Diluted cardiomyopathy (DCM) is characterized by reduced cardiac output, as well as thinning and enlargement of left ventricular chambers. These characteristics eventually lead to heart failure. Current standards of care do not target the underlying molecular mechanisms associated with genetic forms of heart failure, driving a need to develop novel therapeutics for DCM. To identify candidate therapeutics, we developed an in vitro DCM model using induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) deficient in B-cell lymphoma 2 (BCL2)-associated athanogene 3 (BAG3). With these BAG3-deficient iPSC-CMs, we identified cardioprotective drugs using a phenotypic screen and deep learning. From a library of 5500 bioactive compounds and siRNA validation, we found that inhibiting histone deacetylase 6 (HDAC6) was cardioprotective at the sarcomere level. We translated this finding to a BAG3 cardiomyocyte–knockout (BAG3<sup>−/−</sup>) mouse model of DCM, showing that inhibiting HDAC6 with two isoform-selective inhibitors (tubastatin A and a novel inhibitor TYA-018) protected heart function. In BAG3<sup>−/−</sup> and BAG3<sup>E455K</sup> mice, HDAC6 inhibitors improved left ventricular ejection fraction and reduced left ventricular diameter at diastole and systole. In BAG3<sup>−/−</sup> mice, TYA-018 protected against sarcomere damage and reduced Nppb expression. Based on integrated transcriptomics and proteomics and mitochondrial function analysis, TYA-018 also enhanced energetics in these mice by increasing expression of targets associated with fatty acid metabolism, protein metabolism, and oxidative phosphorylation. Our results demonstrate the power of combining iPSC-CMs with phenotypic screening and deep learning to accelerate drug discovery, and they support developing novel therapeutics that address underlying mechanisms associated with heart disease.

**INTRODUCTION**

Diluted cardiomyopathy (DCM) is a form of heart muscle weakness characterized by reduced cardiac output, as well as thinning and enlargement of left ventricular chambers (1, 2). DCM affects about 1 of 2500 adults (2), accounts for 30 to 40% of all heart failure cases in clinical trials, and is a major cause of heart transplants (1, 3). Current treatments for heart failure include angiotensin–converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, aldosterone antagonists, vasodilators, angiotensin receptor–neprilysin inhibitors, and sodium-glucose cotransporter 2 inhibitors. However, these treatments mainly ameliorate symptoms and do not target the underlying molecular mechanisms associated with genetic forms of heart failure (4). Therefore, we critically need therapeutics that precisely target the genetic causes of heart failure and prevent worsening function of cardiac muscle (5).

About one-third of individuals with DCM have an inherited form of the disease. Familial DCM accounts for 30 to 50% of all DCM cases and has an autosomal-dominant mode of inheritance (1). Genetic forms of DCM have been associated with more than 50 genes, and more than 50% of patients with DCM have at least one mutation in one of these genes (1, 2). Several of these DCM-associated genes code for central regulators of protein quality control, and mutations in these genes lead to protein aggregation and accumulation of misfolded proteins (6, 7).

One gene that is essential to maintaining protein quality control is B-cell lymphoma 2 (BCL2)-associated athanogene 3 (BAG3). BAG3 is a stress response gene, and it acts as a heat shock protein 70 (HSP70) co-chaperone in a complex with small HSPs to maintain cardiomyocyte function (8–12). BAG3 is highly expressed in cardiac and skeletal muscle, and it can localize to the Z-disk (13). BAG3 has also been proposed to protect myocytes from mechanical damage and proteotoxic stress (9, 14).

Mutations in BAG3 have been linked to DCM. In adults over 40 years old, loss-of-function BAG3 mutations show 80% penetrance in DCM (1, 2). Familial BAG3 mutations are autosomal dominant, suggesting a heterozygous loss-of-function mechanism (2, 9, 15). BAG3 mutations that result in BAG3 loss of function account for ~3% of variant distribution in DCM genes (16). Most mutations in BAG3 are deleterious (e.g., E455K), but a cardioprotective variant (C151R) has also been reported (2). This finding suggests that the BAG3 chaperone complex may acquire a gain-of-function phenotype that protects against proteotoxic stress and mechanical damage in the heart. In addition, mutations in BAG3 led to cardiac-related phenotypes both in vivo and in vitro, in zebrafish (17, 18), mice (6, 13), and human induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) (9). In the BAG3 mouse models (6), cardiac output shows a steady decline, which creates an ideal window of opportunity to intervene with a small molecule. Moreover, BAG3 expression was reduced in patients with idiopathic DCM, implying that BAG3 must be expressed at certain amounts to maintain its chaperone function in protein quality control (19, 20). Therefore, BAG3 is an attractive target for developing small-molecule therapeutics for...
BAG3 myopathies. These efforts could also lead to interventions for other genetic causes of DCM and nongenetic forms of heart failure (7).

In this study, we aimed to identify small-molecule therapeutic targets for BAG3-related DCM that could enable drug development for genetically defined cardiomyopathies that disrupt protein quality control. Previously, we showed the feasibility of high-content phenotypic screening using deep learning with iPSC-CMs (21). We hypothesized that we could use a similar approach to identify cardioprotective small-molecule targets in BAG3-deficient iPSC-CMs that would translate to therapeutic benefits in a mouse model of DCM.

RESULTS

Loss of BAG3 in iPSC-CMs leads to sarcomere damage

To develop a robust and reproducible loss-of-function model of DCM, we expressed small interfering RNAs (siRNAs) targeting BAG3 in human iPSC-CMs. First, using scramble (SCR) siRNA labeled with red fluorescent protein, we optimized conditions for iPSC-CM seeding density and transfection to ensure more than 95% transfection efficiency (fig. S1A). Next, using a pool of four siRNAs targeting BAG3, we obtained ~90% knockdown of BAG3 mRNA in iPSC-CMs as measured using quantitative polymerase chain reaction (qPCR; Fig. 1A) and RNA sequencing (RNA-sequencing; fig. S1B). We also showed that the quantity of BAG3 protein was reduced by 70 to 90% using Western blot, flow cytometry, and immunostaining (Fig. 1, B and C, and fig. S1, C to E). BAG3 knockdown (BAG3KD) in iPSC-CMs remained suppressed for at least 10 days after transfection. This finding is likely due to the low proliferative capacity of iPSC-CMs that prevented their recovery even with 5-azacytidine (5-aza) or adenoviral expression of hTERT (fig. S1A). We showed that BAG3 siRNA knockdown model showed stronger and more consistent sarcomere damage and reduced batch-to-batch experimental variability. In addition, our model using BAG3 siRNA was amenable to high-content screening because it provided the ideal dynamic range to enable robust hit identification (fig. S2).

