

1 **Mechanical dysfunction induced by a hypertrophic cardiomyopathy mutation is the**
2 **primary driver of cellular adaptation**

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27 **Abstract**

28 Familial hypertrophic cardiomyopathy (HCM), a leading cause of sudden cardiac death,
29 is primarily caused by mutations in sarcomeric proteins. The pathogenesis of HCM is
30 complex, with functional changes that span scales from molecules to tissues. This makes
31 it challenging to deconvolve the biophysical molecular defect that drives the disease
32 pathogenesis from downstream changes in cellular function. Here, we examined a HCM
33 mutation in troponin T, R92Q. We demonstrate that the primary molecular insult driving
34 the disease pathogenesis is mutation-induced alterations in tropomyosin positioning,
35 which causes increased molecular and cellular force generation during calcium-based
36 activation. We demonstrate computationally that these increases in force are direct
37 consequences of the initial molecular insult. This altered cellular contractility causes
38 downstream alterations in gene expression, calcium handling, and electrophysiology.
39 Taken together, our results demonstrate that molecularly driven changes in mechanical
40 tension drive the early disease pathogenesis, leading to activation of adaptive
41 mechanobiological signaling pathways.

42 **Introduction**

43 Hypertrophic cardiomyopathy (HCM) is the leading cause of sudden cardiac death
44 in people under age 30. HCM is characterized by hypertrophy of the left ventricular wall
45 and the intraventricular septum, myocyte disarray, fibrosis, and diastolic dysfunction.
46 HCM is also associated with marked alterations in cardiomyocyte functioning, including
47 changes in electrophysiology, contractility and calcium handling (1). Large scale
48 sequencing of families has revealed that HCM is caused by mutations in sarcomeric
49 proteins involved in cardiac contraction, including troponin T (2).

50 Disease presentation in HCM is quite complex, with functional differences seen at
51 scales ranging from molecules to tissues; however, at a fundamental level, the molecular
52 trigger that drives the disease pathogenesis is alterations in the abundance, stability,
53 and/or functioning of the mutant protein (3). This initial trigger leads to downstream
54 adaptive and maladaptive processes, some of which can take years to decades to
55 manifest, including ventricular remodeling, and eventually symptomatic cardiac
56 dysfunction. Given the inherent complexity of HCM, it has been challenging to link the
57 molecular and cellular phenotypes and to dissect the initial biophysical trigger from
58 secondary adaptive processes.

59 To better understand the connection between the initial molecular insult and
60 cellular dysfunction in the early disease pathogenesis of HCM, we examined a point
61 mutation in troponin T, R92Q (Fig. 1A), identified in several unrelated families, that causes
62 pronounced ventricular hypertrophy and a relatively high incidence of sudden cardiac
63 death (2). R92Q has been studied in several model systems, including feline (4) and rat
64 (5) cardiomyocytes, rabbit skeletal myofibrils (6), quail myotubes (7), and transgenic mice

65 (8). These studies have resulted in conflicting conclusions about the effects of the
66 mutation, at least in part due to phenotypic differences between species. For example,
67 the widely studied transgenic mouse model of R92Q (8) recapitulates some, but not all,
68 aspects of the disease phenotypes seen in humans. Elegant experiments by the Tardiff
69 lab have shown that the disease presentation in mice depends on the myosin heavy chain
70 isoform expressed, with different phenotypes seen when using the faster (*MYH6*) isoform
71 found in mouse ventricles or the slower (*MYH7*) isoform found in human ventricles (9).
72 These studies highlight the need to study the mutation in humanized systems.

73 Troponin T is part of the troponin complex, which, together with tropomyosin,
74 regulates the calcium-dependent interactions between myosin and the thin filament that
75 drive muscle contraction. Three models have been put forward to describe the initial
76 molecular insult that drives the disease pathogenesis of R92Q (Fig. 1B). 1) R92Q could
77 affect the cycling kinetics of myosins that are bound to the thin filament (9). In this model,
78 one would expect to observe a change in the amount of time that myosin remains bound
79 to the thin filament during crossbridge cycling in the mutant. 2) R92Q could increase the
80 calcium affinity of the troponin complex, leading to altered calcium buffering by
81 myofilaments that directly disrupts calcium homeostasis (10-12). In this model, one would
82 expect to observe an increased binding affinity for calcium in the troponin complex
83 containing R92Q. 3) R92Q could alter the distribution of positions assumed by
84 tropomyosin along the thin filament, leading to changes in the fraction of bound myosin
85 crossbridges (13). In this model, one would expect to see changes in the equilibrium
86 constants that define the positioning of tropomyosin along the thin filament. The

87 mechanistic differences between these models have important implications for the design
88 of therapeutic strategies.

89 Here, we set out to identify the initial molecular insult in troponin T caused by the
90 R92Q mutation, and to link the molecular defect to observed derangements in cellular
91 function. To do this, we developed a human R92Q model in gene-edited human induced
92 pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). We show here that the initial
93 biophysical insult is altered positioning of tropomyosin along the thin filament. The altered
94 positioning of tropomyosin directly affects cellular tension, leading to secondary adaptive
95 changes in calcium homeostasis, gene expression, and electrophysiology. Our results
96 implicate mechanobiological signaling as a primary driver of disease pathogenesis in
97 HCM.

98 **Results**

99 *Generation of gene-edited stem cell-derived cardiomyocytes*

100 We used CRISPR/Cas9 to generate two independent human induced pluripotent
101 stem cell (hiPSC) lines that are homozygous for the R92Q mutation (Supplementary Fig.
102 S1). Homozygous lines were used to facilitate direct correlation of the molecular insult
103 with alterations in cellular function. Heterozygous lines would better mimic the disease
104 seen in humans but would contain complex mixtures of wild type (WT) and mutant
105 proteins, confounding the correlation of the molecular and cellular results. Both WT and
106 R92Q hiPSCs were derived from the same parent line and are therefore isogenic except
107 for the mutation. We previously showed, by whole exome sequencing of the parent line,
108 that it has no known variants associated with cardiomyopathy (14). Gene-edited hiPSCs
109 have normal karyotypes (Fig. S1B) and are pluripotent, as assessed by
110 immunofluorescence (Supplementary Fig. S2). hiPSCs were differentiated to hiPSC-CMs
111 through temporal modulation of WNT signaling (15, 16), and our efficiency of
112 differentiation using this procedure is >90% (14).

113

114 *R92Q hiPSC-CMs generate increased force, power, and contraction speed compared to* 115 *WT cells*

116 To test whether R92Q hiPSC-CMs show the altered contractility seen in some
117 model systems, we measured the contractility of single hiPSC-CMs using traction force
118 microscopy. hiPSC-CMs were seeded onto rectangular extracellular matrix (ECM)
119 patterns on polyacrylamide hydrogels of physiological stiffness (10 kPa) (14). This
120 patterning on physiological stiffness hydrogels promotes hiPSC-CM maturation and

121 sarcomeric alignment (17). The force, speed of contraction, and power were calculated
122 from the displacement of beads embedded in the hydrogel (18). Data were plotted as
123 cumulative distributions of single cells to account for cell-to-cell variability (14). R92Q
124 hiPSC-CMs generate more force, power, and have a higher contractile speed compared
125 to the WT (Fig. 2).

126

127 *Intracellular calcium transients are reduced in R92Q cells*

128 Previous studies using R92Q transgenic mice showed altered cardiomyocyte
129 calcium handling (10, 11, 19, 20). To examine calcium dynamics in hiPSC-CMs, cells
130 were patterned onto rectangular ECM patterns on 10 kPa hydrogels and loaded with the
131 ratiometric fluorescent calcium indicator dye, Fura Red. Line scans of the fluorescence of
132 spontaneously beating cells were collected at 1.9 ms intervals. As can be seen, hiPSC-
133 CMs display well-defined calcium transients (Fig. 3A); however, the amplitudes of the
134 transients are lower ($p < 0.002$) in R92Q (0.56 ± 0.13 ; $n=18$), compared to WT ($0.84 \pm$
135 0.11 ; $n=19$), cells. Therefore, despite generating increased force, R92Q hiPSC-CMs
136 show reduced calcium transient amplitudes compared to WT cells.

137

138 *R92Q cells show alterations in expression of calcium-handling genes*

139 The observed changes in calcium handling could come from a variety of sources,
140 including changes in transcription, protein expression, and/or post-translational
141 modifications of proteins that regulate calcium homeostasis. To explore a possible role
142 for transcriptional remodeling, we performed qPCR analyses of the expression of
143 transcripts encoded by key genes involved in the regulation of calcium homeostasis in

144 cardiomyocytes. Specifically, we examined the expression levels of transcripts encoding
145 phospholamban (*PLN*), sarcoendoplasmic reticulum calcium-ATPase (*ATPA2*), voltage-
146 gated calcium channel subunits (*CACNA1C*, *CACNA1G*, *CACNA1H*), IP3 receptor
147 (*ITPR2*), calsequestrin (*CASQ2*), calcium-calmodulin dependent kinase 2 (*CAMK2D*),
148 sodium-calcium exchanger (*SLC8A1*), and the ryanodine receptor (*RYR2*). We found
149 marked upregulation of *CASQ*, *CAMK2D*, and *SLC8A1* and downregulation of *CACNA1H*
150 in R92Q, compared with WT, hiPSC-CMs (Fig. 3B, Supplementary Table S2),
151 demonstrating that the expression levels of key genes associated with calcium handling
152 are altered in R92Q hiPSC-CMs.

153

154 *R92Q cells show altered action potentials and reduced inward calcium current densities*

155 The observed reductions in the calcium transients observed in spontaneously
156 beating R92Q cells could reflect changes in transmembrane calcium influx. To determine
157 directly if membrane excitability is altered in R92Q cells, we obtained whole-cell current
158 clamp recordings of spontaneous action potentials in WT and R92Q mutant hiPSC-CMs
159 patterned onto rectangular ECM patterns on 10 kPa hydrogels (Fig. 4A-B). Analyses of
160 the data obtained in these experiments revealed that the maximum diastolic potential (the
161 most negative membrane potential achieved between action potentials in spontaneously
162 firing cells) is more depolarized in R92Q hiPSC-CMs than in WT hiPSC-CMs (Fig. 4B). In
163 addition, the frequency of spontaneous action potential firing is higher, upstroke velocities
164 (i.e., the rate of membrane depolarization) are lower, and action potential durations,
165 measured at 50% repolarization (APD_{50}), are shorter in R92Q hiPSC-CMs, compared
166 with WT cells (Fig. 4B, Supplementary Table S3).

167 To better understand the mechanism(s) contributing to the reductions in the APD₅₀
168 seen in spontaneously beating R92Q cells, we examined the waveforms of evoked action
169 potentials of hiPSC-CMs hyperpolarized to a membrane potential of -80 mV. Although
170 similar hyperpolarizing currents were required to render R92Q and WT hiPSC-CMs
171 electrically silent and similar currents were required to evoke action potentials in WT and
172 mutant cells, the durations of evoked action potentials are significantly shorter in R92Q,
173 than in WT, cells (Fig. 4C-D).

