

Myosin-binding protein composition tunes vertebrate superfast contractility

Glenda Comai

comai@pasteur.fr

Paris-Saclay Institute of Neuroscience <https://orcid.org/0000-0003-3244-3378>

Amaury Korb

Stem Cells & Development Unit CNRS UMR 3738, Institut Pasteur, Université Paris Cité

<https://orcid.org/0000-0003-2602-3190>

Stephane Fischer

Institut Pasteur

Matthieu Dos Santos

UTSW <https://orcid.org/0000-0002-7113-102X>

Carole Dabadie

Institut NeuroMyoGène, Pathophysiology and Genetics of Neuron and Muscle, CNRS UMR5261, INSERM U1315, Université Claude Bernard Lyon 1

Kathleen Woulfe

Department of Medicine, Division of Cardiology, University of Colorado Anschutz Medical Campus

<https://orcid.org/0000-0001-9885-7026>

Nicolas Maillet

Institut Pasteur

Hannah Cizauskas

Department of Cell and Molecular Physiology, Loyola University Chicago <https://orcid.org/0009-0004-7760-1143>

Anita Kneppers

Institut NeuroMyoGène, Pathophysiology and Genetics of Neuron and Muscle, Université Claude Bernard Lyon 1, CNRS UMR5261, INSERM U1315 <https://orcid.org/0000-0003-4901-5468>

Valérie Seffer

Institut Pasteur

Carolina Moraes Cabe

Institut Pasteur <https://orcid.org/0000-0001-7718-159X>

Charlot Philips

Tissue Engineering Lab, Department of Development and Regeneration, KU Leuven campus Kulak

<https://orcid.org/0000-0001-6834-0763>

Ryoto Iwai

Laboratory Animal Resource Center in Transborder Medical Research Center, and Department of Anatomy and Embryology, Institute of Medicine, University of Tsukuba

Ghislain Banos

IMRB, INSERM, EFS, Université Paris Est Créteil <https://orcid.org/0009-0008-1628-2512>

Nathalie Didier

IMRB, INSERM, EFS, Université Paris Est Créteil

Lieven Thorrez

KU Leuven <https://orcid.org/0000-0002-5896-6823>

Pascal Maire

Institut Cochin <https://orcid.org/0000-0001-7795-0029>

Rémi Mounier

CNRS <https://orcid.org/0000-0003-3464-3322>

Bruno Allard

Université Claude Bernard Lyon 1 <https://orcid.org/0000-0002-7131-7828>

Ryo Fujita

University of Tsukuba

Iris Adam

University of Southern Denmark <https://orcid.org/0000-0001-7561-7636>

David Barefield

Department of Cell and Molecular Physiology, Loyola University Chicago <https://orcid.org/0000-0002-6166-640X>

Shahragim Tajbakhsh

Institut Pasteur, Paris <https://orcid.org/0000-0003-1809-7202>

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5 Amaury Korb¹, Stephan Fischer², Matthieu Dos Santos³, Carole Dabadie⁴, Kathleen Woulfe⁵,
6 Nicolas Maillet², Hannah Cizauskas⁶, Anita Kneppers⁴, Valerie Seffer⁷, Carolina Moraes⁷, Charlot
7 Philips⁸, Ryoto Iwai⁹, Ghislain Banos¹⁰, Nathalie Didier¹⁰, Lieven Thorrez⁸, Pascal Maire¹¹, Rémi
8 Mounier⁴, Bruno Allard⁴, Ryo Fujita¹², Iris Adam¹³, David Barefield⁶, Shahragim Tajbakhsh¹ and
9 Glenda Comai^{1,14*}

10 **Affiliations:**

11 ¹ Stem Cells & Development Unit CNRS UMR 3738, Institut Pasteur, Université Paris Cité;
12 Paris, 75015, France.

13 ² Bioinformatics and Biostatistics Hub, Institut Pasteur, Université Paris Cité ; Paris, 75015,
14 France.

15 ³ Department of Molecular Biology, The University of Texas Southwestern Medical Center,
16 Dallas, TX, USA; Hamon Center for Regenerative Science and Medicine, The University of
17 Texas Southwestern Medical Center, Dallas, TX, USA; Senator Paul D. Wellstone Muscular
18 Dystrophy Specialized Research Center, The University of Texas Southwestern Medical
19 Center, Dallas, TX, USA.

20 ⁴ Institut NeuroMyoGène, Pathophysiology and Genetics of Neuron and Muscle, CNRS
21 UMR5261, INSERM U1315, Université Claude Bernard Lyon 1; Lyon, 69100, France.

22 ⁵ Department of Medicine, Division of Cardiology, University of Colorado Anschutz Medical
23 Campus; Aurora, CO, USA.

24 ⁶ Department of Cell and Molecular Physiology, Loyola University Chicago; Maywood, IL,
25 USA.

26 ⁷ Single Cell Biomarkers UTechS, Institut Pasteur, Université Paris Cité ; Paris, 75015, France

27 ⁸ Tissue Engineering Lab, Department of Development and Regeneration, KU Leuven campus
28 Kulak; Kortrijk, 53 850, Belgium.

29 ⁹ Laboratory Animal Resource Center in Transborder Medical Research Center, and
30 Department of Anatomy and Embryology, Institute of Medicine, University of Tsukuba;
31 Ibaraki 305-8575, Japan.

32 ¹⁰ IMRB, INSERM, EFS, Université Paris Est Créteil; Créteil, 94010, France.

33 ¹¹ Institut Cochin, CNRS, INSERM, Université Paris Cité; Paris, 75014, France.

34 ¹² Division of Regenerative Medicine, Transborder Medical Research Center, Institute of
35 Medicine, University of Tsukuba; Ibaraki, 305-8575, Japan.

36 ¹³ Vocal Neuromechanics lab, Department of Biology, University of Southern Denmark;
37 Odense, M 5230, Denmark.

38 ¹⁴ Institut des Neurosciences Paris-Saclay, CNRS UMR 9197, Université Paris-Saclay ;
39 Saclay, 91400, France.

40 * Corresponding author, email: comai@pasteur.fr

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Abstract

Skeletal muscles all rely on actin-myosin cross-bridge cycling, and harbor sarcomeres as basic contractile units, yet display extreme functional diversity. Superfast muscles that drive sound production and rapid eye movements in vertebrates cycle almost two orders of magnitude faster than locomotory muscles. Accelerated detachment of myosin cross-bridges has been proposed to underlie this speed and attributed solely to specialization of the myosin motor¹⁻³. Using single-nucleus multiome profiling of chromatin accessibility and gene expression in the mouse, we identify myosin-binding protein H-like (Mybphl), until now considered heart-restricted⁴⁻⁶, as a core component of superfast sarcomeres. We show that Mybphl is selectively expressed in the fastest fibres of superfast muscles and is required for accelerated relaxation through the phase attributed to crossbridge detachment. Gain- and loss-of-function experiments demonstrate that Mybphl expression is controlled by the transcription factor *Tbx15*, which acts as a molecular switch within a canonical fast-fibre program. Phylogenetic analysis reveals recurrent myosin-binding protein ratios in vertebrate superfast muscles, with *Tbx15* also consistently enriched. Our work uncovers a conserved mechanism for extreme contractile specialization, in which a single transcriptional regulator deploys a cardiac protein to enable unprecedented contractile speeds.

Main Text

“Superfast” skeletal muscles constitute a specialized class whose contractile frequency surpasses conventional fast-twitch kinetics. Superfast muscles are mainly present in vertebrate sound-producing tissues including mammalian intrinsic laryngeal muscles (ILM), bat cricothyroid muscles involved in echolocation, birdsong syringeal muscles, rattlesnake shaker muscle, and “sonic” swimbladder muscles in some teleost fishes^{1-3,7,8}. Extraocular muscles (EOM), which empower high-frequency saccadic eye movements, are generally classified as superfast⁹ although there is no clear consensus about the minimal contraction frequency required for such categorization. Superfast muscles are found in most major vertebrate lineages, but many of their essential features remain poorly understood, including the transcriptional networks that establish superfast identity, the specific rate-limiting determinants of extreme contractile speed, and the extent to which these molecular features are conserved across vertebrates. Whether superfast contractility represents quantitative enhancement of the canonical fast-fiber program or instead constitutes a qualitatively distinct, mutually exclusive design is also unresolved. Superfast muscles have probably evolved multiple times³, and understanding how the traits underlying superfast performance evolved is an open question.

Muscle contraction results from attachment-detachment cycles between myosin heads and actin. This process is regulated by Ca^{2+} release from the sarcoplasmic reticulum and by troponin conformational changes that allow actomyosin crossbridge formation and generation of force¹⁰. Three major factors were proposed to confer superfast contractility: 1) rapid Ca^{2+} release and reuptake, 2) fast Ca^{2+} dissociation from troponin, and 3) accelerated crossbridge detachment rate¹. The first two are well-characterized at the cellular level³, but the molecular basis for rapid myosin detachment remains unresolved. The field has long attributed superfast cross-bridge kinetics (50-fold faster than in rabbit fast-twitch muscle) to specialized myosin heavy chain (MyHC) isoforms¹. MYH13 is known as the “superfast” myosin because it is enriched in EOM, ILM, and the syringeal muscles used for birdsong⁹, and its isolated motor domain detaches from actin unusually rapidly^{11,12}. However, MYH13 cannot fully explain superfast contractility: the gene is either missing or not expressed in several vertebrates that possess superfast muscles and,

conversely, it is present in some non-superfast muscles^{3,9,13–15}. These observations indicate that additional molecular adaptations must exist.

