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Supplemental Information

Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function

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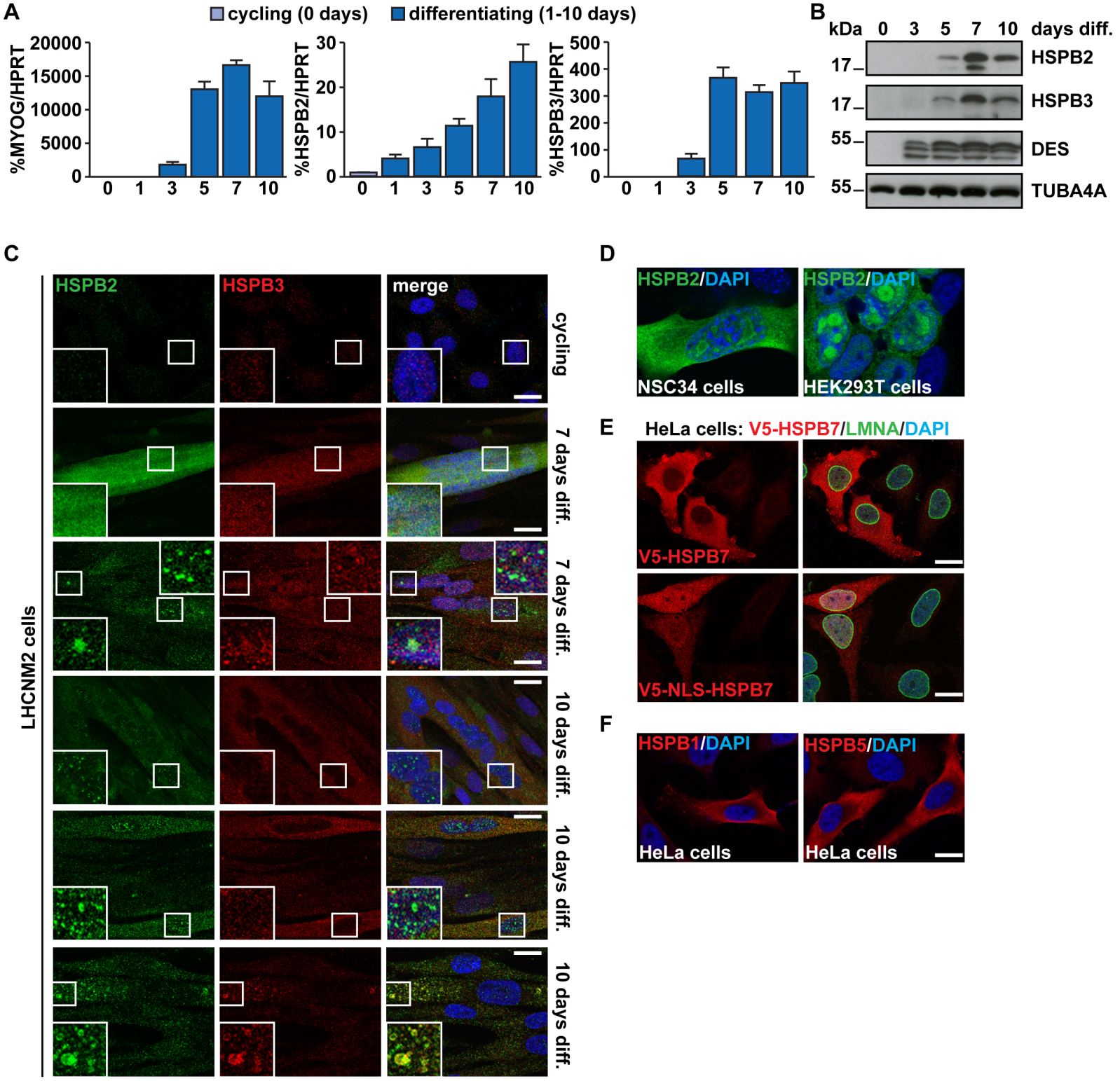

Figure S1: HSPB2 forms nuclear foci in differentiating myoblasts.
Related to Figure 1.

A: RT-qPCR showing the expression levels of myogenin, HSPB2 and HSPB3 in LHCNM2 cells differentiated for 0 days (cycling), 1, 3, 5, 7 or 10 days. HPRT was used as reference gene for qPCR normalisation. **B**: Immunoblot showing the expression levels of HSPB2, HSPB3 and desmin in LHCNM2 cells differentiated for the indicated days (0-10). TUBA4A was used as loading control. **C**: Immunofluorescence showing the subcellular distribution of HSPB2 and HSPB3 in LHCNM2 cells differentiated for 0 days (cycling), 7 or 10 days. **D**: Immunofluorescence showing the subcellular distribution of overexpressed HSPB2 in NSC34 and HEK293T cells. **E**: Immunofluorescence showing the subcellular distribution of overexpressed V5-tagged HSPB7 or NLS-HSPB7 in HeLa cells. Cells were stained for V5, LMNA and nucleic acid (DAPI). **F**: Immunofluorescence showing the subcellular distribution of overexpressed HSPB1 and HSPB5 in HeLa cells. Scale bars 15 µm.

