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JCB Article

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

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The localization of Oskar at the posterior pole of the *Drosophila* oocyte induces the assembly of the pole plasm and therefore defines where the abdomen and germ cells form in the embryo. This localization is achieved by the targeting of *oskar* mRNA to the posterior and the localized activation of its translation. *oskar* mRNA seems likely to be actively transported along microtubules, since its localization requires both an intact microtubule cytoskeleton and the plus end–directed motor kinesin I, but nothing is known about how the RNA is coupled to the motor. Here, we describe *barentsz,* a novel gene required for the localization of *oskar* mRNA. In contrast to all other mutations that disrupt this process, *barentsz*-null mutants completely block the posterior localization of *oskar* mRNA without affecting *bicoid* and *gurken* mRNA localization, the organization of the microtubules, or subsequent steps in pole plasm assembly. Surprisingly, most mutant embryos still form an abdomen, indicating that *oskar* mRNA localization is partially redundant with the translational control. Barentsz protein colocalizes to the posterior with *oskar* mRNA, and this localization is *oskar* mRNA dependent. Thus, Barentsz is essential for the posterior localization of *oskar* mRNA and behaves as a specific component of the *oskar* RNA transport complex.

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

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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 *osk* 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 $TmII^{gs}$ 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 $TmII^{gs}$ 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^1) 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^1 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 Novaya Zemlya during an attempt to find a passage past the North pole. btz^1 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^1/*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.

