Circulation Research

ORIGINAL RESEARCH

EGFR/IGF1R Signaling Modulates Relaxation in Hypertrophic Cardiomyopathy

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BACKGROUND: Diastolic dysfunction is central to diseases such as heart failure with preserved ejection fraction and hypertrophic cardiomyopathy (HCM). However, therapies that improve cardiac relaxation are scarce, partly due to a limited understanding of modulators of cardiomyocyte relaxation. We hypothesized that cardiac relaxation is regulated by multiple unidentified proteins and that dysregulation of kinases contributes to impaired relaxation in patients with HCM.

METHODS: We optimized and increased the throughput of unloaded shortening measurements and screened a kinase inhibitor library in isolated adult cardiomyocytes from wild-type mice. One hundred fifty-seven kinase inhibitors were screened. To assess which kinases are dysregulated in patients with HCM and could contribute to impaired relaxation, we performed a tyrosine and global phosphoproteomics screen and integrative inferred kinase activity analysis using HCM patient myocardium. Identified hits from these 2 data sets were validated in cardiomyocytes from a homozygous *MYBPC3*$_{c.2373insG}$ HCM mouse model.

RESULTS: Screening of 157 kinase inhibitors in wild-type (N=33) cardiomyocytes (n=24 563) resulted in the identification of 17 positive inotropes and 21 positive lusitropes, almost all of them novel. The positive lusitropes formed 3 clusters: cell cycle, EGFR (epidermal growth factor receptor)/IGF1R (insulin-like growth factor 1 receptor), and a small Akt (α-serine/threonine protein kinase) signaling cluster. By performing phosphoproteomic profiling of HCM patient myocardium (N=24 HCM and N=8 donors), we demonstrated increased activation of 6 of 8 proteins from the EGFR/IGFR1 cluster in HCM. We validated compounds from this cluster in mouse HCM (N=12) cardiomyocytes (n=2023). Three compounds from this cluster were able to improve relaxation in HCM cardiomyocytes.

CONCLUSIONS: We showed the feasibility of screening for functional modulators of cardiomyocyte relaxation and contraction, parameters that we observed to be modulated by kinases involved in EGFR/IGF1R, Akt, cell cycle signaling, and FoxO (forkhead box class O) signaling, respectively. Integrating the screening data with phosphoproteomics analysis in HCM patient tissue indicated that inhibition of EGFR/IGF1R signaling is a promising target for treating impaired relaxation in HCM.

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

Key Words: cardiomyopathy, hypertrophic ■ epidermal growth factor receptor ■ insulin-like growth factor receptor ■ high-throughput screening ■ left ventricular diastolic dysfunction ■ myocytes, cardiac ■ protein kinase inhibitors

In This Issue, see p 373 | Meet the First Author, see p 374

The healthy heart is exquisitely attuned to meet the body's wide-ranging metabolic demands. Heart rate and the degree and kinetics of contraction and relaxation are the factors that determine cardiac output, which is the main determinant of exercise capacity. Impaired contraction and relaxation form the basis of almost all cardiac pathologies, with the latter observed in heart failure with preserved ejection fraction and hypertrophic cardiomyopathy (HCM).[1] The prevalence of HCM in the general adult population is 1:200 to

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Novelty and Significance

What Is Known?

- Impaired relaxation lies at the heart of diseases such as heart failure with preserved ejection fraction and hypertrophic cardiomyopathy (HCM).
- Cardiomyocyte relaxation is mediated by (1) diastolic calcium concentration, (2) titin isoforms and phosphorylation, and (3) the sensitivity of the contractile machinery to calcium.
- Our understanding of signaling pathways that modulate relaxation is not comprehensive, which limits our ability to develop therapeutic targets.

What New Information Does This Article Contribute?

- The first ever screening for compounds that improve relaxation in cardiomyocytes showed that inhibition of kinases belonging to a cell cycle, EGFR (epidermal growth factor receptor)/IGF1R (insulin-like growth factor 1 receptor), and an Akt (α-serine/threonine protein kinase) signaling cluster enhances cardiac relaxation in healthy mouse cardiomyocytes.
- HCM patient heart tissue shows activation of kinases in the EGFR signaling cluster.
- Inhibition of a subset of kinases from this cluster improves relaxation in HCM mouse cardiomyocytes.

Only fairly recently has it been recognized that impaired filling is the most common cause of heart failure and is an early pathological mechanism in patients with HCM. Although we understand the factors that directly cause relaxation, we have a far from complete understanding of the signaling pathways that modulate cardiac relaxation. Moreover, studying the entirety of these signaling pathways in physiologically relevant models has been hampered by the low throughput of existing experimental methods. We address these limitations by optimizing and using a high-throughput contractility system to study the effects of a library of kinase inhibitors on cardiac relaxation parameters in isolated healthy adult mouse cardiomyocytes. We found 21 compounds that could enhance cardiac relaxation and which can be clustered as (1) EGFR/IGF1R signaling, (2) cell cycle kinases, and (3) Akt signaling. Interestingly, our phosphoproteomics screen shows activation of the EGFR/IGF1R cluster in HCM patient myocardium. We, therefore, defined the therapeutic potential of a subset of compounds from this cluster in HCM mouse cardiomyocytes, thereby validating the role of 3 kinases. These findings show that a wide variety of kinases, including those involved in the cell cycle, may regulate cardiac contraction and relaxation. We highlight that a subset of these kinases could be a significant therapeutic approach in improving impaired relaxation of failing cardiomyocytes.

Nonstandard Abbreviations and Acronyms

Akt	α-serine/threonine protein kinase
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
FS	fractional shortening
HCM	hypertrophic cardiomyopathy
IGF1R	insulin-like growth factor 1 receptor
TTB70	time to baseline 70%
WT	wild type

1:500, and it is considered the most common familial heart disease.[1] HCM is defined by an increased wall thickness, but impaired relaxation is observed even before the onset of hypertrophy.[2,3] In addition, the ejection fraction in HCM is typically slightly increased, indicating a hypercontractile phenotype.[4–6] Mavacamten—a novel therapeutic myosin inhibitor—reduces this hypercontractile state and has been shown to be effective in increasing exercise capacity and reducing symptom load.[7] While diastolic dysfunction drives cardiac remodeling and may advance into heart failure, current treatment options for patients with HCM are ineffective at ameliorating relaxation deficits.[8] This partly stems from an incomplete understanding of the modulators of cardiomyocyte relaxation.

On a cellular level, cardiac relaxation is mediated by (1) the kinetics of $Ca2^+$ removal from the cytosol, (2) titin isoform composition and phosphorylation, and (3) the sensitivity of myofilaments to calcium.[1] Kinase signaling can modulate these steps, thereby allowing for swift changes in cardiac relaxation. A clear example of this is the binding of adrenaline to the β-adrenergic receptor and subsequent activation of PKA (protein kinase A) during the fight-or-flight response. PKA phosphorylates (1) phospholamban to speed up calcium reuptake into the sarcoplasmic reticulum,[9,10] (2) titin to increase muscle compliance,[11] and (3) cardiac troponin I and cardiac myosin-binding protein C to reduce $Ca2^+$ sensitivity of the myofilaments.[12,13] Cumulatively, this leads to faster relaxation. In addition to PKA, a small group of other kinases are known to modulate relaxation, such as the related kinase PKG (protein kinase G)[14] or CaMKII ($Ca2^+$/calmodulin-dependent kinase

II). However, only a small set of kinases have been studied to date.

