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UNC-83 Is a KASH Protein Required for Nuclear Migration and Is Recruited to the Outer Nuclear Membrane by a Physical Interaction with the SUN Protein UNC-84

Matthew D. McGee,* Regina Rillo,* Amy S. Anderson, and Daniel A. Starr

The Center for Genetics and Development and the Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA 95616

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UNC-84 is required to localize UNC-83 to the nuclear envelope where it functions during nuclear migration. A KASH domain in UNC-83 was identified. KASH domains are conserved in the nuclear envelope proteins Syne/nesprins, Klarsicht, MSP-300, and ANC-1. *Caenorhabditis elegans* UNC-83 was shown to localize to the outer nuclear membrane and UNC-84 to the inner nuclear membrane in transfected mammalian cells, suggesting the KASH and SUN protein targeting mechanisms are conserved. Deletion of the KASH domain of UNC-83 blocked nuclear migration and localization to the *C. elegans* nuclear envelope. Some point mutations in the UNC-83 KASH domain disrupted nuclear migration, even if they localized normally. At least two separable portions of the C-terminal half of UNC-84 were found to interact with the UNC-83 KASH domain in a membrane-bound, split-ubiquitin yeast two-hybrid system. However, the SUN domain was essential for UNC-84 function and UNC-83 localization in vivo. These data support the model that KASH and SUN proteins bridge the nuclear envelope, connecting the nuclear lamina to cytoskeletal components. This mechanism seems conserved across eukaryotes and is the first proposed mechanism to target proteins specifically to the outer nuclear membrane.

INTRODUCTION

A variety of cellular and developmental processes, including fertilization, cell division, cell migration, and establishment of polarity, depend on positioning the nucleus to a specific location within the cell. For example, in budding yeast the nucleus must migrate to the bud neck before the onset of mitosis. Also, nuclei actively follow the leading edge of migratory cells, such as those in the developing cerebral cortex. Nuclear migration defects in these two examples lead to the missegregation of chromosomes or the neurological disease lissencephaly, respectively (reviewed in Morris, 2000). The role of microtubules and associated dynein and kinesin motors in nuclear migration are well established (reviewed in Reinsch and Gonczy, 1998). Although less established, actin also plays an important role in many nuclear positioning events (reviewed in Starr and Han, 2003). Recently, a definitive role for actin networks has been described in nuclear migration during NIH 3T3 cell polarization (Gomes *et al*., 2005). It remains relatively unknown how the nucleus connects to the cytoplasmic cytoskeleton during nuclear migration. Furthermore, it is not clear how the forces involved in nuclear positioning are transferred across both membranes of the nuclear envelope from the cytoskeleton to the nuclear matrix.

We have previously proposed that two *C. elegans* proteins, UNC-84 and UNC-83, function to control nuclear migration by bridging the nuclear envelope, connecting the cytoskeleton with the nuclear matrix (Starr *et al*., 2001; Lee *et al*., 2002; Starr and Han, 2003). Mutations in *unc-83* or *unc-84* disrupt nuclear migration in at least three cell types: embryonic hypodermal hyp7 precursors, larval hypodermal P-cells, and embryonic intestinal primordial cells (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Malone *et al*., 1999; Starr *et al*., 2001). Failure of hyp7 precursor nuclear migration results in easily observed nuclei in the dorsal cord of the newly hatched animal. Failure of P-cell nuclear migration leads to death of the cell. The resulting P-cell lineage defects lead to a lack of vulval and neural cells, which result in egg-laying and uncoordinated phenotypes (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981).

UNC-83 and UNC-84 localize to the nuclear envelope (Malone *et al*., 1999; Starr *et al*., 2001; Starr and Han, 2002). UNC-83 is a novel protein with a predicted transmembrane spanning domain 18 residues from its C terminus; it is not clearly conserved outside of nematodes. Monoclonal antibodies against UNC-83 localize to the nuclear envelope of a subset of cells. Many of the nuclei with UNC-83 at the nuclear envelope partake in nuclear migration events at specific stages of development (Starr *et al*., 2001). Although UNC-83 is essential for nuclear migration in many developmental events, its molecular mechanism and interacting partners remain unknown. UNC-84 is a novel protein with a

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* These authors contributed equally to this work.

Address correspondence to: Daniel A. Starr (dastarr@ucdavis.edu).

Abbreviations used: KASH, Klarsicht, ANC-1, and Syne homology; SUN, Sad1p and UNC-84 homology; MbYTH, split-ubiquitin membrane yeast two-hybrid.

predicted transmembrane domain near the center of the protein and a conserved SUN (*S*ad1p and *UN*C-84) domain at its C terminus. SUN domains are conserved across all eukaryotes and include *S. pombe* Sad1p, *C. elegans* UNC-84 and SUN-1, four human Sun proteins, and even a homologue in the most basal eukaryote *Giardia* (GenBank EAA41593) (Hagan and Yanmagida, 1995; Malone *et al*., 1999, 2003; Hodzic *et al*., 2004; Padmakumar *et al*., 2005; Starr and Fischer, 2005). UNC-84 localizes to the nuclear envelope in nearly all somatic cells from the 24-cell stage on in development (Lee *et al*., 2002). One important role for UNC-84 is to target other proteins, including UNC-83 and ANC-1, to the nuclear envelope (Starr *et al*., 2001; Starr and Han, 2002). Through this mechanism, UNC-84 functions to control nuclear migration and anchorage.

Three lines of genetic data predict an interaction between UNC-83 and the SUN protein UNC-84. First, in animals with a null *unc-84* mutation, UNC-83 fails to localize to the nuclear envelope. More specifically, missense mutations in the SUN domain of UNC-84 block localization of UNC-83, implicating an important, but unknown, role for the SUN domain in targeting UNC-83 to the nuclear envelope (Starr *et al*., 2001). The same mutations in *unc-84* also disrupt the localization of ANC-1, a large protein that functions to link the outer nuclear membrane to actin filaments to anchor nuclei (Starr and Han, 2002). Second, *unc-84; unc-83* double mutant lines have the same nuclear migration phenotype as either single mutant does on its own, suggesting they function in the same pathway. Finally, screens for proteins essential for postembryonic nuclear migration are likely saturated. More than 20 alleles of *unc-83* and *unc-84* have been isolated (Malone *et al*., 1999; Starr *et al*., 2001). The lack of additional complementation groups suggests that there is not an intermediate protein.

The cell biological and genetic data mentioned above suggest a nuclear envelope bridging model. In this model, UNC-84 localizes to the inner nuclear membrane with its N terminus associated with the nuclear lamina and its C-terminal SUN domain facing the lumen of the nuclear envelope. We hypothesized that the SUN domain of UNC-84 would then recruit UNC-83 to the outer nuclear membrane through direct interactions in the lumen of the nuclear envelope. Such a mechanism would allow the bulk of UNC-83 to extend into the cytoplasm while attached specifically to the outer nuclear membrane. Together, the proteins would span the nuclear envelope, connecting the nuclear matrix to unknown components in the cytoskeleton. Here, we test parts of the bridging model. Specifically, we identify a conserved domain in UNC-83 that is required for targeting to the outer nuclear membrane and characterize a direct physical interaction between UNC-83 and the C terminus of UNC-84. This interaction is likely the central link in the nuclear envelope bridging mechanism and represents a conserved mechanism for recruiting proteins to the outer nuclear membrane.

MATERIALS AND METHODS

C. elegans Strains and Maintenance and Phenotypic Characterization

C. elegans were cultured using standard conditions (Brenner, 1974). The Bristol N2 strain was used for wild type and to generate all other strains. The null alleles *unc*-84(n369) and unc-83(e1408) as well as the SUN domain missense alleles *unc*-84(sa61 S988F) and unc-84(n399 G1002D) were described previously (Horvitz and Sulston, 1980; Malone *et al*., 1999; Starr *et al*., 2001). Transgenic lines were created by standard DNA microinjection techniques either using pRF4, a plasmid encoding dominant *rol-6(su1006)* allele, or pTG96, a *sur-5::gfp* construct as a transformation marker (Mello *et al*., 1991; Yochem *et al*., 1998).

Dorsal cord nuclei were counted in L1 larvae using differential interference contrast (DIC) optics and a 63× PLAN APO 1.40 objective on a Leica DM 6000 compound microscope. In all cases, dorsal cord nuclei were counted in at least 15 transgenic animals. All counts were blind, in that the animal was only identified as transgenic or not after counting (using the *sur-5::gfp* coinjection marker). Thus, hyp7 nuclei were counted in >250 nontransgenic *unc-84(e1408)* L1 animals.

