Integrins regulate epithelial cell differentiation by modulating Notch activity

M. Jesús Gómez-Lamarca¹, Laura Cobreros-Reguera, Beatriz Ibáñez-Jiménez¹, Isabel M. Palacios² and María D. Martín-Bermudo¹,³

1. Centro Andaluz de Biología del Desarrollo CSIC-Univ. Pablo de Olavide, Sevilla 41013, Spain.
2. The Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK.
3. Correspondence should be addressed to: M.D.Martín-Bermudo CABD CSIC-Univ.Pablo de Olavide, Ctra Utrera km1, Sevilla 41013, Spain.

Phone: + 34 954348674
Fax: + 34 954349376
e-mail: mdmbarber@upo.es

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Abstract

Coordinating exit from the cell cycle with differentiation is critical for proper development and tissue homeostasis. Failure to do so can lead to aberrant organogenesis and tumorigenesis. However, little is known about the developmental signals that regulate the cell cycle exit-to-differentiation switch. Signals downstream of two key developmental pathways, Notch and Salvador-Warts-Hippo (SWH), and of myosin activity regulate this switch during the development of the follicle cell epithelium of the Drosophila ovary. Here, we have identified a fourth player, the integrin signaling pathway. We find that elimination of integrin function blocks mitosis-to-endocycle switch and differentiation in posterior follicle cells (PFCs), via regulation of the CDK inhibitor dacapo. In addition, we show that integrin mutant PFCs show defective Notch signalling and endocytosis. Furthermore, integrins act in PFCs by modulating the activity of the Notch pathway, as reducing the amount of Hairless, the major antagonist of Notch, or misexpressing Notch intracellular domain rescues the cell cycle and differentiation defects. Altogether, our findings reveal a direct involvement of integrin signalling on the spatial and temporal regulation of epithelial cell differentiation during development.
Introduction

The processes of cell proliferation and differentiation have been studied independently, but the cell cycle is intimately entwined with differentiation, as the latter is usually accompanied by irreversible cell cycle exit. However, in some cell types, a modified cell cycle occurs during differentiation in which the DNA is replicated without concomitant cell division, resulting in an increase in nuclear DNA content. This process, called endoreplication, occurs in many cell types including plant, invertebrate and vertebrate epidermis and in platelet-producing megakaryocytes and placental trophoblast cells in mammals. Furthermore, recent work indicates that progression through the endoreplication cycle is an important aspect of cell fate acquisition and cell differentiation in Arabidopsis (Bramsiepe et al., 2010). However, although considerable progress has been made to understand how the core cell cycle machinery is modified to convert a mitotic cycle into an endocycle, little is known about the developmental signals that couple mitosis-to-endocycle switch with differentiation.

The follicular epithelium (FE) of the *Drosophila* ovary constitutes an excellent model system for the *in vivo* study of the molecular and cellular mechanisms regulating the mitotic to endocycle switch and its association with cell differentiation. The *Drosophila* ovary is composed of about 15 ovarioles, each containing a gerarium at their proximal end and progressively older egg chambers at their distal end. Each egg chamber is composed of 15 nurse cells and one oocyte surrounded by a single layer of follicle cells (FCs), which constitute the FE (King, 1970). FCs originate from the asymmetric division of the Follicle Stem Cells (FCSs), which are located in the gerarium (Margolis and Spradling, 1995). At the time that the egg chamber buds from the gerarium, approximately 80 FCs surround the germline cyst (King, 1970). FCs continue to divide mitotically until stage 6 (S6). At this point, FCs exit the mitotic cycle and switch to an endocycle (Calvi et al., 1998). Between S7-10, FCs undergo three rounds of endoreplication, become polyploid and increase their size. At S10B, FCs undergo their third cell cycle regimen, gene amplification, by which four regions containing genes encoding chorion proteins are locally amplified (Calvi et al., 1998). These switches in cell cycle are coupled with changes in cell growth and differentiation.

A major pathway controlling cell proliferation and differentiation in FCs is the Notch pathway. The Notch pathway is evolutionary conserved and operates iteratively in an
enormous diversity of developmental and physiological processes (Andersson et al., 2011; Bray, 2006). This indicates that despite its simple molecular design, the Notch pathway must be able to elicit a large variety of appropriate responses in many spatially and temporally distinct cellular contexts. This versatility in signalling output is in part achieved by modulation of the pathway at different levels in the signal transduction cascade, such as post-translational modifications and trafficking of both the receptor itself and its ligands (reviewed in (Bray, 2006). Notch activity is also highly sensitive to chromatin modification and histone re-arrangements (reviewed in (Andersson et al., 2011; Bray, 2006). Finally, a number of reports have indicated that crosstalks with other signalling pathways also influence the Notch signalling output, adding additional levels of regulation (reviewed in (Guruharsha et al., 2012). In the context of this work, the Notch pathway has been shown to regulate the mitotic-to-endocycle switch, as well as the transition from undifferentiated to mature FCs (reviewed in (Klusza and Deng, 2011). At around S6, increased Delta (Dl) expression in the germ line activates Notch in FCs promoting the mitotic-to-endocycle switch (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Loss of function of Notch in FCs, or Dl in the germ line, leads to prolonged mitosis at the expense of endocycles and to a block in cell differentiation. Notch signalling regulates negatively the Drosophila Cdc25 phosphatase String (Stg). Because Stg activates the CycA/Cdk1 and CycB/Cdk1 complexes and an inhibitor of CycE/Cdk2 complexes, the p27Cip/Kip CKI protein Dacapo (Dap), Notch signalling allows CycE to oscillate driving cells into the endocycle. In addition, Notch regulates positively Fizzy-related (Fzr), an adaptor for the APC/C E3 ligase, which allows the degradation of the mitotic cyclins, thereby ensuring complete transition to endocycle (Schaeffer et al., 2004; Shcherbata et al., 2004). One pathway that has been shown to modulate Notch activity in this context is the SWH pathway (Yu et al., 2008). SWH mutant FCs continue proliferating, fail to entry into the endocycle and remain undifferentiated (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). Furthermore, the SWH pathway has been proposed to promote Notch signalling in FCs by regulating Notch endocytic trafficking (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). The Notch pathway can also be modulated by myosin activity in FCs. As it is the case for SWH, elimination of PPIβ, a phosphatase that dephosphorylates and inactivates the non-muscle myosin II light chain, also results in defective Notch signalling due to defects in the endosomal trafficking of the receptor (Sun et al., 2011).
Interestingly, myosin activity does not affect the SWH pathway. These results highlight the complexity of the genetic circuitry that affects Notch signalling during FC differentiation.

