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ELYS is a dual nucleoporin/kinetochore protein required for nuclear pore assembly and proper cell division

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Nuclear pores span the nuclear envelope and act as gated aqueous channels to regulate the transport of macromolecules between the nucleus and cytoplasm, from individual proteins and RNAs to entire viral genomes. By far the largest subunit of the nuclear pore is the Nup107–160 complex, which consists of nine proteins and is critical for nuclear pore assembly. At mitosis, the Nup107–160 complex localizes to kinetochores, suggesting that it may also function in chromosome segregation. To investigate the dual roles of the Nup107–160 complex at the pore and during mitosis, we set out to identify binding partners by immunoprecipitation from both interphase and mitotic *Xenopus* egg extracts and mass spectrometry. ELYS, a putative transcription factor, was discovered to copurify with the Nup107–160 complex in *Xenopus* interphase extracts, *Xenopus* mitotic extracts, and human cell extracts. Indeed, a large fraction of ELYS localizes to the nuclear pore complexes of HeLa cells. Importantly, depletion of ELYS by RNAi leads to severe disruption of nuclear pores in the nuclear envelope, whereas lamin, Ran, and tubulin staining appear normal. At mitosis, ELYS targets to kinetochores, and RNAi depletion from HeLa cells leads to an increase in cytokinesis defects. Thus, we have identified an unexpected member of the nuclear pore and kinetochore that functions in both pore assembly at the nucleus and faithful cell division.

Nup107–160 complex | MEL-28 | Nup133 | mitosis

Essential for cell survival, nuclear pore complexes are large multiprotein assemblages, ≈30 times the size of the ribosome. Structurally, nuclear pores are comprised of three major domains inserted in the nuclear membranes. These domains include a massive central scaffold, cytoplasmic filaments, and a nuclear basket (1). Nuclear pores consist of multiple copies of ≈30 different proteins termed nucleoporins (Nups) (2). A third of these contain phenylalanine-glycine (FG) repeat domains, believed to be key sites for interaction with transport receptors (3).

During vertebrate mitosis, the nuclear pore disassembles into approximately a dozen subunits, concurrent with the breakdown of the nuclear envelope. Most diffuse throughout the mitotic cytoplasm, playing no role in mitotic progression identified to date. However, a small number of nuclear pore proteins, including the Nup107–160 complex, localize to regions of the mitotic kinetochore and/or spindle, pointing toward a function in mitotic chromosome segregation (4–15). We now know that, *in vitro*, the Nup107–160 complex is required for spindle assembly (15).

Nuclear reassembly, which begins in late anaphase and continues through telophase, occurs at the chromatin periphery. During this time, the nuclear pore subunits reassemble, stepwise, into pore complexes within the double nuclear membrane. The Nup107–160 complex, by far the largest of the pore subunits, has been shown to play a critical role in nuclear pore assembly. The Nup107–160 complex consists to date of nine proteins (Fig. 1C: Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1) and is part of the pore's central scaffold domain (5, 6, 8, 11). Immunodepletion of the Nup107–160 complex from *in vitro* nuclear reconstitution extracts, derived from *Xenopus* eggs, results in the assembly of nuclei completely devoid of nuclear pores (8, 9). Partial

knockdown of members of the Nup107–160 complex in vertebrate tissue culture cells by RNAi also results in severe nuclear pore assembly defects in the nuclear envelope (4, 8, 9, 14). This complex is one of the first nuclear pore subunits recruited to the reforming nuclear envelope during pore assembly (5). Thus, the Nup107–160 complex is an essential and early determinant of nuclear pore assembly (5, 8, 9). However, its immediate binding partners within the vertebrate pore, as well as its kinetochore partners, remain speculative.

To begin to dissect the roles of the Nup107–160 complex in nuclear pore assembly and kinetochore function, a search for its protein-binding partners in both interphase and mitosis was initiated. We identified the protein ELYS, a putative transcription factor, to be a highly abundant binding partner of the Nup107–160 complex at both nuclear pores and kinetochores. We show that ELYS is essential not only for correct nuclear pore assembly but also for cell division.

