

1 **Title**

2 AMPK regulates cell shape of cardiomyocytes by modulating turnover of microtubules
3 through CLIP-170

4

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35 **Running title**

36 AMPK regulates shape of cardiomyocytes

37 **Summary**

38 AMP-activated protein kinase (AMPK) is a multifunctional kinase that regulates microtubule
39 (MT) dynamic instability through CLIP-170 phosphorylation; however, its physiological
40 relevance in vivo remains to be elucidated. In this study, we identified an active form of
41 AMPK localized at the intercalated discs in the heart, a specific cell-cell junction present
42 between cardiomyocytes. A contractile inhibitor, MYK-461, prevented the localization of
43 AMPK at the intercalated discs, and the effect was reversed by the removal of MYK-461,
44 suggesting that the localization of AMPK is regulated by mechanical stress. Time-lapse
45 imaging analysis revealed that the inhibition of CLIP-170 Ser-311 phosphorylation by AMPK
46 leads to the accumulation of MTs at the intercalated discs. Interestingly, MYK-461 increased
47 the individual cell area of cardiomyocytes in CLIP-170 phosphorylation-dependent manner.
48 Moreover, heart-specific CLIP-170 S311A transgenic mice demonstrated elongation of
49 cardiomyocytes along with accumulated MTs, leading to progressive decline in cardiac
50 contraction. In conclusion, these findings suggest that AMPK regulates the cell shape and
51 aspect ratio of cardiomyocytes by modulating the turnover of MTs through homeostatic
52 phosphorylation of CLIP-170 at the intercalated discs.

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56 **Keywords**

57 AMPK, microtubule, CLIP-170, intercalated disc

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63 Introduction

64 AMP-activated protein kinase (AMPK) can sense the increase of intracellular AMP or ADP
65 concentration, and is fully activated by the phosphorylation of conserved Thr residue in the
66 activation loop by upstream kinases, including LKB1 or Ca²⁺/calmodulin-activated protein
67 kinase kinases, CaMKK2 [1]. Canonical stimulation known to activate AMPK is energetic
68 stress, and this explains why AMPK switches on downstream signaling pathways involved in
69 ATP production while switching off the anabolic pathways. However, AMPK can be activated
70 by various stimuli other than energetic stresses including Ca²⁺ increase, oxidative stress, or
71 genotoxic stress. The downstream effects of AMPK are not just restricted to the regulation of
72 metabolism. It has also been demonstrated that AMPK is a multifunctional kinase known to
73 regulate cell cycle, polarity, membrane excitability, and a variety of cellular functions by
74 phosphorylating specific sets of substrates, presumably in a spatiotemporal manner [1–3].

75 As such, we previously demonstrated that AMPK controls directional cell migration by
76 modulating microtubule (MT) dynamic instability through direct phosphorylation at CLIP-170
77 Ser-311 in Vero cells [4]. MTs are noncovalent polymers comprised of tubulin heterodimers,
78 and one of the major constituents of cytoskeleton. Although the term of cytoskeleton
79 suggests static structure, MTs are in fact highly dynamic, especially the plus end of MTs,
80 which exhibits a behavior called as dynamic instability; individual MT ends fluctuate between
81 polymerization and depolymerization phase [5]. CLIP-170 is one of the MT plus end tracking
82 proteins (+TIPs), and its role is to bind MT plus end in order to protect them from
83 depolymerizing factors, thereby MT polymerization is accelerated. Conversely, MT
84 depolymerization is promoted when CLIP-170 departs from plus end [6,7]. This dissociation
85 of CLIP-170 has been shown to be regulated by AMPK-mediated phosphorylation of
86 CLIP-170 Ser 311 residue [8,9]. In migrating Vero cells, the inhibition of AMPK or the
87 expression of CLIP-170 Ser 311-to-Ala mutant (CLIP-170 S311A), which is a
88 non-phosphorylatable mutant, leads to an increase in the amount of stable MTs and
89 disturbed cell polarity, thereby resulting in the impairment of free or directional cell migration
90 [4]. We originally found CLIP-170 as a novel substrate of AMPK from mouse heart
91 homogenates, the relevance of AMPK-CLIP-170 on MT dynamic instability in vivo, however,
92 remains to be elucidated.

93 One of the most important findings regarding cardiac MTs is that density of MTs increases
94 during end-stage heart failure regardless of their etiology [10,11]. In a mouse
95 pressure-overload heart failure model, the expression of MTs in the heart was shown to be
96 increased [12]. Interestingly, the treatment with MT depolymerizer, colchicine, reversed the
97 MT accumulation and improved cardiac function and survival rate [13]. Conversely,
98 anti-cancer drug, paclitaxel, which has been shown to stabilize MTs, was reported to induce

99 cardiac dysfunction as a side effect [14]. Moreover, increasing MT stability impairs
100 contraction and thus is associated with human heart failure [15]. These findings suggest that
101 effective reversal of cardiac MT stability will have therapeutic potential for the treatment of
102 heart failure [11,16]. However, upstream and downstream mechanisms of MT stabilization in
103 vivo heart are still not well understood.

104 As an integral component of cytoskeleton, cardiac MTs are also important for the
105 maintenance of cell shape, that is, aspect ratio (length/width ratio). Cardiomyocytes adapt to
106 elasticity of the extracellular matrix and modulate their aspect ratio in such manner that they
107 can maximize its systolic performance. When cardiomyocytes changes its aspect ratio on
108 stiff gels, MT polymerization increases, however, other cytoskeleton components are not
109 involved in changing the cell shape [17].

