Wnt pathway modulation is required to correctly execute multiple independent cellular dynamic programs during cranial neural tube closure

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Abstract

Defects in cranial neural tube closure are among the most common and deleterious human structural birth defects. Correct cranial closure requires the coordination of multiple cell dynamic programs including cell proliferation and cell shape change. Mutations that impact Wnt signaling, including loss of the pathway co-receptor LRP6, lead to defects in cranial neural tube closure indicating that this pathway is an important mediator of this critical morphogenetic event, but the cellular dynamics under control of the Wnt pathway during closure remain unclear. Here, we use mice mutant for LRP6 to examine the consequences of conditional and global reduction in Wnt signaling, as well as conditional inactivation of APC to examine the consequences of pathway hyperactivation. Strikingly, we find that regulated Wnt signaling is required for two independent events during cranial neural tube closure. First, global reduction of Wnt leads to a surprising hyperplasia of the cranial neural folds driven by excessive cell proliferation at early pre-elevation stages. The increased tissue volume presents a mechanical blockade to efficient closure despite normal apical constriction and cell polarization at later stages. Conversely, conditional hyperactivation of the pathway at elevation stages prevents apical constriction and neural fold elevation but has no impact on cell polarization or proliferation. Together these data reveal that Wnt signaling levels must be modulated to restrict proliferation at early stages and allow for apical constriction later at elevation stages to promote efficient closure of the cranial neural tube.

Keywords: Wnt, Lrp6, Apc, neural tube closure, apical constriction, proliferation

Introduction

Formation of the brain requires the conversion of the cranial neural plate, a sheet of neuroepithelial cells, into the closed tube that will form the structural basis of the cranial nervous system. This process is known as cranial neural tube closure and defects in its execution are among the most common and deleterious human birth defects, resulting in invariable pre- or perinatal lethality (Copp et al., 2013; Wallingford et al., 2013; Wilde et al., 2014; Zaganjor et al., 2016). This process exhibits complex genetic requirements, with well over a hundred genes implicated in cranial closure (Harris and Juriloff, 2007; Harris and Juriloff, 2010; Lee and Gleeson, 2020; Wilde et al., 2014); however, the specific function of the majority of these genes in cranial tissue remodeling remains unknown.

Cranial closure (schematized in Figure 1A) begins with the induction of the anterior neural tissues, which completes by day 7.5 (E7.5) in the mouse embryo (Harland, 2000; Levine and Brivanlou, 2007; Ozair et al., 2013; Rhinn et al., 1998). The cranial neural plate continuously

grows via proliferation during and after induction, resulting in the formation of a wide, outwardly curved morphology, with the lateral edges below the level of more medial regions (Jacobson and Tam, 1982; Juriloff and Harris, 2018; Morriss-Kay, 1981). Beginning at E8, the neural fold tissues undergo a curvature inversion that will bring these lateral edges above the midline, a process known as neural fold elevation. Subsequently, the lateral edges will deflect inward to meet above the midline of the tissue during apposition. Finally, the neural folds will fuse, as will the overlying surface ectoderm, completing closure and internalizing the tissue.

The tissue scale morphogenetic events of cranial closure require dynamic activities of individual cells, including shape changes such as apical constriction to drive the curvature change of neural fold elevation, cell rearrangement to alter the aspect ratio of the tissue, and polarized cell division which is thought to drive anisotropic growth along the anteroposterior axis (Devenport, 2016; Martin and Goldstein, 2014; Miller and Davidson, 2013; Nikolopoulou et al., 2017; Vijayraghavan and Davidson, 2017; Zallen and Goldstein, 2017). These individual cellular activities must be coordinated in space and time to effectively reshape the tissue; however, the upstream controls on their spatial and temporal deployment often remain opaque. Developmental morphogens, such as Wnt and Shh are likely to be key players in ensuring that cell dynamics are correctly integrated over time and tissue length scales, as defects in the activity and reach of these pathways lead to gross defects in neural tube closure (Allache et al., 2014; Brooks et al., 2020; Brown et al., 2020; Carter et al., 2005; Gray et al., 2013; Harris and Juriloff, 2007; Harris and Juriloff, 2010; Kimura-Yoshida et al., 2015; Misra and Matise, 2010; Patterson et al., 2009; Pinson et al., 2000; Ybot-Gonzalez et al., 2002). In the case of Wnt, mutations that either inhibit or hyperactivate the pathway lead to defects in cranial closure (Brown et al., 2020; Gray et al., 2013; Kimura-Yoshida et al., 2015; Pinson et al., 2000). However, the impact of Wnt dysregulation on the spatial and temporal patterns of cell behavior required for closure are not fully understood. This confusion likely reflects the relative complexity of Wnt signaling in the tissue, where multiple Wnt ligands are expressed (Bally-Cuif et al., 1995; Bouillet et al., 1996; Parr et al., 1993; Yamaguchi et al., 1999), as well as the fact that the Wnt pathway can exert control over multiple classes of cellular dynamics including proliferation, cell shape change, and planar polarized rearrangement (Butler and Wallingford, 2017; Fumoto et al., 2017; Reya and Clevers, 2005; Steinhart and Angers, 2018; Sutherland et al., 2020; Yoon et al., 2023).

Here, we seek to systematically address the role of Wnt signaling in control of cellular dynamics during cranial neural tube closure. To do so we use a conditional mutant of the critical pathway co-receptor *Lrp6* to reduce Wnt signaling either globally, or specifically within the future midbrain tissues beginning at neural fold elevation stages. This analysis reveals that Wnt inhibition impacts the earliest stages of neural tube closure but not later elevation, as we find that patterned apical constriction and planar polarization are normal in *Lrp6* mutants. Closure defects instead stem from neural fold hyperplasia caused by excessive proliferation just after the completion of anterior neural induction. We next ask if hyperactivation of Wnt signaling also leads to defects in closure, using conditional truncation of APC specifically at neural fold elevation stages, and find that this results in defects in apical constriction and failure of neural fold elevation. Thus, both reduction and hyperactivation of Wnt activity result in failures in cranial neural tube closure, but do so by interfering with distinct cellular mechanisms. Together, our data demonstrate that Wnt signaling levels are key determinants of the success of multiple independent patterns of cell behavior required for robust cranial neural tube closure.

Results

Global, but not elevation stage specific, reduction of Wnt signaling leads to failure of cranial neural tube closure

To begin, we wanted to understand the developmental window during which Wnt signals were required for cranial closure by using a conditional allele of *Lrp6* (*Lrp6*^{fl} (Joeng et al., 2011)).To inhibit Wnt globally, we used *Sox2-Cre* to ablate function throughout the epiblast (Hayashi et al., 2002), as well as to drive recombination of the *Lrp6* locus in the germline to create a heritable global loss of function allele (*Lrp6*$^{\Delta}$). In all analyses reported below, no difference was observed between embryos with epiblast specific ablation (*Lrp6*$^{\Delta/fl}$; *Sox2-Cre)* or global germline ablation (*Lrp6*$^{\Delta/\Delta}$), indicating that phenotypes arise from the epiblast. We therefore refer to these genotypes collectively as *Lrp6* mutants. Additionally we ablated *Lrp6* function specifically in the future midbrain tissues using *Wnt1-Cre2* (Chen et al., 2017; Lewis et al., 2013), which turns on in the embryo beginning at early elevation stages (~E8.25).

Global *Lrp6* loss led to exencephaly with an 80% penetrance (8/10 mutants, Figure 1B), in line with though modestly higher than previously reported *Lrp6* mutants (Gray et al., 2010; Gray et al., 2013; Pinson et al., 2000; Zhou et al., 2010). Notably, despite the clear exencephalic phenotypes, the cranial neural folds appeared well elevated. Conversely, none of the conditional *Lrp6*$^{fl/fl}$*; Wnt1-Cre2* mutant embryos we recovered showed failures of cranial closure (Figure 1C, 0/4 mutants). Together, these data demonstrate that LRP6 function is broadly required for cranial neural tube closure, and loss of function specifically in the midbrain during neural fold elevation stages is not sufficient to drive exencephaly. We therefore focused the rest of our analysis on global *Lrp6* mutants.

To better understand the consequences of *Lrp6* loss on Wnt signaling levels specifically in the cranial neural plate, we examined expression of a previously described transgenic reporter of Wnt activity comprised of H2B-GFP under the control of Tcf/Lef binding sites (Ferrer-Vaquer et al., 2010). In cranial neural plates of control embryos at elevation stages (E8.5), this reporter recapitulated the expected pattern of Wnt signaling (Brown et al., 2020), showing high expression at the lateral edges of the tissue, and a midbrain specific stripe of expression in the Wnt1 domain (Figure 2A). *Lrp6* mutant embryos retained a similar spatial pattern of Wnt signaling but demonstrated apparent diminishment of reporter activity at all positions. We therefore quantified the relative Wnt signaling strength at progressive distances from the tissue midline within the midbrain stripe. This analysis revealed a graded Wnt response, with the highest levels of signaling observed at the lateral tissue edge (Figure 2B, C). In *Lrp6* mutants, pathway response was significantly decreased at several mediolateral positions. Together, these data indicate that *Lrp6* mutants show a quantitative reduction in, but not elimination of, Wnt signaling in the cranial neural folds.

Lrp6 mutants show tissue scaling defects despite normal cell remodeling

To better understand what tissue-level mechanisms could be driving terminal closure defects, we examined *Lrp6* mutants at stages ranging from E8.0-E8.5 when the neural folds are undergoing critical morphogenetic events including anteroposterior growth and elongation, as well as the curvature changes associated with neural fold elevation (Figure 1A). At these stages, *Lrp6* mutants showed a striking tissue scaling defect in the cranial neural folds (Figure 3A-D)

with a doubling of the tissue width but no change in the length of cranial tissues relative to controls at elevation stages (E8.5, Figure 3D). This tissue scaling phenotype was apparent as early as pre-elevation stages (E8.0, 0-3 somites, Figure 3B). To our knowledge this is the first report of cranial neural fold hyperplasia at these stages, representing a potentially unique tissue-level etiology for closure defects.

We next considered potential cellular mechanisms that could increase the width of the tissue. First, apical constriction reduces the apical surface area of the tissue to drive the curvature changes required for neural fold elevation (Brooks et al., 2020; Haigo et al., 2003; Martin and Goldstein, 2014; Nikolopoulou et al., 2017), and severe defects in this process could result in an apparent increase in tissue width. We therefore used our previously reported *in toto* confocal imaging and computational segmentation approaches (Brooks et al., 2020) to examine cell apical areas (Figure 4A-C). These analyses revealed no difference in the average apical cell area (Figure 4D) or the distribution of apical cell areas (Figure 4E) within the constrictive domains between control and *Lrp6* embryos, indicating that apical constriction is normal despite the observed reduction in Wnt activity. The presence of normal apical constriction in these mutants is also consistent with the observed robust neural fold elevation (Figure 1A, yellow arrowheads). We next considered failures in pseudo-stratification and apicobasal thickening of the tissue, which are also increases known to increase tissue width and block closure (Grego-Bessa et al., 2016). However, tissue thickness was unchanged between control and *Lrp6* mutant embryos (Figure 4F), indicating this process occurs normally in these mutants. Together, these data demonstrate that impaired Wnt signaling upon loss of *Lrp6* does not alter the patterned cell remodeling programs driving neural fold elevation and therefore, defects in these cell dynamics cannot explain the cranial closure phenotypes of mutant embryos.