In this work, we have identified a fourth pathway regulating cell proliferation-to-differentiation switch in FCs, signalling downstream of integrins. Integrins are αβ heterodimeric cell-surface receptors that connect the extracellular matrix (ECM) to the cell’s cytoskeleton (Hynes, 2002). While in vertebrates there are at least 8 β subunits and 18 α subunits, in Drosophila, there are only two β subunits, βPS and βn, and five α subunits, αPS1 to αPS5. We have previously shown that elimination of myospheroid (mys), the gene encoding the βPS subunit, the only β subunit present in the ovary, from FCs results in multilayering and polarity defects at both poles of the egg chamber (Fernandez-Minan et al., 2008). Here, we report a new role for integrins in FCs, the control of the coupling of mitosis-to-endocycle switch with differentiation. We show that elimination of integrin function results in aberrant PFC endoreplication and differentiation. Furthermore, we show that integrins regulate proper exit from the cell cycle and cell differentiation by regulating the expression of the CDK inhibitor dacapo. Our results also demonstrate that integrins act in FCs by modulating the activity of the Notch pathway through the regulation of its intracellular trafficking and/or processing. In summary, we believe our work reveals that cell-ECM interactions mediated by integrins contribute to the temporal coupling of cell-cycle arrest with differentiation during development.

Results

Integrins regulate FC differentiation

We have previously reported that mosaic egg chambers containing mys mutant FC clones very often grew extra layers at both poles of the egg chamber (Fernández-Miñán et al., 2007). In addition, we have shown that mys mutant FCs located at the posterior pole, hereafter referred to as mys PFCs, showed an aberrant distribution of polarity markers (Fernandez-Minan et al., 2008). Interestingly, this polarity defect was only observed in mys PFCs located in the ectopic layers, while mys PFCs in contact with the germline were correctly polarized (Fernandez-Minan et al., 2008). Recently, we noticed that the nuclear diameter of mys PFCs (as identified by the absence of GFP) was significantly smaller
than their wild type counterparts (Fig. 1A, B). As it was the case for the polarity defects, the nuclear size phenotype was only observed in mutant PFCs that have lost contact with the germline (Fig.1A, white arrow), while mys PFCs in contact with the germline show no phenotype (Fig.1, white arrowhead). Small nuclear size is a potential indication of a failure to differentiate and to transit into the endocycle. Thus, we checked whether integrins were required for proper FC differentiation and mitosis-to-endocycle switch. In order to do this, we generated FC clones for two unrelated null alleles mys11 and mys10 and analysed the expression of markers normally found in undifferentiated cells, FasIII and Eyes Absent (Eya), or in differentiated PFCs, pointed-lacZ (pnt-lacZ) (Bai and Montell, 2002; Gonzalez-Reyes and St Johnston, 1998; Morimoto et al., 1996; Snow et al., 1989). Both alleles gave rise to indistinguishable phenotypes; however, in the text and in the figures we refer only to results obtained with mys11. In wild type egg chambers, FasIII and Eya are detected in all FCs up to S6 (insets in Fig1C and D, respectively). From S6 onwards, they are expressed only in polar cells (PCs) and border (BCs) and stretched (SCs) cells, respectively (Fig 1C, D). Conversely, pnt-lacZ is specifically expressed in wild type PFCs from S6 onwards (Fig 1C, D). However, in contrast to the wild type, we found that in S6 and older egg chambers most mys PFCs express FasIII and Eya (Fig 1E, 83% n=37 and Fig.1F, 80% n=42, respectively) and fail to activate pnt-lacZ (Fig.1E, F, 100% n=31). Again, the effects on FasIII, Eya and pnt-lacZ expression were only observed in mys PFCs located in the ectopic layers (Fig.1E and F, arrows). Finally, we found that the RNA-binding protein Staufen (Stau), which localises to the posterior of the oocyte during S9 (St Johnston et al., 1991), Supplementary Fig.1A), was properly localised in mosaic egg chambers containing mys PFCs (Supplementary Fig.1B, arrowhead), most likely due to the normal differentiation of mys PFCs in contact with the germ line. Altogether, these results strongly suggest that integrins are required for proper PFC maturation when contact with the germline is lost.

**Integrins are required in FCs for the mitosis-to-endocycle switch and for gene amplification**

Differentiation and withdrawal from the mitotic cycle are coupled in FCs. However, whether initiation of the differentiation program in FCs depends on exit from cell cycle has not yet been formally proven. Here, we decided to directly address this by blocking
exit from the cell cycle through the simultaneous overexpression of E2F, DP, CycE and Stg. This combination is sufficient to abrogate cell cycle exit in Drosophila eye and wing disc cells, where cell cycle arrest is not required for terminal differentiation (Buttitta et al., 2007). Here, we found that overexpression of those four genes in FCs was also sufficient to bypass cell cycle exit, as visualized by ectopic Phospho-Histone 3 (PH3) staining after S6 (Sup. Fig.2B). However, in contrast to the wing and eye, differentiation in FCs was blocked, as revealed by the maintenance of high levels of FasIII expression after S6 (Sup. Fig.2B). These results strongly suggest that FCs need to exit the mitotic cycle to differentiate.

