# A micrococcal nuclease homologue in RNAi effector complexes

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**RNA interference (RNAi) regulates gene expression by the cleavage of messenger RNA, by mRNA degradation and by preventing protein synthesis. These effects are mediated by a ribonucleoprotein complex known as RISC (RNA-induced silencing complex)[1]. We have previously identified four *Drosophila* components (short interfering RNAs[1], Argonaute 2 (ref. 2), VIG and FXR[3]) of a RISC enzyme that degrades specific mRNAs in response to a double-stranded-RNA trigger. Here we show that Tudor-SN (tudor staphylococcal nuclease)—a protein containing five staphylococcal/micrococcal nuclease domains and a tudor domain—is a component of the RISC enzyme in *Caenorhabditis elegans*, *Drosophila* and mammals. Although Tudor-SN contains non-canonical active-site sequences, we show that purified Tudor-SN exhibits nuclease activity similar to that of other staphylococcal nucleases. Notably, both purified Tudor-SN and RISC are inhibited by a specific competitive inhibitor of micrococcal nuclease. Tudor-SN is the first RISC subunit to be identified that contains a recognizable nuclease domain, and could therefore contribute to the RNA degradation observed in RNAi.**

Exposure of cells to double-stranded RNA (dsRNA) can elicit various types of sequence-specific gene silencing[4]. A signature of these silencing events is the involvement of small RNAs of approximately 22–25 nucleotides (nt) that guide the selection of silencing targets[1,5,6]. These short interfering RNAs (siRNAs) or microRNAs (miRNAs) are generated by the processing of silencing triggers by an RNaseIII family nuclease, Dicer[7]. Small RNAs join multicomponent ribonucleoprotein (RNP) complexes, known generically as RISCs, which enforce silencing.

Both to address the nature of the RNAi effector machinery in detail, and to examine the relationship between the different effector mechanisms of RNAi, we have biochemically purified a RISC complex from *Drosophila* that degrades its mRNA target, and have sought to identify its protein and RNA components. In multiple, independent purifications of RISC, we identified, along with previously characterized proteins, a potentially novel component corresponding to a *Drosophila* candidate gene, CG7008 (Supplementary Fig. 1). This evolutionarily conserved 103 kDa protein contains five repeats of a staphylococcal/micrococcal nuclease domain (Supplementary Fig. 2). Four of these repeats are intact, whereas the fifth repeat is fused at its amino terminus to a tudor domain, which has been implicated in the binding of modified amino acids[8]. On the basis of this characteristic domain structure, we named the protein Tudor-SN, for tudor staphylococcal nuclease. Through each purification step, Tudor-SN co-fractionated with known RISC components (Fig. 1a, b and Supplementary Fig. 3).

Orthologues of Tudor-SN are found in plants (*Arabidopsis*[9]), *C. elegans*[9,10], mammals[10,11] and *Schizosaccharomyces pombe* (A.M.D., unpublished observations), but not in *Saccharomyces cerevisiae*. To investigate whether a role for Tudor-SN orthologues in RNAi is evolutionarily conserved, we carried out biochemical fractionation of extracts from *C. elegans* and mammalian cells.

We began by preparing cytosolic extracts from synchronized cultures of wild-type *C. elegans*. As in *Drosophila*, a large fraction of the miRNA population can be extracted from the ribosomes (Supplementary Fig. 4). Size fractionation of extracts derived from adult animals revealed that miRNAs eluted from the column in two peaks, representing ~500 kDa and ~250 kDa complexes (Fig. 1c), similar to what had been observed previously in extracts from *Drosophila* S2 cells[3]. Three different miRNAs—*lin-4*, *let-7* and *mir-52*—behaved identically in this assay. By contrast, size fractionation of *C. elegans* egg extract, and examination of complexes containing *mir-40* and *mir-52*, suggested the presence of only the 500 kDa complex (Fig. 1c). Thus, it seems that miRNAs in *C. elegans* can inhabit multiple, distinct RNP complexes, and that the partitioning of miRNAs between these complexes may depend on both the identity of the miRNA and the developmental stage of the organism.

