Drosophila Ana2 is a conserved centriole duplication factor

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Introduction

The centriole is composed of a radial array of nine microtubule (MT) triplets, doublets, or singlets depending on species and cell type. Centrioles are required to make two important cellular structures: centrosomes and cilia. The centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM) and is the major MT organizing center in many animal cells (Rieder et al., 2001; Doxsey et al., 2005). Cilia are formed when the centriole pair migrates to the cell cortex, and the older, mother, centriole forms a basal body that nucleates the ciliary axoneme. Many different cell types possess cilia, and they have multiple roles in development (Gerdes et al., 2009).

To ensure their inheritance by each daughter cell, centrioles duplicate precisely once per cell cycle. This process must be tightly regulated. Failure in centriole duplication leads to catastrophic errors during embryogenesis in both worms and flies (O’Connell et al., 2001; Stevens et al., 2007), and an increasing number of human diseases have been linked to defects in centrosome and/or cilia function (Badano et al., 2005; Sharma et al., 2008). Centriole overduplication can be equally damaging, as excess centrioles are frequently observed in human tumors (Nigg, 2002), and there appears to be a direct causative relationship between centriole overduplication and tumorigenesis in flies (Basto et al., 2008).

In canonical centriole duplication, a new daughter centriole grows at a right angle to the mother centriole. A series of genome-wide RNAi and genetic screens in worms have found just five proteins essential for centriole duplication: SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4. Functional orthologues of all but SAS-5 have been found in other species. In Drosophila melanogaster and humans, Sak/Plk4, DSas-6/hSas-6, and DSas-4/CPAP—orthologues of ZYG-1, SAS-6, and SAS-4, respectively—are required for centriole duplication. Strikingly, all three fly proteins can induce the de novo formation of centriole-like structures when overexpressed in unfertilized eggs. Here, we find that of eight candidate duplication factors identified in cultured fly cells, only two, Ana2 and Asterless (Asl), share this ability. Asl is now known to be essential for centriole duplication in flies, but no equivalent protein has been found in worms. We show that Ana2 is the likely functional orthologue of SAS-5 and that it is also related to the vertebrate STIL/SIL protein family that has been linked to microcephaly in humans. We propose that members of the SAS-5/Ana2/STIL family of proteins are key conserved components of the centriole duplication machinery.
role in centriole duplication in other systems. In Drosophila, for example, the kinase Sak, which is related to ZYG-1, and the homologues of SAS-6 (DSas-6) and SAS-4 (DSas-4) are required for centriole duplication (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a). Recently, however, several additional proteins have been identified in cultured fly cells that are potentially involved in centriole duplication (Goshima et al., 2007; Dobbelraere et al., 2008). Here, we set out to identify which of these potential duplication factors are likely to function as upstream regulators of centriole formation.

Results and discussion

Ana2 and Asterless (Asl) can drive the de novo formation of centriole-like structures

Genome-wide RNAi screens in cultured fly cells identified just 18 proteins that, when depleted, gave a reduced number of centrioles (Goshima et al., 2007; Dobbelraere et al., 2008). This list includes Sak, DSas-6, and DSas-4, as well as eight other proteins that specifically localize to centrosomes (Ana1, Ana2, Ana3, Asl, DCP110, DCEp135/Bld10, Dce97, and Rcd4); these eight are therefore good candidates to play a direct role in centriole duplication.

GFP-Sak, GFP–DSas-6, and DSas–4–GFP share the unique ability to drive de novo formation of centriole-like structures in unfertilized eggs when highly overexpressed from the upstream activation sequence (UAS) promoter (Peel et al., 2007; Rodrigues-Martins et al., 2007b). UAS-GFP-Sak and UAS-GFP–DSas-6 induce these structures in ~95% of unfertilized eggs, whereas UAS–DSas–4–GFP does so in ~60% of unfertilized eggs (Peel et al., 2007). We wondered if we could use this assay to identify other components likely to function upstream in the centriole duplication pathway. We therefore generated transgenic lines carrying GFP fusions to all eight potential duplication factors under the control of the UAS promoter, which allowed us to overexpress them in unfertilized eggs (Fig. S1). Strikingly, only Ana2 (in 97% of eggs) and Asl (in 33% of eggs) were able to drive de novo formation of centriole-like structures (Fig. 1).

