Mechanisms of Congenital Heart Disease Caused by NAA15 Haploinsufficiency

Tarsha Ward, Warren Tai, Sarah Morton, Francis Impens, Petra Van Damme, Delphi Van Haver, Evy Timmerman, Gabriela Venturini, Kehan Zhang, Min Young Jang, Jon A.L. Willcox, Alireza Haghhighi, Bruce D. Gelb, Wendy K. Chung, Elizabeth Goldmuntz, George A. Porter, Jr, Richard P. Lifton, Martina Brueckner, H. Joseph Yost, Benoit G. Bruneau, Joshua Gorham, Yuri Kim, Alexandre Pereira, Jason Homsy, Craig C. Benson, Steven R. DePalma, Sylvia Varland, Christopher S. Chen, Thomas Arnesen, Kris Gevaert, Christine Seidman, J.G. Seidman

RATIONALE: NAA15 (N-alpha-acetyltransferase 15) is a component of the NatA (N-terminal acetyltransferase complex). The mechanism by which NAA15 haploinsufficiency causes congenital heart disease remains unknown. To better understand molecular processes by which NAA15 haploinsufficiency perturbs cardiac development, we introduced NAA15 variants into human induced pluripotent stem cells (iPSCs) and assessed the consequences of these mutations on RNA and protein expression.

OBJECTIVE: We aim to understand the role of NAA15 haploinsufficiency in cardiac development by investigating proteomic effects on NatA complex activity and identifying proteins dependent upon a full amount of NAA15.

METHODS AND RESULTS: We introduced heterozygous loss of function, compound heterozygous, and missense residues (R276W) in iPSCs using CRISPR/Cas9. Haploinsufficient NAA15 iPSCs differentiate into cardiomyocytes, unlike NAA15-null iPSCs, presumably due to altered composition of NatA. Mass spectrometry analyses reveal ≈80% of identified iPSC NatA targeted proteins displayed partial or complete N-terminal acetylation. Between null and haploinsufficient NAA15 cells, N-terminal acetylation levels of 32 and 9 NatA-specific targeted proteins were reduced, respectively. Similar acetylation loss in few proteins occurred in NAA15 R276W induced pluripotent stem cells. In addition, steady-state protein levels of 562 proteins were altered in both null and haploinsufficient NAA15 cells; 18 were ribosomal-associated proteins. At least 4 proteins were encoded by genes known to cause autosomal dominant congenital heart disease.

CONCLUSIONS: These studies define a set of human proteins that requires a full NAA15 complement for normal synthesis and development. A 50% reduction in the amount of NAA15 alters levels of at least 562 proteins and N-terminal acetylation of only 9 proteins. One or more modulated proteins are likely responsible for NAA15-haploinsufficiency mediated congenital heart disease. Additionally, genetically engineered induced pluripotent stem cells provide a platform for evaluating the consequences of amino acid sequence variants of unknown significance on NAA15 function.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: congenital heart defects ■ haploinsufficiency ■ induced pluripotent stem cells ■ proteins ■ proteomics ■ ribosomes
sequencing of CHD probands and their parents have enabled the identification of recurrent damaging variants in multiple genes that likely are critical for normal cardiac development.3 Functional studies are necessary to further define these gene functions and the pathogenetic mechanisms of damaging variants. While loss of function (LoF) variants infer that haploinsufficiency of the encoded protein contributes to CHD, the consequence of missense variants on protein function are less readily interpreted, often leading to classification of these variants of unknown significance although some may contribute to the CHD.2

We previously reported 2 patients with CHD with de novo heterozygous LoF variants in NAA15 (N-alpha-acetyltransferase 15), which encodes a protein subunit of the NatA (N-terminal acetyltransferase) complex.2–4 In addition to CHD, these patients had extra-cardiac disorders including neurodevelopmental deficits.2,3 Prior studies have reported damaging NAA15 variants in patients with other congenital malformations and neurodevelopmental abnormalities.5–7

Acetylation of the N-terminus of proteins is a prevalent modification that occurs in \( \approx 85\% \) of yeast and human proteins.8,9 The effect of Nt-acetylation on proteins is diverse and includes changes to protein stability, complex formation, protein folding, and aggregation.10 The NatA complex, one of 8 NAT types, is essential in most, if not all, eukaryotes and is responsible for the majority of Nt-acetylation.10,11 This complex binds the ribosome and was shown to acetylate nascent polypeptide chains at specific Nt amino acids (Ser-, Thr-, Ala-, Val-, Gly-, and Cys-) after the initiating methionine is removed.4,10,12,13 The NatA complex is formed by the catalytic subunit NAA10 and the auxiliary unit NAA15. HYPK (Huntingtin interacting protein K) is a chaperone protein that attaches to the NatA complex along with subunit NAA50.14–17 NAA50 also forms part of the NatE complex, which displays a distinct substrate...
specificity compared with NatA.\textsuperscript{10,18} NAA15 is a subunit of both the NatA and NatE complexes, and its role is to position the catalytic subunits in close vicinity to the nascent polypeptides; in the case of NAA10, it also modulates its substrate specificity.\textsuperscript{10,19–21} Additionally, NAA15 interaction with NAA10 and HYPK has been implicated in regulation of protein folding and Nt-acetylation fidelity.\textsuperscript{15,22} Abnormal NatA complex function has been previously associated with human cancers and neurological disorders.\textsuperscript{5,23,24} To date, the relationship between Nt-acetylation, NAA15, and CHD has not been investigated.

We studied human isogenic induced pluripotent stem cell (iPSC) lines that were engineered to contain NAA15 variants identified in patients with CHD and predicted to be damaging. We evaluated whether iPSCs with NAA15 variants differentiated into cardiomyocytes. By assessing both Nt-acetylation and protein levels by mass spectrometry (MS), we demonstrate that NAA15 haploinsufficiency perturbs normal function of undifferentiated iPSCs. We identify proteins that require the full complement of NAA15 to preserve the integrity of these stem cells for cardiac development.

METHODS

Data Availability

All data and materials have been made publicly available. Further details are provided in the Major Resources Table located in the Data Supplement.

Study Cohort With CHD

CHD subjects (n=4511) were recruited to the Congenital Heart Disease Network Study of the Pediatric Cardiac Genomics Consortium (CHD GENES; URL: https://www.clinicaltrials.gov; Unique identifier: NCT01196182) or the DNA biorepository of the Single Ventricle Reconstruction trial after approval from Institutional Review Boards as previously described.\textsuperscript{1,25,26} All subjects or their parents provided informed consent. Clinical diagnoses, including cardiac and noncardiac congenital anomalies, were obtained from review of patient charts and family interview.

CRISPR Gene Editing and Mutation Confirmation

Isogenic personal genome project 1 iPSCs were modified using CRISPR/Cas9 technology to create NAA15 LoF or nonsense mutation cell lines.\textsuperscript{27} Further details provided in Methods in the Data Supplement.

Label-Free Quantitative Shotgun Proteomics and Data Analysis

Two independent iPSC lines of both wild type (WT) cells and NAA15 mutant cells were grown in 10 cm petri dishes until near confluency. iPSCs were detached and collected in 15 cm centrifuge tubes. Cells were centrifuged (Beckman) in a 15 mL tube at 1000 rpm for 5 minutes. Cells were washed with PBS and pelleted by centrifugation. Eight samples of each genotype was collected, and a total of 24 samples were prepared for LC-MS/MS analysis. Further details provided in Methods in the Data Supplement.