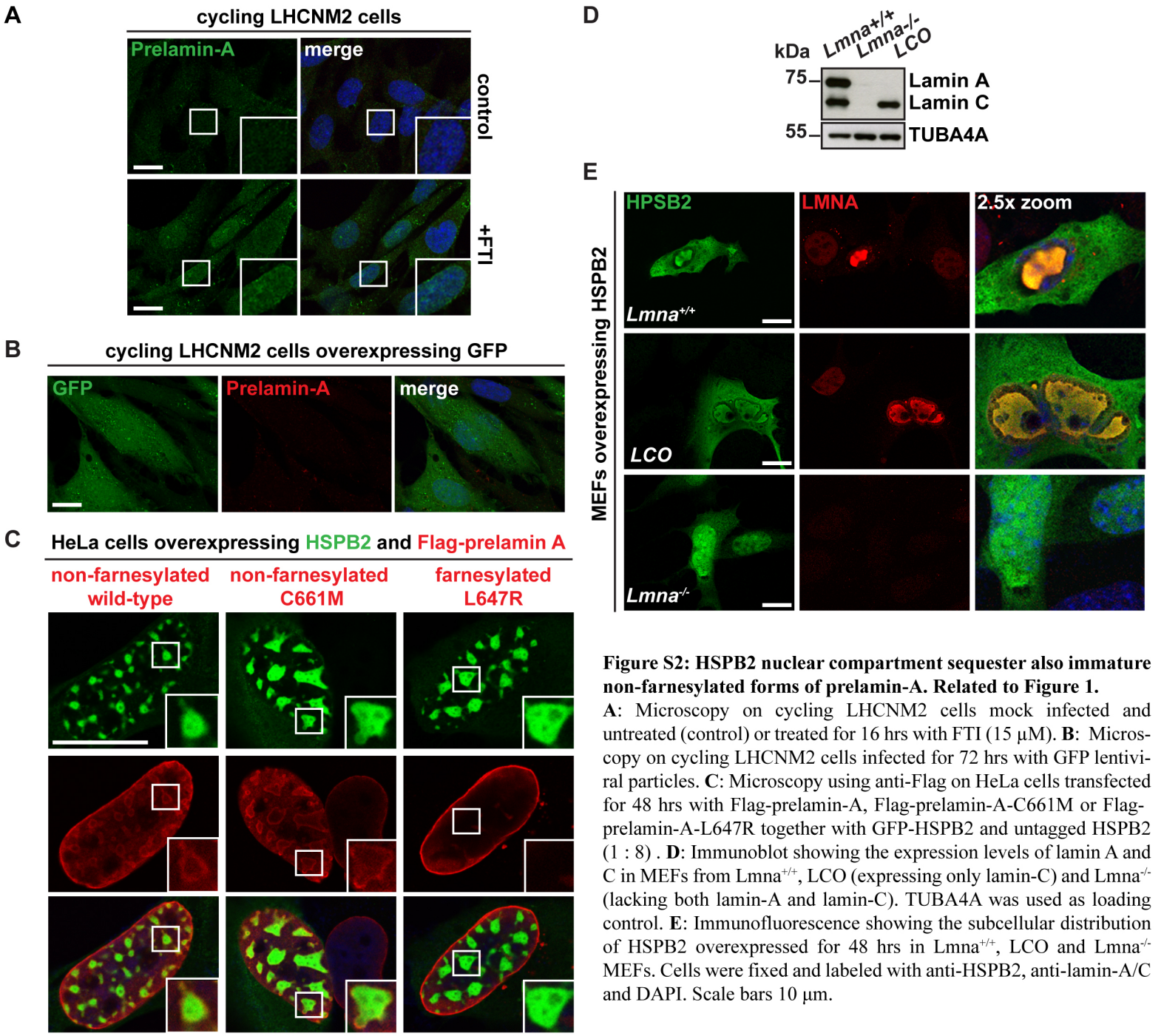

Figure S2: HSPB2 nuclear compartment sequester also immature non-farnesylated forms of prelamin-A. Related to Figure 1.
A: Microscopy on cycling LHCNM2 cells mock infected and untreated (control) or treated for 16 hrs with FTI (15 μM). **B**: Microscopy on cycling LHCNM2 cells infected for 72 hrs with GFP lentiviral particles. **C**: Microscopy using anti-Flag on HeLa cells transfected for 48 hrs with Flag-prelamin-A, Flag-prelamin-A-C661M or Flag-prelamin-A-L647R together with GFP-HSPB2 and untagged HSPB2 (1 : 8) . **D**: Immunoblot showing the expression levels of lamin A and C in MEFs from Lmna$^{+/+}$, LCO (expressing only lamin-C) and Lmna$^{-/-}$ (lacking both lamin-A and lamin-C). TUBA4A was used as loading control. **E**: Immunofluorescence showing the subcellular distribution of HSPB2 overexpressed for 48 hrs in Lmna$^{+/+}$, LCO and Lmna$^{-/-}$ MEFs. Cells were fixed and labeled with anti-HSPB2, anti-lamin-A/C and DAPI. Scale bars 10 μm.

