An actomyosin network organizes niche morphology and responds to feedback from recruited stem cells

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Summary

Stem cells often rely on signals from a niche, which in many tissues adopts a precise morphology. What remains elusive is how niches are formed, and how morphology impacts function. To address this, we leverage the *Drosophila* gonadal niche, which affords genetic tractability and live-imaging. We have previously shown mechanisms dictating niche cell migration to their appropriate position within the gonad, and the resultant consequences on niche function. Here, we show that once positioned, niche cells robustly polarize filamentous actin (F-actin) and Non-muscle Myosin II (MyoII) towards neighboring germ cells. Actomyosin tension along the niche periphery generates a highly reproducible smoothened contour. Without contractility, niches are misshapen and exhibit defects in their ability to regulate germline stem cell behavior. We additionally show that germ cells aid in polarizing MyoII within niche cells, and that extrinsic input is required for niche morphogenesis and function. Our work reveals a feedback mechanism where stem cells shape the niche that guides their behavior.

Keywords

Drosophila, Stem cell, testis, niche, feedback, actomyosin contractility, morphogenesis

Introduction

In many tissues, stem cells reside in a specialized niche, where they receive signals to properly balance self-renewal and differentiation. Imbalances in renewal or differentiation can lead to tissue degeneration or tumor formation[1,2], so it is vital to understand mechanisms of niche function. Niches often have a precise, reproducible architecture[3–6], but only recently has attention turned to how a niche forms its shape and how shape contributes to function[7–9]. The experiments to date often rely on whole-tissue manipulation or organoid models. Therefore, it is necessary to establish models to interrogate mechanisms controlling niche morphogenesis with cell-type specificity and in developing tissue.

We have leveraged the genetically tractable *Drosophila* testis, as the cells comprising the niche and associated stem cells are well-defined[10]. This niche is anchored at the apex of the testis[11], and is comprised of quiescent somatic cells organized in a reproducible sphere[12–14]. Functionally, this niche supports two stem cell lineages[10,15]: germline stem cells (GSCs), which produce differentiating germline cells, and Cyst stem cells (CySCs), which produce somatic cells that support the germline through differentiation[16,17].

The niche regulates multiple aspects of stem cell behavior to maintain tissue homeostasis. For example, the niche secretes the chemokine Unpaired (Upd) and activates the Jak-STAT signaling pathway in adjacent cells[18]. Activation promotes self-renewal in the CySCs, and aids in GSC self-renewal by promoting their adhesion to the niche[19,20]. The niche also regulates stem cell division orientation. GSCs and CySCs generally divide perpendicular to the niche to ensure that one daughter remains accessible to renewal signals, while the other is displaced away from the niche and differentiates[21–26].

While much is known about how the niche supports its stem cells at steady state, little is known about the establishment of a functional niche. Early in gonadogenesis, the Jak-STAT pathway is active in all primordial germ cells (PGCs) and somatic gonadal precursors (SGPs) within the gonad[27,28]. However, during late embryogenesis, the niche forms at the gonad anterior[12,13], where it selectively contacts and restricts signaling to only a subset of PGCs and SGPs. Only these cells will gain stem-like properties, with PGCs becoming GSCs, and later, SGPs becoming CySCs[27,28]. Because the critical niche-stem cell connections become defined concomitantly with niche morphogenesis, it begs the question of how proper niche shape affects its ability to define and regulate the stem cell pool.

In a major advance, we developed techniques to capture the behavior of niche cells and prospective stem cells through live imaging during this previously elusive period of gonadogenesis[29]. This, together with fixed analyses, has defined two dynamic processes during niche formation: 1) assembly and 2) compaction[12,13]. During assembly, pro-niche cells migrate to form a cap at the gonad anterior. Next, during compaction, niche cells reorganize to present a smoothened boundary to the surrounding cells. We have previously shown that errors in assembly lead to defects in niche function, niche cell polarity, and cell cycling[30]. The requirement for compaction in establishing final niche shape and function has not been explored.

Here, we identify a role for polarized actomyosin contractility (AMC) in establishing the final shape and function of the niche. We find that Non-muscle Myosin II (MyoII) within niche cells establishes increased tension along the niche-GSC boundary and is required to shape niche architecture at that interface. When niche structure is altered, we find defects in both signaling and establishment of proper orientation of GSC divisions. Furthermore, compaction timing correlates with the recruitment of actively dividing GSCs, and our data suggests that GSC divisions exert forces on the niche. In fact, inhibiting GSC divisions disrupted niche MyoII polarity, consequently disrupting niche shape and function. Thus, our analyses identify the cytoskeletal mechanics required to form a functional niche and reveal a feedback mechanism in which GSCs shape the niche that guides their behavior.

Results

Niche compaction is characterized by changes in niche shape and size

To characterize compaction dynamics, we live-imaged embryonic stage (ES) 16 gonads *ex vivo*, when niche cells had assembled but had not compacted[12,29]. We expressed fluorescent transgenic fusion constructs either in all cells, or restricted to germline or soma depending on the experiment. To examine niche contour, we used a somatic marker for filamentous actin (F-actin; hereafter referred to as Six4-Moe::GFP). We showed previously that Six4-Moe::GFP is expressed highly in male specific SGPs (msSGPs) located posteriorly, permitting identification of niche cells due to their increased fluorescence compared to the remainder of somatic gonadal cells and location opposite the msSGPs[12]. To visualize nuclear movements, we utilized a ubiquitously expressed chromatin marker, histone 2Av::mRFP (His2Av::mRFP1). Imaging onset revealed niche cells at the gonad anterior, forming a jagged contour between themselves and adjacent germ cells (Figure 1A). Within a few hours, niche cells moved closer together and the contour facing the germline adopted a more circular profile (Figs. 1A'-A'''). This morphogenesis is consistent with previous observations in fixed preparations and our earlier live-imaging analyses[12,13].

To further confirm our results, we analyzed niche morphology at pre-compaction (ES16) and post-compaction (ES17) stages in fixed preparations using Fasciclin III (FasIII) as a niche-specific marker (Figs. 1B, B', C, C'). We quantified changes to the niche contour by comparing circularity of the niche outline, where a value of 1 represents a perfect circle and lower values indicate a more jagged contour. Like our *ex vivo* results, niche cells *in vivo* reorganized from pre- to post-compaction, generating a more circular form (Fig. 1D). 3-Dimensional analysis revealed that compact niches were smaller in surface area compared to uncompact niches (Fig. 1E). This was partially explained by niche cells moving closer together, as evidenced by a decrease in niche cell internuclear distance (Fig. 1F). We also noticed a variable and slight decrease in niche cell number from pre- to post-compaction (Fig. 1G). Thus, morphological compaction of the niche occurs both *ex vivo* and *in vivo,* suggesting this process can be used to identify factors required for niche morphogenesis. Below, all *ex vivo* experiments were performed on ES16 gonads to visualize compaction, whereas *in vivo* fixed experiments were performed on late ES17 gonads for endpoint analyses.

F-actin and MyoII polarize along the niche periphery during compaction

Due to the role of AMC in many morphogenetic events shaping cell and tissue contours[7,8,31–35], we hypothesized that AMC drives niche compaction. To visualize cytoskeletal dynamics of F-actin and MyoII during compaction, we live-imaged gonads containing Six4-Moe::GFP and a ubiquitously-expressed MyoII regulatory light chain (MyoII RLC) tagged with mCherry (MyoII:mCherry; Fig. 2A). We found F-actin localized along the niche periphery throughout compaction (Fig 2A'). However, MyoII progressively enriched over a few hours during compaction, appearing as puncta along the niche-germline interface (Fig. 2A'', arrow). These trends were confirmed by quantification of enrichment along the niche-germline boundary at 0h, 2.5h, and 5h post-dissection to represent early-, mid-, and post-compaction, respectively (Figs. 2B-C). The correlation of MyoII enrichment with morphogenesis suggests a functional role for AMC in niche compaction.

Enrichment along the niche boundary might reflect a symmetric contribution from the niche cell cortex and the adjacent germline, or an asymmetric contribution from one lineage. To distinguish these possibilities, we independently labeled F-actin or MyoII in a somatic- or germline-specific manner (Fig. 2D-G). For somatic labeling, we used Six4-Gal4 to drive either an F-actin marker (F-Tractin::tdTomato; Fig. 2D), or a MyoII heavy chain marker (MyoII HC::GFP; Fig. 2E). For germline labeling, we used the germline-specific F-actin marker, Nanos-Moesin::GFP (Fig. 2F), and the germline-specific Gal4 driver, Nanos-Gal4::VP16, to express MyoII HC::GFP (Fig. 2G). Quantitative analysis revealed F-actin polarization towards the niche-GSC interface is

derived from both somatic niche cells and germline cells (Figs. 2D, F, H, J). Conversely, only when labeled within niche cells did MyoII HC polarize to the niche-GSC interface (Fig. 2E,G,I,K). The asymmetric recruitment of MyoII in niche cells suggests that tension specifically within the niche cell cortex is required for compaction.

AMC induces tension along the niche-GSC interface

To test whether AMC generates tension along the niche-GSC boundary during compaction, we dissected gonads for *ex vivo* live-imaging, and identified gonads mid-compaction, correlating with when MyoII polarizes along the niche-GSC interfaces (Fig. 2C). Using a laser, we severed the actomyosin network either at the niche-GSC interface or at a niche-niche interface (Figs. 3A-B). As a proxy for tension, we calculated the initial retraction velocity of vertices flanking a cut by measuring the displacement of each vertex within 5s post-cut (Fig. 3D; ref.[36]). This revealed higher tension along niche-GSC interfaces compared to niche-niche interfaces, suggesting a correlation between MyoII polarization and tension. To assess whether the tension along the niche-GSC interfaces is induced by AMC, we treated gonads with the H-1152 Rho Kinase inhibitor (ROKi) to inhibit AMC, then severed niche-GSC interfaces (Figs. 3C-D). ROKi treatment reduced tension along the niche-GSC interface (Fig. 3E), confirming that AMC induces polarized tension during compaction.

AMC is required for niche morphogenesis

To address whether niche morphology is controlled by AMC, we monitored compaction live in gonads cultured in the presence or absence of ROKi (Figs. 4A-B). Untreated niches generally exhibited an increase in circularity over time (Fig. 4C; average increase of 20%; median, 8%). In contrast, ROKi treatment administered at compaction onset resulted in mixed circularity changes, with many niches exhibiting decreases in circularity (Fig. 4D; average change of 0.5%; median, -5.8%). These data suggest that contractility is required specifically during compaction to establish niche architecture.

As niche cells contribute to MyoII enrichment at niche-GSC interfaces, we used Six4-Gal4 to challenge MyoII function specifically in somatic gonadal cells (Fig. 2E). Somatic RNAi against MyoII HC successfully depleted MyoII enrichment at the niche-GSC interface (Supplemental Fig. 1), and led to severe compaction defects, as evidenced by a jagged niche-GSC boundary (Fig. 4E, E’, F, F’). We found that niche circularity, but not niche area or cell number, was significantly decreased compared to controls, and observed similar defects using an RNAi to MyoII RLC (Fig. 4G-K). Together, these results show that somatic AMC is required for niche shape.

Proper niche shape limits access to self-renewal signals

The reproducibility of niche morphology suggests that niche shape might contribute to its function. Of the niche-derived signals maintaining the stem cell pool in adult testes, only Upd is known to act at this early stage. Upd expressed from niche cells will activate the JAK-STAT pathway in cells nearest the niche, leading to accumulation of STAT protein, which is routinely used as a reporter for pathway activation[27,28].

In both sibling controls and somatic MyoII HC RNAi gonads, germ cells adjacent to the niche exhibited STAT enrichment compared to germ cells further away (Figs. 5A,A’-B,B’; quantified in C), demonstrating that improperly shaped niches are still able to signal. However, compared with controls, more germ cells contact the niche when the niche is misshapen (supplemental fig. 2A), and most of these germ cells are activated for STAT (Fig. 5D). In fact, we noticed a significant increase in all germ cells of the gonad (Supplemental fig. 2B), likely due to a larger stem cell pool. As a critical role for the niche is establishing an appropriate number of stem cells, these results suggest that niche structure is essential for proper function.

Intriguingly, niches in MyoII HC RNAi are not larger (Fig. 4J), yet they support more stem cells. We hypothesized this contradiction might be explained by increased exposure of niche surface area to the germline. Typically, only a portion of the niche contacts the germline, while some of the niche area is directed away. We used 3D analysis to measure the percentage of the niche surface physically contacting germ cells (Supplemental fig. 2C,C',D,D'), and found an increase in niche surface area contacting the germline in MyoII HC RNAi conditions compared to controls (Fig. 5E). These data suggest that a compact niche is required to limit available surface area to restrict the number of GSCs.