Bioinformatic Analysis and Gene Ontology Analysis

Gene ontology annotation proteome was derived using the R package clusterProfiler.\textsuperscript{28} Proteins were classified by Gene ontology annotation based on biological process, molecular function, and cellular component. Quantified proteins detected by MS of iPSCs were used as a background and other parameters with default. The \( P \) values were adjusted by Bonferroni correction for multiple testing.

Statistics

Single comparisons were analyzed by using the Student \( t \) test, with significance defined as \( P<0.05 \). Multiple comparisons between genotypes were analyzed using 1-way ANOVA with post hoc Tukey HSD, with significance defined as \( P<0.05 \). For proteomics, pairwise SAM \( t \) tests (or pairwise statistical testing using the SAM method\textsuperscript{29}) were performed and significant hits determined using as a cutoff values a permutation based false discovery rate (FDR) of 0.01 (1000 permutations) and a background variance parameter \( s_0 \) of 1. For all experiments analyzed for statistical significance, only within-test corrections were made.

RESULTS

NAA15 Variants Are Associated With CHD and Other Extra Cardiac Anomalies

Whole exome sequencing of 4511 patients with CHD\textsuperscript{1,2,4} identified 4 subjects with a rare LoF variant (allele frequency <0.00005) in the NAA15 gene, resulting in NAA15 haploinsufficiency (Figure 1A). Parental analyses indicated that 3 of these LoF variants (p.Ser761*, p.Lys336Lys fs*6, and p.Arg470*) arose de novo in the probands.\textsuperscript{14} The inheritance of the p.Ala718fs variant is uncertain, as parental samples were unavailable. Among \( \approx 125,000 \) subjects in the gnomAD database,\textsuperscript{12} 14 NAA15 LoF variants are reported, inferring an 8.9-fold higher frequency of NAA15 LoF variants in CHD probands (\( P=0.002 \)).

The CHD phenotypes included tetralogy of Fallot, heterotaxy with d-looped ventricles, transposition of the great arteries, and hypoplastic left or right heart syndrome (Figure 1C and Table I in the Data Supplement). In addition to cardiac anomalies, all 4 patients with CHD with a NAA15 LoF variant had extracardiac anomalies including seizures, neurobehavioral, ophthalmologic, auditory, or orthopedic disorders (Figure 1C and Table I in the Data Supplement).\textsuperscript{5}
We also identified 15 very rare (allele frequency <1.0×10^{-5} or absent from the gnomAD database) inherited NAA15 missense variants among these 4511 patients with CHD (Figure IA and Table I in the Data Supplement). In addition, one missense variant, R276W (Figure 1A and Figure IA and IB in the Data Supplement), could not be assessed for inheritance. The frequency of rare missense alleles in the Pediatric Cardiac Genomics Consortium (PCGC) cohort was 0.0035 (n=16 of 4511 CHD probands), significantly higher than the frequency observed among ≈115 000 Gnomad subjects (frequency, 0.002; n=198; P=0.02; odds ratio, 1.8). CHD probands with rare NAA15 missense variants had notably fewer extracardiac anomalies than CHD probands with NAA15 LoF variants (Table I in the Data Supplement). Hence, despite the observation that these very rare NAA15 missense variants were transmitted from an unaffected parent, we suspected that some of them contribute to CHD.

Genetically Engineered iPSCs Model NAA15 Haploinsufficiency

We introduced NAA15 variants into the iPSC line, personal genome project 1, using CRISPR/Cas9 gene editing (see Methods in the Data Supplement) to create human iPSCs with reduced or no NAA15 protein. Two independent cell lines were constructed with each genotype NAA15+/−, NAA15+/R276W (Figure 1A, Figure IA through IC and Table II in the Data Supplement) and studied as detailed below. NAA15 variants in iPSCs were confirmed both by Sanger sequencing and next generation sequencing of PCR amplified products (Figure IIA through IIC in the Data Supplement).

RNA expression levels for each of the 2 independent iPSC lines in NAA15+/+ and NAA15-mutant iPSCs, were first characterized by RNAseq. NAA15 mRNA levels were reduced by 56% and 24% in the NAA15+/− and NAA15+/R276W iPSCs, respectively (Figure 2A and Table III in the Data Supplement). There was a 99% decrease in NAA15 mRNA in NAA15−/− iPSCs, suggesting that these variants triggered nonsense-mediated decay (Figure 2A). We performed MS-based shotgun proteomics in at least 3 replicates for each of the 2 independent lines. NAA15 protein was near-normal levels in NAA15+/− iPSCs but absent from NAA15−/− iPSCs (Figure 2B). To confirm this hypothesis, NAA15 protein levels were measured in 2 independent biological replicates of both WT and NAA15 mutant iPSCs by Western blotting (Figure IID and IIE in the Data Supplement), which supported this conclusion. NAA15 protein was reduced

Figure 1. NAA15 variants discovered in patients with congenital heart disease patients.
A, Schematic diagram of the NAA15 (N-alpha-acetyltransferase 15) gene. The NAA15 gene consists of tetracopeptide repeats (blue) essential for interaction with the NatA (N-terminal acetyltransferase) complex subunit NAA10 and a HYPK (Huntingtin interacting protein K) interacting domain at the C terminus. Location of variants identified in patients with congenital heart disease (CHD; loss of function [LoF] variants are underlined) and CRISPR/Cas9-derived variants in induced pluripotent stem cell (iPSCs; magenta) are shown. B, The subunit composition of NatA and NatE complexes. NAA15 is the auxiliary unit for both complexes. C, Table of cardiovascular and neuronal clinical phenotypes of patients with CHD. *indicates LoF variants.
in NAA15+/− iPSCs by about 50%, and full length NAA15 protein was not detectable in NAA15−/− iPSCs (Figure IID and IIE in the Data Supplement). Noticeably, HYPK, a NAA15 binding partner and subunit of the NatA complex was significantly reduced in both NAA15+/− and NAA15−/− iPSCs (Figure 2B).

We further explored the functional effects of NAA15+/− and NAA15+/R276W using a yeast assay in which the hNatA (human NatA complex) functionally replaced yeast NatA, as shown by complementation of growth phenotypes with partial rescue of the NatA-specific Nt-acetylome.8 HsNatA D335fs and S761* failed to rescue the temperature-sensitive growth phenotype of yNatA∆ (Figure IIIA through IIIC in the Data Supplement), suggesting that NAA15+/− results in impaired NatA functionality. However, in this yeast assay, human HsNatA R276W rescued yeast growth suggesting at least partial NatA function in yeast (Figure IIIC in the Data Supplement). Noticeably, Schizosaccharomyces pombe NAA15 has a tryptophan residue at position 276 while all mammals have an arginine residue, which suggests that this residue is functionally important in human cells. As such, we suggest that rescue of yeast growth experiments might not fully provide functional assessments of mutant human NAA15 proteins.

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Figure 2. NAA15+/− and NAA15+/R276W induced pluripotent stem cells (iPSCs) develop into cardiomyocytes, while NAA15 (N-alpha-acetyltransferase 15) null iPSCs have reduced viability.

A. Graph shows RNA expression of the NatA (N-terminal acetyltransferase) components in NAA15-mutant iPSCs. NAA15 RNA levels are significantly lowered in iPSCs with NAA15+/− and NAA15−/− variants compared with wildtype iPSCs. Data was collected from 2 independent cell lines for each genotype and as technical replicates for selected lines. Total cell lines analyzed: NAA15+/− (n=3), NAA15−/− (n=4), NAA15+/R276W (n=2). Significance of differences between NAA15 mutant iPSCs and wildtype iPSCs were evaluated by Student t tests, P<0.05 (P values were adjusted by Bonferroni correction; only significant adjusted P values are displayed). All data points are presented and plotted as mean±SEM.

