Capicua integrates input from two maternal systems in *Drosophila* terminal patterning

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We wish to dedicate this paper to the dear memory of Judith Lengyel, a true friend and colleague, who supported us throughout this study.

In *Drosophila*, the maternal terminal system specifies cell fates at the embryonic poles via the localised stimulation of the Torso receptor tyrosine kinase (RTK). Signalling by the Torso pathway relieves repression mediated by the Capicua and Groucho repressors, allowing the restricted expression of the zygotic terminal gap genes *tailless* and *huckebein*. Here we report a novel positive input into *tailless* and *huckebein* transcription by maternal posterior group genes, previously implicated in abdomen and pole cell formation. We show that absence of a subset of posterior group genes, or their overactivation, leads to the spatial reduction or expansion of the *tailless* and *huckebein* posterior expression domains, respectively. We demonstrate that the terminal and posterior systems converge, and that exclusion of Capicua from the termini of posterior group mutants is ineffective, accounting for reduced terminal gap gene expression in these embryos. We propose that the terminal and posterior systems function coordinately to alleviate transcriptional silencing by Capicua, and that the posterior system fine-tunes Torso RTK signalling output, ensuring precise spatial domains of *tailless* and *huckebein* expression.

Introduction

In the *Drosophila* embryo, four maternal coordinate systems act to specify distinct cell fates along the anteroposterior (A/P) and dorsoventral (D/V) axes: the anterior, posterior,

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D/V and terminal systems. The head and thorax are patterned by the activity of the anterior *bicoid* (*bcd*) morphogen, while abdomen formation is directed by a reciprocal posterior gradient of Nanos (Nos). Asymmetrical nuclear distribution of Dorsal governs D/V axis formation, whereas the nonsegmented termini are patterned by the terminal system. The activities of these systems induce regional-specific transcription of zygotic downstream target genes, the products of which eventually assign the different body parts with their prospective identities (St Johnston and Nusslein Volhard, 1992).

Zygotic transcription in the termini of the embryo is controlled by the terminal system. A key component of this system is the gene *torso* (*tor*), which encodes a receptor tyrosine kinase (RTK) (Sprenger *et al*, 1989; St Johnston and Nusslein Volhard, 1992; Lu *et al*, 1993; Duffy and Perrimon, 1994). The Torso (Tor) receptor is uniformly distributed throughout the plasma membrane of the early embryo, but is activated only at the poles by a locally processed ligand, where it transmits a signal via the canonical Ras/Raf/MAP-kinase (MAPK) effectors (Casanova and Struhl, 1989, 1993; Sprenger and Nusslein Volhard, 1992; Lu *et al*, 1993; Duffy and Perrimon, 1994; Casali and Casanova, 2001; Furriols and Casanova, 2003). This signal leads to the restricted expression of two zygotic terminal gap genes, *tailless* (*tll*) and *huckebein* (*hkb*), at the poles of the embryo. *tll* and *hkb* encode transcription factors that, in turn, implement head and tail differentiation programmes (Strecker *et al*, 1986, 1988; Casanova, 1990; Pignoni *et al*, 1990; Weigel *et al*, 1990; Brönner and Jackle, 1991; Brönner *et al*, 1994; Furriols and Casanova, 2003).

Genetic and molecular studies suggest that the activation of *tll* and *hkb* expression by the Tor pathway is indirect, and that Tor signalling allows regional expression of the terminal gap genes by counteracting, at the embryonic poles, general transcriptional repressors (Liaw *et al*, 1995; Paroush *et al*, 1997; Jiménez *et al*, 2000). Two major lines of evidence support this idea. First, a detailed analysis of the *tll* promoter has defined negative *cis*-acting regulatory sequences (designated Torso-response elements; TREs), whose deletion leads to ectopic expression throughout the embryo, indicating that *tll* is normally repressed outside the embryonic poles (Liaw *et al*, 1995; Rudolph *et al*, 1997). Second, mutations in genes encoding maternally contributed transcriptional repressors, such as the nuclear HMG-box protein Capicua (Cic) (Jiménez *et al*, 2000) and the global developmental corepressor Groucho (Gro), result in ectopic, more central, *tll* and *hkb* expression, even in the absence of functional Tor signalling (Paroush *et al*, 1994, 1997; Chen and Courey, 2000). How these factors regulate *tll* and *hkb* transcription is not well understood, although it appears that Cic is one of the key elements regulated by the Tor pathway (Jiménez *et al*, 2000).

At the posterior of the embryo, the activity zone of the terminal system overlaps with that of the posterior system. Posterior group members are localised to the posterior pole, where they function in polar granule assembly and in the formation of the abdomen and germ cells (St Johnston and

Nusslein Volhard, 1992). Assembly of the germ plasm occurs in a stepwise manner, with *oskar* (*osk*) being the key element of this process (Rongo and Lehmann, 1996). Embryos lacking maternal *osk* activity fail to form pole cells and to develop abdominal structures. Further, *osk* dosage dictates the amount of pole plasm assembled, and the consequent number of germ cells produced. Following translation of *osk* mRNA, the Osk protein localises the Vasa (Vas) DEAD-box RNA-binding helicase and the Tudor (Tud) protein to the posterior pole. These three proteins are then required for the execution of all posterior system functions. Downstream of Tud, the posterior group bifurcates: the product of the *germ-cell-less* (*gcl*) gene is required only for pole cell formation, whereas those of *nanos* (*nos*) and *pumilio* (*pum*) regulate abdomen formation by blocking translation of the anterior determinant *hunchback* (*hb*) (Rongo and Lehmann, 1996).

In general, the distinct maternal genetic systems act independently of each other to define discrete portions of the embryonic pattern. Mutations in one maternal coordinate system seem to eliminate a specific body part without grossly affecting the rest of the embryonic pattern. Previous studies, however, have revealed interactions between the anterior, terminal and D/V systems at the most anterior region of the embryo (Pignoni *et al*, 1992; Liaw and Lengyel, 1993). At the posterior pole, on the other hand, the terminal system has long been thought to act alone in regulating zygotic terminal gap gene transcription, given that expression of *tll* and *hkb* is completely absent in embryos devoid of maternal *tor* activity, or lacking any other constituent of this signalling cascade (Weigel *et al*, 1990; Brönner and Jackle, 1991).

