

α -Dystrobrevin-1 recruits α -catulin to the α_{1D} -adrenergic receptor/dystrophin-associated protein complex signalosome

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α_{1D} -Adrenergic receptors (ARs) are key regulators of cardiovascular system function that increase blood pressure and promote vascular remodeling. Unfortunately, little information exists about the signaling pathways used by this important G protein-coupled receptor (GPCR). We recently discovered that α_{1D} -ARs form a "signalosome" with multiple members of the dystrophin-associated protein complex (DAPC) to become functionally expressed at the plasma membrane and bind ligands. However, the molecular mechanism by which the DAPC imparts functionality to the α_{1D} -AR signalosome remains a mystery. To test the hypothesis that previously unidentified molecules are recruited to the α_{1D} -AR signalosome, we performed an extensive proteomic analysis on each member of the DAPC. Bioinformatic analysis of our proteomic data sets detected a common interacting protein of relatively unknown function, α -catulin. Coimmunoprecipitation and blot overlay assays indicate that α -catulin is directly recruited to the α_{1D} -AR signalosome by the C-terminal domain of α -dystrobrevin-1 and not the closely related splice variant α -dystrobrevin-2. Proteomic and biochemical analysis revealed that α -catulin supersensitizes α_{1D} -AR functional responses by recruiting effector molecules to the signalosome. Taken together, our study implicates α -catulin as a unique regulator of GPCR signaling and represents a unique expansion of the intricate and continually evolving array of GPCR signaling networks.

G protein-coupled receptors (GPCRs) are seven-transmembrane spanning proteins that are responsible for communicating information in the form of extracellular stimuli across lipid membranes into distinct intracellular signals with precise accuracy. After ligand binding, GPCRs signal through the canonical heterotrimeric G protein signaling pathway to activate a diverse array of downstream effectors (1). Recently, it has become evident that most GPCRs collaborate with one or more additional proteins at specific points in their lifecycle. These GPCR interacting proteins (or GIPs) are largely receptor subtype and cell context specific, include both membrane and cytosolic proteins, and typically play a highly specific supporting role for GPCR function (i.e., trafficking, ligand binding, enhancing signaling, signal termination, and/or degradation) (2–4).

Recently, we used yeast two-hybrid and proteomic screens to identify GIPs for a clinically important GPCR, the α_{1D} -adrenergic receptor (AR) (5). A member of the adrenergic family (α_1 , α_2 , β), α_{1D} -ARs are ubiquitously expressed on blood vessels and are responsible for increasing blood pressure during exercise, injury, stress, or cardiovascular disease (6). α_{1D} -AR knockout mice are hypotensive and resistant to high salt diet-induced hypertension (7, 8), yet this GPCR has been largely ignored over the past 20 y because after transfection into cell culture α_{1D} -ARs are sequestered in the endoplasmic reticulum (9, 10). Clinical interest in the α_{1D} -AR as a drug target has recently increased with the discoveries that α_{1D} -ARs are the predominant subtype expressed in epicardial coronary arteries (11) and that α_{1D} -AR prostate ex-

pression increases in patients with benign prostatic hypertrophy (12). Through proteomic screening, we discovered that α_{1D} -ARs are scaffolded to the dystrophin-associated protein complex (DAPC) via the anchoring protein syntrophin (10). Coexpression with syntrophins increases α_{1D} -AR plasma membrane expression, drug binding, and activation of $G\alpha_q/11$ signaling after agonist activation. Moreover, syntrophin knockout mice lose α_{1D} -AR-stimulated increases in blood pressure, demonstrating the importance of these essential GIPs for α_{1D} -AR function in vivo (10).

Proper organization of signaling molecules within cells by the DAPC is essential for the maintenance of cellular homeostasis at synaptic junctions (13). Mutations in DAPC result in severe muscle wasting diseases, such as Duchenne muscular dystrophy/Becker muscular dystrophy, and as a result, the role of this complex for proper skeletal muscle function has been thoroughly studied (14). However, the DAPC performs many other functions: it facilitates proper water transport across the blood–brain barrier by anchoring aquaporin (15, 16), clusters nicotinic acetylcholine receptors to ensure signal transmission at parasympathetic synapses (13), and anchors neuronal NOS at the cell membrane in cardiac myocytes to permit cardiodilation (17). We previously demonstrated that α_{1D} -ARs form a complex with the DAPC (10), but why this interaction is necessary for α_{1D} -AR functional coupling is unknown.

