

# Two-Color GFP Imaging Demonstrates Cell-Autonomy of GAL4-Driven RNA Interference in *Drosophila*

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RNA interference (RNAi), the specific inhibition of gene expression by homologous double-stranded RNA (dsRNA) (Fire *et al.*, 1998), has proven to be a broadly applicable approach for assessing loss of gene function in a variety of model organisms. An important characteristic of RNAi in plants (called posttransgenic gene silencing, or PTGS), the nematode *Caenorhabditis elegans*, and invertebrate tissue culture cells is that the inhibitory effect appears to cross cell boundaries. Systemic spreading of RNA interference was first described in plants (Palauqui *et al.*, 1997), where PTGS was shown to transmit from silenced tobacco graft tissue to an unsilenced host. Similar indications of the transmission of dsRNA-mediated gene-silencing have been obtained in animals. For example, gene expression is inhibited in the F<sub>1</sub> progeny of *C. elegans* soaked in dsRNA solutions (Tabara *et al.*, 1998) or fed dsRNA-expressing bacteria (Timmons *et al.*, 2001), and gene expression in the body wall of *C. elegans* can be inhibited by dsRNA transgenically produced in the pharynx (Winston *et al.*, 2002). Gene expression is also inhibited in cultured *Drosophila* cells exposed to conditioned culture medium from independently dsRNA-treated cells (Caplen *et al.*, 2000). In contrast to these results, our work using the GAL4-UAS system (Brand and Perrimon, 1993) to generate tissue-targeted, transgenically induced RNAi in *Drosophila* indicates that the effect in vivo of RNAi is cell-autonomous and confined to cells producing dsRNA.

We have tested the ability of GAL4-driven RNAi to cross cell boundaries in embryonic *Drosophila* by expressing dsRNA transgenically in a subset of cells in every segment and assaying its effect on a homologous, ubiquitously expressed marker transgene. The use of GAL4-based transgenic systems to induce tissue-specific RNAi in *Drosophila* has been described previously (Kenerdell and Carthew, 2000). Although subsequent reports have suggested that GAL4-mediated RNAi is limited to GAL4-expressing body regions (Giordano *et al.*, 2002; Kalidas and Smith, 2002), these have not explicitly investigated the autonomy of RNAi at a single cellular level following localized expression of dsRNA. Characterizing the cellular autonomy of transgenically induced RNAi, however, is critical to understanding both the experimental potential of this technique in *Drosophila* and the

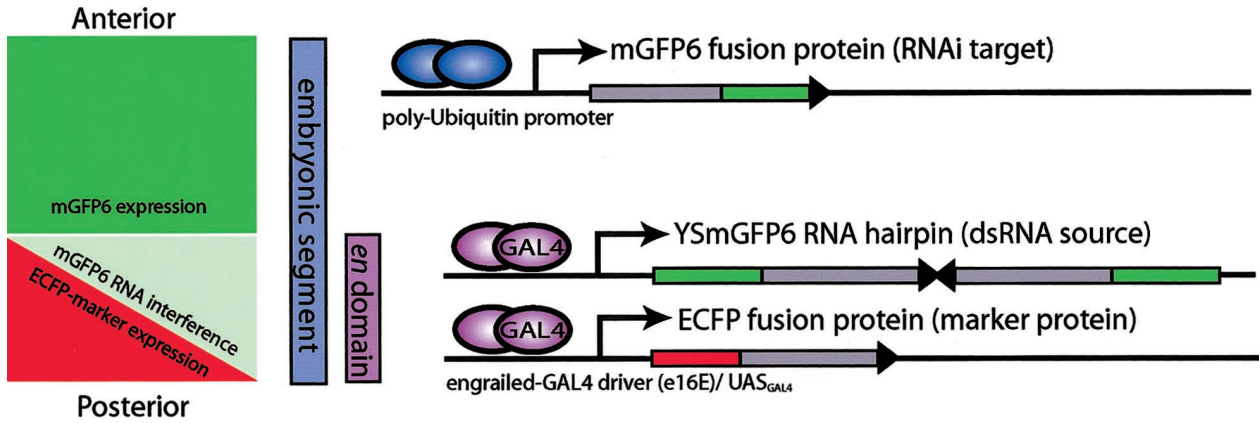
nature of RNA interference mechanisms in higher animals.