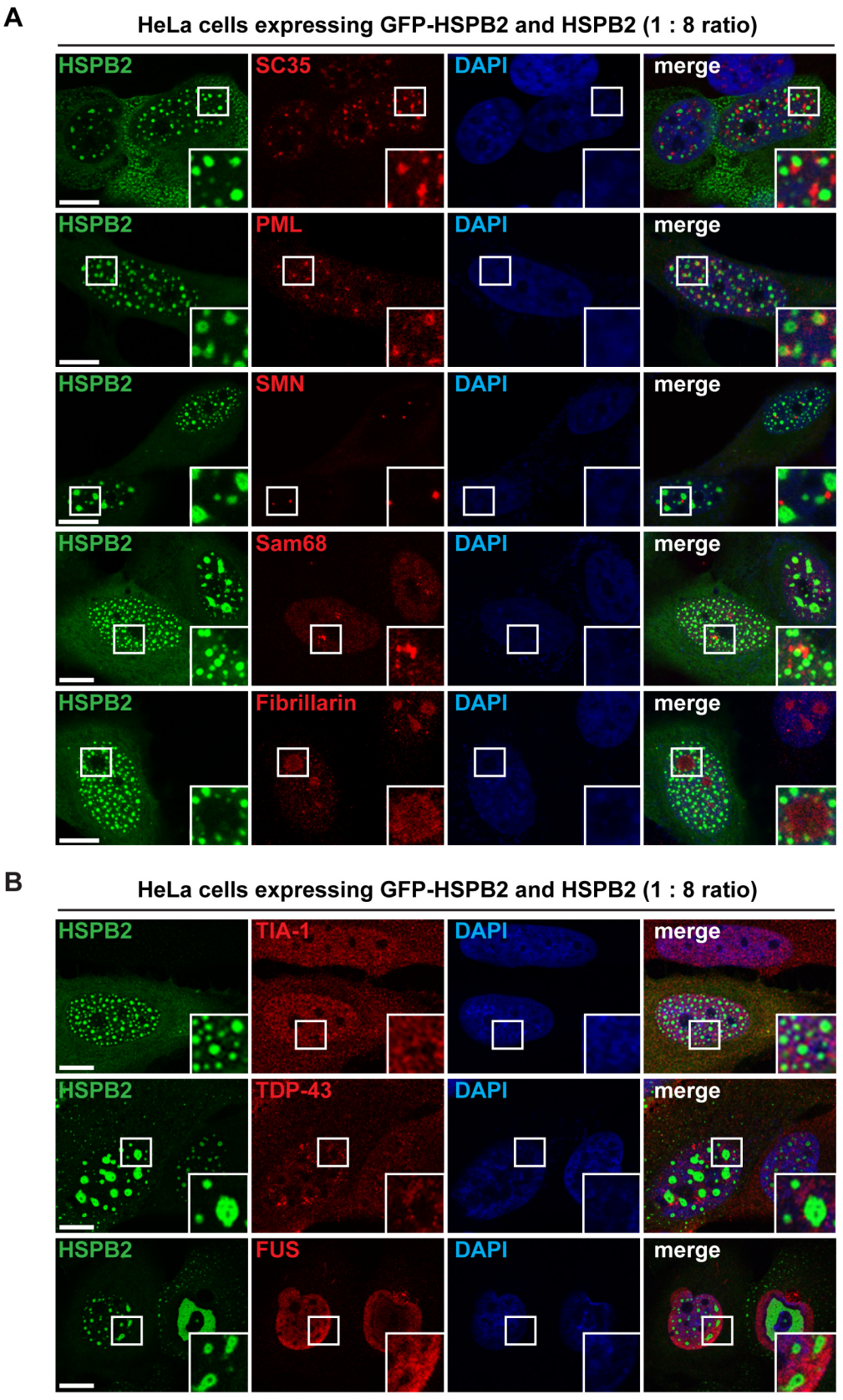

Figure S3: HSPB2 compartments do not colocalize with other known nuclear membrane-less organelles.
Related to Figures 1 and 2.

A: Microscopy on HeLa cells transfected for 48 hrs with GFP-HSPB2 and untagged HSPB2 (1 : 8) using antibodies specific for membrane-less nuclear body markers: speckles (anti-SC35), PML-nuclear bodies (anti-PML), Cajal bodies (anti-SMN), nucleoli (anti-fibrillarin) and Sam68 nuclear bodies (anti-Sam68). **B**: Microscopy on HeLa cells transfected for 48 hrs with GFP-HSPB2 and untagged HSPB2 (1 : 8) using antibodies specific for the RNA-binding proteins TIA-1, TDP-43 and FUS. Scale bars 5 μm.

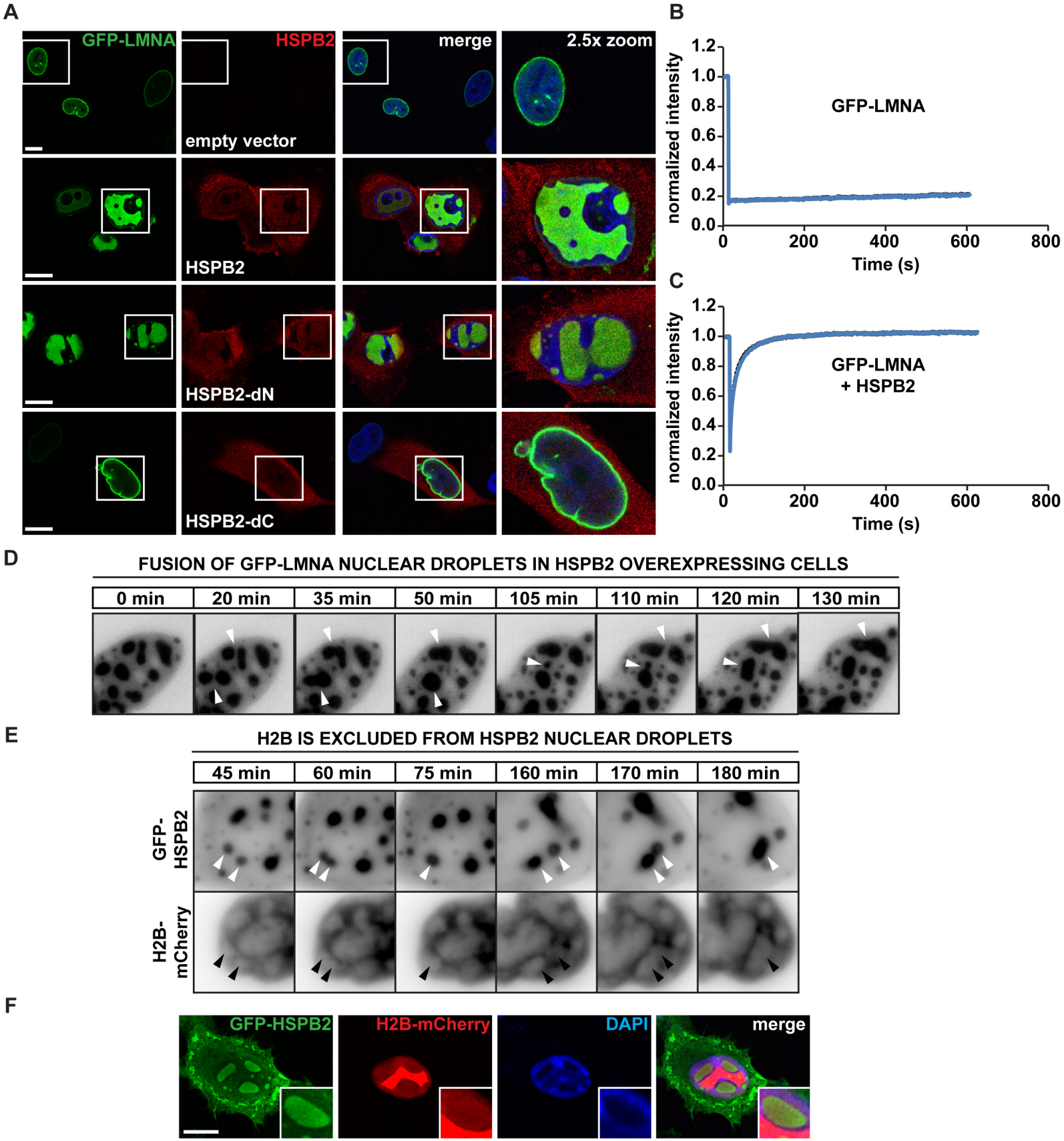

Figure S4: HSPB2 aberrant phase separation affects LMNA and H2B mobility and distribution.
Related to Figures 2, 3 and Movies S5-7.

