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Sox10 and *Itgb1* interaction in enteric neural crest cell migration

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ABSTRACT

SOX10 involvement in syndromic form of Hirschsprung disease (intestinal aganglionosis, HSCR) in humans as well as developmental defects in animal models highlight the importance of this transcription factor in control of the pool of enteric progenitors and their differentiation. Here, we characterized the role of SOX10 in cell migration and its interactions with β1-integrins. To this end, we crossed the *Sox10*$^{lacZ/+}$ mice with the conditional Ht-PA::Cre; *beta1*$^{neo/+}$ and *beta1*$^{fl/fl}$ mice and compared the phenotype of embryos of different genotypes during enteric nervous system (ENS) development. The *Sox10*$^{lacZ/+}$; Ht-PA::Cre; *beta1*$^{neo/fl}$ double mutant embryos presented with increased intestinal aganglionosis length and more severe neuronal network disorganization compared to single mutants. These defects, detected by E11.5, are not compensated after birth, showing that a coordinated and balanced interaction between these two genes is required for normal ENS development. Use of video-microscopy revealed that defects observed result from reduced migration speed and altered directionality of enteric neural crest cells. Expression of β1-integrins upon SOX10 overexpression or in *Sox10*$^{lacZ/+}$ mice was also analyzed. The modulation of SOX10 expression altered β1-integrins, suggesting that SOX10 levels are critical for proper expression and function of this adhesion molecule. Together with previous studies, our results strongly indicate that SOX10 mediates ENCC adhesion and migration, and contribute to the understanding of the molecular and cellular basis of ENS defects observed both in mutant mouse models and in patients carrying *SOX10* mutations.

Introduction

The enteric nervous system (ENS), composed of interconnected ganglia distributed along the length of the gut, is the part of the peripheral nervous system that controls the peristaltic and secretory activity of the gut (Burns and Thapar, 2006; Gershon and Wade, 1994). Mainly derived from vagal neural crest cells, ENS development is dependent on proper proliferation, survival, differentiation, and rostro-caudal migration of enteric neural crest cells (ENCC) along the gut (Heanue and Pachnis, 2007; Obermayr et al., 2012). Alteration of these events can cause an absence of enteric ganglia, usually affecting the colon, and leading to severe constipation or intestinal obstruction, a condition known in humans as Hirschsprung disease (HSCR) (Amiel et al., 2008; Goldstein et al., 2013; Heanue and Pachnis, 2007). HSCR affects 1:5000 live births and requires surgery and re-anastomosis to remove the aganglionic bowel segment (Hotta et al., 2009).

HSCR is a multigenic disorder. Molecular and developmental studies have identified several critical players in HSCR and ENS development, including the RET tyrosine kinase and EDNRB G-coupled receptors and their ligands, L1CAM and β1-integrins adhesion molecules, and various transcription factors including SOX10 (Amiel et al., 2008; Goldstein et al., 2013; Heanue and Pachnis, 2007); however, mutations in HSCR-associated genes account for less than 50% of cases and incomplete penetrance and intrafamilial variability are common. Interactions between HSCR susceptibility loci and modifier genes have been described using genome wide screens and familial studies (Amiel et al., 2008; Gabriel et al., 2002). Similar strategies in mouse and two-locus complementation approaches have identified components contributing to the phenotype variability (see for example Barlow et al. (2003), Heanue and Pachnis (2007), McCallion et al. (2003), Owens et al. (2005), and Wallace and Anderson (2011)). In several studies, *Sox10* mutants were considered as a model of choice (Cantrell et al., 2004; Maka et al., 2005; Owens et al., 2005; Stanchina et al., 2006, 2010; Wallace et al., 2010).

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SOX10 is a member of the high-mobility group-domain SOX family of transcription factors (Bowles et al., 2000; Kelsh, 2006; Wegner, 1999). Mouse model studies have highlighted its crucial function during ENS development (Herbarth et al., 1998; Pingault et al., 1998; Southard-Smith et al., 1998). In *Sox10*$^{lacZ/+}$ heterozygous embryos, mutant ENCC are unable to maintain their progenitor status and acquire pre-neuronal traits, reducing progenitor pool size and resulting in variable enteric defects (Paratore et al., 2002). Extinction of *Sox10* expression is a prerequisite for neuronal differentiation to proceed (Bondurand et al., 2006; Kim et al., 2003). SOX10 function in these processes may be mediated through interactions with *Ret*, *Sox8*, *Zeb2*, or *Edn3*/*Ednrb* (Cantrell et al., 2004; Lang et al., 2000; Lang and Epstein, 2003; Maka et al., 2005; Stanchina et al., 2006, 2010; Zhu et al., 2004). However, the interactions between *Sox10* and *L1cam* (Wallace et al., 2010) and the severe enteric network disorganization observed in *Sox10*; *Zeb2* double mutants (Stanchina et al., 2010) suggested that *Sox10* could also play a central role in cell adhesion and migration.

Gut colonization by ENCC, the mode of ENCC migration, and the organization of the ganglia network can reflect variations in the molecular mechanisms driving ENCC interactions and their progression in response to their environment. ENCC express a large repertoire of adhesion receptors that control their adhesion to the extracellular matrix (ECM) and neighboring cells (Breau et al., 2009; Hackett-Jones et al., 2011; McKeown et al., 2013; Newgreen and Hartley, 1995). Integrins are the main ECM receptors, and the specific combination of α and β subunits determines the ligand recognition and cellular responses (Barczyk et al., 2010; Beauvais-Jouneau and Thiery, 1997; Campbell and Humphries, 2011; Hynes, 2002). ENCC integrins include α4β1, α5β1, α6β1, αVβ1, αVβ3, and αVβ5 (Breau et al., 2009, 2006; Broders-Bondon et al., 2012; McKeown et al., 2013), and ENCC lacking β1-integrin subunit gene *Itgb1* stop migrating before they reach the caecum, generating an HSCR-like phenotype (Breau et al., 2009, 2006). Severe enteric network disorganization along the whole length of the gut was also clearly apparent and defects observed were shown to result from impaired migratory abilities and enhanced aggregation properties of mutant ENCC.

Here, we investigated the interaction between *Sox10* and *Itgb1* in ENS development. The phenotypes of mice carrying combinations of *Sox10* and *Itgb1* mutations were analyzed, focusing on migration and cell adhesion processes. The effect of SOX10 overexpression or haploinsufficiency on β1-integrins expression and function was also examined.

Materials and methods

Animals and tissue collection

Mouse models used in this study are: *Sox10*^{tm1Weg} ((Britsch et al., 2001), referred as *Sox10*^{lacZ} in our study), Gt(ROSA)26 Sor$^{tm1(EYFP)Cos}$ ((Srinivas et al., 2001) referred as R26RYFP), *Itgb1*^{tm1Ref} ((Potocnik et al., 2000), referred as *beta*^{fl}), *Itgb1*^{tm2Ref} ((Fassler and Meyer, 1995), referred as *beta1*^{neo}) and Tg(PLAT-cre) 116Sdu ((Pietri et al., 2003) referred as Ht-PA::Cre). Crossing strategy was as follows: homozygous Ht-PA::Cre mice were crossed with heterozygous *beta1*$^{neo/+}$ mice and subsequently with Sox10$^{lacZ/+}$ heterozygotes to generate Ht-PA::Cre; *beta1*$^{neo/+}$; *Sox10*$^{lacZ/+}$ mutants. These mice were then crossed with *beta1*$^{fl/fl}$; R26RYFP mice to generate eight progeny genotypes, corresponding to five classes of mutants referred as controls, SOX10 heterozygotes (*Sox10*$^{lacZ/+}$), beta1-null, double heterozygotes (DH), and double mutants (DM). The crosses, the eight genotypes generated and the five corresponding classes are reported in Table 1, along

Table 1
Genotypes distribution and viability analysis of animals resulting from Sox10$^{lacZ/+}$ and beta1 mice crosses.

Parents	Crossing strategy and offspring classification			E10.5–E17.5 embryos		Pups					
	Genotypes	Mutant classes	% expected	Number	% observed	Number	% observed	animals dead between P0 and P2	animals dead between P3 and P23	animals dead between P23 and 5 weeks	% mortality over period
Ht-PA::Cre; *beta1*$^{neo/+}$; *Sox10*$^{lacZ/+}$ X *beta1*$^{fl/fl}$; R26RYFP	*beta1*$^{+/fl}$; *Sox10*$^{+/+}$; R26RYFP	Controls	12.5	38	11.48	13	13.98	0	0	0	0.0
	Ht-PA::Cre; *beta1*$^{+/fl}$; *Sox10*$^{+/+}$; R26RYFP		12.5	41	12.39	14	15.05	0	0	0	0.0
	beta1$^{neo/fl}$; *Sox10*$^{+/+}$; R26RYFP		12.5	41	12.39	14	15.05	0	0	0	0.0
	beta1$^{+/fl}$; *Sox10*$^{lacZ/+}$; R26RYFP	Sox10$^{lacZ/+}$	12.5	38	11.48	17	18.28	1	0	0	5.9
	Ht-PA::Cre; *beta1*$^{neo/fl}$; *Sox10*$^{+/+}$; R26RYFP	beta1-null	12.5	58	17.52	7	7.53	2	0	1	42.9
	Ht-PA::Cre; *beta1*$^{+/fl}$; *Sox10*$^{lacZ/+}$; R26RYFP	DH	12.5	40	12.08	8	8.60	0	0	0	0.0
	beta1$^{neo/fl}$; *Sox10*$^{lacZ/+}$; R26RYFP		12.5	52	15.71	12	12.90	0	1	0	8.3
	Ht-PA::Cre; *beta1*$^{neo/fl}$; *Sox10*$^{lacZ/+}$; R26RYFP	DM	12.5	36	10.88	8	8.60	6	2	0	100.0

The crossing strategy, the eight genotypes generated and the five corresponding classes are presented. All genotyped embryos and pups are represented in the expected Mendelian ratio (χ^2 analysis).

