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Stam2 expression pattern during embryo development

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ABSTRACT

STAM2 is a tyrosine-phosphorylated protein suggested to be involved in cargo selection during endocytic pathway, regulation of exocytosis and intracellular signaling. Gene trap method was used to create via insertional mutagenesis a mutant mouse line with integration of promoterless *βgeo* (*lacZ–neomycin phosphotransferase* fusion) gene in the second intron of *Stam2* gene, enabling analysis of its *in vivo* expression and function. The inserted *β-galactosidase* (*lacZ*) reporter gene was used to reveal *Stam2* expression during development. *Stam2 in situ* RNA hybridization and immunostaining confirmed the observed β-galactosidase activity reflecting high *Stam2* expression. The homozygous mutant mice showed no overt phenotypic alterations. *Stam2* expression was detected after E9.5 in the gut, notochord, neural tube and heart. In the nervous system it was located in the floor, roof and basal plates of the developing neural tube, and in the developing cortex, hippocampus and olfactory bulbs. Toward the end of gestation, *Stam2* expression appeared in the testis and ovary, lungs, nasal cavity epithelium, kidneys, urogenital sinus, intestine, pancreas, pituitary and adrenal glands, muscles, brown adipose tissue, skin and epithelium of the tongue and oral cavity.

Proper cellular response in dynamic environment during development requires constant monitoring of the incoming signals, their subsequent processing and appropriate reaction. An important mechanism involved in this task is endocytosis, which through membrane transport regulates the cell sensitivity, modulates the duration of the signal, and assists in its intracellular processing. Molecules on the cell surface together with those from outside could be internalized into early endosomes, and either recycled back to the cell surface or degraded in lysosomes via multivesicular bodies (MVB). The sorting of the right cargo toward these two different pathways is not completely understood, but monoubiquitination and deubiquitination processes could play an important role (Raiborg et al., 2003; Kirkin and Dikic, 2007; Piper and Luzio, 2007).

STAM1 and STAM2 (signal transduction adaptor molecules 1 and 2) are considered to be involved in the endosomal sorting of the ubiquitinated cargo proteins for trafficking toward the lysosome (Bache et al., 2003; Mizuno et al., 2003). Together with HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) they form ESCRT-0 complex (Conibear, 2002; Hurley and Emr, 2006; Williams and Urbé, 2007; Ren et al., 2009), the first of the four protein complexes referred as ESCRT-0, -I, -II, and -III (i.e. endosomal sorting complexes required for transport). They recognize ubiquitinated membrane proteins and direct them toward the MVB pathway (Katzman et al., 2002; Luzio et al., 2009; Raiborg and Stenmark, 2009).

Beside its suggested role in the membrane trafficking during endocytosis, STAM2 is involved in the regulation of exocytosis, as dominant-negative mutants of *Stam2* significantly inhibited IgE receptor (FceRI)-triggered secretory response in RBL-2H3 mast cells (Murai and Kitamura, 2000). In addition, STAM2 seems to connect these processes with intracellular signaling. STAM2 is tyrosine-phosphorylated upon stimulation with a variety of cytokines and growth factors (Endo et al., 2000). Through its ITAM (immunoreceptor tyrosine-based activation motif) domain, STAM2 is associated with JAK2 and JAK3 tyrosine kinases, and it is involved in the regulation of intracellular signal transduction for DNA synthesis and c-myc induction mediated by IL-2 and GM-CSF (Endo et al., 2000; Pandey et al., 2000; Hu et al., 2007).

Loss of function of STAM2 in mouse did not result with any obvious abnormality (Yamada et al., 2002), while loss of STAM1 caused growth retardation in the third week after birth and disappearance of hippocampal CA3 pyramidal neurons (Yamada et al., 2001). Double knockout mice for both *Stam1* and *Stam2* were lethal by embryonic day 11.5 (E11.5), with a defect in ventral folding

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morphogenesis (Yamada et al., 2002) implying that STAMs are indispensable for embryo development.

To explore the possible function of STAM2 during mouse embryo development and to relate it to the observed embryo lethality, we analyzed in detail its *in vivo* expression pattern by tagging it endogenously with *LacZ* reporter via gene trap mutagenesis method. No phenotypic alterations were detected as a consequence of the gene trap mutation. However, the introduced in frame *lacZ* gene allowed us to analyze the developmental expression of *Stam2* demonstrating high *Stam2* expression in the heart and nervous tissue, particularly in the differentiating layers of the telencephalic cortex.

1. Results

1.1. Production and characterization of $Stam2^{Gt1Gaj}$ mouse line

The $Stam2^{Gt1Gaj}$ mouse was produced using a large-scale gene trap approach (Gajović et al., 1998). Gene trap vector used was pKC199βgeo (Thomas et al., 2000) containing the splice acceptor sequence from mouse *Hoxc9* gene located upstream of the promoterless *βgeo* (fused *lacZ* and *neoR*), which generates both β-galactosidase reporter and neomycin resistance activities. The vector was introduced into mouse embryonic stem (ES) cells by electroporation. Chimeric animals were produced from modified ES cell clones, and genes of interest were chosen according to their restricted expression pattern in the developing nervous system at E11.5. The expression was visualized by histochemical detection of β-galactosidase, which was present in the tissue due to the introduced splice acceptor site located upstream of the *lacZ* gene and subsequent *lacZ* transcription driven by endogenous promoter of the modified gene.