Results

The Putative Transcription Factor ELYS Interacts with the Nup107–160 Complex. To identify binding partners of the Nup107–160 complex, extracts of *Xenopus laevis* eggs, prepared in either interphase or mitotic states, were used. Antibodies specific to Nup133 and Nup43, components of the Nup107–160 complex, were used separately to immunoprecipitate the complex from each type of cell cycle extract. The immunoprecipitates were proteolysed and subjected to liquid chromatography tandem MS (15). The MS spectra were searched against the National Center for Biotechnology Information (NCBI) *X. laevis* protein database. Because this database is incomplete, the NCBI human, fish, and reptile protein databases, plus the protein translations of our unpublished *Xenopus* Nup sequencing data, were included in the search. None of the Nup107–160 complex members were found in immunoprecipitations by control rabbit antisera. Seven of the nine Nup107–160 complex members were identified as highly abundant proteins in anti-Nup43 and anti-Nup133 immunoprecipitates from both interphase and mitotic extracts [Nup160, Nup107, Nup85, Nup43, Nup37, Sec13, and Seh1, denoted with dots (Table 1, which is published as supporting information on the PNAS web site)]. A search of the nearly complete NCBI *X.*

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Fig. 1. ELYS coimmunoprecipitates with the Nup107–160 complex. (A) Immunoprecipitations from HeLa cell lysates. Anti-ELYS antibody immunoprecipitates ELYS, together with members of the Nup107–160 complex, such as Nup160, Nup133, and Nup37 (lane 3). Anti-ELYS antibodies do not immunoprecipitate the non-Nup107–160 complex Nup, Nup93, or the transport factor Importin β (lane 3). (B) Nup133 and ELYS are reciprocally coimmunoprecipitated with one another but not with the non-Nup107–160 complex Nup, Nup62 (lanes 6 and 7). (A and B) Immunoprecipitation with rabbit IgG serum (Rbt IgG) was used as a negative control (lanes 2 and 5). Lys, input HeLa cell lysate (lanes 1 and 4). (C) Cartoon representing the vertebrate Nup107–160 complex, including the nine constituents known before this study.

laevis EST database confirmed these seven, as well as the two remaining Nup107–160 complex members, Nup133 and Nup96 whose sequences were not present in the *Xenopus* protein database (data not shown).

A limited set of proteins other than the Nup107–160 complex coimmunoprecipitated with antibodies to Nup43 and Nup133, but not with control antisera. A number were chaperone proteins, such as BiP, gp96, and FK506, likely reflecting a level of unfolding (Table 1). Interestingly, a major and unexpected constituent of the immunoprecipitates was the protein ELYS, which was found in both interphase and mitotic Nup107–160 complex immunoprecipitates and not in the controls.

ELYS (embryonic large molecule derived from yolk sac) is a large protein of ≈270 kDa with a predicted AT-hook DNA-binding motif (PRKRGRPRK). AT-hook motifs bind preferentially to the minor groove of DNA at stretches of AT-rich sequences (16). ELYS was originally identified in a mouse cDNA screen for potential regulatory genes involved in hematopoiesis but was simultaneously recognized to be expressed in a multitude of cell types (17). Because certain regions of ELYS, when fused to a yeast Gal4 DNA-binding domain, activated the transcription of a luciferase reporter gene in cultured cells, ELYS was designated as a putative transcription factor involved in hematopoiesis. In a subsequent mouse knockout study, however, it was found that ELYS-null mice die between embryonic days E3.5 and E5.5, well before the onset of hematopoiesis (day E9.5) (18). This finding suggests that ELYS functions in an unknown process that is essential to early mouse embryonic survival, either in addition to or instead of its function in hematopoiesis.