The mammalian cranial neural plate is planar polarized, but this polarity does not depend on *Lrp6*

We next considered planar polarized cell intercalation, a key driver of tissue narrowing via the process of convergent extension. Planar polarization of many tissues, including the posterior neural plate, is controlled by the Wnt-responsive planar cell polarity (PCP) pathway (Butler and Wallingford, 2017; Nikolopoulou et al., 2017; Sutherland et al., 2020). Defects in this process could theoretically explain the observed increase in tissue width in *Lrp6* mutants, but the relative strength of planar polarization in the mammalian cranial neural folds has not been extensively characterized, confounding analysis of this possibility. To address this gap, we developed an automated approach based on ridge detection algorithms ((Steger, 1998); see materials and methods) to quantitatively examine polarization patterns in the cranial neural folds. We first verified the utility of this method by examining the polarization of phospho-Myosin2 Regulatory Light Chain (pMRLC), a marker for the actively contractile myosin population that remodels cell junctions during cell intercalation. Consistent with our prior manual analysis (Brooks et al., 2020), automated detection showed moderate but highly reproducible polarization, with ~40% of pMRLC+ junctions and multicellular cables oriented within 30º of the mediolateral axis compared with only 25% oriented within 30º of the anteroposteior axis (Figure 5A). Thus, this method accurately captures the known planar polarization of contractility within the tissue. We therefore expanded this analysis to the core PCP component VANGL2, which showed similar levels of planar polarization to pMRLC (Figure 5B). Together with a previous analysis of the PCP component DVL2 (McGreevy et al., 2015), these data argue that the mammalian cranial neural plate shows significant and reproducible levels of planar polarization.

We next tested whether loss of Wnt singling would impair tissue polarization, potentially explaining the increase in cranial neural fold width. However, neither pMRLC nor VANGL2 polarization was decreased in *Lrp6* mutants (Figure 5A-B), and no significant difference in the number of detected cables was found for either staining (Figure S1B-C), indicating that *Lrp6* is not required for planar polarization. This surprising result may reflect the redundancy between LRP5 and LRP6 during Wnt signaling (Joeng et al., 2011; Kelly et al., 2004), an interpretation supported by the perdurance of some Wnt reporter activity in *Lrp6* mutants (Figure 2). Thus, defects in planar polarization of the tissue cannot explain the increased cranial tissue width or closure defects in *Lrp6* mutant embryos.

An early increase in cell proliferation rates in early anterior neural tissues leads to cranial fold hyperplasia and terminal closure defects in *Lrp6* mutants

We next wondered if excessive cell growth or proliferation could explain the increased scale of the cranial neural tissues in *Lrp6* mutants. Apical areas and cell heights showed no differences between control and *Lrp6* mutant embryos (Figure 4A-F), ruling out a large-scale change in cell volumes and resulting hypertrophy of the tissue. Additionally, *Lrp6* mutants did not show changes in proliferative capacity at neural fold elevation stages (E8.5) as assayed by the proportion of phospho-HistoneH3+ cells (Figure 4G-H). Alternatively, changes to the orientation of cell division have been proposed to underlie mediolateral widening of the tissue (Morriss-Kay, 1981), and Wnt signaling influences the orientation of cell division in several contexts (Di Bella et al., 2021; Kim et al., 2013; Schlesinger et al., 1999). We therefore tested whether the pattern of division orientation was changed by loss of *Lrp6*. In controls, mitotic separation events showed a significant bias toward anteroposterior alignment—supporting models proposing anisotropic growth along this axis (Alvarez and Schoenwolf, 1991; Jacobson and Tam, 1982; Morriss-Kay, 1981)—but *Lrp6* mutants maintained this orientation bias (Figure 4I) and exhibited a similar number of anaphase events (Figure S1A).

Given that the known cellular drivers of cranial remodeling and neural fold elevation were all normal, we reasoned that the terminal closure phenotype was likely to be a consequence of defects at earlier stages. This was supported by our observations that elevation stage specific loss of *Lrp6* did not prevent cranial closure (Figure 1B), and that cranial neural plate size was already significantly increased at pre-elevation stages (Figure 3A-B). We therefore investigated two key processes required to appropriately set the initial scale of the neural plate, cranial neural induction and early proliferation.

In the mouse, cranial neural tissues are induced by signals originating from the anterior visceral endoderm (AVE), which include inhibitors that protect early cranial tissues from the posteriorizing activity of Wnt signals (Levine and Brivanlou, 2007; Ozair et al., 2013; Stower and Srinivas, 2014). We reasoned that the reduction in Wnt signaling in *Lrp6* mutants could therefore lead to excessive anteriorization of the neural tissues at the expense of more posterior fates, leading to tissue mis-scaling. To test this hypothesis, we used transverse sectioning to examine the distribution of OTX2, a key anterior fate determinant (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995; Rhinn et al., 1998), after the completion of induction at E7.5 (Figure 6A). In control embryos OTX2 occupied approximately 60% of the diameter of the embryonic cylinder, and we observed no change in proportional occupancy in *Lrp6* mutants (Figure 6C).

Next, we returned to the question of excessive cell proliferation. Despite our data above demonstrating equivalent rates of proliferation between control and *Lrp6* mutants at later stages, an early burst of excessive proliferation could result in early hyperplasia that would then be maintained or expanded by normal ongoing proliferation in the tissue. To test this hypothesis, we examined the proportion of neural plate cells exhibiting phospho-HistoneH3 signatures after tissue induction at E7.5. Strikingly, we observed a near doubling of the anterior proliferative index in *Lrp6* mutants (Figure 6B, D). Increased proliferative capacity was unique to the anterior tissues, as the posterior proliferative index remained equivalent to controls (Figure 6B, E). This increased anterior proliferative capacity, when integrated over the full tissue volume, would result in the addition of a significant number of excessive cells specifically in the cranial neural plate, driving tissue hyperplasia at later stages. Further, this analysis suggests that the role of Wnt in tuning proliferation rates may be unique to anterior tissues, or that loss of Wnt may impair cell cycle control mechanisms specific to the future cranial tissues.

Altogether, our results indicate that loss of *Lrp6* leads to a temporally delimited increase in cell proliferation, which occurs specifically in early anterior neural tissues just after the completion of induction. This window of increased proliferation results in significant tissue hyperplasia prior to the onset of cranial neural fold elevation. Because the other cell dynamics of closure including apical constriction and planar polarization appear normal, and neural fold elevation is robust, the likely genesis of the final cranial neural tube closure defect is an inability for these mechanisms to compensate for the increase is tissue width driven by early excessive proliferation. The increased tissue width results in a failure of neural fold apposition over the midline, blocking fold fusion, representing a unique pathogenic mechanism underlying cranial neural tube defects.

Midbrain specific hyperactivation of Wnt signaling leads to defective apical constriction resulting in failure of neural fold elevation and cranial closure defects.

In addition to the cranial closure defects observed upon reduced Wnt signaling, mutants that increase Wnt activity also prevent closure (Gray et al., 2013; Kimura-Yoshida et al., 2015), but whether pathway reduction and hyperactivation result in equivalent defects in cellular dynamics is unknown. To address this question, we asked if elevating cranial tissues were sensitive to inappropriately increased levels of Wnt signaling, specifically at elevation stages. To do so we used a previously established mouse line (*Apc*^{tm1Tno}*,* (Shibata et al., 1997)*)* to conditionally truncate APC into an inactivate form, hyperactivating Wnt signaling (Azzolin et al., 2014; Buchert et al., 2010; Martinez et al., 2009; Pawlikowski et al., 2013; Radulescu et al., 2013; Sansom et al., 2004; Shibata et al., 1997; Varnat et al., 2010). We again wanted to use *Wnt1-Cre2* to drive APC truncation specifically in the midbrain at neural fold elevation stages. However, *Wnt1-Cre2* is also active in the male germline (Dinsmore et al., 2022) and a previous study showed hypomorphic effects in *Apc*^{tm1Tno} genotypes consisting of one copy of the conditional allele and one copy of the truncated allele (Buchert et al., 2010). Thus, if the sire carried *Wnt1-Cre2,* we would expect potentially confounding hypomorphic effects; therefore we only examined embryos from crosses where the Cre transgene was inherited from the dam.

Embryos with conditional APC inactivation specifically in elevating midbrain neural folds (*Apc*$^{tm1Tno/tm1Tno}$*; Wnt1-Cre2*; hereafter called *Apc-cIN* for APC conditional inactivation) displayed highly penetrant exencephaly (9/13 mutants, ~70%, Figure 7A), confirming that direct hyperactivation of Wnt signaling also blocks cranial closure. Of note, and in contrast to *Lrp6* mutants, *APC-cIN* mutants showed strong defects in neural fold elevation (compare Figure 1B vs 7A). This difference suggested that hyperactivation of Wnt signaling impairs cranial closure

via a different cellular mechanism than that caused by reduced Wnt signaling. This hypothesis was further supported by the absence of cranial neural plate scaling defects in *APC-cIN* embryos (Figure 7B-C).

Because we and others have previously shown that defects in apical constriction lead to failure of cranial neural fold elevation (Brooks et al., 2020; Eom et al., 2011; Grego-Bessa et al., 2015; Lesko et al., 2021; Ohmura et al., 2012; Sulistomo et al., 2019), we asked if *Apc-cIN* mutants displayed defects in this cell behavior. Indeed, these mutants demonstrated significant increases in average cell apical area and changes a shift in the distribution of areas towards larger apical domains (Figure 7C-E). These defects in constriction were equivalent in magnitude to those observed in known mutants with neural fold elevation and cranial closure defects (Brooks et al., 2020), and occurred in the absence of changes to cell height (Figure 7F). Additionally, VANGL2 planar polarity was normal in *Apc-cIN* mutants (Figure 7G-H, Figure S2A), as were proliferation rates (Figure S2B-C). Taken together, these data indicate that hyperactivation of Wnt at elevation stages specifically inhibits apical constriction, preventing cranial neural fold elevation and leading to highly penetrant cranial closure defects.

In sum, our data demonstrate that appropriate Wnt signaling levels are required for at least two distinct patterns of cell behavior required for cranial closure (Figure 9). First, there must be sufficient Wnt activity at post-induction stages to restrict proliferation in early anterior neural tissues. Impaired Wnt signaling, as in *Lrp6* mutants, leads to excessive cell proliferation at these stages and doubles the width of the tissue. The severe hyperplasia in these mutants prevents cranial closure despite the normal activity of other tissue remodeling mechanisms, including apical constriction and planar polarization. This tissue-scaling defect represents a novel cellular basis for cranial closure defects and may be specific to mutants that impact Wnt signaling levels. Conversely, Wnt activity must be restricted at elevation stages, as hyperactivation of the pathway blocks apical constriction and prevents the tissue-wide curvature change required for neural fold elevation. Thus, Wnt levels must be correctly modulated to promote two key temporally independent cell behaviors to reshape the cranial neural folds and promote closure.

Discussion

Cranial neural tube closure is a critical event in vertebrate development, and failures in this process are among the most common and deleterious structural birth defects in humans (Wallingford et al., 2013; Zaganjor et al., 2016). Closure is driven by the active remodeling of the tissue by patterned cell behaviors, including shape change, rearrangement, and proliferation (Nikolopoulou et al., 2017). These patterns are strongly reliant on information provided by spatially restricted signals, such as morphogens, and failure in either these upstream signals or the downstream molecular regulators of cell activity lead to failed closure (Harris and Juriloff, 2007; Harris and Juriloff, 2010; Juriloff and Harris, 2018; Lee and Gleeson, 2020; Wilde et al., 2014). However, the precise consequence of perturbed morphogen signaling for downstream cell behaviors remains opaque in many cases. Here, we showed that mutations leading to either inappropriately reduced or hyperactivated Wnt signaling lead to defects in cranial closure. Intriguingly, defects in these two cases arose from different developmental trajectories. Loss of *Lrp6* led to an increase in cell proliferation specifically in the early cranial tissues, which in turn resulted in a significant expansion of the tissue. Because other known drivers of cranial closure including apical constriction, tissue thickening, and planar polarization were normal, and because *Lrp6* mutants showed strong cranial neural fold elevation, we conclude that the observed tissue hyperplasia creates a mechanical hindrance to final stages of closure that

cannot be fully overcome by the normal function of elevation stage cell behaviors (Figure 9). This model is further supported by the lack of cranial closure defects upon conditional *Lrp6* ablation specifically at elevation stages, indicating that early defects in tissue size, and not direct disruption of elevation mechanisms, underly the terminal closure phenotypes. However, Wnt modulation is still required during elevation, as conditional hyperactivation of the pathway through controlled truncation of APC led to apical constriction defects, failure of neural fold elevation, and cranial closure defects. Thus, Wnt signaling levels are critical determinants of two distinct events during closure, and pathway activity must be appropriately regulated throughout early cranial development to ensure both early tissue scaling, and later elevation occur appropriately.

