In vivo proximity labeling identifies cardiomyocyte protein networks during zebrafish heart regeneration

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Abstract

Strategies have not been available until recently to uncover interacting protein networks specific to key cell types, their subcellular compartments, and their major regulators during complex in vivo events. Here we apply BioID2 proximity labeling to capture protein networks acting within cardiomyocytes during a key model of innate heart regeneration in zebrafish. Transgenic zebrafish expressing a promiscuous BirA2 localized to the entire myocardial cell or membrane compartment were generated, each identifying unique proteomes in adult cardiomyocytes that became altered during regeneration. BioID2 profiling for interactors with ErbB2, a co-receptor for the cardiomyocyte mitogen Nrg1, implicated Rho A as a target of ErbB2 signaling in cardiomyocytes. Blockade of Rho A during heart regeneration, or during cardiogenic stimulation by the mitogenic influences Nrg1, Vegfaa or Vitamin D, disrupted muscle creation. Our findings reveal proximity labeling as a useful resource to interrogate cell proteomes and signaling networks during tissue regeneration in zebrafish.
Introduction

Understanding how and why tissue regeneration occurs is a central objective of developmental biology. Genome-wide transcriptome or chromatin profiling experiments using RNAseq, scRNAseq, ATACseq, or ChiPseq approaches have helped to identify novel factors, mechanisms, and concepts that have informed our understanding of regeneration in many tissues, injury contexts, and species (1-3). Although key for detecting and inferring changes in gene transcription, chromatin organization, or DNA–protein associations, these strategies are limited in their abilities to identify protein and signaling complexes that carry out changes at the cell and tissue level. Co-immunoprecipitations and affinity-purification mass spectrometry (AP/MS/MS) are well-established techniques to probe protein interactions and networks. Yet, they require tissue dissociation and flow cytometry to acquire cell type-specific interactions, and weak interactions between proteins often escape detection (4).

Proximity labeling is a relatively recent technology established to probe protein interactions in specific sub-tissue and subcellular compartments, represented by multiple versions including BioID2, TurboID, and APEX2 (5-7). Here, engineered ligases that attach biotin to lysine residues are fused with a protein of interest such that, when expressed in cells, proteins in close proximity are indiscriminately biotinylated. The biotinylated proteome is isolated by streptavidin affinity, and proteins are identified by quantitative mass spectrometry. Most studies have applied proximity labeling to *in vitro* tissue culture systems, whereas many fewer have explored *in vivo* networks in mice or *Drosophila* (8-12). In the latter studies, proximity labeling captured the proteome within interneuron-neuron interactions, within muscle sarcomeres, and in the ring canal of *Drosophila* oocytes, producing new insights into protein network assemblies and signaling. The principles of proximity labeling should be capable of identifying key protein networks during dynamic cellular processes like embryonic development and tissue regeneration without the need for tissue disruption. Notably, proximity labelling has yet to be applied in central animal models of regeneration such as zebrafish and axolotl salamanders.
Unlike mammals, teleost zebrafish regenerate cardiac muscle after injury with minimal scar formation, based on division of pre-existing cardiomyocytes (13-15). Understanding mechanisms of innate cardiac repair in animals like zebrafish has the potential to define and overcome barriers to cardiac regeneration in humans. Evidence to date indicates that heart regeneration in zebrafish is a relentless process that outcompetes scarring to recover the heart function, rather than a linear series of molecular steps and synchronized cellular progressions. Intrinsic programs in cardiomyocytes and the ability to receive extrinsic proliferative cues prime cardiomyocytes for cell division. Many ligands, receptors, and transcription factors have been shown to be required for heart regeneration (16-21). Three secreted factors, Nrg1, Vegfaa, and Vitamin D, have been demonstrated to have instructive mitogenic effects, characterized by the ability to induce cardiomyocyte proliferation in zebrafish in the absence of injury (22-24). Nrg1/ErbB2 signaling can stimulate cardiomyocyte division in adult mice, most notably when ErbB2 is experimentally expressed in the form of a ligand-independent activator (25-27). ErbB2 signaling was recently reported to work in part through regulation of YAP, a transcriptional activator that can impact cytoskeletal remodeling and cell division in cardiomyocytes during cardiac growth or repair in mice (28-31). In zebrafish, Nrg1/ErbB2 signals in part by promoting glycolysis versus mitochondrial oxidative phosphorylation, and by reducing Tp53 levels through upregulation of its inhibitor Mdm2 (32-34).

Signal transduction mechanisms by which ErbB2 and other mitogenic factors support cardiomyocyte proliferation versus other outcomes, could be illuminated by customized proteomic profiling. Proteomic studies of heart regeneration to date have faced challenges, including lack of cell type specificity and limited detection of changes in poorly represented members of the proteome due to the abundance of sarcomeric proteins. (35-37). Here we applied a transgenic BioID2 strategy to capture cell-specific proteome changes in cardiomyocytes during heart regeneration in adult zebrafish. We show that BioID2 can be used to track changes in protein levels in whole cardiomyocytes or their membrane compartments during regeneration. We also
investigate the proximal proteome of ErbB2 in cardiomyocytes, finding an increase in association with the small GTPase Rho A during regeneration, a protein we find to be essential for injury-induced regeneration and the proliferative response to mitogens. Our findings demonstrate the utility of proximity labeling as a resource to interrogate cell proteomes and signaling networks during tissue regeneration in zebrafish, and they identify a key role of small GTPases in injury-induced cardiogenesis.
Results and Discussion

BioID2 detects cardiomyocyte proteomes in adult zebrafish

BioID2 employs the promiscuous BirA2, which attaches biotin molecules to the lysines of proteins within an approximate distance of 10 nm (5). We reasoned that the lysines of sarcomeric proteins might be less accessible when packed in supermolecular complexes, and therefore less likely to undergo biotinylation. To determine whether BioID2 is functional in cardiomyocytes of adult zebrafish, we generated a transgenic line with a cassette encoding BirA2 fused to GFP and placed downstream of the myosin light chain 7 (cmlc2, or mlc7) promoter (Figure 1A, B). BirA2-GFP was readily detected in adult zebrafish cardiac chambers, where it was distributed throughout the cytoplasm and nuclei of adult cardiomyocytes (Figure 1C). To test whether BirA2 expressed in this manner biotinylates the cardiomyocyte proteome, we supplied biotin either in the aquarium water or via intraperitoneal injections over three consecutive days. We found effective protein biotinylation occurred only when biotin was delivered by injection (Figure 1F, G; Figure 1- figure supplement 1A-E). Thus, transgenic BioID2 systems can biotinylate proteins in cardiomyocytes of adult zebrafish.

