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Fine-tuning of Hh signaling by the RNA-binding protein Quaking to control muscle development

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SUMMARY

The development of the different muscles within the somite is a complex process that involves the Hedgehog (Hh) signaling pathway. To specify the proper number of muscle cells and organize them spatially and temporally, the Hh signaling pathway needs to be precisely regulated at different levels, but only a few factors external to the pathway have been described. Here, we report for the first time the role of the STAR family RNA-binding protein Quaking A (QkA) in somite muscle development. We show in zebrafish that the loss of QkA function affects fast muscle fiber maturation as well as Hh-induced muscle derivative specification and/or morphogenesis. Mosaic analysis reveals that fast fiber maturation depends on the activity of QkA in the environment of fast fiber progenitors. We further show that Hh signaling requires QkA activity for muscle development. By an in silico approach, we screened the 3′UTRs of known Hh signaling component mRNAs for the Quaking response element and found the transcription factor Gli2a, a known regulator of muscle fate development. Using destabilized GFP as a reporter, we show that the *gli2a* mRNA 3′UTR is a functional QkA target. Consistent with this notion, the loss of QkA function rescued slow muscle fibers in *yot* mutant embryos, which express a dominant-negative Gli2a isoform. Thus, our results reveal a new mechanism to ensure muscle cell fate diversity by fine-tuning of the Hh signaling pathway via RNA-binding proteins.

KEY WORDS: Quaking, Hedgehog, Gli2a, Muscle specification, RNA-binding protein, Zebrafish

INTRODUCTION

In zebrafish, motility is initially induced by the contraction of segmental muscles that are located in repeated units – the somites – along each side of the body through the action of motoneurons. Each somite is V-shaped and contains predominantly two differentiated muscle cell types: the superficial slow-twitch fibers and the deep fast-twitch fibers (Devoto et al., 1996; Roy et al., 2001). Slow muscle precursors initially abut the notochord and derive from the so-called adaxial cells. Slow muscle precursors differentiate and elongate along the main body axis to span the entire length of the somite. They then undertake a lateral migration through the fast muscle precursors. During their migration, the slow muscle precursors are thought to trigger maturation of the fast muscle precursors (Henry and Amacher, 2004). These latter precursors elongate to reach the somite boundary and, once this process is completed, the fast muscle cells fuse to create syncytial myotubes (Snow et al., 2008) by an as yet uncharacterized mechanism (Krauss, 2007; Moore et al., 2007; Srinivas et al., 2007).

Within each somite, Hedgehog (Hh) activity emanating from the notochord induces the differentiation of the medially adjacent paraxial mesodermal cells, called adaxial cells, into slow-twitch muscle fibers (Barresi et al., 2000; Blagden et al., 1997; Coutelle et al., 2001; Currie and Ingham, 1996; Currie and Ingham, 1998; Du et al., 1997; Hirsinger et al., 2004; Lewis et al., 1999a; Lewis et al., 1999b; Roy et al., 2001). Overexpression of Hh leads to an excess of slow muscle cells, whereas inhibition of the pathway in the *smo* mutant, which lacks the Hh receptor Smoothened, prevents the differentiation of adaxial cells into their derivatives (Barresi et al., 2000; Hirsinger et al., 2004). Depending on the level of Hh signaling, adaxial cells give rise to two subpopulations: slow muscle cells (low level) and a specialized group of Engrailed-positive muscle cells termed the muscle pioneers (MPs; high level). The MPs populate the medial part of the somite known as the horizontal myoseptum, which separates the ventral from the dorsal half of the somite, and their presence is tightly correlated with the V-shape of the somite (Ekker et al., 1992; Hatta et al., 1991; Roy et al., 2001; Wolff et al., 2003); U-shaped somites often lack MPs. Later, an intermediate level of Hh activity induces the medial fast fibers (MFFs) (Roy et al., 2001), a subpopulation of paraxial mesodermal cells that surround the MPs and also express Engrailed (Wolff et al., 2003).

The activity of the secreted Hh proteins is mediated by their binding to the large seven-multipass transmembrane protein Patched 1 (Ptc1, or Ptch1) (Concordet et al., 1996). Once bound to Hh, Ptc1 ceases to inhibit the twelve-transmembrane receptor Smo (Chen et al., 2001), inducing Hh signaling transduction. The outcome of this pathway is the absence of cleavage and the activation of the Gli family of zinc-finger transcriptional effectors. Gli enters the nucleus to activate the transcription of various target genes, including *ptc1*, and to specify the different muscle cell types (Concordet et al., 1996). As the level and the timing of Hh signaling are critical for the specification of the different muscle cell types, somite morphogenesis is a complex process that requires fine-tuning. Recent studies have revealed a role for miRNA in the control of the Hh pathway, suggesting a level of post-transcriptional regulation, but this area remains largely unexplored (Flynt et al., 2007).

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The post-transcriptional regulation of gene expression plays a fundamental role in the temporal and spatial modulation of developmental processes. Various molecules are involved in this fine-level regulation in addition to miRNAs, including RNA-binding proteins. Within this latter category, Quaking proteins belong to the signal transduction and activation RNA (STAR) family of RNA-binding proteins (Vernet and Artzt, 1997). Their action is mediated by their KH domain, a prevalent RNA-binding domain that recognizes and binds a specific sequence in the 3′UTR of the target mRNAs. STAR proteins function in pre-RNA splicing (Arning et al., 1996; Chawla et al., 2009; Matter et al., 2002; Paronetto et al., 2007; Stoss et al., 2004), mRNA export (Coyle et al., 2003; Larocque et al., 2002; Reddy et al., 2002), mRNA stability (Larocque et al., 2005; Nabel-Rosen et al., 1999) and protein translation (Jan et al., 1999; Lee and Schedl, 2001; Lee and Schedl, 2004; Saccomanno et al., 1999; Schumacher et al., 2005). STAR family members have been identified throughout the evolution of the metazoan lineage and regulate various processes of cell differentiation, such as myelination in mice or tendon differentiation in flies (Ebersole et al., 1996; Nabel-Rosen et al., 1999; Nabel-Rosen et al., 2005; Nabel-Rosen et al., 2002; Sidman et al., 1964; Volk et al., 2008).

In this study, we report the isolation of a new zebrafish mutation, *t31954*, that affects motility. Within the somite, this mutation leads to delayed maturation of fast muscle and specification problems for the Hh-induced muscle cells. Molecular analysis shows that allele *t31954* carries a loss-of-function mutation in *quaking A* (*qkA*), a zebrafish STAR family member that is highly related to vertebrate *Quaking*. We provide evidence that Hh signaling is downregulated in the somitic territory when *qkA* function is lost. *qkA*^{t31954} homozygous mutants are still responsive to overactivation of Hh signaling but the pattern of muscle cell type induction is modified, suggesting that *qkA* acts downstream of the Hh signaling pathway to regulate muscle cell type specification. By in silico analysis, we find that *gli2a* mRNA carries a Quaking response element (QRE) within its 3′UTR. Using a sensor assay, we show that this QRE is essential for *gli2a* translation and mRNA stability. Consistent with this notion, the loss of *qkA* function rescues slow muscle fibers in *yot* mutant embryos, which express a dominant-negative Gli2a isoform. Our results thus uncover a novel role for Quaking as a fine-level post-transcriptional regulator of the Hh signaling pathway that is essential for the proper specification of the various muscle cell types.

MATERIALS AND METHODS

Zebrafish strains and husbandry

The *qkA*^{t31954} allele was isolated in a large-scale ENU mutagenesis screen organized by the ZF-MODELS EU project. Embryos were obtained from natural crosses of either wild-type (WT) or heterozygous *yot*$^{ty119/+}$ or *qkA*$^{t31954/+}$ mutant fish and staged as previously described (Kimmel et al., 1995; Westerfield, 1993).

Genetic mapping of the *qkA* locus

Heterozygous *qkA*$^{t31954/+}$ mutant fish were outcrossed to the polymorphic WIK strain for mapping. Approximate mapping was performed on 48 mutants and siblings from a single F1 cross. The mutation was first mapped to LG17 by bulk segregant analysis. Primer sequences for simple sequence polymorphisms (SSLPs) were obtained from the Boston Massachusetts General Hospital (MGH) zebrafish and ZFIN databases (Fig. 2A). Embryonic genomic DNA from individual zebrafish larvae (n=442 embryos) was prepared as described (Nüsslein-Volhard and Dahm, 2002). PCR products were electrophoresed on 2% MetaPhor plus 1% ultra pure agarose (TEBU-bio) gels.