B. Graph represents fold change ratios of relative protein levels in NAA15 mutant iPSCs compared with wildtype iPSCs for Nat complex subunits. Relative protein abundance was quantified by MaxLFQ algorithms integrated in the MaxQuant software. Data was collected from two independent undifferentiated iPS cell lines for each NAA15 mutant genotype and as technical replicates for each cell line. Total cell lines analyzed: NAA15+/− (n=8), NAA15−/− (n=8), NAA15+/− (n=7), and NAA15+/R276W (n=8). Significant changes indicated by P values were calculated using pairwise comparison SAM t test method and a permutation based false discovery rate (FDR) of 0.01 (1000 permutations) as cutoff values with a background variance parameter s0 of 1. All data points are presented and plotted as mean±SEM.

C. Representative images of NAA15+/− iPSCs and NAA15−/− iPSCs stained with cardiac troponin T antibody (green), α-actinin (magenta), and DAPI for nuclei (blue). A representative image for each cell type is presented. Normal sarcomeres were observed in both NAA15 mutant and wildtype iPSC-CMs. Magnification, 60×; scale bar 25μm.
NAA15<sup>+/−</sup> and NAA15<sup>+/−/R276W</sup> iPSCs Develop Into Contractile Cardiomyocytes, While NAA15<sup>+/−</sup> iPSCs Have Reduced Viability

To test whether NAA15 variants have an effect on iPSC maturation, 2 independent iPSC lines for each genotype (NAA15<sup>+/−</sup>, NAA15<sup>+/−/R276W</sup>, and NAA15<sup>−/−</sup>) were differentiated into cardiomyocytes using a 13-day differentiation protocol to assess for the development and contractility of sarcomeres.30,31 Both NAA15<sup>+/−</sup> and NAA15<sup>+/−/R276W</sup> cells differentiated into cardiomyocytes (Figure 2C, a and b), however, NAA15<sup>−/−</sup> iPSCs grew slowly (data not shown) and failed to differentiate. Cell viability of NAA15<sup>−/−</sup> mutant iPSCs was assessed (see Methods in the Data Supplement). Similar to observations reported in NAA15/NatA yeast knockout and human knockdown cells,19,23,32–34 NAA15<sup>−/−</sup> iPSCs had significantly retarded growth and cell death (P=0.001; average cell death is 12% in NAA15<sup>+/−</sup> iPSCs versus 32% in NAA15<sup>−/−</sup> iPSCs, n=2). NAA15<sup>−/−</sup> iPSC-derived cardiomyocytes (iPSC-CMs) were stained with cardiac troponin T and actinin antibodies to visualize sarcomere structures (Figure 2C, a and b). NAA15 mutant cell sarcomeres were indistinguishable from WT cell sarcomeres (Figure 2C, a and b and Figure IV and Table IV in the Data Supplement).

Contractility of NAA15<sup>+/−</sup> and NAA15<sup>+/−/R276W</sup> iPSC-CMs was monitored by live image analysis (Movies I and II in the Data Supplement). Two biological replicates of NAA15 mutant iPSC-CMs and one of 2 biological replicates for WT iPSC-CMs were incubated with GFP (green fluorescent protein)-actinin lentivirus to enable high fidelity tracking of sarcomere function35(Movies III through V in the Data Supplement). The lengths of GFP-labeled sarcomeres were measured during the contractile cycle. Contractile measurements of unloaded (ie, sarcomeres that were not working against resistance) demonstrated no difference in rates of sarcomere shortening or contraction between mutant and WT cells (Figure VA in the Data Supplement). To better recapitulate native cardiomyocyte architecture and mechanics, the function of loaded sarcomeres from these cells grown as 3-dimensional microarchitecture and mechanics, the function of loaded sarcomeres (Figure 2C, a and b), NAA15<sup>−/−</sup> mutant cell sarcomeres were indistinguishable from WT cell sarcomeres (Figure 2C, a and b and Figure IV and Table IV in the Data Supplement).

Nt-acetylation in NAA15 Haploinsufficient, Null, and R276W Missense iPSCs

We studied Nt-acetylation of 2 biological replicates in iPSCs with NAA15 variants and WT using a MS-based Nt enrichment assay.8,38–40 NatA acetylates N-termini containing Ser<sup>−</sup>, Thr<sup>−</sup>, Ala<sup>−</sup>, Val<sup>−</sup>, Gly<sup>−</sup>, or Cys<sup>10,12</sup> We identified a total of 989 previously annotated N-termini<sup>21</sup> in one or both of the 2 independent cell lines for WT and NAA15 mutant iPSCs (Table V in the Data Supplement); ≈650 N-termini peptides were identified in each cell line (Tables V and VI in the Data Supplement). NatA targeted sequences represented ≈60% of the 989 detected N-termini peptides (Tables V and VI in the Data Supplement); no Cys-starting peptides were detected. In NAA15<sup>−/−</sup> iPSCs, we observed 17 Nt peptides that were partially acetylated (10%–95%) and 359 with complete (>95%) Nt-acetylation (Figure 3A and Tables V and VI in the Data Supplement). Of the completely or partially Nt-acetylated peptides,<sup>8,10</sup> 98% have an alanine, threonine, or serine at position 2 (Figure 3B and Tables VI and VII in the Data Supplement). However, peptides with partial acetylation have a much higher fraction of Nt glycine or valine residues (Figure 3C and Tables VI and VII in the Data Supplement). The preference for acetylation of proteins with Nt alanine and serine residues was preserved in NAA15<sup>−/−</sup>mutant iPSCs as observed in previous studies (Tables VI and VII in the Data Supplement).23

Protein Nt-acetylation in NAA15 mutant iPSCs was compared with Nt-acetylation in WT iPSCs. Only a limited number of putative NatA-type N-termini substrates had >10% difference in acetylation between wild type and mutant iPSCs (Figure 3, Table 1, and Figure VI in the Data Supplement). In NAA15<sup>−/−</sup> iPSCs 32 proteins showed these changes (Figure 3B through 3D and Table 1). Notably, all proteins that had partial acetylation in WT iPSCs lacked Nt-acetylation in NAA15<sup>−/−</sup>. Nine of these proteins were altered in NAA15<sup>−/−</sup> iPSCs and 8 had altered Nt-acetylation in NAA15<sup>+/−/R276W</sup> iPSCs (Figure 3E and 3F, Table 1).

All proteins with reduced acetylation in mutant iPSCs displayed NatA-type substrate specificity (Tables 1 and 2, Table V in the Data Supplement). Greater than 50% of proteins with altered Nt-acetylation had an alanine residue at position 2 and >30% also had an alanine residue at position 3 (Table 2).<sup>23</sup> The distribution of N-terminal residues in proteins with altered Nt-acetylation did not differ significantly from the distribution of N-terminal residues in proteins with unchanged N-terminal acetylation in these cells (not shown). We deduced that NAA15 variants or deficiency altered N-terminal acetylation in a small number of NatA substrate proteins and with no preference for an extended N-terminal amino acid substrate specificity.

We noticed that some proteins reduced in Nt-acetylation (Figure 3D) have functions in cellular proliferation and survival,<sup>22–26</sup> perhaps accounting for the deficits in growth and viability of NAA15<sup>−/−</sup> iPSCs.41–46 To understand whether the change in Nt-acetylation status compromised the function of these proteins and contributed to the phenotype of NAA15<sup>−/−</sup> iPSCs, we used a combination of shotgun MS-based proteomics and pairwise comparison by SAM t test (FDR=0.01; Tables VIII through X in the Data Supplement). Nine proteins with reduced acetylation correlated with changes in protein expression in NAA15<sup>−/−</sup> iPSCs.
Among these, measurable acetylation occurred in 3 proteins in \textit{NAA15}+/− and in one protein in \textit{NAA15}−/− iPSCs (Figure 3E and 3F).

