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Cell Ablation Using Wild-Type and Cold-Sensitive Ricin-A Chain in *Drosophila* Embryonic Mesoderm

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The use of genetic cellular ablation adds an important tool to the armoury of *Drosophila* geneticists. In particular, the gene products encoding the A chains of both Ricin (RTA) (Hidalgo and Brand, 1997; Moffat *et al.,* 1992) and Diphtheria (DTA) (Bellen *et al.,* 1992; Kunes and Steller, 1991; Lin *et al.,* 1995) toxins have been useful in describing a number of developmental phenomena. Cell-specific ablation using an amber suppressible DTA was used to assess the consequences of photoreceptor cell ablation for eye and optic lobe development (Kunes and Steller, 1991). The role of pioneer neurons in the developing CNS of embryos has also been analysed using both RTA (Hidalgo and Brand, 1997) and DTA chains (Lin *et al.,* 1995). In addition, further gene products have been described that will also kill cells by driving them into apoptosis, namely, *ice* (Shigenaga *et al.,* 1997), *hid*, and *reaper* (White *et al.,* 1996). By driving the *ice* gene complete eye ablation was achieved (White *et al.,* 1996). Such ablation systems provide potentially useful alternatives, as the consequences over the fate of a cell killed by DTA or RTA might well be different from those driven into apoptosis. There is, however, very little data to compare the efficacy of these killing methods. Recently, several ablation constructs expressing either *hid*, *rpr*, cold-sensitive Ricin, or the neuronal toxin tetanus have been compared for their ability to block neuronal activity at a behavioural level (Keller *et al.,* 2002). Keller *et al.,* (2002) were able to show that driving with some GAL4 lines *hid* and *rpr* killed flies before eclosion, while other neurons, e.g., leg campaniform sensilla, were largely resistant to their action. Further, they were able to demonstrate the exquisite selectivity of tetanus toxin to block chemical synapses within the central nervous system. In our laboratory we have previously described the isolation of temperature conditional mutations within the Ricin-A chain following mutagenesis in a yeast expression system (Moffat *et al.,* 1992). We reported its use in ablating photoreceptors under the control of a *sevenless* enhancer (Moffat *et al.,* 1992). In addition, we constructed transgenic flies carrying wild-type Ricin-A chain, where the expression of the toxin is conditional on an FLP-mediated event at FRT sites removing an upstream transcriptional stop signal (Smith *et al.,* 1996), similar to the "blue death" constructs reported by Lin *et al.,* (1995).

Here we report the generation of flies bearing the conditional cold-sensitive Ricin-A chain under UAS control and stocks carrying copies of the FLP-recombined version of the wild-type toxin that give stable control of RTA under UAS control. We have compared the activity of the two Ricin transgenes by using a mesoderm-expressing GAL4 line to drive the expression of both *Escherichia coli* LacZ and either cold-sensitive or wild-type RTA and assessing the effects of the RTA genes on expression of the LacZ reporter.

To place the cold-sensitive RAcs2 mutant allele (Moffat *et al.,* 1992) under GAL4 control, it was excised as a 1-kb *Kpn*I fragment from pJG2RAcs2 (Moffat *et al.,* 1992) and cloned into pUAST (Brand and Perrimon 1993) (Fig. 1a). Five transgenic lines were generated in a *w*;+;+ (Canton S) background. The line reported here demonstrated the strongest phenotypes and is referred to as UAS-RAcs2-39 and has a homozygous viable insertion on the third chromosome. To construct UAS-RTA (wild-type) lines, flies carrying UFWRTA (Smith *et al.,* 1996) were crossed to flies bearing an hsFLP source on the X chromosome. UFWRTA carries an RTA gene separated from the UAS controlled promoter by a *white* gene flanked by FRT sites, thus keeping the RTA gene silent. Following larval heat-shocks, white-eyed flies were recovered and stocks made. These flies carry RTA separated from the UAS by a single FRT site (Smith *et al.,* 1996) (Fig. 1b). Two such lines were generated, a homozygous lethal insertion UAS-RTA-1.1 on the second chromosome (used for all experiments reported below) and a homozygous viable insertion UAS-RTA-19 on the third chromosome.

