Phosphorylated Groucho delays differentiation of follicle stem cells by acting as a molecular memory of niche-based EGFR signaling
Phosphorylated Groucho delays differentiation of follicle stem cells by acting as a molecular memory of niche-based EGFR signaling

by

Michael James Johnston

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the
Dedication

This work is dedicated to my family: Dave, Marilyn, and Matthew Johnston. Despite being over a thousand miles from home, I always felt their complete support and encouragement. On tough days, it made all the difference in the world.
Acknowledgements

I have many people to thank for making this work possible.

To Todd Nystul – You have been a great mentor. I aspire to attain your photographic recall of the relevant literature in the field. I relied upon your understanding and willingness to compromise as a supervisor. I will greatly miss our other conversations as friends.

To the rest of the members of the Nystul Lab – Dinara, Mari, Angela, Matt, Yassi, Rebecca, Chelsea, Pankaj, Sumitra, Bryne, and Stephanie – Thank you for all your assistance and patience. Our lab is a great place because of the way we help and listen to each other. I have learned much from you and had some great fun in the process.

To the UCSF research community – Thank you for assisting me through my many technical hurdles. In particular, I would like to thank Mike Kissner and Michael Lee for their assistance in conducting FACS experiments, Greg Fedewa and Chris Williams for answering numerous RNA sequencing questions, and Bree Grillo-Hill for finding a hidden Six4 antibody. I also thank the Jan, O’Farrell, and Brückner labs for many creative discussions and shared fly lines.
Abstract

In the epithelial follicle stem cells (FSCs) of the Drosophila ovary, Epidermal Growth Factor Receptor (EGFR) signaling promotes self-renewal whereas Notch signaling promotes differentiation of the prefollicle cell (PFC) daughters. We have identified two proteins, Six4 and Groucho (Gro), that link the activity of these two pathways to regulate the earliest cell fate decision in the FSC lineage. Our data indicate that Six4 and Gro promote differentiation toward the polar cell fate by promoting Notch pathway activity. This activity of Gro is antagonized by EGFR signaling, which inhibits Gro-dependent repression via p-ERK mediated phosphorylation. We found that the phosphorylated form of Gro persists in newly formed PFCs, which may delay differentiation and provide these cells with a temporary memory of the EGFR signal. Collectively, these findings demonstrate that phosphorylated Gro labels a transition state in the FSC lineage and describe the interplay between Notch and EGFR signaling that governs the differentiation processes during this period.
Table of Contents

Introduction and Background ........................................................................................................... 1
  Stem cells and their niches .............................................................................................................. 1
  Studying stem cells in Drosophila ................................................................................................. 3
  The follicle epithelium .................................................................................................................... 4
  Signaling pathways involved in patterning the follicle epithelium .................................................. 7
  Interactions of the EGFR and Notch signaling pathways ............................................................... 8

Results ............................................................................................................................................... 10
  Live imaging ................................................................................................................................... 10
  Attempted direct isolation of FSCs ................................................................................................. 11
  Isolation of FSC-like pseudo stem cells ....................................................................................... 13
  RNA-seq of early FCs expressing EGFR^{top} identifies transcription factors essential to niche .... 17
  Six4 is required for the specification of polar and stalk cells ....................................................... 21
  Six4 promotes the polar cell lineage via Notch signaling ............................................................. 26
  The global co-repressor groucho promotes prefollicle cell differentiation .................................... 30
  Groucho phosphorylation is enriched in the FSC niche ............................................................... 35
  Gro is required for FSC maintenance while Six4 loss induces hypercompetition ....................... 36

Discussion ......................................................................................................................................... 39
  Live imaging ................................................................................................................................... 39
  Isolation of FSCs ............................................................................................................................. 40
  Identification of transcription factors involved in follicle cell differentiation ............................... 40

Materials and methods ...................................................................................................................... 44
  Fly stocks ...................................................................................................................................... 44
Immunofluorescence: ........................................................................................................46
Live imaging ......................................................................................................................48
Clone induction: ..............................................................................................................49
FSC competition assay .....................................................................................................49
RNA-seq of PFCs expressing EGFR^{top} .........................................................................50
CRISPR generation of Six4^{108} allele ............................................................................52
References .......................................................................................................................54
List of Tables

Table 1. RNAi screen of differentially expressed transcription factors ............................................. 19
Table 2. Competition bias values ........................................................................................................... 39
List of Figures

Figure 1. The *Drosophila* germarium ................................................................. 5
Figure 2. Ex-vivo live imaging of histone-GFP flies allows observation of mitotic events ....... 11
Figure 3. Intersection labeling of FSCs using 109-30 and 13C06 drivers .......................... 12
Figure 4. Expression of EGFR<sup>Δtop</sup> to expand and isolate pseudo-FSCs ...................... 14
Figure 5. Results of FACS isolation of FCs .................................................................. 16
Figure 6. Differential expression of transcription factors by RNA-seq of follicle cells expressing EGFR<sup>Δtop</sup> ........................................................................ 18
Figure 7. Knockdown of Six4 disrupts the follicle epithelium with high penetrance ............ 20
Figure 8. MEME Suite analysis indicates Eip74EF and da binding sites upstream of upregulated genes .................................................................................................................. 21
Figure 9. Six4 staining in the germarium ........................................................................ 23
Figure 10. Six4 is required for polar and stalk cell formation ........................................... 25
Figure 11. Polar cell maintenance is unaffected by Six4 loss or EGFR<sup>Δtop</sup> expression ........ 26
Figure 12. Six4 promotes the polar cell lineage via Notch signaling ................................. 28
Figure 13. Six4 and Gro promote polar cell specification ................................................. 29
Figure 14. Six4 is likely upstream of Notch cleavage ...................................................... 30
Figure 15. Expression of gro<sup>AA</sup> disrupts the follicle epithelium with high penetrance .... 32
Figure 16. Gro is required for the specification of the polar and stalk cell lineages .......... 33
Figure 17. Groucho promotes Notch signaling in the germarium .................................. 34
Figure 18. Groucho is specifically phosphorylated in FSCs and early PFCs ....................... 35
Figure 19. Six4 and Gro are required to maintain neutral FSC competition ...................... 38
**Introduction and Background**

**Stem cells and their niches**

Multicellular organisms must be able to maintain many specialized tissues and cell types throughout the lifetime of the organism. In many tissues, stem cells are responsible for sensing the current state of the tissue and responding appropriately to ensure tissue homeostasis.

Stem cells are defined by their ability to self-renew and to produce progeny that differentiate into one or more cell types. Stem cells typically depend on a stem cell niche for their regulation. The niche provides signaling molecules that promote division and self-renewal of the stem cell, prevent premature stem cell differentiation, and promote differentiation of daughter cells away from the niche. These differentiated daughter cells typically comprise the functional cells of the tissue.

The average citizen is likely most familiar with discussions of human embryonic stem cells. In early human development, cells of the inner cell mass of the blastocyst are capable of dividing and differentiating to generate all cell types of a fully-formed adult human. Cells can be isolated from this inner cell mass, and then maintained in appropriate cell culture conditions to establish an embryonic stem cell line. As embryonic stem cells are pluripotent - they can generate all cell lineages with the exception of placental material - these cells offer broad therapeutic potential if we can understand and control the differentiation of these cells. Studying and experimenting with these cells is politically controversial due to the moral implications of isolating and working with cells that could potentially grow into a human life. Stem cell therapies are frequently discussed in the media as the cutting edge in new biological treatments. The potential to isolate or induce pluripotent stem cells from a patient offers the potential to repair tissues that would otherwise fail to regenerate on their own. The great therapeutic potential
of these cells combined with the potential moral hazard highlights that with great power comes great responsibility.

However, for all the attention given to embryonic stem cells, the study of adult stem cells remains fertile ground for the study of developmental biology, cellular decision-making, and therapeutic intervention. Unlike embryonic stem cells, which exist transiently during the development of the organism, adult stem cells persist throughout the lifetime of the organism and remain responsible for maintaining tissue homeostasis in response to environmental conditions and injuries.

Many epithelial tissues of the human body are maintained by adult epithelial stem cells including the intestine, breast, brain, lung, hair, and others. (Blanpain et al., 2007; Gonzalez-Perez, 2012; Donne et al., 2015). Understanding the regulation of these adult stem cell populations is of great importance considering that dysregulation of stem cells in these tissues is believed to be a causative event in the formation of cancer (Zhu et al., 2016; Tomasetti and Vogelstein, 2015). Most differentiated cell types are limited in the number of times that they can divide. This ensures that as epithelial cells are damaged by environmental insults, they are limited in their potential to propagate a dysregulated cell lineage. However, when a stem cell becomes dysregulated, these safeguarding limits are lost. Not only can compromised stem cells divide indefinitely over the lifespan of an organism, but they can also produce disproportionate numbers of daughter cells that can have negative effects on the organism, such as promoting too much growth or lacking a required cell lineage.

The task of understanding adult epithelial stem cell regulation has proven both technically and biologically challenging. In the case of the mouse intestinal epithelium, despite the identification of label-retaining stem cells over forty years ago (Potten at al., 1974) and many
more recent breakthroughs involving biomarker identification, isolation, and characterization of intestinal epithelial stem cells (Barker et al., 2007; Sangiorgi and Capecchi, 2008; Formeister et al., 2009; Gracz et al., 2012), there remain significant gaps in knowledge regarding the reliability and exclusivity of established biomarkers, the co-existence of multiple functionally-distinct stem cell populations, and the varying degrees of stemness observed within cells of the intestinal niche (Yan et al., 2012; Roche et al., 2015; Gracz and Magness, 2014). Model systems remain an incredibly valuable resource for the exploration and development of current stem cell regulatory models.

**Studying stem cells in Drosophila**

Work on Drosophila neural stem cells was incredibly enlightening toward the idea of the asymmetric division (Knoblich and Jan, 2002; Jan and Jan, 1998). Additional work on the male and female germline cells helped to establish the concepts of promoting self-renewal and inhibiting differentiation with the stem cell niche (McKearin and Ohlstein, 1995; Xie and Spradling, 1998; Xie and Spradling, 2000; Tran et al., 2000; Fuller and Spradling, 2007; de Cuevas and Matunis, 2011). The Drosophila follicle stem cell (FSC) offers certain advantages over these other cell types (Sahai-Hernandez et al., 2012). First of all, the FSC lineage is maintained throughout the lifespan of the organism, unlike the developmental neural stem cells. Additionally, the FSC lineage is somatic in nature. While germline stem cells make many important decisions regarding division, growth, and differentiation, there are many examples of gametes undergoing modes of genetic regulation that are not observed in the soma.

Ultimately, understanding epithelial tissues is of the utmost concern when it comes to addressing stem cell regulation in tissues such as the blood, breast tissue, neurons, or the
intestinal lining where aberrant stem cells are thought to have a role in tumor initiation (Arwert et al., 2012; Costea et al., 2008). Thus, we used the Drosophila FSC to model adult epithelial stem cell regulation.

