Phosphorylation of α-dystrobrevin is essential for αkap accumulation and acetylcholine receptor stability

Received for publication, April 20, 2020, and in revised form, June 8, 2020 Published, Papers in Press, June 12, 2020, DOI 10.1074/jbc.RA120.013952

Po-Ju Chen[1], **Diego Zelada**[2], **Dina Cheryne Belhasan**[1], **and Mohammed Akaaboune**[1,3,*]

From the [1]*Department of Molecular, Cellular and Developmental Biology and the* [3]*Program in Neuroscience, University of Michigan, Ann Arbor, Michigan, USA, and the* [2]*Department of Cell Biology, Universidad de Concepción, Concepción, Chile*

Edited by Roger J. Colbran

The maintenance of a high density of the acetylcholine receptor (AChR) is the hallmark of the neuromuscular junction. Muscle-specific anchoring protein (αkap) encoded within the calcium/calmodulin-dependent protein kinase IIα (CAMK2A) gene is essential for the maintenance of AChR clusters both *in vivo* and in cultured muscle cells. The underlying mechanism by which αkap is maintained and regulated remains unknown. Here, using human cell lines, fluorescence microscopy, and pulldown and immunoblotting assays, we show that α-dystrobrevin (α-dbn), an intracellular component of the dystrophin glycoprotein complex, directly and robustly promotes the stability of αkap in a concentration-dependent manner. Mechanistically, we found that the phosphorylatable tyrosine residues of α-dbn are essential for the stability of α-dbn itself and its interaction with αkap, with substitution of three tyrosine residues in the α-dbn C terminus with phenylalanine compromising the αkap–α-dbn interaction and significantly reducing both αkap and α-dbn accumulation. Moreover, the αkap–α-dbn interaction was critical for αkap accumulation and stability. We also found that the absence of either αkap or α-dbn markedly reduces AChRα accumulation and that overexpression of α-dbn or αkap in cultured muscle cells promotes the formation of large agrin-induced AChR clusters. Collectively, these results indicate that the stability of αkap and α-dbn complex plays an important role in the maintenance of high-level expression of AChRs.

The maintenance of AChR clusters at high density in the postsynaptic membrane is a hallmark of the neuromuscular junction (NMJ) (1). Core proteins such as MuSK (muscle-specific tyrosine kinase), LRP4 (low-density lipoprotein receptor-related protein 4), rapsyn, and Dok7 (dedicator of cytokinesis family member 7) (2–7) are required for initial steps in the formation of AChR clusters, whereas auxiliary proteins such as the dystrophin glycoprotein complex (DGC), neuregulin signaling molecules, and Wnt proteins are essential for the maturation and stability of AChR clusters in the postsynaptic apparatus (8–13).

Recent work has shown that αkap, a non–kinase muscle-specific anchoring protein encoded within the *Camk2a* gene that contains a putative transmembrane domain and an association domain but lacks the catalytic domain (14–16), is also involved in promoting the stability of AChRs (16–18). Knockdown of αkap gene expression with shRNA in cultured myotubes or in mouse sternomastoid muscles resulted in a significant reduction of the postsynaptic receptor density, an alteration in the metabolic stability of AChR, and an impairment of the structural integrity of the synapse (17, 18). Earlier work also reported that αkap plays a role in targeting multiple calcium/calmodulin kinase II isoforms to specific subcellular locations where they can perform their function through its unique hydrophobic N-terminal domain that tightly associates with membranes (16). However, the mechanistic link between αkap and AChR stability remains unknown.

On the other hand, α-dystrobrevin, a component of the DGC that links the intracellular cytoskeleton to the extracellular basal lamina (19), is also essential for the maintenance of the muscle integrity and the maturation and stability of the NMJ (20, 21). NMJs of mice deficient in α-dystrobrevin (α-dbn) bear few receptors and exhibit an abnormal pattern of AChR distribution and a higher turnover rate of AChR. In muscle cells, at least two predominant isoforms of α-dbn (α-dbn 1 and 2) are generated by alterative splicing of the α-dbn gene (22). Full-length α-dbn 1 has a unique C-terminal tyrosine kinase substrate domain and is mainly concentrated at the NMJ, whereas α-dbn 2, which lacks a C terminus, is mainly localized on the sarcolemmal membrane but also present at the synapse (23). It was reported that the phosphorylatable tyrosine residues of α-dystrobrevin are essential for the stability and normal distribution pattern of postsynaptic AChRs (8, 24, 25). α-dbn1, a substrate for tyrosine kinases *in vivo* phosphorylated by neuregulin/ErbB receptor signaling pathway in muscles (8, 26, 27), plays a critical role in the maintenance and the stability of the NMJ, whereas the nonphosphorylated α-dbn 2 is involved in the maintenance of the structural integrity of the muscle (25). Because both αkap and α-dbn are involved in the stability of AChRs, we sought to investigate the interplay between these proteins and their effect on the stability of AChR.

In the present study, we have used muscle cells and heterologous systems to investigate the interplay between α-dbn, αkap, and AChRs accumulation. We found that αkap expression levels, which are critical for promoting accumulation of AChRs, are regulated by α-dbn in a dose-dependent manner. Furthermore, we discovered that the ability of the three tyrosine residues of α-dbn1 to be phosphorylated are critical for the stability of α-dbn and its interaction with αkap. This interaction is essential for αkap accumulation and AChR stability.

* For correspondence: Mohammed Akaaboune, makaabou@umich.edu.

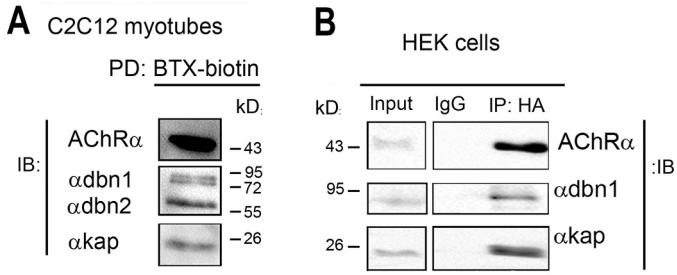

Figure 1. αkap forms complexes with α-dbn and AChR in muscle cells and HEK cells. *A*, lysates from C2C12 myotubes were incubated BTX–biotin, and then biotin-containing complexes were isolated with NeutrAvidin beads. Pulldown proteins (*PD*) were probed with anti-αkap, anti-AChRα, and anti–α-dbn antibodies. *B*, lysates from HEK293T cells co-transfected with AChRα and αkap–HA (hemagglutinin) were immunoprecipitated (*IP*) with anti-HA followed by protein A/G–agarose beads and blotted (*IB*) with anti-αkap, anti-AChRα, and anti–α-dbn anti-antibodies. Western blotting analysis of cell lysate inputs is shown (*left panels*).

Results

αkap forms complexes with α-dystrobrevin and AChRs

Previous studies have shown that αkap forms complexes with AChRs and promotes its stability (17, 18). Because α-dbn is also essential for the stability of AChRs (24), we sought to investigate whether there was a molecular link between αkap, α-dbn, and AChRs. As a first test, we examined whether these proteins are present within the same complex and whether the stability of AChRs requires the presence of both αkap and α-dbn. Lysates from cultured C2C12 myotubes were incubated with BTX–biotin to label AChRs, after which AChR–BTX–biotin complexes were isolated with NeutrAvidin-coated beads. The eluates were subjected to one blotting using anti-AChRα, anti-αkap, and anti-αdbn antibodies. Fig. 1*A* shows that αkap and αdbn were pulled down with AChRs. This indicates that AChRs are present in the same complex as αkap and α-dbn αdbn. It should be noted that neither α-dbn nor αkap have AChR binding sites, suggesting that the pulldown by Neutr Avidin beads is indirect.

Similar results were observed when αkap and AChRα constructs were co-transfected in HEK cells (express endogenous α-dbn) and subjected to HA immunoprecipitation (Fig. 1*B*). Altogether, these results indicate that AChRs, αkap, and α-dbn are present within the same complex.

Because αkap and α-dbn are present within the same complex, we asked whether the promoting effect of αkap on the accumulation of AChRs requires the presence of α-dbn. To investigate this, we used HeLa cells (cells that do not express α-dbn, AChR, or αkap; Fig. 2*A*) that were co-transfected with constructs of AChRα (1 μg) and HA (0.4 μg), with constructs of AChRα (1 μg/μl) and αkap–GFP (0.4 μg/μl), with AChRα (1 μg) and α-dbn–GFP (0.4 μg), or with AChRα (1 μg) and both αkap–GFP and α-dbn–GFP at 0.4 μg, respectively. Twenty-four hours later, lysates from co-transfected cells were immunoblotted with anti-AChRα and anti-GFP antibodies (to probe for αkap–GFP and α-dbn–GFP). Quantification of the intensity bands on Western blots shows that the amount of AChRα expression levels is not significantly different between cells transfected with (1.12, $n = 4$, $p = 0.91$) and without αkap–GFP (set at 100%). In the presence of α-dbn–GFP alone, AChRα levels were slightly increased (153%, $n = 4$, $p < 0.186$) compared with cells transfected with (112%) or without αkap–GFP (100%). However, in the presence of both αkap–GFP and α-dbn–GFP (0.4 μg of plasmids), AChRα levels were significantly increased (240%, $n = 4$) compared with cells transfected with αkap–GFP alone (112%, $n = 4$) or α-dbn–GFP alone (153%, $n = 4$) (Fig. 2, *B* and *C*). These results were further confirmed in HEK cells (cells that express endogenous α-dbn, but not αkap and AChRα; Fig. 2*A*) that were co-transfected with an increasing concentration of αkap construct (0, 0.2, 0.4, or 0.8 μg) and a fixed concentration of AChRα plasmid (1 μg) (Fig. 2*D*). Collectively, these results suggest that high AChRs expression levels require the presence of both αkap and α-dbn.

