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The microRNA pathway regulates the temporal pattern of Notch signaling in *Drosophila* follicle cells

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SUMMARY

Multicellular development requires the correct spatial and temporal regulation of cell division and differentiation. These processes are frequently coordinated by the activities of various signaling pathways such as Notch signaling. From a screen for modifiers of Notch signaling in *Drosophila* we have identified the RNA helicase Belle, a recently described component of the RNA interference pathway, as an important regulator of the timing of Notch activity in follicle cells. We found that loss of Belle delays activation of Notch signaling, which results in delayed follicle cell differentiation and defects in the cell cycle. Because mutations in well-characterized microRNA components phenocopied the Notch defects observed in *belle* mutants, Belle might be functioning in the microRNA pathway in follicle cells. The effect of loss of microRNAs on Notch signaling occurs upstream of Notch cleavage, as expression of the constitutively active intracellular domain of Notch in microRNA-defective cells restored proper activation of Notch. Furthermore, we present evidence that the Notch ligand Delta is an important target of microRNA regulation in follicle cells and regulates the timing of Notch activation through cis inhibition of Notch. Here we have uncovered a complex regulatory process in which the microRNA pathway promotes Notch activation by repressing Delta-mediated inhibition of Notch in follicle cells.

KEY WORDS: miRNA, Oogenesis, Mitotic-to-endocycle switch, Developmental timing, *Drosophila*

INTRODUCTION

The development of multicellular organisms requires the tightly regulated control of many important aspects of cellular behavior, including differentiation and proliferation. Developing organisms govern these processes through the complex spatial and temporal activity patterns of signaling pathways. Much attention has been given to the spatial regulation of signaling pathways (Baker, 2007; Portin, 2002); however, much remains unknown with regard to the mechanisms that control the timing of cell signaling activation and deactivation. It is therefore of great importance to identify the mechanisms by which cells control the timing of cell signaling.

Recently, microRNAs (miRNAs) have emerged as a common mechanism by which animals regulate the activity of many signaling pathways (Inui et al., 2010) and the timing of key developmental events (Rougvie, 2005; Tennessen and Thummel, 2008; Wienholds and Plasterk, 2005). miRNAs have also been shown to regulate the timing of development in plants (Poethig, 2009). The miRNA pathway produces small, 21-24 nucleotide RNA molecules from longer double-stranded RNA precursors that are expressed from endogenous genomic loci. In flies, generation of the mature miRNA from the precursor is achieved by the endonuclease activities of Pasha and Dicer-1 (Dcr-1) (Okamura et al., 2008). Based on sequence complementarity of the miRNA to regions of target mRNA transcripts, the miRNA pathway directs either translational repression or cleavage and degradation of the mRNA (Wu and Belasco, 2008). Functionally, the miRNA pathway has been shown to affect many signaling pathways by regulating the levels of core component proteins or proteins that indirectly promote or inhibit the pathway (Inui et al., 2010). In addition, pioneering work on miRNAs in *C. elegans* revealed the regulation of developmental timing by the let-7 and lin-4 heterochronic miRNAs (Rougvie, 2005; Wienholds and Plasterk, 2005). In *Drosophila*, these same miRNAs, let-7 and miR-125 (the fly homolog of lin-4), have been shown to regulate the timing of many important events during development, including the control of cell cycle exit and differentiation during metamorphosis (Caygill and Johnston, 2008; Sokol et al., 2008).

In animal development, the highly conserved Notch pathway functions repeatedly to regulate many fundamental cellular processes (Portin, 2002). In flies, Notch signaling is activated upon binding of the transmembrane Notch receptor by either of its ligands, Delta or Serrate (Ser). Ligand binding results in proteolytic cleavage of Notch, releasing the intracellular domain of Notch, which translocates to the nucleus to activate target gene transcription (Artavanis-Tsakonas et al., 1995). Because of the importance of Notch signaling during development, it is not surprising that Notch signaling is tightly regulated by a variety of cellular mechanisms (Tien et al., 2009). How these modes of regulation determine the temporal patterns of Notch activity remains largely unexplored.

Drosophila egg chambers constitute a powerful system with which to investigate the temporal regulation of Notch activity because they progress through a series of distinct stages during which Notch is activated and subsequently inactivated (Deng et al., 2001; Klusza and Deng, 2011; Lopez-Schier and St Johnston, 2001; Ruohola et al., 1991; Sun et al., 2008). In the egg chamber,

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the germline cells (nurse cells and oocyte) are surrounded by a monolayer of somatic follicle cells. At stage 7, Notch is activated in follicle cells by germline-expressed Delta, which induces differentiation and a switch from the mitotic cycle to the endocycle (Fig. 1) (Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Ruohola et al., 1991; Sun and Deng, 2007). Later, at stage 10b, Notch is inactivated in the follicle cells overlying the oocyte (Fig. 1), which, in conjunction with ecdysone signaling, results in a second cell cycle transition from endocycle to gene amplification in specific genomic loci (Calvi et al., 1998; Sun et al., 2008).

In *Drosophila*, there have been only a few reports directly linking miRNA regulation and Notch signaling. Two groups have validated several downstream target genes of Notch signaling [the E(spl) and Bearded (Brd) family of transcription factors] as miRNA targets in vivo (Lai et al., 2005; Stark et al., 2003). It has also been reported that miR-1 regulates Notch activity during cardiac development in *Drosophila* by repressing Delta expression (Kwon et al., 2005). More recently, miR-8 was identified as a negative regulator of Notch signaling in eye and wing imaginal discs by targeting the Notch ligand Ser for repression (Vallejo et al., 2011). This relative paucity of examples describing miRNA-based regulation of such a vital signaling pathway as Notch suggests that there is much to discover regarding the role of miRNAs in the control of Notch signaling.

In addition to miRNA regulation of Notch signaling, Notch regulation occurs through ligand-dependent inhibition. Various lines of evidence demonstrate that the ligands Ser and Delta, which act as activating ligands in trans, can also function as inhibitors of Notch in cis (Cordle et al., 2008; de Celis and Bray, 1997; Fiuza et al., 2010; Klein et al., 1997; Li and Baker, 2004; Micchelli et al., 1997; Miller et al., 2009; Sakamoto et al., 2002). Delta inhibition of Notch regulation has been described in tissues such as *Drosophila* imaginal discs, in which it helps to define the spatial pattern of Notch activity (de Celis and Bray, 1997; Li and Baker, 2004; Micchelli et al., 1997). Regulation of Notch signaling by Delta- mediated inhibition has not been reported in follicle cells.

