Xenopus Staufen is a component of a ribonucleoprotein complex containing Vg1 RNA and kinesin

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Summary

RNA localization is a key mechanism for generating cell and developmental polarity in a wide variety of organisms. We have performed studies to investigate a role for the *Xenopus* homolog of the double-stranded RNA-binding protein, Staufen, in RNA localization during oogenesis. We have found that *Xenopus* Staufen (XStau) is present in a ribonucleoprotein complex, and associates with both a kinesin motor protein and vegetally localized RNAs Vg1 and VegT. A functional role for XStau was revealed through expression of a dominant-negative version that blocks localization of Vg1 RNA in vivo. Our results suggest a central role for XStau in RNA localization in *Xenopus* oocytes, and provide evidence that Staufen is a conserved link between specific mRNAs and the RNA localization machinery.

Key words: RNA-binding protein, RNA localization, Polarity

Introduction

From yeast to vertebrates, cytoplasmic RNA transport is a powerful mechanism for spatially restricting protein expression (reviewed by Kloc et al., 2002; Palacios and St Johnston, 2001). Within germ cells, mRNAs are actively transported to specific subcellular destinations, which is an important mechanism to establish regulated cellular asymmetry of gene expression during differentiation and development. One example where RNA localization has developmental consequences is in the frog, *Xenopus laevis*. During oogenesis, maternal RNAs localize to the vegetal cortex of the oocyte and confer germ layer determination and germline specification during later embryonic development (reviewed by Mowry and Cote, 1999). Vegetally localized RNAs can be placed into two classes depending on their timing and mechanism of transport. Maternal mRNAs that are localized by the early localization pathway, also known as the METRO pathway, accumulate in the mitochondrial cloud at the beginning of oogenesis (stage I) and arrive at the vegetal cortex by stage II (Forristall et al., 1995; Kloc and Etkin, 1995). Examples of the early pathway RNAs include Xwnt11, which is a Wnt pathway component (Ku and Melton, 1993); Xdazl, which has been shown to play a role in germ cell determination (Houston and King, 2000); and Xcat2, which encodes a Nanos-like protein and is also thought to act in specification of the germline (Mosquera et al., 1993). The second pathway that functions to localize mRNAs to the vegetal pole was largely defined through studies on Vg1 RNA (Forristall et al., 1995; Kloc and Etkin, 1995; Melton, 1987). RNAs localized by the late pathway are uniformly dispersed in the cytoplasm of stage I oocytes. During mid-oogenesis, the RNAs are transported to the vegetal cortical cytoplasm, where they remain tightly anchored to the vegetal cortex until the end of oogenesis (stage VI) (Alarcon and Elinson, 2001; Elinson et al., 1993; Forristall et al., 1995; Kloc and Etkin, 1995; Yisraeli et al., 1990). An important function of the late pathway is the localization of germ layer determinants to the vegetal hemisphere. Indeed, one mRNA localized through the late pathway is VegT, a T-box transcription factor (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) that is required for endoderm and mesoderm specification (Zhang et al., 1998). Vg1, a member of the transforming growth factor-β superfamily (Weeks and Melton, 1987), has also been implicated in mesoderm and endoderm specification (Dale et al., 1993; Joseph and Melton, 1998; Thomsen and Melton, 1993).

RNA localization relies on cis-acting sequence elements within the RNA that are recognized by trans-acting components of the localization machinery (reviewed by Jansen, 2001). These cis-elements generally reside within the 3′ untranslated region (UTR) of the localized message and are essential for proper transport; examples are numerous, and include *Xenopus* Vg1 RNA (Mowry and Melton, 1992), and *bicoid* (Macdonald and Struhl, 1988; Macdonald and Kerr, 1997; Macdonald and Kerr, 1998) and *oskar* (Kim-Ha et al., 1993) mRNAs of *Drosophila*. Variable in length, the localization elements (LEs) mediate the interaction with trans-acting factors, resulting in formation of a ribonucleoprotein (RNP) complex. RNA-binding proteins have been identified as trans-acting localization factors in many animals, including *Xenopus* and *Drosophila*. The *Xenopus* Vg1 LE interacts with at least six different RNA-binding proteins, some of which have been shown to function in vegetal RNA localization (Cote et al., 1999; Deshler et al., 1998; Deshler et al., 1997; Havin et al., 1998; Kroll et al., 2002; Mowry, 1996; Schwartz et al., 1992; Zhao et al., 2001). In *Drosophila*, genetic approaches have identified a number of RNA-binding proteins required for localization of maternal mRNAs during oogenesis. Examples

include Staufen (St Johnston et al., 1991; St Johnston et al., 1989), Exuperantia (Berleth et al., 1988; Macdonald et al., 1991; Marcey et al., 1991), Swallow (Berleth et al., 1988; Chao et al., 1991; Stephenson et al., 1988), Squid (Norvell et al., 1999) and Modulo (Arn et al., 2003; Perrin et al., 1999), among others. However, the many trans-factors identified are generally non-homologous between vertebrates and invertebrates. This raises the issue of whether the mechanistic strategies for RNA localization are shared among organisms.

One factor that could mechanistically link RNA localization in vertebrates and invertebrates is the double-stranded RNA (dsRNA)-binding protein Staufen. Originally identified in flies, *staufen* mutants exhibited defects in *bicoid* RNA localization (St Johnston et al., 1989). *Drosophila* Staufen was found to be localized to the posterior cortex with *oskar* mRNA in oocytes and to the anterior cortex with *bicoid* mRNA in eggs, suggesting a role for Staufen in localization of RNAs along the anteroposterior axis (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). Moreover, *Drosophila* Staufen was shown to be responsible for asymmetric localization of *prospero* mRNA in neuroblasts (Broadus et al., 1998; Li et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998). Since the identification of *Drosophila* Staufen, several vertebrate homologs have been found (Kiebler et al., 1999; Marion et al., 1999; Wickham et al., 1999). Nonetheless, the functions of known vertebrate Staufen homologs are not yet clear. Mammalian Staufen has been shown to colocalize with polyribosomes and rough endoplasmic reticulum in cultured cells and hippocampal neurons (Kiebler et al., 1999; Marion et al., 1999; Wickham et al., 1999). Also, in hippocampal neurons, mammalian Staufen is colocalized with RNA-containing granules in dendrites (Mallardo et al., 2003; Tang et al., 2001), suggesting a role in RNA localization. However, interactions with specific localized RNAs have not been uncovered, leaving open the question of whether this RNA-binding protein may serve a conserved role in RNA localization.

