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Common *PHOX2B* poly-alanine contractions impair RET gene transcription, predisposing to Hirschsprung disease

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ABSTRACT

HSCR is a congenital disorder of the enteric nervous system, characterized by the absence of neurons along a variable length of the gut resulting from loss-of-function RET mutations. Congenital Central Hypoventilation Syndrome (CCHS) is a rare neurocristopathy characterized by impaired response to hypercapnia and hypoxemia caused by heterozygous mutations of the PHOX2B gene, mostly polyalanine (polyA) expansions but also missense, nonsense, and frameshift mutations, while polyA contractions are common in the population and believed neutral. HSCR associated CCHS can present in patients carrying PHOX2B mutations. Indeed, *RET* expression is orchestrated by different transcriptional factors among which PHOX2B, thus suggesting its possible role in HSCR pathogenesis. Following the observation of HSCR patients carrying in frame trinucleotide deletions within the polyalanine stretch in exon 3 (polyA contractions), we have verified the hypothesis that these *PHOX2B* variants do reduce its transcriptional activity, likely resulting in a down-regulation of *RET* expression and, consequently, favouring the development of the HSCR phenotype. Using proper reporter constructs, we show here that the *in vitro* transactivation of the RET promoter by different HSCR-associated *PHOX2B* polyA variants has resulted significantly lower compared to the effect of PHOX2B wild type protein. In particular, polyA contractions do induce a reduced transactivation of the RET promoter, milder compared to the severe polyA expansions associated with CCHS + HSCR, and correlated with the length of the deleted trait, with a more pronounced effect when contractions are larger.

1. Introduction

The paired-like homeobox 2b gene (*PHOX2B*) encodes a highly conserved homeobox transcription factor essential for the development of the autonomous nervous system [1,2]. Heterozygous mutations of the *PHOX2B* gene are associated with Congenital Central Hypoventilation Syndrome (CCHS), a rare neurocristopathy characterized by hypoventilation during sleep due to an impaired response to hypercapnia and hypoxemia [3]. In most cases (> 90%), CCHS patients show a trinucleotide duplication occurring in a DNA stretch encoding twenty alanine residues. This leads to polyalanine expansions ranging from 4 to 13 additional residues (polyA mutations or PARMs). *PHOX2B* missense, nonsense, and frameshift mutations are much less frequent (non-polyA mutations, or NPARMs) [3–5].

The CCHS phenotype can be found in association with additional neurocristopathies characterized by dysfunctions of the autonomous nervous system. This reflects a generalized alteration of neural crest cells (NCCs) migration and differentiation, with hypoventilation being the common and most severe disease manifestation. CCHS accompanying disorders include Hirschsprung disease (HSCR) (20% of cases) [6,7] and neural crest tumours such as neuroblastoma and ganglioneuroma (5–10% of cases) [8] in addition to ocular defects, decreased heart rate variability, impaired control of body temperature [9–11]. Functional studies have shown that, while PARMs cause PHOX2B to be retained

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within aggregates in the cytoplasmic compartment in a polyA length-dependent manner, NPARMs allow PHOX2B to enter into the nucleus and to exert pathogenic effects likely through anomalous protein-protein interaction or aberrant downstream transcriptional regulation [12].

The most frequent CCHS associated disease, HSCR, is characterized by the absence of parasympathetic ganglia in the submucosal and myenteric plexi of the distal bowel and occurs in 1 in 5000 live births [7]. The genetic etiology of HSCR was recognized three decades ago but its molecular pathogenesis is still elusive. HSCR is a sex dependent low penetrance disease with a complex mode of inheritance. The *RET* proto-oncogene (*REarranged during Transfection*), which encodes a tyrosine kinase receptor, is the main gene involved in HSCR pathogenesis, whose mutations have been identified in 50% of familial patients and in 7–35% of sporadic HSCR cases [7]. HSCR-associated *RET* mutations seem to act mostly by a *loss of function* mechanism [13]. In addition, several Single Nucleotide Polymorphisms (SNPs), belonging to specific haplotypes of the *RET* gene, have been described as under- or over-represented in HSCR patients compared to healthy controls, thus suggesting their role in determining different degrees of disease susceptibility within the population [14]. Conversely, *gain of function* mutations of the *RET* gene are associated with a limited proportion of patients affected with isolated or syndromic autosomal dominant Medullary Thyroid Carcinomas (MTC) [15]. For this reason, *RET* common variants over-represented in HSCR are often under-represented in MTC and *vice versa*, clearly indicating that specific *RET* SNPs may have opposite predisposing/protecting effects. This is the case, among others, of SNPs NM020975.4:c.73 + 9277T > C (rs2435357), NM020975.4:c.2071G > A p.G691S (rs1799939), and NM020975.4:c.*1969T > C (rs3026785) [14,16,17].