\textbf{Altered Protein Expression Due to NAA15 Haploinsufficiency or Deficiency}

Using shotgun proteomics, we studied 2 independent undifferentiated iPSC lines for each \textit{NAA15} mutant genotype and at least 3 technical replicates for each sample to assess protein levels (Table VIII in the Data Supplement). Among 5196 proteins identified by MS, extracts from \textit{NAA15}+/− iPSCs, \textit{NAA15}−/− iPSCs, or both revealed a total of 1209 proteins that were differentially expressed (pairwise comparison by \textit{t} test) compared with WT iPSCs (Figure 4A and 4B, Figure VII and Tables VIII through X in the Data Supplement).

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expressed in both NAA15+/− and NAA15−/− iPSCs compared with WT iPSCs and 505 proteins in NAA15−/− iPSCs but not NAA15+/− iPSCs (Figure 4B, Figure VII and Tables VIII through X in the Data Supplement).

Table 1. Proteins With Altered N-Terminal Acetylation in Wildtype and NAA15 Mutant iPSCs*

| Accession | Description                                                                 | Nt-acetylation percentage | Protein levels (fold change) |
|-----------|------------------------------------------------------------------------------|---------------------------|-----------------------------|
| Q91N52    | COP53                                                                       | +/+ 99.4, +/− 99.9, −/− 83.6, +/+R276W 99.6 | +/+ 1.87, +/− 2.00 |
| Q8TDD1    | ATP-dependent RNA helicase (DDX54)                                           | +/+ 30.8, +/− 0.7, −/− 29.6 | +/+ 2.00, +/− 2.46 |
| O60869    | EDF1                                                                        | +/+ 93.0, +/− 81.6, −/− 99.7 | +/+ 0.38, +/− 0.31 |
| Q9QM65    | Ribosome biogenesis protein (NOP53)                                         | +/+ 70.6, +/− 0.0, −/− 84.0 | +/+ 2.83, +/− 4.92 |
| P16401    | Histone H1.5                                                                | +/+ 96.7, +/− 92.8, −/− 37.1, +/+R276W 98.2 | +/+ 0.20, +/− 0.22 |
| P16403    | Histone H1.2                                                                | +/+ 99.5, +/− 98.4, −/− 50.6, +/+R276W 99.5 | +/+ 0.27, +/− 0.29 |
| P16402    | Histone H1.3                                                                | +/+ 99.6, +/− 99.5, −/− 62.0, +/+R276W 99.6 | +/+ 0.27, +/− 0.29 |
| P10412    | Histone H1.4                                                                | +/+ 97.6, +/− 93.7, −/− 30.9, +/+R276W 98.4 | +/+ 0.27, +/− 0.29 |
| O95139    | NDUFB6                                                                      | +/+ 57.5, +/− 0.0, −/− 7.4 | +/+ 0.33, +/− 0.27 |
| Q866U2    | PABPN1                                                                      | +/+ 99.8, +/− 99.3, −/− 67.6 | +/+ 2.30, +/− 2.30 |
| Q15102    | PAFH1B3                                                                     | +/+ 99.7, +/− 99.6, −/− 84.7, +/+R276W 99.4 | +/+ 3.48, +/− 4.29 |
| Q965P9    | PPWD1                                                                       | +/+ 98.8, +/− 85.6, −/− 98.8 | +/+ 0.33, +/− 0.13 |
| Q9R337    | VPS4A                                                                       | +/+ 98.6, +/− 98.6, −/− 79.4, +/+R276W 99.4 | +/+ 1.00, +/− 0.27 |
| Q99987    | CACYBP                                                                      | +/+ 99.4, +/− 99.6, −/− 67.9, +/+R276W 100.0 | +/+ 0.81, +/− 0.81 |
| P146854   | COX6B1                                                                      | +/+ 99.2, +/− 99.2, −/− 67.3, +/+R276W 99.2 | +/+ 1.62, +/− 1.62 |
| Q02790    | FKB4                                                                        | +/+ 14.3, +/− 11.9, −/− 3.3 | +/+ 14.3, +/− 3.43 |
| Q99561    | elf-2-α kinase activator GCN1                                               | +/+ 62.0, +/− 54.2, −/− 7.3 | +/+ 1.41, +/− 1.15 |
| Q76003    | GLRX3                                                                       | +/+ 98.2, +/− 0.0, −/− 85.3, +/+R276W 94.0 | +/+ 1.15, +/− 1.07 |
| Q00893    | HNRNPU                                                                      | +/+ 99.9, +/− 98.7, −/− 79.9, +/+R276W 96.4 | +/+ 1.52, +/− 1.62 |
| Q89X5     | KLHL7                                                                       | +/+ 96.5, +/− 94.0, −/− 48.7, +/+R276W 98.3 | +/+ 0.76, +/− 0.81 |
| Q99544    | LRRCS9                                                                      | +/+ 60.0, +/− 52.3, −/− 7.9 | +/+ 1.41, +/− 1.62 |
| P62897    | PP4A                                                                        | +/+ 34.3, +/− 31.7, −/− 6.4 | +/+ 1.15, +/− 0.93 |
| P62891    | RPL11                                                                       | +/+ 99.6, +/− 99.3, −/− 77.9, +/+R276W 99.6 | +/+ 1.00, +/− 0.93 |
| P40429    | RPL13A                                                                      | +/+ 95.4, +/− 92.0, −/− 3.5 | +/+ 0.76, +/− 0.76 |
| P25998    | RPS12                                                                       | +/+ 98.0, +/− 99.0, −/− 82.4, +/+R276W 92.9 | +/+ 1.23, +/− 1.23 |
| Q89N27    | THOC2                                                                       | +/+ 99.7, +/− 50.4, −/− 0.0 | +/+ 1.00, +/− 1.07 |
| Q9Y559    | TIMM8B                                                                      | +/+ 95.5, +/− 96.0, −/− 82.7, +/+R276W 0.0 | +/+ 1.62, +/− 1.52 |
| Q94856    | TOMM70A                                                                     | +/+ 98.7, +/− 98.0, −/− 61.0, +/+R276W 98.4 | +/+ 1.41, +/− 1.62 |
| Q99433    | UBXN1                                                                       | +/+ 99.1, +/− 96.7, −/− 41.0, +/+R276W 99.3 | +/+ 0.81, +/− 0.81 |
| P31946    | YWHAB                                                                       | +/+ 95.3, +/− 93.7, −/− 50.9, +/+R276W 96.6 | +/+ 1.32, +/− 1.41 |
| Q994X9    | ALKBH4                                                                      | +/+ 97.5, +/− 44.6, −/− 0.0 | +/+ 0.00, +/− 0.00 |
| Q8NRG0    | CHRC1                                                                       | +/+ 99.4, +/− 88.3, −/− 99.7, +/+R276W 99.1 | +/+ 0.00, +/− 0.00 |
| Q9Y241    | HIG1A                                                                       | +/+ 98.9, +/− 99.4, −/− 87.3, +/+R276W 99.3 | +/+ 0.00, +/− 0.00 |
| P17096    | HMGA1                                                                       | +/+ 99.5, +/− 99.5, −/− 88.8, +/+R276W 99.6 | +/+ 0.00, +/− 0.00 |