Given the central role of BAG3 in maintaining protein quality control, we looked at the quantity of proteins in the BAG3 chaperone complex. In particular, the protein HSPB8 forms a stoichiometric complex with BAG3, and its stability depends on this association (11). Compared to SCR iPSC-CMs, BAG3KD iPSC-CMs expressed less HSPB8 (Fig. 1B), suggesting destabilization of the BAG3 chaperone complex. We also assessed p62, a ubiquitin-binding protein (coded by the SQSTM1 gene) that targets damaged proteins for clearance through the autophagosome (22). Compared to SCR iPSC-CMs, BAG3KD iPSC-CMs showed lower expression of p62 (Fig. 1, B and C), suggesting defects in sarcomere integrity and autophagic flux. We further assessed sarcomere integrity by evaluating cardiac myosin-binding protein C (MYBPC3) staining. MYBPC3 is a cardiac myocyte-specific protein that localizes to the C-zone of the sarcomere (23), and its content and localization highly depend on sarcomere integrity. MYBPC3 also interacts with BAG3 (9). In BAG3KD iPSC-CMs, protein expression of MYBPC3 was unchanged when measured with Western blot (Fig. 1B); however, they were reduced by ~25% (P < 0.00001) when measured with immunostaining (Fig. 1C). Immunostaining of MYBPC3 revealed sarcomere damage in BAG3KD iPSC-CMs (Fig. 1, D and E).

To identify the best time for screening after knockdown, we looked at the extent of sarcomere damage in BAG3KD iPSC-CMs. We manually scored sarcomeres [described in the manual sarcomere scoring method of the Supplemental Materials and Methods (9, 24)] and found that the percentage of damaged sarcomeres increased over time (Fig. 1D). Figure 1E shows representative immunostaining of iPSC-CMs treated with SCR or BAG3 siRNA. The arrows indicate breaks and reduced sarcomere content in BAG3KD iPSC-CMs.

Deep learning reproducibly and accurately identifies healthy and diseased cellular phenotypes

To efficiently and reproducibly quantify sarcomere damage in BAG3KD iPSC-CMs, we adopted an imaging analysis method that uses deep learning (25). As we previously described (21), we used a two-class deep learning model based on healthy (SCR siRNA–treated) and diseased (BAG3 siRNA–treated) iPSC-CMs. We labeled about 1300 images from each class of iPSC-CMs and fed them into the neural network (Fig. 2A). In each training, 20% of the images were reserved for validation. Images were passed through a series of nodes in the hidden layer to build a model that identified features that best separate the two classes of cells. This process was repeated 20 times (epochs) until an optimal model was generated. Figure 2B shows representative accuracy plots from three independent studies. In cases with a robust cellular phenotype, the accuracy of the model was greater than 95%. In cases with a mild or variable phenotype, the accuracy of the model was ~70 to 80%. In cases with no phenotype to separate the classes, the accuracy of the model was ~50%, suggesting a random class designation.

To show the high accuracy of deep learning in identifying subtle and undefined features of cells, six members of the laboratory who routinely conduct iPSC-CM assays blindly scored sarcomeres in 100 randomly selected images (50:50 mix of SCR and BAG3 siRNA–treated). They categorized images on the basis of sarcomere damage to each class. Although this approach achieved an average accuracy of 70 to 75% for each class, deep learning achieved more than 99% accuracy (Fig. 2C). We also saw reproducible sarcomere damage in several pilot studies using individual siRNAs targeting BAG3 (fig. S2). In addition, by titrating the concentration of BAG3 siRNA, we identified intermediate amounts of sarcomere damage using the cardiomyocyte score based on deep learning (fig. S2).

High-content phenotypic screening identifies histone deacetylase and microtubule inhibitors as cardioprotective compounds

To identify candidate therapeutics that protect against sarcomere damage, we conducted an unbiased high-content screen using a library of 5500 bioactive compounds. To this end, iPSC-CMs were seeded, allowed to recover, treated with either SCR or BAG3 siRNA, and then treated with the bioactive compounds at a 1 μM concentration (Fig. 2D). Using deep learning, we determined the sarcomere score for each compound. A high sarcomere score indicated low sarcomere damage; a low sarcomere score indicated high sarcomere damage, and a negative sarcomere score indicated that the compound was toxic (Fig. 2E).

We ranked screening results on the basis of the sarcomere score and designated a hit threshold of 0.3 (false discovery rate of 1%). Results from wells treated with either SCR or BAG3 siRNA were plotted as controls. These data showed that cells treated with SCR siRNA had a sarcomere score ranging from 0.3 to 1, whereas cells treated with BAG3 siRNA had a sarcomere score below 0.3. After manually excluding false-positive hits (due to rare staining and imaging artifacts; fig. S3A), we grouped the top 24 hits from the screen into distinct target classes (Fig. 2F). A list of compounds identified in the primary screen is summarized in table S1. The top


2 of 15
predicted cardioprotective compounds fell under two major classes: histone deacetylase (HDAC) inhibitors and microtubule inhibitors. Among these, the screen identified three compounds that can be broadly classified as “standard-of-care” agents for cardiovascular indications: omecamtiv mecarbil (cardiac myosin activator), sotalol (beta-blocker and K-channel blocker), and anagrelide [phosphodiesterase 3 (PDE3) inhibitor]. These results validate the translational relevance of using iPSC-CMs and deep learning to identify cardioprotective compounds in an unbiased and high-content manner. Representative immunocytochemistry images from the primary screen are shown in fig. S3B.

**HDAC inhibitors do not up-regulate BAG3 expression in iPSC-CMs**

Given the abundance of hit enrichment with HDAC inhibitors, we wanted to ensure that HDAC inhibitors did not prevent sarcomere damage by increasing BAG3 expression in wild-type (WT) iPSC-CMs. To capture a broad spectrum of inhibitors, we used all HDAC inhibitors identified from the screen and additional HDAC inhibitors. On the basis of immunostaining and qPCR, none of the HDAC inhibitors increased BAG3 expression in WT iPSC-CMs (fig. S3, C and D). These data suggest that the HDAC inhibitors did not protect against sarcomere damage by preventing BAG3 knockdown or up-regulating BAG3. Instead, they confer cardioprotection through a different mechanism.

