Essential light chain S195 phosphorylation is required for cardiac adaptation under physical stress

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Aims
Regulatory proteins of the sarcomere are pivotal for normal heart function and when affected by mutations are frequently causing cardiomyopathy. The exact function of these regulatory proteins and how mutations in these translate into distinct cardiomyopathy phenotypes remains poorly understood. Mutations in the essential myosin light chain (ELC) are linked to human cardiomyopathy characterized by a marked variability in disease phenotypes and high incidences of sudden death. Here we studied the role of the highly conserved S195 phosphorylation site of ELC using heterozygous adult zebrafish lazy susan (lazm647) in regulating contractile function in normal physiology and disease.

Methods and results
Echocardiography revealed signs of systolic dysfunction in otherwise phenotypically unremarkable heterozygote mutants. However, after physical stress, heart function of laz heterozygous zebrafish severely deteriorated causing heart failure and sudden death. Mechanistically, we show that upon physical stress, ELCs become phosphorylated and lack of S195 dominant-negatively impairs ELC phosphorylation. In vitro motility analysis with native myosin from adult heterozygous hearts demonstrates that S195 loss, specifically following physical stress, results in altered acto-myosin sliding velocities and myosin binding cooperativity, causing reduced force generation and organ dysfunction.

Conclusion
Using adult heterozygous zebrafish, we show that ELC S195 phosphorylation is pivotal for adaptation of cardiac function to augmented physical stress and we provide novel mechanistic insights into the pathogenesis of ELC-linked cardiomyopathy.

Keywords
Animal models of human disease • Functional genomics • Genetics of cardiovascular disease • Myocardial cardiomyopathy disease

1. Introduction
The heart’s ability to transiently adapt its output to the body’s demand is a unique feature and pivotal for normal heart function. Modulatory proteins adjunctive to actin and myosin, including troponymosin and the myosin light chains, largely facilitate this competence. Two pairs of myosin light chains, the regulatory (RLC) and the essential myosin light chain (ELC) are part of myosin molecules. Mutations in both RLCs and ELCs cause cardiomyopathy in humans.¹,² Hypertrophic cardiomyopathy represents the leading cause of premature death worldwide, often as a consequence of sudden cardiac arrest, e.g. during physical activity.³ While the role of RLCs in cardiac physiology and pathophysiology is well established, the precise function of ELCs in the heart and its contribution to human cardiomyopathy remains incompletely understood. Suitable animal models are needed to evaluate not only the pathogenesis of ELC-linked cardiomyopathy but also to delineate the role of ELC for heart function.

RLCs can be phosphorylated specifically by the myosin light chain kinase, triggering enhanced contractility.⁴ ELCs are similarly subject to phosphorylation and lack of ELC phosphorylation was shown to reduce contractility of rodent cardiomyocytes in vitro and in vivo in larval zebrafish.⁵,⁶ ELC (ELCv in humans) contains two putative and highly
conserved phosphorylation sites, threonine 67 (T66 in zebrafish) and serine 195 (S195) (Supplementary material online, figure S1). In contrast to RLCs, neither the kinase phosphorylating T66/67 and S195, nor the impact of ELC phosphorylation on heart performance is known.

Data exist suggesting that ELC modifications at the N-terminus may modulate myosin – actin cross-bridging, myosin ATPase activity, and calcium utilization, thereby acting as a molecular regulator of muscle force generation. Crystal structure analysis showed that the N-terminus of cardiac ELCs interacts directly with actin during contraction. Cardiomyocyte-specific overexpression of the human N-terminus of ELC 

ELC phosphorylation modulates cardiac adaption following physical stress and might contribute to a better understanding of the pathomechanism in ELC-linked cardiomyopathy. Our study further establishes heterozygous laz as the first stress-induced cardiomyopathy model in adult zebrafish.

2. Methods

A detailed description is available in the Supplementary material online.

All animal experiments have been performed in accordance with the guidelines of the state of Baden-Württemberg and have been approved by the Regierungspärräsidium Karlsruhe (permit number 35-9185.81/G-1/15).

2.1 Physical stress induction and echocardiographic analysis

Adult (aged 6–12 months) heterozygous mutant (laz+/−) and wild-type (wt) zebrafish littermates were subjected to physical stress (pCa), defined as one single session of forced swimming for a total of 10 min in a custom-made tubular swimming basin under optical observation (figure 2A).

Echocardiography in adult zebrafish was conducted as described previously.

2.2 2D gel electrophoresis

Proteins were extracted from zebrafish hearts and dried protein pellets (80 μg) used for performance of isoelectric focusing. Resulting strips were further processed to a second dimension gel and proteins blotted to a PVDF membrane. ELC and RLC protein spots were detected with primary zebrafish custom antibody and secondary HRP-antibody. Denstometric analysis of protein spots was realized with TotalLab Quant v12.5. ImageJ was used to quantify phosphorylation status distribution of ELC and RLC.