Proper niche shape is required to orient GSC divisions

Another critical function of the niche is to orient GSC divisions by anchoring one centrosome to the niche-GSC interface[21–23,25,26]. This ensures an asymmetric outcome in which one daughter will remain a GSC and the other will differentiate. To address whether proper niche shape is necessary to orient divisions, we stained gamma tubulin to label centrosome position. In control gonads, as expected, about 90% of GSCs had proper centrosome positioning (Fig. 5F, H). In contrast, the frequency of mispositioned centrosomes doubled in GSCs from gonads where MyoII was depleted from the niche (Fig. 5G-H). By chance movement, unanchored centrosomes abut the niche ~50% of the time[23]; thus, change of this magnitude is significant. Additionally, this degree of mispositioning is consistent with that caused by early stages of aging in the adult testis[23].

We next live-imaged control and ROKi-treated gonads to determine if germ cells adjacent to a misshapen niche divide aberrantly. We mapped the orientation of condensed chromosomes in dividing GSCs using His2Av::mRFP1 to calculate the trajectory of GSC divisions relative to the niche-GSC interface in 3-dimensions (Supplemental Fig. 3A). In the analysis, lower angles represent those more parallel to the niche-GSC interface, and angles closer to 90 degrees are oriented more away from the niche[37]. A random distribution, with 3-dimensional constraints taken into consideration, would appear as a majority of cells dividing within lower division angles (i.e. 0-45 degrees[37]; Supplemental Fig. 3B). By contrast, we found that control nuclei exhibited division angles predominantly ranging from 30-80 degrees (Fig. 5I-I', K), suggesting that GSC division orientation is highly regulated and biased such that daughter cells are born further from the niche.

However, in ROKi-treated gonads, GSCs primarily divided between 0-45 degrees relative to the niche, significantly differing from the controls (Fig. 5 J,J',K; $p<0.001$, KS test) and consistent with a randomized distribution (Supplemental Fig. 3C; ref[37]). These data suggest that a misshapen niche cannot properly regulate GSC divisions and that contractility within the niche is essential to orient divisions. Because asymmetric cell division is critical for maintaining the balance between self-renewal and differentiation[2], improper division orientations would impede homeostasis.

Taken together, the aberrant features in gonads with misshapen niches suggest that proper niche shape is crucial for maintaining homeostasis in the gonad.

GSCs are required to shape their niche

We next sought to uncover upstream mechanisms contributing to actomyosin polarity within niche cells required for proper niche form and function. Importantly, GSCs are recruited to the niche during morphogenesis[27], and this period coincides with onset of GSC divisions[12]. Since mechanical forces can polarize MyoII[38,39], a potential source of forces on niche cells could be the cell packing or divisions that occur as GSCs are recruited and become adherent to niche cells.

A first hint that forces emanate from GSCs came from the behavior of the niche-GSC interface when laser-severing was carried out during early compaction, prior to MyoII polarization (Fig. 6). When tension along the

niche-GSC interface was severed, the germ cell protruded into the niche (Fig. 6A, A',B), suggesting exertion of positive pressure on the niche. Interestingly, this force appears exclusive to early compaction stages; germ cells did not protrude into niche cell space when interfaces were cut at late timepoints nearing the end of compaction (Fig. 6C, C', D). The timing of the force exerted by germ cells on the niche, and the eventual polarization of MyoII along the niche-GSC interface (Fig. 2A'', C), led us to hypothesize that germ cell force contributed to the polarization required for niche shape.

We therefore selectively ablated germ cells by expressing the pro-apoptosis gene, *head involution defective* (*Hid*), using Nanos-Gal4::VP16 (Fig. 7A-B). We found that upon Hid expression, gonads contained fewer germ cells (Fig.7E) (confirming ablation), and that loss of germ cells led to misshapen niches (Fig. 7B,F).

We suspect that the latent protrusive force exerted by germ cells (Fig. 6A, B) has various origins. One contribution might be from mitotic divisions[40]. To test this, we compromised germ cell divisions by lineage-specific knock down of the Cdc25 phosphatase required for G2-M progression[41]. Total germ cell number was reduced in Cdc25 RNAi gonads compared to controls (Fig. 7E), confirming divisions were inhibited. Similar to germ cell ablation, we observed defects in niche circularity (Fig. 7C,D, F). Neither niche area nor niche cell number changed upon GSC division inhibition or germ cell ablation (Fig. 7G, H), aligning with our findings from knocking down MyoII in the niche (Fig. 4J, K). Together, these data confirm that GSCs play a role in shaping their own niche and suggest that GSC divisions at least partially contribute to the mechanical force required to establish appropriate niche morphology.

GSC divisions aid in polarizing MyoII within the niche

We have established that MyoII polarity is essential to promote proper niche architecture. To assess whether germ cell divisions regulate niche MyoII polarity, we examined Cdc25 RNAi and sibling control gonads carrying MyoII RLC::GFP. In controls, MyoII was primarily enriched along the niche-GSC interfaces (Fig.7 I, I', K). However, in Cdc25 RNAi gonads, enrichment was significantly decreased (Fig. 7J, J', K). Notably, some niche cells appeared to polarize MyoII away from the niche-GSC boundary (Fig. 7J', arrowhead). Further, Cdc25 RNAi gonads exhibited an increase in centrosome mispositioning, suggesting that these niches have functional defects (Fig. 7L-N). Thus, GSC divisions are required to shape their niche by polarizing MyoII to the stem cell-niche interface, which is then critical for niche function.

Collectively, the effects we observed on niche shape and function by depleting germ cells or inhibiting their division strongly suggest that GSCs feedback to shape the niche that guides their behavior.

Discussion

Our work captures the dynamic formation of a functional niche. Prior gonad imaging revealed how pro-niche cells migrate to their proper position[12,30]. Here, we show that morphogenesis of the niche at its appropriate location is dependent on polarization of a force-producing actomyosin network along the niche boundary. If contractility is compromised, the niche adopts an irregular contour and consequently exhibits defects in function. Additionally, the recruited stem cells aid in polarizing MyoII along the forming niche-stem cell boundary. Without this feedback, niche shape and, consequently, niche function is aberrant.

Polarized contractility is required for niche morphogenesis

Cytoskeletal organization is crucial for compacting gonadal niche cells into their final, functional arrangement. Specifically, F-actin and MyoII localize towards, and induce tension along, the interface between niche cells and newly recruited GSCs during compaction. Compromising contractility yielded misshapen niches, reminiscent of the involvement of MyoII for curvature of the mammalian intestinal niche[7], the shape of intestinal stem cells[8], and morphogenesis of the neural stem cell rosette[42]. Other studies benefitted from combining multiple approaches, including whole-tissue knockouts, pharmacological manipulations, and/or *in vitro* culturing, but were limited in terms of cell-type specificity. Our study combines *ex vivo* live-imaging[29] with *in vivo* cell-type specificity. While our pharmacological manipulation does affect all cells, the asymmetry in MyoII contribution suggests that it is the contractile activity in the niche cell cortex that is a main driver in niche morphogenesis. This inference was strongly supported by the misshaping observed when MyoII was inhibited only in somatic cells.

While not currently possible with existing techniques[43], it would be interesting to investigate whether AMC in the GSC cortex also acts in shaping the niche. Indeed, our *ex vivo* experiments with ROKi support such a conclusion. Tissue-level inhibition of AMC induced more severe niche phenotypes than MyoII inhibition just within the soma (Fig. 4C,F,H). Of note, ROKi treatment *both* altered MyoII function in the niche *and* disrupted GSC mitotic divisions. Thus, we suspect the severe niche phenotype of ROKi treated gonads to be caused by a combinatorial effect of both GSC and niche cell involvement in niche morphogenesis.

Niche morphology regulates niche-stem cell signaling

Without proper niche shape, function is aberrant. Our data suggest that the precise contour of the niche is important for modulating its signaling capabilities. We previously reported that blocking niche assembly led to niche cells that signaled poorly, if at all[30]. By affecting compaction, we find significant STAT activation in nearby germ cells, but an increase in the number of those cells contacting the niche. It is possible that MyoII inhibition increases secretion of Upd from niche cells. However, because the pathway receptor polarizes along the niche-GSC interface within GSCs[44], we believe the increase in percentage of niche surface available to the germline likely accounts for the increase in STAT enriched germ cells touching the niche.

Work in intestinal organoids has also shown that curvature of the niche is important for optimizing signaling[8]. It is satisfying that precise niche architecture across different systems is important for tissue homeostasis, highlighting that concepts uncovered in *Drosophila* are applicable to mammalian models.

Centrosome and division orientation are dependent on a precise niche shape

Gonads with aberrant niche morphology exhibit defects in centrosome positioning and an increase in misoriented divisions. Under normal conditions, prior to GSC division, the mother centrosome anchors to the GSC cortex at the interface with the niche via influence from a cluster of proteins that become polarized to

this interface[21,25,26,44]. If centrosome anchoring fails, a "centrosome orientation checkpoint" (COC) is engaged, preventing spindle assembly to avoid aberrant division angles and excess symmetric divisions[22,23].

Prior research on centrosome orientation has exclusively identified and manipulated factors acting in the germline cortex[21,25,26,44]. Our work is the first to reveal a requirement on the niche side of this interface impacting centrosome positioning. An intriguing model is that polarized MyoII-dependent contractility in the niche is required to polarize potentially novel COC players to the niche cortex. Future work will identify the niche-derived factors that influence centrosome anchoring in GSCs.

GSCs feedback to shape their own niche by polarizing MyoII

Importantly, we show that germ cells are required for proper niche shape and function, as their depletion disrupts niche morphology. GSC divisions are specifically required for compaction, and blocking germline divisions causes reduction in MyoII polarization, aberrant niche shape, and defective niche function. We speculate that there is a mechanosensitive pathway between dividing GSCs and the adjacent niche. That pathway could be activated by forces generated by spindle microtubules on the cortex of the GSC[40]. It is also possible that a cell cycle-dependent change to GSC polarization causes the effect we document on niche cell polarity. In the future, force-producing microtubule motors must be manipulated to attribute the niche polarity effects to forces from GSC division.

A critical focus for future studies will be discerning how the niche is able to sense force from the germline to elicit a response in cytoskeletal polarity. Adherens junctions (AJs) are a potential candidate to play this role, as AJs control mechanosensation in other contexts[46,47], and are the main source of adhesion between GSCs and the niche[26,45]. However, we saw no defect in niche shape upon knockdown of E-cadherin (data not shown). Since AJs are comprised of multiple molecules[48], it is possible a combination of AJ components must be inhibited to see an effect on niche morphogenesis. Alternatively, it is possible that other mechanosensory mechanisms, such as mechanosensory ion channels[49] that have yet to be uncovered in the gonad niche, may be involved.

Additionally, division forces are likely not the only source of force production by the germline. Protrusion of a germ cell into the niche upon laser ablation of their cortical cytoskeleton strongly suggests that there is latent pressure from the germline toward the niche. Although we do not know the mechanistic basis for that pressure, the residual MyoII polarization when germline divisions are compromised supports the idea that additional forces generated by GSCs contribute to niche morphogenesis. Indeed, removing many of the germline cells by inducing cell death led to more compromised niche structure than inhibiting divisions (Fig. 7F, compare circularity changes).

Feedback between stem cells and their niche has been seen in other systems, such as the *C. elegans* hermaphroditic gonadal niche, which relies on germline divisions for proper placement[50]. Additionally, murine hair follicle stem cells proliferate and recruit immune cells when the niche is compromised to patch the breached barrier[51,52]. Lastly, neural stem cells within the *Drosophila* central nervous system, send signals to induce remodeling of the glial niche[53]. Therefore, our work further strengthens the paradigm that niche-stem cell relationships are bidirectional and identifies mechanisms of stem cell force-generation to regulate niche form and function, facilitating tissue homeostasis.

Limitations

Our lineage-specific manipulations show somatic requirement for contractility in niche compaction. The only niche cell driver available for these experimental timepoints, Six4-Gal4, is also expressed in the developing

cyst cells. Thus, determining whether cyst cells also impact niche shape in a MyoII-dependent manner is not yet possible. Cyst cells do make connections with the niche but much less extensively than germ cells (c.f. Fig. 2A'; the relative thinness of somatic-to-niche contacts is apparent) [17,33]. We therefore believe it less likely that cyst cells directly affect niche morphogenesis. Nevertheless, while GSCs make direct and extensive contact with niche cells, somatic cells encyst the remainder of the germline surface and could signal or provide force to the GSCs as they shape the niche.

Additionally, the adult testis niche secretes two other ligands, Hedgehog (Hh) and Bone Morphogenetic Protein (BMP), but a role for neither has been described during niche formation. Hh acts exclusively on CySCs, which are recruited later in larval stages[28,54]. In the adult, BMP signaling requires intimate contact between GSCs and niche cells[45,55,56]. While it would be interesting to determine how this pathway is affected by compaction, activation is only robust enough in later larval GSCs for analysis[57].

Lastly, we are curious as to whether mechanisms revealed in building this niche are conserved at steady state. Our preliminary work on the adult testis suggests enrichment for regulators of contractility in the niche. Therefore, approaches available to study the adult testis might provide answers to the questions listed above.

