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Neurons and a Subset of Interstitial Cells of Cajal in the Enteric Nervous System Highly Express *Stam2* Gene

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ABSTRACT

Signal transducing adaptor molecule 2 (STAM2) is a phosphotyrosine protein, which is a member of the endosomal sorting complex required for transport (ESCRT-0) and is involved in the sorting process of the mono-ubiquitinated endosomal cargo for degradation in the lysosome. Analysis of gene trap mice carrying *lacZ* in frame with *Stam2* revealed beta-galactosidase activity in the enteric nervous system (both in the myenteric and submucosal plexus) throughout the digestive tract. STAM2 immunostaining confirmed that the observed beta-galactosidase activity coincided with high *Stam2* expression. To identify cell types with high *Stam2* expression, STAM2 immunostaining was colocalized with the neuronal markers microtubule-associated protein 2 and protein gene product 9.5 and with c-kit as a marker for interstitial cells of Cajal (ICCs). STAM2 and c-kit positive cells comprised a subset of ICCs in the enteric nervous system. Qualitative and quantitative analysis of the morphology of the enteric nervous system in the homozygous mice carrying gene trap insertion in the *Stam2* gene did not reveal phenotype changes; therefore, STAM2 function in the digestive tube remains elusive. Anat Rec, 295:113–120, 2012.

Key words: STAM2; ESCRT; enteric neuron; interstitial cell of Cajal; expression

Many cell surface proteins, including growth factor receptors, are internalized from the cell surface into membrane compartments called early endosomes. The decline in pH in early endosomes causes dissociation of the receptor-ligand complex. Subsequently, the majority of receptors are further directed to late endosomes, often referred to as multivesicular bodies, and delivered to lysosomes for degradation, while others are recycled to the plasma membrane (Katzman et al., 2002; Raiborg et al., 2003). The sorting process within the endosomal system is complex, and the signal for degradation is provided by ubiquitin (Haglund et al., 2003; Huang et al., 2006). Receptors are committed to the lysosomal pathway through four separate protein complexes, called endosomal sorting complex required for transport

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(ESCRT)-0, -I, -II, and -III. The ubiquitinated endosomal cargo is first recognized and bound by ESCRT-0 (Conibear, 2002; Hurley and Emr, 2006; Williams and Urbé, 2007), which consists of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and two signal transducing adaptor molecule (STAM) isoforms, signal transducing adaptor molecule 1 (STAM1) and signal transducing adaptor molecule 2 (STAM2) (Asao et al., 1997; Endo et al., 2000). STAMs have previously been identified as proteins that are highly phosphorylated on tyrosine residues in response to cytokine and growth factor stimulation and are therefore thought to be involved in intracellular signaling as well (Takeshita et al., 1997). They are considered to link membrane transport with intracellular signaling and have a characteristic functional domain organization: Vps-27/HRS/STAM (VHS), ubiquitin-interacting motif (UIM), Src homology (SH3), coiled coil (CC), STAM-specific motif (SSM), and immunoreceptor tyrosine-based activation motif (ITAM) domain (Endo et al., 2000; Kato et al., 2000; Lohi and Lehto, 2001; Mizuno et al., 2003, 2004). Double knockout of both *Stam1* and *Stam2* is embryonically lethal, while either *Stam1* or *Stam2* knockout mice show no abnormalities at birth. Nevertheless, *Stam1* knockout adult mice were shown to display loss of neurons in the CA3 region of the hippocampus (Yamada et al., 2002). In general, mutations in components of the ESCRT machinery have recently been linked to many diseases, in particular neurodegenerative diseases and cancer (Stuffers et al., 2009).

Although our initial reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Stam2* mRNA indicated that *Stam2* mRNA is ubiquitously expressed (Curlin et al., 2006), as such confirming the Northern blotting data of Takata et al. (2000), subsequent fine analysis obtained through gene trap mice carrying *lacZ* reporter in frame with *Stam2* specifically revealed strong *Stam2* expression in the enteric nervous system. This somewhat unexpected finding led us to perform this study. A variety of signaling pathways and molecules have proven necessary for the normal development and function of the enteric nervous system. Moreover, the unknown significance of the cell signaling and endosome sorting for the maintenance of a healthy gut was another important reason to study *Stam2* in the enteric nervous system.

We demonstrated high *Stam2* expression in the enteric nervous system, in neurons and in interstitial cells of Cajal (ICCs). Still, the gene trap mutation of *Stam2* did not cause phenotype changes of the enteric nervous system.

