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Genetics

A modifier screen in the eye reveals control genes for *Krüppel* activity in the *Drosophila* embryo

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ABSTRACT ***Irregular facets* (*If*) is a dominant mutation of *Drosophila* that results in small eyes with fused ommatidia. Previous results showed that the gene *Krüppel* (*Kr*), which is best known for its early segmentation function, is expressed ectopically in *If* mutant eye discs. However, it was not known whether ectopic *Kr* activity is either the cause or the result of the *If* mutation. Here, we show that *If* is a gain-of-function allele of *Kr*. We then used the *If* mutation in a genetic screen to identify dominant enhancers and suppressors of *Kr* activity on the third chromosome. Of 30 identified *Kr*-interacting loci, two were cloned, and we examined whether they also represent components of a natural *Kr*-dependent developmental pathway of the embryo. We show that the two genes, *eyelid* (*eld*) and *extramacrochaetae* (*emc*), which encode a Bright family-type DNA binding protein and a helix-loop-helix factor, respectively, are necessary to achieve the singling-out of a unique *Kr*-expressing cell during the development of the Malpighian tubules, the excretory organs of the fly. The results indicate that the *Kr* gain-of-function mutation *If* provides a tool to identify genes that are active during eye development and that a number of them function also in the control of *Kr*-dependent developmental processes.**

The gene *Krüppel* (*Kr*) encodes a zinc finger-type transcription factor (1, 2) expressed in spatially and temporally restricted patterns throughout *Drosophila* embryogenesis (3). It functions as a gap gene required for the proper segmentation of the central region of the embryo during early blastoderm stage (reviewed in refs. 4–6). Within the segmentation gene cascade, *Kr* protein functions mainly as a repressor of other gap genes and pair-rule genes (6–8), restricting their localized activities along the anterior–posterior axis of the embryo (9). After blastoderm stage, *Kr* is expressed functionally during the following development of the larval visual system (10), formation of the central and stomatogastric nervous system (11, 12), muscle differentiation (13, 14), and generation of the kidney-like Malpighian tubules (15–17).

In addition to the multiple activity patterns of *Kr* during embryogenesis, *Kr* mis-expression had been observed in the eye imaginal discs of the dominant *Irregular factes* (*If*) mutation (18), which results in reduced adult eyes lacking the regular array of ommatidia (19). However, the question of whether the notable ectopic *Kr* expression is the cause of the *If* mutant eye phenotype or just a peripheral consequence of the mutation has not been addressed. Here, we show that *If* is a dominant gain-of-function allele of *Kr* that causes mis-expression of the gene in the developing eye imaginal disc. We used the dosage-sensitive *If* mutation in a genetic screen to isolate dominant enhancers and suppressors of *Kr* activity that are located on the third chromosome. We identified 30 loci that modulate the eye phenotype generated by the ectopic *Kr* activity. A more detailed analysis of two genes demonstrates that the modifier screen involving the *If* mutation provides a tool to isolate factors that act, in addition to being expressed and possibly required during eye development, in a *Kr*-dependent developmental pathway during embryogenesis. The two genes described here, *eyelid* (*eld*) and *extramacrochaetae* (*emc*), code for a member of the Bright family of DNA binding proteins and for a helix-loop-helix protein, respectively (20–22). They control specific aspects of *Kr* expression required for the proper allocation of a unique *Kr*-dependent cell fate within the Malpighian tubule primordium.

MATERIALS AND METHODS

***Drosophila* Strains, Mutagenesis, and Mutant Embryos.** *Drosophila* strains were kept under standard conditions. The ethylmethane sulfonate (EMS) screen on *If* mutants was done as described (1). The F2 progeny was inspected for embryonic segmentation defects. Twelve *Kr* alleles were obtained, all of which suppressed the *If* phenotype to some degree. In the modifier screen, *If* homozygous females were crossed to males bearing a mutant third chromosome in trans over balancer chromosomes *TM3* or *TM6B*. F1 progeny carrying a mutant third chromosome in the *If* mutant background were scored for an altered *If*/+ eye phenotype. The screen involved 335 lethal P-element insertion lines of the Berkeley Genome Project (23) and deficiency chromosomes (Umea and Bloomington stock collection) that uncover the regions of the third chromosome as shown in Fig. 2 (black bars; details in the legend).

