SUN-1 and ZYG-12, Mediators of Centrosome–Nucleus Attachment, Are a Functional SUN/KASH Pair in *Caenorhabditis elegans*

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Klarsicht/ANC-1/Syne/homology (KASH)/Sad-1/UNC-84 (SUN) protein pairs can act as connectors between cytoplasmic organelles and the nucleoskeleton. *Caenorhabditis elegans* ZYG-12 and SUN-1 are essential for centrosome–nucleus attachment. Although SUN-1 has a canonical SUN domain, ZYG-12 has a divergent KASH domain. Here, we establish that the ZYG-12 mini KASH domain is functional and, in combination with a portion of coiled-coil domain, is sufficient for nuclear envelope localization. ZYG-12 and SUN-1 are hypothesized to be outer and inner nuclear membrane proteins, respectively, and to interact, but neither their topologies nor their physical interaction has been directly investigated. We show that ZYG-12 is a type II outer nuclear membrane (ONM) protein and that SUN-1 is a type II inner nuclear membrane protein. The proteins interact in the luminal space of the nuclear envelope via the ZYG-12 mini KASH domain and a region of SUN-1 that does not include the SUN domain. SUN-1 is hypothesized to restrict ZYG-12 to the ONM, preventing diffusion through the endoplasmic reticulum. We establish that ZYG-12 is indeed immobile at the ONM by using fluorescence recovery after photobleaching and show that SUN-1 is sufficient to localize ZYG-12 in cells. This work supports current models of KASH/SUN pairs and highlights the diversity in sequence elements defining KASH domains.

INTRODUCTION

Current models of nuclear positioning are based on coupling of the nucleoskeleton to the cytoskeleton via protein partners that bridge the nuclear envelope (Starr and Fischer, 2005; Crisp *et al.*, 2006). One partner is an outer nuclear membrane (ONM) protein that binds to a component of the actin or microtubule cytoskeleton and the second is an inner nuclear membrane (INM) protein that binds to the luminal domain of the ONM protein as well as an immobile nuclear structure, like the lamina. These ONM–INM membrane protein pairs can thus connect structural components of the nucleus and cytoplasm across the double membrane of the nuclear envelope, and they have a critical and evolutionarily conserved role in moving or anchoring nuclei within the cytoplasm (Starr, 2007).

In each case where a pair of proposed INM and ONM partner proteins has been identified, the ONM candidate contains a Klarsicht/ANC-1/Syne/homology (KASH) domain, which consists of transmembrane domain and ~30 amino acids that follow it at the C terminus of the protein (Starr and Han, 2002; Starr and Fischer, 2005). Similarly, the INM protein partners contain a conserved domain; the Sad-1/UNC-84 (SUN) domain is localized at the C terminus of the INM partner. The model for topology of these proteins suggests that the KASH domain resides in the space between the INM and ONM where it mediates the interaction with the INM partner. The SUN domain is also proposed to

lie in the intermembrane space. According to the model, the N termini of SUN domain proteins lie in the nucleus and are important for attachment of the complex to the lamina or chromatin. Whereas several ONM–INM protein pairs are proposed to fit this model for bridging the nuclear envelope and various aspects of this model have been tested for some of the KASH–SUN partners (Padmakumar *et al.*, 2005; Crisp *et al.*, 2006; Haque *et al.*, 2006; McGee *et al.*, 2006), the localization, topology, and binding interactions of a SUN–KASH pair have rarely been thoroughly and simultaneously examined.

To investigate the SUN–KASH model for bridging the nuclear envelope, we have focused on the SUN-1 and ZYG-12 pair. We previously reported that *zyg-12* mediates the essential attachment of the centrosome to the nucleus in early *Caenorhabditis elegans* embryos (Malone *et al.*, 2003). *zyg-12* mutants also showed nuclear positioning, migration, and chromosome segregation defects. Based on the observation that ZYG-12 localizes to the nuclear envelope and interacts with cytoplasmic dynein, it is proposed to localize to the ONM, with access to the cytoplasm (Malone *et al.*, 2003). ZYG-12 is also observed on the centrosome, and ZYG-12 homo-dimerizes. Thus, we hypothesize that ZYG-12 mediates the association between the nucleus and the centrosomes through homotypic interaction. Given that the outer nuclear envelope is thought to be contiguous with the endoplasmic reticulum, ONM-specific localization of ZYG-12 is critical for its function. However, the molecular mechanism which restricts ZYG-12 to the ONM has not been fully elucidated.