The follicle epithelium

The development of eggs within the Drosophila ovary was first described by King (King, 1970). The Drosophila ovary is comprised of many long strands of developing follicles termed ovarioles. Many ovarioles join together to form the ovary in a shape reminiscent of a large bunch of bananas (Fig. 1A). At the anterior tip of each ovariole, there is a small peanut-shaped structure termed the gerarium, which contains two FSCs. Within the gerarium, a population of stromal inner gerarial sheath cells (IGSCs, also known as escort cells) support germ cell development in Regions 1 and 2a, and provide niche factors that anchor the FSCs at the Region 2a/2b border to promote self-renewal (Fig. 1B, D).
**Figure 1. The Drosophila germarium.** (A) Ink drawing of the Drosophila ovary, adapted from Miller, 1950. Boxed yellow region indicates the germarium. (B) Schematic presentation of a germarium and the most anterior budded follicles. The regions of the germarium and stages of follicle development are indicated below. Cell are color coded to match the lineages indicated in panel C. Early germline and IGSCs have been faded to highlight the beginning of the follicle epithelium. (C) Map of cell lineages in the follicle epithelium including some of the known signaling inputs. EGFR promotes FSC self-renewal, Notch promotes the polar fate, and Upd promotes the stalk fate. Expression of Cas or Eya is indicated on each
(D) Morphology of a wildtype germarium. Fas3 (red) staining outlines cell membranes in early follicle cells. Vasa (green) staining marks the germline cysts of the developing follicles. In this and all subsequent images, DAPI is in blue. (E) Differentiation status of wildtype follicle cells as monitored by staining for Cas (green) and Eya (red). Undifferentiated prefollicle cells express both Cas and Eya (solid line), while main body cells express only Eya, and polar/stalk cells (arrowheads) express only Cas.

One of the challenges of working with the FSC niche is that, unlike other model stem cell populations that have reproducible markers that labels the stem cell population (e.g. Lgr5+ cells of stem cells within the mouse intestinal crypts; Barker, 2014), the FSC niche does not have markers that uniquely and consistently label the stem cells within the tissue. Previous research in our lab has identified two signaling pathway readouts that are detected with high frequency in FSCs but not downstream daughters. Sahai showed that Wingless signaling, as monitored by the Wingless signaling reporter Wf-WRE, is present in stem cells 65% of the time and is not found in downstream daughter cells (Sahai-Hernandez and Nystul, 2013). Castanieto showed that an antibody which detects phosphorylated Extracellular-signal Related Kinase (pERK) is active in 90% of FSCs, however pERK signal can also be frequently observed in IGSCs and 13% of PFCs just downstream of the niche (Castanieto et al., 2014). Additionally, both of these markers are intracellular, which precludes antibody detection and subsequent cell sorting based on these markers.

The best molecular markers to monitor PFC differentiation into stalk, polar, or main body follicle cells are the combined staining pattern of Castor (Cas) and Eyes Absent (Eya). The Montell Lab has published that FSCs and undifferentiated follicle cells in the germarium are Cas+Eya+ whereas mature main body follicle cells are Cas−Eya+, and both stalk and polar cells are Cas+Eya− (Fig. 1B,C,E; Chang et al, 2013).
**Signaling pathways involved in patterning the follicle epithelium**

The niche that supports FSC self-renewal in this position has a very limited range, resulting in the activation of the Wingless (Wg) and EGFR pathways in the FSCs but not in the immediately adjacent prefollicle cell (PFC) daughters (Castanieto et al., 2014; Sahai-Hernandez and Nystul, 2013). Wg and EGFR signaling are required for FSC self-renewal but do not appear to be required for PFC differentiation (Castanieto et al., 2014; Song and Xie, 2003). However, constitutive activation of either pathway inhibits PFC differentiation and, in the case of EGFR signaling, increases the propensity of mutant cells to occupy the FSC niche and self-renew (Castanieto et al., 2014; Song and Xie, 2003). These findings demonstrate that Wg and EGFR pathway activity are part of an FSC specific program that is absent in the PFCs immediately downstream from the niche.

An orderly series of events directs the differentiation of PFCs into each of the three main follicle cell types: polar cells, stalk cells, or main body follicle cells (Fig. 1C). First, approximately 2-3 divisions downstream from the FSC division, a subset of PFCs receive a Delta signal from the germline that activates Notch signaling and initiates differentiation toward the polar cell fate (Lopez-Schier and St Johnston, 2001; Nystul and Spradling, 2010). Next, these newly-specified polar cells secrete the Jak-Stat ligand Unpaired (Upd) to initiate the differentiation of other PFCs into stalk cells (Assa-Kunik et al., 2007). It is unclear when the main body follicle cell fate is specified, but a single PFC division can produce one daughter cell that differentiates into a polar or stalk cell and another daughter that differentiates into a main body follicle cell, suggesting that PFCs do not commit to an exclusively main body follicle cell fate prior to polar cell specification (Chang et al., 2013; Nystul and Spradling, 2010). Much less is understood about the signaling that occurs after newly-produced PFCs have exited the niche.
and prior to the initiation of Notch signaling. PFCs that have exited the niche do not receive self-renewal signals and yet retain the ability to re-enter the niche and assume the stem cell fate. Additionally, these cells contact the germline almost immediately after they exit the niche, yet Notch activation and polar cell differentiation is delayed.

**Interactions of the EGFR and Notch signaling pathways**

As our lab has previously published that EGFR is necessary for stem cell maintenance (Castanieto et al., 2014) and Notch signaling is thought to be the earliest differentiation signal that follicle cells receive (Lopez-Schier and St Johnston, 2001), it is important to understand how these pathways operate and potential interactions between their signaling activities (reviewed in Hasson and Paroush, 2006; Doroquez and Rebay, 2006). Briefly, EGFR is a single-pass transmembrane receptor that homodimerizes and self-phosphorylates upon exposure to an EGF ligand (Dawson et al., 2005). In *Drosophila*, there is one known EGFR, while there are four activating EGF ligands – *gurken, keren, spitz*, and *vein* (Shilo, 2003). The precise source and nature of the EGF ligand(s) in the germarium is unknown, but is an active area of research within our lab. Ligand binding causes conformational changes which lead to the phosphorylation of the cytoplasmic tail of EGFR which subsequently promotes many additional phosphorylation events (Herbst, 2004). Among many other downstream EGFR phosphorylation targets, the sequential phosphorylation of Ras (or *Ras1; Ras85D*), Raf, MEK (or *Dsor1*), and then ERK (or *rl; MAPK*) constitute the canonical kinase cascade responsible for propagating and amplifying the signal of EGFR activation within the cell (Shilo, 2003; Herbst, 2004).

Notch signaling is also initiated by ligand-receptor interactions at the cell surface. However, unlike EGF ligands which are able to travel through intercellular space, the Notch
ligands Delta and Serrate are membrane tethered, meaning that they are only able to activate Notch signaling in cells that they directly contact. In the gerarium, the source of Delta ligand is the anterior face of the developing germline cyst (Lopez-Schier and St. Johnston, 2001; Torres et al., 2003).

In the absence of ligand binding, transcription of Notch target genes is inhibited by a repressive complex including Suppressor of Hairless (Su(H)). Su(H) binds to the DNA and recruits the global co-repressor groucho (Gro) to maintain repression of these target genes (Barolo et al., 2002). Upon ligand binding, the intracellular domain of the Notch receptor (N\text{intra}) is cleaved (Bray, 2006). The glycosyltransferase Fringe (fng) is able to glycosylate the Notch receptor to enhance interactions between the Notch receptor and the ligand Delta, thus sensitizing signaling activity of the Notch pathway (Panin et al., 1997). Once N\text{intra} is released from the membrane, it relocates to the nucleus where it binds to Su(H). Together, Su(H) and N\text{intra} form an activating complex that promotes the transcription of downstream Notch targets, including the Enhancer of Split (E(spl)) family of genes (Bray, 2006). The E(spl) genes bind to DNA at different genomic targets, and are capable of recruiting Gro to repress these targets (Bray, 2006). Thus, one outcome of Notch signaling activation is to switch Gro from repressing Notch target genes to repressing E(spl) target genes.

Finally, an established point of cross-talk between these pathways is the molecule Gro (Hasson et al., 2005). Briefly, the E(spl) family of genes are the primary mediators of Notch signal activation, and they depend on interactions with Gro to subsequently repress their target genes. Therefore, Gro is an essential downstream component of Notch signaling activation. Interestingly, EGFR activation causes phosphorylation of ERK (pERK), and pERK is capable of phosphorylating Gro (pGro). This phosphorylated form of Gro is greatly diminished in its ability
to act as a transcriptional repressor. Therefore, by promoting the conversion of Gro to pGro, EGF signaling activity removes a key downstream component of Notch signaling. Therefore, active EGFR signaling prevents complete Notch signaling activation.

With this study, we sought to identify transcriptional targets of EGFR signaling that influence FSC fate decisions. Our focus was to find genes that maintain the undifferentiated population by preventing precocious differentiation of FSCs and early PFCs. We were also interested in genes that promoted differentiation specifically in the late PFC population by modulating the activity of fate-determining signaling pathways.

**Results**

**Live imaging**

The most specific and consistent markers of FSC fate are Wf-WRE and pERK, which indicate activity of the Wg and EGFR pathways, respectively. However, neither of these markers is observed 100% of the time in both FSCs. Based on this data, we hypothesized that the activities of the Wg, EGFR, or other pathways might be transient due to varying environmental signals or cell cycle influences on signaling pathway responsiveness. Ex-vivo live imaging of the gerarium has recently been published (Morris and Spradling, 2011), and thus we sought to optimize live imaging conditions within our own lab, with the hope of eventually monitoring signal pathway reporters in real time. There are many transgenic fly lines available that contain genetically encoded fluorescent proteins that are ideal for live imaging. Some of these include histone-GFP (to label all nuclei), shotgun-mTomato (to label all adherens junctions), fax-GFP (labels cell membranes in gerarium), act>S-G2-M-GFP (to transiently label cells depending on their progression through the cell cycle), and dad-RFP (to report BMP signaling in FCs).
Imaging of the histone-GFP fly line offered the best signal intensity, and allowed for the observation of mitotic events (Fig. 2). Other reporter lines were imaged, but the results were less informative. Membrane markers such as shotgun-mTomato and fax-GFP offered lower signal intensity. The act>S-G2-M-GFP was not ideal as cells progressing beyond mitosis would no longer be visible to be imaged. The dad-RFP reporter appeared to work, but there were no regions of the germarium with notably higher intensity, nor cycling intensity.

**Figure 2.** Ex-vivo live imaging of histone-GFP flies allows observation of mitotic events. (A) Early germ cell before division. (B) Condensed germ cell chromosomes. (C) Separation and decondensation of chromosomes. (D) Early follicle cell before division. (E) Condensed chromosomes during mitosis. (F) Separation of chromosomes during telophase. Images taken on 12m intervals for 5h imaging session.