Asl has recently been shown to be essential for centriole duplication in flies (Blachon et al., 2008), whereas, of the six proteins unable to induce de novo centriole formation, two, DCEp135/Bld10 and Ana3, are now known not to be essential for centriole duplication in flies (Mottier-Pavie and Megraw, 2009; Stevens et al., 2009). These findings indicate that our overexpression assay can identify those proteins likely to be most intimately involved in centriole duplication. As Asl has already been shown to be required for centriole duplication (Blachon et al., 2008), we focused on investigating the function of Ana2.

Ana2 is an important regulator of canonical centriole duplication

Ana2 can drive de novo formation of centriole-like structures as efficiently as DSas-6 and Sak (Peel et al., 2007; Rodrigues-Martins et al., 2007b). We wanted to verify, however, that it also has a role in canonical centriole duplication. Overexpressing GFP-Sak or GFP–DSas-6 from the ubiquitin (Ubq) promoter induces centriole overduplication in brains and embryos, respectively (Peel et al., 2007). Surprisingly, however, overexpression of Sak, DSas-6, or DSas-4 cannot drive centriole overduplication in primary spermatocytes (Peel et al., 2007), which suggests that another duplication protein is limiting. To test if Ana2 might be this limiting factor, we generated Ubq-GFP-Ana2 transgenic lines. Strikingly, we found that in spermatocytes expressing Ubq-GFP-Ana2, in addition to the normal centriole pairs (doublets), we observed centriole triplets, quadruplets, and even quintets (Fig. 2, A–G). The extra centrioles in these clusters appeared to be fully functional; they separated from one another by the end of meiosis I (as centriole doublets normally do), and the extra centrioles inherited by secondary spermatocytes recruited PCM and nucleated MT asters, and so formed multipolar spindles during meiosis II (Fig. 2, H and I).

We did not observe centriole overduplication in embryos or brain cells expressing Ubq-GFP-Ana2 (unpublished data), which is consistent with DSas-6 and Sak levels, respectively, limiting centriole formation in these tissues. Nevertheless, that Ana2 overexpression can drive centriole overduplication in spermatocytes demonstrates that it is an important regulator of canonical centriole duplication.

Ana2 shows a unique asymmetric localization to the daughter centriole

We next wanted to compare the localization of Ana2 with that of the other Drosophila centriole duplication factors. DSas–4–GFP, GFP–DSas-6, and GFP-Sak are all enriched at the proximal and distal ends of the large spermatocyte centrioles (Peel et al., 2007). We found that, likewise, Ana2-GFP localized preferentially to the proximal and distal centriole tips. Strikingly, however, Ana2-GFP (and GFP-Ana2) also exhibited a unique asymmetric distribution, consistently localizing preferentially along one centriole barrel (Fig. 3, A and B).

In primary spermatocytes, it is possible to distinguish mother and daughter centrioles, as the daughter can often be observed associating end-on with the side of the mother (Blachon et al., 2008). In 25 centriole pairs where we could unambiguously distinguish mother and daughter centrioles, Ana2-GFP was always enriched on the daughter (Fig. 3 A). Mother and daughter centrioles can show important differences in their behavior in vertebrate cells (Piel et al., 2000) and during asymmetric stem cell divisions in Drosophila (Rebollo et al., 2007; Rusan and Peifer, 2007; Yamashita et al., 2007). Although mother and daughter centrioles are morphologically and molecularly distinguishable in vertebrates (see, for example, Vorobjev and Chentsov, 1982; Chang et al., 2003; Gromley et al., 2003; Graser et al., 2007), this is not the case in Drosophila (Callaini and Riparbelli, 1990; Callaini et al., 1997; Vidwans et al., 2003). To our knowledge, Ana2-GFP is the first fly protein shown to localize asymmetrically to mother and daughter centrioles in this manner.

Interestingly, as spermatocytes progressed through meiosis I, this centriolar asymmetry became less pronounced, and this appeared to reflect the selective loss of GFP-Ana2 from the daughter centriole, bringing its levels down to that of the mother (compare Fig. 3 B, showing a G2 centriole pair, to Fig. 3 C,
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• Stevens et al. (D-PLP; Martinez-Campos et al., 2004), continue to localize along the basal body. In contrast, Ana2, like the conserved duplication proteins (Blachon et al., 2009), was undetectable along the basal body (Fig. 3 D). Ana2 did, however, colocalize with GFP–DSas-6 at the proximal centriole-like structure (Fig. 3 D), a small nodule adjacent to the basal body that has been proposed to be an early intermediate in centriole formation (Blachon et al., 2009).

