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Differential regulation of epiboly initiation and progression by zebrafish Eomesodermin A

Susan Du [a], Bruce W. Draper [b], Marina Mione [c], Cecilia B. Moens [d], Ashley Bruce [a,*]

[a] Department of Cell and Systems Biology, University of Toronto, 25 Harbord Street, Toronto, ON Canada M5S 3G5
[b] Molecular and Cellular Biology, University of California, Davis, One Shields Avenue, Davis, CA, 95616, USA
[c] IFOM, Istituto FIRC di Oncologia Molecolare, Via Adamello 16, Milan, I-20139, Italy
[d] Howard Hughes Medical Institute, Division of Basic Science, Fred Hutchinson Cancer Research Center, P.O. Box 19024, 1100 Fairview Avenue, North Seattle, WA, 98109–1024, USA

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ABSTRACT

The T-box transcription factor Eomesodermin (Eomes) has been implicated in patterning and morphogenesis in frog, fish and mouse. In zebrafish, one of the two Eomes homologs, Eomesa, has been implicated in dorsal-ventral patterning, epiboly and endoderm specification in experiments employing over-expression, dominant-negative constructs and antisense morpholino oligonucleotides. Here we report for the first time the identification and characterization of an Eomesa mutant generated by TILLING. We find that Eomesa has a strictly maternal role in the initiation of epiboly, which involves doming of the yolk cell up into the overlying blastoderm. By contrast, epiboly progression is normal, demonstrating for the first time that epiboly initiation is genetically separable from progression. The yolk cell microtubules, which are required for epiboly, are defective in maternal-zygotic *eomesa* mutant embryos. In addition, the deep cells of the blastoderm are more tightly packed and exhibit more bleb-like protrusions than cells in control embryos. We postulate that the doming delay may be the consequence both of overly stabilized yolk cell microtubules and defects in the adhesive properties or motility of deep cells. We also show that Eomesa is required for normal expression of the endoderm markers *sox32*, *bon* and *og9x*; however it is not essential for endoderm formation.

Introduction

The T-box transcription factor Eomesodermin (Eomes) has been implicated in patterning and morphogenesis in frog, fish and mouse. In *Xenopus*, where Eomes was first identified, ectopic expression in animal caps leads to a concentration-dependent induction of mesodermal gene expression, with higher doses inducing expression of more dorsal mesodermal markers (Ryan et al., 1996). In zebrafish, there are two *eomes* genes (*a* and *b*) (Takizawa et al., 2007), with *eomesa* being the more intensively studied. Over-expression of Eomesa leads to ectopic expression of dorsal organizer genes and secondary axes are induced when Eomesa is expressed ventrally (Bruce et al., 2003). In addition, Eomesa has been shown to play a role in induction of the endoderm gene *sox32* (Bjornson et al., 2005). More recent work has also shown that Eomesa acts in combination with the transcription factor FoxH1 to specify mesendoderm (Slagle et al., 2011). In the mouse, Eomes mutants completely lack definitive endoderm, while mesodermal patterning is relatively unaffected (Arnold et al., 2008; Russ et al., 2000).

Eomes is also important for normal gastrulation movements. Expression of dominant-negative Eomes constructs in *Xenopus* embryos leads to gastrulation arrest (Ryan et al., 1996). In zebrafish embryos, a similar Eomesa construct produces abnormal epiboly, which is the first coordinated cell movement during development (Bruce et al., 2005; Lepage and Bruce, 2010; Warga and Kimmel, 1990). Knockout of Eomes in the mouse epiblast results in gastrulation defects due to blocked migration of prospective mesoderm away from the primitive streak (Arnold et al., 2008; Russ et al., 2000). This migratory defect appears to be due to the failure to down-regulate expression of the adhesion molecule E-Cadherin (Arnold et al., 2008).

