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smyd1 and *smyd2* are expressed in muscle tissue in *Xenopus laevis*

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Abstract Epigenetic modifications of histone play important roles for regulation of cell activity, such as cell division, cell death, and cell differentiation. A SET domain consisting of about 130 amino acids has lysine methyltransferase activity in the presence of the cosubstrate S-adenosyl-methionine. More than 60 SET domain-containing proteins have been predicted in various organisms. One of them, the SMYD family genes which contain a SET domain and a zinc-finger MYND domain are reported to regulate cell cycle and muscle formation. Here we examined the expression and function of *smyd1* and *2* in *Xenopus*. s*myd1* and *2* were expressed in various muscle tissues. While *smyd1* expression was observed mainly in cardiac muscle and skeletal muscle, *smyd2* expression was done abundantly in skeletal muscle and face region. Moreover, by loss-of-function experiments using antisense morpholino oligonucleotides, it was suggested that *smyd1* and *2* related to muscle cells differentiation.

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Introduction

Epigenetic modifications of histone regulate chromatin structure and gene expression in various cellular functions, such as cell cycle, cell death, maintenance of cell state, and cell differentiation. One of epigenetic modifications, methylation of histone tail is thought to play an important role for cell fate determination (Zhang and Reinberg 2001; Jenuwein and Allis 2001; Kouzarides 2002). In recent studies, it is reported that Polycomb group (PcG) and trithorax group (TrxG) regulate differentiation state of embryonic cells by methylating histone H3 lysine 27 (H3K27), H3K4 and H3K9 of differentiation factor genes locus, such as homeobox (Hox), Distal-less homeobox (Dlx) paired box (Pax) and sine-oculis-related homeobox (Six) gene locus (Caretti et al. 2004; Szutorisz et al. 2005; Azuara et al. 2006; Boyer et al. 2006; O'Neill et al. 2006; Rinn et al. 2007; Reik 2007). The SET domain is a catalytic domain conserved among lysine methyltransferases proteins and more than 60 SET protein genes are identified in various organisms (Jenuwein et al. 1998; Trievel et al. 2002; Qian and Zhou 2006). For examples, Ezh2 which is a member of PcG regulates myogenesis by histone H3-K27 methylation (O'Carroll et al. 2001; Czermin et al. 2002; Caretti et al; 2004). Mll and Mll2

involved in TrxG sustain Hox genes regulation in normal development (Yu et al. 1995; Yu et al. 1998; Hanson et al. 1999; Glaser et al. 2006). Furthermore, Set9 and Smyd2 can methylate not only histone but also p53 and regulate the cell cycle (Chuikov et al. 2004; Huang et al. 2006). However, functions of many of SET proteins have not resolved yet.

Among SET proteins, SMYD proteins which contain MYND (named from myeloid translocation protein 8, Nervy, and DEAF-1) and SET domains. The MYND domain is a conserved zinc binding domain and defined by seven conserved cysteine residues and a single histidine residue that are arranged in a C4-C2HC consensus (Spadaccini et al. 2006). The 5 varieties of *smyd* genes in *M. musculus* and *H. sapiens* have been cloned. It is well established that SMYD1 plays a role in normal development of heart in mouse (Hwang and Gottlieb 1997; Gottlieb et al. 2002; Phan et al. 2005) and muscle in zebrafish (Du et al. 2006; Tan et al. 2006). Also, SMYD2 and SMYD3 are reported to might be involved in repression and activation of cell cycle (Brown et al. 2006; Tsuge et al. 2005; Hamamoto et al. 2004).

In this study, we have characterized the expression patterns of *smyd1* and *2* in *Xenopus laevis* embryos. s*myd1* and *2* were expressed in various muscle tissues. While *smyd1* expression was observed mainly in cardiac muscle and skeletal muscle, *smyd2* expression was done abundantly in skeletal muscle and face region. Taken together with the loss-of-function experiments, it was suggested that *smyd1* and *2* are related to muscle tissue development.

