F for examples of transduction efficiency.

Primers and sequences for making Ad-βcat, -trβcat, -TCF4 and -dnTCF4 adenovirus are available in Supplemental Materials and Methods.

Immunoprecipitation and western blot

Individual control or *βcat*^{EKO} E12.5 hearts were cultured to isolate primary epicardial cells as described (Mellgren et al., 2008). After 48 hours, heart explants were removed, and epicardial cells were expanded for five days. Lysates from hearts of each genotype were combined and processed for immunoprecipitation with 1 μg of primary antibody. Antibodies used for immunoprecipitation and western blots include Numb (1:1000); β-catenin (1:1000); N-Cadherin (1:500); pan-Cadherin (1:500); Par3 (1:250) and β-tubulin (1:1000).

Time-lapse imaging

E12.5 hearts from *PDGFRα*$^{GFP/+}$ mice (Hamilton et al., 2003) were harvested and embedded in low melt agarose in glass bottomed 35 mm dishes. Ventricles were seated for two hours and then subjected to microscopy using a spinning disk confocal system (Ultraview ERS; PerkinElmer) set up on a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with an Orca Hamamatsu camera. Epicardial cell division was followed by imaging 9 planes with 2 μm per plane using a 40X lens for 8 hours with 6 minutes per

time point. Cultures were maintained at 37°C, 5% CO_2 and saturating levels of humidity. Images were acquired by Volocity (Version 5.0.3) and edited by Imaris (Version 6.2). Target cells were reviewed in movie replay after data concatenation into time-lapse stacks. To generate the AVI videos, data were collected over all continuous planes and projected into one image per time point.

Quantitative evaluation of the orientation of the epicardial cell division plane

Coronal, sagittal, and transverse sections of E12.5 and E13.5 hearts were stained with α, β, or γ-tubulin. Spindle orientations of left and right ventricular epicardial cells in late metaphase, anaphase or early telophase, where both centrioles or centrosomes are in the same focal plane, were quantified. The orientation of the spindle is determined by the angle between the spindle axis and a basement membrane reference line. Division planes positioned at 60–90° to the basement membrane were classified as perpendicular and those that were oriented at 0–30° were classified as parallel.

For whole mount, hearts were fixed in 4% PFA overnight, then permeabilized by 0.5% Triton-100 in PBS (PBT) for at least 2 hours. After permeabilization, hearts were blocked in 10% FBS for at least 2 hours, then incubated with primary antibody overnight. After removing primary antibody by washing with PBT, hearts were incubated with secondary antibody for at least two hours. Hearts were washed with PBT, and then cleared with glycerol:PBS. To determine the spindle orientation of left and right ventricular epicardial cells in E12.5 and E13.5 whole mount stained hearts, *Z*-stack images were acquired using a Zeiss LSM 510 Meta confocal laser-scanning microscope with one μm per section. Three-dimensional images were reconstructed using Imaris software. The spindle orientation was measured by the angle between spindle axis, determined by the two centrosomes, and the *x-y* plane of heart surface (Toyoshima and Nishida, 2007). To determine the spindle orientation of epicardial cells in the time lapse imaged hearts, Z-stack images were acquired using spinning disk confocal in a time-lapse manner as described above. Three-dimensional images were reconstructed and smashed to relative real size of the epicardial cells using Imaris. Spindle orientation was determined by angle between the spindle axis, determined by the two nuclei labeled by the H2b–GFP, and the *x-y* plane of heart surface (Toyoshima and Nishida, 2007). As described above, divisions whose angles are between 0–30° were classified as parallel, while 60–90° were classified as perpendicular.

