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# Dazl can bind to dynein motor complex and may play a role in transport of specific mRNAs

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**Male germ cell development includes mitotic and meiotic cell divisions that are followed by dramatic morphological changes resulting in the production of spermatozoa. Genetic evidence has indicated that the *DAZ* family genes are critical for successful male germ cell development in diverse animals as well as humans. In the present study, we investigated the cellular functions of Dazl in the mouse male germ cells. We identified a specific interaction of Dazl with the dynein light chain, a component of the dynein–dynactin motor complex. The subcellular distribution of Dazl was microtubule-dependent and a selected number of Dazl-bound mRNAs could accumulate in the perinuclear area. Based on these results, we propose that Dazl may play a role in transport of specific mRNAs via dynein motor complex. The Dazl-bound mRNAs may be stored at specific sites and would be available for future developmental processes. Our study revealed the presence of an active mRNA transport system in mouse male germ cells.**

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## Introduction

A significant proportion of the azoospermic male infertility patients have micro-deletions at specific loci called *AZF* in the Y chromosome, suggesting that some genes located at these loci are critical for spermatogenesis (Tiepolo and Zuffardi, 1976). Of all the genes located at the *AZF* loci, *DAZ* has been considered to be a male infertility gene (Reijo *et al*, 1995). Genetic evidence indicates that the *DAZ* family genes are critical for germ cell development in diverse animals as well as in humans. The disruption of the *Dazl* gene in mice leads to loss of germ cells and complete absence of gamete production (Ruggiu *et al*, 1997). A careful examination of the *Dazl*-deficient mice revealed that the leptotene–zygotene stage of meiotic prophase I was the final point reached in the male germ cell development (Saunders *et al*, 2003). A study on the *Drosophila boule* mutant revealed the primary defect in the meiotic cell division of male germ cells (Eberhart *et al*, 1996). In *Caenorhabditis elegans*, the loss of DAZ-1-function caused female sterility due to the blockage of oogenesis at the pachytene stage of meiotic prophase I (Karashima *et al*, 2000).

There are three *DAZ* family genes in the human genome. Four copies of the *DAZ* gene are located at the *AZFc* locus in the Y chromosome, and *DAZL* and *BOULE* are autosomal genes (Reijo *et al*, 1996; Saxena *et al*, 2000; Kuroda-Kawaguchi *et al*, 2001). The Y chromosomal *DAZ* is found only in humans and Old World primates, while the autosomal *DAZ* family genes are found to be present in all the tested organisms (Xu *et al*, 2003). Genetic rescue experiments were carried out to determine the functional redundancy among the DAZ family proteins. Male germ cell development in the *Dazl*-deficient mice was rescued partially by human *DAZ* or *DAZL* genes (Slee *et al*, 1999; Vogel *et al*, 2002). Similarly, the introduction of *Xenopus Dazl* or human *BOULE* into the *boule* mutant fly led to the completion of meiosis in male germ cells (Houston *et al*, 1998; Xu *et al*, 2003). These results suggest that the DAZ family proteins play related, yet distinct roles in male germ cell development.

The presence of an RNA-binding domain within the Dazl protein suggested its involvement in the RNA metabolism. Diverse strategies have been adopted to identify the mRNA species that are specifically bound to Dazl. A consensus sequence for the Dazl binding-element was defined and from among the candidate mRNA species, *Cdc25C* was confirmed to interact with Dazl (Venables *et al*, 2001). The mRNA species that interact with the GST-Dazl fusion protein was isolated and *Cdc25A* and *Tpx1* from among candidate mRNAs were suggested to specifically interact with Dazl (Jiao *et al*, 2002). Fox *et al* (2005) identified the mRNAs to which both Dazl and a Dazl-interacting protein Pum2 were bound simultaneously, and *Sdad1* was selected from the candidate mRNAs to be characterized further. Finally, the mRNA species that were coimmunoprecipitated with the endogenous Dazl protein were identified, and *Mvh* from among the candidate mRNAs was confirmed to interact with Dazl *in vivo* (Reynolds *et al*, 2005). These results indicate that Dazl interacts with a specific set of mRNA species and probably controls their expression.

The active transport of mRNAs along the cytoskeletal network has been described in selected model systems (reviewed in St Johnston, 2005). Perhaps, the best understood example of this process may be the transport of the *ASH1* mRNA to the bud tip in *Saccharomyces cerevisiae*, thus repressing mating-type switching in daughter cells. This transport is actin-dependent and requires Myo4-a type V myosin-and additional coupling proteins such as She2 and She3 (reviewed in Cosma, 2004). Microtubule-dependent

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transport of mRNAs has been reported in *Drosophila* oocytes (Januschke *et al*, 2002). The *oskar* mRNA is transported from nurse cells to the posterior end of the oocytes by kinesin, a plus-end-directed microtubule motor protein (Brendza *et al*, 2000). On the other hand, the *bicoid* mRNA is transported and anchored to the anterior end of the oocytes. Dynein is responsible for *bicoid* transport, and a number of additional proteins such as Exuperantia, Swallow, γ-tubulin37C, Grip75 and Staufen are also involved in the transport and retention of *bicoid* at the anterior end of the oocytes throughout oogenesis (Ferrandon *et al*, 1994; Schnorrer *et al*, 2000, 2002; Cha *et al*, 2001). In particular, Swallow was known to interact with the dynein light chain (Dlc), suggesting its function as an adaptor for *bicoid* mRNA to dynein (Schnorrer *et al*, 2000). Other examples may be *gurken* mRNA that are moved within the oocytes in two dynein-dependent steps (MacDougall *et al*, 2003), and a group of pair-rule mRNAs that are transported in association with dynein and accumulated in the apical region of the syncytial blastoderm (Wilkie and Davis, 2001; Delanoue and Davis, 2005). The active transport of mRNAs through microtubules was also observed in *Xenopus* oocytes and mammalian neurons (reviewed in St Johnston, 2005; Carson and Barbarese, 2005). The primary goal of the active mRNA transport is the localization of mRNAs in a specific region of the cell. Translational regulation is always coupled with the active transport mechanisms.

The regulation of gene expression at translational levels has been emphasized in germ cell development (reviewed in Kuersten and Goodwin, 2003). In the present study, we report that Dazl interacts with Dlc, thereby suggesting that Dazl functions as an adaptor for a specific set of mRNAs on the dynein motor complex. Our study revealed the presence of an active mRNA transport system in mouse male germ cells.

