A genetic approach to study the role of nuclear envelope components in nuclear positioning

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Abstract. In many cell types, the nucleus is positioned to a specific location. Our work and that of others has demonstrated that several integral nuclear envelope proteins function to move the nucleus and to anchor it in place. Our forward genetics approach has identified three components of the nuclear envelope involved in nuclear positioning. ANC-1 consists of two actin-binding calponin domains, a huge central coiled domain, and a nuclear envelope targeting a domain termed the KASH domain. ANC-1 functions to physically tether the actin cytoskeleton to the outer nuclear membrane. UNC-83 is a novel protein that functions in an unknown manner during nuclear migration. UNC-83 contains a domain with weak homology to the KASH domain of ANC-1. UNC-84 is a SUN protein that is required for both nuclear migration and anchorage. UNC-84 recruits both UNC-83 and ANC-1 to the nuclear envelope. We propose a model where UNC-84 is an integral component of the inner nuclear membrane, with its SUN domain in the perinuclear space. The SUN domain then recruits ANC-1 and UNC-83, through interactions with their KASH domains, to the outer nuclear envelope. Together these proteins function to bridge the two membranes of the nuclear envelope, connecting the nuclear matrix to the cytoskeleton.

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The nuclear envelope (NE) is a unique structure that performs multiple essential functions. It consists of two membranes; the inner nuclear membrane (INM) is closely associated with the nuclear lamina and is contiguous with the outer nuclear membrane (ONM) through the nuclear pores. The ONM is contiguous with the endoplasmic reticulum (ER). In addition to separating the nucleoplasm from the cytoplasm, the NE also controls the movement of molecules between these two compartments, gives physical structure to the nucleus, organizes chromatin, and functions in multiple other roles. Despite all these functions, fewer than 15 integral membrane nuclear envelope components have been

studied in any detail. Other chapters of this book go into great detail on many of these proteins and functions. Here we discuss the role of the NE in respect to nuclear positioning within the cell. Specifically, how does the NE function in the regulation of its own movement from one location to another in the cytoplasm? And, after the establishment of a specific location, how does the NE function to anchor the nucleus in place?

We have taken a genetic approach using the relatively simple model organism *Caenorhabditis elegans* to study nuclear positioning. Three integral components of the NE, UNC-83, UNC-84, and ANC-1 are essential for proper nuclear positioning within the cell. At least two of these proteins are conserved in mammals. Our data suggest a model where these proteins function to bridge both membranes of the nuclear envelope, effectively connecting the nuclear matrix to the cytoskeleton.

Results and discussion

Genetic screens for mutations disrupting nuclear migration and anchorage

C. elegans is an excellent choice for a non-biased genetic approach to study nuclear positioning. Since the entire cell lineage for the development of *C. elegans* and the normal position for all nuclei have been precisely documented (Sulston & Horvitz 1977, Sulston et al 1983), genetic screens for defects in the position of nuclei are simple to carry out. Horvitz and Sulston isolated the first mutants that disrupted nuclear migrations more than 20 years ago (Horvitz & Sulston 1980, Sulston & Horvitz 1981). They screened for mutants that altered the normally invariant cell lineage of *C. elegans* and found a range of phenotypic classes. One class that is relevant to our work consists of two complementation groups, *unc-83* and *unc-84*, which disrupt nuclear migration in three different cell types (Fig. 1A–C). Defects in the nuclear migration of P cells, which normally migrate from a lateral position to the ventral cord during the first larval stage, lead to cell death. Therefore, the normal descendants of P cells are missing, which results in egg laying defects and uncoordinated movement (unc) in *unc-83* and *unc-84* mutants (Sulston & Horvitz 1981). Since these initial studies, nearly 20 alleles of both *unc-83* and *unc-84* have been isolated from various screens, but no third complementation class has been identified (Malone et al 1999, Starr et al 2001).

Hedgecock and Thomson identified the first mutants disrupting nuclear anchorage in a screen for defects of nuclear positioning (1982). Five alleles of a single complementation group, *anc-1* (for anchorage defective), caused nuclei of the syncytial hypodermis to float freely through the cytoplasm, often forming large clumps of nuclei (Fig. 1D). In fact, *anc-1* mutants probably disrupt the anchorage of nuclei in all somatic, post-embryonic cells (Hedgecock & Thomson 1982). *unc-84* mutants were found to have similar defects in nuclear anchorage

A.
P cell migration
lateral view
Wild-type
forms vulva
and neurons
unc-83
unc-84 25°C
ventral cord
Egl and Unc
animals
B.
Hyp7 migration
dorsal view
Wild-type
unc-83
unc-84
dorsal cord
C.
Intestinal polarization
dorsal view
Wild-type
unc-83
unc-84 15°C
intestinal midline
D.
Adult hypodermal syncytia
lateral view
Wild-type
anc-1
unc-84

FIG. 1. *unc-83*, *unc-84*, and *anc-1* mutant phenotypes. *unc-83* and *unc-84 m*utations disrupt the nuclear migrations of three cell types. (A) Left lateral view of P-cell nuclear migration in wild-type and *unc-83* or *unc-84* mutant larvae at 25 °C (nuclear migration is nearly normal at 15 °C). P-cell cytoplasm is gray and nuclei are white. White X marks dying nuclei where nuclear migration has failed. (B) The dorsal surface of a pre-elongation embryo illustrating intercalation and nuclear migration of hyp7 precursors in wild-type and *unc-83* or *unc-84* mutant embryos. Cytoplasm of the hyp7 precursors is gray, nuclei that migrate from right to left are black, and nuclei that migrate from left to right are white. Anterior towards left, right is upwards. Nuclei that fail to migrate are abnormally located in the dorsal cord. (C) A dorsal view, through the middle of a pre-elongation embryo, of nuclear migration during intestinal polarization during the E16 stage in wild-type and *unc-83* or *unc-84* mutant embryos raised at 15 °C. Cytoplasm of embryonic intestinal cells is gray, nuclei right of the midline are black and nuclei left of the midline are white. Anterior is leftwards. In *unc-83* or *unc-84* mutants, the nuclei do not localize to the midline. (D) *anc-1* and *unc-84* mutations disrupt nuclear anchorage. A lateral view of the adult syncytial hypodermis in wild-type and *anc-1* or *unc-84* mutant animals. Cytoplasm of the four syncytia that cover the entire mid-body of an adult animal is gray, and nuclei are white. Normally nuclei are evenly spaced, but in the mutants, they are unanchored, and often cluster. Parts of this figure (A–C) have been reproduced with permission from Starr et al 2001.