The following fly stocks were used in this work: Oregon R, *l(3)00090, l(3)04539, l(3)05592* (23), emc^{E12} (24), $E(Spl)^{X1}$, $E(Spl)^{8D06}$, da^{1}, Kr^{9}, Kr^{25} (19), brm^{2} (25), and *b pr cn wx If* (obtained from the Tübingen stock collection). The two *eld* alleles (eld^{dust55} and eld^{dust31}) were recovered in a standard EMS mutagenesis screen (26). *eld* alleles were used in trans over the *l(3)00090* P-element insertion, shown to cause an *eld* mutation (20). Complementation analysis showed that *l(3)00090* and *l(3)04539* are allelic. The Malpighian tubules were examined in trans-heterozygotes of the respective EMS alleles and the *l(3)00090* P-insertion. Ectopic expression of *Kr* in the eye imaginal disc was induced by using the Gal4/UAS system (27) comprised of the *sevenless* heatshock promoter (gift of K. Basler) or the *eyeless*-Gal4 (U.W., unpublished work) and UAS-Kr (M.H. and H.J., unpublished work) transgenes.

SEM, Immunocytochemistry, and Molecular Procedures. Flies were prepared for SEM as described (20), and micrographs were obtained by using a Philipps XL 20 electron

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Abbreviation: EMS, ethylmethane sulfonate.
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microscope. Immunological stainings of whole-mount embryos were carried out as described (28) by using the Vectastain ABC Elite horseradish peroxidase kit (Vectastain, Vector). After staining, embryos were examined as described (29). Antibody dilutions were: rabbit anti-Krüppel 1:20 (3); Mab22C10 1:20 (Hybridoma Bank); mouse anti-β-galactosidase 1:1000 (Cappel). Whole-mount *in situ* hybridizations were performed with digoxigenin-labeled DNA probes (30) or digoxigenin-labeled RNA probes (8). As probes, we used *lacZ*, *emc* (obtained from J. Modolell), and *eld* cDNA fragments. Stained embryos were examined and photographed by using a Zeiss Axiophot microscope.

Plasmid rescue for obtaining genomic DNA from the lines *l(3)00090*, *l(3)04539*, and *l(3)05592* as well as for the other P-element modifier lines identified was performed as described (32). The screening of genomic and cDNA libraries, the handling of DNA, and the preparation of probes were as described in ref. 33. The sequences of the genomic DNA of *If*, the *If* revertant alleles, and the cDNAs as well as the DNA surrounding the P-element insertion sites were determined by using the dideoxynucleotide method (34). Sequence comparison and database searches [National Center for Biotechnology Information database (25)] revealed that the P-elements were inserted in the first intron of the previously identified *eld* gene and in the 5′-untranscribed region of the *emc* gene, respectively. A 3.5-kb *Hin*dIII plasmid rescued of line *l(3)00090* (see Fig. 2) was used to screen for *eld* cDNAs.

RESULTS AND DISCUSSION

Irregular Facets Is a Dominant *Krüppel* Gain-of-Function Mutation. *If* was identified as a spontaneous dominant mutation that causes a severe adult eye phenotype (19). Heterozygous adults bearing the dominant *If* mutation develop small and narrow eyes that are pointed ventrally; the facets are irregularly arranged, sometimes fused or absent in ventral portions (Fig. 1 *A* and *B*). In homozygous *If* mutants, eyes are further reduced in size and form narrow slits with a glossy surface lacking most of the ommatidia (Fig. 1*C*). The eye defects can be traced back to an irregular arrangement of differentiating photoreceptor cells during eye imaginal disc development (35) and correlate with ectopic expression of *Kr* in the *If* mutant discs, as has been described (18).

We reasoned that the ectopic *Kr* expression de-regulates gene activities during eye imaginal disc development. We tested this proposal by misexpression of *Kr* by the UAS/GAL4 system (27). Misexpression of *Kr* during eye development resulted in an *If* mutant-like phenotype (Fig. 1*D*; details in the legend). Furthermore, we also found the *If* phenotype with flies that carry the *If*-bearing chromosome in trans to a *Kr* deficiency or to a chromosome bearing a *Kr* lack-of-function allele (data not shown). These results suggest that *If* is a neomorphic allele of *Kr*. This proposal is supported by the identical cytological location of both *If* and *Kr* at 60F3 and by the lack of recombinants between *If* and *Kr* (36).