From a screen for modifiers of Notch signaling we found that Belle (Bel), a recently identified component of the RNA interference (RNAi) pathway (Ulvila et al., 2006; Zhou et al., 2008), promotes proper timing of Notch activity in follicle cells, and that the miRNA pathway contributes to Notch signaling in a similar fashion. We also provide evidence that the relevant miRNA target is the Notch ligand Delta, operating through cis-inhibition in follicle cells. Together, these findings link these two mechanisms of cell signaling regulation to reveal a complex regulatory process in which the miRNA pathway promotes Notch activation by repressing the expression of Delta in follicle cells, where it acts as a repressor of Notch signaling.

MATERIALS AND METHODS

Identification of new *bel* alleles

To map the location of the P-element insertions that serve as the mutagen in the Szeged stocks, we performed inverse PCR using the primers and protocols suggested by the Berkeley Drosophila Genome Project (BDGP) for the P(lacW) element used in the Szeged stocks. Both the 74407 and 47110 stocks mapped to the first intron of *CG9748*, *bel*. For the experiments described in this paper we used the bel^{74407} allele, unless otherwise indicated.

Fly stocks and genetics

Loss-of-function follicle cell clones were generated by heat shock-inducible Flippase (hsFLP) as previously described (Yu et al., 2008). The fly stocks used in loss-of-function analyses were:

hsFLP,Stau:GFP;FRT82B ubiGFP/TM3,Sb;
hsFLP,Stau:GFP;FRT82B arm-lacZ/TM6b;
hsFLP;Sp/Cyo;FRT82B ubi-RFP/TM6b;
hsFLP,Stau:GFP;FRT42D ubi-GFP/Cyo;
hsFLP;hisGFP FRT40A/Cyo;
FRT82B bel^{74407}*/TM3,Sb*;
FRT82B bel^{47110}*/TM3,Sb*;
FRT82B bel^{L4740}*/TM3,Sb*;
FRT82B bel^{eke}*/TM3,Sb*;
FRT82B $Dcr\text{-}1^{Q1147X}$*/TM3,Sb*;
FRT82B $pasha^{KO}$*/TM3,Sb*;
FRT82B $Dcr\text{-}1^{Q1147X}$,$pasha^{KO}$*/TM3,Sb*;
FRT42D $Dcr\text{-}2^{L811fsX}$*/Cyo*;
piwi[1] FRT40A/Cyo;
FRT82B $Delta^{revF10}$*/TM3,Sb*;
FRT82B $Delta^{M1}$*/TM3,Sb*;
FRT82B $Delta^{revF10}$,$Dcr\text{-}1^{Q1147X}$*/TM3,Sb*;
FRT82B bel^{74407},$Delta^{revF10}$*/TM3,Sb*;
FRT82B Ser^{rx106};
E(spl)mβ-CD2/Cyo; and
Delta 3′UTR-GFP sensor/Cyo.

Knockdown of Bel by RNAi used a UAS-driven *bel*-specific hairpin. To generate mosaic egg chambers and wing discs expressing this construct, *UAS-bel RNAi* (VDRC #6299) flies were crossed to the *flip-out Gal4* stock *hsFLP;actin5C<CD2<Gal4,UAS-GFP/TM3,Sb*. For wing disc clones, first instar larvae were heat shocked at 37°C for 45 minutes and dissected at third instar. Follicle cell clones were generated by a 30-minute adult heat shock followed by dissection 4 days later.

Delta overexpression clones were also generated by crossing *UAS-Delta* (BDSC #5614) to *hsFLP;actin5C<CD2<Gal4,UAS-GFP/TM3,Sb*.

The overexpression of NICD in *Dcr-1* mutant cells was performed using the MARCM technique (Lee and Luo, 1999). Flies of the following genotype were heat shocked under the same conditions as the loss-of-function clones: *hsFLP;UAS-NICD/act-Gal4,UAS-GFP;FRT82B* $Dcr\text{-}1^{L811fsX}$*/FRT82B tub-Gal80*.

We did not include the polar follicle cells in our scoring of Cut and Hnt expression because of their unique expression patterns for these markers.

Immunocytochemistry

Antibody staining was performed as previously described (Deng et al., 2001). Antibodies from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA) were Cut (1:50), CycA (1:10), CycB (1:10), Delta (1:20) and Hnt (1:15). Other antibodies used were PH3 (rabbit, 1:1000; Upstate), CD2 (mouse, 1:50; Serotec), Bel (rabbit, 1:500; gift from Paul Lasko, McGill University, Montreal, Quebec, Canada), β-Gal (rabbit, 1:1000; Sigma) and Ser [rabbit, 1:20; gift from Ed Giniger, NIH (NINDS), Bethesda, MD, USA]. Actin was stained by phalloidin (1:500, Alexa). Nuclei were stained with DAPI (1:1000, Invitrogen). Images were captured on a Zeiss LSM-510 confocal microscope in the Biological Science Imaging Resource Facility at FSU or on a Zeiss LSM-5 Pascal confocal microscope at UNC-Chapel Hill. Figures were processed and arranged in Adobe Photoshop.

Generation of a *Delta* 3′UTR sensor

The entire 3′UTR of *Delta*, including 158 bp downstream of the polyA site, was directionally cloned downstream of GFP driven by the *tubulin* promoter, similar to previously described protocols (Silver et al., 2007) (tub-GFP vector generously provided by Eric Lai). We used the *Not*I and *Xho*I sites (underlined) of the tub-GFP vector to design the following primers (5′ to 3′) for the PCR reaction: GTGCGGCCGCCTC-CAAAAATCCGGAAGGGCTCC and GTCTCGAGCTGCAATAC-CCATACCACGTTCT. The purified PCR product was cut and ligated into the tub-GFP vector and the resulting plasmid was used to create transgenic flies (GenetiVision, Houston, TX, USA). The insertion sites were mapped and the transgene was crossed into the appropriate mutant backgrounds using standard *Drosophila* genetics.