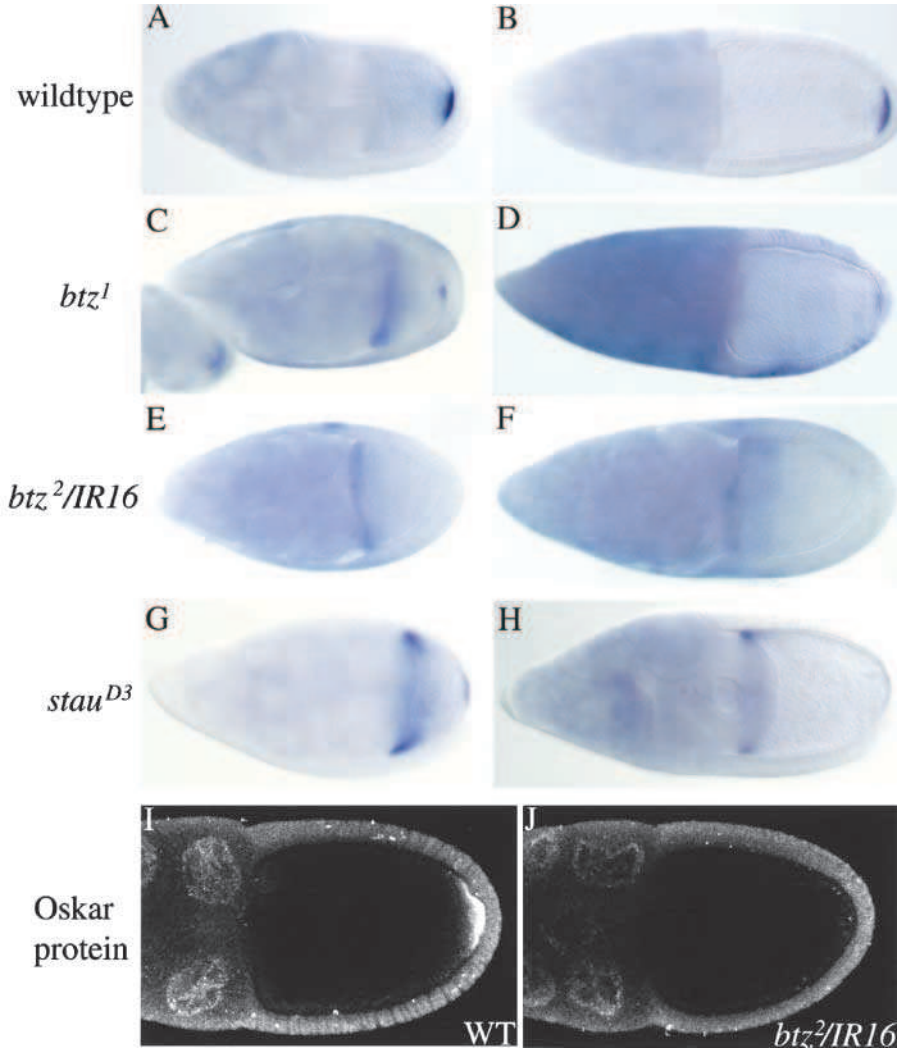

Figure 1. ***barentsz* is required for the localization of *oskar* mRNA and protein.** (A–H) Localization of *oskar* mRNA at stage 9 (left) and stage 10 (right) of oogenesis in wild-type (A and B), btz^1 (C and D), $btz^2/Df(3R)IR16$ (E and F), and $stau^{D3}$ ovaries (G and H). In wild-type egg chambers, *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 *barentsz*, btz^2, and *oskar* mRNA is only detected at the anterior pole, even when the in situ is overstained. In the weak allele btz^1, some *oskar* mRNA is localized to the posterior at stage 9 and remains anchored at stage 10. A small amount of *oskar* mRNA also localizes to the posterior at stage 9 in a *staufen*-null allele, $stau^{D3}$, but the RNA is not anchored at the posterior and disappears by stage 10. (I and J) Antibody staining for Oskar protein in wild-type stage 10B ovaries (I) and $btz^2/Df(3R)IR16$ (J). Oskar protein is never detected at the posterior of *barentsz* mutant oocytes.

We have subsequently isolated further alleles of *barentsz* as described below, and one of these, btz^2, appears to be a null mutation in the locus by molecular criteria. In mutants for other genes involved in *oskar* mRNA localization, such as *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 *staufen*, where traces of *oskar* mRNA can bee seen at the posterior pole at stage 9 but not later in oogenesis (Fig. 1, G and H). In contrast, btz^2 homozygotes or $btz^2/Df(3R)IR16$ females show a completely penetrant *oskar* mRNA localization phenotype at all temperatures. *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 *barentsz*-null mutant oocytes (Fig. 1, I and J). *oskar* mRNA shows a transient localization at the anterior of wild-type oocytes before it localizes to the posterior pole, and the persistent anterior localization seen in *barentsz* mutants suggests that Barentsz is specifically required for the transport of *oskar* mRNA from the anterior to the posterior of the oocyte.

Table I. ***barentsz* posterior patterning and *oskar* mRNA localization defects**

	btz^2	btz^1 (18°C)	btz^1/DF(3R)IR16 (18°C)
		%	
Abdominal defects	53	1	34
No pole cells	100	65	100
osk mRNA mislocalized	100[a]	68[b]	100[a]

At least 60 samples analyzed in each case.
[a]*oskar* mRNA is never detected at the posterior.
[b]This figure includes egg chambers that show a similar or stronger defect in *oskar* mRNA localization to that shown in Fig. 1 C.

The posterior localization of *oskar* mRNA and protein defines where the abdominal and germline determinants are localized, and *staufen* mutant females therefore give rise to embryos that lack both the abdomen and the pole cells. Surprisingly, the embryos laid by *barentsz* mutant females show much milder phenotypes. Embryos from btz^2 homozygous females lack pole cells but develop an average of 5.4 ± 3.1 denticle belts. Furthermore, embryos from $btz^2/Df(3R)IR16$ flies form a very similar number of 4.8 ± 3.1 denticle belts ($n = 43$), indicating that btz^2 is a null mutation or a very strong hypomorph (Table I). The embryonic phenotype of 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

