Global phosphoproteomic profiling reveals perturbed signaling in a mouse model of dilated cardiomyopathy

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Phospholamban (PLN) plays a central role in Ca^{2+} homeostasis in cardiac myocytes through regulation of the SERCA2A Ca^{2+} pump. An inherited mutation converting arginine residue 9 in PLN to cysteine (R9C) results in dilated cardiomyopathy (DCM) in humans and transgenic mice, but the downstream signaling defects leading to decompensation and heart failure are poorly understood. Here, we used precision mass spectrometry to study the global phosphorylation dynamics of 1887 cardiac phosphoproteins in early affected heart tissue in a transgenic R9C mouse model of DCM compared to wild-type littermates. Dysregulated phosphorylation sites were quantified after affinity capture and identification of 3908 phosphopeptides from fractionated whole heart homogenates. Global statistical enrichment analysis of the differential phosphoprotein patterns revealed selective perturbation of signaling pathways regulating cardiovascular activity in early stages of DCM. Strikingly, dysregulated signaling through the Notch-1 receptor, recently linked to cardiomyogenesis and embryonic cardiac stem cell development and differentiation but never directly implicated in DCM before, was a prominently perturbed pathway. We verified alterations in Notch-1 downstream components in early symptomatic R9C transgenic mouse cardiomyocytes compared to wild-type by immunoblot analysis and confocal immunofluorescence microscopy. These data reveal unexpected connections between stress-regulated cell signaling networks, specific protein kinases, and downstream effectors essential for proper cardiac function.

phospholamban | proteomic | bioinformatics | heart disease

INTRODUCTION

Cardiovascular diseases (CVDs) leading to systolic/diastolic heart failure (HF), such as hypertensive/diabetic heart disease, stroke and vascular atherosclerosis, are leading causes of death in the developed world (1). Many CVDs are associated with genetic predispositions. For example, in humans, the R9C substitution in phospholamban (PLN) has been shown to result in dilated cardiomyopathy (DCM) presenting in adolescence, leading to rapid deterioration of heart function and premature death (2). However, the etiology and molecular mechanisms of progression of DCM and other CVDs leading to HF are complex and still poorly understood, further complicating clinical assessment and management. From a biological and clinical perspective, the identification and characterization of clinically relevant, potentially druggable, pathways driving the maladaptive response in affected heart tissue is a key challenge to improved diagnostic and therapeutic tools for earlier detection and preventative treatment of both inherited and chronic CVDs.

Cardiac muscle contraction is controlled by Ca^{2+} flux and signaling relays, which are perturbed in HF. Internal stores of Ca^{2+} required for the proper functioning of cardiomyocytes (CMs) are normally maintained through the function of the sarco(endo)plasmic (SR) reticulum Ca^{2+}-ATPase 2 (SERCA2) (3), which is responsible for the sequestration of Ca^{2+} resulting in muscle relaxation. SERCA2 activity is regulated through a reversible inhibitory interaction with PLN, which can be relieved by phosphorylation of PLN by protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (3).

Proteomic analyses have revealed changes in the abundance of other effector proteins in diverse biochemical pathways in DCM. Notably, shotgun proteomic analysis of membrane protein expression dynamics in heart microsomes isolated from mice overexpressing a superinhibitory (I40A) mutant of PLN revealed changes in G-protein coupled receptor mediated pathways leading to activation of protein kinase C (PKC) (4). We previously reported quantitative changes in protein and cognate mRNA expression levels in cardiac ventricular tissue at different time points in the development of DCM in R9C-PLN mice representing clear clinical stages in the progression to HF (5). We showed that the latter maladaptive response was driven by elevated activity of MAPK signaling by the protein kinases p38 and JNK, in part through down-regulation of pro-survival microRNAs (6). However, the underlying upstream and downstream signaling events preceding HF were not fully explored.

In the present study, we report the first systematic, large-scale quantitative phosphoproteomic analysis of dysregulated protein phosphorylation-dependent signaling occurring at the early symptomatic stages of DCM progression in whole hearts from R9C mutant mice compared to wild-type littermates.