In light of the above, we hypothesized that cardiac relaxation is regulated by multiple unidentified signaling molecules and pathways. By expanding our newly developed high-throughput system to measure cardiomyocyte function,[15] we were able to perform a compound screening in cardiomyocytes for the first time. A library of kinase inhibitors targeting a wide range of signaling pathways was screened in isolated adult cardiomyocytes derived from wild-type (WT) mice and our recently established HCM mouse model carrying a homozygous HCM-causing *MYBPC3* mutation.[16] We studied the effects of these compounds on cardiac relaxation parameters with the aim to identify novel signaling molecules and pathways that modulate cardiomyocyte relaxation kinetics in healthy cardiomyocytes and define their potential to correct impaired relaxation. By performing phosphoproteomic profiling of HCM patient myocardial tissue, we show the activation of specific signaling pathways that may underlie impaired relaxation observed in these patients.

METHODS

Please refer to the Supplemental Material for a detailed description of the Materials and Methods, and see the Major Resources Table in the Supplemental Material.

Data Availability

Additional phosphoproteomic data that are not provided in this article are available from the corresponding author upon reasonable request.

RESULTS

Fetal Bovine Serum Exposure to Uncover Effects of Inhibitors

We assessed the contractile effects of a library of kinase inhibitors (see Table S2 for compounds used and their targets) in isolated WT adult mouse cardiomyocytes to find novel regulators of contraction and relaxation. Because inhibitors can only function when the kinase they target is activated and cardiomyocyte measurements are performed in Tyrode solution that contains no cytokines or growth factors, we optimized our treatment protocol using varying concentrations of fetal bovine serum (FBS) in the absence and presence of the known negative inotrope, the β-adrenergic receptor blocker propanolol. FBS contains a wide range of cytokines and will activate a multitude of intracellular signaling cascades. As illustrated in Figure 1A, β-adrenergic receptor antagonist propranolol showed a small reduction in fractional shortening (FS; 16%) without FBS. The addition of FBS greatly increased the effect size, with the most clear effect seen at 2% FBS (41% reduced FS compared with the tyrode with 2% FBS). At 3% FBS, increased arrhythmic behavior was observed, so we chose tyrode with 2% FBS to perform our compound screening.

Controlling for Rundown

For our screening procedure, we performed isolations in 34 WT animals, with an average yield of 41.8±1.8% live cells. Following each isolation, cells were plated on 2 laminin-coated 24-well plates (12 wells were used per plate), and living cells were allowed to attach for 1 hour. The medium of the first plate was then changed to Tyrode solution +2% FBS and measured after 1 hour to acclimatize and to reach the target temperature of 37 °C (±0.5 °C). For our screening experiments, we measured contractile function for ≈2 to 8 hours after cardiomyocyte isolation and plating. To correct for this rundown, we alternated between control measurements (dimethylsulfoxide [DMSO]) and compound measurements (Figure 1B). In doing so, we were able to measure the effects of every compound on the contractile parameters directly before or after a control and normalize the data accordingly. We observed a gradual rundown of contraction (measured as FS; Figure 1C) over time.

Targeting of Kinase-Dependent Contractile Parameters Using a Compound Screening

Using the above adjustments, we next performed the compound screening in accordance with the 3 rounds depicted in Figure 2A. For the initial screening, each of the 157 kinase inhibitors were measured in cardiomyocytes isolated from 2 different WT mice. On average each compound in this round was measured in 59±14 cells (see Table S4 for absolute values and Table S5 for normalized values). We selected compounds as outlined in the following criteria: an increase or decrease of at least 20% in FS, 10% in time to peak 70%, 10% in time to baseline 70% (TTB70), 30% in departure velocity, or 30% in return velocity when compared with controls. The variability from a previous large-scale study was used to perform a power calculation to determine the effect size that we could reliably measure using ≈60 cells (see Table S1 for power calculations). Combined compound averages (ie, averages based on different mice) that met 1 of these selection criteria moved onto the validation round (Figure 2A) when their contractile parameters met at least 1 of these selection criteria, whereas the compounds negative for these criteria were rejected. Seventy-two compounds were selected and measured a third time. If the mean of these 3 measurements was positive for at least 1 of the selection criteria, compounds were considered as positive hits. Of the 72 compounds that entered

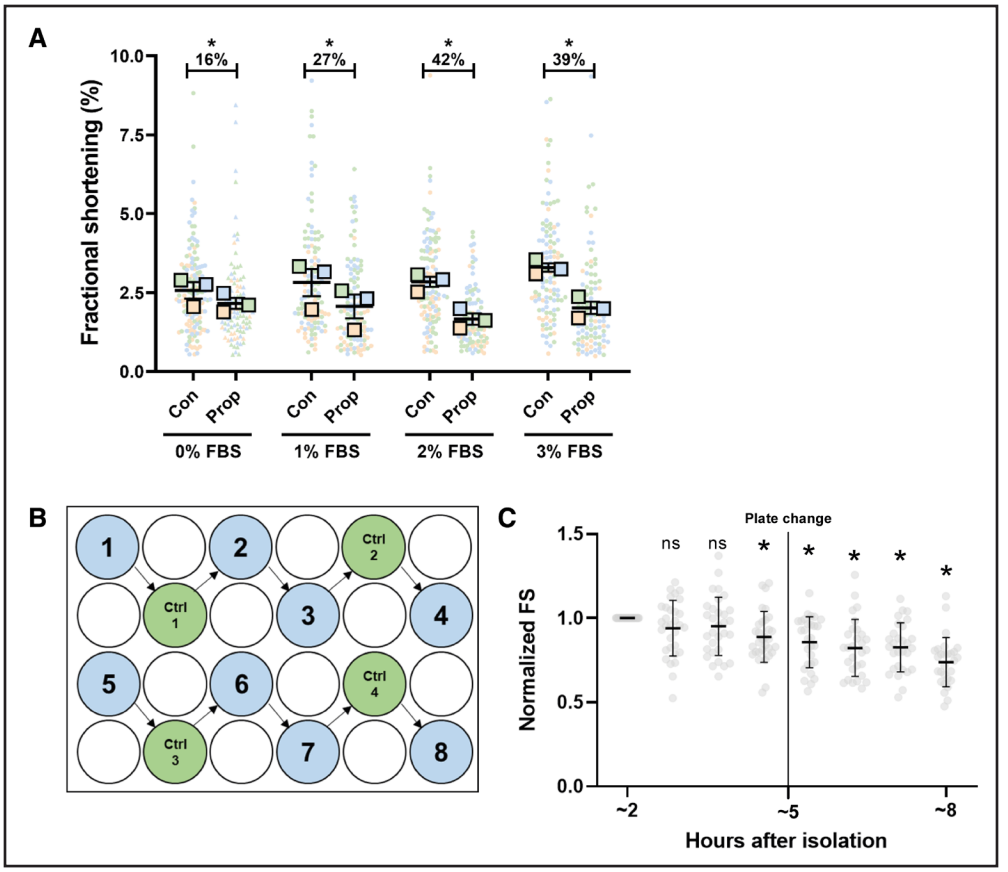

Figure 1. Experimental design and rundown over time.

