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STATE-OF-THE-ART REVIEW

# Use of Proximity Labeling in Cardiovascular Research

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## HIGHLIGHTS

- **Protein interaction networks are of critical importance in regulating cardiac physiology, including adrenergic signaling.**
- **The proximity labeling approach is based on the enzymatic-dependent conjugation of biotin to endogenous proteins in intact cells, enabling their selective enrichment and identification.**
- **Proximity labeling and mass spectrometry have been used to identify novel protein-protein interactions in cardiac and vascular cells.**
- **The use of proximity proteomics is highlighted in the discovery of the mechanism underlying adrenergic regulation of $Ca^{2+}$ influx in the heart.**

## SUMMARY

Protein-protein interactions are of paramount importance in regulating normal cardiac physiology. Methodologies to elucidate these interactions in vivo have been limited. Recently, proximity-dependent biotinylation, with the use of BioID, TurboID, and ascorbate peroxidase, has been developed to uncover cellular neighborhoods and novel protein-protein interactions. These cutting-edge techniques have enabled the identification of subcellular localizations of specific proteins and the neighbors or interacting proteins within these subcellular regions. In contrast to classic methods such as affinity purification and subcellular fractionation, these techniques add covalently bound tags in living cells, such that spatial relationships and interaction networks are not disrupted. Recently, these methodologies have been used to identify novel protein-protein interactions relevant to the cardiovascular system. In this review, we discuss the development and current use of proximity biotin-labeling for cardiovascular research.
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Proximity proteomic methodologies have been developed to enzymatically label nearby proteins while still maintaining information about the proteins' location and interactions with other proteins within a cell (1–6). With the rapid advancement of proximity labeling technologies comes the ability to collect substantial data and answer longstanding scientific questions that would otherwise have been impossible. In this review, we discuss using these techniques for cardiovascular research, highlighting the use of proximity labeling and quantitative mass spectrometry to identify the

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mechanisms underlying adrenergic regulation of $Ca^{2+}$ current in the heart (7).

## DEVELOPMENT OF PROXIMITY-DEPENDENT BIOTINYLATION METHODOLOGY

With the advent of transcriptomics and the development of extensive lists of candidate genes, identifying critical protein interactors has remained a serious bottleneck in advancing our understanding of physiology and pathophysiology. To elucidate the components of macromolecular complexes, biochemical fractionation followed by mass spectrometry can be performed (8–10). Another approach uses affinity purification of a protein, typically by antibody, followed by mass spectrometry (11). These methodologies, however, have important limitations (12,13). Some proteins, especially membrane-bound proteins, are relatively inaccessible and difficult to solubilize. Coimmunoprecipitations require high-quality antibodies that are often not available. Important interactions may have fast-off kinetics and therefore will not persist after solubilization and during affinity purification (14). Also true of yeast-hybrid approaches, the biological process of interest may only operate in the proper milieu, as part of a larger more extensive protein interaction network that only exists in terminally differentiated mammalian cells. In addition, tissue-specific post-translational modifications can reduce the effectiveness of these methods.

Cognizant of these limitations, Roux et al. (15) adapted an *Escherichia coli* biotin ligase, BirA, to perform in situ labeling in eukaryotic cells. This system has several advantages. Posttranslational modification with biotin, that is, vitamin $B_7$, was chosen in part for its low toxicity and frequent use, alongside its ultra-high-affinity binding partner streptavidin, in molecular biology and biochemistry. There is also a relative paucity of biotin in eukaryotic cells: generally, there is only a single biotin ligase per species and these are known for their exquisite specificity, targeting only a single lysine residue in up to 5 different protein targets depending on the species (16). Roux et al. (15) fused different variants of BirA to lamin A, a nuclear envelope intermediate filament protein, mutation of which can cause a number of diseases, including dilated cardiomyopathy (17). In HeLa and human embryonic kidney (HEK) 293 cells, BirA R118G, also known as BirA* and named BioID for "biotin identification," was especially promiscuous. This variant was capable of a marked increase in nonspecific but proximity-based biotin ligation with the addition of excess biotin to the culture media (**Figure 1A**) (15). Combined with streptavidin-based affinity purification of biotinylated proteins and liquid chromatography/mass spectrometry, numerous known and novel interactors of lamin A were identified (**Central Illustration**). Depending on the experimental conditions and labeling time, the proximity-labeling radius may be as low as a few nanometers (4). For these reasons, BioID has been used in diverse model systems, and within 4 years of its introduction it was coupled with injection of adeno-associated virus to characterize a tissue-specific subcellular proteome in mice in vivo: postsynapse cortical inhibitory neurons in newborn mice (18).

The next generation of biotin ligases overcame at least some of the limitations of BioID, especially its slower kinetics, which requires longer labeling times. Branon et al. (4) used error-prone polymerase chain reaction and 5 generations of yeast display-based directed evolution to produce a more promiscuous and faster BirA, TurboID, capable of markedly greater biotin labeling in heterologous expression and in live *Caenorhabditis elegans* and *Drosophila melanogaster*. An alternate fifth-generation BirA contained an N-terminal truncation with slightly less activity than the full-length form but also decreased labeling in the absence of biotin supplementation (referred to as miniTurbo). This has important implications in the development of transgenic animals. For example, the fraction of fruit flies expressing miniTurbo surviving to adulthood did not differ from control; however, fewer than one-half of TurboID-expressing flies survived to adulthood, an effect that was completely eliminated with dietary supplementation of biotin.

One important limitation of BioID proximity labeling is the time required to detectably biotinylate neighboring proteins—hours to days of biotin supplementation. This limitation has been somewhat reduced with the advent of TurboID, although this engineered biotin ligase still requires a minimum of several minutes to biotinylate protein neighbors (4).

The introduction of soybean ascorbate peroxidase (APEX) and its engineered derivatives represented a major advance in the field with the generation of significant enzyme-catalyzed proximity labeling within seconds, allowing the capture of dynamic signaling events at high resolution with subcellular localization (1,2). APEX2 was engineered with yeast display-based evolution to improve its catalytic efficiency, increasing sensitivity in cells with lower transgene expression. In the presence of biotin-

### ABBREVIATIONS AND ACRONYMS

**APEX** = ascorbate peroxidase

**BioID** = biotin identification

**ER** = endoplasmic reticulum

**HEK** = human embryonic kidney

**HRP** = horseradish peroxidase

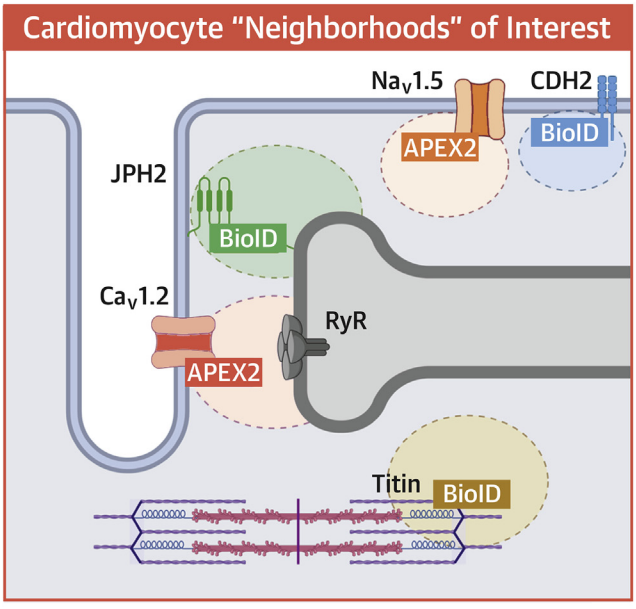
**JAM-C** = junctional adhesion molecule C