MATERIALS AND METHODS

Animals

Experiments were performed on 4- to 8-month-old male mice from the gene trap mouse line $Stam2^{Gt1Gaj}$ and the wild type inbred strain C57Bl/6NCrl.

The transgenic mouse line $Stam2^{Gt1Gaj}$ was generated by the gene trap method (Skarnes et al., 1992; Thomas et al., 2000). Embryonic stem cells were modified with a nonhomologous DNA vector pKC199βgeo containing a splice acceptor sequence from the mouse *Hoxc9* gene and fused promoterless *lacZ* and *neoR* genes. The obtained insertion was in the *Stam2* gene, in the intron between exons 2 and 3 (Curlin et al., 2006). The $Stam2^{Gt1Gaj}$ transgenic mice were kept as heterozygous on a C57Bl/6NCrl genetic background through 24 generations. Homozygous animals were obtained by intercrossing of heterozygous. Transgenic mice were genotyped by PCR of tail genomic DNA.

All experiments were approved by the institutional Ethical Committee and were in agreement with the Croatian Society for Laboratory Animal Science and the International Council for Laboratory Animal Science.

Detection of β-Galactosidase Activity

The mice were anesthetized with intraperitoneal injections of Avertin (0.5 g/kg) and then perfused transcardially sequentially with phosphate-buffered saline (PBS) and fixative containing 2% formaldehyde and 0.2% glutaraldehyde (Sigma-Aldrich) in 0.01 M PBS (pH 7.4). Esophagus, stomach, duodenum, and distal colon were dissected and immersion fixed with the same fixative for 30 min on ice. After rinsing in PBS, 3–4 cm-long tissue slabs were incubated in the staining solution, containing 0.5 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.01% Igepal (Sigma-Aldrich) in 0.01 M PBS (pH 7.4) under light protection at 37°C overnight. The specimens were rinsed with PBS and cleared in ascending concentrations of glycerol in PBS at 4°C. One part of the tissue was visualized under the stereomicroscope (Olympus SZH10) as a whole mount of the digestive tube; the other part was embedded in paraffin and 20 μm-thin histological sections were prepared and counterstained with nuclear fast red (Chroma-Gesellschaft Schmid Gmbh & Co.).

Unlike the wholemounts of the total digestive wall, those of the muscle layer were prepared by separating the muscle layer from the other layers. These animals were not perfused, but the muscle layer was isolated from unfixed tissue, subsequently fixed by immersion and processed as above.

Histology

For conventional histology, animals were fixed by perfusion in 4% paraformaldehyde. The selected parts of the gastrointestinal tract were isolated and fixed further by immersion in the same fixative, for 1 hr on ice. Esophagus, stomach, duodenum, and distal colon were embedded in paraffin and 7 μm-thin sections were prepared and stained with hematoxylin–eosin.

Immunohistochemistry

Segments of stomach, duodenum, and distal colon used for immunohistochemical analysis were collected after fixation by perfusion in 4% paraformaldehyde followed by immersion in the same fixative for 3 hr on ice. Each segment was rinsed in PBS, and then transferred to 10% sucrose followed by 30% sucrose in PBS at 4°C. A total of 20 μm-thin frozen sections were cut on a cryostat and immunolabeled on SuperFrost microscope slides (Menzel-Glaser) with primary antibodies against: STAM2 (rabbit polyclonal, diluted 1:150, Santa Cruz Biotechnology, sc-98681), STAM1 (goat polyclonal, diluted 1:100, Santa Cruz Biotechnology, sc-6919), HRS (mouse monoclonal, diluted 1:100, Santa Cruz Biotechnology,

sc-166843), microtubule-associated protein 2 (MAP2; chicken polyclonal, diluted 1:700, Abcam, ab5392), protein gene product 9.5 (PGP9.5; mouse monoclonal, diluted 1:20, Abcam, ab8189), glial fibrillary acidic protein (GFAP; chicken polyclonal, diluted 1:50, Abcam, ab4674), c-kit (goat polyclonal, diluted 1:200, Santa Cruz Biotechnology, sc-1494), β-galactosidase (chicken polyclonal, diluted 1:200, Abcam, ab9361), and early endosome antigen 1 (EEA1; mouse monoclonal, diluted 1:500, BD Transduction Laboratories, 610457). Incubation with primary antibodies was performed at 4°C overnight. The secondary antibodies were: Alexa Fluor 488 goat anti-rabbit (Invitrogen, A11008), Alexa Fluor 488 donkey anti-rabbit (Invitrogen, A11056), Alexa Fluor 546 goat anti-chicken (Invitrogen, A11040), Alexa Fluor 546 goat anti-mouse (Invitrogen, A11003), and Alexa Fluor 546 donkey anti-goat (Invitrogen, A21206). All secondary antibodies were diluted at a concentration 1:500, and incubation was performed for 2.5 hr at room temperature. After final rinsing, sections were coverslipped in Fluoromount (Sigma-Aldrich) and analyzed by confocal microscopy (Zeiss LSM 510 Meta).

