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RAF1 mutations in childhood-onset dilated cardiomyopathy

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Dilated cardiomyopathy (DCM) is a highly heterogeneous trait with sarcomeric gene mutations predominating. The cause of a substantial percentage of DCMs remains unknown, and no gene-specific therapy is available. On the basis of resequencing of 513 DCM cases and 1,150 matched controls from various cohorts of distinct ancestry, we discovered rare, functional *RAF1* mutations in 3 of the cohorts (South Indian, North Indian and Japanese). The prevalence of *RAF1* mutations was ~9% in childhood-onset DCM cases in these three cohorts. Biochemical studies showed that DCM-associated RAF1 mutants had altered kinase activity, resulting in largely unaltered ERK activation but in AKT that was hyperactivated in a BRAF-dependent manner. Constitutive expression of these mutants in zebrafish embryos resulted in a heart failure phenotype with AKT hyperactivation that was rescued by treatment with rapamycin. These findings provide new mechanistic insights and potential therapeutic targets for *RAF1*-associated DCM and further expand the clinical spectrum of *RAF1*-related human disorders.

DCM is characterized by left ventricular dilation with systolic dysfunction and affects approximately 1 person in 250. This disorder is highly genetically heterogeneous, with mutations identified in 40 different genes, including many encoding sarcomeric and other structural proteins[1–5]. The underlying genetic causes of roughly 50–60% of DCM cases remain unknown. Of the known genes mutated in DCM, many are also implicated in hypertrophic cardiomyopathy (HCM), including the genes encoding titin, cardiac actin, β-myosin heavy chain, cardiac troponin T and α-tropomyosin[3–6].

Signaling through mitogen-activated protein kinases (MAPKs) has crucial roles in myocardial biology[7]. Germline mutations in genes encoding RAS-MAPK pathway members lead to several overlapping developmental syndromes termed the RASopathies, including a high prevalence of HCM[8–12]. RASopathy-related genes do not seem to have a causal role in non-syndromic HCM[13]. On the basis of mouse genetic models, the altered activities of certain proteins relevant for signaling through MAPKs, such as loss of SHP-2 or RAF1 or increased p38 activation, can induce DCM[7]. However, inherited abnormalities in RAS-MAPK signaling have not previously been implicated in humans with DCM.

To explore whether mutations in genes encoding proteins in the RAS-MAPK pathway contribute to non-syndromic DCM, we first screened DNA from 218 individuals with isolated DCM from South India (group 1) for mutations in 9 RAS-MAPK genes (*PTPN11*, *HRAS*, *KRAS*, *RAF1*, *BRAF*, *SOS1*, *MEK1*, *MEK2* and *SHOC2*) and identified 5 new *RAF1* missense variants with the following predicted amino acid substitutions: p.Pro332Ala, p.Leu603Pro (2 cases), p.His626Arg and p.Thr641Met (NM_002880.3; **Fig. 1a** and **Supplementary Fig. 1**). No change was observed in the other eight genes. Each *RAF1* variant altered an amino acid that was evolutionarily conserved among the proteins encoded by vertebrate *RAF1* orthologs (**Fig. 1b**), and all variants were absent from 500 ancestry-matched normal South Indian individuals. We also sequenced all coding exons of *RAF1* in 420 South Indians (100 of the 500 controls, 190 individuals with solid cancers,

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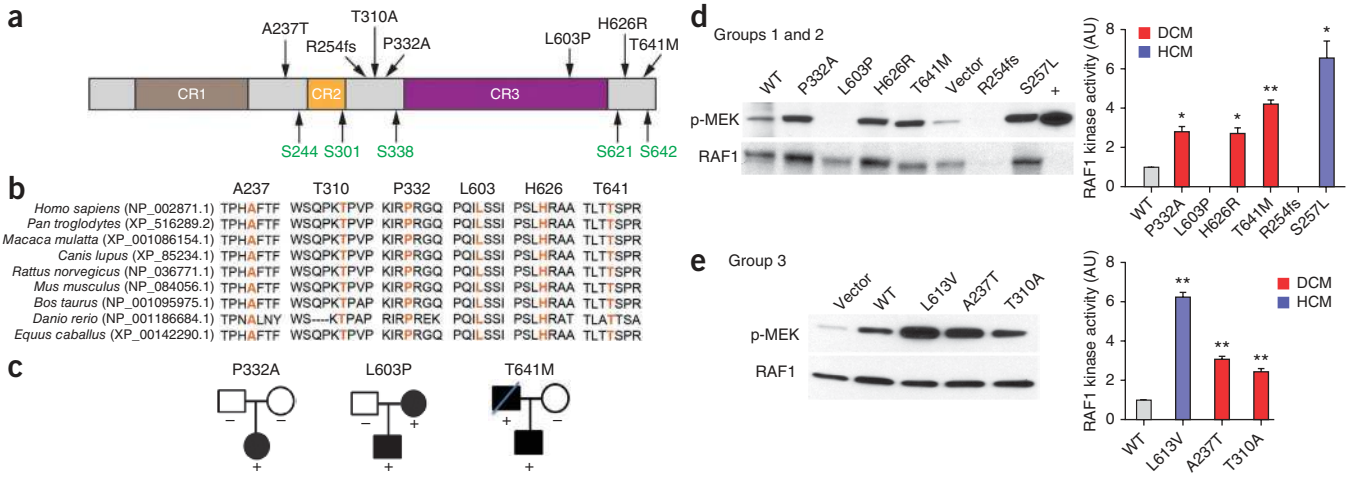