ALKBH4 indicates α-ketoglutarate-dependent dioxygenase; CACYBP, calcyclin-binding protein; CHRC1, chromatin accessibility complex protein 1; COP53, COP9 signalosome complex subunit 3; COX6B1, cytochrome c oxidase subunit 6B1; EDF1, endothelial differentiation-related factor 1; FKB4, peptidyl-prolyl cis-trans isomerase; GLRX3, glutaredoxin 3; HIG1A, HIG1 domain family member 1A, mitochondrial; HMGA1, high mobility group protein HMGI-α/HMG-Y; HNRNPU, heterogeneous nuclear ribonucleoprotein U; iPSC, induced pluripotent stem cell; KLHL7, Kelch-like protein 7; LRRCS9, leucine-rich repeat-containing protein 59; NDUFB6, NADH dehydrogenase (ubiquinone) 1β subcomplex subunit 6; PABPN1, polyadenylate-binding protein 2; PAFH1B3, platelet-activating factor acetylhydrolase IB subunit γ; PPIA, peptidyl-prolyl cis-trans isomerase A; PPWD1, peptidyl-prolyl isomerase domain and WD repeat-containing protein 1; RPL11, 60S ribosomal protein L11; RPL13A, 60S ribosomal protein L13a; RPS12, 40S ribosomal protein S12; THOC2, THO complex subunit 2; TIMM8B, mitochondrial import inner membrane translocon subunit; TOMM70A, mitochondrial import receptor subunit; UBXN1, UBX domain-containing protein 1; VPS4A, vacuolar protein sorting-associated protein 4; and YWHAB, 14-3-3 protein beta/alpha.

*Altered acetylation levels= >10% deviation.
†Protein levels could not be determined or quantified.

had a NatA-target residue at position 2 of the mature protein (Table XI in the Data Supplement). NatA-target residues, alanine and serine, were frequently observed at position 2. Similar frequencies are seen in proteins with no expression changes (Table XI in the Data Supplement).
Supplement). Despite the large numbers of differentially expressed proteins, there were very few, if any, significant differences in mRNA levels, as assessed by RNAseq analysis (Tables III and IV in the Data Supplement).

Among differentially expressed proteins identified in NAA15<sup>−/−</sup> iPSCs and NAA15<sup>+/−</sup> iPSCs, the levels of HYPK, a component of the NatA complex that interacts with NAA15, were significantly reduced (Figure 1C).<sup>14</sup>–<sup>17</sup> A total of 29 out of 54 known interactors of HYPK were detected in the WT iPSCs (Table XII in the Data Supplement, and Figure 4D). Affected small ribosomal subunits (RPS18, RPS25, RPS19, RPS20) were clustered at the head of the small ribosomal subunit<sup>53</sup> (Figure 5C). Large ribosomal units<sup>53</sup> (RPL5 [ribosomal protein L5], RPL13, RPL15, RPL19, RPL30, RPL31, RPL35, RPL23A, RPL39) with low protein levels were clustered at the exit tunnel of the ribosome near the NAA15/NatA docking site (Figure 5B).

Fetal Heart Expression and Association With CHD of Proteins Perturbed in NAA15-Haploinsufficient iPSCs

Because extensive protein expression studies of human and mouse fetal heart are not available, we assessed expression of the RNAs encoding these proteins in the mouse and human fetal heart<sup>46</sup>; ≈50% of the 562 proteins are expressed in the mouse and human fetal heart (unpublished). Of these 562 proteins, 4 (DHCR7 [7-dehydrocholesterol reductase], MAP2K2 [mitogen-activated protein kinase kinase 2], NSD1 [nuclear receptor binding SET domain protein 1], and RPL5; Table XIII in the Data Supplement) are encoded by genes known to cause autosomal dominant CHD (Table XIII in the Data Supplement). RNA encoding all 4 of these proteins are found in multiple cell types in the human fetal heart (Figure VIII in the Data Supplement).

DISCUSSION

Exome sequence analyses of 4511 CHD subjects with extra-cardiac phenotypes identified 20 subjects with rare

| Table 2. Second and Third Amino Acid Identities of Peptides With Altered Nt-Acetylation in NAA15 Mutant iPSCs |
|-----------------------------------------------|
| Amino acid residue | /-/- | /+- | /+R276W |
|-------------------|------|-----|--------|
| AA                | 10   | 6   | 5      |
| AD                | 0    | 1   | 0      |
| AE                | 5    | 1   | 1      |
| AQ                | 2    | 0   | 1      |
| AS                | 2    | 0   | 0      |
| SE                | 5    | 0   | 0      |
| SG                | 1    | 0   | 0      |
| SS                | 1    | 0   | 0      |
| ST                | 1    | 0   | 0      |
| TA                | 1    | 0   | 0      |
| TG                | 1    | 1   | 1      |
| TK                | 1    | 0   | 1      |
| TM                | 1    | 0   | 0      |
| TT                | 1    | 0   | 0      |
| VN                | 1    | 0   | 0      |

A indicates alanine; D, aspartic acid; E, glutamic acid; G, glycine; iPSC, induced pluripotent stem cell; K, lysine; M, methionine; N, asparagine; Q, glutamine; S, serine; T, threonine; and V, valine.
inherited or de novo variants that are predicted to perturb NAA15 (Table I and Figure I in the Data Supplement, Figure 1A). Similar NAA15 variants occur in patients with neurological abnormalites.5,7 We explored the consequences of NAA15 variants using human iPSCs by both transcriptome (RNAseq) and proteomic analyses (Figure 2; Figure II and Tables III, IV, VIII, IX, and X in the Data Supplement).

NAA15+/− cells had ≈50% of normal levels but like NAA15+/R276W iPSCs, these differentiated into cardiomyocytes. NAA15+/− iPSC-CMs displayed normal unloaded contractility, but impaired contractility when loaded (ie, working against resistance; Figure V in the Data Supplement). NAA15+/−/ ipcscs produced no NAA15 and like yeast cells lacking NAA15, grew slowly (Figure 2D).19 NAA15-deficiency had minimal effect on the transcriptome but significantly altered the proteome consistent with its role in protein modification.

Because NAA15 is a component of the NatA complex, we assessed the level of N-terminal protein acetylation in iPSCs carrying NAA15 variants using positional proteomics. Approximately 650 proteins with N-terminal sequences that were predicted to be NatA targets could be assessed. Only 32 and 9 proteins had altered Nt-acetylation in NAA15 null and haploinsufficient cells, respectively. By contrast, levels of 562 proteins were altered in both NAA15-null and NAA15-haploinsufficient cells suggesting a possible role for NAA15 and NatA in translation efficiency and protein stability in addition to Nt-acetylation. If NAA15 was the only available ribosome anchor for NatA complex in human cells as it is in yeast cells,8,55,58 NAA15 null cells should present with a complete lack of Nt-acetylation of all NatA substrates. The explanation for the partial effect observed may be due to the presence of the NAA15 paralogue NAA16 in human cells; however, the low expression of NAA16 in iPSCs suggests the need for additional studies to measure whether the paralogue to NAA15 had any effect on N-terminal acetylation. It has been reported that NAA16-NAA10 complexes may perform NatA-type Nt-acetylation in human cells and partially act as a backup system for the NAA15-NAA10 NatA complex.63

The levels of 18 of the 81 identified ribosomal proteins were altered in NAA15-haploinsufficient cells. NAA15 anchors the NatA complex to the ribosomal large subunit via RPL25 (L23A).55–58,64 Consistent with this model, some of these affected proteins clustered

Figure 4. Proteins differentially expressed in NAA15+/− and NAA15−/− induced pluripotent stem cells (iPSCs).

