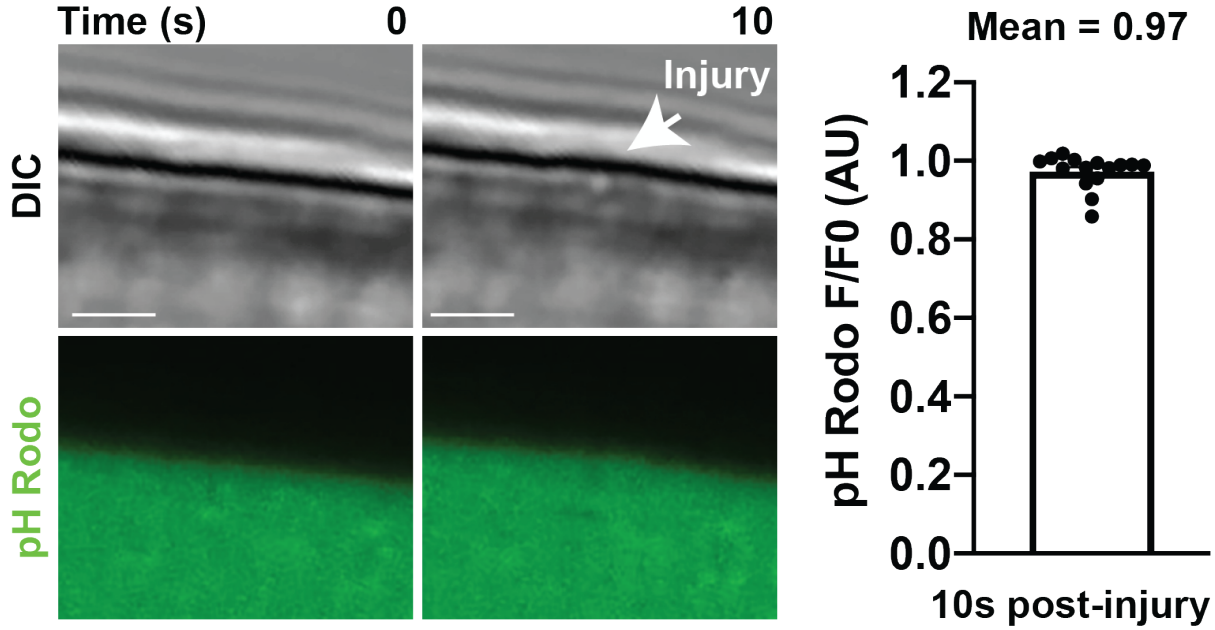
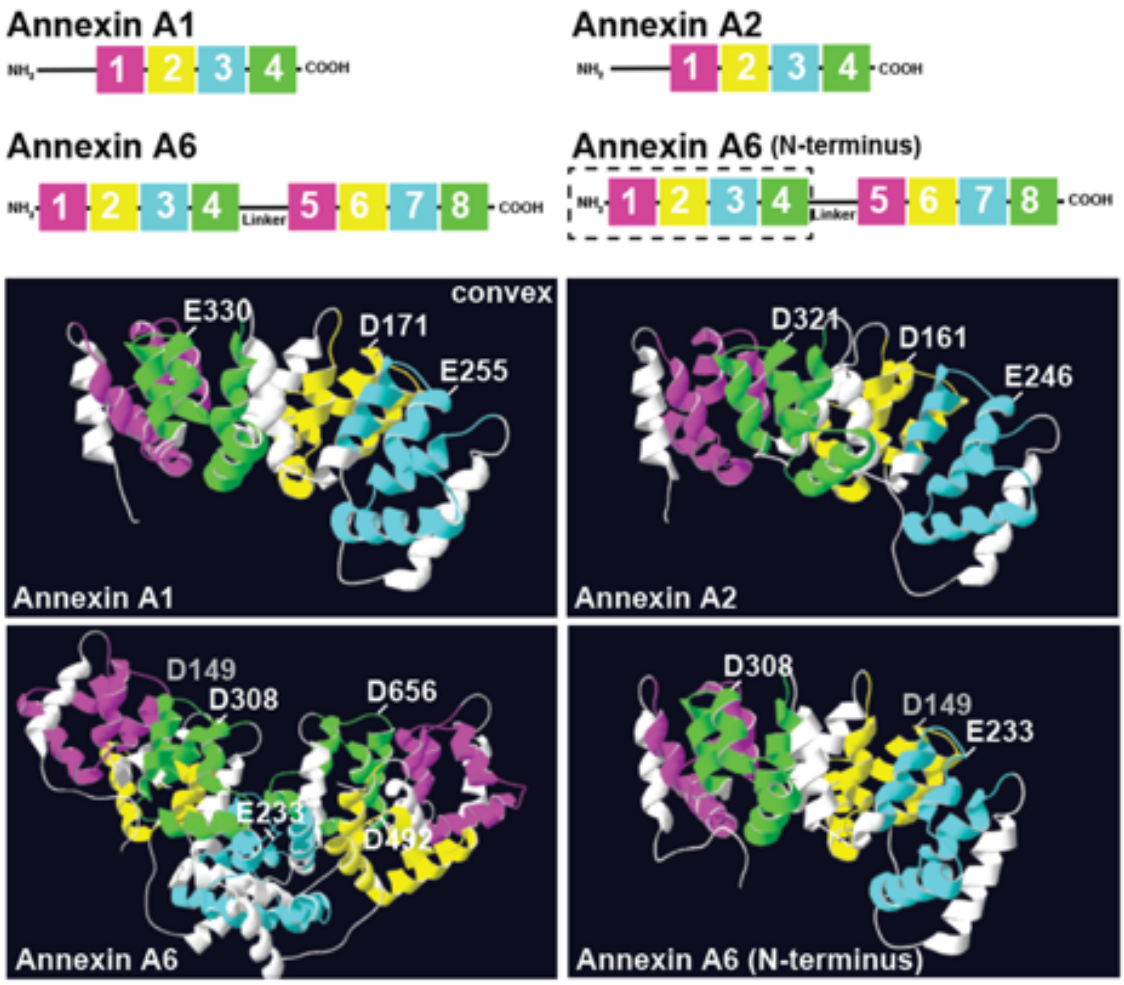
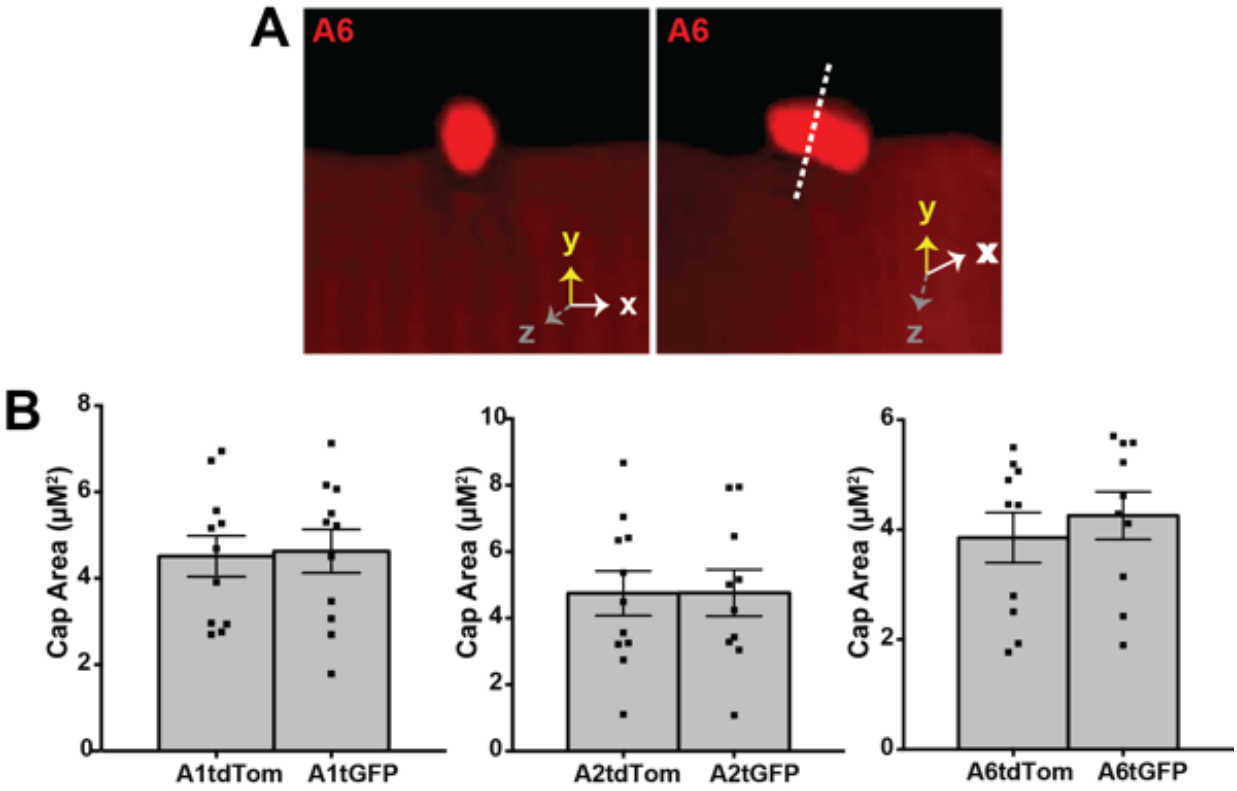
SUPPLEMENTAL FIGURES LEGENDS FOR
Demonbreun et al. Recombinant annexin A6 promotes membrane repair and protects against muscle injury



