Supporting Online Material for

A Mitotic Lamin B Matrix Induced by RanGTP Required for Spindle Assembly

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Published 16 March 2006 on Science Express
DOI: 10.1126/science.1122771

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This Supplementary Material consists of Experimental Procedures and six figures with figure legends.

Experimental Procedures:
Expression constructs, proteins, and antibodies

The expression construct for EGFP-tagged human LB1 is from Dr. Jan Ellenberg (1). 6His-tagged LB3 or ΔNLB3 was made by cloning the full-length LB3 or ΔNLB3 (lacking the first 32 amino acids of LB3) into the NcoI and HindIII sites of pET30a. The resulting fusion proteins have 6His at both N- and C-termini. The plasmids for 6His-tagged LB3T were previously described (2, 3). LB3T(-)nls was made by mutagenizing RGKKRKLD (the NLS of LB3) to RGASSKLDE using Quickchange II (Stratagene) resulting in LB3T(-)nls that does not bind to importin α. LB3T and LB3T(-)nls were expressed in bacteria with IPTG induction at 25°C for 4 hr, while LB3 and ΔNLB3 were expressed at 13 °C for 12 hours without IPTG (the low level of expression from the promoter in the absence of induction keeps the lamins soluble). The proteins were purified using Ni-agarose (Qiagen) in a Tris buffer (50mM Tris-HCl pH 8.0, 25% sucrose, 1% TritonX-100, 1 mM PMSF, and 5 mM imidazole). The purified proteins were exchanged into XB buffer (10 mM Hepes, pH 7.7, 50 mM sucrose, 100 mM KCl, 0.1 mM CaCl₂, and 5mM EGTA) using a desalting column (PD-10 Columns, GE Healthcare) and concentrated to 1 mg/ml using Amicon Ultra Centrifugal Filter Devices (Millipore). Purification of RanL43E, RanQ69L, RanT24N, and importin α and β were described (4, 5). All proteins were snap-frozen in liquid nitrogen as small aliquots and stored at −80 °C. The monoclonal antibody to Xenopus LB3 (L6 5D5) and polyclonal antibodies to Xenopus NuMA were from Drs. Reimer Stick and Andreas Merdes, respectively. Rabbit antibodies to Eg5 and LB3 were raised with the C-terminus of Xenopus Eg5 (amino acids: 811-1091), the full-length LB3 fusion protein, or a peptide corresponding to LB3 (amino acids: 6-21), respectively. Antibodies to Xenopus Aurora A, TPX2, and XMAP215 were described (4, 6). Antibodies to the following proteins were purchased: LB1 (sc-6216, Santa Cruz Biotechnology), LB2 (ab8983, Abcam), PAR
RNAi experiments in HeLa cells

Regular (Dharmacon) or Stealth (Invitrogen) siRNAs corresponding to LB1 (aagcugcagaucgacgucgc from Dharmacon and uuccaucaaucaauuuucuucga from Invitrogen) and LB2 (aagaggaggagaagccgagu from Dharmacon and gaggucaacaagcgcagcagaaga from Invitrogen) were used to down-regulate the respective LB in HeLa cells. SiRNAs corresponding to luciferase (aacguacgcggaauacuucga from Dharmacon) or Stealth negative control siRNA from Invitrogen were used for controls. 170 nM of Dharmacon siRNA or 4 nM of Invitrogen Stealth siRNA was used to transfect HeLa cells with Lipofectamine (Invitrogen). Over 100 mitotic cells were analyzed 48 or 72 hrs after transfection using a Nikon E800 microscope equipped with a Micromax CCD camera. For live cell imaging, HeLa cells were treated with siRNA for 48 hours followed by imaging on a temperature-controlled stage at 3 min intervals for 12-16 hrs using a Hoffman modulation contrast objective lens (10x) on a Nikon TE200 microscope equipped with an Orca-2 camera. The appearance of a bar of chromosomes at the middle of the cell shows the beginning of chromosome alignment in prometaphase. Metaphase to anaphase transition was clearly detected as the chromosomal bar separated into two. We quantified the time elapsed from the appearance of the chromosomal bar to the splitting of the bar. 50-100 mitotic cells were analyzed for each siRNA treatment. All LB siRNAs gave similar phenotypes. Quantifications shown in Figure 2 B and D are representatives from at least 5 independent siRNA experiments.

