Figure S1

A

Hoechst  cPKC  NPC
Late embryo extract
Late embryo extract + WGA

20 µm

B

Nuclear size

|                | Buffer control | Late embryo extract | Late embryo extract + WGA |
|----------------|----------------|----------------------|---------------------------|
| Cross-sectional nuclear area | 1.0            | 0.7                  | 1.0                       |

C

cPKC staining intensity

|                  | Late embryo extract | Late embryo extract + WGA |
|------------------|----------------------|---------------------------|
| Nuclear cPKC staining intensity | 1.0               | 0.6                       |
Figure S2

A
Recombinant GFP-LB3 treated with cPKC

Phosphorylation-shifted GFP-LB3 analysed by mass spectrometry

Unshifted GFP-LB3

Coomassie-stained gel

B

| Reference | Peptide                      | Ascore | Site       |
|-----------|------------------------------|--------|------------|
| LB3       | R.QFRSQTK.R                  | 11.3   | T571       |
| LB3       | R.NFSAKLENAQLAAAK.N          | 1000   | S267       |
| LB3       | R.LLNNTEDHSLHGWWVR.R         | 12.9   | S464       |
| LB3       | R.KRHDTIRVIEDSGR.R           | 54.8   | T223       |
| LB3       | R.KLEETGRSVTK.R              | 11.6   | T420       |
| LB3       | R.HDTRVEIDSGR.R              | 110.8  | T223       |
| LB3       | R.EHASAAPSPGSPTISR.M         | 4.8    | S26        |
| LB3       | R.AQIAAGLESSLRDTTK.Q         | 0      | S173,S174  |
| LB3       | K.LSPSPSQRSTVSR.A            | 13.3   | S393       |
| LB3       | K.LSPSPSQRSTTVSR.A           | 0, 7.4 | S393,T397  |
Figure S3

A. LB3 staining and Hoechst images for control oligo and LB3 morpholinos.

B. LB3 staining intensity comparison between control oligo and LB3 morpholinos.

C. Nuclear size comparison between control oligo and LB3 morpholinos.

D. LB3 protein levels based on western blots comparison between control oligo and LB3 morpholinos.

E. Nuclear GFP-LB3 intensity comparison for different microinjected GFP-LB3 mRNA variants.
Figure S4

**A**
HeLa cells transiently transfected with PKC plamids

|          | α | βI | βII | control |
|----------|---|----|-----|---------|
| PKC western |   |    |     |         |

**B**
HeLa cells transiently transfected with PKC siRNA

|          | ζ | α | β |
|----------|---|---|---|
| PKC western |   |   |   |

|          | PKC | Ran |
|----------|-----|-----|

**C**
HeLa siRNA

| HeLa siRNA | control | LA |
|------------|---------|----|
| LA western |         |    |

**D**
HeLa cells transiently transfected with GFP-LA plamids

| HeLa cells transiently transfected | control | wt | S268A | S268E |
|------------------------------------|---------|----|-------|-------|
| GFP-LA western |         |    |       |       |

|          | GFP-LA | actin |
|----------|--------|-------|

**E**
Endogenous LA RNAi and GFP-LA plasmid co-transfections in HeLa cells

![Graph showing cross-sectional nuclear area](image)

|       | Cross-sectional nuclear area (PMA-treated/DMSO-treated) |
|-------|--------------------------------------------------------|
| LA-WT | ***                                                      |
| LA-S268A | NS                                      |
| LA-S268E | NS                                      |
Supplemental Figure Legends

Figure S1. Nuclear shrinking and cPKC nuclear localization are inhibited by WGA. (A) The nuclear shrinking assay was performed as shown in Fig. 1A. Where indicated, late embryo extract was supplemented with 0.2 mg/ml WGA. Fixed nuclei were visualized by immunofluorescence using antibodies against the NPC (mAb414 that recognizes FG-repeat nucleoporins) and cPKC. Representative images are shown. WGA binds to glycosylated FG-repeat nucleoporins, explaining the reduced mAb414-staining intensity of WGA-treated nuclei. Bar, 20 µm. (B) For each sample, the cross-sectional nuclear area of at least 215 nuclei was quantified from NPC-stained nuclei, averaged, and normalized to the buffer control (set at 1.0). Cumulative data from 2 independent experiments are shown. (C) Nuclear cPKC was visualized by immunofluorescence. For each sample, total nuclear cPKC fluorescence intensity was quantified for at least 50 nuclei, averaged, and normalized to the late embryo extract sample (set at 1.0). Cumulative data from 3 independent experiments are shown. ***, P < 0.005; NS, not significant. Error bars represent SD.

