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Molecules and Cells

The Expression Profile and Function of Satb2 in Zebrafish Embryonic Development

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The present study shows the expression profile and function of the homeobox gene, *satb2* during zebrafish embryonic development. *Satb2* was ubiquitously expressed from the 1 cell stage to the 10-somite stage in zebrafish embryos. *Satb2* showed stage-specific expression profiles such as in the pronephric duct at 24 hpf, the branchial arches at 36 hpf, and the ganglion cell layer of the retina and fins at 48 hpf. Additionally, *satb2* knockdown embryos were arrested at 50-60% epiboly, and transplantation experiments with *satb2* knockdown cells showed migration defects. Interestingly, *satb2* knockdown cells also exhibited down-regulation of dynamin II and VAMP4, which are involved in exocytosis and endocytosis, respectively. Furthermore, *satb2* knockdown cells have a disorganized actin distribution and an underdeveloped external yolk syncytial layer, both of which are involved in epiboly. These results suggest that satb2 has a functional role in epiboly. This role may potentially be the regulation of endo-exocytic vesicle transport-dependent cell migration and/or the regulation of the development of the yolk syncytial layer.

INTRODUCTION

Special AT-rich sequence binding protein 2 (Satb2) belongs to a Cut family of homeodomain proteins, and has one satb domain for dimerization, two cut domains, and one homeodomain for DNA binding (FitzPatrick et al., 2003). The cut domain binds to specific AT-rich matrix attachment regions (MARs) that associate with the intranuclear skeletal frame known as nuclear matrix (Dickinson et al., 1997). The periodic attachment of MARs to the nuclear matrix separates chromatin into topologically independent loop domains where different genetic processes take place, such as transcription and replication (Ciejek et al., 1983; Vaughn et al., 1990). Satb2 binds to multiple MARs where chromatin is fastened forming loop domains and modifies the structure of those domains, thereby orchestrating the transcription of tissue type-specific genes (Dobreva et al., 2006).

Satb2 was originally identified in human cleft palate only (CPO) patients by high resolution FISH mapping (FitzPatrick et al., 2003). Translocation of the chromosomal region 2q32-q33 causes a breakpoint and interrupts one allele of the *satb2* gene, which in the haplo-sufficient condition induces a CPO phenotype. A patient with a truncated form of satb2 protein, which results from a pathogenic nonsense mutation, shows generalized osteoporosis, profound mental retardation, epilepsy, as well as craniofacial dysmorphisms, that includes maxillary malformation, mandibular hypoplasia, and cleft palate (Leoy-klang et al., 2007). In accordance with human patients, *satb2* knockout mice also exhibit craniofacial abnormalities and defects in osteoblast differentiation (Dobreva et al., 2006). During osteoblast differentiation, satb2 recognizes MAR-like sequences, and binds to and regulates target genes that are critical components of osteoblast differentiation (Dobreva et al., 2006). Recently, it has been reported that *satb2* is expressed in the developing mouse and rat neocortex, and that it is involved in regulating neuronal specification and the migration of projection neuron. These satb2 functions may be related to the mental retardation and epilepsy phenotype in *satb2* nonsense mutation patients (Alcamo et al., 2008; Britanova et al., 2005; 2006a; 2008; Leoyklang et al., 2007; Szemes et al., 2006).

In the present study, we cloned the zebrafish *satb2* gene and examined its expression pattern during early embryonic development by whole mount *in situ* hybridization. We further analyzed the function of satb2 with anti-sense morpholino (*satb2-MO*) injection and transplantation experiments.

MATERIALS AND METHODS

Fish care

Zebrafish were maintained as described in the Zebrafish book (Westerfield, 1995).

Cloning and sequence analysis

We performed sequence comparison in the ensemble MultiBlastView engine (http://www.ensembl.org/Multi/blastview) using mouse *satb2* cDNA. Based on this comparison, putative zebrafish *satb2* cDNA sequences were identified. We designed the primers S1F (5′-ATG GAG CGT CGA GGA GAG AGT-3′) and

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S1R (5′-AGC CAC GTC TAC ACC GCC GCC CTC-3′), and performed reverse transcriptase-polymerase chain reaction with 24 h post fertilization (hpf) total RNA in order to clone zebrafish *satb2* cDNA. All PCR reactions consisted of 25 cycles and were performed with Pfu Turbo (Stratagene). PCR products were subcloned into pBluescript SK and confirmed by sequence analysis.

