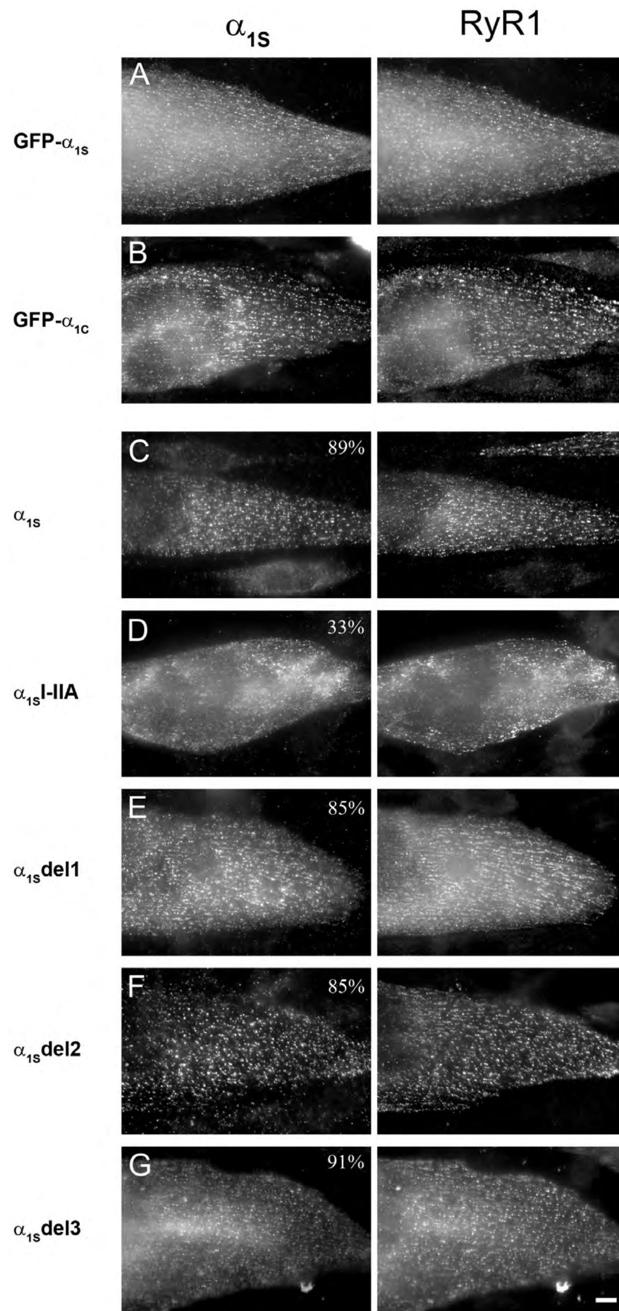


Fig. S1. Normal incorporation of heterologously expressed α_1 constructs in t tubule/SR junctions of dysgenic myotubes. Double immunofluorescence labeling of α_1 subunits (A,B anti GFP, C-G mAb1A) and RyRs shows that GFP-α_{1S}, GFP-α_{1C}, α_{1S}, α_{1S}I-IIA, and α_{1S}del1-3 subunits can colocalize with RyRs in clusters, presumably representing triad junctions and peripheral couplings. The number at the right shows the percentage of transfected myotubes in which the expressed α_1 subunit isoform/chimera achieved a clustered distribution indicative of correct triad targeting. Note that the percentage of transfected myotubes in which α_{1S}I-IIA achieved a clustered distribution was dramatically reduced compared to the other α_{1S} subunits (cf. Fig. 4B). Bar, 10 µm.