In this study, we postulated that molecules necessary for α_{1D} -AR signaling are recruited by the DAPC. Using a sequential proteomic screening approach, we identified α -catulin as a unique member of the α_{1D} -AR signalosome. The goal of these experiments was to understand how α -catulin integrates into this growing GPCR protein complex and to decipher the purpose of this relatively unstudied protein in GPCR signaling networks.

Results

α -Catulin: Unique Member of the α_{1D} -AR/DAPC Signalosome. We previously demonstrated that syntrophins are required for α_{1D} -AR function in vitro and in vivo by anchoring α_{1D} -ARs to the DAPC (10). Our working hypothesis is that the DAPC facilitates α_{1D} -AR function by acting as a multiprotein scaffold to arrange signaling molecules in close proximity to the receptor. To test this hypothesis, we fused tandem-affinity purification (TAP) epitopes

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containing streptavidin/calmodulin-binding proteins to the α_{1D} -AR and all members of the DAPC, including, α -syntrophin, β_1 -syntrophin, β_2 -syntrophin, α -dystrobrevin-1 (α -DB1), and α -DB2. HEK293 cell lines stably expressing each individual clone were created. TAP-tagged and associated proteins were purified from lysates of each stable cell line, digested with trypsin, and the tryptic peptides subjected to liquid chromatography (LC) tandem mass spectrometry (MS/MS).

Analysis of the peptides identified by LC-MS/MS revealed both predicted and unique components of the scaffold (Fig. 1A and Table S1). The core components of the scaffold (dystrophin, utrophin, α/β -DB, and $\alpha/\beta_1/\beta_2$ -syntrophin) were cross-identified in each of the individual pulldowns. Our experiments identified known interactors [i.e., CASK and LIN7C with β_2 -syntrophin, G α subunits with α -syntrophin (18, 19)] for each bait protein, confirming the validity of our experimental data sets. One protein, α -catulin (CTNNA1) was consistently identified in the majority of our proteomic screens (Table S1).

Next, we confirmed these findings using immunoprecipitation experiments of full-length proteins in living cells. Shown in Fig. 1B, lysates from HEK293 cells stably expressing TAP- α_{1D} -ARs were precipitated and probed for α -catulin, α -DB1, and syntrophin. Both endogenous α -catulin and transfected flag-tagged α -catulin bound to TAP- α_{1D} -ARs, along with other members of the α_{1D} -AR/DAPC signalosome. Immunoprecipitation and TAP/MS analysis revealed that α -catulin does not interact with the α_{1A} -AR subtype (Fig. S1 and Dataset S1). Combined with our TAP/MS and biochemical analysis, these findings implicate α -catulin as a unique member of the α_{1D} -AR/DAPC signalosome.

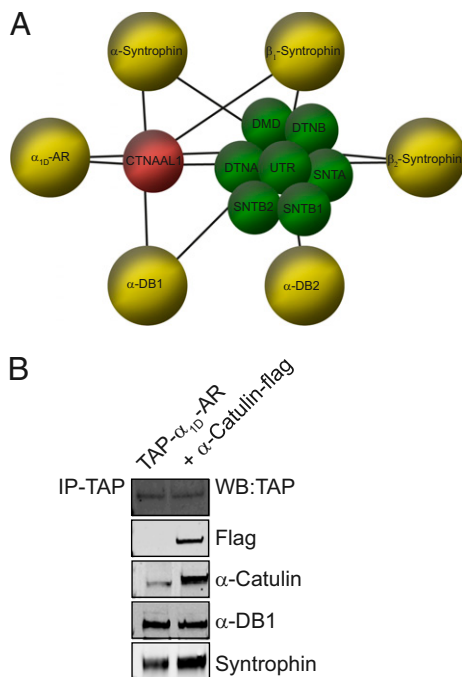


Fig. 1. α -Catulin is a unique member of the α_{1D} -AR/DAPC signalosome. (A) TAP-tagged α_{1D} -AR, $\alpha/\beta_1/\beta_2$ -syntrophin, and α -DB1/2 were expressed in HEK293 cells, purified, and subjected to LC/MS. Bait proteins are indicated by yellow circles. Identified proteins known to be members of the DAPC are shown in green circles (DMD, dystrophin; UTR, utrophin; DTNA, α -DB; DTNB, β -DB; SNTA, α -syntrophin; SNTB1, β_1 -syntrophin; SNTB2, β_2 -syntrophin). α -Catulin (CTNNA1, in red) was identified by our TAP/MS screening as a unique member of the signalosome. (B) TAP- α_{1D} -AR coimmunoprecipitated endogenous α -catulin, α -catulin-flag, α -DB1, and syntrophins in HEK293 cells.