## Results

### *Dazl interacts with Dlc*

In order to elucidate the biological functions of Dazl in male germ cells, we carried out yeast two-hybrid screenings with Dazl as the bait and isolated *Dlc1* clones most predominantly. In addition, multiple clones of *DAZAP2* (Tsui *et al*, 2000a) were also identified. The physical interaction of Dazl with Dlc1 was examined by the GST pull-down assay (Figure 1A). The results showed that the Flag-Dlc1 protein in the 293T cell lysates was coprecipitated with GST-Dazl, and the HA-Dazl protein was coprecipitated with GST-Dlc1. When the mouse testicular lysates were incubated with GST-Dazl, the endogenous Dlc was coprecipitated (Figure 1A). The physical association of endogenous Dazl and Dlc1 proteins was also confirmed by coimmunoprecipitation experiments (Figure 1B). When testicular lysates were incubated with antibodies against Dazl or Dlc, both the proteins were immunoprecipitated, thereby indicating a physical interaction between Dazl and Dlc in the mouse male germ cells.

In order to define the Dazl sequence that is responsible for the interaction of Dazl with Dlc1, we performed GST pull-down and coimmunoprecipitation assays with truncated Dazl mutant proteins. The mouse Dazl protein contains an RNA recognition motif and a so-called DAZ domain (Figure 1C). Truncated Dazl mutants tagged with HA or GFP were transfected into the 293T cells and subjected to GST pull-down

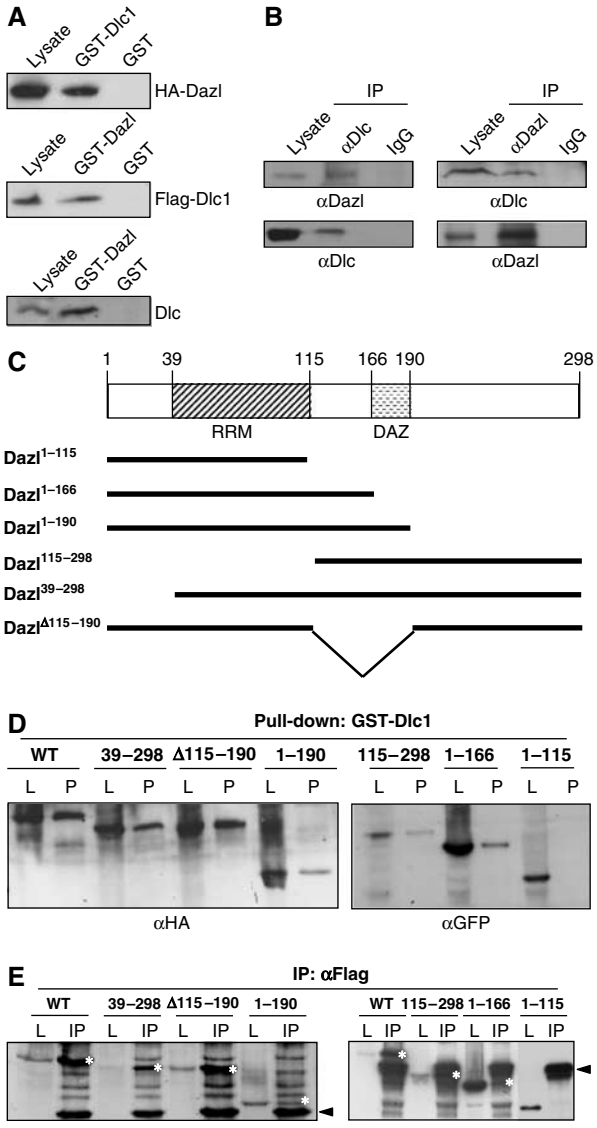


**Figure 1** Interaction of Dazl with Dlc (**A**) GST pull-down assay. The cell lysates of the 293T cells in which the HA-Dazl or Flag-Dlc1 proteins were ectopically expressed were incubated with the GST-Dlc1 or GST-Dazl fusion protein and the coprecipitated proteins were detected using antibodies against the HA or Flag epitopes. Mouse testicular lysates were incubated with GST-Dazl, and the coprecipitated Dlc protein was detected using the Dlc antibody. (**B**) Coimmunoprecipitation assay. The endogenous Dazl or Dlc protein in the testicular lysates was immunoprecipitated, and the coimmunoprecipitated proteins were detected using the specific antibodies. IgG was used as a control for immunoprecipitation. (**C**) Truncated mutants of the mouse *Dazl* gene. The mouse Dazl protein contains an RNA-recognition motif (RRM) for RNA binding and a DAZ repeat (DAZ) that is characteristic to the DAZ family proteins. (**D**) The 293T cells were transfected with the expression vectors of truncated Dazl mutants tagged with HA (*pHA-Dazl*, *pHA-Dazl*$^{39-298}$, *pHA-Dazl*$^{\Delta 115-190}$ and *pHA-Dazl*$^{1-190}$) or with GFP (*pGFP-Dazl*$^{115-298}$, *pGFP-Dazl*$^{1-166}$ and *pGFP-Dazl*$^{1-115}$) and were subjected to GST pull-down assays (P) with the GST-Dlc1 fusion protein. Antibodies against the HA or GFP epitopes were used for the detection of the Dazl mutant proteins. The cell lysates (L) were simultaneously loaded on the gels as the size markers of the Dazl mutant proteins. (**E**) The 293T cells cotransfected with the *pFLAG-Dlc1* and *Dazl* mutant vectors were subjected to immunoprecipitation (IP) assays with the Flag antibody. The Dazl proteins were detected using antibodies against the HA or GFP epitopes. The cell lysates (L) were simultaneously loaded on the gels as the size markers of the Dazl mutant proteins. The Dazl protein bands are marked as white asterisks and the heavy and light chains of IgG are indicated with arrow heads.

assays using GST-Dlc1 fusion proteins. The results showed that all the tested Dazl mutants, except Dazl$^{1-115}$, were coprecipitated with the GST-Dlc1 fusion protein (Figure 1D). Coimmunoprecipitation assays conducted with the same set of Dazl mutants also demonstrated positive physical interactions with Dlc1, except in the case of the Dazl$^{1-115}$ mutant protein (Figure 1E). These results indicate that Dlc interacts with the C-terminal end of the Dazl protein. This binding activity is distinct from that of poly(A)-binding protein (PABP), which interacted with neither Dazl$^{1-115}$ nor Dazl$^{115-298}$ mutant proteins (Collier *et al*, 2005).