Statistics—Data shown as mean ± standard deviation, and p values were determined by Student's t test or one way ANOVA using Prism 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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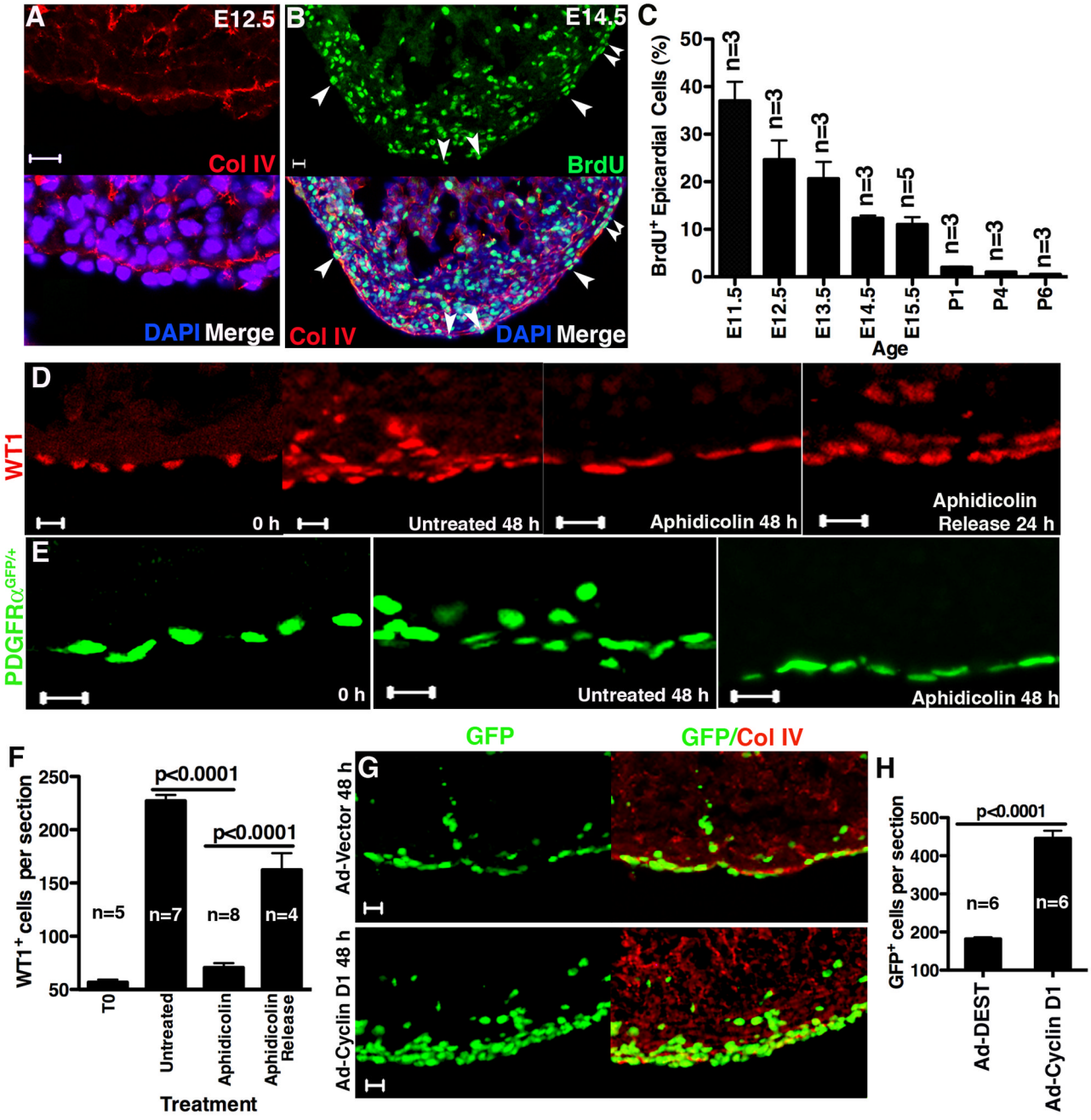

Figure 1. Cell proliferation is required for epicardial cell EMT

(A) The basement membrane of the epicardium was identified by staining for collagen IV (Col IV). The layer of mesothelial cells attached to the basement membrane on the outer surface of the heart is the epicardium. (B) BrdU labeling indicates epicardial cell proliferation. Arrowheads point to epicardial cells that are $BrdU^+$. Scale bar = 20 μm. (C) Proliferation rate of epicardial cells at indicated time points. Six midcoronal sections per heart for each age were quantified. (D–E) WT1 and GFP from *PDGFRα*$^{GFP/+}$ mice were used to visualize epicardial cell location with ex vivo culture and aphidicolin treatment. (F) Quantification of $WT1^+$ cells present within myocardium after treatment. (G) *PDGFRα*$^{GFP/+}$ hearts were used to visualize epicardial cell location with ex vivo culture and

cyclin D1 over-expression at 48 hours. (H) Quantification of GFP^+ within the myocardium after 48 hours of culture with control adenovirus or cyclin D1 adenovirus. Six midcoronal sections per heart were quantified. Scale bar = 10 μm. See also figure S1.

