Myogenin promotes myocyte fusion to balance fibre number and size

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Each skeletal muscle acquires its unique size before birth, when terminally differentiating myocytes fuse to form a defined number of multinucleated myofibres. Although mice in which the transcription factor Myogenin is mutated lack most myogenesis and die perinatally, a specific cell biological role for Myogenin has remained elusive. Here we report that loss of function of zebrafish myog prevents formation of almost all multinucleated muscle fibres. A second, Myogenin-independent, fusion pathway in the deep myotome requires Hedgehog signalling. Lack of Myogenin does not prevent terminal differentiation; the smaller myotome has a normal number of myocytes forming more mononuclear, thin, albeit functional, fast muscle fibres. Mechanistically, Myogenin binds to the myomaker promoter and is required for expression of myomaker and other genes essential for myocyte fusion. Adult myog mutants display reduced muscle mass, decreased fibre size and nucleation. Adult-derived myog mutant myocytes show persistent defective fusion ex vivo. Myogenin is therefore essential for muscle homeostasis, regulating myocyte fusion to determine both muscle fibre number and size.
R
gulation of tissue size requires balancing cell number and
cell size. In skeletal muscle, tissue size depends on gen-
erating the correct number of multinucleated muscle fibres
with an appropriate number of nuclei in each; how these pro-
cesses are controlled in vertebrates is mysterious. Formation of
syncytial muscle fibres is a three-step process: commitment as a
myoblast, terminal differentiation into a myocyte, defined here as
irreversible cell cycle exit and expression of muscle-specific actin,
myosin and other genes, and finally myocyte fusion and growth to
form a mature multinucleate myofibre. In specialised cir-
sstances, such as the mononucleate slow myofibres of larval
zebrafish, myocytes mature into functional innervated and con-
tractile muscle fibres without fusion. The Myogenic Regulatory
Factor (MRF) family of transcription factors (Myod, Myf5, Mrf4
and Myog) are key players in orchestrating each of these steps in
skeletal muscle development. We
find that Myog is dispensable for
muscle regeneration18,20. Hence, despite strong evidence for roles for
Myog in later myogenesis not compensated for by other MRFs7,8,
mRNA in slow muscle
mRNA in fast muscle
Myog−/− myoblasts efficiently contribute to multinucleated fibres
in genetic mosaic experiments11. Furthermore, depletion of Myog
after birth reduces myofibre size and affects overall body home-
ostasis, although without perturbing muscle histology12–14.
However, given that other MRFs can and do bind the same DNA
motifs as Myog15, the precise role(s) of Myog remain ill-defined.
Knockdown of zebrafish myog has minor effects on initial
events in myogenesis16–19, whereas combined knockdown of
Myog and Myod strongly reduces myogenesis of fast myofibres17.
These findings were confirmed using a zebrafish mutant
(myog fh265) bearing a stop mutation downstream of the bHLH
domain, which also shows delayed muscle regeneration18,20.
However, as a similar truncation of mouse Myog has residual
activity, we previously suggested that myog fh265 is
hypomorphic18,21. Hence, despite strong evidence for roles for
Myog in later myogenesis not compensated for by other MRFs7,8,
a specific evolutionarily-conserved function in vivo is unclear.
Here we create null alleles in zebrafish myog and reveal a
specific function for Myog in myocyte fusion during skeletal
muscle development. We find that Myog is dispensable for
myoblast terminal differentiation, expression of many muscle-
pecific markers, myofibre elongation across the somite, sarco-
meric assembly, innervation and generation of functional con-
tractile muscle. However, lack of Myog prevents most myocyte
fusion and leads to supernumerary mononucleated muscle fibres.
Myog is required for the expression of membrane proteins
involved in cell fusion, such as Myomaker22–25. Despite gross
myocyte fusion defects, zebrafish myog mutants survive to
adulthood with more but thinner muscle fibres and reduced
overall body size. Adult muscle precursor cells lacking Myog
show a persistent fusion defect ex vivo. Interestingly, residual
fusion in myog mutants occurs primarily in the deep myotome
and is dependent upon Hedgehog signalling, indicating the
existence of two pathways to myocyte fusion.
Myogens differentiate and muscle functions without Myo-
genin. To examine muscle differentiation, mutants and sibs were
compared for mulpf and smyc1 expression that distinguish fast and
slow muscle26–28. ISH analysis revealed no difference at 15 somite stage (ss) (Supplementary Fig. 1b). In this and sub-
sequent experiments, no differences were observed between wild
type and heterozygous sibs, consistent with their similar Myog
level (Supplementary Fig. 1a). At 22 ss, strong mulpf mRNA in
fast muscle in anterior somites and smyc1 mRNA in slow muscle
extend more posteriorly were also unaltered in myog mutants
(Supplementary Fig. 1c). At this stage, slow myofibres have
migrated to the lateral surface of the myotome and remain
mononucleated, whereas the more abundant multinucleated fast
muscle fibres are located deeper in the myotome29–31. Moreover,
no obvious difference was observed, either in motility or in fast
and slow myosin heavy chain (MyHC) immunoreactivity at 20
hpf, 1 day post-fertilisation (dpf) or 2 dpf, when embryos have
hatched and make short bursts of controlled swimming (Fig. 2b,c
and Supplementary Fig. 1c–e). Slow myofibre number and
thickness were not affected in mutants (Fig. 2b and Supplemen-

tary Fig. 1e). Thus, without Myogenin, specification and early
development of slow and fast muscles appears normal.
α-Actinin, Titin, F-actin and Acetylcholine Receptor staining
also showed that fibre formation, sarcomere assembly and
innervation had occurred properly in mutants (Fig. 2d–h). At 2
dpf, mutant myofibres were correctly positioned and elongated
across the length of the somite. However, fast muscle appeared
mildly disorganised and slightly reduced in extent (Fig. 2f).
Nevertheless, motor function at 5 dpf assayed by time spent
swimming, total travelled distance and average speed did not
differ between myog mutants and sibs. Irrespective of genotype,
some larvae were consistently more active than others throughout
the 30 min measurement period (Fig. 2i). To test fibre integrity
and anchorage, fish were swam in a viscous methyl-cellulose
(MC) solution, which led to a general decrease of swimming
performance. Despite this challenging environment, mutants did
not perform significantly worse than their siblings (Fig. 2i). When
sib and mutant larvae were grown in MC from 5 to 8 dpf, a
procedure known to damage defective muscle32, myog mutants
retained good muscle morphology (Supplementary Fig. 1f). Thus,
Myogenin is dispensable for the initial phases of myogenesis and
generation of strong functional muscle in zebrafish.

