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Detecting cardiovascular protein-protein interactions by proximity proteomics

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Abstract

Rapidly changing and transient protein-protein interactions regulate dynamic cellular processes in the cardiovascular system. Traditional methods, including affinity purification and mass-spectrometry (MS), have revealed many macromolecular complexes in cardiomyocytes and the vasculature. Yet these methods often fail to identify *in vivo* or transient protein-protein interactions. To capture these interactions in living cells and animals with subsequent MS identification, enzyme-catalyzed proximity labeling techniques have been developed in the past decade. Although the application of this methodology to cardiovascular research is still in its infancy, the field is developing rapidly, and the promise is substantial. In this review, we outline important concepts and discuss how proximity proteomics has been applied to study physiological and pathophysiological processes relevant to the cardiovascular system.

INTRODUCTION

Resolving spatial organization and dynamic protein interaction networks within the cell has long been an aspirational goal. Traditional techniques, namely immuno-precipitation (IP) coupled with mass spectrometry (MS), have yielded important insights (Fig. 1A). These studies, however, are limited because they require high-quality antibodies and may miss transient or low-affinity interactions, in part because the purification is performed after detergent solubilization. Moreover, capturing local protein-interaction dynamics from

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COMPETING INTERESTS

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homogenized cell lysates is often impossible. Crosslinking can stably fix some of these interactions [1], but complicates MS analysis due to chemical protein modification and background crosslinking. Subcellular fractionation followed by mass spectrometry has offered insights into the constituents of various cellular compartments (Fig 1B), but information on protein-protein interactions in specific sub-cellular compartments remained limited [2]. The Contaminant Repository for Affinity Purification (CRAPome) [3] and Significance Analysis of INTeractome (SAINT) [4] have cataloged many putative false positives using these approaches.

Proximity-dependent labeling has been developed to resolve the nano-environment (a.k.a. "neighborhood") of target proteins *in situ* [5, 6]. In most studies, the protein of interest is expressed as a fusion to the labeling enzyme in cultured cells or organisms [5, 7–11], although some approaches have been developed using an antibody against the protein of interest to target biotin conjugation to adjacent proteins in fixed cells, primary tissues and zebrafish [12, 13]. For proximity-dependent approaches, proteins near the protein of interest are labeled with biotin, with the labeling radius primarily dependent on the reactivity of the biotinylating agent: biotin-phenoxy radicals for peroxidases and biotinoyl-AMP esters for biotinylation-enzyme derivatives. Labeling also depends in part on the steric accessibility of reactive amino acids. Peroxidase-catalyzed labeling can be rapidly stopped and quenched, allowing short 30-60 second labeling pulses. Biotinylated proteins can be efficiently affinity-purified with streptavidin and quantified by mass spectrometry. Compared to traditional antibody affinity purification, proximity labeling can capture weak and transient interactions, even of trans-membrane proteins that require membrane insertion to maintain an interaction, which are disrupted by detergent solubilization [14–17]. In contrast to antibody- or epitope-based affinity purifications, enrichment of biotinylated proteins is compatible with harsh protein extraction, denaturing and stringent wash conditions since labeling is completed *in situ* prior to enrichment and biotin-streptavidin affinity is very high. In this review, we describe the rapid adoption of proximity-dependent labeling for quantification of the molecular protein environment, focusing on studies that have used the methodology to reveal composition and dynamics of macromolecular complexes and signaling pathways in the cardiovascular system.

FOUNDATIONAL STUDIES OF PROXIMITY LABELING

There are two classes of enzymes used for proximity labeling, derivatives of biotin-ligases, and peroxidases, both of which make use of biotin to covalently modify nearby proteins (Fig. 2A–B). Biotin, also known as vitamin B7, is a cofactor for carboxylase enzymes that transfer carbon dioxide to organic acids and is essential for all life forms from archaea to eukaryotes [18, 19]. Three different classes of enzymes rely on biotin as cofactor: carboxylases, including CoA carboxylases, decarboxylases and transcarboxylases [19]. In eukaryotes, these enzymes are in the cytoplasm and mitochondria and participate in carbohydrate, lipid, and amino acid metabolism and energy transduction. Native biotin ligases display high lysine specificity of only few biotinylation-target proteins [5, 18]. Due to the limited number of endogenous biotinylated proteins and the exceptional strength of the non-covalent biotin-avidin interaction ($K_d = 10^{-14}$), an early iteration of enzyme catalyzed proximity labeling

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used a mutant *Escherichia coli* biotin ligase BirA R118G, also known as BirA* and named BioID for “biotin identification” [5] (Table 1).

BirA requires both biotin and ATP to generate a reactive biotinoyl-5’AMP ester intermediate, which it uses to transfer biotin to specific lysine residues on bacterial carboxylase proteins. The R118G mutation of BirA loosens a loop normally preventing diffusion of biotinoyl-5’AMP, markedly reducing the enzyme’s affinity for this reactive intermediate, thus enabling the promiscuous, substrate-nonspecific labeling of lysine residues on proximal proteins (Fig. 2A) [20]. BirA* was expressed as a fusion protein with lamin-A, an intermediate filament protein, that is a constituent of the nuclear lamina in the nuclear envelope [5]. BirA*-lamin-A was expressed in HeLa cells and was used to perform *in situ* labeling. Upon the addition of 50 μM biotin to the tissue culture medium, BirA* was capable of substrate-nonspecific but proximity-dependent biotin ligation within a ~10 nm radius [5, 21]. Numerous protein-protein interactors, including novel ones such as SLAP75, were identified by combining streptavidin-based affinity purification of biotinylated proteins with liquid chromatography/mass spectrometry [5]. BirA* fusion, which adds 321 amino acids to the protein of interest, may influence trafficking or function of the target protein. A smaller biotin ligase from *Aquifex aeolicus* was modified for superior expression in human cells and mutated to create BioID2, which was shown to alter localization to a lesser extent and displayed robust biotinylation at lower biotin concentrations [22] (Table 1). BioID has been applied in diverse models, including the heart [11].

BioID is not optimal for the study of fast dynamic processes in the seconds to minute range because of its low catalytic efficiency and relatively long labeling time. A directed-evolution variant of BioID, called TurboID, was developed to overcome some of these limitations of BioID and specially to improve labeling kinetics [9] (Table 1). Removing the N-terminus of TurboID generated an even smaller version of TurboID, termed miniTurbo. Compared to BioID and miniTurbo, TurboID has faster kinetics, which confers increased temporal resolution for resolving evolving changes in the domain of interest. Potential drawbacks for TurboID are increased protein instability, an increase in the labeling radius, and persistent biotinylation in the absence of exogenously supplemented biotin with potentially harmful effects on organism viability [9, 25]. This was noted in the improved viability of drosophila pupae given supplemental biotin, presumably complementing biotin depletion by TurboID conjugation [9].

Peroxidase-catalyzed proximity labeling is based on the expression of either horseradish peroxidase (HRP) or an engineered soybean ascorbate peroxidase (APEX) and its engineered derivatives such as APEX2 (Table 1) [6–8, 26]. HRP is inactive when expressed in mammalian cytosol, likely due to the reduction of its disulfide bonds and Ca^{2+}-dependence [6]. After the addition of H_2O_2 for 1 minute in cells or tissues loaded with the substrate biotin-phenol (biotin-tyramide), the peroxidase generates biotin-phenoxy radicals that react with electron-rich side chains of amino acids, preferentially tyrosines, of nearby proteins (Fig. 2B). The phenoxy radical, which is believed to be membrane impermeant, has a short half-life of less than 1 ms, limiting the radius of labeling of approximately 20 nm for APEX. The fast-labeling kinetics of APEX and the limited labeling intervals can be exploited to probe very rapid and dynamic events during cell signaling [14, 17, 27].

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Mitochondrial matrix-targeted APEX was initially used to identify proteins within the human mitochondrial matrix. Using stable isotope labeling of experimental and control samples and tandem mass spectrometry, 495 proteins were identified including 31 proteins not previously linked to mitochondria [26]. Although the depth of coverage was high, approximately 15% of known mitochondrial proteins were not identified, likely because they were sterically inaccessible to phenoxy radicals [26]. Thereafter, APEX was used to characterize the mitochondrial intermembrane space proteome, which was previously inaccessible with traditional purification approaches [7]. To discriminate protein proximity-specific biotinylation from unwanted background, a stable isotope labeling by amino acids in cell culture (SILAC) based ratiometric tagging strategy was developed. The ratio of biotinylation by two different APEX fusion proteins, one within the region of interest, namely the mitochondrial intermembrane space, and one outside of the region of interest, cytosolic APEX, was utilized [7]. With this approach a high-quality mitochondrial intermembrane space proteome of 127 proteins, with >94% specificity and 65% coverage, was identified.