To induce RNAi in specific tissues, we use transgenic *Drosophila* carrying an invert-repeat (“hairpin”) sequence encoding both a variant of GFP (YSmGFP6; Haseloff, 1999) and an endogenous *Drosophila* gene (FKBP12, not relevant to this experiment) under the transcriptional control of UAS<sub>GAL4</sub> binding sites (*UAS-invert repeat(YSmGFP6-FKBP12)*). We drive expression of this sequence in embryonic stripes using an engrailed promoter-GAL4 driver line, en-GAL4<sup>c16E</sup> (AHB, K. Yoffe, N. Perrimon, unpublished; Fietz *et al.*, 1995). We assess the efficacy of induced RNAi by imaging the effect of YSmGFP6 dsRNA on expression of an mGFP6-fusion protein driven in all cells from the poly-ubiquitin promoter (*PUBq-D-TACC-mGFP6*; Gergely *et al.*, 2000). We simultaneously marked all dsRNA-expressing cells by using GAL4 to coexpress a blue-shifted cyan fluorescent protein fusion transgene (*UAS-ECFP-β-actin*), which due to codon alteration of ECFP should be unaffected by RNAi against mGFP6. This experimental approach is diagrammed in Figure 1.

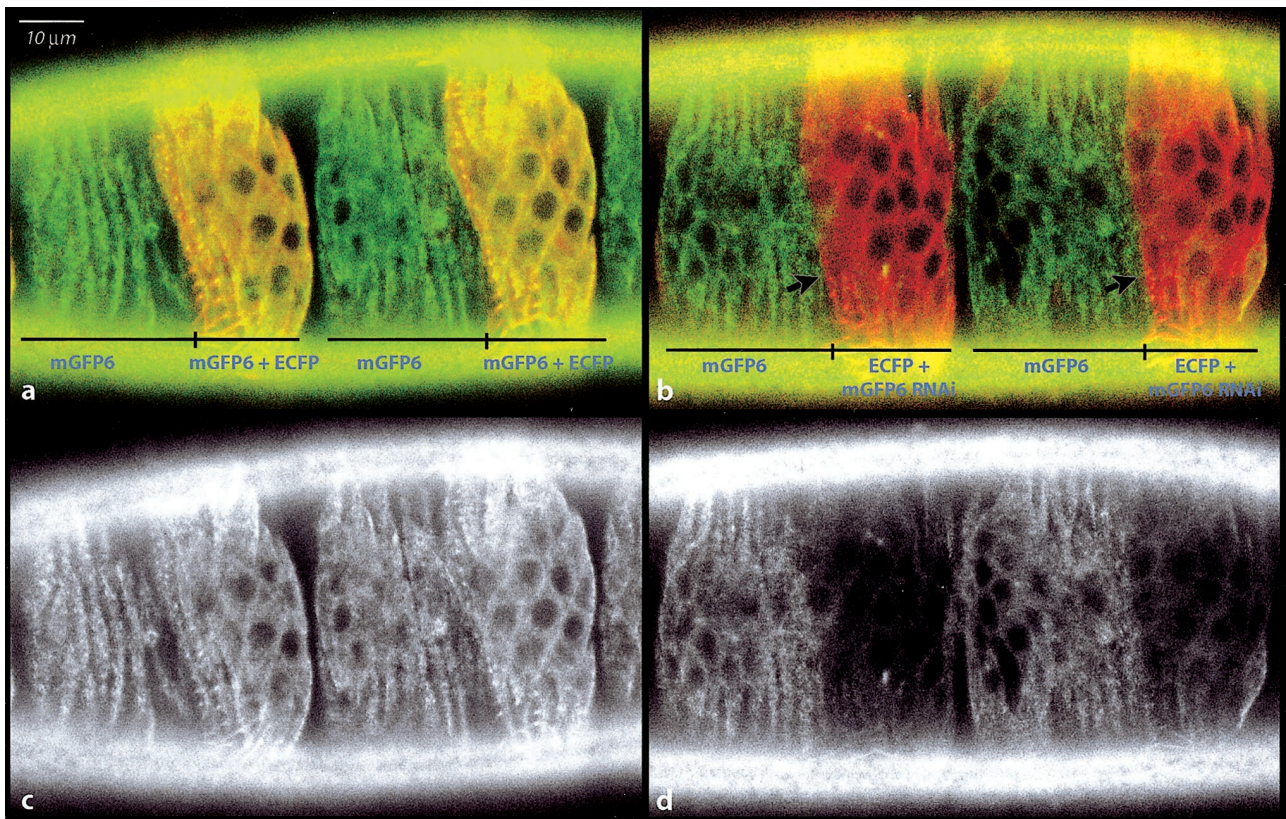
YSmGFP6 mRNA shares 98% nucleotide identity with mGFP6, differing at only 15 bases, and we observed accordingly that expression of *D-TACC-mGFP6* is potentially inhibited in YSmGFP6 dsRNA-expressing cells. In contrast, although the human codon-optimized coding sequence of ECFP (ClonTech, Palo Alto, CA) is 77% identical to that of YSmGFP6, ECFP-actin expression is not strongly affected by YSmGFP6 dsRNA (Fig. 2a,b). This confirms previously reported data on the high sequence-specificity of RNA interference in *Drosophila* embryos (Yang *et al.*, 2000). Furthermore, ECFP is blue-shifted in both excitation and emission spectra and can be imaged independently of mGFP6. All cells expressing dsRNA can thus be marked unambiguously. Independent