RESULTS

Comparative phosphoproteome analysis. To achieve a comprehensive survey of cardiac signaling cascades impacted by DCM, we performed global quantitative phosphopeptide profiling on

Significance

The present study demonstrates the utility of global phosphoproteomic profiling of diseased cardiac tissue to identify signaling pathways and other biological processes disrupted in cardiomyopathy. Perturbed Notch-1 signaling was identified by bioinformatics analyses of phosphoprotein patterns present in affected cardiac tissue in a transgenic mouse model system of dilated cardiomyopathy and by complementary molecular biology and microscopy techniques. In addition, dozens of other disturbed signaling pathways offer an opportunity for novel therapeutic and/or diagnostic clinically applicable targets. Although this study was performed in mice, only minor adjustments to the experimental approach would be required for comparative analysis of analogous samples from human cardiac patients, potentially leading to even more clinically relevant data.

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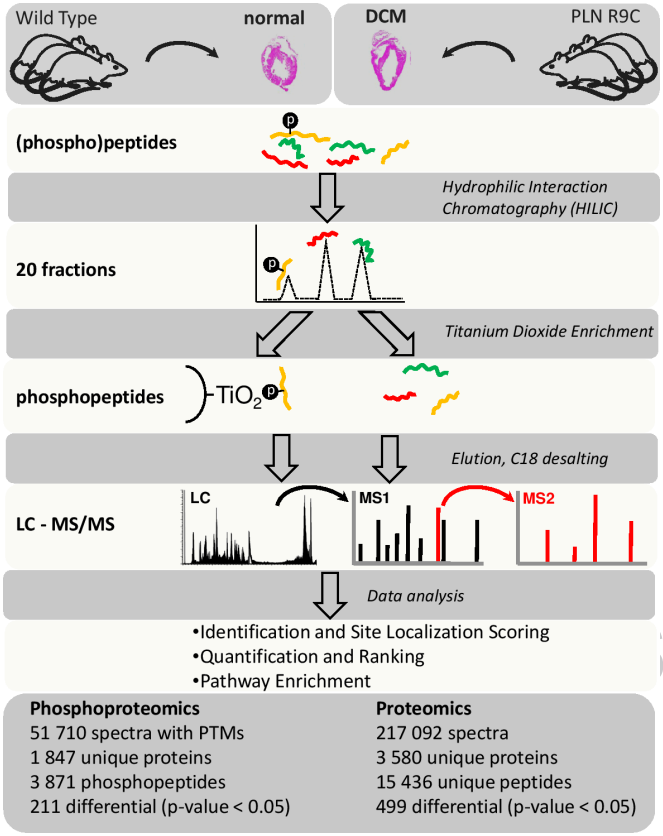

Fig. 1. General phosphoproteomics workflow. Three pooled hearts from R9C PLN transgenic and wild type littermates were analyzed by quantitative precision LC-MS/MS.

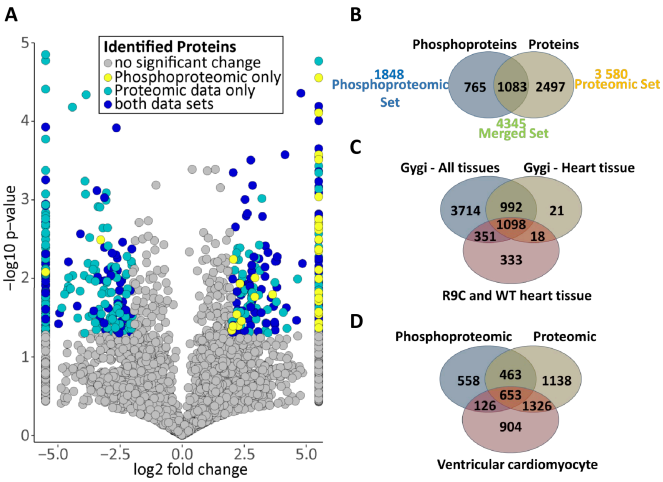

Fig. 2. **(A)** Volcano plot indicating significantly altered proteins and phosphoproteins identified in the combined data sets. Log-transformed p-values (t-test) associated with individual peptides and phosphopeptides plotted against log-transformed fold change in abundance between the PLN R9C and wild type hearts. **(B)** Venn diagram depicting the total number of proteins identified in the corresponding proteomic and phosphoproteomic datasets. **(C)** Venn diagram depicting phosphoprotein overlap with mouse tissue phosphoproteomes from Huttlin et al. (63). **(D)** Venn diagram depicting overlap of R9C and WT combined phosphoproteome and proteome with ventricular cardiomyocyte proteome from Sharma et al. (12).