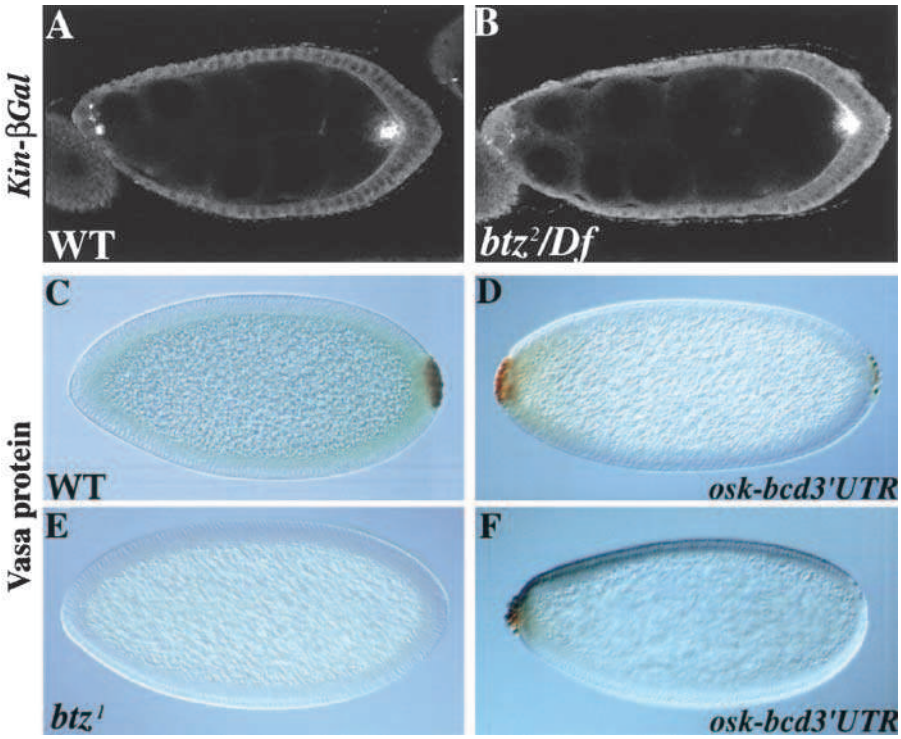

Figure 2. ***barentsz* mutants do not affect the organization of the microtubule cytoskeleton or downstream steps in pole plasm assembly.** (A and B) Localization of kinesin-βGal in wild-type egg chambers (A) and *btz²/Df(3R)IR16* (B). In both cases, kinesin-βGal localizes to the posterior pole, indicating that the plus ends of the microtubules are correctly positioned in *barentsz* mutants. (C–F) Vasa protein labeling of the pole cells in wild-type blastoderm embryos (C), *osk-bcd* 3′UTR (D), *btz¹* (E), and *osk-bcd* 3′UTR, *btz*1 (F). The *osk-bcd* 3′UTR transgene directs Oskar protein expression at the anterior of the oocyte independently of the normal *oskar* localization and translation signals and leads to the formation of anterior pole cells. The formation of anterior pole cells in *btz¹* embryos expressing *osk-bcd* 3′UTR (F) shows that Barentsz is not required for the steps in pole plasm assembly downstream of Oskar protein. *btz¹* embryos lack posterior pole cells because *oskar* mRNA is not localized to the posterior.

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 *gurken* 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 (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 *btz*^{1} 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 *btz*^{1} between two RFLPs that define a 10.7-kb interval containing three genes, CG12876, CG12878, and carnitine 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 *btz*^{1} 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 *btz* mutant phenotypes.

The exact molecular nature of *btz*^{1} has not been determined: the coding sequence of *barentsz* is not affected, but PCR analysis of homozygous *btz*^{1} 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}l(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, *btz*^{2}, 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 NH_2-terminal 332 amino acids of the *barentsz*-coding region, including the conserved domains described below. Homo- or hemizygous *btz*^{2} 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 pI of 5.2 but contains no known protein motifs. However, a region of 184 amino acids near its NH_2 terminus shows homology to three other proteins: human MLN51 (sequence data available from Genbank/EMBL/DDBJ under accession no. 015234), which is overexpressed in breast cancer cell lines, a predicted protein from *Caenorhabditis elegans* (sequence data available from Genbank/EMBL/DDBJ under accession no. CAB07130) and partial sequence from two overlapping mouse ESTs (sequence data available from Genbank/EMBL/DDBJ under accession nos. AI876771 and AW496349) (Tomasetto et al., 1995). Apart from their homologies to each other, these proteins show no significant similarities to any other proteins in the databases, suggesting that they may represent a new but divergent protein family.

Barentsz localizes to the posterior of the oocyte with *oskar* mRNA

To investigate the function of Barentsz in *oskar* mRNA localization, we raised a polyclonal antiserum in rabbits against the NH_2 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 *btz*^{2}/*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 *btz*^{2}/*Df(3R)IR16* ovaries and wild-type ovaries stained with preimmune 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.