Together with previous studies, our work helps clarify the complex nature of the role of Wnt signaling in cranial tissue morphogenesis. Previous studies of *Lrp6* have shown that both loss of function and gain of function alleles of LRP6 show defects in cranial and spinal neural tube closure (Allache et al., 2014; Andersson et al., 2010; Carter et al., 2005; Gray et al., 2013; Pinson et al., 2000; Zhao et al., 2022). Our data indicate that hypomorphic and hypermorphic LRP6 states likely represent different impacts on cranial closure, with hypermorphs hewing closer to the defects in apical constriction observed in our *Apc-cIN* mutants, while loss of function leads to early tissue scaling defects. Previous studies have also suggested that LRP6 plays a role in convergent extension during gastrulation (Tahinci et al., 2007) and its loss can exacerbate spinal neural tube defects in *Vangl2* heterozygotes (Allache et al., 2014). Additionally, *Snx3* mutants with impaired Wnt signaling show defects consistent with convergent extension (Brown et al., 2020). Together, these studies suggest that Wnt levels are important regulators of planar polarity in posterior neural tissues, but our data suggest that neither reduction nor hyperactivation of the Wnt pathway result in defective planar polarization of cells within the cranial neural tissues. Thus, the functions of Wnt signals in polarization could differ between the cranial and spinal domains, perhaps reflecting recent work demonstrating that these two populations have fundamentally different dynamic properties, despite being continuous with one another (Baldwin et al., 2022; Christodoulou and Skourides, 2022). However, given that loss of *Lrp6* alone is not sufficient to completely abrogate Wnt signaling, studies of stronger loss of function mutants will be required to fully define the role of the pathway in patterned cell remodeling and planar polarization within cranial tissues. These same studies may also be useful in determining the relative contribution of polarized cell intercalation to cranial morphogenesis, which remains an important open question.

The fact that loss of LRP6 and concomitant reduction in Wnt signaling leads to excessive proliferation in early stages is noteworthy given that Wnt activity is generally pro-proliferative, including in neural tissues (Ikeya et al., 1997; Kalani et al., 2008; Reya and Clevers, 2005; Steinhart and Angers, 2018). In line with this, *Lrp6* mutants which show reduced Wnt signaling demonstrate a slight reduction in proliferation just after closure (Gray et al., 2013). Why then do early stages in these mutants show increased proliferative capacity? In one model, decreased Wnt signaling leads to an anteriorization of the tissue at a level downstream of the broad early patterning by OTX2, possibly by driving over expression or expansion of one of the anterior homeobox factors that control early neural patterning. Given that these homeobox factors can also influence proliferation rates (Andreazzoli et al., 2003; Gestri et al., 2005), this may explain the tissue hyperplasia of *Lrp6* mutants. In another model, reduced Wnt signaling could alter the timing or efficacy of the transition from epiblast to pro-neural fates, perhaps incorrectly maintaining a more pluripotent and proliferative state. Finally, studies in other developing tissues

and during tumorigenesis have shown that there is significant cross talk between the Wnt and Hippo signaling pathways (Azzolin et al., 2012; Azzolin et al., 2014; Doddihal et al., 2024; Heallen et al., 2011; Varelas et al., 2010), and the proliferation and tissue scaling defects in *Lrp6* could result from secondary impacts on Hippo pathway activity due to reduced Wnt activity.

To our knowledge, no other mutants have been reported with excessive cell proliferation in the cranial neural plate. This includes our own observations of mutants with increased Sonic Hedgehog signaling (Brooks et al., 2020), which do not show altered proliferation rates despite this pathway being generally pro-mitogenic (Corrales et al., 2004; Fink et al., 2018; Ishibashi and McMahon, 2002). Mutants that inhibit apoptosis, e.g. *Jnk1/2* doubles (Kuan et al., 1999; Sabapathy et al., 1999), may result in similar tissue scaling defects, as defects in this process result in neural tube hyperplasia at later stages (Cecconi et al., 2008), but the stages of hyperplasia onset in these mutants have not been identified. Another potentially interesting comparison comes from studies of hemifacial hypertrophy, a rare human developmental disease characterized by unilateral overgrowth of facial tissues (Dattani and Heggie, 2021). This disease has been proposed to stem from an increase in the number of neural crest cells due to unilateral hyperplasia of a single cranial neural fold (Pollock et al., 1985), though it remains unknown if this is a unifying etiology for the disease or if Wnt signaling is altered in these patients. Nonetheless, together with our data, these results suggest that proliferation is actively modulated in cranial tissues during closure stages, though it is unclear whether cell division actively drives morphogenesis of these tissues. Experiments directly increasing or decreasing proliferative capacity will help delineate the role(s) of cell division during closure.

Another interesting finding from this study is that excessive Wnt signaling blocks apical constriction, akin to the consequences of excessive Shh signaling (Brooks et al., 2020).This is somewhat surprising given that Wnt and Shh are antagonistic in neural patterning (Kicheva and Briscoe, 2023; Ulloa and Martí, 2010), and we and others have recently shown that Shh activation inhibits transcription of several Wnt pathway members in neural tissues at closure stages (Brooks et al., 2024; Kim et al., 2019). Based on these observations one of our initial hypotheses was that Wnt would act as a positive signal for apical constriction in opposition to Shh as a negative signal. However, our data here indicate that high levels of activity in either pathway instead inhibit constriction. This suggests that each signal likely acts to set cell morphology in its own unique domain—the lateral edge of the tissue for Wnt and the midline for Shh—but must be restricted in the intervening domain. This model is further supported by our observation that reduced Wnt signaling in *Lrp6* mutants did not result in defects in apical constriction. Therefore, the positive signal for apical constriction in this tissue remains unclear, though given the significant redundancy of LRP5 with LRP6 (Joeng et al., 2011; Kelly et al., 2004; Ren et al., 2021), we cannot rule out the requirement for a role for basal levels of Wnt in promoting apical constriction. Alternatively, signals from other morphogen systems, such as BMP, may act positively to induce apical constriction, or constriction may simply be the default behavior of induced cranial tissues when not overwritten by exposure to high Wnt or Shh activity.

To conclude, this study lays the groundwork for several avenues of further investigation. First, it will be important to understand if and how Wnt signaling levels change in space and time as the tissue proceeds from induction to closure to distinguish between models for how proliferation is controlled. Second, examination of the intersection between Shh and Wnt signaling may reveal the molecular logic that governs the unique pattern of apical constriction observed in cranial

tissues. Finally, further investigation of the Wnt pathway may provide insights into how tissues are appropriately scaled during development. This is a critical concern, as the cellular mechanisms driving morphogenesis must be accurately scaled to underlying tissue dimensions in order to appropriately reshape tissues for function during embryogenesis, but the mechanisms of this scaling are not fully understood.

Materials and Methods

Mouse handling

Mice were bred and housed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and an approved Institutional Animal Care and Use Committee protocol (22-124) of North Carolina State University.

Timed pregnant mice were euthanized at E7.5-E10.5. Noon on the day of the vaginal plug was considered E0.5, and individual embryos were staged by counting the number of somites, or by Thieler criteria in stages prior to the appearance of somites. Strains used in this study included the following previously reported lines: the conditional *Lrp6*^{fl} line [Lrp6$^{tm1.1Vari}$, MGI ID: 5299216] (Joeng et al., 2011); the *Apc*^{tm1Tno} line [Apc^{tm1Tno}, MG ID: 1857966] (Shibata et al., 1997); the Tcf/Lef H2B-GFP transgenic insertion [Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J, MGI ID: 4881506] (Ferrer-Vaquer et al., 2010); and the *Wnt1-Cre2* transgenic insertion line [E2f1$^{Tg(Wnt1-cre)2Sor}$, MGI ID: 5485027] (Lewis et al., 2013). Genotyping was performed in accordance with reported protocols for each strain. Mice were received and maintained on a mixed background. Control embryos were wild-type and heterozygote littermates for global *Lrp6* analyses, or wild-type, heterozygote, and Cre-negative littermates for *Lrp6; Wnt1-Cre2* and *Apc-cIN* analyses.

Whole-mount immunostaining

Embryos were dissected in ice-cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for either 1 hour at room temperature or overnight at 4 ºC. Embryos were then washed 3x for 30 minutes in PBS + 0.1% Triton X-100 (PBTriton) at room temperature, blocked for 1 hour at room temperature in PBS containing 10% bovine serum albumin and 0.1% Triton X-100 (blocking solution), and incubated with primary antibodies in blocking solution overnight at 4 ºC. Embryos were then washed 3x 30 minutes in PBTriton at room temperature, incubated with secondary antibodies in blocking solution for either 1h at room temperature or overnight at 4 ºC, and finally washed 3x 30 minutes in PBTriton. Primary antibodies used in this study were: rabbit anti-N-cadherin (1:500, Cell Signaling #13116), mouse anti-phospho-HistoneH3 (1:500, Cell Signaling #9706), rabbit anti-phospho-Myosin Regulatory Light Chain 2 (1:100, Cell Signaling 3674), rat anti-VANGL2 (1:100, Millipore MABN750), and goat anti-OTX2 (1:500, R&D Systems AF1979). Alexa conjugated Phalloidin and DAPI (ThermoFisher) were used as counterstains.

Cryosectioning

E7.5 Embryos were dissected and fixed in 4% paraformaldehyde for 1 hour at room temperature and washed 3x 30 minutes in PBTriton and were transferred to 15% sucrose overnight. Embryos were then placed in cryoblocks containing OCT (Tissue-Tek) and frozen on dry ice and stored at -80 ºC until sectioning. Embryos were sectioned on a cryostat (Leica) in 10-14 µm increments and sections were adsorbed onto Superfrost slides (Fisher). Slides were

washed 3x 5 minutes in PBTriton at room temperature, blocked for 15 minutes with blocking solution (see above), incubated with primary antibodies for 30 minutes, washed 3x 5 minutes with PBTriton, incubated with secondary antibodies for 30 minutes, and washed 3x 5 minutes in PBTriton. Stained sections were then mounted in Prolong Gold antifade reagent (ThermoFisher).

Brightfield imaging and morphometric analysis

Embryos were placed in a petri dish with a layer of 2% agarose on the bottom and filled with PBTriton. Small gouges were made in the agarose to gently restrain embryos in the desired orientation, and images were taken using a Zeiss Lumar V12 Steroescope equipped with a CCD camera. Cranial neural plate morphometrics were measured using FIJI/ImageJ (Schindelin et al., 2012; Schneider et al., 2012). The length of the cranial tissues was measured as a segmented line along the neural plate border from the anterior neural ridge to the otic sulcus. The width of the tissue was measured by drawing a line between the lateral neural plate borders at the widest position in the future midbrain.

Confocal Microscopy

For whole mount imaging, embryos were mounted dorsal side down on #1.5 coverglass in PBTriton in Attofluor chambers (ThermoFisher A7816) using a small fragment of broken cover glass with dabs of vacuum grease to gently hold the embryo against the glass. Embryos were imaged on a Zeiss LSM 980 confocal microscope equipped with a Plan-NeoFluar 40x/1.3 oil immersion objective. Images were captured using tile-based imaging to acquire contiguous z-stacks of 50-100 µm depth, with an optical slice thickness of 0.6 µm and z-steps of 0.3 µm. Tile stitching and maximum intensity projection were performed in Zeiss ZEN Blue software. Imaging of cryosections was performed on the same microscope using a 20x/0.8 air immersion objective, with z-stacks of 10-14 µm depth imaged with a 1.8 µm optical slice thickness and a 0.9 µm z-step.

