LETTER

A Compact *unc45b*-Promoter Drives Muscle-Specific Expression in Zebrafish and Mouse

Steven Rudeck,[1] Christelle Etard,[2] Muzamil M. Khan,[1,3,5] Wolfgang Rottbauer,[4] Rüdiger Rudolf,[2,3,5] Uwe Strähle,[2] and Steffen Just[1]*

[1]Molecular Cardiology, Department of Internal Medicine II, University of Ulm, Ulm, Germany

[2]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Karlsruhe, Germany

[3]Institute of Molecular and Cell Biology, Hochschule Mannheim, Mannheim, Germany

[4]Department of Internal Medicine II, University of Ulm, Ulm, Germany

[5]Interdisciplinary Center for Neurosciences, University Heidelberg, Heidelberg, Germany

Received 20 April 2016; Revised 2 June 2016; Accepted 8 June 2016

Summary: Gene therapeutic approaches to cure genetic diseases require tools to express the rescuing gene exclusively within the affected tissues. Viruses are often chosen as gene transfer vehicles but they have limited capacity for genetic information to be carried and transduced. In addition, to avoid off-target effects the therapeutic gene should be driven by a tissue-specific promoter in order to ensure expression in the target organs, tissues, or cell populations. The larger the promoter, the less space will be left for the respective gene. Thus, there is a need for small but tissue-specific promoters. Here, we describe a compact *unc45b* promoter fragment of 195 bp that retains the ability to drive gene expression exclusively in skeletal and cardiac muscle in zebrafish and mouse. Remarkably, the described *unc45b* promoter fragment not only drives muscle-specific expression but presents heat-shock inducibility, allowing a temporal and spatial quantity control of (trans)gene expression. Here, we demonstrate that the transgenic expression of the *smyd1b* gene driven by the *unc45b* promoter fragment is able to rescue the embryonically lethal heart and skeletal muscle defects in *smyd1b*-deficient *flatline* mutant zebrafish. Our findings demonstrate that the described muscle-specific *unc45b* promoter fragment might be a valuable tool for the development of genetic therapies in patients suffering from myopathies. genesis 54:431–438, 2016. © 2016 The Authors. Genesis Published by Wiley Periodicals, Inc.

Key words: *unc45b*; *smyd1b*; muscle; muscle diseases; zebrafish

In the context of heart and skeletal muscle disorders, the SET and MYND domain containing protein 1 (SMYD1) was identified as disease causing when mutated or defective (Abaci *et al*., 2010; Gottlieb *et al*., 2002; Just *et al*., 2011; Nagandla *et al*., 2016; Stewart *et al*., 2016). SMYD1 is exclusively expressed in cardiac and skeletal muscle cells from zebrafish and mice (Just *et al*., 2011; Nagandla *et al*., 2016) where it locates to the cell nucleus, as expected for a histone methyltransferase (HMT), and to the sarcomeric M-band (Just *et al*., 2011). In zebrafish, Smyd1b was shown to be crucial to

*Correspondence to: Steffen Just, Molecular Cardiology, Department of Internal MedicineII, University of Ulm Albert-instein-llee 23, 89081 Ulm, Germany. E-mail: steffen.just@uniklinik-ulm.de

S. Rudeck and C. Etard contributed equally to this work.

Abbreviations: AAV, adeno-associated virus; AP, alkaline phosphatase; GFP, green fluorescent protein; HCM, hypertrophic cardiomyopathies; HMT, histone methyltransferase; HRE, heat shock factor response elements; HSF1, heat shock factor 1; MCK, muscle creatine kinase; RSV, Rous sarcoma virus; SMYD1, SET and MYND domain containing protein 1; TA, tibialis anterior.

Contract grant sponsor: Deutsche Forschungsgemeinschaft, Contract grant number: DFG Str439/5-1 and DFG RO2173/5-1; Contract grant sponsor: Bundesministerium für Bildung und Forschung, Contract grant number: 01ZX1407A; Contract grant sponsor: Association Française contre les Myopathies, Contract grant number: EU-IP ZF-HEALTH

Published online 9 July 2016 in
Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/dvg.22953

orchestrate thick filament assembly in cardiomyocytes and fast-twitch skeletal muscle cells and when defective leads to severe cardiac and skeletal muscle dysfunction due to impaired myofibrillogenesis (Just *et al.*, 2011; Li *et al.*, 2013; Prill *et al.*, 2015; Tan *et al.*, 2006). Consistently, nullizygous SMYD1 mice die at embryonic day E10.5 due to severe heart malformations and cardiac dysfunction (Gottlieb *et al.*, 2002). Conditional ablation of murine SMYD1 at the myoblast stage results in impaired myoblast differentiation accompanied with fewer myofibers and a reduction of the expression of muscle-specific genes (Nagandla *et al.*, 2016). By contrast, targeted SMYD1 elimination after myoblast differentiation leads to pronounced myopathy with severe myofibrillar disarray (Stewart *et al.*, 2016). Interestingly, mutations in human SMYD1 were implicated to be involved in the development of hypertrophic cardiomyopathies (HCM) (Abaci *et al.*, 2010).

To date, many genetic muscle diseases such as cardiomyopathies or muscular dystrophies, targeted gene therapy/transfer might be a promising strategy to attenuate pathological symptoms or even to cure the disease. Gene therapy often aims at reintroducing the wild-type sequence of a deficient gene in the affected organs, tissues or cell populations. Therefore, the target sequence needs to be driven by effective tissue-specific promoters, and packed into appropriate vectors such as viruses for their efficient delivery to and into the target cells (Kotterman and Schaffer, 2014).