2.3 In vitro motility assay

Zebrafish skeletal muscle actin was isolated according to Pardee and Spudich. Cardiac myosin was extracted directly into the IVMA flow cells from permeabilized zebrafish ventricles. Rhodamine-Phalloidin labelled zebrafish actin was added and myosin motor function started by the presence of calcium and ATP. Filament sliding (see Supplementary material online, figure S2E) was recorded and average sliding speed of filaments determined. Additionally, histograms for single records were averaged and analysed.

2.4 pCa–force measurements

Zebrafish hearts were processed immediately after organ removal and clamped into a force transducer (SI-Heidelberg, Heidelberg, Germany). Hearts were permeablized, prestretched, and optimal length determined. Force measurement data were acquired and mean relative passive forces at different pCa (6.2–4.5) were plotted and pCa50 and nHill determined.

2.5 Calcium-transients

Cardiomyocytes were isolated according to Breite et al., seeded onto imaging dishes, and incubated 30 min with 10 μM Fluor-4 AM (Molecular Probes). Five consecutive stimulations via platinum electrodes were given to induce single twitch (18 V for 10 ms) and RLC.

2.6 Statistical analyses

Results were expressed as median ± 95% confidence interval (CI) and significant differences between wt and laz+/− zebrafish before and after physical stress, if not stated otherwise. Most data are depicted as boxplots with the lower limit of the box representing the 25 percentile, the line in the box representing the median, the upper limit of the box being the 75 percentile. Whiskers are chosen to reach down to the 5 percentile and up to the 95 percentile, respectively. Statistical differences were determined by
3. Results

3.1 Adult laz heterozygous zebrafish display signs of cardiac dysfunction with preserved cardiac output

Adult heterozygous laz (laz$^{+/−}$) zebrafish were considered unaffected, since life span and reproductive capability are comparable to their wt littermates. However, as shown by echocardiography analyses, adult laz$^{+/−}$ zebrafish displayed a significantly increased heart rate compared with their wt littermates (HR; laz$^{+/−}$ 118.0 ± 7.17 b.p.m., N = 11 vs. wt 86.00 ± 6.90 b.p.m., N = 14; P < 0.0001; median ± 95% CI) (Figure 1A). Noticeably, systolic function was significantly decreased, as measured by ejection fraction (EF; laz$^{+/−}$ 37.39 ± 10.45, N = 8 vs. wt 52.33 ± 8.26, N = 12; P < 0.0001) (Figure 1B). Cardiac output (CO) was not different between laz$^{+/−}$ and wt littermates, suggesting that the increased HR compensated for the systolic dysfunction (Figure 1C). We next assessed the expression of the heart failure indicator genes anp and bnp in control and laz$^{+/−}$ hearts. Interestingly, we could not detect any significant difference in the levels of anp and bnp transcripts (Figure 1D).

3.2 Cardiac performance deteriorates in response to cardiac stress

Next, we subjected wt littermates (pswt) and laz$^{+/−}$ adult zebrafish (p$laz^{+/−}$) to a physical stress (ps) protocol in the flow basin with an initial flow rate of 45 mL/s (equivalent to 3 average body lengths (BL)/s) for the first 5 min and of 70 mL/s (or 4–5 BL/s) for the following 5 min. This is in agreement with a flow-rate of 2–5 BL/s in an exercise-study and far below the critical swimming speed of up to 18 BL/s$^{20,21}$

Interestingly, already during the first session of increased flow rate p$laz^{+/−}$ zebrafish showed significant signs of fatigue as indicated by the rate of rest per minute (laz$^{+/−}$ 1.21 events/min CI: 0.00–4.34, N = 6 vs. wt 0.0 events/min CI: 0.00–0.59, N = 5; P < 0.0026; Figure 2B, Supplementary material online, Movies S1 and S2). Further, after two swimming sessions on consecutive days, we observed a significantly increased mortality in p$laz^{+/−}$ (N = 22 vs. wt N = 8; P = 0.038), where 92% of the p$laz^{+/−}$ survived the first and 67% the second session (Figure 2C). Importantly, all wt animals survived all swimming sessions without any signs of overexertion.