Image analysis and quantification

All quantifications were performed on unprocessed images. To quantify Wnt response, Tcf/Lef-H2B-GFP intensity was normalized to DAPI in a contiguous series of 20x100 µm boxes along the midbrain stripe of expression in maximum intensity projections. We were unable to directly analyze the impact of APC truncation on Wnt signaling levels as, despite numerous crosses, we never recovered embryos that had both homozygous APC truncation and a copy of the Tcf/Lef:H2B-GFP reporter. This could indicate that Wnt hyperactivation in combination with this reporter transgene is embryotoxic, perhaps due to increased metabolic burden.

Apical cell area was quantified in 100 µm x 100 µm regions in maximum intensity projections in a pair of regions on either side of the midline, approximately halfway between the midline and lateral edge, and halfway between the pre-otic sulcus and the cranial flexure. Cells entirely contained within these regions were segmented using CellPose (Stringer et al., 2021). Cell areas were quantified and cell area maps were generated using the MorphoLibJ plugin (Legland et al., 2016) in FIJI/ImageJ (Schindelin et al., 2012; Schneider et al., 2012). Tissue thickness was measured in orthogonal reconstructions of z-stacks, midway between the midline and lateral edge of the tissue.

Planar polarization of pMRLC and VANGL2 was measured by first using a ridge detection algorithm (Steger, 1998) in FIJI/ImageJ to identify linear junctional signals in a 100 µm x 100 µm region, selected as above, of maximum intensity projections of these stainings. After segmentation, a fit ellipse was generated for each detected object. In this analysis 90º is anterior, -90º is posterior, and after taking the absolute value, reported values range from 0-90º. The frequency of objects falling into one of three angular bins (anteroposterior, 60-90º; intermediate 30-60º; and mediolateral 0-30º) was calculated.

For elevation stages, the proportion of proliferative cells was measured by normalizing the number of phospho-Histone H3 positive cells to the total number of cells in a 100 µm x 100 µm region as described above. Anaphase angle separation was quantified in DAPI staining and all obvious chromosomal separation events in one lateral half of the cranial neural plate were quantified. The frequency of objects falling into one of three angular bins (anteroposterior, 60-90º; intermediate 30-60º; and mediolateral 0-30º) was calculated. For pre-elevation stages, the relative percentage of the tissue showing OTX2 staining was calculated by drawing a segmented line in FIJI/ImageJ, and sections were subsequently divided into two regions, anterior (OTX+) and posterior (OTX2-) and the number of PHH3+ cells to the total cells, specifically within the neuroepithelial tissues, was calculated independently in these regions.

Statistics

Statistical analysis and graph generation were performed in Prism software (GraphPad). All results are reported as mean ± standard deviation. Summary significance levels are: ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Statistical tests were the unpaired students t-test for comparing two conditions, and two-way ANOVA with either Tukey's multiple comparisons or Sidak's multiple comparisons. Details on the statistical tests, n values, and p values for each experiment can be found in Table S1. No formal power analyses were conducted, an n of 3-6 embryos per genotype was targeted for each analysis.

Figure Assembly

Display images in figures were linearly adjusted to increase brightness and contrast in either FIJI/ImageJ or Photoshop (Adobe). Figures were assembled in Illustrator (Adobe).

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Figure Legends

Figure 1: Cranial closure defects occur in global but not cranial neural fold specific *Lrp6* mutants. (**A**) Schematics representing the *en face* (left) and transverse morphology of the midbrain region of the cranial neural tissues at post-induction (top), elevation (middle) and apposition (bottom) stages of closure. (**B**) Side and dorsal views of control and *Lrp6* global mutants. Mutants exhibit failed cranial closure from the forebrain to the hindbrain-spinal cord juncture (dashed yellow line) at 80% penetrance. Notably, however, the neural folds appear well elevated in these mutants (yellow arrowheads).

(**C**) Side and dorsal views of a control embryo and an embryo where *Lrp6* function has been conditionally ablated specifically in the future midbrain tissues at the onset of neural fold elevation via the activity of the *Wnt1-Cre2* transgene. No recovered embryos of this genotype displayed cranial neural tube closure defects. Scale bar, 100 μm; yellow arrowheads show the position of the lateral edge of the cranial neural tissues.

Figure 2: *Lrp6* mutants show reduced Wnt signaling in the midbrain during neural fold elevation. (**A**) A maximum intensity projection of control and *Lrp6* embryos expressing the Tcf/Lef>H2B-GFP reporter of Wnt signaling. The control embryo demonstrates the spatial pattern of Wnt response in the midbrain and hindbrain tissues of the cranial neural plate. Pathway response is high laterally at all anteroposterior positions, with expansion laterally occurring specifically in the future midbrain. The gross pattern of Wnt response is not altered in *Lrp6* mutants but shows apparent diminishment in all domains. Dashed box represents the region analyzed in B-C. (**B**) The midbrain stripe of Wnt response, with DAPI as a counterstain, is shown in control (top) and *Lrp6* mutant (bottom) embryos. (**C**) Quantification of the Wnt signaling response, after normalization to DAPI, is shown at increasing distances from the midline. Both control and *Lrp6* mutant embryos show increasing response in more lateral domains, however mutants show a significant reduction in response at several positions along the mediolateral axis (two-way ANOVA with Tukey's multiple comparisons, n = 5 control embryos, 3 *Lrp6* embryos, see Table S1 for p values). Anterior is up in all images, scale bars represent 100 μm. *, $p < 0.05$, **, $p < 0.01$.

Figure 3: *Lrp6* mutants show enlargement of the cranial neural plate. (**A**) Brightfield images of cranial neural plates from control and *Lrp6* mutant at E8 (3 somites), a stage prior to the onset of strong cranial neural fold elevation. Note that a visible increase in tissue size is already apparent at these stages (compare white dashed line in the control to yellow dashed line in the mutant). The apparent lengthening of *Lrp6* tissue is an artifact caused by delay in formation of the cranial flexure between the forebrain and midbrain tissues. (**B**) Quantification of the length and width of the cranial neural plate in control and *Lrp6* embryos at 0-3 somite stages. Length is defined as the distance between the anterior neural ridge and the otic sulcus. There is no significant difference in this value between control and *Lrp6* mutants (control, mean ± SD: 452.7 ± 41.87 μm; *Lrp6*: 499.1 ± 76.00 μm. n = 7 control and 6 *Lrp6* embryos, p = 0.1905 by unpaired t-test). Width is defined as the length of a line drawn from one lateral edge of the tissue to the other across the widest portion of the future midbrain. This value is significantly larger in *Lrp6* mutans (control, 561.4 ± 40.86 μm; *Lrp6*: 713.9.1 ± 115.6.00 μm. n = 7 control and 6 *Lrp6* embryos, p = 0.0073 by unpaired t-test). (**C-D**) Tissue enlargement in *Lrp6* mutants persists into elevation stages (E8.5, 6-7 somites) and continues to be driven by tissue widening (control, 688.6 ± 108.3 μm; *Lrp6*: 1097 ± 100.9 μm. n = 5 control and 7 *Lrp6* embryos, $p < 0.0001$ by unpaired t-test) but not lengthening (control, 1035 ± 175.7 μm; *Lrp6*: 978.0 ± 98.83 μm. n = 5 control and 7 *Lrp6* embryos, p = 0.4845 by unpaired t-test). Scale bars represent 100 μm, dashed outlines indicate edges of the neural plate. ns, not significant; **, $p < 0.01$, ***, $p < 0.001$. Anterior is up in all images.

Figure 4: *Lrp6* mutants do not show defects in apical constriction, apicobasal elongation, or proliferation during elevation stages. (**A-B**) Maximum intensity projections of tiled confocal images of the future midbrain and hindbrain regions from control and *Lrp6* mutants at cranial neural fold elevation stages (E8.5, 5-7 somites). The apparent lengthening of the tissue in the *Lrp6* embryo is an artifact caused by delay in formation of the cranial flexure between the forebrain and midbrain tissues. (C) The apical areas of

cells within the 100 μm x 100 μm boxed regions in A-B are shown by staining for the apical junctional marker N-Cadherin (top) and computational segmentation (bottom). (**D**) The average apical area of cells does not differ between control and *Lrp6* mutant embryos (control, 23.63 ± 4.59 μm^2; *Lrp6*: 24.35 ± 2.81 μm. n = 4 control and 7 *Lrp6* embryos, p = 0.7492 by unpaired t-test, each dot represents the average of cell areas for a single embryo). (**E**) The distribution of cell apical areas does not differ between control and *Lrp6* mutants. (**F**) The apicobasal thickness of the lateral cranial tissues does not differ between control and *Lrp6* mutant embryos (control, 38.96 ± 6.40 μm; *Lrp6*: 40.44 ± 3.22 μm. n = 6 control and 6 *Lrp6* embryos, p = 0.6242 by unpaired t-test). (**G**) Staining for phospho-histone H3 (PHH3) in a 100 μm x 100 μm region of the lateral midbrain are shown. (**H**) No difference in the proportion of PHH3+ cells was observed between control and *Lrp6* mutants (control, 4.12 ± 0.55 percent; *Lrp6*: 4.11 ± 0.55 percent. n = 4 control and 7 *Lrp6* embryos, p = 0.9855 by unpaired t-test). (**I**) No difference in the orientation of anaphase separation angles was observed between control and *Lrp6* mutants by two-way ANOVA analysis with Sidak's multiple comparisons, see Table S1 for n and p values. Anterior is up in all images, scale bars represent 100 μm in A-B, and 20 μm in C and G. ns, not significant; A, anterior; P, posterior; L, lateral; M, medial.

Figure 5: Planar polarization of cranial neural tissues is normal in *Lrp6* mutants. (**A**) Staining for phosphorylated myosin regulatory light chain (pMRLC, top) and segmented cables that are color coded according to angular orientation (bottom; -90º is posterior, 90º is anterior). Images are 100 μm x 100 μm lateral regions, similar to those analyzed in Figure 4. (**B**) The fraction of pMRLC cables in three angular bins, after taking the absolute value of the individual angles, is plotted for control and *Lrp6* mutants. Note that both control and *Lrp6* mutants show an equivalent bias toward mediolaterally aligned cables (0-30º, Ctl: 0.41; *Lrp6*: 0.40) over intermediate (30-60º, Ctl: 0.31; *Lrp6*: 0.32) and anteroposterior (60-90º, Ctl: 0.27; *Lrp6* 0.28) alignment. (**C**). Staining for the planar cell polarity component VANGL2 is shown in 100 μm x 100 μm lateral regions (top), and segmented cable orientation (bottom). (**D**) The fraction of VANGL2 cables in three angular bins, after taking the absolute value of the individual angles, is plotted for control and *Lrp6* mutants. Note that both control and *Lrp6* mutants show an equivalent bias toward mediolaterally aligned cables (0-30º, Ctl: 0.43; *Lrp6*: 0.40) at frequencies similar to that observed for pMRLC staining and biased over intermediate (30-60º, Ctl: 0.33; *Lrp6*: 0.34) and anteroposteriorly (60-90º, Ctl: 0.24; *Lrp6* 0.26) aligned ones. See Table S1 for n and p values. Anterior is up in all images, scale bars represent 20 μm. ns, not significant; A, anterior; P, posterior; L, lateral; M, medial.

Figure 6: *Lrp6* mutants show excessive proliferation in anterior neural tissues at early neural plate stages. (**A-B**) Transverse sections through control and *Lrp6* mutant embryos at E7.5, stained with (A) an antibody against OTX2 and counterstained with Phalloidin (F-actin) and, or (B) the S-M phase marker phospho-HistoneH3. Anterior is to the left, dashed white lines indicate the OTX2+ neuroepithelial region. (**C**) Quantification of the percentage of the epiblast tissue marked with OTX2 shows that anterior fates are not expanded in *Lrp6* mutants (control: 61.67 ± 6.03 %; *Lrp6*: 63.67 ± 3.06 %; n = 3 control, 3 *Lrp6* embryos at E7.5; p = 0.6352 by unpaired t-test). (**D**) *Lrp6* mutants show a significantly increase in the proportion of proliferative cells in the anterior (OTX2+) neural tissues as compared to controls (control: 5.23 ± 0.97 %; *Lpr6*: 9.38 ± 1.25%; n = 3 control, 3 *Lrp6* embryos; p = 0.0105 by unpaired t-test). (**E**) No difference is observed in the proportion of proliferative cells between control and *Lrp6* mutants in the posterior (OTX2-) neural tissues (control: 5.74 ± 2.08 %; *Lrp6*: 6.33 ± 0.95 %; n = 3 control, 3 *Lrp6*

embryos; p = 0.6783 by unpaired t-test). Scale bars represent 100 μm; A, anterior; P, posterior.