To accomplish directional transport of localized RNAs within the cytoplasm, roles for motor proteins have long been sought (reviewed by Wilhelm and Vale, 1993), but only recently have the identities of some of these come to light (reviewed by Tekotte and Davis, 2002). In yeast, a myosin motor is responsible for the localization of ASH1 mRNA to budding daughter cells (Bobola et al., 1996). During embryogenesis in flies, dynein has been shown to be necessary for apical localization of pair-rule transcripts (Bullock and Ish-Horowicz, 2001; Wilkie and Davis, 2001). In *Drosophila* oocytes, kinesin I has been implicated in transport of *oskar* mRNA to the posterior during oogenesis (Brendza et al., 2000), but this role may be indirect (Cha et al., 2002). In other systems, including *Xenopus*, inhibition of RNA localization by cytoskeletal disruption also provided clues towards involvement of molecular motors (Knowles et al., 1996; Yisraeli et al., 1990). While motor proteins have been implicated in RNA sorting in vertebrate neurons (Carson et al., 1997), and most recently in vegetal RNA localization in *Xenopus* (Betley et al., 2004), the mechanisms by which specific motor proteins may be targeted to localized RNAs is not at all clear.

We have identified a *Xenopus* homolog of Staufen (XStau) as a component of the RNA localization machinery in frog oocytes. We present evidence that XStau interacts with specific localized RNAs during oogenesis and is expressed in a spatial and temporal pattern consistent with a role in RNA localization. Importantly, we show that XStau is in an RNP complex that associates with a kinesin motor and that a mutant version of XStau blocks RNA localization in oocytes. These results suggest that XStau is an integral component of the machinery necessary to localize RNA to the vegetal cortex and that XStau may mediate interactions between localized RNAs and motor proteins that are crucial for transport.

Materials and methods

Cloning and production of recombinant protein

Search of GenBank database using human STAU1 sequence (Wickham et al., 1999) yielded an expressed sequence tag (EST) clone (GenBank Accession Number BG486325) containing partial sequence of *Xenopus* Staufen (XStau). PCR using degenerate primer (forward 5′-ATGAARCTIGGAAARAARCCAAT-3′), designed from the conserved region of dsRBD2a of human STAU1 and mouse Stau1, and gene-specific primer 1 (GSP1) (reverse 5′-GGCATCGGCAA-GGATGT GGAGTCC-3′) within the EST amplified a product of 1.5 kb. The amplicon was ligated into pCR4-TOPO using the TOPO-TA kit (Invitrogen). 3′ RACE was conducted using the GSP2 (forward 5′-CGGCCCTCTGAGCAATTAAATTACC-3′) and the GeneRacer kit (Invitrogen), and 5′ RACE was conducted using GSP3 (reverse 5′-GGGCCACAGGAAATGGGTAGAAGTACCGTGGG-3′) and the SMART RACE kit (Clontech). The resulting RACE fragments contained the full coding sequence of XStau. To obtain the full-length XStau cDNA, N-terminal and C-terminal primers (forward 5′-AACAGATCTATGGACGTCACCATGTCTCAAGC-3′ and reverse 5′-AACATCGATGGCTGTGCTGTCCAGTGTTTG-3′) were used to amplify a 2.2 kb product from total oocyte cDNA which was ligated into pCR4-TOPO vector (Invitrogen).

To generate the GST-fusion clone pGEX-RBD2C, XStau sequence encoding dsRBD2 to the C terminus was PCR amplified using primers (forward 5′-ACGCGTCGACTCATGAAGCTGGGAAAGAAACC-AATATAC-3′ and reverse 5′-AAGGAAAAAGCGGCCGCGGC-TGTGCTGTCCAGTGTTTGCCC-3′) and cloned into pGEX4T-1 (Amersham Pharmacia) at *Sal*I and *Not*I sites. FLAG-tagged XStau constructs were generated by PCR amplification of full-length XStau using primers RBD1-5′ (forward 5′-GCAGATCTATGGACGTCA-CCATGTCTCAAGCT-3′) and RBD5-3′ (reverse 5′-AGCTCGAG-GCTGTGCTGTCCAGTGTTTG-3′), and XStau234 using primers RBD2-5′ (forward 5′-GCAGATCTATGCCTGTACGCGACAGC-ATCACG-3′) and RBD4-3′ (reverse 5′-AGCTCGAGGGCGGA-GGGACTTTAAAACCTAG-3′), followed by ligation into pSP64TSNRLMCSFLAG at *Bgl*II and *Xho*I sites to generate pSP64TSNXStauFLAG and pSP64TSNXStau234FLAG.

Antibody production and immunoblotting

pGEX-RBD2C was transformed into *E. coli* strain BL21 CodonPlus(DE3)-RIL (Stratagene). Expression and purification of GST-XStau peptide has been described previously (Coligan et al., 1995), with minor modifications. Lysis buffer contained 50 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.1 μg/ml leupeptin, 0.1 μg/ml antipain, 0.1 μg/ml trypsin inhibitor, 0.4 M Pefabloc and 200 μg/ml lysozyme. After sonication, lysed cells were centrifuged at 40,000 ***g*** for 20 minutes, passed through a 0.45 μm filter and loaded onto a glutathione column. Eluted fusion protein was verified on 10% SDS PAGE, quantified using Bradford assay, and concentrated using Centriplus YM-50 (Amicon). XStau-GST fusion peptides (500 mg) were injected along with an equal volume of complete Freund's adjuvant into New Zealand white rabbits to generate polyclonal anti-XStau antibodies.

Immunoblotting was performed as described previously (Denegre

et al., 1997). XStau antibodies were used at 1:2000; rpS6 antibodies (E-13, Santa Cruz Biotechnology) at 1:800; dynein intermediate chain antibodies (DIC, Sigma) at 1:4000; tubulin antibodies (Sigma) at 1:2000; SUK4 antibodies (Ingold et al., 1988) at 1:1000; hnRNP I antibodies (Kress et al., 2004) at 1:2000; Vg1RBP antibodies (Zhang et al., 1999) at 1:30,000; and FLAG antibodies (Sigma) at 1:2000 dilutions in BLOTTO (250 mM NaCl, 50 mM Tris pH 7.5, 5% dried milk, 0.1% Tween 20). Anti-rabbit secondary was used at 1:160,000, anti-mouse secondary at 1:100,000, anti-goat secondary at 1:8000 dilutions. All secondary antibodies used were peroxidase-conjugated (Sigma) and detection was by enhanced chemiluminescence.

