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bicoid RNA localization requires specific binding of an endosomal sorting complex

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Summary paragraph:

bicoid mRNA localises to the anterior of the *Drosophila* egg, where it is translated to form a morphogen gradient of Bicoid protein that patterns the head and thorax of the embryo. Although *bicoid* was the first identified localised cytoplasmic determinant1-4, little is known about how the mRNA is coupled to the microtubule-dependent transport pathway that targets it to the anterior, and it has been proposed that it is recognised by a complex of many redundant proteins, each of which binds to the localisation element in its 3'UTR with little or no specificity5. Indeed, the only known RNA-binding protein that co-localises with *bicoid* mRNA is Staufen, which binds non-specifically to dsRNA in vitro6, 7. Here we show that mutants in all subunits of the ESCRT-II complex (Vps22, Vps25 and Vps36) abolish the final Staufen-dependent step in *bcd* RNA localisation. ESCRT-II is a highly conserved component of the pathway that sorts ubiquitinated endosomal proteins into internal vesicles8, 9, and functions as a tumour-suppressor by removing activated receptors from the cytoplasm10, 11. However, the role of ESCRT-II in *bicoid* localisation appears to be independent of endosomal sorting, because mutations in ESCRT-I and III components have no effect of the targeting of *bicoid* mRNA. Instead, Vps36 functions by binding directly and specifically to stem-loop V of the *bicoid* 3'UTR through its N-terminal GLUE domain12, making it the first example of a sequence specific RNA-binding protein that recognises the *bicoid* localisation signal. Furthermore, Vps36 localises to the anterior of the oocyte in a *bicoid* mRNA-dependent manner, and is required for the subsequent recruitment of Staufen to the *bicoid* complex. This novel function of ESCRT-II as an RNA-binding complex is conserved in vertebrates, and may explain some of its roles that are independent of endosomal sorting.

Genetic screens for mutations that disrupt anterior-posterior patterning of the *Drosophila* embryo have identified a few genes that are required at different stages for the anterior localisation of *bicoid* mRNA, but most of these appear to play an indirect role in the process. Mutations in *exuperantia (exu)* abolish all stages of *bicoid* mRNA localisation13, 14, but its function is unclear, since Exu protein is a component of a translational repression complex that co-purifies with *oskar* mRNA, but not with *bicoid* itself15. Swallow, γ-Tubulin37C, Dgrip75, Dgrip128, and Minispindles are necessary for the localisation of *bicoid* mRNA from stage 10B of oogenesis onwards, and function to nucleate anterior microtubules that direct localisation at this stage16-18. Finally, Staufen is required for *bicoid* mRNA localisation at the end of oogenesis and in the early embryo19, 20. Unlike the other trans-acting factors, Staufen is a dsRNA-binding protein, and associates with *bicoid* mRNA at the oocyte anterior from stage 10B onwards6.

bicoid mRNA is localised by distinct and partially redundant mechanisms at different stages of oogenesis, which may explain why genetic screens have missed many of the essential

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trans-acting factors. In mutants that only disrupt early localisation, the localisation of the mRNA in late oocytes can rescue anterior development, whereas mutants that only disrupt late localisation result in a gradient of mRNA that induces some anterior patterning, unless *bicoid* translation is also impaired21-23. To circumvent this problem, we performed a direct visual screen in germline clones for mutants that alter the localisation of *bicoid* mRNA in living oocytes using GFP-Staufen as a marker24. This screen identified one complementation group, called *larsen*, that is required for the anterior localisation of *bicoid* mRNA. GFP-Staufen fails to localise to the anterior cortex of the oocyte in homozygous germline clones of both *larsen* alleles (Fig.1 a, b, d, e), whereas its posterior localisation with *oskar* mRNA is unaffected. The stronger allele, lsn^{5F3-8}, is homozygous lethal, and females with a homozygous mutant germline do not lay eggs. However, the weaker allele, lsn^{2B63}, is only semi-lethal, and mutant germline clones result in eggs in which *bicoid* mRNA forms a gradient across the anterior half of the embryo (Fig.1 f), a phenotype very similar to that seen *staufen* mutants6.