Quantitative Morphological Analysis

Whole-mount preparations of the muscle layer (described above; prepared by separating the muscle layer from the other layers) stained with X-gal solution were used to estimate the volume density of the X-gal-labeled structures (Van Ginneken et al., 2002). Samples of the muscle layer (3 cm^2) were taken from two animals per genotype (homozygous and heterozygous). In each sample, 10 fields, each of 1.3 mm^2 were randomly chosen and analyzed. Volume densities were calculated by dividing the number of grid points that overlapped the space of interest ($P_{(I)}$, X-gal stained area) by the number of grid points hitting the reference space ($P_{(ref)}$, muscle layer) of 1.3 mm^2. The density of the stereological grid (number of points), the number of sections, and the number of sample fields were chosen to give a coefficient of error (CE) of the estimation that was less than 0.1 (Gundersen and Jensen, 1987).

Samples of distal colon (1 cm each) were taken from two animals per genotype (homozygous and wild type) and seven randomly chosen cryosections per each animal were immunostained and used for analysis. The density of the stereological grid, the number of sections, and the number of sample fields were chosen to give a coefficient of error (CE) of the estimation that was less than 0.1 (Gundersen and Jensen, 1987). Volume density of the immunoreactive MAP2 areas in the 0.2 mm^2 reference space of muscle layer was calculated by dividing the number of grid points hitting the space of interest ($P_{(I)}$, MAP2 positive area) by the number of grid points hitting the reference space ($P_{(ref)}$, muscle layer), as previously described (Van Ginneken et al., 2002).

Statistical Evaluation

All measurements of the volume densities of the X-gal-stained areas and MAP2-positive areas from both genotypes (i.e., homozygous and heterozygous, and homozygous and wild type, respectively) were used to test the normality of sample distribution by the Kolmogorov–Smirnov test and the Shapiro–Wilk test. The samples which followed normal distribution (X-gal-stained areas) were statistically analyzed by the Student *t* test ($P < 0.1$) and samples, which did not follow normal distribution (MAP2-positive areas) were analyzed by nonparametric Mann–Whitney U test ($P < 0.1$). Analyses of variance (ANOVA) or Kruskal–Wallis ANOVA were used to compare the means of single animals to confirm the equality of the variances calculated by the comparison of two genotype groups.

RESULTS

β-Galactosidase Activity is Present in the Enteric Nervous System of *Stam2*^{Gt1Gaj} Mice

Visualization of *Stam2* expression in the enteric nervous system was achieved with the genetically modified mouse line generated by the gene trap screen. As a consequence of the gene trap modification, *lacZ* gene was in frame with *Stam2* gene, allowing us to assess *Stam2* expression by histochemical detection of β-galactosidase activity via its substrate X-gal, which formed a blue precipitate.

X-gal staining was analyzed on whole-mount fragments of the digestive tube and on cryosections. β-galactosidase activity was present in the myenteric and submucosal plexus of both heterozygous and homozygous animals at all levels of the digestive tract (Fig. 1A–F). Only in the esophagus, where the submucosal plexus is lacking in mice, was the staining restricted to the myenteric plexus only. The cell morphology or the abundance of X-gal staining was essentially the same in heterozygous and homozygous mice (Fig. 1G–I).

STAM2 is Present in β-Galactosidase Positive Cells

To verify whether *lacZ* transgene expression pattern reflects endogenous *Stam2* expression, cryosections of heterozygous *Stam2*$^{+/Gt1Gaj}$ large intestine were double-labeled with STAM2 and β-galactosidase antibody. A strong fluorescence (of both STAM2 and β-galactosidase) was present at the level of the myenteric and submucosal plexus. The observed overlap in the distribution of STAM2 and β-galactosidase proteins (Fig. 2A) suggests that the X-gal staining reliably identified *Stam2*-expressing cells. Additionally, a very weak STAM2 signal was spread throughout the gut, indicative of low levels of STAM2 in all tissues (i.e., epithelial, connective, and muscle tissue), which was in accordance with previously described northern blotting and RT-PCR results (Takata et al., 2000; Curlin et al., 2006).