For the successful delivery of the gene product into post-mitotic cells such as cardiomyocytes or skeletal muscle cells, adeno-associated virus (AAV)-based vectors have emerged as safe and effective technologies. In this context, serotype AAV9 shows high cardiac- and skeletal muscle tropism (Asokan *et al.*, 2012; Katwal *et al.*, 2013; Riaz *et al.*, 2015), maximizing transgene expression. Nevertheless, since systemic application of AAV9 vectors also lead to the transduction of other organ systems, tissue-specific promoters are essential to minimize off-target effects or overall toxicity of the transgene (Wang *et al.*, 2008). For instance, dogs injected with AAV-derived vectors harboring a Rous sarcoma virus (RSV) promoter driving alkaline phosphatase (AP) reporter show widespread AP expression in every muscle, but also in many other organs, like kidney, pancreas, and peripheral nerves (Yue *et al.*, 2015). Most muscle-specific promoters such as that of the *muscle creatine kinase* (MCK) gene are very large (6.5 kb), and thus incompatible with AAVs having a 4.5 kb capacity (Wu *et al.*, 2010). Efforts have been made to shorten the MCK promoter, giving rise to the dMTK (509 bp) or tMCK (720 bp) promoter that show skeletal muscle-specific expression (Yue *et al.*, 2015) and the MHCK7 (770 bp) promoter that drives cardiac and skeletal muscle expression (Salva *et al.*, 2007).

Unc45b is a cardiac and skeletal muscle-specific myosin chaperone shown to be essential for proper thick filament assembly in *C. elegans*, zebrafish, and mice (Chen *et al.*, 2012; Etard *et al.*, 2007, 2015; Hoppe *et al.*, 2004). In 2013, a 503 bp fragment driving *unc45b* endogenous expression was described but no regulatory sequences were discovered (Berger and Currie, 2013). We recently identified the relevant regulatory sequences recapitulating its endogenous expression (Etard *et al.*, 2015). By systematically shortening the promoter sequence, we found that a 195 bp *unc45b* promoter fragment in combination with a *gata2* minimal promoter drives expression of a reporter in the same patterns as that of the endogenous *unc45b* gene.

Here, we show that the described 195 bp *unc45b* promoter fragment without the *gata2* minimal promoter is able to direct highly specific reporter-gene expression in skeletal and cardiac muscle in transgenic zebrafish. Furthermore, we find that the promoter fragment is heat-shock inducible allowing quantity control of transgene expression in a temporal and spatial manner. Remarkably, *unc45b* promoter-driven expression of wild-type zebrafish *smyd1b* in cardiomyocytes and skeletal muscle cells was sufficient to reconstitute heart and skeletal muscle development and function in the *smyd1b*-deficient zebrafish mutant *flatline* (*fla*). Furthermore, we find that the *unc45b* promoter fragment also effectively mediates reporter gene expression in transduced mouse muscle. In summary, our findings suggest that the described compact *unc45b* promoter might have a high potential in AVV-mediated gene transfer approaches to treat monogenic muscle diseases.

RESULTS

Very recently, we identified the regulatory sequences of the *unc45b* gene promoter driving reporter gene expression specifically in cardiomyocytes and skeletal muscle under basal conditions and after further induction in response to misfolded myosin (Fig. 1A) (Etard *et al.*, 2015). We found that a 195 bp fragment termed *−505-310(unc45b)gata2:gfp* efficiently mediates green fluorescent protein (GFP) expression in skeletal and cardiac muscle when inserted in front of the heterologous *gata2* minimal promoter in transient as well as in stable expression analyses (Fig. 1B,C) (Etard *et al.*, 2015).

Here, we further characterized these *unc45b* regulatory sequences and assessed a potential use of the minimal *unc45b* promoter for gene transfer/therapy approaches to treat cardiac and skeletal muscle diseases. We first tested in transient expression analyses whether the 195 bp *unc45b* fragment can act as promoter by inserting it upstream of the teal fluorescent protein coding region (*(−505-310(unc45b):tfp*) hereafter named *unc45b^{min}:tfp*). This construct lacks the *gata2* minimal promoter. Injection of the *unc45b^{min}:tfp* containing vector construct into