A: Immunofluorescence on HeLa cells overexpressing for 48 hrs GFP-LMNA and an empty vector, HSPB2, HSPB2-dN or HSPB2-dC. **B**: Quantification of the fluorescence intensity recovery after bleach of GFP-LMNA expressed alone. The time scale of a bleach experiment is shown. n = 39, +/- SEM. **C**: Quantification of the fluorescence intensity recovery after bleach of GFP-LMNA nuclear droplets in HeLa cells overexpressing GFP-LMNA with HSPB2. The time scale of a bleach experiment is shown. n = 43, +/- SEM. **D**: Inverted black and white images of GFP-LMNA in HeLa cells overexpressing GFP-LMNA with HSPB2. Fusion events of GFP-LMNA nuclear droplets are indicated by arrowheads. **E**: Inverted black and white images of GFP-HSPB2 and H2B-mCherry in HeLa cells. Fusion events of GFP-HSPB2 nuclear droplets and exclusion of H2B-mCherry are indicated by arrowheads. **F**: Immunofluorescence on HeLa cells overexpressing for 48 hrs GFP-HSPB2 and H2B-mCherry and showing that GFP-HSPB2 displaces H2B-mCherry and DNA (DAPI). Scale bars 10 µm.

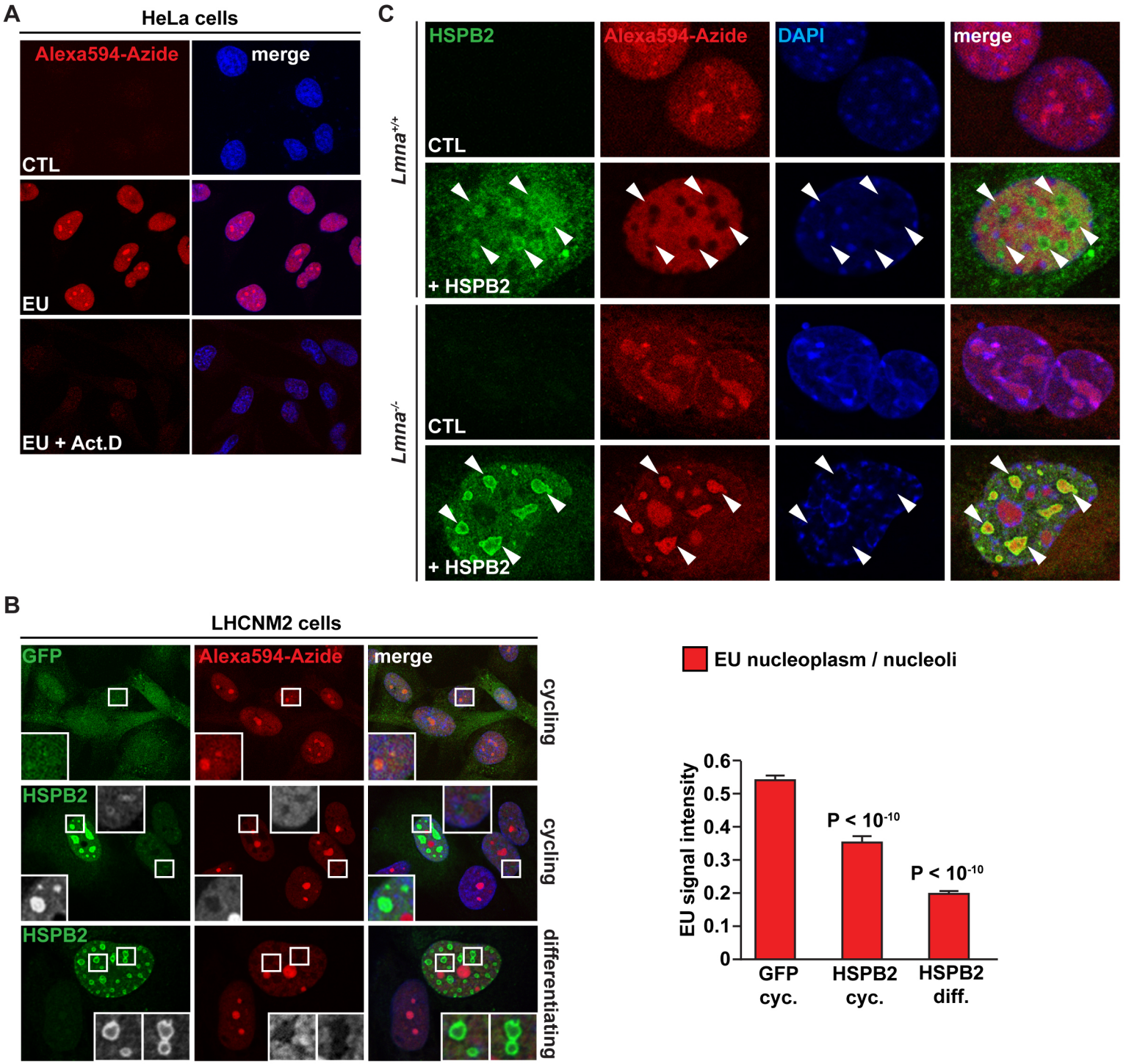

Figure S5: HSPB2 phase separation inhibits RNA transcription.
Related to Figure 3.

A: HeLa cells were left untreated (CTL) or incubated with 5-ethynyl uridine (EU; 200 µM) alone or with actinomycin D (EU + Act.D; 2 µM) for 6 hrs. Cells were then fixed and stained with Alexa594-Azide to visualize newly synthetized RNAs.

B: Cycling and differentiating LHCNM2 cells overexpressing GFP or HSPB2 were incubated with EU (200 µM) for 6 hrs. Cells were fixed, stained with Alexa594-Azide and subsequently, where indicated, for HSPB2. The signal intensity of EU was quantified in the nucleoli and in the nucleoplasm and nucleoplasm/nucleoli ratio was calculated. Quantitation of EU signal intensity is reported. n = 105-117, +/- SEM; $P < 10^{-10}$.