Whole-mount *in situ* hybridization and immunohistochemistry

Whole-mount *in situ* hybridization and immunohistochemistry were performed as previously described (Yeo et al., 2001). For *in situ* hybridizations, antisense riboprobes were synthesized from *sox17, no tail, sox3* and *satb2* cDNA as previously described (Alexander and Stainier, 1999; Schulte-Merker et al., 1994; Yeo et al., 2001; Zhang et al., 2004). For immunohistochemistry the following primary antibodies were used: zo-1 (1:1000, Zymed), dynamin II (1:1000, Santa Cruz) and VAMP4 (1:1000, Stressgen).

Microinjections of morpholinos

Microinjections of morpholinos were performed on one-cell stage wild-type embryos as previously described (Yeo and Chitnis, 2007). Morpholinos (MOs) (Gene Tools, LLC) were designed between intron2 and exon3 of the *satb2* gene, stored at a stock concentration of 10 mg/ml at -20°C, and injected at the dose of 4 ng. The sequences of the MOs used were 5′-GCAGTGTTGAACTCACCATGAGCCT-3′ for *satb2* (*satb2-MO*), and 5′-GCACTGTTCAACTCAGCATGAGGCT-3′ for the control (*con-MO*).

Polymerase chain reaction

Total RNA from MOs-injected 10 hpf embryos was extracted with Trizol Plus RNA Purification System (Invitrogen Corp.). RNA templates were reverse transcribed with oligo dT by 200 units of MMLV reverse transcriptase and amplified using *satb2* primers between exon2 and exon3. The following primers were used: *Satb2* up: 5′-TACTCTCACAGTTCTGCAGCC-3′, *Satb2* down: 5′-TGGTTCCACTGCTCAGACGGA-3′, *EF1* up: 5′-GC CACACTGCTCACATCGCCT-3′ and *EF1* down: 5′-TGGCAA CAGGTGCAGTTCTAA-3′

Transplantation experiments

Transplantation experiments were performed as follows: either 4 ng of *satb2-MO* with 100 pl of 0.5% fluorescein-dextran (MW 10,000, Molecular Probes), or 4 ng of *con-MO* with 100 pl of 0.5% rhodamine-dextran (MW 10,000, Molecular Probes) were injected into the donor yolk sac cell at the one-cell stage. Each donor embryo was grown until 4 hpf. A small population of cells (10-20 cells) from each donor embryo (containing fluorescein or rhodamine) was aspirated using a micropipette. The donor blastomere cells were mixed together and transplanted into wild-type embryos. The locations of the labeled donor cells were monitored by observation through a LSM510 META confocal microscope (Zeiss).

RESULTS

Expression profiles of zebrafish *satb2* during embryonic development

Using mouse *satb2* cDNA, we performed an *in silico* sequence comparison using the ensemble MultiBlastView engine (http://www.ensembl.org/Multi/blastview). Based on this comparison, the zebrafish *satb2* cDNA sequence was identified and isolated by RT-PCR (see experimental procedures). Zebrafish satb2 has a high sequence homology to the mouse sequence, especially in the functional domains, including the satb domain (86%), the cut domain (92%), and the homeo domain (97%) (Supplementary Fig. 1). A partial N-terminal cDNA fragment was cloned by RT-PCR and used as a probe for whole mount *in situ* hybridization (see experimental procedures).

Satb2 was expressed ubiquitously from the 1-cell to the 10-somite stage embryo, which implies a functional role for satb2 in early embryonic development (Figs. 1A-1H). Specific expression of satb2 in early stage embryos was confirmed by RT-PCR analysis (Supplementary Fig. 2). At 24 hpf, *satb2* was expressed in the pronephric duct (Figs. 1I and 1I'). At 36 hpf, s*atb2* was strongly expressed in the branchial arches, which is consistent with the expression of mouse and human *satb2* during craniofacial development (Fig. 1J). At 48 hpf, *satb2* was specifically expressed in the retina and fins (Fig. 1K). At 72 hpf, *satb2* was expressed in the ganglion cell layer of retina and in the brain (Fig. 1L). These diverse expression patterns of *satb2* suggest that it has stage-specific developmental roles, and that there are multiple functions for satb2 during zebrafish embryonic development.