STAM2 Expressing Cells are Neurons and ICCs

To identify the cell types that expressed *Stam2*, cryosections of stomach, and small and large intestine of wild type mice were double-labeled with STAM2 antibody and neuronal markers (the neuron-specific cytoskeletal marker MAP2, and the neuronal cell body and axon marker PGP9.5), an enteroglial marker (GFAP) or a marker for ICCs (c-kit).

The majority of *Stam2*-positive cells costained with the neuronal markers MAP2 and PGP9.5, indicating that *Stam2*-expressing cells are largely neurons (Fig. 2B,C). STAM2 staining was visible throughout the soma

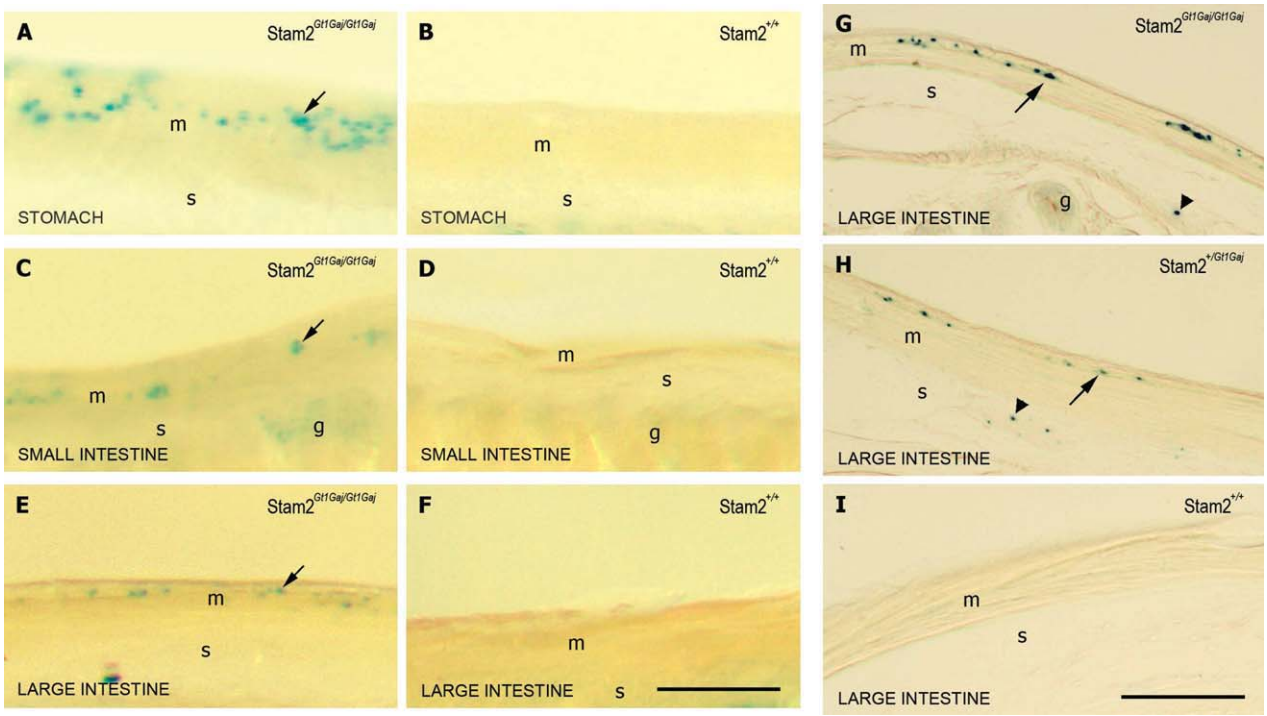

Fig. 1. β-galactosidase as a marker of *Stam2* expression was present in the enteric nervous system. (**A–F**) 500 μm-thin coronar sections of stomach (A,B), small intestine (C,D), and large intestine (E,F) visualized under the stereomicroscope. A,C,E samples were from $Stam2^{Gt1Gaj/Gt1Gaj}$ and B,D,F from $Stam2^{+/+}$ animals. β-galactosidase activity revealed a blue precipitate (arrows) in the myenteric plexus. (**G–I**) 20 μm-thin cryosections of the large intestine visualized under the microscope. β-galactosidase activity revealed a blue precipitate in the myenteric plexus (arrows) and in the submucosal plexus (arrowhead); the abundant presence of the precipitate was comparable in both homozygous (G, $Stam2^{Gt1Gaj/Gt1Gaj}$) and heterozygous (H, $Stam2^{+/Gt1Gaj}$) animals and absent in the control $Stam2^{+/+}$ mice (I); g, glands in mucosa layer; m, muscle layer; s, submucosal layer. Bars = 100 μm.

and nerve fibers, showing a punctuate appearance and high fluorescence intensity. STAM2 was also present in neuronal nuclei, but the signal was weaker and homogenous (e.g., Fig. 2C). To visualize ICCs, anti-c-kit immunohistochemistry was chosen as the most reliable microscopic method (Komuro et al., 1996). ICCs were observed around the myenteric plexuses and in the muscle layer. Double staining with STAM2 showed that STAM2 was present in ICCs in the myenteric plexus, albeit only in a subset of those cells (Fig. 2E). Enteroglial cells visualized by GFAP antibody had weak immunostaining signal for STAM2 protein, similar to the weak staining observed in other gut structures (Fig. 2D).

