Rediscovery of the nucleolinus, a dynamic RNA-rich organelle associated with the nucleolus, spindle, and centrosomes

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The nucleolinus is an RNA-rich compartment, closely apposed to or embedded within the nucleolus. Discovered over 150 y ago, fewer than two dozen articles have been published on the nucleolinus, probably because complex histochemical stains are required for its visualization in the great majority of cells. The nucleolinus has been reported in invertebrate oocytes, mammalian and amphibian epithelial cells, neurons, and several transformed cell lines. A prominent nucleolinus, clearly visible with transmitted light microscopes at 10× magnification, is present in each oocyte of the surf clam, *Spisula solidissima*. We observed a consistent relationship between the nucleolinus and the developing meiotic apparatus following *Spisula* oocyte activation. Through sonication and sucrose gradient fractionation of purified oocyte nuclei, we isolated nucleolini, extracted their RNA, and prepared an in situ riboprobe (NLi-1), which is associated specifically with the nucleolinus, confirming its unique composition. Other in situ observations revealed a NLi-1 and nucleolinar association with the developing spindle and centrosomes. Laser microsurgery that targeted the nucleolinus resulted in failed meiotic cell division in parthenogenetically activated oocytes and failed mitosis in fertilized oocytes. Although the nucleolinus may be a forgotten organelle, its demonstrated role in spindle formation suggests it deserves renewed attention.

cell division | mitosis | meiosis

The nucleolinus was described by Montgomery in eggs of the nudibranch mollusk, *Montagua*, in his 1898 monograph, "Comparative Cytological Studies, with Especial Regard to the Morphology of the Nucleolus" (1). As the title indicates, the study focuses on the appearance and behavior of the nucleolus. In it, Montgomery presents his own thoughts on the significance of the nucleolinus as well as the observations of other investigators, including Louis Agassiz, over the previous 40 y (2–4). These range from the attachment of no morphological significance to the structure to suggestions that the nucleolinus is a microorganism enclosed in the nucleus and a direct morphological progenitor of the centrosome. Twenty-two years later, in his "Observations on an Intra-Nucleolar Body," Carleton experimented with Cajal's formol-silver nitrate technique to visualize the nucleolinus in cat and frog intestinal epithelial cells (5). In some cases, he reported, the nucleolinus was the only silver-impregnated structure within the entire nucleus. As of 90 y ago, then, the nucleolinus was clearly distinguished from the surrounding or adjacent nucleolus, but only via histochemistry. Carleton, on the basis of morphological observations of fixed, sectioned tissues, suggested that the nucleolinus divided into two during prophase. Allen (6, 7) subsequently proposed a "spindle forming role" for the nucleolinus, on the basis of his observations of live *Spisula* oocytes, but no photomicrographs or other data were presented to directly support this hypothesis.

The most comprehensive analysis of the nucleolinus appears in a series of reports by Love and colleagues from the late 1950s to the early 1970s (8–14). Love and Liles developed a method for the differentiation of nucleoprotein complexes by deamination and staining with a combination of toluidine blue and ammonium molybdate (8). Using a variety of normal and tumorigenic mammalian cells, histochemical studies showed that the nucleolinus stained metachromatically against the uniformly green background of the nucleolus. The authors observed that the metachromatic compartment increased significantly during prophase and then disappeared through anaphase and telophase. Love and Wildy later reported that the first detectable abnormality in herpes virus-infected HeLa cells was an enlargement of nucleolini followed by their extrusion from the nucleolus into the nucleoplasm (9). In Ehrlich ascites and other cells, changes in nucleolini were correlated with perturbations in cell division (9). As did Allen (6, 7), the authors speculated on a functional relationship between the nucleolinus and cell division. Love (10) reported that changes in nucleolinar morphology occur in aneuploid and virally transformed cells, and Mironescue et al. (15) observed similar results in rat liver cells after administration of liver carcinogens. A few other reports discussing the nucleolinus can be found in the literature, but for the most part the organelle is mentioned only in passing (16–18).

The nucleolinus as described by Carleton, Love, Allen, and others has thus been identified in many cell types and species. In mammals alone, the list includes corneal and gut epithelium, sympathetic neurons, amnion, liver, kidney, pancreas, and more. Yet, our lack of knowledge regarding nucleolinar composition (other than to say it contains RNA and no detectable DNA, ref. 13) and function make it impossible to know what role(s) the organelle plays in cell physiology. An understanding of nucleolinar dynamics, bioactivity, and relationship to other nuclear bodies would likely have fundamental impact on cell biology. With this report, we introduce a unique specific molecular marker for the nucleolinus, test the long-standing hypothesis that it functions in cell division, and aim to reinvigorate the study of this little known and enigmatic organelle.