UNC-83 and UNC-84 Immunofluorescence in C. elegans Embryos

A new antibody against the UNC-84 peptide TEADNNFDTHEWKSC was raised in rats. The peptide was conjugated to KLH and injected into rats five times at 3-wk intervals (done at Covance, Denver, PA). Sera from rats CA2608 and CA2609 were found to behave similarly by enzyme-linked immunosorbent assay and immunofluorescence and used for subsequent immunofluorescence studies at a dilution of 1/1000. Serum from rat 3A against ANC-1 was used as described previously (Starr and Han, 2002). The UNC-83 mouse monoclonal antibody1209D7D5 was used as described previously; tissue culture media from clone 1209D7D5 was used undiluted (Starr *et al*., 2001). Goat anti-mouse or anti-rat IgG conjugated to Cy3 at a 1/200 dilution was used for the secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were collected using the Leica DM 6000 with a 63× PLAN APO 1.40 objective, a Leica DC350 FX camera, and Leica FW4000 software. Images were uniformly enhanced using the levels and contrast commands in Adobe Photoshop (Adobe Systems, Mountain View, CA).

Expression and Topology of UNC-83 and UNC-84 in Mammalian Tissue Culture Cells

The BamHI/XhoI fragment of the *unc-83* cDNA yk230e1 and the ApaI/XbaI fragment of the *unc-84* cDNA yk402g1 (Kohara, 1996) were engineered to express from the strong mammalian cytomegalovirus (CMV) promoter in the plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA) to create pDS59 and pDS60, respectively. Human embryonic kidney (HEK) 293 cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Before transfections, cells were grown to 60–80% confluence on acid-washed glass coverslips precoated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO). Lipofectamine Plus reagent (Invitrogen) was used, according to the manufacturer's recommendations. HEK 293 cells were transfected with either pDS59 or pDS60, along with the cytosolic green fluorescent protein (GFP) marker pFM112 (Green *et al*., 2005; provided by Frank McNally, UC Davis, Davis, CA) to allow for identification of transfected cells.

Twenty-two hours after transfection, the cells were washed twice in phosphate-buffered saline (PBS) and fixed for 10 min in 3% paraformaldehyde in PBS. After fixation, all subsequent steps were done on ice. Cells were permeabilized with prechilled 0.5% Triton X in PBS or 40 μg/ml digitonin (Sigma-Aldrich) in PBS for a total of 15 min, blocked with 5% milk in PBS for 30 min, and then washed three times with the same solution used in permeabilizing step (Adam *et al*., 1992). Permeabilized cells were then incubated with the appropriate primary antibodies for 1 h: undiluted mouse anti-UNC-83 monoclonal antibody (mAb) (as described above), rat anti-UNC-84 antibody used at a dilution of 1/1000 (as described above), mouse anti-nuclear pore complex monoclonal 414 antibody used at a dilution of 1/1000 (Covance) (Davis and Blobel, 1986), and goat anti-lamin B antibody (Santa Cruz Biotechnology, Santa Cruz, CA) used at a dilution of 1/100. After rinsing the cells three times with permeabilization buffer, a 1-h incubation with the appropriate secondary antibodies followed: donkey anti-mouse antibody conjugated to Cy3, donkey anti-rat antibody conjugated to Cy3, or donkey anti-goat antibody conjugated to Cy2 (Jackson ImmunoResearch Laboratories). Chromatin was stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 10 min. Images were captured as described above.

unc-83 and unc-84 Rescuing and Mutant Constructs

The UNC-83 KASH domain point mutations and deletions were constructed in the minimal *unc-83* rescuing construct pDS22, a 10.5-kb EagI-to-HpaI fragment of cosmid W01A11 in pBS (Stratagene, La Jolla, CA) (Starr *et al*., 2001). To do this, the 3′ end of *unc-83*, a 1.8-kb PstI-to-KpnI fragment was subcloned from pDS22 into pBS to create pSL2. Deletion of the coding sequence for the C-terminal 17 residues of UNC-83 and single point mutations was created using the QuikChange XL system (Stratagene) according to the manufacturer's protocol. Alternatively, mutations were created using standard PCR SOEing techniques (Horton *et al*., 1990). The sequences of the resulting subclones were verified (University of California, Davis, sequencing facility or SeqWright, Houston, TX) and subcloned back into pDS22.

A minimal UNC-84 rescuing construct was created by amplifying a 7.5-kb genomic fragment around *unc-84* corresponding to 9684–17190 of cosmid F54B11. The genomic fragment was cloned into the pCR-XL-TOPO vector (Invitrogen) to create pSL38. To delete the SUN domain, the QuikChange mutagenesis system (Stratagene) was used to delete the last 177 amino acids

of UNC-84 and replace them with a PacI site immediately before the stop codon. The UNC-84ΔSUN construct was named pSL47.

Split-Ubiquitin Yeast Two-Hybrid System

A detailed methods paper for using the membrane-bound split-ubiquitin yeast two-hybrid (MbYTH) system has been published previously (Fetchko and Stagljar, 2004). To create the UNC-84 bait construct, the sequence encoding the C-terminal 633 residues of UNC-84 was amplified using *Pfu*Ultra high-fidelity DNA polymerase (Stratagene) from the cDNA yk402g1 (a gift from Y. Kohara, National Institute of Genetics, Mishima, Japan) with overhanging NcoI and NheI restriction sites. The PCR product was cloned in frame and downstream of the LexA/VP16 hybrid transcription factor (TF) and the C-terminal half of ubiquitin (Cub) in the vector pTFB1 (Dualsystems Biotech, Zurich, Switzerland) to create pSL36, which expresses a TF::Cub::UNC-84 fusion protein and a LEU2 selectable marker. The series of UNC-84 deletions were made by an analogous manner to delete the last 278 (UNC-84Δ278 or ΔSUNa), 329 (UNC-84Δ329 or ΔSUNb), or 369 (UNC-84Δ369 or ΔSUNc) residues (the SUNa and SUNb deletions were a gift from I. Minn and C. Malone, The Pennsylvania State University, University Park, PA). For the UNC-83 prey construct, DNA encoding the last 294 residues of UNC-83 was amplified with *Pfu*Ultra from yk230e1 (a gift from Y. Kohara) with overhanging NcoI and PstI sites. The product was cloned into pNubG-HA-prey (a gift from I. Stagljar, University of Zurich-Irchel, Zurich, Switzerland) to create pSL37, which expresses aNubG::UNC-83 fusion protein and the TRP1 marker. The pNubI vector was used as a control to confirm proper orientation of the bait constructs in the membrane (gift from I. Stagljar) (Fetchko and Stagljar, 2004). The UNC-84 point mutations were made using pSL36 as a template and PCR SOEing mutagenesis (Horton *et al*., 1990). Point mutations were introduced into the KASH domain of UNC-83 by two alternative methods. For the Y967A mutation, the QuikChange kit (Stratagene) was used to mutagenize a base in the wild-type prey construct pSL37. For the N969A and P973A mutations, the sequence encoding the C-terminal KASH domain was amplified with *Pfu*Ultra using primers that incorporated the point mutations, and cloned into pNubG-HA-prey as described above.

Bait and prey vectors were transformed into the yeast strain L40 (*MATa his3-200 trp-901, leu2-3 112 ade2, LYS2::(4xlexAop)-HIS3, URA3::(8xlexAop)-lacZ GAL4*) (Fetchko and Stagljar, 2004). Controls were done to ensure that neither the bait nor prey constructs self-activated the reporters and that the bait was expressed in the proper orientation (Figure 5; Fetchko and Stagljar, 2004). The *HIS3* reporter construct was assayed by growth on SD −trp −leu −his dropout plates supplemented with 2 mM 3-amino-1,2,4-triazole (Sigma-Aldrich) to prevent leakiness of the *his3-200* gene. Qualitative assessment of the *lacZ* reporter was done by filter lift β-galactosidase assays (Fetchko and Stagljar, 2004).