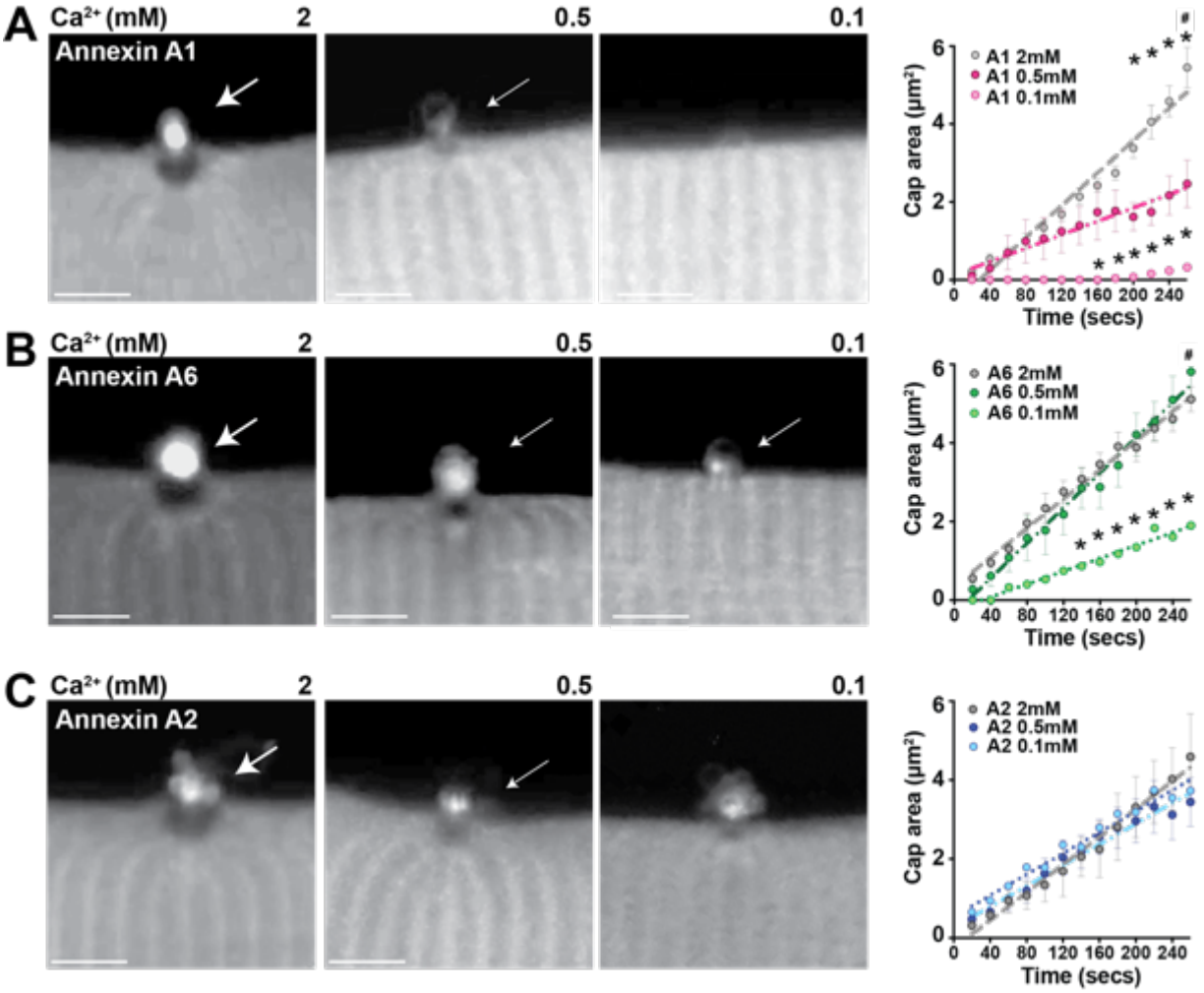
Supplemental Figure 1. Lack of visible pH change immediately after membrane injury. Myofibers were laser injured in the presence of the pH fluorescence indicator pH Rodo AM. The pH remained unchanged between pre injury (0s) and post-injury (10s) expressed as F/F0 mean = 0.97, where a value of 1 is identical.