If *If* is a neomorphic allele of *Kr*, point mutations within the coding region of the gain-of-function allele *If* should cause a reversion of the dominant eye mutant phenotype and generate a *Kr* segmentation phenotype in homozygousity. To obtain *If* revertants, we performed an EMS mutagenesis screen and examined whether the sequence of the *Kr* gene is affected in such *If* revertants (details in Materials and Methods). One *If* reversion, termed "If^{R1}," was left with subtle eye defects, meaning that the severeness of the *If* mutation is substantially reduced, almost to wild type (Fig. 1*E*). We also obtained a series of weaker revertants of the *If* mutant phenotype, indicating that the reversion was less effective than in the first case. One such weaker revertant, termed "If^{R2}," is shown in Fig. 1*F*. All of the *If* revertants are recessive embryonic lethals and cause a typical *Kr* mutant segmentation phenotype (not shown). Moreover, although *If* complements *Kr* mutations (ref. 36; unpublished work), the *If* revertants fail to do so. Thus, the *If* revertants are alleles of *Kr*; the dominant *If* mutation is a *Kr* gain-of-function allele whereas the reversions cause *Kr* loss-of-function alleles. To establish this link between *If* and *Kr* firmly, we determined the molecular lesions generated in If^{R1} and If^{R2}.

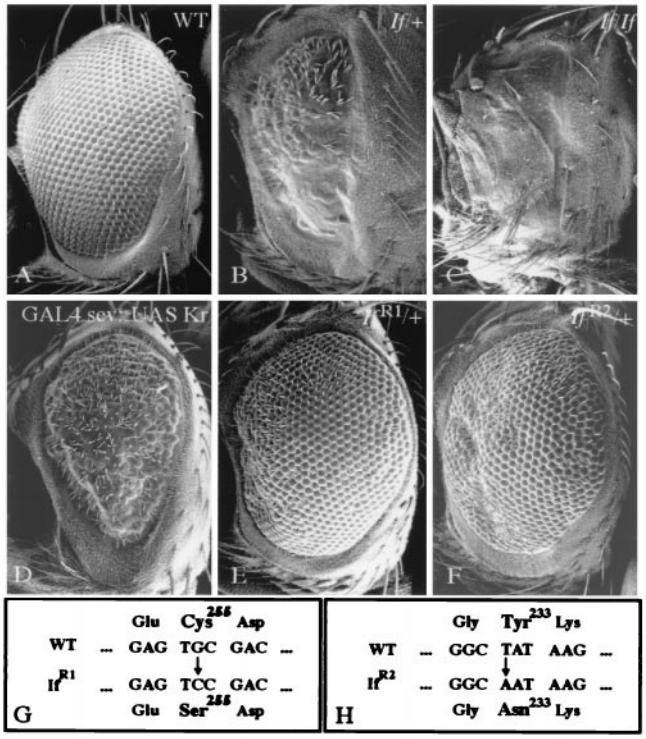

FIG. 1. SEM of wild-type and *If* mutant *Drosophila* eyes. (*A*) Wild-type eye. (*B*) *If*/+ mutant eye. Note a reduction of the size of eyes; the ommatidia are fused or absent in ventral portions of the eye (*Upper*) and irregularly arranged dorsally (*Lower*). (*C*) Eye remnant of a homozygous *If* mutant fly. (*D*) Eye phenotype caused by ectopic *Kr* expression provided by the *sevenless*/heatshock-GAL4/UAS-*Kr* transgene combination (see *Materials and Methods*). Note the *If*-like symptoms such as a reduction of the eye and the irregular arrangement of the ommatidia. A similar although less severe eye phenotype was caused by *eyeless* -GAL4. The difference in the severity is likely to be due to differences between the ectopic promoter in *If* and the *eyeless* or *sevenless*/heat shock promoter used to misexpress *Kr* (see *Materials and Methods*). (*E*) $If^{R1/+}$ mutant eye indicating that the *If* mutant phenotype is almost reversed to wild type. (*F*) $If^{R2/+}$ mutant eye indicating that the *If* mutant phenotype is weakened significantly. (*G*) Molecular lesion generated in the If^{R1} DNA. The G/C transversion causes a replacement of cysteine^{255} by serine within the second zinc finger motif of Krüppel (37). This lesion causes a strong *Kr* segmentation phenotype (38). (*H*) Molecular lesion of the If^{R2} allele; T/A transversion resulting in a replacement of tyrosine^{233} by asparagine within the first zinc finger motif of Krüppel that causes a weak *Kr* segmentation phenotype (39). For details see text.

Sequencing and comparison of *If* and If^{R1} DNA revealed a *Kr* wild-type coding region in *If* DNA and a single missense mutation causing a replacement of cysteine 255 by a serine residue of the If^{R1} protein (Fig. 1*G*). This replacement causes a disruption of the second zinc finger motif of Krüppel, explaining the strong *Kr* segmentation phenotype in mutant embryos (37, 38). The weak eye defects that were left with the revertant If^{R1} (Fig. 1E) argue for some residual *Kr* activity that may interfere with eye development. The *Kr* sequence of the revertant If^{R2} shows a different single base pair exchange that results in a replacement of tyrosine 233 within the first Krüppel zinc finger motif by asparagine (Fig. 1*H*). This finding is in agreement with a weak *Kr* mutant segmentation defect gen-

erated by such a replacement (39), and it argues that *Kr* function is not as strongly impaired as in the first case. The molecular analyses of two of several EMS-induced *Kr* revertants establish unequivocally allelism between *If* and *Kr*.