Using an anti-hELYS antibody, we found that Nup107–160 complex members consistently coimmunoprecipitated with ELYS in human cell lysates (Fig. 1A, lane 3). Nups not present in the Nup107–160 complex, such as Nup93 and Nup62, as well as the import receptor importin β, failed to immunoprecipitate
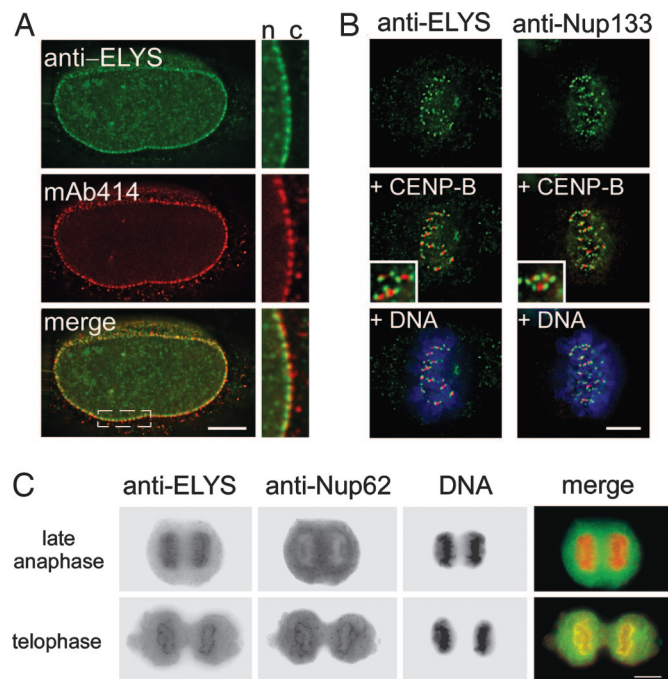
Fig. 2. ELYS localizes to the nuclear pore in interphase and to kinetochores during mitosis. (A) Double immunofluorescence on permeabilized, then fixed, HeLa cells with anti-ELYS antibody (*Top*, green) and the anti-FG Nup monoclonal antibody, mAb414 (*Middle*, red), revealing that both localize to the nuclear rim in a punctate pattern characteristic of nuclear pores. Superposition of the signals (*Bottom*) and a 250-fold magnification (see *Insets* to the right) show that ELYS colocalizes with the FG Nups. The nuclear (n) and cytoplasmic (c) sides of the nuclear envelope are indicated. (B) Immunofluorescence on mitotic HeLa cells extracted with PHEM buffer. Insets show a 250-fold magnification of kinetochore stains. ELYS (left column, green) and Nup133 (right column, green) show similar localization and bracket CENP-B (red) on the kinetochores. The DNA is stained with DAPI (blue). (C) During nuclear assembly in HeLa cells, ELYS (left column and red) associates with the chromatin periphery in late anaphase, whereas Nup62 (center column and green) associates in telophase. (Scale bars: *A* and *B*, 5 μm; *C*, 10 μm.)

with anti-ELYS antibody (Fig. 1 *A* and *B*), although a small amount of Nup358 and Nup153 were occasionally detected (data not shown). Importantly, anti-Nup133 antibody reciprocally immunoprecipitated ELYS (Fig. 1*B*, lane 6).

In summary, ELYS can be found in close association with the Nup107–160 complex throughout the cell cycle, and this interaction is evolutionarily conserved in vertebrates.

ELYS Localizes to both the Nuclear Pores and Nuclear Interior During Interphase. In the mouse study, ELYS was found to localize rather generally to the cytoplasm and nucleus with the authors' polyclonal antibody (17). When we performed immunofluorescence on HeLa cells using an affinity-purified commercially prepared anti-ELYS antibody, fixed either before or after Triton X-100 permeabilization, we did not see a significant cytoplasmic stain (Fig. 2*A*; and see Fig. 5, which is published as supporting information on the PNAS web site). Instead, ELYS was located at the nuclear rim in a punctate pattern typical of a nuclear pore stain as well as in the nuclear interior (Fig. 2*A*). Consistent with this finding, ELYS clearly colocalized with the FG Nups (mAb414) at the nuclear pore complexes (Fig. 2*A*).