Figure 1 RAF1 mutants observed in DCM. (**a**) Schematic of the RAF1 structure and the locations of the residues altered in DCM cases. CR1, CR2 and CR3 represent the conserved regions of RAF1. Regulatory serine residues are shown in green. (**b**) Alignment of RAF1 protein sequences from different species, with the residues altered in DCM shown in orange. (**c**) Pedigrees of families with DCM with their RAF1 amino acid changes indicated. Filled symbols represent affected individuals. Plus and minus signs indicate the presence and absence of the alteration, respectively. (**d**) RAF1 kinase assays. Vector alone, full-length wild-type RAF1 (WT), HCM-associated RAF1 mutants (Leu613Val and Ser257Leu) and DCM-associated RAF1 mutants (Ala237Thr, Thr310Ala, Pro332Ala, Leu603Pro, His626Arg, Thr641Met and Arg254fs) were expressed in HEK293 cells as indicated. RAF1 was immunoprecipitated from cells stimulated with EGF for 15 min along with a positive control constituting a truncated form of active RAF1 (+). Linked kinase assays were performed using inactive MEK1 as the substrate. RAF1 and phosphorylated MEK1 (p-MEK) were detected. Kinase activity (p-MEK/RAF1) is expressed as relative expression compared to levels in cells expressing wild-type RAF1 (AU, arbitrary units). Data are the mean values ±s.d. from two independent biological replicates. $*P < 0.05$, $**P < 0.01$, comparison to wild-type RAF1.

100 individuals with coronary artery disease and 30 individuals with atrial septal defects), finding only 2 synonymous alleles (affecting c.543T ($n = 1$) and c.638T ($n = 9$)) in these exons and their splice sites. The frequency of missense *RAF1* mutations was significantly higher in the South Indian DCM cohort than in the controls (5/436 versus 0/840; $P = 0.0046$). None of the nonsynonymous variants of *RAF1* had been observed previously in Noonan syndrome with or without HCM, as a somatic change associated with cancer or in the 13,600 CEU (European) and African-American alleles in the Exome Sequencing Project (ESP)[10,11]. The individuals positive for *RAF1* mutations screened negative for mutations in 12 known cardiomyopathy-related genes (*MYH7*, *MYH6*, *MYBPC3*, *TNNT2*, *TPM1*, *MYL3*, *MYL2*, *TNNI3*, *PRKAG2*, *LMNA*, *PLN* and *TTN*) (**Supplementary Table 1**). *In silico* functional analyses showed that the DCM cases (**Supplementary Table 2**) had significantly more variants predicted to alter RAF1 function than the population-matched controls (PolypPhen-2: probably damaging, 4/436 versus 0/840, $P = 0.0135$; pMut: pathological, 5/436 versus 0/840, $P = 0.0046$). We were able to analyze the families of three probands with DCM harboring a *RAF1* missense mutation. For one affected individual with a *RAF1* allele encoding p.Pro332Ala who had a family history negative for DCM, analysis of parental DNA confirmed paternity and showed an absence of the *RAF1* variant in both parents, consistent with a *de novo* change. The family histories for the two other probands suggested that additional relatives were affected, and the relevant *RAF1* variants were observed in other symptomatic individuals in these families (**Fig. 1c**).

