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even skipped is required to produce a *trans*-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone

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SUMMARY

Development of a multicellular organism requires precise coordination of cell division and cell type determination. The selector homeoprotein Even skipped (Eve) plays a very specific role in determining cell identity in the *Drosophila* embryo, both during segmentation and in neuronal development. However, studies of gene expression in *eve* mutant embryos suggest that *eve* regulates the embryonic expression of the vast majority of genes. We present here genetic interaction and phenotypic analysis showing that *eve* functions in the *trol* pathway to regulate the onset of neuroblast division in the larval CNS. Surprisingly, Eve is not detected in the regulated neuroblasts, and culture experiments reveal that Eve is required in the body, not the CNS. Furthermore, the effect of an *eve* mutation can be rescued both in vivo and in culture by the hormone ecdysone. These results suggest that *eve* is required to produce a *trans*-acting factor that stimulates cell division in the larval brain.

Key words: *eve*, *trol*, Ecdysone, Neuroblast, Proliferation, *Drosophila*

INTRODUCTION

Proper spatiotemporal regulation of cell cycle progression is essential for the successful development of multicellular organisms. Many evolutionarily conserved cell cycle regulators have been identified in both vertebrate and invertebrate systems. Although their functions in cell cycle progression have been extensively analyzed, relatively little is known about how the cell cycle is regulated during development, especially in vivo. Several studies in *Drosophila melanogaster* provide us with a glimpse into the developmental regulation of the cell cycle. The coordination of embryonic cell division in mitotic domains foreshadows cell fate domains, as revealed by fate mapping studies (Foe, 1989). The timing of cell division coincides with the patterning and differentiation of imaginal discs (reviewed by Edgar and Lehner, 1996). Alterations of the cell cycle period or the number of cycles can change the expression patterns of genes that determine neuronal identity (Cui and Doe, 1995; Weigmann and Lehner, 1995). Thus, regulation of the cell cycle is closely related to the regulation of pattern formation and to differentiation during development.

The central nervous system (CNS) of the fruit fly, *Drosophila melanogaster*, is an excellent system in which to study genetic control of the cell cycle in the context of development. The CNS contains several populations of neuronal precursor cells called neuroblasts with characteristic profiles of cell cycle progression during development (Hofbauer and Campos-Ortega, 1990; Ito and Hotta, 1991; Truman and Bate, 1988; White and Kankel, 1978). The adult CNS is formed by periods of neurogenesis during embryonic and larval stages. Larval phase neurogenesis occurs in a stereotyped spatial and temporal pattern as mitotically quiescent larval neuroblasts reactivate cell cycle progression (Fig. 1). Both observations of quiescent neuroblasts soon after hatching (Truman and Bate, 1988) and classical mammalian studies (Pardee, 1989), suggest that neuroblasts may initially be arrested in G0, and activate division by proceeding into G1 and then into S phase. The regulated neuroblasts are divided into sub-groups, depending on the developmental fate of their progeny and their kinetics of proliferation. Optic lobe neuroblasts stop cell division at embryonic cell cycle 17 (Campos-Ortega and Hartenstein, 1985). They remain quiescent until late first instar when they reactivate cell division and continue to divide until the pupal stage (White and Kankel, 1978). The central brain neuroblasts start cell division by mid-embryogenesis and become quiescent in late embryogenesis. They reenter the cell cycle at late first instar (Campos-Ortega and Hartenstein, 1985). Only the four mushroom body neuroblasts and one lateral neuroblast located at the ventrolateral side of each hemisphere begin cell division in embryogenesis and continue to divide through larval life (Ito and Hotta, 1991).

Several genes have been identified that affect neuroblast proliferation (Datta and Kankel, 1992; Ebens et al., 1993; Lipshitz and Kankel, 1985; Prokop and Technau, 1994),

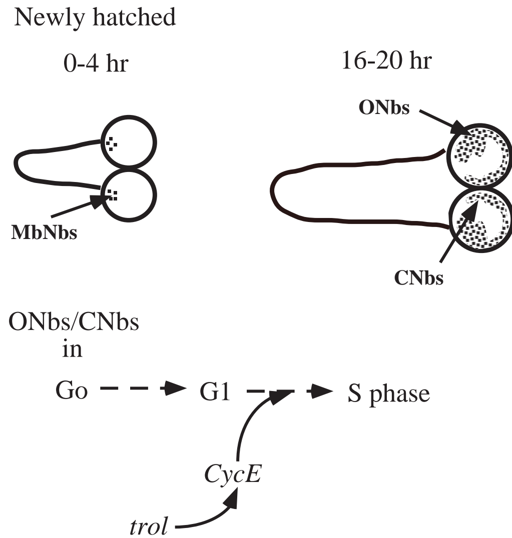

Fig. 1. Neuroblast division in the larval CNS. (A) Mushroom body neuroblasts (MbNbs) divide at 0-4 hours post hatching (ph). (B) Central brain (CNbs) and optic lobe (Onbs) neuroblasts divide by 16-20 hours ph. (C) *trol* is postulated to stimulate cell cycle progression in CNbs and Onbs by increasing expression of the cell cycle regulator Cyclin E (CycE).

including *anachronism* (*ana*), *terribly reduced optic lobes* (*trol*) and *even skipped* (*eve*). *trol* was originally identified in a genetic screen for abnormal larval brain morphology that was due to defective patterns of neuroblast proliferation in the larval brain (Datta and Kankel, 1992). Mutations in *trol* cause a dramatic decrease in the reactivation of proliferation from mitotic quiescence (Datta, 1995). Recent studies suggest that *trol* may regulate this reactivation of neuroblast proliferation by stimulating the G1/S transition through upregulation of *Cyclin E* (*CycE*) expression (Caldwell and Datta, 1998). Several studies on *trol* and *ana* have led to the hypothesis that *trol* is required to overcome the repression of neuroblast cell division imposed by *ana* (Datta, 1995; Datta and Kankel, 1992; Ebens et al., 1993). *eve*, a homeodomain-containing transcriptional repressor, was identified in a screen for enhancers of the hypomorphic allele $trol^{b22}$ (Park et al., 1998). Mutations in *eve* enhanced both the $trol^{b22}$ proliferation phenotype and the associated lethality, indicating that *eve* may regulate transcription of cell cycle genes in the *trol* pathway.

eve plays a key role in many cell fate decisions in the developing embryo, ranging from segmentation to neuronal identity. While the role of *eve* in the determination of specific neuronal identity in the embryonic CNS appears to be part of a cell-autonomous cascade of transcription factors, its function during earlier embryonic segmentation is mediated, in part, by regulation of the localized signaling factors Hedgehog and Wingless (reviewed by Akam, 1987). Other studies have suggested that *eve* plays a direct role in controlling transcription of several genes, including *Adh* and *ry* (Liang and Biggin, 1998). The contrasting views of *eve* as a specific developmental regulator versus *eve* as a general transcriptional factor have yet to be resolved.

Another factor implicated in the developmental coordination of cell division is the hormone ecdysone. Ecdysone plays a role in the initiation of imaginal histoblast division and the proliferation of post-embryonic neuroblasts in *Manduca* and *Drosophila* (Champlin and Truman, 1998). Ecdysone is also required for the activation of mitotically quiescent neuroblasts in explanted *Drosophila* larval CNS, but addition of ecdysone does not rescue the proliferation phenotype of *trol* mutant CNS in culture (Datta, 1999).

We present evidence that *eve* is required for production of a *trans*-acting signal that regulates activation of neuroblast proliferation and can be mimicked by ecdysone. We demonstrate that loss of *eve* function produces increased lethality and cell cycle arrest that is consistent with *eve* function in the *trol* pathway, and that *eve* function requires an intact C-terminal domain, in addition to the homeodomain and a repression domain. Importantly, both Eve distribution within the larval CNS and neuroblast division in a heterogenetic explant/extract system show that *eve* expression is not required in the regulated neuroblasts, or even in the larval CNS, to stimulate cell division, but instead is required in some other tissue(s). Furthermore, addition of ecdysone either in vitro or in vivo rescues the defective neuroblast proliferation caused by a mutation in *eve*. These studies reveal that heterozygous mutations in *eve* in one part of the larva can affect the generation of a signal that impacts cellular events in a separate organ of the developing fruit fly.