110 Cardiomyocytes exhibit unique property of cellular polarity through which they are
111 connected to the neighboring cardiomyocytes only at the short side of the cells, which is
112 referred to as the intercalated disc. The intercalated disc is a special form of cell-cell
113 junctions in cardiomyocytes, which consists of three types of cell-cell junctions, namely
114 adherence junctions, gap junctions, and desmosomes. They cooperatively reinforce the
115 synchronized cardiac contraction by producing of mechanical stability, transmission of
116 forces generated by myofibrils, and electrical coupling [18,19]. Furthermore, it has been
117 demonstrated that a transient receptor potential, vanilloid family type 2 (TRPV2) cation
118 channel localizes at the intercalated discs and serves as a mechanoreceptor to maintain
119 cardiac structure and function [20]. The structure of intercalated discs modulate in response
120 to hemodynamic stress; therefore, mechano-signaling has to be involved in such feedback
121 system [18,19]. Although the intercalated discs play an important role in cardiac
122 homeostasis and pathophysiology, the mechanism of their maintenance in cardiomyocytes
123 and details of mechano-signaling initiated from them are largely unknown.

124 In this study, we revealed that AMPK is localized at the intercalated disc in the heart,
125 where it regulates MT dynamics through CLIP-170 phosphorylation. The localization of
126 AMPK was regulated by mechanical stress. Inhibition of CLIP-170 phosphorylation resulted
127 in the accumulation of MTs and an increase in individual cell area. Our data also revealed
128 the important association between mechano-signaling and regulation of cell shape through
129 MT dynamics, which is regulated by AMPK at the intercalated discs.

130

131 **Results**

132 **AMPK and phosphorylated CLIP-170 are localized at the intercalated discs in murine** 133 **heart**

134 In order to gain an insight into the relevance of AMPK and CLIP-170 in the heart, we first

135 assessed and compared phosphorylation levels of AMPK and CLIP-170 in both heart and
136 liver in different developmental stages from embryonic day 15 to 8 weeks after the birth of
137 mouse (Fig. 1A and B). In the liver, the phosphorylation levels of AMPK and acetyl-CoA
138 carboxylase (ACC), which is a crucial substrate of AMPK known to be involved in the
139 regulation of metabolism, increased simultaneously with mouse development, implying an
140 important role of AMPK in the regulation of systemic metabolism. Conversely,
141 phosphorylation levels of CLIP-170 were not changed in the liver throughout the mouse
142 development (Fig. 1B). However, in contrast to the liver, phosphorylation levels of both
143 AMPK and CLIP-170 were significantly elevated at 8 weeks after birth in the heart.
144 Importantly, phosphorylation level of ACC in the heart did not correlate with the increase of
145 AMPK phosphorylation levels after the birth (Fig. 1A). These data suggest that AMPK in the
146 heart might have a distinct role other than metabolism, especially after the birth.

147 Next, we examined the localization of AMPK through immunohistostaining in an adult
148 murine heart. Surprisingly, majority of phosphorylated AMPK was localized at the
149 intercalated discs, which was confirmed through its co-localization with plakoglobin (Fig. 1C).
150 We also confirmed the localization of AMPK β 2 at the intercalated discs using a
151 subunit-specific antibody (Supplemental Fig. 1A). To eliminate non-specific signals in
152 immunohistostaining, we confirmed the localization of AMPK at the cell-cell junctions using
153 the following two methods. In the first method, mCherry-fused AMPK α 2, which was
154 expressed in cultured cardiomyocytes, was used to demonstrate AMPK localization at the
155 cell-cell junctions (Supplemental Fig. 1B). Next, we assessed the activity of AMPK at the
156 plasma membrane in cardiomyocytes by using previously described organelle-specific
157 AMPK activity probe, ABKAR [21]. Cardiomyocytes demonstrated significantly higher AMPK
158 activity at the cell-cell junctions compared to HeLa cells (Supplemental Fig. 1C and D).
159 Moreover, the majority of phosphorylated CLIP-170 (Fig. 1D) and LKB1 (Supplemental Fig.
160 1A), an upstream kinase of AMPK, were found to be localized at the intercalated discs. The
161 intercalated discs are absent in embryonic stages and are eventually formed 7 to 8 weeks
162 after the birth [18]. It is noteworthy that the levels of phosphorylation of AMPK and CLIP-170
163 assessed by Western blotting were upregulated following the same time course as that of
164 intercalated disc formation (Fig. 1B). Altogether, these findings indicate that AMPK localizes
165 at the intercalated discs in the heart, where it phosphorylates its potential substrate,
166 CLIP-170.

167

168 **The localization of AMPK is regulated by contraction of cardiomyocytes.**

169 In the heart, proper mechanical stress is essential for maintaining homeostasis,
170 development and cellular function. An individual cardiomyocyte always undergoes

171 mechanical stress due to spontaneous beating, and thus protracted decrease in mechanical
172 stress induces atrophy and cell death in the cardiomyocytes [18]. Cardiomyocytes, or cells
173 in general, possess a mechanical stress sensing system that detects stress or strain.
174 Although the precise molecular mechanism remains unclear, there are several cellular
175 components, including cell membrane and sarcomere-related, which are supposedly
176 involved in mechanical stress sensing [22]. The intercalated disc is one such component,
177 which has been shown to be critical for detecting the mechanical stress generated through
178 myocyte contraction [20].

179 Therefore, in order to examine whether contraction of cardiomyocytes influences the
180 activity of AMPK at the cell-cell junctions, we performed immunostaining with the
181 anti-phosphorylated AMPK α antibody, which is an indicator of activated AMPK, in rat
182 primary cardiomyocytes. After 2 hours of treatment with MYK-461, a myosin ATPase
183 inhibitor which suppress the contraction of cardiomyocytes, phosphorylated AMPK α signals
184 at the cell-cell junctions were found to be significantly reduced, although the levels of
185 N-cadherin, an adherens junction component, did not change. However, 4 hours after
186 removal of MYK-461, phosphorylated AMPK α reappeared at the cell-cell junctions along
187 with the re-initiation of cardiac beating (Fig 2A). The signal corresponding to a subunit of
188 holoenzyme, AMPK β 2, at the cell-cell junctions depleted within 2 hours of MYK-461
189 treatment, while the sarcomere-like signal remained unchanged. Similar to the
190 phosphorylated AMPK α , AMPK β 2 reappearance was observed upon removal of MYK-461
191 (Fig 2B). The localization of connexin43 or plakoglobin, both of which are the components of
192 the cell-cell junctions, was not changed upon MYK-461 treatment (Fig 2C). LKB1 also
193 localized at the cell-cell junctions, but did not disappear upon MYK-461 treatment (Fig 2D).