Next, we screened 200 North Indians (genetically distinct from South Indians), 35 Japanese and 60 Italians who were probands with

Table 1 Clinical features of *RAF1* mutation–positive subjects

Amino acid change	P1 p.Pro332Ala	P2A[a] p.Leu603Pro	P2B[a] p.Leu603Pro	P3 p.His626Arg	P4 p.Thr641Met	P5 p.Leu603Pro	P6[b] p.Arg254fs	P7 p.Thr641Met	P8 p.Ala237Thr	P9 p.Thr310Ala
Age (years)	21	40	4	20	15	21	3	21	44	2
Age of onset (years)	13	24	3	10	7	10	1	16	40	2
Sex	F	F	M	M	F	F	M	M	M	F
Origin	South India						North India		Japan	
NYHA III or IV	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ST-T change	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Ventricular arrhythmia	Yes	No	Yes	No	Yes	No	Yes	Yes	No	No
LVIDd (mm)	52	55	74	70	72	68	71	65	85	63
LVEF (%)	31	27	17	22	24	20	18	25	18	12
Mitral regurgitation	Moderate	Mild	Severe	Moderate	Moderate	Moderate	Severe	Moderate	Mild	Moderate

Clinical features refer to the time of presentation. Of note, no person had other features of a RASopathy (facial dysmorphism, short stature, webbed neck, chest deformity or mental retardation). LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diastolic dimension; NYHA, New York Heart Association; ST-T change, change of the ST segment (after the QRS wave and before the T wave) and the T wave on the electrocardigram; M, male; F, female.

[a]P2A is the father of P2B (**Fig. 1c**). [b]P6 is deceased.

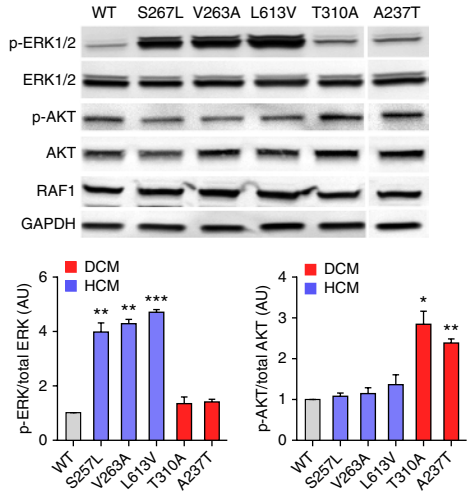
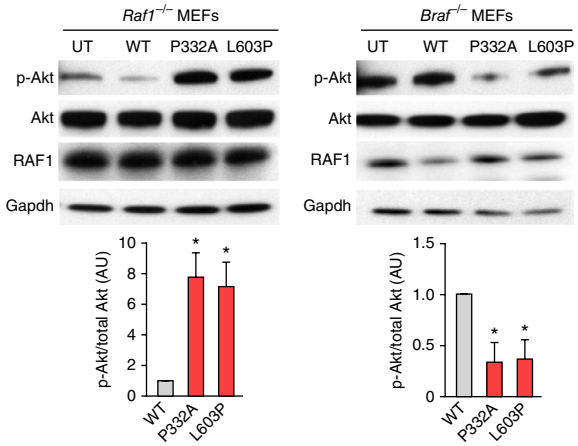


Figure 2 DCM-associated RAF1 mutants activate AKT. Representative immunoblot with total lysates from HEK293 cells expressing wild-type RAF1, HCM-associated RAF1 mutants (Ser257Leu, Val263Ala and Leu613Val) and DCM-associated RAF1 mutants (Thr310Ala and Ala237Thr) that were stimulated with EGF for 15 min. The blot was probed with antibodies to phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated AKT (p-AKT) and AKT. Levels of phosphorylated protein were normalized to respective total protein levels and are expressed as relative expression compared to levels in cells expressing wild-type RAF1. GAPDH levels were used as a loading control. Data are the means ± s.d. from four independent experiments assayed in duplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, comparison to wild-type RAF1.

DCM (groups 2, 3 and 4, respectively) for *RAF1* mutations. In group 2, we identified two additional *RAF1* sequence variants: a single-base deletion leading to protein truncation (p.Arg254fs) and a missense mutation predicted to encode a p.Thr641Met substitution. Neither variant was identified in 350 ancestry-matched North Indian controls (**Fig. 1a**,**b** and **Supplementary Fig. 1**). In group 3, we identified two additional new *RAF1* missense mutations (encoding p.Ala237Thr and p.Thr310Ala substitutions). Neither variant was detected in 300 Japanese controls. Both of the altered residues are evolutionarily conserved (**Fig. 1a**,**b**). Although both variants were predicted to be tolerated (**Supplementary Table 2**), each had functional consequences when assessed *in vitro*. None of these missense variants was observed in public databases (dbSNP, 1000 Genomes Project and ESP), and no other damaging allele (nonsense or frameshift) was described. In group 4, we did not observe any *RAF1* missense or deletion variants. Combining all groups (1–4), the frequency of *RAF1* missense or damaging mutations was significantly higher in DCM cases than in ESP cohorts (9/1,026 versus 29/13,006; $P = 0.0004$). Thus, we concluded that *RAF1* variants were strongly associated with non-syndromic DCM (all DCM cases versus population-unmatched ESP controls: odds ratio (OR) = 3.96, 95% confidence interval (CI) = 1.87–8.39; South Indian DCM cases versus population-matched controls: OR = 21.42, 95% CI = 1.18–388.41).

