© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 1937-1947 doi:10.1242/dev.143727

Escort cells generate a dynamic compartment for germline stem cell differentiation via combined Stat and Erk signalling

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ABSTRACT

Two different compartments support germline stem cell (GSC) self-renewal and their timely differentiation: the classical niche provides maintenance cues, while a differentiation compartment, formed by somatic escort cells (ECs), is required for proper GSC differentiation. ECs extend long protrusions that invade between tightly packed germ cells, and alternate between encapsulating and releasing them. How ECs achieve this dynamic balance has not been resolved. By combining live imaging and genetic analyses in *Drosophila*, we have characterised EC shapes and their dynamic changes. We show that germ cell encapsulation by ECs is a communal phenomenon, whereby EC-EC contacts stabilise an extensive meshwork of protrusions. We further show that Signal Transducer and Activator of Transcription (Stat) and Epidermal Growth Factor Receptor (Egfr) signalling sustain EC protrusiveness and flexibility by combinatorially affecting the activity of different RhoGTPases. Our results reveal how a complex signalling network can determine the shape of a cell and its dynamic behaviour. It also explains how the differentiation compartment can establish extensive contacts with germ cells, while allowing a continual posterior movement of differentiating GSC daughters.

KEY WORDS: Germline stem cells, Escort cells, *Drosophila*, Stat, Erk, Rho, Cdc42, Protrusion

INTRODUCTION

Balancing stem cell maintenance and differentiation is fundamental for correct embryonic development and tissue homeostasis. However, although the signalling cross-talk accompanying stem cell self-renewal and differentiation is amply addressed, the dynamic nature of this homeostatic process is less clear. In the fly germarium, the switch between germline stem cell (GSC) maintenance and differentiation overlaps a switch from a maintenance niche to a differentiation compartment. Here, we address the dynamic nature of this GSC differentiation zone.

The germarium is located at the most anterior part of the fly ovary and harbours two or three GSCs, which self-renew and generate differentiating daughters (Fig. 1A). These daughter cells undergo four cycles of incomplete division, resulting in the production of germline cysts. Cysts can be identified by the presence of a branched intracellular structure, the fusome, whereas GSCs and their immediate daughters harbour a spherical fusome (de Cuevas and Spradling, 1998). GSCs are maintained by various signals, with primary contribution by Decapentaplegic (Dpp), a BMP family ligand emanating from somatic terminal filament (TF) and cap cells, which together make the GSC niche (Xie and Spradling, 1998, 2000). Dpp signalling represses the major differentiation factor Bag of marbles (Bam), thus maintaining GSCs in an undifferentiated state (Chen and McKearin, 2003a,b; Song et al., 2004).

Once GSC daughters leave the niche, they are encapsulated by somatic ECs, which line the germarium towards the posterior. Importantly, the tight association with ECs is a prerequisite for germline differentiation and cyst formation (Kirilly et al., 2011; Maimon et al., 2014; Schulz et al., 2002). One model accounting for this requirement suggests that the tight contact between ECs and differentiating germ cells shields the germline from the self-renewal cues that emanate from the niche (Kirilly et al., 2011; Liu et al., 2010; Lu et al., 2015; Luo et al., 2015; Wang et al., 2015). Alternatively, ECs may play an instructive role in GSC differentiation (Gancz et al., 2011; Li et al., 2015; Maimon et al., 2014).

Several signalling pathways are implicated in maintaining EC shape (Konig et al., 2011; Liu et al., 2015; Lu et al., 2015; Maimon et al., 2014; Morris and Spradling, 2012; Schulz et al., 2002; Upadhyay et al., 2016; Wang et al., 2015). Specifically, germ cells lacking the Egfr ligands or the enzyme Stet, which is required for processing the Egfr ligand Spitz, fail to induce Egfr signalling in ECs, resulting in EC extension loss and a subsequent failure in GSC differentiation (Liu et al., 2010; Schulz et al., 2002). Similar defects were observed when ablating Stat (Stat92E – FlyBase) pathway components, including the associated transcription factor Without Children (Woc), and the downstream target Zfh1 from ECs (Maimon et al., 2014).

Despite their clear phenotypic consequences, the mechanistic aspects of how the Stat and Egfr pathways control protrusions remain elusive. On the one hand, ECs must invade between tightly packed germ cells and maintain tight germ cell encapsulation. On the other hand, differentiating cysts must pass through the germarium, transferring from one EC to the next. ECs must therefore exercise recurrent cycles of germ cell capture and release. Here, we provide a first detailed characterisation of the unique cytoskeleton shapes ECs display when they encapsulate germ cells. We used live imaging to follow the dynamic behaviour of ECs and show how they interact to form an intricate meshwork of protrusions to engulf the germline. We further uncover the mechanistic basis for the elaborate shapes and dynamic behaviour of ECs; both these traits rely on integrating the constitutive activity of the Stat and Egfr pathways. This cooperative activity regulates downstream actin remodelling factors – the small Rho-GTPases Cdc42 and Rho1. The superimposition of a composite signalling network on a Rho-

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Received 13 August 2016; Accepted 21 April 2017

Development (2017) 144, 1937-1947 doi:10.1242/dev.143727

GTPase network is likely to have general implications for various motile cells, including cancer cells.

RESULTS

Escort cells form a tight meshwork that dynamically encapsulates germ cells

The differentiation of GSC daughters within the germarium relies on tight contacts with the somatic ECs. ECs send long protrusions, which traverse the germarium and encapsulate germ cells (Kirilly et al., 2011; Liu et al., 2010; Maimon et al., 2014; Morris and Spradling, 2011; Schulz et al., 2002). However, the mechanisms allowing ECs to invade between germ cells and engulf them are unknown. To better define EC behaviour, we first characterised their structural components and the *in vivo* behaviour of their extensions using high-resolution and live-imaging approaches. Previous studies suggested EC protrusions contain both actin- and tubulin-based filaments (Kirilly et al., 2011; Morris and Spradling, 2011). Indeed, labelling of actin filaments using anti-Coracle (Cora) antibodies and of microtubules using a GFP fusion of End-binding 1 (Eb1:GFP) showed colocalisation of both types of filaments in EC extensions (Fig. 1B, inset). To reveal the precise arrangement of these filaments within extensions, we performed high-resolution imaging, using stochastic optical resolution microscopy (STORM) (Rust et al., 2006). STORM imaging revealed that microtubules

