Barentsz is essential for the posterior localization of oskar mRNA and colocalizes with it to the posterior pole

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Introduction

The establishment of cellular asymmetries and cell polarity requires the targeting of proteins to specific cellular regions, and this is often achieved by localizing the corresponding mRNAs (St Johnston, 1995). For example, the polarized movement of fibroblasts requires the localization of β-actin mRNA, whereas Ash1 mRNA localization to the bud tip in Saccharomyces cerevisiae ensures that only the mother cell can switch mating type (Kislauskis et al., 1994; Long et al., 1997; Takizawa et al., 1997). In many organisms, mRNA localization plays an important role in axis formation through the targeting of cytoplasmic determinants to particular regions of the egg (Bashirullah et al., 1998). For example, in Drosophila the localization of oskar mRNA to the posterior pole of the oocyte defines where the pole plasm forms and thus where the abdomen and germ line will develop, whereas the localization of bicoid mRNA to the anterior specifies where the head and thorax form (Ephrussi and Lehmann, 1992; Driever, 1993; Lasko, 1999).

Although several mechanisms can target transcripts to a particular region of the cell, this is often thought to involve active transport along the cytoskeleton. This has been most clearly demonstrated in the case of Ash1 mRNA, which is transported to the bud tip along actin filaments (Bertrand et al., 1998; Beach et al., 1999). Ash1 mRNA is recognized by She2p, which then links it via the adaptor protein She3p to the myosin motor Myo4p, which moves this RNA–protein complex along actin cables (Bohl et al., 2000; Takizawa and Vale, 2000). Much less is known about mRNA localization in higher eukaryotes, but drug inhibitor studies have implicated the cytoskeleton in the localization of several mRNAs. For example, the localization of β-actin RNA in fibroblasts is disrupted by actin-destabilizing drugs, whereas many other transcripts are localized in a microtubule-dependent manner including bicoid and oskar mRNAs in Drosophila (Clark et al., 1994; Pokrywka and Stephenson, 1995). However, it still remains to be proven that any of these RNAs are localized by active transport, and almost nothing is known about how they are coupled to the motors that are presumed to transport them.

One case which is very likely to involve active transport along microtubules is the localization of oskar mRNA to the posterior of the Drosophila oocyte. During stage 9 of oogenesis, oskar mRNA accumulates transiently at the anterior of...
the oocyte before moving to form a crescent at the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). This anterior to posterior movement is disrupted by colchicine treatments, indicating that it requires an intact microtubule cytoskeleton (Clark et al., 1994). Furthermore, the site of oskar RNA localization correlates with the arrangement of the microtubules in the oocyte. During stages 7–9 of oogenesis, the oocyte microtubule cytoskeleton is reorganized in response to a polarizing signal from the posterior follicle cells, and a diffuse microtubule-organizing center at the anterior pole nucleates an anterior to posterior gradient of microtubules (Ruohola et al., 1991; Theurkauf et al., 1992). The polarity of these microtubules has been analyzed by expressing fusion proteins that contain microtubule motor domains: the plus end–directed motor kinesin fused to β-galactosidase has been observed to localize to the posterior of the oocyte at exactly the same stage as oskar mRNA, whereas a nod–βGal fusion localizes to the anterior (Clark et al., 1994, 1997). Thus, the minus ends of the microtubules seem to lie at the anterior of the oocyte with the plus ends extending towards the posterior pole. Furthermore, oskar mRNA still colocalizes with the plus ends of the microtubules in mutants that disrupt the polarity of the oocyte. For instance, oskar mRNA localizes to the center of the oocyte in gurken and PKA mutants, which led to the formation of a symmetric microtubule cytoskeleton with minus ends at both poles and the plus ends in the middle (González-Reyes et al., 1995; Lane and Kalderon, 1995; Roth et al., 1995). Finally, it has been shown recently that mutants in the heavy chain of kinesin I block the posterior localization of oskar mRNA and cause it to accumulate instead at the anterior of the oocyte (Brendza et al., 2000). Since these mutants do not appear to affect the organization of the microtubules, this motor seems to be directly involved in localizing oskar mRNA. It is therefore very attractive to propose that kinesin I transports oskar mRNA along microtubules from the anterior towards the plus ends at the posterior pole.

Besides the kinesin heavy chain, mutants in staufen, mago nashi, and tropomyosin II (TmII) have also been found to specifically block the posterior localization of oskar mRNA without affecting the organization of the microtubules (Ephrussi et al., 1991; Kim-Ha et al., 1991; Newmark and Boswell, 1994; Erdélyi et al., 1995; Tetzlaff et al., 1996). Staufen contains five copies of the dsRNA-binding domain and colocalizes with oskar mRNA throughout oogenesis in wild-type egg chambers and in all mutants that have been examined so far (St Johnston et al., 1991, 1992). Furthermore, the posterior localizations of Staufen and oskar mRNA are mutually dependent and require the RNA-binding activity of Staufen protein (Ferrandon et al., 1994; Ramos et al., 2000). Thus, it seems probable that Staufen binds directly to oskar mRNA and is required in some way to couple this complex to the translocation machinery. It is very important that Oskar protein is only produced at the posterior of the oocyte, and the translation of unlocalized oskar mRNA is therefore repressed through the binding of factors, such as Bruno protein, to sites within the 5′ and 3′ untranslated regions (UTRs)* (Kim-Ha et al., 1995; Gunkel et al., 1998). This repression must be relieved once oskar has been localized, and Staufen, which remains associated with the RNA at the posterior pole, performs a second function in the activation of oskar translation (Micklem et al., 2000).