Identification of the *qkA*^{t31954} mutation and cloning of *qkA* and *prdm1a*

RNA was extracted from 24-hpf embryos with TRIzol (Molecular Probes), then reverse transcribed with the AccuScript High Fidelity First-Strand cDNA Synthesis Kit (Stratagene). The cDNA was PCR amplified using the qkA_forward 5′-AAAGAGCACGCCAGACAGAGAC-3′ (5′UTR) and qkA_reverse 5′-GGCGGCTCATCATTGGGTGAAG-3′ (3′UTR) primers. cDNA products were cloned into pSPE3-Rfa. *prdm1a* was amplified with prdm1a_Fwd 5′-ctactcgagACTAGCAGTGGGACAAGACAGG-3′ and prdm1a_Rev 5′-ctatctagaCGGCCTACATTAAAAATCAACC-3′ and cloned into pBluescript SK^{-} (Stratagene).

Genotyping of mutant embryos

Genomic DNA from single *qkA*^{t} embryos was amplified by PCR using primers 5′-CAGAGTTTCCACACTGTTAGTGG-3′ and 5′-ACATCA-AAGTACAAGCCTCAAGC-3′. PCR products were digested with *Bcl*I and resolved on a 3% agarose gel. *yot*^{ty119} genotyping was performed as described (Vanderlaan et al., 2005).

mRNA synthesis and morpholinos

Capped mRNAs were synthesized from pSPE3-Rfa and pCS2^{+} linearized with *Kpn*I and *Not*I, respectively, and transcribed with the T7 (*qkA*) and SP6 (*GFP* and *kaede*) RNA polymerases (Ambion) then injected at 50 ng/μl (*GFP*), 10 ng/μl (*kaede*) or 100 ng/μl (*qkA*). Morpholinos (MOs) were obtained from Gene Tools and included: *qkA* exon 1-intron 1 splicing site MO (*qkA*$^{gt\text{-}MO}$, 5′-TCAGATTTACACACACATACCTTCG-3′); *qkA* translation-blocking MO (*qkA*$^{ATG\text{-}MO}$, 5′-CCTTCACCTCCATCTCC-CCGACCAT-3′); and *gli2a* translation-blocking MO (*gli2a*$^{ATG\text{-}MO}$, 5′-GAGGTGGGACTTGTGGTCTCCATGA-3′). To ensure local MO delivery (Fig. 3), MO was injected together with *GFP* RNA into one cell of the 8-cell embryo and sorted at gastrulation, selecting embryos with labeled clones restricted to the margin.

Transplantations

To target muscle cells, ~20-50 labeled cells were removed from WT donors (sphere stage) and transplanted to the margin of unlabeled hosts according to Hirsinger et al. (Hirsinger et al., 2004). Genotypes of donor and/or host embryos derived from heterozygous intercrosses were determined by PCR and/or by fast startle escape-touch response.

Whole-mount in situ hybridization and antibody labeling

Probe synthesis and in situ hybridization were performed following standard protocols (Hauptmann and Gerster, 1994).

Immunohistochemistry was performed as follows on at least 50 mutant and WT sibling embryos. In brief, embryos were fixed in 4% paraformaldehyde (PFA) in PBS and stored in methanol at –20°C. After progressive rehydration, 24- or 48 hpf-embryos were incubated in proteinase K (10 μg/ml) for 15 or 60 minutes, respectively, washed three times with PBS containing 0.1% Tween 20 and post-fixed with 4% PFA for 20 minutes at room temperature. After four washes, blocking with 1% DMSO, 1% BSA, 10% normal goat serum in PBS containing 1% Triton X-100 for 1 hour was followed by incubation with primary antibodies overnight at 4°C, then incubation with secondary antibodies at room temperature for 2.5 hours. Primary antibodies were: F59 (1/100), F310 (1/100), MF20 (1/100), Pax7 (1/100) and 4D9 (1/50) (all DSHB); Prox1 (Reliatech, 1/200); and GFP (Torrey-Pines, 1/1000). Secondary antibodies included Alexa Fluor 488- or 594-conjugated anti-mouse IgG1 or anti-mouse IgG2b and Alexa Fluor 488- or 568-conjugated anti-rabbit (Invitrogen) at 1/1000. Specimens were visualized by laser-scanning inverted confocal microscopy.

Sensor assay

The full 3′UTRs of *wnt11* and *gli2a* were fused to the 3′ end of *d2egfp* using *Xho*I and *Xba*I (Giraldez et al., 2005). Sequences were amplified from genomic DNA with the following primers and cloned into double-digested pCS2+d2egfp: 3′UTR-*wnt11*_Fwd 5′-CTACTCGAGCAGACCG-TCTTCACCAATAGACCT-3′ and 3′UTR-*wnt11*_Rev 5′-CTATCTAGA-CAGGCTGAAATGCATGGCTTTCAT-3′; 3′UTR-*gli2a*_Fwd 5′-CTA-CTCGAGTAATTGAAGGCATTACTCTA-3′ and 3′UTR-*gli2a*_Rev 5′-CTATCTAGAGGTCATACAGTGAAAGTCAA-3′. The resulting

constructs were linearized by *Not*I and then RNAs transcribed using SP6 RNA polymerase (Ambion). RNAs were co-injected at the one-cell stage with *kaede* mRNA. Individual embryos were prepared for western blot analysis (trunk) and genotyping (head).

Western blot

Each sample was incubated with RIPA solution (Flynt et al., 2007) containing protease inhibitors and then an equal volume of Laemmli 2× loading buffer containing β-mercaptoethanol was added. After genotyping, three embryos were pooled and resolved on a 10% SDS-polyacrylamide gel, blotted onto a Hybond ECL membrane (GE Healthcare) and blocked with 5% non-fat dried milk in TBS (Hauptmann and Gerster, 1994) containing 0.1% Tween 20. Immunodetection was carried out using rabbit anti-GFP antibody (Torrey Pines, 1/5000-1/10,000), anti-Kaede (MBL, 1/1000) and anti-Actin (Sigma, 1/1000) as primary antibodies and HRP-conjugated anti-rabbit secondary antibody (Roche, 1/2000). Detection was carried out using the Lumi-Light chemiluminescence reagent (Roche) and ChemiCapt apparatus (Vilber Lourmat). The intensities of all bands were normalized to Kaede signal and Actin levels (three independent experiments).

RESULTS

t31954 and *qkA*

The recessive lethal embryonic mutation *t31954* exhibits impaired motility at 24 hours post-fertilization (hpf). This feature was used to distinguish mutant embryos from WT siblings and allowed us to map the *t31954* mutation to the locus referred to as *qkA* (see below). From now on, we refer to this mutation as qkA^{t31954} or, in short, qkA^{t}.

qkA is required for slow and fast muscle fiber patterning and morphogenesis

Consistent with the impaired motility, qkA^{t} homozygous embryos exhibited somite defects. First, the border of the somite segments was not V-shaped but U-shaped (Fig. 1A-D) and slightly irregular. However, early and late boundary markers, including Fibronectin, Laminin and Dystrophin, did not show marked discontinuity indicating that the border is present (data not shown). In addition, somite muscles were affected. Mononucleated slow-twitch fibers are aligned in a parallel array of ~20 fibers along the dorsoventral axis in WT 27-hpf embryos and encompass the entire length of the somite (Fig. 1A,A′, white arrow). In qkA^{t} homozygotes, they were reduced in number, the arrays were disorganized and exhibited gaps (Fig. 1B,B′, white asterisk), branching and abnormal fiber direction (Fig. 1B, white arrowhead), resulting in the invasion of the fast muscle domain (Fig. 1B′). Consistent with the U-shaped somite, the MPs, a subpopulation of medial slow muscle cells in the immediate vicinity of the notochord that specifically co-expresses Prox1 and the homeoprotein Engrailed (Ekker et al., 1992; Glasgow and Tomarev, 1998; Halpern et al., 1993; Hatta et al., 1991; Roy et al., 2001), were also reduced (Fig. 1E,F, white arrows; Table 1). In addition, the MPs were mislocated and situated away from the notochord in some cases, suggesting that they were pseudo-MPs (see Fig. 5D-F; data not shown) (Wolff et al., 2003).

