**Supporting Information for**

# Nanobar Array Assay Revealed Complimentary Roles of BIN1 Splice Isoforms in Cardiac T-tubule Morphogenesis

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**Supporting Information-Figures:**

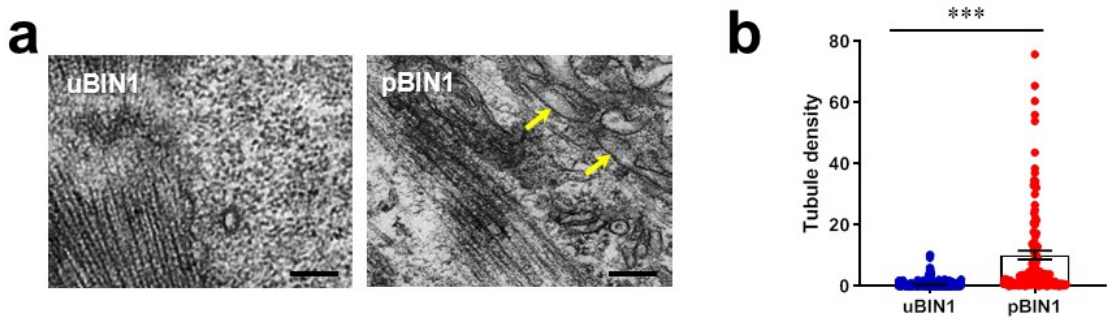


**Figure S1. Tubules were induced by pBIN1 but not by uBIN1.** (**a)** Typical transmission electron microscopic images of cardiomyocytes transfected with eGFP-tagged uBIN1 and pBIN1 splices. Yellow arrows indicate tubular structures. Scale bar, 200 nm. (**b)** The volume density of tubular structures was measured using the stereological morphometric method. More than 1700 $\mu m^2$ of image area was analyzed in each group. ***$P < 0.001$ *vs* uBIN1 group by *t*-test.

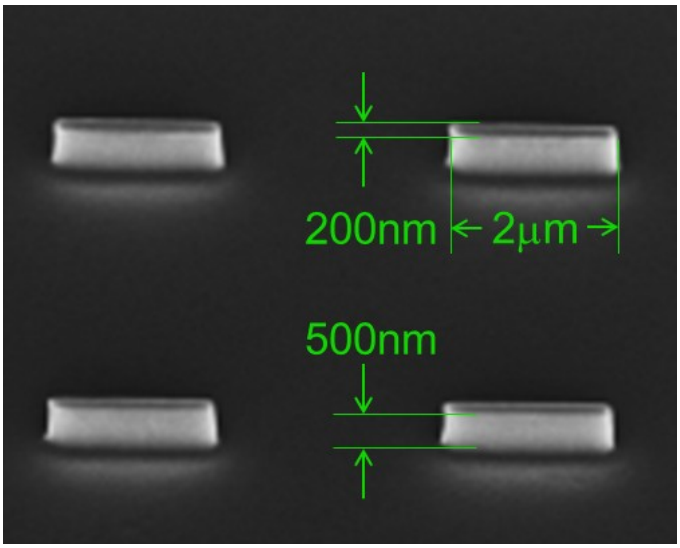


**Figure S2. The morphology of engineered nanobars.** The scanning electron microscopic image shows individual nanobar of 200 nm width, 2 μm length and 500 nm height.