In the case of Mago nashi and TmII, it has not yet been possible to establish a clear link between the molecular nature of the proteins and their effects on the localization of oskar mRNA. Mago is a mainly nuclear protein that has been extremely highly conserved during evolution and plays several roles during oogenesis (Newmark and Boswell, 1994; Micklem et al., 1997; Newmark et al., 1997). In the weakest mutant combinations, oskar mRNA remains at the anterior of the oocyte and never reaches the posterior pole. This phenotype is not due to the defect in microtubule organization, suggesting that Mago also has specific function in oskar mRNA localization (Micklem et al., 1997). Consistent with this, small amounts of green fluorescent protein (GFP)-Mago colocalize with oskar mRNA at the posterior pole at stage 9 (Newmark et al., 1997). oskar mRNA also fails to localize to the posterior in TmI<sup>P</sup> homozygotes. This phenotype has been difficult to explain because TmII encodes a cytoplasmic tropomyosin that is known to function in the regulation of the actin rather than microtubule cytoskeleton. This analysis is further complicated by the fact that the TmI<sup>P</sup> mutation that disrupts oskar mRNA localization is not a null allele, but its exact effect on tropomyosin expression is not clear (Erdélyi et al., 1995).

Here, we report the phenotypic and molecular characterization of a novel locus called barentsz, which is specifically required for the movement of oskar mRNA from the anterior to the posterior of the oocyte and analyze the behavior of Barentz protein during oogenesis. These results suggest that Barentsz is an essential and specific component of the oskar mRNA localization complex.

Results

Barentsz is required for the posterior localization of oskar mRNA

The initial allele of barentsz (btz<sup>1</sup>) was identified as a second hit on a female sterile chromosome in a screen of the Tübingen stock collection for mutants with defects in localization of Staufen protein. In most btz<sup>1</sup> homozygous egg chambers at 18°C, both Staufen protein and oskar mRNA fail to localize to the posterior of the oocyte at stage 9 and remain instead at the anterior until the beginning of stage 10B. However, occasionally small amounts of oskar mRNA localize to the posterior pole at stage 9 and are anchored there throughout oogenesis (Fig. 1, A–D). Based on this phenotype where the RNA becomes trapped at the anterior and fails to reach the posterior pole, we named the gene after Willem Barentsz, a seventeenth-century explorer whose boat became frozen in the ice off Novaia Zemlya during an attempt to find a passage past the North pole. btz<sup>1</sup> seems to be a cold-sensitive partial loss-of-function mutation, since the phenotype is stronger over Df(3R)IR16 (Table I), a deletion for the locus, but weaker at higher temperatures: at 25°C, many btz<sup>1</sup>/Df(3R)IR16 egg chambers have some oskar mRNA at the posterior pole, although this is always much less than wildtype.

*Abbreviations used in this paper: GFP, green fluorescent protein; RFLP, restriction fragment length polymorphism; UTR, untranslated region.
We have subsequently isolated further alleles of \textit{barentsz} as described below, and one of these, \textit{btz}^2, appears to be a null mutation in the locus by molecular criteria. In mutants for other genes involved in \textit{oskar} mRNA localization, such as \textit{Tropomyosin II}, a small amount of RNA is often detectable at the posterior pole. Although it has not been reported previously, this is also the case for null mutants in \textit{staufen}, where traces of \textit{oskar} mRNA can be seen at the posterior pole at stage 9 and remain anchored at stage 10. A small amount of \textit{oskar} mRNA also localizes to the posterior at stage 9 in a \textit{staufen-null} allele, \textit{stauD3}, but the RNA is not anchored at the posterior and disappears by stage 10. Antibody staining for Oskar protein in wild-type stage 10B ovaries (I and J). Oskar protein is never detected at the posterior of \textit{barentsz} mutant oocytes.

Table 1. \textit{barentsz} posterior patterning and \textit{oskar} mRNA localization defects

|        | \textit{btz}^2 | \textit{btz}^2 (18°C) | \textit{btz}^2/DF(3R)IR16 (18°C) |
|--------|---------------|----------------------|----------------------------------|
| Abdominal defects | 53 | 1 | 34 |
| No pole cells | 100 | 65 | 100 |
| oskar mRNA mislocalized | 100^a | 68^b | 100^a |

At least 60 samples analyzed in each case.

^a\textit{oskar} mRNA is never detected at the posterior.

^bThis figure includes egg chambers that show a similar or stronger defect in \textit{oskar} mRNA localization to that shown in Fig. 1 C.