C: $Lmna^{+/+}$ and $Lmna^{-/-}$ (lacking both lamin-A and lamin-C) MEFs were transfected with an empty vector (CTL) or HSPB2. 48 hrs post-transfection, cells were incubated with 5-ethynyl uridine (EU; 200 µM) for 6 hrs, fixed and stained with Alexa594-Azide, anti-HSPB2 and DAPI. Confocal microscopy shows that in $Lmna^{+/+}$ MEFs HSPB2 nuclear compartments are all devoid of EU (257 HSPB2-overexpressing cells analyzed). Instead, in $Lmna^{-/-}$ MEFs, HSPB2 nuclear compartments all colocalize with newly synthetized RNAs (196 HSPB2-overexpressing cells analyzed).

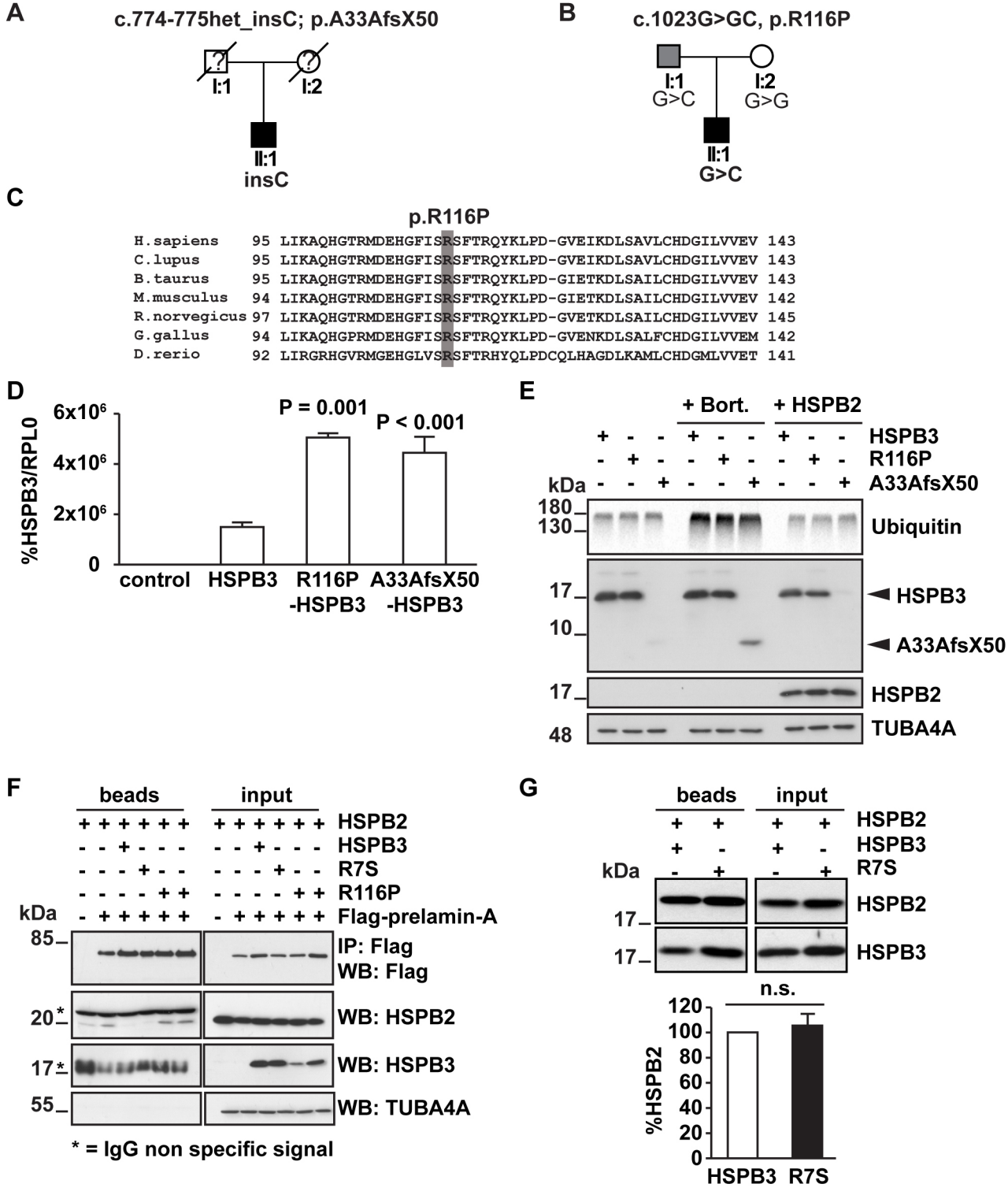

Figure S6: Sequencing analysis identified two putative mutations in HSPB3 in two patients affected by myopathy. Related to Figures 4, 5 and 6.

A, B: Black symbols = affected individuals; grey symbol = asymptomatic individual; white symbols = unaffected individuals, diagnonal line = person diseased, and question marks = diagnosis unavailable. **C**: Comparison of the HSPB3 protein sequence in different species shows the conservation of the R116 residue. R116 is located in the a-crystallin domain of HSPB3. **D**: RT-qPCR analysis of myc-tagged HSPB3, HSPB3-R116P and HSPB3-A33AfsX50 mRNA expression levels after overexpression for 48 hrs in HeLa cells. n = 3, +/- SEM. $P < 0.001$. **E**: Immunoblot showing the expression levels of myc-tagged HSPB3, HSPB3-R116P and HSPB3-A33AfsX50 in HeLa cells transfected for 48 hrs. Where indicated cells were co-transfected with HSPB2 (+ HSPB2) or treated with Bortezomib (+ Bort.; 100 nM) overnight prior to protein extraction. TUBA4A was used as loading control. **F**: HEK293T cells were transfected with cDNAs encoding for Flag-prelamin-A and an empty vector or HSPB2 alone or combined with myc-HSPB3, myc-R7S-HSPB3 or myc-R116P-HSPB3. Due to myc-R116P-HSPB3 high aggregation propensities and in order to reach in the supernatant fraction used for co-immunoprecipitation expression levels of myc-R116P-HSPB3 similar to the ones of myc-HSPB3 or myc-R7S-HSPB3, two increasing amounts of cDNAs encoding for myc-R116P-HSPB3 were transfected. 24 hrs post-transfection, cells were subjected to immunoprecipitation with a Flag antibody. * indicates IgG or nonspecific signal. **G**: HeLa cells were transfected with vectors encoding for his-HSPB2 and myc-HSPB3 or myc-R7S-HSPB3. 24 hrs post-transfection, cell lysates were subjected to NiNTA pull-down. Quantitation of data of five independent samples is shown. n.s: non-significant. **F, G**: Total cell lysates (input) and immunocomplexes (beads) were processed for western blot.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture, Transfection and Treatments