Defects in epibolic movements of *satb2* knockdown cells

To investigate the function of satb2 during zebrafish embryonic development, we generated an anti-sense morpholino against *satb2*, *satb2-MO*. This morpholino was designed to interfere with the splicing that generates alternative transcripts, which should result in non-functional proteins. A 4 base-pair mismatched *con-MO* was used as controls. RT-PCR analysis showed that the *satb2-MO* completely blocks the splicing of *satb2* in the developing zebrafish embryos (Fig. 2A). Embryos injected with *satb2-MO* had an elongated ellipsoidal shape with arrested epiboly and died at 13 hpf (Fig. 2B). Whole mount *in situ* hybridization with *sox17*, a marker for endoderm, showed no difference between *con-MO* and *satb2-MO* injected embryos (Fig. 2C). *Satb2-MO* injected embryos, however, did show defects in the migration of axial mesoderm cells, marked by the expression of *no tail,* and in the development of the neural ectoderm, marked by the expression of *sox3* (Fig. 2C). Taken together, these results suggest a role for satb2 in the movement of embryonic cells during gastrulation.

To test whether satb2 induces the cell movement defects, we performed a transplantation experiment using cells from zebrafish embryos injected with either *con-MO* or *satb2-MO*. Cells from *satb2-MO* and fluorescein-dextran co-injected embryos were mixed with cells from *con-MO* and rhodamine-dextran co-injected 4 hpf embryos, and then transplanted into the same place of a wild-type 4 hpf embryo. The migration patterns were monitored by time lapse imaging with a confocal microscope (see Fig. 3A for the detailed experimental scheme). As development proceeded, *con-MO* injected cells migrated toward the vegetal poles of injected embryos, which follow normal epiboly, but cells injected with *satb2-MO* migrated in random directions (Figs. 3B and 3C, Supplementary Fig. 3). These data support the idea that the movement defects previously observed in the developing embryos were caused by the knockdown of *satb2*.

To gain insight into the satb2 knockdown phenotype, we performed whole-mount immunohistochemistry using markers associated with cell to cell communication. Recent reports suggest that cell-cell contacts are important for establishing the membrane polarity necessary for directed migration, and that endocytic machinery facilitates cell migration and invasion (Britanova et al., 2006a; Fink and Cooper, 1996; Kruchten and McNiven, 2006). Based on these reports, we used phalloidin as a marker for actin, zo-1 antibody as a marker for tight junctions,