ZYG-12 nuclear envelope localization requires the SUN domain protein, SUN-1 (Malone *et al.*, 2003). Because SUN-1 interacts with nuclear lamins, it is likely to localize to the INM (Fridkin *et al.*, 2004). We propose that the ZYG-12/

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Figure 1. Conserved mini KASH domain of ZYG-12 B and C together with a transmembrane domain and a part of coiled coil domain (52 amino acids) are sufficient for nuclear envelope localization. (A) Alignment of KASH domains from various proteins. *C. elegans* ZYG-12 is divergent but contains the important proline repeat motif at the C-terminal end. TMAP (Persson and Argos, 1994) predicts the transmembrane domain of ZYG-12 B from 743 to 768 amino acids resulting in nine amino acid (-SALNAPPNA) peptide stretch at the C terminus of the protein. Shading indicates identity and boxes outline conserved residues. (B) Diagrams of ZYG-12 B and ZYG-12 C isoforms and GFP-tagged truncations. CC, coiled-coil domain; TM, transmembrane domain; K, KASH domain. ZYG-12 C is almost identical to ZYG-12 B, it lacks only 16 amino acids in the region before the TM domain. Black bar (RNAi) represents the region of ZYG-12 targeted via RNAi to deplete the endogenous ZYG-12. Numbers indicate the amino acid positions of each construct. (C) Localization of ZYG-12 GFP-tagged truncations in the embryo. Fourth row DIC images demonstrate successful depletion of endogenous ZYG-12 via RNAi as indicated by the detached centrosomes (asterisks). Bar, 10 μ m.

SUN-1 pair is localized to the nuclear envelope and functions to mediate centrosome-nucleus attachment. Although there is evidence that ZYG-12/SUN-1 are a bona fide KASH/SUN pair, direct evidence of their interaction and their topologies is missing. Thorough investigation of the localization, topology, and interactions of the two proteins will provide important insight into the model for function of SUN-KASH pairs as well as the molecular mechanism for linkage of the centrosome and the nucleus. ZYG-12 is also an interesting model for SUN-KASH pair investigations because the KASH domain of ZYG-12 is significantly shorter than other identified family members and contains only four of the eight absolutely conserved residues in other KASH domains (Figure 1A). Thus, our experiments also lend important insight into the potential diversity of KASH domain sequences.

We took advantage of in vivo nuclei that express endogenous ZYG-12 and SUN-1 in combination with in vitro assays to determine that ZYG-12 resides in the outer membrane of the nuclear envelope in vivo and directly interacts

with inner nuclear membrane protein SUN-1 using a fully functional, although divergent, mini KASH domain. We further show that ZYG-12 has restricted mobility at the nuclear membrane by using fluorescence recovery after photobleaching (FRAP) analysis and that SUN-1 is sufficient for ZYG-12 localization via ectopic expression of both proteins in mammalian cells.

MATERIALS AND METHODS

C. elegans Culture and Transgenes

N2 is the wild-type strain. All strains were grown under standard conditions at 20°C (Brenner, 1974) except *qals3502[unc-119(+)] + pie-1::YFP::LMN-1 + pie-1::CFP::H2B*, which was grown at 25°C. We used the *pie-1* promoter and enhancer system (vector pFJ1) to express green fluorescent protein (GFP) reporter proteins in the germ line and early embryos (Strome *et al.*, 2001). See Supplemental Table S1 for vector construction. We performed transformations by using the microparticle bombardment system (Praitis *et al.*, 2001, Strome *et al.*, 2001). We performed RNA interference (RNAi) by feeding (see Supplemental Data).

Proteinase K Protection Assay

We cloned full-length *sun-1* cDNA into pCITE2b (Novagen, Madison, WI) (details upon request) and used linearized vector for *in vitro* transcription using Megascript 7 (Ambion, Austin, TX). We synthesized SUN-1 using Flexi rabbit reticulocyte lysate (Promega, Madison, WI) in the absence or presence of canine microsomal membranes (Promega). Five microliters of translated protein was digested with 4 μ g/ml proteinase K on ice for 30 min followed by sequential addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 30 μ l of 1 $\times$ SDS-polyacrylamide gel electrophoresis sample buffer containing dithiothreitol. The protected fragments were analyzed by Western blotting.

Epitope Accessibility Assay (EAA)

Gonads of wild-type animals were cut open on coverslips and immobilized on poly-L-lysine-coated slides. We fixed gonads with 4% formaldehyde in phosphate-buffered saline (PBS) and immunostained using α -SUN-1 antibody, which was produced against bacterially expressed protein containing the C-terminal 242 amino acids of SUN-1 (generous gift from Abby Dernburg, University of California–Berkeley) or α -ZYG-12 rat polyclonal antibodies (Malone *et al.*, 2003) in the absence or presence of 1% Triton X-100. Samples were blocked with 0.5% bovine serum albumin, incubated with primary antibodies at 4°C overnight, washed with cold PBS, and incubated with secondary antibodies (Alexa Fluor 488 and 568; Invitrogen, Carlsbad, CA) for 1 h at room temperature. We acquired images using ECLISPE 90i wide-field microscopy (Nikon, Melville, NY) and SimplePCI software (Compix, Irvine, CA) and processed images using PhotoShop software (Adobe Systems, Mountain View, CA).

Fluorescence Protease Protection (FPP) Assay

Gonads from *qals3502* and wild-type hermaphrodites were cut, immobilized on poly-L-lysine-coated coverslips and fixed with 4% formaldehyde. They were washed with cold PBS and incubated with 1 mg/ml trypsin in PBS for 10 min at 4°C. Samples were then washed with cold PBS containing 1 mM PMSF and 1 μ g/ml aprotinin and transferred to poly-L-lysine-coated slides. We immunostained in the presence of Triton X-100 by using 3E6 monoclonal antibodies against GFP (Invitrogen), α -SUN-1, and α -ZYG-12 antibodies as described above.