Results

The Nucleolinus Is Spatially Related to the Forming Spindle Axis and Pole. Mature *Spisula* oocytes are arrested in prophase I of meiosis. They contain a large tetraploid germinal vesicle (nucleus) and a prominent nucleolus (Fig. 1). Closely apposed to the nucleolus is a prominent nucleolinus. There are no centrosomes in unactivated oocytes; neither centrosomes, spindle, nor microtubules are detectable by light, immunofluorescence, or electron microscopy until several minutes *after* activation (19). When

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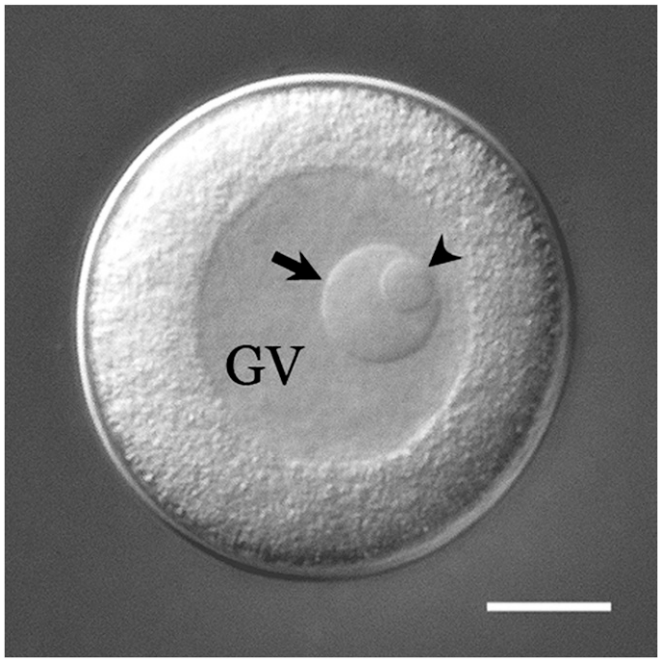

Fig. 1. DIC image of the nucleolinus within an unactivated *Spisula* oocyte. A large tetraploid nucleus (germinal vesicle, GV) is present in the unactivated oocyte, within which lie a prominent, spherical nucleolus (arrow) and nucleolinus (arrowhead). The nuclear envelope begins to disintegrate within the first 2 min of activation and is indistinguishable by 10–12 min postactivation. The nucleolus becomes morphologically indistinct within approximately 5 min postactivation, and the nucleolinus persists for several minutes beyond that time (see Figs. 2 and 3 for examples). (Scale bar, 15 μm.)

meiosis is resumed after fertilization or parthenogenetic activation, the nuclear envelope breaks down and the nucleolus becomes morphologically indistinct. The nucleolinus persists as a discrete structure for several minutes after dissipation of the nucleolus, and then, in agreement with observations on somatic cells (8), eventually becomes undetectable by phase contrast or differential interference contrast (DIC) microscopy before meiosis I spindle assembly (Figs. 2 and 3). During this process, centrosomes are thought to form de novo in the oocyte. To effect unequal cleavage for polar body formation, the spindle is positioned eccentrically in the oocyte cytoplasm. One spindle pole and centrosome come to lie in the oocyte interior, and the second spindle pole and centrosome migrate to the cell periphery, just beneath the plasma membrane. The point at which the polar bodies are ejected marks the first cleavage plane in *Spisula* and the animal pole of the embryo for many mollusks.

We tracked the nucleolinus after oocyte activation by time lapse DIC microscopy and consistently observed that its last visible position in the cytoplasm coincided with the interior spindle pole (Fig. 2 and Movie S1). Because its movement after activation usually consisted of only localized oscillations, spindle position and the eventual site of polar body extrusion could be approximated by the position of the nucleolinus before oocyte activation. However, in some instances the nucleolinus was transported over striking distances in a brief time. Fig. 3 and Movie S2 show an example in which the nucleolinus was translocated over 12 μm in less than 1 min. Again, it was the last visible position of the nucleolinus and not its starting point that correlated with spindle location. Although results such as these are only correlative, they nevertheless provide a unique, living view of nucleolinar dynamics. Taken together with descriptions made by earlier investigators (6, 7), they support the hypothesis that the nucleolinus is spatially associated with the developing spindle and spindle pole.

The Nucleolinus Is a Unique Cellular Compartment Containing Centrosome- and Spindle-Associated RNAs. It has been reported that specific RNAs (cnRNAs) are associated with centrosomes (20–22). In *Spisula* oocytes, most of these are not detectable before oocyte activation, but several were found to be present in unactivated oocytes as a distinct hybridization patch associated with the nucleolinus (21). As the nuclear envelope breaks down, the newly formed centrosomes are found to be embedded within, or closely apposed to, the nucleolinar patch. We now report on NLi-1 RNA, a specific molecule uniquely isolated from and localized to the nucleolinus in situ.

Nucleolinar RNA was extracted from isolated nucleolini obtained in a two-step process described in detail in *Materials and Methods*. Importantly, the first step is isolation of intact nuclei, which eliminates a large proportion of cytoplasmic contamination. At this stage of the preparation there is virtually no debris, and the only structures visible by DIC microscopy are the nuclear envelope, within which lie chromosomes and intact nucleolini (Fig. S1 *A* and *B*). The nucleolus dissipates early in the process, during initial cell lysis. After mechanical disruption of isolated nuclei, the highly dense nucleolini are isolated through several sucrose gradient steps and washes. The final preparation contains intact nucleolini and a small amount of debris which, by their appearance and comparison with intermediate steps in the preparation, are likely to be damaged nucleolini (Fig. S1*C*).

