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A. Lopez · D. Higuet · R. Rosset · J. Deutsch
F. Peronnet

corto genetically interacts with Pc-G and trx-G genes and maintains the anterior boundary of *Ultrabithorax* expression in *Drosophila* larvae

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Abstract In *Drosophila melanogaster*, segment identity is determined by specific expression of homeotic genes (Hox). The Hox expression pattern is first initiated by gap and pair-rule genes and then maintained by genes of the Polycomb-group (Pc-G) and the trithorax-group (trx-G). The *corto* gene is a putative regulator of the Hox genes since mutants exhibit homeotic transformations. We show here that, in addition to previously reported genetic interactions with the Pc-G genes *Enhancer of zeste*, *Polycomb* and *polyhomeotic*, mutations in *corto* enhance the extra-sex-comb phenotype of *multi sex combs*, *Polycomb-like* and *Sex combs on midleg*. *corto* also genetically interacts with a number of trx-G genes (*ash1*, *kismet*, *kohtalo*, *moira*, *osa*, *Trithorax-like* and *Vha*55). The interactions with genes of the trx-G lead to phenotypes displayed in the wing, in the postpronotum or in the thoracic mechanosensory bristles. In addition, we analyzed the regulation of the Hox gene *Ultrabithorax* (*Ubx*) in *corto* mutants. Our results provide evidence that *corto* maintains the anterior border of *Ubx* expression in third-instar larvae. We suggest that this regulation is accomplished through an interaction with the products of the Pc-G and trx-G genes.

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A. Lopez · J. Deutsch · F. Peronnet (✉)
Equipe Développement et Evolution,
Biologie du Développement, UMR 7622 CNRS,
Université Pierre et Marie Curie, 9,
Quai Saint-Bernard, 75252 Paris Cedex 05, France
E-mail: Frederique.Peronnet@snv.jussieu.fr
Tel.: +33-1-44272739
Fax: +33-1-44273258

D. Higuet
Laboratoire Dynamique du Génome et Evolution,
Institut Jacques Monod, UMR 7592 CNRS,
Université Pierre et Marie Curie – Université Denis Diderot,
2, Place Jussieu, 75251 Paris Cedex 05, France

R. Rosset
Laboratoire de Génétique et Physiologie du Développement,
IBDM, Université de la Mediterranée,
163, Avenue de Luminy, 13288 Marseille Cedex 9, France

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Introduction

Cell fate is determined at very early stages of the ontogenesis of multicellular organisms and is precisely maintained throughout development in order to achieve the appropriate organization of the adult body. The homeotic (Hox) genes have been extensively described as the master genes in the determination of the identity of body regions. These selector genes encode homeodomain-containing transcription factors that orchestrate the spatial expression of effector genes (see Gehring 1985 for review). In *Drosophila melanogaster*, gap and pair-rule gene products establish the initial pattern of Hox gene expression during embryogenesis (see Akam 1987 for review). Later on, two sets of proteins encoded by the Polycomb-group (Pc-G) and the trithorax-group (trx-G) of genes are recruited to maintain the spatial domains of Hox expression throughout development (for reviews, see Pirrotta 1995; Simon 1995). Perturbance of the dosage of these genes induces homeotic phenotypes. Pc-G and trx-G genes encode proteins with antagonistic roles: Pc-G proteins ensure that homeotic genes are kept repressed, whereas Trx-G proteins maintain them in an active state (for review, see Van Lohuizen 1999), and these functions have been conserved throughout evolution (Hanson et al. 1999). Combinations of two mutations from the same group produce more severe phenotypes than either single mutation alone, while combinations of mutations from each group suppress the phenotype. Both protein classes act through multimeric complexes which control the chromatin structure (Pirrotta 1998). The Pc-G proteins are thought to package DNA into heterochromatin-like structures, thus rendering it inaccessible to transcription factors. The spreading of these Pc-G complexes along the chromatin fiber has recently been shown to be blocked by specific insulators (Van der Vlag et al. 2000).

The trx-G proteins act at many levels of gene regulation (for review, see Kingston et al. 1996). *brahma* (*brm*) encodes an ATPase which belongs to the SWI/SNF chromatin remodeling complex (Tamkun et al. 1992). Moira (MOR), the homologue of the human BAF170 and BAF155 factors and of the yeast SWI3 protein, is an additional component of this SWI/SNF complex (Crosby et al. 1999) and interacts with the transcriptional machinery (Van der Knaap et al. 1997). Also associated with the BRM-SWI/SNF complex is OSA, whose ARID DNA-binding domain may anchor the complex to target genes. OSA is also a pleiotropic transcription regulator (Vazquez et al. 1999). Trithorax-like (TRL), the *Drosophila* GAGA factor, belongs to another chromatin remodeling complex named NURF and has been shown to bind to insulator DNA (Farkas et al. 1994; Tsukiyama et al. 1995; Ohtsuki and Levine 1998).

The Pc-G and Trx-G proteins are not only required for the expression of the Hox genes but also for the expression of other genes. For example, the Pc-G proteins maintain the expression pattern of the segmentation gene *engrailed* (Moazed and O'Farrell 1992), BRM and MOR are required for ovogenesis as well as for the correct expression of *engrailed* (Brizuela et al. 1994). Some Trx-G proteins have been shown to interfere with signaling pathways: OSA functions as a downstream repressor of the Wingless pathway (Treisman et al. 1997) and KIS may be involved in the Notch pathway (Go and Artavanis-Tsakonas 1998).

Mutants that are defective in the *corto* gene of *D. melanogaster* (also named *ccf* for *centrosomal and chromosomal factor*) display homeotic transformations reminiscent of the phenotypes associated with mutations in some Pc-G or trx-G genes (Kodjabachian et al. 1998). Thus, adults homozygous for the $corto^{420}$ null allele show additional sex combs on the second tarsomere of the first leg. This corresponds to the homeotic transformation of the second to the first tarsomere of the leg, a phenotype shared with mutants for some Pc-G genes like *crm*, *E(z)*, *mxc*, *ph* and *pho* (Jones and Gelbart 1990; Girton and Jeon 1994; Santamaria and Randsholt 1995; Yamamoto et al. 1997). Moreover, a partial loss-of-function of *corto* enhances the extra sex comb phenotype of *Pc*, *ph* or *E(z)* mutants (Kodjabachian et al. 1998). Flies that overexpress *corto* under the control of a *Gal4/UAS* system show transformation of arista into a leg extremity, reminiscent of trx-G mutant phenotypes. These mutants also exhibit weak transformation of halteres into wings, which, by contrast, is reminiscent of Pc-G mutant phenotypes. Corto may thus be recruited either as an activator or as a repressor of the homeotic genes depending on the developmental stage and/or the target gene considered. Defects in neuroblast chromosome condensation, another characteristic of *corto* mutants, suggest that it might also be involved in cell division (Kodjabachian et al. 1998). Hence, we speculated that *corto* might be a Pc-G or trx-G gene involved in the transmission of the open or closed chromatin state from one cell cycle to the next. This process of cellular memory is still poorly understood. To assess the involvement of *corto* in Pc-G gene silencing and/or trx-G gene activation, we examined the phenotypes of double heterozygous mutants carrying either a Pc-G or a trx-G mutant allele together with a loss-of-function allele of *corto*. Furthermore, we directly addressed the question of a role for *corto* in the regulation of Hox genes by following the expression of *Ultrabithorax* (*Ubx*) in *corto* mutants.

