MicroRNA Transgene Overexpression Complements Deficiency-Based Modifier Screens in *Drosophila*

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ABSTRACT Dosage-sensitive modifier screening is a powerful tool for linking genes to biological processes. Use of chromosomal deletions permits sampling the effects of removing groups of genes related by position on the chromosome. Here, we explore the use of inducible microRNA transgenes as a complement to deficiency-based modifier screens. miRNAs are predicted to have hundreds of targets. miRNA overexpression provides an efficient means to reduces expression of large gene sets. A collection of transgenes was prepared to allow overexpression of 89 miRNAs or miRNA clusters. These transgenes and a set of genomic deficiencies were screened for their ability to modify the bristle phenotype of the cell-cycle regulator *minus*. Sixteen miRNAs were identified as dominant suppressors, while the deficiency screen uncovered four genomic regions that contain a dominant suppressor. Comparing the genes uncovered by the deletions with predicted miRNA targets uncovered a small set of candidate suppressors. Two candidates were identified as suppressors of the *minus* phenotype, *Cullin-4* and *CG5199*/*Cut8*. Additionally, we show that *Cullin-4* acts through its substrate receptor *Cdt2* to suppress the *minus* phenotype. We suggest that inducible microRNA transgenes are a useful complement to deficiency-based modifier screens.

GENETIC modifier screens have proven to be a powerful means with which to identify genes in a common biological process. Modifier screens can be carried out with high efficiency by chemical mutagenesis, but recombination mapping to identify the affected loci can be tedious. In *Drosophila*, a popular alternative has been to use a collection of deletion mutant strains known as the "deficiency kit" (St Johnston 2002), which consists of mapped chromosomal deletions that remove large blocks of genes. This approach allows sampling of the effects of removing one copy each of hundreds of genes in a single genetic cross. The deficiency kit currently available from the Bloomington Drosophila Stock Center covers 97.8% of annotated euchromatic genes (Cook *et al.* 2010). The advantage of the approach is that screens for autosomal modifiers can be performed with fewer than 400 crosses. An offsetting disadvantage is that genetic heterogeneity in these strains can affect the results.

In this context we decided to explore the possibility of using microRNAs (miRNAs) as an alternative means of downregulating hundreds of genes concurrently. miRNAs are small noncoding RNAs that post-transcriptionally silence gene expression. Target prediction algorithms suggest the existence of hundreds of targets per miRNA (reviewed in Bartel 2009; Thomas *et al.* 2010), recently including sites in protein-coding regions of the genes (Schnall-Levin *et al.* 2010). miRNA overexpression can cause simultaneous reduction of the expression levels of hundreds of genes (Lim *et al.* 2005; Easow *et al.* 2007; Baek *et al.* 2008; Selbach *et al.* 2008). Potential advantages of the use of miRNAs include the following:

1. miRNA targets may allow access to genes not covered by the deficiency kit, as well as facilitating access to genes on the X chromosome. There are 176 annotated *Drosophila* miRNAs (Kozomara and Griffiths-Jones 2011).
2. Overexpression studies can be conducted in a spatiotemporally controlled manner through the use of the GAL4–UAS system (Brand and Perrimon 1993). Potential drawbacks of the miRNA approach include: (a)

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miRNAs do not target all protein coding genes and (b) miRNAs do not regulate those they target with equal efficiency.

3. Identification of biologically significant targets is imperfect due to limitations in target prediction algorithms.

To explore the potential of the miRNA-based screening approach we made use of the *minus* mutant, which exhibits a "small bristle" phenotype that is sensitive to genetic background (Szuplewski *et al.* 2009). *Drosophila* adult mechanosensory bristles have been used as a model system to study cell determination and asymmetric cell division (Abdelilah-Seyfried *et al.* 2000; Mummery-Widmer *et al.* 2009). Each bristle is composed of four cells: the neuron, its sheath cell, and two external cells forming the shaft and socket. Growth of the outer cells requires endoreplication, a variant cell cycle in which cells become polyploid through DNA replication without subsequent cell division (Weng *et al.* 2003). Therefore, bristles provide a model to study endoreplication. We reported previously that Minus influences cyclin E degradation and that reduction of *Cyclin E* gene dosage could rescue the *minus* mutant bristle endoreplication defect (Szuplewski *et al.* 2009). This phenotype does not affect viability or fertility of the fly, so *minus* mutants provide a suitable genetic background for modifier screens to identify regulators of the endocycle.

Material and Methods

Molecular biology

pUAST.attB-SLIC (sequence and ligation-independent cloning) and pUASP.attB-SLIC were produced as follows: Two synthetic oligonucleotides (SLIC-fw, 5′-GCGGCCGCATGTTTAAACGCTTAATTAAGCCCTAGGGCTAGCGGCCGGCCGGCGCGCCATACCGGTGCATTTAAATCGTCTAGA-3′, and SLIC-rv, 5′- TCTAGACGATTTAAATGCACCGGTATGGCGCGCCGGCCGGGCCGCTAGCCCTAGGGCTTAATTAAGCGTTTAAACATGCGGCCGC-3′) were annealed, treated with *Taq* polymerase for 15 min at 72°, and cloned into PCR2.1-TOPO. The insert (SLIC-linker) was excised using *Not*I/*Xba*I and ligated into *Not*I/*Xba*I-digested pUASTattB (Bischof *et al.* 2007) to generate pUAST.attB-SLIC and into *Not*I/*Xba*I-digested pUASP2 (Rorth 1998) to generate pUASP-SLIC. The pUASP-cassette was PCR amplified from pUASP-SLIC (forward primer, 5′−CTAGCGGATCCGGGAATTGGGAATTCTAGAATTGGCCGCTCTAGCC-3′; reverse primer, 5′-AGTGGATCTCTAGAGGTACCCTCGAGTCCGTGGGGTTTGAATTAAC-3′) and inserted into *Eco*RI/*Xho*I-digested pattB using the SLIC to generate pUASP.attB-SLIC (Bischof *et al.* 2007; Li and Elledge 2007). To generate UAS–microRNA overexpression vectors, genomic fragments containing the miRNA stem loop plus ~100 nucleotides upstream and downstream were PCR amplified using primers with extensions homologous to the SLIC-linker sequence (extension of the forward primer, 5′GTTTAAACGCTTAATTAAGCCCTAGG-3′; extension of the reverse primer, 5′- CGATTTAAATGCACCGGTATGGCGCGCC-3′) and subsequently SLIC recombined (Li and Elledge 2007) into *Avr*II/*Asc*I-digested pUAST.attB-SLIC and pUASP.attB-SLIC to generate RT–miR and RP–miR transgenes, respectively. Primer sequences are available upon request. The subset of miRNAs chosen for the miRNA overexpression library is in Supporting Information, Table S1. This collection was completed by cloning some additional miRNAs in pUAS-T-DSred2 for random insertion into fly genome (Table S1; Stark *et al.* 2003).

