Supplemental Figure legends:

Supplemental Figure 1: Amino acid sequence for AnkB-188

Supplemental Figure 2: Amino acid sequence for AnkB-212

Supplemental Figure 3: AnkB and NCX expression in HeLa-T7 cells. (**A**) HeLa cells expressed endogenous AnkB (~190 kD) as demonstrated by immunoblot analysis. (**B**) AnkB-188-mediated NCX membrane enrichment was quantified by densitometry from 3 separate experiments. NCX membrane expression was normalized to pan-actin and graphed as relative units (RU) ***P*-value < 0.05. (**C**) NCX mRNA expression was measured by qt-PCR in HeLa cells transiently expressing GFP, B188-GFP, or B212-GFP and graphed as cycle threshold values (C_T).

Supplemental Figure 4: Expression of pan-AnkB and AnkB-212 in isolated AnkB$^{+/-}$ adult cardiomyocytes. (**A**, **C**) Two subpopulations of ankyrin-B are expressed in AnkB$^{+/-}$ cardiomyocytes. The less abundant population (white arrowheads) co-localizes with α-actinin at the Z-line (**A**), while the more abundant population (white arrows) co-localizes with myomesin at the M-line (**C**). There is one population of AnkB-212 (white arrows) that co-localizes with myomesin (**D**), but not with α-actinin (**B**). For all antibody conditions, 6-8 myocytes per condition were imaged. Scale bar is 2 μm.

Supplemental Figure 5: AnkB-212 CTD ΔE46 lacks obscurin-binding and M-line targeting. (**A**) Diagram of two tagged (flag and GFP) constructs of AnkB-212 CTD that lack the first obscurin binding site in *Ank2* exon 46. (**B**) A GST-fusion protein of obscurin CTD (containing the ankyrin-binding sites) does not precipitate AnkB-212 CTD ΔE46. (**C**) GFP-tagged AnkB-212 CTD ΔE46 does not co-localize with myomesin in virally transduced cardiomyocytes.

Supplemental Figure 6: AnkB-188 knockdown decreases NCX Z-line localization. (**A**) Endogenous AnkB-212 co-localizes with the M-line marker myomesin. AnkB-212 siRNA treatment reduces AnkB-212

expression at the M-line. (**B**) Both the M-line and Z-line populations of ankyrin-B are visualized in the untransfected and scramble controls. AnkB-188 siRNA treatment diminishes the AnkB subpopulation at the Z-line. (**C**) Expression and localization of NCX and ankyrin-B are not changed by scramble siRNA transfection. (**D**) NCX expression at the Z-line (α-actinin) is not affected by either scramble or AnkB-212 siRNA treatment. In contrast, AnkB-188 siRNA treatment dramatically reduces the expression and striated patterning of NCX.

Supplemental Figure 7: Epinephrine enhances contraction rate and variability in cardiomyocytes treated with siRNAs to AnkB-188 or AnkB-212. The left panel presents representative contraction rhythms in control and transfected (scramble, B188-siRNA, B212-siRNA) cardiomyocytes following epinephrine stimulation (1 μM). The right panel presents the summary data for each condition. Box-and-whisker plots represent the difference in time between individual contractions and the average contraction time over a 30-second interval. Sample size is untransfected + epi: 5, scramble + epi: 5, B188-siRNA + epi: 7, B212-siRNA + Epi: 5. #$p>0.05$ and *$p<0.05$ when compared to the untransfected + epi control.

Supplemental Table 1: siRNA sequences targeting rat Ank2

Target	Oligo Name	Sequence (5'-3')
E12	B188-siRNA sequence #2 sense	CUGCCUUCAUGGGCCACUU[dT][dT]
	B188-siRNA sequence #2 anti-sense	AAGUGGCCCAUGAAGGCAG[dT][dT]
E17	B188-siRNA sequence #1 sense	CCAUGUUGCUGCUCAUUAU[dT][dT]
	B188-siRNA sequence #1 anti-sense	AUAAUGAGCAGCAACAUGG[dT][dT]