### *Dlc mediates the association of Dazl with the dynein–dynactin complex*

We examined the association of Dazl with the dynein–dynactin complex. When the testicular lysates were immunoprecipitated with the Dazl antibody, the dynein intermediate chain (Dic) and p150$^{Glued}$ were coimmunoprecipitated, thus indicating that Dazl is associated with the dynein–dynactin complex in the mouse male germ cells (Figure 2A). A GST pull-down assay was carried out with GST-Dazl and ectopically expressed Dic proteins. The results showed that HA-Dic1 was coprecipitated with GST-Dazl only in the presence of Flag-Dlc1 (Figure 2B). The indirect interaction of Dic1 with Dazl was also confirmed by the coimmunoprecipitation assay using the 293T cells co-transfected with a combination of *pMyc-Dazl*, *pHA-Dic1* and *pFlag-Dlc1*. The HA-Dic1 proteins were coimmunoprecipitated with Myc-Dazl only in the presence of Flag-Dlc1 (Figure 2C). Identical results were obtained on the same set of experiments conducted with Dic2 (data not shown). These results strongly suggest that Dlc mediates the association of Dazl with the dynein–dynactin complex.

### *Dazl travels via the microtubule network*

*In vivo* association of Dazl with dynein–dynactin complex allowed us to propose that Dazl travels via the microtubule network. In the COS7 cells, the ectopic Dazl proteins were located in the perinuclear area in either a diffused or an aggregated form and endogenous Dlc colocalized with the Dazl proteins (Figure 3A). When nocodazole was added to the culture medium to disrupt the microtubule network, it caused the dispersion of both ectopic Dazl and endogenous Dlc proteins throughout cytoplasm; this suggests that Dazl is linked to the microtubule network in cells (Figure 3A). Little change was observed in the subcellular distribution of Dazl in the cytochalisin D-treated cells; this suggests that the Dazl distribution is not actin-dependent (Figure 3A). The overexpression of dynamintin, a component of the dynactin complex, has been known to induce the disruption of the dynein–dynactin complex from the microtubule network (Echeverri *et al*, 1996). As expected, HA-Dazl had a scattered distribution throughout the cytoplasm in the *dynamitin*-overexpressed COS7 cells (Figure 3B). Finally, endogenous Dazl coprecipitated with taxol-stabilized microtubules along with Dic, while Rbm, another male infertility protein, did not (Figure 3C). Taken together, these results indicate that Dazl is associated with the microtubule network via the dynein–dynactin complex.

The real-time movement of the ectopic Dazl protein was measured in the cultured cells. The CFP-Dazl protein moved along straight or curvilinear tracks intermittently, whereas

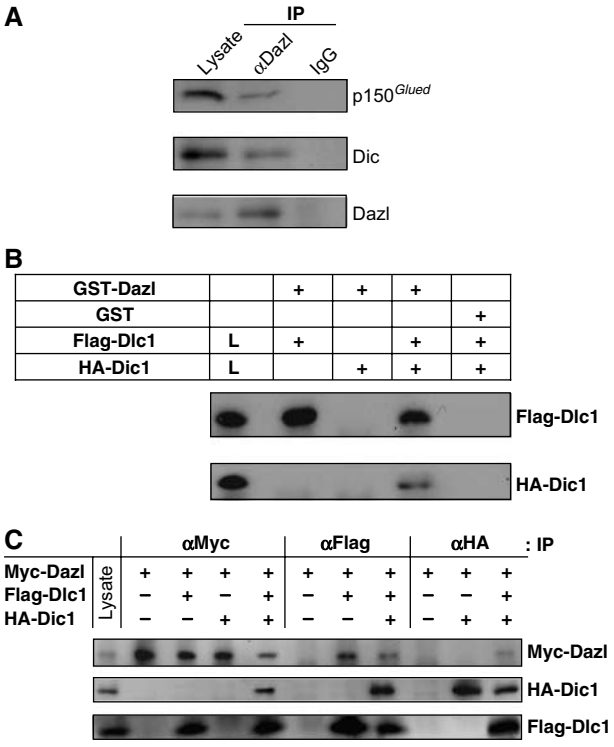


**Figure 2** Association of Dazl with the dynein–dynactin complex. (**A**) *In vivo* interaction of Dazl with Dic and p150$^{Glued}$ in the testis. The endogenous Dazl protein in the testicular lysates was immunoprecipitated (IP), and the coimmunoprecipitated proteins were detected using the specific antibodies against p150$^{Glued}$ and Dic. IgG was used as a control for immunoprecipitation. Testicular lysates were used for detection of the endogenous p150$^{Glued}$, Dic and Dazl proteins in the immunoblots. (**B**) Indirect interaction of Dazl with Dic: GST pull-down assay. The 293T cell lysates transfected with *pFlag-Dlc1* and/or *pHA-Dic1* were incubated with the GST-Dazl fusion protein or with the GST protein as a control. The precipitated Dlc1 and Dic1 proteins were detected using the Flag and HA antibodies, respectively. The HA-Dic1 protein was precipitated with GST-Dazl only in the presence of Flag-Dlc1. (**C**) Indirect interaction of Dazl with Dic: immunoprecipitation assay. The 293T cell lysates cotransfected with *pFlag-Dlc1* and/or *pHA-Dic1* along with the Dazl expression vector (*pMyc-Dazl*) were immunoprecipitated, followed by immunoblotting using the indicated antibodies. The cell lysates were loaded on the gels to confirm the expression of Myc-Dazl, HA-Dic1 and Flag-Dlc1 proteins in the cells. The HA-Dic1 protein was coimmunoprecipitated with Myc-Dazl only in the presence of Flag-Dlc1.

the GFP-Dazl$^{1-115}$ mutant protein remained stationary (Figure 4A and Supplementary videos). The polarity and speed of movement of Dazl and Dazl$^{1-115}$ were quantitated. Dazl moved in a retrograde direction more frequently, while the Dazl$^{1-115}$ mutant protein remained stationary and did not have any directional preferences (Figure 4B). The median speed of the CFP-Dazl particles was approximately 0.3 μm/s (Figure 4C); this is comparable to that of the dynein motor complex (Gross *et al*, 2000). These results are consistent with the hypothesis that Dazl travels via the microtubule network in association with the cytoplasmic dynein motor complex.

