Proximodistal subdivision of *Drosophila* legs and wings: the *elbow-no ocelli* gene complex

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Summary

Appendages are thought to have arisen during evolution as outgrowths from the body wall of primitive bilateria. In *Drosophila,* subsets of body wall cells are set aside as appendage precursors through the action of secreted signaling proteins that direct localized expression of transcription factors. The *Drosophila* homeodomain protein Distal-less is expressed in the leg primordia and required for formation of legs, but not wings. The homeodomain protein Nubbin is expressed in the wing primordia and required for formation of wings, but not legs. Given that insect legs and wings have a common developmental and evolutionary origin, we sought to identify genes that underlie the specification of all appendage primordia. We present evidence that the zinc-finger proteins encoded by the *elbow* and *no ocelli* genes act in leg and wing primordia to repress body wall-specifying genes and thereby direct appendage formation.

Key words: Homothorax, Distal-less, Nubbin, Limb development, Imaginal disc

Introduction

The progenitors of the adult body regions in *Drosophila* are set aside in the embryonic ectoderm as discrete groups of cells known as imaginal discs. The disc primordia contain the progenitors of both the adult body wall and the appendages; the wing imaginal disc for example forms the wing and the dorsal thorax (reviewed by Cohen, 1993). Subdivision of the imaginal discs into presumptive appendage and body wall territories results from the activity of conserved signaling proteins (summarized in Fig. 1B). The combined activities of Wingless (Wg) and Decapentaplegic (Dpp) specify proximal and distal domains in the leg imaginal discs, thereby defining the appendage fields (Campbell and Tomlinson, 1995; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). In the wing disc, signaling by the EGFR ligand Vein (Vn) antagonizes Wg activity to limit the appendage-forming region to the ventral part of the imaginal disc (Wang et al., 2000; Zecca and Struhl, 2002a; Zecca and Struhl, 2002b), whereas the appendage-forming region is centered in the leg disc.

Wg and Dpp signaling activities restrict the expression of the homeodomain protein Homothorax and the zinc-finger transcription factor Teashirt to the presumptive body wall by repressing them in distal domains (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; Wu and Cohen, 2000; Wu and Cohen, 2002). Wg and Dpp also activate expression of genes required for appendage formation, including *Distal-less* (*Dll*) in the distal leg primordium (Cohen and Jürgens, 1989a; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997) and *vestigial* (*vg*) and *nubbin* (*nub*) in the distal wing primordium (wing pouch) (Ng et al., 1995; Ng et al., 1996; Wu and Cohen, 2002). Thus, different transcription factors appear to be used in leg and wing primordia to confer distal identity.

Given that there is a common evolutionary origin of legs and wings (Averof and Cohen, 1997), and that legs and wings derive from a common imaginal disc primordium in the *Drosophila* embryo (Cohen et al., 1993), we might expect that Wg and Dpp would act through the same transcription factor(s) to specify the appendage-forming region in the leg and wing discs. In this context we became interested in the zinc-finger proteins encoded by the *elbow* (*el*) and *no ocelli* (*noc*) loci. We present evidence that Elbow and Noc proteins are expressed in the presumptive distal cells of both leg and wing imaginal discs, where they are required to repress the expression of the body wall genes *homothorax* (*hth*) and *teashirt* (*tsh*) and promote appendage formation.

Materials and methods

Drosophila strains

ap-gal4 (Calleja et al., 1996)*; dpp-gal4, UAS-wg*, *UAS-sgg*, and *UAS-brinker* are described in FlyBase. el^{1} is described by Davis et al. (Davis et al., 1997). *EP(2)2039* directs Gal4-dependent expression of El protein (not shown) and produces a wing defect with $scalloped^{Gal4}$ (not shown). The enhancer trap line *el-gal4* has a Gal4-containing P-element 950 bp upstream of the transcriptional start of *el*. NocGal4 contains a Gal4-containing P-element 285 bp upstream of the transcriptional start of *noc*. The loss-of-function mutant $el^{3.3.1}$ was recovered by EMS-induced reversion of this phenotype. $el^{3.3.1}$ contains a stop codon in exon 2, leading to truncation of the protein at Q262. $noc^{\Delta 64}$ is a 848 bp deletion within the Noc ORF (Dorfman et al., 2002). The double mutant chromosome was generated by