E48	B212-siRNA sequence #1 sense	CAGCUAUUCCAAAGUGAUA[dT][dT]
	B212-siRNA sequence #1 anti-sense	UAUCACUUUGGAAUAGCUG[dT][dT]
E49	B212-siRNA sequence #2 sense	GUUUCAGGCGGAACCAGUA[dT][dT]
	B212-siRNA sequence #2 anti-sense	UACUGGUUCCGCCUGAAAC[dT][dT]-
	Scramble sense	GCUCCCAGCUCGUCUAUGU[dT][dT]
	Scramble anti-sense	ACAUAGACGAGCUGGGAGC[dT][dT]

Supplemental Table 2: *ANK2* qt-PCR primer sets

Primer set	5' primer	3' primer	Size (bp)	T_m (C°)	Primer eff. (%)
Hu-E31/32	GCATGGATGAAG/tactggatag	ctgctcagtacacctccttc	151	64	91
Hu-E45/51	CAGCTTTTGAAAAG/gacaacaatgag	caagtcctccttgcagaaatg	180	62.5	90
Hu-E50/51	GGATCAATAATTAAAAG/gacaacaatgag	caagtcctccttgcagaaatg	183	57	94
Mu-E31/32	CATGGATGAAG/tgctggacag	cactgtgctgctcagtactc	158	67	90
Mu-E45/51	CGCTTTTCAAAAG/gacaacaatgcg	gaaggagttgctggagatctc	157	59	97
Mu-E50/51	GATCAATAATTAAGAG/gacaacaatgcg	gaaggagttgctggagatctc	160	62	90
Ra-E12/13	GGTTTTACTCCACTGCACATTG	gttagtgacatctggagaggc	192	64.5	107
Ra-E31/32	CATGGATGAAG/tgctggacag	gacagcttgcacctgtgatac	178	64.5	101
Ra-E50/51	GATCAATAATTAAGAG/gacaacaatgag	gaaggagttgctggagatctc	159	57	101

Columns represent: name of the primer set, sequence for 5' and 3' primers, size of PCR product, optimal annealing temperature, and primer efficiency. Primer efficiency was determined using a standard curve of C_t values acquired from a 10-fold dilution of heart cDNA (e.g. 1, 1:10, 1:100, 1:1000).

2. Supplementary Methods

2.4 Quantitative real-time (qt)-PCR analysis of *ANK2* transcripts

Exon-exon boundary spanning primers were designed to the unique splice junctions encoding the C-terminal domains of AnkB-188 kD (exon junction 45 to 51) and AnkB-212 kD (exon junction 50 to 51). Primers were optimized based on nucleotide sequence and annealing temperatures such that primer efficiencies were between 90 – 110%. cDNAs were generated from ventricular and atrial mRNA that was isolated from three human and three mouse hearts. Relative expression of *ANK2* transcripts was measured in triplicate by qt-PCR using SYBR Green dye (Bio-Rad) and experiments were replicated three times. Individual C_t values were normalized to the average of the C_t values of *ANK2* transcripts with exon junction 31 to 32 (which encodes the minimal spectrin-binding domain). Expression of *ANK2* transcripts corresponding to AnkB-188 kD and AnkB-212 kD were expressed as a percentile of *ANK2* transcripts with exon junction 31 to 32, which was set to 100%. Error bars represent standard deviation with a sample set of three.

2.6 Immunoblot assay: cellular fractionation

HeLa-T7 cells stably expressing NCX lentivirally transduced with GFP, AnkB-188-GFP, or AnkB-212-GFP were washed with cold PBS, lysed in 200µL of homogenization buffer (10mM Tris pH 7.5, 5mM $MgCl_2$, 1mM EGTA, 0.003mg aprotinin, 0.1mM Na_3VO_4, 0.033mg leupeptin, and 1mM DTT), and homogenized with 1mL-syringe and 23G1¼ needle with repeated strokes. The homogenate was centrifuged 1500 x g for 10 minutes at 4°C and the supernatant was removed for ultracentrifugation at 100,000 x g for 40 minutes at 4°C. The resulting supernatant (cytosolic fraction) was placed in a new tube, and the pellet (membrane fraction) was washed and resuspended in homogenization buffer by sonication for 1 min at 4°C. Protein contents for both fractions were measured and same protein quantities were separated by

SDS-PAGE and immunobloted with the following primary antibodies: NCX (1:250, Swant, Switzerland), GFP (1:500, Santa Cruz), and pan-actin-5 (1:10,000; Thermo Scientific).