NLi-1 is a >1-kb contig assembled from isolated, cloned nucleolinar RNA (Fig. S2; GenBank accession HM004235). The contig comprised over 50% of all nonribosomal clones sequenced. NLi-1 contains a potential 555 nt ORF encoding a highly basic polypeptide of 184 amino acids with a predicted molecular mass

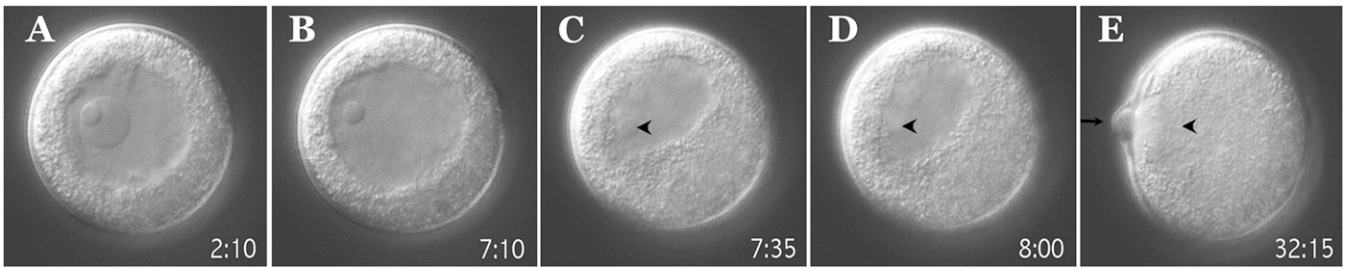

Fig. 2. Spatial relationship of the nucleolinus to the developing spindle and pole in a living oocyte. (*A*) At approximately 2 min postactivation, the nucleus (germinal vesicle, GV), already appearing ragged around the edges, the nucleolus, and nucleolinus are all clearly visible. (*B*) By 7 min the nucleolus is no longer visible, but the nucleolinus still persists as a well-defined structure. (*C* and *D*) The disintegration of the nucleolinus (arrowhead) is traced through to its last visible position as cytoplasm continues to infiltrate the central area of the oocyte. During this time the nucleolinus oscillates slightly in all three planes, but remains in the same vicinity despite significant cytoplasmic rotation (Movie S1). (*E*) The emerging polar body (arrow) together with the site mark for the nucleolinus (arrowhead) delineate the axis and poles of the first meiotic spindle. Elapsed time in minutes and seconds is indicated in each frame.

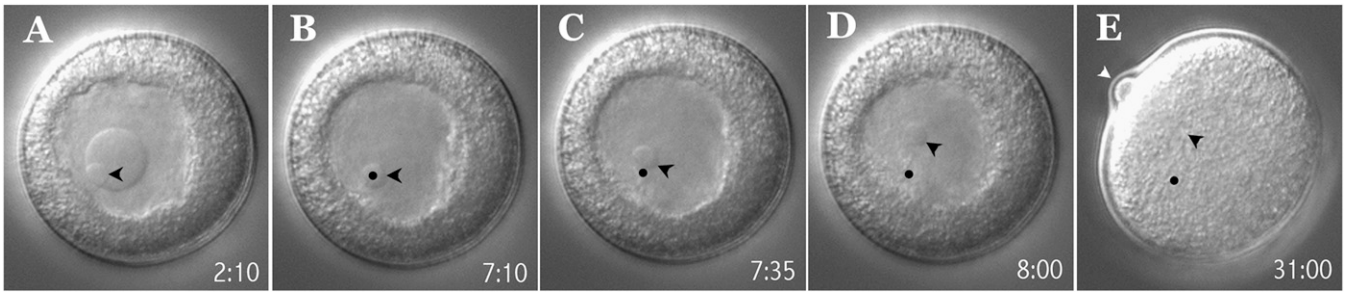

Fig. 3. Transport of the nucleolinus through the cytoplasm. In this case the nucleolinus (black arrowhead) was transported from its original position (marked by the black dot in *B–E*) and, along with the emerging polar body (white arrowhead in *E*), delineate the long axis of the spindle. During the 50-s time span shown in *B–D*, the nucleolinus moved over 12 μm (>0.24 μm/s) through the cytoplasm. This series of time frames is taken from Movie S2.

of 20 kDa and a pI of 10.5. It is unclear at present whether this gene is translated into protein in oocytes. No significant conserved domains or orthologs were found in nucleic acid or protein databases. The apparent lack of homology is not a function of *Spisula* divergence or under-representation of mollusk entries in sequence databases, because BLAST analysis of other *Spisula* sequences readily reveals orthologs across a broad spectrum of phylogenetic groups (20). It is also important to highlight the absence of similarity to rRNA. There are a number of database entries for *Spisula* rRNAs as well as rRNAs derived from other mollusks. The lack of identity in our analysis, at even low stringency, indicates that NLi-1 is not rRNA.