**HDAC6 inhibition protects against BAG3 loss of function in iPSC-CMs**

We performed a secondary validation of the top hits from the primary screen, the results of which are summarized in Fig. 3A. These results highlighted that HDAC and microtubule inhibitors are putative cardioprotective compounds. HDAC inhibitors show varying types of polypharmacology for different HDAC isoforms. For example, class I HDACs (HDAC1, 2, 3, and 8) are predominantly located in the nucleus and target histone substrates. Inhibiting these isoforms activates global or specific gene expression programs (26). We further interrogated all HDACs individually using siRNA to co-knockdown BAG3 and individual HDAC isoforms (HDAC1 through HDAC11). In independent studies (two to seven biological replicates), co-knockdown of HDAC6 with BAG3 prevented sarcomere damage induced by BAG3 knockdown as measured by the cardiomyocyte score (Fig. 3B). Representative immunostainings of BAG3 siRNA–treated cells showed damaged sarcomeres, which appeared reduced by knockdown of HDAC6 (Fig. 3C).

We further validated these findings using siRNAs that independently target HDAC1 through HDAC11. We found that two siRNAs (1 and 3) targeting HDAC6 protected against sarcomere damage in the BAG3KD model when used separately or pooled (fig. S4A) and did not affect

---

**Fig. 1. Development of BAG3 loss-of-function model in iPSC-CMs.**

(A) qPCR analysis of iPSC-CMs treated with SCR or BAG3 siRNA, analyzed 10 days after knockdown. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. (B) iPSC-CMs treated with SCR and BAG3 siRNA were harvested 2 to 11 days after treatment. Total protein was extracted from samples and analyzed using Western blot. KD, knockdown. (C) Protein quantification using immunostaining of iPSC-CMs treated with SCR and BAG3 siRNA. (D) Quantification of sarcomere damage in iPSC-CMs treated with SCR or BAG3 siRNA. (E) Representative immunostaining of iPSC-CMs treated with SCR or BAG3 siRNA and then stained with an antibody against MYBPC3. Arrows indicate breaks and reduced sarcomere content in BAG3 knockdown cells. Scale bars, 50 μm. N > 3 technical replicates. Data are shown as means ± SD. Multiple comparisons were made using one-way analysis of variance (ANOVA). *P < 0.05, **P < 0.01, and ****P < 0.0001. ns, not significant.
agents were identified: sotalol (beta-blocker and K-channel blocker), omecamtiv mecarbil (cardiac myosin activator), and BAG3 expression (fig. S4B). To further confirm which HDAC is a target for tubulin, we also measured acetylated tubulin (Ac-Tubulin) intensity in these knockdown studies. We found that Ac-Tubulin intensity was significantly greater in knockdowns of HDAC3 and HDAC6 versus the SCR control ($P < 0.01$; fig. S4C).

**HDAC6 inhibition or knockout leads to tubulin hyperacetylation**

Previous studies showed that HDAC6 is located in the cytoplasm (27, 28). Here, we verified that HDAC6 is predominantly cytoplasmic (≈90%) in iPSC-CMs (fig. S5, A and B). Using CRISPR-Cas9, we overexpressed human HDAC1 through HDAC11 (tagged with a green fluorescent protein reporter at the C-terminal domain) in HDAC6$^{KO}$ iPSC-CMs and measured Ac-Tubulin intensity. Overexpression of HDAC6 in HDAC6$^{KO}$ iPSC-CMs reduced Ac-Tubulin (fig. S5, E and F), confirming that HDAC6 is a tubulin deacetylase (27). HDAC6$^{KO}$ iPSC-CMs have about three times higher Ac-Tubulin intensity than HDAC6$^{WT}$ iPSC-CMs. We treated HDAC6$^{WT}$ iPSC-CMs with an HDAC6 inhibitor (tubastatin A), which increased Ac-Tubulin to an intensity similar to HDAC6$^{KO}$ iPSC-CMs (fig. S5G).

**TYA-018 is a highly selective HDAC6 inhibitor**

Hydroxamic acids are zinc chelators that have been used extensively to develop pan- and HDAC-selective inhibitors. However, hydroxamic acids are known to cause genotoxicity (29); in addition, they either lack the desired selectivity or show poor bioavailability with an unfavorable pharmacokinetic profile (30, 31). Thus, we developed a novel, exquisitely selective HDAC6 inhibitor (TYA-018) by using a fluoroalkyl oxadiazole as the zinc-binding group (Fig. 4A). We ensured high selectivity of TYA-018 using a biochemical assay and measured potency against HDAC6 and selectivity against HDAC1 (Fig. 4B). As controls, we used a pan-HDAC inhibitor (givinostat) and a well-known HDAC6-specific inhibitor (tubastatin A). Tubastatin A is selective for HDAC6; however, at high concentrations, it has off-target activity with HDAC1, HDAC8 (30), and HDAC10 (32). We also assessed on-target activity of TYA-018 by measuring Ac-Tubulin in iPSC-CMs (Fig. 4, C to E). The data suggest that TYA-018 is more potent and selective than tubastatin A. We further interrogated TYA-018 in a cell-based assay by measuring acetylated lysine on histone H3 and H4. We did not detect any off-target activity of TYA-018 on nuclear HDACs, indicating high selectivity (Fig. 4E).