α-Dystrobrevin promotes the expression levels of αkap in a concentration-dependent manner

Given the fact that αkap, α-dbn, and AChRα are present within the same complex and that the promoting effect of αkap on AChRs necessitates the presence of α-dbn, we asked whether expression levels of αkap are regulated by α-dbn. In the first set of experiments, we examined the effect of α-dbn on αkap expression levels. For this, we used three independent cell lines: nonmuscle HeLa cells (no endogenous expression of both α-dbn and αkap), HEK cells (endogenous expression of α-dbn only), and muscle C2C12 myoblasts (endogenous expression of both α-dbn1 and αkap). All of these cells were co-transfected with αkap–GFP and α-dbn–GFP. Twenty-four hours later, cell lysates were immunoblotted with anti-αkap and anti–α-dbn antibodies to probe for the endogenous proteins and anti-GFP antibody to probe for the exogenous expression of αkap–GFP and α-dbn–GFP. We found that αkap levels were significantly higher in all three cell lines where α-dbn is exogenously and/or endogenously expressed compared with cells transfected with αkap alone (Fig. 3, *A–G*). In C2C12 muscle cells, the overexpression of α-dbn–GFP also enhanced expression levels of endogenous αkap (Fig. 3, *F* and *G*). We also asked whether α-dbn and αkap are present in the same intracellular organelles. To examine this, HeLa cells were co-transfected with α-dbn–GFP (*green*) and αkap–mCherry (*red*) constructs, and 24 h later, the cells were fixed and imaged with the confocal microscope. As shown in Fig. 3*C*, we found that ~50%, green and red punctas co-localized in the same vesicles ($n = 250$ vesicles analyzed, number of cells = 25). Interestingly, it appears that the size of the intracellular vesicles in which both green and red co-localized are larger than vesicles in cells transfected by αkap–mCherry only (Fig. 3*C*).

We next asked whether the expression level of αkap depends on α-dystrobrevin expression levels. To examine this, HeLa cells (neither αkap nor α-dbn are expressed) were co-transfected with the same amount (1 μg) of αkap–GFP construct and increasing α-dbn–GFP construct doses (0, 0.2, and 0.4 μg). The HA control plasmid was co-transfected with αkap and α-dbn plasmids, so that all transfections contained the same amount of plasmid DNA. We found that in HeLa cells co-transfected with 0.2 μg of α-dbn–GFP and 1 μg of αkap plasmid, the expression level of αkap was increased ~2-fold compared with αkap alone (in absence of α-dbn–GFP). In cells co-transfected

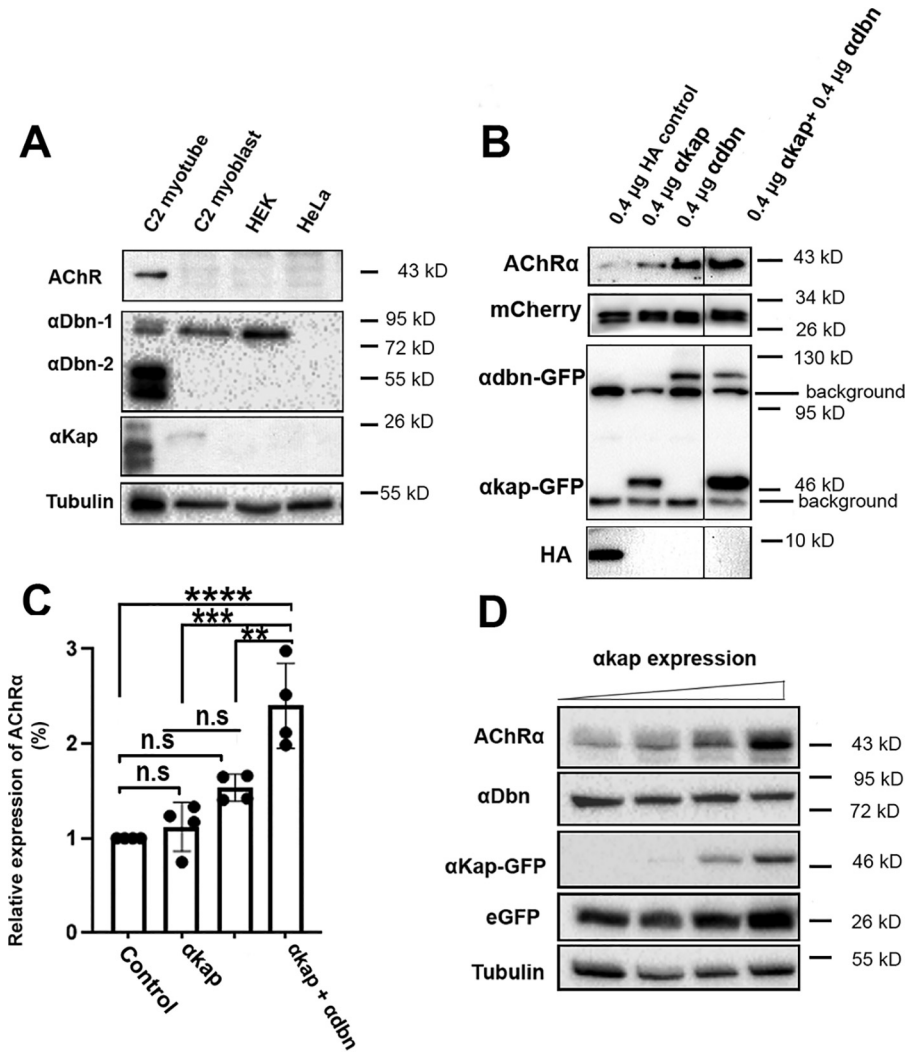

Figure 2. AChRα expression is enhanced in presence of α-dystrobrevin and αkap. *A*, Western blots of αkap, α-dbn, and AChRα expression in C2C12 myoblasts, myotubes, HEK cells, and HeLa cells. Note that HeLa cells do not express these proteins, whereas HEK cells express only α-dbn1. C2C12 myotubes express all these proteins (AChR, α-dbn (1 and 2), and αkap), whereas myoblasts express only α-dbn and αkap. Tubulin was used as a loading control. *B*, HeLa cells were transfected with the same amount of AChRα (1 μg) construct and either individual αkap–GFP (0.4 μg), α-dbn–GFP (0.4 μg), or both (0.4 μg of each) constructs. mCherry was used as a control for the transfection efficiency and for the loading of total proteins. *C*, scatter plot summarizing quantification from four independent blots (data are presented as means ± S.D.). Statistical analysis was done by one-way ANOVA ($F = 22.30$) followed by Tukey's post hoc multiple comparisons test. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. *n.s*, not significant. *D*, HEK cells were transfected with the same amount of AChRα (1 μg) and with increasing amounts of αkap–GFP (0, 0.2, 0.4, and 0.8 μg). Enhanced GFP (*eGFP*) was used as a control for the transfection efficiency and for the loading of total proteins with tubulin. Note that in the presence of αdbn, the expression levels of AChRα subunit depend on αkap concentration.

with 0.4 μg of α-dbn–GFP plasmid, the expression levels of αkap were increased 1.5-fold compared with 0.2 μg of α-dbn–GFP and ~3-fold compared with αkap alone (Fig. 4, *A* and *B*). In C2C12 muscle cells (in which both αkap and α-dbn are endogenously expressed) that were co-transfected with 1 μg of αkap–GFP and α-dbn–GFP constructs (0 and 0.2 μg), we found that both exogenous and endogenous αkap expression levels were also increased (Fig. 4, *C* and *D*). These results strongly suggest that α-dbn is involved in promoting the expression of αkap in a concentration-dependent manner. Conversely, when HeLa cells were co-transfected with the same amount (1 μg) of α-dbn and increasing αkap constructs doses (0, 0.2, and 0.4 μg), the expression levels of α-dbn remained unchanged (Fig. 4, *E* and *F*), indicating that expression of α-dbn is not controlled by αkap. Altogether, these results indicate that αkap expression depends on α-dbn expression, whereas α-dbn expression is independent of αkap.

Effect of phosphorylatable tyrosine residues of α-dystrobrevin on the expression of α-dystrobrevin and αkap

We wanted to know the mechanism by which α-dbn controls αkap accumulation. First, we confirmed that the fused α-dbn1–GFP protein underwent tyrosine phosphorylation. HEK cells were transfected with either WT α-dbn1–GFP or the non-phosphorylatable α-dbn–GFP mutant (the three tyrosine residues were changed to phenylalanine; 3Y/F) and treated with peroxivanadate before lysis to inhibit tyrosine phosphatases. Lysates were then incubated with anti-GFP antibodies to immunoprecipitate α-dbn, and eluates were subjected to