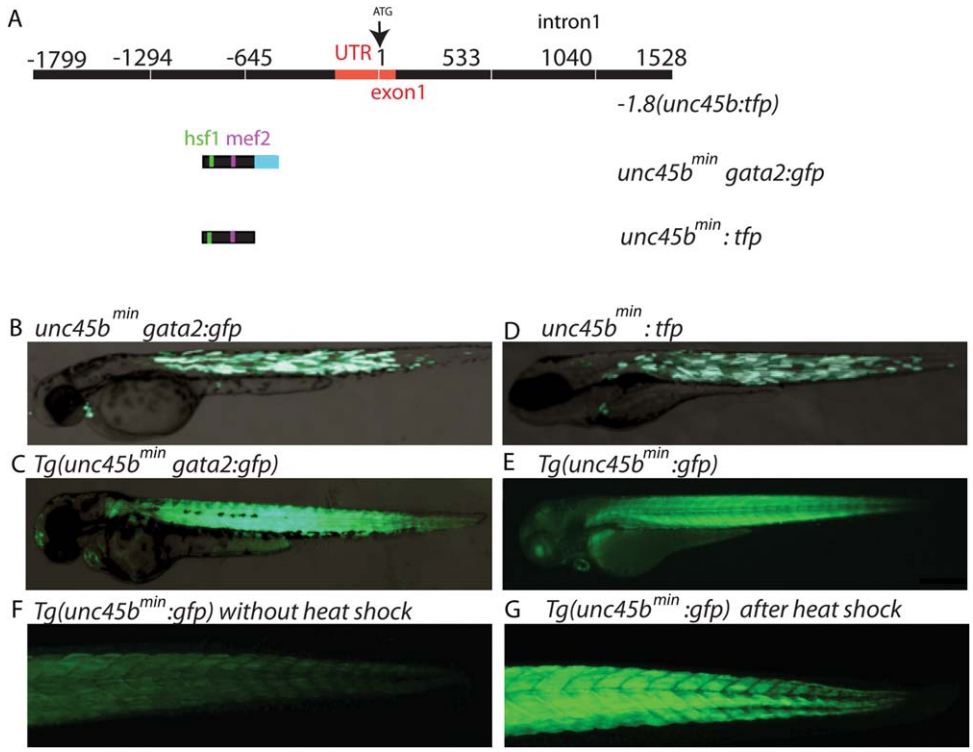

FIG. 1. A small 195 bp *unc45b* promoter fragment drives cardiac and skeletal muscle expression. (A) Schematic representation of the full-length *unc45b* promoter *−1.8(unc45b:tfp)*, a 195 bp *unc45b* promoter fragment associated with *gata2* (*unc45b*^{min}*gata2:gfp*) and the 195 bp *unc45b* promoter fragment alone (*unc45b*^{min}*:gfp*). (B) Injection of *unc45b*^{min}*gata2:gfp* into wild-type embryos reveals GFP expression within cardiac and skeletal muscle (72 hpf). (C) Transgenic *unc45b*^{min}*gata2:gfp* embryos *Tg(unc45b*^{min}*gata2:gfp)* show a restricted expression in skeletal and cardiac muscles at 72 hpf. (D) Microinjection of *unc45b*^{min}*:tfp* into wild-type embryos also reveals a restricted expression in skeletal and cardiac muscles (72 hpf). (E) *Tg(unc45b*^{min}*:gfp)* transgenic zebrafish embryos show heart- and skeletal muscle-specific GFP expression (72 hpf). (F, G) Heat shock significantly increases GFP levels in *Tg(unc45b*^{min}*:gfp)* embryos (G) compared to untreated embryos (F) at 72 hpf.

fertilized zebrafish oocytes at the one-cell stage led to a specific and strong TFP expression in skeletal muscle cells and cardiomyocytes (Fig. 1D), demonstrating that the used 195 bp *unc45b*^{min} fragment can act as promoter fragment and is able to drive strong reporter gene expression in a pattern identical to the endogenous *unc45b* promoter. To further prove this finding, we next generated a transgenic zebrafish line using the pDest-Tol2pA2 vector backbone including the *unc45b*^{min}*:gfp* construct (*Tg(unc45b*^{min}*:gfp)*). Consistently, *Tg(unc45b*^{min}*:gfp)* zebrafish embryos exhibit GFP expression specifically in skeletal muscle cells and cardiomyocytes at 48 (data not shown) and 72 h post fertilization (hpf) (Fig. 1E). Additionally, we evaluated whether reporter gene expression can be induced in *Tg(unc45b*^{min}*:gfp)* embryos as observed in *Tg(unc45b*^{min}*-gata2:gfp)* fish (Etard *et al*., 2015). Indeed, we found that incubation of 48 h old *Tg(unc45b*^{min}*:gfp)* embryos with pre-heated E3 medium (39°C) for 1 h significantly increased GFP expression compared to untreated *Tg(unc45b*^{min}*:gfp)* embryos (Fig. 1F,G). These findings clearly demonstrate that the compact 195 bp *unc45b*^{min} promoter fragment is able to recapitulate spatial expression of the full-length zebrafish *unc45b* promoter (Etard *et al*., 2015) and that transcriptional activity can be efficiently induced by heat shock. Very recently, we demonstrated that heat shock factor response elements (HRE) in the *unc45b* promoter are indispensable for transcriptional inductivity. Furthermore, we showed that misfolded myosin seems to be the major trigger for the induction of the *unc45b* promoter mediated by the activation and recruitment of heat shock factor 1 (HSF1) proteins to its binding sites within the *unc45b* promoter (Etard *et al*., 2015).

Next, to assess whether the identified minimal *unc45b* promoter is able to direct sufficient wild-type gene expression to rescue muscle mutant embryos, we used the *smyd1b*-deficient *flatline* mutants, which display severe cardiac and skeletal muscle dysfunction due to impaired myofibrillogenesis (Just *et al*., 2011). We first generated a transgenic zebrafish line expressing a Smyd1b-GFP fusion protein under the control of the compact 195 bp *unc45b*^{min} promoter (*Tg(unc45b*^{min}*:-smyd1b-gfp)*). As shown in Figure 1E, we found that Smyd1b-GFP is specifically expressed in cardiomyocytes and skeletal muscle cells (Fig. 2A,B) and that the transgene localizes to the sarcomere in an alternating pattern to α-actinin, an established Z-disk marker (Fig. 2C).