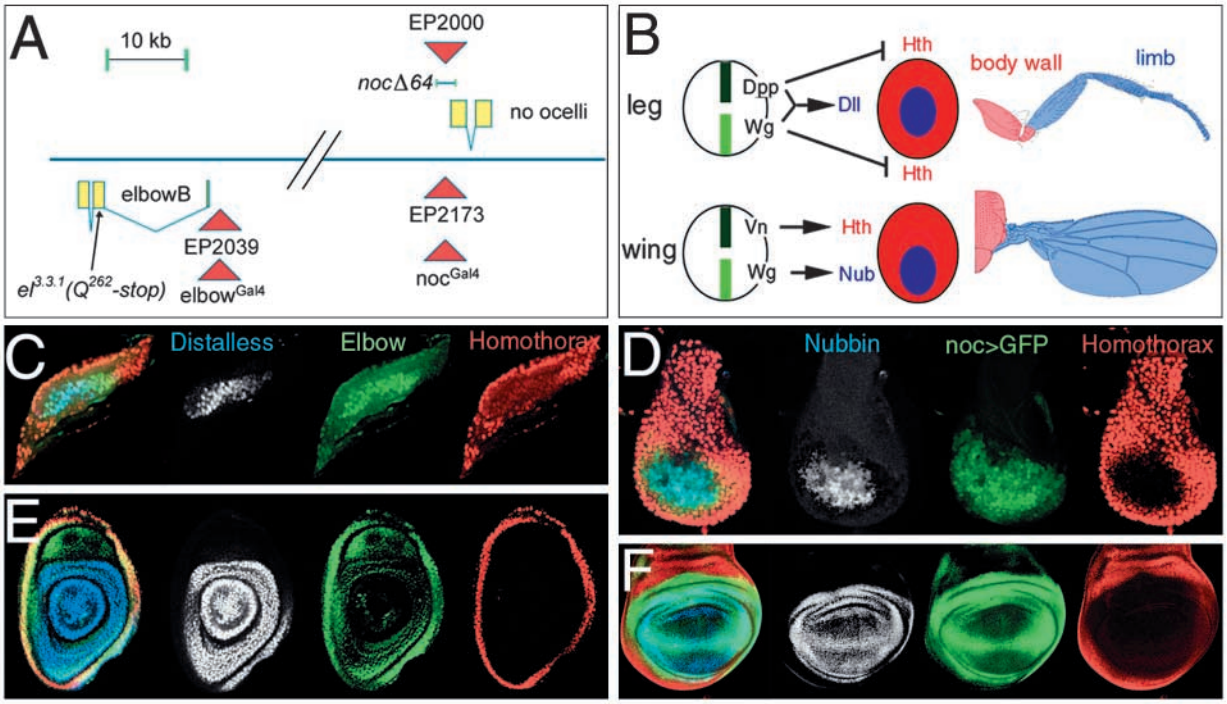

Fig. 1. *el* and *noc* expression in presumptive appendages. (A) Genomic organization of the *elbow* and *no ocelli* loci. The *el* transcript consists of three exons spanning 3.3 kb [EST clone RE67722 (Dorfman et al., 2002)]. *noc* is located 83 kb downstream of *el* and consists of two exons. The el^{1} deletion removed ~25 kb of DNA between the two coding regions and specifically affects expression of both genes in the wing imaginal disc, suggesting that it removes a common regulatory element shared by the two loci. *el* and *noc* were coexpressed at all stages examined. (B) Diagram of the proximal-distal subdivision of both wing and leg primordia by signaling molecules. In the leg imaginal disc, the combined activity of Dpp and Wg induces the expression of Dll in the presumptive appendage primordium (blue) and restricts the expression of Hth to the periphery of the disc, which will give rise to the adult body wall (red). In the wing imaginal disc, Wg activity induces the expression of Nub in the wing primordium (blue) and Vein (Vn) activity induces the expression of Hth in the periphery of the disc, which will give rise to the adult body wall (red). (C) Second instar leg disc labeled with antibodies to visualize Dll protein (blue), El protein (green) and Hth protein (red). Noc expression was identical to El expression (not shown). (D) Second instar wing disc labeled to visualize noc^{Gal4} using *UAS-GFP* (green), Nub protein (blue) and Hth protein (red). Note that the expression domain of Noc is slightly broader than the domain of Nub and overlaps Hth expression. Hth repression lags behind Tsh repression and thus underestimates the size of the wing field at early stages (Wu and Cohen, 2002). (E) Mature third instar leg imaginal disc labeled as in C. Note the change in the relative expression pattern of El and Dll with respect to the earlier stage in C. (F) Mature third instar wing disc labeled as in (D). El and Noc are expressed in a ring corresponding to the wing hinge and in wedge shaped domains centered on the dorsal-ventral boundary.

inducing male-specific recombination in a S^{1} $noc\Delta64/L^{rm}$ $el^{3.3.1}$ *EP(2)2039; Δ2,3 Sb* genotype (Preston et al., 1996). Recombinants lacking both dominant markers were stocked and analyzed by PCR for the presence of the *noc* deletion and the *el* point mutation. The integrity of the recombination region was verified by PCR (data not shown). UAS-el and UAS-noc were constructed by cloning full-length cDNAs into pUAST (Cheah et al., 1994; Dorfman et al., 2002).

Antibodies

El and Noc protein expression was visualized using polyclonal antibodies raised against full-length proteins in rat (El) or guinea pig (Noc). The protein expression patterns were faithfully reflected by the noc^{Gal4} and el^{Gal4} enhancer trap lines, which were useful for double-labeling experiments.

Rat anti-Hth, Rabbit anti-Tsh, mouse anti-Nub and rat anti-Dll were described previously (Wu and Cohen, 1999; Wu and Cohen, 2000; Wu and Cohen, 2002). Other antibodies are commercially available.

Genotypes of larvae used for genetic mosaic analysis

hs-FLP (I);$noc^{\Delta64}$ *FRT40/arm-lacZ FRT40.*
hs-FLP (I); $el^{3.3.1}$ $noc^{\Delta64}$ *FRT40/arm-lacZ FRT40.*
hs-FLP (I); $el^{3.3.1}$ $noc^{\Delta64}$ *FRT40/ M(+) arm-lacZ FRT40.*
f^{36a}*hs-FLP (I);* $el^{3.3.1}noc^{\Delta64}$ *FRT40/P(f+) FRT40.*
f^{36a}*hs-FLP (I);* $el^{3.3.1}noc^{\Delta64}$ *FRT40/ P(f+) M(+) FRT40.*

Clones were generated by giving a 1 hour heat shock at 38°C at the indicated stages.

