Par-1 regulates *bicoid* mRNA localisation by phosphorylating Exuperantia

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Summary

The Ser/Thr kinase Par-1 is required for cell polarisation in diverse organisms such as yeast, worms, flies and mammals. During *Drosophila* oogenesis, Par-1 is required for several polarisation events, including localisation of the anterior determinant *bicoid*. To elucidate the molecular pathways triggered by Par-1, we have performed a genome-wide, high-throughput screen for Par-1 targets. Among the targets identified in this screen was Exuperantia (Exu), a mediator of *bicoid* mRNA localisation. We show that Exu is a phosphoprotein whose phosphorylation is dependent on Par-1 in vitro and in vivo. We identify two motifs in Exu that are phosphorylated by Par-1, and show that their mutation abolishes *bicoid* mRNA localisation during mid-oogenesis. Interestingly, *exu* mutants in which Exu phosphorylation is specifically affected can to some extent recover from these *bicoid* mRNA localisation defects during late oogenesis. These results demonstrate that Par-1 establishes polarity in the oocyte by activating a mediator of *bicoid* mRNA localisation. Furthermore, our analysis reveals two phases of Exu-dependent *bicoid* mRNA localisation: an early phase that is strictly dependent on Exu phosphorylation and a late phase that is less phosphorylation dependent.

Key words: *Drosophila*, *bicoid*, Par-1, Exn, Polarity, mRNA localisation

Introduction

The Ser/Thr kinase Par-1 is involved in several polarisation events in the *Drosophila* female germline. During early oogenesis, one of the 16 germline cells is selected to become the oocyte and the other 15 cells develop as nurse cells, providing the oocyte with mRNAs and proteins essential for its growth and patterning (for a review, see Riechmann and Ephrussi, 2001). The maintenance of oocyte cell fate is the earliest known function of Par-1 during oogenesis. In germline clones of a *par-1* null allele, the oocyte fails to polarise during early oogenesis and reverts to a nurse cell fate, resulting in egg chambers with 16 nurse cells and no oocyte (Cox et al., 2001; Huynh et al., 2001). Analysis of hypomorphic *par-1* alleles that allow oocyte development past early oogenesis revealed that *par-1* is required for repolarisation of the oocyte during mid-oogenesis (Shulman et al., 2000; Tomancak et al., 2000). At this stage *par-1* is required for polarisation of the oocyte microtubule network, and for localisation of *oskar* mRNA, which encodes the posterior determinant of the fly. In wild-type oocytes, microtubules nucleate from the anterior and lateral cortex, their plus ends directed towards the posterior pole of the oocyte (Cha et al., 2001), and *oskar* mRNA is localised in a kinesin-dependent process to the posterior pole (Brendza et al., 2000). By contrast, in *par-1* mutants microtubules nucleate from the entire oocyte cortex, including the posterior pole, directing their plus ends towards the centre of the oocyte. In these mutants, *oskar* mRNA is mislocalised to the centre of the oocyte (Shulman et al., 2000; Tomancak et al., 2000). As *oskar* mRNA localisation is kinesin dependent, the mislocalisation of *oskar* mRNA is most likely the consequence of the aberrantly polarised microtubule network (Shulman et al., 2000). Finally, during the late stages of oogenesis, Par-1 accumulates at the posterior pole of the oocyte. Here, Par-1 guarantees the maintenance of posterior polarity by phosphorylating, and thus stabilising, Oskar protein (Riechmann et al., 2002).

Recently, it has been reported that *par-1* is also involved in localisation of the anterior determinant *bicoid* mRNA (Benton et al., 2002). *bicoid* mRNA localisation occurs in two phases (St Johnston et al., 1989; Schnorrer et al., 2002): in the early phase, the mRNA is transported to the anterior margin of the oocyte, resulting in its distribution in a ring-shaped pattern. Anterior localisation of *bicoid* mRNA is microtubule dependent (Pokrywka and Stephenson, 1991), and is most probably mediated by plus end-directed motors. It is unclear, however, how the *bicoid* mRNA localisation complex distinguishes between microtubules nucleating from the lateral cortex and those nucleating from the anterior cortex of the oocyte. It has been speculated that anterior-nucleating microtubules might be qualitatively different from lateral microtubules and that this difference might be perceived by the *bicoid* mRNA-transport complex (Cha et al., 2001). In the late phase, *bicoid* mRNA redistributes along the entire anterior cortex, resulting in a disc-shaped distribution of the mRNA. This localisation is dependent on the function of *γTub37C* and *Dgrip75* (*Grip75* – FlyBase), suggesting that it requires the formation of an anterior microtubule organising centre

(MTOC) (Schnorrer et al., 2002). In hypomorphic *par-1* mutants, the early phase of *bicoid* mRNA localisation is disrupted, such that the mRNA is not restricted to the anterior of the oocyte, but rather is distributed down the lateral cortex towards the posterior pole (Benton et al., 2002).

Another gene essential for *bicoid* mRNA localisation is *exu*. In *exu* mutants, *bicoid* mRNA localisation is affected beginning from the early phase, when the mRNA fails to localise specifically at the anterior and is dispersed throughout the oocyte cytoplasm (Berleth et al., 1988; St Johnston et al., 1989). Exu protein contains no known domains, with the exception of a region with weak homology to an RNA-binding motif (Macdonald et al., 1991; Marcey et al., 1991). However, this domain is dispensable for the *bicoid* mRNA localisation function of Exu (Wang and Hazelrigg, 1994). By electron microscopy, Exu is detected in large electron-dense structures in the nurse cells, the sponge bodies, where Exu is thought to associate with *bicoid* mRNA (Wilsch-Bräuninger et al., 1997). A series of RNA injection experiments has led to the hypothesis that, in the nurse cells, Exu promotes the recruitment of anterior-targeting factors to *bicoid* mRNA. This assembly of the *bicoid* mRNA localisation complex would render the mRNA competent for transport to the anterior margin of the oocyte during the early phase localisation (Cha et al., 2001).

We have identified Exu in a proteomic screen for direct targets of Par-1 kinase. We show that Exu is a phosphoprotein whose phosphorylation is dependent on Par-1. In the nurse cells, Exu and Par-1 colocalise in patches that also contain *bicoid* mRNA. We have determined Par-1 phosphorylation sites in Exu, and show that mutation of these sites abolishes the ability of Exu to mediate *bicoid* mRNA localisation during mid-oogenesis. These mutants, which specifically affect phosphorylation of Exu protein, allow us differentiate between two phases of *exu*-dependent *bicoid* mRNA localisation: an early phase during mid-oogenesis, which is strictly dependent on Exu phosphorylation; and a late phase at the end of oogenesis, in which the requirement for Exu phosphorylation is less stringent.

Materials and methods

An in vitro screen for Par-1 substrates

To identify Par-1 substrates, we screened the first release (DGC-1) of the *Drosophila* Unigene collection of Expressed Sequence Tags (EST) produced by the Berkeley *Drosophila* Genome Project (BDGP). Plasmid DNAs of the ESTs were prepared from bacterial glycerol stocks using a miniprep robot. Pools of eight ESTs were expressed in vitro and ^{35}S-methionine labelled, using the coupled transcription-translation system (Promega TNT system). Protein pools were split and incubated either with purified Par-1 kinase and kinase buffer [40 mM HEPES (pH 7.2), 3 mM $MgCl_2$, 5 mM EGTA, 1 mM ATP], or with buffer alone, for 2 hours at 37°C. Proteins were separated on polyacrylamide gels, in which SDS is only provided in the running buffer but not in the gel itself (Anderson gels). We used 15 ml 30% Acrylamide, 2.58 ml 1% bis-Acrylamide 7.5 ml 1.5 M Tris (pH 8.8), 4.74 ml H_2O, 150 μl 10% Ammonium Persulfate and 15 μl TEMED to prepare a 30 ml resolving gel; 2.5 ml 30% Acrylamide, 2 ml 1% bis-Acrylamide 1.875 ml 1.5 M Tris (pH 6.8), 8.625 ml H_2O, 150 μl 10% Ammonium Persulfate and 15 μl TEMED for a 15 ml-stacking gel; and 60 g Tris-base, 288 g Glycine, 10 g SDS to prepare 2 litres of 5×running buffer. After SDS-PAGE and autoradiography, pools containing Par-1 substrates were identified by scoring for proteins with altered electrophoretic mobility. Identification of the cDNAs of interest within a positive pool was achieved by translating each of the cDNAs separately, and running the eight translation products in parallel with the original pools. We confirmed the identified cDNAs as Par-1 substrates by performing the kinase assay with each cDNA individually. Among the 5849 screened cDNAs, 133 (2.2%) encode proteins that showed altered mobility after incubation with Par-1. Those were analysed using data provided by the BDGP and FlyBase. Based on the presence of certain protein domains, homologies to proteins from other species and functional data, seven groups of substrates were classified: Cytoskeletal proteins (11), proteins involved in different aspects of signal transduction (35), DNA-associated proteins (34), RNA-associated proteins (11), enzymes (9), novel proteins (25) and others (9).

