Online Supplement

Cardiomyocyte orientation modulated by the Numb Family Proteins-N-Cadherin axis is essential for ventricular wall morphogenesis

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Running Title: NFPs modulate morphogenesis via N-Cadherin trafficking

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Supplemental materials and methods

Generation of the mCherry:Numb knockin line

The mCherry:Numb knockin line (mCherry:NumbKI), in which the coding sequences of Flag and mCherry were inserted after the start codon of *Numb* (Fig. 6A), was generated with the help of Biocytogen. The precise insertion of mCherryFlag was confirmed by multiple methods including southern blot and sequencing. The genotyping primers are included in the supplemental figure legends. The expressed mCherry:NumbKI in the knockin line was functional, as the homozygotes (mCherry:NumbKI) were able to survive to adulthood without any obvious morphologic defects.

Generation of the αMHC-cN-Cadherin transgenic line

Transgenic lines overexpressing chicken N-Cadherin (cN-Cadherin), driven by the αMHC promoter[1], were generated with the help of Cyagen. Three lines were chosen and were proven to express cN-Cadherin specifically in the heart.

Frozen section immunofluorescence

Immunofluorescence staining was performed as described[2]. Primary antibodies are: BrdU (1:50; Becton Dickinson, 347583), collagen IV (1:1000; Chemicon, AB756P), β-tubulin (1:1000; BD Pharmingen, 556321), MF20 (1:100; Developmental Studies Hybridoma Bank (DSHB)), Cardiac Troponin I (1:300, abcam, ab56357), Cardiac Troponin T (1:300, abcam, ab115134), PECAM (1:50, BD Pharmingen, 550274), Endomucin (1;100, Santa Cruz Biotechnology, sc-65495), GFP (1:1000, abcam, ab290), mCherry (1:200, Biorbyt, orb66657), tdTomato (1;200, Biorbyt, orb182397), acetylated α-Tubulin (1:300, Sigma Aldrich,T6793-.2ML), γ-Tubulin (1:300, Sigma Aldrich, T5192-.2ML), N-Cadherin (1;200, Proteintech, 22018-1-AP), N-cadherin (1:200, BD Transduction, 610920), N-cadherin (chick) (1:200, Invitrogen, 13-2100), EEA1 (1:100, Cell Signaling, 3288S), Rab5 (1:200, Cell Signaling, 3547), Rab7 (1:100, Cell Signaling, 9367) and Rab11 (1:100, Cell Signaling, 5589), LAMP1 (1:100, Cruz Biotechnology, SC-20011), and β-catenin (1:500, BD PharMingen, 61053) and Histone H3 (1:100, Upstate, 06-755).

Whole embryo immunofluorescence staining, clearing and imaging

Whole embryos were stained as described [3-5]. Briefly, whole embryos were fixed for 2-4 hours in 4% PFA, permeabilized for 2 hours in PBS-Tween 20, blocked with 3% BSA, and then incubated with the primary antibody for ~24 hours. After three washes, the embryos were incubated with the secondary antibody for 24 hours, followed by three more washes with PBS. After staining, embryos were cleared using Scale[5] or RapiClear® 1.52 (SunJin Lab, #RC152001) and then 3D imaged. Z-stack images were acquired using a Zeiss 880-META NLO confocal scope equipped with multi-photon excitation at 1-3 μm per section. Three-dimensional images were reconstructed using Imaris software.

Quantitative evaluation of the orientation of the cardiomyocyte division plane

Orientation of the cardiomyocyte division plane was determined by imaging heart sections or cleared whole hearts (described below). Sections or whole hearts of *Nkx2.5*$^{Cre/+}$;*Nb*$^{fl/fl}$; *Nl*$^{fl/fl}$*; mTmG* and *Nkx2.5*$^{Cre/+}$;*Nb*$^{fl/+}$; *Nl*$^{fl/fl}$*; mTmG* at E9.25 were stained with acetylated α-tubulin to determine the spindle orientation. Z-stack images were acquired using a Zeiss 880-META NLO confocal scope equipped with multi-photon excitation at 1-3 μm per section. Three-dimensional images were reconstructed using Imaris software. Spindle orientations of left ventricular cardiomyocytes in anaphase or early telophase where both centrosomes or nuclei were in the same focal plane were

quantified. The spindle orientation was determined by the angle between the spindle axis, determined by the two centrosomes, and the basement membrane or heart surface reference line[2, 6]. Division planes positioned at 60–90° to the basement membrane were classified as perpendicular, those oriented at 0–30° were classified as parallel, and those oriented between 30-60° were considered non-classified[2]. The data shown are a combination of separate analyses from two investigators.

Quantitative evaluation of the orientation of cardiomyocytes

Orientation of the cardiomyocyte plane was determined by imaging heart sections or cleared whole hearts. Cardiomyocytes of *Nkx2.5$^{Cre/+}$;Nb$^{fl/fl}$; Nl$^{fl/fl}$; mTmG* and *Nkx2.5$^{Cre/+}$;Nb$^{fl/+}$; Nl$^{fl/fl}$; mTmG* heart at E9.25 were labeled with membrane GFP, and whether a cell is oriented can be determined by the length to width of the cell. If the ratio of length to width of a cell is greater than 1.2, then this cell is oriented. The cellular orientation was determined by the angle between the longitudinal axis of an oriented cell to the heart surface reference line. Cell orientations with the angles of 60–90° are classified as perpendicular, those that are oriented at 0–30° are classified as parallel and those oriented between 30-60° are considered non-classified[2]. The data shown are a combination of separate analysis from three hearts. Only the cellular orientations of cardiomyocytes from the same regions of hearts from the same litter were compared.

Inducible lineage tracing and mosaic analysis

Heterozygous *Rosa26^{CreERT2};Nb$^{fl/+}$;Nl$^{D/D}$* males were crossed with homozygous *Nb$^{fl/fl}$;Nl$^{D/D}$;Conf* females to produce control embryos (*Rosa26^{CreERT2};Nb$^{fl/+}$;Nl$^{D/D}$*) or iDKO (*Rosa26^{CreERT2};Nb$^{fl/fl}$;Nl$^{D/D}$*). Tamoxifen (T5648, Sigma), dissolved in sunflower oil (S5007, Sigma), was gavaged to pregnant females 7.75 days after coitus, with a dose of tamoxifen at 20 μg per gram body weight (20 μg/g). The embryos were harvested at the indicated age and whole mount stained, and then cleared as described above. The embryos were then transferred to a glass bottom plate and imaged using the confocal system. Z-stack images of the whole heart were acquired using a Zeiss LSM 880-NLO confocal scope equipped with multi-photon excitation at 1-3 μm per section. The clones were imaged and reconstructed by Imaris software, and were then analyzed for cell count and distance (z) from the heart surface to the innermost cell of the clone.

***Ex vivo* culture system**

The *ex vivo* culture protocol was developed by Dr. C.P. Cheng, and was utilized as reported[4]. Briefly, embryos with the intact heart exposed to medium were cultured in medium containing Ade-Hist:Numb or vehicle for 24 hours. Then, the embryos were whole mount imaged.

Imaging

The following systems were used: for confocal imaging, Zeiss LSM 880-NLO confocal microscope system with an Airyscan detector with FAST module on a Zeiss Axio observer Z1 inverted microscope equipped with an internal spectral QUASAR detector; for color imaging, Zeiss Observer Z1 with a Hamamatsu ORCA-ER camera; for fluorescence imaging, Zeiss Observer Z1 with an AxioCam MRm camera. 3D images were produced by Zeiss LSM 880-NLO with 1-3 μm per section and reconstructed with Imaris software (Bitplane). Stereo images of the heart or embryos were harvested by a stereoscope (Leica M205 FA) equipped with GFP, CFP and RFP filters. All histology analysis and immune-fluorescent staining analysis were quantitated in a blinded way by at least two investigators.