**Attempted direct isolation of FSCs**

To study the molecular components active in stem cells, one must first separate these stem cells from the rest of the cells in the tissue. As there are no known FSC-specific gene expression markers, direct isolation of these cells is difficult.
To attempt direct isolation of FSCs, we combined two transgenic constructs whose expression pattern intersects to specifically label FSCs. Earlier work in the Nystul lab showed that the driver 13C06-Gal4 expresses in posterior IGSCs and FSCs (Sahai-Hernandez, 2013). The driver 109-30-Gal4 expresses FSCs and early FC daughters (Hartman, 2010). The intersection of these two markers should exclusively contain the FSC population. With the goal of isolating the FSC population in mind, I generated a fly line that contains the 13C06 promoter directly driving GFP. I subsequently generated a fly line with the genotype 109-30-Gal4, UAS-RFP, 13C06-GFP to visualize both drivers simultaneously. Intersection labeling using these two drivers appears to label FSCs and the most posterior IGCSs.

However, in this genetic system, there is no UAS-Gal4 genetic amplification of GFP transcription, and the 13C06 promoter region only allowed for very limited expression of GFP. The resulting fluorescence was very dim, proved difficult to detect on a microscope, and impossible to detect on a FACS machine. Ultimately, due

Figure 3. Intersection labeling of FSCs using 109-30 and 13C06 drivers. (A) Overlapping expression of 109-30 and 13C06 occurs at the border between regions 2a and 2b of the gerarium. This includes FSCs and the most posterior IGCSs. (B) 109-30 fluorescence alone. (C) 13C06 fluorescence alone.
to insufficient label intensity, this approach was abandoned, as well as our attempts to specifically purify wildtype stem cells.

**Isolation of FSC-like pseudo stem cells**

As direct isolation of FSCs proved technically challenging, we sought to artificially expand the population of FSC-like cells in the germarium. Previous studies of the female germline have successfully utilized overexpression of the stem cell self-renewal factor Dpp to prevent germline differentiation. Subsequently, the population of undifferentiated germ cells was expanded, isolated, and studied (Kat et al, 2005). In an analogous approach, we overexpressed a constitutively active construct of EGFR termed λ-top (EGFR\textsuperscript{λtop}). Expression of EGFR\textsuperscript{λtop} in the germarium inhibits follicle cell differentiation, as evidenced by an expansion in the number of Cas\textsuperscript{+} Eya\textsuperscript{+} cells (Fig. 4A,B). An overexpression construct of UAS-mCD8-GFP was then included in this genotype to allow for fluorescence-activated cell sorting (FACS) of the 109-30 population (Fig. 4C,D).
Figure 4. Expression of $EGFR^{\text{top}}$ to expand and isolate pseudo-FSCs. (A-B) The population of Cas$^+$ Eya$^+$ cells is expanded in in number and in distance from the niche, as indicated by the yellow line at the edge to the germaria. (C-D) Expression of $mCD8$-$GFP$ along with $EGFR^{\text{top}}$ causes these cells to fluoresce green, and tethers an $mCD8$ peptide fragment to the extracellular side of the plasma membrane.
For many months, we attempted to isolate the expanded population of Ltop-expressing PFCs by use of FACS. Consistently, the scatter plot of the flow cytometry data indicated high amounts of debris, while analysis of the RNA isolated from the sorted cell fraction indicated very low RNA concentrations, and very low RNA quality.

Considering that a previously described FACS method for isolating FCs includes a specific low-pressure FACS protocol (Borghese et al., 2006), it is likely that our FACS procedure was too harsh and that the cells were lysing or shearing apart within the machine. Despite many attempts with different FACS conditions, we were unable to emulate the successful FACS isolation of FCs demonstrated by the Rørth Lab.

A gentler alternative to FACS is magnetic-activated cell sorting (MACS), which has previously been used to isolate follicle cells (Wang et al., 2006). The cells expressing EGFR\textsuperscript{Ltop} and mCD8-GFP contain an external CD8 fragment that is available for antibody detection. By exposing dissociated cells to an anti-CD8 magnet-conjugated antibody and then passing the cells over a strong magnetic column, only the desired cells are maintained on the column. We used this method to isolate both 10930\textsuperscript{Ls}>CD8-GFP+EGFR\textsuperscript{Ltop} cells, as well as 10930\textsuperscript{Ls}>CD8-GFP control cells. The RNA yield and quality was drastically improved.
Figure 5. Results of FACS isolation of FCs. (A) Representative scatter plot displaying fluorescence intensity measured by the red and green detectors by flow cytometry. Red fluorescence serves as a negative control as this only captures autofluorescence of the cells (blue region). Green fluorescence should only be observed in the presence of GFP expression (green region). No green fluorescence is measured in the yw control flies. Many green cells are detected in the EGFR\textsuperscript{λtop} flies. However, this cell population is not completely resolved from the autofluorescent population in the middle of the plot. (B) Representative Bioanalyzer trace of the sizes of the total RNA species isolated by FACS of FCs. The fluorescence intensity (y-axis) is low as the RNA yield was low. Total RNA in a high quality sample should be overwhelmingly comprised of the large ribosomal RNA subunits, which occur as a twin peak in Drosophila (elution time ~47s). Many smaller RNA species are evident in this trace (elution times 25-45s), indicative of RNA degradation. (C) Representative Bioanalyzer trace of the sizes of the total RNA species isolated by MACS of FCs. RNA yield is much greater (y-axis scale is much larger), and RNA species smaller than the ribosomal RNAs are virtually absent, indicating high quality RNA. Note that the calibration of RNA elution time and RNA size from Bioanalyzer traces is often variable, and therefore only relative, not absolute, comparisons should be made.
RNA-seq of early FCs expressing EGFR\textsuperscript{\lambda top} identifies transcription factors essential to niche

As EGF signaling is required for FSC maintenance in the niche (Castanieto et al., 2014), we sought to identify transcriptional targets of EGF signaling by performing massively parallel RNA sequencing. Using MACS, we isolated follicle cells with 109-30\textsuperscript{ts} driving the expression of CD8::GFP either alone or in combination with EGFR\textsuperscript{\lambda top}. Duplicate samples were collected for both genotypes for a total of four samples. The mRNA from each sample was then purified and prepared for sequencing on an Illumina HiSeq device. Output sequencing reads were aligned to Drosophila genome using Tophat2, and testing for statistically significant differential expression was performed using both CuffDiff and DESeq2. We identified 2286 genes with significant differences in expression (p-adj<0.01, DESeq2 method).

As the EGF pathway is expected to have many downstream targets, we initially chose to focus transcription factors, as this class of proteins is known to modulate transcriptional activities of many genes simultaneously. We limited our search to those classified by FlyTF.org as “trusted” transcription factors. To identify transcription factors that regulate PFC differentiation, we performed an RNAi screen using 109-30\textsuperscript{ts} and RNAi lines from the TRiP collection (Ni et al., 2011) for 26 of the 40 transcription factors with the most statistically significant differences in gene expression (Figure 6; Table 1). Ovarioles were examined for gross disruption of the follicle epithelium, as monitored by staining for Fas3 to mark cell membranes of early follicle cells, and Vasa to mark germline cysts of the developing follicles. The most severe and highly penetrant follicle cell phenotype we observed was caused by knockdown of Six4 (Figure 7; Table 1).
Figure 6. Differential expression of transcription factors by RNA-seq of follicle cells expressing EGFR$^{\lambda_{top}}$. Plot of expression counts (FPKM: Fragments Per Kilobase of transcript per Million mapped reads; as determined by Cuffdiff) observed in EGFR$^{\lambda_{top}}$ overexpression vs WT follicle cells. Each dot represents a distinct transcription factor. Black dots are insignificant while white dots are significant at p-adj < 0.01 as determined by DESeq2. The line y=x represents a zero-fold change.
<table>
<thead>
<tr>
<th>symbol</th>
<th>FBgnID</th>
<th>DE_padj</th>
<th>DElog2FC</th>
<th>RNAi line tested (Bloomington ID#)</th>
<th>Phenotype penetrance in FCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>abd-A</td>
<td>FBgn0000014</td>
<td>2.50E-03</td>
<td>-0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alh</td>
<td>FBgn0261238</td>
<td>9.80E-03</td>
<td>-0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cf2</td>
<td>FBgn0000286</td>
<td>1.00E-04</td>
<td>-1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG6272</td>
<td>FBgn0036126</td>
<td>1.00E-04</td>
<td>-0.97</td>
<td>33652 none</td>
<td></td>
</tr>
<tr>
<td>cnc</td>
<td>FBgn0262975</td>
<td>0.00E+00</td>
<td>-1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>FBgn0000412</td>
<td>2.00E-04</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>da</td>
<td>FBgn0000413</td>
<td>1.70E-03</td>
<td>-0.92</td>
<td>38382 none</td>
<td></td>
</tr>
<tr>
<td>dl</td>
<td>FBgn0260632</td>
<td>8.90E-03</td>
<td>-0.71</td>
<td>34938 none</td>
<td></td>
</tr>
<tr>
<td>Dref</td>
<td>FBgn0015664</td>
<td>6.60E-03</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E(spl)m3-HLH</td>
<td>FBgn0002609</td>
<td>1.20E-03</td>
<td>1.37</td>
<td>25977 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>E(spl)m7-HLH</td>
<td>FBgn0002633</td>
<td>2.00E-04</td>
<td>1.41</td>
<td>35703 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>E(spl)mgamma-HLH</td>
<td>FBgn0002735</td>
<td>1.90E-03</td>
<td>1.66</td>
<td>51762 none</td>
<td></td>
</tr>
<tr>
<td>Eip74EF</td>
<td>FBgn0000567</td>
<td>0.00E+00</td>
<td>-1.09</td>
<td>29353 none</td>
<td></td>
</tr>
<tr>
<td>ftz-f1</td>
<td>FBgn0001078</td>
<td>1.00E-04</td>
<td>-1.16</td>
<td>33625 none</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>FBgn0001168</td>
<td>4.00E-03</td>
<td>-0.90</td>
<td>34326 none</td>
<td></td>
</tr>
<tr>
<td>hkb</td>
<td>FBgn0261434</td>
<td>2.00E-04</td>
<td>1.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hr39</td>
<td>FBgn0261239</td>
<td>9.40E-03</td>
<td>-0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hr46</td>
<td>FBgn0000448</td>
<td>3.00E-04</td>
<td>-1.46</td>
<td>51442 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>lms</td>
<td>FBgn00034520</td>
<td>8.50E-03</td>
<td>-1.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mef2</td>
<td>FBgn0011656</td>
<td>4.00E-04</td>
<td>-1.07</td>
<td>38247 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>Mrtf</td>
<td>FBgn0052296</td>
<td>0.00E+00</td>
<td>-2.14</td>
<td>42537 ~50%</td>
<td></td>
</tr>
<tr>
<td>mTTF</td>
<td>FBgn0028530</td>
<td>3.00E-04</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>org-1</td>
<td>FBgn0021767</td>
<td>1.00E-04</td>
<td>-1.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pnt</td>
<td>FBgn0003118</td>
<td>0.00E+00</td>
<td>-1.29</td>
<td>35038 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>Rel</td>
<td>FBgn0014018</td>
<td>0.00E+00</td>
<td>-1.32</td>
<td>33661 none</td>
<td></td>
</tr>
<tr>
<td>schlank</td>
<td>FBgn0040918</td>
<td>8.30E-03</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sd</td>
<td>FBgn0003345</td>
<td>6.10E-03</td>
<td>-0.93</td>
<td>35481 none</td>
<td></td>
</tr>
<tr>
<td>sim</td>
<td>FBgn0004666</td>
<td>0.00E+00</td>
<td>-1.95</td>
<td>32488 none</td>
<td></td>
</tr>
<tr>
<td>sima</td>
<td>FBgn0015542</td>
<td>5.70E-03</td>
<td>-0.85</td>
<td>33894 none</td>
<td></td>
</tr>
<tr>
<td>Six4</td>
<td>FBgn0027364</td>
<td>0.00E+00</td>
<td>1.70</td>
<td>30510 &gt;90%</td>
<td></td>
</tr>
<tr>
<td>Sox14</td>
<td>FBgn0005612</td>
<td>0.00E+00</td>
<td>-1.09</td>
<td>34794 none</td>
<td></td>
</tr>
<tr>
<td>srp</td>
<td>FBgn0003507</td>
<td>0.00E+00</td>
<td>-1.53</td>
<td>35813 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>Ssb-c31a</td>
<td>FBgn0015299</td>
<td>0.00E+00</td>
<td>1.63</td>
<td>35437 none</td>
<td></td>
</tr>
<tr>
<td>Stat92E</td>
<td>FBgn0016917</td>
<td>4.00E-04</td>
<td>1.03</td>
<td>35600 none</td>
<td></td>
</tr>
<tr>
<td>su(Hw)</td>
<td>FBgn0003567</td>
<td>2.00E-04</td>
<td>0.80</td>
<td>33906 &lt;15%</td>
<td></td>
</tr>
<tr>
<td>Su(var)205</td>
<td>FBgn0003607</td>
<td>8.20E-03</td>
<td>0.91</td>
<td>33400 ~25%</td>
<td></td>
</tr>
<tr>
<td>TFAM</td>
<td>FBgn0038805</td>
<td>0.00E+00</td>
<td>1.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tgo</td>
<td>FBgn00264075</td>
<td>1.90E-03</td>
<td>-0.61</td>
<td>53351 none</td>
<td></td>
</tr>
<tr>
<td>ush</td>
<td>FBgn0003963</td>
<td>0.00E+00</td>
<td>-1.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vvl</td>
<td>FBgn0086680</td>
<td>5.30E-03</td>
<td>-1.64</td>
<td>50657 none</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. RNAi screen of differentially expressed transcription factors. Padj refers to the p-value corrected for a 5% false discovery rate. DE refers to the statistical significance values output by DESeq2.
Figure 7. Knockdown of Six4 disrupts the follicle epithelium with high penetrance. The penetrance of follicle cell morphology phenotypes (shown in Fig 2A) in wildtype ovarioles and ovarioles with 109-30\textsuperscript{ts} driving expression of Six4 RNAi. N = 3 replicates; error bars represent the 95% confidence interval; *** p < 0.001 using a two-tailed t-test.