In the unactivated oocyte, NLi-1 is exclusively localized to the nucleolinus (Fig. 4*A*). There is an absence of label from all other cytoplasmic and nuclear structures including the nucleolus and chromosomes. This is in firm agreement with histochemical studies suggesting the nucleolinus is a distinct cellular compartment (5, 8). Following oocyte activation, as the nucleolinus begins to dissipate and centrosomes are formed, NLi-1 is seen as a discrete, although slightly more diffuse patch in the cytoplasm (Fig. 4*B*). Centrosomes are often seen to be resting upon (or sometimes encased within) the NLi-1 hybridization patch or in its immediate proximity. Slightly later, at 8–10 min postactivation, NLi-1 often appears as a narrow streak coinciding with the developing spindle axis. By 10 min postactivation, the developing spindle is situated within the expanded NLi-1 zone, giving the impression of an NLi-1 spindle matrix (Fig. 4*C*). The identification of a specific nucleolinar RNA intimately associated with the developing spindle serves as a molecular correlate of the time-lapse images obtained from living oocytes and summons the idea posed by early investigators that the nucleolinus functions in spindle formation and/or cell division. We next performed a series of laser microsurgery experiments to directly test this long-standing hypothesis.

The Nucleolinus Is Required for Normal Cell Division. The effect of laser microsurgery on the nucleolinus was examined by DIC microscopy to determine whether this disrupts meiosis. We use the term "targeted" and not "ablated" because, although clear cell division defects (described below) and a visible welt were produced, gross nucleolinar structure was not destroyed in these experiments. Cells were labeled with bisbenzimide and observed by fluorescence microscopy to track the fate of chromatin. Oocytes in which the nucleolus was targeted, approximately one nucleolinus diameter (~5 μm) away from the experimental target site, were used as controls. In addition, nonlasered oocytes were used as controls to visualize normal meiotic progression and also as event timers (i.e., cells were fixed at the time nonlasered cells exhibited polar bodies).

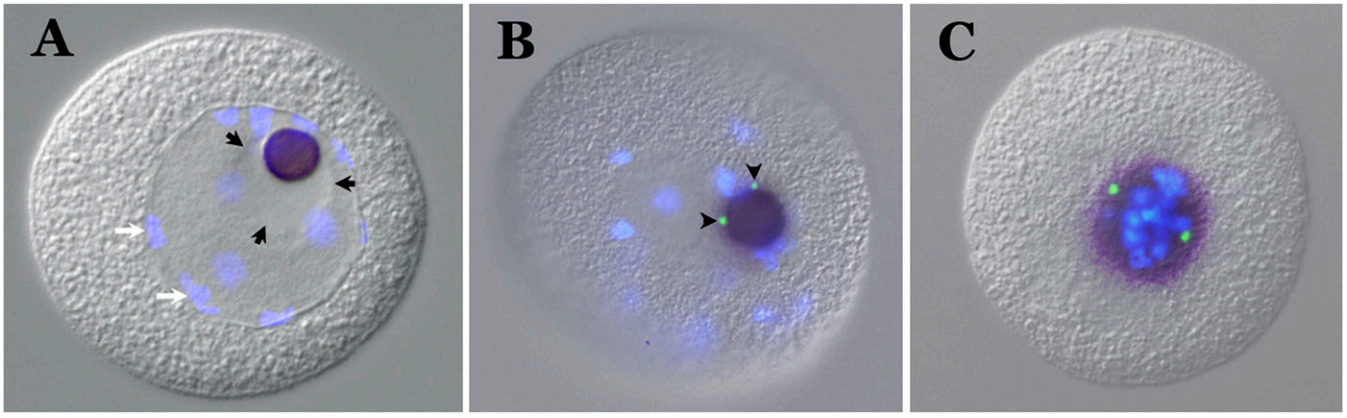

Fig. 4. In situ localization of NLi-1 RNA. (*A*, unactivated oocyte) The nucleolinus labels intensely with the NLi-1 RNA probe (purple). The borders of the nucleolus are difficult to distinguish after the 3-d hybridization regime, and are therefore highlighted with black arrows. Chromosomes are visualized with bisbenzimide (smaller, pale-blue structures, two of which are labeled with white arrows). Observations on nonhybridized cells labeled with bisbenzimide and hybridized cells not labeled with bisbenzimide confirm that there is no overlap between these two compartments. That is, nucleolini contain no detectable DNA [as reported earlier by Love and Walsh (13)] and chromosomes contain no detectable NLi-1 signal. As the nucleolinus begins to "dissolve" at 6 min postactivation (*B*), a strong but slightly more diffuse hybridization patch remains in the oocyte. γ-Tubulin immunofluorescence in the same cell (green) reveals the newly formed centrosomes (arrowheads) closely apposed to the nucleolinus. By 12 min postactivation (*C*), the entire cell division apparatus is associated with an NLi-1 "matrix." Spindle microtubules are present at this time, but cannot be visualized in these oocytes due to lability of α-tubulin antigenicity following the hybridization regimen (including 3 d at 60 °C). (*A–C*) Overlays of black and white DIC images with color brightfield images (alkaline phosphatase staining) and immunofluorescence. "Sense" probes were used for negative controls in all in situ hybridizations and were completely devoid of label.