Endosomal and ESCRT-0 Proteins are Present in the Enteric Nervous System and Colocalize With STAM2

To verify in the gut whether STAM2 is associated with the early endosomes, immunolabeling with EEA1 (a protein present in the early endosome membrane) was performed. Double staining with STAM2 and EEA1 antibody confirmed that STAM2 was localized in the punctuate structures positive for EEA1 (Fig. 2F).

To examine the regional distribution of ESCRT-0 members (STAMs and Hrs) in the enteric nervous system, we performed immunohistochemical analysis with the other STAM isoform, STAM1, and with HRS. STAM1 and HRS were present in all layers of the gut, although the staining was stronger in the submucosal and myenteric plexus of the enteric nervous system. Double labeling immunohistochemistry with STAM2/STAM1 and STAM2/HRS showed that STAM1 and HRS colocalize with STAM2 in the cells of the enteric nervous system (Fig. 2G,H).

The Phenotype Analysis of the Enteric Nervous System Did Not Reveal Any Difference Between Homozygous ($Stam2^{Gt1Gaj/Gt1Gaj}$) and Wild Type Mice

To gain insight into a possible specific STAM2 function in the enteric nervous system, we performed a phenotypic analysis of the homozygous mice carrying the gene trap insertion ($Stam2^{Gt1Gaj/Gt1Gaj}$). Histological analysis of $Stam2^{Gt1Gaj/Gt1Gaj}$ mice revealed normal morphology of the digestive tube. Hematoxylin & eosin staining of esophagus, stomach, and small and large intestine sections showed no morphological differences between $Stam2^{Gt1Gaj/Gt1Gaj}$ and wild type mice. This general observation was substantiated by the following quantitative measurements.

To verify whether gene trap mutation influenced the morphology of the *Stam2*-expressing structures, the *lacZ*-positive areas in the muscle layer of the large

