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

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Summary

remains elusive is how niches are formed, and how morphology impacts function. To address this, we let the *Drosophila* gonadal niche, which affords genetic tractability and live-imaging. We have previously she mechanisms 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
cons the Drosophila gonadal incite, which affords genetic tractability and the imaging. We have previously shown
mechanisms dictating niche cell migration to their appropriate position within the gonad, and the resultant
conseq consequences on niche function. Here, we show that once positioned, niche cells robustly polarize filament
actin (F-actin) and Non-muscle Myosin II (MyoII) towards neighboring germ cells. Actomyosin tension along
the niche 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 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 ge misshapen and exhibit defects in their ability to regulate germline stem cell behavior. We additionally show
that germ cells aid in polarizing Myoll within niche cells, and that extrinsic input is required for niche
morpho that germ cells aid in polarizing Myoll 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 tha
guides their morphogenesis and function. Our work reveals a feedback mechanism where stem cells shape the nic
guides their behavior.
Keywords
Drosophila, Stem cell, testis, niche, feedback, actomyosin contractility, morphogenesis guides their behavior.
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Keywords

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Drosophila, Stem cell, $\frac{1}{2}$ Drosophila, Stem cell, testis, niche, feedback, actomyosin contractility, morphogenesis Drosophila, Stem cell, testis, niche, feedback, actomyosin contractility, morphogenesis

Introduction

differentiation. Imbalances in renewal or differentiation can induce tissue degeneration or tumor formation^{1,2}, so it is vital to understand mechanisms of niche function. Niches often have a precise morphology³⁻⁶, but

so it is vital to understand mechanisms of niche function. Niches often have a precise morphology², but only recently has attention turned to how a niche is shaped and how shape contributes to function^{7–9}. Recent work
 recently has attention turned to how a niche is shaped and how shape contributes to function' ". Recent work
often relied on whole-tissue manipulation or organoid models. Therefore, it is necessary to establish models
to i to interrogate niche morphogenesis with cell-type specificity and in developing tissue.
We have leveraged the genetically tractable *Drosophila* testis, as the niche and associated stem cells are well
defined¹⁰. This nic The have leveraged the genetically tractable *Drosophila* testis, as the niche and associant defined¹⁰. This niche is anchored at the testis apex¹¹, and is comprised of quiescent so a reproducible sphere¹²⁻¹⁴. This n We have leveraged the genetically tractable Drosophia testis, as the methe and associated stem cells are well
defined¹⁰. This niche is anchored at the testis apex¹¹, and is comprised of quiescent somatic cells organize defined⁴⁰. This niche is anchored at the testis apex¹⁴, and is comprised of quiescent somatic cells organized in
a reproducible sphere^{12–14}. This niche supports two stem cell lineages^{10,15}: germline stem cells (GSC

a reproducible sphere^{42–44}. This niche supports two stem cell lineages^{40,23}: germline stem cells (GSCs), which
produce differentiating germline cells, and Cyst stem cells (CySCs), which produce somatic cells supportin produce the chemokine displaces of stem cell behavior to maintain homeostasis. For example, the niche
secretes the chemokine Unpaired (Upd) and activates the Jak-STAT pathway in adjacent cells, ¹⁸ directly
promoting CyS secretes the chemokine Unpaired (Upd) and activates the Jak-STAT pathway in adjacent cells,¹⁸ directly
promoting CySC renewal and indirectly promoting GSC renewal^{19,20}. The niche also regulates GSC division
orientation

promoting CySC renewal and indirectly promoting GSC renewal^{22,20}. The niche also regulates GSC division orientation to ensure one daughter remains accessible to renewal signals, while the other is displaced awa
from the 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 embryonic gonadogenesis, t establishment of a functional niche. Early in embryonic gonadogenesis, the Jak-STAT pathway is active in al
primordial germ cells (PGCs) and somatic gonadal precursors (SGPs)^{27,28}. Later, the niche forms at the gona
ant primordial germ cells (PGCs) and somatic gonadal precursors (SGPs)^{27,28}. Later, the niche forms at the gonad
anterior^{12,13}, where it selectively contacts and restricts signaling to a subset of cells. Only these cells primordial germ cells (PGCs) and somatic gonadal precursors (SGPs)^{27,20}. Later, the niche forms at the gonad
anterior^{12,13}, where it selectively contacts and restricts signaling to a subset of cells. Only these cells g

anterior^{12,12}, where it selectively contacts and restricts signaling to a subset of cells. Only these cells gain stem-
like properties, with PGCs becoming GSCs, and later, SGPs becoming CySCs^{27,28}. Because critical nic like properties, with PGCs becoming GSCs, and later, SGPs becoming CySCs^{27,22}. Because critical niche-stem cell
connections become defined concomitantly with niche morphogenesis, it begs the question of how niche
shape a shape affects its ability to define and regulate the stem cell pool.

In a major advance, we developed techniques to capture niche and stem cell behavior through live-imagin

during this previously elusive period of gonad In a major advance, we developed techniques to capture niche are during this previously elusive period of gonadogenesis²⁹. This, tog dynamic processes during niche formation: 1) assembly and 2) co cells migrate towards during this previously elusive period of gonadogenesis²⁹. This, together with fixed analyses, has defined two
dynamic processes during niche formation: 1) assembly and 2) compaction^{12,13}. During assembly, pro-niche
cel dynamic processes during niche formation: 1) assembly and 2) compaction^{-12,2}. During assembly, pro-niche
cells migrate towards the gonad anterior. Next, during compaction, niche cells reorganize to present a
smoothened b

smoothened boundary to the surrounding cells. We have previously shown that errors in assembly lead
defects in niche polarity, quiescence, and function³⁰. The requirement for compaction in niche function
not been explore defects in niche polarity, quiescence, and function³⁰. The requirement for compaction in niche function has
not been explored.
Here, we identify a role for polarized actomyosin contractility (AMC) in establishing the fin defects in niche polarity, quiescence, and function³⁰. The requirement for compaction in niche function has not been explored.
Here, we identify a role for polarized actomyosin contractility (AMC) in establishing the fin Here, we identify a
function of the nich
tension along the ni
we find defects in b
with the recruitmer
niche shape and fur
cvtoskeletal mechal function of the niche. We find that Non-muscle Myosin II (MyoII) within niche cells establishes increased tension along the niche-GSC boundary required to smoothen that interface. When niche structure is alt we find defect tension along the niche-GSC boundary required to smoothen that interface. When niche structure is alte
we find defects in both signaling and GSC centrosome orientation. Furthermore, compaction timing corre
with the recruit we find defects in both signaling and GSC centrosome orientation. Furthermore, compaction timing correlate
with the recruitment of actively dividing GSCs, and GSC divisions aid in polarizing niche Myoll required for
niche with the recruitment of actively dividing GSCs, and GSC divisions aid in polarizing niche Myoll required for
niche shape and function, likely through a force dependent mechanism. Thus, our analyses identify
cytoskeletal me niche shape and function, likely through a force dependent mechanism. Thus, our analyses identify
cytoskeletal mechanics required to form a functional niche and reveal a feedback mechanism in which GS(
shape the niche that cytoskeletal mechanics required to form a functional niche and reveal a feedback mechanism in wh
shape the niche that guides their behavior.
 cytoskeletal mechanics required to form a functional nichelectric mechanism in which GSCs
shape the niche that guides their behavior. shape the niche that guides their behavior.

Niche compaction is characterized by changes in niche shape and size

Niche composition
To chara
16, whe
somatic
identific
location 16, when niche cells had assembled but not compacted^{12,29}. To examine niche contour, we expressed a somatic marker for filamentous actin (F-actin; Six4-Moe::GFP). We previously showed Six4-Moe::GFP permit identification 16, when niche cells had assembled but not compacted^{22,29}. To examine niche contour, we expressed a somatic marker for filamentous actin (F-actin; Six4-Moe::GFP). We previously showed Six4-Moe::GFP perior identification identification of niche cells due to their increased fluorescence compared to all cells with the exception of, an
location opposite to, the male specific SGPs (msSGPs)¹². To visualize nuclear movements, we utilized a
ubi identification opposite to, the male specific SGPs (msSGPs)¹². To visualize nuclear movements, we utilized a
ubiquitously expressed histone marker (His2Av::mRFP1). Imaging onset revealed niche cells at the gonad
anterio location opposite to, the male specific SGPs (msSGPs)¹². To visualize nuclear movements, we utilized a
ubiquitously expressed histone marker (His2Av::mRFP1). Imaging onset revealed niche cells at the gona
anterior, formi

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 pro
(Figure 1A'-A hours, niche cells moved closer together and the contour facing the germline adopted a more circular prof
(Figure 1A'-A'''), consistent with previous observations^{12,13}.
To confirm our results *in vivo*, we analyzed niche (Figure 1A'-A'''), consistent with previous observations^{12,13}.
To confirm our results *in vivo*, we analyzed niche morphology at pre-compaction (ES16) and post-compaction
(ES17) stages in fixed preparations using the nic (Figure 1A'-A'''), consistent with previous observations^{22,25}.
To confirm our results *in vivo*, we analyzed niche morpholog
(ES17) stages in fixed preparations using the niche-specific
quantified niche contour changes To commit our results in vivo, we analyzed incher incrpinating y at pre-compaction (ES17) and post-compaction

(ES17) stages in fixed preparations using the niche-specific marker Fasciclin III (FasIII) (Figure 1B, B', C, C quantified niche contour changes 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 vi* a perfect circle and lower values indicate a more jagged contour. Like our ex vivo results, niche cells in vivo
evolved a more circular form from pre- to post-compaction (Figure 1D). 3-Dimensional analysis revealed that
ni a perfect circle and fower values indicate a more jagged contour. The our ex vivo results, mene eens in vivo
evolved a more circular form from pre- to post-compaction (Figure 1D). 3-Dimensional analysis revealed the
niches niches decrease in surface area (Figure 1E). This was partially explained by niche cells moving closer, as
evidenced by a decrease in niche cell internuclear distance (Figure 1F). We also noticed a variable and slight
decr evidenced by a decrease in niche cell internuclear distance (Figure 1F). We also noticed a variable and s
decrease in niche cell number from pre- to post-compaction (Figure 1G). Overall, compaction of the nicl
occurs both decrease in niche cell number from pre- to post-compaction (Figure 1G). Overall, compaction of the niche
occurs both *ex vivo* and *in vivo*, suggesting this process can be used to identify factors required for niche
morph occurs both *ex vivo* and *in vivo,* suggesting this process can be used to identify factors required for niche
morphogenesis. Below, all live *ex vivo* experiments were performed on ES16 gonads to visualize compactic
wher