Genotypes of larvae used for ectopic expression of el and noc

$ap^{\gamma\alpha\lambda4}$*/+; uas-el uas-noc.*
dpp-gal4/uas-el uas-noc.

Results

el and *no ocelli* expression in appendage primordia

The *elbow (el)* and *no ocelli (noc)* genes are located in close proximity on chromosome 2L and encode closely related zinc finger proteins (Fig. 1A). The predicted El and Noc proteins are 50% identical. We became interested in the functions of El and Noc because of their expression patterns in the developing wing and leg imaginal discs. In second instar larvae El and Noc proteins are co-expressed in the presumptive distal regions of the leg and wing imaginal discs where the appendages are

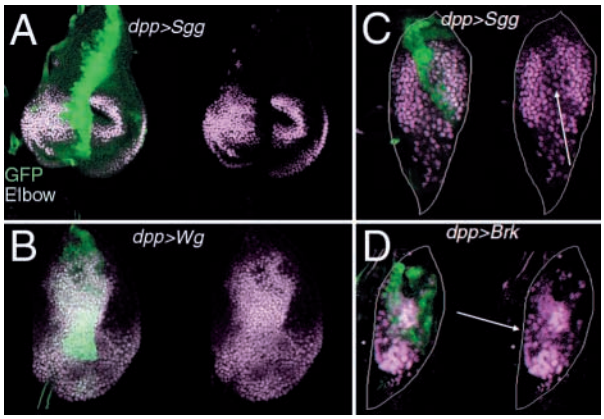

Fig. 2. Regulation of El and Noc at early stages. (A) dpp^{Gal4}-driven expression of the Wg-pathway inhibitor GSK3/Shaggy (Sgg) in an early third instar wing disc. Anti-El staining (purple) is reduced in the *dpp* expression domain (GFP, green). (B) dpp^{Gal4}-driven expression of Wg in a late second instar wing disc. El staining is ectopically induced in body wall regions in a non-autonomous manner close to the dpp expression domain. (C) dpp^{Gal4}-driven expression of the Wg-pathway inhibitor GSK3/Shaggy in an early third instar leg disc. El is reduced in the *dpp* expression domain (arrow). (D) dpp^{Gal4}-driven expression of the Dpp-pathway inhibitor Brinker (Brk) in an early third instar leg disc. El is reduced in the *dpp* expression domain (arrow).

specified (Fig. 1C,D). In second instar leg discs, El and Noc expression coincided with Dll (Fig. 1C), which marks the leg-forming part of the disc at this stage (Cohen and Jürgens, 1989a; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). At the earliest stages, the El and Noc domain appears slightly broader than the Dll domain, perhaps reflecting a slight delay in the onset of Dll expression relative to El and Noc. A similar result was observed in second instar wing discs, where the El and Noc expression domain included the nascent Nub domain, where Hth was beginning to be repressed (Fig. 1D). Later in development the expression patterns of El and Noc change (Fig. 1E,F), suggesting that they are used again under different regulatory control to fulfill secondary functions (see below).

The early onset and position of El and Noc was consistent with their genes being early targets of Wingless (Wg) and Decapentaplegic (Dpp), which specify the appendage primordia. Expression of GSK3/Shaggy, a repressor of the Wg signaling pathway (Diaz-Benjumea and Cohen, 1994), or Brinker, a repressor of the Dpp signaling pathway (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999), under control of *dpp-Gal4*, repressed El and Noc expression (Fig. 2A,C,D and not shown). Likewise ectopic expression of Wg caused ectopic expression of El and Noc (Fig. 2B and not shown). These observations indicate that Wg and Dpp signaling are required for induction of *el* and *noc* expression in early leg and wing discs. The late expression of *el* and *noc* in the wing disc (Fig. 1F) is also under Dpp and Wg control, with Wg signaling inducing their expression and Dpp repressing it (data not shown).

el and *noc* define the appendage-forming domain of the imaginal discs

We sought to analyze the functions of *el* and *noc* by producing mutants. A mutant allele of *el* was produced in an F_1 screen by EMS-induced reversion of a phenotype caused by *el* overexpression (using an EP-element inserted at the *el* locus, see Materials and methods). $el^{3.3.1}$ truncates the protein before the zinc-finger domain and is probably a null allele (Fig. 1B). Nonetheless, flies homozygous for the $el^{3.3.1}$ mutation were viable without obvious defects in phenotype from wild type (data not shown). For *noc*, we made use of a small deletion that removes exon 1 of the gene and is embryonic lethal (Dorfman et al., 2002) (Fig. 1B). Clones of cells homozygous for the $noc^{\Delta 64}$ allele develop without any observable defect in legs and wings (data not shown). As *el* and *noc* encode homologous proteins, this suggested that they might function redundantly in imaginal disc development. Consequently, it was necessary to generate an *el noc* double mutant chromosome and examine clones of cells lacking both. Although the two loci are located only 83 kb apart, this was possible using P-element-mediated male recombination (Preston and Engels, 1996; Preston et al., 1996), because the $el^{3.3.1}$ mutant was induced on a chromosome carrying an EP-element. P-transposase induced recombinants between the EP2039 $el^{3.3.1}$ and the $noc^{\Delta 64}$ FRT chromosome were recovered and tested for the presence of both alleles by PCR. The double mutant chromosomes were analyzed by PCR to ensure that no additional alterations were induced adjacent to the P-element in the course of recombination. Antibody labeling confirmed that the El and Noc proteins were not expressed in clones of $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant cells (not shown).