Results

Generation of myogenin mutant alleles. To create a null myog mutant, we targeted genome editing far upstream of bHLH region and obtained two nonsense alleles (Fig. 1a). Myogfh128 has an insertion of 1 bp (A), whereas myog fh265 has a deletion of 3 bp (TCA). Both mutations create a stop codon in an identical position (Y377), producing a truncated protein lacking both basic and
HLH domains. In situ mRNA hybridisation (ISH) for myog on
myog fh128/+ and myog fh265/+ incross lays at 18 h post fertilization
(hpf) showed reduced signal in mutant embryos compared to
siblings (sibs), presumably by nonsense-mediated decay (NMD)
(Fig. 1b). mRNA downregulation was confirmed by qPCR at 20
hpf (Fig. 1c). Congruently, mutant embryos lacked Myog immu-
noreactivity, whereas F-actin accumulation and overall number of
nuclei per myotome was indistinguishable from wild-type (wt)
(Fig. 1d). Heterozygote and wt siblings showed similar levels of
Myog (Supplementary Fig. 1a). No compensatory upregulation of
other MRFs was noted at 20 hpf. Indeed, lack of Myog sig-
ificantly reduced expression levels of myf5 (40%) and myf4
(54%), whereas myod remained unchanged (Fig. 2a). These results
demonstrate that homozygous mutant alleles myog fh128 or
myog fh265 block Myog mRNA and protein accumulation.

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Muscle size reduction and myofibre number increase in myog mutant. Although functional muscle was formed, our data suggested a reduction in myotome size in mutants (Fig. 2). Measurement of the dorso-ventral extent of ISH staining of fast mylpfa and slow smyhc1 revealed a reduction of mutant myotome size, both at 1 and 2 dpf (Fig. 3a, b and Supplementary Fig. 2a). Nevertheless, the body length of mutants and sibs was comparable, suggesting that muscle reduction was not due to reduced overall body size or delayed development (Fig. 3c and Supplementary Fig. 2b).

To analyse the defect in cellular detail, myogkg128 and myogkg125 were bred onto Tg(Ola.Actb:Hsa.HRAS-EGFP)vu119, in which EGFP targets plasma membranes of all cells (β-actin:EGFP hereafter). Confocal sections of β-actin:EGFP;myogkg128/+ incross larvae confirmed that reduction in myotome volume was present at 2 dpf in mutants and persisted until at least 5 dpf (Fig. 3d, f). Although myotome cross-sectional area was consistently reduced in mutants, myotome length was unaffected (Fig. 3g; Supplementary Fig. 2b). A reduction of fast fibre cross-sectional area in mutant fish was observed (Fig. 3e). Indeed, quantitative analysis at 2 dpf revealed a 50% increase of fast fibre number accompanied by a 45% reduction in mean fibre volume in mutant embryos (Fig. 3h, i). myogfh265 mutants did not have reduced size or altered cellularity and we did not observe any other phenotype in un-manipulated embryos, larvae or adults, confirming that this allele is hypomorphic (Supplementary Figs 2c–e; 5a). We conclude that Myog controls fast myofibre number and size.
Myogenin is required for normal myocyte fusion. Two hypotheses could explain the overabundance of fast muscle fibres in myog<sup>kg128</sup> and myog<sup>kg125</sup> mutant embryos: increased frequency of terminal differentiation of muscle progenitor cells (MPCs) into myocytes or reduced fusion of differentiating myocytes into multinucleate fibres. Most, if not all, fast myofibre nuclei derive from MPCs expressing either Pax3 or Pax7<sup>34–42</sup>. We found no differences between myog mutants and sibs in position or number of Pax3/7 positive cells at 1–2 dpf (Fig. 4a, b). Nor was there a change in the number of nuclei in the myotome (Fig. 4h). Thus, no evidence supported the possibility that myocyte formation from MPCs was increased.

Next, we tested the ability of Myog to promote myocyte fusion. We injected DNA encoding CAAX-mCherry at the 1-cell stage into myog<sup>kg128</sup>/+ incross embryos to label mosaically the plasma membrane of single fibres. At 2 dpf the majority of labelled fibres in mutant embryos were mononucleated, whereas those in sibs were multinucleate (Fig. 4c). To quantify this defect, we injected...
H2B-mCherry mRNA into β-actin-EGFP;myog\textsuperscript{fl125} embryos to label nuclei and plasma membranes uniformly. Strikingly, over 92% of muscle fibres in myog \textit{mutants} were mononucleate, compared to 33% in sibs (Fig. 4d, e; Supplementary Fig. 3a). The fraction of nuclei in multinucleate fibres dropped from 50% in sibs to 16% in mutants (Fig. 4f; Supplementary Fig. 3a). This phenotype persisted at least until 6 dpf (Supplementary Fig. 3b). Nuclei in mononucleated myofibres preferentially located near the centre of the myocyte (Fig. 4d). Despite the twofold reduction in myonuclei per myofibre, the total number of nuclei within each myocyte was not altered in mutants, paralleling the increase in myofibre number (Figs. 3h, 4g, h).