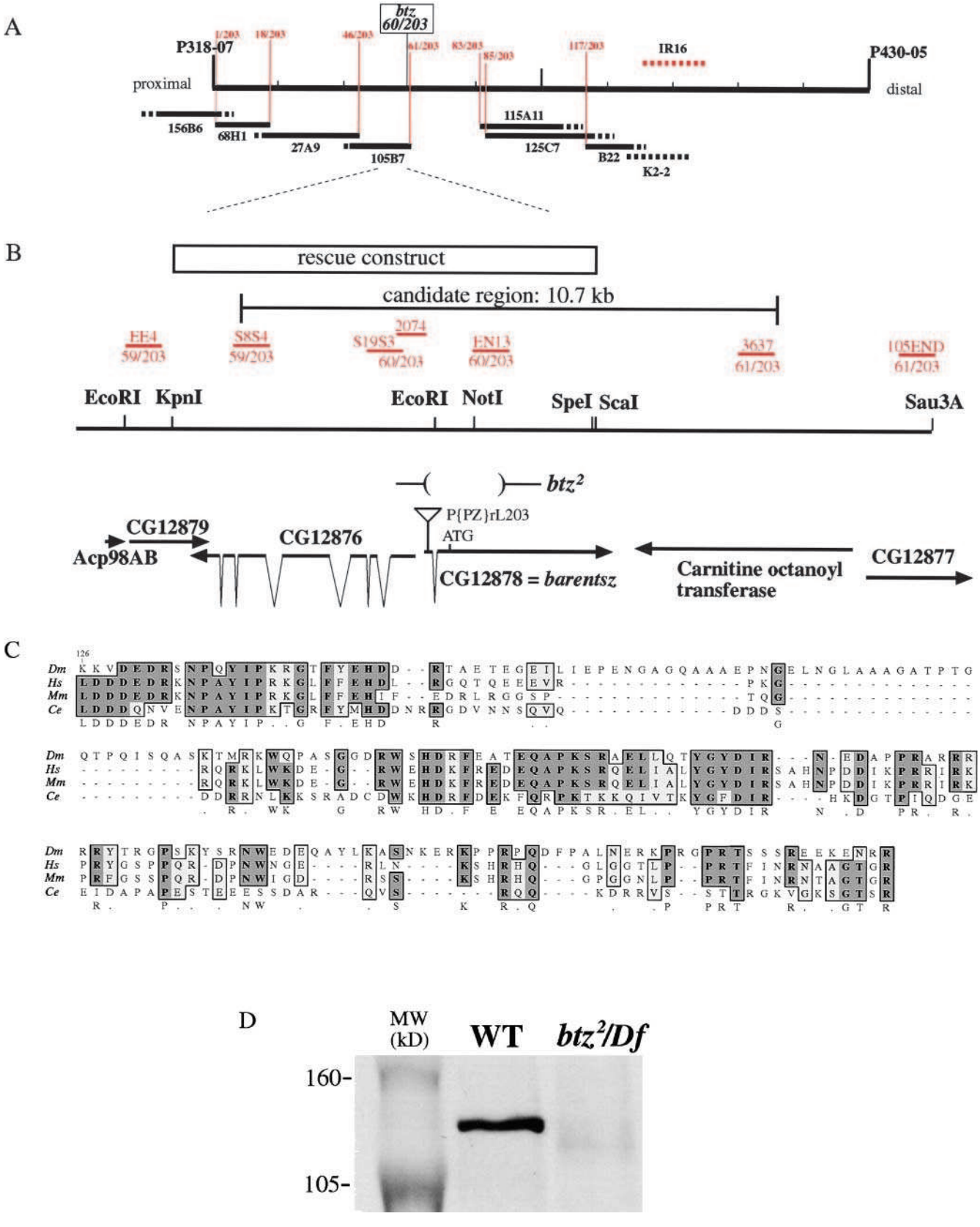

Figure 3. **Cloning of *barentsz* and identification of Barentsz protein.** (A) Diagram of the genomic region between the two P elements that flank *barentsz*. The position of the distal breakpoint of *Df(3R)IR16* is shown in red dashes above the line, and the cosmids from two genomic walks in the interval 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 *btz*^{1} 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