Oocytes and microinjection

Oocytes were obtained surgically from *Xenopus laevis* and microinjection was performed as previously described (Gautreau et al., 1997). VLE RNA was transcribed from pSP73-340 (Mowry, 1996) in reactions containing 0.5 mM ATP and CTP, 100 μM GTP, 1 mM diguanosine triphosphate, 450 μM UTP, 50 μM Alexa-546-14-UTP (Molecular Probes), 1 μCi of ^{32}P-UTP. Stage III albino oocytes were injected with 150 pg of RNA and incubated at 18°C in OCM [50% L15 medium, 15 mM HEPES (pH 7.6), 1 mM glutamine and 1 μg/ml insulin] for 18-24 hours, fixed in MEMFA and either imaged directly, or subjected to immunofluorescence as described below using anti-XStau and Alexa-647-conjugated secondary antibody (Molecular Probes). For interference assays, oocytes were injected with RNA transcribed from pSP64TSNXStauFLAG or pSP64TSNXS-tau234FLAG using the mMessage mMachine kit (Ambion) and incubated for 16 hours prior to VLE injection (as above).

Immunolocalization

Oocytes were defolliculated by treatment with collagenase as described previously (Mowry, 1996) and manually sorted into stages I-II, III-IV and V-VI (Dumont, 1972). After fixation in MEMFA for 1 hour, whole-mount immunofluorescence was performed as described previously (Sive et al., 2000). XStau antibodies were used at 1:250 dilution in PBT (1×PBS, 2 mg/ml BSA and 0.1% Triton X-100) overnight at 4°C. Oocytes were washed three times in PBT for 2-4 hours at room temperature. Goat anti-rabbit Alexa-568- or -647-conjugated secondaries (Molecular Probes) were added at 1:100 dilution in PBT overnight at 4°C. Oocytes were washed extensively as above and dehydrated in methanol. Oocytes were cleared and analyzed by confocal microscopy (Denegre et al., 1997). For colocalization analysis, oocytes were analyzed on a Leica TCS SP2 inverted confocal microscope; excitation to detect Alexa-546 labeled RNA was at 543 nm, with emission at 557-607 nm. To detect XStau immunofluorescence with Alexa-647, excitation was at 633 nm and emission was at 664-756 nm.

Subcellular fractionation

To prepare S10 lysates, total or staged oocytes were homogenized in an equal volume of XB (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1 μg/ml leupeptin, 0.1 μg/ml antipain, 0.1 μg/ml trypsin inhibitor and 0.4 mM Pefabloc). The homogenate was centrifuged twice for 10 minutes at 10,000 ***g*** to remove any insoluble material. The supernatant (50-100 μl) (S10 lysate at ~20 μg/μl protein) was applied to a 2 ml 5-40% sucrose gradient in XB with protease inhibitors. After centrifugation for 2 hours at 237,000 ***g*** at 17°C, 100 μl fractions were collected and either processed for immunoprecipitation (below) or precipitated in 10% TCA and resolved by SDS-PAGE.

Immunoprecipitation

Protein G sepharose beads (2.5 mg, Amersham) were combined with 50 μg of XStau or 30 μg of SUK4 antibody in a total volume of 500 μl of NET-150 [150 mM NaCl, 50 mM Tris (pH 8.0) and 0.05% NP-40] and mixed for 2 hours. The beads were washed four times in NET-150 and mixed with S10 lysate. After binding for 2 hours, the beads were washed four times in NET-150, and samples were boiled in the presence of SDS buffer and resolved on SDS-PAGE as above. For co-immunoprecipitation, NET-75 [75 mM NaCl, 50 mM Tris (pH 8.0) and 0.05% NP-40] was used in place of NET-150. FLAG immunoprecipitations were preformed as described previously (Kress et al., 2004) using αFLAG-conjugated beads (Sigma).

RIP assay and RT-PCR

RNP immunoprecipitation (RIP) was performed as in Niranjanakumari et al. (Niranjanakumari et al., 2002) with modifications as follows: fractions from 5-40% sucrose gradients containing the 20S Staufen complex were pooled and crosslinked in 0.1% to 0.2% formaldehyde for 15 minutes at room temperature. Reactions were quenched in 0.25 M glycine and incubated for 15 minutes. Antibodies bound to beads were mixed with 20 μg of competitor RNA for 30 minutes. XStau complexes were immunoprecipitated for 1 hour and washed four times in NET-150. Crosslinking was reversed by incubation at 70°C for 45 minutes. RT-PCR was performed according to a previously published method (LaBonne and Whitman, 1994) with minor modifications. Each sample was denatured for 10 minutes at 95°C, followed by 30 cycles (25 cycles for EF1α) of: 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. Primers used are as follows: Vg1 (forward 5′-CGATGACATCCACCCAACAC-3′, reverse 5′-GAGGGTCACAGT-CAGCAAGG-3′); VegT (forward 5′-CAAGTAAATGTGAG-AAACCGTG-3′, reverse 5′-CAAATACACACACATTTCCCGA-3′) (Zhang and King, 1996); Xcat2 (forward 5′-GGCTGCGGGTT-CTGCAGGAG-3′, reverse 5′-GCCAGTCCCCCGAGGAGCCC-3′); Xwnt11 (forward 5′-ACAAAATGCAAGTGCCACGG-3′, reverse 5′-TTGACAGCGTTCCACGATGG-3′) (Schroeder et al., 1999); and EF1α (forward 5′-CAGATTGGTGCTGGATATGC-3′, reverse 5′-ACTGCCTTGATGACTCCTAG-3′) (Wilson and Melton, 1994). The accession number for the *Xenopus* Staufen sequence is AY342402.

Results

Xenopus Staufen is expressed during oogenesis

In order to investigate potential conservation of localization mechanisms, we have identified a *Xenopus* homolog of the dsRNA-binding protein Staufen (St Johnston et al., 1992). A search of the *Xenopus* database identified an EST (GenBank Accession Number BG486325) containing a partial Staufen sequence. Using the EST sequence information, we performed both 5′ and 3′ rapid amplification of cDNA ends (RACE) from *Xenopus* oocyte cDNA to obtain the full-length coding sequence of *Xenopus* Staufen (XStau). The complete ORF translates a protein of 712 amino acids with a predicted molecular mass of 78 kDa (Fig. 1). Amino acid sequence comparison of XStau with other known Staufen homologs (Fig. 1) shows that, like *Drosophila* Staufen (St Johnston et al., 1992), XStau possesses five double-stranded RNA-binding domains. However, XStau carries an insert in dsRBD1. Similar to its mammalian homologs (Kiebler et al., 1999; Marion et al., 1999; Wickham et al., 1999), XStau also possesses a tubulin-binding domain (TBD), which was not evident in *Drosophila* Staufen (St Johnston et al., 1991). Notably, the regions of highest sequence homology were apparent in the dsRBDs and the TBD, suggesting conservation of function among the homologs. Overall, XStau shares greatest similarity with human STAU1 (Wickham et al., 1999), with 64% identity and 72% similarity in amino acid composition.