Figure 1. \textit{barentsz} is required for the localization of \textit{oskar} mRNA and protein. (A–H) Localization of \textit{oskar} mRNA at stage 9 (left) and stage 10 (right) of oogenesis in wild-type (A and B), \textit{btz}^1 (C and D), \textit{btz}^2/DF(3R)IR16 (E and F), and \textit{stauD3} ovaries (G and H). In wild-type egg chambers, \textit{oskar} mRNA localizes to the posterior pole of the oocyte during stage 9 and remains anchored there throughout oogenesis. This localization is completely abolished in ovaries that are mutant for a null of \textit{barentsz}, \textit{btz}^2, and \textit{oskar} mRNA is only detected at the anterior pole, even when the in situ is overstained. In the weak allele \textit{btz}^1, some \textit{oskar} mRNA is localized to the posterior at stage 9 and remains anchored at stage 10. In contrast, \textit{btz}^2 homozygotes or \textit{btz}^2/DF(3R)IR16 females show a completely penetrant \textit{oskar} mRNA localization phenotype at all temperatures. \textit{oskar} mRNA is transported from the nurse cells into the oocyte as in wild-type, but the RNA is never detected at the posterior pole at stage 9 or later and persists instead at the anterior of the oocyte (Fig. 1, E and F). Consistent with this, Oskar protein is never visible at the posterior of \textit{barentsz-null} mutant oocytes (Fig. 1, I and J). \textit{oskar} mRNA shows a transient localization at the anterior of \textit{wild-type} oocytes before it localizes to the posterior pole, and the persistent anterior localization seen in \textit{barentsz} mutants suggests that Barentsz is specifically required for the transport of \textit{oskar} mRNA from the anterior to the posterior of the oocyte.

The posterior localization of \textit{oskar} mRNA and protein defines where the abdominal and germline determinants are localized, and \textit{staufen} mutant females therefore give rise to embryos that lack both the abdomen and the pole cells. Surprisingly, the embryos laid by \textit{barentsz} mutant females show much milder phenotypes. Embryos from \textit{btz}^2 homozygous females lack pole cells but develop an average of 5.4 ± 3.1 denticle belts. Furthermore, embryos from \textit{btz}^2/DF(3R)IR16 flies form a very similar number of 4.8 ± 3.1 denticle belts (\(n = 43\)), indicating that \textit{btz}^2 is a null mutation or a very strong hypomorph (Table I). The embryonic phenotype of \textit{btz}^1 is even weaker. At 18°C, almost all embryos lack pole cells (Fig. 2, C and E, and Table I) but have a normal number of denticle belts, but most embryos form a few pole cells.
and develop into viable and fertile adults if the mothers are kept at 25°C. As a consequence, the reduced number of pole cells, about a third of the adult female progeny, have only one gametic ovary, presumably because there were too few germ cells to populate both gonads.

**Barentsz** is not required for the polarization of the microtubule cytoskeleton

Mislocalization of oskar mRNA can result from earlier defects in the patterning of the follicular epithelium, signaling from the posterior follicle cells to polarize the anterior–posterior axis of the oocyte or the organization of the oocyte microtubule cytoskeleton, and we therefore examined whether any of these upstream events are disrupted in barentsz mutants. Firstly, **barentsz** is not required for cell fate determination in the follicle cell layer, since the enhancer trap lines slbo, L53b, and A62-GAL4, which mark specific populations of anterior or posterior follicle cells, show wild-type expression patterns in a btz1 mutant background (unpublished data). Consistent with this, follicle cell clones of **barentsz** have no phenotype, whereas germline clones produce a fully penetrant oskar mRNA localization defect. Second, unlike mutants that affect the signaling pathway from the posterior follicle cells to the oocyte, **barentsz** mutants do not disrupt the anterior localization of bicoid mRNA or the positioning of the oocyte nucleus and **gerken** mRNA at the dorsal/anterior corner of the oocyte. Finally, we examined the organization of the microtubule cytoskeleton by looking at the localization of tau-GFP to label the microtubules directly, and kinesin-βGal to mark where the plus ends of the microtubules are concentrated (Clark et al., 1994; Micklem et al., 1997). In both btz1 and btz2 mutant ovaries, the arrangement of the microtubules appears normal, and kinesin-βGal localizes to the posterior of the oocyte at stage 9 as it does in wildtype (Fig. 2, A and B). Thus, **barentsz** is required for the microtubule-dependent transport of oskar mRNA to the posterior pole but not for the polarization of the microtubule cytoskeleton itself.

Given the role of **barentsz** in oskar mRNA localization, we wondered whether it might also be involved in the assembly of the pole plasm once Oskar protein is translated at the posterior. To test this possibility, we took advantage of an osk-bcd 3′UTR transgene in which the bicoid 3′UTR directs the anterior localization and translation of Oskar protein (Ephrussi and Lehmann, 1992). In a wild-type background, the ectopic Oskar produced from the transgene directs the formation of anterior pole plasm and pole cells, whereas normal pole cells develop at the posterior under the control of the endogenous oskar mRNA (Fig. 2, C and D). When this transgene is crossed into btz1 homozygotes at 18°C, the anterior pole cells still form, although the posterior ones are missing because oskar mRNA is not localized to the posterior (Fig. 2, E and F). Although btz1 is not completely null at the restrictive temperature, this result strongly suggests that **barentsz** is also not required for any events in pole plasm assembly downstream of Oskar protein.

**Cloning of barentsz**

The analysis of the **barentsz** phenotype indicated that the gene has a unique and specific role in oskar mRNA localization, and we therefore decided to characterize it molecularly. Initial mapping placed **barentsz** at 98A-B on the right arm of the third chromosome in the region uncovered by the deficiency Df(3R)IR16 but not by other deletions in the area.

