# Centrin 2 Localizes to the Vertebrate Nuclear Pore and Plays a Role in mRNA and Protein Export<sup>⊽</sup>†

Karen K. Resendes, Beth A. Rasala, and Douglass J. Forbes\*

Section of Cell and Developmental Biology, Division of Biological Sciences 0347, University of California—San Diego, 9500 Gilman Drive, La Jolla, California 92093-0347

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Centrins in vertebrates have traditionally been associated with microtubule-nucleating centers such as the centrosome. Unexpectedly, we found centrin 2 to associate biochemically with nucleoporins, including the *Xenopus laevis* Nup107-160 complex, a critical subunit of the vertebrate nuclear pore in interphase and of the kinetochores and spindle poles in mitosis. Immunofluorescence of *Xenopus* cells and in vitro reconstituted nuclei indeed revealed centrin 2 localized at the nuclear pores. Use of the mild detergent digitonin in immunofluorescence also allowed centrin 2 to be clearly visualized at the nuclear pores of human cells. Disruption of nuclear pores using RNA interference of the pore assembly protein ELYS/MEL-28 resulted in a specific decrease of centrin 2 at the nuclear rim of HeLa cells. Functionally, excess expression of either the N-or C-terminal calcium-binding domains of human centrin 2 caused a dominant-negative effect on both mRNA and protein export, leaving protein import intact. The mRNA effect mirrors that found for the *Saccharomyes cerevisiae* centrin Cdc31p at the yeast nuclear pore, a role until now thought to be unique to yeast. We conclude that in vertebrates, centrin 2 interacts with major subunits of the nuclear pore, exhibits nuclear pore localization, and plays a functional role in multiple nuclear export pathways.

The nuclear pore complex (NPC) is the sole mediator of traffic between the nucleus and cytoplasm (3, 15, 16, 27, 39, 66, 134). The vertebrate NPC is a massive 125-MDa complex composed of  $\sim 30$  different proteins in multiple copies per pore (18, 102). Together these proteins, or nucleoporins, create a structure composed of three distinct domains: the cytoplasmic filaments, the central scaffold with eight large spokes, and the nuclear basket (120). The overall structure of the vertebrate NPC exhibits a striking similarity to the smaller yeast NPC (129, 140). The Saccharomyces cerevisiae and vertebrate nucleoporins, despite the fact that their protein components have extensive sequence divergence, show many structural and functional similarities, with few exceptions (18, 84, 103). One major exception was thought to be the integral membrane proteins that anchor the NPC to the nuclear envelope, which were thought to differ completely (14, 24, 45, 85). The recent discovery of the vertebrate Ndc1, a homologue for yeast Ndc1, now provides a common integral membrane pore protein between the species (63, 81, 114). The second exception was the yeast centrin protein, Cdc31, which was designated a nucleoporin based on its presence in highly purified yeast nuclear pores (31, 103). Despite extensive purification of rat nuclear pores (18, 84) and the existence of multiple human centrin genes, there has been no experimental evidence to date that links centrin to the vertebrate nuclear pore. Here, we address the unusual finding of centrin in the yeast nuclear pore and its apparent absence in the vertebrate pore.

As a group, centrins are small calcium-binding proteins traditionally associated with essential cellular structures responsible for nucleating microtubules (105, 136). This nucleation function for centrin is evolutionarily conserved, from the flagella of algae to the spindle pole body of yeast to their vertebrate equivalent, the centrosome (10, 29, 65, 105, 107, 113). For this reason, it was unexpected to find centrin in the yeast nuclear pore (103).

In humans, three different centrin genes have been identified: human centrin 1 (*Homo sapiens Cen1* [Hs*Cen1*]), centrin 2 (Hs*Cen2*), and centrin 3 (Hs*Cen3*) (29, 65, 87). Hs*Cen1* and Hs*Cen2* show substantial identity with each another (84%), but only 54% identity with Hs*Cen3* (87). Hs*Cen1* is distinct in that it exhibits tissue-specific expression, being expressed primarily in the testes and retina, which have flagella and cilia, respectively (48, 137, 138). In contrast, Hs*Cen2* and Hs*Cen3* are ubiquitously expressed throughout the body, where they have been demonstrated to play critical roles in centriole/centrosome duplication and cell division (86, 108, 138).

Despite centrin's known localization to the centrosome, however, most human centrin 2 is in fact not centrosomeassociated but is present in both the cytosol and the nucleus, as shown by cell fractionation and immunofluorescence (94). Thus, it is highly possible that centrin 2 has further functions, ones outside microtubule nucleation. At least one other role for centrin 2 has indeed been found in higher eukaryotes. In both humans and *Arabidopsis thaliana*, centrin 2 has been shown to be a functional part of the xeroderma pigmentosum group C (XPC) complex which initiates nucleotide excision repair as a part of a global DNA repair pathway (4, 72, 90, 92). In addition, as stated above, the single yeast centrin homologue, Cdc31, has been identified as a component of the yeast nuclear pore (103). Indeed, Cdc31 has a role in yeast mRNA export, interacting with the Sac3-Thp1-Sus1 mRNA export

<sup>\*</sup> Corresponding author. Mailing address: Room 2124A Pacific Hall, 9500 Gilman Drive, La Jolla, CA 92093-0347. Phone: (619) 972-6493. Fax: (858) 534-0555. E-mail: dforbes@ucsd.edu.

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complex. Strikingly, Cdc31 mutants are severely defective in mRNA export (31).

Within the vertebrate nuclear pore, a number of nucleoporins have been found to be essential for mRNA export. These nucleoporins include multiple phenylalanine-glycine (FG)containing nucleoporins (Nup358, Nup214, Nup153, and Nup98), which interact with the mRNA export cargo as it transits the pore (8, 13, 34, 43, 52, 98, 112, 127, 133). In addition, a critical subcomplex of the scaffold of the nuclear pore, the Nup107-160 complex, has been implicated in mRNA export. Overexpression of a specific fragment of either Nup133 or Nup160, a component of the Nup107-160 complex, causes strong defects in mRNA export (128). Similarly, mutations of the yeast homologues of these proteins (*S. cerevisiae* Nup133 and Nup120) were among the first of the yeast nucleoporins to show defects in mRNA export (2, 7, 22, 23, 38, 50, 70).

Globally, a subset of nucleoporins, including the vital Nup107-160 complex, has also been shown to have mitotic functions. These nucleoporins move to the kinetochore and/or spindle poles during mitosis (5, 11, 12, 25, 56, 74, 79, 93, 104, 119, 142). Focusing specifically on the Nup107-160 complex, this large 9- to 10-member complex, essential for mRNA export, pore assembly, and structure, is also absolutely required for correct spindle assembly (93), likely due to its mitotic location at the kinetochores and spindle poles (11, 47, 93, 96, 100, 111, 128, 131).

In the present study, we observed the interaction of centrin 2 with the vertebrate Nup107-160 complex in both *Xenopus laevis* and human cells. Strikingly, we found centrin 2 to be strongly enriched at the vertebrate nuclear pore. Moreover, misexpression of centrin 2 led to defects in nuclear export. Our results demonstrate not only that vertebrate centrin 2 interacts with the nuclear pore but that this interaction has a role in vertebrate mRNA and protein export.