Whole-mount in situ staining

Antibody staining of ovaries and embryos was performed as described (Tomancak et al., 2000). In situ hybridisation was performed as described by Wilkie et al. (Wilkie et al., 1999), and an RNA probe corresponding to full-length *bicoid* mRNA was generated by in vitro transcription using an Ambion Megascript kit. Double staining for *bicoid* mRNA and Exu GFP was performed as described by Vanzo and Ephrussi (Vanzo and Ephrussi, 2002) using a mouse anti-GFP antibody (Roche) in a 1:200 dilution. For double staining for Exu-GFP and Par-1, the same antibody was used in combination with a rabbit anti-Par-1 antibody (Tomancak et al., 2000) at a 1:40 dilution. Anti-Bicoid (Kosman et al., 1998) antibody was used at a 1:600 dilution.

Western blotting

Preparation of ovarian extracts and western blotting was performed as described (Riechmann et al., 2002). An anti-Exu antibody from rabbit was used at a 1:10.000 dilution. For the blot shown in Fig. 1B, Exu was detected with Enhanced Chemiluminescence using an anti-rabbit antibody coupled to horseradish peroxidase. For the blot in Fig. 4E, a fluorochrome-coupled anti-rabbit antibody was used, and signal was detected using an Odyssey scanner (LI-CORE).

In vitro mutagenesis and generation of transgenes

exu mutants were generated by mutating the original EST from the Unigene collection using the Quick change mutagenesis kit from Stratagene according to the manufacturer's protocol. Mutant cDNAs were confirmed by sequencing, and in vitro translated for kinase assays with Par-1. *exu* transgenes are based on a previously described P-element transformation vector designed for the expression of Exu-GFP (Wang and Hazelrigg, 1994). The vector contains the *exu* upstream regulatory sequences and the *exu* open reading frame (ORF) supplemented at its 3′ end with a sequence encoding GFP. To remove the GFP sequence and to introduce the mutations, we excised a *Not*I-*Bam*HI fragment that includes the 3′ end of the ORF and the GFP sequence and replaced this fragment with a *Not*I-*Bam*HI fragment bearing the mutation.

Fly stocks

Following fly stocks were used: exu^{XL} and exu^{VL} (protein null alleles), $par\text{-}1^{W3}$ (a null allele), and $par\text{-}1^{9A}$ and $par\text{-}1^{574}$ (hypomorphic alleles). All females used for staining were grown at 18°C.

Results

Exu phosphorylation is dependent on *par-1*

To elucidate the pathways by which Par-1 establishes polarity during oogenesis, we modified an assay developed by Stukenberg et al. (Stukenberg et al., 1997) to identify proteins phosphorylated by Par-1 kinase. This assay is based on the fact that phosphorylation usually alters the mobility of proteins on SDS polyacrylamide gels. We screened the *Drosophila* genome

for Par-1 substrates, by testing in vitro-translated and radiolabelled proteins for mobility shifts after incubation with purified Par-1 (see Materials and methods). Exu, an essential mediator of *bicoid* mRNA localisation (Berleth et al., 1988; St Johnston et al., 1989; Macdonald et al., 1991), was among the substrates identified in the screen. Migration of in vitro translated Exu protein changed dramatically after incubation with Par-1, suggesting phosphorylation of the protein (Fig. 1A, lanes 1,2). We confirmed by phosphatase treatment that the altered mobility was due to phosphorylation (Fig. 1A, lane 3). To test if Exu phosphorylation is also dependent on Par-1 in vivo, we compared the migration of Exu in ovarian extracts of females possessing different levels of Par-1 activity. Remarkably, overexpression of Par-1 causes hyperphosphorylation of Exu (Fig. 1B, lane 4), while the majority of Exu protein is unphosphorylated in *par-1* mutants. In addition, the degree of Exu phosphorylation is dependent on the strength of the *par-1* allele, as strong mutants show a stronger reduction in Exu phosphorylation than weak mutants (Fig. 1B, lanes 2 and 3). These data show that the phosphorylation of Exu is dependent on Par-1, suggesting that Par-1 phosphorylates Exu during oogenesis.

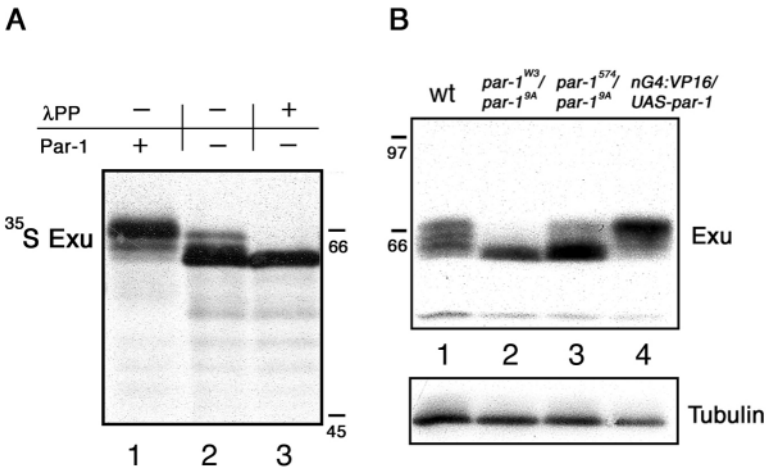

Fig. 1. Par-1 phosphorylates Exu in vitro and in vivo. (A) In vitro translated and ^{35}S radiolabelled Exu protein subjected to SDS-PAGE and autoradiography. Exu protein incubated in kinase buffer alone migrates as three bands consisting of a major band at 60 kDa and two weaker protein bands that migrate more slowly (lane 2). As phosphorylation usually reduces the electrophoretic mobility of a protein, the presence of the two weak bands of Exu suggests that the protein is phosphorylated by kinases present in the in vitro translation extract. The absence of the slowly migrating protein bands after 2 hours treatment with lambda phosphatase shows that Exu is phosphorylated (lane 3). Phosphorylation is dramatically increased after incubation with Par-1 kinase, as revealed by the absence of the 60 kDa fast migrating band and the appearance of a slowly migrating protein doublet at 66 kDa (lane 1). (B) Western blot of ovarian extracts of females of the indicated genotypes probed with an antibody against Exu (upper panel) or Tubulin (lower panel) as a loading control. *par-1^{W3}* is a null allele, and *par-1^{9A}* and *par-1^{574}* are hypomorphic alleles. *par-1* overexpression in ovaries was achieved by placing *par-1* under the control of the Gal4 UAS system and expressing a *UAS-par-1* transgene in the germline using the *nanos*Gal4:VP16 driver.

par-1 and *exu* are required for *bicoid* mRNA localisation

In wild type oocytes, two phases of *bicoid* mRNA localisation can be distinguished by in situ hybridisation (St Johnston et al., 1989; Schnorrer et al., 2002). First, *bicoid* mRNA forms a ring at the anterior margin of stage 9-10a oocytes (Fig. 2A,D). Next, at stage 10b, the ring of *bicoid* mRNA evolves into a disk, such that the mRNA is present along the entire anterior surface of the oocyte (Fig. 2G). As a first step towards the analysis of the role of Exu phosphorylation by Par-1, we re-examined the distribution of *bicoid* mRNA in *exu* and *par-1* mutants. Consistent with previous reports (Berleth et al., 1988; St Johnston et al., 1989), we found that in *exu* null mutants (*exu^{XL}/exu^{VL}*) (Marcey et al., 1991) *bicoid* mRNA localisation is severely affected and the anterior ring does not form (Fig. 2B). Interestingly however, *bicoid* mRNA localisation is not completely abolished in *exu* mutants, as the mRNA still concentrates in the anterior region of stage 9 oocytes (Fig. 2B,E). In addition, residual amounts of *bicoid* mRNA are detected at the anterior cortex of disk stage oocytes (Fig. 2H). Residual anterior localisation of *bicoid* mRNA was also observed in to other *exu* alleles (*exu^{QR}* and *exu^{PJ}*), confirming that *exu* does not completely abolish *bicoid* mRNA localisation to the anterior of the oocyte.

As *par-1* null mutants arrest oogenesis before *bicoid* mRNA is localised, we analysed *bicoid* mRNA distribution in a combination of *par-1* alleles that retains some Par-1 activity (*par-1^{W3}/par-1^{9A}*) (Cox et al., 2001; Huynh et al., 2001). In these mutants, formation of the anterior *bicoid* ring is severely affected, as revealed by spreading of the mRNA along the lateral cortex of the oocyte (Benton et al., 2002) (Fig. 2C,F). However, this defect recovers during stage 10 and only trace amounts *bicoid* mRNA are mislocalised (Fig. 2I). In conclusion, both *exu* and *par-1* mutants show strong *bicoid* mRNA localisation defects during the early phase of *bicoid* mRNA localisation. These defects, together with the finding that Par-1 phosphorylates Exu, raises the possibility that Par-1 acts via Exu phosphorylation in *bicoid* mRNA localisation.