Cardiomyocyte and MEF isolation

Neonatal cardiomyocytes were isolated according to the protocol of the Pierce™ Primary Cardiomyocyte Isolation Kit (#88281; Thermo Scientific). Briefly, p0 or p1 neonatal hearts with the genotype of *Rosa26^{CreERT2};Nb$^{fl/+}$;Nl$^{D/D}$* or *Rosa26^{CreERT2};Nb$^{fl/fl}$;Nl$^{D/D}$* were harvested and placed in ice cold HBSS. The HBSS was aspirated, the enzyme was added from the kit, and the heart was incubated for 15 minutes at 37°. After digestion, the enzyme solution was gently removed and 0.5mL of Complete DMEM for Primary Cell Isolation was added to each tube. The heart tissue was broken up by pipetting up and down 25-30 times using a sterile 1.0mL pipette tip. After the tissue was rendered into primarily a single-cell suspension, cells were plated on 60 mm dishes and cultured to 60–80% confluency. Cultured cardiomyocytes were infected with different viruses or induced with hydroxytamoxifen as indicated in different experiments for 72 hours. Mouse embryonic fibroblasts (MEFs) were isolated from E13.5-E14.5 embryos with the genotype of *Rosa26^{CreERT2};Nb$^{fl/+}$;Nl$^{D/D}$* or *Rosa26^{CreERT2};Nb$^{fl/fl}$; Nl$^{D/D}$* as described previously. Cultured MEFs were infected with different viruses or induced with hydroxytamoxifen as indicated in different experiments for 72 hours,

Western blot analysis

Western blot was performed as described[4, 7]. The antibodies used in the study include Numb (1:1000, Cell Signaling, 2756S), GAPDH (1:1000, Santa Cruz Biotechnology, sc-25778), N-Cadherin (1;1000, Proteintech, 22018-1-AP), N-cadherin (1: 1000, BD Transduction, 610920), N-cadherin (chick) (1: 1000, Invitrogen, 13-2100), EEA1 (1:1000, Cell Signaling, 3288S), Rab5 (1: 1000, Cell Signaling, 3547), Rab7 (1: 1000, Cell Signaling, 9367) and Rab11 (1: 1000, Cell Signaling, 5589), Clathrin (1:1000, Cell Signaling, 4796), and α-Tubulin (1:200, Developmental Studies Hybridoma Bank (DSHB), AA4.3).

Biotin–IP-based N-cadherin internalization and recycling assay and Western blot-based detection

This experiment was performed according to the published protocol[8]. Briefly, to determine if NFPs play roles in N-Cadherin internalization and trafficking to the membrane, control and iDKO cardiomyocytes, 72 hours after Cre induction, were used in the Biotin–IP-based internalization and recycling assay following the protocol provided by the kit EZ-link cleavable sulfo-NHS-SS-biotin (#21331; Thermo Scientific). Membrane N-Cadherin was labeled by the sulfo-NHS-SS-biotin 0.5mg/ml for 30 minutes at 4°, and the labeled N-Cadherin was allowed to internalize for 20 minutes at 37°. The remaining biotin at the cell surface was removed with 60 mM MesNa (M1511-5G, sodium 2-mercaptoethanesulfonate: Sigma) in MesNa buffer (50 mM Tris–HCl, pH 8.6, 100 mM NaCl) for 30min at 4°, followed by quenching with 100 mM iodoacetamide (I1149-5G, Sigma) for 15min on ice. Cells were washed three times with cold PBS, and then lysed. NeutrAvidin Agarose Resins (Cat: 29200, Thermo Scientific) were used to pull down the cell lysates overnight at 4 °C. The Biotin labeled N-Cadherin inside the cytoplasm was detected and quantified in the control and knockout by Western blot. Similarly, to determine if NFPs play a role in N-Cadherin trafficking, Biotin labeled N-Cadherin was allowed to internalize for 30 minutes at 37° and recycle back to the membrane for 20 minutes, and the remaining N-Cadherin in the cytoplasm was quantified.

Antibody-based N-cadherin internalization and recycling assay

Cardiomyocytes were plated on a µ-Slide (chambered coverslip) with 8 wells (ibidi; Cat: 80826) to 60–80% confluency. The plate was cooled on ice and the cell surface was labeled for N-cadherin with Alexa Fluor 488-conjugated N-cadherin in cardiomyocyte

culture medium supplemented with 30 mM HEPES (pH 7.4) for 1 hr on ice. After labeling, the staining medium was aspirated, the cells were washed and the medium was replaced with fresh culture medium with 30 mM HEPES (pH 7.4). To start internalization, plates were placed in a 37°C water bath and incubated for 20 min. After internalization, plates were cooled on ice. The fluorescence on the cell surface was quenched by adding anti-Alexa Fluor 488 antibody and incubating for 1 h on ice. Cells were fixed in 4% PFA for 15 min at RT, and internalized N-cadherin fluorescent intensities were measured using a Zeiss 880-META NLO confocal scope. To measure N-Cadherin recycling, cardiomyocytes were plated and cell surface N-cadherin was labeled as above. Then labeled N-cadherin was allowed to internalize for 30 min at 37°C. The surface was quenched as above. Cells were re-incubated at 37°C for 20 min for internalized N-Cadherin to recycle back to the membrane. After re-incubation, the surface signal was re-quenched and cells were fixed. The fluorescent dots were quantified of control and knockout cells using a Zeiss 880-META NLO confocal scope.

Membrane and cytoplasmic fractionation via Sulfo-NHS-SS-Biotin kit

Neonatal cardiomyocytes were cultured to 70-80 % confluence in 60 mm tissue culture dishes. Cells were washed with HBSS and then the membrane proteins were labeled by the sulfo-NHS-SS-biotin 0.5mg/ml for 30 minutes at 4°C. Cells were washed three times with cold HBSS, and then lysed. NeutrAvidin Agarose Resins (Cat: 29200, Thermo Scientific) were used to pull down the cell lysates overnight at 4 °C. The agarose resins were centrifuged at 500 x g for 10 minutes, and the supernatant was transferred as the cytosolic fraction. The agarose resins were washed five times with lysis buffer, and boiled in laemmli sample buffer. The tube was spun at top speed for 5 minutes and the supernatant was kept as the membrane fraction. The resulting cytoplasmic and membrane fractions were used to determine the protein level of N-cadherin by western blot. The experiments were repeated three times and quantification data were shown in the main text.

Cardiomyocyte proliferation assay via BrdU pulse labeling

Pregnant females were intraperitoneally injected with BrdU for one hour before harvesting the embryos. The proliferation rate was assessed by percentage of BrdU positive cardiomyocytes out of total cardiomyocytes following our previous protocol [2, 4]. Cardiac troponin T and Endomucin were stained to distinguish cardiomyocytes from non-cardiomyocytes.

Single mRNA molecule in situ hybridization (ISH) and Immuno-fluorescent staining

ISH and IFS were performed according to the protocol of the kit RNAscope 2.5 HD (RED) Assay (Cat. No. 322360), which can detect single mRNA molecules. Briefly, 24 hours after fixation, the embryos were frozen embedded in OCT compound. Sections of the frozen embedded samples were processed following the step-by-step protocol of the kit. The expressional level of mRNA in each cell was based on the number of mRNA molecules or signal intensity using the confocal scanned pictures, and three scanned sections for each cell were quantified.

Duolink proximity ligation assay (DPL) fluorescence protocol

The experiment was performed according to the protocol provided by the kit DPL (Sigma, DUO92101-1KT). Briefly, cardiomyocytes isolated from p0-2 hearts were cultured on the μ-Slide (chambered coverslip) with 8 wells (ibidi; Cat: 80826) and 48 hours after culture, the cells were fixed, penetrated and then blocked with the blocking

buffer from the kit. The cells were then incubated with primary antibody diluted in diluent, then with DPL probe for one hour. The plus and minus probes were ligated and then amplified with polymerase. The sample was then mounted using a minimal volume of DPL mounting medium with DAPI and was imaged using the LSM880 confocal.