three independent biological replicates of whole heart tissue isolated from 8-week old (early symptomatic) R9C mice and wild-type littermates. A major experimental consideration was preservation of phosphorylation site integrity, through use of phosphatase inhibitors, low temperature, and harsh denaturing sample processing conditions. To achieve the deepest possible coverage, we performed chromatographic sample pre-fractionation steps, using hydrophilic interaction liquid chromatography (HILIC) to separate peptides bearing negatively charged phosphate moieties (7). Given the low relative abundance of phosphopeptides and their tendency to be undetectable due to ion competition/suppression during MS analysis, we performed differential affinity capture with immobilized metal oxide affinity chromatography (TiO_2) to enrich for phosphopeptides. To measure differences in relative abundance, we applied label-free quantification, comparing the extracted parent ion current intensities recorded in high-energy collision dissociation (HCD) spectra using a high precision Orbitrap instrument. In parallel, we measured total protein levels by shotgun sequencing.

In total, our stringent data analysis workflow (**Fig S1**) mapped 94,956 and 217,092 spectra to mouse reference protein sequences for the phosphoproteomic and background proteomic data sets, respectively. From this, we derived a set of high-confidence sequence matches (FDR ~1% at both protein and peptide levels) corresponding to over 1,800 cardiac phosphoproteins and 3,900 phosphopeptides in normal and DCM hearts, and 15,436 unique peptides mapping to 3,580 unique proteins in the background proteomic data set (**Fig S1**, **Fig 2A/B**). For the former, we confidently (site localization probability >0.7 as described in **Supp. Methods**) identified 7,589 unique putative phosphorylation sites (i.e. sites on identified phosphopeptides with supporting MS/MS data and localization probability greater than 0.7) of which 6,855 mapped to serine, 674 to threonine, and 60 to tyrosine residues (consistent with the expected 90:9:1% cellular distribution ratios) on 1,848 distinct cardiac proteins (**Fig 2A/B**; see **Supp. Datasets S1** and **S2**). Our coverage is comparable to a pioneering study of the mouse cardiac phosphoproteome by Lundby et al. (8) and a recent phosphoproteomic analysis of *in vivo* effects of CaMKII inhibition in mouse hearts (9). Phosphoproteins identified in our study also showed considerable overlap with heart and other tissue profiles in the mouse phosphoproteomic atlas reported by the Gygi group (10), but 333 were uniquely identified in our study; 96 of which contained phosphopeptides significantly altered and/or solely identified in R9C hearts (**Fig 2C**). A high degree of overlap with the mouse ventricular myocyte proteome by Sharma et al. (11) was also observed (**Fig 2D**).

Quantification and Ranking. Based on reproducible measurements of precursor ion intensity, we scored both the individual phosphopeptides and their consolidated cognate phosphorylation sites for differential relative abundance between the healthy and diseased samples. Based on a two-tailed student's t-test ($p < 0.05$), the abundance of 211 phosphopeptides was differentially altered (elevated or reduced) between the R9C and WT hearts (**Fig 2A**; see **Supp. Dataset S1**), with 86% of these predominantly higher in the disease state. In comparison, 499 proteins showed differential expression, consistent with our previous report (5).

Systematic evaluation of the corresponding biological annotations revealed the aberrant phosphorylation patterns occurred on proteins linked to disparate subcellular compartments, ranging from membrane-associated receptors to nuclear-localized proteins with established links to heart development, contractile function and/or cardiomyopathy (**Supp. Data 1**). Consistent with expectation, the predominant phosphoform of PLN detected preferentially in R9C hearts was phosphorylated on serine 16, a site critical for inhibition of SERCA2 activity (3). We also detected alterations in the phosphorylation pattern of central kinases involved in cardiac signaling like protein kinase A (PKA) . Additionally, the S112 site on the PKA Type II regulatory beta

Table 1.

motifs	Kinase	MOTIF SCORE	matches	fold increase
...RS.S.....	CamKII, PKA, PKC, CK2	27.34	59	4.76
.....SD.E...	CK2	24.66	34	6.77
....SPT.....	GSK3, ERK1/2, CDK5, CK2, DNA-PKcs	23.7	27	7.75
.R..S.S.....	CamK2, PKA, PKC, CK2	23.62	34	5.64
....SDS.....	CK2, BARK	17.65	21	6.41
.....SP....	GSK3, ERK1/2, CDK5	16	275	2.34
...S..S.....	CK1, CK2	16	258	1.74
...R..S.....	CamK2, PKA, PKC	12.45	123	2.01
.....TP.....	GSK3, ERK1/2, CDK5	9.46	63	2.35
..S...S.....	GPCRK1, MAPKAPK2K, GSK3	8.1	120	1.7

Top 10 ranked identified consensus motifs on phosphopeptides significantly altered and/or exclusively detected in R9C heart tissue with kinases predicted to target these sequences. Number of matches in the query set and fold increase over the normal occurrence of consensus sequence is also provided.

