

Dicer-1-dependent Dacapo suppression acts downstream of Insulin receptor in regulating cell division of *Drosophila* germline stem cells

Jenn-Yah Yu^{1,*†}, Steven H. Reynolds^{1,*}, Steve D. Hatfield^{1,‡}, Halyna R. Shcherbata^{1,§}, Karin A. Fischer¹, Ellen J. Ward¹, Dang Long², Ye Ding² and Hannele Ruohola-Baker^{1,¶}

It is important to understand the regulation of stem cell division because defects in this process can cause altered tissue homeostasis or cancer. The cyclin-dependent kinase inhibitor Dacapo (Dap), a p21/p27 homolog, acts downstream of the microRNA (miRNA) pathway to regulate the cell cycle in *Drosophila melanogaster* germline stem cells (GSCs). Tissue-extrinsic signals, including insulin, also regulate cell division of GSCs. We report that intrinsic and extrinsic regulators intersect in GSC division control; the Insulin receptor (InR) pathway regulates Dap levels through miRNAs, thereby controlling GSC division. Using GFP-*dap* 3' UTR sensors in vivo, we show that in GSCs the *dap* 3' UTR is responsive to Dicer-1, an RNA endonuclease III required for miRNA processing. Furthermore, the *dap* 3' UTR can be directly targeted by *miR-7*, *miR-278* and *miR-309* in luciferase assays. Consistent with this, *miR-278* and *miR-7* mutant GSCs are partially defective in GSC division and show abnormal cell cycle marker expression, respectively. These data suggest that the GSC cell cycle is regulated via the *dap* 3' UTR by multiple miRNAs. Furthermore, the GFP-*dap* 3' UTR sensors respond to InR but not to TGF- β signaling, suggesting that InR signaling utilizes Dap for GSC cell cycle regulation. We further demonstrate that the miRNA-based Dap regulation may act downstream of InR signaling; Dcr-1 and Dap are required for nutrition-dependent cell cycle regulation in GSCs and reduction of *dap* partially rescues the cell cycle defect of *InR*-deficient GSCs. These data suggest that miRNA- and Dap-based cell cycle regulation in GSCs can be controlled by InR signaling.

KEY WORDS: Cell cycle, Dacapo, Stem cells, *Drosophila*

INTRODUCTION

Stem cells are a class of specialized cells that can divide for long periods of time to produce both new stem cells and daughter cells with the potential to differentiate into a specific cell lineage. They play important roles in maintaining homeostasis of tissues by providing new cells and replacing lost cells. The cell cycle is carefully controlled so that stem cells may respond properly to different physiological conditions including tissue injury, nutrition status and aging. Slow division or premature differentiation of stem cells may deplete the stem cell pool, whereas excessive proliferation may contribute to tumorigenesis. Therefore, it is important to understand cell cycle regulation in stem cells. Many adult stem cells reside in a niche, a restricted microenvironment with supporting cells (Fuller and Spradling, 2007; Morrison and Spradling, 2008; Yamashita et al., 2005). Extrinsic signaling between stem cells and niche cells is thought to play important roles in controlling intrinsic factors in stem cells, thereby affecting self-renewal, cell division and differentiation.

MicroRNAs (miRNAs) are endogenous, short (~21 nucleotide) non-coding RNAs that regulate gene expression through incomplete sequence complementarity with miRNA response elements (MREs) in the 3' UTR of target mRNAs. Recent studies have shown that miRNAs serve important regulatory roles in a variety of tissues, including stem cells (Ambros, 2004; Bartel, 2004; Carrington and Ambros, 2003; Du and Zamore, 2005; Stadler and Ruohola-Baker, 2008; Yi et al., 2008). Mutations in, or misexpression of, miRNAs are found in several human cancers, indicating that miRNAs may also function as oncogenes or tumor suppressors (Croce and Calin, 2005; Esquela-Kerscher and Slack, 2006).

Analysis of essential miRNA biogenic factors [Dicer, Dgcr8 (Pasha – FlyBase)] has guided our understanding of the functions of miRNAs in vivo. *Dicer1* knockout mice die as embryos owing to depletion of pluripotent stem cells (Bernstein et al., 2003) and the proliferation of mouse embryonic stem (ES) cells that are deficient for Dicer1 or Dgcr8 is reduced (Murchison et al., 2005; Wang et al., 2007). In *Drosophila*, disruption of *Dcr-1* in germline stem cells (GSCs) also leads to a significant reduction of cell division and to the disruption of GSC maintenance (Hatfield et al., 2005; Jin and Xie, 2007; Park et al., 2007; Shcherbata et al., 2007). Expression of the *Drosophila* cyclin-dependent kinase inhibitor (CKI) Dacapo (Dap), a p21/p27 (Cdkn1a/Cdkn1b) homolog, is increased in *Dcr-1* mutant GSCs, suggesting that miRNAs might regulate the cell cycle of GSCs by repressing Dap (Hatfield et al., 2005). Other components involved in miRNA biogenesis and function, such as *loquacious* (*loqs*) or *Argonaute-1* (*Ago1*), are also required for GSC maintenance (Forstemann et al., 2005; Park et al., 2007; Yang et al., 2007). These results suggest that miRNAs play crucial roles in stem cell maintenance and division. Furthermore, conservation between miRNA function in stem cells is highlighted

¹Department of Biochemistry, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA. ²Wadsworth Center, New York State Department of Health, 150 New Scotland Avenue, Albany, NY 12208, USA.

*These authors contributed equally to this work

[†]Present address: Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei 112, Taiwan

[‡]Present address: F. M. Kirby Center for Molecular Ophthalmology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

[§]Present address: Research Group Gene Expression and Signaling, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, Goettingen 37077, Germany

[¶]Author for correspondence (e-mail: hannele@u.washington.edu)

by miRNAs regulating p21^{cip1} in mouse ES cells and Dap in *Drosophila* GSCs (Sinkkonen et al., 2008; Wang et al., 2008; Hatfield et al., 2005).

Extrinsic signaling is crucial in regulating stem cells. For example, neural stem cell maintenance in mammals requires hedgehog signaling (Ahn and Joyner, 2005; Balordi and Fishell, 2007). Similarly, the chemokine Cxcl12 (Sdf1) is required to maintain bone marrow hematopoietic stem cells (Kollet et al., 2006; Sacchetti et al., 2007; Sugiyama et al., 2006). In *Drosophila*, TGF- β and insulin signaling regulate cell division of GSCs (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005; Xie and Spradling, 1998). Activation of the Insulin receptor (InR; Insulin-like receptor – FlyBase) in GSCs by *Drosophila* Insulin-like peptide (Dilp; Ilp1 – FlyBase) is required for the nutrient-dependent regulation of cell division (LaFever and Drummond-Barbosa, 2005). However, the molecular mechanisms downstream of InR signaling that directly regulate the GSC cell cycle remain unknown. Evidence from mammalian cells suggests that the CKIs p21^{cip1} and p27^{kip1} might regulate the cell cycle downstream of the InR signaling cascade through Foxo, a member of the forkhead transcription family (Medema et al., 2000; Nakae et al., 2003; Seoane et al., 2004; Alvarez et al., 2001; Burgering and Kops, 2002; Kops et al., 1999). Since InR signaling also regulates the cell cycle in *Drosophila* GSCs and miRNAs affect cell division by negatively regulating Dap levels in these cells, we explored the possibility that these pathways might interact to control the GSC cell cycle.

The *Drosophila* CKI Dap can inhibit the Cyclin E-Cdk2 (Cdc2c) complex that is required for the G1–S phase transition (de Nooij et al., 2000; Lane et al., 1996). Previous studies have shown increased Dap expression in *Dcr-1* mutant GSCs. Furthermore, reduction of *dap* partially rescues the cell cycle defects in *Dcr-1* mutant GSCs. This suggests that Dap acts downstream of miRNAs to regulate the cell cycle (Hatfield et al., 2005). We now show that the *dap* 3'UTR responds to miRNA activities in GSCs using heterologous reporters (sensors) consisting of a tubulin promoter driving the green fluorescent protein (GFP) gene fused to the *dap* 3'UTR. Using luciferase assays, we identified miRNAs that can directly target the *dap* 3'UTR, including *miR-7*, *miR-278* and *miR-309*. GSCs deficient for *miR-278* or *miR-7* show mild division defects or abnormal expression of cell cycle markers, respectively. It is therefore possible that the control of cell division through Dap in GSCs requires the simultaneous function of multiple miRNAs. We further show that GFP-*dap* 3'UTR sensors respond to InR but not to TGF- β activity. Consistent with these findings, GSCs deficient for *InR* show a *Dcr-1*-like cell division defect: slow kinetics, increased frequency of staining for Cyclin E and Dap and decreased frequency of staining for Cyclin B. The genetic evidence places the miRNAs and Dap downstream of InR signaling in regulating cell division: cell division of *Dcr-1* or *dap* mutant GSCs does not respond to nutrition, and reduction of *dap* partially rescues the cell cycle defects of *InR* mutant GSCs. Thus, our results suggest that InR can regulate the *Drosophila* GSC cell cycle through miRNAs and Dap.

