

## Expression analysis and essential role of the putative tyrosine phosphatase His-domain-containing protein tyrosine phosphatase (HD-PTP)

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**ABSTRACT** The putative tyrosine phosphatase HD-PTP, encoded by the protein-tyrosine-phosphatase-n23 (*Ptpn23*) gene, has been described as a tumor suppressor candidate gene. However, its physiological roles and detailed expression profiles are poorly defined. To investigate HD-PTP functions, we generated a mouse model in which the *Ptpn23* locus was disrupted by an in-frame insertion of a  $\beta$ -galactosidase-neomycin-phosphotransferase II ( $\beta$ -geo) cassette. This insertion leads to the expression of a catalytically inactive truncated protein preserving only the uncharacterized N-terminal BRO1-like domain in fusion with  $\beta$ -geo under the control of the endogenous promoter. Here we report that homozygous gene deletion is lethal around embryonic day 9.5, suggesting that *Ptpn23* is an essential requirement for early stages of embryonic development. Taking advantage of the  $\beta$ -galactosidase insertion into the *Ptpn23* locus, we define the precise *Ptpn23* expression pattern by performing X-gal staining at different stages of mouse development. Our results show that *Ptpn23* is expressed early during mouse development and that its expression is maintained in adult tissues, markedly in the epithelial cells of many organs.

**KEY WORDS:** HD-PTP, *Ptpn23*, gene-trap, expression pattern, embryonic lethality

The protein-tyrosine-phosphatase-n23 (*PTPN23*) gene is a tumor suppressor gene candidate encoding a poorly characterized protein tyrosine phosphatase called His-domain-containing protein tyrosine phosphatase (HD-PTP) (Cao *et al.*, 1998; Toyooka *et al.*, 2000). The *PTPN23* gene is located on the 3p21.3 tumor suppressor gene cluster frequently deleted in human kidney, breast, lung and cervical tumors and its overexpression inhibits *ras*-mediated transformation of NIH-3T3 cells (Cao *et al.*, 1998; Toyooka *et al.*, 2000; Imreh *et al.*, 2003; Ji *et al.*, 2005; Hesson *et al.*, 2007). The HD-PTP protein encompasses four main domains: a functionally uncharacterized BRO1-like domain (BRO), a histidine-rich domain (HIS), a classical protein tyrosine phosphatase domain (PTP) and a proteolytic degradation targeting sequence (PEST motif) (Toyooka *et al.*, 2000). Potential functions for HD-PTP in inhibition of endothelial migration and EGFR endosomal trafficking were recently reported, but its role *in vivo* is still undefined (Castiglioni *et al.*, 2007; Doyotte *et al.*, 2008; Mariotti *et al.*, 2008; Miura *et al.*, 2008). The high level of conservation of HD-PTP among multi-

cellular organisms (human, monkey, mouse, rat, chicken, frog and fly) suggests a significant and common biological function, as does its ubiquitous mRNA expression in adult human and rat organs (Cao *et al.*, 1998; Mariotti *et al.*, 2006). However, the developmental and cell type specific expression pattern remains unreported.

In order to verify the physiological functions of HD-PTP, we generated a mouse model containing a  $\beta$ -galactosidase-neomycin-phosphotransferase II ( $\beta$ -geo) insertion into the *Ptpn23* locus, leading to the expression of a catalytically inactive truncated protein in fusion with  $\beta$ -geo, under the control of the *Ptpn23* promoter (Nord *et al.*, 2006). Our results show that *Ptpn23* is essential for early mouse embryonic development

*Abbreviations used in this paper:*  $\beta$ -geo,  $\beta$ -galactosidase-neomycin-phosphotransferase II; BRO, BRO1-like domain; E, embryonic day; HD-PTP, His-domain-containing protein tyrosine phosphatase; HIS, histidine-rich domain; PTP, protein tyrosine phosphatase; PTPN23, Protein-tyrosine-phosphatase-n23.