MATERIALS AND METHODS

Genetic strains and transgenes

Flies were grown in standard medium at 25°C. Markers and balancer chromosomes are described in Lindsley and Zimm (Lindsley and Zimm, 1992). $trol^{b22}$ and $trol^{sd}$ have been previously described (Datta, 1995; Datta and Kankel, 1992; Shannon et al., 1972). The $trol^{4}$ and $trol^{8}$ alleles were isolated from independent mutageneses (S. D., M. C. C., M. M. R., C. R., Y. P. and S. M., unpublished). $trol^{b22}$, $trol^{4}$, $trol^{8}$ and $trol^{sd}$ mutant animals were obtained from *y* $trol^{b22}$ stock and from *y* $trol^{x}$ *w/Binsn* stocks. *y* $trol^{b22}$*; hs-CycE* flies were constructed in our laboratory from a *y* $trol^{b22}$ and a *hs-CycE* stock described previously (Caldwell and Datta, 1998). Hemizygous *y* $trol^{x}$ *w/Y;* eve^{3}/+ larvae were obtained by mating of *y* $trol^{x}$ *w/Binsn* virgin female flies to eve^{3}*/CyO* y^{+} male flies. Elliott Goldstein has generously provided *eve* mutations $eve^{58\text{-}11}$, $eve^{14\text{-}10}$, $eve^{20\text{-}35}$ and $eve^{10\text{-}5}$. The eve^{5}*/CyO, hb-lacZ* stock was used to distinguish homozygous eve^{5} from heterozygous embryos.

The parental *eve* transgene, *P[eve+]*, capable of fully rescuing *eve* null mutants, was described previously (Fujioka et al., 1999). Derivatives of this construct (Kobayashi, et al., 2001) contain the following alterations in the LFKPY motif near the C terminus of the protein-coding region (see Table 3): EGNΔLFK, a STOP codon inserted just before LFKPY, which removes the C-terminal 10 amino acids; EGNHA, the Hairy family Groucho interaction motif WRPW in place of FKPY; EGNPA in place of LFKP<u>A</u>; EGNAY in place of LFK<u>A</u>Y.

trol lethality screening

Virgin homozygous *y* $trol^{b22}$ females were mated to males carrying different *eve* mutations. The progeny of each population were counted, and the ratio of *y* $trol^{b22}$*/Y;* eve^{x}/+ to *y* $trol^{b22}$*/Y; CyO/+* was calculated. The closer the ratio is to 0, the stronger the enhancement of $trol^{b22}$ lethality. Because heterozygosity at *eve* alone could impact viability, males from the same genotype were also crossed to *Canton S* females as a control. Virgin homozygous *y* $trol^{b22}$*; hs-CycE* females were mated to males from eve^{3}*/CyO,* y^{+} to ask if ectopic *CycE*

expression can rescue the enhanced lethality of hemizygous *y trol*^{b22}/*Y; eve*^{3}/+ animals.

BrdU incorporation and neuroblast counting

5′-bromodeoxyuridine (BrdU) incorporation was analyzed as previously described (Datta, 1995; Park et al., 1998). The average number of BrdU-labeled neuroblasts in sibling controls was calculated and used for normalization of neuroblast proliferation levels between experiments. Control normalized proliferation ranged from 0.8 to 1.3 in almost all genotypes. The number of labeled neuroblasts in each mutant population was also normalized by the average of sibling controls to determine if mutations in specific genes result in proliferation defects. If mutation(s) cause a proliferation phenotype, the population distribution shifts to lower values. At least three independent crosses were analyzed for each genotype.

Embryo analysis and cuticle preparation

In situ hybridization was performed as described (Tautz and Pfeifle, 1989) using digoxigenin-labeled antisense *engrailed* probe visualized via the alkaline phosphatase reaction with NBT/CIBP. In situ hybridization was followed by staining with anti-Eve antiserum (provided by M. Frasch). The antibody was visualized via the horse radish peroxidase reaction with DAB, as described (Mullen and DiNardo, 1995). For cuticle preparations, after devitellinization, embryos were mounted in a 1:1 mixture of Hoyer's reagent and 30% lactic acid, then cleared by incubation at 55°C.

Sequence analysis, immunohistochemistry and Western analysis of *eve*^{5}

PCR was done as previously described (Park et al., 1998). PCR products of mutated *eve*^{5} DNA were isolated and cut with *Eco*RI and *Spe*I. The resulting PCR fragments were cloned into the pBluescript vector and sequenced on both strands.

Expression of Eve proteins in larval brains and embryos was monitored by immunohistochemical analysis with mouse monoclonal 2B8 antibody (provided by N. Patel) and guinea pig anti-Eve polyclonal antibody (provided by D. Kosman). Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115-035-003) or Alexa™ 488-conjugated goat anti-mouse IgG (Molecular Probe, A-11001). Homozygous *eve*^{5} embryos were identified by the lack of staining with rabbit anti-β galactosidase antibody (Chemicon, AB986) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-003). Western analysis of Eve proteins in embryos was done as follows: 2-4 hour old embryos were dechorionated, protease inhibitor cocktail (Roche, 1-836-153) and 2× Laemmli sample buffer were added to 1× strength, and the sample was homogenized. Whole homogenate was centrifuged for 30 seconds at 14,000 ***g*** and the supernatant analyzed. Eve was detected either by guinea pig anti-Eve polyclonal antibody or by mouse monoclonal 3C10 antibody (DSHB).

Ecdysone feeding

1 mg/ml 20-hydroxyecdysone (Sigma) in BrdU-containing medium (Caldwell and Datta, 1998; Datta, 1995; White and Kankel, 1978) was used for ecdysone feeding and BrdU labeling from 16-20 hours posthatching. Plain media was used from 0-16 hours post hatching. For ecdysone feedings from 0-16 hours post hatching, 20 μl of 1 mg/ml 20-hydroxyecdysone in 10% isopropanol were mixed with 1 mg of dry yeast. Newly hatched larvae were placed in the middle of the yeast paste to maximize probability of feeding.

Preparation of larval extract and explant culture

Larval extracts from stocks of different genotypes were prepared from first instar larvae as previously described (Datta, 1999). Extracts were heat-treated after preparation and stored at –70°C. Explant culture analysis of different genotypes was as described (Datta, 1999).

Statistical analysis

Standard error of the mean was calculated. The significance of differences between the mean response indices of two different populations was evaluated using the Student's *t*-test.

RESULTS

eve transheterozygotes show defective neuroblast proliferation

We have previously shown that *eve* is a dominant enhancer of *trol*^{b22}, and that heterozygosity for strong *eve*^{3} or *eve*^{4} mutations does not cause defective proliferation (Park et al., 1998), suggesting that *eve* is in the *trol* pathway. If so, homozygous *eve* mutations might cause a proliferation phenotype in the larval CNS. Complementation analysis showed that a very small number of *eve*^{1}/*eve*^{5} transheterozygous flies survived to adulthood (O'Brien et al., 1994). This enabled us to ask if *eve*^{1}/*eve*^{5} larval brains exhibit defective proliferation. *y w/Y; eve*^{1}/*CyO, y*$^{+}$ flies were crossed to *y w; eve*^{5}/*CyO, y*$^{+}$ females, and *eve*^{1}/*eve*^{5} mutants were selected as *yellow* larvae. *eve*^{1}/*eve*^{5} transheterozygotes show decreased BrdU labeling, resulting in a shift of normalized BrdU incorporation from control values (ranging from 0.8 to 1.2 in sibling *eve*^{1}/+ *or eve*^{5}/+ animals, Fig. 2A,C) to lower values (ranging from 0.4 to 0.9 in *eve*^{1}/*eve*^{5} mutants, Fig. 2B,D). Comparison of average normalized proliferation reveals a significant reduction in *eve*^{1}/*eve*^{5} transheterozygotes (0.57±0.04, *n*=19) versus controls (1.00±0.02, *n*=24).

