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Widespread but tissue-specific patterns of interferon-induced transmembrane protein 3 (IFITM3, FRAGILIS, MIL-1) in the mouse gastrula

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ABSTRACT

Interferon-induced transmembrane protein 3 (IFITM3; FRAGILIS; MIL-1) is part of a larger family of important small interferon-induced transmembrane genes and proteins involved in early development, cell adhesion, and cell proliferation, and which also play a major role in response to bacterial and viral infections and, more recently, in pronounced malignancies. IFITM3, together with tissue-nonspecific alkaline phosphatase (TNAP), PRDM1, and STELLA, has been claimed to be a hallmark of segregated primordial germ cells (PGCs) (Saitou et al., 2002). However, whether IFITM3, like STELLA, is part of a broader stem/progenitor pool that builds the posterior region of the mouse conceptus (Mikedis and Downs, 2012) is obscure. To discover the whereabouts of IFITM3 during mouse gastrulation (~E6.5-9.0), systematic immunohistochemical analysis was carried out at closely spaced 2-4-h intervals. Results revealed diverse, yet consistent, profiles of IFITM3 localization throughout the gastrula. Within the putative PGC trajectory and surrounding posterior tissues, IFITM3 localized as a large cytoplasmic spot with or without staining in the plasma membrane. IFITM3, like STELLA, was also found in the ventral ectodermal ridge (VER), a posterior progenitor pool that builds the tailbud. The large cytoplasmic spot with plasma membrane staining was exclusive to the posterior region; the visceral yolk sac, non-posterior tissues, and epithelial tissues exhibited spots of IFITM3 without cell surface staining. Colocalization of the intracellular IFITM3 spot with the endoplasmic reticulum, Golgi apparatus, or endolysosomes was not observed. That relatively high levels of IFITM3 were found throughout the posterior primitive streak and its derivatives is consistent with evidence that IFITM3, like STELLA, is part of a larger stem/progenitor cell pool at the posterior end of the primitive streak that forms the base of the allantois and builds the fetal-umbilical connection, thus further obfuscating practical phenotypic distinctions between so-called PGCs and surrounding soma.

A variety of cell lineages is formed in the posterior region of the mouse conceptus, yet only the primordial germ cells (PGCs) have undergone long-standing scrutiny there (reviewed in Hayashi et al., 2007; McLaren, 2000). Over the past decade, efforts have intensified to identify genes that regulate the segregation of PGCs from surrounding soma. The current view is that PGC progenitors arise in proximal epiblast, and express PR domain zinc finger protein 1 (*Prdm1*; also *Blimp1*) (Ohinata et al., 2005) as well as interferon-induced transmembrane protein 3 (*Ifitm3*; also *Fragilis*, *Mil-1*) (Lange et al., 2003; Saitou et al., 2002; Tanaka and Matsui, 2002), making them distinct from surrounding soma. *Prdm1/Ifitm3*-expressing epiblast cells then move into the primitive streak, from which they translocate with streak mesoderm into the base of the allantois and begin to co-express tissue non-specific alkaline phosphatase (*Tnap*; Lawson and Hage, 1994) and *Stella* (*Dppa3*, *PGC7*). Thus, a small cluster of *Prdm1/Ifitm3/Tnap/Stella*-positive PGCs (Ohinata et al., 2005; Saitou et al., 2002) is located within the midline of the base of the allantois. However, none of these PGC hallmark gene products, if absent, has demonstrated effects on fertility (Bortvin et al., 2004; Lange et al., 2008; Macgregor et al., 1995; Payer et al., 2003; Vincent et al., 2005).

Abbreviations: ACD, allantoic core domain; AP, alkaline phosphatase; CALR, careticulin; EB, early (allantoic) bud; ES, early streak; EHF, early headfold; ER, endoplasmic reticulum; Flk1, fetal liver kinase 1; GOLGA1, Golgi autoantigen; IF, immunofluorescence; IHC, immunohistochemistry; IFITM2, interferon-induced transmembrane protein 2; IFITM3, interferon-induced transmembrane protein 3; IPS, intraembryonic posterior primitive streak; LAMP1, lysosomal-associated membrane protein 1; LB, late (allantoic) bud; LHF, late headfold; LS, late streak; MS, mid-streak; OB, no (allantoic) bud; PCC, Pearson correlation coefficient; PGCs, primordial germ cells; PRDM1, PR domain zinc finger protein 1; PVE, posterior visceral endoderm; Runx-1, runt-related transcription factor 1; s, somite pairs; TNAP, tissue-nonspecific alkaline phosphatase; VCM, ventral cuboidal mesothelium; VER, ventral ectodermal ridge; XPS, extraembryonic posterior primitive streak.