To test whether BioID2 can detect proteins that are compartmentalized in cardiomyocytes, we generated transgenic zebrafish with BirA2-GFP expected to be localized to membranes via a CAAX motif (Figure 1A, D-E). We found that the majority of BirA2-GFP-CAAX signal associated with the cardiomyocyte plasma membrane in cmlc2:birA2-GFP-CAAX hearts (Figure 1E; Figure 1- figure 1 supplement 2A, B). Although BirA2-GFP-CAAX was enriched at membranes, direct staining for biotin revealed signals at membranes and cytosol, suggesting release of labeled proteins from the membrane over the 3-day incubation period (Figure 1- figure supplement 2C-G). To capture the membrane proteome of cardiomyocytes, we conducted the BioID2 assay on adult cmlc2:bira2-GFP or cmlc2:bira2-GFP-CAAX hearts (Figure 1I). Quantitative BioID2 analysis captured 1113 unique proteins, of which 343 proteins showed a 2.5-fold enrichment in BirA2-CAAX samples when compared to BirA2-GFP samples (Table 1). The threshold of 2.5-fold has
previously been used in in vivo BioID2 studies in mice (38). Gene ontology analysis for cellular components of the 343 proteins revealed enrichment for proteins associated with cellular junctions, plasma membrane, and membrane-bound organelles (Figure 1J). Taken together, our results indicate that in vivo proximity labeling using BioID2 is functional in zebrafish and can profile proteomes of highly specialized cells like cardiomyocytes.

**BioID2 detects proteome changes in cardiomyocytes during heart regeneration**

To capture the changes in the protein network of cardiomyocytes during regeneration, we combined our BirA2 system with a transgenic system to ablate ~60% of cardiomyocytes through the tamoxifen (4-HT)-inducible expression of a Cre-induced diphtheria toxin A gene (39). We injected biotin for 3 days and collected hearts at 14 days post incubation (dpi), followed by purification of the biotinylated proteome (Figure 2A, B). Proliferation of cardiomyocytes in this injury model is most prominent around 7 dpi, with regeneration of the myocardium essentially complete by 28 dpi (Figure 2 – figure supplement 1A-C). We assessed cmlc2:bira2-GFP hearts in two different conditions, uninjured (treated with 4-HT but no CreER transgene) and 14 dpi. Quantitative mass spectrometry analysis of pooled samples revealed that 208 proteins showed a 1.5-fold or greater change during regeneration when compared to uninjured hearts (Figure 2D, Table 2).

It is common in proximity labeling assays to normalize levels of the protein of interest to those of a diffuse BirA2-labeled protein. However, this requires a high threshold for protein changes to avoid false positives, diminishing the gating of proteins with subtle fold changes. Normalizing the regenerating heart dataset of a transgenic strain to the uninjured control of the same strain allowed a low threshold. Gene ontology analysis (over-representation test) for biological processes characterized the proteins with increased levels during regeneration in the whole-cell BioID2 set as involved in wound healing, cardiac development, muscle cell differentiation and response to wounding, whereas decreased proteins were enriched for metabolites and energy.
precursors (Figure 2D). The latter category includes the generation of ADP to ATP and oxidative phosphorylation, implying that these processes are dampened during dedifferentiation and proliferation of cardiomyocytes, as suggested by recent publications (33, 34). The top 10% of increased proteins included ErbB2 and the mTOR signaling protein Rictora (Figure 2 – figure supplement 1D), each previously implicated in heart regeneration (22, 40). We compared the BioID2 dataset to published RNA-seq or ChIP-seq datasets, which each identified a much greater number of differential products than BioID2 (1,10,41,42). Correlations were generally weak, with the highest being an RNA-seq dataset from 14 dpi hearts at r = 0.39 (Figure 2 – figure supplement 2A-E) (41). We suspect that any post-transcriptional regulation incorporated in BioID2 data contributes to these weak correlations. Beyond this, there are several possible technical explanations, including differences in assessed timepoints, injury models, and cell types, and limitations of BioID2 in capturing targets. Regardless, our results indicate that proximity labeling with BirA2 can identify biological processes that are involved in tissue regeneration and proteins that have been reported to regulate cardiac repair.

To investigate how the membrane proteome changes after heart injury, we examined cmlc2:bira2-GFP-CAAX samples at 14 dpi (Figure 2A, B; Figure 2 – figure supplement 3A-C). We identified 173 proteins with a 1.5-fold or greater change in membrane-associated proteins during regeneration when normalized to uninjured hearts (Figure 2C, E; Table 3), with the majority of these proteins reducing presence during regeneration. This contrasts with BirA2-GFP samples that showed more proteins increasing levels during regeneration than those decreasing (Figure 2D vs. 2E). Proteins with reduced membrane levels during regeneration showed overrepresentation of involvement in transmembrane transport, muscle development and energy production (electron transport and glycolysis), while proteins increased at the membrane during regeneration were involved in protein complex assembly and organelle organization (Figure 2E). Intracellular restructuring is known to occur in dividing cardiomyocytes (43). Upon comparison of the top and bottom 10% of the membrane profiling data with BirA2-GFP data (Figure 2 – figure
supplement 3D vs Figure 2 – figure supplement 1D), very few proteins were found in both datasets, suggesting specificity of the membrane BioID2 data.

Comparison of proteins found in BirA2-GFP and BirA2-GFP-CAAX datasets identified 44 shared proteins (Figure 2C). Of these, 16 proteins showed opposing changes in levels at the membrane or in the whole cell (Figure 2F). To visualize dynamism of proteins for which there were available candidate antibodies during regeneration, we assessed some of these proteins by immunofluorescence in a model of regeneration in which ~20% of the ventricle is resected (13). Erythrocyte membrane protein band 4.1 like 5 (Epb41l5) is involved in animal organ development and has been implicated as a positive regulator of Notch signaling, a pathway involved in heart regeneration (44, 45) (Figure 2F, G). Epb41l5 was predominantly localized at the plasma membrane (Figure 2G, arrows) and increased presence throughout cardiomyocytes during regeneration, consistent with the BioID2 data. Talin2, an adapter protein that couples integrin focal adhesions to the actin cytoskeleton (Figure 2 – figure supplement 4A, B) displayed low levels in uninjured hearts (Figure 2 – figure supplement 4B). Upon injury, Talin2 levels increased near the wound site as well as in ventricular muscle away from the injury, with a punctate pattern appearing that resembled focal adhesions (Figure 2 – figure supplement 4B). These results are consistent with a remodeling of focal adhesions during myocardial stress induction that has been reported for Talin 1 (46). To assess BioID2 results implicating proteins that increase levels at the cell membrane during regeneration, we identified antibodies against SET nuclear proto-oncogene a (Seta), which is involved in nucleosome assembly (Figure 2F, H; Figure 2 – figure supplement 4A, C). Seta levels were undetectable in uninjured hearts, yet became prominent in compact muscle and epicardial tissue in injured ventricles (Figure 2H, Figure 2 – figure supplement 4C). The Seta signal was perinuclear and colocalized with Wheat germ agglutinin (WGA), which indicates that Seta associates in part with the nuclear membrane and endomembranes after heart injury (Figure 2H, arrows; Figure 2 – figure supplement 4C). As Seta has been proposed to silence Histone acetyltransferase (HAT)-dependent transcription, its recruitment to nuclear and
endomembranes could impact this function (47). Taken together, our data indicate that BioID2 can capture changes in the proteomes of cardiomyocytes during heart regeneration, providing a resource for candidate effectors.