To assess their role in early wing development, clones of $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant cells were generated in early second instar larvae and their distribution was compared with their wild-type twins in the wing disc (e.g. Fig. 3A). The homozygous mutant cell and its wild-type "twin" are products of a single cell division, so the two clones are normally recovered at equal frequency and in close proximity to one another after a period of growth. This was the case for *el* and *noc* double mutant clones in the presumptive body wall portion of the wing disc (Fig. 3B, 26/26 pairs). When the clone pair was close to the edge of the endogenous Nub expression domain, mutant clones were recovered outside the Nub domain while their twin clones were found in the Nub expression domain (8/8 pairs). In some cases the mutant clone was separated from its twin (Fig. 3A), suggesting that the mutant clone had been displaced to the edge of the Nub domain. In other cases, the mutant clones appear to have been lost. Only five double mutant clones were recovered for 31 wild-type twins within the Nub domain. These observations suggested that *el* and *noc* double mutant clones sorted out from the Nub domain.

Although few clones of *el* and *noc* double mutant cells were recovered in the wing pouch, those that were examined had lost expression of Nub and showed ectopic expression of Tsh, which is normally limited to the body wall (Fig. 3C; Hth was also misexpressed; not shown). These clones rounded-up and appeared to extrude from the wing epithelium, suggesting that affinity differences were causing them to sort out. In some cases clones of mutant cells were recovered as vesicles of mutant tissue between the wing surfaces (data not shown). We next produced discs with very large areas of *el* and *noc* mutant tissue, using the Minute technique to give mutant cells a relative growth advantage (Morata and Ripoll, 1975). Despite

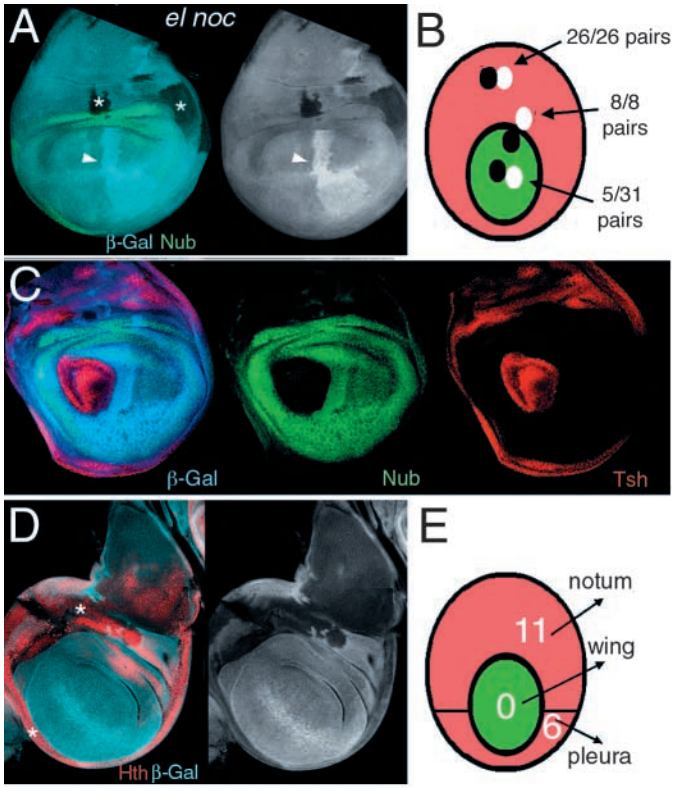

Fig. 3. Early *el noc* double mutant clones in the wing. (A,C) Wing discs with clones of cells lacking *elbow* and *no ocelli* ($el^{3.3.1}$ $noc^{\Delta 64}$) at an early stage. Nub protein is shown in green; Tsh protein in red. Clones are marked by the absence of β-gal (blue). (A) Mutant clones (asterisks) sorted out from the Nub-expressing wing pouch. Twin clones (arrowheads) remain in the Nub-expressing domain. (B) Summary of the relative positions of clones of cells lacking *el* and *noc* (white circles) and their twins (black circles) when born in the wing primordium (green) or in the body wall (red). (C) Wing disc with a large $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clone in the wing pouch. Tsh (red) was ectopically expressed and Nub (green) was lost in the clone. Comparable clones also showed ectopic Hth expression (not shown). (D) Wing disc with large Minute$^+$ $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clones. Asterisks mark clones abutting the wing pouch and remaining in the body wall region. Hth protein is shown in red. (E) Summary of the location of Minute$^+$ $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clones in the body wall region of wing imaginal discs.