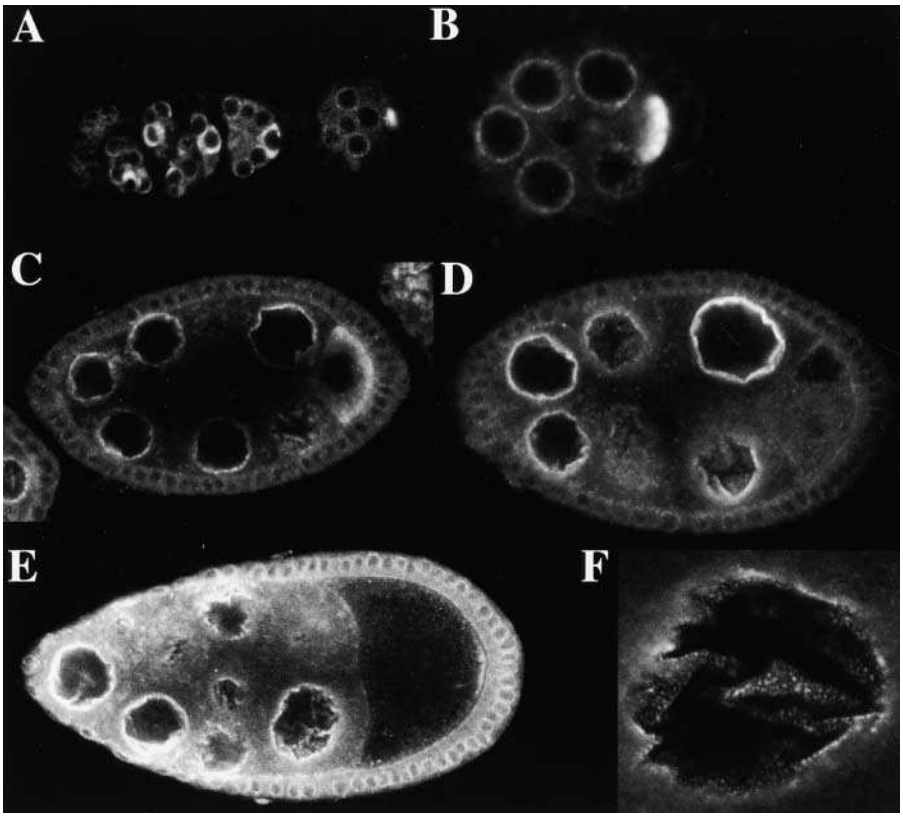

Figure 4. **Distribution of Barentsz during oogenesis.** (A) α-Barentsz staining of a wild-type germarium. Barentsz protein is first expressed in region 2 of the germarium and accumulates in the presumptive oocyte in region 2b. (B) Stage 4. Barentsz localizes to the posterior of the oocyte. The protein is also concentrated around the nuclei of the nurse cells. (C) Stage 6. The posterior enrichment of Barentsz starts to disappear. The protein is now also expressed in the follicle cells and concentrates around the nuclei. (D) Stage 8. Barentsz shows a uniform distribution in the oocyte cytoplasm. (E) Stage 9. Barentsz localizes to the posterior cortex of the oocyte. (F) Surface view of a nurse cell nucleus, showing the punctate distribution of Barentsz, suggesting that it associates with nuclear pores.

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 trangenes 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 osk^{54}, 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 $stau^{D3}$ 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 $mago^{1}/Df$ or $mago^{2}/Df$ 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

region of 10.7 kb. The transcripts in this interval and the site of the P{PZ}rL203 insertion are shown below. Mobilization of this P element generated the btz^{2} deletion, confirming that CG12878 corresponds to *barentsz*. The initial transformation construct that rescues *barentsz* is shown at the top. (C) An alignment of amino acids 126–311 of *Drosophila* Barentsz protein (Dm) with the homologous regions of human MLN51 (Hs), a composite of two partial mouse ESTs (Mm), and the predicted *C. elegans* protein CAB07130 (Ce). (D) Western blot of wild-type and btz^{2} mutant ovary extracts ($btz^{2}/Df(3R)IR16$) probed with α-Barentsz antiserum. The protein markers are in the left lane (MW [kD]). In wild-type extracts, the antibody recognizes a single band of 125 kD, which is absent in the btz^{2} mutant extract.