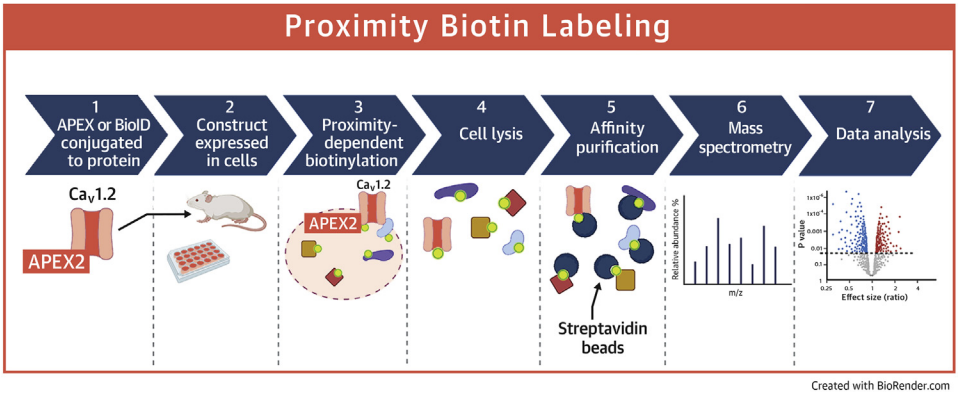
**Jph2** = junctophilin 2

**PKA** = protein kinase A

**TMT SPS $MS^3$** = tandem mass tag triple-stage mass spectrometry

**CENTRAL ILLUSTRATION** **Proximity Labeling Approaches Used in Cardiovascular Research**

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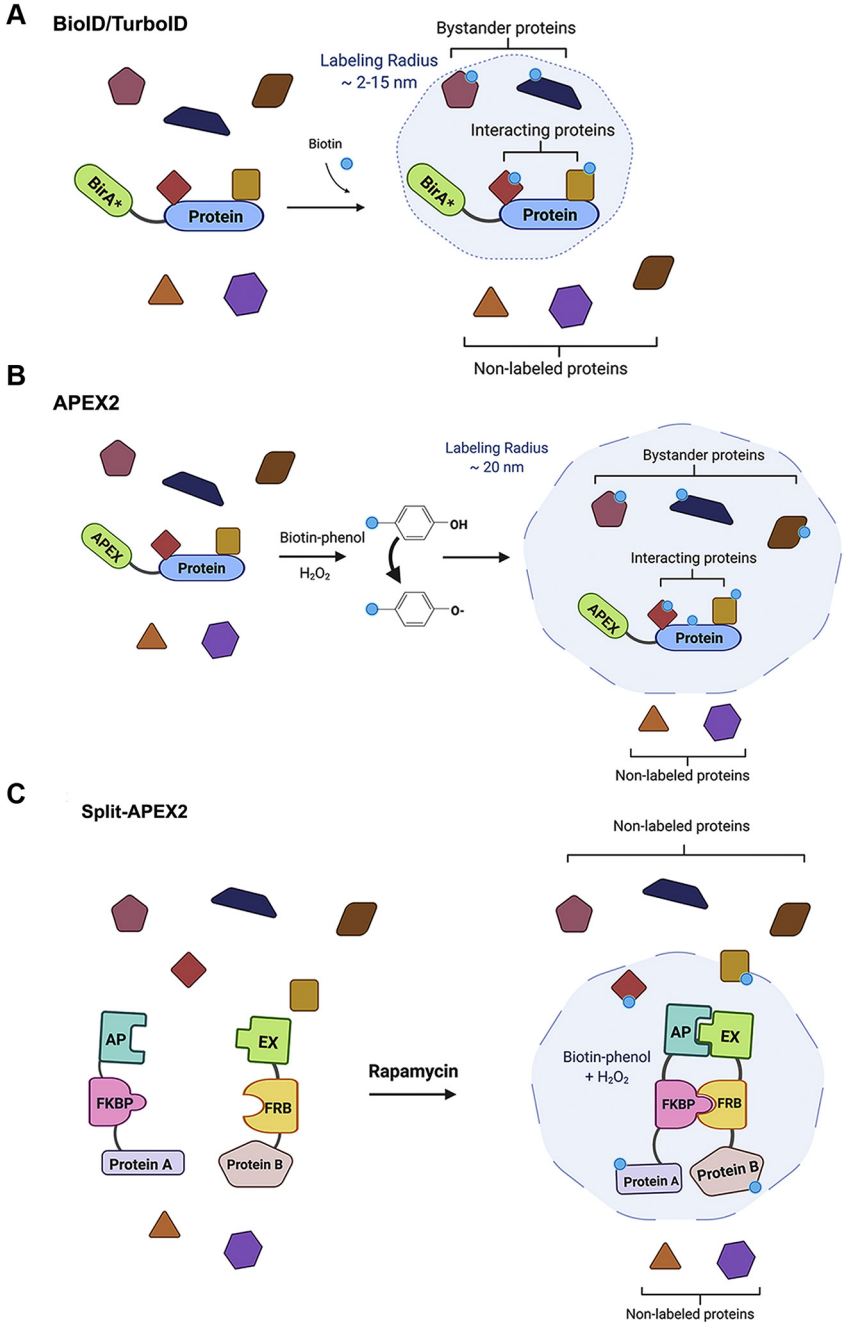
Proximity labeling and quantitative mass spectrometry have been used in cardiomyocytes to elucidate the interactome and neighborhood of proteins such as voltage-gated $Ca^{2+}$ and $Na^{+}$ channels ($Ca_V1.2$ and $Na_V1.5$, respectively), junctophilin (JPH2), N-Cadherin (CDH2), and titin. They have also been used to elucidate the interactome of junctional adhesion molecule C (JAM-C) and $K^{+}$ channels (Kir2.1) in cultured cells (not pictured). A workflow schematic for proximity labeling experiments, with $\alpha_{1C}$-APEX as an example, follows.

tyramide (biotin-phenol) and hydrogen peroxide, these peroxidases form biotin-phenoxyl radicals that form covalent linkages to electron-rich residues (primarily tyrosines; **Figure 1B**) (1). The short half-life of these radicals (<1 ms) and their rate of diffusion ensures that labeling occurs only within ~20 nm of APEX (19). Addition of an antioxidant cocktail quenches the reaction. The membrane impermeability of these radicals ensures that proximity labeling is limited to membrane-contained or membrane-excluded spaces.