(Malone et al 1999). Mutations in *anc-1* also disrupt the anchorage of mitochondria (Hedgecock & Thomson 1982, Starr & Han 2002). We have isolated five additional alleles of *anc-1* in screens for un-anchored nuclei, but none of *unc-84* (unpublished results). Due to the large target size of *anc-1* (see below) it is not known if this screen has been saturated.

UNC-84, UNC-83, and ANC-1 are components of the NE

unc-84 encodes a 1111 residue protein with a predicted transmembrane domain in the middle and a conserved C-terminal SUN domain (for Sad1p, UNC-84 homology; Malone et al 1999). Both the C-terminal SUN domain and the unique N-terminal domain are required for nuclear migration (Malone et al 1999). Both GFP-tagged UNC-84 and antibodies against UNC-84 localize to the nuclear envelope of nearly all somatic cell nuclei (Fig. 2A,B; Lee et al 2002, Malone et al 1999). *unc-83* encodes a completely novel protein; the only identified motif is a predicted transmembrane region 17 residues from the C-terminus (Starr et al 2001). Monoclonal antibodies against UNC-83 localize to the nuclear envelope, where they co-localize with antibodies against UNC-84. Unlike UNC-84, which is found at the nuclear envelope of nearly all somatic cell nuclei from the 24-cell stage through to adulthood, UNC-83 is found on only a subset of nuclei. UNC-83 is first observed at the NE of migrating hyp7 nuclei during embryogenesis (Fig. 2C,D; Starr et al 2001). Later it is also localized to the NE of P cells and intestinal cells; both populations of these nuclei fail to migrate in *unc-83* and *unc-84* mutations. UNC-83 was also observed in a limited number of other somatic cells (Starr et al 2001).

anc-1 encodes a huge protein of 8546 residues (Starr & Han 2002). The bulk of the protein is repetitive and is predicted to form a long helical fibrous domain. Antibodies against this domain localize to the cytoplasm, are excluded from the nucleus, and are enriched at the NE (Fig 2E-F; Starr & Han 2002). The N-terminus of ANC-1 contains two $\sim$100 amino acid domains with calponin homology (Gimona et al 2002); it binds F-actin *in vitro* and localizes with actin *in vivo* (Starr & Han 2002). The C-terminal 60 residues of ANC-1 are highly conserved (40-60% identity) with the C-termini of *Drosophila* Klarsicht and human Syne-1 and Syne-2. We term this the KASH domain (Klarsicht, ANC-1, Syne homology); it consists of a predicted transmembrane domain followed by 39 residues. When ectopically expressed, the C-terminal 350 residues of ANC-1, including the KASH domain, are sufficient for NE localization and cause a dominant negative nuclear anchorage phenotype (Starr & Han 2002). Our model for ANC-1 function is that the N-terminus binds to the actin cytoskeleton, while the C-terminus binds to the ONM. The long fibrous middle then extends between these two structures, effectively tethering the nucleus to the actin cytoskeleton (Starr & Han 2002, 2003).