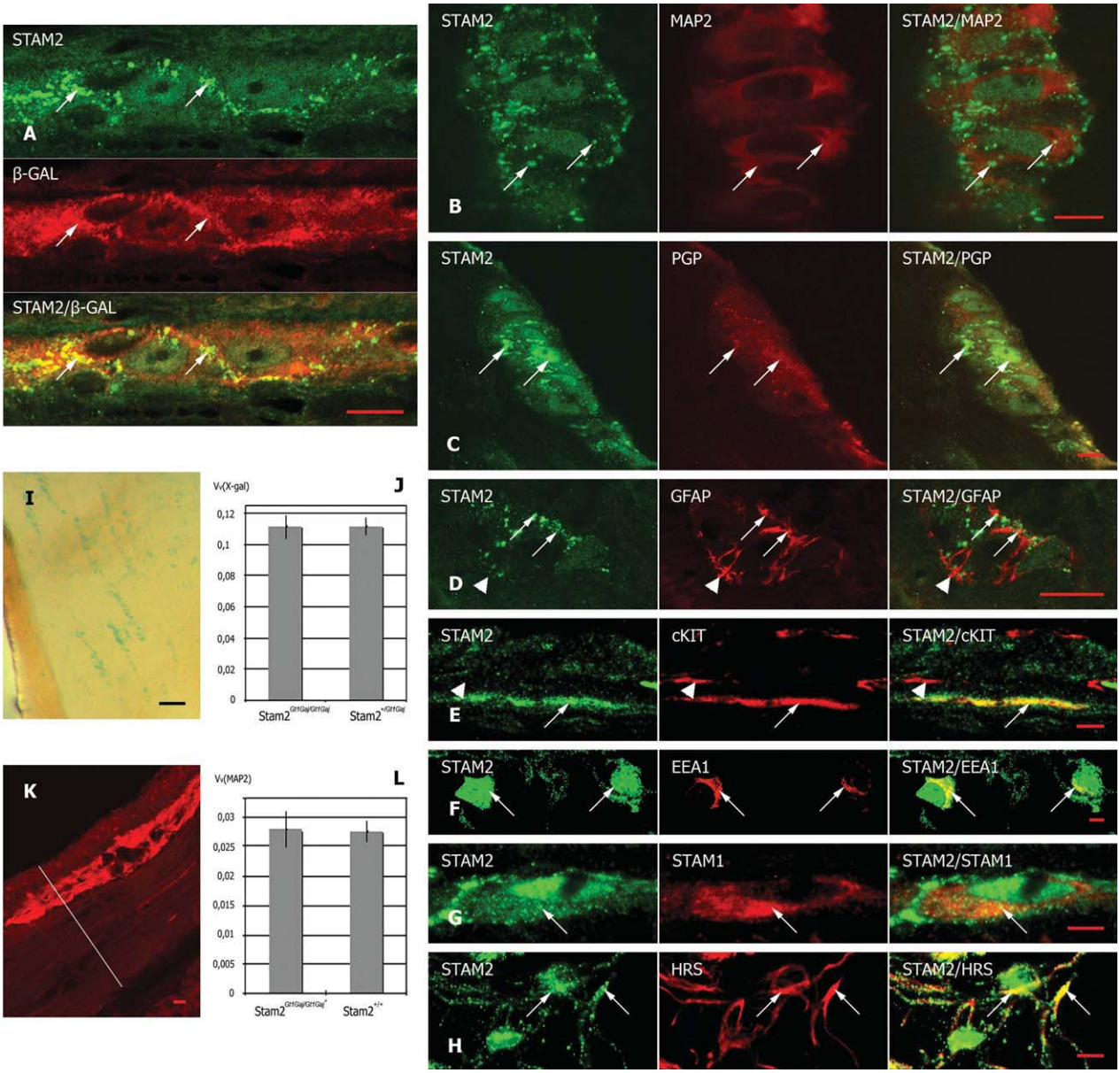

Fig. 2. *Stam2* expression in different cell types of the enteric nervous system and analysis of its influence on the morphology of the myenteric plexus. (**A**) Confocal photomicrographs showing the colocalization of STAM2 and β-galactosidase in the myenteric plexus of the large intestine of heterozygous mice. Overlap in distribution of STAM2 protein and β-galactosidase (arrows) confirmed that *lacZ* insertion in the *Stam2* gene reflected *Stam2* expression. (**B-H**) Confocal photomicrographs showing *Stam2* expression in neurons, enteroglial cells, and ICCs at the level of the myenteric plexus of the large intestine of wild-type mice. (B) The highest expression of *Stam2* was found in neurons (arrows), which were demonstrated with the neuronal marker MAP2. (C) Double staining of STAM2 and the neuronal marker PGP9.5 showed *Stam2* expression in the cytoplasm but also in the nucleoplasma (arrows). (D) A very weak STAM2 signal (arrowheads) was found in GFAP-positive cells (arrows), indicating the absence of high *Stam2* expression in enteroglia. (E) Detection of STAM2 antibody in some of the c-kit-positive cells showed *Stam2* expression in some of the ICCs (arrows) but not in all of them (arrowheads). (F) STAM2 was costained with the EEA1 antibody (arrows) indicating that the STAM2 protein was localized mainly to the early endosome membrane. (G, H) STAM2 was double-stained with other members of the ESCRT-0 complex. Arrows indicate the colocalization of STAM2 with STAM1 (G) and HRS (H) at the level of the myenteric (G) and submucosal (H) plexus of large intestine. Bars in A–H are 10 μm. (**I**) X-gal-positive areas (blue precipitates) in the myenteric plexus (arrows) of a whole-mount preparation of the large intestine of *Stam2*$^{Gt1Gaj/Gt1Gaj}$ mouse. Quantification of X-gal-positive areas was done by stereological measurement of their volume density in a reference space of the wall of the colon with the detached mucosa. Bar = 100 μm. (**J**) Means of the volume densities of X-gal-positive areas in the colon of homozygous and heterozygous mice, showing that they were abundantly present in both *Stam2*$^{Gt1Gaj/Gt1Gaj}$ and *Stam2*$^{+/Gt1Gaj}$ mice. (**K**) Confocal photomicrograph of the muscle layer of the large intestine (the width of the muscle layer is depicted by a white line) with MAP2-immunoreactive areas. Bar = 10 μm. (**L**) Means of the volume densities of the MAP2-immunoreactive areas in a reference space of the muscle layer of homozygous and wild-type mice. No significant differences were observed between both mouse strains ($P < 0.1$). Vertical lines in both charts show standard error of the mean.

intestine of homozygous ($Stam2^{Gt1Gaj/Gt1Gaj}$) and heterozygous ($Stam2^{+/Gt1Gaj}$) mice were compared. Quantitative analysis of the *lacZ*-positive areas was performed in wholemounts of the large intestine with detached mucosa stained with X-gal. Two samples per genotype (homozygous and heterozygous) of distal colon (3 cm^2 each) were taken and 10 randomly chosen fields (1.3 mm^2 each) were analyzed. No significant differences in volume density of the X-gal-stained areas in the muscle layer were found between homozygous and heterozygous mice, showing that the morphology of the enteric nervous system in the transgenic mice was not affected due to the modified *Stam2* gene (Fig. 2I,J).