At 27 hpf, WT fast muscle cells, labeled by F310 (Crow and Stockdale, 1986)], are elongated and arranged in three diagonal arrays of multinucleated myotubes that are anchored to the anterior and posterior somite boundary and exhibit cortical labeling (Henry et al., 2005; Roy et al., 2001; Snow et al., 2008; Snow and Henry, 2009). By contrast, qkA^{t} mutant F310-positive cells were not structured like fibers, appeared immature, with predominantly cytosolic fMLC labeling, and were not organized into recognizable arrays or adopted an array disposition parallel to the main axis (Fig. 1C,D). Moreover, cortical labeling with β-catenin antibody showed that a large portion of the mutant fast muscle fibers do not span the entire somite length and are mononucleated, suggesting that cell fusion failed to occur (compare Fig. 1G with 1H, arrows). Similar to MPs, MFFs were also reduced in number in qkA^{t} mutants (Fig. 1F; Table 1).

The requirement for *qkA* could be first traced during early to mid-somitogenesis. At the 10-somite stage, qkA^{t} mutant adaxial cells were not all juxtaposed to the notochord, in contrast to WT (Fig. 1I,J). At early stages, *myod* (*myod1* – Zebrafish Information Network) expression appeared unaffected, indicating that the specification of the myogenic lineage was properly initiated (see Fig. S1 in the supplementary material).

Thus, qkA^{t} homozygous embryos exhibit a severe and persistent muscle phenotype that affects the morphogenesis of slow and fast fibers as well as maturation of the latter (data not shown). In addition, slow muscle cells, including the MPs and MFFs, are reduced. Together, our results indicate that *qkA* is required to control both slow and fast muscle cell fates as well as their maturation (fast) and morphogenesis (fast and slow).

Mapping and positional cloning of *t31954*

qkA^{t31954} was meiotically mapped to linkage group (LG) 17 using MGH polymorphic markers, at 1.5 cM from z9633 and 0.11 cM from z21435 (Fig. 2A). Closest to the latter marker, the gene *qkA* was found in the Zv7 Ensembl assembly of the zebrafish genome. *qkA* cDNA was cloned and sequenced from WT, heterozygous and qkA^{t} homozygous mutant embryos. *qkA* contains eight exons that span 84.2 kb of genomic DNA (Fig. 2B). Sequencing of the cDNA obtained from the qkA^{t} homozygote showed a T-to-A transversion in position +467 relative to the ATG start codon, which was confirmed on genomic DNA (Fig. 2B,C). This mutation leads to the loss of the *Bcl*I site at +464, which was confirmed on PCR-amplified genomic DNA (Fig. 2D), allowing us to easily genotype qkA^{t} mutant embryos.

RT-PCR of zebrafish *qkA* cDNA generated two splice variants: *qkA1* and *qkA2*. Zebrafish *qkA1* includes all the exons, whereas *qkA2* lacks the 3′ part of exon 6 (Fig. 2B). *qkA1* and *qkA2* mRNAs encode highly conserved 383 and 342 amino acid RNA-binding proteins, respectively (Fig. 2E), that belong to the STAR family (Hardy et al., 1996). Proteins of this family include a KH domain surrounded by two sites, QUA1/NK and QUA2/CK, which together form the STAR domain. The protein can homo/heterodimerize through the NK/CK domains, whereas the KH domain mediates direct protein-mRNA interaction in order to upregulate or downregulate mRNA expression (Cox et al., 1999; Larocque et al., 2005; Larocque et al., 2002; Larocque and Richard, 2005; Nabel-Rosen et al., 2002; Noveroske et al., 2002; Wu et al., 2002). Moreover, one of the mouse quaking (*Qk*) transcripts, *QkI-5* (Ebersole et al., 1996), contains a nuclear localization signal (NLS) sequence at the C-terminus, which is also detected in zebrafish QkA1/2 [366 to 372 (QkA1) and 325 to 331 (QkA2)]. Additionally, there is a significant conserved synteny between regions containing zebrafish *qkA* and mouse *Qk*. We therefore conclude that zebrafish *qkA* is the orthologue of mouse *Qk* (see Fig. S2 in the supplementary material). The T-to-A transversion in qkA^{t} leads to a change from a highly conserved isoleucine to asparagine (I156N) in the KH domain, which probably results in a misfolding of this domain that is likely to alter QkA function (Fig. 2E; see red box in Fig. S2 in the supplementary material).

The qkA^{t} mutation was phenocopied by morpholino antisense oligonucleotide (MO) (Nasevicius and Ekker, 2000) injections, either splice blocking ($qkA^{gt\text{-}MO}$; Fig. 2F-H,J-L) or translation

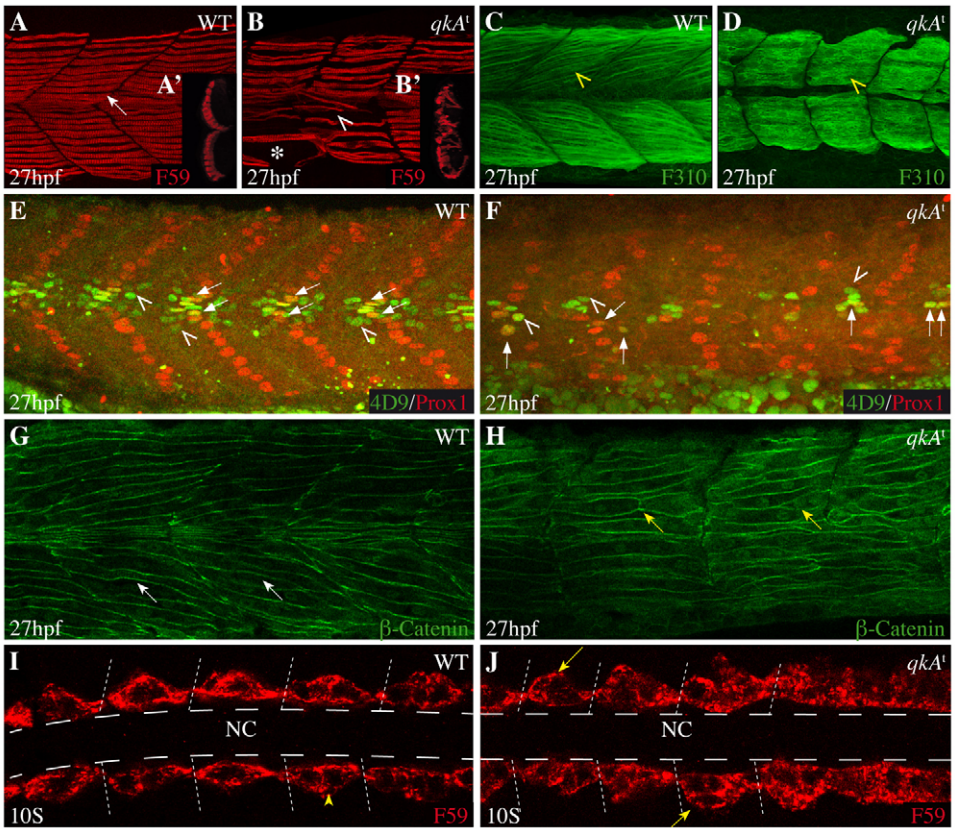

Fig. 1. Requirement for *qkA* during somite development. (**A-H**) Lateral views from 27-hpf zebrafish embryos of the indicated genotypes (upper right) immunostained with the indicated markers (lower right) and analyzed by confocal imaging. Anterior is to the left. (A-B′) Slow muscle cells labeled by F59 (arrow) are disorganized in *qkA*^{t} homozygotes (arrowhead), are less numerous, exhibit gaps (asterisk) and invade the fast muscle domain (see optical transverse sections in insets A′,B′). (C,D) Fast muscle fibers (yellow arrowhead) appear immature in *qkA*^{t} homozygotes, with predominantly cytosolic staining and imprecise contours. (E,F) Muscle subpopulations are reduced in *qkA*^{t}. Embryos were stained for Prox1 [slow muscle cells, including muscle pioneers (MPs)] and Engrailed [MPs (arrows) and medial fast fibers (MFFs; arrowheads) by 4D9 antibody]. Note the reduction in all these cell types in the *qkA*^{t} homozygotes. (G,H) *qkA* is required for fast myotube formation. β-catenin staining reveals a large number of short mononucleated cells (yellow versus white arrows) in the fast muscle domain, indicating retarded fusion. (**I,J**) Early requirement for *qkA* in slow muscle development. Ten-somite stage embryos stained for slow muscle, dorsal view. At this stage, adaxial cells abut the notochord in wild type (WT) (arrowhead), whereas mutant somites often exhibit several mislocated cells (arrows). Dashed lines indicate the position of the notochord. NC, notochord.

blocking (*qkA*$^{ATG\text{-}MO}$; data not shown). *qkA*$^{gt\text{-}MO}$ (Fig. 2B) induced a truncated QkA isoform devoid of the STAR domain (Fig. 2E; see Fig. S3 in the supplementary material). The phenotypes of the *qkA*^{t} mutant or morphants were efficiently rescued by *qkA1* or *qkA2* mRNA overexpression, leading, at 27 hpf, to a striking recovery of both slow and fast muscle fiber morphology (Fig. 2I,M; data not shown). However, overexpression of *qkA1* or *qkA2* in WT embryos did not noticeably affect either slow or fast fiber development (data not shown). Thus, our data show that the muscle phenotypes observed in *qkA*^{t} homozygous embryos result from the loss of *qkA* zygotic function and *qkA* therefore appears to be a permissive factor for muscle development.

