Structural and functional differences in PHOX2B frameshift mutations underlie isolated or syndromic congenital central hypoventilation syndrome

Simona Di Lascio1 | Roberta Benfante1,2 | Eleonora Di Zanni3* | Silvia Cardani1 | Annalisa Adamo3† | Diego Fornasari1,2 | Isabella Ceccherini3

1Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Milan, Italy
2CNR- Neuroscience Institute, Milan, Italy
3UOC Genetica Medica, Istituto Giannina Gaslini, Genoa, Italy

Correspondence
Tiziana Bachetti, UOC Genetica Medica, Istituto Giannina Gaslini, Largo G. Gaslini 5, 16148 Genova, Italy.
Email: tiziana.bachetti@tin.it
Simona Di Lascio, Department of Medical Biotechnology and Translational Medicine, University of Milan Via Vanvitelli 32, 20129 Milan, Italy.
Email: simona.dilascio@unimi.it

Contract Grant Sponsors: Telethon Foundation (GGP13055); AIRC Foundation (MFG No. 11501); Associazione Italiana per la Sindrome da Ipoventilazione Centrale Congenita (A.I.S.I.C.C.);
* Present address: Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Via De Marinis 6, 16149 Genova, Italy.
† Present address: Stem Cell Research Laboratory, Section of Hematology, Department of Medicine, University of Verona, Verona, Italy.
Communicated by George P. Patrinos

Abstract
Heterozygous mutations in the PHOX2B gene are causative of congenital central hypoventilation syndrome (CCHS), a neurocristopathy characterized by defective autonomic control of breathing due to the impaired differentiation of neural crest cells. Among PHOX2B mutations, polyalanine (polyAla) expansions are almost exclusively associated with isolated CCHS, whereas frameshift variants, although less frequent, are often more severe than polyAla expansions and identified in syndromic CCHS. This article provides a complete review of all the frameshift mutations identified in cases of isolated and syndromic CCHS reported in the literature as well as those identified by us and not yet published. These were considered in terms of both their structure, whether the underlying indels induced frameshifts of either 1 or 2 steps ("frame 2" and "frame 3" mutations respectively), and clinical associations. Furthermore, we evaluated the structural and functional effects of one "frame 3" mutation identified in a patient with isolated CCHS, and one "frame 2" mutation identified in a patient with syndromic CCHS, also affected with Hirschsprung's disease and neuroblastoma. The data thus obtained confirm that the type of translational frame affects the severity of the transcriptional dysfunction and the predisposition to isolated or syndromic CCHS.

KEYWORDS
congenital central hypoventilation syndrome, dominant-negative, frameshift mutations, gain-of-function, Hirschsprung's disease, loss-of-function, neuroblastoma, PHOX2B

INTRODUCTION
The paired-like homebox 2B (PHOX2B) gene encodes a key homeobox transcription factor that is involved in the development of the autonomic nervous system (ANS) and the neuronal structures controlling breathing (Pattyn, Morin, Cremer, Goridis, & Brunet, 1999; Pattyn, Hirsch, Goridis, & Brunet, 2000). It is the disease-defining gene of congenital central hypoventilation syndrome (CCHS; MIM# 209880), a very rare neonatal neurocristopathy caused by the defective migration and/or differentiation of neural crest derivatives. CCHS is characterized by an abnormal ventilatory response to hypoxia and hypercapnia due to a failure of autonomic respiratory control that leads to hypoventilation during sleep (in less severe forms) and during both sleep and while awake in the most severe cases.

CCHS may be isolated or associated with other neurocristopathies, including tumors originating from the sympathetic nervous system (TSNS) such as neuroblastomas (NBs; MIM# 613013), ganglioneuromas and ganglioneuroblastomas (GNBs), autonomic dysfunctions of the enteric nervous system such as the aganglionic megacolon...
Hirschsprung’s disease (HSCR), the association of CCHS and HSCR is known as “Haddad syndrome”; MIM# 209880) and/or milder intestinal phenotypes (gastroesophageal reflux, constipation), and symptoms that can be attributed to the defective differentiation of the neural crest cells (cardiac arrhythmias, ocular, and endocrinological disorders) (Amiel et al., 2003; Matera et al., 2004; Weese-Mayer et al., 2010). Such complex phenotypes are due to the many genes expressed in different districts of the ANS that have been identified as PHOX2B transcriptional targets, including dopamine beta hydroxylase (DBH), paired-like homebox 2A (PHOX2A), REarranged during Transfection (RET), T-Cell Leukemia Homebox 2 (TLX-2), and PHOX2B itself (Adachi, Browne, & Lewis, 2000; Bachetti, Borghini, Ravazzolo, & Ceccherini, 2005a; Borghini et al., 2006; Cargnin, Flora, Di Lascio, Battaglioni, Longhi, Clementi, & Fornasari, 2005; Flora, Lucchetti, Benfante, Goridis, Clementi, & Fornasari, 2001).