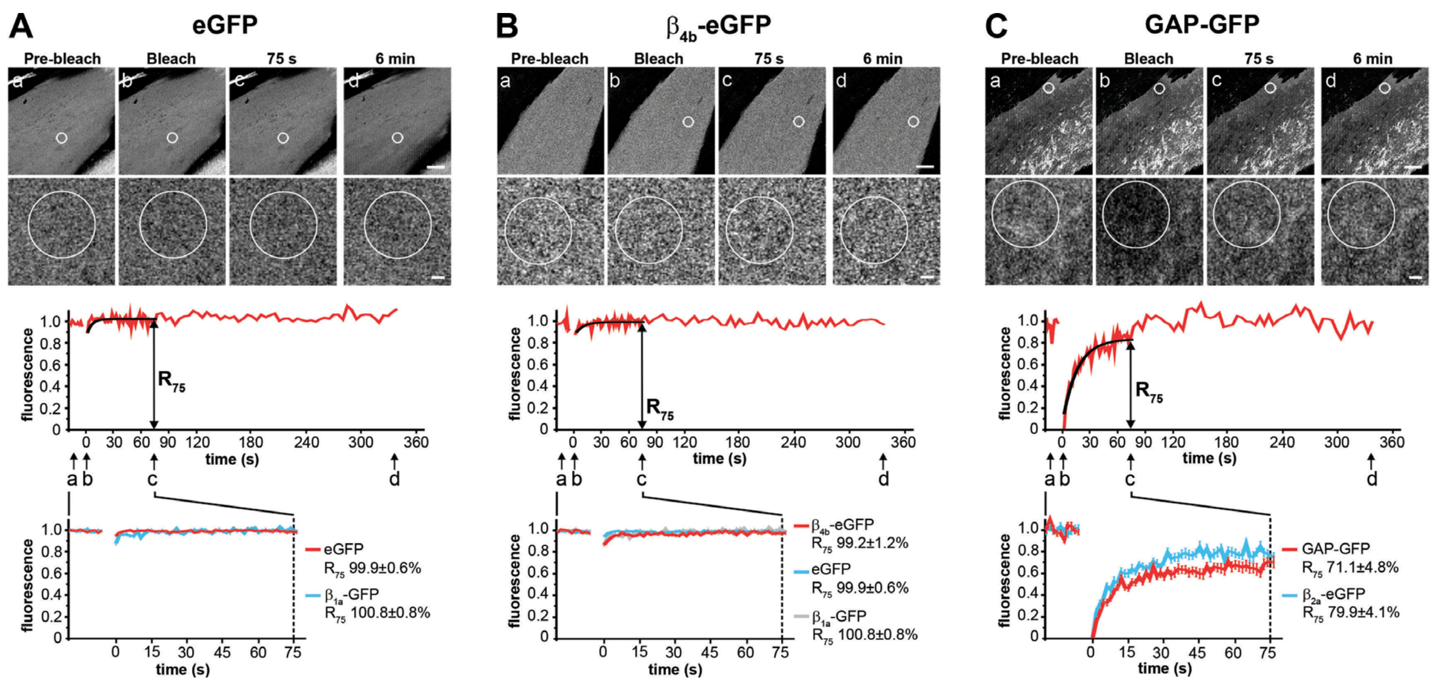


Fig. S2. FRAP analysis of eGFP, β_{4b}-eGFP, and GAP-GFP. A: soluble eGFP expressed in dysgenic myotubes is diffusely distributed and its fluorescence recovers instantaneously after photobleaching (red, mean±SE, N=1 n=10), similar to when β_{1a}-GFP is expressed without an α_1 subunit (blue, from Fig. 2B´). B: β_{4b}-eGFP expressed without an α_1 subunit in dysgenic myotubes is also diffusely distributed and its fluorescence recovers within few seconds after photobleaching (red, mean±SE, N=2 n=15), similar to eGFP (blue) and β_{1a}-GFP (gray, from Fig. 2B´). In contrast GAP-GFP, a palmitoylated GFP containing the 20 aa membrane anchoring sequence of GAP-43, is localized in the plasma membrane and its fluorescence fully recovers within 6 min after bleaching (red, mean±SE, N=1 n=7), similar to when the palmitoylated β_{2a}-eGFP subunit is expressed without an α_1 subunit (blue, from Fig. 3B). Upper bar, 10 μm; lower bar, 1 μm.