194 These data indicate that the localization of AMPK in cardiomyocytes is regulated in
195 response to the contraction or mechanical stress of cardiomyocytes.

196

197 **AMPK regulates MT plus end dynamics through CLIP-170 phosphorylation in** 198 **cardiomyocytes**

199 As we previously demonstrated that AMPK regulates MT dynamic instability in migrating
200 Vero cells [4], in this study, we assessed the MT dynamics focusing at the intercalated discs
201 in cardiomyocytes. After 3 days of primary culture of cells isolated from rat neonatal heart,
202 cardiomyocytes were found to be connected with the neighboring cardiomyocytes by
203 forming cell-cell junctions composing gap junction proteins, desmosomal proteins and
204 adherens junction proteins, which are the basic constituents of the intercalated disc.
205 However, free plasma membrane without intercellular connections do not possess these
206 junction proteins, suggesting that the heart-specific polarity was established in these primary

207 cultured cells, although they were not completely mature. First, we assessed the
208 intracellular dynamics of CLIP-170 by a time-lapse image analysis in rat neonatal
209 cardiomyocytes. In EGFP-CLIP-170 WT transfected cardiomyocytes, CLIP-170 migrated
210 from the cell's interior to the periphery with short comet at the plus end of MTs. In
211 cardiomyocytes with rectangular-like shape, the majority of CLIP-170 migrated longitudinally
212 towards the cell-cell junctions (Fig 3A and Movie S1). These results are distinct from the
213 previously published results that CLIP-170 radially moved from the microtubule organizing
214 center to the periphery in migrating cells [4]. After addition of Compound C, an AMPK
215 inhibitor, EGFP-CLIP-170 WT signals became elongated and formed lines, which
216 specifically accumulating at the cell-cell junctions (Fig 3A and Movie S1). To exclude the
217 possibility that the observed change was mediated by non-specific inhibition of other
218 kinases by Compound C, we examined EGFP-CLIP-170 WT dynamics in AMPK α 1 α 2
219 knockdown (KD) cardiomyocytes using short interfering RNAs (siRNAs). CLIP-170 comets
220 became elongated and accumulated at the cell-cell junctions in AMPK α 1 α 2 KD
221 cardiomyocytes (Fig 3C and D), as observed in Compound C treatment. Moreover, in
222 cardiomyocytes transfected with EGFP-CLIP-170 S311A, which is non-phosphorylatable
223 mutant [4], CLIP-170 also accumulated at the cell-cell junctions with elongated comet as
224 shown in Compound C treated cells, or in AMPK α 1 α 2 KD cells (Fig 3A and B,
225 Supplemental Fig. 2 and Movie S2).

226 These results suggest that MT turnover at the cell-cell junctions is regulated through
227 CLIP-170 Ser 311 phosphorylation by AMPK in cardiomyocytes.

228

229 **AMPK controls cell size and shape by regulating MT dynamics through CLIP-170** 230 **phosphorylation of in cardiomyocytes**

231 We noticed that a fraction of cardiomyocytes treated with MYK-461 became elongated in the
232 same direction as that of MT migration during the time-lapse image analysis (Fig. 4A).
233 These results directed us to measure the individual cell size of cardiomyocytes treated with
234 MYK-461. Analysis of the imaging data using IN Cell Analyzer revealed that MYK-461
235 treatment for 2 h significantly increased the cell area of cardiomyocytes (Fig. 4B and C). In
236 order to further elucidate the specific role of CLIP-170 Ser 311 phosphorylation by AMPK,
237 we compared the phenotypes of cardiomyocytes transiently transfected with CLIP-170 WT
238 and two of the Ser 311 mutants of CLIP-170, namely CLIP-170 S311A and CLIP-170 S311D
239 (a phosphomimetic mutant) [4]. The expression of CLIP-170 S311A led to the cell area
240 expansion at baseline, while CLIP-170 S311D mutant had no effect (Fig. 4C). Addition of
241 MYK-461 had no further effect in the cell size in CLIP-170 S311A-expressing
242 cardiomyocytes (Fig. 4C). Conversely, expression of CLIP-170 S311D mutant was refractory

243 to the action of MYK-461 (Fig. 4C). These data suggest that MT dynamics is critical for the
244 maintenance of cell size and shape of the beating cardiomyocytes, which is further
245 dependent on CLIP-170 phosphorylation.

246

247 **Inducible heart-specific *Clip-170* S311A overexpressing transgenic mice exhibit**
248 **cardiac dysfunction**

249 Thereafter, to investigate the physiological relevance of AMPK-CLIP-170 in vivo, we
250 generated tamoxifen inducible, cardiomyocyte-specific CLIP-170 S311A overexpressing
251 transgenic (TG) mice. In adult CLIP-170 S311A flox/+; MerCreMer+/- mice, we successfully
252 confirmed CLIP-170 S311A protein expression in cardiac muscle from these mice 2 weeks
253 after the initiation of tamoxifen administration. We established two lines of CLIP-170 S311A
254 TG mice (Supplemental Fig. 3), we present the data of line 3 hereafter. We found that they
255 were phenotypically similar. To check the influence of overexpression of CLIP-170 S311A in
256 cardiac function, we performed serial echocardiography measurements. Two weeks after
257 tamoxifen induction, CLIP-170S311A TG mice showed a mild, but significant decline in
258 fractional shortening, known as an indicator of cardiac function, compared to the control
259 mice. Over 1 year after tamoxifen administration, CLIP-170 S311A TG mice exacerbated
260 cardiac dysfunction (Fig. 5A). To further analyze both ventricles, we performed cardiac MRI
261 in these mice. Both left and right ventricle were found to be significantly dilated and with
262 reduced contraction significantly in CLIP-170 S311A TG mice (Fig. 5B and C). The
263 histological assessment of CLIP-170 S311A TG mice after 3 months of tamoxifen treatment
264 showed that there was no tissue degeneration, however the cardiac function was impaired.
265 However, CLIP-170 S311A TG mice over 1 year after tamoxifen treatment showed
266 significant tissue fibrosis compared to the control (Fig. 6A and B). Immunohistostaining
267 revealed enhanced accumulation of tubulin in CLIP-170 S311A TG mice (Fig. 6C). Next, we
268 checked the individual cell size in the heart of CLIP-170 S311A TG mice by wheat germ
269 agglutinin (WGA) staining, a lectin that stains cell membrane. CLIP-170 S311A TG mice
270 showed that the length of individual cells was significantly elongated in a long axis direction
271 compared to the control (Fig. 6D and E).