To identify the **barentsz** gene in this interval, we employed a novel positional cloning strategy that uses high resolution meiotic mapping between two nearby P element insertions. We first used P-mediated male recombination to map **ba**
rentsz between PlacW 318-07 in 98A6 and PlacW 430-05 in 98B. We then generated btz1 PlacW430/PlacW318 females and used the miniwhite eye color phenotypes of the P elements to select for progeny in which recombination had occurred between them. Out of ~16,000 flies, we recovered 203 lines in which recombination had occurred between the two P elements, corresponding to approximately one recombination event per 2 Kb (Fig. 3). At the same time, we extended two molecular walks into this region and identified restriction fragment length polymorphisms (RFLPs) between the two chromosomes. These enabled us to map btz1 between two RFLPs that define a 10.7-kb interval containing three genes, CG12876, CG12878, and carotine octanyl transferase (Berkeley Drosophila Genome Project; http://www.fruitfly.org) (Fig. 3 B).

To determine which of these genes is barentsz, we generated a transformation construct that contains a genomic DNA fragment, spanning CG12876 and CG12878. Since this construct completely rescues all of the btz1 mutant phenotypes, we then made two further constructs in which one or the other of these genes is mutated. Only the construct in which CG12878 is intact rescues the barentsz phenotype, identifying this as the barentsz gene. Furthermore, a transformation construct that contains the coding region of barentsz fused to the GFP (BtzGFP) also rescues all of the btz1 mutant phenotypes. The exact molecular nature of btz1 has not been determined; the coding sequence of barentsz is not affected, but PCR analysis of homozygous btz1 mutants suggests that an insertion has occurred in the 3’UTR or immediately downstream of the transcription unit. This leads to reduced expression of barentsz RNA and protein as determined by in situ and Western blot analysis (unpublished data). We also found that the P-element P[PZ][3]rL203 is inserted in the 5’UTR of btz but does not cause a phenotype, and we therefore used imprecise excision of this P element to generate new barentsz alleles. One of these, btz2, is very likely to be a null mutation in the gene, since the first 1,221 nt of the transcription unit are deleted, but the expression of the adjacent transcription unit CG12876 is unaffected as determined by whole-mount in situ analysis (Fig. 3 B). This deletion removes the NH2-terminal 332 amino acids of the barentsz-coding region, including the conserved domains described below. Homo- or hemizygous btz2 mutants are mostly pharate lethal, and the few adults that eclose die within a few days. Thus, barentsz seems to have a zygotic function in addition to its maternal role in oskar mRNA localization.

The sequence of barentsz was obtained from the Berkeley Drosophila Genome Project database (http://www.fruitfly.org) and was confirmed by direct sequencing of the longest EST. barentsz encodes a very hydrophilic protein with a predicted molecular weight of 83.7 kD and a pl of 5.2 but contains no known protein motifs. However, a region of 184 amino acids near its NH2 terminus shows homology to three other known protein motifs. However, a region of 184 amino acids near its NH2 terminus shows homology to three other known protein motifs. However, a region of 184 amino acids near its NH2 terminus shows homology to three other known protein motifs.

To investigate the function of Barentsz in oskar mRNA localization, we raised a polyclonal antiserum in rabbits against the NH2 terminus of the protein. On Western blots of wild-type ovary extracts, affinity purified antibody recognizes a single 125-kD band that is absent from btz1/ Df(3R)IR16 extracts, confirming that it is Barentsz protein (Fig. 3 D). The antibody also specifically recognizes Barentsz in whole-mount stainings of ovaries, since all of the specific staining patterns described below are absent in btz1/Df(3R)IR16 ovaries and wild-type ovaries stained with pre-immune serum.

Barentsz is expressed very early in oogenesis and localizes to the presumptive oocyte as soon as it can be identified in region 2B of the germarium (Fig. 4 A). The protein then concentrates at the posterior of the oocyte between the nucleus and the posterior follicle cells during stages 1–6 of oogenesis, before becoming dispersed throughout the oocyte cytoplasm at stage 8 (Fig. 4, B–D). In addition, Barentsz shows a punctate staining of the nuclear membrane in all cells of the egg chamber except the oocyte itself, suggesting an association with nuclear pores (Fig. 4 F). The localization of Barentsz within the oocyte correlates with the behavior of the oocyte microtubule-organizing center, which moves to the posterior at around stage 1 and persists until stage 7 and is identical to that observed for oskar mRNA. However, these localizations appear to be independent of each other, since barentsz mutants have no effect on the early localization of oskar mRNA.

Given its role in the transport of oskar mRNA, the most significant aspect of the Barentsz distribution during oogenesis is that it localizes to the posterior of the oocyte at stage 9 (Fig. 4 E). However, unlike Staufen and oskar mRNA, Barentsz only accumulates transiently at the posterior, and this localization disappears at stage 10. Thus, Barentsz only localizes to the posterior during stages when oskar mRNA is being transported there, and it is not anchored in the same way as Staufen/oskar RNA complexes.