#### MATERIALS AND METHODS

Antibodies. Two commercial antibodies were used to detect human and Xenopus centrin 2. Centrin antibody (Ab) A, raised to an undisclosed sequence located within amino acid (aa) sequence 50 to 100 of human centrin 2, cross-reacts with human centrin 1 and 2 but not with centrin 3 (Santa Cruz Biotechnology, Santa Cruz, CA). Centrin Ab B, raised to the C terminus of human centrin 1 (aa 152 to 172), similarly cross-reacts with human centrin 1 and 2 but not with centrin 3 (Sigma, St. Louis, MO). Other antibodies used include anti-hNup160, anti-xNup160, anti-hNup133 (128), anti-hNup37, anti-xNup37 (93), MAb414 anti-FG Nups; (Covance, Berkeley, CA), anti-xNup155 (47), anti-mNup53, anti-xNup50 (a gift from V. Delmar), and anti-myc (Calbiochem/EMD Biosciences, San Diego, CA).

**GST pulldowns.** Fifty micrograms of glutathione *S*-transferase (GST) or GSTxNup160 C terminus was cross-linked to CNBr beads in phosphate-buffered saline (PBS) (8 g/liter NaCl, 0.2 g/liter KCl, 0.14 g/liter Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g/liter KH<sub>2</sub>PO<sub>4</sub>). The beads were then incubated with 50 µl of *Xenopus* cytosol with or without 10 µM RanQ69L-GTP in a final volume of 500 µl of PBS, 50 mM NaF, 50 mM β-glycerophosphate, and 1 mM NaVO<sub>4</sub> plus protease inhibitors (catalog no. P3840; Sigma Aldrich, St. Louis, MO) for 1 h at room temperature. The reaction mixtures were washed three times with PBS, eluted with 0.1 M glycine (pH 2.5), and neutralized with 100 mM Tris (pH 7.9). The eluate was then subjected to liquid chromatography-tandem mass spectrometry (101).

**Immunoprecipitation.** HeLa cells grown to 80% confluence in 10-cm dishes were washed with  $1 \times$  PBS and lysed at 4°C in 1 ml of 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.25% sodium deoxycholate supplemented with aprotinin and leupeptin (final concentration, 10 µg/ml) for 30 min. Cell lysates were vortexed briefly and spun at 14,840 × g for 10 min. Immunoprecipitations were performed by adding 2 to 5 µg of anticentrin (Ab A

or Ab B, where indicated), anti-hNup160, or nonimmune rabbit or goat immunoglobulin G (IgG), followed by the addition of protein A (for rabbit IgG) or protein G (for goat IgG) Sepharose beads (Amersham Biosciences, Piscataway, NJ). Centrin Ab coimmunoprecipitation of myc-tagged proteins was performed as described above, except that HeLa cells were transfected 24 h prior to lysis with the indicated constructs, using Lipofectamine (Invitrogen, Carlsbad, CA).

Immunofluorescence. For indirect immunofluorescence, HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA)/10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA). Xenopus XL177 cells were grown in L-15 medium (Mediatech, Herndon, VA)/15% FCS at room temperature on coverslips for 1 to 2 days. Cells were washed with  $1 \times PBS$  and fixed with 2% formaldehyde for 10 min. Cells were then permeabilized with 0.005% digitonin in transport buffer (20 mM HEPES, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA [pH 7.4]) for 5 min at 4°C, followed by a 20-min wash in transport buffer, or they were permeabilized in 0.5% Triton X-100 in  $1 \times$  PBS for 10 min at room temperature. Cells were blocked for a period ranging from 2 h to overnight in PBS containing 2% FCS, 2% bovine serum albumin, and 0.02% NaN3. Where indicated, the fixation step was carried out after permeabilization (see Fig. S3 in the supplemental material). Cells were stained with anticentrin Ab (1:750, Sigma; or 1:100, Santa Cruz Biotechnology) or with anti-lamin B Ab (Santa Cruz Biotechnology) together with MAb414 (Covance) for 1 h. Staining was detected with Alexa Fluor 568-labeled goat anti-rabbit IgG (for the centrin Ab) or with Alexa Fluor 568-labeled donkey anti-goat IgG (for the lamin B Ab), each with Alexa Fluor 488-labeled donkey anti-mouse IgG (for MAb414) (Molecular Probes/Invitrogen, Carlsbad, CA). Coverslips were mounted on Vectashield (Vector Laboratories, Burlingame, CA) and visualized with an Axiovert 200 M microscope (Carl Zeiss, Thornwood, NY) at a magnification of ×63, using an oil objective with a 1.3 numerical aperture at 23°C, with Immersol 518F (Carl Zeiss) as the imaging medium. Images were recorded using a Coolsnap HQ camera (Photometrics, Tucson, AZ) and Metavue software (Molecular Devices Corporation, Downingtown, PA). Trace images were produced using the trace contour function of Adobe Photoshop.

Nuclear reconstitution. Cytosolic and membrane vesicle fractions of *Xenopus* egg extracts were prepared as described previously (99). The membranes were stored in 10- $\mu$ l aliquots at  $-80^{\circ}$ C and used as a 20× stock. Nuclei were reconstituted by mixing *Xenopus* egg membrane and cytosolic fractions at a 1:20 ratio with an ATP regeneration system and sperm chromatin (80). For indirect immunofluorescence, reconstituted nuclei were formaldehyde fixed, pelleted onto poly-L-lysine-coated coverslips (15 min at 750 rpm) and probed with MAb414 and either of the anticentrin antibodies described above. Where indicated, pelleted nuclei were permeabilized with Triton X-100 (see Fig. S3 in the supplemental material). Both centrin Ab A and B exhibited NPC staining of reconstituted nuclei in the absence or presence of Triton X-100 treatment; however, NPC staining was more pronounced if Triton X-100 was not used.

Constructs. The C terminus of Xenopus Nup160 was obtained by PCR from a non-full-length xNup160 cDNA clone (primer 1, CCCGAATTCCAGCCGGTA TCAGGAGCTGTG; primer 2, TTTCTCGAGTTACGCCCGTAGAGGCTTC) (128). This fragment, containing the last  $\sim$ 297 aa of the Xenopus Nup160 C terminus, was subcloned into a pGEX-4T-1 GST-tagged vector (GE Healthcare, Piscataway, NJ). The xNup160 sequence is homologous to aa sequence 1144 to 1429 of human Nup160. A full-length cDNA clone of HsCen2 (Entrez nucleotide accession no. NM\_004344.1) was obtained from Origene (Rockville, MD). Oligonucleotides were used to amplify either the HsCen2 N terminus (aa 1 to 98) or the HsCen2 C terminus (aa 94 to 172), which were subcloned as EcoRI-Kpn1 fragments into a pCDNA3.1 myc-tagging transfection vector (Invitrogen, Carlsbad, CA). For the myc tag transfection experiments, a cDNA of human Nup160 aa 912 to 1436 was reverse transcribed from HeLa total RNA, amplified by PCR, and cloned into the pBluescript vector (Stratagene, La Jolla, CA). Nup160 aa 1146 to 1436 from this cDNA clone was then subcloned as an XhoI-BamHI fragment into pcDNA 3.1.

**RNAi and transfection.** For the RNA interference (RNAi) experiments, HeLa cells plated on coverslips were transfected for 48 to 60 h by using 0.84 µg of short interfering RNA (siRNA) duplexes to ELYS (target, exon 28 Silencer predesigned siRNA; catalog no. 108720; Ambion, Austin, TX) (60 h) or centrin 2 (target, 5'-AAGAGCAAAAGCAGGAGATCC-3; Ambion, Austin TX) (48 h) (108) and Silencer negative control no. 1 siRNA (Ambion) in Oligofectamine (Invitrogen, Carlsbad, CA) as described in reference 101.