272 These data indicate that the physiological CLIP-170 phosphorylation by AMPK at the
273 cell-cell junctions is important for homeostatic MT dynamics, thereby maintaining the cell
274 shape and cardiac function of cardiomyocytes.

275

276

277 **Discussion**

278

279 In this study, we demonstrated that cardiac MTs are dynamic and undergo constant turnover
280 at the intercellular junctions, as revealed by time-lapse image analysis. Most of the AMPK in
281 beating cardiomyocytes is localized at the cell-cell junctions where it phosphorylates
282 CLIP-170, thereby regulating MT turnover. It is noteworthy that the myosin ATPase inhibitor,
283 MYK-461, prevents the localization of AMPK at the cell-cell junctions, and this effect is
284 reversed by the removal of MYK-461. The inhibition of CLIP-170 phosphorylation or
285 MYK-461 treatment, which alters subcellular localization of AMPK, results in an increase in
286 the cell area of cardiomyocytes. Moreover, the heart-specific CLIP-170 S311A
287 overexpressing TG mice showed the elongation of cardiomyocytes with accumulated MTs,
288 which ultimately resulted in a progressive decline in cardiac contractile function.

289 Cardiac MTs have drawn attention in cardiovascular research as a modulator of
290 intracellular stiffness. Accumulation of MTs has been associated with human heart failure
291 and rodent models of hypertrophy, myocardial ischemia-reperfusion, catecholamine-induced
292 myocardial injury, heart failure [11,12,15,16,23]. Recently, Prosser lab elegantly revealed
293 that tubulins with increased post-translational modification (PTM) confer mechanical
294 resistance to contraction and regulate the viscoelastic properties of myocyte [15].
295 Detyrosinated MTs are found to be elevated in human or rodent heart failure models,
296 suggesting that PTM of cardiac MTs has a relevance in the pathology of heart diseases,
297 however, the mechanisms that are responsible for the accumulation of tubulins or PTM in
298 heart diseases are not well understood.

299 Cardiac MTs have been recognized as static constituents of the cellular
300 cytoskeleton, especially in highly differentiated cells, including cardiomyocytes. Our findings
301 suggest that MTs in cardiomyocytes are rather dynamic and their turnover is regulated by
302 CLIP-170 phosphorylation, which is mediated by specially localized AMPK in the
303 intercalated discs. CLIP-170 is an MT plus end tracking protein (+TIPs), which is involved in
304 maintaining the equilibrium of MT dynamics towards extension rather than catastrophe.
305 Once CLIP-170 is phosphorylated by the upstream kinases, for instance, AMPK in this case,
306 its affinity to MT is decreased [8]. Therefore, it is reasonable that the affinity of CLIP-170 to
307 MTs is decreased upon phosphorylation at the intercalated discs, where MT polymerization
308 is supposed to terminate. We previously demonstrated that the inhibition of CLIP-170
309 phosphorylation by AMPK increases detyrosinated MTs, leading to accumulation of MTs [4].
310 In this study, CLIP-170 S311A TG mice showed decreased cardiac function upon
311 accumulation of MTs, which is often found in heart failure models. It is thus possible that the
312 perturbation of CLIP-170 phosphorylation or inhibition of AMPK at the intercalated discs in

313 cardiomyocytes is involved in the pathogenesis of heart diseases, which could be mediated
314 by an increase in detyrosinated MTs [16]. It has been shown that AMPK is activated in a
315 rodent and human heart failure samples [24,25], although another study showed decreased
316 activity in a rat spontaneous hypertensive model [26]. Considering the fact that we revealed
317 specific subcellular localization of AMPK at the intercalated discs in an adult murine heart, it
318 will be interesting to assess AMPK activity in each subcellular compartment using heart
319 failure models in future studies.

320 Inhibition of cardiac contraction by MYK-461 or inhibition of phosphorylation, as
321 shown by using the CLIP-170 S311A mutant, surprisingly led to increase in cell size. It is
322 noteworthy that cardiomyocytes in the CLIP-170 S311A TG mice became elongated and
323 showed an increase in the aspect ratio of individual cells. A previous report demonstrated
324 that cardiomyocytes adapt to the elasticity of extracellular matrix and modulate their cell
325 shape and length in order to maximize their systolic performance, and also that MT is a key
326 component of cardiomyocytes [17]. In other words, cardiomyocytes have an intrinsic
327 mechanism to adjust their cell shape or aspect ratio through change in MT polymerization.
328 Therefore, it is likely that perturbation in MT dynamics leads to failure in maintaining the
329 optimal aspect ratio of the cardiomyocytes, resulting in decreased contraction, which might
330 be the consequence of what we observed in the CLIP-170 S311A TG mice. Elongation of
331 cardiomyocytes is often observed in heart failure models or human end-stage heart failure
332 [17,27–29]. And it is considered a part of the vicious cycles in the pathogenesis of heart
333 failure. Thus, maladaptation of cardiomyocyte cell shape through altered MT dynamics
334 could be a domain that needs further consideration in future cardiovascular research. From
335 another view point, there are many reports suggesting that MTs play an important role as an
336 endogenous factor regulating the contractile force of cardiomyocytes through modulating
337 intracellular stiffness [15,16]. Therefore, in our CLIP-170 S311A TG mice, the accumulation
338 of MTs themselves may explain the reduced contractility observed in these mice.