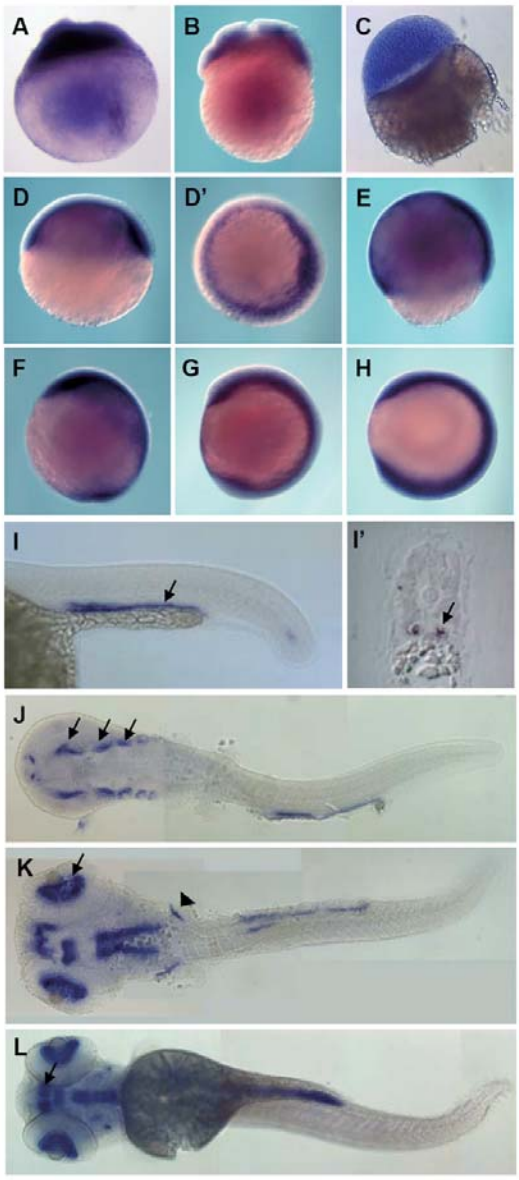

Fig. 1. Developmental stage- and tissue-specific expression of zebrafish *satb2*. Lateral views (A-I), dorsal view (D'), and ventral views (J-L) are shown. (A-H) *Satb2* is ubiquitously expressed in the 1-cell (A), 4-cell (B), dome (C), shield (D, D'), 75% epiboly (E), bud (F), 3-somite (G), and 10-somite (H) embyos. (I-L) *Satb2* is expressed in the pronephric duct (arrow) at 24 hpf (I, I'), in branchial arches (arrows) at 36 hpf (J), in the ganglion cell layer of the retina (arrow) and fins (arrowhead) at 48 hpf (K), and in the brain (arrow) at 72 hpf (L).

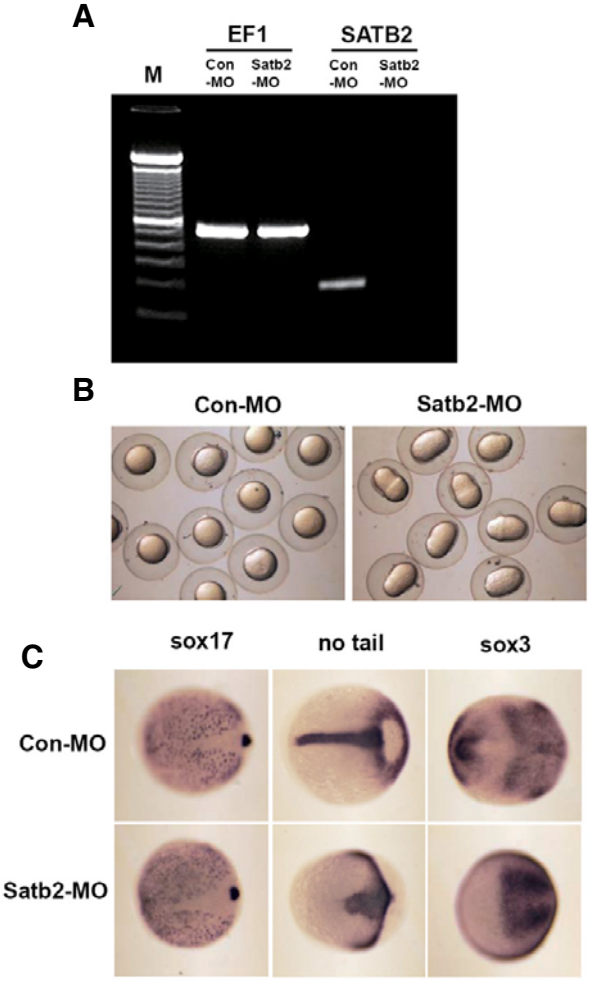

Fig. 2. Inhibition of *satb2* causes defects in epibolic movements. (A) Specificity of *satb2* morpholino is shown. Four nanograms of *satb2-MO,* targeting the junction between intron 2 and exon 3, were injected into the embryos. Total RNA was extracted from *con-MO* and *satb2-MO* injected embryos at 10 hpf and subjected to RT-PCR with *satb2* primers. *Satb2* was not detected in *satb2-MO* injected embryos. *EF1* was used as a positive control. (B) The morphology of *con-MO* and *satb2-MO* injected embryos is shown. *Satb2-MO* injected embryos arrest at the epiboly stage with an elongated ellipsoidal shape. (C) Expression of sox17, no tail, and sox3 in *con-MO* and *satb2-MO* injected embryos at 13 hpf. Lateral view (B) and dorsal view (C) are shown.

dynamin II antibody as a marker for endocytosis, and VAMP4 antibody as a marker for exocytosis. Actin distribution was disorganized in *satb2-MO* injected embryos compared to *con-MO* injected embryos, which is consistent with the idea that cell polarity is involved in directed cell migration (Fig. 4A). No difference, however, was observed in the expression and distribution of zo-1, a tight junction related protein (Fig. 4A). Interestingly, dynamin II, which is involved in rapid endocytosis, was expressed in localized regions in control cells, but its expression was dramatically reduced in cells from *satb2-MO* injected embryos (Fig. 4A). Furthermore the expression of vesicle associated membrane protein 4 (VAMP4) was also dramatically reduced in *satb2-MO* injected embryos (Fig. 4A). These results imply that cellular polarity and endo-exocytic processes, which may be important during migration, are impaired in *satb2* knockdown embryos.