The results establish that the phenotype of the spontaneous *If* mutation is caused by a dominant gain-of-function of *Kr* activity in eye imaginal discs. One possibility that would explain the mutational event is that *If* may have acquired additional cis-acting enhancer sequence elements due to a small chromosomal rearrangement or a transposition event that conducts a new *Kr* expression domain during the eye imaginal disc development. This proposal is consistent with the notion that DNA fragments outside the 18-kb cis-acting *Kr* control region (40) of *If* and wild-type flies are different in a sense that *If* DNA, but not *Kr* wild-type DNA, contains repetitive DNA (41). This suggests that the spontaneous *If* mutation could be caused by a transposon insertion resulting in the misexpression of the *Kr* gene.

Dominant Modifiers of Ectopic *Kr* Activity in the Eye Imaginal Disc. Homozygous *If* eyes are more severely affected than heterozygous *If* eyes. Furthermore, a small reduction of *Kr* activity during eye development, as exemplified with the If^{R2} mutation, reduces the strength of the *If* phenotype. These observations suggest that the *If* mutant phenotype is sensitive to the dose and activity of *Kr* in the eye disc and may thereby provide a means to identify modifiers of *Kr* activity. We therefore conducted a genetic screen, searching for mutations on the third chromosome that dominantly increase (enhance) or decrease (suppress) the severity of the *If* mutant eye phenotype. For this, we examined the eye phenotypes of heterozygous *If* mutants in combination with chromosomal deficiencies or lethal P-element enhancer trap insertions (Berkeley collection; see *Materials and Methods*). The chromosome combinations examined cover ≈75% of the third chromosome, and 30 modifier loci (12 enhancers, 18 suppressors) were identified. The results of the screen and the by now few molecularly characterized candidate genes are summarized in Fig. 2. Here, we will focus on two modifiers of ectopic *Kr* activity, *eld* and *emc* (Fig. 3), showing that their activities also are required in a natural context during *Kr*-dependent embryonic organ development.

Identification of *eld* and *emc* as *Kr* Interacting Genes. We molecularly characterized two modifiers of *If* that were generated by the P-element insertions *l(3)00090* and *l(3)05592*, respectively (23). After plasmid rescue (32), we cloned genomic DNA fragments adjacent to the P-element insertion sites, determined its sequence and identified the transcription units affected by the P-element insertion. The results showed that the P-element insertions were localized in the 5′ regions of the previously characterized genes *eld* (20) (Fig. 3*C*) and *emc* (21, 22) (Fig. 3*D*), respectively. Transcript mapping combined with cDNA sequencing and complementation analysis with known *eld* and *emc* alleles confirmed that the *If* enhancer is *eld* (Fig. 3*A*) whereas the *If* suppressor is *emc* (Fig. 3*B*). Furthermore, previously characterized alleles of the two loci were found to modify the *If* mutant phenotype, indicating that the effects on ectopic *Kr* activity in the eye disc were not allele-specific (not shown).

Both modifier genes code for transcription factors. As shown recently, *eld* encodes a Bright family-type DNA binding protein (20), whereas *emc* codes for a helix-loop-helix transcription factor (21, 22) required for the proper specification of many cell types in the embryo (24, 42). As expected from their interference with ectopic *Kr* activity (see above), both genes are expressed in the developing eye imaginal discs (23) (data not shown). In addition, they are expressed at multiple other sites during embryogenesis, including the Malpighian tubules, which develop in a *Kr*-dependent manner (see below). This allowed us to examine whether the two modifiers of ectopic *Kr*