To determine whether XStau is expressed in *Xenopus* oocytes, we generated antibodies against recombinant XStau. The XStau antisera recognizes a protein of ~80 kDa (Fig. 2A,

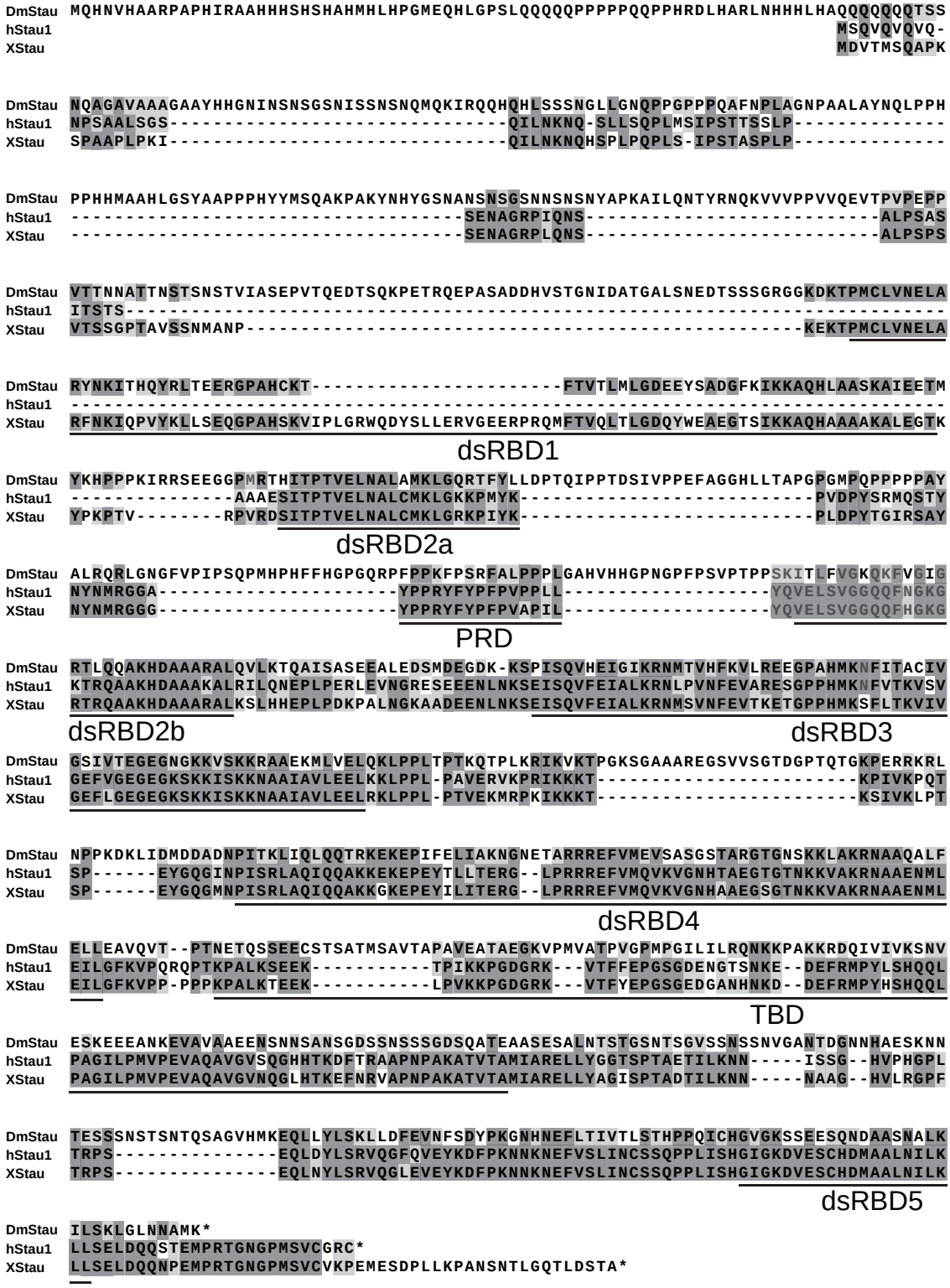

Fig. 1. Comparison of Staufen sequences. Amino acid sequence of *Xenopus* Staufen (XStau) is aligned with human Staufen isoform 1 (hStau1), and *Drosophila* Staufen (DmStau). Conserved amino acids are boxed in dark gray, and similar amino acids are in light gray; stop codons are denoted by *. Identified domains of Staufen are underlined: dsRBD denotes double-stranded RNA-binding domain, PRD denotes proline rich domain, and TBD denotes tubulin-binding domain.

lane 2) from an immunoblot analysis of total oocyte S10 lysate, which correlates well with the size predicted from the cDNA (78 kDa). To examine the expression of XStau during oogenesis, oocytes were sorted according to stage (I-VI) (Dumont, 1972) and S10 lysates were prepared. As shown in Fig. 2B, XStau is expressed throughout oogenesis, with

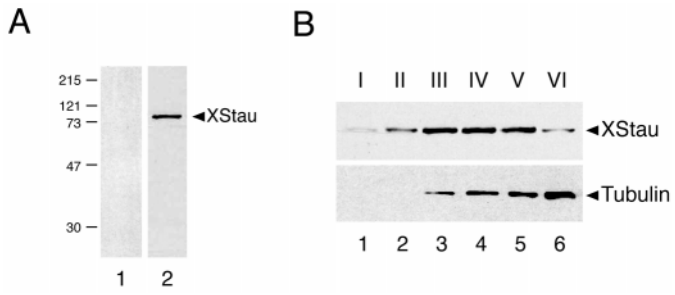

Fig. 2. XStau is expressed in *Xenopus* oocytes. (A) Total oocyte S10 lysate was immunoblotted with either preimmune sera (lane 1) or XStau antibodies (lane 2). A single band of ~80 kDa is recognized by XStau antibodies (lane 2). Molecular weight markers are indicated at the left. (B) Oocyte S10 lysates were prepared from each stage of oogenesis [I-VI (Dumont, 1972)]. One oocyte equivalent of S10 lysate per lane was resolved by SDS-PAGE and immunoblotted using XStau antibodies (above) or tubulin antibodies (below).

increasing levels through stages III-IV (mid-oogenesis) and declining in expression later in oogenesis. Only trace amounts of XStau were detectable in the insoluble (P10) fractions (data not shown). These results demonstrate that a Staufen homolog, XStau, is expressed during *Xenopus* oogenesis.