We confirmed the selectivity of TYA-018 in a full set of biochemical assays using HDAC1 through HDAC11 (fig. S6A). TYA-018 had more than 2500-fold selectivity compared with other HDAC6 knockout (HDAC6$^{KO}$) iPSCs. We successfully differentiated these cells to cardiomyocytes, as described previously (21), which showed expression of sarcomeric markers [cardiac troponin T (TNNT2) and MYBPC3] and hyperacetylation of tubulin (fig. S5, C and D).
Figure 3. Target validation studies show inhibiting HDAC6 alone protects against sarcomere damage in BAG3-deficient iPSC-CMs. (A) Top compound classes (HDAC inhibitors and microtubule inhibitors) from the library screen and two cardiovascular standard-of-care agents (omecamtiv mearcaril (Omecamtiv) and sotalol) identified from the screen were validated at a 1 μM dose using the cardiomyocyte score. Data from two to four independent biological replicates. Cntrl, control. (B) Further validation using siRNAs to knock down HDAC6 in BAG3 KO iPSC-CMs using the cardiomyocyte score with the deep learning algorithm. Data from two to four independent biological replicates. N = 4 to 16 technical replicates per biological replicate. (C) Representative immunostaining of anti-MYBPC3 and Hoechst counterstain in iPSC-CMs treated with SCR, BAG3, or BAG3 + HDAC6 siRNA. Arrows indicate sarcomere damage. Scale bar, 50 μm. Data are shown as means ± SD. Multiple comparisons were made using one-way ANOVA. ****P < 0.0001.

zinc-dependent HDACs (fig. S6B). Using immunostaining for acetylated lysine, we confirmed that TYA-018 does not have off-target HDAC6 activity (fig. S6C). Next, we interrogated cellular toxicity of TYA-018 in human embryonic kidney cells and showed that a lethal dose (LD₅₀) is greater than 50 μM (fig. S6D). Last, with a pro-B-type natriuretic peptide (ProBNP) assay, we found that TYA-018 did not dose-dependently increase ProBNP concentration, as seen with givinostat and tubastatin A (fig. S6E).

Next, we performed RNA-seq on WT iPSC-CMs treated with TYA-018, givinostat, and two HDAC6-selective inhibitors (tubastatin A and ricolinostat). Raw RNA-seq data are available on the Gene Expression Omnibus (GEO) database (GSE180248) and provided in table S2. With increasing selectivity, we reduced the number of genes with altered transcript expression in iPSC-CMs. These findings further confirm the high selectivity of TYA-018 for HDAC6, ensuring that the activity of TYA-018 is not associated with transcriptional activation in WT iPSC-CMs (fig. S7, A to C). In addition, treating HDAC6 KO cells with TYA-018 does not increase the intensity of Ac-Tubulin (fig. S7, D and E).

**Cardiomyocyte-specific KO of BAG3 in mice leads to heart failure**

We used a cardiomyocyte-specific BAG3-KO mouse (BAG3\textsuperscript{−/−}) as a general model of DCM (fig. S8A) (6) and to test the efficacy of HDAC6 inhibitors. Although BAG3 loss of function accounts for only ~3% of variant distribution in DCM genes (16), we chose this model because BAG3 is a central node in protein quality control for sarcomeric proteins, and it is involved in maintaining myocyte function (6, 9, 20). In addition, as reported by Fang and colleagues (6), this mouse model shows a steady decline in heart function and death because of heart failure. We found that by 5 months old, the mice showed an average ejection fraction of ~30% and a survival rate of ~50% (fig. S8, B and C). We also found that left ventricular internal diameter at diastole (LVIDd), left ventricular internal diameter at systole (LVIDs), and left ventricular mass were significantly greater in BAG3\textsuperscript{−/−} mice versus their WT littermates (P < 0.05; fig. S8D). M-mode echocardiography tracings from BAG3\textsuperscript{−/−} mice showed a steady decline in heart function from 1 to 5 months old (fig. S8E). This steady decline in heart function gives an ideal window of opportunity to intervene with a therapeutic small molecule.

**Givinostat and tubastatin A prevent progression of heart failure in BAG3\textsuperscript{−/−} mice**

To evaluate the translatability of our findings from in vitro screening to an in vivo model, we conducted an efficacy study in BAG3\textsuperscript{−/−} mice using a pan-HDAC inhibitor (givinostat) and HDAC6-selective inhibitor (tubastatin A). Previous literature suggested that pan-HDAC inhibitors provide protection in various heart failure models (33–35). First, we used both inhibitors to assess what percentage of efficacy comes from inhibiting only HDAC6 and whether inhibiting other HDACs has additional benefits. Both givinostat and tubastatin A have similar biochemical and cell-based potencies for HDAC6 inhibition (Fig. 4, B and C).

We started administering daily doses of givinostat (30 mg/kg by oral gavage) and tubastatin A (50 mg/kg by intraperitoneal injection) when mice were 1 month old (Fig. 5A). At this age, BAG3\textsuperscript{−/−} mice show significantly lower (~13%, P < 0.0001) heart function, as measured by ejection fraction, than WT littermate controls (Fig. 5, B and H).
Fig. 4. TYA-018 is a highly selective HDAC6 inhibitor. (A) Chemical structures of givinostat, tubastatin A, and TYA-018. (B) Biochemical assays using recombinant human HDAC6 and HDAC1 deacetylase activity showing inhibition curves after treatment with givinostat (pan-HDAC inhibitor), tubastatin A (HDAC6-selective inhibitor), and TYA-018 (HDAC6-selective inhibitor). (C) Cell-based assay in iPSC-CMs showing the dose-response curve of tubulin acetylation (Ac-Tubulin) with drug concentrations. EC50, half maximal effective concentration. (D) Immunostaining of iPSC-CMs treated with 5.5 μM each drug stained with anti–Ac-Tubulin antibody. Scale bar, 200 μm. MW, molecular weight (E) Western blot of iPSC-CMs treated with TYA-018 (HDAC6-specific inhibitor) stained with monoclonal anti–Ac-Lysine. Giv, givinostat (pan-HDAC inhibitor control). Data are shown as means ± SD.
Over the 10-week treatment, daily administration of both givinostat and tubastatin A prevented the progression of heart failure (Fig. 5, B to E and H to K). In addition, LVIDd and LVIDs were significantly reduced in BAG3\(^{cKO}\) mice treated with givinostat (P < 0.05 for LVIDd and P < 0.001 for LVIDs; Fig. 5, F and G) or tubastatin A (P < 0.01 for LVIDd and P < 0.001 for LVIDs; Fig. 5, L and M). On the basis of these efficacy studies, inhibiting HDAC6 alone provided cardioprotection against heart failure in BAG3\(^{cKO}\) mice. In addition, polypharmacology associated with a pan-HDAC inhibitor did not provide further cardioprotection in these mice.