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

occurs both ex *vivo* and *in vivo,* suggesting this process can be used to identify factors required for incheremorphogenesis. Below, all live ex vivo experiments were performed on ES16 gonads to visualize compact whereas morphogenesis. Below, all live ex vivo experiments were performed on ES10 gonads to visualize compaction,
whereas in vivo fixed experiments were performed on late ES17 gonads for endpoint analyses.
F-actin and Myoll polari F-actin and Myoll polarize along the niche periphery during compaction

Due to the role of AMC in many morphogenetic events^{7,8,31–35}, we hypothesized that AMC drive

compaction. To visualize F-actin and Myoll dynamics, w Due to the role of AMC in many morphogenetic events^{7,8,31}–37, we hypothesized that AMC drives niche
compaction. To visualize F-actin and Myoll dynamics, we live-imaged gonads containing Six4-Moe::GFP
Myoll regulatory lig Myoll regulatory light chain (Myoll RLC) tagged with mCherry (Myoll:mCherry; Figure 2A). We found F-actin localizes along the niche periphery throughout compaction (Figure 2A'). However, Myoll progressively enriches over a localizes along the niche periphery throughout compaction (Figure 2A'). However, MyoII progressively
enriches over a few hours during compaction, appearing as puncta along the niche-germline interface (Figure 2A'', arrow). enriches over a few hours during compaction, appearing as puncta along the niche-germline interface (2A", arrow). These enrichment trends were confirmed by quantification along the niche-germline bourdh, 2.5h, and 5h post-2A", arrow). These enrichment trends were confirmed by quantification along the niche-germline boundary at
Oh, 2.5h, and 5h post-dissection, representing early-, mid-, and post-compaction, respectively (Figure 2B-C).
The c

2At the sention of Myoll enrichment with morphogenesis suggests a functional role for AMC in niche
2B-C). The correlation of Myoll enrichment with morphogenesis suggests a functional role for AMC in niche
2B-C).
2At the co 0.5h, and 5h post-dissection, representing early, that parapeter enaptating (Figure 2B-C).
The correlation of Myoll enrichment with morphogenesis suggests a functional role for AMC in niche
compaction.
Enrichment along the Compaction.

Enrichment along the niche boundary might reflect a symmetric contribution from the niche cortex and

adjacent germline, or an asymmetric contribution from one lineage. To distinguish these possibilities,

ind Enrichment
Enrichment a
adjacent gerr
independent
used Six4-Ga
HC::GFP; Figu
(Figure 2F), a
Quantitative adjacent germline, or an asymmetric contribution from one lineage. To distinguish these possibilities, we
independently labeled F-actin or Myoll in a lineage-specific manner (Figure 2D-G). For somatic labeling, we
used Six independently labeled F-actin or Myoll in a lineage-specific manner (Figure 2D-G). For somatic labeling, wused Six4-Gal4 to drive an F-actin (F-Tractin::tdTomato; Figure 2D) or Myoll heavy chain marker (Myoll HC::GFP; Figu used Six4-Gal4 to drive an F-actin (F-Tractin::tdTomato; Figure 2D) or Myoll heavy chain marker (Myoll
HC::GFP; Figure 2E). For germline labeling, we used the germline-specific F-actin marker, Nanos-Moesin::GF
(Figure 2F), HC::GFP; Figure 2E). For germline labeling, we used the germline-specific F-actin marker, Nanos-Moesir
(Figure 2F), and the germline-specific Gal4 driver, Nanos-Gal4::VP16, to express MyoII HC::GFP (Figure 2
Quantitative a (Figure 2F), and the germline-specific Gal4 driver, Nanos-Gal4::VP16, to express Myoll HC::GFP (Figure 2G).
Quantitative analysis revealed F-actin polarizes towards the niche-GSC interface in both niche cells and
germline (Figure 2F), and the niche-GSC interface in both niche cells and germline cells (Figure 2D, F, H, J). Conversely, only when labeled within niche cells did MyoII HC polarize to to niche-GSC interface (Figure 2E, G, I, K). C germline cells (Figure 2D, F, H, J). Conversely, only when labeled within niche cells did Myoll HC polarize
niche-GSC interface (Figure 2E,G,I,K). The asymmetric recruitment of Myoll in niche cells suggests that t
specific priche-GSC interface (Figure 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.
specifically within the niche cell c niche-GSC interface (Figure 2E) (Figure 2E) is equived to compact the MyoII in niche capacity within the niche cell cortex is required for compaction. specifically within the niche cell cortex is required for compaction.

AMC induces tension along the niche-GSC interface

To test this, we ussected gonads, and identified inters find-compaction, correlating with when Myoni
polarizes along the niche-GSC interfaces (Figure 2C). Using a laser, we severed the actomyosin networ
at the niche-GSC in at the niche-GSC interface or at a niche-niche interface (Figure 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 5 at the niche-GSC interface or at a niche-niche interface (Figure 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 5 post-cut (Figure 3D; ref.³⁶). This revealed higher tension along niche-GSC interfaces compared to niche-niclenterfaces, suggesting a correlation between Myoll polarization and tension. To assess whether the tension along post-cut (Figure 3D; ret.³⁶). This revealed higher tension along niche-GSC interfaces compared to niche-niche
interfaces, suggesting a correlation between Myoll polarization and tension. To assess whether the tension
alo interfaces is induced by AMC, we used the H-1152 Rho Kinase inhibitor (ROKi) to inhibit
AMC, then severed niche-GSC interfaces (Figure 3C-D). ROKi treatment reduced tension along the niche-GSC
interface (Figure 3E), confir

AMC is required for niche morphogenesis

along the nice-GSC interfaces (Figure 3C-D). ROKI treatment reduced tension along the niche-GSC
interface (Figure 3E), confirming that AMC induces polarized tension during compaction.
AMC is required for niche morphogenesi interface (Figure 3E), confirming that AMC induces polarized tension during compaction.
 AMC is required for niche morphogenesis

To address the role of polarized AMC in niche morphogenesis, we monitored compaction live and is required for niche morphogenesis
To address the role of polarized AMC in niche morphogenesis, we monitored compaction
cultured in the presence or absence of ROKi (Figure 4A-B). Untreated niches generally exl
circula cultured in the presence or absence of ROKi (Figure 4A-B). Untreated niches generally exhibited an incre
circularity over time (Figure 4C; average increase of 20%; median, 8%). Conversely, ROKi treatment
administered at co circularity over time (Figure 4C; average increase of 20%; median, 8%). Conversely, ROKi treatment
administered at compaction onset resulted in mixed circularity changes, with many niches exhibiting
decreases in circularit

administered at compaction onset resulted in mixed circularity changes, with many niches exhibitine
decreases in circularity (Figure 4D; average change of 0.5%; median, -5.8%). These data suggest that
contractility is requ decreases in circularity (Figure 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 My contractility is required specifically during compaction to establish niche architecture.
As niche cells contribute to Myoll enrichment at niche-GSC interfaces (Figure 2E), we used Six4-Gal4
challenge Myoll specifically in As niche cells contribute to Myoll enrichment at niche-GSC interfaces (Figure 2E), we underleading Myoll specifically in somatic gonadal cells. Somatic RNAi against Myoll HC su
Myoll at the niche-GSC interface (Figure S1), challenge MyoII specifically in somatic gonadal cells. Somatic RNAi against MyoII HC successfully deplet MyoII at the niche-GSC interface (Figure S1), and led to severe compaction defects, as evidenced by a jainche-GSC bou MyoII at the niche-GSC interface (Figure S1), and led to severe compaction defects, as evidenced by a jagg
niche-GSC boundary (Figure 4E, E', F, F'). Niche circularity, but not niche area or cell number, significantly
decr Myoniche-GSC boundary (Figure 4E, E', F, F'). Niche circularity, but not niche area or cell number, significantly
decreases compared to controls, and we observed similar defects using Myoll RLC RNAi (Figure 4G-K). These
re

Proper niche shape limits access to self-renewal signals

ntercases compared to controls, and we observed similar defects using Myoll RLC RNAi (Figure 4G-K). The
results show that somatic AMC is required for niche shape.
Proper niche shape limits access to self-renewal signals
 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 shape contributes to function. Of the niche-derived
 Proper niche shape limits access to self-renewal signals
The reproducibility of niche morphology suggests that shap
signals maintaining stem cells in adult testes, only Upd acts
activates the JAK-STAT pathway in cells near

Signals maintaining stem cells in adult testes, only Upd acts at this early stage. Upd expressed from niche continuates the JAK-STAT pathway in cells nearest the niche, leading to accumulation of STAT, which is routinused signals maintaining stem cells maintained from the stem cells and tractivates the JAK-STAT pathway in cells nearest the niche, leading to accumulation of STAT, which is routinely used as a reporter for pathway activation² used as a reporter for pathway activation^{27,28}.
In both sibling controls and somatic Myoll HC RNAi gonads, germ cells adjacent to the niche exhibited STAT
enrichment compared to germ cells further away (Figure 5A, A', B, used as a reporter for pathway activation^{27,28}.
In both sibling controls and somatic Myoll HC I
enrichment compared to germ cells further aw
improperly shaped niches can signal. However
niches (Figure S2A), and most of t enrichment compared to germ cells further away (Figure 5A, A', B, B'; quantified in C), demonstrating that
improperly shaped niches can signal. However, compared with controls, more germ cells contact misshapen
niches (Fig improperly shaped niches can signal. However, compared with controls, more germ cells contact misshape
niches (Figure S2A), and most of these germ cells are activated for STAT (Figure 5D). In fact, we noticed a
significant inches (Figure S2A), and most of these germ cells are activated for STAT (Figure 5D). In fact, we noticed a
significant increase in all germ cells of the gonad (Figure S2B), likely due to a larger stem cell pool. As a crit

significant increase in all germ cells of the gonad (Figure S2B), likely due to a larger stem cell pool. As a cri
role for the niche is establishing an appropriate number of stem cells, these results suggest that niche
str significant increase increase increase increase increases that niche
structure is essential for proper function.
Intriguingly, Myoll HC RNAi niches are not larger (Figure 4J), yet they support more stem cells. We
hypothesi structure is essential for proper function.
Intriguingly, Myoll HC RNAi niches are not larger (Figure 4J), yet they support more stem cells. We
hypothesized this might be explained by increased niche surface area accessibl Intriguingly, Myoll HC RNAi niches are not
hypothesized this might be explained by in
portion of the niche contacts the germline
contacting germ cells (Figure S2C, C', D, D
(Figure 5E). These data suggest that a com
number hypothesized this might be explained by increased niche surface area accessible to germline. Typic
portion of the niche contacts the germline. We measured the percentage of niche surface physical
contacting germ cells (Fig frition of the niche contacts the germline. We measured the percentage of niche surface physically
contacting germ cells (Figure S2C, C', D, D'), and indeed found an increase under Myoll HC RNAi conditions
(Figure 5E). The proportacting germ cells (Figure S2C, C', D, D'), and indeed found an increase under Myoll HC RNAi cond
(Figure 5E). These data suggest that a compact niche is required to limit available surface area to rest
number of GSC (Figure 5E). These data suggest that a compact niche is required to limit available surface area to restrict th
number of GSCs.
 (Figure 5. These data suggests that a compact nicheless that a compact nicheless that are surface as $\frac{1}{2}$ and $\frac{1}{2}$ are strict the surface area to restrict the surface area to restrict the surface area to restrict