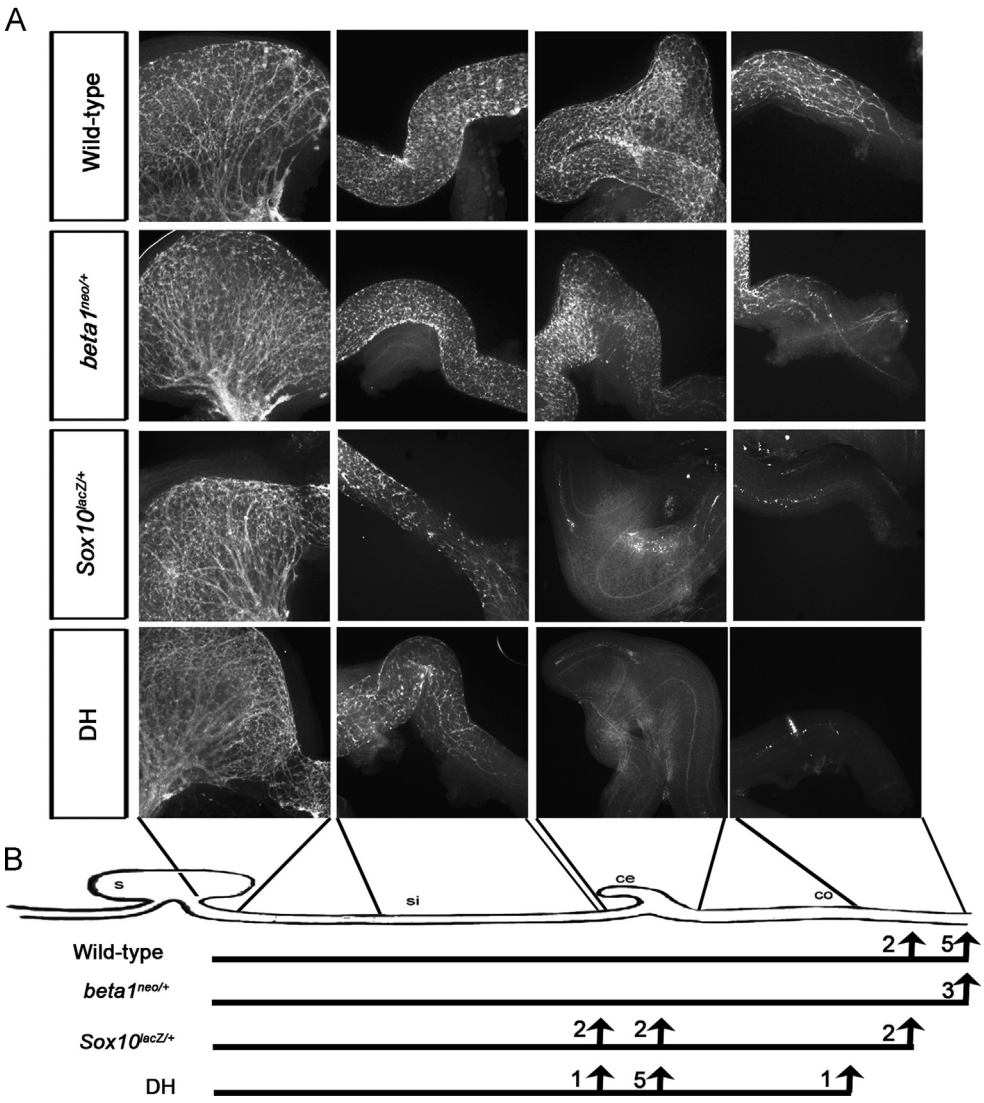

Fig. 1. Genetic interaction between *Sox10* and *Itgb1*. (A) Whole-mount TUJ1 immunohistochemistry on E13.5 guts from wild-type, *beta1*$^{neo/+}$, *Sox10*$^{lacZ/+}$, and DH embryos. Panels (left to right) show staining in the distal stomach, middle of small intestine, caecum, and colon, respectively. (B) Schematic representation of the gut. The areas marked at the top represent the regions of the gut shown in A. Below the schematic, the lines and perpendicular arrows indicate the extent of colonization for each class of embryos. The number of embryos presenting with a defined defect is indicated to the left of each arrow. s, stomach; si, small intestine; ce, caecum; co, colon.

with the total number of embryos (ranging from E10.5 to E17.5) of each genotype collected during the study, showing that up to E17.5, all genotypes are represented in the expected Mendelian ratio. Death rate of each class of mutant postnatally is also reported in Table 1.

Experiments were performed in accordance with the ethical guidelines of the INSERM and CNRS. Embryos were obtained from timed pregnancies. Dissected guts were used for explant or acute cultures, video-microscopy, and flow cytometry analysis. Alternatively, guts or embryos were fixed, sectioned and/or used to perform various labelings.

Organotypic cultures, Immunostainings and X-Gal staining

Ex-vivo cultures of guts were carried out as in Breau et al. (2009). Immunostainings of sections, embryos, guts, acute or 2D cultures were performed as described (Bondurand et al., 2003; Broders-Bondon et al., 2012), using the primary and secondary antibodies shown in Supplementary Table 1. Immunostained samples were examined using an Olympus SZH10 stereo microscope coupled to Visilog, a Zeiss Axioplan 2 confocal microscope coupled to Metamorph, at the Nikon Imaging Center of Curie Institute (NIMCE@IC-CNRS). Focal adhesions were quantified using ImageJ software. X-Gal staining followed standard procedures.

Enteric network quantification

Image stacks were acquired at the proximal or median part (first third) of small intestine as well as at the migratory wave front (variable region depending on the phenotypes observed). To quantify the ENCC density, the area devoid of YFP$^+$ cells (ENCC) and TUJ1$^+$ cells (neurons) was analyzed as in Broders-Bondon et al. (2012), and the proportion of ENCC-free area per image determined. Segmentation was applied on the maximum intensity projection of 3–6 confocal slices taken from stained guts using a self-developed imageJ macro (Broders-Bondon et al., 2012) based on K-means clustering (Dima et al., 2011).

Video time-lapse imaging

Video time-lapses of *ex-vivo* gut cultures, individual ENCC tracking within the gut tissue, speed of locomotion, directionality and persistence measurements were performed as in Breau et al. (2009) and Broders-Bondon et al. (2012).