Figure 1. Overexpression of Ana2 or Asl drives de novo formation of centriole-like structures. (A and B) Unfertilized eggs laid by UAS-Ana2-GFP (A) or UAS-Asl-GFP (B) mothers containing numerous MT asters (stained for tubulin). Arrows indicate the polar bodies. (C and D) Single asters from UAS-Ana2-GFP (C) or UAS-Asl-GFP (D) eggs stained for tubulin (blue) and DSas-4 (red). GFP is in green. Each aster contains several structures that stain for centriole markers. Bars: (A and B) 20 µm; (C and D) 2 µm.

showing a centriole pair separating at the end of meiosis I). As overexpression of Ana2 can lead to centriole overduplication, Ana2 levels presumably must normally be tightly regulated to prevent the formation of extra centrioles.

After exit from meiosis II, each spermatid inherits a single centriole, which acts as a basal body to nucleate the flagellar axoneme. Structural components of the centriole, like Ana3 (Stevens et al., 2009) and Drosophila pericentrin-like protein (D-PLP; Martinez-Campos et al., 2004), continue to localize along the basal body. In contrast, Ana2, like the conserved duplication proteins (Blachon et al., 2009), was undetectable along the basal body (Fig. 3 D). Ana2 did, however, colocalize with GFP–DSas-6 at the proximal centriole-like structure (Fig. 3 D), a small nodule adjacent to the basal body that has been proposed to be an early intermediate in centriole formation (Blachon et al., 2009).
similar in size and have a single central coiled-coil domain, leading Goshima et al. (2007) to suggest that Ana2 could be the Drosophila equivalent of SAS-5 (Goshima et al., 2007). As SAS-5 interacts with SAS-6 in worms (Leidel et al., 2005), we looked for a genetic interaction between Ana2 and DSas-6 in flies.

Ana2 is the likely functional orthologue of Caenorhabditis elegans SAS-5

Intriguingly, Drosophila homologues have been identified for all the C. elegans centriole duplication factors except SAS-5, which has no clear homologues outside worms. Ana2 and SAS-5 are

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**Figure 2. Overexpression of Ana2 drives centriole overduplication in spermatocytes.** (A and B) Centriole number (A) and conformation (B) in G2 primary spermatocytes expressing either the centriole marker RFP-PACT alone or both RFP-PACT and GFP-Ana2. Centrioles were counted in a total of 109 RFP-PACT cells and 138 GFP-Ana2 RFP-PACT cells from seven testes per condition. (C and D) G2 primary spermatocytes expressing either RFP-PACT (red) alone (C) or both RFP-PACT and GFP-Ana2 (D). DNA is in blue. The cell in C has the normal two centriole pairs. Overexpression of GFP-Ana2 induces centriole triplets and quadruplets (D). [E–G] Magnified images of RFP-PACT–labeled doublet (E), triplet (F), and quadruplet (G) centriole groups. (H and I) Secondary spermatocytes in meiosis II expressing either RFP-PACT (red) alone (H) or both RFP-PACT and GFP-Ana2 (I). Tubulin is in green and DNA in blue. The cell in H has the normal two centrioles whereas the one in I has three centrioles forming a tripolar spindle. Bars: (C and D) 10 µm; (H and I) 5 µm.
Interestingly, the centriole-like structures produced by overexpressing UASp-GFP–DSas-6 differ significantly from those resulting from the overexpression of GFP-Sak, DSas-4–GFP, Asl-GFP, or Ana2-GFP in that they are much larger and often appear ring-shaped, and that only one structure is contained within each aster (Fig. 4 E; Peel et al., 2007; Rodrigues-Martins et al., 2007a). The structures in the eggs from females expressing both Ubq-GFP–DSas-6 and Ubq-Ana2-GFP were similar to this DSas-6 type (Fig. 4, D and E). Importantly this interaction was specific to Ana2 and DSas-6. In eggs from mothers carrying one copy of either Ubq-Ana2-GFP or Ubq-GFP–DSas-6 together with one copy of either Ubq-GFP-Sak, Ubq-Asl-GFP, or Ubq–DSas-4–GFP, we observed at most a very small number of asters in very few eggs (Fig. 4 A).
Figure 4. **Ana2 and DSas-6 functionally and physically interact.** (A) Percentage of unfertilized eggs laid by mothers of the given genotypes that contained MT asters. All transgenes were GFP fusions with a Ubq promoter. Eggs from mothers expressing one or two copies of Ubq-GFP–DSas-6 and Ubq-Ana2-GFP were analyzed; all combinations expressed one copy of each transgene. n > 80 eggs per genotype (for values, see Materials and methods). (B and C) Almost all unfertilized eggs from mothers expressing one copy of Ubq-GFP–DSas-6 and one copy of Ubq-Ana2-GFP assemble large numbers of
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Ana2 is related to vertebrate STIL. Schematic of human STIL, Drosophila Ana2, and C. elegans SAS-5. All three proteins have a central, coiled-coil domain (green) and a conserved region near the C terminus (blue): the STAN motif. An alignment of the STAN motif is shown in full, with an alignment including SAS-5 below. Both are colored according to the Blosum62 coloring scheme, where dark blue indicates a match to the consensus sequence and light blue indicates a positive Blosum62 score. Asterisks indicate residues that are identical in all aligned sequences, colons indicate conserved substitutions, and periods indicate semiconserved substitutions.