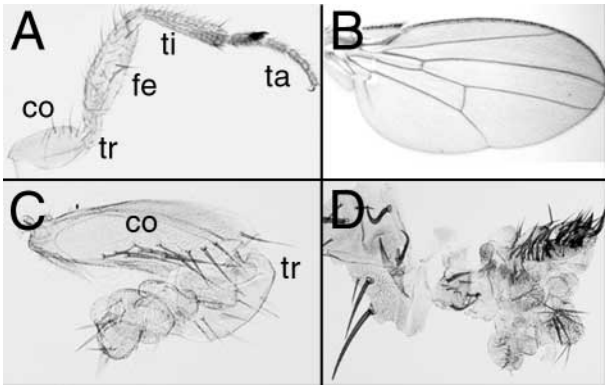

Fig. 4. Truncated appendages. (A) Cuticle preparation of a wild-type leg. (B) Cuticle preparation of a wild-type adult wing. (C) Cuticle preparation of a leg with Minute$^+$ $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clones marked with *forked*. induced early in development (60 hours AEL). Distal elements are deleted (co, coxa; tr, trochanter; fe, femur; ti, tibia; ta, tarsal segments). (D) Cuticle preparation of a wing carrying large Minute$^+$ $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clones marked with *forked*, induced early in development (60 hours AEL). The remaining wing tissue is heterozygous. The notum was not affected.

the large size of the double mutant clones, they were always located outside the domain of Nub expression, in the Hth expression domain (Fig. 3D,E). In some cases the Nub-expressing area was very small, but all mutant cells expressed Hth. We examined adult flies from these crosses to evaluate the effects on the adult wing. Although few larvae with many large clones survived to adulthood, those that did displayed a nearly complete loss of wing tissue (Fig. 4B,D). In such cases all the remaining wing elements were composed of heterozygous cells expressing one wild-type copy of *el* and *noc*. In the thorax, clones of mutant tissue were recovered without defects (not shown).

The behavior of *el* and *noc* double mutant clones was similar in the leg imaginal disc. When induced early in development, double mutant clones were recovered at normal frequencies in the proximal region of the disc (Fig. 5A,B; 21/21 pairs). Double mutant clones were not recovered in the distal-most part of the disc (0 clones for 6 twins). When induced in late second instar, only four double mutant clones were recovered distally. These clones ectopically expressed Hth, rounded up and sorted out from the epithelium (e.g. Fig. 5E). These observations again suggest that El/Noc activity is required downstream of Wg and Dpp to repress Hth expression in the leg disc. In contrast to the wing, ectopic expression of Tsh or Hth does not repress Dll expression in distal leg (Abu-Shaar and Mann, 1998; Wu and Cohen, 2000), and consequently Dll expression was not lost in the *el* and *noc* double mutant clones (not shown). Ectopic expression of Tsh and Hth can in some cases cause ectopic expression of Dll (Wu and Cohen, 2002), as in the example in (Fig. 5E).

Double mutant clones given a growth advantage were excluded from the presumptive tarsus (Fig. 5C,D). The relationship between proximal and distal domains of gene expression differs somewhat in the leg from that in the wing. Lineage tracing experiments have shown that cells born in the body wall portion of the leg disc (tsh^{Gal4} expression domain) normally contribute significantly to forming the femur and tibia segments of the leg, but do not contribute to the tarsus (Weigmann and Cohen, 1999). To do so the cells must lose proximal gene expression (e.g. *hth*) and acquire distal gene expression [e.g. *Dll* and/or *Dac* (Wu and Cohen, 1999)]. In this context it is interesting that *el* and *noc* double mutant clones occupied large proximal areas and extended distally into femur and tibia segments (Fig. 5C,D), but were not recovered in the tarsal segments of the adult leg (Fig. 5A). Apparently, during third instar, *el* and *noc* mutant cells can migrate distally and contribute to formation of femur and tibia. This correlates with a distal-ward retraction of the Dll expression domain (Campbell and Tomlinson, 1998). The finding that *el* and *noc* mutant cells do not contribute to wing at all may suggest that comparable distal migration of mutant tissue does not happen in the wing. Lineage tracing of proximal cells in the wing has

