

Downregulation of protein 4.1R impairs centrosome function, bipolar spindle organization and anaphase

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Centrosomes nucleate and organize interphase MTs and are instrumental in the assembly of the mitotic bipolar spindle. Here we report that two members of the multifunctional protein 4.1 family have distinct distributions at centrosomes. Protein 4.1R localizes to mature centrioles whereas 4.1G is a component of the pericentriolar matrix surrounding centrioles. To selectively probe 4.1R function, we used RNA interference-mediated depletion of 4.1R without decreasing 4.1G expression. 4.1R downregulation reduces MT anchoring and organization at interphase and impairs centrosome separation during prometaphase. Metaphase chromosomes fail to properly condense/align and spindle organization is aberrant. Notably 4.1R depletion causes mislocalization of its binding partner NuMA (Nuclear Mitotic Apparatus Protein), essential for spindle pole focusing, and disrupts ninein. During anaphase/telophase, 4.1R-depleted cells have lagging chromosomes and aberrant MT bridges. Our data provide functional evidence that 4.1R makes crucial contributions to centrosome integrity and to mitotic spindle structure enabling mitosis and anaphase to proceed with the coordinated precision required to avoid pathological events.

INTRODUCTION

Centrosomes nucleate and organize interphase microtubules (MTs) and are instrumental in assembly of mitotic bipolar spindles responsible for accurate chromosome segregation. Precise distribution of duplicated chromosomes to daughter cells is of paramount importance since aberrant cell division is associated with genetic diseases and aneuploidy is characteristic of many cancers. To date the structural components of centrosomes and their functions are only partially characterized. The multifunctional structural protein 4.1 formerly was only categorized as a crucial red cell adaptor protein stabilizing spectrin-actin lattices and anchoring them to plasma membrane proteins. However, in nucleated cells protein 4.1 is newly recognized as an important structural component of centrosomes and mitotic spindle poles, as well as of the midbody and the nucleus (De Carcer et al., 1995; Delhommeau et al., 2002; Krauss et al., 1997b; Krauss et al., 1997a; Mattagajasingh et al., 1999).

As adaptor proteins integrating centrosomal components, 4.1 isoforms could be crucial for centrosomal functions such as mitotic spindle assembly, cytokinesis, and MT organization governing cell shape, polarity, motility and intracellular transport (Hinchcliffe and Sluder, 2001a; Kellogg et al., 1994; Khodjakov and Rieder, 2001; Piel et al., 2001). Centrosomes consist of a cylindrical tubulin-rich centriole pair, the more mature centriole having “appendages” at its distal end appearing to anchor MTs (Mogensen et al., 2000). Proteins such as EB1, p150/glued, cenexin/ODF2 and centriolin differentiate mature from immature centrioles as well as distal appendage proteins, including ninein and ϵ -tubulin (Bornens, 2002; Chang et al., 2003; Gromley et al., 2003; Lange and Gull, 1995; Nakagawa et al., 2001). Surrounding centrioles is pericentriolar material (PCM) containing many protein complexes such as pericentrin scaffolds contacting γ -tubulin ring complexes (γ -TURCs) which nucleate MT growth (Dictenberg et al., 1998; Moritz et al., 1995; Schnackenberg et al., 1998; Zheng et al., 1995). Proteins such as PCM-1 likely mediate MT anchoring via assembly of subsets of centrosomal proteins (Dammermann and Merdes, 2002). Centrosomes duplicate, separate and mature in stages that correlate with cell cycle progression. During G1, each centrosome contains a mature and an immature centriole. During S, each centriole spawns a procentriole such that by S/G2 centrosomes contain two centriole pairs which separate to form the spindle poles during mitosis.

Previously, we characterized protein 4.1 as a “core” component of mammalian centrosomes and present in basal bodies of murine, porcine and *Xenopus* sperm (Krauss et al., 1997b; Krauss et al., 2004). By confocal microscopy, we detected 4.1 colocalized with centriolar tubulin, in surrounding PCM and on fibers connecting centriolar pairs in mammalian cells. At high resolution whole mount EM, 4.1 has a polar distribution on centrioles and decorates fibrous structures extending into the PCM (Krauss et al., 1997b). Using a cell-free *Xenopus* egg extract system we showed by depletion/addback that 4.1 is essential for spindle, centrosome aster and self-assembled MT aster assembly. Furthermore, dominant negative 4.1 peptides impaired MT dynamics and organization (Krauss et al., 2004). Significantly, 4.1 has binding sites for MTs (Perez-Ferreiro et al., 2001), the spindle pole focusing protein Nuclear Mitotic Apparatus protein (NuMA) (Mattagajasingh et al., 1999), and CPAP (Centrosome Protein 4.1-Associated Protein (Hung et al., 2000), a regulator of MT dynamics.

Recently a 4.1 gene family was discovered necessitating refinement in identifying common as well as unique functions of its members. The prototypical 4.1, now called 4.1R (red cell), is abundant both in erythroid tissues and non-erythroid cells, 4.1G is generally distributed (Parra et al., 1998a; Walensky et al., 1998), 4.1N is predominantly neural (Walensky et al., 1999); 4.1B is detected mostly in brain (Parra et al., 1998b; Yamakawa et al., 1999). The new genes have

some conserved subdomains in common (Parra et al., 2004) as well as unique regions, raising the important question if they have unique, redundant or synergistic functions.

We report here that both 4.1R and 4.1G localize to centrosomes but have different subcentrosomal distributions during the cell cycle. We establish that 4.1R is a mature centriole component and 4.1G is resident in the PCM. Furthermore, by abrogating 4.1R function using RNA interference (RNAi)-mediated depletion, we characterized a pleiotropic phenotype involving altered centrosomal structure, centrosomal and mitotic spindle dysfunction, and impaired anaphase demonstrating important roles of 4.1R in centrosomal structure and function.