New *eve* alleles define a threshold for enhancement of *trol*^{b22} lethality

Four new *eve* alleles (Bour et al., 1995) were assayed for their ability to enhance the lethality of *trol*^{b22}. The phenotypic strengths of the new alleles were assayed by examining both their embryonic cuticular phenotypes and the expression patterns of *engrailed* (*en*) transcripts at earlier stages (Fig. 3). *eve*$^{58-11}$ showed a null cuticular phenotype (Fig. 3C) and a severely defective *en* expression pattern (Fig. 3D), consistent with the lack of Eve staining, and also strongly enhanced *trol*^{b22} lethality when heterozygous (Table 1). *eve*$^{14-10}$ showed a hypomorphic cuticular phenotype, clearly weaker than that of *eve*$^{58-11}$ (Fig. 3E), and the expression patterns of *en* and Eve showed correspondingly milder defects (Fig. 3F). Both *eve*$^{10-5}$ and *eve*$^{20-35}$ showed weak hypomorphic cuticular

Table 1. Dominant enhancement of *trol*^{b22} lethality by new *eve* alleles

Genotype of cross (male × female)	Male			Female		
	eve^{x}/+	*CyO*/+	Ratio*	*eve*^{x}/+	*CyO*/+	Ratio*
eve$^{58-11}$/*CyO* × *trol*^{b22}	0	74	0.00	28	357	0.08
eve$^{58-11}$/*CyO* × CS	66	81	0.81	84	92	0.91
eve$^{14-10}$/*CyO* × *trol*^{b22}	283	295	0.96	977	958	1.02
eve$^{14-10}$/*CyO* × CS	253	238	1.06	272	294	0.93
eve$^{10-5}$/*CyO* × *trol*^{b22}	118	119	0.99	347	325	1.07
eve$^{10-5}$/*CyO* × CS	90	89	1.0	106	114	0.93
eve$^{20-35}$/*CyO* × *trol*^{b22}	67	73	0.92	310	318	0.91
eve$^{20-35}$/*CyO* × CS	94	99	0.95	77	110	0.70

*Ratio, (number of *eve*^{x}/+ flies)/(number of *CyO*/+ flies).

phenotypes (Fig. 3G,I), and the *en* and Eve patterns were closer to wild type (Fig. 3H,J). eve^{10-5}, eve^{20-35} and eve^{14-10} did not enhance $trol^{b22}$ lethality. Taken together, the embryonic phenotypes and the interactions with $trol^{b22}$ suggest that enhancement of $trol^{b22}$ lethality requires a major reduction in *eve* function, close to 50% (i.e. that produced by a null/+ *eve* genotype).

eve^3 enhances the proliferation phenotype of two *trol* alleles

We tested two *trol* alleles of intermediate strength, $trol^4$ and $trol^8$, for their ability to be enhanced by mutations in *eve*. $trol^4$ and $trol^8$ are independent lethal alleles, and both $trol^4/trol^{b22}$ and $trol^8/trol^{b22}$ are viable (S. D., M. C. C., M. M. R., C. R., Y. P. and S. M., unpublished). Analysis of larval neuroblast proliferation in *y* $trol^4$ *w/Y* brains revealed a bimodal distribution. BrdU labeling was within control values in 43% of the samples (19 out of 44, ranging from 0.78 to 1.2), and was decreased in 57% of the samples (25 out of 44, ranging from 0.29 to 0.76, Fig. 2E). In contrast, heterozygosity of eve^3 in a $trol^4$ background caused defective proliferation in 100% of the samples (total of 22 brains, Fig. 2F). Hemizygous *y* $trol^8$ *w/Y* animals had a much weaker proliferation phenotype than did $trol^4$ mutants. Only 23% (6/26) exhibited defective proliferation, with ratios ranging from 0.28 to 0.76 (Fig. 2G). The remaining samples (20/26) showed proliferation consistent with wild-type levels. Introduction of a heterozygous eve^3 mutation in a $trol^8$ background increased the number of brains with defective proliferation to 88% (21/24) (Fig. 2H). Thus, like $trol^{b22}$, both intermediate *trol* alleles are significantly enhanced by eve^3.

Ectopic expression of *cyclin E* can rescue the enhanced neuroblast proliferation phenotype

Ectopic expression of *cyclin E* from a *hs-CycE* transgene consistently rescues the defective neuroblast proliferation in $trol^{sd}$ first instar larval CNSs (Caldwell and Datta, 1998). To determine if expression of *CycE* would also rescue the increased neuroblast arrest of *y* $trol^{b22}/Y$; $eve^3/+$ animals, we examined the effect of expression from a *hs-CycE* transgene. In *y* $trol^{b22}/Y$; $eve^3/+$ animals, weak over expression of *CycE* (no heat shock) partially rescued the neuroblast phenotype. Without heat induction only 35% (7/20) of *y* $trol^{b22}/Y$; $eve^3/+$ animals (Fig. 4E,F) carrying the *hs-CycE* transgene showed defective proliferation compared to 96% (26/27) of *y* $trol^{b22}/Y$; $eve^3/+$ animals without the transgene (Fig. 4C,D). Furthermore, strong induction of *CycE* expression with a 30 minute heat shock rescued the neuroblast phenotype almost completely (96%, 22/23; Fig. 4G,H). Interestingly, weak induction of *CycE* did not rescue the enhanced lethality of *y* $trol^{b22}/Y$; $eve^3/+$ animals.

eve protein is not expressed in larval brain neuroblasts

We examined the distribution of *eve* protein in the brains of wild-type early, mid and late first instar larvae using immunohistochemistry. The distribution of Eve protein within the CNS did not change appreciably during first instar. Eve protein is expressed in the CNS mainly in the thoracic region (Fig. 5A,B). *eve*-expressing cells appear to be mostly ganglion mother cells and neurons as characterized by their smaller size in comparison with thoracic neuroblasts and their more internal position on the ventral side of the thoracic ganglion. A few ganglion mother cells and neurons in the central brain region