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**FIG. 1.** Experimental approach. The poly-ubiquitin promoter drives expression of an mGFP6 fusion protein in all cells of an embryonic segment (blue bar), while GAL4, expressed in the posterior of every segment by the *engrailed* (*en*) enhancer (purple bar), drives expression of a UAS transgene encoding a “hairpin” invert-repeat construct containing sequence homologous to mGFP6. The dsRNA produced by this transgene blocks mGFP6 expression. A second UAS transgene, also driven by GAL4, encodes an enhanced cyan fluorescent protein (ECFP) fusion protein. ECFP is codon-altered and thus not significantly inhibited by RNA interference against mGFP6. ECFP can be imaged independently of mGFP6 and serves to mark cells expressing dsRNA.



**FIG. 2.** dsRNA expression targeted by GAL4 to the posterior of all embryonic segments yields highly sequence-specific, cell-autonomous RNA interference (RNAi). **a:** Two segments of a Stage 16 embryo expressing an mGFP6 fusion protein (green) in all cells and an enhanced cyan fluorescent protein fusion protein (red) in the posterior engrailed domain of every segment (anterior is to the left). Overlapping expression appears yellow. **b:** Embryo similar to **a**, but also expressing dsRNA homologous to mGFP6 in all engrailed domain cells, making area of overlapping expression appear red only. **c:** Same embryo as shown in **a**, mGFP6 channel only (bleedthrough of ECFP signal makes engrailed domain cells slightly brighter). **d:** Same embryo as shown in **b**, mGFP6 channel only. mGFP6 expression is strongly reduced in engrailed domain, demonstrating effective RNAi. A sharp border (arrows in **b**) can be seen between those cells expressing dsRNA, where mGFP6 expression is reduced, and those not expressing dsRNA, where no reduction in mGFP6 expression is evident, thus confirming the cell-autonomy of induced RNAi. The effective expression of ECFP in mGFP6 RNAi-affected cells also demonstrates the sequence specificity of RNAi, as ECFP shares 77% nucleotide identity with the YSmGFP6-encoding dsRNA transgene, yet is not significantly affected by the dsRNA expression.

imaging of ECFP and mGFP6 reveals a clear border between ECFP- $\beta$ -actin marked cells expressing *YSmGFP6* dsRNA, in which *D-TACC-mGFP6* expression is reduced, and adjacent, non-ECFP marked cells, in which *D-TACC-mGFP6* expression is unaffected (Fig. 2d). Control embryos without the *UAS-YSmGFP6-FKBP12* transgene show no inhibition of D-TACC-mGFP6 expression in ECFP- $\beta$ -actin marked cells (Fig. 2c).

We have obtained similar results in the embryonic central nervous system of *Drosophila*, indicating that cell-autonomy is not a specific characteristic of epidermal cells, and we have obtained similar results showing cell-autonomy when using hairpin UAS transgenes other than the *YSmGFP6-FKBP12* construct described here (not shown). It remains possible that dsRNA might act non cell-autonomously when expressed in cells joined by specialized intercellular connections. Gap junctions, however, are present between ectodermal and epidermal cells in *Drosophila* embryos from Stage 6 onwards (Tepass and Hartenstein, 1994) and appear insufficient to mediate RNAi spreading. It has been reported that injection of dsRNA into the adult *Drosophila* abdomen can mediate RNA interference in the adult nerve cord (Dzitoyeva *et al.*, 2001). While our results do not preclude the presence in certain tissues of channels for the nonspecific uptake of dsRNA or other molecules mediating RNA interference, they demonstrate that the RNAi effect induced by targeted expression of dsRNA is cell-autonomous in our experimental context. Cell nonautonomy cannot be considered a general characteristic of dsRNA-mediated gene silencing.

## MATERIALS AND METHODS

### Transgene Construction

#### UAS-ECF- $\beta$ -actin.

BglII/BamHI-flanked PCR product amplified from pECFP (ClonTech) was ligated to BglII/BamHI-cut DNA encoding human cytoplasmic  $\beta$ -actin (pEGFP-human- $\beta$ -actin, ClonTech) and subcloned into BglII-cut pUASp (Rorth, 1998) to generate pUASp-ECFP- $\beta$ -actin.