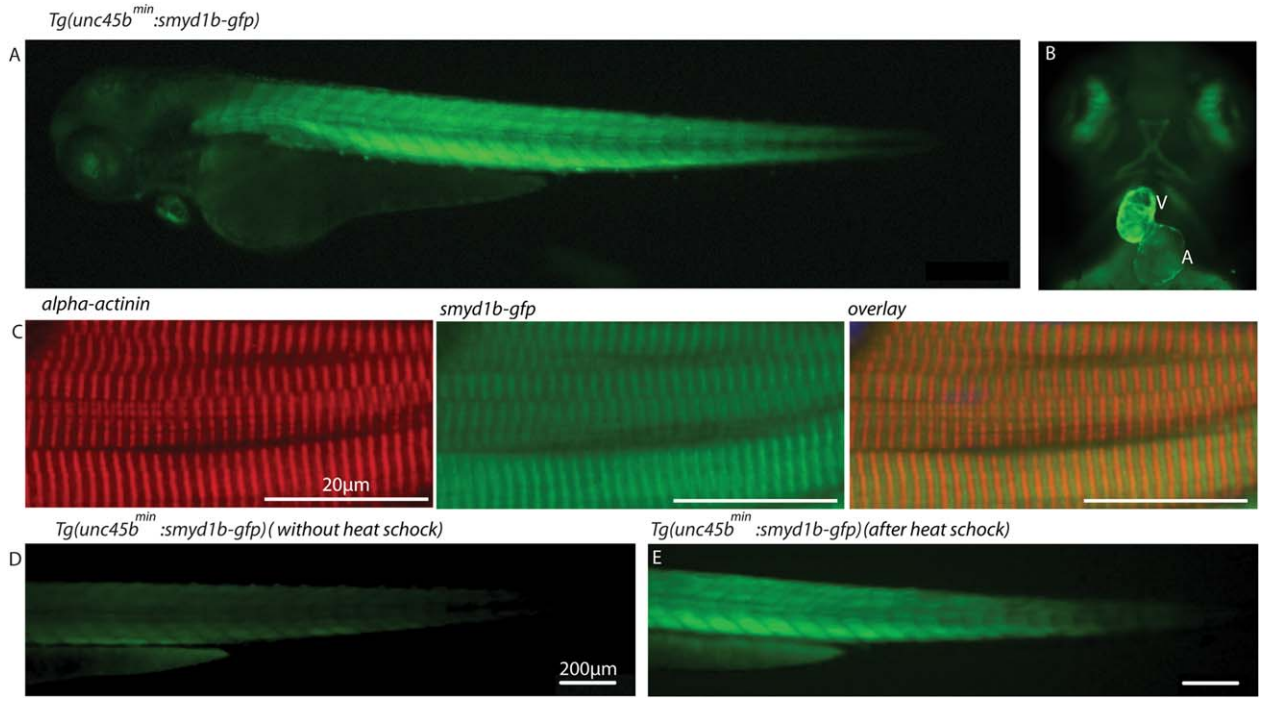

FIG. 2. The transgenic zebrafish line *Tg(unc45b^{min}:smyd1b-gfp)* exhibits Smyd1b expression specifically within cardiac and skeletal muscles. (A, B) At 72 hpf, *Tg(unc45b^{min}:smyd1b-gfp)* transgenic embryos show strong and restricted expression of Smyd1b-GFP fusion proteins within heart and skeletal muscles. A (Atrium), V (Ventricle). (C) α-actinin-specific immunostaining of *Tg(Unc^{min}45b:smyd1b-gfp)* transgenic embryos reveals an alternating distribution of *Smyd1b*-GFP and α-actinin, suggesting Smyd1b localization at the sarcomeric M-line (72 hpf). (D, E) *smyd1b-gfp* expression was significantly enhanced by 1 h heat shock of the transgenic line *Tg(unc45b^{min}:smyd1b-gfp)* (E) compared to untreated transgenic embryos (D) at 72 hpf.

This finding suggests that similar to the subcellular localization of endogenous Smyd1b, transgenic Smyd1b-GFP properly localizes to the sarcomeric M-band (Just *et al*., 2011). Furthermore, similar to the situation in *Tg(unc45b^{min}:gfp)* zebrafish embryos, 1 h heat shock significantly increased GFP fluorescence intensity in *Tg(unc45b^{min}:smyd1b-gfp)* embryos, indicating that the 195 bp *unc45b^{min}* promoter fragment can be utilized to titrate transgene expression levels *in vivo* (Fig. 2D,E) (Etard *et al*., 2015). Next, we crossed homozygous *Tg(unc45b^{min}:smyd1b-gfp)* fish with heterozygous *flatline* (*fla$^{+/-}$*) mutant fish and raised their offspring to adulthood. *smyd1b*-deficient homozygous *fla* mutant embryos (*fla$^{-/-}$*) are characterized by severe cardiac and skeletal muscle dysfunction caused by defective myofibrillogenesis in cardiomyocytes and skeletal muscle cells as depicted by an α-actinin-specific immunostaining and birefringence analyses (Fig. 3A–D) (Just *et al*., 2011). After genotyping and transgene detection, we incrossed *Tg(unc45b^{min}:smyd1b-gfp)/(fla$^{+/-}$)* fish and evaluated their offspring. Usually, homozygous *flatline* mutant embryos (*fla$^{-/-}$*) can be detected already at 24 hpf by the lack of cardiac contractions and complete paralysis (Just *et al*., 2011). None of the investigated embryos exhibited the typical *fla* heart and skeletal muscle phenotype as demonstrated by measuring cardiac and skeletal muscle functionality (Fig. 3E–I, movie). Next, all individuals were first subjected to *in vivo* birefringence analysis, second to α-actinin immunostainings to evaluate sarcomere organization and finally to a genotyping assay to identify homozygous *flatline* mutant embryos (*fla$^{-/-}$*). Remarkably, we found that all *fla$^{-/-}$* embryos carrying the *smyd1b-gfp* transgene driven by the compact *unc45b^{min}* promoter show bright birefringence signals (Fig. 3G), suggesting that sarcomere organization is preserved in these individual fish. Indeed, α-actinin immunostaining revealed regular sarcomerogenesis in *fla$^{-/-}$* embryos (Fig. 3J). Furthermore, Smyd1b-GFP regularly locates to the sarcomeric M-band in the rescued *fla$^{-/-}$* embryos (Fig. 3J). Together, these findings clearly demonstrate that *unc45b^{min}* promoter driven expression of *smyd1b* is able to structurally and functionally rescue the heart and skeletal muscle defects in *smyd1b*-deficient *fla* mutant zebrafish embryos. Moreover, we found that *Tg(unc45b^{min}:smyd1b-gfp)/(fla$^{-/-}$)* fish can be raised to adulthood without showing an overt muscle phenotype (data not shown).