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Alkaline phosphatase (AP) activity (Benham et al., 1983; Bernstine et al., 1973) and STELLA (Bowles et al., 2003; Carter et al., 2008) are individually associated with pluripotency, while PRDM1 (Horsley et al., 2006; Mould et al., 2012; Nishikawa et al., 2010; Turner et al., 1994) and AP activity (Jaiswal et al., 1997; Lewinson et al., 1982) have each been associated with progenitor cell populations. All of these gene products are found within a self-propagating stable stem/progenitor cell population in the base of the allantois, called the allantoic core domain (ACD; Downs et al., 2009). In eutherian mammals, the allantois is the source of the umbilical cord which, through its connection to the fetus and yolk sac, ensures a vascular continuum throughout the conceptus.

Intriguingly, DiI fate mapping the ACD has revealed that it is both a self-propagating midline population, similar to the node at the anterior end of the streak (Beddington, 1994), and a source of the distal region of the allantois (Downs et al., 2009). In the absence of the ACD, the allantois fails to elongate and vascularize (Downs et al., 2009; Inman and Downs, 2006).

Following the discovery of the ACD, we re-investigated the whereabouts of STELLA. Careful spatiotemporal histological analysis at closely-spaced intervals and fate mapping demonstrated that, from the precursor ACD, STELLA spreads to the intraembryonic primitive streak (IPS); both the ACD and IPS make overlapping and distinct STELLA-positive contributions to the fetal-umbilical connection (Mikedis and Downs, 2012), including the allantois, ventral hindgut, posterior mesoderm, and ventral ectodermal ridge (VER). Thus, STELLA is not restricted to the putative germ line but is part of a broader pool of posterior primitive streak stem/progenitor cells that build the posterior region of the eutherian mammal, ensuring proper development of the fetal-umbilical connection. These results beg the question of what is and is not a primordial germ cell.

Here we now report the whereabouts of IFITM3, from the earliest stages of primitive streak formation through elaboration of the hindgut (~E6.5-9.0). All other tissues within the mouse gastrula were analyzed, and localization of IFITM3 to them is also reported. We have found that IFITM3 localization as a cytoplasmic spot with cell surface staining, previously associated with the putative PGCs (Saitou et al., 2002), is unique to the posterior region but is not restricted to the putative PGC trajectory. Additional IFITM3, primarily as a cytoplasmic spot without cell surface staining, was localized throughout the conceptus.

1. Results

1.1. Specificity of IFITM3 antibody

The commercially available antibody used to identify IFITM3 was raised against a synthetic peptide derived from within the first 50 residues of the mouse IFITM3 N-terminal region. To preliminarily confirm antibody specificity, we used the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify mouse proteins that share sequence similarity to this synthetic peptide sequence (proprietary information; contact technical support at Abcam for further details). The only protein with sequence similarity to the IFITM3 synthetic peptide was family member IFITM2 ($E < 0.01$). For all other protein matches, $E > 0.01$; thus, no other proteins exhibited significant sequence similarity to the synthetic peptide.

As IFITM2 and IFITM3 have predicted molecular weights (MW) of 15.7 and 15.0 kDa (UniProtKB), respectively, they cannot be reliably distinguished as distinct bands on a Western blot. Nor can immunohistochemical methods be used to determine whether the antibody exclusively identifies IFITM3, as *Ifitm2* and *Ifitm3* are expressed in similar tissues in the mouse conceptus (Lange et al., 2003). Therefore, to test whether anti-IFITM3 detects IFITM2, our overall plan was to carry out Western blotting on mouse IFITM2-transfected 293T protein extract, using IFITM2-negative 293T cells as a negative control and mouse embryonic fibroblast NIH 3T3 cells as a positive control for the presence of IFITM3 (Bailey et al., 2012; Brass et al., 2009).