To evaluate, whether cardiac dysfunction may cause the reduction in exercise capacity following forced cardiac stress, we investigated p$laz^{+/−}$ and pwt littermates briefly after swimming by echocardiography, an equivalent to the human stress echo. We found no significant difference in HR (Figure 2D). However, p$laz^{+/−}$ showed indication for advanced heart failure with severe systolic dysfunction indicated by a dramatic reduction in EF (p$laz^{+/−}$ 22.56 ± 5.73, N = 15 vs. pwt 62.16 ± 6.45, N = 9; P < 0.0001; median ± 95% CI) and CO (p$laz^{+/−}$ 7.68 ± 4.92 μL/min, N = 5 vs. pwt 16.649 ± 10.10, N = 5; P < 0.0001)

Figure 1 Adult heterozygous laz (laz$^{+/−}$) zebrafish show signs of systolic dysfunction but no increase in heart failure markers. (A–C) Echocardiography quantification of heart rate (HR) (A), ejection fraction (EF) (B), and cardiac output (CO) (C) of adult heterozygous mutant (laz$^{+/−}$) and wt littermates. (D) Relative mRNA levels of anp and bnp in wt and laz$^{+/−}$ hearts. For data in (A–C) differences were determined by Student’s t-test; $^{*}P < 0.0026; ^{**}P < 0.0001; N = 8–15$ fish per genotype. Data in (D) are represented as mean ± SD, N = 24 from three independent experiments. Significant differences were determined using the Holm–Sidak method with multiple t-tests compared with wt. n.s., not significant.
Figure 2 Physical stress exacerbates cardiac dysfunction in laz^{+/−} mutants. (A) Schematic of setup to induce physical stress (top). Swimming protocol with indicated flow rates and durations (bottom). (B) Quantification of exhaustion as events of rest (events/min) during one session in wt and laz^{+/−} zebrafish. (C) Kaplan–Meier survival curve after indicated quantity of physical stress sessions. Log-rank test indicated significantly increased death events in pLaz^{+/−}. (D–F) Directly after physical stress, heart rate (HR) (D), ejection fraction (EF) (E), and cardiac output (CO) (F) were quantified. Changes of echocardiography parameters in wt and laz^{+/−} at baseline and indicated time-points after physical stress were quantified (G–I): EF (G), fractional shortening (FS) (H), and stroke volume (SV) (I). Statistical differences in (B), (D–F) were determined by Student’s t-test; *P < 0.05; **P < 0.01; N = 15 for each condition from three independent experiments; in G–I via 2-way ANOVA with post hoc Tukey’s modification for multiple testing. Horizontal lines: significant intergroup differences; asterisk indicates vertical interclass differences, *P < 0.05; N = 5.

(Figure 2E and F). Follow-up examinations, immediately (0 min) and at 30 and 120 min after physical stress further underlined pLaz^{+/−} disability to adapt to augmented cardio-circulatory demand in contrast to their wt littermates (Figure 2G–I). pLaz^{+/−} were able to transiently adapt cardiac function, indicated by enhanced EF, FS, and stroke volume (SV) directly after physical stress (0 min) and during the early recovery
period (30 min) with complete return to basal functional levels 120 min post physical stress. Contrarily, \( p^{\text{ps laz}^{+/+}} \) zebrafish demonstrated a marked decline in EF, FS, and SV directly post stress and at 30 min (Figure 2G–I). Noticeably, \( p^{\text{ps laz}^{+/+}} \) animals similarly returned to their basal cardiac activity by 120 min post physical stress.

These data indicate that under resting conditions \( \text{laz}^{+/+} \) zebrafish display signs of cardiac dysfunction and an increased HR, which compensates for the contractile dysfunction. Physical stress, however, induces marked cardiac dysfunction, which can no longer be compensated by elevation of HR but leads to progressive heart failure and cardiac death.

### 3.3 Physical stress affects phosphorylation of wild-type and mutant ELC protein

Since \( \text{laz} \) results in a 11 amino acid truncation of ELC including the removal of S195, we next investigated whether physical stress influenced phosphorylation of this site, which was shown to be of pivotal importance for heart contraction.\(^6\) To assess changes in phosphorylation of wt and \( \text{laz} \) mutant ELC protein in response to physical stress, we performed 2D gel electrophoresis and western blotting using an antibody to specifically detect ELC protein from zebrafish on cardiac protein extracts derived from wt and \( \text{laz}^{+/+} \) zebrafish under resting conditions and after physical stress. 2D gel electrophoresis analysis enabled us to clearly distinguish both wt and \( \text{laz} \) mutant protein in \( \text{laz}^{+/+} \) hearts as evident by the shift in size and isoelectric point (pI) caused by the 11 amino acid truncation (Figure 3A and B). Interestingly, \( \text{laz} \) mutant ELC protein appeared to be present in slightly higher levels than the wt protein (Figure 3A). We found that in wt zebrafish under basal conditions, ELC was predominantly present in its unphosphorylated (0P; 58.2% ± 8.3 SD) and monophosphorylated (1P; 41.8% ± 8.3 SD) form (Figure 3C and D). In response to physical stress, 1P of ELC in wt hearts increased slightly (43.2% ± 11.4 SD) and dual-phosphorylation