RET expression, essential during development for adequate intestinal innervation [18], is orchestrated by different transcriptional factors such as PHOX2A, PHOX2B, MASH1, HOX11L1, SOX10 and PAX3 [19–21]. No physical binding of PHOX2B to the *RET* promoter has been proven, therefore PHOX2B seems to act as an element recruiting other transcriptional factors [19,21]. Additional key factors, RARB and GATA2, have recently been demonstrated to bind two *cis*-acting RET enhancers, whose activity is reduced by HSCR risk variants [22]. Several observations suggest the involvement of the *PHOX2B* gene in the pathogenesis of HSCR. Indeed, *PHOX2B* point mutations/deletions have been reported in patients presenting neuroblastoma (NB), HSCR or both, without any CCHS sign [23–25]. In particular, isolated HSCR seems to associate with deletions of the whole *PHOX2B* gene leading to null alleles [23,25]. In the mouse, *Phox2b* expression starts as soon as enteroblasts invade the foregut mesenchyme and is maintained throughout the development into enteric neurons. For this reason, *Phox2b* knockout mice show the HSCR phenotype [2].

In this study, starting from the observation of HSCR patients carrying in frame trinucleotide deletions within the polyalanine stretch in exon 3 (polyA contractions), we have verified the hypothesis that these *PHOX2B* variants reduce its transcriptional activity, thus inducing a down-regulation of *RET* expression and, consequently, favouring the development of the HSCR phenotype.

2. Materials and methods

2.1. Mutational screening of the polyA coding PHOX2B region

To amplify the *PHOX2B* exon 3 region encompassing the polyA coding stretch we used the forward primers 22F 5′-ACTGACCCGGACAGCACT-3′ and the reverse primer 279R 5′-GAGCCCAGCCTTGTCCAGG-3′ reported by Matera et al. [5].

PCR reactions specific for GC rich templates were set up in a total volume of 25 μl containing 200 ng of genomic DNA, 200 nM of each primer, 1 × AccuPrime™ GC-Rich buffer A, and 1 U of AccuPrime™ GC-Rich DNA Polymerase (AccuPrime™ GC-Rich DNA Polymerase, Invitrogen), and run for 35 cycles at 95 °C (45 s), 60 °C (60 s), 72 °C (45 s), and 72 °C (7 min). PCR fragments were checked through 4% agarose gel electrophoresis to distinguish the wild-type allele from the deletion allele. Anomalous samples were then analyzed by direct sequencing using the Big Dye Terminator cycle sequencing kit (Applied Biosystems) on ABI 3100 DNA automated Sequencer. PCR products were purified before sequencing (*Sap*I-*Exo*III) and incubate to 37 °C (40 min), and 80 °C (15 min).

2.2. Construction of expression plasmids

Three regions containing *in-frame* deletions within the stretch of *PHOX2B* polyA (del15, del21, del39) were subcloned in the expression vector pcDNA3.1TOPO (Invitrogen), in order to produce − 5Ala, − 7Ala and − 13Ala PHOX2B proteins. In particular, the entire exon 3 of the *PHOX2B* gene of patients with the deletions was amplified using the forward primer 10F: 5′-TGCTTCACCGTCTCTCCTTCC-3′ and the reverse primer 3.3R: 5′-TACCCGCTCGCCCACTCG-3′. PCR reactions specific for GC rich templates were set up in a total volume of 25 μl as reported above. At the end, 2 U of Taq DNA Polymerase (Invitrogen) were added to each sample, and run for 10 min at 72 °C. PCR products were cloned in the vector pCR2.1-TOPO using “TA Cloning” system (TOPO TA Cloning, Invitrogen) following the manufacturer's instructions.

To generate pcDNA3.1TOPO-del15, pcDNA3.1TOPO-del21, and pcDNA3.1TOPO-del39 constructs, a 270 bp region containing the deleted polyA stretch was isolated by *Ppu*MI enzymatic digestion from pCR2.1-TOPO-del15, pCR2.1-TOPO-del21, pCR2.1-TOPO-del39 respectively, and cloned into pcDNA3.1TOPO-wild-type PHOX2B, after removing the corresponding region. Constructs containing GFP-fused deleted PHOX2B (pcDNA3.1/CT-GFP-TOPOdel15, pcDNA3.1/CT-GFP-TOPOdel21, and pcDNA3.1/CT-GFP-TOPOdel39), were generated, through the above protocol, after substituting the insert of the pcDNA3.1/CT-GFP-TOPO-wild-type PHOX2B construct with those of the three clones above. All constructs were checked by DNA sequencing.

The generation of pcDNA3.1TOPO-wild-type PHOX2B, pcDNA3.1TOPO-dup15, pcDNA3.1TOPO-dup21, pcDNA3.1TOPO-dup27, pcDNA3.1TOPO-dup39 (encoding for WT and + 5, + 7, + 9 and + 13Ala PHOX2B proteins) and pcDNA3.1/CT-GFP-TOPO-wild-type PHOX2B have been described elsewhere [12].