339 Our results suggest that the activity of the AMPK, which is specifically localized at
340 the intercalated discs, does not correlate with energy metabolism (sensing AMP/ATP ratio),
341 but it does correlate with mechanical stress. Although the localization of AMPK in other
342 tissues have not been fully examined yet, it was reported that AMPK activity is involved in
343 cell-cell junctions in lung epithelium and alveolar development in response to repeated
344 respiration-induced physical stretching [30]. In fact, AMPK and its upstream LKB1 ortholog
345 have been found to exist in animals lower than mammalian order, however, there is no such
346 report that these enzymes are involved in energy metabolism [9]. In budding yeast, the
347 SNF1 complex corresponding to the AMPK ortholog has been shown to be activated upon
348 glucose-starved state, but is not allosterically activated by AMP [31]. The AMP/ADP-sensing

349 property of AMPK is considered to have been acquired, at least in mammals [32],
350 suggesting that the ancestral regulation and/or function of AMPK might be different.
351 Certainly, it has been reported that fructose-1,6-diphosphate and aldolase mediate glucose
352 sensing by AMPK localized in lysosomes occurs without being activated by AMP/ADP [33].
353 It is possible that the mechanosensing property of AMPK is an evolutionary descendant of
354 the ancestral AMPK, and it may be found in other tissues in mammals or other organisms.

355 In this study, we demonstrated that AMPK localization is dynamically regulated by
356 the beating of cardiomyocytes, suggesting the involvement of mechanical stress. LKB1 is
357 one of the upstream kinases of AMPK, which is constitutive active. Interestingly, we found
358 that LKB1 was co-localized at the cell-cell junctions along with AMPK; however, MYK-461
359 treatment did not influence its localization (Fig. 2D). Therefore, it seems subcellular
360 localization of AMPK is crucial for its mechanical signaling sensing. AMPK is a
361 multifunctional kinase with multiple substrates and cellular outcomes [21]. Thus, it is
362 conceivable that specific subcellular localization enables AMPK to play distinct roles in
363 different cell types. AMPK has been shown to be activated by mechanical stretch in skeletal
364 muscle cells [34], or in the lung epithelial cells [35]. Additionally, LKB1 has been shown to be
365 recruited to the cadherin adhesion complex in response to force and thereby activates
366 AMPK in epithelial cells [36,37]. From these reports and our findings, it is possible that
367 AMPK is involved in mechanotransduction; however, LKB1-AMPK regulation in response to
368 mechanical stress could depend on types of cells or stimulation. We were not able to reveal
369 the molecular mechanism involved in the translocation of AMPK in response to mechanical
370 signaling in cardiomyocytes, and thus further investigation is required to be performed in
371 future studies.

372 The number of patients with chronic heart failure has increased globally, and there
373 is an urgent need to develop effective treatment strategies for heart failure with novel
374 mechanisms of action. Previous studies have provided strong evidences that cardiac MTs
375 play crucial role in the pathogenesis of heart failure [16]. Thus, it is extremely important to
376 understand the molecular mechanism that regulates MT dynamics in the physiological and
377 pathological heart conditions so that the molecular targets can be identified to establish
378 effective treatment strategies.

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380
381

382 **Methods**

383

384 Plasmids and viral constructs

385 To create adenoviral vectors expressing full-length mouse CLIP-170 WT/S311A/S311D
386 fused with enhanced green fluorescent protein (EGFP) [4,38], the corresponding cDNAs
387 were subcloned into pENTR for further Gateway recombination in adenoviral expression
388 plasmids, pAdCMV/V5/DEST (Invitrogen). Recombinant adenoviral vectors were produced
389 and purified using HEK293A cells according to manufacturer's protocol (ViraPower
390 Adenoviral Expression System; Invitrogen, AdenoPACK 20, Vivapure; Sartorius AG).

391

392 Generation of rabbit polyclonal antibodies specific for the phosphorylated S311 of CLIP-170.

393 A phospho-specific polyclonal antibody to CLIP-170 (Ser 311) was generated by Scrum Inc.
394 as follows. Ser-phosphorylated or non-phosphorylated peptides surrounding S311 (amino
395 acids 305–316, SLKRSP(pS)ASSLS) was synthesized. Rabbits were immunized 5 times
396 with the keyhole limpet hemocyanin–phosphopeptide conjugates mixed with Freund's
397 complete adjuvant, and bled 7 days after the last immunization. Phosphopeptide-reactive
398 antibody was captured and eluted by a column containing phosphopeptide-conjugated
399 sepharose. Then, non-specific fraction was removed using a column containing
400 non-phosphorylated peptides. Specific reactivity with the targeted phosphoserine sequence
401 was confirmed by an ELISA in which phosphorylated or non-phosphorylated peptides were
402 coated.

403

404 Cell culture, plasmid transfection, and siRNA

405 Primary cultures of neonatal cardiomyocytes were prepared from 1- to 3- day-old Wistar
406 rats as described previously [39,40]. Briefly, harvested hearts were incubated in 0.25%
407 trypsin/EDTA (Sigma) at 4°C overnight and then digested with collagenase type II
408 (Worthington). The cardiomyocyte fraction was collected after differential plating for 70 min
409 at 37°C, counted, and seeded onto plates or collagen-coated glass-bottom dishes.
410 Cardiomyocytes were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS
411 (Sigma-Aldrich), penicillin and streptomycin (Gibco) at 37°C in a 5% CO₂ atmosphere at
412 constant humidity.

413 For the time-lapse imaging, cardiomyocytes seeded on collagen-coated 35-mm glass
414 dishes were transfected with adenovirus expressing EGFP-CLIP-170 WT/S311A/S311D at
415 48 hours after isolation and observed at 24 hours after transfection.