To increase spatial specificity, reduce background labeling from endogenous peroxidase activity, and conditionally control proximity ligation activity, split enzymes for APEX2, BioID and TurboID approaches have been developed [10, 28–30]. When N- and C-terminal paired fragments of the enzymes are complemented, enzymatic activity is restored (Fig. 2C). Split-APEX2 and split-TurboID have been used to characterize the proteome of the contact region between mitochondria and endoplasmic reticulum [10, 28, 29].

Distinguishing interacting proteins from bystanders

Several methods have been developed to distinguish interacting proteins from bystanders, which are compartment-specific proteins residing in the local neighborhood but not physically interacting with the bait. Two approaches were used to elucidate signaling through G-protein coupled receptors, which are critical regulators of physiological responses. In theory, agonist-induced changes in G-protein coupled receptor signaling would induce dynamic changes in the interactome but likely not in the abundance of bystanders. One study focused on quantification of dynamic changes of the proximity proteome by isobaric tandem mass tag (TMT) MS, without distinguishing constant interactors from constant bystanders [17]. Proximity labeling with biotin-phenol, denaturing streptavidin purification and TMT labeling of multiple individual samples in parallel was followed by pooling of samples and analysis in a single mass spectrometry experiment to achieve accurate relative quantification of protein abundance, enabling the recording of time courses of GPCR signaling [17]. Dynamic and rapid changes in the interactome could be resolved because multiplexed TMT-MS allows massive parallel and time-resolved quantitative analysis [31, 32], now in up to 18 channels [33]. In HEK293 cells, type 1 angiotensin II receptor and β2-adrenergic receptor (β2-AR) were expressed as APEX2 fusion proteins. Using a time course of agonist ligand binding and proximity labeling, followed by TMT-MS, the tracking of activation and internalization of these receptors and the identification of novel and functionally relevant interaction partners, such as LMBRD2, after ligand binding was achieved [17]. Thus, the combined use of APEX2 proximity labeling and multiplexed quantitative mass spectrometry is ideally suited to study changes in perturbed biological

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systems [16]. These studies formed the basis of our utilization of APEX2-based proximity labeling to elucidate the molecular mechanisms underlying adrenergic regulation of $Ca_V1.2$ in the heart [14].

Similarly, APEX2 was used to capture snapshots of β2-AR and the δ-opioid receptor interactome [27]. In an alternative approach to distinguish the specific protein-protein interaction network from bystanders, spatially specific APEX reference labeling probes were developed: Lyn_{11} (plasma membrane targeting), 2xFYVE (early endosome targeting), and GFP (cytoplasmic). It is critically important to select the correct spatial reference and ensure achievement of the anticipated localization pattern. For instance, the known receptor network components for β2-adrenergic receptor could not be distinguished from bystanders using GFP-APEX or 2xFYVE-APEX at 1 minute after agonist stimulation, when receptors should still be in the plasma membrane. Ten minutes after agonist stimulation, the endosomal 2xFYVE-APEX spatial reference differentiated the β2-adrenergic receptor network from bystanders. Thus, label-free mass spectrometry-based quantification with spatial references can be used to distinguish an interaction network from bystanders.

Off-the-shelf proximity-based biotinylation methods

The “standard” approaches for proximity-based labeling rely on the knock-in or expression via plasmids of the biotinylation enzyme fused to the bait protein in target cells of interest. Creating animals expressing proximity-based labeling enzymes is labor- and resource-intensive and carries the risk that conjugation of the APEX2 enzyme alters bait protein function. Moreover, proximity-based labeling using the expression of a fusion gene cannot be used on primary human tissue samples. Several methods have been developed to facilitate biotin-based proximity labeling without the need for genetic engineering or transfection of cultured cells. Prior to the development of TurboID or APEX, proximity labeling using local biotin conjugation was based on antibody-guided targeting of HRP [34–37]. This approach was further developed to study fixed and permeabilized tissue samples, in which a primary antibody binds to the target protein and in the presence of biotin-phenol and H_2O_2, a secondary HRP-conjugated antibody creates biotin-phenoxy radicals that covalently conjugate to nearby proteins [13], which can be identified and quantified using mass spectrometry. Another approach is to use Protein A fused to the TurboID enzyme (ProtA-Turbo). In permeabilized fixed or non-fixed mammalian cells, including clinical samples, the ProtA-Turbo enzyme can be specifically targeted to bait proteins using antibodies [38]. Upon the addition of exogenous biotin, proteins proximal to the bait are subsequently biotinylated and analyzed by MS [38].

The applicability of proximity labeling in animals has been limited by the necessity to tag each protein of interest with an engineered biotin ligase or peroxidase, and then generate transgenic organisms. A more adaptable system was developed by combining the ability to use existing transgenic zebrafish expressing GFP-tagged proteins and a biotin-ligase conjugated to a GFP-binding nanobody for proteomic comparison of different tissues and/or different proteins (Fig. 2D) [12, 39]. Initial attempts to use BirA* and BioID2 in zebrafish were unsuccessful and did not demonstrate detectable *in vivo* biotinylation [12] (although see [40], in which BioID2 was successfully used in zebrafish, discussed below). In contrast, TurboID

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induced strong biotin labeling as detected by streptavidin blotting. In a novel approach to labeling, a TurboID-conjugated nanobody directed at GFP was created, in which the GFP-binding nanobody is degraded when not bound to GFP ('destabilized GFP binding protein' or dGBP) (Fig. 2D). Transgenic zebrafish were created with expression, under the control of a broadly expressing actin promoter, of a TurboID-dGBP fusion with a P2A- red-fluorescent protein element (termed "biotin labeling in tagged zebrafish" or BLITZ) [12]. Double-positive TurboID-dGBP and GFP-transgenic fish were selected by fluorescence. In double-positive fish treated with supplemental biotin, *in vivo* biotinylation co-localized with the same subcellular distribution of the GFP-tagged proteins. Streptavidin affinity purification was able to precipitate known interactors of the GFP-tagged proteins. Streptavidin affinity purification of double-positive transgenic embryos expressing GFP-tagged Cavin4B, a protein associated with striated muscle and transverse (T)-tubules, enriched muscle specific proteins including SPEG, LDB3 and CMYA5, which have also been enriched in dyad protein proximity proteomes from transgenic BioID and APEX2 mouse model systems [14, 41, 42].

CARDIOVASCULAR APPLICATIONS OF PROXIMITY PROTEOMICS

Proximity proteomics to identify Kir2.1 macromolecular complexes in HEK cells

The strong inward rectifying potassium (K^+) current imparted by Kir2.1 is an important regulator of the resting membrane potential. KCNJ2 gain-of-function mutations cause short QT syndrome, which increases the risk of sudden cardiac death [43]. $Kir2.1^{WT}$ and $Kir2.1^{\Delta 314\text{-}315}$, an Andersen-Tawil Syndrome-associated mutant that blocks Kir2.1 Golgi export, were conjugated to BirA* and stably expressed in HEK293T cells [44]. HEK cells expressing a yeast transmembrane domain conjugated to BirA* (TM-CTRL) were used as a negative control. The proteomic analysis, which did not utilize mass tag quantification, resulted in the identification of 218 high-confidence Kir2.1 BioID hits. Of 24 previously identified Kir2.1 interactors, 10 (~42%) were identified by BioID as well. Of the 218 proteins identified, 75 interacted preferentially with $Kir2.1^{WT}$, 66 interacted preferentially with $Kir2.1^{\Delta 314\text{-}315}$ and the remaining 77 hits interacted with both $Kir2.1^{WT}$ and $Kir2.1^{\Delta 314\text{-}315}$. $Kir2.1^{WT}$ channel interactors were enriched for protein families involved in cell adhesion, and the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways included arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy, and hypertrophic cardiomyopathy. The $Kir2.1^{\Delta 314\text{-}315}$ that is retained in the Golgi preferably interacts with protein families involved in intracellular transport, ESCRT-0, and lysosomal processes, suggesting that the ATS1 mutation leads to increased targeting of Kir2.1 to lysosomal degradation. The functional role of one Kir2.1 interactor, Plakophilin 4 (PKP4), was validated as a neighbor and functional interactor, respectively, using co-immunofluorescence in isolated rat ventricular myocytes, and in a stable HEK293 cell line expressing Kir2.1, in which PKP4 expression increased current density, whereas depletion of PKP4 reduced current density. Thus, proximity labeling identified a new Kir2.1 interactor PKP4, which functions as a positive regulator of Kir2.1 density [44].