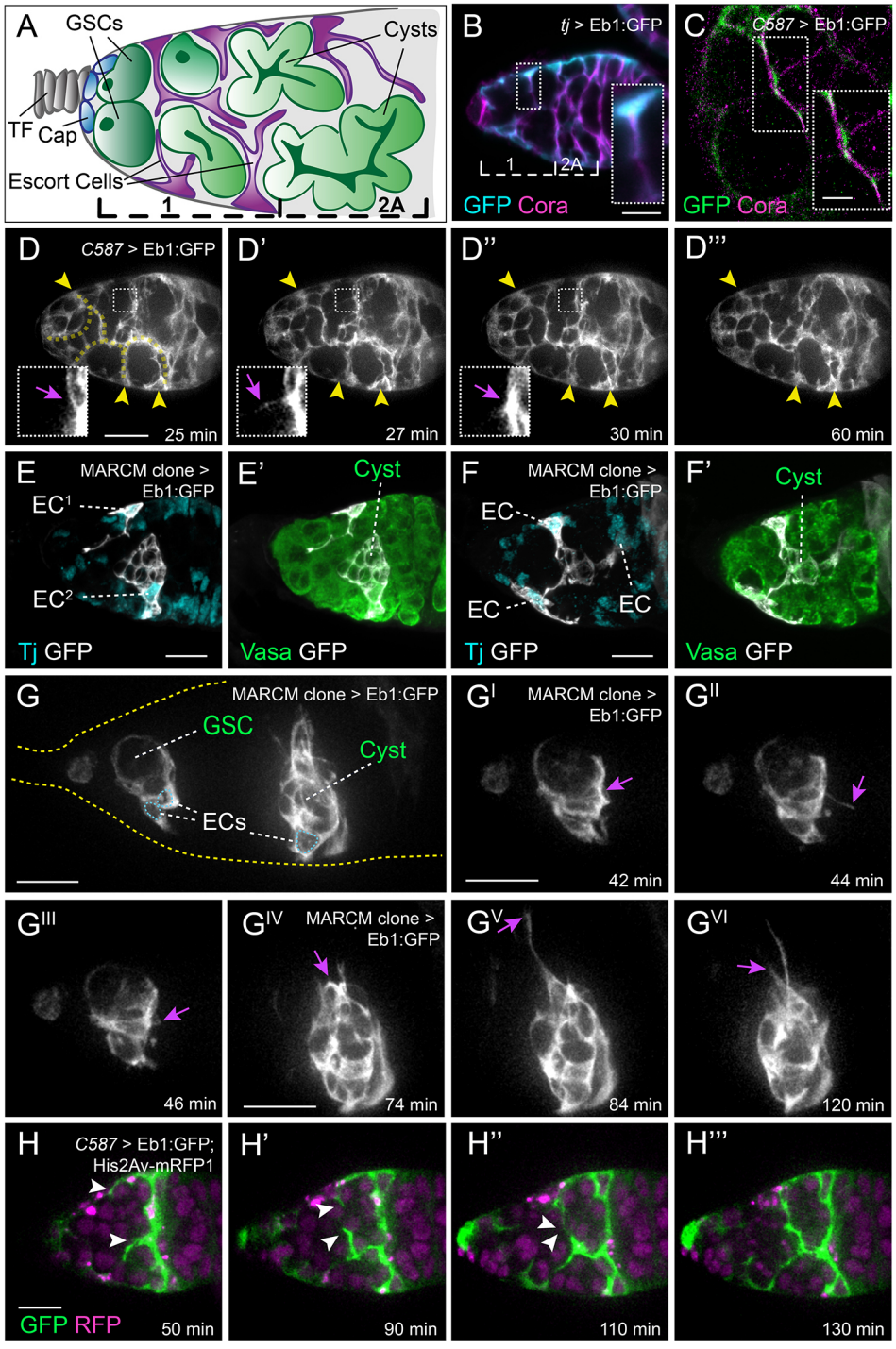

Fig. 1. Escort cells cooperate to dynamically encapsulate germ cells. (A) Schematic representation of the anterior part of a germarium; regions 1 and 2A are indicated by brackets. Somatic terminal filament (TF) and cap cells support germline stem cells (GSCs). Somatic escort cells (ECs) send long protrusions to engulf differentiating germline cysts. GSCs and their immediate daughters carry round fusomes (dark-green dots), whereas differentiating cysts contain branched fusomes (dark-green lines). (B-H‴) Microtubules are marked by Eb1:GFP. (B,C) Anti-Cora antibody marks actin filaments in ECs. (B) Wild-type germarium; regions 1 and 2A are indicated by brackets. Extensions traverse the germarium between germline cysts. Inset: actin and tubulin staining colocalise in an EC protrusion. (C) STORM images of EC extensions. Scale bar: 2 µm. Inset shows actin filaments and microtubules wrapped around each other. (D-D‴) Movie stills illustrating the behaviour and dynamics of EC extensions. Individual EC protrusions stably engulf germ cells (yellow arrowheads and dotted line). ECs also generate short and highly dynamic side-branches (insets, purple arrows, *n*=20 germaria). (E,F′) Confocal images of Eb1:GFP-labeled MARCM clones showing partial (EC^1 in E), full (EC^2 in E) and simultaneous (F,F′) engulfment of germ cells. EC nuclei are labelled using anti-Tj antibodies; germ cells are stained using anti-Vasa antibodies. (G-G^{VI}) Movie stills of Eb1:GFP MARCM-labelled EC clones. (G) An overview of a germarium (yellow line) containing three labelled ECs engulfing germ cells. Position of associated germ cells and ECs are given; EC nuclei are indicated (blue). (G^{I}-G^{III}) In addition to major protrusions, ECs displayed short and highly dynamic projections (purple arrows, 4.07±1.5 µm in length; 5.0±3.2 min persistence; from 32 projections). (G^{IV}-G^{VI}) Ten percent of central-to-posterior ECs also displayed long and more-persistent side-branches (purple arrows, 13.65±1.7 µm length; 29±15 min persistence). (H-H‴) Stills from a movie illustrating EC cooperation during germ cell cyst engulfment. Cell nuclei are labelled by His2Av-mRFP. Extensions from two ECs (arrowheads) protrude and connect to each other to stably engulf a cyst. Scale bars: 10 µm. Stills from movies are maximum intensity projections covering 20 µm (D-D‴,H-H‴) or 5 µm (G-G^{VI}); confocal images are maximum projections covering the full volume of ECs; Vasa labelling covers only *z*-planes of associated germ cells.

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wound around the actin bundles (Fig. 1C, 87.5% of analysed extensions, *n*=8), a conformation that might aid in efficient cyst engulfment. Indeed, actin and microtubule cable 'bundling' was previously suggested to allow for dynamic protrusive activity in migrating cells (Rodriguez et al., 2003). Thus, the specific organisation of EC cytoskeleton components may underlie the motility and invasiveness that ECs must practice while encapsulating germ cells.

To analyse the dynamic aspects of EC extensions, we followed them *in vivo* using a live-imaging approach. The strong Eb1:GFP signal allowed us to image germaria with high temporal resolution for at least 2 h without significantly damaging germ cells or somatic cells (Fig. S1A,B). We could capture the shape of ECs in fine detail, observe how they engulf germ cells and track the movement of their extensions. Live imaging revealed two types of EC extension: long and persistent protrusions as well as small and highly dynamic projections. The major protrusions persisted for several hours, ensuring stable germline engulfment (Fig. 1D, yellow arrowheads and dotted line, Movie 1).

Next, we used single cell labelling, employing the MARCM technique (Lee and Luo, 1999), which allowed us to monitor individual EC shapes. To determine how these individual ECs might encapsulate germline cysts, we additionally labelled germline cysts and EC nuclei. This analysis revealed different EC-germline cysts relationships: First, a single EC could encapsulate a cyst partially (EC^1 in Fig. 1E,E′). Indeed, ECs could jointly encapsulate germline cysts (Fig. 1F,F′, Fig. S1L). Alternatively, ECs could apply prominent extensions and generate elaborate 'basket-like' structures that wholly encapsulate cysts (EC^2 in Fig. 1E,G, Fig. S1J and Movies 2-4). Occasionally, ECs could form more than one such 'basket' and were thus able to associate with more than one cyst at a time (Fig. S1K and Movie 5). In most cases (40 out of 60 EC clones), we detected ECs as they encapsulate cysts. However, rarely (five out of 60 EC clones, Movie 6), we also observed ECs disengaging from a cyst. Combined, our observations suggest that germ cell encapsulation is maintained by ECs in a 'hand-me-down' fashion, even as cysts move down the germarium.

ECs fell into three major categories that varied greatly in shape and size, depending on their location within the germarium. The anterior-most ECs displayed short and less elaborate extensions (Fig. $1G^{I\text{-}III}$ and Fig. S1C). In the central region of the germarium, ECs showed highly branched and dynamic protrusions (Fig. $1G^{IV\text{-}VI}$ and Fig. S1D-F), while posterior ECs had gradually longer and more stable extensions (Fig. S1G). In addition to the major protrusions, short and highly dynamic side branches were also observed (Fig. $1E^{I\text{-}III}$ and Fig. S1C-G). Consistent with the amount of extensions displayed by each class of EC, the degree of germ cell full engulfment increased along the AP axis: It was rarely observed for ECs positioned at the anterior (6%) or at the centre (30%) of the germarium. However, 65% of posterior ECs did fully engulf neighbouring cysts (Fig. S1H).

ECs contacted not only germ cells, but also each other (Fig. S1K, L and Movie 5), suggesting that ECs might cooperate during cyst engulfment. Indeed, long-term imaging revealed that EC-EC interactions could lead to *de novo* encapsulation of germline cysts that persisted for several hours (Fig. 1H and Movie 7). Thus, EC-EC connections may contribute to the stability of germline encapsulation.

In summary, cyst encapsulation by ECs involves a unique arrangement of their cytoskeleton to build elaborate 'baskets' that enclose germ cells. While EC cell bodies were stationary, their protrusions displayed a highly motile and diverse behaviour. Stable protrusions secure proper germ cell engulfment, while dynamic projections likely probe the immediate environment of the ECs.