PHOX2B is a protein of 314 amino acids that is highly conserved in mammals and has two short and stable polyalanine (polyAla) repeats of nine and 20 residues in the C-terminus. Over 90% of patients with CCHS are heterozygous for in-frame triplet duplications within the sequence stretch coding for 20 alanine amino acids, and the consequent polyAla repeat expansion mutations (PARMs) produce 4-13 additional alanine residues; the remaining 10% have heterozygous non-PARM mutations (NPARMs) in exon 1, 2, or 3 that include missense (MS), nonsense (NS), and frameshift (FS) mutations, the last of which lead to an aberrant C-terminal region (Amiel et al., 2003; Amimoto, Okada, Nakano, Sasaki, Hayasaka, & Odajima, 2014; Bachetti & Ceccherini, 2013; Berry-Kravis, Zhou, Rand, & Weese-Mayer, 2006; Buchan, Minneci, Nugent, Bryson, & Jones, 2013; Holzinger et al., 2005; Matera et al., 2004; Raabe et al., 2008; Rand, Carroll, & Weese-Mayer, 2014; Sasaki et al., 2003; Szymońska et al., 2015; Trochet et al., 2005b, 2008; Weese-Mayer et al., 2003, 2010, 2017).

Previous studies have revealed the following genotype–phenotype correlations: (1) the longer the stretch of alanine, the worse the respiratory phenotype; and (2) PARMs are almost exclusively identified in isolated CCHS, whereas NPARMs are often associated with syndromic forms in which the disease occurs together with HSCR and/or a NB, or other less frequent ANS disorders. However, NPARMs may have widely variable phenotypic expressivity, with the majority causing very severe forms of syndromic CCHS, and a small subset causing milder phenotypes with reduced penetrance that may represent isolated CCHS, isolated HSCR and/or NB (Berry-Kravis et al., 2006; Matera et al., 2004; McConville, Reid, Baskcomb, Douglas, & Rahman, 2006; Mosse, Laudenslager, Khazi, Carlisle, Winter, Rappaport, & Maris, 2004; Rand, Carroll, & Weese-Mayer, 2014; Trochet et al., 2005b, 2008; van Limpmt et al., 2004). One further genotype–phenotype correlation in the case of NPARMs suggests that translation frame 2 (resulting from the insertion of triplets +1 nucleotide or the deletion of triplets +2 nucleotides) may predispose to a severe and fully penetrant phenotype, whereas frame 3 (resulting from the insertion of triplets +2 nucleotides or the deletion of triplets +1 nucleotide) may contribute to a less severe and incompletely penetrant phenotype (Weese-Mayer et al., 2010). In line with this, in vitro studies have shown that the transactivation of the DBH and TLX-2 promoters is drastically impaired when PHOX2B carries frame 2 FS mutations, but only partially compromised in the presence of PHOX2B frame 3 mutants (Bachetti, Matera, Borghini, Di Duca, Ravazzolo, & Ceccherini, 2005b; Borghini et al., 2006; Trochet et al., 2005a, 2009). However, despite these observations, the molecular mechanisms underlying a predisposition to the development of severe associated diseases such as HSCR and NB in NPARM-carrying CCHS patients need further investigation.

In an attempt to elucidate the degree of association with other neurocristopathies, we classified reported and still unreported NPARMs on the basis of their different translational frames. Furthermore, we analyzed the consequences of two FS mutations (one frame 2 and one frame 3) by predicting protein structural changes and characterizing their functional effects in terms of promoter transactivation of the genes involved in ANS development and, probably, the pathogenesis of HSCR and NbDs. The obtained data confirm that the type of translational frame affects the severity of the transcriptional dysfunction and the predisposition to isolated or syndromic CCHS, thus reinforcing the correlation with the disease phenotype.