Both *larsen* alleles correspond to mutations in the highly conserved protein, Vps22 (supplementary Fig.1). Vps22p, together with Vps25p and Vps36p, forms the ESCRT-II (for endosomal sorting complex required for transport), one of four complexes (Vps27/Hrs complex, ESCRT-I, -II and -III) that act in a linear pathway to sort mono-ubiquitinated transmembrane proteins within the endosomal compartment into internal vesicles, leading to the formation of multivesicular bodies (MVBs)9, 25. Mutants in *Drosophila* ESCRT-I and ESCRT-II components have been shown to cause a tumorous phenotype, because activated receptors accumulate on endosomes, rather than being removed from the cytoplasm and degraded10, 11, 26. Mammalian ESCRT-II was independently identified as a complex that binds the RNA polymerase II elongation factor, ELL27, 28, while the fission yeast Vps 22/ Larsen homologue regulates the expression of centriolar proteins during meiosis29. However, the relationship between these functions and endosomal protein sorting remains unclear.

To test whether the role in *bcd* mRNA localisation is specific for Larsen/Vps22 or whether the whole ESCRT-II is involved in the process, we examined mutants in the other two components of this complex, *vps25* and *vps36*. Homozygous germline clones of *vps25* and *vps36* show an identical phenotype to lsn^{5F3-8}: GFP-Stau does not localise to the anterior of the oocyte (Fig.1g-j), and the flies do not lay eggs. We therefore tested whether other MVB sorting mutants also disrupt *bcd* mRNA localisation. However, *bicoid* mRNA localises normally in germ line clones mutant for *hrs (vps27)*, the ESCRT-I components, *vps28* and *ept*, and the ESCRT-III component, *vps32* (Fig.2c-f). Thus, the ESCRT-pathway appears to be dispensable for *bcd* mRNA localisation, indicating that ESCRT-II has another function in addition to the sorting of ubiquitinated proteins into MVBs.

Given the role of ESCRT-II in *bicoid* mRNA localisation, we tested whether any of the proteins of the complex interact with the *bicoid* localisation signal, using a yeast 3-hybrid assay30. Both Staufen and Vps36, but not Vps22 or Vps25, interact with the *bicoid* 3′UTR in this assay, resulting in growth of the yeast cells on plates lacking histidine and in the expression of α-galactosidase (Fig.3a). To determine whether Vps36 protein binds directly to *bcd* RNA, we performed a UV-cross linking assay with purified recombinant Vps36 and *in vitro* transcribed *bicoid* 3'UTR. Vps36 cross-links efficiently to the *bcd* 3′UTR in this assay, indicating that it contacts single-stranded regions of the RNA directly (Fig.3b). Furthermore, this interaction is specific, since the binding is competed by excess unlabelled *bcd* 3′UTR, but not by control RNAs (Fig.3b, d, e and data not shown). Given the high degree of conservation of ESCRT-II, we tested whether *Xenopus laevis* Vps36 also interacts with RNA, and found that its N-terminal GLUE domain12 binds specifically to the *bcd*

3'UTR, indicating that the RNA-binding activity of Vps36 is conserved in vertebrates (Fig. 3c).

To map the binding site within the RNA more precisely, we tested the ability of single stem-loops of the *bicoid* 3'UTR to compete for binding to Vps36. Stem-loop V competed as well as the full-length 3'UTR, whereas stem-loops III, IVb and the distal part of stem-loop V did not compete (Fig.3b, d). We then performed a random mutagenesis to map the Vps36 binding site within stem-loop V, and found that none of the distal mutations affect the interaction, whereas three base changes in the proximal stem and the central loop-region strongly reduce Vps36 binding (Fig.3e and supplementary Fig.2). Thus, Vps36 binds specifically to the proximal part of stem-loop V, and recognises specific bases, most probably in the context of the RNA structure of this region.

We next examined whether Vps36 associates with *bicoid* mRNA in vivo by generating transgenic flies expressing Vps36-YFP under the control of its endogenous promoter. Vps36-YFP is ubiquitously expressed in the ovary, and shows a general cytoplasmic localisation with some clouds in the nurse cells (Fig.4a and a′). From stage 10B/11 of oogenesis, the protein localises to the anterior cortex of the oocyte in the same region as *bicoid* mRNA (Fig.4b, b' and c). This anterior accumulation is abolished in *exu* mutants, which block *bicoid* mRNA localisation at an earlier stage (Fig.4d), and in *sry δ* mutants, in which *bcd* RNA is not transcribed (Fig.4e). Thus, Vps36 co-localises with *bicoid* mRNA at the anterior, and this localisation is *bicoid* mRNA-dependent, indicating that the protein binds directly to the RNA in vivo, as it does in vitro.

Both ESCRT-II and Staufen are recruited to *bicoid* mRNA at stage 10B, and are then required for its localisation during the final stages of oogenesis. *staufen* mutants have no effect on the anterior recruitment of Vps36 (Fig.4f), however, whereas mutants in *larsen/vps22* and *vps36* abolish the anterior recruitment of Staufen protein at this stage (Figs.1 and 2). ESCRT-II therefore binds to *bicoid* mRNA independently of Staufen, and is required for the subsequent recruitment of the latter to form a functional *bicoid* mRNA localisation complex.