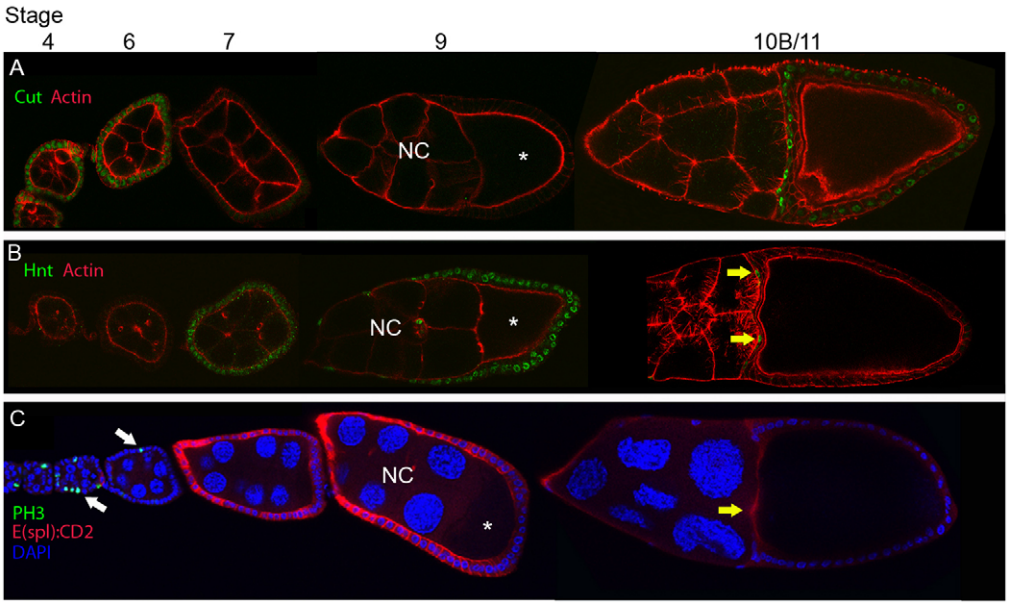


Fig. 1. Notch activity pattern and markers in *Drosophila* oogenesis. (**A-C**) An egg chamber is composed of a layer of follicle cells that surrounds the germline cells: the nurse cells (NC) and oocyte (asterisk). The oocyte resides at the posterior end of the egg chamber (posterior is to the right in all images). (A) In early stage egg chambers (before stage 7), follicle cells express the immature fate marker Cut (A). At stage 7, Notch is activated in follicle cells, resulting in downregulation of Cut (A) and upregulation of Hnt (B) as well as in upregulation of the Notch activity reporter E(spl):CD2 (C). Notch activation at stage 7 induces the switch from mitotic divisions to endocycle. Therefore, dividing follicle cells express mitotic markers such as PH3 before stage 7 (white arrows), but once Notch is activated at stage 7 no more divisions occur and thus no PH3 staining is observed. Notch remains active until stage 10b, when Notch signaling declines dramatically in follicle cells overlying the oocyte, resulting in the downregulation of Hnt and E(spl):CD2 at stage 10b (B,C), as well as in the reappearance of Cut expression (A). As revealed by prolonged expression of Hnt and E(spl):CD2, Notch signaling appears to remain active in anterior follicle cells even after stage 10b (yellow arrows). Nuclei are labeled with DAPI (C).

RESULTS

Identification of Belle as a Notch regulator

To identify new genes involved in the regulation of Notch signaling, we conducted a mosaic screen using the Flippase-Flippase recognition target (FLP-FRT) (Golic, 1991) technique in follicle cells, and tested for defects in the expression of Notch-dependent markers. We tested several hundred lines from the Szeged Stock Center library of lethal P-element insertions, including those in the right arm of the third chromosome (FRT82B). These stocks were crossed to *hsFLP;FRT82B ubi-GFP* to generate follicle cell clones (homozygous mutant cells) that were then screened for defects in the expression pattern of the Notch target Cut, a homeodomain transcription factor (Sun and Deng, 2005). Cut is normally expressed in early oogenesis (stages 1-6), then downregulated at stage 7 by Notch signaling in the follicle cells (Fig. 1A) (Sun and Deng, 2005). We could therefore use the downregulation of Cut as a marker of Notch activity; failure to downregulate Cut at stage 7 suggests a defect in Notch signaling. From this screen, two stocks (74407 and 47110) showed prolonged expression of Cut in follicle cell clones after stage 7 (Fig. 2A,B). The P-element insertion sites for these two lines were subsequently mapped by inverse PCR to the first intron of *belle* (*bel*), a DEAD-box RNA helicase. Bel was recently found to be necessary for small interfering RNA (siRNA) silencing in *Drosophila* cell culture and to be in a complex with several components of both the siRNA and miRNA pathways, including the small non-coding RNAs that mediate both pathways (Zhou et al., 2008). Bel has also been linked to other cellular processes and this might be related to its role in RNAi (Ambrus and Frolov, 2010; Ambrus et al., 2007; Worringer et al., 2009).

To confirm that the phenotypes generated by bel^{74407} and bel^{47110} were caused by mutations in the *bel* gene, we first performed complementation analyses testing bel^{74407} and bel^{47110} against other known lethal alleles (bel^{eke}, bel^{b10}, bel^{L4740} and bel^{6}) (Ambrus et al., 2007; Bender et al., 1987; Johnstone et al., 2005; Jones and Rawls, 1988). Both bel^{74407} and bel^{47110} failed to complement any of the other *bel* alleles, indicating that these two lines represent strong mutations in *bel*. We then examined the expression of Bel protein from bel^{74407} and bel^{47110} using a Bel polyclonal antibody (Johnstone et al., 2005). We observed a complete loss of Bel staining in imaginal disc clones of bel^{74407} and bel^{47110}, suggesting that both alleles are protein null (see Fig. S1A in the supplementary material; data not shown). Furthermore, germline clones of bel^{74407} and bel^{47110} arrested in mid-oogenesis (data not shown), similar to previously described *bel* mutants (Johnstone et al., 2005).

To confirm that the Notch phenotypes associated with these new *bel* alleles were in fact due to the mutations in *bel*, we first generated follicle cell clones of two known *bel* alleles (bel^{L4740} and bel^{eke}) and found that they displayed similar Notch-like defects as those we observed for bel^{74407} and bel^{47110} (see Fig. S2 in the supplementary material). We also tested the role of Bel in Notch signaling by expressing a *UAS-bel RNAi* construct under the control of the *flip-out Gal4* driver, which significantly reduces Bel protein levels in imaginal disc cells (see Fig. S3A in the supplementary material). When this *UAS-bel RNAi* construct was expressed in follicle cells, we observed similar Notch-like phenotypes as in the mutant *bel* clones (see Fig. S3B,C in the supplementary material). Together, these experiments indicate that 74407 and 47110 are alleles of *bel* and that disruption of *bel* is responsible for the Notch-related phenotypes we observed.