Myosin-binding proteins (MyBPs) represent another class of sarcomeric regulators. While MyBP-C family members (MYBPC1-3) slow cross-bridge detachment and enhance Ca²⁺ sensitivity through N-terminal interactions^{16–18}, the truncated MyBP-H subfamily (MYBPH, MYBPHL) lacks these regulatory domains⁵. MYBPH is highly expressed in developing fast-twitch skeletal muscle but downregulated in adulthood^{19,20}, while MYBPHL has only been reported in the mammalian heart^{4,6}. Whether members of the MyBP-H subfamily contribute to superfast contractility is unknown.

To dissect the molecular basis of superfast contractility in syncytial skeletal muscle cells, we performed single-nucleus transcriptomic and epigenomic profiling. We identify *Mybphl*, a sarcomeric protein long considered heart-restricted, as a crucial determinant of accelerated relaxation speed in mammalian superfast muscles. Using gene regulatory network analysis, gain- and loss-of-function experiments, we show that *Mybphl* expression is gated by the transcription factor *Tbx15*, in fibres that already run the canonical fast-twitch program. Sampling a broad spectrum of vertebrates, we find that an elevated stoichiometric ratio of MyBP-H to MyBP-C transcripts is a conserved molecular signature of superfast muscles. Our work identifies a conserved molecular adaptation that tunes relaxation kinetics to meet extreme physiological demands, along with the regulatory threshold that switches it on.

Results

***Mybphl* is a sarcomeric protein specific to the fastest fibers of extraocular and laryngeal muscles**

To identify regulators of superfast contractility, we applied single nucleus RNA sequencing (snRNA-seq) to mouse EOM, which enable high frequency eye movements, and to the *Tibialis Anterior* (TA), a conventional fast limb muscle (Fig. 1A and B; Suppl. Fig. 1A-J, Extended Data Table 1 and 2). Because skeletal muscle fibers contain many nuclei, snRNA-seq can resolve individual myonuclear transcriptomes rather than averaging signals across fiber types. Unexpectedly, this approach revealed few sarcomeric transcripts specific to EOM, including *Myh13*, the superfast myosin, and *Mybphl*, a heart-specific sarcomeric regulator (Fig. 1C).

Despite its "superfast" designation, *Myh13* was mainly detected in *Myh1*-expressing myonuclei (Fig. 1D and E), which correspond to fibers with slower contractile kinetics (20). *Mybphl*, by contrast, was present in faster *Myh4+* myonuclei in EOM (Fig. 1D and E). To test *Mybphl* specificity to superfast muscles and assess the relative proportions of all MyBPs in a variety of striated muscles, we re-analyzed published bulk RNA-seq data²¹. This analysis validated that, among skeletal muscles, *Mybphl* mRNA is restricted to EOM and ILM, i.e., the two superfast muscle groups present in rodents (Fig. 1F; Suppl. Fig. 1K), in addition to previously documented expression in cardiac atria^{4,6}. Superfast muscles also featured a reduced proportion of *Mybpc2* (Fig. 1F), the canonical MyBP of fast, force generating, sarcomeres²².

Using a specific *Mybphl* mouse antibody⁶, we confirmed protein expression in EOM and ILM (Fig. 1G-L). In EOM, *Mybphl* colocalized with fast (*Myh4+*) fibers of the global muscle layer (GL; i.e. facing the globe) but was excluded from fibers of the orbital layer (OL; i.e. closer to the orbit) and slow (*Myh7+*) myofibers (Suppl. Fig. 2A-C). In ILM, *Mybphl* was detected in all

muscles with exception of the cricothyroid (CT) muscle (Fig. 1I-J, L), correlating with Myh4 expression at the mRNA and protein level as in EOMs (Suppl. Fig. 2D-F). The relationship between Mybphl and Myh13 differed between muscles: in the larynx, Mybphl broadly overlapped with Myh13⁺ fibers, while in EOM it was excluded from Myh13⁺ orbital layer fibers (Suppl. Fig. 2A1, D1). These patterns demonstrate that Mybphl, rather than Myh13, consistently marks the fastest Myh4⁺ fibers in superfast muscles.

To assess conservation, we examined primate muscles, which display inherent slower contractile kinetics than rodents²³. Using a human MYBPHL antibody⁵, we detected robust protein expression in *Macaca fascicularis* EOM and TAM, as well as in the global layer of human EOM (Suppl. Fig. 2G-I). Notably, these are the only muscles reported to express *MYH4* mRNA^{24,25}, indicating that MYBPHL expression in primates is correlated with the myofibers displaying the “fast” program, despite the lack of MYH4 protein²⁶.

In summary, we show Mybphl to be a new sarcomeric component specifically associated with the fast *Myh4*⁺ myonuclei of mammalian superfast muscles where it coincides with markedly reduced levels of Mybpc2.

Mybphl accelerates sarcomere relaxation in superfast muscles

We then evaluated the precise location of Mybphl on superfast sarcomeres using super-resolution structured illumination microscopy. Immunostaining against Mybphl showed a doublet pattern around the M-line, with a spacing coherent with C-zone localization (Fig. 2A and B). To determine the functional role of Mybphl in superfast muscles, we analyzed Mybphl knockout mice⁴. While MyHC expression remained unchanged (Suppl. Fig. 3A-C), quantitative mass spectrometry revealed compensatory upregulation of Mybph and Mybpc2, in absolute and relative levels, in mutant EOM and TAM (Suppl. Fig. 3D-G).

To test whether Mybphl loss and changes in MyBP abundance affect contractile kinetics in EOM and ILM, we assessed contractile kinetics using a subcellular myofibril assay under controlled Ca²⁺ conditions²⁷ (Fig. 2C-E, Suppl. Fig. 4A and B). Myofibril relaxation occurs in two distinct phases: an initial slow linear (isometric) phase due to actomyosin crossbridge detachment, followed by a fast exponential phase driven by inter-sarcomere dynamics²⁸. In both mutant muscles, the linear relaxation phase ($T_{REL, LINEAR}$) was significantly prolonged, and the kinetics of this phase ($K_{REL, LINEAR}$) reduced, indicating a specific conserved role for Mybphl in accelerating crossbridge detachment in mammalian superfast muscles (Fig. 2E-G, Suppl. Fig. 4B). The maximal force (F_{max}) and rate of activation (K_{ACT}) were unaffected in the mutant (Suppl. Fig. 4C and D). The kinetics of the exponential phase of relaxation and rate of tension redevelopment after re-stretch (K_{TR}), which is an indicator of crossbridge cycling, were reduced in only ILM (Suppl. Fig. 4C and D). The selective impairment of the linear phase of relaxation is in line with altered strain-dependent crossbridge detachment; phenotype observed in N-terminally truncated MyBP-C variants¹⁸, and further supported by our computational modeling approach (Suppl. Fig. 4E-H).

To confirm these findings, we overexpressed mouse *Mybphl* in hindlimb muscles via intramuscular AAV delivery and performed whole-muscle *ex vivo* contractility assays in isolated fast-twitch *Extensor Digitorum Longus* (EDL) muscle²⁹ (Fig. 2H, Suppl. Fig. 4I). Mybphl gain-of-function caused a rightward shift of the force-frequency curve compared to controls (Fig. 2I and J), which reflects accelerated relaxation in superfast muscles¹ and mirrors N-terminal-truncated MyBP-Cs^{17,18}. Taken together, our data point to a specific function of Mybphl to enable

the high-frequency contractile cycling characteristic of superfast muscles (Fig. 2K), a function antagonized in muscles expressing only MyBP-C isoforms.

Integrated multiome profiling identifies *Tbx15* as a regulator of *Mybphl*

To identify regulators of *Mybphl*, we performed single nucleus multiome sequencing (10X Genomics) to simultaneously profile gene expression (snRNA-seq) and open chromatin landscapes (snATAC-seq) in individual myonuclei on superfast EOM and ILM tissues. We compared this with publicly available data on fast limb muscles³⁰ (Fig. 3A; Suppl. Fig. 5A-J, Extended Data Table 3). We found that chromatin accessibility landscapes at the *Mybphl* locus, particularly within the first intron rich in H3K27ac marks (Suppl. Fig. 6A), are very similar in both EOM and ILM fast (*Myh4+*) myonuclei (Fig. 3B), suggesting that common cis-regulatory mechanisms govern superfast identity.

To dissect the transcriptional regulation of *Mybphl*, we performed gene regulatory network (GRN) analysis using a custom Pando-based pipeline³¹. We identified several common transcription factors associated with *Mybphl* regulatory elements, including *Maf* and *Tbx15* (Fig. 3C and D, Suppl. Fig. 6B, Extended Data Table 4), which are known regulators of the canonical fast-twitch limb myofiber program^{30,32–36}. *Tbx15* expression and regulon activity correlated with *Mybphl* chromatin accessibility and expression (Fig. 3D; Suppl. Fig. 6C-F, Extended Data Table 5), suggesting suprathreshold *Tbx15* levels are required for *Mybphl* induction.

While *Maf* levels were comparable across muscles, *Tbx15* was enriched in EOM and ILM (Fig. 3E and F, Suppl. Fig. 6C). *Tbx15* expression and regulon activity correlated with *Mybphl* chromatin accessibility and the ON/OFF *Mybphl* pattern across superfast *versus* fast muscles (Fig. 3D; Suppl. Fig. 6C-F). Altogether, our *in silico* analysis suggests that suprathreshold *Tbx15* levels (i.e., levels exceeding those in conventional fast muscle), and not merely the presence of *Tbx15*, are necessary for *Mybphl* induction.

To confirm these findings, we examined *Tbx15* and *Maf* triple knockout (TKO) mice. As expected, Mybphl protein persisted in *Maf* TKO EOM and ILM (Suppl. Fig. 7A-C), indicating that Maf transcription factors are only partially required for *Mybphl* expression (Suppl. Fig. 7B, C). *Tbx15* was unchanged in the TKO, placing *Tbx15* outside direct Maf control (Suppl. Fig. 7B, C). In contrast, analysis of *Tbx15* knockout mice at birth showed a strong reduction of Mybphl in EOM and ILM (Fig. 3G and H). These data demonstrate that *Tbx15* is required for *Mybphl* activation during postnatal development of superfast muscles.