2.3. Cell cultures, transient transfection and Luciferase assay

The neuroblastoma cell line SK-N-BE(2) was grown in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco, New Zealand) and 1% L-glutamine and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. 7.5×10^4 cells were plated in 24 multiwell plate. Transfections were performed using Fugene6 Transfection Reagent (Roche), with 1 μg of expression vector pcDNA3.1/V5-His-TOPO encoding for the different PHOX2B variants (750 ng for pcDNA3.1/V5-His-TOPO/Empty), 500 ng of pGL3basic-RET promoter expressing the Firefly *luciferase* reporter gene downstream of a fragment of the human c-RET 5′ flanking region subcloned in the pGL3basic vector (Promega), and 50 ng of pRL-SV40 expressing the *Renilla luciferase* gene. pRL-SV40 was used as an internal control of each sample to normalize for the transfection rate. Forty-eight hours after transfection, cells were assayed for Luciferase activity (Dual-Luciferase Reporter Assay System, Promega) using a TD-20/20 Luminometer following the manufacturer's instructions.

2.4. Fluorescence microscopy analysis

To define the localization of the PHOX2B protein, HeLa cells were cultured in a 96 well-plate (cellcarrirer96, Perkin Elmer) and transfected with 200 ng of each expression construct encoding for WT or

mutant PHOX2B with GFP protein fused to the C-terminal (Fugene6 Transfection Reagent, Roche). 48 h after transfection cells were fixed with MetOH/Acetone, stained for DAPI and observed by an inverted fluorescence microscope equipped with motorized control and acquisition (Eclipse Ti-E Nikon). Acquisition and analysis were performed with NIS-Element 4.2 AR platform implemented/integrated with Jobs software (Nikon). Software was programmed to automatically capture 24 fields for each well with a 20 × magnification after sequential excitation of GFP and DAPI. Aggregates quantification was performed by the assessment of PHOX2B intensity fluorescence in the nuclear and in the cytoplasm area after applying a mask to define nuclear area marked by DAPI signal. The percentage of GFP intensity present outside of the nuclear area was regarded as due to aggregate. The experiment was performed in triplicate to allow statistical analysis.

2.5. Patients and statistical analyses

The genetic study of patients affected with Hirschsprung disease has been approved by the IGG Ethical Committee, and all patients recruited afterward gave their informed consent. Luciferase assay data are presented as mean ± SD. Statistical differences between means were evaluated by two-tailed *t*-test with Bonferroni correction. Statistical significance was assumed at $P < 0.05$. Student's *t*-test was also performed to compare PHOX2B localization value in microscopy assay.

3. Results

3.1. In frame deletions of the PHOX2B gene in HSCR patients

A heterozygous *in frame* deletion of 15 bp, affecting the homopolymeric trait of *PHOX2B* coding for a stretch of 20 alanine residues, thus leading to deletion (hereon defined "contraction") of 5 alanine residues, was found to segregate with intestinal motility defects in a family whose proband was suspected to be affected with CCHS. The patient had a neonatal respiratory distress, that resolved spontaneously, and showed also Hirschsprung disease (HSCR). Because of the respiratory phenotype, we were asked to exclude a CCHS condition by screening for *PHOX2B* mutations. No other genes were sequenced, except for the *RET* gene where no pathogenic variant was detected. The deletion was transmitted from the mother, who displayed chronic constipation during childhood. A maternal aunt died five days after birth, while a second cousin died when he was three years old, by undetermined causes. Based on these observations, we have hypothesized that contractions of the 20 alanine stretch of the *PHOX2B* gene could lead to HSCR, in addition to mild neonatal respiratory disorders.

To verify our hypothesis, we screened for *PHOX2B* polyA length variations a panel of 254 HSCR patients and 255 healthy individuals, finding 8 and 5 *in frame* deletions of 15, 21 and 39 bp, leading to a protein lacking of 5Ala, 7Ala and 13Ala. These contractions were more frequent in HSCR patients (1,6%) than controls (0,98%), though the difference did not result statistically significant (Table 1), with − 5Ala, − 7Ala, and − 13Ala present in both patients and controls. Interestingly, the ExAC project website (http://exac.broadinstitute.org/) reports a total of 40 polyA in frame deletions found in 6329 European (Non-Finnish) individuals tested. In particular, 6 are deletions of 39 bp corresponding to lack of 13aa (246–259 Ala residues) while 34 are deletions of 21 bp corresponding to lack of 7aa (252–259 Ala residues).

Table 1
PHOX2B polyA contraction observed in healthy individuals and in HSCR patients.

	Subjects tested	PHOX2B contractions	Allele frequency	*P*-value
Controls	255	5	0,0098	$P = 0,417$
HSCR patients	254	8	0,0160	

The frequency of contracted polyA PHOX2B alleles is therefore 40/12658 = 0.00316, a proportion significantly much lower than the one assessed in our Control samples (5/255 = 0.0196; $P < 0.0001$). At present, the reason why control samples in our hands have such a higher frequency of PHOX2B polyA contractions is inexplicable.