HeLa cells were transfected 24 h prior to the immunoprecipitation or  $poly(A)^+$  RNA assay with the indicated constructs, using Lipofectamine 2000 (Lipofectamine to DNA ratio of 2.5:1; Invitrogen, Carlsbad, CA). Where immunofluorescence was performed, successfully transfected cells were identified by positive staining for the myc epitope, using fluorescein isothiocyanate (FITC)-

labeled anti-myc Ab (Santa Cruz Biotechnology, Santa Cruz, CA). *Xenopus* XL177 cells were transfected 48 h prior to poly(A) plus RNA assays using Lipofectamine at an increased ratio (5:1, Lipofectamine to DNA) at room temperature. XL177 cells were changed to fresh L-15 medium four hours following transfection.

Poly(A) + RNA nuclear accumulation assay. Cells were grown on coverslips for 1 day and then transfected for ~24 h with control plasmids or plasmids encoding Nup160 fragments or HsCen2 fragments in pCDNA3.1, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (128). For siRNA experiments, cells were transfected with the indicated siRNA 48 h before performing the poly(A)<sup>+</sup> RNA accumulation assay. Cells were fixed (3% formaldehyde in PBS; 20 min on ice), permeabilized (0.5% Triton X-100 in PBS), incubated for 5 min with PBS plus 1 mM vanadyl ribonucleoside complexes (VRC) and then for 5 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus VRC (0.3 M NaCl, 0.03 M sodium citrate [pH 7]), and then prehybridized with 50% formamide,  $2\times$ SSC, 1 mg/ml bovine serum albumin, 1 mM VRC, and 10% dextran sulfate (1 h at 37°). The cells were hybridized with Cy3-oligo(dT)<sub>50</sub> (GeneLink, Hawthorne, NY) at 100 pg/µl in the same buffer (overnight at 37°C), washed three times in  $2 \times$  SSC (at 37°C for 5 min each), and then refixed with 3% formaldehyde in PBS for 20 min on ice. The expression of transfected proteins was detected with FITC-labeled anti-myc Ab (1:100; Santa Cruz Biotechnology, Santa Cruz, CA).

Nuclear protein import and export. Cells were grown on coverslips for 1 day and then cotransfected for ~16 h with the Rev-glucocorticoid-green fluorescent protein (GFP) (pXRGG) plasmid (44, 75, 128) and either (i) the control plasmid encoding malate dehydrogenase, (ii) the plasmids encoding Nup160 fragments, or (iii) the HsCen2 fragments in pCDNA3.1, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). All of the last plasmids were myc tagged. For siRNA experiments, cells were transfected with RGG and the indicated siRNA 48 h before performing the import/export assay. After the cells were transfected, they were treated with dexamethasone (final concentration 1 µM) (Sigma-Aldrich, St. Louis, MO) for 60 min to induce RGG import. In parallel, an identical set of transfected cells were treated with dexamethasone for 60 min to induce RGG import, washed, and then incubated with medium lacking dexamethasone (for 2 h at 37°C) to promote RGG export. Cells were fixed (with 3% formaldehyde in PBS for 15 min on ice), permeabilized (with 0.5% Triton X-100 in PBS for 10 min), and blocked (with 5% FBS in PBS for 10 min). The transfected expressed proteins were detected with tetramethyl rhodamine isocyanate (TRITC)-labeled anti-myc Ab (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or, in the case of RGG, by its GFP moiety.

## RESULTS

Centrin 2 interacts with the Nup107-160 complex. Many of the vertebrate nucleoporins exhibit low homology to their yeast counterparts ( $\leq 25\%$ ). Despite this, the vertebrate Nups are found in subcomplexes akin to the subcomplexes derived from the yeast nuclear pore. The yeast homologue of the Nup107-160 complex, the Nup84 complex, exhibits a Y-shaped structure, as determined by electron microscopy and in vivo reconstitution (77, 78, 110). Fluorescence resonance energy transfer (or FRET) experiments between Nup120 (the yeast homologue of Nup160) and an adjacent central component of the Nup84 complex suggest that the C terminus of Nup120 faces away from the center of the Y and is, thus, free to interact with other proteins outside of the Y-shaped complex (20). In vertebrates, the homologue Nup160 is ~300 amino acids longer than the yeast Nup120, thus increasing this protein's extension away from the core of the Nup107-160 complex (128).

Higher metazoan nuclear pores experience unique situations that yeast nuclear pores do not, such as mitotic nuclear pore disassembly and subsequent reassembly. Thus, for multiple reasons, we sought to identify novel protein binding partners for the C terminus of vertebrate Nup160. We used *Xenopus* egg extracts as a source of cellular proteins (46, 88). *Xenopus* Nup160 C-terminal fragment pulldowns were performed in the presence or absence of 10  $\mu$ M RanQ69L-GTP. Pulldowns with GST alone served as negative controls. The proteins bound were identified by mass spectrometry.

One intriguing candidate found to bind to the GST-xNup160 C terminus was *Xenopus laevis* centrin. Six full-length *Xenopus* centrin protein sequence isolates are present in the Entrez protein database at this time. Four sequences contain the three peptides identified by our mass spectrometry screen and are either identical to each another or contain three-amino-acid differences. These four sequences are designated in the database as *Xenopus* centrin 2 or, more generically, centrin. The fifth and sixth sequences are clearly *Xenopus* homologues of centrin 3, as they share only 58% identity to the sequences above but 88% identity with human centrin 3. No *Xenopus* centrin 1 has yet been identified.

We thus believe that the interacting protein that we have found is *Xenopus* centrin 2. It has 85% identity to human centrin 2, a ubiquitously expressed centrin, versus 81% identity to human centrin 1, the testis/retina-specific centrin. While human centrin 1 and 2 are similar, in the sequence locations where they differ, *Xenopus* centrin more closely resembles human centrin 2 than centrin 1 (14 versus 6 identities [see Fig. S1 in the supplemental material]). In consequence, we conclude that this interacting protein is *Xenopus* centrin 2.

To further analyze the Xenopus centrin 2 interaction with Nup160, two commercially prepared antibodies to human centrin proved to be of value (Fig. 1A). Centrin Ab A, raised to an internal region of human centrin 2, is known to recognize human centrin 1 and 2 but not centrin 3 (Fig. 1A) (Santa Cruz Biotechnology). Similarly, centrin Ab B, raised to the C terminus of human centrin 1, recognizes both human centrin 1 and 2 but not centrin 3 (Fig. 1A) (Sigma Aldrich). In HeLa cells, centrin 2 is the only protein that should be recognized by each of these antibodies, as the testis/retina-specific centrin 1 protein should not be present (138). Both antibodies detected a protein of the appropriate size in human HeLa cell lysates (Fig. 1B, lanes 6 and 7). These anticentrin antibodies also recognized an identically sized protein of 20 kDa on immunoblots of Xenopus egg cytosol and lysates of Xenopus XL177 cultured cells (Fig. 1B, lanes 1 to 4).

To confirm the mass spectrometry interaction between the C termini of xNup160 and centrin 2, we again performed a GST-xNup160 C-terminal pulldown from *Xenopus* egg cytosol and probed with centrin Ab B (Fig. 1C). A centrin band was indeed observed for the Nup160 C-terminal pulldown assay (Fig. 1C, lanes 3 and 5) but not in control GST pulldowns (Fig. 1C, lanes 2 and 4).

We next looked for evidence that centrin 2 interacts with the full Nup107-160 complex. Centrin Ab B was used to immunoprecipitate *Xenopus* centrin 2 from interphase egg cytosol (Fig. 2A, lane 2). Importantly, this centrin Ab B also coimmunoprecipitated all tested members of the Nup107-160 complex, including Nup160, Nup133, Nup85, and Nup43 (Fig. 2A, lane 2). Similar results were observed with centrin Ab A (data not shown). However, Nup93, which is not a member of the Nup107-160 complex (40), did not coimmunoprecipitate significantly with either centrin Ab (Fig. 2A, lane 2, and data not shown).