Materials and methods

Embryos manipulation

Xenopus laevis embryos were obtained and cultured by standard methods (Sive et al. 2000). Embryos were allowed to develop in 1× Steinberg's solution and staged according to the normal table of Nieuwkoop and Faber (1967). Animal cap cells were dissected from embryos at stage 9 and cultured in 1× modified Barth's saline (MBS) supplemented with 0.1% BSA. Subsequently, these were treated with 5 ng/ml activin A.

smyd1 and *2* antisense morphlono oligonucleotides (Smyd1MO and Smyd2MO) were designed and supplied by Gene Tools LCC as follows, Smyd1MO: 5′-catggctgttccgcttctaccctgt-3′, and Smyd2MO: 5′-gttccagaccctcgggctgtcccat-3′. Microinjection was performed in 1× MBSH containing 5% ficoll solution. Morpholino oligonucleotide were microinjected into the animal side of stage 1 embryos.

RNA extraction and RT-PCR

RNAs of each animal cap and embryo were extracted using TRIzol reagent (Invitrogen). cDNA was

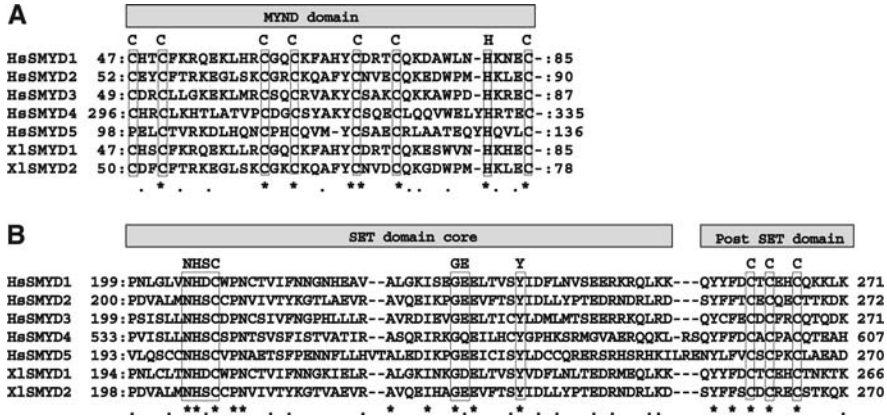

Fig. 1 Comparison of SMYD proteins sequence among *H. sapiens*, SMYD1 (HsSMYD1, NM_198274), SMYD2 (HsSMYD2, NM_020197), SMYD3 (HsSMYD3, NM_022743), SMYD4 (HsSMYD4, NM_052928) and SMYD5 (HsSMYD5, NM_006062), and *X. laevis*, SMYD1 (XlSMYD1) and SMYD2 (XlSMYD2). (**a**) MYND domain sequences. (**b**) SET domain and post-SET domain sequences. Identical amino acids are indicated by asterisks. Conserved domain residues are boxed

synthesized using M-MLV reverse transcriptase (Invitrogen). Oligo-dT primed reverse-transcription was performed using 1 μg total RNA as a template. Each cDNA was amplified by PCR using the following primer pairs and under the following cycling conditions; *pax3* (NM_001095524), forward: 5'-tttacccg ttactcatggatagtgt-3′, reverse: 5′-aatgtcacataaaatc-caaaaagga-3′ for 30 cycles, *myoD* (NM_203641), forward: 5′-tctctccagcatcgtcgagc-3′, reverse: 5′-ggaatt cattgtccgtttgg-3′ for 30 cycles, *myf-5* (X56738), forward: 5′-ccatgagagaacggagaagg-3′, reverse: 5′-cgggg tgatagagtctggaa-3′ for 30 cycles, *smyd1* (MGC80131), forward: 5′-cgtgttgtgaaagaggtg-3′, reverse: 5′-gggt tcatccatgacttg-3′ for 30 cycles, *smyd2* (MGC82991), forward: 5′-tgatgcacctccctttg-3′, reverse: 5′-caaaccgt aaaccaagctc-3′ for 30 cycles and an internal control, ornithine decarboxylase (*odc*, NM_001086698), forward: 5′-gcgggcaaaggagcttaatg-3′, reverse: 5′-taacgcc agaatctgctggg-3′ for 25 cycles. Five micro liters of each PCR product was resolved by 1% agarose gel electrophoresis and observed with ethidium bromide.