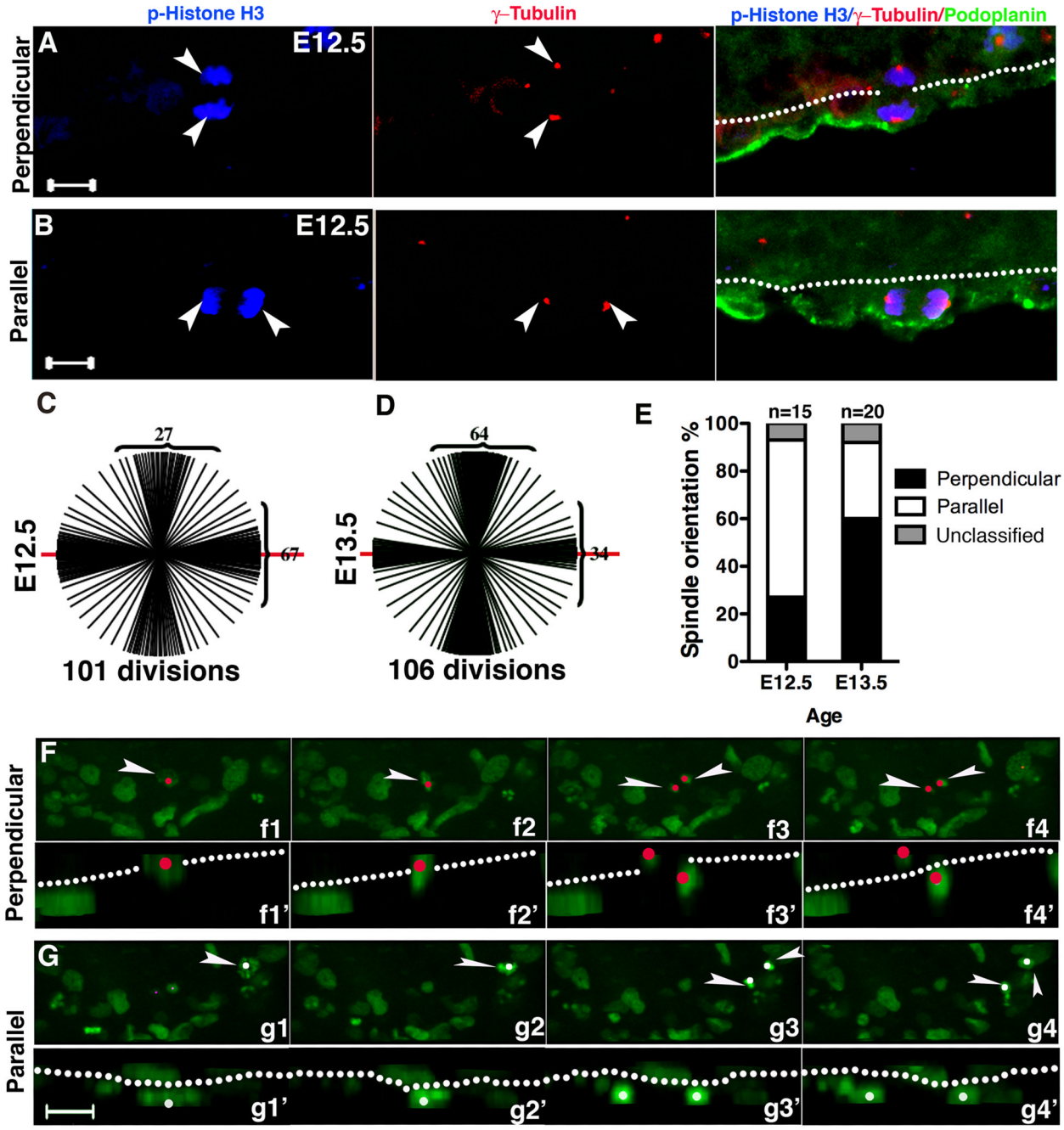

Figure 2. Mitotic spindle orientation of epicardial cells

(A–B) E12.5 hearts stained with podoplanin, p-Histone H3 and γ-tubulin indicate epicardial mitotic spindles are either (A) perpendicular or (B) parallel to the basement membrane indicated by dotted line. Arrowheads point to epicardial cells positive for p-Histone H3 labeled centrosomes. (C–E) Quantification of epicardial cell spindle orientations at E12.5 and E13.5. Spindles were visualized by staining for γ-tubulin (as in A and B) or α-tubulin (as in Fig. S2). The basement membrane was considered 0° and is represented by the red line. Each line represents the spindle orientation of a dividing epicardial cell. Numbers in brackets quantify the divisions that occur between 0–30° and 60°–90°. (E) Ratio of spindle orientations at each embryonic stage. (F,G) Time lapse and Z stack images of E12.5 hearts

cultured ex vivo. Tracing GFP positive cells, we observed divisions that were either (F) perpendicular or (G) parallel to the basement membrane. f1–f4 are consecutive images that were taken every 6 minutes. Arrowhead points to a cell that starts in (f1) pro-metaphase, then moves to (f2) metaphase, (f3) telophase, and (f4) produces two daughters. g1–g4 is similar to f1–f4 except this cell completed a parallel division. Images indicated by the primes are side views, and nuclei are indicated by red and white dots, respectively. Dotted lines indicate the basement membrane. Scale bar = 10 μm. See also figure S2.