3.2. RET mutations/SNPs in HSCR patients with in-frame PHOX2B deletions

The *RET* gene and its *loss of function* mutations have been identified as the main cause of HSCR development [7,26]. In addition, protective and predisposing *RET* alleles have been described [14,16,27–29], confirming the complex inheritance of the disease. One of the most significant predisposing HSCR haplotypes includes two SNPs in the promoter, at − 5 and − 1 nucleotides from the transcription start codon (− 5A and − 1C), the synonymous "A" variant allele of the *RET* exon 2 SNP (SNP2: c.135G > A; p.A45A), spanning the whole intron 1 and the so called RET + 3 SNP (rs2435357) [14]. This haplotype is over-represented in HSCR patients and it is associated with a lower *RET* expression, further confirming the haploinsufficiency/*loss of function* of the RET protein in HSCR pathogenesis [30,31] and also contributing to the genetic susceptibility of most of the HSCR patients without apparent causative *RET* mutations [14].

Therefore, to unravel whether the disease pathogenesis could be ascribed to specific *RET* variants, rather than to *PHOX2B* polyA contractions, we searched for both *RET* mutations and protective or susceptibility SNPs in the patients carrying *PHOX2B in-frame* deletions. As reported in Table 2, only HSCR patient 468 had neither a *RET* mutation nor the susceptibility haplotype, revealed by the "A" allele of SNP2. HSCR patients 519 and 547 showed in addition the presence of a protective variant allele of the synonymous "T" variant allele of the RET exon 14 SNP (SNP14: c.2508C > T, p.S836S), already reported to be under-represented in and untrasmitted to HSCR patients [16], further supporting an involvement of *PHOX2B* in HSCR pathogenesis. Indeed, this variant is underrepresented in HSCR patients compared to controls and shown to be in Linkage Disequilibrium with a variant located in the 3′ untranslated region (UTR) of the *RET* gene affecting the physiological mRNA decay of the gene transcript which results more abundant [16]. Two HSCR patients displayed different *RET* mutations. HSCR 100 had a not-reported mutation, while HSCR 584 showed a common missense variant whose pathogenicity is unlikely [32,33]. Moreover, HSCR 584 carried the variant allele c.2071G > A of exon11, a *RET* SNP whose variant allele might account for enhanced signaling transduction explaining its over-representation in MTCs [17]. Only HSCR 132 showed a *RET* intronic mutation (c.1879 + 1G > A), likely interfering with splicing and therefore responsible for the HSCR phenotype. Overall, we have 5 "A" risk alleles for HSCR out of 16 alleles, with an excess of "GG" homozygotes compared to what expected (χ^2 test, $P = 0.0014$). This strengthens the hypothesis that RET risk alleles are dispensable in the presence of *PHOX2B* polyA contractions. In other words, our results show that the presence of the deletions in the polyA coding stretch of *PHOX2B* may actually be responsible for the HSCR phenotype, especially when no other causative mutation or predisposing factor is present in the *RET* gene. In addition, the length of *PHOX2B* deletions and the presence of the *RET* predisposing haplotype seem to be inversely correlated: the longer the deleted trait, the higher the chance of having HSCR, even in the absence of any other predisposing genetic factor.

3.3. HSCR-associated PHOX2B mutations reduce the in vitro activation of RET promoter

The expression regulation of the *RET* proto-oncogene involves the dynamic interaction of different transcriptional factors, such as NKX2-1, RARB, GATA2, SOX10 and PAX3 [2,20,22,34]. Among these, also PHOX2B plays a role in the *RET* expression regulation, though

Table 2
Molecular characterization of HSCR patient screened for *RET* and *PHOX2B* gene.

HSCR patient (ID)	PHOX2B deletion	RET mutation	genotype at synonymous RET SNP c.135G > A (rs1800858)	Heterozygote for protective RET SNPs
583	c.763_777del15 (− 5Ala)	−	AA	−
468	c.763_777del15 (− 5Ala)	−	GG	−
97	c.739_753del15 (− 5Ala)	−	AA	−
132	c.739_753del15 (− 5Ala)	c.1879 + 1G > A	GG	−
519	c.754_774del21 (− 7Ala)	−	GG	c.2508C > T (p.S836S) (rs1800862)
100	c.754_774del21 (− 7Ala)	c.539G > C (p.R180P)	AG	−
547	c.736_774del39 (− 13Ala)	−	GG	c.2508C > T (p.S836S) (rs1800862)
584	c.736_774del39 (− 13Ala)	c.2944C > T (p.R982C) (rs17158558)	GG	c.2071G > C (p.G691S) (rs1799939)

Table 3
PolyA PHOX2B mutations analyzed for their effect on *RET* promoter.