To be able to examine the localization of Barentsz protein and Staufen simultaneously, we generated transformants that express a BtzGFP fusion protein under the control of a germ line–specific promoter. BtzGFP is functional, since it rescues the barentsz mutant phenotype and shows an identical localization in the germ line to that revealed by antibody staining. Labeling BtzGFP transgenic ovaries with anti-Staufen antibody revealed that BtzGFP and Staufen colocalize at the posterior from stage 9 onwards but that colocalization is lost after stage 10 (Fig. 5, A–F). Furthermore, the two proteins colocalize to the center of the oocyte in gurken mutants, suggesting that they are localized as part of the same complex (Fig. 5, G–I). Since oskar mRNA is not translated in gurken mutants, this observation also indicates that Barentsz localization is independent of Oskar protein.
Figure 3. Cloning of barentsz and identification of Barentsz protein. (A) Diagram of the genomic region between the two P elements that flank barentsz. The position of the distal breakpoint of Df(3R)IR16 is shown in red dashes above the line, and the cosmids from two genomic walks in the interval are shown below. The red vertical lines indicate the molecular positions of the RFLPs used to map barentsz, and the numbers show their meiotic map positions between the two P elements (proportion of recombinants between the two P elements that occurred proximal to the RFLP). 60 out of 203 recombination events occurred proximal to btz1 in this meiotic mapping experiment, placing barentsz close to the distal end of cosmid 105B7. (B) A restriction map of the distal end of cosmid 105B7. The RFLPs (red) define a barentsz candidate region: 10.7 kb.
The posterior localization of Staufen protein depends on oskar mRNA, and we therefore asked whether this is also the case for Barentsz (Ferrandon et al., 1994). Compared with wild-type females, females that carry two extra copies of oskar on transgenes produce twice as much mRNA, which localizes to the posterior pole. This increase in the amount of localized oskar mRNA produces a corresponding increase in the amount of Barentsz that localizes to the posterior at stage 9 (Fig. 5, J–L). Thus, the localization of Barentsz seems to require oskar mRNA and vice versa.

Localization of Barentsz in other mutants that disrupt oskar mRNA transport

Barentsz protein shows an identical phenotype to oskar mRNA and Staufen protein in oskar protein-null mutants, such as osknull, in tropomyosin II and kinesin heavy chain mutants (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Erdélyi et al., 1995; Brendza et al., 2000). In the former, the protein forms a diffuse posterior cloud rather than a tight crescent because Oskar protein is required for the posterior anchoring of its own mRNA (Fig. 6, A and B) (Markussen et al., 1995; Rongo et al., 1995). In tropomyosin II mutants and in kinesin heavy chain germ line clones, Barentsz accumulates at the anterior margin of the oocyte and fails to translocate to the posterior pole (Fig. 6, C and F).

In contrast to the mutants above, the colocalization of oskar mRNA and Barentsz is partially lost in staufen mutants. In staufnull mutant egg chambers, Barentsz shows a weak and variable posterior localization, which is similar to the small amounts of oskar mRNA that localize to the posterior of these ovaries at stage 9 (Fig. 6 E). However, Barentsz shows no significant accumulation at the anterior where the vast majority of the oskar mRNA remains.

The final gene we examined is mago nashi, which in addition to its role in the polarization of the oocyte cytoskeleton has second and apparently independent function in the transport of oskar mRNA to the posterior pole (Newmark and Boswell, 1994; Micklem et al., 1997; Newmark et al., 1997). In mago1/Df or mago2/Df ovaries at permissive temperature, the microtubule organization appears normal, but oskar mRNA remains at the anterior of the oocyte. Furthermore, small amounts of myc- or GFP-tagged Mago protein can be detected at the posterior of the oocyte at stage 9, suggesting that Mago localizes with oskar mRNA (Newmark et al., 1997). Since both the phenotype and distribution of mago resembles that of barentsz, we generated flies expressing a GFP-Mago transgene to compare the localizations of two proteins in wild-type ovaries. As previously reported, GFP-Mago shows a weak localization to the posterior of the oocyte at stage 9 that can be detected by GFP fluorescence.
but not by anti-Mago antibodies (Fig. 7 A). Furthermore, Mago and Barentsz colocalize at the posterior pole, and both proteins accumulate only transiently at this site, unlike oskar mRNA and Staufen. However, the vast majority of Mago protein is nuclear, and it also appears to colocalize with Barentsz at the nurse cell nuclear membranes.

In antibody stainings of mago1/Df ovaries, Barentsz shows no localization to the posterior of the oocyte, and both the staining at the nuclear envelope and the cytoplasm appear to be strongly reduced (unpublished data). Given its nuclear localization, this observation raised the possibility that Mago is required for the transcription of barentsz, and we therefore examined the distribution of Barentsz-GFP in these mutant ovaries, since this is expressed from a heterologous promoter. As observed with the antibody, Barentsz-GFP fails to localize to the posterior of the oocyte and shows a strong reduction in signal elsewhere (Fig. 7 B). However, Western blot analysis of size and stage-matched young ovaries (ovaries which do not contain egg chambers older than stage 10B) does not reveal any significant changes in the level of Barentsz protein in mago mutants (Fig. 7 C). This suggests that the reduced level of Barentsz staining is mainly the result of dispersion of the protein throughout the developing egg chamber. Thus, mago mutants also uncouple the localizations of Barentsz and oskar mRNA, since the latter accumulates at the anterior of the oocyte in these ovaries.

The effect of barentsz mutations on GFP-Mago localization is less dramatic: the protein still accumulates normally in the nuclei but fails to localize to the posterior pole (Fig. 7 D). Thus, Mago is required for the posterior localization of Barentsz and vice versa.

