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TECHNOLOGY REPORT

RNAi-Mediated Inhibition of Gene Function in the Follicle Cell Layer of the *Drosophila* Ovary

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Summary: RNA-mediated interference (RNAi) has been reported to be an effective reverse genetic approach for studying gene function in various organisms. To assess RNAi as a means of examining genes expressed in ovarian follicle cells for their involvement in embryonic dorsal–ventral patterning, we tested the ability of transgenically expressed double-stranded RNA (dsRNA) directed against the dorsal group gene *windbeutel* to generate phenotypic effects in the progeny of expressing females. We observed that expression in follicle cells under the control of Gal4 transcribed from the strong and widely expressed *alphaTub84B* or *Actin5C* promoters led to efficient dorsalization of progeny embryos. Surprisingly, a variety of strongly expressed follicle cell-specific Gal4 enhancer trap lines failed to elicit an RNAi phenotype in combination with the *windbeutel*-specific dsRNA. These results stress the importance of careful choice of expression system and of conditions for use in transgenic RNAi-mediated studies of gene function. genesis 40: 101–108, 2004. © 2004 Wiley-Liss, Inc.

Key words: oogenesis; dorsal-ventral patterning; dorsal group; *windbeutel*; *pipe*; RNAi; RNA interference; double-stranded RNA; dsRNA; *Drosophila melanogaster*

First observed in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998), double-stranded RNA-mediated interference (RNAi) is a widely conserved process in eukaryotes which may participate in the host response to RNA viral infections and in stabilizing the genome against the effects of repetitive elements (Hannon, 2002). RNAi acting through dsRNA leads to the degradation of complementary mRNAs in a process that can be divided mechanistically into two steps (Cerutti, 2003). In the initiation step, the long dsRNA is processed into small (21–23 nucleotides) double-stranded interfering RNAs (siRNAs) by an RNAse III-like dsRNA-specific ribonuclease called Dicer. In the subsequent effector step, the large multiprotein RNA-induced silencing complex (RISC) unwinds the strands of the siRNAs and uses the single strands as probe for complementary RNAs which are then cleaved and degraded.

RNAi has become a powerful reverse genetic tool for analysis of the phenotypic consequences of interfering with gene expression. In *C. elegans*, for which a fully sequenced genome is available, several large-scale screening strategies have taken advantage of the fact that worms fed on bacteria expressing worm gene-targeted dsRNAs often exhibit a phenotype consistent with loss-of-function for the gene of interest (Kamath and Ahringer, 2003). RNAi has also proven a unique means of elucidating the function of sequenced genes in organisms for which other genetic approaches to the study of gene function are not available (Hughes and Kaufman, 2000).

The application of RNAi technology to the study of *Drosophila* has received considerable attention because current technologies for isolation of mutations can be cumbersome and time-consuming. Moreover, the availability of methods for conditionally expressing transgenic constructs (Duffy, 2002) provides a potential utility for RNAi in instances where it is of interest to assess the tissue-specific requirement for the function of genes whose expression in other tissues or at earlier times during development is essential for fly viability.

The application of RNAi technology to *Drosophila* is subject to limitations that can reduce its usefulness. Although direct injection of dsRNA into embryos has been shown to eliminate the function of genes expressed during embryogenesis, the effect does not persist to later stages of fly development and is not inherited (Kennerdell and Carthew, 1998). To overcome these problems, a number of investigators have attempted RNAi-mediated gene targeting by expressing dsRNA in transgenic *Drosophila*. One approach has been to express a transgene that is transcribed in both sense and antisense orientations by promoters that flank the cloned insert specific to the gene of interest (Giordano *et al.*, 2002). An alternative approach has been to express

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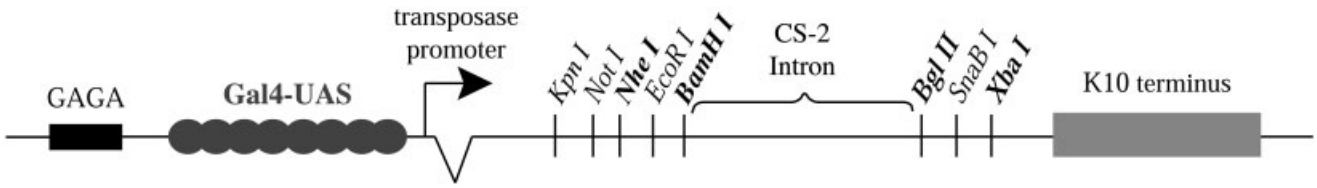