Whole mount in situ hybridization

smyd1 and *2* PCR products were inserted into pBluescript II (SK-) (TOYOBO) and then these were prepared to construct probes for whole mount in situ hybridization. Digoxigenin-labeled antisense RNA probes were in vitro transcribed with T7 or T3 polymerase (Roche Molecular Biochemicals) from template cDNA, for *smyd1* and *2*. Whole mount in situ hybridization was performed according to the method of Harland (Sive et al. 2000), except that the chromogenic reaction was done using BM purple as the substrate (Roche Molecular Biochemicals).

Results and discussion

Sequence comparison

We found that AAH72803 and AAH73650 correspond to SMYD1 and 2, respectively, by BLAST search and domain analysis (Fig. 1a). The post-SET domain shown in Fig. 1b is not essential for methylation activity, but it associates with the SET domain and exerts histone methyltransferase activity (Rea et al. 2000).

Expression pattern of *smyd1* and *2* during early development

We examined the expression pattern of *smyd1* and *2* in *X. laevis* early development by using RT-PCR (Fig. 2a). We used embryos at stage 2, 10, 12, 16, 25, 29/30, 33/34 and 40 according to the normal table of Nieuwkoop and Faber (1967). *smyd2* expression was identified at stage 2, indicating that *smyd2* mRNA existed maternally, and was persistent through stage 40 (Fig. 2a). On the other hand, *smyd1* expression was not identified maternally, but began to increase after the gastrula stage.

Next, we analyzed the spatial expression patterns of *smyd1* and *2* by using whole mount in situ

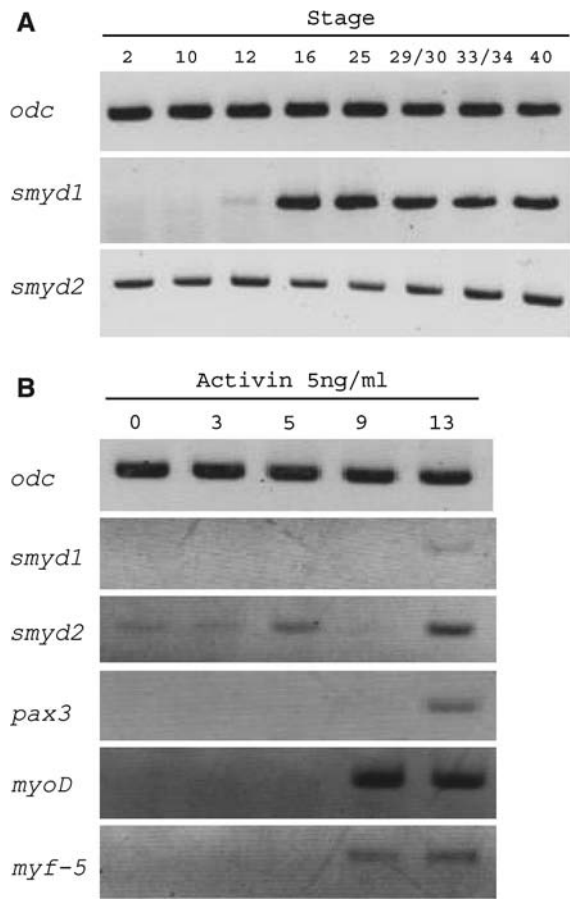

Fig. 2 Gene expression patterns of *smyd1* and *2*. (**a**) RT-PCR analysis from whole embryo RNA at the indicated stage according to Nieuwkoop and Faber (1967). Ornithine decarboxylase (*odc*) was used as control. (**b**) RT-PCR analysis from animal cap assay. *myoD*, *myf-5* and *pax3* were used as control of myogenesis