Exu and Par-1 colocalise with *bicoid* mRNA in the nurse cells

To further investigate the connection between Exu and Par-1, we compared their distributions by detecting Exu as a GFP fusion protein and Par-1 with an antibody. Both proteins have previously been detected in the cytoplasm of the nurse cells (Wang and Hazelrigg, 1994; Theurkauf and Hazelrigg, 1998; Shulman et al., 2000; Tomancak et al., 2000). Strikingly, the proteins show a near-identical localisation in patches in the apical regions of the nurse cells (Fig. 3A). It has previously been shown that Exu-GFP colocalises with *bicoid* mRNA in the nurse cells (Cha et al., 2001). We therefore tested whether *bicoid* mRNA is also present in these patches in the nurse cells. *bicoid* mRNA is enriched in the apical patches, although a substantial amount of the mRNA is also detected outside of the patches (Fig. 3B). The extensive colocalisation of Par-1 and Exu-GFP in these patches suggests phosphorylation of Exu by Par-1 may occur at these sites. The presence of *bicoid* mRNA in the apical patches supports our hypothesis that Exu

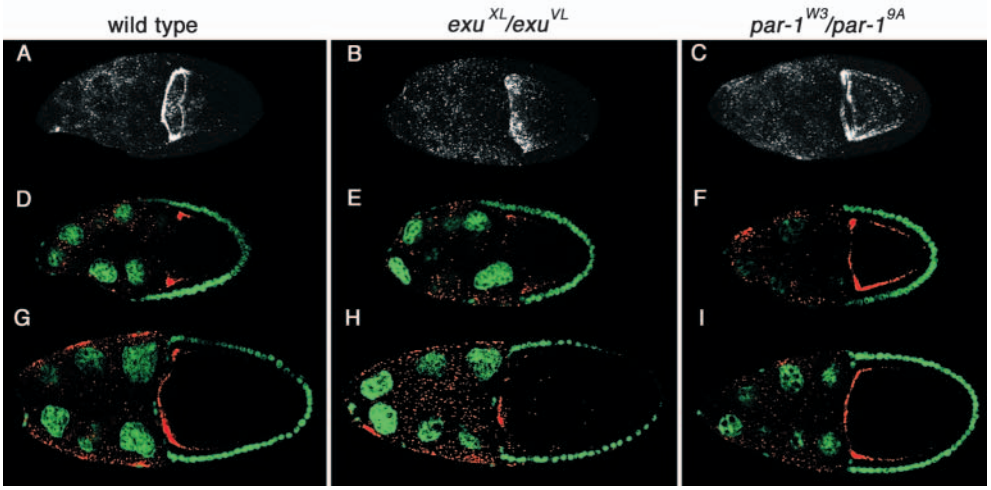

Fig. 2. *exu* and *par-1* are required for *bicoid* mRNA localisation. (A-C) In situ hybridisation showing *bicoid* mRNA localisation in entire egg chambers, by projection of eight confocal sections. (D-I) Single confocal sections in the middle of the oocyte showing *bicoid* mRNA (red) and DNA (green) at stage 9 (D-F) and 10b (G-I) of oogenesis. Anterior is leftwards, and posterior is rightwards. Genotypes are indicated. All females were grown at 18°C.

phosphorylation is involved in the localisation of *bicoid* mRNA.

Mapping and mutagenesis of Exu phosphorylation sites

To test the hypothesis that Par-1 regulates *bicoid* mRNA localisation by phosphorylating Exu, we searched for potential Par-1 phosphorylation sites in Exu protein. Within the C-terminal part of Exu, we identified two sites resembling 14-3-3 binding motifs, which are known targets of Par-1 phosphorylation (Benton et al., 2002) (Fig. 4A). We mutated all relevant serines within these sites, produced the mutant proteins by in vitro translation and analysed their phosphorylation state by SDS-PAGE analysis after incubation with Par-1 kinase. Remarkably, mutations in both sites cause an increased mobility of Exu protein, indicating reduced phosphorylation of the mutant proteins. Although mutations in site A cause only a modest increase in Exu mobility (Fig. 4B, A6) mutations in site B lead to a dramatic increase (Fig. 4B, B3). An even stronger reduction in Exu phosphorylation, resembling the situation after phosphatase treatment, is observed when all Serines within sites A and B are mutated simultaneously (compare B3 and A6B3 in Fig. 4D). By mutating single Serine residues in site A and B, we mapped two serines in site A (S438 and S440, see Fig. 4C, A3 and A4) and one serine in site B (S457, see Fig. 4D, B2), the mutation of which causes a significant reduction of Exu phosphorylation. A triple mutant, in which these three serines were simultaneously mutated shows a slightly greater degree of phosphorylation than a mutant in which all

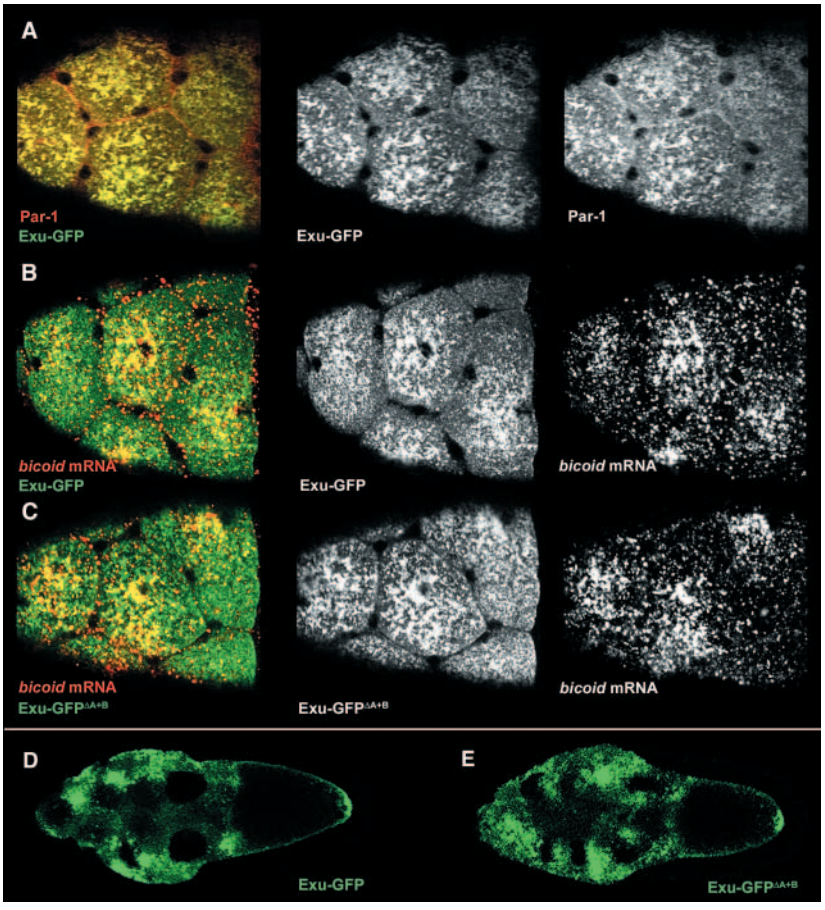

Fig. 3. Par-1, Exu-GFP and *bicoid* mRNA localise to patches in the nurse cells. (A-C) Confocal section in the apical region of the nurse cells of egg chambers expressing Exu-GFP. (A) Par-1 protein is visualised with an anti-Par-1 antibody (red) and Exu-GFP with an antibody against GFP in the wild type. Both proteins accumulate in patches and colocalise almost throughout the whole nurse cell cytoplasm. Par-1 is also detected at the membranes of the nurse cells. (B) Double staining for Exu-GFP (green) detected with antibody against GFP and *bicoid* mRNA (red) in the wild type. (C) *exu* null mutants (exu^{XL}/exu^{VL}) expressing Exu-GFP protein in which all phosphorylation sites are mutated (Exu-$GFP^{\Delta A+B}$) double stained for GFP (green) and *bicoid* mRNA (red). (D,E) Confocal sections through the middle of a wild type egg chamber expressing wild-type Exu-GFP (D), and an *exu* null mutant egg chamber expressing Exu-$GFP^{\Delta A+B}$ (E). Exu-GFPs are visualised with fluorescence of the GFP.