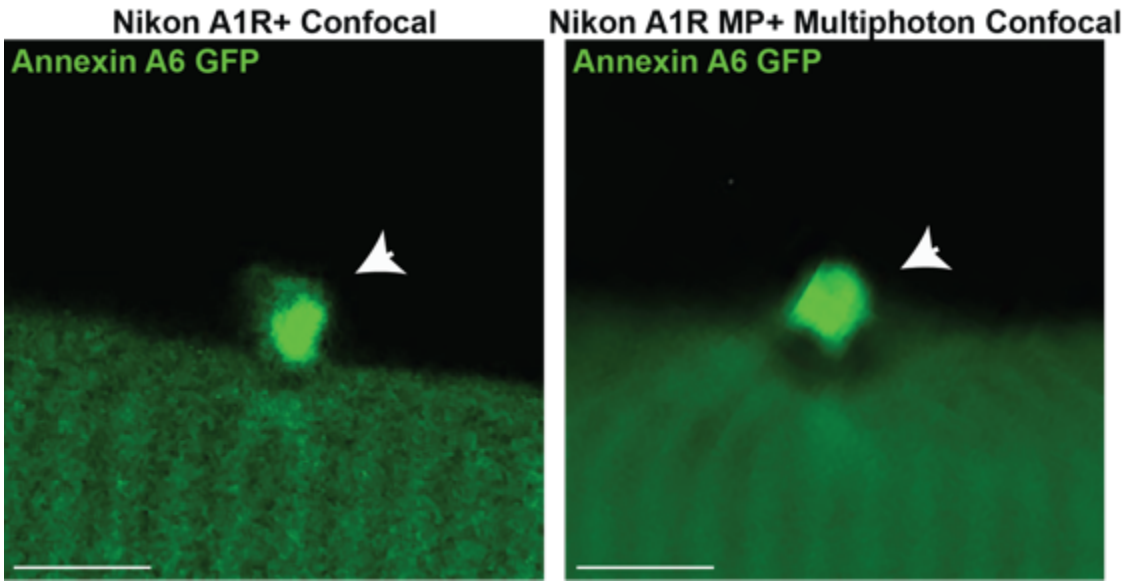
Supplemental Figure 2. Domain modeling of annexin's multiple Ca^{2+} binding sites. Annexins typically contain four annexin domains (pink, yellow, blue and green) with the exception of annexin A6, which is duplicated to have 8 annexin domains. Annexin A1, A2, and A6 coordinate multiple Ca^{2+} ions and bind membrane on the convex face, positioned facing the top of each ribbon diagram. The D171 residue in annexin A1 and the D161 residue in annexin A2 were previously described as necessary residues to coordinate Ca^{2+} binding (43). Both D171 and D161 localize in the second (yellow) annexin domain in annexin A1 and A2, respectively. The orthologous residue in annexin A6, D149, which falls into the first half of annexin A6, was not observed to coordinate Ca^{2+} in bovine A6 (44). www.rcsb.org. In this study, we examined D149 in annexin A6 as well as E233, which fall into the second (yellow) and third (blue) annexin domains, respectively.