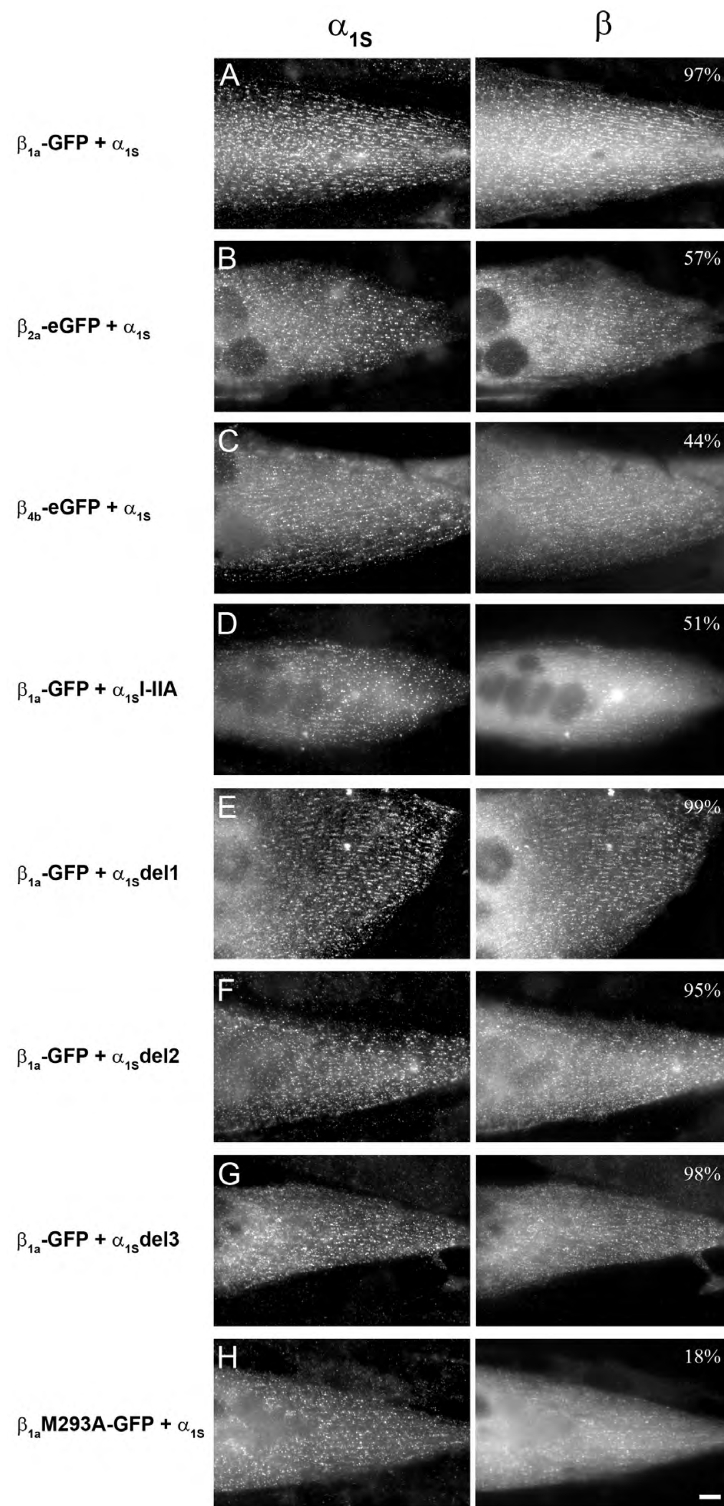


Fig. S3. Clustering of different α_1-β subunits combinations. Double immunofluorescence labeling of α_{1S} (mAB1A) and β (anti-GFP) subunits shows that different β isoforms can co-cluster with all the used α_{1S} channels. The number at the right shows the percentage of α_{1S} clustered myotubes in which the expressed β subunit co-clustered with the α_1 subunit. Note that lower co-clustering corresponds to higher mobility of the β subunit (cf. Fig. 2C, 2D, 4F), with the exception of β_{1a}-GFP + α_1I-IIA. Bar, 10 µm.