Supplemental figure legends:

Figure S1. NFPs regulate cardiomyocyte orientation in an autonomous manner.
(A&B) Most of the cardiomyocytes in the myocardium at 3 somite stages (st) labeled by *Nkx2.5$^{Cre/+}$; mTmG* (A) or *cTnT-Cre; mTmG* at 6 st (B) do not display orientations. * indicates a cardiomyocyte that does not display orientation. (C&D) Cardiomyocytes stained by MF20 and DAPI showed a perpendicular orientation in the control (C), but a parallel orientation in the MDKO (D). (E&F) The shapes of trabecular cardiomyocytes were examined with reconstructed Z-stack images via Imaris software. The surface module of Imaris software was used to create 3D-reconstructed cells and then to determine the cell shape (Suppl. Movie 3&4). The length, width and height of the reconstructed trabecular cells were measured in both control and MDKO hearts. The cell is considered as a cylinder and its volume was calculated. The volumes of control cells are significantly greater than MDKO cells. (G&H) MDKO embryos at E9.25 were cultured with Adenovirus-Numb:Histidine (H) or Ade-GFP as a control (G) with hearts exposed to medium and 24 hours later, the embryos were fixed and stained with Histidine or GFP and imaged. The picture shown in G or H is one section from a heart. Scale bars in A&B, C&D and G&H are 20 μm.

Figure S2. Labeled single cardiomyocyte undergoes OCD or directional migration to initiate trabecula and form a clone.
(A) Scheme of cardiomyocytes that undergo perpendicular OCD or directional migration (DM) that contributes to trabecular initiation. (B) The myocardium at E7.75 is a monolayer, and after trabecular initiation, the myocardium contains compact zone and trabecular zone beginning at around E9.5. (C-G) shows four different clonal patterns. The clones were labeled at about E7.75 and examined at E10.5. Top image of each item (D-G) is a representative section from a Z stack of the same clone. The Z stack was reconstructed to a 3D image (bottom image). (D) A surface clone. (E) A compact clone. (F) A trabecular clone. (G) A transmural clone. (H) A scheme of the Numb mRNA to show the probes for ISH and primers for Q-PCR. (I) shows that primers of NbRTF1&NbRTR1 can detect the deleted exon 4, while NbRTF2&NbRTR2 can not determine whether exon 4 was deleted or not. Similarly, only probes that are complement to exon 4 but not exon 9 can detect the deletion of exon 4 (J&K). Scale bars in D-G and J&K are 30 μm.

Figure S3. Schemes of clonal patterns of cells labeled at about E7.75 and examined at E10.5 or at E12.5.
The clonal patterns examined at E10.5 and E12.5 are different. At E10.5, there are four types including surface (blue), trabecular (green), transmural (red) and compact (pink). At E12.5, there are only three types including trabecular (green), transmural (blue) and compact (purple). Possibly, the surface clone at E10.5 might change to compact clone due to the proliferation of cardiomyocytes in the compact zone or the translocation of cardiomyocytes from the surface to the inner compact zone and trabecular zone.

Figure S4. N-Cadherin regulates cellular orientation and organization.
(A&B) shows one section from a whole mount stained and imaged control (*Nkx2.5$^{Cre/+}$; Cdh2$^{fl/+}$; mTmG*) or knockout heart (*Nkx2.5$^{Cre/+}$; Cdh2$^{fl/fl}$; mTmG*). The cardiomyocytes in the inner layer in control hearts display perpendicular orientation indicated by double arrows, while cardiomyocytes in *Nkx2.5$^{Cre/+}$; Cdh2$^{fl/fl}$* hearts display random or no orientation indicated by *. (C) Numb variants with different domain or motif deleted Numb were used to rescue the N-Cadherin protein level and we found that Cent domain,

proline rich domain and DPF/NPF motifs are required to maintain N-Cadherin protein level. (D) The growth of compound heterozygous mouse was retarded. (E) Compound heterozygous hearts (*Nkx2.5$^{Cre/+}$;Nb$^{fl/fl}$; Nl$^{fl/+}$;Cdh2$^{fl/+}$* or *Nkx2.5$^{Cre/+}$; Nb$^{fl/+}$; Nl$^{fl/fl}$; Cdh2$^{fl/+}$*) display thicker and less dense trabecula compared to the control hearts, while the single heterozygotes are not significantly different from control. Scale bars in A&C are 20 μm and in E 40 μm.

Figure S5. mCherry:NumbKI line generation and confirmation by Southern blot.
(A) mCherryFlag:Numb knockin mouse line (mCherry:NumbKI) was generated via CRISPR/Cas9. Sequences of mCherry and Flag with linker sequences on both sides were inserted after the start codon of Numb via CRISPR-Cas9. The probes and southern blot strategy for the mCherry:NumbKI knockin line confirmation; (B) Result of southern blot. The data shows that pups 1EE26-47, 1EE26-48, 1EE26-49, 1EE26-50, 1EE26-51, 1EE26-52, 1EE26-53 and 1EE26-54 were positively confirmed by Southern blot. Meanwhile, they have no random insertion demonstrated by Southern blot using internal probe. (C) Numb did not show obvious asymmetric localization in perpendicular oriented cardiomyocytes indicated by arrows of mCherry:NumbKI heart. (D) mCherry:NumbKI is highly expressed in epicardium. Numb is polarized and enriched in migrating mouse embryonic fibroblasts (E). Scale bars in C&D are 10 μm.

Figure S6. Numb localization and its effect on early endosome biogenesis.
(A-D) Numb co-localizes with EEA1, RAB7, LAMP1 and RAB11 via the mCherry co-staining. The MDKO heart displays reduced early endosomes indicated by the EEA1 staining (E&F). Scale bars in A-D are 10 μm, and in E&F are 100 μm.

Figure S7. Numb and N-Cadherin interact in cardiomyocytes via DPL assay.

(A) *ROSA26$^{CreERT2/+}$; Nb$^{fl/fl}$; Nl$^{D/D}$* cardiomyocytes from p0-p2 neonatal hearts were cultured and then treated with Hydroxytamoxifen (4-OHT). N-Cadherin level was not significantly reduced at 72 hours after 4-OHT additions. The levels of both Numb and N-cadherin were reduced at 96 hrs. (B) Histone H3 is expressed in the nuclei of cardiomyocytes identified by cTni. (C) Both mCherryNumb and N-Cadherin do not interact with Histone H3, consistent with previous reports that Numb or N-Cadherin does not localize to nucleus, suggesting that the DPL signal is specific. (D) Numb and N-Cadherin interact at the cortex indicated by the arrows and also inside the cytoplasm based on DPL assay. Scale bars in B-D are 10 μm,

Figure S8. N-Cadherin overexpression transgenic line generation and confirmation.
(A) shows the First round of PCR and the primers used include
Transgene PCR primer F1: TAGCCCACACCAGAAATGACAGAC
Transgene PCR primer R1: TCGAGCAGACACTACAAACTCCG
Internal control PCR primer F1: CAACCACTTACAAGAGACCCGTA
Internal control PCR primer R1: GAGCCCTTAGAAATAACGTTCACC
The expected PCR products:
Transgene PCR product size: 398 bp
Internal control PCR product size: 632 bp
(B) shows the second round of PCR and the primers used include
Transgene PCR primer F2: GATTGGTCTCCCAGCCTCTGCTAC
Transgene PCR primer R2: GTTGCTTTTATCGGTGCCTGCTGC
The expected transgene PCR product size is 521 bp.

Figure S9. Model of NFPs regulating N-Cadherin recycling, cellular orientation and trabecular morphogenesis.
(A) The cardiomyocytes in the control heart at E12.5 display special cellular orientations. Most cells in the trabecular zone, being of a long spindle shape, orient parallel to the trabeculae. However, cells in the MDKO heart lose this pattern and display no or random orientation. (B) The abnormal orientation in the MDKO hearts is possibly caused by reduced N-Cadherin membrane localization. The reduced N-Cadherin membrane localization affects the OCD, cellular orientation and organization. (C) N-Cadherin endocytosis was not affected in the NFP null cardiomyocytes, but N-Cadherin post-endocytic recycling to the membrane is attenuated, and the transition to LE is affected. EE: Early Endosome, LE: Late Endosome.

Legends for supplemental movie:
Supplemental movie 1&2: Trabecular cardiomyocytes in MDKO hearts display defects in cellular organization.

Control (*Nkx2.5$^{Cre/+}$;Nb$^{fl/+}$;Nl$^{fl/fl}$*)(Suppl. Movie 1) and MDKO (*Nkx2.5$^{Cre/+}$;Nb$^{fl/fl}$;Nl$^{fl/fl}$*) (Suppl. Movie 2) hearts were harvested at E11.5. The hearts were whole mount stained with PECAM and then cleared. The cleared hearts were 3D imaged via confocal microscopy with 3 μm/section and the cellular orientation and organization was shown.