Finally, to evaluate whether the zebrafish *unc45b^{min}* promoter is also capable to drive gene expression in

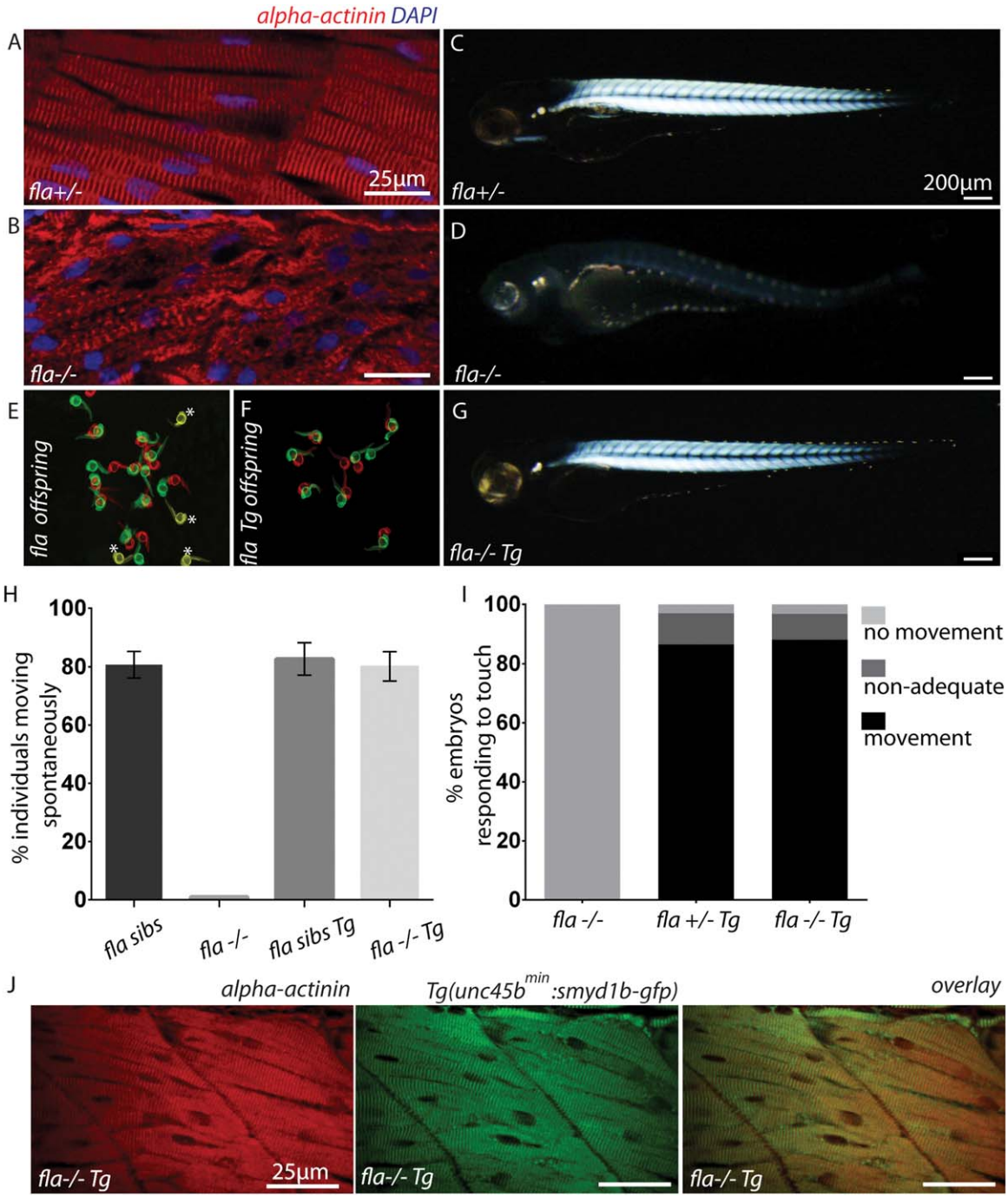

FIG. 3. Functional and structural rescue of *fla* mutant embryos by the transgenic reintroduction of Smyd1b. (A, B) Immunostaining of *fla* siblings (A) and mutants (B) with α-actinin-specific antibodies (red) and DAPI (blue). Normal sarcomeric striation was visible in siblings, whereas complete lack of sarcomeric organization was found in homozygous *fla* mutant embryos. (C, D) *fla* siblings show strong birefringence signals at 72 hpf (C) whereas *fla* mutants lack proper sarcomeric organization and thereby birefringence signal (D). (E, F) Representative overview of spontaneous movement assays (at 24 hpf) with false-colored and superimposed pictures of *fla* offspring derived from intercrossing heterozygous *fla* fish. Genotyping of these embryos revealed that all non-moving (yellow*) embryos are homozygous *fla* mutants (E). By contrast, all homozygous *fla* mutant embryos carrying the transgene (*Tg(unc45b^{min}:smyd1b-gfp)*) are able to move (F) (red to green shift). Red pictures 0 s, green pictures 10 s. (G) Transgenic reintroduction of Smyd1b (*Tg(unc45b^{min}:smyd1b-gfp)*) into homozygous *fla* mutant embryos leads to normal birefringence signal. (H) Statistical analysis of the spontaneous movement assay. 80 ± 3.6% of genotyped *fla* siblings (*fla*$^{+/+}$ and *fla*$^{+/-}$) showed normal motility after 24 hpf ($n = 111$; three independent experiments). By contrast, homozygous *fla* mutant embryos are completely paralyzed ($n = 35$; three independent experiments). In comparison, 82.7 ± 5.6% of *fla* sibs (*fla*$^{+/+}$ and *fla*$^{+/-}$) ($n = 98$; three independent experiments) and 80.7 ± 4.6% of homozygous *fla* mutants carrying the transgene that expresses *smyd1b* showed normal spontaneous movements ($n = 32$; three independent experiments). Error bars indicate sd. (I) Quantification of the touch-evoked flight response at 72 hpf. Homozygous *fla* mutants are completely paralyzed and do not flight upon tactile stimulation ($n = 47$; three independent experiments). 