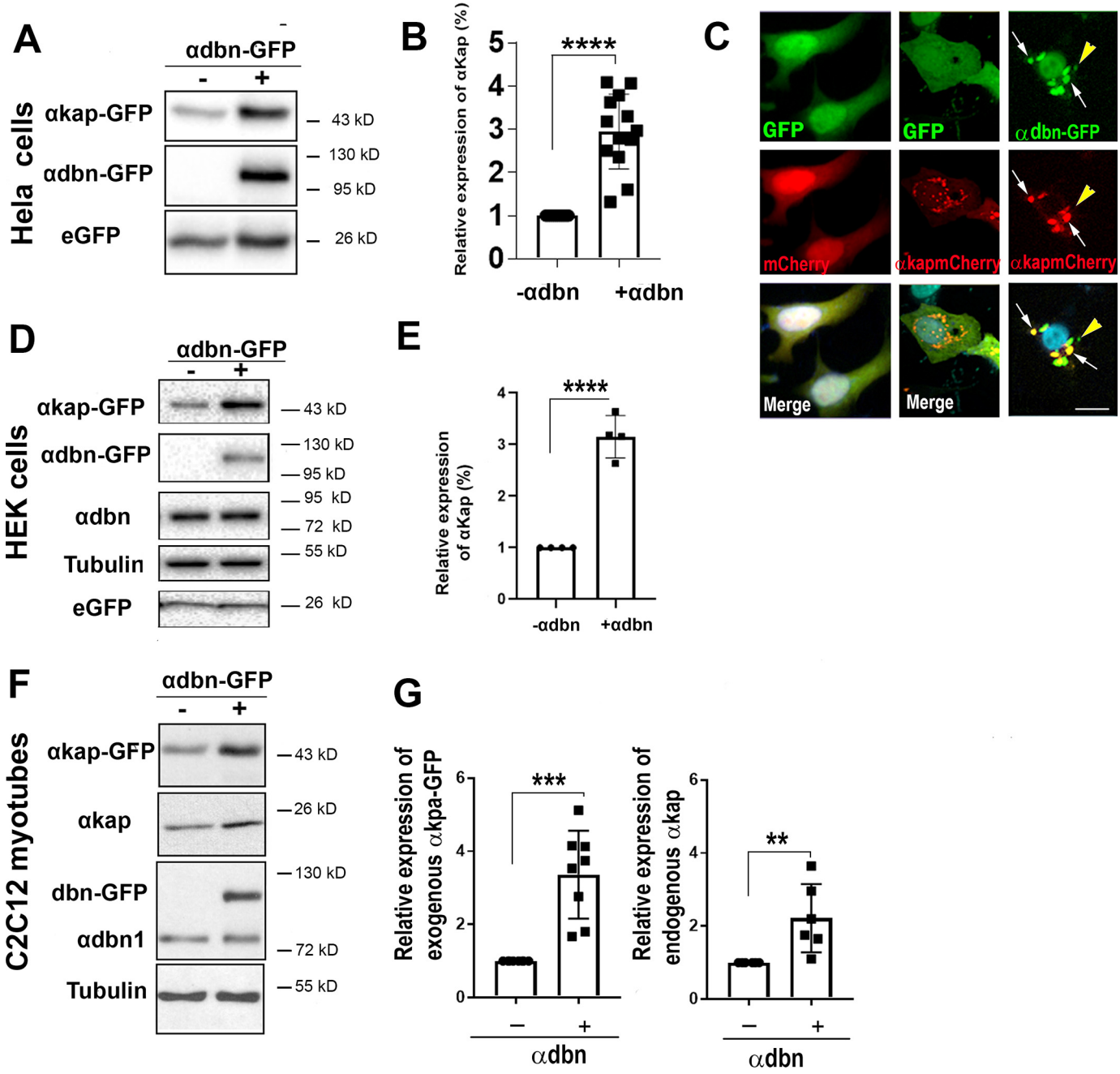

Figure 3. α-Dystrobrevin-1 promotes the expression levels of αkap in transfected cell lines. HeLa, HEK, and C2C12 cells were co-transfected with αkap–GFP (1 μg) and α-dbn–GFP (1 μg) plasmids, and 24 h later lysates from these cells were probed with anti-GFP, anti–α-dbn, and anti-αkap antibodies. *A*, Western blots of lysates from HeLa cells. Enhanced GFP (*eGFP*) was used as a control for the transfection efficiency and for the loading of total proteins. *B*, quantification of blots from independent replicates of experiments as in *A* ($n = 13$). *C*, examples of HeLa cells co-transfected with mCherry and GFP constructs (*left column*), GFP and αkap–mCherry (1 μg) constructs (*middle column*), and αkap–mCherry (1 μg) α-dbn–GFP (1 μg) (*right column*). Note the co-localization (*yellow*) between (*red*) αkap–mCherry and (*green*) α-dbn–GFP (*white arrows*) in intracellular vesicles. *Arrowheads* indicate the presence of *green* only (absence of co-localization). *D*, Western blotting of lysates from HEK cells. *E*, quantification of blots as in *D* ($n = 4$). *F*, Western blotting of lysates from C2C12 myotubes. *G*, quantification of blots showing an increase of both exogenous αkap–GFP ($n = 8$) and endogenous αkap ($n = 6$) expression levels. Statistical analysis was done by Student's *t* test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Western blotting with anti–α-dbn antibody, anti-phosphotyrosine antibody, and anti-GFP antibodies. As shown in Fig. 5*A*, the α-dbn1–GFP WT showed the presence of tyrosine phosphorylation sites but not the mutant one.

We next examined whether the phosphorylatability of the three tyrosine residues of α-dbn is essential for α-dbn stability. HeLa cells were transfected with either WT GFP-α-dbn or mutant GFP-α-dbn (3Y/F) and then harvested 18 and 24 h later. Western blotting analysis of proteins with anti-GFP or anti–α-dbn antibodies showed that the expression levels of α-dbn mutant (α-dbn–3Y/F) is significantly reduced compared with WT (Fig. 5*B*). To determine whether the reduction in the amount of α-dbn1 mutant was due to a decrease in its transcript, we analyzed RT-PCR products specific to α-dbn of both WT and

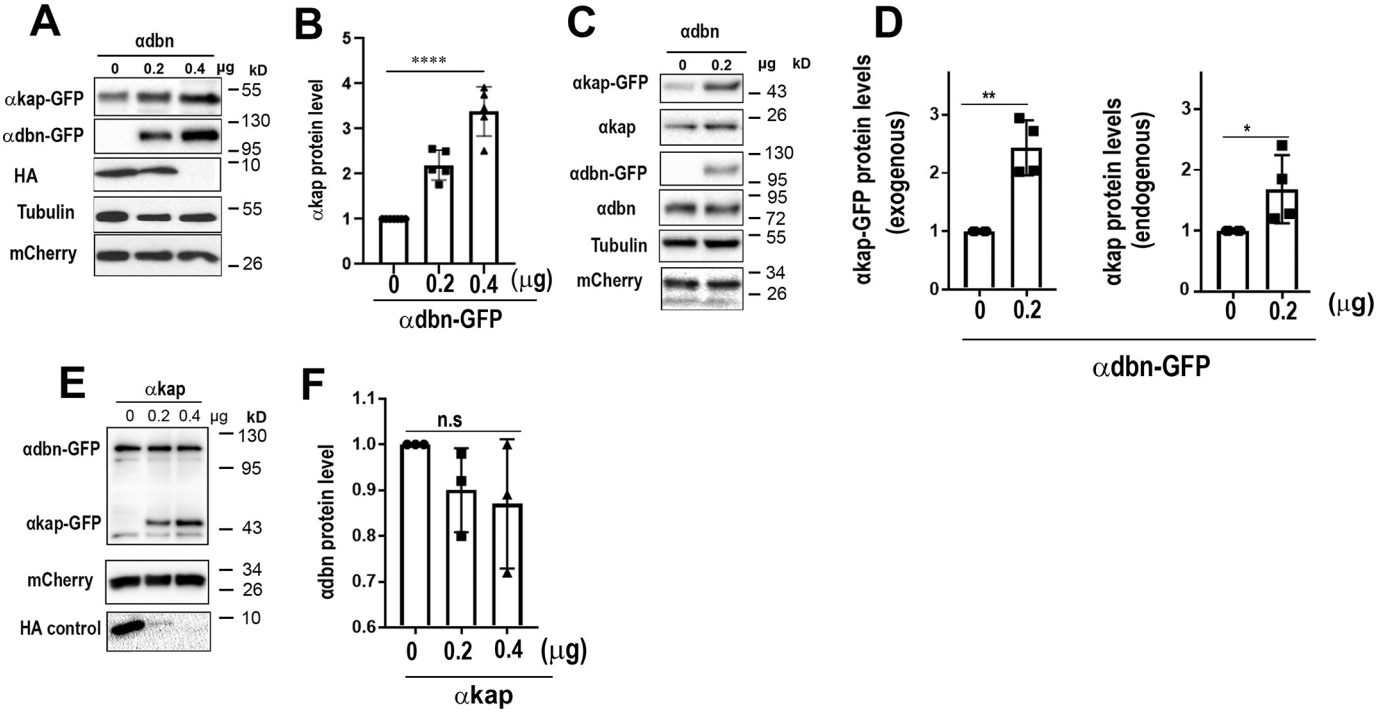

Figure 4. α-Dystrobrevin promotes αkap accumulation in a concentration-dependent manner. HeLa cells and C2C12 muscle cells were co-transfected with 1 μg of αkap–GFP and either with 0, 0.2, or 0.4 μg of α-dbn–GFP. HA-control plasmid was co-transfected to make up the total plasmid amount (0.4 μg of HA for 0 μg of α-dbn–GFP or 0.2 μg of HA for 0.2 μg of α-dbn–GFP). 24 h later, the lysates were probed with anti-GFP, anti-αkap antibodies. Anti-HA antibody was used to confirm the expression of αkap. mCherry was used as a control for the transfection efficiency and as a loading control with tubulin. *A*, Western blots of lysates from HeLa cells. *B*, quantification of five blots as in *A* (means ± S.D.). Statistical analysis was done by one-way ANOVA ($F = 52.32$) followed by Tukey's post hoc multiple comparisons test. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. *C*, Western blots of lysates from C2C12 myoblasts. *D*, quantification of four blots as in *C*. Statistical analysis was done by Student's *t* test. Note that both exogenous and endogenous αkap showed an increase in their expression levels. αkap–GFP was normalized to mCherry, and αkap was normalized to tubulin. *E*, HeLa cells were co-transfected with 1 μg of α-dbn–GFP and either with 0, 0.2, or 0.4 μg of αkap–GFP and HA plasmid to make up for the total transfected plasmid. 24 h later, homogenates were probed with anti-GFP. *F*, quantification of three blots as in *E*. Statistical analysis was done by one-way ANOVA ($P = 0.32$; $F = 1.37$) followed by Tukey's post hoc multiple comparisons test (individual means of each columns were compared with the mean of every other column). *n.s*, not significant.

mutant. Fig. 5*C* showed that there was no significant difference in the amount of α-dbn mRNA products between cells transfected with the phosphorylatable and nonphosphorylatable α-dbn constructs, suggesting that post-translational modifications of α-dbn by the phosphorylation of the three tyrosine residues play an important role in its accumulation/stability.