#### UAS-invert-repeat(FKBP12-YSmGFP6).

A cDNA encoding FKBP12 (LD11775) was obtained from the Berkeley *Drosophila* Genome Project (BDGP). A BglII/HindIII PCR product amplified from LD11775 was ligated to HindIII/XhoI-cut DNA encoding YSmGFP6 (Haseloff, 1999) and subcloned into BglII/XhoI-cut pUAST (Brand and Perrimon, 1993), yielding pUAS-YSmGFP6-FKBP12. From this product EcoRI/XhoI and PstI/XhoI fragments of YSmGFP6-FKBP12 were subcloned into EcoRI/PstI-cut pBluescript SKII (Stratagene, La Jolla, CA). The resulting invert-repeat sequence was excised as a BglII fragment and ligated into BglII-cut pUAST, generating pUAS-invert-repeat(FKBP12-YSmGFP6). Recombination-deficient SURE *E. coli* (Stratagene) were used for propagating plasmids containing invert-repeat sequences. PCR primer sequences and further

cloning details are available from the authors upon request.

### Transgenesis and *Drosophila* Stocks

Transgenic *Drosophila* were generated by standard P-element transformation procedures (Spradling, 1986). The genotype of the experimental embryos was: y w, PUBq-D-TACC-mGFP6 / y w, UAS-invert-repeat(YSmGFP6-FKBP12); en-GAL4<sup>c16E</sup> / UASp-ECFP- $\beta$ -actin. Control embryos were: y w, PUBq-D-TACC-mGFP6 / Y; en-GAL4<sup>c16E</sup> / UASp-ECFP- $\beta$ -actin. All embryos were raised at 25°C.

### Two-Color Fluorescent Protein Imaging

ECFP and mGFP6 images were collected sequentially on a Leica TCS-SP laser scanning confocal microscope using a 63 $\times$  1.4 NA lens. ECFP was imaged with 458 nm laser excitation, a 465 nm longpass filter, and an emission collection window of 470–510 nm. mGFP6 was imaged using 488 nm laser excitation, a 525 nm longpass filter, and an emission collection window of 510–600 nm. Differential excitation is not sufficient for complete separation of ECFP and mGFP6 signals: some bleed-through ECFP is observed in the mGFP6 channel, and a small degree of bleed-through of mGFP6 is recorded in the ECFP channel.

### ACKNOWLEDGMENT

*Drosophila* carrying *PUBq-D-TACC-mGFP6* were a kind gift of Jordan Raff.

### LITERATURE CITED

- Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
- Caplen NJ, Fleenor J, Fire A, Morgan RA. 2000. dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene* 252:95–105.
- Dzitoyeva S, Dimitrijevic N, Manev H. 2001. Intra-abdominal injection of double-stranded RNA into anesthetized adult *Drosophila* triggers RNA interference in the central nervous system. *Mol Psychiatry* 6:665–670.
- Fitz J, Jacinto A, Taylor AM, Alexandre C, Ingham PW. 1995. Secretion of the amino-terminal fragment of the hedgehog protein is necessary and sufficient for hedgehog signalling in *Drosophila*. *Curr Biol* 5:643–650.
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–811.
- Gergely F, Kidd D, Jeffers K, Wakefield JG, Raff JW. 2000. D-TACC: a novel centrosomal protein required for normal spindle function in the early *Drosophila* embryo. *EMBO J* 19:241–252.
- Giordano E, Rendina R, Peluso I, Furia M. 2002. RNAi Triggered by symmetrically transcribed transgenes in *Drosophila melanogaster*. *Genetics* 160:637–648.
- Haseloff J. 1999. GFP variants for multispectral imaging of living cells. *Methods Cell Biol* 58:139–151.
- Kalidas S, Smith DP. 2002. Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. *Neuron* 33:177–184.
- Kennerdell JR, Carthew RW. 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat Biotechnol* 18:896–898.

- Palauqui JC, Elmayan T, Pollien JM, Vaucheret H. 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J* 16:4738-4745.
- Rorth P. 1998. GAL4 in the *Drosophila* female germline. *Mech Dev* 78:113-118.
- Spradling AC. 1986. P-element mediated transformation. In: Roberts DB, editor. *Drosophila*, a practical approach. Oxford: IRL Press. p 175-198.
- Tabara H, Grishok A, Mello CC. 1998. RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282:430-431.
- Tepass U, Hartenstein V. 1994. The development of cellular junctions in the *Drosophila* embryo. *Dev Biol* 161:563-596.
- Timmons L, Court DL, Fire A. 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263:103-112.
- Winston WM, Molodowitch C, Hunter CP. 2002. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295:2456-2459.
- Yang D, Lu H, Erickson JW. 2000. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr Biol* 10:1191-1200.