RESULTS

UNC-83 Has a KASH Domain That Is Required for Nuclear Envelope Localization

Mutations in *unc-84* disrupt the localization of both UNC-83 and ANC-1 to the nuclear envelope (Starr *et al*., 2001; Starr and Han, 2002). This suggested a common mechanism for the localization of these two proteins. We therefore examined UNC-83 and ANC-1 for similarities. It was previously thought that UNC-83 was not conserved outside of closely related nematodes; the only identified motif was a predicted transmembrane domain ending just 18 residues from the C terminus of UNC-83 (Starr *et al*., 2001). On closer examination of the C-terminal 18 residues of UNC-83, a KASH domain (for *K*larsicht, *A*NC-1, and *S*yne *h*omology) was identified. Ten of the C-terminal 18 residues (56%) of UNC-83 were identical to the KASH domain of ANC-1 (Figure 1A). Together, the high level of identity and the requirement of UNC-84 for localization to the nuclear membrane suggest that UNC-83 contains a conserved KASH domain.

To test the function of the KASH domain in UNC-83, we engineered a version of UNC-83 lacking the KASH domain (UNC-83Δ17) and compared it with a wild-type minimal rescuing fragment (Starr *et al*., 2001). To assay nuclear migration, we counted the number of hyp7 nuclei in the dorsal cord. Normally, nuclei in hyp7 precursors migrate contralaterally during the comma stage of embryogenesis (Sulston *et al*., 1983). If nuclear migration fails, the hyp7 nuclei end up in the dorsal cord (Starr *et al*., 2001). In the nontransgenic starting strain *unc-83(e1408)*, an average of 12.8 nuclei/dorsal cord was observed. In the three independent transgenic lines with the wild-type minimal rescuing construct, only averages of 0.7, 2.7, and 4.7 hyp7 nuclei were counted (Figure 1, B and D). Although not as low as wild type (0.45 hyp7 nuclei/dorsal cord), this baseline represents what we termed the rescued *unc-83* phenotype. The construct encoding UNC-83Δ17 was injected into null *unc-83(e1408)* hermaphrodites to create extrachromosomal transgenic lines. This UNC-83Δ17 construct failed to rescue the hyp7 nuclear migration defect in transgenic *unc-83(e1408)* animals; averages of 12.7, 12.9, and 13.3 hyp7 nuclei were observed in the dorsal cords of animals from three independent transgenic lines (Figure 1, C and D). These data show that the KASH domain of UNC-83 is necessary for UNC-83 function during nuclear migration.

We further examined the UNC-83Δ17 lines for UNC-83 localization by immunofluorescence. In wild-type comma stage embryos, UNC-83 localizes to the nuclear envelope of P-cells, hyp7 precursors, and intestinal primordial cells (Figure 1E) (Starr *et al*., 2001). When the minimal rescuing fragment was expressed in *unc-83(e1408)* embryos, UNC-83 normally localized to the nuclear envelope of hyp7 precursors and P-cells (Figure 1F). In these rescued animals, UNC-83 also localized at much higher levels to unidentified structures in the cytoplasm of a large number of cells in the anterior half of the embryo (Figure 1, F and G) (note that the exposure times for the images in Figure 1, E and F, and H were equal). Because the minimal rescuing fragment is missing 10–15 kb of regulatory sequences, we hypothesize that the extra staining seen around the developing pharynx is due to a lack of repressors in the minimal genomic rescuing fragment. Nonetheless, this extra staining was useful as a marker to confirm that the transgene expressed UNC-83 as detected by the anti-UNC-83 mAb (Starr *et al*., 2001). In transgenic lines expressing UNC-83Δ17, UNC-83 was not detectable at the nuclear envelope in hyp7 or P-cells. The strong, nonspecific anterior staining was seen in these animals, confirming that UNC-83Δ17 was being expressed (Figure 1H). We therefore conclude that the KASH domain of UNC-83 is required for its proper localization to the nuclear envelope.

Topology of UNC-83 and UNC-84 in the Nuclear Membrane

Given the homology of the UNC-83 KASH domain and the UNC-84 SUN domain with other mammalian proteins (Figure 1A) (Malone *et al*., 1999), we tested whether conserved targeting mechanisms existed. *C. elegans* UNC-83 or UNC-84 was transiently expressed in mammalian HEK 293 cells. When stained with the anti-UNC-83 mAb or a newly raised anti-UNC-84 antibody, both UNC-83 and UNC-84 were detected at the nuclear envelope of independently transfected cells. As a control, no epitope was detected in neighboring untransfected cells (Figure 2). These data suggest that SUN proteins from *C. elegans* to humans are targeted to the nuclear envelope by conserved proteins, presumably through lamin interactions with the N terminus of SUN proteins (Hodzic *et al*., 2004). Furthermore, *C. elegans* and mammalian KASH proteins are targeted to the nuclear envelope through conserved mechanisms.

Our nuclear envelope bridging model makes clear predictions of the topology of UNC-83 and UNC-84 in the nuclear envelope. Using the transfected 293 system, we were able to test the topology of the transfected *C. elegans* proteins. Digitonin is a detergent that selectively permeabilizes the plasma membrane but not the nuclear envelope. In a digitonin-treated cell, outer nuclear membrane epitopes facing