Assays for spindle assembly in egg extracts

*Xenopus* M-phase egg extracts, *Xenopus* sperm, and AurA-beads were prepared as described (6-8). Egg extracts were made by a crushing spin at 10 krpm (for sperm-spindle assembly) or 12.5 krpm (for AurA-beads and RanGTP-spindle assembly). Spindle assembly reactions were incubated at room temperature for 60 to 90 min with sperm chromatin or for 10 to 30 min with AurA-beads and RanGTP. To immunodeplete LB3, monoclonal antibody or polyclonal antibody to LB3 was coupled to 100 µl of protein A-
coupled magnetic bead suspension (~10^9 beads/ml, Dynal). This is used to deplete proteins in 100 µl of egg extracts. IgG from non-immunized mouse or rabbit (Jackson Laboratory) was used for mock-depletion. To rescue LB3 depletion, purified 6His-LB3 was added to the egg extracts that were depleted of LB3 using the polyclonal antibody (raised against the full length LB3) to a final concentration of 0.2 µM. The egg extracts were incubated on ice for 10 min before spindle assembly. To inhibit LB3 assembly with dominant negative LB3 mutants, LB3T and LB3T(-)nls were added at 10µM final concentration (3), ∆NLB3 and wild-type LB3 (9) were added at 2 µM final concentration to the egg extracts and incubated for 20 min on ice before spindle assembly assays. Quantifications shown in Figure 2F and Figure 6 are representative results from at least 3 independent experiments.

Detection of lamin B

To detect LB1 and LB2 in HeLa cells, the cells were fixed with methanol (from -20 °C) at room temperature for 20 min followed by immunostaining with antibodies against LB1, LB2, and tubulin. To detect EGFP-tagged LB1, HeLa cells were transfected with EGFP-LB1 expression vector using Lipofectamine 2000 (Invitrogen) in the absence of serum for 8 to 12 hours, followed by incubation in fresh medium containing serum for 24 hours. The cells were fixed in methanol as described above and immunostained with tubulin antibody. To detect LB3 associated with spindles assembled in egg extracts, spindles were spun onto coverslips through a cushion consisting of BRB80 buffer (80 mM Pipes pH 6.8, 1 mM EGTA, 1 mM MgCl2) and 40% glycerol and fixed with methanol for 5 min before immunostaining.

Detection and assay for LB3-matrices in egg extracts

Two methods were used to assay for the LB3-matrix assembled in M-phase egg extracts. In the first method, spindle assembly was induced with either sperm chromatin or AurA-beads plus RanGTP. After incubation, 10 µl of egg extract was diluted into 1 ml XB buffer containing nocodazole (10 µM final concentration) and incubated at room temperature for 10-15 min to depolymerize MTs. In the second method, egg extracts were incubated with either sperm chromatin or AurA-beads plus RanGTP in the presence
of 10 μM nocodazole at room temperature for the same time duration as for spindle assembly reactions. After incubation, 10 μl egg extracts were diluted into 1 ml BRB80 buffer containing 30% glycerol. LB3-matrices assembled using both methods were spun onto coverslips through 3 ml BRB80 buffer containing 40% glycerol in the same manner as the spin-down for spindles. The LB3-matrices were fixed with methanol and stained with antibodies.