To eliminate the possibility of CRISPR off-target effects, we re-expressed Myog mosaically in myog \textit{mutant} larvae by injection of a plasmid containing the zebrafish myog promoter driving wt zebrafish Myog-ires-GFP expression. myog-MyogCDS-ires-GFP rescued fusion in fast fibres, whereas a control myog-GFP-only vector did not (Fig. 4i, j). Myog knockdown with a morpholino fully recapitulated the mutant phenotype with increased number of mononucleon fibres and decreased somite growth (Supplementary Fig. 3c–e). We conclude that Myogenin is essential for most myocyte fusion.

To examine the cell autonomy of the need for Myogenin we analysed myog \textit{mutant} larvae with mosaic Myog-ires-GFP re-expression further. Myog-ires-GFP significantly rescued fusion; Myog-expressing fibres in myog \textit{mutants} contained a range of nuclear numbers approaching the distribution in controls (compare Fig. 4e, i). Adjacent unlabelled fibres remained mononucleate (Fig. 4j). As cells expressing Myog-ires-GFP occurred at a rate of about 2–3 per somite and were well-scattered, this finding indicates that only a single fusin partner needs to express Myog to permit fusion. Moreover, although mosaic Myog expression rescued mutants, it did not induce more fusion than observed in wt in either mutants or sibs (Fig. 4i, j; Supplementary Fig. 4a–c). Importantly, Myog expression (either in myog \textit{mutants} or sibs) failed to elicit fusion of the normally-mononucleate slow fibres, even though the slow fibres were adjacent to fast fibres and their MHC precursors, indicating that the low level of myog mRNA in wt slow MHCs is not the only reason for their lack of fusion (Supplementary Fig. 4d,e). However, both mutant and sib slow fibres overexpressing Myog-ires-GFP showed significantly reduced myofibrillar width (Supplementary Fig. 4e,f). Thus, Myog is required in at least one of two fusing fast myocytes to permit fusion.

Expression of fusogenic genes reduced in myog mutants. Fusion of myocytes is a key feature of skeletal myogenesis and requires several transmembrane proteins\textsuperscript{13}. We hypothesised that Myog regulates these genes. Myomaker (mymk)\textsuperscript{22–25,44} mRNA was strongly reduced (72%) in myog null mutant embryos at 20 hpf, during initial myocyte fusion, and was also mildly affected in myog\textsuperscript{265} hypomorphs (Fig. 5a, b; Supplementary Fig. 5a, b), paralleling the previously observed myog nonsense-mediated mRNA decay in this hypomorphic allele\textsuperscript{18}. Myomaker was also reduced in myog\textsuperscript{261} mutant in proportion to myog mRNA reduction and loss of fast muscle\textsuperscript{18,45} (Supplementary Fig. 5a, b). Reduction of mymk mRNA thus parallels lack of myocyte fusion. Myomixer/myomergen/minion, a micropeptide recently described to enhance myoblast fusion\textsuperscript{44,46,47}, was also reduced (34%; Fig. 5b). Moreover, jam3b mRNA was significantly reduced (22%) in mutants, but jam2a\textsuperscript{48} and kirrel3\textsuperscript{49} were unaffected (Fig. 5a, b).

The extent of reduction of myomaker expression in mutants argues for direct transcriptional regulation by Myogenin. To test whether Myog directly regulates mymk transcription in zebrafish, we scanned 3 kb of putative promoter region upstream of the myomaker 5′-UTR and found two E-box elements (E-box 1 and E-box 2, Fig. 5c). ChIP-qPCR assay on 20 hpf embryos revealed that Myog binds both E-box elements, with significant enrichment of Myogenin binding to E-box 1 compared to two different negative controls. The more proximal E-box 1 showed greater binding than E-box 2 (Fig. 5d). Combined, these data support a role for Myogenin in governing myocyte fusion through direct transcriptional upregulation of mymk and other fusogenic factors.

Hedgehog drives residual fusion and mymk expression. Myog mutants retain small numbers of multinucleate fibres in the medial somite (Fig. 4e; Supplementary Fig. 3a,b). Residual mymk mRNA also persists in myog mutants (Fig. 5a, b), showing that other factors drive mymk expression in some cells. Residual mymk mRNA is preferentially enriched in the medial region of mutant myotome, adjacent to the notochord (Fig. 6a). Notochord-derived Hedgehog (Hh) signals promote differentiation of slow and a medial subset of fast muscle in zebrafish\textsuperscript{19,30,35,37,50–54}. Treatment of myog mutant embryos with the Hh inhibitor cyclopamine (CyA) led to an additional 54% reduction of mymk mRNA, leaving < 20% of the original mymk expression compared to vehicle-treated wt siblings (Fig. 6b; Supplementary Fig. 5c,d). CyA-treated sibs also showed a 22% mymk reduction (Supplementary Fig. 5c–e) compared to controls. Congruently, when a β-actin-EGFP;myog\textsuperscript{fl125}/+ incross was treated with CyA, residual fusion in myog mutants at 2 dpf was largely lost (Fig. 6c). Blockade of Hh signalling had no detectable effect on fusion in sibs, although reducing both sib and mutant fast muscle growth, as previously reported\textsuperscript{15} (Fig. 6c). These observations show that in myog mutants Hh signalling sustains residual mymk expression and myocyte fusion in the deep/medial myotome close to the notochord.
Adult myog mutants have small muscle with reduced fibre size. Both putative null myog alleles are homozygous viable. By 4 months (120 dpf), compared to their co-reared sibs, adult mutants showed a reduction in standard weight, a measure that compensates for length changes (Fig. 7a, b; Supplementary Fig. 6a, b). In contrast, myogfh265 mutants were similar to sibs, consistent with the lack of larval phenotype (Fig. 7b; Supplementary Fig. 6a). The new mutants showed a 35-40% reduction in weight and lower 'body mass index'. These data show that muscle bulk reduction is independent of, and may cause, the observed length reduction (Supplementary Fig. 6a). Muscle reduction persisted also in 15 month old mutants (Supplementary Fig. 6c).
Adult muscle phenotype examined in transverse cryosections of 120 dpf myogkg128 incross fish revealed a decreased myotome area, reduced fibre cross-sectional area and increase in fibre number per unit area of muscle in myogkg128 mutants, compared to matched sibs (Fig. 7c–f; Supplementary Fig. 6b). The total number of fibres in single body cross-sections of a mutant (4097) and a heterozygote sib (3657) were similar and were approximately 40-fold those in larvae, indicating that fibre formation had persisted (Supplementary Fig. 6b). The number of nuclei in mutant fibre cross-sections was reduced threefold compared to sibs, but a few fibres with several nuclei were still present (Fig. 7g, h; Supplementary Fig. 6d, e). The total area of both slow and fast muscle also appeared reduced (Fig. 7i; Supplementary Fig. 6b).