Materials and methods

Fly strains and culture conditions

Flies were grown on standard corn-meal, sugar and yeast medium with methyl hydroxy-4-benzoate as a mold inhibitor and benzyl benzoate-coated paper as an anti-mite agent. Stocks were maintained at 25°C, as were all crosses, except when otherwise indicated. The strains used for the analysis of genetic interactions between *corto* and Pc-G or trx-G genes are listed in Table 1. Effects of *corto* mutations on *Ubx* regulation were analyzed using the 35UZ-3, line which contains a *P{35UZ}* element localised at 34D (Irvine et al. 1991). The $corto^{Scer\backslash UAS.cKa}$ allele, in which the *corto* coding region is subcloned downstream of *GAL4* UAS sequences (Kodjabachian et al. 1998), was combined with $GAL4^{daG32}$ (Wodarz et al. 1995) or $GAL4^{69B}$ (Brand and Perrimon 1993) chromosomes to overproduce Corto. Both lines express GAL4 ubiquitously. The balancer chromosomes used are described in Flybase (The Flybase Consortium 1999). The $corto^{420}$ null allele and the *Df(3R)6-7* allele were also used in this study.

Genetic interactions

To test for genetic interactions between *corto* and Pc-G or trx-G genes, flies that were heterozygous for the $corto^{420}$ null allele were crossed with flies which were heterozygous for one Pc-G or one trx-G mutant allele. About five virgin females and five males were mated per vial. They were successively transferred three times into a new vial before being discarded. Two independent crosses were performed. All the progeny were observed, except when otherwise indicated. All crosses were done reciprocally to detect possible maternal effects. The offspring of *corto* × Pc-G mutant crosses was examined for the appearance of extra sex combs in males and the percentage of extra sex combs per leg was calculated and analyzed by Fisher's exact tests. Double heterozygous males were compared to their control siblings mutated in the Pc-G locus only. The offspring of *corto* × trx-G mutant crosses was carefully examined for any transformation potentially related to a defect in homeotic gene activity. Defects were scored and analyzed by Chi-square and Fisher's exact tests. For Fisher's exact tests and Chi-square tests, significances were determined using sequential Bonferroni correction (Rice 1989), to take account of the number of comparisons performed.

Generation of 35UZ; *corto* flies

$GAL4^{daG32}$ or $GAL4^{69B}$/*TM6B* flies, were crossed with *35UZ3*; $corto^{Scer\backslash UAS.cKa}$ flies to produce embryos and larvae overexpressing *corto* and carrying one allele of the *P{35UZ}* element. Crosses with $GAL4^{69B}$/*TM6B-Tb* flies were performed at 29°C. Flies of the genotype *35UZ-3/35UZ-3;* $corto^{420}$/*TM3-hb::lacZ* were mass-mated with *Df(3R)6-7/TM3-hb::lacZ* or $corto^{420}$/*TM3-hb::lacZ* flies to obtain either *35UZ-3/+;* $corto^{420}$/*Df(3R)6–7* or *35UZ-3/+;* $corto^{420}/corto^{420}$ embryos. They were distinguished from their balanced siblings by the absence of *hb*-driven β-galactosidase

Table 1 *corto*, Pc-G and trx-G alleles used in this study

Gene	Allele	Type of mutation	Reference
corto	$corto^{420}$	Null	Kodjabachian et al. (1998)
	Df(3R)6-7	Deficiency including *corto*	Castrillon et al. (1993)
Pc-G			
Additional sex combs	Asx^{XF23}	Amorph	Simon et al. (1992)
cramped	crm^{7}	Unknown	Yamamoto et al. (1997)
	crm^{sa}	Unknown	Yamamoto et al. (1997)
Enhancer of Polycomb	$E(Pc)^{1}$	Unknown	Moazed and O'Farrell (1992)
Enhancer of zeste	$E(z)^{61}$	Temperature sensitive, loss of function	Wu et al. (1989)
	$E(z)^{62}$	Antimorph	Jones and Gelbart (1990)
extra sex combs	esc^{e5}	Unknown	Lindsley and Zimm (1992)
	esc^{r4}	Unknown	Lindsley and Zimm (1992)
multi sex combs	mxc^{M1}	Hypomorph	Santamaria and Randsholt (1995)
pleiohomeotic	pho^{b}	Amorph	Girton and Jeon (1994)
Polycomblike	Pcl^{XM3}	Amorph	Tearle and Nusslein-Volhard (1987)
	Pcl^{11}	Amorph	Kennison and Russell (1987)
Posterior sex combs	$Psc^{vg\text{-}D}$	Deficiency including *Psc* and *vg*	Simon et al. (1992)
	Psc^{1}	Hypomorph	Adler et al. (1989)
Sex combs extra	Sce^{1}	Unknown	Breen and Duncan (1986)
Sex combs on midleg	Scm^{D1}	Amorph	Breen and Duncan (1986)
	Scm^{4}	Amorph	Jürgens (1985)
super sex combs	sxc^{1}	Amorph	Lindsley and Zimm (1992)
trx-G			
absent, small, or homeotic discs 1	$ash1^{vv183}$	Amorph	Shearn et al. (1971)
	$ash1^{B1}$	Hypomorph	Gindhart and Kaufman (1995)
absent, small, or homeotic discs 2	$ash2^{1}$	Amorph	Adamson and Shearn (1996)
brahma	brm^{2}	Amorph	Tamkun et al. (1992)
kismet	kis^{2}	Hypomorph	Gindhart and Kaufman (1995)
kohtalo	kto^{1}	Hypomorph	Gindhart and Kaufman (1995)
	$Df(3L)kto^{2}$	Deficiency including *kto* and *ash1*	Lindsley and Zimm (1992)
moira	mor^{1}	Hypomorph	Brizuela and Kennison (1997)
osa	osa^{2}	Hypomorph	Kennison and Tamkun (1988)
trithorax	trx^{E2}	Amorph	Kennison and Tamkun (1988)
Trithorax-like	Trl^{13C}	Hypomorph	Liaw et al. (1995)
	Trl^{R85}	Hypomorph	Hagstrom et al. (1997)
Vacuolar H^{+}-ATPase 55kD B subunit	$Vha55^{7}$	Unknown	Gausz et al. (1979)
	$Vha55^{14}$	Unknown, embryonic lethal	Gausz et al. (1979)
urdur	urd^{2}	Hypomorph	Gindhart and Kaufman (1995)

expression. Larvae carrying a *P{35UZ}* element and lacking *corto* were generated by mating *35UZ-3/35UZ-3;* $corto^{420}$*/TM6B-Tb* females with $corto^{420}$*/TM6B-Tb* or *Df(3R)6-7/TM6B-Tb* males. They were selected on the basis of their Tb^{+} phenotype.

X-gal staining and immunochemistry

35UZ3 embryos were fixed and stained with X-gal as described by Hazelrigg (2000). β-Galactosidase expression in third-instar larvae was detected according to Yanicostas et al. (1995). Embryonic expression of *Ubx* in germline clones was detected with a mouse monoclonal antibody (1:100, a kind gift of Dr. Y. Graba) as described in Rothwell and Sullivan (2000) and visualized with NBT/BCIP.