α -Catulin Is Recruited to the α_{1D} -AR Signalosome by α -DB1. We detected α -catulin as a positive interactor in the α_{1D} -AR, $\alpha/\beta_1/\beta_2$ -syntrophin, and α -DB1 proteomic screens, making it difficult to predict which protein directly binds and recruits α -catulin to the α_{1D} -AR/DAPC signalosome. An important clue was that α -catulin was not detectable in the α -DB2 screen (Fig. 1A and Table S1). α -DB1 and α -DB2 are C-terminal splice variants of the α -DB gene (20). Following their common 504-aa core, α -DB1 has a unique 184-aa C terminus, whereas α -DB2 has a 9-aa unique C terminus. Because α -catulin interacts with α -DB1 but not with α -DB2, we suspected that α -catulin is most likely recruited by association with the α -DB1 C-terminal domain.

To test this possibility, we used HEK293 cells stably expressing TAP- α -DB1 or TAP- α -DB2. Coimmunoprecipitation experiments revealed that TAP- α -DB1 interacts with both endogenous and transiently transfected α -catulin-flag, whereas TAP- α -DB2 failed to coimmunoprecipitate either protein (Fig. 2A). These results were corroborated by experiments demonstrating that α -catulin-flag coimmunoprecipitates specifically with endogenous α -DB1 but not α -DB2 (Fig. 2B).

Next, we used nitrocellulose blot overlay assays to test whether the interaction between α -DB1 and α -catulin is direct. TAP- α -DB1, TAP- α -DB2, or untransfected HEK293 cell lysates were separated by SDS/PAGE, transferred to nitrocellulose, and incubated with GST- α -catulin purified from *Escherichia coli*. Membranes were probed with antibodies directed against GST or dystrobrevins. Secondary antibodies conjugated to IRDye 680 and 800 permitted coanalysis of blot data using a LiCor Odyssey system. As shown, GST- α -catulin bound endogenous and TAP- α -DB1 (Fig. 2C, Top). GST- α -catulin failed to bind either endogenous or TAP- α -DB2 (Fig. 2C, Middle), consistent with our TAP/MS analyses and coimmunoprecipitations. However, we observed a direct interaction between GST- α -catulin and β -DB (Fig. 2C, Bottom), which shares considerable homology with α -DB1 in the C-terminal region (Fig. S2). GST- α -catulin was unable to bind GST and other members of the DAPC, indicating that α -catulin directly and selectively interacts with α -DB1 and β -DB (Fig. S3). Thus, we conclude from our TAP/MS, coimmunoprecipitation, and nitrocellulose blot overlay analyses that α -catulin is recruited to the α_{1D} -AR signalosome through a direct interaction with the C-terminal domain of α -DB1.

Protein domain analysis revealed three regions in α -catulin homologous to vinculin (Fig. S4). We tested whether these regions represent potential α -DB1 interaction domains by introducing stop codons along the borders of each vinculin homology domain in α -catulin (Fig. 2D). Truncation mutants were expressed in and purified from HEK293 cells to ensure equivalent expression levels and proper predicted size of each construct (Fig. 2D). Biochemical analysis revealed that α -DB1 retained the ability to coimmunoprecipitate with each C-terminal α -catulin mutant (T1-4), indicating that vinculin type domains 2 and 3 are not necessary for interactions with α -DB1. However, TAP- α -catulin lacking N-terminal amino acids 1-277 resulted in a dramatic loss in α -DB1 association, indicating that vinculin homology domain 1 is essential for this interaction.

To determine whether α -catulin cellular localization overlaps with DAPC members, we immunostained HEK293 cells for endogenous α -catulin and either syntrophins (Fig. S5) or α -dystrobrevins (Fig. 3). As shown, α -catulin codistributes with dystrobrevins in HEK293 cells (Pearson coefficient, 0.38) but displays little codistribution in the cytoplasm. Because the localization seen might be caused by artifacts from folded or perturbed membranes, we also examined cross-sections of murine quadriceps. α -Catulin displays strong codistribution with α -dystrobrevins in nerve bundles and blood vessels in these slices (Fig. 3). Taken together with our MS, coimmunoprecipitation, and blot overlay results, these data suggest that α -catulin and α -DB1 associate together, presumably in complex with the