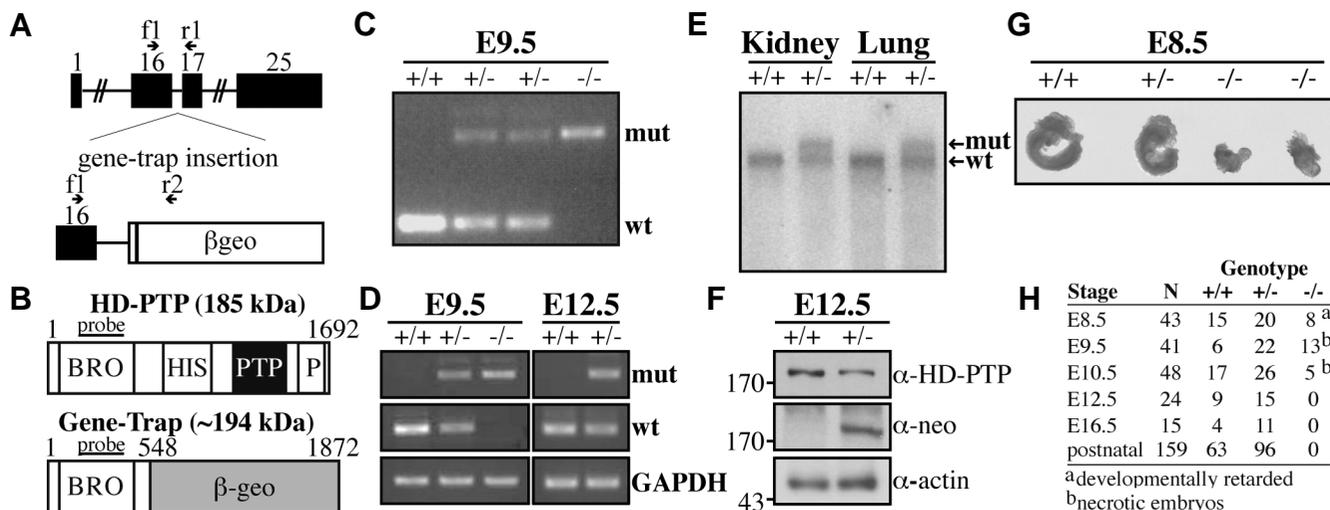
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**Fig. 1. Generation of a *Ptpn23* disrupted mouse.** (A) Schematic representation of the 25 exons of the mouse *Ptpn23* locus and the  $\beta$ -galactosidase-neomycin phosphotransferase II ( $\beta$ -geo) gene-trap insertion site between exons 16 and 17. Forward (f1) and reverse (r1 and r2) primers used for genotyping are indicated. (B) Schematic representation of the 185 kDa mouse HD-PTP protein and its domains (upper panel) and the predicted 194 kDa gene-trap fusion protein (lower panel). Numbers correspond to amino acids and the location of the probe used for the northern blot is indicated. BRO: BRO1-like domain, HIS: histidine-rich domain, PTP: protein-tyrosine-phosphatase domain, P: proteolytic degradation targeting sequence also known as PEST motif. (C) DNA genotyping at embryonic day (E) 9.5, using primers amplifying the mutated (mut) or the wild type (wt) allele. (D) RT-PCR performed on RNA extracts from embryos (E9.5 or E12.5) using primers amplifying the mutated (mut), the wild type (wt) and the endogenous GAPDH transcripts. (E) Northern blot analysis performed on adult kidney and lung RNA extracts using a probe that recognizes both wild type (wt) and mutated (mut) transcripts. (F) Western blot analysis of E12.5 *Ptpn23*<sup>+/+</sup> and *Ptpn23*<sup>+/-</sup> embryos using anti-HD-PTP and anti-neomycin ( $\alpha$ -neo) antibodies. Actin levels were monitored as loading control. (G) Representative pictures of E8.5 *Ptpn23*<sup>+/+</sup>, *Ptpn23*<sup>+/-</sup> or *Ptpn23*<sup>-/-</sup> embryos. (H) Detailed genotyping analysis of embryos. N, number of genotyped individuals.

since the homozygous deletion of the region encoding the HIS, PTP and PEST domains is lethal around embryonic day 9.5. Furthermore, this model allowed us to characterize the *Ptpn23* expression pattern during development and in adult tissues. Here we show that even though *Ptpn23* is widely expressed, its expression is not uniform but rather restricted to epithelial cells of several organs such as kidney, lung, digestive tract, liver and skin.