Induction of *corto*-deficient germline clones

yw hs::FLP; FRT^{82B} $corto^{420}$*/TM3* females were crossed with *w**, $P\{ry^{+t7.2}=neoFRT\}^{82B}$ $P\{w^{+mC}=ovo^{D1\text{-}18}\}3R/TM3$ males. Larvae (late L2; young L3) were heat shocked at 37°C for 2 h in a water bath over a period of 2 days (Chou and Perrimon 1996). Then, *yw hs::FLP/w**; FRT^{82B} $corto^{420}$*/FRT* $P\{ovo^{D1}\}$ females were crossed with $corto^{420}$*/TM3-hb::lacZ* males. Embryos were collected and immunostained. *corto* null embryos were distinguished from their balanced siblings by the absence of *hb*-driven β-galactosidase expression.

Results

Phenotypes of $corto^{420}$ mutants

The $corto^{420}$ allele has been shown to lack the entire *corto* coding region (Kodjabachian et al. 1998). About 9% of the heterozygous adults displayed defects in thoracic macrochaetes (Fig. 1B). Abnormalities included duplication of the whole organ, duplication of the bristle alone, disappearance of the bristle leaving the socket alone, or loss of the whole organ. Similar defects have also been reported in some trx-G mutants such as *brm*, *ash2* and *osa* (Adamson and Shearn 1996; Elfring et al. 1998; Collins et al. 1999). Moreover, about 11% of the $corto^{420}$ heterozygotes also exhibited defects in the postpronotum and the humeral bristles (Fig. 2B). These defects included a stunted postpronotum, folded

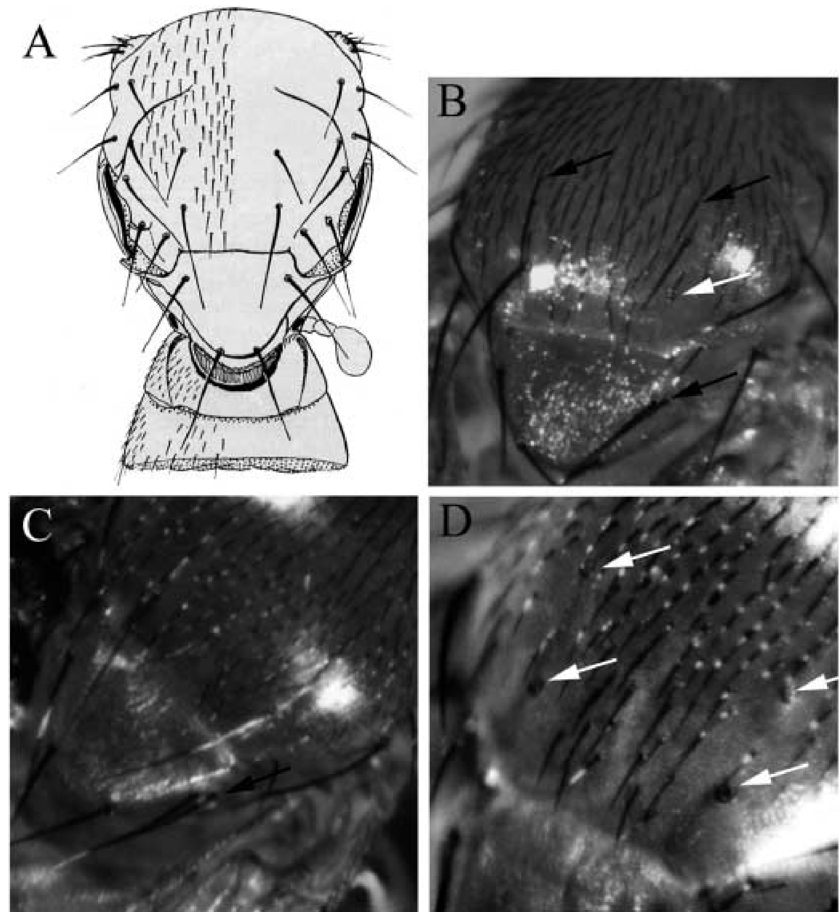

Fig. 1A–D Defects in thoracic mechanosensory bristles. **A** In wild-type *Drosophila* (Demerec et al. 1950), the thoracic macrochaetes (22 long bristles) are organised in a highly compex and reproducible pattern. Defects in these mechanosensory organs (duplication or loss of the whole organ, twinning of the bristle or loss of the bristle) occur in $corto^{420}/+$ flies as well as in some trx-G mutants. **B** $corto^{420}/+$; the *white arrow* indicates the deletion of one macrochaete leaving only the socket; the black arrows point to extra macrochaetes. **C** $trx^{E2}/+$; the *black arrow* indicates a case of bristle twinning. **D** $trx^{E2}/corto^{420}$; the *white arrows* indicate sockets without bristles

bristles, thick and short bristles or missing bristles. Complete loss-of-function of *corto* ($corto^{420}/corto^{420}$ or $corto^{420}/Df(3R)6$-7) led to lethality at various times throughout development. Only about 10% of the progeny survived to adulthood. All the adult homozygous escapers displayed extra pieces of vein tissue projecting from the posterior crossvein of the wing (Fig. 3B). Some of them presented a partially or totally deleted anterior crossvein. In addition, about 50% of the male escapers displayed an additional sex comb on the second tarsomere of the first leg. Such a homeotic transformation has previously been observed in Pc-G mutants such as *crm*, *E(z)*, *mxc*, *ph* and *pho* (Jones and Gelbart 1990; Girton and Jeon 1994; Santamaria and Randsholt 1995;Yamamoto et al. 1997).

Interactions between *corto* and Pc-G mutants

A previous study has shown that loss of one dose of *corto* enhances the extra sex comb phenotype of Pc^{K}, ph^{410} and $E(z)^{60}$ heterozygotes (Kodjabachian et al. 1998). We extended this analysis to 18 other Pc-G alleles (Table 1). Flies heterozygous for Asx^{XF23}, crm^{7}, crm^{sa}, $E(Pc)^{1}$, $E(z)^{61}$, $E(z)^{62}$, esc^{e5}, esc^{r4}, mxc^{M1}, pho^{b}, Pcl^{XM3}, Pcl^{11}, $Psc^{vg\text{-}D}$, Psc^{1}, Sce^{1}, Scm^{D1}, Scm^{4} or sxc^{1} were crossed with $corto^{420}$ heterozygotes in both reciprocal fashion. All the male progeny of these crosses were examined for the appearance of extra sex combs. The percentage of extra sex combs per leg, i.e. the penetrance of the phenotype, was scored in double heterozygotes and in their single heterozygous control siblings. $corto^{420}/+$ heterozygotes never displayed extra sex combs. Thus, the percentage of extra sex combs in ($Pc\text{-}G^{-}/+$; $corto^{420}/+$) flies was compared to the percentage of extra sex comb in $Pc\text{-}G^{-}/+$ flies and the results were subjected to Fisher's exact test. No significant modification of the number of extra sex combs was observed in double heterozygotes ($Pc\text{-}G^{-}/+$; $corto^{420}/+$) vs. single heterozygotes ($Pc\text{-}G^{-}/+$) when $corto^{420}$ was combined with Asx^{XF23}, crm^{7}, crm^{sa}, $E(Pc)^{1}$, $E(z)^{61}$, esc^{e5}, esc^{e5}, esc^{r4}, pho^{b}, $Psc^{vg\text{-}D}$, Psc^{1}, Sce^{1} , sxc^{1} or Scm^{4} (data not shown). On the other hand, enhancement of the extra sex combs phenotype was observed in double heterozygotes ($Pc\text{-}G^{-}/+$; $corto^{420}/+$) relative to the single heterozygotes ($Pc\text{-}G^{-}/+$) in the progeny of crosses between $corto^{420}$ and $E(z)^{62}$, mxc^{M1}, Pcl^{XM3}, Pcl^{11} or Scm^{D1} Scm^{D1} (Table 2). Two independent effects were observed. First, enhancement of the transformation of second and third thoracic legs into first thoracic legs was observed in double heterozygotes as compared to single heterozygotes for the Pc-G alleles mxc^{M1}, Pcl^{XM3}, Pcl^{11} and Scm^{D1}. For Pcl^{XM3} and Scm^{D1}, this enhancement occurred only when the mother was heterozygous for