Escort cells lacking Stat or Erk signalling fail to stably engulf germ cells

Our live-imaging data describe ECs as supporting an elaborate and dynamic cytoskeletal network. This must require complex regulation. We have previously shown that Stat signalling and the transcription factor Without Children (Woc) collaborate to enhance Zfh1 expression and EC extensions (Maimon et al., 2014). Using our live-imaging set-up, we asked which aspect of EC behaviour is affected by the Stat/Woc pathway. We first examined *woc*-deficient ECs in *woc*-RNAi germaria, in which all ECs contain reduced Woc levels. Such ECs still extended small dynamic protrusions (Fig. 2A′-A‴, inset and arrows, Movie 8). However, the stable extensions that penetrated deep into the tissue were lacking (96% of analysed germaria, *n*=24).

The inability of *woc*-deficient ECs to form stable connections with other *woc*-deficient ECs allowed us to probe the contribution of EC-EC connections to germline encapsulation. We generated single *woc*- or *stat*-mutant EC clones, which were in contact with wild-type neighbours. As expected, both *woc*- and *stat*-mutant EC clones were less efficient in encapsulating germ cells than WT ECs, with less than 10% of *stat*-mutant and 25% of *woc*-mutant ECs achieving full germline engulfment (Fig. 2B-G). Surprisingly, the majority of these clones were able to at least partially engulf cysts (Fig. 2B-G and Movies 9 and 10). These results, together with our observations in *woc*-RNAi ovaries, in which mutant ECs could not encapsulate cysts (Fig. 2A) (Maimon et al., 2014), suggest that wild-type neighbours stabilised mutant EC extensions and that encapsulating germ cells is a communal phenomenon.

In addition to Woc/Stat, Egfr signalling also promotes EC extensions and GSC differentiation (Liu et al., 2010; Schulz et al., 2002). Indeed, removal of *Drosophila* Mek and Erk (Dsor1 and Rolled, respectively) from ECs by RNAi resulted in loss of EC extensions and defects in GSC differentiation (Fig. S2). Interestingly, live imaging showed that ECs in *dsor1*-RNAi germaria behaved similarly to ECs in *woc*-RNAi germaria (*n*=9 germaria); in both cases, ECs could not maintain stable long protrusions, but did support active small protrusions (Fig. 2H, Movie 11). This suggests that both Erk and Stat/Woc signalling promote extension elongation and stabilisation, and that the pathways may cooperate.

The Stat and Erk signalling cascades act in parallel to promote EC extensions

To test possible interactions between Stat and Erk in ECs, we investigated whether defects in one pathway could be rescued by activating the other. To quantify these genetic interactions, EC nuclei were stained using anti-Traffic Jam (Tj) antibodies and their extensions using anti-Cora antibodies. The extent of EC defects was scored by analysing regions 1 and 2A of individual germaria (see Fig. 1A,B, bracketed) for normal, partial or complete lack of protrusions (Fig. S3A-C). By this analysis, overexpression of the major Stat/Woc ovarian target Zfh1 (Leatherman and Dinardo, 2008; Maimon et al., 2014) re-established wild-type extensions in 47% of *dsor1*-RNAi germaria and reduced the fraction of germaria with no extensions from 31% to 0%. Similar results were obtained for *rolled*-RNAi germaria (Fig. 3A-E, Fig. S3D-G). Concomitant with extension re-establishment, GSC differentiation was also restored, in accordance with the role of EC-germ cell contacts in inducing GSC differentiation (Fig. S3H). Zfh1 levels were normal

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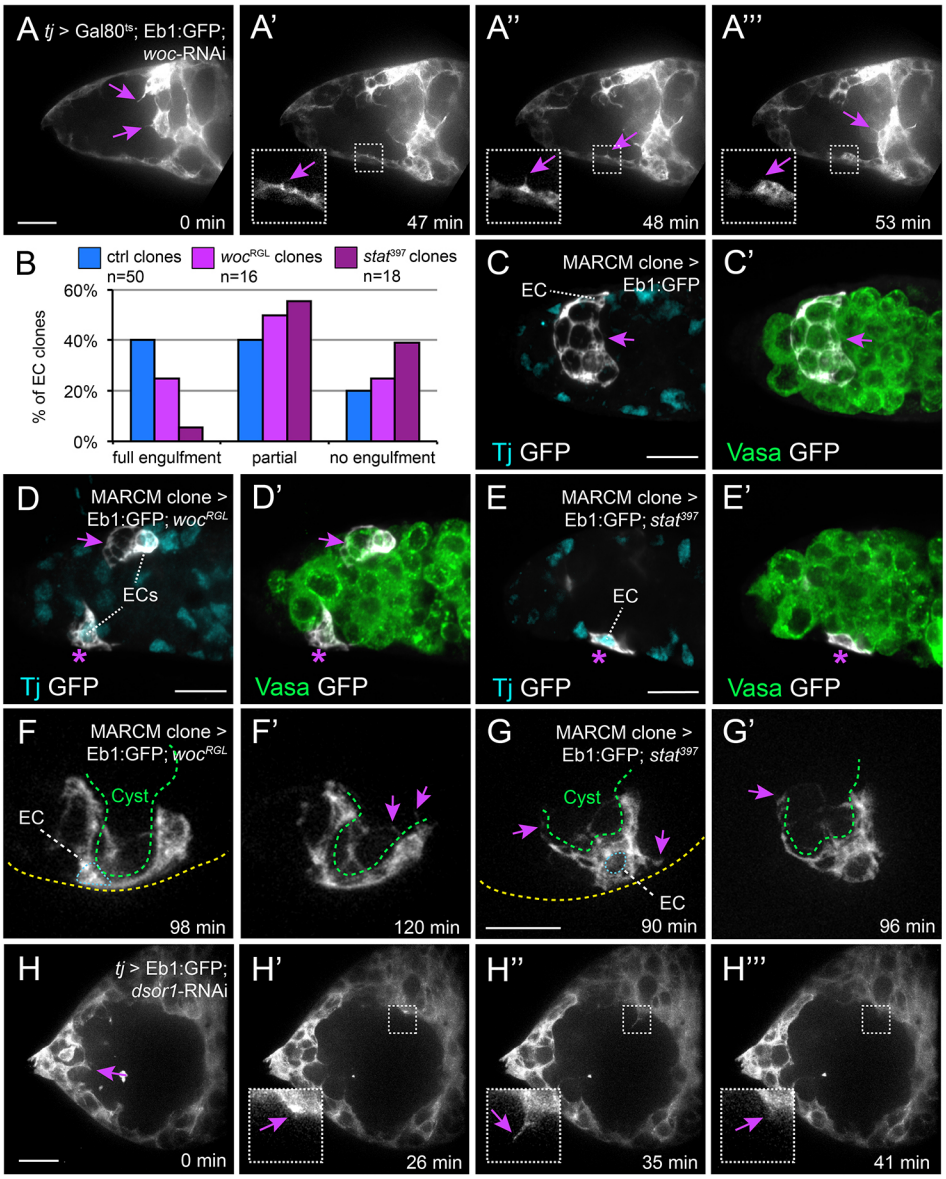

Fig. 2. Stat and Erk signalling promote elongation and stability of EC extensions. (A-A‴,F-H‴) Movie stills show ECs labelled using Eb1-GFP. (A-A‴) All ECs are *woc* deficient and display only short dynamic protrusions (insets, purple arrows). (B) The extent of germline engulfment by ECs comparing wild-type, *woc*- and *stat*-mutant EC clones, which were followed live for 2 h. *P*-values calculated using Fisher's exact test are 0.099 for woc^{RGL} and 0.003 for $stat^{397}$ compared with control. (C-E′) Confocal images show wild-type (C,C′) *woc*-mutant (D,D′) or *stat*-mutant (E,E′) EC clones in germaria co-labelled with anti-Tj antibodies to mark EC nuclei and Vasa to mark germ cells. Arrows indicate ECs that fully (C,C′) or partially (D-E′) engulf germ cells; asterisks indicate ECs not engulfing germ cells. (F-G′) Still images from movies depicting *woc*- (F,F′) or *stat*- (G,G′) mutant MARCM clones. Mutant ECs send short dynamic protrusions (arrows) but are unable to engulf a neighbouring cyst (green outline); yellow line outlines the germarium; EC nuclei are indicated in blue. (H-H‴) *dsor1*-deficient ECs generate only short dynamic protrusions (arrows in inset, *n*=9 germaria); long extensions are completely lacking. Scale bars: 10 µm. Stills from movies are maximum intensity projections covering 20 µm; confocal images are maximum projections covering the full volume of ECs; Vasa labelling covers only *z*-planes of associated germ cells.

in *dsor1*- and *rolled*-RNA germaria, suggesting that the Zfh1 rescue was not a trivial correction of Zfh1 levels (Fig. S3I). Importantly, pErk levels remained low in *dsor1*-RNAi ECs following Zfh1 expression, suggesting that the Zfh1 rescue was downstream of Erk activity (Fig. 3F).