Yeast Two-Hybrid Assay

We used a split-ubiquitin based yeast two-hybrid system (Fetchko and Stajgar, 2004). See Supplemental Data for details.

Fluorescence Recovery after Photobleaching

We used GFP fusions to endoplasmic reticulum (ER) resident protein signal peptidase SP-12 (Rolls *et al.*, 2002; Poteryaev *et al.*, 2005), to all isoforms of ZYG-12 (Malone *et al.*, 2003), and to SUN-1 (see above) for FRAP analysis. We examined nuclei of developing oocytes in the region of the gonad just proximal to the turn with a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Thornwood, NY) with the Plan-Apocromat 63 $\times$ 1.4 oil differential interference contrast (DIC) objective (Carl Zeiss MicroImaging). We excited with the 488-nm line of an Ar laser and detected emitted light with a 505-nm long pass filter. We placed extruded gonads on a 2% agarose pad and bleached a rectangular portion of the nucleus by using a 488-nm laser line at 100% power. The nuclei were monitored at 10-s intervals up to 300 s. We measured the intensity of total and bleached regions by using NIH ImageJ (National Institutes of Health, Bethesda, MD) and normalized as described earlier to account for the bleaching during monitoring (Ostlund *et al.*, 2006) using the formula $I_{rel} = T_0 I_t / I_0$, where T_0 is total fluorescence of the image before bleaching, I_0 is fluorescence from the bleached region before bleaching, I_t is total fluorescence of the image at time point t , and I_t is fluorescence from the bleached region at time point t . At least six independent bleaching experiments were combined for each GFP reporter, and the normalized values are means $\pm$ SD.

HeLa Cell Culture and Transfection

HeLa cells were maintained in DMEM/Ham's F-12 with 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Mediatech, Herndon, VA). pECFP-ER (Clontech, Mountain View, CA) was used as the ER marker. Full-length *sun-1* and *zyg-12 B* and *C* cDNAs were cloned into pEYFP C1 and pECFP C1, respectively (Supplemental Table S3). Plasmid DNAs were transfected into HeLa cells by using Effectene Transfection Reagent (QIAGEN, Valencia, CA). One microgram of DNA in 150 μ l of EC buffer was mixed with 8 μ l of Enhancer and incubated for 5 min at room temperature (RT). After adding 25 μ l of Effectene Transfection Reagent and incubating for 10 min at RT, DMEM/Ham's F-12 with 10% FBS was added to the mixture. Subsequently, the mixture was transferred to 5×10^5 HeLa cells seeded onto a six-well culture plate 1 d prior and incubated at 37°C for 4 h. Cells were washed with PBS and incubated for 2 d in DMEM/Ham's F-12 with 10% FBS. Cells were observed using Axiovert 200M microscope (Carl Zeiss MicroImaging) with Chroma 41028 filter for yellow fluorescent protein (YFP) and Chroma 31044 V2 filter for cyan fluorescent protein (CFP) (Chroma

Technology, Brattleboro, VT). The image stacks of the Z-axis were taken and deconvolved using AxioVision software (Carl Zeiss MicroImaging) and processed using PhotoShop CS software (Adobe Systems).

RESULTS

The ZYG-12 B and C Mini KASH with Part of the Coiled Coil Is Sufficient for Nuclear Envelope (NE) Localization

All known ONM proteins require a KASH domain for targeting. For example, deletion of the highly conserved last four amino acids (-PPPT) of human Syne-2 or Syne-3 α KASH domains results in the loss of nuclear membrane specific localization (Padmakumar *et al.*, 2005; Ketema *et al.*, 2007). *C. elegans* ZYG-12 has three isoforms (A, B, and C) (Malone *et al.*, 2003). Two of the three ZYG-12 isoforms (B and C) contain transmembrane domains and are proposed to localize to the ONM (Malone *et al.*, 2003) but do not contain all the hallmarks of known KASH domains. The third isoform, A, has a similar N terminus, but it has no transmembrane domain and is localized exclusively to the centrosome. Like ONM-localized KASH domain proteins, ZYG-12 B and C have a predicted transmembrane domain near the C terminus, followed by a short tail. However, the tail contains only four residues that are found in other KASH domains (Figure 1A). In addition, the mini KASH domain contains only two prolines of the -PPPT motif that is required for the nuclear envelope localization of human Syne-2. To test whether the divergent mini KASH domain of ZYG-12 B and C is functional and sufficient to localize the protein to the nuclear envelope, we expressed GFP-tagged truncations of ZYG-12 containing the mini KASH domain in embryos (Figure 1B). ZYG-12 B Δ 2 and ZYG-12 C Δ 2 containing the mini KASH domains of ZYG-12 B and C, respectively, localized to the plasma membrane. However, ZYG-12 B Δ 1 and ZYG-12 C Δ 1 containing the mini KASH and 52 amino acids of predicted coiled-coil domain of ZYG-12 B and C, respectively, localized to the nuclear envelope and plasma membrane (Figure 1C). This result indicates that the mini KASH domain together with a partial coiled-coil domain is sufficient for the nuclear envelope localization of ZYG-12 B and C. Because the coiled-coil domain of ZYG-12 is responsible for self-dimerization (Malone *et al.*, 2003), we tested the possibility that ZYG-12 B Δ 1 and ZYG-12 C Δ 1 might interact with endogenous ZYG-12 at the nuclear envelope to achieve nuclear envelope localization. We introduced double-stranded (ds)RNA that specifically targets the N-terminal sequence of ZYG-12 to reduce endogenous ZYG-12 without affecting the ZYG-12::GFP truncations (Figure 1B). Depletion of endogenous ZYG-12 was successful as shown by the detached centrosome phenotype (Figure 1C, asterisk). Nonetheless, nuclear envelope localization of ZYG-12 B Δ 1 and ZYG-12 C Δ 1 was maintained when endogenous ZYG-12 was depleted. None of the truncations tested localized to the centrosome in contrast to the full-length ZYG-12 B and C that localize to both the nuclear envelope and the centrosome (Malone *et al.*, 2003). This indicates that the N terminus of the ZYG-12 is required for centrosome localization.