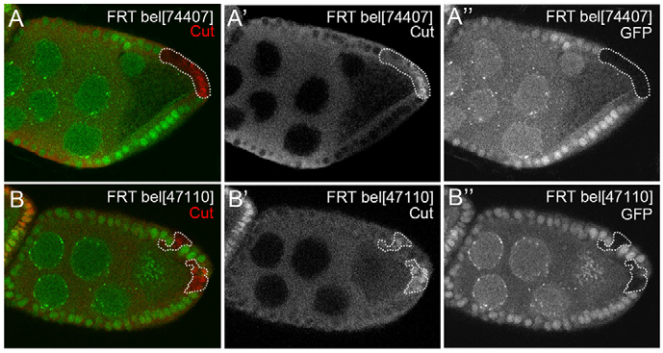
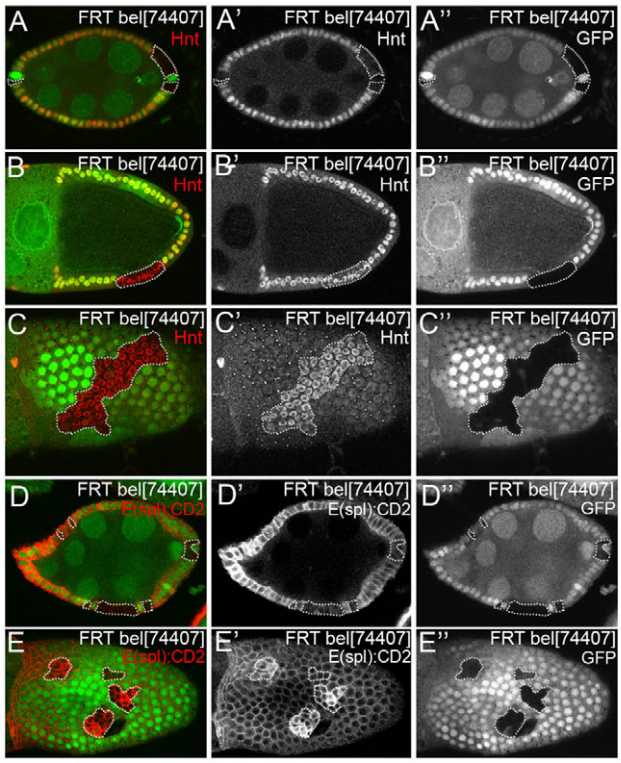


Fig. 2. Bel is required for downregulation of Cut. (**A-B″**) After stage 6, wild-type follicle cells (GFP positive) downregulate Cut, as shown in these stage-7/8 *Drosophila* egg chambers. However, follicle cell clones (GFP negative) of two new alleles of *bel*, 74407 (A-A″) and 47110 (B-B″), exhibit prolonged expression of Cut. Clones are outlined with dotted white lines. A and B are a merge of the Cut (A′,B′) and GFP (A″,B″) stainings.

Bel regulates the timing of Notch activation in follicle cells

As mentioned above, follicle cells mutant for *bel* displayed defects in the downregulation of Cut after stage 6, suggesting a failure to activate Notch signaling (Sun and Deng, 2005). We therefore tested a positively regulated target of Notch signaling in follicle cells: the zinc-finger transcription factor Hindsight (Hnt; Pebbled – FlyBase). Hnt is upregulated at stage 7 by Notch and is downregulated after stage 10a in response to the decline in Notch activity that occurs at this stage (Sun and Deng, 2007) (Fig. 1B). In stage-7 *bel* clones, however, only a small fraction of mutant cells expressed Hnt (37%, *n*=120 clone cells; compared with 100% in wild-type cells, *n*=307) (Fig. 3A,F). This block on Hnt expression in *bel* clones was temporary, as Hnt was expressed in the majority of *bel* clones by stage 8 (65%, *n*=87) and in almost all clones by stages 9/10a (92%, *n*=130) (Fig. 3B,F). Intriguingly, we also observed that Hnt is frequently expressed in *bel* clones beyond stage 10a, when it should normally be downregulated in response to waning Notch activity levels (Fig. 3C). We also used a Notch activity reporter transgene to directly assay the state of Notch activation in *bel* clones. The *E(spl):CD2* construct contains the 1.5 kb regulatory sequence of the *E(spl)* gene, a well-characterized direct target of Notch signaling, fused to the rat *CD2* gene (de Celis et al., 1998). Based on comparisons with the expression patterns of other known Notch targets, E(spl):CD2 is an excellent reporter of Notch activity patterns in follicle cells. In *bel* mosaic egg chambers expressing *E(spl):CD2*, we observed significantly reduced levels of CD2 staining in stage-7 clones (Fig. 3D), indicating that Notch had not successfully activated this promoter. We also noticed expression of this reporter after stage 10a in *bel* clones (Fig. 3E), consistent with the concept of delayed Notch signaling in follicle cells mutant for *bel*.

A key function of Notch signaling in follicle cells is regulation of the switch from the mitotic cycle to endocycle at stage 7. To investigate the possibility that *bel* clones affect Notch-induced transitions in the cell cycle, we examined three important hallmarks of the mitotic cycle: Cyclin A (CycA), Cyclin B (CycB) and phospho-Histone H3 (PH3). Because wild-type follicle cells are in asynchronous mitotic cycles prior to stage 7, these proteins display oscillating patterns of expression. After stage 6, Notch signaling induces follicle cells to enter the endocycle and expression of these proteins is lost for the remainder of oogenesis (Sun and Deng, 2005). In *bel* clones, however, we found that all three markers were sometimes present in stages 7-8, indicating that these cells remained mitotically active (see Fig. S4A-C in the supplementary material). In addition, *bel* clones frequently possessed smaller



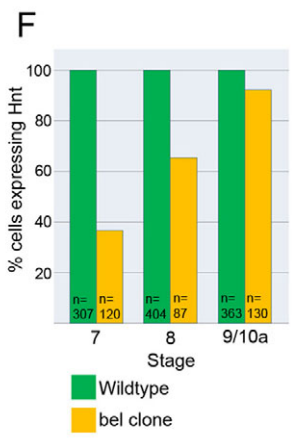


Fig. 3. Bel is required for proper timing of Notch activity. (**A-E″**) In wild-type *Drosophila* follicle cells (GFP positive), Hnt is upregulated in response to Notch signaling at stage 7 (A-A″). *bel* clones (GFP negative) infrequently express Hnt at stage 7, although they do eventually express Hnt by stage 9/10 (B-B″; see F). Interestingly, *bel* clones show prolonged expression of Hnt after stage 10a (C-C″), when Notch signaling and Hnt expression are normally downregulated in wild-type cells. Similar results were observed for the Notch activity reporter E(spl)-CD2 in *bel* clones (D-E″). (**F**) The delay of Notch activation in *bel* clones. Shown is the percentage of *bel* clone cells that express Hnt compared with their wild-type counterparts in mid-oogenesis. Wild-type expression is 100% at all stages. *bel* clones: stage 7, 37%; stage 8, 65%; stage 9/10a, 92%. The total number of cells scored for each genotype at each stage is indicated (*n*). $P<0.01$ (χ^2 analysis) for *bel* clones expressing Hnt at stage 7 compared with stage 8, and for stage 8 compared with stage 9/10a. Clones are outlined with dotted white lines. Note that the Hnt-negative cells with strong GFP expression at the anterior and posterior tips of the egg chamber in A are polar cells and were not included in our analyses.

nuclei (Fig. 3C), indicative of a failure to enter the endocycle, which causes the nucleus to grow in size. These findings agree with the differentiation marker data indicating that Notch signaling is not properly activated in *bel* clones at stage 7.