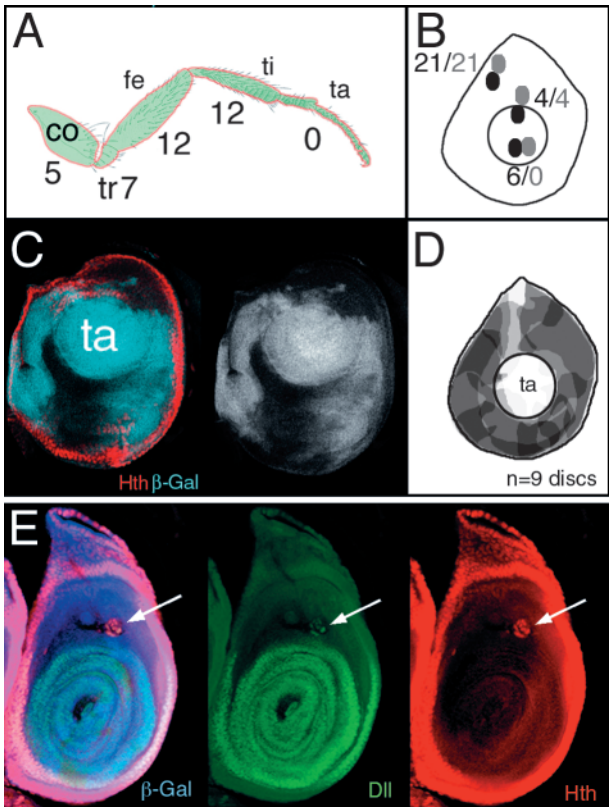

Fig. 5. Early *el noc* double mutant clones in the leg. (A) Schematic representation of a leg showing the distribution of Minute[+] $el^{3.3.1}$ *nocΔ64* double mutant clones induced early in development (60 h AEL). Co, Coxa; Tr, Trochanter; Fe, Femur; Ti, Tibia; Ta, tarsal segments. (B) Summary of the relative position of clones of cells lacking *el* and *no ocelli* (grey circles) and their twins (black circles) when born in the proximal or distal leg. ta, tarsal segments. (C) Leg disc with Minute[+] $el^{3.3.1}$ $noc^{\Delta 64}$ double mutant clones. Mutant cells were found in femur and tibia, but not in the tarsal segments (ta). Hth protein (red). Clones are marked by the absence of βGal (blue). (D) Summary of the locations of Minute^{+} $el^{3.3.1}$ *nocΔ64* double mutant clones in the proximal region of leg imaginal discs. The position of each clone is depicted as one semi-transparent layer of grey. The darker an area is, the more clones were found in that region. (E) Leg disc with an $el^{3.3.1}$ *nocΔ64* double mutant clone induced early in development (60 hours AEL). Hth (red) was ectopically expressed in some clones (arrow). Note that the twin spot cannot be seen in this picture because the $el^{3.3.1}$ *nocΔ64* double mutant clones sort out from the epithelium and are therefore found in a different focal plane. Clones induced earlier were not recovered.

shown that cells born proximally in the hinge region normally do not contribute to the wing blade (K. Weigmann and S.M.C., unpublished data)

Few larvae in which we generated large *el* and *noc* double mutant clones survived to adulthood and we did not recover adult flies in which all derivatives of the leg disc were mutant. The most severe defects recovered corresponded to nearly complete loss of distal leg tissue, including reduction of femur and tibia (Fig. 4A,C). Although El and Noc activity are not strictly required in femur- or tibia-producing cells, the defects observed in these segments in discs with large areas of mutant tissue presumably reflect a requirement at earlier stages. As for Dll the domain in which El and Noc are required may retract distally as the disc develops (Campbell and Tomlinson, 1998; Cohen et al., 1989; Cohen and Jürgens, 1989a; Gorfinkiel et al., 1997). Dll is required early for formation of tibia and femur as well as tarsus, even though Dll mutant clones induced later can be recovered in tibia and femur.

These observations suggest that the combined activity of El and Noc is required to prevent Tsh and Hth expression in the presumptive appendage-forming region of the early leg and wing imaginal discs. As neither gene produces a defect when mutated alone, it appears that either protein is sufficient to mediate repression of Hth and Tsh. We have attempted to test this by Gal4-dependent ectopic expression of UAS-noc or UAS-el constructs. To our surprise, ectopic expression of El or Noc or both together, resulted in a reduced expression of Nub and up-regulation of Tsh (data not shown). As these effects are the same as those produced by the loss-of-function mutant clones, we infer that overexpression of either protein causes a paradoxical dominant negative effect, reducing net activity. While unexpected, this is not unprecedented. For example, when overexpressed, dLDB/CHIP, a cofactor of Apterous, behaves as a dominant negative, apparently by disrupting the stoichiometry of a multi-protein complex that is required for transcription factor activity (Fernandez-Funez et al., 1998; Milan and Cohen, 1999).

Taken together our observations suggest that the early activities of *el* and *noc* are required for specification of both, wings and legs in *Drosophila*. In the wing disc, repression of Hth and Tsh is required to allow Wg and Dpp to induce Nub expression in the early wing primordium. In the leg Wg and Dpp repress Hth and Tsh and independently induce Dll and Dac (Abu-Shaar and Mann, 1998; González-Crespo et al., 1998; Wu and Cohen, 1999; Wu and Cohen, 2000). We suggest that Wg and Dpp act through *el* and *noc* to define the appendage-forming region of the early wing disc by repression of body-wall specific genes. Cells lacking El/Noc activity appear to adopt proximal identity by default, by virtue of not being able to repress Hth and Tsh.

Late functions of *el* and *no ocelli*

Transformation of *el* and *noc* mutant cells toward body wall identity occurred when clones were induced in first or early second instar. Clones induced later did not alter their proximal-distal identity, indicating that the role of *el* and *noc* in repressing body wall-specific genes is transient. This is consistent with the alteration in their patterns of expression as development proceeds (Fig. 1E,F). In the wing disc, El and Noc expression shifts to a ring near the base of the wing pouch and to a wedge shaped stripe along the dorsal-ventral boundary. Consistent with these changes, clones induced during early third instar lost proximal (hinge) structures (Fig. 6B,C). These phenotypes resemble those seen in the wings of the homozygous viable el^{1} mutant (compare Fig. 6C and D) (Davis et al., 1997). el^{1} is a deletion that removes approximately 25 kb of DNA from the region between the *el* and *noc* genes (Ashburner et al., 1999; Davis et al., 1997), but does not affect the coding region of either gene. By antibody labeling, we found that the el^{1} deletion causes loss of El and Noc protein expression in the wing pouch, while their expression in the hinge region seems to be broader than in wild-type discs (Fig. 7). On this basis we conclude that el^{1} deletes a common