RISC complexes in *C. elegans* have not previously been characterized. We therefore probed whether *Drosophila* RISC components co-fractionated with miRNAs in *C. elegans* extracts. We raised antibodies to F56D12.5 (VIG-1), the worm homologue of *Drosophila* VIG, and F10G7.2 (TSN-1), the worm orthologue of Tudor-SN. Both VIG-1 and TSN-1 were enriched in the fractions that contained 250 kDa miRNA (Fig. 1c). By contrast, VIG-1 or TSN-1 did not appear in fractions containing 500 kDa miRNA complex.

To test whether putative RISC components are present in

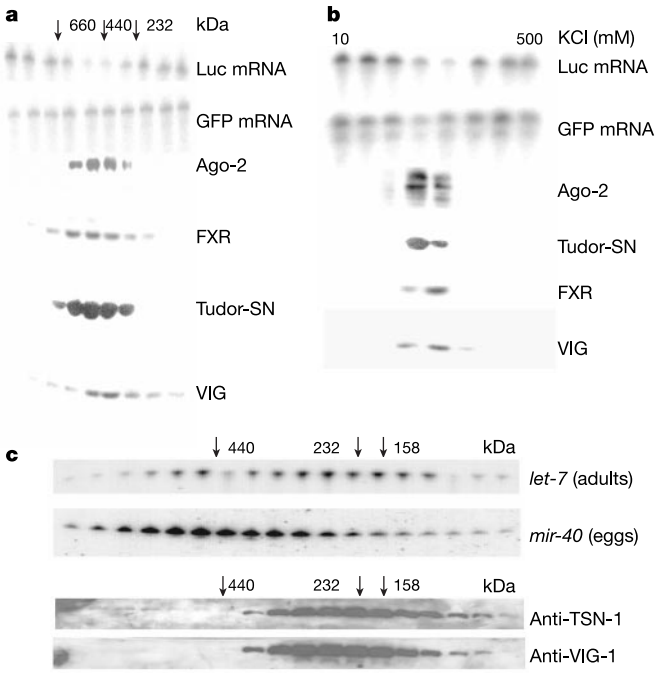


**Figure 1** Identification and confirmation of Tudor-SN as a component of RISC complexes. **a**, *Drosophila* RISC activity was extracted from ribosomes and fractionated on Superose 6. The migration of size markers is indicated. RISC assays and controls for nonspecific activity were carried out as in ref. 1. Western blots were done using antibodies to Ago-2, VIG, FXR and Tudor-SN. **b**, The active gel-filtration fractions from **a** were further chromatographed on Source Q. Fractions were analysed as in **a**. **c**, Size fractionation of *C. elegans* ribosome-associated extracts prepared from both eggs and adults. The fractions were analysed by northern blotting. Egg-derived extract is probed for *mir-40*, the adult extract for *let-7*. Similar results are obtained when these blots are probed for *mir-52*. Below, size-fractionated extract from adult animals, analysed by western blotting as indicated for TSN-1 and VIG-1. GFP, green fluorescent protein; Luc, luciferase.

the same complex, we used antibodies to immunoprecipitate individual components. In *Drosophila*, antibodies to either FXR or VIG co-immunoprecipitated Argonaute 2 (Ago-2; Fig. 2a), as was predicted by our previous findings using epitope-tagged versions of these proteins[3]. Similar amounts of Ago-2 were also co-immunoprecipitated using antibodies directed against Tudor-SN (Fig. 2a). Furthermore, antibodies directed against FXR and VIG co-immunoprecipitate Tudor-SN (Fig. 2a), and VIG and Tudor-SN antisera conversely recover FXR (Fig. 2a), indicating that all four proteins are present in a single complex. In *C. elegans*, VIG-1-specific antibodies can co-immunoprecipitate TSN-1 (Fig. 2b), indicating a similar association of the *C. elegans* orthologues of *Drosophila* RISC proteins.

In naive mammalian cells, we had difficulty detecting interactions between orthologues of *Drosophila* RISC components (Fig. 2c). However, the formation of a complex containing these proteins was induced if we first triggered an RNAi response by transfection with siRNAs. Specifically, we showed interactions between an Argonaute family protein, AGO2 (human GERp95/EIF2C2/AGO2; reviewed in ref. 12), the fragile X mental retardation protein (FMRP) and the mammalian Tudor-SN homologue, p100 (Fig. 2c). Notably, complex formation occurred without changes in the expression levels of individual RISC components, indicating that association of pre-existing proteins is nucleated when a siRNA becomes available in the cell (Fig. 2c).