Figure 5. Ana2 is related to vertebrate STIL. Schematic of human STIL, Drosophila Ana2, and C. elegans SAS-5. All three proteins have a central, coiled-coil domain (green) and a conserved region near the C terminus (blue): the STAN motif. An alignment of the STAN motif is shown in full, with an alignment including SAS-5 below. Both are colored according to the Blosum62 coloring scheme, where dark blue indicates a match to the consensus sequence and light blue indicates a positive Blosum62 score. Asterisks indicate residues that are identical in all aligned sequences, colons indicate conserved substitutions, and periods indicate semiconserved substitutions.
amplified from either cDNA (Ana1, Ana2, Asl, DCP110, DCep135, and DSas-6, Ubq-Ana2-GFP, Ubq-GFP-Ana2, and Ubq-Asl-GFP. The Ubq promoter drives moderate expression in all tissues (Lee et al., 1988), whereas the Ubasp lines were crossed to V32a, which expresses a Gal4/VP16 fusion protein from a maternal tubulin promoter; this drives a very high-level over-expression in the female germline (Peel et al., 2007).

We also used the previously described transgenic lines Ubq-GFP–DSas-6, Ubq-GFP-Sak, Ubq-DSas-4-GFP, and Ubq-MRP–pericentrin/AKAP450 centrosomal-targeting domain (PACT; Peel et al., 2007).

Generation and use of Ana2 antibodies
A maltose-binding protein (MBP, New England Biolabs, Inc.) fusion of an 1–201 of Ana2 was purified according to the manufacturer’s instructions, and antisera were raised in two rabbits by Eurogentec. To affinity purify antibodies, the antisera was first depleted of anti-MBP antibodies by passing over an AminoLink MBP column (Thermo Fisher Scientific). Specific antibodies were then purified by passing the antisera over a column of MBP-Ana2(1–201) fusion protein. The column was washed with PBS + 0.5 M KCl, and antibodies were eluted in 0.1 M glycine, pH 2.1. The antibodies were neutralized with 1 M Tris, pH 8.5, and glyceral was added to 50%, then materials were stored at −20°C.

The antibody was used at 1:250 for immunofluorescence experiments. It weakly stained centrosomes in embryos and the proximal centriole-like structure in spermatids, but it did not stain primary spermatocyte centrioles. To investigate why this was the case, we used the antibody to stain Ana2-GFP-expressing spermatocytes. Here, the antibody stained the distal tips of the centrosomes, but not the proximal ends of the centrioles or the single centriole barrel, even though the Ana2-GFP labeling was clearly visible at these sites. This suggests that the antibody does not stain spermatocyte centrioles for a combination of reasons. First, endogenous Ana2 must be present at centrioles at very low levels, as we cannot detect it even in the distal portion of the centrioles where we can detect Ana2-GFP. Second, endogenous Ana2 is probably not easily accessible to antibodies at the proximal end of the centrioles and along the centriole barrel, as we cannot detect Ana2-GFP with the antibody at these sites even though Ana2-GFP is localized there. For other uses see the “Electrophoresis and immunoblotting” and “Immunoprecipitation” sections.