Because a significant amount of α-dbn mutant proteins are degraded, we wanted to know whether the degradation of α-dbn mutant is mediated by the proteasome. The cells were transfected with the phosphorylatable tyrosine WT or nonphosphorylatable the mutant α-dbn and treated for 6 h with MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), a widely used proteasome inhibitor that can prevent degradation of many ER-associated degradation substrates (28). Lysates from transfected cells were subjected to Western blots using anti-GFP antibodies, and α-dbn expression levels of WT and mutant were analyzed. As shown in Fig. 5 (*D* and *E*), we found that in the presence of MG132, the expression levels of the mutant were significantly increased (~93.1% of WT, S.D. 0.09 $n = 4$, $p = 0.163$), comparable with the phosphorylatable WT α-dbn, which suggests that the degradation of mutant α-dbn is mediated by the proteasome.

Fig. 4 showed that α-dbn promotes αkap accumulation in a concentration-dependent manner. Thus, we wanted to know the mechanism by which α-dbn regulates αkap expression levels. We hypothesized that the three tyrosine phosphorylatable sites of α-dbn are essential for the interaction with αkap and that interaction is critical for αkap accumulation/stability. To test this, HeLa cells were co-transfected with αkap-HA construct and either the 1) phosphorylatable tyrosine residues α-dbn1–GFP (WT) or 2) α-dbn–GFP mutant and then treated with MG132, so the same amount of expression levels of WT and mutant α-dbn1 would be used for immunoprecipitation. Lysates were incubated with anti-GFP antibody to immunoprecipitate α-dbn–GFP, and eluates were subjected to Western blotting analysis with anti-αkap, anti–α-dbn, and anti-HA antibodies. Fig. 6*A* shows that αkap was pulled down successively and specifically enriched in lysates transfected with the phosphorylatable α-dbn1. However, in lysates from cells co-transfected with nonphosphorylated α-dbn–GFP mutant, αkap was barely visible (or not detectable), indicating that the interaction between αkap and nonphosphorylated α-dbn was impaired. These results suggest that the phosphorylatability of α-dbn1 is essential for the interaction with αkap.

We next wanted to know whether the interaction between αkap and phosphorylatable α-dbn1 leads to αkap accumulation. Lysates from HeLa cells co-transfected with a fixed amount of αkap and WT α-dbn–GFP or α-dbn–3Y/F–GFP mutant constructs were subjected to Western blotting analysis with anti-αkap and anti–α-dbn antibodies. As shown in Fig. 6

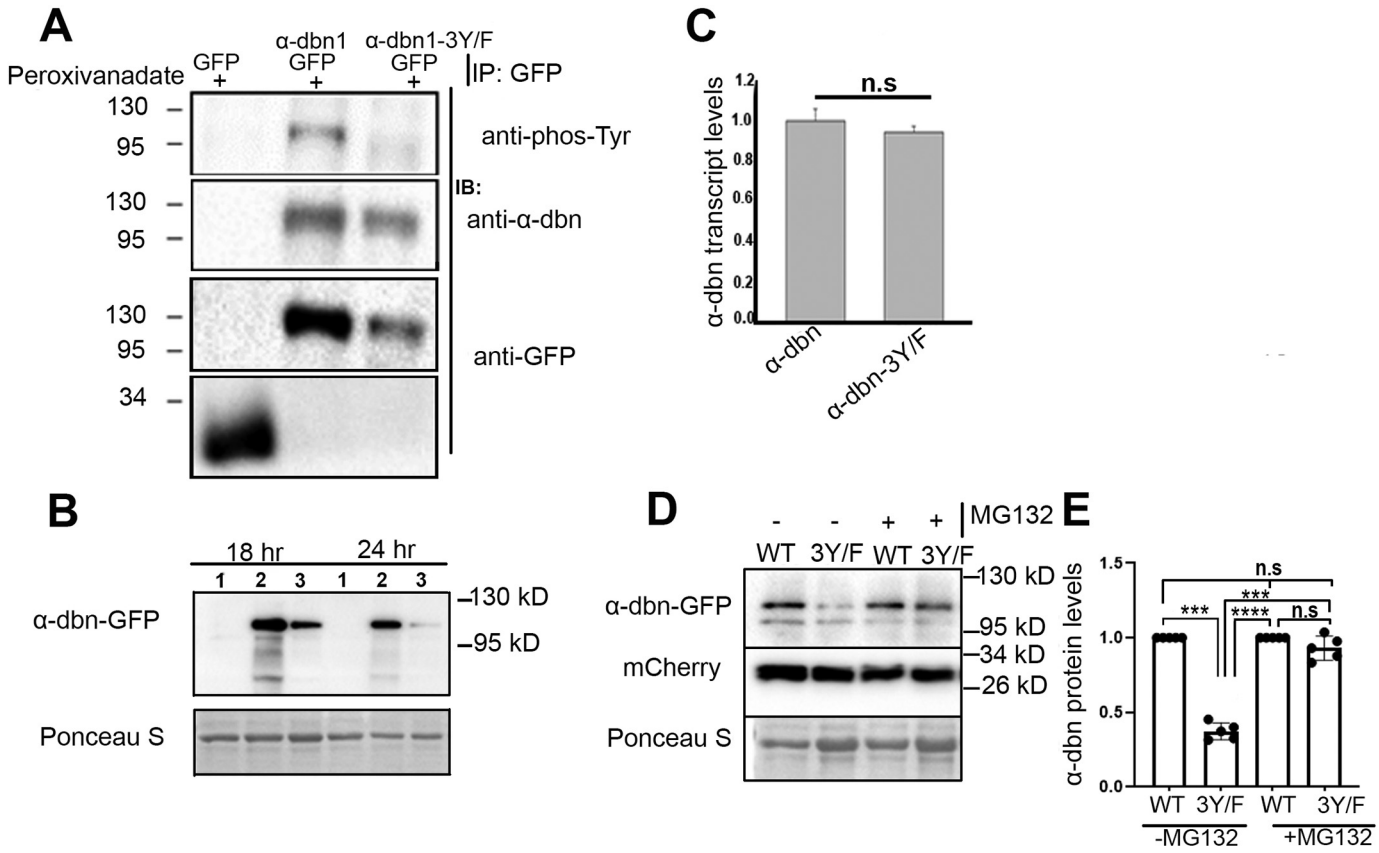

Figure 5. Phosphorylatable tyrosine residues of α-dystrobrevin are critical for α-dystrobrevin accumulation levels. *A*, immunoblot (*IB*) of HEK cells transfected with WT α-dbn1–GFP, mutant α-dbn (3Y/F)–GFP, or GFP constructs (1 μg). 18 h later and 15 min prior to lysis, the cells were treated with peroxivanadate to inhibit tyrosine phosphatases. Lysates were then incubated with GFP antibody, and immunoprecipitate (*IP*) proteins were probed with anti-GFP, anti-αdbn or an anti-phosphotyrosine antibody. *B*, representative Western blots of α-dbn1–GFP and Ponceau stain of protein extracts from transfected HeLa cells. *Lanes 1*, control plasmid; *lanes 2*, α-dbn–WT–GFP; *lanes 3*, α-dbn–3Y/F–GFP. Note that expression levels of α-dbn-WT-GFP were significantly increased compared with nonphosphorylated mutant α-dbn–3Y/F–GFP. *C*, graph shows that real-time RT-PCR of WT and mutant α-dbn1 mRNA expression levels are similar. *D*, HeLa cells transfected with phosphorylated WT and nonphosphorylated mutant α-dbn were treated with the proteasome inhibitor MG132. Lysates were immunoblotted with anti-GFP antibody. mCherry was used as a control for the transfection efficiency and as a loading control with Ponceau S. *E*, quantification of four independent experiments as in *D* (data are presented as means ± S.D.). The α-dbn signals were normalized to Ponceau S signals. Note that in the presence of the proteasome inhibitor MG132, the expression level of nonphosphorylated α-dbn mutant was significantly increased (93.1%, comparable with WT (100%)). In the absence of MG132 treatment, however, the expression level of α-dbn mutant was significantly low (37.3% of WT (100%)). Statistical analysis was done by two-way ANOVA (interaction P value = 0.0120; $F = 8.747$) followed by Tukey's post hoc multiple comparisons test. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. *n.s*, not significant.

(*B* and *C*), the amount of αkap was significantly higher in cells co-transfected with the phosphorylatable α-dbn1 isoform compared with cells co-transfected with nonphosphorylated α-dbn mutant. These results were further confirmed in cells that were co-transfected with a fixed amount of αkap, and an increasing amount of WT and mutant α-dbn. Fig. 6 (*D* and *E*) showed that αkap expression levels increased with WT α-dbn concentration as expected, whereas an increase in mutant α-dbn has no significant effect on αkap accumulation. These results indicate that the phosphorylation of the three tyrosine residues of α-dbn is essential for the interaction and the stability of αkap in HeLa cells.