XStau is localized to the vegetal cortex during mid-oogenesis

To examine the intracellular distribution of XStau during oogenesis, we carried out immunofluorescence with early (I-II), middle (III-IV) and late (V-VI) stage oocytes (Fig. 3). XStau was not apparent in the nuclei of all oocytes examined, but the cytoplasmic XStau pattern was dynamic through oogenesis. During early oogenesis, XStau exhibits uniform cytoplasmic staining (Fig. 3A) and by the end of oogenesis (Fig. 3C), the staining is largely cortical. In stage III-IV oocytes, however, XStau is asymmetrically concentrated near the oocyte cortex (Fig. 3B). Notably, late pathway RNA localization occurs during stages III-IV, and the XStau distribution observed at those stages is reminiscent of that seen for protein factors that participate in this RNA localization pathway (Cote et al., 1999; Zhang et al., 1999). Thus, the temporal and spatial localization of XStau is intriguing and could suggest an interaction with late pathway RNAs such as Vg1.

To test whether XStau might be colocalized with Vg1 RNA, stage III oocytes were injected with fluorescently labeled Vg1 LE (VLE) RNA (Mowry and Melton, 1992). The oocytes were incubated overnight to initiate localization of the injected RNA, followed by immunofluorescence using XStau antibodies and confocal microscopy to determine localization of endogenous XStau and injected VLE RNA. Strikingly, XStau distribution in the vegetal hemisphere cytoplasm (Fig. 4A) is coincident with the injected VLE RNA (Fig. 4B). The overlapping signal is represented in white (Fig. 4C,D), and significant colocalization is evident. Control injections with β-globin RNA gave no evidence of similar colocalization (not shown). The wedge-like pattern observed for XStau in the vegetal hemisphere cytoplasm (Fig. 4A,E) is coincident with the injected VLE RNA that is undergoing localization (Fig. 4B-D), and is distinct from the distribution observed in uninjected oocytes (Fig. 4F). These results suggest that VLE RNA may recruit endogenous XStau to RNP complexes upon injection. Moreover, XStau and Vg1 are both localized to the same region of the vegetal cytoplasm, suggesting that they may interact with one another.

XStau interacts with localized RNAs

The possibility of an interaction between XStau and localized RNAs led us to investigate whether XStau might be present in an RNP complex. To analyze potential XStau complexes, oocyte S10 lysate was fractionated by sucrose gradient centrifugation. We observed two distinct XStau-containing complexes, of ~80S and ~20S (Fig. 5A). Upon treatment of the lysate with RNase A, we saw the loss of both the 80S and 20S complexes, suggesting that the complexes contain RNA (data not shown). To further examine the composition of the 80S XStau complex, the immunoblot shown in Fig. 5A (top) was re-probed with antibodies against ribosomal protein S6 (rpS6). Indeed, rpS6 co-migrates with the 80S XStau complex (Fig. 5A, bottom), and rpS6 can be immunoprecipitated with anti-XStau (not shown). These results suggest that the 80S XStau complex represents association of a population of XStau with ribosomes.

To characterize the 20S XStau-containing RNP complex, we tested for potential association of localized RNAs. For this, we used an RNP immunoprecipitation (RIP) assay (Niranjanakumari et al., 2002) to capture the RNA components of the 20S XStau RNP. After sucrose gradient centrifugation, 20S XStau-containing fractions (bracket, Fig. 5A) were pooled and subjected to RIP using XStau antibodies. RNA isolated from the immunoprecipitated complex was analyzed for the presence of specific localized RNAs by RT-PCR. As shown in Fig. 5B, Vg1 and VegT RNAs were both immunoprecipitated by anti-XStau. By contrast, RNAs localized by the early pathway, such as Xcat2 or Xwnt11, and a non-localized RNA, EF1α were not present. Similar experiments with the 80S XStau-containing fractions did not reveal enrichment for localized RNAs (not shown). These results indicate that the 20S population of XStau associates with specific localized RNAs that use the late pathway.

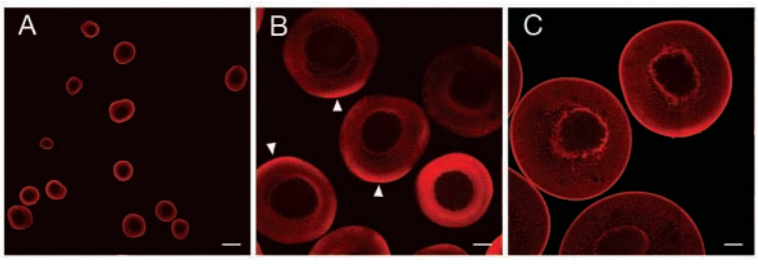

Fig. 3. Distribution of XStau during oogenesis. Immunofluorescence was performed using XStau antibodies and Alexa-568 conjugated secondary antibody to detect the distribution of XStau in oocytes of the following stages: (A) stages I-II, (B) stages III-IV and (C) stages V-VI (Dumont, 1972). Confocal images are shown. Scale bars: 100 μm. Arrowheads show enrichment of XStau in the vegetal cortex of stage III-V oocytes.

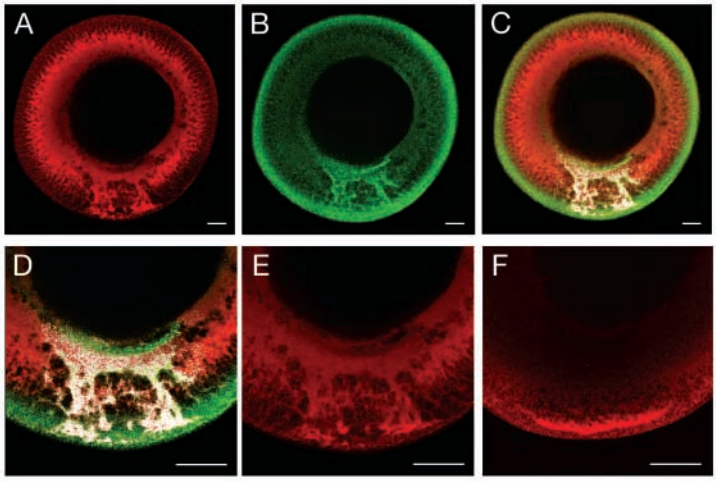

Fig. 4. XStau and Vg1 RNA are colocalized in *Xenopus* oocytes. (A-D) Stage III-IV oocytes were microinjected with Alexa-546 labeled VLE RNA, followed by immunofluorescence using XStau antibodies and Alexa-647 secondary antibodies. (A) Localization of endogenous XStau, shown in red. (B) Injected VLE RNA, shown in green. (C) Colocalization of endogenous XStau and injected VLE RNA, digitally represented in white. (D) High-magnification image of the oocyte shown in C. (E) High-magnification image of the oocyte shown in A. (F) Distribution of XStau in vegetal hemisphere of an uninjected stage III oocyte, determined by immunofluorescence using XStau antibodies and Alexa-568-conjugated secondary antibodies. For all panels, representative confocal sections are shown. Scale bars: 20 μm.