174 Additional voltage-clamp experiments were conducted to determine directly if
175 voltage-gated inward calcium current densities were altered in R92Q, compared with WT,
176 cells. With outward potassium currents blocked, we recorded whole-cell voltage-gated
177 calcium currents evoked on membrane depolarization in WT and R92Q hiPSC-CMs. As
178 illustrated in Figure 4E, these experiments revealed that inward calcium current densities
179 are markedly reduced in R92Q, compared to WT hiPSC-CMs (Fig. 4F).

180

181 *Determination of the molecular mechanism of R92Q*

182 The cellular studies describe above clearly show changes in cellular mechanics,
183 calcium handling, gene expression, and electrophysiology in R92Q, compared with WT,
184 hiPSC-CMs. However, it is difficult to deconvolve the initial driver of the disease
185 pathogenesis from downstream effects in the inherently complicated cellular context. At
186 the molecular scale, the initial insult that drives the disease pathogenesis is mutation-
187 induced alterations in protein function. Therefore, we set out to determine the molecular
188 mechanism of the R92Q mutation in troponin T.

189 Troponin T is part of the troponin complex, which, together with tropomyosin,
190 regulates the calcium-dependent interactions between myosin and the thin filament that
191 power force generation in muscle. Biochemical (21) and structural (22) measurements
192 have demonstrated that tropomyosin can lie in three states along the thin filament, termed
193 blocked, closed, and open, and, in addition, that myosin can bind either weakly or strongly
194 to the thin filament when tropomyosin is in the open position (Fig. 1B). In the absence of
195 calcium, tropomyosin lies in the blocked position and inhibits the binding of force-
196 generating actomyosin crossbridges. When calcium binds to troponin C on a thin filament
197 regulatory unit, tropomyosin shifts to the closed position. The tropomyosin can then be
198 pushed into the open position either by thermal fluctuations or myosin binding. Myosin is
199 then able to isomerize into a strong binding state, generating force. The number of
200 strongly bound, force-generating myosin crossbridges at a given calcium concentration
201 determines the amount of force developed.

202 To examine the molecular effects of the R92Q mutation, WT and R92Q human
203 troponin T were expressed and reconstituted into functional troponin complexes for
204 biochemical and biophysical measurements. All assays were conducted using
205 recombinant human tropomyosin and troponin complex. β -cardiac ventricular cardiac
206 myosin (*MYH7*) and cardiac actin were purified from porcine hearts. The porcine β -
207 cardiac myosin isoform has 97% identity with human β -cardiac myosin (compared to 92%
208 with murine ventricular myosin), and it has very similar biophysical properties, including
209 the kinetics of the myosin ATPase cycle and mechanics measured in the optical trap (23-
210 25).

211 We examined the effect of the R92Q mutation on thin filament regulation using an
212 *in vitro* motility assay. In this assay, fluorescently labeled reconstituted regulated thin
213 filaments are translocated over a bed of myosin in the presence of ATP and varying
214 concentrations of calcium (26). The speed of translocation was measured as a function
215 of calcium concentration, and normalized data were fitted with the Hill equation, as
216 previously described (27). As can be seen from the data (Fig. 5A), R92Q-regulated thin
217 filaments show a shift towards activation at submaximal, but physiologically relevant,
218 calcium concentrations (pCa_{50} for WT = 6.12 ± 0.02 versus 6.37 ± 0.03 for R92Q; $p <$
219 0.001). There is no change in cooperativity, as determined by the Hill coefficient ($3.8 \pm$
220 0.6 for WT versus 3.4 ± 0.7 for R92Q; $p = 0.75$).

221
222 *The R92Q mutation does not change myosin detachment kinetics or calcium binding*
223 *affinity*

224 The shift towards submaximal calcium activation observed for R92Q in the *in vitro*
225 motility assay stems from changes in the function of the troponin-T protein. Given the role
226 of troponin T in regulating calcium-dependent muscle contraction, three models have
227 been proposed to explain the molecular mechanism of the R92Q mutation (Fig. 1B): 1)
228 R92Q could affect the cycling kinetics of myosins that are bound to the thin filament (9).
229 In this model, one would expect to observe a change in the amount of time that myosin
230 remains bound to the thin filament during crossbridge cycling in the mutant. 2) R92Q
231 could increase the calcium affinity of the troponin complex, leading to altered calcium
232 buffering by myofilaments that directly disrupts calcium homeostasis (10-12). In this
233 model, one would expect to observe an increased binding affinity for calcium in the

234 troponin complex containing R92Q. 3) R92Q could alter the distribution of positions
235 assumed by tropomyosin along the thin filament, leading to changes in the fraction of
236 bound myosin crossbridges (13). In this model, one would expect to see changes in the
237 equilibrium constants that define the positioning of tropomyosin along the thin filament.
238 We set out to test these three models.

239 First, we tested whether the mutation affects the kinetics of myosin detachment
240 from the thin filament by using stopped-flow kinetics to measure the rate of ADP release
241 from actomyosin (i.e., the transition that limits actomyosin dissociation and myosin's
242 unloaded sliding velocity) (28), as we have done previously (14). We found that the rate
243 of ADP release from myosin bound to regulated thin filaments is not affected by the R92Q
244 mutation ($75.8 \pm 3.9 \text{ s}^{-1}$ for R92Q versus $76.3 \pm 5.0 \text{ s}^{-1}$ for WT; $p = 0.88$) (Fig. 5B).
245 Therefore, changes in myosin detachment kinetics cannot explain the shift towards
246 submaximal calcium activation seen in the *in vitro* motility assay.

247 Next, we measured whether the calcium binding affinity to the troponin complex is
248 affected by the mutation. We used an IAANS-labeled form of troponin C to characterize
249 calcium binding to the troponin complex (29, 30). The fluorescence intensity of this probe
250 changes upon calcium binding to troponin C (29-32). We used it to spectroscopically
251 measure the affinity of calcium binding to regulated thin filaments (Fig. 5C) (29). We saw
252 that the calcium concentration required for half maximal activation, Ca_{50} , is not
253 significantly different for the WT ($0.66 \pm 0.18 \mu\text{M}$) and R92Q mutant ($0.67 \pm 0.19 \mu\text{M}$; $p =$
254 0.93) proteins. Similar results were seen at 15°C (Fig. 5C) and 20°C (Supplementary Fig.
255 S3). These results demonstrate that changes in the affinity of calcium binding to troponin

256 C cannot explain the shift towards submaximal calcium activation seen in the *in vitro*
257 motility assay (Fig. 5A).

258

259 *The initial biophysical insult of R92Q is increased thin filament activation due to*
260 *repositioning of tropomyosin along the thin filament*

261 To test whether the shift in calcium sensitivity can be explained by a change in the
262 distribution of positions assumed by tropomyosin along the thin filament (Fig. 6A), we
263 measured the equilibrium constants that define the fraction of thin filament regulatory
264 units in each state (21, 33). The equilibrium constant between the blocked and closed
265 states, K_B , was determined by rapidly mixing fluorescently labeled regulated thin filaments
266 together with myosin and then measuring the rate of myosin binding (seen as quenching
267 of the fluorescence signal) in the presence and absence of calcium (see Materials and
268 Methods for details). At low calcium, when tropomyosin is primarily in the blocked state,
269 the rate of myosin binding to the thin filament is slower than at high calcium, when the
270 blocked state is less populated. The ratio of the rates of binding at low and high calcium
271 were used to calculate K_B (Eq. 1, Fig. 6B). As can be seen from the fluorescence
272 transients, the rate of myosin binding to regulated thin filaments is similar for the WT and
273 R92Q mutant proteins at high calcium (pCa 4); however, at low calcium (pCa 9), the rate
274 of binding for the mutant is much faster than for the WT, consistent with lower population
275 of the blocked state. When we calculate K_B , we see that it is significantly larger in the
276 mutant compared to the WT (1.02 ± 0.26 for R92Q vs. 0.40 ± 0.15 for WT, $p=0.003$),
277 meaning that the population of the more inhibitory blocked state is reduced while the
278 population of the closed state is increased. The increased K_B value means that, at low

279 calcium levels, the thin filament will be more activated in the mutant, consistent with the
280 *in vitro* motility measurements (Fig. 5A).

281 Next, we considered whether the mutation affects the equilibrium constant for the
282 transitions between the closed and open states, K_T , or the equilibrium constant between
283 the open and myosin weakly bound states, K_w . To do this, we performed titrations of
284 fluorescently labeled regulated thin filaments with increasing concentrations of myosin
285 and measured the quenching of the fluorescence as the myosin binds to the regulated
286 thin filaments (Fig. 6C). The data, analyzed using a modification of the method of McKillop
287 and Geeves (21, 33), show that there are no significant differences in K_T between the WT
288 and R92Q (Fig. 6). There is a statistically significant increase in K_w ; however, this is small,
289 and the magnitude is insufficient to explain the shift in the *in vitro* motility assays. This
290 demonstrates that the primary molecular defect in R92Q is partial activation of the thin
291 filament at low calcium levels due to reduced population of the inhibitory blocked state.
292 Based on this result, one would expect increased contractility in the mutant, compared to
293 the WT, during a calcium transient.

294

295 *Computational modeling demonstrates that altered tropomyosin positioning with R92Q is*
296 *sufficient to explain the increase in cellular contractility*

297 To test whether the observed change in K_B is sufficient to explain the shift towards
298 submaximal calcium activation seen in the *in vitro* motility assay (Fig. 5A), we used a
299 computational model of thin filament activation developed by Campbell et al (34). In this
300 model, the user inputs a set of equilibrium constants, and the model predicts several
301 parameters, including the force per sarcomere as a function of calcium. When we use the

302 default parameters of the model, but proportionally increase the value of K_B to match the
303 fractional change seen in our biochemical experiments, we find that this change alone
304 produces a shift towards submaximal calcium activation similar to the shift observed in
305 the *in vitro* motility experiments (Fig. 7A). This finding validates that the primary effect of
306 the R92Q mutation on motility can be explained by reduced population of the thin filament
307 blocked state.

308 Our data with hiPSC-CMs demonstrate that R92Q has increased force production
309 (Fig. 2A), but reduced calcium transient amplitudes (Fig. 3A). To see whether the reduced
310 population of the blocked state, observed in our molecular studies (Fig. 6A), is sufficient
311 to explain the hypercontractility seen in cells despite the reduction in calcium transient
312 amplitude, we used the same computational model to calculate the expected force per
313 sarcomere in response to a calcium transient. In the modeling, the amplitude of the
314 calcium transient for R92Q was reduced to 67% of the value seen in the WT, as observed
315 in our cellular measurements (Fig. 3A). As above, we proportionally increased K_B for the
316 mutant to match the relative difference seen in our biochemical experiments. Consistent
317 with our cellular experiments, the model predicts that the mutant will generate more force
318 in response to a calcium transient than the WT, despite having a smaller amplitude
319 calcium transient (Fig. 7B). Taken together, our molecular experiments demonstrate that
320 the initial molecular insult of the R92Q mutation is decreased population of the thin
321 filament blocked state, leading to increased force generation during a calcium transient.