The UAS ricin transgenes were crossed to the embryonic mesodermal driver GAL4-24B (Brand and Perrimon, 1993), which also carried a single copy of UAS-*lacZ*

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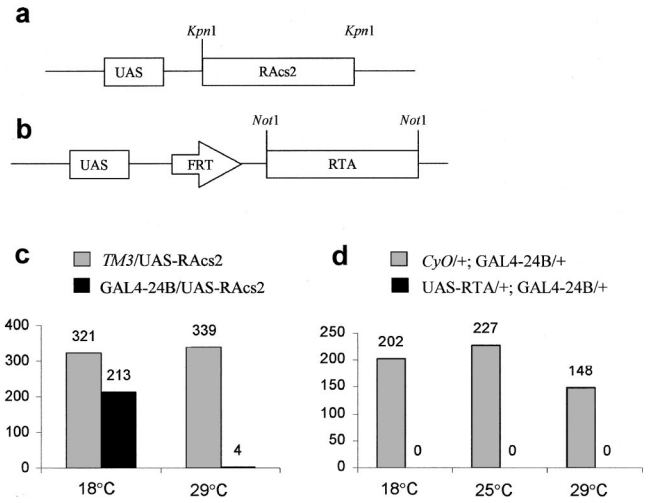

FIG. 1. a: Diagram showing the UAS-RAcs2 ablation construct used. **b:** Diagram of the UAS-RTA construct used after FLP-mediated recombination of UFWTRA (Smith *et al.,* 1996) to bring RTA under UAS control. **c:** Survival rates of flies expressing RAcs2. Flies of genotype *w*; GAL4-24B, UAS-*lacZ*/*TM3*, *Sb* were crossed to *w*; UAS-RAcs2/*TM3, Sb* and adult progeny counted. Numbers of progeny containing RAcs2 with either *TM3* (orange-eyed, Stubble) or GAL4-24B (red-eyed, non-Stubble), expected in equal numbers, are plotted. **d:** Survival rates of flies expressing RTA. Flies of genotype *w;* UAS-RTA/*CyO* were crossed to *w*; GAL4-24B, UAS-*lacZ*/*TM3*, *Sb* and progeny counted at 18, 25, and 29°C. Progeny containing GAL4-24B with either *CyO* or UAS-RTA, expected in equal numbers, are plotted. The latter class are clearly absent at all temperatures.