MATERIALS AND METHODS

Plasmids and constructs

The following primers (5' to 3'; sequence according to NM_057600) were used for cloning the *dap* 3'UTR:

dapL forward, GCTCTAGATTCGCTGGCCAACCC and reverse, GCTCTAGAATAGGCTCTGCCTATGT;

dapS forward, GCTCTAGACTCGTAACCAGTAATTAG and reverse, GCTCTAGAGCCCAGAGATCATAGCAA;

dapF forward, GCCTCTAGAACAACTAATGCTCCAGA and reverse, GCGACTAGTATTATGTACTACCAAC.

Fragments of *dap* 3'UTR were amplified by PCR from *Drosophila* genomic DNA. Fragments of *dapF* (1185 bp), *dapL* (866 bp) and *dapS* (630 bp) were ligated into the *tub-GFP* plasmid. *tub-firefly-luciferase (tub-luc)* and *tub-renilla-luciferase (tub-rLuc)* plasmids were provided by the Cohen laboratory (Stark et al., 2003). *dapF* was ligated into the *tub-fLuc (tub-fLuc-dapF)* vector.

tub-miR-1, *tub-miR-7* and *tub-miR-309* cluster expression vectors were obtained from the Cohen laboratory (Stark et al., 2003); all other miRNA expression vectors were constructed from amplicons of 400–1000 bp containing pre-miRNA sequence and inserted into a tubulin-driven expression vector. The primers for primary miRNAs were:

miR-289 forward, CACGAAGGATCCAGTCTGTGCCAG and reverse, CAGCAATCTAGAACCACCTCCAGCAC;

bantam forward, ATAGCGGCCGCGTTAACTGGCAGCATATA-ATTTTC and reverse, ATTCTAGATTATAGGCAGATTTAACATGTGG;

miR-8 forward, ATAGCGGCCGCGGTCACACGCACATTT-CAATA and reverse, ATTCTAGAAATGGGAATTGGGAACGATCTCGC;

miR-303 forward, ATAGCGGCCGCTGCATTTCGAAAGGCCAGG-TGAA and reverse, ATTCTAGATTGTCCAGGATCTAACATGATTTTCGT; *let-7* (including pre-*miR-125*) forward, ATAGCGGCCGCGAAGATCAACAGCGATCCATTAACA and reverse, ATTCTAGATTGCGGATACTTGTGCCTTGA;

miR-34 forward, ATAGCGGCCGATTTGGCTTGCACACACT and reverse, ATTCTAGATTCGTTGTTTCAGGCGTCTGGTT;

miR-278 forward, ATAGCGGCCGCTTGGCGCATTAACCGA-CGCTTT and reverse, ATTCTAGATCCTTGTGACTCCCAGAAA.

Mutagenesis of the miRNA MRES

dapF was cut from *tub-fLuc-dapF* and inserted into the CS2p plasmid (Turner and Weintraub, 1994). Mutagenesis was by PCR using the following primers (introduced restriction sites in parentheses; mutated sequences underlined):

miR-7 mutation (*NcoI*) forward, GAATATTAATCGTTCCATGGCA-ACTACTCGTAACCAGTA and reverse, TTACGAGTAGTTGCCAT-GGAACGATTAATATTCGCAACT;

miR-8 mutation (*NcoI*) forward, CTGCGATTGTGTCCATGGTCTCTA-ATTTTTATTACGAACC and reverse, GTAATAAAAAATTAG-GACCATGGACACAATCGCAGTGGCTT;

miR-309 mutation (*BglII*) forward, CTCATTTCTTAAAGATCTC-TAAAAATGTCTTTTTATGATTTG and reverse, CATAAAGACATTTT-TAGAGATCTTTAAGAAATGAGAGCG;

miR-278 mutation (*BglII*) forward, CGCTGGCCAAAGATCTGAAT-TGCAATTTGTAATTTTATTTTAC and reverse, TACAAATT-GCAATTCAGATCTTTGGCCAGCGAATCTGGAGC.

Mutated *dapF* fragments were then cut from the CS2p plasmids and inserted into the *tub-fLuc* plasmid.

The *bantam* mutation was introduced by PCR using 5'-TAAAGAT-CTCTAAAAATGTCTTTTTATGATTTGCTATCCATGGTGGGCAAATT-ATGAAAAC-3' (with *NcoI*) and a T7 primer with the CS2p-*dapF-miR-309* mutant as the template.

For *UASp-miR-7*, 432 nt containing the *miR-7* precursor was amplified from *pUAST-miR-7* with the following primers and ligated into *pUASP*: forward, CACGAAGGATCCGCTAACCACCCATCCCCACAA and reverse, CAGCAATCTAGAATGGGAGGGTACTGGGGAGTTC.

S2 cell culture, transfection and luciferase assay

S2 cells were cultured in Schneider's *Drosophila* Medium (Gibco) with 10% heat-inactivated FBS and penicillin/streptomycin at room temperature (20°C). Transfection used Cellfectin (Invitrogen) according to the manufacturer's instruction. For the luciferase assays in Fig. 2, 1 μ g of each miRNA expression plasmid, 100 ng of firefly luciferase reporter plasmid with or without *dap* 3'UTR, and 100 ng of renilla luciferase reporter plasmid were transfected into cells in a well of a 12-well plate. For the luciferase assays in Fig. S5 in the supplementary material, the amount of miRNA expression vector added in each group was: 0.6 μ g *miR-1*; 0.2 μ g *miR-278* and 0.4 μ g *miR-1*; 0.2 μ g *miR-278*, 0.2 μ g *miR-309* and 0.2 μ g *miR-1*; 0.2 μ g *miR-278*, 0.2 μ g *miR-309* and 0.2 μ g *miR-7*. Combined miRNA expression vectors and 50 ng of firefly luciferase reporter plasmid with or

without *dap* 3'UTR, and 50 ng of renilla luciferase reporter plasmid were transfected into cells in a well of a 24-well plate. Luciferase assays (Dual Luciferase System, Promega) were performed 2 days after transfection. Renilla luciferase activity provided normalization for firefly luciferase activity. The relative luciferase activities of the cells transfected with *tub- β Luc-dapF* and the different *tub- β Luc-mutant-dapF* constructs were further normalized to the relative luciferase activities of *tub- β Luc* for each miRNA.

Generation of GFP-*dap* 3'UTR sensors and *UASp-miR-7* transgenic lines

Transgenic flies were generated by injection of purified plasmid DNA into *w¹¹¹⁸* *Drosophila* embryos (Rainbow Transgenic Flies, Newbury Park, CA, USA). These flies were crossed with *w¹¹¹⁸* and transformants were selected based on eye color. Twelve, nine, one and nine lines were generated for *tub-GFP:dapF*, *tub-GFP:dapL*, *tub-EGFP:dapS* and *UASp-miR-7*, respectively.

Recombination for *FRT42DmiR-278^{KO}*, *FRT42DmiR-278^{Gal4KI}* and *FRT42DmiR-278^{Gal4KI},miR-7^{Δ1}/CyO*

Two *miR-278* mutations were recombined into the *FRT42D* chromosome using standard meiotic recombination protocols (Xu and Rubin, 1993). *FRT42DmiR-278^{Gal4KI}* was further recombined into the *FRT42DmiR-7^{Δ1}* chromosome.