416 To knockdown AMPK α 1 and α 2, cardiomyocytes were transfected with siRNAs (Silencer®
417 Select siRNA; AMPK α 1 siRNA ID : s134808 (30 nM), AMPK α 2 siRNA ID : s134962 (10 nM),

418 Thermo Fisher Scientific) using lipofectamine RNAi MAX (Invitrogen) at 3 hours after
419 isolation.

420 As a negative control, cells were transfected with siControl Non-Targeting siRNA#1
421 (B-bridge).

422 Isolation of mRNA and protein experiments were performed at 72 hours after transfection.
423 For immunostaining, the same procedures of siRNA transfection were performed in one-fifth
424 scale on Lab-Tek Chamber Slides (nunc).

425

426 Immunoblotting

427 Protein concentration was determined using the BCA protein assay kit (Thermo Fisher
428 Scientific). Equal amounts was fractionated by SDS-PAGE, transferred to a PVDF
429 membrane by electroblotting, and processed for Immunoblotting, as described elsewhere
430 [4]. Blots were probed with the appropriate specific antibodies (Anti-AMPK α , 1:1000;
431 Anti-pAMPK, 1:1000; Anti-pACC, 1:1000; Anti-CLIP170, 1:1000; Anti-pCLIP170, 1:1000;
432 Anti- α -Tubulin, 1:5000 dilution), followed by secondary antibodies, and developed by ECL
433 chemiluminescence (GE Healthcare).

434

435 Immunostaining and immunohistology

436 Cardiomyocytes were seeded on a collagen-coated 35-mm glass dishes (Iwaki). After cells
437 firmly attached to the dish, they were washed once with warm PBS and fixed with methanol
438 for 15 min at 20°C. Next, the cells were permeabilized with 0.1% Triton X-100 in PBS for
439 5 min at room temperature and then blocked with 1% BSA and 5% goat serum for 15 min at
440 room temperature. Samples were immunostained with primary antibodies (1:200 in 1% BSA
441 and 5% goat serum, overnight). The next day, for secondary reactions, species-matched
442 Alexa Fluor 488- or Alexa Fluor 568- or Alexa Fluor 647-labelled secondary antibody was
443 used (1:400 in 1% BSA, 30 min). Fluorescence images of EGFP, Alexa Fluor 488, Alexa
444 Fluor 546, Alexa Fluor 568 and Alexa Fluor 647 were recorded using an Olympus FV1000-D
445 confocal laser scanning microscope (Olympus Corporation) equipped with a cooled
446 charge-coupled device CoolSNAP-HQ camera (Roper Scientific, Tucson, AZ, USA) and a
447 PLAPO x60 oil-immersion objective lens.

448 The mouse heart was perfused with ice-cold PBS, then removed, cut and embedded in
449 O.C.T. compound and frozen in isopentane chilled in liquid nitrogen. The frozen tissue
450 sections (7-10 μ m thick) were fixed with MeOH at -20°C for 10 min. After permeabilization
451 with PBS containing 0.1% of TritonX100 for 5 minutes at the room temperature, and the
452 non-specific antibody- binding sites were pre-blocked with the blocking buffer (PBS plus 5%
453 of goat serum and 1% of bovine serum albumin). The primary antibodies were applied

454 overnight at 4°C. After rinsing 3 times for 5 minutes in PBS, the sections were next
455 incubated with appropriate fluorophore-conjugated secondary antibodies and
456 4',6-diamidino-2-phenylindole (DAPI) in the blocking buffer for 30 min at room temperature.
457 The primary antibodies used in this study are as follows;
458

Primary antibodies	Host	dilution	
phosphorylated AMPK α	rabbit	1/100	Cell signaling
Connexin43	rabbit	1/200	abcam
Plakoglobin	guinea pig	1/400	PROGEN
AMPK β 2	rabbit	1/100	HPA
N-cadherin	mouse	1/200	Santa Cruz
α -actinin	mouse	1/200	abcam
AMPK α	rabbit	1/500	Cell Signaling
CLIP-170	mouse	1/500	HPA
α -Tubulin	rabbit	1/1000	Cell Signaling
phosphorylated CLIP	rabbit	1/500	Custom made
phosphorylated ACC	rabbit	1/1000	Cell Signaling
LKB1	rabbit	1/200	Cell signaling

459

460 Time-lapse imaging and tracking

461 The time-lapse imaging was performed as described previously [4]. Briefly, fluorescence
462 images were recorded by using the same experimental equipment and software described
463 in the section above. An EGFP image was obtained every second through a U-MNIBA2 filter
464 (Olympus), which had a 470–495 excitation filter and a 510–550 emission filter. To achieve
465 high temporal resolution, we had to limit the exposure time to 200 msec. CLIP-170 kinetics
466 were analyzed on 16-bit depth images after subtraction of the external background. We
467 measured the fluorescence intensity values within the line of 1 pixel in width along the
468 CLIP-170–EGFP tracks over time. We determined the beginning of a comet as the point at
469 which fluorescence intensity showed a rapid rise and the end of a comet as the point at
470 which fluorescence intensity reached baseline. MetaMorph was used to convert a series of
471 time-lapse images to video format and obtain tracking images in this analysis.

472

473 The quantitative evaluation of surface area of cardiomyocytes

474 We performed the quantitative evaluation of surface area of cardiomyocytes by using IN
475 Cell Analyzer (GE healthcare). α Actinin positive cardiomyocytes with nuclei (Hoechst) were
476 selected and cell bodies were decided on the basis of intensity. The mean cell surface area

477 of each cardiomyocyte was calculated as a numerical value.

478

479 Animals

480 All animal experiments were approved by the Animal Research Committee of Osaka
481 University, and were performed in accordance with institutional guidelines.