Supplemental movie 3&4: Trabecular cardiomyocytes in MDKO hearts display defects in cellular orientation and cell size.
Control (*Nkx2.5$^{Cre/+}$;Nb$^{fl/+}$;Nl$^{fl/fl}$*)(Suppl. Movie 3) and MDKO (*Nkx2.5$^{Cre/+}$;Nb$^{fl/fl}$;Nl$^{fl/fl}$*) (Suppl. Movie 4) hearts were harvested at E11.5. The hearts were whole mount stained with PECAM and then cleared. The cleared hearts were 3D imaged via confocal microscopy with 0.54 μm/section. The shapes of trabecular cardiomyocytes were examined with reconstructed Z-stack images via Imaris 9.1.2 software (Bitplane, Oxford Instruments). The surface module of Imaris software was used to create 3D-reconstructed cells and then to determine the cell shape (Suppl. Movie 3&4).

Supplemental movie 5-8: Four different clonal patterns were shown with each movie showing one clonal pattern. The clones were labeled at E7.75 and examined at E10.5. The hearts were cleared and then stained with PEAM and DAPI.
Suppl. Movie 5: A surface clone was shown; Suppl. Movie 6: A compact clone was shown; Suppl. Movie 7: A trabecular clone was shown; Suppl. Movie 8: A transmural clone was shown.

Supplemental movie 9&10: The distribution of cells in a compact clone.
A single clone that was induced to be labeled at E7.75 was examined at E12.5. The heart was stained with PECAM and then cleared. The cells of the clone were 3D imaged via a confocal microscope (Zeiss LSM880) and reconstructed via Imaris. Suppl. Movie 9 shows the sections from the top to the bottom of the clone. Suppl. Movie 10 shows the 3D reconstructed clone. In the compact clone, there are some cells at the surface of the heart, some cells in the compact zone and a few cells at the base of a trabecula.

Supplemental movie 11&12: The distribution of cells in a trabecular clone and in a transmural clone.

Suppl. Movie 11 & 12 shows the 3D reconstructed trabecular and transmural clone, respectively. In the trabecular clone, all of the cells are inside the trabecular zone, while in the transmural clone, there are some cells at the surface of the heart, some cells in the compact zone and many cells in trabecular zone.

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Figure S1. NFPs regulate cardiomyocyte orientation in an autonomous manner.

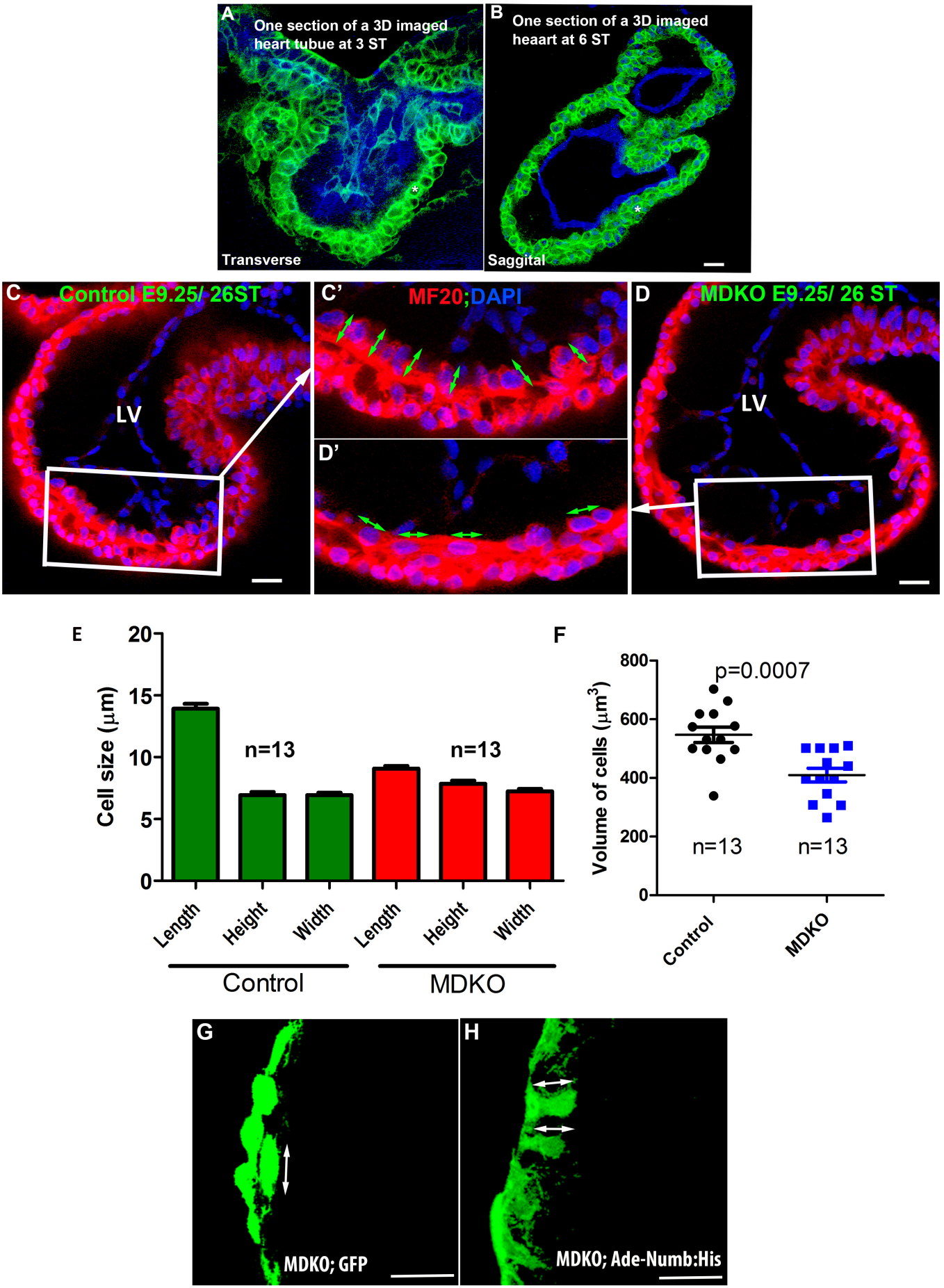
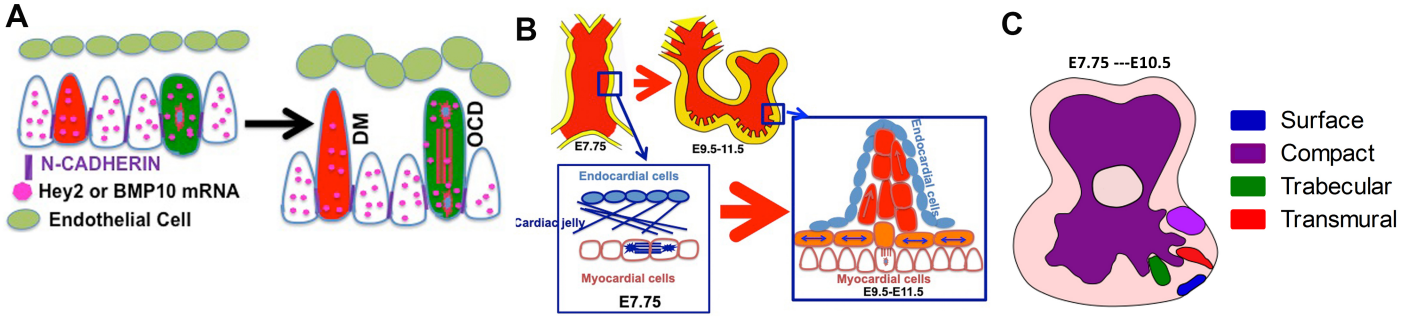
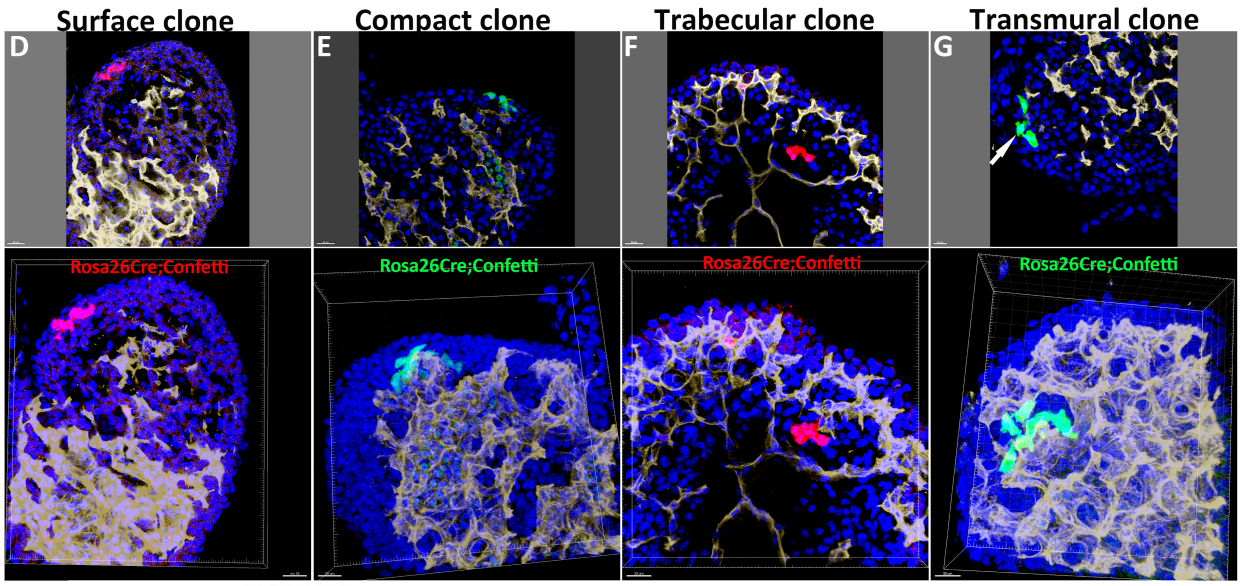
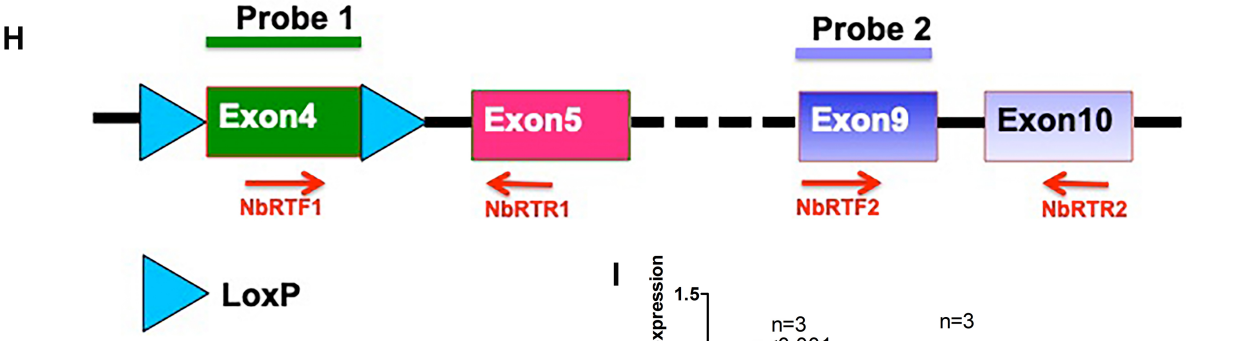
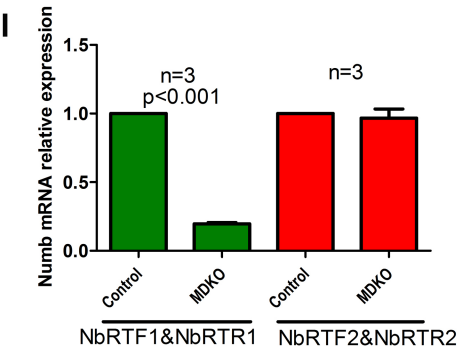