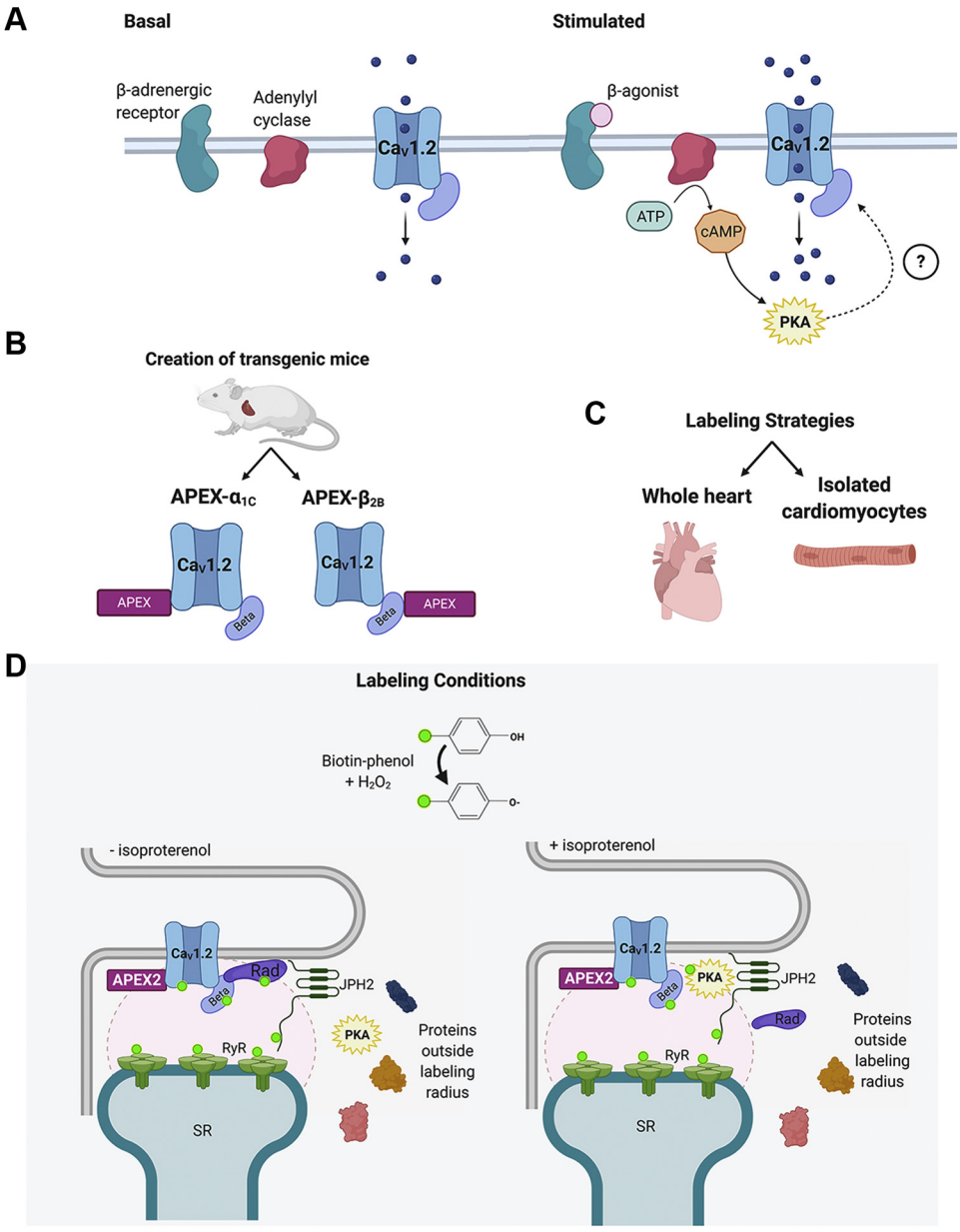
There are several limitations, namely that the labeling by APEX2 requires hydrogen peroxide, which can induce brief oxidative stress, and that the high labeling activity of APEX2 may preclude the precise spatial resolution required to identify the local interaction network of a target protein (20). For example, clouds of APEX2-generated biotin-phenoxyl

**FIGURE 1** **Proximity Proteomics Labeling Methods Used in Cardiovascular Studies**




**(A)** Biotin labeling of sterically available basic residues by the *Escherichia coli* biotin ligase BirA, or engineered derivatives (BirA* [BioID], BioID2, TurboID, and miniTurbo). BirA* is conjugated to the protein of interest. With biotin supplementation, biotin is covalently linked to both interacting and neighboring proteins. **(B)** Soybean ascorbate peroxide (APEX) or its derivative (APEX2) is conjugated to the protein of interest, which catalyzes the generation of biotin phenoxyl radicals that form covalent linkages with sterically available electron-rich residues. Proteins within the "cloud" of biotin phenoxyl radicals are labeled with biotin, whereas those outside of the radius are not labeled. **(C)** Complementation of N- and C-terminal APEX2 fragments, "AP" and "EX," respectively, induced by rapamycin-mediated interaction between FK506 binding protein (FKBP) and its binding partner, the FKBP-rapamycin binding (FRB) domain of the kinase mammalian target of rapamycin. Biotin labeling of neighboring proteins is enabled in cellular locations where both protein A and protein B interact. Figure created with BioRender.com.

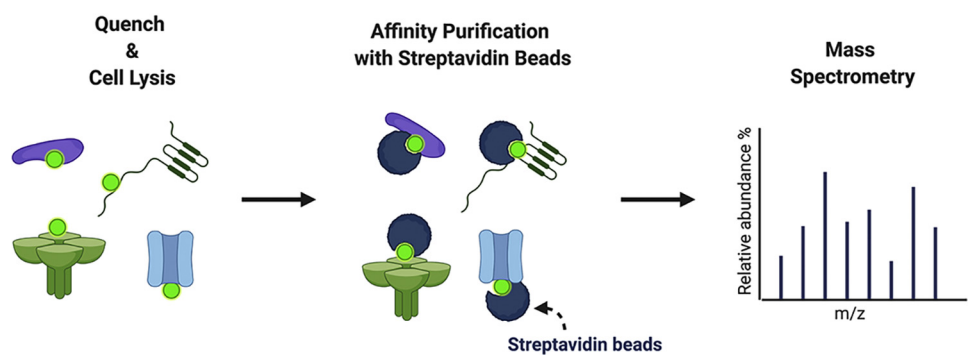
**FIGURE 2 Proximity Labeling Methodology to Define Mechanisms of Adrenergic Modulation of $Ca^{2+}$ Channels**




**(A)** Schematic depicting adrenergic regulation of $Ca_V1.2$ in heart. A β-agonist binds to the β-adrenergic receptor, thereby activating protein kinase A (PKA). Before discovering the elusive protein via proximity proteomics, the target of PKA was unknown. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate. **(B)** Generation of transgenic mice with ascorbate peroxide (APEX2) fused to the N-terminus of either the $\alpha_{1C}$ or the $\beta_{2B}$ subunits. **(C)** Proximity labeling can be completed in isolated myocytes or in intact whole heart ex vivo through retrograde perfusion of biotin-phenol and $H_2O_2$. **(D)** Schematic shows the localization of APEX2-conjugated $Ca_V1.2$ channels in the dyadic space of cardiomyocytes. After β-adrenergic receptor activation, PKA phosphorylation of Rad depletes Rad from the $Ca_V1.2$ complex, making it less accessible to APEX2-mediated biotinylation. Figure created with BioRender.com.

radicals may surround APEX2 fusion proteins in various subcellular compartments, not limited to the ultimate trafficking destination of the protein of interest, tagging bystander proteins along the way (e.g., ribosome and rough endoplasmic reticulum [ER], smooth ER, Golgi network, and so on). Proteins in these compartments may be important for folding, trafficking, and/or degradation, but deconvolving

**FIGURE 3** Post-Biotinylation Processing and Analysis




After ascorbate peroxide–mediated biotinylation, the biotinylation is quenched and cells are lysed. Biotinylated proteins are affinity purified with the use of streptavidin magnetic beads and washed with denaturing conditions. Peptides are identified by means of mass spectrometry. Figure created with Bio.Render.com.

these neighborhoods is challenging. To address these limitations, quantitative proteomics were combined with either spatial references (20) or isobaric tandem mass tags (21) to enable tracking the activation, internalization, and subcellular protein complexes of G-protein–coupled receptors when expressed in HEK cells.