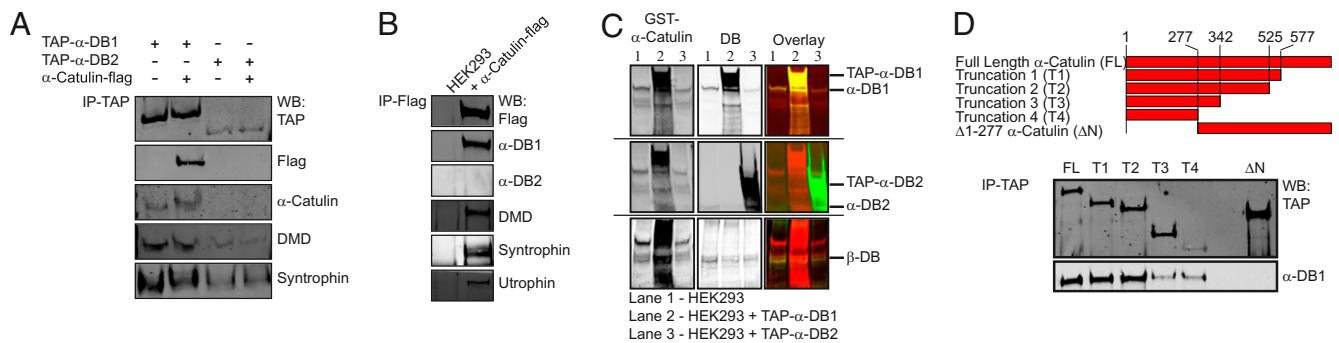


Fig. 2. The N terminus of α -catulin interacts directly with the C terminus of α -DB1. (A) TAP- α -DB1 or TAP- α -DB2 were expressed with (+) or without (-) α -catulin-flag in HEK293 cells. TAP-proteins were immunoprecipitated and analyzed by Western blotting as indicated. (B) α -Catulin-flag coimmunoprecipitates α -DB1, dystrophin (DMD), syntrophin, and utrophin in HEK293 cells. (C) Untransfected, TAP- α -DB1, or TAP- α -DB2-expressing HEK293 cell lysate was run on SDS/PAGE, transferred to nitrocellulose, blocked, and incubated with GST- α -catulin expressed and purified from *E. coli*. Blots were washed and simultaneously Western blotted for GST and either α -DB1 (Top), α -DB2 (Middle), or β -DB (Bottom). Overlays of the GST and dystrobrevin images are shown in the far right column, with GST signals in red and dystrobrevin signals in green. (D) Upper: TAP- α -catulin deletion constructs. Deletion constructs were expressed in HEK293 cells, purified using streptavidin Sepharose, washed, and analyzed by SDS/PAGE. Lower: Blots were Western blotted for both TAP- α -catulin deletions and α -DB1.

DAPC and membrane receptors [i.e., α_{1D} -AR, SLO-K1 (21), and 5-hydroxytryptamine-2 receptor (22)].

α -Catulin Supersensitizes α_{1D} -AR G α q/11 Signaling. What is the functional purpose of α -catulin in the α_{1D} -AR/DAPC signalosome? Previous studies indicate that α -catulin may facilitate intracellular signaling mechanisms by scaffolding essential downstream effectors in close proximity to one another (23–25). To determine whether α -catulin performs a similar function in the α_{1D} -AR/G α q/11 signaling pathway, we examined the effects of overexpressing or knocking down α -catulin on α_{1D} -AR signaling. In agreement with previous studies, α_{1D} -AR displays low levels of functional activity with rate-limiting concentrations of syntrophins in cells (Fig. 4A) (9, 26). To circumvent this technical issue, we used an α_{1D} -AR-syntrophin fusion protein separated by a six-glycine linker (10). We previously demonstrated that this molecular construct increases α_{1D} -AR receptor density and enhances signaling (10). Next, we examined the effects of α -catulin on α_{1D} -AR or α_{1D} -AR-6G-syntrophin signaling by either overexpressing TAP- α -catulin or selectively knocking down endogenous α -catulin expression with siRNA (Fig. S6). As shown in Fig. 4A, cotransfecting TAP- α -catulin resulted in a significant

enhancement of α_{1D} -AR-6G-syntrophin coupling to IP₃ production (Fig. 4A) and ERK1/2 phosphorylation (Fig. 4B), but not binding site formation (Fig. S1). Interestingly, siRNA α -catulin knockdown significantly decreased α_{1D} -AR-stimulated ERK1/2 phosphorylation (Fig. 4B) but not IP₃ production (Fig. 4A).