HeLa, Lmna$^{+/+}$, Lmna$^{-/-}$ (Sullivan et al., 1999), LCO (Fong et al., 2006) MEFs, NSC34 and HEK293T cells were cultured in DMEM (ECB7501L; EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% Fetal Bovine Serum (F7524; Sigma-Aldrich, Milan, Italy) in a 37°C incubator with 5% CO_2. Lmna$^{+/+}$, Lmna$^{-/-}$, LCO MEFs were kindly provided by Dr. J. Lammerding (Cornell University). Cycling LHCNM2 cells were kindly provided by Prof. E. Pegoraro and were cultured in HAM's F12 (ECB7502L; EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 20% FBS (Gibco 10106-169; Invitrogen, USA) and 25 ng/mL of rh FGF-b/FGF-2. Differentiated LHCNM2 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 2% horse serum (Gibco 26050-088; Life Technologies, USA) and 30 µg/mL of insulin. We thank Dr. E. Galletta, Dr. C. Stewart and Dr. S. Young for providing LHCNM2, Lmna$^{-/-}$ and LCO cells, respectively. Stable HeLa-Kyoto cells expressing C-terminally mCherry-tagged human H2B were generated by random integration into the genome via BAC recombineering technology (Poser et al., 2008). H2B-mCherry cells were kept under selection using Puromycin (Sigma-Aldrich P8833, 0.5 µg/ml). Transfections were performed using the calcium phosphate method as previously described (Carra et al., 2005). Unless otherwise indicated, cells were transfected with cDNAs for 48 hrs.
Cells were treated with the following drugs at the concentrations indicated here: arsenite 0.5 mM; actinomycin D (ActD) 2 µM; bortezomib (Bort) 100 nM; 5-Ethynyl Uridine (EU) 200 µM.

Generation of cDNAs

The generation of the human HSPB plasmid library was described before (Vos et al., 2010). The sequence encoding for human non-tagged HSPB2, HSPB5 and HSPB3 wildtype, myc-HSPB1, myc-HSPB3, myc-R7S-HSPB3, myc-R116P-HSPB3, myc-R7S-HSPB3 and myc- A33AfsX50-HSPB3 were subcloned by PCR into the pCDNA5.1-FRT/TO.
FLAG-tagged plasmids containing wild-type prelamin A, the non-farnesylatable mutant LA-C661M and the farnesylated mutant LA-L647R were previously described (Mattioli et al., 2011).

Immunofluorescence on cultured cells

HeLa, Lmna$^{+/+}$, Lmna$^{-/-}$, LCO and HEK293T cells were grown on polylysine-coated glass coverslip, while NSC34 cells were grown on uncoated glass coverslip. Cycling LHCNM2 cells were grown on coverslips coated with gelatin (0.1%), while differentiating LHCNM2 cells were grown on plastic chamber slides. Cells were washed with cold PBS and fixed with 3.7% formaldehyde in PBS for 9 minutes at room temperature, followed by permeabilization with cold acetone for 5 minutes at -20°C. Alternatively, cells were fixed with ice-cold methanol for 10 minutes at -20°C. Blocking and incubation with primary and secondary antibodies were performed in PBS containing 3% BSA and 0.1% Triton X-100. The primary and secondary antibodies used are listed in the section of supplemental experimental procedures. Analyses of the cells were done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems) and a 63x oil-immersion lens.

Reagents and Antibodies

The reagents used in this study include: 5-Ethynyl Uridine (E10345, Life Techologies); actinomycin D (A1410, Sigma-Aldrich); bortezomib (S1013, Selleck Chemicals); DAPI (sc-3598, Santa Cruz Biotechnology); insulin (I1882, Sigma-Aldrich); polybrene (107689, Sigma-Aldrich); sodium arsenite (Carlo Erba Reagents, Cornaredo, Italy); rh FGF-b/FGF-2 (11343625,ImmunoToos); Farnesyltransferase inhibitor (BML-G242; Enzo Life Sciences).

The antibodies used in this study are: mouse monoclonal anti-HSPB2 (sc-136339, Santa Cruz Biotechnology); rabbit polyclonal anti-HSPB3 (SAB1100972, Sigma-Aldrich); rabbit polyclonal anti-ubiquitin (Z 0458, DakoCytomation); mouse monoclonal anti-α-tubulin (T6074, Sigma-Aldrich); goat polyclonal anti-lamin B (sc-6217, Santa Cruz Biotechnology); rabbit polyclonal anti-lamin A/C (sc-20681, Santa Cruz Biotechnology); goat polyclonal anti-lamin A (sc-6214, Santa Cruz Biotechnology); rabbit polyclonal anti-Desmin (sc-14026, Santa Cruz Biotechnology); rabbit polyclonal anti-Myogenin (sc-576, Santa Cruz Biotechnology); mouse monoclonal anti-V5 (R960-25, Life Technologies); mouse monoclonal anti-SC-35 (S4045, Sigma-Aldrich); mouse monoclonal anti-myc (9E10; sc-40, Santa Cruz Biotechnology; used for western blotting); mouse monoclonal anti-myc (9E10; kindly provided by Prof. R. Tanguay; used for western blotting and immunofluorescence); mouse monoclonal anti-αB Crystallin (SMC-159A, StressMarq Biosciences Inc); rabbit polyclonal anti-PML (sc-5621, Santa Cruz Biotechnology); mouse monoclonal anti-SMN (sc-365909, Santa Cruz Biotechnology); goat polyclonal anti-TIA-1 (sc-1751, Santa Cruz Biotechnology);mouse monoclonal anti-TDP-43 (60019-2-Ig, Proteintech); mouse monoclonal anti-FUS/TLS (sc-373698, Santa Cruz Biotechnology); mouse monoclonal

anti-Fibrillarin (sc-374022, Santa Cruz Biotechnology); rabbit polyclonal anti-Prelamin A (ANT0045, Diatheva); mouse monoclonal anti-SAM68 (sc-1238, Santa Cruz Biotechnology); mouse monoclonal anti-Flag (F1804, Sigma-Aldrich). Rabbit polyclonal anti-SUN2 was kindly provided by Prof. S. Marmiroli; rabbit polyclonal anti-HSPB1 and anti-HSPB8 are homemade antibody kindly provided by Prof. J. Landry.