Figure 7: Cranial neural fold specific Wnt hyperactivation leads to neural fold elevation and cranial closure defects. (**A**) Conditional hyperactivation of Wnt signaling by forced truncation of APC specifically in the midbrain prevents cranial neural tube closure from the forebrain to the hindbrain-spinal cord juncture (dashed yellow line) at a high penetrance (~70%). These *Apc-cIN* (short for APC conditional inactivation) mutants are distinct from *Lrp6* mutants due to strong defects in neural fold elevation (compare yellow arrowheads here and in Fig 1A). (**B-C**) Hyperactivation of Wnt signaling by conditional APC truncation in *Apc-cIN* mutants does not lead to tissue scaling defects in either length (control, 1259 ± 208.6 μm; *Lrp6*: 1191 ± 214.7 μm. n = 8 control and 6 *Apc-cIN* embryos, p = 0.5604 by unpaired t-test) or width (control, 602.1 ± 41.09 μm; *Lrp6*: 616.2 ± 56.29 μm. n = 8 control and 6 *Apc-cIN* embryos, p = 0.59674 by unpaired t-test). Scale bars represent 100 μm, dashed outlines indicate edges of the neural plate. ns, not significant; **, p < 0.01, ***, p < 0.001. Anterior is up in all images.

Figure 8: *Apc-cIN* mutants show failure of apical constriction but not planar polarization or proliferation. (**A-B**) Maximum intensity projection of tiled confocal images of the midbrain and hindbrain regions of (A) control and (B) *Apc-cIN* mutant embryos at 8 somites. Dashed boxes indicate the areas analyzed in C and G. (**C**) The apical areas of cells within the 100 μm x 100 μm boxed regions in A-B are shown by staining for the phallodin (F-actin, left) and computational segmentation (right). Note the increase in cells displaying very large apical areas in *Apc-cIN* mutants. (**D**) Quantification of the average apical cell area of embryos. *Apc-cIN* mutants show a significant enlargement of apical area (Ctl: 13.36 ± 2.29 μm^2; *Apc-cIN*: 18.78 ± 1.07 μm^2; n = 3 control and 3 *Apc-cIN* mutants; p = 0.0293). (**E**) Increases in the average apical area of cells in *Apc-cIN* mutants are driven by a reduction in the number of cells < 15 μm^2 and an increase in the number of cells > 25 μm^2. (**F**) *Apc-cIN* mutants do not display changes to tissue thickness (Ctl: 50.61 ± 5.77 μm; *Apc-cIN*: 51.10 ± 5.92 μm; n = 3 control and 3 *Apc-cIN* mutants; p = 0.9238). (**G**) Staining for the planar cell polarity component VANGL2 is shown in 100 μm x 100 μm lateral regions (left), and segmented cables that are color coded by their angular orientation (right, 90º is anterior). (**H**) Control and *Apc-cIN* mutants show equivalent planar polarization of VANGL2 staining (two-way ANOVA with Sidak's multiple comparisons, see Table S1 for n and p values).

Figure 9: Model for the role of Wnt signaling levels in cranial neural tube closure. Schematic representation of the impact of Wnt reduction (*Lrp6* mutants) or hyperactivation (*Apc-cIN*) on elements of cranial neural tube closure. Events that are comparable with controls are highlighted in green, while those that are defective are highlighted in magenta. In *Lrp6* mutants, neural induction is normal, but early tissues show roughly double the rate of cell proliferation. This leads to a doubling of tissue width at elevation stages, and a resulting failure to bring the neural folds together over the midline despite the normal activity of apical constriction and neural fold elevation. Wnt hyperactivation in *Apc-cIN* mutants does not impact early events or tissues scale, but blocks apical constriction resulting in a failure of neural fold elevation. Thus, both conditions lead to cranial closure defects but do so via disruption of unique cellular mechanisms.

Figure S1: *Lrp6* mutants do not show changes to the number of anaphase events, pMRLC cables, or VANGL2 cables. (**A**) Quantification of the number of anaphase

separation events observed in DAPI staining on one half of the cranial neural plate in control and *Lrp6* mutant embryos. (control: 16.4 ± 1.7; *Lrp6*: 15.8 ± 1.6 ; n = 5 control, 5 *Lrp6* embryos; p = 0.5830 by unpaired t-test). (**B**) Quantification of the number of pMRLC cables observed in a 100 µm x 100 µm region in control and *Lrp6* mutant embryos. (control: 229.0 ± 28.6; *Lrp6*: 234.3 ± 46.0 ; n = 5 control, 3 *Lrp6* embryos; p = 0.8432 by unpaired t-test). (**B**) Quantification of the number of VANGL2 cables observed in one 100 µm x 100 µm region in control and *Lrp6* mutant embryos. (control: 258.6 ± 33.7.6; *Lrp6*: 309.0 ± 63.3; n = 5 control, 3 *Lrp6* embryos; p = 0.1823 by unpaired t-test).

Figure S2: *Apc-cIN* mutants do not show altered number of VANGL2 cable number or proliferative capacity. (**A**) Quantification of the number of VANGL2 cables detected in a 100 µm x 100 µm region in control and *Apc-cIN* mutant embryos (Ctl: 299.3 ± 8.6; *Apc-cIN*: 304.3 ± 11.2; n = 3 control, 3 *Apc-cIN*; p = 0.5694 by unpaired t-test). (**B**) Images showing staining for phospho-HistoneH3 in 100 µm x 100 µm lateral regions of control and *Apc-cIN* mutants. (**C**) Quantification of the proportion of proliferative cells shows no difference between control and *Apc-cIN* mutant embryos (Ctl: 3.53 ± 0.93%; *Apc-cIN*: 3.63 ± 1.04%; n = 3 control, 3 *Apc-cIN*; p = 0.9072 by unpaired t-test).

Table Legends

Table S1: Summary of statistical tests, n, and p values. For each formal statistical test reported here, the relevant figure, test type, n values, mean and standard deviation (when applicable), and p values are reported.

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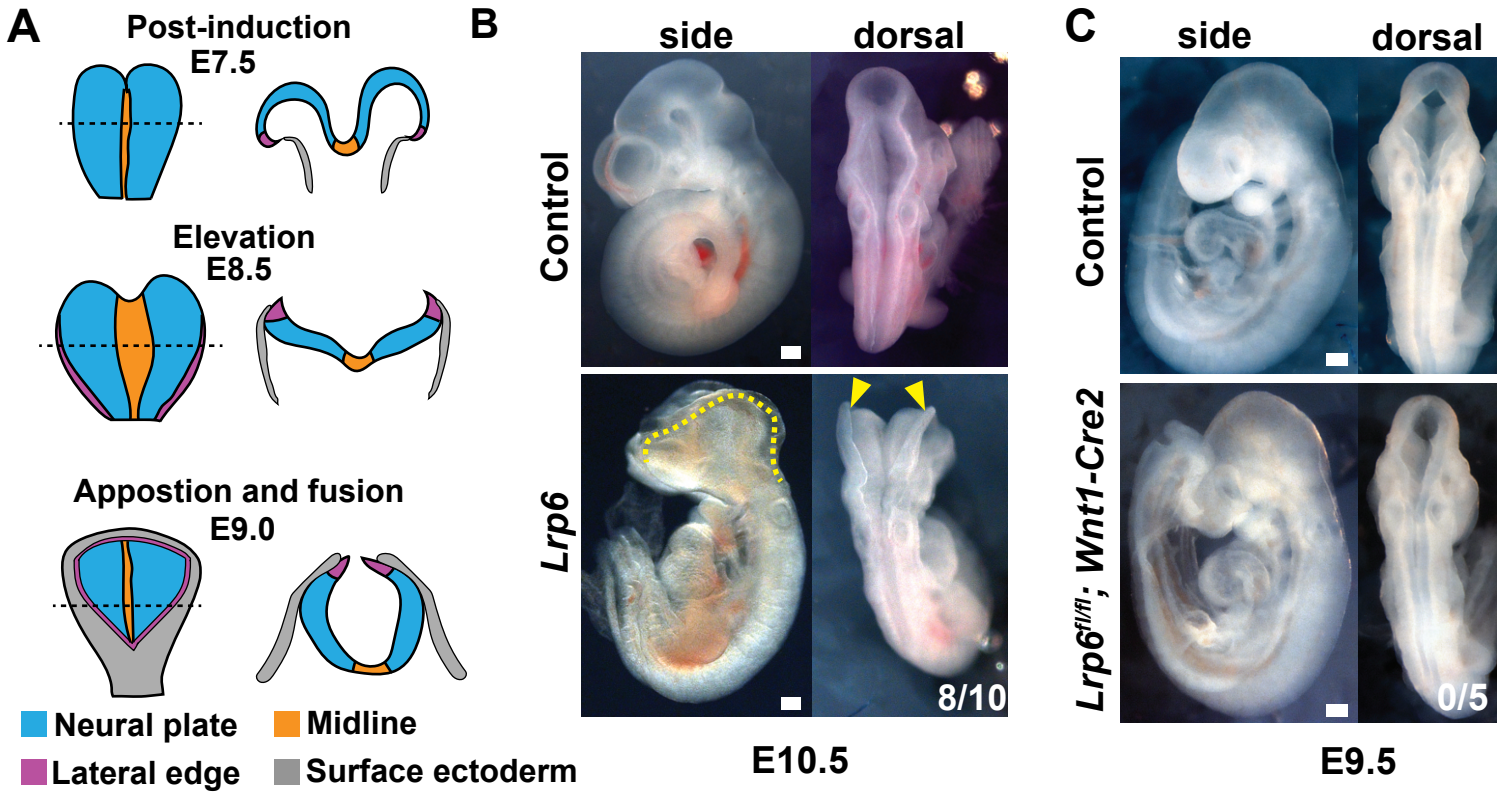
A
Post-induction
E7.5
Elevation
E8.5
Appostion and fusion
E9.0
Neural plate
Midline
Lateral edge
Surface ectoderm
B
side
dorsal
Control
Lrp6
8/10
E10.5
C
side
dorsal
Control
Lrp6fl/fl; Wnt1-Cre2
0/5
E9.5