An additional function of Notch-induced differentiation of follicle cells is the establishment of anterior-posterior polarity in the oocyte (Poulton and Deng, 2007; Ruohola et al., 1991). Proper differentiation of the posterior follicle cells (PFCs) of the egg chamber (those overlying the oocyte) is necessary to generate the polarizing signal from the PFCs to the oocyte. Disruption of Notch signaling in PFCs has been shown to prevent formation of the anterior-posterior axis in the oocyte (Poulton and Deng, 2007). We examined the localization of a key anterior-posterior polarity marker, Staufen:GFP (see Fig. S5A in the supplementary material) (Schuldt et al., 1998; St Johnston et al., 1991), in stage-9 egg chambers in which the PFCs were mutant for *bel*. Consistent with a role for Bel in Notch signaling, egg chambers with PFC clones of *bel* resulted in mislocalization of Staufen:GFP from the posterior cortex to the center of the oocyte (see Fig. S5B in the supplementary material). Together, these Notch-related defects in differentiation, oocyte polarity and cell cycle transition confirm that Bel regulates the timing of Notch signaling in follicle cells.

The miRNA pathway promotes Notch signaling in follicle cells

Bel was recently linked to both the miRNA and siRNA pathways in *Drosophila* (Ulvila et al., 2006; Zhou et al., 2008). Determining whether either pathway is involved in regulating Notch signaling is a relatively straightforward process because *Drosophila* uses two distinct Dicer proteins (Dcr-1 and Dcr-2) to generate the different short RNAs utilized in the miRNA and siRNA pathways, respectively (Lee et al., 2004). To test a role for the siRNA pathway, we generated follicle cell clones of the *Dcr-2*^{L811fsX} mutant (Lee et al., 2004). Follicle cell clones of *Dcr-2* showed no discernible defects in expression of the Notch target Hnt, indicating that the siRNA pathway is not required for proper Notch signaling in follicle cells (see Fig. S6A,B in the supplementary material). We also considered the possibility that Bel might function in a third small RNA pathway mediated by Piwi-interacting RNAs (piRNAs); however, clones of a core piRNA component *piwi*^{1} also showed no defects in Hnt expression in follicle cells (see Fig. S6C,D in the supplementary material) (Aravin et al., 2007; Cox et al., 1998).

By contrast, when we generated follicle cell clones of *Dcr-1*^{Q1147X} (Lee et al., 2004), we observed defects in Hnt expression similar to those of *bel* clones. Specifically, *Dcr-1* clones did not properly express Hnt at stage 7, suggesting that Notch was not activated in these cells (only 63% of clone cells expressed Hnt, *n*=85) (Fig. 4A). Similar to *bel* clones, *Dcr-1* clones exhibited a shift in the timing of Notch activity, as most clones did express Hnt by stage 8/9 (90%, *n*=88), and some showed prolonged expression of Hnt after stage 10a (Fig. 4B). To confirm that the *Dcr-1* defects were caused by disruption of the miRNA pathway, we tested a second component of the miRNA biogenesis pathway, Pasha. *pasha*^{KO} (Martin et al., 2009) clones also displayed defects in Notch signaling, as indicated by prolonged Cut expression after stage 6 (see Fig. S7A in the supplementary material) and lack of E(spl):CD2 expression in stage-7 clones (see Fig. S7B in the supplementary material). To further reduce miRNA production we generated *Dcr-1,pasha* double-mutant clones, and found that they also disrupted Notch signaling as evidenced by prolonged Cut staining after stage 6 (see Fig. S7C in the supplementary material).

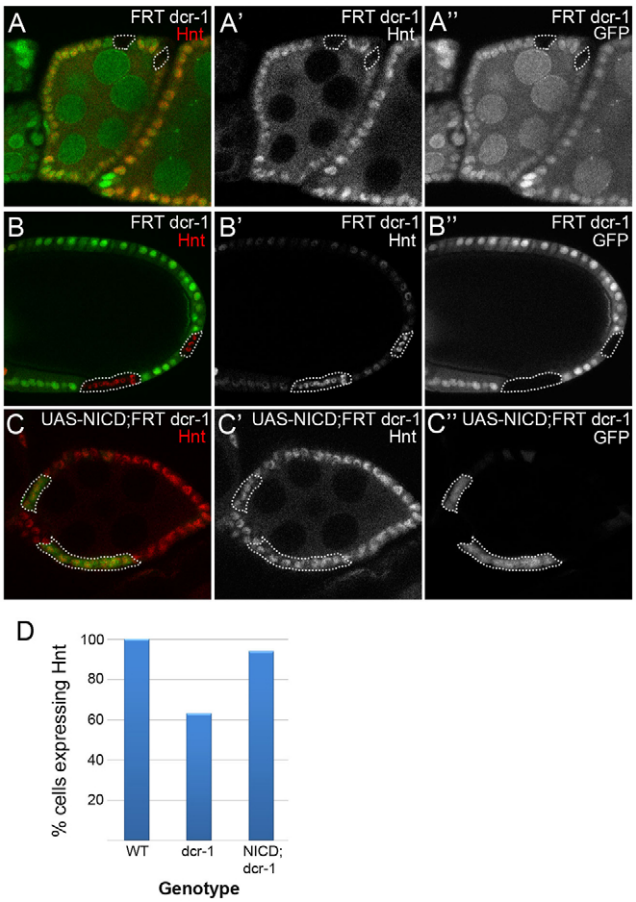


Fig. 4. Dcr-1 regulates the temporal pattern of Notch activity. (**A-A″**) In *Drosophila Dcr-1* clones (GFP negative), Hnt is not upregulated at stage 7, in contrast to wild-type cells (GFP positive). (**B-B″**) *Dcr-1* clones also display prolonged expression of Hnt after stage 10a. (**C-C″**) Expression of constitutively active Notch (NICD) in *Dcr-1* mutant clones significantly restores the proper expression of Hnt at stage 7 (GFP positive; outlined with white dotted line). (**D**) Quantitative analysis of stage-7 cells with correct Hnt expression in *Dcr-1* clones (63%, *n*=85) as compared with *Dcr-1* clones with NICD expression (94%, *n*=89) indicates that the difference in Hnt expression is significant ($P<0.001$ by χ^2 analysis).

To determine at which level in the Notch pathway miRNAs act, we used the MARCM technique to express a constitutively active form of Notch [UAS-Notch intracellular domain (NICD)] in *Dcr-1* clones (Lee and Luo, 1999). The *NICD* transgene mimics cleaved Notch and bypasses any requirement for ligand binding (Rauskolb et al., 1999). If expression of NICD can reduce or eliminate the Notch defects caused by mutation of *Dcr-1*, it would indicate that loss of *Dcr-1* disrupts Notch activity upstream of Notch cleavage. If, by contrast, NICD cannot restore proper Notch activity in *Dcr-1* mutant cells, it would suggest that loss of *Dcr-1* affects Notch signaling downstream of receptor activation and cleavage. Consistent with the former, we found that expression of NICD in *Dcr-1* clones significantly relieves the defects in Hnt expression typical of *Dcr-1* single-mutant clones [6% of clone cells lacked Hnt (*n*=89) as compared with 37% in *Dcr-1* single-mutant clones; $P<0.001$ by χ^2] (Fig. 4C,D). Together, these data indicate that the miRNA pathway promotes the correct timing of Notch activity in follicle cells by acting at, or prior to, Notch receptor cleavage.