Proper niche shape is required for GSC centrosome orientation
Another critical niche function is to orient GSC divisions to ensure one daughter will remain a GSC near the
niche, and the other will be moved away and diff niche, and the other will be moved away and differentiate^{21–23,25,26}. To assess the role of niche shape one division orientation, we live-imaged control and ROKi-treated gonads, since this treatment affects niche shape (Figure 4A-D). ROKi will also arrest cytokinesis³⁷, and indeed we captured completed GSC cytokinesis in one
third of the control gonads (11/32), but never in ROKi-treated gonads (0/26). Whether cytokinesis completed
or or not, we could map the orientation of nuclear divisions using His2Av::mRFP1 to calculate chromatin
trajectory relative to the niche-GSC interface (Figure S3A). In these analyses, higher angles represent divisions
oriente trajectory relative to the niche-GSC interface (Figure S3A). In these analyses, higher angles represent doriented away from the niche³⁸. Control nuclei divided predominantly from 30-80 degrees (Figure S3E), indicating t oriented away from the niche³⁸. Control nuclei divided predominantly from 30-80 degrees (Figure S3B, B', D, E), indicating that GSC division orientation is regulated and biased so daughter cells are born further from th

niche. In contrast, division orientation of ROKi-treated cells significantly differed from controls, with GSCs
primarily dividing between 0-45 degrees relative to the niche (Figure S3C, C', D; p<0.001, KS test; Figure S3F) primarily dividing between 0-45 degrees relative to the niche (Figure S3C, C', D; p<0.001, KS test; Figure S3
which is consistent with a randomized distribution with 3-dimensional constraints taken into consideration
A lim which is consistent with a randomized distribution with 3-dimensional constraints taken into consideration³⁸.
A limitation is that pharmacological treatment affects all cells. To determine whether changes in niche shape
 which is consistent with a randomized distribution with 3-dimensional constraints taken into consideration³⁰.
A limitation is that pharmacological treatment affects all cells. To determine whether changes in niche shape
 affect GSC behavior non-autonomously, we manipulated niche shape in a lineage-specific manner. Division
orientation is controlled via anchoring of one GSC centrosome at the niche-GSC interface^{21–23,25,26}. We
therefore an orientation is controlled via anchoring of one GSC centrosome at the niche-GSC interface^{21–23,25,26}. We
therefore analyzed centrosome positioning in fixed tissue after somatic Myoll knockdown. In controls, as
expected, orientation is controlled via anchoring of one GSC centrosome at the niche-GSC interface^{21–23,25}. We therefore analyzed centrosome positioning in fixed tissue after somatic MyoII knockdown. In controls, expected, about 9 expected, about 90% of GSCs had proper centrosome positioning (Figure 5F, H). In contrast, the frequence
mispositioned centrosomes doubled in GSCs from gonads exhibiting somatic MyoII knockdown (Figure 5G
By chance movemen expection of Cases are interesting to GSCs from gonads exhibiting somatic Myoll knockdown (Figure 5G-H).
By chance movement, unanchored centrosomes abut the niche ~50% of the time²³; thus, change of this
magnitude is sig By chance movement, unanchored centrosomes abut the niche ~50% of the time²³; thus, change of this magnitude is significant.
Taken together, niche shape is crucial in regulating GSC centrosome orientation and in limitin

By chance movement, unanchored centrosomes abut the niche ~50% of the time~; thus, change of this
magnitude is significant.
Taken together, niche shape is crucial in regulating GSC centrosome orientation and in limiting se magnitude is significant.

Taken together, niche shape is crucial in regulating GSC centrosome orientation and in limiting self-renewal

signals to a subset of cells.
 GSCs are required to shape their niche

We sought to

GSCs are required to shape their niche

Surfare to a subset of cells.
 GSCs are required to shape their niche

We sought to uncover mechanisms contributing to actomyosin polarity within the niche required for proper

form and function. Importantly, GSCs are r Signals to a state of the completed to shape
Signals to uncover med
form and function. Importa
with GSC division onset¹². S
be the packing or cell divisi

form and function. Importantly, GSCs are recruited to the niche during morphogenesis²⁷, and this coincides
with GSC division onset¹². Since mechanical forces can polarize Myol^{139,40}, a potential force on niche cells with GSC division onset⁺⁺. Since mechanical forces can polarize Myoll^{39,40}, a potential force on niche cells could
be the packing or cell divisions that occur as GSCs are recruited to the niche.
Evidence that forces em be the paramger can antertracture that cooking to the nicher burden in the series experimental compaction, prior to Myoll polarization (Figure 6). When tension along the nithe germ cell protruded into the niche (Figure 6A, compaction, prior to Myoll polarization (Figure 6). When tension along the niche-GSC interface was severed, the germ cell protruded into the niche (Figure 6A, A', B), suggesting exertion of positive pressure on the niche (the germ cell protruded into the niche (Figure 6A, A', B), suggesting exertion of positive pressure on the niche
Interestingly, this force appears exclusive to early compaction, as no protrusion was observed when cuts wer
 Interestingly, this force appears exclusive to early compaction, as no protrusion was observed when cuts were
performed nearing the end of compaction (Figure 6C, C', D). The timing of the force exerted by germ cells on
the performed nearing the end of compaction (Figure 6C, C', D). The timing of the force exerted by germ cells on
the niche, and the eventual polarization of Myoll along the niche-GSC interface, led us to hypothesize that
germ

performed near the eventual polarization of Myoll along the niche-GSC interface, led us to hypothesize that
germ cell force contributes to polarization required for niche shape.
We therefore selectively ablated germ cells germ cell force contributes to 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 (Figure 7A-B). We therefore selectively ablated germ cells by expressing the pro-ap
(*Hid*), using Nanos-Gal4::VP16 (Figure 7A-B). We found that upon Hit
germ cells, confirming ablation (Figure 7E), and had misshapen niche
We suspect tha

We therefore selectively ablated germ cells by expressing the pro-apoptosis gene, *head involution* defective
(*Hid*), using Nanos-Gal4::VP16 (Figure 7A-B). We found that upon Hid expression, gonads contained fewer
germ ce (Ha), using Nanos-Gal-4::VT 16 (Figure 7A-B). We found that upon Hid expression, gonads contained fewer
germ cells, confirming ablation (Figure 7E), and had misshapen niches (Figure 6A, B) has various origins. One
contrib We suspect that the latent protrusive force exerted by germ cells (Figure 6A, B) has v
contribution might be from mitotic divisions⁴¹. We compromised germ cell divisions k
down of Cdc25⁴² via RNAi. Upon Cdc25 knockdow contribution might be from mitotic divisions⁴¹. We compromised germ cell divisions by lineage-specific knock
down of Cdc25⁴² via RNAi. Upon Cdc25 knockdown, germ cell number decreased (Figure 7E), confirming
down of Cd down of Cdc25⁺² via RNAi. Upon Cdc25 knockdown, germ cell number decreased (Figure 7E), confirming
Exponent of Cdc25⁺²
In Read of the Cdc of the Mumber decreased (Figure 7E), confirming

divisions were inhibited, and observed defects in niche circularity (Figure 7C, D, F). Neither niche area nor
niche cell number changed upon GSC division inhibition or germ cell ablation (Figure 7G, H), which parallels
our our findings from MyoII knockdown in the niche (Figure 4J, K). These data confirm that GSCs act to shape the niche and suggest that GSC divisions at least partially contribute to the mechanical force required to establis
a

GSC divisions aid in polarizing MyoII within the niche

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 appropriate niche morphology.
 GSC divisions aid in polarizing Myoll within the niche

We have established that Myoll polarity is essential for niche architecture. To assess whether germline

divisions regulate this pola **GSC divisions aid in polarizing N**
We have established that Myoll
divisions regulate this polarity, \
Myoll was enriched along the ni
Cdc25 RNAi gonads (Figure 7J, J'
boundary (Figure 7J', arrowhead divisions regulate this polarity, we visualized MyoII RLC::GFP in Cdc25 RNAi and sibling controls. In cont
MyoII was enriched along the niche-GSC interfaces (Figure 7 I, I', K), but this was significantly decreased
Cdc25 R Myoll was enriched along the niche-GSC interfaces (Figure 7 I, I', K), but this was significantly decreased in
Cdc25 RNAi gonads (Figure 7J, J', K). Notably, some niche cells polarized MyoII away from the niche-GSC
boundar Cdc25 RNAi gonads (Figure 7J, J', K). Notably, some niche cells polarized Myoll away from the niche-GSC
boundary (Figure 7J', arrowhead). Further, Cdc25 RNAi gonads exhibited an increase in centrosome
mispositioning, sugg boundary (Figure 7J', arrowhead). Further, Cdc25 RNAi gonads exhibited an increase in centrosome
mispositioning, suggesting that these niches have functional defects (Figure 7L-N). Thus, GSC divisions ar
required to shape

mispositioning, suggesting that these niches have functional defects (Figure 7L-N). Thus, GSC division
required to shape their niche by polarizing Myoll to the stem cell-niche interface, which is critical for
function.
Adh misposition, suggesting that the that the theorem in the tell-niche interface, which is critical for niche
function.
Adherens junctions (AJs) are a main source of niche-GSC adhesion^{26,45} and can control mechanosensation Function.
Adherens junctions (AJs) are a main source of niche-GSC adhesion^{26,45} and can control mechanosensation in
other contexts^{43,44}. To test whether cadherins could be the sensor for germline forces on niche cells Adherens
other con
knocked c
mechano
Collective
inhibiting other contexts^{43,44}. To test whether cadherins could be the sensor for germline forces on niche cells, we knocked down E-cadherin, but observed no defect in niche shape (Figure S4). Thus, factors underlying mechanotransd other contexts^{43,44}. To test whether cadherins could be the sensor for germline forces on niche cells, we knocked down E-cadherin, but observed no defect in niche shape (Figure S4). Thus, factors underlying mechanotransd

mechanotransduction remain to be discovered in this system.
Collectively, however, the effects we observed on niche shape and function by depleting germ cells or
inhibiting their division strongly suggest that GSCs feedbac .
Collectively, however, the effects we observed on niche shape
inhibiting their division strongly suggest that GSCs feedback to
. Collectively, however, the effects we observed on the effects we observed on a perform game inhibiting their division strongly suggest that GSCs feedback to shape the niche that guides their behave inhibiting their divisio inhibiting their division strongly suggest that GSCs feedback to shape the nichele that guide state at nichele
Expansion strongly suggest that guide strongly suggest that guide strongly support that guide strongly support