αkap and α-dystrobrevin promote the formation of agrin-induced AChR clusters

Because α-dbn promotes αkap accumulation, which in turn enhances AChR stability, we asked whether overexpressing α-dbn and αkap in muscle cells would increase the size of AChR clusters. C2C12 muscle cells were transfected with either αkap–mCherry (*red*) or α-dbn–GFP (*green*) or co-transfected with both αkap–mCherry and α-dbn–GFP, and differentiated myotubes were treated with agrin overnight, fixed, and bathed with fluorescently tagged BTX (Alexa 647, Alexa 488, or Alexa 594) to label only surface AChRs. Because transfection efficiency was only ~50%, we were able to use the same culture dish and quantify the area and length of AChR clusters on both transfected and nontransfected myotubes. Fig. 7 shows that both the area and length of agrin-induced AChR clusters were significantly increased on muscle transfected with either αkap (area, 35.1 ± 2.8 μm^2; length, 10.4 ± 0.6 μm), or α-dbn1 (area, 31.9 ± 1.9 μm^2; length, 9.9 ± 0.4 μm) compared with nontransfected cells with αkap (area, 19.4 ± 2.8 μm^2; length, 7.5 ± 0.4 μm) or α-dbn clusters (area, 17.5 ± 1.7 μm^2; length, 6.5 ± 0.4 μm). However, the averages of area (41.2 ± 3.9 μm^2) and length (11.2 ± 0.7 μm) of AChR clusters in muscles overexpressing both α-dbn and αkap were not significantly different from myotubes expressing either α-dbn or αkap (Fig. 7; see "Discussion"). However, because most of the myotubes do not form clusters in the absence of agrin (at least in our hand), the effect

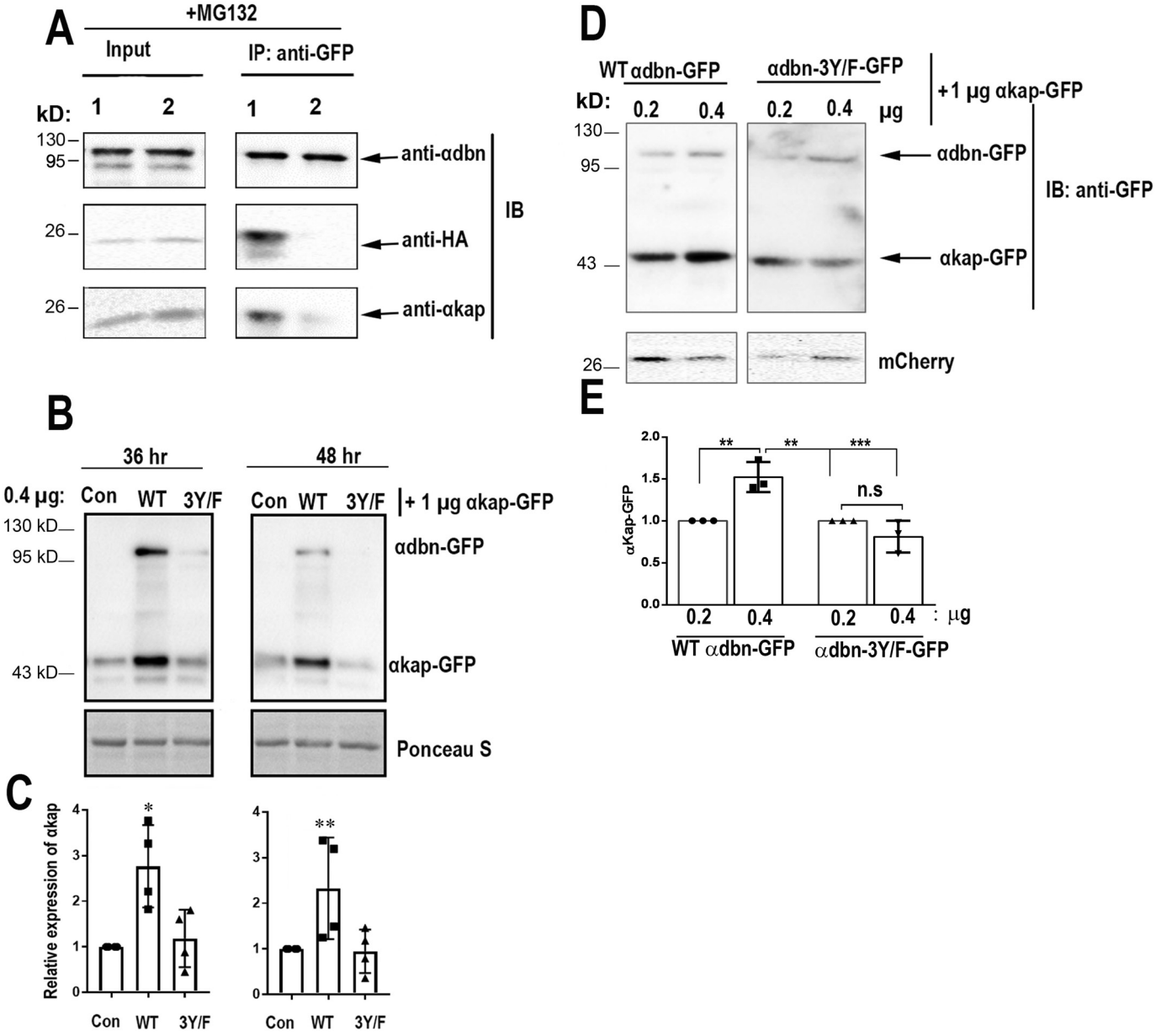

Figure 6. The three phosphorylatable tyrosine residues are essential for the interaction of α-dbn1 with αkap. *A*, HeLa cells were co-transfected with αkap-HA and either phosphorylatable (*lanes 1*) or nonphosphorylated α-dbn1–GFP mutant (*lanes 2*). The cells were then treated with MG132 for 6 h to prevent the degradation of α-dbn1 mutant so the same amount of WT, and mutant α-dbn1 would be immunoprecipitated with anti-GFP. The precipitates were immunoblotted with anti–α-dbn, anti-αkap, and anti-HA antibodies. Note the presence of positive immunoblotting for α-dbn and αkap in lysates co-transfected with WT phosphorylated α-dbn and αkap. However, lysates from cells transfected with αkap and nonphosphorylated α-dbn showed little to no positive immunoblotting for αkap. *B*, Western blots of lysates harvested from HeLa cells co-transfected with either αkap–GFP and WT α-dbn–GFP or αkap–GFP and α-dbn1–GFP mutant (36 and 48 h after transfection). *C*, quantification of four blots as in *B* (means ± S.D.). Statistical analysis was done by one-way ANOVA (at 36 h: *, $P < 0.035$; $F = 4.97$; at 48 h: **, $P < 0.006$; $F = 9.34$) followed by Dunnett's post hoc multiple comparisons test. **, $P < 0.01$; *, $P < 0.05$. *D*, Western blots of lysates from HeLa cells co-transfected with 1 μg of αkap–GFP and either (0.2 and 0.4 μg) WT or mutant α-dbn–GFP. Note that the expression level of αkap–GFP has increased as the concentration of WT α-dbn1 increased, but not in mutant. *E*, quantification of blots as in *D* (means ± S.D., $n = 3$). Statistical analysis was done by two-way ANOVA (interaction p value = 0.0015; $F = 22.47$) followed by Tukey's post hoc multiple comparisons test. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. *n.s*, not significant.

of α-dbn or αkap overexpression on AChR clusters size and length was not assessed.

Discussion

This work demonstrates that α-dystrobrevin plays an important role in maintaining high expression of αkap and subsequently AChRs' accumulation. The major findings are: 1) α-dbn, αkap, and AChRα are present within the same complex; 2) α-dbn promotes the expression levels of αkap in a concentration-dependent manner, whereas αkap has no regulatory effect on α-dbn; 3) the promoting effect of αkap on AChRα accumulation requires the presence of α-dbn; 4) the tyrosine phosphorylation of α-dbn is critical for its stability and its interaction with αkap; 5) a stable interaction of phosphorylated α-dbn isoform with αkap is essential for αkap accumulation;