	Associated phenotypes	References
15 nt *in frame* deletion (− 5Ala)	HSCR	[11]
21 nt *in frame* deletion (− 7Ala)	HSCR	[11,25]
39 nt *in frame* deletion (− 13Ala)	HSCR	[11]
Various *in frame* deletions	SIDS	[43]
15 nt *in frame* duplication (+ 5Ala)	CCHS	[3,4,5,11]
21 nt *in frame* duplication (+ 7Ala)	CCHS ± HSCR	[3,4,5,11]
27 nt *in frame* duplication (+ 9Ala)	CCHS ± HSCR	[3,4,5,11]
39 nt *in frame* duplication (+ 13Ala)	CCHS + HSCR	[3,4,5,11]

indirectly as no PHOX2B binding site has ever been identified in the *RET* promoter [19,35]. Hence, we sought to verify whether *PHOX2B* alterations could lead to reduced *RET* expression, impairing the enteric neurogenesis and causing HSCR. To this end, the ability of different PHOX2B mutants to transactivate the *RET* promoter was assessed, testing HSCR-associated and not-associated variants. In particular, we compared the effects on the *RET* promoter of three *in frame* deletions of the polyA tract (− 5Ala, − 7Ala and − 13Ala) and of four *in frame* duplications (+ 5Ala, + 7Ala, + 9Ala, + 13Ala) (Table 3).

According to our hypothesis, all variants tested, including the three polyA contractions, did induce a transactivation of the *RET* promoter significantly lower than PHOX2B wt (Fig. 1A and Table 4). Moreover, we observed that the longer the deleted tract, the more pronounced the reduction of *RET* promoter transactivation, suggesting an inverse correlation between the deletion length and RET transcription (Fig. 1B).

3.4. Subcellular localization of PHOX2B carrying polyA deletions

As PHOX2B polyA tract duplications (polyA expansions) show a defective transactivation of target genes as a consequence of subcellular mis-localization, with retention in cytoplasmic aggregates that impair proteins translocation in the nuclear compartment [12], we sought to investigate whether the effects of PHOX2B carrying polyA deletions may be due to the same mechanism.

To this end, polyA contractions were cloned into a vector expressing GFP fused PHOX2B proteins whose localization could be successively assessed by fluorescence microscopy followed by quantitative analysis of protein aggregation. As shown in Fig. 2A, similarly to the wild type protein and in contrast with polyA expansions, already known to aggregate in the cytoplasm, PHOX2B polyA contractions localized in the nucleus. This is confirmed by a quantitative analysis of PHOX2B aggregates that do not show significant differences between the wild type protein and the − 5Ala, − 7Ala and − 13Ala PHOX2B contractions (Fig. 2B). Conversely, the polyA expansion tested (+ 13Ala) has revealed, as already reported [12], a significant extent of aggregates.

Overall, these results suggest that different molecular mechanisms can account for the inability of mutant PHOX2B versions to transactivate the *RET* gene.

4. Discussion

HSCR is characterized by the absence of parasympathetic ganglia in the submucosal and myenteric plexi of the distal bowel. Heterozygous *RET* mutations and common Single Nucleotide Polymorphisms (SNPs) of the same gene, acting through *loss of function* or haploinsufficiency mechanisms, account for disease predisposition only in a proportion of HSCR cases. On the other hand, additional genes have been identified with mutations in a total of 5% of mostly syndromic HSCR patients. Incomplete penetrance and variable expressivity of *RET* mutations, in addition to a high proportion of sporadic cases, confirm the complex inheritance of HSCR disease [7,26]. As still unrecognized known or novel genes may contribute to the control of RET expression, and consequently to HSCR pathogenesis, here we sought to deepen into the role of PHOX2B in the development of HSCR disease.

Heterozygous *PHOX2B* mutations are responsible for Congenital Central Hypoventilation Syndrome (CCHS) [3,5,11]. While the vast majority of CCHS associated *PHOX2B* mutations is represented by in frame duplications within a stretch of trinucleotide repeats leading to polyalanine expansions (PARMs), a less frequent group of non-polyalanine repeat mutations (NPARMs) has been found in syndromic CCHS patients showing additional neurocristopathies, such as Hirschsprung disease (HSCR) and NB [8,11]. Additional observations have contributed to refine the genotype-phenotype correlation. Indeed, the CCHS + HSCR syndromic association is often reported also for the largest polyA expansions, including the 21 bp duplication leading to + 7Ala genotype, whereas, no association with HSCR can be observed with the shortest polyA expansions (+ 4Ala, + 5Ala), which usually lead to either low penetrant, mild or late onset CCHS [11]. Interestingly, additional *PHOX2B* defects such as whole gene interstitial deletions and in frame deletions of the *PHOX2B* polyA stretch (polyA contractions), have been identified in isolated HSCR, but not in CCHS patients so far [25,36].