To test if high levels of *Tbx15* are sufficient to activate *Mybphl*, we overexpressed *Tbx15* in adult wildtype mouse hindlimbs using AAV intramuscular delivery (Fig. 3I). Three weeks post-injection, Mybphl protein was detected exclusively in *Tbx15*-transduced limb muscles (Fig. 3J and K), without altering the overall fiber type composition (Suppl. Fig. 7D and E). Interestingly, *Tbx15* overexpression induced Mybphl exclusively in *Myh4+* fibers, while Mybphl overexpression itself can occur in all fiber types (Fig. 3J, Suppl. Fig. 7F and G). This data indicates that *Tbx15* requires a fast *Myh4+* fiber context to trigger *Mybphl* expression. Functionally, AAV-*Tbx15* mimicked the contractile phenotype of AAV-Mybphl, with a rightward shift of the force frequency curve (Suppl. Fig. 7H and I), pointing towards accelerated relaxation kinetics.

Together, these results demonstrate the *Tbx15* acts as a molecular switch for *Mybphl* within a fast-fiber context and that complete rewiring of the contractile apparatus is not required to achieve accelerated relaxation (Fig. 3L).

Concomitant expression of MyBPs of the H subfamily and *Tbx15* are conserved features of superfast muscles across vertebrates

As different superfast muscles are powered by different motor proteins argues strongly in favor of the muscles evolving independently³, but the existence of other common sarcomeric components enabling superfast contractility is unknown. Given our findings that *Mybphl* is a specific component of the superfast mammalian skeletal sarcomere, we sought to assess the evolution proteins of the MyBP-H subfamily using an unbiased phylogenetic analysis of all UNIPROT protein sequences of mouse *Mybphl* and *Mybph* orthologs (Fig. 4A). We confirmed the existence of an ancestral gene that underwent two independent duplication events in vertebrates: one within actinopterygians (ray-finned fishes), resulting in *mybpha* and *mybphb*, and a separate duplication within mammals, generating *MYBPH* and *MYBPHL*. Sauropsids possess a single gene called *MYBPH*, while agnathans lack it entirely.

Across vertebrates, superfast muscles trade-off force for speed^{1,2,7,8}. In canonical fast-twitch muscles, *Mybpc2* is essential for maximal speed and force of contraction, sarcomere integrity, and calcium sensitivity in fast-twitch muscle³⁷. The co-occurrence of a high ratio of *Mybphl/Mybpc2* in mammalian superfast muscles prompted us to test whether this pattern is conserved across vertebrates. We analyzed transcriptomic datasets from fast and superfast sound-producing muscles in five species, alongside our own mouse multiome data (Fig. 4B-K). *Pteronotus parnellii* is a bat species that uses superfast CT muscles for ultrasonic vocalizations during echolocation but lacks *MYH13* expression³. Mirroring the mouse larynx, *MYBPHL/MYBPC2* ratio was significantly higher in bat CT than in fast breast muscle, as were *TBX15* levels (Fig. 4B-E).

Songbirds rely on superfast syringeal muscles that express *MYH13*^{38,39}. Zebra finch (*Taeniopygia guttata*) syringeal muscles exhibited a much higher proportion of *MYBPH* mRNA than limb muscle, whose composition was dominated by *MYBPC2* transcripts (Fig. 4F). Reanalysis of proteomic data³⁸ confirmed *MYBPH* as the most abundant MyBP in the male syrinx (Suppl. Fig. 8A). *TBX15* mRNA was enriched in syringeal muscles compared to limb muscles (Fig. 4G). Similarly, in rattlesnake, which lack *MYH13*¹⁵, superfast tail shaker muscles had substantially higher levels of *MYBPH* and *TBX15* than those in cranial muscles (Fig. 4H-I). *MYBPC2* transcripts were virtually absent in tail shaker muscles.

Within actinopterygians, the small teleost *Danionella cerebrum* can produce “clicks” at a frequency up to 120 Hz by accelerating a specialized cartilage with their “drumming” muscle^{40,41}. In this fish, *mybphb* reaches ~97% of all MyBPs transcripts in drumming muscles, compared to 58% in fast trunk muscles (Fig. 4J). By contrast, *mybpc2b* represents only ~2% of MyBPs in drumming muscles, but 37% in trunk muscles. Drumming muscle MyBP composition mirrors that of zebrafish larval muscles that power high-frequency swimming activity¹⁹. Notably, adult zebrafish EOM, which display superfast behaviour, express high ratio of *mybpha* and *mybphb* compared trunk muscles, with *mybpc2b* being fully absent (Suppl. Fig. 8B). In both fish species, *tbx15* transcripts are enriched in all superfast muscles (Fig 4K, Suppl. Fig. 8C).

Together, our data reveal a conserved signature across vertebrate superfast muscles with higher levels of either: 1) a specific member of the *MyBP-H* subfamily (*MYBPHL* in mammals, *mybpha* and/or *mybphb* in fish) or 2) the unduplicated ancestral *MYBPH* gene (in sauropsids), both at the expense of *MYBPC2* isoforms usually associated with force production. What seems to recur across vertebrate superfast muscles is therefore not the identity of the motor protein, which differs between lineages, but a universal “speed module” dependent on the stoichiometry of the MyBPs and *Tbx15* levels.

Discussion

Here we identify *Mybphl* as a sarcomeric regulator that is, among skeletal muscles, specific to the fastest mammalian fibers, and implicate *Tbx15* as a regulator of this phenotype, in a role that appears to be that of a molecular switch. These are new roles for both proteins, and may explain both the occurrence and evolutionary trajectory of superfast muscles.

While accelerated crossbridge detachment rate is recognized as the most important factor for superfast performance^{1,3}, previous work mostly focused on MyHC adaptations to support this function. We show that *Mybphl* is required for accelerated relaxation, likely through competitive displacement of MyBP-C isoforms on the thick filament. Our findings demonstrate that MYBPHL regulates the linear phase of relaxation, consistent with studies using N-terminally truncated forms of MyBP-C^{17,18}. However, while *Mybphl* accelerates relaxation in EOM and ILM, it paradoxically slows relaxation in the atria^{5,42}. This tissue-specific discrepancy likely arises from differences in MyBP-C isoforms (e.g., by phosphorylation) across muscle types⁴³. Since MYBPHL and MyBP-Cs compete for the same myosin-binding sites on the C-zone, their relative abundance and biochemical properties may determine the net effect on relaxation kinetics.

While mammals have two genes of the MyBP-H subfamily - with MYBPHL expression restricted to superfast muscles - many other vertebrates possess only a single MyBP-H gene. We propose that a high ratio of MyBP-H/MyBP-C isoforms, and not merely the presence of MYBPH or MYBPHL, is a distinctive molecular feature of superfast muscles that predates the diversification of specialized fast MyHC isoforms such as *MYH13* in birds³. Previous phylogenetic analysis of human, mouse, rat, and zebrafish suggested that these proteins arose from the duplication of an ancestral MyBP-C gene which subsequently lost its N-terminal domain¹⁹. How gene duplications could have led to further specialization of MyBP-H paralogs in mammals to support different contractile properties is an interesting future question.

In the mouse, *Tbx15* was already known to specify fast, glycolytic fibre identity in limb muscle³². What distinguishes superfast muscle is not the presence of *Tbx15* but its level on top of an existing fast *Myh4+* program to further refine relaxation kinetics. It is also striking that *Mybpc2*, the MyBP isoform historically associated with the fastest skeletal fibers, constitutes a small proportion of all MyBPs in superfast muscles. We hypothesize that Maf-dependent fast-twitch myonuclei can adopt either a canonical “fast” *Mybpc2*-dependent or a “superfast” MyBP-H subfamily identity, with *Tbx15* serving as a molecular switch between these two profiles. While the upstream factors that trigger high levels of *Tbx15* in specific muscle groups remain unknown, the presence of high levels of *Tbx15* in both head and tail muscles rules out a common cranial versus trunk developmental origin for these expression differences. Comparative study of ILM development in bats and other mammals could reveal evolutionary changes in gene regulation that led to high levels of *Tbx15*. Rather than evolving entirely new sarcomeric components, superfast muscles may have achieved their extreme performance by repurposing and changing the proportions of existing proteins, guided by transcription factors already present in fast-twitch fibers.