The *Cul-4* 3′-UTR luciferase reporter was generated by cloning the 551-bp 3′-UTR from *Cul-4* into a vector directing firefly luciferase under control of the tubulin promoter. The *Cul-4* 3′-UTR reporter with *miR-5* sites mutated was generated by PCR amplification using primers designed to incorporate the mutations. miRNA overexpression vectors for S2 cell transfections were generated by cloning genomic DNA fragments containing the *miR-5* hairpin into pJB25 (*tub* > *MCS_SV40 polyA*) (Stark *et al.* 2005) to generate *pJB25-miR-5*. The UAS–Cul-4 transgene lacking the 3′-UTR binding site was generated by PCR amplification using the following primers: aaaaagcggccgcGGCGTCGAAGGAACCCCA and tttg gtaccTCGGTGTTCGGATTTCAC.

Drosophila stocks and genetics

The following stocks and all the deficiencies were obtained from Bloomington Stock Center: $CycE^{Ar95}$, *UAS–GFP*, *sca–GAL4*, *pnr–GAL4*, $Cul\text{-}4^{KG02900}$ (Table 1 and Table S2). *UAS–RNAi-Rac2* (construct 17536), *UAS–RNAi-CG5199* and *UAS–RNAi-minus* lines were from the Vienna Drosophila RNAi center. $Cul4^{11L}$, $Cul4^{6AP}$ were provided by R. J. Duronio (Hu *et al.* 2008) and $Cul4^{G1\text{-}3}$, $Cul4^{L2\text{-}1}$, $Cul4^{G3\text{-}5}$ by C.-T. Chien (Lin *et al.* 2009). $l(2)dtl^{c02261}$ was from the Exelixis Collection at Harvard Medical School. *11295R-1* and *11295R-4* were from NIG–FLY. Transgenic strains were prepared carrying RT–miR and RP–miR constructs integrated into defined landing-sites (see Table S2 for insertion sites), all obtained from the Bloomington Stock Center. To perform the screen, at least 5 males from each *UAS–miRNA* line were crossed to at least 10 *sca–GAL4*, *UAS–RNAi-mi/CyO* virgin females. All crosses were performed at 25°. Phenotypes were screened in female flies, which are larger and have longer bristles. A deficiency or miRNA overexpression transgene was scored as a suppressor when at least 50% of the progeny had obviously wild-type or near wild-type bristles.

Quantitative PCR (qPCR)

Ten pairs of salivary glands were dissected from prewandering third instar larvae expressing either an UAS–GFP or an UAS–miR-4-5 transgene driven by *patched-Gal4* (*ptc-Gal4*). RNA was isolated using TRIZOL reagent (Invitrogen) and DNaseI treated (Promega) using manufacturer's recommendations. After quantification, 800 ng total RNA per sample was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). qPCR was performed on two biological replicates with three independent primer pairs covering

Table 1 Deletions used to locate dominant suppressor of *minus* loci

Region	Deficiency	Breakpoints	Suppression
35D1; 35E2	Df(2L)TE35BC-24	35B4; 35E2	*Su(mi)*
	Df(2L)r10	35D1; 36A6–7	*Su(mi)*
44A4; 44C1	Df(2R)cn9	42E; 44C1	No suppression
	Df(2R)H3C1	43F; 44D8	*Su(mi)*
	Df(2R)H3E1	44D1; 44F12	No suppression
	Df(2R)BSC267	44A4; 44C4	*Su(mi)*
59D11; 59F8	Df(2R)vir130	59B; 59D8–E1	No suppression
	Df(2R)or-BR6	59D5; 60B8	*Su(mi)*
	Df(2R)Chig230	60A3–60A7; 60B4	No suppression
	Df(2R)bw-HB132	59D11; 59F8	*Su(mi)*
77C3; 77D1	Df(3L)ED4858	76D3; 77C1	No suppression
	Df(3L)rdgC-co2	77A1; 77D1	*Su(mi)*
	Df(3L)ri-79c	77B–C; 77F–78A	No suppression
	Df(3L)BSC796	77C3; 77E4	*Su(mi)*

different exon junctions in *cullin-4* mRNA and *RP49*-specific primers for normalization.

Cell transfection and luciferase assays

S2 cells were transfected in 24-well plates with 200 ng empty pJB25 or *pJB25-miR-5* vector, 80 ng firefly luciferase with or without intact or mutant *Cul-4* 3′−UTR, and 80 ng Renilla luciferase DNA as transfection control. Dual luciferase assays were performed 60 hr post-transfection with the Dual-Glo luciferase kit (Promega).

Results

Generation of UAS–miRNA strains

To generate a library of fly strains for conditional overexpression of microRNAs under GAL4 control, we assembled two vectors suitable for recombination-based SLIC cloning (Li and Elledge 2007): pUAST.attB-SLIC and pUASP.attB-SLIC (Figure 1). Both vectors allow site-specific integration into the genome (Bischof *et al.* 2007) and contain a common SLIC polylinker for recombination-based cloning. In pUAST.attB-SLIC, the polylinker is flanked by the *hsp70* promoter and the SV40 3′-UTR. Because pUAST-based constructs express poorly in the germ line, pUASP.attB-SLIC was based on the germ line-competent pUASP vector (Rorth 1998). Genomic fragments spanning miRNA hairpins by ∼100 bp upstream and downstream were amplified and cloned into the UAS vectors to generate RT–miR and RP–miR plasmids (Table S1). The *UAS–miRNA* collection includes several lines described previously and others cloned into pUAST-DsRed2 (Stark *et al.* 2003) for random insertion into the genome. In total 109 miRNAs were overexpressed (Table S1).

Characterization of a sensitive minus background

minus mutants show a reduction in the size of the large thoracic mechanosensory bristles (Szuplewski *et al.* 2009). *Cyclin E* (*CycE*) mutants behave as dominant suppressors of the *minus* bristle phenotype in that they restore bristle size to normal in a *minus* mutant background. We designed a genetic modifier screen to identify suppressors with the aim of uncovering regulators of endoreplication. To simplify the screen, we sought to reduce *minus* mRNA levels by expression of a UAS–RNAi transgene. This would allow a F1 screens for the effects of coexpressing UAS–miRNA transgenes or introduction of a genomic deficiency. Expression of *UAS–RNAi-minus* under control of *scabrous-GAL4* (*scaG4*) or *pannier-GAL4* (*pnrG4*) (Klaes *et al.* 1994; Calleja *et al.* 1996) recapitulated the *minus* mutant phenotype (Figure 2, A, B, D, and E). In both combinations, removing one copy of *CycE* suppressed the RNAi phenotype, as observed previously with the *minus* mutant (Compare Figure 2, B, C, E, and F). On this basis, we chose the *scaG4 > UAS–RNAi-mi* background for an F1 miRNA modifier screen.