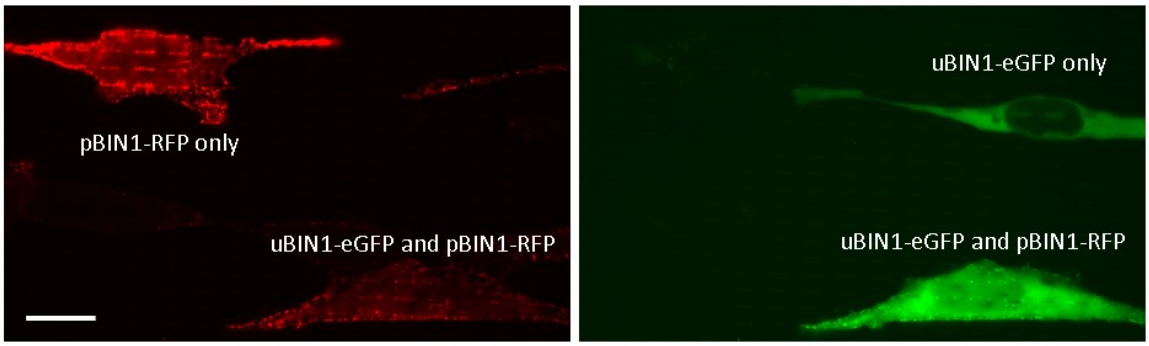


**Figure S3. Independence of florescence between RFP (left) and eGFP (right) channels.** In this experiment, cells expressed with pBIN1-RFP only, uBIN1-eGFP only and uBIN1-eGFP combined with pBIN1-RFP were mixed together and imaged in both eGFP and RFP channels. Bar size 20 μm. Note that the RFP signal did not cause any detectable signal in the eGFP channel, and *vice versa*.