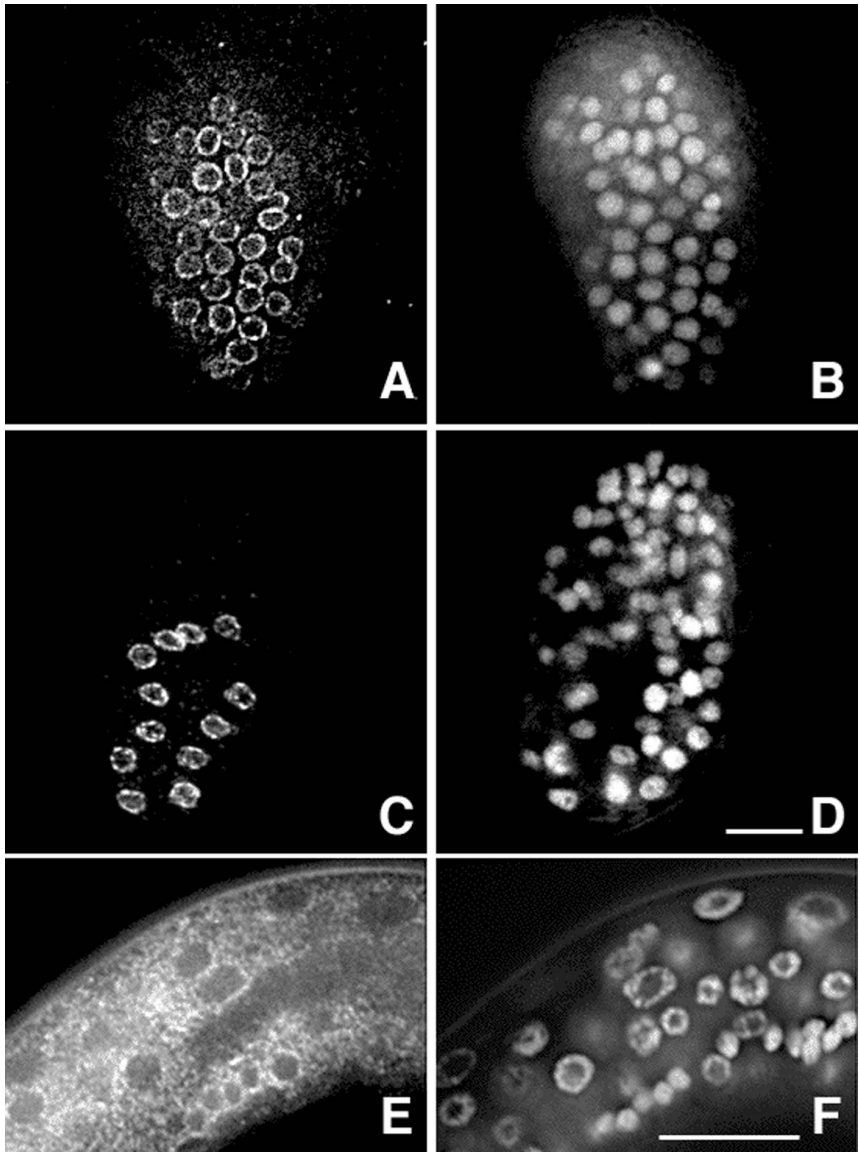

FIG. 2. UNC-84, UNC-83, and ANC-1 localize to the NE. (A–B) An embryo stained with anti-UNC-84 antibodies showing UNC-84 (A) at the NE of all DAPI-stained nuclei (B). (C–D) A similar stage embryo stained with anti-UNC-83 antibodies showing UNC-83 (C) at the NE of only a few (the migrating hyp7 cells) DAPI-stained nuclei (D). Scale bar for A-D is 10 microns. (E–F) The midbody of an L4 larvae showing localization of anti-ANC-1 antibodies (E) and DAPI stained nuclei (F). Scale bar is 10 μm. Parts of this figure (C–F) have been reproduced with permission from Starr et al (2001) and Starr & Han (2002).

The SUN domain of UNC-84 recruits UNC-83 and ANC-1 to the NE

unc-83(lf), *unc-84(lf)*, or double mutant animals have the same nuclear migration phenotype, suggesting that these two proteins function in a single pathway. We therefore determined whether they require each other for localization to the NE. UNC-83 fails to localize to the NE in an *unc-84(null)* mutant embryo. Moreover, missense mutations in the SUN domain of UNC-84 disrupt UNC-83 localization, while missense mutations in the N-terminus of UNC-84 do not, despite their defect in nuclear migration (Starr et al 2001). Mutations in *unc-83* do not disrupt UNC-84 localization (Lee et al 2002). Additionally, point mutations in the N-terminal or SUN domains of UNC-84, which disrupt nuclear migration, do not disrupt the localization of UNC-84 to the NE (Lee et al 2002). These data suggest that UNC-84 recruits UNC-83 to the NE through a genetic interaction with the SUN domain. Only when both UNC-84 and UNC-83 are at the NE, can nuclear migration proceed normally. This suggests that UNC-83 then recruits or controls additional factors required for migration. The molecular mechanisms of UNC-83 are under investigation.

unc-84(lf) and *anc-1(lf)* mutants have indistinguishable nuclear anchorage phenotypes. Given that the SUN domain of UNC-84 recruits UNC-83, we tested whether UNC-84 might act as a docking site for ANC-1. ANC-1 failed to localize to the NE in an *unc-84(null)* mutant and in *unc-84* alleles with missense mutations in the SUN domain (Starr & Han 2002). Thus, the SUN domain of UNC-84 is required for the localization of both ANC-1 and UNC-83 to the NE. It is therefore likely that UNC-84 helps control the switch between migration and anchorage of nuclei. A simple model is that UNC-83 and ANC-1 compete with one another for limited numbers of UNC-84 docking sites. This is unlikely because overexpression of ANC-1 does not lead to mislocalization of UNC-83, nor does the overexpression of UNC-83 lead to nuclear anchorage defects (unpublished data). We propose that other unidentified proteins participate in this important developmental switch between migration and anchorage.

SUN domains have been found in *C. elegans* UNC-84, *S. pombe* Sad1p, *Drosophila* predicted protein CG18584, and two human proteins, SUN1 and SUN2; these SUN domains are between 34 and 47% identical to one another (Hagan & Yanmagida 1995, Malone et al 1999). Sad1p, which is required for setting up the mitotic spindle, localizes to the spindle pole body and, when overexpressed, to the NE (Hagan & Yanmagida 1995). Recently, a divergent SUN domain (26% identity to UNC-84) was identified in *C. elegans* SUN-1. SUN-1 is required to recruit ZYG-12 to the outer nuclear membrane in the early embryo; ZYG-12 then functions to attach the centrosome to the NE (Malone et al 2003). Epitope-tagged versions of human SUN1 localize in transfected tissue culture cells to the nuclear envelope (Dreger et al 2001). Although SUN proteins clearly localize to the NE, the

topology of UNC-84/SUN in the NE remains to be determined. Since lamin is required for the localization of UNC-84 (Lee et al 2002), one model suggests that UNC-84 is an integral component of the inner nuclear membrane. In this model, the SUN domain of UNC-84 extends into the perinuclear space where it could interact with UNC-83 and ANC-1, effectively targeting these proteins to the outer nuclear envelope (Starr & Han 2003).