Results of the screens

We first screened 111 deletions on the second chromosome and 139 deletions on the third chromosome (Table S2). Four chromosomal regions that suppressed the *minus* bristle phenotype were uncovered. Table 1 shows the deletions that were used to narrow down the locations of the suppressor loci. On the second chromosome two newly identified loci were located in the intervals 44A4–44C1 and 59D11–59F8, in addition to the region where the previously identified suppressor, *CycE*, is located (35D1–35E2). One new suppressor was identified in the interval 77C3–77D1 on the third chromosome.

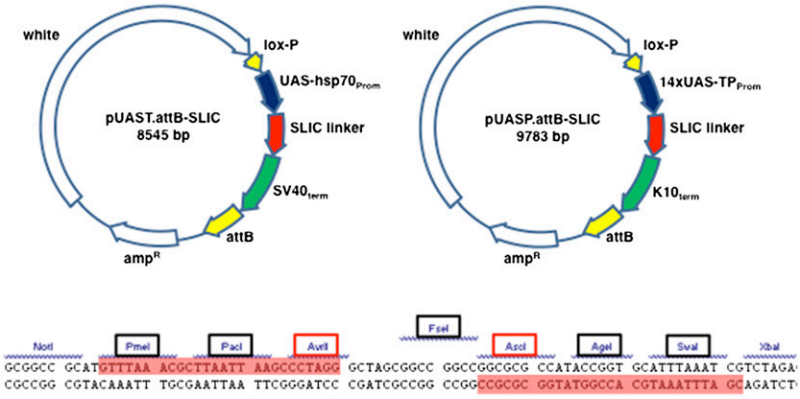

Figure 1 The miRNA overexpression vectors. Schematic representation of pUAST.attB-SLIC for somatic miRNA overexpression and pUASP.attB-SLIC optimized for germ line miRNA overexpression. The sequence of the SLIC linker is indicated below. All restriction sites of the SLIC linker are unique in pUAST.attB-SLIC. Unique sites for both vectors are boxed. When *Avr*II and *Asc*I are used to linearize the vectors for SLIC cloning (Li and Elledge 2007), the sequences marked in red should be added to the forward (*Avr*II) and reverse (*Asc*I) primers.

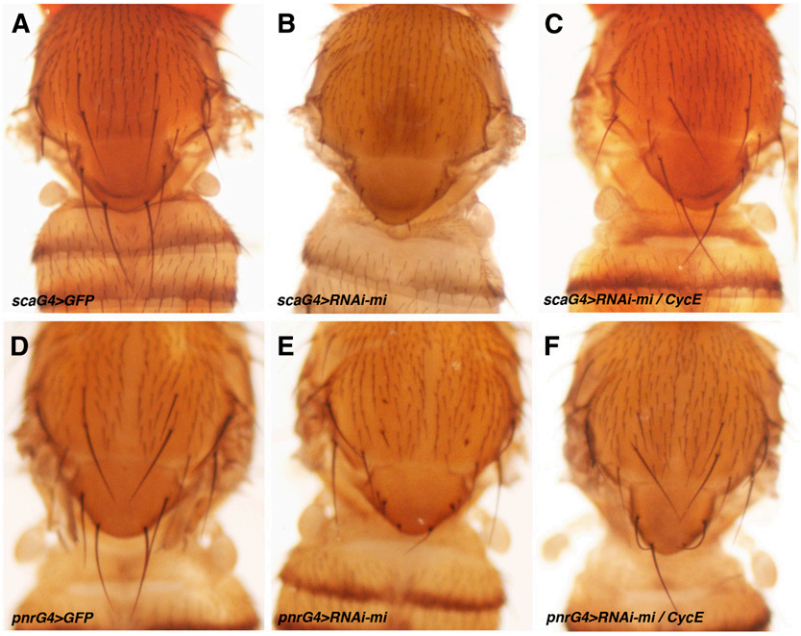

Figure 2 *minus* RNAi phenotype sensitive to *CycE* dosage. Images of the dorsal thorax of adult female flies of the indicated genotypes. (A) *sca–GAL4/UAS–GFP, scaG4* control. (B) *sca–GAL4, UAS–RNAi-mi/+* caused a size reduction of thoracic macrochaete compared to control. (C) *sca–GAL4, UAS–RNAi-mi/CycE^{Ar95}*: reduction of cyclin E gene dosage rescued the RNAi phenotype. (D) *pnrG4* control: *UAS–GFP/+; pnr–GAL4/+* (E) *pnr–GAL4: UAS–RNAi-mi/+* caused a size reduction of thoracic macrochaete compared to control. (F) *CycE^{Ar95}/+; pnr–GAL4, UAS–RNAi-mi/+*: reduction of cyclin E rescued the phenotype.

The UAS–miRNA screen identified 13 transgenes that suppressed the *minus* phenotype (Table 2 and Table S1). Members of the *miR-2* seed family figure prominently in this group. The *miR-2* family consists of 8 genes at six loci (Table 2). All six loci proved to be effective suppressors of *minus* when the miRNAs were overexpressed (Table 2 and Figure 3, A–D). Overexpression of the *miR-4-5* cluster, *miR-284*, *miR-929*, or *miR-986* also suppressed the *minus* phenotype (Figure 3, E–H).

In addition to modifiers of the *UAS–RNAi-mi* phenotype this screen revealed bristle phenotypes associated with miRNA overexpression that were unrelated to the sensitized background (Table S1). Overexpression of *miR-2* family miRNAs resulted in occasional additional bristles, as reported previously (Lai *et al.* 2005). This phenotype was suggested to reflect regulation of Notch activity and asymmetric division of the progenitors, a process distinct from endoreplication. Similarly, overexpression of *miR-7* and *bantam* have been previously reported to result in additional bristles (Abdelilah-Seyfried *et al.* 2000; Lai *et al.* 2005). We observed additional bristles when *miR-7* and *bantam* were coexpressed with *UAS–RNAi-mi*, but all the bristles had the small size caused by depletion of *minus*, suggesting that there was no suppression of the *minus* phenotype by these two miRNAs (Figure 3, I and J). We also observed cases in which miRNA overexpression caused bristle loss, which precluded analysis of the *minus* phenotype. *miR-957*, *miR-92a*, or *miR-92b* appeared to enhance the *UAS–RNAi-mi* phenotype (Figure 3, K and L); however, bristle loss was observed when these miRNAs were expressed alone under *scaG4* control. *miR-9a* overexpression also results in a loss of scutellar bristles when expressed under *apterous-GAL4* control (Li *et al.* 2006). Similarly, a number of UAS–miRNA transgenes were lethal when coexpressed with the *UAS–RNAi-mi* transgene under *scaG4* control, precluding analysis of their potential as modifiers in this screen (Table S1).