In this work, we identify a surprising, novel role for maternal posterior group genes in embryonic terminal patterning. We show that *tll* and *hkb* expression patterns are spatially reduced in several posterior group mutants, suggesting that posterior group members positively contribute to the transcription of zygotic terminal gap genes. Our data indicate that the positive input from posterior group genes, in particular Nos, into *tll* and *hkb* expression converges on the Tor pathway. Thus, minimal TRE sequences, through which terminal system effectors regulate *tll* expression, also respond to the lack, or to the overactivation, of the posterior system. We further demonstrate that posterior genes act upstream or at the level of Cic, as ectopic accumulation of the Cic repressor is seen in the termini of posterior group mutants, accounting for the reduced *tll* and *hkb* expression domains in these embryos. Hence, multiple maternal inputs are required to effectively antagonise ubiquitous negative regulators at the posterior pole, in order to generate the precise patterns of *tll* and *hkb* expression. We propose that the posterior system acts to refine Tor RTK signalling output, ensuring that terminal gap gene expression patterns are properly established and consequently cell fates are correctly specified.

Results

Terminal gap gene expression is reduced in oskar, vasa and tudor mutant embryos

By the beginning of cellularisation, *tll* and *hkb* are expressed in a nested-set pattern at the pole regions of wild-type *Drosophila* embryos (Weigel *et al*, 1990; Brönner *et al*, 1994). At this developmental stage, the dynamic *tll* RNA expression pattern has resolved into smaller domains at

both the anterior and posterior ends. *tll* is expressed at the anterior cap in a horseshoe-shaped stripe that extends about two-thirds down along the D/V axis, whereas, posteriorly, *tll* covers a region comprising approximately 0–16% egg length (EL; 0% being the coordinate of the posterior tip) (Figure 1A) (Pignoni *et al*, 1990). *hkb*, on the other hand, is transcribed in the presumptive head region as a cap and, at the posterior, its expression domain overlaps with that of *tll*, covering about 0–12% EL (Figure 1E) (Brönner and Jackle, 1991).

We find that the posterior expression domains of both terminal gap genes are reduced in embryos laid by *osk* mutant females (Figure 1). Thus, *tll* and *hkb* expression is spatially reduced in stage 4 embryos derived from osk^{166} and osk^{346} homozygous females, and from null $osk^{54}/Df(3R)p$-$XT103$ mutant females (Figure 1B, C, F and G; Table I). Retracted terminal gap gene expression domains are also seen in older, stage 5 embryos (not shown). In these and subsequent experiments, we minimised the effects caused by differential probe concentrations and/or duration of staining reactions by first mixing and then simultaneously fixing and processing wild-type embryos together with mutants, the latter distinguishable by their lack of pole cells. In all cases, the spatial reduction is reproducible and statistically significant (Table I). Thus, we conclude that the posterior group gene *osk* is required for the full extent of terminal gap gene expression at the posterior pole.

We also examined if mutations in other posterior group genes elicit similar alterations in terminal gap gene patterns. The *vasa* (*vas*) gene product is required for the activation of Osk translation (Rongo *et al*, 1995). We find that vas^{PD}/vas^{011} mutant embryos show a reduction in *tll* and *hkb* expression, comparable to that seen in *osk* mutants (data not shown; Table I), implying that, like *osk*, *vas* also normally contributes to terminal gap gene regulation.

Next we examined the effect of Tud, a posterior group member that mediates the transport of RNA from the mitochondria to polar granules (Amikura *et al*, 2001). In embryos derived from tud^{WC}/tud^{B36} mothers, the posterior *tll* expression domain is reduced, although *hkb* expression is not significantly altered, perhaps due to the use of hypomorphic alleles (Figure 1D and H; Table I).

In all of the above mutant backgrounds, a lower staining intensity relative to that seen in wild-type embryos (Figure 1) suggests a decline in transcript abundance. This weakened staining is specific to *tll* and *hkb* transcripts, as other genes are expressed at normal levels in the posterior and elsewhere of *osk* mutant embryos (D Gur-Wahnon, unpublished results). Notably, the anterior *tll* expression domain is also reduced in all of the above posterior group mutants (see Discussion). Thus, the posterior group genes *osk*, *vasa* and *tud* are positively required for the expression of the terminal gap genes *tll* and *hkb*.

Nanos positively regulates tailless and huckebein expression

We next established the expression patterns of *tll* and *hkb* in embryos mutant for posterior genes acting downstream of *osk*, *vas* and *tud* (Figure 5I) (Rongo and Lehmann, 1996). In embryos produced by *gcl* homozygous mothers, the *tll* and *hkb* expression domains are both normal (data not shown; Table I), suggesting that *gcl* is not required for terminal gap gene regulation. Given that pole cells do not form in *gcl*
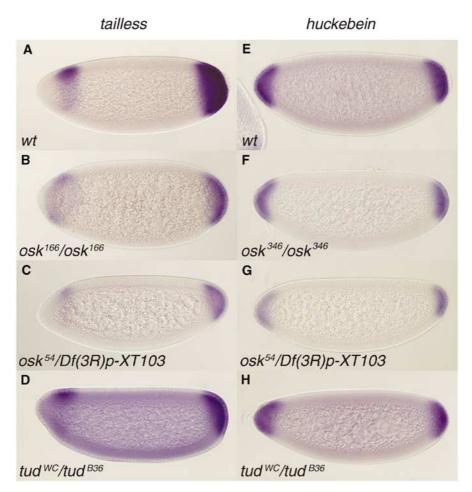
Figure 1 Terminal gap gene expression domains are reduced in *oskar* and *tudor* mutant embryos. Spatial distribution of *tll* (**A–D**) and *hkb* (**E–H**) transcripts in wild-type (A, E) and in posterior group mutant embryos (B–D, F–H), detected by *in situ* hybridisation as described in Materials and methods. Embryos shown are at the syncytial blastoderm stage (stage 4), and are derived from the following females: (A, E) wild type; (B) osk^{166}/osk^{166}; (F) osk^{346}/osk^{346}; (C, G) $osk^{54}/Df(3R)p$-$XT103$; (D, H) tud^{WC}/tud^{B36}. Note the reduced posterior *tll* and *hkb* expression domains in *osk* and *tud* mutants, compared to wild-type embryos. The anterior expression domains of *tll* and *hkb* are also affected by the absence of *osk* (B, C, F, G), *tud* (D, H) and *vasa* (not shown) (see Discussion). For quantification of the spatial extent of the posterior terminal gap gene expression domains in these and other mutants, see Table I. Control and mutant embryos were mixed, then fixed and stained simultaneously. In all figures, the views are lateral, anterior is to the left and dorsal side up.