### *Dazl controls the subcellular distribution of a specific set of mRNAs*

Dazl was reported to bind to a specific set of mRNAs (Venables *et al*, 2001; Jiao *et al*, 2002; Fox *et al*, 2005;

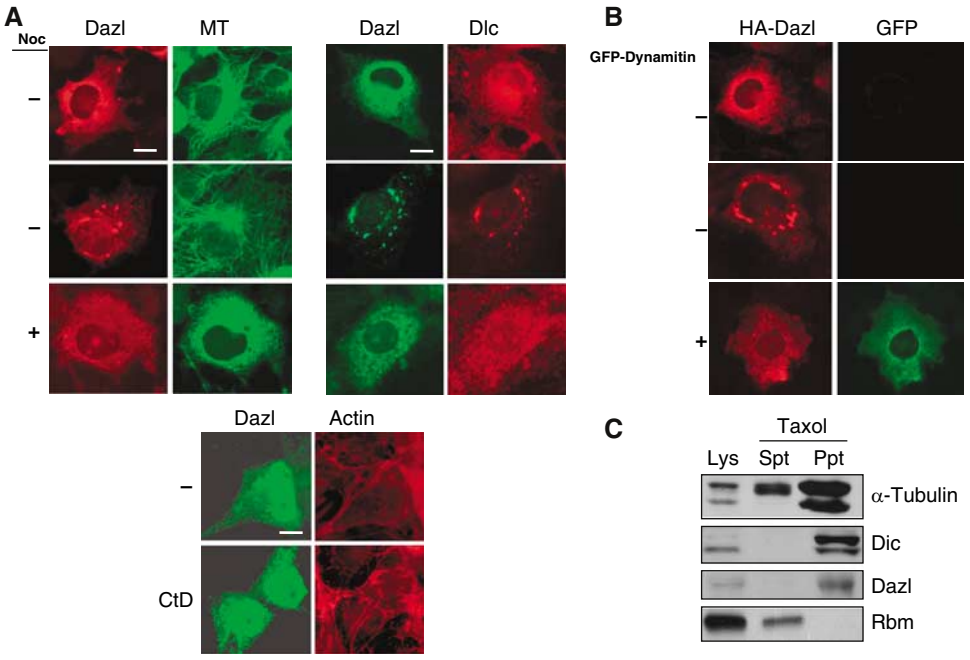


**Figure 3** Dazl travels via the microtubule network. (**A**) The COS7 cells transfected with *pHA-Dazl* were treated with either 10 μM nocodazole (Noc) or 1 μM cytochalasin D (CtD) for 1 h and immunostained with antibodies against Dazl, α-tubulin and Dlc. Actin was stained with phaloidin. Scale bars, 10 μm. (**B**) Subcellular distribution of HA-Dazl in the COS7 cells was observed in the absence or presence of GFP-Dynamitin. Scale bar, 10 μm. (**C**) Association of Dazl with microtubules. The soluble fraction of the mouse testicular lysates (Lys) was treated with 20 μM taxol and 0.5 mM GTP and centrifuged to separate the precipitate (Ppt) from the supernatant (Spt). Immunoblotting analyses were performed using antibodies against α-tubulin, Dic, Dazl and the Rbm proteins.

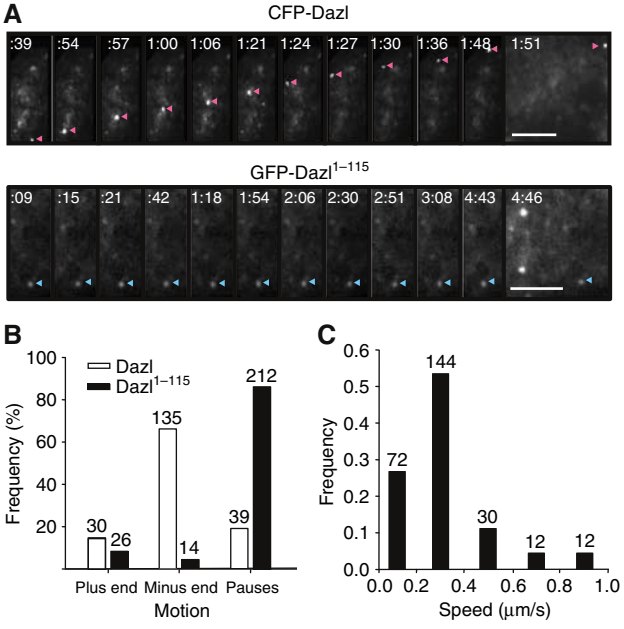


**Figure 4** Live cell images of the Dazl proteins in cultured cells. (**A**) The COS7 cells were transfected with *pCFP-Dazl* or *pGFP-Dazl*$^{1-115}$. The movement of the CFP-Dazl and GFP-Dazl$^{1-115}$ proteins was recorded 24 h after the transfection. The Dazl particles are indicated by arrowheads. The elapsed times of sequences was recorded (ms). Scale bars, 5 μm. (**B**) Directional movement of the Dazl proteins in cultured cells. The directions of the Dazl protein movements were analyzed using the COS7 cells transfected with *pCFP-Dazl* (blank bar) or *pGFP-Dazl*$^{1-115}$ (solid bar). Signals slower than 0.05 μm/s were considered to be in pause. The number of signals that moved toward the periphery (plus end) or nucleus (minus end) was counted and indicated at the top of the bars. (**C**) Velocity distribution of the retrograde Dazl movements. The velocity distribution of the CFP-Dazl protein was analyzed in the COS7 cells. The number of particles counted was indicated on the top of the bar.

Reynolds *et al*, 2005). In order to examine whether Dazl functions as an mRNA transporter in cells, we determined the subcellular distribution of the target mRNAs by using a system involving the MS2-GFP fusion protein (Fusco *et al*, 2003). This protein was located in the nucleus due to the presence of a nuclear localization signal within the protein (Figure 5A (a–d)). In the presence of an mRNA with the MS2-binding sites, a fraction of the MS2-GFP fusion proteins was transported to the cytoplasm, following the MS2-fusion mRNA to which it bound (panels e, i, and m). In the presence of Dazl, the fluorescent signals aggregated in the perinuclear area of cells with the *MS2-Tpx1* and *MS2-Cdc25C* fusion mRNAs (panels f and n) but not in cells with the *MS2-Cdc25A* fusion mRNA (panel j). The Dazl protein was also colocalized in the *MS2-Tpx1* and *MS2-Cdc25C* aggregates (data not shown). Such perinuclear aggregates were absent in cells with Dazl$^{1-115}$ that does not bind to Dlc1 (panels g and o) as well as in cells with Dazl$^{115-298}$ that lacks the RNA-binding motif (panels h and p). These results suggest that the Dazl-bound mRNAs such as *Tpx1* and *Cdc25C* are linked to the cytoplasmic dynein motor complex and accumulate at specific sites within a cell.