The clinical features of *RAF1*-associated DCM are notable (**Table 1**). Of the ten subjects with a *RAF1* mutation and known age of onset, eight presented in childhood or adolescence. The average age at presentation was 12.6 years, a younger age than the approximate average of 20 years associated with DCM caused by sarcomeric genetic mutations. Consistent with this observation, screening of DNA from 60 Italian DCM cases with an age at diagnosis of >18 years who were negative for mutations in the 9 known DCM-associated genes (group 4) identified no disease-associated *RAF1* mutation.



Of the 218 cases in the South Indian cohort, 33 had childhood-onset disease with an age at diagnosis of <18 years, including all 5 individuals with *RAF1* mutations. Similarly, 30 of the 200 North Indian cases had childhood-onset DCM, including both individuals with *RAF1* mutations. *RAF1* mutations in Indian subjects presented during childhood significantly more frequently than expected by chance (7 of 63 total Indian childhood-onset cases (11%) compared to 0 of 355 adult-onset cases; $P < 0.0001$). Including the 1 *RAF1* mutation identified in the 25 Japanese childhood-onset cases, 8 of 88 individuals with childhood-onset DCM (9%) harbored *RAF1* mutations ($P < 0.0001$). Thus, *RAF1* is the first gene strongly associated with isolated DCM to be predominantly associated with pediatric-onset disease. Of note, HCM cases associated with RASopathies also present early in life, as have the rare cases of apparently isolated HCM with RAS-MAPK mutations identified so far[10,11,13].

To understand the functional consequences of the DCM-associated *RAF1* mutations and how they differ from the HCM-associated *RAF1* mutations observed in RASopathies, we transiently expressed several RAF1 mutants in human embryonic kidney (HEK293) cells and assessed their kinase activity and extracellular signal–regulated kinase (ERK) activation. Five DCM-associated RAF1 missense mutants (Ala237Thr, Thr310Ala, Pro332Ala, His626Arg and Thr641Met) had kinase activities that were mildly increased compared to wild-type RAF1 but were less augmented in comparison to the HCM-associated RAF1 mutants tested (Leu613Val and Ser257Leu) (**Fig. 1d**,**e**). ERK activation engendered by the DCM-associated RAF1 mutants was similar to that observed with wild-type RAF1 and was significantly less than with the HCM-associated mutants ($P < 0.05$; **Fig. 2** and **Supplementary Fig. 2**). One DCM-associated missense mutant (Leu603Pro) had impaired kinase activity and reduced ERK activation, as did the truncated RAF1 protein (Arg254fs). Of note, Leu603 is located in the kinase domain, and its substitution with a proline residue could therefore plausibly render RAF1 non-functional (**Fig. 1d** and **Supplementary Fig. 2**).

Figure 3 DCM-associated RAF1 mutants require Braf for Akt activation. Representative immunoblots with total lysates from MEFs from *Raf1* and *Braf* knockout mice expressing wild-type human RAF1 and two representative DCM-associated mutants (Pro332Ala and Leu603Pro). Levels of phosphorylated Akt were normalized to the total levels of Akt and are expressed as relative expression compared to levels in cells expressing wild-type RAF1. Gapdh levels were used as a loading control. Data are the means ± s.d. from four independent experiments assayed in duplicate. $*P < 0.05$, comparison to wild-type RAF1. UT, untransfected cell lysates.