Figure 1

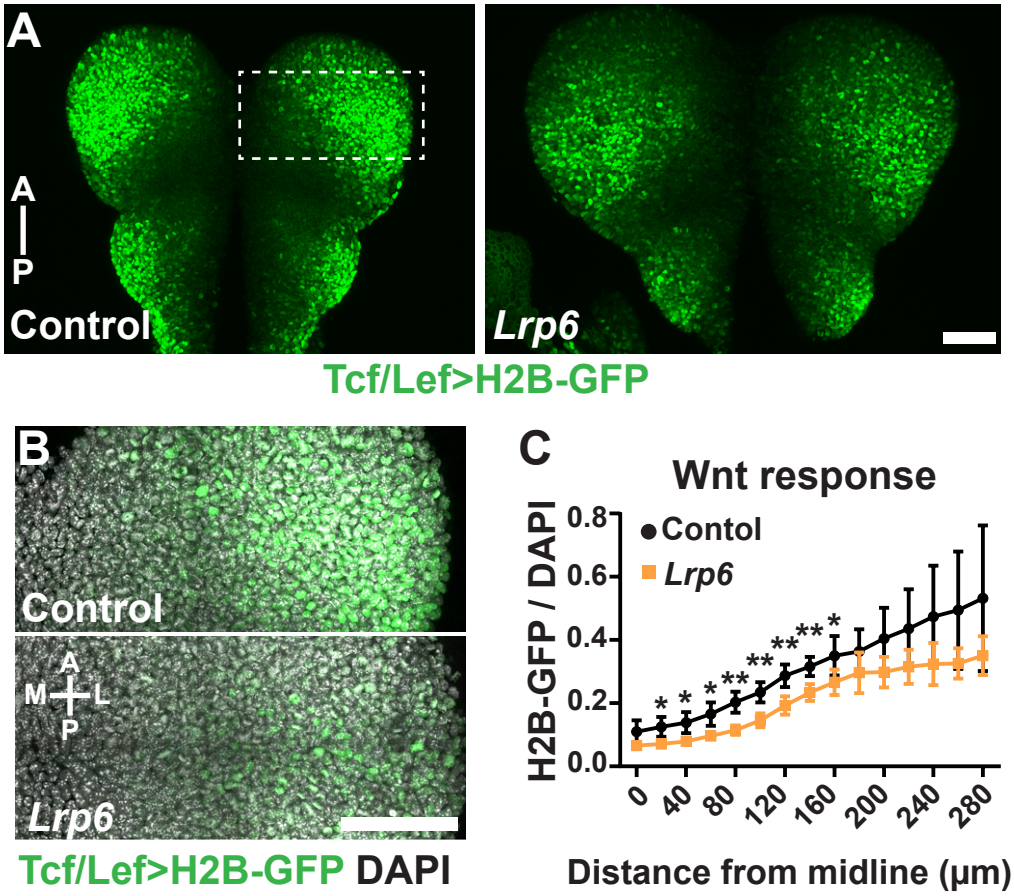
A
A
P
Control
Lrp6
Tcf/Lef>H2B-GFP
B
Control
A
M
L
P
Lrp6
Tcf/Lef>H2B-GFP DAPI
C
Wnt response
Contol
Lrp6
H2B-GFP / DAPI
0.8
0.6
0.4
0.2
0.0
0
40
80
120
160
200
240
280
Distance from midline (μm)

Figure 2

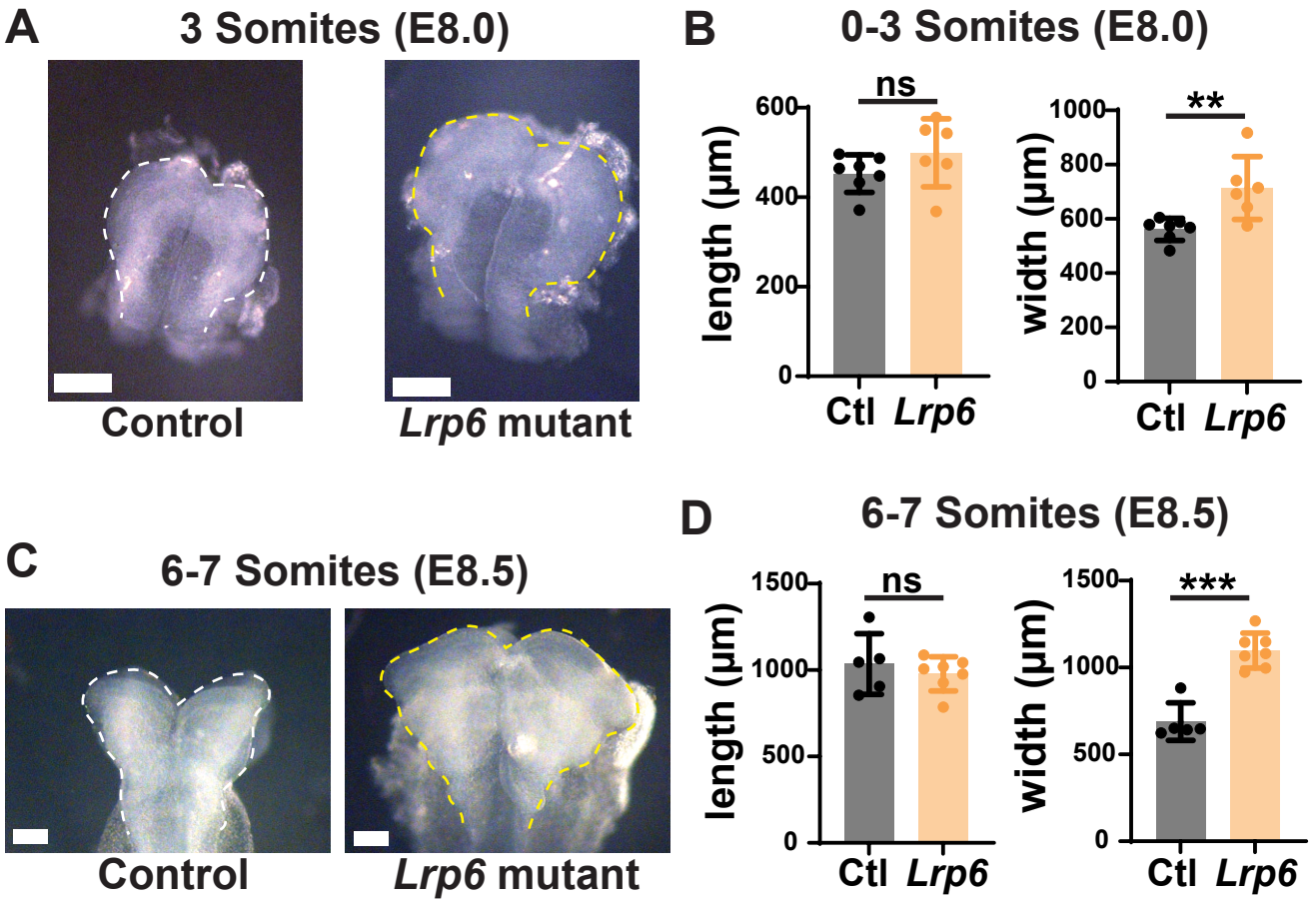
A
3 Somites (E8.0)
Control
Lrp6 mutant
B
0-3 Somites (E8.0)
ns
**
length (µm)
width (µm)
600
400
200
0
1000
800
600
400
200
0
Ctl
Lrp6
Ctl
Lrp6
C
6-7 Somites (E8.5)
Control
Lrp6 mutant
D
6-7 Somites (E8.5)
ns
***
length (µm)
width (µm)
1500
1000
500
0
1500
1000
500
0
Ctl
Lrp6
Ctl
Lrp6

Figure 3

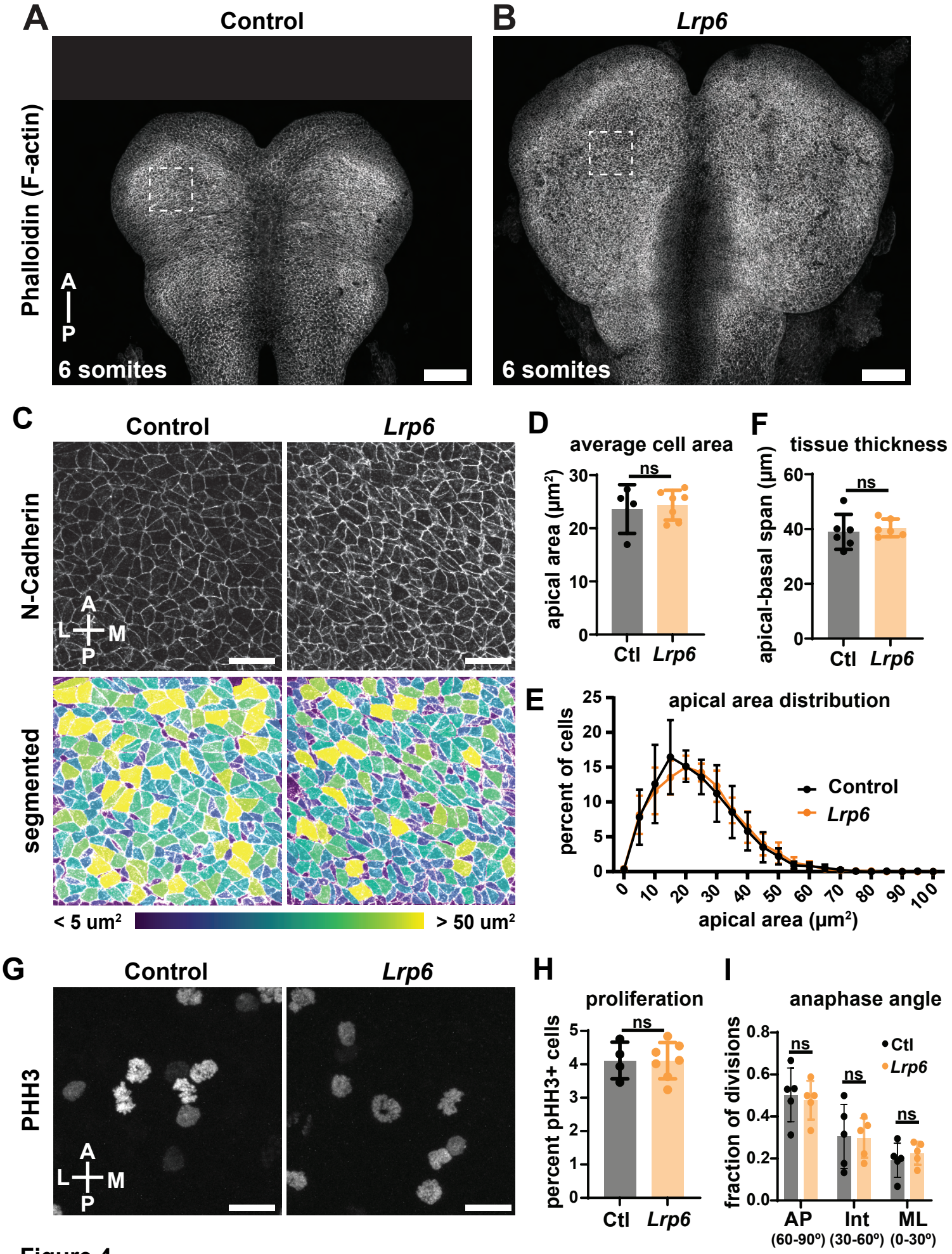
A
Control
Phalloidin (F-actin)
A
P
6 somites
B
Lrp6
6 somites
C
Control
Lrp6
N-Cadherin
A
L
M
P
segmented
< 5 um²
> 50 um²
D
average cell area
ns
apical area (µm²)
30
20
10
0
Ctl
Lrp6
F
tissue thickness
ns
apical-basal span (µm)
60
40
20
0
Ctl
Lrp6
E
apical area distribution
percent of cells
25
20
15
10
5
0
Control
Lrp6
0
10
20
30
40
50
60
70
80
90
100
apical area (µm²)
G
Control
Lrp6
PHH3
A
L
M
P
H
proliferation
ns
percent pHH3+ cells
5
4
3
2
1
0
Ctl
Lrp6
I
anaphase angle
ns
ns
ns
Ctl
Lrp6
fraction of divisions
0.8
0.6
0.4
0.2
0.0
AP
(60-90°)
Int
(30-60°)
ML
(0-30°)