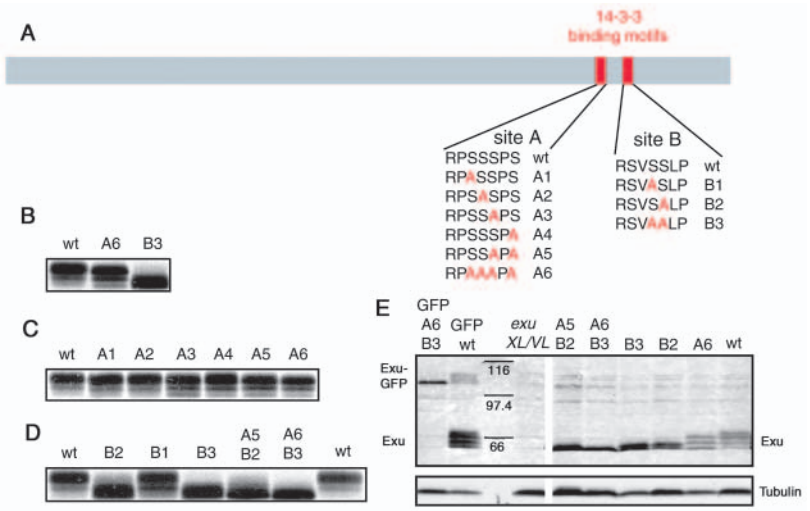

Fig. 4. Mapping and mutation of Exu phosphorylation sites. (A) Position of the two potential 14-3-3 binding sites (red) in Exu protein (grey bar). Sequences of site A (amino acids 434-440) and B (amino acids 453-459) and of the corresponding mutations (A1-A6 and B1-B3) are shown below. (B-D) Autoradiographic images of SDS polyacrylamide gels showing in vitro translated and radiolabelled wild-type or mutant Exu proteins after incubation with Par-1. Mutations in site A and B are indicated above the lane. (E) Western blot of ovarian extracts probed with an antibody against Exu (upper panel) or Tubulin (lower panel) as a loading control. Exu protein was expressed from transgenes using the endogenous *exu* promoter in an *exu*-null background (exu^{XL}/exu^{VL}). Transgenes encode either wild-type or mutant Exu protein. The nature of the mutation is indicated above the corresponding lane. The first lane shows extracts from *exu* null mutant ovaries expressing Exu-GFP protein, in which all Par-1 phosphorylation sites are mutated (GFP A6B3). This mutant protein runs in a single band, indicating absence of phosphorylation. The second lane shows extracts from wild-type ovaries expressing Exu-GFP. Exu-GFP is phosphorylated and migrates as a triplet around 100 kDa while untagged Exu migrates around 66 kDa.

serines were mutated, indicating that the other serine residues are also subject to phosphorylation (Fig. 4D, A5B2 and A6B3).

After identifying Par-1 target sites in Exu in vitro, we generated transgenes to express the mutant Exu proteins in ovaries under the control of the *exu* promoter (Wang and Hazelrigg, 1994). The transgenes were crossed into an *exu* protein null background to assess the phosphorylation state of the mutant Exu proteins by western blot analysis. As in the in vitro assay, a knockout of site A causes only a slight decrease in Exu phosphorylation (Fig. 4E, A6), while a knockout of site B leads to a dramatic reduction (Fig. 4E, B3). We observed the same strong decrease in phosphorylation when only S457 of site B was mutated as when both relevant serines in B were mutated (Fig. 4E, B2 and B3) confirming that this Serine is especially important for Exu phosphorylation. Most importantly, upon mutation of all serines in A and B ($Exu^{\Delta A+B}$), the protein migrates as a single band, indicating absence of any phosphorylation (Fig. 4E, A6B3). Hence, the motifs mediating phosphorylation of Exu by Par-1 in vitro also mediate Exu phosphorylation in vivo. Together with the finding that Exu phosphorylation is dependent on Par-1 during oogenesis, these data strongly suggest that Par-1 phosphorylates Exu on sites A and B.

Phosphorylation does not affect Exu localisation or mobility

Different aspects of Exu function have been revealed by analysis of an Exu-GFP fusion protein, which, when expressed under the control of the endogenous promoter, rescues the *exu* phenotype (Wang and Hazelrigg, 1994). Exu-GFP can be detected at the anterior of the oocyte and accumulates at the posterior of stage 9-10 oocytes (Fig. 3D). Within the nurse cells, Exu-GFP forms particles that move through the ring canals into the oocyte (Theurkauf and Hazelrigg, 1998). To examine whether one of these properties of Exu is phosphorylation dependent, we expressed a mutant Exu-GFP, in which all serines in site A and B are mutated in ovaries of *exu* null mutant females. First, we confirmed by western analysis that the mutant Exu-GFP is not phosphorylated (Fig. 4E, A6 B3-GFP). Next, we analysed the distribution of mutant Exu-GFP protein in egg chambers and found that neither its subcellular localisation in the oocyte nor in the nurse cells is affected (Fig. 3C,E). Consistent with the finding that the overall distribution of the protein is not altered, we could not detect any differences in the mobility of unphosphorylated and phosphorylated Exu-GFP particles (data not shown). Finally, we tested if Exu phosphorylation affects the ability of the protein to colocalise with *bicoid* mRNA in the nurse cells. Notably, *bicoid* mRNA is still enriched in the Exu-GFP containing apical patches in the nurse cells (Fig. 3C). In conclusion, our Exu-GFP analysis suggests that phosphorylation of Exu is neither required for its localisation, nor its mobility, nor its ability to colocalise with *bicoid* mRNA.

Exu phosphorylation is required for anterior patterning of the embryo

A Bicoid protein gradient controls the hierarchy of segmentation genes at beginning of embryogenesis (Driever, 1993). The mislocalisation of *bicoid* mRNA in embryos from *exu* females disturbs this gradient, resulting in head defects (Schüpbach and Wieschaus, 1986; Frohnhöfer and Nüsslein-Volhard, 1987). To test whether phosphorylation of Exu is required for anterior patterning of the embryo, we first attempted to rescue the head defects of *exu* null mutant embryos using the $exu^{\Delta A+B}$ transgene, which expresses unphosphorylated Exu. More than one-third of the embryos produced by exu^{VL}/exu^{XL}; $exu^{\Delta A+B}$ females develop head defects typical of *exu* mutants and fail to hatch (data not shown), indicating that the Bicoid protein gradient is affected when Exu is unphosphorylated. Next, we directly analysed the Bicoid gradient in embryos produced by $exu^{\Delta A+B}$ mothers, using an anti-Bicoid antibody. We could detect no Bicoid gradient in approximately one-third of the embryos (Fig. 5E). In the rest of the embryos, a gradient of Bicoid protein was detected, however, its extent appears reduced compared with the wild type (Fig. 5E). These results suggest that the head defects observed in one third of the embryos produced by

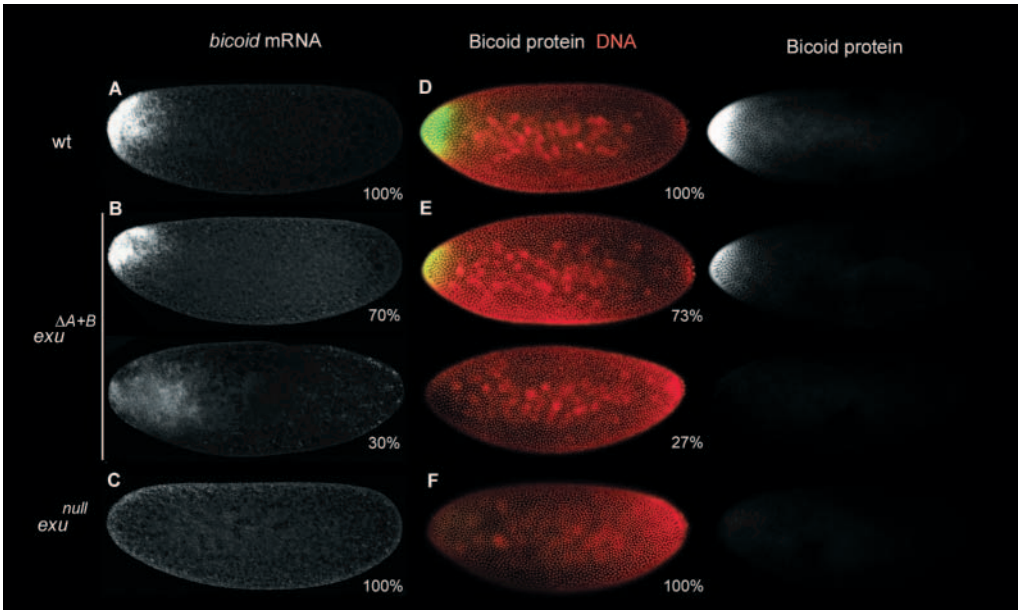

Fig. 5. Exu phosphorylation is required for proper formation of the Bicoid protein gradient. (A-C) Confocal sections through the middle of early cleavage stage embryos hybridised with a *bicoid* RNA probe. (A) In all wild-type embryos (*n*=20), *bicoid* mRNA was localised anteriorly. (B) In embryos from females expressing the $exu^{\Delta A+B}$ transgene in an *exu*-null mutant background, only 70% of the embryos (*n*=32) show anteriorly localised *bicoid* mRNA, while the other 30% show no anterior signal. (C) In embryos from *exu*-null mutants (exu^{XL}/exu^{VL}) all embryos (*n*=19) lack anterior *bicoid* mRNA. (D-F) Blastoderm embryos stained for Bicoid protein (green) and DNA (red). Right panel shows Bicoid protein alone. All embryos were fixed and stained in parallel, and pictures were taken with the same setup with a digital camera and a light microscope. (D) In all wild-type embryos (*n*=27), a clear Bicoid gradient with similar extension was observed. (E) In embryos from females expressing the $exu^{\Delta A+B}$ transgene in an *exu* null mutant background, no Bicoid was detected in 27% of the embryos, while in all of the remaining embryos the extension of the gradient is reduced compared with wild-type embryos (*n*=30). (F) In all *exu*-null mutants examined (*n*=34) no Bicoid protein was detectable.