Figure Legends

Figure 1: Niche compaction is characterized by a change in niche shape and size

A-A''') 5-hour timelapse of Six4-Moe::GFP (green); His2Av::mRFP1 (magenta) gonad undergoing niche compaction *ex vivo*. The niche cortex and nuclei reveal organizational changes through time. A) 5 niche cells in view reveal an elongated niche at the anterior A') Niche cells have rearranged with their neighbors moving closer together. The niche contour is jagged. A'') 6 niche cells in view present a more rounded contour. A''') 7 niche cells in view are yet closer and present a more circular contour. B-C) Head on views of niches from fixed Six4-Moe::GFP, His2Av::mRFP1 ES16 (B) or late stage ES17 (C) gonads developed *in vivo.* B'-C') Niche marker, Fasciclin III. D-G) 3D analysis of niches developed *in vivo.* Niche circularity (D) increases post-compaction. Niche surface area (E), niche cell internuclear distance (F), and niche cell number (G), each decrease post-compaction. (** p <0.01, ****p<0.0001, Mann-Whitney). For graphs D, E, and G, each dot represents one gonad pre- (n=17) and post- (n=31) compaction. In F, each dot represents one niche cell pre- (n=277) or post- (n=435) compaction. Unless stated otherwise, for all figures: scale bars = 10 microns; images = single Z slice; yellow dashes outline entire gonad; white dashes outline niche.

Figure 2: F-actin and MyoII are enriched along the niche-GSC interface during compaction

A) Timelapse of Six4-Moe::GFP; MyoII::mCherry gonad undergoing niche compaction *ex vivo*. A') Somatic F-actin. A'') MyoII RLC. B-C) Quantifications of F-actin (B) and MyoII (C) along niche-GSC interfaces (magenta symbols) relative to non-niche somatic interfaces (blue symbols) at 0h, 2.5h, and 5h post-dissection. Each symbol represents normalized intensity along one interface (n=27 for both). D-G) Fixed and stained images of cell-type-specific F-actin or MyoII. D) Somatic F-actin (Six4-Gal4 > UAS-F-Tractin::TdTomato.) E) Somatic MyoII (Six4-Gal4::VP16 > MyoII HC::GFP). F) Germ cell F-actin (Nanos-Moesin::GFP). G) Germ cell MyoII (Nanos-Gal4::VP16 > MyoII HC::GFP). H-K) Quantification of polarization towards the niche-GSC interface (pink symbols) compared to niche-niche interfaces (blue symbols, H-I) or GSC-GSC interfaces (blue symbols, J-K). Equal numbers of niche-GSC interfaces were analyzed compared to the respective controls (H: n=57, I: n=52, J: n=57, K: n=79) (**p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney). Arrows = polarization towards niche-GSC interface. Arrowhead = polarization away from niche-GSC interface. Asterisk = niche.

Figure 3: AMC induces tension along the niche-GSC interface

A-C) Six4-Moe::GFP gonads developing *ex vivo.* Outlines highlight interfaces selected for laser ablation of the cytoskeleton pre-cut (A, B, C; red line = interface targeted) and 5s post-cut (A', B', C'). A''-C'') Montages representing timepoints pre-cut and at 5s intervals post-cut. Dashed lines show location of vertices over time. A) Niche-niche interface of untreated control gonad. B) Niche-GSC interface of control gonad. C) Niche-GSC interface of gonad treated with ROKi. D) Initial retraction velocity along niche-niche (blue, n=17) and niche-GSC (magenta, n=15) interfaces in controls. E) Initial retraction velocity along niche-GSC interfaces in untreated gonads (magenta; same as panel D) compared to ROKi treated gonads (black, n=11). (*p<0.05, *** p<0.001 Mann-Whitney). Scalebars for panels A''-C'' represent 1 micron.

Figure 4: AMC is required for niche morphogenesis

A-B) 5-hour timelapse of Six4-Moe::GFP; His2Av::mRFP1 gonads to visualize somatic F-actin (green) and nuclei (Magenta). Gonads were either untreated (A) or treated with ROKi during imaging (B). C-D) Niche circularity measurements at 0h and 5h of untreated (C, n=15 gonads; previously shown in ref.[12]) or ROKi treated gonads (D, n=15 gonads). Lines pair 0-5h measurements taken from the same niche. (**p<0.01, Wilcoxon test). E-H) Six4-moe::GFP, His2Av::mRFP1 control (E, G) or Six4-Gal4 > MyoII RNAi gonads (F = HC RNAi; H = RLC RNAi)

developed *in vivo.* E'-H') Niche cell marker, FasIII. I) Niche circularity is decreased when either MyoII HC or RLC is depleted (***p<0.001, ****p<0.0001, Mann-Whitney). J-K) Niche surface area (J) and niche cell number (K) show no difference between RNAi treatments and respective controls (Mann-Whitney). For graphs I-K, n=17 for MyoII HC Controls, MyoII HC RNAi, and MyoII RLC controls; n= 21 for MyoII RLC RNAi.

Figure 5: Proper niche shape is required to regulate GSC behavior

A-B) Control (A) or Six4-Gal4 > MyoII HC RNAi (B) gonads stained for Vasa (magenta) and FasIII (white). A'-B') Single channel for STAT. Asterisk = niche. Solid outlines = niche-adjacent germ cells ('GSC') enriched for STAT. Dotted outlines = posterior germ cells ('GC') exhibiting lower STAT. C) Quantification of STAT enrichment in GSCs under control (n= 87 GCs and 145 GSCs) and RNAi (n=108 GCs and 180 GSCs) conditions. D) Number of STAT+ germ cells contacting the niche is increased in MyoII HC RNAi gonads (n= 27 gonads) compared to controls (n=23 gonads). E) Percentage of niche area that contacts the germ line is increased in MyoII HC RNAi gonads. (*p<0.05, ***p<0.001, ****p<0.0001, Mann-Whitney). F-G) Control (F) and MyoII HC RNAi (G) gonads stained for gamma tubulin (green) to mark centrosomes, Vasa (magenta), and FasIII (white). F) A GSC (solid outline) with two centrosomes: one anchored at the niche (arrow), and one located opposite (arrowhead). G) A GSC (solid outline) with two centrosomes (arrowheads) that are both oriented away from the niche. H) Percentage of cells with mispositioned centrosomes doubled from 9.4 to 20.5% in MyoII HC RNAi gonads compared to controls (**p<0.01, Chi-Square). I-J) Live-images of Six4-Moe::GFP, His 2::RFP gonads to track nuclear division orientation of control (I) or ROKi treated (J) cells. I) Early anaphase nucleus (arrow). J) Prometaphase nucleus (arrow) I'-J') Late telophase nuclei of the respective cells from panels I and J (arrowheads). K) Distribution of division angles, binned in 10-degree increments, from untreated (blue symbols, n=75 nuclear divisions) or ROKi treated gonads (magenta symbols, n=52 nuclear divisions; ****p<0.001, KS Test).

Figure 6: Germ cells protrude into the niche during early compaction

A, C) *Ex vivo* images of Nanos-Moe::GFP gonads during early (A) or late compaction stages (C). Asterisk = niche. The contour of a niche-GSC interface was monitored before laser cut (pre-cut), during cut (red line, 0s), and 10 and 20s post-cut. A', C') Inset of analyzed cell. Yellow dashed line = the contour pre-cut; magenta = extent of protrusion post-cut, if any. A, A') Severing the niche-GSC interface during early compaction led to germ cell protrusion into the niche (10s and 20s). C, C') Severing the niche-GSC interface during late compaction revealed little to no protrusion. B, D) Quantifications of protrusion into the niche, where each line represents the same interface at early (0sec) and late (20sec) times after severing. Germ cells significantly protrude into the niche during early (B, n=8 cells), but not during late compaction (D, n=8 cells). (**p<0.01, Wilcoxon Test).

Figure 7: GSC divisions are required to shape their niche

A-B) Six4-Moe::GFP control (A) or Nanos-Gal4::VP16 > hid.Z gonad (B) developed *in vivo*, and labeled with F-actin (green), Vasa (magenta), and FasIII (A'-B'). C-D) Six4-Moe::GFP; His2AV::mRFP1 control gonad (C) or Nanos-Gal4::VP16 > Cdc25 RNAi gonad (D) developed *in vivo,* and labeled with F-actin (green), DNA (magenta) and FasIII (C'-D'). Panels A, C, and D are maximum projections of ~10 slices with 0.5-micron intervals. E) Total germ cell number is reduced by expressing hid.Z (n=19 gonads) or Cdc25 RNAi (n= 11 gonads) in the germline compared to respective controls (n=21 and 11, respectively). F) Niche circularity is decreased by germline expression of hid.Z or Cdc25 RNAi (*p<0.05, **p<0.01, Mann-Whitney). G-H) Niche surface area (G) and niche cell number (H) show no difference between manipulated conditions (n=18 hid.Z gonads and 21 Cdc25RNAi gonads) and respective controls (n= 20 and 22, respectively). I-J) Control and Cdc25 RNAi gonads expressing MyoII RLC::GFP and stained for Vasa (magenta) and FasIII (white). I'-J') Single channel MyoII RLC::GFP shows enrichment in controls towards niche-GSC interfaces (I', arrow), and decreased but residual polarity towards

the niche-GSC interface in Cdc25 RNAi gonads (J', arrow). MyoII is sometimes mis-polarized away from the niche-GSC interface (J', arrowhead). K) Quantifications show germline Cdc25 RNAi leads to decreased MyoII enrichment at the niche-GSC interface (n=54 control = interfaces, n=72 Cdc25 RNAi interfaces, ***$p<0.001$, Mann-Whitney). L) Percentage of cells with mispositioned centrosomes is increased from 8% in controls to 26% in Cdc25 RNAi gonads (**$p<0.01$, Chi-Square). M-N) Control (M) and Cdc25 RNAi (N) gonads used for centrosome analysis, stained for gamma tubulin (green), Vasa (magenta), and FasIII (white). M) A GSC (solid outline) with two centrosomes: one anchored at the niche (arrow), and one located opposite (arrowhead). N) A GSC (solid outline) with two centrosomes (arrowheads) both oriented away from the niche.

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STAR Methods

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen DiNardo (sdinardo@pennmedicine.upenn.edu).

Materials Availability
Six4-Gal4::VP16 *Drosophila* stocks will be provided upon request to the lead contact.

Data and Code Availability
All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Transgenic Fly stocks:
Gal4 Stocks
Nanos-Gal4::VP16 was used to restrict expression of UAS-responder transgenes to germline cells[58]. To restrict expression to somatic gonadal cells, we utilized Six4-Gal4 and Six4-Gal4::VP16 transgenic lines. For knockdown experiments, we utilized the previously described Six4-Gal4[12], aging embryos for 15-17 hours at 29 degrees C to increase knockdown efficiency, capitalizing on the inherent temperature sensitivity of full length Gal4. To label cytoskeletal proteins with cell-type specificity, we generated a stronger line where the Gal4 DNA binding domain was fused to a VP16 transactivation domain (Six4-Gal4::VP16).

To construct the Six4-Gal4::VP16 line, a previously identified enhancer from the third intron of Six4[59] was amplified from genomic DNA with primers listed in the Reagent Table, and cloned into the pENTR/D-Topo entry vector (Invitrogen; K240020). The enhancer was transferred into the destination vector pBPGal4.2::VP16Uw (ref.[60]; Addgene, # 26228 ; RRID:Addgene_26228) via an LR clonase reaction. Transgenic flies were generated by The Best Gene, using PhiC-31 integration into an attP host (RRID:BDSC_8622).

Fluorescent labeling
For cytoskeletal or nuclear labeling, we utilized multiple fluorescent transgenic constructs. Nuclear chromatin was labeled using a His2Av::mRFP1 transgene (FBtp0056035). Somatic F-actin was labeled by P{D-six4-egfp:moesin (here called Six4-Moe::GFP; ref.[61]) or Six4-Gal4 > UASp-F-Tractin::TdTomato (a recombinant of Six4-Gal4 and RRID:BDSC_58989), whereas Germ cell F-actin was labeled by Nanos-Moesin::GFP[62]. To visualize MyoII with lineage specificity, we utilized a UAS-GFP::Zipper construct[34] driven by either a somatic or germline Gal4. Ubiquitous MyoII was visualized using either sqh-Sqh::mCherry (ref.[31]; RRID:BDSC_99923; RRID:BDSC_59024) or sqh-Sqh::GFP3x[63].

Transgenic manipulation
The following transgenes expressing shRNAs were used in knock down experiments: MyoII HC (RRID:BDSC_65947), MyoII RLC (RRID:BDSC_33892), Cdc25 (RRID:BDSC_34831), and E-Cadherin (RRID:BDSC_32904). P(UAS-hid.Z)2 (RRID:BDSC_65403) was used to ablate germline cells. Knockdown of the MyoII heavy chain was confirmed by measuring fluorescence intensity of Sqh-Sqh::GFP3x along the niche-GSC interface normalized to unmanipulated GSC interfaces (see cytoskeletal polarity analysis section for more

details). Germ cell ablation or division inhibition were confirmed by quantifying the number of germ cells in manipulated gonads compared to sibling controls and analyzed via a Mann-Whitney test.