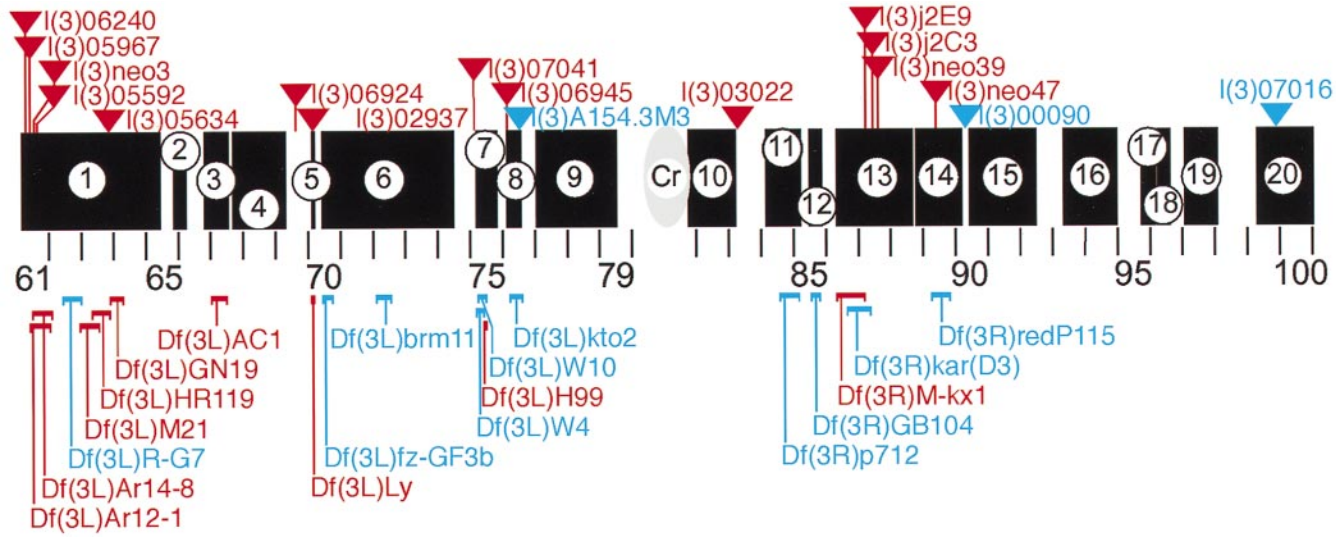

FIG. 2. Schematic representation of the third chromosome of *Drosophila* (section 61–100) showing the regions that were screened with deficiencies (black bars; nos. 1–20). Deficiencies that enhance (blue) or suppress (red) are indicated below the sections; P-element-tagged loci that enhance (blue) or suppress (red) the *If* phenotype are indicated by triangles above the sections. The following deficiencies were tested: (1) region 61A to 65C: *Df(3L)emc-E12*, *Df(3L)Ar12–1*, *Df(3L)Ar14–8*, *Df(3L)R-G5*, *Df(3L)R-G7*, *Df(3L)Aprt-1*, *Df(3L)GN19*, *Df(3L)GN34*, *Df(3L)GN24*, *Df(3L)M21*, *Df(3L)HR370*, *Df(3L)HR232*, *Df(3L)HR119*, *Df(3L)GN50*, *Df(3L)ZM47*; (2) region 65F3 to 66B10: *Df(3L)pbl-X1*; (3) 66F5–67D7–13: *Df(3L)29A6*, *Df(3L)AC1*; (4) region 67F2–3 to 69B4–5: *Df(3L)BK9*, *Df(3L)lxd6*, *Df(3L)vin2*, *Df(3L)vin6*, *Df(3L)vin7*; (5) region 70A2–3 to A5–6: *Df(3L)Ly*; (6) region 70C1 to 74C: *Df(3L)fzGF3b*, *Df(3L)fzD21*, *Df(3L)st-f13*, *Df(3L)st7*; (7) region 75B3–6 to 75F1: *Df(3L)W10*, *Df(3L)Cat*, *Df(3L)W4*, *Df(3L)H99*; (8) region 76A3-B2 to76D5: *Df(3L)VW3*, *Df(3L)kto2*; (9) region 77A1 to 79C9: *Df(3L)rdgC*, *Df(3L)ri79c*, *Df(3L)Pc-Mk*; (10) region 81F to 83A: *Df(3R)ME15*, *Df(3R)4–75*, *Df(3R)P-93*, *Df(3R)2–2*; (11) region 84A1–2 to 85B6: *Df(3R)Scr*, *Df(3R)Antp17*, *Df(3R)p712*, *Df(3R)pXT103*, *Df(3R)p819*; (12) region 85D8 to 85F6: *Df(3R)by10*, *Df(3R)GB104*, *Df(3R)by62*; (13) 86C1;88E5–6: *Df(3R)M-Kx1*, *Df(3R)karD3*, *Df(3R)ry506–85c*, *Df(3R)red1*; (14) 88F;90A: *Df(3R)Po4*, *Df(3R)redP115*, *Df(3R)C4*; (15) region 90C2-D1 to 92D3–6: *Df(3R)P14*, *Df(3R)ChaM7*, *Df(3R)D1-BX12*; (16) region 93B3–5 to 94: *Df(3R)e-R1*, *Df(3R)e-N19*; (17) region 95E8-F1 to 96A17–18: *Df(3R)crbS87–4*, *Df(3R)crbS87–5*, *Df(3R)XS*; (18) region 96B-D: *Df(3R)XTA1*; (19) region 97A to 98A1–2: *Df(3R)TI-P*; and (20) region 99B to 100F: *Df(3R)L127*, *Df(3R)B81*, *Df(3R)awd-KRB*. Cr: position of centromer. Details in *Materials and Methods*.