ELYS Localizes to the Kinetochores in Mitosis. A fraction of the Nup107–160 complex is known to localize to the kinetochores from prophase to late anaphase (5, 8, 11). To determine whether ELYS is also present at kinetochores during mitosis, HeLa cells
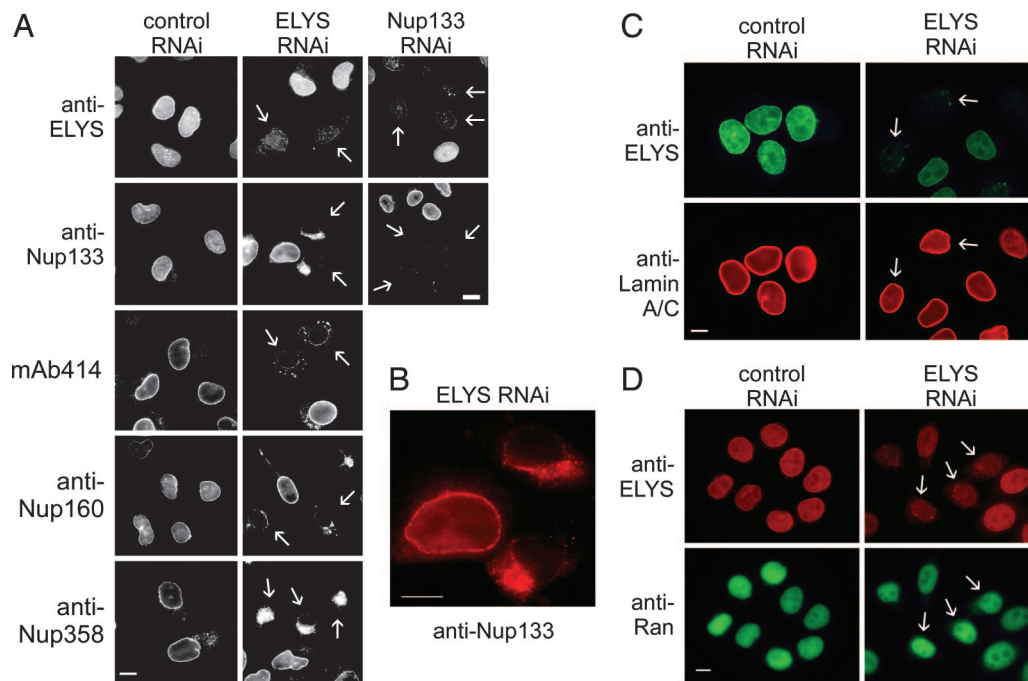
Fig. 3. ELYS is required for proper nuclear pore assembly. Immunofluorescence on HeLa cells transfected with control, ELYS, or Nup133 siRNA duplexes for 48–60 h. Cells were Triton X-100-extracted and then fixed and stained with the antibodies shown. Arrows indicate transfected cells. (*A*) ELYS RNAi results in a knockdown of ELYS and mislocalization of Nup133, whereas Nup133 RNAi similarly results in a knockdown of Nup133 and a reduction of ELYS in the nuclear envelope. ELYS RNAi leads to the mislocalization of the FG-Nups (mAb414), Nup160, and Nup358 from the nuclear rim to cytoplasmic aggregates (center column). (*B*) A magnification of ELYS-depleted cells clearly shows that Nup133 is greatly reduced in nuclear envelope pores and is mislocalized to cytoplasmic aggregates. (*C* and *D*) ELYS RNAi did not significantly affect lamin A/C localization to the nuclear lamina (*C*), or the nuclear accumulation of the transport factor Ran (fixed before permeabilization) (*D*). (Scale bars: 10 μm.)

were subjected to double immunofluorescence by using antibodies to ELYS and to the inner kinetochore protein, CENP-B. Strikingly, anti-ELYS antibodies stained mitotic cells in the paired dot-like pattern characteristic of kinetochore localization (Fig. 2*B* top left). Overlays of anti-ELYS and anti-CENP-B signals showed that ELYS brackets the CENP-B signal in a manner similar to an outer kinetochore protein (Fig. 2*B left*) and does so from prophase to late anaphase (Fig. 6, which is published as supporting information on the PNAS web site). A direct comparison of ELYS and Nup133 revealed that these proteins both localize to an identical region of the kinetochore in mitosis (Fig. 2*B*, compare left and right).

ELYS Is Recruited Early in the Nuclear Envelope Reassembly Process. To analyze the timing of ELYS recruitment during nuclear envelope reassembly, we compared its behavior to that of Nup62 and the Nup107–160 complex member, Nup133. Nup62, a component of the nuclear pore central channel domain, is recruited relatively late in the nuclear envelope reassembly process, i.e., during telophase (19, 20), whereas the Nup107–160 complex is recruited early (5). Immunofluorescence on HeLa cells revealed that ELYS begins to associate with the chromatin/ nuclear periphery in late anaphase, well before Nup62 (Fig. 2*C*), in a manner similar to Nup133 (5) (see also Fig. 7, which is published as supporting information on the PNAS web site). Thus, ELYS is recruited early in the nuclear assembly process, with similar timing to that of the Nup107–160 complex.

ELYS Is Essential for Nuclear Pore Assembly. To investigate the role of ELYS at the nuclear pore, we depleted HeLa cells of ELYS by RNAi. HeLa cells transfected with ELYS siRNA oligonucleotides showed a drastic decrease in ELYS at both the nuclear rim and nuclear interior as compared with cells transfected with

control oligonucleotides (Fig. 3*A Top*). Indeed, immunoblot analysis of lysates from cells transfected with ELYS siRNA oligonucleotides showed that the protein levels of ELYS were knocked down to very low levels (Fig. 8*A*, lane 2, which is published as supporting information on the PNAS web site).