Table I Extent of posterior *tailless* and *huckebein* expression domains in posterior group mutants

Maternal genotype	*tailless*		*huckebein*	
	Stage 4	Stage 5	Stage 4	Stage 5
osk^{166}/osk^{166}	0.82***	ND	ND	ND
$osk^{54}/Df(3R)p$-$XT103$	0.74***	0.61***	0.78**	0.85***
vas^{PD}/vas^{011}	0.88**	ND	ND	ND
tud^{WC}/tud^{B36}	0.61***	0.66***	1.05	1.07
nos^{L7}/nos^{L7}	0.89	0.96	ND	ND
nos^{BN}/nos^{BN}	0.72***	0.83*	0.93	0.84*
nos^{RC}/nos^{BN}	0.58***	0.71***	0.52***	0.80***
pum^{680}/pum^{680}	1.26***	1.04	ND	ND
$pum^{ET1}/In(3R)Msc$	0.99	0.98	1.24***	1.02
$gcl^{\Delta49}/gcl^{\Delta49}$	0.94	1.04	1.19*	1.18***
osk^{Ak}/osk^{Ak} (4xosk)	1.75***	1.07	1.34**	1.45***
bicS/bicS	1.39***	1.63***	1.39***	ND

Values below or above 1 represent a reduction or expansion of gap gene expression domains, respectively, while ratios of around 1 denote normal extent (see Materials and methods). ND: not determined. Statistically significant results are marked by asterisks: ***$P<0.01$, **$P<0.05$ and *$P<0.1$.

mutants (Jongens *et al*, 1992), we conclude that the lack of germ cells in *osk*, *vas* and *tud* embryos is not the basis for the reduced *tll* and *hkb* expression in these genotypes.

Embryos derived from *nos* and *pum* mutant mothers possess pole cells, but exhibit abdominal defects resulting from the failure of these RNA-binding proteins to repress the

translation of maternal *hb*. We find that Nos participates in *tll* and *hkb* regulation. In stage 4 embryos derived from nos^{RC}/nos^{BN} or homozygous nos^{BN} mutant females, the *tll* and *hkb* expression domains are significantly reduced (Figure 2B and E; data not shown; Table I), implying that the Nos protein is a positive regulator of terminal gap gene expression. Double *in situ* hybridisation staining shows that the posterior expression of *tll*, but not that of the central *Krüppel* (*Kr*) stripe, is reduced in nos^{RC}/nos^{BN} embryos compared to wild type (Figure 2M and N), confirming the specificity of this effect. In contrast, we find that pum^{ET1}/*In(3R)Msc* females lay embryos with normal *tll* and *hkb* expression (Figure 2C and F; Table I), indicating that *pum* may not be involved in this regulation.

The differential effects of Nos and Pum on *tll* and *hkb* expression are surprising, given that these two proteins act as partners in abdominal patterning and in other settings. However, two lines of evidence support this conclusion. First, the mutant *nos* and *pum* allelic combinations tested show a similar loss of posterior denticle belts (Figure 2K and L) (Irish *et al*, 1989; Murata and Wharton, 1995) and the absence of abdominal gap gene expression (e.g., the posterior *giant* stripe; Figure 2H and I), as reported for posterior group mutants (Kraut and Levine, 1991). Second, we find that *tll* and *hkb* expression is unaffected in homozygous nos^{L7} mutant embryos (Table I). This particular allele encodes a Nos protein lacking its carboxy-terminal tail, the domain required for the recruitment of Nos into the Pum/*hb* mRNA ternary complex (Sonoda and Wharton, 1999). Thus, nos^{L7} renders Nos inactive as a repressor of *hb* mRNA translation (a Pum-dependent activity), yet it does not impair Nos' other functions, for example in oogenesis (Arrizabalaga and Lehmann, 1999). Nos could therefore be acting in terminal patterning in conjunction with some other maternally provided factor, perhaps an RNA-binding protein. Given that the pum^{ET1}/*In(3R)Msc trans*-allelic combination might still possess some Pum activity, however, it is premature to completely rule out Pum's contribution to terminal gap gene regulation.

Taken together, our results suggest that Nos is a key posterior group member required for accurate terminal gap gene expression.

Overexpression of oskar or nanos leads to expanded terminal gap gene expression

If posterior group genes have a positive input into terminal gap gene regulation, then the overexpression of these genes should cause broadening of the *tll* and *hkb* posterior expression domains. To test this idea, terminal gap gene expression patterns were assessed in offsprings of females with increased *osk* dosage, or that uniformly express *nos*. Embryos overexpressing *osk* were derived from two maternal genetic backgrounds, each containing two extra copies of *osk*: homozygous females, which harbour a small tandem duplication of the *osk* locus (referred to herein as *bicaudalS*; *bicS*), and homozygous females carrying an *osk* transgene (4x*osk* females) (Ephrussi and Lehmann, 1992). To express *nos* uniformly, we used a *nos* transgene, in which the *nos* 3'UTR was replaced with that of *tubulin* (Gavis and Lehmann, 1994). In all cases, embryos show cuticular phenotypes attributable to *osk* or *nos* overexpression: either a pronounced reduction of head structures, or a bicaudal appearance, with posterior

terminal telson structures replacing the anterior acron (data not shown). Significantly, all combinations result in expanded posterior domains of *tll* and *hkb* (Figure 3; Table I), supporting the notion that *osk*, *nos* and other posterior group genes fulfil a positive role in terminal gap gene regulation.