2 MATERIALS AND METHODS

2.1 Patient samples

From 2004 to date, samples of 305 patients with symptoms suggestive of hypventilation have been recruited at the Gaslini Hospital to undergo PHOX2B testing. CCHS-related PHOX2B mutations were identified by sequencing in 116 (38%) cases. The remaining 189 patients with no detectable PHOX2B mutation are clinically heterogeneous, with twenty of them who received a different clinical diagnosis afterward.

The parental DNA of CCHS patients with identified PHOX2B mutations was screened by direct DNA sequencing in 102 families (96 pairs of parents and 6 single parents).

2.2 Mutational screening of the PHOX2B gene

All three exons of the PHOX2B coding region were analyzed using published protocols (Bachetti et al., 2005b; Matera et al., 2004), in which their amplification was followed by product purification and direct Sanger sequencing. Mosaicism was analyzed by modifying a previously reported protocol: the region spanning the polyAla stretch was amplified using a 6′FAM-conjugated oligonucleotide and AccuPrime GC-Rich DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and the PCR product was run using the capillary electrophoresis method (see Bachetti, Parodi, Di Duca, Santamaria, Ravazzolo, & Ceccherini, 2011 for details).

GenBank sequence NG_008243.1 was used as reference for the PHOX2B gene while NM_003924.3 and NP_003915.2 were used as reference sequences for PHOX2B cDNA and protein respectively.

The PHOX2B variants identified in our cohort of patients have been submitted to the locus-specific database (LSDB) at https://www.lovd.nl/PHOX2B.
2.3 | Cell cultures, transient transfections, and Luciferase assays

The HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Lonza, Basel, Switzerland) and the SK-N-BE and U251-MG cells were grown in RPMI (Euroclone, Pero, Milan, Italy), with the addition of 10% fetal calf serum, penicillin 100 units/ml, streptomycin 100 μg/ml, and l-glutamine (Gln) 2 mM (Sigma–Aldrich, St. Louis, MO, USA). The transfection and co-transfection experiments were performed by means of lipofection as previously described (Di Lascio, Belperio, Benfante, & Fornasari, 2016) using 5 × 10^4 cells. The luciferase assays were carried out using the Dual Luciferase Reporter Assay System as previously described (Bachetti et al., 2005b). All of the transfections were performed in triplicate, and each construct was tested in at least three independent experiments using different batches of plasmid preparation.

2.4 | Generation of the expression and reporter constructs

In expression plasmids, the full name of mutant proteins derived from c.618del (p.Ser207Alafs*102) and c.930dup (p.Ser311Glufs*49) cDNAs has been shortened in p.Ser207fs and p.Ser311fs, respectively and they were prepared as follows.

The pcDNA3.1Myc-PHOX2B WT and the pcDNA3.1Myc-p.Ser207fs (c.618del) expression constructs were generated as follows: the entire cdna region was amplified starting from the already available pcDNA3.1TOPO-PHOX2B WT and the pcDNA3.1TOPO-p.Ser207fs (c.618del) constructs (named pcDNA3.1TOPO-c.614_618 delC in Bachetti et al., 2005b), using a forward primer (Pr. FW: 5′-AGCCACCTTCTCCATATCC-3′) and the following reverse primers: Pr. REV1 (5′-CGAACATACTGCTCTTCACTAAGG-3′) for PHOX2B WT and Pr. REV2 (5′-CAGGCCGCTTTGCCAGCAGG-3′) for PHOX2B p.Ser207fs; the PCR products lacking the stop codon were subcloned in the pcCR2.1 vector (TOPO TA Cloning; Invitrogen, Waltham, MA, USA) and ligated in the pcDNA3.1Myc vector after EcoRI digestion.

The pcDNA3.1 Myc-p.Ser311fs (c.930dup) plasmid was generated by means of site-specific mutagenesis starting from the pcDNA3.1TOPO-PHOX2B WT, containing the entire 3′UTR, as template. The entire p.Ser311fs coding region was obtained by means of the whole amplification of the two fragments containing the mutation, and produced using the following primers: Pr. FW fragment 1 (5′-AGGCCACCTTCTCCATATCC-3′) and Pr. REV fragment 1 (5′-TACTGCTCTTCTCCTAAGGCGG-3′); Pr. FW fragment 2 (5′-CGCCTCTATTTAGGAGCAGTA-3′) and Pr. REV fragment 2 (5′-CGCCTCTATTTAGGAGCAGTA-3′); after sequencing, the PCR products underwent the process described above before being inserted into the pcDNA3.1Myc vector.