86.6% of *fla* siblings (*fla*$^{+/+}$ and *fla*$^{+/-}$) carrying the transgene ($n = 61$; three independent experiments) show regular motility at 72 hpf. Similarly, 88.2% of homozygous *fla* mutant embryos carrying the transgene ($n = 26$; three independent experiments) are able to respond regularly upon tactile stimulation. (J) α-actinin-specific immunostainings of skeletal muscles reveal normal sarcomeres in *fla* mutants carrying *Tg(unc45b^{min}:smyd1b-gfp)*, indicating a complete structural rescue by the transgenic reintroduction of Smyd1b in homozygous *fla* mutants.

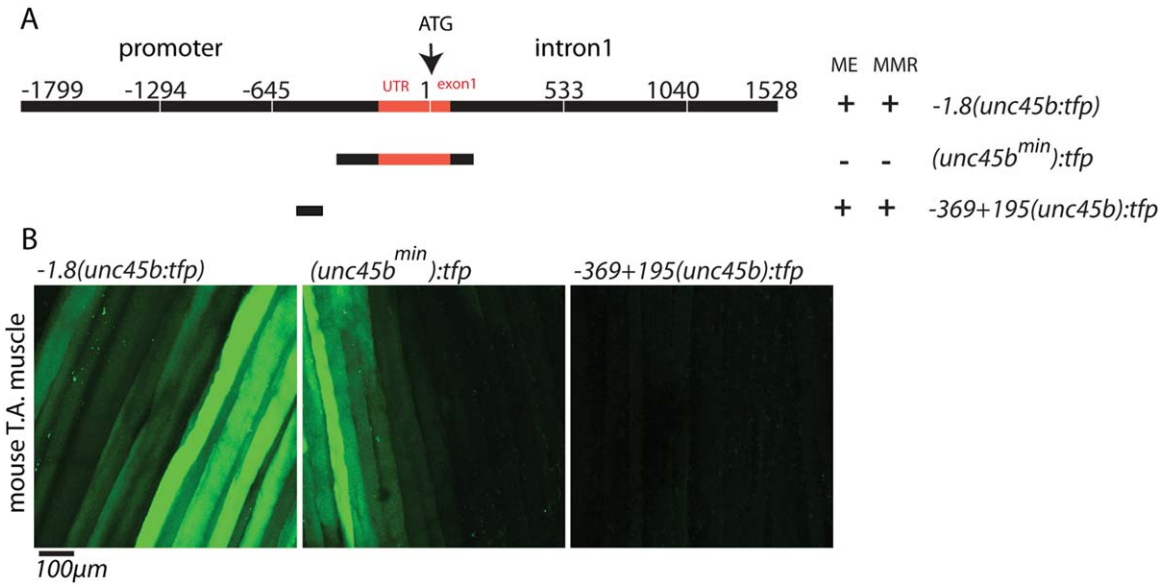

FIG. 4. *−505-310(unc45b)tfp* drive TFP expression within mouse muscle. (A) Scheme showing different *unc45b* promoter constructs used for electroporation into mouse muscles. (B) Electroporation of full-length *−1.8(unc45:tfp)* and *(unc45b^{min}):tfp* constructs show expression of GFP in skeletal muscle fibers of mouse tibialis anterior (T.A) muscle, whereas *−369 + 195(unc45b):tfp* is not able to drive GFP expression.

mammalian myocytes, we transfected mouse hind limb tibialis anterior (TA) muscles with cDNA constructs encoding TFP under the control of different *unc45b* promoter fragments such as the full-length *unc45b* promoter (*−1.8(unc45b:tfp)*), the compact 195 bp fragment (*unc45b^{min}:tfp*) and a 570 bp *unc45b* (*−369 + 195(unc45b):tfp*) fragment (Fig. 4A) (Etard *et al.*, 2015). Ten days after electroporation, the mouse was anaesthetized, TA muscle was exposed and then examined by *in vivo* confocal fluorescence microscopy as described previously (Rudolf *et al.*, 2012). Corroborating the observations in zebrafish, the truncated 195 bp fragment drove GFP expression with similar efficiency to that of the full-length *unc45b* promoter (Fig. 4B), while the control promoter *−369 + 195(unc45b):tfp* (lacking the 195 bp fragment) did not mediate detectable GFP expression (Fig. 4B) (Etard *et al.*, 2015). These findings demonstrate that the zebrafish 195 bp *unc45b^{min}* promoter fragment is able to drive reporter gene expression in mammalian muscle cells thereby suggesting a high potential of this compact muscle-specific promoter for therapeutic gene transfer approaches in diseased muscles.