A, Scatterplot displays log2 fold changes of differentially expressed proteins in NAA15+/− and NAA15−/− iPSCs compared with wildtype (WT) iPSCs. Approximately 1200 proteins are differentially expressed, 9 and 3 proteins of which have reduced Nt-acetylation in NAA15−/− iPSCs and NAA15+/− iPSCs, respectively. Data was collected from 2 independent undifferentiated iPSC cell lines for each NAA15 (N-alpha-acetyltransferase 15) mutant genotype and at least 3 technical replicates for each sample. B, Comparison of differentially expressed proteins in NAA15+/− and NAA15−/− iPSCs. Differential expression was considered for log2 fold change [1]. Identified proteins: 5196, quantified proteins: 3911 proteins detected by mass spectrometry (C) gene ontology (GO) enrichment of differentially expressed proteins in both NAA15+/− and NAA15−/− iPSCs. A total of 41 proteins are localized to the ribosome. Raw P value presented in data table. D, Bar graph represents log2 fold change of ribosomal proteins that are differentially expressed in both NAA15+/− and NAA15−/− iPSCs.
near the NAA15/NatA docking site. Presumably, NAA15 binding to one or more of these proteins protects them from subsequent degradation either during ribosome assembly or subsequent ribosomal activity. Previous studies have demonstrated that ribosomal subunit deficiency leads to a variety of pathological states. Damaging human variants in ribosomal subunits RPS19, RPL5, and RPL35A have been identified in patients with CHD (PCGC consortium data not shown) and also contribute to Diamond-Blackfan anemia, a disorder that is accompanied by CHD in 30% of patients.

Among differentially expressed proteins in NAA15 haploinsufficient cells, 4 (DHCR7, MAP2K2, NSD1, and RPL5) are encoded by known CHD genes (Table XIII and Figure VIII in the Data Supplement) and are most likely responsible for NAA15-haploinsufficient mediated CHD. As the precise mechanisms by which each haploinsufficiency of each of these genes is defined, we anticipate the mechanism(s) will be shared by NAA15 haploinsufficient patients.

Exome analyses of CHD probands also identified NAA15 missense variants, including R276W, a variant of uncertain significance. iPSCs carrying the NAA15 R276W variant had eight proteins with altered Nt-acetylation, of which 4 had altered Nt-acetylation in NAA15-haploinsufficient cells. These data suggest that the
R276W variant likely impairs NAA15 function, and consequently NaTα function, and like heterozygous LoF variants contributes to CHD. Future studies of protein levels in genetically engineered iPSCs carrying other NAA15 variants found in CHD probands, will help identify those variants contributing to cardiac abnormalities and those variants that are likely benign.

In this study, we use genetically engineered iPSC models to recapitulate NAA15 LoF and missense variants discovered in CHD patients. We observe that aberrant protein expression of NAA15 alters Nt-acetylation of a small number of proteins. Most importantly, a reduction of the NAA15 protein interrupts its interaction with the ribosome. The failed interaction causes ribosomal deficiency and mis-expression of a large number of proteins most likely involved in heart development. Protein expression changes are possibly due to obstructed ribosomal machinery and defects in protein synthesis. One or more proteins affected in both NAA15-mutant iPSCs have been documented as a cause of congenital heart defects and are likely candidates required for normal cardiac developmental processes.

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Affiliations
Genetics (T.W., WT, SM, G.V., MYJ, JALW, AH, J.G., YK, AP, JH, CCB, S.RD, C.S., J.G.S) and Howard Hughes Medical Institute (AH, CS), Harvard Medical School. Division of Newborn Medicine, Boston Children’s Hospital (SM). VIB Center for Medical Biotechnology, B-9000 Ghent, Belgium (FL, D.V.H., ET, K.G). VIB Proteomics Core, B-9000 Ghent, Belgium (FL, D.V.H., ET). Biomolecular Medicine (FL, D.V.H., ET, K.G.) and Biochemistry and Microbiology (PvV.D), Ghent University, B-9000 Ghent, Belgium University of Sao Paulo (G.V.). Bio- medical Engineering, Boston University, MA (KZ, C.S.C.). The Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA (KZ, C.S.C.). Medicine, Brigham and Women’s Hospital (AH, CS). Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York (BD.G.). Pediatrics and Medicine, Columbia University Medical Center, New York (WK.C). Cardiology, Children’s Hospital of Philadelphia, Department of Pediatrics, The Perelman School of Medicine, University of Pennsylvania, Philadelphia (E.G.). Pediatrics, University of Rochester Medical Center (G.A.P). Genetics, Yale University School of Medicine, New Haven (R.P.L., MB). Laboratory of Human Genetics and Genomics, Rockefeller University, New York (R.P.L.). Pediatrics, Yale University School of Medicine, New Haven (MB). Molecular Medicine Program, University of Utah, Salt Lake City (HJ.Y). Gladstone Institutes, San Francisco, CA (GB.B). Division of Cardiovascular Medicine, Brigham and Women’s Hospital (Y.K). Biomedicine (S.V., TA) and Biological Sciences (S.V., TA), University of Bergen, N-5002 Bergen, Norway. Donnelly Centre for Cellular and Molecular Research, Toronto, Canada (S.V.). Surgery, Hakelund University Hospital, N-5021 Bergen, Norway (TA).

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Disclosures
None.

Supplemental Materials
Expanded Online Materials and Methods
Online Figures I–VIII
Online Movies I–VIII
Data Set of Online Tables I–XIII
References 72–76

REFERENCES
1. van der Linde D, Konings EE, Slager MA, Witsenburg M, Helbing WA, Talenkenberg J, Roos-Henrickson JW. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. J Am Coll Cardiol. 2011;58:241–247. doi: 10.1016/j.jacc.2011.08.025
2. Homsy J, Zaidi S, Shen Y, Ware JS, Samocha KE, Karczewski KJ, DePalma SR, McKean D, Wakiimoto H, Gorham J, et al. De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies. Science. 2015;350:1262–1266. doi: 10.1126/science.aac9396
3. Zaidi S, Cho M, Wakiimoto H, Ma L, Jiang J, Overton JD, Romano-Adesman A, Bjornson RD, Breitbart RE, Brown KK, et al. De novo mutations in histone-modifying genes in congenital heart disease. Nature. 2013;498:229–233. doi: 10.1038/nature12141
4. Jin SC, Homsy J, Zaidi S, Lu Q, Morton S, DePalma SR, Zeng X, Qi H, Chang W, Sierant MC, et al. Contribution of rare inherited and de novo variants in 2,871 congenital heart disease probands. Nat Genet. 2017;49:1593–1601. doi: 10.1038/ng.3970
5. Cheng H, Dharmadhikari AV, Varland S, Ma N, Domingo D, Kleyner R, Rope AP, Yoon M, Stray-Pedersen A, Pose JE, et al. Truncating variants in NAA15 are associated with variable levels of intellectual disability, autism spectrum disorder, and congenital anomalies. Am J Hum Genet. 2018;102:985–994. doi: 10.1016/j.ajhg.2018.03.004
6. Guo H, Wang T, Wu H, Long M, Coe BP, Li H, Xun G, Ou J, Chen B, Duan G, et al. Inherited and multiple de novo mutations in autism/developmental delay risk genes suggest a multifactorial model. Mol Autism. 2018;9(6):4. doi: 10.1186/s13229-018-0247-z
7. Zhao JJ, Halvorsen J, Zander CS, Zaghloul A, Georgii-Hemming P, Månsson E, Brandberg G, Sävmarker HE, Frykholm C, Kuchinskaya E, et al. Exome sequencing reveals NAA15 and PUF60 as candidate genes associated with intellectual disability. Am J Med Genet B Neuropsychiatr Genet. 2017;178:10–20. doi: 10.1002/ajmg.b.32574
8. Arnensen T, Van Damme P, Polevoda B, Helbo H, Eijernt R, Coiera N, Varhaug JE, Vandekerckhove J, Lillehaug JR, Varhaug JE, Vandekerckhove J, Lillehaug JR, Sherman F, et al. Proteomic analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci U S A. 2009;106:8167–8162. doi: 10.1073/pnas.091931109.
10. Draczik A, Myklebust LM, Ree R, Arnesen T. The role of protein acetylation. *Biochim Biophys Acta*. 2016;1864:1372–1401. doi: 10.1016/j.bjbaap.2016.06.007