Another approach used to identify transcription factors involved in FC development included analysis of regulatory sequences upstream of differentially expressed genes. The top fifty up-regulated and down-regulated genes were analyzed separately. 1000 base pairs of sequence upstream of the transcription start site of each gene was downloaded from Ensembl BioMart. This collection of sequences was analyzed using http://meme-suite.org/. First, MEME was used to generate motifs commonly found in these upstream sequences, then TOMTOM was used to compare these generated motifs with known transcription factor binding sites.

The upstream sequences of the downregulated genes did not produce any motifs with identifiable transcription factor binding sites. The upstream sequences of the upregulated genes produced a motif that contained elements associated with two notable transcription factors, Eip74EF and daughterless (da).
Six4 is required for the specification of polar and stalk cells

Six4 is a well-conserved member of the SIX (SIme oculus homeoboX) family of transcription factors (Kumar, 2009). The founding member of this family, *sine oculis*, was initially studied in *Drosophila*, where the gene was named for the severe eye loss phenotypes that its alleles can produce. Analysis of conserved sequences identified six *sine oculis* homologs in mouse, which were named SIX1-6 (Oliver et al., 1995; Kawakami et al., 1996a; Kawakami et al., 1996b). The *Drosophila* Six4 gene was also identified by this approach, and subsequently named for having closest homology with the mammalian SIX4 gene (Seo et al., 1999). Further studies established that SIX family proteins contain two evolutionarily conserved domains: (1)
the homeodomain which allows for targeted DNA binding and (2) the SIX domain which mediates binding to other proteins, such as the transcriptional co-regulator Eya (Pignoni et al., 1997).

There are three known members of the SIX family in *Drosophila*: sine oculis, optix, and Six4. Proper eye development depends on the functions of optix and sine oculis (Pignoni et al., 1997). Six4, however is not required for eye development (Clark et al., 2006; Kirby et al., 2001) but is required for mesoderm development and in follicle cells at late stages of oogenesis (Borghese et al., 2006; Clark et al., 2006; Clark et al., 2007; Kirby et al., 2001). To determine the expression pattern of Six4 in the gerarium, we performed immunofluorescence staining with an anti-Six4 antibody (Hwang and Rulifson, 2011). We detected uniform staining in all follicle cells of the gerarium, nuclear staining in the main body follicle cells of later stages, and no signal in stalk cells (Fig. 9A). This signal was absent in follicle cell clones expressing Six4 RNAi, which confirms that the antibody is specific for Six4 and that RNAi knockdown is efficient (Fig. 9B). Our RNA-seq data indicated a 3.2-fold increase in Six4 transcript levels in the population of follicle cells expressing *EGFR*\textsuperscript{top} (Fig. 6; Table 1). However, Six4 may not be a direct target of EGFR signaling, as we do not observe elevated Six4 staining in FSCs where pERK is detected (Castanieto et al., 2014). Instead, because constitutive activation of EGFR signaling blocks PFC differentiation (Fig. 4A,B) and mature stalk cells downregulate Six4 (Fig 9A), the relative increase in Six4 transcript levels may be due to the lack of mature stalk cells in the *EGFR*\textsuperscript{top} expressing population.
**Figure 9. Six4 staining in the germarium.** (A) Six4 staining is uniform in the follicle cells of Region 2b and 3 of the germarium (solid lines), nuclear in the main body follicle cells of budded follicles (dotted lines), and absent from stalk cells (arrowheads). (B) GFP+ cells expressing Six4 RNAi (solid line) do not exhibit nuclear staining with Six4 antibody, as compared to non-clonal cells. (C) GFP+ cells homozygous for the \( \text{Six4}^{108} \) allele (solid line) do not exhibit nuclear staining with Six4 antibody, as compared to non-clonal cells.
We found that RNAi knockdown of Six4 using 109-30ts prevents stalks from forming, resulting in partially fused egg chambers (Fig. 10A). In addition, we consistently observed Cas+ Eya+ dual positive cells located outside of the germarium, typically near the interface between adjacent follicles (Fig. 10C), which is never observed in wildtype ovarioles. Notably, consistent with studies of Six4 in follicle cells at later stages of oogenesis (Borghese et al., 2006), knockdown of Six4 increased the levels of Eya in the cytoplasm (compare Fig. 10C to Fig. 1E). We next used CRISPR (Bassett and Liu, 2014; Gratz et al., 2014) to induce a frameshift mutation after the first 36 codons (position 108) of Six4 in a stock containing FRT2A. This new allele, Six4108, is homozygous lethal and fails to complement Six4289 (Kirby et al., 2001). In addition, FSC clones that are homozygous mutant for Six4108 exhibit all of the phenotypes we observed by RNAi, including a lack of signal with the Six4 antibody and failure to form stalk-like structures (Fig. 9C). Thus, we conclude that Six4108 behaves as a functional null and the Six4 RNAi phenotypes we observed are due to loss of Six4.

Overexpression of Six4 in early follicle cells caused an excess of Cas+ Eya− cells to accumulate in the stalk regions and extend onto the surface of the adjacent follicles (Fig. 10D). The phenotypes caused by knockdown or overexpression of Six4 resemble the follicle cell phenotypes caused by downregulation (Keller Larkin et al., 1999; Lopez-Schier and St Johnston, 2001) or constitutive activation (Larkin et al., 1996; Lopez-Schier and St Johnston, 2001; Vied and Kalderon, 2009) of Notch signaling, respectively. Indeed, RNAi knockdown of Notch caused fused egg chambers and the perdurance of Cas+ Eya+ cells downstream from the germarium (Fig. 10E), whereas constitutive activation of Notch signaling by expression of the Notch intracellular domain (Nintra) in early follicle cells caused an accumulation of Cas+ Eya−
cells in the stalk regions (Fig. 10F). These observations indicate that both Notch and Six4 are required for the establishment of the polar and stalk cell fates, and are sufficient to ectopically induce a polar/stalk-like cell fate in a subset of PFCs.

**Figure 10. Six4 is required for polar and stalk cell formation.** (A) Knockdown of Six4 prevents stalk formation between adjacent follicles (arrowheads). (B) Overexpression of Six4 causes the accumulation of extra cells between adjacent follicles (arrowheads). (C-F) Knockdown of Six4 (C) or Notch (E) by RNAi causes some follicle cells (arrowheads) to remain Cas⁺ Eya⁺ in follicles that have budded from the germarium. Overexpression of Six4 (D) or N intra (F) leads to ectopic Cas⁺ Eya⁻ cells (arrowheads) between adjacent follicles. Insets in C-F are enlarged to the right of each panel.
To determine whether *Six4* is also required for the maintenance of the polar cell fate, we expressed *Six4* RNAi using *unpaired-Gal4* (*upd-Gal4*), which is expressed in mature polar cells but not stalk cells or PFCs within the germarium (Assa-Kunik et al., 2007). Interestingly, this did not cause any discernable phenotype (Fig. 11A). Polar and stalk cells were present in the proper positions with normal morphology and were Cas+ Eya-. Additionally, expression of *EGFR*\textsuperscript{lop} using *upd-Gal4* also failed to produce a phenotype (Fig. 11B). This suggests that polar cells become refractory to these perturbations once they are established.