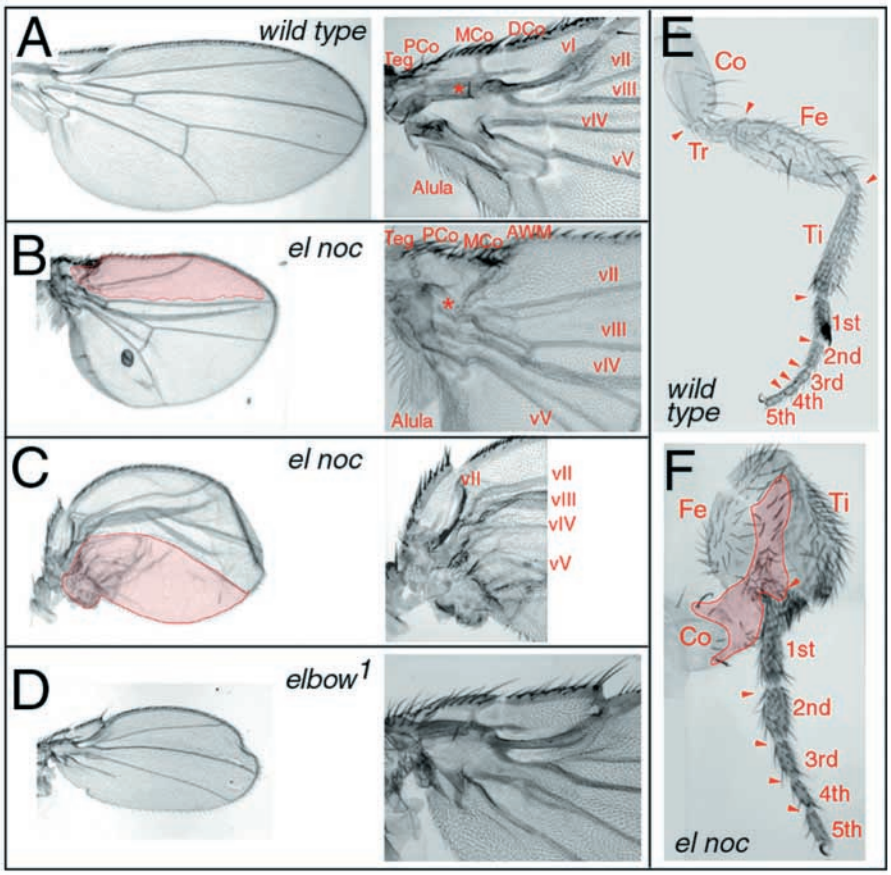

Fig. 6. Phenotypes of later *el noc* double mutant clones. (A) Cuticle preparation of a wild-type wing (left) and magnification of the wing hinge (right). PCo, MCo and DCo indicate proximal, medial and distal costa; Teg, tegula; veins are numbered vI-V. (B,C) Cuticle preparations of wings with large Minute^{+} *el$^{3.3.1}$ nocΔ64* double mutant clones induced at 84 hours (early third instar time in the *Minute* genotype). Clone marked by *forked* (shaded pink). Note the reduced size of the wings compared to the wild-type wing in A (same magnification). Right: detail of the wing hinge regions. Note deletion of hinge structures. (D) Cuticle preparation of an *el^{1}* mutant wing (left) and amplification of the wing hinge (right). Note deletion of hinge structures and reduced wing size compared to wild-type wings. (E) Cuticle preparation of a wild type adult leg. (F) Cuticle preparation of a leg carrying a double mutant clone extending through coxa, femur and tibia, but not tarsal segments. The trochanter is missing. Mutant tissue is shaded pink. Arrowheads indicate joints (not visible at this magnification; Co, Coxa; Tr, Trochanter; Fe, Femur; Ti, Tibia; 1st-5th, tarsal segments).

regulatory element that controls expression of *el* and *noc* in the wing pouch. As such *el^{1}* should be considered a regulatory allele of both loci and not just as an allele of *el* (Davis et al., 1997). The broader ring of El and Noc expression in the hinge might explain the adult hinge phenotype (Fig. 6).

We also noted that *el noc* double mutant clones had a non-autonomous effect on growth of the wing, which was similar to that described previously for *nub* mutant clones in the wing (Cifuentes and Garcia-Bellido, 1997; Ng et al., 1995). It is possible that the effects observed in *el noc* double mutant clones resulted from loss of Nub expression in the proximal part of the wing, where Nub is directly required for patterning proximal wing elements and indirectly for normal growth of the wing pouch.

In the leg, large *el* and *noc* double mutant clones extended from coxa, through femur and tibia and caused failure of leg segmentation (Fig. 6E,F). These defects resembled those produced by *hth* or *exd* mutant clones, which fail to maintain an affinity border between body wall and leg (González-Crespo and Morata, 1996; Wu and Cohen, 1999). Thus the *el* and *noc* genes appear to serve distinct functions in proximal wing and proximal leg later in development that differ from their primary early role in appendage specification.

Discussion

Insect legs and wings have a common developmental and evolutionary origin. The appendage primordia are specified by the Wg and Dpp signaling proteins, which subdivide leg and wing imaginal discs into presumptive body wall (proximal) and presumptive appendage regions (distal) during the second instar. Initially the entire discs express both Tsh and Hth, and their repression by Wg and Dpp signaling is required for subdivision of the disc into proximal and distal domains (Abu-Shaar and Mann, 1998; Dong et al., 2001; González-Crespo et al., 1998; Wu and Cohen, 1999; Wu and Cohen, 2000). We have provided evidence that the *el* and *noc* genes serve as mediators of the function of the Wg and Dpp signaling systems in specification of the appendage field within the imaginal discs. El and Noc are induced by Wg and Dpp and are required to repress the proximally expressed proteins Hth and Tsh. Previous work had identified *Dll* as a gene required for appendage formation in leg and antenna, and *nub* as a gene required for wing. This report identifies El and Noc as a pair of zinc-finger proteins that function in both ventral and dorsal appendages. However, there are interesting differences in the way that they do so, when examined in detail.