Remarkably, overexpression of *osk* can rescue some of the morphological defects seen in terminal group mutants. Specifically, the terminal filzkorper (FK) structure is never observed in hypomorphic *torso-like* (tsl^{691}) mutant embryos, but is partially rescued in embryos harbouring this allele that also concomitantly overexpress *osk* (*bicS tsl*; Figure 3I and J). Thus, overactivation of the posterior system appears to compensate in part for compromised terminal system activity.

We note that the anterior *tll* stripe retracts anteriorly when *osk* or *nos* are overexpressed (Figure 3B–D), similar to what is seen in *bcd* mutants (Pignoni *et al*, 1992) (in both cases, anterior *hkb* expression does not change). This aspect of *tll* misexpression likely stems from translational inhibition of *bcd* mRNA by Nos, which is mislocalised to the anterior in these embryos (Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1994).
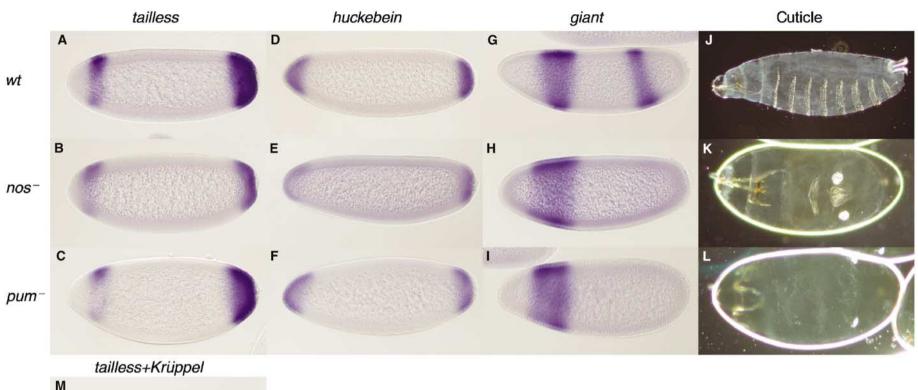
Posterior group genes regulate tailless at the transcriptional level

None of the posterior group members encode for transcription factors, so we next asked whether the products of these genes regulate *tll* and *hkb* expression at the transcriptional level, or whether they do so post-transcriptionally, for example by stabilising terminal gap gene mRNA. To address this point, we made use of a reporter construct (*P1*), the expression of which is controlled by extensive *tll* promoter sequences and resembles that of the endogenous gene (Figure 4A) (Liaw and Lengyel, 1993). We find that *P1 lacZ* expression is reduced in *osk* mutants (Figure 4B), while it expands into central parts in embryos laid by females overexpressing *osk* (Figure 4C), in accordance with endogenous *tll* expression (Figures 1B and 3C). Moreover, the anterior *P1 lacZ* stripe is also affected in 4x*osk* embryos, similar to endogenous *tll*. Thus, the positive regulatory input from posterior group genes must be acting, presumably indirectly, on *tll* transcription rather than at a post-transcriptional level.

Torso-response elements in the tailless promoter respond to alterations in maternal oskar dosage

We next investigated if posterior group genes act in parallel to, and independently of, the *tor* signalling cascade, or whether they feed into the terminal pathway. Molecular dissection of the *tll* promoter has defined two *cis*-acting TRE sequences that respond to both the lack and the constitutive activation of the *tor* pathway (Liaw *et al*, 1995). These DNA regulatory elements mediate transcriptional repression, specifically by the Cic repressor complex, as mutations in either the TREs or in *cic* lead to derepression (data not shown) (Liaw *et al*, 1995; Jiménez *et al*, 2000). We reasoned that if the posterior system regulates terminal gap gene expression independently of the Tor pathway, then its effects are likely to be exerted via distinct *cis*-acting elements in the *tll* promoter. If, on the other hand, this maternal system acts jointly with the terminal pathway, then input by the posterior system is expected to converge on the previously defined TREs.

Figure 2 Terminal gap gene expression domains are reduced in *nanos* but not in *pumilio* mutants. (**A–I, M, N**) *In situ* hybridisation. (**J–L**) Larval cuticular preparations. (A, D, G, J, M) Wild type; (B, E, H, K, N) *nos^RC^/nos^BN^*; (C, F, I, L) *pum^ET1^/In(3R)Msc*. Expression domains of *tll* (A–C), *hkb* (D–F), *gt* (G–I) and *tll + Kr* (M, N) in stage 4 embryos. Note that the posterior *tll* and *hkb* expression domains are reduced in *nos*, but not in *pum*, mutant embryos. In both *nos* and *pum* mutants, abdominal segmentation is defective, as evident by the absence of the posterior *giant* stripe (H, I; cf. with G) and by the lack of abdominal denticle belts (K, L; cf. with J). The *Kr* stripe expands posteriorly in *nos* (cf. N with M) and *osk* mutant embryos (not shown) (Hulskamp *et al*, 1990).
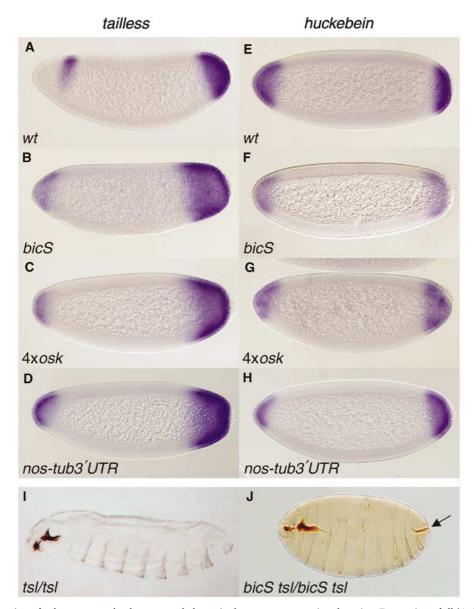
tailless   huckebein