We show here that mys PFCs display differentiation defects. This could reflect a failure to exit the mitotic cycle or a problem to differentiate. To test between these possibilities, we analysed the expression of the cell cycle regulator Cyclin B (CycB). CycB is normally expressed in mitotic FCs from S4 to S6, when it is downregulated (Lehman et al., 1999) and inset in Fig.2A). However, we found that mys PFCs located in ectopic layers showed prolonged expression of CycB after S6 (Fig.2B, 62%, n=34, arrow). We next analysed the expression of the Broad-Complex (BR-C), an early ecdysone-response gene that is essential for endoreplication and gene amplification. In wild type egg chambers, the BR-C is activated by ecdysone in all FCs from S6 onwards (Tzolovsky et al., 1999), Fig.2C), where it drives gene amplification, as visualised by the incorporation of BrdU in four discrete spots that represent amplification of the chorion genes in S10B follicle cells (Fig.2E). However, we did not detect BR-C expression in mys PFCs located in ectopic layers after S6 (Fig. 2D, 100%, n=39, arrow). As expected, these mys PFCs also fail to undergo gene amplification, as shown by BrdU incorporation (Fig.2F, 75%, n=38, arrow). Thus, instead of the foci-like pattern of BrdU incorporation typical of FCs undergoing gene amplification (Fig.2E), some S10B mys PFCs show a pattern characteristic of cells in mitosis, where BrdU labels the whole nuclei (Fig.2F, arrow). CycB and BR-C expression patterns were not affected in mys PFCs in contact with the germline (arrowheads in B and D).

Taken altogether, our results show that mys PFCs located in ectopic layers fail to undergo proper mitotic-to-endocycle switch and cell differentiation. As mentioned in the introduction, a key pathway regulating cell proliferation and differentiation in FCs is the Notch pathway. Thus, we next decided to analyse whether elimination of integrins could affect the Notch pathway.
**Notch signalling is disrupted in mys PFCs**

To examine whether Notch signalling was affected in mys PFCs, we analysed the expression of Cut, a homeobox protein that is downregulated by Notch signalling in FCs after S6 (Sun and Deng, 2005); Fig.3A, and that of Hindsight (Hnt), a zinc-finger protein that is induced by Notch after S6 (Sun and Deng, 2007); Fig.3C). We found that, in contrast to the wild type, mys PFCs that have lost contact with the germline showed continued expression of Cut (Fig.3B, n=27, arrow) and failed to activate Hnt (Fig.3D, n=29, arrow) in 82% and 80% of S6 and older egg chambers analyzed, respectively. These results suggest that Notch signalling is affected in the absence of integrin function.

To further confirm this, we measure Notch activity using the Gbe-Su(H)-lacZ reporter, which yields high levels of expression in cells where Notch is active (Furriols and Bray, 2001); Fig3E). Normally, the activity of this reporter is increased in FCs upon Notch activation during S7-10A, although its expression is not uniform along the FE, being highest in PFCs (Fig.3E). We found that the expression of this reporter was significantly reduced in mys PFCs located in ectopic layers in 75% of the egg chambers analyzed (Fig.3F, n=28, arrow). These results show that Notch signalling is indeed defective in mys PFCs that have lost contact with the germline.

**Integrins are required to strengthen Notch signalling**

An intriguing aspect of the mys phenotype is the fact that the defects in Notch signalling are only observed in mutant PFCs located in ectopic layers, while those mys mutant cells in contact with the germline develop normally. In this scenario, since the ligand for the Notch receptor in the ovary, Delta (Dl), is produced in the germline (Deng et al., 2001), the mutant cells adjacent to the germline could receive a normal signal, activating the Notch pathway properly. In contrast, those mutant cells in the ectopic layers, placed away from the source of ligand, would receive much less ligand input and consequently develop Notch mutant phenotypes. Therefore, the Notch-like defects could just be a secondary consequence of the multilayering phenotype. Alternatively, integrins may themselves be required to strengthen Notch signalling. This hypothetical integrin modulation of Notch signalling would become more critical in a sensitized background,
such as cells located in the ectopic layers. To test between these two possibilities, we performed four sets of experiments. First, we tested whether increasing Notch signalling in mys PFCs could rescue the phenotype. In order to do this, we removed one copy of Hairless (H), the major antagonist of Notch signalling in Drosophila (reviewed in (Maier, 2006), in mys mosaic egg chambers. Removing one copy of H in a wild type background did not affect FC differentiation (Fig.4A). In addition, halving the dose of H in a mys background did not have any effect on the onset or formation of the multilayering (Fig.4C). However, interestingly, we found that it rescued the differentiation (Fig.4C and G, n=32) and nuclear size defects (arrow in Fig.4C’ and H, n=28) of mys PFCs, as now only 23% of mosaic egg chambers containing mys PFCs and heterozygous for H (mys; H/+) showed FasIII expression after S6, in contrast to the 83% observed in mosaic egg chambers containing mys PFCs (Fig.4B and G). Second, we analysed whether halving the amount of Dl, by heterozygosity of a Dl loss-of-function allele, which on its own did not cause any effect, would enhance the integrin phenotype. Indeed, we found that in 87% of mosaic egg chambers containing mys PFCs and heterozygous for Dl (mys; Dl/+, n=23), the mutant PFCs in contact with the germline display differentiation defects, as monitored by FasIII expression (Fig.4D, arrowhead). Third, we tested whether failure to maintain contact with the germ line was on its own sufficient to affect proper differentiation. To this end, we analyzed the expression of FasIII and Eya in mosaic egg chambers containing bazzoka (baz) mutant FCs, which very often grew extra layers at both poles of the egg chamber. We did not find any alteration on the expression of FasIII or Eya in the mutant cells located on the ectopic layers (Fig.4E, F, arrows). And fourth, we tested whether increasing integrin signalling without rescuing the multilayer phenotype would rescue the differentiation defects. To this end, we made use of a chimeric integrin that allows constitutive integrin signalling in absence of adhesion, the Torβp/βcyt (Martin-Bermudo and Brown, 1999). We have previously shown that expression of this construct in mosaic egg chambers containing mys PFCs results in a partial rescue of the multilayer phenotype (Fernández-Miñán et al., 2007). Here, we found that it rescued the differentiation (Fig.5C and D, n=21) and nuclear size defects (Fig.5E and G, n=26) in 100% of the cases, including those in which the stratification phenotype was not rescued. Altogether, these results strongly suggest that the cell differentiation phenotype is not just a consequence of the multilayering and that integrin-mediated signalling is required to enhance Notch signalling in PFCs.
Finally, we tested whether elimination of other focal adhesion components, such as Talin, would also affect proper follicle cell differentiation. In order to do this, we generated mosaic egg chambers containing FCs mutant for *rhea*, the gene encoding Talin in *Drosophila* (Brown et al., 2002). We have previously shown that removal of *rhea* in FCs cause polarity and stratification defects identical to that of loss of *mys* (Fernandez-Minan et al., 2008) and Fig. 5F). However, we found that both the expression of FasIII and the nuclei size of all *rhea* PFCs, including those located in ectopic layers, were similar to wild type (Fig.5F, H). These results argue against the view that the differentiation defects observed in *mys* PFCs are mainly due to the multilayering and polarity phenotypes and strongly support our conclusion that integrins strengthen Notch signalling.