Fly stocks

We used *Drosophila* stocks carrying the *tub-GFP:2x(miR-7)* reporter, control *tub-GFP* reporter (Stark et al., 2003), *tub-GFP:dapL* reporter, *tub-GFP:dapF* reporter, *tub-GFP:dapS* reporter, *hsFLP;;UAS-GFPact>CD2>Gal4/TM3Sb, eyFlp;;FRT82BDcr-1^{Q1147X}/TM3, FRT82BInR^{ex52.1}/TM6B, FRT82BInR^{ex15}/TM3, eyFlp;FRT82B, hsFlp;;FRT82BarmilacZ/TM3, hsFlp;;FRT82BUbi-GFP/TM3, FRT42Bdap⁴/CyO, FRT42B, eyFlp;FRT42D, hsFlp;FRT42BUbi-GFP/CyO, hsFlp;FRT42DUbi-GFP/CyO, FRT42DmiR-278^{KO}/CyO, FRT42DmiR-278^{KI-gal4}/CyO, FRT42DmiR-7^{Δ1}/CyO, P{EP}bl^{EP954}, w⁻;FRT82Bput¹³⁵/TM3, yw;Mad¹²FRT40A/CyO, *dap*[2x10]/CyO, *dap*[g36]/CyO, *ftz-lacZ* +; *dap^{5gm.T.HsapMYC}*; +, w⁻; *Sp*/CyO; *FRT82BInR^{EX52.1}*/TM6B, yw *hsFLP*; *Sp*/CyO; *FRT82BUbi-GFP/TM6B*.*

Generation of clones

Clones of GSCs were induced using the heat shock FLP-FRT system (Dang and Perrimon, 1992; Xu and Rubin, 1993). To generate GSCs clones during third instar larval or pupal stages, flies were heat shocked for 1 hour at 37°C for 2 consecutive days. To generate GSCs clones at the adult stage, newly eclosed flies (1-2 days) were collected and heat shocked twice per day for 30 minutes at 37°C for 2 consecutive days.

Antibodies

The following were used: mouse anti-Adducin [clone 1B1, Developmental Studies Hybridoma Bank (DSHB)]; mouse anti-Cyclin B (DSHB); guinea pig anti-Cyclin E (T. Orr-Weaver, Whitehead Institute); mouse anti-Dap (I. Hariharan, University of California, Berkeley); Alexa 488-conjugated rabbit anti-GFP (Molecular Probes); Alexa 488, 555, 568 or 633-conjugated goat anti-mouse, anti-rabbit and anti-guinea pig antibodies (Molecular Probes).

Immunostaining and fluorescence microscopy

Ovaries were fixed as described previously: ovaries were dissected in PBS and fixed in 5% paraformaldehyde in PBS, then sequentially incubated in primary and secondary antibodies overnight at 4°C, followed by DAPI (1 μg/ml) for 15 minutes. Confocal microscopy and two-photon laser-scanning imaging were performed with a Leica TCS SP/MP or Leica SPE microscope.

Starvation procedure

The starvation condition was adapted from previous reports (Drummond-Barbosa and Spradling, 2001). For poor food conditions, adult flies were collected into plastic bottles containing molasses plates providing moisture and sugar, whereas for rich food conditions this was supplemented with yeast. Ovaries were dissected 10 or 14 days later.

Division frequency analysis

Only germaria containing both GFP (or β-gal)-positive and GFP (or β-gal)-negative GSCs were analyzed for cell division. The average sum of cystoblasts and cysts generated by individual GFP (or β-gal)-negative GSCs in region 1-2A of a germarium was normalized to that of individual GFP (or β-gal)-positive control heterozygous GSCs to obtain the division index.

Quantitation of GFP in GSCs

MetaMorph (Molecular Devices) software was used to quantify GFP fluorescence intensity in GSCs imaged on a Leica SP1 confocal microscope. Images of the brightest GFP optical slice were quantified by drawing an elliptical field of identical dimensions for each cell and reading the average intensity in the field.

Quantification of cell cycle markers in GSCs

Quantification of cell cycle marker levels in GSCs was determined with the histogram function in Adobe Photoshop. For a given GSC, intensity was determined by averaging intensities from three different regions within the cell. A background germline intensity value was determined by averaging intensities obtained from three different regions within region 1 of the germarium having low-level staining or background staining. For each antibody, the intensity fold above background was determined as the average GSC intensity divided by the average background intensity.

miRNA quantitative RT-PCR (qPCR)

RNA was isolated from 10-20 ovaries using Trizol (Invitrogen) and treated with RNase-free DNaseI (Fermentas). The extracted RNA (0.5 μg) was reversed transcribed with Omniscript reverse transcriptase (Qiagen). miRNA levels (*dme-miR-8* and *dme-miR-278*) were quantified using TaqMan MicroRNA Assays (Applied Biosystems) as per manufacturer's instruction, using 10 ng of total RNA on an ABI 7300 real-time PCR system.

RESULTS

The *dap* 3'UTR is regulated in GSCs by miRNAs

To test whether miRNAs regulate the *dap* 3'UTR in GSCs, we generated two transgenic lines containing different regions of the *dap* 3'UTR inserted downstream of GFP. *dapL* contains most of the *dap* 3'UTR, whereas some predicted MREs are deleted in the truncated *dapS* construct (Fig. 1A). A transgenic GFP line that lacks significant 3'UTR served as a control (control sensor; Fig. 1A). The GFP intensity was used to detect endogenous regulation through the *dap* 3'UTR. Reduction of miRNA production in *Dcr-1* mutant (*Dcr-1^{Q1147X}*) GSCs led to significant upregulation of GFP intensity of the *dapL* and *dapS* sensors, but not of the control sensor (Fig. 1B). Image quantification analysis showed that the GFP intensity of *dapL* and *dapS* sensors increased an average of 1.65±0.065-fold and 1.31±0.051-fold, respectively, in *Dcr-1* null GSCs as compared with the neighboring control heterozygous GSCs. The control sensor showed no significant difference (Fig. 1C). Similarly, no significant difference was detected between two adjacent *Dcr-1^{Q1147X}*/+ heterozygous GSCs for *dapL* or *dapS* (Fig. 1C). These results suggest that miRNAs can directly regulate Dap levels through the *dap* 3'UTR in *Drosophila* GSCs.

miR-7, *miR-278* and *miR-309* can target the *dap* 3'UTR directly

To identify which miRNAs repress Dap directly through the *dap* 3'UTR, we used luciferase assays in S2 cells. Computational algorithms based on sequence complementarity, homology across species and RNA secondary structure predict that many miRNAs target the *dap* 3'UTR, including *miR-7*, -8, -34, -278, -289, -303, -309, *let-7* and *bantam* (Fig. 2A,B) (Enright et al., 2003; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Grun et al., 2005; Long et al., 2007; Ruby et al., 2007). To test whether these predicted miRNAs are sufficient to regulate Dap, the full-length 3'UTR of

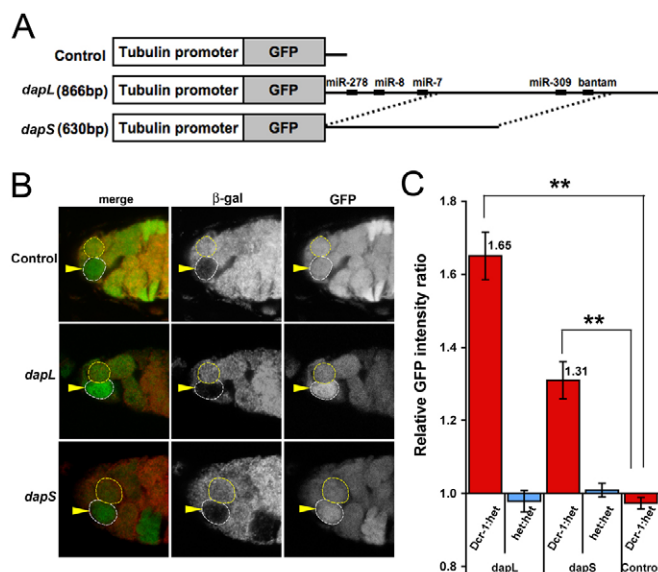


Fig. 1. The *Drosophila* *dap* 3'UTR is repressed by miRNAs in the germline stem cells. (A) The control sensor lacks significant 3'UTR. The *dapL* and *dapS* sensors contain 866 bp and 630 bp of *dap* 3'UTR, respectively. Predicted miRNA response elements (MREs) are shown. (B) GFP expression was unchanged in *Dcr-1* germline stem cells (GSCs) (arrowhead) in the control sensor background as compared with the neighboring control GSC, but was upregulated in the *dapL* and *dapS* sensor lines. (C) The *dapL* and *dapS* sensors were upregulated in *Dcr-1* GSCs. Mean \pm s.e.; $n \geq 23$ pairs of GSCs. Student's *t*-test; ** $P < 0.01$.

dap, *dapF*, was fused downstream of a firefly luciferase gene. Partial precursor sequences of these miRNAs were cloned into an expression vector driven by a tubulin promoter. *miR-1* was used as a control as there is no predicted *miR-1* MRE in the *dap* 3'UTR. Whereas expression of *miR-7*, *miR-8*, *miR-278*, *miR-309* and *bantam* inhibited luciferase activity significantly, *miR-34*, *miR-289*, *miR-303* and *let-7* did not repress the activity (Fig. 2C). It is notable that *let-7* and *miR-289* increased luciferase activity. One possible explanation for this result is that the negative control miRNA, *miR-1*, might still mildly repress the *dap* 3'UTR even though there is no predicted MRE for *miR-1* and, therefore, *let-7* and *miR-289* increased luciferase activities after normalization to *miR-1*. It is also possible that *let-7* and *miR-289* increased the expression of luciferase through the *dap* 3'UTR (Vasudevan et al., 2007). Further experiments are required to test this hypothesis.