RISC is an RNP complex that can contain either siRNAs or miRNAs[1–3,13]. Consistent with their roles as components of RISC, both miRNAs (Fig. 2d) and siRNAs (Fig. 2e) can be co-immunoprecipitated from *Drosophila* cells using antisera that recognize FXR, VIG and Tudor-SN. Similarly, in *C. elegans*, TSN-1 and VIG-1 immunoprecipitates contain *let-7* RNA (Fig. 2f). In addition, we find the mammalian *let-7* miRNA in immunoprecipitates of p100, and in parallel confirm the previously demonstrated association between miRNAs and AGO2 (refs 13–15; Fig. 2g). We also detect miRNAs in association with members of the fragile X family in mammalian cells, including FMRP, FXR1 and FXR2 (S.M.H., unpublished observations). Considered together, our results point to a common architecture for RISC in animals as a complex that contains a small RNA (miRNA or siRNA) and protein components that include an Argonaute family member, VIG, Tudor-SN and, at least in *Drosophila* and mammals, a fragile X family member.

The mammalian homologue of Tudor-SN, known as p100, has

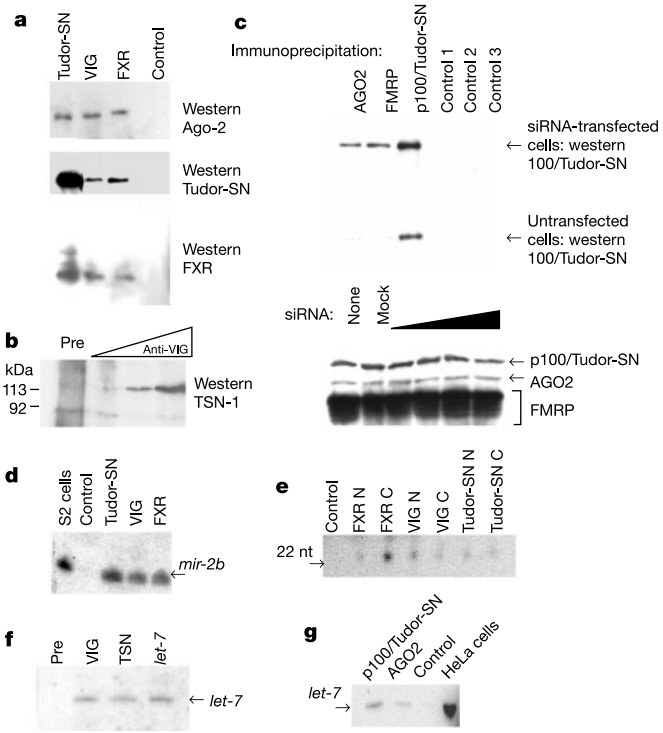


**Figure 2** Immunoprecipitations from multiple organisms confirm association between Tudor-SN and components of RISC. **a**, Immunoprecipitations from *Drosophila* S2 cells were done using the indicated affinity-purified antibody or a control antibody—affinity-purified antibody to the Drosha protein. Western blots of the immunoprecipitates were as indicated. **b**, Immunoprecipitations from *C. elegans* were performed with a nonspecific serum (Pre) or an increasing amount of VIG-1-specific antibody (2, 5 and 10 μl anti-VIG). Precipitates were western-blotted using an anti-TSN-1 antibody. **c**, Immunoprecipitations from 293 cells were done using the indicated antibodies, and precipitates were blotted for p100/Tudor-SN. The control antibodies were as follows: control 1, non-immunized rabbit serum; control 2, irrelevant mouse ascites; control 3, non-immunized guinea pig serum. As indicated, the top panel of immunoprecipitations was done using cells previously transfected with siRNAs corresponding to luciferase, the bottom panel was done with untransfected cells. Below, westerns as indicated on cells untransfected (none), mock-transfected (mock) or transfected with siRNAs at a concentration of 10–100 nM. **d**, RNA was prepared from the indicated *Drosophila* immunoprecipitates as in **a**, and northern blotted for *mir-2b*, a miRNA expressed in S2 cells. **e**, *Drosophila* S2 cells were transfected with radiolabelled dsRNA. Two days after transfection, immunoprecipitations were performed as in **a**, and RNA was analysed on a 15% denaturing polyacrylamide gel. **f**, Immunoprecipitation from *C. elegans* was performed with a nonspecific serum (Pre), VIG-1-specific antibodies or TSN-1-specific antibodies. RNA was isolated from immunoprecipitated material and blotted for *let-7*. **g**, RNA was prepared from the indicated HeLa cell immunoprecipitates and blotted for *let-7*.