322

323 **Discussion**

324 Here, we elucidated the molecular and cellular consequences of the R92Q
325 mutation in troponin T that causes HCM, R92Q. We show that the initial molecular insult
326 that drives disease pathogenesis is increased thin filament activation at physiologically
327 relevant micromolar calcium levels due to destabilization of the blocked state of
328 tropomyosin. We demonstrate computationally and experimentally that this increased
329 activation directly causes increased mechanical force produced by hiPSC-CMs. We show
330 that this initial insult of altered mechanical forces leads to downstream changes in the
331 expression of genes associated with calcium handling, altered calcium transients, and
332 alterations in cellular electrophysiology. Taken together, our results highlight the role of
333 mechanobiology in driving the early disease pathogenesis.

334

335 *Defining the primary molecular driver of the disease pathogenesis*

336 Previous *in vivo* and *in vitro* cellular studies have demonstrated that the R92Q
337 mutant protein is expressed and properly integrated into sarcomeres, suggesting that the
338 driver of the disease is changes in protein biochemistry and biophysics, rather than
339 haploinsufficiency (4, 7, 8, 35). To better understand these changes in protein function,
340 we conducted *in vitro* motility assays which demonstrated that R92Q causes a shift
341 towards submaximal calcium activation (Fig. 5A). This finding is consistent with some (6,
342 9, 35-39), but not all (5, 7), previous measurements in muscle fibers and in biochemical
343 assays using non-cardiac muscle protein isoforms. The shift towards submaximal calcium
344 activation could potentially come from changes in actomyosin dissociation kinetics, the

345 affinity of calcium binding to troponin C, and/or the positioning of tropomyosin along the
346 thin filament (Fig. 1B).

347 Our biophysical studies clearly demonstrate that the mutation does not affect the
348 binding of calcium to troponin C or the kinetics of actomyosin dissociation in the absence
349 of load (Fig. 5). The results show, however, that the mutation causes a pronounced
350 increase in the equilibrium constant between the blocked and closed states, K_B (Fig. 6B).
351 This change would favor the closed state over the blocked state, effectively lowering the
352 energy barrier required for thin filament activation at physiologically relevant (pCa 5-7
353 range (40)) calcium concentrations. Our computational modeling (Fig. 7) demonstrates
354 that the observed change in this equilibrium constant is sufficient to explain the shift
355 towards submaximal calcium activation seen in our *in vitro* motility measurements (Fig.
356 5A). Our data support a model in which the initial insult that drives disease pathogenesis
357 is altered positioning of tropomyosin along the thin filament, with a greater fraction of
358 regulatory units in the closed, than in the more inhibitory blocked, position at low calcium.
359 This shift would lower the energy barrier for activation of the thin filament, leading to
360 submaximal calcium activation.

361 The R92Q mutation has been studied in many model systems, including quail
362 myotubes (7), transfected rat cardiomyocytes (5), skinned rabbit muscle fibers (6),
363 transgenic mice (8), and transfected cat cardiomyocytes (4). While these studies have
364 greatly advanced our understanding of the mutation, they have also shown that the effects
365 of the mutant protein present differently depending on the model system used. In
366 addition, previous work using transgenic mice demonstrated that disease presentation
367 varies depending on whether proteins with biophysical properties similar to human

368 isoforms are used (9). The use of all cardiac proteins with biochemical and biophysical
369 properties similar to human proteins is especially important for studies of thin filament
370 mutations, since the activation of the thin filament depends on both myosin and calcium
371 binding (Fig. 1B). In our molecular studies, we used human cardiac troponin and
372 tropomyosin and porcine cardiac myosin and actin. Porcine cardiac myosin (*MYH7*) is
373 97% identical to the human protein, and displays biochemical kinetics, mechanical step
374 sizes, and load-dependent kinetics that are indistinguishable from the human isoform (23-
375 25). Therefore, we believe that our model system reliably mimics the molecular phenotype
376 in humans.

377 Interestingly, the R92 residue is in the region of troponin T that interacts with
378 tropomyosin, near where two tropomyosin molecules overlap in a head-to-tail fashion
379 (41). Two other HCM-causing mutations have been identified at the R92 site, R92W and
380 R92L, and this has led to the suggestion that it is a hotspot for HCM mutations. To date,
381 there are no atomic-resolution structures of this region of the thin filament. Structural
382 studies, however, have shown that troponin T plays a role in stabilizing the blocked state
383 in the absence of calcium (42). In addition, molecular dynamics simulations have shown
384 that mutations in the R92 region can lead to changes in the distance between troponin T
385 and tropomyosin (43) and biochemical experiments have shown that the R92L mutation
386 decreases the affinity of troponin for tropomyosin (44). We speculate that the R92Q
387 mutation has a similar effect on the interactions between troponin T and the thin filament,
388 leading to destabilization of the blocked state.

389 It has previously been proposed that R92Q causes an increase in calcium affinity
390 for the troponin complex on the thin filament which would affect the buffering of calcium

391 by myofilaments, leading to disrupted calcium homeostasis (10, 12). While our cellular
392 data reveal disrupted calcium homeostasis, our molecular work shows no change in the
393 affinity of calcium for R92Q troponin, demonstrating that this change in calcium
394 homeostasis is a downstream consequence of the primary molecular insult. This result is
395 consistent with work from the Molkenkin lab (45), which showed that the development of
396 HCM correlates with changes in tension, rather than calcium handling.

397 Recent studies of HCM-causing mutations in thick filament proteins, including β -
398 cardiac myosin (*MYH7*), myosin binding protein C (*MYBPC3*), myosin regulatory light
399 chain, and myosin essential light chain, have demonstrated that many of these mutations
400 disrupt the autoinhibited super relaxed state of myosin, leading to the recruitment of more
401 crossbridges and thus hypercontractility (46-51). It has been proposed that increased
402 crossbridge recruitment correlates with the hyperdynamic cardiac function seen in HCM
403 (48). Our studies with R92Q, a thin filament mutation, demonstrate a similar net effect of
404 increased crossbridge recruitment at physiologically relevant calcium levels, suggesting
405 altered recruitment of crossbridges in HCM as a common theme for both thin and thick
406 filament mutations.

407

408 *Connecting the molecular and cellular phenotypes in R92Q*

409 Our data clearly demonstrate that the primary molecular driver of early disease
410 pathogenesis is altered positioning of tropomyosin along the thin filament. At the cellular
411 level, R92Q shows both an increase in cellular force production (Fig. 2A) and a reduction
412 in the amplitude of the calcium transient (Fig. 3A). These seemingly conflicting findings
413 can be reconciled by our computational modeling (Fig. 7B), which reveals that the shift

414 towards thin filament activation at submaximal calcium leads to cellular hypercontractility,
415 despite the reduction in the amplitude of the calcium transient. The hypercontractile
416 effects of this shift are relevant at physiological (micromolar) concentrations of calcium
417 (52). Importantly, our results demonstrate that the cellular hypercontractility can be
418 explained by our molecular mechanism.

419 At the cellular level, we see disrupted calcium homeostasis with R92Q, which is a
420 downstream consequence of the primary hypercontractile phenotype. Calcium
421 homeostasis in the myocardium is a complicated process which depends on many
422 factors, including gene expression and post-translational modifications of signaling and
423 contractile proteins (52). While a complete dissection of this mechanism is beyond the
424 scope of the current study, our work provides insights into potential transcriptional
425 mechanisms. We observed changes in the expression of several genes involved in
426 calcium handling (Fig. 3B), including calsequestrin (*CASQ2*), calcium-calmodulin kinase
427 (*CAMK2D*), the sodium-calcium exchanger (*SLC8A1*), and a voltage-gated calcium
428 channel subunit (*CACNA1H*). Interestingly, overexpression of *CASQ* or *CAMK2D* in
429 transgenic mice drives the development of heart failure and arrhythmogenesis (53, 54).
430 We recognize that changes in transcript expression do not always correlate with protein
431 function. Regardless, our data demonstrate that altered mechanics at the molecular level
432 can drive changes in cellular gene expression, showing a mechanobiological link between
433 these processes in HCM.

434 At a functional level, our single-cell electrophysiological experiments reveal that
435 action potential durations are shorter in R92Q, compared with WT cells, due in part to
436 reduced inward L-type calcium current densities (Fig. 4). These changes would be

437 expected to be arrhythmogenic and could contribute to the increased incidence of
438 arrhythmias and sudden cardiac death in individuals harboring the R92Q mutation. We
439 observe normal expression levels of the transcripts encoding L-type channel subunits,
440 and therefore, the reduced current density could be due to alterations in signaling
441 pathways and/or post-translational modifications of channel subunit proteins. The
442 reduction in inward calcium current densities observed in our electrophysiological
443 measurements (Fig. 4) would be expected to reduce calcium-induced calcium release
444 from intracellular calcium stores, potentially contributing to the observed reductions in
445 calcium transient amplitudes (Fig. 3A).

446 Our results clearly show that molecular hypercontractility drives downstream
447 changes in cellular calcium handling and electrophysiology in single hiPSC-CMs. We
448 propose that mutation-induced changes in cellular tension alter mechanosensitive
449 signaling pathways in cardiomyocytes (55). Consistent with this idea, recent work from
450 our lab demonstrated that a dilated cardiomyopathy mutation in troponin T, Δ K210,
451 affects molecular mechanosensing, which helps to drive the disease progression (14).
452 Such a mechanism is also consistent with the model of Davis et al., who proposed that
453 alterations in cellular tension correlate with the hypertrophic response (45). In fact,
454 increases in cardiac tension stemming from external sources such as hypertensive
455 disease and aortic stenosis can promote pathological hypertrophy. Deciphering the
456 specific mediators of mechanobiological pathways in cardiomyocytes is an active field of
457 research (55-58). In the broader context of the myocardium, hypercontractility of
458 cardiomyocytes can impose aberrant stretch on fibroblasts, activating the transition to

459 myofibroblasts (55). Such a mechanism could contribute to the diffuse myocardial fibrosis
460 frequently seen with HCM.

461

462 *Implications for modeling and treating HCM*

463 The goal of our study was to connect the initial molecular insult with the early
464 disease pathogenesis in human cells. We therefore used genome-edited hiPSC-CMs,
465 which are excellent tools for dissecting early disease pathogenesis (59, 60). These
466 experiments were conducted using isogenic cells, making it easier to understand the
467 direct consequences of the point mutation on a controlled genetic background (61). The
468 results obtained demonstrate that these hiPSC-CMs recapitulate important aspects of
469 HCM-induced changes in contractility (8, 9, 37), altered electrophysiology (10, 20), and
470 calcium dysfunction (10, 11, 19) seen in other model systems. While our hiPSC-CM
471 model recapitulates some aspects of the early disease pathogenesis, it cannot fully
472 capture the clinical phenotype seen in patients for several reasons. First, hiPSC-CMs are
473 developmentally immature, and they lack many of the physiological cues present
474 in patients (59, 60). As such, they do not capture some aspects of clinical HCM, including
475 fibrosis, tissue hypertrophy, and ventricular arrhythmias. Moreover, while patients are
476 typically heterozygous for the R92Q mutation, our studies were conducted using
477 homozygous cell lines to facilitate connecting the molecular and cellular phenotypes.
478 Work in transgenic mice has shown that disease phenotypes vary with mutant gene
479 dosage (8, 62) and that the homozygous mutation is embryonic lethal. Therefore, care
480 should be taken when extrapolating from these studies to the clinical phenotype.