Figure 4

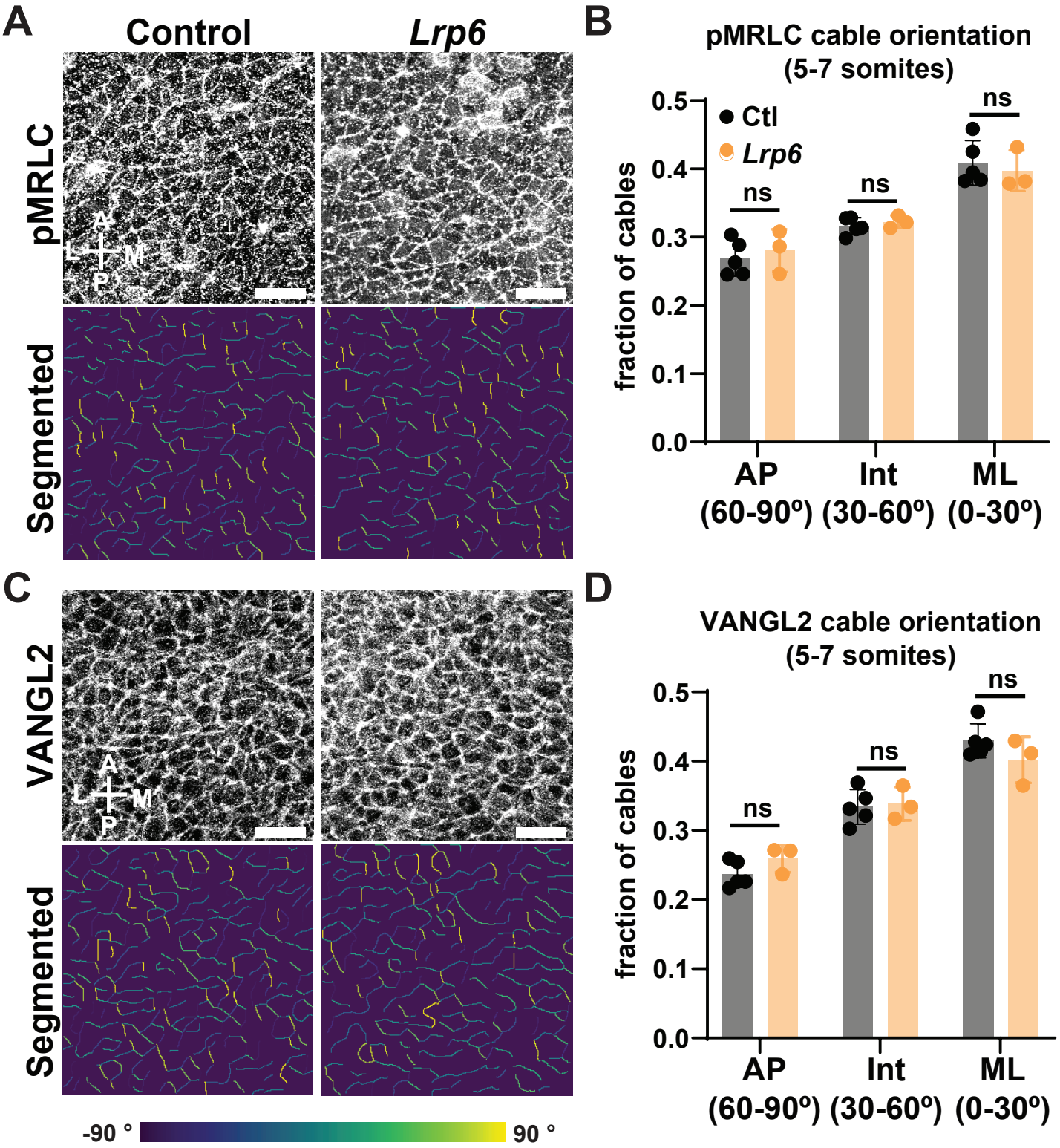
A
Control
Lrp6
pMRLC
Segmented
A
L
M
P
B
pMRLC cable orientation
(5-7 somites)
Ctl
Lrp6
ns
fraction of cables
0.5
0.4
0.3
0.2
0.1
0.0
AP
(60-90º)
Int
(30-60º)
ML
(0-30º)
C
VANGL2
D
VANGL2 cable orientation
(5-7 somites)
-90 °
90 °

Figure 5

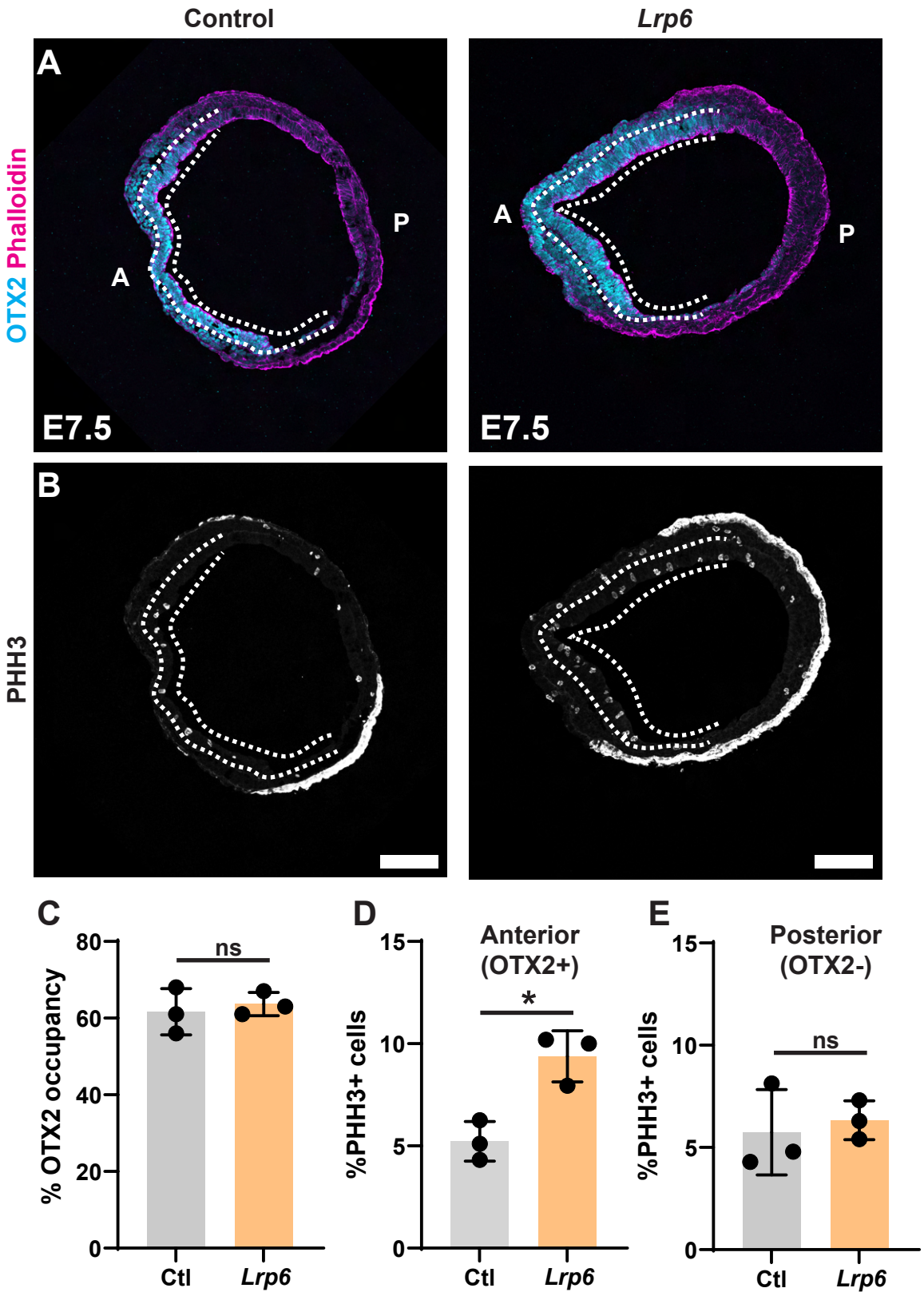
Control
Lrp6
A
OTX2 Phalloidin
A
P
E7.5
A
P
E7.5
B
PHH3
C
% OTX2 occupancy
80
60
40
20
0
ns
Ctl
Lrp6
D
%PHH3+ cells
15
10
5
0
Anterior
(OTX2+)
*
Ctl
Lrp6
E
%PHH3+ cells
15
10
5
0
Posterior
(OTX2-)
ns
Ctl
Lrp6

Figure 6

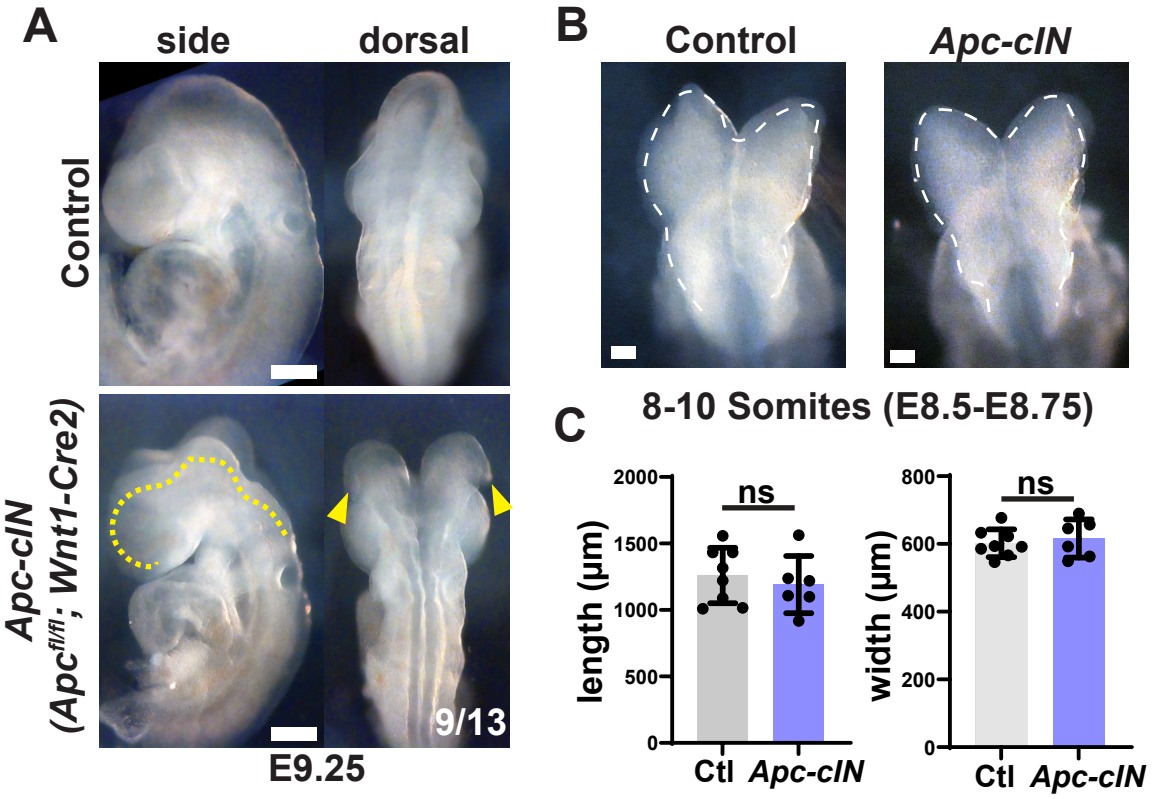
A
side
dorsal
Control
Apc-cIN
(Apc^fl/fl; Wnt1-Cre2)
9/13
E9.25
B
Control
Apc-cIN
C
8-10 Somites (E8.5-E8.75)
ns
length (µm)
2000
1500
1000
500
0
Ctl
Apc-cIN
ns
width (µm)
800
600
400
200
0
Ctl
Apc-cIN