Dazl-binding elements have been defined in selected mRNAs. The (GUn)n stretch located between nucleotides 123–160 of the 5′UTR of *Cdc25C* was designated as a possible binding site of Dazl (Venables *et al*, 2001). Dazl-binding elements were also defined in the first 300 nucleotide residues of the 3′UTR of the *Mvh* mRNA (Reynolds *et al*, 2005). In order to confirm that the perinuclear aggregation of mRNAs depends on their specific recognition by Dazl, we prepared *MS2* mRNAs linked to the 5′UTR of *Cdc25C* or the 3′UTR of the *Mvh* sequences and determined their distribution. The results showed that only the mRNA species that possessed

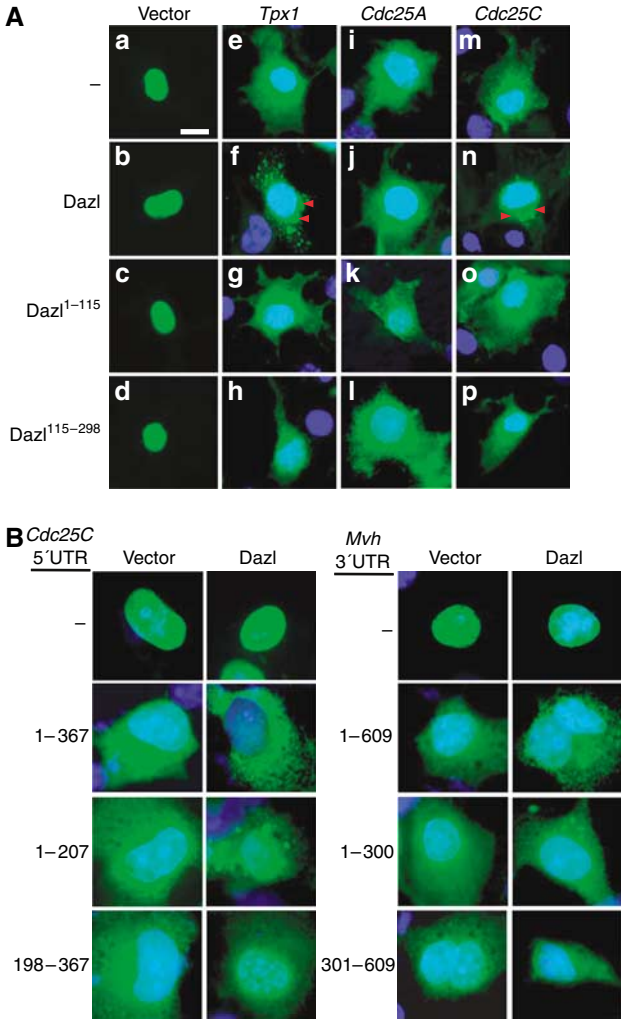


**Figure 5** Specific association of Dazl with the *Cdc25C*, *Tpx1* and *Mvh* mRNAs. (**A**) The COS7 cells were transfected with *pSL-MS2* linked to *Tpx1 3′UTR* (*pMS-Tpx1*), *Cdc25A 3′UTR* (*pMS2-Cdc25A*) or *Cdc25C 5′UTR* (*pMS2-Cdc25C*), along with *pHA-Dazl*, *pHA-Dazl*$^{1-115}$, or *pHA-Dazl*$^{115-298}$. The subcellular localization of the fusion mRNAs was traced using the fluorescent signals of the MS2-GFP protein. The *MS2–Tpx1* and *MS2-Cdc25C* fusion mRNAs formed aggregates in the perinuclear area of the cells (arrowheads) scale bar, 10 μm. (**B**) The COS7 cells were transfected with *pSL-MS2* linked to *Cdc25C 5′UTR* of full-length (1–367) or truncated (1–207 and 198–367) sequences or to *Mvh 3′UTR* of full-length (1–609) or truncated (1–300 and 301–609) sequences along with *pHA* or *pHA-Dazl*. The subcellular localization of the fusion mRNAs was traced as described.

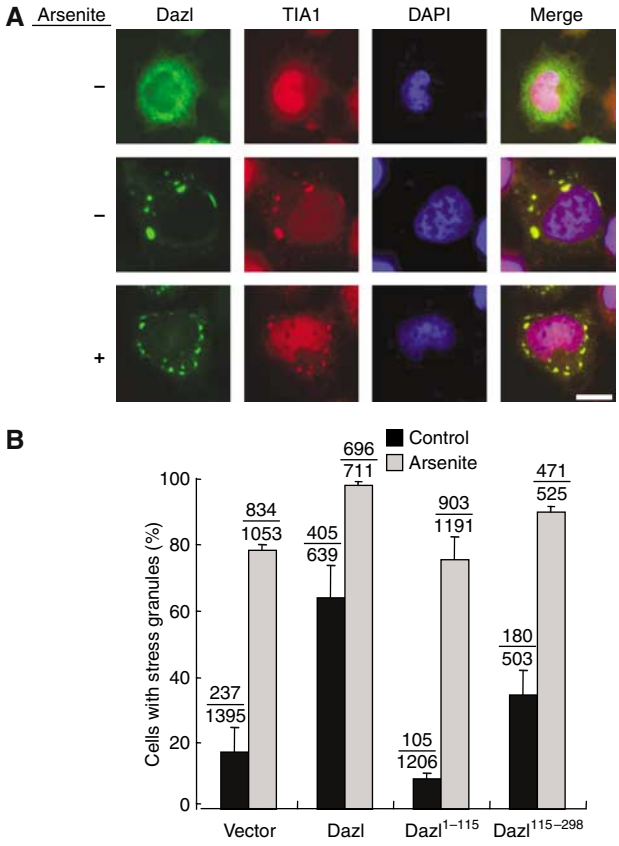


**Figure 6** Subcellular localization of Dazl. (**A**) The HeLa cells with HA-Dazl were coimmunostained with antibodies against HA and TIA1, a stress-granule marker. The cells were treated with arsenite (0.5 mM) to induce the formation of the stress granules. The DNA was stained with DAPI. Scale bar, 10 μm. (**B**) The HeLa cells transfected with HA-Dazl, HA-Dazl$^{1-115}$ and HA-Dazl$^{115-298}$ were cultured in the presence or absence of 0.5 mM arsenite for 45 min and immunostained with the TIA1 and HA antibodies. These experiments were repeated three times. Total number of the cells with stress granules is indicated on the top of the bars.

the Dazl-binding elements could form perinuclear aggregates (Figure 5B). These results confirmed that Dazl binding is required for the specific accumulation of mRNAs in perinuclear areas.