481 Limitations aside, hiPSC-CMs are a unique tool to study the connection between
482 the initial molecular insult and the early disease pathogenesis in human cells. Our
483 identification of altered cellular mechanics and downstream mechanobiological signaling
484 pathways as key drivers of the disease pathogenesis has important implications for
485 treatment. There is currently an outstanding need to develop new therapeutics to treat
486 HCM. The current therapeutic regimen is the use of agents to prevent further myocardial
487 remodeling, and in extreme cases, myectomy or cardiac ablation. Our findings suggest
488 that approaches which target mechanobiological signaling pathways in cardiomyocytes
489 could be useful in the treatment of HCM.

490 Recently, there was a report of an HCM mutation in α -actinin that causes
491 prolongation of the action potential due to an increase in the calcium current density (63).
492 In this case, the patient was successfully treated with the L-type calcium channel blocker,
493 diltiazem. In R92Q, we observed a reduction in the calcium current density (Fig. 4), and
494 therefore, a different therapeutic would be necessary. These differences between cellular
495 phenotypes in these two HCM mutations highlights the need to understand the underlying
496 changes in molecular and cellular function, and it demonstrates the need to consider a
497 personalized medicine approach for HCM.

498

499 *Conclusions*

500 The results here demonstrate that the initial insult of the R92Q mutation in troponin
501 T is molecular and cellular hypercontractility at physiologically relevant calcium
502 concentrations, which leads to alterations in mechanobiological signaling pathways that
503 regulate calcium homeostasis, gene expression, and cellular electrophysiology. Taken

504 together, the data presented suggest that these mechanobiological adaptations play a
505 central role in the early disease pathogenesis, and they suggest that targeting these
506 pathways could open new avenues for treating this devastating class of diseases.

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517

518 **Conflict of interest statement:**

519 All experiments were conducted in the absence of any commercial or financial
520 relationships that could be construed as potential conflicts of interest.

521

522 **Author contributions:**

523 S.R.C. purified proteins and performed and analyzed the stopped flow and fluorescence
524 experiments. P.E.C. performed and analyzed the traction force microscopy experiments
525 with the stem cell derived cardiomyocytes. W.W. performed and analyzed
526 electrophysiological experiments. L.G. purified proteins, implemented the cell-based
527 assays, performed and analyzed experiments with stem cell derived cardiomyocytes,
528 performed qPCR measurements, and performed calcium imaging experiments. W.T.S.
529 designed tools for microcontact printing. P.A. performed *in vitro* motility assays. J.M.N.

530 oversaw the electrophysiological experiments and analyzed data. M.J.G. oversaw the
531 project, performed simulations, generated mutant proteins, implemented biochemical
532 assays, analyzed data, and drafted the manuscript. All authors contributed to the writing
533 and/or editing of the manuscript.

534 **Methods**

535 **Protein modification and purification**

536 Cardiac myosin and actin were purified from cryoground porcine ventricles (Pelfreez) as
537 previously described (14). S1 myosin was prepared by chymotrypsin digestion as
538 previously described (14). Recombinant human cardiac tropomyosin, troponin I, troponin
539 T, and troponin C were expressed in *E. coli* and purified from BL21-CodonPlus cells
540 (Agilent) as described previously (14). Purified tropomyosin was reduced in 50 mM DTT
541 at 56°C for 5 minutes and ultracentrifuged to remove aggregates immediately before
542 being used in each assay. The R92Q mutation was introduced into troponin T using
543 QuikChange Site-Directed Mutagenesis (Agilent) and the presence of the mutation was
544 verified by sequencing.

545 For the studies of calcium binding, we used IAANS (6-((4-((2-
546 iodoacetyl)amino)phenyl)amino)-2-naphthalenesulfonic acid)-labeled troponin C (32).
547 IAANS was custom synthesized by Toronto Research Chemicals. Troponin C^{T53C} was
548 labeled with five-fold molar excess IAANS dye overnight, and the reaction was quenched
549 with DTT. Excess dye was dialyzed out with 4 dialysis buffer changes of 1 mM DTT,
550 0.01% NaN₃, 50 μM CaCl₂, 1 mM MgCl₂, 3 M Urea, 1 M KCl, 5 mg/L TPCK, 5 mg/L TLCK,
551 0.3 mM PMSF (32). The IAANS-labeled troponin C^{T53C} was then purified over a MonoQ
552 column and complexed with the troponin T and I as done previously (14).

553

554 ***In vitro* motility assays**

555 *In vitro* motility assays were conducted using thin filaments containing R92Q troponin T
556 as previously described (14). Data for WT troponin T are from (14). Briefly, enzymatically
557 inactive full-length porcine cardiac myosin was removed by cosedimentation with
558 phalloidin-stabilized F-actin in the presence of ATP. Flow cells were loaded with 1 volume
559 (50 μ L) of 200 nM myosin, 2 volumes of 1 mg/mL BSA, 1 volume of 1 μ M F-actin, 2
560 volumes of KMg25 (25 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 60 mM MOPS pH
561 7.0) + 1 mM MgATP, 4 volumes of KMg25, and 1 volume of 40 nM rhodamine-phalloidin-
562 labeled thin filaments. After loading 2 volumes of activation buffer (KMg25 with 4 mM
563 MgATP, 1 mg/mL glucose, 192 U/mL glucose oxidase, 48 μ g/mL catalase, 2 μ M troponin
564 and tropomyosin, 0.5% methyl cellulose), flow cells were imaged for 20 frames. Individual
565 motile filaments were manually tracked using the MTrackJ plugin in Fiji ImageJ (64), and
566 each point shows the average and standard deviation of the speed from 3 separate
567 experiments.

568

569 **Stopped-flow transient kinetic measurement of K_B and ADP release**

570 An SX-20 stopped flow apparatus (Applied Photophysics) was used. K_B was determined
571 as previously described (14, 33). WT data are from (14). At both low (pCa 9) and high
572 calcium (pCa 4), 5 μ M phalloidin-stabilized pyrene actin, 2 μ M tropomyosin, 2 μ M
573 troponin, and 0.04 U/mL apyrase were rapidly mixed with 0.5 μ M S1 myosin and 0.04
574 U/mL apyrase. Performed at 20°C, each experiment was the average of at least 3
575 separate mixes and the data were fit by a single exponential curve. K_B was calculated
576 from:

577
$$\frac{k_{obs}(-Ca^{2+})}{k_{obs}(+Ca^{2+})} = \frac{K_B}{1+K_B}$$
 Equation 1

578 The reported K_B is the average of at least three different experiments. The p-value was
579 calculated from a 2-tailed Student's t-test.

580 The rate of ADP release from myosin bound to regulated thin filaments (20°C) was
581 measured as previously described. (14) The average and standard deviation of the rate
582 of at least four experiments was calculated and the p-value was derived using a two-tailed
583 Student's t-test.

584

585 **Fluorescence titrations to measure K_W , K_T , and n**

586 A SX-20 stopped flow fluorometer was used for all fluorescence titrations. The values of
587 K_W , K_T , and n (the cooperativity) were determined for R92Q and WT using fluorescence
588 titrations as previously described (14, 33). The WT data is from (14). Our MATLAB-based
589 computational tool was used for hypothesis testing and uncertainty estimation, as
590 previously described (33).

591

592 **Measurement of calcium binding to troponin C**

593 The calcium affinity for the troponin complex (Tn^{IAANS}) was determined by titrating
594 regulated thin filaments with increasing calcium concentrations and measuring the
595 change in fluorescence in IAANS-labeled troponin C upon calcium binding (32). Tn^{IAANS}
596 was excited at 330 nm and fluorescence emission was detected using a 395 nm long-
597 pass filter. 0.15 μ M Tn^{IAANS} complex, 0.45 μ M tropomyosin, and 2 μ M actin were mixed

598 with increasing concentrations of calcium in 10 mM MOPS pH 7.0, 150 mM KCl, 3 mM
599 MgCl₂, and 1 mM DTT. Each buffer was balanced to give the desired free calcium, free
600 magnesium, and ionic strengths using MaxChelator (65). The solution was allowed to
601 equilibrate for 1 minute after mixing with constant stirring before the fluorescence intensity
602 was measured. The titration curve was fit by the logistic sigmoid function, which is
603 mathematically equivalent to the Hill equation:

$$604 \quad Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + \exp[-H(X - pCa_{50})]} \quad \text{Equation 2}$$

605 where Y_{max} and Y_{min} are the maximum and minimum IAANS fluorescence, X is the
606 negative logarithm of $[Ca^{2+}]_{free}$, pCa_{50} is the negative log of the concentration of free
607 calcium producing half-maximal fluorescence, and H is the cooperativity (proportional to
608 the Hill coefficient) (66). Titrations were performed at both 15°C (Fig. 5C) and 20°C
609 (Supplementary Fig. S3) to facilitate comparison with previous measurements using
610 different proteins (29).

611

612 **Computational modeling of sarcomeric contractility**

613 To simulate the effects of the experimentally determined changes in equilibrium constants
614 on force production, we used the computational model developed by Campbell et al. (34)
615 based on McKillop and Geeves (21), as we have done previously (14). Briefly, in this
616 model, 9 sarcomeres are simulated, where the equilibrium constants between states and
617 a coupling constant describing cooperativity define the probability of switching between
618 biochemical states. The steady-state force is calculated from the equilibrium distribution
619 of states at a given calcium concentration. Our biochemical experiments demonstrated

620 that the primary change at the molecular scale with the mutation is an increase in K_B ,
621 such that K_B (R92Q) = 2.56 * K_B (WT). To simulate the WT, we used the default model
622 parameters. To simulate the mutant, we decreased the reverse rate constant that defines
623 K_B , so that K_B (R92Q) = 2.56 * K_B (WT). To simulate the force per sarcomere in response
624 to a calcium transient for the WT, we used the default calcium transient. To simulate the
625 response of R92Q, we changed K_B as described above and we reduced the amplitude of
626 the default calcium transient to 67% of its value to match our measurements in hiPSC-
627 CMs.

628

629 **Stem cell line derivation**

630 R92Q stem cells were derived and the quality control was performed using procedures
631 described in depth previously (14). Briefly, the parent human BJ fibroblast stem cell line
632 (BJFF.6, ATCC) was reprogrammed to stem cells by the Genome Engineering and iPSC
633 Center (GEiC) at Washington University in St. Louis. Two independent isogenic stem cell
634 lines with the R92Q hTNNT2 point mutation were also generated at the GEiC using the
635 CRISPR/Cas9 system (67). The oligo used to generate the gRNA was
636 CCTTCTCCATGCGCTTCCGGNGG and the mutation was introduced by homology
637 directed repair. This gRNA was selected to minimize off target effects. The presence of
638 the homozygous mutation was verified by sequencing. Karyotype (G-banding) analysis
639 was performed by Cell Line Genetics (Supplementary Fig. S1). Mycoplasma testing and
640 immunofluorescence staining for pluripotency markers were performed by the GEiC.