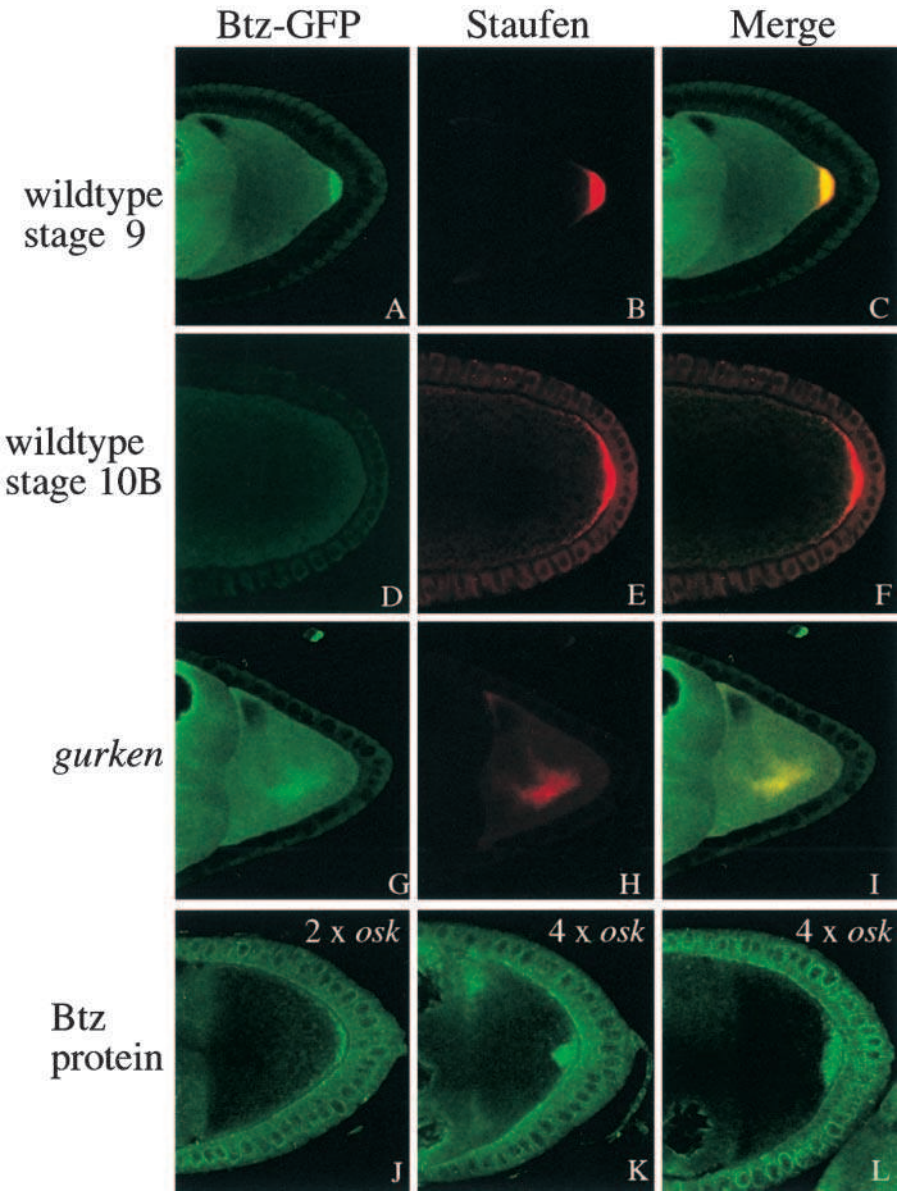

Figure 5. **Barentsz colocalizes with Staufen and *oskar* mRNA.** The localization of Barentsz-GFP (A, D, and G), Staufen protein (B, E, and H), and the merged images of the two (C, F, and I). (A–C) Barentsz-GFP colocalizes with Staufen at the posterior of the oocyte at stage 9. (D–F) The posterior enrichment of Barentsz-GFP has disappeared by stage 10B, whereas Staufen protein remains anchored at the posterior pole. (G–I) In grk^{2B6}/grk^{2E12} egg chambers, Barentsz and Staufen colocalize to the center of the oocyte at stage 9. (J–L) α-Barentsz antibody staining of wild-type egg chambers (J) and in egg chambers that express two extra copies of *oskar* from a trangene (K and L). The increase in *oskar* gene dosage to four copies causes a corresponding increase in the localization of Barentsz to the posterior pole.

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 $mago^{1}/Df$ ovaries, Barentsz shows no localization to the posterior of the oocyte, and the levels of 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