**Figure 11. Polar cell maintenance is unaffected by Six4 loss or EGFR\textsuperscript{lop} expression.** Using the polar cell driver *upd-Gal4*, neither knockdown of *Six4* nor overexpression of *EGFR*\textsuperscript{lop} cause follicle cell phenotypes, indicating that polar cell maintenance is unaffected by these inputs.

*Six4* promotes the polar cell lineage via Notch signaling

Previous studies have shown that specification of the stalk cell fate requires the secretion of *Upd* from polar cells (Assa-Kunik et al., 2007; Torres et al., 2003). Accordingly, mutations that disrupt polar cell specification subsequently disrupt stalk cell specification as well. To determine where *Six4* functions in the progression toward the polar and stalk cell fates, we measured the expression of an early polar cell reporter, *neuralized-lacZ* (*neur-lacZ*) (Lopez-Schier and St Johnston, 2001), in the context of *Six4* knockdown or overexpression using the 109-30\textsuperscript{ts} driver. In wildtype tissue, *neur-lacZ* is weakly expressed in 2-4 cells between the cysts in
Regions 2b and 3 of the germarium and more strongly expressed in pairs of polar cells on budded follicles (Fig. 12A). Knockdown of Six4 caused a loss of neur-lacZ+ cells throughout the germarium and in budded follicles (Fig. 12B). Conversely, overexpression of Six4 caused neur-lacZ expression to expand beyond the developing polar cell region to include some stalk cells and cells in the main body of the follicle (Fig. 12C). Thus, Six4 functions in undifferentiated PFCs to promote specification of the polar cell fate choice, which accounts for the loss of polar and stalk cells upon Six4 knockdown (Fig. 12B).

The expression of neur-lacZ in PFCs requires Notch signaling (Lopez-Schier and St Johnston, 2001). Therefore, we next investigated whether Six4 is required for the activation of Notch target genes using a Notch pathway activity reporter, NRE-GFP (Saj et al., 2010). Expression of NRE-GFP in wildtype ovarioles is detectable in PFCs contacting the anterior face of the germline in Regions 2b and 3 of the germarium, and is then restricted to pairs of mature polar cells of early follicles in Stages 2-5 (Fig. 12D). At Stage 6, a separate wave of Notch signaling activates NRE-GFP expression in all follicle cells (Lopez-Schier and St Johnston, 2001). We found that the effect of Six4 knockdown or overexpression on NRE-GFP expression closely paralleled the effects we observed on neur-LacZ expression. Specifically, upon RNAi knockdown of Six4, the NRE-GFP signal was undetectable in follicle cells throughout the germarium, and in the polar cell regions of most Stage 2-5 follicles (Fig. 12E) whereas overexpression of Six4 caused an expansion in the region of Notch-responsive cells at the poles of each follicle (Fig. 12F; Fig. 13). These results indicate that Six4 is necessary and sufficient to promote Notch signaling in the PFCs of the germarium and the subset of follicle cells at the poles of early follicles.
Figure 12. Six4 promotes the polar cell lineage via Notch signaling. (A) Expression of neur-lacZ (red, arrowheads) in wildtype. Expression is low in 2-4 PFCs on the anterior face of a Region 3 follicle and high in pairs of polar cells on subsequent stages. (B) Knockdown of Six4 by RNAi eliminates neur-lacZ reporter expression in cells positioned to become polar cells (arrowheads). (C) Overexpression of Six4 causes ectopic activation of neur-lacZ (red) in main body (arrowheads) and stalk cells (arrow) near the polar cells, but not in all follicle cells. (D) Expression of NRE-GFP (green) in wildtype. GFP is detected in 4-6 cells at the anterior face of a follicle budding from the gerarium but is restricted to pairs of polar cells in later stages (arrowheads). (E) Knockdown of Six4 by RNAi causes loss of NRE-GFP activity in cells positioned to become polar cells (arrowheads). Occasional follicle cells can still activate NRE-GFP (arrow), but fail to induce stalk formation. (F) Overexpression of Six4 causes ectopic activation of NRE-GFP (green) in many cells near the border between adjacent follicles but not all follicle cells.
To determine where in the Notch signaling pathway Six4 exerts its influence, we established an epistatic relationship by coexpressing Six4 RNAi and $N_{intra}$ using 109-30ts. This caused an accumulation of Cas$^+$ Eya$^-$ cells between follicles (Fig. 14A,B), which resembles the phenotype caused by overexpression of $N_{intra}$ alone (Fig. 10F), rather than a loss of Cas$^+$ Eya$^-$ cells, as caused by knockdown of Six4 (Fig. 10C), indicating that Six4 is not required downstream of Notch cleavage in Notch signaling and polar cell specification.
**Figure 14. Six4 is likely upstream of Notch cleavage.** (A-B) The phenotype of \( N^{\text{intra}} \) expression is epistatic to Six4 RNAi. Many ectopic Cas\(^{+}\) Eya\(^{-}\) cells are observed (arrowheads).

The global co-repressor groucho promotes prefollicle cell differentiation

Our finding that Six4 promotes Notch signaling and polar cell differentiation prompted us to search for other genes that may participate in the process. Although little is known about Six4, the founding SIX family member *sine oculis* has been extensively studied (Kumar, 2009). Interestingly, Sine oculis functions in conjunction with a broadly expressed and highly conserved transcriptional repressor, Gro (Kumar, 2009) that is an effector of many signaling pathways, including EGFR and Notch signaling (Hasson et al., 2005). In wing and notal bristle
patterning, Gro functions as a positive effector of Notch signaling by cooperating with Enhancer-of-split proteins, and is antagonized by ERK-mediated phosphorylation of Gro (Hasson et al., 2005).

To examine potential roles for Gro in the gerarium, we first stained wildtype ovarioles using an anti-Gro antibody (Delidakis et al., 1991). We found that Gro is expressed strongly in all follicle cells of the gerarium and more weakly in germ cells and IGS cells (Fig. 16A). Knockdown of gro in follicle cells produced a phenotype that strongly resembles the phenotype caused by overexpression of $EGFR^{iiope}$. Specifically, upon expression of gro RNAi with $109-30^i$, we observed an accumulation of follicle cells in the gerarium and an absence of stalks between follicles (Fig. 16B) in 100% ($n = 64$) of ovarioles. In addition, as with overexpression of $EGFR^{iiope}$, follicle cells remained Cas$^+$ Eya$^+$ beyond the gerarium (Fig. 16D). In combination with previous studies (Hasson et al., 2005; Helman et al., 2011), these observations suggest that the inhibition of follicle cell differentiation caused by ectopic activation of EGFR signaling in PFCs may be due to ERK-mediated phosphorylation and inhibition of Gro.

To investigate the role of Gro phosphorylation in FSCs and early PFC differentiation, we examined the phenotypes caused by overexpression of either wildtype gro or alleles of gro with point mutations at the ERK target sites. We found that overexpression of an allele of gro ($gro^{AA}$) that is refractory to ERK inhibition (Helman et al., 2011) caused excessive differentiation toward the polar/stalk lineage, resulting in elongated and multilayered stalks with extra Cas$^+$ Eya$^-$ cells in 78 ± 11% of germaria (Fig. 15; Fig. 16C,E). In contrast, overexpression of wildtype gro produced less severe phenotypes with lower penetrance (15 ± 2% of ovarioles had at least one elongated or multilayered stalk, Fig. 15). Overexpression of phosphomimetic gro ($gro^{DD}$), which
behaves as if it is constitutively repressed by ERK-mediated phosphorylation, produced these phenotypes with a similar low penetrance (10 ± 8%, Fig. 15).

The strong phenotype caused by groAA expression indicates that the ERK target sites of Gro are important for its function in promoting polar and stalk cell differentiation, and suggest that overexpression of EGFR\textsuperscript{ztop} inhibits polar and stalk cell formation by repressing Gro. To test this possibility, we investigated whether expression of groAA could restore polar/stalk cell differentiation in ovarioles expressing EGFR\textsuperscript{ztop}. Indeed, we found that 75 ± 6% of ovarioles expressing both groAA and EGFR\textsuperscript{ztop} had polar/stalk-like Cas\textsuperscript{+} Eya\textsuperscript{−} cells between follicles, compared to just 16 ± 6% of ovarioles expressing EGFR\textsuperscript{ztop} alone (Fig. 16F,G).
Figure 16. Gro is required for the specification of the polar and stalk cell lineages. (A) Gro is expressed most strongly in follicle cells, but also in IGS and germ cells. (B, D) Knockdown of gro causes severe multilayering, fusion of adjacent follicles and ectopic Cas⁺ Eya⁺ cells (arrowheads) beyond the germainrium, indicative of excess undifferentiated follicle cells and a lack of stalk cells. (C, E) Overexpression of groAA causes elongated, multilayered stalks and elicits the accumulation of excess Cas⁺ Eya⁻ cells between adjacent follicles (arrowheads). (F, G) Stalk-like Cas⁺ Eya⁺ cells (arrowheads) are present between follicles in ovarioles expressing groAA and EGFRtop⁺. *** p < 0.001 using a two-tailed t-test.
Next, we examined NRE-GFP expression to test whether gro regulates Notch pathway activity. Indeed, we found that RNAi knockdown of gro eliminated NRE-GFP activity in the germarium and early stage egg chambers (Fig. 17A), whereas overexpression of groAA ectopically activated NRE-GFP activity throughout the FSC lineage in the germarium, including the FSCs (Fig. 17B). Follicle cells expressing groAA also exhibit ectopic expression of the polar cell reporter, neur-lacZ, in cells typically positioned to become stalk cells (Fig. 17C). Collectively, these results indicate that Gro promotes Notch signaling and polar cell differentiation, and that, as in other tissues, Gro function is antagonized by ERK-mediated phosphorylation of Gro at ERK target sites.

**Figure 17. Groucho promotes Notch signaling in the germarium.** (A) Expression of gro RNAi causes loss of NRE-GFP activity in early stage follicles (arrowheads). A separate wave of Notch activation occurs surrounding Stage 6 follicles (solid line), which is beyond the range of 109-30 expression. (B) Expression of groAA, which is refractory to ERK-mediated phosphorylation, causes ectopic Notch activity throughout the early follicle cell lineage, including FSCs (arrowhead). (C) Expression of groAA causes ectopic activation of neur-lacZ in stalk cells (arrowheads).
Groucho phosphorylation is enriched in the FSC niche

We hypothesized that, if Gro undergoes phosphorylation in response to EGFR signaling as the above results suggest, then it should be detected in its phosphorylated state in cells with active EGFR signaling. To visualize the pattern of Gro phosphorylation in the FSC lineage, we performed immunofluorescence with an antibody that specifically recognizes Gro protein that has been phosphorylated at the ERK target sites (Helman et al., 2011). Interestingly, we found that phosphorylated Gro (p-Gro) is highly enriched not only in FSCs and IGS cells, both of which have active EGFR signaling, but also in newly-produced PFCs, located within approximately three cell diameters from the FSCs (Fig. 18). Beyond this stage, p-Gro staining is still detectable in follicle cells but at substantially lower levels. We confirmed this result using a staining protocol that allows for simultaneous and mutually exclusive detection of the phosphorylated and non-phosphorylated forms of Gro (Cinnamon et al., 2008). This co-staining revealed very low Gro signal in the p-Gro+ FSCs and PFCs (Fig. 18C), suggesting that the majority of Gro protein is phosphorylated in these cells.