641

642 **Stem cell and hiPSC-CMs culture**

643 Stem cell culture and differentiation to hiPSC-CMs were done as previously described
644 (14). Briefly, stem cells were maintained in feeder-free culture. To differentiate the stem
645 cells to hiPSC-CMs, we used small-molecule manipulation of WNT signaling (15, 16).
646 hiPSC-CMs were enriched using metabolic selection (68). All functional experiments were
647 conducted at least 30 days after the initiation of differentiation. Experiments were
648 conducted using 2 independently derived cell lines for the R92Q mutant. All experiments
649 were repeated using at least two independent differentiations.

650

651 **Microcontact patterning of hiPSC-CMs on glass and hydrogels**

652 Fabrication of rectangular (7:1 aspect ratio) PDMS stamps for micropatterning of hiPSC-
653 CMs on both glass and 10 kPa hydrogels was done as previously described (14, 17).
654 Cells were patterned onto 10 kPa polyacrylamide hydrogels containing stamped Geltrex
655 (Thermo Fisher) in rectangular patterns as in (14).

656

657 **Traction force microscopy**

658 Traction force microscopy was conducted on 10 kPa hydrogels as previously described
659 (14) and analyzed using the computational tool developed in (18). Data were analyzed
660 and 95% confidence intervals of the mean were calculated as described previously (14,
661 33).

662

663 **Measurement of calcium transients in live cells**

664 Live-cell imaging was conducted using the ratiometric fluorescent calcium indicator dye
665 Fura Red AM (Thermo Fisher). The use of a ratiometric dye is important since the
666 mutation could affect the uptake of dye into the cells, and the ratiometric dye normalizes
667 the calcium-induced changes in fluorescence to the total amount of dye taken up by the
668 cell. hiPSC-CMs were patterned on hydrogels as described above. After 5-7 days on the
669 patterns, the cells were loaded with 10 μ M Fura Red AM dye and 0.01% Pluronic F-127
670 (Invitrogen/ThermoFisher) in RPMI-B27 with insulin media for 20 min at room
671 temperature. The cells were washed twice and incubated with Tyrode's solution (1.8 mM
672 CaCl_2 , 135 mM NaCl, 4 mM KCl, 1 mM MgCl_2 , 5 mM glucose and 10 mM HEPES, pH 7)
673 for 15-20 minutes at 37°C to allow de-esterification of the dye. Calcium transients were
674 recorded with a Nikon A1Rsi confocal microscope in line scan mode using a 40X objective
675 and the Ex2Em1 microscope setting. Fura Red AM loaded cells were excited at both 405
676 nm and 488 nm, and the emission fluorescence signal was collected at 595nm. Line scans
677 were acquired at a sampling rate of 512 pixels x 1.9 ms per line (total 10,000 lines per
678 recording). Each cell was recorded along with a line scan of the background fluorescence
679 outside the cell area.

680

681 **Analysis of calcium transients**

682 The calcium transient fluorescence counts were converted to ratios using Fiji software
683 (64). The averaged background fluorescence was subtracted from each recording and
684 a montage was created from the image stacks. The ratio of fluorescence at 405 nm /
685 488 nm was then calculated in Excel. The resulting ratiometric calcium fluorescence
686 traces were then analyzed using a custom MATLAB script to calculate the amplitude of

687 the calcium transient. Traces with fewer than 3 peaks were not analyzed. Briefly, the
688 data were smoothed over a 100-point sliding window using a Savitsky-Golay filter. The
689 locations of peaks and minima in the fluorescence signal were determined using a
690 peak-finding algorithm. Statistical significance was tested using a 2-tailed Student's t-
691 test.

692

693 **Measurement of the expression of transcripts encoding key calcium-handling**
694 **proteins**

695 hiPSC-CMs were grown on Matrigel-coated (Corning) 10 kPa PrimeCoat elastic substrate
696 culture dishes (35 mm) (ExCellness Biotech SA, Lausanne, Switzerland) for 10 days.
697 Total RNA was isolated using RNeasy Mini Kit (Qiagen) with on-column DNase I
698 treatment according to the manufacturer's instructions. cDNA was generated using iScript
699 Reverse Transcription Supermix (Biorad) according to the manufacturer's instructions.
700 qPCR reactions were performed in triplicate using iTaq Universal SYBRGreen Supermix
701 (Biorad) and using the ViiA 7 System (Applied Biosystems). Primers for all genes were
702 obtained from IDT PrimeTime qPCR Primers. Primer product numbers from IDT are listed
703 in Supplementary Table S1. Three separate biological samples were evaluated for both
704 WT and R92Q homozygous hiPSC-CMs. The relative levels of mRNA were calculated
705 using the comparative threshold cycle (Δ Ct) method (69). GAPDH and HPRT1 were used
706 as endogenous controls, and Rox dye present in the master mix was used to normalize
707 background fluorescence. Δ Ct values are plotted in Supplementary Table S2. The
708 statistical significance of differences in Δ Ct values was evaluated using a two-tailed
709 Student t-test.

710

711 **Cellular electrophysiological studies**

712 Whole-cell current- and voltage-clamp recordings were obtained at room temperature
713 (22~24°C) from hiPSC-CMs plated on hydrogel-coated coverslips using a Dagan 3900A
714 (Dagan Corporation) amplifier interfaced to a Digidata 1332A A/D converter (Axon) and
715 the pClamp 10.3 software (Axon). For current-clamp recordings, recording pipettes
716 contained 135 mM KCl, 5 mM K₂ATP, 10 mM EGTA, 10 mM HEPES and 5 mM glucose
717 (pH 7.2; 310 mOsm). The bath solution contained 136 mM NaCl, 4 mM KCl, 2 mM MgCl₂,
718 1 mM CaCl₂, 10 mM HEPES and 10 mM glucose (pH 7.4; 300 mOsm). For recordings of
719 voltage-gated Ca²⁺ currents (I_{Ca}), pipettes contained 5 mM NaCl, 90 mM Cs CH₃SO₃, 20
720 mM CsCl, 4 mM MgATP, 0.4 mM Tris-GTP, 10 mM EGTA, 10 mM HEPES and 3 mM
721 CaCl₂ (pH 7.2; 310 mOsm), and the bath solution contained 20 mM NaCl, 110 mM TEA-
722 Cl, 10 mM CsCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES and 10 mM glucose (pH 7.4;
723 300 mOsm). In all experiments, pipette resistances were 2-3 MΩ.

724 Electrophysiological data were acquired at 10 or 100 kHz, and signals were low pass
725 filtered at 5 kHz before digitization and storage. After the formation of a gigaohm seal
726 (>1GΩ) and establishment of the whole-cell configuration, brief (10 ms) ± 5 mV voltage
727 steps from a holding potential (HP) of -70 mV were presented to allow measurements of
728 whole-cell membrane capacitances (C_m), input resistances (R_{in}), and series resistances
729 (R_s). Mean ± SEM C_m values were 32 ± 2 pF and 47 ± 2 pF (*P* < 0.001) in WT (*n* = 96)
730 and R92Q (*n* = 67), hiPSC-CMs, respectively; mean ± SEM R_{in} values were 1665 ± 125
731 MΩ and 1551 ± 162 MΩ (*P* > 0.05) in WT (*n* = 96) and R92Q (*n* = 67), hiPSC-CMs,
732 respectively. Whole-cell C_m and R_s were electronically compensated by 85%. Voltage

733 errors resulting from the uncompensated R_s were always <2 mV and were not corrected.

734 Leak currents were always <50 pA and also were not corrected.

735 In current-clamp recordings, spontaneous action potentials were recorded on establishing
736 the whole-cell configuration. To record evoked action potentials, small ($-10 \sim -100$ pA)
737 current injections were made to hyperpolarize the membrane potential to -80 mV and to
738 stop spontaneous firing. Individual action potentials were then evoked by brief (4 ms)
739 depolarizing current (600 pA) injections. In voltage-clamp experiments, whole-cell I_{Ca} ,
740 evoked in response to depolarizing (300 ms) voltage steps to test potentials between -45
741 and $+15$ mV (in 5 mV increments at 1 s intervals) from a holding potential of -50 mV, were
742 recorded.

743

744 **Analysis of electrophysiological data**

745 Electrophysiological data were compiled and analyzed using Clampfit 10.3 (Axon) and
746 GraphPad (Prism). C_m values were determined by integration of the capacitive transients
747 recorded during ± 5 mV voltage steps from -70 mV. Current amplitudes in each cell were
748 normalized to the C_m and current densities are reported (pA/pF). All data are presented
749 as means \pm SEM. The statistical significance of observed differences between WT and
750 R92Q hiPSC-CMs was evaluated using two-tailed Student's t-test or two-way ANOVA; p-
751 values are presented in Supplementary Table S3.

752

753

754 **Figure legends**

755 **Figure 1. R92Q mutation in troponin T causes hypertrophic cardiomyopathy. (A)**

756 Cartoon of the troponin complex based on (70). R92Q is located in the region of troponin
757 T that interacts with tropomyosin, near the tropomyosin overlap region. (B) Models for the
758 molecular mechanism of R92Q.

759

760 **Figure 2. R92Q causes cellular hypercontractility in hiPSC-CMs.** Single hiPSC-CMs

761 were seeded on rectangular patterns on 10 kPa hydrogels for traction force microscopy.
762 Cumulative distributions reveal that R92Q hiPSC-CMs have a (A) greater total force, (B)
763 contraction speed, and (C) contraction power compared to the WT. Values from the
764 analysis, 95% confidence intervals, and p-values are listed in the table.

765

766 **Figure 3. R92Q hiPSC-CMs show altered calcium transients and gene expression.**

767 (A) Representative fluorescence traces showing calcium transients. Single hiPSC-CMs
768 were seeded on rectangular patterns on 10 kPa hydrogels and loaded with the ratiometric
769 calcium dye, Fura Red. R92Q hiPSC-CMs calcium transients have lower amplitudes than
770 the WT cells. (B) Expression of key calcium-handling genes measured using qPCR. Data
771 show significant increases in the expression of CASQ2, CAMK2D, and SLC8A1, and a
772 decrease in CACNA1H. Δ Ct values are shown in Supplementary Table S2. Statistics were
773 performed on the Δ Ct values; however, we show the log-fold change. Red lines show the
774 means, boxes show the quartiles, and error bars show the standard deviations. Data are
775 collected from 3 biological replicates, each of which contained 3 technical replicates. *
776 denotes Δ Ct values with $p < 0.05$ compared to the WT.

777

778 **Figure 4. Spontaneous and evoked action potentials are altered in R92Q hiPSC-**

779 **CMs and I_{Ca} densities are reduced.** (A) Representative whole-cell spontaneous action

780 potentials recorded from WT and R92Q hiPSC-CMs are illustrated; dotted black lines

781 indicate 0 mV. (B) Firing frequencies, maximum diastolic potentials (MDP), maximum

782 upstroke velocities (dV/dt_{max}) and action potential durations at 50% repolarization

783 (APD_{50}), measured in individual WT ($n = 58$) and R92Q ($n = 29$) hiPSC-CMs are plotted;

784 mean values are also indicated and are provided in Supplementary Table S3. (C)

785 Representative whole-cell action potential waveforms evoked from a hyperpolarized

786 membrane potential, as described in Material and Methods, in WT and R92Q cells are

787 shown; dotted black lines indicate 0 mV. (D) dV/dt_{max} and APD_{50} values measured in

788 individual WT ($n = 58$) and R92Q ($n = 29$) cells are plotted; mean values are also indicated

789 and are provided in Supplementary Table S3. (E) Representative voltage-gated Ca^{2+}

790 current (I_{Ca}) waveforms, elicited by voltage steps to test potentials between -40 and +15

791 mV (in 5 mV increments) from a holding potential (HP) of -50 mV, in WT and R92Q hiPSC-

792 CMs are shown. (F) Mean \pm SEM peak I_{Ca} densities in R92Q ($n = 12$) and WT ($n = 15$)

793 hiPSC-CMs are plotted as a function of the test potential.