To test whether the predicted MREs are required for the repression by miRNAs, the sequences of putative MREs cognate to the miRNA seed regions were mutated individually to disrupt miRNA binding (Fig. 2B). The miRNA-dependent repression of luciferase activity was relieved significantly when the MRE site was disrupted for *miR-7*, *miR-278* or *miR-309*, but not for *bantam*. When the MRE site for *miR-8* was disrupted, the repression of luciferase activity was mildly relieved (Fig. 2C). These results suggest that *miR-7*, *miR-278* and *miR-309* can directly target the *dap* 3'UTR, whereas *miR-8* and *bantam* may either target the *dap* 3'UTR indirectly or through cryptic binding sites that are not predicted by the current algorithms. To test the cooperative effect of multiple miRNAs on the *dap* 3'UTR, we co-transfected multiple miRNA expression constructs with the *dap* 3'UTR luciferase reporter (see

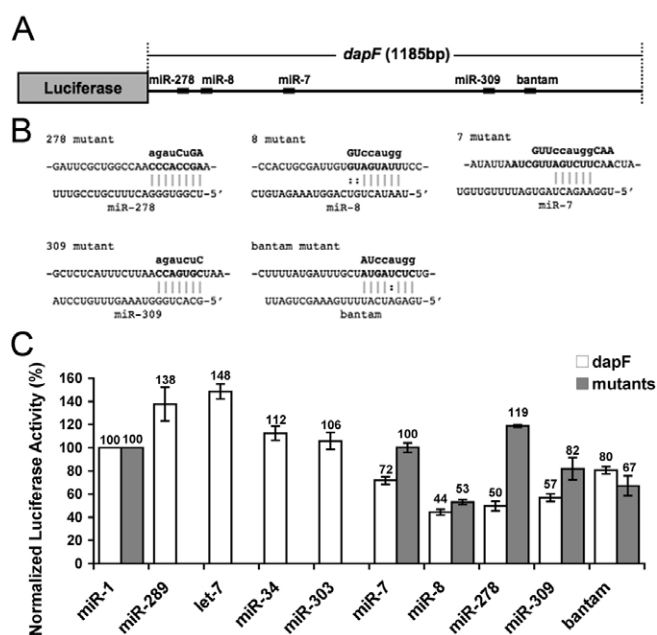


Fig. 2. The *dap* 3'UTR is targeted directly by *miR-7*, *miR-278* and *miR-309*. (A) The *luciferase-dapF* reporter consists of tubulin promoter-driven luciferase and the 3'UTR of *dap*. Predicted MREs are shown. (B) Mutations in the *dap* 3'UTR predicted MREs are shown in lowercase. miRNA/MRE complementarity is also shown. (C) Normalized luciferase activity upon co-expression of miRNAs. *miR-1* served as a control. *miR-7*, *-8*, *-278*, *-309* and *bantam* repress luciferase activity, and the predicted MREs for *miR-7*, *-278* and *-309* are required for repression. Mean \pm s.e. of at least three repeats.

Fig. S5 in the supplementary material). Expression of *miR-7*, *miR-278* and *miR-309* simultaneously repressed the luciferase activity significantly more than expression of *miR-278* alone, suggesting that multiple miRNAs might act on *Dap* expression.

***miR-278* and *miR-7* affect the cell cycle of GSCs**

We further examined whether *miR-278* and *miR-7* might regulate GSC division. Two *miR-278* null lines, *miR-278^{KO}* and *miR-278^{Gal4KI}*, were examined (Teleman et al., 2006). Quantitative RT-PCR (qPCR) was used to detect mature miRNAs in *FRT42D* control or *FRT42DmiR-278^{KO}* mutant ovaries. *miR-8* was used as a control miRNA as it is expressed in the GSCs (Shcherbata et al., 2007). *miR-278* expression was more than 300-fold higher in *FRT42D* control ovaries than in *miR-278^{KO}* ovaries. By contrast, the control miRNA *miR-8* was expressed at similar levels in the ovaries in both animals (Fig. 3A; see Table S1 in the supplementary material). These results show that *miR-278* is expressed in the ovary.

We generated GSC clones deficient for *miR-278* using the heat shock FLP-FRT system (Fig. 3B). GSCs can be identified by their position adjacent to the cap cells, and by the shape and position of the fusome stained by anti-Adducin (Add; Hu li tai shao – FlyBase) antibody (Fig. 3B). After generation of clones, the sum of cystoblasts and cysts generated by each mutant GSC in regions 1-2A of the germarium was counted and compared with that generated by the neighboring control GSCs (see Materials and methods). The *miR-278* mutant GSCs showed a 21-25% reduction in division index, whereas the *FRT42D* control clones divided normally (Fig. 3C; see Table S2 in the supplementary material). Similarly, GSCs in transheterozygous (*miR-278^{KO}/miR-278^{Gal4KI}*)

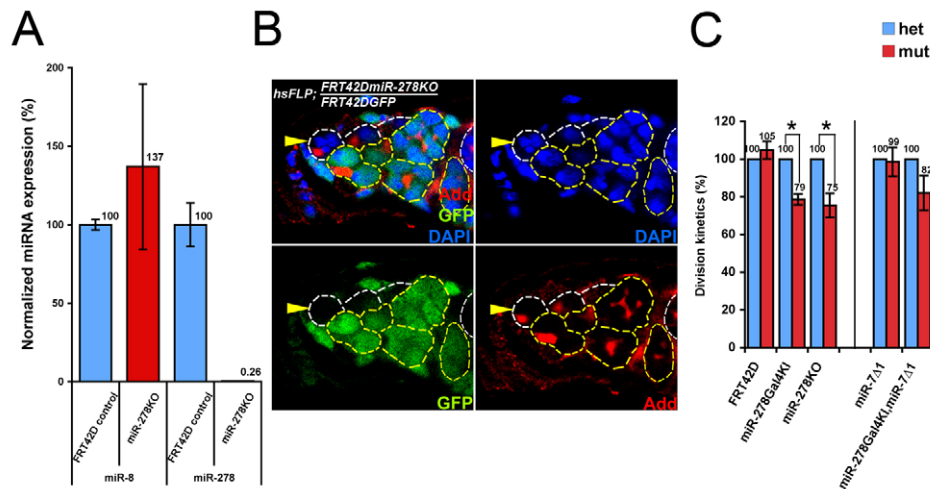


Fig. 3. *miR-278*-deficient GSCs proliferate more slowly than control GSCs. (A) qPCR analysis of the mature miRNA expression level of *miR-8* and *miR-278* in the whole ovary. RNA from *miR-278*^{KO} ovaries served as negative control for the expression of *miR-278*. The miRNA expression level is calculated from the cycle threshold (CT) (see Table S1 in the supplementary material) by $2^{CT(FRT42D\ control) - CT(miR-278KO)}$. Mean \pm s.e. for at least two repeats. (B) *miR-278*-deficient GSCs produce fewer progeny than control GSCs. A mutant GSC (arrowhead) and its progeny are marked by the lack of GFP. Add, Adducin. (C) The division kinetics of *miR-278* GSCs was reduced to 75-79% as compared with the neighboring heterozygous control GSCs or the *FRT42D* control GSC clones. Division kinetics of *miR-7* GSCs were unaffected by comparison with control. The division kinetics of GSCs mutant for both *miR-7* and *miR-278* were reduced to 82% as compared with the control. Mean \pm s.e. of three repeats. Student's *t*-test; **P* < 0.05. The number of homozygous GSCs counted: *hsFLP;FRT42D*, *n* = 32; *hsFLP;FRT42DmiR-278*^{KO}, *n* = 42; *hsFLP;FRT42DmiR-278*^{Gal4KI}, *n* = 35; *hsFLP;FRT42DmiR-7*^{Δ1}, *n* = 55; *hsFLP;FRT42DmiR-278*^{Gal4KI}; *miR-7*^{Δ1}, *n* = 41.

flies showed a 28% reduction in the number of cystoblasts and cysts at region 1-2A in germaria as compared with GSCs in heterozygous (*miR-278*^{KO/+}) animals 13-14 days after eclosion. These results suggest that *miR-278* plays a role in regulating the cell division of GSCs.