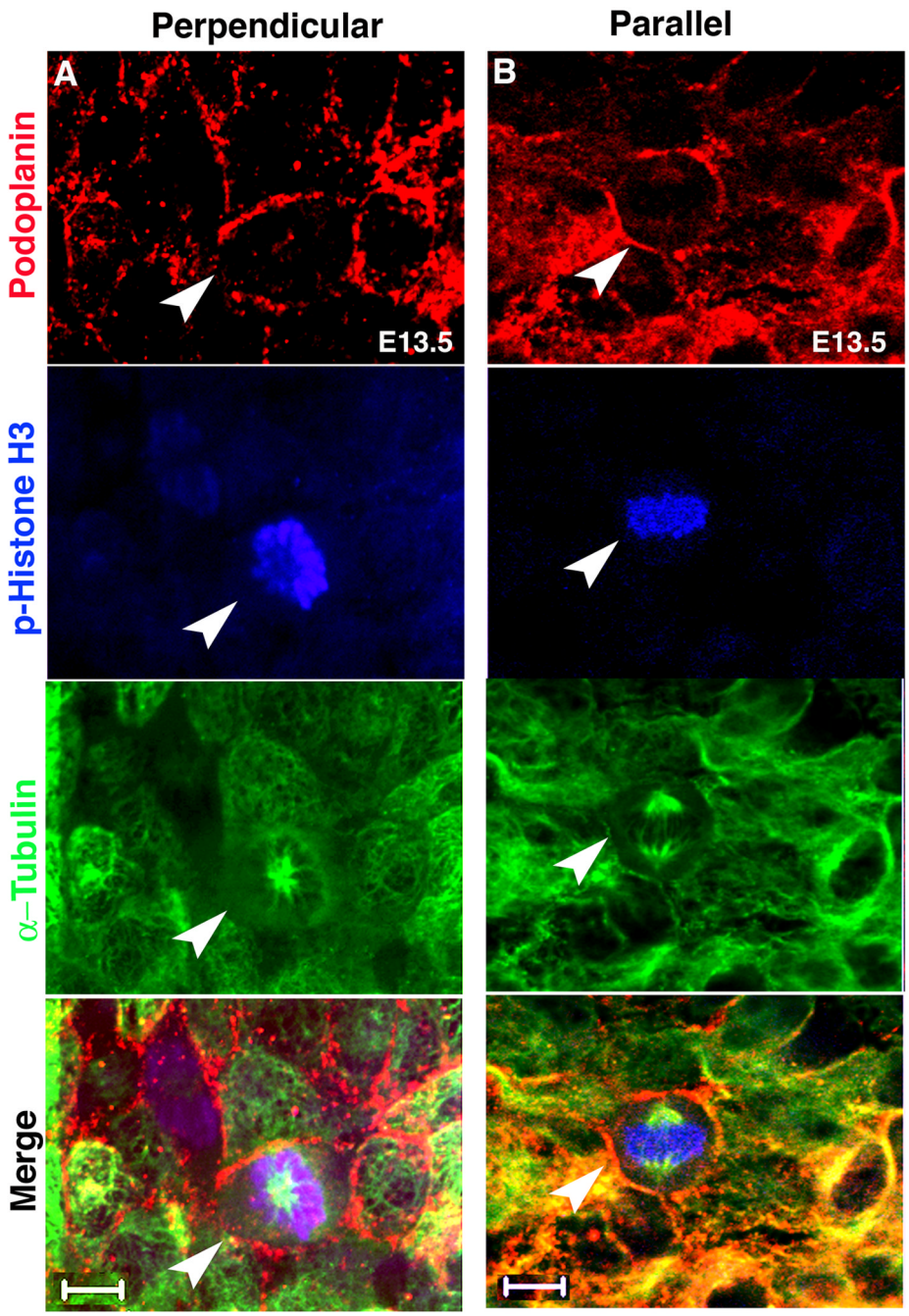

Figure 3. Whole mount staining of dividing epicardial cells

(A) Perpendicular and (B) parallel divisions can be observed in whole mount stained hearts. Podoplanin, p-Histone H3 and α-Tubulin stained: epicardial cells, mitotic cells and spindles respectively. Arrowheads point to the mitotic cells. Scale bars = 10 μm. See Table S1 for additional information.