2.7 Isolation and cultures of neonatal rat cardiomyocytes (NRVM)

NRVM were isolated from 1-2 day old Sprague-Dawley rat hearts as previously described with a minor modification [14]. In accordance with the animal protocol approved by the Animal Welfare Committee at University of Texas Health Science Center at Houston, neonatal rat pups were euthanized by decapitation. Briefly, hearts were exposed, excised, minced, and digested through a series of agitations in buffer containing collagenase type II (Worthington, Collagenase type II 305U/mg) and pancreatin (Sigma). Cells from each digestion were collected and pooled, and pre-plated on Nunc plates (Thermo Fisher Scientific) to reduce fibroblasts and enrich for cardiomyocytes. Cells were plated on either Primaria plates (Corning) or fibronectin-coated glass-bottom MatTek plates (MatTek Corporation). Media was changed 24-hours after plating and experiments started 2 days after plating to ensure homogenous cultures. Following 3 days in culture, cardiomyocytes were transduced with lenti-viral constructs of GFP-tagged full-length AnkB-188 or AnkB-212, and imaged 4 days later by confocal microscopy.

2.8 Isolation of individual adult mouse cardiomyocytes

Adult mouse cardiomyocytes were isolated as described previously [14]. In accordance with the animal protocol approved by the Animal Welfare Committee at University of Texas Health Science Center at Houston, 3 month-old mice were anesthetized with intraperitoneal injection of tribromoethanol-Avertin (Sigma T48402) at 250 mg/kg in 1X PBS and euthanized by removing the heart. Hearts from wild type or ankyrin-$B^{+/-}$ mice were placed in ice-cold saline and the aorta was cannulated. Hearts were first perfused with warm perfusion buffer for a few minutes, followed by perfusion with digestion buffer containing collagenase (Worthington, Collagenase type II 305U/mg). Once digested, hearts were minced and

triturated, then centrifuged at 300 rpm x 5 minutes at 4˚C. Supernatant was removed and cells were immediately fixed in ice-cold 100% ethanol, and kept in -20˚C until use.

2.10 Fluorescent immunocytochemistry and image quantification

Cells were washed in ice-cold phosphate-buffered saline (PBS, pH 7.4) 3x. Cells were then blocked with 5% normal goat serum and 0.075% TritonX-100 for 30 minutes at room temperature then incubated in primary antibodies overnight at 4˚C. Primary antibodies used were: ankyrin-B (1:500, [1]), ankyrinB-212 (1:900), α-actinin (1:1000, Sigma), myomesin (1:500, Developmental Studies Hybridoma Bank, University of Iowa), MyBPC3 (1:250, Santa Cruz), GFP (1:250, UC Davis/NIH NeuroMab Facility). Secondary antibodies used were goat anti-rabbit conjugated to Alexa Fluor 488 and goat anti-mouse conjugated to Alexa Fluor 568 (1:500, LifeTechnologies). Hoechst 33258 (1:1000, LifeTechnologies) was used for nuclear staining after removal of the secondary antibody. Images were obtained with a Nikon A1 confocal microscope (Nikon, Melville, NY) equipped with 100X oil, numerical aperture 1.4 objective. Fluorescence intensities for ankyrin-B populations and sarcomeric markers were analyzed with ImageJ (version 1.47, NIH, Bethesda, MD) and Excel (Microsoft, Bellevue, WA). Intensities were obtained by the mean pixel intensity of the magnified image.