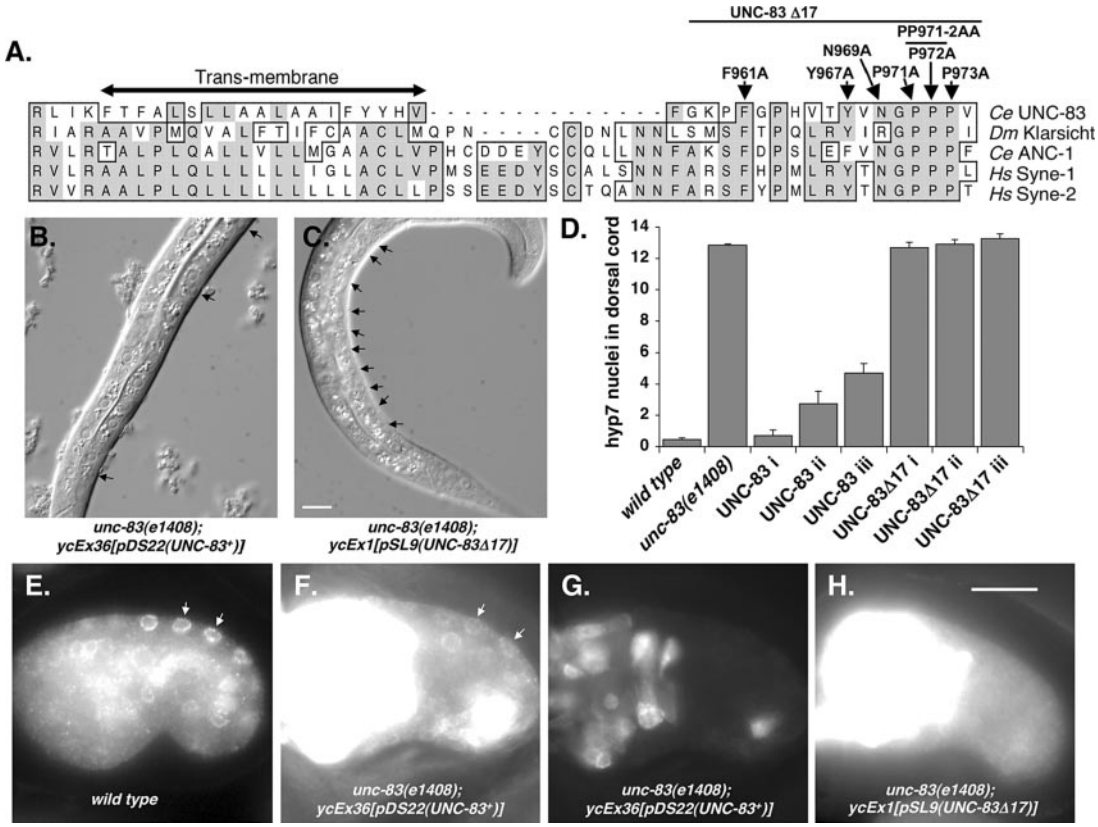

Figure 1. UNC-83 has a KASH domain that is necessary for nuclear localization. (A) The C-terminal fragment of UNC-83 is aligned with the C-terminal KASH domains from *D. melanogaster* Klarsicht, *C. elegans* ANC-1, and human Syne-1 and Syne-2 (also known as nesprins). Similar residues are boxed and identical residues are shaded. Dashes represent a gap in UNC-83 and Klarsicht. The predicted transmembrane span is marked. Point mutations in the KASH domain used throughout this article are marked here for reference. (B) DIC images showing the dorsal cord in an *unc*-83(e1408); ycEx36[pDS22(UNC-83$^+$)] rescued animal and (C) an *unc*-83(e1408); ycEx1[pSL9(UNC-83Δ17)] transgenic animal. Arrows point out hyp7 nuclei that failed to migrate and were observed in the dorsal cord of L1 larvae. Bar, 10 μm. (D) The mean number of hyp7 nuclei found in the dorsal cord of wild type (*N2*), *unc-83(e1408)* null animals, three lines each of *unc-83(e1408);* Ex[pDS22(UNC-83$^+$)] (UNC-83 i–iii) and unc-83(e1408); Ex[pSL9(UNC-83Δ17)] (UNC-83Δ17 i–iii) animals. Three independent transgenic lines are shown for each construct ($n > 15$ for each line). SE bars are shown. (E–H) Immunolocalization of UNC-83 in comma stage embryos. Anti-UNC-83 immunofluorescence with the mAb is shown. (E) Wild type (*N2*). (F) unc-83(e1408); ycEx36[pDS22(UNC-83$^+$)] rescued embryo. (G) The same embryo as in F but at a much lower exposure to see the strong expression of UNC-83 in the pharynx region from the transgene. (H) unc-83(e1408); ycEx1[pSL9(UNC-83Δ17)] embryo. Examples of clear hyp7 nuclear envelope staining in the wild-type or rescued animals are marked by arrowheads (F and G). Images in E, F, and H were taken from parallel experiments at the same exposure. Anterior is left, dorsal is up; bar, 10 μm (E–H).

the cytoplasm are available to the primary antibody, but epitopes of the inner nuclear membrane facing the nucleoplasm are not (Adam *et al*., 1992). We used antibodies against nuclear pore complexes (mAb414) or lamin B as controls for outer and inner nuclear membrane markers, respectively (Adam *et al*., 1992; Ganeshan *et al*., 2001). In all cases, data were collected from at least two experiments; because the data were consistent, they were pooled. The mAb414 epitope was detected in 99.3% ($n = 134$) of the digitonin and 100% of the Triton X-100–extracted cells ($n = 148$). The lamin B epitope was only detected in 3.8% ($n = 1092$) of the digitonin-extracted cells and 96.7% ($n = 181$) of the Triton X-100–extracted cells (Figure 2, A and B). Thus our digitonin extraction conditions are appropriate to distinguish between inner and outer nuclear membrane components.

After a 3- to 4-h transfection and 22 h to express either UNC-83 or UNC-84, cells were extracted with digitonin or Triton X-100. Transfected cells were identified using a cytoplasmic GFP cotransfection marker. Although UNC-84 was detected at the nuclear envelope in 95.5% of the Triton X-100–treated cells, UNC-84 was only detected in the outer nuclear membrane in 39.8% of digitonin-treated cells (Figure 2, C–E). It is likely that the UNC-84–detected represents overexpression because when extracted 42 h after transfection, the UNC-84 epitope was detected in 62.9% ($n = 135$) of UNC-84–transfected cells treated with digitonin. In contrast to UNC-84, UNC-83 was detected at the outer nuclear membrane in 93.1% of the digitonin-treated transfected cells (Figure 2F). These data are consistent with our nuclear envelope bridging model where UNC-84 is an integral component of the inner nuclear membrane and UNC-83 is a component of the outer nuclear membrane.

Identifying Conserved Residues in the UNC-83 KASH Domain

To further study the role of KASH domains and to identify important residues of the KASH domain of UNC-83, we engineered a number of point mutations in the C terminus of UNC-83. Specifically, constructs were made encoding mutant proteins with alanines in place of conserved residues

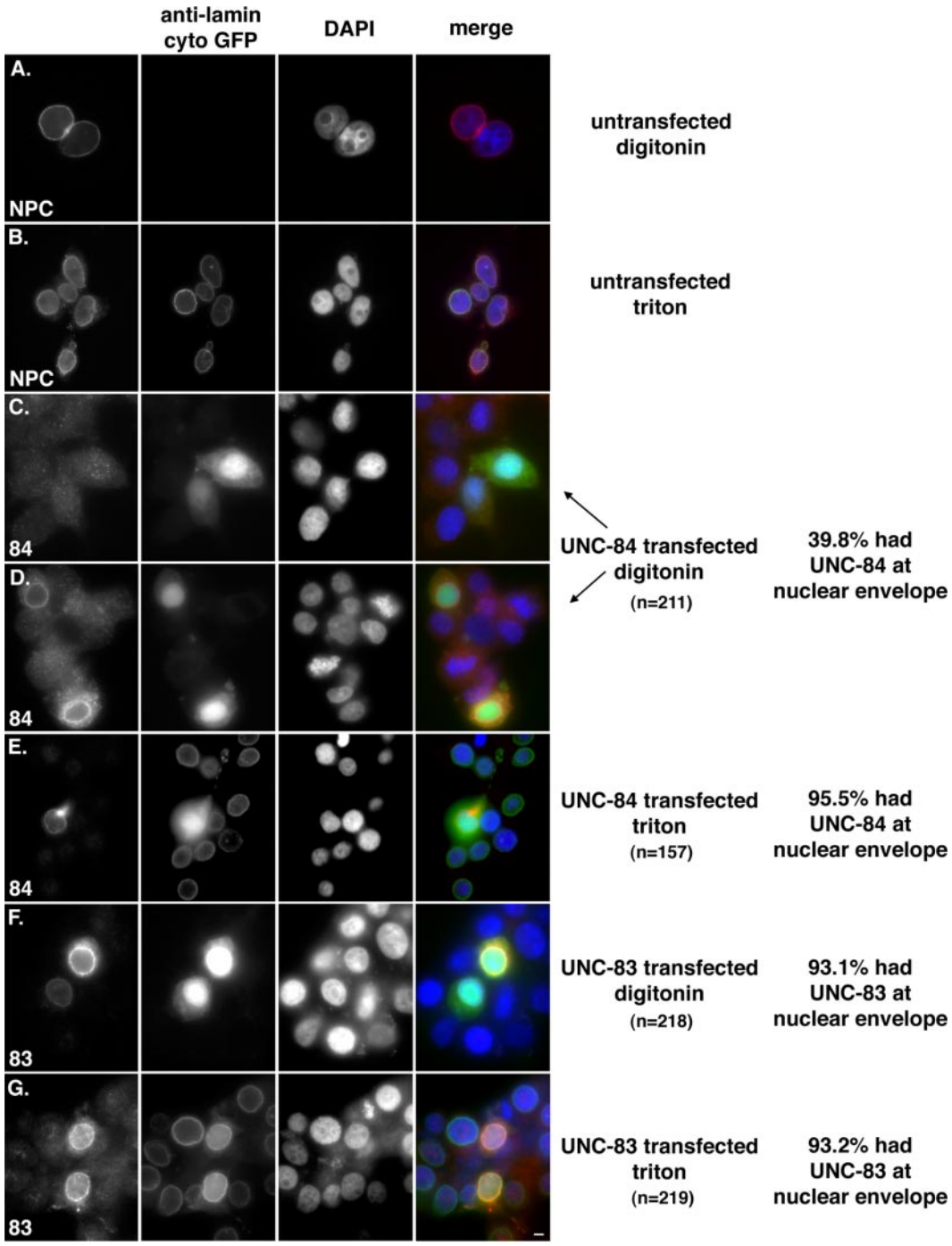

Figure 2. UNC-83 localizes to the outer nuclear membrane and UNC-84 to the inner nuclear membrane in transfected mammalian tissue culture cells. The same field of HEK 293 cells are shown in three different channels and merged in the fourth column. In the first column (red in the merge), cells were stained with either mAb414 to detect outer membrane nuclear pore complexes (NPC) (A and B), anti-UNC-84 (C–E), or anti-UNC-83 (F and G). In the second column (green in the merge), cells are imaged for both anti-lamin antibodies and a GFP marker to identify transfected cells. In the third column (blue in the merge), cells are imaged for DAPI staining to detect chromatin. (A and B) Untransfected control cells treated with digitonin so only outer nuclear envelope components can be detected (A) or treated with Triton to expose all antigens (B). (C–E) Cells transiently transfected for 22 h with the cDNA for *C. elegans* UNC-84 under the control of the CMV promoter. (F and G) Cells transiently transfected for 22 h with the cDNA for *C. elegans* UNC-83 under the control of the CMV promoter. Cells were extracted after fixation with digitonin to expose outer nuclear membrane epitopes including NPC and UNC-83 (A, C, D, and F) or Triton X-100 to expose all nuclear envelope antigens including lamins and UNC-84 (B, E, and G). The data are summarized at the right and detailed in the text. Bar, 5 μm.