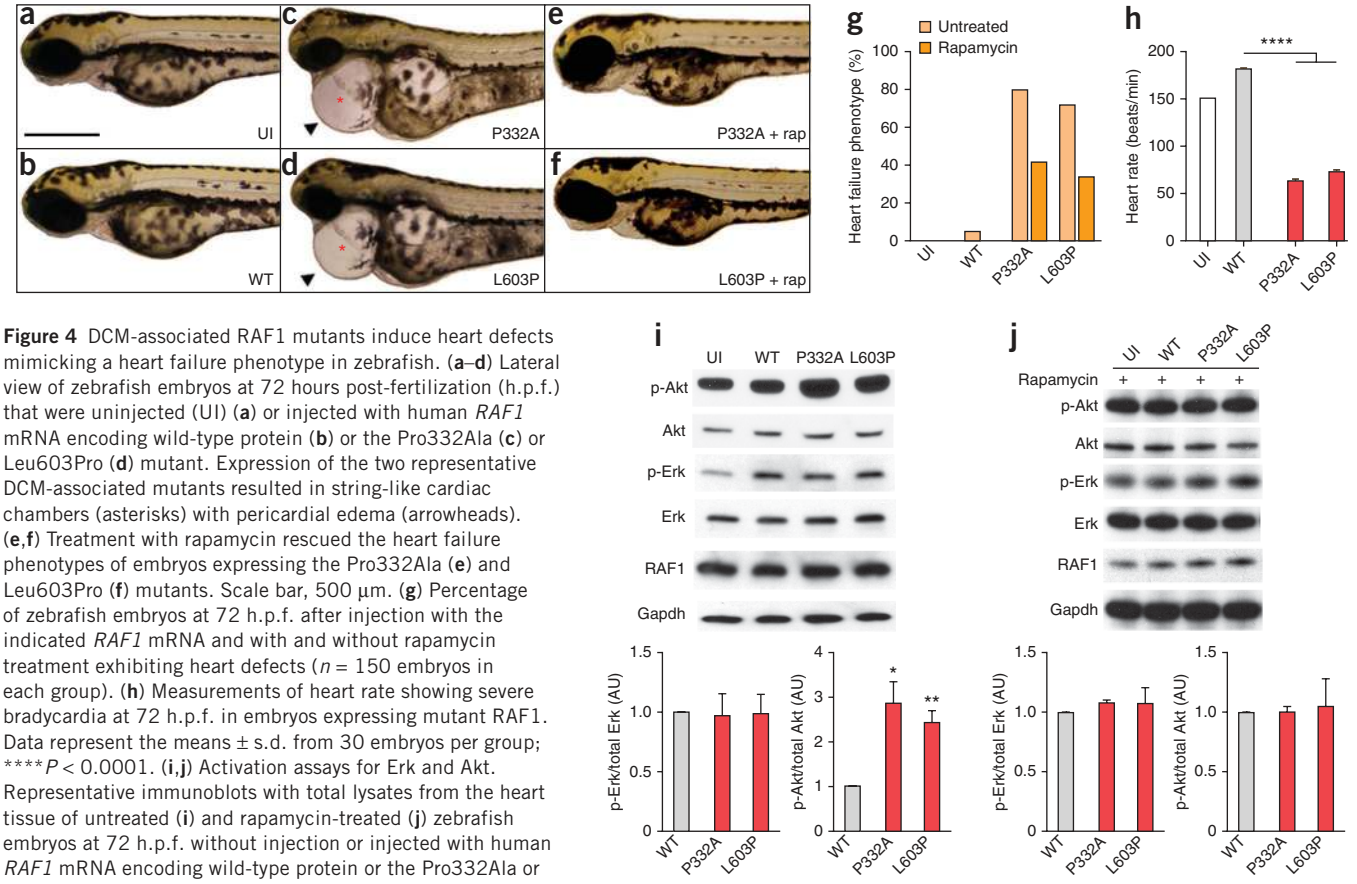


Figure 4 DCM-associated RAF1 mutants induce heart defects mimicking a heart failure phenotype in zebrafish. (**a**–**d**) Lateral view of zebrafish embryos at 72 hours post-fertilization (h.p.f.) that were uninjected (UI) (**a**) or injected with human *RAF1* mRNA encoding wild-type protein (**b**) or the Pro332Ala (**c**) or Leu603Pro (**d**) mutant. Expression of the two representative DCM-associated mutants resulted in string-like cardiac chambers (asterisks) with pericardial edema (arrowheads). (**e**,**f**) Treatment with rapamycin rescued the heart failure phenotypes of embryos expressing the Pro332Ala (**e**) and Leu603Pro (**f**) mutants. Scale bar, 500 µm. (**g**) Percentage of zebrafish embryos at 72 h.p.f. after injection with the indicated *RAF1* mRNA and with and without rapamycin treatment exhibiting heart defects ($n = 150$ embryos in each group). (**h**) Measurements of heart rate showing severe bradycardia at 72 h.p.f. in embryos expressing mutant RAF1. Data represent the means ± s.d. from 30 embryos per group; $^{****}P < 0.0001$. (**i**,**j**) Activation assays for Erk and Akt. Representative immunoblots with total lysates from the heart tissue of untreated (**i**) and rapamycin-treated (**j**) zebrafish embryos at 72 h.p.f. without injection or injected with human *RAF1* mRNA encoding wild-type protein or the Pro332Ala or Leu603Pro mutant. Levels of phosphorylated protein were normalized to respective total protein levels and are expressed as relative expression compared to levels in cells expressing wild-type RAF1. Gapdh levels were used as a loading control. Data are the means ± s.d. from four independent experiments assayed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, comparison to wild-type RAF1.

Thus, the DCM-associated RAF1 mutants had biochemical profiles that were distinct from the ones observed for the RASopathy-associated mutants causing HCM.