In MyoII HC RNAi experiments, some gonads were defective in niche assembly, as evidenced by multiple niche aggregates. This is likely because timing of the somatic Gal4 driver cannot be controlled as accurately as, for example, our pharmacological ROKi treatment. Gonads exhibiting niche assembly defects were excluded from analysis.

Fluorescent balancers
For all transgenic manipulation experiments, utilization of marked balancers allowed unambiguous identification of embryonic gonads that expressed both the Gal4 and UAS construct, as well as sibling controls that lacked either the Gal4 or the UAS construct, as identified by the presence of the appropriate fluorescent balancer (TM3, P{w[+mC]=Gal4-twi.G}2.3, P{UAS-2xEGFP} AH2.3, Sb[1], Ser[1], FBst0006663; TM6B, P{Dfd-GMR-nvYFP}4, Sb[1] Tb[1] ca[1], RRID:BDSC_23232; or CyO, P{w[BmC]=Dfd-EYFP.w[BmC]}2, RRID:BDSC_8623). For simplicity, we refer to the latter two balancers as DfdGFP.

Sex identification:
Male embryos, as well as gonads dissected from male embryos, were identified by the presence of male-specific, msSGPs, visualized by high-level expression of the Six4-Moe::GFP transgene, or by the accumulation of Vasa protein as previously described[12].

METHOD DETAILS

Fixed gonad preparation and staining:
Embryonic gonad dissections and Fixation
Unless otherwise noted, embryos were collected and aged in a humid 25 degree C chamber. Embryos were either aged 15-17 hours after egg lay (ES16) for pre-compaction niche analysis or 22-24 hours (late ES17) for post-compaction niche analysis. Embryos were dechorionated, staged based on gut morphology, hand-devitellinized and dissected in ~500uL of Ringers solution (5mM HEPES, pH 7.3; 130mM NaCl; 5mM KCl; 2mM $MgCl_2$; 2mM $CaCl_2$) as previously described [12]. Tissue was fixed in 4% formaldehyde, Buffer B (16.7mM KPO_4, pH 6.8; 75mM KCl; 25mM NaCl; 3.3mM $MgCl_2$), and 0.1% Triton-X-100 for 15 minutes, then washed in PBS (10mM Na_2 HPO_4; 1.8mM KH_2PO_4; 2.7mM KCl; 137mM NaCl; pH 7.4) plus 0.1% Triton-X-100 (PBS-Tx). Tissue was then blocked for 1 hour at room temperature in 4% normal donkey serum in PBSTx.

Immunostaining
Primary antibodies were used overnight at 4C. We used Goat antibody against Vasa 1:400 (Santa Cruz, dC-13, now discontinued); Rabbit antibody against STAT92E 1:350 (gift from E. Bach, NYU); and RFP 1:1000 (Abcam, ab62341); mouse antibody against Fasciclin III 1:50 (DSHB, 7G10); and Gamma Tubulin 1:200 (Sigma, GTU-88); Rat antibody against E-Cadherin (DSHB, DCAD2); and chick antibody against GFP 1:1000 (Aves Labs, GFP-1020). Secondary antibodies were used at 3-4ug/ml (Alexa488, Cy3, orAlexa647; Molecular Probes and Jackson ImmunoResearch) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2ug/ml for 5 minutes. Tissue was equilibrated overnight in 50% glycerol and 50% Ringers, then mounted with 2% propyl-gallate in 80% glycerol. Images of fixed gonads were acquired on a Zeiss Imager with Apotome using a 40x, 1.2 N.A. lens.

***Ex-vivo* experiments:**
Live-imaging
Dissection and live-imaging was performed as previously described[29]. Embryos were aged in a humid container in a 25-degree incubator for 14-17 hours after egg lay, and staged based on gut morphology to select stage 16

embryos. For analysis of gonads before and after compaction, we imaged for 5 hours. We only imaged gonads with niches that have been assembled at the gonad anterior but the contours of which have not yet become rounded. For some experiments, we selected gonads based on their stage within compaction (early, mid, or late) depending on the relative smoothness of the niche boundary. Imaging was carried out on gonads carrying either a somatic (Six4-Moe::GFP) or germline (Nanos-Moe::GFP) F-actin marker, and in some cases a MyoII marker (sqh-Sqh::mCherry). Gonads were imaged using an IX7 Olympus spinning disk confocal, using a 63x, NA 1.2 water immersion, or a 100x, NA 1.4, oil immersion lens, and captured with an EMCCD camera (Hamamatsu photonics, model C9100-13) controlled by MetaMorph software.

Laser ablation

Laser ablation experiments were carried out on dissected gonads carrying either a somatic (Six4-Moe::GFP) or germline (Nanos-Moe::GFP) marker. We prepared gonads for live-imaging as described above. However, we imaged gonads at two different stages of compaction: early or late. Identification of niches undergoing early compaction is described above. To analyze late-compaction stages, such as in figures 3 and 6, we again selected stage 16 embryos, but only those whose niches were both assembled and rounded.

To identify prospective interfaces to be targeted for ablation, a single time point z-stack was acquired for each gonad. Usually only one or two interfaces would be selected for treatment per gonad to limit any potential effects of global relaxation. The ablating beam generated by a MicroPoint laser emanating from a 405 nm dye cell was focused to the interface through a 100x, 1.4NA lens, using Andor IQ3.2 software. The micropoint laser settings were optimized at each session, selecting the minimum power required for junction severing. Simultaneously, Metamorph software was set to stream single color (488 excitation) images from the plane of the interface at 250 milliseconds intervals. Streaming acquisition was begun, and then the laser fired to treat that interface selectively. Post-treatment acquisition would continue for 1–3 minutes.

Rho Kinase Inhibitor treatment

For inhibitor treatments, a single timepoint z-stack was obtained for each gonad to establish a pretreatment standard. Then, the potent and selective Rho Kinase inhibitor, H-1152, was added to 10 µM final concentration with thorough but gentle pipetting (Santa Cruz, sc-203592; Ki = 1.6 nM for Rho Kinase compared with Ki = 140 nM for the inhibitor Y-27632). Time-lapse imaging was carried out for 5 hours, with inhibitor replenished every 90 minutes[64]. In experiments where laser ablation of the cortical cytoskeleton was carried out, the ablations were begun within 15-30 minutes after inhibitor addition.

QUANTIFICATION AND STATISTICAL ANALYSIS

Fixed Images were analyzed via the Image J Blind Analysis Tools plugin.

Cytoskeletal polarity analysis

Fluorescent gonads were dissected and immunostained either with an antibody against GFP or RFP, depending on the fluorescent transgene present. Niche interfaces were visualized by anti FasIII. To extract the fluorescence intensity of F-actin or MyoII, a 3-pixel wide line was drawn over the target niche-GSC interface and its mean grey value returned in Image J. In most cases, the control interfaces were, respectively, either niche-niche or GSC-GSC interfaces depending on whether niche cells or germ cells were being analyzed. While confirming MyoII knockdown, however, GSC-GSC interfaces were utilized for normalization, since these interfaces should not have been affected by MyoII knockdown. Mean gray values were background subtracted by drawing a line where no tissue was present, and niche-GSC interface values were normalized to an average of the respective control interfaces taken for that gonad. Normalized values were plotted, and niche-GSC intensity values were either compared between genotypes, or compared to control interfaces of the same genotype, using a Mann-Whitney test.

Niche phenotypic analysis
For analysis of niches from *ex vivo* cultured gonads, niche circularity was measured as described previously [12]. For fixed samples, three dimensional images were displayed using Imaris software. Niche area was measured by generating two surfaces in Imaris. The first surface was manually created by using FasIII and Six4-Moe::GFP as a guide to outline the niche on multiple z-planes. Creating this first surface is essential to isolate the niche from surrounding tissue. However, the manually drawn surface is a rough outline of the niche, and therefore needed to be refined to accurately recapitulate the curvature of the niche. To make a more refined surface, we made a mask of Six4-moe::GFP fluorescence from the first, rough surface. Using the masked Six4-moe::GFP fluorescence, we generated the second surface via Imaris's automatic surface generation protocol. We smoothed the surface with a 0.5-micron surface detail, and pixel-value thresholds were determined manually to ensure that the entire niche boundary was included in the surface. Niche surface area measurements were extracted from the second, refined surface for all samples by using the Imaris vantage function.
To measure the percentage of niche area that contacted germ cells, we additionally created a surface of all germ cells in the same way we made our niche surface (see above), but instead used Vasa as our marker to generate the surface. We used a surface-surface contact Xtension to generate the percentage of the niche surface that was contacting the germline surface.

Niche cell counts were extracted in Imaris using either Hoechst or His2Av::mRFP1 to visualize individual cell nuclei and FasIII to visualize cell outlines. Nuclei were also marked to extract internuclear distance between each cell, and generate the average nuclear distance between a niche cell and its 3 nearest neighbors. Nuclear distance between the 3 nearest neighbors were extracted from all samples using the Imaris vantage function.

Finally, the 3-dimensional image was rotated to orient the niche head-on, and a screenshot was captured to measure circularity using Image J. To measure circularity, an ROI was drawn using the freehand selection tool to trace the niche boundary labeled by Six4-moe::GFP, and circularity was extracted using the 'shape descriptors' tool.

Mann-Whitney tests were used to compare niche parameters between different genotypes. A Wilcoxon-test was used for paired analysis when measuring niche circularity of gonads live-imaged *ex vivo* at two separate timepoints.

Quantification of retraction velocity
After live-acquisition, images were imported into Image J, with the treated interface identified, oriented vertically, and the time stack cropped. A montage was then created that consisted of one pre-treatment frame, followed by 60 seconds worth of post-ablation frames, with each frame in the montage at 5 second intervals; analyses carried out at 1 second intervals generated the same results. Using the segmented line tool, the junction above and below the cut interface of the niche cell and germline cell was marked throughout the montage. The X-Y coordinates for the mapped vertices were exported to a spreadsheet. Peak retraction velocities were determined from vertical displacement over time, as presented in the respective scatter plots. Peak retraction velocities between treatment conditions or interfaces were compared via a Mann-Whitney test.

GSC protrusion quantification
In those cases where severing the cortical cytoskeleton led to pronounced invasion of niche cell territory by the germ cell, the extent of invasion was quantified in the Nanos-Moe::GFP background by measuring the length of the protrusion from its base by tracing the arcing germ cell outline along the protruded part and comparing that to the vertical distance at the base of that protrusion. In this manner, no or little protrusion

would yield a value close to one, while protrusions would yield values greater than one. Level of protrusion was compared between GSCs at early and late compaction timepoints with a Wilcoxon test.

Quantification of STAT accumulation

STAT intensity was extracted in Image J from anti STAT-stained gonads using regions of interest (ROI) drawn to include the Vasa signal of germline cells. In Figure 5C, for each gonad, we sampled 5 GSCs defined as those germ cells in contact with the niche, and 3 posterior germ cells located more than one cell diameter from the niche. We background-subtracted the average fluorescence intensity from each GSC and GC by drawing an ROI directly outside of the gonad. Relative STAT enrichment values were obtained by dividing the background-subtracted value of each GSC or GC by an average of the 3 background-subtracted GC values from that particular gonad. For Figure 5D, STAT positive cells were quantified by measuring the normalized fluorescence intensity of each germ cell directly contacting the niche as described. To establish a threshold over which a cell would be scored as STAT-positive, we took the average STAT enrichment (2.29 fold) and standard deviation (0.85) of all control cases. We then counted a cell touching the niche as positive if STAT levels were greater than 1.44-fold enriched (average enrichment minus 1 standard deviation). Data was obtained from sibling control and MyoII HC RNAi conditions and analyzed with a Mann-Whitney test.

Centrosome orientation analysis

Centrosomes were visualized with immunofluorescence against Gamma tubulin. GSCs contacting the niche were only scored for centrosome position if they had 2 clearly discernible centrosomes, and therefore had undergone centrosome duplication. For each such GSC, we scored whether one of the two centrosomes was located at the niche, as evidenced by cortical localization (visualized with Vasa) and proximity to the niche (visualized with FasIII). GSCs with one centrosome located along the niche-GSC interface were scored as "anchored," whereas GSCs with neither centrosome located at the niche-GSC interface were scored as "unanchored." Data was analyzed via Chi-squared analysis.