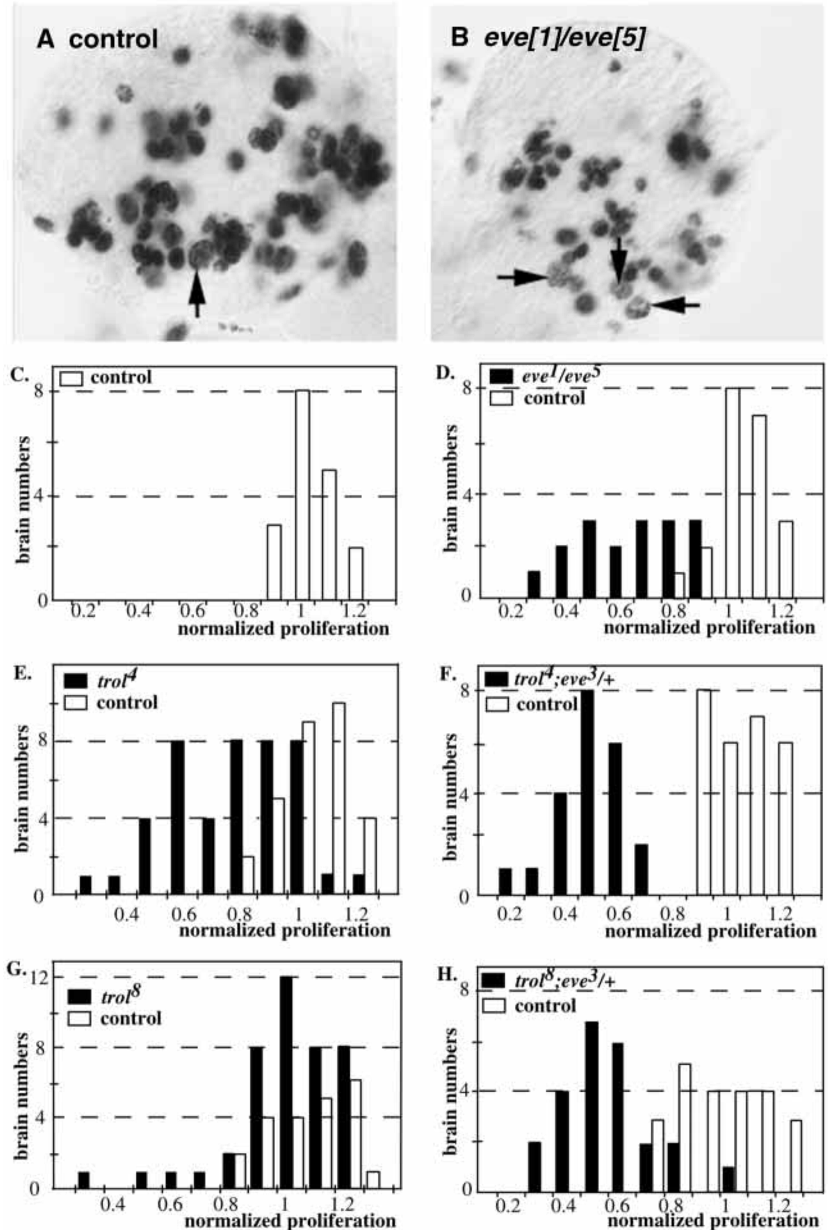

Fig. 2. Proliferation in *eve*, *trol* and *trol;eve/+* mutants. BrdU incorporation from 16-20 hours ph. Arrows indicate labeled neuroblasts. (A) Control brain lobe. (B) eve^1/eve^5 brain lobe. Quantitation of the number of BrdU-labeled neuroblasts in mutant and control samples. White bars indicate control samples. Black bars indicate mutant samples. (C) Control brains. (D) eve^1/eve^5. (E) $trol^4$. (F) $trol^4;eve^3/+$. (G) $trol^8$. (H) $trol^8;eve^3/+$.

expressed Eve protein, but no cells in the optic lobe proliferation center stained positively for Eve (Fig. 5A, B). Cells in the brain lobes do stain for engrailed protein (Fig. 5C), indicating that penetration of the tissue is not an issue. Thus, Eve does not appear to be expressed in larval neuroblasts close to the time when their proliferation is affected by *eve* and *trol* mutations.

In an explant proliferation assay, *eve* function is required in the larval extract

The expression pattern of *eve* in the larval brain suggested that Eve protein might act in some other tissue to affect neuroblast proliferation in the brain itself. This hypothesis was tested in vitro using a brain explant culture system (Table 2) (Datta, 1999). When wild-type (CS) brains were cultured with wild-type extract, 39% (7/18) of the samples had numbers of dividing neuroblasts that were similar to those observed in vivo. When *y* $trol^{b22}/Y; eve^3/+$ CNSs were cultured with extract from *y* $trol^{b22}/Y; eve^3/+$ first instar larvae, just 7.8±1.3% of the samples (4/53 total) showed in vivo levels of neuroblast proliferation, a percentage comparable to that observed in *y* $trol^{b22}/Y; eve^3/+$ animals in vivo. Similarly, only 6.3±1.3% (4/66 total) of brains derived from *y* $trol^{b22}/Y; eve^3/+$ animals showed normal neuroblast proliferation when cultured with $eve^3/+$ extract, consistent with a locus of action of *trol* within the neuroblasts. In sharp contrast, 36% (9/25) of *y* $trol^{b22}/Y; eve^3/+$ brains cultured with wild-type (CS) extract had levels of neuroblast proliferation within normal in vivo levels. These levels are close to those of wild-type brains cultured with wild-type extract (above), suggesting that *eve* is not functioning within the brain to affect proliferation. Strikingly, when $trol^{b22}$ mutant brains (wild type for *eve*) were cultured in extract derived from $eve^3/+$ first instar larvae (wild type for *trol*), none of the samples (0/42 total) showed normal activation of neuroblast division, showing that *eve* affects the extract.

Addition of ecdysone rescues defective proliferation

Previously, we have shown that the addition of ecdysone to the medium in the brain explant culture system is required for the activation of neuroblast proliferation (Datta, 1999). This led us to ask if ecdysone would suppress the *eve*-mediated loss of neuroblast proliferation. When *y* $trol^{b22}/Y; eve^3/+$ brains were cultured in *y* $trol^{b22}/Y; eve^3/+$ extract, to which 10 μg/ml ecdysone had been added, 40±2.7% (27/67 total, Table 2) of the samples revealed normal activation of neuroblast division. This is strikingly similar to the results for wild-type brains cultured with wild-type extract (see above). These results were confirmed in vivo by feeding studies. When *y* $trol^{b22}/Y; eve^3/+$ animals were fed 1 mg/ml ecdysone from 0-20 hours after hatching, the number of samples showing neuroblast proliferation within the control range rose from 4% (1/27) (Fig. 6A,B) to 90% (27/30) (Fig. 6E,F). Interestingly, while 68% (15/22) of *y* $trol^{b22}/Y; eve^3/+$ animals fed ecdysone from 0-16 hours (Fig. 6C,D) showed normalized neuroblast proliferation levels that were within the control range (0.8-1.2), all of the samples (14/14) from animals fed ecdysone from 16-20 hours showed normal proliferation levels, indicating that ecdysone addition is most effective during the last four hours of the treatment period.

Table 2. Non-autonomous *eve* function is required for neuroblast proliferation

Brain	30% Larval extract	% of Normal proliferation*
CS	CS	38.9
$trol^{b22}; eve^3/+$	$trol^{b22}; eve^3/+$	7.8±1.3
$trol^{b22}; eve^3/+$	$eve^3/+$	6.3±1.4
$trol^{b22}$	$eve^3/+$	0±0
$trol^{b22}; eve^3/+$	$trol^{b22}; eve^3/+$ with 10 μg/ml 20E	39.8±2.7

*% of normal proliferation, (number of samples with normal proliferation)/(total number of samples) in independent batches of tissue explant experiments±s.e.m.

Structural analysis of the eve^5 allele

To investigate the structural requirements for Eve function we analyzed the molecular lesion in the eve^5 allele. It was previously shown that the eve^5 mutation removes the 3′ portion

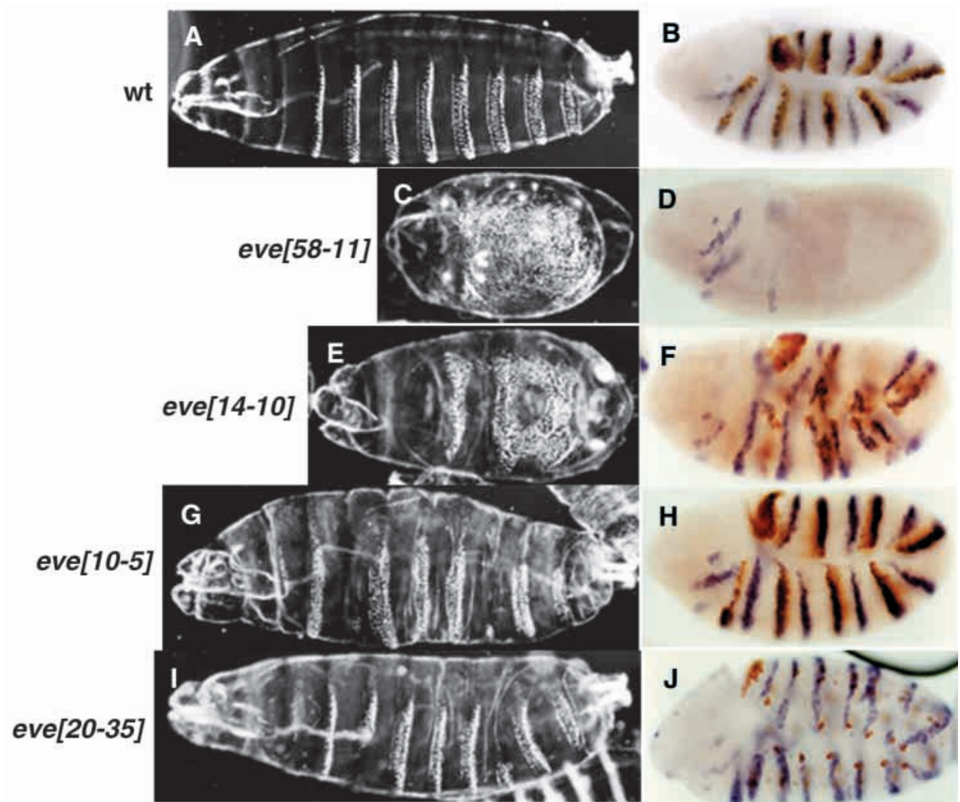

Fig. 3. Embryonic phenotypes of *eve* alleles. Cuticular phenotypes. (A) Control. (C) eve^{58-11}. (E) eve^{14-10}. (G) eve^{10-5}. (I) eve^{20-35}. *engrailed* expression at stage 10. (B) Control. (D) eve^{58-11}. (F) eve^{14-10}. (H) eve^{10-5}. (J) eve^{20-35}. In all panels anterior is towards the left, ventral is downwards.