Details of these experiments are provided in *Materials and Methods*. The results show a significant disparity between experimental and control groups (Fig. 5 *A–C*). Only 19% of oocytes in which the nucleolinus was targeted successfully completed meiosis. Interestingly, a single small membrane bleb the size of a normal polar body was sometimes observed in these oocytes, but this "polar body" was devoid of chromatin. We do not know whether these blebs were the result of aborted cytokineses or whether they were an experimental artifact. As will be noted below, the spindle in these oocytes was often grossly disorganized, so we could not determine with confidence any spatial relationship or polarity in relation to these membrane blebs. In nucleolus-targeted (control) cells, a chromatin-containing polar body of normal appearance was evident in 91% of cases.

In subsequent experiments, oocytes were fixed at different times after laser microsurgery and KCl activation to determine the morphological basis of the cell division defect. Samples were stained with anti-γ tubulin antibody to assess the presence of centrosomes, anti-α tubulin to examine spindle morphology, and bisbenzimide to visualize chromosome congression and segregation. The effects of microsurgery were multiple and manifested primarily at later time points (Fig. 5 *D–K*). Approximately two-thirds of cases involved gross malformation of the meiotic figure. Chromatin often appeared as a disorganized cluster associated with a tangle of microtubules. In other cases the meiotic spindle appeared somewhat normal in shape, but was diminished in size and microtubule content as judged by anti-tubulin immunofluorescence. In a few cases progression through meiosis appeared to be arrested in metaphase at times when control cells had advanced from late anaphase through telophase and cytokinesis. It is possible that this range of results is due to slight variability in laser targeting, but we consider it more likely the results are due to real, pleiotropic effects of nucleolinar perturbation. At earlier time points the differences between control and experimental groups were subtle and difficult to substantiate; however, supernumerary and sometimes smaller, less intensely stained centrosomes were observed.

Unperturbed, parthenogenetically activated oocytes complete meiosis but do not enter mitosis. By fertilizing oocytes rather than activating with KCl, we could address the questions of whether meiotic spindle organization could be restored by sperm-derived elements and whether laser microsurgery on the nucleolinus had effects upon mitosis as well as meiosis. The results were clear in addressing both questions. First, we found that oocytes laser targeted on the nucleolinus and then fertilized were able to proceed through meiosis I and II (Fig. 6). This occurred in eight of eight cases. However, after completing meiosis, zero of eight of these zygotes formed mitotic centrosomes or spindles. In controls (nucleolar targeted), eight of nine oocytes contained a normal complement of centrosomes and formed a mitotic spindle. In total, the results reported here indicate that the nucleolinus is required for progression through meiosis in activated oocytes. When fertilized with sperm, however, elements of the nucleolinus are not required for meiosis, but are required for entry into the subsequent rounds of mitosis.

Discussion

On the basis of morphological observations of nucleolinar dynamics, investigators spanning the years between the 1870s and 1970s proposed a role for this structure in cell division. Some reported centrosome-like behavior of the nucleolinus, including self-divisional properties, disregulation related to aneuploidy, and proximity to the spindle pole. The more recent discovery that some RNAs later found in centrosomes localize to the nucleolinus *before* centrosomes assemble suggested that it may even serve as a cytological precursor of the division center (21), a view first expounded over a century ago. As quoted from Montgomery (1): "Lavdovsky (4) concludes that the nucleolinus