## Results and Discussion

### Generation of a *Ptpn23* mutant mouse

We generated a *Ptpn23* mutant mouse using an embryonic stem cell clone containing an in-frame  $\beta$ -galactosidase-neomycin-phosphotransferase II ( $\beta$ -geo) insertion between exons 16 and 17 of the mouse *Ptpn23* locus (Fig. 1A) (Nord et al., 2006). This gene-trap insertion leads to the expression of a fusion protein encompassing mouse HD-PTP amino acid residues 1 to 548 (out of 1692 amino acids) fused to  $\beta$ -geo under the control of the *Ptpn23* promoter (Fig. 1B). Chimeric mice were generated, germ line transmission was confirmed by PCR and genomic DNA sequence analysis using a  $\beta$ -geo specific primer confirmed the location of the insertion (not shown). Heterozygous mice were intercrossed and the Mendelian transmission of the  $\beta$ -geo cassette insertion between exons 16 and 17 was verified by PCR (Fig. 1C) and RT-PCR (Fig. 1D). For simplicity, we will refer to the gene-trapped allele as null (-). We detected wild type (*Ptpn23*<sup>+/+</sup>), heterozygous (*Ptpn23*<sup>+/-</sup>) and homozygous (*Ptpn23*<sup>-/-</sup>) embryos at embryonic day (E) 9.5 (Fig. 1 C,D)

but only *Ptpn23*<sup>+/+</sup> and *Ptpn23*<sup>+/-</sup> at E12.5 (Fig. 1D). Northern blot analysis of the *Ptpn23* RNA transcripts expressed in adult kidney and lung extracts confirmed the presence of a full length *Ptpn23* wild type (wt) transcript in *Ptpn23*<sup>+/+</sup> organs and the existence of a second larger transcript in the *Ptpn23*<sup>+/-</sup> organs, corresponding to the fusion of the *Ptpn23* exons 1-16 with the  $\beta$ -geo cassette (Fig. 1E). Finally, western blot analysis using an antibody generated against the HD-PTP HIS domain, absent in the gene-trap fusion protein (see Fig. S1 for the characterization of the antibody), shows a decrease in the HD-PTP protein level in the *Ptpn23*<sup>+/-</sup> E12.5 embryos (Fig. 1F). As expected, an anti-neomycin ( $\alpha$ -neo) antibody recognizes a band only in the *Ptpn23*<sup>+/-</sup> sample at the expected size of the fusion protein. All together, these data confirm that the gene-trap cassette is inserted between exons 16 and 17, is transcribed in fusion with the 5' portion of *Ptpn23* and that a HD-PTP (amino acids 1-548)- $\beta$ -geo fusion protein is expressed.

### Deletion of the 3' portion of the *Ptpn23* gene leads to embryonic lethality

Since we never found *Ptpn23*<sup>-/-</sup> embryos at postnatal stages (n=159), detailed genotyping analysis were performed on embryos obtained from *Ptpn23*<sup>+/-</sup> intercrosses (Fig. 1 G,H). Although we did not detect any *Ptpn23*<sup>-/-</sup> embryos after E12.5, interestingly we found *Ptpn23*<sup>-/-</sup> embryos as disorganized mass of cells surrounded by maternal hemorrhagic tissue at E9.5 and E10.5 (Fig. 1H). None of the *Ptpn23*<sup>-/-</sup> embryos (n=18) showed recognizable embryonic structures at E9.5 and E10.5. In addition, all the *Ptpn23*<sup>-/-</sup> embryos found at E8.5 (n=8) were