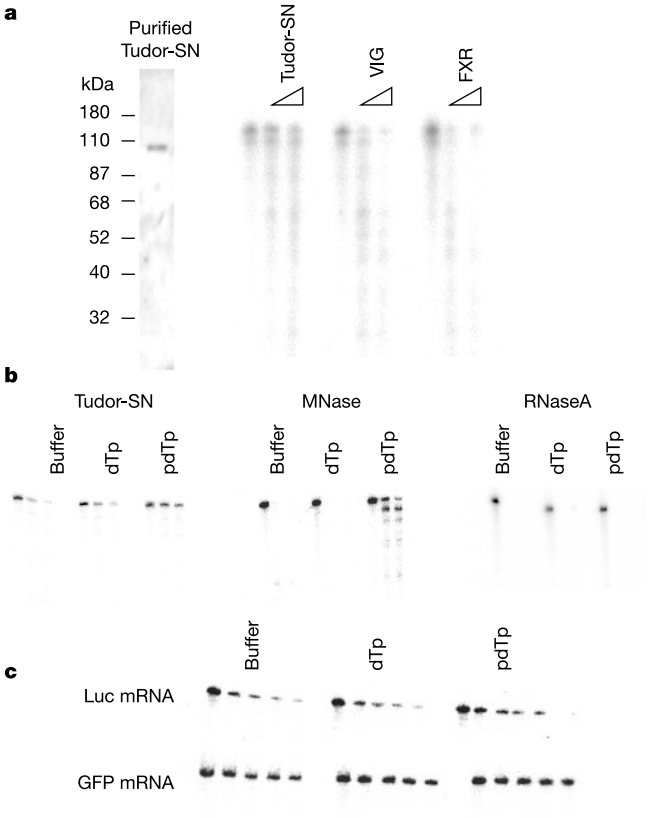


**Figure 3** Tudor-SN has nuclease activity. **a**, 6×His N-tagged *Drosophila* Tudor-SN was purified to homogeneity. A silver-stained gel is shown. Purified Tudor-SN fractions were immunodepleted with the indicated antibodies. The immunodepleted supernatants were tested for activity against single-stranded RNA. **b**, Purified Tudor-SN was treated with 100 μM pdTp, dTp or a buffer control, substrate RNAs were added, and reaction time points of 0, 30 and 60 min were taken. **c**, Fractions containing partially purified RISC were treated for 15 min with 100 μM pdTP, dTp or a buffer control, substrate RNAs were added, and reaction time points of 0, 15, 30, 45 and 60 min were taken.

been implicated as a co-activator for an Epstein–Barr virus transcription factor, EBNA-2 (ref. 10). An exclusively nuclear localization of Tudor-SN would be inconsistent with a role in RNAi, as many studies of RNAi in *C. elegans*, *Drosophila*, *Neurospora* and mammals have shown that post-transcriptional gene silencing by RNAi occurs largely in the cytoplasm. In both *Drosophila* and mammalian cells, Tudor-SN/p100 was present predominantly in cytoplasmic fractions (Supplementary Figs 5, 6). Examination of Tudor-SN immunoreactivity also showed a predominantly cytoplasmic localization (Supplementary Fig. 7; J.M.S., unpublished observations; ref. 16). In *C. elegans*, TSN-1 is found in significant amounts both in the nucleus and in the cytosol (not shown).