GSC division angle analysis

We surmised that the spatial constraints imposed by the spheroidal gonad might influence the possible division angles compared to that reported for the adult testis. That caution appeared justified, as using the rigorous 3-dimensional analysis described next, Figure 5K indeed showed that the distribution of angles was slightly broader than expected from previously published 2-dimensional centrosome and spindle analyses[21,25,65]. To preserve the 3-dimensional nature of the division coordinates, we used the model from ref.[37]. Therefore, we calculated division angles from live-imaged Six4-Moe::GFP;His2AV::mRFP1 gonads, based on two parameters as described by ref[37]: 1) a plane of best-fit to represent the niche interface, and 2) the division trajectory. First, a plane representing the niche-GSC interface was approximated as follows. The plane was highlighted by Six4-Moe::GFP, and five points were chosen along the interface confronting a dividing GSC (visualized by His2Av::mRFP1 and identified by condensed chromosomes), with points taken from two or more z-steps. The extracted x, y, z values were used as input in orthogonal distance regression to derive a best-fit plane. Second, for dividing GSCs, we extracted two sets of x, y, z coordinates to represent the division trajectory for anaphase, telophase, or metaphase nuclei. The coordinates were taken by marking the vertex of each chromosome complement, or for a metaphase figure, by marking a point on either side of the chromosome complex located midway on its long axis. The division angle was calculated as the angle between the best-fit plane and this inferred division trajectory. In all cases, division angle distribution between controls and ROKi-treated gonads were compared via a KS-test.

We also tested if control or ROKi divison angle distributions deviated from what might be expected as random. Note that from the perspective of an external reference plane, such as the niche-GSC interface, for a spherical cell, not all division angles are equally likely in a random model. If spindle position is not regulated in the cell, the distribution of spindles will exhibit a bias toward shallow angles, as shown in Supplementary Figure 2E, F,

and as described in ref[37]. This is because the probability of orientation falling within a particular range is dependent on the fraction of the inside cortical surface available for spindle attachment. There is much more surface area to attach to away from the reference interface. Hence, more representation of lower division angles. We therefore calculated the probability of cells dividing within 10-degree intervals from 0-90 degrees, following the equation from ref[37]. We then graphed the actual percentage of control or ROKi cells that divided within these intervals compared to the expected percentages of a random distribution.

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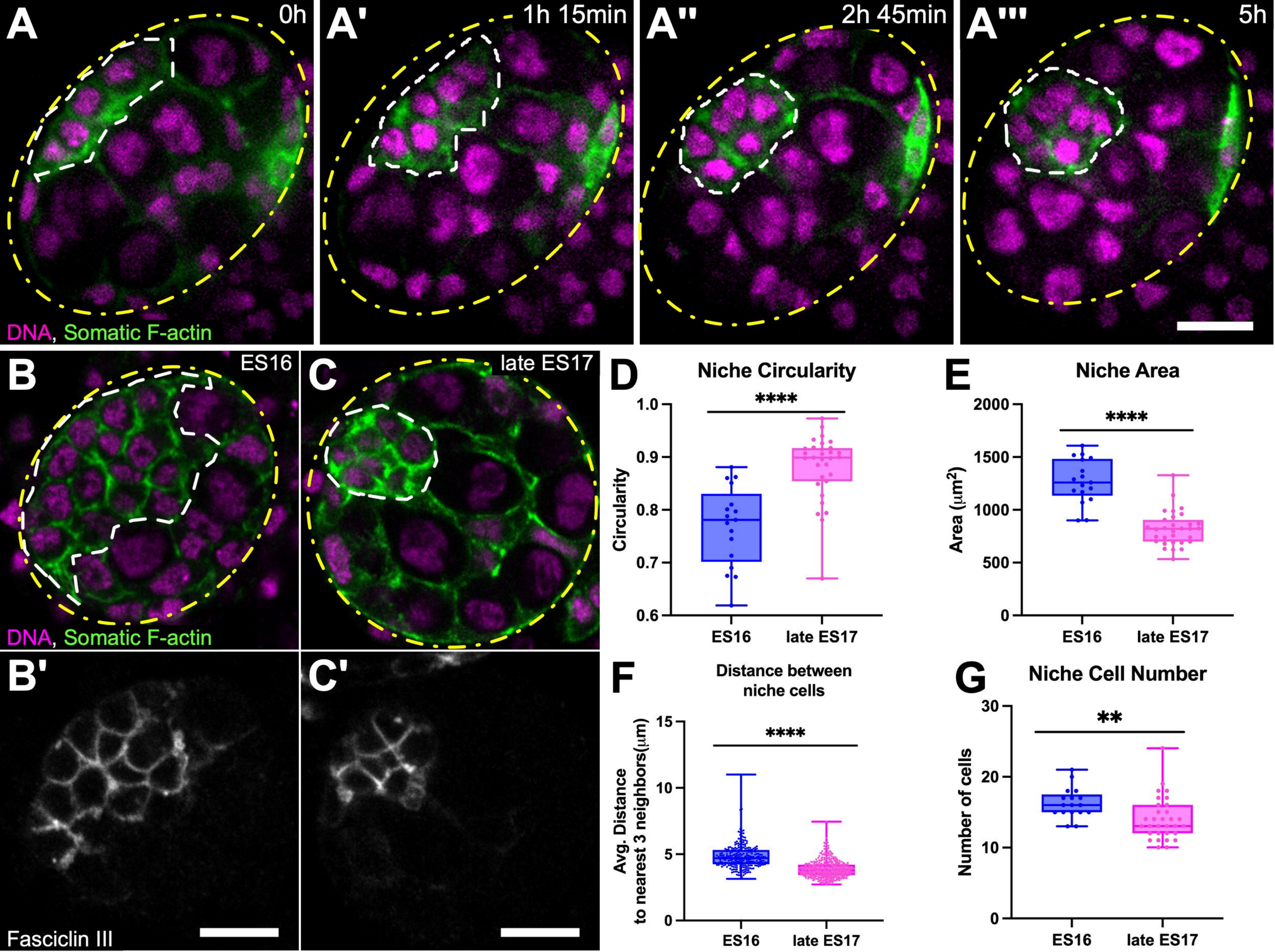
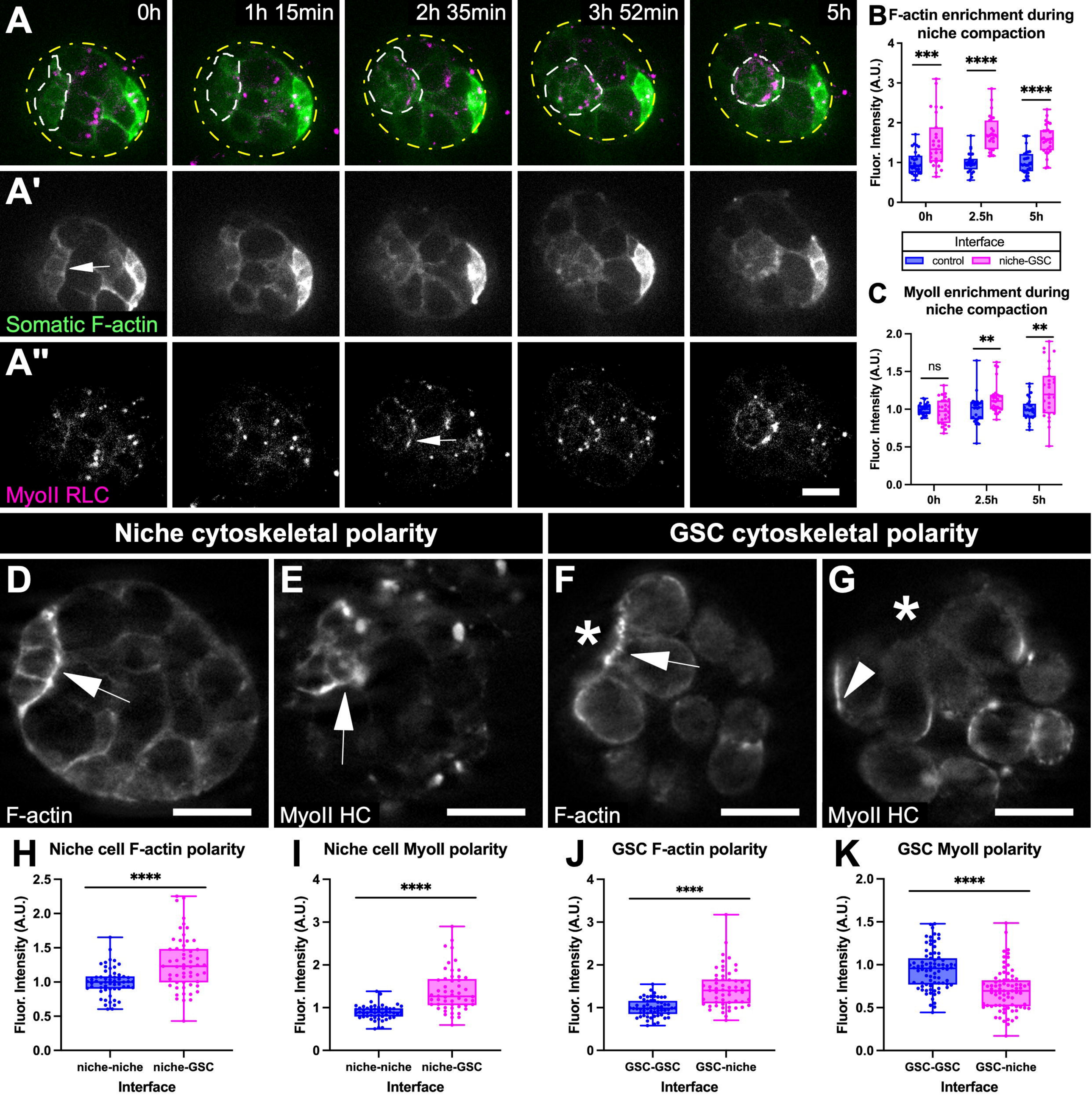
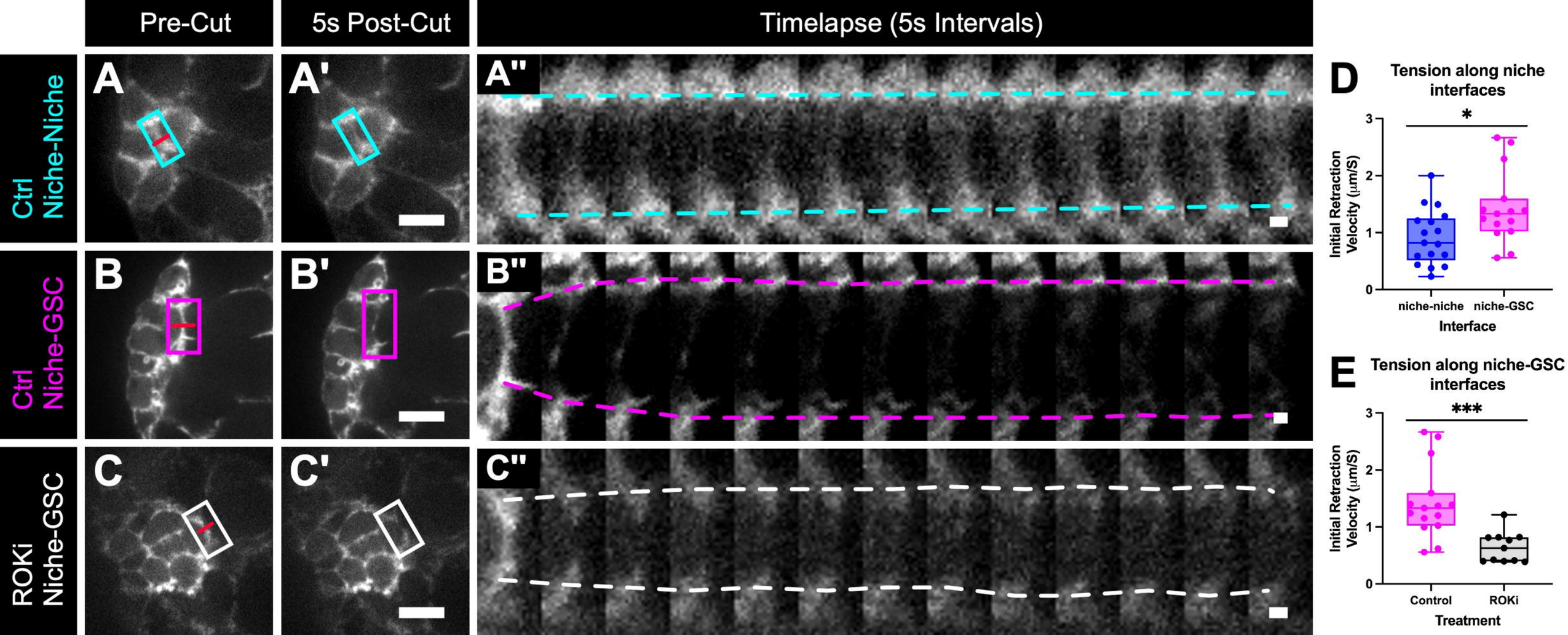
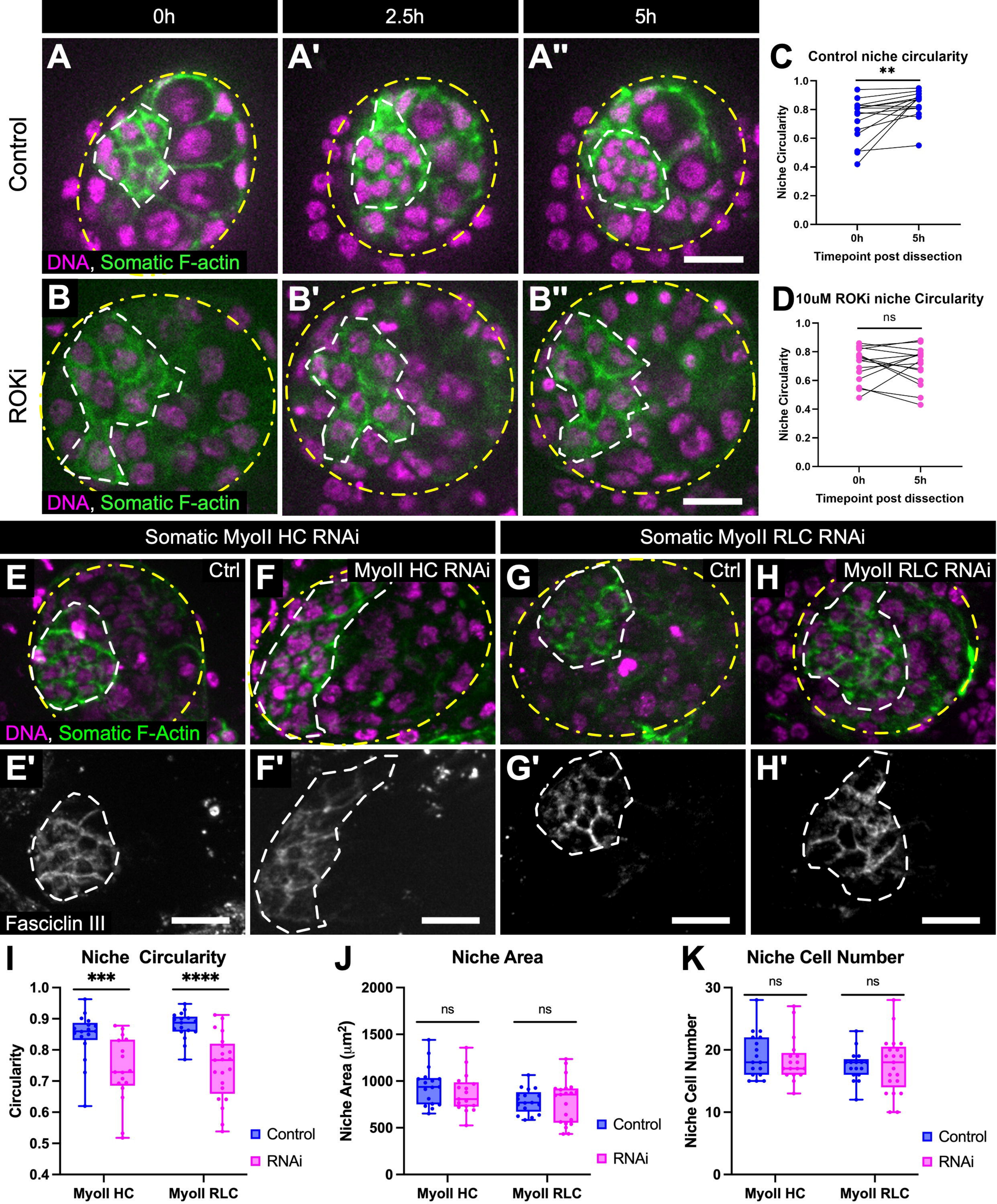
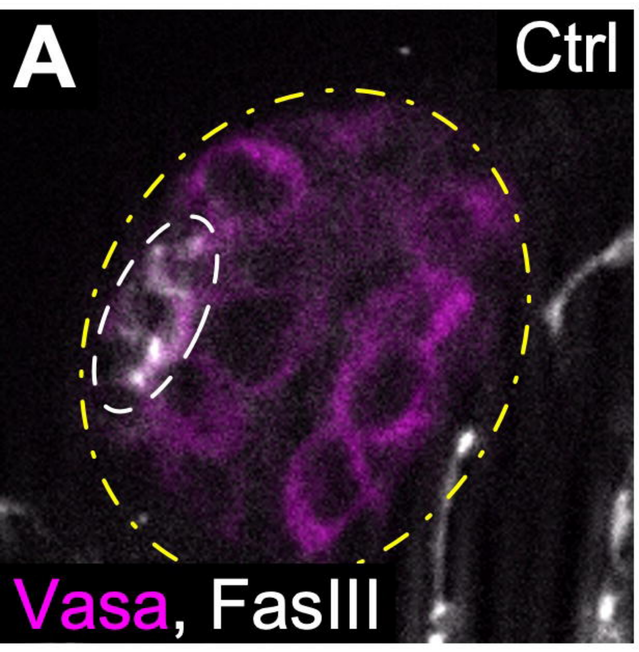