482

483 Generation of cardiomyocytes specifically CLIP-170 S311A-overexpressing mice

484 CLIP-170 S311A was subcloned into CAG-loxp-CAT-loxp vector, and transgenic strain by
485 pronuclear injection in mouse zygotes. Mice bearing the CLIP-170 S311A flox/+ allele were
486 crossed with a transgenic line expressing Cre recombinase under the control of the
487 α -myosin heavy chain promoter (MerCreMer: provided by Dr Molkenin) in a
488 tamoxifen-inducible cardiomyocyte-specific manner to produce CLIP-170 S311A flox/+;
489 MerCreMer+/- mice [41]. CLIP-170 S311A flox/+; MerCreMer-/- littermates were used as
490 age-matched controls. The CLIP-170 flox/+; MerCreMer+/- mice were genotyped by PCR
491 using primers for CAT gene and Cre recombinase. In adult CLIP-170 S311A flox/+;
492 MerCreMer+/- mice treated with tamoxifen for 6 days (daily dose of 20 mg kg⁻¹), Cre
493 recombination was confirmed by checking CLIP-170 S311A protein levels in cardiac muscle
494 from these mice 14 days after the onset of tamoxifen treatment. We established and
495 analyzed 2 lines of S311A mice (Supplementary figure 3). We presented the data of line 3;
496 their phenotypes were basically similar.

497

498 Cardiac MRI

499 Serial MRI was conducted using a horizontal 7.0 T Bruker scanner (BioSpec 70/30 USR,
500 Bruker Biospin). All MRI experiments were performed under general anaesthesia using 1%–
501 2% isoflurane administered via a mask covering the nose and mouth of the animals.
502 Respiratory signals, body temperature, and heart rate were monitored using a physiological
503 monitoring system (SA Instruments, Inc.). Body temperatures were continuously maintained
504 at 36.0 ± 0.5°C by circulating water through heating pads throughout all experiments [42].
505 The center of the imaging slice was carefully positioned at the mouse hearts. First, a
506 three-plane sequence was performed for the definition of slice orientation using self-gated
507 cine imaging with navigator echo. Next, six consecutive scans of the short axis from the
508 apex to the base of hearts were obtained in the long axis four-chamber and long axis
509 two-chamber views. These eight scans were used for fast low-angle shots with navigator
510 echo (IntraGate, Bruker) using the following parameters: repetition time/echo time = 6.0/2.2
511 ms, flip angle = 10 degrees, field of view = 2.56 × 2.56 cm, matrix = 256 × 256, slice
512 thickness = 1.0 mm, number of repetitions = 300, four concomitant slices covering the whole

513 heart from the apex to base, 10 phases per cardiac cycle, expected heart rate = 400 beats
514 per minute (bpm), expected respiratory rate = 60 bpm, in-plane resolution per pixel = 100 μ m,
515 acquisition time = 3 minutes 50 seconds per scan, total acquisition time = approximately
516 35 min, and a total anesthesia time = approximately 40 min.

517

518 MRI data analysis

519 In short-axis images, end-diastolic and end-systolic frames were selected according to
520 maximal and minimal ventricular diameter. The epicardial border was manually outlined and
521 the LV cavity was segmented in both frames using software ImageJ. The respective
522 volumes were calculated as the area of each compartment multiplied by the slice thickness
523 (1.0 mm). Based on end-systolic and end-diastolic volumes [ESV (μ l) and EDV (μ l),
524 respectively), all parameters characterizing cardiac function, such as stroke volume [SV
525 (μ l) = EDV - ESV] and ejection fraction [EF (%) = SV/EDV] were calculated.

526

527 Statistical analyses

528 Data are expressed as means \pm S.D. The two-tailed Student's t-test was used to analyze
529 differences between two groups. Differences among multiple groups were compared by
530 one-way ANOVA, followed by a post hoc comparison using the Tukey method with Prism 6
531 (GraphPad). For the histological assessment of CLIP-170 S311A mutant mice, comparison
532 was made by Cochran-Armitage trend test. $P < 0.05$ was considered statistically significant.

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541

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546

547

548 Disclosures

549

550 The authors declare that they have no conflict of interest.

551

552

553 Author contributions

554

555 YS conceived and designed the research; S. Yashirogi conducted most of the experiments
556 in this study; TK, TN, YN, TSM, SS, H. Kioka, YM, IY, KY contributed data collection,
557 analysis and figure preparation. HU, OY provided the resources and experimental advice.
558 YN, OT, S. Yamazaki, H. Kato, KY, ST, discussed the data from expert knowledge; S.
559 Yashirogi, S. Yamazaki and YS wrote the manuscript.

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563

564 **Figure legend**

565

566 **Figure 1: Phosphorylation levels of AMPK significantly increased at the intercalated**
567 **discs in adult mouse heart together with its substrate CLIP-170.**

568 (A, B) Immunoblot analysis of the phosphorylation level of CLIP-170, AMPK and ACC in
569 heart (A) and liver (B) along with the developmental stages. α -Tubulin was used as a
570 loading control. (C, D) Immunostained images of adult mouse heart tissue. These were
571 stained with a phosphorylated AMPK antibody (C, left), a phosphorylated CLIP-170 antibody
572 (D, left) and a plakoglobin antibody (C, D, center). Scale bar, 20 μ m (C, D).

573

574 **Figure 2: The localization of AMPK was regulated by contraction of the**
575 **cardiomyocytes**

576 (A, B) Immunostained images of neonatal rat cardiomyocytes 2 hours after treatment with
577 0.01 % DMSO (Control, upper row) or 4 μ M MYK-461 (MYK-461, middle row) and 4 hours
578 after washing out of MYK-461 (Wash out, bottom row). These cells were stained with a
579 phosphorylated AMPK antibody, a AMPK β 2 antibody and a N-cadherin antibody. (C, D)
580 Immunostained images of neonatal rat cardiomyocytes 2 hours after treatment with 0.01 %
581 DMSO (Control, upper row) or 4 μ M MYK-461 (MYK-461, bottom row) and stained with a
582 connexin43 antibody, a plakoglobin antibody, a LKB-1 antibody and an N-cadherin antibody.
583 Scale bar, 20 μ m (A-D).