To deepen into the possible role of *PHOX2B* polyA anomalies in HSCR pathogenesis, we performed a mutation screening finding more contractions in HSCR patients than in controls. However, as the difference was not statistically significant, and contraction lengths similarly distributed between HSCR and controls (data not shown), these data led to the exclusion of *PHOX2B* contractions as causative variants in HSCR. Given the complex genetic inheritance of HSCR, *PHOX2B* polyA contractions might still concur to the HSCR phenotype, particularly when no other causative mutation or predisposing factor is present in the RET gene. Indeed, as observed in the present study, co-segregation of *PHOX2B* contractions with *RET* mutations has suggested that the shortest polyA contractions are not sufficient for HSCR to develop and other predisposing factors, among which *loss-of-function* RET variants, are needed. Conversely, the largest polyA contractions seem to be causative even without the well-known predisposing

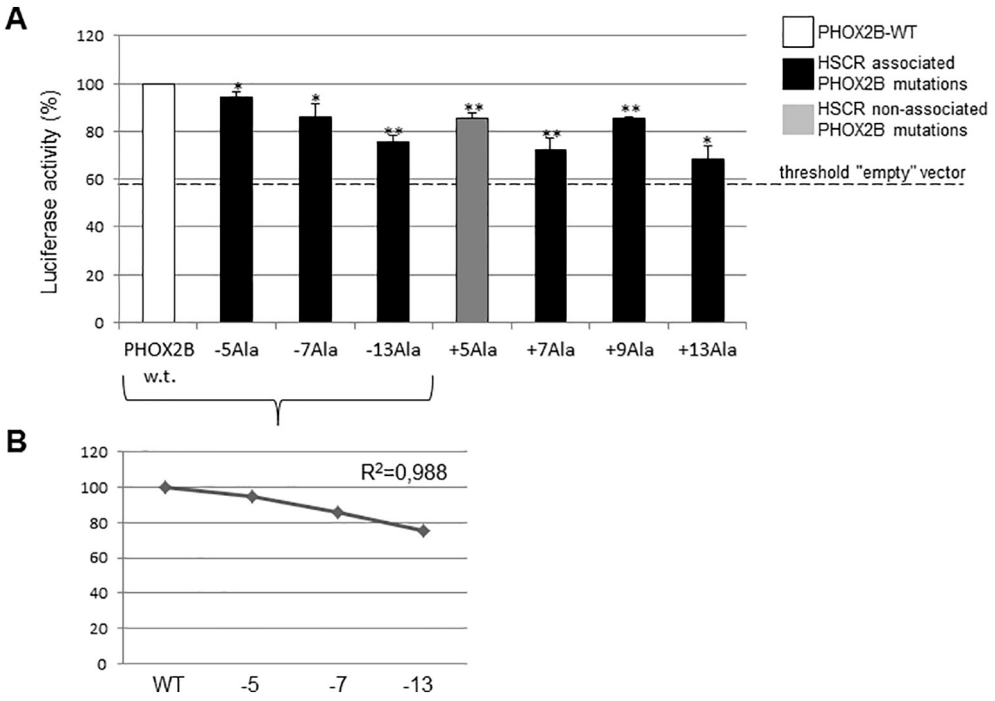

Fig. 1. Transcriptional effect of PHOX2B mutant on *RET* promoter activation. A. *RET* promoter driven *luciferase* activities, induced by PHOX2B carrying contractions or expansions mutations, are expressed as ratio between activities of mutant *versus* wild-type proteins. Each Luciferase activity has been normalized with an internal control (pRL-CMV). The results are the mean values ± SE (error bar) of at least three independent experiments performed in triplicate in SK-N-BE(2) cells. The dotted line at 58% value represents the mean of the activity induced by the empty vector. Black bars: HSCR associated PHOX2B mutations; gray bar: HSCR not associated PHOX2B mutations. Asterisks indicate significance level of the Student's *t*-test, corrected according to Bonferroni (* = $P < 0.05$; ** = $P < 0.01$). B. the regression test performed between the deletion length and levels of RET transcription suggests an inverse correlation with Y = − 8,2366X + 109,55.

RET + 3 intronic mutation, lying in a HSCR susceptibility haplotype [14,22,28]. Moreover, the − 7Ala and − 13Ala are still associated with HSCR despite the presence in the corresponding patients of known protective variants at the *RET* locus, less frequent in HSCR than in healthy individuals and consistently predisposing to Medullary Thyroid Carcinoma [16,17]. These observations have prompted us to hypothesize an involvement of *PHOX2B* polyA contractions in HSCR pathogenesis.

In addition, it has been shown that impairment of PHOX2B physiological role in the development of the enteric neuronal cell population does cause HSCR. In mice, *Phox2b* expression starts as soon as enteroblasts invade the foregut mesenchyme and is maintained throughout the development to the enteric neurons so that homozygous deletion of the *PHOX2B* gene results in the absence of the enteric ganglia. Interestingly, there is no RET expression in Phox2b mutant embryos, suggesting that *RET* expression is regulated by the PHOX2B protein [2,19,37]. However, no putative *PHOX2B* binding site has been identified in the *RET* promoter implying that PHOX2B may act as an indirect factor which recruits other transcriptional regulators [19,35].