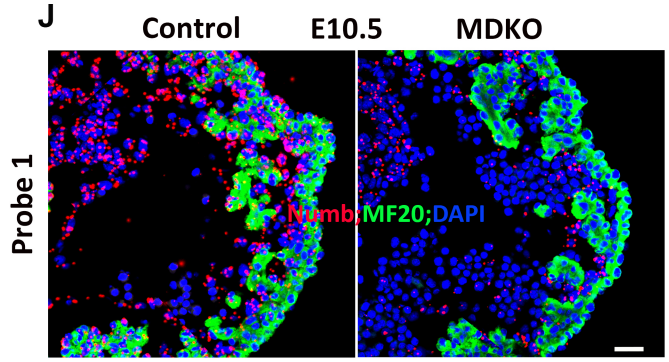
Figure S2. Labeled single cardiomyocyte undergoes OCD or directional migration to initiate trabecula and form a clone .











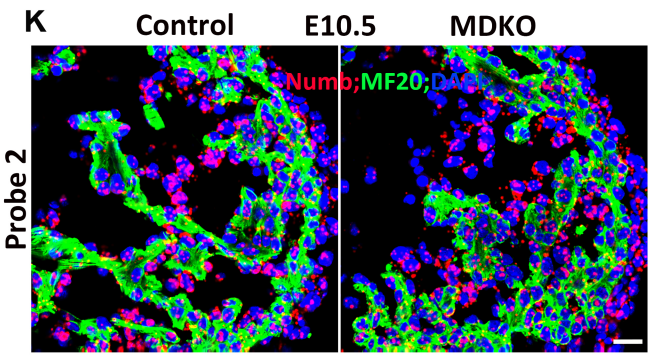


Figure S3 Schemes of clonal patterns of cells labeled at E7.75 and examined at E10.5 or E12.5.