We further tested whether the localization of ZYG-12 B Δ 1 and ZYG-12 C Δ 1 to the nuclear membrane requires SUN-1. We depleted SUN-1 via RNAi and found that nuclear envelope localization of ZYG-12 B Δ 1 and ZYG-12 C Δ 1 was completely lost, whereas plasma membrane localization was still intact. These results indicate that ZYG-12 truncations are targeted to the nuclear envelope by SUN-1 and that plasma membrane localization is probably nonspecific. We conclude that addition of the coiled-coil domain to the mini

Figure 2. C termini of ZYG-12 and SUN-1 are oriented toward the lumen of the nuclear envelope. (A) Diagrams of SUN-1 and ZYG-12. TM, transmembrane domain; CC, coiled-coil domain; K, KASH domain. Black bar indicates C-terminal 242-amino acid fragment of SUN-1 and N-terminal 236-amino acid fragment of ZYG-12 used for raising the α -SUN-1 and α -ZYG-12 polyclonal antibodies, respectively. Numbers represent the amino acid positions of predicted domains. (B) Fluorescence protease protection assay using syncytial gonads from animals expressing YFP::LMN-1 (*qals3502[unc-119(+)] + pie-1::YFP::LMN-1 + pie-1::CFP::H2B*). Immunostaining was performed using α -GFP mAb 3E6 (Invitrogen) to detect YFP::LMN-1 and α -SUN-1 antibody (Fig. 2 A) to detect SUN-1. Signals from

KASH domain is sufficient to localize ZYG-12 to the nuclear envelope and that this localization is dependent on SUN-1. These results suggest that the divergent mini KASH domain of ZYG-12 B and C functions like other KASH domains.

ZYG-12 and SUN-1 Are Type II Integral Membrane Proteins That Localize to the Outer and Inner Membrane of the Nuclear Envelope, Respectively

We predict that the ZYG-12 C-terminal mini KASH domain interacts with the C terminus of SUN-1 in the lumen between the INM and ONM. To test whether the proteins are type II integral membrane proteins that are inserted in the membrane with their C termini in the lumen, we performed three independent assays.

First, we examined the membrane orientations of ZYG-12 and SUN-1 at the outer and the inner membranes of the nuclear envelopes, by using an FPP assay (Lorenz *et al.*, 2006). Trypsin is a small protease that can freely diffuse into nuclei through the nuclear pore complex resulting in digestion of proteins on the outside and the inside of the nuclear envelope. Regions of proteins in the lumen between the ONM and INM are protected from trypsin cleavage. Thus, analysis of protein fragments protected from trypsin cleavage reveals information about the topology of proteins. We extruded gonads from wild-type (Figure 2C) animals and animals expressing YFP::LMN-1 (Figure 2B) and used intrinsic fluorescence to detect YFP::LMN-1 and antibodies directed against C-terminal epitopes to detect SUN-1 (Figure 2A). Without trypsin treatment, both laminin:YFP and SUN-1 were detectable (Figure 2B, first column). Trypsin treatment reduced Laminin:YFP to undetectable levels, as expected. However, the C-terminal fragment of SUN-1 was protected from protease cleavage (Figure 2B, second column), indicating that the C terminus of SUN-1 resides in the lumen of the nuclear envelope. In similar experiments using the SUN-1 antibody and the ZYG-12 antibody (Figure 2A), we confirmed that the N terminus of ZYG-12 is accessible to trypsin (Figure 2C). Thus, the C termini of both ZYG-12 and SUN-1 reside in the nuclear envelope lumen.