How does α -catulin regulate α_{1D} -AR signal transduction? Previous studies suggest that α -catulin scaffolds important signaling proteins to the signalosome such that, after agonist stimulation, liberated G α subunits rapidly activate downstream effectors, before being sequestered by reassociation with G $\beta\gamma$ or through signal termination by enhanced GTP hydrolysis by RGS proteins (27). To test this, HEK293 cells stably expressing TAP- α -catulin were created for TAP/MS proteomic analysis. α -Catulin and associated proteins were purified from cell lysates, digested with trypsin, and subjected to LC-MS/MS. Bioinformatic analysis of the resulting data sets revealed ~30 α -catulin interacting proteins (Fig. 4C and Table S2). As expected, we identified all known members of the α_{1D} -AR signalosome, including dystrophin, utrophin, α -dystrobrevin-1, and all three syntrophin isoforms previously shown to bind the α_{1D} -AR C-terminal PDZ domain: α -, β_1 -, and β_2 -syntrophin (5). Our attention was drawn to one protein of particular interest: phospholipase C isoform β_2 (PLC β_2), which is a key effector molecule in the canonical G α q/11 signaling pathway (28). After agonist stimulation, GTP-bound G α q/11 binds and activates PLC β_2 , which then cleaves the membrane phospholipids phosphatidyl-inositol-4,5-bisphosphate into signaling molecules IP₃ and diacylglycerol (29). Coimmunoprecipitation (Fig. 4D) and blot overlay (Fig. 4E) experiments reveal that α -catulin directly associates with PLC β_2 , whereas TAP- α -DB1 immunoprecipitation confirmed that PLC β_2 is recruited to the DAPC/ α -catulin signalosome (Fig. 4F). Thus, these data clearly identified α -catulin as a unique member of the α_{1D} -AR/DAPC signalosome, provided a functional purpose for α -catulin in the complex, and revealed a previously unknown and unique mechanism by which PLC β_2 is recruited to GPCRs.

Discussion

Recent studies show that GPCRs are tightly organized in cells through interactions with a highly diverse and selective group of GIPs, which facilitate downstream signaling, effect the pharmacological properties of ligand binding, and terminate receptor activity after prolonged agonist stimulation (3, 4). Our focus is on a particularly complex GPCR, the α_{1D} -AR, which must bind syntrophins to become functional (5, 10). Previously, we discovered that syntrophins link α_{1D} -ARs to the DAPC (10) but had

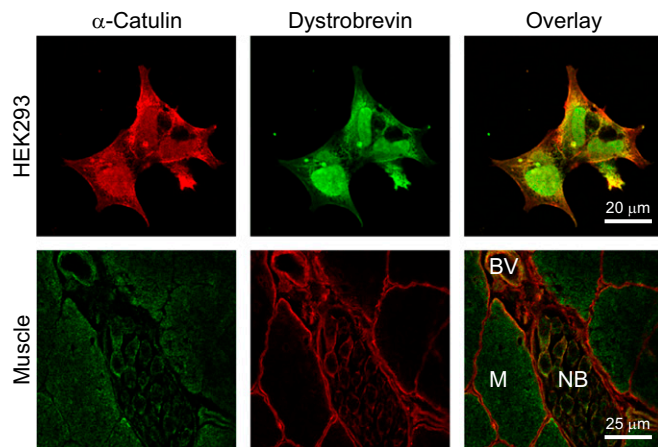


Fig. 3. α -Catulin colocalizes with α -dystrobrevin. HEK293 and mouse quadriceps muscle slices were stained for α -catulin and α -dystrobrevin-1. Blood vessel (BV), nerve bundle (NB), and muscle (M) sections of quadriceps are indicated.