A Nup107-160 complex interaction with centrin 2 was also observed with human cells. Of the anticentrin antibodies, centrin Ab A proved the most efficient at the immunoprecipitation



FIG. 1. Scheme of proteins and antibodies used. (A) *Xenopus* centrin 2 and human centrin 2 both consist of 172 amino acids and share 85% identity. Two antibodies were used to recognize both human and *Xenopus* centrin 2 in this study. Centrin Ab A recognizes a region between residues 50 and 100 of human centrin 2; *Xenopus* centrin 2 shares 80% identity with human centrin 2 in this domain. Centrin Ab B was raised to C-terminal residues 152 to 172 of human centrin 1, which because of high homology, also recognizes human centrin 2. *Xenopus* centrin 2 differs from human centrin 1 at only one position, from aa 152 to 172. (B) Both anticentrin antibodies recognize an apparent single band in *Xenopus* egg extract (lanes 1 and 2), *Xenopus* XL177 cell lysate (lanes 3 and 4), and HeLa cells (lanes 6 and 7). Control IgG did not recognize this band (lane 5). Hatch marks on the left indicate the molecular mass markers (from top: 150, 100, 75, 50, 37, 25, 20, and 15 kDa). (C) The GST-tagged C terminus of xNup160 interacts with *Xenopus* centrin 2, as well as with members of the Nup107-160 complex in the presence or absence of endogenous importin  $\beta$ , a negative inhibitor of the interaction. We observed no effect of RanQ69L-GTP addition here, other than the removal of peripheral importin  $\beta$ . GST alone was used as a negative control (lanes 2 and 4). Cyt indicates a fraction of input *Xenopus* egg cytosolic extract (lane 1).

of centrin 2 from HeLa cell lysates prepared by Triton X-100/ deoxycholate lysis (Fig. 2B, lane 2). When analyzed, this Ab consistently coimmunoprecipitated all tested members of the human Nup107-160 complex (Fig. 2B, lane 2).

Importantly, the Ab to Nup160 reciprocally coimmunoprecipitated centrin 2 from HeLa cells (Fig. 2C, lane 2). Nup93 and several other nucleoporins not in the Nup107-160 subcomplex, such as Nup205, Nup155, Nup53, and Nup50, were not coimmunoprecipitated by the centrin Ab (Fig. 2B; also see Fig. S2 in the supplemental material), although the FG proteins Nup358, Nup214, and Nup62 were sometimes observed in small amounts (data not shown). Thus, centrin 2 and the Nup107-160 complex show a specific interaction.

Testing other nucleoporins involved in mRNA export. Mass spectrometry of the *Xenopus* proteins pulled down by the GSTxNup160 C terminus also revealed Nup153 (data not shown), a known Nup107-160 complex interacting partner that plays an important role in mRNA export (8, 91, 112, 127, 128). We therefore asked whether centrin 2 interacts with Nup153 or other nucleoporins involved in mRNA export. Centrin Ab A did indeed coimmunoprecipitate Nup153 from HeLa cell and *Xenopus* egg extracts (Fig. 2D, lane 2, and data not shown). However, Nup98, which is also involved in mRNA export, did not coimmunoprecipitate with the centrin Ab (Fig. 2D, lane 2). In summary, we have identified interactions between centrin 2 and specific nucleoporins involved in mRNA export, i.e., the Nup107-160 complex and Nup153.

Centrin 2 is found at the nuclear pores of *Xenopus* reconstituted nuclei and cultured cells. The interaction between *Xenopus* centrin 2 and specific nucleoporins suggested that cen-

trin 2 could be located at the nuclear pore as previously seen in yeast (103). However, our previous finding of the Nup107-160 complex at the spindle poles (93) made it equally possible that the centrin–Nup107-160 interaction could in fact be occurring at the spindle pole and not at the nuclear pore. To address this, immunofluorescence was performed with nuclei assembled in vitro in *Xenopus* interphase egg extracts (30, 46, 73, 109, 126, 141). This system provides a powerful tool by which robust assembly of functional nuclei occurs around chromatin templates in vitro. Using immunofluorescence with both centrin Ab A and B, we found that *Xenopus* centrin 2 was indeed localized at the nuclear rim and to a lesser extent in the nuclear interior (Fig. 3A). Moreover, the centrin 2 at the nuclear rim was observed with a punctate pattern that closely colocalized with that of FG nucleoporins (Fig. 3A, 5×).

When immunofluorescence was performed on *Xenopus* XL177 cultured cells, centrin Ab A and B again consistently gave a nuclear pore stain (Fig. 3B and data not shown). Centrin 2 was also observed in the cytoplasm of XL177 cells, consistent with the previous finding (94) that the majority of centrin 2 in animal cells is not associated with the centrosome but is cytoplasmic and nuclear. A specific centrosomal stain was not seen, likely because the cytoplasmic stain obscures it. Taken together, the above results demonstrate that *Xenopus* centrin 2 is clearly present at the nuclear pore.

**Centrin 2 is at the nuclear pores of human cells.** In human and mouse cells, vertebrate centrin has long been observed at the centrosome, when either immunofluorescence or GFP tagging was used (21, 29, 49, 51, 65, 76, 86, 87, 105, 108, 135). In prior studies where centrin 2 was identified visually as located



FIG. 2. Centrin 2 Ab coimmunoprecipitates nucleoporins involved in mRNA export from human cells and *Xenopus* egg extracts. (A) Anticentrin Ab B (AbB) immunoprecipitates *Xenopus* centrin 2 (open arrowhead), along with members of the Nup107-160 complex, such as Nup160, Nup133, Nup85, and Nup43 (filled arrowhead, lane 2) from *Xenopus* egg extract. Nup37 was also occasionally immunoprecipitated by centrin 2 Ab B (data not shown). Centrin Ab B did not immunoprecipitate the non-Nup107-160 complex member Nup93 (lane 2). Immunoprecipitation with rabbit IgG was used as a negative control (lane 3). Cyt indicates a fraction of input *Xenopus* egg cytosolic extract. Centrin Ab A also coimmunoprecipitated both centrin 2 and Nup160 (data not shown). Because of its superior efficiency, centrin Ab B was used for the majority of subsequent *Xenopus* centrin 2 immunoprecipitation experiments. (B) Anticentrin Ab A (AbA) immunoprecipitates human centrin 2 (open arrowhead) and all tested members of the Nup107-160 complex (Nup160, Nup133, Nup85, Nup43, and Nup37, filled arrowheads) from HeLa cells but does not immunoprecipitate the noncomplex member Nup93 (lane 2). Immunoprecipitation with goat IgG (lane 3) was used as a negative control. Lys indicates a fraction of the input HeLa cell lysate (B to D). Centrin Ab B also coimmunoprecipitated both centrin 2 and Nup160 (data not shown); however, because of the superior levels of human centrin 2 immunoprecipitated human centrin 2 (lane 2). Immunoprecipitation experiments. (C) Nup160 Ab reciprocally coimmunoprecipitated human centrin 2 (lane 2). Immunoprecipitation of HeLa immunoprecipitate s a negative control (C and D). (D) Anticentrin Ab A immunoprecipitates Nup153 (filled arrowhead) but not Nup 98 from HeLa cell lysate.

primarily at the centrosome, with no NPC localization, the human cells were either methanol fixed or formaldehyde fixed and then Triton X-100 permeabilized (29, 62, 94, 108). When we fixed human HeLa cells with formaldehyde, permeabilized them with Triton X-100, and then performed immunofluorescence with centrin Ab A in the traditional manner, we saw no centrin staining of any sort (data not shown). Notably, even yeast Cdc31 could not be visualized at the NPC by immunofluorescence (31). When we performed formaldehyde fixation and permeabilized the cells with Triton X-100 and used centrin Ab B on HeLa cells, we saw distinct centrosomal staining and little of any other stain (Fig. 4A), as observed previously by others. However, if HeLa cells were permeabilized with the milder detergent digitonin and immunofluorescence was performed with anti-centrin Ab A or B, we observed centrin 2 with a punctate stain at the nuclear rim (Fig. 4B; also see Fig. S3B in the supplemental material). This stain was seen colocalizing with that of the FG nucleoporins. Nuclear pore staining was observed whether formaldehyde fixation preceded (Fig. 4B) or followed (see Fig. S3B in the supplemental material) digitonin permeabilization of the HeLa cells. Some nuclear and cytoplasmic centrin 2 staining was additionally observed, but the nuclear pore stain was prominent.