FIG. 1. The organization of the cloning region in the RNAi expression vector pUASp-NBa-CS2-BgX. The positioning of the promoter/enhancer region, *CS-2* intron and useful cloning sites in the vector are shown. This figure is modified from Rorth (1998).

a transgene with an inverted repeat confirmation, enabling the expressed RNA to "snap-back" into a double-stranded hairpin configuration (Fortier and Belote, 2000; Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Martinek and Young, 2000). Using both of these approaches, suppression has been reported to be variable in its effectiveness depending on the gene function examined. Kalidas and Smith (2002) described a novel strategy for expressed RNAi that utilizes a genomic DNA/cDNA hybrid construct. Following splicing of RNA derived from the genomic DNA segment of the hybrid, these molecules were predicted to form hairpin dsRNA that can suppress expression of mRNA target molecules in specific tissues. The potency of these constructs, even when present as a single transgenic insert, led the authors to speculate that spliced RNAi constructs are more efficiently processed and transported from the nucleus and therefore accumulate to higher levels in the cytoplasm. However, this technique is limited to genes with an amenable intron/exon structure, and its effectiveness is likely dependent on the efficiency of splicing of each particular RNAi hybrid generated.

A more generally applicable technique has been developed by Reichart *et al.* (2002) and by Lee and Carthew (2003), using "splice-activated" hairpin RNAs that mediate effective RNAi-mediated knockout of target genes. In this approach, coding sequences corresponding to the gene of interest are cloned in IR orientation upstream and downstream of a DNA fragment containing a known intron that undergoes efficient splicing. Following expression of RNAi constructs generated in this way, Reichart *et al.* (2002) observed phenotypes resulting from the suppression of several target genes including *necrotic, Drosomycin, dFADD,* and *forked*. Similarly, Lee and Carthew (2003) observed effective suppression of the *white* gene using a splice-activated dsRNA-expressing transgene.

We used the strategy of Reichart *et al.* (2002) to construct an expression vector for the production of splice-activated dsRNAs for use in studies of genes involved in the maternal control of embryonic dorsal–ventral polarity. pUASp-NBa-CS2-BgX was designed to enable the convenient cloning of DNA fragments in IR orientation for subsequent inducible expression in *Drosophila* tissues. The vector is based on pUASp, generated by Rorth (1998), an expression vector enabling Gal4-dependent expression in germline and somatic tissues, due to the presence of 14 UAS Gal4 sites and the basal promoter from the P-element transposase gene. pUASp-NBa-CS2-BgX contains the 816 nucleotide long seventh intron from the *CS-2* gene (Gagou *et al.*, 2002), flanked by the restriction enzyme target sites in the arrangement shown in Figure 1. This arrangement of sites enables convenient two-step cloning of a DNA fragment with one BamHI/BglII/BclI compatible end and one XbaI/NheI/SpeI compatible end in IR orientation, on either side of the *CS-2* intron sequences.

We examined the ability of dsRNA expressed from the pUASp-NBa-CS2-BgX vector to eliminate function of *windbeutel (wind*), a maternal effect gene whose expression in the ovarian follicle cell layer is required for the formation of progeny embryos with correct dorsal–ventral (DV) polarity (Konsolaki and Schüpbach, 1998). The determination of pattern elements along the DV axis of the *Drosophila* embryo requires the function of 11 maternal-effect loci known collectively as the dorsal group (Morisato and Anderson, 1995). Females homozygous for loss-of-function alleles of these genes produce embryos that exhibit an expansion of dorsal pattern elements at the expense of ventral and lateral elements. The products of the dorsal group genes comprise a signal transduction pathway that originates in the epithelial layer of follicle cells which surrounds the developing egg during oogenesis. Once initiated in the follicle cell layer, DV polarity is transmitted to the developing embryo via a signal that controls the localized activation of a serine proteolytic cascade that generates a ligand for the Toll receptor, which is located in the embryonic membrane. Ventrally restricted processing of the precursor form of the ligand, Spätzle, under the control of this protease cascade is responsible for the ventral activation of the Toll receptor and for the control of zygotic gene expression along the DV axis. The ventrally localized expression of the *pipe* gene in the follicle cell layer is the primary cue that controls the spatial specificity of Toll activation (Sen *et al.*, 1998), presumably through regulation of the serine proteolytic cascade. The *pipe* locus has the capacity to express multiple protein isoforms, produced by alternative splicing, with sequence similarity to vertebrate heparan sulfate 2-O-sulfotransferase (Sen *et al.*, 1998; Sergeev *et al.*, 2001). One of these isoforms appears to function specifically in the DV patterning pathway. These observations suggest the existence of a critical glycosaminoglycan or related glycan molecule whose modification by Pipe in ventral follicle cells is essential for the initiation of dorsal group signal-