Second, we performed an epitope accessibility assay by using the gonads of wild-type animals to determine whether fragments of ZYG-12 and SUN-1 are exposed to the outside of the nucleus. Syncytial gonads were physically cut open to expose nuclei for immunostaining. We detected endogenous proteins using α -ZYG-12 N terminus and α -SUN-1 C terminus antibodies (Figure 2A) in the absence and the presence of Triton X-100. In the presence of Triton X-100, the ZYG-12 and SUN-1 epitopes were accessible to antibody as expected, resulting in clear detection of signal at the nuclear envelope (Figure 3, A, C, and E). In contrast, in the absence of the detergent, only the ZYG-12 epitope was accessible to the antibody (Figure 3, B, D, and F), indicating that the N terminus of ZYG-12 is on the outside of the nucleus. The lack of SUN-1 signal in the absence of Triton X-100 is consistent with a luminal localization of its C terminus.

Finally, we performed a proteinase K protection assay to determine how many transmembrane domains are present in SUN-1 (Figure 2A). Topology prediction using transmem-

LMN-1 after trypsin treatment were captured using 30-fold longer exposure relative to those without trypsin treatment. Bar, 10 μ m. (C) Fluorescence protease protection assay using syncytial gonads from wild-type animals. Signals from ZYG-12 after trypsin treatment were captured using 20-fold longer exposure relative to those without trypsin. Bar, 10 μ m.

Figure 3. ZYG-12 is a type II transmembrane protein of the outer membrane of the nuclear envelope. Epitope accessibility assay using wild-type gonads. Syncytial gonads were physically cut, opened and the immunostained using α -ZYG-12 (A and B) and α -SUN-1 (C and D) polyclonal antibodies in the presence (A, C, and E) or absence (B, D, and F) of Triton X-100. E and F are the merged images. Bar, 10 μ m.

brane topology prediction (TMAP) (Persson and Argos, 1994) and hidden Markov model for topology prediction (HMMTOP) (Tusnady and Simon, 1998, 2001) predict only an N-terminal transmembrane domain (TM) domain for SUN-1, whereas TMPred (Hoffman *et al.*, 1993) predicts a second C-terminal TM domain. We synthesized full-length SUN-1 in vitro in the absence or presence of canine pancreatic microsomal membranes and treated with proteinase K in the absence or presence of Triton X-100. The protected fragments of SUN-1 were then visualized by Western blot analysis using α -SUN-1 C terminus (Figure 2A). The size of the protected fragment of SUN-1 was $\sim$ 40 kDa (Figure 4D, arrow), which is consistent with the size of the portion of SUN-1 to the C terminus of the first predicted TM (Figure 4A). The exclusive use of TM2 would have produced a 23-kDa fragment, and use of both TMs would produce 13- and 25-kDa fragments (Figure 4C). The protected fragment was not detectable from the reactions without microsomes or with Triton X-100, indicating that microsomes prevent digestion. This result indicates that the first TM domain of SUN-1 is used, and SUN-1 is a type II integral membrane protein with the C terminus in the lumen.

SUN-1 Interacts Directly with ZYG-12

SUN-1 is required for ZYG-12 localization to the nuclear envelope (Malone *et al.*, 2003), and we showed that the C termini of ZYG-12 and SUN-1 are localized to the lumen between the ONM and INM. Thus, an interaction between ZYG-12 and SUN-1 in the lumen is a possible mechanism for ONM-specific localization of ZYG-12. We used a yeast two-hybrid assay to test whether SUN-1 physically interacts with ZYG-12. Because SUN-1 and ZYG-12 are integral membrane proteins, which potentially interact in the luminal space of the nuclear envelope, we took advantage of the DUALmem-

Figure 4. SUN-1 is type II transmembrane protein and the first predicted TM is functional. (A–C) Predictions of membrane topology of SUN-1 in liposome. TM, transmembrane domain; N, N terminus; C, C terminus. (D) Western blot analysis of microsomes. Lane 1, SUN-1 translated with microsomes without proteinase K treatment; lane 2, SUN-1 translated in the absence of microsomes and treated with proteinase K; lane 3, SUN-1 translated with microsomes and treated with proteinase K. The arrow indicates the 42-kDa protected fragment of SUN-1 that would include the first TM. The fraction of the protein protected relative to the amount of input probably reflects inefficiencies in protein incorporation into microsomes; lane 4, SUN-1 translated with microsomes and treated with proteinase K in the presence of Triton X-100.

brane yeast two-hybrid system based on split-ubiquitin (Stagljär *et al.*, 1998; Fetchko and Stagljär, 2004).