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Use of proximity labeling in cultured neonatal cardiomyocytes

i. Identifying the adherens junction proteome using N-cadherin-BioID—The adherens junction (AJ), the primary anchor for myofibrils and linker of actin filaments from adjacent cells, senses mechanical forces, enables cells to maintain shape, and transduces signals related to the actin cytoskeleton [45]. The transmembrane protein N-cadherin is the main component of the AJs, homodimerizing with N-cadherin from adjacent cells in the extracellular space. To identify novel components of cardiomyocyte AJs, a fusion protein of N-cadherin-BioID2 was expressed using adenovirus in cultured primary cardiomyocytes isolated from neonatal (P1-P3) mice [46]. The cadherin-BioID2 fusion proteins trafficked to the AJ, like endogenous cadherin. Upon biotin supplementation in the culture media, biotinylated proteins were observed along cell-cell contacts and, to a lesser extent, at Z-disks. 917 biotinylated proteins were identified by mass spectrometry. Excluding proteins with a single unique peptide reduced the list to 487 proteins. The list was further refined by including only proteins enriched by ≥ 10-fold, compared to control, non-infected cardiomyocytes, further reducing it to 365 proteins. The most abundant proteins were known components of nearby macromolecular complexes: the AJ, including β-, Δ-, and α-catenin, and junction plakoglobin; desmosomal proteins, desmoglein2 and plakophilin2, reflecting the proximity of AJs and desmosomes; and actin-binding proteins, vinculin and afadin, reflecting the importance of these proteins linking the AJ to the myofibril network. Comparing this refined list of cadherin-BioID2 enriched proteins with the published epithelial cadherin (E-cadherin) interactome in epithelia, one finds many of the closest N-cadherin interactors are shared with E-cadherin, indicating that the core cardiomyocyte AJ protein interactome is similar to that of epithelia, whereas specialization seems to occur in the less-enriched, deeper, N-cadherin specific interaction network.

In the N-cadherin proteome network, ~140 curated intercalated disk proteins were not detected including connexin 43 [46]. The absence of these proteins could be due to the range of biotinylation, the absence of surface lysine residues, the occlusion of access to biotinylation by other macromolecular components, or the specifics of the method (for instance, the use of cultured neonatal versus freshly isolated cardiomyocytes). Of the 185 N-cadherin hits, only 13 are curated intercalated disk proteins. As in other proximity labeling studies, some hits may not be located at the intercalated disk but rather reflect that biotin-labeling can occur through all stages of the fusion-protein life cycle.

ii. Defining the caveolin 3 interactome—Caveolae, small invaginations in the lipid bilayer, are created by the oligomerized scaffolding protein caveolin [47, 48]. They have important roles as scaffolding proteins acting on intracellular signal transduction, endocytosis, and mechano-modulation. To identify caveolin 3 interactions in cardiomyocytes, N-terminal tagged V5-APEX2-caveolin 3 was expressed using adenovirus in cultured neonatal rat ventricular myocytes [49]. Expression of V5-APEX2 via adenovirus served as a control. Ratiometric proteomic analysis was performed using 3-state isotope labeling by amino acids in cell culture (SILAC) labeling for 13 days. Adenoviral expression of either V5-APEX2-caveolin 3 or V5-APEX2 occurred on day 11. Biotinylated proteins were enriched by affinity purification and MS identified 1131 bound proteins, of which 101 proteins were significantly enriched by V5-APEX2-caveolin 3. All essential

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and muscle-specific components of the core caveolar complex were identified including cavin1 and cavin4. Other proteins identified included caveolin 1, myosin light chain, actin, troponin, Na^+-K^+ ATPase α1 and β1 subunits, and Na^+-Ca^{2+}-exchanger. Proteins that are involved in transmembrane substrate transport were also detected, including monocarboxylate transporter (McT1) and transferrin receptor (TfR1), which were speculated to be new caveolin 3 proximity candidates. The monocarboxylate transporter is a major pathway for transmembrane lactate and pyruvate transport in the heart [50]. To validate these findings, caveolin 3 knockout human iPSC-CMs were created. Surface expression of the monocarboxylate transporter was decreased in the knockout cardiomyocytes, causing reduced extracellular acidification through the lactate/proton shuttle. These results demonstrate the power of proximity labeling in identifying new caveolin partners [51].

iii. Defining the phospholamban proteomic subdomain—Calcium handling in the heart requires the coordinated activities of ion channels and pumps. The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} from the cytosol into the SR during diastole. Its activity is regulated by the inhibitory protein, phospholamban (PLB). Phosphorylation of PLB relieves the inhibition of PLN, leading to accelerated relaxation and increased contraction [52, 53]. PLN is phosphorylated at Ser16 by PKA and at Thr17 by Ca^{2+}-calmodulin-dependent kinase (CaMKII) [54].

To explore the proteomic neighborhood of SERCA and PLB, proximity labeling and mass spectrometry with APEX2-conjugated PLB was performed in neonatal rat cardiomyocytes [55]. The cardiomyocytes were cultured for 13 days in heavy, medium, and light SILAC (stable isotope labeling by amino acids in cell culture) medium and thereupon transduced with adenoviral vectors for expression of V5-APEX2-PLB, V5-APEX2 fused to green fluorescent protein (negative controls for background binding to avidin) or V5-APEX2 fused to PLB lacking its N-terminal domain (non-specific APEX2-mediated labeling). Although PLB, protein phosphatase 1 and Hsp20 were enriched, SERCA was not detected. Moreover, six of seven 14-3-3 proteins were labeled, consistent with precipitation studies showing a complex between 14-3-3 and phosphorylated PLB. The binding of 14-3-3 to phosphorylated PLB prevents the rapid dephosphorylation of PLB, stabilizing the increased SERCA activity. The authors propose that 14-3-3 binding to PLB creates a molecular memory of kinase activity.

Use of proximity labeling in cultured iPSC-CMs.

Actinin, an integral member of the actin cytoskeleton, interacts with multiprotein complexes at Z-disks and focal adhesions, which both function as hubs for mechanical force generators and sensors. Using CRISPR, BirA* (BioID) was fused with a hemagglutinin (HA) tag to the C-terminus of endogenous *ACTN2* in a wildtype human iPSC line and a troponin T2 gene knock-out ($TNNT2^{ko/ko}$) iPSC line (Fig. 3A). While both iPSC lines can be differentiated to cardiomyocytes, $TNNT2^{ko/ko}$ cardiomyocytes exhibit sarcomere structural and functional deficits such that Z-disk assembly is blocked at the Z-body stage, and twitch force does not occur. Using these two models, actinin nano-environments were studied [56]. Biotin supplementation (50 μM) induced strong biotinylation, and actinin proximity partners were identified by TMT quantitative mass spectrometry. 324 neighboring proteins were enriched,

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principally involved in cell adhesion, anchoring junction, actin cytoskeleton, contractile fiber, and RNA binding. 24 actinin partners were exclusive to the Z-disk stage and one was exclusive to the Z-body stage. Using gene ontology (GO) analysis, the study also identified 99 proteins with general RNA-binding functions, such as ribosomal assembly, transcription/translation and RNA localization and metabolism. Many of these RNA-binding proteins [56], excluding IGF2BP2, were also identified in the $Ca_V1.2$-APEX list [14].

Proximity labeling methods can also be applied to elucidate subcellular RNA localization either by direct RNA-labeling [57], or through proximity labeling of RNA-binding proteins followed by RNA precipitation and sequencing (RIP-seq) [58]. To reveal gene transcripts bound to the RNA-binding proteins in the actinin sub-proteome, biotinylated RNA-binding proteins were affinity purified using streptavidin followed by RIP-seq (Fig. 3B). Differential expression analysis identified 945 transcripts bound to biotinylated RNA-binding proteins, and GO analysis revealed enrichment of electron transport chain (ETC) and ribosome transcripts. Additional biochemical and protein interaction studies revealed that Insulin Like Growth Factor 2 mRNA Binding Protein 2 (IGF2BP2), an RNA-binding protein with metabolic functions, directly interacted with actinin, and IGF2BP2 knock-down impaired ETC transcript localization to Z-disks but not overall transcript stability (Fig. 3C). To study the function of actinin-IGF2BP2 interactions in cardiomyocytes, the actinin missense mutation glutamic acid at residue 445 substituted for alanine (E445A) was studied because it disrupted actinin-IGF2BP2 interactions without disrupting actinin dimerization and sarcomere assembly. Cardiomyocytes expressing E445A actinin exhibited diminished oxygen consumption rates and cell survival following pathological sarcomere activation induced by a hypertrophic cardiomyopathy-causing mutation in *TNNT2*. Overall, the use of proximity labeling in human iPSC-CMs enabled the identification of new actinin interaction partners including through sarcomere assembly, and their essential role in the metabolic responses to pathological sarcomere activation [56].