**Tubastatin A protects against heart failure in BAG3\(^{E455K}\) mice**

To mimic patient-specific mutations, we used a second mouse model containing a human mutation of BAG3 (BAG3\(^{E455K}\); fig. S9A) (6). Mutations in this domain of BAG3 disrupt the interaction between BAG3 and HSP70, destabilizing the chaperone complex needed to maintain protein quality control and homeostasis in the cell (6). Because only HDAC6 inhibition provided sufficient cardioprotection, we began a third efficacy study in BAG3\(^{E455K}\) mice. To test whether later interventions could protect against heart failure, we treated these mice with tubastatin A (50 mg/kg by intraperitoneal injection) at 3 months old. After 6 weeks of treatment, tubastatin A provided cardioprotection, including improved ejection fraction (fig. S9, B to G), in BAG3\(^{E455K}\) mice. Tubastatin A also reduced the mortality rate due to heart failure in BAG3\(^{E455K}\) mice (fig. S9H). Protection against death due to heart failure was more pronounced in male mice (fig. S9I), supporting studies that found that males are more susceptible to BAG3 loss of function (36).

**TYA-018 selectively inhibits HDAC6, reducing Nppb expression and sarcromere damage and preventing heart failure in BAG3\(^{cKO}\) mice**

We tested the efficacy of the novel, highly selective HDAC6 inhibitor TYA-018 in BAG3\(^{cKO}\) mice. In this fourth efficacy study, we treated mice daily with TYA-018 (15 mg/kg by oral gavage) starting at 2 months old (Fig. 6A). Similar to the efficacy studies with givinostat and tubastatin A, TYA-018 conferred cardioprotection in these mice during the 8-week dosing period, as indicated by the increased ejection fraction (Fig. 6, B to E) and significantly reduced LVIDd (P < 0.05; Fig. 6, F and G). Because TYA-018 is highly selective for HDAC6 (fig. S6) and structurally distinct from tubastatin A (Fig. 4A), this efficacy study confirms that HDAC6 inhibition exclusively drives the cardioprotection. The cardioprotective effects of TYA-018 were not tested in BAG3\(^{E455K}\) mice.

At 4 months old, after 8 weeks of dosing with TYA-018, we collected and processed hearts from mice in the efficacy study. qPCR analysis of brain natriuretic peptide [a cardiac stress heart failure biomarker (37–39) encoded by the Nppb gene] showed an about threefold increase in BAG3\(^{cKO}\) mouse hearts. TYA-018 treatment significantly reduced Nppb expression to near WT (P < 0.001; Fig. 6H). Heart tissue also showed a slightly increased percentage (1.3%) of trichrome-positive tissue in BAG3\(^{cKO}\) mice versus their WT littermates, suggesting that BAG3\(^{cKO}\) mice at 4 months old do not have substantial fibrosis in the heart. TYA-018 treatment reduced fibrosis in BAG3\(^{cKO}\) mice, albeit not significantly (P = 0.555) (Fig. 6I). Heart tissues also showed higher filamin-C (FLNC), phosphatase and tensin homolog-induced kinase 1 (PINK1), and voltage-dependent anion channel 2 (VDAC2) and lower HSPB8 and p62 in BAG3\(^{cKO}\) mice versus WT mice (Fig. 6I). In BAG3\(^{cKO}\) mice, TYA-018 treatment partially restored protein expression of FLNC, PINK1, VDAC2, and p62 to amounts similar to WT mice. In addition, alpha-actinin-2 (ACTN2) and hematoxylin and eosin staining show sarcomere damage, reduced content, and myofibril disarray in hearts from BAG3\(^{cKO}\) mice versus those from WT mice and BAG3\(^{cKO}\) mice treated with TYA-018 (Fig. 6, K and L). TYA-018 treatment significantly reduced the percentage of cardiomyocytes with damaged and reduced sarcromeres in BAG3\(^{cKO}\) mice (P < 0.01; Fig. 6M).

**TYA-018 treatment reduces mitochondrial content, decreases apoptotic nuclei, and increases LC3 puncta in hearts of BAG3\(^{cKO}\) mice**

Immunohistochemistry with a mitochondrial-specific antibody showed greater mitochondrial content in BAG3\(^{cKO}\) mouse hearts (Fig. 7A), suggesting a high energy demand potentially due to increased sarcomere damage and myofibril disarray. In BAG3\(^{cKO}\) mice, TYA-018 significantly reduced mitochondrial content to amounts similar to WT mice (P < 0.05; Fig. 7, A to E). Using immunohistochemistry, we saw higher fragmented and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling–positive (TUNEL\(^{+}\)) nuclei in BAG3\(^{cKO}\) mice versus their WT littermates (P < 0.05). However, TYA-018 did not reduce fragmented and TUNEL\(^{+}\) nuclei in BAG3\(^{cKO}\) mice (Fig. 7, F to H, and fig. S10, A and B). In addition, with immunostaining using microtubule-associated protein light chain 3 (LC3), which regulates autophagosome formation, we observed greater LC3 puncta and a higher percentage of LC3\(^{+}\) areas in hearts from BAG3\(^{cKO}\) mice treated with TYA-018 (Fig. 7I). The percentage of LC3\(^{+}\) areas correlated with ejection fraction (Fig. 7I), suggesting that activating autophagy and clearing damaged mitochondria may be part of the cardioprotective mechanism of HDAC6 inhibitors. However, single-agent and co-drug treatment studies in iPSC-CMs did not indicate activation of autophagic flux based on ratios of LC3II/LC3I and p62 (fig. S10, C and D). This result suggests that a more mature cell system may be needed to detect activation of autophagic flux in iPSC-CMs. As part of our mechanistic studies, we tested whether TYA-018 affects acetylation of the antioxidant peroxiredoxin 1 (PRDX1). The acetylation status of PRDX1 did not differ after TYA-018 treatment (fig. S10E).