We expressed full-length ZYG-12 B and C and SUN-1 and various SUN-1 truncations and mutants as bait constructs (Figure 5A). We expressed C-terminal portions of ZYG-12 B (86 amino acids) and C (70 amino acids) and SUN-1 as prey. We showed previously that full-length ZYG-12 A binds to itself by using the internal coiled-coil domain (Malone *et al.*, 2003). Full-length ZYG-12 B and C contain coiled-coil domains identical to ZYG-12 A and bind to each other (data not shown) but not to ZYG-12 B, CA1 that lack N terminus (Figure 5A). SUN-1 fragments (SUN-1 Δ 3, Δ 4, and Δ 5) that lack C-terminal region including the conserved SUN domain were able to interact with ZYG-12 B, CA1 (Figure 5A). Interestingly, SUN-1 fragments that had only 40 more amino acid residues after the first transmembrane domain (SUN-1 Δ 5) were able to interact with ZYG-12 truncations. To further define the region of SUN-1 that interacts with ZYG-12, we created SUN-1 bait fragment that has only five additional amino acid residues after first transmembrane domain (SUN-1 Δ 6) and a bait with an internal deletion that retains the five amino acids and the conserved SUN domain (SUN-1 Δ 7). Neither of these was able to interact with ZYG-12 truncations. Other internal deletions (SUN-1 Δ 8, Δ 9, and Δ 10) that maintain 40 amino acids and SUN domain interacted with ZYG-12. These results indicate that the interaction with ZYG-12 requires the internal domain of SUN-1 (residues 123–325) and does not require the conserved SUN domain. In addition, to test whether residues 123–163 are necessary for the interaction with ZYG-12 truncation, we made SUN-1

Figure 5. ZYG-12 directly interacts with SUN-1. Split-ubiquitin-based yeast two-hybrid assay. (A) Various ZYG-12 and SUN-1 constructs were cloned into pTFB1 bait vector containing the C-terminal half of ubiquitin (Cub), the LexA DNA binding domain, and the VP16 transactivation domain. SUN-1 Δ1 to Δ11 lack parts of the proteins as illustrated. ZYG-12 B,CΔ1 that contains KASH domain were cloned into the prey vector pNubGHAX containing N-terminal half of ubiquitin (Nub) that harbors a single missense mutations to prevent spontaneous interaction with Cub. pAlg5-NubG is the negative control prey vector. The interactions between ZYG-12 B,CΔ1 and bait constructs were measured by growth on -Leu, -Trp, -His media containing 40 mM 3-amino 1,2,4-triazole. SUN-1Δ2 self-activated. (B) Self interaction of SUN-1. SUN-1 bait constructs were tested for the interaction with full-length SUN-1 in the prey vector.

fragment (SUN-1Δ11) that lacks this region. This fragment was still able to interact with ZYG-12 truncations, indicating that the 40-amino acid stretch after the first transmembrane domain is not the sole requirement for the interaction. One

explanation of the data is that there are two regions within the internal domain that can each independently interact with ZYG-12, one region comprises amino acids 123–163, and the other is between amino acids 163 and 325. The data

Figure 6. ZYG-12 is immobile at the outer membrane of the nuclear envelope. (A) We performed FRAP analysis on SP-12::GFP, SUN-1::GFP, and ZYG-12::GFP. We bleached using a 488-nm laser at 100% power (dotted boxes) and captured images at 10-s intervals. The ER resident transmembrane protein SP-12 showed rapid fluorescence recovery, which SUN-1 and ZYG-12 did not. Bar, 10 μ m. (B) We quantified and normalized images and prepared the graph using R software. We calculated normalized fluorescence values using the formula $I_{rel} = T_0 I_t / T_t I_0$, where T_0 is total fluorescence of the image before bleaching, I_0 is fluorescence from the bleached region before bleaching, T_t is total fluorescence of the image at time point t , and I_t is fluorescence from the bleached region at time point t .

do not support any interaction between the SUN domain and ZYG-12.

SUN-1 Δ 2, which lacks the N-terminal 158 amino acids, including the first predicted transmembrane domain, self-activated, as expected from a bait protein that cannot target to the ER membrane and can thus enter the nucleus and activate the target genes. In contrast, SUN-1 Δ 1, which preserves the first putative transmembrane domain, did not self-activate. This finding is consistent with our proteinase K protection assay, indicating the first predicted transmembrane domain of SUN-1 is the only functional one. We conclude that SUN-1 directly interacts with ZYG-12 and the interaction occurs between the internal domain of SUN-1 and the mini KASH domain of ZYG-12.

Because SUN-1 has two putative coiled-coil domains, we examined whether SUN-1 can bind to itself. SUN-1 constructs that contained the coiled-coil domains interacted with full-length SUN-1 in the prey, whereas SUN-1 truncations that lack this region did not (Figure 5B). This result suggests that SUN-1 binds to itself using the internal coiled-coil domains.

ZYG-12 Is Immobile at the Outer Membrane of the Nuclear Envelope

We have shown that ZYG-12 localizes to the outer membrane of the nuclear envelope in a SUN-1-dependent manner. In addition, ZYG-12 does not diffuse throughout the ER even though the outer membrane of the nuclear envelope is contiguous with the ER. We hypothesized that if ZYG-12 were restricted to the outer membrane of the nuclear envelope by an interaction with the inner nuclear membrane protein SUN-1, ZYG-12 would behave like an inner membrane protein in mobility tests. To test this prediction, we performed FRAP analysis (Figure 6). As expected, the ER resident transmembrane protein signal peptidase (SP-12) quickly recovered within 30 s after bleaching. In contrast, the inner nuclear membrane protein SUN-1 did not recover after bleaching, indicating that its movement in the membrane is restricted like other inner membrane proteins. Similarly, we found that ZYG-12 at the outer nuclear membrane was not able to recover after bleaching. These results indicate that ZYG-12 mobility in the outer nuclear membrane is restricted

and support our model that ZYG-12 is specifically localized to the outer membrane of the nuclear envelope through the interaction with inner nuclear membrane protein SUN-1.