Expression of proximity labeling enzyme-conjugated proteins in hearts of mice

i. Elucidating the molecular mechanism of adrenergic $Ca_V1.2$ modulation—

Voltage-gated Ca^{2+} channels in heart cells mediate the initiation of cardiac excitation-contraction coupling, control action potential duration, and regulate gene expression. Cardiac $Ca_V1.2$ channels are prominently up-regulated by β-adrenergic agonists via activation of PKA [59, 60]. This regulation is believed to contribute to the increased contractility of the heart during exercise, and as a component of the physiological 'fight-or-flight' response. The elucidation of mechanisms underlying β-adrenergic regulation of cardiac Ca^{2+} channels has resisted decades of investigation, marked by the inability, until recently, to recapitulate this regulation in heterologous expression systems [61]. Since phosphorylation of the core α_{1C} and β_2 subunits were shown to be dispensable in the process [14, 62–65], we speculated that other members of the $Ca_V1.2$ macromolecular complex were the functional protein kinase A (PKA) target(s). A long-standing barrier to discover this basic mechanism regulating Ca^{2+} in the heart was the inability to reconstitute this regulation in the heart, precluding initial investigations using heterologous expression. To explore the nano-domain of the $Ca_V1.2$ macromolecular complex in heart, we created two transgenic mouse lines, one with inducible, cardiomyocyte-specific

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expression of dihydropyridine-resistant, APEX2 and V5-epitope-tagged α_{1C} subunit and the other with inducible, cardiomyocyte-specific expression of APEX2 and V5-epitope-tagged β_{2B} subunit [14]. Importantly, fusing APEX2 to either α_{1C} or β_{2B} altered neither trafficking nor function of $Ca_V1.2$ channels, nor adrenergic stimulation of Ca^{2+} channels. We reasoned that the use of two APEX2-conjugated target proteins, one transmembrane and the other cytoplasmic, would report intersecting and distinct neighboring proteins. Instead, the proteomic compositions were remarkably similar [14]. Using triple-stage mass spectrometry, we quantified hundreds of biotinylation-enriched proteins although many are possibly bystanders rather than physically interacting proteins. Many of these proteins could theoretically be the functional PKA target and identifying the correct one is challenging. Furthermore, a substantial number of these proteins may not be located within the macromolecular complex of a fully matured channel at the surface membrane. Proximity labeling may occur in several cellular compartments and likely reflects, to some extent, synthesis, maturation and trafficking with or adjacent to $Ca_V1.2$ channel subunits.

The speed of APEX2-mediated labeling has enabled quantitative analysis of G-protein coupled receptor (GPCR) signaling, allowing parallel time-resolved measurement of the dynamic changes in the proximity of hundreds to thousands of proteins [17, 27]. Work on β2AR-APEX functioned as a proof of concept showing that it is possible to identify signal transduction components via target-APEX2 fusion even in the absence of prior knowledge in signaling effectors [17]. We reasoned that β-adrenergic agonist induced PKA-dependent stimulation of Ca^{2+} current in the heart, which occurs rapidly, could alter the macromolecular complex of $Ca_V1.2$ such that PKA phosphorylation of an activator or inhibitor could be either recruited or depleted respectively. Following biotin-phenol incubation, we induced proximity labeling with H_2O_2 during the exposure of isoproterenol to either isolated cardiomyocytes or retrograde perfused heart (Fig. 4A–B). We confirmed that in the presence of biotin-phenol and H_2O_2, PKA signaling was preserved by assessing the phosphorylation status of phospholamban, and for the retrograde-perfused hearts, the effects of isoproterenol on the heart rate. The biotinylated proteins were affinity-purified under denaturing conditions using streptavidin and their relative abundance was quantified by tandem mass tag synchronous precursor selection triple stage MS (TMT SPS MS^3). β-adrenergic stimulation induced changes in the biotinylation of several proteins, including an increased biotinylation of the PKA catalytic subunit and decreased biotinylation of Rad (Ras associated with diabetes) [14], a member of the RGK (Rad, Rem, Rem2, Gem/Kir) Ras-family of proteins (Fig. 4C). These findings indicate that more PKA catalytic subunit and less Rad are near $Ca_V1.2$ channels upon exposure to isoproterenol.

Further studies revealed the functional relevance of these dynamic changes in the macromolecular complex of $Ca_V1.2$ channels [14, 66]. At baseline, the association of Rad with $Ca_V1.2$ inhibits a subpopulation of Ca^{2+} channels. Upon PKA phosphorylation of Rad, in response to β-adrenergic agonists, the interaction of Rad with the channel is diminished, releasing the inhibition of Ca^{2+} channels and thereby increasing Ca^{2+} influx. Co-expression of multiple classes of voltage-gated Ca^{2+} channels with Rad in HEK cells demonstrated increased Ca^{2+} current in response to stimulation with forskolin, an adenylate cyclase and PKA activator. Thus, through the use of proximity labeling and quantitative

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mass-spectrometry, the long-sought mechanism underlying adrenergic control of Ca^{2+} influx in the heart was discovered [14, 61, 66].

ii. Identifying the cardiac dyadic proteome—Junctophilin 2 (JPH2) spans the cleft from the transverse tubule (t-tubule) to the sarcoplasmic reticulum, tethering these two membrane structures to facilitate normal cardiac Ca^{2+} handling. Mutations in *JPH2* are associated with cardiomyopathy in humans [67–69]. Mice with BioID2 fused to the Jph2 coding sequence were created using a CRISPR-Cas9 knock-in strategy [42]. The 3XFLAG-BioID2-Jph2 proteins were expressed at levels equivalent to Jph2 protein. Saturation of proximity labeling was achieved in homozygous knock-in mouse hearts after several days of intraperitoneal injection of biotin. Using label-free tandem MS, 550 biotinylated proteins were identified including RyR2 and the $Ca_V1.2$ subunits, α_{1C} and β_2. Gene ontology enrichment analysis showed that the top cellular components were sarcolemma, cation channel complexes and t-tubule. A comparison with an IP-MS analysis of Jph2 interactors in a transgenic mouse line overexpressing Jph2-HA fusion protein showed that of the 18 proteins suggested to be direct interactors of Jph2 [70], only 2 were found in the enriched Jph2-BioID2 list (Fig. 5B and 5D), namely RyR2 and Striated Muscle Preferentially Expressed Protein Kinase (SPEG). The study demonstrates that a BioID2 knockin can be used to identify potentially novel protein-protein interactions in the heart.

iii. Identifying the sarcomere proteome using titin-BioID—Using homologous recombination BioID was inserted into exon 28 of the titin (*ttn*) gene, corresponding to a location on the titin filament located at the transition from the Z-disc to I-band (Fig. 5A) [41]. Mice tolerated this insertion and displayed normal growth and heart size. The most prominently biotinylated protein was titin with the expected hotspot of biotinylated sites near the BioID insertion site, yielding an estimated radius of biotinylation of 7-15 nm. In addition to biotinylated titin, which accounted for > 90% of total biotinylated peptides, other proteins were identified related to muscle filament assembly, myocyte function, α-actinin binding, and metabolism. In adults, 478 sarcomeric or sarcomere-associated proteins were identified in heart and skeletal muscle – more than twice the number of known sarcomeric proteins. BioID-mediated labeling can be applied in unique ways leading to new insights into positioning and function of titin, greater understanding of myofilament dynamics, and, by comparing the neonatal versus adult sarcomere proteome, capturing a snapshot of the shift to adult energy metabolism. For instance, the distribution of biotinylation sites suggested that titin forms a hairpin as the sarcomere contracts, which may contribute to the elastic properties of the sarcomere [41].

Interaction networks in zebrafish

Given the high energy requirements and low rate of proliferation of post-natal mammalian cardiomyocytes, the heart is an organ with limited capacity for regeneration after injury. Proximity labeling can identify protein networks during development and in animal models of tissue regeneration. Two transgenic zebrafish lines were created with cardiac-specific expression driven myosin light chain 7 promoter; one with BioID2 fused to GFP and the other with BioID-GFP fused to a membrane-localizing CAAX motif [40]. Robust biotinylation was not detected by streptavidin blotting of hearts from fish in biotin-supplemented water.

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Rather adult fish 3-8 months old were given three sequential daily intraperitoneal injections of biotin (3.7μg biotin in total), demonstrating that effective biotinylation in zebrafish only occurs after injection. Homogenates of pooled BioID expressing fish hearts were affinity purified on neutravidin beads and exposed to stringent detergent and denaturing conditions prior to quantitative mass spectrometry. From this study 1113 proteins were quantified, 343 of which were enriched at least 2.5-fold in the transgenic fish with BioID2-CAAX fusion [40].

BioID2 fish were crossed with transgenic fish with tamoxifen-inducible expression of diphtheria toxin A in the heart, which has been shown to ablate 60% of myocytes [71]. Fish were biotin supplemented 11-13 days after ablation and the BioID2 and BioID2-CAAX proteomes were examined during cardiac regeneration. Here 173 proteins were changed at least 1.5-fold in the injured compared to BioID-expressing uninjured hearts. Validating this approach, Seta, a protein expressed in injured heart, was enriched only in the BioID2-CAAX hearts after injury. Similarly, the abundance of Epb41l5 was reduced in recovering BioID2-CAAX heart but increased in BioID2 fish without CAAX. Confocal microscopy confirmed localization of this protein changes from membrane-restricted to global after injury. Gene ontology term analysis showed injured hearts expressed more proteins involved in wound healing, cardiac development, and muscle differentiation while there were diminished proteins involved in energy metabolism, particular oxidative phosphorylation, identified.