**The Notch intracellular domain (NICD) can rescue the Notch signalling phenotype of *mys* mutant PFCs**

To investigate how integrin function regulates Notch signalling, we tested whether the expression of various Notch constructs in *mys* PFCs could rescue the differentiation defects. As a result of ligand binding, Notch is proteolytically processed and the extracellular domain (NECD) is transendocytosed into the ligand-sending cell. The remaining receptor undergoes two successive proteolytic cleavages by ADAM and γ-secretase, respectively, the second step requiring Notch endocytosis. This is followed by the release of the Notch intracellular domain (NICD) and its translocation to the nucleus, where it regulates the transcription of its downstream targets (reviewed in (Fortini and Bilder, 2009). We found that when NICD was expressed in *mys* PFCs, both the nuclear size defect and the differentiation phenotype were rescued, as FasIII expression after S6 was only detected in 13% of the egg chambers carrying *mys* PFCs in ectopic layers (Fig.6A, arrow), in contrast to the 83% observed in mosaic egg chambers containing *mys* PFCs without NICD (Fig. 1E). Interestingly, neither full-length Notch (N) nor NECD were able to rescue the differentiation phenotype (Fig.6B and data not shown). This result suggests that elimination of integrins disrupts Notch signalling probably at the level of the final Notch cleavage, as this generates the functional NICD. This cleavage is regulated at the level of endosomal trafficking. Thus, to further investigate the consequences of eliminating integrin function on the Notch pathway, we compared the
expression and localization pattern of the Notch receptor itself in wild type and *mys* PFCs. In wild type FCs up to S6, the Notch receptor accumulates at high levels on the apical side. From this stage onwards, upon Notch activation by DI from the germ line, the levels of Notch in the membrane are strongly reduced (Lopez-Schier and St Johnston, 2001; Fig.6C, arrowhead). In *mys* PFCs located in ectopic layers, we found significant accumulations of both NICD and NECD at or below the plasma membrane (Fig.6D, n=32 and Sup. Fig.3B, C, arrows), suggesting that full-length Notch is present in excess amounts in these mutant cells. Furthermore, part of the ectopic Notch protein was visible in punctate cytoplasmic concentrations, indicating that some of the Notch protein was accumulating in vesicles (white arrows in Fig.6D’ and in Sup. Fig.3B, C). In fact, we found that several of the ectopic Notch-positive vesicles present in *mys* PFCs were also positive for the early endosomal marker, Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate, Fig.6E, arrow). This result suggested a potential defect in the processing and/or endocytic trafficking of Notch in *mys* PFCs. The aberrant Notch distribution observed in *mys* PFCs could just be a consequence of the multilayering and/or polarity defects found in *mys* PFCs. Alternatively, integrins may be required for proper endosomal trafficking of Notch. To distinguish between these two possibilities, we performed two sets of experiments. First, we tested whether halving the amount of DI, which on its own does not affect Notch distribution (data not shown), will affect Notch trafficking in *mys* PFCs that contact the germline. Indeed, we found a punctate distribution of Notch in the cytoplasm of *mys; Dl/+* PFCs in contact with the germline (Fig.6F, arrowhead), which resembled that observed in *mys* PFCs located in ectopic layers (Fig.6D, arrow). And second, we analysed Notch distribution in *baz* FCs, which very often show both multilayering and polarity defects. We found that, in contrast to what happens in *mys* PFCs, there is no increased accumulation of Notch at or below the plasma membrane in *baz* PFCs located in ectopic layers (Supplementary Fig.3D). These results strongly suggest that the changes in Notch distribution found in *mys* PFCs are not just a consequence of the multilayering or polarity defects and that integrins contribute to the proper endosomal trafficking and/or processing of the Notch receptor.