The KASH domain specifies NE localization

KASH domains (for Klarsicht, ANC-1, Syne homology) have been found in a number of proteins that have been shown to localize to the NE. They consist of a predicted transmembrane stretch followed by about 40 amino acids and are found at the C-termini. The founding member of the family is *Drosophila* Klarsicht, which is required for nuclear migration in the developing eye disc and lipid droplet migration in the embryo (Mosley-Bishop et al 1999, Welte et al 1998). ANC-1 is a member of a family of huge proteins that have calponin domains at the N-terminus, a KASH domain at the C-terminus, and a large helical central rod domain. *Drosophila* MSP-300 and mammalian Syne-1 and Syne-2 (also published as myne-1 and -2, nesprin-1 and -2, and NUANCE) are the other identified members of this family. The central rod domains of MSP-300 and the Syne proteins consist of spectrin repeats (Apel et al 2000, Mislow et al 2002a, Starr & Han 2002, Zhang et al 2001, 2002, Zhen et al 2002).

The KASH domain likely acts as an NE targeting signal. Klarsicht localizes to the NE by a lamin-dependent mechanism and is required for centrosome-to-NE attachment in migrating nuclei in the *Drosophila* eye disc (Mosley-Bishop et al 1999, Patterson et al 2004). As discussed above, the C-terminus of ANC-1 is sufficient for localization to the NE (Starr & Han 2002). Likewise, the 60 residue KASH domains of Syne-1 and Syne-2 have been shown to be necessary and sufficient for localization to the NE (Zhang et al 2001). Whether the localization of KASH domains is to the ONM or the INM remains a point of debate. Our ANC-1 cytoplasmic localization data (Starr & Han 2002), and the digitonin extraction experiments of Zhen et al (2002) strongly suggest that ANC-1 and Syne-1 localize to the ONM. If true, this is the first protein to our knowledge that specifically binds to the ONM but not the ER. However, blot overlay experiments suggest that Syne-1 binds to lamin and emerin, implicating Syne-1 at the INM (Mislow et al 2002b). It is possible that alternatively spliced products of Syne-1 and Syne-2 may be localized to different membranes. However, the exact localization of KASH domain proteins within the NE requires further study.

We wanted to test if Syne-1 behaves in a mammalian system in a manner similar to ANC-1 in *C. elegans*. Since the overexpression of the C-terminus of ANC-1 leads to a strong dominant negative phenotype (Starr & Han 2002), we are carrying out a

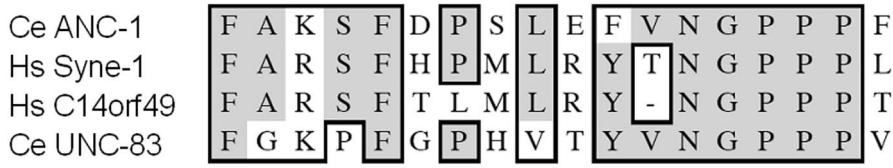

FIG. 3. UNC-83 has a C-terminal KASH domain. A clustalW protein alignment of the C-terminal regions of *C. elegans* ANC-1 and UNC-83, and human Syne-1 and C14orf49 is shown. Identical residues are shaded, and similar residues are boxed.

similar experiment in mice. At the mouse neuromuscular junction (NMJ), a cluster of about six muscle nuclei normally forms immediately beneath the NMJ. These nuclei express specific transcripts to respond to signals across the NMJ (Sanes & Lichtman 2001). We have generated a transgenic line that expresses the C-terminal 350 residues of Syne-1 in skeletal muscles and are currently collaborating with R. Grady and J. Sanes (Washington University, St. Louis, MO) to study the role of of Syne-1 in positioning of nuclei at the NMJ.

Since ANC-1 and UNC-83 both require UNC-84 to localize to the NE, it is possible that they are localized by similar mechanisms. We therefore examined the sequence of UNC-83 for a KASH domain. The C-terminus of UNC-83 has a predicted transmembrane domain followed by 18 amino acids; 50% of these residues are conserved in other KASH domains, suggesting that UNC-83 does in fact have a divergent KASH domain (Fig. 3). We are currently testing the hypothesis that the divergent KASH domain of UNC-83 is required for nuclear localization and an interaction with the SUN domain of UNC-84. Our extensive genomic searches identified one other potential KASH domain containing protein in humans, C14orf49 (Fig. 3). Interestingly, this protein was identified as a probable integral membrane component of the NE in a recent proteomic study (Schirmer et al 2003). We are currently testing the hypothesis that C14orf49 is a true NE component and whether C14orf49 might be a homologue of UNC-83.

Summary

A model for the role of the NE in nuclear positioning

We propose a model (Fig. 4) where proteins bridge the two membranes of the nuclear envelope. This bridge can then act as part of a connection between two relatively stable structures: the nuclear matrix and the cytoplasmic cytoskeleton. In this model, the predicted transmembrane region of UNC-84 would be in the inner nuclear membrane. Presumably, UNC-84 would be recruited to the inner nuclear membrane through an interaction with the lamina. In fact, localization of UNC-84 to the NE requires lamin (Lee et al 2002), although it is not known how

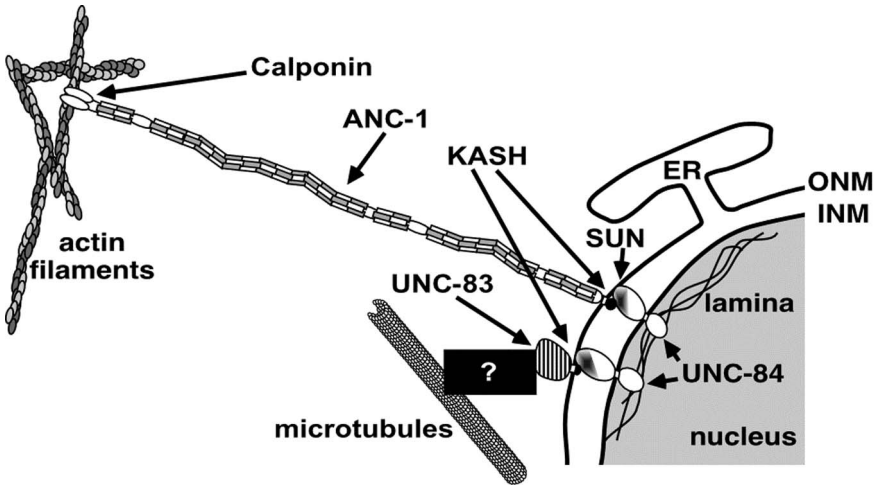

FIG. 4. A model for nuclear positioning is shown. See text for details.