A, Contractility measurements are performed in Tyrode solution. As no ligands are present in that solution, cytokine and growth factor–rich fetal bovine serum (FBS) was added to activate intracellular kinases. Mouse cardiomyocytes were incubated with a known negative inotrope, the β-adrenergic receptor blocker propranolol (Prop, 5 μM) in Tyrode solution with different concentration FBS (0%–3%). Even though a significant reduction could be found in all conditions, the effect size (stated above the line, as percentage reduction from corresponding control) was the biggest in the 2% FBS condition. For every condition, N=3 mice were measured and n≈100 cells were measured for each condition. Data are presented as a superplot with individual cells as dots and averages per day in squares and mean±SEM. Data were analyzed using hierarchical clustering method with Bonferroni post hoc test for the individual groups. **B**, Schematic depiction of how compound and control measurements were alternated to allow for normalization of the data to a neighboring control. From 1 isolation, 16 compounds and 8 controls using 2 plates were measured. After plating (1 hour) and 1-hour acclimatization in Tyrode solution with 2% FBS, measurements start. For each well, compound or dimethylsulfoxide (DMSO) is added before measurements, followed by 1-minute incubation. Pacing (2 Hz) is started 1 minute before contractility measurements start, and only the well that is measured is paced. The second plate is kept in culture medium until 1 hour before use, when Tyrode with 2% FBS is added. **C**, The high number of control wells was used because rundown of cells occurs after isolation. The graph shows fractional shortening (FS) of all 8 control wells measured per isolation (n=34) normalized to the first control well of that isolation. FS shows a slow decay over time and becomes statistically different from control 4. Around 4 to 5 hours after isolation, the 24-well plate is changed, which is indicated by the vertical line in the graph. Every gray dot represents an individual cell. Data are presented as mean±SD. Rundown data were analyzed with an ANOVA and Bonferroni post hoc tests between timepoints. *P<0.05.

the validation round, 17 compounds displayed consistent but 55 compounds demonstrated inconsistent results in 1 of the 3 measurements (ie, both increased and decreased values for 1 selection criterion) and were designated to a fourth measurement. Following this, a total of 19 compounds were identified as having 4 measurements that on average were positive for at least 1 of the selection criteria. In total, this resulted in 36 positive hits. During this screening process, 28 397 cardiomyocytes were measured from 34 WT mice, of which, following the data filtering steps illustrated in Figure 2C, 24 563 cardiomyocytes were included in our final analysis.

Positive Hits Exert Both Lusitropic and Inotropic Effects

Our screening resulted in 36 kinase inhibitors that showed an effect on contraction, relaxation, or both, of which many kinases have never been ascribed a role in modulating contraction/relaxation before. When only considering the degree of contraction (FS; Figure 3A), more kinase inhibitors were able to increase FS (12 compounds) than decrease it (3 compounds). The strongest inotropic responses were observed upon inhibition of ATR (ataxia-telangiectasia and Rad3-related protein) and ATM (ataxia-telangiectasia mutated) serine kinases

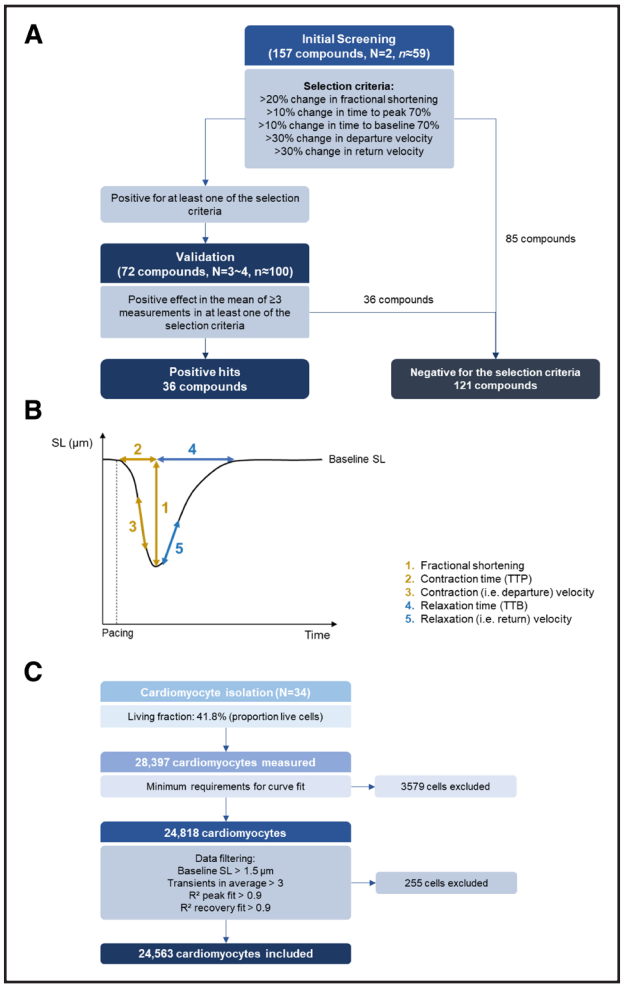

Figure 2. Flowchart of compound screening.
A, One hundred fifty-seven compounds were tested in cardiomyocytes isolated from 2 different wild-type (WT) mice in the initial screening round. We selected compounds for the validation round when their contractile parameters met at least 1 of the depicted selection criteria, whereas the compounds negative for these criteria were rejected. Seventy-two compounds were selected and measured a third time in the validation round. If the mean of these 3 measurements was positive for at least 1 of the selection criteria, compounds were considered as positive hits. Compounds that demonstrated inconsistent results in the 3 measurements were designated for a fourth measurement. In combination with the compounds that met the selection criteria in the validation round, this resulted in 36 compounds as positive hit. **B**, Schematic representing an exemplary trace, with which the contractile parameters are quantified. Every number corresponds to one of the selection criteria presented in **A**. **C**, Flowchart of WT cardiomyocyte selection. TTP, time to peak; TTB, time to baseline.

(193% increase; compound 33), IGF1R (insulin-like growth factor 1 receptor; 48% increase; compound 37), and IκB (inhibitor of nuclear factor kappa B) kinase (39% increase; compound 29). SYK (spleen-associated tyrosine kinase; compound 24) inhibition led to a 24% decrease in FS. When looking at the most important relaxation parameter (relaxation time; Figure 3B), we found 12 compounds that shortened relaxation time, while 5 prolonged it. The strongest effects on time-to-baseline shortening, that is, faster relaxation, were observed upon inhibition of TTK (monopolar spindle 1 kinase; 12% decrease; compound 131), the checkpoint kinases CHK1 (checkpoint kinase 1) and Wee1 (11% decrease; compound 34), and IGF1R (11%; compound 37), while SYK (50% increase; compound 24) and mTOR (mammalian target of rapamycin; 42% increase; compound 80) showed the strongest effect in slowing down relaxation.

We validated that short-term incubations with inhibitors for IGF1R (compound 37) and p38 MAPK (mitogen-activated protein kinase; compound 40) decreased activity of the kinases in mouse cardiomyocytes (Figure S5A and S5B).

The Table categorizes the compounds by means of their ability to increase or decrease any of the 5 assessed contractile parameters. The compounds are ordered according to their effects, thereby arranging compounds with the largest effect at the top and the smallest effect at the bottom of every list. Multiple compounds altered >1 parameter. We, therefore, categorized them into compounds that are either positive or negative lusitropes and inotropes or compounds that are both lusitropic and inotropic (Figure 4). Seventeen compounds can be classified as positive inotropes, as they increase FS, decrease time to peak 70%, or increase departure velocity. Seven compounds can be categorized as negative inotropes, as they decrease FS, increase time to peak 70%, or decrease departure velocity. Positive lusitropes are compounds decreasing TTB70 or increasing return velocity. Twenty-one of our positive hits are positive lusitropes, whereas 5 compounds are negative lusitropes, which means that they increase TTB70 or reduce return velocity. Twelve of the 21 compounds with a positive lusitropic effect also induced positive inotropy evident from the increase in FS. Overall, data show that many positive hits present with both inotropic and lusitropic effects in isolated intact cardiomyocytes, as these contractile features were not mutually exclusive.