of the Eve-coding region, leaving intact the DNA-binding domain and a transcriptional repression domain (Park et al., 1998). To identify the specific sequences deleted, PCR products amplified from *eve*^{5} genomic DNA were cloned. Sequence analysis showed (Fig. 7A) that the deletion starts at the first base of codon ala-255 and ends at the second base of the pro-326 codon, resulting in deletion and frameshift mutations from amino acid 255 to amino acid 376, the C terminus of the wild-type Eve protein. The deletion eliminates PEST sequences found between amino acids 283 and 335, suggesting that the Eve^{5} protein may have enhanced stability. This predicted increase in stability is in agreement both with comparative immunohistochemistry on *eve*^{5} mutant and wild-type embryos, and with western analysis. Staining for Eve protein at embryonic stages 6, 8 and 10 in *eve*^{5} mutant and wild-type embryos revealed a dramatic increase in the quantity of Eve detected in the *eve*^{5} embryos at all three stages (Fig. 7C). Consistent with the lack of obvious mutations in transcriptional control regions (Park et al., 1998), the spatial pattern of Eve protein in the mutant embryos appears normal, as does the level of *eve* mRNA (data not shown). The putative increase in stability of the Eve^{5} protein is also supported by western analysis. Wild-type Eve is sufficiently unstable that it cannot be detected on western blots even in samples prepared in the presence of high levels of protease inhibitors from 2-4 hour embryos, which show maximum levels of Eve immunostaining (Fig. 7B). In contrast, the Eve^{5} protein can easily be detected in samples from heterozygous *eve*^{5}/CyO embryos by immunoblotting both with the monoclonal antibody 3C10 and with polyclonal anti-Eve antiserum (Fig. 7B). The band identified by each of the anti-Eve antibodies corresponds to approximately 40 kDa in size, while the predicted size of the unmodified Eve^{5} protein is about 33 kDa, suggesting that Eve may be post-translationally modified in vivo. Eve protein expressed in cultured *Drosophila* cells also migrates significantly slower than expected from its molecular weight (Han and Manley, 1993).

Transgenes with a deletion of the Groucho-interacting domain rescue most of *eve* function in the *trol* pathway

The C-terminal frameshift mutation in the *eve*^{5} allele eliminates a Groucho-interacting motif, LFKPY, that is required for full Eve function in segmentation (Kobayashi et al., 2001). We showed previously that *eve*^{5} causes weak *trol*^{b22} lethality and a corresponding enhancement of the proliferation phenotype (Park et al., 1998). This suggests that the Groucho-interacting domain may be required for *eve* function as a cell cycle regulator. Therefore, we tested the ability of three independent transgenes with insertions on the second chromosome to rescue the enhancement of *trol* lethality by *eve*^{3} (Table 3). The EGNΔLFK transgene, which contains a small deletion of the Groucho-interacting motif, fully rescued the *trol*^{b22} lethality enhanced by heterozygosity at *eve*; i.e. the transgene supported viability in a *trol*^{b22}; *eve*/+ background

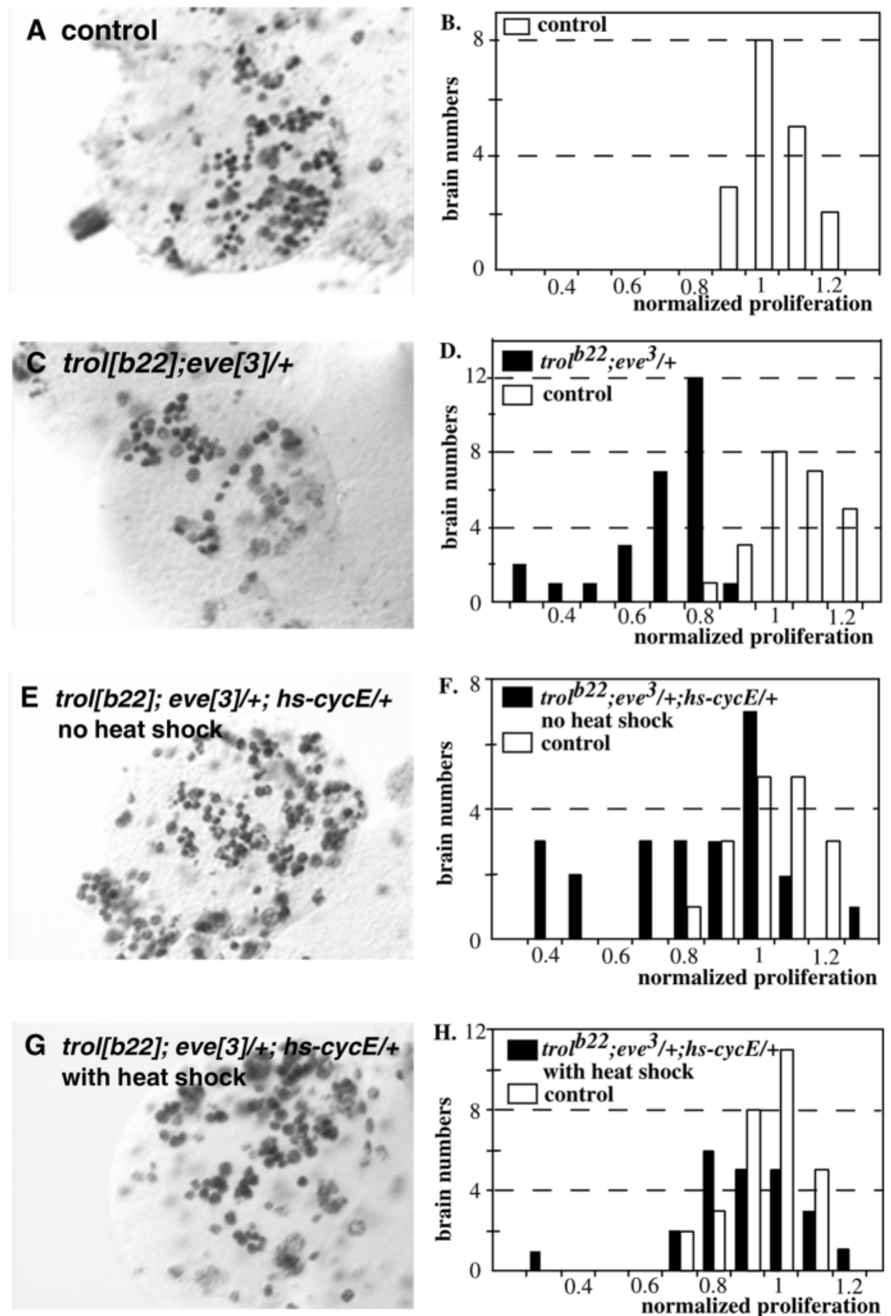

Fig. 4. Rescue of neuroblast phenotypes by expression of Cyclin E. BrdU incorporation in mutant and control brain lobes from 16-20 hours ph. (A) Control. (C) *trol*^{b22}*;eve*^{3}/+. (E) *trol*^{b22}*;eve*^{3}*/+;hs-CycE/*+, no heat shock. (G) *trol*^{b22}*;eve*^{3}*/+;hs-CycE/*+, with heat shock. Quantitation of the number of BrdU-labeled neuroblasts. (B) Control. (D) *trol*^{b22}*;eve*^{3}/+. (F) *trol*^{b22}*;eve*^{3}*/+;hs-CycE/*+, no heat shock. (H) *trol*^{b22}*;eve*^{3}*/+;hs-CycE/*+, with heat shock.