To compare the distribution of neurons in the myenteric plexus of the large intestine between wild type and homozygous $Stam2^{Gt1Gaj/Gt1Gaj}$ mice, volume densities of MAP2-immunoreactive areas in the muscle layer of the distal colon were calculated on cryosections. Seven randomly chosen sections per animal (two homozygous and two wild-type mice) were used to measure the volume density of the MAP2-immunoreactive areas in a reference space of the muscle layer (one reference space per section, $A_{(ref)} = 0.2$ mm^2). Averages per genotype of the volume densities of the MAP2-immunoreactive areas in the colon showed that the distribution of neurons in the myenteric plexus of the large intestine was not significantly different ($P < 0.1$) between homozygous $Stam2^{Gt1Gaj/Gt1Gaj}$ and wild-type mice (Fig. 2K,L).

DISCUSSION

To determine *Stam2* expression and function in the mouse, we used a previously generated mouse model with gene trap modification of *Stam2* gene. Analysis of these mice indicated that *Stam2* was strongly expressed in the enteric nervous system, both in the submucosal and in the myenteric plexus. All cells in which ß-galactosidase immunoreactivity was detected, also showed STAM2-specific immunostaining, which confirmed that *lacZ* expression coincided with *Stam2* expression, at least in the digestive tract. In addition, this finding demonstrated that the gene trap mouse line $Stam2^{Gt1Gaj}$ can be reliably used in *Stam2* expression studies of the enteric nervous system. The observation that ß-galactosidase activity was restricted only to a subset of the gut structures, might appear at first sight in contradiction with previous data obtained by northern blotting and RT-PCR, which suggest ubiquitous *Stam2* expression (Takata et al., 2000; Curlin et al., 2006). Previously, restricted β-galactosidase activity was demonstrated in 11.5-day mouse embryos, in particular in the developing heart and the central nervous system, which was the main reason to select the gene trap mouse line for further investigations of *Stam2* (Gajovic et al., 1998; Curlin et al., 2002). The data presented here resolve this inconsistency between molecular analysis and expression of *lacZ* transgene, as they showed both, high and low levels of *Stam2* expression in the gut. The most prominent illustration of the observed two levels of *Stam2* expression (low and ubiquitous vs. high and restricted) was obtained by immunostaining with STAM2 antibody. This demonstrated a low level of protein presence (comparable with background staining) all over the digestive tube, whereas the high levels of STAM2 always colocalized with transgene derived ß-galactosidase.

Our hypothesis was that analyzing the regions of high expression would disclose a previously unknown specific function of STAM2 in the enteric nervous system. This approach necessitated the identification of enteric cells with high levels of *Stam2* expression. These cells turned out to be neurons and ICCs. We found that only a subset of the ICCs, namely those present in the myenteric plexus colocalized with STAM2 immunoreactivity. ICCs are specialized mesenchymal cells in the gastrointestinal tract that are neither neurons nor smooth muscle cells; they might play a pacemaker role in intestinal peristalsis in that they generate spontaneous, rhythmic electrical oscillations called slow waves (Ward et al., 1994). Synaptic contacts have been demonstrated between ICCs and enteric nerve terminals. Specific subpopulations of enteric ICCs are involved in conducting and amplifying neuronal signals from excitatory cholinergic and inhibitory nitrergic motor neurons (Ward et al., 1998; Iino et al., 2004). It has been proposed previously (Komada and Kitamura, 2001) that STAM2, in complex with HRS, is implicated in the neurotransmitter release in neurons. It is also well known that ICCs express a wide variety of receptors for enteric neurotransmitters, and many of the same receptors are expressed by enteric neurons (Beckett et al., 2005). Thus, it is not surprising expression of *Stam2*, both in neurons and ICCs.