We first verified the presence of IFITM2 in IFITM2-transfected 293T cell lysate using an antibody that detects both IFITM2 and IFITM3 (anti-IFITM2/3); IFITM3-positive mouse embryonic fibroblast NIH 3T3 cell lysate was used as a positive control. Anti-IFITM2/3 detected a protein band at ~15.0 in IFITM2:293T lysate (Fig. 1A$_1$, lane 2, below asterisk) and NIH 3T3 lysate (Fig. 1A$_1$, lane 4), but did not identify any bands in the negative control, 293T lysate (Fig. 1A$_1$, lane 3). By contrast, anti-IFITM3 did not detect IFITM2 in the IFITM2:293T lysate (Fig. 1A$_1$, lane 5, below asterisk) or negative control, 293T lysate (Fig. 1A$_1$, lane 6), but did detect it in the positive control, NIH 3T3 lysate (Fig. 1A$_1$, lane 7). Although anti-IFITM3 detected higher MW protein bands in IFITM2:293T lysate at ~31.0 and ~66.0 kDa (Fig. 1 A$_1$, lane 5), these bands were also present in IFITM2-negative 293T lysate (Fig. 1A$_1$, lane 6). Therefore, despite the sequence similarity between IFITM2 and the immunogen used to produce anti-IFITM3, the IFITM3 antibody does not identify IFITM2.

We then confirmed anti-IFITM3 specificity in mouse conceptuses by Western blot analysis of total protein at combined EHF-6-s stages (~E7.75-8.5), when our preliminary experiments revealed that IFITM3 was present. Four reactions were carried out: (i) fresh primary antibody (Fig. 1A$_2$, lanes 9, 10 and Fig. 1A$_3$, lanes 14, 15 representing two biological replicates for both the embryonic cell lysate and the IFITM3-positive NIH 3T3 cell lysate); (ii) elimination of the primary anti-IFITM3 (Fig.1A$_2$, lanes 11, 12); (iii) pre-binding primary anti-IFITM3 alone with its cognate control peptide sequence for 1 h at room temperature (Fig. 1A$_3$, lanes 16, 17); and (iv) primary anti-IFITM3 alone, incubated for 1 h at room temperature (Fig. 1A$_3$, lanes 18, 19). In the embryonic and IFITM3-positive NIH 3T3 lysates, fresh anti-IFITM3 identified one band slightly above the 14.4 kDa MW mark that is consistent with IFITM3's predicted MW of 15.0 kDa (Fig. 1A$_2$, lanes 9, 10; Fig. 1A$_3$, lanes 14, 15). In addition, the IFITM3-positive NIH 3T3 cell lysates had an additional band just below the 14.4 kDa MW mark (Fig. 1A$_1$, lane 7; Fig. 1A$_2$, lane 10; Fig. 1A$_3$, lane 15), which may represent a degradation product of IFITM3. The Western blot analysis also revealed one (total embryonic lysate) or three (IFITM3-positive NIH 3T3 lysate) bands above the predicted 15.0 kDa that appeared to be approximate multiples of 15 kDa (Fig. 1A$_2$, lanes 9, 10; Fig. 1A$_3$, lanes 14, 15); these are consistent with polyubiquitination of IFITM3 at up to four lysine residues (Yount et al., 2012). As the MW of ubiquitin is 8.5 kDa (reviewed in Hasselgren and Fischer, 1997), the approximately 15.0 kDa intervals between IFITM3 bands suggests that two (17.0 kDa) or four (34.0 kDa) molecules of ubiquitin are bound to embryonic IFITM3. All of these bands were eliminated when the blots were incubated in the absence of IFITM3 antibody (Fig. 1A$_2$, lanes 11, 12) and in the presence of pre-bound anti-IFITM3 (Fig. 1A$_3$, lanes 16, 17), with the exception of the robust band slightly above the 14.4 kDa standard band in the lane containing IFITM3-positive NIH 3T3 lysate treated with pre-bound primary antibody, which was nevertheless greatly diminished (Fig. 1A$_3$, lane 17). Incubated IFITM3 not pre-bound to the control peptide identified the same bands as the fresh primary antibody without any discernable decrease in signal (Fig. 1A$_3$, lanes 18, 19). Together, these data provide convincing evidence for specificity of anti-IFITM3.

IFITM3 antibody specificity was further verified in histological sections at 4-s (~E8.25), one of the stages during which IFITM3-positive cells were abundant in the allantois (Fig. 1B; see the following sections for details). Four reactions were carried out: (i) fresh primary anti-IFITM3 (Fig. 1B); (ii) elimination of the primary