Figure S2. PKC phosphorylation site mapping on lamin B3. (A) Recombinant GFP-lamin B3 (GFP-LB3) was incubated with recombinant cPKC as described in Fig. 1B and Materials and Methods, with the only difference being that the reaction was scaled up 4-fold to 100 µl. GFP-LB3 was isolated from the reaction using Ni-NTA beads (Qiagen) and resolved on a SuperSep Phos-tag gel (Wako). The gel was stained with Coomassie. The upper band was excised for phosphorylation site mapping by mass spectrometry. (B) All results from phosphorylation site mapping are shown, with predicted phosphorylation sites highlighted in red. Ascores indicate the confidence with which phosphorylation sites can be assigned (Beausoleil et al., 2006). Sites with an Ascore greater than 19 can be considered confidently assigned, and a score of 1000 indicates an unequivocal localization.

Figure S3. Knockdown of LB3 and nuclear import of LB3 mutants in Xenopus embryos. (A) One-cell embryos were microinjected with either a standard control oligo (Gene Tools) or a combination of two lamin B3 (LB3) morpholinos, as described in Materials and Methods. Embryos were allowed to develop to stage 12. Nuclei were isolated from embryo extracts, fixed, and processed for immunofluorescence using a LB3 antibody. Representative images are shown. Bar, 50 µm. (B) For each sample, at least 30 embryos were microinjected and the LB3 fluorescence intensity of at least 100 nuclei was quantified, averaged, and normalized to the control oligo (set at 1.0). Cumulative data from 2 independent experiments are shown. (C) For each sample, at least 30 embryos were microinjected and the cross-sectional nuclear area of at least 100 nuclei was quantified, averaged, and normalized to the control oligo (set at 1.0). Cumulative data from 2 independent experiments are shown. (D) Western blots were performed using the embryo extracts described in (A). For each sample 25 µg of total protein were separated on a 10% SDS-PAGE gel, transferred to PVDF, and probed with a LB3-specific antibody. Band intensities were quantified by infrared fluorescence and normalized to the control oligo (set at 1.0). Cumulative data from 5 independent experiments are shown. ***, P < 0.005. Error bars represent SD. (E) One-cell stage X.
laevis embryos were co-microinjected with morpholinos to knockdown endogenous LB3 levels and equivalent amounts of mRNA (500 pg per embryo) expressing GFP-LB3 phosphorylation site mutants. Stage 11.5-12 nuclei were isolated and GFP-LB3 images were acquired with the same exposure time. For each sample, the GFP-LB3 fluorescence intensities of >160 nuclei were quantified, averaged, and normalized to LB3-wt (set at 1.0). Cumulative data are shown for at least 2 different fertilizations each with a minimum of 20 embryos. ***, P < 0.005; NS, not significant. Errors bars represent SD. The phospho-null LB3 mutants showed similar intranuclear levels as LB3-wt. The fact that nuclear import efficiency was unaffected, and in particular did not increase, for the LB3-S267A phospho-null mutant demonstrates that this mutant increases nuclear size through a mechanism that does not involve its increased import. On the other hand, the LB3-S267E phosphomimetic exhibited reduced nuclear localization. This could be because the nuclear import efficiency is reduced for this mutant or because the mutant LB3 is more dynamic. In the latter case, the association of LB3-S267E with the nuclear lamina would be reduced leading to less mutant GFP-LB3 protein being retained within the nucleus after fixation and isolation, as observed. While we cannot definitively state why the nuclear localization of LB3-S267E is reduced, we have established that the phosphorylation state of LB3-S267 affects nuclear size, mediated at least in part by altering nuclear lamina dynamics as supported by our FRAP studies (Fig. 2).