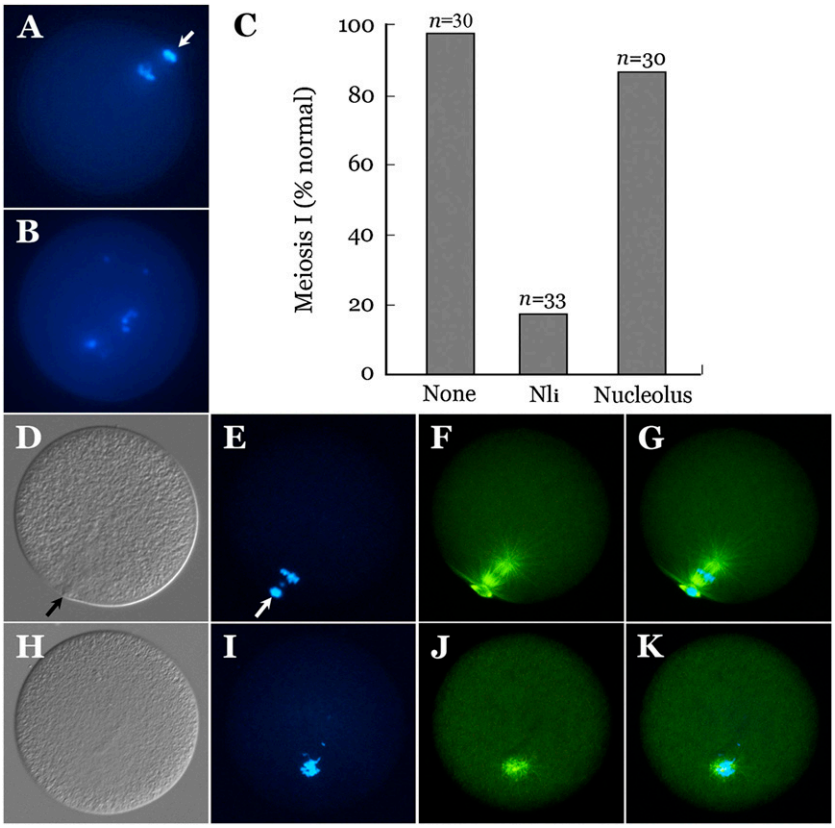

Fig. 5. Laser ablation of the nucleolinus results in failed meiotic cell division. (*A*) Example of successful meiosis I in a control (nucleolar targeted) oocyte fixed at 31 min postactivation and labeled with bisbenzimide. Polar body chromatin is clearly observed on the cell periphery (arrow), segregated from the remaining chromatin of the 2° oocyte. (*B*) Oocyte targeted at the nucleolinus and fixed 31 min postactivation. In this case, chromatin is scattered in the oocyte cytoplasm, but in other examples (*I*) remained clustered. (*C*) Percentage of cells determined to successfully complete meiosis I (using images such as these) following laser ablation of the nucleolinus (Nli) compared with those receiving no laser treatment (non) or control cells in which the nucleolus was targeted. (*D–K*) Oocytes that have been double labeled with bisbenzimide (blue) and anti-α-tubulin (green) to reveal spindle configuration in control (*D–G*) and nucleolinus-targeted (*H–K*) oocytes. Arrows in *D* (DIC) and *E* (bisbenzimide) indicate the newly formed first polar body. *F* (anti-tubulin) and *G* (overlay of *E* and *F*) show a normal metaphase II configuration. In the nucleolinar-targeted cell shown in *H–K*, clustered chromatin is associated with a disorganized knot of microtubules. No polar body is evident.