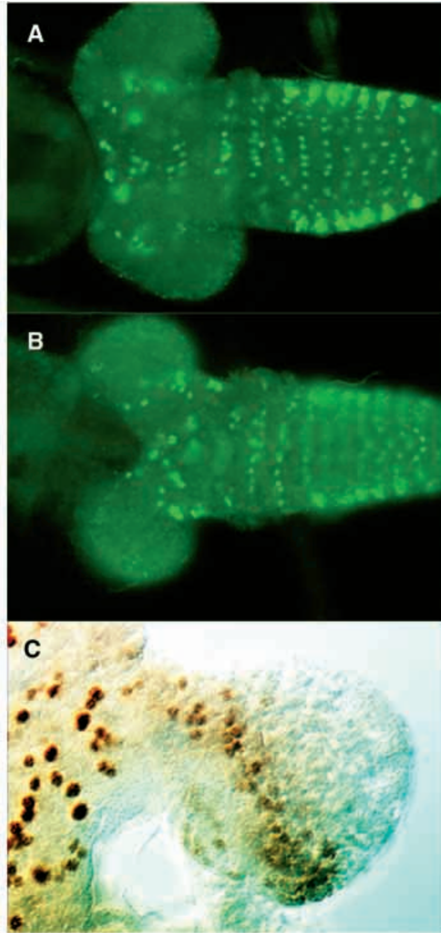

Fig. 5. Eve localization in the larval brain. Eve localization in late first instar brain. (A) Ventral view. (B) Dorsal view. (C) Engrailed localization in a late first instar brain. In panels A and B anterior is towards the left. In panel C anterior is to the top.

Table 3. Effect of LFKPY amino acid sequence on $trol^{b22}$; eve^3/+ lethality

Parents (male × female)	Male		
	eve^3, *P*/+	*CyO*/+	Ratio*
EGNΔLFK (A-2) × $trol^{b22}$	240	119	2.1±0.3 (*n*=3)
EGNΔLFK (A-2) × CS	343	282	1.2±0.06 (*n*=2)
EGNHA (C-2) × $trol^{b22}$	404	176	2.7±0.61 (*n*=3)
EGNHA (C-2) × CS	539	407	1.4±0.14 (*n*=2)
EGNHA (F-3) × $trol^{b22}$	223	139	1.7±0.23 (*n*=3)
EGNHA (F-3) × CS	462	392	1.2±0.04 (*n*=2)
	eve^3/+; *P*/+	*CyO*/+; *P*/+	Ratio‡
$P[eve^+]$ × $trol^{b22}$	253	31	0.12±0.02 (*n*=3)
$P[eve^+]$ × CS	85	83	0.98
EGNPA (F-2) × $trol^{b22}$	312	180	0.53±0.08 (*n*=3)
EGNPA (F-2) × CS	513	417	0.82±0.04 (*n*=2)
EGNAY (D-1) × $trol^{b22}$	270	98	0.30±0.07(*n*=4)
EGNAY (D-1) × CS	293	249	0.84±0.08 (*n*=2)
EGNAY (E-1) × $trol^{b22}$	279	91	0.31±0.03 (*n*=3)
EGNAY (E-1) × CS	354	368	1.1±0.1 (*n*=2)

*Ratio, (number of eve^3, *P*/+ flies)/(number of *CyO*/+ flies).
‡Ratio, (number of *CyO*/+; *P*/+ flies)/(number of eve^3/+; *P*/+flies).
Contents inside parentheses of each genotype refer to independent transgenic lines.

comparable with that of CS controls. Two independent EGNHA transgenes, containing a *hairy* Groucho-interacting motif (*WRPW,* Fisher et al., 1996) instead of the *eve* Groucho-interacting motif, showed rescue to an extent similar to that of the EGNΔLFK transgene. Three other independent transgenes inserted on the third chromosome, which have amino acid substitutions that disrupt the Groucho interaction in yeast two-hybrid assays and are similarly defective in segmentation function (Kobayashi et al., 2001) also showed rescue of the enhanced $trol^{b22}$ lethality (Table 3). However, in the absence of an *eve* mutation, none of the transgenes resulted in enhancement of $trol^{b22}$ lethality that was due to *eve* overexpression to the same extent as did a wild-type *eve* transgene (expressing a normal Eve protein with an intact Groucho-interacting motif) (Park et al., 1998). These results are consistent with the partial loss of *eve* segmentation function caused by these disruptions of the Eve-Groucho interaction, which is about 50% (Kobayashi et al., 2001), and with the fact that a reduction of close to 50% in *eve* function is required to enhance the *trol* phenotype. They also suggest that the Eve-Groucho interaction contributes to Eve function in the *trol* pathway (see Discussion).

DISCUSSION

eve is part of the *trol* pathway

Heterozygous *eve* mutations dramatically increase $trol^{b22}$ lethality and interact synergistically with $trol^{b22}$ to uncover a proliferation phenotype (Park et al., 1998). These data suggest that *eve* is a part of the *trol* pathway. If so, then a stronger reduction in Eve activity alone might also cause a proliferation phenotype. Fig. 2 clearly shows that eve^1/eve^5 transheterozygous animals have a neuroblast proliferation defect, consistent with the interpretation that *eve* is in the *trol* pathway.

A corollary of the hypothesis that *eve* is part of the *trol* pathway is that neuroblasts arrested in a *y* $trol^{b22}/Y$; eve^3/+ animal are in the same cell cycle phase as those arrested in $trol^{sd}$ mutants and can be rescued by expression of *CycE*, as was previously shown for $trol^{sd}$ (Caldwell and Datta, 1998). In fact, the proliferation defect in some *y* $trol^{b22}/Y$; eve^3/+ mutant brains is rescued by low levels of *CycE* expression, while higher levels result in rescue in virtually every individual (Fig. 4). The partial rescue by low levels of *CycE* expression indicates that the *trol* pathway in *y* $trol^{b22}/Y$; eve^3/+ animals is less compromised than that in $trol^{sd}$ mutants, which require high levels to obtain rescue, and further supports the hypothesis that *trol* and *eve* function in a common proliferation pathway.

The *eve*-*trol* interaction is not allele specific

Known mutations in both the *eve* homeodomain (eve^1 and eve^2) and a transcriptional repression domain (eve^4) enhance $trol^{b22}$ phenotypes, suggesting that $trol^{b22}$ enhancement is due to *eve* function as a DNA-binding transcriptional repressor. However, *eve* mutations do not enhance the strongest *trol* allele, $trol^{sd}$. This led us to ask whether $trol^{b22}$ enhancement by *eve* was due to a partial loss of function of *eve* and *trol* or to allele-specific interactions. We tested several new *eve* alleles (Table 1), and found a good correspondence between the ability to enhance *trol* and the severity of the *eve* embryonic phenotype, strongly

supporting the hypothesis that the *trol*^{b22}-*eve* interaction is due to a general loss of *eve* function. The lack of *trol* enhancement by weak *eve* mutations also indicates that enhancement requires a reduction in Eve activity of close to 50%. However, *trol* enhancement is not due to a defect in segmentation. In addition to the fact that heterozygous *eve* mutants produce few segmentation defects, this is supported indirectly by observations on another transcriptional regulator, *ftz*, which was also identified as a *trol* enhancer (C. D. Hough and S. D., unpublished). Heterozygous *ftz* mutations enhance *trol*^{b22} lethality and produce a proliferation phenotype similar to that of *eve* mutations. A *ftz* transgene that restores the early pair rule expression of *ftz* (but not CNS expression) cannot rescue enhancement (Y. P. and S. D., unpublished), implying that segmentation defects are not the cause of *trol* enhancement by *ftz*.