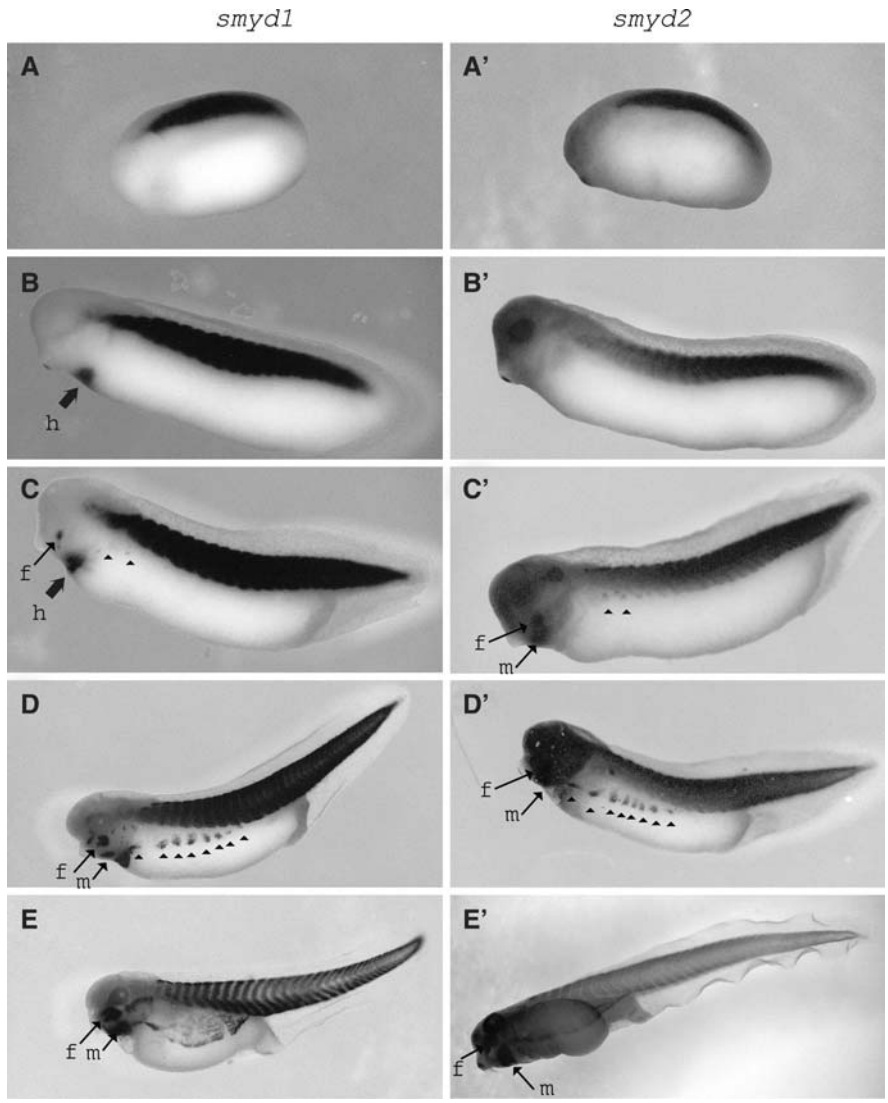

Fig. 3 Spatial expression patterns of *smyd1* and *2* in early development. *smyd1* and *2* mRNA localized in somite at stage 23 (**a** and **a**′). At stage 28, *smyd1* expression was observed in the presumptive heart (**b**) but *smyd2* was not (**b**'). After that, when the ventral body wall cells began to migrate from the somite at stage 33-34 (**c** and **c**′) and expanded completely by stage 41(**e** and **e**′), *smyd1* and *2* expression was observed in these migrating cells (**c**–**d** and **c**′–**d**′, arrow heads). h; heart, f; face and m; mandibular

hybridization. The expression of *smyd1* and *2* was observed distinctly in the prospective muscle tissue regions, somite, at stage 22 (Fig. 3a and a′). Subsequently, expression of both *smyd1* and *2* was observed also at the prospective ventral body wall, which began to migrate from the somite at later stage (Fig. 3c–e and c′–e′). These migrating cells correspond to dermomyotome.

Furthermore, we identified the differences between *smyd1* and *2* expression. At first, only *smyd1* expression was detected in the heart (Figs. 3 and 4). This expression is coincident with the fact that *smyd1* expression in heart is important for beating cardiac muscle in zebrafish (Du et al. 2006; Tan et al. 2006). Secondly, in the section at the anterior somite, while *smyd1* was observed within the entire region of the dermomyotome (Fig. 4c), *smyd2* expression was concentrated more within the dorsomedial lip (Fig. 4 c′). Finally, in the face region, *smyd2* expression was more widely than *smyd1* (Fig. 3c, d, c′ and d′). Also, in mandibular regions, following the smyd2 expression at stage 33/34, *smyd1* expression began at stage 37/38 (Fig. 3d, e c′, and e′).