Comparison of the two screens

The screens each identified many interaction candidates: genes located in a deletion and those predicted to be miRNA targets (Table S3). The Microcosm (Griffiths-Jones *et al.* 2008) and TargetScan (Ruby *et al.* 2007) databases were searched for predicted targets of the miRNAs of interest (Table 2). Genes uncovered by the relevant deletions and predicted as miRNA targets are listed in Table 3. This approach identified seven candidate genes for the interval 35D1–35E2, eight candidates for 44A4–44C1, nine candidates for 59D11–59F8, and only four candidates for 77C3–77D1 (Table S4). Overlapping the putative miRNA targets therefore reduced the number of candidates to 10–15% of the number identified on the basis of the overlapping deficiency intervals alone (Table 3).

The interval 35D1–35E2 contains *Cyclin E*, an already known suppressor of *minus*; therefore we focused on the

Table 2 List of miRNAs whose overexpression suppressed *minus* bristle phenotype

Su(mi)	Cytogenetic location
miR-2a-1, miR-2a-2, miR-2b-2	37F2
miR-2b-2	37F2
miR-2c, miR-13a, miR-13b-1	88F4
miR-6-1, miR-6-2, miR-6-3	56E1
miR-11	93E9
miR-308	50E4
miR-4, miR-5	56E1
miR-284	87C1
miR-929	82A1
miR-986	44D1

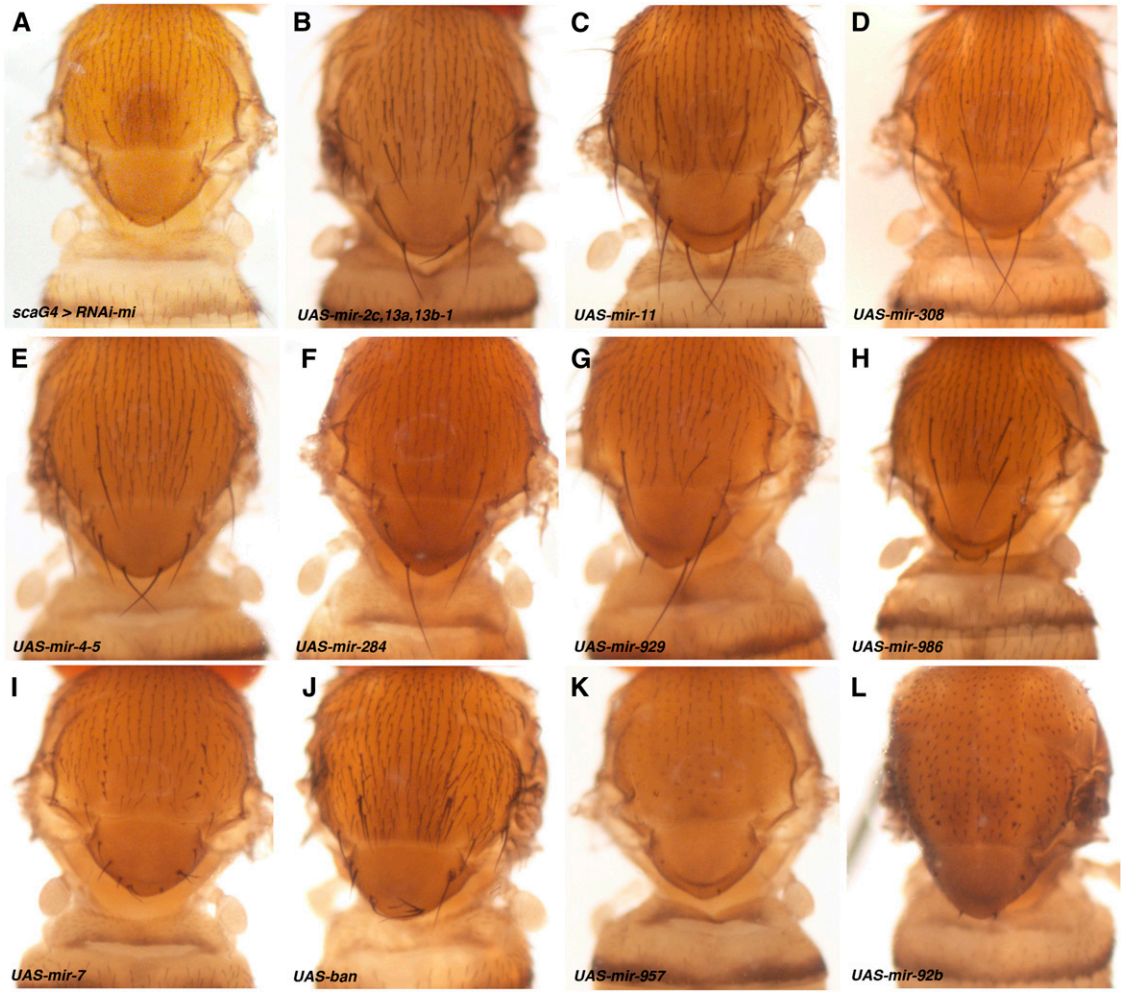

Figure 3 Examples of phenotypes observed in the screen. Images of the dorsal thorax of adult female flies of the indicated genotypes. (A) *sca–GAL4, UAS–RNAi-mi/+* phenotype shown for comparison. (B) *UAS–mir-2c,13a,13b/+*; *sca–GAL4, UAS–RNAi-mi/+*. (C) *sca–GAL4, UAS–RNAi-mi/+*; *UAS–mir-11/+* (note the extra macrochaete on the notum for B and C). (D) *sca–GAL4, UAS–RNAi–mi/UAS–mir-308* (note the extra scutellar bristles in B–E). (E) *sca–GAL4, UAS–RNAi-mi/UAS–miR-4, 5*. (F) *sca–GAL4, UAS–RNAi-mi/+*; *UAS–mir-284/+*. (G) *sca–GAL4, UAS–RNAi-mi/+*; *UAS–mir-929/+*. (H) *sca–GAL4, UAS–RNAi-mi/+*; *UAS–mir-986/+*. (I and J) Absence of suppression was oberved following *miR-7* or *bantam* overexpressison: (I) *UAS–mir-7/+*; *sca–GAL4, UAS–RNAi-mi/+*. (J) *sca–GAL4, UAS–RNAi-mi/+*; *UAS–ban/+*. (K) Absence of suppression and loss of bristles, sometimes associated with an absence of sockets, was observed following *miR-957* overexpression: *sca–GAL4, UAS–RNAi-mi/UAS–mir-957*. (L) Enlarged shaft and bristle cells of macro- and microchaete were caused by *miR-92b* overexpressison. Genotype: *sca–GAL4, UAS–RNAi-mi/+*; *UAS–mir-92b/+*.

other three regions. We noted, however, that none of the miRNAs identified as suppressors are predicted to target *Cyclin E*.