Similar to the rescue of the Erk pathway by the Stat target Zfh1, we found that defects in the Woc/Stat pathway could be rescued by activating Erk signalling. Expression of a constitutively active Egfr ($Egfr^{CA}$) alone had some deleterious effect on EC extensions (Fig. 3H,K). However, expression of $Egfr^{CA}$ in *woc*-RNAi ovaries still achieved a significant rescue, and reduced the fraction of germaria containing no extensions from 76% to 17% (Fig. 3G-K). Introduction of $Egfr^{CA}$ could also alleviate the phenotype of other Stat signalling components, such as *stat* itself or *zfh1* (Fig. S3J-N). pErk levels were normal in *stat*- and *woc*-deficient ECs, suggesting that the $Egfr^{CA}$ rescue was not a mere correction of Erk activity (Fig. S3O-P). Importantly, the levels of Woc protein remained low in *woc*-RNAi ovaries that expressed $Egfr^{CA}$, suggesting Egfr activity did not rescue *woc* deficiency by restoring Woc expression (Fig. S3Q-R). Likewise, Zfh1 levels remained low in rescued ECs (Fig. 3L), suggesting that $Egfr^{CA}$ rescued the Stat pathway defects downstream of Zfh1.

In conclusion, our data indicate that the Erk and Stat/Woc pathways act in parallel to promote EC extensions and GSC differentiation. However, the reciprocal functional rescues suggest that these pathways must converge downstream to promote the protrusive activity observed in ECs.

Differential rescue of *woc*- and *dsor1*-deficient ECs by $Cdc42^{CA}$ and Rok^{DN}, respectively

Signalling pathways are known to affect cell shape and motility by controlling cytoskeleton dynamics (Bear and Haugh, 2014; Mogilner and Keren, 2009; Pocha and Montell, 2014; Tybulewicz and Henderson, 2009). Possible targets for such effects are the small RhoGTPases: Rho1, Rac1 and Cdc42. We therefore asked whether manipulating RhoGTPases could counter-balance the defects caused by Stat/Woc and Erk deficiencies. To perform these experiments, we introduced either constitutively active (CA) or dominant-negative (DN) versions of RhoGTPases into *woc*-RNAi expressing adult ECs in a temporally controlled manner. In these

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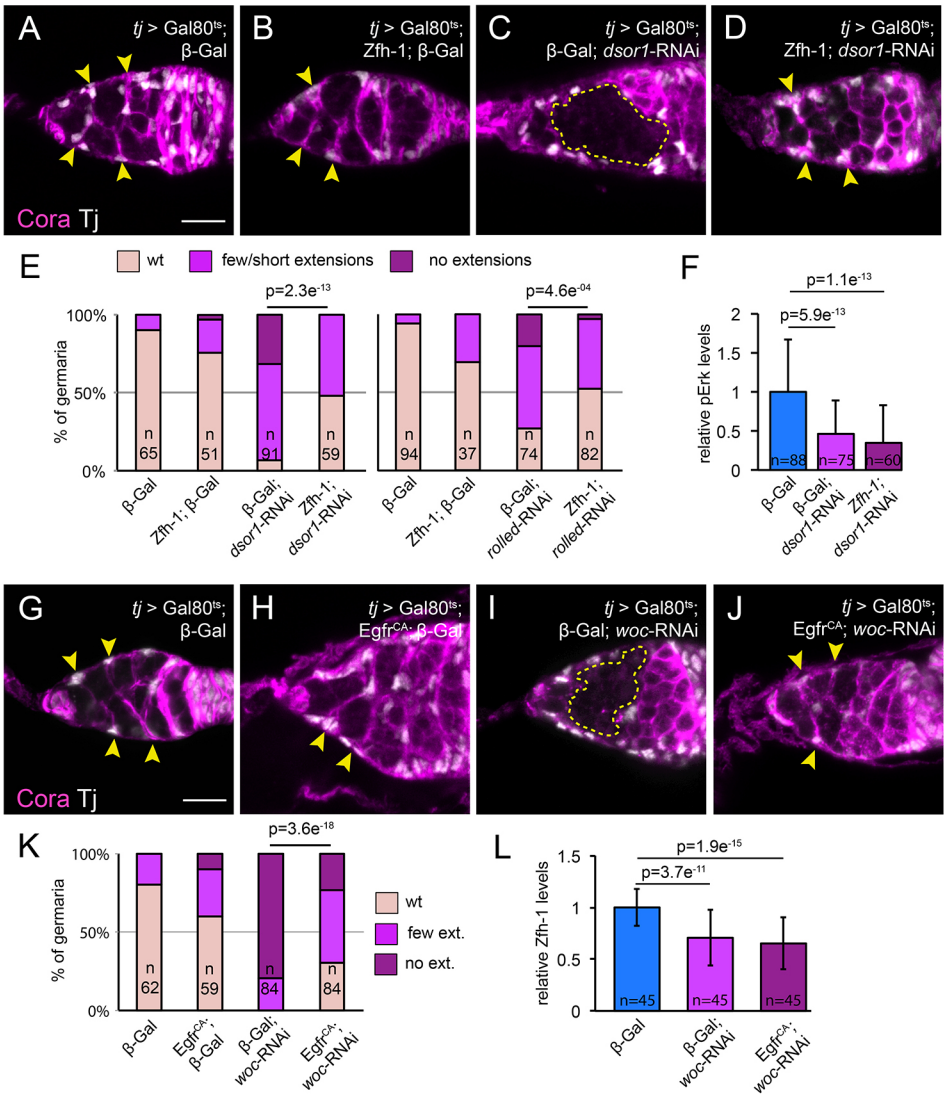

Fig. 3. Stat and Erk signalling independently regulate EC extension stability. (A-D,G-J) EC nuclei (white) are labelled using anti-Tj antibodies; anti-Cora antibodies label the actin cytoskeleton (magenta). Arrowheads mark nuclei of ECs that send long protrusions. Areas devoid of EC extensions are outlined. (A-D) Zfh1 overexpression can rescue protrusions in ECs lacking *dsor1*. (E) Quantification of protrusions in *dsor1*- and *rolled*-mutant ECs that are rescued by Zfh1. (F) Quantification of relative pErk levels in EC nuclei. Zfh1-mediated rescue of *dsor1*-RNAi does not involve elevated pErk levels. (G-J) Rescue of *woc*-deficient EC extensions by $Egfr^{CA}$ introduction. (K) Quantification of the results presented in G-J. (L) Quantification of relative Zfh1 levels in EC nuclei. *woc*-RNAi rescue by $Egfr^{CA}$ does not involve elevated Zfh1 levels. Error bars represent s.d.; *P*-values are calculated using the Mann-Whitney *U*-test (F,L) or Fisher's exact test (E,K). Scale bars: 10 µm.

experiments, EC extensions were compared three ways: the DN or CA form of each RhoGTPase on its own, *woc*-RNAi on its own or a combination of the two (Fig. 4A). Introduction of $Rho1^{DN}$ into control germaria disrupted EC extensions, as described previously (Kirilly et al., 2011). Similar disruption occurred by expressing $Rho1^{CA}$ in ECs. Manipulation of Rac1 activity resulted in 50% of germaria entirely lacking germ cells (Fig. 4A), a severity of phenotype suggesting additional roles for Rac1 in ECs. Finally, using CA and DN constructs to manipulate Cdc42, which is a key player in promoting filopodia-like extensions in various cell types (Hall, 2005; Heasman and Ridley, 2008), affected ECs only mildly (Fig. 4A). Cdc42 is, however, an important regulator of EC protrusions, as expression of *cdc42*-RNAi in ECs could abolish EC extensions (Fig. S4A-C). Significantly, introducing $Cdc42^{CA}$ into *woc*-deficient ECs did not restore Woc protein expression (Fig. S4D-F), but did result in a substantial rescue of EC extensions; the fraction of *woc*-RNAi germaria completely lacking extensions was reduced from 57% to 6% upon $Cdc42^{CA}$ co-expression (Fig. 4A-D). Introduction of no other RhoGTPase, in either its CA or DN form, could achieve such a rescue (Fig. 4A-E).