Fibre typing for oxidative metabolism revealed that fast muscle fibres in single body cross-sections of a mutant (4097) expressed desmin and MyHC, elongated and aligned similarly to control cells (Fig. 8a, b; Supplementary Fig. 6g). These data show expressed desmin and MyHC, elongated and aligned similarly to control cells (Fig. 8a, b; Supplementary Fig. 6g). These data show

**Discussion**

The data presented radically change the interpretation of the evolutionarily conserved role of Myog in skeletal muscle development through four major findings. First, Myog is not required for terminal differentiation of most myoblasts into myocytes. Second, Myog has a major conserved role in driving the fusion of myocytes into multinucleate fibres. Third, that a second, Myog-independent, pathway to muscle fusion exists and, in the zebrafish trunk, is promoted by Hh signalling. Lastly, that Myog is required for normal myogenesis throughout life and that its loss leads to poor muscle and whole body growth and a persistent functional fusion defect in adult satellite cell-derived muscle progenitors.

In mice lacking Myog, myoblasts can form myocytes expressing proteins of the contractile apparatus5–8. However, a major deficit of early muscle formation was reported, with dramatic downregulation of MyHC at e12.5 in both trunk and limb muscle and a worsening deficit in neonates7,8. This led to the view5, that ‘Myogenin knockout has a … complete absence of functional skeletal muscle’. Our data from zebrafish contradict this view; we observe differentiated muscle fibres, normal sarcomere formation and normal numbers of nuclei within fibres in the larval myotome of mutants. Nevertheless, although to our knowledge no compelling images of fusion in the absence of Myog in vivo have been published, cultured myoblasts and satellite cells from Myog mutant mice are reported to fuse and myocytes lacking Myog can fuse with wild-type myocytes in vivo in murine chimaeras5,11. We conclude that Myog is dispensable for terminal differentiation of myoblasts into post-mitotic myocytes and contractile myofibres in zebrafish (Fig. 8e).

Early reports suggested that murine myotomal Myog protein does not accumulate until after muscle differentiation, despite the earlier presence of Myog mRNA36,57. Moreover, embryonic MyHC (Myh3) mRNA is almost normally expressed in e14.5 Myog mutants, whereas maturation to expression of perinatal MyHC (Myh8) mRNA is dramatically reduced at e18.57,8. The widespread accumulation of desmin protein, hitherto taken as an indication of a myoblast state7,8,38, could instead reflect myocyte formation. The ability of myogenic cells from Myog−/− mice to form myotubes in culture also argues for unimpaired myocyte formation7,8. Consistent with murine data8, we find that balance of zebrafish slow and fast fibre formation is unaffected by loss of Myog. However, our finding that several genes required for later steps in myocyte differentiation (e.g. fusion) are down-regulated in mutants indicates that a subset of muscle differentiation genes, the ‘Myog-module’ are regulated by Myog in zebrafish (Fig. 8e).

Zebrafish Myog is not essential for, but can promote, muscle terminal differentiation. We previously reported that loss of both Myf5 and Myod ablates all skeletal myogenesis, whereas combined reduction of Myod and Myog severely reduces fast muscle17,18. These findings show that, in the absence of MyoD, Myf5 requires Myog to drive fast myogenesis17. Interestingly, even hypomorphic myogkg265 mutants that lack a developmental fusion defect show poor muscle regeneration and apoptosis of myf5-marked cells20. Hence, we cannot exclude the possibility that a non-essential subset of myoblasts requires Myog for terminal differentiation in older zebrafish. Lack of Myog is not compensated by increased expression of myod and leads to downregulation of myf4 mRNA, as observed in mice lacking Myog5,7,8,12. Surprisingly, we found that myog mutant embryos accumulate less myf5 mRNA, suggesting that Myog may promote myf5 expression or be required for the production of myf5-expressing cells.
Myog mutants have a severe lack of fusion, despite efficient myocyte differentiation. We find that myog activity is essential for normal expression of a subset of fusogenic genes, mymk, mymx and jam3b, mutation of which causes fusion defects strikingly similar to the myog mutant phenotype. We propose, therefore, that early terminally-differentiated fast myocytes are primed for fusion, but lack sufficient expression of critical Myog-module components until Myog becomes active (Fig. 8e). Interestingly, mymk mRNA was more highly reduced than any other gene analysed, which could account for the fusion defect observed. However, as jam3b mRNA is more widely expressed, its lesser reduction may reflect strong reduction in.
muscle and unaltered expression elsewhere. Thus, the extent of the Myog-module functionally required for fusion remains to be determined.