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**Figure 3** Physical stress causes aberrant phosphorylation of ELC in \( \text{laz}^{+/+} \) mutants. (A) Schematic illustration of the localization of the wt (ELC\(^{\text{wt}}\)) and mutant (ELC\(^{\text{laz}}\)) unphosphorylated (0P), monophosphorylated (1P), and dual-phosphorylated (2P) isoforms on a 2D gel-electrophoresis according to size and the isoelectric point (pI). Hash indicates size difference between ELC\(^{\text{wt}}\) and ELC\(^{\text{laz}}\). (B) Two representative 2D gel western blots and densitometric analysis (small numbers above and below) of \( \text{laz}^{+/+} \) cardiac protein detecting ELC pretreated with phosphatase to remove all phosphate modifications. (C) 2D gel western blot and densitometric analysis (small numbers above and below) of wt (top) and \( \text{laz}^{+/+} \) (bottom) ELC (upper lane) and regulatory light chain (RLC; lower lane) proteins derived from resting (the two left lanes) hearts and after physical stress (the two right lanes). Two representative blots from four independent experiments (\( N = 18 \) wt hearts; \( N = 18 \) \( \text{laz}^{+/+} \) hearts) for each condition are shown. Monophosphorylation 1P and dual-phosphorylation 2P indicated by black arrows. (D) Quantification of unphosphorylated (0P), monophosphorylated (1P), and dual-phosphorylated (2P) ELC\(^{\text{wt}}\) and ELC\(^{\text{laz}}\) distribution. A total of four experiments with a total of 36 hearts (\( N = 18 \) wt hearts; \( N = 18 \) \( \text{laz}^{+/+} \) hearts) was included in the analysis. (E) Quantification of unphosphorylated (0P), monophosphorylated (1P), and dual-phosphorylated (2P) RLC distribution.
ELC phosphorylation modulates cardiac adaption

(2P: 24.2% ± 6.7 SD) became detectable. As an indicator for physical stress, we assayed changes in RLC phosphorylation, which is well known to be phosphorylated under physical stress conditions.\(^{22,23}\) We observed that after physical stress in both wt and laz hearts 1P and 2P RLC were increased (Figure 3C and D). In laz\(^{+/−}\) hearts we found that under resting conditions 1P of wt ELC protein was decreased dramatically compared with wt ELC from wt hearts (10.9% ± 4.8 SD in laz\(^{+/−}\) vs. 41.8% ± 8.3 SD in wt), while 0P was robustly present (Figure 3C and D). After physical stress 1P of wt ELC protein from laz\(^{+/−}\) hearts was noticeably increased (19.6% ± 5.2 SD), while phosphorylation of laz mutant ELC protein did not change (Figure 3C and D). Under all conditions, laz mutant ELC protein was detectable in its 0P and 1P form and, as expected, never in a 2P form due to the lack of S195 phosphorylation site. In the 2D gel from laz\(^{+/−}\) hearts, the 2P of wt ELC protein converted with 0P of laz mutant protein at similar size and pl. The results suggest that the dramatic decrease in 1P of wt protein, when compared with the phosphorylation of ELC in wt control hearts, is not due to a massive shift towards 2P. Our results indicate that ELC phosphorylation changes upon physical stress and thereby might be involved in adapting the heart function to physical stress and that loss of the single phosphorylation site S195 in heterozygotes impairs cardiac stress response. Further analysis is summarized in Supplementary material online, Figure S3 quantifying distinct combinations of phosphorylation in the wt and laz ELC isoforms.