Non-autonomous requirement for QkA in fast muscle cells

qkA is expressed throughout paraxial mesoderm (Baxendale et al., 2009; Tanaka et al., 1997) (data not shown) and could be required within fast muscle progenitor cells or in their

Table 1. Quantification of the different muscle cell types in the absence and presence of Shh

	Uninjected embryos		Shh-injected embryos	
Cell type	WT	*qkA*^{t}	WT	*qkA*^{t}
Muscle pioneers (Eng^{+}/Prox1^{+})	11.9±0.61	5.5±0.91	50±3.74	42±4.76
Medial fast fibers (Eng^{+}/Prox1^{-})	27±1.12	12.9±1.40	3.8±1.01	75.6±3.69
Slow fibers (Eng^{-}/Prox1^{+})	26.5±1.56	14.8±0.98	44.8±3.18	34±2.83

Values represent the mean number of nuclei per somite (± s.e.).
In each case, four somites and five (except six for uninjected *qkA*^{t}) embryos were counted.
Eng, Engrailed.

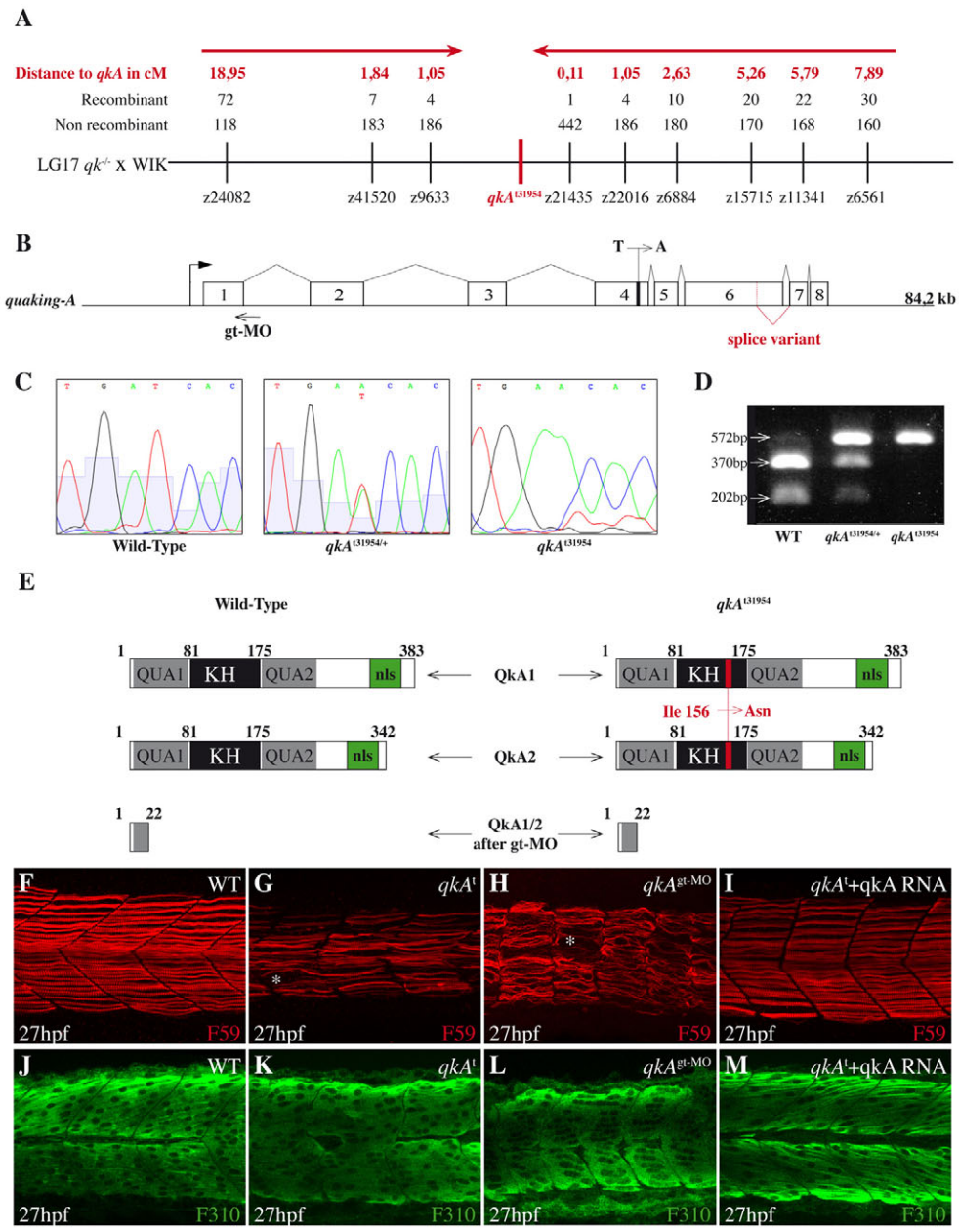

Fig. 2. Molecular and functional characterization of the *qkA* locus. (**A**) Genetic mapping of zebrafish *quaking A* (*qkA*). On chromosome 17, the qkA^{t31954} mutation is linked to markers z9633 and z21435, delineating a candidate genomic region of ~100 kb that contains *qkA*. Red arrows indicate the directions of mapping progression. (**B**) *qkA* gene structure. *qkA* includes eight exons. The alternative splicing site and the positions of the qkA^{t31954} mutation in exon 4 and of the $qkA^{gt\text{-}MO}$ are indicated. (**C**) Chromatogram showing the single base change in qkA^{t31954} mutants. (**D**) qkA^{t31954} mutation leads to the loss of an RFLP site (*BclI*). The *qkA* region was PCR amplified from genomic DNA and digested with *BclI*, which normally cuts a 572 bp fragment into 370 bp and 202 bp fragments. (**E**) Structure of predicted QkA protein isoforms in WT, mutant and $qkA^{gt\text{-}MO}$ embryos. The *t31954* mutation leads to the Ile156Asn amino acid change (red) in the KH domain. KH, K homology domain; nls, nuclear localization signal. (**F-M**) qkA^{t31954} mutation can be rescued by *qkA* mRNA injection and is phenocopied by $qkA^{gt\text{-}MO}$. WT, qkA^{t31954} mutants or *qkA* morphants, untreated or injected with *qkA* mRNA, were fixed at 27 hpf and processed as in Fig. 1A-D. Asterisks, gaps in slow muscle cells.

environment. To address this issue, *qkA* function was blocked locally with $qkA^{ATG\text{-}MO}$ (Fig. 3A). The presence of the MO within a large group of fast muscle progenitors did not affect their capacity to fuse, to elongate, nor to adopt a diagonal arrangement (Fig. 3E-G, *n*=9/10). A similar result was obtained when $qkA^{ATG\text{-}MO}$ was restricted to the notochord/floor plate domain (Fig. 3B-D, *n*=8/8). In sharp contrast, presence of the $qkA^{ATG\text{-}MO}$ in a domain that included slow muscle cell progenitors but excluded fast muscle progenitors led to the typical qkA^{t} immature fast muscle phenotype (Fig. 3H-J, *n*=9/11). Hence, QkA function is not required in fast muscle cells, but in their environment. Consistent with this result, WT fast muscle cell progenitors grafted into a qkA^{t} mutant fast muscle domain, or qkA^{t} mutant fast muscle cell progenitors grafted into WT fast muscle domain, developed as host tissue, confirming the non-autonomous requirement for QkA in fast muscle cells (Fig. 3K-M, *n*=22/22).