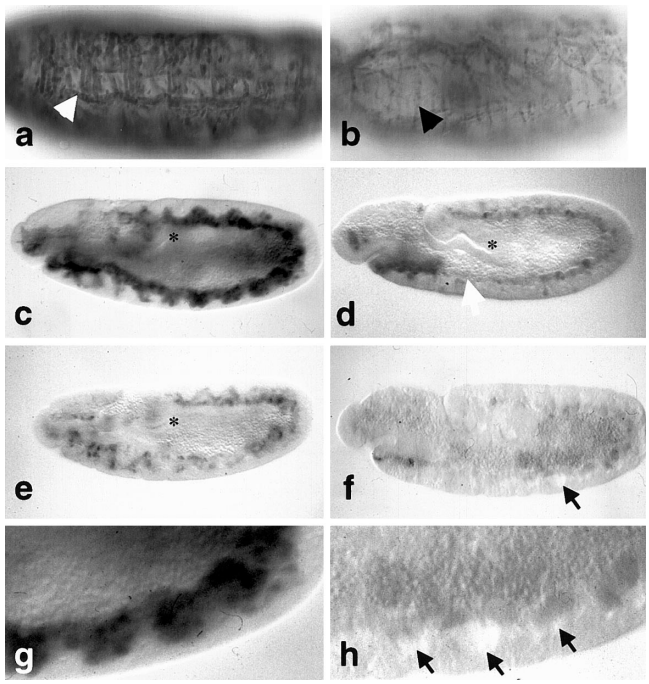

FIG. 2. Mesodermal *lacZ* expression in ablated embryos. Embryos from the crosses detailed in Figure 1 were processed for immunocytochemistry and LacZ was detected using rabbit anti-LacZ antibody (Cappel) and biotinylated goat antirabbit secondary antibody (Vector, Burlingame, CA) followed by horseradish peroxidase and diaminobenzidine detection. **a:** GAL4-24B, UAS-*lacZ* control embryo at stage 17 showing expression of the *lacZ* reporter in musculature of the mature embryo. The white arrowhead points to the ventral internal/external oblique muscles, visible laterally on the body wall just below the pleural external transverse muscles. **b:** GAL4-24B, UAS-*lacZ*/UAS-RAcs2 embryo, raised at 29°C, at stage 17. There is an obvious reduction in LacZ expression, although some is clearly still detectable. Arrow shows what appears to be the pleural internal transversal muscle along the segment borders. The ventral oblique muscles, however, appear to be missing. **c:** GAL4-24B, UAS-*lacZ* control embryo, stage 11. **d:** GAL4-24B, UAS-*lacZ* UAS-RTA embryo, stage 11, 18°C. **e:** GAL4-24B, UAS-*lacZ* UAS-RTA embryo, stage 11, 25°C. **f:** GAL4-24B, UAS-*lacZ* UAS-RTA embryo, stage 11, 29°C. **g:** Close-up of the control embryo from (**c**) showing LacZ expression in the mesoderm of the posterior ventral part of the embryo. **h:** Close-up of ablated embryo in (**f**), showing clear presence of vacuoles, marked with arrows. Stage 11 was judged by the appearance of the parasegmental furrows and the position of the posterior midgut (*marked). In (**d**) and (**e**) there is already a marked reduction in levels of LacZ product (white arrow), at 29°C in (**f**) there is hardly any detectable. At 29°C embryos appear not to have any mesoderm, as judged by the lack of LacZ expression. They are marked with the presence of vacuoles, seen more clearly in (**h**); compare to (**g**).

recombined on the same chromosome. Expression of UAS-RAcs2 in this line killed all but a few adult escapers at 29°C (<1%), while at 18°C about 66% of expected numbers were recovered (Fig. 1c). Earliest lethal phase analysis revealed that at 29°C flies were dying as embryos. A small number of embryos (51) from a cross of GAL4-24B/TM3 × UAS-RAcs2 collected at 25°C and shifted immediately to 29°C for the remainder of embryogenesis led to a 47% hatch rate. The surviving larvae (24) were then collected and placed at 18°C for the remainder of development. All these larvae survived to adulthood and were of the genotype GAL4-24B/TM3, implying that the consequence of embryonic mesodermal expression at the permissive temperature for RAcs2 is embryonic lethal. However, killing is not complete, as adult flies counted from this cross at 29°C, in a large sample, showed a survival rate of about 1% of that expected in the absence of any toxicity (Fig. 1c). No survivors were recorded after expression of UAS-RTA at any temperature (Fig. 1d) and we presumed this was due to embryonic lethality as well.

Analysis of LacZ expression was used to assess the extent of mesoderm development after expression of the cold-sensitive and wild-type RTA genes. Development appeared normal when RAcs2 was expressed continually at 18°C. However, continual expression of the RAcs2 toxin at 29°C resulted in a severe loss of musculature, as judged by LacZ immunocytochemistry in late stage (16+) embryos (Fig. 2b). This explained the lack of hatching larvae as no obvious defects were seen in the mesoderm of early embryos. In contrast, expression of RTA caused a severe reduction of LacZ protein in embryonic mesoderm at the extended germband stage at all temperatures. The germband failed to retract and often

the majority of the mesoderm appeared to be absent at 29°C (Fig. 2f). Ablation by RTA showed temperature sensitivity with progressively less severe defects found at 25°C and 18°C (Fig. 2d,e). Thus, the most likely explanation is that the expression of the RTA toxin is affected by the increased activity of the GAL4 protein at higher temperatures.

In summary, the RAcs2 protein is most likely a weakly active toxin (as judged here and by biochemical assays in yeast and tissue culture cells; data not shown), compared to its native counterpart, whose expression is also presumably affected by temperature effects on GAL4 activity. Our data suggest the killing time for RAcs2 is thus likely to be on the order of many hours or days. In other experiments using an adult brain driver in the mushroom bodies, P{GAL4}A107, a reduction in LacZ activity was seen only after several days at the permissive temperature for the RAcs2 toxin (data not shown). In contrast, UAS-RTA is a more powerful cell ablation tool. The detection of expression of the LacZ reporter from the GAL4-24B driver begins at late stage 9 / early stage 10, while in flies coexpressing RTA the mesodermal cells are largely removed by estimated early stage 11. This gives an estimated killing time of less than an hour, albeit without the ability for activity of the protein to be modulated by temperature.

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