Strikingly, in ELYS-depleted cells, Nup133 was mislocalized from the nuclear pores to cytoplasmic aggregates (Fig. 3 *A* and *B*). Moreover, RNAi depletion of Nup133 led to a much reduced level of ELYS at the nuclear envelope (Fig. 3*A*). Thus, ELYS and Nup133 are codependent for proper localization to the pore complexes of the nuclear envelope.

Further analysis revealed that RNAi depletion of ELYS affected the nuclear localization of all of the Nups we tested, including the FG Nups, the central scaffold proteins Nup93 and Nup53, the Nup107–160 complex members Nup85 and Nup160, and the cytoplasmic filament protein, Nup358 (Fig. 3*A* and data not shown). All exhibited reduced nuclear rim staining as well as increased cytoplasmic aggregate staining. Pom121, one of the few transmembrane pore proteins, and Tpr, a nuclear basket protein, also exhibited reduced nuclear rim staining upon ELYS depletion but showed few or smaller cytoplasmic aggregates (Fig. 8*B*). Clearly, with defects in all three domains of the nuclear pore complex (filaments, scaffold, and basket), ELYS is critical for the assembly and maintenance of nuclear pores.

Although the knockdown of ELYS by RNAi drastically affected nuclear pores, other cellular structures examined remained intact. ELYS RNAi did not substantially affect the nuclear lamina, (lamin A/C, Fig. 3*C*) or localization of the transport factor Ran to the nucleus, indicative of an intact nuclear envelope (Fig. 3*D*). Similarly, the microtubule cytoskeleton, as visualized by anti-tubulin staining, did not appear altered (Fig. 8*C*). Thus, our knockdown of ELYS does not have a global deleterious effect on cellular structure.
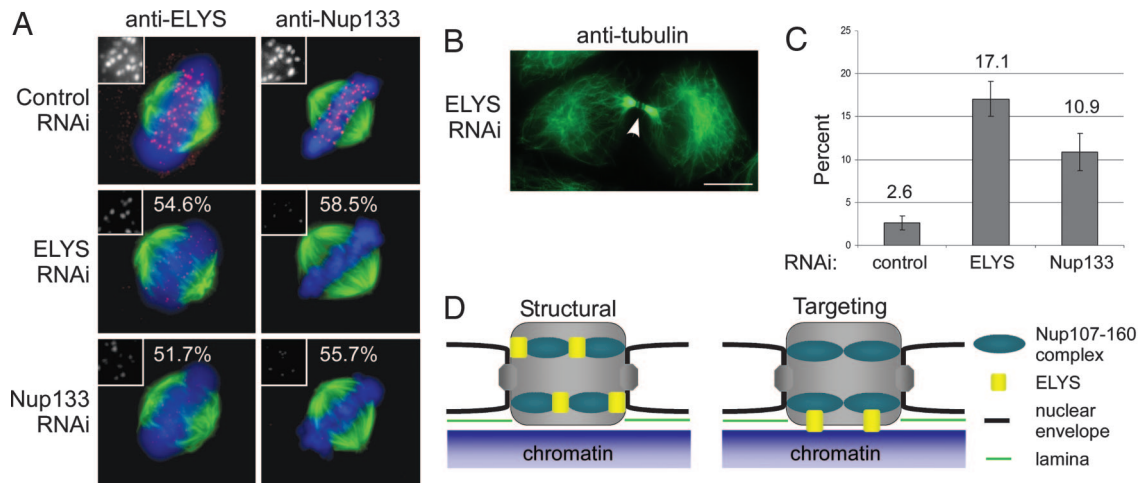
Fig. 4. Knockdown of ELYS leads to cell division defects. HeLa cells were transfected with control, ELYS or Nup133 siRNA duplexes for 48 h. (*A*) ELYS RNAi in HeLa cells leads to reduced levels of both ELYS and Nup133 (red, and *Insets*) at the kinetochores during mitosis, as compared with control-treated cells. Similarly, Nup133 RNAi leads to mitotic HeLa cells with reduced levels of both Nup133 and ELYS (red, and *Insets*) at the kinetochore. Numbers indicate the percentage of reduction of fluorescent intensities compared with those in the control RNAi transfections. (*B*) A large proportion of ELYS-depleted cells contain a midbody (arrowhead). (Scale bar: 10 μm.) (*C*) Percentage of cells with a midbody. Quantitation of the number of cells found in cytokinesis shows a significant increase in the occurrence of cells with midbody microtubules after ELYS and Nup133 RNAi, demonstrating a temporal block at this stage of the cell cycle, in comparison with transfections with the control siRNA duplex. (*D*) Two potential models for ELYS function. In the first model, ELYS serves as a core structural protein of the nuclear pore, one required for formation of the structure of nuclear pores. In the second model, ELYS is a nuclear pore-associated targeting protein that recruits Nups, such as the Nup107–160 complex, to assemble nuclear pores at the chromatin periphery. In its absence, pores would not be found at the nuclear rim.