Discussion

Our work captures the dynamic formation of a functional niche. Prior imaging revealed how pro-niche cells
migrate to their proper position^{12,30}. Here, we show that its continued morphogenesis is dependent on
polarization migrate to their proper position^{12,30}. Here, we show that its continued morphogenesis is dependent on
polarization of a force-producing actomyosin network. If contractility is compromised, the niche adopts
irregular cont

Polarized contractility is required for niche morphogenesis

prendiant contour and exhibits defects in function. Additionally, recruited stem cells aid in polarizing Myoll
along the forming niche-stem cell boundary. Without this feedback, niche shape and function are aberrant.
Polar increases and function are aberrained in polarized to the shape and function are aberrant
along the forming niche-stem cell boundary. Without this feedback, niche shape and function are aberrant
Polarized contractility is actin and Myoll induce tension along the interface between niche cells and newly recruited GSCs during
compaction. Compromising contractility yielded misshapen niches, reminiscent of the involvement of Myoll
for mammalian morphogenesis. This inference was strongly supported by the misshaping observed when Myoll was depleted compaction. Compromising contractility yielded misshapen niches, reminiscent of the involvement of My
for mammalian intestinal niche curvature⁷, intestinal stem cell shape⁸, and neural stem cell rosette
morphogenesis⁴ for mammalian intestinal niche curvature⁷, intestinal stem cell shape⁸, and neural stem cell rosette
morphogenesis⁴⁶. Other studies benefitted from whole-tissue knockouts, pharmacological manipulations,
and/or *in v* and/or *in vitro* culturing, but were limited in cell-type specificity. Our study combines *ex vivo* live-imaging²⁵
with *in vivo* cell-type specificity. While pharmacological manipulation affects all cells, the asymmetr

with *in vivo* centrype specificity. While pharmacological manipulation affects an cells, the asymmetry in Myon
contribution suggests that it is the contractile activity in the niche cell cortex that is a main driver in ni morphogenesis. This inference was strongly supported by the misshaping observed when MyoII was deple
only in somatic cells.
It would be interesting to investigate whether AMC in the GSC cortex also shapes the niche, but th only in somatic cells.

It would be interesting to investigate whether AMC in the GSC cortex also shapes the niche, but this is not

currently testable⁴⁷. Indeed, our ROKi experiments support such a conclusion. Tissue-le [']
It would be interestin
currently testable⁴⁷. I
induced more severe
altered Myoll functio
ROKi treated gonads
morphogenesis. currently testable⁴⁷. Indeed, our ROKi experiments support such a conclusion. Tissue-level inhibition of AM induced more severe phenotypes than somatic Myoll inhibition (Figure 4C, F, H). Of note, ROKi treatment lattered currently testable"'. Indeed, our ROKi experiments support such a conclusion. Tissue-level inhibition of AMC
induced more severe phenotypes than somatic Myoll inhibition (Figure 4C, F, H). Of note, ROKi treatment *bo*
alte induced more severe phenotypes than somatic Myon inhibition (Figure 4C, F, H). Of note, NOKI treatment both
altered MyoII function in the niche *and* disrupted GSC cytokinesis. We suspect the severe niche phenotype of
ROKi

Niche morphology regulates niche-stem cell signaling

altered Myon Function in the niche and disrupted GSC cytokinesis. We suspect the severe inche phenotype of
ROKi treated gonads is caused by a combinatorial effect of GSC and niche cell involvement in niche
morphogenesis.
N Miche morphology regulates niche-stem cell signaling
Roki treated and niche morphology regulates niche-stem cell signaling
Dur data suggest that the contour of the niche is important for modulating signaling. We previously Morphogenesis.
Niche morpholo,
Our data suggest
that blocking nic
significant STAT :
niche. It is possik
pathway recepto that blocking niche assembly led to niche cells that signaled poorly, if at all³⁰. By affecting compaction, we find
significant STAT activation in nearby germ cells, but an increase in the number of those cells contactin niche. It is possible that Myoll inhibition increases Upd secretion from niche cells. However, because the pathway receptor polarizes along the niche-GSC interface within GSCs⁴⁸, we believe the increase of niche surface pathway receptor polarizes along the niche-GSC interface within GSCs⁴⁸, we believe the increase of niche
surface available to the germline likely accounts for the increase in niche-adjacent STAT-positive cells. We
intest surface available to the germline likely accounts for the increase in niche-adjacent STAT-positive cells. Work in
intestinal organoids has also shown that niche curvature helps optimize signaling⁸, highlighting that conc

Centrosome orientation is dependent on a precise niche shape

intestinal organoids has also shown that niche curvature helps optimize signaling⁸, highlighting that concepts
uncovered in *Drosophila* are applicable to mammals.
Centrosome orientation is dependent on a precise niche Centrosome orientation is dependent on a precise n
Gonads with aberrant niche morphology exhibit defecentrosome anchors to the GSC cortex at the interface
interface^{21,25,26,48}. If centrosome anchoring fails, a "ce
preven centrosome anchors to the GSC cortex at the interface with the niche via influence of proteins polarized to
interface^{21,25,26,48}. If centrosome anchoring fails, a "centrosome orientation checkpoint" (COC) is engaged,
pr interface^{21,25,26,48}. If centrosome anchoring fails, a "centrosome orientation checkpoint" (COC) is engaged,
preventing spindle assembly to avoid symmetric divisions^{22,23}.
Prior centrosome orientation research has exc

interface^{21,25,26,48}. If centrosome anchoring fails, a "centrosome orientation checkpoint" (COC) is engaged,
preventing spindle assembly to avoid symmetric divisions^{22,23}.
Prior centrosome orientation research has excl preventing spindle assembly to avoid symmetric divisions^{22,23}.
Prior centrosome orientation research has exclusively manipul
cortex^{21,25,26,48}. Our work is the first to reveal a requirement or $\text{cortex}^{21,25,26,48}$. Our work is the first to reveal a requirement on the niche side of this interface imported factors action of the niche side of this interface imported factors and \mathbb{R}^2 . cortex^{21,25,26,48}. Our work is the first to reveal a requirement on the niche side of this interface impacting
interface impacting
the first of a requirement on the niche side of this interface impacting.

GSCs feedback to shape their own niche by polarizing MyoII

required to polarize yet-to-be-identified, novel COC players to the niche cortex.
 GSCs feedback to shape their own niche by polarizing MyoII

Importantly, germ cells are required for proper niche shape and function. Spe GSCs feedback to shape their own niche by polarizing Myoll
Importantly, germ cells are required for proper niche shape and function. Specif
required for niche Myoll polarization, and consequently proper niche shape and
is required for niche Myoll polarization, and consequently proper niche shape and function. We speculate
is a mechanosensitive pathway between dividing GSCs and the niche, perhaps activated by force-produc
spindle microtubule

is a mechanosensitive pathway between dividing GSCs and the niche, perhaps activated by force-producing
spindle microtubules on the GSC cortex⁴¹. It is also possible that a cell cycle-dependent change to GSC
polarization spindle microtubules on the GSC cortex⁴¹. It is also possible that a cell cycle-dependent change to GSC
polarization causes the effect on niche cell polarity.
Future work must discern how the niche senses force to elici polarity and the niche senses force that
Future work must discern how the niche senses force
Cadherin is not essential (Figure S4), perhaps a coml
niche morphogenesis. Alternatively, other mechanis
involved.
Additionally,

compromised supports the idea that additional forces generated by GSCs contribute to niche morphogenesis. Cadherin is not essential (Figure S4), perhaps a combination of AJ components²² must be inhibited to affect
niche morphogenesis. Alternatively, other mechanisms, such as mechanosensory ion channels,⁵⁰ may be
involved.
 niche morphogenesis. Alternatively, other mechanisms, such as mechanosensory ion channels," may be
involved.
Additionally, division forces are likely not the only source of force from the germline. Protrusion into the
upon Additiona
upon cort
know the
comprom
Indeed, re
inhibiting upon cortical laser severing suggests there is latent germline pressure toward the niche. Although we do not
know the mechanistic basis for that pressure, the residual Myoll polarization when germline divisions are
compro know the mechanistic basis for that pressure, the residual Myoll polarization when germline divisions are
compromised supports the idea that additional forces generated by GSCs contribute to niche morphogenesis
indeed, rem

compromised supports the idea that additional forces generated by GSCs contribute to niche morphogene
Indeed, removing many of the germline cells by inducing cell death led to more severe niche phenotypes i
inhibiting divi Indeed, removing many of the germline cells by inducing cell death led to more severe niche phenotypes than inhibiting divisions (Figure 7F, compare circularity changes).

Feedback between stem cells and their niche has be inhibiting divisions (Figure 7F, compare circularity changes).
Feedback between stem cells and their niche has been seen in other systems, such as the *C. elegans*
hermaphroditic gonadal niche⁵¹, the murine hair follicle Feedback between stem cells and their niche has been seen
hermaphroditic gonadal niche⁵¹, the murine hair follicle sten
Therefore, our work strengthens the paradigm that niche-ste
identifies mechanisms of stem cell force Feedback between stem cans and their methe has been seen in other systems, such as the C. elegans
hermaphroditic gonadal niche⁵¹, the murine hair follicle stem cell niche^{52,53}, and the *Drosophila* glial r
identifies m

Limitations

hermaphroditic gonadal niche⁵², the murine hair follicle stem cell niche^{52,53}, and the *Drosophila* glial niche⁵⁴.
Therefore, our work strengthens the paradigm that niche-stem cell relationships are bidirectional and Therefore, and therefore, our contract interest in the paradigm that niche-stem cell force-seneration to regulate niche form and function.
 Limitations