Table 2.

Pathway	Identified components	Cardiac Context
Notch-1 signaling	**hdac7(s178), tle3(s216), snw1(s224,s232), tmed2, ncor2*, tbl1xr1, tbl1x**, ncor1(s2351), ep300, adam10, crebbp, rbx1, numb, hdac5(s652), arrb1, rps27a, mib1	dilated cardiomyopathy, left ventricular non-compaction, cardiac development (36, 40)
VIP signaling	**map3k1(s915), ppp3cb(s469), plcg1(s1263), nfatc2(s136), prkar2b(s112), prkacb**, prkar2a, prkar1a, nfkb1, ppp3ca	myocardial fibrosis, diabetic cardiomyopathy, ischemia-reperfusion (41)
H3-K4 Histone methylation	**ogt, kmt2a, snw1(s224,s232), nipbl(s2652), bcor(s423), men1, kdm1a, mt2b, dnmt1***, pml(s449), pygo2(t264), ctbp1, arrb1, ube2n, wbp2, lpin1	hypertrophic cardiomyopathy, fetal gene program (42)
BCR signaling, beta cell activation and regulation	**mif, irs2(s1165), thoc1(s560), sos1*, orai1, fas*, sash3*, ptprc(s801), lyn(s19), syk, itpr1(s1573), cd81, stim1*, ptpn6, nfatc2(s136), inpp5d(t701,s709), sh3kbp1(s193), aplf(s41), cbl, plcg1(s1263), tcf3(s517), bad(s112), itpr2**, calm1, pawr, itpr3, bmi1, nck1	heart failure, hypertrophy, myocardial fibrosis, myocardial infraction, ischemia-reperfusion (43, 44)
TGFb/smad signaling	**glg1, pdpk1(s117), jun(s63), lemd3, fbn2, men1, zeb1, aspn, eng, snx6**, zfyve9, cav3, sptbn1, bcl9l, strap, cav2*, ppm1a, htra1	dilated cardiomyopathy, heart failure, fibrosis, myocardial infraction, cardiac remodeling (24, 45)
PPARa signaling	**med19(s226), txnrd1, ankrd1, wwtr1*, abca1(s2234), yap1, med24*, tbl1xr1, alas1, cdk19, tbl1x**, apoa1, acox1, apoa2, smarcd3, agt, ncoa3*, ncoa2, nfya(s187), fhl2, sp1, ncoa6, acadm, acsl1, cd36, crebbp, slc27a1, cpt2, ncor1(s2351), plin2, me1, ep300	dilated cardiomyopathy, heart failure, diabetic cardiomyopathy (24, 46)
TLR signaling	**hsp90b1(s306), lgmn, ikbkb, map2k1, app, nfkb2(t425), dhx9(s137), dnm2, ppp2r5d*, unc93b1, dnm1(s774), itgb2, rps6ka3, eea1**, dusp3, tlr3, ctsb, nfkb1, ube2n, ppp2ca, cd36, mef2a, ppp2r1a	dilated cardiomyopathy, heart failure, ischemia-reperfusion, hypertrophy, viral myocarditis (24, 47)
Wnt signaling	**scyl2(s253), dab2ip(s854), ranbp3, cdh2, wwtr1*, rapgef1*, gsk3a, ppp2r3a, mcc*, dab2, limd1, ctnnb1, dact3(s165), bicc1, gsk3b, stk3(s246), ctnnd1(s813), g3bp1**, cav1, ppp2r5a, pi4k2a(s462), cul3, mapk14*, dvl3*, hdac2(s394)	dilated cardiomyopathy, fibrosis, heart failure, myocardia infraction, arrhythmia (24, 48)

List of select cardiac-relevant signaling pathways (from the Cytoscape analysis), along with their relevant protein components, altered in R9C PLN hearts. Phosphopeptides/proteins increased in R9C hearts are shown in bold, while components identified but showed a decrease in R9C are not bolded. The identified phosphorylation site of a particular protein is indicated in brackets. Proteins annotated with * are 'ambiguous' as described in methods. For a full list of protein/gene names associated with each pathway, refer to Supp. Data.