$exu^{\Delta A+B}$ mothers are caused by the absence of a Bicoid gradient in the embryo at the beginning of embryogenesis. The fact that two third of the embryos hatch indicates that, in those embryos, the Bicoid gradient is sufficiently strong to support anterior patterning.

To investigate more precisely the role of Exu phosphorylation in formation of the anterior-posterior axis of the embryo, we made use of our allelic series of *exu* transgenes encoding Exu proteins with different phosphorylation levels. For this analysis, we chose those transgenic lines that express equivalent levels of Exu protein as revealed by western blot analysis (Fig. 4E). We first examined the segmentation of the embryos produced by exu^{VL}/exu^{XL} mothers expressing Exu proteins mutant in the different Par-1 phosphorylation sites, staining for the protein product of the pair-rule gene *even skipped* (*eve*). Mutations in either site A or B, as well as the triple mutation of serines 438, 440 and 457, cause a weak anterior shift of embryonic segments typical of embryos produced by females in which *bicoid* activity is slightly reduced (Frohnhöfer and Nüsslein-Volhard, 1987) (Fig. 6B′-D′). We observed two types of patterning defects in embryos from $exu^{\Delta A+B}$ females: (1) embryos with a stronger anterior shift of Eve stripes than that observed in mutants in which Par-1 phosphorylation is merely reduced and (2) embryos that, in addition to an anterior extension of the first Eve stripe also show an enlargement of the first two stripes and a compression of the posterior stripes (Fig. 6E′). To determine whether there was a correlation between the reduction of Exu phosphorylation and the segmentation defects, we compared the extent of anterior shift of the first Eve stripe in the different mutants. In Table 1, the average extension of the first Eve stripe in the different phosphorylation defective backgrounds is indicated, and in Fig. 6 those embryos with the maximal extension of the first Eve stripe are shown. Strikingly, the more Exu phosphorylation is reduced, the more the first Eve stripe extends anteriorly. Thus, there is a direct correlation between the degree of reduction of Exu phosphorylation and the position of the first Eve stripe. In summary, we observed two classes of phenotypes in the embryos of the phosphorylation defective mutants. First, in one third of the *exuΔA+B* mutants we found a severe phenotype in which the Bicoid protein

Table 1. Quantification of anterior patterning defects in phosphorylation defective *exu* mutants

Genotype	Average position of first Eve stripe	Variation of first Eve stripe among scored embryos
exu wild type	33.4%	32-35%
exu A6	30%	28-31%
exu B2	30.3%	28-32%
exu A5 B2	29.5%	27-31%
exu A6 B3 ($exu^{\Delta A+B}$)	28.9%	23-31%
exu^{XL}; exu^{VL}	26.9%	21-32%
Wild type	32.7%	32-36%

Embryos form an overnight collection were fixed, stained with an antibody against Eve protein and embedded in a plastic dish containing araldite. Ten cellular blastoderm embryos (stage 6) of each genotype were randomly chosen with a needle under a stereomicroscope and mounted individually on a slide. Photographs were taken using a digital camera and a light microscope (Axioplan). The extension of the first Eve stripe in each embryo was measured using Photoshop and is indicated as a percentage of egg length. The values obtained were then used to calculate the the average position of the first Eve stripe for each genotype. The values for the positions of the first Eve stripe in the embryos with the maximal and minimal extension are also indicated to show the variation within each genotype. The transgenes encoding wild type *exu* and phosphorylation defective *exu* mutants (*exu* wild type, *exu* A6, *exu* B2, *exu* A5 B2, *exu* A6 B3) were all expressed in an *exu* null mutant background (exu^{XL}; exu^{VL}). Equivalent levels of expression of these transgenes was revealed by western blot analysis (see Fig. 4E).

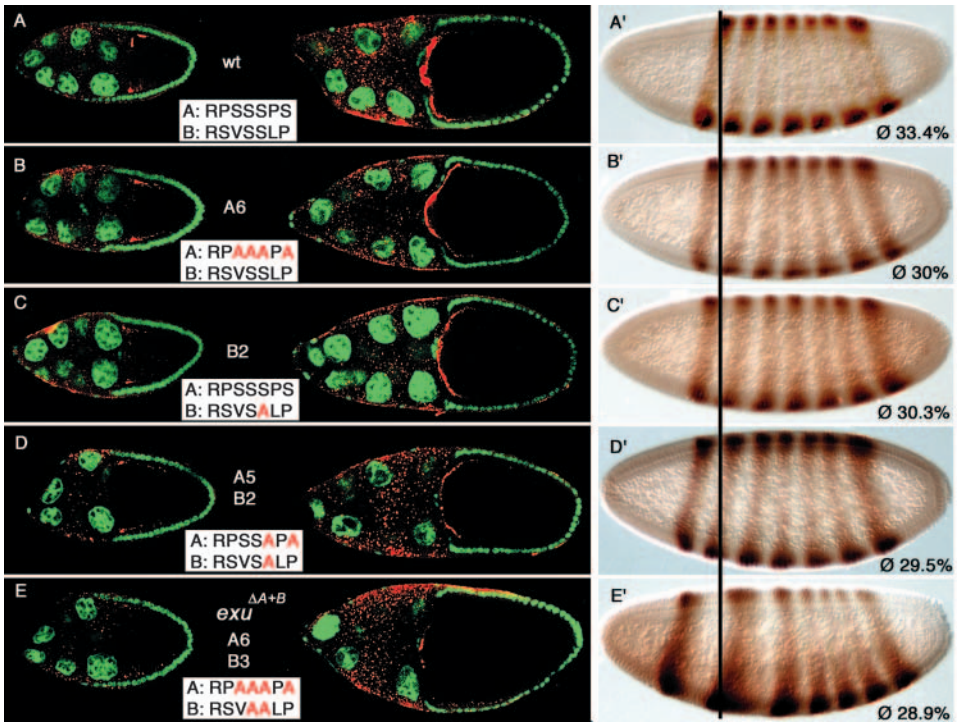

Fig. 6. Exu phosphorylation is required for *bicoid* mRNA localisation and embryonic patterning. (A-E) In situ hybridisation showing *bicoid* mRNA (red) in confocal sections of stage 9 (left) and stage 10b (right) egg chambers. DNA is shown in green. Wild-type (A) or mutant (B-E) Exu protein was expressed from transgenes in an *exu*-null mutant background (exu^{XL}/exu^{VL}). Sequences of site A and B are indicated, and mutations are shown in red. All mutations affect *bicoid* mRNA localisation at stage 9 (B-E). *bicoid* mRNA localisation defects recover at stage 10b when only site A or B is mutant (B,C), and partially recovers when serines 438, 440 and 457 are mutant (D). No recovery is observed when all relevant serines in site A and B are simultaneously mutated. (A′-E′) Embryos in the right panel are derived from females of the same genotype as egg chambers in the left panel. Embryos were stained with anti-Eve antibody to visualise segmentation. Ten embryos from each genotype were randomly chosen for analysis with the light microscope. Those embryos that show maximal anterior extension of the first Eve stripe are shown. The average position of the first Eve stripe of the ten analysed embryos is indicated as percentage of egg length. The average position of the first Eve stripe in *exu* null mutants (exu^{XL}/exu^{VL}) is at 26.9% egg length. Black line indicates the position of first Eve stripe in embryos expressing wild type Exu. The extension of the anterior shift of the first Eve stripe in the mutants (B′-E′) corresponds to the severity of the *bicoid* mRNA localisation defects during oogenesis (B-E). All females were grown at 18°C. Anterior is leftwards; posterior is rightwards.

gradient is abolished, resulting in strong segmentation defects and lethality. Second, in two thirds of the $exu\Delta A+B$ mutants and in the mutants that affect only a subset of the Exu phosphorylation sites we observed a milder phenotype reflected by a reduction of the Bicoid gradient and an extension of the first Eve stripe.