SUN-1 Is Sufficient to Localize ZYG-12

We showed previously that SUN-1 is required for ZYG-12 nuclear envelope localization (Malone *et al.*, 2003). To determine whether interaction with SUN-1 is sufficient to localize ZYG-12, we tested whether SUN-1 could recruit ZYG-12 in a heterologous system. We independently expressed ZYG-12 and SUN-1 in human HeLa cells (Figure 7, B–D) and then coexpressed the proteins (Figure 7, E–J). When full-length ZYG-12 B or ZYG-12 C is expressed in HeLa cells alone, it localizes diffusely to ER and the nuclear envelope (Figure 7, C and D). This localization pattern is very similar to that of an ER marker protein (Figure 7A). Unlike human Sun1, which is evenly distributed around the nuclear membrane when ectopically expressed in HeLa cells (Padmakumar *et al.*, 2005), full-length CeSUN-1 tagged with YFP at its N terminus is clustered in patches near the HeLa nuclear membrane (Figure 7B). When SUN-1 is coexpressed with ZYG-12, ZYG-12 B and C are recruited to juxtanuclear patches where CeSUN-1 is localized, and the ER localization of ZYG-12 B and C was dramatically reduced to an almost undetectable level (Figure 7, E–J). The CeSUN-1 localization around the nuclei was unchanged during coexpression with ZYG-12. These results indicate that SUN-1 is sufficient to colocalize ZYG-12 and restrict its subcellular localization when they are coexpressed.

DISCUSSION

ZYG-12 Has a Mini KASH Domain That Functions in Nuclear Envelope Targeting with Part of the Coiled-Coil Domain

We show that ZYG-12 B and C have a functional mini KASH domain. C-Terminal fragments of ZYG-12 B and C containing the mini KASH domain and an additional 52-amino acid coiled-coil domain (ZYG-12 B, C Δ 1) were sufficient for the localization to the nuclear envelope, and SUN-1 was required for this localization. Although we subsequently found that the luminal domain of ZYG-12 binds directly to

Figure 7. ZYG-12 B and C colocalize with SUN-1 in HeLa cells. (A) The ER marker (pECFP-ER; Clontech) shows outer nuclear membrane and ER localization patterns. (B) pEYFP::SUN-1 localizes to juxtannuclear patches around the nucleus. (C) pECFP::ZYG-12B and (D) pECFP::ZYG-12 C show almost identical localization patterns with the ER marker. (E–G) Coexpression of (E–G) pEYFP::SUN-1 and pECFP::ZYG-12 B and (H–J) pEYFP::SUN-1 and pECFP::ZYG-12 C. (E) EYFP::SUN-1 and (F) ECFP::ZYG-12 B and (H) EYFP::SUN-1 and (I) ECFP::ZYG-12 C colocalize, whereas the ER localization of ZYG-12 B and C were disappear. (G and J) Merged images. Bar, 10 μ m.

SUN-1, this region of the protein was not sufficient to localize a short fusion of the transmembrane domain with GFP to the NE. It is possible that short fusions of the C terminus of ZYG-12 to GFP do not insert correctly into membranes, or ZYG-12 may need part of the coiled coil to dimerize in order to localize to the NE.

Topology of ZYG-12 and SUN-1

The model (Figure 8) of KASH-SUN protein pairs coupling nucleoskeleton and cytoskeleton predicts that these localize to the outer and inner membrane of the nuclear envelope, respectively, with their C termini in the luminal space of the nuclear envelope where they bind one another. We used three assays to rigorously test whether ZYG-12 and SUN-1 adopt the topology and localization that fits this model. The most advantageous aspect of our approach was that we used

nuclei from *C. elegans* gonads that express endogenous ZYG-12 and SUN-1 for two of the assays. Because the gonad is syncytial, we could avoid treating cells with digitonin to selectively permeabilize plasma membrane and could instead cut open the gonad to expose the nuclear surface. The EAA and FPP assays clearly show that endogenous ZYG-12 and SUN-1 are type II integral membrane proteins that localize to the outer and inner membrane of the nuclear envelope, respectively. There are several observations that support these findings. First, ZYG-12 has single transmembrane domain and is required for cytoplasmic motor protein Dynein nuclear envelope localization (Malone *et al.*, 2003). Second, ZYG-12 also interacts with Dynein light intermediate chain. Third, SUN-1 binds to the nuclear lamina (Fridkin *et al.*, 2004). Fridkin *et al.* (2004) reported that SUN-1/Matefin has two predicted transmembrane domains and localizes