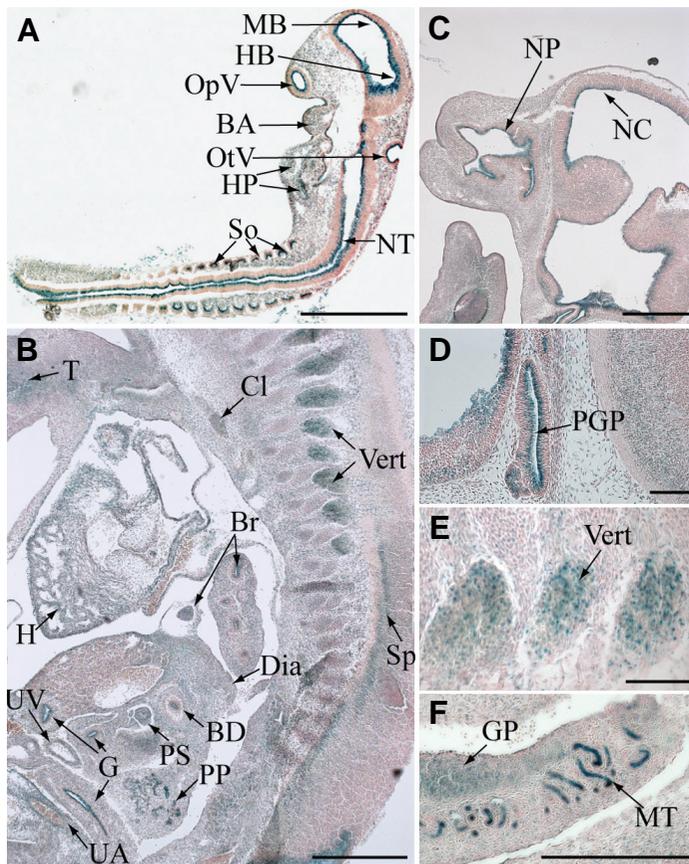
significantly smaller than normal embryos and their appearance were similar to E6.5 or early E7.5 (late egg cylinder stage) but never reached the E8.5 stage (Fig. 1 G,H). These observations indicated the importance of *Ptpn23* functions during early embryogenesis before E8.5. However, as expected from Mendelian inheritance ratios, a total of 21% (26/132) of embryos were detected as *Ptpn23*<sup>-/-</sup> between E8.5 to E10.5, suggesting the fertilization and implantation (~E4.5) steps were not affected in *Ptpn23*<sup>-/-</sup> embryos. Thus, our results suggest that *Ptpn23* plays a critical role in early stages of development between E4.5 ~ E7.5, where the period of gastrulation takes place and embryonic ectoderm differentiates to form the neural plate.

**Developmental expression profile of Ptpn23**

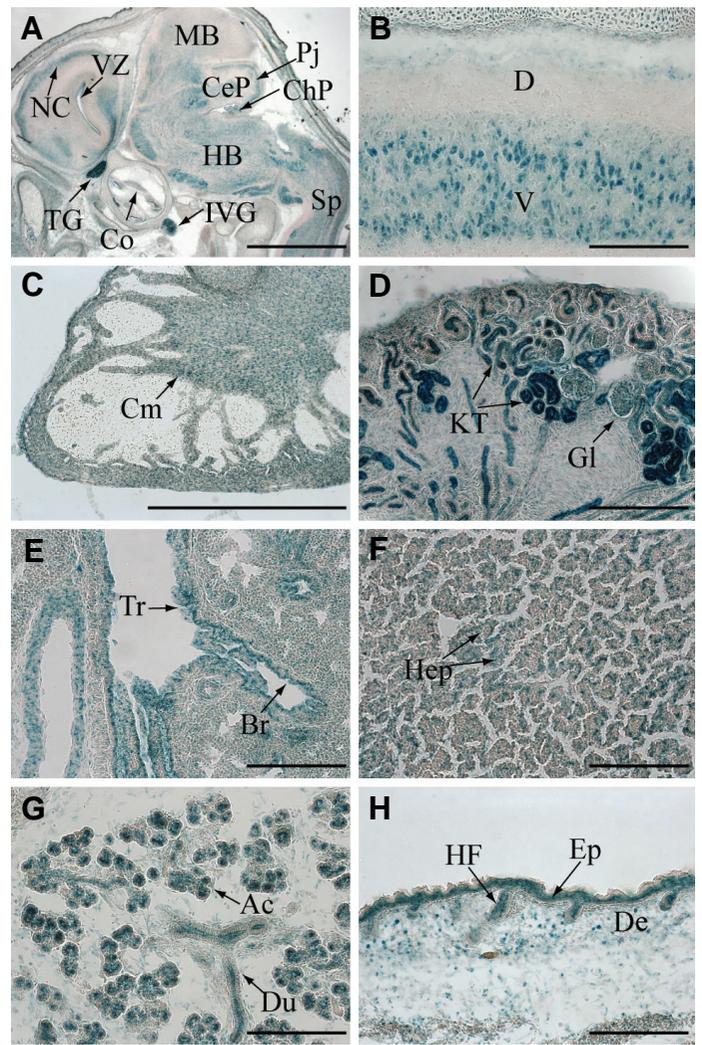
While *Ptpn23* expression was described as ubiquitous in adult human and rat organs by northern blot analysis, its cell type and developmental expression profile has never been described (Cao *et al.*, 1998; Mariotti *et al.*, 2006). Since the  $\beta$ -galactosidase reporter is expressed under the control of the