Figure 18. Groucho is specifically phosphorylated in FSCs and early PFCs. (A,B) Phosphorylated Gro (p-Gro) is observed only in FSCs and PFCs within three cell diameters of the niche (arrowheads). (C) Co-staining for Gro (green) and p-Gro (red) indicates FSCs and early PFCs are p-Gro+ Gro− (arrowheads), whereas later PFCs are p-Gro− Gro+ (arrows).
Gro is required for FSC maintenance while Six4 loss induces hypercompetition

Our findings thus far demonstrate a clear role for Six4 and gro in promoting differentiation of PFCs toward the polar cell fate. To test whether either of these transcriptional regulators are also part of the programs that promote FSC self-renewal and occupancy of the niche, we performed an FSC competition assay (Kronen et al., 2014). This assay compares the fitness of a mutant FSC lineage to a wildtype FSC lineage in the same germarium. Mutations that disrupt a function required for FSC self-renewal or niche occupancy cause “hypocompetition” in which the mutant FSCs are lost at an increased rate and replaced by daughters of the wildtype FSC lineage. Conversely, other mutations cause “hypercompetition” in which the mutant FSC lineage expands at the expense of the wildtype lineage. The causes of hypercompetition are not fully understood but, in the FSC lineage, the phenotype is associated with mutations that delay PFC differentiation (Kronen et al., 2014).

We measured the proportion of germaria containing 0 (unlabeled), 1 (single-labeled), or 2 (double-labeled) GFP+ FSC clones at 7, 14, and 21 days post clone induction (Fig. 19A). In germaria that are single-labeled at the time of clone induction, the replacement of one FSC by a daughter cell of the other FSC results in a decrease in the proportion of single-labeled germaria with a concomitant increase in either the unlabeled or double-labeled population. Thus, the changes in the proportion of germaria with 0, 1, or 2 labeled FSCs can be used to calculate rates of FSC turnover as well as a competitive bias value (b) which describes the fitness of a mutant stem cell relative to wildtype stem cell (Kronen et al., 2014). Bias values range from +100% to -100% with positive values indicating that the mutant is hypercompetitive for the niche relative to wildtype, negative values indicating that the mutant is hypocompetitive relative to wildtype, and 0% indicating that mutant and wildtype are equally competitive (neutral competition).
We found that loss of *Six4*, either by RNAi knockdown or homozygosity for *Six4*<sup>108</sup>, caused a significant hypercompetition phenotype (*b* = 50 ± 23% and 52 ± 30%, respectively; *p* < 0.05 for the null hypothesis that *b* = 0; Table 2). This indicates that *Six4* is not necessary for FSC self-renewal and is consistent with a role for Six4 in PFC differentiation since mutant PFCs that fail to differentiate may be more competitive for the niche. Despite the essential role for Six4 in PFC differentiation, overexpression of *Six4* did not result in a significant niche competition phenotype (*b* = -9 ± 5%; *p* = 0.73; Table 2), indicating that it is not sufficient to cause FSCs to differentiate prematurely. In contrast, overexpression of *gro*<sup>AA</sup> caused a severe hypocompetition phenotype (*b* = -100% ± 0; *p* < 0.001; Table 2), indicating that phosphorylation of Gro at ERK target sites is essential for FSC self-renewal. Unexpectedly, RNAi knockdown of *gro* also caused a severe hypocompetition phenotype (*b* = -100% ± 0; *p* < 0.001; Table 2), suggesting that *gro* has other functions in FSC self-renewal or survival that are not repressed by ERK phosphorylation.
Figure 19. Six4 and Gro are required to maintain neutral FSC competition. (A) FSC competition assay using MARCM clones. The proportion of germaria that were double-labeled, single-labeled, or unlabeled are indicated by the black, dashed, or white bars, respectively. Timepoints observed were 7, 14, and 21 days after clone induction. At least 250 ovarioles scored for each genotype and time point. (B) Rates of FSC clonal extinction or expansion. Error bars indicate the 95% confidence intervals. (C) Competitive bias for the indicated genotypes. FRT 2A and FRT 40A controls exhibit neutral competition. Six4 loss, either by Six4 RNAi or homozygosity for the Six4<sup>108</sup> allele, causes hypercompetition. Six4 overexpression is neutral. Expression of either gro RNAi or gro<sup>AA</sup> cause strong FSC hypocompetition. Error bars indicate the 95% confidence intervals. The p-value for the null hypothesis that \( b = 0 \) is indicated above each bar in the graph. Asterisk indicates \( p < 0.05 \). (D) Interactions of Six4 and Gro in the FSC niche. Within the niche, EGFR maintains Gro phosphorylation to inhibit Notch signaling, and thus differentiation. Cells that have recently left the niche temporarily maintain p-Gro, thus resisting differentiation to allow for replacement events and transit amplifying divisions. As the prevalence of p-Gro decreases, non-phosphorylated Gro becomes available to engage in Notch signaling. Six4 and Gro both promote Notch signaling to initiate differentiation of PFCs specifically toward the polar cell fate.
Table 2. Competition bias values. Lists the bias values, standard error, and p-values associated with the competition assay.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bias</th>
<th>Standard Error</th>
<th>p-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT2A</td>
<td>-26%</td>
<td>13%</td>
<td>0.29</td>
</tr>
<tr>
<td>FRT40A</td>
<td>28%</td>
<td>14%</td>
<td>0.20</td>
</tr>
<tr>
<td>Six4RNAi</td>
<td>50%</td>
<td>23%</td>
<td>0.01</td>
</tr>
<tr>
<td>Six4[108]</td>
<td>52%</td>
<td>30%</td>
<td>0.02</td>
</tr>
<tr>
<td>Six4OE</td>
<td>-9%</td>
<td>5%</td>
<td>0.73</td>
</tr>
<tr>
<td>GroRNAi</td>
<td>-100%</td>
<td>0%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gro[AA]</td>
<td>-100%</td>
<td>0%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Discussion**

**Live imaging**

Overall, this effort proved that germaria can be sustained in ex-vivo culture for up to 12 hours. Unfortunately, FSCs only divide approximately every 10-14 hours. Thus, in the optimal scenario of FSC fitness not decreasing in ex-vivo culture, each germarium imaged offers the opportunity to observe a maximum of two FSC divisions. In practice, mitotic FCs were very uncommon, and mitotic FSCs were incredibly rare. The low throughput of this assay, combined with the rarity of visible division / replacement events led to a low return on effort from these experiments.

In future attempts at live imaging, it would be important to include a fluorescent protein that achieves fluorescence rapidly after translation and is whose signal degrades rapidly in the absence of continued transcription. Lengthy times for protein folding or fluorescence maturation can cause a delay in the readout of signal activation (Hebisch et al., 2013). Rapid degradation, such as that observed in destabilized fluorescent proteins (Li et al., 1998; Housden et al., 2012), allows a rapid transition towards the signal reporter being turned off rather than waiting for the endogenous rate of protein turnover.
Isolation of FSCs

Our struggle to isolate wildtype FSCs highlighted that good markers and separation techniques are essential for the study of stem cell biology. As stem cells make up a small percentage of the total number of cells in a tissue, it is often difficult to identify or isolate stem cells to study their properties independently of the differentiated daughter cells which surround them. Typically, a stem cell lineage must be identified by the presence of a combination of molecular markers that is unique to the stem cell. Strategies to subsequently isolate these cells include FACS (Fluorescence-activated cell sorting) or laser microdissection. Novel strategies for investigating stem cells, and indeed all cell populations, include single-cell sequencing platforms. A single-cell approach could be very informative in the gerarium, where consistent and specific markers remain scarce.

Identification of transcription factors involved in follicle cell differentiation

Although the motif-based analysis of transcription factors was never directly followed up on, Eip74EF and da could be interesting targets to investigate in the future. We have generated preliminary data that Eip74EF expression is enriched near the niche, however, this would need to be repeated before we could be confident in this assertion. Additionally, da is known to have important roles in the somatic cells of the ovary (Smith et al., 2002). Follow-up experiments would be warranted with either of these genes.

In the primary focus of this work, we identified two transcriptional regulators, Six4 and gro, to be important in early PFC differentiation. PFCs contribute cells to the polar, stalk, and main body cell lineages with the earliest identified signaling input being Notch-mediated
specification of polar cells. Both Six4 and gro are required for Notch signaling, and thus polar cell formation. Overall, our findings support a model (Fig. 19D) in which EGFR signaling inhibits FSC differentiation within the niche by phosphorylating Gro to prevent it from participating in Notch signal propagation. Outside of the niche, newly-produced PFCs enter a transition state defined by the lack of exposure to self-renewal cues such as active Wg and EGFR signaling (Castanieto et al., 2014; Sahai-Hernandez and Nystul, 2013) but a dependence on continued Gro inhibition to resist differentiation. During embryonic development, the perdurance of p-Gro in the neuroectoderm allows the effects of transiently active signaling from receptor tyrosine kinases such as EGFR to persist beyond the window of pathway activation (Helman et al., 2011). Likewise, the perdurance of p-Gro may provide early PFCs with a molecular memory of the niche signaling, thus delaying differentiation and allowing these cells to participate in stem cell replacement or to expand in number before committing to a cell fate choice. Following a prolonged absence of EGFR signaling, p-Gro is gradually replaced by an active, unphosphorylated form of Gro that is able to promote Notch signaling in some PFCs, causing them to differentiate into polar cells. It remains interesting that some FCs remain resistant to Notch signaling, even in the presence of strong genetic manipulations such as the expression of N\textsuperscript{intra}. This implies that there are additional, regulatory mechanisms in place that can specifically disable canonical Notch signaling in certain contexts, highlighting the overall robustness of cell fate decision processes.

Our model (Fig. 19D) builds on previous findings that p-ERK is detectable in FSCs (Castanieto et al., 2014), that Gro is a direct substrate of p-ERK, and that p-ERK phosphorylation inhibits the activity of Gro (Cinnamon et al., 2008; Hasson et al., 2005; Helman et al., 2011). Our immunofluorescence assays (Fig. 18) confirmed that the phosphorylated form
of Gro predominates in FSCs, while the strong hypocompetition phenotype caused by overexpression of \( gro^{AA} \) (Fig. 19A-C; Table 2) demonstrates the importance of phosphorylation at these sites for FSC self-renewal. Interestingly, our finding that \( NRE-GFP \) is active in these FSCs (Fig. 17B) suggests that the phosphorylation is important to prevent Notch-mediated differentiation in FSCs. Consistent with this possibility, overexpression of \( N^{\text{intra}} \) increases the rate of FSC loss (Vied and Kalderon, 2009). Our observations that loss of \( gro \) or \( Six4 \) attenuated Notch pathway activation and polar cell specification while overexpression of \( gro^{AA} \) or \( Six4 \) had the opposite effect, strongly support the idea that both genes promote Notch-mediated differentiation of PFCs toward the polar cell fate. In addition, our finding that overexpression of \( gro^{WT} \) or \( gro^{DD} \) had a much weaker effect on PFC differentiation than overexpression of \( gro^{AA} \) indicates that loss of phosphorylation at these sites is important for the pro-differentiation function of Gro.