Since the *miR-7* mutant is homozygous lethal (Li and Carthew, 2005), qPCR analysis of homozygous *miR-7* mutant ovaries (comparable to the *miR-278* analysis shown in Fig. 3A) could not be performed. Therefore, in order to determine whether *miR-7* is expressed in the GSCs, we analyzed a sensor line with a constitutively driven GFP transgene bearing two perfectly matched target sites for *miR-7* (Li and Carthew, 2005; Stark et al., 2003). Compared with a control sensor driven by the same promoter but lacking miRNA complementarity, the GFP-*miR-7* sensor was strongly downregulated in the germline cells in the anterior of germaria, from the GSCs to region 3, as well as in the somatic tissue of the germarium (Fig. 4A). Furthermore, the GFP-*miR-7* sensor was upregulated in *Dcr-1* mutant GSCs in comparison with the heterozygous neighboring control GSCs (Fig. 4B). Image quantification analysis showed that the GFP level was 1.528 ± 0.065 -fold higher (mean \pm s.e. of three repeats; *n* = 71 germaria) in the *Dcr-1* mutant GSCs than in neighboring control (heterozygous) GSCs. These results showed that the *miR-7* sensor in the GSCs is responsive to *Dcr-1* activity, suggesting that *miR-7* is expressed in the GSCs.

To characterize the effect of *miR-7* on the GSC cell cycle, we examined the expression of the cell cycle marker Cyclin E (CycE) in *miR-7* mutant (*FRT42DmiR-7*^{Δ1}) GSCs (Li and Carthew, 2005). It has been shown that Dap can trap the CycE-Cdk2 complex in a stable but inactive form (de Nooij et al., 1996). Increased levels of Dap result in cell cycle arrest at the G1-S transition and prolonged expression of CycE protein (Shcherbata et al., 2004). We detected an increase in the frequency of CycE staining in *miR-7*^{Δ1} GSCs relative to the heterozygous neighboring control GSCs (Fig. 4C). However, the defects observed in the *miR-7* mutant GSCs were

insufficient to cause an obvious reduction in division index (Fig. 3C; see Fig. S1 and Table S2 in the supplementary material). Since *miR-7* resides in an intron of a host gene, *bancal* (Charroux et al., 1999), we asked whether the elevated frequency of GSCs positive for CycE was due to the loss of *bancal* or *miR-7*. Expressing *miR-7* with *UASp-miR-7* driven by a germline-specific *nanos-Gal4* driver returned the frequency of GSCs that stained positive for CycE to wild-type levels, thereby showing that it is the loss of *miR-7* and not *bancal* that is responsible for the increased frequency of CycE staining (Fig. 4C). We found further evidence that *miR-7* is sufficient to regulate Dap expression: follicle cell clones in stage 2-4 egg chambers overexpressing *miR-7* with an enhancer trap driver, *P{EP}bl^{EP954}* (Li and Carthew, 2005), exhibited a decreased frequency of Dap staining compared with wild-type control cells (Fig. 4D,E).

Although disruption of *miR-278* or *miR-7* showed mild cell division defects or abnormal cell cycle marker expression, respectively, neither alone nor in combination displayed as dramatic a perturbation of the cell cycle as *Dcr-1*-deficient GSCs (Fig. 3C). These results suggest that regulation of the GSC cell cycle might require miRNAs in addition to *miR-7* and *miR-278*, and possibly a combination of multiple miRNAs.

The *dap* 3'UTR is regulated by InR but not TGF- β signaling

GSCs, like many other stem cells, change their division rate in response to extrinsic factors such as nutrition-dependent InR and TGF- β signaling from the niche cells (LaFever and Drummond-Barbosa, 2005; Xie and Spradling, 1998). To analyze whether these signaling pathways affect cell division through miRNA-based Dap regulation, we tested the responsiveness of the GFP-*dap* 3'UTR sensor lines to both TGF- β and InR activity. Interestingly, the GFP intensity of the *dap* 3'UTR sensors *dapL* and *dapF* was upregulated in *InR* mutant GSCs (Fig. 5A). By contrast, the GFP intensity in

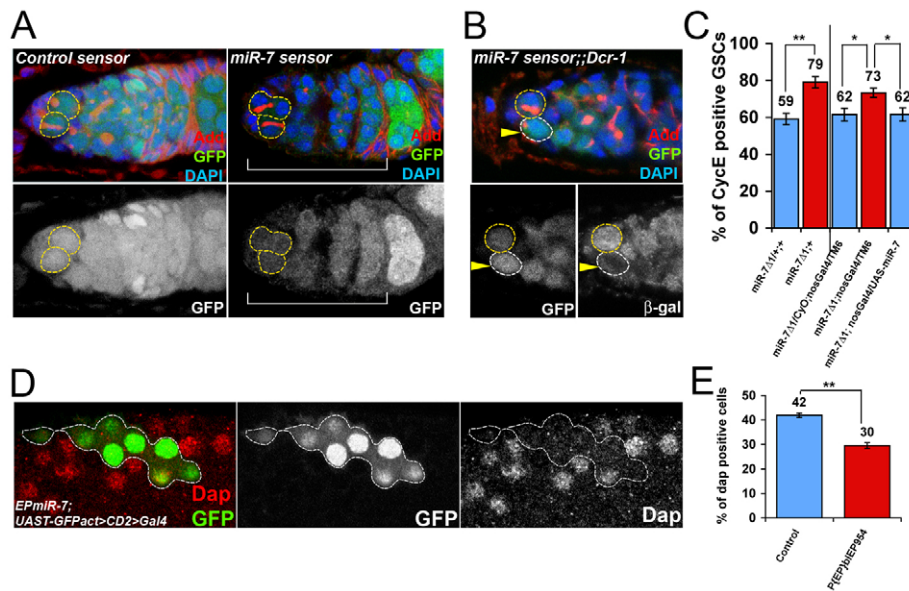


Fig. 4. *miR-7* expression in GSCs. (A) The GFP-*miR-7* sensor is repressed in the anterior-most germline (bracket), as compared with control GFP sensor lacking significant 3'UTR content. Adducin (Add), red; DAPI, blue; GFP, green. (B) *Dcr-1* GSC clones lacking β -gal (arrowhead) exhibit elevated GFP-*miR-7* sensor expression relative to neighboring heterozygous cells. (C) *miR-7^{Δ1}* GSC clones exhibit an elevated frequency of CycE-positive staining compared with heterozygous GSCs. Ectopic expression of *miR-7* in *miR-7^{Δ1}* mutant GSCs returns the frequency of CycE staining to near control levels. Mean \pm s.e. of three repeats. Student's *t*-test; **P*<0.05, ***P*<0.01. The number of GSCs counted: *FRT42DmiR-7^{Δ1}/CyO;nosGal4/TM6*, *n*=523; *FRT42DmiR-7^{Δ1};nosGal4/TM6*, *n*=87; *FRT42DmiR-7^{Δ1};nosGal4/UAS-miR-7*, *n*=110. (D,E) Ectopic expression of *miR-7* in the GFP-positive follicle cell clones (*y w hsFLP;EP[954];pUAST-GFP-act>CD2>Gal4*) decreases the frequency of Dap staining compared with the neighboring wild-type cells. Mean \pm s.e. of three repeats. Student's *t*-test; ***P*<0.01.

GSCs deficient for *Mad* or *punt*, two key components in the TGF- β pathway, was not affected in the GFP-*dap* 3'UTR sensor *dapL* (Fig. 5A,B). This result demonstrates that InR signaling, but not TGF- β signaling, regulates the *dap* 3'UTR, suggesting that InR signaling might affect Dap expression in GSCs through miRNAs. Quantitation showed that the GFP intensity of the *dapL* and *dapF* sensor in *InR* mutant GSCs increased 1.39-fold and 1.23-fold, respectively, in comparison with the neighboring control heterozygous GSCs (Fig. 5B). Interestingly, the GFP intensity of the *dapS* sensor was not significantly affected in *InR* mutant GSCs (Fig. 5A,B), suggesting that the InR-responsive *dap* 3'UTR region is absent from the *dapS* construct.