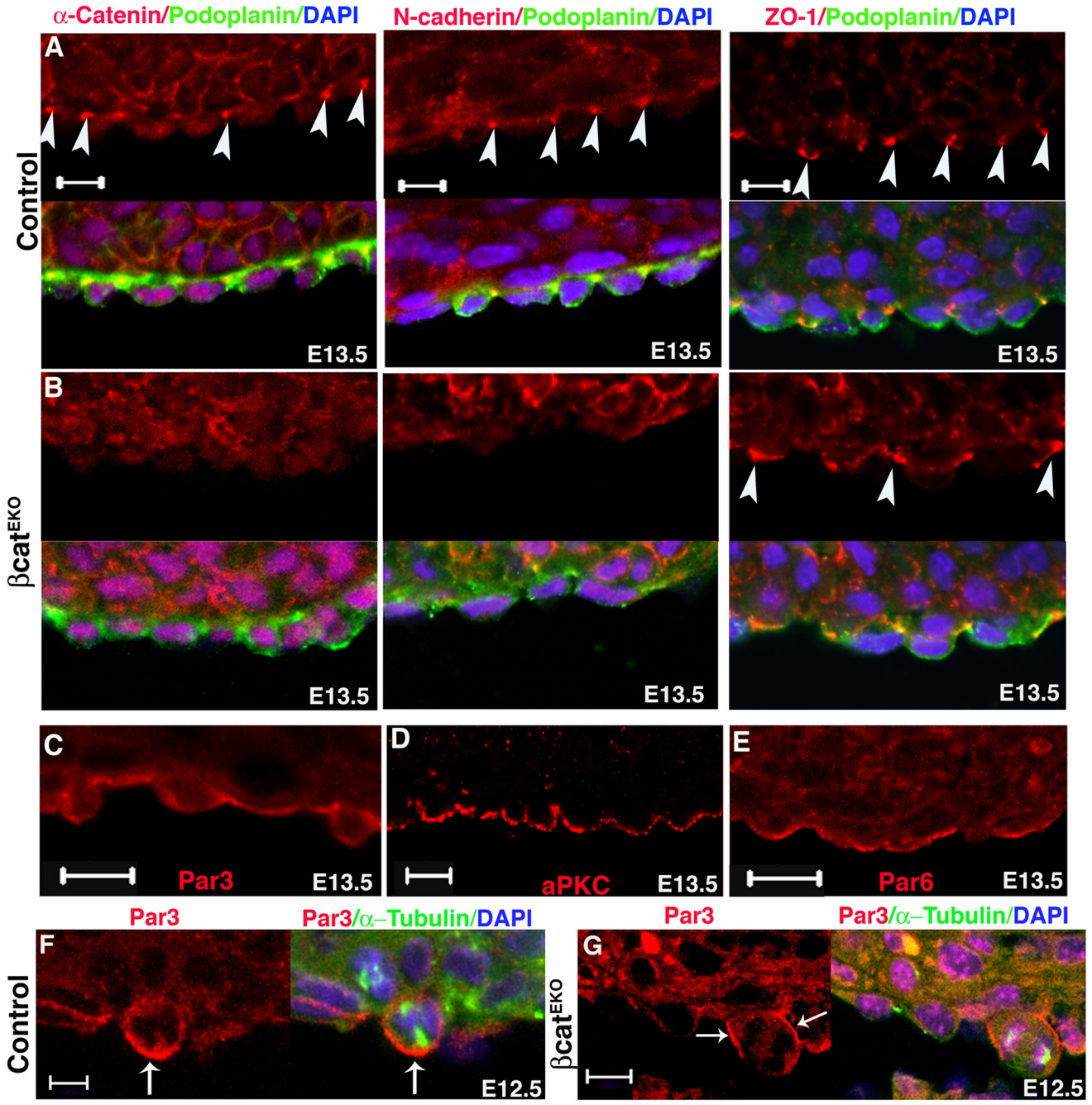

Figure 4. Epicardial cell junctions and polarity

Formation of adherens junctions (α-catenin and N-cadherin) and tight junctions (ZO-1) in (A) control and (B) *βcat*^{EKO}. Arrowheads point to the junctions. Podoplanin marked epicardial cells. (C–E). Wild type hearts were stained for the indicated cell polarity proteins. (F–G) Par3 localization in (F) control and (G) *βcat*^{EKO} mitotic epicardial cells. Arrow indicates accumulated Par3. Scale bar = 10 μm.