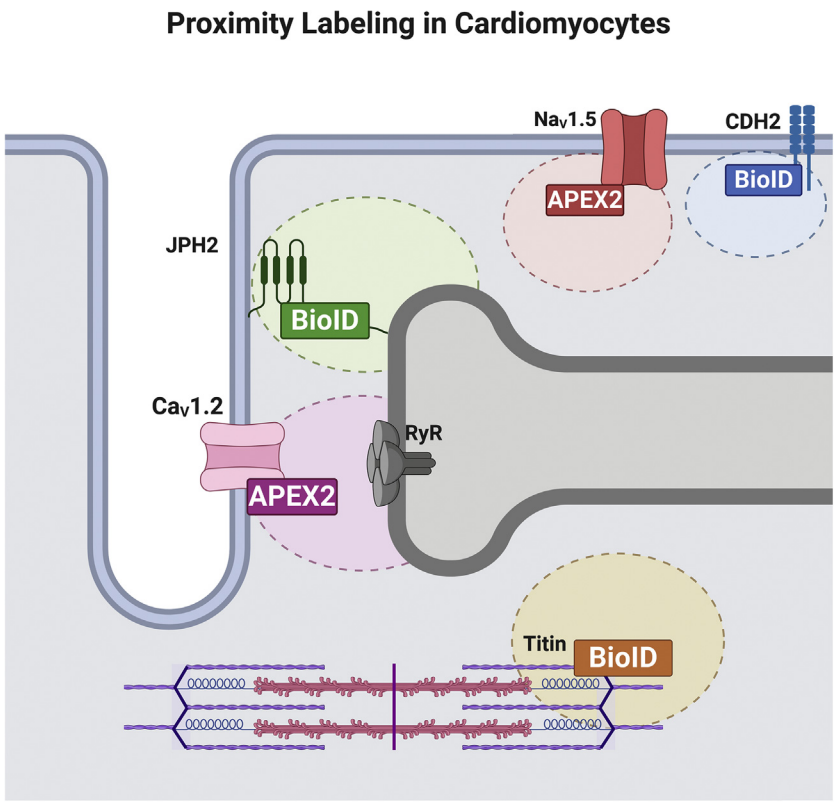
## USE OF APEX-BASED PROXIMITY PROTEOMICS IN THE HEART

$Ca^{2+}$ channels, which are the major pathway for entry of $Ca^{2+}$ ions into excitable cells, transduce membrane depolarization into multiple cellular functions (22). In the heart, the influx of $Ca^{2+}$ is essential for activation of excitation-contraction coupling and modulating pacemaker activity in sinoatrial nodal cells. In adult ventricular cardiomyocytes, the predominant type of $Ca^{2+}$ channel, $Ca_V1.2$, localizes to transverse tubules in close proximity to ryanodine receptors at dyadic junctions (23). Despite considerable research, the molecular mechanisms responsible for adrenergic stimulation of $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels have remained stubbornly obscure for decades (24–30). For many years, the mechanism was thought to involve protein kinase A (PKA) phosphorylation of residues on $Ca_V1.2$ $\alpha_{1C}$- and/or $\beta_{2B}$-subunits (31–37). More recent studies in mice demonstrated, however, that adrenergic stimulation of $Ca_V1.2$ did not involve direct phosphorylation of these residues on $\alpha_{1C}$ or $\beta_2$ subunits (**Figure 2A**) (7,38–44).

There are many hundreds of proteins localized to the dyadic space, many of which could transiently interact with the $Ca^{2+}$ channel and serve as the functional PKA target (**Figure 2A**). Based on the observations that β-adrenergic regulation of $Ca_V1.2$ cannot be reconstituted when the core subunits are heterologously coexpressed (45), that PKA phosphorylation of the $\alpha_{1C}$- and/or $\beta_{2B}$ subunit is not sufficient (43), and that $Ca_V1.2$ is the epicenter of a large macromolecular complex, we aimed to identify proteomic neighbors of $Ca_V1.2$ in the heart that may be the true PKA target. The specialized ultrastructure of the cardiac dyad is spanned and maintained by junctophilin-2 (Jph2), which brings the sarcoplasmic reticulum and its $Ca^{2+}$ release channels within 12–18 nm of the transverse tubules and its membrane-bound $Ca_V1.2$, permitting excitation-contraction coupling with every single heart beat (46–49). It is therefore a membrane-enclosed space well suited to proximity-based proteomic characterization with the use of proximity labeling.

Transgenic mice with doxycycline-inducible cardiomyocyte-specific expression of dihydropyridine-resistant $\alpha_{1C}$ or $\beta_{2B}$ proteins with APEX2 and a V5 epitope conjugated to the N-terminals were generated (**Figure 2B**). This inducible transgenic methodology (50,51) was perfectly suited for these experiments as we could both temporally and spatially limit expression of modified channels in cardiomyocytes (38,52,53). The subcellular

**FIGURE 4 Proximity Proteomics Performed in Cardiomyocytes**




Schematic of dyadic space of cardiomyocyte, showing position of T-tubules, sarcoplasmic reticulum, and actin-myosin filaments and titin. Proximity-labeled studies have been performed with the use of ascorbate peroxide derivative (APEX2)-conjugated $Ca_V1.2$ channels, BioID-conjugated junctophilin (Jph2), N-cadherin (CDH2), and titin. RyR = ryanodine receptor. Figure created with Bio.Render.com.

localization and function of $Ca_V1.2$ in cardiomyocytes was not affected by fusing APEX2 to $\alpha_{1C}$ and $\beta_{2B}$ (7). Importantly, the $Ca^{2+}$ currents imparted by these APEX-conjugated $Ca^{2+}$ channels were stimulated by β-adrenergic agonists, implying that conjugating APEX2 to the channels did not perturb PKA-mediated regulation of this process. Incubating isolated ventricular cardiomyocytes with biotin-phenol for 30 minutes followed by exposure to hydrogen peroxide for 1 minute induced biotinylation of proteins in a striated Z-disk pattern, consistent with proper localization of $Ca^{2+}$ channels within the T-tubules of cardiomyocytes (**Figures 2C and 2D**). Affinity purification of the biotinylated proteins was then performed with the use of streptavidin-sepharose (**Figure 3**). The high affinity of biotin and avidin enables washing of the avidin-sepharose under denaturing conditions (3). Via mass spectrometry, we identified nearly all previously reported members of the cardiac $Ca_V1.2$ complex, as well as many proteins known to be in close proximity to $Ca_V1.2$ (7). A detailed description of sample preparation has been thoroughly reviewed elsewhere and is beyond the scope of this review (2,3,54).