direct this interaction is. Once at the inner nuclear membrane, we predict that the conserved SUN domain of UNC-84 faces the perinuclear space. From there, the SUN domain can recruit additional proteins to the outer nuclear membrane. The NE localization of both UNC-83 and ANC-1 require UNC-84 (Starr & Han 2002, Starr et al 2001). Specifically ANC-1 localizes to the NE by way of its KASH domain (Starr & Han 2002). Here we show that UNC-83 also has a KASH domain, suggesting that UNC-83 and ANC-1 localize to the outer nuclear membrane through a common mechanism, interaction with the SUN domain. Once recruited to the NE, ANC-1 functions to tether the nucleus to the actin cytoskeleton, while UNC-83 functions through an unknown mechanism to control migration. We propose that this conserved KASH/SUN interaction is a general mechanism to recruit proteins to the outer nuclear membrane, but not the ER.

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References

Apel ED, Lewis RM, Grady RM, Sanes JR 2000 Syne-1, a dystrophin- and Klarsicht-related protein associated with synaptic nuclei at the neuromuscular junction. J Biol Chem 275:31986–31995

Dreger M, Bengtsson L, Schoneberg T, Otto H, Hucho F 2001 Nuclear envelope proteomics: novel integral membrane proteins of the inner nuclear membrane. Proc Natl Acad Sci USA 98:11943–11948

Gimona M, Djinovic-Carugo K, Kranewitter WJ, Winder SJ 2002 Functional plasticity of CH domains. FEBS Lett 513:98–106

Hagan I, Yanmagida M 1995 The product of the spindle formation gene sad1+ associates with the fission yeast spindle pole body and is essential for viability. J Cell Biol 129:1033–1047

Hedgecock EM, Thomson JN 1982 A gene required for nuclear and mitochondrial attachment in the nematode C. elegans. Cell 30:321–330

Horvitz HR, Sulston JE 1980 Isolation and genetic characterization of cell-lineage mutants of the nematode Caenorhabditis elegans. Genetics 96:435–454

Lee KK, Starr DA, Cohen M, Liu J, Han M, Wilson KL, Gruenbaum Y 2002 Lamin-dependent localization of UNC-84, a protein required for nuclear migration in C. elegans. Mol Biol Cell 13:892–901

Malone CJ, Fixsen WD, Horvitz HR, Han M 1999 UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during C. elegans development. Development 126:3171–3181

Malone CJ, Misner L, Le Bot N et al 2003 The C. elegans Hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. Cell 115:825–836

Mislow JM, Kim MS, Davis DB, McNally EM 2002a Myne-1, a spectrin repeat transmembrane protein of the myocyte inner nuclear membrane, interacts with lamin A/C. J Cell Sci 115:61–70

Mislow JM, Holaska JM, Kim MS et al 2002b Nesprin-1alpha self-associates and binds directly to emerin and lamin A in vitro. FEBS Lett 525:135–140

Mosley-Bishop KL, Li Q, Patterson L, Fischer JA 1999 Molecular analysis of the klarsicht gene and its role in nuclear migration within differentiating cells of the Drosophila eye. Curr Biol 9:1211–1220

Patterson K, Molofsky AB, Robinson C, Acosta S, Cater C, Fischer JA 2004 The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the Drosophila eye. Mol Biol Cell 15:600–610

Sanes JR, Lichtman JW 2001 Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805

Schirmer EC, Florens L, Guan T, Yates JR 3rd, Gerace L 2003 Nuclear membrane proteins with potential disease links found by subtractive proteomics. Science 301:1380–1382

Starr DA, Han M 2002 Role of ANC-1 in tethering nuclei to the actin cytoskeleton. Science 298:406–409

Starr DA, Han M 2003 ANChors away: an actin based mechanism of nuclear positioning. J Cell Sci 116:211–216

Starr DA, Hermann GJ, Malone CJ et al 2001 unc-83 encodes a novel component of the nuclear envelope and is essential for proper nuclear migration. Development 128:5039–5050

Sulston JE, Horvitz HR 1977 Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol 56:110–156

Sulston JE, Horvitz HR 1981 Abnormal cell lineages in mutants of the nematode Caenorhabditis elegans. Dev Biol 82:41–55

Sulston JE, Schierenberg E, White JG, Thomson JN 1983 The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol 100:64–119

Welte MA, Gross SP, Postner M, Block SM, Wieschaus EF 1998 Developmental regulation of vesicle transport in Drosophila embryos: forces and kinetics. Cell 92:547–557

Zhang Q, Skepper JN, Yang F et al 2001 Nesprins: a novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. J Cell Sci 114:4485–4498

Zhang Q, Ragnauth C, Greener MJ, Shanahan CM, Roberts RG 2002 The nesprins are giant actin-binding proteins, orthologous to Drosophila melanogaster muscle protein MSP-300. Genomics 80:473–481

Zhen YY, Libotte T, Munck M, Noegel AA, Korenbaum E 2002 NUANCE, a giant protein connecting the nucleus and actin cytoskeleton. J Cell Sci 115: 3207–3222

DISCUSSION

Collas: I'm interested in the signalling from the cytoplasm to the nucleus, and possibly the other way round. Howard Worman touched on this with TGFβ, and you touched upon this. Has anyone looked at whether Syne1 has binding sites for kinases and phosphatases?