The Alexa-conjugated secondary antibodies used in this study were from Life Technologies and include: donkey-anti-goat-Alexa488, donkey-anti-goat-Alexa647, donkey-anti-mouse-Alexa594, donkey-anti-rabbit-Alexa594, donkey-anti-got-Alexa594, donkey-anti-rabbit-Alexa488, donkey-anti-mouse-Alexa488, goat-anti-rabbit-Alexa488, goat-anti-rabbit-Alexa594, goat-anti-mouse-Alexa488, goat-anti-rabbit-Alexa594 or goat-anti-mouse-Alexa647. For click-chemistry on EU-treated cells we used Alexa fluor 594 Azide (A10270, Life Technologies). Mouse and rabbit HRP-conjugated secondary antibodies for western blot were from GE Healthcare Europe GmbH.

Preparation of samples for western blotting

For the preparation of total protein extracts, cells were lysed in Laemmli sample buffer and homogenized by sonication. Protein samples were boiled for 3 minutes at 100 °C, reduced with β-mercaptoethanol and separated by SDS-PAGE, followed by western blot.

5-Ethynyl Uridine (EU) Detection by Click Chemistry

The protocol for EU labelling was performed basically as described by Jao and Salic (2008). Briefly, 48 hrs post-transfection cells were incubated at 37°C with 5% CO_2 with 200 µM of EU for 6 hrs. After EU labelling, cells were fixed (125 mM Pipes pH 6,8, 10 mM EGTA, 1 mM magnesium chloride, 0.2% Triton X-100, 3.7% formaldehyde) for 30 minutes at room temperature and processed for Alexa Fluor 594 Azide staining (100 mM Tris pH 8,5, 1 mM $CuSO_4$, 10 µM Alexa Fluor 594 Azide, 100 mM ascorbic acid) for 30 minutes at room temperature and in dark condition. Cells were next processed for immunofluorescence microscopy as described above.

Viral vector production and lentiviral vectors

Lentiviral particles were generated by transient co-transfection using the calcium phosphate method of HEK293T cells with the 3rd generation packaging systems (pMDlg/pRRE #12251; pRSV-Rev #12253; pMD2.G #12259; Addgene) and lentiviral vectors encoding for Human HSPB2 (EX-Q0523-Lv105; Tebu-bio), Human myc-HSPB3 wt (EX-T1904-Lv107; Tebu-bio) or myc-HSPB3 R116P (CS-T1904-Lv107-01). For lentiviral particles expressing GFP the modified pLKO.1 GFP plasmid (Benatti et al., 2011) and the 2nd generation packaging systems (pCMV-dR8.74 #22036; pMD2.VSVG #12259; Addgene) were used. Shcontrol (RHS4346; Dharmacon) and shHSPB3 (VGH5518-200215240; Dharmacon) lentiviral particles were generated using GIPZ™ Lentiviral shRNA (Dharmacon) according to the manufacturer's instructions. Cell medium was replaced 16 hrs after transfection. 32 hrs later, the medium containing the lentiviral particles was harvested, filtered by 0.45 µm filter and stored at -80°C or ultracentrifuged at 19.000 rpm for 2 hrs and 30 minutes at 4°C on SW-28 rotor in a Beckman refrigerated centrifuge. Then the viral pellet was re-suspended with sterile DMEM and stored at -80°C.

In vitro transduction

Cycling LHCNM2 were transduced by adding viral supernatant to infection medium (HAM's F12, 2 mM L-glutamine, 10% heat-inactivated FBS, 25 ng/mL of rh FGF-b/FGF-2) supplemented with 8 µg/mL polybrene. 24 hrs post-transduction, the medium was replaced with fresh cycling medium, and left for 48 hrs prior to fixation. For experiments under differentiation conditions, 24 hrs post-transduction, the medium was replaced with differentiation medium and left for ca. 120 hrs prior to fixation. For lentiviral particles expressing HSPB2 or HSPB3, the infection efficiency was calculated via immunofluorescence, while it was calculated both via immunofluorescence and FACS for GFP expressing lentiviral particles. For western blot analysis, 24 hrs post-transduction, LHCNM2 cells infected with lentiviral particles expressing HSPB2 or HSPB3 were selected with puromycin (4 µg/mL). 48 hrs after, cells were lysed in Laemmli buffer.

Immunoprecipitation assay and Ni-NTA purification assay

24 hrs post-transfection cells were lysed in lysis buffer (150 mM NaCl, 0.5% NP40, 1.5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 3% glycerol, 1 mM DTT, complete EDTA-free, Roche). The cell lysates were centrifuged and cleared with A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C for 1 h. Mouse TrueBlot beads (88-7788-31; tebu-bio) at 4°C for 1 h. Mouse TrueBlot beads (88-7788-31; Tebu-bio) complexed with specific antibodies were added to the precleared lysates. After incubation for 1 h at 4 °C, the immune complexes were centrifuged. Beads were washed four times with the lysis buffer; both co-immunoprecipitated proteins and input fractions were resolved on SDS-PAGE.

For the purification of His-tagged HSPB2 with Ni-NTA, 24h post-transfection cells were lysed in lysis buffer (150 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 0.5% NP40, 1.5 mM $MgCL_2$, 3% glycerol, 0.9 mM DTT and protease inhibitors). Lysates were centrifuged at 14.000 rpm for 15 minutes at 4°C. An aliquot of the supernatant was kept as input fraction. Ni-NTA agarose beads (Qiagen) were added to the rest of the supernatant and incubated at 4°C for 1 h with slow agitation. The beads were washed once with lysis buffer and four times with washing buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, 0.5% NP40, 1.5 mM $MgCl_2$, 3% glycerol).
Proteins bound to the beads were recovered by boiling in 2% SDS laemmli buffer supplemented with β-mercaptoethanol and processed for SDS-PAGE followed by western blot.