These expression patterns in somite migration cell, heart and face regions are similar to those of *myoD* and *myf-5*, which are homeobox domain transcription factors and are referred to myogenic regulatory factors (Martin and Harland 2001). Taken together,

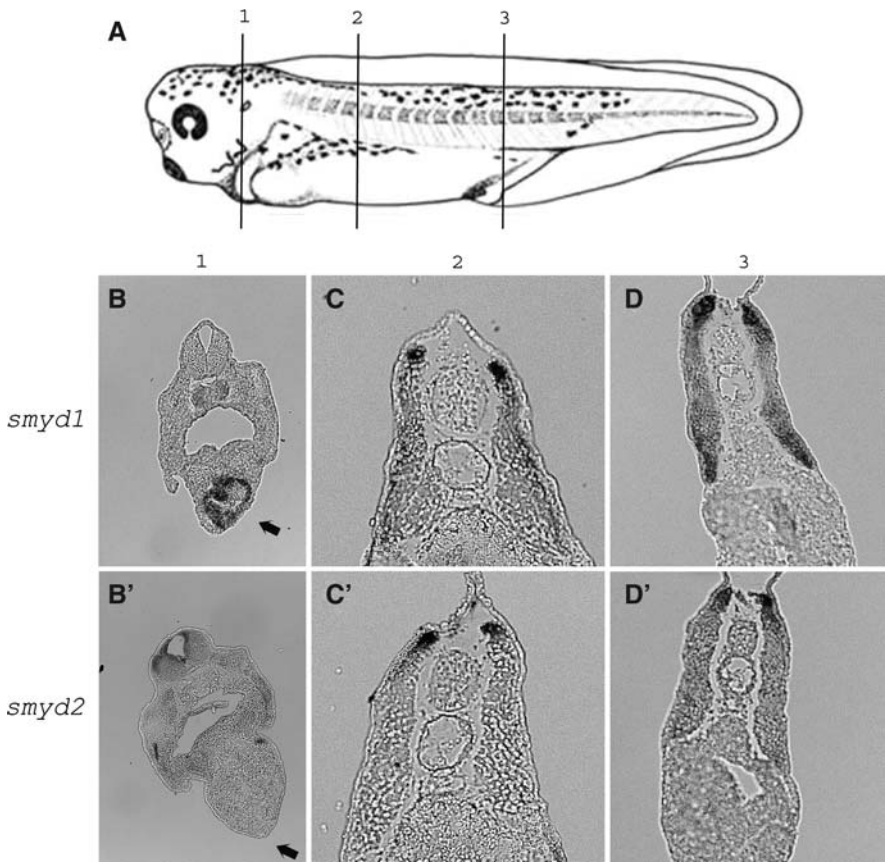

Fig. 4 *smyd1* and *2* expression in somite region. (**a**) Position of the partial sections (stage 37/38). *smyd1* expression was observed in the heart (**b**, arrow) but *smyd2* was not (**b**′). *smyd1* was observed within dermomyotome (**c**). *smyd2* was observed within dorsomedial lip (**c**′). In middle of somite, both *smyd1* and *2* mRNA existed in dermomyotome (**d**, **d**′)

we suggest that the expression of *smyd1* and *2* may correlate with muscle tissue formation during *X. laevis* early development.

Expression during muscle differentiation

To examine the expression *smyd1* and *2* during myogenesis, we carried out animal cap assays. Animal caps cells are multipotent cells and are able to differentiate into various types of cells by responding to differentiation inducing factors. When animal cap cells are treated with activin A, they become mesodermal tissues including muscle cells (Tamai et al. 1999). The animal cap cells were dissected from blastula embryo (stage 9), treated with 5 ng/ml activin A, and then used for RT-PCR. As *myoD*, *myf-5* and *pax3*, which are known as myogenic regulatory factor, were expressed by treatment of activin A (Fig. 2b), the animal cap cells differentiated into muscle lineage cells. *smyd1* and *2* expression also increased after the treatment with activin A (Fig. 2b 13 h). The existence of *smyd2* mRNA from 0 to 3 hrs after treatment with activin A (Fig. 2b) may be due to the occurrence of its mRNA as a maternal substance (Fig. 2a). This indicated that *smyd1* and *2* are related to myogenesis process.