794

795 **Figure 5. Molecular studies of R92Q demonstrate that R92Q does not change the**

796 **rate of unloaded actomyosin dissociation or calcium binding affinity to troponin C.**

797 (A) *In vitro* motility assays using cardiac myosin and reconstituted regulated thin

798 filaments. The speed of motility was measured as a function of calcium. R92Q shows a

799 shift towards submaximal calcium activation. Error bars are standard deviations from 3

800 separate experiments. (B) The rate of ADP release from myosin attached to regulated
801 thin filaments was measured using stopped-flow kinetics (fluorescence transients are
802 shown). Myosin bound to ADP and pyrene-labeled regulated thin filaments was rapidly
803 mixed with ATP and the fluorescence increases as myosin dissociates from the thin
804 filament. The rate of actomyosin dissociation was unchanged by the R92Q mutation. (C)
805 The affinity of calcium binding to the troponin complex. IAANS-labeled troponin C was
806 reconstituted into regulated thin filaments. Titrations with increasing calcium were
807 conducted, and there is no difference in calcium binding affinity between the WT and
808 R92Q. Error bars show the standard deviation of 5 experiments. Values derived from fits,
809 standard errors in the fits, and p-values are shown.

810

811 **Figure 6. R92Q alters the positioning of tropomyosin along the thin filament. (A)**
812 Kinetic scheme for thin filament activation. (B) Measurement of the equilibrium constant
813 K_B using stopped-flow kinetics methods (fluorescence transients are shown). Pyrene-
814 labeled regulated thin filaments were rapidly mixed with myosin at high (pCa 4) or low
815 (pCa 9) calcium, and the rate of myosin binding was measured by quenching of the
816 fluorescence. K_B is calculated as described in the Methods. The rate of myosin binding is
817 similar for the WT and R92Q at pCa 4, but much faster for R92Q at pCa 9, consistent with
818 destabilization of the blocked state. K_B for R92Q is significantly larger than the WT. (C)
819 Measurement of the parameters K_T and K_W using equilibrium titrations of myosin with
820 regulated thin filaments (see Methods). Fitting reveals no significant differences for R92Q
821 for either K_T or K_W compared to the WT. Error bars show the standard deviation of 5

822 experiments. The average value, 95% confidence intervals, and p-values are shown in
823 the table.

824

825 **Figure 7. Computational modeling reveals that altered tropomyosin positioning is**
826 **sufficient to explain the hypercontractility seen with R92Q.** (A) Using the
827 computational model developed by (34) and the measured equilibrium constants for thin
828 filament activation, the steady state force per sarcomere was calculated (see Materials
829 and Methods). Changing K_B alone is sufficient to reproduce the shift towards submaximal
830 calcium activation seen in the *in vitro* motility experiments (Figure 5A). (B) Using the same
831 model, the equilibrium constants measured *in vitro*, and the calcium transients measured
832 in hiPSC-CMs, the twitch force (solid line) in response to a calcium transient (dashed line)
833 was calculated. Consistent with our cellular measurements, the simulations demonstrate
834 that despite having a reduced calcium transient, R92Q produces a larger force in a twitch
835 due to changes in tropomyosin positioning.

836

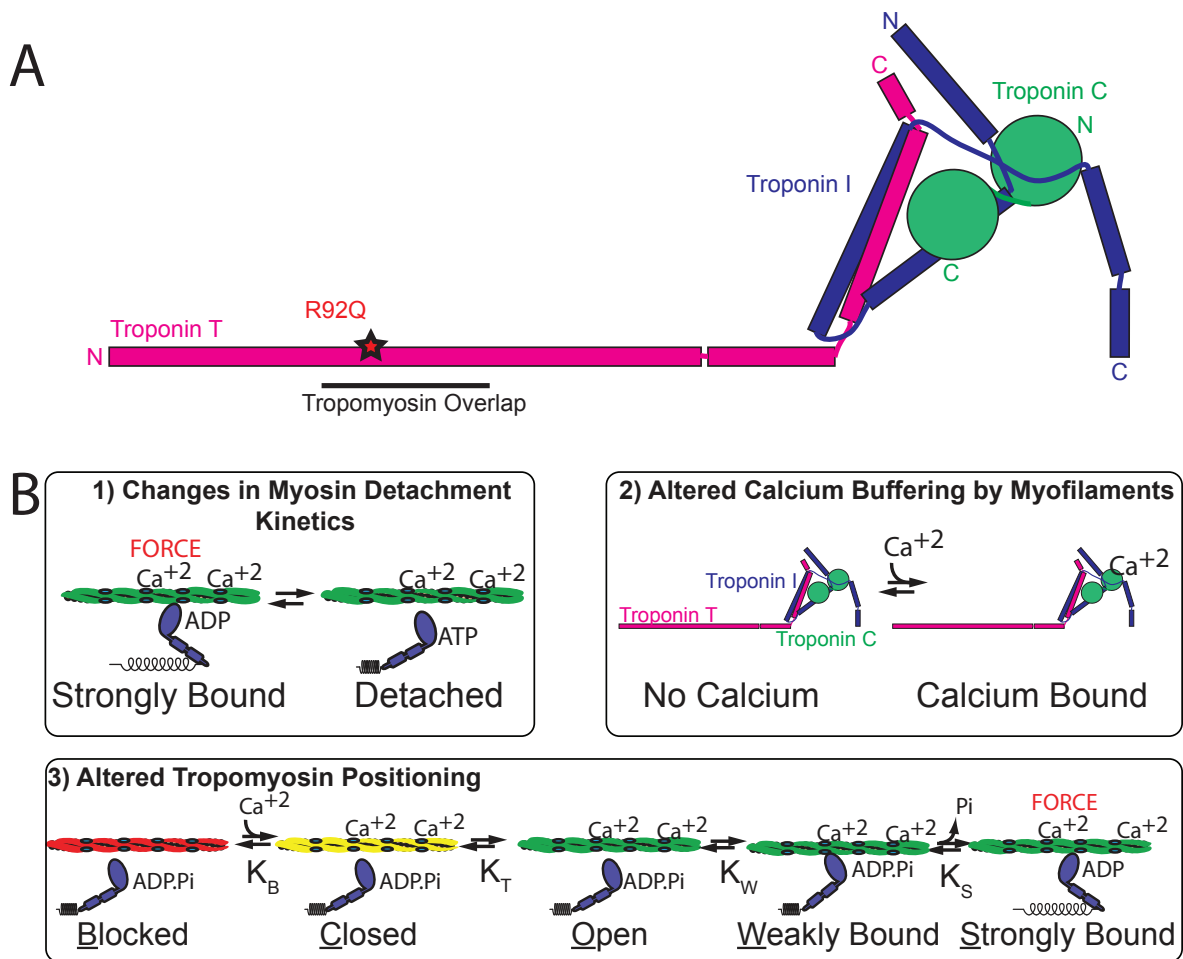
837 **Supplementary Figure S1. Generation of gene edited hiPSC-CMs.** (A) CRISPR/Cas9
838 targeting of R92Q in troponin T. The R92Q mutation was added via homology directed
839 repair. The guide RNA sequence for targeting was CCTTCTCCATGCGCTTCCGGNGG.
840 From our screen, 21% of the cells were homozygous for the R92Q mutation (CGG ->
841 CAA). (B) Karyotyping of R92Q gene edited cells reveals a normal karyotype.

842

843 **Supplementary Figure S2. Pluripotency staining of R92Q cells.** R92Q gene edited
844 cells are pluripotent as assessed by immunofluorescence staining for the markers
845 SSEA4, OCT4, SOX2, and TRA-1-60.

846

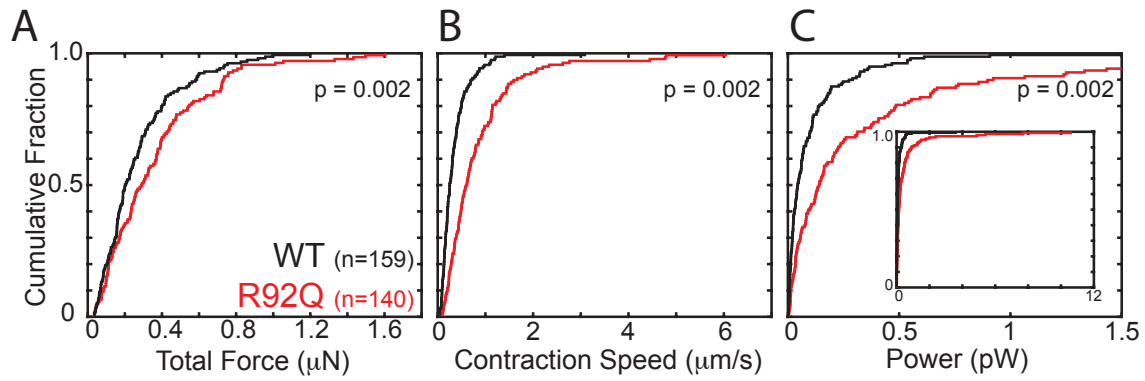
847 **Supplementary Figure S3. The affinity of calcium binding to the troponin complex**
848 **at 20°C.** IAANS-labeled troponin C was reconstituted into regulated thin filaments.
849 Titrations with increasing calcium were conducted. Error bars show the standard deviation
850 of 5 experiments. Values derived from fits, standard errors in the fits, and p-values are
851 shown.