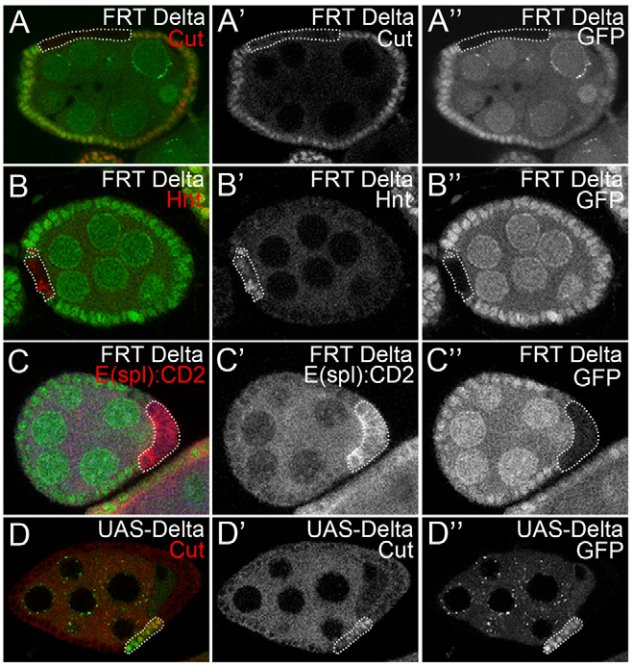


Fig. 5. Delta negatively regulates Notch activity in follicle cells. (**A-A″**) In *Drosophila Delta* clones (GFP negative), a negatively regulated target of Notch signaling, Cut (red), is prematurely downregulated before stage 7. (**B-C″**) Consistent with early activation of Notch in *Delta* clones, we observed precocious expression of Hnt and of the Notch reporter E(spl)-CD2 before stage 7. (**D-D″**) Overexpression of Delta (GFP-positive cells) was sufficient to cause prolonged expression of the early follicle cell marker Cut. Clones are outlined with dotted white lines.

The miRNA pathway promotes Notch activity by repressing Delta in follicle cells

Because mutations in miRNA pathway components disrupt Notch activity in follicle cells, it stands to reason that there must be a gene targeted by miRNAs that encodes a repressor of Notch signaling. Loss of miRNA function in *Dcr-1* or *pasha* clones would leave these cells unable to repress the expression of the target gene transcripts, resulting in more of this protein in these cells, thereby inhibiting proper activation of the Notch pathway. To determine the protein involved in this process, we cross-referenced known negative regulators of Notch signaling against a list of predicted miRNA targets (TargetScan 5.1) (Ruby et al., 2007), and found that *Delta* is a putative target of several miRNAs. In fact, *Delta* has been validated as a target of miR-1 in *Drosophila* cardiac development (Kwon et al., 2005). Although Delta functions as an activating ligand of Notch in trans, in some contexts Delta can also inhibit Notch activation in cis (Cordle et al., 2008; de Celis and Bray, 1997; Fiuza et al., 2010; Li and Baker, 2004; Micchelli et al., 1997; Miller et al., 2009; Sakamoto et al., 2002).

To determine whether Delta is an important regulator of Notch signaling in follicle cells we examined the effects of loss of follicle cell Delta on Notch activity. If Delta functions to repress Notch activity, we would expect to observe premature activation and/or upregulation of Notch signaling in *Delta* mutant follicle cells. We first examined the early follicle cell marker Cut, which is downregulated by Notch activity at stage 7. Indeed, we did observe downregulation of Cut in $Delta^{revF10}$ clones prior to stage 7 (Fig. 5A), suggesting that Notch signaling is active in these cells. To confirm that Delta is inhibiting Notch activity, we then stained *Delta* clones for Hnt and E(spl):CD2, which are upregulated at stage 7 in response to Notch activation. In agreement with the hypothesis that Delta is functioning as a repressor of Notch activity, *Delta* mutant clones precociously expressed both Hnt and E(spl):CD2 before stage 7 (Hnt upregulated in 67% of clone cells, n=73) (Fig. 5B,C). The *Delta* mutant clones also tended to have larger nuclei than those of neighboring wild-type cells, which is indicative of early entry into the endocycle (see Fig. S8A in the supplementary material). We confirmed this role of Delta by examining a second *Delta* mutant allele, $Delta^{M1}$ (de Celis et al., 1991), clones of which, like those of $Delta^{revF10}$, displayed early expression of Hnt and larger nuclei (see Fig. S8B in the supplementary material). Interestingly, in the mid-stages of oogenesis, when Notch is normally active, *Delta* clones occasionally displayed higher levels of E(spl):CD2 expression than their wild-type counterparts, suggesting that Delta was also functioning at these stages to limit the level of Notch activity (see Fig. S8C in the supplementary material). We then used the *flip-out Gal4* driver to overexpress Delta in follicle cells and observed prolonged expression of Cut after stage 6 (44%, n=257 clone cells) (Fig. 5D), indicating that expression of Delta alone is sufficient to repress activation of Notch in follicle cells. Together, these data demonstrate that follicle cell Delta acts as a potent inhibitor of Notch signaling.

We then tested the hypothesis that *Delta* is a relevant miRNA target in follicle cells by assaying Notch activation in *Dcr-1,Delta* double-mutant clones. We reasoned that if loss of miRNAs caused by mutated *Dcr-1* leads to inhibition of Notch activity through increased Delta protein levels, then removing Delta should prevent this block on Notch activation and the dominant phenotype in these cells should be similar to that of *Delta* clones, that is, premature Notch activation. If *Delta* is not the miRNA target, then the relevant target that is upregulated in *Dcr-1* clones would still be present in the *Delta,Dcr-1* double-mutant clones and activation of Notch signaling at stage 6 would be inhibited, as we observe in *Dcr-1* clones. Consistent with the hypothesis that *Delta* is an important miRNA target and repressor of Notch in follicle cells, we found that the *Delta,Dcr-1* double-mutant cells displayed premature Notch activity (76% of clone cells express Hnt at stages 5/6, n=64) (Fig. 6B), similar to *Delta* single-mutant clones (67%) (Fig. 5B). We also observed premature downregulation of Cut in *Delta,Dcr-1* clones, supporting the hypothesis that Notch is activated early in these cells (Fig. 6C).