**Rho A interacts with ErbB2 during heart regeneration**

Nrg1 ligand has been reported to bind its receptor ErbB4 on cardiomyocytes, causing heterodimerization of ErbB4 and its co-receptor ErbB2 (25, 48). To probe the network of ErbB2-signaling during heart regeneration in zebrafish, we generated the transgenic line cmic2:erb2- birA2-HA-P2A-GFP (Figure 3A), with BirA2 fused to the C-terminal of ErbB2. We detected ErbB2-BirA2-HA via western blotting analysis, although we could not visualize it by immunofluorescence (Figure 3 – figure supplement 1A). We also detected biotinylation of cardiac proteins in this line in the presence of injected biotin (Figure 3 – figure supplement 1A). To assess dynamic ErbB2 networks during heart regeneration, we crossed cmic2:erb2-birA2-HA-P2A-GFP with ZCAT animals to ablate cardiomyocytes as described above and collected ventricles at 14 dpi (Figure 3B, Figure 3 – figure supplement 1B-D). Quantitative mass spectrometry analysis identified 667 proteins, of which 108 showed a greater than 1.5-fold change during regeneration when normalized to uninjured control hearts (Table 4). Using STRING and BioGRID databases, we identified 9 known ErbB2 interactors in our dataset. Three of these proteins showed increased association with ErbB2 during regeneration, whereas two had decreased association (Figure 3C, yellow and blue circles, respectively). ErbB4, encoded by erbb4a and b paralogs was not detected in any of the BioID2 datasets, which might either indicate that ErbB4 does not associate with ErbB2 in the zebrafish heart, or that their C-terminal regions are not in close proximity, or a limitation in sensitivity of the assays. The strongest detected increase was an association with the Rho GTPase Rho A-b, and pathway analyses indicated enrichment for proteins involved in cytoskeletal regulation by Rho GTPases (Figure 3C, D). Rho A activation by ErbB2 has been previously reported to promote pro-metastatic and invasive behavior of human breast cancer, and...
to contribute to mammary adenocarcinoma tumorigenesis (49, 50). To test whether ErbB2-Rho A association is increased during regeneration, we conducted co-immunoprecipitations (Figure 3E). Interestingly, Rho A was pulled down with ErbB2-BirA2-HA in regenerating heart samples, but not from uninjured myocardium, suggesting a context-dependent association (Figure 3E).

As many direct interactors of Rho A have been identified, we searched our dataset of whole cardiomyocyte changes at 14 dpi for these proteins. Levels of myocardial Rho A proteins detected by BioID2 were similar in uninjured and regenerating hearts, consistent with previous published transcriptome and epigenome data sets (Table 5) (1, 20). Yet, all known Rho A interactors detected in our cmilc2:birA2-GFP data set showed elevated levels after injury, including ErbB2 (59.2-fold; Figure 3F). Most of the detected Rho A interactors are involved in cytoskeletal organization such as Moesin a, WASP-like actin nucleation promoting factor a, profilin 1, Catenin δ1 and ROCK2a, or cell adhesion such as Scribble and Integrin-linked kinase (Figure 3F). The signal transducer Rho GTPase activating protein 21b, the Guanine nucleotide binding protein (G protein) alpha 13a, which is involved in heart field formation, and Reticulon 4a, which plays a role in EMT-transition, were also found to be increased after injury in the whole cell BioID2 data set (Figure 3F)(51, 52). Rho A signaling is known to facilitate cytoskeletal changes via Rho-associated protein kinases (ROCK), which have been reported to regulate the DNA binding activity of the cardiogenic transcription factor Gata4 and nuclear localization of SRF in cell culture (53-55). ROCK2a levels were elevated in our whole cell BioID2 dataset, further confirmed by western blotting (Figure 3G), consistent with the idea that ErbB2–Rho A association could impact ROCK2 levels in cardiomyocytes after injury. Thus, BioID2 interrogation of the co-receptor ErbB2 captured the network of ErbB2 during heart regeneration, a potential resource to uncover novel ErbB2-interacting proteins, and revealed increased association of Rho A and ErbB2 in regenerating cardiomyocytes.

_Rho A activity is essential for mitogen-stimulated cardiomyocyte proliferation_
To evaluate a potential role for Rho A signaling during cardiac repair, we treated zebrafish with the Rho A inhibitor Rhosin for 3 consecutive days after partial ventricular resection and assessed indicators of cardiomyocyte proliferation (Figure 4A, B). In these experiments, Rhosin reduced the proliferation index by ~68% (Figure 4C). To test whether regeneration is inhibited or delayed when Rho A signaling is blocked, we generated a transgenic line that enables tamoxifen-inducible expression of a dominant-negative Rho A cassette in cardiomyocytes. (Figure 4F, top panel). In animals expressing a dominant-negative Rho A for 30 days after resection (Figure 4F), injury sites remained deficient in cardiac myofibers, whereas in control hearts had re-established a contiguous wall of muscle (Figure 4F). Proteomic analyses of our whole cell BioID2 dataset also implicated the small GTPases Rac1 and Cdc42 in heart regeneration; although protein levels remained unchanged, pathway analyses showed enrichment for signaling by Rho GTPases and its effectors (Figure 4D, E). Similar to Rho A, the interactome maps of Rac1b and Cdc42 revealed many known interactors increased in the whole cell dataset after injury (Figure 4 – figure supplement 1A, B). Using inducible dominant-negative gene cassettes in transgenic animals, we found that Rac1 and Cdc42 are similarly required for normal heart regeneration (Figure 4F, middle and bottom panels). Thus, our data implicate each member of the small GTPase trio - Rho A, Rac1 and Cdc42 – in zebrafish heart regeneration.

To further test whether Rho A function is required for the effects of ErbB2 signaling, we used transgenic fish enabling tamoxifen-inducible overexpression of Nrg1 ligand in cardiomyocytes (Figure 5A) (22). Depending on the duration of Nrg1 overexpression, these animals display overt cardiomyocyte hyperplasia and thickening of the ventricular wall (Figure 5C). We examined Rho A levels from hearts that ectopically expressed Nrg1 for 14 days, and unlike during ablation-induced heart regeneration where Rho A levels are unchanged, we found an ~85% increase in total Rho A protein (Figure 5 – figure supplement 1A, B). Moreover, 3 daily injections of zebrafish with the Rho A inhibitor Rhosin reduced the cardiomyocyte proliferation index by ~71% in the
presence of ectopic Nrg1 expression (Figure 5B, C, F). These data indicate that Rho A function is an essential component of mitogenic Nrg1 /ErbB2 signaling in cardiomyocytes.

To examine whether Rho A is key for the activity of other cardiomyocyte mitogens, we tested Rho A levels and the effects of its inhibition during tamoxifen-induced myocardial vegfaa overexpression or cardiomyocyte proliferation induced by the vitamin D analog Alfacalcidol (Figure 5A, B). Western blot analysis revealed that vegfaa-expressing hearts elevated cardiac Rho A levels, whereas Alfacalcidol-treated hearts displayed no significant change (Figure 5 – figure supplement 1A, B). Inhibition of Rho A by Rhosin reduced cardiomyocyte proliferation by ~68% and ~65% during vegfaa overexpression and Alfacalcidol treatment, respectively (Figure 5E, H). In total, our data indicate that Rho A signaling is a major target during heart regeneration, required for normal cardiomyocyte proliferation in response to presence of Nrg1, Vegfaa, or Vitamin D.