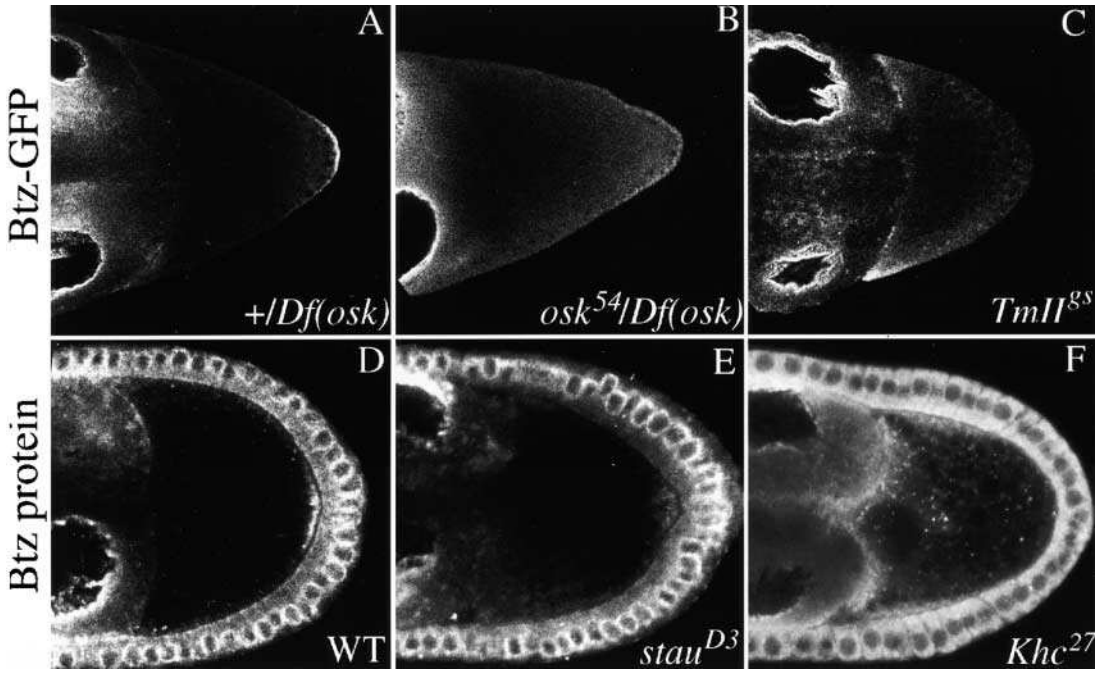

Figure 6. **Posterior localization of Barentsz requires *oskar, kinesin I,* and *tropomyosin II* but not *staufen.*** (A) Barentsz-GFP shows a wild-type posterior localization in +/Df(osk) egg chambers.*osk*^{54}*/Df(osk).* (B) Barentsz-GFP accumulates in the posterior cytoplasm of the oocyte in *oskar* nonsense mutants but fails to form a clear cortical crescent. (C) *TmII*^{gs}. 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) *stau*^{D3}. 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 *TmII*^{gs} and *Khc*^{27} mutants. (F) *Khc*^{27}. Barentsz accumulates at the anterior of the oocyte.

cloning of the *barentsz* locus by conventional means. We therefore developed a novel strategy in which we first mapped *btz*^{1} between two nearby Pw^{+} insertions using male recombination and then selected for female meiotic recombinants between these P elements using *btz*^{1} Pw^{+} 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*^{1} 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*^{2} 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*