MATERIAL AND METHODS

Fish Stock

Fish were bred and raised as previously described (Westerfield, 1993). The following mutant alleles were used: *smyd1b$^{zf340/zf340}$* (Just *et al.*, 2011).

Microinjection

Microinjection was carried out as described (Müller *et al.*, 1999), briefly, 1.5–2 nl of injection solution containing 20 ng/µl reporter plasmid DNA and 15 ng/µl Tol2 transposase mRNA, supplemented with 0.1% phenol red (injection marker), was injected into zebrafish eggs using a FemtoJet microinjector (Eppendorf).

Cloning

For *−1.8(unc45b:tfp)*, *−510-310(unc45b)tfp*, and *−369 + 195(unc45b)tfp* construct, see Etard *et al.* (2015).

The *−505-310(unc45b)* promoter construct was amplified out of genomic DNA and cloned into the gateway 5′ entry p5E-MCS. A full-length cDNA encoding zebrafish *smyd1b* was amplified and cloned into pDONRzeo (Invitrogen). For transgenesis pDest-Tol2pA2 was used as backbone for multisite reaction with p3E-EGFPpA as 3′ entry (Kwan *et al.*, 2007). For each construct, at least six transgenic lines were identified and used for further analysis.

Histology, Immunohistochemistry, and Microscopy

The non-invasive birefringence and touch-evoke escape response analysis were carried out as described (Buhrdel *et al.*, 2015). False-colored and superimposed overviews of 24 hpf embryos were analyzed for spontaneous movement assays. Whole-mount fluorescent immunostaining were carried out as described in (Inoue and Wittbrodt, 2011), embedded in JB-4

(Polysciences) and 5 μm sections were cut. As primary antibodies we used: polyclonal rabbit anti-GFP (1:200, Thermo Fisher Scientific, #A11122) and monoclonal mouse anti-α-actinin (1:10, Sigma Aldrich, #A7811). As secondary antibodies we used: goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 555 (1:200, Thermo Fisher Scientific).

Heat shocks were performed for 1 h at 39°C with 48 hpf embryos. Heat shock images were taken at the Keyence BZ-9000E, whole mount stainings at the Zeiss LSM710.

Mouse Skeletal Muscle Transfection and In Vivo Microscopy

All animals were kept and treated according to EU directives and national law. Adult male and female C57BL/10J were used for experiments. For cDNA electroporation into tibialis anterior muscle, the animals were anaesthetized under Isofluorane and the muscle was exposed for injection of a cDNA solution containing 10 μg of cDNA encoding TFP under the full-length *unc45b* promoter (*−1.8(unc45b:tfp)*), short 195 bp fragment (*unc45b^{min}:tfp*), and 570 bp *unc45b* (*−369 + 195(unc45b:tfp)*) (Fig. 4A) (Etard *et al.*, 2015). Post-cDNA solution injection, electrodes connected to an electric pulse generator were placed under and above the T.A muscle and the muscles were subjected to five electric shocks, each of 20 V intensity, 20 ms duration, and 200 ms apart (Dona *et al.*, 2003). Next the surgical opening of skin was sutured and 10 days post-transfection, for *in vivo* microscopy anesthesia and preparation of mice were performed as described (Choi *et al.*, 2012).

For the microscopic analysis of the TFP expression in T.A muscles, we used a DMRE TCS SP2 confocal microscope equipped with a KrAr laser (488 nm, 514 nm) and a 20×/0.7 N. A. HC PL APO CS IMM/CORR UV (immersion medium, Visc-Ophtal gel; Dr. Winzer Pharma).

ACKNOWLEDGMENTS

We thank the Core Facility Confocal and Multiphoton Microscopy Ulm for their support. This research work is part of the project "Molecular Interaction Engineering: From Nature's Toolbox to Hybrid Technical Systems," which is funded by the German Federal Ministry of Education and Research (BMBF), funding code 031A095 C.

LITERATURE CITED

Abaci N, Gulec C, Bayrak F, Komurcu Bayrak E, Kahveci G, Erginel Unaltuna N. 2010. The variations of BOP gene in hypertrophic cardiomyopathy. Anatol J Cardiol 10:303–309.

Asokan A, Schaffer DV, Samulski RJ. 2012. The AAV vector toolkit: Poised at the clinical crossroads. Mol Ther 20:699–708.

Berger J, Currie PD. 2013. 503unc, a small and muscle-specific zebrafish promoter. Genesis 51:443–447.

Buhrdel JB, Hirth S, Kessler M, Westphal S, Forster M, Manta L, Wiche G, Schoser B, Schessl J, Schroder R, Clemen CS, Eichinger L, Furst DO, van der Ven PF, Rottbauer W, Just S. 2015. In vivo characterization of human myofibrillar myopathy genes in zebrafish. Biochem Biophys Res Commun 461:217–223.