Starr: It would be a great candidate to be a scaffold in the system, but we haven't looked.

Gruenbaum: I'm very pleased to see that our bridging model is catching up. Also, your data doesn't take into account the possibility that there are shorter forms of nesprin that might be nuclear, as was seen in mammalian cells. Your blot of expressed nesprins showed many lower molecular weight bands. Is it possible that one or some of these bands could represent a nuclear ANC-1 protein?

Starr: The evidence I have that ANC-1 would be on the outer nuclear membrane is that most of my staining is cytoplasmic. But there could be some staining at the inner nuclear envelope that I can't distinguish. One explanation is that there are shorter isoforms, which are inside the nucleus. I can't exclude this. We have not looked in *C. elegans* at what types of smaller isoforms might exist, but given the abundant evidence in mammalian systems that there exist a number of different sizes of transcripts of these proteins, there are likely to be different sized transcripts in *C. elegans*. I did a Western and looked at the lower portion of the gel and I didn't see any obvious bands.

Young: Do you think the situation in your transgenic mice is just due to variegation of transgene expression — so that some nuclei express your transgene and others don't — or do you think there is something more complicated happening, like you suggested?

Starr: This is a good question. One problem is that we only have one transgenic line. The second problem is that it's difficult to imagine that most nuclei in a myotube could be expressing something but that for some reason it cannot be detected on other nuclei in the same syncytium. Perhaps this can be explained if the transgenic protein actually has to be expressed very close to where it ends up. For example, it needs to be expressed by the very nucleus it is localized to. Of course, this is difficult to test. Another possibility is that perhaps these nuclei aren't underneath the NMJ. They could be muscle satellite cells.

Shackleton: Do you have any evidence that UNC-84 is on the inner nuclear membrane as opposed to the outer.

Gruenbaum: When lamin is down-regulated, UNC-84 is displaced to the cytoplasm similar to most known inner nuclear membrane proteins. Although we don't have direct EM evidence for UNC-84 presence in the inner nuclear membrane, we have analysed another protein with a SUN domain, which is present at the inner nuclear membrane.

Wilson: We tested UNC-84 and failed to see any direct interaction between it and lamins.

Bonne: Did your transgenic mice express any visible phenotype? What do the NMJs look like when they don't have any nuclei underneath?

Starr: We saw few (16%) NMJs with no nuclei underneath. This could explain why we don't see a gross phenotype. On average there were 2.3 nuclei under each NMJ. Perhaps this is enough for them to function. Grossly, the bungarotoxin staining looks like it does in wild-type.

Davies: Have you done any electrophysiology?

Starr: No.

Worman: Does anyone know the width of the perinuclear space? Is it small enough that the luminal domains of two proteins can interact with each other?

Starr: Yes.

Goldman: I would argue that from a cell biological viewpoint we can't measure this distance accurately. We can only make an assumption knowing that EMs have the potential for fixation artefacts.

Gerace: In the Fawcett textbook it is about 50–70 nm. This raises the question of the predicted secondary structure for the luminal domain.

Starr: There's nothing obvious.

Malone: Both *C. elegans* SUN proteins have coiled-coil domains that may be long enough to span the luminal domain.

Shumaker: In *Xenopus* nuclei the luminal distance has been measured as 40–130 nm depending on fixation.

Shanahan: Since you found another KLS domain protein in *C. elegans*, UNC-83, does this have any homology with *Drosophila* Klarsicht in any other domain, or with any other proteins?

Starr: The bulk of UNC-83 has no homology with anything. The bulk of Klarsicht has no homology with anything. There's also another protein that I have identified in mammalian systems that has a KASH domain and then a large domain that doesn't have homology to anything. Those three proteins are all predicted to be highly helical. Klarsicht is required to connect the centrosome to the nuclear membrane (Patterson et al 2004), whereas UNC-83 is not.

Gruenbaum: You showed two interesting observations that may shed light on UNC-84 organization. Mutations in the UNC-84 N-terminus can complement mutations in the UNC-84 C-terminus. The other is that the mutations in the

UNC-84 N-terminus do not affect the localization of UNC-83. These observations could imply that UNC-84 self-dimerizes. Has anyone looked at this?

Starr: That is the prediction but we haven't looked.

Shackleton: I have been working with mouse SUN1. This protein seems to be quite different. Perhaps it is a more evolutionarily advanced version of UNC-84, because it seems to have several transmembrane domains. From the work I have done, the SUN domain would appear to be on the exterior of the nucleus. Whether it has evolved to fulfil the function of two proteins, I don't know.

Wilson: So you are modelling the SUN domain as located outside the luminal space.

Goldman: What are your criteria for binding?

Shackleton: We pulled it out in a yeast two-hybrid screen originally, and then we did various GST pull-downs.

Goldman: Which domain of lamin does it bind to?

Shackleton: The globular domain. We didn't even use the helical domain in the two-hybrid screen for lamin.

Goldman: So does it bind to the C- or N-terminus of lamin?

Shackleton: The C-terminus.

Wilson: With mice that overexpress the myc-tagged KASH domain, did you quantify nuclei? Were there any clusters of non-expressing nuclei outside the neuromuscular junction? In other words, were non-expressing nuclei all located near the neuromuscular junction?

Starr: We have tried to quantitate this. It looks like there are some nuclei far from the neuromuscular junction that have no transgenic protein. The problem is, we can't be sure that those nuclei are in the myotube, a satellite cell or associated cells of some sort. We don't have the proper markers.