in the consensus KASH sequence FxPxxxYxNGPPPx of UNC-83 (Figure 1A). These constructs were then injected into the *unc-83(e1408)* null background to examine whether the KASH domain point mutations could function. We assayed for function by two means. First, we used our monoclonal anti-UNC-83 antibody to confirm expression and to

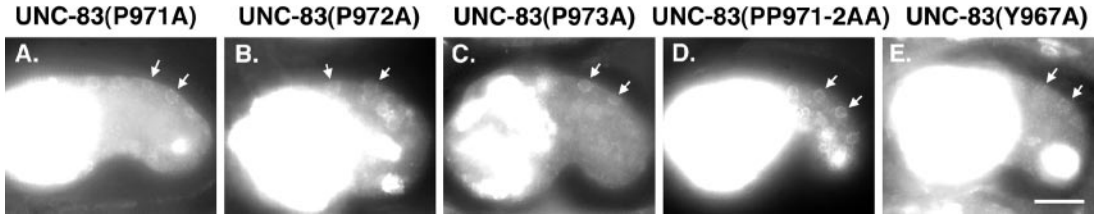

Figure 3. Localization of mutant UNC-83 KASH domains. Anti-UNC-83 antibodies were used to stain transgenic embryos. Fluorescent images taken at equal exposure times are shown. A comma stage embryo is shown from a transgenic line of each construct. Arrowheads point out clear examples of hyp7 nuclear envelope localization of UNC-83. Anterior is left, dorsal is up; bar, 10 μm.

determine whether versions of UNC-83 with point mutations in its KASH domain were able to properly localize to the nuclear envelope (Figure 3). Second, we analyzed whether the mutant KASH domain proteins disrupted the function of UNC-83 during nuclear migration of hyp7 precursors by counting numbers of hyp7 nuclei in the dorsal cord because of failed nuclear migrations (Figure 4). Transgenic lines of most constructs had ectopic, strong anti-UNC-83 staining in the head (Figure 3), suggesting that the transgene was expressed. In multiple lines of the UNC-83 N969A mutation or the F961A mutation, the transgene was expressed at very low levels (our unpublished data). Therefore, we cannot make any conclusions about the in vivo function of the N969A or F961A mutant proteins. All other lines had high levels of expression of the transgene.

In one class of UNC-83 KASH domain point mutations, the P971A, P972A, and P973A mutations, mutant UNC-83 localized to the nuclear envelope and rescued (at least partially) hyp7 nuclear migration (Figures 3, A–C, and 4, D–F). Even changing two of the prolines at the same time to alanines did not disrupt nuclear envelope localization (Figure 3D). Significantly more nuclei migrated normally in the UNC-83 PP971-2AA lines than in the null background (Figure 4, A and G), suggesting at least partial function during hyp7 nuclear migration. However, localization of mutant UNC-83 at the nuclear envelope did not always accurately predict function during hyp7 nuclear migration. Three independent lines expressing Y967A localized normally to the nuclear envelope; nonetheless, nuclear migration in hyp7 precursors completely failed (Figures 3E and 4H). This suggests that perhaps a stronger or different interaction is required for nuclear migration than for nuclear envelope localization.

The KASH Domain of UNC-83 Interacts with UNC-84

Genetic evidence suggests that UNC-83 and UNC-84 interact (see *Introduction*). We therefore tested for a direct, physical interaction between these proteins. Initial attempts at traditional directed yeast two-hybrid assays and glutathione *S*-transferase pull-down assays did not reveal an interaction (our unpublished data). We hypothesized that to detect a physical interaction between the KASH domain of UNC-83 and UNC-84, the KASH domain would need to be presented in the proper context of a membrane. Thus, we used the split-ubiquitin membrane-bound yeast two-hybrid (MbYTH) system, which allowed us to test for such an interaction in a more natural environment, associated with the lumenal face of the yeast endoplasmic reticulum (ER) membrane.

The MbYTH system is used to study interactions between membrane proteins and uses complementation between two separable domains of the small protein ubiquitin (Figure 5; Fetchko and Stagljar, 2004). The wild-type N-terminal half of ubiquitin (NubI; amino acids 1–34) is able to spontaneously interact with the C-terminal half of ubiquitin (Cub; amino acids 35–76). When NubI and Cub interact, the reconstituted protein recruits ubiquitin-specific proteases, which release ubiquitin from its target before degradation. The MbYTH uses a mutant N-terminal domain of ubiquitin (NubG, which replaces the wild-type isoleucine at position 13 with a glycine) that is not able to bind to Cub. However, by fusing NubG and Cub to interacting proteins X and Y, NubG, and Cub are brought in proximity to one another, are able to partially interact, and recruit ubiquitin-specific proteases. In the MbYTH system, cleavage by the ubiquitin-specific proteases releases a transcription factor containing a LexA DNA binding domain and a VP16 transactivating domain, which activates the *HIS3* and *lacZ* reporter genes. The value of this system is that interacting proteins X and Y can contain signal sequences and transmembrane domains. This allows the interaction of X and Y to take place on the lumenal side of a membrane, which in turn brings NubG and Cub together on the cytoplasmic face of the membrane. The close association of the two halves of ubiquitin recruits the proteases and releases the transcription factor to activate the reporters. If X and Y fail to interact, the transcription factor remains tethered to the cytoplasmic face of the organelle, outside of the nucleus, and the reporter genes are silent.

The C-terminal 633 residues of UNC-84, which contain the predicted transmembrane domain, a linker domain, and the SUN domain, were cloned into a MbYTH bait vector to express a TF::Cub::UNC-84 fusion protein. The C-terminal 294 residues of UNC-83, including the transmembrane and KASH domains, were cloned into a MbYTH prey vector to express NubG:UNC-83. When co-expressed in the yeast reporter strain, the UNC-84 bait and UNC-83 prey strongly interacted, activating both the *HIS3* and *lacZ* reporters (Figure 5). A number of controls were performed to verify the MbYTH interaction and to confirm that the UNC-84 bait was properly targeted to the membrane with the TF::Cub on the cytoplasmic face. First, the UNC-84 bait construct was expressed either alone or with cytoplasmic NubG in the reporter yeast strain. The UNC-84 bait did not self activate the reporters, even in the presence of NubG. This confirmed that the UNC-84 bait was sequestered outside of the nucleus, likely at an ER-derived membrane (Figure 5A, middle). Accordingly, when the empty bait vector with the transcription factor and Cub, but no transmembrane domain, was expressed alone, it activated the reporters. For the second control, to confirm the orientation of the UNC-84 bait, the bait was expressed with the wild-type NubI in the cytoplasm. In this case, the reporters were activated (Figure 5A, left), suggesting that the Cub portion of the UNC-84 bait was in the cytoplasm. We also confirmed that the UNC-83 prey