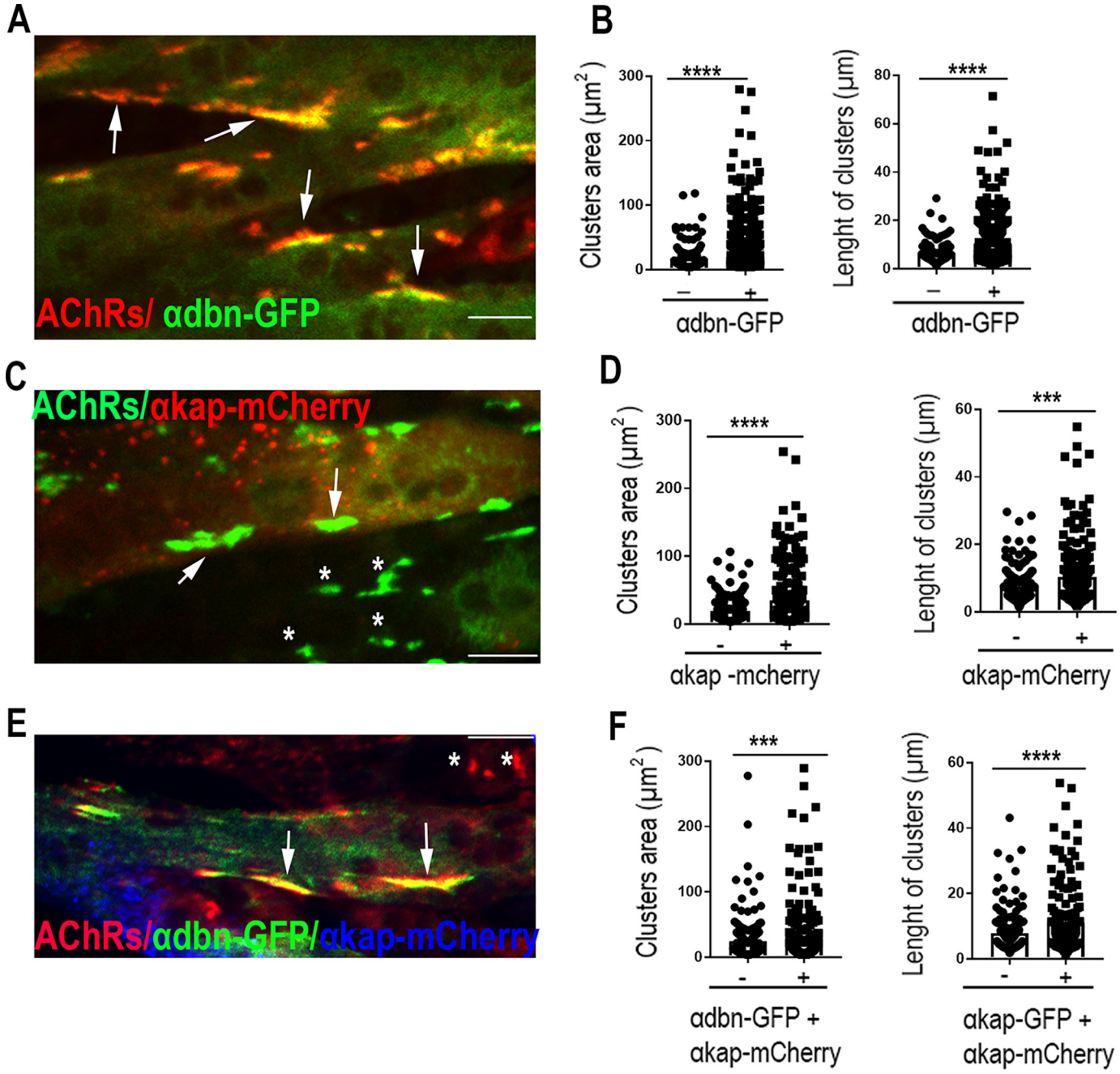

Figure 7. Overexpression of α-dbn and/or αkap in myotubes increased the length and area of agrin-induced AChR clusters. C2C12 myoblasts were transfected with αkap–mCherry and/or α-dystrobrevin–GFP, and differentiated myotubes were treated with agrin overnight, fixed with PFA, and bathed with a fluorescent BTX (Alexa 488 or Alexa 594) to label superficial receptor clusters. AChR clusters on transfected and nontransfected myotubes were imaged, and their area and length were measured and compared with nontransfected myotubes within the same culture dish. *A*, *C*, and *E*, examples of myotubes transfected with α-dbn–GFP (*A*), αkap–mCherry (*C*), and αkap–mCherry and α-dbn–GFP (*E*). *B*, *D*, and *F*, quantification of clusters area and length of AChRs. Note that both the area and length of AChR clusters in transfected group (α-dbn–GFP, αkap–mCherry, and α-dbn–GFP/αkap mCherry; see *arrows*) are significantly increased compared with the neighboring nontransfected group (*asterisks*). The data are presented as means ± S.D. For statistical analyses, unpaired *t* tests were used. ***, $P < 0.001$; ****, $P < 0.0001$.

and 6) high expression of α-dbn and αkap significantly enhances the formation of large agrin-induced AChR clusters. Altogether, these results demonstrate that α-dystrobrevin is essential not only for αkap expression levels but also for the formation of large AChR clusters.

The current experiments show that αkap is present in the same complex with α-dbn and AChRs. However, at AChR clusters, in which α-dbn is concentrated, αkap is not present with AChR/α-dbn. This suggests the possibility that the interaction between αkap and α-dbn may occur in a vesicular complex during their intracellular trafficking, specifically in the ER where αkap has been found to be highly expressed in skeletal and cardiac muscles (18, 29, 30). Interestingly, in the absence of either αkap or α-dbn, AChR accumulation was reduced (Fig. 2). For instance, in cells that do not express endogenous α-dbn, such as HeLa cells, when αkap and AChRα were overexpressed,

αkap has little to no effect on AChR accumulation/stability. Similarly, in HEK cells that express endogenous α-dbn but not αkap, when AChRα was overexpressed, α-dbn has little effect on AChR accumulation. One caveat of this study is that the co-transfection efficiency of multiple plasmids (four AChR subunits (AChRα, β, γ, and δ), αkap, α-dbn, and empty mCherry constructs) into HeLa cells was difficult to obtain, which hinders the analysis of the effect of αkap and α-dbn on the assembly and delivery of the pentameric AChR channel. Of note, in heterologous cells transfected with all receptor subunits, the assembly of AChR subunits into a pentameric complex occurs in the endoplasmic reticulum (35, 37), which is essential for the stability and delivery of functional AChR subunits to the cell surface (36, 38). However, the absence of one or more AChR subunits blocks the transport of other subunits to the cell surface, and the unassembled AChR subunits are presumably targeted to degradation (37). In the ER, AChRα undergoes a conformational change in protein structure that enables it to bind bungarotoxin before assembly with other subunits and that toxin-binding activity increased when all four subunits together were expressed (37). In the present study, we showed that nAChRs associate with αkap and αdbn, perhaps directly or indirectly as part of a complex and that the presence of αkap–α dbn complex substantially increased the amount of unassembled AChRα subunit. Although we have not examined where αkap–αdbn are acting/interacting and stabilizing the AChRα subunit, we think it is likely that increased accumulation of AChRα occurs in the ER/Golgi compartments, because the assembly of AChR and localization of αkap occurs there (18, 29, 35, 37). However, it remains unknown whether the stabilizing effect of the αkap–αdbn complex on nAChRα expression has any impact on the regulation/stability and/or the delivery of the fully assembled pentameric receptor to the cell surface. It is worth mentioning that in our previous studies, we have shown that αkap is present with AChRs in the same complex in cultured myotubes and muscle tissues of mice and that the knockdown of αkap endogenous expression in either cultured muscle cells or in the sternomastoid muscle of living mice with shRNA caused a significant reduction in the number of AChR clusters on cultured myotubes and the postsynaptic receptor density at the neuromuscular junction, in addition to its structural changes (17, 18). On the other hand, NMJs of mice deficient in α-dystrobrevin (a component of the DGC involved in the anchoring of the AChRs in the synaptic membrane) are structurally aberrant and have a reduced level of AChRs and a lower stability of receptors in the postsynaptic membrane (20, 24). Because the abnormal NMJ phenotype of mice deficient in α-dystrobrevin resembles, in some aspects, that in muscles electroporated with shRNA αkap and that αkap and α-dbn are within the same complex, it is possible that αkap and α-dbn interaction is essential for promoting AChR stability and the maintenance of the NMJ.

Interestingly, previous studies have shown that αkap in cultured muscle cells or heterologous system promotes the accumulation of AChRs in a ubiquitin-dependent proteasome mechanism. However, αkap does not share any similarities with known deubiquitinase, suggesting that αkap through an association with deubiquitinases may control the stability of AChRs. Interestingly, it has been reported that α-dbn also forms complex with the deubiquitinases Usp9x (31), and because the complex α-dbn and αkap is indispensable for AChR accumulation, it is conceivable that α-dbn1, through its association with Usp9x, may protect AChRs from degradation.

Several studies have reported that phosphorylation may serve to recruit adaptor/signaling proteins. Along these lines, in the absence of the phosphorylatable tyrosine residues, α-dbn1 expression levels were significantly reduced (Fig. 5). In cells transfected with WT phosphorylatable α-dbn1 and mutant nonphosphorylated α-dbn, expression of mutant isoform was significantly reduced compared with WT, suggesting that phosphorylatable tyrosine residues in the C terminus is essential for the stability of α-dbn1. One plausible possibility is that the three site of tyrosine phosphorylation may affect the folding of α-dbn1, leading to its instability/degradation. Indeed, when cells were treated with a proteasome inhibitor (MG132), there was no significant difference between phosphorylatable WT and nonphosphorylated mutant α-dbn. This suggests that ubiquitination and degradation of α-dbn1 by the ER-associated degradation pathway are likely accelerated in the absence of the phosphorylatable sites in the C terminus of α-dbn1 (32). It is also possible that the interaction of phosphorylated α-dbn with partners may enhance its stability. A recent study identified several WT α-dbn–binding partners that associate with α-dbn through phosphorylation-dependent mechanisms (32). This include scaffold proteins Grb2 and SH3BP2, Rho guanine nucleotide exchange factor Arhgef5, phosphoinositide 3-kinase, and the nonreceptor tyrosine-protein kinase Fyn. Thus, it is possible that when tyrosine phosphorylation residues are mutated, the interaction of α-dbn with its partners is reduced, leading to its instability.

One unexpected result from this work is that α-dbn promotes αkap accumulation in both heterologous nonmuscle cells and cultured muscle cells in a concentration-dependent manner, whereas αkap has no effect on α-dbn accumulation (Figs. 3 and 4). This raises the question of how might α-dbn promote αkap accumulation. The present data show that the phosphorylatable tyrosine residues of α-dystrobrevin are essential for interacting with αkap. In contrast to WT phosphorylatable α-dbn, which forms a complex with αkap, there was little pulldown of αkap when αkap was expressed with the nonphosphorylated mutant α-dbn (3Y/F) in HeLa cells. It is possible that phosphorylation of α-dbn could lead to its conformational changes, leading to enhancing strength of the interaction with αkap. Such strong interactions may enhance the accumulation and stability of αkap. However, when α-dbn is not phosphorylated, the interaction with αkap is weakened, which leads to its destabilization.