Re-establishment of extensions by $Cdc42^{CA}$ was also sufficient to restore proper germ cell differentiation in *woc*-RNAi ovaries. Germ cell differentiation was determined by anti-Hts antibody, which labels the fusome – an intracellular organelle within germ cells. GSCs and their immediate daughters carry a spherical fusome, and differentiating germline cysts contain branched fusomes (Fig. 1A). Whereas wild-type germaria contained two to five single germ cells, *woc*-deficient germaria exhibited a large increase in single germ cells, indicating cyst differentiation failure (Maimon et al., 2014). Re-introduction of $Cdc42^{CA}$ into *woc*-deficient ECs resulted in significant alleviation of the differentiation defects (Fig. 4F-I). Notably, expression of no other form of Rho-GTPase could rescue germ cell differentiation in *woc*-RNAi germaria (Fig. S4G). The rescue of *woc*-RNAi by $Cdc42^{CA}$ is of particular interest as it demonstrates that the signalling properties of *woc*-RNAi ECs are unaffected, and that mere restoration of physical association between such ECs and germ cells is sufficient to support proper GSC differentiation. These results further suggest that the Stat/Woc cascade specifically regulates Cdc42 to promote proper EC form and function.

Studies in the fly testis suggest that Erk signalling might also control EC protrusions via RhoGTPases, as $Rho1^{DN}$ overexpression in cyst cells corrects cyst encapsulation defects caused by reduced Egfr signalling (Sarkar et al., 2007; Schulz et al., 2002). To

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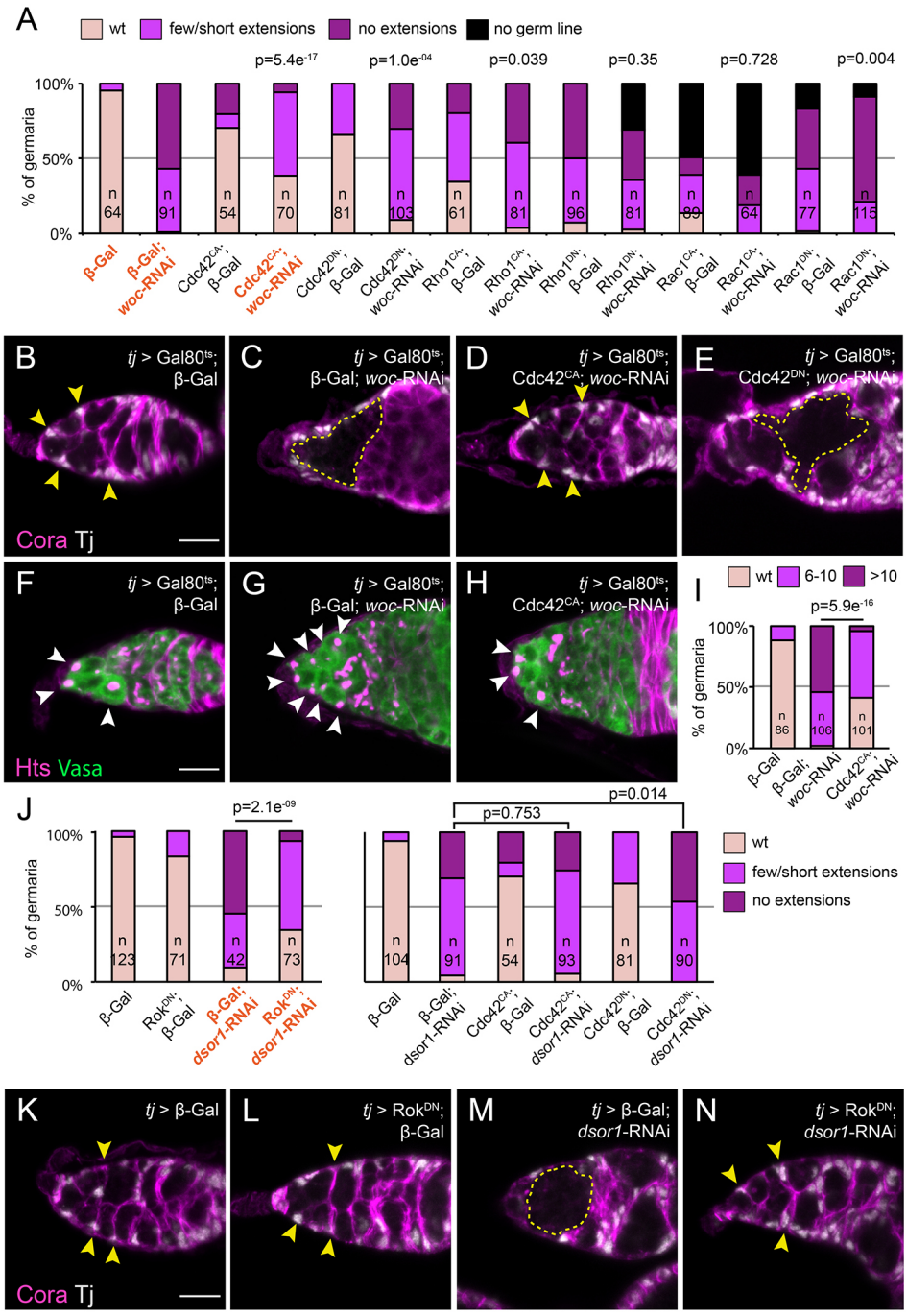

Fig. 4. Rescue of *woc*- or *dsor1*-deficient ECs by differential Rho-GTPase activities. (A) Quantification of EC extensions in *woc*-RNAi germaria following expression of various forms of Rho-GTPases. The most relevant genotypes are in orange; Fisher's exact test *P*-values compare each genotype with β-Gal; *woc*-RNAi controls. (B-E) ECs labelled using anti-Tj antibodies (white) and anti-Cora antibodies (magenta). Arrowheads indicate ECs sending long protrusions. Areas devoid of extensions are outlined. Extensions are abrogated in *woc*-deficient ECs. $Cdc42^{CA}$ overexpression re-establishes extensions, whereas $Cdc42^{DN}$ expression cannot. (F-H) Anti-Vasa antibodies label germ cells (green); anti-Hts antibodies mark fusomes (magenta). Arrowheads indicate undifferentiated germ cells with round fusomes. Expression of $Cdc42^{CA}$ in *woc*-deficient ECs significantly decreases the numbers of undifferentiated germ cells. (I) Quantification of the result presented in F-H. (J) Quantification of EC extension appearance in *dsor-1* RNAi germaria upon expression of the CA or DN versions of Rho-GTPases. The most relevant genotypes are in orange. (K-N) ECs are labelled using anti-Tj antibodies (white) and anti-Cora antibodies (magenta). Arrowheads mark ECs sending long protrusions. Areas devoid of extensions are outlined. EC extensions are lost in *dsor1*-deficient ECs. Rok^{DN} expression rescues EC protrusions. *P*-values are calculated using Fisher's exact test. Scale bars: 10 µm.

determine whether a similar mechanism is in place in ECs, we co-expressed $Rho1^{DN}$ with *dsor*-RNAi. Indeed, some rescue was observed in such ovaries (Fig. S4H). However, the adverse effects of Rho^{DN} on overall germarium morphology complicated our analysis. To gain more insights and to avoid the gross defects caused by $Rho1^{DN}$ expression in ECs, we used a dominant-negative form of the Rho kinase (Rok^{DN}), which is a direct downstream effector of Rho1 (Winter et al., 2001). Although Rok^{DN} on its own had only mild effects on EC extension appearance, its expression in a *dsor*-RNAi background reduced the fraction of germaria containing no extensions from 55% to 7% (Fig. 4J-N). Thus, similar to somatic cyst cells in the male, Erk activity represses Rho1 in ECs to allow for proper protrusive activity. Notably, expression of activated or dominant-negative forms of Cdc42 could not overcome the defects caused by ablation of Dsor1 function in ECs (Fig. 4J), suggesting Erk signalling might regulate Rho1 specifically.