Mosaic Myog re-expression in myog mutant fully rescues fusion. Strikingly, this effect is cell autonomous and fairly efficient, which leads to several important conclusions. Firstly, as two adjacent GFP-marked cells are rare, a single isolated Myog-expressing myocyte appears sufficient to induce fusion to an adjacent cell lacking Myog. This result parallels the chimaera analysis showing fusion of Myog−/− with wild-type cells in murine myogenesis. Secondly, the existence of rescued fibres with more than two nuclei suggests that Myog expression in a binucleate fibre can elicit fusion of adjacent cells lacking Myog. It may be significant in this regard that Jam3b, Mymk and Minion/Myomerger/Myomixer have been shown to be required in only one cell of a fusing pair, although fusion efficiency was reportedly higher when both cells express Mymk.

We observed that Myog occupies E-boxes in the endogenous mymk 5’ proximal region during the period of fusion in vivo. A 3 kb mymk promoter fragment containing these sites drives reporter expression in zebrafish fast muscle. Similarly, in mouse and chick, Mymk expression parallels that of Myog and depends on conserved E-boxes, including one at −41 bp. Moreover, in cultured myocytes, Myog binds to conserved sequences in Mymk, Myx3 and Jam3 (www.encodeproject.org/experiments/ENCSR000AID). Interestingly, the early Xenopus, chick and mouse myotomes are reported to be composed of mononucleate fibres. As the murine Myog mutant shows little early defect, but even partial deletion of a floxed Myog allele after initial fibre formation shows that Myog is essential for late embryonic and neonatal myogenesis, the data on murine Myog mutants are all consistent with a primary fusion defect. Moreover, some reports in C2C12 cells have suggested that Myog expression correlates with, and is required for, myocyte fusion, although conflicting findings exist. We note that zebrafish slow muscle fibres, which remain mononucleate long after their terminal differentiation, accumulate much lower levels of myog mRNA than fast muscle precursors, paralleling their lower levels of mymk and mym34,66,69. However, although overexpression of Myog in slow myocytes reduces their size and alters myofibril organisation, it did not drive their fusion to each other or to adjacent fast myocytes, in contrast to mymk overexpression.

Zebrafish myog mutants show a similar increase in number of myofibres to that reported when fusion is blocked in mutants lacking Jam proteins. In the case of myog mutants, the increase in fibres was quantitative; total nuclear number in myofibres remained constant, suggesting that normal numbers of myoblasts differentiated, survived and made fibres. It seems that during early myotome formation, therefore, either no specific subpopulation of ‘founder’ myoblasts determines fibre number, as occurs in Drosophila embryonic myogenesis, or Myog is required to prevent cells acting as founder cells. Interestingly, previous studies reported that fusion-defective myoblasts elongate and differentiate into mononucleated muscle fibres. We hypothesise that, like fish, murine Myog mutants are blocked in fusion. Perhaps lack of fusion triggers loss of nascent myocytes that fail to form attachments to skeleton or nerve. In addition, myoblast populations such as those in neonatal limb may absolutely require Myog for terminal differentiation.

In myog mutant fish a small group of muscle fibres in the deep myotome undergoes fusion. Residual fusion was also observed in jam3b and jam2a mutants and mymk mutants were also stated to be only ‘predominantly mononucleated’, although the extent and location of residual multinucleate fibres was not reported. We find that Myog-independent fusion requires Hh signalling, probably from adjacent midline tissue, which also up-regulates mymk mRNA in the deep myotome. The low residual level of mymk mRNA observed in CyA-treated myog mutants could reflect incomplete loss of Hh function, or additional controls on mymk expression. As Hh promotes slow and fast muscle differentiation through activation of Myod by a Cdkn1c/p57 positive feedback loop, we speculate that Hh-induced fusion may arise from increase in Myod-driven mymk expression in myocytes adjacent to the midline source of Hh. Our data suggest that distinct myocyte fusion processes contribute to muscle fibre diversity.

Our study quantified the extent of fusion in wild-type and myog mutant fast muscle. Interestingly, a significant minority (~30%) of fast fibres are mononucleated at 2 dpf in wild-type, meaning that ~20% of fast myocytes had not yet fused. Many of these may reflect dermomyotome-derived fast myocytes that had recently undergone terminal differentiation, because they were predominantly located in the lateral myotome. Nevertheless, transplant experiments have reported rates of fusion above 95%, implying that a rare subset of proliferative somite cells generates the mononucleate fast fibres. On the other hand, in myog mutants only about 20% of myocytes fuse. As frequencies of residual fusion are yet to be reported in jam2a, jam3b, mymk and mym3 mutants, it is unclear whether some fusion mutants have more severe defects than others.

Myog mutants are viable but grow less rapidly than their sibs. Early in life, myog mutants have small fibres and reduced myotome size, which might give them a disadvantage in competitive
feeding leading to reduced growth. Alternatively, Myog could be required for some other function, such as synthesis of myokines important to coordinate whole body scaling of tissue size. However, adult mutants have a disproportionate loss of muscle compared to their length and a persistent greatly reduced fibre size and nuclear content throughout life that could reflect defective adult MPC differentiation.

Zebrafish muscle has been shown to contain fibre-associated satellite cells\textsuperscript{41,73,74}. To address the role of Myog in adult life, we developed an MPC culture procedure for zebrafish satellite cells. This method shows that adult MPCs lacking Myog undergo terminal differentiation but fuse poorly ex vivo, indicating that Myog is required throughout life, rather than that the defect in adult fish derives solely from persistence of early developmental defects.