Chen D, Li S, Singh R, Spinette S, Sedlmeier R, Epstein HF. 2012. Dual function of the UNC-45b chaperone with myosin and GATA4 in cardiac development. J Cell Sci 125:3893–3903.

Choi KR, Berrera M, Reischl M, Strack S, Albrizio M, Roder IV, Wagner A, Petersen Y, Hafner M, Zaccolo M, Rudolf R. 2012. Rapsyn mediates subsynaptic anchoring of PKA type I and stabilisation of acetylcholine receptor in vivo. J Cell Sci 125:714–723.

Dona M, Sandri M, Rossini K, Dell'Aica I, Podhorska-Okolow M, Carraro U. 2003. Functional in vivo gene transfer into the myofibers of adult skeletal muscle. Biochem Biophys Res Commun 312:1132–1138.

Etard C, Armant O, Roostalu U, Gourain V, Ferg M, Strahle U. 2015. Loss of function of myosin chaperones triggers Hsf1-mediated transcriptional response in skeletal muscle cells. Genome Biol 16: 267.

Etard C, Behra M, Fischer N, Hutcheson D, Geisler R, Strahle U. 2007. The UCS factor Steif/Unc-45b interacts with the heat shock protein Hsp90a during myofibrillogenesis. Dev Biol 308:133–143.

Gottlieb PD, Pierce SA, Sims RJ, Yamagishi H, Weihe EK, Harriss JV, Maika SD, Kuziel WA, King HL, Olson EN, Nakagawa O, Srivastava D. 2002. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. Nat Genet 31:25–32.

Hoppe T, Cassata G, Barral JM, Springer W, Hutagalung AH, Epstein HF, Baumeister R. 2004. Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in C. elegans. Cell 118:337–349.

Inoue D, Wittbrodt J. 2011. One for all—A highly efficient and versatile method for fluorescent immunostaining in fish embryos. PloS One 6:e19713.

Just S, Meder B, Berger IM, Etard C, Trano N, Patzel E, Hassel D, Marquart S, Dahme T, Vogel B, Fishman MC, Katus HA, Strahle U, Rottbauer W. 2011. The myosin-interacting protein SMYD1 is essential for sarcomere organization. J Cell Sci 124:3127–3136.

Katwal AB, Konkalmatt PR, Piras BA, Hazarika S, Li SS, John Lye R, Sanders JM, Ferrante EA, Yan Z, Annex BH, French BA. 2013. Adeno-associated virus

serotype 9 efficiently targets ischemic skeletal muscle following systemic delivery. Gene Ther 20:930–938.

Kotterman MA, Schaffer DV. 2014. Engineering adeno-associated viruses for clinical gene therapy. Nat Rev Genet 15:445–451.

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. 2007. The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn 236:3088–3099.

Li H, Zhong Y, Wang Z, Gao J, Xu J, Chu W, Zhang J, Fang S, Du SJ. 2013. Smyd1b is required for skeletal and cardiac muscle function in zebrafish. Mol Biol Cell 24:3511–3521.

Müller F, Chang B, Albert S, Fischer N, Tora L, Strahle U. 1999. Intronic enhancers control expression of zebrafish sonic hedgehog in floor plate and notochord. Development 126:2103–2116.

Nagandla H, Lopez S, Yu W, Rasmussen TL, Tucker HO, Schwartz RJ, Stewart MD. 2016. Defective myogenesis in the absence of the muscle-specific lysine methyltransferase SMYD1. Dev Biol 410:86–97.

Prill K, Windsor Reid P, Wohlgemuth SL, Pilgrim DB. 2015. Still heart encodes a structural HMT, SMYD1b, with chaperone-like function during fast muscle sarcomere assembly. PloS One 10:e0142528.

Riaz M, Raz Y, Moloney EB, van Putten M, Krom YD, van der Maarel SM, Verhaagen J, Raz V. 2015. Differential myofiber-type transduction preference of adeno-associated virus serotypes 6 and 9. Skeletal Muscle 5:37.

Rudolf R, Hafner M, Mongillo M. 2012. Investigating second messenger signaling in vivo. Methods Enzymol 505:363–382.

Salva MZ, Himeda CL, Tai PW, Nishiuchi E, Gregorevic P, Allen JM, Finn EE, Nguyen QG, Blankinship MJ, Meuse L, Chamberlain JS, Hauschka SD. 2007. Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. Mol Ther 15:320–329.

Stewart MD, Lopez S, Nagandla H, Soibam B, Benham A, Nguyen J, Valenzuela N, Wu HJ, Burns AR, Rasmussen TL, Tucker HO, Schwartz RJ. 2016. Mouse myofibers lacking the SMYD1 methyltransferase are susceptible to atrophy, internalization of nuclei and myofibrillar disarray. Dis Models Mech 9: 347–359.

Tan X, Rotllant J, Li H, De Deyne P, Du SJ. 2006. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. Proc Natl Acad Sci USA 103:2713–2718.

Wang B, Li J, Fu FH, Chen C, Zhu X, Zhou L, Jiang X, Xiao X. 2008. Construction and analysis of compact muscle-specific promoters for AAV vectors. Gene Therpy 15:1489–1499.

Westerfield M. 1993. The zebra fish book. Eugene: University of Oregon Press.

Wu Z, Yang H, Colosi P. 2010. Effect of genome size on AAV vector packaging. Mol Ther 18:80–86.

Yue Y, Pan X, Hakim CH, Kodippili K, Zhang K, Shin JH, Yang HT, McDonald T, Duan D. 2015. Safe and body-wide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus. Human Mol Genet 24:5880–5890.