Additionally, we observed that the external yolk syncytial layer (e-YSL) of *satb2-MO* injected embryos was underdeveloped compared to that of *con-MO* injected embryos (Fig. 4B). The e-YSL is known to be the cell autonomic driving force of epiboly (Betchaku and Trinkaus, 1978). Taken together, these findings suggest that satb2 may be involved in the process of epiboly by regulating the development of the e-YSL. Con-

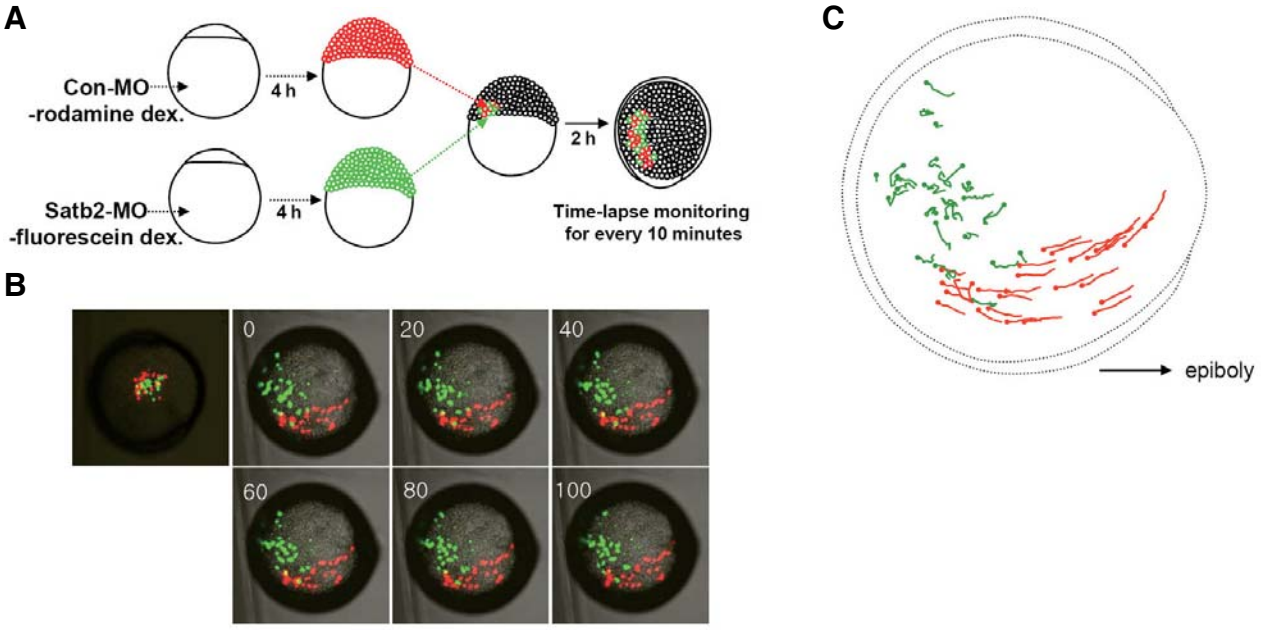

Fig. 3. Migration defects of *satb2* knockdown cells during gastrulation. (A) Schematic representation of the transplantation experiment is shown. *Con-MO* with rhodamine-dextran or *satb2-MO* with fluorescein-dextran was injected into donor one-cell embryos. After 4 h, about 20 cells were removed from each of the donor embryos by micropipette aspiration. Donor cells were mixed together and transplanted into wild-type embryos at the same stage. After stabilizing for 4 h, the movements of the transplanted cells were monitored every 10 min. (B) The time-lapse monitoring of transplanted cells is shown. Dorsolateral views are shown (anterior is left). Pictures of transplanted cells at the time of transplantation, after stabilization for 4 h (0), and 20, 40, 60, 80 and 100 min after monitoring began. (C) Camera lucidal drawing of *con-MO* (red) and *satb2-MO* (green) injected cells. *Con-MO* injected cells migrate in the epiboly direction (arrow), while *satb2-MO* injected cells move irregularly and were stalled in the animal pole regions.