***InR*-deficient GSCs show abnormal cell cycle marker expression**

Since InR signaling regulates the *dap* 3'UTR in GSCs, we further investigated how InR signaling regulates the GSC cell cycle. Previous results have shown that disruption of InR signaling reduces the cell division of GSCs (LaFever and Drummond-Barbosa, 2005). We analyzed the cell cycle characteristics of GSC clones deficient for *InR* with several cell cycle markers (Fig. 6B; see Figs S2 and S3 in the supplementary material). In the *InR* mutant, there was a 2-fold increase in the frequency of CycE- and Dap-positive GSCs and 1.5-fold decrease in the frequency of CycB-positive GSCs (Fig. 6A). *FRT82B* control GSC clones showed a similar frequency of CycE, CycB and Dap staining as neighboring heterozygous GSCs (Fig. 6A). Based on the expression of these cell cycle markers, our results suggest that the cell cycle characteristics of *InR*-deficient GSCs are similar to those of *Dcr-1*-deficient GSCs and are consistent with our hypothesis that InR signaling may regulate the cell cycle through miRNAs and Dap in GSCs (Hatfield et al., 2005).

The *dap-5gm* construct does not respond to InR activity

As shown above, Dap expression, and specifically the *dap* 3'UTR, are responsive to InR activity in GSCs. To test whether other regions of the *dap* gene show responsiveness, we used *dap-5gm*, a genomic construct that contains the *dap* promoter region responsible for GSC expression followed by the *dap* coding sequence fused to six Myc-tag coding sequences and lacking most of the *dap* 3'UTR (Meyer et al., 2002; Hatfield et al., 2005). Expression of *dap-5gm* can be determined with an antibody against Myc. Expression of the Myc tag was the same in *InR*-deficient and neighboring control heterozygous GSCs (Fig. 6A; see Fig. S4 in the supplementary material). These data suggest that the regions of the *dap* gene contained in *dap-5gm*, including the GSC-specific promoter region, are not responsive to InR signaling, supporting our notion that InR regulation of Dap in GSCs occurs through the *dap* 3'UTR. Furthermore, in accordance with the *dap* 3'UTR sensor data (Fig. 6), the full-length *dap* 3'UTR is responsive to InR activity, whereas the *dap* 3'UTR region remaining in *dap-5gm* is not.

miRNAs and *dap* act downstream of InR in regulating cell division

To determine whether miRNAs are required for InR-dependent regulation of cell division, we used a protein-restricted diet to reduce InR signaling (Drummond-Barbosa and Spradling, 2001) and tested whether *Dcr-1* affected the phenotype. We generated GSC clones homozygous for the *Dcr-1* null allele (*Dcr-1^{Q1147X}*), as well as parental control *FRT82B*, during larval/pupal stages. Two days after eclosion, the adult flies were kept under two different diet conditions: rich food with wet yeast and poor food (starvation) without wet yeast. Whereas starvation reduced cell division of the

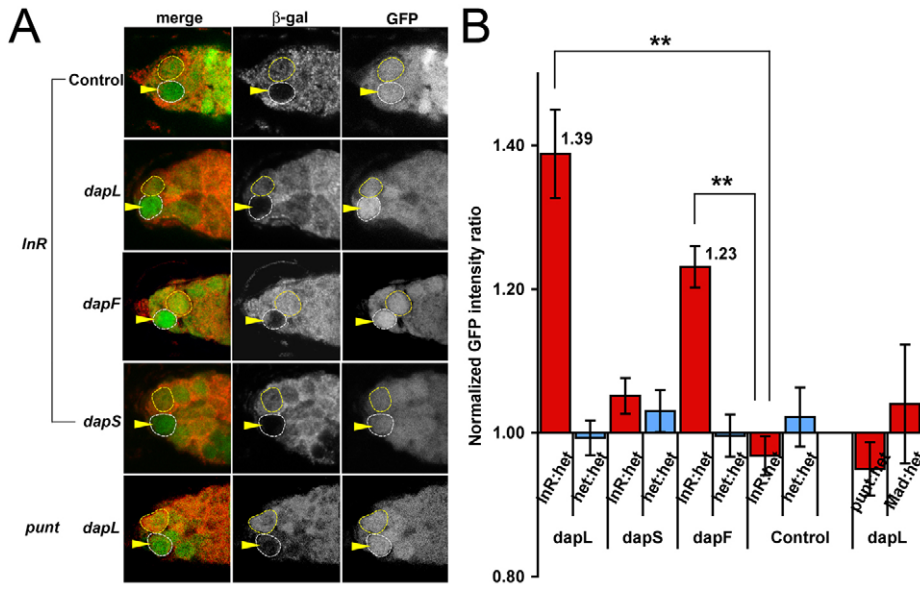


Fig. 5. The *dap* 3'UTR responds to InR but not TGF-β signaling in GSCs. (A) In *InR* mutant GSCs (first four rows, arrowhead), the GFP expression is upregulated in *dapL*, *dapF* and *dapS*, but not in the control sensor. In *punt* mutant GSCs (bottom row, arrowhead), the GFP expression in *dapL* is not upregulated. (B) Quantification of GFP intensity in *InR*, *punt* or *Mad* mutant GSCs compared with the neighboring heterozygous GSCs. The GFP intensity is upregulated in *InR* mutant GSCs with *dapL* and *dapF* by 1.39-fold and 1.23-fold, respectively. Mean±s.e. for all pairs of GSCs (at least 12 pairs for *InR^{ex52.1}* clones, five pairs for *punt¹³⁵* clones, and 24 pairs for *Mad¹²* clones). Student's *t*-test; ***P*<0.01.

control GSCs 1.7-fold, the low cell division index of the *Dcr-1*-deficient GSCs observed under rich food conditions did not change under poor food conditions (Fig. 7A). This result is consistent with our hypothesis that miRNAs act downstream of InR signaling in regulating cell division.

To test whether InR signaling acts through Dap in GSCs, we analyzed cell division kinetics under differing nutritional conditions for GSC clones homozygous for the *dap* null allele *dap⁴* (Lane et al., 1996), with *FRT42B* as a control. The GSC clones were generated in young adults and flies were subsequently housed under different dietary conditions. Whereas starvation reduced cell division of the control GSCs more than 2-fold, the cell division of the GSCs deficient for *dap* was not significantly reduced (Fig. 7B), suggesting that *dap* is required for the starvation-dependent reduction of the cell cycle.

To further examine the interaction between *dap* and InR signaling, we generated GSC clones deficient for *InR* in a *dap⁴* or *dap^{2x10}* heterozygous background. *InR* mutant GSCs show a strong cell division defect (Fig. 7C) (LaFever and Drummond-Barbosa, 2005). This defect can be partially rescued by reducing *dap*: the cell division index of *InR*-deficient GSCs increased from 40 to 60% when *dap* was reduced (*dap⁴*; a 1.5-fold increase) (Fig. 7C). Other strong *dap* mutant alleles provided similar results (*dap^{2x10}*; a 1.4-fold increase) (Fig. 7C, legend). These results suggest that *dap* acts downstream of *InR* in regulating the cell cycle. Together, these data support our hypothesis that the InR pathway regulates the GSC cell cycle by reducing the levels of Dap (Fig. 8).

DISCUSSION

Previous studies have shown that miRNAs may regulate the *Drosophila* CKI Dap, thereby controlling the cell division of GSCs (Hatfield et al., 2005). Here, we show that the *dap* 3'UTR directly responds to miRNA activities in GSCs. Using luciferase assays, we identify *miR-7*, *miR-278* and *miR-309* as miRNAs that can directly repress Dap through the *dap* 3'UTR in vitro. Although *miR-278* and *miR-7* play a role in regulating GSC division and cell cycle marker expression, respectively, neither of these mutants showed as dramatic a defect in the GSC cell cycle