584

585 **Figure 3: AMPK regulated longitudinal microtubule dynamics through the**
586 **phosphorylation of CLIP-170 in cardiomyocytes**

587 (A) GFP time-lapse images of neonatal rat cardiomyocytes expressing EGFP-CLIP-170 WT
588 0 min and 15 min after treatment with 20 μ M Compound C (left side panel) and expressing
589 EGFP-CLIP-170 S311A (right side panel). Higher magnification of white square (upper row)
590 showing CLIP-170 migrated longitudinally toward the cell-cell junctions. (B) Scatter plots of
591 a single comet length of neonatal rat cardiomyocytes expressing EGFP-CLIP-170 WT
592 before (Pre) and 15 min after treatment with 20 μ M Compound C (Cpd. C) and expressing
593 EGFP-CLIP-170 S311A. Data means \pm S.D. Pre: n=32, Cpd. C: n=36, CLIP S311A: n=68, **,
594 P<0.01 versus Pre. (C) GFP time-lapse images of neonatal rat cardiomyocytes expressing
595 EGFP-CLIP-170 WT treated with control siRNA (siCL, left) or siRNA targeting both AMPK α 1
596 and α 2 (siAMPK α 1 α 2, right). White dotted lines in the images showed the connected
597 cardiomyocyte not expressing EGFP-CLIP-170 WT. (D) Scatter plots of a single comet
598 length of cardiomyocytes expressing EGFP-CLIP-170 WT treated with control siRNA or
599 siRNA targeting both AMPK α 1 and α 2. Data means \pm S.D. siCL: n=178, siAMPK α 1 α 2:

600 n=180, **, P<0.01 versus siCL. Scale bar, 10 μ m (A, C).

601

602 **Figure 4: AMPK-CLIP-170 signal at the intercalated disc controlled the cell shape in**
603 **cardiomyocytes**

604 (A) GFP time-lapse images of neonatal rat cardiomyocytes expressing EGFP-CLIP-170 WT
605 0 hour, 1 hour and 2 hours after treatment with 4 μ M MYK-461. (B) Immunostained images
606 of neonatal rat cardiomyocytes 4 hours after treatment with 0.01 % DMSO (Control, upper
607 row) or 4 μ M MYK-461 (MYK-461, bottom row). These cells were stained with an α -Tubulin
608 (green) and a plakoglobin (red) antibody. (C) Bar graphs showing the cell size of a
609 cardiomyocyte 2 hours after treatment with or without 4 μ M MYK-461. Adenovirus
610 expressing EGFP-CLIP-170 (adCLIP) each mutant was used (WT, S311A, S311D). Data
611 means \pm S.D. Control (-/-): n=784, MYK-461: n=939, CLIP WT: n=656, CLIP WT + MYK-461:
612 n=619, CLIP S311D: n=613, CLIP S311D + MYK-461: n=735, CLIP S311A: n=389, CLIP
613 S311A + MYK-461: n=508, **, P<0.01 versus Control, ††, P<0.01 versus CLIP WT, N.S., not
614 significant. Scale bar, 10 μ m (A, B).

615

616

617 **Figure 5: Inducible heart-specific CLIP-170 S311A overexpressing transgenic mouse**
618 **shows cardiac dysfunction**

619 (A) Scatter plots of echocardiographic parameter (fractional shortening) of individual
620 CLIP-170 S311A overexpressing mice and control mice before (Pre), 2 weeks (Tx2w), 8
621 weeks (Tx8w), 26 weeks (Tx26w) and over 1 year (Tx1y) after the tamoxifen induction. Data
622 means \pm S.D. Pre Control: n=9, Pre S311A: n=6, Tx2w Control: n=18, Tx2w S311A: n=12,
623 Tx8w Control: n=21, Tx8w S311A: n=18, Tx26w Control: n=10, Tx26w S311A: n=9, Tx1y
624 Control: n=12, Tx1y S311A: n=13, **, P<0.01 versus each Control, ††, P<0.01 versus Pre
625 S311A. (B) Representative long axis four-chamber cardiac magnetic resonance images of
626 CLIP-170 S311A overexpressing mice and control mice over 1 year after the tamoxifen
627 induction. Left column showed systole images and right column were Diastole images. (C)
628 Bar graphs showing the ejection fraction of CLIP-170 S311A overexpressing mice and
629 control mice over 1 year after the tamoxifen induction. LV, left ventricular, RV, right
630 ventricular. Data means \pm S.D. Control: n=3, S311A: n=3, **, P<0.01 versus each Control.

631

632

633 **Figure 6: CLIP-170 S311A overexpressing transgenic mice showed elongation of the**
634 **cardiomyocytes with MT accumulation.**

635 (A) Masson's trichrome staining of the heart of CLIP-170 S311A overexpressing mice and

636 control mice over 1 year after the tamoxifen treatment. (B) Semi-quantitative scaling of
637 cardiac fibrosis in CLIP-170 S311A overexpressing mice and control mice over 1 year after
638 the tamoxifen treatment. Scaling class, 0, only perivascular fibrosis (white), +1, little
639 interstitial fibrosis (light grey), +2, local interstitial fibrosis more than +1 (grey), +3, extensive
640 interstitial fibrosis (black). *, $P < 0.05$ versus Control. (C) Immunostained images with an
641 α -Tubulin antibody of CLIP-170 S311A overexpressing mice heart and control mice heart
642 over 1 year after the tamoxifen treatment. (D) Representative immunostained images of
643 *Clip-170* S311A overexpressing mice heart and control mice heart over 1 year after the
644 tamoxifen treatment. These were stained with WGA (red) and a plakoglobin antibody
645 (green). (E) Scatter plots of a single cell length of CLIP-170 S311A overexpressing mice
646 heart and control mice heart over 1 year after the tamoxifen treatment. Data means \pm S.D.
647 Control, $n=259$, S311A, $n=326$, **, $P < 0.01$ versus Control. Scale bar, 100 μm (A), 20 μm (C,
648 D).

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