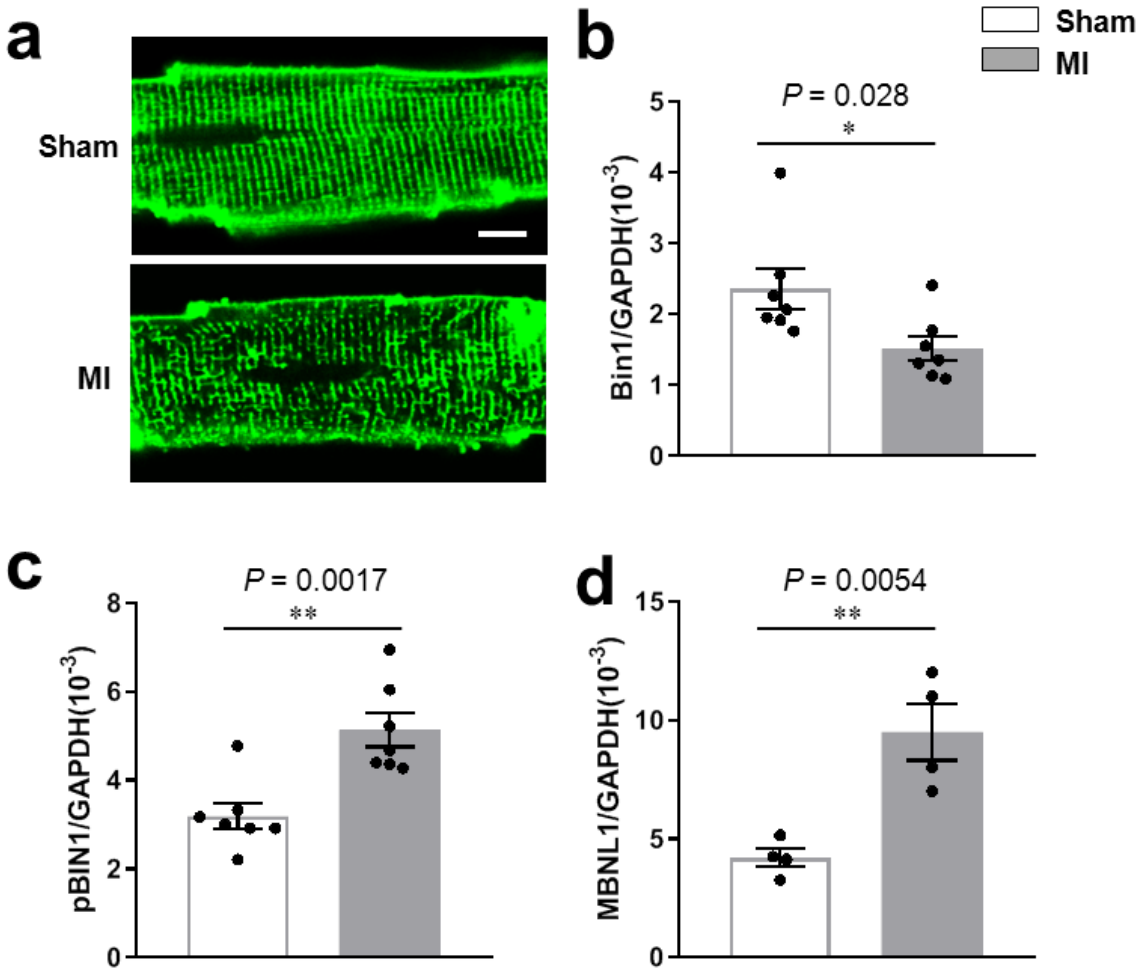


**Figure S4. Altered expression of *BIN1* splice variants in rat MI model.** (**a)** Representative confocal images of TTs stained with Di-8-ANEPPS, showing typically disordered TTs after MI. Scale bar, 10 µm. (**b**) Analysis of the relative expression of *uBIN1* (*Bin1*) mRNA. $N = 7$ / group. **(c)** Analysis of the relative expression of *pBIN1* mRNA. $N = 7$ / group. **(d)** Analysis of the relative mRNA expression of the splicing modulator *MBNL1*. $N = 4$ / group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* sham group by *t*-test.

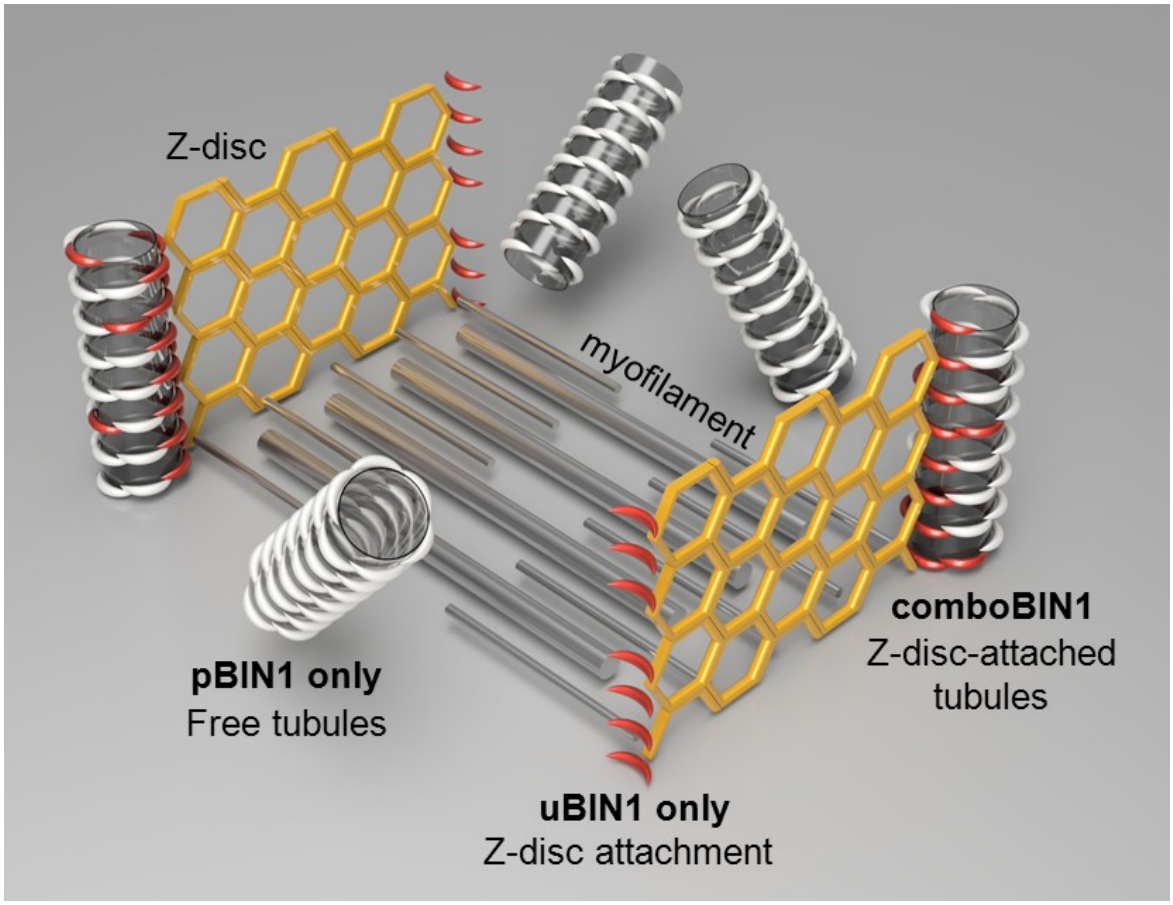


**Figure S5. ComboBIN1 model for the roles of uBIN1 and pBIN1 cooperation in tubule generation along Z-discs.** When expressed alone, pBIN1 molecules organize free membrane tubules, while uBIN1 molecules are attached along Z-discs without membrane tubulation. When pBIN1 and uBIN1 are co-expressed, the heteropolymerization of pBIN1 and uBIN1 renders a comboBIN1 helix with both membrane tubulation and Z-line-positioning capabilities.