Our results demonstrate that ESCRT-II is required for the anterior localisation of *bicoid* mRNA, and is the first identified sequence-specific RNA binding complex that recognises the *bicoid* localisation signal. This novel activity of ESCRT-II seems to be independent of its well-characterised role in endosomal protein sorting, and is conserved, since *Xenopus* Vps36 also interacts specifically with the *bcd* 3'UTR, suggesting that ESCRT-II may play a role in mRNA localisation in vertebrates. In future, it will be important to determine whether this conserved RNA-binding activity of ESCRT-II plays any role in endosomal trafficking, or in the other proposed functions of the complex in transcription elongation and the regulation of centriole assembly.

Methods

A more detailed description of the materials and methods used can be found under supplementary information.

Drosophila Genetics

Flies were grown under standard conditions on corn meal medium. Germline clones were induced using the autosomal DFS technique. For detailed information and the genotypes of the flies used see supplementary information.

Yeast 3-Hybrid assay

For the yeast 3-Hybrid assay we used the system based on the transcription factor GAL 4 and the RNA binding protein RevM10 from HIV-1, as well as its binding element (Rev responsive element, RRE)30.

UV-cross linking

The purified proteins were pre-incubated with the competitor RNAs for 20 min at room temperature in X-link buffer (PBS + 5% Glycerol, 2 mM DTT, 0.2% NP-40, 100 μg/ml heparin and 100 μ/ml tRNA). After addition of the probe and a further 20 min of incubation the cross-linking was performed using a 'Stratalinker 1800' at maximum power for 5 min. The samples were treated with RNaseA (0.5 μg/ml) for 1 h at 37°C and after SDS-PAGE the radioactivity was visualised using a phosphoimager.

Stainings and microscopy

Stainings were performed as described. Pictures were taken using a BioRad 1024 Confocal Microscope and processed with Adobe Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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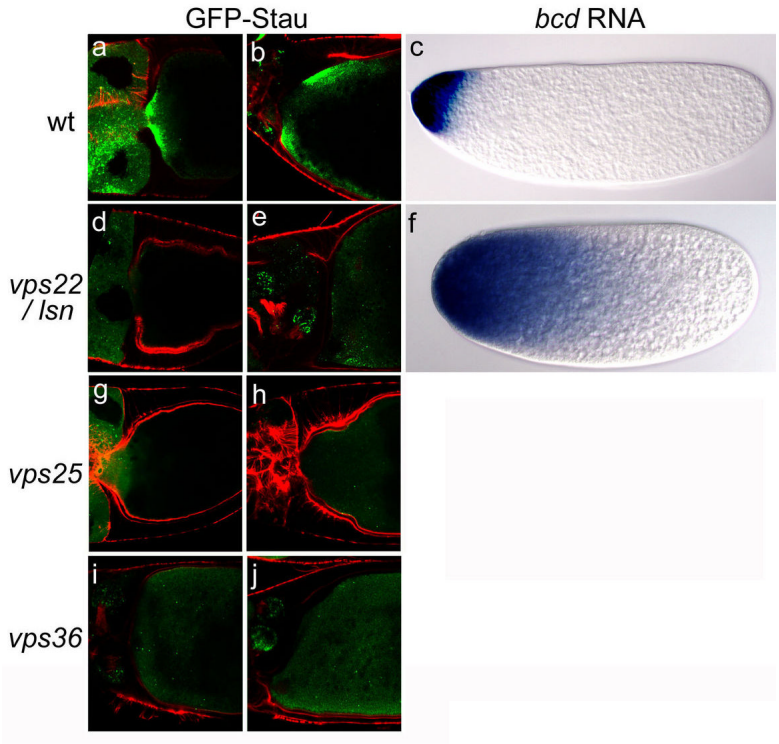

Figure1. Mutations in *lsn* and *vps36* disrupt the anterior localisation of GFP-Stau and *bcd* mRNA

a and **b** GFP-Staufen (green) localisation to the anterior cortex of wildtype stage 11 (**a**) and stage 13 (**b**) oocytes. The actin cortex is labelled with Rhodamine-Phalloidin (red).
c *bcd* mRNA localisation in a freshly-laid wildtype egg.
d and **e** Homozygous *vps22/lsn* germline clones, showing the absence of GFP-Stau at the anterior pole of the oocyte at stage 10B (**d**) and at stage 13 (**e**).
f *bcd* mRNA localisation in an embryo derived from a *vps22/lsn* germline clone.
g and **h** Homozygous $vps25^{Pb2931}$ germline clones, showing the absence of GFP-Stau at the anterior pole of the oocyte at stage 10B (**g**) and at stage 13 (**h**).
i and **j** Homozygous *vps36* (*l(3)L5212*) germline clones, showing the absence of GFP-Stau at the anterior pole of the oocyte at stage 11 (**i**) and at stage 13 (**j**).

