

misty somites, a maternal effect gene identified by transposon-mediated insertional mutagenesis in zebrafish that is essential for the somite boundary maintenance

Tomoya Kotani^{a,1}, Koichi Kawakami^{a,b,*}

^a Division of Molecular and Developmental Biology, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

^b Department of Genetics, The Graduate University for Advanced Studies (SOKENDAI), 1111 Yata, Mishima, Shizuoka 411-8540, Japan

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Abstract

Somite boundary formation is crucial for segmentation of vertebrate somites and vertebrae and skeletal muscle morphogenesis. Previously, we developed a *Tol2* transposon-mediated gene trap method in zebrafish. In the present study, we aimed to isolate transposon insertions that trap maternally-expressed genes. We found that homozygous female fish carrying a transposon insertion within a maternally-expressed gene *misty somites* (*mys*) produced embryos that showed obscure somite boundaries at the early segmentation stage (12–13 hpf). The somite boundaries became clear and distinct after this period and the embryos survived to adulthood. This phenotype was rescued by expression of *mys* cDNA in the homozygous adults, confirming that it was caused by a decreased *mys* activity. We analyzed a role of the *mys* gene by using morpholino oligonucleotides (MOs). The MO-injected embryo exhibited severer phenotypes than the insertional mutant probably because the *mys* gene was partially active in the insertional mutant. The MO-injected embryo also showed the obscure somite boundary phenotype. Fibronectin and phosphorylated FAK at the intersomitic regions were accumulated at the boundaries at this stage, but, unlike wild type embryos, somitic cells adjacent to the boundaries did not undergo epithelialization, suggesting that *Mys* is required for epithelialization of the somitic cells. Then in the MO-injected embryos, the boundaries once became clear and distinct, but, in the subsequent stages, disappeared, resulting in abnormal muscle morphogenesis. Accumulation of Fibronectin and phosphorylated FAK observed in the initial stage also disappeared. Thus, *Mys* is crucial for maintenance of the somite boundaries formed at the initial stage. To analyze the *mys* defect at the cellular level, we placed cells dissociated from the MO-injected embryo on Fibronectin-coated glasses. By this cell spreading assay, we found that the *mys*-deficient cells reduced the activity to form lamellipodia on Fibronectin while FAK was activated in these cells. Thus, we demonstrate that a novel gene *misty somites* is essential for epithelialization of the somitic cells and maintenance of the somite boundary. Furthermore, *Mys* may play a role in a cellular pathway leading to lamellipodia formation in response to the Fibronectin signaling. We propose that the *Tol2* transposon mediated gene trap method is powerful to identify a novel gene involved in vertebrate development.

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Introduction

Somites are morphologically distinct segmental units that are transiently formed during vertebrate embryogenesis and give rise to metameric and fundamental structures such as the ver-

tebrate axial skeleton and their associated muscles. The reiterative pattern of the somite is formed from the anterior to the posterior by a mechanism so-called segmentation clock (Pourquie, 2003). The morphologically distinct boundaries are formed in the intersomitic regions and the somitic cells undergo gross morphological changes to yield the sclerotome, myotome and dermatome (Keynes and Stern, 1988).

In a model vertebrate zebrafish, most somitic cells give rise to muscle fibers. Somitic cells on both side of the intersegmental boundaries undergo a mesenchymal-to-epithelial transition and then elongate to form long muscle fibers that are anchored to the

* Corresponding author. Division of Molecular and Developmental Biology, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. Fax: +81 55 981 5827.

E-mail address: kokawaka@lab.nig.ac.jp (K. Kawakami).

¹ Present address: Laboratory of Molecular and Cellular Interactions, Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan.

boundaries (Devoto et al., 1996; Stickney et al., 2000). Henry et al. defined three stages of the somite boundary formation (Henry et al., 2005). The first stage is the formation of the initial epithelial boundaries that involves accumulation of the extracellular matrix (ECM) components at the intersomitic regions. Fibronectin is a major component of ECM that promotes cell adhesion, cell migration and cytoskeletal organization. During the initial boundary formation, Fibronectin is localized to the intersomitic regions (Crawford et al., 2003). The focal adhesion kinase (FAK) is activated through Fibronectin–Integrin signaling (Burridge et al., 1992; Guan and Shalloway, 1992). At this stage, phosphorylated FAK (an active form of FAK) is also localized at the boundaries (Crawford et al., 2003; Henry et al., 2001). The second stage is a transition stage from the initial somite boundary formation to the myotome boundary formation. The boundaries become chevron-shaped, and muscle precursor cells begin to elongate. The third stage is the formation of the myotome boundary. At this stage, all muscle precursor cells fully elongate to generate the myotome. The myotome boundaries are exceedingly rich in ECM components and phosphorylated FAK. The boundaries that were formed at the first stage are maintained through the later stages.