RESULTS AND DISCUSSION

Protein 4.1R preferentially localizes to mature centrioles and 4.1G to PCM regions

4.1R and 4.1G contain highly homologous spectrin-actin binding and C-terminal domains, two domains we reported as critical for MT organization and dynamics (Krauss et al, 2004; Fig. 1 A). Antibodies specific for 4.1R or 4.1G (Fig. S1 A) detected focal 4.1R and 4.1G immunofluorescent signals at centrosomes in various cultured mammalian cells. Using deconvolution analysis, in asynchronous cells, 4.1R and 4.1G epitopes possessed distinct labeling patterns with partial overlap within centrosomes: 4.1R localized to centrioles while 4.1G localizes to the surrounding PCM network (Fig. 1 B, a-e and Fig. S1 B).

We next compared 4.1R distribution to several key centrosomal proteins. Significantly, 4.1R frequently localized at one or two centrioles labeled by centrin staining in many cells (Fig. 1 B, a, f) or at a subset of supernumerary centrioles in human osteosarcoma U2OS cells (unpublished data). Rotation of reconstructed deconvolved 3D images revealed 4.1R epitopes distributed along the centriole, forming a tube or sleeve around centrin (Fig. 1 B'). Furthermore, 4.1R colocalized extensively with ninein, a signature subdistal appendage protein of mature centrioles (Fig. 1B, g), and was surrounded by p150/glued epitopes, another mature centriole marker (Fig. 1B, h) and part of the dynein/dynactin MT motor complex (Quintyne et al., 1999; Quintyne and Schroer, 2002). By contrast, the 4.1G network partially overlapped and extended beyond pericentrin epitopes, a signature PCM protein (Fig. 1 B, c) (DICTENBERG et al., 1998). Taken together our experiments define 4.1R as a component of mature centrioles whereas 4.1G is a PCM component.

Distributions of centrosomal 4.1R and 4.1G during progression through interphase into prophase

As a component of mature centrioles, 4.1R would be expected to associate with the second maturing centriole during completion of G2. To test this, we analyzed distributions of 4.1R and 4.1G epitopes in synchronized diploid human fibroblasts using the MT anchoring protein ninein as a mature centriole marker. 4.1R co-localized with ninein at a single centriole in a ring-shaped distribution in G1, began to extend to a second ninein-labeled maturing centriole during S phase ("one and a half rings") and colocalized with ninein at both mature centrioles (two ring pattern) during G2 (Fig. 1 C). By contrast, 4.1G epitopes were distributed around ninein-marked mature centrioles in a lattice-like pattern in G1, S and G2. Therefore, while 4.1R accumulates at centrioles as they mature, 4.1G epitopes are detected in the PCM regardless of cell cycle stage. These data suggest that 4.1R functions at mature centrioles while 4.1G has functional interactions within surrounding PCM regions.

4.1R RNAi affects MT organization during interphase

Because 4.1R localized at mature and newly maturing centrioles, we reasoned that centriole morphogenesis and MT organization would be affected in cells depleted of 4.1R induced by 4.1R RNAi. To test this, we surveyed 4.1 family members expressed in various cultured mammalian cells using gene-specific antibodies to identify cells expressing only 4.1R. No cell line tested expressed exclusively 4.1R. However, CaSki cells and human diploid fibroblasts contained only 4.1R and 4.1G, a combination that therefore must be sufficient for cell division. HeLa cells additionally contain a minor amount of 4.1N (Fig. S1 C).

After transfection with a mixture of three RNA duplexes targeting 4.1R mRNA, Western blot analysis of a time course showed that 4.1R expression was significantly depleted (70-85%,

Fig. 2 A) at 72-120 h. No effects on protein levels were detected at 24 hours and only partial inhibition (~50%) was evident at 48 h (unpublished data). Silencing was specific for 4.1R expression since 4.1G expression did not decline relative to controls (Fig. 2 A). Transfection with individual RNAi duplexes showed similar 4.1R-specific depletion. A fourth control duplex with a variant sequence or mock transfection (Lipofectamine without duplex) did not affect 4.1R expression.

Using CaSki or HeLa cells, we specifically downregulated 4.1R expression and immunostained asynchronous 4.1R RNAi-treated cells. Tubulin staining revealed 31% of 4.1R-depleted cells with disorganized whorls of cytoplasmic MTs, in contrast to well-organized radial arrays in 98% of controls (Fig. 2 B, B'). We also observed 26% of 4.1R RNAi-treated cells with abnormally dispersed ninein relative to its characteristic circumscribed distribution in 97% of controls, whereas γ -tubulin localization was not affected (Fig. 2 C, C').

MT anchorage and radial organization depends on many proteins including ninein, EB1, PCM-1, katanin, p150, CEP135 and MIRI. MT nucleation may be a distinct process occurring mainly in the PCM at γ -TURCs, while anchorage is at mature centriolar subdistal appendages and in the PCM. We tested if MT nucleation and/or regrowth were impaired by 4.1R RNAi treatment. Following MT depolymerization by cold and nocodazole exposure/washout, for 0-20 min we found no detectable differences in MT nucleation and regrowth between control and 4.1R RNAi treated cells even when ninein distribution was abnormal (Fig. 2 D, top). However, at 25 min we observed asters in 4.1R RNAi treated cells in which radial MTs were distinctly separated from a centrin-stained centrosome (Fig. 2 D, middle), some even whorled. At longer times there was an increased frequency of MT disorganization and MTs not emanating from a focus of either centrin or perturbed ninein (Fig. 2 D, bottom and 2 D'). Since localization of γ -tubulin, the defining member of the γ -TURC complex, was not perturbed (Fig. 2 C), we speculate that abrogating 4.1R expression may perturb ninein's anchoring but not γ -tubulin MT nucleating functions. In fact, mutations in ninein cause MT anchoring defects without perturbing γ -TURC localization (Delgehyr et al., 2005). Interestingly, overexpression of a variant 4.1R isoform also does not impair MT nucleation/regrowth (Perez-Ferreiro et al., 2004). Future experiments will test direct interactions of 4.1R with ninein as well as with other mature centriole MT anchoring proteins.