Conclusions

Here we have applied the proximity labeling technique BioID2 to investigate cell-specific proteome changes during a key model for innate tissue regeneration, the renewal of cardiomyocytes upon massive injury to the zebrafish heart. We identified changes specific to cardiomyocytes at the level of whole-cell, membrane compartment, and the molecular interface of a key pro-regenerative factor ErbB2. Our profiling and functional experiments implicate the GTPase Rho A as mediating the effects of Nrg1 and other mitogens in cardiomyocytes during regeneration, and they provide a resource for which other candidate regenerative effectors can be assessed. We expect that BioID2 and evolved iterations can be effectively applied in myriad ways to monitor proteome changes during dynamic developmental events like regeneration in many species, tissues, and injury contexts, to provide high-resolution insights into essential networks of regeneration.
Materials and Methods

Zebrafish

Wild-type or transgenic zebrafish of the hybrid EK/AB strain at 3-8 months of age were used for all experiments. All transgenic strains were analyzed as hemizygotes. Published transgenic strains used in this study were cmlc2:CreER (Tg(cmlc2:CreER)^{pdi0}) (14), used with β-act2:BSnrg1 (22), β-act2:BSvegfaa (23) or bactin2:loxP-mCherry-STOP-loxP-DTA (39). Tamoxifen treatment to deplete cardiomyocytes performed as described previously (39). by incubating cmlc2:CreER; βactin2:loxP-mCherry-STOP-loxP-DTA animals in 0.7 μM Tamoxifen (Sigma-Aldrich, St. Louis, MO) for 16 h. Nrg1 and Vegfaa expression were induced as described by incubating adult cmlc2:CreER; β-act2:BSNrg1 or cmlc2:CreER; β-act2:BSVegfaa animals, respectively, in 5 μM Tamoxifen for 24 h. For expression of DN-Rho A, DN-Rac1 and DN-Cdc42, and Cre-negative animals were bathed in 5 μM of 4-HT for 24 h. For treatment with Rho A inhibitor 5 μM of a 250 μm Rhosin (54) solution were injected on 3 consecutive days prior to harvest. Clutch mates were treated with vehicle. EdU injections were performed as described (32). Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Duke University.

Generation of transgenic zebrafish lines

birA2 (a gift from Scott Soderling) was subcloned into the cmlc2-vector (14) into BamHI/Nhel sites to generate cmlc2:birA2. To generate cmlc2:birA2-GFP, an EGFP cassette was subcloned downstream of BirA2 using Nhel/EcoRI sites. A CAAX-tag was inserted into the EcoRI site to generate cmlc2:birA2-GFP-CAAX. erbb2 cDNA was amplified with the following primers and (Forward 5′-GCCACCATGGAGGCGGACAGAAGTTT-3′, Reverse 5′-TCAGGTGTACTCCTTGTGGCCG-3′) and then subcloned into cmlc2-birA2 vector using the BsiWI/NruI sites. HA-P2A-GFP was then inserted into cmlc2:erbb2-birA2 by using Nhel/Xhol sites.
to generate cmlc2:erbb2-birA2-HA-P2A-GFP. Mouse GFP-DN-Rho A (Forward 5’-ACCGCCATGGTGAAGGGCGAAGAG -3’, Reverse 5’-TCTGGTTGCCTTGTCTTGAGA -3’), GFP-DN-Rac1 (Forward 5’-ACCGCCATGGTGAAGGGCGAAGAG -3’, Reverse 5’-GAAGAGAAAATGCCTGCTGTTTA -3’), and GFP-DN-Cdc42 (Forward 5’-ACCGCCATGGTGAAGGGCGAAGAG -3’, Reverse 5’-CAACCCCCAAAAGGAAGTGCTGTATTTTCTAA -3’) were a gift from Scott Soderling and were each cloned into βactin2:loxp-dsRed-STOP-loxp vector (55) by using the AgeI/NotI sites. Each of these constructs was co-injected with I-Sce-I enzyme mix into one-cell-stage embryos. One founder of each construct was isolated, although they were not maintained as stable lines. The full names of the transgenic lines are as follows Tg(cmlc2:birA2-GFP)pd335, Tg(cmlc2:birA2-GFP-CAAX)pd336, Tg(erbb2-birA2-HA-P2A-GFP)pd337, Tg(βactin2:loxp-BFP-STOP-loxp-DN-Rho)pd68, Tg(βactin2:loxp-BFP-STOP-loxp-DN-Rac)1pd69, and Tg(βactin2:loxp-BFP-STOP-loxp-DN-Cdc42)pd70.

**BioID2 assay and quantitative mass spectrometry analysis**

Twenty hearts from adult zebrafish 3-8 months old were pooled for each sample, and triplicates were used for each condition. Prior to isolation of hearts, 10 μl of a 500 μM biotin/PBS solution was interperitoneally (IP) injected into adult zebrafish on 3 consecutive days. Ventricle and outflow tract but not atrium were extracted, rinsed in cold PBS and collected in ice-cold RIPA buffer. Using an Eppendorf tube pestle, hearts were lysed by 50 strokes, then flash frozen in EtOH/dry ice, incubated on ice for 15 min, and then again homogenized with 50 strokes of an Eppendorf tube pestle. Lysates were then centrifuged at 21,000 rpm for 20 min at 4°C. The supernatant was transferred to a new tube. Meanwhile, NeutrAvidin beads were pre-washed in keratin-free conditions in a laminar flow hood. 25-50 μl of bead slurry were pipetted into a low-protein-binding tube and spun down for 30 sec at 2000 rpm at RT. Supernatant was removed with a 30g needle.
attached to 1 ml syringe. Beads were washed 5 times with 500 ml RIPA buffer. Next, washed beads were resuspended in 100 μl RIPA buffer and added to each sample. Each tube was sealed with parafilm and incubated overnight with rotation in 4°C cold room. The next day, samples were centrifuged for 1 min at 3,000 x g at 4°C to pellet beads, and the supernatant was removed using a 30g needle. 500 μl 2% SDS in 50 mM ammonium bicarbonate in MS-grade water was added to the bead pellet, and the mixture was transferred into a low protein binding tube. Beads were then washed with (1) 2 times with 500 μl 0.5% SDS in 50 Mm ammonium bicarbonate, (2) 2 times with 1% Triton X-100/1% deoxycholate/25 mM LiCl in 50 Mm ammonium bicarbonate, (3) 2 times with 0.5M NaCl in 50 Mm ammonium bicarbonate, and 2 times with 50mM ammonium bicarbonate solution. For each wash step 500 μl wash solution was used and samples were rocked for 10 min at RT. Samples were then centrifuged at 2000 g for 30 sec at RT, and the supernatant was removed with a 30g injection needle. To elute the biotinylated proteome, an elution buffer containing 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, 62.5 mM Tris pH 6.8 (NO bromophenol blue) in MS-grade water was prepared and biotin was added freshly to a final concentration of 2.5 mM. 50 μl elution buffer were added to the washed beads. Samples were boiled for 5 min at 95°C on a heat block. During the elution, samples were slowly vortexed 3 times. Next, samples were centrifuged for 30 sec at 2000 g at RT, and the supernatant was carefully removed using a new 30g needle attached to a 1 ml syringe and transferred to a new low protein-binding tube. Samples were stored at -80°C until delivery to the Duke Proteomics Core Facility (DPCF).