Gerace: Did you say that you used a muscle-specific promoter to drive the expression of your transgene?

Starr: Yes.

Gerace: So you haven't looked at expression in a more general context.

Starr: Others have looked at Syne1 in other tissues so they can address which tissues it is localized in.

Shanahan: It is in every tissue. You can't even consider it as a single gene. It is basically three overlapping genes and we have identified at least 15 different isoforms, ranging in size from 80 kDa to 1 megaDa. I don't think you can talk about it as a single genetic entity.

Goldman: If you are right, then we can't.

Shanahan: Evolutionarily, there seem to be different genes in *C. elegans* and *Drosophila* that have similar functions to Syne1 and 2 but are slightly different. All the different sized isoforms of the nesprins carry out the functions that perhaps Klarsicht and MSP300 effect. In *Drosophila*, MSP300 was identified as

a muscle protein in the Z-lines that, when disrupted, interrupts integrin signalling. The disruption was right up at the N-terminus. This probably corresponds to the larger isoforms of nesprin 1 and 2 that we see in the Z-line and N-line of muscle, and also in mitochondria. We now know that MSP300 does have shorter isoforms from the C-terminus, which would probably be inner nuclear envelope isoforms of nesprins. But they have only just found now that Klarsicht, which was originally identified as a vesicle-moving protein, is actually binding lamin at the nuclear envelope and is involved in nuclear migration.

Starr: That is not a direct interaction. Janice Fischer's (Patterson et al 2004) work shows that there is a genetic interaction, and lamin is required for Klarsicht localization. We would presume that this is an indirect effect.

Worman: Besides the NMJ, if you look at any muscle fibre the nuclei all line up along the side. In dystrophic fibres they are in the middle. Do you think the same things are involved here? Is there a different set of proteins that may be on the outer nuclear membrane that bind to components of the sarcolemma?

Starr: The nuclei do not appear to go into the middle in these mice. That's all I can say.

Davies: Have you tried damaging the muscle to see what happens?

Starr: No.

Davies: What levels of expression do you get from the transgene compared with normal wild-type levels? You could be looking at the limits of expression of the transgene and the distribution could be a result of this.

Starr: We haven't tested that.

Bonne: Howard Worman mentioned that in dystrophic muscle the nuclei are centralized. It is a typical feature, and this is true for every kind of muscular dystrophy, not just laminopathies.

Young: Did *unc-84* mutation also mislocalize the mitochondria?

Starr: No, only mutations in *anc-1* mislocalized mitochondria.

Young: What is it binding to in mitochondria?

Starr: We have no idea. Interesting results have come from Ken Beck's lab (Gough et a 2003) who independently isolated Syne-1. They studied a Golgi-specific protein that turned out to be a Syne-1 isoform. He has dominant negative tissue culture lines in which he overexpresses a piece of the middle of Syne-1 that localizes to and disrupts the structure of the Golgi. Thus, Syne-1 could be acting as a scaffold for a number of different organelles.

Goldman: What does it bind to on the mitochondrial membrane?

Starr: We don't know. We don't know for sure that it directly binds mitochondria, but in the null mutation mitochondria aren't positioned properly. The genetics state that ANC-1 is required for nuclear positioning.

Goldman: That could involve something quite far removed.

Starr: It could be indirect, but it is absolutely required because a null mutation in *anc-1* has severe defects in mitochondrial positioning.

Wilson: The lack of a gross phenotype in the mice is interesting. Do you plan to make a real knockout instead of a dominant disruption?

Starr: Colin Stewart is working on this, as is Min Han. A mouse knockout is not in my plan.

Fatkin: The nuclear position issue has been discussed a lot in skeletal muscle but not cardiac muscle. What would the possible consequences be of abnormal nuclear positioning in cardiac muscle cells?

Starr: Someone else could speculate better on this.

Bonne: Is there an abnormal localization of nuclei in cardiac muscle?

Fatkin: I haven't seen any data on this for cardiac muscle cells.

Bonne: There was a report by Arbustini et al (2002) in Italy. They produced an EM picture of cardiac muscles from patients with mutations. It is the only picture I have seen of cardiac muscle where there was a full disruption of nuclei. In muscle biopsies from patients we have seen very few nuclei with abnormal features—around 5% of the myonuclei. It is quite rare. But we never observed disruption of the membrane. However, the muscle biopsies are not always performed in the most affected muscles of the patients. They are usually made in quadriceps or deltoid muscle, those muscles not being the most affected, so this is not conclusive.

Gasser: What happens if you disrupt lamin in *C. elegans*? Are UNC-83 and UNC-84 properly localized?

Starr: UNC-84 isn't localized in lamin RNAi treated worms but UNC-83 hasn't been looked at. I assume it isn't localized either. Since our genetic experiments show that UNC-84 is required at the nuclear envelope for UNC-83 and ANC-1 localization, I predict that lamin disruption will also mislocalize UNC-83 and ANC-1.

Malone: I study a similar set of proteins, SUN-1 and ZYG-12, earlier in *C. elegans* development. We can clearly detect what the ER looks like in the cell cycle, and it does not look like either of these proteins redistribute to the ER. It is not clear what happens to the ZYG-12 protein when we disrupt localization. I have a question about UNC-84. There is a set of mutations on either side of the protein that both affect nuclear migration, but only one affects UNC-83 localization. Have you any models for how it causes a nuclear migration defect if it doesn't mislocalize UNC-83?

Starr: The model that would fit the best is that N-terminal mutations in UNC-84 would somehow disrupt the interaction between lamin and UNC-84. Then when you start to pull on the outer nuclear membrane, since it is not connected to anything you are not going to move the nucleus.