qkA regulates the Hh signaling pathway

qkA is required for the morphogenesis and/or fate of Hh-induced slow muscle cells and MPs, suggesting that QkA could control these processes by regulating the Hh pathway. Consistent with this notion, somitic expression of the Hh pathway read-outs *ptc1* and *prdm1a* (Baxendale et al., 2004; Concordet et al., 1996) was dramatically reduced in non-axial qkA^{t} tissue during somitogenesis (Fig. 4A,B,D,E, arrows; see Fig. S4A,B,D,E in the supplementary material), but not in the notochord nor presomitic mesoderm (PSM) (Fig. 4C, arrowhead; data not shown). Likewise, the number of Pax7-positive dermomyotomal myogenic precursors (Feng et al., 2006) was higher in qkA^{t} than in WT (Fig. 4G,H; *n*=6 embryos, four somites/embryo), similar to the situation in which Hh signaling is impaired. By contrast, the expression of the Hh-regulated gene *nkx2.2a* was not altered in the central nervous system (data not shown). Thus, *qkA* is required to specifically regulate the Hh pathway within trunk mesoderm.

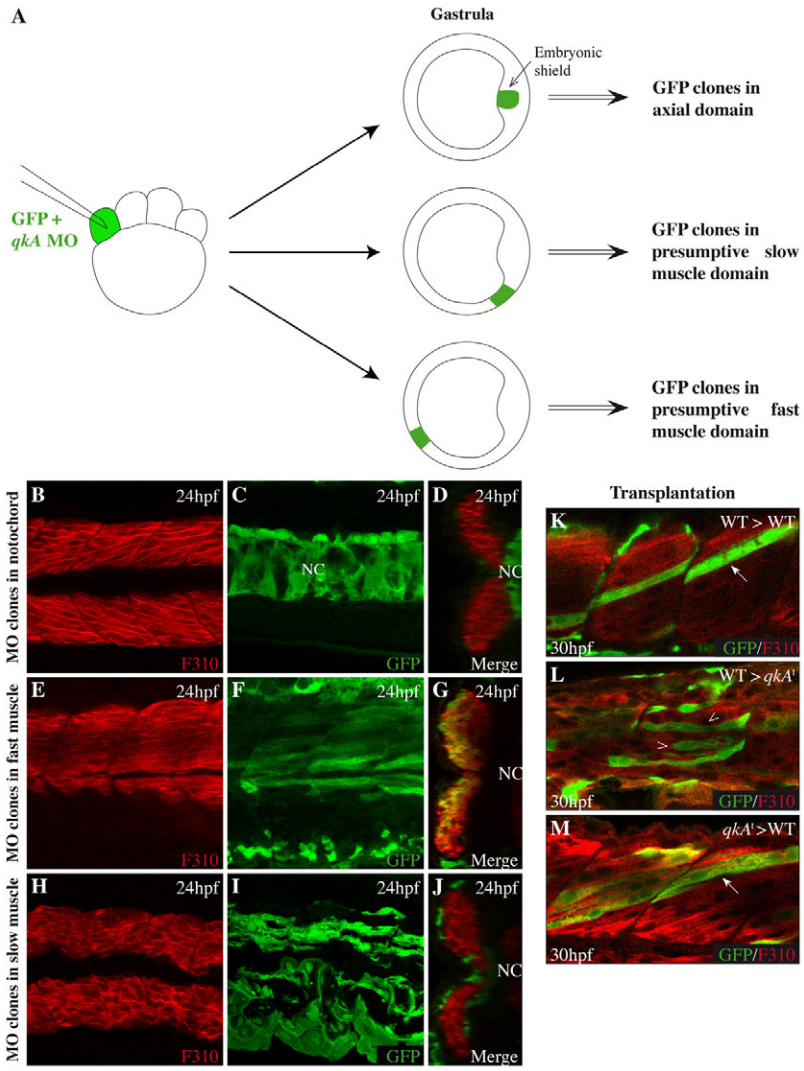

Fig. 3. QkA activity is not required in fast muscle cells. (**A**) Scheme for the generation of morphant clones. $qkA^{gt\text{-}MO}$ was injected together with *GFP* RNA at the 8-cell stage and embryos were sorted for the dorsoventral position of the clone at the onset of gastrulation. (**B-J**) Embryos were immunostained at 24 hpf for fast muscle (F310) and GFP and analyzed by confocal microscopy. (B,C,E,F,H,I) Lateral views, anterior to the left; (D,G,J) corresponding optical sections (merge). (**K-M**) GFP-labeled WT (K,L) or mutant (M) cells were transplanted into WT (arrows; K,M) or qkA^{t31954} homozygotes (arrowheads; L) and analyzed for fast muscle development at 30 hpf. NC, notochord.

If QkA acts within the Hh signaling pathway to control slow muscle/MP morphogenesis, maturation and/or induction, inhibition of the pathway should lead to phenotypes similar to those induced by *qkA* loss of function. The transcription factor Gli2a is required for Hh signal transduction in muscle development. Similar to the effects of *qkA* loss of function, in *gli2a* morphants the expression profile of *ptc1* and *prdm1a* was attenuated during somitogenesis (Fig. 4C,F; see Fig. S4C,F in the supplementary material) and some slow muscle cells were found medial to the bulk of fast muscle cells [compare Fig. 4I-K with 4L-N (qkA^t) and 4O-Q ($gli2a^{MO}$)] (Wolff et al., 2003). Finally, the organization of the fast muscle fibers was impaired in *gli2a* morphants (see Fig. S5 in the supplementary material). Together with the fact that *gli2a* is required for MP induction, our results strongly suggest that *qkA* acts on slow muscle and MP morphogenesis and fate by regulating the Hh signaling pathway.

To directly test the idea that QkA controls the fate of muscle cells by acting on Hh signaling, high levels of Hh protein were induced in the progeny of qkA^t heterozygous carriers by injection of RNA. Slow muscle fibers express the marker Prox1, whereas MPs express both Prox1 and Engrailed (Ekker et al., 1992; Glasgow and Tomarev, 1998; Halpern et al., 1993; Patel et al., 1989; Roy et al., 2001; Wolff et al., 2003), and both can be distinguished from MFFs, which express Engrailed only (Roy et al., 2001; Wolff et al., 2003). In WT or qkA^t heterozygotes (Fig. 5G-I,M; Table 1), *shh* mRNA overexpression led to a twofold increase in both slow muscle fibers and MPs, and to a marked decrease in MFFs. By contrast, *shh* overexpression in qkA^t homozygotes (Fig. 5J-M; Table 1) led to an increase in MPs and slow fibers but to a strong increase in the size of the MFF population. These results are consistent with the idea that Hh signaling is still partially active when QkA activity is downregulated (owing to the persistence of Hh-dependent muscle cells). However, the response to *shh* overexpression in terms of the generation of different muscle cell types is altered in QkA-deficient embryos as the MFF fate is promoted rather than inhibited. Altogether, combined with the downregulation of *ptc1* and *prdm1a* expression and the upregulation of $Pax7^+$ dermomyotomal cells in qkA^t homozygotes, these results show that *qkA* plays a major role in fine-tuning the Hh signaling pathway in the mesoderm.