The proximity proteome of ErbB2, a co-receptor of the mitogen NRG1 shown to stimulate myocyte proliferation in the adult heart [72], was explored using a transgenic zebrafish line expressing the HA-tagged fusion protein ErbB2-BioID2. Crossed with diphtheria toxin A transgenic fish, quantitative mass spectrometry analysis of ErbB2-BioID2 hearts 14 days after myocyte ablation showed 1.5-fold enrichment of 108 proteins among the 667 identified. The most enriched protein was Rho A, a Rho GTPase which was HA-precipitated from injured hearts but not from healthy hearts. Follow-up pharmacologic and genetic inhibition studies showed reduced Rho A function inhibited repair in a resection-model of zebrafish heart injury. Taken together, these proximity proteomic experiments in zebrafish demonstrate that the effects of Nrg1 and other mitogens are mediated by RhoA in cardiomyocytes during regeneration [40].

Proximity labeling to identify secretomes

Proximity-based biotinylation has also been used as a platform to capture tissue-specific secretome. HA-tagged BioID2 with a C-terminal KDEL endoplasmic reticulum (ER) retention sequence (ER-BioID^{HA}) was expressed using lentiviral transduction in cultured endothelial cells [73]. Biotin supplementation of lentivirally transduced cultured endothelial cells resulted in ER-localized anti-HA and streptavidin staining, indicating successful biotinylation of proteins in the ER. In contrast to media from cells transduced with ER-retained GFP (ER-GFP) and supplemented with biotin, streptavidin blotting of electrophoretically resolved media from ER-BioID transduced cells identified multiple proteins. Additionally, ER-BioID was detected in cell lysates but not in the media, suggesting that biotinylation of secreted protein occurred only intracellularly. This technique was used to characterize the changing secretome of an *in vitro* model of the endothelial mesenchymal transition.

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To study the proteome of secreted proteins in animals, transgenic mice with ER-BioID^{HA} expression could be induced by excision of a loxP-flanked enhanced GFP [73]. When these mice were crossed with mice expressing Cre-recombinase in endothelium or skeletal muscle, the enhanced GFP is excised and ER-BioID^{HA} is expressed. From serum of mice supplemented with biotin, biotinylated proteins were purified and analyzed by mass-spectrometry. In mice expressing ER-BioID^{HA} in the endothelium, several well-known proteins were identified from the serum including endothelial cell surface receptors and von-Willebrand factor. Proteins not commonly associated with the endothelium were also identified, such as fibrocystin. To obtain sufficient biotinylation of proteins, mice expressing ER-BioID^{HA} in muscle required biotin-supplementation via multiple routes of administration (intraperitoneal, subcutaneous, and oral feeding) for 5 days. Mice were separated into cages without and with a running wheel to determine how the muscle-derived secretome changed with voluntary exercise. Exercise altered the abundance of several proteins including myostatin which modestly decreased and sarcalumenin which increased [73]. These findings demonstrate the feasibility and sensitivity of using proximity proteomics to detect, in a largely unbiased fashion, alterations in secreted proteins distant from the site of their generation, in changing pathophysiologic states.

CONCLUSIONS AND FUTURE DIRECTIONS

With the rapid advancement of proximity labeling techniques and mass spectrometry, elucidation of novel protein-protein interactions and their dynamics is now feasible. Given the individual strengths of these proximity labeling techniques, the optimal system of choice depends on the biologic question involved (see Table 1). Biotin ligases, with their smaller labeling radiuses and lower catalytic efficiency are well-suited to mapping neighborhoods with relatively high specificity, at different ages, in different genetic backgrounds and in health versus disease. Peroxidase-based labeling, in contrast, is particularly suited to investigate rapid signaling events. For signaling events that are suspected to involve recruitment or removal of an interactor, APEX2 labeling, combined with TMT multiplexing has exceptional specificity—potentially hundreds of neighboring proteins that remain stationary during the perturbation are unchanged during quantification.

To date, we are aware of four studies that have probed the t-tubule and underlying Z-disk with proximity proteomics, examining separately the interaction networks of ACTN2, TTN with BirA* in its Z-disk domain, JPH2, and $Ca_V1.2$, in human iPSC myocytes, knock-in and transgenic overexpression mice (Fig. 5A–B) [14, 41, 42, 56]. BioID proteomes were compiled according to the authors' criteria for significant enrichment, generally comparing biotin supplemented BioID animals or cells versus control. For ACTN2 303 such proteins were identified, 50 for TTN, and 52 for JPH2. For the α_{1C}-APEX mouse cardiomyocyte proteome, we included the top 1000 proteins, which were all at least 4-fold enriched over non H_2O_2 treated controls. Both Z-disk proteins, ACTN2 and TTN identified the other in its nanodomain, as did the all three sarcolemmal proteomes, including that of β_{2B}-APEX (examining its top 1000 β_{2B}-APEX hits, also at least 4-fold enriched). Despite the close-proximity of the bait, there were no proteins in common among all 4 proteomes, though there was abundant overlap (Fig. 5C–D).

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Although APEX2-labeled neighborhoods can be revealing, we found that proximity labeling using $Ca_V1.2$-APEX2 fusion proteins expressed in mice were particularly effective to identify dynamic neighborhoods, namely how β-adrenergic agonists alter the proteomic neighborhood around $Ca_V1.2$ [14]. Through our experiments we identified the Ca^{2+} channel inhibitor Rad as a key mediator in β-adrenergic stimulation of cardiac $Ca_V1.2$ channels. Rad was experimentally confirmed as the regulator of voltage-gated Ca^{2+}-channel that permits their adrenergic regulation, after its identification in the α_{1C}-APEX and β_{2B}-APEX "neighborhood". This, and its absence from other published proteomes, makes detection of Rad an instructive example of the relative of merits of proximity labeling methods. Though this protein's primary site of regulation is the sarcolemma of myocytes, a space that measures a mere 12 nm across, recombinant dyad proteins fused to biotin ligases did not identify significant enrichment of Rad [42, 56]. On the other hand, Rad was found in a list of proteins identified by mass-spectrometry after HA-antibody affinity purification of heart lysates from transgenic HA-tagged junctophilin [70]. Although not compared head-to-head in the same experiments, the greater catalytic efficiency of peroxidase-based systems may lead to the detection of more rare labeling events, at the cost of identifying more bystanders as well.

More broadly, proximity-mediated labeling represents an exciting method for continued probing into the microenvironments of diverse proteins, advancing the discovery of key mediators of cardiac function in health and disease, such as during the development of heart failure and hypertrophy. Techniques and methodological approaches to distinguish interacting proteins from bystanders, and methods for high throughput screening for potential interactions will accelerate adoption and successful implementation of proximity-dependent biotinylation studies.

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Non-standard Abbreviations and Acronyms:

IP	immuno-precipitation
MS	mass spectrometry
HRP	horseradish peroxidase
APEX	ascorbate peroxidase
TMT	tandem mass tag
Rad	Ras associated with diabetes
RGK	Rad, Rem, Rem2, Gem/Kir
PLB	phospholamban

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PKA	protein kinase A
CaMKII	Ca^{2+}-calmodulin-dependent kinase
ER	endoplasmic reticulum
β2-AR	β2-adrenergic receptor
GFP	Green fluorescent protein
dGBP	‘destabilized GFP binding protein
t-tubule	transverse tubule
KEGG	Kyoto Encyclopedia of Genes and Genomes
AJ	adherens junction
E-cadherin	epithelial cadherin
SERCA	sarcoplasmic reticulum (SR) Ca^{2+}-ATPase
HA	hemagglutinin
GO	gene ontology
RIP-seq	RNA precipitation and sequencing
ETC	electron transport chain
IGF2BP2	Insulin Like Growth Factor 2 mRNA Binding Protein 2
GPCR	G-protein coupled receptor
JPH2	Junctophilin 2
CAAX	C is cysteine residue, AA are two aliphatic residues, and X represents any C-terminal amino acid