Exu phosphorylation is required for anterior localisation of *bicoid* mRNA

To test if the embryonic patterning defects we observed could be explained by defects in *bicoid* mRNA localisation during oogenesis, we analysed *bicoid* mRNA distribution in egg chambers by in situ hybridisation. A transgene encoding wild-type Exu rescues all aspects of *bicoid* mRNA localisation in exu^{XL}/exu^{VL} oocytes (Fig. 6A). Strikingly, the transgene expressing Exu protein with a total knockout of phosphorylation, entirely fails to rescue the *bicoid* localisation defects of the *exu* mutant oocytes from stage 9 through stage 10b (Fig. 6E). These *bicoid* mRNA localisation defects are indistinguishable from *exu*-null mutants (Fig. 2E,H) and have been observed in all analysed oocytes (n=31). Exu proteins with intermediate levels of phosphorylation caused by mutations in site A or B are unable to rescue *bicoid* mRNA localisation at stage 9, but possess sufficient residual activity to rescue of *bicoid* mRNA localisation at stage 10 (Fig. 6B,C). Thus, the capacity of Exu protein to localise *bicoid* mRNA correlates with the phosphorylation state of the protein. Therefore, we conclude that Exu phosphorylation is required for localisation of *bicoid* mRNA during mid-oogenesis. Furthermore, these results strongly suggest that the embryonic pattering defects in the phosphorylation defective mutants are the consequence of *bicoid* mRNA mislocalisation during oogenesis.

Different mechanisms mediate *bicoid* mRNA localisation during mid- and late oogenesis

Although *exu*-null and the phosphorylation defective $exu^{\Delta A+B}$ mutants abolish *bicoid* mRNA localisation during mid-oogenesis, the two mutants differ in the strength of their embryonic patterning defects. Although we never observed Bicoid protein at the anterior of the embryos from *exu* null mutants, 73% of the progeny of the $exu^{\Delta A+B}$ mutants form a Bicoid protein gradient (Fig. 5). Though the extension of this gradient is reduced compared with the wild type, its existence in a large proportion of the embryos reveals that unphosphorylated Exu retains some activity. The fact that *exu* null and $exu^{\Delta A+B}$ mutants show identical phenotypes until stage 10b suggests that the residual activity of unphosphorylated Exu protein is required only during the later stages. We therefore compared *bicoid* mRNA localisation in the two mutants after mid-oogenesis. During late oogenesis, $exu^{\Delta A+B}$ oocytes start to accumulate *bicoid* mRNA at the anterior cortex (Fig. 7C), while in *exu* null mutants only traces of *bicoid* mRNA are anteriorly localised, and most of the mRNA is either uniformly distributed in the ooplasm or cortically localised (Fig. 7B). The partial recovery in the $exu^{\Delta A+B}$ mutants shows that *bicoid* mRNA localisation is less phosphorylation dependent during late oogenesis. We conclude that during mid-oogenesis, *bicoid*

mRNA is localised by a mechanism that is strictly dependent on Exu phosphorylation, and that during late oogenesis the mode of *bicoid* mRNA localisation changes to a mechanism that is still dependent on Exu but less dependent on its phosphorylation by Par-1. The fact that the amount of *bicoid* mRNA that is localised during late oogenesis in the $exu^{\Delta A+B}$ mutants is sufficient for the formation of a Bicoid protein gradient in 73% of the embryos shows that mutants, in which localisation is abolished during the early stages of *bicoid* mRNA localisation can recover in late oogenesis. Therefore, the two mechanisms that mediate *bicoid* mRNA localisation during mid- and late oogenesis are redundant, and defects that occur during mid-oogenesis can be compensated in late oogenesis.

An *exu* independent function of *par-1* in *bicoid* mRNA localisation

We have shown that during mid-oogenesis, Exu phosphorylation is required for anterior localisation of *bicoid* mRNA, and have identified Par-1 as the kinase that phosphorylates Exu. Thus, Par-1 regulates *bicoid* mRNA localisation by phosphorylating Exu. However, *par-1* and *exu* null mutants differ in their *bicoid* mRNA localisation phenotypes: *bicoid* mRNA is spread along the lateral cortex of *par-1* mutant oocytes (Fig. 2C,F), while very little *bicoid* mRNA is detected at the cortex of *exu* mutant oocytes (Fig. 2B,E). The interpretation of the *bicoid* mRNA localisation phenotype of the *par-1* mutants is complicated by the fact that *par-1* is not only required for localisation of *bicoid* mRNA, but is also involved in other processes that establish polarity within the oocyte. In particular, *par-1* mutants show defects in polarisation of the microtubule network (Shulman et al., 2000) and, as *bicoid* mRNA localisation is microtubule dependent (Pokrywka and Stephenson, 1991), this defect might well contribute to the localisation defect. We therefore investigated if the *bicoid* mRNA mislocalisation in *par-1* mutants is exclusively due to reduced Exu phosphorylation, or if an Exu-independent process also contributes to the *bicoid* mRNA localisation defect. To this end, we generated an 'activated' version of Exu independent of phosphorylation by Par-1, by replacing (phospho-)serine 457 by the negatively charged residue glutamic acid. Although

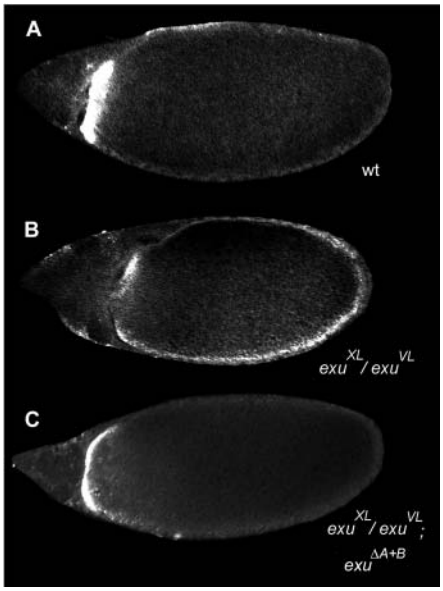

Fig. 7. *bicoid* mRNA localisation during late oogenesis. In situ hybridisation showing *bicoid* mRNA localisation in confocal sections of stage 12 egg chambers. (A) In the wild-type *bicoid* mRNA accumulates at the anterior of the oocyte. (B) Although *bicoid* mRNA is detectable at the anterior of *exu* null mutants most of the mRNA appears to be unlocalised at the cortex or in the ooplasm. (C) In mutants in which Exu phosphorylation is abolished, *bicoid* mRNA is enriched at the anterior indicating partial recovery of the complete loss of *bicoid* mRNA localisation during mid-oogenesis. Genotypes are indicated. All females were grown at 18°C. Anterior is leftwards; posterior is rightwards.

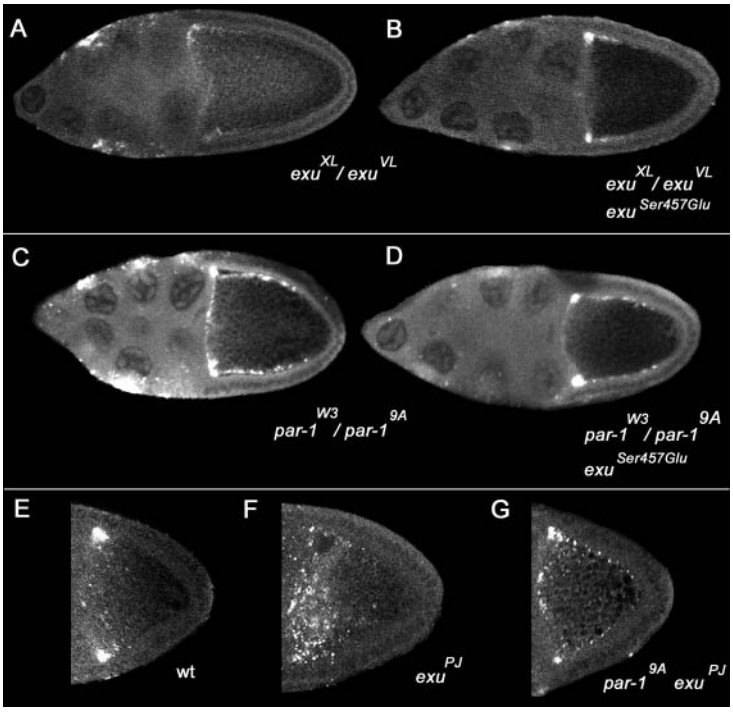

Fig. 8. *exu* independent functions of *par-1* in *bicoid* mRNA localisation. In situ hybridisation showing *bicoid* mRNA localisation in confocal sections of stage 9 egg chambers, in which in the wild-type mRNA is localised as a ring around the anterior margin of the oocyte. (A,B) *exu*-null mutant egg chambers either expressing no transgene (A) or a transgene encoding the activated $Exu^{Ser457Glu}$ (B). Both egg chambers are from siblings from the same cross and have been stained in parallel. The $Exu^{Ser457Glu}$ encoding transgene rescues *bicoid* mRNA localisation completely, as revealed by the restriction of *bicoid* mRNA to the anterior corners of the oocyte. The anterior *bicoid* mRNA ring appears as two discrete dots in the confocal section. (C,D) Egg chambers from *par-1* mutant siblings either expressing no transgene (C) or the $Exu^{Ser457Glu}$ encoding transgene (D). Both egg chambers have been stained in parallel. The $Exu^{Ser457Glu}$ protein is unable to fully rescue the cortical localisation of *bicoid* mRNA as the mRNA is no longer tightly restricted to the anterior corners but spreads along the lateral and anterior cortex of the oocyte (D). (E,F) For clarity only the oocyte of the egg chamber is shown. *bicoid* mRNA is restricted to the anterior corners in the wild type (E) and is dispersed throughout the ooplasm in homozygous exu^{PJ} mutants (F). In homozygous $par\text{-}1^{9A}$ exu^{PJ} double mutants (G), *bicoid* mRNA is cortically localised resembling the situation in *par-1* single mutants.