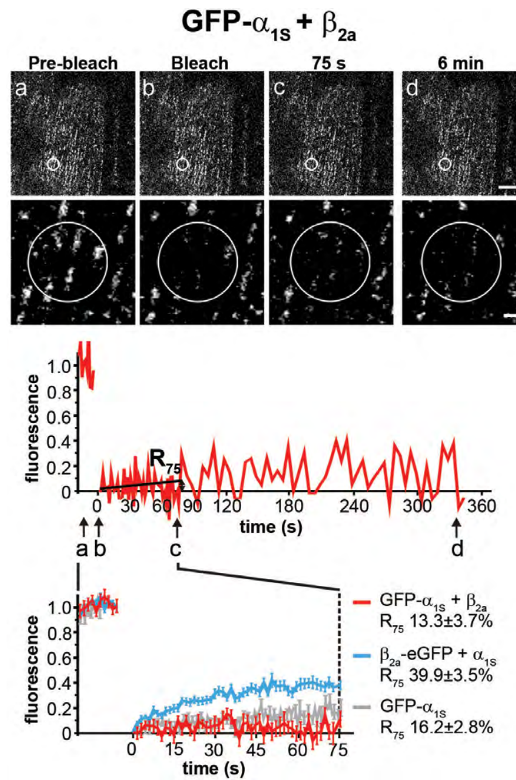


Fig. S4. GFP-α_{1S} clusters are immobile independently of the co-expressed β subunit. Co-expressed with β_{2a}-V5, the fluorescence of GFP-α_{1S} clusters does not recover within 6 min after bleaching (red, mean±SE, N=3 n=11), while β_{2a}–eGFP coexpressed with α_{1S} shows a 3-folds higher FRAP rate (blue, from Fig. 2C'), indicating that the β_{2a} subunit dynamically interacts with the channel complex. The low mobility of GFP-α_{1S} when coexpressed with β_{2a}-V5 is not significantly different from that of GFP-α_{1S} coexpressed with β_{1a} (grey, from Fig. 1A). Upper bar, 10 µm; lower bar, 1 µm.

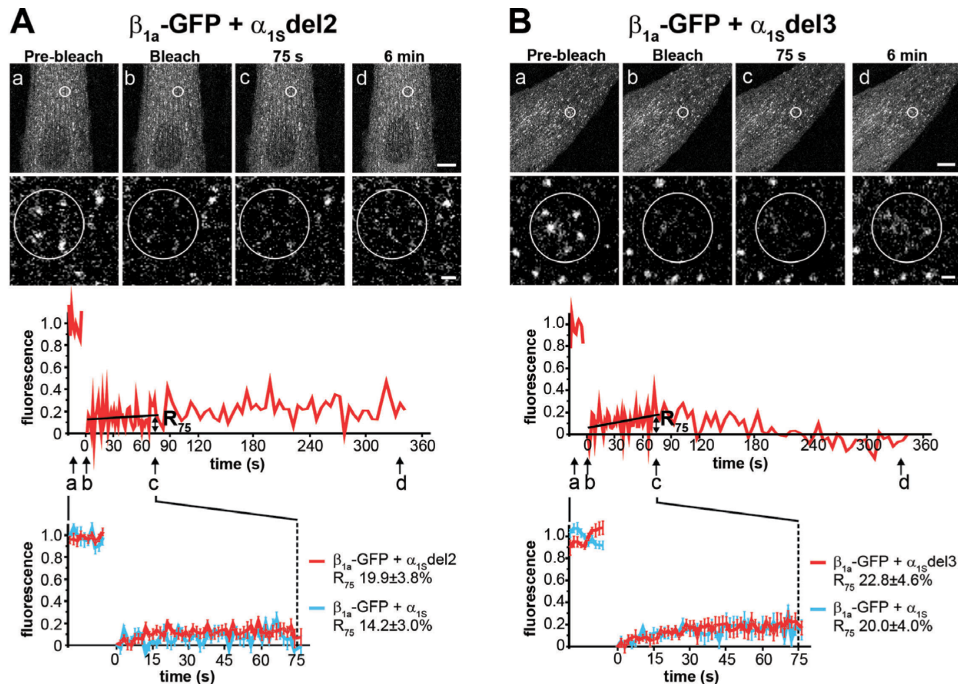
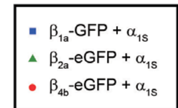


Fig. S5. Altered α_{1S} I-II loop orientation does not affect the mobility of β_{1a}-GFP. In α_{1S}del2 and α_{1S}del3, two or three amino acids in the proximal I-II loop were deleted (similar to α_{1S}del1, Fig. 4A, 4E). When coexpressed with α_{1S}del2 (A) or with α_{1S}del3 (B), β_{1a}-GFP fluorescence did not recover within 6 min after bleaching. With both constructs the mean recovery curves and R_{75} (mean±SE; α_{1S}del2 N=3 n=17, α_{1S}del3 N=3 n=10) were similar to that of β_{1a}-GFP coexpressed with the wildtype α_{1S}. Upper bar, 10 µm; lower bar, 1 µm.

	average cluster area (μm^2)	average cluster intensity (a.u.)	average cell intensity (a.u.)	R_{75} F recovery (%)
β_{1a}-GFP + α_{1S}	0.098 ± 0.004	38193 ± 1087	6467 ± 420	15.4 ± 1.7
β_{2a}-eGFP + α_{1S}	0.156 ± 0.014	38742 ± 1056	9594 ± 826	35.6 ± 3.0
β_{4b}-eGFP + α_{1S}	0.094 ± 0.004	38316 ± 1285	8221 ± 575	32.9 ± 2.4