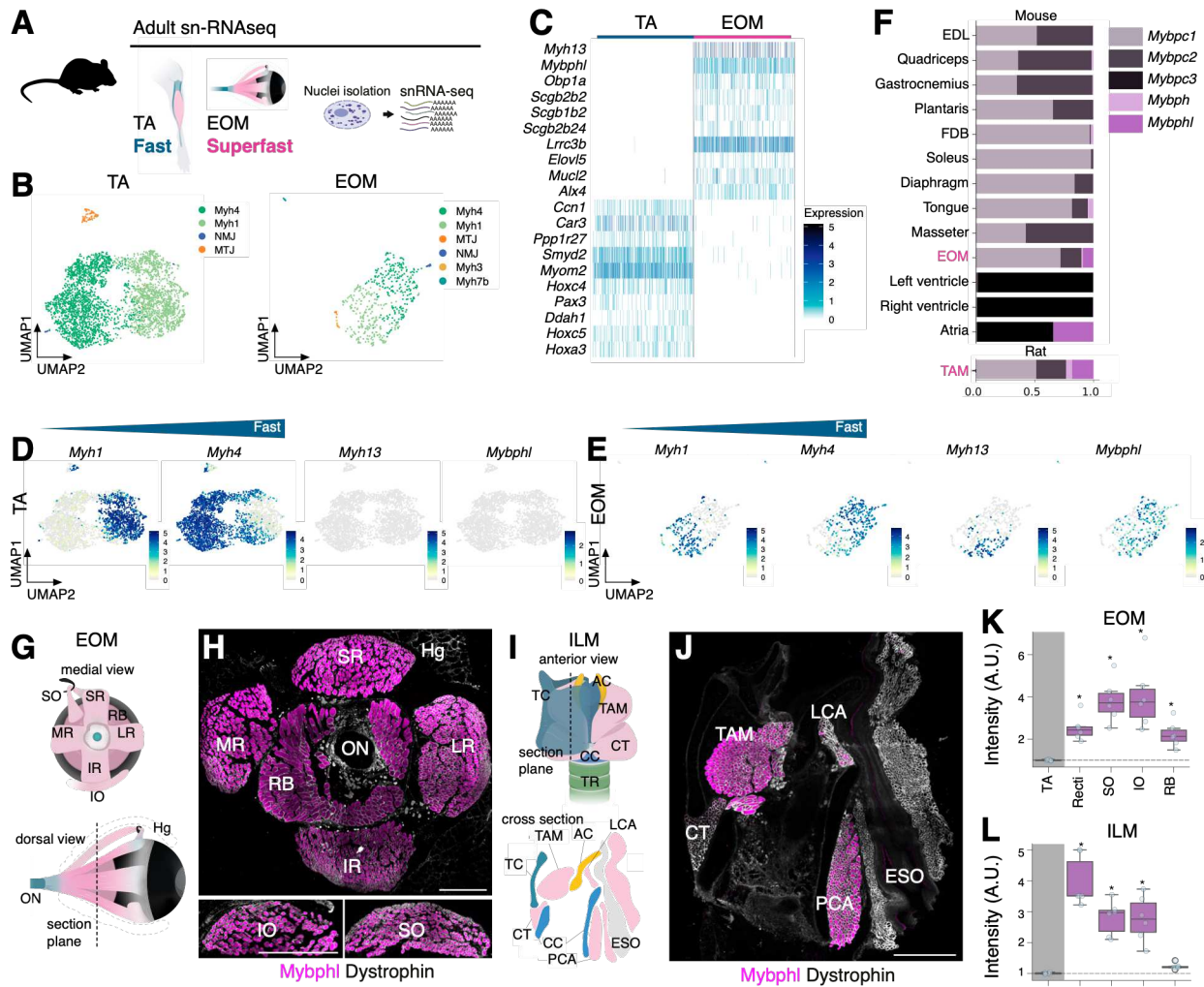


Fig. 1. Mybphl is a new skeletal muscle protein of the fastest fibers of extraocular and laryngeal muscles. (A) Scheme of the experimental approach used for snRNA-seq of adult fast (TA) and superfast (EOM) muscles. (B) Uniform manifold approximation and projection (UMAP) visualization of TA and EOM myonuclear subpopulations. (C) Heatmap of the top 10 differentially expressed genes sorted by log fold change in TA and EOM myonuclei. (D-E) UMAP plots showing expression of several markers to identify different myonuclear types. (F) Relative expression of MyBP transcripts among skeletal and cardiac mouse muscle tissues (re-analysis of ²¹). Superfast muscles highlighted in pink. (G) Diagram of mouse EOM anatomy. (H) Immunostaining of adult mouse EOM cryosections. (I) Diagram of mouse ILM anatomy. (J) Immunostaining of adult mouse laryngeal cryosections. (K-L) Box plot diagram of Mybphl intensity of fluorescence measurements (in arbitrary units, A.U.) in individual EOM and ILM relative to TA intensity (set to 1). N=5 biological replicates. AC, Arytenoid cartilage; CC, cricoid cartilage; CT, cricothyroid muscle; EDL, extensor digitorum longus; EOM, extraocular muscles; ESO, esophagus striated muscle; FDB, flexor digitorum brevis; Hg, Harderian gland; IO, inferior oblique; IR, inferior rectus; LCA, lateral cricoarytenoid; ILM, intrinsic laryngeal muscles; LR, lateral rectus; MR, medial rectus; MyBP, myosin binding protein; ON, optic nerve; PCA, posterior cricoarytenoid muscle; RB, retractor bulbi; SO, superior oblique; SR, superior rectus; TA, tibialis anterior; TAM, thyroarythenoid muscle; TC, thyroid cartilage; TR, trachea. Scale bars, 500µm in H,J.

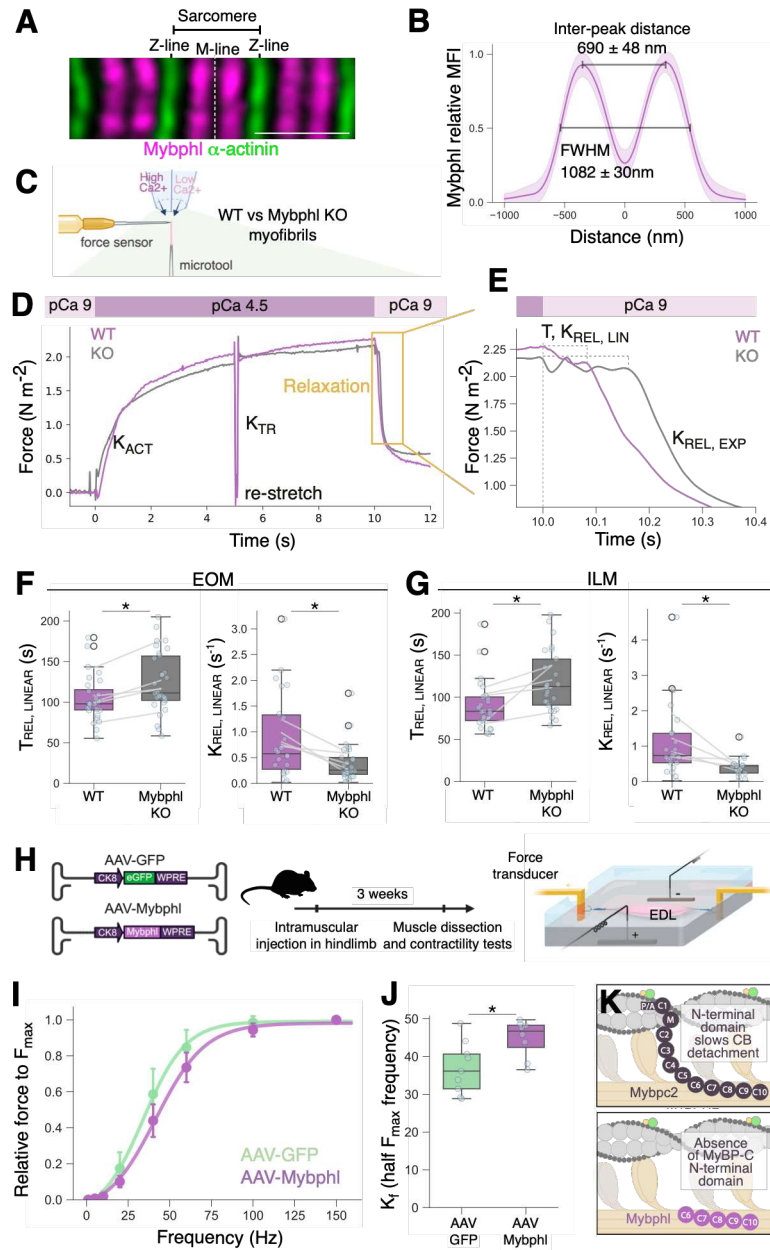


Fig. 2. Mybphl accelerates sarcomeric relaxation in superfast muscles. (A) Representative super-resolution microscopy of ILM myofibres (thyroarythenoid muscle). A doublet pattern of Mybphl is seen between two Z-line stained with anti- α -actinin antibody. (B) Quantification of Mybphl doublet distances centered on M-line in A). (C) Scheme of the setup of the single-myofibril contractility assay. (D) Trace of a representative single myofibril force measurement from wildtype (WT) or Mybphl^{KO} EOM. pCa changes correspond to force activation (k_{ACT}), redevelopment (k_{TR}) and relaxation. (E) Expanded trace of the relaxation phase in C) composed of linear ($T, K_{REL, LIN}$) and exponential ($K_{REL, EXP}$) phases. (F-G) Quantification of single myofibril force measurements from EOM (recti) and ILM (thyroarythenoid muscle) (N=5 biological replicates per muscle and group). Linear mixed model was used with day of experiment as an independent random factor. For EOM, p-values = 0.008 (K_{LIN}) and 0.001 (T_{LIN}). For ILM, p-values = 0.006 (K_{LIN}) and 0.001 (T_{LIN}). (H) Schematic of the experimental pipeline for Mybphl overexpression through intramuscular injection of AAV followed by *ex vivo* EDL contractility assay. (I) Force-frequency curve of AAV injected EDL muscles. Force is relative to the maximal force elicited at

150 Hz. A sigmoid function is fitted on the mean points per condition. **(J)** Frequency eliciting half of the maximal force (K_f). N=9 AAV-GFP and N=8 AAV-Mybphl biological replicates. Mann-Whitney U two-sided test with Benjamini-Hochberg correction, p-value = 0.0420. Same AAV-GFP control samples as in fig. S7I. **(K)** Proposed mechanism of action of Mybphl through competition with MyBP-C. C, globular domain; CB, crossbridge; EOM, extraocular muscles; MyBP, myosin binding protein; TAM, thyroarythenoid muscle. M, M-domain; P/A, proline alanine rich region. Scale bar, 2 μ m in A.