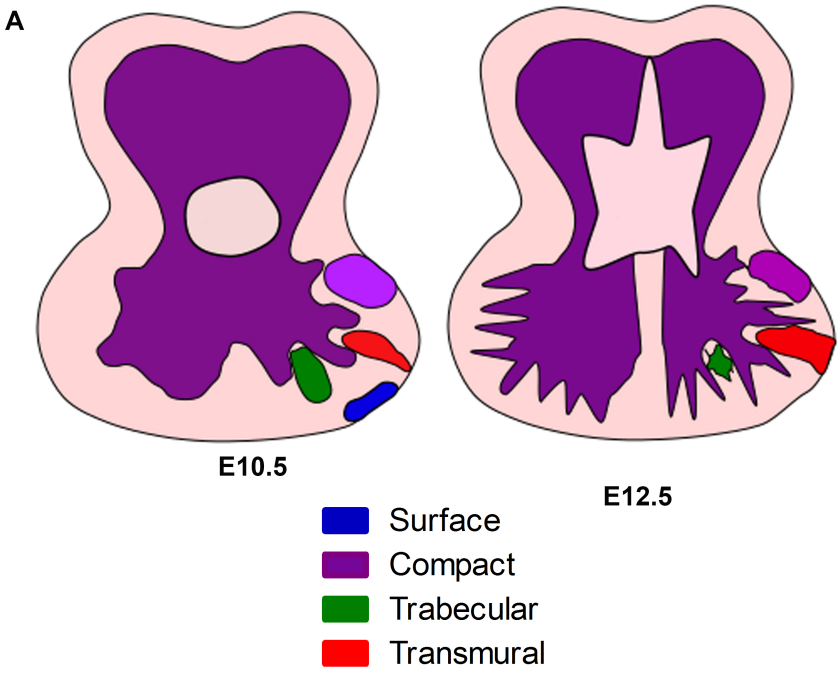
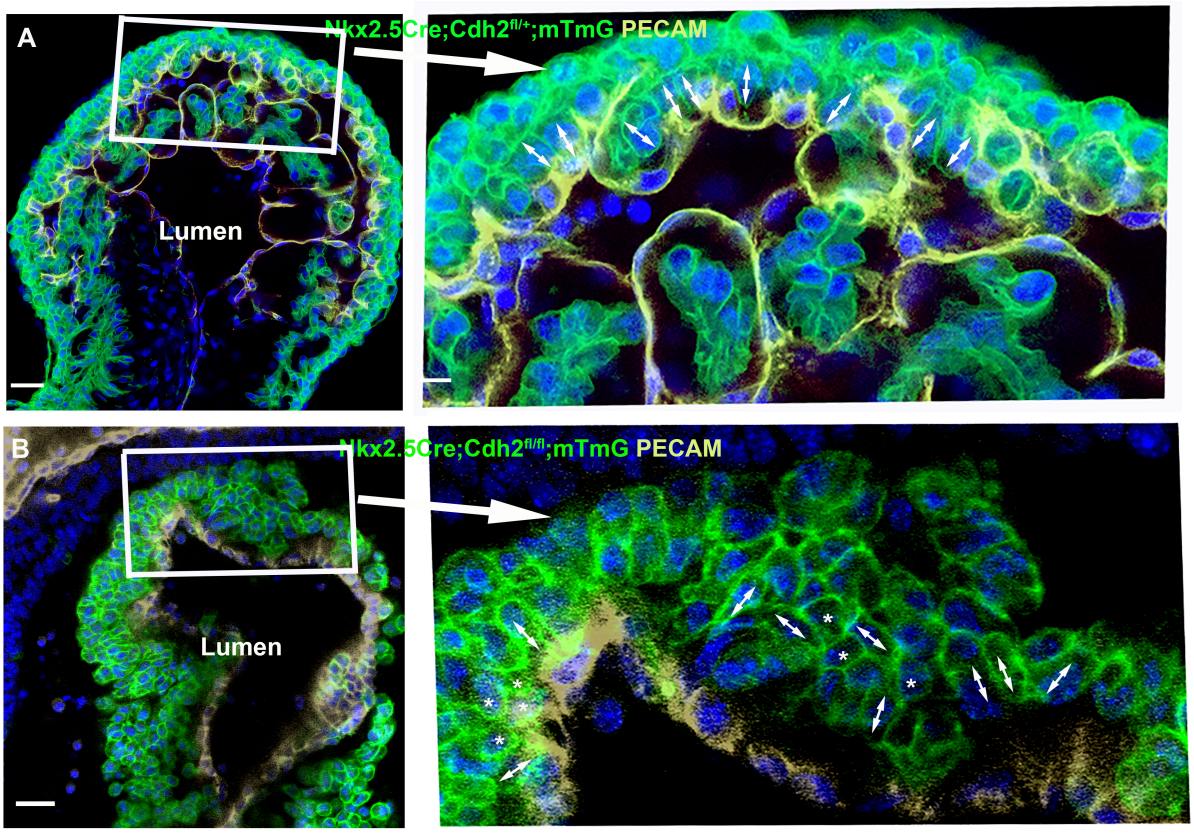
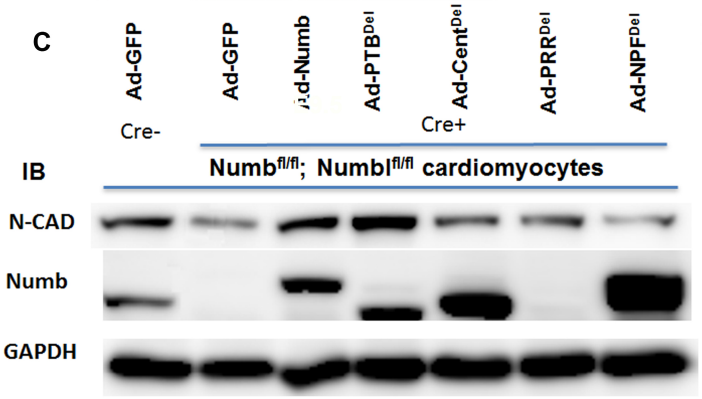
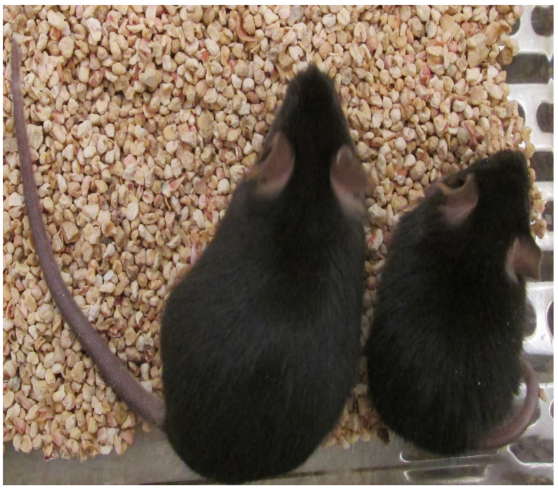


Figure S4. N-Cadherin regulates cellular orientation and organization.







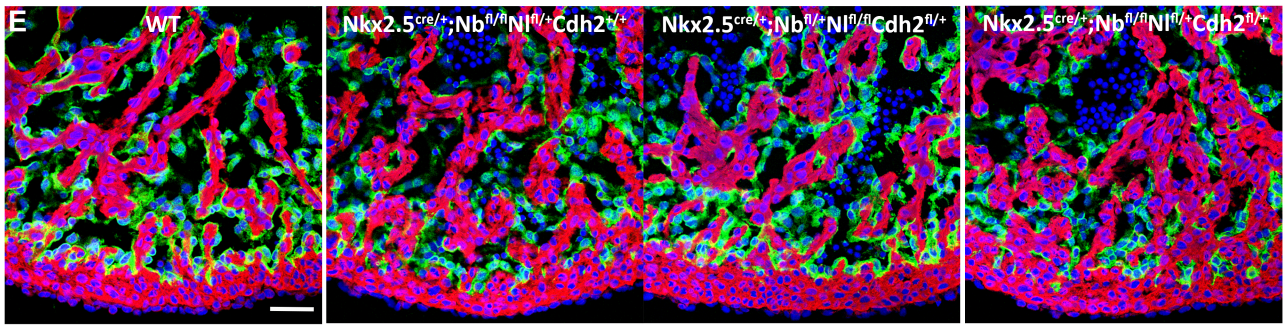
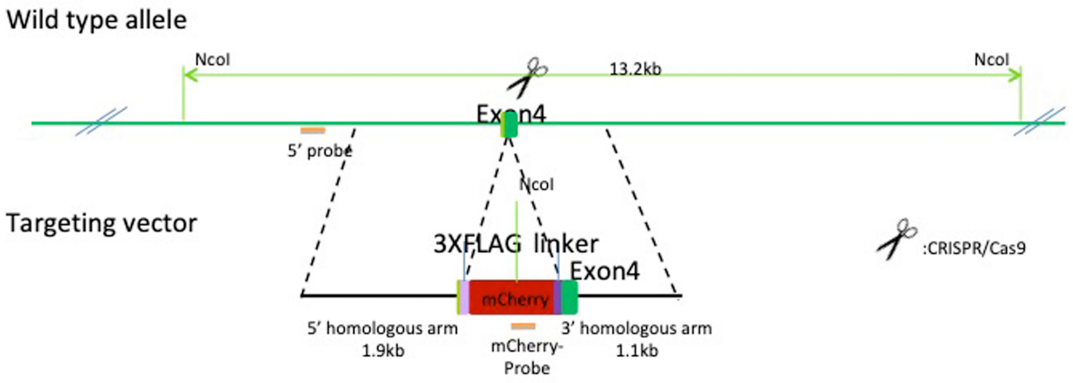


Figure S5. mCherry:NumbKI line generation and confirmation by Southern blot.