**Fig. 5** Myogenin mutants have reduced expression of fusogenic factors. \(a\) ISH on 20 hpf myog\textsuperscript{kg128/++} incross to investigate the expression of genes essential for vertebrate myocyte fusion. Expression of myomaker (mymk) and jam3b, but not of jam2a or kirrel3l, is reduced in myog mutant. Bar = 50 μm. Representative images \(n=10\) mutants, \(n=38\) sibs (mymk); \(n=13\) mutants, \(n=37\) sibs (jam2a); \(n=12\) mutants, \(n=39\) sibs (jam3b); \(n=7\) mutants, \(n=13\) sibs (kirrel3l). \(b\) qPCR analysis of RNA expression levels showing downregulation of mymk, myomixer/myomixer/minion (mymx) and jam3b at 20 hpf on myog\textsuperscript{kg125} mutants, whereas jam2a and kirrel3l remain unaltered compared to wt (myog\textsuperscript{+/+}) sibs. Graphs show mean fold change ± SEM of four independent experiments; paired \(t\) test. Symbol shapes denote wt and mutant samples from paired experiments. \(c\) Schematic of 5’ genomic region of mymk reporting: 5’-UTR and coding sequence (grey and white boxes), position of E-box elements (red boxes), relative bp distance from 5’UTR start (+1), start codon (arrow, ATG). E-box 1 and E-box 2 sequences are shown in red text. \(d\) ChIP-qPCR assay using anti-Myog or mock showing significant Myogenin enrichment on mymk E-box 1 compared to negative controls from the gapdh promoter and a gene-free region on chromosome 14 containing an E-box (Chr14). Mean of percentage of input immunoprecipitated ± SD of two independent experiments, ANOVA.
that are subsequently Myog-independent. Definitive proof of this conclusion will require deletion of myog function in adult fish.

Nevertheless, combined with the defective regeneration of larval muscle in myog hypermorphs, our data strongly suggest that Myog also functions during adult muscle growth and regeneration.

In striking contrast to the myog mutant phenotype, lack of Myod reduces fibre number in larvae without affecting fusion or mymk expression, and the remaining fibres grow larger. In myod mutants, a reduction in the number of fast fibres is accompanied by an increase in MPCs expressing Pax3 and/or...
Given that Myod can activate myog expression and either MRF can drive terminal differentiation of fast fibres,18,45, these opposite effects on fibre number and size suggest that the balance of MRFs influences the mode of muscle growth.

Methods
Zebrafish lines and maintenance. All lines used were reared at King’s College London on a 14:10 h light/dark cycle at 28.5 °C with adults kept at 26.5 °C, with staging and husbandry as described.78 Embryos/larvae were reared at 28.5 °C in the dark, except for periods outside the incubator. 

Imaging and in situ mRNA hybridisation and immunodetection. ISH and immunodetection were performed as described.84 Briefly, fish were fixed in 4% PFA in phosphate-buffered saline (PBS) for 30 min or 3 h at room temperature or overnight at 4 °C. Embryos for ISH were stored in 100% methanol at −20 °C and rehydrated in PBS prior to ISH. Fish for immunostaining were permeabilised in PBS 0.5% Triton X-100 (PBSTx) for 5 min, blocked in Goat Serum 5% (Sigma Aldrich) in PBSTx and incubated with primary antibodies at indicated concentrations at least overnight. Fish were then washed in numerous changes of PBSTx for at least 5 min and incubated and washed similarly with indicated secondary antibodies and prepared for imaging as described below. Primary antibodies against Myog (M-225 Santa Cruz Biotechnology, 1:50), fast MyHC (EB165, 1:100), desmin (Abcam), Titin (T12 (1:10), D. Fürst, University of Bonn, Germany), sarcomeric actin (3A18 1:50), DBH-Mcherry (1:10), DBH-DsRed (1:100), kirrel3L (1:100), FM1-43 Alexa 488 (Agar) or 0.8 M methyl-cellulose (MC, Sigma Aldrich) in PBSTx for at least 30 min. All secondary antibodies are from Invitrogen and Goat anti-Mouse IgA-FITC (Serotec). Digoxigenin-labelled probes were against myog (5′-GAAGAGTACTTGCCGTCATGATC-3′) on the reverse strand using CRISPR/Cas9 method as previously described.79. Mutant lines, myog125 and myog261 mutant alleles18,45, on AB background were genotyped by sequencing as described previously18, myog8125 and myog8126, on the TL background, were genotyped by sequencing or by loss of EcorV site in the mutant alleles, following PCR amplification using primers indicated (Supplementary Table 1). The two new alleles had indistinguishable phenotypes and no differences were detected between wt and heterozygous fish, so we refer to mutants and siblings (sibs), and report the specific myog allele in each experiment in Figures and Supplementary Table 2. Tg(Actb:Hsa.HRAS-EGFP)vu119 (ref. 33) was originally on King’s wild-type background. All experiments were performed on zebrafish derived from 2 or later filial generations, in accordance with licences held under the UK Animals (Scientific Procedures) Act 1986 and later modifications and conforming to all relevant guidelines and regulations.

Embryo manipulation. Myogenin mutants were generated targeting the sequence 5’-GGAGGCTCCTCCTGCTGATACT-3’ on the reverse strand using CRISPR/Cas9 method as previously described.79. Mutant lines, myog125 and myog261, were bred onto Tg(Actb:Hsa.HRAS-EGFP)vu119 (ref. 33) on the TL background, were genotyped by sequencing or by loss of EcorV site in the mutant alleles, following PCR amplification using primers indicated (Supplementary Table 1). The two new alleles had indistinguishable phenotypes and no differences were detected between wt and heterozygous fish, so we refer to mutants and siblings (sibs), and report the specific myog allele in each experiment in Figures and Supplementary Table 2.