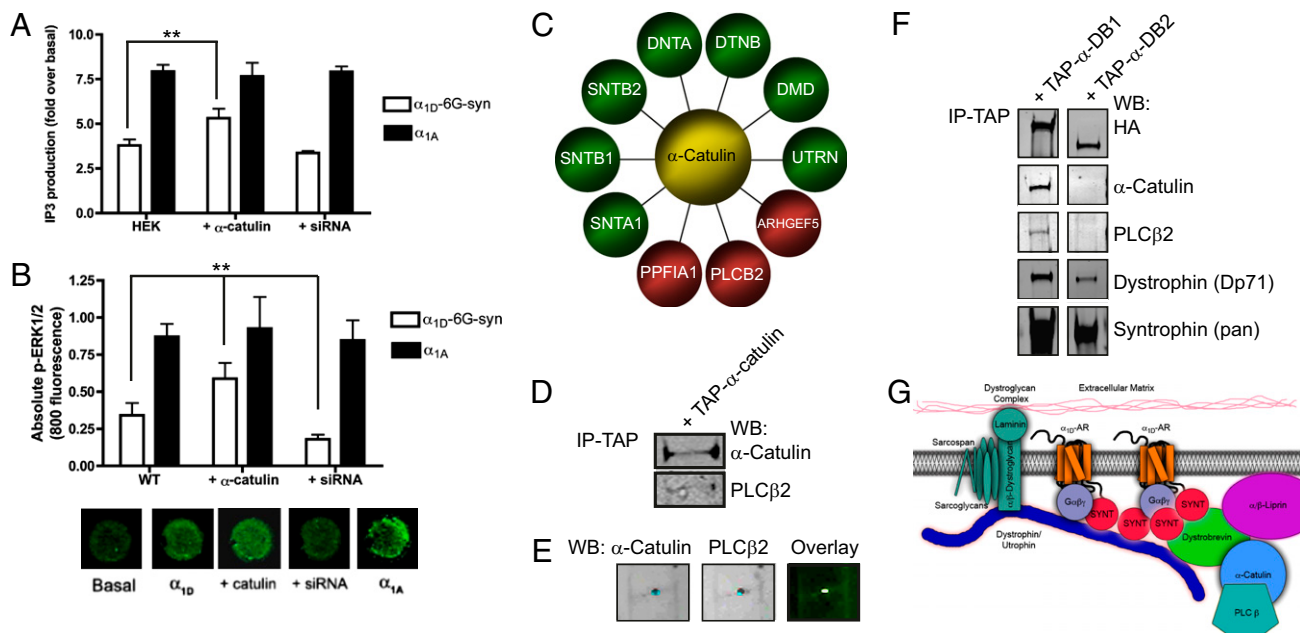


Fig. 4. α-Catulin supersensitizes α_{1D}-AR signaling. Effects of α-catulin knockdown or overexpression on H/F-α_{1D}-AR-6G-syntrophin coupling to IP₃ production (A) and ERK1/2 phosphorylation (B) in HEK293 cells. Representative well staining for phospho-ERK is shown. Statistical analysis was performed using an unpaired *t* test. ****P* < 0.001; ***P* < 0.05. (C) TAP/MS screen of α-catulin interacting proteins purified from HEK293 cells. DAPC proteins are indicated by green circles, and unique proteins are indicated by red circles. (D) TAP-α-catulin coimmunoprecipitated endogenous PLCβ2. (E) Blot overlay of GST-α-catulin indicates that α-catulin binds directly to PLCβ2, although with low affinity. (F) TAP-α-DB1 or TAP-α-DB2 were immunoprecipitated from HEK293 cells, run on SDS/PAGE, and probed for copurification of α-catulin, PLCβ2, and other members of the DAPC. (G) Proposed model of the α_{1D}-AR/DAPC signalosome, including α-catulin, liprins, and PLC-β2.

yet to understand why this interaction is necessary for receptor function. In this study, we identified α-catulin as a unique member of this large GPCR signaling network. Biochemical and proteomic analysis indicates that α-catulin is directly recruited to the signalosome by a splice product of the α-DB gene, α-DB-1, and not the closely related splice variant α-DB2, whereas functional experiments reveal that α-catulin enhances α_{1D}-AR functional coupling by binding and recruiting the Gqα/11 signaling effector PLCβ2 (Fig. 4G).

Why is the DAPC necessary for functional expression of α_{1D}-ARs? One potential explanation is that the DAPC scaffolds α_{1D}-ARs at discrete locations in postsynaptic tissues, and loss of DAPC interactions results in altered α_{1D}-AR spatial orientation in cells and/or loss of functional activity. Indeed, recent studies support the idea that α_{1D}-ARs are expressed at the plasma membrane and intracellularly in native tissues (11, 30), but it remains unclear how the DAPC is regulated to alter α_{1D}-AR localization. One study of particular interest revealed that α_{1D}-ARs are clearly expressed both at the plasma membrane and intracellularly in freshly dissociated aortic smooth muscle cells but become completely intracellular and nonfunctional with culturing (31). Thus, it is likely that unidentified signals are required to maintain sequestration of α_{1D}-ARs at their proper sites *in vivo*. Interestingly, the most prevalent α-catulin interacting protein obtained in our TAP/MS analysis was another scaffolding protein, α-liprin, which is important for clustering a wide variety of signaling molecules in synapses and at the leading edge of focal adhesions, including the tyrosine phosphatase LAR, synaptotagmin, synaptobrevin, and glutamate receptors (32–36). Thus, it may be possible that α-catulin binding of α-liprin clusters α_{1D}-ARs in vascular smooth muscle in close proximity to adjacent sympathetic neurons, where concentrations of norepinephrine are thought to be in high concentration and signals that disrupt liprin function result in loss of α_{1D}-AR clustering and sequestration.