ing. However, no mutations in genes involved in glycan formation have been identified in screens for maternal affect mutations affecting embryonic DV polarity, suggesting that the proteins involved in generating the substrate of Pipe enzymatic activity also participate in the generation of glycan molecules acting in vital processes that occur prior to oogenesis. Induced expression of RNAi provides a possible means for identifying among candidate genes involved in glycan formation ones that may participate in the formation of GAGs or other potential glycan targets of Pipe in the follicle layer, but which are also essential for events occurring at other times during the life of the fly.

To validate the use of RNAi in the follicle cell layer as a means of testing the involvement of candidate genes in embryonic patterning and to test its effectiveness, we examined the ability of dsRNA to eliminate function of *wind*, a dorsal group gene that, like *pipe*, is known to function in the follicle cell layer and encodes an endoplasmic reticulum protein with sequence similarity to protein disulfide isomerase (Kondolaki and Schüpbach, 1998). *wind* activity has been shown to be required for function and correct subcellular localization of Pipe protein to the Golgi (Sen *et al.*, 2000). Thus, RNAi-mediated elimination of *wind* mRNA in the follicle cell layer of females would be expected to lead to a perturbation of Pipe function and to the production of embryos with a dorsalized phenotype.

Our strategy has been to express dsRNA targeting the *wind* transcript under the control of the Gal4/UAS binary system (Brand and Perrimon, 1993). To do this we used a set of transgenic fly stocks that express Gal4 in ovarian follicle cells. These include lines in which Gal4 has been placed downstream of transcriptional signals for *alphaTub84B* promoter (Tubp-Gal4), and *Actin5C* (Act5C-Gal4), which are strongly expressed in the follicle cell layer as well as in a variety of other tissues during fly development. We also obtained a number of Gal4-based enhancer trap lines which exhibit Gal4 expression in the follicle cell layer during oogenesis. For example, the Gal4-CY2, Gal4-T155, and Gal4-3 enhancer trap inserts (Manseau *et al.*, 1997; Queenan *et al.*, 1997; Sen *et al.*, 1998, 2000) have been found to express Gal4 in all follicle cells covering the oocyte from mid-oogenesis on. Representative images of the expression of UAS-lacZ directed in the ovary by the Gal4 drivers used in these studies are shown in Figure 2. Examination of many X-gal-stained egg chambers enabled us to conclude that Tubp-Gal4 and Act5C-Gal4 initiate expression of Gal4 slightly earlier in oogenesis than the other Gal4 drivers investigated. All of the Gal4 drivers exhibited strong uniform expression throughout the follicle cell layers of stage 9/10 and older egg chambers. In addition, the UAST-lacZ;Tubp-Gal4 and Act5C-Gal4/UAST-lacZ females showed consistent staining in the follicle cells of Stage 8 egg chambers with UAST-lacZ;Tubp-Gal4 females producing some egg chambers that stain as early as Stages 6 or 7. In contrast, Gal4-CY2/UAST-lacZ, UAST-lacZ;Gal4-T155, and UAST-lacZ;Gal4-3 females produced few egg chambers that stained with X-gal as early as Stage 8, and those Stage 8 egg chambers that did stain displayed a patchy pattern that was enriched in the posterior half of the egg chamber.