4.1R RNAi affects ninein recruitment to maturing centrioles

In addition to quantitating centrosomal ninein perturbation after 4.1R RNAi, we analyzed 4.1R-depleted cells with normal ninein patterns. Compared to controls, 4.1R RNAi treated populations had more cells with one and a half ninein rings (S phase) and lower amounts of cells with two ninein rings (G2), indicating that progression toward centriole maturation was altered (Fig. 3 A). In addition, in cells with two ninein rings, we quantitated those in which two centrosomes were separated versus juxtaposed. Normally in prophase, mature centrosomes separate and migrate around the nucleus as nuclear envelope breakdown proceeds, culminating in bipolar spindle pole formation. We observed that 4.1R RNAi treated cultures had a ~2.5 increase in cells with two mature centrioles juxtaposed (Fig. 3 B). This result indicates that even when there are two centrosomes present, 4.1R downregulation impacts their capacity to efficiently separate in order to establish a bipolar spindle.

We next asked if perturbed ninein prevents entry into metaphase. By immunostaining phosphohistone H3 (a mitosis marker), we observed 4.1R RNAi-treated cells in early and mid-prophase with irregular ninein patterns (Fig. 3 C). Therefore, an altered distribution of ninein

does not prevent the G2/M transition although, after downregulation of 4.1R, cells have a slower growth rate than controls (Fig S2).

Mitotic spindle defects in 4.1R RNAi treated cells

We previously showed that 4.1 is critical for mitotic spindle assembly and maintenance in *Xenopus* egg extracts (Krauss et al., 2004) and set out to compare effects of silencing 4.1R in mammalian cells. Although defective spindle morphology was previously observed after 24-48 h of 4.1R silencing in 15-20% of HeLa cells, specificity for 4.1R downregulation relative to 4.1G was not tested nor was mislocalization of key spindle proteins assessed (Huang et al., 2004). By immunofluorescence we observed control mitotic cells possessed focused spindle poles capped by NuMA epitopes and bipolar MT arrays emanating toward a metaphase plate with regularly aligned kinetochores (stained by CREST antibody) (Fig. 4, top row). MT interaction with kinetochores is a prerequisite for correct chromosome alignment and segregation (Pinsky and Biggins, 2005). In contrast, 4.1R depletion resulted in mono-, bi- and multi-polar spindles with decondensed chromatin not organized at a midplane between poles and misaligned CREST staining (Fig. 4, bottom). Furthermore, spindle MTs were disorganized, with the 4.1 binding partner NuMA broadly distributed at unfocused poles and mislocalized at non-polar areas (Fig. 4 A, center). Multipolar spindles in 4.1R RNAi-treated populations stained with tubulin or phosphohistone H3 had multiple foci of centrin and ninein instead of only two labeled polar areas (Fig. 4 A, B). Quantitation of 4.1R RNAi treated cells revealed a dramatic increase in mitotic abnormalities by 96 hours (68% abnormal spindles compared with 8% in controls, Fig. 4 C). This spectrum of mitotic defects including MT disorganization, multipolarity and unaligned, poorly-condensed chromatin closely resembles those we observed in *Xenopus* egg extracts in which 4.1 was immunodepleted or functionally disrupted (Krauss et al., 2004). Clearly one major mechanism generating spindle defects during 4.1R depletion in either system is mislocalization of NuMA, a protein required for highly focused spindle poles (Gaglio et al., 1995; Merdes et al., 2000). However, our data further indicate that prior to mitosis in intact cells, immature precursor centrosomes, insufficient centrosome separation (an effector of spindle bipolarity), decreased centrosome-anchored MTs, and decondensed chromatin may also contribute to mitotic spindle defects.

Lagging chromosomes and cytokinesis defects are detected in 4.1R RNAi treated cells

Since centriole maturation, separation, and properly organized spindles are prerequisites for proper cytokinesis, we predicted that 4.1R depletion might produce cytokinesis defects. Indeed, 4.1R RNAi-treated anaphase cells often displayed lagging chromatin trapped between nascent daughters and inappropriately localized spindles (Fig. 5, top and middle panels). Furthermore, while control cells at cytokinesis were connected by short bundled tubulin bridges, 4.1R depleted cells had various cytokinesis defects including elongated tubulin connecting structures, wide intercellular bridges, broken bridges, and tubulin bridges contacting multiple cells (Fig. 5, lower panel). Anaphase defects were apparent in 35% of 4.1R RNAi treated cells. Chromosome missegregation, spindle dysmorphology, and improper bridge formation in 4.1R RNAi treated cells could mechanistically be a consequence of defective centriole structure causing poorly anchored MTs. Several reports document that centrosomal function and completion of cytokinesis are interrelated (Gromley et al., 2003; Hinchcliffe and Sluder, 2001b; Khodjakov and Rieder, 2001) and, more specifically, that the mature centriole moves to the intercellular bridge before the conclusion of cytokinesis (Piel et al., 2001).

In summary, two protein 4.1 family members with unique and highly homologous regions have distinct subcentrosomal localizations. 4.1R localizes on maturing centrioles during cell cycle progression whereas 4.1G localizes in the surrounding PCM. Do 4.1R and 4.1G have separable, redundant or synergistic functions? Coupled with ongoing identification of binding partners, we addressed this question initially by silencing 4.1R alone in cultured cells. This produced a complex phenotype affecting mitotic spindle MT and chromatin organization, centrosome functions, and interphase MT organization as well nuclear defects (unpublished data). This spectrum of pleiotropic defects is strongly predictive of inaccurate chromosome segregation and likely reflects a loss of 4.1R acting as an adaptor protein within several integrated molecular networks responsible for spindle bipolarity, chromosome alignment and completion of cytokinesis. Protein 4.1 at centrosomes or even elsewhere may be key for assembly of critical centrosomal proteins responsible for centrosome maturation, separation and transition to spindle poles. This model is supported by reports of similar phenotypes often inactivating individual centrosome/spindle proteins. As a molecular linker, 4.1R interactions with NuMA, CPAP and potentially ninein and/or other MT anchoring proteins may in turn modulate MT attachment to kinetochores leading to defective chromosome condensation/alignment and cytokinesis. Our investigations highlight roles of the 4.1R and 4.1G multifunctional structural proteins in ensuring the integrity of cell division and suggest that loss or decrements in 4.1R function may lead to as yet unrecognized pathological consequences.