Furthermore, we found evidence supporting the hypothesis that DCM-associated RAF1 mutants signal through the AKT-mTOR pathway. Overexpression of DCM-associated RAF1 mutants in HEK293 cells resulted in excessive activation of AKT and tuberin, a downstream target of the AKT-mTOR pathway, after stimulation of cells with epidermal growth factor (EGF) or insulin-like growth factor (IGF)-1 compared to wild-type RAF1. In contrast, expression of HCM-related RAF1 mutants (Leu613Val, Val263Ala and Ser257Leu) did not excessively activate AKT or tuberin (**Fig. 2** and **Supplementary Figs. 2** and **3**). To determine whether DCM-associated RAF1 mutants depend on BRAF for downstream signaling, as has been documented for the HCM-associated RAF1 mutants[14], we transiently expressed two representative human RAF1 DCM mutants, Pro332Ala (kinase active) and Leu603Pro (kinase impaired), in *Raf1*$^{-/-}$ and *Braf*$^{-/-}$ mouse embryonic fibroblasts (MEFs). After stimulation with EGF, Erk activation in cells expressing these DCM-associated mutants was similar to that with wild-type RAF1 for both types of MEFs (**Supplementary Figs. 4** and **5**). Akt hyperactivation was still observed when the DCM-associated mutants were expressed in the *Raf1*$^{-/-}$ MEFs. In contrast, expression of the DCM-associated mutants in *Braf*$^{-/-}$ MEFs resulted in markedly reduced Akt activation (**Fig. 3**). Collectively, these data suggest that the DCM-associated RAF1 mutants selectively induce AKT hyperactivation that is dependent on BRAF, possibly through heterodimerization.

To determine whether the DCM-associated *RAF1* mutations were sufficient to impair cardiac structure or function, we expressed wild-type RAF1 and two representative DCM-associated mutants, Pro332Ala (kinase active) and Leu603Pro (kinase impaired), in zebrafish embryos via injection with the corresponding human *RAF1* mRNAs at the one-cell stage. Three days after injection, embryos expressing wild-type RAF1 had a cardiac status that was indistinguishable from that of uninjected embryos. In contrast, expression of both RAF1 mutants engendered heart defects mimicking a heart failure phenotype that included elongated ventricular and atrial chambers, profound pericardial edema, blood congestion at the cardiac inflow tract and impaired cardiac contractions ($P < 0.05$; **Fig. 4a**–**h**). Immunoblotting showed that zebrafish hearts expressing the DCM-associated RAF1 proteins had Erk activation that was similar to that of uninjected embryos or embryos expressing wild-type RAF1 but that Akt was significantly hyperactivated ($P < 0.05$; **Fig. 4i**). After treatment of embryos with rapamycin (an AKT-mTOR inhibitor), heart defects in the embryos expressing the DCM-associated RAF1 mutants were partially rescued and Akt activation was normalized (**Fig. 4e**–**g**,**i**,**j**). These results suggest that the cardiac failure induced by DCM-associated mutant RAF1 is partially mediated by increased AKT signaling. Taken together with the genetic and biochemical data, these results provide compelling evidence that *RAF1* mutations have a critical role in DCM. Whereas the role of RAS-MAPK signaling in myocardial biology is well established[7,12], this is the first demonstration, to our knowledge, that its alteration can contribute to isolated DCM in humans.

Notably, RASopathy-associated *RAF1* mutations as a cause of HCM seem to be functionally distinct from the newly identified DCM-associated *RAF1* alleles. The HCM- and DCM-associated *RAF1* mutations are mutually exclusive. Whereas mutations in HCM cluster in two hotspots affecting Ser259 and Ser612, *RAF1* mutations in DCM are more widely distributed and do not alter residues critical for the regulation of RAF1 (with Ser642 being an exception). DCM-associated RAF1 mutant proteins exhibit modestly increased or impaired kinase activity, hyperactivate AKT but not ERK, and induce cardiac phenotypes that are partially rescued by an AKT-mTOR inhibitor in a zebrafish model of disease. In contrast, HCM-associated RAF1 mutants display greatly enhanced kinase activity, leading exclusively to robust ERK activation. Moreover, HCM in a mouse model of a RASopathy-associated *RAF1* mutation (encoding p.Leu613Val) was rescued using a MEK inhibitor, which prevents signaling to ERK1 and ERK2 (ERK1/2)[15]. Thus, the cardiomyopathies associated with *RAF1* mutations are allelic but biologically distinct.