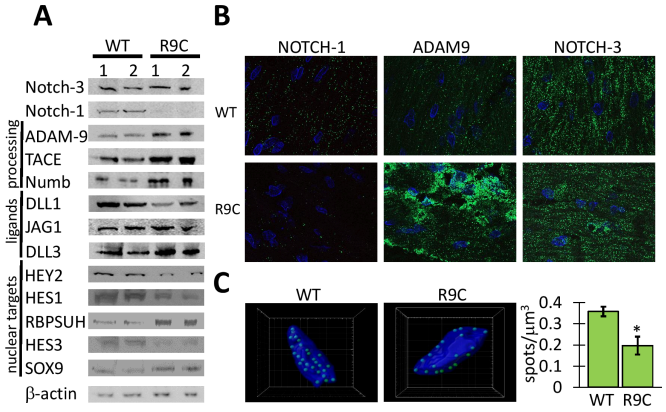

Fig. 3. **(A)** Immunblots of components of the Notch-1 signaling pathway. **(B)** Immunofluorescence images of Notch-1, ADAM9, and Notch-3 in ventricular sections of WT and R9C PLN hearts. **(C)** Representative reconstructed images showing nuclear localization of the Notch-1 intracellular domain and bar graph showing quantification of relative distribution (spots identified/nuclear volume as determined by Hoechst staining) in WT and R9C PLN hearts (p-value = 0.00282).

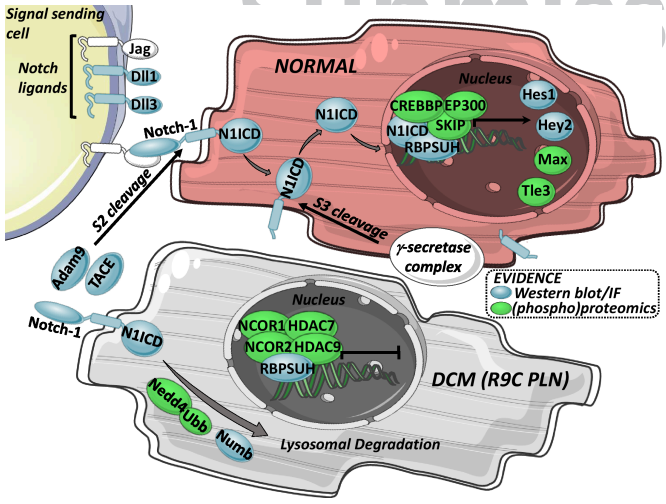

Fig. 4. Proposed schematic model of Notch-1 signaling in normal and DCM cardiomyocytes populated with differential proteins identified with different lines of evidence in whole heart lysates or ventricular sections, including: proteomic data, phosphoproteomic data, immunoblotting, and immunofluorescence.

subunit (PRKAR2B), which results in the lowering of the activation threshold of PKA (12), increased in R9C. Additionally, we detected abundance changes in cardiac stress markers (FHL1), indicators of the fetal gene program (MYH7), markers of ECM remodeling (POSTN, SPARC), and proteins involved in Ca^{2+} modulation (SLMAP, CASQ2) (**Supp. Datasets S1** and **S2**).

Further examples of interest include the highest ranked phosphorylated peptide (S64 + T69) from eIF4E binding protein 1 (EIF4EBP), which negatively regulates translation initiation. Hypophosphorylated EIF4EBP acts as a repressor of eIF4E, whereas its phosphorylation releases eIF4E, thereby upregulating protein translation in response to signaling by diverse cell surface receptors, including insulin, EGF, PDGF and other ligands (13). In heart, EIF4EBP has been implicated in ischemia-reperfusion stress, cardiomyocyte survival, cardiac hypertrophy, and oxidative and nutritional stress responses (13).