Figure 3 Overexpression of *oskar* or *nanos* leads to expanded terminal gap gene expression domains. Expression of *tll* (**A–D**) and *hkb* (**E–H**) in stage 4 embryos. Shown are embryos produced by the following females: (A, E) wild type; (B, F) homozygous *Dp(3;3)bicS (bicS)*; (C, G) homozygous 4x*osk*; (D, H) heterozygous *nos-tub3′UTR*. Note the significant expansion of the *tll* and *hkb* posterior expression domains in lines overexpressing *osk* or *nos*. In these lines, the anterior *tll* stripe is also shifted. (**I, J**) Cuticular phenotypes of embryos produced by homozygous *tsl* (I) or *bicS tsl* double-mutant females (J). The arrow in (J) points to a partially restored filzkorper in a *bicS tsl* embryo, missing in a *tsl* mutant (I).

Transgenes containing the minimal distal (*K11*) and proximal (*G22*) TRE sequences drive *lacZ* expression in a *tll*-like posterior cap, although, as previously published, the intensity of expression is reduced when compared to that of the full-length *tll* promoter construct (*P1*), probably as a result of lost activator binding sites (Figure 4D and G; cf. with Figure 4A) (Liaw *et al*, 1995; Rudolph *et al*, 1997). We find that *lacZ* expression, driven by the distal TRE (*K11*), is reduced in *osk* mutants (Figure 4E) and expanded in 4x*osk* embryos (Figure 4F), similar to the *P1* reporter. When driven by the proximal TRE (*G22*), *lacZ* expression also expands in response to *osk* overexpression, although it is not significantly reduced in an *osk* mutant background (Figure 4H and I; cf. with Figure 4G). Given that besides the minimal TREs there are no apparent sequences common to both constructs,

it appears that posterior group genes act on the same elements in the *tll* promoter that mediate terminal system output.

Input from posterior group genes intersects with the torso pathway upstream of Capicua

To test at what level the crosstalk between the posterior and terminal systems occurs, we first monitored the phosphorylation state of MAPK when *osk* dosage is altered. The doubly phosphorylated form of MAPK (dpERK), which can be reliably detected using a specific antibody (Gabay *et al*, 1997a), makes a perfect read-out for signals transmitted by RTK pathways via the generic Ras/Raf/MAPK module, including that by the Tor receptor. Thus, MAPK activation is diminished in embryos derived from homozygous *tsl* mutant females,
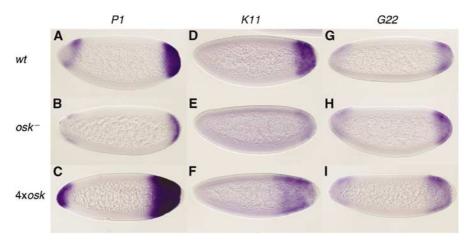
Figure 4 Input by the posterior group converges on minimal TREs in the *tailless* promoter. (**A–I**) *In situ* hybridisation for *lacZ* reporter expression, driven by different *tll* promoter constructs, in stage 4 embryos: (A–C) *P1*, a 5.9 kb full-length *tll* promoter construct; (D–F) *K11*, *tll* promoter subregion −2291 to −2770, oligomerised four-fold; (G–I) *G22*, *tll* promoter subregion −324 to −200, oligomerised four-fold. (A, D, G) In a wild-type background, all constructs drive reporter expression in a posterior cap. The *P1* and *G22* constructs also drive expression in the anterior. Expansion of the *lacZ* expression pattern is apparent for all three constructs when *osk* is overexpressed (4x*osk*; C, F, I), while *lacZ* expression is significantly reduced for the *P1* and *K11* constructs, although not for *G22*, in *osk*[166] homozygous flies (B, E, H).

and is uniformly detected when *tor* is constitutively activated (Figure 5D) (Gabay *et al*, 1997b). In contrast, we find that the pattern of anti-dpERK staining is unaltered in *osk* mutant (Figure 5B) or in *4xosk* (Figure 5C) embryos, suggesting that posterior group genes do not converge with the Tor signal transduction cascade upstream of MAPK.

In terminal patterning, the Tor pathway is required to inhibit the Cic repressor, bringing about its post-transcriptional exclusion from the poles. Cic is normally detected throughout the embryo, except at the termini, where the Tor signal is active. Correspondingly, in *tor* mutants, Cic is found not only in medial regions of the embryo but also at the poles (Jiménez *et al*, 2000). Using anti-Cic antibodies, we followed the accumulation of Cic at the posterior pole of mutant and normal embryos, finding that it is less effectively removed from the terminal regions of stage 5 *osk*, *nos* and *tud* mutants compared to wild-type embryos (data not shown). Equivalent results were obtained using an HA-tagged *cic* transgene that rescues *cic* loss-of-function (see Materials and methods). In normal embryos, tagged Cic is excluded from both the anterior and posterior termini (Figure 5E and G). When introduced into a *nos*[RC]/*nos*[BN] mutant background, however, ectopic staining is found in the terminal nuclei of stage 4 embryos (Figure 5F and H), although not in those nuclei just adjacent to the pole cells. Note that exclusion of both endogenous and tagged Cic from the anterior pole also appears attenuated in *osk* and *nos* mutants (Figure 5F; see Discussion).

These results lead us to conclude that posterior group members act at the level of MAPK or downstream to it, to downregulate the Cic repressor complex (Figure 5I). Importantly, the ectopic accumulation of Cic could account for the reduced *tll* and *hkb* expression patterns observed in posterior group mutants.