In summary, we find that centrin 2 is observed primarily at the centrosome with Triton X-100-permeabilized human cells,



FIG. 3. Centrin 2 is located at the nuclear pore in *Xenopus* nuclei assembled in vitro and in cultured cells. (A) *Xenopus* centrin 2 localizes to the rim of in vitro-reconstituted nuclei. Double immunofluorescence of spun-down-reconstituted nuclei, stained with anti-centrin 2 Ab A or B (left panels) and anti-FG Nup Ab MAb414 (middle panels), with merged images (right panels) are shown. Insets (at far right) show enlarged sections (magnification,  $\times$ 5). See Fig. S3A in the supplemental material for parallel images from this experiment, performed under different conditions. (B) Anticentrin Ab A shows localization of centrin 2 at the nuclear rim of *Xenopus* XL177 cells fixed with formaldehyde and permeabilized with Triton X-100. Double immunofluorescence with anti-FG Nup Ab MAb414 is shown with the merged image at the right. Insets (at far right) show enlarged sections (magnification,  $\times$ 5).

in accordance with previous immunofluorescence studies. However, the milder digitonin treatment conditions after fixation reveals that centrin 2 is clearly located in a punctate pattern at the nuclear rim in human cells, colocalizing with FG nucleoporins.

**Centrin 2 staining is disrupted by the loss of nuclear pores.** To further test the nuclear pore localization of centrin 2, we disrupted the nuclear pores of HeLa cells, using RNAi. It has been shown by us and others that RNAi of the critical nuclear pore assembly protein ELYS/MEL-28 leads to a dramatic loss of nuclear pores at the nuclear envelope in human cells and, instead, promotes the formation of annulate lamellae, cytoplasmic stacks of membranes with pore complexes that contain virtually all nucleoporins tested (17, 19, 36, 101). Furthermore, ELYS RNAi in HeLa cells was previously shown by us to leave the nuclear membranes and the Ran gradient unaltered (103).

We examined the effect of ELYS/MEL-28 RNAi on human centrin 2. ELYS/MEL-28 RNAi of HeLa cells led to a dramatic loss of FG nucleoporins at the nuclear rim and a concomitant increase in large cytoplasmic aggregates containing FG nucleoporins near but not part of the nuclear rim (Fig. 5A, ELYS RNAi, FG Nups panel). Neither of these changes was observed with a control RNAi oligo (Fig. 5A, compare green fluorescence panels). Strikingly, RNAi depletion of ELYS/ MEL-28 led to a decrease in the centrin 2 punctate nuclear rim stain compared to that of the control cells (Fig. 5A, compare red fluorescence panels). Disruption of the nuclear pores caused centrin 2 protein to aggregate in the cytoplasm in locations often coincident with FG nucleoporins (Fig. 5A, Merge panels; see also  $5 \times$  Trace panels). As these FG nucleoporincontaining aggregates have previously been characterized by MOL. CELL. BIOL.



FIG. 4. Centrin 2 is located at the centrosome and the nuclear pore in human cells. (A) Human centrin 2 localizes to the centrosome in HeLa cells fixed with formaldehyde and permeabilized with Triton X-100. Double immunofluorescence of HeLa cells with anticentrin Ab B (left) and the FG Nup Ab MAb414 (middle) is shown with the merged image at the right. (B) Immunofluorescence with centrin Ab B or A performed on HeLa cells fixed with formaldehyde and treated with digitonin. Centrin Ab B reveals more extensive centrin 2 staining with clear human centrin 2 staining present at the nuclear rim in a punctate pattern. Double immunofluorescence of HeLa cells with either anticentrin Ab A or B and anti-FG Nup monoclonal Ab MAb414 is shown with the merged image at the right. Insets (at far right) show enlarged sections (magnification,  $\times$ 5).

electron microscopy to be annulate lamellae, i.e., stacks of cytoplasmic pores (36), our data support the finding that centrin 2 is associated with the pore, both in nuclear pores and in cytoplasmic annulate lamellae pores.

It should be noted that in some ELYS/MEL-28 RNAidepleted cells, a population of centrin 2 also accumulated in the nucleus (Fig. 5A); these nuclear aggregates did not overlap with FG nucleoporins. As expected, ELYS/MEL-28 RNAi did not disrupt the nuclear envelope overall (101), as lamin B staining remained unchanged (Fig. 5B, red fluorescence panels).

We conclude that the disruption of the nuclear pores reduces centrin 2 nuclear pore staining and causes localization of a population of centrin 2 to the FG nucleoporin-containing cytoplasmic aggregates presumed to be annulate lamellae. Thus, the RNAi result is entirely consistent with the localization of centrin 2 at nuclear pores.

Centrin 2 is involved in vertebrate mRNA export. The localization of centrin 2 at the vertebrate nuclear pore suggested that it might have a functional role at the pore. Vertebrate Nup160 has previously been shown to play a role in mRNA export: transfection of a fragment of Nup160, specifically aa 317 to 697, into HeLa cells had a dominant-negative effect on mRNA export, resulting in nuclear poly(A)<sup>+</sup> plus RNA accumulation (128). One possibility is that this fragment of Nup160 might, when overexpressed, sequester centrin away. Thus, we pursued a twofold strategy to determine whether centrin 2 plays a role in mRNA export.

We first asked which domains of human Nup160 are in-



FIG. 5. ELYS/MEL-28 RNAi disrupts centrin 2 at the nuclear pore. Immunofluorescence with HeLa cells transfected with ELYS siRNA duplexes, which is known to disrupt nuclear pore structure, for 48 h. After ELYS or control RNAi, the cells were digitonin permeabilized and fixed with formaldehyde. (A) Double immunofluorescence, performed with anticentrin Ab A and anti-FG Nup monoclonal Ab MAb414, is shown with the merged image at the right. The far right image is a contour trace of the individual Ab signals as a representative of a transfected cell. (B) Double immunofluorescence performed with anti-Iamin B and anti-FG Nup monoclonal Ab MAb414 is shown. ELYS RNAi disrupts centrin 2 at the nuclear rim, while leaving the nuclear lamina unaltered.

volved in the interaction with centrin 2. HeLa cells were transfected either with specific myc-tagged fragments of the Nup160 gene or with a full-length malate dehydrogenase gene as a control. After cells were transfected, they were lysed and subjected to immunoprecipitation with centrin Ab A. Both the myc-tagged C terminus of Nup160 (aa 912 to 1436) and the internal fragment of Nup160 (aa 317 to 697) coimmunoprecipitated with centrin 2 (Fig. 6A, lane 2). The Nup160 fragments may bind directly to centrin 2 or indirectly via a secondary protein. The negative control protein malate dehydrogenase did not immunoprecipitate with centrin 2 (Fig. 6A, lane 2). We conclude that centrin 2 biochemically interacts with at least two regions of human Nup160, and that the C-terminal region of Nup160 appears to be involved in the stronger interaction of the two, as shown by its distinct enrichment (Fig. 6A).