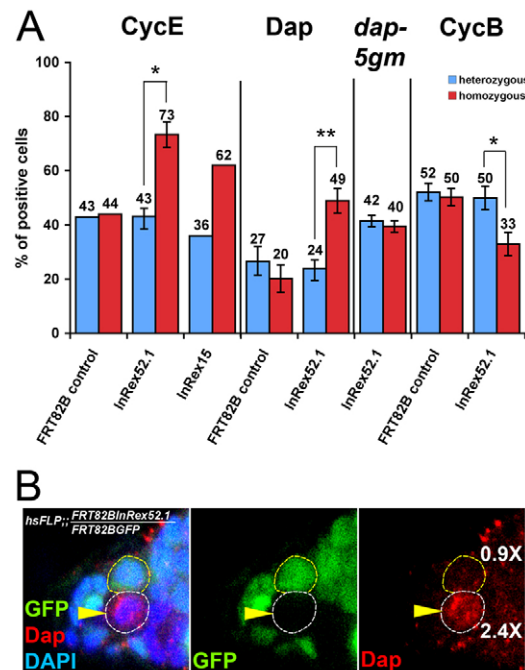


Fig. 6. *InR*-deficient GSCs show abnormal frequencies of cell cycle marker expression. (A) The percentage of CycE-positive GSCs in two different *InR* alleles (*hsFLP*; *FRT82B**InR^{ex52.1}*/*FRT82B**GFP* and *hsFLP*; *FRT82B**InR^{ex15}*/*FRT82B**GFP*) increased 1.7-fold as compared with the control neighboring GSCs. The percentage of Dap-positive GSCs increased 2-fold, whereas the percentage of CycB-positive GSCs decreased 1.5-fold in *InR* mutant GSCs. The percentage of *dap-5gm*-positive GSCs remained the same in *InR* mutant GSCs as in the control heterozygous neighboring GSCs. Flies were dissected 8 or 12 days after larval/pupal heat shock. Mean±s.e. of two to three repeats. Student's *t*-test; **P*<0.05, ***P*<0.01. The number of homozygous GSCs counted was as follows. For CycE staining: *FRT82B*, *n*=14; *InR^{ex52.1}*, *n*=58; *InR^{ex15}*, *n*=47. For Dap staining: *FRT82B*, *n*=58; *InR^{ex52.1}*, *n*=57. For CycB staining: *FRT82B*, *n*=71; *InR^{ex52.1}*, *n*=71. For *dap-5gm*: *InR^{ex52.1}*, *n*=43. (B) Dap expression in an *InR* mutant GSC (yellow arrowhead, expression 2.4-fold higher than background).

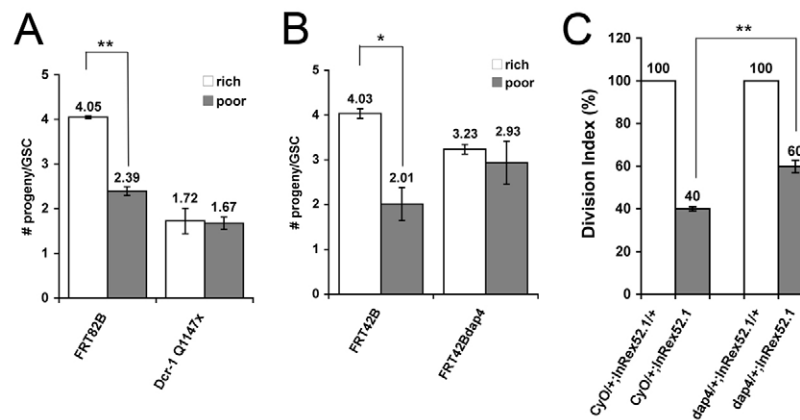


Fig. 7. miRNAs and *dap* act downstream of nutritional/InR signaling to regulate cell division. The average number of progeny in germarial region 1-2A counted under conditions of differing nutrition. (A) The cell division of *FRT82B* control GSCs was reduced dramatically under poor food as compared with rich food conditions, whereas the cell division of *Dcr-1* GSCs was not significantly reduced. (B) The cell division of control GSCs was dramatically reduced under poor food as compared with rich food conditions, whereas cell division of *dap* GSCs was not significantly reduced. The cell division of *dap⁴* GSCs was not significantly increased compared with *FRT42B* control GSCs in poor food conditions ($P=0.14075$, Student's *t*-test). (C) Reduction of *dap⁴* partially rescues cell cycle defects in *InR*-deficient GSCs. Other *dap* alleles gave similar results (*CyO/+;InR^{ex52.1}/+*, 50% division index; *dap²¹⁰/+;InR^{ex52.1}/+*, 68.75% division index). Mean \pm s.e. of three repeats. Student's *t*-test; * $P < 0.05$, ** $P < 0.01$. $n \approx 40$ per condition.

as *Dcr-1*-deficient GSCs (Hatfield et al., 2005). Thus, the *dap* 3'UTR may serve to integrate the effect of multiple miRNAs during cell cycle regulation. It remains possible that some miRNAs involved in this process remain to be identified. We further show that InR signaling controls the *dap* 3'UTR in GSCs. This led us to explore the interaction between InR signaling and miRNA/Dap cell cycle regulation. GSCs deficient for *InR* or *Dcr-1* show similar cell cycle defects. Using starvation to control InR signaling, we show that both *Dcr-1* and *dap* are required for proper InR signaling-dependent regulation of GSC division. Further, reduction of *dap* partially rescues the cell division defect of the *InR* mutant GSCs, suggesting that InR signaling regulates the cell cycle via Dap. Our results suggest that miRNAs and Dap act downstream of InR signaling to regulate GSC division (Fig. 8).

miRNAs target the *dap* 3'UTR

Our data suggest that multiple miRNAs can regulate the 3'UTR of *dap*: *miR-7*, *miR-278* and *miR-309* can regulate the *dap* 3'UTR directly, whereas *bantam* and *miR-8* may regulate it indirectly, or through cryptic MREs in the *dap* 3'UTR. Using GFP sensor assays, we also show that the *dap* 3'UTR may be directly regulated by miRNAs in the GSCs in vivo. However, which specific miRNAs control endogenous Dap levels in *Drosophila* GSCs remains unknown. Mammalian p21^{kip1} has also been shown to be a direct target for specific miRNAs of the *miR-106* family, including *miR-290s* and *miR-372* (Ivanovska et al., 2008; Sinkkonen et al., 2008). Further, the mouse *miR-290* family has recently been identified as regulating the G1-S transition (Wang et al., 2008). In addition, *miR-221* and *miR-222* have been shown to regulate p27^{kip1}, thereby promoting cell division in different mammalian cancer cell lines (Galardi et al., 2007; le Sage et al., 2007; Visone et al., 2007). Neither the *miR-290* nor *miR-220* family is conserved in *Drosophila*. Together, these results indicate that the CKIs (Dap) might be a common target for miRNAs in regulating the cell cycle in stem cells. However, the specific miRNAs that regulate the CKIs might vary between organisms.

miR-7 and *miR-278*

Our study reveals novel regulatory roles for *miR-7* and *miR-278* in the GSC cell cycle. We have shown by luciferase assays that *miR-7* and *miR-278* can directly target Dap. GSCs deficient for *miR-278* show a mild but significant reduction in cell proliferation. Ectopic expression of *miR-7* in follicle cells reduces the proportion of cells that stain positive for Dap. Furthermore, ablation of *miR-7* in GSCs results in a perturbation of the frequency of CycE-positive GSCs. However, the cell division kinetics of *miR-7* mutant GSCs is not reduced, by contrast with the dramatic reduction of cell division in *Dcr-1*-deficient GSCs. It is plausible that *miR-7* and *miR-278* act in concert with other miRNAs to regulate the level of Dap in GSCs and thereby contribute to cell cycle control in GSCs. Recently, the 3'UTR of *nerfin-1*, a *Drosophila* zinc-finger transcription factor gene required for axon pathfinding, has been shown to be regulated by multiple miRNAs in the developing nervous system (Kuzin et al.,

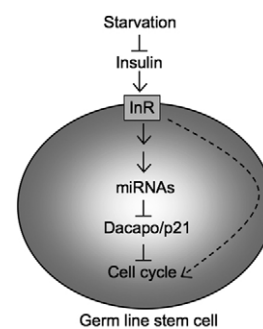


Fig. 8. A model for activation of miRNAs by InR signaling, which inhibits Dap expression and accelerates *Drosophila* GSC division. Reduced InR signaling reduces the levels of miRNAs that repress Dap. Therefore, Dap is upregulated and the cell cycle slows in GSCs. It is also possible that InR signaling regulates GSC division by additional mechanisms (dashed arrow).

2007). Although we have shown that the *dap* 3'UTR lies downstream of the miRNA pathway, it is still possible that some miRNAs control Dap expression indirectly.

The interaction of multiple miRNAs with the *dap* 3'UTR might integrate information from multiple pathways. Further studies will reveal what regulates *miR-7* and *miR-278* expression in GSCs and which other miRNAs might act together in Dap regulation. It is known that *miR-7* and the transcriptional repressor Yan (Anterior open – FlyBase) mutually repress one another in the eye imaginal disk (Li and Carthew, 2005). In this model, Yan prevents transcription of *miR-7* until Erk in the Egfr pathway downregulates Yan activity by phosphorylation, thereby permitting expression of *miR-7*. Conversely, *miR-7* can repress the translation of Yan. Thus, a single pulse of Egfr signaling results in stable expression of *miR-7* and repression of Yan. Whether similar regulation will be observed between *miR-7* and the signaling pathways that regulate GSC division remains to be seen. It has been suggested that *miR-7* might regulate downstream targets of Notch, such as *Enhancer of split* and *Bearded* (Stark et al., 2003). Thus, *miR-7* may have a mild repressive effect on multiple targets in GSCs. Further experiments might illuminate this possibility.

miR-278, on the other hand, has been implicated in tissue growth and InR signaling (Teleman et al., 2006). Overexpression of *miR-278* promotes tissue growth in eye and wing imaginal discs. Deficiency of *miR-278* leads to a reduced fat body, which is similar to the effect of impaired InR signaling in adipose tissue. Interestingly, *miR-278* mutants have elevated insulin/Dilp production and a reduction of insulin sensitivity. Furthermore, *miR-278* regulates *expanded*, which may modulate growth factor signaling including InR. Since InR signaling plays important roles in tissue growth and cell cycle control (Edgar, 2006; Taguchi and White, 2008; Wu and Brown, 2006), it will be interesting to further test how *miR-278* may regulate InR signaling, and whether InR signaling might regulate *miR-278* in a feedback loop in GSCs.