Figure 8. Model for the mechanism of the centrosomal attachment to the nucleus in the *C. elegans* embryo. (A) Centrosomes attach to the nucleus in *C. elegans* embryo. ZYG-12 A localized to the centrosome interacts with ZYG-12 B and/or C at the outer membrane of the nuclear envelope to initiate and maintain the attachment of the centrosomes to the nuclei. ZYG-12 B and C interact with inner nuclear membrane protein SUN-1 in the lumen of the nuclear membrane. (B) ZYG-12 localizes specifically to outer nuclear membrane but not to the ER. ZYG-12 can dimerize. The KASH domain of ZYG-12 directly binds to the internal region of SUN-1 containing the coiled-coil domain. SUN-1 localizes to the inner nuclear membrane as a dimer, and the interaction with ZYG-12 confines the localization of ZYG-12 to the outer membrane of the nuclear envelope. SUN-1 is sufficient for ZYG-12 localization to the membrane. The N-terminal domain of SUN-1 may interact with nuclear lamina and/or chromatin in the lumen of the nuclear envelope.

to the inner nuclear membrane with its N- and C-terminal ends inside of the nucleus. Our data, however, clearly suggest that SUN-1 has a single functional transmembrane domain with its C-terminal part, including the SUN domain, in the lumen of the nuclear envelope.

Direct Interaction between ZYG-12 and SUN-1

Using a yeast two-hybrid assay, we showed that ZYG-12 and SUN-1 directly interact and the interaction takes place between the luminal tail of ZYG-12 and an internal domain of SUN-1 that localizes to the nuclear envelope lumen. We showed that the deletion of SUN domain did not alter the interaction with ZYG-12. In addition, a SUN-1 fragment that lacks the internal domain while maintaining the intact SUN domain failed to interact with ZYG-12. Our findings are consistent with the study showing that mammalian Sun1 uses internal luminal domain without SUN domain to interact with the KASH domain of Nesprin-1 (Padmakumar *et al.*, 2005). Previous studies have provided conflicting evidence on whether the SUN domain is responsible for interaction with the KASH partner. One study showed that SUN domain of human Sun2 is required for the interaction with human Nesprin-2 KASH domain (Stewart-Hutchinson *et al.*, 2008). Our results are more consistent with another study that showed mammalian Sun1 utilizes internal luminal domain without SUN domain to interact with the KASH domain of Nesprin-1 (Padmakumar *et al.*, 2005). Recently, novel *C. elegans* KASH protein KDP-1 was identified (McGee *et al.*, 2009). KDP-1 localizes to the nuclear envelope in the germline in a SUN-1-dependent manner and interacts with SUN-1. It would be very interesting to examine whether SUN-1 uses different domains to interact with different KASH binding partners.

Roles of ZYG-12 (KASH) and SUN-1 (SUN) Pair in Centrosome–Nucleus Attachment

The identification of ZYG-12 as a protein with a fully functional, albeit divergent, KASH domain that lacks previously noted conserved residues means that other KASH domain proteins may have been missed in homology searches. Furthermore, ZYG-12 is the only protein to be identified that has both Hook and KASH domains. Hook proteins are thought to link microtubules and organelles and generally have N-terminal cytoskeleton binding Hook domains, internal coiled-coil domains for homodimerization, and C-terminal cytoplasmic organelle binding domains (Kramer and Phistry, 1999; Sunio *et al.*, 1999). The localization of ZYG-12 to the ONM via its KASH domain and to the centrosome via its N terminus is an interesting twist on organelle–cytoskeleton linkage by Hook proteins—the dimerization of these two populations of ZYG-12 is responsible for centrosome anchoring to the NE in *C. elegans* embryos (Malone *et al.*, 2003). The centrosomal binding partner of ZYG-12 has not yet been identified.

Although there are no obvious homologues of ZYG-12 in other organisms, human hook2 was found to reside at the centrosome by interacting with the centrosomal protein Centriolin/CEP110 by using its C-terminal domain (Szebenyi *et al.*, 2007). Disruption of hook2 resulted in abnormal microtubule organization. Interestingly, the N-terminal region of hook2 is most similar to the N-terminal dynein-binding region of ZYG-12, suggesting a close relationship between these two proteins. However, hook2 does not localize to the ONM and is not used for anchoring the centrosome to the nucleus.

This means that the mechanism of nucleus-centrosome interaction is still unknown in many cells. In mammals, the

INM protein emerin seems to be important as centrosomes in emerin-null or -deficient human dermal fibroblasts from X-linked Emery Dreifuss muscular dystrophy patients were detached from the nucleus (Salpingidou *et al.*, 2007). In addition, emerin was found to be localized to the outer membrane of the nuclear envelope and interact with tubulin. How it would hold on to the centrosome has not been determined. These findings suggest that *C. elegans* and humans may use the same strategy for centrosomal attachment to the nucleus that involves a type II NE protein that can bind to microtubules or microtubule motors.

Using information about centrosome–nucleus interactions it should be possible to identify candidate proteins that mediate this link in other cells and organisms. Any NE protein that binds microtubules or any centrosomal protein that has a transmembrane domain at its C terminus could play a role in this process.

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