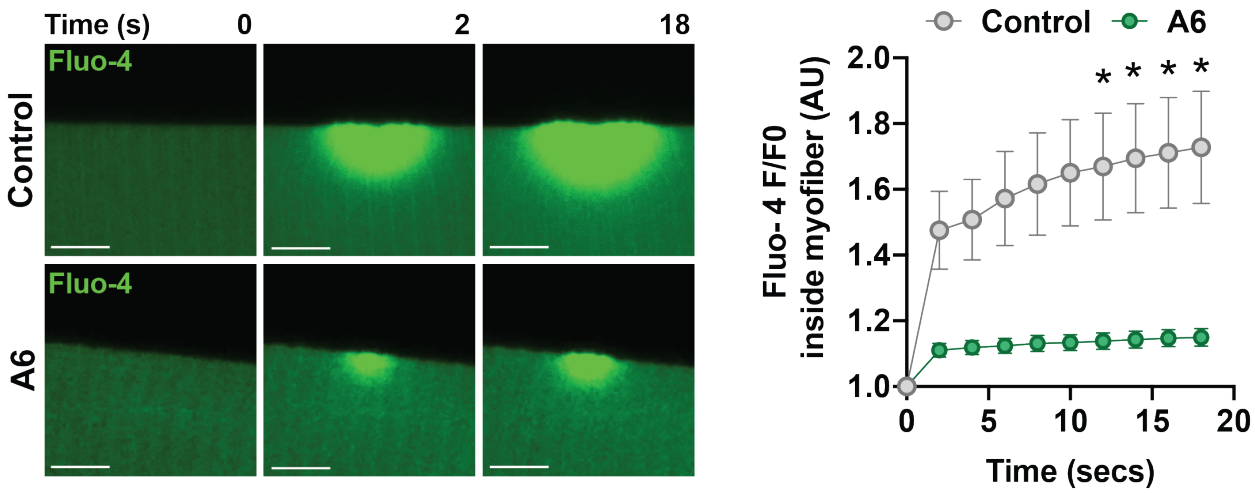
Supplemental Figure 3. A) Max projection of Z-stack imaging of repair cap (left). Rotated Z-stack projection (right). Cap size is measured from the center z-stack 2-D image represented by the white dotted line. **B)** Myofibers were co-electroporated with wildtype annexin-tdTomato and wildtype annexin-turboGFP. Cap size was assessed in both the red and green channels. The type of fluorescent tag did not influence cap size. Data are expressed as mean ± SEM. Differences were tested by two-tailed t-test, ($n \geq 10$ fibers per condition).