Because ELYS has been hypothesized to be a transcription factor, we tested whether the nuclear pore defect might be due to decreased Nup protein levels, resulting from a failure in a possible ELYS-dependent Nup transcription. However, no difference in the protein levels of Nup160, Nup358, Nup214, Nup153, or Nup133 was seen after ELYS RNAi (Fig. 8*A*, lanes 1 and 2). These results indicate that ELYS plays an important and direct role in nuclear pore assembly and/or maintenance at the nuclear envelope.

Knockdown of ELYS Leads to Defects in Cytokinesis. To investigate the role of ELYS in targeting the Nup 107-160 complex to kinetochores, we depleted ELYS from HeLa cells by RNAi and quantitated the mitotic kinetochore signal intensities of ELYS or Nup133 for 46–200 kinetochores per condition. Notably, reducing the kinetochore signal of ELYS by RNAi resulted in a nearly identical reduction of Nup133 at the kinetochores (54.6% and 58.5%, Fig. 4*A*). Conversely, a reduction of the Nup133 kinetochore signal by Nup133 RNAi led to the same level of reduction of ELYS at the kinetochore (55.7% and 51.7%; Fig. 4*A*), indicating that ELYS and Nup133 are codependent for proper kinetochore targeting.

Although the partial loss of ELYS and Nup133 from the kinetochores after RNAi did not result in clear spindle assembly or mitotic chromosome alignment defects, it became quickly apparent that a significant number of the RNAi-depleted cells contained midbody microtubules, as visualized by β-tubulin immunofluorescence (Fig. 4*B*). Remarkably, ≈17% of ELYS-depleted cells and ≈11% of Nup133-depleted cells stained for midbody microtubules compared with only 2.6% of control RNAi-treated cells (Fig. 4*C*). These data indicate that depletion of either ELYS or Nup133 causes a delay in or failure to complete cytokinesis. Thus, ELYS and Nup133, a member of the Nup107–160 complex, are required for proper cell division.

Discussion

In this study, the major subunit of the nuclear pore, the Nup107–160 complex, was tested for molecular binding partners. The putative transcription factor ELYS was found to be such an interactor in both *Xenopus* and mammalian cells. Indeed, ELYS localizes to nuclear pores during interphase and to kinetochores throughout mitosis, in a manner virtually identical to that of the Nup107–160 complex. Both ELYS and the Nup107–160 complex are recruited early in the nuclear pore assembly process, i.e., in late anaphase. RNAi depletion of ELYS from HeLa cells resulted in severely reduced levels of Nups at the nuclear rim. Interestingly, ELYS RNAi often induced large cytoplasmic aggregates containing all of the Nups tested, with the exception of Tpr and, to a lesser extent, Pom121. We conclude that ELYS is an essential component for nuclear pore assembly and/or maintenance at the nuclear rim.

Mammalian ELYS is also a kinetochore-associated protein on mitotic chromosomes from prophase to late anaphase, akin to the Nup107–160 complex. Both bracket the inner kinetochore protein CENP-B and depend on one another for kinetochore localization. Although knockdown of ELYS from the kinetochores by ≈50% does not lead to significant chromosome segregation defects, we think it possible that the remaining protein is sufficient to carry out some ELYS function at the kinetochore. Strikingly, however, RNAi knockdown of ELYS does lead to a significant mitotic cell division defect, increasing the number of cells found detained in cytokinesis with midbody microtubules by ≈600%. A more detailed study of this defect will be needed to determine the precise role of ELYS in vertebrate cell division.