**Discussion**

**Male/female recombination cloning**

Barentsz was originally identified by a single viable allele with a phenotype that can only be clearly detected by antibody stainings or in situ hybridizations, complicating the
cloning of the barentsz locus by conventional means. We therefore developed a novel strategy in which we first mapped btz between two nearby Pw/H11001 insertions using male recombination and then selected for female meiotic recombinants between these P elements using btz Pw/H11001 chromosome generated in the first step. This provides a very efficient selection for a large number of recombination events in a small interval, each of which represents a unique breakpoint between two polymorphic chromosomes, and this allowed us to map btz to a 10-kb region using RFLPs. In principle, this technique can be used to clone any gene identified by mutation and should become increasingly straightforward with the large scale identification of single nucleotide polymorphisms (Teeter et al., 2000).

barentsz is specifically required for oskar mRNA localization

The other genes involved in the posterior localization of oskar mRNA all have other functions: mago nashi is required for the polarization of the oocyte microtubule cytoskeleton (Micklem et al., 1997; Newmark et al., 1997), staufen is required for bicoid mRNA localization and for oskar and bicoid mRNA translation (Ferrandon et al., 1994; Micklem et al., 2000), kinesin I is required for the development of the fertilized egg and is also involved in fast axonal transport (Gho et al., 1992; Hurd et al., 1996), and tropomyosin II is an essential gene required for head development that seems to function in the polarization of the follicle cells (Erdélyi et al., 1995; Tetzlaff et al., 1996). In contrast, barentsz-null mutants block the transport of oskar mRNA from the anterior to the posterior of the oocyte but cause no other discernible phenotypes during oogenesis. In particular, Barentsz does not appear to be required for any other step in oskar mRNA biogenesis because btz mutants do not affect the transcription and export of the mRNA in the nurse cells, its accumulation in the early oocyte and at the anterior pole during stages 8–9, its colocalization with Staufen, or its translational activation. Furthermore, barentsz mutants have a stronger effect on oskar mRNA localization than either TmII or staufen. Very small amounts of oskar mRNA do reach the posterior pole in staufen-null mutant oocytes (Fig. 1 G), but the RNA is not maintained at the posterior for very long. On the other hand, in btz−1 mutant oocytes no oskar mRNA or protein are ever detected at the posterior pole.

Given the severity of the oskar mRNA localization defect in barentsz mutants, it is surprising that the resulting embryos often form a normal abdomen, since this indicates that some Oskar protein must be produced at the posterior pole, although this cannot be detected by antibody staining. The translation of unlocalized oskar mRNA is repressed by factors such as Bruno protein, but this repression is specifically relieved at the posterior (Kim-Ha et al., 1995; Gunkel et al., 1998). Thus, any mRNA that diffuses to the posterior in a barentsz mutant should be translated there, leading to the production of trace amounts of Oskar protein at the posterior, which must be sufficient to induce a normal abdomen in the absence of RNA transport. Unlike Barentsz, Staufen protein is required for the translation of oskar mRNA, and this explains why staufen mutants produce a much stronger abdominal phenotype even though more oskar mRNA is localized to the posterior (Micklem et al., 2000). Thus, oskar

Figure 6. Posterior localization of Barentsz requires oskar, kinesin I, and tropomyosin II but not staufen. (A) Barentsz-GFP shows a wild-type posterior localization in +/Df(osk) egg chambers.oskDf(osk). (B) Barentsz-GFP accumulates in the posterior cytoplasm of the oocyte in oskar nonsense mutants but fails to form a clear cortical crescent. (C) TmIIg8. Barentsz-GFP remains at the anterior of the oocyte. (D) Antibody staining of endogenous Barentsz showing the crescent at the posterior of a wild-type stage 9 oocyte. (E) staD3. Some Barentsz still localizes to the posterior pole in a staufen-null mutant, and the protein does not show the strong persistent anterior localization observed in TmIIg8 and Khe27 mutants. (F) Khe27. Barentsz accumulates at the anterior of the oocyte.
mRNA localization is partially redundant with localized translational activation and is only necessary to produce the high levels of Oskar protein required for pole cell formation. To identify other mutants that specifically block oskar mRNA localization, it will therefore be important to design genetic screens that assay localization directly instead of the resulting embryonic phenotype.

**Barentsz behaves like a component of the oskar RNA localization complex**

Several lines of evidence indicate that Barentsz associates with oskar mRNA and Staufen protein during their movement from the anterior to the posterior of the oocyte. First, Barentsz localizes to the posterior pole at the same time as oskar mRNA and Staufen and colocalizes with Staufen in a posterior crescent at stage 9. However, unlike Staufen Barentsz does not remain at the posterior later in oogenesis and only colocalizes with oskar RNA during the stages when it is being transported to the posterior. Second, Staufen and Barentsz show an identical mislocalization to the center of the oocyte in gurken mutant egg chambers. Since oskar mRNA is not translated in these oocytes, this result argues against a role for Oskar protein in recruiting Barentsz to the complex. Third, like oskar mRNA and Staufen, Barentsz accumulates at the anterior of the oocyte in TmII and in kinesin heavy chain mutants. Thus, Barentsz colocalizes with oskar mRNA both before and after its transport to the posterior of the oocyte. Finally, the posterior localization of Barentsz seems to