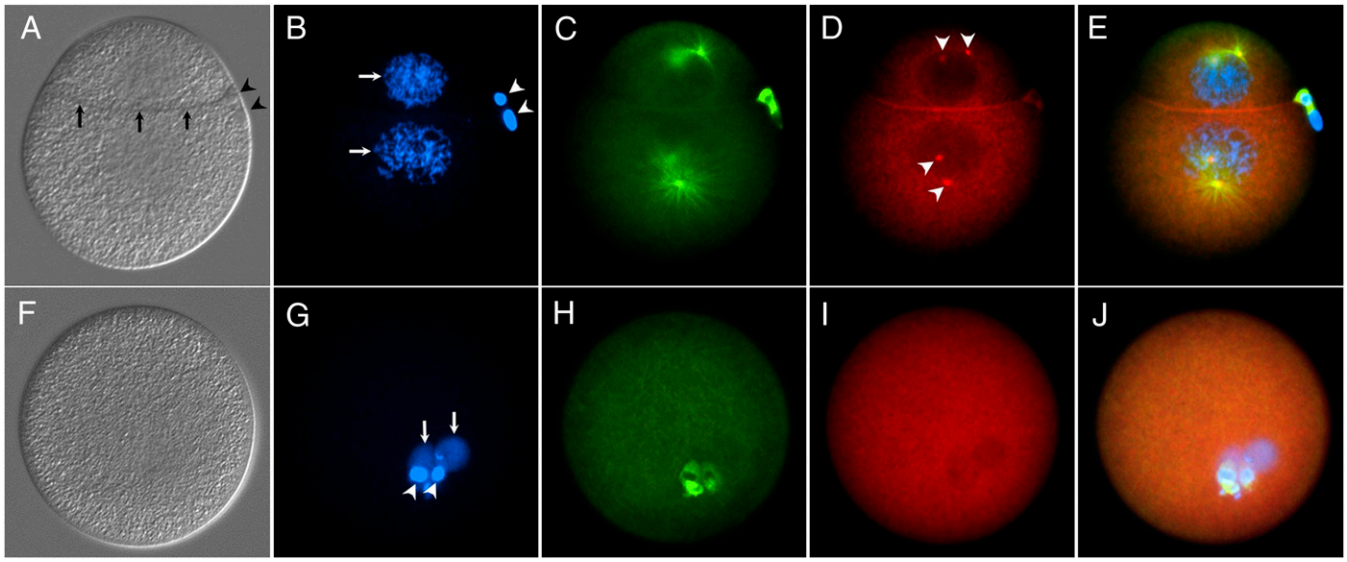

Fig. 6. Mitosis failure in fertilized zygotes following laser microsurgery on the nucleolinus. Oocytes in which nucleolini were laser targeted and then fertilized (vs. KCl activated, as in the previous set of experiments) were able to complete meiosis; sperm components were apparently able to substitute for damaged maternal-nucleolinar components. However, pronuclear fusion and mitotic spindle formation that normally follow polar body ejection were inhibited. (*A–E*) Control zygote. (*F–J*) Zygote targeted on the nucleolinus. (*A*) DIC image showing polar bodies (arrowheads) and a full cleavage furrow (arrows), indicating completion of meiosis and first mitosis. Cleavage does not occur in the nucleolinar-targeted zygote (*F*). (*B* and *G*) Bisbenzimide staining reveals polar bodies (arrowheads) in both control and experimentals, although the latter are not apparent in the DIC image in *F*. Daughter cell nuclei (arrows in *B*) can be seen in control zygotes, small, unfused male and female pronuclei appear to persist in experimentals (arrows in *G*). Anti-α-tubulin staining (green) reveals the developing asters and spindle of second cleavage in controls (*C*), but not in experimentals. Likewise, centrosomes are readily apparent in controls labeled with anti-γ-tubulin (*D*, arrowheads), but are absent in nucleolinar-targeted zygotes (*I*). (*E* and *J*) Composite fluorescence images of *B–D* and *G–I*, respectively.

is the centrosome in the process of formation, but he failed to observe the steps by which the body develops into a centrosome." Although it seems clear that the nucleolinus provides a component or components critical for the normal function of the centrosomes and spindle apparatus, its role is likely more complicated than the straightforward progenitor–progeny relationship proposed by Lavdowsky (4). Otherwise, laser targeting of the nucleolinus should have resulted in the absence of mitotic, as well as meiotic centrosomes.

Also attesting to the complexity of nucleolinar function is the observation that laser microsurgery had no effect on meiosis after fertilization, but still affected the subsequent mitosis (Fig. 6). This is intriquing, and may have bearing on the differences between de novo and replicative modes of centrosome formation, at least in the zygote. Several models may explain this result, but one that is consistent with our observations and others on the early development of *Spisula* is that some components required for biogenesis of meiotic centrosomes are present in the cytoplasm of unactivated oocytes, and others are located in the nucleolinus. Thus, when we lasered the nucleolinus and activated oocytes with KCl, γ-tubulin–containing foci (centrosomes) formed, but apparently did not function to form a spindle. Fertilization, however, allowed sperm-derived elements to either replace maternally derived centrosomes wholesale or substitute for nucleolinar elements that normally complement the cytoplasmic components. Because laser-treated, fertilized oocytes complete meiosis but cannot subsequently undergo mitosis, either (*i*) sperm possess enough of these putative complementing elements for only one round of cell division, (*ii*) sperm-derived complementing elements still require nucleolinar input to function through multiple cell cycles, or (*iii*) they are suppressed by maternal factors normally present until the end of meiosis. This latter possibility is supported by the fact that, following meiosis, the original (meiotic) centrosomes in *Spisula* zygotes are suppressed and the job of organizing subsequent cell divisions is transferred to the replication-competent sperm-derived microtubule organizing center (MTOC) (23).

Whether the nucleolinus should be considered an organelle in its own right must be evaluated using a number of criteria, including structural and functional differentiation from the nucleolus and its ubiquity in cells and across species. It may be closely apposed to, or in some cases embedded within the nucleolus, but our observations lead us to agree with Montgomery (1), Carleton (5), and others (8–15) that it is certainly a unique cellular compartment. Supporting this are behavioral, molecular, and functional data: (*i*) In meiosis as well as during cell fractionation, the nucleolinus persists as a distinct morphological entity after complete dissolution of the nucleolus transpires; (*ii*) the nucleolinus contains molecules (minimally, a subset of cnRNAs, ref. 21, and NLi-1 RNA) that are completely excluded from the nucleolus; and (*iii*) on the basis of our laser irradiation experiments in which the nucleolinus or nucleolus was targeted, the nucleolinus plays a role in cell division that is experimentally separable from nucleolar function (damaging the nucleolinus had profound effects on the cell division apparatus; damaging the nucleolus did not). This is an important point in light of studies indicating the nucleolus, beyond its well-known metabolic functions, plays a direct role in centrosome biogenesis and cell cycle regulation (24, 25). It is possible that cell division defects induced in the work of Gaulden and Perry (24) and Ugrinova et al. (25) are actually attributable to the nucleolinus. The inability to detect its presence in these and most other cell models has certainly left it unaccounted for in the interpretation of past results. Regardless of any role the nucleolus may play in cell cycle regulation, the functional relationship between the nucleolinus and cell division, speculated upon for more than a century, is presently confirmed. To know the precise physiological role of the nucleolinus will require a more thorough knowledge of the nature and fate of its macromolecular components, an area we are only beginning to explore.

Materials and Methods

Gravid *Spisula solidissima* were obtained from the Marine Resources Center at the Marine Biological Laboratory. Gametes were collected by dissection.

Oocytes were rinsed several times in 0.45-μm filtered sea water and activated with 0.14 volumes of 0.5 M KCl. All brightfield and immunofluorescence imaging was performed with a Nikon 90i microscope equipped with DIC, epifluorescence, and a Photometrics CoolSNAP fx digital camera. Anti α- and γ-tubulin immunofluorescence (for microtubules and centrosomes, respectively) were performed as described in an earlier report (20).