Having established that the *eve-trol* interaction is not *eve* allele specific, we proceeded to ask whether other *trol* mutations could also be enhanced by *eve*. Two independent *trol* alleles of intermediate strength also showed *eve* enhancement (Fig. 2), clearly indicating that the *eve-trol* interaction is not due to specific characteristics of the *trol*^{b22} allele or to background mutations in the *trol*^{b22} stock.

We compared the average reduction of normalized proliferation in different mutant populations. For the *trol*^{4} and *trol*^{8} mutations, only the population with a mutant phenotype were included in the calculation. Strong *trol*^{sd} samples led to a reduction in normalized proliferation of 0.66±0.04 (n=13, Y. P. and S. D., unpublished data) of control levels, consistent with published results (Datta, 1995). *eve*^{1}/*eve*^{5} transheterozygotes showed a reduction to 0.57±0.04 (n=19), while *trol*^{4}/*Y; eve*^{3}/+ and *trol*^{8}/*Y; eve*^{3}/+ brains showed a reduction to 0.57±0.03 (n=22) and 0.57±0.03 (n=21), respectively. Thus, while not all animals of each genotype had a proliferation phenotype, the magnitude of the proliferation phenotype, when present, was statistically identical in all the mutant genotypes examined. This consistent reduction of proliferation in different mutant animals suggests that a specific subpopulation of neuroblasts may require the activity of *trol* and *eve* in order to activate cell division, while the remaining neuroblasts use an alternative mechanism, which either is independent of or somehow compensates for a loss of activity in the *trol* pathway.

Eve protein requires several domains to control neuroblast proliferation

We had shown previously that the DNA-binding domain and a transcription repression domain in the Eve protein are likely to be required for neuroblast cell cycle activation (Park et al., 1998). Sequence analysis of the *eve*^{5} mutation suggested that the deletion of PEST sequences within the Eve^{5} protein (Sackerson, 1995) and the resulting protein stability, as demonstrated by both western and immunohistochemical analysis (Fig. 7B,C), might contribute to *eve*^{5} enhancement of *trol*^{b22}. This possibility is consistent with our previous observation that overexpression of *eve* by addition of *eve* transgenes also enhances *trol*^{b22} lethality (Park et al., 1998). Alternatively, deletion of a domain at the C terminus of the Eve^{5} protein, which was recently shown to interact functionally with the corepressor Groucho (Kobayashi et al., 2001), could have diminished its activity as a cell cycle regulator. This interpretation is consistent with the partial-loss-of-function embryonic phenotype of *eve*^{5}. The role of sequences at the C terminus of Eve was investigated using *eve* transgenes with mutant or substituted Groucho-interaction motifs.

Transgenes with truncation and amino acid substitutions in the C-terminal Groucho-interacting motif rescue lethality caused by the loss of Eve activity caused by *eve*^{3}. However,

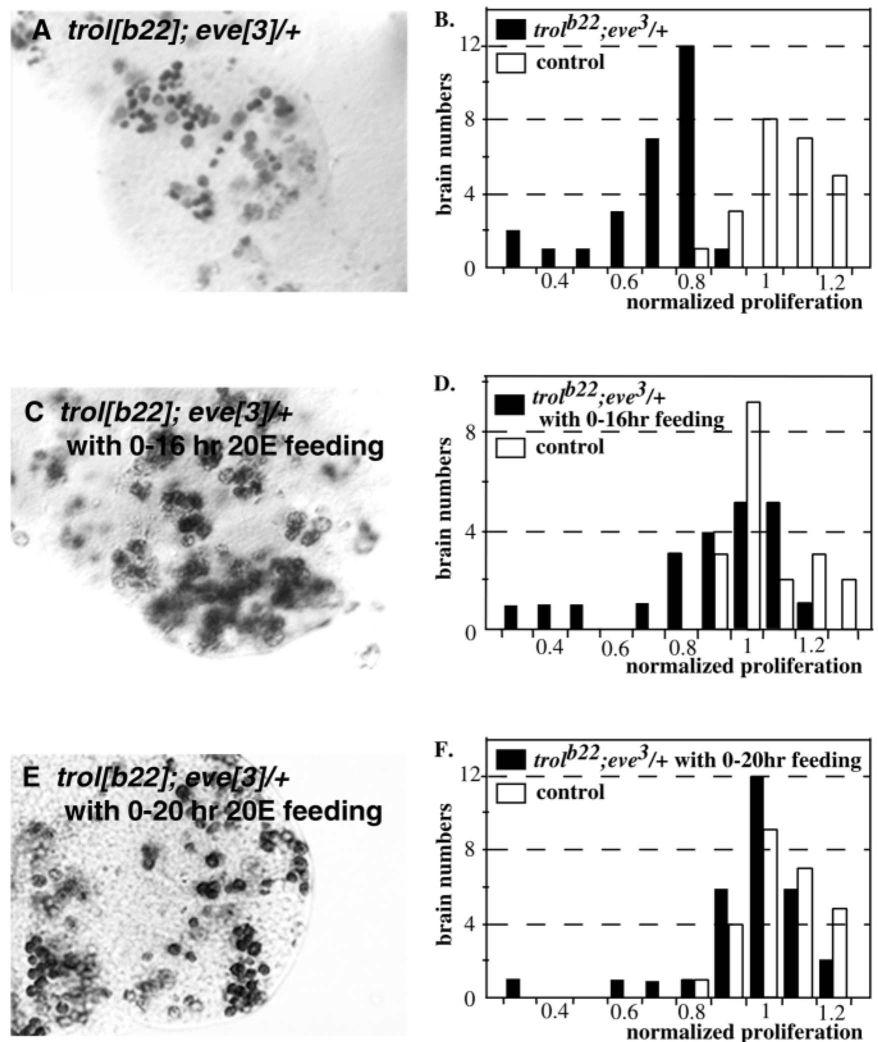

Fig. 6. Rescue of neuroblast proliferation defects by ecdysone. BrdU incorporation in brain lobes of mutant and control brains from 16-20 hours ph. (A) *trol*^{b22}*;eve*^{3}/+. (C) *trol*^{b22}*;eve*^{3}/+ fed ecdysone from 0-16 hours ph. (E) *trol*^{b22}*;eve*^{3}/+ fed ecdysone from 0-20 hours ph. Quantitation of the number of BrdU labeled neuroblasts. (B) *trol*^{b22}*;eve*^{3}/+. (D) *trol*^{b22}*;eve*^{3}/+ fed ecdysone from 0-16 hours ph. (F) *trol*^{b22}*;eve*^{3}/+ fed ecdysone from 0-20 hours ph.

these same transgenes, in the presence of two wild-type *eve* alleles, are not sufficient to enhance $trol^{b22}$ lethality, while transgenes that express the normal Eve protein do cause enhancement. This suggests that the activity of each of the Groucho-motif mutant *eve* transgenes is lower than that of wild-type eve^+ and is consistent with the fact that the transgenes have a reduced function in segmentation (Kobayashi et al., 2001). These data indicate that the Groucho-interaction domain, in addition to the DNA-binding domain and the Groucho-independent transcriptional repression domain, is necessary for the full function of the Eve protein in promoting neuroblast proliferation.

eve acts non-autonomously to control proliferation

All of the genetic evidence to date suggests that *eve* functions as a transcriptional regulator in the *trol* pathway. The most straightforward explanation is that *eve* acts within the regulated neuroblasts to promote *CycE* expression. Surprisingly, while Eve protein is found in the larval CNS (Fig. 5) in a pattern similar to that observed in other insect systems (Duman-Scheel and Patel, 1999), It cannot be detected in the regulated neuroblasts at any time from larval hatching to the point at which the cells have already entered S phase. This unexpected result led to the hypothesis that *eve* might function outside of the larval brain to provide a *trans*-acting signal required for neuroblast proliferation.