Materials and methods

Cells and media

WI38, CaSki and HeLa cells were obtained from American Type Culture collection. WI38 and HeLa were cultured in DME-H21 media (Gibco BRL) and CaSki in RPMI 1640 (Cell Gro) as described previously (Krauss et al., 1997a). WI38 cells were synchronized by double thymidine block for 16-18 hours with 2mM thymidine.

Immunofluorescence

Cells grown on coverslips were fixed and stained by indirect immunofluorescence as described previously (Krauss et al., 1997a). Affinity purified rabbit antibodies against 4.1R were as described previously (Krauss et al., 1997a; Ramez et al., 2003), anti-centrin (20H5) and GFP-centrin were the kind gifts of J. Salisbury (Mayo Clinic Foundation, Rochester MN), mouse anti-ninein was provided by G. Chan (Alberta Cancer Board, Alberta, Canada) and CREST serum was from A. von Hooser (UC-Berkeley, Berkeley CA). Chicken anti-4.1G was raised against a 6XHis peptide encoding the U1 region of human 4.1G (EP41L2) containing 217 amino acids following the AUG1 start site (Parra et al., 1998a). Affinity purified immune IgY was analyzed to confirm no cross-reaction with recombinant 4.1R, 4.1B, 4.1N or with RBC 80kD 4.1R or an irrelevant 6Xhis peptide. Commercial antibodies were: rat anti-tubulin YL1/2 (Accurate Chemical and Scientific Corporation), monoclonal GTU88 against γ -tubulin (Sigma), rabbit anti-pericentrin (Covance), monoclonal anti-p150 (BD Transduction Labs) and rabbit anti-phosphohistone 3 (Upstate). Secondary antibodies with minimal species cross-reactivity were from Molecular Probes or Jackson ImmunoResearch. Parallel samples probed with equal amounts of control non-immune IgG/IgY or without primary antibody showed no fluorescent patterns under conditions used for experimental samples. Images were acquired with a Nikon Eclipse 2000 using a 60x1.4 NA objective equipped with a Retiga Ex camera and ImagePro or a Deltavision microscope on an Olympus IX70 platform with a 100x1.35 NA objective. Images were processed using Adobe Photoshop and volume rendering using Volocity.

4.1R RNAi transfection

Small interfering RNAs against human 4.1R from Dharmacon were: Duplex 1, GAAAGUCUGUGUAGAACAUUU; Duplex 2, UGACACAGUUUAUGAAUGUUU; Duplex 3, GGAUCCAAAUUUCGAUACAUU. Each duplex alone as well as a pool of all three specifically downregulated 4.1R expression. Control cells were transfected with duplex GCUAAGAAAUAUGGAAAGUU, or with Lipofectamine 2000 alone. Each 35mm well containing 2×10^5 cells was transfected with 100nM RNA duplex complexed to Lipofectamine 2000 as per manufacturer's instructions. Downregulation of 4.1R expression by siRNA was detected beginning at 48 hours and was maximal 72-120 hrs. Cells were trypsinized and counted to determine relative growth rates.

MT regrowth

siRNA-transfected and controls cells were incubated for 30 min at 4⁰C and then with 33 μ M nocodazole for 30 min at 37⁰C to completely dissociate MTs. After washing out nocodazole with fresh media, cells were incubated for 2, 5, 10, 15, and 20 min and 2 h at 37⁰C, washed in PBS, pH7.4, containing 2.7 mM KCl, 1.5 mM KH₂PO₄, 1mM MgCl₂, 1 mM EGTA, 137 mM NaCl and 8.1 mM NaHPO₄ and fixed in cold methanol for immunostaining.

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Abbreviations:

MT, microtubule

NuMA, nuclear mitotic apparatus protein

PCM, pericentriolar material

RNAi, RNA interference

γ -TURC, γ -tubulin ring complex

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Figure Legends

Figure 1 Protein 4.1R epitopes are at mature centrioles and 4.1G is distributed in the PCM. (A) Schematic map of 4.1R and 4.1G with 3 unique regions (“U”) and 3 homologous domains: membrane-binding 30-kD/FERM domain, spectrin/actin binding domain (SAB) and C-terminal domain (CTD). Numbers below, degree of homology. Arrows, translation initiation sites. (B) Deconvolution sections of WI38 human fibroblast centrosomes stained for indicated proteins. For 3 colors, cells were transiently transfected with a fluorescent centrin fusion protein. Similar results were obtained using HeLa and CaSki cells. (B’) Rotation analysis of deconvolved sections of 4.1R/centrin recombined in a 3D rendering. 4.1R surrounds centrin signals of one centriole. (C) 4.1R and 4.1G distribution during cell cycle progression in human fibroblasts synchronized by double thymidine block. 4.1R localizes exclusively to centrioles also containing ninein while 4.1G is in the surrounding PCM area. Similar results were obtained using HeLa and CaSki cells. B(a-e), C, bar=0.5 μ . B(f-h): 2X magnification