To isolate LB3-matrices, AurA-beads and RanGTP were added to 1 ml of egg extract followed by incubation at room temperature for 10 min. The egg extract was diluted into 25 ml BRB80 buffer containing 30% glycerol. Spindles associated with AurA-beads were retrieved using a magnet and then washed with 25 ml BRB80 buffer containing 30% glycerol for three times. MTs were then depolymerized by incubating the spindles with 25 ml XB buffer containing 10 μM nocodazole for 10 min at room temperature. The remaining LB3-matrices were washed with 25 ml XB buffer containing 10 μM nocodazole two times, followed by washing with 25 ml XB for two more times to remove nocodazole. To release the matrix from AurA-beads, the beads were resuspended in 60 μl XB buffer and pipetted repeatedly to release the matrices. Finally, the beads were removed using a magnet. The recovery of LB3-matrix is highly dependent on the quality of the egg extracts.

To assay MT assembly on the isolated matrix, 1 μl of matrix preparation was incubated with 10 or 20 μM purified tubulin in 39 μl of XB buffer at 30 °C for 20 min. These preparations were fixed with 1% glutaraldehyde and spun onto coverslips through a glycerol cushion followed by immunostaining.

Representative quantifications shown are from at least 3 (Figure 4 and Figure 5) or 4 (Figures 3) independent experiments.

Detection of lipids on spindles and LB3-matrices

Spindles and LB3-matrices were assembled and spun onto coverslips as described above. The coverslips were incubated in BRB80 containing 20% sucrose and 1 μM Vybrant CM-Dil (Molecular Probes, V22888) for 5 min at room temperature and then washed briefly in BRB80 containing 20% sucrose. The structures were then fixed with methanol as above and then stained for LB3 (Alexa Fluor 488 goat anti-rabbit secondary
antibody) and tubulin (Alexa Fluor 350 goat anti-mouse as secondary antibody). To disrupt the matrix with detergent, the spindles were assembled first and then diluted (100-fold) in XB containing nocodazole and 0.1% Triton X100 for 10 min at room temperature. The structures were spun onto coverslips and then stained with CM-DiI followed by immunostaining with LB3 and tubulin antibodies.

Supplementary Figure Legends:

Fig. S1. Reduction of lamin B by siRNA disrupts chromosome alignment and segregation. Cells were treated with control or LB siRNAs for 48 hrs followed by imaging on a temperature-controlled stage at 3 min intervals for 12 to 16 hrs using a Hoffman modulation contrast objective lens (10x) on a Nikon TE200 microscope equipped with an Orca-2 camera. Images shown are examples of control and lamin B RNAi-treated cells progressing from the beginning (round-up) of mitosis to chromosome separation. The time elapsed from the first sign of chromosome alignment (images with white arrowheads) to chromosome segregation (images with white arrows) was quantified from 50-100 cells in each independent siRNA experiments. Scale bar, 20 µm.

Fig. S2. Purified LB3 does not bind taxol-stabilized MTs or stimulate MT assembly from purified tubulin. (A) MTs were assembled from 10 µM pure tubulin in the presence or absence of 5 µM taxol and 0.5 µM purified LB3 at 30 ºC for 20 min in XB buffer. Polymerized MTs (pellet) were separated from tubulin (supernatant) by centrifugation and then analyzed by SDS-PAGE followed by Coomassie blue staining. Whereas taxol stimulated MT assembly, LB3 failed to do so. However, a fraction of LB3 was found in the pellet. (B) LB3 does not interact with MTs. To determine whether the LB3 that co-pelleted with MTs associated MTs, MTs were polymerized under the same conditions as above. Magnetic beads coated with LB3 antibody were used to immunoprecipitate LB3. The samples were analyzed by Coomassie blue staining. Tubulin did not co-immunoprecipitate with LB3. (C) Tubulin and LB3 polymerize into independent filaments. MTs and LB3 polymerized in the presence of taxol were fixed with 1% gluteraldehyde and then spun onto glass coverslips. The coverslips were post-fixed with
cold methanol. MTs were labeled by rhodamine tubulin, while LB3 was labeled by LB3 antibodies. LB3 aggregates were apparent. However, upon close inspection (see the enlarged images from the boxed areas), filamentous LB3-containing structures were present, and they did not co-localize with MTs. Scale bar, 10 µm.