Fixed analysis of eggs and pupal testes
0–4 h collections of unfertilized eggs were made from mothers expressing UASp-GFP-Ana1 (n = 77), UASp-Ana2-GFP (n = 123), UASp-Ana3-GFP (n = 206), UASp-Asl-GFP (n = 165), UASp-DCP110-GFP (n = 239), UASp-GFP-DCep135 (n = 177), UASp-GFP-DCep97 (n = 172), UASp-GFP-Rcd4 (n = 214), Ubq-GFP-DSas-6 (n = 90), Ubq-GFP-DSas-6/Ubq-Ana2-GFP (n = 92), Ubq-GFP-DSas-6/Ubq-Asl-GFP (n = 84), Ubq-GFP-DSas-6/Ubq-DCep4-GFP (n = 81), Ubq-GFP-DSas-6/Ubq-GFP-Sak (n = 81), Ubq-GFP-DSas-6/Ubq-Asl-GFP (n = 96), Ubq-Ana2-GFP/Ubq-DSas-4-GFP (n = 117), Ubq-Ana2-GFP/Ubq-GFP-Sak (n = 96), and Ubq-Ana2-GFP/Ubq-Asl-GFP (n = 86). Eggs were dechorionated in 60% bleach for 2 min, washed in water + 0.05% Triton X-100, then washed into a small glass bottle with 1 ml heptane. 1 ml methanol + 5% 0.25 M EGTA was added and the bottle was shaken gently until most eggs fell into the lower methanol/EGTA layer. Eggs were stored in methanol at 4°C. For immunostaining, eggs were rehydrated by washing in PBT (PBS + 0.1% Triton X-100), blocked in PBS + 5% BSA, and incubated with primary antibodies in PBS/BSA overnight at 4°C. Eggs were then washed in PBT before incubation with secondary antibodies diluted 1:1,000 in PBT for 4 h at room temperature. After final washes in PBT, eggs were mounted in mounting medium (85% glycerol and 2.5% propilgalliate).

Pupal testes were dissected in PBS, placed on a coverslip, and cut open. A slide was then placed over the coverslip and then flash frozen in liquid nitrogen. Coverslips were removed and the slides were incubated for 5 min in methanol at −20°C, and then in acetone for 1–2 min at −20°C. This was followed by incubation in PBT for 10 min, washes in PBT, and blocking in PBS/BSA (1%). Slides were incubated in primary antibody
(diluted in PBS/BSA) under a mounted coverslip in a moist chamber overnight at 4°C. After washes in PBS, slides were incubated in secondary antibodies (diluted 1:300 in PBS) for 1 h at 25°C. After final washes, slides were mounted in mounting medium.

Slides were observed at room temperature on a spinning disc confocal system (ERS; PerkinElmer), mounted on an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) with a charge-coupled device camera (Orca ER; Hamamatsu), using a 63×/1.4 NA objective (Carl Zeiss, Inc.) with Immersol oil (Carl Zeiss, Inc.). Images were acquired using Ultraview ERS software (PerkinElmer), imported into Photoshop CS2 (Adobe), and adjusted to use the full range of pixel intensities.

Identification of centriole-like structures in eggs

Unfertilized eggs were initially stained for α-tubulin and the centriole markers DSas-4. In D-type WT eggs, the only MTs visible are those surrounding the polar bodies (Peel et al., 2007; Stevens et al., 2009). Under some of the GFP overexpression conditions, MT asters were formed that contained GFP dots at the center, which stained for DSas-4. For these conditions, we went on to stain unfertilized eggs with antibodies against a second centriole marker, DLP, and the PCM proteins centrosomin (Cnn) and γ-tubulin. Structures were considered to be centriole-like if they stained for both of the centriole and both of the PCM markers, and nucleated MT asters. We then quantified the percentage of unfertilized eggs containing these structures for each overexpression condition.

Antibodies

The following antibodies were used: 1:1,000 rabbit anti-DLP (Martinez-Campos et al., 2004), 1:250 rabbit anti–DSas-4 (Basto et al., 2006), 1:500 guinea pig anti-Cnn (Dix and Raff, 2007), 1:1,000 mouse monoclonal anti–α-tubulin (DM1a; Sigma-Aldrich), and 1:1,000 GTU88*, a batch of the mouse monoclonal anti–γ-tubulin GTU88 (Sigma-Aldrich) antibody that cross-reacts with centrioles in flies (Martinez-Campos et al., 2004); Alexa Fluor 488, Cy3, and Cy5 secondary antibodies were obtained from Invitrogen or Jackson ImmunoResearch Laboratories, Inc.