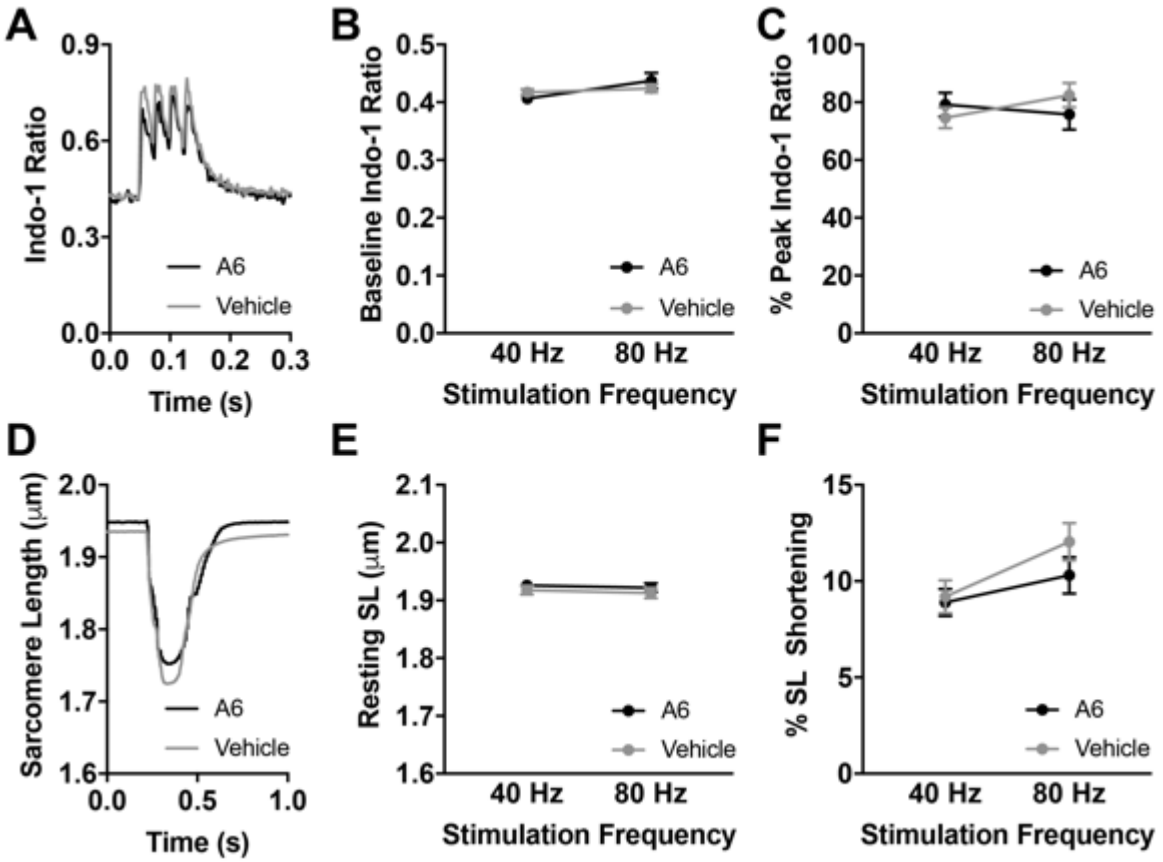
Supplemental Figure 4. Differential Ca^{2+} sensitivity of annexin A1, A2 and A6 in repair cap formation. Myofibers expressing fluorescently tagged annexin A1, A2 or A6 were injured at multiple Ca^{2+} concentrations. **A & B)** Annexin A1 and A6 repair cap size was reduced with decreasing Ca^{2+} concentrations. Annexin A1 and A6 repair cap area and rate of repair cap formation was reduced at 0.5mM Ca^{2+} and 0.1mM compared to 2mM**. C)** Annexin A2 repair cap area was significantly reduced at 0.05mM Ca^{2+}compared to 2mM, 0.5mµM, and 0.1mM Ca^{2+}. The rate of annexin A2 repair cap formation was equivalent at the Ca^{2+} concentrations tested**.** Data from the 0.05mM concentration was not plotted as caps were too small to measure over time. Large arrow indicates large cap. Small arrow indicates small cap. Scale bar 5µm. Data are expressed as mean ± SEM. Differences were tested by 2-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05 cap area or time, #p<0.05 statistically different slopes (n= 4-7 myofibers per condition).