We applied the same approach to test the relationship between Bel and Delta in the regulation of Notch activity and found very similar results. Specifically, follicle cells double mutant for *bel* and *Delta* displayed evidence of premature Notch activation (66% of clone cells expressed Hnt before stage 7, n=112; see Fig. S8D in the supplementary material), similar to the *Delta* single-mutant phenotype, and did not show the delayed Notch activation typical of *bel* single-mutant clones. This suggests that Delta mediates the inhibitory effect of loss of Bel on Notch activation in follicle cells.

We also tested a role for the miRNA pathway in regulating *Delta* by examining Delta protein levels in follicle cell clones of *Dcr-1* and *pasha* single mutants. If *Delta* is normally repressed by the miRNA pathway, we would expect to detect more Delta protein in cells with compromised miRNA function. Although we observed no consistent upregulation of Delta in *Dcr-1* or *pasha* single-mutant clones (data not shown), we did observe significant accumulations of Delta protein in some *Dcr-1,pasha* double-mutant clones, suggesting that *Delta* might be an miRNA target in follicle cells (19% of clone cells possessed increased Delta staining, n=138/709) (Fig. 6D).

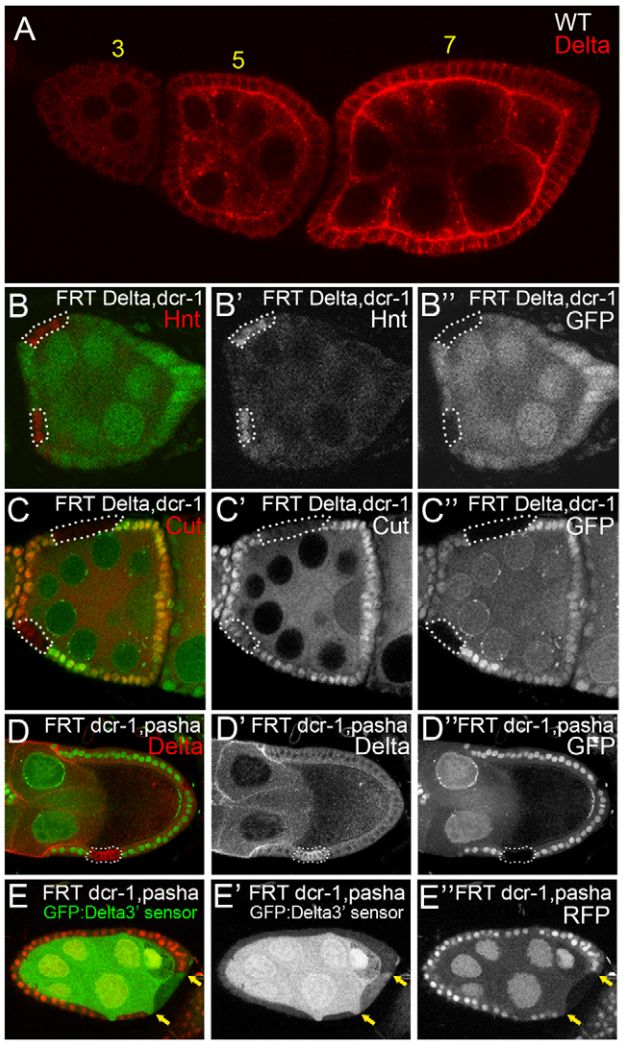


Fig. 6. The miRNA pathway regulates Notch through Delta. (**A**) In wild-type *Drosophila* oogenesis, Delta is noticeably upregulated in the germline cells beginning around stage 5 (stage numbers are given in yellow); however, indicators of Notch activity in the overlying follicle cells are not visible until stage 7 (compare with Fig. 1). (**B-C″**) Similar to *Delta* single-mutant clones (see Fig. 5A,B), double-mutant clones of *Delta* and *Dcr-1* prematurely activate Notch signaling as indicated by premature upregulation of Hnt (B-B″) and downregulation of Cut (C-C″) before stage 7. (**D-D″**) Follicle cells double mutant for *Dcr-1* and *pasha* frequently displayed ectopic accumulation of Delta protein, as seen in this stage-9 egg chamber (19%, *n*=709). (**E-E″**) *Dcr-1,pasha* clones also possessed increased levels of GFP expression from a Delta sensor (47%, *n*=383). Note that the mutant clones in E-E″ were marked as red fluorescent protein (RFP) negative, and the boundaries of the clone cells are indicated by yellow arrows to allow easier visualization of the GFP sensor increase (E′). Clones are outlined with dotted white lines.

To determine whether *Delta* is a direct target of the miRNA pathway, we generated a sensor line to monitor the regulation of *Delta* transcripts by the miRNA pathway (Brennecke et al., 2003; Silver et al., 2007). This sensor line contains GFP ubiquitously expressed under the *tubulin* promoter with the 3′UTR of *Delta* attached downstream of the GFP coding sequence. Because the 3′UTR of *Delta* contains predicted miRNA binding sites, disruption of the miRNA pathway should cause increased GFP expression resulting from derepression of the *Delta* 3′UTR. We therefore generated *Dcr-1,pasha* double-mutant follicle cells in flies that also expressed the Delta sensor and assayed them for any changes in GFP levels. Consistent with the Delta protein data above, we observed a relatively high incidence of increased GFP expression in follicle cells with compromised miRNA function (47% of stage 4-10 *Dcr-1,pasha* clones had increased levels of GFP relative to wild-type neighbor cells, *n*=181/383) (Fig. 6E). These data indicate that *Delta* is an endogenous target of miRNA repression in follicle cells.

DISCUSSION

The strict regulation of important cellular processes, such as the temporal activity of signaling pathways like Notch, is an essential point of control in guiding the development of multicellular organisms. Cells have therefore evolved a complex array of mechanisms to regulate signaling pathways. miRNA regulation of gene expression has rapidly emerged as one of the most important of these regulatory mechanisms. Here, we have shown that the correct timing of Notch activity in follicle cells requires the miRNA pathway and the newly identified RNAi component Bel. Our data suggest that one important target of miRNA-based regulation of Notch signaling in follicle cells is *Delta*, in which Delta acts as a repressor of Notch activity.

Bel and the miRNA pathway control the timing of Notch activity in follicle cells

Our findings that two core components of miRNA production are required to properly initiate the mitotic-to-endocycle switch in follicle cells by promoting Notch signaling describe a novel mechanism by which the miRNA pathway regulates this key developmental event. Interestingly, the miRNA pathway appears to control the overall timing of Notch activity, as disruption of the miRNA pathway results in a delay of Notch activation and inactivation in follicle cells. Previous work has shown that certain miRNAs, known as heterochronic miRNAs, regulate the timing of important developmental processes on a wide biological scale, from changes in cell cycle to the transition from juvenile to adult (Caygill and Johnston, 2008; Rougvie, 2005; Sokol et al., 2008; Tennessen and Thummel, 2008; Wienholds and Plasterk, 2005). Our research identifies a new example of heterochrony mediated by miRNAs, in which cell cycle switches and differentiation are shifted in time as a result of delayed Notch signaling activity.