Positive Lusitropic Hits Cluster in Cell Cycle Kinase and EGFR/IGF1R Signaling Pathways

The aim of our study was to identify novel modulators of relaxation, which could serve as potential therapeutic targets in HCM. Using specific kinase inhibitors to ascribe effects to kinases is always dependent on the off-target effects of the compounds. We used the half maximal effective concentration (EC_{50}) values of the compounds to minimize off-target effects, but this issue was not completely diminished. Some kinases such as EGFR (epidermal growth factor receptor) and TTK had 2 separate inhibitors that both improved relaxation. Having multiple kinases in the same pathway that exert similar effects increases the likelihood that the signaling pathway is a bona fide modulator of cardiac relaxation. The Search Tool for the Retrieval of Interacting

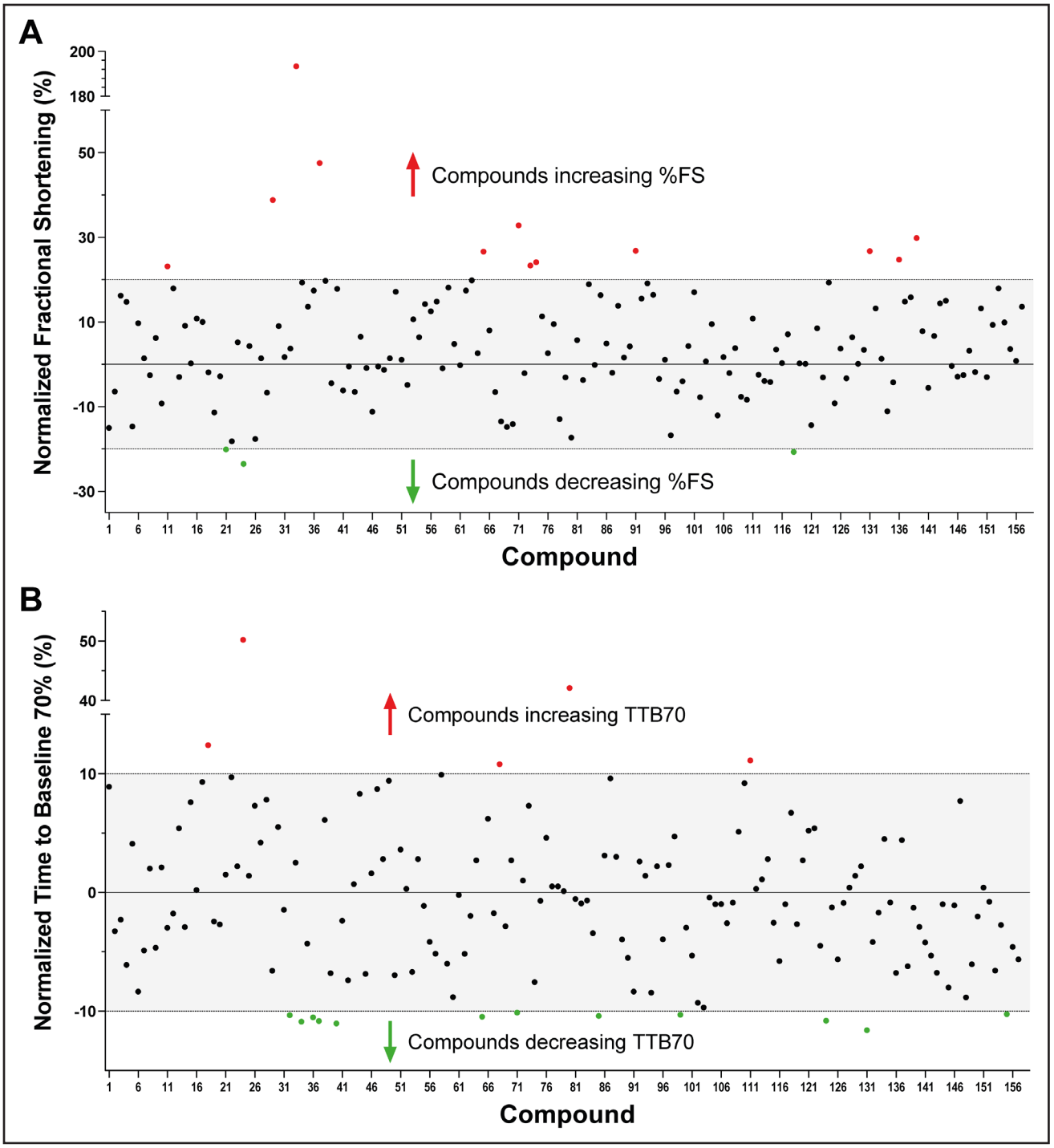

Figure 3. Effects of kinase-inhibiting compounds on contractility.
Effects of kinase-inhibiting compounds on fractional shortening (FS; **A**) and time to baseline 70% (TTB70; **B**) in wild-type murine cardiomyocytes. Every dot represents the average result for each compound, designated by a number. **A**, Gray data points indicate compounds that do not cause more than a 20% change in FS, while red and green dots bring about a >20% increase or decrease in FS, respectively. **B**, Gray data points indicate compounds that do not cause more than a 10% change in FS, while red and green dots bring about a >10% increase or decrease in TTB70, respectively.

Genes/Proteins (STRING) database uses publicly available knowledge and computational modeling to build comprehensive networks of protein-protein interactions (both physical and functional interactions). We used this resource to find whether our 21 positive lusitropes share common signaling pathways and found that they formed 3 distinct clusters (protein-protein interaction enrichment $P<0.001$): cell cycle kinases, EGFR/IGF1R signaling, and a smaller Akt (α-serine/threonine protein kinase) signaling cluster (Figure 5A). Our inotropic hits form a

Table. Overview of Kinase Targets That, When Inhibited, Increase or Decrease the Functional Properties of Isolated Murine Cardiomyocytes

Increase in FS		Increase in TTP70		Increase in TTB70		Increase in Dep Vel		Increase in Ret Vel		Decrease in FS		Decrease in TTP70		Decrease in TTB70		Decrease in Dep Vel		Decrease in Ret Vel	
Target		Target		Target		Target		Target		Target		Target		Target		Target		Target	
33	ATR/ATM kinases	80	mTOR	80	mTOR	33	ATR/ATM kinases	33	ATR/ATM kinases	24	SYK	50	MK2	131	TTK	24	SYK	24	Src kinase
37	Insulin and insulin-like receptors	24	SYK	24	SYK	91	Abl kinase	37	Insulin and insulin-like receptors	21	PKC			40	p38 MAPK				
71	TTK	44	p38 MAPK	18	EGFR	56	Casein kinase 1	91	Abl kinase	118	Rho kinase			99	Src kinases				
29	IκB kinase	37	Insulin and insulin-like receptors	111	Haspin	59	EGFR	29	IκB kinase					34	Checkpoint kinases				
139	LIMK	28	Akt	68	GSK3	37	Insulin and insulin-like receptors	71	TTK					37	Insulin and insulin-like receptors				
91	Abl kinase	33	ATR/ATM kinases			94	B-Raf	94	B-Raf					124	EGFR				
131	TTK	67	Src kinases			136	DYRK	131	TTK					36	Mnk				
65	Akt	139	LIMK			71	TTK	136	DYRK					65	Akt				
136	DYRK					74	Rho kinases	124	EGFR					85	FAK				
74	Rho kinases					124	EGFR	34	Checkpoint kinases					32	SETD8 protein lysine methyltransferases				
73	RSK							139	LIMK					155	VEGFR				
11	Cyclin-dependent protein kinases							65	Akt					71	TTK				
								74	Rho kinases										
								143	EGFR										
								92	GSK3										