Figure S4. Altering LA and PKC expression in HeLa cells. (A) HeLa cells were transiently transfected with mammalian expression vectors (pHACE) expressing constitutively active PKC isoforms. In each case, the N-terminal 29 amino acids encompassing the pseudosubstrate domain had been deleted. 2 days post-transfection, cell lysates were prepared as previously described (Vukovic et al., 2016). Total protein was separated on a 10% SDS-PAGE gel, transferred to PVDF, and probed with a pan-PKC antibody. One representative experiment of two is shown. All transfections were performed in the same way, and equivalent amounts of total protein isolated from equivalent numbers of cells were loaded in each lane. Band intensities were quantified by infrared fluorescence. Compared to endogenous (control) PKC levels set at 1.0, ectopic PKC α, βI, and βII expression was 2.6 ± 2.9, 0.8 ± 0.09, and 7.0 ± 0.2 (average ± SD), respectively. For the experiments shown in Fig. 3B, only PKC α and βII were tested. (B) HeLa cells were transiently transfected with siRNA against the indicated PKC isoforms. 2 days post-transfection, cell lysates were prepared as previously described (Vukovic et al., 2016). Total protein was separated on a 10% SDS-PAGE gel, transferred to PVDF, and probed with pan-PKC and Ran antibodies. One representative experiment of two is shown. Band intensities were quantified by infrared fluorescence, using Ran to normalize. We used siRNA against the exclusively neuronal PKC ζ as a control. Compared to control PKC levels set at 1.0, siRNA against PKC α and β reduced PKC levels to 0.2 ± 0.03 and 0.5 ± 0.3 (average ± SD), respectively. (C) HeLa cells were transiently transfected with a previously described siRNA against the 5'UTR of lamin A (LA) (Lund et al., 2013). 2 days post-transfection, cell lysates were prepared as previously described using equal numbers of cells (Vukovic et al., 2016). Total protein was separated on a 10% SDS-PAGE gel, transferred to PVDF, and probed with a LA-specific antibody. Band intensities were quantified by infrared fluorescence. Consistent with the previous report (Lund et al., 2013), this siRNA reduced LA levels by 81%. (D)
HeLa cells were transiently transfected with the indicated GFP-LA expression plasmids. 2 days post-transfection, cell lysates were prepared as previously described (Vukovic et al., 2016). Total protein was separated on a 10% SDS-PAGE gel, transferred to PVDF, and probed with antibodies against GFP and actin. One representative experiment of two is shown. Band intensities were quantified by infrared fluorescence, using actin to normalize. Compared to LA-wt expression levels set at 1.0, LA-S268A and LA-S268E expression was 1.4 ± 0.6 and 0.8 ± 0.2 (average ± SD), respectively. The wt and mutant proteins are expressed at roughly comparable levels, and variability in expression levels between experiments and constructs likely results from transfection efficiency variability. It is worth noting that in Fig. 5B-C, we analyzed nuclear size and GFP-LA fluorescence on a cell-by-cell basis, thereby accounting for differences in transfection efficiency and GFP-LA expression levels in individual cells. (E) HeLa cells were co-transfected with siRNA against endogenous LA and the indicated GFP-LA expression plasmids. Two days after transfection, cells were treated with 6 nM PMA or DMSO as a control for 90 minutes. Nuclei were visualized with GFP, and cross-sectional nuclear area was quantified for at least 110 nuclei per condition. Cumulative data from 3 independent experiments are shown. For each expressed GFP-LA protein (wt, S268A, or S268E), average cross-sectional nuclear area for PMA-treated cells was normalized to the average cross-sectional nuclear area for DMSO-treated cells (bold horizontal line set at 1.0). For each expressed GFP-LA protein (wt, S268A, or S268E), a Student’s t-test was performed comparing nuclear size data for DMSO-treated and PMA-treated cells. ***P < 0.005; NS, not significant. Error bars represent SD.

**Video Legends**

**Video 1. GFP-LB3 FRAP for nuclei treated with heat inactivated late embryo extract.** Nuclei were assembled in *X. laevis* egg extract at room temperature. 30-40 minutes after initiating nuclear assembly, reactions were supplemented with 28 nM GFP-LB3. After an additional 60-minute incubation, nuclei were isolated and resuspended in late embryo extract that had been heat-inactivated at 56°C for 30 minutes, as depicted in Fig. 1A. Reactions were supplemented with an oxygen scavenging mix. GFP-LB3 was imaged by confocal microscopy, and a 3.6 µm radius circular spot on the surface of the nucleus was photobleached. Two pre-bleach images were acquired. After photobleaching, GFP-LB3 fluorescence recovery was detected by imaging every 5 seconds for a total of 120 seconds.

**Video 2. GFP-LB3 FRAP for nuclei treated with late embryo extract.** Nuclei were assembled in *X. laevis* egg extract at room temperature. 30-40 minutes after initiating nuclear assembly, reactions were supplemented with 28 nM GFP-LB3. After an additional 60-minute incubation, nuclei were isolated and resuspended in late embryo extract, as depicted in Fig. 1A. Reactions were supplemented with an oxygen scavenging mix. GFP-LB3 was imaged by confocal microscopy, and a 3.6 µm radius circular spot on the surface of the nucleus was photobleached. Two pre-bleach images were acquired. After photobleaching, GFP-LB3 fluorescence recovery was detected by imaging every 5 seconds for a total of 120 seconds.
Video 3. GFP-LB3 FRAP for nuclei treated with late embryo extract supplemented with the PKC inhibitor chelerythrine. Nuclei were assembled in X. laevis egg extract at room temperature. 30-40 minutes after initiating nuclear assembly, reactions were supplemented with 28 nM GFP-LB3. After an additional 60-minute incubation, nuclei were isolated and resuspended in late embryo extract supplemented with 0.4 mM chelerythrine to inhibit PKC activity, as depicted in Fig. 1A. Reactions were supplemented with an oxygen scavenging mix. GFP-LB3 was imaged by confocal microscopy, and a 3.6 µm radius circular spot on the surface of the nucleus was photobleached. Two pre-bleach images were acquired. After photobleaching, GFP-LB3 fluorescence recovery was detected by imaging every 5 seconds for a total of 120 seconds.

Supplemental References

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