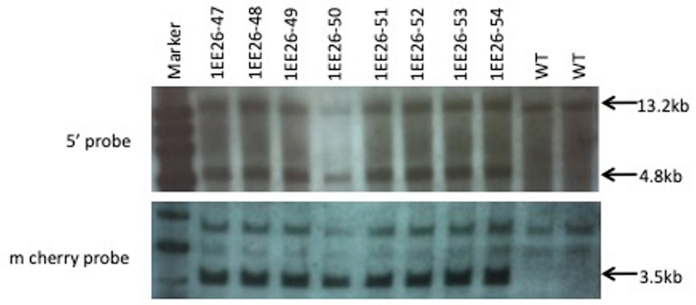
A **Southern blot strategy**

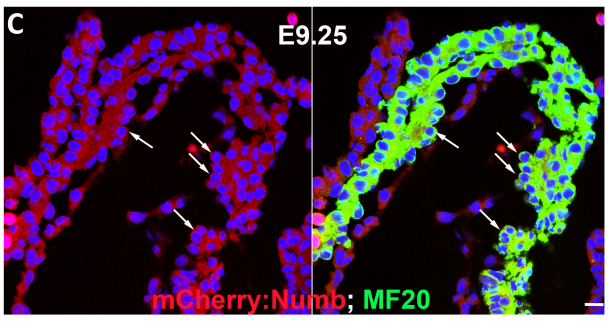


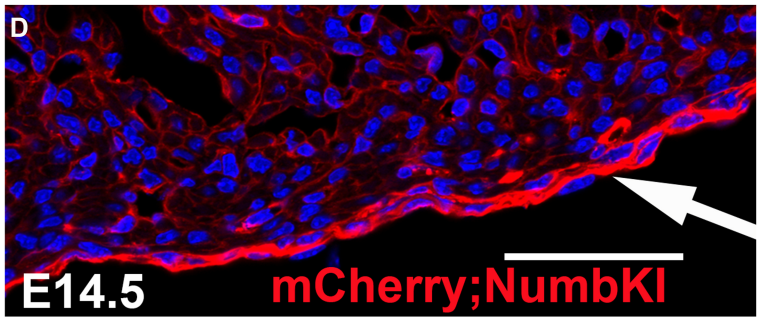
Southern blot strategy			
Restriction enzyme	**Probe**	**WT**	**Targeted**
NcoI	**5'**	**13.2kb**	**4.8kb**
VspI	**3' mcherry**	**--**	**3.5kb**

B **Southern blot: confirmation with 5' and 3'mcherry probes**

Southern blot strategy			
Restriction enzyme	**Probe**	**WT**	**Targeted**
NcoI	**5'**	**13.2kb**	**4.8kb**
VspI	**3' mcherry**	**--**	**3.5kb**







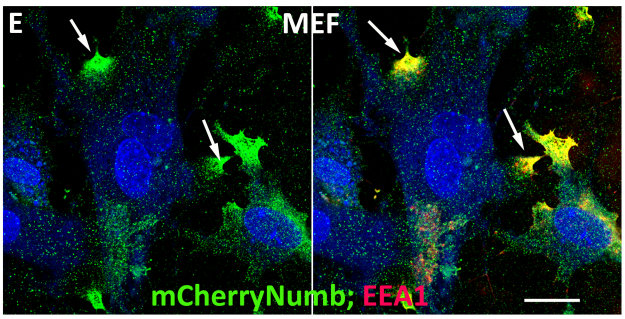


Figure S6. Numb localization and its effect on early endocysome formation.