Figure 2 After downregulation of 4.1R expression, ninein is perturbed and MTs become disorganized. (A) Representative Western blot of 4.1R and 4.1G at 96 h after 4.1R RNAi. 4.1R expression decreased 70-85% in HeLa cells and ~65% in CaSki cells when normalized to actin while 4.1G expression was not markedly altered. (B) Immunofluorescent staining of tubulin and 4.1R. In 4.1R RNAi-treated cells with no detectable 4.1R signals over background, MTs appear whorled in contrast to controls with MTs radiating from a focal point (arrow). Bar=10 μ . (B’) Quantitation of perturbed tubulin. 4.1R RNAi treated CaSki cells, n=1339; controls, n=1016 (3 independent experiments. Similar results in HeLa). (C) Abnormal ninein distribution at centrioles in 4.1R depleted cells. Immunostaining is irregular, dispersed, larger or smaller relative to single or double circular patterns in controls. γ -Tubulin distribution is not affected. (C’) Quantitation of perturbed ninein. 4.1R RNAi, n=803; controls, n=607 (3 independent experiments in CaSki cells. Similar results in HeLa). Bar=1 μ (D) MT nucleation at 37⁰ C in 4.1R RNAi-treated cells and controls is similar up to 20 min after nocodazole exposure/washout. Subsequently MTs in 4.1R RNAi-treated cells become distanced from centrioles (25 min) and disorganized (2h). Centrioles imaged with anti-centrin or anti-ninein (enlarged insets). DNA, DAPI. (D’) Quantitation of decreased focused MT radial arrays (300 HeLa cells per time point). Additional experiments, at 20⁰ C and in CaSki cells, also showed regrowth unaffected in 4.1R depleted cells relative to controls but MTs subsequently became disarrayed. Bar=3 μ .

Figure 3 Downregulation of 4.1R expression affects ninein distribution at maturing centrioles and centrosome separation. Centrioles were scored by ninein immunostaining after 96 h 4.1R RNAi treatment. (A) 4.1R RNAi-treated CaSki cells with apparently normal ninein patterns show similar amounts of G1 cells (1 circle) as controls but more cells with ninein S phase staining (1.5 circles) and less with G2 patterns (2 circles). Error bars are from 3 experiments where 2.9% controls and 28% RNAi-treated cells had abnormal ninein staining. Control, n=442; RNAi, n=462. Asterisk, p=<0.05 (B) Cells exposed to 4.1R RNAi had 6.9% cells versus 21% controls with separated ninein-stained centrosomes. (C) Cells with perturbed ninein after 4.1R depletion can enter metaphase as marked by phosphohistone H3. DNA, DAPI. Left, early prophase and prophase control cells with circular ninein staining at each mature centriole. Right, 4.1R RNAi treated cells in early prophase and prophase having perturbed ninein (insets, ninein). Bar=1 μ

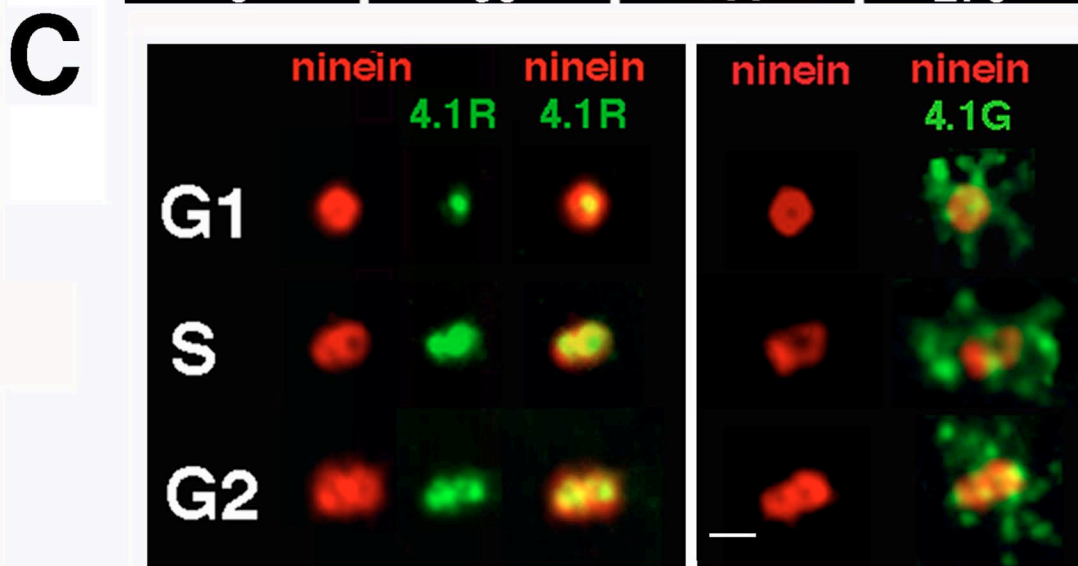
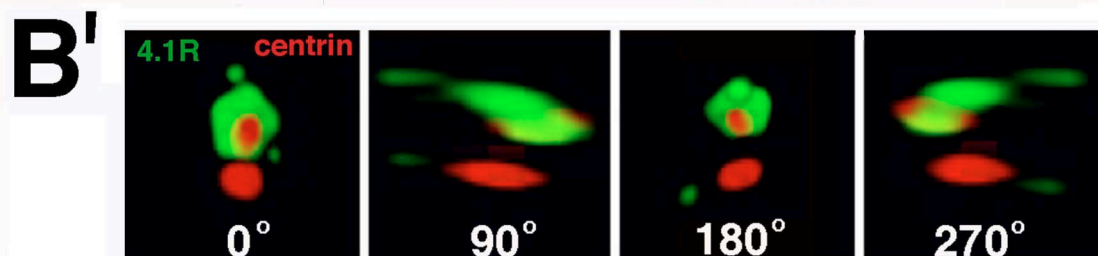
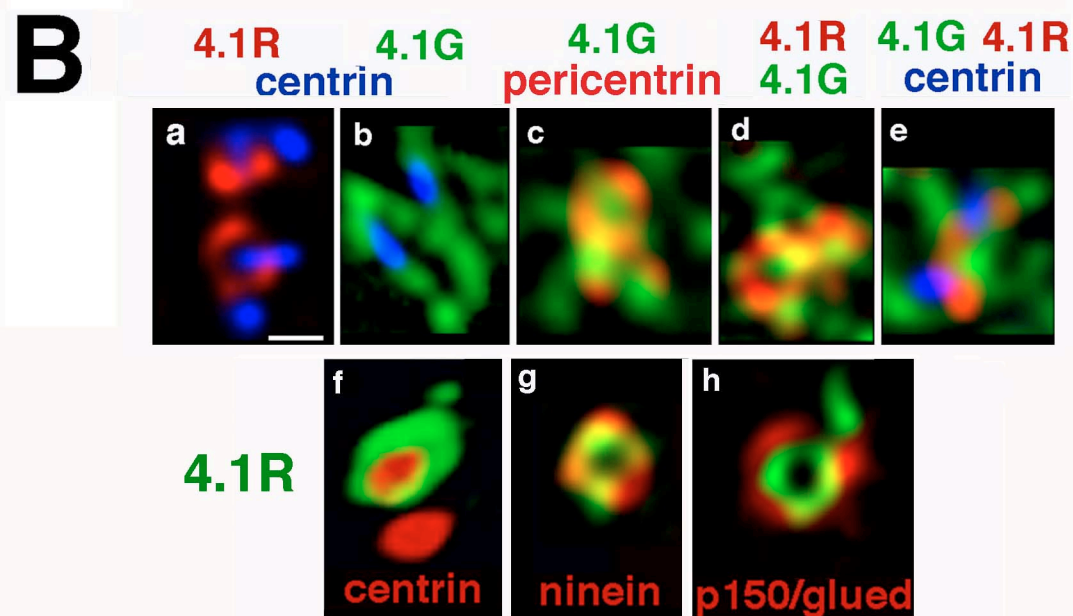
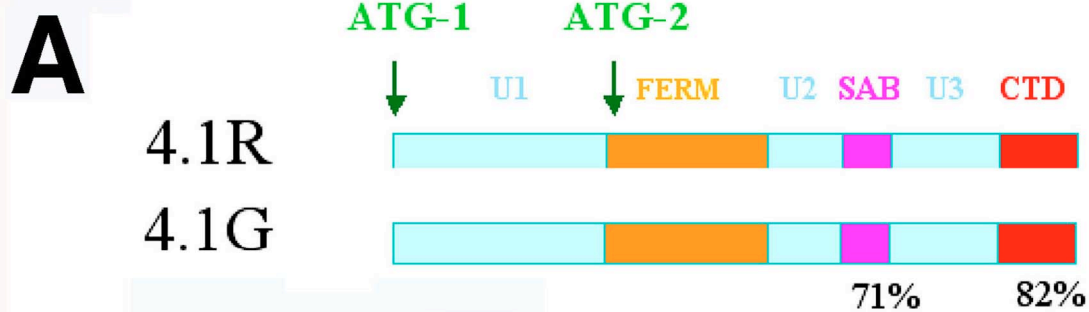
Figure 4 **Mitotic abnormalities after 4.1R RNAi.** (A) Mitotic cells with downregulated 4.1R expression (lower panels) have decondensed chromatin (DAPI), unaligned CREST staining (kinetochores) and mislocalized NuMA. Tubulin staining reveals multi- and mono-polar cells and centrin staining marks multiple poles. Bar=5 μ . (B) Anti-phosphohistone 3 staining of a multipolar cell after 4.1R RNAi depletion shows multiple irregularly sized ninein foci and decondensed DNA in contrast to bipolar mitotic controls. (C) Quantitation of increased abnormal mitosis after 4.1R depletion in 3 experiments. Control, n=222; 4.1R RNAi, n=212.