The compounds are ordered according to their effects, from the largest to the smallest. The compound inhibiting the target kinase is indicated by the number in front of the target. Akt indicates α-serine/threonine protein kinase; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related protein; DYRK, dual-specificity tyrosine-regulated kinase; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FS, fractional shortening; GSK3, glycogen synthase kinase 3; IκB, inhibitor of nuclear factor kappa B; LIMK, LIM kinase-1; MAPK, mitogen-activated protein kinase; MK2, mitogen-activated protein kinase-activated protein kinase-2; mTOR, mammalian target of rapamycin; PKC, protein kinase C; RSK, ribosomal s6 kinase; SYK, spleen-associated tyrosine kinase; and TTK, monopolar spindle 1 kinase.

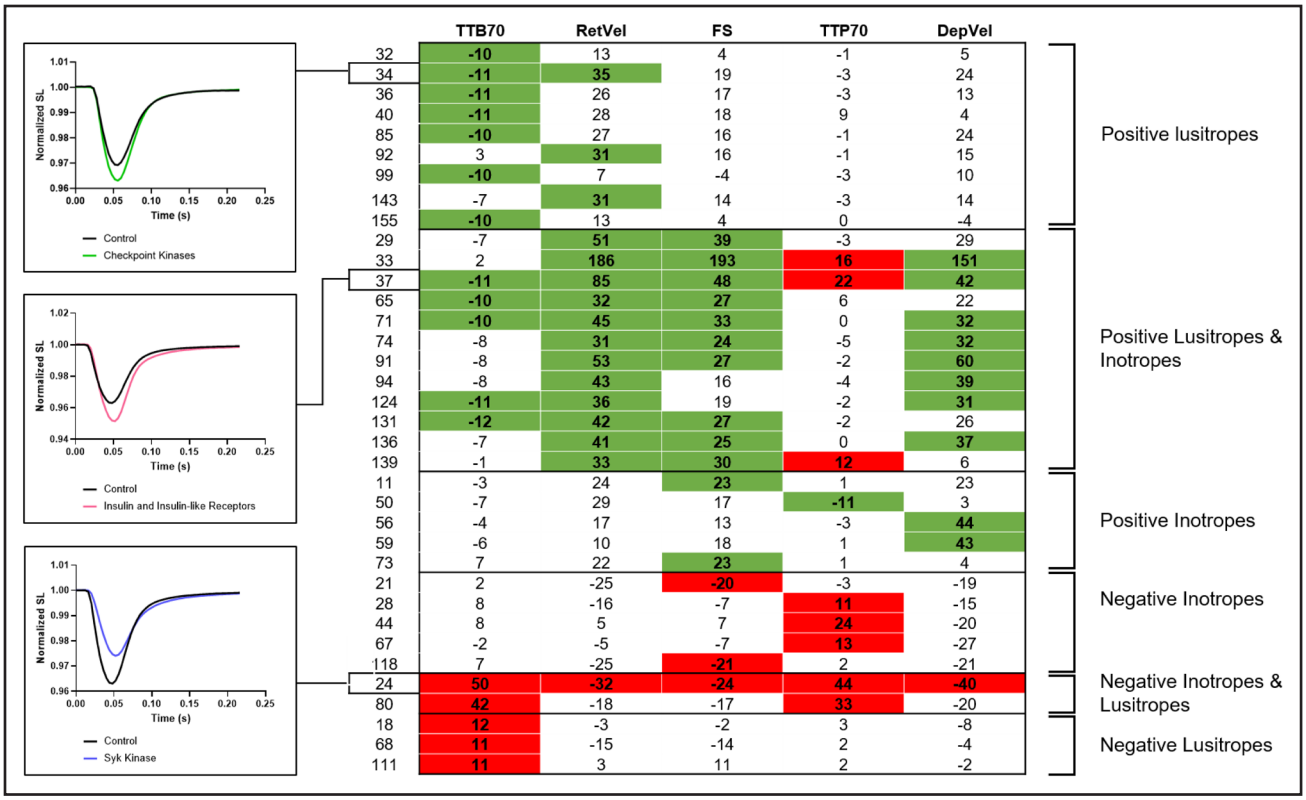

Figure 4. Heat map categorizing positive compound hits into lusitropes and inotropes.
Only fields containing a positive hit (ie, parameter that meets one of our selection criteria) are colored. Green fields indicate a desired effect, whereas red indicates an undesired effect. **Left**, Exemplary traces for normalized sarcomere length (SL) that correspond to the compound surrounded by a box. The green, pink, and blue traces represent a positive lusitrope, a positive lusitrope and inotrope, and a negative lusitrope, respectively. All black traces represent controls (dimethylsulfoxide [DMSO] vehicles).

FoxO (forkhead box class O) signaling cluster (protein-protein interaction enrichment $P<0.001$; Figure 5B).

Phosphoproteomic Analysis in HCM Patient Myocardium Shows Activation of EGFR/IGF1R Signaling

As impaired relaxation is one of the hallmarks of HCM in patients, we subsequently defined whether the newly identified modulators of relaxation play a role in a clinically relevant human model. We, therefore, characterized kinase activity in cardiac tissue from patients with HCM (N=24) compared with nonfailing donor samples (N=8), by performing phosphoproteomic profiling followed by the integrative inferred kinase activity analysis.[17] Phosphopeptides were enriched from tissue lysates by immobilized metal affinity chromatography and phosphotyrosine immunoprecipitation. Integrative inferred kinase activity analysis integrates kinase-centric data, that is, how much a specific kinase is phosphorylated and thus activated, and a phospho-site analysis that provides information on the number of protein target sites of a specific kinase that are phosphorylated in HCM compared with donor samples. This combined analysis enables the quantification of individual kinase activity. Because we found that the inhibition of 21 kinases led to improved relaxation, we hypothesized that some of these kinases would be activated in HCM, leading to impaired relaxation. The kinases that form the cell cycle kinase cluster or Akt cluster (Figure 6A) did not show strong activation in our phosphoproteomics screen, although especially in the cell cycle cluster most kinases were not detected, implying that these kinases are not abundant in the human heart. Our integrative inferred kinase activity analysis shows strong activation of kinases within the EGFR/IGF1R cluster (Figure 6A). Seven of 8 kinases in this cluster were significantly activated in HCM. These newly identified modulators of relaxation could, therefore, contribute to the impaired relaxation in patients with HCM.