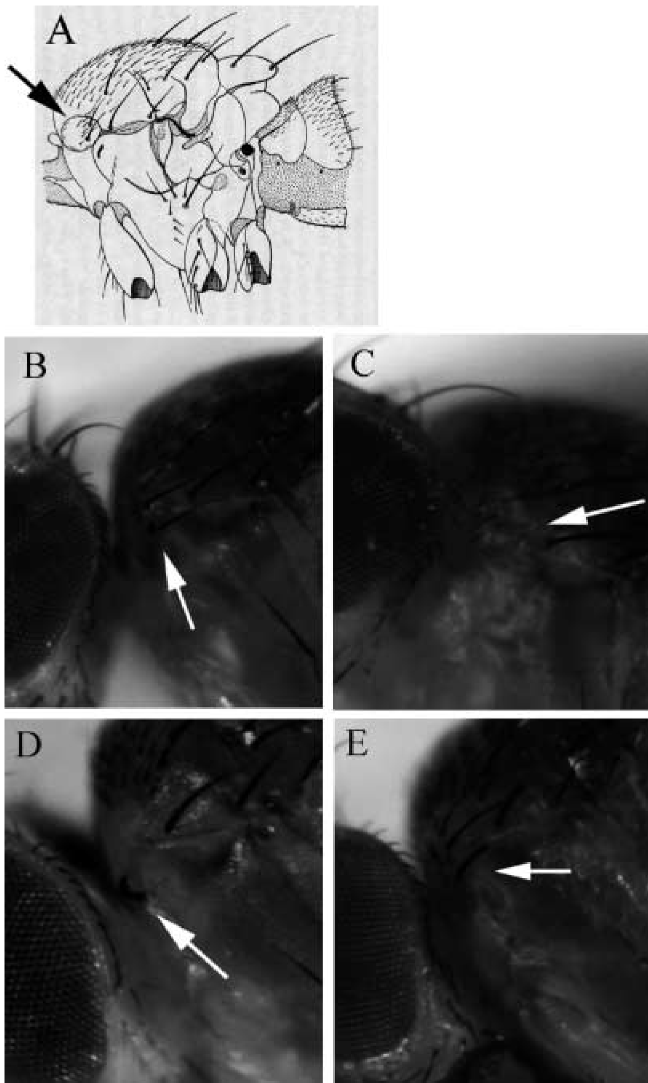

Fig. 2A–E Postpronotum defects. **A** The *black arrow* points to the two long humeral bristles on the wild-type postpronotum (The Flybase Consortium 1999). Postpronotum defects (stunted postpronotum, folded bristles, thick and short bristles or missing bristles) of the humeral bristles occur in $corto^{420}/+$ flies, in some trx-G^{-}/+ flies and in double heterozygotes (trx-G^{-}/+; $corto^{420}/+$). **B** $corto^{420}/+$; one bristle is folded. **C** $brm^{2}/+$; the postpronotum is devoid of long bristles. **D** $ash1^{B1}/+$; the postpronotum bears only one very thick bristle. **E** $urd^{2}/corto^{420}$; one bristle is shorter than in wild-type

$corto^{420}$. Second, a proximo-distal transformation within the first leg, i.e. transformation of the second into the first tarsomere, was observed in double heterozygotes from crosses between $corto^{420}$ and mxc^{M1} and between $corto^{420}$ and $E(z)^{62}$. A strong maternal effect was observed when the reciprocal crosses between $corto^{420}$ and $E(z)^{62}$ were compared; enhancement of the extra sex combs phenotype was observed only when the mother was heterozygous for $corto^{420}$. Taken together, these results led us to conclude that *corto* interacts with a subset of Pc-G genes: *E(z)*, *mxc*, *Pc*, *Pcl*, *ph* and *Scm*.

Interactions between *corto* and trx-G mutants

Following the same procedure, 13 trx-G alleles (Table 1) ($ash1^{B1}$, $ash2^{1}$, brm^{2}, kis^{2}, kto^{1}, mor^{1}, osa^{2}, trx^{E2}, Trl^{13C}, Trl^{R85}, urd^{2}, $Vha55^{7}$ and $Vha55^{14}$) were scored for genetic interactions with $corto^{420}$.

We observed that single heterozygotes for $ash1^{B1}$, $ash2^{1}$, brm^{2}, kto^{1}, osa^{2}, trx^{E2}, Trl^{13C}, Trl^{R85} and urd^{2} displayed the same thoracic macrochaete (Fig. 1) and postpronotum defects (Fig. 2) as $corto^{420}/+$. The penetrance of the phenotype varied depending on the trx-G gene and allele: thoracic macrochaete defects occurred in 4–20% of flies, and postpronotum and humeral bristle defects in 3–32%. This value reached 32% in $brm^{2}/+$ flies, where the postpronotum was quite often naked (Fig. 2C). The penetrance of the macrochaete and postpronotum phenotypes was then scored in flies heterozygotes for both $corto^{420}$ and these trx-G alleles. In all crosses, the same results were obtained, regardless of whatever the mother was mutant for *corto* or for a trx-G gene – unlike the case for the genetic interactions between *corto* and Pc-G genes. The results of reciprocal crosses were therefore pooled. It has been proposed, in the case of multimeric protein complexes (such as those formed by Trx-G proteins) that loss of two components of the complex enhances the phenotype associated with the loss of a single component (Locke et al. 1988; Cheng et al. 1994). Assuming that Corto belongs to such complexes, we tested the independence of $corto^{420}$ and the trx-G alleles according to an additive model. The percentage of double heterozygotes with defects, i.e. the penetrance of the phenotype, was compared to the theoretical percentage (H_0) obtained by summing the percentages of flies with defects among both classes of single heterozygotes (Tables 3 and 4). When the penetrance observed in double heterozygotes (trx-G^{-}/+; $corto^{420}/+$) was higher than the penetrance expected following this model, we inferred a genetic interaction between $corto^{420}$ and the trx-G allele tested. When the penetrance observed in double heterozygotes was equal to, or lower than, the penetrance expected following this model, it was not possible to draw any firm conclusions. The percentage of thoracic macrochaete defects observed was significantly lower than expected under this additive model when $corto^{420}$ was combined with $ash1^{B1}$, $ash2^{1}$, brm^{2}, kto^{1}, trx^{E2}, Trl^{13C}, Trl^{R85} or urd^{2}, but it was higher than expected when $corto^{420}$ was combined with osa^{2} (Table 3). Furthermore, the percentage of postpronotum defects was lower than expected when $corto^{420}$ was combined with $ash1^{B1}$, $ash2^{1}$, brm^{2}, kto^{1}, trx^{E2} or urd^{2}, urd^{2}, equal when combined with osa^{2}, and higher when combined with Trl^{13C} or Trl^{R85} (Table 4). Taken together, these results indicate that there is a synergy between *corto* and *osa* with respect to the differentiation of the thoracic macrochaetes and between *corto* and *Trl* during the differentiation of the postpronotum.