Fig. 6 Hedgehog signalling sustains residual fusion and mymk expression. a ISH for myomaker (mymk) at 20 hpf revealed that residual expression in myog mutant is enriched in the medial region of the somite close to notochord (arrows in transverse sections from indicated axial level, dorsal to top). Note lack of expression in mononucleate slow pioneer fibres (arrowheads, upper panel). Representative images n = 6 mutants, n = 14 wt sibs (mymk) b ISH (lateral view, dorsal to top) and qPCR analysis showing that cyclomycin (CyA) treatment of myog125 embryos almost abolished mymk mRNA compared to ethanol vehicle control. CyA effectiveness is shown by the absence of unstained slow muscle pioneer cells (arrowhead). Mean fold change ± SEM from three independent experiments on embryos from separate lays of myog125 (circles) and myog261 (squares and triangles) analysed on separate days, paired t test statistic. Representative images n = 4 EtOH, n = 6 CyA. c Optical confocal sections of the medial region of somites 17 of β-actin:EGFP, myog8125/+ incross treated with vehicle or CyA. Transverse-section panels show medial position (yellow lines) of respective longitudinal section for each condition. CyA abolished residual fusion in the medial myotome of mutant embryos (arrowheads) but did not detectably affect fusion in sibs. Note that the residual multinucleate fibres in myog125 mutant appear larger than adjacent mononucleate fibres in EtOH but are lacking in CyA. nt: neural tube, nc: notochord. Representative images n = 5 mutants, n = 3 sibs (EtOH); n = 4 mutants, n = 6 sibs (CyA). Bars = 50 μm.
extracted from pools of four embryos of each genotype using Trizol® (Sigma Aldrich) and purified with RNA Clean & Concentrator™-25 (Zymo Research) or RNA Puriﬁcation Plus Kit (Norgen). Total RNA (300 ng) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) following supplier’s instructions. qPCR on technical triplicates for each sample was performed on 5 ng of relative RNA using Takyon Low ROX SYBR 2X MasterMix blue dTTP (Takyon) on a ViiA™7 thermal cycler (Applied Biosystems). For each experimental sample, ΔC<T was calculated by subtracting the CT value for housekeeping gene (actb2) from that of the target gene. ΔΔC<T of each target gene was then calculated by subtracting the average of the ΔC<T obtained in the wt (siblings) samples from ΔC<T for each sample. Relative gene expression was calculated using the 2^-ΔΔC<T formula and the fold change of the expression levels between sibs and mutants were compared using paired Student’s t test. Results are presented as mean ± SEM of fold changes from three or four independent experiments. Primers were purchased from Sigma-Aldrich (KiCqStart® SYBR® Green Primers Predesigned, Sigma Aldrich). All PCRs for genotyping and probe synthesis were performed using Phusion Taq polymerase (Life Technologies) on a T100 thermal cycler (Bio-Rad).

Chromatin Immunoprecipitation and E-box enrichment analysis. 3 Kb of putative promoter region of myonaker, retrieved from UCSC genome browser (GRCz10/danRer10), was scanned for E-box elements using JASPAR 2016 version.
**Fig. 7** Adult Myogenin mutants have reduced muscle with more but smaller myofibres. a Myog mutant and sib at 120 dpf from myog<sup>kg125</sup>/+ incross. Bar = 1 cm. Representative images n = 5 mutants, n = 23 sibs. b Myog<sup>kg125</sup> or myog<sup>kg125</sup> but not myog<sup>fg265</sup> showed reduced standard weight compared to co-reared sibs at 120 dpf. Dots represent individuals. c Laminin immunodetection on cryosections from 120 dpf myog<sup>kg125</sup>/+ incross. Bar = 100 μm. Representative images, n = 3. d-f Number of muscle fibres in 0.1 mm<sup>2</sup> of adult muscle is increased in mutants (d), whereas myofibre cross-sectional area (CSA) is decreased (e) reflecting a shift in CSA frequency distribution compared to sibs. g Fewer myonuclear profiles were present within laminin profiles in adult muscle cross-sections in mutants than in sibs, measured from 107 to 490 fibres at similar medio-lateral and dorso-ventral positions of trunk muscle of three fish per genotype. Mean ± SEM, t test. h Proportions of muscle fibres with indicated number of myonuclei within fibre cross-sectional profile. In sibs, >90% of fibres have more than one nuclear profile, compared with <15% in mutants. Mean ± SEM, χ² test. i NADH tetrazolium reductase stain revealed that in both mutants and sibs three fibre types are present: oxidative/slow (Slow), intermediate (Int) and glycolytic/fast (Fast). Size of more glycolytic myofibres (yellow and green insets) is more reduced than oxidative fibres (cyan). Assay was performed on three 120 dpf adult length-matched fish of each genotype. Representative sib (blue) or mutant (red) fibres are highlighted. Mutant presents smaller slow type fibres ectopically localised in fast domain (red inset). Representative images, n = 3. Bars = 100 μm (except for red, yellow and cyan insets = 10 μm).

**Fig. 8** Mutant adult-derived muscle progenitor cells retain fusion deficit ex vivo. a Immunodetection of desmin (green) and MyHC (red) and nuclei (white, Hoechst) in 15 months old myog<sup>kg125</sup> and sibling myog<sup>kg125</sup>/+ adult-derived MPCs following 5 days of differentiation. Fusion into multinucleated myofibres occurred only in sib (magnified boxes), coloured arrowheads indicate nuclei of each cell. Representative images, n = 3. b Extent of differentiation (Differentiation index) is comparable between mutant and heterozygous MPCs. c Fusion index showing deficit of fusion of mutant myocytes. d Number of nuclei in fused MyHC<sup>+</sup> cells is reduced in mutant, χ² test. Three fish per genotype (three technical replicates each). e Schematic of the role of Myogenin during differentiation, fusion and growth of muscle fibres. During myogenesis, committed MPCs leave the cell cycle, begin to elongate and express early muscle-specific genes during terminal differentiation into myocytes. At this stage, Myogenin (MYOG) promotes the expression of myomaker (MYMK), myomixer (MYMX) and jam3b (JAM3B). These fusogenic proteins prompt myocyte fusion to form muscle fibres. In the absence of Myogenin, myocytes undergo terminal differentiation but fail to express Myog-module genes, remain mononucleated and grow less throughout life. Residual myocyte fusion in Myog mutants in the medial region of the somite (bracket) is sustained by Hedgehog (Hh) signalling.
mulation for sequence genotyping. Standard weight (g) was calculated using Fulton’s formula K = weight (g) × 100 × length3 × 3 (cm)(reviewed in ref. 89). Body mass index (BMI) was calculated as ‘weight (g) × length2 × 3 (cm)’. Three 120 dpf adult male length-matched fish of each genotype were culled using high dose tricaine, eviscerated and skinned. Trunk from just behind gills to 5 mm beyond the dorsal fin was embedded in OCT (CellPath, Fisher Scientific), immersed in freezing iso-pentane (Fisher Scientific) and stored at –80 °C. Cryosections (15 μm) from three anteroposterior positions were immunolabelled for Laminin and counterstained with Hoechst 33342 as described87,88. All signals were normalised for input by percentage input calculation method (www.thermofisher.com).