Next, we examined the *smyd1* and *2* importance in embryogenesis by loss-of-function analysis using the antisense morpholino oligonucleotide (MO). The embryos injected with Smyd1MO had a immature somite (Fig. 5) and could not swim (data not shown). Moreover, injecting with both Smyd1MO and Smyd2MO, the embryos possess not only an abnormal somite, but also an abnormal mandibular tissue (Fig. 5) and the extent of malformations became to be more intensive. When Smyd2MO was injected into embryos, the abnormalities were not observed. Because the difference of *smyd2* expression from *smyd1* was only in face region, it is possible that we could not detect the

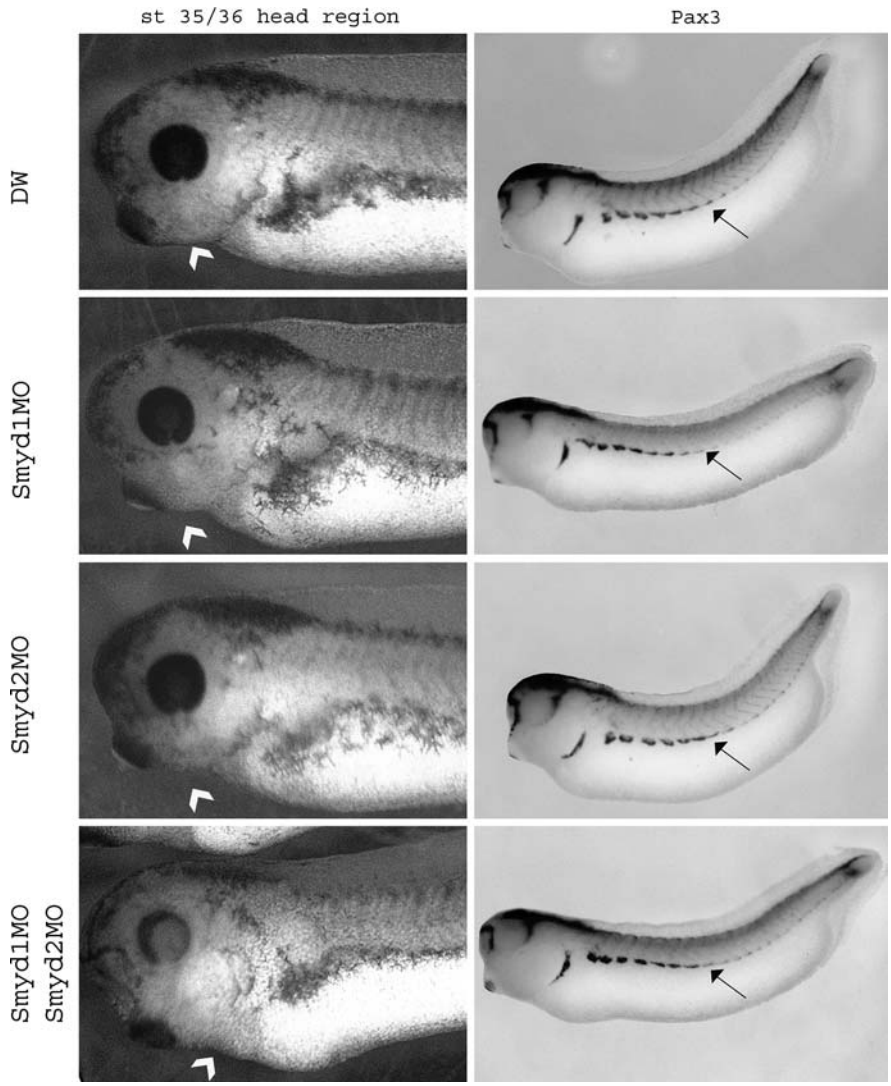

Fig. 5 The loss-of-function analysis of *smyd1* and *2*. DW, Smyd1MO, Smyd2MO and Smyd1MO-Smyd2MO were injected to stage 1 embryos. Right panels: whole mount in situ hybridization by using *pax3* as a probe. The arrow heads show the mandibular and arrow show the somite

malformation of face in this assay. From these results, *smyd1* is essential for the heart and skeletal muscle development.

Conclusion

From the results described here, it is revealed that expression of *smyd1* and *2* plays a role during muscle development. And we suggested that at least *smyd1* is necessary for muscle cell formations. However, there are temporal and anatomical differences between *smyd1* and *2* expression. To clarify the mechanism of muscle tissue differentiation and muscle tissue formation, we have to analyze the roles of *smyd1* and *2* at molecular level.

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