The PHOX2B (HindIII/NcoI), DBH (−993/+1 bp) (NG_008645.1, NM_000787.3), TLX2 (−1,885/+37 bp) (NC_000002.11, NM_0016170.4), GFAP (−2.2 kb + 9 bp) (NG_008401.1, NM_002055.4) and RET (−5,078/+53 bp) (NG_008401.1, NM_002055.4) promoter constructs have been previously described (Bachetti et al., 2005a, 2010; Borghini et al., 2006; Cargnin et al., 2005; Di Lascio, Bachetti, Saba, Ceccherini, Benfante, & Fornasari, 2013).

2.5 | Fluorescence microscopy

HeLa cells plated on 1.7 × 1.7 cm² glass coverslips were grown to 50% confluence and transfected with PHOX2B WT/pcDNA3.1-Myc-His, PHOX2B-p.Ser207fs /pcDNA3.1-Myc-His, PHOX2B p.Ser311fs /pcDNA3-HA, or mock-transfected with the empty vector. Immunofluorescence was performed as previously described (Cargnin et al., 2005). The subcellular localization was analyzed on cells transfected and fixed 48 hr after transfection. The Myc-tagged PHOX2B proteins were detected by means of mouse anti-Myc antibody (1:150; Sigma–Aldrich, St. Louis, MO, USA, #) and the secondary DyLight 549-conjugated goat anti-mouse antibody (1:400; Jackson ImmunoResearch West Grove, PA, USA, #115-505-146). The nuclei were stained with DAPI, and the images were acquired by means of an LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany) with 63x (Nikon, Tokyo, Japan) Apochromat lenses (1.5 NA).

2.6 | Electrophoretic mobility shift assays

The electrophoretic mobility shift assays (EMSAs) were performed following a previously described protocol (Cargnin et al., 2005; Terzano, Flora, Clementi, & Fornasari, 2000). The in vitro expression of wild-type PHOX2B and the mutant variants was obtained using a commercially available rabbit reticulocyte lysate system (TNT Quick-coupled Transcription/Translation System; Promega, Madison, WI, USA) as previously described (Cargnin et al., 2005). The oligonucleotides bearing the ATTA 2 and ATTA 3–4 sites of the PHOX2B promoter have been previously reported (Cargnin et al., 2005; Di Lascio et al., 2013). All of the oligonucleotides were purchased from Sigma–Aldrich. Mouse anti-MYC antibody (Sigma–Aldrich, St. Louis, MO, USA; catalog no. M5546) was used in the EMSA experiments.

3 | RESULTS

3.1 | Mutational screening of the PHOX2B gene in our cohort of CCHS patients

The coding region of the PHOX2B gene has been screened in CCHS probands and their parents, collected since 2004, using previously reported protocols (Bachetti et al., 2011; Matera et al., 2004), and we have identified a total of 116 cases carrying PHOX2B mutations: the vast majority were polyAla expansions (PARMs) containing 5–13 additional residues (98 cases, 84.5%), while FS, MS, and NS mutations (collectively NPARMs) could be detected in a minority of patients (18 cases, 15.5%; Figure 1A).

Fifty-one patients carrying PARMs have been reported previously, 22 with detailed clinical data (Matera et al., 2004; Parodi et al., 2008). In 14 patients we detected the insertion or the deletion of one or more nucleotides, for a total of 11 FS mutations, as summarized in Figure 1A and in Table 1 (in bold). Nine of these mutations have been reported elsewhere (Bachetti & Ceccherini, 2013; Bachetti et al., 2005b;
FIGURE 1  PHOX2B mutations identified in our CCHS panel. A: The bar chart shows the total number of cases carrying PHOX2B mutations belonging to the different classes identified in our CCHS patients (polyAla: polyalanine expansion; FS: frameshift; MS: missense; NS: nonsense). A description of the mutations and, in parenthesis, the corresponding number of carrying samples are reported under each bar. GenBank accession number: NM_003924.3 was used as the reference sequence for the cDNA. # indicates mutations reported here for the first time. B: Percentage of PARM (polyalanine repeat expansion mutation) and NPARM (non polyalanine repeat expansion mutation) carriers affected with isolated or syndromic congenital central hypoventilation syndrome (CCHS) and their distribution among four different categories of phenotypes: NONE (black dots) = CCHS without any associated symptom; HSCR (dark gray) = CCHS and Hirschsprung disease; NB (white) = CCHS and neuroblastoma; HSCR + NB (light gray) = CCHS, Hirschsprung disease and neuroblastoma. On the right, the distribution of NPARMs according to the type of mutation and phenotype. FS, frameshift; MS, missense; NS, nonsense.