In many cell types, Gro functions downstream of Notch target gene expression by interacting with primary Notch targets such as \( \text{Enhancer of Split (E(spl))} \) to carry out the effects of Notch pathway activation (Bailey and Posakony, 1995; Fisher et al., 1996; Lecourtois and Schweisguth, 1995; Paroush et al., 1994). It is unclear if this occurs in the FSC lineage because \( E(spl) \) is not required for follicle cell differentiation (Lopez-Schier and St Johnston, 2001). However, our NRE-GFP data suggest that both Gro and Six4 function upstream of primary Notch target gene expression in PFCs, perhaps by controlling the expression or activity of genes such as \( fringe \) that promote receptivity to Notch ligands (Grammont and Irvine, 2001; Zhao et al., 2000).

Niche signals are commonly thought to promote stem cell self-renewal in part by antagonizing the signals that promote differentiation. In support of this idea, studies of the
epithelial stem cell lineages in the mammalian hair follicle bulge and the intestine found that the stem cell self-renewal and differentiation cues are activated in opposing gradients (Barker, 2014; Rompolas and Greco, 2014; Sato et al., 2011; Tian et al., 2015), but the mechanisms of interaction between the opposing cues in these tissues are unclear. Our findings suggest that Gro may balance the decision between self-renewal and differentiation in the FSC lineage in a manner that is similar to the mechanisms that operate in the Drosophila male and female germline stem cell niches (de Cuevas and Matunis, 2011; Losick et al., 2011; Xie, 2013). Specifically, just as EGFR signaling inhibits Gro in the FSC niche, BMP signaling in the germline stem cell niches inhibits two repressors, bag of marbles (bam) and benign gonial cell neoplasia (bgcn), that promote germ cell differentiation (Chen et al., 2014; Gönczy et al., 1997; McKearin and Ohlstein, 1995; Shen et al., 2009). The structure and function of epithelial stem cell niches are similar in many fly and mammalian tissues, and the structure of both Gro and Six4 are evolutionarily conserved. Moreover, as in the FSC lineage, EGFR and Notch signaling also promote stem cell self-renewal and differentiation in mammalian epithelia such as the intestinal lining, the hair follicle, and the interfollicular epidermis (Aubin-Houzelstein, 2012; Doma et al., 2013; Sato et al., 2011; Tian et al., 2015; Watt et al., 2008). Thus, it will be interesting to determine whether a similar Gro-mediated transition state exists in other epithelial tissues.
Materials and methods

Fly stocks

Fly stocks were maintained on standard molasses food.

Genotypes used in this work were obtained from the Bloomington Stock Center or as indicated below:

(1) UAS-EGFR\textsuperscript{\lambda top} (active EGFR) (Trudi Schupbach)

(2) UAS-Six4.HA (FlyORF; Line ID F000049) (Bischof et al., 2013)

(3) upd-Gal4 (Denise Montell)

(4) y,w, hsFLP, tub-Gal4, UAS-GFP / FM7 ; tub-Gal80 FRT40A / CyO; (Yuh-Nung Jan)

(5) y, w, hsFLP, tub-Gal4, UAS-GFP / FM7 ; ; tub-Gal80, FRT2A / TM3 (Yuh-Nung Jan)

TRiP RNAi lines (Ni et al., 2011) from Bloomington that were used in the main figures are as follows:

(1) UAS-Six4 RNAi (TRiP; BL# 30510)

(2) UAS-gro RNAi (TRiP; BL# 35759)

(3) UAS-Notch RNAi (TRiP; BL# 31383)

The following genotypes were used for the isolation of PFCs for RNA-seq:

(1) Wildtype: +; 109-30-Gal4 / UAS-mCD8::GFP; tub-Gal80\textsuperscript{ts} / +,

(2) EGFR\textsuperscript{\lambda top}: +; 109-30-Gal4 / UAS-mCD8::GFP; tub-Gal80\textsuperscript{ts} / UAS-EGFR\textsuperscript{\lambda top}.

The following genotypes were used in Gal4 experiments:

(1) Wildtype: y, w; + ; +,
(2) 109-30>EGFR\textsuperscript{\lambda top}: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-EGFR\textsuperscript{\lambda top},

(3) 109-30>Six4 RNAi: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-Six4 RNAi,

(4) 109-30>Six4.HA: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-Six4.HA,

(5) 109-30>Notch RNAi: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-Notch RNAi,

(6) 109-30>Notch\textsuperscript{intra}: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-Notch\textsuperscript{intra},

(7) upd>Six4 RNAi: upd-Gal4 / +; +; UAS-Six4 RNAi / +,

(8) upd>EGFR\textsuperscript{\lambda top}: upd-Gal4 / +; +; UAS-EGFR\textsuperscript{\lambda top} / +,

(9) neur-lacZ: +; +; neur-lacZ / +,

(10) 109-30>Six4 RNAi, neur-lacZ: +; 109-30-Gal4 / +; neur-lacZ / UAS-Six4 RNAi,


(12) NRE-GFP: w ; NRE-GFP; +;

(13) 109-30>Six4 RNAi, NRE-GFP: +; 109-30-Gal4 / NRE-GFP; tub-Gal80\textsuperscript{ts} / UAS-Six4 RNAi,

(14) 109-30>Six4.HA, NRE-GFP: +; 109-30-Gal4 / NRE-GFP; tub-Gal80\textsuperscript{ts} / UAS-Six4.HA,

(15) 109-30>gro RNAi: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-gro RNAi,

(16) 109-30>gro\textsuperscript{AA}: +; 109-30-Gal4 / +; tub-Gal80\textsuperscript{ts} / UAS-gro\textsuperscript{AA},

(17) 109-30>gro RNAi, NRE-GFP: +; 109-30-Gal4 / NRE-GFP; tub-Gal80\textsuperscript{ts} / UAS-gro RNAi,

(18) 109-30>gro\textsuperscript{AA}, NRE-GFP: +; 109-30-Gal4 / NRE-GFP; tub-Gal80\textsuperscript{ts} / UAS-gro\textsuperscript{AA},

(19) 109-30>gro\textsuperscript{AA}, neur-lacZ: +; 109-30-Gal4 / +; neur-lacZ / UAS-gro\textsuperscript{AA}.

The following genotypes were used to generate MARCM clones:

(1) Wildtype 40A: hsFlp, tub-Gal4, UAS-GFP / +; tub-Gal80, FRT 40A / FRT 40A; +,

(2) Wildtype 2A: hsFlp, tub-Gal4, UAS-GFP / +; +; tub-Gal80, FRT 2A / FRT 2A,
(3) Six4 RNAi: hsFlp, tub-Gal4, UAS-GFP / +; tub-Gal80, FRT 40A / FRT 40A; UAS-Six4 RNAi / +; +,

(4) Six4^{108}: hsFlp, tub-Gal4, UAS-GFP / +; +; tub-Gal80, FRT 2A / Six4^{108}, FRT 2A,

(5) Six4.HA: hsFlp, tub-Gal4, UAS-GFP / +; tub-Gal80, FRT 40A / FRT 40A; UAS-Six4.HA / +,

(6) gro RNAi: hsFlp, tub-Gal4, UAS-GFP / +; tub-Gal80, FRT 40A / FRT 40A; UAS-gro RNAi / +,

(7) gro^{AA}: hsFlp, tub-Gal4, UAS-GFP / +; tub-Gal80, FRT 40A / FRT 40A; UAS-gro^{AA} / +.

**Immunofluorescence:**

Adult flies were fed wet yeast for at least two days prior to dissection to ensure plump ovaries. Ovaries were dissected in Schneider’s Insect Medium, fixed in phosphate buffered saline (PBS) + 4% paraformaldehyde for 15 min. Tissue was rinsed in PBS + 0.1% Triton X-100 (0.1% PBT) 2x 1-min, prior to incubation with Blocking Solution (0.1% PBT + 0.5% bovine serum albumin) for 15 min. Primary antibodies were diluted in Blocking Solution and incubated with tissue overnight at 4°C while rocking on a nutator. Tissue was rinsed 2x 1-min then washed for 1 hr in 0.1% PBT. Secondary antibodies were diluted in Blocking Solution and incubated with tissue for 2 hr at room temperature while rocking on a nutator. Tissue was rinsed 2x 1-min and washed for 1 hr in PBS, then mounted on a glass slide using Hard Set Vectashield plus DAPI mounting medium (Vector Labs).

All images were acquired using a Zeiss M2 Axioimager with Apotome unit or Nikon C1si Spectral Confocal. For multicolor fluorescence images, each channel was acquired separately. Post-acquisition processing such as image rotation, cropping, brightness or contrast adjustment, stitching of two overlapping fields (in Figure 3F) (Preibisch et al., 2009) and Z-
projections were performed using FIJI (Schindelin et al., 2012). Comparable staging of follicles between samples was accomplished by counter-staining for Fas3 or Vasa to identify the Region 2a/b border in the gerarium, and then using the number of germline cysts posterior to this location, the size and shape of each cyst, and, in the case of budded follicles, its position relative to the gerarium as guides. All scale bars indicate 20 μm.

The following primary antibodies were used: ms α-β-gal (1:1000, Promega Z3781), rb α-Cas (1:5000, a gift from Ward Odenwald) (Kambadur et al., 1998), ms α-Eya (1:100, DSHB 10H6) (Bonini et al., 1993), ms α-Fas3 (1:100, DSHB 7G10) (Patel et al., 1987), gp α-GFP (1:1000, Synaptic Systems 132005), ms α-Gro (1:1000, DSHB anti-Gro) (Delidakis et al., 1991), rb α-p-Gro (1:1000) (Cinnamon et al., 2008), rt α-Six4 (1:1000, a gift from Eric Rulifson) (Hwang and Rulifson, 2011), and rb α-Vasa (1:1000, Santa Cruz sc-30210). The following secondary antibodies were purchased from Thermo Fisher Scientific and used at 1:1000: gt α-gp 488 (A-11073), gt α-rb 488 (A-11008), gt α-rb 555 (A-21428), gt α-ms 488 (A-11029), gt α-ms 555 (A-21424), and gt α-rt 555 (A-21434).