11. Akses H, Draczik A, Marie M, Arnesen T. First things first: vital protein marks by N-terminal acetyltransferases. *Trends Biochem Sci*. 2016;41:746–760. doi: 10.1016/j.tibs.2016.07.005

12. Akses H, Ree R, Arnesen T. Co-translational, post-translational, and non-catalytic roles of N-terminal acetyltransferases. *Mol Cell*. 2019;73:1097–1114. doi: 10.1016/j.molcel.2019.02.007

13. Frottin F, Martinez A, Peynot P, Mitrà S, Holz RC, Giglione C, Mennel T. The proteomics of N-terminal methionine cleavage. *Mol Cell Proteomics*. 2005;6:2353–2349. doi: 10.1074/mcp.M600022-MCP200

14. Arnold RJ, Polevoda B, Reilly JP, Sherman F. The action of N-terminal acetyltransferases on yeast ribosomal proteins. *J Biol Chem*. 1999;274:37035–37040. doi: 10.1074/jbc.274.52.37035

15. Weyer FA, Gumiero A, Lapouge K, Bange G, Kopp J, Sinning I. Structural characterization of yeast NatA and its regulation by chromatin context and mechanism of acetylation by the N-terminal dual enzyme NatA/NtAc. *Nat Struct Mol Biol*. 2008;15:7240–255. doi: 10.1038/nstm.2008.003

16. Ryningen A, Guarner A, Lapouge K, Bange G, Kopp J, Sinning I. Structural basis of NtAc regulating N-terminal acetylation by the NatA complex. *Nat Commun*. 2017:8:15726. doi: 10.1038/s41592-017-0036

17. Deng S, Magin RS, Wei X, Pan B, Petersson EJ, Marmorstein R. Structure and mechanism of acetylation by the N-terminal dual enzyme NatA/NtAc. *Proc Natl Acad Sci USA*. 2019:2:10066–10070.e4. doi: 10.1073/pnas.1905182116

18. Mullen JR, Kayne PS, Moerschell RP, Tsunasawa S, Gribskov M. Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. *EMBO J.* 1989;8:2067–2075.

19. Arnesen T, Anderson D, Baldersheim C, Lanotte M, Varhaug JE, Lillehaug JR. Identification and characterization of the human ARD1–NATH protein complex. *Mol Cell Biol*. 2009;27:1057–1067. doi: 10.1128/MCB.01512-08

20. Arnesen T, Starheim KK, Van Damme P, Evjenth R, Dinh H, Betts MJ, Ohye RG, Sleeper LA, Mahony L, Newburger JW, Pearson GD, Lu M, Gelb B, Brueckner M, Chung W, Goldmuntz E, Kaltman J, Kaski JP, Kim R, Fluge Ø, Bruland O, Akslen LA, Varhaug JE, Lillehaug JR. Identification and characterization of the human Nt-terminal acetyltransferase A induces p53-dependent apoptosis and p53-independent growth inhibition. *J Biol Chem*. 2010;285:2777–2789. doi: 10.1074/jbc.M109.001145

21. Kalvik TV, Arnesen T. Protein N-terminal acetyltransferases in cancer. *Onco. Gene. 2013;32;269–276. doi: 10.1023/A:1012880310041

22. Toepfer CN, Sharma A, Ciconneto M, Garfinkel AC, Macke M, Neyazi M, Willcox JL, Argawal R, Schmid M, Rao J, et al. SarcTrack. *Circ Res*. 2019;2:e201800150. doi: 10.1371/journal.pone.0051415

23. Hinson JT, Chopra A, Nafissi N, Polackow JW, Benson CC, Swift S, Gorham J, Yang L, Schafer S, Scheng, CC, et al. HEART DISEASE. Tnf mutations in iPSC cells define sarcolemme insufficiency as a cause of dilated cardiomyopathy. *Science*. 2015;349:982–986. doi: 10.1126/science.aaa5458

24. Legant WR, Pathak A, Yang MT, Deshpande VS, McRee MK, Chen HS. Microfibrillar tissue gauges to measure and manipulate forces from 3D microtissues. *Proc Natl Acad Sci USA*. 2009;106:10097–10102. doi: 10.1073/pnas.090017105

25. Ohye RG, Sleeper LA, Mahony L, Newburger JW, Pearson GD, Lu M, Gelb B, Brueckner M, Chung W, Goldmuntz E, Kaltman J, Kaski JP, Kim R, Arnesen T, Anderson D, Baldersheim C, Lanotte M, Varhaug JE, Lillehaug JR. Identification and characterization of the human ARD1–NATH protein complex. *Mol Cell Proteomics*. 2005;6:433–443.

26. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA*. 2001;98:5116–5121. doi: 10.1073/pnas.09012461

27. Sharma A, Toepfer CN, Ward T, Wason L, Argawal R, Conner DA, Hu JH, Seidman C. CRISPR/Cas9-mediated fluorescent tagging of endogenous proteins in human pluripotent stem cells. Current protocols in human genetics. 2018;69:11.1–11.20.

28. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 2018;96:21111–21120.

29. Gregory B, Rahman N, Bommakanti A, Shamsuzzaman M, Thapa M, Deshpande VS, McRee MK, Chen HS. Microfibrillar tissue gauges to measure and manipulate forces from 3D microtissues. *Proc Natl Acad Sci USA*. 2009;106:10097–10102. doi: 10.1371/journal.pone.0051415

30. Zhang Y, Cooke M, Panjwiani S, Cao K, Krauth B, Ho PY, Medrzycki M, Berke DT, Pan C, McDevitt TC, et al. Histone h1 deletion impairs embryonic stem cell differentiation. *PloS Genet*. 2012;8:e1002691. doi: 10.1371/journal.pgen.1002691

31. Mangéé A, Coyaud E, Desmetz C, Laurent E, Bègatton B, Coopman P, Raught B, Solassol J. FKB44 connects mTORC2 and PISK to activate the PDK1/Akt-dependent cell survival signaling in breast cancer. *Theranostics*. 2019;9:7003–7015. doi: 10.7150/thno.35561

32. Teng T, Mercer CA, Hesley P, Thomas G, Fumagalli S. Loss of tumor suppressor RPL5/RPL11 does not induce cell cycle arrest but impedes proliferation due to reduced ribosome content and translation capacity. *Mol Cell Biol*. 2013;33:4680–4671. doi: 10.1128/MCB.00174-13

33. Hattasharajee RB, Big JP. Deletion of nuclear poly(A) binding protein PABPN1 produces a compensatory response by cytoplasmic PABP4 and PABP5 in cultured human cells. *PloS One*. 2012;7:e50369. doi: 10.1371/journal.pone.0050369