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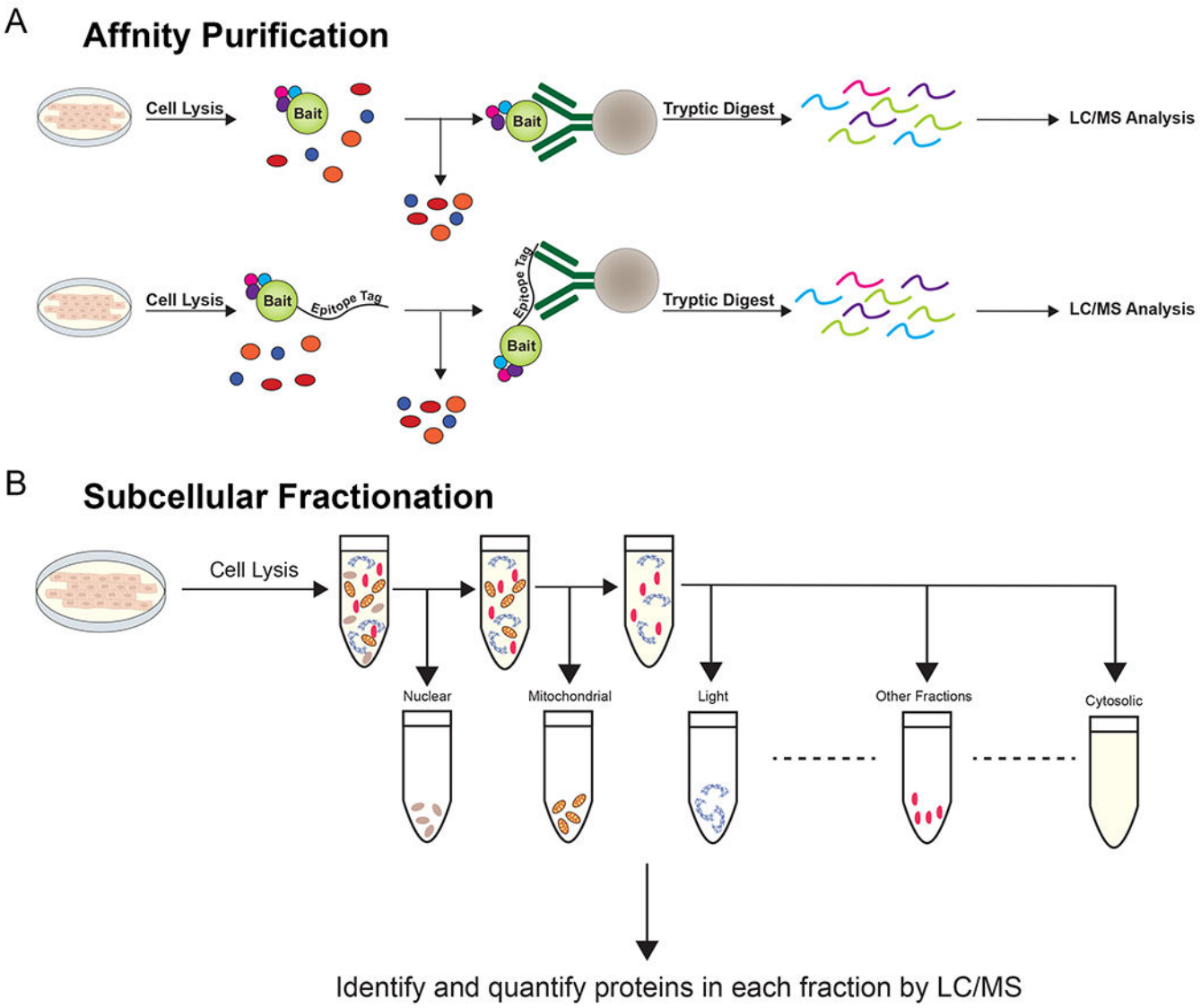

Figure 1. Standard methods for identifying protein-protein interactions.

(A) In affinity purification, cell lysates are exposed to antibodies against the bait protein. These antibodies are precipitated and interacting proteins are digested and identified by LC/MS. To study interactors of bait proteins with universal epitope-tag directed antibodies, the fusion of a standard epitope tag can overcome the need for protein-specific antibodies. **(B)** In lysates subjected to sucrose gradient centrifugation, organelles and subcellular locations of interest are enriched based on their densities.

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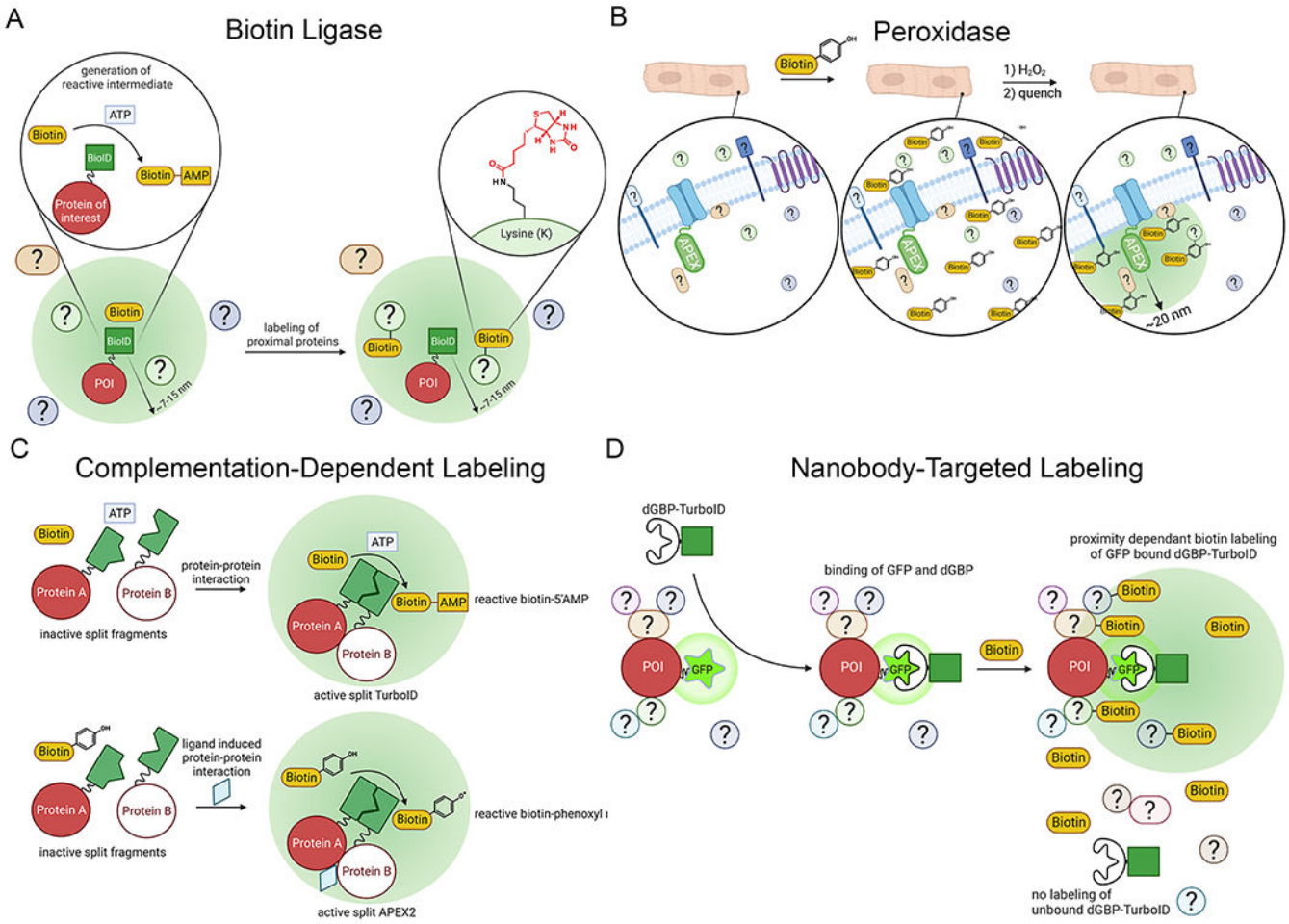

Figure 2. Proteomic mapping by proximity labeling.

(A) In an ATP dependent process, biotin ligases covalently modify basic residues such as lysine with biotin. **(B)** In cells loaded with biotin-phenol and treated with H_2O_2, peroxidases generate biotin-phenoxy radicals which attack nearby electron-rich amino acids, preferentially tyrosine. The labeling radius is determined by the longevity of these radicals prior to their quenching. **(C)** Cleaved or Split-TurboID and Split-APEX2 systems have been developed such that complementation and subsequent biotin-phenol/biotin dependent-proximity labeling occurs when the cleaved enzyme fusions are close together [28, 29]. This complementation increases labeling specificity for the cellular domain where the fused proteins interact. **(D)** In this modular labeling system, a nanobody to GFP that is destabilized when not bound to GFP was conjugated to TurboID (dGFP-TurboID), which is co-expressed with a GFP-fused protein of interest (POI) [12]. The destabilization of the nanobody ensures high labeling specificity for the POI. Illustration Credit: Ben Smith.