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observed in superior and lateral recti EOM at this stage. **(H)** Quantification of Mybphl intensity of fluorescence from EOM and TAM in (G). Mean intensity was measured over all segmented fibers in the superior rectus and normalized based on fluorescence intensity in the levator palpebrae muscle (negative control). For EOM and TAM, N=5 biological replicates for each group. Mann-Whitney U two-sided test with p-value = 0.0079 for EOM and p-value = 0.0119 for TAM. **(I)** Scheme of the experimental approach for Tbx15 overexpression in limb muscles using intramuscular AAV injection. **(J)** Immunostaining for Mybphl on EDL cryosections 3 weeks post injection of AAV-Tbx15 or AAV-GFP (control). **(K)** Quantification of Mybphl intensity of fluorescence from (J). **(K)** Model of Tbx15-dependent Mybphl regulation in mouse skeletal muscles. EOM, extraocular muscles; ILM, intrinsic laryngeal muscles; TF, transcription factors; TSS; transcription start site. Scale bars, 50µm in E; 200µm in G,J.

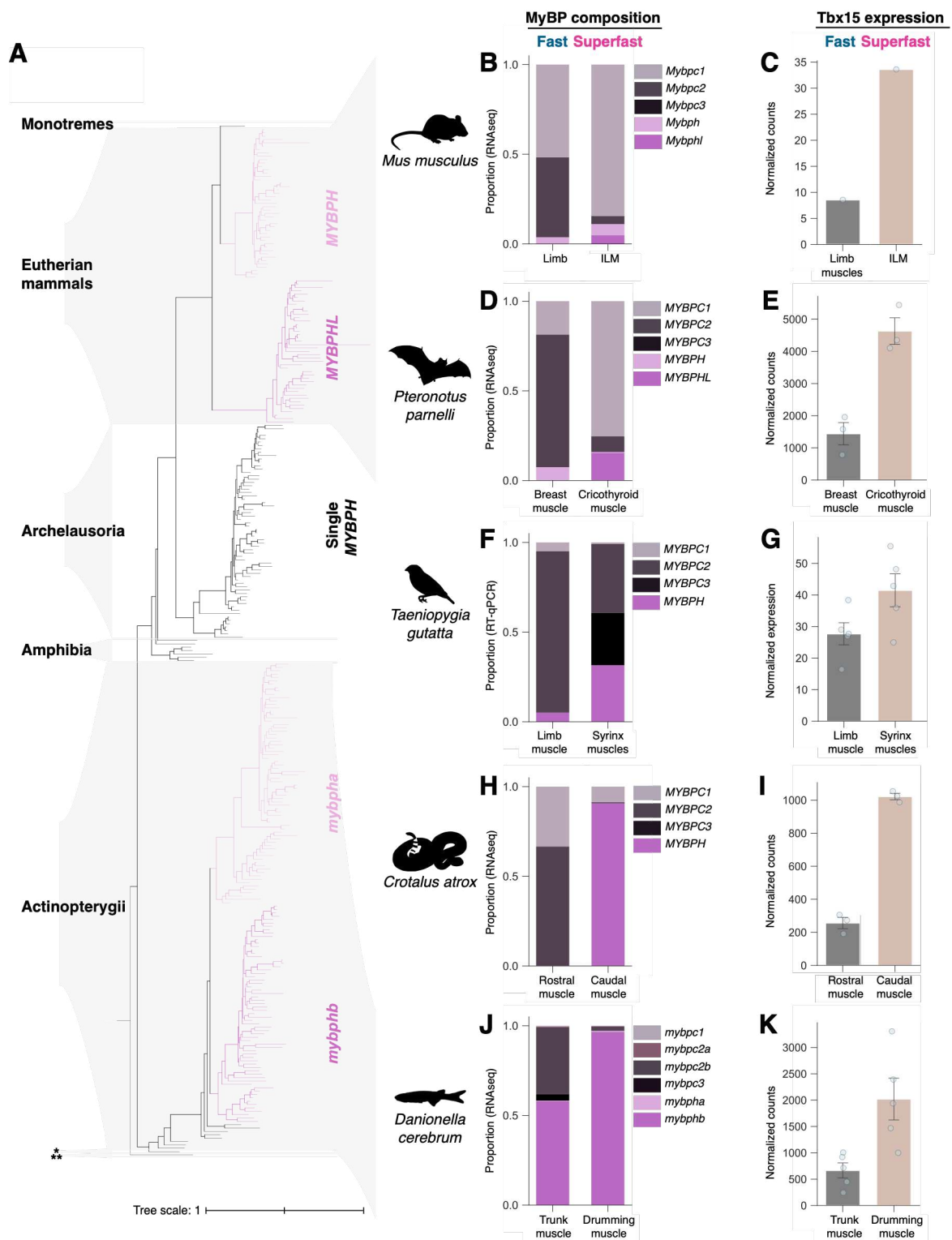


Fig. 4. MyBP-H and Tbx15 are conserved features of superfast muscles in vertebrates. (A) Phylogenetic tree of MyBP-H proteins in vertebrates using BLAST of UNIPROT database. Scale corresponds to number of substitutions per site. * Coelacanthiformes, ** Holocephali. (B) Relative expression of MyBPs in pseudo-bulk of mouse multiome RNA counts. (C) Expression levels of *Tbx15* in pseudo-bulk of mouse multiome RNA counts. (D) Relative expression of MyBPs in bat bulk RNA-seq. N=3 biological replicates per group. (E) Expression levels of *Tbx15* in bat bulk RNA-seq. (F) Relative

376 expression of MyBPs in zebra finch by RT-qPCR. N=5 biological replicates per group. **(G)** Expression
377 levels of *Tbx15* transcripts in zebra finch skeletal muscles by RT-qPCR relative to TBP. **(H)** Relative
378 expression of MyBPs in rattlesnake bulk RNA-seq. N=3 biological replicates per group. **(E)** Expression
379 levels of *Tbx15* in rattlesnake bulk RNA-seq. **(J)** Relative expression of MyBPs in *Danionella cerebrum*
380 bulk RNA-seq. N=5 biological replicates per group. **(K)** Expression levels of *Tbx15* in *Danionella*
381 *cerebrum* bulk RNA-seq. Normalised counts in C, E, I, K are displayed as length-scaled transcripts per
382 million based on Salmon output. EOM, extraocular muscles; MyBP, Myosin binding protein.

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Materials and Methods

Animals

The following strains were previously described: *Tbx15*^{KO} ⁴⁴, *Tg(HSACre);Mafa*^{-/-}; *Maifb*^{flox/flox}; *Maif*^{flox/flox}, designated here as *MaifTKO* ³⁵, *Tg(HSACre)* ⁴⁵, *R26*^{H2BGFP} ⁴⁶, *Mybphl*^{KO} ⁴. Animals were handled according to national and European Community guidelines and Institut Pasteur ethics committee approved the protocols (APAFIS#41051-202302204207082). Except when indicated otherwise, B6D2F1/JRj males and females of 2-4 months were used (Janvier Labs). *Mybphl* mutant animals were housed and sacrificed in accordance with Loyola University Chicago's Institutional Animal Care and Use Committee in adherence to the US National Institutes of Health Guide for Care and Use of Laboratory Animals. Triple *Maif* mutant mice were handled in compliance with the relevant Japanese and institutional laws and guidelines and were approved by the University of Tsukuba Animal Ethics Committee (authorization number: 21-185). Pups were euthanized at P0 by decapitation and adult mice were euthanized by cervical dislocation. *Tbx15*^{KO} mice were analysed at birth due to perinatal lethality ⁴⁴. Genotyping primers are listed in **Suppl. Table 1**.

End-point macaque samples were obtained from Paris Brain Institute Primate facility under projects have been ethically assessed and authorized in these laboratories. Anesthetized animals were perfused with 1X PBS then, with 500mL of PFA 4% and tissues dissected under a chemical hood.

Lab-bred zebra finches (*Taenio-pygia castanotis*) housed at the University of Southern Denmark (SDU) were sacrificed by an isoflurane overdose and immediately perfused transcardially with dissection buffer to remove blood cells. Muscles were dissected on ice, snap frozen in liquid nitrogen and stored at -80°C.

Human EOM samples were collected from fresh cadavers at the Jan Palfijn Anatomy Lab of KU Leuven campus Kulak, within 24 hours post-mortem. All donors provided written consent, and the study received approval from the Institutional Review Board and the Ethical Committee (EC Zorg, NH019-2020-04-02). The EOM of each eye were isolated by performing orbital exenteration following previously described procedures ⁴⁷. Once the muscles were separated from the globe, any residual fat and connective tissue were carefully removed. After embedding in OCT medium (VWR, #361603E), the muscles were snap-frozen in isopentane chilled in liquid nitrogen, shipped to Pasteur Institute and stored at -80 °C until further use.

Quantitative analysis of transcripts using reverse transcription PCR

EOM, ILM, and hindlimb muscles from *MaifTKO* and control mice were dissected from male mice at 10–11 weeks of age immediately after euthanasia. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized from 500 ng of total RNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR was performed with THUNDERBIRD Next SYBR qPCR Mix (Toyobo) on a CFX Opus 384 Real-Time PCR System (Bio-Rad, California, US). Transcript levels were normalized to TATA-box-binding protein (Tbp) mRNA.

EOM, vocal muscles and limb muscles were dissected from adult male and female zebra finches immediately after transcardial perfusion with PBS to flush out blood cells. Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. After RNA-extraction, a second DNase treatment was performed using the DNA-free

DNA Removal Kit (Invitrogen, cat. # AM1907) according to the manufacturer's protocol. First strand cDNA synthesis was performed from 44 ng of total RNA using SuperScript™ IV Reverse Transcriptase (Life Technologies) and random hexamer primers. QPCR reactions were performed on a CFX96 thermocycler (Bio-Rad, Hercules, CA) RealQ Plus 2x Master Mix Green (Ampliqon). A dilution series of a standard plasmid was included for each primer pair for absolute quantification. Primers are listed in **Suppl. Table 2**.

Immunofluorescence

Dissected mouse, human and macaque adult tissues, were embedded in Optimum Cutting Temperature (OCT) compound, snap-frozen in isopentane chilled in liquid nitrogen and stored at -80°C. For macaque samples, perfused muscles were rinsed in PBS for 12h, then equilibrated in sucrose 30% in 1X PBS before embedding.