QkA regulates *gli2a* mRNA activity in paraxial mesoderm

How does QkA act on the Hh signaling pathway? In vertebrates, Quaking regulates different mRNA targets by interacting with their pre-messenger 3′UTR (Larocque et al., 2002; Li et al., 2000;

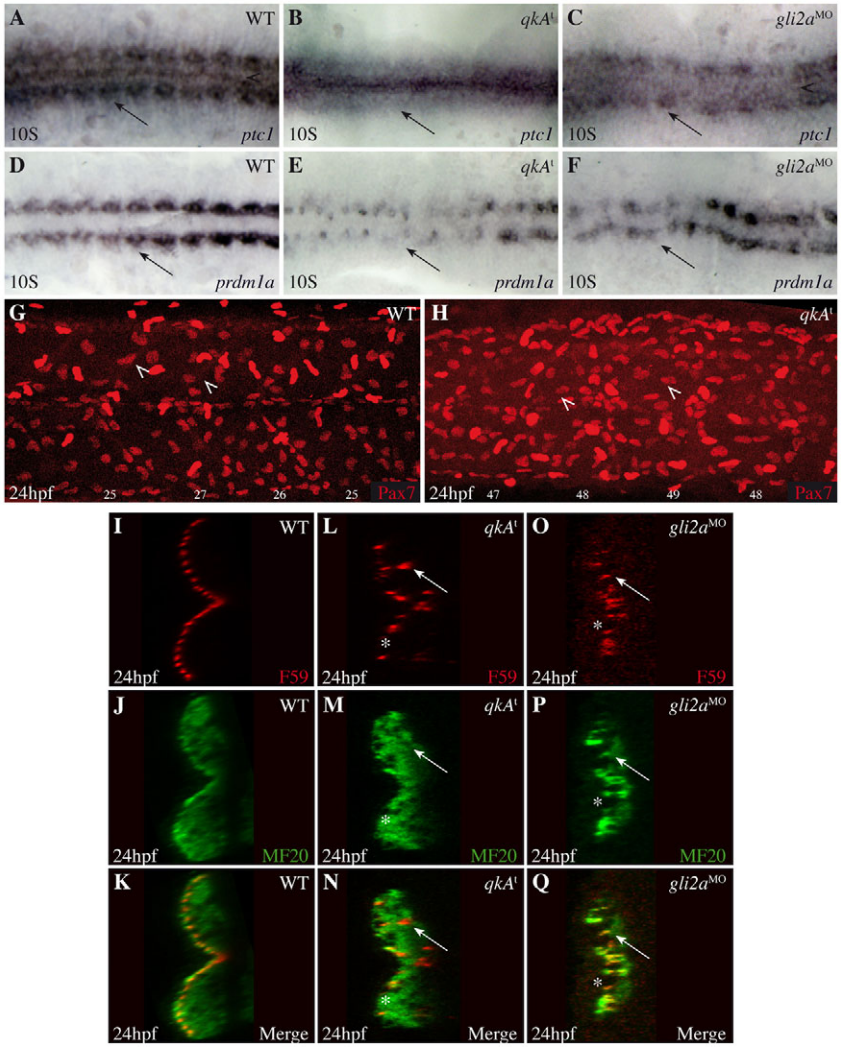

Fig. 4. Convergence of *qkA* and Hh phenotypes. (**A-F**) *qkA*^{t} and *gli2a*^{MO} zebrafish embryos exhibit downregulated Hh signaling. Embryos were processed at the 10-somite stage for in situ hybridization with the indicated probes. Dorsal view. (**G**,**H**) Lateral views of Pax7-immunostained embryos at 24 hpf. Two populations of Pax7-positive cells can be discerned, of which dermomyotomal cells (arrowheads) exhibit the weaker expression. The number of dermomyotomal cells per somite is indicated beneath each somite. (**I-Q**) Slow fiber distal migration requires QkA and Hh activities. Embryos were stained at 24-hpf for slow fibers (F59) and all differentiated muscle fibers (MF20) as indicated, then analyzed by confocal microscopy (transverse sections). Note the presence of gaps (asterisks) and deep slow muscle cells (arrows) medial to the fast muscle cells in *qkA*^{t} mutants and *gli2a* morphants.

Nabel-Rosen et al., 2002; Schachner and Bartsch, 2000) via a QRE (Galarneau and Richard, 2005). A Quaking-binding site has been defined by two motifs, UACUMAY and UAAY, separated by 1-20 nucleotides. This structure has been highly conserved across evolution. The high degree of sequence conservation between mouse Qk and zebrafish QkA KH domains suggested that the QRE is probably conserved in zebrafish as well. We screened in silico, using the Ensembl database, the 3′UTRs of genes encoding the different components of the Hh signaling pathway for the presence of a QRE. *gli2a*, which encodes a transcription factor that promotes *ptc1* transcription and is required for MP, slow muscle and MFF specification (Du and Dienhart, 2001; Roy et al., 2001), includes a sequence that is highly related to the canonical QRE in its 3′UTR (Fig. 6A). QkA could thus regulate the Hh signaling pathway through *gli2a* mRNA binding.

As no antibodies are available for Gli2a, we performed a sensor assay (Leucht et al., 2008) using a destabilized GFP (d2eGFP) as a reporter fused upstream to the 3′UTR of *gli2a* mRNA (d2eGFP-gli2a) or to the 3′UTR of *wnt11* mRNA (d2eGFP-wnt11), which does not carry any obvious QRE, as a control. These constructs were injected at the one-cell stage into the progeny of *qkA*^{t} heterozygotes and d2eGFP expression was assayed by western blotting on mid-somitogenesis embryos. Whereas d2eGFP levels were similar in WT and *qkA*^{t} homozygous d2eGFP-wnt11-injected embryos, d2eGFP expression derived from d2eGFP-gli2a injection was significantly decreased in *qkA*^{t} homozygous versus WT sibling embryos (Fig. 6B, compare left with right panel; Fig. 6C; Table 2). Although this latter effect might seem modest, the assay was performed on whole trunk extracts. Gli2a regulation by QkA might be restricted to adaxial cells and thus swamped by the expression of *gli2a* in surrounding tissues. Thus, this result is consistent with the fact that endogenous *gli2a* mRNA is stabilized, or its translation facilitated, by QkA protein.

To address the role of QkA in *gli2a* mRNA stability more directly, we took advantage of the *yot* mutant allele, which carries a point mutation in the coding region of the *gli2a* transcript that results in the expression of a truncated repressor form of the Gli2a protein (DR-Gli2a). Embryos homozygous for this mutant allele display a complete loss of response to Hh signaling in the myotome, including a loss of all slow muscle fibers as well as MPs (Karlstrom et al., 2003). Consistent with this being due to constitutive Gli2a repressor activity, the phenotype can be suppressed by a translation-blocking *gli2a* MO (Wolff et al., 2003). We reasoned that if QkA stabilizes the *gli2a* transcript, inactivation of QkA should similarly suppress the *yot*^{ty119} phenotype. We injected *yot*^{ty119} homozygous embryos with *qkA*^{MO} and, as predicted, observed significant rescue of slow muscle fiber differentiation (Fig. 6D-F).