Figure 5 **Anaphase/telophase defects after 4.1R RNAi** (A) Anaphase cells with lagging chromosomes (right top panel, arrows), decondensed chromatin with dispersed CREST staining and misaligned spindles (tubulin, middle panel, right) relative to controls (left). In lower right panel, abnormal tubulin bridges formed at cytokinesis in 4.1R RNAi treated cells including broken, tricellular and elongated bridges. (B) Quantitation of anaphase defects. 4.1R RNAi, n=300; controls, n=264. DNA, DAPI. Bar=10 μ .

Figure S1 1 (A) Western blots of recombinant 4.1 proteins probed with affinity purified 4.1R, G, and N gene-specific antibodies. Anti-4.1B specificity has been previously tested (Ramez et al., 2003). (B) Protein 4.1 family members present in cultured mammalian cell lines. Whole cell lysates were probed by Western blot with 4.1 gene-specific antibodies. Human and murine fibroblasts as well as CaSki cells express only 4.1R and 4.1G. (C) Staining of CaSki cell with anti-4.1G showing centrosomal and cytoplasmic staining. Nuclear focal planes showed punctate 4.1G staining. Centrosomal staining with anti-4.1R has been previously reported (Krauss et al., 1997b).

Figure S2

Growth of CaSki and HeLa cells after exposure to 4.1R RNAi relative to control cultures. At the times indicated, cells were trypsinized and counted.