A
0h
A'
1h 15min
A''
2h 45min
A'''
5h
DNA, Somatic F-actin
B
ES16
C
late ES17
DNA, Somatic F-actin
B'
C'
Fasciclin III
D
Niche Circularity
****
Circularity
1.0
0.9
0.8
0.7
0.6
ES16
late ES17
E
Niche Area
****
Area (μm²)
2000
1500
1000
500
0
ES16
late ES17
F
Distance between niche cells
****
Avg. Distance to nearest 3 neighbors(μm)
15
10
5
0
ES16
late ES17
G
Niche Cell Number
**
Number of cells
30
20
10
0
ES16
late ES17

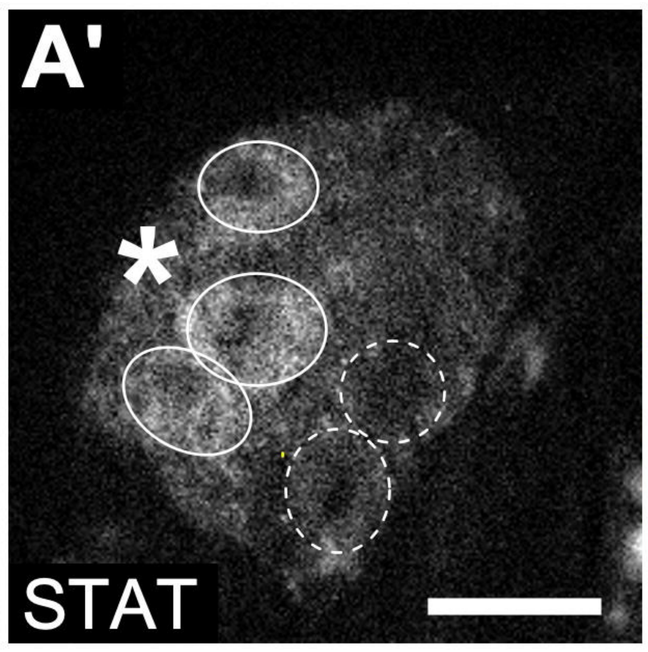

A
0h
1h 15min
2h 35min
3h 52min
5h
A'
Somatic F-actin
A''
MyoII RLC
B F-actin enrichment during niche compaction
Fluor. Intensity (A.U.)
***
****
****
0h
2.5h
5h
Interface
control
niche-GSC
C MyoII enrichment during niche compaction
Fluor. Intensity (A.U.)
ns
**
**
0h
2.5h
5h
Niche cytoskeletal polarity
GSC cytoskeletal polarity
D
F-actin
E
MyoII HC
F
F-actin
G
MyoII HC
H Niche cell F-actin polarity
Fluor. Intensity (A.U.)
****
niche-niche
niche-GSC
Interface
I Niche cell MyoII polarity
Fluor. Intensity (A.U.)
****
niche-niche
niche-GSC
Interface
J GSC F-actin polarity
Fluor. Intensity (A.U.)
****
GSC-GSC
GSC-niche
Interface
K GSC MyoII polarity
Fluor. Intensity (A.U.)
****
GSC-GSC
GSC-niche
Interface

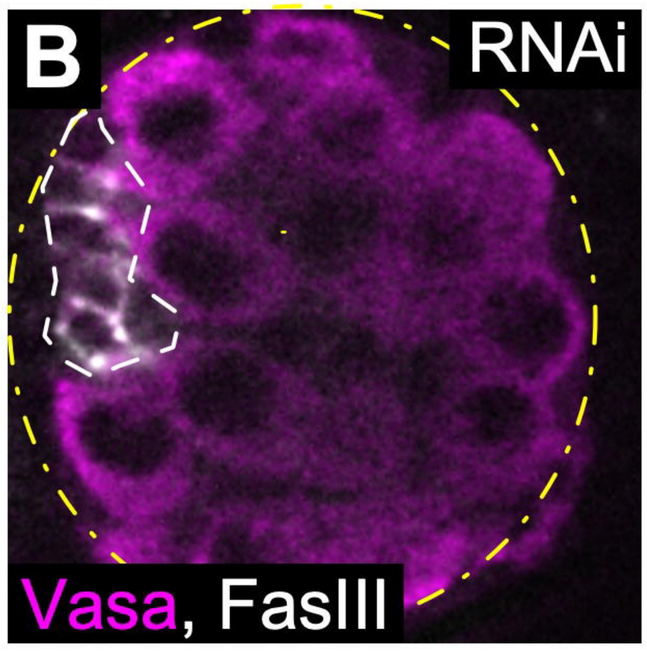

Pre-Cut
5s Post-Cut
Timelapse (5s Intervals)
Ctrl
Niche-Niche
Ctrl
Niche-GSC
ROKi
Niche-GSC
A
A'
A''
B
B'
B''
C
C'
C''
D
Tension along niche interfaces
*
Initial Retraction Velocity (μm/S)
0
1
2
3
niche-niche
niche-GSC
Interface
E
Tension along niche-GSC interfaces
***
Initial Retraction Velocity (μm/S)
0
1
2
3
Control
ROKi
Treatment

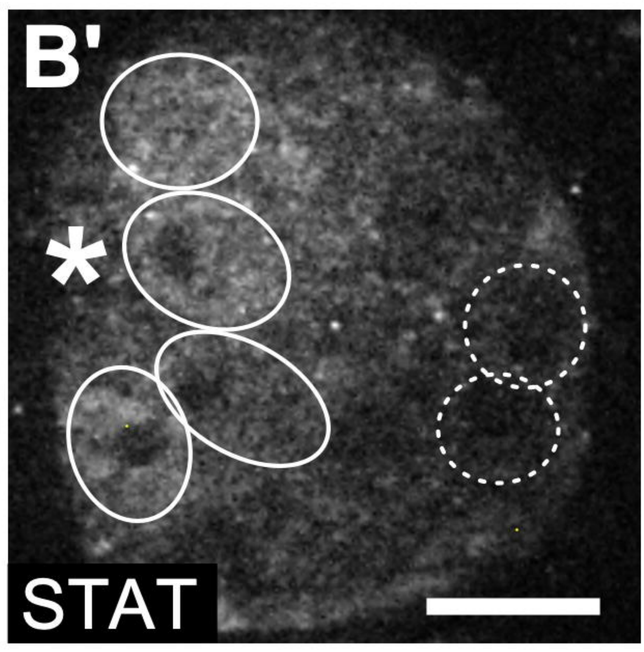

0h
2.5h
5h
Control
ROKi
A
A'
A"
B
B'
B"
DNA, Somatic F-actin
C
Control niche circularity
**
Niche Circularity
Timepoint post dissection
D
10uM ROKi niche Circularity
ns
Somatic MyoII HC RNAi
Somatic MyoII RLC RNAi
E
Ctrl
F
MyoII HC RNAi
G
Ctrl
H
MyoII RLC RNAi
DNA, Somatic F-Actin
E'
F'
G'
H'
Fasciclin III
I
Niche Circularity
***
****
Circularity
Control
RNAi
MyoII HC
MyoII RLC
J
Niche Area
ns
Niche Area (μm²)
K
Niche Cell Number
Niche Cell Number