**Integrins are required for proper downregulation of the expression of the p27Cip/Kip CKI *dacapo* but not that of *string***
An intriguing aspect of the ovarian phenotype of *mys* when compared with that of Notch is that even though, like Notch, integrin mutant PFCs fail to enter in endocycle and to differentiate, in contrast to Notch, they do not continue proliferating. The Notch pathway has been shown to regulate the mitotic-to-endocycle transition by regulating independently three mitotic regulators: downregulating the G2 phosphatase String (Stg) and the G1 CKI p21CIP/Dacapo (Dap) and upregulating the APC activator Fizzyrelated (Fzr) (Shcherbata et al., 2004). To investigate what lies behind the difference between the Notch and *mys* phenotypes, we analysed the expression of these cell cycle regulators in *mys* PFCs using lacZ reporters (Lehman et al., 1999; Shcherbata et al., 2004). *dap-lacZ* and *stg-lacZ* are normally expressed in mitotic FCs from S4 to S6, when they are downregulated (Lehman et al., 1999; Shcherbata et al., 2004); Fig.7A and C). However, we found that even though *mys* PFCs in contact with the germline showed no alteration of *dap-lacZ* pattern of expression (arrowheads in Fig 7B), those located in ectopic layers showed prolonged expression of *dap-lacZ* (arrow in Fig. 7B, 86%, n=39) after S6. In contrast, all *mys* PFCs were able to downregulate *stg-lacZ* (Fig 7D, n=30).

Altogether, our results show that, when contact with the germline is lost, integrins are required in PFCs to downregulate the expression of the CDK inhibitor *dap* but not that of *string*. This may explain why *mys* PFCs do not differentiate properly, yet they do not continue dividing after S6. We next decided to test this by performing two sets of experiments. First, we tested whether a reduction in the amount of *dap* would rescue the differentiation and cell cycle defects found in *mys* PFCs located in ectopic layers. Indeed, we found that removing one copy of *dap* partially rescued the differentiation and Notch signalling defects of *mys* PFCs, as now only 33% (n=26) and 35% (n=24) of mosaic egg chambers containing *mys* PFCs and heterozygous for *dap* (*mys; dap/+*) showed FasIII and Cut expression after S6, respectively (arrows in Fig.7E and F, and 7I) in contrast to the 83% and 82% found in mosaic egg chambers containing *mys* PFCs (Fig.1D, 3B and 7I). In addition, the aberrant pattern of BrdU incorporation and nuclei size of *mys* PFCs were also partially rescued (Fig. 7G, I and J), as now only 35% (n=26) of *mys; dap/+* egg chambers carrying *mys* PFCs showed an aberrant BrdU incorporation pattern, in contrast to the 75% observed in egg chambers containing *mys* PFCs (Fig.7G and I). And second, we analysed whether expression of *string* was sufficient to maintain *mys* PFCs located in ectopic layers within the cell cycle after S6. Using the anti-PH3 antibody, we found that indeed this was the case (Fig.7H, arrow).
Discussion

The coordination of cell proliferation with the gradual differentiation of different cell types is essential for proper development and tissue homeostasis. However, little is known about the signals that couple cell cycle exit to differentiation switch during development. Here, we show that signalling mediated by integrins, the major cell-ECM receptors, contribute to the regulation of this switch in the posterior FCs of the Drosophila ovary. Furthermore, our experiments strongly suggest that one of the mechanisms by which integrins regulate epithelial cell differentiation is by modulating the activity of the Notch pathway through promoting its proper endosomal trafficking and/or processing.

Integrins are known to regulate the cell differentiation and proliferation in other systems. The effects of particular integrins in regulating differentiation vary depending on the epithelial cell type. Thus, while β1 integrin signalling is inhibitory for differentiation in the epidermis (reviewed in Watt, 2002), it is critical for proper differentiation in the mammary gland (Naylore et al., 2005). Interestingly, as we show here for Drosophila PFCs, loss of β1 integrin in the mammary gland leads to an upregulation in the expression of the CDK inhibitor p21cip1 (Li et al., 2005). Furthermore, as it is the case for PFCs, this upregulation is responsible for the proliferation defects observed in β1 mutant mammary glands, since knockdown of p21cip1 rescues the effect of β1 integrin loss. Integrins have been shown to control CDK inhibitors levels in other cell types. Thus, β1 chondrocytes show a defect in G1/S transition, which is accompanied by upregulation of CKIs p16ink4a and p21cip1 (Aszodi et al., 2003). Ablation of β1 in developing cerebellum results in a failure in the expansion of the cerebellar granule cell precursors due to p27kip1 upregulation (Blaess et al., 2004). However, the mechanisms underlying this regulation remain to be determined. In summary, our work extends previous studies demonstrating a key role for integrin signalling in controlling growth/differentiation by regulating CDK inhibitor levels, which suggests that this role is conserved throughout evolution. In addition, our studies unravel one of the mechanisms underlying this regulation, modulation of Notch signalling.

We have found that elimination of integrins results in defective Notch signalling in FCs. However, one puzzling aspect of the integrin phenotype is the fact that the
phenotypes of defective Notch are partial and position-dependent. Similar to Notch, integrin mutant PFCs fail to enter in endocycle and to differentiate, however, unlike Notch, they do not continue proliferating. In addition, and in contrast to Notch, the defects due to absence of integrin function are restricted to PFCs. Position-dependent phenotypes have also been observed in FCs mutants of the SWH pathway or with excessive myosin activity (Meignin et al., 2007; Polesello and Tapon, 2007; Sun et al., 2011; Yu et al., 2008). These position-dependent responses could be due to the involvement of signalling pathways specific to these FC subpopulations, such as the EGFR and the JAK-STAT pathway. Alternatively, they could reflect an intrinsic difference in Notch signalling levels between the PFCs and other FCs, as it has been proposed that the expression of Dl in the oocyte is lower than that in the nurse cells (Yu et al., 2008). Therefore, the intensity of Notch signalling might be weaker in PFCs with respect to other FCs and may depend more on positive modulators such as integrins to achieve adequate levels of activity. In this context, integrin signalling might play a general role in reinforcing Notch activity, being this regulation more critical in a sensitized background. This could explain the restriction of the Notch defects to integrin mutant PFCs located in the ectopic layers, as Notch signalling in these cells could be relatively low being at a greater distance from the source of ligand. In addition, a role for integrins as a positive modulator of Notch signalling could also explain why dacapo but not string expression is affected in integrin mutant PFCs. The reduced Notch activity present in the integrin mutant PFCs might be sufficient to induce normal levels of string expression but not of dacapo. In fact, Notch has been shown to control independently these two cell cycle regulators in FCs (Shcherbata et al., 2004).