### *Ectopic Dazl accumulates at the stress granule*

In response to an assortment of environmental stresses, cells limit translation activity by accumulating mRNAs at discrete cytoplasmic foci called stress granules (Anderson and Kedersha, 2002; Kimball *et al*, 2003). The mRNAs in the stress granules are not degraded; they are stored and are available for re-initiation as soon as the cell recovers from the stress. Since we observed that only specific mRNAs formed granules in the perinuclear area along with ectopic Dazl (Figure 5A (f and n)), we investigated whether the Dazl-mRNA aggregates in cultured cells were indeed stress granules. In HeLa cells with the dispersed Dazl proteins, TIA1, a stress-granule marker, was detected mostly at the nucleus (Figure 6A). However, in cells with Dazl aggregates, TIA1 was found to be colocalized in the perinuclear area along with Dazl (Figure 6A). When the cells were treated with arsenite for the induction of the stress granule, most of the intracellular Dazl formed aggregates along with TIA1 (Figure 6A). In fact, the ectopic expression of Dazl, but not of Dazl$^{1-115}$ or Dazl$^{115-298}$, induced significant stress granule formation (Figure 6B). These results suggest that Dazl transports specific mRNAs to a stress granule-like body in mouse male germ cells.

## Discussion

In the present study, we observed a specific interaction between Dazl and Dlc in mouse male germ cells. Based on the results, we propose that Dazl functions as an adaptor for mRNAs located on the cytoplasmic dynein motor complex. In other words, Dazl recognizes a specific set of mRNAs, links them to the dynein–dynactin complex, and transports them

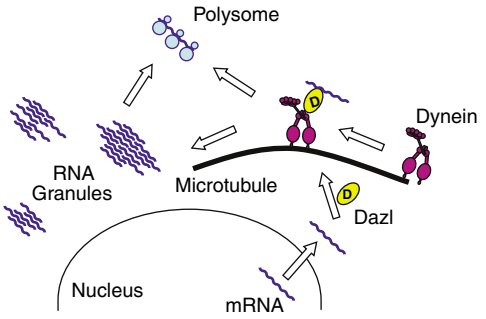


**Figure 7** Model. Dazl recognizes a specific set of mRNAs, links them to the cytoplasmic dynein motor complex and transports them to an RNA granule within the cytoplasm of male germ cells. Some of the Dazl-bound mRNAs may be directly transported to ribosomes.

to an RNA granule that is analogous to the stress granules in male germ cells (Figure 7). The Dazl-bound mRNAs in the RNA granules may be stable but are translationally dormant. When the Dazl-bound mRNAs are released from the RNA granules, they may become translationally active. We do not rule out the possibility that some of these Dazl-bound mRNAs may be directly transported to the ribosomes.

The presence of an active mRNA transport system was rather unexpected in mouse male germ cells wherein the polarized distribution of mRNAs has not yet been reported. Rather, its significance may reside in the translational control of the transported mRNAs. The Dazl-bound mRNAs may be stored to prevent them from being degraded until a proper developmental cue necessitates for their translation. Thus, the spermatocytes can undergo complex and highly elaborate developmental processes such as meiosis with precision.

It remains to be investigated where the Dazl-bound mRNAs are stored within the mouse male germ cells. One candidate site may be the nuage structure, which was initially reported in the oocytes of selected organisms and also in the cytoplasm of the mouse spermatocytes (Fawcett *et al*, 1970; Russell and Frank 1978; reviewed in Ikenishi, 1998). It was known that nuage is essentially an RNA granule with mRNAs for the development and specification of early embryonic cells and proteins for the regulation of mRNA translation and decay (reviewed in Anderson and Kedersha, 2006). The chromatoid body precursor may be the best characterized nuage structure in the mouse male germ cells (Noce *et al*, 2001; Kotaja *et al*, 2006). However, the Dazl-bound mRNAs may be accumulated not only in a single type of RNA granules but also in other types of nuage structures present in spermatocytes, such as Rnf17 granules (Pan *et al*, 2005). Further, we do not rule out the possibility that the Dazl-bound mRNAs accumulate in structures other than RNA granules in male germ cells. Therefore, a thorough investigation of the subcellular distribution of the Dazl-bound mRNAs appears to be critical.

Previous reports in which the Dazl protein was cofractionated with a polysome pool indicated that the Dazl-bound mRNA may be translationally active (Tsui *et al*, 2000b; Maegawa *et al*, 2002). Consistent with this viewpoint, Collier *et al* (2005) reported that Dazl interacted with the PABP and augmented the translation of target mRNAs *in vivo*. On the other hand, the interaction of Dazl with Pum2, a potential translation repressor, was also observed (Moore *et al*, 2003). These results suggest that Dazl functions as a platform for the translational regulation of the bound mRNA. We think that Dazl can perform both functions for the bound mRNAs, that is, active transport and translational regulation. Both these functions may not be required in a competitive situation, as suggested by the fact that the Dazl-binding sites for Dlc are distinct from those for PABP (Collier *et al*, 2005; Figure 1D and E). Therefore, we propose that the translation activity of the Dazl-bound mRNAs may be affected by subcellular location as well as by the translational regulators. For example, the Dazl-bound mRNAs may be translationally active only when a translational activator is in association with Dazl outside the RNA storage granule. Further studies are required to define the physiological role of Dazl in the active transport and translational regulation of mRNA during male germ cell development.

## Materials and methods

### *Yeast two-hybrid screening*

The Matchmaker Two-Hybrid System (Clontech, Palo Alto, CA) was used to isolate the genes that encode the Dazl-interacting proteins. Full-length or truncated mutants of the Dazl proteins were used as baits for screening the mouse testis and HeLa cDNA libraries. Approximately $5 \times 10^6$ colonies were screened with a cotransformation efficiency of approximately $5 \times 10^3$ CFU/μg. The transformants were grown at 30°C for 3–6 days on a medium with the following composition: SD/−His/−Leu/−Trp + 5 mM 3-amino-1,2,4-triazole. Further, the specificity of interaction was tested by a β-galactosidase assay.