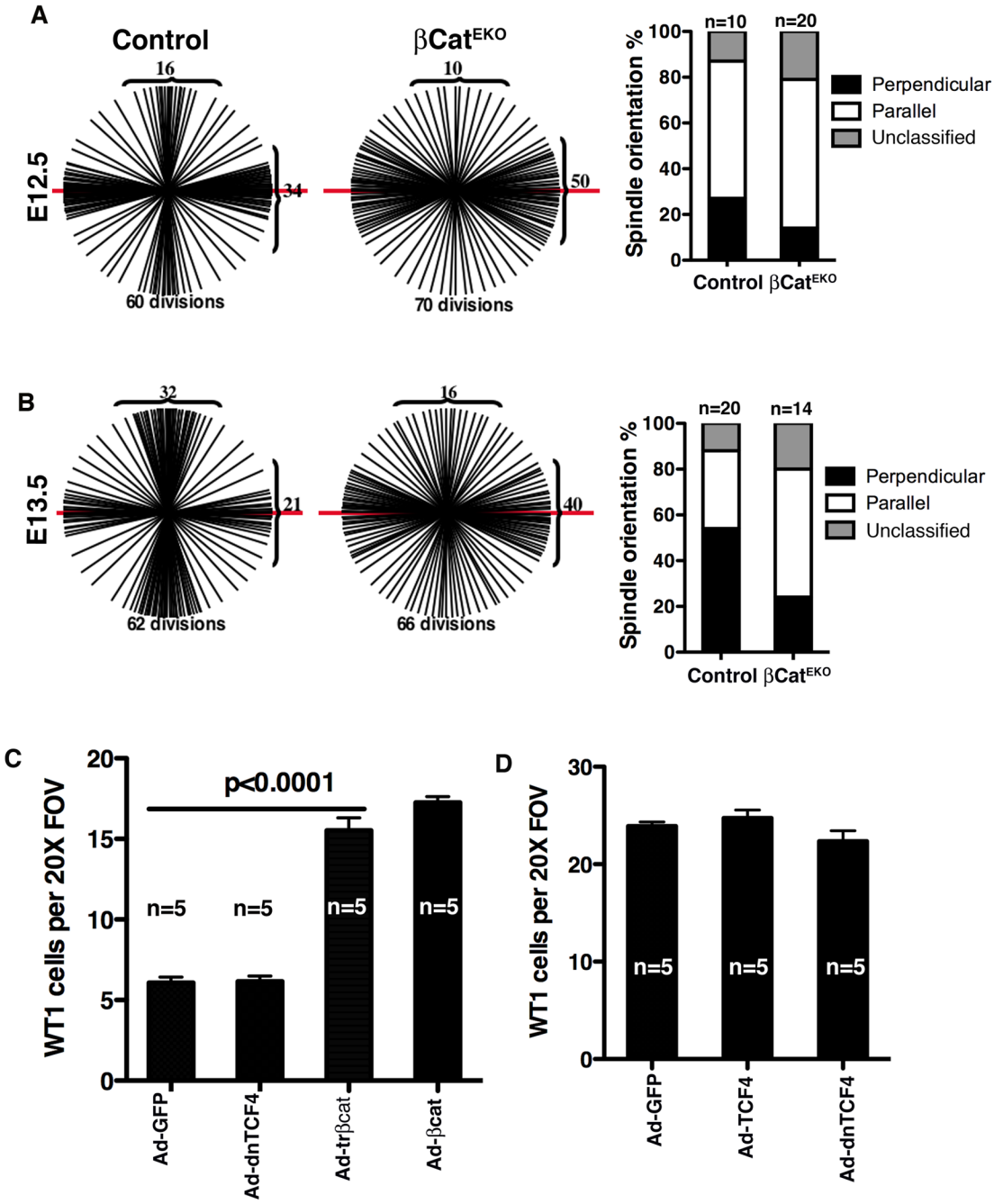

Figure 5. Spindle orientation in *βcat^EKO* epicardial cells and rescue of EMT by a transactivaton domain deficient β-catenin

(A,B) Spindle orientation of control and *βcat^EKO* epicardial cells at E12.5 and E13.5. For illustration purposes, spindle orientation events from only 9 hearts for each genotype and age are shown in the diagrams. Bar graphs at the right indicate the summary of spindle orientations. (C) In *βcat^EKO* hearts, adenoviruses overexpressg full length (Ad-βcat) or transactivation domain deleted β-caten (Ad-trβcat) rescue *βcat^EKO* EMT but Ad-GFP and Ad-dnTCF4 do not. (D) EMT results from wild type hearts transduced with Ad-dnTCF4 or Ad-TCF4. See also figure S3.