We found that proximity labeling of the $Ca^{2+}$ channel neighborhood can also be performed in beating hearts, capturing the full effect of extracellular matrix, myocyte-myocyte, and myocyte-nonmyocyte interactions, the importance of which has become increasingly recognized over time (55,56). For these experiments, Langendorff-perfused hearts were retrograde perfused with Krebs solution for 10 min before the addition of biotin-phenol for 15 min (**Figure 2C**). Electrocardiograms were monitored throughout the infusion protocol to ensure viability of the preparation. Streptavidin-fluorescein isothiocyanate immunofluorescence of tissue sections of Langendorff-perfused hearts also showed biotinylation of proteins in a striated Z-disk pattern (7).

In our APEX2-$Ca_V1.2$ experiment, we also labeled nuclear, mitochondrial, ribosomal, ER, and Golgi proteins (7). This is because $\alpha_{1C}$ and $\beta_{2B}$ APEX fusion proteins not only exist in the transverse tubule, but rather are constantly being created at the ribosomes and trafficked there from the rough ER. Wherever there are transgenic channels in the cell, nearby proteins are biotinylated. Moreover, it is challenging to differentiate direct physical interactors from bystanders even within the subcellular area of interest. Suspecting that the transducer of adrenergic regulation of $Ca^{2+}$ channels would alter its interaction with $Ca_V1.2$ after exposure to β-adrenergic agonist, whereas most bystander proteins would not, we compared the $Ca_V1.2$ neighborhood in the absence and presence of isoproterenol in isolated cardiomyocytes and Langendorff-perfused beating hearts, similarly to studies using heterologously expressed G-protein–coupled receptors (20,21).

We preincubated isolated cardiomyocytes from $\alpha_{1C}$-APEX and $\beta_{2B}$-APEX mice with biotin-phenol for 30 min, and during the final 10 min the cells were also exposed to either isoproterenol or vehicle (**Figure 2D**). The biotinylated proteins were purified and quantified with the use of tandem mass tag triple-stage mass spectrometry (TMT SPS $MS^3$) (**Figure 3**). We also probed the effect of isoproterenol using Langendorff-perfused whole hearts. We were careful only to include in our analysis hearts with demonstrable adrenergic activation, assessed with the use of electrocardiography and phosphorylation of phospholamban. The protein quantification changed for several proteins: We detected an isoproterenol-induced recruitment of the PKA catalytic subunit to $Ca_V1.2$ and a 30% to 50% decrease in the amount of the small Ras-like G protein Rad in the neighborhood of $Ca_V1.2$. Ten-minute exposure of isolated non-transgenic control cardiomyocytes to isoproterenol had a minimal effect on proteins quantified with TMT SPS $MS^3$ and specifically no effect on Rad compared with untreated cardiomyocytes. These experiments indicate that β-adrenergic stimulation depletes Rad from the neighborhood of $Ca^{2+}$ channels, but not from the cell as a whole.

Rad is a member of the Rad, Gem, Kir family of GTP-binding proteins that are well known for their capacity to potently inhibit all high-voltage-activated $Ca^{2+}$ channels (57–63). Applying the adenylate cyclase activator forskolin to HEK293T cells expressing Rad and wild-type $\alpha_{1C}$ and $\beta_{2B}$ increased the maximal conductance and shifted the $V_{50}$ for activation of barium currents, consistent with PKA activation of $Ca_V1.2$. We found that β-adrenergic regulation of $Ca_V1.2$ requires Rad binding to the $Ca_V\beta$ subunit under basal conditions and release of Rad binding to β after adrenergic stimulation. Taken together, Rad potently dampens $Ca_V1.2$ current while phosphorylation of Rad allows channels to operate as though they were devoid of Rad, similar to the way phospholamban regulates sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (64). This study demonstrated the utility of proximity labeling in vivo, especial for defining physiological mechanisms that could not be reconstituted heterologously.

Recently, we generated transgenic mice with doxycycline-inducible cardiomyocyte-specific expression of tetrodotoxin-sensitive $Na_V1.5$ proteins with APEX2 and a V5 epitope conjugated to the N-terminus (**Figure 4**) (65). The $Na_V1.5$ subcellular localization and function, as assessed by cellular electrophysiology and anti-V5 antibody immunofluorescence were not affected by fusing APEX2 to the $Na^+$ channel. We incubated isolated ventricular cardiomyocytes with biotin-phenol and then hydrogen peroxide, which induced robust biotinylation of proteins at the sarcolemma, intercalated disk, and in a striated Z-disk pattern, consistent with the pattern of localization of the $Na_V1.5$ channels. Immunoblots of streptavidin-purified biotinylated proteins showed both calmodulin and fibroblast growth factor (FGF) 13, which was consistent with prior studies showing that calmodulin and FGF13 interact with $Na_V1.5$ (66–69).

## PROXIMITY-DEPENDENT BIOTINYLATION USING BioID IN THE HEART

The dyadic junction proteome was further characterized with in vivo proximity labeling of the Jph2 subdomain proteome (**Figure 4**) (70). Targeted knock-in of engineered BioID (BioID2) fused to Jph2 had no adverse effects on Jph2 expression and function. Intraperitoneal biotin injection once daily for a week at a dose of 24 mg/kg body weight enabled proximity labeling within the dyad. Using label-free mass spectrometry, 550 proteins were identified in the avidin-purified samples from the homozygous knock-in mice. The top cellular components, based on gene ontology enrichment analysis, were sarcolemma, cation channel complexes, and transverse tubules. The authors also identified potentially novel cardiac dyad proteins that may play roles in cardiac dyad formation, maintenance, and function (70).

In another study, BioID was coupled to the structural protein titin (**Figure 4**) (71). Rudolph et al. (71) generated knock-in mice with BioID spliced into *titin* exon 28, corresponding to the location of the Z disk-I

band transition. Mice were continuously supplied with supplemental biotin dissolved in their water. The authors compared this subdomain of the titin proteome, described as the sarcomeric proteome, in quadriceps and heart, finding 100 proteins in common (81% of labeled proteins in the heart and 41% in skeletal muscle). They reported that 5% of isolated protein was biotin labeled and, using titin's immunoglobulin-like domains as reference points, calculated that the BioID labeling radius was 7–15 nm. As in the Jph2-BioID knock-in, no untoward phenotypic or functional effects of the knock-in were noted.

In another study, BioID was fused to N-cadherin and was expressed in neonatal murine cardiomyocytes using an adenovirus (**Figure 4**) (72). The N-cadherin–BioID fusion localized to the cell-cell contacts, as would be expected, and after biotin was added to the cell culture for 24 hours, proteins along the cell-cell contacts and Z disks were labeled. Mass spectrometry identified 917 proteins, which after setting a threshold fold change of ≥10 and $p < 0.001$ was reduced to a final list of 365 proteins, comprising primarily adaptor and adhesion proteins that promote junction specialization (72).