There are different mechanisms by which integrins could modulate Notch signalling. We have shown that defective Notch signalling in mys PFCs can be rescued by expression of NICD, but not by full-length Notch or NEXT. This suggests that elimination of integrins disrupts Notch signalling most likely at the level of the last Notch cleavage, which generates the functional NICD. This step is subject to regulation at the level of endosomal trafficking (reviewed in (Fortini and Bilder, 2009). In fact, we have observed increased punctate distribution of Notch in the cytoplasm of integrin mutant PFCs. Therefore, we propose that a way by which integrins could modulate Notch activity in PFCs could be through the regulation of its intracellular trafficking and/or processing. At what level could integrins and Notch interact? A level of intersection
between Notch and integrins could be at the plasma membrane lipid rafts, a specialized membrane microdomain involved in endocytosis and signalling (Lajoie and Nabi, 2010). Interestingly, mutations in genes required for the biogenesis of sphingolipids, major constituents of lipid rafts, such as lace, ACC genes, cause abnormal accumulation of Notch in endosomes and significant alterations in the pathway (Sasamura et al., 2013). These results have led to suggest that genes involved in the formation and/or function of lipid rafts might influence tissue growth and differentiation by regulating the intracellular trafficking of signalling molecule such as Notch. Integrin signalling has been shown to regulate the location and behaviour of lipid rafts (del Pozo et al., 2004; Norambuena and Schwartz, 2011). Furthermore, the local regulation of these membrane domains by integrins have been proposed to locally regulate signalling proteins thought to associate with these domains, such as the PDGFR or Rac (Baron et al., 2003). Thus, lipid rafts may be a cellular platform to allow for Notch/integrin interactions. Finally, the increased levels of Hrs in cytoplasmic vesicles observed in mys PFCs suggest that the endocytosis defects might not be specific to the Notch receptor, but rather may indicate a more generalized defect in endocytosis.

A temporal coupling of cell-cycle arrest and terminal differentiation is common during development and homeostasis. Furthermore, a loss of coordination between these two processes can lead to aberrant tissue development and tumorigenesis. Here, we have shown that in Drosophila, as it is the case in mammals, integrins can regulate the cell cycle to differentiation switch in epithelial cells by regulating CDK inhibitor levels, suggesting that this role of integrins is conserved. Furthermore, we show that one of the mechanisms by which integrins regulate cell cycle to differentiation switch in FCs is by modulating the activity of the Notch pathway and that this regulation might be achieved by promoting proper endosomal trafficking of Notch. As integrins transmit signals from the ECM inside the cell, our study demonstrates that the ECM environment can “fine tune” the cellular response to pathways regulating cell proliferation and differentiation, such as the Notch pathway, and ultimately determine the cellular state of a specific cell type. In the future, it will be interesting to test whether integrins could interact with other signals known to regulate FC differentiation, such as those downstream of the SWH pathway or myosin activity.
Materials and Methods

To generate follicle cell mutant clones we used the FRT/FLP technique (Chou and Perrimon, 1992). Mutant clones were marked by the absence of GFP. The following mutant alleles and chromosomes were used: \textit{mys}^{11} (also known as \textit{mys}^{XG43} (Bunch et al., 1992)), \textit{e22c-Gal4} UAS-flipase (Duffy et al., 1998), \textit{H}^{2} (Bang and Posakony, 1992), \textit{dap}^{04454}-LacZ, \textit{Dl}^{B2} and \textit{Notch}^{55e11} (Bloomington Stock Center), \textit{Gbe-Su(H)-lacZ} (Furriols and Bray, 2001), \textit{pnt-LacZ} (Morimoto et al., 1996), UAS-Tor^{D}/\beta cyt (Martin-Bermudo and Brown, 1999), \textit{baz}^{815} (Djiane et al., 2005), \textit{rhea}^{79} (Brown et al., 2002) and \textit{ilk}^{l} (Zervas et al., 2001). The \textit{e22c-Gal4} driver is expressed in the follicle stem cells in the germarium and was therefore used in combination with UAS-\textit{flp} to generate large \textit{mys} and \textit{Notch} follicle cell clones. The GR1Gal4 is expressed in all FCs (Gupta and Schupbach, 2003) and it was used to express N full-length, NEXT, NICD (Rauskolb et al., 1999) or \textit{string} (Bloomington Stock Center) in \textit{mys} PFCs in combination with the HS flipase system. Thus, \textit{mys}^{11}/\textit{FRT101}/\textit{FMZ}; UAS-N full length (or UAS-NICD or UAS-NEXT) were crossed to \textit{y w hs-Flipase 122 ubiquitin-GFPFRT101;; GR1Gal4} males. Newly hatched females from this cross were heat-shocked at 37°C for 20 minutes and kept at 25°C for 2 days prior to ovary dissection. To generate \textit{mys} mutant cells expressing Tor^{D}/\beta cyt (Martin-Bermudo and Brown, 1999) in the follicular epithelium, we grew females of the genotype \textit{mys}^{11}/\textit{FRT101}/\textit{ubiquitin-GFPFRT101};\textit{e22c-Gal4}/\textit{UAS-Tor}^{D}/\beta cyt; \textit{tubulin}-Gal80^{0}/+ at 18°C until eclosion. Adult females were kept at 31°C for 3-5 days prior to ovary dissection. To generate clones of cells expressing, CycE, Stg, DP and E2F2, in the follicular epithelium, we used the “flip-out” technique (Struhl et al., 1993). \textit{y w hs-Flipase 122, Act<CD2> Gal4};\textit{UAS-GFP} females were crossed to \textit{UAS-CycE, UAS-Stg; UAS-DP, UAS-E2F2} (Buttitta et al., 2007). Newly hatched females from this cross were heat-shocked at 37°C for 20 minutes and kept at 25°C for 2 days prior to ovary dissection.