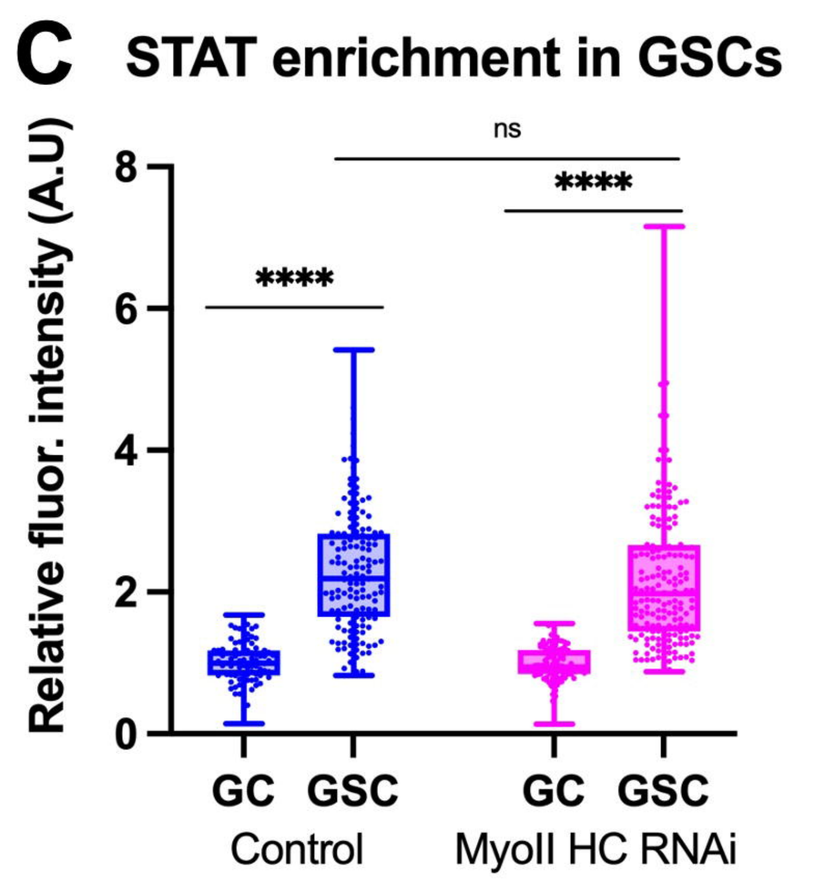
Somatic MyoII HC RNAi

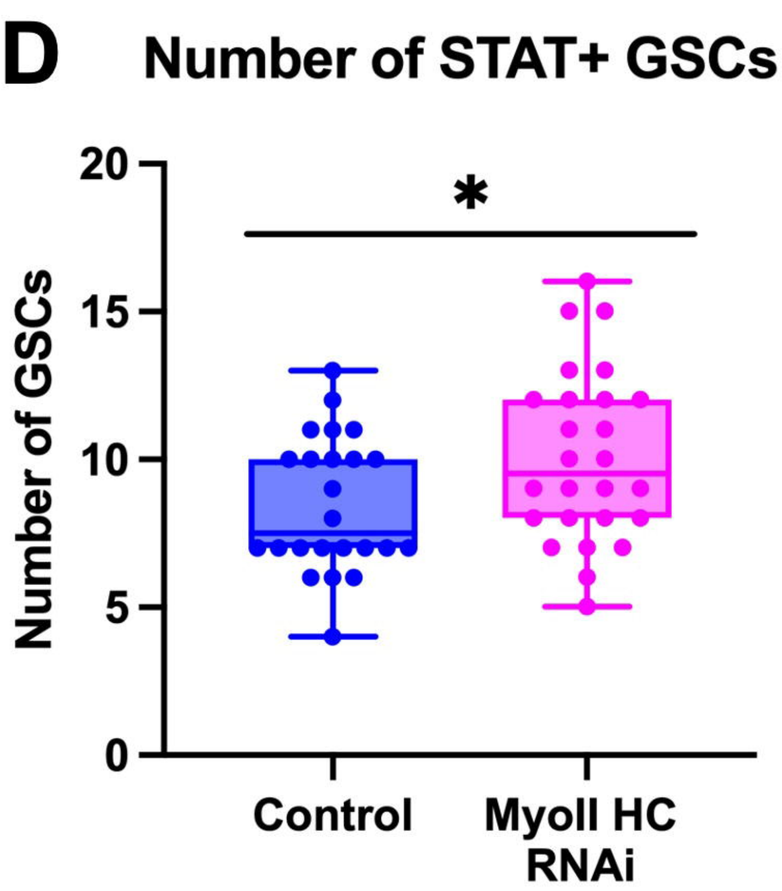


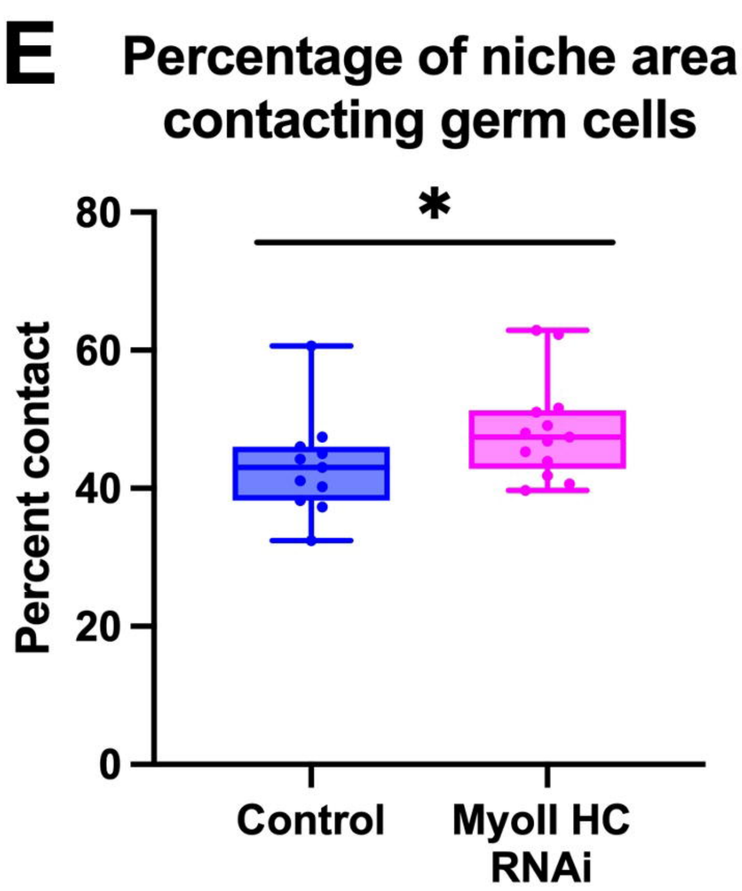




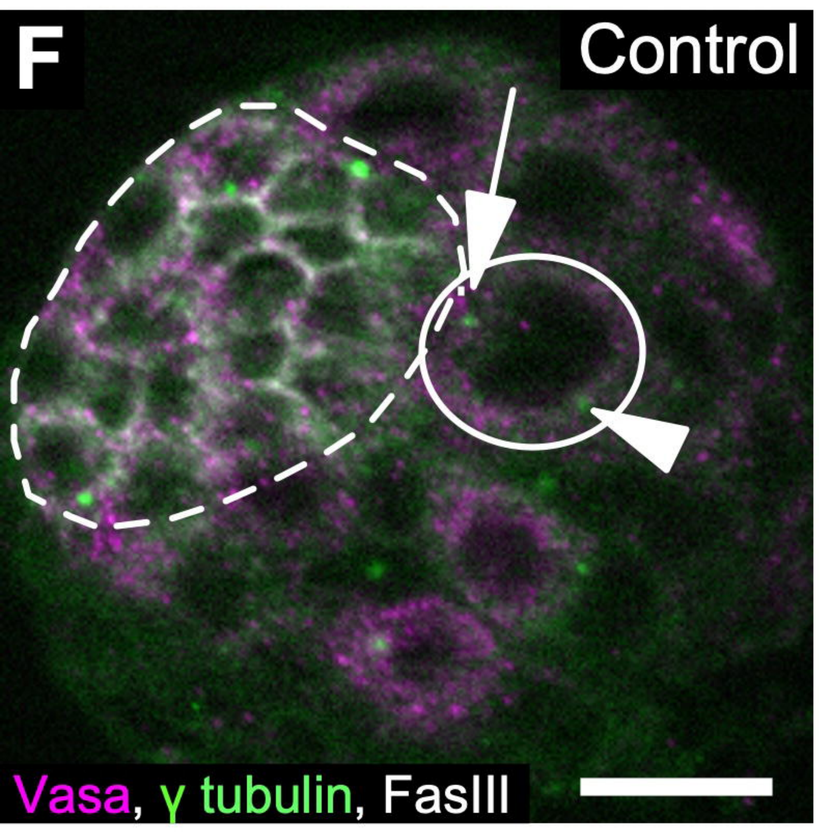


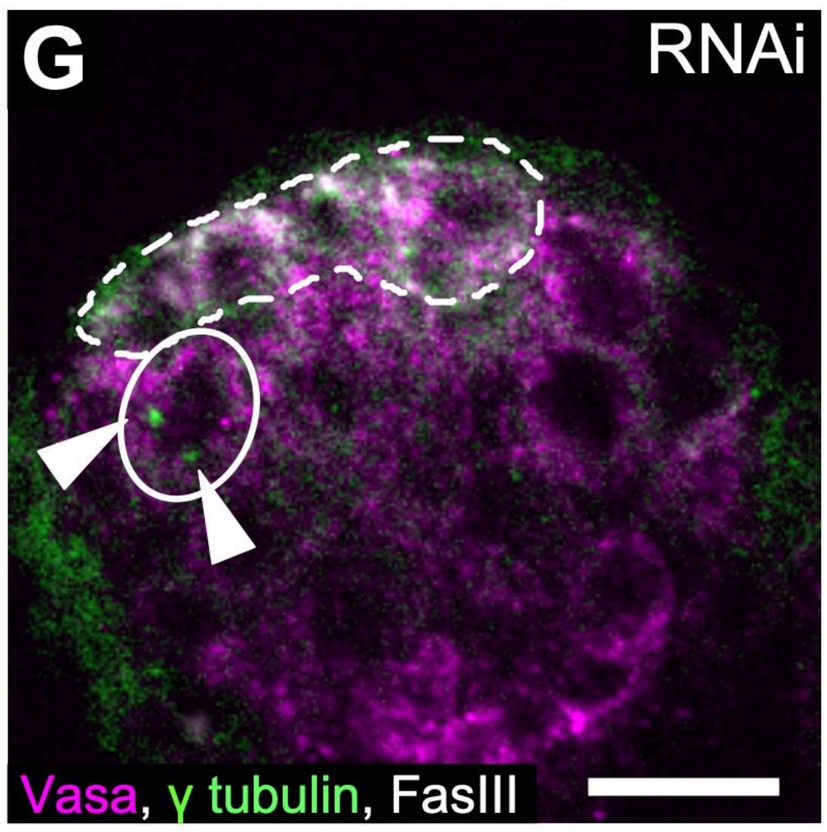


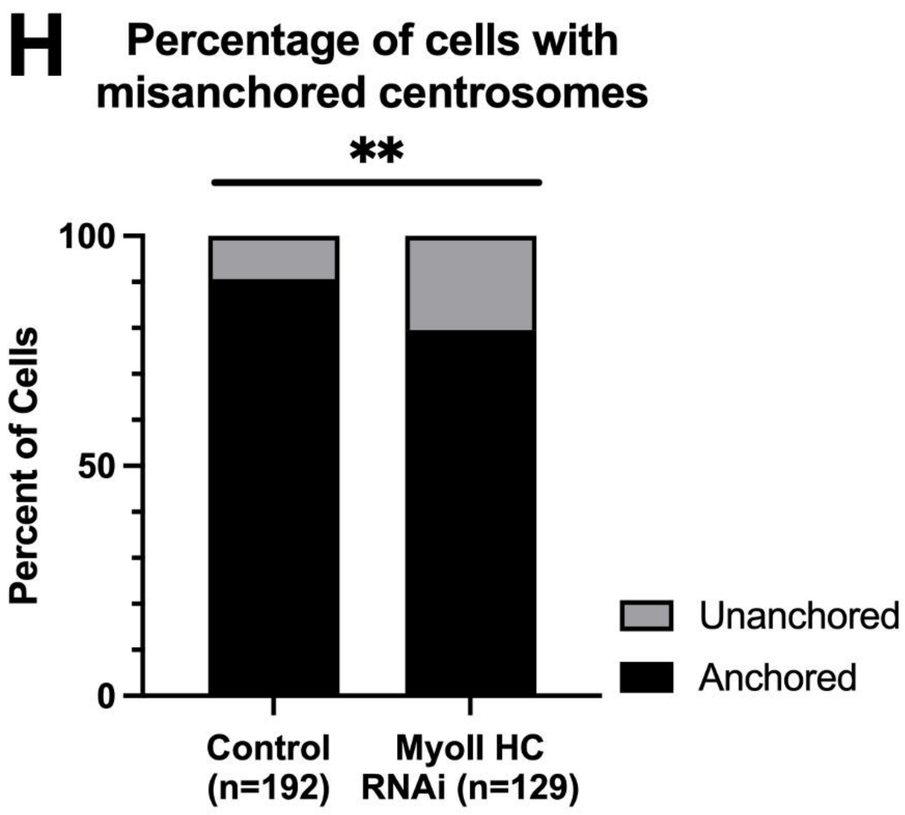


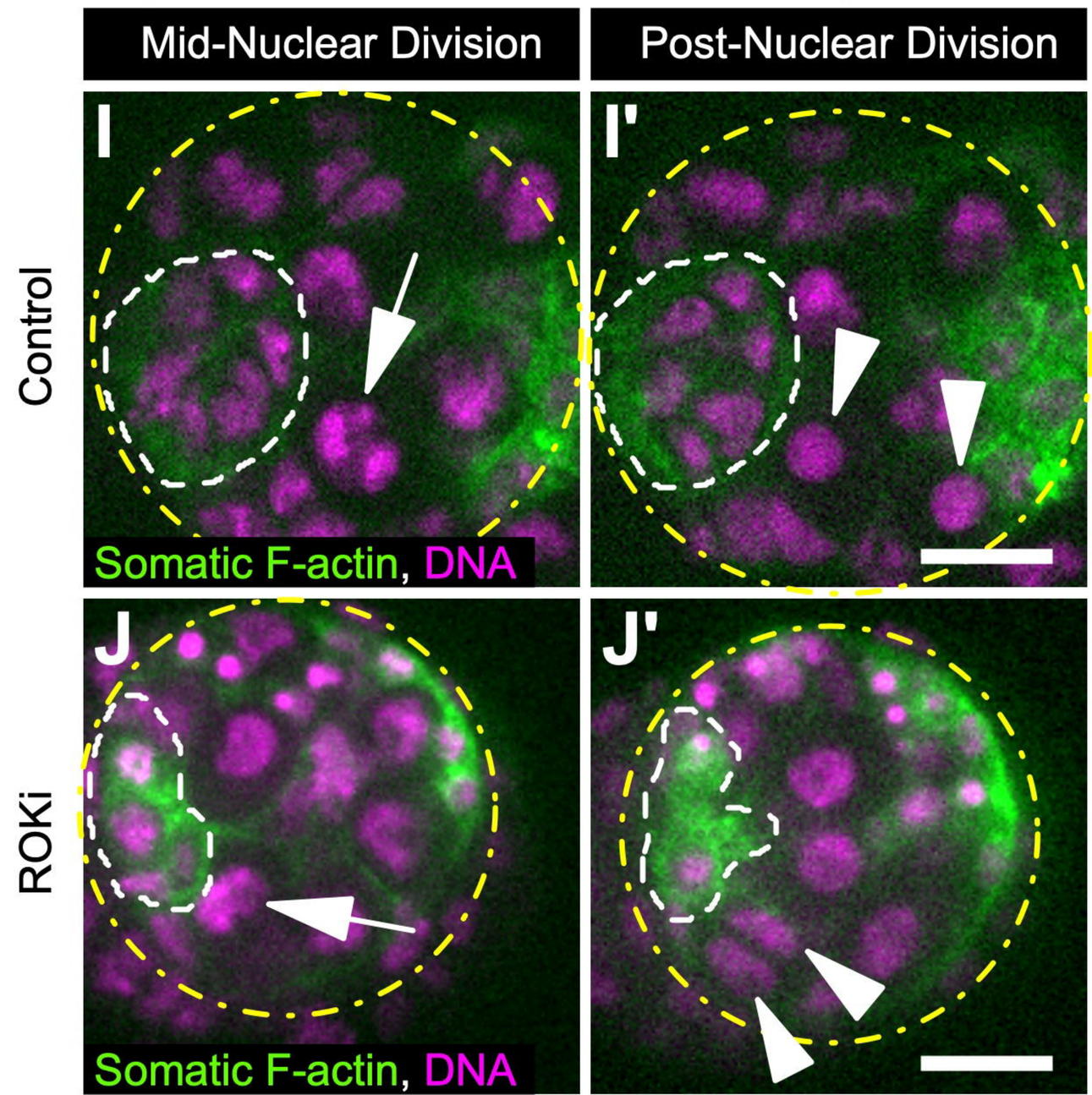


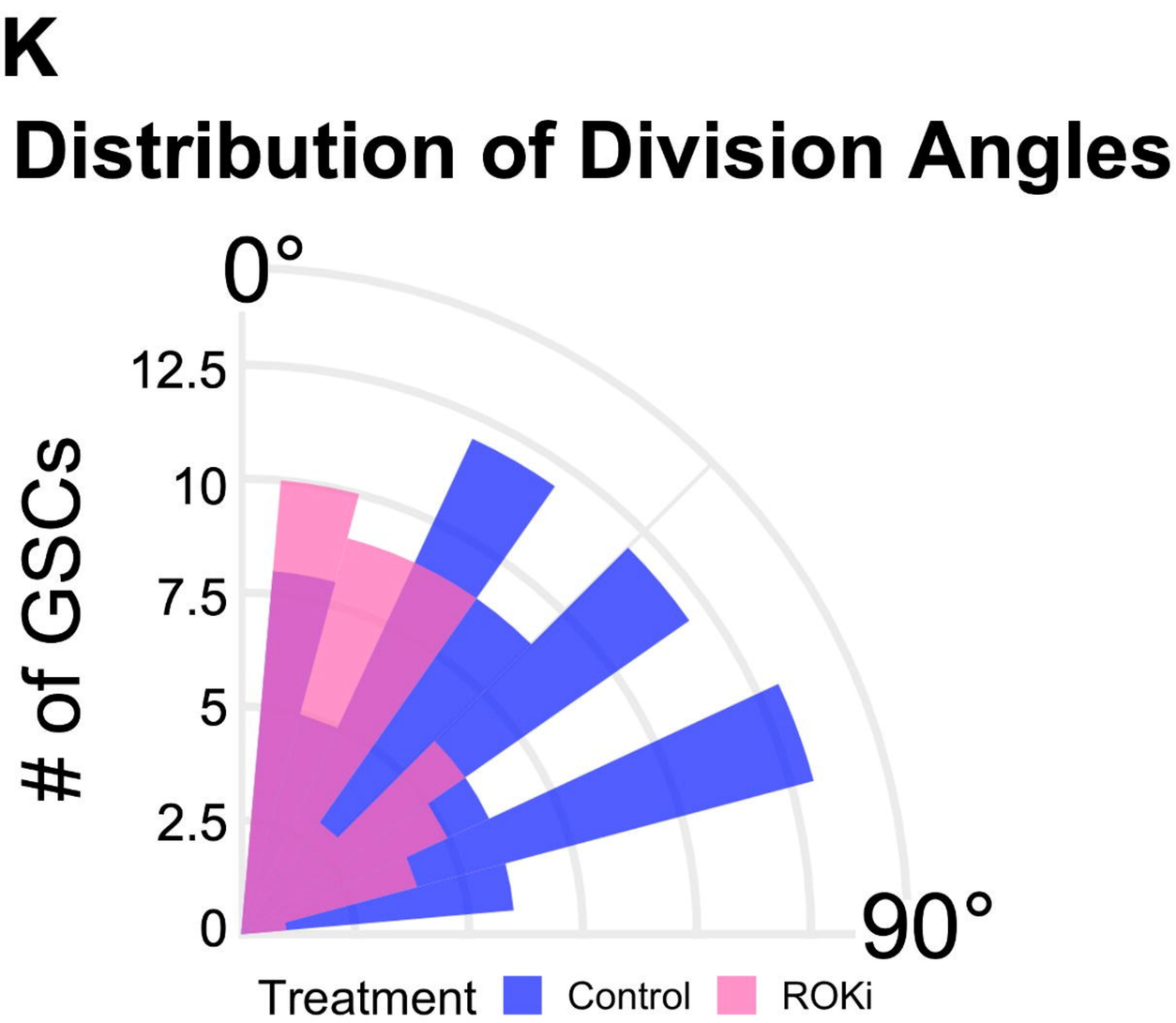
Somatic MyoII HC RNAi

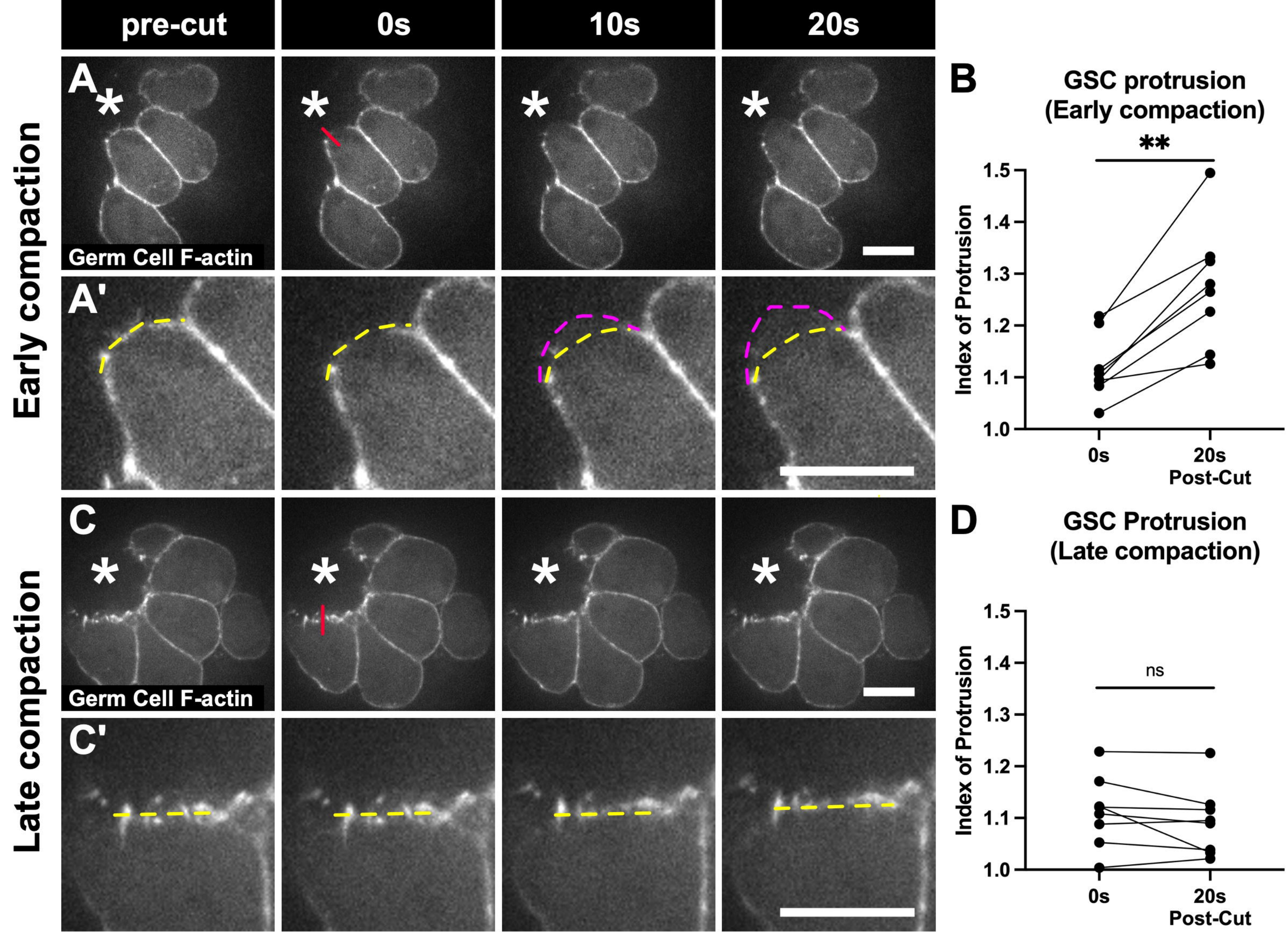











pre-cut
0s
10s
20s
Early compaction
A
Germ Cell F-actin
A'
Late compaction
C
Germ Cell F-actin
C'
B
GSC protrusion
(Early compaction)
**
Index of Protrusion
1.5
1.4
1.3
1.2
1.1
1.0
0s
20s
Post-Cut
D
GSC Protrusion
(Late compaction)
ns
Index of Protrusion
1.5
1.4
1.3
1.2
1.1
1.0
0s
20s
Post-Cut

Germline Hid.Z

Germline Cdc25 RNAi

A Ctrl

Vasa, somatic F-actin

B Hid.Z

Vasa, somatic F-actin

C Ctrl

DNA, somatic F-actin

D Cdc25 RNAi

DNA, somatic F-actin