Repression of tailless is alleviated in CtBP, groucho double-mutant germline clones
We next addressed yet another aspect of *tll* regulation. In *gro* or *cic* maternal mutants, repression of *tll* is hindered and its

expression expands towards the middle of the embryo (Paroush *et al*, 1997; Jiménez *et al*, 2000). Notably, *tll* transcripts are never detected in the centre of these embryos (Figure 6B). What could be the reason for the lack of *tll* expression at the centre of the embryo, even when these essential repressors are removed? One possibility is that activators of *tll* are simply absent from this region. However, several other DNA-binding repressors have been implicated in *tll* silencing (Liaw *et al*, 1995; Chen *et al*, 2002), raising the possibility that repressor activities are redundant. Consistent with the latter possibility, we find that the simultaneous removal of both Gro and the C-terminal Binding Protein (CtBP), a second global corepressor that functions at early stages of embryogenesis (Nibu *et al*, 1998; Poortinga *et al*, 1998), brings about the uniform (albeit weak) expression of *tll* throughout the embryo (Figure 6C). This implies that the *tll* promoter is subjected to multiple repressor mechanisms, at least some of which are *CtBP*-dependent (and perhaps novel), that inhibit *tll* expression from spreading to the middle regions of the embryo, thus allowing correct abdominal development.

Discussion

Terminal gap gene expression must be tightly regulated for the correct specification of terminal cell fates at the nonsegmented poles. Clearly, the Tor pathway plays a key role in driving *tll* and *hkb* transcription, given that terminal gap genes are not expressed at the posterior end of terminal group mutants, and as a result terminal structures such as the FK do not form. In this paper, we reveal a novel biological role for the maternal posterior system, showing that members of this group, in particular Nos, positively regulate transcription of the zygotic subordinate genes of the terminal system. We find that TREs in the *tll* upstream regulatory region, which are derepressed in *cic* mutants, also respond to alterations in maternal *osk* dosage, and that the Cic repressor is not excluded from the termini of posterior group mutants. Our results are consistent with the posterior system feeding into
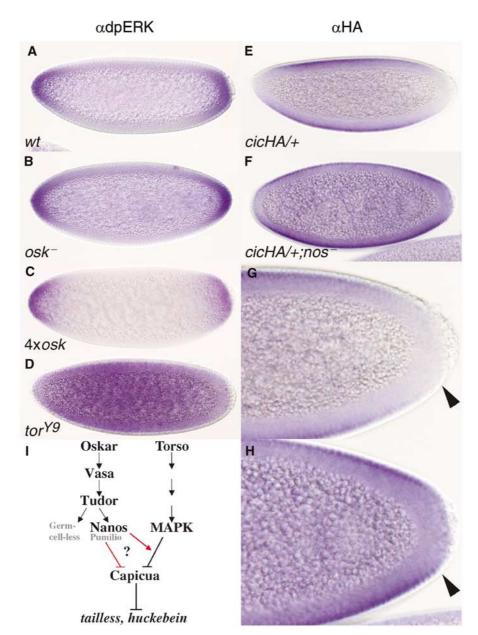
Figure 5 Posterior group genes feed into the Torso pathway upstream of Capicua. (**A–D**) Staining for dpERK (activated MAPK) at stage 4. (**E–H**) Distribution of the HA-tagged Cic protein (CicHA) at stage 4, revealed by anti-HA antibody staining. (A) Wild-type embryos; (B) embryos produced by $osk^{54}/Df(3R)p$-$XT103$ mutant mothers; (C) embryos produced by 4xosk mothers; (D) embryos produced by tor^{Y9} mothers; (E) embryos derived from females heterozygous for the cicHA transgene ($cicHA/+$); (F) embryos produced by $cicHA/+$; nos^{RC}/nos^{BN} mothers; (G, H) larger magnifications of the posterior poles of the embryos shown in (E, F), respectively. The arrowheads in (G, H) indicate the same relative point in the two embryos. dpERK levels in osk and 4xosk mutants are identical to those seen in wild-type embryos; however note the ectopic accumulation of CicHA in the terminal regions of nos mutants compared to its exclusion from these regions in wild-type embryos. Note that while HA staining is observed to the left of the arrowhead in nos mutants (H), it is missing at the corresponding point in the wild type (G). (**I**) Schematic representation of the intersection between the posterior system and the Torso pathway. Nos acts with Pum or some unknown partner, at the level of MAPK or downstream to it, to downregulate the Cic repressor complex at the termini, facilitating tll and hkb transcription (see text for details). Posterior group members that are not involved in terminal gene regulation are presented in light grey.

the Tor signalling pathway, upstream of or at the level of the Cic repressor (Figure 5I). We suggest that the concerted activities of both the terminal and posterior systems, in their spatially overlapping zones of action, generate accurate domains of terminal gap gene expression at the posterior.

Crosstalk between maternal coordinate systems
It was originally proposed that the four maternal systems that pattern the early *Drosophila* embryo act largely indepen-

dently of each other (St Johnston and Nusslein Volhard, 1992). Recent work, however, demonstrated interactions between the Tor pathway and the anterior and D/V systems. For example, *tll* has been shown to respond to the anterior determinant Bicoid (Bcd) even when Tor signalling is genetically blocked. Indeed, *cis*-acting DNA elements responsive to these three maternal systems have been found in the *tll* upstream regulatory region (Liaw and Lengyel, 1993). Our results now link the terminal and posterior systems, pre-
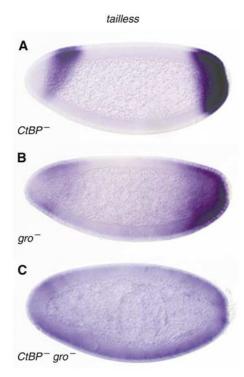
tailless

Figure 6 *tailless* is expressed throughout *CtBP, groucho* double-mutant germline clones. In either *CtBP^{P1590}* (**A**) or *gro^{BX22}* (**B**) single-mutant germline clones, the posterior *tll* expression domain expands anteriorly (only slightly so in embryos devoid of maternal *CtBP*), but never reaches the centre. (**C**) Only when both *CtBP* and *gro* are concomitantly removed (*CtBP^{P1590} gro^{BX22}*) is *tll* uniformly expressed throughout the embryo (although not in pole cells).

viously thought to be independent of each other, in terminal gap gene regulation, reinforcing the idea that maternal systems that pattern the early embryo act in a coordinated manner.