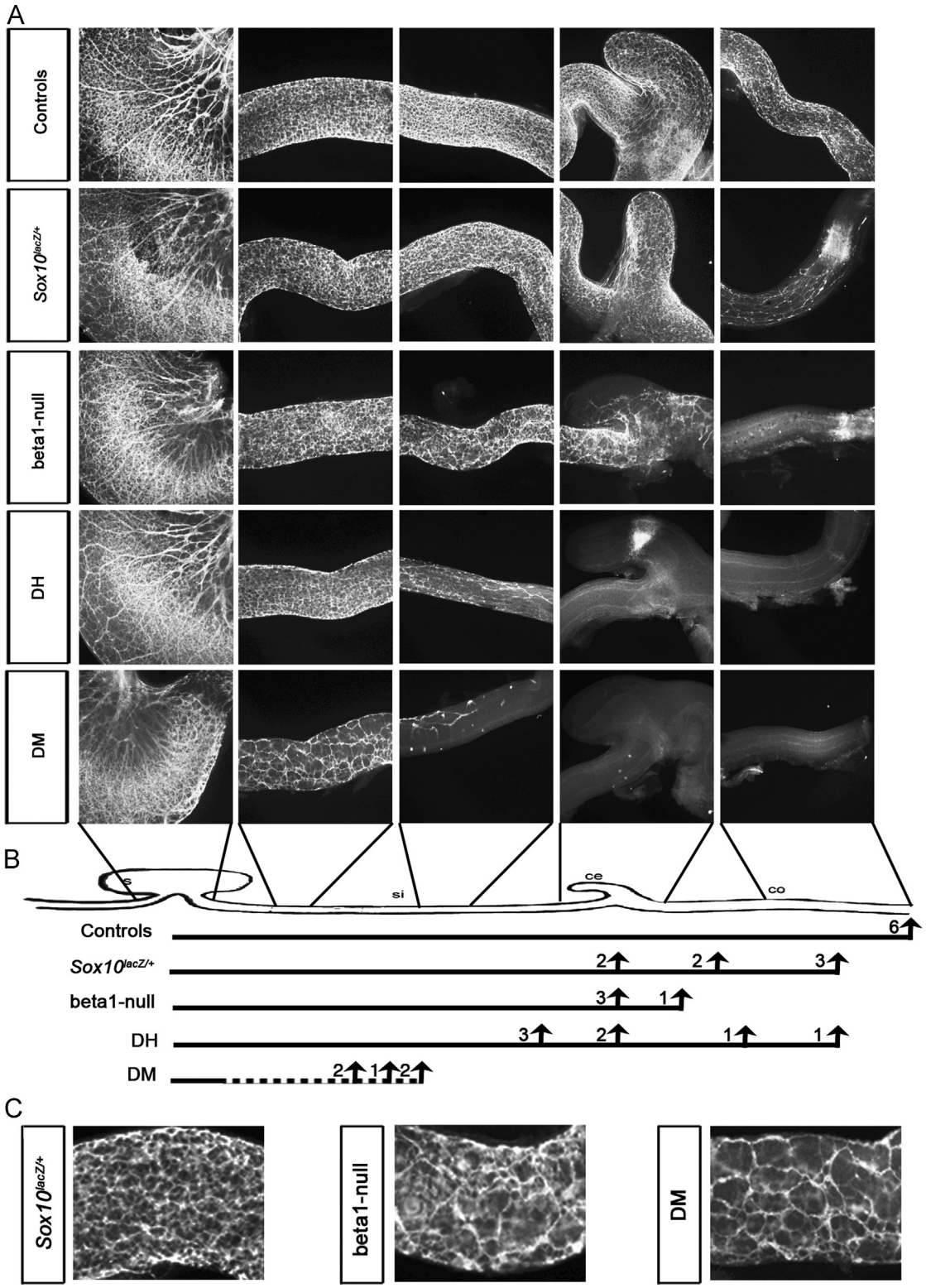

Fig. 2. Genetic interaction between *Sox10* and *Itgb1* controls ENS development. (A) Whole-mount TUJ1 immunohistochemistry on E14.5 guts from controls, $Sox10^{lacZ/+}$, beta1-null, DH, and DM embryos. Panels (left to right) show staining in the distal stomach, proximal and median part of the small intestine, caecum, and colon, respectively. (B) Schematic representation of the gut and phenotypes observed are presented as in Fig. 1. (C) Higher magnification panels of $Sox10^{lacZ/+}$, beta1-null, and DM small intestine showing network disorganization. s, stomach; si, small intestine; ce, caecum; co, colon.

Cell culture, transfection and flow cytometry

Neuro 2a (N2a) neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and transfected with a SOX10-GFP tagged construct under CMV promoter (Rehberg et al., 2002) using Lipofectamine Plus reagents (Gibco BRL).

N2a cells were trypsinised, centrifuged at 1300 rpm for 10 min at 4 °C, and resuspended in DMEM/F12 (Gibco BRL). Embryonic guts were dissociated as described previously (Bondurand et al.,

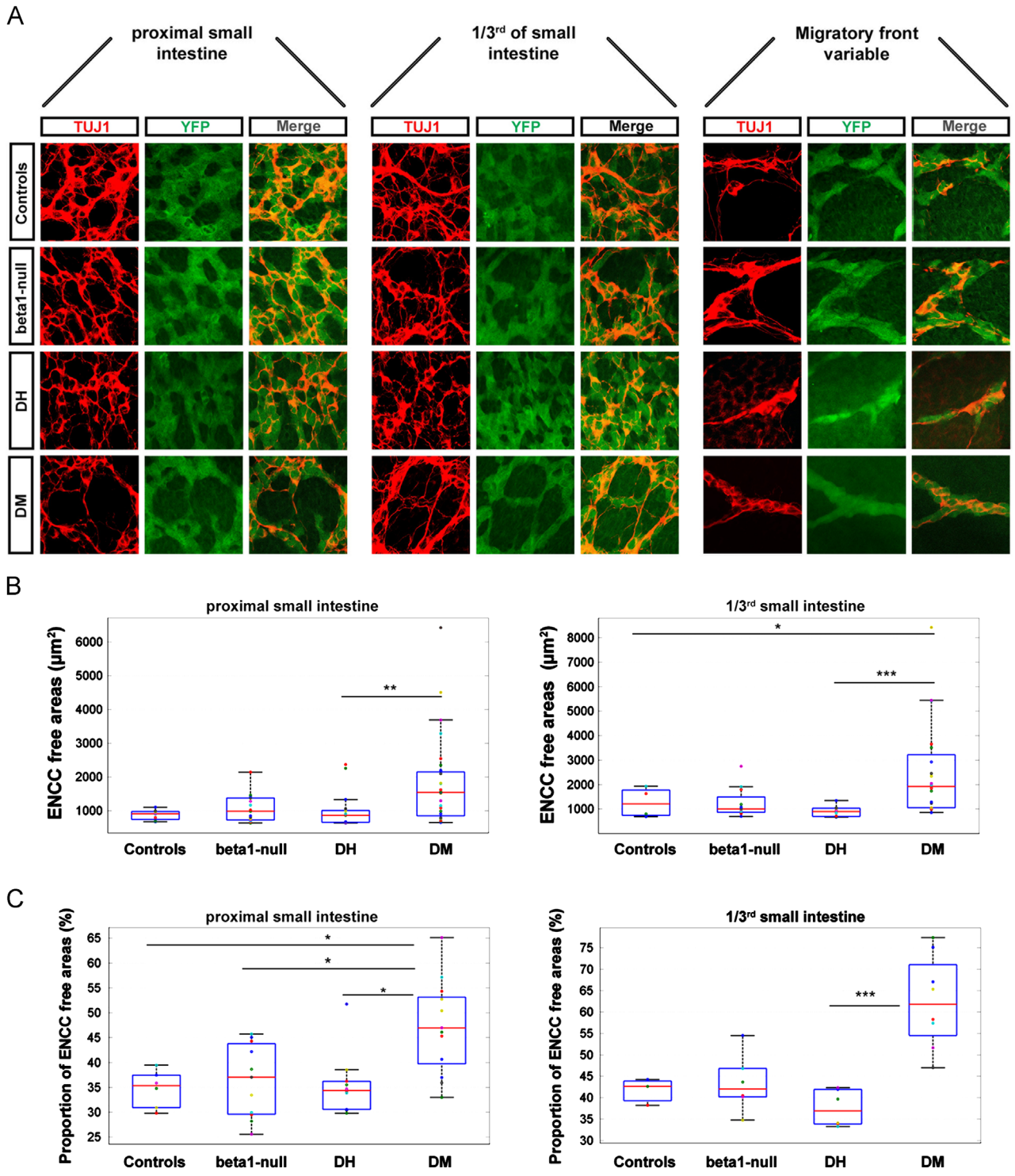

Fig. 3. Comparison of network organization of single and double mutants. (A) Confocal compilation showing neuronal network (TUJ1), ENCC distribution (YFP) and combination (Merge) within the proximal and median (1/3rd) small intestine and migratory wave front, in controls, beta1-null, DH, and DM. (B–C): Quantification of network disorganization within the proximal and median part of the small intestine. (B) Quantification of ENCC-free areas relative to mean area in controls, summarized as box plots. The top and bottom of each box are the 25th and 75th percentiles of the ENCC-free regions areas, respectively. The red line in the middle of the box is the median. (C) Proportion of ENCC-free areas, expressed as percent of the field of view (1024 × 1024 pixels, or 107 × 107 µm) and summarized as box plots, as in (B).

2003). Gut and N2a cell suspensions obtained were incubated with RPE or APC-labeled primary or secondary antibodies directed against the extracellular domain of integrin subunits (β1, or α5) on ice for 30 min, and analyzed with a CyAnADPLX7 instrument (Beckman-Coulter).

Real-time Q-PCR

Total RNA was isolated and reverse transcribed using standard procedures and cDNA were amplified using Fast SYBR® Green Master Mix (Applied Biosystems). PCR analysis was performed in