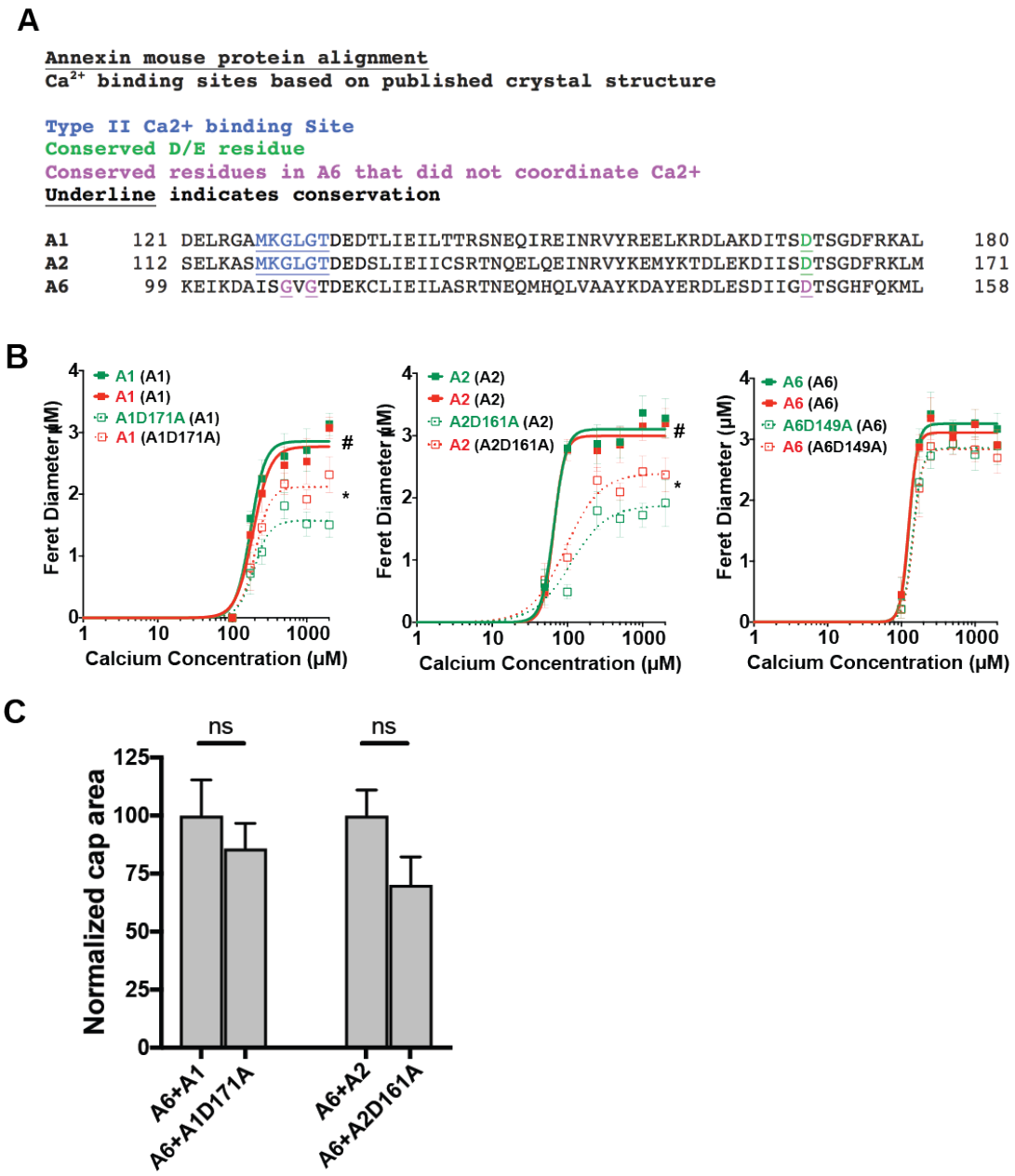
Supplemental Figure 5. Myofiber laser injury on the Nikon A1R+ GaSP confocal and the Nikon A1R MP+ multiphoton confocal induced comparable annexin A6-GFP repair caps (arrowhead) at the site of membrane injury. Scale 5μm, (n≥ 3 mice per condition).



Supplemental Figure 6. Decreased Fluo-4 Ca^{2+} levels at that site of injury with annexin A6 expression. Myofibers were preloaded with the fluorescent Ca^{2+} indicator dye, Fluo-4, and the sarcolemma subsequently injured with a confocal laser. Myofibers overexpressing annexin A6 had significantly decreased levels of internal Fluo-4 Ca^{2+} fluorescence at the site of membrane injury compared to control myofibers, similar to results obtained with the protein-based Ca^{2+} indicator GCaMP5G. Scale bar 5μm. Data are expressed as mean ± SEM. Differences were tested by 2-way ANOVA with Bonferroni's multiple comparisons test * $p<0.05$, (n=3 mice; $n\geq 8$ myofibers per condition).



Supplemental Figure 7. Baseline Ca^{2+} is not changed in myofibers overexpressing annexin A6. Myofibers electroporated with annexin A6-GFP or vehicle control. Isolated myofibers were loaded with Indo-1 AM dye and stimulated to measure Ca^{2+} cycling and cell shortening. **A)** Representative Ca^{2+} transient at 80Hz. **B & C)** Resting Ca^{2+} levels and peak Ca^{2+} transient values were not changed between myofibers electroporated with annexin A6-GFP or vehicle control. **D)** Representative sarcomere length shortening traces. **E & F)** Resting sarcomere length and peak unloaded sarcomere length shortening did not differ between treatment groups. Data are expressed as mean ± SEM. Differences were tested by 2-way ANOVA (A, D) or two-tailed t-test (B, C, E, F), ($n \geq 30$ myofibers from $n \geq 3$ mice per condition).



Supplemental Figure 8. A) The first type II Ca^{2+}-binding site in annexin A1 and A2 is conserved, while it is not conserved in annexin A6. **B)** Myofibers were co-electroporated with wildtype+wildtype or wildtype+mutant annexin constructs and cap size was assessed after sarcolemmal injury. Parentheses indicate protein that is co-expressed in the myofiber, but not visualized within the channel, to determine the effect on the co-expressed annexin. Cap kinetics were plotted as cap feret diameter over a range of Ca^{2+} concentrations, from 0-2mM. Expression of mutant annexin A1D171A and A2D161A, but not A6D149A was sufficient to significantly reduce the co-expressed wildtype annexin cap maximum diameter (D_{MAX}). **C**) Expression of annexin A1D171A and A2D161A was not sufficient to significantly reduce the cap area of co-expressed annexin A6, although annexin A2D161A results were trending. Data are expressed as mean ± SEM. Differences were tested by 2-way ANOVA with Bonferroni's multiple comparisons test (B) or two-tailed t-test (C). * $p<0.05$ #, ns = non-significant, ($n\geq5$ myofibers from $n\geq3$ mice per condition).