The Gal4-expressing lines noted above, and several additional lines exhibiting specific patterns of Gal4 expression during oogenesis, were each crossed to pUASp-windRNAi (see Materials and Methods) and for each cross, females carrying both transposon inserts were recovered and their embryonic progeny examined. Females carrying pUASp-windRNAi together with Tubp-Gal4 or Act5C-Gal4 produced mainly unhatched embryos. Tubp-Gal4 exhibited a more severe effect on fertility, with 98.4% of eggs remaining unhatched (n = 3,026 total eggs counted). Females in which UASp-windRNAi was expressed under the control of the Act5C-Gal4 driver, produced 90.3% unhatched eggs (n = 3,275 total eggs counted). Examination of cuticle preparations of unhatched embryos produced by both types of females showed them to be dorsalized (Fig. 3f,h). This was most easily observed through the absence or disruption of the ventral cuticular denticle bands that are normally present on the ventral side of embryos from wildtype females (Fig. 3b). Tubp-Gal4 directed RNAi targeting of *wind* led to the most severe dorsalization, with some embryos exhibiting a complete lack of ventral and lateral pattern elements, making them indistinguishable from the embryos produced by females homozygous for null alleles of a dorsal group gene (e.g., *pipe*; Fig. 3d). The dorsalized phenotypes of embryos produced by Act5C-Gal4/pUASp-windRNAi females (Fig. 3h) were generally less severe than those generated through Tubp-Gal4 expression (Fig. 3f). This difference in phenotypic severity was quantitatively assessed by determining the relative dorsalization of cuticles of unhatched embryos produced by Tubp-Gal4 or Act5C-Gal4 directed *wind*-targeted RNAi (Table 1), according to the scoring system of Roth *et al.* (1991) (see Materials and Methods). Scored in this way, most of the unhatched embryos produced by Tubp-Gal4 driven *wind*-targeted RNAi were totally (D0, 36%) or strongly (D1, 40%) dorsalized, while the majority of embryos produced by Act5C-Gal4-driven RNAi were moderately (D2, 26%) or weakly (D3, 69%) dorsalized. As an additional confirmation that the embryos produced by RNAi-mediated targeting of *wind* were indeed dorsalized, embryos from females of each of the genotypes described above were stained with an antibody against the product of *twist* (Roth *et al.*, 1989), a gene whose normal ventral expression in the mesodermal anlagen of the wildtype blastoderm embryo (Fig. 3a) is activated by dorsal group signaling (Roth *et al.*, 1989). Embryos from females expressing *wind*-targeted dsRNA under the control of Tubp-Gal4 (Fig. 3e) or Act5C-Gal4 (Fig. 3g) exhibited disruptions of Twist staining, consistent with a dorsalized phenotype.

In contrast to the results observed using Tubp-Gal4 and Act5C-Gal4, the other follicle cell-specific Gal4 enhancer trap lines tested in this study did not lead to the