44A4–44C1: Identification of miR-5 target Cul-4 as a suppressor of minus

Most of the deficiencies identified as suppressors in our initial screen are large and have ill-defined endpoints. Molecularly characterized deficiencies that allow more precise definition of the intervals containing candidate suppressors are now available (Cook *et al.* 2010). In the 44A–C region, the small deficiency *Df(2R)Exel7094* suppressed the *UAS–RNAi-mi* phenotype. This deletion uncovers 20 genes, of which 4 are predicted suppressor miRNA targets (Table S4): *CG8713* (*miR-5*), *CG11210* (*miR-2 family*), *SOCS44A* (*miR-4*), and *Cullin-4 (miR-5)*.

In light of its role in protein turnover, *Cullin-4* (*Cul-4*) stood out as an obvious candidate for further analysis. *Cul-4* encodes a scaffold protein, which is a member of the CRL-4 family of Cullin-4/RING E3 ubiquitin ligases (Jackson and Xiong 2009). The CRL-4 complex consists of a Cullin-4 scaffold, a RING protein catalyzing target ubiquitination, the linker protein DDB1, and a substrate receptor that recruits the target of the ubiquitin ligase complex. *Cul-4* is an attractive candidate to be a suppressor of *minus* because E3 ubiquitin ligases based on this scaffold protein are required for degradation of cell-cycle regulators (Abbas and Dutta 2011; Havens and Walter 2011).

To assess whether *Cul-4* might be the *miR-5* target that interacts with *minus*, we asked whether *miR-5* overexpression could regulate *Cul-4 in vivo*. miRNAs often reduce the transcript level of their targets. For this experiment we made use of larval salivary glands, which like the bristles use

Table 3 Genes in the four genomic regions that are targeted by a suppressor miRNA

Interval	Deficiency	No. genes deleted	miR targets in overlap	% target/overlap
35D1–35E2	Df(2L)TE35BC-24	127		
	Df(2L)r10	122		
	Overlap	68	7	10.29
44A4–44C1	Df(2R)H3C1	136		
	Df(2R)BSC267	53		
	Overlap	53	8	15.09
59D11–59F8	Df(2R)or-BR6	187		
	Df(2R)bw-HB132	65		
	Overlap	65	9	13.85
77C3–77D1	Df(3L)rdgC-co2	87		
	Df(3L)BSC796	35		
	Overlap	31	4	12.90
Total candidates	(Overlap)	217	28	12.90

endoreplication to increase cell growth. Overexpression of *miR-4-5* in salivary glands under *ptc-Gal4* control led to a reduction of *Cul-4* mRNA level, measured by quantitative RT–PCR (Figure 4A), suggesting that *miR-5* can regulate *Cul-4*.

To explore this relationship further, we examined the regulation of *Cul-4* mRNA by *miR-5* in cell-based assays. The Microcosm algorithm predicts one *miR-5* site in the *Cul-4* 3′−UTR (Figure 4B). Further analysis using RNAhybrid (Kruger and Rehmsmeier 2006) revealed another putative *miR-5* site featuring a 7mer seed match and four sites with 5mer seed matches but extensive 3′ pairing (Figure 4B). To test whether *Cul-4* is a direct *miR-5* target, we transfected S2 cells with luciferase reporter constructs that carried the intact *Cul-4* 3′-UTR or a version with mutations in the two 7mer seed sites. Coexpression with *miR-5* reduced *Cul-4* 3′-UTR reporter activity to ∼70% of control levels, indicating that *miR-5* can downregulate *Cul-4* (Figure 4C). The mutant version of the *Cul-4* 3′-UTR reporter was less susceptible to downregulation by *miR-5*. The difference between the degree of downregulation of intact and site mutant UTRs was statistically significant ($P < 0.01$), indicating that the predicted target sites contribute to regulation of *Cul-4* by *miR-5*. The remaining capacity of *miR-5* to act on the double site mutant UTR reporter likely reflects the presence of four additional weaker sites. Together these data provide evidence that *Cul-4* mRNA can be directly targeted by *miR-5*.

Identification of *Cul-4* as a *miR-5* target prompted us to ask whether reducing *Cul-4* levels by an independent genetic means could suppress the *minus* phenotype. *Cul-4* mutant alleles were introduced into the *scaG4 > UAS–RNAi-mi* background. Introducing the hypomorphic allele *Cul-4^{KG02900}* had little or no effect on the severity of the *scaG4 > UAS–RNAi-mi* phenotype (Figure 5, A and B). However, *Cul-4* null alleles proved to be effective (Figure 5, C and D). The *Cul-4^{6AP}* and *Cul-4^{G1-3}* alleles were produced by imprecise excision of independent *P* element insertions so their genetic background is different (Hu *et al.* 2008; Lin *et al.* 2009). Both *Cul-4* null alleles also behaved as suppressors of the *pnrG4 > RNAi-mi* sensitized background (Figure S1). No suppression was observed for an RNAi bristle loss phenotype on the basis of a different transgene (Figure S1), suggesting that the effect is specific for the *minus* RNAi phenotype.