### 3.4 Laz impairs acto-myosin performance but not myosin ATPase activity after cardiac stress

In order to assess whether the laz mutation and the aberrant phosphorylation causes differences in acto-myosin cross-bridge in response to physical stress, we performed IVMA (Figure 4A). Myosin from adult wt and laz\(^{+/−}\) ventricles at rest and immediately after physical stress was extracted directly into IVMA flow chambers and sliding speeds of labelled zebrafish actin filaments were analysed (Supplementary material online, Figure S2A). Importantly, our protein preparations contained all auxiliary and regulatory proteins, including myosin light chains (Supplementary material online, Figure S2B–E). Average sliding velocities of the analysed acto-myosin interactions were in the range of 3.19 ± 0.27 to 4.40 ± 0.16 μm/s and thus in accordance with previously published data for rodent ventricular myosin.\(^{24,25}\) Actin filament speed after physical stress was significantly reduced on ps\(^{wt}\) compared with wt (ps\(^{wt}\) median: 3.28 μm/s, 95% CI of median: 2.18–4.14 vs. wt 3.79 μm/s, CI: 3.36–4.61, n = 11; P < 0.05) (Figure 4B). However, the actin speed on ps\(^{laz\^{+/−}}\) was significantly increased compared with laz\(^{+/−}\) (ps\(^{laz\^{+/−}}\): 4.37 μm/s, n = 11; P < 0.001) (Figure 4B). While in wt zebrafish physical stress tends to decrease the acto-myosin sliding speed (speed-ratio ps/rest: 0.97, CI: 0.73–1.11, n = 11), in laz\(^{+/−}\), physical stress significantly increases the acto-myosin sliding speed (ps/rest: 1.30, CI: 1.18–1.45, n = 11; P < 0.001) (Figure 4C). The two-way ANOVA outcome analysis indicates that the interaction between genotype and physical stress is highly significant ([F(0.05, 1. 40) = 21.11; P < 0.001], confirming that physical stress is responsible for the impaired acto-myosin functionality in laz\(^{+/−}\) hearts. Furthermore, we found a higher degree of heterogeneity in the normalized frequency histograms with all measured actin filaments plotted against their individual sliding speed for laz\(^{+/−}\) hearts compared with wt. These distributions showed that speeds of actin filaments on myosin derived from rested wt ventricles coherently and homogeneously distributed at a higher speed range (95% CI 3.97–4.05 ± 1.13–1.21 μm/s) compared with ps\(^{wt}\) (95% CI 3.56–3.68 ± 0.92–1.04 μm/s) (Figure 4D). Importantly, both wt and ps\(^{wt}\) distributions were significantly different from a Gaussian distribution (D’Agostino & Person Omnibus K2 test; P < 0.0003, n = 11 and P < 0.0001, n = 11 respectively). Physical stress on wt did not impact the distribution of actin filament’s speed (skewness); however, changed the kurtosis, narrowing the variance of the data into the centre of the distribution (Figure 4D). As previously reported, these results indicate higher cumulative contractile force.\(^{24,27}\) Similar analysis was performed for actin filament sliding velocities on myosin isolated from laz\(^{+/−}\) ventricles. At rest, actin filament’s speed was reduced compared with ps\(^{laz\^{+/−}}\) (95% CI, laz\(^{+/−}\): 2.94–3.16 ± 1.78–2.03 vs. ps\(^{laz\^{+/−}}\): 4.66–4.98 ± 2.16–2.51 μm/s). Again, both laz\(^{+/−}\) and ps\(^{laz\^{+/−}}\) distributions were significantly different from Gaussian distribution (P < 0.0001, n = 11 for both laz\(^{+/−}\) and ps\(^{laz\^{+/−}}\)). In contrast to wt results, physical stress in the absence of S195 phosphorylation site on the ELC did neither change the skewness (both right-skewed) nor the kurtosis (both platykurtic) of the actin filament’s velocity distribution, indicating reduced contractile force.\(^{24,27}\) This analysis suggests that ps\(^{laz\^{+/−}}\) zebrafish were not able to appropriately adapt force production to higher demand in response to the physical stress.

To analyse whether the increase in actin sliding velocity on ps\(^{laz\^{+/−}}\) myosin might be due to a higher ATPase activity, we used myosin extracts at different pCa values to measure ATP consumption in the presence of actin. Pi production increased equally in all four groups in parallel to rising [Ca\(^{2+}\)]. This experiment demonstrated uniform ATPase function over the whole range of pCa for both wt and laz\(^{+/−}\) myosin and that physical stress did not alter it (Figure 4E).

### 3.5 Laz mutant ELC impacts actin–myosin calcium binding cooperativity without affecting pCa\(_{50}\) in response to physical stress

Since ELCs are considered to be critical regulators of calcium sensitivity and thereby force generation of muscle fibres,\(^{7–9}\) we conducted pCa–force measurements at different Ca\(^{2+}\)–concentrations in skinned hearts derived from rested and physically stressed wt and laz\(^{+/−}\) zebrafish (Figure 5A). In wt hearts, we observed that physical stress altered neither pCa\(_{50}\) (Figure 5B) nor the steepness of the pCa curve as an indicator for actin–myosin cooperativity (Hill coefficient, n=Hill) (Figure 5C). However, we did detect significantly increased n=Hill in laz\(^{+/−}\) heart muscle when exposed to physical stress (Figure 5C median: 2.51, 95% CI of median: 1.50–2.99, vs. ps\(^{laz\^{+/−}}\): 3.45, CI: 3.20–5.11, N = 5; P < 0.05), indicating increased acto-myosin cooperativity.