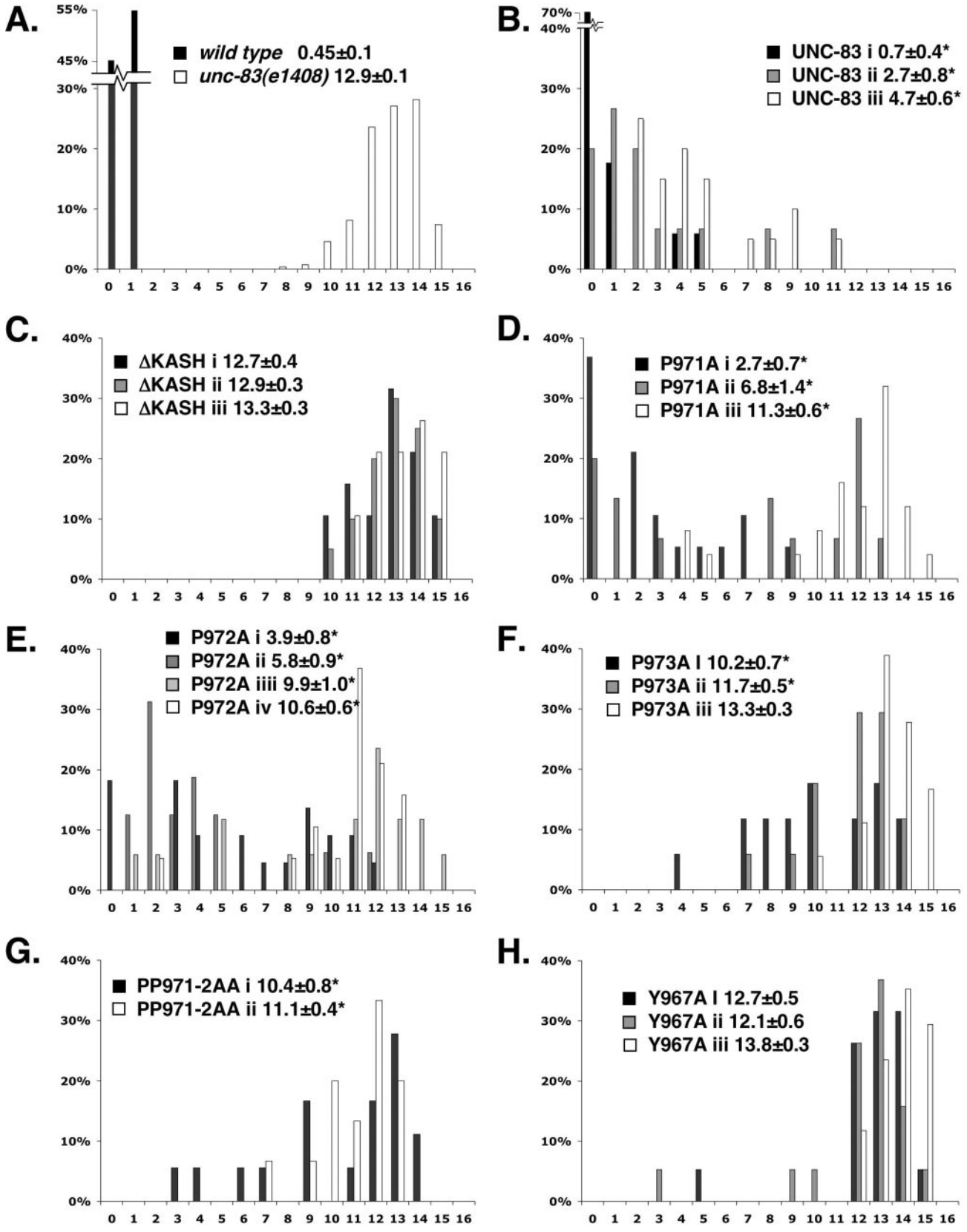

Figure 4. Point mutations in UNC-83 disrupt nuclear migration. For each transgenic line, the number of hyp7 nuclei was counted in dorsal cords of L1 larvae to quantify nuclear migration defects. On the *x*-axis of each plot is the number of hyp7 nuclei observed in an animal. The *y*-axis is the frequency (in percentage) that a particular number of nuclei were counted in a transgenic line. All lines are shown in different shades. The legend for each plot also lists the mean number of nuclei counted in the dorsal cord plus or minus the SE. Asterisks mark means

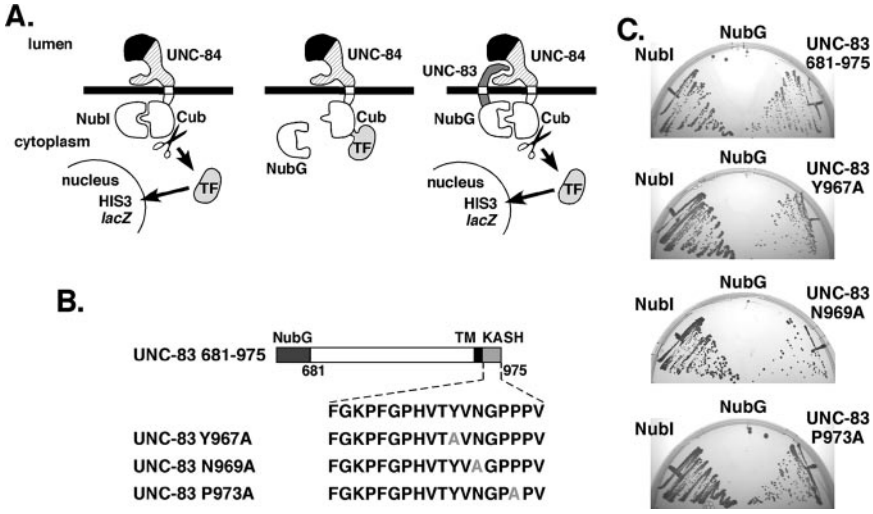

Figure 5. UNC-83 and UNC-84 interact in the MbYTH system. (A) Schematic and controls of the MbYTH system. The bait TF:Cub: UNC-84 in the presence of wild-type NubI, associated the two halves of ubiquitin and released the transcription factor, which activated the reporter genes (left). When the UNC-84 bait was expressed alone or with the mutant NubG, it did not activate the reporters, suggesting it was targeted to the membrane in the correct orientation with Cub in the cytoplasm (middle). When the TF:Cub: UNC-84 was coexpressed with the NubG: UNC-83, the system was activated, meaning the C terminus of UNC-84 interacted with the lumenal KASH domain of UNC-83 (right). (B) The wild-type interaction and point mutations in the KASH domain of UNC-83 that were tested are indicated. (C) Their ability to grow on SD −Trp −Leu −His + 1 mM 3-AT plates is shown.

vector was not able to self-activate the reporters. We therefore conclude that UNC-83 and UNC-84 interact in the MbYTH system.

Once the physical interaction between UNC-83 and UNC-84 was identified using the MbYTH system, we used the system to complement our in vivo molecular genetic experiments on the KASH domain of UNC-83. We expressed three UNC-83 KASH domain missense mutations in the MbYTH system: UNC-83 Y967A, UNC-83 N969A, and UNC-83 P973A. All three were able to interact with the wild-type UNC-84 bait; they grew on histidine dropout plates and activated the *lacZ* reporter when coexpressed with UNC-84 bait (Figure 5, B and C). These data corroborate the in vivo results in that Y967A and P973A were able to localize to the nuclear envelope, regardless of their ability to function during nuclear migration.

The SUN Domain of UNC-84 Is Not Essential for the Interaction with the KASH Domain of UNC-83 in the MbYTH System

As mentioned above, missense mutations in the SUN domain of UNC-84 disrupt nuclear positioning as well as localization of KASH proteins to the nuclear envelope (Malone *et al*., 1999; Starr *et al*., 2001; Starr and Han, 2002). These data suggested that the conserved SUN domain of UNC-84 would overlap with the UNC-83 interaction domain. We used the MbYTH system to characterize the UNC-83 interaction domain within the C-terminal 633 residues of UNC-84. First, mutations to mimic *unc-84(n399)* (UNC-84 G1002D) and *unc-84(sa61)* (UNC-84 S988F) were engineered into our UNC-84 bait construct. Interestingly, mutant proteins containing either amino acid substitution were able to interact with the UNC-83 prey (Figure 6). Initial attempts to quantify the interactions using liquid β-galactosidase assays suggested the mutant forms of UNC-84 interacted at a lower level than the wild-type UNC-84 interaction with UNC-83. However, these results were not statistically significant upon repeating. Thus, we can only use the MbYTH system to qualitatively describe the interaction between UNC-84 and UNC-83.

To precisely map the UNC-83 interaction domain within the C terminus of UNC-84, a series of C-terminal deletions removing the SUN domain and other parts of the linker domain of the UNC-84 bait were created and tested in the MbYTH system (Figure 6A). Deletion of the last 278 or 329 residues of UNC-84, which includes the entire conserved SUN domain, had no qualitative effect on the interaction with UNC-83 (Figure 6). Deletion of just 40 additional residues or more from the C terminus of UNC-84Δ329 (UNC-84Δ369, UNC-84Δ430, or UNC-84Δ490) led to self-activation of the MbYTH system. This suggests that the region between residues 742 and 782 of UNC-84 is important for membrane targeting. Alternatively, the overall size of the UNC-84 bait could be important for membrane targeting. These data allowed us to conclude that the SUN domain of UNC-84 is not required for the interaction with UNC-83 in the MbYTH system. To see whether the SUN domain of UNC-84 is able to interact with UNC-83 on its own, we created a deletion of residues 567–941 in the UNC-84 bait construct to create the UNC-84Δlinker bait. This construct was able to interact with the UNC-83 prey (Figure 6). Therefore, the C-terminal half of UNC-84 contains multiple KASH binding sites, one in the SUN domain and another in the linker domain.