To validate the activity of a subset of clinical hits and to check for a temporal trajectory, we sought to define kinase activity in HCM mice at both 3 and 26 weeks of age. We used a mouse model of overt HCM that we described recently.[16] This model contains the homozygous c.2373insG *MYBPC3* mutation, which is the most common Dutch Founder mutation. We also sought to validate kinase activity in an HCM mouse model carrying a homozygous c.772G>A *MYBPC3* mutation.[18] We observed increased activity IGF1R across all models, although only the $MYBPC3_{c.772G>A}$ mice showed a

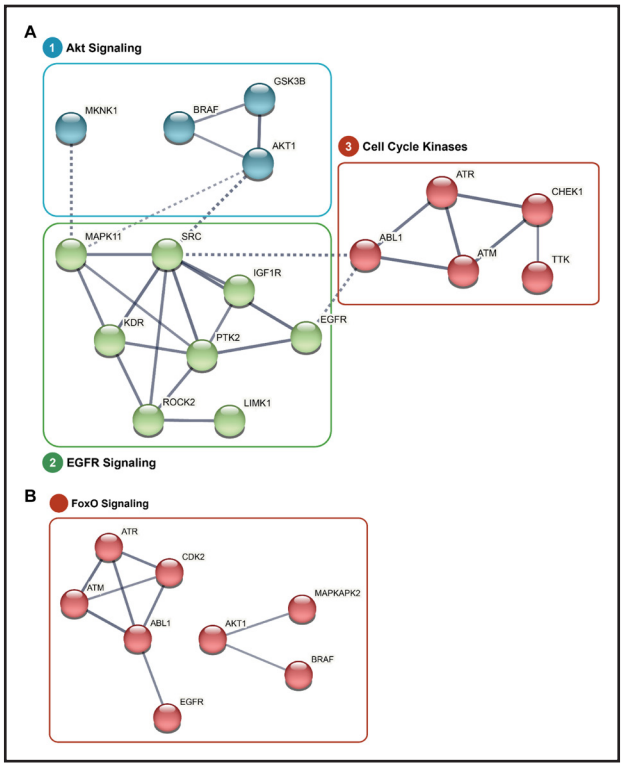

Figure 5. Protein-protein interactions (PPIs) of lusitropic target kinases in wild-type (WT) murine cardiomyocytes reveal 3 major clusters.
A, Postmitotic, WT murine cardiomyocytes present with kinases implicated in the cell cycle (shown in red), in addition to the Akt (α-serine/threonine protein kinase; shown in blue) and EGFR (epidermal growth factor receptor; shown in red) signaling cluster. Gray areas indicate lusitropic nodes, or target kinases, in HOM murine cardiomyocytes. **B**, Network of inotropic target kinases in WT murine cardiomyocytes shows enrichment of FoxO (forkhead box class O) signaling. PPIs are based on a high confidence (minimum required interaction score of 0.7). Line thickness indicates the strength of data support but without text mining evidence.

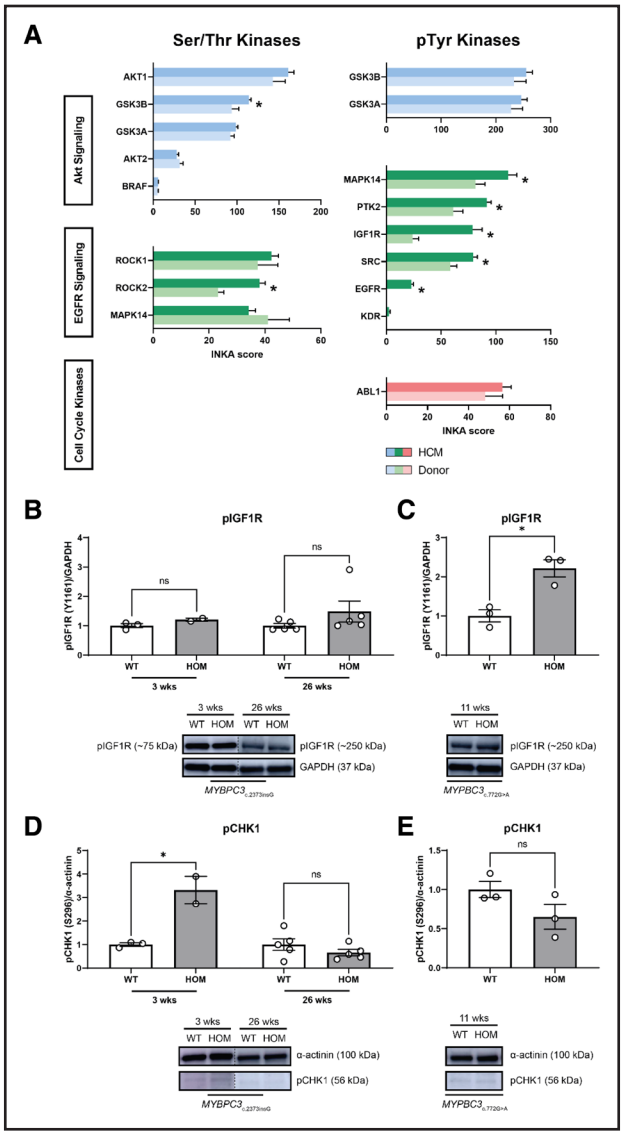

Figure 6. Integrative INKA scoring reveals activation of EGFR (epidermal growth factor receptor)/IGF1R (insulin-like growth factor 1 receptor) signaling in myocardium of patients with hypertrophic cardiomyopathy (HCM).
A, Kinases pertinent to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database–derived clusters shown in Figure 5 were highlighted according to the corresponding signaling cluster. *A significant increase or decrease of kinase activity in HCM compared with donors ($P<0.05$). **B**, Altered kinase activation in HCM mice. **B**–**E**, Homozygous (HOM) 3-week-old mice (*MYBPC3*$_{c.2373insG}$) or 26-week-old mice were compared with their wild-type (WT) counterparts. Additionally, HOM 11-week-old *MYBPC3*$_{c.772G>A}$ mice, which comprise a different model, were compared with WTs. Representative blot images are shown below corresponding graphs; dashed lines indicate lanes that were run on the same membrane but were noncontiguous. Quantified levels of (**B** and **C**) p-IGF1R and (**D** and **E**) p-CHK1. Each dot in the scatter plots represents an individual sample. *$P<0.05$ vs WTs. Measurements are means±SEMs. INKA indicates integrative inferred kinase activity; and p-CHK1, phosphorylated checkpoint kinase 1.

statistically significant increase (Figure 6B and 6C). In addition, CHK1 activity was increased in *MYBPC3*$_{c.2373insG}$ mice at 3 weeks of age but remained unaltered at 11 weeks of age and in the *MYBPC3*$_{c.772G>A}$ mice (Figure 6D and 6E). Thus, with regard to the activation of IGF1R and CHK1, there is a disparity between HCM mouse models and the clinics.