XStau is associated with a kinesin motor

The presence of localized RNAs in the 20S XStau-containing complex suggested that this RNP particle might be involved in RNA localization. To investigate potential roles for motor proteins in late pathway vegetal localization, we further defined the composition of the 20S XStau/Vg1 RNP by immunoblotting gradient fractions for XStau, and for kinesin and dyein motor proteins. As shown in Fig. 6A, XStau (top) co-sediments with kinesin (middle), while dynein (bottom) migrates elsewhere in the gradient. These results suggest a possible association between Staufen and kinesin, and to investigate this interaction more directly, we tested whether XStau and kinesin could be co-immunoprecipitated. We first immunoprecipitated 20S XStau RNP-containing fractions with anti-XStau and immunoblotted for kinesin (Fig. 6B, lane 2; top, SUK4). Indeed, a kinesin protein is co-immunoprecipitated with XStau. Similarly, we immunoprecipitated with sea urchin kinesin antibodies (SUK4), and immunoblotted with anti-XStau (Fig. 6B, lane 4; bottom), and found that XStau is also co-immunoprecipitated with kinesin. The SUK4 antibodies recognize conventional kinesin heavy chain (Ingold et al., 1988; Neighbors et al., 1988; Sawin et al., 1992), suggesting that the XStau-associated kinesin is kinesin I. These results indicate that XStau is associated with a kinesin motor, which may provide a mechanism for active transport of late pathway RNAs.

XStau functions in vegetal RNA localization in *Xenopus* oocytes

To further address potential function of XStau in vegetal RNA localization, we constructed a mutant version of XStau and tested whether it could dominantly inhibit localization of VLE RNA in vivo. Previous analysis of *Drosophila* Staufen had shown dsRBD3 and 4 to have the highest affinity for dsRNA in vitro (Micklem et al., 2000). We reasoned that a mutant XStau containing only dsRBDs 2-4 (Fig. 7A) could potentially bind to VLE RNA but not interact productively with the localization machinery. We injected stage III oocytes with FLAG-tagged RNA transcripts encoding either full-length XStau (WT) or XStau234 (DN). After overnight incubation, injected DN (Fig. 7B, lane 1) and full-length (lane 2, WT) XStau were highly expressed, at levels comparable with endogenous XStau (lane 3). To test for effects on RNA localization (Fig. 7C), we injected fluorescently labeled VLE into oocytes expressing full-length (left, WT) and DN (right) XStau. Vegetal localization of VLE RNA is typified by a wedge-like structure in the vegetal cytoplasm, featured by a sharp cortical crescent staining and finger-like projections that appear as large granules; this was the predominant phenotype exhibited by oocytes expressing wild-type XStau (Fig. 7C, WT). By contrast, most oocytes expressing DN XStau showed no detectable VLE RNA localization (Fig. 7C, DN). The results of localization assays for oocytes expressing full-length and DN XStau are quantified in Fig. 7D: oocytes scored as normal (green) showed a distinct wedge, the presence of a weak cortical or vegetal signal was scored as weak (yellow), and absence of detectable asymmetry was scored as none (red).

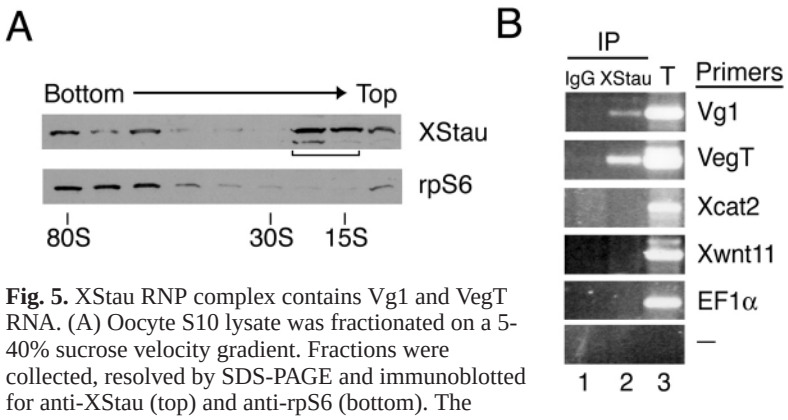

Fig. 5. XStau RNP complex contains Vg1 and VegT RNA. (A) Oocyte S10 lysate was fractionated on a 5-40% sucrose velocity gradient. Fractions were collected, resolved by SDS-PAGE and immunoblotted for anti-XStau (top) and anti-rpS6 (bottom). The bracket denotes the ~20S XStau RNP population, and the positions of size markers are indicated at the bottom. (B) Gradient fractions containing the 20S XStau complex (A, bracket) were pooled, formaldehyde crosslinked and immunoprecipitated with XStau antibodies. The resulting RNA was amplified by RT-PCR (lanes 2, XStau) with specific primer sets for Vg1, VegT, Xcat2, Xwnt11 and EF1α. A control non-relevant antibody was used to determine background interactions (lanes 1, IgG), and input control for each primer set was determined from total oocyte RNA (lanes 3, T).

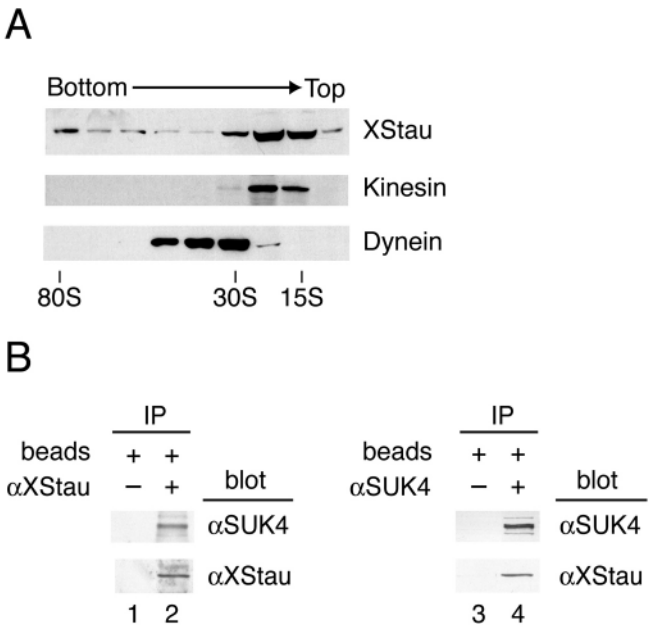

Fig. 6. XStau associates with a kinesin motor. (A) Oocyte S10 lysate was fractionated on a 5-40% sucrose gradient. Fractions were collected, resolved by SDS-PAGE and immunoblotted with XStau (top), kinesin (middle), and dynein (bottom) antibodies. The positions of size markers are indicated at the bottom. (B) Immunoprecipitation was carried out using oocyte S10 lysate with beads only (lanes 1, 3), anti-XStau (lane 2) or SUK4 (kinesin) antibodies (lane 4). After SDS-PAGE, the immunoprecipitates were immunoblotted with either SUK4 (top) or XStau (bottom) antibodies.