Cryosections (14-16µm) were prepared with a Leica Cryostat CM1850 and stored at -80°C. For immunostaining, slides were warmed to room temperature, washed in PBS, and blocked 1 hr in 10% normal goat serum, 3% BSA, 0.5% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. After 3 rounds of 10-15 min washes in PBST (1X PBS with 0.1% Tween20), secondary antibodies were incubated in blocking solution 1 hr at RT together with 1 µg/ml Hoechst 33342 to visualize nuclei. After immunostaining, slides were postfixed for 5 minutes in 4% PFA in PBS, washed three times in PBS and mounted in PBS/70% glycerol. Slides were stored at 4° C until imaging. Antibodies used in this study are listed in **Suppl. Table 3**. Images were taken on a Nikon T2i inverted microscope equipped with a equipped with a Yokagawa CSU-W1 spinning disk (50µm pinhole), 20x objective and Orca Flash 4 (Hamamatsu) camera and NIS Elements Software. Z-stack images were processed with maximum intensity projection using Fiji software ⁴⁸ before further analysis.

RNA-FISH

Dissected muscles were fixed overnight in 4% PFA, washed in PBS 3 × 15 min, equilibrated in 15% and 30% sucrose, embedded in OCT and frozen in isopentane cooled in liquid nitrogen. Tissue blocks were stored at -80°C. 16 µm thick cryosections were collected on Superfrost Plus slides and used within a week.

RNAscope probes against *MmTbx15* (558761) and intronic probes for *Mm-Myh4* (539391-C3) and *MmMybphl* (1846041-C2) were designed commercially by the manufacturer and are available from Bio-technie. In situ hybridization was performed using the RNAscope Multiplex Fluorescent Reagent Kit V2 (Biotechnie, 323100) according to manufacturer's instructions with some modifications. For sample pre-treatments included 10 min at RT with Hydrogen Peroxide treatment, 2 min at 98°C with 1x Target retrieval and 10 min at 40°C with Protease Plus reagent. Probes were detected using TSA Vivid Fluorophore 650 (R&D, 7527/1) and 570 (R&D, 7526/1) (1/1000-1/1500) in RNAscope TSA Buffer (Biotechnie, 322809) After detection steps, slides were washed in PBS, and subjected to immunostaining for dystrophin as above. Slides were mounted on ProLong Gold Antifade Mountant (Fisher Scientific P36934) and scanned on either a Yokagawa CSU-W1 spinning disk or an LSM900 Zeiss Confocal microscope.

Bulk-RNA seq analysis

Published bulk-RNA seq fastq files were downloaded from SRA archives. *Mus musculus*, *Rattus norvegicus*, *Pteronotus parnelli*, *Crotalus atrox* and *Danio rerio* datasets were respectively aligned to GRCm39, mRatBN7, ASM3676855v, ASM1654583v1 and GRCz12tu NCBI genome assemblies using Salmon⁴⁹ index with decoys and quantification with useVBOpt, seqBias and gcBias parameters. Differential Gene Expression analysis was conducted with the DESeq2 package⁵⁰ on Salmon output using tximport. *Danionella cerebrum* dataset was processed as previously described⁴¹. Orthology of *Danionella* genes with zebrafish was established based on the table described in⁵¹.

Single nuclei RNA-seq

For snRNA-seq we used the protocol of⁵². Recti EOM were isolated from 8-week-old male C57Bl6/Rj mice. In the first snRNA-seq 6 TA from 3 mice and 20 EOM from 10 mice together with one harderian gland were sequenced. The second run only had the same number of EOM without harderian gland. Nuclei were then FACS sorted to exclude debris with a BD FACS Aria III and the BDFACSDIVA software. More than 200,000 nuclei were collected in a tube containing 200µl of wash buffer. After FACS sorting, nuclei were centrifuged 5 min at 500g at +4 °C, resuspended in 15µl of wash buffer and counted with a hemocytometer. The concentration of nuclei was then adjusted to 1000 nuclei/µl with wash buffer. Around 4000 nuclei were loaded per condition into the 10x Chromium Chip. Single-Cell 3' Reagent Kit v3 was used according to the manufacturer's protocol. Libraries were then sequenced by pair with a HighOutput flow cell using an Illumina Nextseq 500 with the following mode: 26base-pairs (bp) (10x Index + UMI), 8 bp (i7 Index), and 57 bp (Read 2).

Single nuclear multiome

Nuclei isolation: For EOM, recti muscles were isolated from 23 8-week-old males and females *Tg:HsaCre; R26^{H2BGF}* heterozygote or homozygote for the *R26^{H2BGF}* allele. For ILM, thyroarytenoid, lateral cricoarytenoid and posterior cricoarytenoid muscles from 30 B6D2F1/JRj 8-week-old females in addition to cricothyroid muscles from 6 B6D2F1/JRj 8-week-old females were isolated. Nuclei were extracted using a modified version of a previously described protocol¹³. EOM (without the harderian gland) and ILM were dissected, snap-frozen, and stored in liquid nitrogen. Dissected muscles were minced into small pieces in a petri dish on ice with scissors in 300µl hypotonic buffer (250 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl pH 8.0, 25 mM HEPES pH 8.0, 0.2 mM PMSF and 0.1mM DTT supplemented with 1X protease inhibitor (Merck #11873580001) and 1 U/µl RNase inhibitor (Roche #3335399001) in nuclease free water (Invitrogen #AM9937)). Muscle pieces were transferred with a 1 ml pipette and cut tip to a 2 ml hard tissue homogenizing columns containing six 2.8mm ceramic beads (P000911-LYSK0-A, Bertin Corp). KT03961-1-009.2) containing ceramic beads of mixed size. The dishes and tips were washed with 700µl hypotonic buffer. Samples were incubated on ice for 8 min and homogenized with Precellys 24 tissue homogenizer (Bertin instruments) for 20 sec at 5,000 rpm. Homogenized samples were passed once through a 70µm strainer (Dominique Dutscher, 352350), then once through 40µm strainer (Fisher Scientific 352340) and the filter washed with 300µl hypotonic buffer. The eluate was transferred into a 15 mL Falcon tube, and nuclei pelleted by centrifugation 5 min at 500g at 4°C with a swinging bucket rotor centrifuge (ref). The pellet was

resuspended in 500ul of wash buffer (DPBS (Gibco #14190-094) supplemented with 2% BSA (Sigma-Aldrich #A1595), 0.1mM DTT (Sigma-Aldrich #646563), 1X protease inhibitor (Merck #11873580001) and 1 U/μl RNase inhibitor (Roche #3335399001)) by tapping the tube and 5 smooth up/down with a P1000 tip. In case of WT animals, myonuclei were stained with 2,5μl of PCM1 antibody (Sigma-Aldrich, HPA023374) 10 min on ice. Nuclei were pelleted by centrifugation for 5min at 500 × g at 4 °C, resuspended in 500ul of wash buffer. 1μl of Alexa 555 Goat anti-rabbit secondary antibody was used to detect PCM1 and 2μl of an Alexa 647 conjugated PCNA antibody (Biolegend, 682203) to stain all the nuclei. In the case of *Tg:HS^{Cre}:R26^{H2BGFP}* mice only PCNA antibody was used. After 10 min on ice, nuclei were pelleted by centrifugation for 5 min at 500 × g at 4 °C, resuspended in 300μl of wash buffer and filtered once through a cell-strainer cap (Falcon #352235) placed on top of a 5mL Polypropylene Round-bottom tube (Falcon #352063) that minimizes nuclei adhesion to tube wall.

FACS sorting: Nuclei were FACS sorted to exclude debris with a BD FACSAria III and BD FACSDIVA and FlowJo v10.8.1 software. PCM1+ PCNA+ or GFP+ PCNA+ myonuclei were sorted in a 1.5 ml Eppendorf tube containing 300μl of FACS recovery buffer (DPBS supplemented with 10% BSA, 0.1mM DTT (Sigma-Aldrich #646563), 1X protease inhibitor and 1 U/μl RNase inhibitor). 100k nuclei were obtained per sample upon 15-20min using a 70μm nozzle. EOM nuclei were pelleted as above and then resuspended in 200μl of ATAC lysis buffer (10 mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM MgCl₂, 0.01% Tween-20, 0.01% Nonidet-TM P40, 0.001% Digitonin, 1% BSA, 1 mM DTT, 1U/ul RNase inhibitor in Nuclease-Free Water). After 2 min incubation on ice, 1.3 ml of ATAC wash buffer (10 mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% Tween-20, 1 mM DTT, 1% BSA, 1U/μl RNase inhibitor in Nuclease-Free Water) was added. Nuclei were pelleted by centrifugation for 10 min at 500 × g at 4 °C and resuspended in 20μl of diluted nuclei buffer from 10Xgenomics. For laryngeal muscles, the lysis step was omitted. After FACS, nuclei were pelleted and resuspended directly in 700μl of ATAC wash buffer. Nuclei were pelleted by centrifugation for 7 min at 500 × g at 4 °C and resuspended in 10μl of diluted nuclei buffer from 10Xgenomics. Complete sample lysis, nuclei morphology and number were assessed on a fraction of the nuclei preparation using a Luna FX7 (Logos biosystems) cell counter upon addition of Acridine Orange /Propidium iodide (Logos biosystems F23011) at 1/10 dilution. Nuclei concentration was adjusted to have 3000-4000 nuclei/μl.