For the co-staining of Gro and p-Gro in Figure 4H, ovaries were dissected in PBS and fixed in PBS + 5% formaldehyde for 20 min. Ovaries were rinsed 2x in PBS containing 1% Triton-X-100 (1% PBT), washed in 1% PBT for 10 min, then washed again with 1% PBT for 1 hr. Ovaries were blocked in PBS + 0.3% Triton-X-100 + 1% BSA (PBTB) for 1 h, and then incubated with mouse anti-Gro (1:1000) and rabbit anti-p-Gro (1:1000) antibodies in PBTB overnight at 4°C. Ovaries were washed in PBTB 2x 30-min, and then blocked with PBTB + 5% Normal Goat Serum (NGS, Sigma) for 1 h. Secondary antibodies were donkey α-ms 488 and donkey α-rb Rhodamine Red-X (Jackson Laboratories) diluted 1:500 in PBTB + 5% NGS. Following 2 h incubation with the secondary antibodies, ovaries were washed in 0.3% PBT 3x.
30-min, rinsed twice in PBS, incubated in PBS + DAPI (1:1000) for 5 min, then washed twice in PBS. Finally, ovaries were mounted with Vectashield mounting medium. Images were acquired using a LSM710 confocal microscope.

**Live imaging**

*Preparation:* Flies were fed wet yeast for at least two days prior to dissection. Live Media was prepared containing 15% FBS, 0.2 mg/mL insulin, and 0.5x Pen/Strep. A small disc of agar was prepared by placing a drop of 1.5% low melt agarose onto a microscope slide between two “risers” (two pieces of lab tape at each end of the slide), and then placing a coverslip onto the liquid agar. The coverslip caused the drop to spread out, while the lab taper risers maintained the thickness of the disc. The agar disc was maintained between the microscope slide and coverslip until the dissection was complete to prevent drying.

*Tissue dissection and mounting:* Ovaries were dissected into live media. Individual ovarioles were isolated apart from each other as well as other late stage follicles. Optionally, the muscle sheath could also be removed. This allowed the tissue to be imaged with less motion from muscle contractions, but could also cause follicle cells to leak out of basement membrane of the ovariole after ~4/5 hours of imaging.

Approximately 12 well-separated, in-tact ovarioles were transferred to an agar pad for imaging. The coverslip and agar pad were separated from the microscope slide, leaving the agar pad face-up attached to the coverslip. With a BSA-blocked pipette tip, ovarioles were transferred (in 2-5 μL of Live Media) to the surface of the agar pad. Ovarioles were spread out using blocked pipette tip, taking care to not tear the agar pad. Excess liquid media was removed using the pipette tip. With use of a razor blade, the agar pad with germaria was flipped onto the center
of a fresh coverslip such that the germaria were now touching the new coverslip. Small amounts (2-5 μL of) Live Media were added to the top of the agar pad if it began to appear dry. The coverslip was sealed to the bottom of the live imaging chamber using vacuum grease. A small, harp-shaped ballast was placed on top of the agar pad to prevent sample floating. The rest of the imaging chamber was filled with Live Media.

**Imaging:** Images were acquired using an inverted, spinning-disc confocal microscope. The microscope stage was maintained at room temperature. Images were acquired at approximately a 15-minute time interval. The Live Media in the imaging chamber was replaced throughout the imaging time course with either an automated peristaltic pump or a manual system of syringes containing fresh Live Media.

**Clone induction:**

Flies of the appropriate genotype were cultured and fed wet yeast for 2 days prior to clone induction. Heat shock was performed by transferring flies to empty plastic vials and immersing these vials in a 37°C water bath for 1 hour. Flies were then allowed to recover at 25°C in vials containing supplemental wet yeast. This process was repeated 2x daily for 2 days for a total of 4x 1-hour heat shocks. Control flies were always simultaneously subjected to the same heat shock regimen as experimental flies. Flies were then maintained at 25°C and fed wet yeast daily until dissection.

**FSC competition assay**

In this assay, MARCM clones of various genotypes were induced and the frequency of germaria containing 0, 1, or 2 labeled stem cells was measured at 7, 14, or 21 days after clone
induction. Stem cell labeling counts were analyzed as described previously (Kronen et al., 2014). In brief, replacement events can be measured within the subset of germaria that have 1 stem cell labeled at the initial time point. Over time, single-labeled germaria can become unlabeled or double-labeled, indicating the replacement has occurred. (Germaria containing 0 or 2 labeled stem cells at the earliest timepoint are already homogenous and replacement cannot be detected.)

An increase in the proportion of double-labeled germaria is related to the rate of clone expansion, while an increase in the proportion of unlabeled germaria is related to the rate of clone extinction. Under neutral competition, the rates of extinction and expansion should be equal. Unless otherwise stated, all confidence intervals in this paper are 95% confidence intervals.

**RNA-seq of PFCs expressing EGFR**

109-30ts was used to express UAS-EGFRtop + UAS-mCD8::GFP or UAS-mCD8::GFP alone. Flies were bred and reared to adulthood at 18°C, then transferred to 29°C for 7-10 days prior to dissection. During the incubation at 29°C, flies were provided with wet yeast daily until 24 hr prior to dissection; removal of wet yeast on the final day allows mature egg chambers to clear from the ovary and reduces clogging of cell filters.

For each replicate, over 200 flies were dissected and collected in Schneider’s Insect Medium + 10% FBS. Two replicates were collected for each genotype, for a total of four samples. To maintain tissue health, the total dissection time was limited to 45 mins, and the dissection dish and tissue collection tubes were maintained on ice. Pipette tips used for transferring ovaries to collection tubes should be coated in 35% BSA immediately prior to use to prevent tissue from adhering to the inside of the tips.
Ovaries were rinsed 3x in Cell Dissociation Buffer (CDB; Thermo Fisher Scientific 13151014), then incubated in CDB + 4 mg/mL elastase (Worthington Biochemical LS002292) + 2.5 mg/mL collagenase (Thermo Fisher Scientific 17018029) for 15 min at room temperature to disrupt the tissue. During this 15-minute incubation, tissue was agitated by inverting the tubes and by passing the tissue through a P200 pipette tip. Dissociated cells were passed through a 50µm filter (Partec 04-0042-2317), the dissociation enzymes were quenched by adding 0.5 volumes Scheider’s Insect Medium + 10% FBS, and the cells were maintained on ice. Dissociated cells were pelleted by centrifugation at 1000g for 7 min at 4°C, then resuspended in 90 uL Schneider’s Insect Medium + 10 uL α-CD8a MicroBeads (Miltenyi Biotec 130-049-401) per 15 flies dissected. Dissociated cells were allowed to incubate with the α-CD8 MicroBeads for 15 min at 4°C. The CD8+ cells were then isolated by passing the cells over a magnetic column in an OctoMACS separator (Miltenyi Biotec 130-042-108). Cells were eluted from the column, pelleted by centrifugation at 1000g for 7 min at 4°C, resuspended in ~150 uL Lysis Solution (Thermo Fisher Scientific AM1931), then stored at -80°C until RNA isolation.

RNA was isolated from tissue using the RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher Scientific AM1931), including DNase I treatment. From a preparation of ~200 ovaries, ~100 ng of total RNA was isolated. PolyA selection was performed on the total RNA using oligo-dT Dynabeads (Thermo Fisher Scientific 61006). The ScriptSeqv2 kit (Illumina SSV21106) with Index Primers Set 1 (Illumina RSBC10948) was used to generate indexed paired-end RNA-seq libraries.

Libraries were sequenced at the UCSF Institute for Human Genetics on an Illumina HiSeq 2500 with a High Output PE 2x100bp flow cell. Sequenced reads were trimmed using Scythe (v 0.991, https://github.com/vsbuffalo/scythe) and Sickle (v 1.29,
https://github.com/najoshi/sickle). Quality check was performed using FastQC (v 0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed reads were aligned to the Drosophila melanogaster genome annotation from ENSEMBL (BDGP 5.74; from Illumina iGenomes https://support.illumina.com/sequencing/sequencing_software/igenome.html) using TopHat2 (v 2.0.10) (Kim et al., 2013). Differential expression was of aligned reads was tested by two methods: (1) Cufflinks Cuffdiff (v 2.1.1) (Trapnell et al., 2012) or (2) HTSeq-count (v 0.6.1) (Anders et al., 2015) and DESeq2 (v 1.2.10) (Love et al., 2014). To limit subsequent analysis to transcription factors, only genes referenced in Trusted TFs (v1) on FlyTF.org (http://www.flytf.org/flytfmine/bag.do) were considered. Plotting of FPKM values for transcription factors was performed using matplotlib (Hunter, 2007).

**CRISPR generation of Six4<sup>108</sup> allele**

The following primers were used to generate a chimeric RNA for the CRISPR Cas9 system:

**Sense** CTTCGCGAGCTGAGATTGTCCTGA

**Anti** AAACTCAGGACAATCTCAGC

These primers were annealed and inserted into pU6-BbsI-gRNA according to the U6-gRNA (chiRNA) protocol available at flyCRISPR (Bassett and Liu, 2014; Gratz et al., 2014). This construct was co-injected into flies containing FRT2A along with Cas9 RNA by Rainbow Transgenic Flies, Inc. Injected flies (P<sub>0</sub>) were crossed to (w; Sp / CyO; TM2 / TM6) to introduce balancers. Independent lines were established by backcrossing individual F<sub>1</sub> males to (w ; Sp / CyO; TM2 / TM6). As previously described Six4 alleles are known to be recessive lethal (Clark et al., 2007), lines were screened for lethality. Lines containing a lethal mutation were sequenced.
by first PCR amplifying the region of Six4 targeted for mutation, then sequencing this PCR product using the following primers:

- PCR Forward GACAAGTGAATGCAGTTTAGTG
- PCR Reverse AAATGTGTACTCCTCAGCAG
- Sequencing CTCTGGACTATTTGCACCGA

Of 100+ lines screened, only one line with a Six4 disruption was isolated. Based on the mixed sequencing peaks present, this line likely has a deletion of G108 relative to the ATG start. We have designated this allele Six4$^{108}$. 
References


Assa-Kunik, E., Torres, I., Schejter, E., Johnston, D. and Shilo, B. (2007). Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. Development 134, 1161–1169.


*Development* 124, 3871–3880.


Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H.,
expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7,
562–578.


Wang, X., Bo, J., Bridges, T., Dugan, K. D., Pan, T. C., Chodosh, L. A., and Montell, D. J.
(2006). Analysis of cell migration using whole-genome expression profiling of migratory


Biol.* 2, 261–273.

Xie, T., and Spradling, A. C. (1998). decapentaplegic is essential for the maintenance and
division of germline stem cells in the Drosophila ovary. *Cell.* 94(2), 251-60.

Xie, T., and Spradling, A. C. (2000). A niche maintaining germ line stem cells in the

C., Amieva, M. R., et al. (2012). The intestinal stem cell markers Bmi1 and Lgr5 identify

Publishing Agreement
It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:
I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

[Signature]
Author Signature

Nov. 3, 2016
Date