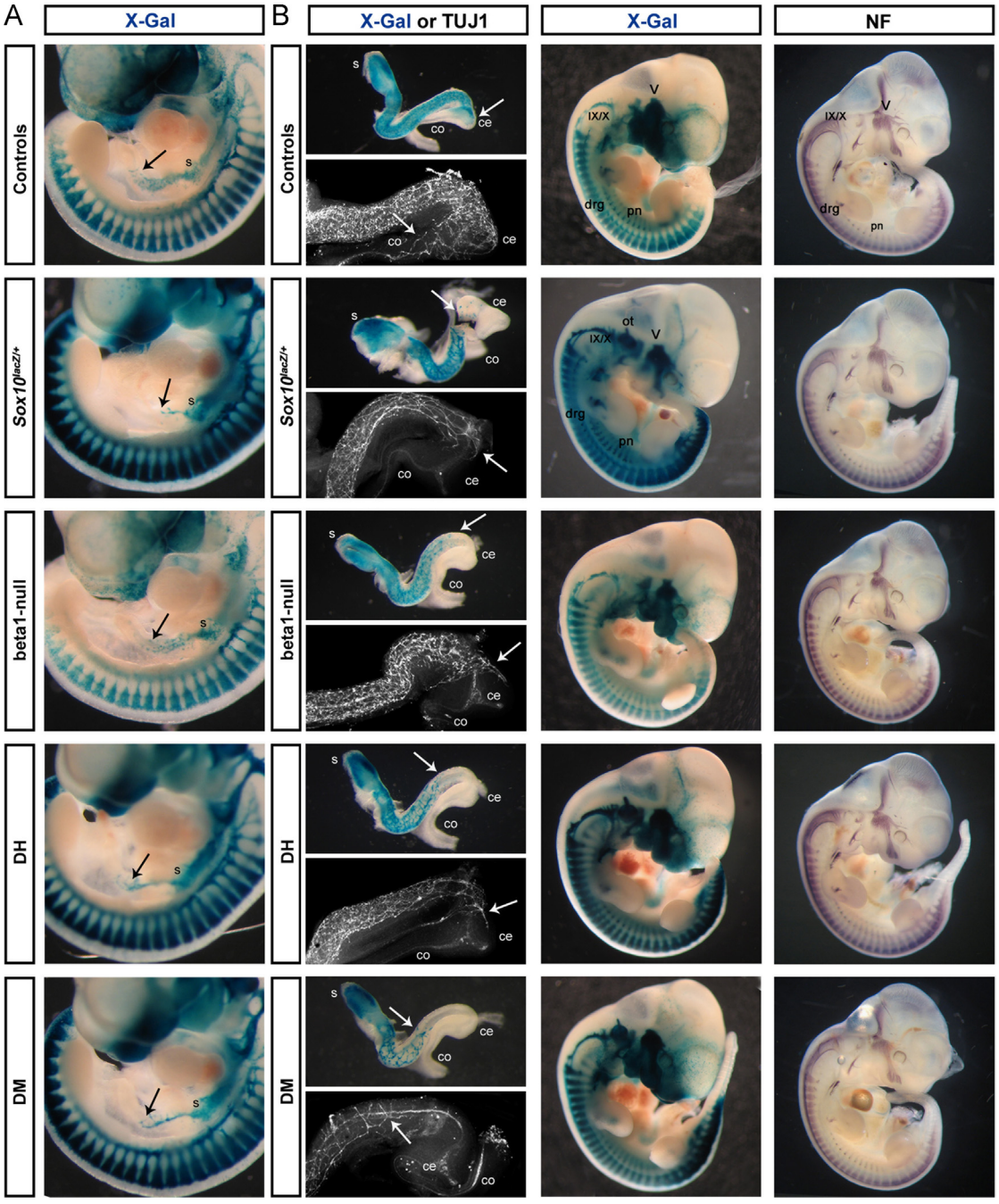

Fig. 4. Timing of ENS defects in controls, *Sox10*$^{lacZ/+}$, beta1-null, DH, and DM embryos. (A) E10.5 whole-mount X-Gal staining showing ENCC migration along the foregut. Note that the lacZ reporter is present in the *Sox10*^{lacZ} allele as well as in the *Itgb1*-floxed locus and therefore shows *Sox10* and/or *Itgb1* in targeted cells, depending of the genotypes of the embryos. (B) Analysis of E11.5 embryos with combined *Sox10* and *Itgb1* mutations. X-gal (E11.5) and TUJ1 (E12.25) staining/labeling performed on whole-mount guts are shown in the first column. X-Gal and neurofilament (NF) staining/labeling of whole embryos are shown in the last two columns. s, stomach; ce, caecum; co, colon; drg, dorsal root ganglia; V and IX/X, cranial ganglia; pn, peripheral nerves; Ot, otic vesicle. Wave front of migration is indicated by black or white arrows in A and B, respectively.

duplicates for three independent experiments and analyzed by the ΔCt method using cyclophilin B as housekeeping gene and GFP- cells for normalization. Primers are available upon request.

Statistical analysis

In Fig. 3, mutant phenotypes were compared to controls using the Kruskal Wallis test for multiple comparisons. For quantification of cell-free area, only the areas above the mean size of the control free areas were compared. Significance levels were: * < 0.05, ** < 0.01, *** < 0.005.

In Figs. 6–8 and Supplementary Fig. 1, results are mean ± SEM. Statistical significance was tested with Student's *t*-test. *P* values: * < 0.05, ** < 0.01, *** < 0.001.

Results

Genetic interaction between Sox10 and Itgb1 controls ENS formation

To test for a genetic interaction between *Sox10* and *Itgb1*, we first crossed *Sox10*$^{lacZ/+}$ mice with *beta1*$^{neo/+}$ animals and compared the

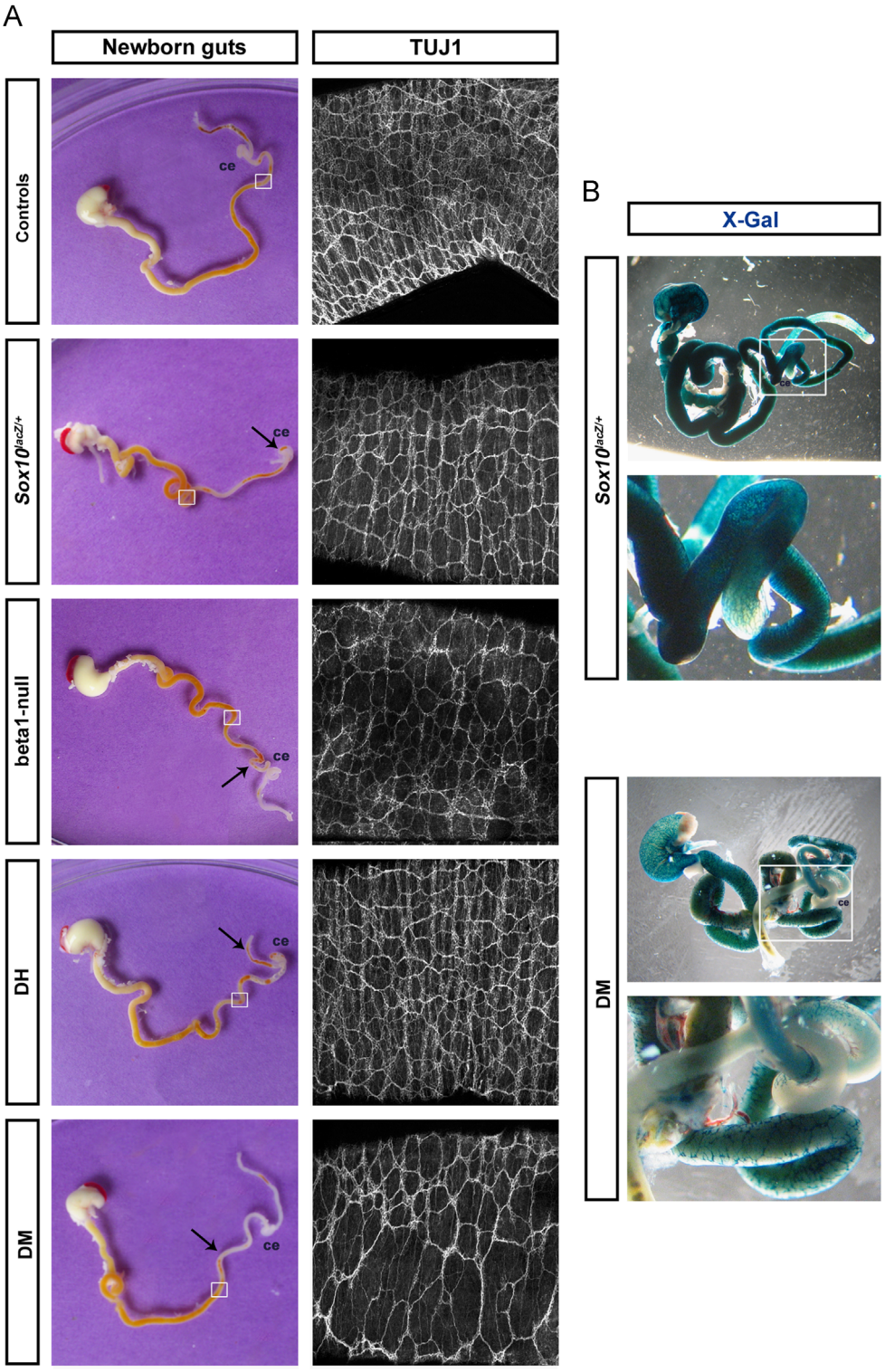

Fig. 5. Phenotypic analysis of guts from newborn mice with *Sox10* and *Itgb1* mutations. (A) Controls, $Sox10^{lacZ/+}$, beta1-null, DH, and DM guts were dissected and photographed (first column). The same guts were used subsequently for TUJ1 immunohistochemistry (second column). White boxes indicate the region presented in the second column. Black arrows indicate the transition zone from stenotic to dilated gut segments. (B) Whole-mount X-Gal staining of guts from $Sox10^{lacZ/+}$and DM showing severe disorganization of the enteric network at birth. White boxes indicate the region shown in higher magnification. In A and B, note the extensive aganglionic segment in the hindgut of DM postnatal mice. ce, caecum.

enteric phenotypes of single and double heterozygotes upon normal complete colonization of the gut by ENCC (E13.5–E14.5) (Barlow et al., 2003; Young et al., 1998). As distribution of enteric neurons reflects the progress of migration and differentiation of ENS progenitors, we compared neurogenesis in the gut of mutant embryos of different genotypes at E13.5 using whole-mount immunostaining with the neuronal class III β-tubulin TUJ1 marker (Barlow et al., 2003; Stanchina et al., 2006). Consistent with previous findings, the guts of wild-type and beta1 heterozygotes were fully colonized whereas the majority of $Sox10^{lacZ/+}$ embryos presented with colonization delay (Fig. 1 and (Breau et al., 2006; Maka et al., 2005; Stanchina et al., 2006, 2010)). No additional