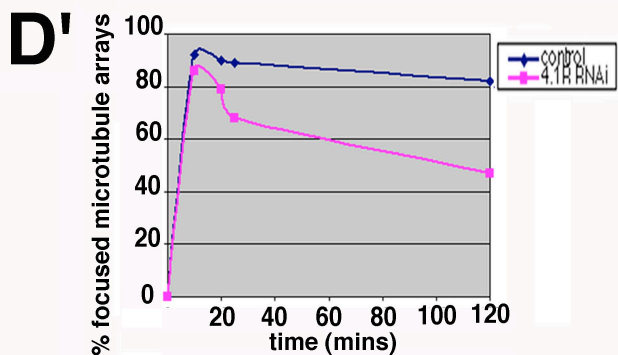
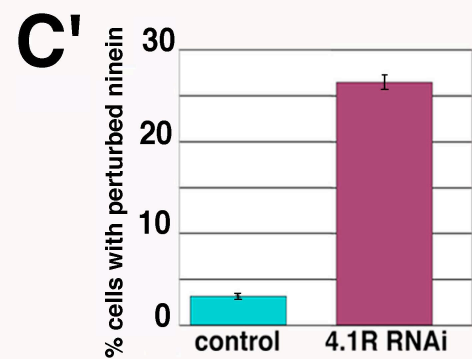
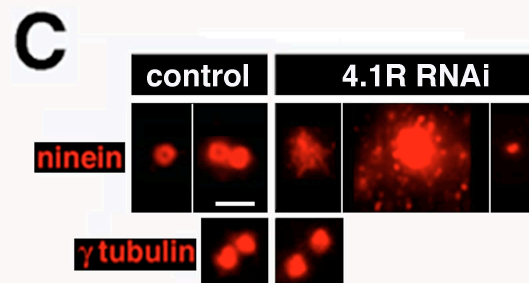
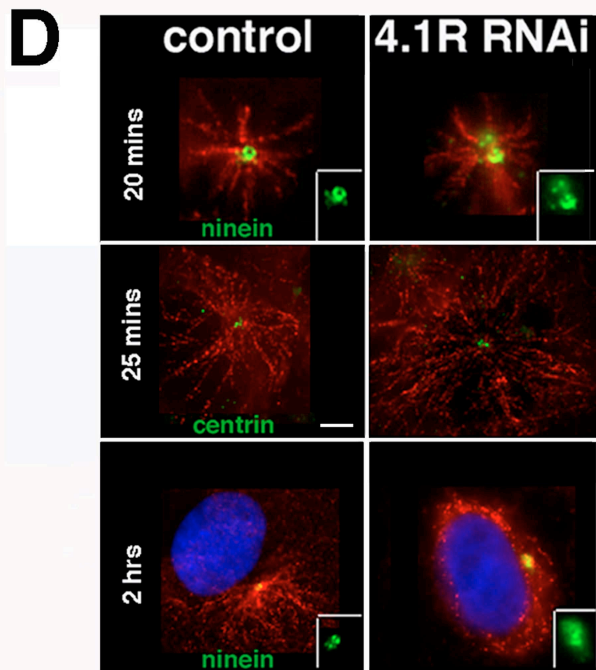
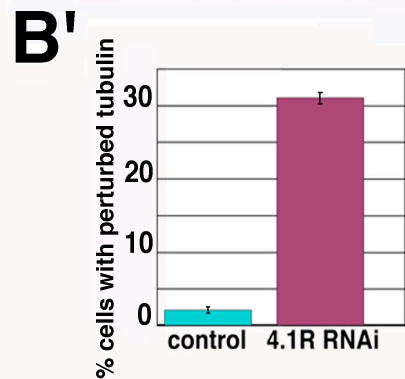
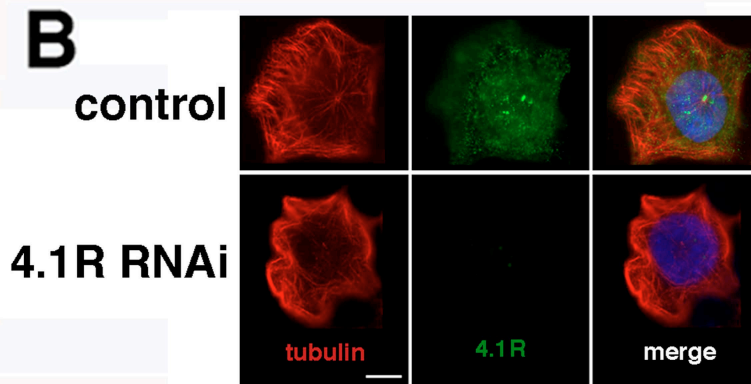
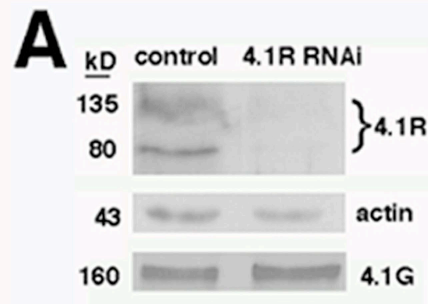
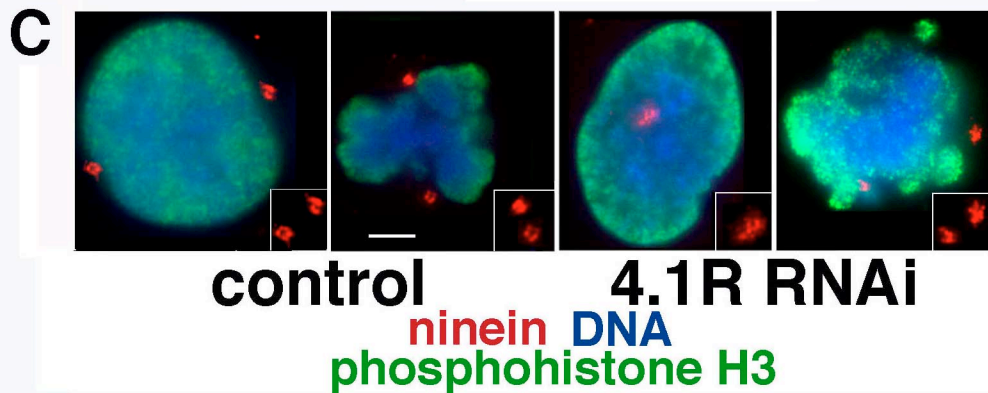
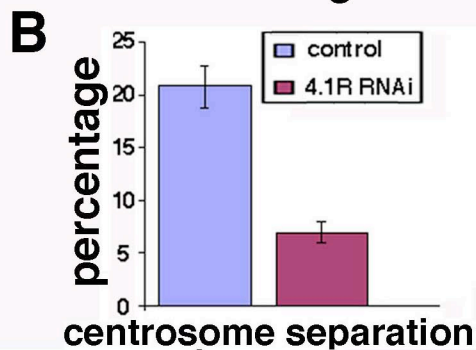
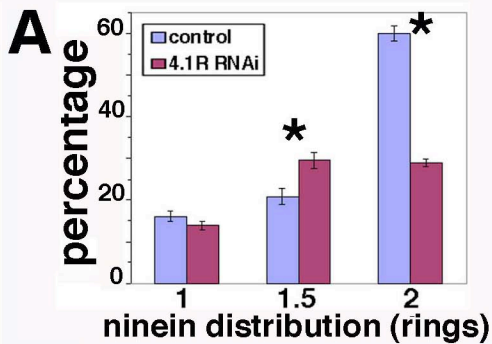