We asked whether overexpression of the strong centrininteracting Nup160 C terminus (aa 912 to 1436) had an effect on mRNA export. Plasmids containing the two different myctagged Nup160 fragments or the malate dehydrogenase were transfected into HeLa cells for 24 h, and poly(A)<sup>+</sup> RNA localization was monitored by hybridization with Cy3-oligo(dT)<sub>50</sub>. Successfully transfected cells were identified with FITC-labeled anti-myc Ab. The location of the poly(A)<sup>+</sup> RNA in the cells was determined with 500 transfected myc-positive cells per experiment and the experiment was done in triplicate. Quantitation of the results indicated that transfection of human cells with the negative control malate dehydrogenase gene inhibited mRNA export in only 2% of the myc-positive transfected cells (Fig. 6B). The expression of Nup160 aa 317 to 697, previously shown to inhibit mRNA export, led to the nuclear accumulation of  $poly(A)^+$  RNA, as expected, in 30% of the myc-positive transfected cells (Fig. 6B). Expression of the strong centrin-binding C terminus of Nup160 (aa 912 to 1436) had an even greater effect, inhibiting mRNA export in nearly 50% of the transfected cells (Fig. 6B). Sample images of the transfected human cells inhibited for mRNA export by the Nup160 fragments are shown in Fig. 6C (where red indicates poly(A)<sup>+</sup> RNA and green indicates myc transfection).

The transfection of *Xenopus* cultured cells, as opposed to human cell transfection, has historically proven difficult. Conditions that allowed the transfection of our myc-tagged constructs into XL177 cells were established (see Materials and Methods). Transfection of each of the human Nup160 fragments into *Xenopus* XL177 cells led to the nuclear accumulation of poly(A)<sup>+</sup> RNA (Fig. 6D). However, due to the low overall transfection efficiency of the XL177 cells, we were unable to precisely quantify and graph this effect. We conclude, however, from the human and *Xenopus* transfection results that the domains of Nup160 that interact with centrin 2 elicit a dominant-negative effect on mRNA export.

Approaching the role of centrin 2 in mRNA export more directly, we asked whether transfection of fragments of the human centrin 2 gene would have an effect on  $poly(A)^+$  RNA export. Centrin 2 protein has been shown by X-ray crystallog-raphy to have a dumbbell-shaped structure consisting of two domains, an N-terminal half containing two Ca<sup>2+</sup> binding EF-hand motifs and a C-terminal half also containing two Ca<sup>2+</sup> binding EF-hand motifs, with the individual halves separated by a helical linker (124). We created separate myc-tagged con-



FIG. 6. Fragments of Nup160 that interact with centrin 2 cause nuclear accumulation of poly(A) plus RNA. (A) Anticentrin Ab A coimmunoprecipitates Myc-tagged Nup160 C terminus (aa 912 to 1436) and Myc-tagged Nup160 aa 317 to 697 (lane 2, filled arrowheads) but not malate dehydrogenase (MD, lane 2, open arrowhead) from transfected HeLa cells. Cells were transfected with the indicated constructs for 24 h and then lysed and immunoprecipitated with anticentrin Ab A. Lane 1 indicates the amount of transfected protein produced. Lane 2 measures whether and how much of the protein is immunoprecipitated by the anticentrin Ab, as revealed by probing with an anti-MycA Ab. Differences in the transfection efficiency or the level of construct protein expression between the constructs were present (lane 1); however, in this experiment, the expression of Myc-Nup160 C terminus was at least as much as that of Myc-malate dehydrogenase (lane 1, compare Myc-MD to Myc-Nup160 C terminus). Immunoprecipitation with goat IgG serum was used as a negative control (lane 3). Lys indicates a lane with 10% of the input transfected HeLa cell lysate shown (lane 1); the remaining 90% was used for the anti-centrin 2 immunoprecipitation (lane 2). (B) Overexpression of Nup160 aa 317 to 697 or Nup160 C terminus (aa 912 to 1436) but not malate dehydrogenase causes nuclear accumulation of poly(A)<sup>+</sup> RNA. HeLa cells were transfected with the myc-tagged Nup160 fragments or malate dehydrogenase 24 h before the poly(A)<sup>+</sup> RNA accumulation assay was performed. Quantitation of nuclear poly(A)<sup>+</sup> RNA accumulation was done with 500 cells per experiment. The percentage of transfected HeLa cells with nuclear poly(A)<sup>+</sup> RNA accumulation was calculated in three independent experiments and averaged. (C) Typical views of HeLa cells successfully transfected with the myc-tagged Nup160 fragments or malate dehydrogenase are shown. The cells were transfected as described for panel B. Left panels show the expression of the myc-tagged constructs, using FITC-labeled myc Ab (green). The center panels are the same cells hybridized with Cy3-oligo(dT)<sub>50</sub> to show nuclear poly( $\hat{A}$ )<sup>+</sup> RNA accumulation (red). Right panels show the complete field of cells by 4',6'-diamidino-2phenylindole (DAPI) DNA staining. (D) Typical views of Xenopus XL177 cells successfully transfected, as described in Materials and Methods, with the myc-tagged Nup160 fragments or the malate dehydrogenase are shown. The cells were visualized as described for panel C.

structs, a human centrin 2 N-terminal half (aa 1 to 98) and a human centrin 2 C-terminal half (aa 94 to 172), based on the published structures of these domains (83, 139). We found that the expression of either half of the human centrin 2 led to nuclear poly(A)<sup>+</sup> RNA accumulation in ~20 to 25% of the transfected cells (Fig. 7A). In contrast, transfection of either of

two myc-tagged negative control genes, malate dehydrogenase (data not shown) or pyruvate kinase (Fig. 7A, PK-Myc), inhibited export in only 5% of transfected cells. The majority of cells transfected with the control constructs showed a largely cytoplasmic stain, indicating that mRNA had been exported. Sample images of the centrin 2-transfected cells with inhibition of



FIG. 7. Overexpression of the N- or C-terminal half of centrin 2 causes nuclear accumulation of  $poly(A)^+$  RNA in human cells. (A) Overexpression of the human centrin 2 N terminus or C terminus, but not pyruvate kinase, causes the nuclear accumulation of  $poly(A)^{+}$ RNA. HeLa cells were transfected with the indicated construct 24 h before the poly(A)<sup>+</sup> RNA accumulation assay was performed. Quantitation of nuclear  $poly(A)^+$  RNA accumulation in cells transfected with either of the two human centrin 2 fragments or the control pyruvate kinase gene was done with 500 cells per experiment. The percentage of transfected HeLa cells with nuclear poly(A)<sup>+</sup> RNA accumulation was calculated in three independent experiments and averaged. (B) Typical views of HeLa cells successfully transfected with the myc-tagged centrin fragments or pyruvate kinase control gene are shown. The cells were transfected as described for panel A. The left panels show expression of the myc-tagged constructs using FITC-labeled myc Ab (green). The center panels are the same cells hybridized with Cy3-oligo(dT)<sub>50</sub> to show nuclear  $poly(A)^+$  RNA accumulation (red). The right panels show the complete field of cells by DAPI DNA staining.

mRNA export are shown in Fig. 7B [red indicates  $poly(A)^+$  RNA and green indicates myc-transfected cells]. We conclude that the overexpression of either the N- or the C-terminal half of human centrin 2 inhibits vertebrate mRNA export in a dominant-negative manner.