Bel is a DEAD-box RNA helicase that was recently identified in two *Drosophila* cell culture screens as necessary for effective siRNA knockdown (Ulvila et al., 2006; Zhou et al., 2008). Precisely how Bel functions in this process is unknown, although data from the Zhou et al. screen suggest that Bel acts downstream of siRNA production and loading (Zhou et al., 2008). Interestingly, although Bel did not significantly disrupt miRNA-based assays in that screen, Bel was found to be in a complex with components of both the miRNA and siRNA pathways, and Bel immunoprecipitation pulled down both miRNAs and siRNAs (Zhou et al., 2008), suggesting that Bel might be involved in both pathways. The similarities we have described between the *bel* mutant phenotype and the phenotypes of the miRNA pathway components *Dcr-1* and *pasha* imply that Bel might function in the miRNA pathway. We attempted to test the role of Bel in the miRNA pathway more directly using the GFP-tagged *Delta* 3′UTR sensor line, the expression of which is regulated by miRNA activity, but the results of these experiments were inconclusive. Although Bel appears to function in the siRNA pathway (Zhou et al., 2008), we found that the siRNA pathway is not involved in regulating Notch in follicle cells. A few reports have also identified several phenotypes associated with disruption of *bel* that indicate that Bel functions in the

G1/S transition in the eye disc by affecting Hedgehog signaling and Dacapo expression (Ambrus and Frolov, 2010; Ambrus et al., 2007), as well as identifying a role for Bel with the zinc-finger protein Zn72D in regulating the splicing and translation of *maleless* transcripts (Worringer et al., 2009). It will be interesting to determine whether the function of Bel in these other important cellular processes is also related to a role in RNAi pathways.

Delta functions as a Notch repressor in follicle cells

Notch can be both activated and inhibited by its ligands. In oogenesis, it is known that Delta from the germline cells functions in trans to activate Notch in the surrounding follicle cells (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Here, we have found that Delta expressed in the follicle cells operates in its repressive capacity to prevent premature activation of Notch. Because Delta is actually upregulated in the germline by stage 5/6 (Fig. 6A), well before the expression of Notch target genes at stage 7, and in light of our data on the inhibitory role of follicle cell Delta, it is likely that the presence of Delta from the germline alone is not what determines the precise timing of Notch activity. Instead, we favor a model in which the timing of Notch activity is determined by a titration of trans-activating germline Delta relative to the cis-inhibitory effects of follicle cell Delta. Therefore, loss of follicle cell Delta, as in our *Delta* mutant clone experiments, allows earlier activation of Notch by the lower levels of Delta presented by the germline before stage 7, as well as higher levels of Notch activity relative to wild-type cells in mid-oogenesis. This antagonistic relationship between germline and follicle cell Delta suggests that there must be a precise balance between these two populations of Delta that determines exactly when Notch is activated during oogenesis; our analysis of the miRNA pathway suggests that miRNAs might help to fine-tune this balance.

The miRNA pathway promotes Notch activity through Delta

Our conclusion that *Delta* is a relevant target of miRNA-based control of Notch activity in follicle cells is supported by the following observations. First, expression of NICD is sufficient to restore proper activation of Notch in the *Dcr-1* mutant, indicating that the relevant miRNA target functions upstream in the Notch pathway (prior to ligand-induced Notch cleavage). Because ligand-based inhibition by Delta acts upstream of Notch cleavage, *Delta* is a logical candidate of miRNA regulation. Second, our *Delta,Dcr-1* double-mutant analysis strongly suggests that *Delta* is an important target of miRNAs. Specifically, in *Dcr-1* single-mutant clones, Notch signaling is delayed, yet removal of Delta along with loss of Dcr-1 leads to premature activation of Notch, as seen in *Delta* single-mutant clones. This indicates that the inhibitory effects on Notch signaling caused by loss of miRNAs requires the presence of Delta. However, we cannot rule out the possibility that the activating effects of loss of Delta on Notch might be stronger than the inhibitory effects of loss of miRNAs on repressing Notch activity through some other miRNA target. Third, *Delta* is an apparent direct target of the miRNA pathway, as indicated by our experiments demonstrating that follicle cell clones of *Dcr-1* and *pasha* result in increased Delta protein and increased expression of a *Delta* 3′UTR sensor. Together, the ectopic expression of Delta protein and of the *Delta* 3′UTR sensor in the *Dcr-1,pasha* clones, in conjunction with the *Delta,Dcr-1* double-mutant analysis, strongly suggest that the miRNA pathway regulates Notch activity by repressing Delta protein levels.

Cis inhibition of Notch has also been described for Ser (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997), and *Ser* mRNA is also a potential target of miRNAs (Vallejo et al., 2011), raising the possibility that Ser might be functioning in follicle cells in a similar capacity to that which we have discovered for Delta. However, *Ser* mutant follicle cell clones possess no defects in Notch activity markers (data not shown). To determine whether Ser is repressed by the miRNA pathway in follicle cells, we examined Ser protein levels in follicle cells double mutant for *Dcr-1* and *pasha* and observed no changes in Ser expression, which in the wild type was essentially undetectable (data not shown). We conclude that Ser does not play a role in regulating Notch activity in follicle cells.

More than two dozen miRNAs are predicted to target *Delta* mRNA. Owing largely to a lack of readily available mutants to conduct a thorough loss-of-function screen for the miRNA(s) involved, it remains unknown which miRNAs are important in governing the timing of Notch signaling in follicle cells. We tested both loss of function and overexpression of miR-1, which has been previously demonstrated to regulate *Delta* in *Drosophila* heart development (Kwon et al., 2005); however, neither produced any phenotype consistent with the Notch defects we have described. As the genetic tools available to investigate the roles of specific miRNAs improve, and our ability to predict which miRNAs target certain transcripts also improves, it should be possible to identify the relevant miRNAs involved in this process.

Our findings describe a complex system by which developing egg chambers regulate the timing of several key events, including cell cycle programs and differentiation. Mechanistically, we have found that the miRNA pathway controls the temporal pattern of Notch activity, apparently by limiting Delta protein levels in follicle cells, in which Delta exerts an inhibitory effect on Notch. Our data support a model in which the timing of Notch activation is determined not just by the expression of germline Delta, but also by a multi-layered regulatory system in which follicle cell Delta prevents premature Notch activation, while miRNAs serve to counter this inhibitory effect by limiting Delta expression. Such a model of miRNA function in follicle cells fits well with the developing theme that miRNAs commonly serve to fine-tune developmental processes by subtle regulation of key regulators (Bartel, 2009). It will be interesting to determine whether miRNAs also regulate Notch signaling in other tissues of the fly through a similar mechanism of ligand-mediated inhibition of Notch, and it will be particularly exciting to investigate whether this regulatory network is utilized in other animals.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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