Differential regulation of Cdc42 by Stat/Woc and of Rho1 by Erk signalling

The reciprocal rescue of *woc* and *dsor1* deficiencies by elevating Cdc42 or reducing Rho1 activities, respectively, suggested to us that the two signalling pathways might differentially regulate RhoGTPases: To ensure proper EC shape and function, Stat/Woc may promote Cdc42 activity while Erk signalling may repress Rho1. To test this model, we investigated whether manipulation of Stat/Woc or the Erk pathway affected Cdc42 and Rho1 activity,

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respectively. A biosensor for Cdc42 (Wasp-RBD-GFP) (Abreu-Blanco et al., 2014) localised to EC bodies and protrusions, demonstrating high Cdc42 activity in these cells (Fig. 5A). As a second marker for Cdc42 activity, we used antibodies that recognise the GTP-bound state of Cdc42. Anti-Cdc42-GTP also stained ECs, confirming the biosensor labelling (Fig. 5B). Quantification of fluorescence levels of this antibody in regions 1 and 2A of the germarium revealed increased labelling when $Cdc42^{CA}$ was expressed in ECs (Fig. 5F). Moreover, overexpression of $Cdc42^{DN}$ resulted in decreased antibody labelling, despite the elevated levels of Cdc42 protein. Thus, the antibody indeed captures the GTP-bound form of Cdc42 (Fig. 5F). Next, we examined how the Stat/Woc pathway affected Cdc42 activity and found significantly reduced levels of Cdc42-GTP in *woc*-RNAi germaria (Fig. 5B-E). Consistent with the rescue of *woc*-RNAi by $Cdc42^{CA}$, Cdc42-GTP levels were elevated upon re-introduction of $Cdc42^{CA}$ to *woc*-RNAi germaria (Fig. 5B-E). These results support a role for Stat/Woc in regulating proper EC protrusive behaviour by promoting Cdc42 activity.

Two biosensors were used to determine Rho1 activity: Capu-RBD-GFP and Dia-RBD-GFP (Abreu-Blanco et al., 2014). Capu-RBD-GFP fluorescence was strong at the posterior of the germarium. In ECs it localised mainly to the EC cell body and only weakly to protrusions (Fig. 5G-G′). Thus, some Rho1 activity is present in ECs, as was also suggested by the phenotypes induced by $Rho1^{DN}$ expression in ECs (see Fig. 4A and Fig. S4G-H). To test how Erk signalling might affect Capu-RBD-GFP localisation, we analysed *rolled*-RNAi germaria that still maintained some extensions. Interestingly, Capu-RBD-GFP mislocalised to EC protrusions in these germaria, suggesting Egfr signalling attenuates Rho1 activity in ECs (Fig. 5H,H′). Expression of Dia-RBD-GFP, a second Rho1 biosensor, could be readily detected in

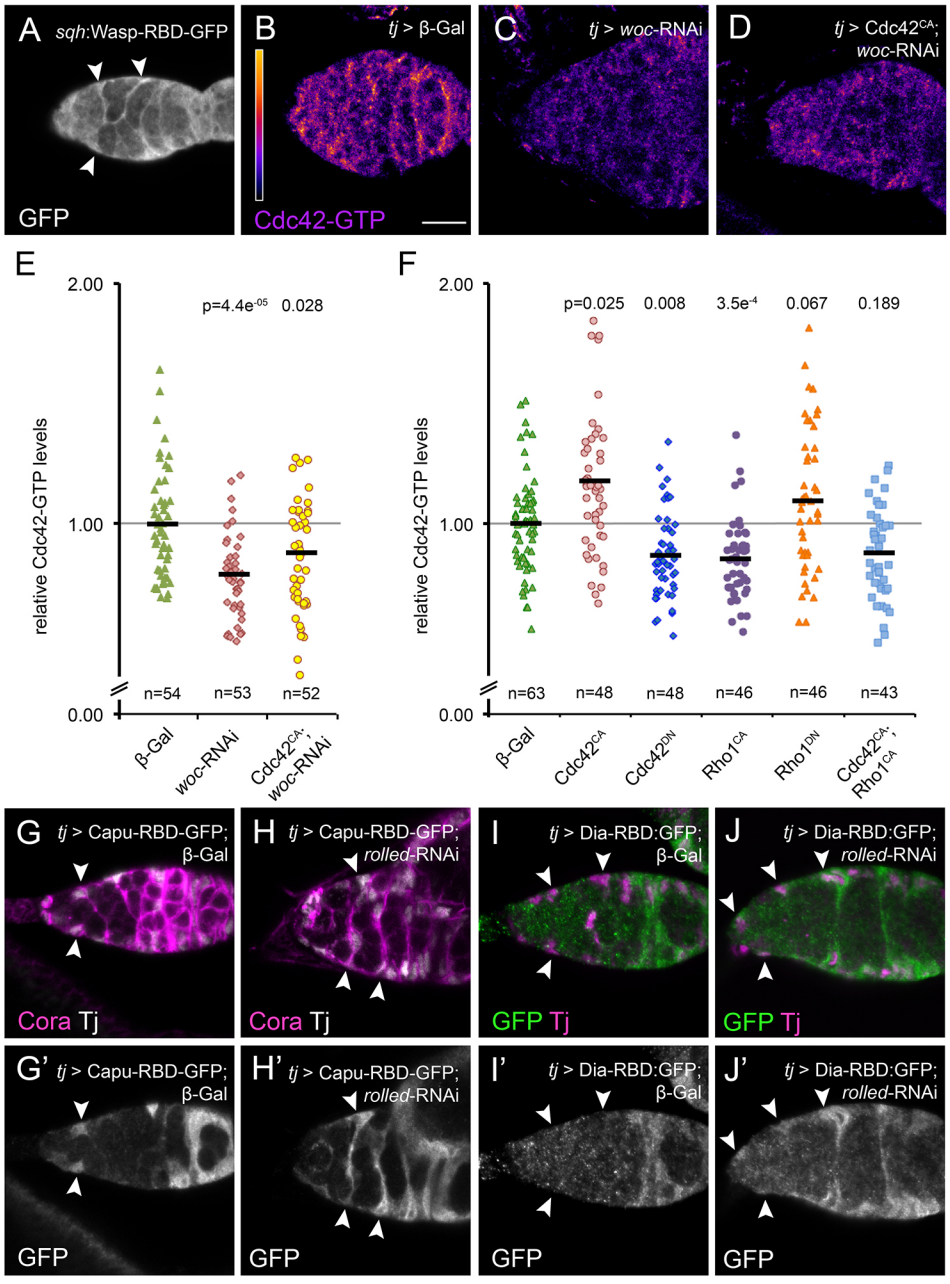

Fig. 5. Combinatorial regulation of Rho-GTPases by Erk and Stat. (A) Expression of the Cdc42 biosensor Wasp-RBD-GFP shows high Cdc42 activity in ECs, including their extensions (arrowheads). (B-D) Anti-Cdc42-GTP labelling (pseudocolour range indicator) is shown; images are background subtracted and scaled (see Materials and Methods). Cdc42 activity decreases in *woc*-RNAi germaria (C) and is restored upon co-expression of activated Cdc42 (D). Colour bar in B denotes low (blue) to high (yellow) labelling of Cdc42-GTP. (E) Quantification of the results presented in B-D. *P*-values are determined using the Mann-Whitney *U*-test. (F) Scatter-plot depicting changes in Cdc42-GTP levels in various genotypes, highlighting the antagonism between Rho1 and Cdc42. *P*-values are determined using the Mann-Whitney *U*-test. (G-J′) Staining of escort cell nuclei (anti-Tj) and EC extensions (anti-Cora) in G-H indicate the localisation of GTPase-specific biosensors (white in G′,H′,I′,J′). (G′-H′) Localisation of the Rho1 activity biosensor Capu-RBD:GFP (white). (G,G′) In 73% of wild-type germaria, no fluorescence from the biosensor was observed in EC extensions. In 27%, weak fluorescence was observed in extensions (*n*=77 germaria). (H,H′) In 86% of *rolled*-RNAi germaria, strong fluorescence was observed in EC extensions (*n*=37). (I-J′) The Rho1 activity biosensor Dia-RBD:GFP (green in I,J, white in I′,J′) shows Rho1 activity only in posterior regions of wild-type germaria (I,I′, 75%, *n*=53), while it is de-repressed in anterior ECs in *rolled*-RNAi germaria (J,J′ 73%, *n*=56). Scale bar: 10 µm (in B for A-D,G-J′).