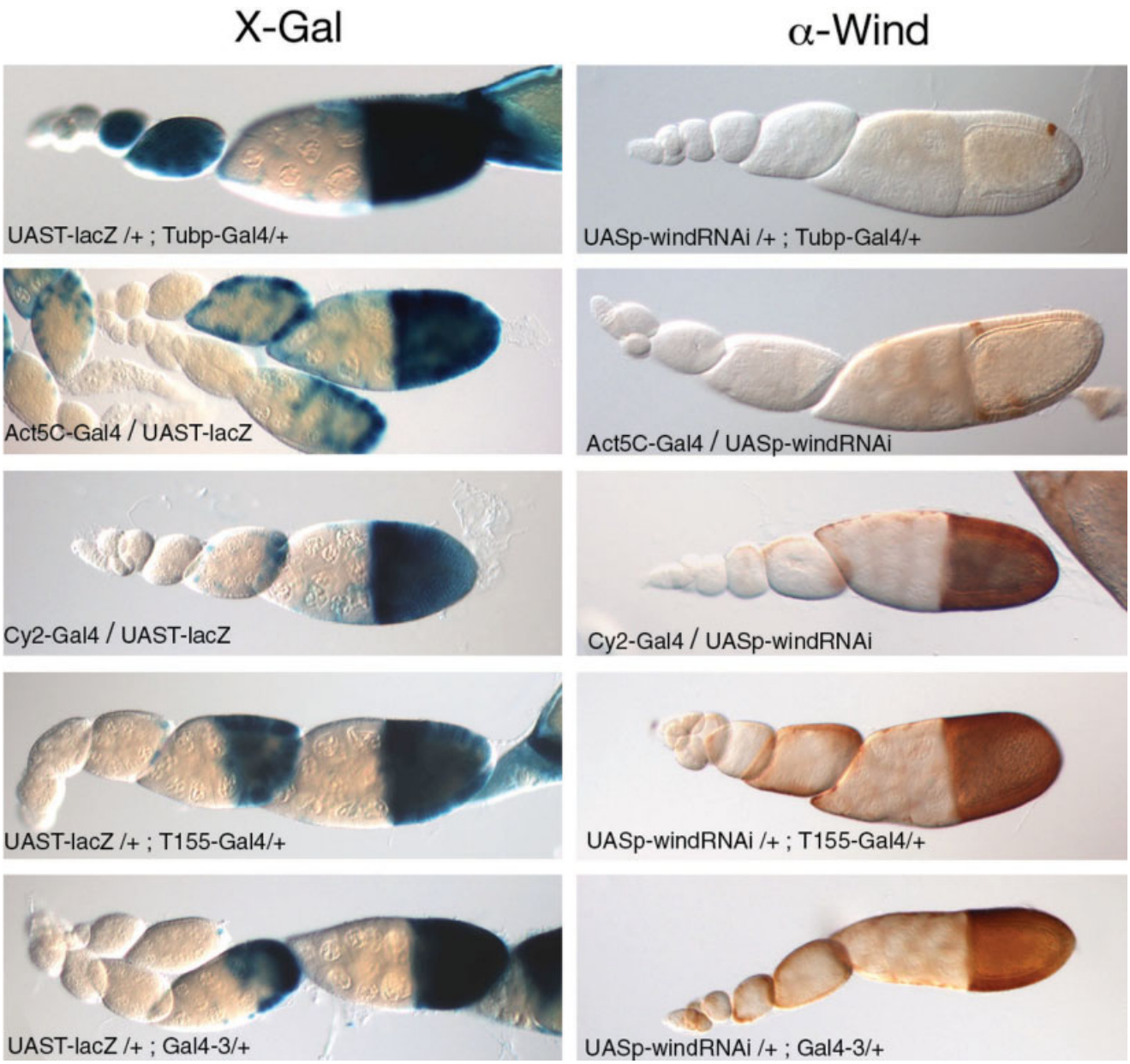

FIG. 2. RNAi-mediated inhibition of Windbeutel expression directed by various Gal4 driver lines. At left, ovarioles from fixed ovaries stained with X-Gal are shown. The blue color indicates the expression of lacZ and, hence, Gal4. At right are ovarioles from fixed ovaries, stained with an antibody directed against Windbeutel. The genotypes of females from which the ovaries were obtained is noted in each panel.

production of dorsalized embryos when combined with the pUASp-windRNAi transgene despite being strongly expressed during Stages 9 and 10 (Fig. 2), the stages at which the *wind* gene has been reported to be expressed during oogenesis (Konsolaki and Schüpbach, 1998). To confirm that the phenotypic effects elicited through Gal4-mediated expression of *wind* dsRNA resulted from an inhibition of Windbeutel expression, we stained ovaries from females of the genotypes described above with an antibody generated against the Windbeutel protein (Ma *et al.*, 2003). Only females in which the *wind* dsRNAi construct was expressed under the control of the Tubp-Gal4 or Act5C-Gal4 drivers exhibited a loss of Windbeutel staining in Stage 9/10 egg chambers (Fig. 2). Interestingly, egg chambers in which Windbeutel expression had been perturbed via Tubp-Gal4- or Act5C-Gal4-directed RNAi often carried a small number of follicle cells that retained expression, demonstrating that the inhibitory effect of RNAi is not uniform throughout the follicle layer.

It is unclear why several enhancer trap lines tested in this work failed to mediate a knockdown of Windbeutel expression. The simplest explanation for the ability of Tubp-Gal4 and Act5C-Gal4, but not the other Gal4 driv-