In zebrafish, unlike *Xenopus*, Eomesa transcript and protein are maternally expressed (Bruce et al., 2003; Ryan et al., 1996). Early development in zebrafish, as in many animals, relies upon maternal stores of mRNA and protein that orchestrate development up to the midblastula transition when zygotic transcription begins (Abrams and Mullins, 2009). Previous work on Eomesa in zebrafish relied upon over-expression, morpholino oligonucleotides, and dominant-negative constructs (Bjornson et al., 2005; Bruce et al., 2005; Bruce et al., 2003; Slagle et al., 2011). Over-expression and dominant-negative constructs can have non-specific effects and morpholinos have no impact on maternal stores of protein. The caveats of these tools are reflected by the fact that there is confusion in the literature

* Corresponding author. Fax: +1 416 978 8532.
E-mail address: ashley.bruce@utoronto.ca (A. Bruce).

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surrounding some aspects of Eomesa expression and function (Bjornson et al., 2005; Bruce et al., 2005; Bruce et al., 2003). Thus our understanding of the role of Eomesa during zebrafish development is far from complete. Here we report the phenotype of a loss of function *eomesa* allele generated by TILLING (Moens et al., 2008). Our characterization of embryos lacking either or both maternal and zygotic Eomesa, as well as the generation of an effective Eomesa antibody, has allowed us to gain new insights into Eomesa function. We find that Eomesa has a strictly maternal role in the initiation of epiboly, while epiboly progression is normal: demonstrating for the first time that epiboly initiation is genetically separable from progression. We also show that Eomesa plays a role in, but is not essential for, endoderm formation.

Material and Methods

Zebrafish

We generated a nonsense allele of *eomesa*, designated *fh105*, by TILLING (Moens et al., 2008). AB and *eomesa*^{fh105} zebrafish were maintained and staged as described (Kimmel et al., 1995). Wild type embryos were obtained from natural matings. Homozygous *eomes*^{fh105} mutant embryos were obtained by in vitro fertilization as described (Westerfield, 1993). Heterozygous *eomes*^{fh105} mutant embryos were obtained by in vitro fertilization using homozygous *eomesa*^{fh105} sperm and wild type eggs. Maternal *eomesa* mutant embryos were obtained by in vitro fertilization using homozygous *eomesa*^{fh105} eggs and wild type sperm. Z*eomesa* mutant embryos were obtained by natural matings of heterozygous individuals and were confirmed by PCR genotyping (see below). Animals were treated in accordance with the policies of the University of Toronto Animal Care Committee.

Generation of Eomesa Antibody

The cDNA sequence encoding amino acids 1–661 was cloned into pETM-14 vector (EMBL Protein Expression and Purification Facility, http://www.helmholtz-muenchen.de/en/pepf/materials/vector-database/bacterial-expression-vectors/index.html) to express a His-tag fusion protein in *E. coli* (BL21 strain DE3). The His-tag protein was isolated according to the protocol provided by the Bjorkman Group, Howard Hughes Medical Institute, California Institute of Technology (http://www.its.caltech.edu/~bjorker/protocols.html), for the extraction of inclusion bodies (Fig. S1) and then stored in 8 M urea.

Two rabbits were immunized with the His-tag fusion protein, using a 3-months standard protocol by Eurogentec S.A., Seraing – Belgium.

In Situ Hybridization

In situ hybridizations were performed as described (Jowett and Lettice, 1994). Antisense riboprobes for *bmp2b* (Martinez-Barbera et al., 1997); *bon* and *sox17* (Alexander and Stainier, 1999); *cdh1* (Kane et al., 2005); *fgf8a* (Reifers et al., 1998); *flh* (Talbot et al., 1995); *gata5* (Rodaway et al., 1999); *gsc* (Stachel et al., 1993); *lfty1* (Bisgrove et al., 1999), *mxtx2* (Hirata et al., 2000); *ndr1* (Erter et al., 1998; Feldman et al., 1998), *ndr2* (Rebagliati et al., 1998; Sampath et al., 1998); *ntla* (Schulte-Merker et al., 1994); *og9x* (Poulain and Lepage, 2002) and *sox32* (Alexander et al., 1999) were generated as described.