While our lineage-specific manipulations show somatic requirement f **Limitations**
While our lineage-specific manipulations show somatic requirement for contractility in nionly niche driver available for these stages, Six4-Gal4, is also expressed in cyst cells. Thus,
whether cyst cells als only niche driver available for these stages, Six4-Gal4, is also expressed in cyst cells. Thus, determining
whether cyst cells also impact niche shape in a Myoll-dependent manner is not yet possible. Cyst cells make
much l whether cyst cells also impact niche shape in a Myoll-dependent manner is not yet possible. Cyst cells much less extensive connections with the niche as do germ cells (c.f. Figure 2A'; the relative thinness of somatic-to-n

much less extensive connections with the niche as do germ cells (c.f. Figure 2A'; the relative thinness of
somatic-to-niche contacts is apparent)^{17,33}. We therefore believe it unlikely that cyst cells directly affect nic somatic-to-niche contacts is apparent)^{17,33}. We therefore believe it unlikely that cyst cells directly affect
morphogenesis. Nevertheless, cyst cells could signal or provide force to the GSCs as they shape the nich
Addit somatic-to-niche contacts is apparent) ^{27,33}. We therefore believe it unlikely that cyst cells directly affect niche
morphogenesis. Nevertheless, cyst cells could signal or provide force to the GSCs as they shape the nic Additionally, Hedgehog and Bone Morphogenetic Protein (BMP) are produced by the adult niche, but a rol
for neither has been described during niche formation. Hedgehog acts exclusively on CySCs, which are
recruited later in For neither has been described during niche formation. Hedgehog acts exclusively on CySCs, which are
recruited later in larval stages^{28,55}. In the adult, BMP signaling requires intimate contact between GSCs and
niche ce recruited later in larval stages^{28,55}. In the adult, BMP signaling requires intimate contact between GSCs niche cells^{45,56,57}. While it would be interesting to determine how niche shape impacts this pathway, acts only

niche cells^{45,56,57}. While it would be interesting to determine how niche shape impacts this pathway, activation
is only robust enough in later larval GSCs for analysis⁵⁸.
Lastly, we are curious as to whether mechanism niche cells^{45,56,57}. While it would be interesting to determine how niche shape impacts this pathway, activation
is only robust enough in later larval GSCs for analysis⁵⁸.
Lastly, we are curious as to whether mechanism is only robust enough in later larval GSCs for analysis⁵⁸.
Lastly, we are curious as to whether mechanisms reveal
Preliminary work on the adult testis suggests enrichmer
approaches available in the adult testis might pro Preliminary work on the adult testis suggests enrichment for contractility regulators in the niche. Therefore,
approaches available in the adult testis might provide answers to the above questions.
. approaches available in the adult testis might provide answers to the above questions.

Therefore, a variable in the nicht testis might provide answers to the above questions. approaches available in the adult testis might provide answers to the above questions.

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Supervision. SD: Conceptu Investigation; Data Curation; Writing-Review-Editing; Visualization. JS: Methodology; Formal Analysis;
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Declaration of Interests:

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The authors declare no competing interests
 $\frac{d\mathbf{r}}{dt}$ The authors declare no competing interests

Figure Legends

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

A-A-YEX VIVO gonadal timelapse (5-hour), revealing niche compaction; SIX4-WOE::GFP (Somatic F-actin, green),
His2Av::mRFP1 (nuclei, magenta). A) 5 niche cells in-view reveal an elongated, jagged niche at the anterior. A')
 Niche cells rearranged with neighbors, moving closer together. A") 6 niche cells in-view present a more
rounded contour. A"') 7 niche cells in-view are yet closer, presenting a circular contour. B-C) Head-on views of
nich rounded contour. A''') 7 niche cells in-view are yet closer, presenting a circular contour. B-C) Head-on v
niches from fixed ES16 (B) or late ES17 (C) gonads developed *in vivo;* Six4-Moe::GFP; His2Av::mRFP1. B'-
FasIII. D niches from fixed ES16 (B) or late ES17 (C) gonads developed *in vivo;* Six4-Moe::GFP; His2Av::mRFP1. B'-C')
Faslll. D-G) 3D analysis of niches developed *in vivo*. Niche circularity (D) increases post-compaction. Niche
s nentes from fixed ES16 (B) or late ES17 (C) gonads developed in vivo, Six4-Moet...GFP; His2Av::mRFP1. B' C)
Fasill. D-G) 3D analysis of niches developed in vivo. Niche circularity (D) increases post-compaction. Niche
surfa Fashit. D-G) 3D analysis of includes developed in vivo. Niche circularity (D) increases post-compaction. Niche
surface area (E), niche cell internuclear distance (F), and niche cell number (G) decrease post-compaction
<0.0 surface area (A), the cell pre- (n=277) or post- (n=17) and post-

surface area (n=31) compaction; in F, each symbol represents one niche cell pre- (n=277) or post- (n=435) compaction.

Unless stated otherwise, for all fig (n=31) compaction; in F, each symbol 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 outlin
enti

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

Unless stated otherwise, for all figures: scale bars = 10 microns; images = single Z slice; yellow dashes outli
entire gonad; white dashes outline niche.
Figure 2: F-actin and Myoll are enriched along the niche-GSC interfa entire gonad; white dashes outline niche.

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

A) *Ex vivo* gonadal timelapse of niche compaction; Six4-Moe::GFP; Myoll::mCherry. A') So Figure 2: F-actin and Myoll are enriched a
A) *Ex vivo* gonadal timelapse of niche com
Myoll. B-C) Quantifications of F-actin (B) a
niche somatic interfaces (blue) at 0h, 2.5h
fluorescence along one interface (n=27 for
Som A) Ex vivo gonadal timelapse of niche compaction; Six4-Moe::GFP; MyoII::mCherry. A') Somatic F-actin. A'')
MyoII. B-C) Quantifications of F-actin (B) and MyoII (C) along niche-GSC interfaces (magenta) relative to non-
nich polarization towards or away from niche-GSC interfaces, respectively. Asterisk = niche. fluorescence along one interface (n=27 for both). D-G) Fixed images of lineage-specific F-actin or Myoll Somatic F-actin (Six4-Gal4 > UAS-F-Tractin::TdTomato.) E) Somatic Myoll (Six4-Gal4::VP16 > Myoll HC::Germline F-actin Somatic F-actin (Six4-Gal4 > UAS-F-Tractin::TdTomato.) E) Somatic Myoll (Six4-Gal4::VP16 > Myoll HC::GFP)
Germline F-actin (Nanos-Moesin::GFP). G) Germline Myoll (Nanos-Gal4::VP16 > Myoll HC::GFP)
Germline F-actin (Nanos-M Germline F-actin (Nanos-Moesin::GFP). G) Germline MyoII (Nanos-Gal4::VP16 > MyoII HC::GFP). H-K)
Fluorescence along the niche-GSC interface (pink) compared to niche-niche (blue, H-I) or GSC-GSC interfaces
(blue, J-K). Equa Fluorescence along the niche-GSC interface (pink) compared to niche-niche (blue, H-I) or GSC-GSC int
(blue, J-K). Equal numbers of niche-GSC interfaces were analyzed compared to respective controls (H:
n=52, J: n=57, K: n (blue, J-K). Equal numbers of niche-GSC interfaces were analyzed compared to respective controls (H: n=57, l)
n=52, J: n=57, K: n=79; **p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney). Arrows and arrowheads =
polarization

(blue, J-K). In=57, K: n=79; **p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney). Arrows and arrowheads =
polarization towards or away from niche-GSC interfaces, respectively. Asterisk = niche.
Figure 3: AMC induces tension polarization towards or away from niche-GSC interfaces, respectively. Asterisk = niche.

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

A-C) Six4-Moe::GFP gonads developing *ex vivo.* Outlines = interfaces se Figure 3: AMC induces tension along the niche-GSC interface
A-C) Six4-Moe::GFP gonads developing ex vivo. Outlines = interfaces selected for severi
line = interface targeted) and 5s post-cut (A', B', C'). A"-C'') Interfac Figure 3: AMC induces tension along the inche-GSC interface
A-C) Six4-Moe::GFP gonads developing ex vivo. Outlines = interface
line = interface targeted) and 5s post-cut (A', B', C'). A"-C'') Int
cut. Dashed lines show di A C) SIX+ Moe.:.GFT gonads developing ex vivo. Outlines - interfaces selected for severing pre-cut (A, B, C, Led
line = interface targeted) and 5s post-cut (A', B', C'). A"-C'') Interface pre-cut and montage of 5s interval Control niche-GSC interface. C) ROKi-treated niche-GSC interface. D) Initial retraction velocities along niche
niche (blue, n=17) and niche-GSC (magenta, n=15) control interfaces show higher tension along niche-GSC
interfa interfaces. E) Initial retraction velocities along niche-GSC interfaces in controls (magenta; same as D) and R
treated gonads show tension is decreased upon AMC inhibition (black, n=11; *p<0.05, *** p<0.001 Mann-
Whitney).

Figure 4: AMC is required for niche morphogenesis $\overline{}$ Figure 4: AMC is required for niche morphogenesis

A-B) 5-hour timelapse of control (A) or ROKi-treated (B) Six4-Moe::GFP (somatic F-actin, green); gonads; previously shown in ref. 12) or ROKi-treated gonads (D, n=15 gonads). Lines pair the same niche at 0 and 5h (**p<0.01, Wilcoxon test). E-H) Six4-moe::GFP, His2Av::mRFP1 control (E, G) or Six4-Gal4 > Myoll RNAi //
Figure 4: A
A-B) 5-hou
His2Av::m
gonads (F :
and 5h (** His2Av::mRFP1 (nuclei, Magenta). C-D) Niche circularity measurements at 0h and 5h of untreate
gonads; previously shown in ref.¹²) or ROKi-treated gonads (D, n=15 gonads). Lines pair the same
and 5h (**p<0.01, Wilcoxon t gonads; previously shown in ref.¹²) or ROKi-treated gonads (D, n=15 gonads). Lines pair the same niche at C
and 5h (**p<0.01, Wilcoxon test). E-H) Six4-moe::GFP, His2Av::mRFP1 control (E, G) or Six4-Gal4 > Myoll RI
gonad gonads; previously shown in ref.**) or ROKi-treated gonads (D, n=15 gonads). Lines pair the same niche at 0
and 5h (**p<0.01, Wilcoxon test). E-H) Six4-moe::GFP, His2Av::mRFP1 control (E, G) or Six4-Gal4 > Myoll RN
gonads gonads (F = HC RNAi; H = RLC RNAi) developed *in vivo.* E'-H') FasIII. I) Niche circularity is decreased when either
MyoII is depleted (***p<0.001, ****p<0.0001, Mann-Whitney; See Figure S1). J-K) Niche surface area (J) an Myoll is depleted $(**p<0.001, ****p<0.0001,$ Mann-Whitney; See Figure S1). J-K) Niche surface area (J) and
Myoll is depleted $(**p<0.001, ****p<0.0001,$ Mann-Whitney; See Figure S1). J-K) Niche surface area (J) and MyoII is depleted (***p<0.001, ****p<0.0001, Mann-Whitney; See Figure S1). J-K) Niche surface area (J) and

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

For graphs I-K, n=17 gonads for all conditions, except n= 21 for Myoll RLC RNAi.