**TYA-018 treatment enriches targets associated with muscle contraction, protein and fatty acid metabolism, and oxidative phosphorylation in BAG3\(^{cKO}\) mice**

We conducted transcriptomics and proteomics analysis of coding genes in hearts harvested from the three arms (WT+Veh, BAG3\(^{cKO}\)+Veh, and BAG3\(^{cKO}\)+TYA-018) in the fourth efficacy study. Raw RNA-seq data are available on the GEO database (GSE179656) and provided in table S3. Principal component analysis showed that WT and BAG3\(^{cKO}\) mice form two distinct clusters, and the BAG3\(^{cKO}\)+TYA-018 mice cluster between WT and BAG3\(^{cKO}\) mice (Fig. 8A). This analysis showed a global correction of BAG3\(^{cKO}\)+TYA-018 coding genes toward their WT littermates. The RNA-seq data showed an about fourfold increase in Nppb expression in BAG3\(^{cKO}\) mice versus WT mice at 4 months old. TYA-018 reduced Nppb expression in BAG3\(^{cKO}\) mice, and Nppb expression was anticorrelated (R < −0.6) with ejection fraction (Fig. 8B). In BAG3\(^{cKO}\) mice treated with TYA-018, the top enriched terms in the biological process category included fatty acid oxidation, respiratory electron transport chain, mitochondrial adenine triphosphate (ATP) transport, and muscle contraction (fig. S11A). The data showed trending correction of key sarcomere
genes (Myh7, Tnnt3, and Myl3) and genes that regulate metabolism and mitochondrial function (Cyc1, Ndufs8, Ndufb8, and Prkag2). The data also showed reduced expression of genes associated with inflammation (Il-1b and Nlrp3) and apoptosis (Casp1 and Casp8) markers (fig. S11B).

Next, we generated a heatmap of genes from the selected enriched Gene Ontology (GO) terms, which showed similar gene expression in WT and BAG3ΔKO mice treated with TYA-018 (Fig. 8C). We also performed GO enrichment analysis of genes positively correlated (R > 0.5) with cardiac function (% ejection fraction) and negatively correlated (R < −0.5) with Nppb expression (fig. S11, C and D, and table S4). We also validated a subset of these targets using qPCR (fig. S11E). The genes up-regulated in control and TYA-018–treated mice encode enzymes that regulate different steps of fatty acid β-oxidation, mitochondrial ATP transport, the respiratory electron transport chain, and muscle contraction.

Differential expression analysis using transcriptomics showed up-regulation of transcripts involved in phagosome formation and maturation, the proteasome complex, and oxidative phosphorylation (Fig. 8D). To determine whether these transcriptional changes
translate to protein changes, we analyzed proteomics in WT, BAG3<sup>cKO</sup>, and BAG3<sup>cKO</sup>+TYA-018 mouse hearts using label-free mass spectrometry. To visualize the biomolecule-interaction network, we used GO enrichment analysis with Cytoscape. We found several pathways commonly altered in BAG3<sup>cKO</sup>+TYA-018 mouse hearts by both transcriptomics and proteomics. These pathways maintain normal cardiac function, muscle contraction, protein folding, energy metabolism through oxidative phosphorylation, protein...
and fatty acid metabolism, and cytoskeleton and mitochondrial organization (Fig 8E and table S5).

**TYA-018 treatment improves mitochondrial membrane potential and function in iPSC-CMs**

To test how TYA-018 affects mitochondrial membrane potential and function, we measured tetramethylrhodamine, methyl ester (TMRM) in iPSC-CMs. TMRM accumulates in the membrane of active mitochondria and is commonly used to indicate mitochondrial health (40). With TMRM fluorescent staining, we found that iPSC-CMs treated with TYA-018 (1 μM) had significantly greater mitochondrial membrane potential than those treated with dimethyl sulfoxide (DMSO; control; P < 0.01; Fig. 8, F and G). In addition, when measuring mitochondrial respiration with the Seahorse Respirometry Mito Stress Test, we found that iPSC-CMs treated with TYA-018 (1 μM) showed no significant difference in basal respiration rate (Fig. 8H). However, iPSC-CMs did show significantly greater reserve respiratory capacity than those treated with DMSO (P < 0.05; Fig. 8I). These results suggest that HDAC6 inhibition improves mitochondrial function in iPSC-CMs and augments their capacity to respond to a metabolic demand.

**DISCUSSION**

In this study, we aimed to identify small-molecule targets that could lead to novel therapeutics for cardiomyopathies, such as BAG3-related DCM. Using high-content screening that combines deep learning with BAG3-deficient iPSC-CMs, we screened 5500 bioactive compounds. We identified HDAC and microtubule inhibitors as two target classes that protect sarcomeres from damage induced by BAG3 knockdown. We also identified heart failure agents [approved by the Food and Drug Administration (FDA) or in late-stage clinical trials] that have shown clinical benefit in heart failure patients (omecamtiv mecarbil, sotalol, and colchicine), supporting the translational relevance of this phenotypic screening strategy. Using siRNAs, we further characterized the polypharmacology associated with HDAC inhibitors. We found that HDAC6 was the only isoform that protects sarcomeres from damage associated with BAG3 loss of function in iPSC-CMs.

We translated these findings to an in vivo setting by treating rodent models of DCM (BAG3 cKO and BAG3 E455K mice) with HDAC6 inhibitors. We used a known HDAC6 inhibitor (tubastatin A) and a highly selective HDAC6 inhibitor with a novel zinc-binding domain (TYA-018). Both tubastatin A (>100-fold selectivity over other HDACs) and TYA-018 (>2500-fold selectivity over other HDACs)
were efficacious in BAG3<sup>cko</sup> mice and protected them from declining heart function compared to vehicle treatment. Tubastatin A also protected BAG3<sup>E455K</sup> mice against declining heart function and reduced their mortality compared to vehicle control. The cardioprotective effects of TYA-018 were not tested in BAG3<sup>E455K</sup> mice.

Several pan-HDAC inhibitors have cardioprotective effects in pressure overload, ischemia/reperfusion, and transgenic rodent models. These inhibitors include suberoylanilide hydroxamic acid (35, 41, 42), trichostatin A (33), and givinostat (34, 43, 44). Because these inhibitors target multiple isoforms of HDACs, it was difficult to distinguish which HDAC isoform was inhibited to confer cardioprotection. Other researchers found that tubastatin A restored impaired left ventricular ejection fraction mediated by angiotensin II and that HDAC6 KO improved cardiac function in mice treated with...
angiotensin II and subjected to transaortic constriction surgery (45). We found that inhibiting HDAC6 alone was cardioprotective in DCM mouse models (BAG3<sup>KO</sup> and BAG3<sup>E455K</sup>). Treatment with the pan-HDAC inhibitor givinostat in BAG3<sup>KO</sup> was not more efficacious than an HDAC6-selective inhibitor, supporting that inhibiting HDAC6 alone provides cardioprotection in a DCM model. Inhibiting HDAC6 prevented declining heart function but did not improve heart function above baseline in BAG3<sup>KO</sup> and BAG3<sup>E455K</sup> mice.