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\textit{Drosophila} females were yeasted for 1-2 days before dissection. Stainings were performed at room temperature according to standard procedures. The DNA dye Hoescht (Molecular Probes™, 1/1000) was added for 10 minutes in PBT (PBS + 0.1% Tween) after the secondary antibody. The following primary antibodies were used: mouse anti-FasIII 1/20, mouse anti-Eya 1/20, mouse anti-cut 1/25, mouse anti-NICD 1/100, mouse
anti-NECD 1/100, mouse anti-Hnt 1/15, mouse anti-cycB 1/20 and mouse anti-Br-C 1/100 (Developmental Studies Hybridoma Bank; DSHB); rabbit anti-Staufen (1:1000) (St Johnston et al., 1991); mouse anti-BrdU 1/20 (Roche); mouse anti-βgal (Promega) 1/10.000; rabbit anti-βgal (CAPPEL™) 1/10.000; rabbit anti-GFP (Molecular Probes™) 1/10.000; mouse anti-GFP (Molecular Probes™) 1/100; mouse anti-myc 1/100 (Oncogen Science) and guinea-pig anti-Hrs (Lloyd et al., 2002). The secondary antibodies used were Alexa fluor 488, (Molecular Probes™) and Cy3 and Cy5 (Jackson ImmunoResearch Laboratories, Inc.) at 1/200. Images were captured with Leica TCS-SPE confocal microscope and processed with ImageJ.

For BrdU incorporation assay, the experiment was performed using the 5-Bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit I from Roche (Cat Num 11296736001). Ovaries dissected in PBS and incubated in BrdU incorporation medium (PBS+10⁻³M BrdU, Roche) for 30 minutes and washed for 5 minutes with PBS. After fixing the ovaries for 20 minutes in 4% PFA, they were blocked with PBT 10 (PBS + 0.1% Tween, 10% BSA Bovine Serum Albumine) for 1 hour. Ovaries were then incubated overnight with anti-GFP antibody in PBT 1 (PBS + 0.1% Tween, 1% BSA Bovine Serum Albumine) to label the clones and washed with PBT 1 for 1 hour. Ovaries were re-fixed in 4% PFA for 20 minutes and treated with HCl 4N in PBS for 7 minutes and washed several times for 30 minutes in PBS, until pH reach 7. Then, ovaries were blocked again with PBT 10 and incubated overnight with Mouse anti-BrdU (Roche) diluted in incubation buffer. After washes with PBT 1 for 1-2 hours, egg chambers were incubated with the secondary antibody in PBT 0.1 (0.1% Tween + 0.1% BSA) for 2-4 hours in the dark. Egg chambers were then washed with PBT for 1-2 hours, and finally mounted in Vectashield (Vector).

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Competing interests

The authors declare no competing financial interests.
Author contributions

M.D. M-B conceived the project, performed experiments, analyzed the data and wrote the paper. M.J. G-L, L. C-R and B. I-J performed experiments, analyzed the data and contributed to the writing of the paper. I. P. contributed to the writing of the paper.

References


Figure Legends

Fig.1 Integrins regulate PFC differentiation.

(A) A S9 mosaic egg chamber carrying mys mutant clones (GFP) stained for anti-GFP and the DNA marker TO-PRO-3 (blue). While mys PFCs in contact with the germline (GFP, arrowhead) have normal nuclei size, the nuclei size of those located in ectopic layers (GFP, arrow) is smaller than that of surrounding wild type cells (GFP⁺). (B) Quantification of the nuclei size. (C-F) S9-10 wild type and mosaic egg chambers carrying mys mutant clones expressing the posterior cell fate marker pnt-lacZ, and stained for anti-GFP (green), anti-β-galactosidase (C-F, blue), anti-FasIII (C, E, red) and anti-Eya (D, F, red). In S9-10 wild type egg chambers, FasIII (C) and Eya (D) are restricted to
polar cells (PCs) and to border cells (BCs) and stretched cells (SCs), respectively, while pnt-lacZ is specifically expressed in PFCs from S6 onward (C and D). However, in mys PFCs located in ectopic layers (GFP⁺, arrow), FasIII (E) and Eya (F) are maintained while pnt-lacZ is inhibited (E, F). mys PFCs in contact with the germline (GFP⁺, arrowhead in E and F) behave as wild type FCs. Upper left panels in C and D show expression of FasIII and Eya in S4 egg chambers, respectively. Anterior is to the left in all figures. (A'-F') magnifications of the white boxes in (A-F), respectively.

**Fig.2 Defective endocycle and CycB expression in integrin mutant PFCs located in ectopic layers.**

(A-D) S9-10 wild type and mosaic egg chambers labelled with anti-GFP (green), anti-CycB (A and B, red), anti-Br-C (C and D, red) and TO-PRO-3 (blue). (A) In wild type egg chambers, CycB is downregulated at S6 (upper left panel shows CycB expression in a S4 egg chamber). (B) However, this expression is maintained at S10 in mys PFCs located in ectopic layers (GFP⁺, arrow). (C) In wild type FCs, Br-C is upregulated after S6. (D) This up-regulation fails to occur in mys PFCs located in ectopic layers (GFP⁺, arrow). Note that in both cases mys PFCs in contact with the germline (GFP⁺, arrowhead in B and D) perform like wild type FCs. (E, F) S11 egg chambers stained with anti-GFP (green) and BrdU (red). While in wild type S11 egg chambers, BrdU incorporates in the four spots of amplification (E), genomic BrdU incorporation was observed in mys PFCs located in ectopic layers (F). (A'-F') magnifications of the white boxes in (A-F), respectively.