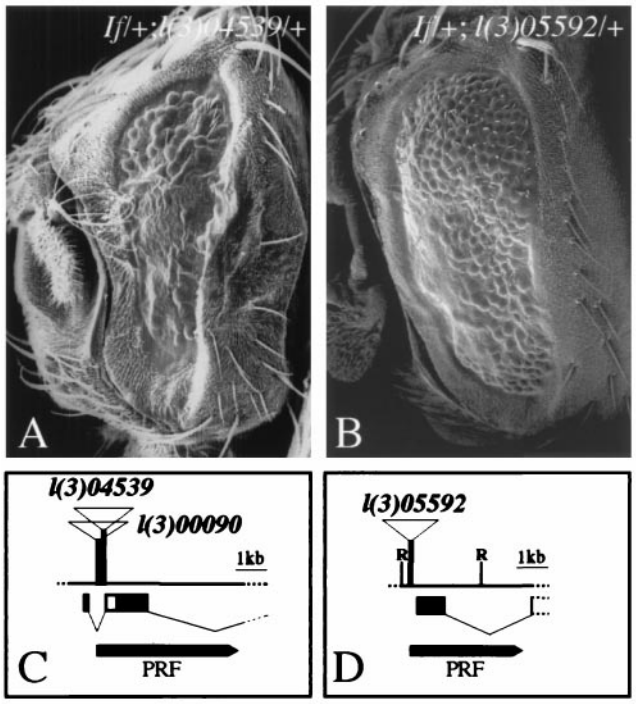

FIG. 3. SEM showing the effect of an enhancer (*eld*) and a suppressor (*emc*) of the *If* mutant eye phenotype and the molecular characterization of the genes. (*A*) *If*/+; *l(3)04539*/+ flies develop smaller eyes and fewer ommatidia than *If*/+ flies, indicating an enhancement of the *If* phenotype (compare with Fig. 1*B*). (*B*) The *If* phenotype is significantly suppressed in *If*/+; *l(3)05592*/+ flies (compare with Fig. 1 *A* and *B*). (*C*) Partial physical map of the *eld* gene (for details see ref. 20), which was identified by complementation analysis of the P-element insertions *l(3)04539* and *l(3)00090* previously shown to be an allele of *eld*. Genomic DNA adjacent to the P-element insertion sites was isolated by plasmid rescue (PRF) and used to screen for cDNAs (see bottom of the physical map). Both P-elements were inserted in the first intron of the previously identified *eld* gene (20). Exons are indicated by black bars below the physical map. (*D*) P-element insertion site of *l(3)05592* in the 5′ region of *emc* gene. *l(3)05592* fails to complement previously identified *emc* mutations indicating that it is an allele of *emc*. Genomic DNA of the *emc* locus was isolated by plasmid rescue (see *Materials and Methods*); the diagnostic sequence 3′ adjacent to the P-element insertion is ACTCCGCCTATCGGATTC. Part of the translated region is shown (black bar; for details, see refs. 21 and 22). Diagnostic restriction site: R, *Eco*RI.

activity act also in a natural *Kr*-dependent developmental pathway.

***emc*-Dependent Singling-Out of the *Kr*-Expressing Mother Tip Cell.** *Kr* expression defines the Malpighian tubule anlage at late blastoderm stage and becomes restricted to a ring of cells at the midgut/hindgut boundary from where *Kr*-expressing Malpighian tubule precursors evert (17). Previous studies have shown that the specification of Malpighian tubule fate and the segregation of the cells depend on *Kr* expression in the Malpighian tubule anlage (17, 43). In *Kr*-deficient embryos, the respective cells become part of the hindgut epithelium (44).