The posterior crossvein defect observed in flies totally lacking Corto ($corto^{420}/corto^{420}$ or $corto^{420}/Df(3R)6\text{-}7$) was also observed in double heterozygotes for $corto^{420}$

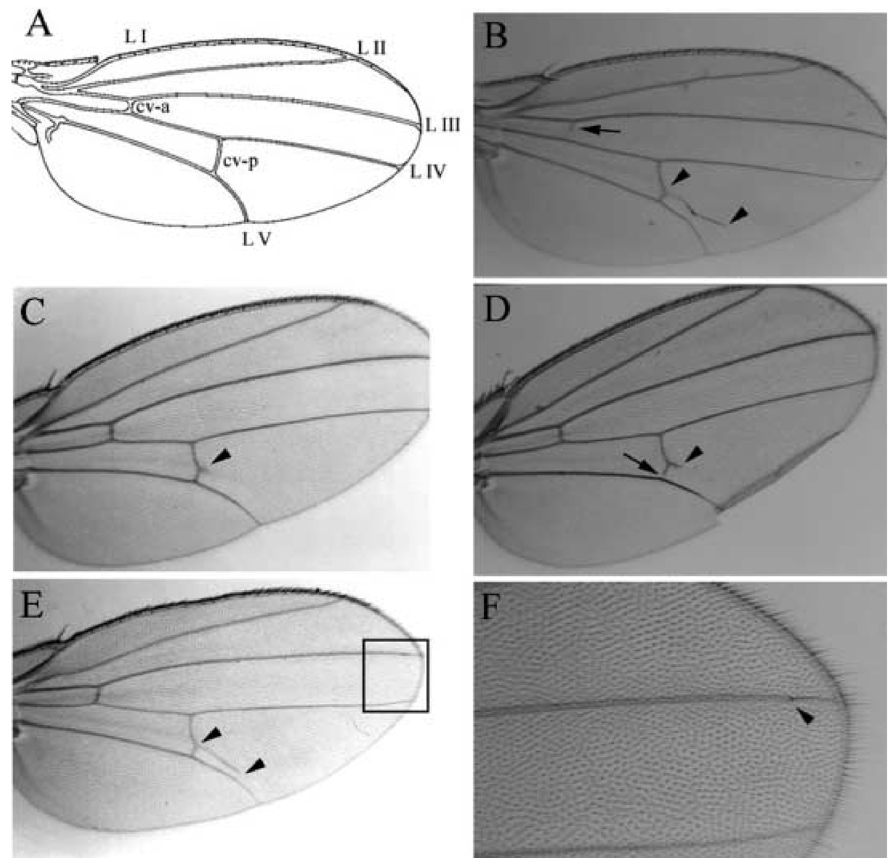

Fig. 3A–F Crossvein defects. **A** Wild-type wing showing the normal pattern of wing veins (The Flybase Consortium 1999). **B** *corto*^{420}/*Df(3R)6-7*; the *black arrowheads* point to the extra vein extending from the posterior crossvein, the *black arrow* to an incomplete anterior crossvein. The extra-vein phenotype was also observed in some trx-G^{-}/+; *corto*^{420}/+ double heterozygotes. **C** *kis*^{2}/+; *corto*^{420}/+ ; the black arrowhead indicates an extra vein projecting from the posterior crossvein. **D** *corto*^{420}/*Vha55*^{7}. **E** *corto*^{420}/*Df(3L)kto*^{2}. **F** Magnification of the area outlined in **E**. The *black arrowhead* indicates an extra sensory bristle

and *ash1*^{B1}, *kis*^{2}, *kto*^{1}, *mor*^{1}, *Vha55*^{7} *or Vha55*^{14} (Fig. 3 and Table 5), whereas this defect was not observed in single heterozygotes. The penetrance of the phenotype was incomplete and varied between 2 and 17%, depending on the trx-G gene and allele. Moreover, the extra pieces of vein tissue were shorter than those in *corto*^{420}/*Df(3R)6-7* flies. As no differences were observed between the reciprocal crosses, the results were again pooled and submitted to Fisher's exact test to compare double heterozygotes (trx-G^{-}/+; *corto*^{420}/+) with single heterozygotes (trx-G^{-}/+). The appearance of the phenotype was significant in the double heterozygotes *ash1*^{B1}/*corto*^{420}, (*kis*^{2}/+; *corto*^{420}/+), *kto*^{1}/*corto*^{420}, *mor*^{1}/*corto*^{420} and *Vha55*^{7}/*corto*^{420}. Taken together, these results suggest that *ash1*, *kis*, *kto*, *mor*, *Vha55* and *corto* are concomitantly involved in a process that lays down wing veins. The weak penetrance and expressivity of the phenotype in double heterozygotes (trx-G^{-}/+; *corto*^{420}/+) may be related to the fact that *ash1*^{B1}, *kis*^{2}, *kto*^{1} and *mor*^{1} are all hypomorphic alleles. To test this hypothesis, we looked for amorphic alleles of these genes. We analyzed the *Df(3L)kto*^{2} deficiency which deletes both *kto* and *ash1*. Surprisingly, the combination of *Df(3L)kto*^{2} and *corto*^{420} was lethal, with only 3% of the *Df(3L)kto*^{2}/*corto*^{420} individuals reaching adulthood. All these escapers exhibited the same posterior crossvein defect as *corto* homozygotes. These extra veins were about the same size as those of *corto*^{420}/*Df(3R)6-7* flies. Moreover, some wings displayed ectopic sensory bristles (Fig. 3F). We also analyzed double heterozygotes for *corto*^{420} and *ash1*^{vv183}, an amorphic allele of *ash1*. No lethality occurred in *corto*^{420}/*ash1*^{vv183} double heterozygotes. This suggests that either extragenic non-complementation between *corto*^{420} and an unidentified locus located within the deficiency, or the cumulative loss of several loci, was responsible for the high lethality in the double heterozygotes *Df(3L)kto*^{2}/*corto*^{420}.

Ubx expression in *corto* mutants

In order to analyze *Ubx* expression in *corto* mutants, we used the 35UZ-3 transgenic line in which an expression pattern very similar to that of *Ubx* throughout development is conferred on *lacZ* via the UCR (upstream control region) of *Ubx* (Irvine et al. 1991). Thus, in 35UZ-3 embryos, *lacZ* is expressed in PS5–PS13 with the highest level of expression in PS6. No modification of this pattern was observed in 35UZ-3/+; *corto*^{420}/+, 35UZ-3/+; *Df(3R)6-7*/+, 35UZ-3/+; *corto*^{420}/*corto*^{420} or 35UZ-3/+; *corto*^{420}/*Df(3R)6-7* embryos (data not shown). However, these embryos had a half dose of maternally inherited Corto protein. To obtain embryos totally lacking the protein, we produced germline *corto* mutants and stained them with an antibody directed against the UBX protein. These *corto* null embryos displayed no modification of the anterior boundary of the *Ubx* expression domain (data not shown). Embryos