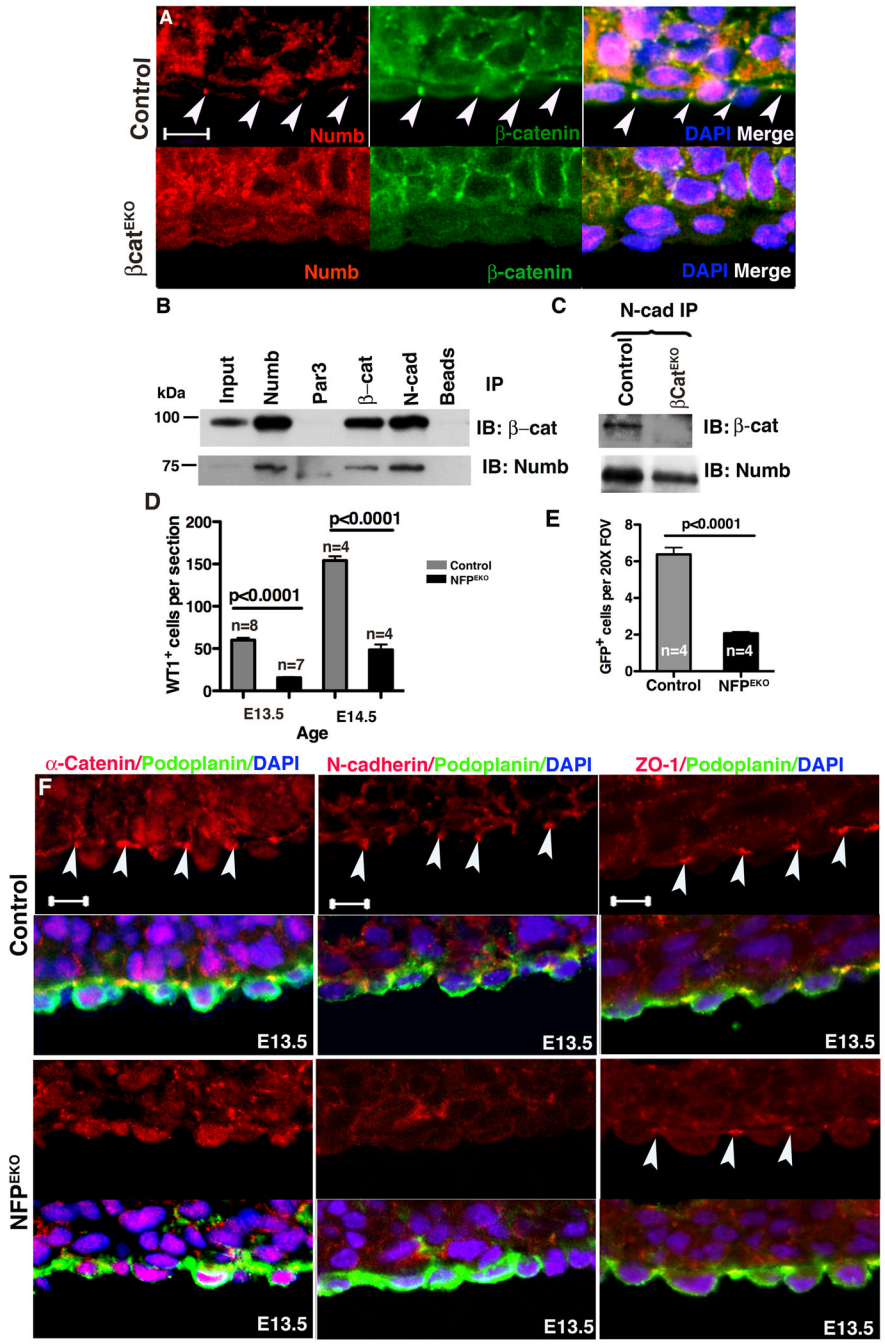

Figure 6. Numb function in adherens junctions and *NFP*^{EKO} epicardial phenotype

(A) Numb localized to adherens junctions in control but not in *βcat*^{EKO} epicardial cells. Arrowheads indicate adherens junctions. (B) Numb, Par3, N-cadherin (N-cad) and β-catenin (β-cat) were immunoprecipitated from primary epicardial cell lysates and blotted for the indicated proteins. (C) N-cadherin was immunoprecipitated from control and *βcat*^{EKO} epicardial cell lysates and blotted for interactions with the indicated proteins. Input was 5% of the lysate. (D) Fewer WT1^{+} cells were present in the myocardium of *NFP*^{EKO} than in control hearts. (E) Ad- GFP was used to trace epicardial cell EMT in an ex vivo culture system. (F) Adherens junctions (α-catenin and N-cadherin) and tight junctions (ZO-1) in

NFP^{EKO} epicardial cells. Arrowheads point to junctions. Podoplanin was used to identify epicardial cells. Scale bar = 10 μm. See also figure S4.