endogenous *Ptpn23* promoter, we took advantage of our mouse model to characterize the *Ptpn23* expression profile during embryonic development and in adult tissues. To proceed, we performed X-Gal staining on heterozygous animal sections, simultaneously with wild-type animals to control for the specificity of the staining.

We first determined the expression of *Ptpn23* at E9.5, as the *Ptpn23* homozygous deletion induces embryonic lethality around that stage. We observed a predominant *Ptpn23* expression in the nervous system, especially in the neuroepithelial lining of the midbrain (Fig. 2A; MB), hindbrain (Fig. 2A; HB), at the inner side of the optic (Fig. 2A; OpV) and otic (Fig. 2A; OtV) vesicles



**Fig. 2 (Left). Early expression profile of *Ptpn23*.** X-Gal staining of sagittal cryosections of *Ptpn23*<sup>+/-</sup> embryos corresponding to areas of *Ptpn23* expression at E9.5 (A) and E12.5 (B-F). See text for details. Bars: A,B, 500  $\mu$ m; D-F, 100  $\mu$ m. BA, branchial arches; BD, bile duct; Br, bronchia; Cl, Clivus; Dia, diaphragm; G, gut; GP, gonad primordium; H, heart; HB, hindbrain; HP, heart precursor; MB, midbrain; MT, mesonephric tubules; NC, neopallial cortex; NP, nasopharynx; NT, neural tube; OpV, optic vesicle; OtV, otic vesicle; PGP, pituitary gland precursor; PP, pancreatic primordium; PS, precursor of the stomach; So, somites; Sp, spinal cord; T, tongue; UA, umbilical artery; UV, umbilical vein; Vert, Vertebrae.



**Fig. 3 (Right). Expression pattern of *Ptpn23* in late embryonic development.** *Ptpn23* expression detected by X-Gal staining performed on *Ptpn23*<sup>+/-</sup> E16.5 sagittal cryosections. See text for details. Bars: A, 2 mm; B-H, 200  $\mu$ m. Ac, acini of the submandibular gland; Br, bronchia; CeP, cerebellum; ChP, choroids plexus; Cm, cardiomyocytes; Co, cochlea; De, dermis; D, dorsal; Du, duct of the submandibular gland; Ep, epidermis; Gl, glomeruli; HB, hindbrain; Hep, hepatocytes; HF, hair follicles; IVG, inferron vagal ganglion; KT, kidney tubules; MB, midbrain; NC, neopallial cortex; Pj, Purkinje cell layer; Sp, spinal cord; TG, trigeminal ganglion; Tr, trachea; V, ventral; VZ, ventricular zone.

and in the ventricular layer of the forebrain (not shown). Strong expression is also observed in the neuroepithelium of the neural tube (Fig. 2A; NT), in somites (Fig. 2A; So) and faint expression is observed in branchial arches (Fig. 2A; BA) and heart precursor (Fig. 2A; HP). At E12.5, *Ptpn23* shows a wider expression, precisely in the tongue (T), heart (H), cartilage primordium of the clivus (Cl) and the vertebrae (Vert), epithelium of the lung bronchia (Br), gut (G), precursor of the stomach (PS) and common bile duct (BD) (Fig. 2B,E). Strong expression is also observed in the spinal cord (Sp), pancreatic primordium (PP), endothelial cell layer of the umbilical vein (UV), artery (UA) and diaphragm (Dia) (Fig. 2B). Pronounced *Ptpn23* expression is detected in the neuroepithelium of the neopallial cortex (NC), epithelium of the nasopharynx (NP) (Fig. 2C), pituitary gland precursor (PGP) (Fig. 2D), gonad primordium (GP) and mesonephric tubules (MT) (Fig. 2F).