Table 2 Genetic interactions between *Pc-G* mutant alleles and *corto*^{420}

Genotype tested[a]	Number of males observed		Percentage of additional sex combs on first leg (T1t2)[b]		Percentage of second leg with sex combs (T2)		Percentage of third leg with sex combs (T3)	
	Pc-G$^-$/Bal mothers	*corto*^{420}/*TM6B* mothers	*Pc-G*$^-$/Bal mothers	*corto*^{420}/*TM6B* mothers	*Pc-G*$^-$/Bal mothers	*corto*^{420}/*TM6B* mothers	*Pc-G*$^-$/Bal mothers	*corto*^{420}/*TM6B* mothers
$E(z)^{62}/corto^{420}$	50	125	0	12*	0	1	0	0
$E(z)^{62}/+$	21	66	0	0	0	1	0	0
$corto^{420}/+$	50	95	0	0	0	0	0	0
$mxc^{M1}/Y; corto^{420}/+$	92		27*		52*		19*	
mxc^{M1}/Y	53		8		17		3	
$corto^{420}/+$	51		0		0		0	
$Pcl^{XM3}/+; corto^{420}/+$	86	89	0	0	4	68*	1	61*
$Pcl^{XM3}/+$	63	84	0	0	2	49	1	17
$corto^{420}/+$	87	95	0	0	0	0	0	0
$Pcl^{11}/+; corto^{420}/+$	50	104	0	0	55*	35*	29*	14*
$Pcl^{11}/+$	39	48	0	0	17	10	14	0
$corto^{420}/+$	50	65	0	0	0	0	0	0
$Scm^{D1}/corto^{420}$	50	163	0	0	0	37*	0	22*
$Scm^{D1}/+$	50	66	0	0	0	16	0	5
$corto^{420}/+$	50	151	0	0	0	0	0	0

[a] *corto*^{420}/*TM6B* flies were crossed with *Pc-G*$^-$/Balancer flies. In each case, a randomly selected set of fifty males or the entire complement of male progeny was examined. Additional sex combs were scored. Two independent crosses were performed. Since no difference was observed, the results were pooled. As *corto*^{420}/+ heterozygotes never displayed extra sex combs, the significance of variation in the percentage of legs bearing extra sex combs between flies carrying only a *Pc-G* mutant allele and flies carrying both a *Pc-G* mutant allele and a *corto*^{420} allele was assessed using Fisher's exact test implemented in the statistical software package SAS (SAS Institute, Cary, N.C., 1990). The *asterisks* indicate significant values

[b] t2, tarsomere 2 of the first leg

overexpressing *corto* (35UZ-3/+; *UAS::corto/GAL4*^{69B} or 35UZ-3/+; *UAS::corto/GAL4*^{daG32}) never exhibited any alteration in the embryonic pattern of *Ubx* expression (data not shown).

In third-instar 35UZ-3 larvae, *lacZ*, like endogenous *Ubx*, is expressed in the posterior compartment of the metathoracic imaginal discs (i.e. third leg and haltere discs) (Fig. 4A). In addition, due to a specific chromosomal position effect, a crescent of *lacZ* expression was observed at the posterior rim of the eye disc, in agreement with previously published results (Docquier et al. 1999). Loss-of-function (35UZ-3/+; *corto*^{420}/*corto*^{420} or 35UZ-3/+; *corto*^{420}/*Df(3R)6-7*), as well as gain-of-function (35UZ-3/+; *UAS::corto/GAL4*^{69B} or 35UZ-3/+; *UAS::corto/GAL4*^{daG32}) mutants frequently exhibited ectopic expression of *lacZ* in the second leg, first leg, wing or eye-antennal imaginal discs, in addition to the 35UZ-3 pattern (Fig 4B, C). Moreover, in haltere and third leg discs, the border of the *Ubx* expression domain was less well defined. The ectopic *Ubx* spots were variable in size and position. In about 27% of deficient larvae and about 47% of gain-of-function larvae, at least one disc exhibited ectopic spots. Moreover, the spots were wider in deficient larvae than in gain-of-function larvae, suggesting that misregulation of *Ubx* arose earlier in the former. Ectopic expression was never observed in 35UZ-3/+; *corto*^{420}/+ imaginal discs.

Discussion

Corto represses *Ubx* expression outside of its normal expression domain in third-instar larvae

Only 10% of *corto* loss-of-function mutants reached adulthood and, in the remaining individuals, mortality occurred throughout development, suggesting that maternally expressed protein present in embryos is responsible for rescuing the lethality of *corto* homozygotes. Furthermore, the longevity and fertility of flies deficient for *corto* were strongly reduced. These observations, as well as the pleiotropic nature of *corto* mutant phenotypes, may reflect a general role for *corto*. Since some phenotypes of *corto* mutants suggest a function in the regulation of homeotic genes, we analyzed *Ubx* expression in these mutants. Modification of the anterior boundary of the *Ubx* expression domain was only observed in *corto* deficient larvae during the third instar, and never in embryos lacking maternal and zygotic Corto. Corto thus seems to repress *Ubx* outside its normal expression in third-instar larvae. Nevertheless, it is not known whether or not Corto acts directly on *Ubx* expression. Intriguingly, anterior expression of *Ubx* is also observed in larvae that overexpress *corto*. One simple explanation is that Corto belongs to some Pc-G multimeric complexes bound to *Ubx* PREs. In this case, excess of Corto could provoke the aggregation of its Pc-G partners before they can bind to chromatin. A similar effect has been reported for *E(z)*, which

Table 3 Genetic interactions between trx-G mutant alleles and *corto*^{420}: thoracic macrochaete defects

Genotype tested[a]	Number of individuals scored	Number of flies with mechanosensory bristle defects	Percentage	Expected percentage (H_0)[b]
corto^{420}/+	429	38	9	–
ash1^{B1}/+	300	25	8	–
ash1^{B1}/*corto*^{420}	313	20	6*	17
ash2^{1}/+	390	26	7	–
ash2^{1}/*corto*^{420}	612	39	6*	16
brm^{2}/+	262	49	19	–
brm^{2}/*corto*^{420}	1189	218	18*	28
kto^{1}/+	234	48	20	–
kto^{1}/*corto*^{420}	317	21	7*	29
osa^{2}/+	328	29	9	–
osa^{2}/*corto*^{420}	1644	397	24*	18
trx^{E2}/+	225	17	7	–
trx^{E2}/*corto*^{420}	1489	173	12*	16
Trl^{13C}/+	227	17	7	–
Trl^{13C}/*corto*^{420}	1324	77	6*	16
Trl^{R85}/+	260	11	4	–
Trl^{R85}/*corto*^{420}	1488	110	7*	13
urd^{2}/+	354	35	10	–
urd^{2}/*corto*^{420}	2438	189	8*	19

[a]Reciprocal crosses between *corto*^{420}/*TM6B* and trx-G^{-}/*Balancer* flies were performed to detect possible maternal effects. The numbers of flies exhibiting thoracic macrochaete defects were scored (duplication and deletion of macrochaetes were both considered as defects). As no difference was observed between the four crosses, the results were pooled. *corto*^{420}/+ and trx-G^{-}/+ flies were obtained by crossing *corto*^{420}/*TM6B* or trx-G^{-}/Balancer with a wild-type strain

[b]We performed an identity chi-square test by comparing the results to an additive model in which the effect observed in the double heterozygotes corresponded to the addition of the effect of the *corto*^{420} allele and the effect of the trx-G mutant allele (H_0 hypothesis). H_0 is the percentage of defective individuals expected in double heterozygotes based on the additive model, i.e. the percentage estimated in *corto*^{420} mutants plus the percentage estimated in trx-G^{-} mutants. The *asterisks* indicate significant differences from the expected values

Table 4 Genetic interactions between *trx-G* mutant alleles and *corto*^{420}: postpronotum defects