852

853 **Figure 1**

854

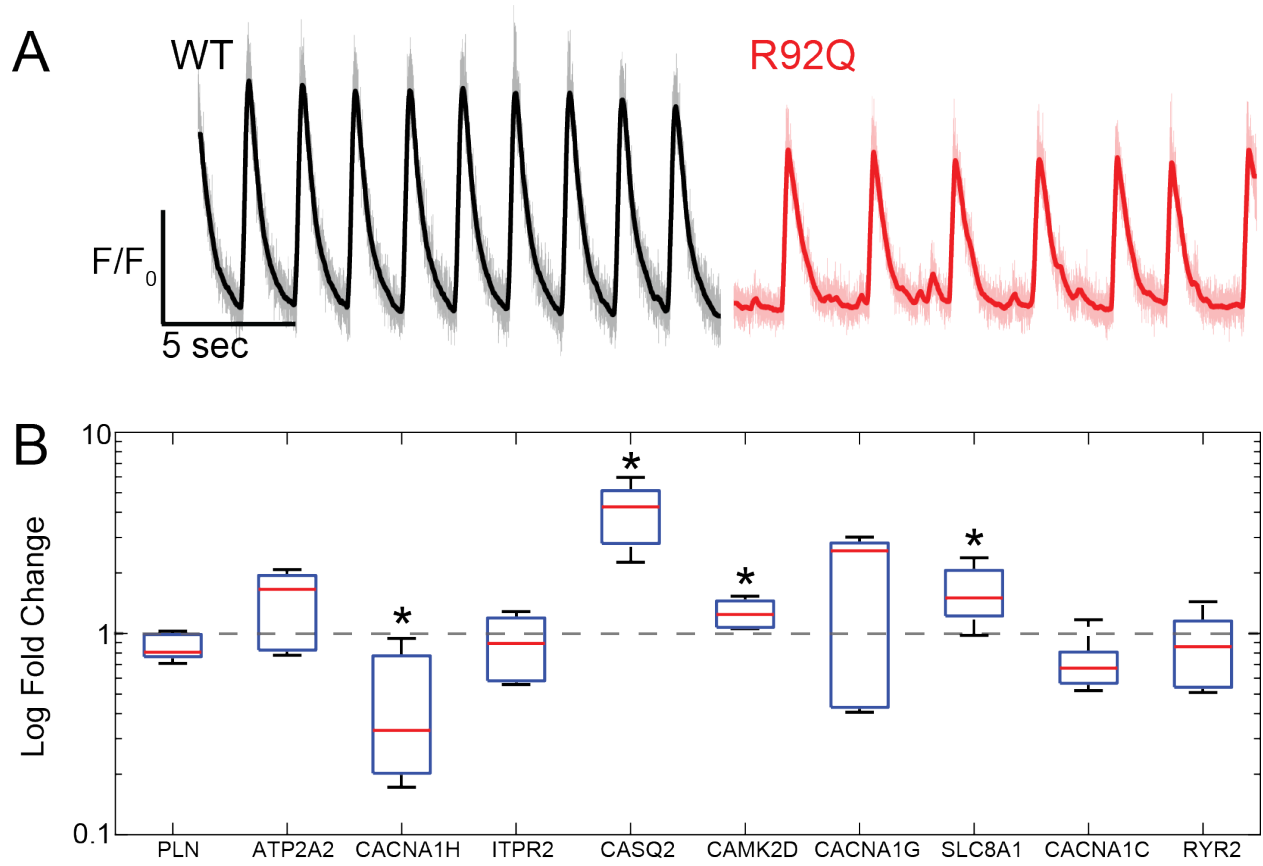


	WT (n=159)	R92Q (n=140)	p
Total force (μN)	0.27 (-0.03/+0.04)	0.36 (-0.04/+0.05)	0.002
Contraction speed (μm/s)	0.35 (-0.05/+0.06)	0.84 (-0.22/+0.23)	0.002
Contraction power (pW)	0.12 (-0.04/+0.06)	0.47 (-0.3/+0.3)	0.002

855

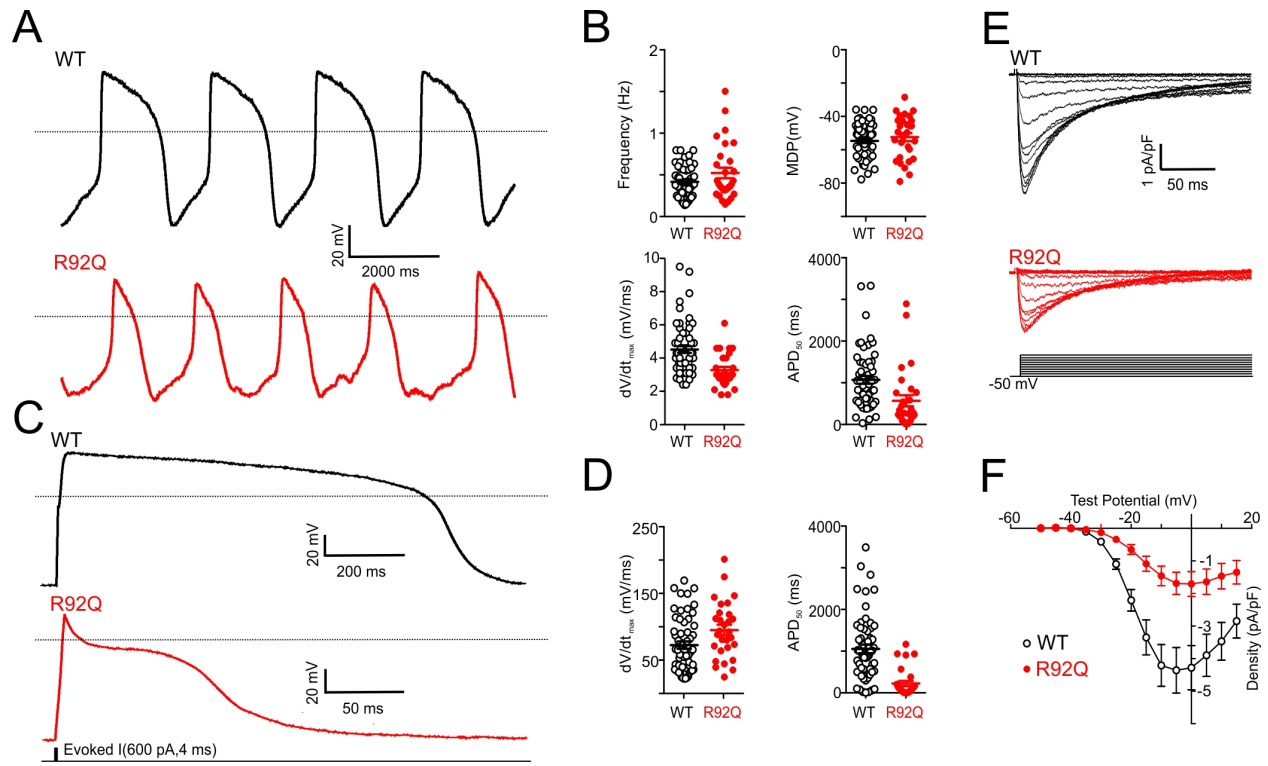
856 **Figure 2**

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858

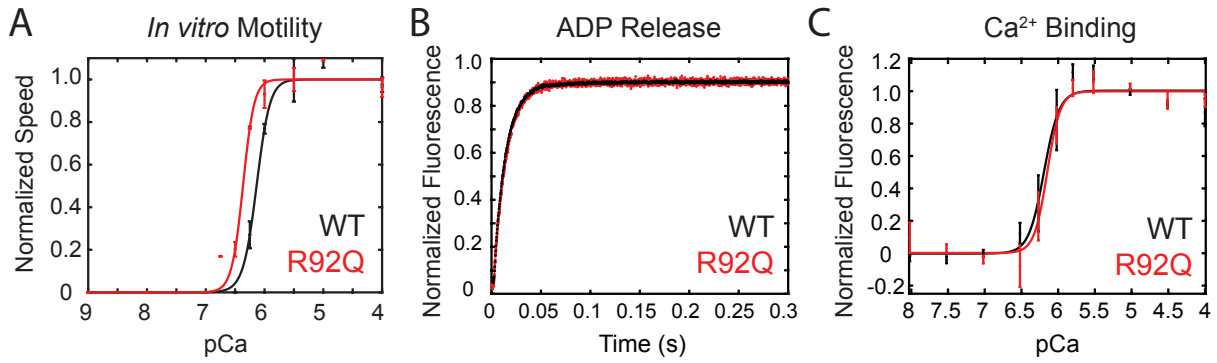
859 **Figure 3**



860

861 **Figure 4**

862

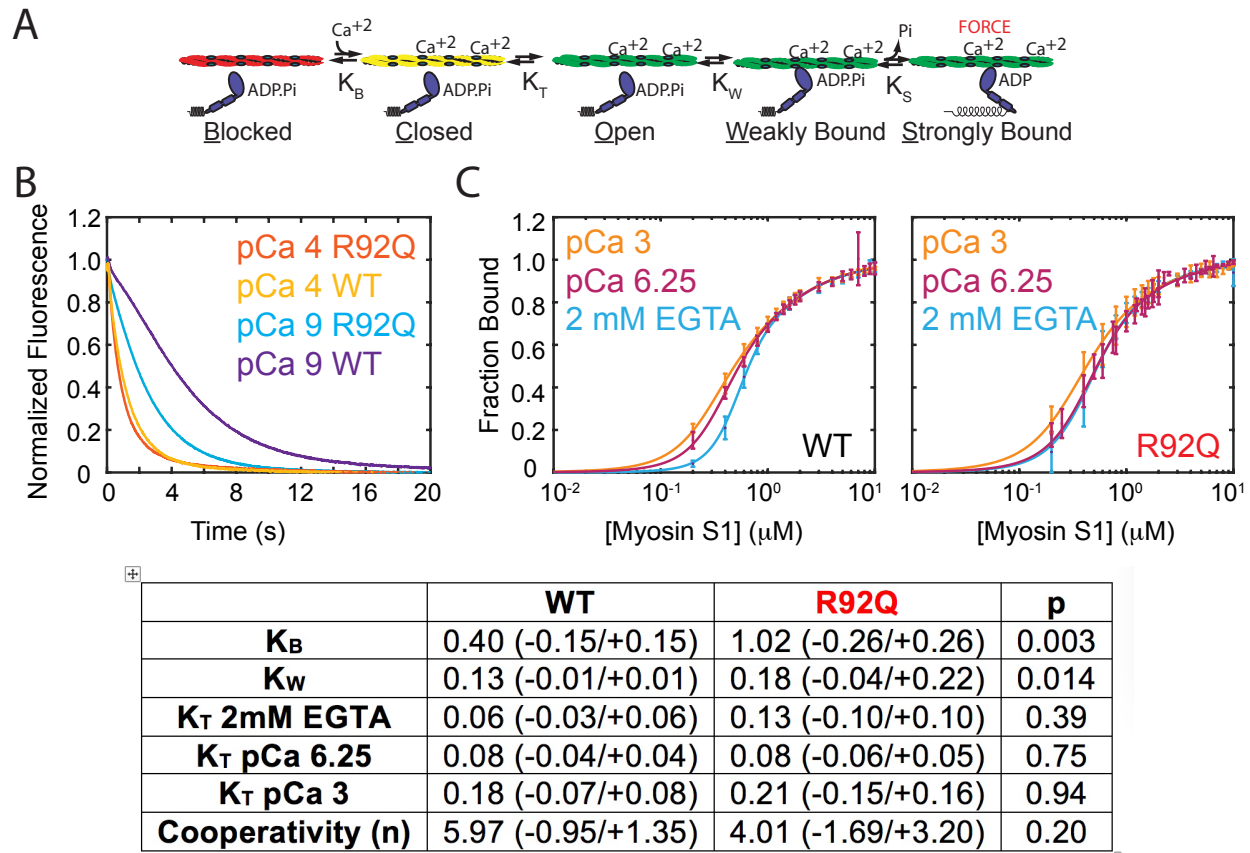


	WT	R92Q	p
Motility pCa50 (n=3)	6.12 (± 0.02)	6.37 (± 0.03)	<0.001
Motility Hill (n=3)	3.8 (± 0.6)	3.4 (± 0.7)	0.75
ADP release (s^{-1}) (n=4)	76.3 (± 5.0)	75.8 (± 3.9)	0.88
IAANs Ca50 (μM) (n=5)	0.66 (± 0.18)	0.67 (± 0.19)	0.93
IAANs Hill (n=5)	2.69 (± 0.40)	3.50 (± 0.39)	0.01