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**Figure 7.** The posterior localizations of Barentsz and Mago nashi depend on each other. (A) GFP-Mago (green) and Barentsz (red) colocalize to the posterior pole of the oocyte at stage 9. Colocalization is shown in yellow in the bottom panel. Most Mago protein is nuclear, and it also appears to colocalize with Barentsz at the nuclear envelope and in diffuse clouds around the ring canals connecting the nurse cells to the oocyte (arrow). (B) Barentsz-GFP in wild-type and mago/Df(2R)F36 egg chambers. Barentsz does not localize to the posterior pole in mago mutants, and its accumulation around the nurse cell nuclei is also strongly reduced. (C) Western blot of stages 1–10 egg chambers from wild-type and mago/Df(2R)F36 females probed with α-Barentsz and α-Staufen antibodies. mago mutant extracts contain wild-type levels of Barentsz, indicating that the loss of localized Barentsz staining is not due to an effect on Barentsz expression or stability. To control for equal loading of the wild-type and mago mutant extracts, a section of the SDS-polyacrylamide gel was silver stained (Silver). (D) Localization of GFP-Mago at stage 9 in wild-type and btz” mutant egg chambers. Mago does not localize to the posterior in barentsz mutants but still accumulates in the nuclei.
depend on oskar RNA. Although it is not possible to examine the localization of Barentsz in oskar RNA-null mutants, overexpression of oskar induces a corresponding increase in the amount of Barentsz that localizes to the posterior pole. In conjunction with the lack of oskar mRNA localization to the posterior in barentsz mutants, these results strongly suggest that Barentsz is an essential component of the oskar RNA transport complex.

Although it has been thought previously that Staufen is essential for oskar mRNA localization, our results show that a very small amount of the RNA can still reach the posterior pole at stage 9 in the complete absence of Staufen protein. Thus, Staufen cannot be the only RNA-binding protein that recognizes oskar mRNA and couples it to the transport machinery. In stauufen mutants, Barentsz shows little if any accumulation at the anterior of the oocyte where the majority of oskar mRNA remains but colocalizes with the tiny fraction of RNA that reaches the posterior pole. Thus, Staufen seems to be required to promote or stabilize the efficient association of Barentsz with oskar mRNA. However, the Barentsz–oskar mRNA complexes that do form in the absence of Staufen still localize to the posterior.

The sequence of Barentsz gives few clues as to its biochemical function, although it appears to have homologues in other species. Some insight into its role may be provided by the comparison of the oskar mRNA localization phenotype in btz mutants with that of mutants in the heavy chain of kinesin 1 (Brendza et al., 2000). In both cases, oskar mRNA does not localize to the posterior and accumulates instead at the anterior of the oocyte. Furthermore, khc mutants block the posterior localization of Barentsz protein, which remains with oskar mRNA at the anterior pole. Since kinesin I is a plus end–directed microtubule motor, a simple explanation for its role in oskar mRNA localization is that it actually transports oskar mRNA to the plus ends of the microtubules at the posterior pole. If this model is correct, the mutant phenotype and localization of Barentsz protein suggest that it acts somewhere between oskar mRNA and the kinesin. For example, Barentsz could play a role in coupling the RNA to the kinesin or in the activation of the motor once the complex has formed. On the other hand, we have been unable so far to detect an interaction between Barentsz and the kinesin heavy chain in Drosophila ovary extracts, although this may be due to the fact that only a fraction of the total Barentsz protein localizes with oskar mRNA, and this only occurs in stage 9 and 10 egg chambers, which represent a small proportion of the egg chambers in the ovary.

Most of our conclusions about Barentsz are also likely to apply to Mago nashi, which seems to serve a closely related function in oskar mRNA localization. mago nashi mutants cause a very similar failure in the translocation of oskar mRNA from the anterior to the posterior of the oocyte (Newmark and Boswell, 1994; Micklem et al., 1997). Furthermore, our results confirm that Mago protein also localizes transiently to the posterior pole, although the amounts are too low to detect by antibody staining (Newmark et al., 1997). Finally, Mago and Barentsz depend on each other for their localization to the posterior, since the localization of Mago is abolished in barentsz mutants and vice versa. Some clue to the relationship between the two may be provided by the fact that mago mutants disrupt the perinuclear localization of Barentsz in the nurse cells. This suggests that Mago may be required for the formation of functional Barentsz and that the two proteins are part of the same complex before they enter the oocyte. Consistent with this, Barentsz and Mago appear to colocalize at the periphery of the nurse cell nuclei and at the ring canals between the nurse cells and the oocyte, although we have been unable so far to detect a direct interaction between them.

Recent results have implicated hnRNP proteins that are predominantly nuclear in the cytoplasmic localization of several RNAs, suggesting that the nuclear history of a transcript may determine its fate in the cytoplasm (Hoek et al., 1998; Cote et al., 1999; Lall et al., 1999; Norvell et al., 1999). In this context, it is interesting to note that most Barentsz is associated with the nuclear membranes of the nurse cells, whereas almost all Mago nashi are found in the nuclei. Since oskar mRNA is transcribed in the nurse cell nuclei, this raises the possibility that Mago associates with the RNA in the nucleus and that Barentsz is then recruited to the complex as it exported into the cytoplasm. Consistent with this, the human homologue of Mago interacts with RBM8/ Y14, a nucleocytoplasmic shuttling protein that binds to spliced mRNAs and remains associated with newly exported transcripts in the cytoplasm (Kataoka et al., 2000; Zhao et al., 2000). Thus, oskar mRNA may provide another example where factors loaded onto a transcript as it exits the nucleus determine its subsequent cytoplasmic localization. Whether Mago or Barentsz is required for oskar mRNA transport from the nurse cells into the oocyte, and they would therefore have to remain associated with the RNA during this phase of its localization before directing its subsequent transport to the posterior of the oocyte.