Genotype tested[a]	Number of individuals scored	Number of flies with postpronotum and humeral bristle defects	Percentage	Expected percentage (H_0)[b]
corto^{420}/+	429	47	11	–
ash1^{B1}/+	300	37	12	–
ash1^{B1}/*corto*^{420}	162	15	9*	23
ash2^{1}/+	390	11	3	–
ash2^{1}/*corto*^{420}	402	26	6*	14
brm^{2}/+	262	84	32	–
brm^{2}/*corto*^{420}	1189	265	22*	43
kto^{1}/+	234	48	20	–
kto^{1}/*corto*^{420}	317	28	9*	31
osa^{2}/+	328	21	6	–
osa^{2}/*corto*^{420}	1210	222	18	17
trx^{E2}/+	225	8	3	–
trx^{E2}/*corto*^{420}	985	60	6*	14
Trl^{13C}/+	227	14	6	–
Trl^{13C}/*corto*^{420}	858	180	21*	17
Trl^{R85}/+	260	7	3	–
Trl^{R85}/*corto*^{420}	914	225	25*	14
urd^{2}/+	354	46	13	–
urd^{2}/*corto*^{420}	1863	108	6*	24

[a]Flies exhibiting postpronotum defects were scored (stunted postpronotum, folded bristles, thick and short bristles and missing bristles were equally considered as defects). Crosses and statistical analysis were performed as described in the footnotes to Table 3. The *asterisks* indicate significant differences from the expected values (see Table 3)

enhances the *esc* phenotypes whenever its dosage is either reduced or increased (Campbell et al. 1995). As a matter of fact, ESC and E(Z) have been shown to belong to the same multimeric complex (Tie et al. 1998; Ng et al. 2000).

corto interacts with some Pc-G genes

Interactions between *corto* and some Pc-G genes *E(z)*, *Pc* or *ph* have been described previously (Kodjabachian et al. 1998). In the present paper, we tested for genetic

Table 5 Genetic interactions between trx-G mutant alleles and *corto*^{420}: posterior crossvein defects

Genotype tested[a]	Number of individuals scored	Percentage of flies with posterior crossvein abnormality[b]
corto^{420}/+	>1000	0
Df(3R)6-7/+	>1000	0
corto^{420}/*corto*^{420}	>1000	100
corto^{420}/ *Df(3R)6-7*	>1000	100
ash1^{B1}/*corto*^{420}	552	3*
ash1^{B1}/+	316	0
kis^{2}/+; *corto*^{420}/+	429	14*
kis^{2}/+	274	0
kto^{1}/*corto*^{420}	381	2*
kto^{1}/+	211	0
mor^{1}/*corto*^{420}	450	17*
mor^{1}/+	526	0
Vha55^{7}/*corto*^{420}	146	6*
Vha55^{7}/+	133	0
Vha55^{14}/*corto*^{420}	147	1
Vha55^{14}/+	81	0
Df(3L)kto^{2}/*corto*^{420}	27	100*
Df(3L)kto^{2}/+	181	0
ash1^{vv183}/*corto*^{420}	184	4
ash1^{vv183}/+	54	0

[a]Reciprocal crosses between *corto*^{420}/*TM6B* and trx-G^{-}/*Balancer* were performed to detect possible maternal effects. Posterior crossvein abnormalities were scored. As no difference was observed between the reciprocal crosses, the results were pooled. *corto*^{420}/+ and trx-G^{-}/+ were obtained by crossing *corto*^{420}/*TM6B* or *trx-G*$^{-}$/ Balancer with a wild-type strain

[b]The penetrance of the phenotype in double heterozygotes (trx-G^{-}/+; *corto*^{420}/+) was compared to the penetrance of the phenotype in single heterozygotes trx-G^{-}/+ by Fisher's exact test using the statistical software package SAS (see footnote to Table 2). The *asterisks* indicate statistical significance

interactions between *corto* and 11 other Pc-G genes, including two other alleles of *E(z)*. Our results show interactions between *corto* and *mxc*, *Pcl* and *Scm*, and confirm interactions with *E(z)*, *Pc* and *ph*. This indicates that *corto* cooperates with a subset of Pc-G genes in the regulation of the Hox gene *Scr*. We failed to detect any interaction with other Pc-G genes. However, whereas the detection of an interaction in such a test is informative, the absence of such an interaction has to be interpreted with caution, given the maternal effects that characterize almost all these genes. *corto* enhances the transformation of the second and third legs into first legs in *mxc*, *Pcl* and *Scm* mutants, as shown previously for *Pc*, *ph* and *E(z)* (Kodjabachian et al. 1998). This implies a common involvement of *corto* and these genes in maintaining the identity of the first thoracic segment. It is noteworthy that enhancement of the antero-posterior tranformation was not observed when *corto*^{420} was combined with all *E(z)* alleles but only with *E(z)*^{62}. However, interactions between Pc-G genes have frequently been shown to be allele-specific. *corto* also enhances the proximo-distal leg transformations seen in *mxc* and *E(z)* mutants. Although this phenotype is not enhanced by combining *mxc*^{MI} and *E(z)*^{62} mutations (Saget et al. 1998), our results suggest a common role of these three proteins in proximo-distal identity determination. Interestingly, enhancement of both proximo-distal and antero-posterior extra-sex-comb phenotypes was nearly always stronger when the chromosome bearing the *corto* mutation was maternally inherited. The requirement for Corto for appropriate expression of *Scr* might therefore arise earlier during development.

Is Corto a part of the Pc-G complexes?

Our data show that Corto might belong to multimeric Pc-G complexes. Few Pc-G complexes have been described in detail so far. It has been suggested that the composition of Pc-G complexes is very flexible and that different Pc-G complexes may be associated with different functions (Strutt and Paro 1997). A Pc-G complex containing ESC and E(Z) has recently been characterized (Ng et al. 2000). The ESC protein is a Pc-G protein that is present only during oogenesis and at the beginning of embryogenesis. It is thought to be involved in the scanning of the Hox state at the end of segment determination (Simon et al. 1992). It has been suggested that the ESC-E(Z) complex initiates repression by other Pc-G complexes (Van Lohuizen 1999). The interaction between *corto* and *E(z)* suggests that Corto might be a member of this so-called initiating complex. A distinct complex termed PRC1 (for Polycomb Repressive Complex 1), containing PC, PSC, PH, SCM and other still unidentified proteins, has recently been described (Shao et al. 1999). This complex is thought to be involved in keeping the Hox genes switched off during development (Van Lohuizen 1999). Since *corto* interacts with *Pc*, *ph* and *Scm*, we suggest that it encodes a member of this PRC1 complex. Although we did not observe any genetic interaction between *corto* and *Psc*, the co-localization of Corto and PSC on polytene chromosomes (Kodjabachian et al. 1998) supports this suggestion. This hypothesis fits with the suggestion that Corto plays a role in the transition between the initiation and maintenance of repression by Pc-G complexes.

corto interacts with trx-G genes

Genetic interactions between *corto* and trx-G genes can be divided into three sets. Firstly, *corto* acts synergistically with *osa* during the development of the thoracic macrochaetes. Second, it acts synergistically with *Trl* in the development of the postpronotum. Third, flies mutated for *corto* and *ash1*, *kto*, *kis*, *mor* or *Vha55* exhibit the posterior crossvein defect previously observed in flies that completely lack *corto*, suggesting that *corto* and these genes act synergistically to regulate the wing vein pattern. *corto* displays extragenic non-complementation with a deficiency that removes *kto* and *ash1*, and the few escapers always display the wing-vein phenotype. This set of interactions might reflect the existence of at least three distinct multimeric complexes specifically involved in different developmental pathways. Corto, ASH1,