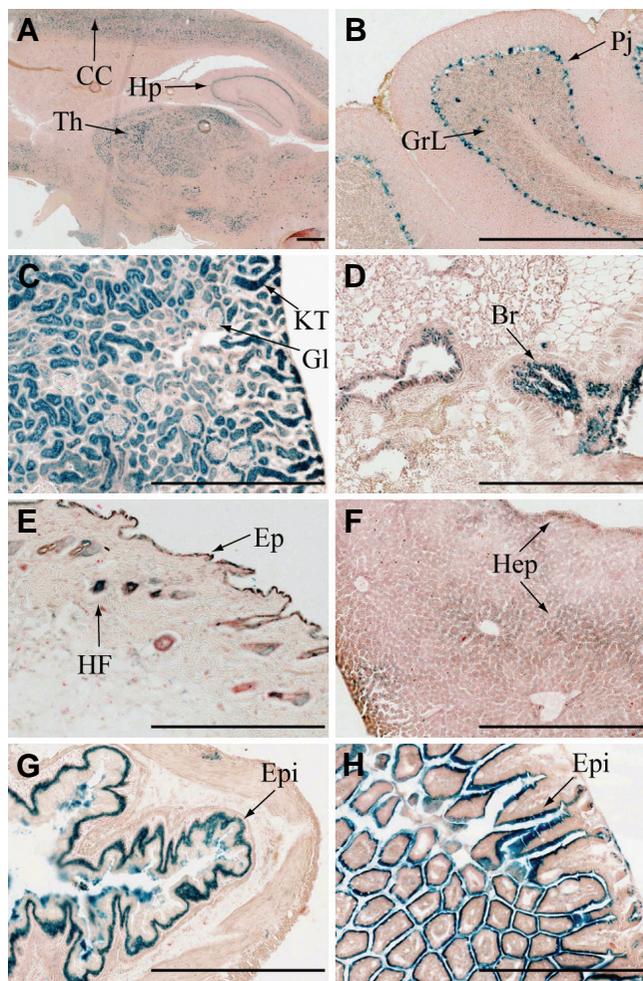
Later during development (E16.5), *Ptpn23* expression is detected in most of the organs, however some cell types and structures described below strongly expressed *Ptpn23*, suggesting a non-uniform expression (Fig. 3). Indeed, *Ptpn23* is markedly expressed in multiple brain regions such as the neopallial cortex (NC) and its ventricular zone (VZ), midbrain (MB), hindbrain (HB), Purkinje cell layer (Pj) of the cerebellum (CeP), choroids plexus (ChP), internal side of the cochlea (Co), as well as trigeminal (TG) and inferior vagal (IVG) ganglions (Fig. 3A). Marked *Ptpn23* expression is also observed in cells at the ventral side (V) of the spinal cord, which are probably motoneurons (Fig. 3A; Sp, B) but not at the dorsal side (D). Consistent with earlier stages, high *Ptpn23* expression is detected in cardiomyocytes (Cm) of the heart (Fig. 3C) and in tubules (KT) and glomeruli (Gl) of the kidney cortex (Fig. 3D). Moreover, substantial expression is observed in epithelial cells of several organs such as the epithelium of the lung trachea (Tr) and bronchia (Br) (Fig. 3E), liver hepatocytes (Fig. 3F, Hep), acini (Ac) and ducts (Du) of the submandibular salivary gland (Fig. 3G) and inner layer of the hair follicles (HF) as well as the epidermal (Ep) and the dermal (De) layer of the skin (Fig. 3H). Thus, the embryonic expression pattern shows that *Ptpn23* is expressed during all stages of organogenesis and is highly enriched in epithelia.