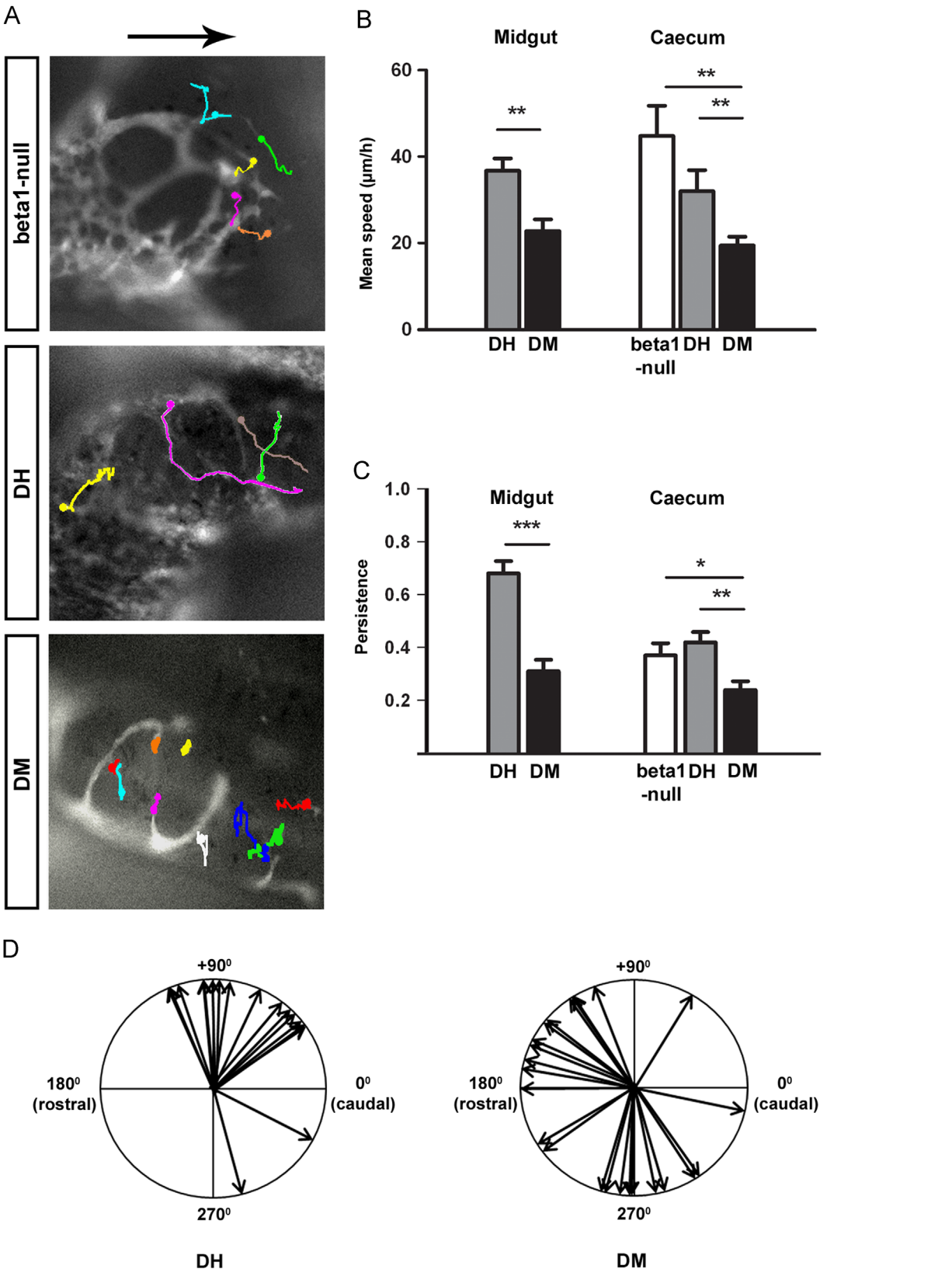

Fig. 6. Migratory properties of ENCC at the migratory front. (A) Individual trajectories of ENCC within the midgut or caecum of E12.5 beta1-null, DH, and DM embryos. The tracks overlay the first image in the time series, with the initial positions of the cells indicated by circles. Average speed (B) and persistence (C) of tracked DH ($n=18$), and DM ($n=27$) ENCC within the midgut and beta1-null ($n=21$), DH ($n=16$), and DM ($n=20$) ENCC within the caecum. (D) Directionality of tracked DH ($n=18$) and DM ($n=24$) ENCC.

defects were observed in DH compared to single mutants (Fig. 1A and B, last lanes). The enteric phenotype of Sox10 heterozygous; beta1-null double mutants (DM) was therefore analyzed.

Because the homozygous *Itgb1* knockout is embryonic lethal, we used a conditional ablation strategy. Using *beta1*^{fl}; R26RYFP and Ht-PA::Cre mouse lines, *Itgb1* was deleted in all neural crest

cells (NCC) as they emerge from the neural tube (Breau et al., 2006). We crossed Ht-PA::Cre; *beta1*$^{neo/+}$; *Sox10*$^{lacZ/+}$ with *beta1*$^{fl/fl}$; R26RYFP mice and analyzed the enteric phenotypes of the five classes of mutants generated at E14.5 (for detailed genotypes see Table 1). A colonization delay was detected in the majority of *Sox10*$^{lacZ/+}$ animals, and beta1-null single mutants (absence of TUJ1^{+} cells from the caecum or from the middle of the colon caudally, Fig. 2A and B). Three of the seven DH embryos had a slight colonization delay compared to single mutants (Fig. 2A and B); however, similar experiments performed at earlier (E12.5) and later (up to E17.5) stages did not confirm this observation. In contrast, guts from DM embryos showed a severe colonization delay. TUJ1 staining stopped within the first half of the small intestine of all embryos analyzed (Fig. 2A and B). Therefore, combined deficits in SOX10 function and β1-integrins-mediated adhesion caused profound ENS defects, arguing for a genetic interaction between the two loci that could rely on cooperative activity or on a successive requirement of these two molecules during ENS development.

As previously described (Breau et al., 2009, 2006), a disorganization of the neuronal network with abnormal aggregates surrounded by enlarged TUJ1-free spaces was also observed along the beta1-null mutant guts. This network disorganization was not observed in *Sox10*$^{lacZ/+}$, but was exacerbated in DM that exhibited larger TUJ1-free regions (Fig. 2C).

Quantitative analysis highlights enteric network disorganization in DM

To determine whether the observed phenotypes arise from alterations affecting all ENCC or result from defects in neuronal differentiation, we took advantage of the *R26RYFP* locus under the control of Ht-PA::Cre present in our lines. Neuronal (TUJ1^{+}) and ENCC (YFP^{+}) populations were analyzed in three regions: the proximal and median part (first third) of the small intestine and the migratory wave front (Fig. 3A). YFP^{+} and TUJ1^{+} cells exhibited a similar pattern throughout, suggesting that enteric defects observed result from the absence of all ENCC along a variable length of the intestine. In colonized regions, organization of both cell types was also very similar, with enlarged ENCC-free spaces observed in DM compared to other genotypes (Fig. 3A), confirming the disorganization of enteric network observed affect all ENCC.

To quantify this defect more carefully, the ENCC distribution was analyzed in the proximal and median part of the small intestine of embryos of different genotypes (Fig. 3B). The areas devoid of cells that are above the mean size of the control cell-free areas were measured. Significantly larger ENCC-free areas were observed in DM compared to DH in both regions (proximal small intestine; 1777 ± 242 and 940 ± 113 respectively, $P < 0.01$ and 1/3rd of small intestine 2341 ± 414 and 797 ± 64, $P < 0.005$, respectively). The proportion of ENCC-free areas was also quantified and expressed as the percentage of the image not containing YFP^{+} cells (Fig. 3C), giving values inversely proportional to density. The proportion of ENCC-free areas was similar for controls, DH, and beta1-null mutants (34.72 ± 1.52, 36.32 ± 2.22 and 35.54 ± 2.00, respectively), but significantly increased within the proximal small intestine of DM (47.04 ± 2.52, $P < 0.05$ in each case). Increased proportion was also observed in the median part of the small intestine of DM, but significantly different upon DM and DH comparison only (62.42 ± 3.81 and 37.51 ± 1.73, $P < 0.005$, respectively; controls: 41.71 ± 1.81; beta1-null: 43.40 ± 2.76, $P < 0.16$). Therefore, in *Sox10*$^{lacZ/+}$ ENCC, depletion of β1-integrins leads to a significant change in the ENS network, with larger meshwork size, and a reduced density of ENCC, indicating that the organization of the ENS is modulated by the interplay between β1-integrins and SOX10.

Timing analysis reveals severe ENS defects from E11.5 onwards

The behavior of vagal NCC at the time of foregut invasion was compared among genotypes. X-Gal staining of E10.5 whole-mount embryos showed stained cells in the stomach region and, irrespective of the genotype of the embryos, cells moving in lines to colonize the midgut (see black arrows, Fig. 4A), suggesting an absence of additional defects in DM compared to single mutants at this stage.

One day later (E11.5), the results were different (Fig. 4B). X-Gal and TUJ1 stainings on whole-mount gut preparations revealed a slight delay in *Sox10*$^{lacZ/+}$, beta1-null, and DH embryos compared to controls, with the front of migrating ENCC in the mutants stopping in the final quarter of the midgut, or just before the caecum (Fig. 4B first column). This delay was clearly exacerbated in DM embryos (cells never reached the second half of the midgut, $n=5$; Fig. 4B). Network disorganization was also visible in colonized regions of the DM gut, suggesting that DM defects are detected by E11.5. The observation of other NCC derivatives such as cranial ganglia, dorsal root ganglia and peripheral nerves by X-Gal staining or neurofilament (NF) whole-mount immunohistochemistry revealed no obvious differences between DM and single mutants at this stage (Fig. 4B) or earlier (data not shown), suggesting that a cooperative requirement of *Sox10* and *Itgb1* is required for ENCC migration along the gut only.

To determine whether ENS defects in DM were overcome after E14.5, guts from newborn mice were photographed and stained for TUJ1 and X-Gal. As shown in Fig. 5, the aganglionic gut segment in DM often extended above the caecum and affected the last third of the small intestine (Fig. 5A, compare position of black arrows). The ENS network was also more disorganized in colonized regions of DM compared to other genotypes (Fig. 5A and B). The postnatal survival of these animals was monitored up to 5 weeks of age (Table 1). Almost all *Sox10*$^{lacZ/+}$ and DH survived, but 43% of beta1-null and 100% of the DM died before weaning. Six of the eight DM died within the first 2 days after birth, but milk was present in the stomach (Fig. 5A), indicating that they started feeding. The remaining DM pups died at 19 and 23 days of unknown causes. Thus, ablation of β1-integrins in *Sox10* heterozygous animals results in more severe ENS defects from E11.5 that are not compensated in later development and reduce postnatal survival.