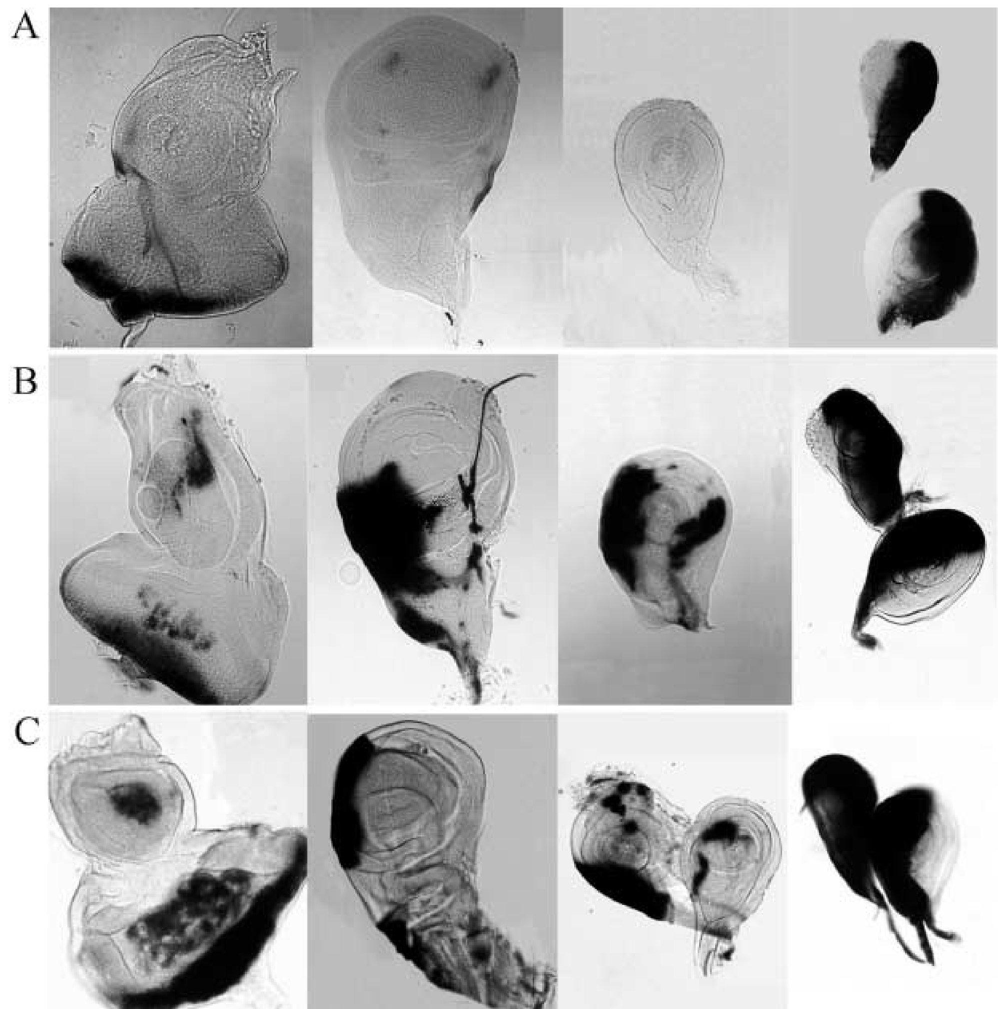

Fig. 4A–C *Ubx* expression in *corto* mutant imaginal discs. Each panel shows (from left to right) an eye-antennal disc, a wing disc, first or second leg discs and leg and haltere discs, respectively. The *35UZ-3* transgenic line was used to study the effects of *corto* mutations on the expression pattern of *Ubx*. **A** In the wild-type background, *lacZ* is expressed like the endogenous *Ubx* gene i.e., in the posterior part of both the third thoracic leg and the haltere discs, but there is no expression in the wing disc or in the first or second leg discs. Moreover, due to a position effect on the transgene, we observed *lacZ* expression in the posterior border of the eye-antennal disc. In flies deficient for *corto* (*35UZ-3/+; corto^{420}/Df(3R) 6-7*) (**B**), as well as in flies that overexpress *corto* (*35UZ-3/+; UAS::corto*/GAL4^{69B}) (**C**), ectopic expression of *Ubx* occurs (either in eye-antennal discs, in wing discs, or in first leg and second leg discs). In third leg and haltere discs of *corto* mutants, the *Ubx* expression domain appears to be broader than in the corresponding wild-type discs

KIS, MOR KTO and VHA55 might belong to a multimeric complex devoted to the regulation of the pathway leading to the wing vein pattern. Moreover, Corto might form both a complex with OSA involved in the development of the thoracic macrochaetes and a complex with TRL involved in the development of the postpronotum. Some results suggest indeed that different Trx-G complexes with different compositions may exist. For instance, OSA is a component of a subset of BRM complexes (Collins et al. 1999). Postpronotum defects may result from a failure to properly express *Antp* (Scott et al. 1983). However, defects in thoracic macrochaetes, as well as inappropriate differentiation of wing-vein tissue, suggest that Corto has other target genes than the Hox genes. It can be reasonably inferred that Corto plays a general role in cell fate control. The thoracic macrochaetes are mechanosensory organs derived from a single precursor cell (SOP) which delaminates from the epidermal layer during the third larval instar and undergoes three asymmetric cell divisions giving rise to the trichogen and the tormogen cell on the one hand, and to the glial cell, the neuron and the thecogen cell on the other hand (for review, see Ghysen and Dambly-Chaudiere 2000). Abnormalities in the sensory organs may result from an alteration of this process. Corto might regulate the activity of one or more neurogenic genes, as proposed for BRM and ASH2 (Adamson and Shearn 1996; Elfring et al. 1998). Defects in the posterior crossvein of the wing as well as in mechanosensory thoracic bristles suggest that Corto might be involved in MAPK signaling cascades. Similar extra veins actually appear in flies bearing a gain-of-function allele of *rolled* (ERK MAPK) and occasional lack of wing veins or ectopic macrochaetes have been observed in flies that overexpress *puckered* (*puc*), a negative feedback mediator of the JNK and ERK MAPK signaling cascades (Martin-Blanco 1998). This hypothesis is consistent with the pleiotropic effect of *corto* mutations.

Dual function of *corto*

Our results provide surprising evidence that *corto* interacts with members of both Pc-G and trx-G classes. The range of interactions and phenotypes observed in double heterozygote combinations leads us to propose that *corto* may perform different functions in conjunction with different subsets of Pc-G or Trx-G proteins.

The same kind of conclusion arises from the analysis of *E(z)*, which can act either as a Pc-G gene or as a trx-G gene, depending on the spatial and temporal stage of development considered (Adamson and Shearn 1996). The ambiguous behavior of E(Z) or Corto may be explained by the close proximity of TRE and PRE. It has been proposed that multimeric complexes localized at both sites might interact (Rozovskaia et al. 1999, 2000). Moreover, the association of a Trx-G factor, the GAGA factor, with Pc-G complexes targeted to PREs reinforces the idea that Pc-G and Trx-G functions overlap (Orlando et al. 1998, Tillib et al. 1999, Horard et al. 2000). Another possibility is that some proteins like Corto, interacting simultaneously with proteins at PRE and at TRE, stabilize multimeric associations. We are currently investigating the protein partners of Corto in order to gain a better understanding of its function.

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