**Supporting Information – Methods:**

## Animals

All animal experimental protocols were approved by Peking University Institutional Committee for Animal Care and Use. The Sprague-Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Company.

## Nanostructures fabrication

Nanobars or nanopillars were fabricated as previous reported.[1-3] First, the quartz wafers were cut into square pieces with a size of 20 mm * 20 mm. Then, the square wafers were cleaned though sonication in acetone (J.T. Baker) and isopropanol (J.T. Baker). Next, the substrates were spin-coated with e-beam resist CSAR 62 (AllResist) which was 275 nm in thickness, followed by coating with Electra 92 (AllResist) to obtain a 110-nm conductive mask. We subsequently applied a JEOL JBX-630 0FS system to carry out E-beam writing. A 100 nm Cr mask were developed on the substrates though sputter deposition method followed by lift-off in acetone. Then, the nanobars or nanopillars were developed though dry etching with a mixture of $CHF_3$ and $O_2$ (AMT 8100 etcher, Applied Materials). We subsequently used $O_2$ plasma to clean the substrates followed by Cr masks depletion though Chromium Etchant 1020 (Transene). The morphology of nanostructure was analyzed by scanning electron microscopy (FEI Nova). For cell culture, the substrates were irradiated by UV to avoid contamination by microorganisms and then coated with 0.1% gelatin (Sigma-Aldrich) for about 30 min at room temperature.

**Cardiac-specific Bin1-over expression rats**

The $\alpha$-$MHC^{CreERT2}$ (Cre) knock-in rats and $rROSA26^{loxp\text{-}stop\text{-}loxp\text{-}eGFP\text{-}Bin1}$ transgenic rats were made in Beijing Biocytogen company to generate inducible and cardiac-specific BOE rats. Rats carrying only $\alpha$-$MHC^{CreERT2}$ (Cre) in the same litters were the WT group rats. All of the adult rats were injected Tamoxifen (Sigma) at consecutive 7 days just before MI surgery at a concentration of 0.05mg/g (weight/body weight).[4]

**Induction of myocardial infarction (MI)**

MI surgery was performed on male WT, $\alpha$-$MHC^{CreERT2}$ (Cre) knock-in rats and $rROSA26^{loxp\text{-}stop\text{-}loxp\text{-}eGFP\text{-}Bin1}$ transgenic rats (8 weeks old) as described.[5] Briefly, rats were anesthetized with avertin (300mg/kg, ip, Sigma). The rats were intubated and ventilated with room air using a small animal ventilator. After performed thoracotomy, an absorbable suture was passed underneath the left anterior descending (LAD) branch of the coronary artery 3 mm from its origin. Next ligate the left coronary artery to produce a large MI in the heart. Successful ligation of the artery was confirmed by blanching of the myocardium. Sham-operated (SHAM) rats underwent the same procedure without coronary artery ligation.

**Cardiomyocytes preparation**

All of the procedures for rat neonatal cardiomyocyte isolation and culture were carried out in a sterile environment. The hearts of neonatal rats (P0) were rapidly excised from the chest. After chopping into small pieces in pre-chilled Hanks Balance Salts solution (HBBS, Sigma), the heart tissues were transferred to a spinner bottle, and then 5 mL of 0.1% trypsin (Gibco) enzyme was added. The bottle was spun at low speed for 5 min at 37°C in a water bath. Afterward, the supernatant enzyme solution containing cardiomyocytes and other cell types was transferred to a fresh conical tube and then mixed with 4 mL of Dulbecco's modified

Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, HyClone). A new 0.1% trypsin enzyme solution was then added to the spinner bottle, and the procedure was repeated 6-8 times. After the collected enzyme solution was centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the cardiomyocyte fraction was re-suspended in DMEM containing 10% FBS. Further purification of cardiomyocytes was attained by pre-plating the cells into 10-cm cell culture dishes and incubating for 2 h in a cell culture incubator to remove the fibroblasts and endothelial cells. Finally, the neonatal cardiomyocytes were cultured in DMEM with 10% FBS at 37°C within a humidified cell incubator containing 95% air and 5% $CO_2$.