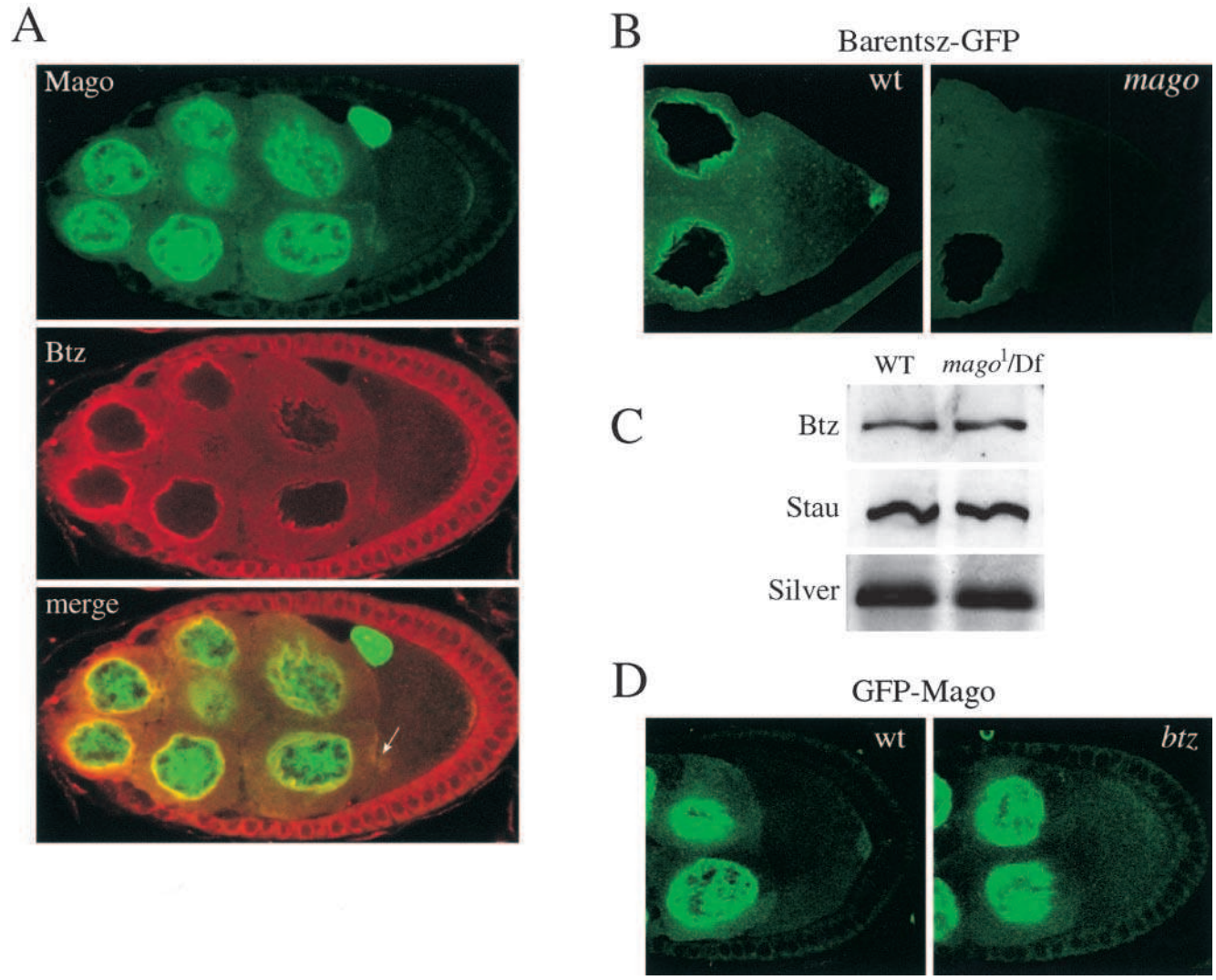

Figure 7. **The posterior localizations of Barentsz and Mago nashi depend on each other.** (A) GFP-Mago (green) and Barentsz (red) colocalize to the posterior pole of the oocyte at stage 9. Colocalization is shown in yellow in the bottom panel. Most Mago protein is nuclear, and it also appears to colocalize 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*^{1}/*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*^{1}/*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*^{2} mutant egg chambers. Mago does not localize to the posterior in *barentsz* mutants but still accumulates in the nuclei.

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

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 *staufen* 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* RNA 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 I (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. Neither 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^{1} was identified in a screen of the Tübingen collection of female sterile mutations as a second hit on a *ru st fch*^{267} *e ca* chromosome (Tearle and Nüsslein-Volhard, 1987). The mutation was initially named *weak localizer*^{1} (*wkl*^{1}) 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*. *Df(3R)IR16* is a deficiency for *barentsz* (Shelton and Wasserman, 1993). We also used the following mutant combinations: *mago*^{1}*/Df(2R)F36* (Boswell et al., 1991), *osk*^{54}/or *osk*^{84}*/Df(3R)p-XT103* (Lehmann and Nüsslein-Volhard, 1986), *nos*^{18}, *stau*^{D3}*/Df(2R)Pcl7B*, *TmII*^{ts} (Erdélyi et al., 1995), and *grk*^{2B6}*/grk*^{2E12} (Neuman-Silberberg and Schüpbach, 1993). Germ line clones of *kinesin heavy chain* (using the mutant chromosome *FRT42B c Khc*^{27}*/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 *slbo* (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: GFPmago17.1 (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* 3′UTR) (Ephrussi and Lehmann, 1992).

Creation of *btz*^{2}

P{PZ}l(3)rL203 (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 *Df(3R)IR16/TM6B* flies, and hemizygous mutants were analyzed by PCR using several primers in the region. One of the alleles, btz^2, has a deletion of the region distal to the P element that contains the NH_2-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 btz^2 deficiency was determined by cloning and sequencing a PCR product spanning the breakpoints.

Whole-mount in situ hybridizations and antibody stainings

Antibody stainings were preformed 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 Grünert, 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 *btz*

Using male recombination, *barentsz* was mapped between two P elements in the 98A-B region, PlacW 318-07 (98A5-10) and PlacW 430-05 (98B) (Preston and Engels, 1996; Deak et al., 1997). To map *barentsz* further, *w; e* btz^1 *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 interval between the two P elements that juxtaposes the proximal part of *e PlacW 318-07* chromosome and the distal part of *e* btz^1 *PlacW 430-05*, whereas flies with white eyes carry neither P element and have undergone recombination in the opposite direction. Stocks of these recombinants were established and tested for the presence of *btz*. 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 that were polymorphic between the *e* btz^1 *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 *ru st e ca* background on which btz^1 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 Df(3R)IR16. The distal end of the first walk was extended twice (27B9,105B7) 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 further 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 (pWRb) (Martin-Bermudo et al., 1997). The orientation of the KpnI fragment was such that CG12878 can use the polyA site encoded by pWRb. This construct was introduced into flies using standard methods and was able to rescue the maternal effect posterior group phenotype of *osk nos* btz^1/btz^1 females. Two mutant constructs were produced: one in which a frameshift was introduced after amino acid 25 in CG12876 using the Stratagene Quickchange kit and the other in which a NotI site in the CG12878 coding sequence was filled in, resulting in a frameshift after amino acid 181. In several independent transformant lines, only the first construct was able to rescue the *barentsz* mutant phenotype, indicating that CG12878 corresponds to *btz*.

Generation of Barentsz-GFP

The barentsz-coding region was amplified and cloned into BamHI/SpeI cut pD277-GFP6 to generate a transformation construct in which the α4-tubulin promoter drives germline-specific expression of Barentsz fused to GFP6. pD277-GFP6 was created by amplifying GFP6 (a gift from A. Brand, Welcome, CRC Institute, and J.V. Haseloff, University of Cambridge, Cambridge, UK) and cloning it between the NotI and XhoI sites of a modified version of pCaTub67MatpolyA (Micklem et al., 1997) in which the tubulin initiation codon had been mutated.

Antibody preparation

Amino acids 2–383 of *barentsz* were amplified and cloned into pQE31 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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