### 3.6 Laz mutant cardiomyocytes present altered calcium handling

Since abnormal calcium handling is a common finding in cardiomyocytes of failing hearts, we analysed calcium-transients in ventricular cardiac myocytes derived from physically stressed and rested wt and laz\(^{+/−}\) hearts loaded with Fluo4-AM and exposed to electrical stimulation (Figure 5D).\(^{28–30}\) We observed no difference in basal [Ca\(^{2+}\)]\(_i\) in cardiac myocytes from laz\(^{+/−}\) and wt under all conditions. Analyses of biophysical parameters (amplitude, peak area and full-width at half maximum (FWHM)) from calcium-transients obtained from ps\(^{wt}\) cardiomyocytes were significantly enhanced compared with wt (Figure 5E–G; peak area (nM*s) wt median: 3.21, 95% CI of median: 2.79–4.32 vs. ps\(^{wt}\) 7.55, CI: 5.83–13.32, P < 0.001; FWHM (ms) wt 372,
Cl: 315−467 vs. \(^{\text{pswt}}\) 479, Cl: 408−761, \(P < 0.05; n = 90\) cells). In contrast, calcium-transients from \(^{\text{laz}}\) zebrafish cardiomyocytes display significantly decreased peak area and FWHM compared with \(^{\text{wt}}\) laz, whereas sliding speed on myosin from \(^{\text{wt}}\) laz, and particularly \(^{\text{pswt}}\) laz, are heterogeneously distributed. (E) \(\text{Ca}^{2+}\)-activated ATPase activity in the presence of thin filament proteins was measured at different pCa values as Pi production (nmoles Pi produced/min/mg myosin). No significant differences were detected between all four conditions. Statistical differences of average sliding speeds in (B) were determined by ANOVA with Bonferroni post hoc pairwise multiple comparisons; difference of ratios in (C) was determined by Student’s t-test. *\(P < 0.05; **P < 0.01; ***P < 0.0001; N = 5, three independent experiments.

4. Discussion

To gain further insights into cardiac ELC function, we here utilized adult heterozygous \(^{\text{laz}}\) zebrafish lacking the highly conserved C-terminal phosphorylation site S195. In contrast to humans, where the atrial ELC becomes re-expressed in the diseased heart and can, at least partially, compensate for the ventricular ELC during decreased heart function, zebrafish only express one ELC isoform in the heart allowing an...
Figure 5. Laz affects acto-myosin cooperativity and impairs calcium handling. (A) pCa–force relationship, normalized to maximal force (at pCa 4.5). The sigmoidal curves were further analysed for pCa_{50} and n_{Hill}. (B) Quantification of pCa_{50} from the pCa–force relationship revealed no significant differences. (C) n_{Hill} was significantly increased for p_laz^{+/−} compared with wt, p_wt, and laz_{−/−} (p < 0.05; N = 5). (D) Intracellular Ca^{2+}-transients were analysed in isolated ventricular cardiomyocytes loaded with Fluo4-AM. Representative brightfield (left) and corresponding fluorescent (middle) and merged (right) images of a single ventricular adult zebrafish cardiomyocyte (scale bar 20 μm). (E) Representative Ca^{2+}-transient traces recorded from wt, p_wt, laz_{−/−}, and p_laz^{+/−}. (F) Peak area and (G) full-width half-maximum (FWHM) obtained from the analyses of Ca^{2+}-transients. Data in (F) and (G) were statistically analysed by using ANOVA with Bonferroni post hoc pairwise multiple comparisons *p < 0.05; **p < 0.01; in A–C: N = 5; in E–G: n = 90 cells.
unhindered evaluation of ELC function in the myocardium.\(^2\) Here we used the zebrafish mutant lazy susan that lacks 11 amino acids, including S195. Importantly, in our previous study using phosphorylation-deficient and phosphomimetic variants of zebrafish ELC, S195A and S195D, respectively, we demonstrated the critical role of the S195 phosphorylation site alone as the cause for the observed lazy susan phenotype, thereby showing that the other missing proportion plays a rather, if any, minor role in ELC function.\(^2\) We thoroughly analysed cardiac function of adult \(laz^{+/−}\) zebrafish by echocardiography and observed that under basal conditions \(laz^{+/−}\) zebrafish display signs of systolic dysfunction accompanied by a significant and likely compensatory increase in heart rate to retain CO.\(^4, 5\) Interestingly, we did not detect an increase in the expression of heart failure markers bnp and anp in \(laz^{+/−}\) zebrafish, suggesting that the observed compensatory increase in HR to stabilize the CO might be sufficient to prevent the activation of maladaptive molecular processes. When subjected to physical stress, \(laz^{+/−}\) developed cardiac failure with reduced systolic function associated with a marked reduction in exercise capacity and a high mortality rate, while wt siblings all survived and showed no obvious signs of overexertion. Interestingly, reported incidences of sudden cardiac death in human ELC-mutation carriers are remarkably high.\(^1, 13\) Follow-up examinations further indicated that \(laz^{+/−}\) hearts are unable to adapt CO appropriately.