ELYS and the Nup107–160 complex plainly act in concert, both in nuclear pore assembly/maintenance and in proper cell division. However, they also differ: a substantial fraction of ELYS has a strong intranuclear presence. It has been shown that certain Nups, such as Nup98, Rae 1, and Nup50, shuttle into the nucleus and reside there part time (13, 20–24). ELYS may resemble these Nups. Alternatively, the intranuclear fraction of ELYS may have a function unrelated to nucleocytoplasmic trafficking. For example, Sec13, a member of the Nup107–160 complex, is both a Nup and a protein integral to the formation of the COPII-coated vesicles involved in cell secretion (2, 8, 11, 25–27). ELYS is referred to in gene databases as a transcription factor (ELYS or AT hook-containing transcription factor 1,

AHCTF1), based largely on the ability of certain regions to activate the transcription of a luciferase reporter (17). An important caveat, however, is that ELYS is a large, acidic protein; acidic domains often have the innate ability to activate transcription in reporter assays (28). A more in-depth analysis of its intranuclear pool is needed to determine whether ELYS is a bona fide transcription factor or has alternative functions inside the nucleus.

The demonstrated essential roles for ELYS in nuclear pore assembly and cytokinesis are consistent with the ELYS-null mouse data, which showed that ELYS is essential for very early embryonic survival. ELYS-null embryos die between days E3.5 and E5.5 (18). Mouse embryos null for Nup214, similarly, die between E4 and E4.5 (29), whereas embryos null for CENP-A, a protein critical for proper kinetochore function, die between E3.5 and E8.5 (30).

In this article, we show that ELYS is a previously uncharacterized resident of the nuclear pore. It is unusual to discover an unexpected member of the nuclear pore, because proteomics on the mammalian nuclear pore complex were completed in 2002 (2). In that study, mass spectrometry was performed on enriched Nup fractions from rat liver nuclei to determine the complete protein composition of the nuclear pore. A feasible explanation for why ELYS was not identified until now is that the human and mouse ELYS protein sequences (gi:17298096 and gi:17298098, respectively; 87% identity to rat ELYS, gi:34881056) were not placed in the NCBI database (September 2002) until after publication of the proteomics study (2). Another possibility is that ELYS was removed by the original rat pore purification protocol (2). We do find ELYS in purified HeLa nuclear pores, although the last purification step removes significant amounts (B.A.R., K. Gustin, and D.J.F., unpublished data).

BLAST searches reveal putative homologs of ELYS from species including *X. laevis* (gi:55250537), *Drosophila* (gi:24643345), and *Caenorhabditis elegans* (gi:42794020) but no obvious yeast homologs. The *C. elegans* gene, *C38D4.3*, which also contains an AT-hook motif, had been linked in a general genome-wide RNAi screen to the nuclear envelope in interphase and possibly to the kinetochore at metaphase (31, 32). Two studies published late after preparation of our report confirm this *C. elegans* link to nuclear pores and kinetochores (33, 34). However, gaps in the nuclear envelope induced by *C38D4.3/MEL-28* RNAi may have led to additional severe defects in the *C. elegans* embryos. Of note, the link to nuclear pores and kinetochores in worms is entirely consistent with our current findings in mammalian cells that ELYS RNAi disrupts the nuclear pore and cell division.

Considering the essential role of ELYS for nuclear pore assembly and maintenance, at least two basic models are possible (Fig. 4D). In the first, ELYS is an essential structural protein of the pore, associated with the Nup107–160 complex. The depletion of ELYS by RNAi in this model would result in no pores forming at any location within the cell. In the second model, ELYS is a nuclear pore-associated targeting protein that specifically recruits Nups, such as the Nup107–160 complex, to assemble into nuclear pores at the nuclear envelope rather than in membranes in cytoplasmic locations. Precedence for cytoplasmic pore complexes exists in annulate lamellae (AL), which are cytoplasmic structures that contain numerous nuclear pore-like complexes within stacks of double membranes. AL are present in cells but are not generally abundant (35). RNAi depletion of ELYS in this second model would also result in a clear loss of pores at the nuclear envelope. A potential secondary outcome, however, could be assembly into newly forming annulate lamellae pores. This possibility shows some support from the cytoplasmic aggregates of Nups that appear in ELYS RNAi-depleted cells (Figs. 3 *A* and *B* and 8*B*). Further work will be required to distinguish such models of ELYS function in nuclear pore assembly.

In summary, ELYS and the Nup107–160 complex biochemically interact and share strikingly similar localization and RNAi-depletion phenotypes. ELYS is a component of both the nuclear pore and the kinetochore as well as being intranuclear. RNAi confirms that ELYS is required for pore assembly at the nuclear envelope and for cell division. If it is a DNA-binding protein, as its AT-hook domain might suggest, ELYS could potentially use this domain to bind DNA and recruit pore subunits to the surface of the chromatin during mitosis. In interphase, ELYS could function similarly in new pore assembly or, alternatively, be involved in potential targeting of active vertebrate genes to the nuclear pore. As yet, gene targeting to the nuclear pore has been observed only in yeast (36–38), occurring through a set of specific yeast Nups.