Samples were reduced with 10 mM dithiolthreitol for 30 min at 80°C and alkylated with 20 mM iodoacetamide for 30 min at room temperature. Next, they were supplemented with 15 μL of 20% SDS in 50 mM TEAB, a final concentration of 1.2% phosphoric acid, and 555 μL of S-Trap (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins were trapped on the S-Trap, digested using 20 ng/μl sequencing grade trypsin (Promega) for 1 hr at 47C, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All samples were then lyophilized and
resuspended in 12 μl 1%TFA/2% acetonitrile containing 12.5 fmol/μl yeast alcohol dehydrogenase (ADH_YEAST). From each sample, 3 μl was removed to create a QC Pool sample which was run periodically throughout the acquisition period. Quantitative LC/MS/MS was performed on 3 μl of each sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Thermo Fusion Lumos high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 20 mm × 180 μm trapping column (5 μl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8 μm Acquity HSS T3 C18 75 μm × 250 mm column (Waters Corp.) with a 90-min linear gradient of 5 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55°C. Data collection on the Fusion Lumos mass spectrometer was performed in a data-dependent acquisition (DDA) mode of acquisition with an r=120,000 (at m/z 200) full MS scan from m/z 375 – 1500 and a target AGC value of 2e5 ions with a 2 sec cycle time. Ion trap MS/MS scans were acquired with a Rapid scan rate, 100 ms max injection time, and a target AGC value of 5e3 ions. A 20s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each sample injection was approximately 2 hours.

Following UPLC-MS/MS analyses, data were imported into Proteome Discoverer 2.2 (Thermo Scientific Inc.), and analyses were aligned based on the accurate mass and retention time of detected ions (“features”) using Minora Feature Detector algorithm in Proteome Discoverer. Relative peptide abundance was calculated based on area-under-the-curve (AUC) of the selected ion chromatograms of the aligned features across all runs. The MS/MS data was searched against the TrEMBL D. rerio database (downloaded in May 2018) with additional proteins, including yeast ADH1, bovine serum albumin, as well as an equal number of reversed-sequence “decoys”) false discovery rate determination. Mascot Distiller
and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion spectra and to perform the database searches. Database search parameters included fixed modification on Cys (carbamidomethyl) and variable modifications on Meth (oxidation) and Asn and Gln (deamidation). Peptide Validator and Protein FDR Validator nodes in Proteome Discoverer were used to annotate the data at a maximum 1% protein false discovery rate.

Relative peptide abundance of triplicates was averaged for each protein, and proteins were confirmed as positive with $p < 0.05$. As biotinylation of proteins depends on lysine availability at the contact site, proteins with 1 unique peptide were included in the data if relative abundance fit the outlined criteria above. Injured hearts were normalized to uninjured hearts, and a fold change of 1.5-fold was used as a threshold for protein level change. For the membrane proteome, uninjured BirA2-GFP-CAAX expressing hearts were normalized to BirA2-GFP hearts, and proteins with a 2.5-fold change were considered to be membrane-specific.

Cytoscape was used to create interactome maps. BioGRID and Scaffold were used to search for interactors of ErbB2, Rho A, Rac1b and Cdc42. Panther gene list analysis was used for over-representation tests in cellular components, signaling pathways and reactome analysis of the BioID2 data sets.

BioID2 MS proteomics datasets have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier XXXX, and are also summarized in Table 6 and Table 7.

**Immunofluorescence and imaging**

Primary and secondary antibody staining were performed as described (58). EdU/Mef2 staining of unfixed sections of ventricles was performed as follows: Fish were injected intraperitoneally with 10 mM EdU on 3 consecutive days prior to harvest. For tissue preparation, hearts were
removed from animals and placed into ice-cold 30% sucrose/PBS, before mounting. Hearts were
flash-frozen in ETOH/Dry Ice and stored in -80°C. Cryosections were preformed to generate 10
μm thick sections, and slides were air-dried for 30 min and then stored at -20°C. For EdU/Mef2
staining, slides were incubated on a slide warmer for 10 min and then fixed in 3.7 %
formaldehyde/PBS at RT for 15 min. Next slides were washed 3 times 5 min in PBS. EdU staining
was performed by mixing 600 μl 1M Tris pH 8.5, 6 μl 1< CuSO₄, 6 μl 10 mM azide, and 600 μl
0.5 M ascorbic acid (made fresh). Slides were incubated for 30 min at RT. Next, washes were
performed in 2% Triton-X-100/PBS 4 times each 5 min at RT, and slides were blocked for 1 h at
RT in 2% Triton-X-100/PBS, 5% Goat serum, and 1 %BSA. Primary antibody anti-Mef2 (Abcam,
ab197070) was incubated 1:100 in 2% Triton-X-100/PBS and 5% goat serum overnight at 4°C.
The next day, slides were washed in 2% Triton-X-100/PBS 4 times each for 5 min at RT, and
stained with the secondary antibody for 2h at RT. Lastly. four 5 min washes in 2% Triton-X-
100/PBS were performed, and slides were mounted in Vectashield hard set DAPI reagent.

A Zeiss 700 confocal microscope was used to image slides. For EdU/Mef2 staining, images
were acquired of the three largest sections from each ventricle. Mef2⁺ and Mef2⁺/EdU⁺ cells were
counted manually using Fiji (ImageJ) from a 100 μm cortical area. Values from the sections were
averaged to compute a proliferative index for each animal. Five to 6 animals were sampled for
each condition.

Biochemical analysis

Co-Immunoprecipitations of Erbb2-HA in whole zebrafish hearts was performed as follows. Hearts
from 20 cmclc2:erbb2-birA2-HA-P2A-GFP; cmclc2:CreER; βactin2:lox-p-mCherry-STOP-loxp-DTA
animals treated with tamoxifen, or from 20 controls without cmclc2:CreER, were extracted 14 days
after treatment. Ventricle and outflow tract were isolated, rinsed in cold PBS, and collected in ice-
cold RIPA buffer. Using an Eppendorf tube pestle, hearts were lysed by 50 strokes, flash-frozen
in EtOH/dry ice, incubated on ice for 15 min, and then again homogenized with 50 stokes of an
Eppendorf tube pestle. Lysates were then centrifuged at 21,000 rpm for 20 min at 4°C, and the supernatant was transferred to a new tube. 50 μl were saved as an input control and kept on ice during the entire Co-IP protocol. Heart lysates were first pre-cleared with 20 μl of packed protein A beads (Sigma) per lysate on a rocker or tumbler at 4°C overnight. The next day, 1 μl of the anti-HA antibody (1 μl IgG rabbit for control) was incubated with 20 μl of protein A beads for 1 h on a rocker or tumbler at 4°C. Next, antibody-bead mix was added to the pre-cleared heart lysates and incubated for 2 h at on a rocker or tumbler at 4°C. Samples were three times washed with 500 μl lysis and spun down at 800 rpm for 1 min at 4°C. After the last wash, 50 μl of lysis buffer was added to the samples, and samples were boiled with Laemmli buffer and β-Mercaptoethanol at 95°C for 10 min. Samples were stored at -20°C before Western Blot analysis.