Video-microscopy demonstrates altered migration properties of DM ENCC

To determine the cellular mechanism underlying the ENS defect observed, we first compared the proliferation and survival capacities of ENCC of different genotypes. To this end, E12.5 guts were dissociated, plated in acute culture over a short period of time, fixed and used for immunolabelling. Cell death was quantified by counting the activated caspase-3 positive cells among the SOX10 positive population and no statistical difference was found. Indeed, the percentage of apoptosis in controls, *Sox10*$^{lacZ/+}$, beta1-null, DH and DM ENCC was $0.37 \pm 0.09\%$, $0.52 \pm 0.22\%$, $0.58 \pm 0.16\%$, $0.51 \pm 0.13\%$ and $0.72 \pm 0.15\%$, respectively ($n > 4$; DM vs. controls ($P=0.07$), *Sox10*$^{lacZ/+}$ ($P=0.47$), beta1-null ($P=0.56$), DH ($P=0.33$)). Proliferation was quantified by counting the Phospho-Histone H3 positive cells among the SOX10 population and no significant difference was observed either. Indeed, proliferation rates in controls, *Sox10*$^{lacZ/+}$, beta1-null, DH and DM ENCC were $10.5 \pm 1.4\%$, $9.4 \pm 0.7\%$, $11.6 \pm 1.3\%$, $9.2 \pm 1.2\%$ and $9.5 \pm 0.5\%$, respectively ($n=3$; DM vs. controls ($P=0.51$), *Sox10*$^{lacZ/+}$ ($P=0.94$), beta1-null ($P=0.12$), DH ($P=0.81$)). As gut colonization defects in beta1-null mice were previously shown to result from impaired migratory abilities and enhanced aggregation properties

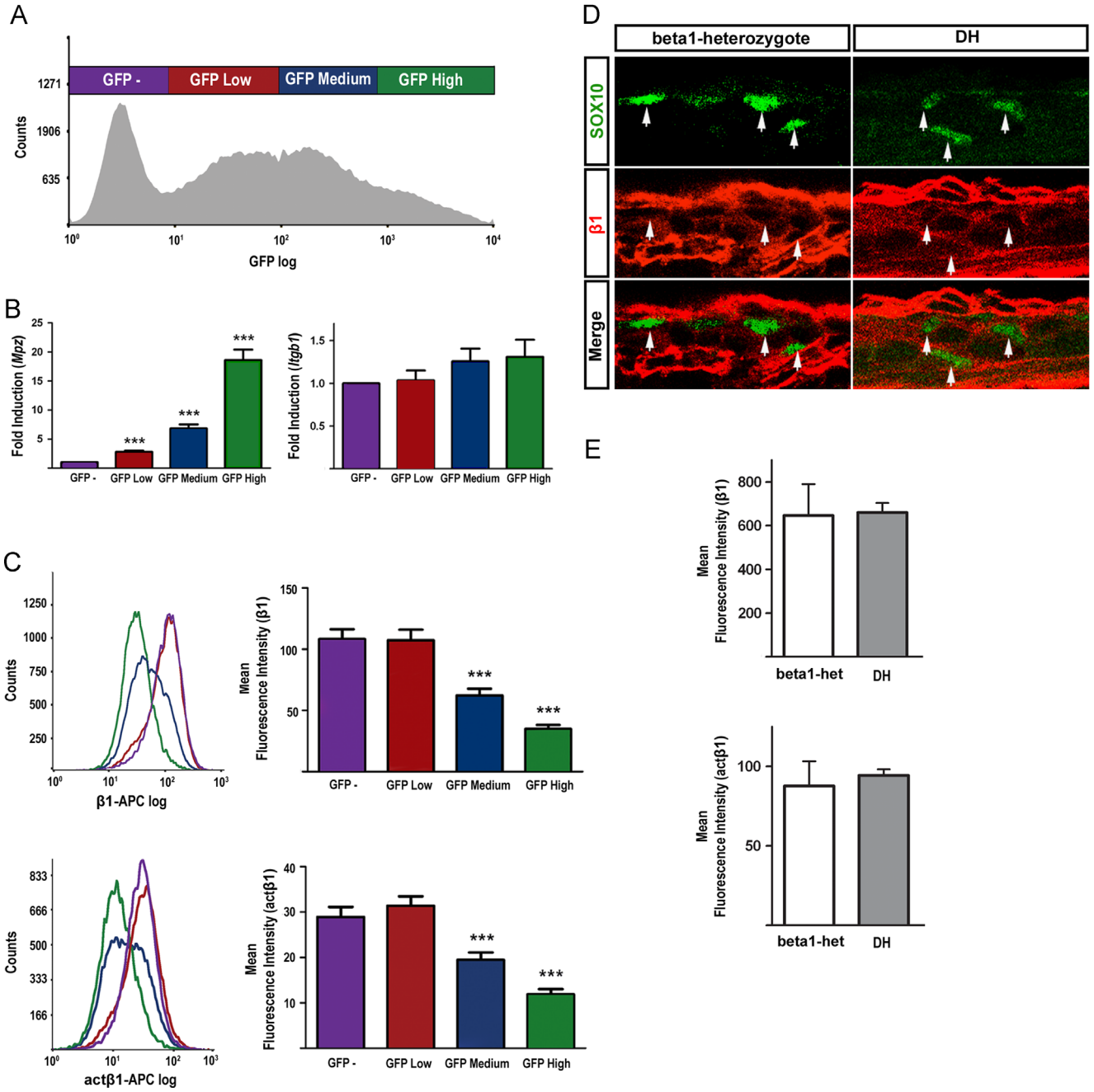

Fig. 7. Effect of SOX10 overexpression or *Sox10* haploinsufficiency on *Itgb1*/β1-integrins expression. N2a cells were transfected with a SOX10-GFP expression vector. (A) Four chosen cell populations expressing increasing amounts of SOX10-GFP were used for (B) Real time quantitative-PCR analysis of the expression of *Mpz* (graph on the left) and *Itgb1* (graph on the right), and for (C) flow cytometry analyses of β1 integrins expression. In (B), results are presented as relative *Mpz* and *Itgb1* expression (fold induction) compared to the GFP-cells. In (C), β1 and activated β1 (actβ1) –APC mean fluorescence intensity are presented as raw data or relative mean intensity of 3 experiments. (D) E17.5 gut sections from beta1 heterozygotes and DH were labeled with SOX10 and β1-integrins antibodies (E) Beta1 heterozygotes (beta1-het) and DH E14.5 guts were dissociated and used for flow cytometry analysis to quantify β1- and actβ1 integrin associated-RPE mean fluorescence intensity among the YFP$^+$ population.

of mutant ENCC (Breau et al., 2009), the dynamic behavior of ENCC of different genotypes was examined.

The migration of YFP$^+$ cells in E12.5 gut tissues in *ex-vivo* cultures was imaged using time-lapse fluorescence microscopy. Individual cell trajectories showed that DM cells are unable to migrate for long distances (but rather form aggregates) compared to DH and beta1-null cells (Fig. 6A). The other genotypes did not express YFP and the controls beta1 heterozygotes have already reached the hindgut by E12.5 and were not tracked.

The migration speeds of individual cells were measured for beta1-null, DH and DM at the migratory front. The beta1-null ENCC had invaded the ceacum at this stage and their mean speed of locomotion was found to be 44.80 ± 6.96 μm/h ($n=21$). Due to phenotype variability, DH ENCC speed was measured in the midgut or ceacum region depending on the embryos analyzed. ENCC mean speed was very similar in both regions (36.73 ± 2.87 μm/h ($n=18$) and 31.96 ± 3.48 μm/h ($n=16$), respectively), and not significantly different from that of beta1-null cells ($P=0.11$). In most cases, DM ENCC were found migrating in the midgut region and their mean speed (22.80 ± 2.69 μm/h, $n=27$) was significantly reduced, indicating that the more severe ENS defect is partly due to altered cell migration capacities (Fig. 6B).