2.11 Binding studies

AnkB-188-GFP and AnkB-212-GFP were expressed in HeLa-T7 cells and purified using an affinity-purified GFP antibody coupled to protein A-agarose beads as previously described [15] and incubated with *in-vitro* translated ^{35}S-radiolabeled fragments of the sodium-calcium exchanger (Asp-253 to Lys-615, NM_021097) or the C-terminal domain of human obscurin (Leu-6148 to Asn-6460, NM_052843) (TnT T7-Coupled Reticulate Lysate System, Promega). Binding reactions occurred in 500µL binding buffer (50mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 150mM NaCl, 0.1% TritonX-100) at 4^{o}C over night. Reactions were washed in wash buffer (binding buffer with 500 mM NaCl and 1% TritonX-100), pelleted, re-suspended in SDS-

sample buffer, separated by SDS-PAGE, and visualized with autoradiography film (HyBlot CL, Denville Scientific).

2.12 Patch-clamp recording

Whole-cell patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, CA)at room temperature (22–24 °C) on the stage of an inverted phase-contrast microscope equipped with an appropriate filter set for green fluorescence protein visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument Company, Novato, CA) with a Sutter P-97 pipette puller had resistances of 2–4 MΩ when filled with pipette solution containing (mM) 120 CsCl, 20 NaCl, 5 Na_2ATP, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 EGTA with pH 7.3 and 315 mOsm l^{-1} in osmolarity. The extracellular solution for whole-cell recording contains (mM) 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 mM glucose, and 5 mM HEPES (the pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to 340 mOsm l^{-1} with sucrose). The whole-cell membrane currents were recorded using a voltage ramp (from +80 to -100 mV at 100 mV/s) following 100-ms step depolarization to +80 mV from a holding potential of -40 mV. Data were acquired and analyzed using pClamp 10 software (Molecular Devices, CA). Currents were filtered at 2 kHz and digitized at 10 kHz with Digidata 1440A acquisition system (Molecular Devices, CA). Membrane capacitance was directly read from the membrane test function of pClamp 10. Current density was obtained by dividing the current amplitude by cell capacitance.

2.13 Recording and analysis of NRVM contraction rates

Contracting NRVM syncytium was located on light microscope (Micromaster; Fisher) with 10X magnification eyepiece and 20X objective lens. A camera with 10 megapixel resolution (HTC One M8) was mounted on the microscope using Snapzoom Universal Digiscoping Adapter (Snapzoom). Contractions were recorded for 2 to 2.5 minutes. The same recording method was used following stimulation with 1μM epinephrine.

Using Video Spot Tracker (VST) program (http://cismm.cs.unc.edu/downloads) and the tracking method described by Fassina *et. al*. [16] we placed a marker on the contracting syncytium and the program tracked the marker displacement frame by frame (30 frames per second) registering the spatial-temporal coordinates x, y, (expressed in pixels), and t (expression in frame number that converts to second). Coordinates were plotted in Excel (Microsoft) with each peak corresponding to an active syncytial contraction. We assessed rhythmicity of the syncytial contractions by randomly picking a consecutive 30-second interval and measuring the time between contractions. The average contraction time for each video was calculated and the time difference between each contraction and the average was plotted on GraphPad Prism 6 (GraphPad Software) using box-and-whisker plots.

Wu, Supplemental Figure 1

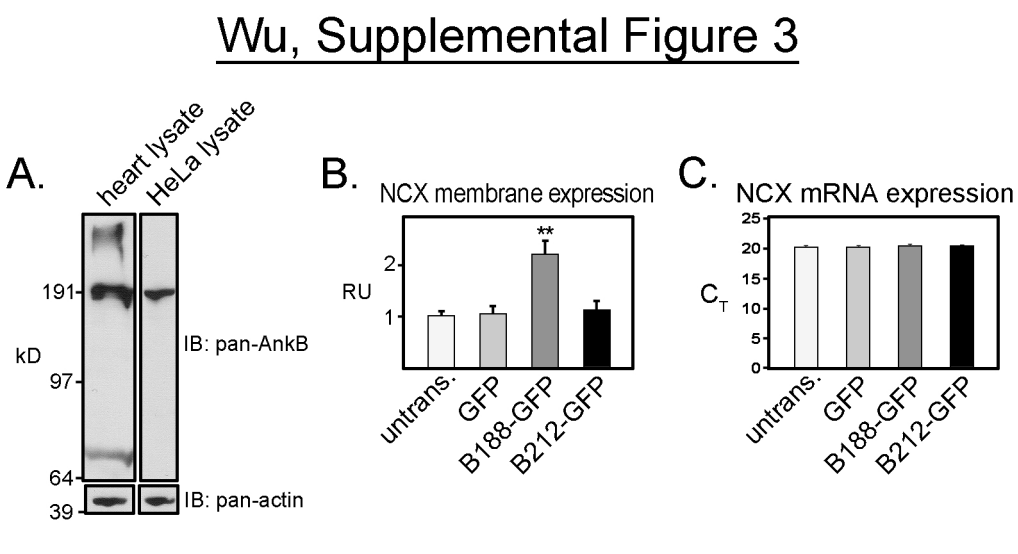
AnkB-188 kD:

Wu, Supplemental Figure 2

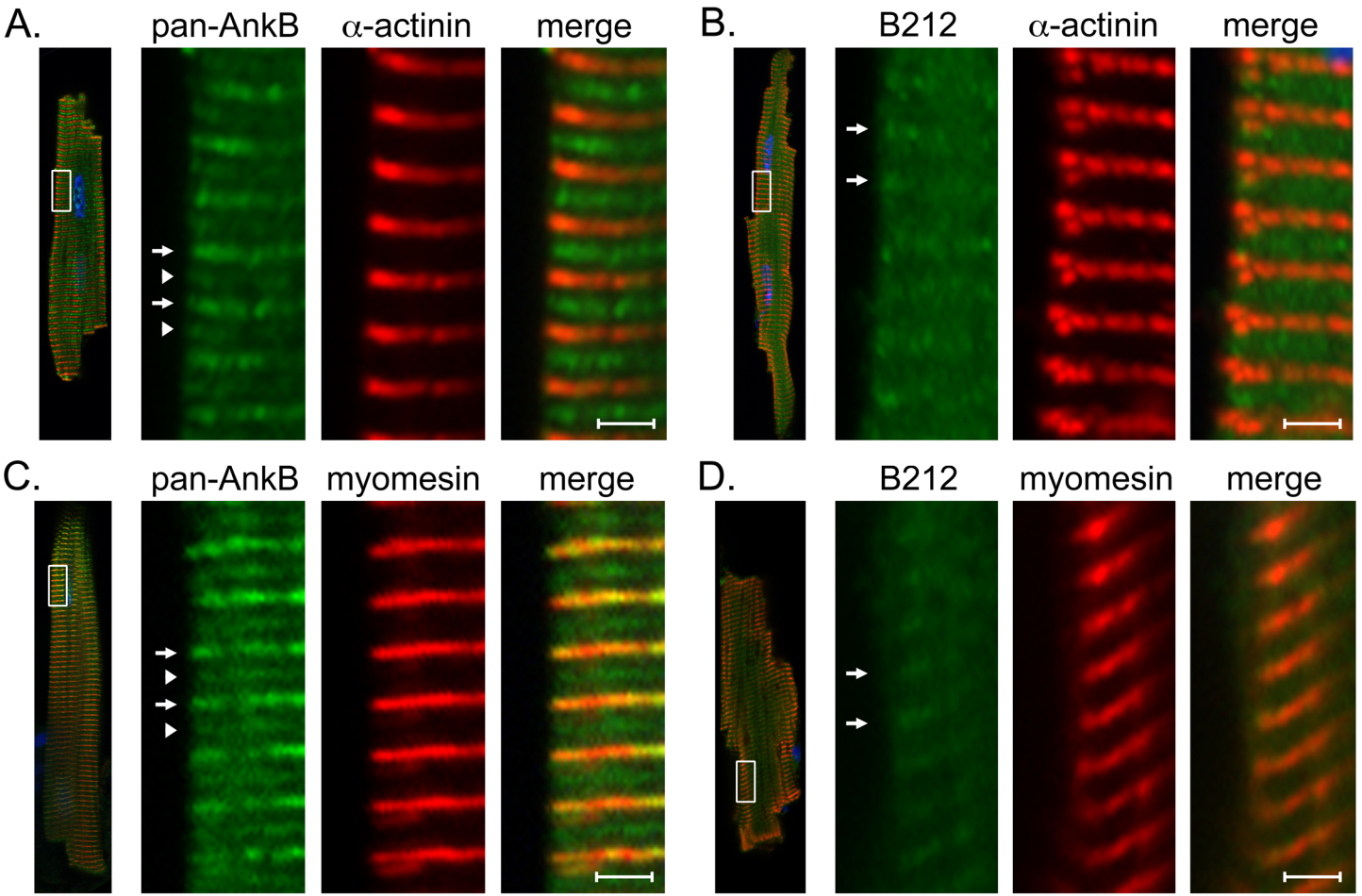
AnkB-212 kD:

```
MTTMLQKSDSNASFLRAARAGNLDKVVEYLKGGIDINTCNQNGLNALHLAAKEGHVGLVQELLGRGSSVDSATKKGNTALHIASLAGQAEVVKVLVKEGANINAQSQNGFTPLY
MAAQENHIDVVKYLLENGANQSTATEDGFTPLAVALQQGHNQAVAILLENDTKGKVRLPALHIAARKDDTKSAALLLQNDHNADVQSKSGFTPLHIAAHYGNVNVATLLLNRGA
AVDFTARNGITPLHVASKRGNTNMVKLLLDRGGQIDAKTRDGLTPLHCAARSGHDQVVELLLERGAPLLARTKNGLSPLHMAAQGDHVECVKHLLQHKAPVDDVTLDYLTALHV
AAHCGHYRVTKLLLDKRANPNARALRGETALHMAARAGQVEVVRCLLRNGALVDARAREEQTPLHIASRLGKTEIVQLLLQHMAHPDAATTNGYTPLHISAREGQVDVASVLLE
AGAAHSLATKKGFTPLHVAAKYGSLDVAKLLLQRRAAADSAGKNGYTPLHIAAKKNQMQIASTLLNYGAETNIVTKQGVTPLHLASQEGHTDMVTLLLDKGANIHMSTKSGLTS
LHLAAQEDKVNVADILTKHGADQDAHTKLGYTPLIVACHYGNVKMVNFLLKQGANVNAKTKNGYTPLHQAAQQGHTHIINVLLQHGAKPNATTANGNTALAIAKRLGYISVVDT
LKVVTEEVTTTTTTITEKHKLNVPETMTEVLDVSDEEALKQFGDHFIDGEALSDSGDDTMTGDGGEYLRPEDLKELGDDSLPSSQFLDGMNYLRYSLEGGRSDSLRSFSSDRSH
TLSHASYLRDSAVMDDSVVIPSHQVSTLAKEAERNSYRLSWGTENLDNVALSSSPIHSGRASPCLERDNSSFLVSFMVDARGGAMRGCRHNGLRIIIPPRKCTAPTRVTCRLVK