Also significantly more highly phosphorylated in R9C hearts was a previously uncharacterized T187 site in EMMPRIN (BSG/CD147), an extracellular metalloprotease inducing member of the immunoglobulin superfamily, consistent with its role in inflammatory processes in cardiac remodeling (14). In addition, the S2 phosphosite on transcription factor MAX (Myc associated factor X) (15), a positive regulator of cardiac alpha-myosin heavy-chain gene expression in cardiomyocytes, was significantly altered in R9C mice. Phosphorylation of S2 inhibits DNA binding by MAX homodimers and association with MYC, but is not known to affect its interaction with another cofactor, transcription enhancer factor 1 (TEF-1, TEAD1), or regulation of cardiac alpha-myosin heavy chain (16). In contrast, when phosphorylated, the S73 site of transcription factor JUN, likewise more highly phosphorylated in the R9C mutant mice, is linked to increased transcription of JUN target genes encoding components of a myriad of signaling pathways (17).

Phosphosites on two intercalated disc-associated Xin repeat proteins (XIRP1, XIRP2) with roles in cardiac development were also increased significantly in R9C hearts. XIRPs interact with catenins and ion channels in mature cardiac muscle and while the significance of phosphorylation is unclear, gene knock out mice display early onset cardiomyopathy (18).

Kinase Target Motif Analysis. We used the MotifX algorithm (19) to identify consensus motifs overrepresented in the set of phosphopeptides identified exclusively in R9C heart tissue and/or those showing significant ($p < 0.05$) differential expression between R9C and wild-type mice (**Table 1** and **Supp. Dataset S1**). Candidate consensus sequences were processed using PhosphoMotif Finder (20) to identify cognate kinases known or predicted to phosphorylate these sequences.

We found central kinases involved in cardiac contractility through transient Ca^{2+} regulation among the most overrepresented compared to the whole mouse proteome. Two consensus motifs identified were targeted by PKA and CaMKII (**Table 1**), one enriched 4.76-fold (59 matches) over background and the other 5.64-fold (34 matches). Phosphorylation of PLN by PKA on S16, or by CaMKII on T17, reverses PLN-mediated inhibition of SERCA2a activity up to 3-fold during β-adrenergic stimulation (3). Upregulation of these kinases is expected as a compensatory mechanism considering that R9C-PLN mutant molecules effectively trap PKA, inhibiting phosphorylation of wild-type protein and relieving SERCA2a inhibition (2).

Two consensus sequences targeted by extracellular signal regulated kinases (ERK1/2) were identified on 275 significantly altered phosphopeptides (**Table 1**). ERK1/2 becomes activated in cardiac myocytes in response to a variety of stimuli, including progression of DCM (21). Likewise, we found consensus motifs associated with phosphorylation by casein kinases and GSK3 kinases, previously linked to cardiac pathology (22).

Pathway Network Enrichment Analysis. For each phosphoprotein (and corresponding gene), the most differential peptide or modification site was selected for a systematic pathway enrichment analysis (see Methods). Phosphopeptides were initially ranked based on the level of statistical significance (differential *p*-value) rather than direction (up/down) of observed fold change. The focus of this analysis was to identify pathway-level alterations present in R9C mice compared to controls.

Three sets of enrichment analyses were performed to gain the most complete coverage, based on: (i) ranking the phosphoproteomic data set alone, (ii) the background proteomic data set alone, and (iii) a merged combination of both (**Fig 2A/B** and **Supp. Fig S1**), which included the significant differentially abundant proteins derived from both the proteomic background and differential phosphorylation events. This merged set of 4,345 unique proteins/genes (**Fig 2B**) was included since signaling perturbations do not necessarily consist only of changes in phosphorylation state or protein expression alone, but often rather both in concert. The most differentially expressed peptides or phosphorylation events (based on *p*-value) from each protein

were used to represent individual protein/gene products. The majority of enriched pathways found in the merged set (**Supp. Dataset S3**) reflected the presence of many proteins exclusively in the background proteome. Of the 2,039 gene sets enriched in the merged set, many (1,427) were likewise enriched in the global proteomic data alone (**Supp. Fig S1**).

We chose to focus on a subset of 612 pathways and processes that reflected contributions from both datasets, rather than merely differential protein levels. To simplify analysis by minimizing redundancy in the annotation term labels (**Supp. Data S1**), we combined the 182 enrichments from the phosphoproteomic data set (not in the background proteome) with the 526 gene sets unique to the merged set (**Supp. Fig S1** and **Dataset S3**). By visualizing the resulting 708 enriched gene sets as a single graphical Enrichment Map (23), we identified several dysregulated pathways of notable relevance to cardiac pathology (**Table 2, Supp. Fig S2**). For instance, reflecting a central role in cardiac physiology and pathology, dysregulated pathways associated with activation PKA and cAMP-dependent kinases were prominently overrepresented in all gene set enrichment analyses (**Supp. Datasets S1 and S3**). Considering the ubiquity of many signaling pathways across different tissues and cell types, when examined as a whole (rather than individual components), it is not surprising that most pathways have associations with other disorders or processes. This is also partly a bias of existing annotations in curation databases.