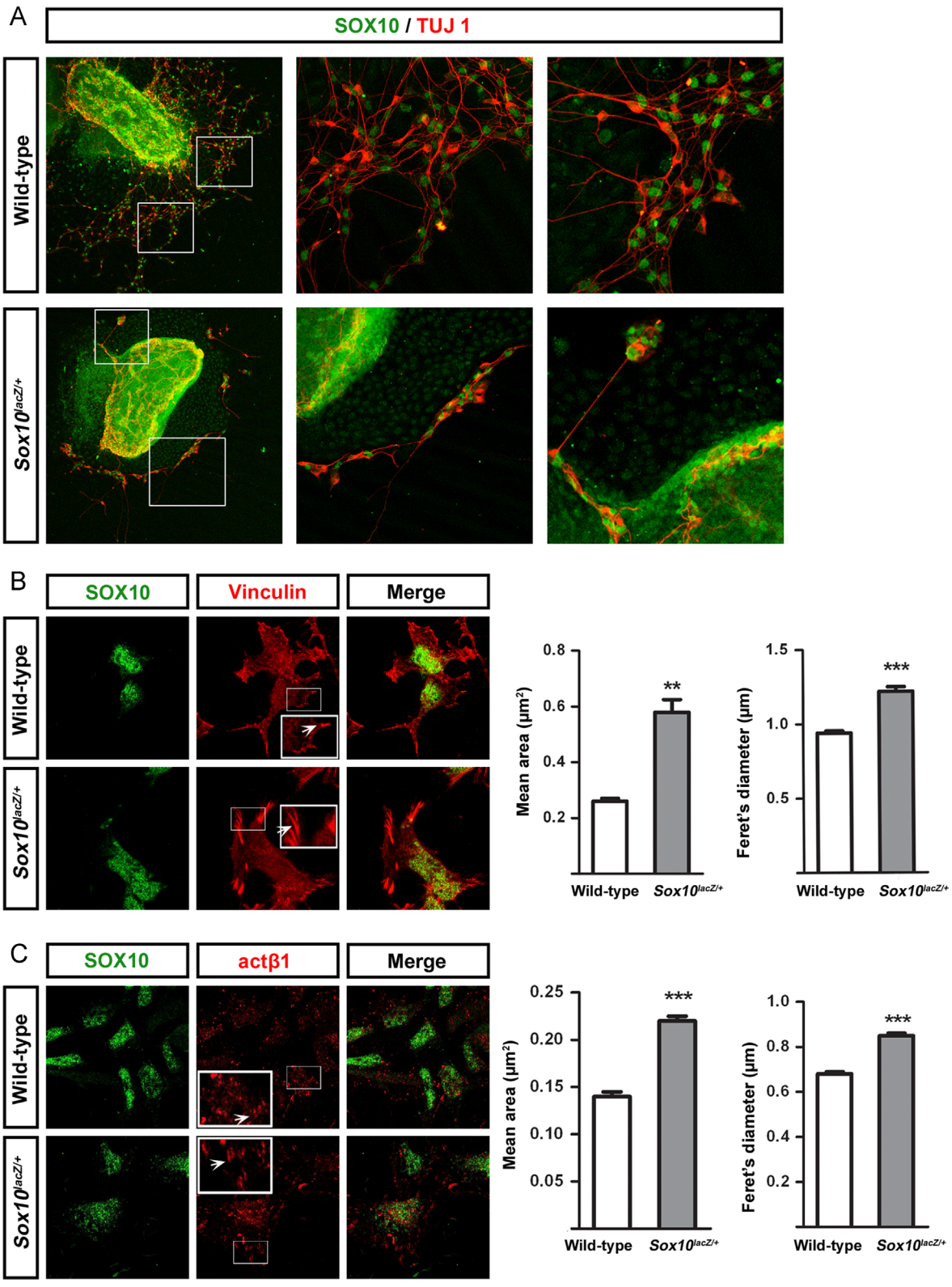

Fig. 8. Effect of *Sox10* haploinsufficiency on cell adhesion in-vitro. (A) Gut cross-sections from E12.5 Wild-type and *Sox10*$^{lacZ/+}$ midgut were placed on FN-coated and labeled with SOX10/TUJ1 antibodies. Low magnification images provide an overview of the explant morphology. White boxes in (A) indicate the region shown in higher magnifications, providing detail. (B) Quantification of focal adhesions (FA) in wild-type and *Sox10*$^{lacZ/+}$ ENCC measured by localization of Vinculin and SOX10 using antibodies provided in Supplementary Table 1. (C) Quantification of activated β1-integrins (act β1) in FAs. In (B) and (C) graphs show the mean area and mean Feret's diameter indicating the size of FAs. To generate data presented in A, B and C, note that three independent experiments were carried out using 2 to 3 embryos of each genotype.

In the only one DM sample in which cells were found to reach the caecum, ENCC presented a mean speed of migration of 19.41 ± 2.08 (n=20), displaying a decreased velocity compared to beta1-null and DH ENCC (P=0.0018 and P=0.0041 respectively, Fig. 6B).

The same cell trackings were used to analyze the persistence (calculated by dividing the distance between its initial and final positions by the total distance covered by the cell, Fig. 6C) and the directionality (evaluated by measuring the angle between the

rostro-caudal axis of the gut and the straight line separating the initial and final positions of the cell, Fig. 6D). These measurements confirmed the severe defects in DM. In the midgut, mutant cells showed decreased persistence compared to DH (0.31 ± 0.044 vs. 0.68 ± 0.047, respectively, $P < 0.0001$) (Fig. 6C). The one DM sample in which cells were found to reach the caecum also showed decreased persistence compared to other genotypes (0.24 ± 0.033, 0.42 ± 0.039, and 0.37 ± 0.046 for DM, DH and beta1-null, respectively; DM vs. DH and beta1-null, $P=0.0014$ and $P=0.0289$, Fig. 6C). Finally, the directionality of migration of DM cells was found erratic too compared to DH, indicating that most of the leading cells migrated in the wrong (caudo-rostral) direction (Fig. 6D). Other DM cells were almost static. Altogether, our results highlight an essential role of SOX10 in cell migration that may depend on β1-integrins.

Effect of SOX10 overexpression or Sox10 haploinsufficiency on β1 and α integrins expression

SOX10 regulates the expression of several genes during ENS and other neural crest derivatives development, including *Ednrb*, *L1cam*, *Ret* and *Mpz* (Lang et al., 2000; Peirano et al., 2000; Wallace et al., 2010; Zhu et al., 2004). *Itgb1* expression was therefore analyzed in N2a cells upon SOX10 overexpression. We used N2a neuroblastoma cells for our analyses as these tumor cells are derived from and still exhibit some similarities to neural crest cells. Quantitative PCR analysis of four cell populations expressing increasing amount of SOX10-GFP (Fig. 7A) revealed that SOX10 strongly activates *Mpz* transcription as previously described (Peirano et al., 2000), but has no significant impact on *Itgb1* expression (Fig. 7B).

In parallel, we tested the effect of SOX10 overexpression on β1-integrins membrane protein levels (Fig. 7C). Flow cytometry analysis using APC-coupled antibodies directed against the total or activated form of the β1-integrin subunit revealed a shift in the mean fluorescence intensity upon SOX10 overexpression, suggesting that this transcription factor drastically reduce β1-integrins expression and activation in a dose-dependent manner (Fig. 7C).

In parallel, the expression of β1-integrins in E17.5 gut sections were examined in embryos with different combinations of *Sox10* and *Itgb1* mutant alleles (Fig. 7D). β1-Integrins were not expressed in beta1-null mutants or DM (data not shown). While β1-integrins showed lower expression in enteric ganglia compared to muscle at E17.5, β1-integrins were detected in $SOX10^+$ cells of wild-type, beta1 heterozygotes, $Sox10^{lacZ/+}$, and DH embryos (Fig. 7D and data not shown). The level of β1-integrins in ENCC (YFP^+) from E14.5 dissociated gut cell suspensions of beta1 heterozygotes (controls) and DH was also quantified by flow cytometry. The YFP^+ population represented 6.08 ± 0.68 and 5.14 ± 0.33 of the total population analyzed in controls and DH cells respectively, suggesting similar numbers of ENCC between the two genotypes. No significant difference in total or activated β1-integrins-RPE mean fluorescence intensity was detected (Fig. 7E), suggesting that *Sox10* haploinsufficiency does not affect β1-integrins expression.

We also tested whether SOX10 overexpression or haploinsufficiency could modify other integrins protein levels and focused on α5 and αv, since these subunits dimerize with β1, β3, or β5, and interactions between *L1cam* and *Sox10* (Wallace and Anderson, 2011; Wallace et al., 2010), αvβ3, αvβ1, and α5β1 (Felding-Habermann et al., 1997) have been reported. SOX10 overexpression reduced αv and α5-integrins levels in a dose dependent manner (Supplementary Fig. 1A). In contrast, immunofluorescent stainings of gut sections revealed no major change in the expression of either αv or α5 in embryos of various genotypes (Supplementary Fig. 1B). Altogether, our results suggest that SOX10 overexpression in-vitro but not *Sox10* haploinsufficiency in-vivo could affect expression of β1 and other integrins.

SOX10 haploinsufficiency alters cell adhesion properties

The effect of *SOX10* haploinsufficiency on cell adhesion was examined. Gut explant cultures using rings of E12.5 midgut were established. Fibronectin (FN) was used as a permissive substratum for adhesion and migration of ENCC. After 24 h of culture, explants were stained for SOX10 (to detect progenitors) and TUJ1 (to detect neurons). In control cultures, both cell types formed scattered networks around the explants, at the periphery of smooth muscle cells. In contrast, few $Sox10^{lacZ/+}$ ENCC were found outside the explants, and often formed aggregates containing both neurons and progenitor cells (Fig. 8A). These data suggest that $Sox10^{lacZ/+}$ ENCC are either unable to migrate or interact efficiently with their environment, and/or have modified intercellular adhesion properties.

The adhesion sites formed by wild-type or $Sox10^{lacZ/+}$ ENCC on the FN-coated surface (Fig. 8B) were examined by labeling with antibodies against SOX10 to visualize ENCC and vinculin to identify focal adhesions (FA). The number, area, and Feret's diameter of FA were quantified. Feret's diameter corresponds to the longest length of the focal adhesion signal, independently of its orientation in the cell. Wild-type and $Sox10^{lacZ/+}$ ENCC had similar numbers of FA (data not shown). However, mutant ENCC displayed a significant increase of the area and Feret's diameter per FA (0.58 ± 0.045 and 1.22 ± 0.03, respectively) compared to wild-type (0.26 ± 0.01 and 0.94 ± 0.015, respectively; Fig. 8B). The change in FA size suggests modifications in their dynamics in $Sox10^{lacZ/+}$ ENCC that may affect the migratory properties. These results also suggest that $Sox10^{lacZ/+}$ ENCC adhere more strongly to FN, possibly by increasing recruitment of β1- or β3-integrins at the FN contact sites. Of interest, *Sox10* heterozygotes also displayed a significant increase in the FA area and Feret's diameter visualized by activated β1-integrin subunit antibody (Fig. 8C). These results highlight an essential function of SOX10 in cell–ECM and/or cell–cell adhesion, events that are dependent on proper integrins function and β1, in particular.