#### Expression pattern of *Ptpn23* in adult mouse organs

To determine if the developmental expression pattern of *Ptpn23* is preserved after birth, we investigated its expression in adult organs (Fig. 4). Results show that *Ptpn23* expression persists at the adult stage and is observed in several brain regions such as cerebral cortex (CC), hypothalamus (Hp) and thalamus (Th) (Fig. 4A). *Ptpn23* is also substantially expressed in the Purkinje cells (Pj) layer of the cerebellum and some intensely stained cells in addition with uniform and faint staining are detected in the granular layer (GrL) (Fig. 4B). Strongest expression is detected in the renal tubules of the kidney cortex (KT), but in contrast with the E16.5 pattern, no expression was observed in glomeruli (Gl) (Fig. 4C). Similarly to the E16.5 expression profile, *Ptpn23* expression is detected in the epithelial cells of the trachea (not shown), bronchia (Br) (Fig. 4D), hair follicles (HF), epidermal layer (Ep) of the skin (Fig. 4E) and hepatocytes (Hep) of the adult liver (Fig. 4F). Moreover, pronounced and specific expression has been detected in the

epithelium (Epi) of the stomach (Fig. 4G), small intestine (not shown) and colon (Fig. 4H). We did not observe  $\beta$ -gal expression in adult heart, spleen and muscle, suggesting a weak expression below detection limit or an absence of *Ptpn23* expression in these tissues (not shown).

In summary, we demonstrate an essential role for *Ptpn23* during mouse development, since the homozygous inactivation by gene-trap insertion leads to embryonic lethality around E9.5. Because we observed abnormal but late cylinder stage in *Ptpn23*<sup>-/-</sup> embryos at E8.5, deletion of *Ptpn23* might affect the process of neurulation, which normally occurs after gastrulation around E7.5. Indeed, our results demonstrate that *Ptpn23* is expressed extensively in ectoderm-derived tissues such as neuroepithelium at E9.5, implicating a function in neurulation. Alternatively, absence of *Ptpn23* may lead to defects in circulatory system development. Interestingly, *Ptpn23* expression has been reported to be upregulated by the angiogenic factor



**Fig. 4.** *Ptpn23* expression pattern in adult mouse organs. X-Gal staining performed on cryosections of brain (A), cerebellum (B), kidney (C), lung (D), skin (E), liver (F), stomach (G) and colon (H) from 6 month old *Ptpn23*<sup>+/-</sup> mice, corresponding to the location of *Ptpn23* expression. See text for details. Bars, 500  $\mu$ m. CC, cerebral cortex; Hp, hypothalamus; Th, thalamus; GrL, granular layer of the cerebellum; Pj, Purkinje cell layer of the cerebellum; KT, kidney tubules; Gl, glomeruli; Br, bronchia; HF, hair follicle; Ep, epidermis; Hep, hepatocytes; Epi, epithelium.

Tat and to regulate endothelial cell migration, suggesting a potential role in angiogenesis (Mariotti *et al.*, 2006; Castiglioni *et al.*, 2007; Mariotti *et al.*, 2008). The gene-trap insertion results in a protein containing the complete BRO1-like domain fused to  $\beta$ -geo. This insertion leads to a deletion of the HIS, PTP and PEST domains of HD-PTP. Interestingly, the tumor suppressor activity of the rat ortholog of HD-PTP in *ras*-mediated NIH-3T3 transformation was abrogated when a protein truncated for the PTP and PEST domains was expressed (Cao *et al.*, 1998). Moreover, the expression of a truncated HD-PTP protein containing only the BRO1-like domain result in a non-functional protein with respect to intracellular sorting of endocytosed cargo and tumor suppression, suggesting that the C-terminal domains of HD-PTP are essential for these activities (Doyotte *et al.*, 2008; Gingras *et al.* unpublished data). *PTPN23* expression has been previously described as ubiquitous in adult human and rat tissues (Cao *et al.*, 1998; Mariotti *et al.*, 2006). Our detailed histological analysis demonstrates that *Ptpn23* is widely expressed during mouse embryonic development and in adulthood however its expression pattern is restricted to specific cell types. In fact, *Ptpn23* is preferentially expressed in the epithelium of multiple organs and marked *Ptpn23* expression has been detected in the renal tubules, in the lung bronchia and in multiple brain compartments. Interestingly, most of the renal, lung and cervical tumors that are frequently deficient for the 3p21.3 tumor suppressor cluster where HD-PTP is encoded, originates from epithelial cells that strongly expressed HD-PTP. The specificity of HD-PTP expression in epithelia suggests that it could be involved in signaling pathways activated in epithelial cells. Consistently, a role for HD-PTP in the endosomal transport of the EGF receptor, which is highly expressed in tissues from epithelial and neuronal origins, and resulting in the enhancement of its downstream signaling has recently been described (Miura *et al.*, 2008).