RHRLATMPPMVEGEGLASRLIEVGPSGAQFLGPVIVEIPHFAALRGKERELVVLRSENGDSWKEHFCDYTEDELNEILNGMDEVLDSPEDLEKKRICRIITRDFPQYFAVVSRI
KQDSNLIGPEGGVLSSTVVPQVQAVFPEGALTKRIRVGLQAQPMHSELVKKILGNKATFSPIVTLEPRRRKFHKPITMTIPVPKASSDVMLNGFGGDAPTLRLLCSITGGTTPA
QWEDITGTTPLTFVNECVSFTTNVSARFWLIDCRQIQESVTFASQVYREIICVPYMAKFVVFAKSHDPIEARLRCFCMTDDKVDKTLEQQENFAEVARSRDVEVLEGKPIYVDC
FGNLVPLTKSGQHHIFSFFAFKENRLPLFVKVRDTTQEPCGRLSFMKEPKSTRGLVHQAICNLNITLPIYTKESESDQEQEEEIDMTSEKNPQDEQERIEERLAYIADHLGFSW
TELARELDFTEEQIHQIRIENPNSLQDQSHALLKYWLERDGKHATDTNLVECLTKINRMDIVHLMETNTEPLQERISHSYAEIEQTITLDHSEGFSVLQEELCTAQHKQKEEQA
VSKESETCDHPPIVSEEDISVGYSTFQDGVPKTEGDSSATALFPQTHKEQVQQDFSGKMQDLPEESSLEYQQEYFVTTPGTETSETQKAMIVPSSPSKTPEEVSTPAEEEKLYL
QTPTSSERGGSPIIQEPEEPSEHREESSPRKTSLVIVESADNQPETCERLDEDAAFEKELTEELGELEASSDEEAMVTTRVVRRRVIIQGDDMPEIPPETVTEEEYIDEHGHTV
VKKVTRKIIRRYVSSEGTEKEEIMVQGMPQEPVNIEEGDGYSKVIKRVVLKSDTEQSEVTLCEPSILSSTSQFQAEPVEGRRVSKVVKTTVVLGERMEKHLGDSSLATDLPSAK
DDFEEALSYTGSHMKVHLPSLVENEILKASEDGSIIKRTTMSKAITQKRAVVKDQHGKRIDLEHLEDVPEALDQDDLQRDLQQLLRHFCKEDLKQEAK
```