Matera et al., 2004) and two of them have also been reported by others (Amiel et al., 2003; Berry-Kravis et al., 2006; Holzinger et al., 2005; Sasaki et al., 2003; Trochet et al., 2005b). The c.722_759dup and c.807_825dup mutations are reported here for the first time.

In the remaining four patients carrying NPARMs, we detected a MS mutation in the homeodomain (HD) (c.419C>A), two in the stop codon (c.944G>T and c.945A>C), and one NS mutation in the first exon (c.18T>G), already reported elsewhere (Bachetti & Ceccherini, 2013; Parodi et al., 2008).

In 20 patients (18 carrying PARMs and two NPARMs), the mutation was inherited by one parent, while in the remaining patients for whom DNA from both parents was available (76 cases) the mutation arose de novo. In six cases, for whom only one parental DNA sample was available, turned out to be negative for the mutation, the de novo/inherited
| ID | Exon | Mutation description | Mutant protein | Effect on protein | Length of unaltered protein (AA) | Total length of protein (AA) | Mutation-positiona | G-S rich tractb | Q-R rich tractb | 7 poly-Alab | CCHSc | Report associated phenotypesd | Frame | Inheritancee | References | Number of probands with mutation |
|----|-----|---------------------|----------------|------------------|-------------------------------|-----------------------------|------------------|-----------------|-----------------|----------------|-----------|-----------------------------|-------|-------------|-----------|--------------------------------|
| 1  | 1   | c.203del p.(Gly68Alafs*66) | Truncated | 67 | 1.32 | UP | – | – | – | – | Yes | None | 3 | Denovo | Bachetti & Ceccherini (2013) and authors’ unpublished data | 1 |
| 2  | 2   | c.284_291del p.(Lys95Thrfs*80) | Truncated | 94 | 1.73 | UP | – | – | – | – | No | NB | 2 | Denovo | van Limpt et al. (2004) | 1 |
| 3  | 2   | c.391del p.(Leu131Trfps*3) | Truncated | 130 | 1.32 | UP | – | – | – | – | Yes | ±HSCR | 3 | Inherited | Buchetti et al. (2013) | 1 |
| 4  | 3   | c.519del p.(Lys175Serfs*134) | Slightly truncated (7AA) | 174 | 3.07 | UP | + | – | – | + | – | Yes | None | 3 | n.a. | Bachetti & Ceccherini (2013) and authors’ unpublished data | 1 |
| 5  | 3   | c.577del p.(Asp193Thrfs*116) | Slightly truncated (7AA) | 192 | 3.07 | UP | + | – | – | + | – | Yes | HSCR | 3 | Inherited | Berry-Kravis et al. (2006) | 1 |
| 6  | 3   | c.590del p.(Gly197Alafs*112) | Slightly truncated (7AA) | 196 | 3.07 | UP | + | – | – | + | – | Yes | None | 3 | n.a. (‘) | Amimoto et al. (2014) | 1 |
| 7  | 3   | c.600del p.(Asn201Ilefs*108) | Slightly truncated (7AA) | 200 | 3.07 | UP | + | – | – | + | – | No | TSNS | 3 | n.a. | Mccormik et al. (2006) | 1 |
| 8  | 3   | c.608dup p.(Asn203Lysfs*157) | Elongated | 202 | 3.58 | UP | + | – | – | + | – | Yes | HSCR (L) | 2 | Inherited | Trochet et al. (2005b) | 1 |
| 9  | 3   | c.618del p.(Ser207Iles*102) | Slightly truncated (7AA) | 206 | 3.07 | UP | + | – | – | + | – | Yes | None | 3 | Inherited | Matera et al. (2004) | 1 |
| 10 | 3   | c.618dup p.(Ser207Glnfs*153) | Elongated | 206 | 3.58 | UP | + | – | – | + | – | Yes | HSCR+ TSNS | 2 | Denovo/ inherited | Amiel et al. 2003; Trochet et al. (2005a) | 2f |
| 11 | 3   | c.617_618insT p.(Ser207Glnfs*153) | Elongated | 206 | 3.58 | UP | + | – | – | + | – | Yes | HSCR | 2 | Denovo | Berry-Kravis et al. (2006) | 1 |
| 12 | 3   | c.633_670del p.(Gly216Alafs*131) | Elongated | 215 | 3.45 | UP | + | – | – | – | No | NB | 2 | Tumor-specific | van Limpt et al. (2004) | 1 |
| 13 | 3   | c.663dup p.(Gly222Trfps*138) | Elongated | 221 | 3.58 | UP | + | – | – | + | – | Yes | HSCR | 2 | n.a. | Bachetti & Ceccherini, (2013) and authors’ unpublished data | 1 |
| 14 | 3   | c.676del p.(Ala226Argfs*83) | Slightly truncated (7AA) | 225 | 3.07 | UP | + | – | – | + | – | No | ±HSCR+ ±NF1+NB | 3 | Inherited | Mose et al. (2004) | 1 |
| 15 | 3   | c.692del p.(Gly231Alafs*78) | Slightly truncated (7AA) | 230 | 3.07 | UP | + | – | – | + | – | Yes | None | 3 | Inherited | Trochet et al. (2005b, 2008) | 1 |