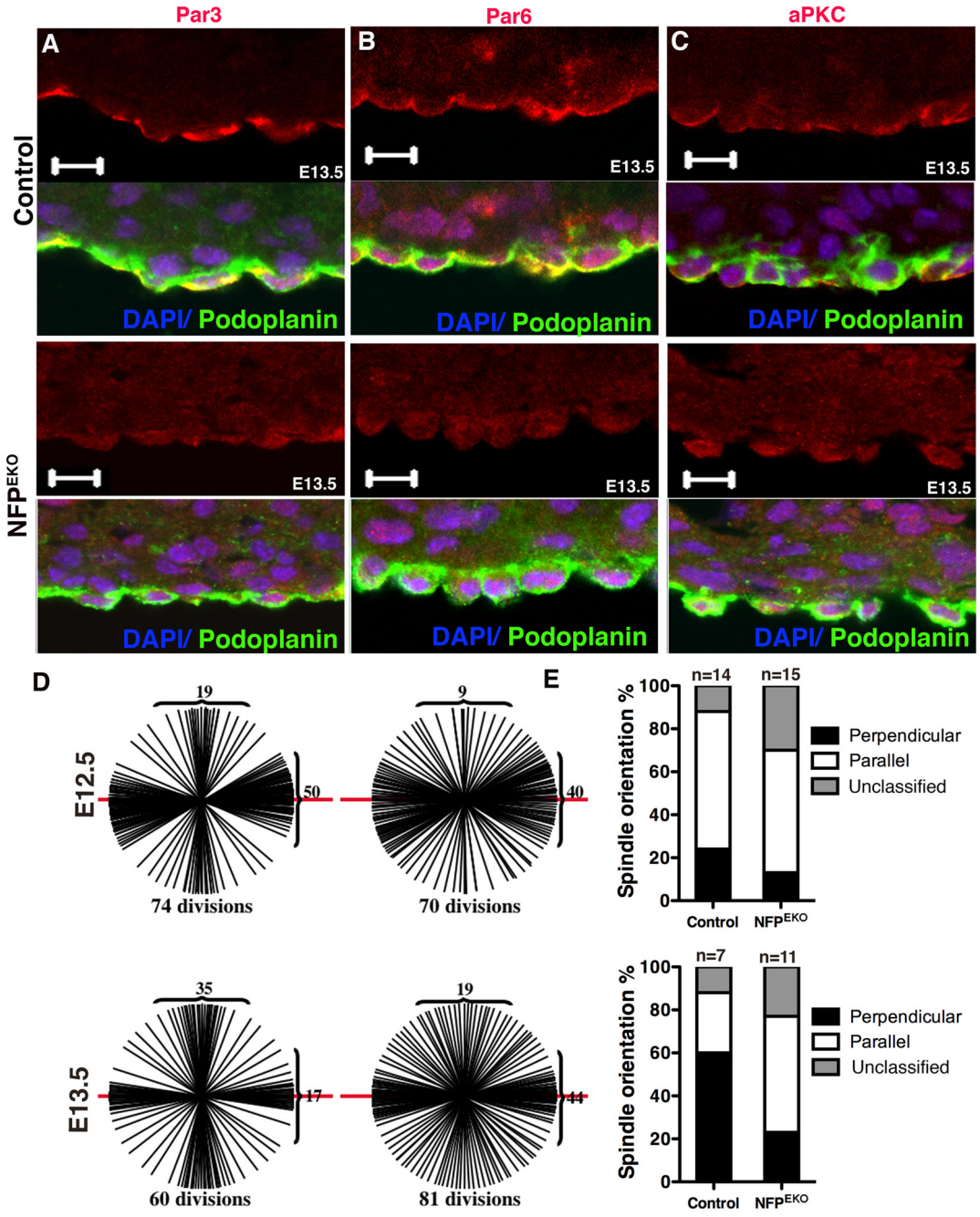

Figure 7. NFP mutant hearts exhibit disrupted epicardial polarity and spindle orientation

(A–C) *NFP*^{EKO} epicardial cells displayed abnormal apical basal polarity based on Par3, Par6 and aPKC localization. (D) Spindle orientation of control and *NFP*^{EKO} epicardial cells. Spindle orientation events from 9 hearts for each genotype, except control at E13.5 which used 7 hearts. (E) Indicates the ratio of spindle orientations at each embryonic stage. Scale bar = 10 μm. See also figure S5.