The SUN Domain of UNC-84 Is Required for In Vivo Localization of UNC-83 and ANC-1

To confirm our MbYTH results that suggest the SUN domain of UNC-84 is dispensable for the full UNC-83 interaction, we tested the in vivo ability of a SUN-less form of UNC-84 to recruit UNC-83 to the nuclear envelope. We cloned a 7.5-kb genomic fragment that was able to rescue the hyp7 precursor nuclear migration defect of null *unc-84(n369)* animals (Figure 7A). We next deleted the region encoding the C-terminal 177 residues of UNC-84 from our minimal rescuing construct and injected it into *unc-84(n369)* animals to express UNC-84ΔSUN in an otherwise null *unc-84(n369)*

Figure 4 (cont). of hyp7 nuclei in the dorsal cord that were significantly below the *unc-83(e1408)* starting strain ($p < 0.0001$). For each line, a least 15 animals were scored. Because animals were scored before determining whether they were transgenic, >250 animals of the *unc-83(e1408)* starting line were scored. The distributions of the number of nuclei in the dorsal cord are shown for the following strains: wild-type (*N2*) and *unc-83(e1408)* null animals (A), unc-83(e1408); ycEx36,28,32[pDS22(UNC-83^{+})] rescued animals (B), ycEx1,37,38[pSL9(UNC-83Δ17)] (C), ycEx23,27,35[pSL21(UNC-83P971A)] (D), ycEx24,29,31,40[pSL22(UNC-83P972A)] (E), ycEx13,15,39[pSL59(UNC-83P973A)] (F), ycEx25,34[pSL23(UNC-83PP972-3AA)] (G), and ycEx10,11,26[pSL58(UNC-83Y967A)] (H).

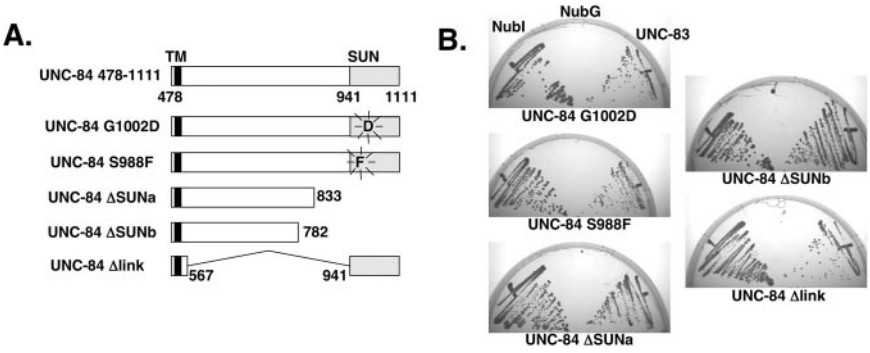

Figure 6. Mapping the KASH interaction domain of UNC-84. (A) Various constructs for the UNC-84 bait were tested, including point mutations in the SUN domain and indicated deletions. (B) Growth on SD −Trp − Leu −His + 3-AT plates indicates an interaction in the MbYTH system.

background. To follow the localization of the mutant UNC-84 transgenic protein, we raised a polyclonal antibody in rats against the N terminus of UNC-84. Our antibodies from rats CA2608 and CA2609 behave similarly to the antibody from rat 3595 as described previously (Lee *et al*., 2002) (Figure 7, C and E). Both the minimal rescuing construct and the UNC-84ΔSUN construct localized normally to the nuclear envelope, showing that the SUN domain is not required for nuclear envelope localization of UNC-84 (Figure 7, D and F). However, the UNC-84ΔSUN construct was not functional. It failed to rescue either the hyp7 nuclear migration defect (Figure 7B) or the nuclear anchorage defect (our unpublished data) of *unc-84(n369)* animals. Furthermore, UNC-83 and ANC-1 were not detected at the nuclear envelope in transgenic animals expressing UNC-84ΔSUN (compare Figure 7, J and N with I and M). As a control, UNC-83 and ANC-1 were detected at the nuclear envelope in wild-type and *unc-84(n369)*-rescued animals (Figure 7, G, H, K, and L). Thus, unlike the SUN-less experiments in the MbYTH system, the SUN domain of UNC-84 is necessary in vivo for even a basal interaction with KASH proteins.

DISCUSSION

Here, we present in vivo molecular genetic data that show UNC-83 has a KASH domain that is required for outer nuclear membrane localization of UNC-83 and therefore nuclear migration. Furthermore, our membrane-associated yeast two-hybrid results show that the KASH domain of UNC-83 directly interacts with the C-terminal half of UNC-84. Together, these results strongly support our nuclear envelope bridging model. They suggest that a physical interaction between UNC-83 and UNC-84 in the intermembrane space of the nuclear envelope functions as the central link of the nuclear envelope bridge.

The Function of KASH Domains

The interaction between UNC-83 and UNC-84 is the likely mechanism for targeting UNC-83 to the nuclear envelope. In this manner, any KASH domain-containing protein could be recruited to the nuclear envelope. KASH domains are found in a variety of proteins that target to the nuclear envelope (Starr and Fischer, 2005). In all tested cases, the KASH domain is sufficient for nuclear envelope localization (Starr and Han, 2002; Zhang *et al*., 2002; Fischer *et al*., 2004). Moreover, a naturally occurring isoform of Klarsicht without the KASH domain does not localize to the nuclear envelope (Guo *et al*., 2005). KASH-domain proteins have been shown to play roles in positioning of nuclei in mammalian skeletal muscles, *Drosophila melanogaster* photoreceptor cells, and *C. elegans* syncytial cells (Starr and Han, 2002; Zhang *et al*., 2002; Patterson *et al*., 2004; Grady *et al*., 2005; Guo *et al*., 2005). These studies have implicated that KASH-domain containing proteins function at the outer nuclear membrane and likely represent the first group of proteins to localize specifically to the outer nuclear membrane but not the rest of the ER (reviewed in Starr and Fischer, 2005). Given that *C. elegans* UNC-83 also localized to the outer nuclear membrane of transfected mammalian tissue culture cells, the mechanism of targeting KASH proteins to the outer nuclear membrane is likely conserved.

Refining the Nuclear Envelope Bridging Model

Two recent studies show that the SUN domain of human orthologues of UNC-84, Sun1 and Sun2, are integral membrane components of the inner nuclear membrane, with their SUN domains extended into the lumen of the nuclear envelope (Hodzic *et al*., 2004; Padmakumar *et al*., 2005). The digitonin extraction data presented in Figure 2 is consistent with our previous hypothesis that UNC-83 is a component of the outer nuclear membrane with its KASH domain in the lumen (Starr *et al*., 2001; Starr and Han, 2003). We therefore believe that we have characterized the central link of the nuclear bridging model by demonstrating a direct, physical interaction between an inner nuclear membrane protein (UNC-84) and an outer nuclear membrane component (UNC-83) (Figure 8). Through this central link, forces could be transferred from the cytoplasm to the nuclear matrix during nuclear migration.

We propose that the strength of the interaction between UNC-83 and UNC-84 controls the function of UNC-83. A relatively weak interaction is required to localize KASH-domain proteins to the nuclear envelope. However, a much stronger interaction between the KASH domain of UNC-83 and UNC-84 is required to transfer forces across the membrane during nuclear migration. This model would explain our data. Specifically, the UNC-83 Y967A mutant protein preserves the initial weak interaction required for localization, but it fails to transfer the larger forces required during nuclear migration.