### *Plasmid construction*

Mouse *Dazl*, *Dic1* and *Dic2* genes were subcloned into the *pcDNA3.1-HA* vector (Invitrogen) and the mouse *Dlc1* gene was subcloned into the *p3XFLAG-CMV-10* vector (Sigma). Truncated *Dazl* mutants were subcloned into *pGEM-T*, *pCMV-Tag3B* and *pcDNA3.1-HA*, or *pEGFP-C1* (Clontech). The mouse *Dynamitin* gene was subcloned into *pEGFP-C1* (Clontech). The *Cdc25A* 3′UTR, *Cdc25C* 5′UTR, *Tpx1* 3′UTR and *Mvh* 3′UTR sequences were subcloned into the *pSL-MS2-6X* and *pSL-MS2-12X* plasmids (Fusco *et al*, 2003). The *pMS2-GFP* and *pSL-MS2* plasmids were generously provided by Dr R Singer (Albert Einstein College of Medicine, USA).

### *Antibodies*

The Dazl polyclonal rabbit antibody was raised against the GST-Dazl fusion protein and was affinity-purified. Monoclonal Dlc antibodies were purchased from Alexis (Lausanne, Switzerland) and BD Transduction Laboratories (NJ, USA). Monoclonal antibodies against the Flag and HA epitopes were purchased from Sigma (St Louis, MO, USA) and the Berkeley Antibody Company (Richmond, CA, USA), respectively. Antibodies against GFP, Dic, α-tubulin, Myc, TIA1 and $p150^{Glued}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *Cell culture and transfection*

HeLa, 293T and COS7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained in 5% $CO_2$ at 37°C. The plasmids were transfected into the 293T cells by the calcium phosphate method and into the COS7 and HeLa cells by using the LipofectAMINE Plus™ reagent in accordance with the manufacturer's instruction (Invitrogen).

### *Immunoprecipitation, GST pulldown and microtubule precipitation analyses*

For immunoprecipitation, the cells were lysed in the RIPA buffer and centrifuged at 5000 *g* for 30 min at 4°C. Next, 1 mg protein from the supernatant was incubated with the indicated antibodies and precipitated with protein A-Sepharose (Amersham Pharmacia, Buckinghamshire, UK), as recommended by the manufacturer.

For GST pull-down analysis, 5 μg of the GST fusion proteins were incubated with the cell lysates for up to 3 h at 4°C and precipitated with glutathione-Sepharose beads. The beads were washed three times with the lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl,

 

1% NP-40, 25 mM NaF, 2 mM EDTA, 1 mM $Na_3VO_4$, 10% glycerol, 100 mM PMSF and protease inhibitor cocktails). The precipitated proteins were resolved by 10–15% SDS–PAGE and immunoblotted using the indicated antibodies.

The microtubule precipitation assay was carried out by a previously described method (Tavares *et al*, 1996).

#### *Immunocytochemistry and stress granule formation*

The cells were fixed with methanol at −20°C and washed with phosphate-buffered saline. The samples were then incubated with the primary antibodies in 3% BSA for 1 h, washed three times, incubated with fluorescein- or rhodamine-conjugated secondary antibodies, mounted with a mounting solution with DAPI, and observed under a fluorescence microscope (Olympus IX51). The images were photographed using a CoolSnap-Hq CCD camera (Roper Scientific).

In order to induce stress granule formation, HeLa cells were treated with 0.5 mM sodium arsenite for 45 min. The cells were fixed with 3% paraformaldehyde followed by −20°C methanol for 10 min each and immunostained as described.

#### *Live cell imaging of the minus-end-directed proteins*

The COS7 cells containing CFP-Dazl or GFP-$Dazl^{1-115}$ were immersed in a phenol red-free medium and imaged on an Olympus IX71 inverted microscope equipped with differential interference contrast optics, a ×60 fluorite objective (NA 1.2) and a 100 W mercury lamp. Images were acquired at every 2- or 3-s interval using the CoolSnap-Hq CCD camera driven by the MetaMorph imaging software (Universal Imaging, West Chester, PA, USA). The imaging chambers were prepared on glass slides with a border of silicone grease; the slides were sealed with nail polish. The samples were maintained at 37°C by a heater that directed the heat flow across the microscope stage. Image analysis and generation of video clips were performed using the MetaMorph software.

#### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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## References

Anderson P, Kedersha N (2002) Stressful initiations. *J Cell Sci* **115:** 3227–3234

Anderson P, Kedersha N (2006) RNA granules. *J Cell Biol* **172:** 803–808

Brendza RP, Serbus LR, Duffy JB, Saxton WM (2000) A function for kinesin I in the posterior transport of *oskar* mRNA and Staufen protein. *Science* **289:** 2120–2122

Carson JH, Barbarese E (2005) Systems analysis of RNA trafficking in neural cells. *Biol Cell* **97:** 51–62

Cha B, Koppetsch BS, Theukauf WE (2001) *In vivo* analysis of *Drosophila bicoid* mRNA localization reveals a novel microtubule-dependent axis specification pathway. *Cell* **106:** 35–46

Collier B, Gorgoni B, Loveridge C, Cooke HJ, Gray NK (2005) The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. *EMBO J* **24:** 2656–2666

Cosma MP (2004) Daughter-specific repression of *Saccharomyces cerevisiae HO*: Ash1 is the commander. *EMBO Rep* **10:** 953–957

Delanoue R, Davis I (2005) Dynein anchors its mRNA cargo after apical transport in the *Drosophila* blastoderm embryo. *Cell* **122:** 97–106

Eberhart CG, Maines JZ, Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*. *Nature* **381:** 783–785

Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB (1996) Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* **132:** 617–633

Fawcett DW, Eddy EM, Phillips DM (1970) Observations on the fine structure and relationships of the chromatoid body in mammalian spermatogenesis. *Biol Reprod* **2:** 129–153

Ferrandon D, Elphick L, Nusslein-Volhard C, St Johnston D (1994) Staufen protein associates with the 3′UTR of *bicoid* mRNA to form particles which move in a microtubule-dependent manner. *Cell* **79:** 1221–1232