Zebrafish is an excellent model animal to identify developmental genes by forward genetics approaches. Large-scale mutagenesis screens by using a chemical mutagen ENU have identified zygotic mutations that affect early somitogenesis (Julich et al., 2005a; Koshida et al., 2005; van Eeden et al., 1996). Studies of these mutants have revealed that genes involved in the Notch pathway; *after eight/deltaD* (Holley et al., 2000), *deadly seven/notch1a* (Holley et al., 2002), *mind bomb* (E3 ubiquitin ligase) (Itoh et al., 2003) and *beamter/deltaC* (Julich et al., 2005b), genes involved in the Fibronectin–Integrin signaling pathway; *integrin α 5* and *fibronectin* (Julich et al., 2005a; Koshida et al., 2005), and *fused somites/tbx24* (Nikaido et al., 2002) are crucial for somitogenesis. Although these mutants show different types of defects in the somite boundary formation, namely the Notch pathway mutants are defective in the posterior somites, the Fibronectin–Integrin pathway mutants are defective in the anterior somites and the *tbx24* mutant is defective in both anterior and posterior somites, all of them showed defects during the formation of the initial epithelial boundary formation, the first stage of the somite boundary formation defined by Henry et al. (2005). Thus, little is known about genes involved in the maintenance of the epithelial boundaries through the transition and myotome boundary formation stages.

The large-scale chemical mutagenesis screens in zebrafish have estimated that only 2,400 genes are zygotically essential (Driever et al., 1996; Haffter et al., 1996) although there may be 30,000 or more genes in the zebrafish genome. Also a large-scale insertional mutagenesis screen using a pseudotyped retrovirus has estimated that the zebrafish genome contains only 1,400 zygotically essential genes (Amsterdam et al., 2004). On the other hand, chemical mutagenesis screens for maternal effect mutants have been carried out, and it was shown that maternal factors also regulate various developmental processes as well as zygotic factors (Dosch et al., 2004; Kishimoto et al.,

2004; Pelegri et al., 1999; Wagner et al., 2004). These maternal effect mutants should be valuable sources to discover novel genes that had not been discovered by the zygotic screens. Identification of mutated genes however are laborious and time-consuming, especially for maternal mutations, since it requires genetic mapping and positional cloning.

We have developed the transposon-mediated transgenesis method in zebrafish by using the medaka fish *Tol2* transposable element (Kawakami et al., 1998, 2000; Kawakami and Shima, 1999). Recently, we have successfully performed a gene trap screen by using the *Tol2* transposon system and created a number of fish expressing GFP in temporally and spatially restricted patterns (Kawakami et al., 2004). In these fish, insertions of the gene trap transposon construct captured endogenous transcripts and interfered with their normal splicing partially or nearly completely (Kawakami et al., 2004; Kotani et al., 2006), suggesting that the gene trap method is applicable to insertional mutagenesis. In this study, we aimed to isolate maternal effect mutants by performing the *Tol2*-mediated gene trap approach. We hypothesized that, if the gene trap construct was integrated in maternally expressed genes, GFP expression should be detected in fertilized eggs. Therefore, we “prescreened” insertions for GFP expression at the one cell stage, and created female fish homozygous for such insertions. Then we crossed these female fish and analyzed their progeny for developmental phenotypes. By this strategy, we isolated a maternal effect mutant, *misty somites*. We will describe the identification and characterization of a novel gene, *misty somites*, and the discovery of its role in maintenance of the initial epithelial somite boundary.

Materials and methods

Fish

SAG14A, SAG20A, SAG86A, SAGm11A, SAGm11B, SAGm18B, SAGp4A and SAGp53B, were isolated previously (Kawakami et al., 2004). SAGm11C, SAGm11D and SAGm11E were newly identified during outcrosses of SAGm11A and SAGm11B (Kawakami, 2005). SAGn10A, SAGn15C, SAGn25A and SAGn28C were created in this study. GFP expression was analyzed under a fluorescent stereoscope MZ 16 FA (Leica). Photos were taken by using DFC300FX (Leica). TL, Tuebingen (Tu) and TAB, a hybrid between Tu and AB were used as wild type fish.

Primers, Southern blot hybridization, inverse PCR, linker-mediated PCR, 5'RACE, 3' RACE and RT-PCR

Primers used in this study are shown in Table S1. Southern blot hybridization, inverse PCR, linker-mediated PCR, 5' RACE, 3' RACE and RT-PCR were carried out as described previously (Kawakami, 2004; Kawakami et al., 2004; Kotani et al., 2006). The PCR products were purified from a gel or cloned by TA cloning kit (Invitrogen), and sequenced by using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem). Oligo-dT30 Super mRNA Purification Kit (Takara) was used to purify poly(A)⁺ RNA.

Construction of *Tol2*–*mys* transgenic fish

1305 bp of cDNA containing the open reading frame for the *Mys* protein was amplified by RT-PCR using *mys*-f1-ATG and *mys*-r9, and cloned between the *Bam*HI and *Cla*I sites of T2KXIGΔin (Urasaki et al., 2006), resulting in *Tol2*–*mys*. Four fish injected with a plasmid containing *Tol2*–*mys* and the

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