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# Completion of the epithelial to mesenchymal transition in zebrafish mesoderm requires Spadetail

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ABSTRACT

The process of gastrulation is highly conserved across vertebrates on both the genetic and morphological levels, despite great variety in embryonic shape and speed of development. This mechanism spatially separates the germ layers and establishes the organizational foundation for future development. Mesodermal identity is specified in a superficial layer of cells, the epiblast, where cells maintain an epithelioid morphology. These cells involute to join the deeper hypoblast layer where they adopt a migratory, mesenchymal morphology. Expression of a cascade of related transcription factors orchestrates the parallel genetic transition from primitive to mature mesoderm. Although the early and late stages of this process are increasingly well understood, the transition between them has remained largely mysterious. We present here the first high resolution *in vivo* observations of the blebby transitional morphology of involuting mesodermal cells in a vertebrate embryo. We further demonstrate that the zebrafish *spadetail* mutation creates a reversible block in the maturation program, stalling cells in the transition state. This mutation creates an ideal system for dissecting the specific properties of cells undergoing the morphological transition of maturing mesoderm, as we demonstrate with a direct measurement of cell–cell adhesion.



## Introduction

Gastrulation lays down the organizational foundation of the embryo by separating the endodermal, mesodermal and ectodermal germ layers (Keller, 2002; Shook and Keller, 2008). Cells in each germ layer undergo a characteristic series of molecular and morphological changes following their initial specification. In the case of zebrafish mesoderm, for example, cells first migrate collectively as an epithelioid sheet towards a site of involution, where they migrate underneath the outer cell layer (the epiblast) to populate a deeper layer (the hypoblast). Cells in the hypoblast exhibit a mesenchymal morphology, extending actin-based protrusions (lamellipodia and filopodia) as well as occasional blebs, which form when pressure in the cell inflates a blister of membrane at sites where the membrane and cortical actin are uncoupled (reviewed in Charras and Paluch, 2008; Fackler and Grosse, 2008). Our work, and that of others, demonstrated that regulated blebbing is a crucial aspect of embryonic cell migration, most notably in the case of germ cells, which migrate exclusively by blebbing (Blaser et al., 2006; Kardash et al., 2010; Weiser et al., 2007, 2009). These various protrusive activities act coordinately to allow zebrafish mesendodermal cells to converge toward the dorsal side of the embryo during gastrulation.

An extensive body of literature describes the pathways that initially specify mesodermal identity in the epiblast (reviewed in Kimelman, 2006; Schier and Talbot, 2005), and the factors that regulate cell behavior in the hypoblast are increasingly well understood (Arboleda-Estudillo et al., 2010; Kai et al., 2008; Keller, 2005; Myers et al., 2002; Ulrich et al., 2005; von der Hardt et al., 2007). Elegant studies have demonstrated the means by which the directed migration of many mesodermal cells shapes the gastrulating embryo (Carmany-Rampey and Schier, 2001; Concha and Adams, 1998; Warga and Kimmel, 1990; Yin et al., 2009). Such studies have focused on the movement of cells within the hypoblast or epiblast. However, the transition between epiblast and hypoblast has remained mysterious, both on the molecular level and in understanding the process of involution. Because this transition represents such a dramatic and crucial change in cell morphology we wanted to dissect the regulatory mechanisms at work. Two barriers to dissecting the transition state are the brief time each cell spends moving between the epiblast and hypoblast, and the difficulty of making *in vivo* observations. Zebrafish embryos have several advantages for studying this dynamic process including optical clarity, rapid and external development, and thinness of the tissue. Using time-lapse DIC microscopy we made high resolution observations of cells transitioning from epiblast to

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hypoblast and observed a dramatic change in behavior in which cells undergoing involution pass through a transition state in which they bleb extensively.

We reasoned that the transition state between epiblast and hypoblast would be more tractable for study if we could find a way to hold cells in this state. The *spadetail* (*spt*) mutation was a good candidate to introduce a temporary blockade in the morphogenetic transition from epiblast to hypoblast (Kimmel et al., 1989). Mesodermal cells that contribute to the somites express specific T-box transcription factors in series as they progress from specification through their differentiation program (Amacher et al., 2002; Goering et al., 2003; Griffin and Kimelman, 2002). These mesodermal progenitors express the *brachyury* factor *no tail* (*ntl*) from the onset of their fate specification, then subsequently activate *spt* and *tbx6* as they begin the differentiation process, entering a region that we have called the maturation zone (Griffin and Kimelman, 2002). As cells leave the maturation zone and enter the presomitic mesoderm, they turn off *ntl*, and activate *tbx24*. We previously showed by examining gene expression that *spt* mutant cells enter the maturation zone state but remain trapped there, retaining expression of progenitor genes but failing to activate downstream genes such as *tbx24*. These results, however, did not explain why *spt* mutant cells fail to migrate properly, although defective cell adhesion has been a commonly held view (Warga and Nusslein-volhard, 1998; Yamamoto et al., 1998). Here we show that mesodermal cells in *spt* mutant embryos enter the blebby transition state as normal but are unable to complete the morphological transition of normal cells in the hypoblast. Whereas normal cells reduce blebbing as they leave the transition state and migrate away, *spt* cells continue the rapid blebbing and fail to move away from the transition zone. Crucially we show that this phenotype represents a temporary, reversible interruption in the maturation program rather than a permanent change in cell fate. Thus, mesodermal cells pass through a morphological as well as genetic transition stage between epiblast and hypoblast, with Spadetail required to complete the transition.