Dll expression is required for the formation of all leg and antenna elements in the ventral (leg) discs, and until this work Dll was the earliest known marker for the distal region leg disc (Cohen et al., 1989; Cohen and Jürgens, 1989a; Diaz-Benjumea et al., 1994; Gorfinkiel et al., 1997; Panganiban et al., 1994). Previous work has shown that repression of Hth and Tsh by Dpp and Wg was not required for expression of Dll in the leg, nor could Dll repress Hth and Tsh (Wu and Cohen, 1999). Thus an essential mediator of the effects of Wg and Dpp was missing. Our results present evidence that El and Noc serve this function, as their removal leads to ectopic expression

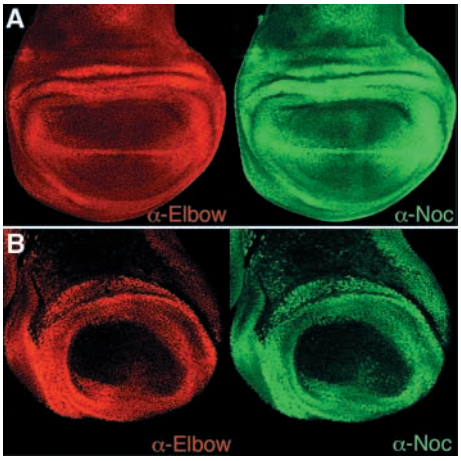

Fig. 7. *elbow*^{1} is a regulatory double mutant of both *el* and *noc*. (A) Immunostaining of wild-type third instar wing imaginal discs with anti-El (red) and anti-Noc (green) antibodies. (B) Immunostaining of *el*^{1} homozygous mutant third instar wing imaginal discs with anti-El (red) and anti-Noc (green) antibodies. Note that the mutant does not show the wedge-like staining along the dorsal-ventral boundary that is characteristic of wild-type third instar wing discs. Since both, El and Noc stainings are affected, we conclude that *el*^{1} is a regulatory double mutant affecting both genes.

of Hth and Tsh. Removal of El and Noc does not cause loss of Dll expression, so we conclude that Wg and Dpp act independently to induce El and Noc expression and Dll to define the distal region of the leg disc.

The situation differs slightly in the wing. Repression of Tsh is the earliest marker for specification of the distal wing region (Wu and Cohen, 2002), preceding the onset of Hth repression or of Nub induction (Azpiazu and Morata, 2000; Casares and Mann, 2000; Ng et al., 1996). Loss of Tsh and Hth are required to allow Nub expression. We observed ectopic expression of Hth and Tsh and loss of Nub in clones lacking El and Noc activity. Thus in the wing, expression of the distal marker Nub cannot be demonstrated to be independent of El and Noc (because ectopic Hth can repress Nub, but not Dll). The *vestigial* gene is also important for wing development and has been proposed to be a wing specifying gene (Kim et al., 1996; Simmonds et al., 1998; Williams et al., 1991). However, Vestigial is expressed all along the DV boundary of the wing, both in the wing primordium and in the body wall. This leads us to suggest that while Vestigial is essential for wing development, its expression cannot be taken as a molecular marker for wing identity per se, particularly at early stages [as discussed previously (Wu and Cohen, 2002)]. For this reason we have not focussed on analysis of the relationship between El, Noc and Vestigial in this report.

Is the repression of trunk genes needed to specify appendage as opposed to the body wall in wing and leg discs? In the wing disc the answer appears to be yes; repression of 'trunk genes' like *hth* is necessary to make the remaining part of the disc competent to form the appendage. However, in the leg the situation is more complex. Coexpression of Dll and Hth does not disrupt proximal-distal axis formation, but leads to homeotic transformation of leg tissue into antennal tissue (Casares and Mann, 1998; Dong et al., 2001). Hth is not repressed and limited to proximal areas in the antenna. However, loss of *el* and *noc* activities in the leg disc leads to loss of distal leg tissue without any evident transformation into antennal tissue. Thus, El and Noc may regulate the expression of other 'trunk genes', whose restricted expression are required to make the remaining leg and antenna disc competent to form the appendage.

The regional requirements for El and Noc highlight another interesting difference between leg and wing disc development. *el noc* double mutant cells were excluded from contributing to the tarsal region of the leg but not from contributing to the femur and tibia. As summarized above, lineage tracing has shown a considerable net flux of cells from the proximal (Tsh-expressing domain) into femur and tibia (Weigmann and Cohen, 1999). While there is no boundary of lineage restriction separating these domains, cells must be able to change from expressing the proximal marker Hth to expressing the distal marker Dll in order to move from one territory to the other (Wu and Cohen, 1999). The wing in contrast does not appear to normally exhibit this large net flux of cells from proximal to distal (K. Weigmann and S.M.C., unpublished data) and the *el noc* double mutant cells were excluded from contributing to the entire wing region. Our clonal analysis has suggested that *el noc* double mutant cells attempt to sort out toward proximal territory, or if that fails they can be lost from the disc, apparently by sorting out perpendicular to the epithelium. These observations suggest that El and Noc activity may contribute to the production of proximal-distal differences in cell affinities and thereby may help to maintain segregation of these cell populations during development.

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