Our study indicates that inhibiting HDAC6 alone does not lead to histone deacetylation nor alter the transcriptional profile in iPSC-CMs. This important finding excludes a mechanism that alters cellular transcriptional programs, which could affect expression of key developmental, regulatory, and ion-channel genes (46–49). HDAC6-selective inhibitors have lower cytotoxicity because of the cytoplasmic location of HDAC6 substrates and reduced effects on nuclear targets and global transcription (50). Our studies also indicate that treating with givinostat (pan-HDAC inhibitor) and ricolinostat (partially selective HDAC6 inhibitor) induces transcriptional changes in iPSC-CMs. Pan-HDAC inhibitors acetylate histones and transcription factors, leading to pleiotropic transcriptional changes that affect cellular functions, such as cell growth, apoptosis, and differentiation (47). In addition, pan-HDAC inhibitors can be highly cytotoxic (51), as most pan-HDAC inhibitors were developed for cancer therapeutics (47, 52). Therefore, a highly selective HDAC6 inhibitor may enable safe and efficacious inhibition of HDAC6 as a therapeutic target for DCM.

Although more translational work is needed to investigate the cardioprotective effects of HDAC6-specific inhibitors in other DCM and large animal models of heart failure, our data support that HDAC6 inhibitors may be promising drug candidates for treating cardiomyopathies. In addition, future studies may be needed to assess whether a combined treatment of an HDAC6 inhibitor with a standard of care (53–55) or gene therapy (20, 56) may synergistically improve heart function in a heart failure model.

Tubulin is a known substrate for HDAC6, and Ac-Tubulin is a posttranslational modification used as a pharmacodynamic marker of HDAC6 activity (27). Long-lived microtubules in the heart undergo frequent buckling because of compressive loads in the heart, and pathogenic modifications and remodeling on tubulin stiffens myocytes and reduces compliance. In myocytes, microtubules are anchored at the Z-disk via desmin (57). Thus, with buckling, microtubules exert force back on the Z-disk, causing long-term mechanical damage at the Z-disk (57, 58). Ac-Tubulin protects microtubules against this mechanical damage (59), implicating that HDAC6 inhibition may directly stabilize and protect microtubules against damage, improve myocyte compliance, and protect the Z-disk. Because sarcomere damage, myofibril disarray, and impaired sarcomere turnover are hallmarks of DCM (1, 16, 20), our study supports that inhibiting HDAC6 provides protection at the sarcomere level in DCM.

Our integrated RNA-seq and proteomics data revealed that HDAC6 inhibition enhanced cardiac energetics. Specifically, our data showed that TYA-018 increased expression of targets associated with fatty acid and protein metabolism, as well as oxidative phosphorylation. These mechanisms could underlie the improved cardiac function in BAG3<sup>KO</sup> mice treated with TYA-018. We also found that HDAC6 inhibition enhanced mitochondrial membrane potential and reserve respiratory capacity, supporting our integrated omics data and indicating greater ATP production capacity in human iPSC-CMs. Although more work is needed to fully understand the mechanism underlying the enhanced cardiac energetics and reduced sarcomere damage, one possible mechanism may involve activation of autophagic flux through chaperone-assisted selective autophagy (20). In addition, studies have shown that HDAC6 inhibition signals recruitment of the microtubule-associated protein LC3 that assembles autophagosomes and transports damaged and misfolded proteins (7, 60) and mitochondria (61, 62). In cardiomyopathies, in which protein quality control pathways are impaired, activation of autophagic flux may lead to clearance of damaged mitochondria and misfolded proteins (60, 61, 63).

In summary, our results demonstrate the power of combining iPSC-CMs with phenotypic screening and deep learning to identify therapeutic targets for heart disease. With this approach, we identified HDAC6 as a target in DCM and showed that its inhibition prevented declining heart function in BAG3<sup>KO</sup> mice. Although future work will need to assess the safety and efficacy of HDAC6 inhibitors in larger animal models and patient populations with cardiomyopathies, HDAC6 inhibitors may be promising candidates for treating cardiomyopathies and other forms of heart failure.

## MATERIALS AND METHODS

### Study design

The objective of this study was to identify cardioprotective targets using high-content screening and human iPSC-derived cardiomyocytes. The DMSO (5,5-dimethyl-oxazolidine-2,4-dione) concentration was controlled in all studies. The number of biological and technical replicates (N) per experiment is noted in each figure legend. Mice were randomized before being assigned to either vehicle or treatment groups. At least six mice were assigned for each group. An individual blinded to the genotype and treatment performed echocardiography and another individual analyzed the data. No outliers were removed from the data.

### iPSC-CM culture for high-content screening

iCell Cardiomyocytes<sup>2</sup> were thawed according to the manufacturer’s instructions (FUJIFILM Cellular Dynamics) directly onto Matrigel-coated 384-well plates at a density of 20,000 cells per well. The next day, the medium was switched to CDI maintenance media (FUJIFILM Cellular Dynamics) for 6 days before screening.

### siRNA knockdown

To knock down endogenous expression, iPSC-CMs were transfected with FlexiTube GeneSolution siRNAs (QIAGEN) at a final concentration of 5 nM using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in RPMI+B27 media (Thermo Fisher Scientific). When not specified, a siRNA pool consisting of four independent siRNAs was used. Forty-eight hours after transfection, medium was removed and replaced with fresh CD1 media.

### qPCR analysis

Real-time qPCR was performed using the TaqMan universal PCR master mix (Thermo Fisher Scientific) with the TaqMan probes listed in table S6. Additional details about this method are provided in Supplementary Methods.