Materials and Methods

Antibodies. The antibodies used included affinity-purified anti-hELYS (aa2220–2302; Bethyl Laboratories, Montgomery, TX), anti-hNup160, anti-hNup133 (6), anti-xNup43, anti-hNup37 (15), anti-Nup93 (39), anti-Nup358 (M. Dasso, National Institutes of Health, Bethesda, MD), anti-Tpr (40), anti-rat Pom121 (S. Tugendreich and D.J.F., unpublished data), anti-*Xenopus* importin β (R. Chan and D.J.F., unpublished data), anti-FG Nup antibody, mAb414 (Covance, Berkeley, CA), anti-CENP-B, [D. Cleveland, University of California at San Diego (UCSD)], anti-tubulin (A. Desai, UCSD), anti-hsp70, anti-Ran, and anti-Nup62 (BD Transduction Laboratories, Lexington, KY).

MS Analysis of Mitotic and Interphase Nup107–160 Complex Immunoprecipitations. Immunoprecipitation and MS analysis of the Nup107–160 complex from *Xenopus* egg extract were performed as recently published (15), except that the MS/MS spectra were searched against a combined forward–reverse database of NCBInr (National Center for Biotechnology Information nonredundant, version 1/10/2005) protein database limited to human, fish, and reptile taxonomies (345,250 sequences).

Immunoprecipitation from HeLa Cells. HeLa cells grown to 80% confluency in 10-cm dishes were washed twice with ice-cold PBS and 1 mM EDTA, lysed at 4°C in 1 ml of 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, and supplemented with 1 mM PMSF and a protease inhibitor mixture (P8340; Sigma, St. Louis, MO) for 30 min. Cell lysates were sonicated briefly and spun at 14,840 $\times$ *g* for 15 min. Immunoprecipitations were performed by adding 2–5 μg of anti-hNup133, anti-ELYS, or nonimmune rabbit IgG (Calbiochem/EMD Biosciences, San Diego, CA) coupled to protein A Sepharose beads (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence and RNAi. For indirect immunofluorescence, HeLa cells were grown in DMEM/10% FCS on coverslips for 3 days. Cells were either 4% formaldehyde-fixed for 5 min or permeabilized with PHEM buffer (60 mM Pipes/20 mM Hepes, pH 6.9/10 mM EGTA/4 mM MgSO$_4$/0.2% Triton X-100) for 5 min, methanol-fixed for 10 min at −20°C or 4% formaldehyde-fixed for 10 min, and rehydrated with TBS-Tx (10 mM Tris, pH 7.4/150 mm NaCl/0.1% Triton X-100). The cells were processed for immunofluorescence as described (6). Images were acquired by using an optical sectioning deconvolution microscope (DeltaVision; Applied Precision, Issquah, WA; Figs. 2 *A* and B and 4*A*) or an Axioskop fluorescence microscope (Zeiss, Thornwood, NY) (Figs. 2*C*, 3, and 4*B*). Measurements of kinetochore intensity were conducted on nondeconvolved DeltaVision images. We believe that the ≈50% reduction in kinetochore staining (Fig. 4*A*), compared with the extensive reduction in total ELYS protein (Fig. 8A)

and ELYS at nuclear pores (Fig. 3 *A*, *C*, and *D*) after RNAi, derives from the finding that only a small percent of the Nup107–160 complex is found localized to the kinetochores at mitosis even in normal cells (5, 8, 11). Extensive cellular RNAi depletion would likely leave sufficient ELYS for the kinetochore stain observed here. All images used identical exposure settings, and scaling and intensities were determined by using Metamorph software (Universal Imaging, Downingtown, PA).

For the RNAi experiments, HeLa cells plated on coverslips were transfected for 48–60 h by using 0.84 μg of siRNA duplexes to ELYS (target: Exon 28 Silencer Pre-Designed siRNA #108720; Ambion, Austin, TX), Nup133 (target: AAGTCGAT-GACCAGCTGACCA) or Silencer Negative Control #1 siRNA (Ambion) in Oligofectamine (Invitrogen, Carlsbad, CA).

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