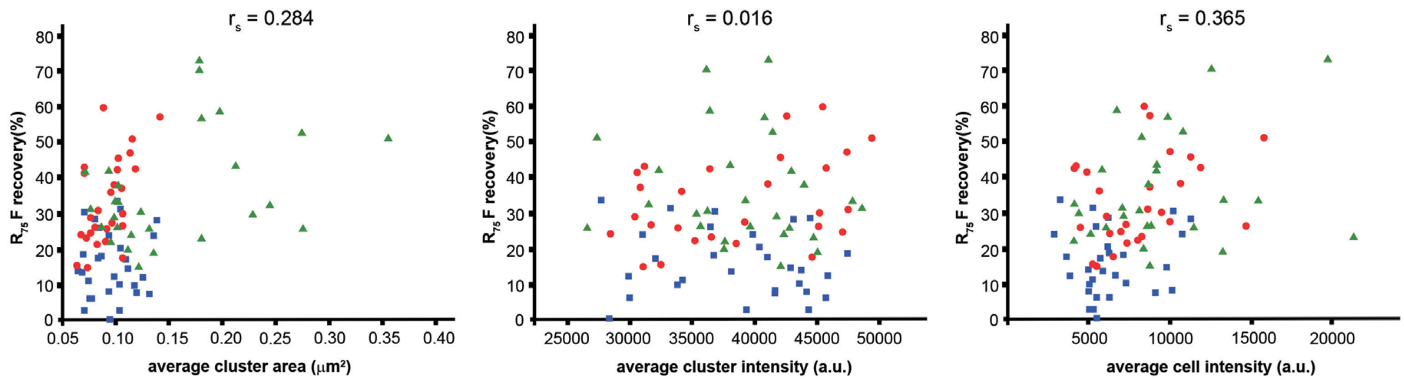


Fig. S6. FRAP rates do not correlate with cluster size and expression level of β subunits. To examine whether the variability of expression levels or differences in the subcellular distribution of the constructs influenced the FRAP data we measured and compared the average fluorescence intensity of the recorded myotubes, as well as the average size and fluorescence intensity of the clusters. Images of the myotubes used in FRAP experiments ($n_{\beta1a\text{-}GFP+\alpha1S} = 32$, $n_{\beta2a\text{-}eGFP+\alpha1S} = 34$, $n_{\beta4b\text{-}aGFP+\alpha1S} = 28$) were cluster-thresholded using the automatic multilevel thresholding method by Yen (Image J, National Institues of Health, Bethesda). Images in which automatic thresholding failed to highlight clusters were excluded ($n_{\beta1a\text{-}GFP+\alpha1S} = 3$, $n_{\beta2a\text{-}eGFP+\alpha1S} = 7$, $n_{\beta4b\text{-}aGFP+\alpha1S} = 1$). The thresholded images were calibrated and an integrated morphometric analysis of cluster area and intensity was performed using Methamorph software. Expression levels were estimated by analyzing the average fluorescence intensity (background subtracted) of each recorded cell and calculating the mean ± standard error (SE) for each construct in MS Excel. Anova with Tukey post-hoc analysis of the average cluster area, average cluster intensity and average cell intensity and R_{75} values were performed using SPSS statistical software (SPSS Inc., Chicago IL, USA) and Spearman correlation tests of each of the three parameters against the R_{75} values were performed. The table shows that the means of the average cluster area and of the average cell intensity, but not of the average cluster intensity, vary between the three examined β subunits. The values of average cluster area of the palmitoylated/membrane associated β_{2a} is significantly ($p<0.001$) higher than those of β_{1a} and β_{4b}, and the average cell intensity of β_{2a} is significantly ($p=0.002$) higher than that of $\beta_{1a.}$ Note that the means of the two non-palmitoylated isoforms β_{1a} and β_{4b} were not significantly different from each other, even though their fractional FRAP were significantly different ($p<0.001$). Conversely, the mean cluster size of β_{2a} was significantly higher than that of β_{4b} even though their FRAP values were similar. These findings do not indicate a correlation of FRAP and cluster size or expression levels. Also a correlation analysis of each of the three measured parameters with the fractional FRAP (R_{75}) values recorded in the same cell did not reveal any correlation between overall expression levels, cluster size or fluorescence intensity of clusters and the obtained FRAP values. The scatter blots and correlation coefficients below 0.4 indicate weak to no correlation.