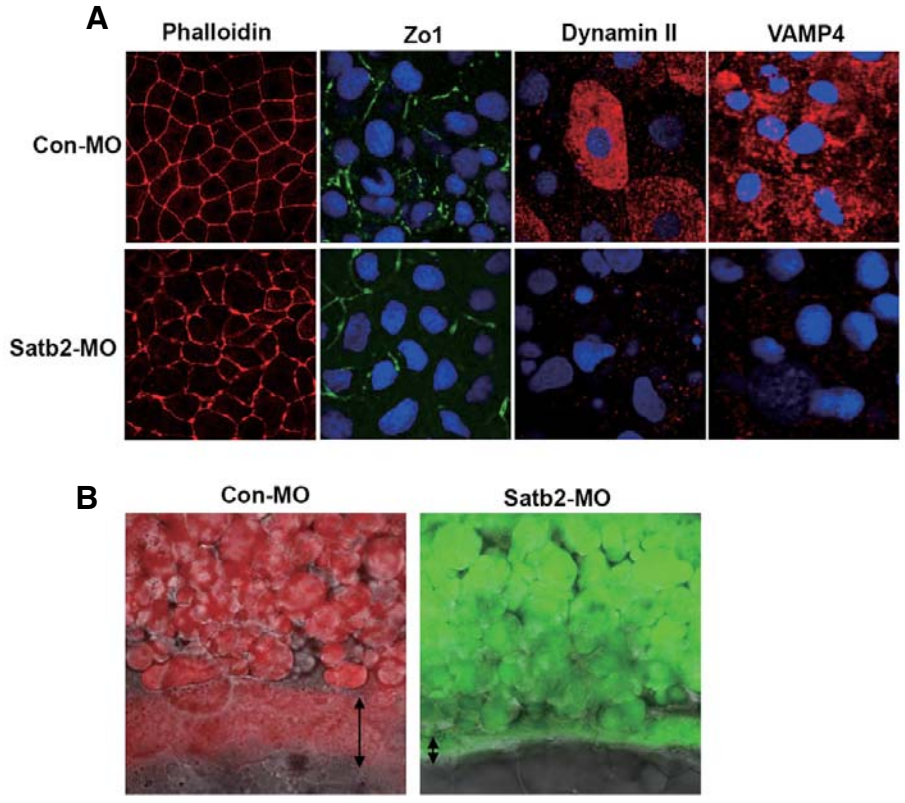

Fig. 4. (A) The morphology of actin organization and expression of zo-1, dynamin II, and VAMP4 in the *con-MO* and *satb2-MO* injected embryos is shown. Actin distribution, stained by phalloidin, was disorganized in the *satb2-MO* injected embryos. Endo-exocytic vesicle-related dynamin II and VAMP4 protein levels are decreased in the *satb2-MO* injected embryos. The nucleus was stain by DAPI. (B) The e-YSL (double arrows) of the *satb2-MO* injected embryo is underdeveloped compared to that of the *con-MO* injected embryo.

versely, satb2 may be involved in the process of epiboly by regulating other factors that are indirectly or directly involved in the formation of e-YSL. These two proposed roles for satb2, however, are not mutually exclusive. The precise role of satb2 during e-YSL development may be elucidated by conducting additional experiments in the future.