The Role of the SUN Domains in Nuclear Positioning

Our two-hybrid data show that UNC-84 contains multiple UNC-83 interaction domains. We propose that the SUN domain of UNC-84 (black in Figure 8) and the linker domain between the transmembrane and the SUN domain (cross hatched in Figure 8) form two faces or a pocket to interact with the KASH domain of UNC-83 (gray in Figure 8). Surprisingly, either one of these two domains alone can efficiently bind the UNC-83 KASH domain in the MbYTH system (Figure 6). A similar result was obtained in a study

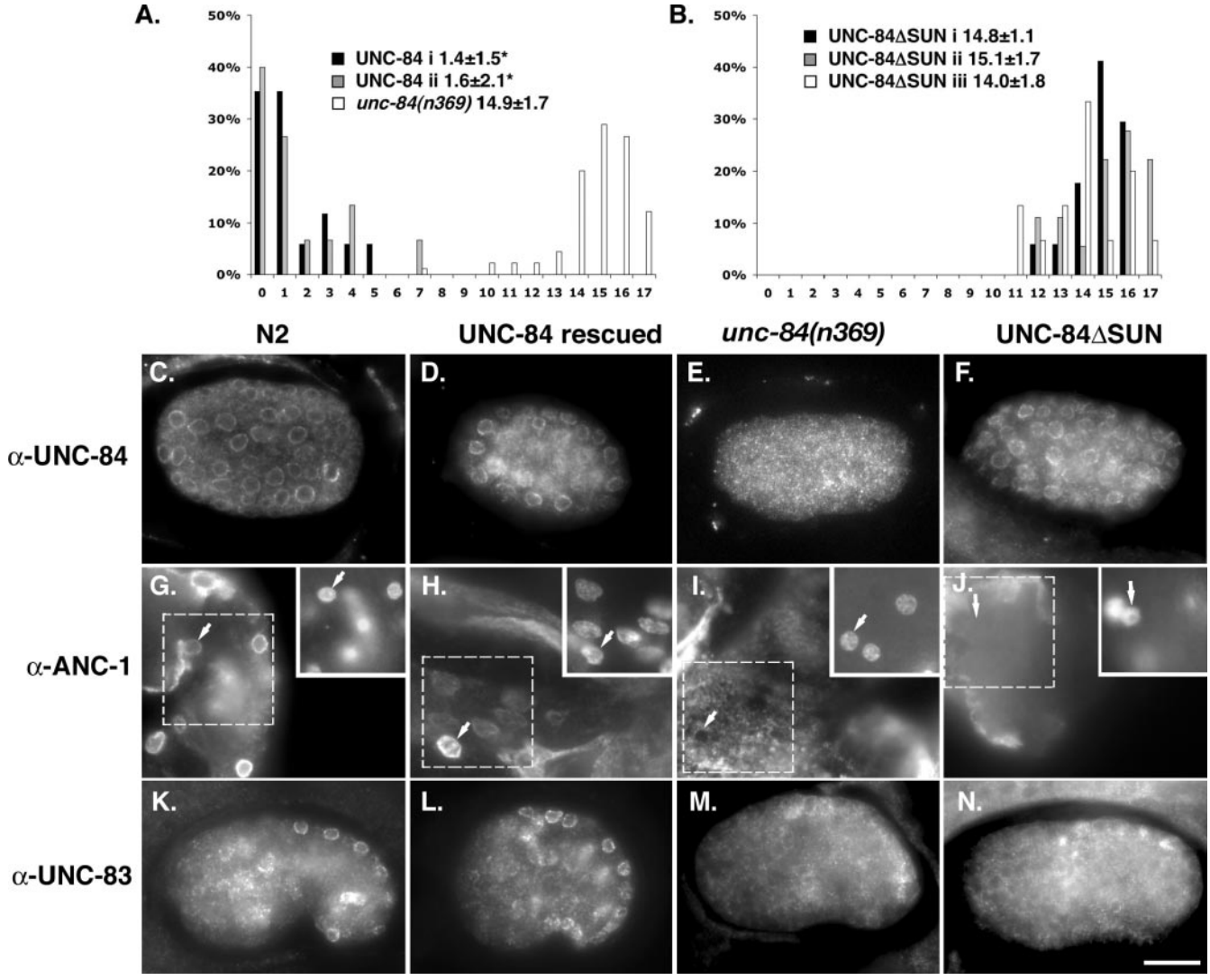

Figure 7. Role of the SUN domain of UNC-84 in *C. elegans*. (A and B) Nuclear migration in hyp7 cells is presented as in Figure 4. The legend for each plot also lists the mean number of nuclei counted in the dorsal cord plus or minus the SE. Asterisks mark means of hyp7 nuclei in the dorsal cord that were significantly below the *unc*-84(n369) starting strain ($p < 0.0001$). The distributions of the number of nuclei in the dorsal cord are shown for the following strains: *unc-84(n369)* null animals and *unc*-84(n369); ycEx12,14[pSL38(UNC-84$^+$)]-rescued animals (A), and ycEx42,43,44[pSL47(UNC-84ΔSUN)] animals that are not rescued (B). Staining with anti-UNC-84 antibody in embryos (C–F), anti-ANC-1 antibody in extruded adult somatic gonad tissue (G–J), and anti-UNC-83 antibody in comma-staged embryos (K–N). The immunofluorescence was done in wild-type N2 animals (C, G, and K), rescued *unc-84(n369)* transgenic animals expressing the wild-type construct ycEx12or13[pSL38(UNC-84$^+$)] (D, H, and L), unc-84(n369) null animals (E, I, and M), or *unc-84(n369)* transgenic animals expressing the SUN-deleted construct ycEx41[pSL47(UNC-84ΔSUN)] (F, J, and N). For each antibody, exposures are roughly the same. The insets in G–J show the corresponding region stained with DAPI to identify uterine cell nuclei; the arrowheads in G–J point out uterine nuclei (inset) that normally stain brightly with anti-ANC-1 (G–H) but fail to stain with ANC-1 at the nuclear periphery in the *unc-84* null animals (I) or in animals expressing the SUN-deleted rescuing construct (J) (main picture). Bar, 10 μm.

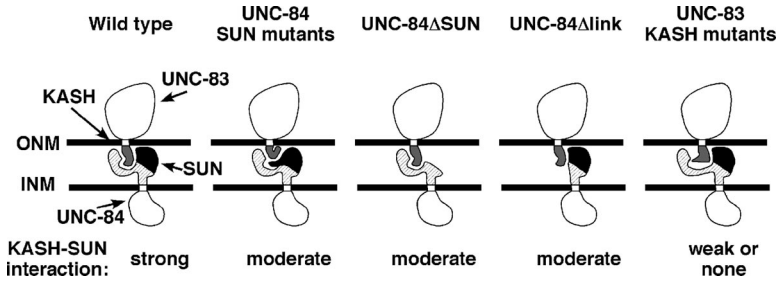

Figure 8. Model for KASH–SUN interactions. Our model for how UNC-83 and UNC-84 bridge the nuclear envelope is shown. Various point mutations that disrupt the central KASH–UNC-84 interaction are depicted. See text for details.

describing the two-hybrid interaction of human Sun1 and nesprins (Padmakumar *et al*., 2005).

To test the in vivo implications of our yeast two-hybrid data that suggest the SUN domain might be dispensable for certain functions of UNC-84, we created transgenic lines that deleted the SUN domain of UNC-84. The SUN deletion did not affect UNC-84 localization. However, neither ANC-1 nor UNC-83 was recruited at detectable levels to the nuclear envelope in the SUN-deleted transgenic lines. Furthermore, the null *unc-84* phenotypes were not rescued. Thus, *unc-84* null, the SUN deletion, and the SUN missense mutations (G1002D or S988F) all have the same loss-of-function phenotypes. We therefore conclude that the SUN domain of UNC-84 is essential for the function of UNC-84 in nuclear positioning. The exact mechanism of the SUN–KASH physical interaction remains unknown. Future studies to determine the crystal structure these proteins should clarify our model.

Conservation and Roles of SUN–KASH Protein Interactions

As stated above, SUN and KASH domains are found in a number of proteins conserved across a variety of eukaryotes (reviewed in Starr and Fischer, 2005). Thus, it is likely that the interaction between KASH and SUN domains is also conserved. A recent study has characterized a similar interaction between the human proteins Syne-2 (Nesprin-2) and Sun1 (Padmakumar *et al*., 2005).

Surprisingly, none of the UNC-83 point mutations studied here completely disrupted KASH function. They all localized normally to the nuclear envelope, although they functioned to varying degrees during nuclear migration. This suggests that the exact sequence of KASH domains might not be essential. Furthermore, at least one KASH-binding site of UNC-84 (the linker domain) is not obviously conserved, suggesting that divergent KASH-binding proteins might remain to be identified. Thus, there remains an exciting possibility that there could be a variety of other nuclear envelope components that interact with SUN proteins through KASH-like domains too divergent to be recognized using current technologies.

The nuclear envelope plays many essential roles in eukaryotic cells. Minor defects in the nuclear envelope, including mutations in lamin and emerin, lead to a variety of human diseases termed laminopathies (reviewed in Mounkes *et al*., 2003). Furthermore, the Syne-1 and Syne-2 KASH domain proteins have been found to interact with emerin and lamin (Mislow *et al*., 2002; Libotte *et al*., 2005; Zhang *et al*., 2005). Likewise, SUN proteins Sun1 and Sun2 interact with lamin (Hodzic *et al*., 2004; Padmakumar *et al*., 2005). It will therefore be interesting to characterize KASH–SUN interactions in a number of different systems and to determine whether defects in KASH or SUN proteins contribute to laminopathies.

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