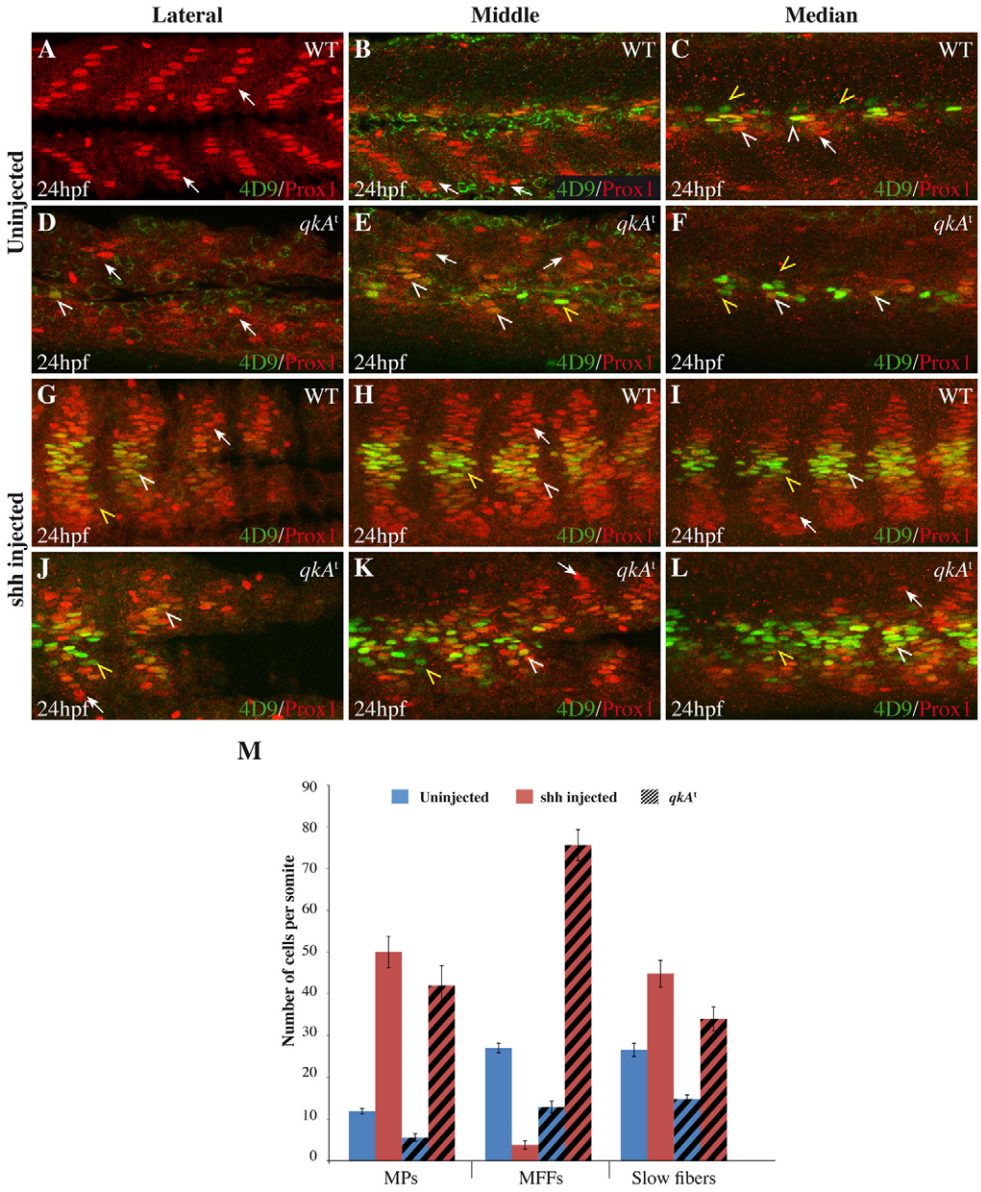

Fig. 5. Requirement for *qkA* in Hh signaling. (**A-L**) WT or *qkA*^{t} mutant zebrafish embryos were injected with *shh* RNA (G-L) or left untreated (A-F), then fixed at 24 hpf and stained for Prox1 and Engrailed (4D9) as in Fig. 1 revealing slow fibers (arrows), MPs (white arrowheads) and MFFs (yellow arrowheads). Different mediodistal sections are shown, as indicated above each column. (**M**) The numbers of each fiber type per somite (based on the expression of Prox1 and/or Eng) plotted according to the genotype and the treatment (*n*=5-6 embryos per condition; see Table 1). Error bars indicate s.d.

Altogether, our data reveal a novel and essential role for QkA proteins in the regulation of the Hh signaling pathway and in the specification of the different muscle cell types in the zebrafish embryo.

DISCUSSION

We have identified and characterized *qkA*, which encodes a highly conserved member of a large family of RNA-binding proteins, as a new locus required for somitic muscle development. We also present the first direct evidence that the post-transcriptional regulator QkA acts on muscle development in zebrafish by regulating the Hh signaling pathway via the transcription factor Gli2a.

qkA is required for muscle morphogenesis

Both slow and fast muscle fibers require *qkA* function. *qkA* loss of function leads to both a reduction in the number of slow muscle cells, including MPs (see below), and in altered morphogenesis for the general population of slow muscle cells. In particular, their parallel horizontal alignment is disrupted at 24 hpf and slow cells invade the fast muscle domain. Although it is difficult at this stage to trace the origin of the altered alignment, the invasion of the fast muscle domain suggests that the distal migration of slow muscle cells could have been hampered. This process is known to rely on the complementary expression of n- and m-Cadherins (Cortes et al., 2003), so *qkA* might interact with these latter pathways. Consistently, some adaxial cells do not contact notochord in *qkA*^{t} mutants, suggesting that adhesion is altered. Alternatively, *qkA* could be involved in the regulation of early movements controlled by the PCP components *knypek* and *trilobite* (*glypican 4* and *vang-like 2* – Zebrafish Information Network). A detailed analysis of adaxial cell movements during somitogenesis is required to clarify this issue.

Fast fibers express the appropriate differentiation markers (F310, MF20) at 27 hpf in *qkA* mutants/morphants and many of them exhibit the classical multinucleated morphology. However, unlike their WT counterparts, MF20 and F310 stainings appear diffuse, progenitors often fail to undergo fusion and, although they are aligned along the main body axis, fast muscle cells do not adopt a tense fiber-like aspect. As myogenic markers are expressed as in

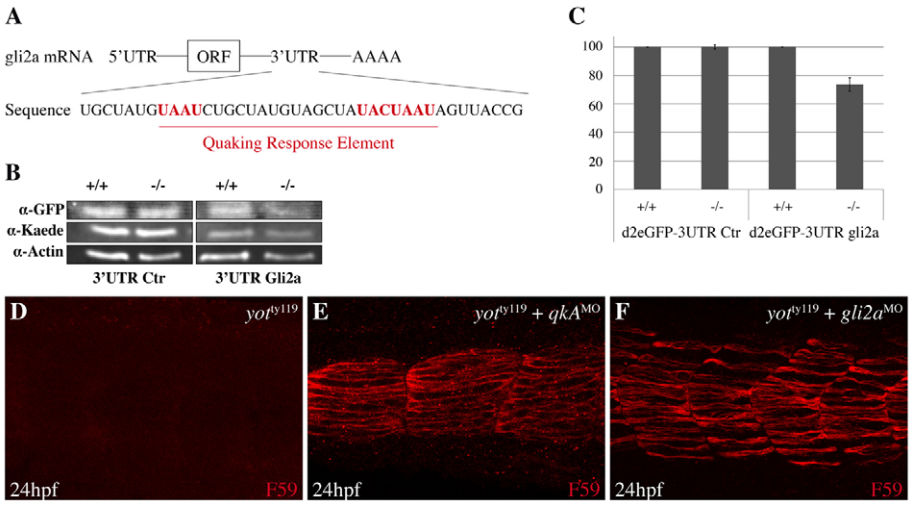

Fig. 6. Analysis of QkA requirement for *gli2a* mRNA stability. (**A**) The region of the zebrafish *gli2a* 3′UTR mRNA that contains the Quaking response element (QRE). Consensus sequences are in red. (**B**,**C**) Biosensor assay for QkA activity. RNA encoding d2eGFP flanked by the *gli2a* 3′UTR or a control (Ctr) 3′UTR were injected, together with *kaede* RNA as a control, into WT (+/+) or qkA^{t} mutants (–/–). (B) Proteins from each embryo were extracted at the 10-somite stage and probed by western blot. (C) Protein expression values from B were normalized to controls (see Materials and methods) and are plotted according to the genotype. Error bars indicate s.d. (**D-F**) Slow muscle fiber staining in yot^{ty119} embryos injected with $qkA^{ATG\text{-}MO}$ or $gli2a^{MO}$.

the WT, cell type specification seems to occur properly. However, at 48 hpf, fast fibers recover partially (see Fig. S6 in the supplementary material), suggesting that QkA is required for the on-time maturation of the fast muscle fibers. How is *qkA* required for fast muscle maturation? Both local inhibition of *qkA* within fast muscle progenitors and transplantation experiments demonstrate that *qkA* is not required within fast muscle cells but in their environment. Fast muscle cells or their precursors are known to require interaction with their environment/neighbors to properly develop. Notochord is required for slow muscle development as a source of the Hh morphogen (Devoto et al., 1996; Halpern et al., 1993; Krauss et al., 1993). However, creating a large clone of *qkA*-negative cells in notochord does not delay fast muscle maturation. Similarly, muscle cells require an interaction with somite/myotome boundaries for their development. However, although the boundary might appear morphologically altered, the expression of segment boundary matrix components appears, in general, normal, making it unlikely that QkA acts on fast muscle cells via the segment boundary. Finally, fast muscle progenitors are known to require slow muscle cells for their maturation (Henry and Amacher, 2004). Slow muscle cells do differentiate and most of them migrate through the field of fast muscle progenitors when *qkA* function is inhibited. So one intriguing possibility is that *qkA* is required for the action of slow muscle cells on the maturation of fast muscle cells. Alternatively, other cell/tissue types, such as the dermomyotome (Feng et al., 2006), could be involved. Restoration of *qkA* activity in specific regions of *qkA*-deficient embryos and analysis of the resulting fast muscle phenotype is required to answer this point.