RISC is a nuclease that catalyses endonucleolytic cleavage of substrates, as directed by the associated siRNA. In many cases, siRNAs also trigger mRNA degradation, and the results of our biochemical purification are consistent with an association between RISC and a nuclease that catalyses complete destruction of targeted mRNAs[1]. To assess the possibility that Tudor-SN might contribute to catalysis by RISC, we examined its intrinsic nuclease activity. All five staphylococcal nuclease domains of Tudor-SN contain mutations that alter the canonical active site, as derived from comparisons of bacterial family members and from structural data[9,10]. A large panel of mutations has been made in staphyloccocal nuclease, some of which are similar to those that alter Tudor-SN domains away from the active-site consensus[17]. Generally, these mutations lower the reaction rate but do not abolish catalysis.

We expressed and purified recombinant *Drosophila* Tudor-SN from *Escherichia coli* (Fig. 3a). Nuclease activity precisely co-fractionated with the recombinant protein (not shown), and monospecific carboxy-terminal Tudor-SN anti-peptide antibodies selectively depleted activity from purified Tudor-SN preparations (Fig. 3b). In these depletion experiments, nuclease activity was recovered on antibody–sepharose complexes (not shown). Micrococcal nucleases show broad substrate specificity, cutting both RNA and DNA. Similarly, Tudor-SN can cleave both RNA and DNA substrates (not shown).

3′,5′-Deoxythymidine bisphosphate (pdTp), known to be a specific competitive inhibitor of staphylococcal nucleases[18], inhibits Tudor-SN at 100 μM concentrations, whereas dTp (3′-deoxythymidine monophosphate) does not inhibit Tudor-SN activity (Fig. 3d). Importantly, RISC activity is also inhibited by pdTp (Fig. 3e). These data are consistent with the possibility that Tudor-SN contributes at least some of the nuclease activity observed in RNAi effector complexes, but are also consistent with other interpretations (see below).

To examine the role of Tudor-SN in dsRNA-mediated silencing *in vivo*, we made use of a reporter system in *C. elegans*. A *lacZ–lin-41* fusion transgene expresses LacZ in the seam cells under the translational control of *let-7* (ref. 19). In wild-type animals, LacZ staining in the seam cells can be observed from the L1 stage through to L4. The staining is absent in adult animals as a consequence of *let-7*-mediated repression (Fig. 4a, b). As expected, suppression of *alg-1* and *dcr-1* by RNAi resulted in persistence of LacZ staining in adults[20–22] (Fig. 4c, d). Next, we used RNAi to analyse the involvement of VIG-1 and TSN-1 in *let-7* function. RNAi against either VIG-1 or TSN-1 results in persistent expression of the LacZ reporter in the seam cells of adult animals (Fig. 4e, f). This shows that VIG-1 and TSN-1 are required for proper function of the *let-7* miRNA *in vivo*. By contrast, after RNAi against VIG-1, TSN-1 and DCR-1, we do not see an effect on RNAi efficiency. The effects we observe after RNAi against VIG-1, TSN-1 and DCR-1 (refs 20–22) probably reflect a partial reduction of function, as other phenotypes associated with *let-7* loss of function (vulva and alae defects[23]) are not observed.

Our data strongly indicate that Tudor-SN is a bona fide RISC component. This is reflected by the co-purification of Tudor-SN and RISC in *Drosophila*, *C. elegans* and mammalian cells. However, it remains open to question whether Tudor-SN is a catalytic engine of RNAi. Despite the aforementioned data, there are potential inconsistencies. First, purified, recombinant Tudor-SN is non-sequence-specific, in contrast to RISC, which shows a high degree of selectivity for its mRNA targets. Second, Tudor-SN will cleave both RNA and DNA, whereas we detect no DNase activity in RISC (data not shown). Third, several investigators have detected specific cleavage of mRNAs within the siRNA–mRNA hybrid, and this is difficult to rationalize with the known activities of Tudor-SN and related enzymes[6,13,24,25]. It is certainly consistent with our biochemical data to suppose that RISC contains multiple nucleases, only one of which (the putative Slicer) can catalyse site-specific mRNA cleavage. In this scenario, Tudor-SN might act to degrade the remainder of the mRNA. In accord with this idea, targeting of an mRNA by a single siRNA often results in complete degradation of the mRNA[26,27]. Alternatively, it is possible that Tudor-SN does not have a catalytic role in the RISC complex. Indeed, pdTp is a competitive inhibitor that engages the potential nucleic-acid-binding domains of Tudor-SN. Thus, inhibition of RISC by pdTp may reflect a block in the ability of Tudor-SN to engage RNAs, possibly including the mRNA target, in the context of the RISC complex. Answers to these questions will come only from understanding RISC in sufficient detail to allow reconstitution of its native activity from purified components such that we can study in detail the individual contributions of each to the varied roles of the RNAi effector machinery. □