replacement of serine 457 by alanine results in severe *bicoid* mRNA localisation defects at the ring stage (Fig. 6C), its replacement by glutamic acid fully rescues *bicoid* mRNA localisation in *exu*-null mutants (Fig. 8B and Fig. 2D). If reduced Exu phosphorylation were solely responsible for the *bicoid* mRNA localisation defect in *par-1* mutant oocytes, then the activated Exu protein should rescue this defect in *par-1* mutants. However, the $Exu^{Ser457Glu}$ transgene does not rescue the *par-1* phenotype completely, and *bicoid* mRNA is aberrantly distributed along the lateral cortex of the oocyte and in the middle of the anterior cortex at stage 9 (the 'ring' stage) (Fig. 8D). This indicates that Par-1 has Exu-independent functions which, when they fail in *par-1* mutants, contribute to the mislocalisation of *bicoid* mRNA in these oocytes. The predominant cortical localisation of *bicoid* mRNA in *par-1* oocytes expressing the activated Exu protein suggests that the cortical mislocalisation defect occurs upstream of *exu* function.

To test the genetic hierarchy of *par-1* and *exu,* we recombined the two mutations and compared the distribution of *bicoid* mRNA in the double-mutant with that in the single *exu* and *par-1* mutants. Strikingly, *bicoid* mRNA in *par-1 exu* double-mutant oocytes is mainly cortical and resembles the distribution of *bicoid* mRNA in *par-1* oocytes, confirming that the cortical localisation defect of *par-1* occurs upstream of *exu* function (Fig. 8G). We conclude that *par-1* has two functions in *bicoid* mRNA localisation: a first Exu-independent function, which – when it fails – causes cortical accumulation of *bicoid* mRNA; and a second function, which is to phosphorylate Exu and render the protein competent for anterior targeting of *bicoid* mRNA.

Discussion

We have shown that Par-1 has two distinct functions in *bicoid* mRNA localisation. First, Par-1 is necessary for the release of *bicoid* mRNA from the oocyte cortex. This role of Par-1 is epistatic to its second function in regulating anterior localisation of *bicoid* mRNA by phosphorylating Exu. By generating mutants that abolish Exu phosphorylation, we could further distinguish two phases of Exu dependent *bicoid* mRNA localisation. An early phase, in which *bicoid* mRNA localisation is abolished when Exu is unphosphorylated and a late phase, in which the requirement for Exu phosphorylation is less stringent. Thus, our results show that *bicoid* mRNA localisation is a multi-step process, and that redundant mechanisms are used to ensure the anterior accumulation of *bicoid* mRNA.

A role for Exu phosphorylation in recruiting anterior targeting factors to *bicoid* mRNA

Exu protein is an essential mediator of *bicoid* mRNA localisation. In this study we have shown that Par-1 kinase phosphorylates Exu, and that this phosphorylation is necessary for anterior localisation of *bicoid* mRNA during mid-oogenesis. We have also shown that Exu phosphorylation does not affect Exu localisation, its ability to form mobile particles, or its colocalisation with *bicoid* mRNA. How then might Par-1 phosphorylation enable Exu to mediate *bicoid* mRNA localisation? Experiments in which fluorescently labelled *bicoid* mRNA was microinjected into living egg chambers have revealed that Exu is required in the nurse cells for anterior localisation of *bicoid* mRNA within the oocyte. These experiments have led to a model whereby Exu associates in the nurse cells with *bicoid* mRNA and mediates the recruitment of additional nurse cell factors required for targeting of *bicoid* mRNA to the anterior of the oocyte (Cha et al., 2001). Our finding that mutation of Exu phosphorylation sites results in a phenotype that is, during mid-oogenesis, indistinguishable from that of *exu*-null mutants suggest that Exu phosphorylation is involved in the recruitment of these anterior-targeting factors in the nurse cells. Phosphorylation might increase the binding affinity of Exu for these nurse cell factors, promoting their association with *bicoid* mRNA. The colocalisation of Exu-GFP, Par-1 and *bicoid* mRNA in patches in the nurse cells suggests that this is where the *bicoid* RNP complexes assemble.

Cortical *bicoid* mRNA localisation precedes its anterior transport in the oocyte

The consequences of *exu* and *par-1* mutations on *bicoid* mRNA localisation are distinct. Although loss of *exu* function results in diffuse *bicoid* mRNA distribution in the ooplasm, a reduction in *par-1* function causes cortical localisation of the mRNA. We have generated an Exu protein that localises *bicoid* mRNA independent of phosphorylation by Par-1 and rescues *exu* mutants, but that is unable to rescue *bicoid* mRNA localisation in *par-1* mutants. Therefore, the cortical mislocalisation of *bicoid* mRNA in *par-1* mutant oocytes is independent of Exu function. What might be the other function of Par-1 in localisation of *bicoid* mRNA? The fact that *bicoid* localisation requires the microtubule cytoskeleton (Pokrywka and Stephenson, 1991), together with the report that oocyte microtubules are improperly polarised in *par-1* mutants (Shulman et al., 2000), suggests that cortical localisation in the mutants is caused by a microtubule defect. It has been proposed that microtubules of different qualities may nucleate from different regions of the oocyte cortex (Cha et al., 2001). A simple explanation for the aberrant localisation of *bicoid* mRNA in *par-1* oocytes would be that the subset of microtubules nucleating from the anterior corners of the oocyte and serving as tracks for anterior transport of *bicoid* mRNA are not restricted to the anterior corners, but spread along the cortex, resulting in the lateral cortical localisation of *bicoid* mRNA. However, this model is not supported by our genetic epistasis experiments, which indicate that the *exu* independent function of *par-1* acts at a step upstream of *exu* in *bicoid* mRNA localisation. Therefore, we favour a different model, in which in wild-type oocytes *bicoid* mRNA first localises cortically preceding its targeted transport along microtubules. In this model, most of the *bicoid* mRNA entering the oocyte moves in a nonpolar fashion, either passively or by active transport, to the oocyte cortex. Only after this cortical localisation does the targeted transport of *bicoid* mRNA to the anterior corners of the oocyte commence. In *par-1* mutants, the improperly organised microtubule cytoskeleton prevents release of the mRNA from the cortex to the (anterior-targeting) microtubules and the mRNA remains cortically localised. In *exu* mutants, the polarity of the microtubules is normal and *bicoid* mRNA is released from the cortex. However, its targeted transport to the anterior is impaired and the mRNA is diffusely distributed in the ooplasm.

A trapping mechanism for *bicoid* mRNA localisation in late oogenesis

The requirement for Exu phosphorylation in *bicoid* mRNA localisation decreases during the later stages of oogenesis. This is revealed by the partial recovery of *bicoid* mRNA localisation in *exu* mutants that abolish phosphorylation. These mutants are indistinguishable from *exu*-null mutants through stage 10b of oogenesis, but during early embryogenesis two-thirds of the mutants localise enough *bicoid* mRNA at the anterior to support formation of a Bicoid protein. This indicates that the mechanism of *bicoid* mRNA localisation changes after stage 10b of oogenesis, from an early phase that is strictly dependent on Exu phosphorylation, to a late phase that is less dependent on phosphorylation. Stage 10b is the stage at which ooplasmic streaming commences, providing a possible mechanism for localisation of *bicoid* mRNA in mutants in which Exu phosphorylation cannot occur. Before stage 10b, anterior targeting of *bicoid* mRNA could be mediated solely by directed transport of *bicoid* mRNA complexes along microtubules, a process that is strictly dependent on Exu phosphorylation. After stage 10b, this directed transport might be complemented or replaced by a passive trapping mechanism, which has also been postulated for the localisation of *oskar* and *nanos* mRNAs during late oogenesis (Glotzer et al., 1997; Forrest and Gavis, 2003). This mechanism relies on the movements generated by ooplasmic streaming, which could bring *bicoid* mRNA complexes into contact with the anterior cortex of the oocyte, where the mRNA could be trapped by localised anchoring molecules. This change in the mechanism of *bicoid* mRNA localisation would occur at the time of assembly of the anterior MTOC that is essential in the late phase of *bicoid* mRNA localisation (Schnorrer et al., 2002), suggesting that the MTOC might be involved in the trapping mechanism. Such a trapping mechanism would be differentially affected in Exu-null mutants and in mutants that specifically abolish Exu phosphorylation. It is possible that Exu provides *bicoid* mRNA not only with factors required for anterior targeting, but also with factors required for anchoring of *bicoid* mRNA. Unphosphorylated Exu might be inactive in recruiting the factors for anterior targeting, but be competent for binding of factors required for anchoring.