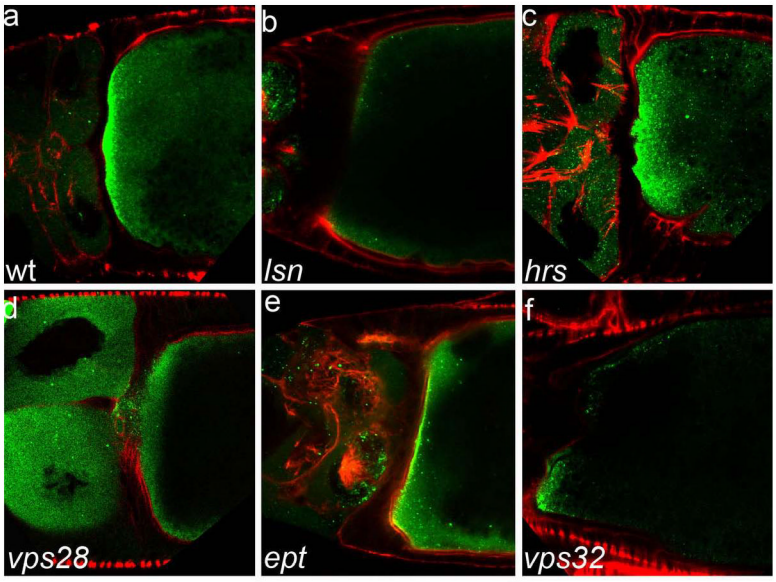

Figure 2. Localisation of GFP-Stau in oocytes mutant for different ESCRT components

a The anterior localisation of GFP-Stau in a wildtype stage 11 oocyte.

b-f GFP-Staufen localisation in *lsn*$^{2B6\text{-}3}$ (**b**), *hrs*^{D28} (**c**), *vps28* (*l(2)k16503*) (**d**), *ept*^{P26} (**e**) and *vps32* (*KG01481*) (**f**) germline clones.

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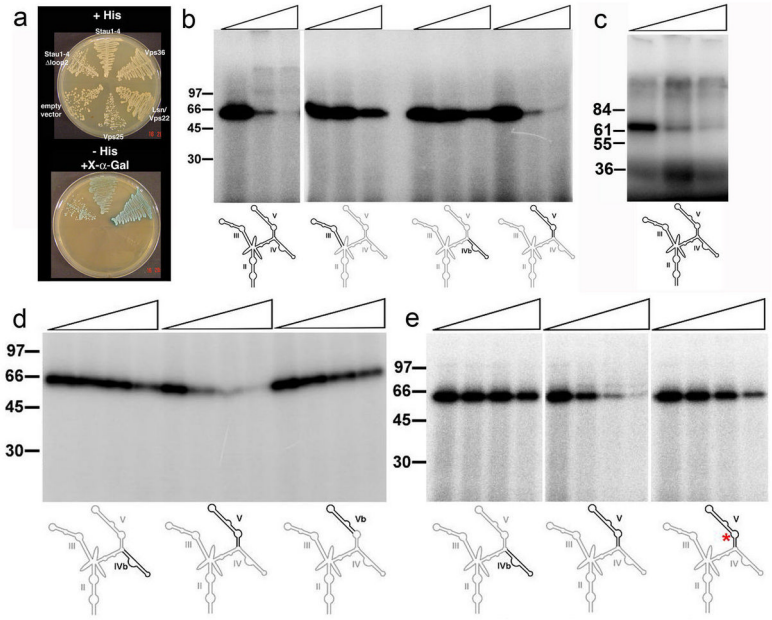

Figure 3. Vps36 binds specifically to stem-loop V of the *bicoid* 3'UTR

a 3-Hybrid assay using the full-length *bcd* 3′UTR as an RNA bait. Growth on media lacking histidine and α-galactosidase activity (blue staining) indicates an interaction between the protein and the RNA. Vps36 interacts with *bcd* RNA in this assay, as does the region of Staufen containing the first four dsRNA-binding domains with or without the insert in dsRBD2 (Δloop2). In contrast, Lsn/Vps22 and Vps25 show no interaction.