Library construction: 16.000 and 20.000 nuclei per EOM and laryngeal muscles were respectively loaded into the 10x Chromium Chip (10X Genomics). The Next GEM single-cell Multiome ATAC+ Gene expression kit was used following the manufacturer's instructions. Briefly, the nuclei suspension was used for the transposase step and then loaded onto a controller Chromium instrument to generate gel beads in emulsion (GEM). After GEM generation, reverse transcription incubation was performed to produce barcoded cDNA, which was then amplified by PCR for RNA-seq and ATAC-seq libraries construction. The quality of the prepared libraries was assessed using the Bioanalyzer High Sensitivity kit (Agilent) for qualitative analysis and the Qubit dsDNA HS Assay Kit (ThermoFisher) for quantification. Gene expression libraries were then sequenced with a P2 flow cell using an Illumina Nextseq2000 with a loading concentration of 660pM and with the following mode: Read 1 N – 28 cycles, i7 index – 10 cycles, i5 index – 10 cycles, Read 2 N – 90 cycles. ATAC-seq libraries were then sequenced with a P2 flow cell using an Illumina Nextseq2000 with a loading concentration of 660pM and with the following mode: Read 1 N – 50 cycles, i7 index – 8 cycles, i5 index – 24 cycles, Read 2 N – 49 cycles.

Uniform processing of single-nuclear multiome data: We computed count matrices and ATAC fragment files for newly collected data (EOM and larynx) using CellRanger (v7.0.0, mm10-2020-

A, for RNA data) or CellRanger-ARC (v2.0.2, mm10-2020-A-2.0.0, for multiome-data). Count matrices for limb multiome samples were obtained from the authors from the original study ³⁰, along with the annotations used in the original publication. To obtain matching ATAC fragment files, we downloaded the corresponding BAM files from the SRA database (accession SRP392860) and converted them using sinto (v0.10.1, <https://timoast.github.io/sinto>). Count matrices and fragment files were imported into Seurat ⁵³ and Signac ⁵⁴ for QC assessment and further processing. All ATAC count matrices were recomputed from fragment files using FeatureMatrix and we computed doublet scores for each modality (RNA and ATAC) using scDblFinder ⁵⁵. By default, we used the following criteria for cell inclusion in RNA assays: #genes $\geq$ 200, #UMIs $\leq$ 20,000, MT $\leq$ 10%, doublet score $\leq$ 0.75. In ATAC assays: 500 $\leq$ #fragments $\leq$ 50,000, FRiP $\geq$ 20%, blacklisted fragments $\leq$ 5%, nucleosome score $\leq$ 2, TSS enrichment $\geq$ 1, doublet score $\leq$ 0.75. For the limb dataset, we allowed for a slightly lower FRiP content (down to 10%) due to slightly lower ATAC quality in this dataset.

RNA and ATAC assays were then processed separately using the standard Seurat (v5.2.1) and Signac (v1.14) pipelines. RNA data were CP10K normalized and ATAC data TF-IDF normalized. Due to insufficient ATAC data quality, the final clustering and annotations were based solely on the RNA data. We extracted highly variable genes, computed the 30 top PCs, and clustered the data using default parameters. We performed a first round of annotation to identify myonuclei clusters, then repeated the clustering process on myonuclei only. During this second phase of clustering and annotation, we adapted the clustering to the complexity of the data to ensure uniform granularity across datasets: we removed clusters of low-quality myonuclei, adjusted the number of PCs and neighbors in the SNN graph according to the number of cells left, and subclustered clusters with clear subpopulation structure according to known myonuclei markers.

Cross-tissue gene regulation inference: To compare multiome data across tissues, we computed a set of consensus peaks (daisy chaining of overlapping peaks, removing peaks larger than 10kb) and recomputed ATAC count matrices. To mitigate data sparsity and batch effects due to differences in quality levels, we aggregated the data at the metacell level. We used the default Seurat clustering pipeline on the first 30PCs and increased the clustering resolution until we obtained on average 50 to 70 cells per metacell, which was found to be an optimal trade-off for gene regulation inference ⁵⁶ (resolution 10 for EOM and larynx, 5 for limb). To compute transcription factor activity, we identified transcription factor binding sites using motifmatchr (<https://doi.org/10.18129/B9.bioc.motifmatchr>) on the JASPAR2024 motif database ⁵⁷ then ran ChromVAR ⁵⁸ to identify per-cell accessibility scores for each motif. We then inferred a gene regulatory network (GRN) using a two-step strategy. In the first step, we initiated the GRN by using linear relationships between TF expression, candidate cis-regulatory elements (cCREs) accessibility and target expression to identify a large set of putative regulatory relationships. In the second step, we used a non-linear algorithm to prune this initial GRN and obtain a restricted set of high-confidence regulons.

To initiate the GRN, we first selected a subset of target genes (detection rate $>$ 5%, standardized cell-type variability $>$ 1, all myosin heavy chain genes) and TFs (detection rate $>$ 1%). For each target, we identified a set of putative cCREs using a strategy inspired by Signac's LinkPeaks function. We collected all peaks on the target gene body, up to 100kb upstream and, importantly, 100kb downstream of the gene body (Signac only takes 100kb downstream of the TSS, which can be problematic for long genes). We then computed the cCRE score as the Spearman correlation between the normalized accessibility of each peak and the normalized target expression at the single-cell level (one score per tissue). To account for biases in peak detection rates, we built a

background distribution by recomputing this score for 1000 peaks with similar detection rate (we grouped peaks into 20 equally sized bins according to detection rate), keeping only peaks that are on a different chromosome than the target. The cCRE score was then converted to a z-score by subtracting the mean background score and dividing by the background standard deviation. To select putative cCREs, we kept peaks with an average (across tissues) absolute z-score ≥ 1.5 , an average detection rate $\geq 1\%$, then selected the top 20 peaks with the highest average raw cCRE scores (putative enhancers) and the top 20 peaks with the lowest scores (putative silencers).

To identify putative regulatory TFs, we computed the Spearman correlation between TF expression and cCRE accessibility at the metacell level across tissues, then extracted the top 100 TFs most correlated to each cCRE (with a minimum required correlation of 0.05). At this stage, we did not require a TF motif to be detected, as we found that some highly expressed TFs are likely to use degraded motifs (motif detection and scores were assessed post hoc).

To prune the GRN and extract regulons, we used Pando³¹ with the non-linear XGB algorithm⁵⁹. We initiated Pando using the GRN described above and combined multi-ome data at the metacell level. For tractability, we kept only TF-cCRE links when a TF motif was detected with a motif score > 0 . We extracted regulons for each TF using the `find_modules` function (top 50 targets with highest association score). We computed per-cell regulon scores by computing an adapted AUCell score⁶⁰ (AUC of target genes found in the top 5% of a cell's most expressed genes). To compare regulon scores at the cell type level, we used the non-parametric Mann-Whitney test (as implemented in the MetaMarkers package) for significance and reported the average difference in AUC as the effect size.

Mass Spectrometry

Mouse EOM and TAM tissue samples were used for protein extraction, trypsin digestion, peptide purification, and peptide quantification prior to mass spectrometry. Protein was extracted in urea buffer (8 M urea, 50 mM ammonium bicarbonate, pH 7.8). Protein was digested using mass spectrometry grade trypsin protease (Thermo Scientific 90058) following the user guide with the following modifications: Protein reduction was performed using a 1% v/v ratio of dithiothreitol, 5 mM final concentration, incubated for 30 minutes at 56 °C. Protein alkylation was performed using acrylamide at a 4% v/v, 20 mM final concentration. Finally, quenching was performed using a 5% v/v of dithiothreitol, 25 mM final concentration and incubated at room temperature for 30 minutes. Peptide purification was performed using C18 spin columns (G-Biosciences 786-930) following their protocol. Dried samples were resuspended in resuspension buffer (0.1% formic acid, 2.5% acetonitrile) and peptide quantification was performed using a quantitative colorimetric assay (Thermo Scientific 23275), modified for a 385 well plate using one tenth of the volumes indicated in the protocol. 1 μ g of peptide per sample was loaded on a Vanquish Neo ultra-high performance lipid chromatography system (Thermo Fisher). Spectra were acquired with an Orbitrap Eclipse Tribrid mass spectrometer with FAIMS Pro interface (Thermo Fisher) running Tune 3.5 and Xcalibur 4.5 and run as previously described⁶¹. Raw data were analyzed using Proteome Discoverer 2.5 (Thermo Fisher) using Sequest HT search engines. The data were compared with Uniprot protein sequence database (Mus musculus Proteome ID UP000000589). Percolator peptide spectrum match validation was used with the following parameters: strict false discovery rate of 0.01, relaxed false discovery rate of 0.05, maximum Δ Cn of 0.05, and validation based on q-value. Other parameters were consistent with prior publications⁴².

Single Myofibril Measurements

Myofibril mechanical function was assessed using the fast solution switching method as described⁵. EOM myofibrils were prepared as previously described⁵, using the midbelly region of recti muscles. For ILM, thyroarythenoid muscles in bath solution were placed into Eppendorf tubes with 3 to 4 2mm zirconium beads and homogenized in a Bullet Blender Tissue Homogenizer at max speed for 10 seconds, repeated three times. For myofibril analysis, bundles of myofibrils were mounted between a calibrated cantilever force probe and length controller at 15 °C in relaxing solution (pCa 9.0). Myofibril length was set at ~2.0 μm. Mounted myofibrils were measured by rapidly switching the perfusion between full activating calcium concentration solution (pCa 4.5) and relaxation solution. Data were collected and analyzed using LabView software. Parameters measured are defined as follows: resting tension (mN/mm²), myofibril basal tension in fully relaxing condition; maximal tension (mN/mm²), maximal tension generated at full activating calcium activation (pCa 4.5); the rate constant of tension development following maximal calcium activation (K_{ACT}); the rate constant of tension redevelopment following a release-restretch applied to the activated myofibril (K_{TR}); duration of early slow force decline measured from onset of solution change to the beginning of the exponential force decay ($T_{REL, LINEAR}$); the rate constant of the first linear phase of relaxation ($K_{REL, LINEAR}$); the rate constant of the final exponential phase of force decline ($K_{REL, EXPONENTIAL}$). 3 to 7 single myofibrils from *Mybphl*^{KO} and control mice were measured in the same experimental session in a genotype blind manner.