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

A-B) Control (A) or Six4-Gal4 > Myoll HC RNAi (B) stained gonads; Vasa (g Figure 5: Proper niche shape is required to regulate GSC behavior
A-B) Control (A) or Six4-Gal4 > MyoII HC RNAi (B) stained gonads; Vasa (germline
B') STAT antibody. Asterisk = niche. Solid outlines = niche-adjacent germ c B') STAT antibody. Asterisk = niche. Solid outlines = niche-adjacent germ cells ('GSC') enriched for STAT. Dotte
outlines = posterior germ cells ('GC') exhibiting lower STAT. C) STAT enrichment in GSCs under control (n= 87 outlines = posterior germ cells ('GC') exhibiting lower STAT. C) 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 nic 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 n the niche is increased in Myoll 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 Myoll HC RNAi gonads. (*p<0.05,
p<0.001, *p<0. 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; See Figure S2). F-G) Control (F) and MyoII HC RNAi (G) stai
gonads; centrosomes ($***p<0.001$, $***p<0.0001$, Mann-Whitney; See Figure S2). F-G) Control (F) and MyoII HC RNAi (G) st
gonads; centrosomes (gamma tubulin, green), Vasa (germline, magenta), and FasIII (white). F) A GSC (
outline) with one centr gonads; centrosomes (gamma tubulin, green), Vasa (germline, magenta), and FasIII (white). F) A GSC (solid
outline) with one centrosome oriented at the niche (arrow), and one opposite (arrowhead). G) A GSC (solid
outline) w outline) with one centrosome oriented at the niche (arrow), and one opposite (arrowhead). G) A GSC (solid
outline) with two centrosomes (arrowheads) both oriented away from the niche. H) Percentage of cells wit
misposition outline) with two centrosomes (arrowheads) both oriented away from the niche. H) Percentage of cells with
mispositioned centrosomes doubled from 9.4 to 20.5% comparing control and Myoll HC RNAi (**p<0.01, Ch
Square; See Fi

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

mispositioned centrosomes doubled from 9.4 to 20.5% comparing control and Myoll HC RNAi (**p<0.01, Chi
Square; See Figure S1 and S3).
Figure 6: Germ cells protrude into the niche during early compaction
A, C) Gonads ex viv protrusion post-cut, if any. A, A') Severing the niche-GSC interface during early compaction led to germ cell Square; See Figure S1 and S3).

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

A, C) Gonads ex vivo expressing Nanos-Moe::GFP (germline F-actin); early (A) or late compaction (C). Asterisk =

niche. A, C) Gonads ex vivo expressing Nanos-Moet.CFF (germline F-actin), early (A) or late compaction (C). Asterisk =
niche. Niche-GSC interface contour 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 = 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 pro protrusion post-cut, if any. A, A') Severing the niche-GSC interface during early compaction led to germ contrusion into the niche (10s and 20s). C, C') Severing the niche-GSC interface during late compaction revealed lit 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 represen
the sa revealed little to no protrusion. B, D) Quantifications of protrusion into the niche, where each line reprothe same interface 0s and 20s after severing. Germ cells significantly protrude into the niche during ear
but not l

Figure 7: GSC divisions are required to shape their niche

revents interface Os and 20s after severing. Germ cells significantly protrude into the niche during early (B)
but not late compaction (D; n=8 cells each; **p<0.01, Wilcoxon Test).
Figure 7: GSC divisions are required to s but not late compaction (D; n=8 cells each; **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*; F-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)

(magenta), and FasIII (A'-B'). C-D) Six4-Moe::GFP; His2AV::mRFP1 cont

Cdc25 RNAi gonad (D) A B) SIX+ Moe.:GFF control (A) or Nanos-Gal-1:VF16 > Ind.2 gonad (B) developed in vivo, F-actin (green), vasa
(magenta), and FasIII (A'-B'). C-D) Six4-Moe::GFP; His2AV::mRFP1 control gonad (C) or Nanos-Gal4::VP16 >
Cdc25 R (magenta), and Fashi (A'-B'). C-D') Six4-Moet..GFT; HiszAV:...IIINT I control gonad (c) or Nanos-Gal4::VTD2
Cdc25 RNAi gonad (D) developed in vivo; F-actin (green), DNA (magenta) and FasIII (C'-D'). A, C, and D are
maximum edezs RNAi gonad (D) developed *in Vivo*; F-actin (green), DNA (magenta) and Fasin (C-D-). A, C, and D are
maximum projections of ~10 slices (0.5-micron intervals). E) Total germ cell number is reduced by express
hid.Z (n= 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 upon germline expression of hid.Z or Cdc25 RNAi (*p<0.05,
p< 11, respectively). F) Niche circularity is decreased upon germline expression of hid.Z or Cdc25 RNAi ($*p<0.05$, $p<0.01$, Mann-Whitney). G-H) No difference in niche surface area (G) nor niche cell number (H) between con **p<0.01, Mann-Whitney). G-H) No difference in niche surface area (G) nor niche cell number (H) between
conditions (n=18 hid.Z and 21 Cdc25RNAi gonads) and respective controls (n= 20 and 22, respectively). I-J)
Control (I conditions (n=18 hid.Z and 21 Cdc25RNAi gonads) and respective controls (n= 20 and 22, respectively). I-J)
Control (I) and Cdc25 RNAi (J) gonads expressing Myoll RLC::GFP; Vasa (magenta), FasIII (white). I'-J') Myoll
RLC:: Control (I) and Cdc25 RNAi (J) gonads expressing Myoll RLC::GFP; Vasa (magenta), Faslll (white). I'-J') Myoll
RLC::GFP shows enrichment in controls towards niche-GSC interfaces (I', arrow), and decreased but residual
polar RLC::GFP shows enrichment in controls towards niche-GSC interfaces (I', arrow), and decreased but residua
polarity towards the niche-GSC interface in Cdc25 RNAi gonads (J', arrow). MyoII is sometimes mis-polarized
away fro polarity towards the niche-GSC interface in Cdc25 RNAi gonads (J', arrow). Myoll is sometimes mis-polarized
away from the niche-GSC interface (J', arrowhead). K) Quantifications show germline Cdc25 RNAi leads to
decreased polarity of 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 decreased Myoll enrichment at the niche-GSC interface (n=54 control = interfaces, n=72 Cdc25 RNAi
interfaces, ***p<0.001, Mann-Whitney; see Figure S4). L) Percentage of cells with mispositioned centroso
tripled from 8 to 2 interfaces, ***p<0.001, Mann-Whitney; see Figure S4). L) Percentage of cells with mispositioned centripled from 8 to 26% comparing control and Cdc25 RNAi gonads (**p<0.01, Chi-Square). M-N) Control and Cdc25 RNAi (N) gonad tripled from 8 to 26% comparing control and Cdc25 RNAi gonads (**p<0.01, Chi-Square). M-N) Control (M) and Cdc25 RNAi (N) gonads; gamma tubulin (green), Vasa (magenta), Fasili (white). M) A GSC (solid outline) with one ce and Cdc25 RNAi (N) gonads; gamma tubulin (green), Vasa (magenta), FasIII (white). M) A GSC (solid outline)
with one centrosome anchored at the niche (arrow), and one located opposite (arrowhead). N) A GSC (solid
outline) w and Case Rimar(1, gamma tubulin (green), The (magenta), Then (mate), M, Then (state summa),
with one centrosome anchored at the niche (arrow), and one located opposite (arrowhead). N) A GSC (solid
outline) with two centros with one centrosome anthorem and nichel (arrow), and one located opposite (arrowing), α , α goes (contained). Note that the niche outline) with two centrosomes (arrowheads) oriented away from the niche. outline) with two centrosomes (arrowheads) oriented away from the niche.

STAR Methods

l, RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the

Materials Availability

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

Data and Code Availability

Further information and request for the lead contact, Stephen DiNardo (sdinardo@pennmedicine.upenn.edu).
Furtherials Availability
Six4-Gal4::VP16 *Drosophila* stocks will be provided upon request to the lead contact.
Data Materials Availability
Six4-Gal4::VP16 *Drosophila* stocks will be provided upon request to the
Data and Code Availability
All data reported in this paper will be shared by the lead contact upon
original code. Any addition 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 p
original code. Any add 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

EXPERIMENTAL MODEL AND SUBJECT DETAILS

֦ Transgenic Fly stocks.

From the lead contact upon request.
 EXPERIMENTAL MODEL AND SUBJECT DETAILS
 Transgenic Fly stocks:

Manos-Gal4::VP16 was used to restrict expression of UAS-responder transgenes to germline cells⁵⁹. To rest

expressi EXPERIMENTAL MODEL AND SUBJECT
Transgenic Fly stocks:
Gal4 Stocks
Nanos-Gal4::VP16 was used to restric
expression to somatic gonadal cells, vexperiments. we utilized the previou 【
| 1 (
| 6 (Odia Stocks
Nanos-Gal4:
expression t
experiments
to increase l
label cytoske
domain was
To construct Nanos-Gal4::VP16 was used to restrict expression of UAS-responder transgenes to germline cells⁻⁻. To restrict
expression to somatic gonadal cells, we utilized Six4-Gal4 and Six4-Gal4::VP16 transgenic lines. For knockdowr experiments, we utilized the previously described Six4-Gal4¹², 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
 experiments, we utilized the previously described Six4-Gal4**, 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
la

The cytoskeletal proteins with cell-type specificity, we generated a stronger line where the Gal4 DNA bindir
domain was fused to a VP16 transactivation domain (Six4-Gal4::VP16).
To construct the Six4-Gal4::VP16 line, a pr 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 Six4⁶⁰ was
amplified from genomic DNA with primers lis To construct the Six4-Gal4::VP16 line, a previously identified enhancer
amplified from genomic DNA with primers listed in the Reagent Table, a
entry vector (Invitrogen; K240020). The enhancer was transferred into
pBPGal4.2 - 「 こ 6 ド 仟
| 「 *|* To construct the Six4-Gal4::VP16 line, a previously identified enhancer from the third intron of Six4^{vo} was
amplified from genomic DNA with primers listed in the Reagent Table, and cloned into the pENTR/D-Topo
entry vect 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.⁶¹

pBPGal4.2::VP16Uw (ref.⁶¹; 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 l Fluorescent labeling