We utilized mesodermal cells lacking Spadetail to probe aspects of the epiblast-to-hypoblast transition state. Using a single-cell adhesion assay we demonstrate that non-axial mesoderm lacking Spadetail is significantly more adhesive than wild-type mesoderm, ruling out the possibility that cells fails to leave the maturation zone because of an inability to adhere to their neighbors. In support of this, we show that surface levels of the classical cadherins, the major adhesion factors in the early embryo, are not affected by a loss of Spadetail. Interestingly, we also observed identical levels of phosphorylated (activated) myosin in cells with and without Spadetail. This result is surprising since previous work showed that zebrafish mesodermal cells adopt a highly blebby state in response to increases in myosin phosphorylation (Weiser et al., 2009). We conclude that wild-type cells activate the highly blebby state as they enter the maturation zone, and that Spadetail inhibits this activity in a myosin-independent manner. Our results demonstrate that *spt* mutant embryos are a valuable system for probing the dynamics of the epiblast to hypoblast transition since they reversibly hold cells in the transition state.

## Materials and methods

### *Zebrafish lines, heat shocks and morpholinos*

The *Tg(HS:spt, eGFP)* line was created by placing the coding sequence of zebrafish *spt* (Spadetail-myc fusion, a kind gift from David Grunwald) on one side of a multimerized heat shock promoter (Bajoghli et al., 2004) with a Green Fluorescent Protein (eGFP) gene on the opposite side (Fig. 4A). This was flanked by two Tol2 elements and used to generate stable transgenics in the WIK/AB background according to Kawakami (2004). Heat shocks were at 40.5 °C for thirty minutes, in pre-warmed embryo rearing media (EM). *spt* morpholinos were the same as in Lewis and Eisen (2004). A mixture of 1.5 ng of MO1 and 0.75 ng MO2 was injected into each embryo.

### *Induced ventral/lateral mesoderm*

To induce ventral and lateral mesoderm, embryos were injected with 5 pg synthetic *cyc* mRNA at the 1-cell stage. At dome stage, embryos were treated with the GSK3 inhibitor BIO (CalBiochem) at 3 μM.

### *Surface biotinylation, immunoprecipitation and Western blotting*

Surface-exposed proteins were biotinylated immediately after the onset of gastrulation in induced ventral/lateral mesoderm embryos. Dechorionated embryos were gently de-yolked by pipetting, leaving the blastoderms intact. They were incubated in 1 mg/mL EZ-Link Sulfo-NHS-Biotin (Pierce) in 0.1X MMR without EDTA (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8). Biotinylation was carried out at 4 °C for 10 min, then quenched with two washes of 5 mM glycine in embryo rearing media. Cells were lysed in buffer containing 1% Triton X-100 and HALT protease/phosphatase inhibitor cocktail (Pierce; 1.5 μL/embryo lysed). This method was adapted from Chen et al. (1997).

Total embryo lysate was separated by polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and probed with anti-actin (MAB1501R, Chemicon) or anti-pMLC2 (Cell Signaling, 3671) antibodies. Biotinylated proteins were immunoprecipitated with E-cadherin or N-cadherin antibodies (raised against synthetic peptides: DKDLPPFAGPFKVEPQGDTSKN for Cdh1 (as described in Babb and Marrs, 2004), and CNAGPYAFELPNRPSDIRRNWTL for Cdh2), and detected on Western blots with an anti-biotin antibody (Thermo Pierce, MA1-37172).

### *In situ hybridization and immunohistochemistry*

Single probe whole-mount in situ hybridization was performed as described in Griffin, et al. (1995).

### *Cell transplantation*

Donor embryos obtained from an outcross of *Tg(HS:spt, eGFP)* hemizygotes to WIK/AB wild-types were injected with *spt* morpholinos and 1% rhodamine dextran at the 1-cell stage. Transplants were from donors at sphere stage into hosts at shield stage, targeted to the ventral mesoderm. Hosts were heat shocked at the 1-somite or 8-somite stage and photographed at 36 hpf.

### *Analysis of cell movement*

An Axiovert 200 M microscope (Zeiss) and 40× objective with DIC optics were used to make *in vivo* observations of lateral mesodermal cells (mesoderm 90° from the shield) at 55–60% epiboly. Observations were made of at least four separate embryos for all conditions. Images were acquired every three seconds to observe cell protrusions, or every 15 s for cell movement tracking. Tracking was performed using ImageJ (Rasband, 1997–2009).

### *Dual Micropipette Aspiration Assay*

The Dual Micropipette Aspiration Assay was performed as previously described (Daoudi et al., 2004). Cells were prepared as by Krieg et al. (2008) from embryos induced to ventral/lateral mesoderm, with and without prior injection of *spt* MOs. The cells were manipulated at 25 °C with two micropipettes, each one held by a micromanipulator connected to a microfluidic pump (Fluigent). Micropipettes with an internal diameter of 6–7 μm were pulled