Gruenbaum: For the first time I think that we can talk about the cytoskeleton and include the nucleoskeleton. Now we have good evidence that every component in

the cell is somehow bound to the others and is required to maintain cell integrity. Bob Goldman, I would like to hear your comments on IF localization near the nuclear envelope and whether you think it is physically connected.

Goldman: If you look for cytoskeletal interactions with the nuclear surface, there is some evidence for actin on the outer nuclear surface. Microtubules seem to be concentrated in the centrosomal region, which is close to the nucleus. However, in the axopods of heliozoans Keith Porter and Lew Tilney showed that microtubules looked like they were growing off the nuclear surface. The bulk of cytoskeletal protein on the outside of the nucleus in mammalian cells is invariably IFs. It would be interesting to know whether nesprin can self-associate to form an oligomeric complex. You are showing linkages of nesprin going from the nuclear surface to the cell surface, so can it polymerise into long chains? If you go back to the old literature you will find that there are many ways to nucleate actin *in vitro* which may not reflect normal physiological conditions.

Wilson: Even though the actin binding domain of nesprins is far from the membrane-binding region, there may be additional domains that bind indirectly to actin or stimulate actin events. We can't assume that the middle parts of these long proteins are featureless. They may have interesting functions.

Goldman: It might be interesting to look at the smaller variants of bacterial-expressed nesprin. One might be able to obtain sufficient protein to carry out biochemical experiments.

Shanahan: Using an antibody to the calponin homology (CH) domain region we have found that it is present in foci in the nucleus. We haven't been able to identify what isoform with the CH domain would find its way into the nucleus. But there definitely are foci of CH domain-containing nesprin proteins in the nucleus.

Goldman: How do you know it is nesprin?

Shanahan: There are other proteins in the nucleus with this domain as well. It's not just a nesprin with a CH domain that can get into the nucleus.

Goldman: There are many actin binding proteins. I think we should be excited but cautious. Let's limit our excitement to reality.

Starr: There was an interesting result from Elena Korenbaum's group (Zhen et al 2002). They were the fourth group to identify Syne-1 and they were the first group to identify the longest isoforms, which they named NUANCE. They took the C-terminal KASH domain and bound it to the N-terminal calponin domains. They transfected tissue culture cells and induced an actin cage around the outside of the nuclear envelope.

Goldman: Has the length of nesprin been measured using rotary shadowed preparations for electron microscopy? This should be done. As an aside, we also need to decide what we are going to call this fascinating protein. We need to come to grips with this nomenclature problem.

Shanahan: A lot of the problem is that some of the papers have come out later and the authors have refused to even acknowledge that there are other isoforms. It makes it too complicated for them to discuss their data when they only have one antibody.

Goldman: Few of us can name the person who named tubulin. No one should worry about their reputation on the basis of naming a protein. We call it nesprin because we know Elizabeth McNally and she sent us nesprin antibodies.

Starr: I call it Syne-1 because that is the name that appeared first in the literature — by 14 months (Apel et al 2000).

Gerace: When you did your Western blot to characterize ANC-1, there were a lot of bands recognized. How many alternative splice forms are seen at the mRNA level? Since there are a lot of bands, considering the cytoplasmic localization, is it possible that *in vivo* proteolysis is releasing soluble forms that can move around? Have you used the most rigorous conditions possible to avoid *in vitro* proteolysis?

Starr: We haven't done any of these things. The bands could be because of any of these. This is an important question that needs to be addressed.

Gruenbaum: How many introns are there in *anc-1*?

Starr: Not a lot for *C. elegans* and for a protein of this size. There are about 30. Most of the introns are fairly small. Many other genes that have multiple transcripts in *C. elegans* tend to have larger introns. This one has some larger introns at the 5′ end, but not the middle or at the 3′ end. Also, there is no evidence of SL1 *trans* splicing which is usually associated with multiple start sites.

Hutchison: Can I ask about your model? Your model implies that you need to bring the nucleus to a position in order to help propagate a signal transduction pathway. Are you at the stage where you are prepared to invoke a forced transmission signal transduction mechanism?

Starr: That is completely hypothetical.

Gasser: Is there any evidence that any of this nuclear migration requires actin polymerization?

Starr: It is hard to tell in *C. elegans* whether actin is required or not. The drug studies are hard to do in a multicellular organism.

Goldman: In nuclear migration studies, when the two pronuclei move together, there is literature showing that microtubule-based motors are involved.

Gasser: The same is true in yeast.

Gruenbaum: There were two cases in *Drosophila* where mutations in lamin Dm0 were shown to affect cytoplasmic organization. One was the abnormal cytoplasmic RNA distribution of Gurken RNA, which leads to the dorsalization of the embryo. The other was the disruption of directed outgrowth of cytoplasmic extensions from terminal cells of the tracheal system. We have to keep in mind that there are other examples where nuclear lamins probably affect the cytoplasmic organization.

References

Apel ED, Lewis RM, Grady RM, Sanes JR 2000 Syne-1, a dystrophin- and Klarsicht-related protein associated with synaptic nuclei at the neuromuscular junction. J Biol Chem 275:31986–31995

Arbustini E, Pilotto A, Repetto A et al 2002 Autosomal dominant dilated cardiomyopathy with atrioventricular block: a lamin A/C defect-related disease. J Am Coll Cardiol 39:981–990

Gough LL, Fan J, Chu S, Winnick S, Beck KA 2003 Golgi localization of Syne-1. Mol Biol Cell 14:2410–2424

Patterson K, Molofsky AB, Robinson C et al 2004 The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the Drosophila eye. Mol Biol Cell 15:600–610

Zhen YY, Libotte T, Munck M, Noegel AA, Korenbaum E 2002 NUANCE, a giant protein connecting the nucleus and actin cytoskeleton. J Cell Sci 115:3207–3222