Once the tubules evert, *Kr* expression becomes restricted to a single cell, termed the "tip mother cell" (Fig. 4*A*). The singling-out process of this cell from an equivalence group of Malpighian tubule precursors involves the activated *Notch* pathway (15), which restricts the proneural bHLH proteins encoded by the *achaete-scute*-complex (*ASC*) genes (45) to the tip mother cell. In this cell, the ASC proteins act in concert with bHLH protein encoded by *daughterless* (*da*) (45–47) to maintain *Kr* expression (M.H. and H.J., unpublished work). The tip

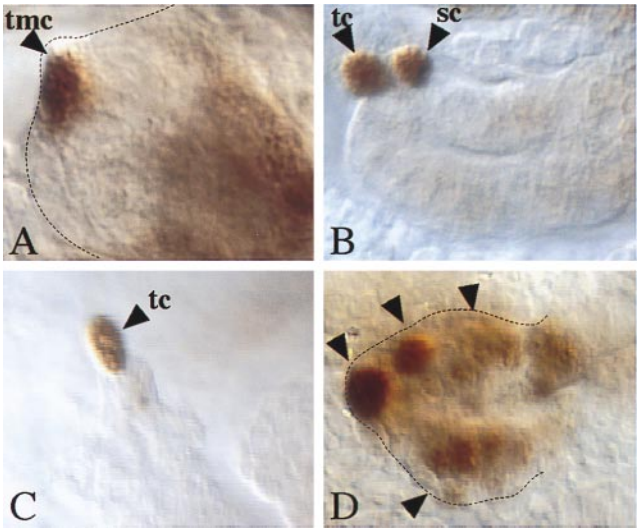

FIG. 4. *emc* represses *Kr* expression in the Malpighian tubules. (*A–C*) Wild-type embryos. (*D*) Homozygous *emc* mutant embryo (*emc*^{E12}) stained with anti-Krüppel antibodies. (*A*) Restricted *Kr* expression in the tip mother cell (tmc). (*B*) The tip mother cell divides and gives rise to the tip cell (tc) and the satellite cell (sc) (15). (*C*) *Kr* expression is maintained only in the tip cell (tc). (*D*) In *emc* mutants, *Kr* is expressed in many cells along the tubule (arrowheads).

mother cell divides once, and the daughters give rise to the tip cell, which controls proliferation during tubule elongation (44) and differentiates neuronal characteristics (15), and an excretory cell, termed "satellite cell" (Fig. 4*B*). The satellite cell loses *Kr* expression in a *Notch*-dependent manner (15), whereas *Kr* expression is maintained in the tip cell until the end of embryogenesis (Fig. 4*C*).

emc expression accompanies Malpighian tubule development in a manner similar to *Kr* expression. However, once the

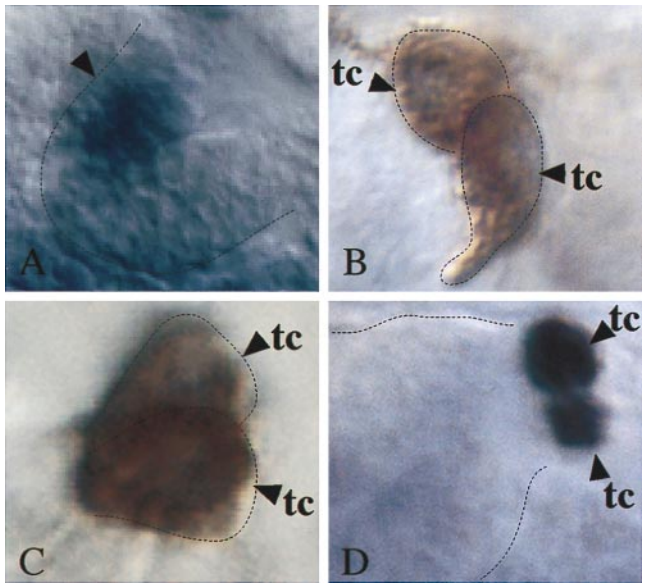

FIG. 5. *eld* represses *Kr* expression in the sibling cell. (*A*) RNA *in situ* hybridization of wild-type embryos with an *eld* probe (see *Materials and Methods*) showing expression in a group of cells (arrowhead) corresponding to the cluster of cells in the outgrowing tubules from which the tip mother cell is selected. (*B* and *D*) Embryos transheterozygous for the *eld* P-insertion *l(3)00090* and *eld*^{dust55} and (*C*) for *l(3)00090* and *eld*^{dust31}. (*B* and *C*) Mab22C10 antibody staining, characteristic for tip cells (15), shows that two instead of a single tip cell (tc) are formed. (*D*) Formation of two tip cells corresponds to maintained *Kr* expression in the satellite cell, as revealed by anti-Krüppel antibody stainings (arrowheads). Details in the text.