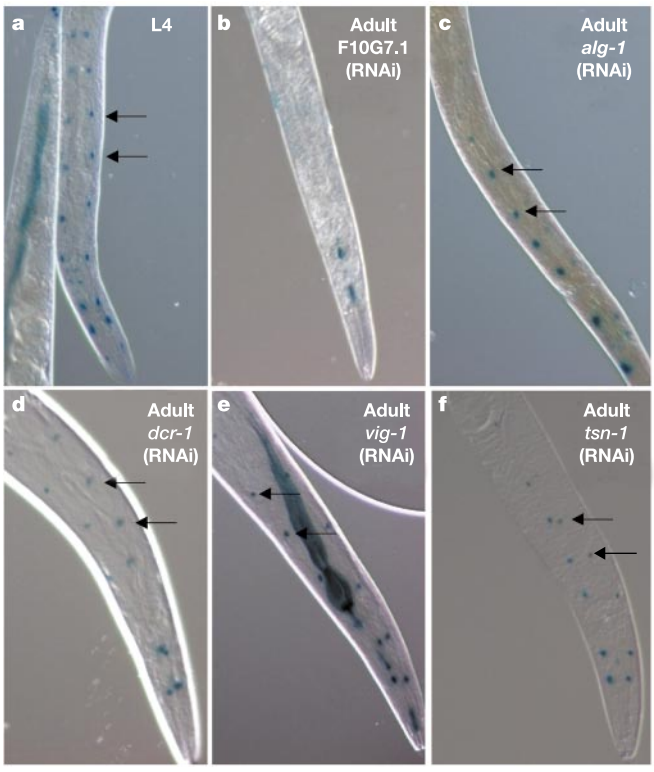


**Figure 4** Staining of animals carrying a transgene expressing LacZ in the seam cells, under the translational control of *let-7*. In L4-stage animals, LacZ is expressed (**a**), but in adult animals the LacZ expression is repressed, also after performing RNAi against a control gene that is in the same operon as TSN-1 (22 of 23 animals silenced) (**b**). RNAi against genes that are involved in miRNA function and processing (*alg-1* and *dcr-1*) results in the re-expression of LacZ in adult animals (**c**, 24 of 28 animals; **d**, 15 of 29 animals). RNAi against *vig-1* and *tsn-1* also results in re-expression of LacZ in adults (**e**, 17 of 33 animals; **f**, 14 of 32 animals).

## Methods

### *Drosophila* cell culture and extract preparation

S2 cells were soaked with luciferase dsRNA at 3 mg l$^{-1}$ and harvested after 7 days. Hypotonic

extracts were spun at 200,000*g* for 3 h to pellet ribosomes, which were resuspended and extracted in 400 mM potassium acetate. Resultant soluble protein was precipitated by 1:4 dilution in hypotonic buffer and redissolved in buffer A (20 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 2 mM dithiothreitol and 0.5% octyl glucoside) with 400 mM KCl. This protein was then fractionated on two Superose-6 HR10/10 columns (Pharmacia) in series. Fractions were assayed for RISC activity as described[1]. The fraction showing peak activity was diluted 1:5 in buffer A and fractionated over a Mono Q HR5/5 column (Pharmacia) with a linear gradient elution of buffer A with 0–500 mM KCl. The RISC fraction with peak activity from the Mono Q column was used for the EDTA/EGTA experiments described here. The purification of RISC for Tudor-SN identification was essentially as described[2].