High *Stam2* expression was found in all neurons of the enteric nervous system. STAM2 immunostaining mainly appeared as punctuate structures in the soma and neuronal processes. We confirmed that these punctuate structures in the cytoplasm are mainly early endosomes and that STAM2 localization in the gut is related to the other members of the ESCRT-0 complex, STAM1 and HRS (Takata et al., 2000; Bache et al., 2003; Mizuno et al., 2003). The high expression of STAM2 in neurons indicates that STAM2-related endosomal activity could be very high in neurons. Endosomes are indeed highly engaged in neuronal activities, including formation of synaptic vesicles, retrograde axonal transport, growth factor-mediated cell signaling, and receptor sorting and down-regulation (Vance et al., 2000). Compared with other cell types, neurons are particularly vulnerable to defects in the endosomal–lysosomal system, and aberrant endosomal trafficking has been linked to neurodegenerative diseases (Saksena and Emr, 2009; Stuffers et al., 2009). For example, amyotrophic lateral sclerosis and frontotemporal dementia are characterized by the accumulation of ubiquitin-positive protein inclusions in the nervous system, which suggests that the mechanism that scavenges such aggregates may be impaired in the affected neurons (Skibinski et al., 2005; Parkinson et al., 2006). The presence of STAM2 and the other proteins involved in ESCRT-0 complex, STAM1 and HRS, in the enteric nervous system and in the brain (unpublished data) provides additional evidence that enteric neurons could relate to those affected by neurodegenerative processes in the brain.

Nevertheless, it should be noted that we found a weak but homogenous STAM2 signal in neuronal nuclei, which was not the case for STAM1 or HRS. In addition to endosomal activity STAM2 is also involved in cytokine signaling, which might well contribute to its

intracellular distribution and specific nuclear expression within the enteric neurons (Endo et al., 2000). Therefore, future studies are needed to verify STAM2 localization in neuronal nuclei throughout the nervous system and to determine the subcellular distribution of other proteins, which interact with STAM2 to disclose possible STAM2 functions in various cell compartments of the neurons, in particular in the cell nuclei of the neurons.

The specific phenotypes of mice lacking *Stam1*, *Hrs,* and *Amsh* (i.e., a molecule associated with the SH3 domain of STAM), which are all *Stam2*-associated molecules, indicate the importance of STAM2-interacting proteins in the nervous system. Loss of function of *Stam1* and *Hrs*-specific conditional knock-out cause the disappearance of hippocampal CA3 pyramidal neurons (Yamada et al., 2001: Tamai et al., 2008); similarly, *Amsh* knockout leads to neuronal loss in the CA1 subfield of the hippocampus (Ishii et al., 2001). Therefore, we assumed that similar morphological consequences could occur if *Stam2* would be missing in the enteric nervous system.

Whether high expression of *Stam2* in the enteric nervous system is related to its specific function could not be confirmed. Histological, histochemical, and immunohistological qualitative and quantitative examinations of homozygous $Stam2^{Gt1Gaj/Gt1Gaj}$ mice revealed a normal morphology of the enteric nervous system. The gene trap event occurred between exons 2 and 3; therefore, it is expected that 86% of the normal STAM2 protein is missing from the C-terminal of the mutant protein as a consequence of the gene trap mutation (Curlin et al., 2006). We regard as highly unlikely the possibility that a truncated protein containing only 14% of STAM2 could maintain its function. The remaining part contained only part of the VHS region, while all other functional domains (i.e., UIM, SH3, CC, SSM, and ITAM) were missing. A possible reason for the lack of phenotypic changes might be that the gene trap mutation did not completely abolish STAM2 function due to the incomplete efficiency of the introduced splice acceptor and the subsequent production of wild type mRNA and protein regardless of gene trap mutation. The most plausible explanation, however, seems to lie in a compensatory function of the *Stam1* and *Stam2* genes, that is, the *Stam1* gene compensates for the loss of *Stam2* in the analyzed mice. This was substantiated by an overlap in the expression pattern of both STAM proteins that was evident within the enteric nervous system. The finding that loss of both STAM proteins is embryonically lethal in mice, stresses the importance of STAM proteins as early as during embryonic development (Yamada et al., 2002). In addition, the double knockdown of both *Stam1* and *Stam2* using the siRNA method led to increased HeLa cell death (Rismanchi et al., 2009). Together, these data show the importance of STAM proteins in the maintenance of healthy cells and indicate that their function in the enteric nervous system can only be disclosed after conditional double *Stam1* and *Stam2* knockout.

In conclusion, we showed that *Stam2* is highly expressed in neurons and in a subset of ICCs located in the enteric nervous system. However, analysis of the homozygous carriers of gene trap mutation of *Stam2* gene failed to reveal STAM2 function in these cells.

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