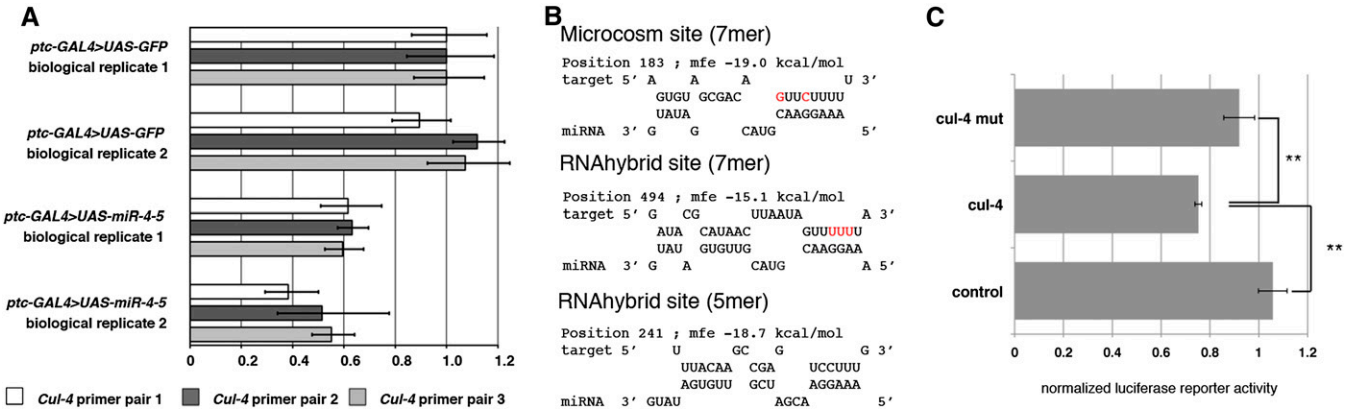

Figure 4 *Cul-4* is a *miR-5* target. (A) *Cul-4* RNA levels measured using three sets of *Cul-4* primer pairs. RNA was extracted from salivary glands expressing GFP (*ptc-GAL4 > UAS–GFP*) or *miR-4-5* cluster (*ptc-GAL4 > UAS–miR-4-5*). Data represent mean ±SD for three technical replicates. Results of two independent experiments are shown (1 and 2). (B) Predicted *miR-5* target sites in the *Cul-4* 3′−UTR. Minimal free energy (mfe) is calculated by RNAhybrid. Nucleotides changed to generate the target site mutant UTR are in red. The predicted RNAhybrid 5mer shown is the example with the highest mfe in this category. (C) Luciferase assays showing regulation of a *Cul-4* 3′-UTR reporter. Luciferase reporter activity is normalized to the *Renilla* transfection control and to empty miRNA overexpression vector control. Data represent mean ±SD. (**) $P < 0.01$ (Student's *t*-tests).

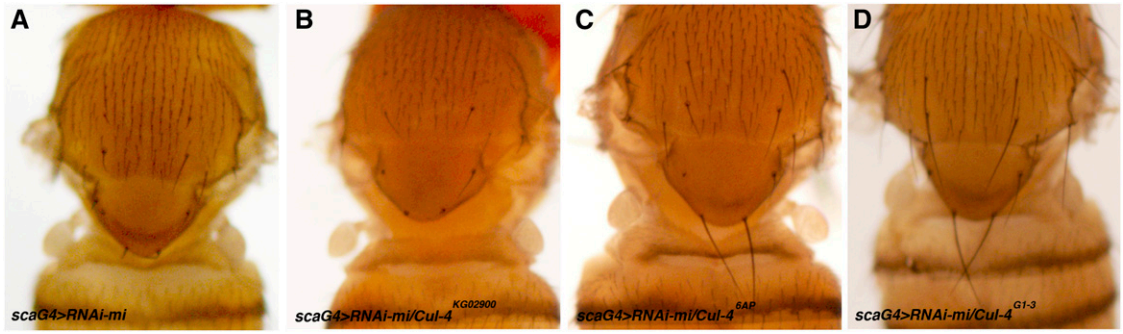

Figure 5 *Cul-4* dosage reduction suppresses *minus* bristle phenotype. Images of the dorsal thorax of adult female flies of the indicated genotypes. (A) *sca–GAL4, UAS–RNAi-mi/+*. (B) *sca–GAL4, UAS–RNAi-mi/Cul-4^{KG02900}*. (C) *sca–GAL4, UAS–RNAi-mi/Cul-4^{6AP}*. (D) *sca–GAL4, UAS–RNAi-mi/Cul-4^{G1-3}*. The *minus* mutant bristle phenotype was not suppressed by the hypomorphic allele *Cul-4^{KG02900}* but was suppressed by two independent *Cul-4* null alleles.

To further evaluate the specificity of *Cul-4* as the mediator of the interaction between *minus* and *miR-5* we asked whether restoring *Cul-4* expression could offset the suppressive effects of *miR-5*. To enable this test to be done, we generated a *UAS–Cul-4* transgene in which the endogenous 3′−UTR was replaced by the SV40 3′-UTR. Use of this transgene ensured that Cul-4 could be coexpressed with *miR-5*, without being subject to downregulation by *miR-5*. It also alleviates the concern that overexpressing a target would serve as a miRNA sponge sequestering the miRNA and thereby reducing its effectiveness for reasons independent of *Cul-4* protein coding sequence. The modified *UAS–Cul-4* transgene was introduced into the *scaG4 > UAS–RNAi-mi*; *UAS–miR-4-5* background and was found to abrogate the suppressive effect of *UAS–miR-4-5* on *scaG4 > UAS–RNAi-mi* (Figure 6, A–C). Four independent *UAS–Cul-4* insertions produced equivalent results, arguing against an effect of integration site. Expression of the *UAS–Cul-4* transgene on its own had little or no effect on bristle size (Figure 6D). These results provide evidence that downregulation of *Cul-4* is a major contributor to the ability of *miR-5* to suppress the *minus* phenotype and identify *Cul-4* as a bona-fide modifier of the *minus* bristle phenotype.

59D11–59F8: Reduced l(2)dtl dosage suppresses the minus bristle phenotype

A subset of CRL-4 family E3 ligases recognize their targets through the substrate receptor Cdt2 and these CRL-4–Cdt2 complexes seem to function exclusively in S phase of the cell cycle (Abbas and Dutta 2011; Havens and Walter 2011). Interestingly, the *Drosophila* Cdt2 ortholog *l(2)dtl* is located in the genetic interval 59D11–59F8, which includes a suppressor of *minus* (Table 1). We therefore asked whether *l(2)dtl* behaves as suppressor of *minus*. The *l(2)dtl^{c02261}* allele contains a *piggyBac* transposon inserted in *l(2)dtl* coding sequence, and may be a functional null allele (Thibault *et al.* 2004). Introducing one copy of *l(2)dtl^{c02261}* suppressed the bristle phenotypes of *scaG4 > UAS–RNAi-mi* (Figure 7, A and B) and *pnrG4 > UAS–RNAi-mi* (Figure S1, A and C). In addition, coexpression with a *UAS–RNAi* transgene targeting *l(2)dtl* suppressed the *scaG4 > UAS–RNAi-mi* (Figure 7C) and *pnrG4 > UAS–RNAi-mi* (Figure S1, A and C) phenotypes. The control for effects on the RNAi machinery again proved negative (Figure S1, G and H). Thus, reduction of CRL4-Cdt2 activity specifically suppressed the *minus* phenotype.