A major unanswered question in the DAPC field involves the functional significance of α-DB splice variants *in vivo* (20). The α-dystrobrevin gene produces five splice variants capable of interacting with the DAPC, and the most well studied of the splice variants, α-DB1 and α-DB2, differ only in the length of their C-terminal domain (α-DB2 is truncated at position 570, resulting in a loss of amino acids 570–766). Both isoforms are ubiquitously expressed and interact with the DAPC along with known binding partners syncoilin, dysbindin, and DAMAGE (20), yet a conclusive study addressing the necessity of α-DB splice variants remained elusive. It has been suggested that the α-DB1 C terminus acts as a regulatory domain that is modulated by tyrosine phosphorylation, or that the C terminus imparts specialized localization patterns in the neuromuscular junction (20). With this discovery of an α-DB1 splice variant-specific binding partner, it will be interesting in future studies to reveal how the α-catulin/DAPC complex regulates neuromuscular junction signaling (13).

Although few studies have examined the functional role of α-catulin, accumulating evidence strongly suggests it acts as a multipurpose scaffolding protein. For example, α-catulin has been shown to directly bind and modulate the enzymatic activity of Lbc-Rho guanine exchange factor (GEF), IκB kinase, and Ras (23–25). More recently, siRNA screens performed in *Caenorhabditis elegans* suggest that α-catulin facilitates proper localization and function of the BK channel SLO-1 (21, 37), and it is likely that α-DB1 recruits α-catulin to the BK channel, considering the high sequence homology in α-DB1 C-terminal domains across species (Fig. S2). A parallel study revealed that α-catulin bridges 5-hydroxytryptamine-2 receptor Gqα/11 coupling to activation of the Lbc-RhoGEF/RhoA/ROCK pathway in pulmonary artery smooth muscle cells (22). Thus, these combined results implicate α-catulin as a unique regulator of membrane receptor function, and herein we provide a detailed molecular mechanism by which

α -catulin links these membrane signaling proteins to the DAPC through α -DB1.

Our data suggest that α -catulin supersensitizes α_{1D} -AR functional responses by recruiting PLC β 2 and/or RhoGEFs. Interestingly, α_1 -AR functional responses become supersensitized in vivo after sympathetic denervation (38, 39). Because the α_{1D} -AR is the only α_1 -AR subtype that interacts with the DAPC/ α -catulin complex (5, 10), the findings of this study may provide a mechanistic explanation to the mystery of GPCR supersensitivity, a physiological process observed in hypertension and benign prostatic hypertrophy (12). Additionally, it is interesting to note that all identified α -catulin binding proteins are modulated by G α q/11 signaling (22, 29). Thus, we propose that α -catulin is a key component of G α q/11 signaling complexes, where it functions to recruit activator and effector proteins in close proximity to each other. Given the ubiquitous expression of α -catulin throughout the body, the large number of GPCRs containing PDZ domains in their C-terminal tails (40, 41), and the broad array of cellular functions controlled by G α q/11 signaling, it is likely that α -catulin plays an important role in numerous GPCR signaling networks.

Materials and Methods

Reagents. Antibodies generated against α -DB1, α -DB2, β -DB, pan-DB, utrophin, and syntrophin were described previously (42–44). Other antibodies were purchased from commercial sources: anti-dystrophin and anti-flag (Sigma), anti-HA (Cell Signaling), and anti- α -catulin (Abnova).