Finally, the complexities of signal transduction in myocardial biology are highlighted by the finding that loss-of-function *PTPN11* alleles underlying another RASopathy, Noonan syndrome with multiple lentigines (NSML; formerly, LEOPARD syndrome), potentially with dominant-negative properties, result in HCM. On the basis of a knock-in mouse model, an NSML-associated *Ptpn11* allele results in hyperactivation of Akt but not Erk1/2 in the myocardium, leading to HCM earlier in life that transforms into DCM at older ages[16]. Of note, an mTOR inhibitor prevented or rescued the HCM phenotype in these mice, leading to the suggestion that a clinical trial of a rapamycin analog is indicated for patients with LEOPARD syndrome and HCM. If subsequent preclinical studies, most likely with a mouse model of a DCM-associated *RAF1* mutation, recapitulate cardiac disease and drug efficacy, a similar route to therapy can be envisioned for this genetic form of DCM.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.S.D. conceived the project, with input from B.D.G. P.S.D., M.A.R., U.M., S.K., D.S.R., G.L., A.B., M.K., A.R., E.F., M.T., T.Y., M.F., T. Nishizawa, T. Nakanishi, P.G., B.R., R.M. and K.T. collected and sequenced the various cardiomyopathy cases and controls. P.S.D., M.A.R., J.J.E. and I.R. performed the functional studies, with the help of J.S., S.P. and S.M.-N. J.R., R.J.H., D.L., K.C.S., K.T. and B.D.G. provided reagents for the study. P.S.D. drafted the manuscript, with input from B.D.G.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Clinical evaluations. A total of 513 hospitalized, unrelated individuals with DCM (groups 1–4) from (i) Madurai Rajaji Hospital, Madurai, India, (ii) the Sri Chitra Tirunal Institute of Medical Sciences and Technology, Trivandrum, India, and Government Medical College Hospital, Kozhikode, India (representing South India as group 1); (iii) the Post-Graduate Institute of Medical Education and Research, Chandigarh, India, and (iv) the Seth GS Medical College and KEM Hospital, Mumbai, India (representing North India as group 2); (v) Tokyo Women's Medical University, Tokyo, Japan (representing Japan as group 3); and (vi) Monaldi Hospital, Second University of Naples (SUN), Naples, Italy (representing Italy as group 4) were recruited with written informed consent. In addition, a total of 320 registered South Indian cases of various other diseases (190 individuals with solid cancers, 100 individuals with coronary artery disease and 30 individuals with atrial septal defects) were obtained from the referral centers outlined above. The institutional review boards of the study centers approved the protocol.

Diagnostic criteria for the index subjects. A standard international protocol was followed in diagnosing the DCM cases based on published criteria[5,6,17]. Accordingly, individuals were diagnosed with DCM when their echocardiograms showed depressed left ventricular systolic function (left ventricular ejection fraction (LVEF) < 0.45 and/or fractional shortening < 0.25) and a dilated left ventricle (left ventricular end diastolic dimension > 117% of the predicted value corrected for age and body surface area) in the absence of other cardiac or systemic causes, including coronary and valvular diseases.

Details of the subject and control cohorts. In group 1, the cases and controls were matched with respect to geographic region, ancestry (self-reported), sex (67% and 64% male, respectively) and age (40.02 ± 22 years and 45 ± 17.2 years). In group 2, the cases and controls were matched for geographic region (as outlined for group 1), ancestry (self-reported), sex (64% and 68% male, respectively) and age (47.22 ± 22 years and 49 ± 17.2 years). In group 3, the cases and controls were matched for geographic region (as outlined for group 1), ancestry (self-reported), sex (60% and 62% male, respectively) and age (40 ± 19 years and 40 ± 11 years). The average LVEF for the cases in groups 1, 2 and 3 at the time of enrollment was 29 ± 11%, 32 ± 10% and 25 ± 14%, with the average age of onset being 37 ± 12 years, 40 ± 13 years and 39 ± 11 years, respectively. The controls were apparently healthy volunteers with no family history or symptoms of cardiovascular diseases with normal electrocardiogram (ECG) and echocardiography parameters from the hospitals mentioned and were unrelated to the individuals with cardiomyopathy. Population stratification analysis was performed earlier using 50 ancestry-informative markers as described[17].

Assessment of family members. The family members of three unrelated index subjects with *RAF1* mutations from South India were invited to participate in the present genetic study. The family members who participated in the present study were assessed by ECG and echocardiography. For two of the three families, additional affected individuals were identified. Further details of the affected family members are given in **Table 1**. Apart from these families, family members of the other 510 unrelated index subjects were not assessed.

Sequencing and mutation analysis. Genomic DNA from individuals with DCM and controls was isolated from peripheral blood lymphocytes. The exons and flanking intronic boundaries of *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *SOS1*, *MEK1*, *MEK2*, *SHOC2* and *RAF1* were amplified from genomic DNA. Amplified PCR products were isolated from gels with a QiaxII Gel Extraction kit (Qiagen), sequenced using the BigDye Terminator kit (Applied Biosystems) and analyzed on an ABI3730 DNA Analyzer (PerkinElmer, Applied Biosystems).