A model for *bicoid* mRNA localisation

In summary, our data supplement the models for *bicoid* mRNA localisation previously presented by Cha et al. (Cha et al., 2001) and Schnorrer et al. (Schnorrer et al., 2002) in the following way: in the first phase of *bicoid* mRNA localisation, the mRNA is transported to the anterior corners of the oocyte, resulting in a ring-like distribution. This targeted transport requires the formation of RNP complexes that contain *bicoid* mRNA and specific anterior-targeting factors that allow the RNPs to identify those microtubules that nucleate from the anterior corners of the oocyte. Assembly of this complex takes place in the nurse cells and requires the phosphorylation of Exu by Par-1. Upon entry of the complex into the oocyte, a specific proportion of the RNP complexes encounter the microtubules that nucleate from the anterior corners, and these complexes are directly transported to their final destination. However, a large proportion of the complexes does not find these microtubules directly, and moves first to the oocyte cortex. The transfer of these cortically localised complexes to microtubules nucleating from the anterior corners solely requires a properly polarised microtubule network. Only at this stage can the nurse cell factors assembled on the mRNA act to transport the cortically localised complexes to the anterior corners of the oocyte. During the second phase of *bicoid* mRNA localisation, the ring-shaped distribution changes to a disc-shaped distribution and a MTOC forms at the anterior of the oocyte. The third phase of *bicoid* mRNA localisation begins after the onset of ooplasmic streaming. In this late phase, the mechanism of *bicoid* mRNA localisation changes from targeted transport to passive trapping, mediated by ooplasmic streaming, and the mRNA is anchored at the anterior margin. The generation of *exu* mutants that abolish phosphorylation allows us to distinguish between the early and the late mechanisms of *bicoid* mRNA localisation, as the two mechanisms differ in their sensitivity to Exu phosphorylation.

Par-1 establishes polarity in the oocyte by different mechanisms

We have previously shown that Par-1 controls posterior patterning by phosphorylating Oskar (Riechmann et al., 2002). Here, we have shown that Par-1 regulates anterior patterning by phosphorylating Exu. Although Oskar is an intrinsically unstable protein whose stability is increased by Par-1 phosphorylation, Par-1 phosphorylation does not affect Exu stability (Fig. 1B, Fig. 3C,E; data not shown) but does affect its ability to mediate *bicoid* mRNA localisation. Thus, Par-1 uses at least two different mechanisms to generate polarity within the same cell. Interestingly, these two Par-1 substrates, Oskar and Exu, are unique to Diptera, showing that during evolution Par-1 gained fly-specific mediators of cell polarisation as substrates. Par-1 is therefore flexible in the mechanisms and in the targets by which it mediates cell polarisation. This is in striking contrast to the PDZ-containing proteins Par-3 and Par-6, which appear to establish polarity by the assembly of a conserved protein complex.

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References

Benton, R., Palacios, I. M. and Johnston, D. S. (2002). *Drosophila* 14-3-3/PAR-5 is an essential mediator of PAR-1 function in axis formation. *Dev. Cell* **3**, 659-671.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7**, 1749-1756.

Brendza, R. P., Serbus, L. R., Duffy, J. B. and Saxton, W. M. (2000). A function for kinesin I in the posterior transport of *oskar* mRNA and Staufen protein. *Science* **289**, 2120-2122.

Cha, B. J., Koppetsch, B. S. and Theurkauf, W. E. (2001). In vivo analysis of *Drosophila bicoid* mRNA localization reveals a novel microtubule-dependent axis specification pathway. *Cell* **106**, 35-46.

Cox, D. N., Lu, B., Sun, T.-S., Williams, L. T. and Jan, Y. N. (2001). *Drosophila par-1* is required for oocyte differentiation and microtubule organization. *Curr. Biol.* **11**, 75-87.

Driever, W. (1993). Maternal control of anterior development in the *Drosophila* embryo. In *The Development of* Drosophila melanogaster, Vol. 1 (ed. M. Bate and A. Martinez-Arias), pp. 301-324. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Forrest, K. M. and Gavis, E. R. (2003). Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for *nanos* mRNA localization in *Drosophila*. *Curr. Biol.* **13**, 1159-1168.

Frohnhöfer, H. G. and Nüsslein-Volhard, C. (1987). Maternal genes required for the anterior localisation of *bicoid* activity in the embryo of *Drosophila*. *Genes Dev.* **1**, 880-890.

Glotzer, J. B., Saffrich, R., Glotzer, M. and Ephrussi, A. (1997). Cytoplasmic flows localize injected *oskar* RNA in *Drosophila* oocytes. *Curr. Biol.* **7**, 326-337.

Huynh, J.-R., Shulman, J., Benton, R. and St Johnston, D. (2001). Par-1 is required for the maintenance of oocyte fate in *Drosophila*. *Development* **128**, 1201-1209.

Kosman, D., Small, S. and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. *Dev. Genes Evol.* **208**, 290-294.

Macdonald, P. M., Luk, S. K. and Kilpatrick, M. (1991). Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes Dev.* **5**, 2455-2466.

Marcey, D., Watkins, W. S. and Hazelrigg, T. (1991). The temporal and spatial distribution pattern of maternal exuperantia protein: evidence for a role in establishment but not maintenance of *bicoid* mRNA localization. *EMBO J.* **10**, 4259-4266.

Pokrywka, N. J. and Stephenson, E. C. (1991). Microtubules mediate the localization of *bicoid* RNA during *Drosophila* oogenesis. *Development* **113**, 55-66.

Riechmann, V. and Ephrussi, A. (2001). Axis formation during *Drosophila* oogenesis. *Curr. Opin. Genet. Dev.* **11**, 374-383.

Riechmann, V., Gutierrez, G. J., Filardo, P., Nebreda, A. R. and Ephrussi, A. (2002). Par-1 regulates stability of the posterior determinant Oskar by phosphorylation. *Nat. Cell Biol.* **4**, 337-342.

Schnorrer, F., Luschnig, S., Koch, I. and Nüsslein-Volhard, C. (2002). Gamma-tubulin37C and Gamma-tubulin ring complex protein 75 are essential for *bicoid* RNA localization during *Drosophila* oogenesis. *Dev. Cell* **3**, 685-696.

Schüpbach, T. and Wieschaus, E. (1986). Maternal-effect mutations altering the antero-posterior pattern of the *Drosophila* embryo. *Rouxs Arch. Dev. Biol.* **195**, 302-317.

Shulman, J. M., Benton, R. and St Johnston, D. (2000). The *Drosophila* homolog of *C. elegans* PAR-1 organizes the oocyte cytoskeleton and directs *oskar* mRNA localization to the posterior pole. *Cell* **101**, 377-388.

St Johnston, D., Driever, W., Berleth, T., Richstein, S. and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Development* **107**, 13-19.

Stukenberg, P. T., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J. and Kirschner, M. W. (1997). Systematic identification of mitotic phosphoproteins. *Curr. Biol.* **7**, 338-348.

Theurkauf, W. E. and Hazelrigg, T. I. (1998). In vivo analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway. *Development* **125**, 3655-3666.

Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K. C., Kemphues, K. J. and Ephrussi, A. (2000). A *Drosophila melanogaster* homologue of *Caenorhabditis elegans par-1* acts at an early step in embryonic-axis formation. *Nat. Cell Biol.* **2**, 458-460.

Vanzo, N. F. and Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the *Drosophila* oocyte. *Development* **129**, 3705-3714.

Wang, S. and Hazelrigg, T. (1994). Implications for *bcd* mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* **369**, 400-403.

Wilkie, G. S., Shermoen, A. W., O'Farrell, P. H. and Davis, I. (1999). Transcribed genes are localized according to chromosomal position within polarized *Drosophila* embryonic nuclei. *Curr. Biol.* **9**, 1263-1266.

Wilsch-Bräuninger, M., Schwarz, H. and Nüsslein-Volhard, C. (1997). A sponge-like structure involved in the association and transport of maternal products during *Drosophila* oogenesis. *J. Cell Biol.* **139**, 817-829.