We also determined the effect of centrin 2 depletion on mRNA export by using a specific siRNA oligo known to knock down centrin 2 (108) (see Fig. S4A to C in the supplemental material). Because centrin 2 RNAi is known to disrupt centriole duplication and lead to mitotic defects, we were only able to look for the potential effects of centrin 2 knockdown upon mRNA export in those cells which were not arrested in mitosis (without nuclei) or were multinucleate (as a result of inaccu-

rate exit from mitosis). This made approximately half the transfected cells available for  $poly(A)^+$  RNA assessment. Centrin 2 RNAi depletion led to  $poly(A)^+$  RNA accumulation in ~20% of this group of cells. We thus conclude that the over-expression of either the N- or C-terminal half of human centrin 2, as well as the depletion of the centrin 2 protein via RNAi, inhibits vertebrate mRNA export.

Dominant-negative fragments of centrin 2 block protein export but not import. Nuclear protein import and export can be measured with an NES/NLS-bearing protein construct, RGG, which contains the human immunodeficiency virus (HIV) protein REV with its NES, the ligand binding domain of the glucocorticoid receptor which contains a hormone-dependent NLS and GFP (44, 75). When transfected alone, the RGG protein is cytoplasmic until the introduction of dexamethasone, which induces its import into the nucleus (Fig. 8A). Upon removal of dexamethasone, the RGG construct protein is exported, which is presumed to occur through the Crm-1 export receptor. Cotransfection of the RGG construct with a control malate dehydrogenase gene had no effect on either RGG protein import or export (Fig. 8B to E). Cotransfection of RGG with either the Nup160 aa 317 to 697 or the Nup160 C terminus (aa 912 to 1436) also had no effect on nuclear RGG protein import or export induced by dexamethasone (Fig. 8B to E). Similarly, each half of centrin 2 showed no effect on RGG protein import (Fig. 8B and D). However, both centrin fragments exhibited a dominant-negative effect on RGG protein export (Fig. 8C and E). Specifically, the expression of either the N or C terminus of human centrin 2 led a block in RGG protein export in  $\sim 20$  to 30% of the transfected cells (Fig. 8E). siRNA depletion of centrin 2 led to a similar block, disrupting RGG protein export in 23 to 32% of depleted cells without mitotic defects, while leaving protein import intact (see Fig. S4D to F in the supplemental material). Strikingly, this effect on RGG protein export appears to mirror exactly the effect of the centrin halves on mRNA export (Fig. 7A). In this context, it is significant to the consideration of the mechanism of action to note that Rev NES protein export uses the receptor Crm-1, while mRNA export employs a different set of transport factors which include Tap1/Mex67 and associated proteins (6, 28, 33, 35, 54, 57, 68, 69, 117, 118). Thus, while the overexpression of fragments of Nup160 only affect mRNA export (Fig. 6 and 8) (128), our results support the fact that centrin 2 acts on both protein and mRNA export pathways.

# DISCUSSION

A new role for vertebrate centrin at the nuclear pore has been identified in this study. Despite the observation in 2000 that centrin was a structural component of the yeast nuclear pore (103) and the fact that its functional role was revealed later (31), the intervening years have never shown a hint that centrin has any connection to the vertebrate nuclear pore, either structurally or functionally. We have now observed the interaction between vertebrate centrin 2 and the critical Nup107-160 complex of the nuclear pore from *Xenopus* to human cells (Fig. 1 and 2). Indeed, although centrin 2 was previously visualized to be a centrosomal protein by immunofluorescence, we have found that centrin 2 colocalizes extensively with nuclear pores in human and *Xenopus* cultured cells,



FIG. 8. Overexpression of the N- or C-terminal half of centrin 2 blocks protein export but not import. (A) HeLa cells were transfected with the Rev-glucocorticoid ligand binding domain-GFP (RGG) plasmid for 16 h before the addition of dexamethasone (DEX) to induce RGG import (which occurred exactly as described in references 44, 75, and 128). (B) Overexpression of the human centrin 2 N terminus or C terminus, Nup160 as 317 to 697, Nup160 C terminus (aa 912 to 1436), or malate dehydrogenase, have no effect on RGG protein import. HeLa cells were

as well as with the pores of nuclei reconstituted in vitro in *Xenopus* egg extracts (Fig. 3 and 4). Although the association of centrin 2 with NPCs is readily apparent in Xenopus cells and in vitro-reconstituted nuclei, the substitution of digitonin for Triton X-100 is required to observe the NPC association in HeLa cells. Disruption of the nuclear pore targeting/assembly protein ELYS/MEL-28 by RNAi, which is known to lead to a loss of nuclear pores, causes a dramatic decrease in centrin 2 at the nuclear pore under conditions where the nuclear lamina remains unaltered (Fig. 5). Instead, in ELYS RNAi-treated cells, centrin 2 relocates to FG nucleoporin-containing cytoplasmic aggregates (annulate lamellae), demonstrating that centrin 2 interacts with both nuclear and cytoplasmic pore complexes (Fig. 5). We additionally identified a functional role for centrin 2 at the nuclear pore. Overexpression of either the N-terminal or C-terminal Ca2+ binding EF-hand domain of human centrin 2 leads to the nuclear accumulation of  $poly(A)^+$ RNA, consistent with a disruption of mRNA export (Fig. 7). This mRNA export defect mirrors the nuclear  $poly(A)^+$  RNA accumulation caused by overexpression of Nup160 fragments, which are either direct or indirect binding sites for centrin 2 (Fig. 6), and also mirrors the Cdc31 defect observed with yeast (31). In addition, overexpression of either half of human centrin 2 has a dominant-negative effect on RGG protein export but not its import (Fig. 8). Depletion of centrin 2 by RNAi causes similar disruptions of both mRNA and protein export (see Fig. S4 in the supplemental material). We conclude that centrin 2 interacts with a major subunit of the nuclear pore, exhibits a nuclear pore localization, and has a functional role in mRNA and protein export.

Centrins have long been associated structurally with the centrosome (105). Traditional immunofluorescence with human cells, using methanol or formaldehyde/Triton X-100, has previously revealed centrin 2 to be localized at the centrosome (29, 94, 108). RNAi experiments demonstrated that centrin 2 is required for centriole duplication (108). Moreover, a physical interaction between centrin 2 and the centrosomal protein Sfi-1, observed for both yeast and human cells, plays a key role in the regulation of centrosome structure and centriole duplication (59, 71, 82, 106). It is, thus, clear that centrins are an essential component of the vertebrate centrosome and yeast spindle pole body. A single higher eukaryotic, noncentrosomal nuclear function for centrin 2 was previously observed for the

XPC nucleotide excision repair complex, as described in the introduction (4, 72, 90, 92).

Our identification of vertebrate centrin 2 as a novel binding partner for the Nup107-160 complex in immunoprecipitations originally suggested a possible mitotic, centrosome-related role for the interaction. Indeed, the Nup107-160 complex moves to the kinetochores and spindle poles at mitosis (11, 25, 74, 93, 142), and we previously demonstrated that it is required for correct bipolar spindle assembly: immunodepletion of the Nup107-160 complex from *Xenopus* mitotic extracts results in severely defective spindle assembly in vitro (93).