The BioID methodology has also been applied to elucidating the interactome of heterologously expressed wild-type Kir2.1 and a trafficking-deficient type 1 Andersen-Tawil syndrome mutant Kir2.1$^{\delta 314-315}$ channel in HEK cells (73). Kir2.1 is an inward rectifying $K^+$ current, playing important roles in setting the membrane potential and controlling excitation and action potential duration of ventricular cardiomyocytes (74). Mass spectrometry identification of avidin-purified biotinylated proteins revealed a total of 218 high-confidence Kir2.1 neighbors, including 75 proteins that were preferentially proximate with wild-type channels, 66 proteins that were preferentially proximate with the mutant Kir2.1 channels, and 77 proteins that were proximate with both wild-type and mutant Kir2.1 channels (73). Based on these BioID-identified interaction studies (73), the authors concluded that the pathogenesis associated with the Kir2.1$^{\Delta 314-315}$ mutation involves malfunctions in the mechanisms governing Kir2.1 trafficking from the Golgi to the cytoplasmic membrane, in agreement with prior studies (75–77). Several desmosome-associated proteins, including plakophilin-4 (PKP4) were found to interact with both wild-type and Kir2.1$^{\Delta 314-315}$ channels in HEK cells. Kir2.1 and PKP4 colocalize at intercalated disks and Z disks in cardiomyocytes, and PKP4 is a positive regulator of Kir2.1 current density when coexpressed in HEK cells (73). Taking these findings together, this study suggested that proximity labeling may reveal how and why specific mutations affect the trafficking and function of ion channels.

## COMPARISON OF BioID AND APEX LABELING METHODS

Whereas we identified more than 3,800 proteins and multiple peptides for over 3,000 proteins using APEX2-conjugated $Ca^{2+}$ channels (7), Feng et al. (70) identified 550 proteins with at least 2 unique peptides. These differences speak to the promiscuity of APEX-catalyzed biotin-phenoxyl radicals diffusing out to sterically available or "exposed" tyrosines, relative to the BioID approach where the proximity-labeling enzyme itself must directly tag nearby basic residues, such as lysine. The labeling substrate in APEX experiments is biotin-tyramide, which can diffuse easily across membranes, although the radicals are membrane impermeable. The available substrate for BioID labeling depends on the vehicle, route of administration, and pharmacokinetics in the model system. Biotin is a carboxylic acid and is less membrane permeable than biotin-tyramide. Our experimental approach made use of mice with doxycycline-inducible cardiac-specific overexpression of the labeling enzyme (7). Feng et al. (70) instead used a knock-in approach with the native gene dosage of their gene of interest. As noted before, APEX experiments permit the characterization of rapid subcellular changes during signaling events and it is feasible to label in a Langendorff preparation ex vivo, whereas BioID can take several days to fully label the proteome subdomain. On the other hand, the BioID approach should not alter reactive oxygen species signaling that may occur in APEX-labeled cells, although any changes related to exposure to hydrogen peroxide in APEX labeling would likely be minimal because they would have to occur during the 1-minute labeling interval before quenching of the labeling reaction.

## USE OF HORSERADISH PEROXIDASE–BASED PROXIMITY LABELING

Horseradish peroxidase (HRP) is inactive in a reducing environment such as the cytosol of a cell (78). HRP is active in the oxidizing secretory pathway (Golgi and ER lumen) and extracellular surface, and catalyzes the same labeling chemistry as APEX2 with biotin-phenol (19,79). HRP was fused to the extracellular region of the junctional adhesion molecule C (JAM-C), designed to define the receptors localized with JAM-C at the cell junction and to determine which ones co-traffic with JAM-C in endothelial cells (80). Transfected human umbilical vein endothelial

cells were exposed to biotin-phenol for 30 min, and proteins neighboring JAM-C were biotinylated during a 1-minute incubation with hydrogen peroxide. To specifically identify neighboring proteins within intracellular stores of JAM-C, ascorbate, a membrane-impermeant inhibitor of HRP, was used to block the proximity-labeling reaction at the extracellular side of the membrane. In addition, APEX2 was conjugated to the intracellular domain of wild-type JAM-C or a mutant JAM-C with 4 arginine substitutions for ubiquitinated lysine residues. Using these multiple proximity-labeling approaches, the authors concluded that JAM-C cotraffics with receptors associated with changes in permeability, such as vascular endothelial cadherin, neuropilin (NRP) 1, NRP-2, and some integrin subunits, but not with junctional proteins associated with transmigration of leukocytes, such as the lateral border recycling compartment components (80). This study demonstrated the full utilization of proximity labeling to explore both extracellular and intracellular neighborhoods of a protein of interest as well as the trafficking of the protein.

## NEXT-GENERATION PROXIMITY LABELING

Recent innovations in proximity labeling, primarily complementation, can help to address the issue of "false positives." Han et al. identified 24 potential "cut sites" in the wild-type APEX (5). These 24 pairs were conjugated with FK506-binding protein (FKBP) and FKBP-rapamycin-binding domain to permit rapamycin-induced complementation. Ultimately, the 200 amino acid N-terminal fragment pairs and 50 amino acid C-terminal pairs were found to have the most favorable characteristics and were referred to as "AP" and "EX," respectively (**Figure 1C**). These underwent multiple rounds of positive and negative selection with yeast display-based directed evolution to increase the strength of labeling and to ensure that labeling occurs only with complementation. Han et al. (5), with the use of rapamycin induction, were able to label the proteome of the mitochondrial-ER interface, and through fusing AP and EX to ribonucleoproteins specific to a particular noncoding RNA, label the proteome of this particular RNA. Although several split BioIDs have been developed, a faster split TurboID, developed and validated in the same manner as split APEX, is now available, allowing for rapid in vivo proximity labeling with high spatial specificity (81).

## CONCLUSIONS

Proximity proteomics, an avenue of investigation developed only in the past 10 years, holds great promise for advancing our understanding of cardiovascular biology. As these methods continue to mature, several concepts should be kept in mind to ensure high-quality work and worthwhile investment in time and resources. A worthwhile question might be, "What will we do with these results?" Situations well suited to these methods may involve suspected though missing interacting elements that have avoided detection, and after labeling identification can be evaluated in some manner. We found that having a functional or biochemical readout to confirm the effect of our perturbation (β-adrenergic stimulation)—heart rate elevation and phospholamban phosphorylation in our case—allowed us to cull undertreated samples and increase the sensitivity of the method to detect changes in signaling. Examining the pathophysiological alterations of the proteome in vivo lends itself particularly well to biotin ligase labeling, whereas more acute changes, such as signal transduction, may favor APEX labeling.

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## REFERENCES

**1.** Rhee HW, Zou P, Udeshi ND, et al. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 2013;339:1328–31.

**2.** Lam SS, Martell JD, Kamer KJ, et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat Methods 2015;12:51–4.

**3.** Hung V, Udeshi ND, Lam SS, et al. Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. Nat Protoc 2016;11:456–75.

**4.** Branon TC, Bosch JA, Sanchez AD, et al. Efficient proximity labeling in living cells and organisms with TurboID. Nat Biotechnol 2018;36:880–7.

**5.** Han Y, Branon TC, Martell JD, et al. Directed evolution of split APEX2 peroxidase. ACS Chem Biol 2019;14:619–35.

**6.** Cho KF, Branon TC, Udeshi ND, Myers SA, Carr SA, Ting AY. Proximity labeling in mammalian

cells with TurboID and split-TurboID. Nat Protoc 2020;15:3971–99.

**7.** Liu G, Papa A, Katchman AN, et al. Mechanism of adrenergic $Ca_V1.2$ stimulation revealed by proximity proteomics. Nature 2020;577:695–700.

**8.** Itzhak DN, Tyanova S, Cox J, Borner GH. Global, quantitative and dynamic mapping of protein subcellular localization. Elife 2016;5:e16950.