In vivo administration of AAVs

All vectors were constructed and packaged in AAV9 capsids by VectorBuilder Inc (Chicago, IL, USA), with vector IDs as follows: VB250120-1536jea (mTbx15), VB251211-1415tqk (mMybphl) and VB250703-1475wrm as GFP control. The muscle-specific creatine kinase promoter with deletion of poorly conserved regions (CK8e) was used in all constructs. Viral titers were determined by quantitative PCR by the manufacturer.

For intramuscular delivery, two months old B6D2F1/JRj female mice were anesthetized by intraperitoneal injection of ketamine (Imalgene 1000, 100 mg/kg) and xylazine (Rompun 2%, 12 mg/kg) cocktail in 1X PBS. Intramuscular injection was done in the anterior lower hindlimb muscle using U-100 insulin needle. Numbers of AAV9 viral particles per muscle were 1.10^{11} for AAV-GFP and AAV-Tbx15 and 1.5×10^{11} for AAV-Mybphl diluted in 1X PBS, for a total volume of 75, 70 and 50 μL respectively. 3 weeks after injection, TA and EDL muscles were dissected and processed for contractile assays (EDL) or directly for immunofluorescence (TA).

Ex vivo contractility measurements

Experiments were conducted as in²⁹. Both limbs were cut before dissecting out EDL and TA in 1X PBS. Silk sutures (Ethicon #FK889) were attached to the tendons at each extremity of EDL. EDL were then kept in warm DMEM high glucose GlutaMAX medium (Gibco #31966021) in a humidified incubator at 37°C and 5% CO₂ for at least 10 minutes. Muscles were attached with silk sutures loops to a force transducer (Grass #FT03) on one side and to an immovable hook on the other side. Field stimulation was delivered using a constant current stimulator (Digitimer #DS7AH) and a pair of carbon electrodes flanking the muscles at a 0.5 cm distance. Measurements were made at 25°C.

Optimal current intensity was determined by using single-twitch stimulation and progressively increasing the current intensity until maximal twitch force was reached ($582 \pm 56\text{mA}$). After a 3 min rest period, optimal muscle length was established by incrementally increasing the length until no further increase in twitch force was observed ($14.3 \pm 0.7\text{mm}$). Muscles were allowed to rest for 5 min before starting the protocol. Stimulation pulses of 0.2ms were delivered at supramaximal intensity with increasing frequency at 1, 5, 10, 20, 40, 60, 100 and 150Hz for 500ms with resting time from 1min to 1.30min (from 40Hz onwards). Signal was acquired with PowerLab/8SP and LabChart software (AD Instruments), then further processed with custom Python scripts. Force-frequency curve was built with maximal forces elicited for each frequency fitted with a sigmoid function. EDLs transduced with different viral vectors we measured in the same experimental session in a condition blind manner. At the end of the protocol, muscles were detached and directly snap-frozen in liquid nitrogen or embedded in OCT and processed for immunofluorescence.

Protein phylogeny

To obtain the most complete and representative set of Mybphl homologs, we used a query composed of the human MyBP-H (uniprot id: Q13203), human MyBP-HL (A2RUH7), mouse MyBP-H (P70402), mouse MyBP-HL (Q5FW53), chicken MyBP-H (Q05623), zebrafish MyBP-H(a) (Q6GMJ2) and zebrafish MyBP-H(b) (A6H8Q3). We used the easy-search pipeline of MMseqs2 v18-8cc5c⁶² to find all sequences in Uniprot (in date of March 25) similar to at least one of the aforementioned sequences. This search was performed with high sensitivity (-s option at 7.5) and resulted in 2,546 hits. We then only retained the 2,296 hits with a query coverage, a target coverage and a proportion of identical hits all above 50%. Note that at this step We also manually removed 3 sequences: 2 supposedly from the mosquito (A0A8D8GDS6 and A0A8D8F9Q2) and one from the ostrich (A0A093H4F3) as they unambiguously appeared as wrongly annotated sequences in the final classification.

To reduce sequence redundancy while maintaining species diversity, we grouped sequences by species and used CD-HIT v4.8.1⁶³ on each species with a sequence identity of at least 70% (-c option set to 0.7), keeping only one representative per cluster. This resulted in 761 sequences. Considering that our result contains different group of paralogous proteins, we clustered all the 761 sequences using MMSeqs2 and the Greedy Set cover algorithm, with an identity threshold of 40% and a coverage threshold of 50% (--min-seq-id option at 0.4 and -c option at 0.5). We obtained 2 clusters. We again reduced redundancy on each cluster using CD-HIT and a higher threshold (-c option set to 0.9) to only remove highly similar sequences sharing at least 90% of identity. We gathered all representatives of all clusters and added 3 divergent chicken sequences to form an out-group: MYBPC3 (Q90688), MYBPC2 (P16419) and MYBPC1 (A0A8V0Y5D1).

We then aligned all these 310 sequences using MAFFT v7.525⁶⁴ and L-INS-i algorithm. This allows to align all sequences around one alignable domain, ignoring flanking regions than might differ a lot. We removed the sites of the alignment containing more than 20% of gaps using goalign v0.3.6⁶⁵. Using IQ-TREE2 v2.4.0⁶⁶ we determined that the best-fit model according to the BIC criterion was the LG+G4. Then, using this best-fit model, a hundred of trees were generated using a thorough NNI search (allnni option set) and only the one with the best likelihood was kept. We finally inferred 1000 bootstrap trees to calculate the support values using the transfer bootstrap expectation method using gotree v0.4.4.

We uploaded the tree to iTOL⁶⁷, rooted it with the out-group that was then removed from the final Figure (<https://itol.embl.de/tree/1579910120109331758627437>).

840

841 **Image analysis**

842 Antibody fluorescence intensity was measured using a custom Python script. Individual fibers
843 were segmented using Cellpose package ⁶⁸ segmentation tool on Laminin or Dystrophin antibody
844 staining, then selected based on their presence within a drawn region of interest. Mean intensity
845 in the channel of interest was then computed for each fiber, and mean density was computed for
846 the whole ROI across all fibers. Mean intensity was then divided by a common factor in a way
847 that the mean intensity across all negative controls is equal to one.

848 RNA-FISH spots density was measured using a custom Python script. Individual fibers were
849 segmented using Cellpose package ⁶⁸ segmentation tool on Laminin or Dystrophin antibody
850 staining, then selected based on their presence within a drawn region of interest. For RNA
851 detection, images were processed using a median blur and difference of Gaussians. RNA spots
852 were detected using the FISH-quant v2 package ⁶⁹ with a common threshold and decomposition
853 of high intensity spots. Spots were then attributed to individual fibers and mean density of spots
854 per μm^2 was computed for each fiber. Mean density was computed for the whole ROI across all
855 fibers. Nuclear localization of Mybphl and Myh4 intronic probes was done manually on Fiji and
856 Imaris. Mybphl doublet pattern was analysed using the MyoProfiler package developed by
857 Campbell and colleagues (<https://github.com/Campbell-Muscle-Lab/MyoProfiler>).

858

859 **Statistics**

860 Data analysis, statistics and visualizations were performed using python package statsmodels ⁷⁰,
861 matplotlib ⁷¹ and seaborn ⁷². Statistical tests are described in Figure legends.

862

863 **Modeling single-myofibril contractility**

864 Simulations were performed using the FiberSim (v2.3.0) ⁷³ package reproducing a single myofibril
865 k_{TR} experiment. Crossbridge detachment was modeled using the embedded `exp_wall` function:

866
$$\text{crossbridge detachment rate} = a \cdot e^{-\frac{k_{cb} \cdot (x + x_{ps}) \cdot b}{k_b \cdot T}} + \frac{\text{max rate}}{(1 + e^{-d \cdot (x - c)})}$$

867

868 With k_{cb} the stiffness of a crossbridge link, x the distance from binding site, x_{ps} the length of
869 crossbridge extension, k_b the Boltzmann constant and T the temperature. High and low strain-
870 induced crossbridge detachment corresponds respectively to $\{c = 10, d = 5\}$ and $\{c = 15, d =$
871 $10\}$. Model and setup files are available in <https://github.com/GComaiLab/Mybphl2026>.
872

873 **Methods References**

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935

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Methodology: AK, SF, GC

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 990 under accession number GSE322891 and GSE322890 respectively. Mybphl KO vs control
 991 mass spectrometry based proteomic data is available at
 992 <https://figshare.com/s/c786c35539d8cb212c31>. Code used for gene regulatory network
 993 inference is and sn-RNAseq analysis is available at
 994 link <https://github.com/GComaiLab/Mybphl2026>. Any additional information required to
 995 reanalyze the data reported in this paper is available from the lead contact upon request.
 996
 997 **Additional information**
 998 **Supplementary figures (separate files)**
 999 Suppl. Fig. 1 to 8
 1000 **Supplementary tables (separate files)**
 1001 Suppl. Table 1 to 3
 1002 **Extended data tables (separate files)**
 1003 Extended Data Table 1 to 5
 1004
 1005

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Suppdata2markersmyonucleisnRNAseq.csv](#)
- [Suppdata2markersmyonucleisnRNAseq.csv](#)
- [Suppdata4mybphlregulatorsinference.csv](#)
- [Suppdata4mybphlregulatorsinference.csv](#)
- [SupplementarymaterialsKorbNature.pdf](#)
- [SupplementarymaterialsKorbNature.pdf](#)
- [Suppdata5regulonstbx15maf.csv](#)
- [Suppdata5regulonstbx15maf.csv](#)
- [Suppdata3markersmyonucleimultiome.csv](#)
- [Suppdata3markersmyonucleimultiome.csv](#)
- [Suppdata1degEOMTAsnRNAseq.csv](#)
- [Suppdata1degEOMTAsnRNAseq.csv](#)