Cloning. *RAF1* mutations were introduced into a human *RAF1* construct encoding Flag-tagged protein by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit, Stratagene). For zebrafish studies, pcDNA3 constructs encoding human *RAF1* mRNA for wild-type protein and two DCM-related mutants with p.Pro332Ala and p.Leu603Pro alterations were amplified and subcloned into the pCR8/GW/TOPO TA Cloning kit (Invitrogen) in combination with Gateway-compatible vectors to create an mRNA expression vector appropriate for zebrafish embryos encoding six copies of a Myc tag at the N terminus of RAF1. The sequences of all of the mutant constructs were verified.

Expression and assessment of mutant RAF1 proteins. Routinely used HEK293 cells as well as *Raf1*$^{-/-}$ and *Braf*$^{-/-}$ MEFs were transfected with plasmids encoding wild-type or mutant RAF1 using Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were switched to serum starvation medium for 16 h. After stimulation with EGF (10 ng/ml) for the indicated intervals at 37 °C, cells were lysed in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1× protease inhibitor cocktail). Protein concentrations for cell lysates were measured using the Bradford method, and approximately 30 µg of total protein was loaded onto gels, separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in 5% nonfat milk and incubated with primary antibodies overnight at 4 °C. Membranes were then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce), and signal intensities were visualized by chemiluminescence (Pierce). The following primary antibodies were from Cell Signaling Technology: phosphorylated ERK1/2 (4370), ERK1/2 (4695), phosphorylated AKT (4060), phosphorylated TSC2 (3617) and β-actin (4967). Antibodies to total RAF1 (610152), AKT (ab64148) and GAPDH (SAB2701826) were obtained from BD Biosciences, Abcam and Sigma, respectively. All cell lines tested negative for mycoplasma contamination, and antibodies were diluted according to the manufacturers' instructions.

RAF1 kinase activity assays. Protein samples were prepared from HEK293 cells transfected with expression constructs for wild-type or mutant RAF1 as detailed. Lysates (containing 800 µg to 1 mg of protein) were incubated with 4 µg of antibody to Flag overnight at 4 °C. Lysates were further incubated with 40 µl of protein G–Sepharose beads (Roche) for 2 h at 4 °C. Bead-associated immune complexes were washed three times with chilled immunoprecipitation wash buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Triton X-100, 1× protease inhibitor cocktail) and once with RAF1 assay reaction buffer (RAF1 Kinase Assay kit; Upstate, 17-357). Beads were incubated with inactive MEK1 (provided with the kit) at 30 °C for 1 h with shaking. Reactions were stopped by adding SDS loading buffer, and samples were boiled for 5 min, separated by SDS-PAGE and transferred to PVDF membrane. Products were detected on a protein blot using antibody to phosphorylated MEK (Upstate, 17-357; 1:2,000 dilution) and goat secondary antibody to rabbit. RAF1 was detected with antibody to Flag (Sigma, F3165; 1:2,000 dilution).

***In silico* analysis.** Analysis of the likelihood of a pathogenic effect for each *RAF1* variant was carried out using three bioinformatics tools: PolyPhen-2, SIFT and PMut. Amino acid conservation across species was analyzed by comparing the protein sequences of various vertebrate species using ClustalW2 software.

Zebrafish maintenance and embryo injection. Adult male and female wild-type (TAB14, AB and TAB5) zebrafish (*Danio rerio*) were maintained on a 14-h light/10-h dark cycle at 28 °C. Transgenic fish expressing GFP in cardiomyocytes under the control of the *myl7* promoter (*Tg*(*myl7*:*EGFP*)) were generated using constructs from the tol2 kit[18]. Fertilized embryos collected after natural spawning were cultured at 28 °C in fish water (0.6 g/l Crystal Sea Marinemix, Marine Enterprises International) containing methylene blue (0.002 g/l). Needles were calibrated to inject 4 nl per embryo using a Narishige IM-300 microinjector. mRNA encoding Myc-tagged human RAF1 was obtained using mMessage mMachine (Ambion). By titrating mRNA concentrations, 75 ng of RNA injected per embryo was identified as the maximal tolerable and minimal effective concentration. Injections with mRNAs encoding wild-type RAF1 and two mutants (Pro332Ala and Leu603Pro) were carried out in embryos at the one- to four-cell stage. Subsequent experiments were neither randomized, nor were investigators blinded to the mRNA constructs with which the zebrafish embryos were injected. The Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai approved the necessary ethical protocols regarding zebrafish maintenance, handling and care.

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Statistical analysis. Data were expressed as mean values ± s.e.m. Differences between experimental groups were evaluated for statistical significance using Student's *t* tests. *P* values of ≤0.05 were considered to be statistically significant.

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