### Compound library screening

A library containing 5500 bioactive compounds consisting of FDA-approved drugs, tool compounds, and preclinical drug candidates...
was sourced from Selleck Chemicals and TargetMol. After thawing, iPSC-CMs recovered for 6 days in CDI media (FUJIFILM Cellular Dynamics). Then, BAG3 siRNAs were added to the cells (day 0) to induce sarcomere damage. Two days later (day 2), medium was removed and replaced with fresh CDI media. Four days later (day 6), the compound library was added at 1.0 µM in RPMI+B27 media (Thermo Fisher Scientific). After 4 days with the compound library (day 10), cells were fixed in 4% paraformaldehyde for 15 min, stained with MYBPC3 antibody (table S7), and imaged.

Construction of deep learning models and neural network architecture

Deep learning artificial intelligence models were built using the PhenoLearn platform (www.Phenolearn.com) as described (21). We used PyTorch as the framework for the deep learning library and ResNet50 architecture, a 50-layer-deep convolutional neural network. A two-class deep learning model was developed by inputting about 1300 images from either SCR- or BAG3-siRNA–treated conditions. Each input image was divided into 12-square sub-images with sizes ranging from 224 × 224 to 300 × 300 pixels (64). We used 80% of the images to construct the neural network and the remaining 20% to validate the deep learning model. For each training, the final neural network was selected from the epoch with the highest validation accuracy. Z-factor was calculated using the following formula

\[ Z\text{-factor} = 1 - \frac{3(\sigma_p - \sigma_n)}{|\mu_p - \mu_n|} \]

Animal studies

Animal studies were performed according to Tenaya Therapeutics’ animal use guidelines. The animal protocols were approved by the Institutional Animal Care and Use Committee and accredited by the Association for Accreditation and Accreditation of Laboratory Animal Care (IACUC number: 2020.007).

Mouse models

BAG3-floxed (BAG3flo) mice and BAG3E455K+/− (equivalent to the mouse E460K) knock-in mice were obtained from J. Chen’s laboratory (University of California San Diego School of Medicine) (6). To generate BAG3 cardiomyocyte–specific KO mice, BAG3flo mice were crossed with α-myosin heavy chain–transgenic (αMHC-Cre) mice (strain code: 011038; the Jackson Laboratory). To generated cardiomyocyte–specific BAG3E455K+/− (αMHC-Cre; BAG3E455K+) mice, BAG3E455K+/− mice were crossed with BAG3Ko (αMHC-Cre; BAG3Ko) mice. Littermate BAG3flo+/+ αMHC-Cre–negative mice were used as controls. Both male and female mice older than 2 months were used in the study.

Statistical analysis

The number of technical and biological replicates and animals for each experiment are indicated in the figure legends. Statistical analyses were performed using Prism 9. Student’s t test was used to analyze two unpaired groups and one-way analysis of variance (ANOVA) with Tukey post hoc test was used to compare more than two groups. Significant differences were defined as P < 0.05. Error bars in all biochemical and cell-based assays indicate SD. Error bars in all in vivo studies represent SEM. The number of samples and subject-level data are listed in data file S1.

REFERENCES AND NOTES


**Acknowledgments:** We thank C.R. Herron for editing the manuscript. We thank B.R. Conklin and D.V. Goeddel for guidance on experimental design and valuable discussion. We thank J. Chen and X. Fang for providing the BAG3 mouse models. We thank F. Liang and M. Garcia for assistance with screening and target validation studies. We thank members of Tenaya’s in vivo pharmacology and drug discovery teams for technical assistance and helpful comments on the manuscript. **Funding:** No external funding was received for this work. **Author contributions:** M.A.M. conceived the idea and designed the experiments. M.A.M., J.Y., and T.H. supervised the studies. M.A.M., J.Y., F.G., A.B., M.C., and E.X. performed the experiments. M.A.M., J.Y., F.G., S.R., A.B., F.F., E.X., J.H., S.P., and T.H. interpreted the results of the experiments. S.R. and F.F. performed the bioinformatics analysis. K.E.L. and M.M. developed the PhenoLearn platform. M.A.M., J.Y., F.G., S.R., and F.F. curated the data. J.M., D.S., and S.P. performed medicinal chemistry. M.A.M. wrote the manuscript with support from all authors. **Competing interests:** J.Y., F.G., A.B., E.X., M.C., S.R., F.F., J.H., S.P., T.H., and M.A.M. have stock holdings at Tenaya Therapeutics. M.M. and K.E.L. are affiliated with Dana Solutions. J.M. and D.S. are affiliated with R2M Pharma. M.A.M., S.P., J.M., and D.S. are inventors on patents WO/2021/127643 and WO/2021/067859 held by Tenaya Therapeutics that covers chemical structures of HDAC6 inhibitors. **Data and materials availability:** All data associated with this study are included in the paper or the Supplementary Materials. RNA-seq data are available on the GEO database: GEO submission GSE179656 (mouse RNA-seq data) and GSE180248 (human iPSC-CM RNA-seq data).

Submitted 22 July 2021
Resubmitted 28 February 2022
Accepted 27 May 2022
Published 6 July 2022
10.1126/scitranslmed.abi5654
Phenotypic screening with deep learning identifies HDAC6 inhibitors as cardioprotective in a BAG3 mouse model of dilated cardiomyopathy

Jin Yang, Francis Grafton, Sara Ranjbarvaziri, Ana Budan, Farshad Farshidifar, Marie Cho, Emma Xu, Jaclyn Ho, Mahnaz Maddah, Kevin E. Loewke, Julio Medina, David Sperandio, Snahel Patel, Tim Hoey, Mohammad A. Mandegar

Sci. Transl. Med., 14 (652), eabl5654. • DOI: 10.1126/scitranslmed.abl5654

Screening to save sarcomeres
Loss-of-function mutations in BAG3 are associated with dilated cardiomyopathy (DCM). Here, Yang and colleagues performed high-content phenotypic screening in human induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) lacking BAG3 expression. Using deep learning, they identified histone deacetylase (HDAC) and microtubule inhibitors as potential cardioprotective agents. HDAC6 inhibition reduced sarcomere damage in cells and protected against heart failure in BAG3 cardiomyocyte–specific knockout mice and mice harboring a mutation in BAG3 linked to DCM. TYA-018, an HDAC6-specific inhibitor, reduced sarcomere damage and apoptosis and improved mitochondrial function in mice and human iPSC-CMs, supporting the translational potential of HDAC6 inhibition for DCM.

View the article online
https://www.science.org/doi/10.1126/scitranslmed.abl5654
Permissions
https://www.science.org/help/reprints-and-permissions