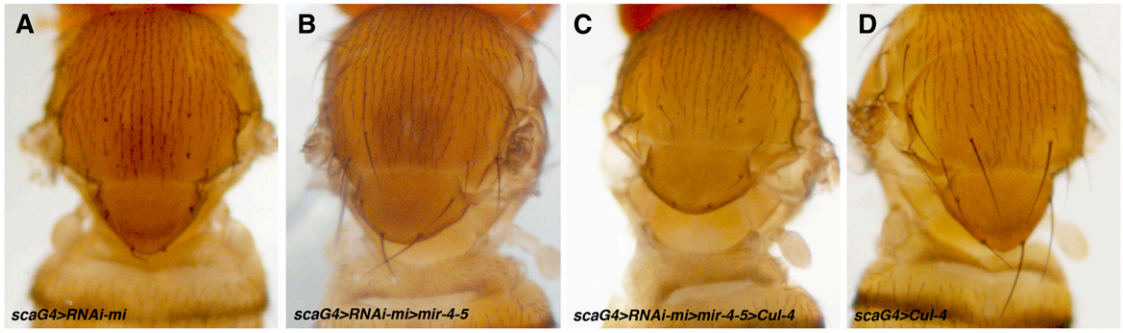

Figure 6 *Cul-4* downregulation is responsible for the *Suppressor of minus* behavior caused by *miR-5* overexpression. Images of the dorsal thorax of adult female flies of the indicated genotypes. (A) *sca–GAL4, UAS–RNAi-mi/+*, and (B) *sca–GAL4, UAS–RNAi-mi/UAS–miR-4-5*, show the *minus* RNAi phenotype and the suppressed phenotype as controls for C. (C) *sca–GAL4, UAS–RNAi-mi/UAS–miR-4-5*; *UAS–Cul-4/+*. Coexpression of *Cul-4* and the *miR-4-5* cluster in the *sca–UAS–RNAi-mi* sensitized background restored the mutant phenotype. (D) *sca–GAL4/+*; *UAS–Cul-4/+*. Expression of *Cul-4* alone with *sca–GAL4* caused only a slight reduction of some bristles.

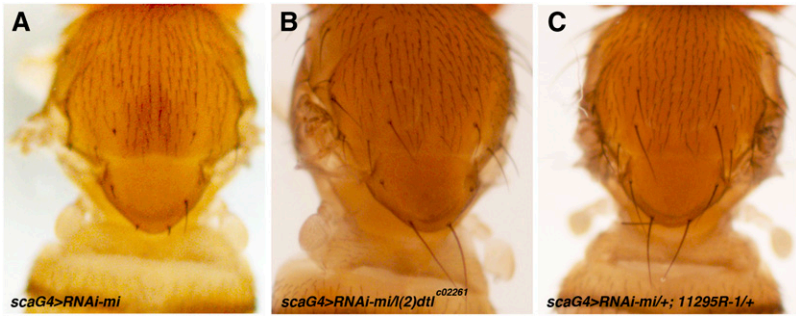

Figure 7 *l(2)dtl* dosage reduction. Images of the dorsal thorax of adult female flies of the indicated genotypes. (A) *sca–GAL4, UAS–RNAi-mi/+*; shows the *minus* RNAi phenotype as a control for the suppression shown by reduction of *l(2)dtl* in B. (B) *sca–GAL4, UAS–RNAi-mi/ l(2)dtl*^{c02261}. (C) *sca–GAL4, UAS–RNAi-mi/11295R-1* shows the effect of depleting *l(2)dtl* by RNAi.

77C3-77D1: miR-284 target CG5199/Cut8 is a suppressor of minus

The genomic region defined by the overlap of the deficiencies *Df(3L)rdgC-co2* and *Df(3L)BSC796* contains 31 genes, of which four are predicted targets of the suppressor miRNAs: *CG5199* and *CG5969* by *miR-284*, *CG13250* by the *miR-2 family*, *CG5104* by *miR-4* (Table 3 and Table S4). *CG5199* is related to fission yeast Cut8 (Takeda and Yanagida 2005). In yeast, Cut8 is conditionally required for destruction of a mitotic cyclin (Cdc130) and in both organisms mediates localization of the proteasome to the nuclear periphery (Tatebe and Yanagida 2000). As *minus* was demonstrated to influence cyclin E degradation (Szuplewski *et al.* 2009) and as the identification of the CRL4–Cdt2 implicates proteasomal degradation as relevant to *minus* function, we asked whether *CG5199* was the relevant suppressor in 77C3–77D1. Coexpression with a *UAS–RNAi* transgene targeting *CG5199*, *P{GD11574}v27372* suppressed the *scaG4 > UAS–RNAi-mi* phenotype, suggesting that *CG5199* is the relevant suppressor within the 77C3–77D1 genomic region. No additional *CG5199* alleles or RNAi lines are available, so we could not evaluate this interaction further.

Discussion

microRNA overexpression screens for genetic modifiers

This study illustrates the potential of a genetic modifier screen based on inducible miRNA-expressing transgenes. The utility of this approach lies in the fact that there are many predicted miRNA targets, so the probability of intersection between the target list of any miRNA and the list of genes uncovered by any deletion is quite high, approaching 50%. Most of the genes that are uncovered by an interacting deletion are unlikely to be responsible for the interaction phenotype. Similarly, most of the predicted targets of a miRNA are unlikely to be causally linked to the genetic interaction with the miRNA. This study identified four deletion intervals and six miRNA families that interacted with *minus*. Use of the predicted targets of these six miRNAs narrowed down the number of candidate genes to 10–15% of the total number of genes in the four deletion intervals to produce a short list for further analysis (Table 3). Together with *CycE*, which was known previously to interact with *minus*, the predicted miRNA targets *Cul-4*, and very likely *CG5199/Cut8*, each account for a dominant suppressor locus in two of the four chromosomal regions identified by the deficiency interaction screen. Similarity in function to these, two allowed for identification of *l(2)dtl* as an interacting locus in the fourth deficiency, although *l(2)dtl* was not a predicted miRNA target.

A limitation inherent in the miRNA overexpression approach is its dependence on miRNA target prediction. Current algorithms differ in their specificity and sensitivity and often show limited overlap in the targets they predict (See Table S3; Thomas *et al.* 2010). Improvements in these methods should enhance the utility of the miRNA-based screening approach in future. A second inherent limitation to this approach is that miRNAs do not target all genes in the genome, nor do they act with comparable efficiency on those they do target. A third limitation is that some miRNAs produce phenotypes that may preclude their use in a particular screen. We have highlighted a few examples for screens involving bristle size and morphology. In the most extreme form, this limitation is exemplified by miRNAs that were lethal when overexpressed. This problem might be solved by using the temperature-sensitive inhibitor of GAL4 (McGuire *et al.* 2003), Gal80^{ts}, in combination with a suitable Gal4 driver to control when and where the transgenes are expressed.