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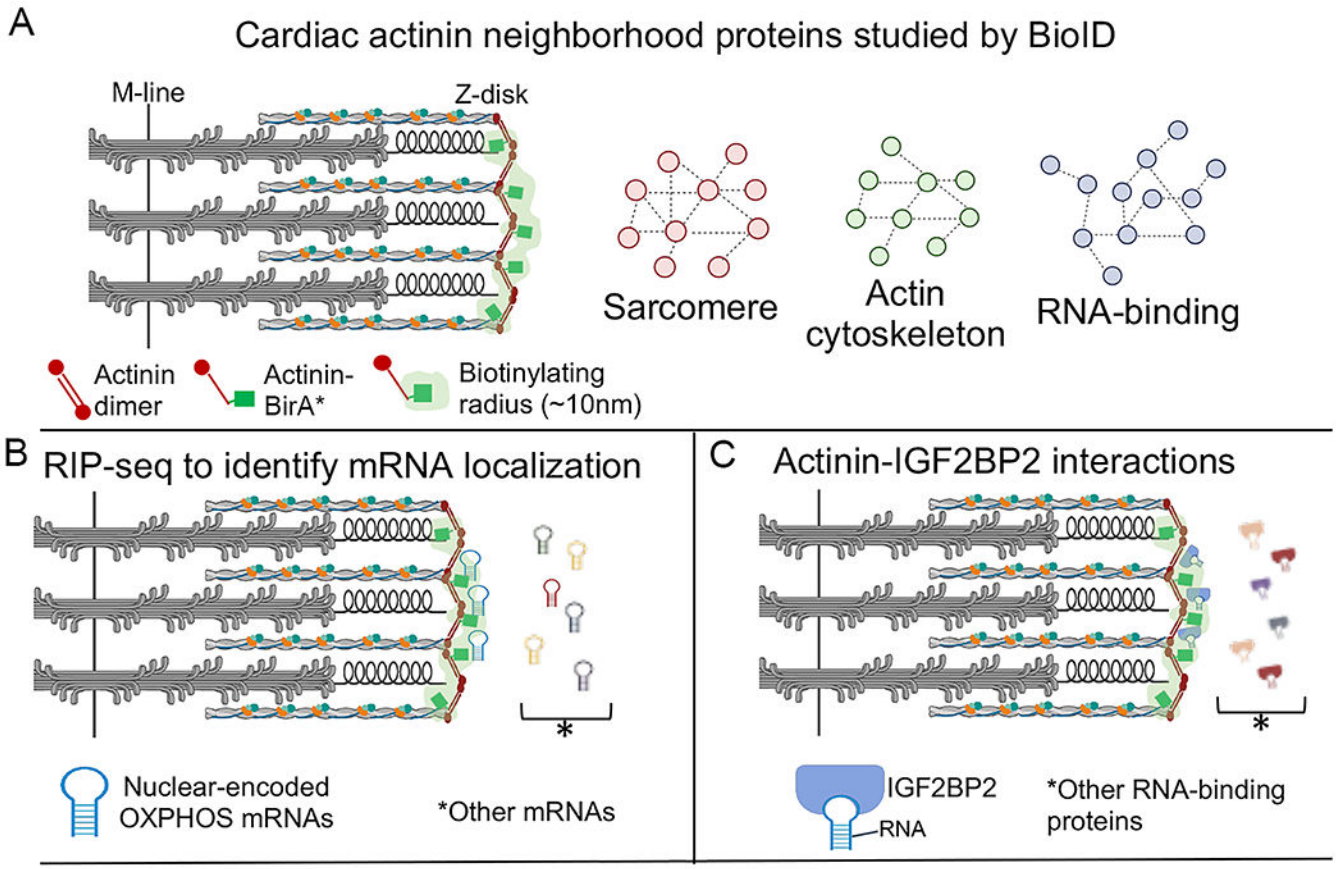

Figure 3. Use of BioID to probe the cardiac actinin neighborhood.

(A) Actinin-BirA* was expressed, via CRISPR knock-in, in human inducible pluripotent stem cell derived cardiomyocytes. Biotinylated proteins were purified and identified by mass spectrometry. Gene ontology analysis of the proteins revealed functions in actin cytoskeleton, sarcomere, and RNA binding. The light-green shading indicates an estimate of the zone of biotinylation. **(B)** RNA precipitation followed by sequencing (RIP-seq) using streptavidin affinity purification to pull-down RNA-binding proteins that were biotinylated within ~10 nm of actinin-BirA*. Gene ontology analysis indicated enrichment of oxidative phosphorylation (OXPHOS) transcripts. **(C)** Actinin interacts directly with insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2), localizing IGF2BP2 to Z-disks. Adapted from [56].

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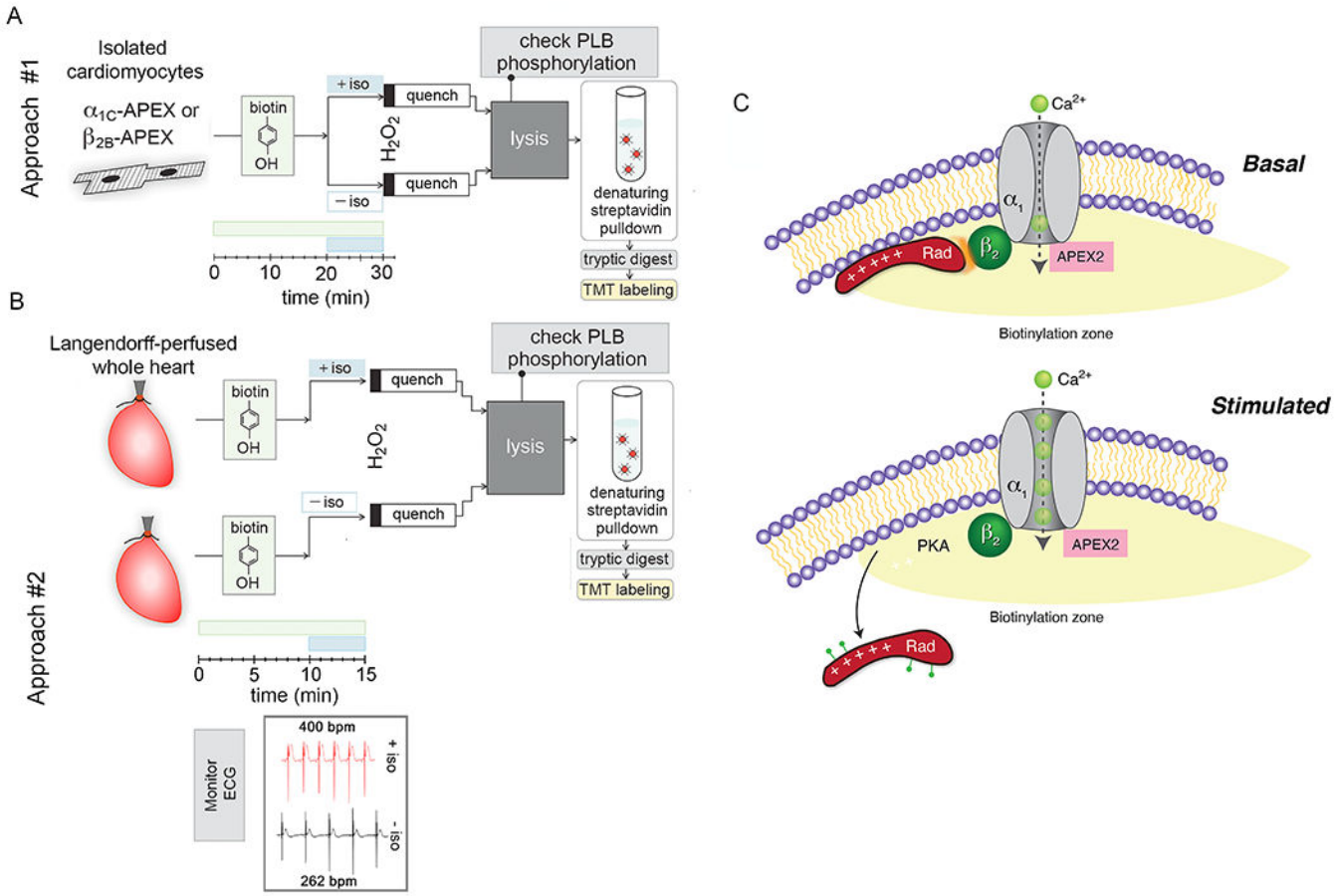

Figure 4. APEX2-based proximity labeling identifies mechanism underlying adrenergic regulation of $Ca_V1.2$ in heart.