Other miRNAs or miRNA-dependent mechanisms might also play roles in *Drosophila* GSCs. For example, the miRNA *bantam* is required for GSC maintenance (Shcherbata et al., 2007). A recent study has shown that the Trim-NHL-containing protein Mei-P26, which belongs to the same family as Brain tumor (Brat), affects *bantam* levels and restricts cell growth and proliferation in the GSC lineage (Neumuller et al., 2008). Interestingly, most miRNAs are upregulated in *mei-P26* mutant flies. By contrast, overexpression *mei-P26* in *bag of marbles* (*bam*) mutants broadly reduces miRNA levels. This suggests that Mei-P26 regulates proliferation and maintenance of GSC lineages via miRNA levels. Since InR signaling cell-autonomously regulates GSC division but not maintenance, the possible interaction between Mei-P26 and InR signaling might be complex.

InR signaling regulates Dap and the cell cycle cell-autonomously

The systemic compensatory effect of insulin secretion in mammals with defective InR signaling is well documented. Insulin levels in mice with liver-specific *InR* (*Insr* – Mouse Genome Informatics) knockout are ~20-fold higher than those of control animals owing to the compensatory response of the pancreatic β -cells and impairment of insulin clearance by the liver (Michael et al., 2000). Knockout of the neuronal *InR* also leads to a mild hyperinsulinemia, indicating whole-body insulin resistance (Bruning et al., 2000). Furthermore, the knockout of components in the InR signaling pathway, such as Akt2 and the regulatory and catalytic subunits of PI3 kinase, also leads to hyperinsulinemia and glucose intolerance

(Brachmann et al., 2005; Cho et al., 2001; MacDonald et al., 2004; Ueki et al., 2002). Therefore, a systemic decrease in InR signaling may lead to compensatory responses.

To understand the roles of InR signaling in the GSCs while avoiding any systemic compensatory effect we analyzed the phenotypes of GSC clones. Using a panel of cell cycle markers, we find that *InR* mutant GSCs show cell cycle defects similar to those of *Dcr-1* mutant GSCs: a reduction of cell division rate, an increased frequency of cells staining positive for Dap and CycE, and a decreased frequency of cells staining positive for CycB. Using GFP-*dap* 3'UTR sensors, we show that the *dap* 3'UTR responds to InR signaling in GSCs, suggesting that InR signaling can regulate Dap expression through the *dap* 3'UTR. This, together with our genetic data indicating that InR/starvation-dependent cell cycle regulation requires *Dcr-1* and *dap*, led us to propose the hypothesis that InR signaling regulates the cell cycle through miRNAs that further regulate Dap levels (Fig. 8). Since a reduction in *dap* only partially rescues the cell cycle defects of *InR* mutant GSCs, it is possible that InR signaling might also regulate GSC division by additional mechanisms (Fig. 8, dashed arrow).

Starvation, InR signaling and cell cycle control

InR signaling regulates the cell cycle through multiple mechanisms, mainly through the G1–S, but also partly through the G2–M, transition. Recent work has shown a delay in the G2–M transition in GSCs during *C. elegans* dauer formation (Narbonne and Roy, 2006). Starvation and *InR* deficiency may also affect the G2–M checkpoint in *Drosophila* GSCs (Hsu et al., 2008). Here we dissect one possible molecular pathway that InR signaling utilizes to regulate the *Drosophila* GSC G1–S transition and show that InR signaling can control the cell cycle through miRNA-based regulation of Dap.

Many studies have connected InR and CKIs to Tor (Target of rapamycin) or Foxo pathways downstream of InR signaling. In *S. cerevisiae*, the yeast homolog of p21/p27 is upregulated when Tor signaling is inhibited (Zinzalla et al., 2007). Foxo, a transcription factor that can be repressed by InR signaling, is known to play important roles in nutrition-dependent cell cycle regulation by upregulating p21 and p27 (Medema et al., 2000; Nakae et al., 2003; Seoane et al., 2004) and by repressing cyclin D1/D2 (Park et al., 2005; Schmidt et al., 2002). In *C. elegans*, starvation causes L1 cell cycle arrest mediated by InR (*daf-2*) and Foxo (*daf-16*): InR represses the function of Foxo, thereby downregulating the CKI (*cki-1*) and upregulating the miRNA *lin-4* (Baugh and Sternberg, 2006). We have now shown that a miRNA-based regulation of Dap can be coordinated by InR in *Drosophila* GSCs.

Insulin and insulin-like growth factors (Igf1 and Igf2) are known to play important roles in regulating metabolic and developmental processes in many stem cells (Mourkioti and Rosenthal, 2005; Saltiel and Kahn, 2001; Ye and D'Ercole, 2006). In mammals, Igf signaling is required by different stem cell types, including human and mouse ES cells for survival and self-renewal (Bendall et al., 2007; Hallmann et al., 2003; Rubin et al., 2007; Wang et al., 2007), neural stem cells for expediting the G1–S transition and cell cycle re-entry (Hodge et al., 2004), and skeletal muscle satellite cells for promoting the G1–S transition via p27^{kip1} downregulation (Chakravarthy et al., 2000). Here we have dissected the molecular mechanism of the InR pathway in another adult stem cell type, *Drosophila* GSCs, showing that InR signaling can regulate stem cell division through miRNA-based downregulation of the G1–S inhibitor Dap. Further studies will reveal whether miRNAs also mediate InR signaling in other stem cell types.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/9/1497/DC1>

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Table S1. Cycle threshold (CT) of qPCR detecting expression levels of mature miRNAs *miR-8* and *miR-278*

Cycle threshold	<i>miR-8</i>	<i>miR-278</i>
<i>FRT42D</i>	22.95±0.039	30.64±0.156
<i>FRT42DmiR-278^{KO}</i>	22.55±0.569	39.22±0.390*

Mean±s.e. is shown.

*The maximum PCR cycle is 40. If the fluorescent signal of the PCR products is not higher than the threshold after 40 cycles, the CT is assigned as 40.

Table S2. Number of progeny generated by each GSC in regions 1-2A of germarium and number of progeny produced by the heterozygous neighboring control GSC

Genotypes (days after larval/pupal heat shock)	Cysts/GSC*
<i>FRT42D/FRT42DGFP</i> (14 d)	3.45±0.27
<i>FRT42D/FRT42D</i> (14 d)	3.60±0.14
<i>FRT42DmiR-278^{KO}/FRT42DGFP</i> (14 d)	3.29±0.09
<i>FRT42DmiR-278^{KO}/FRT42DmiR-278^{KO}</i> (14 d)	2.47±0.15
<i>FRT42DmiR-278^{Gal4K1}/FRT42DGFP</i> (14 d)	3.38±0.02
<i>FRT42DmiR-278^{Gal4K1}/FRT42DmiR-278^{K1Gal4}</i> (14 d)	2.66±0.11
<i>FRT42DmiR-7^{Δ1}/FRT42DGFP</i> (8 d)	4.40±0.11
<i>FRT42DmiR-7^{Δ1}/FRT42DmiR-7^{Δ1}</i> (8 d)	4.33±0.29
<i>CyO/+;FRT82BlnR^{ex52.1}/+</i> (8 d)	4.21±0.16
<i>CyO/+;FRT82BlnR^{ex52.1}/FRT82BlnR^{ex52.1}</i> (8 d)	1.68±0.09
<i>FRT42Bdap⁴/+;FRT82BlnR^{ex52.1}/+</i> (8 d)	3.95±0.13
<i>FRT42Bdap⁴/+;FRT82BlnR^{ex52.1}/FRT82BlnR^{ex52.1}</i> (8 d)	2.37±0.20

*Mean±s.e. of three repeats. At least ten mosaic germaria were counted in each repeat.