Fox M, Urano J, Reijo RA (2005) Identification and characterization of RNA sequences to which human PUMILIO-2 (PUM2) and deleted in Azoospermia-like (DAZL) bind. *Genomics* **85:** 92–105

Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard JM, Singer RH, Bertrand E (2003) Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr Biol* **13:** 161–167

Gross SP, Welte MA, Block SM, Wieschaus EF (2000) Dynein-mediated cargo transport *in vivo*: a switch controls travel distance. *J Cell Biol* **148:** 945–956

Houston DW, Zhang J, Maines JZ, Wasserman SA, King ML (1998) A *Xenopus DAZ*-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila boule*. *Development* **125:** 171–180

Ikenishi K (1998) Germ plasm in *Caenorhabditis elegans*, *Drosophila* and *Xenopus*. *Dev Growth Differ* **40:** 1–10

Januschke J, Gervais L, Dass S, Kaltschmidt JA, Lopez-Schier H, St Johnston D, Brand AH, Roth S, Guichet (2002) Polar transport in the *Drosophila* oocyte requires dynein and kinesin I cooperation. *Curr Biol* **12:** 1971–1981

Jiao X, Trifillis P, Kiledjian M (2002) Identification of target messenger RNA substrates for the murine deleted in azoospermia-like RNA-binding protein. *Biol Reprod* **66:** 475–485

Karashima T, Sugimoto A, Yamamoto M (2000) *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. *Development* **127:** 1069–1079

Kimball SR, Horetsky RL, Ron D, Jefferson LS, Harding HP (2003) Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes. *Am J Physiol Cell Physiol* **284:** C273–C284

Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, Sassone-Corsi P (2006) The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. *Proc Natl Acad Sci USA* **103:** 2647–2652

Kuersten S, Goodwin EB (2003) The power of the 3′UTR: translational control and development. *Nat Rev Genet* **4:** 626–637

Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC (2001) The *AZFc* region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat Genet* **29:** 279–286

MacDougall N, Clack A, MacDougall E, Davis I (2003) *Drosophila gurken* (TGFα) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps. *Dev Cell* **4:** 307–319

Maegawa S, Yamishita M, Yasuda K, Inoue K (2002) Zebrafish DAZ-like protein controls translation via the sequence 'GUUC'. *Genes Cells* **7:** 971–984

Moore FL, Jaruzelska J, Fox MS, Urano J, Firpo MT, Turek PJ, Dorfman DM, Reijo Pera RA (2003) Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ-Like protein. *Proc Natl Acad Sci USA* **100:** 538–543

Noce T, Okamoto-Ito S, Tsunekawa N (2001) Vasa homolog genes in mammalian germ cell development. *Cell Struct Funct* **26:** 131–136

Pan J, Goodheart M, Chuma S, Nakatsuji N, Page DC, Wang PJ (2005) RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development* **132:** 4029–4039

Reijo R, Lee T-Y, Salo P, Allagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, de la Chapelle A, Silber S, Page DC (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet* **10:** 383–393

Reijo R, Seligman J, Dinulos MB, Jaffe T, Brown LG, Disteche CM, Page DC (1996) Mouse autosomal homolog of *DAZ*, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. *Genomics* **35:** 346–352

Reynolds N, Collier B, Maratou K, Bingham V, Speed RM, Taggart M, Semple CA, Gray NK, Cooke HJ (2005) Dazl binds *in vivo* to specific transcripts and can regulate the pre-meiotic translation of *Mvh* in germ cells. *Hum Mol Genet* **14:** 3899–3909

Ruggiu M, Speed R, Taggart M, KcKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ (1997) The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* **389:** 73–77

Russell L, Frank B (1978) Ultrastructural characterization of nuage in spermatocytes of the rat testis. *Anat Rec* **190:** 79–97

Saunders PT, Turner JM, Ruggiu M, Taggart M, Burgoyne PS, Elliott D, Cooke HJ (2003) Absence of m*Dazl* produces a final block in germ cell development at meiosis. *Reproduction* **126:** 589–597

Saxena R, de Vries JWA, Repping S, Alagappan RK, Skaletsky H, Brown LG, Ma P, Chen E, Hoovers JMN, Page DC (2000) Four *DAZ* genes in two clusters found in the *AZFc* region of the human Y chromosome. *Genomics* **67:** 256–267

Schnorrer F, Bohmann K, Nusslein-Volhard C (2000) The molecular motor dynein is involved in targeting swallow and *bicoid* RNA to the anterior pole of *Drosophila* oocytes. *Nat Cell Biol* **2:** 185–190

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

Slee R, Grimes B, Speed RM, Taggart M, Maguire SM, Ross A, McGill NI, Saunders PT, Cooke HJ (1999) A human *DAZ* transgene confers partial rescue of the mouse *Dazl* null phenotype. *Proc Natl Acad Sci USA* **96:** 8040–8045

St Johnston D (2005) Moving messages: the intracellular localization of mRNAs. *Nat Rev Mol Cell Biol* **6:** 363–375

Tavares AA, Glover DM, Sunkel CE (1996) The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule-associated substrates in *Drosophila* embryo extracts. *EMBO J* **15:** 4873–4883

Tiepolo L, Zuffardi O (1976) Localisation of factors controlling spermatogenesis in the non-fluorecent portion of the human Y chromosome long arm. *Hum Genet* **34:** 119–124

Tsui S, Dai T, Roettger S, Schempp W, Salido EC, Yen PH (2000a) Identification of two novel proteins that interact with germ-cell-specific RNA-bindind proteins DAZ and DAZL1. *Genomics* **65:** 266–273

Tsui S, Dai T, Warren ST, Salido EC, Yen PH (2000b) Association of the mouse infertility factor DAZL1 with actively translating polyribosomes. *Biol Reprod* **62:** 1655–1660

Venables JP, Ruggiu M, Cooke HJ (2001) The RNA-binding specificity of the mouse Dazl protein. *Nucleic Acids Res* **29:** 2479–2483

Vogel T, Speed RM, Ross A, Cooke HJ (2002) Partial rescue of the *Dazl* knockout mouse by the human *DAZL* gene. *Mol Hum Reprod* **8:** 797–804

Wilkie GS, Davis I (2001) *Drosophila wingless* and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. *Cell* **105:** 209–219

Xu EY, Lee DF, Klebes A, Turek PJ, Kornberg TB, Reijo Pera RA (2003) Human *BOULE* gene rescues meiotic defects in infertile flies. *Hum Mol Genet* **12:** 169–175

 