Discussion

This study demonstrates an essential role for SOX10 in the control of migration and shows that the coordinated action of SOX10 and the β1-integrin subunit is required for proper ENCC migration along the gut and enteric neuronal network organization. Our observations thus extend the SOX10 interaction network and open new research areas concerning its function.

Analysis of ENS development in *Sox10*; *Itgb1* mutant revealed that complete removal of β1-integrins function in the context of *Sox10* heterozygosity leads to an increase in enteric phenotype severity. Both timing of ENCC colonization of the gut and quality of the ENS network were affected, highlighting a cooperative or successive requirement for SOX10 and cell–ECM adhesion receptors during ENS development.

An interaction between SOX10 and another adhesion molecule, L1cam was previously reported (Wallace et al., 2010). Authors clearly showed that *L1cam*; *Sox10* double mutants defects observed resulted from excessive cell death of neural crest cells prior to entering the gut. In contrast, and similar to *Sox10*; *Zeb2* DH (Stanchina et al., 2010), severe ENS defects in *Sox10*; *Itgb1* embryos were observed from E11.5 onwards, suggesting that cooperativity between SOX10 and β1-integrins is critical as ENCC migrate along the gut. However, we cannot exclude the possibility that crosstalk between SOX10 and β1-integrins occurs earlier, but that the

conditional ablation strategy used here prevented its observation. β1-integrins were shown to be barely detectable at the cell surface of beta1-null ENCC when they start to invade the foregut, at E9.5, and completely absent from E11.5 onwards (Breau et al., 2006). Minor amounts of β1-integrins on the cell surface at early stages, or the activity of αvβ3 integrin, also expressed by ENCC, may be sufficient, and explain the absence of ENS defect in DM before E11.5.

Previous analysis suggested that β1-integrins activity was crucial for caecum colonization only (Breau et al., 2006). By contrast, the affected gut segment in DM extended beyond the ileo-caecal junction through the small intestine, suggesting that migration within the midgut is under the control of coordinate action of SOX10 and β1-integrins or requires molecules acting in synergy with β1-integrins. Whether this function is mediated through FN or other ECM components in-vivo remains to be determined.

In addition to the ENS, *Sox10* and *Itgb1* are expressed in other NCC derivatives and their removal appears deleterious during the formation of some derivatives, Schwann cells in particular (Britsch et al., 2001; Mollaaghababa and Pavan, 2003; Pietri et al., 2004; Wegner, 2009). The formation and differentiation of other NCC derivatives in E10.5 and E11.5 DM was therefore examined, but no obvious additional defect was found, suggesting that the cooperative requirement for SOX10 and β1-integrins could be specific to ENS development. Alternatively, functional redundancy could explain the absence of other defects. Indeed, SOX9, SOX8, SOX2, and other integrins have been shown to play key roles in some NCC derivatives and could compensate for the partial or complete absence of SOX10 or β1-integrins in those cells (Beauvais-Jouneau and Thiery, 1997; Bronner-Fraser, 1986; Crump et al., 2004; Delannet et al., 1994; Desban and Duband, 1997; Kil and Bronner-Fraser, 1996; Le et al., 2005; Stolt and Wegner, 2009; Testaz and Duband, 2001; Wegner, 2009; Wegner and Stolt, 2005). However, we cannot exclude that interactions in other NCC derivatives could take place beyond the stages analyzed. We believe the ENS defects observed may not be the cause of the early postnatal mortality observed in DM. The presence of milk in the stomach shows that they are able to breathe and feed. SOX10 and β1-integrins involvement in the control of other NCC derived tissues such as lung or heart could explain early death, but further experiments are needed to test these possibilities.

To decipher the cellular basis of the ENS defects, migration of mutant ENCC was observed by video-microscopy. Similar migration speeds in DH and beta1-null ENCC suggested that SOX10 is involved in control of cell migration. Changes in cell adhesion properties were also observed in $Sox10^{lacZ/+}$ cells, as shown by the formation of cell aggregates upon coating on permissive substratum, suggesting heterozygous cells are unable to migrate or interact efficiently with their environment, and/or have modified intercellular adhesion properties. In addition, strong alterations in directionality, persistence and speed of migration observed in DM, indicated that the severe ENS defects observed are partly due to altered cell migration, and that SOX10 and β1-integrins act synergistically to control this process. To understand the molecular mechanisms underlying the *Sox10*/*Itgb1* genetic interaction, the effect of SOX10 overexpression or *Sox10* haploinsufficiency on integrins expression and function was examined. No effect of SOX10 overexpression on *Itgb1* transcription was found, suggesting that *Itgb1* is not a SOX10 target gene. In contrast, β1-integrins membrane protein levels and activation were clearly downregulated by SOX10 overexpression. β1-Integrins membrane protein levels were unchanged in $Sox10^{lacZ/+}$ and DH mutant ENCC, but increased Vinculin and activated β1-integrins clustering were observed at cell–FN adhesion sites revealed by larger FA. Integrins mediate the interaction of cells with ECM. During the development of cell–matrix adhesions, these receptors are activated and recruit structural and signaling proteins, which contribute to maturation of nascent adhesion sites into FA, making the link between ECM and the actin cytoskeleton and stimulating cell migration and contractility (for reviews see Wehrle-Haller (2012) and Zamir and Geiger (2001)). The change in the size of FA reflects modifications in either their maturation or dynamics, two processes that regulate cell migration.

The severe increase in ENS defects in DM suggests that other molecules whose signaling is in direct or indirect connection with β1-integrins might be affected by changes in *Sox10*. Immunohistochemistry and flow cytometry analyses of other α subunits showed that α5 and αv may also be under SOX10 control. The effect on other β chains remains to be determined.

Alternatively, SOX10 and β1-integrins could be part of a common signaling pathway. Signaling by vascular endothelial growth factor, platelet-derived growth factor receptor, and tyrosine kinase receptors such as c-Kit, EGFR, ErbBs are modulated by integrins. The essential function of ErbBs in ENS development, as well as their possible regulation by SOX10, suggests that SOX10 function in ENS migration could rely on *ErbB* upregulation and activation of β1-integrins dependent signaling cascades (Adelsman et al., 1999; Barczyk et al., 2010; Chalazonitis et al., 2011; Crone et al., 2003; Goodman and Picard, 2010; Streuli and Akhtar, 2009; Yamashita et al., 2010). SOX10, in synergy with its known cofactors, could also induce *Ret* or *Ednrb* expression (Lang et al., 2000; Lang and Epstein, 2003; Zhu et al., 2004) and subsequent RET- or EDNRB-β1-integrins dependent signaling cascades (Cockburn et al., 2010; Lange et al., 2007) and thus control cell migration. Finally, SOX10 has recently been shown to regulate other genes involved in migratory processes, some of them expressed in gut and interacting with components of β1-integrins signaling pathways (Finzsch et al., 2008; King et al., 2011; Lee et al., 2008; Veevers-Lowe et al., 2010; You and Lin-Chao, 2010). The defects observed could therefore be due to changes in crosstalk mediated by these genes.

Diverse human pathologies involve integrins-mediated cell adhesion, including thrombotic disease, inflammation (including inflammatory bowel disease), cancer, fibrosis, and infectious diseases (Goodman and Picard, 2010). *Itgb1* variations have not been reported in human pathology so far. The severe phenotype observed upon ablation of this gene (null mutants die soon after implantation due to inner cell mass defects) offers an explanation for the absence of human mutations. However, similar to *ITGAV*, *ITGA2*, and *ITGB3*, polymorphisms or common haplotypes could influence the phenotype resulting from mutations in other genes (Barczyk et al., 2010; Goodman and Picard, 2010; Napolioni et al., 2011). HSCR penetrance is incomplete in patients carrying *SOX10* mutations, and even within HSCR affected patients, the length of the aganglionosis and the nature of the transition zone can vary. Some patients with *SOX10* mutations have chronic intestinal pseudo-obstruction instead of HSCR (for review see Pingault et al. (2010)). The molecular basis for this phenotype is currently unknown. Here, DM not only present with an increase in the length of the non-colonized region, but also with an altered organization of the enteric network in the colonized intestine. Whether the SOX10/β1-integrins cooperativity contributes to the variability of phenotypes in patients with *SOX10* mutations remains to be clarified. It may also be of interest to search for variations in the *Itgb1* gene in patients carrying *SOX10* mutations and presenting with altered gut function without distal aganglionosis (pseudo-obstruction).

In conclusion, our study suggests that SOX10 is not only required to control stem cell maintenance and cell differentiation, but also is crucial in controlling cell migration and adhesion. Interestingly, *SOX10* is not the only *SOX* gene known to interact

with ECM. During chondrogenesis, SOX9 regulates collagen and aggrecan genes, suggesting a strong link between *SOX* genes and ECM formation (Guth and Wegner, 2008). Besides further our understanding of the molecular and cellular bases of ENS defects caused by SOX10 mutations in humans and mice, our results may contribute to the development of therapeutic strategies for HSCR and intestinal pseudo-obstruction.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.04.013.

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