(A-B) Workflow for processing proximity-labeling in isolated cardiomyocytes and retrograde-perfused hearts. V5 epitope and APEX2 cDNA were conjugated to the N-terminus of dihydropyridine-resistant α_{1C} and wild-type β_{2B} subunits. Transgenic mice with non-targeted insertion of tetracycline-regulated cDNAs were bred with cardiac-specific codon-optimized reverse transcriptional transactivator (rtTA) mice [74]. Transgene expression did not require doxycycline due to low basal binding of rtTA to the *Tet* operator sequences. In approach #1, ventricular myocytes were isolated by enzymatic digestion and proximity labeling was performed as previously described for isolated cells except for the addition of 1 μM isoproterenol for 10 minutes prior to labeling with H_2O_2. For each heart, samples were split into 2 groups, one with isoproterenol and one without isoproterenol. After lysis, the effect of PKA on phospholamban (PLB) was determined by western blotting to ensure viability of cardiomyocytes during labeling. In approach #2 (B), samples could not be paired, and hearts were labeled in the absence or presence of isoproterenol. Incubation with biotin-phenol was reduced to 15 minutes and isoproterenol for 5 minutes. Bpm= beats per minutes. **(C)** Schematic demonstrating zone of biotinylation around $Ca_V1.2$ channels. PKA is recruited to and Rad is depleted from the channel neighborhood after isoproterenol. Adapted from [14].

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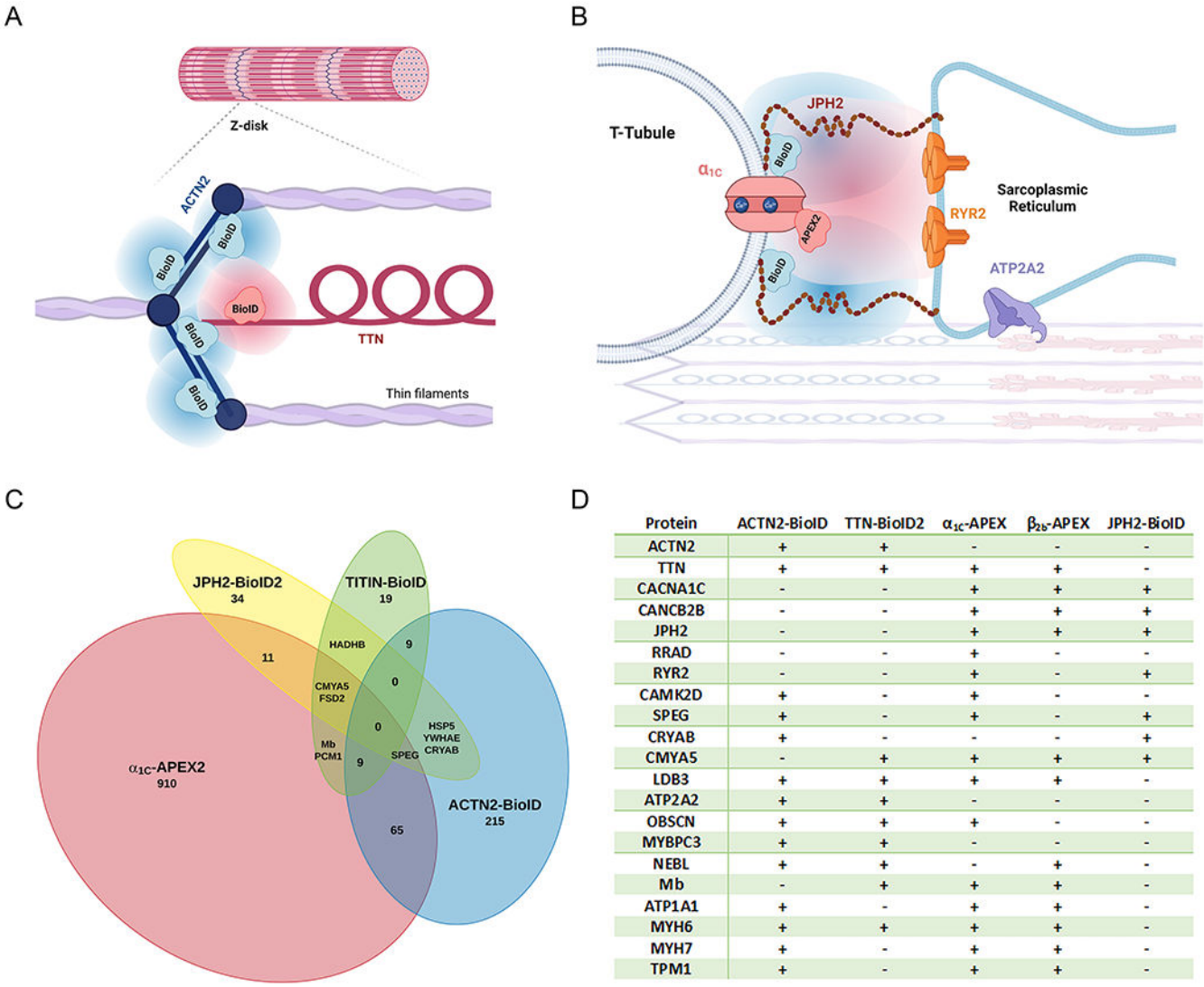

Protein	ACTN2-BioID	TTN-BioID2	α_{1C}-APEX	β_{2b}-APEX	JPH2-BioID
ACTN2	+	+	-	-	-
TTN	+	+	+	+	-
CACNA1C	-	-	+	+	+
CANCB2B	-	-	+	+	+
JPH2	-	-	+	+	+
RRAD	-	-	+	-	-
RYR2	-	-	+	-	+
CAMK2D	+	-	+	-	-
SPEG	+	-	+	-	+
CRYAB	+	-	-	-	+
CMYA5	-	+	+	+	+
LDB3	+	+	+	+	-
ATP2A2	+	+	-	-	-
OBSCN	+	+	+	-	-
MYBPC3	+	+	-	-	-
NEBL	+	+	-	+	-
Mb	-	+	+	+	-
ATP1A1	+	-	+	+	-
MYH6	+	+	+	+	-
MYH7	+	-	+	+	-
TPM1	+	-	+	+	-

Figure 5: Proximity proteomes of the cardiac Z-disk and cardiac dyad.

(A-B) Schematics illustrating the foci of published proteomic mapping studies located at the Z-disk (A) and the overlying sarcolemma (B). **(C)** Overlapping proteomic mapping of sarcolemmal and Z-disk protein neighborhoods. Proteins included were described by authors as showing significant enrichment in the neighborhood of their protein of interest. *In* vivo proximity labeling occurred in knock-in mice with BioID fused to *Ttn*, BioID2 fused to *Jph2*, human iPSC-derived cardiac myocytes with knock-in of BioID fused to *ACTN2*. Live *in-situ ex-vivo* proximity labeling was examined in mice with inducible transgenic overexpression of the $Ca_V1.2$ α_{1C} subunit fused to APEX2. **(D)** Enrichment of proteins critical for cell structure, Ca^{2+} homeostasis, contraction and signaling were noted across multiple proximity proteomes, including those from isolated myocytes from mice overexpressing $Ca_V1.2$ β_{2B}-APEX2. Created in part with LucidChart and Biorender.com [14, 41, 42, 56].

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Table 1:

Proximity-dependent biotinylation methods [5, 6, 8, 10, 15, 22–24].

Enzymes	**APEX2[8]**	**HRP[6, 23]**	**BioID[5]**	**BioID2[22]**	**TurboID[9]**
Type	Peroxidase	Peroxidase	Biotin ligase	Biotin ligase	Biotin ligase
Size (kDa)	28	44	35	27	35
Active region	intracellular	extracellular, secretory pathway, inactive in cytosol	intracellular	intracellular	intracellular
Model organisms	mammalian cell culture, bacteria, yeast, flies, mice	mammalian cell culture, flies, primary human tissue	mammalian cell, culture, yeast, flies, plants, mice	mammalian cell culture, mice	mammalian cell, culture, yeast, flies, plants, worms
Labeling time	30 sec-1 min	1 min	18 h	18 h	10 min
Modification sites	preferentially Tyr, also Trp, Cys, His	Tyr, Trp, Cys, His	Lys	Lys	Lys
Substrates for protein labeling	biotin-phenol, H_2O_2	biotin-phenol, H_2O_2	biotin, ATP	biotin, ATP	biotin, ATP
Half-life of labeling	<1msec	<1msec	minutes	minutes	minutes
Advantages	High temporal resolution; versatility for both protein and RNA labeling	High temporal resolution; versatility for both protein and RNA labeling	Non-toxic for *in vivo* applications	Non-toxic for *in vivo* applications	Highest activity biotin ligase; non-toxic for *in vivo* applications
Limitations	Limited application *in vivo* because of the toxicity of H_2O_2 and low biotin-phenol permeability. H_2O_2 quickly decomposes.	Limited application *in vivo* because of the toxicity of H_2O_2; limited to secretory pathway and extracellular applications	Poor temporal resolution because of low catalytic activity	Poor temporal resolution because of low catalytic activity	Potentially less control of labeling window because of high biotin affinity, potential toxicity in long-term experiments
Notes	Can be used for electron microscopy	Can be used for electron microscopy; can be used when conjugated to antibodies	Reduced activity below 37 °C	Reduced activity below 37 °C	Can be used when conjugated to antibodies