Reduction of CRL4–Cdt2 activity suppresses the minus mutant phenotype

The finding that reduction of CRL4–Cdt2 activity suppresses the *minus* mutant phenotype suggests a contribution of elevated of E3 ubiquitin ligase activity. Three substrates for CRL4–Cdt2 have ben identified in *Drosophila*: the cyclin-dependent kinase inhibitor Dacapo (Dap, the ortholog of mammalian p21), the Drosophila orthologs of Cdt1 replication factor, Double parked (Dup), and the *Drosophila* E2F oncogene ortholog (Higa *et al.* 2006a,b; Shibutani *et al.* 2008; Lee *et al.* 2010). All three are cell-cycle regulators involved in G1 or S phase that possess a PCNA-interacting polypeptide box degron required for their CRL4–Cdt2-dependent proteasomal degradation. Therefore, it seems reasonable to assume that reduced CRL4–Cdt2 activity leads to elevated levels of Dap, Dup, and E2F. However, introduction of mutant alleles of *dap*, *dup*, or *E2F* did not modify the bristle phenotype in the *l(2)dtl / sca–GAL4 > UAS–RNAi-mi* background (data not shown). This suggests that excessive

downregulation of these genes is unlikely to explain the effects of elevated CRL4–Cdt2 activity in the *minus* mutant. However, the possibility exists that concurrent downregulation of all three might produce a different experimental outcome than from removing one at a time. An alternative hypothesis is that CRL4–Cdt2 may act via another, yet to be identified, substrate in this context.

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GENETICS

Supporting Information
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MicroRNA Transgene Overexpression Complements Deficiency-Based Modifier Screens in *Drosophila*

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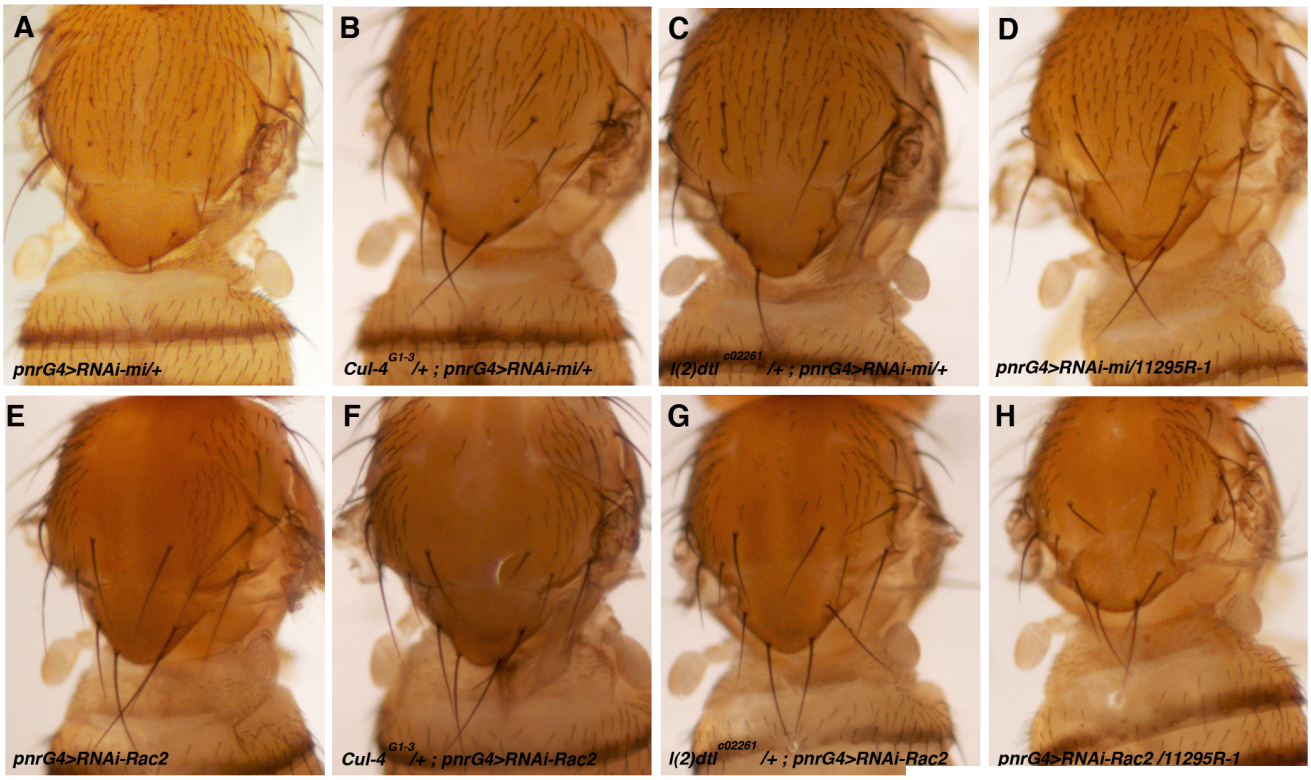

Figure S1 Additional controls.
Heterozygosity for *Cul-4* (B: *Cul-4*$^{G1-3}$*/+; pnr-GAL4, UAS-RNAi-mi/+*) or for *l(2)dtl* (C: *l(2)dtl*^{c022613}*/+; pnr-GAL4, UAS-RNAi- mi/+*) alleles or expression of a RNAi directed against *l(2)dtl* (F: *pnr-GAL4, UAS-RNAi-Rac2/11295R-1*) suppress *minus* mutant bristle size reduction phenotype (A: *pnr-GAL4, UAS-RNAi-mi/+*), confirming the specificity to *UAS-RNAi-mi*. Expression of *RNAi-Rac2* with the *pnr-GAL4* driver results in a quasi-absence of microchaete and an absence of dorso-anterior macrochaete in the notum central part (E: *pnr-GAL4, UAS-RNAi-Rac2/UAS-GFP*). These phenotypes are not suppressed by heterozygosity for *Cul-4* (F: *pnr-GAL4, UAS-RNAi-Rac2/Cul-4*$^{G1-3}$) or for *l(2)dtl* (C: *pnr-GAL4, UAS-RNAi-Rac2/l(2)dtl*^{c022613}) alleles or by expression of a RNAi directed against *l(2)dtl* (F: *pnr-GAL4, UAS-RNAi-Rac2/11295R-1*), ruling out that *Cul-4* or *l(2)dtl* dosage reduction simply affects the RNAi pathway and abrogates *GAL4>UAS-RNAi-mi* function.

Tables S1-S4 are available for download at
http://www.genetics.org/content/suppl/2011/11/18/genetics.111.136689.DC1 as excel files.

Table S1 Detailed results of the UAS-miRNA screen.
Nomenclature: P/R miRNA name@ landing site with P= pUASP.attB-SLIC and R=pUASP.attB-SLIC. Used landing sites are Fb= M{3xP3-RFP.attP}ZH-86Fb, P2= P{CaryP}attP2, P16= P{CaryP}attP16, VK= PBac{yellow[+]-attP-3B}VK00037.
UAS-(0/dsred2/GFP) - miRNA name - chromosome of insertion (X/II/III/? (not mapped)) – transgenic line.

Table S2 Collections of deficiencies used for the screen.

Table S3 A-D Numbers of candidate genes predicted to be targeted by suppressor miRNAs and uncovered by suppressor deficiencies.

Table S4 Shortlisted candidates derived from intersecting both screens.
Genes highlighted in grey are uncovered by Df(2R)Exel7094.