tip cell is formed, the patterns of expression become complementary, meaning that *emc* expression continues in all cells of the elongating Malpighian tubules except in the tip cell (ref. 24; unpublished work). To test whether the complementary patterns of *Kr* and *emc* expression reflect a regulatory effect of *emc* on *Kr*, as indicated during eye development in the *If* mutant, we examined *Kr* expression in the Malpighian tubules of *emc* mutant embryos. Fig. 4*D* shows multiple *Kr*-expressing cells in *emc* mutant Malpighian tubules. This finding is consistent with the previous finding that *emc* mutant embryos develop multiple tip cells and that each of them continues to express *achaete* (24). Virtually the same observations have been made previously with *Notch* mutants, and it was shown that *Notch* acts toward restricting the activity of the proneural bHLH proteins, which are required to maintain *Kr* expression first in the tip mother cell and subsequently in the tip cell (15). However, although the activated *Notch* pathway acts through transcriptional repression of the *ASC* genes, *emc* protein antagonizes proneural bHLH activities by sequestering the proteins as heterodimers that are incapable of binding to DNA (48, 49). Our results are therefore consistent with the proposal that *emc* functions in the control of *Kr* expression by antagonizing proneural bHLH activities that are required to maintain *Kr* expression in the tip mother cell.

***eld* Antagonizes *Kr*-Dependent Tip Cell Differentiation.** The *eld* protein shows a nuclear localization, consistent with its suspected function as a transcription factor (20). It appears to act in multiple signaling pathways because it antagonizes *wingless* activity, suppresses *Ras1* activity in the eye (50), and blocks *Notch*-dependent neuronal differentiation (20). During Malpighian tubule development, *eld* is expressed in a restriced area of the everting precursors that corresponds to the equivalence group of cells expressing the proneural genes (Fig. 5*A*).

eld mutant embryos exert a distinct phenotype during Malpighian tubule development that is linked to *Kr* activity. Whereas the anlage and the four tubules evert normally (data not shown), each tubule develops two instead of the normal one tip cell (Fig. 5 *B* and *C*). Tip cell development is under the control of *Kr* activity (M.H. and H.J., unpublished work), so we next asked whether and when *Kr* expression is altered in *eld* mutant embryos. In correspondence with the mutant phenotype, the initial expression of *Kr*, including its restriction to the tip mother cell, appears to be normal (not shown). However, once the tip mother cell has undergone division, two instead of only one of the daughter cells maintain *Kr* expression (Fig. 5*D*). This indicates that *eld* activity is necessary to prevent *Kr* expression in the sibling of the tip cell and allows for its differentiation into a satellite cell. Thus, although *emc* is necessary for the restriction of *Kr* to the tip mother cell, *eld* functions specifically at the subsequent step during Malpighian tubule development where an alternative and *Kr*-dependent cell fate decision is taken between the daughters of the tip mother cell.

Notch signaling recently was shown to be required first for the selection of the tip mother cell and subsequently for the distinction between its daughters to either develop a tip cell or a satellite cell (15). Consistently, in *Notch* mutant embryos, all cells of the proneural equivalence group develop first into tip mother cells; these cells divide and subsequently develop into the multiple tip cells that continue *Kr* expression (15). In contrast, only two tip cells were found in *eld* mutants. This finding implies that, if *eld* acts in a *Notch*-dependent manner and/or mediates *Notch* signaling (20), its activity is required only for the second of the two *Notch*-dependent differentiation steps during Malpighian tubule development. Thus, *eld* participates as an optional component in the *Notch*-signaling pathway and is needed to prevent, directly or indirectly, the maintenance of *Kr* expression in the satellite cell that would otherwise develop into a second tip cell.

CONCLUSIONS

The results presented here demonstrate that gene activities that were identified via an artificial experimental situation, namely the ectopic expression of *Kr* in the developing eye disc, can lead to the identification of integral components of a *Kr*-dependent developmental pathway during embryogenesis. In the eye imaginal disc, *emc* suppresses *Kr* activity whereas *eld* has an opposite effect, but both act during embryonic Malpighian tubule development as negative regulators of *Kr*. We have no explanation for this phenomenon. It could mean, in negative terms, that the *Kr* misexpression screen turned up dosage-sensitive genes affecting cell fate that were several steps downstream from *Kr* activity and thus have no direct interaction with *Kr*. Thus, each gene identified in the modifyer screen represents a candidate gene that needs to be evaluated critically through additional criteria as outlined here for *eld* and *emc*. The additional screening is essential to distinguish between direct *Kr* interactors and genes that mediate different read-outs of the *Kr* pathway in cells that have a different organ or tissue competence. However, in view of the fragmentary information concerning the spatial and temporal control of postblastodermal *Kr* expression (17, 40) and in view of the fact that the few *Kr* target genes of *Kr* were identified by molecular approaches (13, 51), our experimental strategy to assess components of a *Kr*-dependent regulatory circuitry seems a valid one.

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