Positive Lusitropic Hits From EGFR/IFGR1 Cluster Demonstrate Varying Effects on Relaxation Kinetics in Homozygous *MYBPC3*$_{2373insG}$ Murine Cardiomyocytes

To test the therapeutic potential of the positive lusitropes within the EGFR/IGF1R cluster, we used our HCM mouse model. Isolated cardiomyocytes from this mouse model presented with normal contractile function but impaired cardiomyocyte relaxation, as is indicated by the increased TTB70 and reduced relaxation velocity shown in Figure 7A. We set out to study the effect of 7 of our EGFR/IGF1R-clustered positive lusitropes in homozygous cardiomyocytes (Figure S2), as is depicted in Figure 7B and 7C. In these cardiomyocytes, 2 compounds targeting insulin and insulin-like receptors and EGFR reduced TTB70

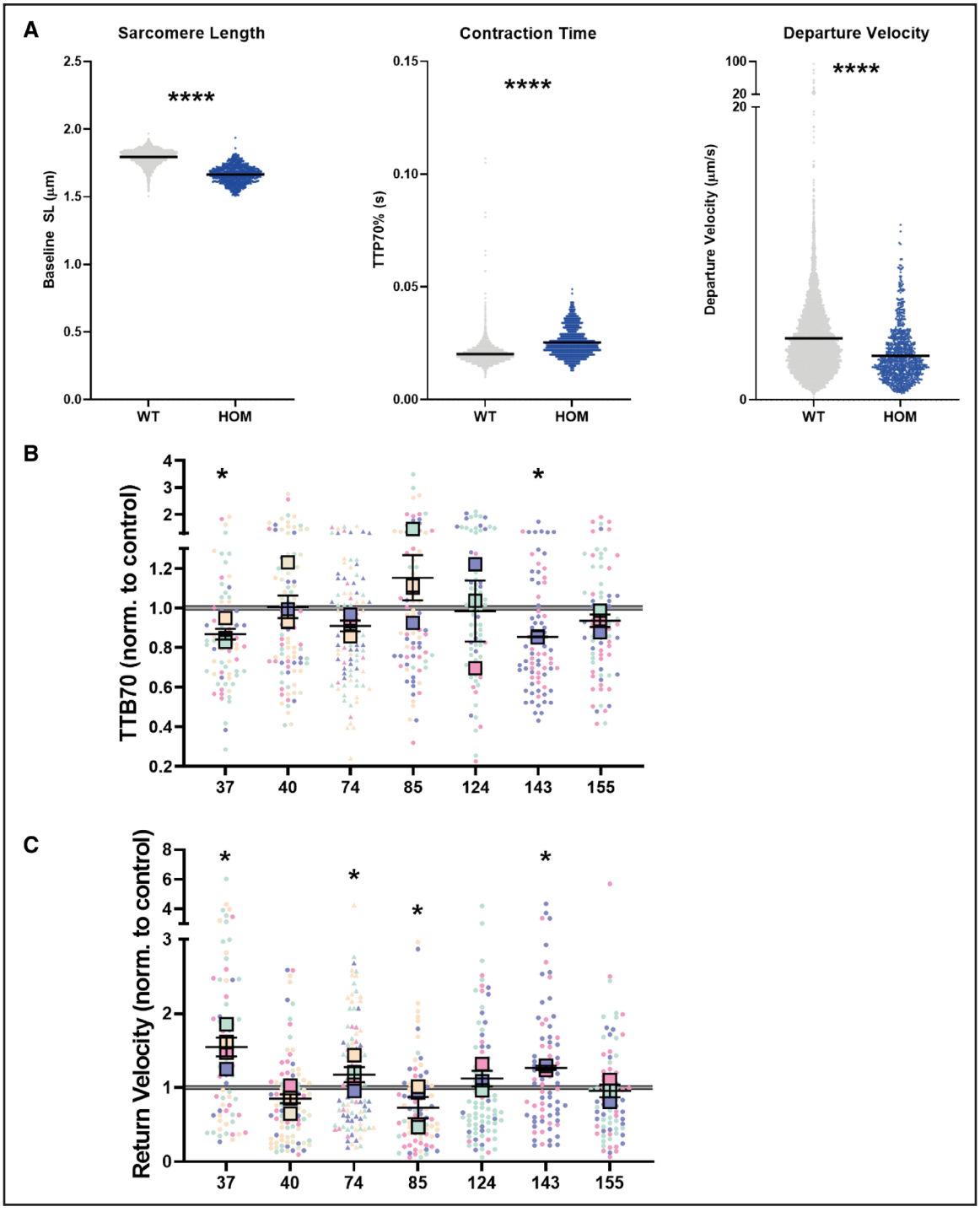

Figure 7. Validation of positive lusitropes from the EGFR/IGF1R cluster in *MYBPC3*$_{2373insG}$ mouse cardiomyocytes.
Positive lusitropic EGFR (epidermal growth factor receptor)/IGF1R (insulin-like growth factor 1 receptor) hits demonstrate varying effects on time to baseline 70% (TTB70; **B**) and return velocity (**C**) in homozygous (HOM) *MYBPC3*$_{2373insG}$ murine cardiomyocytes as compared with wild-type cardiomyocytes. **A**, Impaired relaxation in HOM *MYBPC3*$_{2373insG}$ murine cardiomyocytes as compared with wild-type (WT) cardiomyocytes. Every dot represents an individual cell, with gray and blue dots representing WT (n=6186 and N=33) and HOM (n=896 and N=12) cells, respectively. **P*<0.05 vs WT. Compound effects in HOM cardiomyocytes for TTB70 (**B**) and return velocity (**C**) normalized to control. Cardiomyocytes (n=69–98) derived from 2 to 5 isolations were incubated with lusitropic compounds or dimethylsulfoxide (DMSO; control, n=1190). Each well was normalized to neighboring control. Data are presented as a superplot with individual cells as dots and averages per day in squares and mean±SEM. Line and shading is mean±SEM of control cells. **P*<0.05. Compound effect data were analyzed using the hierarchical clustering method with Bonferroni post hoc test for the individual groups.

(12.4% and 14.5%; compounds 37 and 143, respectively; Figure 7B; $P<0.05$). They also increased the return velocity of these cardiomyocytes (61.8% and 27.5%, respectively; Figure 7C; $P<0.05$), in addition to compound 74, which targets Rho kinases (19.8%; Figure 7C; $P<0.05$). Inhibition of IGF1R and EGFR also increased contraction or contraction kinetics (Figure S2B through S2D). Noteworthy, compound 85, which targets FAK (focal adhesion kinase), increased TTB70 in cardiomyocytes by 10.5% and decreased their return velocity by 21.9% (Figure 7B and 7C). As this aggravates diastolic dysfunction instead, it indicates that results from WT mice, including those that are FBS dependent, cannot automatically be translated into disease models.

DISCUSSION

For the first time, a compound screening was performed to find novel modulators of contraction/relaxation in adult cardiomyocytes. Our experimental approach allowed us to assess the contractile phenotype of acute inhibition with 157 kinase inhibitors by measuring >25 000 cardiomyocytes. This uncovered 36 regulators of contraction and relaxation, almost all of which are novel. The 21 positive regulators of relaxation clustered together in 3 signaling cascades: cell cycle kinases, EGFR, and Akt signaling. To test the translational potential of the identified regulators of relaxation, we performed phosphoproteomics profiling in myocardial tissue from patients with HCM. We then tested the EGFR/IGFR1 cluster of these kinases in cardiomyocytes from an HCM mouse model with diastolic dysfunction. We validated 3 compounds that accelerated relaxation in mouse HCM cardiomyocytes and demonstrated the involvement of the EGFR/IGFR1 signaling cluster in the HCM pathophysiology.