qkA mutation may perturb RNA binding

We provide several lines of evidence that *qkA* is responsible for the different muscle cell type phenotypes observed in qkA^{t} mutants. First, the mutation maps to the *qkA* linkage group. Second, *qkA* harbors a missense mutation in the *qkA* KH domain, at a highly conserved residue. Third, the mutation can be rescued by *qkA* mRNA injections. Fourth, qkA^{MO} injections mimic the *t31954* phenotype. Altogether, these results allow us to unambiguously assign the phenotype to a loss of *qkA* function. Consistent with this notion, the *t31954* allele is recessive. Thus, *qkA* is required for somitic muscle development. How can it fulfill its function? STAR family members act as RNA-binding proteins that are able to regulate different biological processes by either preventing translation (Saccomanno et al., 1999) or interfering with RNA transport/splicing (Larocque et al., 2002). In this process, RNA binding requires the KH domain, as well as a consensus sequence UACUAAY-$N_{(1\text{-}20)}$-UAAY (Galarneau and Richard, 2005). In this study, we showed that the mutation in the qkA^{t} RNA-binding KH domain involves a change from the hydrophobic Ile to the polar Asn. In mice, a mutation involving a similar change in a neighboring position within the Qk KH domain has been analyzed at the molecular level. The mouse Qk^{k2} allele causes a transformation from Val to Glu, which leads to a loss of RNA-binding activity but not hetero/homodimerization (Cox et al., 1999; Larocque et al., 2002; Noveroske et al., 2000), most likely by perturbing the hydrophobic core of the Qk KH domain. The *t31954* mutation should therefore also affect the QkA RNA-binding activity.

Fine-tuning of the Hh signaling pathway by QkA

We present several lines of evidence that QkA interacts with the Hh signaling pathway. First, inhibition of *qkA* function leads to a reduction in the size of cell populations that are derivatives of the adaxial cells, which are classically induced by Hh signaling. Second, the number of $Pax7^{+}$ dermomyotomal cells is increased. Third, slow muscle cells invade the fast muscle domain in both qkA^{t} mutants and when Hh signaling is inhibited ($gli2a^{MO}$). Fourth, expression of *ptc1* and *prdm1a*, two major read-outs for Hh activity, is markedly reduced in the trunk of qkA^{t}. Interestingly, this reduction is observed in paraxial but not in axial mesoderm, nor in PSM,

Table 2. d2eGFP activity in the presence of the *wnt11* or *gli2a* 3′UTR

	wnt11 3′UTRs		*gli2a* 3′UTR	
Cell type	WT	qkA^{t}	WT	qkA^{t}
d2eGFP activity (%)	100±0	100±1.43	100±0	73.6±4.70

The d2eGFP reporter fused to the 3′UTR of *gli2a* or *wnt11* (control) mRNA was injected into WT and qkA^{t} embryos and d2eGFP expression assayed by western blotting. Values indicate the percentage of d2eGFP expression relative to WT uninjected control (± s.e.). *n*=3 experiments.

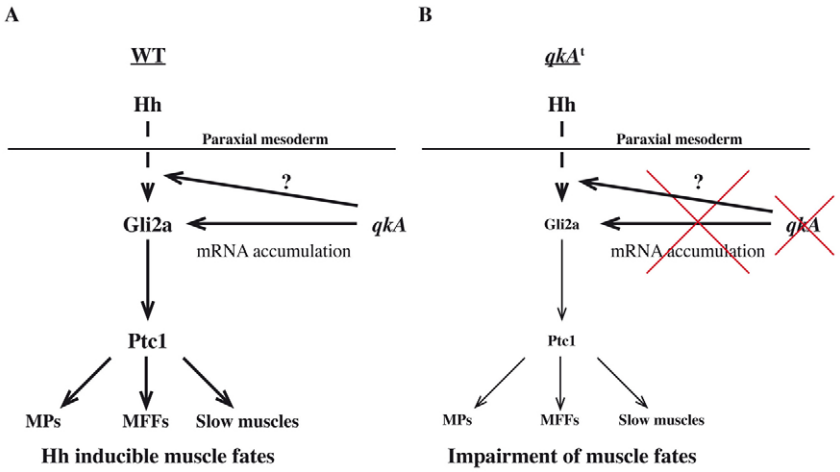

Fig. 7. Model of post-transcriptional regulation of *gli2a* mRNA by QkA. (**A**) In the WT zebrafish paraxial mesodermal cell, Shh interacts with its receptor Patched, allowing transduction of the Hh signal via Smoothened. Concomitantly, *gli2a* mRNA is transcribed and stabilized by QkA protein through an interaction with its 3′UTR. (**B**) In a *qkA*^{t} context, *gli2a* mRNA is destabilized and the quantity of Gli2a protein is dramatically reduced, such that the three muscle types are no longer correctly specified in number. The question mark indicates potential additional QkA targets.

indicating that *qkA* is required only locally and temporally for full activation of the Hh pathway. Thus, *qkA* appears to be required for the proper regulation of the Hh signaling pathway within paraxial mesoderm. When the Hh pathway is overactivated in *qkA*^{t}, adaxial derivatives are induced to a similar level as in WT, suggesting that either the *t31954* allele is not null or that *qkA* acts redundantly with other genes to regulate muscle fates. The slow muscle phenotype is stronger in *qkA* morphants than in *qkA*^{t} mutants, suggesting that *qkA*^{t} does not completely abolish *qkA* function, although the redundancy hypothesis cannot be discounted.

Hyperactivation of the Hh pathway, which normally leads to a reduction in the MFF population, results in a dramatic increase in the MFF population when *qkA* is attenuated. Two mechanisms could explain such an increase. First, Hh activity represses the fast muscle (and thus MFF) fates and QkA could act by preventing this repression. Alternatively, *qkA* could act on the timing of MFF induction by lengthening the competence period of fast muscle progenitors to Hh signals. Further experiments are required to resolve these issues.

How does QkA act on the pathway? Among the different Hh signaling pathway members available in the zebrafish Ensembl database, only *gli2a* exhibits a canonical QRE. Moreover, *gli2a* appears to be a good candidate target mRNA for QkA as it is required for slow muscle induction. Furthermore, we show that the *gli2a* 3′UTR confers sensitivity to QkA in vivo (sensor assay) and provide evidence for in vivo functional interaction between QkA and *gli2a* (the analysis of the *yot* mutant). Interestingly, the capacity of mouse Qk and the related *C. elegans* GLD-1 to regulate *Gli1* (or *C. elegans tra-1*) by binding to their QREs and repressing translation has been described (Lakiza et al., 2005). Our data are consistent with an action at the translational level, although they support an activation rather than a repression, potentially stemming from a difference in the QRE-encompassing region or structural differences between zebrafish QkA and GLD-1. Furthermore, our data suggest that the regulation of the Hh pathway by Qk-gli interaction is highly conserved throughout evolution and provide direct evidence for the in vivo implication of such an interaction in a developmental process. However, the muscle phenotype resulting from *qkA* loss of function cannot be entirely explained by an action through *gli2a* mRNA. In particular, the MFF population is reduced in *qkA*^{t} but increased in *gli2a* morphants (Wolff et al., 2003). Although the mechanism of action of Gli2a is rather complex because of the existence of activator and repressor states, a reduction in *qkA* translation would be expected to lead to a decreased capacity of activation. Thus, other QkA-regulated genes need to be evoked to explain its action. It is conceivable, for instance, that Gli1 is an indirect target of QkA, considering the fact that combined loss of function for *gli1* and *gli2a* leads to a complete loss of Engrailed-positive cells in the somite (Wolff et al., 2003). Alternatively, other QRE-negative genes might be involved.

We propose the following model (Fig. 7). In WT embryos, *gli2a* is transcribed and the pre-messenger is stabilized by QkA activity. Then, *gli2a* mRNA is exported to the cytoplasm and translated. In response to Shh, the activated form of Gli2a transduces Hh signaling by entering into the nucleus and inducing the transcription of target genes, including *ptc1* (Fig. 7A). When *qkA* function is inhibited, the stabilization of *gli2a* mRNA is not ensured, leading to reductions in the *gli2a* transcript and Gli2a protein concentration (Fig. 7B). Alongside the negative regulation of the Hh transduction component Sufu by miRNAs (Flynt et al., 2007), our results provide a novel regulator of this pathway that allows fine-tuning to generate the different muscle cell populations of the somite.

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Competing interests statement
The authors declare no competing financial interests.

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