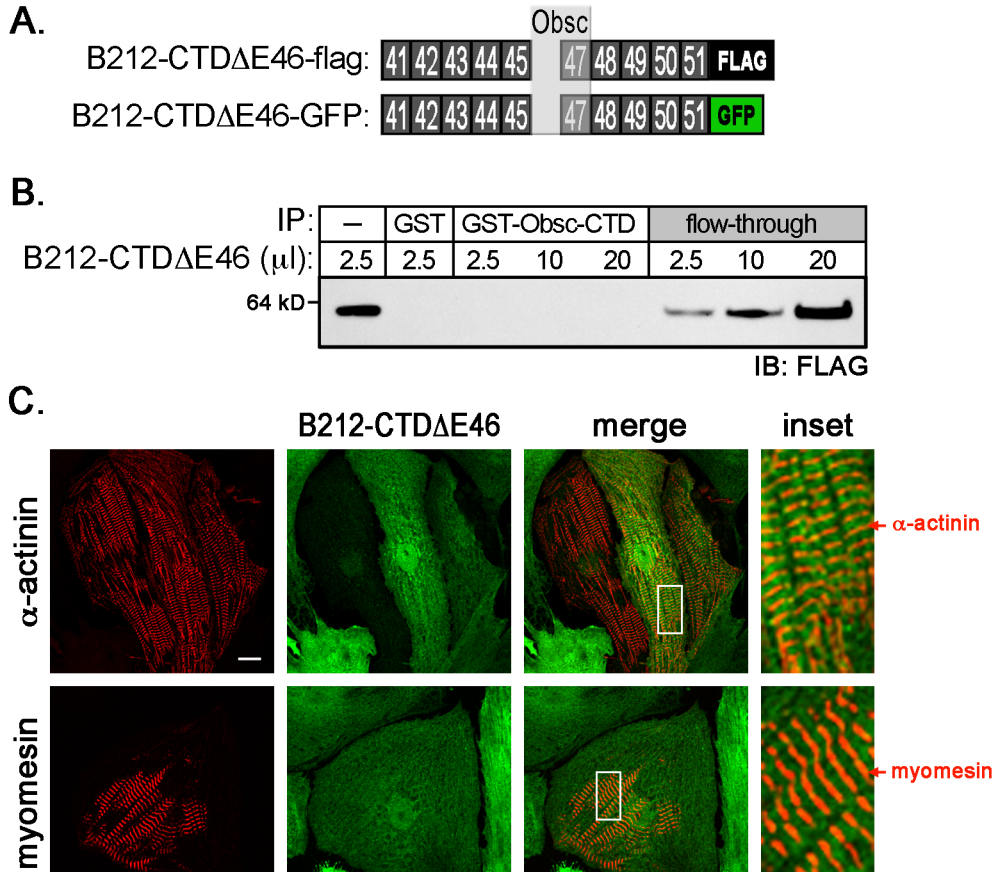
Wu, Supplemental Figure 3



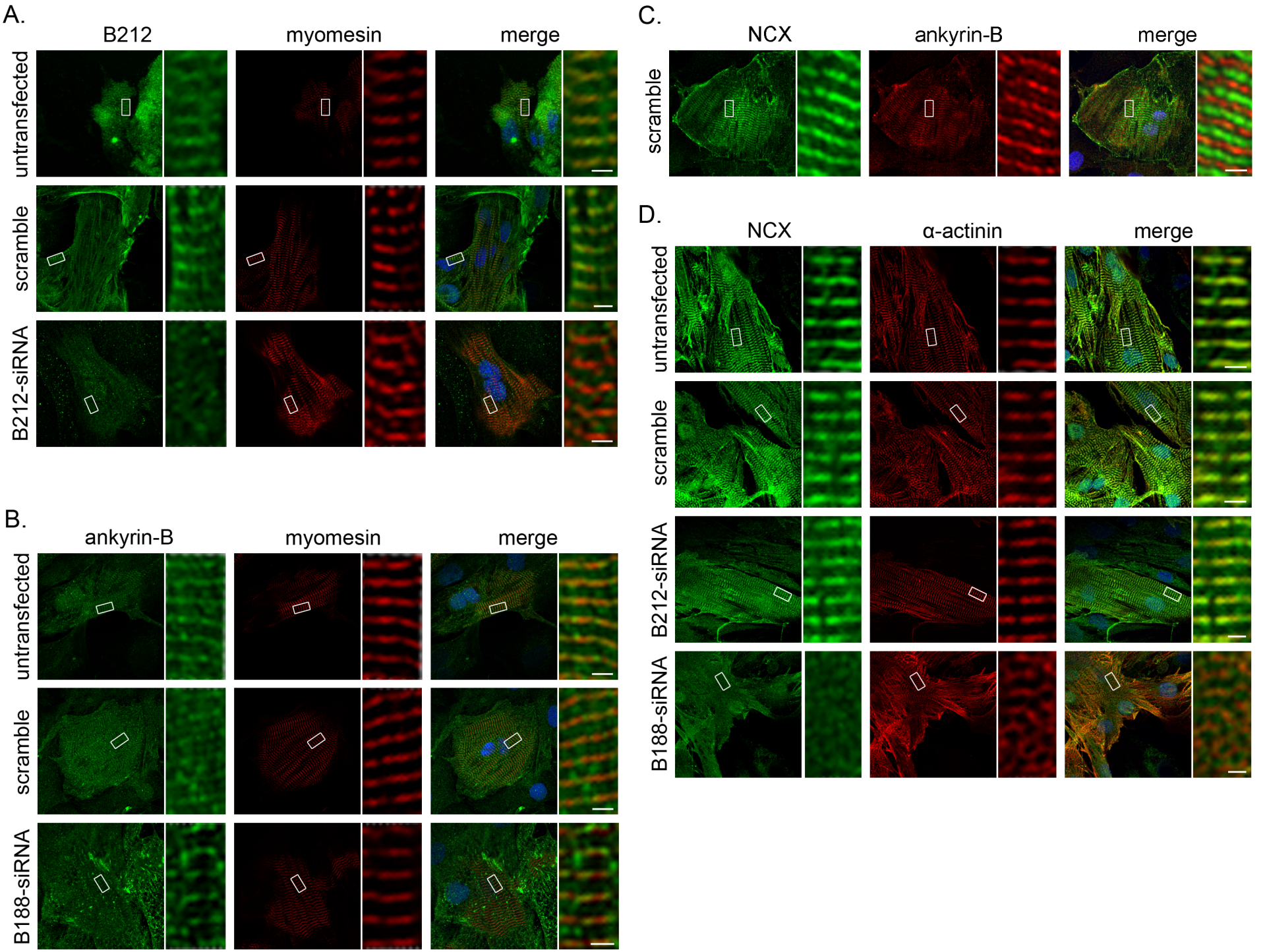
Wu, Supplemental Figure 4



Wu, Supplemental Figure 5



Wu, Supplemental Figure 6



Wu, Supplemental Figure 7