For whole heart lysates, the first part of the Co-IP protocol was followed until lysates were centrifuged at 21,000 rpm for 20 min at 4°C. The supernatant was isolated and a Bradford assay (BioRad) was performed to determine protein concentration. Then, samples were boiled with Laemmli buffer and β-Mercaptoethanol at 95°C for 10 min. 25 μg protein from each whole heart sample was used in Western Blot analysis.

**Antibodies and reagents**

Primary antibodies used in this study: anti-Mef2 (Abcam, ab197070, 1:100), anti-Rho A (Santa Cruz, 26C4,1:1000), anti-epb41L5 (RPL39, Proteintech, 1:100), anti-Talin 2 (Boster Biological Technology, PB9961, 1:100), anti-Seta (Antibodies online, ABIN2786762, 1:100), anti-ROCK2 (Bioss Antibodies, BS-1205R, 1:500), anti-HA (Abcam, ab9110, 1:1000), anti-CT3 (DSHB, 1:200), and anti-MHC (DSHB, F59-S, 1:200). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific, 1:200), Alexa Fluor 547 goat anti-mouse IgG (H + L) (Thermo Fisher Scientific, 1:200), EdU probes (Thermo Fisher Scientific, E10187), HRP goat anti-mouse IgG (Thermo Fisher Scientific, 31430, 1:50000), HRP goat anti-rabbit IgG (Thermo Fisher Scientific, 65-6120, 1:50000), and HRP goat streptavidin IgG (Pierce, 21130, 1:5000). Reagents
used in this study were Biotin (Sigma-Aldrich, B4501), Rhosin (Calbiochem, 555460), EDU (Life technology, A10044), Protein A sepharose beads (Sigma-Aldrich, P3391). Neutravidin Resin (Piere, Thermo Fisher Scientific 29202), and WGA alexa fluor 633 conjugate (Invitrogen, W21404, 1:100).
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Figure 1: In vivo BioID2 captures the proteome in cardiomyocytes of adult zebrafish hearts. (A) Schematic representation of a zebrafish heart. The heart consists of a ventricle, atrium and outflow tract (B) BirA2-GFP is expressed in cardiomyocytes via the cmlc2 promoter. (C) Section of ventricle from cmlc2:birA2-GFP transgenic animal. BirA2 is distributed throughout the
cardiomyocytes including the nucleus. DAPI staining and native GFP signal are shown. (D) BirA2-GFP-CAAX is expressed in cardiomyocytes via the cmlc2 promoter. (E) Section of ventricle from cmlc2:bira2-GFP-CAAX transgenic animal. The CAAX-tag localizes BirA2 to the membranes of cardiomyocytes. (F) Timeline of biotin administration by intraperitoneal injections (IP injections). (G) Western Blot analysis of biotinylation activity of BirA2-GFP expressing ventricles. BirA2 is functional and biotinylates sufficiently after 3 daily biotin injections. Endogenous biotinylated carboxylase was detected in untreated and biotin treated hearts. (H) Western Blot analysis of BirA2-GFP-CAAX-expressing ventricles. BirA2 is functional and biotinylates sufficiently after 3 daily injections with biotin, WT = wild-type. (I) Schematic summary of BioID2 assay on uninjured zebrafish hearts. (J) Over-representation test for cellular components. BioID2 for BirA-GFP-CAAX enriches for membrane associated proteins. 343 total proteins were gated at a 2.5-fold change when normalized to BirA-GFP. p < 0.001, FDR < 0.015%; dpt = days post treatment.
Figure 2: BioID2 identifies changes in membrane protein levels during heart regeneration.  
(A) Schematic overview of experimental workflow. *cmlc2:birA2-GFP* or *cmlc2:bira2-GFP-CAAX* 
vventricles were collected as uninjured samples or 14 days after induced cardiomyocyte ablation 
(dpi). (B) Timeline of injury and biotin injections. (C) Venn diagram comparing proteins captured 
by either the whole cell BioID2 assay or the membrane BioID2 data set that display at least a 1.5-
fold change. (D) Summary of BioID2 analysis of cmlc2:birA2-GFP hearts during regeneration. Left: Heatmap of proteins found in triplicates of quantitative mass spectrometry analysis. 208 proteins displayed a 1.5-fold change during regeneration when compared to uninjured hearts (p < 0.05). Of these, most protein levels increase during heart regeneration. Protein level changes in log10 scale. Right: Gene ontology analysis of BirA2-GFP BioID2 data set. Over-representation test of biological processes for at least 1.5-fold increased (yellow) and at least 1.5-fold decreased proteins (blue). Fold enrichment is shown, protein number is indicated in red. p < 0.001, FDR < 0.04%. (E) Summary of BioID2 on cmlc2:bira2-GFP-CAAX hearts. Left: Heatmap of proteins found in triplicates of quantitative mass spectrometry analysis. 173 proteins displayed an at least 1.5-fold change when compared to uninjured hearts (p < 0.05). Of these, most proteins decreased at the membrane during heart regeneration. Right: Gene ontology analysis of BirA2-GFP-CAAX BioID2 data set. p < 0.001, FDR < 0.05% (F) Heat map of proteins that have been identified in the BioID2 whole cell and membrane data sets and that display opposing changes in levels. Those shown changed at least 1.5-fold, p < 0.05. (G) Immunofluorescence against indicated proteins in ventricles. Epb41l5 is localized to the plasma membrane (marked by wheat germ agglutinin (WGA) staining) in uninjured hearts, with cytoplasmic fluorescence signals increasing during regeneration of resected tissue (14 dpa). (H) Seta is poorly detected in uninjured hearts, rising 7 days after resection injury in the epicardium and compact layer of the heart. Seta localizes around the nucleus and colocalizes with the membrane marker WGA. Scale bar in images 50 μm, magnification scale bar: 10 μm.
Figure 3: BioID2 identifies Rho A as a downstream target of ErbB2.
(A) ErbB2-BirA2-HA-P2A-GFP is expressed in cardiomyocytes via the cmhc2 promoter. (B) Summary of experiment and timeline, cmhc2:erbb2-birA2-HA-P2A-GFP ventricles were collected as uninjured samples or 14 days after induced cardiomyocyte ablation (dpi). (C) Known direct interactors of ErbB2 that were captured in the ErB2 BioID2 assay. 108 proteins showed a change greater than 1.5-fold when normalized to uninjured cmhc2:erbb2-birA2-HA-P2A-GFP ventricles. These data were analyzed for known ErbB2 interactors. Colors and size of interactor correspond to fold changes identified during regeneration. Green: no change; blue: levels decrease; yellow: levels increase. Tns1=Tensin-1 (1.57-fold), Hspb2= Heat Shock Protein beta 2 (-2.43-fold), Gapdh = Glyceraldehyde 3-phosphate dehydrogenase (p > 0.05), Acta1b = Actin alpha 1 (p >
0.05), Ezrb = Ezrin b (p > 0.05), Ctnnd1 = Catenin δ1 (-4.59-fold), Rhoab = Rho A-b (13-fold), Scrib = Scribble (p > 0.05), Anxa2a = Annexin A2a (7.31-fold). (D) Over-representation test - Pathway analysis of proteins increased 1.5-fold or more in ErbB2-BirA data set. P < 0.001, FDR < 0.005%. Fold enrichment is shown, protein number in red. (E) Co-immunoprecipitation of ErbB2-BirA2-HA from uninjured or regenerating cmic2:erbb2-birA2-HA-P2A-GFP hearts. Rho A association with ErbB2 is increased after injury. Anti-HA antibody was used for ErbB2 detection, Troponin T and IgG were used as loading controls. (F) Analysis of known Rho A interactors in BirA2-GFP BioID2 data set. All known direct interactors were found to be increased when normalized to uninjured hearts. Size of circles indicate fold change; proteins are sorted clockwise after fold change from high to low. Scrib = Scribble (90.9-fold), ErbB2 = Erb-b2 receptor tyrosine kinase 2 (52.9-fold), Gna13 = Guanine nucleotide binding protein alpha 13a (25.8-fold), Rtn4a = Reticulon 4a (8.82-fold), ROCK2a = Rho-associated, coiled-coil containing protein kinase 2a (5.05-fold), Ilk = integrin-linked kinase (3.91-fold), Pfn1 = Profilin 1 (3.25-fold), Arhgap21b = Rho GTPase activating protein 21b (2.65-fold), Wasla = WASP like actin nucleation promoting factor a (2.5-fold), Lrp5 = low density lipoprotein receptor-related protein 5 (2.21-fold), Ctnnd1 = Catenin δ1 (2.2-fold), Msna = Moesin a (1.9-fold). (G) Western Blot analysis of ROCK2 levels in uninjured and regenerating hearts. ROCK2 levels are increased during heart regeneration.
Figure 4: Small GTPases are required for cardiac regeneration. (A) Timeline of experiment for inhibiting Rho A function in 14 dpa hearts. (B) Immunofluorescence images of sections of 14 dpa ventricles vehicle or Rhosin treated and stained for EdU incorporation, an indicator of cardiomyocyte proliferation. Mef2 staining marks cardiomyocyte nuclei. Dashed line, approximate resection plane. Arrowheads, Mef2+/EdU+ cardiomyocytes. (C) Quantification of Mef2/EdU assays. Inhibition of Rho A by Rhosin reduces cardiomyocyte (CM) proliferation. n = 5 animals for each condition, 2 independent experiments. Data show mean ± SEM. (Mann–Whitney U test) (D) Heat map of indicated proteins from the whole cell BioID2 data set. Of these, only levels of ROCK2a changed consistently (5.1-fold; p < 0.05) during regeneration. (E) Over-representation test of Reactome pathways. Signaling and effectors of Rho GTPases were found to be over-represented. Fold enrichment as indicated, protein number in red. p < 0.001, FDR < 0.02%. (F) MHC (green) staining ventricles from animals with induced
myocardial specific dominant-negative Rac, Rho, or Cdc42, along with vehicle treated ventricles, at 30 dpa. Five to 8 animals were assessed in each group treated with vehicle, with none of these animals displaying regeneration defects. Four of 5 ventricles with induced dominant-negative Rac, and all ventricles with induced dominant-negative Rho (n = 7/7), or Cdc42 (n = 9/9), showed obvious areas of missing myocardium. Fisher Irwin exact test, p < 0.05. Dashed line, approximate resection plane. Scale bars: 50 µm. Data show mean ± SEM. (Mann–Whitney U test)
Figure 5: Rho A activity is required for cardiogenic responses to mitogens.
(A) Summary of transgenic animals used in Rho A inhibition experiments. (B) Timeline of experiments for Nrg1-overexpression (OE, blue), Vegfaa-OE (red) and Alfacalcidol treatment (orange). (C-E) Immunofluorescence images of ventricles stained for Mef2/EdU from animals overexpressing Nrg1 (C) or Vegfaa (D) in cardiomyocytes, or injected with Alfacalcidol. Hearts were treated with either vehicle or Rhosin. Scale bar 50 μm. (F-H) Inhibition of Rho A by Rhosin reduces cardiomyocyte (CM) proliferation. Quantification of Mef2/EdU staining. Five to six animals were assessed for each group in 2 independent experiments. Data show mean ± SEM. (Mann–Whitney U test).
Supplemental Information