The tyrosine phosphorylation of α-dbn1 has been shown to play an important role in the maintenance of the structural integrity of the synapse (25). Similarly, the knockdown of αkap both in culture muscle cells and *in vivo* (17, 18) dramatically reduced AChR expression levels and altered the structural integrity of the postsynaptic apparatus of the NMJs. The current experiments show that the overexpression of either αkap or α-dbn in C2C12 muscle cells significantly increased the number and size of AChR clusters (Fig. 7). However, when both

αkap and α-dbn are co-expressed in cultured muscle cells, the size of AChR clusters is not significantly different from AChR clusters on muscle cells overexpressing either αkap or α-dbn (Fig. 7). One possible explanation is that when α-dbn is overexpressed, the endogenous expression of αkap is also significantly increased (Fig. 3). However, it must be acknowledged that, despite the fact that the C2C12 differentiated myotube is a widely used experimental model for studying the underlying mechanism for the formation of receptor clusters, it does have several limitations compared with innervated muscle cells, in which nerve impulses are critical for the stability and the formation of the neuromuscular in a timely manner. Thus, further *in vivo* studies are warranted.

In summary, the present work suggests a model by which the complex α-dbn–αkap controls AChR stability during intracellular trafficking. Phosphorylated α-dbn recruits a deubiquitinase Usp9x and/or chaperon proteins. The complex interacts and stabilizes αkap. αkap, through its unique hydrophobic N-terminal domain, targets the complex to the ER (where αkap has been found to be highly expressed) where it promotes AChR stability by a ubiquitin-dependent mechanism. In the absence of phosphorylated α-dbn and αkap interaction, most of AChRα subunits are targeted to degradation in a proteasome-dependent mechanism.

Materials and methods

Plasmid constructs and reagents

The plasmids of the mouse AChR subunit α was a generous gifts from Dr. William Green at the University of Chicago. Plasmids of αkap–GFP and α-dystrobrevin–GFP were described in our previous work (17, 18). α-Dystrobrevin-1–GFP is the full-length phosphorylatable isoform; α-dystrobrevin–3Y/F–GFP is the mutated isoform in which the C-terminal phosphorylatable three tyrosine residues were changed to phenylalanine (Y705F, Y713F, and Y730F). αkap–mCherry was cloned by swapping GFP (from the αkap–GFP) to mCherry (from pmCherry-N3) with 5′ EcoRI and 3′ KpnI sites. Both α-dystrobrevin–3Y/F–GFP and αkap–mCherry were verified by sequencing, transfection, and Western blots. The proteasome inhibitor MG132 (Enzo Life Sciences) was used at 2.5 μM as described by Mouslim *et al.* (18) to determine whether the degradation of α-dbn–GFP mutant is mediated by the proteasome.

Cell culture, transfection, and cell imaging

HeLa, HEK 293T, and C2C12 cells (ATCC, Manassas, VA, USA) were used in this study. HeLa and HEK 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) (11995-065; Gibco) and supplemented with 10% fetal bovine serum (S11050; Atlanta Biologicals). C2C12 myoblasts (ATCC) were maintained in DMEM with 20% fetal bovine serum. To differentiate C2C12 myoblasts into myotubes, C2C12 myoblasts at 80% confluency were switched to DMEM supplemented with 5% horse serum (16050; Gibco) for 5–7 days.

To examine the effect of overexpression of αkap and α-dbn on the formation of AChR clusters, cells (at 80% confluency) were transfected with appropriate plasmids using Lipofectamine 2000 (1467572; Invitrogen) according to the manufacturer's instructions. Differentiated myotubes were treated with C-terminal agrin (550-AG; R&D Systems) at a concentration of 500 ng/ml overnight. The cells were then fixed by 2% paraformaldehyde in PBS for 20 min and then washed with PBS three times. The cells were incubated with fluorescent BTX-Alexa (Molecular Probes) for 2 h at room temperature, followed by four washes in PBS and 4[prime],6[prime]-diamino-2-phenylindole Fluoromount-G mounting medium (0100-20; Southern Biotech). The cells were then imaged using the Olympus spinning-disk confocal microscope (Andor/Olympus IX81). The *z*-stacks were then collapsed, and the contrast was adjusted with Photoshop (Adobe Systems).

To examine the co-localization between α-dbn and αkap, HeLa cells were co-transfected with α-dbn–GFP (*green*) and αkap–mCherry (*red*) constructs, and 24 h later, the cells were fixed and imaged with the confocal microscope. The z-stacks were then collapsed, and the contrast was adjusted with Photoshop (Adobe Systems).

Quantification of AChR clusters

Spinning-disk images of myotubes were processed and analyzed by using ImageJ (National Institutes of Health, version 1.49). Labeled clusters of AChRs with fluorescent BTX-Alexa (either 647, 594 or, 488) on transfected and nontransfected myotubes were quantified using the following procedure: images were set to 8-bit binary, and a threshold that clearly identified clusters and excluded background was used. The area (μm^2) of AChR clusters and their lengths (μm) were measured. At least 30 myotubes from 3 independent plates were quantified from each experimental condition.

Pulldown and Western blotting

C2C12 myotubes were harvested in lysis buffer containing 100 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor mixture (11873580001; Roche) and then processed by sonication and centrifugation (8,000 × *g* for 5 min at 4 °C.). To pulldown AChRs, lysates were incubated with BTX–biotin overnight followed by a 2-h incubation with NeutrAvidin beads (Thermo Fisher Scientific). The beads were collected by centrifugation, washed in PBS four times, and subjected to boiling in reducing 2× lithium dodecyl sulfate buffer and DTT (Invitrogen). The released bound proteins were separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane, as described previously (18, 34). The membranes were then incubated with mouse anti-αkap (1:200; clone A1, Santa Cruz Biotechnology), anti-AChRα MAB210 (1:500); Covance), or anti-αdbn (1:3000) overnight at 4 °C. After extensive washing, the membranes were then incubated with secondary horseradish peroxidase–conjugated antibodies (1:10,000; Jackson ImmunoResearch) and developed using SuperSignal West Femto maximum sensitivity substrate or West Pico (Thermo Fisher Scientific).

In another series of experiments, HeLa and HEK cells at 90–100% confluence were washed with cold PBS, lysed with lysis buffer (100 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 5 mM Na_3VO_4, 2.5 mM $Na_4P_2O_7$, 1% Nonidet P-40,

0.5% sodium deoxycholate, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor mixture), and centrifuged for 10 min at 10,000 [times] *g*. The lysates were resuspended in LDS reducing sample buffer and boiled for 5 min prior to Western blotting. The proteins were separated by SDS-PAGE (10%) and transferred to PVDF membranes. The membranes were stained with Ponceau S for 1 h, imaged, washed three times with PBST (PBS with 0.05% Tween 20), and then bathed in a blocking solution (5% dry milk and 0.05% Tween 20 in TBS, 10× TBS: 170-6435; Bio-Rad) for 1–3 h. The membranes were incubated with primary antibodies in blocking solution overnight at 4 °C. After three washes with PBST, the membranes were incubated with secondary horseradish peroxidase–conjugated antibodies and developed using SuperSignalWest Pico solutions (34077; Thermo Fisher Scientific) or Clarity Western ECL substrate (1705060; Bio-Rad). Mouse anti-HA (clone 16B12, MMS-101R; Covance) and mouse anti-αkap (calcium/calmodulin kinase IIα A-1, sc-13141; Santa Cruz Biotechnology) were diluted at 1:2000; mouse anti–α-dystrobrevin (610766; BD Biosciences) was diluted at 1:4000; rabbit anti-GFP (A6455; Invitrogen), rat anti-AChRα (MAB210; Covance), and mouse anti-tubulin (AA4.3-s; Developmental Studies Hybridoma Bank) were diluted at 1: 5000. All horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch) were diluted at 1:10,000. Western blotting band signals were captured using either the Bio-Rad ChemiDoc MP imaging system or FluorChem M FM0467. We first used the auto-exposure mode to determine the optimum exposure time to avoid pixels' saturation (according to the manufacturers' handbook). Within in that time range, we captured at least two images to use for quantification. Only the nonsaturated bands in the images were quantified. Between the two images, the samples were assigned proportional values to ensure that the signals fell within the ECL linear exposure range. Because of inconsistencies and variations in signal intensity between the same protein bands in different gel lanes, we normalized the proteins bands to its transfection efficiency indicator, housekeeping proteins, or total protein identified by Ponceau S. In this way, errors related to variations in sample loading and transfers were corrected. Western blotting bands were quantified with ImageJ (National Institutes of Health, version 1.49). The intensity of bands was quantified by using the rectangular selection tool and keeping the area uniform, which covered each band's active area, where a straight line was used to cut off nonspecific bands, and the wand tool selected the target peak. These were compared with equal area baseline selections of an empty lane nearby (no protein loaded) to subtract background. If the background was not uniform across the blot, the background subtraction would be individually selected for each lane, for which the criteria is a no-signal, slightly upper or lower active band area. For all data presented as histograms, results from several blots were averaged, with the number indicated in the figure legends.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 8.2.0). The experimental results were analyzed by Student's *t* test (compare differences between two groups) and either by one-way ANOVA or two-way ANOVA followed by Tukey's or Dunnett's post hoc comparison tests where appropriate. The data are expressed as means ± S.D. Significance was defined as $p < 0.05$ for all analyses.

Data availability

All the data for this study are available within the article.

Acknowledgments—We thank Drs. Richard Hume, Amy Chang, and Jianming Li (University of Michigan) for helpful comments on this manuscript and the Akaaboune laboratory members for technical assistance.

Author contributions—P.-J. C. data curation; P.-J. C. formal analysis; P.-J. C. validation; P.-J. C. visualization; P.-J. C., D. Z., and D. C. B. methodology; P.-J. C., D. Z., and M. A. writing-original draft; D. C. B. and M. A. writing-review and editing; M. A. conceptualization; M. A. supervision; M. A. funding acquisition; M. A. project administration.

Funding and additional information—This work was supported by NINDS, National Institutes of Health Grants NS-047332 and NS-103845 (to M. A.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AChR, acetylcholine receptor; α-dbn, α-dystrobrevin; NMJ, neuromuscular junction; shRNA, short hairpin RNA; HA, hemagglutinin; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; ANOVA, analysis of variance; DGC, dystrophin glycoprotein complex.