To identify disease signatures specifically relevant to DCM, we integrated and compared these pathways against a recent in depth RNAseq-based gene expression study of the R9C PLN model (**Supp. Datasets S2 and S6**) (24). Specifically, differential mRNA expression modules in the Seidman study corresponding to cardiomyocyte and non-myocyte cardiac cell populations at different stages of disease (represented by six individual nodes) were tested for overlap with our enrichment map using a stringent Mann-Whitney test cutoff ($p<0.01$). Strikingly, we observed highly significant overlap between most of the disrupted biological processes and signaling pathways noted by the two studies, including (but not limited) to: innate immunity, glucose metabolism, TGF beta signaling, PPAR signaling, Wnt/β-catenin signaling, and TLR signaling. Notably, the general pathways involved in metabolic and pro-fibrotic processes determined by the Seidman study to differentiate between DCM and hypertrophic cardiomyopathy (HCM) were likewise detected as differentially perturbed in R9C hearts in our merged data.

Notch-1 Signaling. Distinct from the Seidman study, however, we identified evidence for significant disruption in homeostatic Notch-1 signaling in R9C hearts, with most phosphosites never reported before in the context of DCM. For instance, uncharacterized sites on SNW1 and CREBBP, members of the Notch-1 transcription co-activator complex (25), were hyperphosphorylated in the R9C mice. Similarly, we detected previously unreported phosphorylation sites on both MAX and NCOR1 and NCOR2, components of a downstream transcriptional corepressor complex known to inhibit transcription of Notch target genes (26).

To directly determine the differential state of Notch receptor signaling, we performed immunoblots (**Fig 3A**) using a panel of antibodies targeting different Notch receptor isoforms (Notch1/3), activating and inhibiting ligands (DLL1/4, JAGG1/2, NUMB), processing enzymes (ADAM9/17), and key downstream effector transcription factors (HEY2, HES3, SOX9). Strikingly, a very substantive and specific decrease in total Notch-1 receptor levels was seen in the R9C hearts, while levels of Notch-3 receptor were unchanged (Notch-2 was undetectable, as was the cleaved Notch-1 intracellular domain).

Of the activating Notch ligands, DLL1 was downregulated in R9C, while JAGG1 showed no change (DLL4 and JAGG2 were not detected). Conversely, DLL3 and NUMB, which can suppress Notch signaling, were upregulated in R9C hearts. Two metalloproteinases (ADAM9 and TACE/ADAM17) required for extracellular processing and/or degradation of the Notch-1 receptor were similarly upregulated in R9C. Conversely, the downstream Notch-1 regulated transcription factors HES1 and HEY2 were downregulated in R9C.

Immunofluorescence visualization of components of the Notch signaling pathway in ventricular wall of R9C and wild-type hearts (**Fig 3B**) established downregulation of Notch-1 receptor expression in R9C cardiomyocytes along with upregulation of the ADAM9 metalloproteinase (Notch-3 was unchanged). Imaris-based quantification (**Fig 3C**) of signal intensity and localization of the Notch-1 intracellular domain (N1CID) in cardiomyocyte nuclei, indicative of Notch-1 activation, revealed a significant decrease ($p < 0.003$) in overall nuclear localization of N1CID in cells in the ventricular sections of R9C hearts compared to controls (due to gross changes in tissue morphology in R9C hearts, a large proportion of nuclei showed no N1CID colocalization). These results verify Notch-1 receptor signaling inhibition in affected heart tissue during early-stage disease (**Fig 4**).

DISCUSSION

During pathological cardiac remodeling, including DCM, disruptions in various signaling cascades have been reported, including AKT, ERKs, GSK-3, JAKs, MAPKs, PI3K, PKA, PKC, and TGFβ. Activation or inhibition of signaling pathways can ultimately result in changes to cardiomyocyte cell growth, differentiation, proliferation and/or survival (27). Yet despite advances in understanding the molecular basis of cardiac disease, a clear global picture of system wide signaling network perturbations does not exist for most conditions, including DCM. To address this gap, we applied quantitative precision mass spectrometry to glean the complex interplay of signaling events associated early during disease progression with a genetic cause of cardiac HF.