Figure 1- figure supplement 1: Evaluation of biotinylation in BirA2-GFP expressing transgenic zebrafish. (A-E) Immunofluorescence images of sections of ventricles with native BirA2-GFP signal, stained for DAPI (nuclei). Animals were injected intraperitoneally with Biotin for 1, 2, 3 or 4 days. dpt = days post treatment. Scale bars: 50 µm.
Figure 1 – figure supplement 2: Evaluation of BirA2-GFP-CAAX localization and biotinylation.

(A) BirA2-GFP-CAAX is expressed in cardiomyocytes via the cmic2 promoter. (B) BirA2-GFP-CAAX is localized to the cell membrane. Membrane staining with wheat germ agglutinin (WGA) showed colocalization with the BirA2-GFP-CAAX signal. Scale bar: 10 µm. (C-G) Immunofluorescence images of sections of ventricles with native BirA2-GFP-CAAX signal stained for DAPI. Animals were injected intraperitoneally with Biotin for 1, 2, 3 or 4 days. dpt = days post treatment. Scale bars: 50 µm.
Figure 2 – figure supplement 1: Cardiomyocyte ablation in *cmlc2:birA2-GFP* fish, and summary of proteins that display largest changes in levels.

**(A-C)** Immunofluorescence images of sections of ventricles of fish expressing BirA2-GFP crossed to the Z-CAT cardiomyocyte ablation model. Animals were treated with 4-HT to induce expression of DTA toxin. Ventricle were stained for DAPI, Actin and Biotin. Scale bars: 50 µm. **(D)** Top 10% and Bottom 10% of proteins that were captured in the whole cell BioID2 assay and show the largest changes in levels after injury in BirA2-GFP; Z-CAT fish.
Figure 2 – figure supplement 2: Comparison of the BioID2 dataset with published transcriptome or epigenome datasets.