DISCUSSION

Expression patterns of *satb2* during zebrafish embryonic development

This study showed expression patterns of *satb2* in the pronephric duct, branchial arches, ganglion cell layer of the retina, and fins during zebrafish embryonic development. The expression of zebrafish *satb2* in the branchial arches and fins is consistent with the expression patterns of human and mouse *satb2*. Comparative studies show that the inactivation of *satb2,* by translocation and genetic ablation results in a cleft palate and craniofacial malformations in humans and mice (Beaty et al., 2006; Britanova et al., 2006b; Dobreva et al., 2006; FitzPatrick et al., 2003; Leoyklang et al., 2007; Van Buggenhout et al., 2005). These findings suggest that satb2 may play a similar role in the craniofacial development of zebrafish. The transcription factor runx2 is essential for osteoblast differentiation, and it is also known to be involved in zebrafish craniofacial skeleton and fin development (Flores et al., 2006; Smith et al., 2006). Interestingly, it has been reported that satb2 augments *runx2* expression, and that it binds to and enhances runx2 function during mouse craniofacial development and osteoblast differentiation (Dobreva et al., 2006). These findings suggest that zebrafish satb2 may be involved in craniofacial and fin development by regulating or interacting with runx2.

Satb2 was uniquely expressed in the ganglion cell layer of the retina in the zebrafish. Ganglion cells are the projection neurons that connect the eye to the brain. A recent study identified a number of factors involved in several processes such as ganglion cell specification, migration to form the ganglion cell layer, and projection of axons to the brain (Godinho et al., 2005; Kay et al., 2001; Pujic et al., 2006). The genetic circuitry that orchestrates these processes, however, is largely unknown. Satb2 is one potential candidate that may orchestrate these processes, considering its unique mechanism for regulating transcription. The SATB family contains novel-type transcription factors whose mechanism for regulating transcription is different from those mechanisms utilized by classical transcription factors. Classical transcription factors bind to individual target genes in order to regulate transcription, and consequently, tissue-type-specific transcription is controlled by a group of transcription factors that activate or suppress the expression of target genes in a given tissue. SATB family members, however, bind to multiple sites within chromatin loop domains and modulate their organization and structure. This mechanism gives them the ability to orchestrate the transcription of multiple genes in a given tissue type (Alvarez et al., 2000; Dobreva et al., 2006).

Involvement of satb2 in epiboly

Our results show that dynamin II and VAMP4 expression are dramatically reduced, and that actin distribution is disorganized in *satb2-MO* injected embryos. These findings suggest that there are impairments in the endo-exocytic process and in cellular polarity, both of which are important for cell migration. Consistent with this proposal, *satb2* knockdown embryos are arrested near 50-60% epiboly.

Dynamin II, known for its role in endocytosis, has recently been implicated as a facilitator of cell migration. De Camilli and colleagues provided the first functional link between components of the actin cytoskeleton and the previously categorized endocytic protein, dynamin II (Ochoa et al., 2000). The matrix-degrading structures at the base of cells are formed from tubular invaginations of the membrane, and they appear to require both actin and dynamin II (Ochoa et al., 2000). Additionally, more recent observations link dynamin II to other processes that are also essential for cell motility. For example, its roles in actin polymerization, membrane deformation and vesiculation, and focal adhesion dynamics are all important for proper cell motility (Kruchten and McNiven, 2006). These reports provide a possible explanation for our observations. The impairment of endocytosis may cause the disorganization of actin distribution in *satb2* knockdown embryos, thereby resulting in cell migration defects in *stab2-MO* injected embryos.

The relationship between the decreased expression of dynamin II and VAMP4 and the arrested epibolic movement in the *satb2* knockdown embryos is not yet clear. Recent papers have shown that the endo-exocytic process is involved in polarizing plasma membrane proteins, such as ECM receptor molecules, and that this polarity is required for directed migration (Jones et al., 2006). Taking these findings into consideration, it is likely that satb2 regulates the expression of exo-endocytic vesicular transport-related proteins that enhance epibolic movements. However, we cannot exclude the possibility that cell-cell communication with neighboring cells is important for embryonic cell migration, and that it may orchestrate gastrulation movements.

Additionally, we also found an underdevelopment of the e-YSL in s*atb2* knockdown embryos for reasons unknown. Recent papers, however, provide important information about the molecular relationship between epiboly and the e-YSL (Cheng et al., 2004; Koppen et al., 2006). Cells at the margin of the EVL are tightly connected to the e-YSL, and actin accumulates at the connection site within the e-YSL. The accumulated actin, which appears as a ring-like band at the marginal equator of the epibolic movements, serves as a pulse string. When the pulse string tightens, it pulls both the e-YSL and EVL toward the vegetal pole (Cheng et al., 2004). The molecular mechanism of the involvement of satb2 in actin accumulation along this band remains to be elucidated.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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