Annexin A6 Clustal protein alignment

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Homo        MAKPAQGAKYRGSIHDFPGFDPNQDAEALYTAMKGFGSDKEAILDIITSRSNRQRQEVCQ     60
Macaca      MAKPAQGAKYRGSIHDFPGFDPNQDAEALYTAMKGFGSDKEAILDIITSRSNRQRQEICQ     60
Canis       -MAKPQGVKYRGSIHDFPNFDPNQDAEALYTAMKGFGSDKEAILELITSRSNRQRQEISQ     59
Rattus      MAKIAQGAMYRGSVHDFADFDANQDAEALYTAMKGFGSDKESILELITSRSNKQRQEICQ     60
Mus         MAKIAQGAMYRGSVHDFPEFDANQDAEALYTAMKGFGSDKESILELITSRSNKQRQEICQ     60
                 **. ****:***  ** ******************:**::******:****:.*

Homo        SYKSLYGKDLIADLKYELTGKFERLIVGLMRPPAYCDAKEIKDAISGIGTDEKCLIEILA     120
Macaca      SYKSLYGKDLIADLKYELTGKFERLIVGLMRPPAYCDAKEIKDAISGIGTDEKCLIEILA     120
Canis       SYKSLYGKDLIADLKYELTGKFERLIVGLMRPLAYCDAKEIKDAISGIGTDEKCLIEILA     119
Rattus      SYKSLYGKDLIADLKYELTGKFERLIVNLMRPLAYCDAKEIKDAISGIGTDEKCLIEILA     120
Mus         NYKSLYGKDLIEDLKYELTGKFERLIVNLMRPLAYCDAKEIKDAISGVGTDEKCLIEILA     120
            .********** ***************.**** **************:************

Homo        SRTNEQMHQLVAAYKDAYERDLEADIIGDTSGHFQKMLVVLLQGTREEDDVVSEDLVQQD     180
Macaca      SRTNEQMHQLVAAYKDAYERDLEADIIGDTSGHFQKMLVVLLQGTREEDDVVSEDLVQQD     180
Canis       SRTNEQIHQLVAAYKDAYERDLEADIIGDTSGHFQKMLVVLLQGTREQDDVVSEDLVQQD     179
Rattus      SRTNEQIHQLVAAYKDAYERDLESDIIGDTSGHFQKMLVVLLQGTRENDDVVSEDLVQQD     180
Mus         SRTNEQMHQLVAAYKDAYERDLESDIIGDTSGHFQKMLVVLLQGTRENDDVVSEDLVQQD     180
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Homo        VQDLYEAGELKWGTDEAQFIYILGNRSKQHLRLVFDEYLKTTGKPIEASIRGELSGDFEK     240
Macaca      VQDLYEAGELKWGTDEAQFIYILGNRSKQHLRLVFDEYLKTTGKPIEASIRGELSGDFEK     240
Canis       VQDLYEAGELKWGTDEAQFIYILGNRSKQHLRLVFDEYLRTTGKPIEASIRGELSGDFEK     239
Rattus      VQDLYEAGELKWGTDEAQFIYILGNRSKQHLRLVFDEYLKTTGKPIEASIRGELSGDFEK     240
Mus         VQDLYEAGELKWGTDEAQFIYILGNRSKQHLRLVFDEYLKTTGKPIEASIRGELSGDFEK     240
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Homo        LMLAVVKCIRSTPEYFAERLFKAMKGLGTRDNTLIRIMVSRSELDMLDIREIFRTKYEKS     300
Macaca      LMLAVVKCIRSTPEYFAERLFKAMKGLGTRDNTLIRIMVSRSELDMLDIREIFRTKYEKS     300
Canis       LMLAVVKCIRSTPEYFAERLFKAMKGLGTRDNTLIRIMVSRSELDMLDIREIFRTKYEKS     299
Rattus      LMLAVVKCIRSTPEYFAERLFKAMKGLGTRDNTLIRIMVSRSELDMLDIREIFRTKYEKS     300
Mus         LMLAVVKCIRSTPEYFAERLFKAMKGLGTRDNTLIRIMVSRSELDMLDIREIFRTKYEKS     300
            ************************************************************

Homo        LYSMIKNDTSGEYKKTLLKLSGGDDDAAGQFFPEAAQVAYQMWELSAVARVELKGTVRPA     360
Macaca      LYSMIKNDTSGEYKKSLLKLCGGDDDAAGQFFPEAAQVAYQMWELSAVARVELKGTVRPA     360
Canis       LYSMIKNDTSGEYKKALLKLCGGDDDAAGQFFPEAAQVAYQMWELSAVARVELKGTVRPV     359
Rattus      LYSMIKNDTSGEYKKALLKLCGGDDDAAGQFFPEAAQVAYQMWELSAVSRVELKGTVRAA     360
Mus         LYSMIKNDTSGEYKKALLKLCGGDDDAAGQFFPEAAQVAYQMWELSAVSRVELKGTVCAA     360
            ***************:****.**************************:********  .