As the integration of gene trap vector into mouse ES cell genome is random, the endogenous gene affected by the gene trap mutation had to be identified. The known sequence of the inserted gene trap vector enabled us to amplify and identify the targeted gene by 5′ and 3′RACE (rapid amplification of cDNA ends) methods (Frohman et al., 1988; Ćurlin et al., 2002). BLAST search has shown that the obtained cDNA sequence represents an already known gene, referred as *Stam2* (Genbank NM019667) or *Hbp* (Hrs binding protein; Genbank AB012611; Takata et al., 2000). According to the restricted expression pattern in the developing neural tube and heart, the mouse line with modified *Stam2* gene was investigated further. Heterozygous carriers of the mutation were crossed to C57Bl/6NCrl mice, currently for 17 generations, in order to obtain a congenic mouse line that differs from the inbred strain only in the modified locus.

Sequence analysis of the vector insertion site revealed that it was integrated in the second intron of *Stam2* gene (Fig. 1A). Southern blot analysis with a vector specific probe excluded the possibility of multiple gene trap vector insertions and confirmed that the used mouse line contained only specific *Stam2* mutation (Fig. 1B). As a consequence of the mutation, 86% of the C-terminal wild type STAM2 protein was expected to be missing from the mutant protein, leaving only 41 N-terminal amino acid residues (of the total of 523 amino acids; the product of the first two of total of 14 exons) fused to β-galactosidase and neomycin phosphotransferase protein encoded by the inserted *βgeo* gene.

The homozygous carriers of the gene trap mutation were produced by intercrossing heterozygous mice. They were identified by Southern blotting with *Stam2* specific probe or by PCR (Fig. 1A, C and D). Both heterozygous and homozygous $Stam2^{Gt1Gaj}$ mice carrying the gene trap mutation were born with the expected Mendelian ratio (χ^2 test $p > 0.1$), had no macroscopic abnormalities and were viable, fertile and had a normal life span.

1.2. β-Galactosidase activity in the developing mutant mouse embryos

To determine the expression pattern of *Stam2* gene, whole-mount histochemical X-gal staining to visualize β-galactosidase activity (i.e. to visualize in-frame-inserted *lacZ* expression pattern) was performed on the mutant and, as a control, on the wild type mouse embryos from E8.5 to E18.5 (Table 1, Fig. 2). Cryosections of E16.5 embryos were also prepared and stained by X-gal (Fig. 3B).

1.2.1. Stage E8.5

At stage E8.5, no β-galactosidase activity was found.

1.2.2. Stage E9.5

The earliest β-galactosidase activity was detected at E9.5, when it was present in the hindgut (Fig. 2A).

1.2.3. Stage E10.5

At E10.5, the X-gal staining was not visible in the gut; however the β-galactosidase activity was present in the heart, where it persisted afterwards throughout gestation (Figs. 2B and C, 3A and B).

1.2.4. Stage E11.5

At E11.5, in addition to the heart, β-galactosidase was detected in the notochord and neural tube (Fig. 2B and D). In the neural tube it was located in the floor plate and its ventral part corresponding to the basal plates (Fig. 2D).

1.2.5. Stage E12.5

The basic pattern of β-galactosidase activity observed at stage E11.5 was slightly modified at stage 12.5 and later. At 12.5 the β-galactosidase activity was present in the heart and neural tube, but disappeared in the notochord. In the neural tube, the expression was present in the basal plates, floor plate and additionally in the roof plate. As a background the newly formed choroid plexus showed diffused positive X-gal staining. This background staining persisted afterwards throughout the gestation.

1.2.6. Stage E13.5

Together with the expression in the heart and developing spinal cord, β-galactosidase activity was detected in the floor plate of the brain stem (medulla oblongata and pons). In the developing spinal cord the expression included not only the floor plate but as well the future raphe region. It was not any more present in the basal plates. Low β-galactosidase activity was detected in the testes. Background X-gal staining appeared in the stomach and intestinal mucosa and continued there until the end of gestation.

1.2.7. Stage E14.5

At E14.5 β-galactosidase activity was present in the developing spinal cord (the floor plate, raphe region, the roof plate and weakly in the gray matter). In the medulla oblongata and pons, the β-galactosidase was present in the floor plate, in the most ventral part of the raphe and in the gray matter (Figs. 2E, 3A). At this stage a β-galactosidase activity appeared in the telencephalon, i.e. in the intermediate zone of the neopallial cortex, olfactory bulbs, trigeminal and dorsal root ganglia, pituitary, lungs, nasal cavity epithelium, pancreas, urogenital sinus, skin and muscles in the head and neck, tongue, back and limbs. The activity in the testes was visible in the future seminiferous tubules.

1.2.8. Stage E15.5

The regions of β-galactosidase activity in E15.5 embryos corresponded to the regions of its activity detected in E14.5 embryos, but the staining was generally stronger. The staining in the heart was more intensive in the atrium than in ventriculum.