## Materials & Methods

### Ptpn23 mouse model

The mouse YHB-130 embryonic stem cell line containing an in-frame  $\beta$ -geo ( $\beta$ -galactosidase and neomycin phosphotransferase II fusion) insertion between exons 16 and 17 of the mouse *Ptpn23* gene was obtained from the International Gene-Trap Consortium (www.genetrap.org). The YHB-130 clone (129 s/v background) was injected into C57BL/6 blastocysts, chimeric mice were generated and intercrossed to obtain *Ptpn23*<sup>+/-</sup> mice. For the embryonic genotyping analysis, *Ptpn23*<sup>+/-</sup> mice were mated and embryos at different stages were dissected in cold PBS and the yolk sacs were kept for genotyping analysis (described below). Representative pictures of E8.5 *Ptpn23*<sup>+/+</sup>, *Ptpn23*<sup>+/-</sup> and *Ptpn23*<sup>-/-</sup> embryos were taken using a Stemi SV11 stereomicroscope equipped with a CCD camera (Zeiss).

### Genotyping, RT-PCR, Northern blot and Western blot analyses

Genomic DNA or RNA was isolated from tail biopsies, complete embryos or yolk sacs and PCR or RT-PCR were performed using a forward primer located in *Ptpn23* exon 16 and reverse primers located in *Ptpn23* exon 17 or  $\beta$ -geo. For RT-PCR analysis, GAPDH was amplified as control of the RNA levels. Primer sequences are available in figure S2. Northern blot analysis was performed on *Ptpn23*<sup>+/-</sup> or *Ptpn23*<sup>-/-</sup> adult kidney and lung RNA extracts using a human cDNA *PTPN23* probe (human amino acids 231 to 403 = 98% homology with mouse sequence) that binds specifically to both wild type and mutant

transcripts. Western blot analyses were performed on protein extracts from E12.5 *Ptpn23*<sup>+/-</sup> and *Ptpn23*<sup>-/-</sup> embryos using our specific anti-HD-PTP rabbit polyclonal antibody generated against its HIS domain (human amino acids 400 to 800) that only recognizes the wild type protein, an anti-neomycin antibody ( $\alpha$ -neo: rabbit polyclonal anti-neomycin phosphotransferase II antibody, Upstate) and an anti- $\beta$ -actin antibody (mouse monoclonal AC74, Sigma-Aldrich) as loading control.

### X-Gal staining

*Ptpn23*<sup>+/-</sup> females were mated with C56BL/6 males, embryos were dissected and yolk sacs were used for genotyping as described above. Dissected *Ptpn23*<sup>+/-</sup> or *Ptpn23*<sup>+/-</sup> adult tissues (6 months) or complete embryos were fixed (0,2% glutaraldehyde) and embedded in OCT (Tissue-Tek). The E16.5 embryos were manually hemisected halfway and arm and legs were removed to permit efficient fixation. Frozen tissues and embryos were sectioned into 10  $\mu$ m slices; the  $\beta$ -galactosidase activity was detected by X-Gal staining and counter stained with Nuclear Fast Red (Sigma-Aldrich) according to the instructions of the International Gene-trap consortium. Staining was terminated before background signal was detected in *Ptpn23*<sup>+/+</sup> samples and was performed on three to five different individuals from each genotype at each stage. Representative pictures were taken using a XT Scan Scope Imaging System (Aperio).

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