**Fig.3 The Notch pathway is disrupted in integrin PFCs.**

Egg chambers stained for anti-GFP (green, A-F), anti-Cut (red, A, B), anti-Hnt (red, C, D), anti-β-galactosidase (red, E, F) to detect the reporter of the Notch activity Gbe-Su(H)-lacZ and TO-PRO-3 (blue, A-F). In wild type egg chambers, Notch signalling induces downregulation of Cut (A) and upregulation of Hnt (C) from S6 onwards (upper left panel in A shows expression of Cut in a S4 egg chamber). In contrast, mys PFCs located in ectopic layers (GFP⁺, arrow) show prolonged Cut expression after S6 (B) and fail to upregulate Hnt at S7 (D). (E) In wild type egg chambers, expression of the reporter of the
Notch activity, *Gbe-Su(H)-lacZ* is upregulated after S6 in PFCs. (F) This upregulation fails to take place in *mys* PFCs in ectopic layers (GFP, arrow). *mys* PFCs in contact with the germline (GFP, arrowhead in D and F) behave as wild type FCs. (A’-F’) magnifications of the white boxes in (A-F), respectively.

**Fig.4 Integrins are required to strengthen Notch signalling**

S9-10 Egg chambers labelled with anti-GFP (green), anti-FasIII (A-E, red), anti-Eya (F, red) and TO-PRO-3 (blue). (A) In a H/+ control egg chamber, FasIII (red) is restricted to PCs, as it is the case in wild type. (B) S10 mosaic egg chambers showing the abnormal expression of FasIII (red) in *mys* PFCs located in ectopic layers. (C) Reducing the amount of *H* restores FasIII expression and nuclear size in *mys* PFCs located in ectopic layers (GFP, arrow). This is quantified in G and H, respectively. (D) *mys* PFCs in contact with the oocyte (arrowhead) show high levels of FasIII when Delta (Dl) function is reduced. (E, F) FasIII (E) and Eya (F) expression patterns are not affected in *baz* PFCs located in ectopic layers. (A’-F’) magnifications of the white boxes in (A-F), respectively.

**Fig.5 Integrin-mediated signalling is required for PFC maturation.**

Egg chambers stained for anti-GFP (green, A-F), anti-FasIII (red, A, C and F), anti-Cut (red, B, D), anti-myc (blue, C, D) to detect the TorD/βcyt chimeric integrin and TO-PRO-3 (blue). (A, B) S9-S10 mosaic egg chambers showing abnormal expression of FasIII (A, red) and Cut (B, red) in *mys* PFCs located in ectopic layers (GFP, arrow). (C-E) Ectopic expression of TorD/βcyt (blue) in these *mys* PFCs restores the normal expression of FasIII (C, red), Cut (D, red) and nuclei size (E, G). (F) FasIII expression is not affected in *rhea* PFCs located in ectopic layers (GFP, arrow). (G, H) Quantification of the nuclei size of the indicated genotypes. The nuclei size of *rhea* PFCs is similar to that of wild type FCs (H). In all cases, *mys* PFCs in contact with the germline (GFP, arrowhead in A-E) behave as wild type FCs.

**Fig.6 Integrins modulate the Notch pathway by regulating its intracellular trafficking and/or processing**
Egg chambers stained for anti-GFP (green, A-F), anti-FasIII (red, A, B), anti-NICD (red, C-F), anti-Hrs (green, E) and TO-PRO-3 (blue). Expression of NICD (A) but not full length Notch (B) restores the normal expression of FasIII and nuclear size of mys PFCs in ectopic layers (GFP, arrow). (C) NICD expression in wild type egg chambers (arrow). (D) mys PFCs located in ectopic layers (GFP’, arrow) contain discrete cytoplasmic and membrane-associated accumulations of NICD. (E) Some of the ectopic NICD is found to colocalize with Hrs (arrow). Note that mys PFCs in contact with the germline show normal NICD localization (GFP, arrowhead in D). (F) However, reduction of Dl function in these mutant PFCs (arrowhead) leads to an accumulation of NICD similar to that found in mys PFCs in ectopic layers (arrow in D). (A’-F’) magnifications of the white boxes in (A-F), respectively.

**Fig. 7 Integrins regulate PFC maturation by regulating *dap* expression.**

Egg chambers stained for anti-GFP (green, A-H), anti-β-galactosidase (A-D, red) to monitor the expression of the *dap-lacZ* (A, B) and *stg-lacZ* (C, D) reporters, anti-FasIII (red, E), anti-Cut (red, F), anti-BrdU (red, G), anti-PH3 (red, H) and TO-PRO-3 (blue, A-H). (A, C) In wild type egg chambers, *dap* (A) and *stg* (C) are downregulated at S6. Upper left panels show expression of these markers in S4 egg chambers from the same ovarioles. (B, D) In mys PFCs located in ectopic layers (GFP’, arrow), *dap* expression is maintained after S6 (B) while *stg* is properly downregulated (D). mys PFCs in contact with the germline show normal *dap* and *stg* expression (GFP, arrowheads in B and D). (E-G) Reducing *dap* levels in mosaic egg chambers carrying mys PFCs restores normal expression of FasIII (E, I), Cut (F, I), BrdU incorporation pattern (G, I) and nuclei size (J). (H) Ectopic expression of *stg* in mys PFCs located in ectopic layers (GFP’, arrow) is sufficient to maintain these mutant cells within the cell cycle. (A’-H’) magnifications of the white boxes in (A-H), respectively.
Figure 4
Figure 5
Figure 7