For cytoskeletal or nuclear labeling, we utilized multiple fluorescent transgenic constructs. Nuclear chr

was labeled using a His2Av::mRFP1 transgene (FBtp0056035). Somatic F-actin was labeled by P{ りり くらい For cytoskeletal or nuclear labeling, we utilized multiple fluorescent transgenic constructs. Nuclear chromatin was abeled using a His2Av::mRFP1 transgene (FBtp0056035). Somatic F-actin was labeled by P{D-six4-
egfp:moesin (here called Six4-Moe::GFP; ref.⁶²) or Six4-Gal4 > UASp-F-Tractin::TdTomato (a recombinant of
Six4-Gal4 and R egfp:moesin (here called Six4-Moe::GFP; ref.⁶²) 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::GFP⁶³. To visual Myoll wi Six4-Gal4 and RRID:BDSC_58989), whereas Germ cell F-actin was labeled by Nanos-Moesin::GFP°°. To visualize
Myoll with lineage specificity, we utilized a UAS-GFP::Zipper construct³⁴ driven by either a somatic or germline

MyoII with lineage specificity, we utilized a UAS-GFP::Zipper construct³⁴ driven by either a somatic or germline
Gal4. Ubiquitous MyoII was visualized using either sqh-Sqh::mCherry (ref.³¹; RRID:BDSC_99923;
RRID:BDSC_5 Gal4. Ubiquitous Myoll was visualized using either sqh-Sqh::mCherry (ref.³¹; RRID:BDSC_99923;
RRID:BDSC_59024) or sqh-Sqh::GFP3x⁶⁴.
Transgenic manipulation
The following transgenes expressing shRNAs were used in knock RRID:BDSC_59024) or sqh-Sqh::GFP3x°⁻.
T*ransgenic manipulation*
The following transgenes expressing shR
(RRID:BDSC_65947), Myoll RLC (RRID:BD
(RRID:BDSC_32904). P(UAS-hid.Z)2 (RRID
Myoll heavy chain was confirmed by mea
 ה
| | |
| | | Transgenic manipulation
The following transgenes expressing shRNAs were used in knock down experiments: Myoll HC (RRID:BDSC_65947), MyoII RLC (RRID:BDSC_33892), Cdc25 (RRID:BDSC_34831), and E-Cadherii
(RRID:BDSC_65947), MyoII RLC (RRID:BDSC_33892), Cdc25 (RRID:BDSC_34831), and E-Cadherii
(RRID:BDSC_32904). P(UAS-hid.Z)2 (RRID:BDSC_65 (RRID:BDSC_32904). P(UAS-hid.Z)2 (RRID:BDSC_65403) was used to ablate germline cells. Knock
MyoII heavy chain was confirmed by measuring fluorescence intensity of Sqh-Sqh::GFP3x along
interface normalized to unmanipulated (RRID:BDSC). Note that the set of the state of the state of the state of the state is the state of Squad in the state of Squad interface normalized to unmanipulated GSC interfaces (see cytoskeletal polarity analysis sectio My Chain Was confirmed by measuring fluorescence intensity of Sqh-Sqh: Capital along the niche-GSC
interface normalized to unmanipulated GSC interfaces (see cytoskeletal polarity analysis section for more interface normalized to unmanipulated GSC interfaces (see cytoskeletal polarity analysis section for more

manipulated gonads compared to sibling controls and analyzed via a Mann-Whitney test.
In Myoll HC RNAi experiments, some gonads were defective in niche assembly, as evidenced by multiple nicl
aggregates. This is likely bec in Myoll HC RNAi experiments, some gonads were defective in niche assembly, as evidency
aggregates. This is likely because timing of the somatic Gal4 driver cannot be controlled as
example, our pharmacological ROKi treatme | a c c c c *|* 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
analy

aggregates in the somaly accurate multiple in the somalistic sexting niche assembly defects were excluded from
analysis.
Fluorescent balancers
For all transgenic manipulation experiments, utilization of marked balancers al examples.
Examples:
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 con /
Fluoresce
For all tra
identifica
that lacke
balancer
GMR-nvY
For simpl /「it」
11
「it」 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 siblir
that lacked either the Gal4 or the UAS construct, as identified by the presence of the appropriate flu
balancer (TM3, P{w identification:

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 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 r

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 hig Sex identification:
Sex identification:
Male embryos, as well as gonads dissected from male embryo
specific, msSGPs, visualized by high-level expression of the Six-
of Vasa protein as previously described¹².
METHOD DETAI SI
Islam
F Sex identification:
Male embryos, as well as gonads dissected from male embryos, were identified by the presence of male-

METHOD DETAILS

Fixed gonad preparation and staining:

Embryonic gonad dissections and Fixation

embryome gonad assections and Fixation
Unless otherwise noted, embryos were collected and aged in a humid-25 degree C chamber. Embryos we of Vasa protein as previously described¹².
**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 ch of Vasa protein as previously described**.
METHOD DETAILS
Fixed gonad preparation and staining:
Embryonic gonad dissections and Fixation
Unless otherwise noted, embryos were co
either aged 15-17 hours after egg lay (ES1
no **『 『 』**
「 】 (「 「 (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 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
MgCl₂; 2mM CaCl₂ MgCl₂; 2mM CaCl₂) as previously described ¹². Tissue was fixed in 4% formaldehyde, Buffer B (16.7mM KPO₄, pH 6.8; 75mM KCl; 25mM NaCl; 3.3mM MgCl₂), and 0.1% Triton-X-100 for 15 minutes, then washed in PBS (10mM MgCl₂; 2mM CaCl₂) as previously described ²². Tissue was fixed in 4% formaldehyde, Buffer B (16.7mM KPO₄,
pH 6.8; 75mM KCl; 25mM NaCl; 3.3mM MgCl₂), and 0.1% Triton-X-100 for 15 minutes, then washed in PBS
(10mM

Immunostaining

nimunostaming
Primary antibodies were used overnight at 4C. We used Goat antibody against Vasa 1:400 (Santa Cruz. ((10mM Na₂ HPO₄; 1.8mM KH₂PO₄; 2.7mM KCl; 137mM NaCl; pH 7.4) plus 0.1% Triton-X-100 (PBS-Tx). Tissu
was then blocked for 1 hour at room temperature in 4% normal donkey serum in PBSTx.
Immunostaining
Primary antibod 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 discontinu Immunostaining
Primary antibodies were used overnight at 4C. We used Goat antibody against Vasa 1:40
now discontinued); Rabbit antibody against STAT92E 1:350 (gift from E. Bach, NYU); and
ab62341); mouse antibody against F 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 antib 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-102C
Secondary 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 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. Tiss ImmunoResearch) for 1 hour at room temperature. DNA was stained with Hoechst 33342 (Sigma) at 0.2L
for 5 minutes. Tissue was equilibrated overnight in 50% glycerol and 50% Ringers, then mounted with 2%
propyl-gallate in 80 Impuno Book and the Conservation of the Conservation of the Conservation of the Conservation of the Propyl-gallate in 80% glycerol. Images of fixed gonads were acquired on a Zeiss Imager with Apotome using a

10x, 1.2 N.A. propyl-gallate in 80% glycerol. Images of fixed gonads were acquired on a Zeiss Imager with Apotome usir
40x, 1.2 N.A. lens.
Ex-vivo experiments:
Live-imaging
Dissection and live-imaging was performed as previously descr

Ex-vivo experiments:
Live-imaging

propylingallate in 80% glues were acquired to fixed and a stronger with proton and $40x$, 1.2 N.A. lens.
Ex-vivo experiments:
Dive-imaging
Dissection and live-imaging was performed as previously described²⁹. Embryos were Ex-vivo experimer
Live-imaging
Dissection and live
in a 25-degree incu $\frac{1}{2}$ Live-imaging Dissection and live-imaging was performed as previously described²⁹. 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
— in a 25-degree incubator for 14-degree incubator for 14-17 hours after egg lay, and select stage 14-17 hours a
The 14-17 hours after each stage 16-degag lay, and stage 16-degag lay, and select stage 16-degag lay, and selec

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) depend 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 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 (s carrying either a somatic (Six4-Moe::GFP) or germline (Nanos-Moe::GFP) F-actin marker, and in some c
Myoll marker (sqh-Sqh::mCherry). Gonads were imaged using an IX7 Olympus spinning disk confocal, u
63x, NA 1.2 water imme Myoll 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 phot

Laser ablation

My 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
germline (Nanos-Moe: (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::GFF
germline (Nanos-Moe::GFP) mark (Hamaniana photonics, model C21) contracted y model photonical photonical caser ablation
Laser ablation experiments were carried out on dissected gonads carrying eithe
germline (Nanos-Moe::GFP) marker. We prepared gonads f 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

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 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 interfa 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 fo
gonad. Usually only one or Selected stage 16 embryor, but only three interestments who is the therm and remains the comparation of the interfaces to be targeted for ablation, a single time point z-stack was a gonad. Usually only one or two interface 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 wa get fects of global relaxation. The ablating beam generated by a MicroPoint laser emanating from a 405 nm d
cell was focused to the interface through a 100x, 1.4NA lens, using Andor IQ3.2 software. The micropoint la
settin 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.
Simultaneo 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 Simultaneously, Metamorph software was set to stream single color (488 excitation) images from the pof the interface at 250 milliseconds intervals. Streaming acquisition was begun, and then the laser fired treat that inte

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 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 Rho Kinase Inhibitor treatment
For inhibitor treatment
For inhibitor treatments, a single timepoint z-stack was obtained for each gonad to establish
standard. Then, the potent and selective Rho Kinase inhibitor, H-1152, w |
| | s
| c
| c 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 μ M final
concentration with thorough but gentle pipetting (Santa Cruz, sc-203592; Ki = 1.6 nM for Rho Kinase
compared with Ki = 140 standard. The potential of the potential of the potential or the concentration with thorough but gentle pipetting (Santa Cruz, sc-203592; Ki = 1.6 nM for Rho Kin
compared with Ki = 140 nM for the inhibitor Y-27632). Timecompared with Ki = 140 nM for the inhibitor Y-27632). Time-lapse imaging was carried out for 5 hour
inhibitor replenished every 90 minutes⁶⁵. In experiments where laser ablation of the cortical cytoskel
carried out, the inhibitor replenished every 90 minutes⁶⁵. In experiments where laser ablation of the cortical cytoskeleton wi
carried out, the ablations were begun within 15-30 minutes after inhibitor addition.
QUANTIFICATION AND STATIS inhibitor replenished every 90 minutes¹³. In experiments where laser ablation of the cortical cytoskeleton was
carried out, the ablations were begun within 15-30 minutes after inhibitor addition.
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QUANTIFICATION AND STATISTICAL ANALYSIS

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

COUANTIFICATION 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 ag (
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|} Cytoskeletal polarity analysis
Fluorescent gonads were dissected and immunostained either with an a
on the fluorescent transgene present. Niche interfaces were visualized l
fluorescence intensity of F-actin or Myoll, a 3-p (Fcfcrci 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 Fasill. To extract the
fluorescence intensity of F-actin or Myoll, a 3-pixel wide line was drawn over the target niche-GSC interface
and its me fluorescence intensity of F-actin or Myoll, a 3-pixel wide line was drawn over the target niche-GSC i
and its mean grey value returned in Image J. In most cases, the control interfaces were, respectively
niche-niche or GSC 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. Whi
confir niche-niche or GSC-GSC interfaces depending on whether niche cells or germ cells were being analyzed. Wh
confirming Myoll knockdown, however, GSC-GSC interfaces were utilized for normalization, since these
interfaces shoul confirming Myoll knockdown, however, GSC-GSC interfaces were utilized for normalization, since these
interfaces should not have been affected by Myoll knockdown. Mean gray values were background subtracted
by drawing a lin interfaces should not have been affected by MyoII knockdown. Mean gray values were background subt
by drawing a line where no tissue was present, and niche-GSC interface values were normalized to an av
of the respective co 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 by 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, usin of the respective control interfaces taken for the gonal control interest control interfaces of the same
intensity values were either compared between genotypes, or compared to control interfaces of the same
genotype, usin intersity values were either compared between genotypes, using a Mann-Whitney test.