According to the evidence, *PHOX2B* mutations act by impairing PHOX2B ability to transactivate the promoter of target genes such as *DBH*, *PHOX2A*, and *TLX2* [12,38–40]. Moreover, the longer the polyA expansions the stronger their mis-localization and aggregation in both nuclear and cytoplasmic compartments. The involvement of also a fraction of the wild-type protein in this pathogenic process further accounts for reduced PHOX2B transcriptional activity [12,39]. Conversely, CCHS-associated *PHOX2B frameshift* mutations do interfere with the transcriptional activity of the protein but not with its nuclear localization [12,41]. In the light of the above considerations, we have hypothesized that mutations reducing PHOX2B transcriptional activity lead to a down-regulation of *RET* expression and, consequently, to a failure of the development of enteric ganglia and to HSCR.

Consistently, the *in vitro* transactivation of RET promoter by different HSCR-associated *PHOX2B* mutations has resulted significantly lower compared to the effect of PHOX2B wild type protein. In particular, polyA contractions do induce a reduced transactivation of the RET promoter which correlates with the length of the deleted trait, with a more pronounced effect when contractions are larger (Fig. 2 and Table 4). This is slightly different from the effect of polyA contractions already reported on the DBH promoter: despite the reduction of the transactivation activity, with respect to the wild type protein, no correlation with the polyA contraction length could be observed in that case [42,43]. Such a lack of contraction length effect could depend on either the different cell lines used (SK-N-BE(2) *vs* HepG2), a more

Table 4
Statistical analysis of transcriptional activity induced by different PHOX2B mutant constructs on RET promoter reporter plasmid.

Wild type	–							
− 5Ala	0,03088	–						
− 7Ala	0,04460	0,15258	–					
− 13Ala	**0,00001**	0,01079	0,10407	–				
+ 5Ala	0,00468	0,00985	0,92596	0,03997	–			
+ 7Ala	0,00480	**0,00052**	0,10112	0,18079	0,01101	–		
+ 9Ala	0,00599	0,02222	0,96378	0,06353	0,95361	0,04197	–	
+ 13Ala	0,01259	**0,00034**	0,05010	0,05996	0,03246	0,34243	0,07513	–

Student's *t*-test values obtained by comparing the effect of the different constructs. Significant *P*-values are indicated in bold ($P < 0.05$, Bonferroni's correction: 0.05/28 = 0.0018).