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all posterior follicle cells, but in only very few ECs (Fig. 5I,I′). By contrast, increased numbers of ECs with prominent Dia-RBD-GFP fluorescence were detected upon *rolled* removal (Fig. 5J-J′). Similar tendencies were observed using an antibody directed against Rho1-GTP (Fig. S5). The data thus reveal that Erk functions to restrict Rho activity in the anterior regions of the germarium.

EC protrusions depend on Woc/Stat promoting Cdc42 activity and on Erk signalling attenuating Rho1 activity. One likely explanation for this double requirement is an inherent antagonism between Cdc42, which promotes filopodia-like protrusion, and Rho1, which represses this activity (Kutys and Yamada, 2014; Vaughan et al., 2011). To test this option, we examined whether Rho1 activity might dampen Cdc42 activity in ECs. As expected, Cdc42 activity increased upon $Cdc42^{CA}$ expression and decreased by $Cdc42^{DN}$ expression (Fig. 5F). Manipulating Rho1 affected Cdc42 in an opposite manner: $Rho1^{CA}$ expression repressed Cdc42 activity, whereas $Rho1^{DN}$ increased it (Fig. 5F). This increase was mild, as Rho1 activity is naturally low in ECs. Importantly, elevating Rho1 activity was as efficient in reducing active Cdc42 as expression of $Cdc42^{DN}$ itself (Fig. 5F). Finally, co-expression of both $Cdc42^{CA}$ and $Rho1^{CA}$ resulted in a significant reduction of Cdc42-GTP levels compared with $Cdc42^{CA}$ ($P=1.9\times10^{-5}$), suggesting that Rho1 activity antagonises that of Cdc42 and that, to ensure continuous Cdc42-mediated support of EC protrusions, Rho1 activity must be continually restrained.

DISCUSSION

GSC daughters differentiate to form germline cysts as they travel down the germarium. This process requires tight association with ECs, and also that ECs periodically release the moving cysts. Here, we show that EC shape and motility are position dependent. We further show that the Stat and Erk signalling act in concert to generate EC shapes and protrusive activity. Stat signalling promotes Cdc42 activity and long filopodia-like extensions, while Egfr attenuates the activity of Rho1, which is an antagonist of Cdc42. Thus, the two pathways function in a ‘gas and brake’ mode, allowing ECs to encapsulate germ cells (Fig. 6).

ECs dynamically associate with and induce germ cell differentiation

Utilising our live-imaging protocol, we explored the dynamics of a system that has mostly been studied in fixed samples. Analysis of single-labelled ECs revealed a highly complex and dynamic organisation of EC extensions. ECs varied greatly in shape, size and their association with germ cells. These differences correlated with EC position within the germarium. The apparent morphological and behavioural differences in the EC pool suggest that the different EC classes might be functionally diverse. In support of this, ECs closest to the Cap cells were shown to participate in GSC maintenance (Rojas-Rios et al., 2012). In addition, it was demonstrated that the position of mutant ECs within the germarium affected the resulting phenotype (Eliazer et al., 2014). Further studies investigating these apparent EC differences and their genetic basis would be of great interest.

ECs encapsulate germline cysts either singly or in cooperation with neighbouring ECs, suggesting that germ cell encapsulation by ECs has a communal component. Indeed, the presence of wild-type ECs was sufficient to partially rescue the ability of *stat*- and *woc*-mutant ECs to encapsulate cysts (Fig. 2D). This may explain why GSC differentiation defects in *stat* and *woc* mutant germaria could only be observed when all ECs within a particular germarium were mutant (Maimon et al., 2014). Wild-type ECs can rescue GSC differentiation in two ways: first, wild-type protrusions can encourage cyst encapsulation by mutant ECs; second, the hand-me-down nature of EC-cyst interactions ensures that in a germarium with mixed wild-type and mutant ECs, germ cells will encounter wild-type ECs that can promote their differentiation.

Our findings regarding EC-cyst interactions may help resolve the two models that currently vie to explain how ECs promote germline cyst differentiation. The first model suggests that ECs block maintenance cues from the niche. This blockage could be either physical, as was suggested for cyst differentiation in males (Fairchild et al., 2015), or biochemical, by modulating the spread of Dpp (Kirilly et al., 2011; Liu et al., 2010; Lu et al., 2015; Luo et al., 2015; Wang et al., 2015). The second model suggests that ECs promote cyst differentiation via an unknown differentiation factor (Li et al., 2015; Maimon et al., 2014). Our observations suggesting recurrent capture and release of cysts from ECs contest a physical barrier function for ECs, as complete blocking of cysts from their environment during these cycles is hard to achieve. Similarly, the fact that a few wild-type ECs within an otherwise mutant germarium are sufficient to rescue GSC differentiation cannot easily be reconciled with a biochemical barrier. On the other hand, the capture-and-release model is compatible with an inductive role for ECs in GSC differentiation, as it predicts that the serial contacts of the few wild-type ECs with many cysts could overall rescue germ cell differentiation.

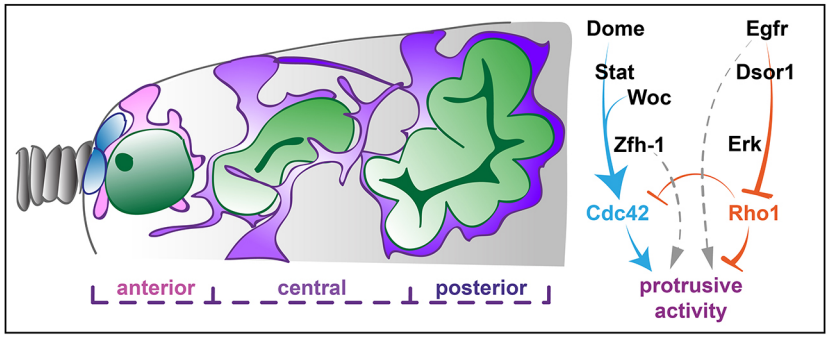

Fig. 6. ECs form a dynamic differentiation niche regulated by superimposition of Stat and Erk signalling onto a GTPase network. ECs differ in their shapes, size and association with germ cells according to their position within the germarium. The highly dynamic ECs capture and release germ cells as they progress towards the posterior of the germarium. Adaptability and flexibility of ECs is secured by dual regulation involving the Stat and Egfr pathways. Continuous Stat signalling in conjunction with Woc and Zfh1 promotes Cdc42 activity and drives proper EC protrusive activity. Egfr signalling through the Dsor1-Erk cascade attenuates Rho1 activity in ECs to ensure proper EC shape and function. Egfr and Zfh1 can also influence protrusive behaviour by alternative routes (grey arrows, see Discussion).

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In support of an inductive role for ECs, three groups have reported the existence of 'cystoblast tumours' (Kirilly et al., 2011; Li et al., 2015; Maimon et al., 2014). These 'tumours' consist of *bam*-expressing cells that cannot differentiate further to make cysts. A barrier function cannot be invoked in these cases, as germ cells have already escaped niche signals and embarked on the differentiation path. Indeed, even forced Bam expression cannot induce cyst formation in *woc*-RNAi germaria (Maimon et al., 2014). This strongly suggests that ECs provide a differentiation cue to allow cystoblasts to further differentiate into germline cysts.

Combinatorial Stat/Woc and Erk signalling controls EC cell shape and protrusive behaviour

The germarium is packed with germline cysts, and EC protrusions must invade between and engulf them. This requires constant Egfr and Woc/Stat activity. Activation of Egfr and/or Stat has been linked to motile behaviour in various cell types (Caric et al., 2001; Duchek and Rorth, 2001; Kawata, 2011; Niwa et al., 2005; Schafer et al., 2004; Silver et al., 2005; Silver and Montell, 2001; Soldevila et al., 2004; Yue et al., 2012). However, how these two pathways interface to promote motility has not been resolved. In ECs, Erk and Stat signalling promote protrusiveness by targeting the activity of specific Rho-GTPases, favouring a state where Cdc42 activity is high and Rho1 activity is low. Keeping a correct balance between Rho1 and Cdc42 may be an essential aspect of controlling EC elongation, retraction or stabilisation.