b-e UV cross-linking assay using purified recombinant *Drosophila* Vps36 protein or purified GST-GLUE domain from *Xenopus* (**c**) and full-length ^{32}P-labelled *bcd* 3′UTR as a probe. **b** Vps36 binding to the full-length 3'UTR in the presence of increasing amounts of competitor RNA (first lane: no competitor, second lane: 10-fold excess, third lane: 100-fold excess). The binding of Vps36 is inhibited by an excess of the full-length 3′UTR (lanes 1-3) and stem-loop V (lanes 10-12), but not by stem-loops III or IVb (lanes 4-9).

c The *Xenopus* GLUE domain can be cross-linked to the *bcd* 3'UTR (first lane: no competitor, second lane: 10-fold excess, third lane: 100-fold excess).

d The distal region of Stem-loop V does not compete for to Vps36 binding, indicating that the binding site must include part of the proximal region (first lane: no competitor, second lane: equal amount, third lane: 5-fold excess, fourth lane: 25-fold excess).

e Stem-loop V carrying three base changes in the central loop region shows an impaired ability to compete for Vps 36 binding. The probe is stem-loop V, and the competitors are Stem-loop IVb, Stem-loop V, and Stem-loop V with the following three mutations: 419 A->G, 427 A->C, 428 C->A (numbering starts with the first nucleotide after the Stop codon, see supplementary figure 2).

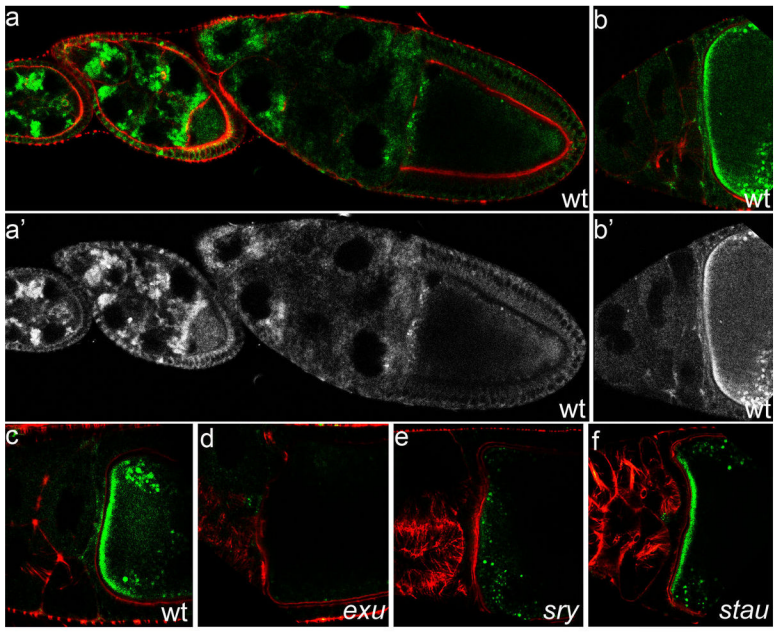

Figure 4. Vps36 is recruited to the anterior of the oocyte by *bicoid* mRNA

a-f Expression of a YFP -Vps36 fusion protein (green) under the control of the endogenous *vps36* promoter. **a** Vps36 is expressed in both the germ line and the somatic follicle cells of the ovary. The actin cytoskeleton has been counter-stained in red with Rhodamine–Phalloidin (**a'** shows the YFP channel alone). The protein shows a uniform distribution in the cytoplasm of the oocyte and the follicle cells, with a patchy distribution in the nurse cells, which becomes more disperse as oogenesis progresses. At stage 10B, Vps36 begins to accumulate at the anterior pole of the oocyte. **b** and **b'** Close-up of the anterior of a stage 11 egg chamber, showing the strong enrichment of Vps36 at the anterior cortex. The green spheres in the oocyte cytoplasm in this panel and panels D and E are not due to YFP-Vps36, but the background autofluorescence of yolk granules at late stages. **c –f** Localisation of YFPV-ps36 at stage 11 in wildtype **(c)**, exu^{VL}/exu^{SC} (**d**), $sry\text{-}\delta^{14}/$ *Df(3R)X3F,P{ry*$^{+}$*,sryDB56}* (**e**), and homozygous $stau^{D3}$ (**f**) oocytes. The fusion protein does not localise to the anterior cortex in *exu* mutants, which disrupt all stages of *bicoid* mRNA localisation, or in *sry* mutants, which prevent *bicoid* transcription, but localises normally in *staufen* mutants.