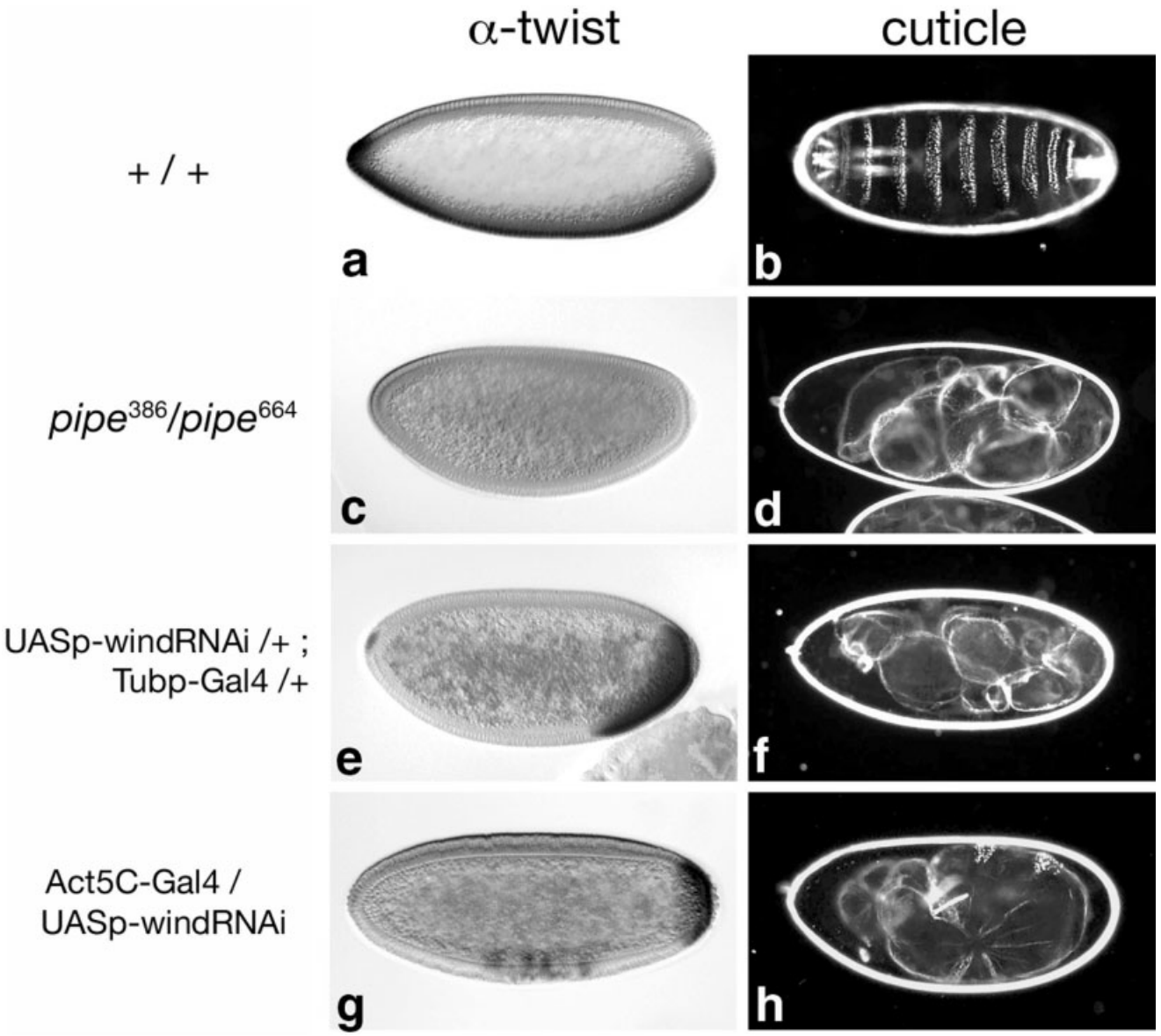

FIG. 3. Dorsalized phenotypes produced by RNAi-mediated disruption of *wind* function. Blastoderm embryos stained with an antibody against the ventral-specific marker Twist (**a,c,e,g**) and cuticle preps (**b,d,f,h**) are shown. **a,b:** Embryos from wildtype females. **c,d:** Dorsalized embryos produced by *pipe*^{386}/*pipe*^{664} transheterozygous females. **e,f:** Dorsalized embryos produced by females expressing *wind* dsRNA under the control of Tubp-Gal4. The embryo in **f** exhibits a D0 phenotype. **g,h:** Dorsalized embryos produced by females expressing *wind* dsRNA under the control of Act5C-Gal4. The embryo in **h** exhibits a D2 phenotype. The maternal genotypes of females producing the embryos shown is noted to the left of each pair of panels.

Table 1
Degree of Dorsalization of Embryonic Cuticles Generated by wind-*Targeted RNAi*

	% Unhatched embryos				
Maternal genotype	No cuticle (unscored)	D0	D1	D2	D3
UASp-windRNAi/+; Tubp-Gal4/+ (n = 192 embryos)	6	36	40	12	6
Act5C-Gal4/UASp-windRNAi (n = 249 embryos)	3	0	2	26	69

Degree of dorsalization defined as D0 = completely dorsalized, D1 = strongly dorsalized, D2 = moderately dorsalized, D3 = weekly dorsalized. (See Materials and Methods for detailed explanation).