One third of all cardiac proteins are known or predicted to be phosphorylated. Here, we mapped 7,589 phosphopeptides on 1,848 cardiac proteins, of which 211 were differentially abundant in the R9C model, providing unprecedented insights into the global dysregulation of signaling cascades in early-stage DCM. Many sites we identified are novel and of unknown biological significance. While annotated repositories of experimental phosphoproteomic datasets have been established (e.g. PhosphoSitePlus) (28), establishing relevance to heart biology and CVD is not straight-forward. Our present study aimed to first identify significantly altered individual phosphosites and then use the corresponding associated protein/gene annotations in conjunction with information gained from global proteomic analysis. This design was motivated by computational tools for signaling network analysis and visualization, which historically focused on gene expression datasets rather than individual phosphosites.

One especially intriguing finding was identification of Notch-1 signaling as prominently altered in DCM. The Notch pathway is a widely studied system crucial to "cell fate" determination (29). Mammals express four Notch receptors (Notch 1-4) and five ligands (DLL-1, -3, -4, JAGG-1, -2) (30). Notch-1 is expressed across a broad variety of cell types, including cardiomyocytes. All members of the Notch family are translated as single chains, but are subsequently processed in the Golgi system to produce extracellular and transmembrane subunits that remain bound together at the plasma membrane by non-covalent bonds. Generally, ligand binding triggers the selective cleavage and removal of the extracellular subunit by members of the ADAM protease family, followed by cleavage of the membrane-bound intracellular subunit by γ-secretase to release N1ICD, which translocates to the nucleus to modulate transcription (**Fig 4**). However, this oversimplification does not reflect cell context as different ligands

can act as Notch inhibitors, while processing enzymes can oppose Notch activation (29).

Changes in phosphorylation of Notch signaling components included an uncharacterized phosphorylation site on the polyubiquitin protein UBB required for homeostatic recycling of Notch-1, which is upregulated in R9C mice (31), as well as members of the histone deacetylase family (HDAC-4, -7, -9) and others (32). However, due to incomplete functional annotation, our cautious conclusion was that Notch-1 pathway was disrupted but not the precise nature of the perturbation (i.e. activation/inhibition).

We verified changes in Notch-1 signaling by immunoblots and immunofluorescence. We demonstrated downregulation of Notch-1 and DLL1 in R9C. DLL3, a Notch-1 ligand previously shown as a cis-acting inhibitor (33), was upregulated along with NUMB, whose association with Notch-1 leads to proteasomal degradation (34). ADAM9 and TACE/ADAM17, normally necessary for activation of Notch-1, were also upregulated in R9C. However, excessive expression of these proteases has also been shown to inhibit Notch signaling, while deficiency of a major associated inhibitor, TIMP3, leads to dilated cardiomyopathy in mice (35). Furthermore, expression and nuclear localization of the N1ICD domain of Notch-1 were significantly greater in wild-type cardiomyocytes compared with R9C. Together, these results show that Notch-1 signaling is downregulated in myocardium in early-stage DCM.

Our findings are consistent with previous reports of development of DCM *in vitro* and in newborn mice treated with an inhibitor of Notch-1 dependent signaling (36), or more pronounced cardiac dysfunction, fibrosis and apoptosis in adult hearts from an agonist-induced hypertrophy model or Notch-1 cardiac-specific null mice (37). Similarly, transgenic mice overexpressing Notch ligand, JAGGED1, were protected from pressure overload-induced cardiac hypertrophy (38).

Considering this unprecedented phosphoproteomics data, it is clear that a more complete understanding of Notch-1 signaling and other potentially relevant pathways in adult heart following injury, including human patients, is warranted (39). Indeed, uncoupling of Notch-1 signaling in R9C (**Fig 4**) suggests that Notch-1 activating compounds (i.e. activating antibodies, polypeptides, small molecules) may be clinically beneficial under DCM conditions, similar to that observed in hypertrophy (38) and potentially other pathological contexts.

MATERIALS AND METHODS

Transgenic mice carrying the R9C mutation in the PLN gene were previously described (2). Workflows for heart sample preparation, processing and (phospho)proteomic profiling, including chromatographic fractionation, MS data generation, phosphosite localization and scoring, statistical enrichment analyses, motif prediction, Western blotting, and immunofluorescence, are detailed in the **Supp. Methods** available online.

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