Homo        NDFNPDADAKALRKAMKGLGTDEDTIIDIITHRSNVQRQQIRQTFKSHFGRDLMTDLKSE     420
Macaca      NDFNPDADAKALRKAMKGLGTDEDTIIDIITHRSNAQRQQIRQTFKSHFGRDLMSDLKSE     420
Canis       DNFNPDADAKALRKAMKGLGTDEDTIIDIITHRSNAQRQQIRQTFKSHFGRDLMADLKSE     419
Rattus      NDFNPDADAKALRKAMKGIGTDEATIIDIITQRSNAQRQQIRQTFKSHFGRDLMADLKSE     420
Mus         NDFNPDADAKALRKAMKGIGTDEATIIDIVTHRSNAQRQQIRQTFKSHFGRDLMADLKSE     420
            ::*****************:**** *****:*:***.******************:*****

Homo        ISGDLARLILGLMMPPAHYDAKQLKKAMEGAGTDEKALIEILATRTNAEIRAINEAYKED     480
Macaca      ISGDLARLILGLMMPPAHYDAKQLKKAMEGAGTDEKALIEILATRTNAEIRAINEAYKED     480
Canis       ISGDLARLILGLMMPPAHYDAKQLKKAMEGAGTDEKALIEILATRTNAEIRAICEAYKED     479
Rattus      ISGDLARLILGLMMPPAHYDAKQLKKAMEGAGTDEKALIEILATRTNAEIRAINEAYKED     480
Mus         ISGDLARLILGLMMPPAHYDAKQLKKAMEGAGTDEKTLIEILATRTNAEIRAINEAYKED     480
            ************************************:*************** ******

Homo        YHKSLEDALSSDTSGHFRRILISLATGHREEGGENLDQAREDAQVAAEILEIADTPSGDK     540
Macaca      YHKSLEDALSSDTSGHFRRILISLATGNREEGGENLDQAREDAQVAAEILEIADTPSGDK     540
Canis       YHKSLEDALSSDTSGHFRRILISLATGNREEGGEDRNQAREDAQVAAEILEIADTPSGDK     539
Rattus      YHKSLEDALSSDTSGHFKRILISLATGNREEGGENRDQAQEDAQVAAEILEIADTPSGDK     540
Mus         YHKSLEDALSSDTSGHFRRILISLATGNREEGGENRDQAQEDAQVAAEILEIADTPSGDK     540
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Homo        TSLETRFMTILCTRSYPHLRRVFQEFIKMTNYDVEHTIKKEMSGDVRDAFVAIVQSVKNK     600
Macaca      ASLETRFMTILCTRSYPHLRRVFQEFIKMTNYDVEHTIKKEMSGDVRDAFVAIVQSVKNK     600
Canis       TSLETRFMTILCTRSYPHLRRVFQEFVKMTNYDVEHTIKKEMSGDVRDVFVAIVQSVKNK     599
Rattus      TSLETRFMTVLCTRSYPHLRRVFQEFIKKTNYDIEHVIKKEMSGDVKDAFVAIVQSVKNK     600
Mus         TSLETRFMTVLCTRSYPHLRRVFQEFIKKTNYDIEHVIKKEMSGDVKDAFVAIVQSVKNK     600
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Homo        PLFFADKLYKSMKGAGTDEKTLTRIMVSRSEIDLLNIRREFIEKYDKSLHQAIEGDTSGD     660
Macaca      PLFFADKLYKSMKGAGTDEKTLTRIMVSRSEIDLLNIRREFIEKYDKSLHQAIEGDTSGD     660
Canis       PLFFADKLYKSMKGAGTDDKTLTRIMVSRSEIDLLNIRREFIEKYDKSLHQAIEGDTSGD     659
Rattus      PLFFADKLYKSMKGAGTDEKTLTRVMVSRSEIDLLNIRREFIEKYDKSLHQAIEGDTSGD     660
Mus         PLFFADKLYKSMKGAGTDEKTLTRVMVSRSEIDLLNIRREFIEKYDKSLHQAIEGDTSGD     660
            ******************:*****:**********************************

Homo        FLKALLALCGGED 673
Macaca      FLKALLALCGGED 673
Canis       FLKALLAICGGED 672
Rattus      FMKALLALCGGED 673
Mus         FMKALLALCGGED 673
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Percent Identity Matrix - created by Clustal2.1

	1	2	3	4	5
1: Homo Sapien (AAH17046.1)	100.00	98.96	95.54	94.65	94.35
2: Macaca mulatta (AFE65315.1)	98.96	100.00	95.98	95.10.	94.80
3: Canis lupus familiaris (XP_00519331.1)	95.54	95.98	100.00	93.90	93.30
4: Rattus norvegicus (NP_07707.2)	94.65	95.10	93.90	100.00	98.37
5: Mus musculus (NP_038500.2)	94.35	94.80	93.30	98.37	100.00

Supplemental Figure 9. Amino acid conservation of annexin A6.

SUPPLEMENTAL MOVIE LEGENDS

Supplemental Movie 1. Shown is tdTomato-labeled annexin A6 (red) co-expressed with the Ca^{2+} indicator GCaMP5G (green) highlighting large GCaMP5G-positive, external blebs extruding from the site of injury.

Supplemental Movie 2. Overexpression of GFP-labeled annexin A2 (green) in myofibers, is sufficient to induce dynamic, annexin A2-positive, external bleb formation at the site of injury.