ers tested, to affect an RNAi phenotype is a quantitative difference in the levels of Gal4 protein (and consequently, *wind* dsRNA) produced by the driver lines at mid-oogenesis. In that scenario, the levels of Gal4 protein (and *wind* dsRNAi) generated by the Gal4-CY2, Gal4-T155, and Gal4-3 would not meet a threshold necessary to perturb Windbeutel expression and DV patterning. Alternatively, the slightly earlier expression of Gal4 protein directed by the Tubp-Gal4 and Act5C-Gal4 drivers might provide them with a "head start" (compared to the other Gal4 drivers) in accumulating sufficient *wind* dsRNA to facilitate the destruction of endogenous wind mRNA produced at later oogenic stages. In order to distinguish between these possibilities, it would be necessary to determine directly the concentration of *wind* dsRNA produced by the Gal4 driver lines at various stages of oogenesis.

Our results indicate that it is possible to accomplish effective transgenic dsRNA-mediated suppression of gene expression in the follicle cell layer, leading to detectable phenotypes in progeny embryos. However, the Gal4 drivers with which we were able to observe such an effect were two that are also expressed at other times in the life of the fly. Although *wind* is expressed during embryogenesis (Sen *et al.*, 2000), its function is not essential for fly viability, so that RNAi-mediated elimination of *wind* function prior to adulthood would not be expected to affect survival. However, for genes that are essential for viability the necessity of using strong and widely expressed Gal4 drivers in order to accomplish efficient dsRNA expression might limit the utility of this approach for studies of gene function at late times in development, owing to early RNAi-mediated lethality. However, while the vast majority of embryos produced by the strongest RNAi genotype described here (Tubp-Gal4; pUASp-windRNAi) were dorsalized, we did observe a background of hatching escaper embryos, indicating that Gal4-directed RNAi is not 100% effective. Moreover, because of the intrinsic temperature sensitivity of the Gal4 protein, which is more active at higher temperatures, it should be possible to obtain flies carrying Tubp-Gal4 or Act5C-Gal4 together with pUASp-RNAi constructs targeting vital genes by carrying out the crosses at low temperature (18°C), then shifting the flies to higher temperature (25–29°C) once adults have been obtained. In additional experiments, we observed Tubp-Gal4/pUASp-RNAi-target gene combinations which are lethal at 25°C but viable at 18°C.

Taken together, our results stress the importance of optimizing conditions for RNAi. In particular, for investigations in which inducible RNAi is to be used to test the function of a candidate gene of interest in a specific tissue, it is essential to validate that the chosen expression system is capable of accomplishing RNAi in that tissue by testing for RNAi-mediated elimination of a gene known to function there.

MATERIALS AND METHODS

Fly Stocks and Maintenance

All stocks were maintained and crosses carried out employing standard conditions and procedures. For studies of RNAi-mediated inhibition of *wind* gene function, flies were cultured at 25°C. Larval cuticles were prepared according to Van der Meer (1977). For scoring the degree of dorsalization in embryonic cuticles, the classification of Roth *et al.* (1991) was used. D0 embryos are completely dorsalized, lacking Filzkörper (posterior tracheal structures) and ventral denticles. Strongly dorsalized D1 embryos lack ventral denticles but carry the dorsolaterally derived Filzkörper. Moderately dorsalized D2 embryos carry Filzkörper and ventral denticles of reduced width, compared to that seen in wildtype embryo. The weakly dorsalized D3 embryos exhibited fusions of ventral denticle bands, characteristic "twisted" phenotypes, or head defects.

Fly stocks carrying the Tubp-Gal4 and Act5C-Gal4 driver lines were obtained from Dr. Joe Duffy. The Gal4-CY2 and Gal4-T155 transgenic lines were from Dr. Trudi Schüpbach. Dr. Leslie Stevens provided the Gal4-3 transgenic line. Details regarding additional follicle-cell-expressed Gal4 enhance trap driver lines tested in this study are available upon request. The UAST-Gal4 reporter-containing line was provided by Dr. Norbert Perrimon. The $pipe^{386}$ and $pipe^{664}$ mutant stocks used in the generation of dorsalized embryos are described in Sen *et al.* (1998). The wildtype strain used in these studies is a *w/w* derivative of Oregon R.