We have previously demonstrated that a CNS mutant for $trol^{sd}$ shows a defect in neuroblast proliferation even when cultured with wild-type whole-body extract, suggesting that *trol* is required only within the larval CNS for normal activation of neuroblast division (Datta, 1999). We used the same system to determine whether *eve* was required in the CNS or the body for normal proliferation to occur. As shown in Table 2, when $trol^{b22}$ brains are cultured with extract derived from $eve^3/+$ animals, a proliferation defect is observed. The magnitude of the defect is similar to that of $trol^{b22}/Y;\ eve^3/+$ brains cultured with $trol^{b22}/Y;\ eve^3/+$ extract, and comparable with the defect in $trol^{b22}/Y;\ eve^3/+$ animals in vivo. Thus, a decrease in *eve* activity within the body can precipitate a lack of neuroblast proliferation within a sensitized *trol* larval brain.

Eve-mediated enhancement of defective neuroblast proliferation can be rescued by ecdysone

Analysis of explants had previously shown that ecdysone enabled activation of neuroblast division and could substitute for larval extract (Datta, 1999). Furthermore, addition of ecdysone did not rescue the proliferation phenotype of cultured $trol^{sd}$ mutant brains, implying that ecdysone acts upstream of *trol*. Addition of ecdysone to extract from $trol^{b22}/Y;\ eve^3/+$ animals produced normal proliferation in cultured $trol^{b22}/Y;\ eve^3/+$ mutant brains (Table 2). Thus, ecdysone can overcome the lack of *eve*-induced activity in the extract. The ability of ecdysone to compensate for low *eve* expression was also seen in vivo in the rescue of proliferation defects of $trol^{b22}/Y;\ eve^3/+$ animals by ecdysone feeding (Fig. 6). Interestingly, almost complete rescue was obtained when animals were fed ecdysone from 16-20 hours posthatching, indicating that the time between ecdysone action and S phase entry is at most four hours.

eve, ecdysone and activation of imaginal neuroblast division

Mutations in *eve* produce specific defects in embryonic segmentation and the determination of neuronal identities in the embryonic CNS. Analyses of these phenotypes has led to the elucidation, especially for segmentation, of a complex molecular circuit that controls the expression of specific genes to set the body plan of the embryo. Yet some studies have also suggested that mutations in *eve* result in changes in the spatial pattern of expression of 87% of the genes in the embryo, even those such as *ry*, whose linkage to changes in segment identity are not obvious (Liang and Biggin, 1998). So how does a gene like *eve* with such apparent specificity in mutant phenotype and expression pattern affect the expression of a majority of genes in such a global fashion? Biochemical analyses indicate that Eve binds throughout the length of many genes, although how that binding might regulate gene expression is not clear (Walter et al., 1994). Another possibility is that *eve* is also required for the formation of an organismal-level *trans*-acting signal that affects the expression of other genes.

The genetic interaction between *eve* and *trol* has all the characteristics expected for two components of a common pathway: (1) the *eve*-*trol* interaction is not allele specific and

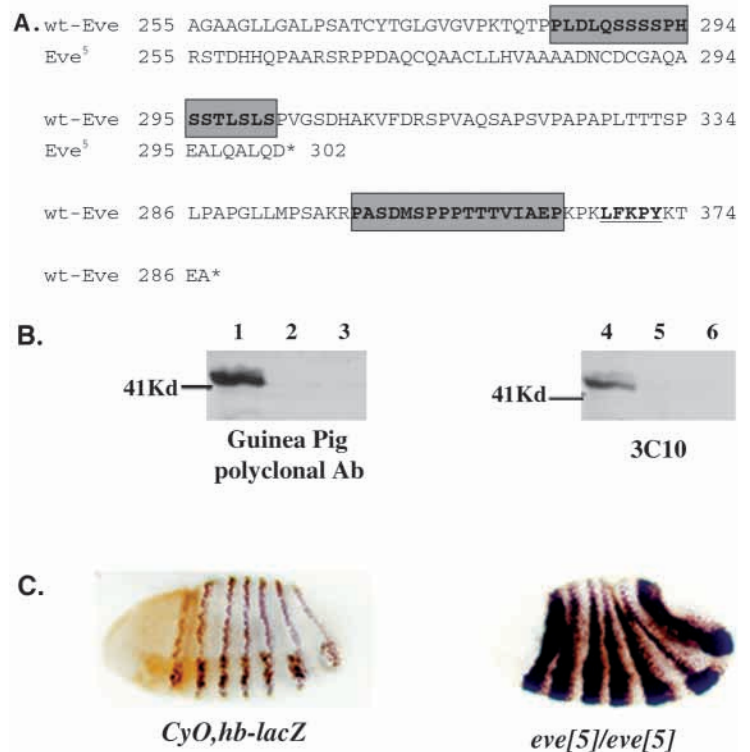

Fig. 7. Molecular analysis of eve^5. (A) Nucleotide and predicted protein sequence comparison of eve^5 with wild type. Shaded boxes indicate predicted PEST sequences. Underline indicates Groucho interacting motif. (B) Western blot of $eve^5/+$ and control samples. Lanes 1, 2 eve^5/CyO; 2, 5 eve^4/CyO; 3, 6 CS. (C) Immunohistochemical detection of wild-type Eve at late stage 6 of embryogenesis (left), when wild-type Eve protein levels are decreasing and Eve^5 protein at early stage 7 (panel).

the known functional domains of Eve are implicated in the interaction; (2) the strength of the interaction mirrors the strength of the *eve* allele in segmentation; (3) *eve* mutants themselves have the predicted proliferation phenotype; and (4) neuroblasts arrested in $trol^{b22}$*;eve/+* can be rescued by expression of *CycE*, as can the neuroblasts arrested in a strong *trol* mutant. The latter is especially revealing, as induction of *CycE* expression in *trol* mutants results in the activation of cell division ONLY in the number of neuroblasts appropriate to the developmental stage of the induction (Caldwell and Datta, 1998). That is, not all mitotically quiescent neuroblasts are arrested at the same cell cycle phase, and the extent to which CycE is a limiting factor is developmentally controlled. Therefore, as in embryonic segmentation and determination of neuronal identity, *eve* appears to function in a specific genetic pathway to affect the behavior of specific cells at specific times.

However, Eve is not detectable in regulated neuroblasts at any time during first instar. Furthermore, *eve* function is not required within the larval CNS, but is required within the larval body from which extracts are prepared. Moreover, low levels (10-20%) of extract made from eve^+ (CS) animals will not support activation of neuroblast division while higher concentrations will (Y. P. and S. D., data not shown). This concentration dependence indicates that *eve* does not inhibit production of a *trans*-acting proliferation repressor that is produced at higher levels in a *eve* mutant, as dilution of such a repressor would allow neuroblast division at lower rather than higher extract concentrations. These results strongly suggest that *eve* function is required for the production of a *trans*-acting factor that stimulates neuroblast division.

Is ecdysone the *trans*-acting factor produced in response to *eve*? Ecdysone can rescue *eve*-dependent proliferation defects both in vivo and in vitro (Fig. 6, Table 2), but not the proliferation defect of *trol* mutants in vitro (Datta, 1999). This suggests that ecdysone acts upstream of *trol*, as would be expected if it is the *eve*-dependent *trans*-acting signal, and *trol* acts within the receiving cells. However, while the ecdysone receptor has been detected in a few neurosecretory cells of the first instar CNS, it has not been detected in neuroblasts (Truman et al., 1994). This may indicate that only a few high-affinity receptors are required to transduce the ecdysone signal, or that ecdysone acts indirectly through the products of the neurosecretory cells. However, as Eve is not detectable in the neurosecretory cells in wild-type brain lobes (Fig. 5), it is unlikely that the added ecdysone rescues mutant animals by compensating for a loss of Eve activity in those cells. In each of these cases, *eve* could be acting through ecdysone production. Alternatively, ecdysone may act through a parallel pathway to that stimulated by an (unknown) *eve*-dependent signal. While the relationship between *eve* and ecdysone is not yet clear, it seems likely that *eve* is required for the production of an organismal-level *trans*-acting signal that is specifically required to stimulate larval neuroblast proliferation.

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