**Fig. S3.** The fibrillar-granular LB3-matrices contain lipids and are completely disrupted by 0.1% Triton X100. (A) Two images in Figure 3A and B are enlarged here to show the details of the fibrillar-granular LB3-matrices (green) associated with sperm chromatin (blue) or AurA-beads (red). (B) Detection of lipids on spindles assembled with AurA-beads and RanGTP. Lipid, MTs, and LB3 were labeled with DM-DiI (green), tubulin (red), and LB3 (blue) antibodies. The images are pseudo colored. (C) Detection of lipids on LB3 matrices. (D) Disruption of LB3-matrices by Triton X100. Scales: white bar, 10 µm; magnetic beads, 2.8 µm.

**Fig. S4.** Assembly time-course of LB3-matrix and MTs induced by *Xenopus* sperm chromatin. Sperm chromatin (blue) were incubated with egg extracts for the indicated time duration. LB3-matrices (green) and MTs (red) assembled independently near the sperm chromatin at the earliest time point (3 min). At this time point, MTs, which were short and disorganized, often did not associate with LB3-matrices. The association of LB3-matrices and MTs coincided with the organization of MTs into astral arrays. LB3-matrices often appeared to extend beyond MTs. Monoclonal antibody to LB3 was used for immunostaining, which stained both the LB3-matrices and the sperm-specific LB4. In this particular egg extract, aster assembly occurred by 5 min of incubation. Scale bar, 10 µm.

**Fig. S5.** Effects of Importin α and β on LB3-matrices that contain SAFs. (A) Binding of the nuclear localization signal (NLS) in the C-terminus of LB3 to importin α. LB3T and LB3T(-)nls were expressed and purified as S-tagged (a 15-meric peptide from ribonuclease S-protein) fusion proteins. SDS-PAGE and Coomassie blue staining showed that LB3T, but not LB3T(-)nls, was associated with importin α. (B) Sensitivity of the interaction between LB3 and importin α and β in the egg extract to RanGTP. Control IgG
or LB3-IgG was used to immunoprecipitate proteins from egg extract in the presence or absence of RanGTP. More importin α and β were co-immunoprecipitated with LB3 in the absence of RanGTP than in the presence of RanGTP. (C) Disruption of matrices by importin α and β. Spindle assembly was induced with AurA-beads and RanGTP. The reaction was diluted 100-fold in XB containing nocodazole and purified importins α and β (2 μM each) or nocodazole and purified RanGTP (5 μM) with importins α and β (2 μM each). The graph on the right shows quantification of beads associated with LB3-matrices under these conditions. Similar percentages were observed for matrices containing SAFs. Scale, (magnetic beads), 2.8 μm.

**Fig. S6.** Mutant LB3 disrupts the localization of Eg5 and XMAP215 to MTs. Spindle assembly was induced with sperm in the presence of wild-type or mutant LB3. MTs (red) were labeled with rhodamine tubulin. Eg5 or XMAP215 was detected by immunostaining with their respective antibodies (green). In the presence of LB3 mutants, Eg5 and XMAP215 often formed aggregates that did not localize to MTs. Scale bars, 10 μm.

**References**

Fig. S1

control siRNA

lamin B siRNA
Fig. S2

A
Pellet | Supernatant
------|--------
LB3   | - - + +
taxol | + - + -
tubulin| + + + +

B
LB3-Ab-beads | + + + +
LB3          | - - + +
taxol      | + + + +
tubulin    | - - - +

C
microtubule

merge
Fig. S3

A  Sperm chromatin

B  MT  LB3
    CM-DiI  Merge

C  MT  LB3
    CM-Dil  Merge

D  MT  LB3
    CM-Dil  Merge
Fig. S4

3 min | 5 min | 10 min | 15 min
---|---|---|---
DNA | | | |
LB3 | | | |
MT | | | |
Merge | | | |
Fig. S6