Generation of pUASp-NB-CS2-BgX and pUASp-windRNAi

pUASp-NBa-CS2-BgX was constructed according to a strategy outlined by Reichart *et al.* (2002) for splice-activated RNAi. The two deoxyribonucleotides: 5′-GTATACGCGGCCGCTAGCGAATTCGGATCCAGGTAAGTGGGAGTCGC-3′ and 5′-GCTCTAGATACGTAAGATCTCCTGAAAAAAAAAACAG-3′ were used for PCR-mediated amplification of the 816 nucleotide-long seventh intron from the *CS-2* gene. The PCR fragment generated was digested with NotI and XbaI and subcloned into similarly digested pUASp. This led to incorporation of the *CS-2* intron, flanked by the restriction enzyme target sites in the arrangement shown in Figure 1. This arrangement enables convenient two-step cloning of a DNA fragment with one BamHI/BglII/BclI compatible end and one XbaI/NheI/SpeI compatible end in IR orientation, on either side of the *CS-2* intronic sequences.

For the generation of pUASp-windRNAi, the two oligonucleotides: 5′-GAAGATCTGCATATTTTGGTGACTCTGCTCC-3′ and 5′-GCTCTAGAGTTCCTCCTTTTCCGGCGCTG-3′ were used for PCR-mediated amplification of a 766 nucleotide-long segment of the coding region from the *windbeutel* gene. The PCR fragment was cut with BglII and XbaI and subcloned, in antisense orientation,

into BamHI/NheI-digested pUASp-NBa-CS2-BgX, upstream of the *CS-2* intron. The resultant plasmid was digested with BglII and XbaI and the BglII/XbaI-cut PCR fragment was cloned downstream of the *CS-2* intron, in sense orientation. Our decision to focus on pUASp in these studies, rather than the more commonly used expression vector pUAST (Brand and Perrimon, 1993), was based on the potential utility of pUASp for expression of dsRNAs in the germ-line, a tissue in which pUAST cannot be effectively employed.

The pUASp-windRNAi vector was introduced into the *Drosophila* genome by conventional P-element-mediated transformation, following microinjection (Rubin and Spradling, 1982). Two of four independent insertions of the pUASp-windRNAi vector obtained led to the generation of dorsalized embryonic phenotypes in combination with the Tubp-Gal4 and Act5C-Gal4 driver lines. An insertion on the second chromosome was used in the generation of all ovaries and embryos shown in this study. In additional investigations, a NotI/XbaI fragment from pUASp-windRNAi, corresponding to the *CS-2* intron flanked by *wind*-derived sequences, was subcloned into the P-element-based expression vector, pUAST (Brand and Perrimon, 1993). The resultant plasmid, pUAST-windRNAi, was introduced into the *Drosophila* genome as described above. Four of six independent insertions of UAST-windRNAi obtained were observed to produce dorsalized embryos, in combination with Tubp-Gal4 and Act5C-Gal4, but not with the other enhancer trap lines examined in this work (data not shown). Insofar as the UAST expression vector has been shown to be incapable of supporting the expression of cloned DNAs in the *Drosophila* germline, these observations support the notion that the RNAi-dependent phenotypes that we have observed are due to the expression of *wind* dsRNA in the follicle cell layer, where both UASp and UAST are efficiently expressed. The pUASp-windRNAi and pUAST-windRNAi transgenic lines that did not produce RNAi phenotypes are likely to carry transgene insertions at genomic locations that do not support high levels of expression.

Antibody Staining of Embryos

Whole-mount antibody staining of embryos was carried out as described by Roth *et al.* (1989). The rabbit polyclonal antibody directed against the Twist protein (Roth *et al.*, 1989) was used at a dilution of 1:5,000. The rabbit antibody directed against the Windbeutel protein (Ma *et al.*, 2003) was used at a dilution of 1:2,000. The primary antibodies were used in conjunction with a biotinylated goat antirabbit (1:500 diluted, preabsorbed against wildtype embryos) secondary antibody visualized with avidin/HRP complex (Vector Laboratories, Burlingame, CA).

β-Galactosidase Staining of Ovaries

Dissected ovaries from yeast-fed females expressing the UAST-directed lacZ under the control of various Gal4 drivers were fixed in 1% glutaraldehyde in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.2) for 15 min, followed by extensive washing in PBT (PBS, containing 0.1% Tween 20). Ovaries were then incubated in staining solution (150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 1 mM $MgCl_2$, 3.1 mM potassium ferrocyanide, 3.1 mM potassium ferricyanide, 0.3% Triton X-100, 0.2% X-Gal). Stained ovaries were mounted in a 1:1 mix of glycerol:PBS for photography.

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