A'

FasIII

B'

FasIII

C'

FasIII

D'

FasIII

E Number of germ cells

Number of cells

50 40 30 20 10 0

*

*

control hid.Z control Cdc25 RNAi

F Niche Circularity

Circularity

1.0 0.9 0.8 0.7 0.6 0.5

**

*

control hid.Z control Cdc25 RNAi

G Niche Area

Niche Area (μm²)

1500 1000 500 0

ns

ns

Control hid.Z control Cdc25 RNAi

H Number of niche cells

Number of cells

30 20 10 0

ns

ns

control hid.Z control Cdc25 RNAi

Germline Cdc25 RNAi

I Ctrl

Vasa, FasIII, MyoII RLC

I' Cdc25 RNAi

Vasa, FasIII, MyoII RLC

K MyoII enrichment along niche-GSC interfaces

niche-GSC/niche-niche fluor. intensity ratio

6 4 2 0

***

Control Cdc25 RNAi

M Cdc25 RNAi

Vasa, γ tubulin, FasIII

J

MyoII RLC

J'

MyoII RLC

L Percentage of cells with misanchored centrosomes

Percent of Cells

100 50 0

**

Control (n=63) Cdc25 RNAi (n=82)

Unanchored

Anchored

N Ctrl

Vasa, γ tubulin, FasIII