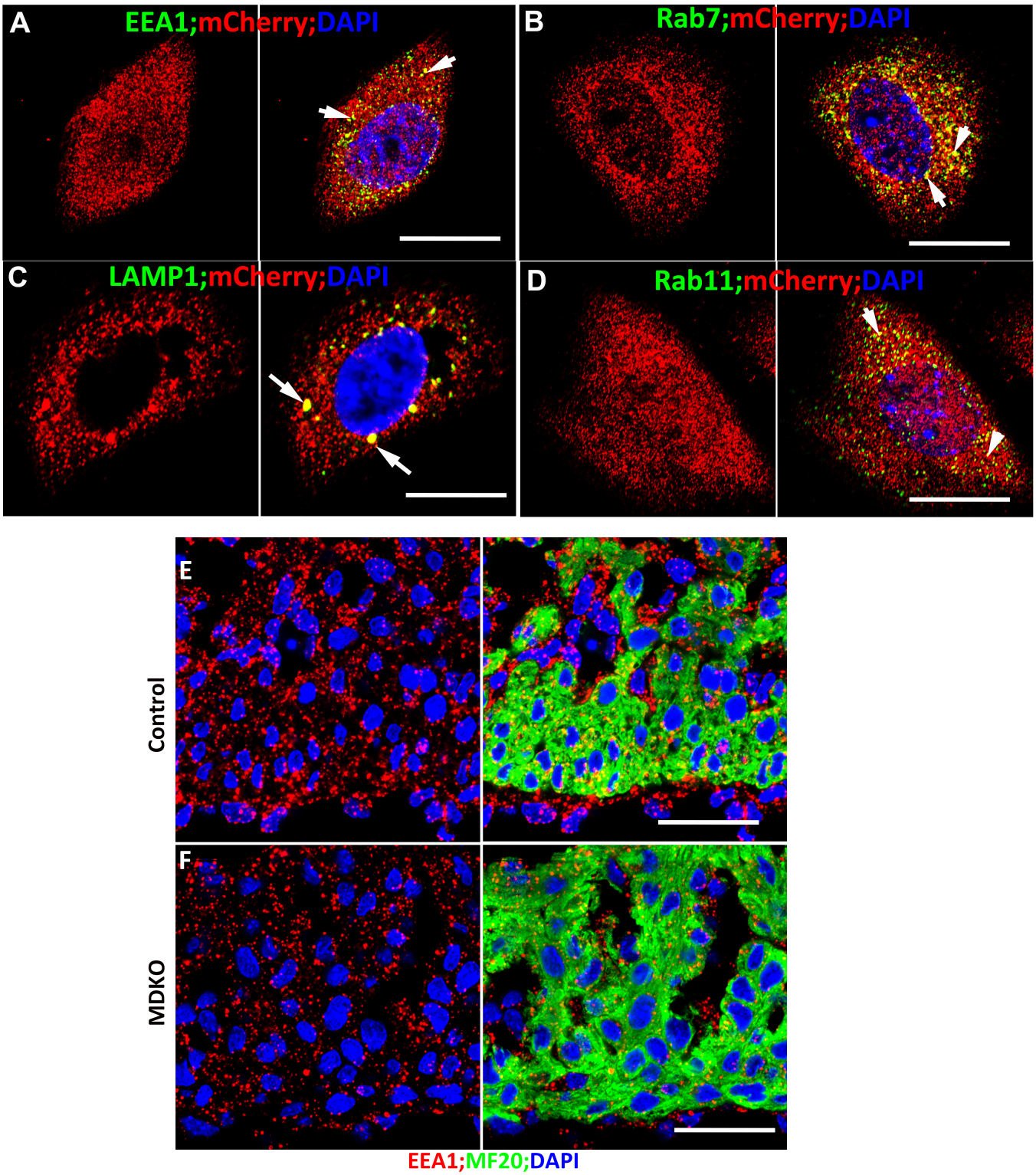


Figure S7. Numb and N-cadherin interact in cardiomyocytes via DPL assay

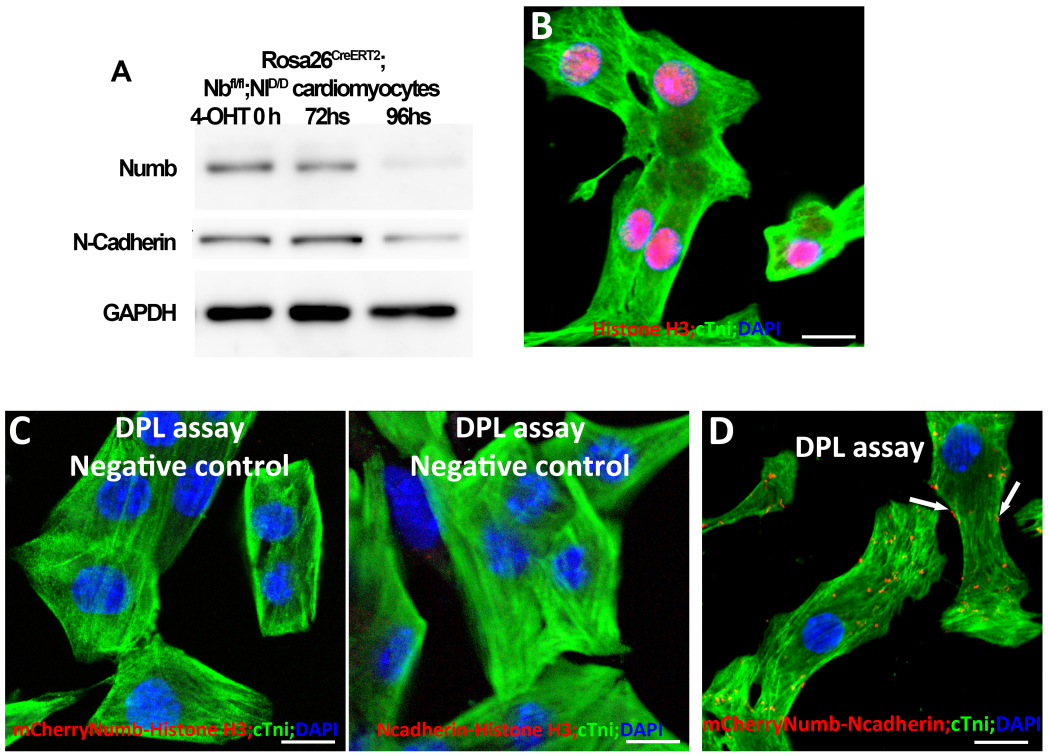
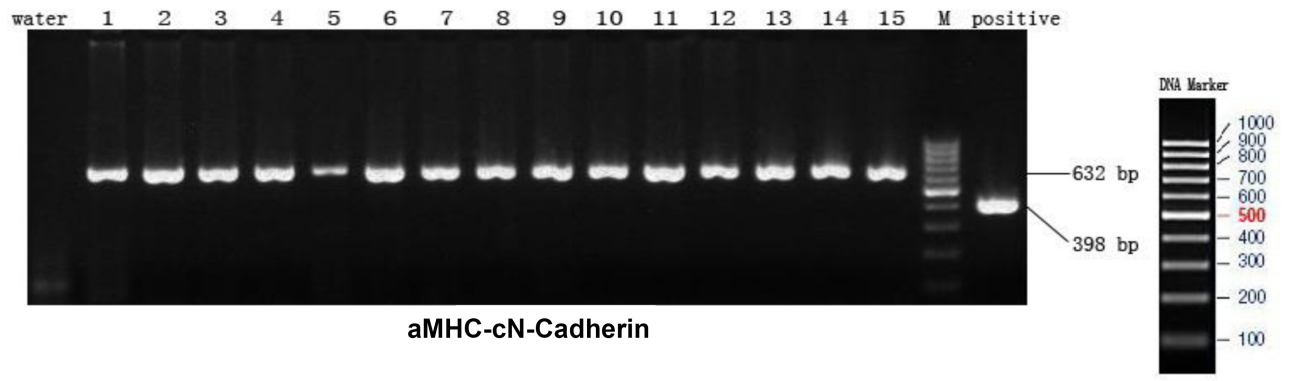
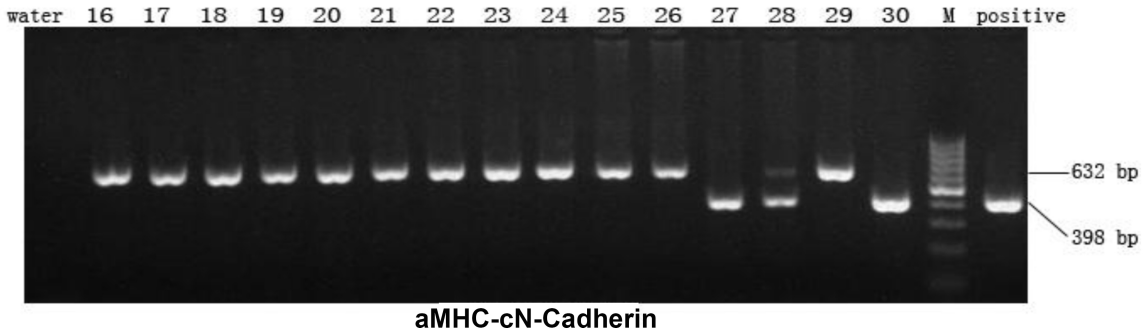


Figure S8. cN-Cadherin transgenic line generation

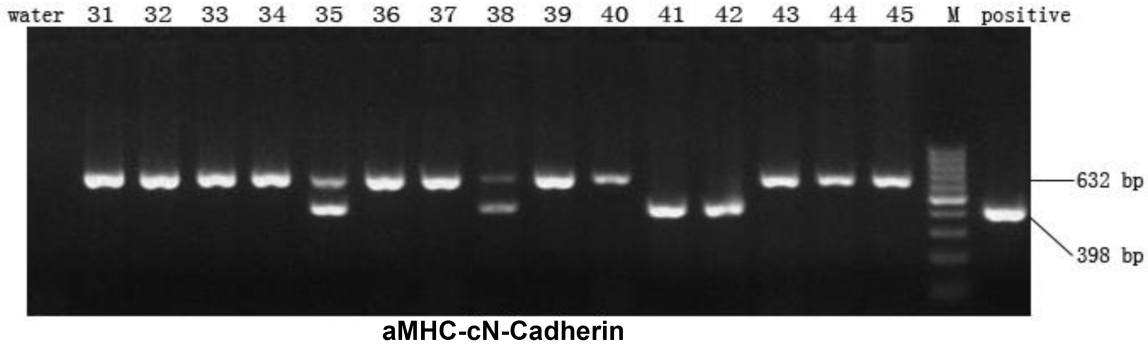
A



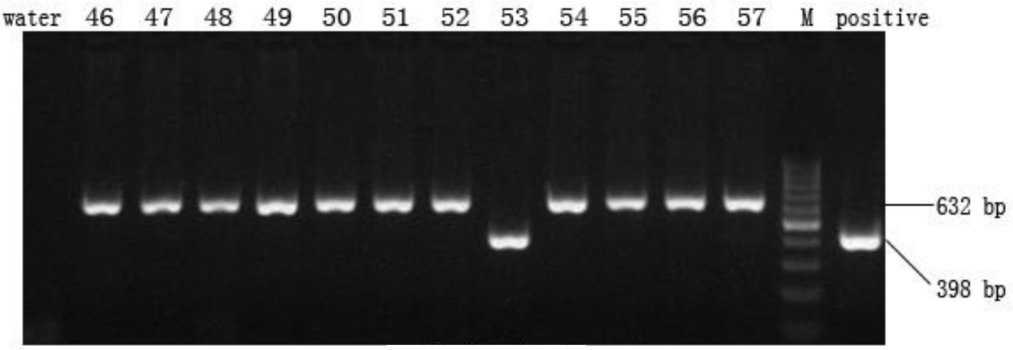
aMHC-cN-Cadherin



aMHC-cN-Cadherin

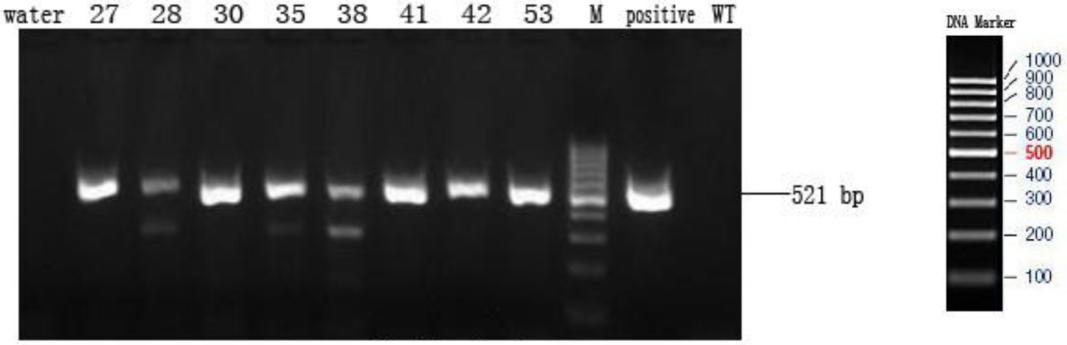


aMHC-cN-Cadherin



aMHC-cN-Cadherin

B



aMHC-cN-Cadherin

Figure S9. Model of NFPs regulating N-Cadherin recycling, cellular orientation and trabecular morphogenesis.