For adult cardiomyocyte isolation, Sprague-Dawley rats (male, 8 weeks old) were anaesthetized by isoflurane (Hebei Yipin). After rapidly excising the heart from the chest, the heart was attached to a Langendorff apparatus and digested with 1 mg/ml type II collagenase (Worthington) in $O_2$-saturated Tyrode's solution (in mmol/L: 130 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 25 HEPES, 0.33 $NaH_2PO_4$, 22 glucose, with the pH adjusted to 7.35 with NaOH at room temperature). After the heart tissue became soft, the hearts were removed and minced to harvest the isolated cardiomyocytes.

For the WT sham and MI rats, the whole hearts were removed from the Langendorff apparatus and minced to harvest the isolated cardiomyocytes. For acute $\alpha$-$MHC^{CreERT2}$ (WT) knock-in MI rats and $rROSA26^{loxp\text{-}stop\text{-}loxp\text{-}eGFP\text{-}Bin1}$ transgenic MI rats, the border zones of the heart were separated after the heart was removed from the Langendorff apparatus. Then the border zone tissues were minced to harvest isolated cardiomyocytes. According to previous reports,[6] the area comprising 10% of the immediate myocardium around infarcted zone was defined as the border region.

**Molecular Cloning**

The total RNA was extracted from rat and human heart tissues using Trizol reagent (Invitrogen) and reverse-transcribed into cDNA. PCR amplification was applied to identify the *BIN1* variants. The cloning primers were designed within exons 10 and 18 to cover the whole alternatively spliced region. The following primers were used: rat forward 5'-GAGAAGCAACACGGGAGCAACA-3', reverse 5'-AGAAGGTCTCCACCACCACAGC-3'. The cDNA PCR products were then cloned into the pEASY-T1 plasmid (Transgen Biotech). We randomly picked 200 clones for sequencing to identify the *BIN1* splice variants.

We cloned the full-length of the *BIN1* variants though PCR amplification of the rat heart cDNA. The primers for rat full-length *BIN1* primers were: forward 5'-ATGGCAGAGATGGGGAGC-3', reverse 5'-TCACTGCACCCGCTCTGTG-3'. The PCR products were cloned into the peGFP-C1 plasmid. Again, we randomly picked 200 clones to sequence and obtained the full-length cDNA clones of 6 different *BIN1* variants in rat hearts. We substituted the eGFP sequence in peGFP-C1-*Bin1+11* with RFP sequences to obtain the RFP-*Bin1+11* plasmid.

**Quantitative RT-PCR analysis of *BIN1* isoforms**

The total RNA was extracted from cardiac samples using the Trizol reagent (Invitrogen) and reversed transcribed into cDNA. Quantitative RT-PCR amplification was performed to profile the absolute or relative *BIN1* variant expression. For real-time RT-PCR amplification, 10 ng of cDNA was applied with Ex Cell Bio QPCR master mix (Genetimes Technology) on the Mx3000p Real-Time PCR System (Stratagene). The primers for various genes were the

following: rat *pBIN1* forward 5'-CTATGAGTCTCTTCAAACCGCCA-3', reverse 5'-GCGCAGCCGTGAGAACAGTTTAG-3';

rat *Bin1:* forward 5'-CAAGGCCCAGCCCAGTGACAG-3', reverse 5'-GAGAGAGCTCTGAGATGGGGACTTG-3'; rat *Mbnl1* forward 5'-GTTGCACCAAGCTTAGCCAC-3', reverse 5'-CCAGTCTGTCTGTCCGCATT-3'; rat *Gapdh* forward 5'-ATCAAGAAGGTGGTGAAGCA-3', reverse 5'-AAGGTGGAA GAATGGGAGTTG-3'. The amplification protocol was as follows: one denaturation step at 95°C for 10 min, 40 cycles of amplification 95°C for 1 min, 60°C for 30 s, 72°C for 30 s and a final step at 72°C for 5 min.