### *C. elegans* culture and extract preparation

Nematodes were grown in liquid culture. At the appropriate stage, the animals were collected by sedimentation on ice, and purified by flotation on 30% sucrose. After washing, the animals were resuspended in 0.5 volumes of hypotonic buffer: 10 mM HEPES (pH 7.1), 5 mM $MgCl_2$, 2 mM DTT and protease inhibitors (Complete protease inhibitor tablets; Roche). The suspension was dropped into liquid nitrogen, and the resulting balls were ground in a mortar. The powder was allowed to thaw on ice, and was centrifuged for 10 min at 14,000*g*. The supernatant was used as crude extract. The crude extract was then spun at 117,000*g* for 2 h to obtain a non-ribosome-associated supernatant fraction (S100) and a ribosome-pellet fraction (P100). The P100 was resuspended in hypotonic buffer supplemented with 500 mM NaCl, and mixed at 4 °C for 2 h. This was again separated in a ribosome-pellet fraction and a ribosome-associated supernatant fraction (S100H) by centrifugation at 117,000*g* for 2 h. The S100H fraction was used in most experiments. For egg extracts, animals were grown on bacteria that expressed *pos-1* dsRNA. Eggs were purified from sucrose-purified gravid adults by bleach treatment, and the resulting egg pellet was washed extensively in M9 buffer before extract preparation as described above.

Extracts were size-fractionated on a Superdex 200HR10/30 column (Amersham). Fractions of 250 μl were collected. We used the following size markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and albumin (67 kDa).

### Expression and purification of full-length Tudor-SN

The complementary DNA was amplified with appropriate restriction sites, cloned into pENTR11 (Invitrogen), and transferred by means of Gateway (Invitrogen) to pDEST17, which has a 6×His N-terminal tag. The plasmid was transformed into BL21 pLysS (Stratagene). Cells were grown to a density of ~0.5 and induced using 1 mM IPTG for 1 h. Cells were freeze-thawed for lysis, suspended in 300 mM NaCl and 50 mM $NaPO_4$ (pH 7), spun for 20 min at 20,000 *g* to clear, and loaded onto a HR 10/10 Talon column (Clontech). The peak fractions were diluted with five volumes of buffer B (buffer A plus 2 mM $CaCl_2$) and eluted from Source Q on a gradient of buffer B from 0 to 1 M KCl. The peak from Q was chromatographed in the same manner on Source S, and finally purified by gel-filtration on Superdex 200, the column being developed using buffer B with 150 mM KCl.

### Antibodies

Peptides were synthesized corresponding to eight amino acids from the N and C termini of FXR, VIG and Tudor-SN. Peptides were conjugated to KLH (Pierce) and injected into rabbits. The antisera were affinity-purified using agarose-coupled peptide (Pierce). The FMRP antibody was purchased from Covance.

### Immunoprecipitation

For *Drosophila* immunoprecipitations, cells were lysed in 150 mM NaCl, 0.1% NP-40, 20 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM DTT and protease inhibitors (Roche). Primary complexes were formed for 1–4 h, and immunoprecipitates were collected using a mix of Protein A–agarose (Pierce) and Protein G–agarose (Roche). Washes were with the same buffer, except that NaCl was adjusted to 400 mM. For HeLa and HEK 293 immunoprecipitation, the buffer was modified to contain 0.5% NP-40, and washes were in the same buffer. For immunoprecipitation–western experiments, cells were previously transfected with siRNAs (Invitrogen). *C. elegans* immunoprecipitations were performed using S100H extract. Extract was incubated with antibody and protein A/G Plus-agarose beads (Santa Cruz) for a period ranging from 5 h to overnight. After extensive washing, the beads were put in SDS–polyacrylamide gel electrophoresis loading buffer, or RNA was isolated using Trizol.

### Fractionation of nuclei and cytoplasm

For *Drosophila* cells, fractionation was as described in ref. 28. For 293 cells, fractionation was performed as described in ref. 29. Histone H3 antibody was purchased from Upstate; dynein IC antibody was purchased from Covance.

### Immunodepletion

Purified Tudor-SN fractions from Superdex were subjected to two rounds of immunoprecipitation using approximately 10 μg of affinity-purified antibody and protein A/G beads.

### Immunofluorescence

Immunofluorescence was carried out using a modification of the protocol described in ref. 30. The concentration of paraformaldehyde used was 0.5%.

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