Isolation and Cloning of NLi-1 RNA. Nucleolini were isolated in a two-phase process. The first phase is isolation of oocyte nuclei on the basis of methods described by Maul and Avdalovic (26). Starting with 8–10 mL of packed, unactivated oocytes distributed between four 50-mL conical centrifuge tubes, the cells were resuspended by addition of 20 mL glycerol phosphate buffer (1 M glycerol, 20 mM $NaPO_4$, pH 8.0), incubated at room temperature for 3 min, and sedimented at 1,750 × *g* for 15 s. The oocytes were resuspended to 40 mL in ice cold lysis buffer (0.5 M hexylene glycol, 0.75 mM $MgCl_2$, 1 mM hepes, pH 7.5) and incubated at room temperature with occasional mixing by inversion. The oocytes were monitored until complete lysis of cells, leaving only intact nuclei. Unlike the nucleolinus, the nucleolus is unstable in lysis buffer and rapidly dissolves. Nuclei were stabilized by addition of 8 mL ice cold 40% sucrose with gentle mixing, and the tubes were placed on ice. The lysate was underlaid with 1.5 mL 10% sucrose buffer (equal parts lysis buffer and 20% sucrose) and centrifuged for 8 min at 780 × *g*, 4 °C, in a swinging bucket rotor. The resultant soft, white pellet was collected by pipetting into a microcentrifuge tube, frozen in liquid nitrogen, and stored at −80 °C for later use.

Nucleolini were then harvested from isolated nuclei. Eight frozen nuclear pellets were thawed on ice, resuspended with 1 mL 10% sucrose buffer each, pooled, divided into two 15-mL conical centrifuge tubes, and disrupted by sonication on ice with three bursts of 5 s each at 11 W with a microprobe sonicator. The samples were underlaid with 2 mL 40% sucrose buffer (40% sucrose in lysis buffer) and centrifuged for 10 min at 500 × *g*. The resulting pellet was washed twice by resuspension in ice cold PBS and sedimentation in a microcentrifuge for 1 min at maximum speed. RNA was extracted using Qiagen RNeasy reagents, reverse transcribed using universal primers described previously (20), and blunt-cloned into Invitrogen PCR-script plasmid. Over 3,500 clones have been sequenced to date, ≈10% of which are nonribosomal. Characterization of isolated nucleolini and complete analysis of all potential nucleolinar RNAs and proteins is ongoing.

In Situ Hybridization. Methods for in situ hybridization and immunofluorescence were similar to those described in an earlier report (20) with an important modification. In the present study, oocytes were fixed for 1 h at room temperature in 4% formaldehyde containing 0.6% Brij 58, 5 mM EGTA, and 1 mM $MgCl_2$, buffered with 100-mM pipes, pH 6.8 (27). They were then rinsed once briefly in PBS with 0.1% Tween-20 (PBST), equilibrated in PBST for 30 min, dehydrated in a series of ethanols, and stored at −20 °C in 70% ethanol. Previously, the nucleolus and nucleolinus were not visible by DIC at the end of the hybridization protocol. The new fixation method preserved nucleolar and nucleolinar structure so that RNA label could be correlated with DIC-visible structures. Samples were hybridized with digoxygenin-labeled nucleolinus-1 RNA probe for 3 d at 60 °C. Alkaline phosphatase reaction product was visualized after development at 4 °C with gentle rocking.

Laser Microsurgery. For laser microsurgery, 8–10 oocytes were transferred into a 3-μL drop of 0.45-μm filtered sea water (buffered with 10 mM Tris, pH 8.0) on a 1.0-mm thick glass slide with 0.2-mm spacers and a 1.2-mm thick cover. A 150-μs pulsed 1,480 nm laser set at 300 mW was directed into a 5-μm spot through the ×40 objective using a Hamilton Thorne XYClone laser system mounted on a Nikon 90i microscope. A visible lightbeam sight guide of the same diameter was used to effect precise targeting. These conditions were selected in preliminary experiments using a graded series of pulse parameters and determined mild enough so that no overt physical damage to the oocyte was observed (as confirmed by controls). Controls were irradiated using these same parameters, but at 1 nucleolinus diameter away from the experimental target, striking within the nucleolus. Following irradiation, oocytes were quickly transferred to sea water containing 70 mM KCl to activate meiosis (or sperm to fertilize). A similar number of untreated oocytes were transferred to a separate KCl-containing well to serve as nonlasered controls and event timers. Activated oocytes were fixed at various times and labeled with bisbenzimide to visualize chromatin segregation and/or prepared for immunohistochemistry as described above. Because these cells are nearly spherical, it was necessary to rotate oocytes during observation to avoid mistaking an end-on view of a normal spindle for a malformed cluster of chromatin and microtubules. We reiterate here for clarity that at the time of laser irradiation (unactivated oocyte stage), no centrosomes or spindle are present. These elements arise only *after* fertilization or parthenogenetic activation.

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