Our finding that centrin 2 is present at the nuclear pore in interphase and is involved in mRNA and protein export now shows that centrin 2 has another important novel interphase function in vertebrates. This conclusion brings an unexpected but remarkable symmetry to the studies of yeast and vertebrate centrins. The single centrin of S. cerevisiae, Cdc31, is an essential component of the yeast centrosome equivalent, the spindle pole body, and an established member of the yeast nuclear pore complex (31, 53, 103, 113). Indeed, a functional role for yeast Cdc31 in mRNA export was identified; the expression of a mutant Cdc31 allele (Cdc31-151) or the absence of Cdc31 expression leads to nuclear accumulation of  $poly(A)^+$  RNA (31). Here, we find that the overexpression of either half of the human centrin 2 has a dominant-negative effect on mRNA export, in both human and Xenopus cultured cells (Fig. 7). Moreover, centrin 2 affects Crm-1 mediated protein export but not at least one major type of protein import (Fig. 8). Thus, our data indicate for the first time that vertebrate centrin 2 is at the nuclear pore and plays a role in mRNA and nuclear protein export during interphase.

In yeast, the function of Cdc31 in mRNA export appears to be mediated through the interaction with an mRNA export complex, the yeast Sac3-Thp1-Sus1 complex (31, 130). Specifically, Cdc31 interacts with yeast Sac3p, a 150-kDa protein (9, 31). Vertebrate centrin 2 might also possibly bind and/or function through an as yet undiscovered vertebrate Sac3-Thp1-Sus1 complex.

Three potential vertebrate homologues of Sac3 have been identified, GANP, MCM3AP, and Shd1 (1, 58). B-cell germinal center-associated protein (GANP) and MCM3 associated protein (MCM3AP) are derived from the same gene by alternative splicing (1). GANP is 210 kDa, 60 kDa of which has

cotransfected with the indicated constructs plus the RGG construct 16 h before the addition of dexamethasone to induce RGG protein import. Typical views of HeLa cells successfully transfected with the myc-tagged constructs and the RGG construct following induced protein import are shown. The top panels show expression of the myc-tagged constructs using tetramethyl rhodamine isocyanate-labeled myc Ab (red). The bottom panels are the same cells showing the location of the RGG protein via its GFP tag (green). Typically, the RGG protein targets the nucleoli within the nucleus (44, 75, 128) as observed here. (C) Overexpression of the human centrin 2 N terminus or C terminus, but not Nup160 aa 317 to 697 or C terminus (aa 912 to 1436) and malate dehydrogenase, blocks RGG protein export. Cells were transfected and treated with dexamethasone as described for panel B. Following the RGG protein import, the cells were switched to fresh medium lacking dexamethasone for 2 h to induce RGG protein export. Typical views of HeLa cells successfully transfected with the myc-tagged proteins and RGG construct following induced protein export. Typical views of HeLa cells successfully transfected with the myc-tagged proteins and RGG construct following induced protein export. Typical views of HeLa cells successfully transfected with the myc-tagged proteins and RGG construct following induced protein export. (D) Quantitation of the RGG protein import in cells cotransfected cells, 24% were inhibited for RGG export by the N-terminal half of centrin 2 and 22% by the C-terminal half of centrin 2, while only 5% of control malate dehydrogenase transfected cells showed inhibition of export. (D) Quantitation of the RGG protein import in cells cotransfected with the human centrin 2 N terminus or C terminus, Nup160 aa 317 to 697 or C terminus (aa 912 to 1436), or malate dehydrogenase is shown. The cells were transfected as described for panel B. The percentage of transfected HeLa cells with blocked RGG protein import was calculated in three ind

23% homology with a region of yeast Sac3 (aa 129 to 803). This region of yeast Sac3 contains its centrin-interaction domain (1, 60, 61). GANP also has several N-terminal degenerate FG motifs (32), a motif found almost exclusively in nucleoporins. However, the large GANP protein and its smaller alternatively spliced C-terminal isoform, MCM3AP, contain other non-Sac3 domains, including a GCN5-related N-acetyltransferase domain and a DNA primase activity, both of which are required for their function in DNA replication (60, 61, 121–123).

The third vertebrate relative of Sac3p, the Sac3 homology domain protein 1 (SHD1) is one-third the size of yeast Sac3p and lacks the yeast-centrin interaction domain (58). However, SHD1 localizes to centrosomes, and RNAi of SHD1 causes abnormalities in centrosome duplication and spindle formation (58). Thus, while both Shd1 and GANP/MCM3AP contain similarities to yeast Sac3, they contain unrelated additional domains. We cannot rule in or out a role for these proteins at the nuclear pore in mRNA export. As yet they have no direct connection to vertebrate centrins.

Our work identifies interactions between centrin 2 and several major nucleoporins involved in mRNA export. In addition to the Nup107-160 complex, we observed centrin 2 interacting with the FG nucleoporin Nup153 (Fig. 2). Interestingly, yeast Sac3 is known to bind to the yeast FG nucleoporin Nup1, a distant homologue of Nup153 (31, 32, 55, 67). Our observed interaction of vertebrate centrin 2 with Nup153 may be direct or indirect and involve a vertebrate Sac3 equivalent, such as Shd1. Alternatively, the interaction between vertebrate centrin 2 and Nup153 may use the Nup107-Nup160 complex as an intermediary, since we have shown this complex binds both centrin 2 and Nup153 (Fig. 2) (128, 132).

Structurally, the major domains of centrins are comprised of  $Ca^{2+}$  binding EF-hand motifs. It has been observed that mutations that specifically affect the calcium binding of yeast Cdc31 cause nuclear accumulation of poly(A)<sup>+</sup> RNA (31). Other actions of centrins are also altered by calcium. The affinity of Cdc31 for its spindle pole body partner, Kar1, increases 10-fold with calcium and affects its role in spindle pole body duplication (37). The affinity of human centrin 2 for XPC similarly increases 28-fold in the presence of calcium, although how this affects XPC activity has not yet been determined (92, 97).

Calcium has been implicated separately as a possible regulator of the nuclear pore in a handful of studies (see references 26 and 125 and references therein). For example, atomic force microscopy observations showed structural changes in the pore with calcium addition, described as a calcium-induced, iris-like opening of the nuclear basket ring, the first pore structure encountered by proteins and mRNAs before export (116). Specific changes in nucleoporin localization include the FG domain of Nup153, which changes its position within the pore with the addition of 2 mM Ca<sup>2+</sup> (95), a Ca<sup>2+</sup> change comparable to that of typical calcium flux from the endoplasmic reticulum (ER).

Calcium changes have been observed to influence not only nuclear pore structure but also pore function. Thapsigargin, which depletes the ER calcium stores, is seen to disrupt passive diffusion and nuclear transport through the vertebrate NPC in living cells (41, 64). Because the integral membrane pore protein, gp210, contains luminal calcium-binding motifs, and the expression of an Ab to this portion of gp210 within the ER lumen inhibits both passive diffusion and signal-mediated transport, one hypothesis has been that gp210 could be a calcium sensor that triggers such conformational NPC changes (26, 42). However, our identification of centrin 2 at the vertebrate nuclear pore introduces a potential more centrally located calcium sensor for the NPC.

In summary, we conclude that a population of vertebrate centrin 2 interacts with nuclear pore subcomplexes, localizes to the nuclear pore, and plays a role in both mRNA and protein export. Thus, we have identified a new and distinct interphase function for vertebrate centrin 2. Possible interaction of centrin 2 in an mRNA export complex, such as the yeast Sac3-Thp1-Sus1-Cdc31 complex, which is as yet undiscovered in vertebrates, may be of future interest. In addition, the ability of centrin 2 to bind calcium provides interesting potential roles for this protein as a regulatory or structural component of the nuclear pore.

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