**Western blot analysis**

Tissues were homogenized in RIPA buffer (Beyotime) containing phenylmethylsulfonyl fluoride (PMSF, Thermo Fisher). The lysates were warmly agitated and centrifuged at 4°C. The supernatant protein mixture was separated by 12% SDS-PAGE analysis and then examined by Western blot analysis with a custom-made rabbit polyclonal antibody against the PI motif (amino acid sequence RKKSKLFSRLRRKKN) encoded by *BIN1* exon 11 (Genscript, 1:1000). The total BIN1 antibody was anti-BIN1 from Abcam (ab185950,1:1000). The horseradish peroxidase-conjugated goat anti-rabbit antibody (ThermoFisher, 1:5000) was used for detection. The horseradish peroxidase-conjugated GAPDH antibody (Shanghai Kangchen) was used to detect GAPDH content as the loading control.

**Membrane labeling and imaging**

Freshly isolated ventricular cardiomyocytes from adult rat hearts were incubated with 10 μmol/L Di-8-ANNEPS for 20 min at room temperature or with CellMask Deep Red for 10 min at 37°C. After washing, the cells were imaged with confocal microscopy (Zeiss).

**Immunofluorescence imaging**

After a 48-h transfection of BIN1 plasmids, rat neonatal cardiomyocytes were fixed by 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100 (Ameresco) in PBS for 15 min at room temperature. After blockading with 5% normal goat serum (Gibco) in PBS for 30 min, the cells were labeled with mouse monoclonal antibody α-actinin (Sigma) at 1:200 overnight at 4°C. Finally, cells were incubated with Alexa Fluor 647-conjugated goat anti-mouse antibody (ThermoFisher, 1:100) at room temperature for 1 h. The confocal or structured illumination microscopic was applied to take the florescence images.

**Immnuno-electron microscopy**

Immnuno-electron labeling for pBIN1 was performed following a previous report.[7,8] The male rat cardiomyocytes was fixed in 4% paraformaldehyde (Sigma) and 1% SDS buffered in PBS for 30 min at room temperature. After permeablized with 0.3% Triton X-100 (Ameresco), the samples were blocked with 0.3% Triton X-100 and 2% BSA in PBS. The samples were then incubated with rabbit anti-PI motif antibody (1:50) overnight at 4°C and with 1.4 nm immuno-gold conjugated anti-rabbit secondary antibodies (1:50, Sigma) for 1 h at room temperature. The HQ SILVER™ enhancement kit (Nanoprobes) was applied to enhance the gold particles to improve the signal. Next, the samples were post-fixed in 0.1% OsO4 (Ted Pella) buffered by 0.1 mol/L cacodylate for 30 min. Then, the samples were dehydrated in 50%, 70%, 80%, 90%, 100%, and 100% alcohol solutions for 10 min one by one. The samples were embedded in spurr resin (SPI) and polymerized at 65°C for 24 h at last. Ultrathin sections (80 nm) were imaged in a FEI Tecnai G2 20 Twin system operated in 120 kV.

**Transmission electron microscopy (TEM) imaging and analysis**

Rat neonatal cardiomyocytes were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 mol/L sodium cacodylate (Biolink) buffer (pH 7.4). To specifically stain the membrane, the samples were post-fixed in a mixture of 0.8% potassium ferrocyanide and 2% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for 30 min. The samples were then stained with 4% uranyl acetate for 1 h. After dehydration in a graded series of alcohol, the samples were embedded in Spurr resin and sectioned with a glass knife on a Leica Ultracut R cutter. The images were obtained using a FEI Tecnai G2 Twin system operated at 120 kV.

**BIN1 curvature-sensing assay**

The U2OS cells were cultured on gelatin-coated nanopillar arrays in DMEM (Invitrogen) with 10% FBS (HyClone) in a humidified incubator containing 95% air and 5% $CO_2$ at 37°C. The cells were transfected with vectors of different BIN1 isoforms labeled with EGFP. After cultured overnight on nanopillars, cells were imaged using epi-fluorescence microscopy (Leica). Fiji software was used for quantitative analysis of colocalization between uBIN1 and pBIN1.

**Statistics**

All data are presented as the mean ± S.E.M. Statistical analysis was performed using Student's *t*-test. A value of $P < 0.05$ was considered significant.

**Reference:**

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