Constructs. pRESpuro-GLUE (TAP vector) was kindly provided by Randal Moon (University of Washington, Seattle, WA). Mouse β_1 -syntrophin was amplified by PCR to add 5' EcoRI and 3' NsiI restriction sites for subcloning into pRESpuro-GLUE. Mouse β_2 -syntrophin was amplified by PCR to add 5' EcoRI and 3' PstI restriction sites for subcloning into pRESpuro-GLUE. The 5' end of mouse α -dystrobrevin (shared by both α -DB1 and -2) was amplified using a primer containing an Asc I site in frame and an internal primer downstream of the endogenous BglII site. The PCR product and the C-terminal-specific regions of each isoform were cloned into pRESpuro-GLUE. Human α -catulin-DDK/myc was purchased from OriGene. Human α -catulin cDNA was amplified by PCR to add 5' BamHI and 3' NotI for subcloning into pGEX-4T-2 (GE Healthcare) to generate GST- α -catulin. Human α -catulin cDNA was amplified by PCR to add 5' NotI and 3' BamHI for subcloning into pRESpuro-GLUE to generate TAP- α -catulin. TAP- α -catulin truncations 1–4 were generated by using QuikChange Mutagenesis (Agilent Technologies) to introduce stop codons at base pairs 831, 1026, 1575, and 1731 of α -catulin. Δ 1–277 TAP- α -catulin was generated by introducing an EcoRV restriction site at amino acid 831, followed by subcloning with EcoRV to remove base pairs 1–831 of α -catulin. TAP- α_{1D} -AR, TAP- α -syntrophin, H/F- α_{1D} -A,R and H/F- α_{1D} -AR-6G- α -syntrophin have been described previously (10, 26).

Cell Culture and Transfection. HEK293 cells were propagated in DMEM containing 10% FBS, 10 mg/mL streptomycin, and 100 U/mL penicillin at 37 °C in 5% CO₂. The constructs were transfected using FuGENE HD transfection reagent (Roche Applied Science) when cells were \approx 80% confluent.

Tandem Affinity Purification/Mass Spectrometry. TAP purification of all constructs has been described previously (10). MS on final eluates was done on

a linear ion trap Fourier transform or linear trap quadrupole at the Fred Hutchinson Cancer Research Center (Seattle, WA).

Immunoprecipitations and Western Blotting. For TAP- α -DB1/2, TAP- α -catulin, and flag (DDK), immunoprecipitations were performed as previously described (10).

Blot Overlays. HEK293, TAP- α -DB1-expressing HEK293, and TAP- α -DB2-expressing HEK293 lysate was run on an 8% SDS/PAGE gel (Thermo Fisher Scientific) per the manufacturer's instructions. Gels were transferred to nitrocellulose by using a wet transfer system per the manufacturer's instructions. Membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature, followed by two brief washes in overlay buffer [150 mM NaCl, 2 mM MgCl₂, 20 mM Hepes (pH 7.5), 3% BSA, and 1 mM DTT]. GST- α -catulin was diluted to 200 nM in overlay buffer and incubated with membranes overnight at 4 °C with rocking. GST- α -catulin was removed and membranes were washed 2 \times 10 min in overlay buffer, followed by two brief washes in TBST. Western blotting procedures described above were then used to detect proteins.

Immunohistochemistry. HEK293 cells were fixed in ice-cold 4% paraformaldehyde in PBS for 10 min at 25 °C. Cells were permeabilized in PBS containing 1% Triton X-100 for 10 min. Blocking, antibody incubation, and wash steps were done in cold PBS containing 1% BSA and 1% fish gelatin. Antibodies were incubated at 25 °C for 1 h and washed three times for 10 min. Invitrogen Alexa Fluor secondary antibodies were incubated overnight at 4 °C. Nuclear stain DRAQ5 (Axxora Platform) or actin stain phalloidin 635 (Invitrogen) were added for 20 min at 25 °C, and coverslips were then mounted on glass slides with Invitrogen ProLong Gold. Cells were imaged on a Zeiss LSM 510 META confocal microscope using a 100 \times oil immersion lens. Staining of wild-type mouse quadriceps was performed as previously described (42). Data analysis was performed with ImageJ software.

IP3 Assay. To knock down α -catulin expression, HEK293 cells were treated with 20 nM siRNA against α -catulin (Invitrogen Stealth RNAi siRNA). Twenty-four hours later both siRNA-treated and untreated cells were transfected with constructs as indicated. Twenty-four hours later siRNA cells were retreated with 20 nM siRNA. Cells were then seeded into 24-well plates with 2 mCi/mL ³H-myo-inositol (American Radiolabeled Chemicals). Twenty-four hours later cells were stimulated with 100 μ M phenylephrine, and total inositol phosphates were purified as described previously (45). Total inositol phosphates purified were normalized to incorporated counts and analyzed in Prism software (GraphPad Software).

Radioligand Binding and ERK1/2 Assays. Radioligand binding assays and ERK1/2 phosphorylation assays have been previously described (10, 45).

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