Materials and methods

Fly stocks

btz was identified in a screen of the Tübingen collection of female sterile mutations as a second hit on a ru st lc84CG107 e ca chromosome (Tearle and Nüsslein-Volhard, 1987). The mutation was initially named weak localized’ (wlk) because of the reduction in the amount of localized oskar mRNA, but the discovery that a null allele abolishes all oskar mRNA localization made this name inappropriate, and we renamed the gene barentsz. DI3IR16 is a deficiency for barentsz (Shelton and Wasserman, 1993). We also used the following mutant combinations: mago/DI2RFl36 (Boswell et al., 1991), osk+/DI3IRpXT103 (Lehmann and Nüsslein-Volhard, 1986), nos18/+; sta/+; DI2R;Pc17B, TmP0 (Erdélyi et al., 1995), and grk2600/grk2601 (Neuman-Silberberg and Schupbach, 1993). Germ line clones of kinesin heavy chain using the mutant chromosome FRT42B c Khc72/CyO; Brendza et al., 2000) and both germ line and follicle cell clones of barentsz were generated using the FLP recombinase system (Chou et al., 1993; Chou and Perrimon, 1996). Mutant clones in follicle cells were generated by heat shocking pupae for 2 h at 37°C during three consecutive days. Germ line clones were generated by heat shocking third instar larvae as described above. Enhancer trap lines sbo (Montell et al., 1992), L53b (Fasano and Kerridge, 1988), and A62-GAL4 (Yeh et al., 1995) were stained for Xgal as described in Montell et al. (1992). The following transgenic stocks were used: GFMago10.1 (second chromosome; unpublished data and Micklem, 1997), BtzGFP24B, BtzGFP8 (both second), BtzGFP26C1, BtzGFP26C2 (both third), and BtzGFP2 X, KZ32 kinesin βGal (Clark et al., 1994), ob42 (osk-bcd D’UTR) (Ephrussi and Lehmann, 1992).

Creation of btz

P(Flip2X)3rd1.203 Berkeley Drosophila Genome Project; http://www.fruitfly.org) was mobilized using TM3 Δ2-3 (Robertson et al., 1988), and eight of the resulting ry excision events were found to be new alleles of btz. These
were crossed to D63R1R16/TM6B flies, and hemizygous mutants were analyzed by PCR using several primers in the region. One of the alleles, bzt
\(^{\text{a}}\), has a deletion of the region distal to the P element that contains the NH1-terminal portion of Barentsz without affecting the expression of the adjacent proximal gene CG12876 as determined by whole-mount in situ analysis. The precise extent of the bzt deficiency was determined by cloning and sequencing a PCR product spanning the breakpoints.

**Whole-mount in situ hybridizations and antibody stainings**

Antibody stainings were performed as described in St Johnston et al. (1991). The following antibodies were used: affinity purified anti-Stau (1:1,000; St Johnston et al., 1991) anti-Osk (1:1,000; unpublished data and Grüßner, S., personal communication), anti-Barentsz (1:500–1:1,000; this paper), anti-βGal (1:2,000; Cappel). In situ hybridizations were performed as described in Tautz and Pfeifle (1989).

**Cloning of bzt**

Using male recombination, barentsz was mapped between two P elements in the 98A-B region, PlacW 318-07 (98AS-10) and PlacW 430-05 (98B) (Preston and Engels, 1996; Deak et al., 1997). To map barentsz further, \( e \) bzt \( C \) PlacW 430-05/\( e \) PlacW 318-07 females were created and crossed to \( w \) males. Meiotic recombination events in the interval between the P elements were selected on the basis of the red or white eye color of the F1 progeny. Flies with red eyes carry a third chromosome that contains both P elements and have therefore undergone a recombination event in the progeny. Flies with red eyes carry a third chromosome that contains both P elements and have therefore undergone a recombination event in the interval between the two P elements that juxtaposes the proximal part of \( e \) PlacW 318-07 chromosome and the distal part of \( e \) bzt PlacW 430-05, whereas flies with white eyes carry the neither P element and therefore have undergone recombination in the opposite direction. Stocks of these recombinants were established and tested for the presence of bzt. The position of barentsz was then refined by mapping it relative to RFLPs in two genomic walks in the region. Restriction fragments in these walks were polymorphic between the \( e \) bzt \( C \) PlacW 430-05 and the PlacW 318-07 chromosomes were discovered by generating 400–1,000-bp PCR products from each chromosome, digesting these with a battery of restriction enzymes with 4-bp recognition sequences and screening for differences between the two restriction digests on agarose gels. 75% of the PCR products that we tested showed a clear polymorphism between the two chromosomes, indicating that the \( rs \) st e ca background on which bzt \( ^{\text{a}} \) was induced is sufficiently different from that of the PlacW stocks to allow such an approach. The first walk contained cosmids 156B6 and 68H1, and the second started from the breakpoint of D63R1R16. The distal end of the first walk was extended twice (278B9,105B7) using gridded cosmid filters from the United Kingdom Human Genome Mapping Project Resource Centre, and the last cosmid was found to extend beyond the barentsz locus. Mapping barentsz relative to the other RFLPs in this cosmid placed the mutation in a 10.7-kb interval that contains three predicted genes. Primer sequences and information on polymorphic enzymes are available on request (FvE).

A 7.3-Kb BamHI/Sca genomic fragment containing CG12876 and CG12878 was cloned in the BamHI/EcoRV sites of pBluescriptKS (Stratagene) and recloned as a KpnI fragment into the pWhite Rabbit transformation vector (QIAGEN). The fusion protein was produced as described and expressed in M15[pRep4] cells (QIAGEN). The fusion protein was purified using the Amersham Pharmacia Biotech His-trap kit, and polyclonal antisera were produced in rabbits by Eurogentec.

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