We hypothesized that physical stress affects ELC phosphorylation and thereby its role in adapting the heart’s activity. Using 2D gel analyses from native cardiac protein extracts, we show that physical stress in wt animals markedly induced ELC mono- and dual-phosphorylation. However, in \(laz^{+/−}\) hearts loss of the S195 phosphorylation site not only eliminated the ability for dual-phosphorylation of the mutant ELC protein fraction, as expected after physical stress, but unexpectedly also affected phosphorylation of the wt ELC protein. Wt ELC protein under resting conditions was present much less phosphorylated in \(laz^{+/−}\) hearts than in wt hearts. Physical stress only induced phosphorylation in a small proportion of wt ELC in \(laz^{+/−}\) hearts. Possible changes in ELC phosphorylation at both sites are rather complex and are summarized in Supplementary material online, Figure S3. Our results support the hypothesis that ELC phosphorylation thus is essential to adapt cardiac function in response to physical stress. Impaired phosphorylation due to, e.g. mutations in the ELC gene even when present in heterozygosis can exert dominant-negative effects on overall ELC phosphorylation and thereby on ELC function.

Previous studies often used recombinant proteins harbouring the respective disease mutation for IVMA. However, mutant proteins reside in the native tissue of heterozygous mutation carriers or respective animal models in varying and sometimes unpredictable ratios when compared with their wt form.\(^3\) To address the molecular consequence of physical stress in wt animals markedly induced ELC mono- and dual-phosphorylation.\(^4, 5\) We hypothesized that physical stress affects ELC phosphorylation and thereby its role in adapting the heart’s activity. Using 2D gel analyses from native cardiac protein extracts, we show that physical stress in wt animals markedly induced ELC mono- and dual-phosphorylation. However, in \(laz^{+/−}\) hearts loss of the S195 phosphorylation site not only eliminated the ability for dual-phosphorylation of the mutant ELC protein fraction, as expected after physical stress, but unexpectedly also affected phosphorylation of the wt ELC protein. Wt ELC protein under resting conditions was present much less phosphorylated in \(laz^{+/−}\) hearts than in wt hearts. Physical stress only induced phosphorylation in a small proportion of wt ELC in \(laz^{+/−}\) hearts. Possible changes in ELC phosphorylation at both sites are rather complex and are summarized in Supplementary material online, Figure S3. Our results support the hypothesis that ELC phosphorylation thus is essential to adapt cardiac function in response to physical stress. Impaired phosphorylation due to, e.g. mutations in the ELC gene even when present in heterozygosis can exert dominant-negative effects on overall ELC phosphorylation and thereby on ELC function.

Previous studies often used recombinant proteins harbouring the respective disease mutation for IVMA. However, mutant proteins reside in the native tissue of heterozygous mutation carriers or respective animal models in varying and sometimes unpredictable ratios when compared with their wt form.\(^3\) To address the molecular consequence of the heterozygosity of S195 phosphorylation on myofilament function in the \(laz^{+/−}\) model, we directly extracted native myosin including regulatory proteins from zebrafish ventricles to measure acto-myosin interaction in IVMA. We observed that averaged thin filament sliding speed decreased on \(laz^{+/−}\) myosin compared with wt myosin. Physical stress induced an opposite effect in \(laz^{+/−}\) animals, where the average speed of actin filaments was significantly higher in \(laz^{+/−}\) compared with resting \(laz^{+/−}\) individuals. This finding of increased speed is consistent with an earlier study of an HCM patient carrying an ELC mutation.\(^3\) The actin sliding speed is a result of changes in actin–myosin interaction and kinetics, specifically by the myosin attachment and detachment rate.\(^3\) An increase in the myosin attachment rate results in enhanced and stabilized myosin head binding to the actin leading to decreased sliding speeds, thereby enabling production of stronger working strokes.\(^3\) Our data thus support an increase of the myosin’s detachment rate independent of its ATPase activity that results in a reduced binding of myosin heads to actin, consequently leading to increased sliding speeds but weaker power strokes in \(laz^{+/−}\).\(^3\) Structural modelling supported by experimental in vivo transgenic mice models demonstrated an important modulatory role of the N-terminus of ELCs on cross-bridging activity and myosin function.\(^4, 5\) The N-terminal ELC extension is a 91 Å rod-like domain that functions as a stabilizing bridge between the ELC core at the myosin neck and the binding site of the ELC on the actin filament, thereby enhancing myosin–actin attachment.\(^4, 5\) Our data might represent a potential modulatory impact of the C-terminal S195 phosphorylation on the N-terminal function, as schematically modelled and described in Figure 6. Consistent with that, previous work demonstrated that N-terminal truncation of the ELC affected neither the muscle’s \(Ca^{2+}\) sensitivity nor its myosin ATP-ase activity, characteristics we also found upon loss of S195.\(^4, 5\) Lack of S195 hence would result in destabilization of the myosin to actin binding, causing an increase in myosin detachment rate leading to an increase in sliding speed, as we observed, and consequently to weaker stroke power as evident in the decrease in heart contractility measured by echocardiography in \(laz^{+/−}\) fish (Figure 6).\(^4, 5\)