Y2H assay

pDEST22 (prey) and pDEST32 (bait) vectors containing full-length Ana2, full-length DSas-6, DSas-6 NT (aa 1–210), DSas-6 M (aa 104–317), DSas-6 CA (aa 224–414), Ana2 NT (aa 1–200), or Ana2 CT (aa 201–420) were generated as described for the GFP constructs in the “Generation of GFP fusions and transgenic lines” section. Prey vectors were transformed into Y8800 (a) and bait vectors into Y8930 (b) (Boxem et al., 2004). Yeast containing bait or prey constructs were arrayed into 96-well plates. Baits were tested on −Leu, −His medium with different 3-aminoatrazole (3AT) concentrations for autoactivation. Only full-length Ana2 showed strong autoactivation. For the final screen, the yeast strains were mated overnight on yeast extract, peptone, adenine, and dextrose (YPD) plates, and diploids were selected twice on −Leu, −Trp plates. Diploids containing both bait and prey vectors were then screened on −Leu, −Trp, −His with 5 mM or 20 mM 3AT −Leu, −Trp, −Ade plates and by performing an X-gal assay (Invitrogen) according to manufacturer’s instructions. Krev1 was used as a control bait, and interaction was tested with RafGDS-WT (positive) and RafGDS-m2 (negative) according to manufacturer’s instructions (ProLab2; Invitrogen).

Drosophila cell culture

S2 cells were cultured in Schneider’s medium (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen). pUbq-GFP–DSas-6 and pUbq-Ana2-GFP vectors were transfected into S2 cells using cellfectin (Invitrogen) using the pCocktail vector for selection. After 3 wk of selection with blasticidin, stable cell lines were obtained.

Immunoprecipitation

An 80-cm² flask of cells was grown for 3 d and harvested by centrifugation (5 min at 10,000 rpm). Cells were washed once in immunoprecipitation (IP) buffer (PBS, 5 mM EDTA, 1 mM PMSF, 1 mM protease inhibitor [Roche], 25 mM NaF, 1 mM Na3VO4, 20 mM β-glycerolphosphate, and 1× phosphatase inhibitor cocktail [Sigma-Aldrich]) and resuspended in 500 µl of IP buffer. Keeping the cells on ice, they were broken by syringing up and down 20 times using a G24 syringe. Extracts were then centrifuged twice for 15 min at 15,000 rpm. 30 µl of Dynabeads (Invitrogen) were prepared and coupled with 10 µg of antibody (Ana2, DSas-6 [Peel et al., 2007], and random rabbit IgG) using the cross-linker BS3 (Thermo Fisher Scientific) according to the manufacturer’s instructions. 150 µl of extract was added to 30-µl beads and incubated overnight at 4°C. The beads were washed five times with IP buffer and then resuspended in 60 µl of loading buffer. 15 µl was then loaded on a 4–12% gradient precast NuPAGE (Invitrogen) acrylamide gel and analyzed by Western blotting.

Electrophoresis and immunoblotting

WT, UAS-GFP-Ana1, and UAS-Ana2-GFP methanolixed extracts were rehydrated, and 30 µg were selected. Extracts were then boiled in SDS sample buffer. The proteins were separated in a 4–12% gradient precast NuPAGE (Invitrogen) acrylamide gel and transferred to a Hybond-P membrane (GE Healthcare). After transfer, the membrane was blocked in ECL advance blocking solution (PBST [PBS + 0.1% Tween-20] and 2% blocking agent [GE Healthcare]) before incubation with primary antibody overnight at 4°C (diluted to 5 ng/ml for Ana1 and 1 ng/ml for Ana2 in blocking solution). The membrane was then washed in PBST and incubated with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) diluted 1:1,500,000 in PBST for 1 h at room temperature. Finally, the membrane was washed in PBST, incubated with ECL advance chemiluminescent substrate (GE healthcare) according to manufacturer’s instructions, and exposed to x-ray film.

Identification of Ana2 homologues and sequence alignments

The position-specific iterated BLAST (PSI-BLAST) algorithm (Altschul et al., 1997) from the National Center for Biotechnology Information was used to search for homologues of Ana2. Multiple sequence alignments were performed using ClustalW2 (larkin et al., 2007) and visualized in Jalview (Waterhouse et al., 2009) using the Blossum2 coloring scheme.

Online supplemental material

A Western blot showing that both UAS-GFP-Ana1 and UAS-Ana2-GFP are overexpressed at very high levels in unfertilized eggs. Fig. S2 shows the raw data from our Y2H analysis of Ana2 and DSas-6. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200910016/DC1.

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