Figure 3



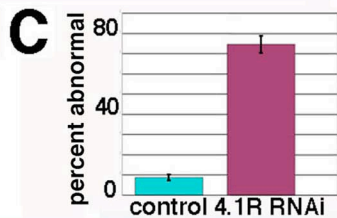
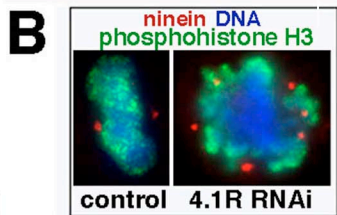
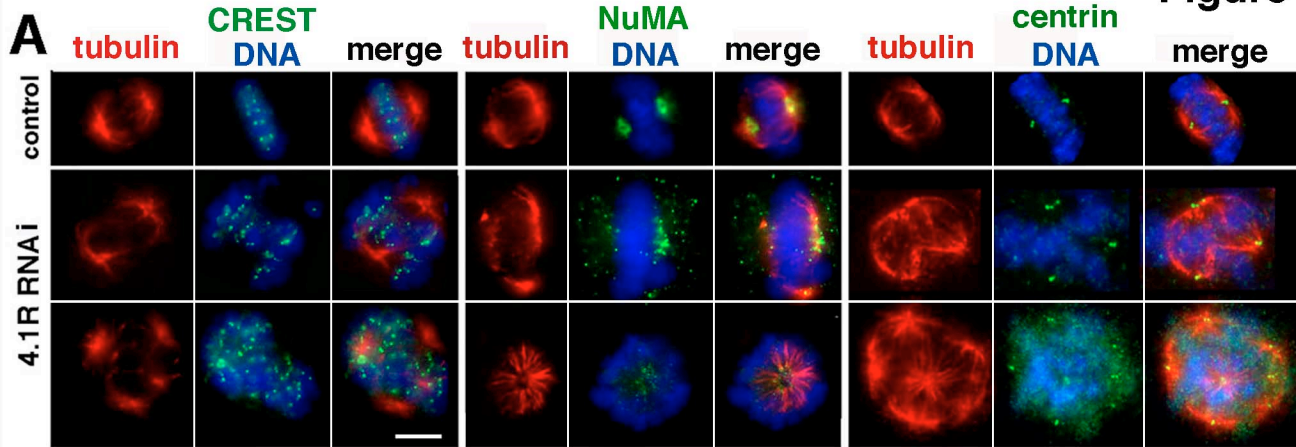
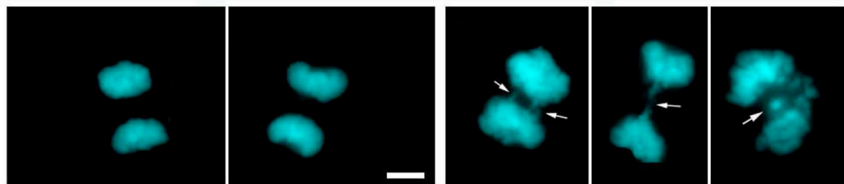


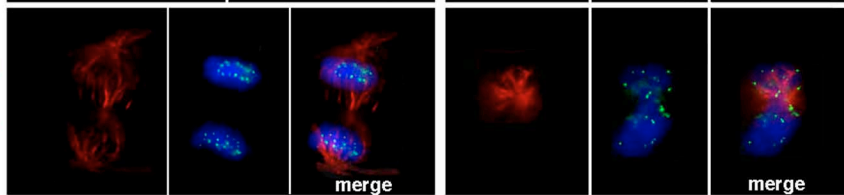
Figure 5

A

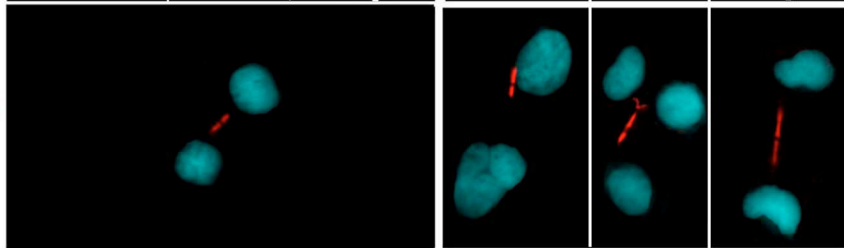
DNA



tubulin
DNA
CREST



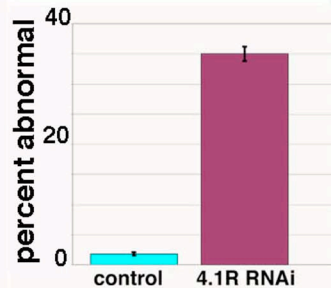
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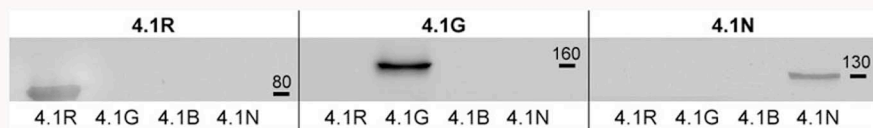
control

4.1R RNAi

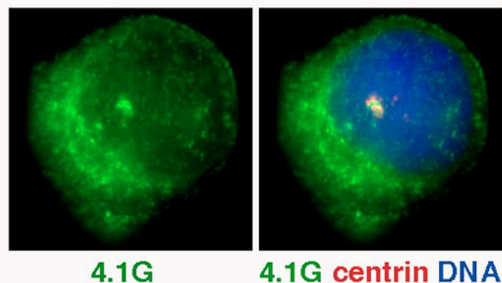
B



A



B



C

	4.1R	4.1G	4.1N	4.1B
Diploid Human Fibroblasts	++	++	-	-
Murine Embryonic Fibroblasts	++	++	-	-
CaSki	++	++	-	-
HeLa	++	++	+	-
U2OS	++	++	+	-
Murine Thymocytes	++	++	-	+

Figure S2