Whole-mount Immunohistochemistry

Antibody and phalloidin staining were performed as described (Bruce et al., 2001; Topczewski and Solnica-Krezel, 1999). Antibodies were used as follows: anti-Eomesa (1: 500), anti–α–Tubulin (1:500; Sigma Aldrich), anti-E-Cadherin (1:2500) (Babb and Marrs, 2004) and goat-anti-mouse Alexa 488 secondary (1:1000, Invitrogen). Embryos were mounted in ProLong Gold Antifade reagent (Invitrogen) in agarose wells on a glass bottom dish (MatTek Corporation) for analysis on a Zeiss LSM510 confocal microscope or analyzed on a Leica MZ16F stereomicroscope.

Western Blots

Embryos at sphere stage were dechorionated and blastoderm caps were manually dissected off the yolk cell. Thirty blastoderm caps were homogenized in RIPA lysis buffer (Sigma Aldrich) supplemented with EDTA (1 μM) inhibition, and protease inhibitor cocktail (Roche). Two to fifteen embryo equivalents were loaded into each lane. Protein extracts were run on a 10% SDS denaturing protein gel. Gels were transferred onto nitrocellulose filter papers using the Bio-Rad semi-dry transfer apparatus and blocked in 5% milk in PBT (0.1% Tween −20 in PBS). Blots were incubated in primary antibodies diluted in 5% milk overnight at 4 °C, washed in PBT (5 x 10 minutes) and incubated in secondary antibodies for 1 hr at room temperature. The following primary antibodies were used: rabbit anti-E-Cadherin (1:2500) (Babb and Marrs, 2004), anti-Eomesa antibody (1: 750), anti-α–Tubulin (1: 2000; Sigma Aldrich) and anti-β–Catenin (1:250; Sigma Aldrich).

PCR Genotyping and RT-PCR

Genotyping for the presence of the *eomes*^{fh105} allele was performed as described (Zebrafish International Resource Center, www.zebrafish.org). For RT-PCR, RNA was prepared using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA synthesis was performed using the AffinityScript cDNA synthesis kit (Stratagene). PCR was performed for 28 cycles using the cycling parameters and primer sequences from the genotyping assay.

Microinjections and Constructs

Microinjections were performed and RNA for *eomesa*-VP16, *eomesa*-eng, *eomesa* and *gfp* were made as described previously (Bruce et al., 2003). 50 pg of the RNAs were injected per embryo. Alexa 488 conjugated Histone H1 (Invitrogen) was injected as described (Carvalho et al., 2009). The splice blocking morpholino used to test the specificity of the Eomesa antibody targets the exon/intron 2 boundary and has the following sequence: GTAATGCTTCATTTCTTACCTGCC (GeneTools, LLC). RT-PCR using an exon 2 forward primer (GACGCGCGTAAAAGTTCTC) and an exon 3 reverse primer (CTTGATGTTGTTGTCCGCTTTC) was used to confirm the specificity of the splice blocking morpholino. In splice blocking morpholino injected embryos a band of 760 base pairs (bp) instead of the expected 600 bp was PCR amplified from cDNA. Sequencing of the product revealed retention of intron 2, which contains several stop codons. This corresponded to the truncated protein fragment of approximately 25 kDa shown in Fig. 1C.

Imaging

Confocal data were acquired using a Zeiss LSM 510 microscope. Fixed embryos were mounted in wells made in 2% low melt agarose (Sigma Aldrich) in glass bottom culture dishes (MatTek, Ashland, MA).

Results

Characterization of the eomesa^{fh105} *mutant allele*

The *eomesa*^{fh105} allele was generated by TILLING and it contains a point mutation that changes a tyrosine at position 100 to a stop codon