Additionally, we found a higher degree of heterogeneity in the sliding speed distribution of actin filaments driven by \(laz^{+/−}\) myosin when compared with wt. A possible explanation might be the broader variety of ELC subtype combinations on the basic molecular organization level, namely the myosin hexamers (Supplementary material online, Figure S3). These functional differences may further contribute to a reduced contraction efficiency of the whole organ, leading to the observed overall heart dysfunction particularly in response to physical stress. Detailed analysis of the normalized frequency histograms revealed an actin speed distribution in wt hearts significantly different from a Gaussian distribution, indicating the presence of basal force level. The decrease of actin speed after physical stress and the change in the histogram’s shape suggest enhanced contraction force.\(^2, 6, 7\) Contrarily, the histogram shape of \(laz^{+/−}\) actin’s speed distribution after physical stress did not change compared with the resting condition, suggesting that the acto-myosin interaction did not increase force production, hence further explaining the observed overall systolic dysfunction particularly in response to physical stress.\(^2, 6, 7\)

We found that lack of S195 phosphorylation affects the actin–myosin binding cooperativity specifically after physical stress without affecting calcium-sensitivity of the heart in general as evident by the steeper pCa-force relationship curve without changes in pCa50. There are two known cooperativity mechanisms in muscle involving cross-bridge-troponin and troponin-C cooperativity.\(^6, 5\) While troponin-C cooperativity is responsible for changes in \(Ca^{2+}\)-dependent force generation, cross-bridge-troponin cooperativity modulates acto-myosin interaction and kinetics with no difference in the pCa–force relationship.\(^45\) The observed increase in cross-bridge-troponin cooperativity in \(laz^{+/−}\) most likely represents a compensatory mechanism to account for decreased acto-myosin interaction. Increasing cooperativity enables the muscle to generate higher force by only slightly increasing the systolic intracellular calcium.\(^45\) Consequently, increased cooperativity combined with decreasing systolic calcium would obliterate the beneficial effect and would in contrast result in lower stroke force. To test, whether \(laz^{+/−}\) cardiomyocytes display changes in intracellular \(Ca^{2+}\)- handling during systole, we measured calcium transients in isolated and electrically stimulated cardiomyocytes in both resting state and after
physical stress conditions. As expected, Ca\(^{2+}\)-transients were significantly higher in wt after physical stress compared with rested wt zebrafish. Ca\(^{2+}\)-transients determined from rested \(\text{laz}^{+/−}\) cardiomyocytes were not significantly different from rested wt controls, suggesting that the activities of major excitation-contraction-coupling proteins, including L-type calcium channel and SERCA, are probably normal in \(\text{laz}^{+/−}\). Analysis of \(\text{ps}^{\text{laz}^{+/−}}\) cardiomyocytes quickly after a single swimming session revealed a decreased systolic Ca\(^{2+}\)-release indicated by Ca\(^{2+}\)-transients with significantly decreased amplitude, smaller peak area, and a shortened FWHM. Since all experiments were conducted briefly after physical stress, alterations in the protein level seem to be unlikely. The observed effects may thus rather indicate transient changes in protein activity caused by post-translational modifications, including phosphorylation. In light of the observed increase in cooperativity, these results of decreased systolic calcium would lead to decreased stroke force compared with controls. This assumption is in concert with the echocardiography data documenting decreased systolic activity in \(\text{ps}^{\text{laz}^{+/−}}\).

Our study provides evidence on the function of ELC in the heart, where ELC S195 phosphorylation is pivotal to adapt cardiac contractility specifically during physical stress. Additionally, we show that physical stress triggers cardiac failure in a before phenotypically rather unremarkable individual. Under resting conditions, the heart appears to be able to compensate for impaired ELC function. When subjected to intense physical stress, cardiac function in the \(\text{laz}^{+/−}\) animals considerably deteriorates. At the molecular level, our study suggests that ELC S195 phosphorylation might modulate the detachment rate during acto-myosin cross-bridging. One possible explanation is a role of S195 in stabilizing myosin head binding to actin through the ELC N-terminal domain. Previous studies demonstrated that pseudo-phosphorylation of RLC and ELC can efficiently rescue the development of cardiomyopathy in animal models carrying human disease mutations, highlighting the importance of myosin light chain phosphorylation. Considering the altered RLC phosphorylation in \(\text{laz}\) animals under basal conditions, it would be interesting to evaluate in the future if pseudo-phosphorylation of RLC similarly can rescue S195-ELC loss in \(\text{laz}\) animals. Taken together, our study presents a new model to understand a proportion of the high incidences of sudden cardiac death in human ELC mutation carriers and establishes adult zebrafish as a valuable model to understand complex genotype-phenotype relationships. To fully integrate ELC in the network regulating cardiac stress response, it will be pivotal in future studies to identify the kinase responsible for ELC phosphorylation. At the same time, identification of the phosphatase that terminates ELC-mediated...
adaptation post-stress by dephosphorylating ELC will be similarly important.

### Supplementary material
Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest
none declared.

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### References