**9.** Havugimana PC, Hart GT, Nepusz T, et al. A census of human soluble protein complexes. Cell 2012;150:1068–81.

**10.** Salas D, Stacey RG, Akinlaja M, Foster LJ. Next-generation Interactomics: considerations for the use of co-elution to measure protein interaction networks. Mol Cell Proteomics 2020;19:1–10.

**11.** Gingras AC, Raught B. Beyond hairballs: the use of quantitative mass spectrometry data to understand protein-protein interactions. FEBS Lett 2012;586:2723–31.

**12.** Samavarchi-Tehrani P, Abdouni H, Samson R, Gingras AC. A versatile lentiviral delivery toolkit for proximity-dependent biotinylation in diverse cell types. Mol Cell Proteomics 2018;17:2256–69.

**13.** Iacobucci I, Monaco V, Cozzolino F, Monti M. From classical to new generation approaches: an excursus of -omics methods for investigation of protein-protein interaction networks. J Proteomics 2021;230:103990.

**14.** Rees JS, Li XW, Perrett S, Lilley KS, Jackson AP. Protein neighbors and proximity proteomics. Mol Cell Proteomics 2015;14:2848–56.

**15.** Roux KJ, Kim DI, Raida M, Burke B. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. J Cell Biol 2012;196:801–10.

**16.** Chapman-Smith A, Cronan JE Jr. The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. Trends Biochem Sci 1999;24:359–63.

**17.** Worman HJ. Nuclear lamins and laminopathies. J Pathol 2012;226:316–25.

**18.** Uezu A, Kanak DJ, Bradshaw TW, et al. Identification of an elaborate complex mediating postsynaptic inhibition. Science 2016;353:1123–9.

**19.** Martell JD, Deerinck TJ, Sancak Y, et al. Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat Biotechnol 2012;30:1143–8.

**20.** Lobingier BT, Huttenhain R, Eichel K, et al. An approach to spatiotemporally resolve protein interaction networks in living cells. Cell 2017;169:350–60.e12.

**21.** Paek J, Kalocsay M, Staus DP, et al. Multidimensional tracking of gpcr signaling via peroxidase-catalyzed proximity labeling. Cell 2017;169:338–49.e11.

**22.** Bers DM. Cardiac excitation-contraction coupling. Nature 2002;415:198–205.

**23.** Scriven DR, Dan P, Moore ED. Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. Biophys J 2000;79:2682–91.

**24.** Bean BP, Nowycky MC, Tsien RW. Beta-adrenergic modulation of calcium channels in frog ventricular heart cells. Nature 1984;307:371–5.

**25.** Reuter H, Scholz H. The regulation of the calcium conductance of cardiac muscle by adrenaline. J Physiol 1977;264:49–62.

**26.** Cachelin AB, de Peyer JE, Kokubun S, Reuter H. $Ca2^+$ channel modulation by 8-bromocyclic AMP in cultured heart cells. Nature 1983;304:462–4.

**27.** Yue DT, Herzig S, Marban E. Beta-adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. Proc Natl Acad Sci U S A 1990;87:753–7.

**28.** Weiss S, Oz S, Benmocha A, Dascal N. Regulation of cardiac L-type $Ca^{2+}$ channel $Ca_V1.2$ via the β-adrenergic–cAMP-protein kinase A pathway: old dogmas, advances, and new uncertainties. Circ Res 2013;113:617–31.

**29.** Hofmann F, Flockerzi V, Kahl S, Wegener JW. L-Type CaV1.2 calcium channels: from in vitro findings to in vivo function. Physiol Rev 2014;94:303–26.

**30.** Wang X, Tsien RW. Suspect that modulates the heartbeat is ensnared. Nature 2020;577(7792):624–6.

**31.** Hell JW, Yokoyama CT, Wong ST, Warner C, Snutch TP, Catterall WA. Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel alpha 1 subunit. J Biol Chem 1993;268:19451–7.

**32.** de Jongh KS, Murphy BJ, Colvin AA, Hell JW, Takahashi M, Catterall WA. Specific phosphorylation of a site in the full-length form of the α1 subunit of the cardiac L-type calcium channel by adenosine 3′,5′-cyclic monophosphate–dependent protein kinase. Biochemistry 1996;35:10392–402.

**33.** Hulme JT, Westenbroek RE, Scheuer T, Catterall WA. Phosphorylation of serine 1928 in the distal C-terminal domain of cardiac $Ca_V1.2$ channels during β1-adrenergic regulation. Proc Natl Acad Sci U S A 2006;103:16574–9.

**34.** Fuller MD, Emrick MA, Sadilek M, Scheuer T, Catterall WA. Molecular mechanism of calcium channel regulation in the fight-or-flight response. Sci Signal 2010;3:ra70.

**35.** Fu Y, Westenbroek RE, Yu FH, et al. Deletion of the distal C terminus of $Ca_V1.2$ channels leads to loss of beta-adrenergic regulation and heart failure in vivo. J Biol Chem 2011;286:12617–26.

**36.** Fu Y, Westenbroek RE, Scheuer T, Catterall WA. Phosphorylation sites required for regulation of cardiac calcium channels in the fight-or-flight response. Proc Natl Acad Sci U S A 2013;110:19621–6.

**37.** Hell JW. Beta-adrenergic regulation of the L-type $Ca^{2+}$ channel $Ca_V1.2$ by PKA rekindles excitement. Sci Signal 2010;3:pe33.

**38.** Yang L, Katchman A, Samad T, Morrow J, Weinberg R, Marx SO. beta-adrenergic regulation of the L-type $Ca^{2+}$ channel does not require phosphorylation of α1C Ser1700. Circ Res 2013;113:871–80.

**39.** Ganesan AN, Maack C, Johns DC, Sidor A, O'Rourke B. Beta-adrenergic stimulation of L-type $Ca^{2+}$ channels in cardiac myocytes requires the distal carboxyl terminus of α1C but not serine 1928. Circ Res 2006;98:e11–8.

**40.** Lemke T, Welling A, Christel CJ, et al. Unchanged beta-adrenergic stimulation of cardiac L-type calcium channels in $Ca_V1.2$ phosphorylation site S1928A mutant mice. J Biol Chem 2008;283:34738–44.

**41.** Miriyala J, Nguyen T, Yue DT, Colecraft HM. Role of $Ca_V\beta$ subunits, and lack of functional reserve, in protein kinase A modulation of cardiac $Ca_V1.2$ channels. Circ Res 2008;102:e54–64.

**42.** Brandmayr J, Poomvanicha M, Domes K, et al. Deletion of the C-terminal phosphorylation sites in the cardiac beta-subunit does not affect the basic beta-adrenergic response of the heart and the $Ca_V1.2$ channel. J Biol Chem 2012;287:22584–92.

**43.** Yang L, Katchman A, Kushner J, et al. Cardiac $Ca_V1.2$ channels require beta subunits for beta-adrenergic-mediated modulation but not trafficking. J Clin Invest 2019;129:647–58.

**44.** Katchman A, Yang L, Zakharov SI, et al. Proteolytic cleavage and PKA phosphorylation of α1C subunit are not required for adrenergic regulation of $Ca_V1.2$ in the heart. Proc Natl Acad Sci U S A 2017;114:9194–9.

**45.** Shuja Z, Colecraft HM. Regulation of microdomain voltage-gated L-type $Ca^{2+}$ channels in cardiac health and disease. Curr Opin Physiol 2018;2:13–8.