34. Choudhary KR, Bucha S, Baks S, Mukhopadhyay D, Bhattacharya NP. Chaperone-like protein HYPK and its interacting partners augment autophagy. *Eur J Cell Biol*. 2016;95:182–194. doi: 10.1016/j.ejcb.2016.03.003

35. Zheng J, Mugler CF, Kellison AM, Khan ME, Wei M, Mertins P, Regov A, Jovanovic B, Brar GA. Small and large ribosomal subunit deficiencies lead to distinct gene expression signatures that reflect cellular growth rate. *Mol Cell*. 2019;73:36–47.e10. doi: 10.1016/j.molcel.2018.10.032

36. Gregory B, Rahman N, Bommakanti A, Shamsuzzaman M, Thapa M, Lesure AE, Zengel ML, Lindahl L. Small and large ribosomal subunits depend on each other for stability and accumulation. *Life Sci Alliance*. 2019;2:e201800150.
50. Narla A, Ebert BL. Ribosomopathies: human disorders of ribosome dysfunc-
   tion. Blood 2010;115:3196–3205. doi: 10.1182/blood-2009-10-178129

51. Peisker K, Braun D, Wölfe T, Hentschel J, Fünfschilling U, Fischer G,
   Sickmann A, Rospert S. Ribosome-associated complex binds to ribosomes in
   close proximity of Rpl31 at the exit of the polypeptide tunnel in yeast. Mol
   Cell Biol 2008;18:5279–5288. doi: 10.1007/mcb-06-0661

52. Steffen KK, McCormick MA, Pham KM, Mackay VL, Delaney JR, Murakami
   CJ, Kaeberlein M, Kennedy BK. Ribosome deficiency protects against ER
   stress in Saccharomyces cerevisiae. Genetics. 2012;191:107–118. doi:
   10.1534/genetics.111.136549

53. Khatter H, Mysnikov AG, Nachbar SF, Klaholz BP. Structure of the human
   ribosome. Nature 2008;103:492–508. doi: 10.1002/jcb.21418

54. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol
   Graph 1996;14:33–38. doi: 10.1065/jmg/2.1583-5

55. Gautschi M, Just S, Mun A, Ross S, Rücknagel P, Dubaquié Y,
   Ehrenhofer-Murray A, Rospert S. The yeast N(alpha)-acetyltransferase
   NatA is quantitatively anchored to the ribosome and interacts with nascent
   polypeptides. Mol Cell Biol 2003;23:7403–7414. doi: 10.1128/mcb.
   23.2003.7403-7414.2003

56. Kramer G, Boehringer D, Ban N, Bukau B. The ribosome as a platform
   docking site on the ribosome. Mol Cell Proteomics 2014;13:2513–
   2526. doi: 10.1074/mcp.M113.031591

57. Polevoda B, Brown S, Cardillo TS, Rigby S, Sherman F. Yeast N(alpha)-
   acetyltransferase is associated with ribosomes. J Cell Biol. 2008;135:
   499–508. doi: 10.1083/jcb.21418

58. Varland S, Arnesen T. Investigating the functionality of a ribosome-bind-
   ing mutant of NAA15 using Saccharomyces cerevisiae. BMC Res Notes.
   2018;11:404. doi: 10.1186/s13104-018-3513-4

59. Kramer G, Boehringer D, Ban N, Bukau B. Ribosome-NatA architecture reveals that rRNA expan-
   sion segments coordinate N-terminal acetylation. Nat Struct Mol Biol
   2019;26:35–39. doi: 10.1038/s41594-018-0165-y

60. Cui Y, Zheng Y, Liu X, Yan L, Fan X, Yong J, Hu Y, Dong J, Li Q, Wu X,
   Wilson DN, Nierhaus KH. Ribosomal proteins in the spotlight.

61. Kramer G, Rauch T, Rist W, Vorderwülbecke S, Patzelt H, Schulze-Specking
   et al. Single-cell transcriptome analysis maps the developmental track
   of the human heart. Circ Res. 2019;112:1582–1592. doi: 10.1182/blood-2008-02-140012

62. Gaza HT, Sheen MR, Vlachos A, Choesmel V, O’Donohue MF, Schneider H,
   Darras N, Hasman C, Seiff CA, Newburger PE, et al. Ribosomal protein L5
   and L11 mutations are associated with cleft palate and abnormal thumbs in
   Diamond-Blackfan anemia patients. Am J Hum Genet. 2008;83:769–780.

63. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A,
   et al. Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of
   Genetic Medicine, Johns Hopkins University, Baltimore. Accessed April
   2020. https://omim.org/

64. Altshuler D, Gabriel S, Tされていた
   fastQ data to high confidence variant calls: the Genome Analysis Toolkit
   best practices pipeline. Curr Protoc Bioinformatics. 2013;43:11.10.1–
   11.10.33. doi: 10.1002/0471250953.bi1110s43

65. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G,
   Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, et al. From
   short to long read sequence alignment using Bespin. Genome Res. 2010;20:
   1297–1303. doi: 10.1101/gr.107524.110

66. van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G,
   Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, et al. From
   short to long read sequence alignment using Bespin. Genome Res. 2010;20:
   1297–1303. doi: 10.1101/gr.107524.110

67. Oyston J. Online Mendelian Inheritance in Man. Anesthesiology. 1998;89:
   811–812. doi: 10.1097/00000542-199809000-00006

68. Vlachos A, Osorio DS, Atsidafos E, Kang J, Lababidi ML, Selden HS,
   Gruber D, Glader BE, Ondel K, Farrar JE, et al. Increased prevalence of congenital
   heart disease in children with Diamond Blackfan anemia suggests
   unrecognition Diamond Blackfan anemia as a cause of congenital
   heart disease in the general population: a report of the Diamond Blackfan
   Anemia Registry. Circ Genom Precis Med. 2018;11:e002044. doi:
   10.1161/CIRCGENETICS.117.002044

69. Maceth L. [Genetic diseases on the Internet: OMIM]. Ann Dermatol Venereol
   1998;125:645.

70. Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of
   Genetic Medicine, Johns Hopkins University, Baltimore. Accessed April
   2020. https://omim.org/

71. Mutley-Davidson WJ, Miller DJ, Henderson JR, Gage D, Nagle SM, Dresseda
   M, et al. Ribosomal deficiency protects against ER stress in Saccharomyces cerevisiae. Cell Rep.
   2019;26:1934–1950.e5. doi: 10.1016/j.celrep.2019.01.079

72. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A,
   et al. Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of
   Genetic Medicine, Johns Hopkins University, Baltimore. Accessed April
   2020. https://omim.org/

73. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A,
   et al. Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of
   Genetic Medicine, Johns Hopkins University, Baltimore. Accessed April
   2020. https://omim.org/

74. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G,
   Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, et al. From
   short to long read sequence alignment using Bespin. Genome Res. 2010;20:
   1297–1303. doi: 10.1101/gr.107524.110

75. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G,
   Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J, et al. From
   short to long read sequence alignment using Bespin. Genome Res. 2010;20:
   1297–1303. doi: 10.1101/gr.107524.110

76. Dong C, Wei P, Jian X, Gibbs R, Boerwinkle E, Wang K, Liu X. Comparison
   and integration of deleteriousness prediction methods for nonsynonymous
   SNVs in whole exome sequencing studies. Hum Mol Genet. 2015;24:2125–
   2137. doi: 10.1093/hmg/ddu733

77. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate proteome-
   wide label-free quantification by delayed normalization and maximal peptide
   ratio extraction, termed MaxLFQ. Mol Cell Proteomics. 2014;13:2513–
   2526. doi: 10.1074/mcp.M113.031591