Why has the positive input, by posterior group genes into terminal patterning, been largely overlooked to date? Classical segmentation studies mostly involved phenotypic analyses at the cuticular level. For this reason, and when taking into account the primary contribution of the terminal system, the delicate input by the posterior group has gone unnoticed. Thus, the unextended FK that develops in posterior group mutant background, which may arise from decreased terminal gap gene expression, had largely been attributed to pleiotropic effects arising from abdominal defects. We have been able to detect the relatively subtle changes in *tll* and *hkb* gene expression patterns only by investigating terminal gap gene regulation at the molecular level. In fact, at least one other molecular study had previously reported reduced terminal gap gene expression in *osk* mutant embryos (Brönner and Jackle, 1991).

Posterior group input impinges on RTK signalling
One emerging concept is that, for the refinement of the expression levels and spatial extents of RTK signalling targets, it is also imperative to integrate accurately information originating from other, non-RTK sources (Simon, 2000). In many cases this integration occurs at the level of target gene enhancers, with various effectors of distinct signalling pathways binding to specific DNA elements to regulate transcription (Flores *et al*, 2000; Halfon *et al*, 2000; Xu *et al*, 2000). For

example, *D-Pax2* expression in the cone and pigment cells of the developing eye is regulated by effectors of the EGFR RTK pathway, such as Pointed P2 and Yan, and also by the Notch signalling component Suppressor of Hairless, as well as by the transcription factor Lozenge (Flores *et al*, 2000). Here we have shown that terminal gap gene expression requires not only Tor RTK pathway activity but also a contribution from the posterior system. In this instance, inputs from these two maternal coordinate systems are interpreted and linked not at the level of terminal gap gene promoters but at the level of the Cic repressor. Thus, Cic functions as an integrator of multiple regulatory inputs, with both the posterior and terminal systems acting to relieve transcriptional silencing mediated by this repressor.

Regulation of terminal gap gene expression by Nanos
Surprisingly, we find that anterior *tll* and *hkb* expression is also reduced in posterior group mutants (Figures 1 and 2). Similarly, others have reported prolonged *bcd* expression and head defects in *pum* mutants (Gamberi *et al*, 2002). We can only speculate that low levels of Osk and Nos, which escape translational repression, similarly regulate terminal gap gene expression via Cic removal at the anterior. In accordance with this, the dismissal of Cic from the anterior pole of posterior group mutants is also ineffective (Figure 5F).

How does Nos, which has been assigned the role of a translational repressor, positively regulate *tll* and *hkb* transcription? Our results suggest that Nos does so indirectly, by downregulating the accumulation of the Cic repressor at the termini. The exact mechanism by which the Tor pathway mediates the exclusion of Cic from terminal regions has not been established, but one model argues that phosphorylation of Cic by MAPK causes degradation of the protein, as in the case of Yan (Rebay and Rubin, 1995; Jiménez *et al*, 2000). Thus, Nos could be affecting this process in one of several possible ways, at the level or downstream of MAPK. For example, Nos could be facilitating the translocation of phosphorylated MAPK into the nucleus. In posterior group mutants, then, activated MAPK would remain in the cytoplasm rather than enter the nucleus, impeding Cic phosphorylation and degradation. Alternatively, Nos may be modulating MAPK activity, or regulating adaptor proteins that promote Cic phosphorylation by nuclear MAPK. Nos may also be controlling the translation of factors that are involved in the nuclear trafficking (import/export) or degradation of Cic, or perhaps may even be acting on the *cic* message itself. Future studies will distinguish between these possibilities, and may shed new light on the molecular mechanisms underlying Nos' role in other developmental processes, for example, the establishment/maintenance of transcriptional quiescence in pole cells (Deshpande *et al*, 1999).

Multiple layers of terminal gap gene regulation
We view the positive input by the posterior group genes as evolving to modulate terminal pathway activity, merging with other varied modes of Tor regulation to ultimately ensure accurate *tll* and *hkb* expression and, consequently, precise cell fate determination.

The Tor signal transduction pathway is under multiple tiers of regulation, outside and inside the nucleus. For instance, internalisation and trafficking of the activated Tor receptor to the lysosome for degradation attenuates the signal, as evident

by the spatial broadening and temporal prolonging of Tor activation in mutants for *hrs*, a component of the endosomal recycling machinery (Lloyd *et al*, 2002). Yet another level of control is provided by the tyrosine phosphatase *corckscrew*, which sharpens the gradient of Tor activity (Cleghon *et al*, 1998). Additionally, multiple cytoplasmic adaptor proteins take part in transducing the Tor signal (Luschnig *et al*, 2000), conceivably buffering against surplus or deficiency in signalling.

In the nucleus, *tll* and *hkb* are subjected to silencing by several repressors. Derepression of *tll* is observed in *grainyhead* and *tramtrack69* (*ttk69*) mutants, and the proteins encoded by these genes bind *tll* promoter sequences (Liaw *et al*, 1995; Chen *et al*, 2002). Cic and Gro appear to play a leading role in terminal gap gene silencing, given that mutations in *cic* and *gro* bring about a significant expansion of the *tll* and *hkb* expression domains (Figure 6B) (Paroush *et al*, 1997; Jiménez *et al*, 2000). Intriguingly, however, *tll* expression never reaches the middle of the embryo in these mutants. We find that *tll* is uniformly expressed, albeit weakly, throughout the embryo only when both the developmental corepressors Gro and CtBP are removed concomitantly (Figure 6C). This broadened *tll* expression likely stems from the fact that there is a redundancy in the activities that normally restrict terminal gap gene transcription from inappropriately spreading into the central portion of the embryo; by jointly removing the Gro and CtBP coregulators, activity of the above repressors is compromised. Alternatively, CtBP might be acting in conjunction with a novel, unidentified repressor that prevents *tll* transcription in the middlemost region of the embryo.