Same of the s

Same of the same of the sa generation and Mann-Whitney test.

ノトト にっかい Niche phenotypic analysis
For analysis of niches from ex vivo cultured gonads, niche circularity was measured as described previously ¹². For analysis of niches from *ex vivo* cultured gonads, niche circularity was measured as described previously ¹⁴.
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::GFF
as a guide to outline the niche on multiple z-planes. Creating this first surface is essential to isolate the n by a sa 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 there 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 mad meeded 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::GF
fluoresc we made a mask of Six4-moe::GFP fluorescence from the first, rough surface. Using the masked Six4-moe::G
fluorescence, we generated the second surface via Imaris's automatic surface generation protocol. We
smoothed the sur 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 smoothed the surface with a 0.5-micron surface detail, and pixel-value thresholds were determined ma
to ensure that the entire niche boundary was included in the surface. Niche surface area measurement:
extracted from the 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 lmaris vantage function.
To measure the per extracted from the second, refined surface for all samples by using the lmaris vantage function.
To measure the percentage of niche area that contacted germ cells, we additionally created a surface of all
germ cells in the To measure the percentage of niche area that contacted germ cells, we additionally created a su
germ cells in the same way we made our niche surface (see above), but instead used Vasa as our
generate the surface. We used a

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 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 gurface that was contacting the germline surface.

Niche cell counts were extracted in Imaris using either Hoechst or His2Av::mRFP1 to visualize individual cel

nuclei and FasIII to visualize cell outlines. Nuclei were als Niche cell counts were extracted in Imaris using ei
nuclei and FasIII to visualize cell outlines. Nuclei w
each cell, and generate the average nuclear distan
distance between the 3 nearest neighbors were ex
Finally, the 3-| r e
| c
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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. Nucle
di each cell, and generate the average nuclear distance between a niche cell and its 3 nearest neighbors. Nucle
distance between the 3 nearest neighbors were extracted from all samples using the Imaris vantage function
Finall 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 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 tr ー
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オ Finally, the 3-dimensional image U. To measure circularity, an ROI was drawn using the freehand selection too to trace the niche boundary labeled by Six4-moe::GFP, and circularity was extracted using the 'shape descriptors

measure 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 the scriptors' tool.

Mann-Whitney tests were used to compare niche parameters between different genotypes. A Wilcox

was used for paired analysis when measuring niche circularity of gonads live-imaged ex vivo at two se

t Mann-Whitney te
Mann-Whitney te
was used for pair
timepoints.
Quantification of
After live-acquisit
vertically, and the | \
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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, wit was used for paired analysis when measuring mene encurantly or gonads live-imaged ex vivo at two separate time
points.
Quantification of retraction velocity
After live-acquisition, images were imported into Image J, with t Marepoints
After live-ac
After live-ac
vertically, ar
frame, follo
intervals; an
the junction
montage. Th Quantification of retraction velocity
After live-acquisition, images were in
vertically, and the time stack croppe
frame, followed by 60 seconds wort
intervals; analyses carried out at 1 so
the junction above and below the 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 secor
intervals; analy frame, followed by 60 seconds worth of post-ablation frames, with each frame in the montage at 5 secore intervals; analyses carried out at 1 second intervals generated the same results. Using the segmented lint
the junctio intervals; analyses carried out at 1 second intervals generated the same results. Using the segmented line t
the junction above and below the cut interface of the niche cell and germline cell was marked throughout t
montag 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 w 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 retra retraction velocities were determined from vertical displacement over time, as presented in the
scatter plots. Peak retraction velocities between treatment conditions or interfaces were com
Mann-Whitney test.
GSC protrusio

retraction velocities between treatment conditions or interfaces were compared via a
Mann-Whitney test.
Mann-Whitney test.
In those cases where severing the cortical cytoskeleton led to pronounced invasion of niche cell te Saction of the protrusion and the continuity of the basic interfaces were compared in those cases where severing the cortical cytoskeleton led to pronounced invasion of niche cell territory in those cases where severing th *GSC protrusion quan*
In those cases where
the germ cell, the exi
length of the protrus
comparing that to th (I
I
I
C 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 t the germ can, the entriest in the extent of plantified in the nanos-model in the Lange can by measuring the pro
length of the protrusion from its base by tracing the arcing germ cell outline along the protruded part and
co comparing that to the vertical distance at the base of that protrusion. In this manner, no or little protrusion
comparing that to the vertical distance at the base of that protrusion. In this manner, no or little protrusio comparing that to the vertical distance at the base of that protrusion. In this manner, no or little 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 interes Quantification of STAT accumulation

STAT intensity was extracted in Image J from anti STAT-stained gonads using regions of inter

include the Vasa signal of germline cells. In Figure 5C, for each gonad, we sampled 5 GSCs C vii ke r c v 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
nich germ cells in contact with the niche, and 3 posterior germ cells located more than one cell diameter from th
niche. We background-subtracted the average fluorescence intensity from each GSC and GC by drawing an
directly ou or inche. We background-subtracted the average fluorescence intensity from each GSC and GC by drawing an RC
directly outside of the gonad. Relative STAT enrichment values were obtained by dividing the background-
subtracte 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. particular gonad. For Figure 5D, STAT positive cells were quantified by measuring the normalized fluore
intensity of each germ cell directly contacting the niche as described. To establish a threshold over whic
would be sc 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 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 (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

Centrosome orientation analysis

than 1.44-fold enriched (average enrichment minus 1 standard deviation). Data was obtained from sibling
control and Myoll HC RNAi conditions and analyzed with a Mann-Whitney test.
Centrosome orientation analysis
Centrosome Than 1.44-fold enring in the MAI conditions and analyzed with a Mann-Whitney test.

Centrosome orientation analysis

Centrosomes were visualized with immunofluorescence against Gamma tubulin. GSCs contacting the niche

wer Centrosome orientation analysis
Centrosomes were visualized with immunofluorescence against Gamma tubulin
were only scored for centrosome position if they had 2 clearly discernible centr
undergone centrosome duplication. F 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 wa
located at the undergone centrosome duplication. For each such GSC, we scored whether one of the two centrosomes walocated at the niche, as evidenced by cortical localization (visualized with Vasa) and proximity to the niche (visualized 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
"anchore (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

"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 constr "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 th GSC division angle analysis.
We surmised that the spatial constraints imposed by the sp
division angles compared to that reported for the adult test
rigorous 3-dimensional analysis described next, Figure 5K in
slightly bro (ヽ c r c こ 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 t
rigorous 3-dimensional analysis described next, Figure 5K indeed showed that the distribution of angles
slightly b 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^{21,25,66}. slightly broader than expected from previously published 2-dimensional centrosome and spindle
analyses^{21,25,66}. To preserve the 3-dimensional nature of the division coordinates, we used the model from
ref.³⁸. Therefore slightly analyses^{21,25,66}. To preserve the 3-dimensional nature of the division coordinates, we used the moref.³⁸. Therefore, we calculated division angles from live-imaged Six4-Moe::GFP;His2AV::mRFP1 g
on two paramet ref.³⁸. Therefore, we calculated division angles from live-imaged Six4-Moe::GFP;His2AV::mRFP1 gonads, based
on two parameters as described by ref³⁸: 1) a plane of best-fit to represent the niche interface, and 2) the
 on two parameters as described by ref³⁸: 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 p
was on two parameters as described by ref³⁰: 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 p
was 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 (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
plan (visual) 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
traje 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
each chromo plane trajectory for anaphase, telophase, or metaphase nuclei. The coordinates were taken by marking the veach chromosome complement, or for a metaphase figure, by marking a point on either side of the chromosome complex l the 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 a

entromosome complex located midway on its long axis. The division angle was calculated as the angle
the best-fit plane and this inferred division trajectory. In all cases, division angle distribution betwee
and ROKi-treate 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 dist 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 pla We also tested if control or ROKi divison angle distribut
Note that from the perspective of an external reference
cell, not all division angles are equally likely in a random
the distribution of spindles will exhibit a bia 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 th Note that from the perspective of an external reference plane, such as the niche-GSC interface, in the cell,
cell, not all division angles are equally likely in a random model. If spindle position is not regulated in the c che distribution of spindles will exhibit a bias toward shallow angles, as shown in Figure S2E, F, and as
we distribution of spindles will exhibit a bias toward shallow angles, as shown in Figure S2E, F, and as the distribution of spin distribution of spin distribution of spin distribution $\mathcal{L}_\mathcal{S}$

described in ref³⁸. 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 surfac 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 equ therefore calculated the probability of cells dividing within 10-degree intervals from 0-90 degrees, followi
the equation from ref³⁸. We then graphed the actual percentage of control or ROKi cells that divided with
these the equation from ref³⁸. 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.
 Supplemental Information:

Doc the equation from ref³⁹. 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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Supplemental Information:
Document S1. Figures S1-S4 Supplemental Information:

Document S1. Figures S1-S4

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Pre-Cut

5s Post-Cut

Timelapse (5s Intervals)

ROKI

Somatic Myoll HC RNAi

Somatic Myoll RLC RNAi

DNA, Somatic F-Actin H^o $\overline{}$ bioRxiv pre<mark>pr</mark>int doi: <mark>https://doi.org/10.1101/2023.09.08.556877</mark>; this version posted August 7, 2024. The copyright holder for this preprint (which was **not certi<u>fi</u>ed by peer review) is the a**uthor/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under [aCC-BY-NC-ND 4.0 International license.](http://creativecommons.org/licenses/by-nc-nd/4.0/) **Fasciclin III**

Somatic Myoll HC RNAi

Number of STAT+ GSCs

Percentage of niche area contacting germ cells

F

RNAi (n=129)

ction

compa

ate

Germline Hid.Z

Germline Cdc25 RNAi

Germline Cdc25 RNAi