(A-D) The 14 dpi BirA2-GFP BioID2 dataset (normalized to uninjured, p < 0.05) was compared to three RNA-seq datasets (20, 41, 42) and one ChIP-seq (H3.3) dataset (1). For RNA-seq datasets (A-C), expression levels have been normalized to uninjured controls and genes with differences of significance p < 0.05 were considered. (A) RNA-seq dataset from Kang et al., 2016 (41), where 14 dpi whole hearts were used. Thirty-seven genes were identified in both datasets, with a correlation coefficient r = 0.39. (B) RNA-seq dataset from Ben-Yair et al., 2019 (42), in which purified gata4-expressing cells were examined for transcriptome changes at 5 dpa. Seventy-five genes were represented in both datasets, with a correlation coefficient r = 0.18. (C) RNA-seq data set from Wu et al., 2016 (20), where 7 days post cryoinjury (dpci) whole hearts (border zone) were used. One hundred and sixty genes were identified among both datasets, with a correlation coefficient r = 0.2. (D) ChIP-seq dataset from Goldman et al., 2016 (1), in which genes with changes in enrichment for a cardiomyocyte-restricted, tagged H3.3 protein at promoters were used in comparison. Promoters were defined as between 5 kb upstream and 2 kb downstream from the gene start site. FDR < 0.05 for significant differential sites and p < 0.05. Thirty genes where identified among both datasets.
Figure 2 – figure supplement 3: Cardiomyocyte ablation in cmlc2:bira2-GFP-CAAX fish, and summary of proteins that display largest changes levels. (A-C) Immunofluorescence images of sections of ventricles of fish expressing BirA2-GFP-CAAX crossed to the Z-CAT ablation model. Animals were treated with Tamoxifen to induce expression of DTA toxin. Ventricle were stained for DAPI, actin and biotin. Scale bars: 50 µm. (D) Top 10% and Bottom 10% of proteins that were captured in the whole cell BioID2 assay and show the largest changes in levels after injury.
Figure 2 – figure supplement 4: Protein levels and localization from BioID2 comparison of whole cardiomyocytes and membranes.

(A) Heat map of proteins identified in the BioID2 whole cell and membrane datasets and that display opposing changes in levels. Changes 1.5-fold or greater with p < 0.05. Log 10 scale is used. (B-D) Immunofluorescence for indicated proteins in ventricular sections. (B) Talin 2 levels are low and display a punctate distribution in the uninjured heart. During regeneration, Talin levels rise in the cytoplasm. (C) Seta-positive cells are localized to the compact layer and epicardium in 14 dpi hearts injured by the Z-CAT ablation model. Scale bar: 50 μm; magnification scale bar: 10 μm.
Figure 3 – figure supplement 1: Biotinylation in *cmic2:erbb2-birA2-HA-P2A-GFP* ventricles

(A) Western Blot analysis of ErbB2-BirA2-HA assessed for biotin and HA in zebrafish expressing *cmic2:erbb2-birA2-HA-P2A-GFP*. Troponin T is used as a loading control. (B-D) Immunofluorescence images of sections of ventricles of fish expressing ErbB2-BirA2-HA crossed to the Z-CAT ablation model. Animals were treated with Tamoxifen to induce expression of DTA toxin. Ventricle were stained for DAPI, actin and biotin. Scale bars: 50 µm.
Figure 4 – figure supplement 1: Interactome map of Rac1 and Cdc42.

(A) Analysis of known Rac1b interactors in cmlc2:birA2-GFP BioID2 data set. Except for Jupb, all known interactors were found to be increased during heart regeneration. Size of circles indicate fold change. Proteins are sorted clockwise after fold change from high to low. Gna13 = Guanine nucleotide binding protein alpha 13a (25.8-fold), Gab1 = GRB2-associated binding protein 1 (8.34-fold), Ephb2 = Ephrin receptor B2 (5.72-fold), Pfn1 = Profilin 1 (3.25-fold), Arhgap21b = Rho GTPase activating protein 21b (2.65-fold), Wasla = WASP like actin nucleation promoting factor a (2.5-fold), Ctnnd1 = Catenin δ1 (2.2-fold), Ctnna1 = Catenin α1 (1.68-fold), Flii = Flightless I actin remodeling protein (1.72-fold), Jupb = Junctional plakoglobin b (192-fold).

(B) Analysis of known Cdc42 interactors in cmlc2:birA2-GFP BioID2 data set. See (A) for fold changes of proteins found also in Rac1b interactome. Mapre1b = microtubule-associated protein EB 1b (200-fold), Anxa2a = Annexin A2a (20.5-fold), Tln1 = Talin 1 (7.56-fold), ROCK2a = Rho-associated, coiled-coil containing protein kinase 2a (5.05-fold), Ilk = integrin-linked kinase (3.91-fold), Msna = Moesin a (1.9-fold).
Figure 5 – figure supplement 1: Rho A levels in hearts of cardiac mitogen-treated animals. (A) Western Blot analysis of Rho A levels in ventricles of zebrafish with Nrg1-OE, Vegfaa-OE or vitamin D receptor activation via Alfacalcidol. (B) Quantification of Western Blot shown in (A). In untreated hearts, Rho A levels were set to 100%. Levels were normalized to loading control GAPDH.
Table Legends

Table 1: Normalized levels of BioID2 proteins from uninjured cmlc2:birA2-GFP-CAAX hearts.
List of proteins from quantitative mass spectrometry analysis of cmlc2:birA2-GFP-CAAX ventricles. Uninjured BirA2-GFP-CAAX protein levels were normalized to uninjured BirA2-GFP levels. Data are sorted by fold change. Accession number, gene name, description of gene, total unique peptide count, and fold change (FC) with p-values are shown.

Table 2: Normalized levels of BioID2 proteins from regenerating cmlc2:birA2-GFP hearts.
List of proteins from quantitative mass spectrometry analysis of cmlc2:birA2-GFP ventricles. Regenerating BirA2-GFP protein levels were normalized to uninjured BirA2-GFP levels. Data are sorted by fold change. Accession number, gene name, description of gene, total unique peptide count, and fold change (FC) with p-values are shown.

Table 3: Normalized levels of BioID2 proteins from regenerating cmlc2:birA2-GFP-CAAX hearts.
List of proteins from quantitative mass spectrometry analysis of cmlc2:birA2-GFP-CAAX ventricles. Regenerating BirA2-GFP-CAAX protein levels were normalized to uninjured BirA2-GFP-CAAX levels. Data are sorted by fold change. Accession number, gene name, description of gene, total unique peptide count, and fold change (FC) with p-values are shown.

Table 4: Normalized levels of BioID2 proteins from regenerating cmlc2:erbb2-birA2-HA-P2A-GFP hearts.
List of proteins from quantitative mass spectrometry analysis of cmlc2:erbb2-birA2-HA ventricles. Regenerating ErbB2-BirA2-HA protein levels were normalized to uninjured ErbB2-BirA2-HA levels. Data are sorted by fold change. Accession number, gene name, description of gene, total unique peptide count, and fold change (FC) with p-values are shown.

Table 5: Rho A gene expression levels in published transcriptome and epigenome datasets.

Table 6: Raw data from BioID2 assays used to generate Tables 1-3.

Table 7: Raw data from Erbb2-HA-BirA2-P2A-GFP BioID2 assays used to generate Table 4.