Figure 7

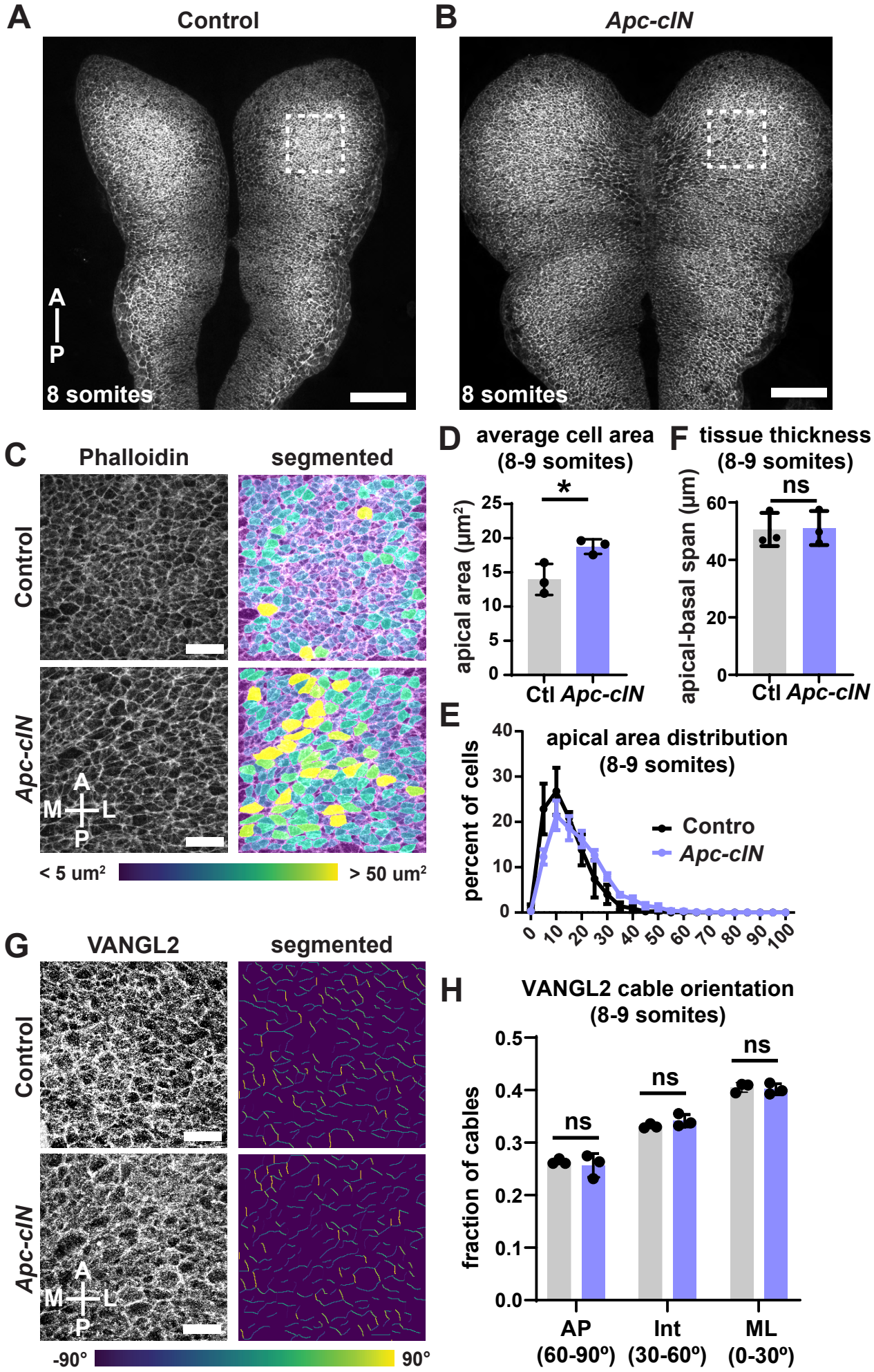
A
Control
A
P
8 somites
B
Apc-cIN
8 somites
C
Phalloidin
segmented
Control
Apc-cIN
A
M
L
P
< 5 um²
> 50 um²
D
average cell area
(8-9 somites)
*
apical area (µm²)
25
20
15
10
5
0
Ctl
Apc-cIN
F
tissue thickness
(8-9 somites)
ns
apical-basal span (µm)
60
40
20
0
Ctl
Apc-cIN
E
apical area distribution
(8-9 somites)
percent of cells
40
30
20
10
0
Contro
Apc-cIN
0
10
20
30
40
50
60
70
80
90
100
G
VANGL2
segmented
Control
Apc-cIN
A
M
L
P
-90°
90°
H
VANGL2 cable orientation
(8-9 somites)
fraction of cables
0.5
0.4
0.3
0.2
0.1
0.0
ns
ns
ns
AP
(60-90º)
Int
(30-60º)
ML
(0-30º)

Figure 8

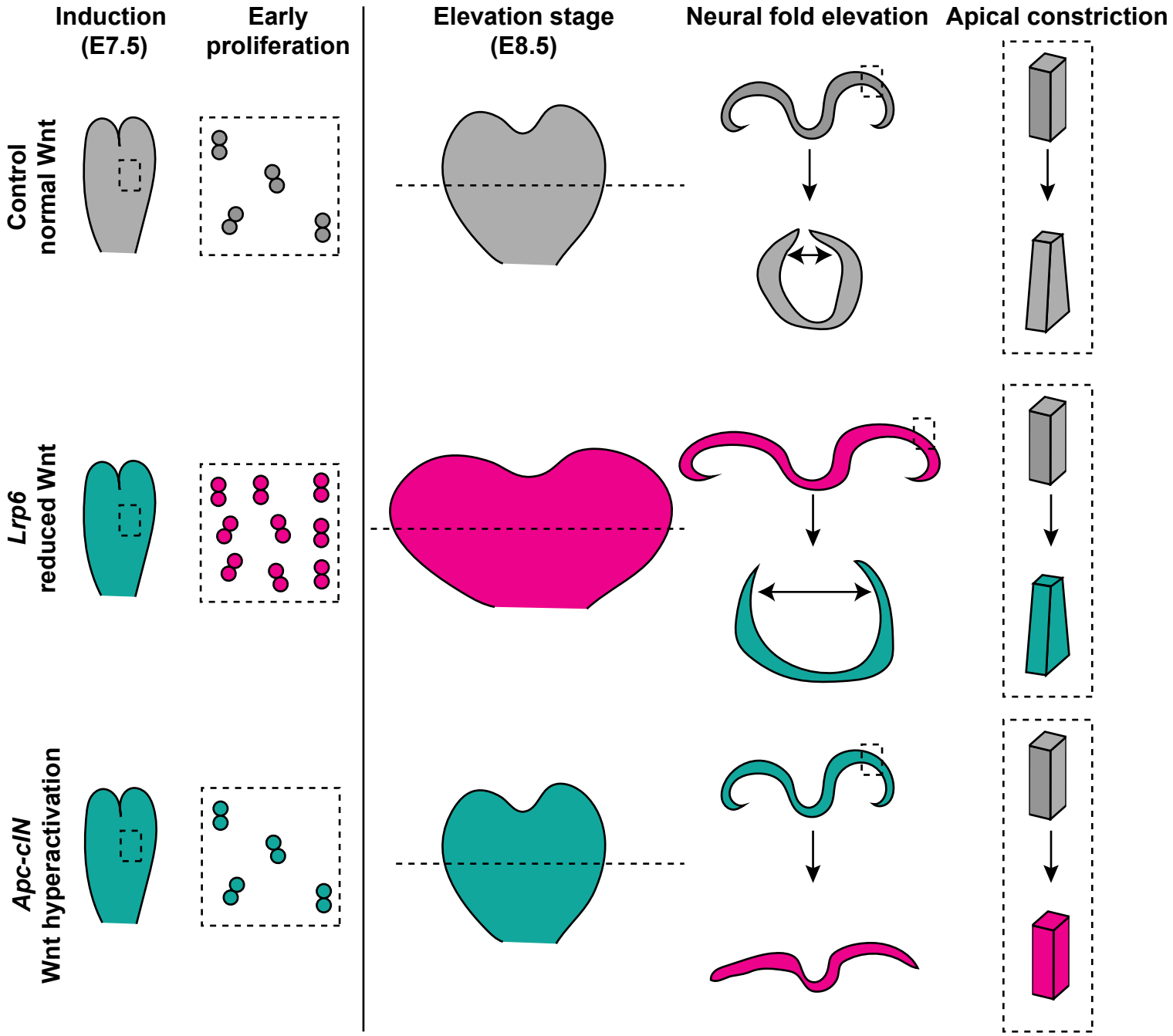
Induction
(E7.5)
Early
proliferation
Elevation stage
(E8.5)
Neural fold elevation
Apical constriction
Control
normal Wnt
Lrp6
reduced Wnt
Apc-cIN
Wnt hyperactivation

Figure 9

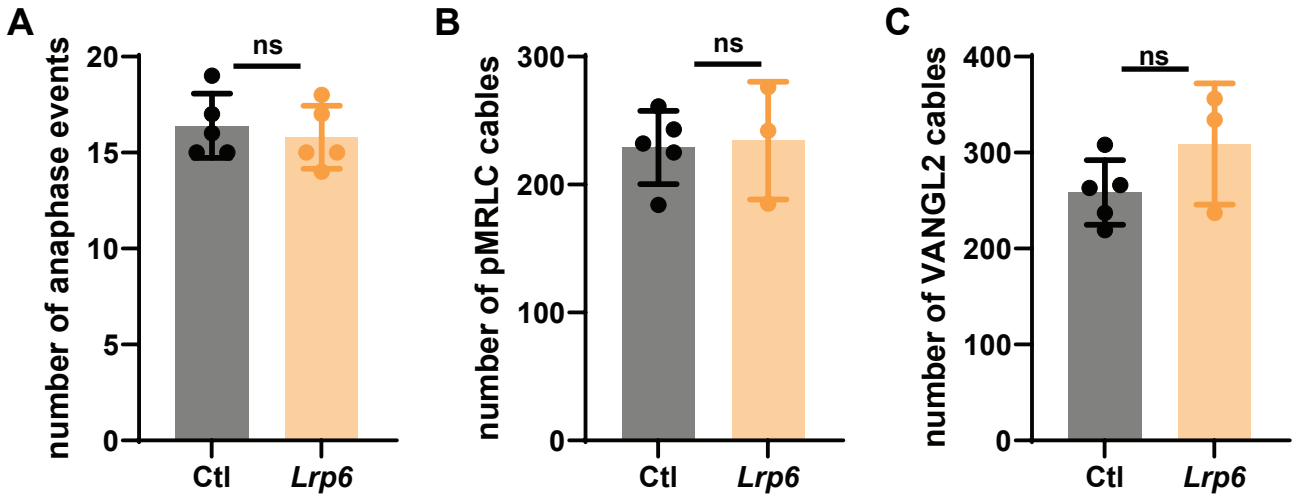
A
number of anaphase events
20
15
10
5
0
ns
Ctl
Lrp6
B
number of pMRLC cables
300
200
100
0
ns
Ctl
Lrp6
C
number of VANGL2 cables
400
300
200
100
0
ns
Ctl
Lrp6

Figure S1

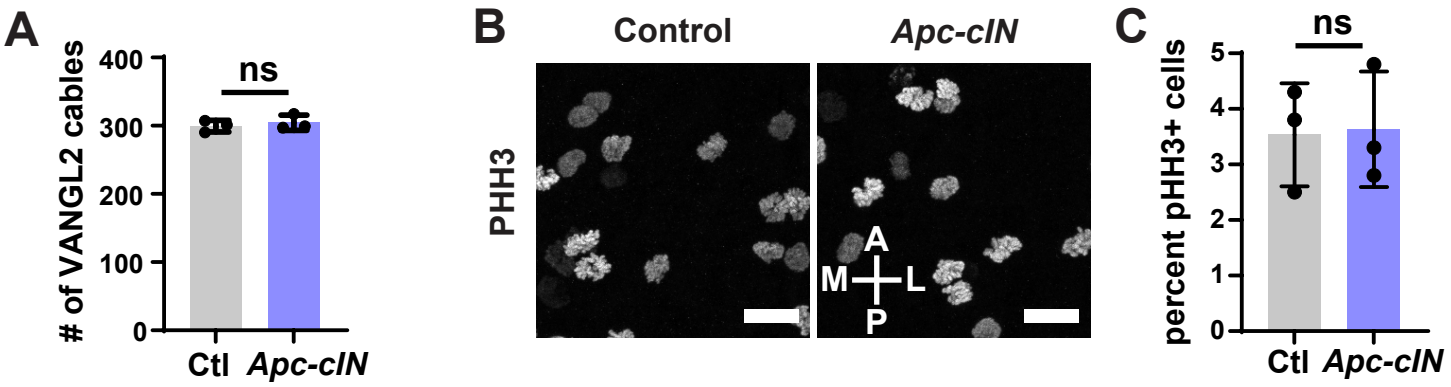
A
# of VANGL2 cables
400
300
200
100
0
ns
Ctl
Apc-clN
B
Control
Apc-clN
PHH3
A
M
L
P
C
percent pHH3+ cells
5
4
3
2
1
0
ns
Ctl
Apc-clN

Figure S2