Egfr and Stat are at the top of a cascade that branches to control multiple targets, and they likely affect cytoskeleton dynamics by multiple means (Fig. 6). Indeed, reciprocal rescue between the Egfr and Stat pathways was possible at the level of Zfh1 and Egfr (Fig. 3), but not at the level of the RhoGTPases themselves (Fig. 4). Clearly, both Egfr and Stat coordinate a multi-target system that balances cytoskeleton dynamics. The identity of the other targets should be the aim of further investigations (Fig. 6).

Interestingly, border cells, an additional group of ovarian somatic cells, which are studied as a model for invasive migration, also rely on continuous Erk and Stat signalling for their motility (Duchek and Rorth, 2001; Montell et al., 2012; Prasad and Montell, 2007; Silver et al., 2005; Silver and Montell, 2001). Rac1 is the driving force of motility in border cells (Montell et al., 2012; Murphy and Montell, 1996), with Rho1 and Cdc42 playing only minor roles (Bastock and Strutt, 2007; Llense and Martin-Blanco, 2008; Murphy and Montell, 1996). It is possible that the difference in RhoGTPase identity reflects the nature of cellular protrusions each cell type generates (Ridley, 2015).

Constitutive activation of Egfr and Stat, which underpins protrusive behaviour in ECs, has also been associated with malignant transformation and with metastasis. Indeed, simultaneous activation of the Stat and Erk pathways in *Drosophila* results in highly aggressive tumours (Herranz et al., 2012; Wu et al., 2010). This particular combination of signalling pathways therefore seems specifically suited to control cell motility and invasiveness.

A network of RhoGTPAses regulates EC protrusive behaviour

The protrusive activity of ECs is regulated by a network of RhoGTPases, which control cytoskeletal rearrangements in many cells. In general, Cdc42 activity is associated with assembly of parallel actin bundles and filopodia-like protrusions. On the other hand, Rho1 and Rac1 regulate actomyosin filaments and branched actin structures (Hall, 2005; Heasman and Ridley, 2008; Ridley, 2015). Indeed, while Cdc42 affected EC protrusions specifically, manipulating Rho1 and Rac1 activity in ECs affected the whole structure of the germarium, and could even result in loss of germ cells. This suggests a more-general structural role in actin organisation for Rho1 and Rac1 within ECs. Such function does affect extension formation, likely by modulating the cytoskeletal arrangement to an extent that disrupts filopodial outgrowth (Mattila and Lappalainen, 2008). We suggest that EC extensions are modulated by the interplay between high Cdc42 activity, which promotes protrusions, and low Rho activity, which serves to fine-tune or modify it. Consistent with this idea, we were able to show that Cdc42 activity is sensitive to changes in Rho1 activation (Fig. 5F). A similar antagonistic relationship between these two RhoGTPases occurs in neuronal growth cones (Hall and Lalli, 2010) and in migrating fibroblasts (Kutys and Yamada, 2014).

Notably, a study in the fly testis linked Rho1 and Rac1 to germ cell encapsulation by cyst cells: the male analogues of ECs (Sarkar et al., 2007). It remains to be seen whether Cdc42 plays a similar role in male cyst cells and if the observed defects arising from Rho1 and Rac1 manipulations result from a direct impairment of protrusion generation or indirectly by affecting the general actin-cytoskeleton architecture. Alternatively, the different nature of the interactions between male cyst cells, which stably adhere to the same germline cyst cell throughout its development, and the more transient interactions of ECs and germ cells, may require a different mode of cytoskeleton activation. Female ECs emerge therefore as an interesting model for studying how external cues modulate cell shape and motility in a dynamic manner.

MATERIALS AND METHODS

Antibody preparation and staining

Antibodies were used in the following concentrations throughout this study: mouse monoclonal anti-Hts (1b1, 1:20) and anti-Coracle (1:200) from the Developmental Studies Hybridoma Bank (DSHB); mouse anti-Cdc42-GTP (1:100, #21010) and anti-Rho-GTP (1:75, #26904) from New East Biosciences; rabbit anti-Vasa (1:5000) and anti-Zfh1 (1:5000) were a gift from Dr Ruth Lehmann (HHMI, New York University, USA); rabbit anti-Woc (1:2000) was a gift from Dr Maurizio Gatti (Università di Roma, Italy); guinea pig anti-Tj (1:7000) was a gift from Dr Dorothea Godt (University of Toronto, Canada); rabbit anti-pERK (1:200, #4370) was from Cell Signaling; and rabbit anti-GFP (1:1000, #ab290) was from Invitrogen. Secondary antibodies were from Invitrogen and were used according to the manufacturer's instructions. Secondary antibodies used for STORM were Alexa 647 anti-mouse (1:1500 or 1:5000) from Life Technologies. Cy3b (1:100) was conjugated to a secondary IgG antibody. For the conjugation method and PI staining, see supplementary Materials and Methods.

Imaging

For live and STORM imaging, germaria were mounted in 0.8% low-melting point agarose (NuSieveGTG from Lonza) in 35 mm glass-bottomed culture dishes (with 14 mm Microwell No.1.0 coverglass; 0.13-0.16 mm) filled with imaging buffer [7 µM glucose oxidase (Sigma), 20 mM cysteamine (Sigma), 150 mM β-mercaptoethanol (Sigma), 50 mM Tris, 10 mM NaCl, 56 nM catalase (Sigma) and 10% glucose (pH 8)]. STORM imaging was performed on a Vutara SR200 microscope. Live imaging was performed on a Zeiss Axio Observer.Z1 equipped with a CSU-X1 Spinning Disc Unit or on a Zeiss Lightsheet Z.1 SK-2 system. Confocal imaging was performed on Zeiss LSM 710, on a Zeiss Observer.Z1 or a Zeiss LSM 800. For detailed experimental methods, see supplementary Materials and Methods section.

Statistical analyses

All experiments were performed with at least three biological repeats. For all experiments, over 25 germaria from at least 10 different animals were examined. Statistical significance was determined either using a Mann-Whitney *U*-test or a Freeman Halton extension of the Fisher's Exact test, as indicated. Error bars represent s.d.

Fly stocks

Fly stocks used in this study are listed in Table S1.

MARCM clones were generated using *c587*-Gal4; Eb1-GFP; *tubP*-Gal80,FRT82B crossed to hsflp;;neoFRT82B, hsflp; FRT82B,$stat^{397}$ or hsflp; FRT82B,woc^{RGL}. Somatic clones for intensity measurements were generated using *c587*-Gal4,UASflp;; FRT82B,nls-GFP. The exact regimens for each temperature shift experiment are specified in the supplementary Materials and Methods.

Acknowledgements

We thank R. Lehmann, B. Shilo, S. Parkhurts, E. Bach, O. Schuldiner, D. Godt and M. Gatti for their kind donation of fly stocks and reagents. We thank the TRiP at Harvard Medical School for providing transgenic RNAi fly stocks used in this study. We thank the Bloomington, FlyTrap, DGRC and VDRC stock collections for the stocks used in this study. We acknowledge the FlyBase team for their exceptional work on the *Drosophila* database. Super-resolution microscopy was performed at the Irving and Cherna Moskowitz Center for Nano and Bio-Nano Imaging at the Weizmann Institute of Science. We also thank the image analysis unit at the Weizmann Institute for assistance and the Microscopy Core of the NYU Langone Medical Center.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.U.B., I.M., L.G.; Methodology: T.U.B., L.G.; Investigation: T.U.B., I.M.; Writing - original draft: T.U.B., L.G.; Writing - review & editing: T.U.B., L.G.; Visualization: T.U.B., T.D.; Supervision: L.G.; Funding acquisition: T.U.B., L.G.

Funding

This work was supported by the Israel Cancer Research Fund (12-3073-PG), and by the Helen and Martin Kimmel Institute for Stem Cell Research (HMKISCR). T.U.B. was supported by an European Molecular Biology Organization long-term fellowship (ALTF 47-2014).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.143727.supplemental

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