VLE localization was robust in the presence of exogenous full-length XStau (WT); 66% localized strongly (normal) and 34% showed either weak or no localization, whereas the localization phenotype most prevalent in oocytes expressing DN XStau was weak (32%) or none (52%). Inhibition of VLE RNA localization in DN XStau injected oocytes was most pronounced 6-8 hours after the VLE injection, with greater than 80% of the oocytes showing defects (weak and none) in localization phenotype. These results reveal a functional role for XStau in RNA localization in *Xenopus* oocytes.

To further characterize the nature of the dominant-negative phenotype, we asked whether XStau234 was impaired in its ability to interact with other localization factors. We have previously shown that XStau interacts with hnRNP I and Vg1RBP/vera in a cytoplasmic RNP (Kress et al., 2004), and we compared the ability of XStau234 (DN) and full-length XStau (wild type) to interact with these RNA binding proteins. We prepared S10 lysates from oocytes expressing either DN or wild-type XStau, immunoprecipitated with anti-FLAG and tested for the presence of hnRNP I or Vg1RBP/vera in the complexes by immunoblotting with anti-hnRNPI and anti-Vg1RBP/vera, respectively. As shown in Fig. 7E, full-length XStau (lane 2, WT) interacts with both Vg1RBP/vera (top) and hnRNP I (bottom); and XStau234 (DN, lane 1) interacts with Vg1RBP/vera. However, interaction with hnRNP I (bottom) is

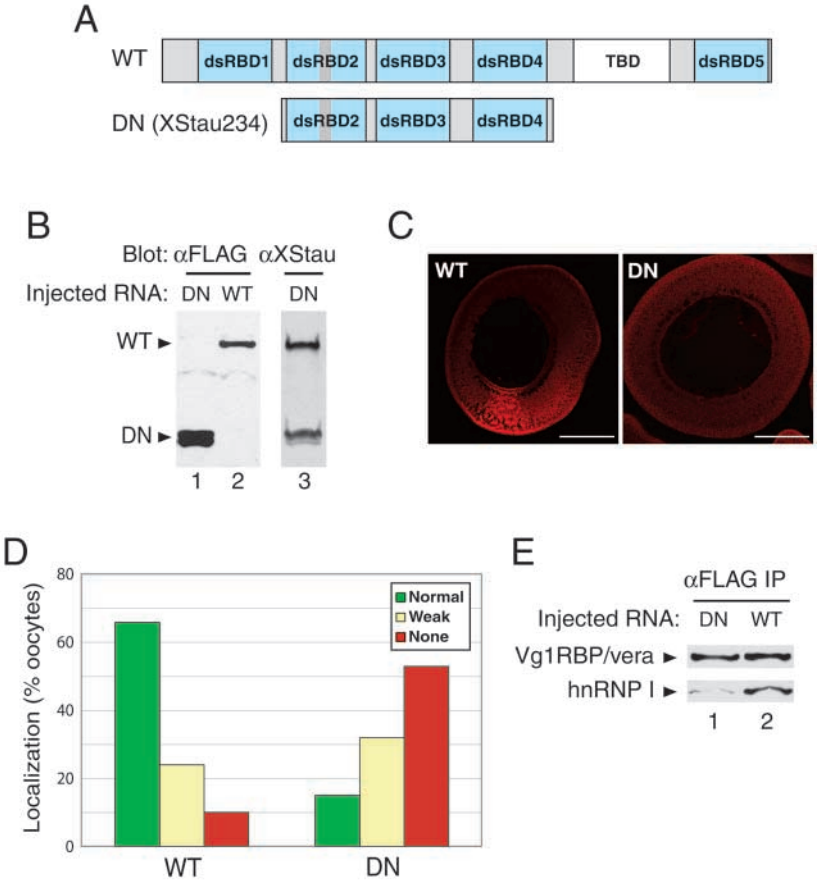

Fig. 7. Dominant-negative XStau blocks VLE localization in vivo. (A) Full-length XStau (WT, above) and XStau234 (DN, below) are shown. The double-stranded RNA binding domains (dsRBDs) are shown in blue and are numbered, and the tubulin-binding domain (TBD) is shown in white. (B) RNA encoding either XStau234 (DN, lanes 1, 3) or full-length XStau (WT, lane 2) was injected into stage III oocytes, and S10 lysates were prepared after 16 hour incubation. Expression of injected RNA was analyzed by immunoblotting with FLAG antibodies (lanes 1, 2); immunoblotting with XStau antibodies (lane 3) was used to compare expression of XStau234 (DN, below) to that of endogenous XStau (above). (C) Fluorescently labeled VLE RNA was injected into oocytes expressing either full-length XStau (WT, left) or XStau234 (DN, right). Examples are shown of the predominant phenotypes observed for oocytes expressing full-length XStau (WT), showing localization of VLE RNA (red) and XStau234 (DN), exhibiting no detectable localization. Scale bars: 100 μm. (D) Localization was scored for VLE-injected oocytes expressing either WT XStau (left, *n*=151) or DN XStau (right, *n*=117). The graph shows the percentage of oocytes that exhibited strong vegetal localization (green), weak vegetal localization (yellow) or no detectable localization (red). (E) S10 lysates were prepared from oocytes expressing either XStau234 (DN, lane 1) or full-length XStau (WT, lane 2), and immunoprecipitation was performed using FLAG antibodies. Bound proteins were separated by SDS-PAGE and immunoblotted with Vg1RBP/vera antibodies (above) and hnRNP I antibodies (below).

significantly impaired for XStau234 (Fig. 7E, lane 1). These data suggest that one cause of the observed dominant-negative effect on VLE RNA localization could be a failure of XStau234 to interact productively with specific components of the localization machinery.

Discussion

Developmental polarity can be achieved by localization of maternal determinants in the form of mRNA. In recent years, much progress has been made toward understanding the molecular mechanisms underlying the process of RNA localization through studies in a variety of systems. It has become clear that localized RNAs carry localization signals that interact with trans-acting factors to direct localization. Many of these factors have been identified (reviewed by Jansen, 2001), but few are conserved among the many organisms studied. A lack of conservation of RNA transport machineries could suggest that mechanistic strategies for RNA localization vary among different organisms. However, one localization factor does appear to be conserved among different organisms; Staufen may be the first RNA-binding protein to provide a mechanistic link between invertebrates and vertebrates in RNA localization.