**46.** Fabiato A, Fabiato F. Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. J Physiol 1975;249:469–95.

**47.** Fabiato A, Fabiato F. Calcium and cardiac excitation-contraction coupling. Annu Rev Physiol 1979;41:473–84.

**48.** Franzini-Armstrong C, Protasi F, Ramesh V. Shape, size, and distribution of $Ca^{2+}$ release units and couplons in skeletal and cardiac muscles. Biophys J 1999;77:1528–39.

**49.** Sun XH, Protasi F, Takahashi M, Takeshima H, Ferguson DG, Franzini-Armstrong C. Molecular architecture of membranes involved in excitation-contraction coupling of cardiac muscle. J Cell Biol 1995;129:659–71.

**50.** Sanbe A, Gulick J, Hanks MC, Liang Q, Osinska H, Robbins J. Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. Circ Res 2003;92:609–16.

**51.** Hambleton M, York A, Sargent MA, et al. Inducible and myocyte-specific inhibition of PKCα enhances cardiac contractility and protects against infarction-induced heart failure. Am J Physiol Heart Circ Physiol 2007;293:H3768–71.

**52.** Avula UMR, Abrams J, Katchman A, et al. Heterogeneity of the action potential duration is required for sustained atrial fibrillation. JCI Insight 2019;5.

**53.** Wan E, Abrams J, Weinberg RL, et al. Aberrant sodium influx causes cardiomyopathy and atrial fibrillation in mice. J Clin Invest 2016;126:112–22.

**54.** Kalocsay M. APEX peroxidase-catalyzed proximity labeling and multiplexed quantitative proteomics. Methods Mol Biol 2019;2008:41–55.

**55.** van Deel ED, Najafi A, Fontoura D, et al. In vitro model to study the effects of matrix stiffening on $Ca^{2+}$ handling and myofilament

function in isolated adult rat cardiomyocytes. J Physiol 2017;595:4597–610.

**56.** Hulsmans M, Clauss S, Xiao L, et al. Macrophages facilitate electrical conduction in the heart. Cell 2017;169:510–22.e20.

**57.** Yada H, Murata M, Shimoda K, et al. Dominant negative suppression of Rad leads to QT prolongation and causes ventricular arrhythmias via modulation of L-type $Ca^{2+}$ channels in the heart. Circ Res 2007;101:69–77.

**58.** Wang G, Zhu X, Xie W, et al. Rad as a novel regulator of excitation-contraction coupling and beta-adrenergic signaling in heart. Circ Res 2010;106:317–27.

**59.** Manning JR, Yin G, Kaminski CN, et al. Rad GTPase deletion increases L-type calcium channel current leading to increased cardiac contraction. J Am Heart Assoc 2013;2:e000459.

**60.** Xu X, Marx SO, Colecraft HM. Molecular mechanisms, and selective pharmacological rescue, of Rem-inhibited $Ca_V1.2$ channels in heart. Circ Res 2010;107:620–30.

**61.** Chang DD, Colecraft HM. Rad and Rem are noncanonical G-proteins with respect to the regulatory role of guanine nucleotide binding in $Ca_V1.2$ channel regulation. J Physiol 2015;593:5075–90.

**62.** Finlin BS, Crump SM, Satin J, Andres DA. Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. Proc Natl Acad Sci U S A 2003;100:14469–74.

**63.** Levitan BM, Manning JR, Withers CN, et al. Rad-deletion phenocopies tonic sympathetic stimulation of the heart. J Cardiovasc Transl Res 2016;9:432–44.

**64.** Kranias EG, Garvey JL, Srivastava RD, Solaro RJ. Phosphorylation and functional modifications of sarcoplasmic reticulum and myofibrils in isolated rabbit hearts stimulated with isoprenaline. Biochem J 1985;226:113–21.

**65.** Abrams J, Roybal D, Chakouri N, et al. Fibroblast growth factor homologous factors tune arrhythmogenic late $Na_V1.5$ current in calmodulin binding-deficient channels. JCI Insight 2020;5:e141736.

**66.** Kim J, Ghosh S, Liu H, Tateyama M, Kass RS, Pitt GS. Calmodulin mediates $Ca^{2+}$ sensitivity of sodium channels. J Biol Chem 2004;279:45004–12.

**67.** Gabelli SB, Boto A, Kuhns VH, et al. Regulation of the $Na_V1.5$ cytoplasmic domain by calmodulin. Nat Commun 2014;5:5126.

**68.** Wang C, Chung BC, Yan H, Lee SY, Pitt GS. Crystal structure of the ternary complex of a $Na_V$ C-terminal domain, a fibroblast growth factor homologous factor, and calmodulin. Structure 2012;20:1167–76.

**69.** Wang C, Hennessey JA, Kirkton RD, et al. Fibroblast growth factor homologous factor 13 regulates $Na^+$ channels and conduction velocity in murine hearts. Circ Res 2011;109:775–82.

**70.** Feng W, Liu C, Spinozzi S, Wang L, Evans SM, Chen J. Identifying the cardiac dyad proteome in vivo by a BioID2 knock-in strategy. Circulation 2020;141:940–2.

**71.** Rudolph F, Fink C, Huttemeister J, et al. Deconstructing sarcomeric structure-function relations in titin-BioID knock-in mice. Nat Commun 2020;11:3133.

**72.** Li Y, Merkel CD, Zeng X, et al. The N-cadherin interactome in primary cardiomyocytes as defined using quantitative proximity proteomics. J Cell Sci 2019;132:jcs221606.

**73.** Park SS, Ponce-Balbuena D, Kuick R, et al. Kir2.1 interactome mapping uncovers PKP4 as a modulator of the Kir2.1-regulated inward rectifier potassium currents. Mol Cell Proteomics 2020;19:1436–49.

**74.** Nichols CG, Makhina EN, Pearson WL, Sha Q, Lopatin AN. Inward rectification and implications for cardiac excitability. Circ Res 1996;78:1–7.

**75.** Plaster NM, Tawil R, Tristani-Firouzi M, et al. Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. Cell 2001;105:511–9.

**76.** Ma D, Taneja TK, Hagen BM, et al. Golgi export of the Kir2.1 channel is driven by a trafficking signal located within its tertiary structure. Cell 2011;145:1102–15.

**77.** Bendahhou S, Donaldson MR, Plaster NM, Tristani-Firouzi M, Fu YH, Ptacek LJ. Defective potassium channel Kir2.1 trafficking underlies Andersen-Tawil syndrome. J Biol Chem 2003;278:51779–85.

**78.** Hopkins C, Gibson A, Stinchcombe J, Futter C. Chimeric molecules employing horseradish peroxidase as reporter enzyme for protein localization in the electron microscope. Methods Enzymol 2000;327:35–45.

**79.** Martell JD, Yamagata M, Deerinck TJ, et al. A split horseradish peroxidase for the detection of intercellular protein-protein interactions and sensitive visualization of synapses. Nat Biotechnol 2016;34:774–80.

**80.** Kostelnik KB, Barker A, Schultz C, et al. Dynamic trafficking and turnover of JAM-C is essential for endothelial cell migration. PLoS Biol 2019;17:e3000554.

**81.** Cho KF, Branon TC, Rajeev S, et al. Split-TurboID enables contact-dependent proximity labeling in cells. Proc Natl Acad Sci U S A 2020;117:12143–54.

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