So what is the purpose of the input by the posterior group genes into *tll* and *hkb* transcription? Quantitative differences in Tor receptor activity have to be eventually interpreted and translated into distinct cell fates at the termini. Strong Tor activation induces both *hkb* and *tll* expression, whereas weaker Tor activation only brings about *tll* expression. We surmise that the precision endowed by the Tor RTK cascade may not suffice for the complex patterning of the termini, given that mere two-fold fluctuations in Tor signalling result in defective embryonic development (Strecker *et al*, 1989; Furriols *et al*, 1996; Greenwood and Struhl, 1997). For example, mutants with reduced Tor RTK activity show partial *tll* expression and the complete loss of *hkb*. These mutants consequently develop incomplete terminal structures and die at the larval stage. Conversely, overactivation of the Tor pathway leads to anterior expansion of the posterior *tll* expression domain, perturbing segmentation in central body parts, likely as a result of downregulation of abdominal gap genes by the Tll protein (Steingrímsson *et al*, 1991; Paroush *et al*, 1997). Thus, the precise spatial confinement of terminal gap gene expression domains requires the coordinated integration of regulatory inputs, coming from two maternal systems and converging on the same effector protein, Cic.

Materials and methods

Fly culture
Flies were cultured and crossed on standard yeast–cornmeal–molasses–malt extract–agar medium at 25°C.

Fly stocks and germline clones
The following mutant alleles were used: osk^{166}, osk^{54}, osk^{346}, vas^{PD}, vas^{011}, tud^{WC}, tud^{B36}, gcl^{A49}, nos^{RC}, nos^{BN}, nos^{L7}, *nos-tub3′UTR*, pum^{ET1}, pum^{680}, tor^{Y9} and tsl^{691} (FlyBase). *Df(3R)p-XT103* and *In(3R)Msc* are deficiencies that uncover the *osk* and *pum* loci, respectively (FlyBase). *OregonR* and *yw* stocks served as wild-type controls.

Flies carrying the *P1*, *G22* and *K11 tor-RE-lacZ* transgenes (Liaw *et al*, 1995; Rudolph *et al*, 1997) were a kind gift from Judith Lengyel. For increasing maternal *osk* dosage, two lines were used: osk^{AK} (4x*osk*), kindly provided by Anne Ephrussi (Ephrussi and Lehmann, 1992), and *Dp(3;3)bicS*, a small tandem duplication of the *osk* locus, which will be described in detail elsewhere.

Embryos expressing HA-tagged Cic were obtained from females carrying a modified version of the rescuing construct containing the complete *cic* coding sequence (Jiménez *et al*, 2000). The tagged transgene is identical to the parental construct, except that it includes three tandem copies of the HA (also known as Flu) epitope inserted at the C-terminal region of the protein. Details on the construction of the plasmid are available on request.

Embryos lacking maternal *gro* and/or *CtBP* activities were derived from mosaic gro^{mat-} (gro^{E48} or gro^{BX22}) and $CtBP^{mat-}$ ($CtBP^{P1590}$) single- and double-mutant germ lines, obtained using the FLP-DFS technique (Chou *et al*, 1993). Standard recombination techniques were used to generate the double-mutant chromosomes FRT[82B] $CtBP^{P1590}$ gro^{BX22}, FRT[82B] $CtBP^{P1590}$ gro^{E48} and $bicS^{3014}tsl^{691}$.

In situ hybridisation and antibody staining of Drosophila embryos
Wild-type or mutant embryos (1–3.5 h collections) were dechorionated in bleach and fixed in 4% formaldehyde/PBS/heptane for 15–20 min. Expression patterns were visualised by whole-mount *in situ* hybridisation using digoxygenin-UTP-labelled antisense RNA probes and anti-digoxygenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim).

Immunohistochemical detection of activated MAPK, in freshly fixed embryos (10% formaldehyde/PBS/heptane buffer), was achieved with preabsorbed monoclonal antibodies against the diphosphorylated form of Erk (dpERK) (1:100; Sigma). Secondary antibodies were conjugated to biotin (1:2000; Chemicon), and visualised by addition of streptavidin alkaline phosphatase (1:500; Chemicon). For viewing the endogenous Cic protein, a preabsorbed polyclonal antibody (1:1000) was used as previously described (Jiménez *et al*, 2000). In this case, a preabsorbed alkaline phosphatase-coupled secondary antibody was utilised (1:1500; Jackson). HA-tagged Cic was followed by using an anti-HA monoclonal antibody (1:200; Convance). Incubations of primary and secondary antibodies were performed in 0.2% NaN_3. Blocking was performed in a 0.1% PBS, 0.1% Tween, 10% BSA, 5% normal goat serum and 0.2% NaN_3 buffer.

In Table I, the spatial extent of the posterior terminal gap gene expression domains, in embryos at stages 4 (syncytial blastoderm) and 5 (cellular blastoderm), was calculated as follows: for a given embryo, *tll* and *hkb* expression domains were measured, then divided by the embryo's length to calculate the domain's size as percent of EL. Values were averaged for the respective mutant and corresponding wild-type embryos, and are represented as a mutant to wild-type ratio. Thus for each genetic background, values below or above 1 represent a reduction or expansion of gap gene expression domains, respectively, while ratios of around 1 denote normal extent. Please note that differences between the strengths of alleles used for each mutation likely account for the varying degrees of reduction/expansion of *tll* and *hkb* expression domains.

Cuticle preparation
Unhatched larvae (24–48 h) were dechorionated in bleach, transferred into 50% lactic acid and 50% hoyers medium and baked at 70°C for 2 h.

Acknowledgements

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