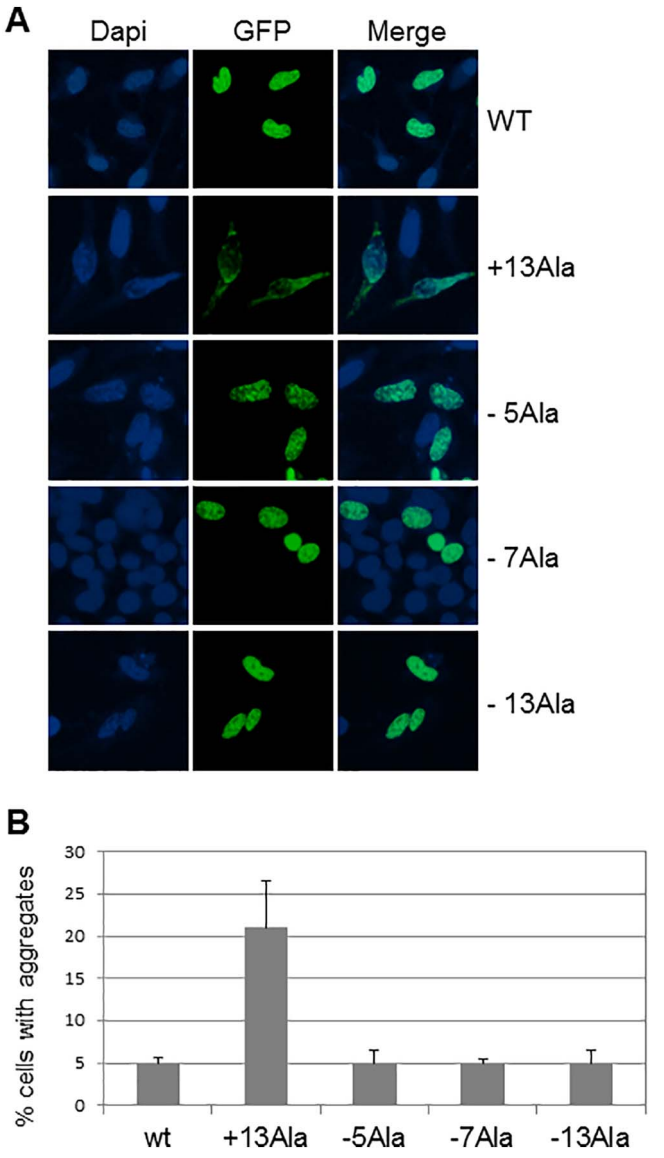

Fig. 2. Localization of WT and mutant PHOX2B proteins. (A) Representative fluorescence images of HeLa cells transfected with GFP-tagged wild type PHOX2B or GFP-tagged mutant PHOX2B. Nuclei were stained with DAPI and the right images in each raw represent the merge of the two adjacent pictures. Acquisition and analysis were performed with NIS-Element 4.2 AR platform integrated with Jobs software (Nikon) (20 × magnification). (B) Bar graph reporting the proportion of cells characterized by a mis-localization of PHOX2B with formation of aggregates. Significant differences are indicated by asterisks (Student's *t*-test, $*P < 0.05$, $**P < 0.01$). In particular *P*-values obtained by comparing each mutant with PHOX2B wt are: dup15 = 0,410; dup21 = 0,054; dup39 = 0,007; del15 = 0,118; del21 = 0,06; del39 = 0,110; deltaC = 0,249; c.930insG = 0,1711.

specific effect of polyA contractions on the RET promoter compared to DBH promoter, or a different sensitivity to protein conformation rather than recruitment of transcriptional co-factors.

All the PHOX2B polyA expansions tested have led to a reduction of transcriptional activity of PHOX2B. In particular, + 13Ala and + 7Ala mutants, which are more often associated with HSCR/CCHS, consistently show a remarkable decrease of the transactivation of the RET promoter, while the + 9Ala mutant, less frequent in CCHS + HSCR patients, shows a weaker effect on RET promoter. Indeed, this latter variant may become crucial for HSCR pathogenesis only in the presence of other predisposing factors which synergistically reduce RET expression levels. Interestingly, such a length-independent effect on the RET promoter resembles the effects polyA expansions have shown to exert on the promoter of the TLX-2 gene, also expressed in the enteric nervous system [38], thus suggesting that in the developing ENS PHOX2B transcriptional activity depends on still undisclosed additional factors. Controversial results are available for the + 5Ala mutant, which on one hand has never been associated with HSCR but still showed a reduction of RET transactivation compared to the RET levels induced by the PHOX2B WT construct (Fig. 2). We cannot exclude that a fraction of the + 5Ala mutant does aggregate into the cytoplasmic compartment, without any dominant negative effect on the wild type PHOX2B protein which is therefore able to move to the nucleus and guarantee a given, though low, level of RET promoter transactivation. In this light, the PHOX2B + 5Ala mutant can be considered as a HSCR predisposing rather than a causative factor. This hypothesis is supported also by an auto-regulatory mechanism that allows PHOX2B to control and sustain its own expression [44]. Indeed, we cannot exclude that a low availability of PHOX2B wt, retained in aggregates by the largest polyA expansions, leads to a decreased expression of PHOX2B itself and of RET too.

Overall, our data indicate that HSCR-associated PHOX2B alterations impair the ability of the PHOX2B protein to regulate the transcription of the RET gene, although at different extent, further confirming the PHOX2B regulation of RET expression and therefore the likely involvement of different PHOX2B variants in HSCR pathogenesis.

However, as several genes have been implicated to date in HSCR development [7,26], we cannot exclude that HSCR patients carrying a PHOX2B polyA contraction, reported in Table 2, do carry also mutations in other untested HSCR genes. In any case, PHOX2B impairment, as achieved in the presence of in frame deletions of the polyalanine tract, can be still regarded as one of the genetic conditions predisposing and contributing to HSCR. Similarly to RET rs2435357 SNP, shown to be a modifier of the PHOX2B gene in HSCR associated CCHS phenotype [45], PHOX2B polyA contractions may act as either modifiers in RET dependent HSCR development or as causative/predisposing factors in the absence of RET predisposing alleles.

Last but not least, our results raise the question of the structural and functional roles of polyA tracts in proteins. Though these homopolymers are considered to be flexible spacers essential for protein conformation, and for DNA–protein and protein–protein interactions [46,47], recent findings indicate that the complete deletion of the polyalanine tract leads to the production of a protein with a normal nuclear localization and partial transactivation activity [48]. On the other hand, the expansion of the polyalanine tract would impair the folding of the C-terminal domain, thus also influencing the homeodomain and its functions [49]. Therefore, the polyalanine tracts may influence the structure and spatial orientation of the remaining portion of PHOX2B protein, and their length changes become harmful only when inducing dominant negative effect, such as in the case of the expansions, while contractions would have a milder outcome. Indeed, it has been suggested that central ventilatory neurons are more sensitive to PHOX2B mutations that affect protein misfolding, oligomerisation or gain-of-function mutations (*i.e.* alanine expansion), whereas the enteric phenotype is associated with gene dosage or missense mutations which retains some transcriptional activity but do not present oligomerisation properties, as would be the case for the mutation reported here [23,25]. This strengthens the hypothesis that PHOX2B haploinsufficiency cannot ensure a correct ENS development, thus acting as predisposing factor for intestinal developmental and functional defects.

Transparency document

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