863

864 **Figure 5**

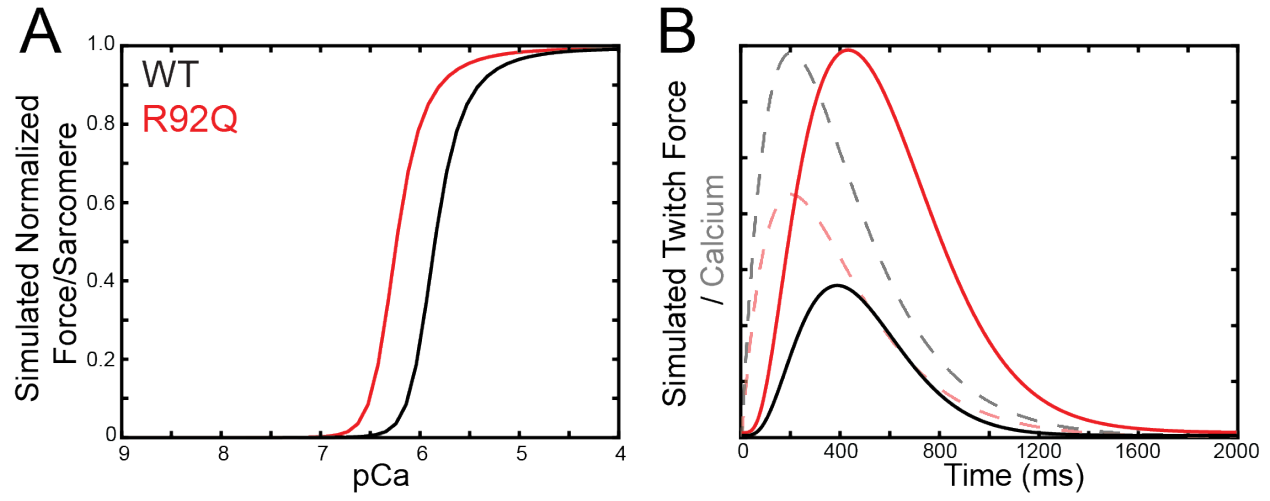
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866

867 **Figure 6**

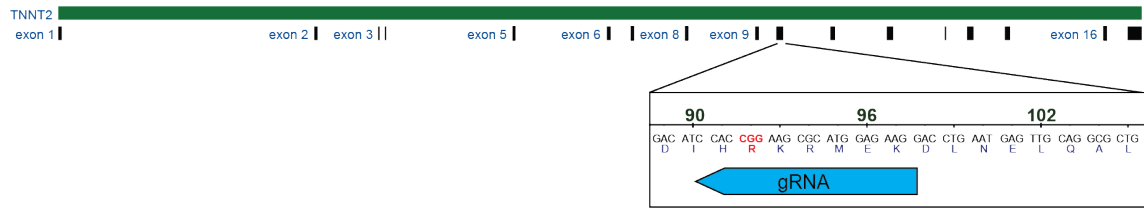
868



869

870 **Figure 7**

A



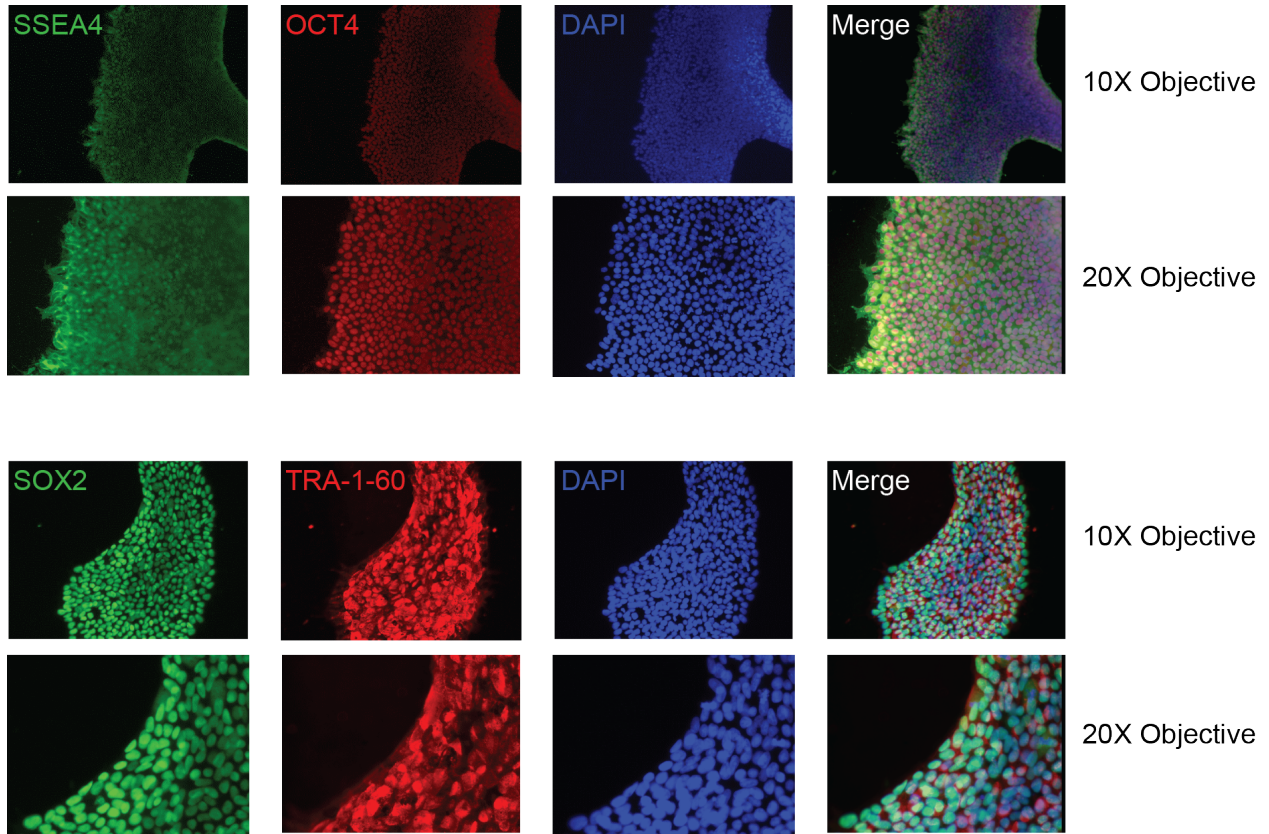
B



871

872 **Supplementary Figure S1**

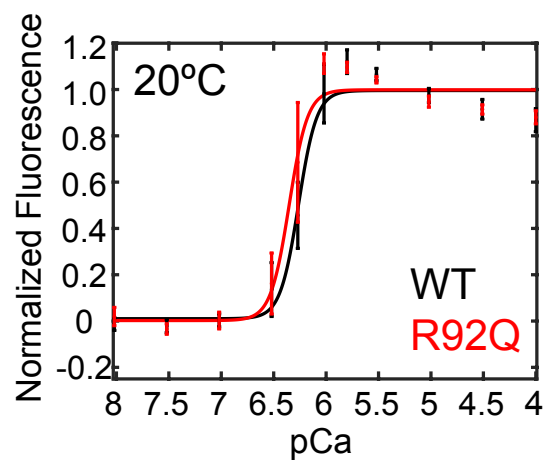
873



874

875 **Supplementary Figure S2**

876



	WT (20°C)	R92Q (20°C)	p
IAANs Ca50 (μM) (n=5)	0.55 (± 0.10)	0.49 (± 0.09)	0.36
IAANs Hill (n=5)	3.60 (± 1.16)	6.59 (± 2.20)	0.04

877

878 **Supplementary Figure S3.**

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880

Gene Symbol	Gene Name	Product Number
PLN	Phospholamban	Hs.PT.58.23189767
ITPR2	Inositol 1,4,5-trisphosphate receptor type 2	Hs.PT.58.3479603
RYR2	Ryanodine receptor 2	Hs.PT.58.502763
CACNA1C	Calcium voltage-gated channel subunit alpha1 C	Hs.PT.58.14979004
CACNA1G	Calcium voltage-gated channel subunit alpha1 G	Hs.PT.58.4441520
CACNA1H	Calcium voltage-gated channel subunit alpha1 H	Hs.PT.58.814570
CASQ2	Calsequestrin 2	Hs.PT.56a.219158
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca ²⁺ transporting 2	Hs.PT.56a.39859858.g
SLC8A1	Solute carrier family 8 member A1	Hs.PT.58.40534466
CAMK2D	Calcium/calmodulin dependent protein kinase II delta	Hs.PT.56a.25723872.g
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Hs.PT.58v.45621572
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs.PT.39a.22214836

881

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Supplementary Table S1. qPCR Gene Names and Primers

Gene	WT ΔCt	WT ΔCt S.D.	R92Q ΔCt	R92Q ΔCt S.D.	Fold Change	p-value
PLN	-4.83	0.27	-4.59	0.20	0.85	0.05
ATP2A2	-1.18	0.26	-1.65	0.60	1.39	0.05
CACNA1H	6.69	1.28	8.15	0.96	0.36	0.01
ITPR2	6.48	0.36	6.73	0.50	0.84	0.25
CASQ2	2.87	0.09	0.92	0.51	3.86	1.2E-06
CAMK2D	-0.10	0.26	-0.43	0.21	1.25	0.01
CACNA1G	8.86	1.36	8.32	1.35	1.46	0.41
SLC8A1	4.97	0.31	4.36	0.45	1.54	0.004
CACNA1C	1.91	0.67	2.45	0.37	0.69	0.06
RYR2	1.34	0.92	1.60	0.57	0.83	0.48

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Supplementary Table S2. qPCR Results

Action Potentials	Cells	dV/dt _{max} (mV/ms)	APD ₅₀ (ms)	Frequency (Hz)	MDP (mV)
Spontaneous	WT	4.5 ± 0.2	1076 ± 93	0.41 ± 0.02	-55 ± 1
	R92Q	3.3 ± 0.2*	571 ± 134 [‡]	0.52 ± 0.06 ⁺	-50 ± 2 ⁺
Evoked	WT	73 ± 5	1053 ± 103	na	na
	R92Q	95 ± 8 ⁺	230 ± 61 [*]	na	na

886 Values are means ± standard error of the means determined in WT (n = 58) and R92Q (n = 29)
887 cells, where n = number of cells; dV/dt_{max} = maximal rate of change of the membrane voltage during
888 the rising phase of the action potential; APD₅₀ = action potential duration at 50% repolarization;
889 MDP= maximum diastolic potential; na = not applicable. ⁺, [‡], ^{*}Values in R92Q mutant cells are
890 significantly different from those in WT cells at the ^{*}P < 0.05, [‡]P < 0.01 and ^{*}P < 0.001 levels.

891

892

893 **Supplementary Table S3. Electrophysiology Results**

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