References

1. Sanes, J. R., and Lichtman, J. W. (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* **2,** 791–805 CrossRef Medline
2. DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., Kinetz, E., Compton, D. L., Rojas, E., Park, J. S., Smith, C., DiStefano, P. S., Glass, D. J., Burden, S. J., and Yancopoulos, G. D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation *in vivo*. *Cell* **85,** 501–512 CrossRef Medline
3. Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R., and Merlie, J. P. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377,** 232–236 CrossRef Medline
4. Kim, N., Stiegler, A. L., Cameron, T. O., Hallock, P. T., Gomez, A. M., Huang, J. H., Hubbard, S. R., Dustin, M. L., and Burden, S. J. (2008) Lrp4 is a receptor for agrin and forms a complex with MuSK. *Cell* **135,** 334–342 CrossRef Medline
5. Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M., Akiyama, T., Iwakura, Y., Higuchi, O., and Yamanashi, Y. (2006) The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science* **312,** 1802–1805 CrossRef Medline
6. Weatherbee, S. D., Anderson, K. V., and Niswander, L. A. (2006) LDL-receptor–related protein 4 is crucial for formation of the neuromuscular junction. *Development* **133,** 4993–5000 CrossRef Medline

7. Zhang, B., Luo, S., Wang, Q., Suzuki, T., Xiong, W. C., and Mei, L. (2008) LRP4 serves as a coreceptor of agrin. *Neuron* **60,** 285–297 CrossRef Medline
8. Schmidt, N., Akaaboune, M., Gajendran, N., Martinez-Pena y Valenzuela, I., Wakefield, S., Thurnheer, R., and Brenner, H. R. (2011) Neuregulin/ErbB regulate neuromuscular junction development by phosphorylation of α-dystrobrevin. *J. Cell Biol.* **195,** 1171–1184 CrossRef Medline
9. Barik, A., Zhang, B., Sohal, G. S., Xiong, W. C., and Mei, L. (2014) Crosstalk between Agrin and Wnt signaling pathways in development of vertebrate neuromuscular junction. *Dev. Neurobiol.* **74,** 828–838 CrossRef Medline
10. Koles, K., and Budnik, V. (2012) Wnt signaling in neuromuscular junction development. *Cold Spring Harb. Perspect. Biol.* **4,** a008045 CrossRef Medline
11. Tintignac, L. A., Brenner, H. R., and Rüegg, M. A. (2015) Mechanisms regulating neuromuscular junction development and function and causes of muscle wasting. *Physiol. Rev.* **95,** 809–852 CrossRef Medline
12. Messeant, J., Ezan, J., Delers, P., Glebov, K., Marchiol, C., Lager, F., Renault, G., Tissir, F., Montcouquiol, M., Sans, N., Legay, C., and Strochlic, L. (2017) Wnt proteins contribute to neuromuscular junction formation through distinct signaling pathways. *Development* **144,** 1712–1724 CrossRef Medline
13. Belhasan, D. C., and Akaaboune, M. (2020) The role of the dystrophin glycoprotein complex on the neuromuscular system. *Neurosci. Lett.* **722,** 134833 CrossRef Medline
14. Bayer, K. U., Löhler, J., and Harbers, K. (1996) An alternative, nonkinase product of the brain-specifically expressed Ca^{2+}/calmodulin-dependent kinase IIα isoform gene in skeletal muscle. *Mol. Cell Biol.* **16,** 29–36 CrossRef Medline
15. Bayer, K. U., De Koninck, P., and Schulman, H. (2002) Alternative splicing modulates the frequency-dependent response of CaMKII to Ca^{2+} oscillations. *EMBO J.* **21,** 3590–3597 CrossRef Medline
16. Bayer, K. U., Harbers, K., and Schulman, H. (1998) αKAP is an anchoring protein for a novel CaM kinase II isoform in skeletal muscle. *EMBO J.* **17,** 5598–5605 CrossRef Medline
17. Martinez-Pena, Y. V. I., Aittaleb, M., Chen, P. J., and Akaaboune, M. (2015) The knockdown of αkap alters the postsynaptic apparatus of neuromuscular junctions in living mice. *J. Neurosci.* **35,** 5118–5127 CrossRef Medline
18. Mouslim, C., Aittaleb, M., Hume, R. I., and Akaaboune, M. (2012) A role for the calmodulin kinase II–related anchoring protein (αkap) in maintaining the stability of nicotinic acetylcholine receptors. *J. Neurosci.* **32,** 5177–5185 CrossRef Medline
19. Ervasti, J. M., and Campbell, K. P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122,** 809–823 CrossRef Medline
20. Grady, R. M., Zhou, H., Cunningham, J. M., Henry, M. D., Campbell, K. P., and Sanes, J. R. (2000) Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin–glycoprotein complex. *Neuron* **25,** 279–293 CrossRef Medline
21. Grady, R. M., Grange, R. W., Lau, K. S., Maimone, M. M., Nichol, M. C., Stull, J. T., and Sanes, J. R. (1999) Role for α-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. *Nat. Cell Biol.* **1,** 215–220 CrossRef Medline
22. Blake, D. J., Nawrotzki, R., Peters, M. F., Froehner, S. C., and Davies, K. E. (1996) Isoform diversity of dystrobrevin, the murine 87-kDa postsynaptic protein. *J. Biol. Chem.* **271,** 7802–7810 CrossRef Medline
23. Peters, M. F., Sadoulet-Puccio, H. M., Grady, M. R., Kramarcy, N. R., Kunkel, L. M., Sanes, J. R., Sealock, R., and Froehner, S. C. (1998) Differential membrane localization and intermolecular associations of α-dystrobrevin isoforms in skeletal muscle. *J. Cell Biol.* **142,** 1269–1278 CrossRef Medline
24. Akaaboune, M., Grady, R. M., Turney, S., Sanes, J. R., and Lichtman, J. W. (2002) Neurotransmitter receptor dynamics studied *in vivo* by reversible photo-unbinding of fluorescent ligands. *Neuron* **34,** 865–876 CrossRef Medline
25. Grady, R. M., Akaaboune, M., Cohen, A. L., Maimone, M. M., Lichtman, J. W., and Sanes, J. R. (2003) Tyrosine-phosphorylated and nonphosphorylated isoforms of α-dystrobrevin: roles in skeletal muscle and its neuromuscular and myotendinous junctions. *J. Cell Biol.* **160,** 741–752 CrossRef Medline
26. Balasubramanian, S., Fung, E. T., and Huganir, R. L. (1998) Characterization of the tyrosine phosphorylation and distribution of dystrobrevin isoforms. *FEBS Lett.* **432,** 133–140 CrossRef Medline
27. Wagner, K. R., Cohen, J. B., and Huganir, R. L. (1993) The 87k-postsynaptic membrane-protein from torpedo is a protein-tyrosine kinase substrate homologous to dystrophin. *Neuron* **10,** 511–522 CrossRef Medline
28. Lee, D. H., and Goldberg, A. L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* **8,** 397–403 CrossRef Medline
29. Nori, A., Lin, P. J., Cassetti, A., Villa, A., Bayer, K. U., and Volpe, P. (2003) Targeting of α-kinase–anchoring protein (αKAP) to sarcoplasmic reticulum and nuclei of skeletal muscle. *Biochem. J.* **370,** 873–880 CrossRef Medline
30. Singh, P., Salih, M., and Tuana, B. S. (2009) α-Kinase anchoring protein αKAP interacts with SERCA2A to spatially position Ca^{2+}/calmodulin-dependent protein kinase II and modulate phospholamban phosphorylation. *J. Biol. Chem.* **284,** 28212–28221 CrossRef Medline
31. Gingras, J., Gawor, M., Bernadzki, K. M., Grady, R. M., Hallock, P., Glass, D. J., Sanes, J. R., and Proszynski, T. J. (2016) Dystrobrevin recruits Grb2 and α-catulin to organize neurotransmitter receptors at the neuromuscular junction. *J. Cell Sci.* **129,** 898–911 CrossRef Medline
32. Amm, I., Sommer, T., and Wolf, D. H. (2014) Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. *Biochim. Biophys. Acta* **1843,** 182–196 CrossRef Medline
33. Sun, Z. H., and Brodsky, J. L. (2018) The degradation pathway of a model misfolded protein is determined by aggregation propensity. *Mol. Biol. Cell* **29,** 1422–1434 CrossRef Medline
34. Martinez-Pena y Valenzuela, I., Mouslim, C., and Akaaboune, M. (2010) Calcium/calmodulin kinase II–dependent acetylcholine receptor cycling at the mammalian neuromuscular junction *in vivo*. *J. Neurosci.* **30,** 12455–12465 CrossRef Medline
35. Gu, Y., Forsayeth, J. R., Verrall, S., Yu, X. M., and Hall, Z. W. (1991) Assembly of the mammalian muscle acetylcholine receptor in transfected COS cells. *J. Cell Biol.* **114,** 799–807 CrossRef Medline
36. Blount, P., Smith, M. M., and Merlie, J. P. (1990) Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.* **111,** 2601–2611 CrossRef Medline
37. Smith, M. M., Lindstrom, J., and Merlie, J. P. (1987) Formation of the α-bungarotoxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. *J. Biol. Chem.* **262,** 4367–4376 Medline
38. Claudio, T., Paulson, H. L., Green, W. N., Ross, A. F., Hartman, D. S., and Hayden, D. (1989) Fibroblasts transfected with torpedo acetylcholine receptor β-, γ-, and δ-subunit cDNAs express functional receptors when infected with a retroviral α recombinant. *J. Cell Biol.* **108,** 2277–2290 CrossRef Medline