To perform a compound screen in isolated cardiomyocytes, a number of experimental challenges had to be overcome. We wanted to test the effect of a wide variety of kinase inhibitors. To be able to observe the effect of an inhibitor, the target protein must be active. We show that the β-adrenergic receptor antagonist propranolol has no effect on contractile function in isolated cardiomyocytes. By using the growth factor and cytokine-rich FBS, however, we were able to measure the expected antagonizing effect. The precise effects of FBS on cardiomyocytes have not been defined yet. Ideally, a specific agonist upstream of each kinase is used, but this was not feasible. The exact composition of FBS, however, is unknown, and often there is batch-to-batch variability,[19] which is why we used a single batch for the current study. Another experimental issue that needed to be overcome to allow a maximal output per isolation to perform this large-scale screening was high-quality measurements for an extended period after isolation (up to 8 hours after isolation). We observed a slow decay of FS over time, which is indicative of rundown (Figure 1B).[20,21] To account for this rundown, compounds were assigned to their wells at random and were measured blindly and before or after a control well. This allowed us to normalize all data and reduce variability.

In our compound screening of 157 kinase inhibitors, 22.9% (36 compounds) had a functional effect. Oftentimes, compounds were both positive inotropes and lusitropes (12/36; Figure 4). This was expected as the best known modulator of contractility, PKA also regulates both contraction and relaxation, as do other known regulators such as PKG. We showed that our approach worked as inhibition of protein kinase C using compound 21 showed decreased contraction and PKC is a known enhancer of contractility.[22] In addition to already known mediators, we identified novel modulators of inotropy. Compounds 24 and 118, which target SYK and Rho kinases, respectively, are negative inotropes in WT cardiomyocytes. We further show that the Akt signaling pathway, which was shown to be implicated in HCM in a previous study by Ackerman et al,[23] and cell cycle kinases were also implicated in the regulation of contractile kinetics in our WT murine cardiomyocytes, but how these clusters modulate cardiac contraction remains to be determined. Overall, we were able to detect both known and unknown modulators of contraction.

Assigning a specific role for an individual kinase that is inhibited by a compound is dependent on the specificity of that compound and the native expression level of the target kinase in cardiomyocytes. To increase the likelihood that kinases are true modulators of contractility, we made sure that when that information was available (144/157 compounds; Table S2) the compound was used at its EC_{50} value, which limits the lower affinity off-target effects. Furthermore, we reasoned that if signaling molecules are involved in modulating an effect, kinases involved in the same signaling pathway would have a similar effect. We focused on lusitropic effects, as patients with HCM show impaired relaxation. Therefore, we entered our positive lusitropic hits into the STRING database. This database clustered our positive lusitropic target kinases into (1) Akt signaling, (2) EGFR/IGFR1 signaling, and (3) the cell cycle kinases, with a high confidence depicted for the protein-protein interactions (Figure 5A).

Another observation in these mouse cardiomyocytes, which are postmitotic, encompasses the involvement of cell cycle kinases in both contraction and relaxation. Apart from a recently suggested role for ATM in cardiac dysfunction[24,25] and both ATM and ATR in DNA damage[25,26] (compound 33), the TTK (compounds 71 and 131), checkpoint kinases (compound 34), SETD8 protein lysine methyltransferases (compound 32), and DYRK (dual-specificity tyrosine-regulated kinase; compound 136) have not been described in the context of cardiac contractility before. How these cell cycle kinases regulate cardiac contraction remains to be determined. An

intriguing possibility is that the lusitropic effects of these kinases are mediated through microtubules, as they play a key role in both the cell cycle and, as we and others have previously shown, cardiomyocyte relaxation.[16,27]

As patients with HCM present with impaired relaxation, we tried to establish whether changes in activity of kinases involved in lusitropy could partly explain the relaxation deficits. We processed interventricular septal tissue to perform a phosphoproteomics screen using HCM and donor tissue. We show that using the integrative inferred kinase activity analysis, our screen reveals an increased activation of kinases within the EGFR/IGF1R cluster in HCM patient tissue. As inhibition of kinases in this cluster led to increased relaxation, we argued that activation would lead to impaired relaxation. Thus, this raises the possibility that the EGFR/IGFR1 cluster is an especially potent target to treat diastolic dysfunction in HCM, which aligns with the previous observation that EGFR signaling is implicated in an HCM mouse model.[28]

The EGFR signaling cluster comprises regulators of cardiac contractility that are less well studied. The receptor tyrosine kinases EGFR and IGFR1 phosphorylate and activate the downstream targets FAK (PTK2 [protein tyrosine kinase 2]), Src, and ROCK2 (Rho associated coiled-coil containing protein kinase 2).[29,30] Another target within this cluster comprises p38 MAPK. We found a marked negative inotropic effect of the p38 MAPK-inhibiting compound 44, whereas previous studies show that the activation of p38 MAPK has negative inotropic effects in murine cardiomyocytes, though this may be due to the usage of different compounds.[31,32] We also observed such contrasting effects with the compounds targeting Rho kinases (compounds 74 and 118), which are linked to LIMK (LIM kinase-1; compound 139), but the role of these kinases in cardiac contraction has not been explored yet. Although the precise mechanism remains unclear, we identify a notable role in cardiac contraction and relaxation for the EGFR/IGFR1-signaling cluster.

In these analyses, we only tested acute effects of our compounds. Nevertheless, our phosphoproteomics screen does predict the involvement of kinases proposed by our compound library screen. Another limitation is that we could not always assign a compound as an exclusive lusitrope or inotrope because compounds targeting 1 kinase sometimes presented opposing effects. For example, we demonstrate that the inhibition of Akt using compounds 28 and 17 has negative inotropic and lusitropic effects, but we also find that the Akt inhibiting compounds 65 and 102 are positive inotropes and lusitropes in our WT murine cardiomyocytes. In line with the latter, Christian et al[33] have previously demonstrated that the chemical inhibition of Akt, using another compound, had positive inotropic and lusitropic effects in both intact murine cardiomyocytes and isolated hearts. The different effects of the multiple compounds targeting a single kinase might be caused by the specificity of the compounds and off-target effects on other kinases or nonkinases.[34] We, therefore, predict the involvement of a kinase based on the general effect of a given cluster, though future studies will be needed to ascertain these effects.

The current study is limited by screening for compounds in healthy cells, which may lead to missing disease-specific targets. We also aimed to provide broad-scale activation of kinase activity with FBS, but any kinase that is not inherently active or that is not activated by FBS will not respond to an inhibitor. Kinases whose inhibitors did not show an effect on contractility could still have a modulatory role. Another limitation is that we performed our screening data based on effect sizes (eg, ≥20% increase in FS). This was based on both having a strong enough biological effect to be interesting but also that we are able to measure such an effect with ≈60 cells. Some compounds yielded variations that were bigger than anticipated, resulting in β-values above our preset β-value of 0.2, despite having reached the biological effect size. The vast majority of compounds, however, did show consistent results. Finally, we did not perfuse the cardiomyocytes for this study, which may lead to hypoxia or the buildup of reactive oxygen species, in turn affecting our contractility measurements. Nevertheless, our cardiomyocytes did not show a decline in contractile performance over a period of 16 minutes and in the absence of perfusion (Figure S8).

CONCLUSIONS

By optimizing and substantially increasing the throughput of cardiomyocyte functional measurements, we showed that cardiac contraction and relaxation kinetics are regulated by multiple unexplored kinases, including those involved in the cell cycle and EGFR/IGFR1 signaling pathways. By combining this knowledge with phosphoproteomics data of kinase activation state in human HCM myocardium, we could show that especially EGFR/IGF1R signaling activation contributes to impaired relaxation. Given the clinical value of positive lusitropes, our hit compounds need to be validated in follow-up studies and translated into the clinic.

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Disclosures

E. Manders is an employee of CytoCypher BV. M. Helmes is the Director of CytoCypher BV. The other authors report no conflicts.

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