In this study, we have identified a *Xenopus* homolog of Staufen (XStau) and provide evidence of a role in RNA localization during frog oogenesis. We found that XStau is present in an RNP complex and associates with localized RNAs and a kinesin motor. An interaction between Vg1 and XStau is demonstrated by the findings that XStau is both colocalized with Vg1 RNA and co-immunoprecipitated with Vg1 RNA (Figs 4, 5). Specificity of the biochemical interaction is indicated as RNAs (Vg1 and VegT) transported by the late pathway in *Xenopus* oocytes are co-immunoprecipitated with XStau, while RNAs that localize through a different pathway (the METRO pathway) are not (Fig. 5). The subcellular distribution of XStau is also provocative; enrichment of XStau at the vegetal cortex is coincident with the timing of late pathway RNA localization (Fig. 3B). Strikingly, upon injection of VLE RNA into stage III oocytes, XStau adopts a distribution that is overlapping with the injected RNA undergoing localization to the vegetal cortex. We interpret colocalization of XStau and VLE RNA as resulting from recruitment of endogenous XStau to active localization complexes. More importantly, we obtained functional evidence of a role for XStau in vegetal RNA localization through in vivo interference experiments (Fig. 7). We found that expression of a dominant-negative (DN) version of XStau blocked VLE RNA localization. The DN XStau contained dsRBDs 2-4 (Fig. 7A), and dsRBDs 3 and 4 are predicted to bind RNA most strongly (Micklem et al., 2000). Thus, the dominant effect of this protein may be due to binding of VLE RNA by DN XStau, which may be defective in interaction with other components of the RNA localization machinery. Indeed, we have found that XStau234 exhibits impaired interaction with hnRNP I (Fig. 7F), a late pathway localization factor (Cote et al., 1999). Our results provide evidence for interaction of XStau with late pathway RNAs both in vitro and during localization in vivo, and reveal a role for XStau in vegetal RNA localization in *Xenopus* oocytes.

Motor proteins have been implicated in transport of late pathway RNAs because of the requirement for intact microtubules (Yisraeli et al., 1990). However, the nature of any such motors had remained elusive. Our results have revealed that XStau is associated with kinesin I (Fig. 6), and kinesin II has recently been implicated with a role in Vg1 RNA localization (Betley et al., 2004). Certainly, roles for kinesin I and kinesin II in vegetal RNA localization are not mutually exclusive; each motor could have distinct functions in RNA localization. Multiple kinesin-like proteins are expressed in *Xenopus* (Boleti et al., 1996; Houliston et al., 1994; Le Bot et al., 1998; Tuma et al., 1998; Vernos et al., 1993; Vernos et al., 1995), and we have identified the XStau-associated kinesin as kinesin I based both on its size (~120 kDa) and recognition by kinesin I-specific antibodies. The antibodies we used to detect the XStau-associated kinesin, anti-sea urchin SUK4, are specific for kinesin I (Ingold et al., 1988), and have been shown to recognize a kinesin I doublet of ~120 kDa in *Xenopus* extracts (Neighbors et al., 1988; Sawin et al., 1992). In addition, a second kinesin I-specific antibody (Romberg et al., 1998) recognized the XStau-associated kinesin as well (not shown). Based on these findings, we propose that XStau interacts with kinesin I. In rat hippocampal neurons, Staufen has been shown to co-sediment with kinesin I in gradients (Mallardo et al., 2003), and kinesin I has also been linked to Staufen through genetic studies in *Drosophila*. In the latter studies, mislocalization of Staufen protein and *oskar* RNA resulted from deletion of kinesin heavy chain (Brendza et al., 2000). Our biochemical results indicate that XStau and kinesin I interact within an RNP complex that also contains vegetally localized RNAs. We propose that interactions between Staufen and kinesin motors may be a conserved theme in RNA localization pathways.

We have suggested that Staufen is a conserved feature in RNA localization; paradoxically, however, the RNA targets appear rather unrelated. In flies, Staufen has been suggested to interact with *bicoid*, *oskar* and *prospero* RNAs (Broadus et al., 1998; Ephrussi et al., 1991; Ferrandon et al., 1994; Kim-Ha et al., 1991; Li et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998; St Johnston et al., 1991). In *Xenopus*, we have provided evidence of association with the late pathway RNAs Vg1 and VegT. One cause for the range of RNAs recognized by Staufen probably lies in the nature of the interaction between dsRBDs and dsRNA, which is generally non-sequence specific (St Johnston et al., 1992). Vg1 and VegT contain potentially double-stranded regions, but they are specifically bound by XStau in vivo (Fig. 5B). So the question remains as to how Staufen could interact specifically with disparate RNA targets. We propose that there are two classes of RNA-binding factors involved in RNA localization. One class recognizes and binds to RNA localization elements in a sequence-specific manner. Examples of such factors in *Xenopus* include Vg1 RNA-binding proteins hnRNP I (Cote et al., 1999) and Vg1RBP/vera (Deshler et al., 1998; Deshler et al., 1997; Havin et al., 1998; Schwartz et al., 1992). This class of factors may be cell-type specific and act to establish a core ribonucleoprotein complex for transport. The other class of factors, such as XStau, may act not at the level of sequence-specific RNA recognition, but rather, recognize the core RNP complex and mediate the interaction with the localization machinery. In such a model, some dsRBDs would interact in a non-sequence specific manner with double-stranded regions of RNA presented on the RNP, while other dsRBDs could

interact with protein components of the core RNP. Consistent with this idea, dsRBD2 and dsRBD5 of *Drosophila* Staufen do not bind RNA in vitro, whereas dsRBD1, dsRBD3 and dsRBD4 bind dsRNA sequence nonspecifically (Micklem et al., 2000; St Johnston et al., 1992). We have shown (Fig. 7) that dominant-negative XStau234 is defective in interaction with hnRNP I, suggesting that XStau dsRBD1 or dsRBD5 could potentially facilitate interaction between XStau and hnRNP I. We suggest that this interaction is in the context of an RNP, and we have previously shown that hnRNP I and Vg1RBP/vera associate with Vg1 and VegT RNAs in the nucleus, prior to recruitment of XStau to the cytoplasmic RNP (Kress et al., 2004). Our observed biochemical interaction between XStau and kinesin (Fig. 6) could further suggest a role for XStau in motor recruitment, although this remains an issue for future investigation. Thus, Staufen may represent a central component of the RNA localization machinery, perhaps linking the localized RNP cargoes with the motors that move them.

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