Fractionation of cytoplasmic and nuclear proteins
For the fractionation of cytoplasmic and nuclear proteins, cells were harvested and homogenized in a buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, DTT 1 mM, NP40 0.15% and 1% complete EDTA-free (Roche). Cell lysates were centrifuged at 12.000 rpm for 30 seconds at 4°C, to separate the supernatant (cytosolic fraction) from the pellet. The pellet fraction was lysed in buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1 mM DTT, 0.5% NP40 and 1% complete EDTA-free (Roche), sonicated and centrifuged. The supernatant (nuclear fraction) was transferred into a new tube. Laemmli buffer (to a final 2% SDS concentration) was added to the cytosolic and nuclear fractions prior to SDS-PAGE followed by western blot.

RNA extraction, RT-PCR and Real-Time PCR
Total RNA was isolated using Trizol reagent (15596-026; Life Technologies) and subsequently treated with DNase I according to the manufacturer's instructions. First-strand cDNA was generated using High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) according to the manufacturer's instructions. The relative changes in the levels of mRNAs for human HSPB2, HSPB3, myogenin and hypoxanthine guanine phosphoribosyltransferase (HPRT), used as housekeeping gene, were determined using CFX96 Touch Thermal cycler (Bio-Rad, Hercules, CA, USA) in combination with SYBR green master mix. The primers used were all purchased from Sigma-Aldrich and are listed below: HSPB2 For (CATGGTCCACAATGTATGGT); HSPB2 Rev (ATTTGGGTTTATTCAGCTCCAC); HSPB3 For (GACTAAGTGACATCGTATCGG); HSPB3 Rev (ACAAACATTCTCGTAGTACCAG); myogenin For (CACTCCCTCACCTCCATCGT); myogenin Rev (CATCTGGGAAGGCCACAGA); HPRT For (CGTCGTGATTAGCGATGATGA); HPRT Rev (TCCAAATCCTCGGCATAATGA). The real-time PCR was performed as follows: one cycle of denaturation (95°C for 3 minutes) and 40 cycles of amplification (95°C for 10 seconds, 60°C for 30 seconds). A triplicate of each sample was analyzed. Data were analyzed with Bio-rad CFX Manager 3.1 (Windows 7.0).

Electron Microscopy (EM)
Cell pellets were washed with PBS and fixed with 2.5 M glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. After post-fixation with 1% osmium tetroxide (OsO_4) in cacodylate buffer 0.1 M for 1h at 4°C, the pellets were dehydrated in an ethanol series, infiltrated with propylene oxide and embedded in Epon resin. Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate and were observed with a JEOL JEM 1011 trasmission electron microscope, operated at 100kV. At least 200 cells were examined for each sample.

Live imaging
For live-imaging, every 3 min for GFP-HSPB2 and every 5 min for GFP-LMNA and H2B-mCherry, 6 sections with 800-nm spacing were acquired and the maximum intensity projections were created in Fiji (www.Fiji.sc).

Fluorescence recovery after photobleaching (FRAP) and fluorescence density analysis
Using a 100x oil immersion objective a region of approximately 2.09 x 2.09 μm^2 was bleached for 50 ms using a laser intensity of 20% at 405 nm (2.3 mW for GFP). Recovery was recorded for 510-600 time points after bleaching (600-1020 s). Analysis of the recovery curves were carried out with the FIJI/ImageJ.
The flow of the protein within the droplet was measured by quantifying the recovery of the bleached area at the cost of the unbleached region by a custom written FIJI/ImageJ routine. During image acquisition, the bleached region was corrected for general bleaching. We quantified the molecules that move from the unbleached region to the bleached region, leading to the recovery of the bleached region.
Prior to FRAP analysis the images were corrected for drift using the StackReg plug-in function of the FIJI software suite. FRAP analysis was carried out using the following equation: $((I_{bleach} - I_{background}) / (I_{bleach(t0)} - I_{background(to)})) / ((I_{total} - I_{background}) / (I_{total(t0)} - I_{background(to)}))$, where I_{bleach} is the fluorescence intensity in the bleach area, $I_{background}$ the background the camera offset and I_{total} is the fluorescence intensity of the entire cellular structure. Mean and standard deviation were obtained from averaging FRAP curves.

Analysis of fluorescent density was performed using FIJI/ImageJ and selecting specific ROI (region of interest). Briefly, for the analysis of HSPB2 critical threshold, HSPB2 fluorescence intensity in the ROI (nucleoplasm or nuclear droplets) was divided for the HSPB2 fluorescence density in the cytoplasm, selecting a region where no HSPB2 phase separation occurred. For the analysis of EU incorporation, Alexa594-Azide fluorescence intensity in the ROI (nucleoplasm) was divided for the Alexa594-Azide fluorescence intensity in the nucleoli.

Sequencing of *HSPB2* and *HSPB3*

The genomic DNA of all participating subjects in this study was used to amplify the *HSPB2* and *HSPB3* coding regions (GenBank accession numbers: NM 001541 and 006308) by PCR. The coding exons were amplified by a standard PCR using the following primers for HSPB3: HSPB3-For 3' GACTGAAGGCAGTGGAAGGT '5 and HSPB3-Rev 3'TGTGGGTAATACAAACATTCTCG and for HSPB2: HSPB2_FOR: GGCACTATTTTGGGTGGTGT; HSPB2_REV: AGGGGATGAGGGGTAGTCTG; HSPB2_1FOR: CTGTGCCAGCCTCATCCT; HSPB2_1REV: GATGCTGCTACCTCTGGAGTG. The resulting amplicons, including the intron-exon boundaries, were screened for mutations via Sanger sequencing on an ABI 3700 (Applied Biosystems). The DNA sequences were analyzed using Mutation Surveyor software (Softgenetics).

SUPPLEMENTAL REFERENCES

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