**Adult fish analysis.** Siblings (120 dpf or 15 mpf) from heterozygote incrosses were anaesthetised with tricaine (Sigma Aldrich), blotted dry, weighed on an Ohaus YA102A balance, and repositioned with collagenase (C0130, Sigma Aldrich). 1% Penicillin/Streptomycin DMEM at 25.8 °C for at least 2 h. Single muscle fibres were released by trituration using heat-polished glass pipettes and washed three times with DMEM. 90–100 myofibers per fish were plated on Matrigel (Invitrogen) coated 24-well plates and cultured in 20% Foetal Bovine Serum in 1% Penicillin/Streptomycin DMEM for 7 days. Cells were washed twice with PBS to remove muscle fibres and induced to differentiate in 2% Horse Serum 1% Penicillin/ Streptomycin/DMEM for 5 days at 28.5 °C in 5% CO2 with medium change every 48 h, then fixed with 4% PFA, processed for immunofluorescence and imaged at 20X using an Axiovert 200 M microscope (Zeiss) equipped with LD A-plan ×20/0.85 objective. Fibre cross-sectional area (CSA) was measured in each image and averaged. Nuclei/fibre were scored as nuclei within laminin rings at three trunk positions in each fish of each genotype. The data are presented as the mean of averaged values from each individual fish. For digital whole section reconstruction several images where taken on iRiS® Digital Cell Imaging System, using ×4 objective, and merged using Photoshop CSS5.1.

**NADH Tetrozolium Reductase.** NADH-TR protocol was adapted from (https://neuromuscular.wustl.edu/pathol/histol/nadt.htm). Briefly, 15 μm unfixed cryosections, from three 120 dpf adult male length-matched fish of each genotype, were incubated in a 1:1 solution of NBT (Nitro-blue tetrazolium, 2 mg/ml, N6876, Sigma) and NADH (1.6 mg/ml, N8129, Sigma) in 0.05 M Tris HCl pH 7.6 at RT for 2 h. Sections were then washed three times with deionized water (dH2O), serially immersed in acetone-water 30%, 60%, 90%, 60%, 30%, ×3 dH2O, glycerol mounted and imaged with Axiohot microscope (Zeiss) equipped with Olympus DP-70 camera.

**Isolation and culture of zebrafish MPCS from adult tissue.** Isolation and culture of zebrafish adult muscle fibres was adapted from ref. 90. Briefly, adult fish were culled in high dose tricaine, washed in PBS, then 70% ethanol, eviscerated and skinned. Trunk muscle was incubated in 0.2% Collagenase (C0130, Sigma Aldrich). 1% Penicillin/Streptomycin DMEM at 28.5 °C for at least 2 h. Single muscle fibres were released by trituration using heat-polished glass pipettes and washed three times with DMEM. 90–100 myofibers per fish were plated on Matrigel (Invitrogen) coated 24-well plates and cultured in 20% Foetal Bovine Serum in 1% Penicillin/Streptomycin DMEM for 7 days. Cells were washed twice with PBS to remove muscle fibres and induced to differentiate in 2% Horse Serum 1% Penicillin/ Streptomycin/DMEM for 5 days at 28.5 °C in 5% CO2 with medium change every 48 h, then fixed with 4% PFA, processed for immunofluorescence and imaged at 20X using an Axiovert 200 M microscope (Zeiss). At least five random fields were acquired for analysis of the technical replicates of each fish. Six 15 months old adult male (three myogk125c/– heterozygotes and three myogk125c/– mutants) were dissected for the analysis. Differentiation index = nuclei in MyHC+ myocytes (MF20 and A4.1025) × 100/nuclei in desmin+ cells. Fusion index = Nuclei in myocytes with ≥ 2 nuclei × 100/ total nuclei in MyHC+ myocytes.

**Statistical analyses.** Quantitative analysis on images was performed with Fiji software (NIH, Fiji.sc). Statistical analyses used GraphPad (Prism 6) for unpaired two-tailed Student’s t test or Statplusmac v5 for ANOVA with Bonferroni or Tukey post-hoc tests to assess significant differences between mutant and sibling groups, unless otherwise stated. χ2 test was used to analyse difference between distributions using raw values. All data are expressed as mean ± standard error of the mean (SEM). Unless otherwise stated, numbers on columns represent number of fish analysed. p values for rejection of the null hypothesis of no difference between groups are indicated above columns.

**Data availability** The authors declare that all the data supporting the findings of this study are available within the Article and its Supplementary Information files or from the corresponding author upon reasonable request.
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Author contributions
M.G. and S.M.H. conceived the project and designed the experiments. Y.H. designed CRISPR strategy and generated myog mutant lines. M.G. performed all the experiments and analysis. S.B. designed and performed ChIP-qPCR assay, contributed to embryo genotyping and sample preparation for qPCR. M.G., H.F.O.Q. and P.S.Z. established the protocol for single fibre isolation and culture of zebrafish adult MPCs. S.M.H. and M.G. wrote the paper with contributions from all other authors.

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