(Continues)
| ID | Exon | Mutation description | Mutant protein | Effect on protein | Length of unaltered protein (AA) | Total length of protein (AA) | Mutation-position<sup>a</sup> | 9 Ala<sup>b</sup> | 20 Ala<sup>b</sup> | G-S rich tract<sup>b</sup> | Q-R rich tract<sup>b</sup> | 7 Poly-Ala<sup>b</sup> | CCHS<sup>c</sup> | Reported associated phenotypes<sup>d</sup> | Frame | Inheritance<sup>e</sup> | References | Number of probands with mutation |
|----|-----|----------------------|----------------|-------------------|---------------------------------|-----------------------------|-----------------------------|----------------|----------------|-----------------|-----------------|----------------|----------------|-----------------------------|------|-----------------|----------------|------------------------------|
| 16 | 3   | c.691_698dup         | p.(Gly234Alafs*78) | Slightly truncated (AAA) | 233                            | 310                         | UP                          | +              | −              | −               | −               | −               | −               | ±HSCR ± NB               | 3    | Inherited      | Raabe et al. (2008); Trochet et al., (2005a) | 2    |
| 17 | 3   | c.693_700del         | p.(Pro232Argfs*125) | Elongated         | 231                            | 355                         | UP                          | +              | −              | +               | −               | +               | Yes             | HSCR (TCA) + NB       | 2    | Inherited      | Trochet et al. (2005b) | 1    |
| 18 | 3   | c.699_706del         | p.(Gly234Argfs*123) | Elongated         | 233                            | 355                         | UP                          | +              | −              | +               | −               | +               | Yes             | HSCR + NB              | 2    | Denovo         | Szymonik et al. (2015) | 1    |
| 19 | 3   | c.702_714dup         | p.(Gly239Argfs*125) | Elongated         | 238                            | 362                         | UP                          | +              | −              | +               | −               | +               | No              | NB             | 2                | Tumor-specific | van Limpt et al. (2004) | 1    |
| 20 | 3   | c.721_740del         | p.Ala241Argfs*112  | Elongated         | 240                            | 351                         | IN                          | +              | −              | +               | −               | +               | No              | NB             | 2                | SK-N-SH cell line specific | van Limpt et al. (2004) | 1    |
| 21 | 3   | c.722_738del         | p.(A1a241Glyfs*113) | Elongated         | 240                            | 352                         | IN                          | +              | −              | +               | −               | +               | Yes             | HSCR (TCA)               | 2    | Denovo/ Inherited | Bachetti & Ceccherini (2013); Holzmünger et al. (2005) and authors’ unpublished data | 2    |
| 22 | 3   | c.722_738dup         | p.(A1a247Glnfs*68) | Slightly truncated (1AA) | 246                            | 313                         | IN                          | +              | 6A1a           | −               | +               | −               | Yes             | HSCR (TCA) + NB        | 3    | Denovo         | van Limpt et al. (2004) | 1    |
| 23 | 3   | c.722_756del         | p.(A1a241Glyfs*107) | Elongated         | 240                            | 346                         | IN                          | +              | −              | +               | −               | +               | Yes             | HSCR (TCA); ± NB       | 2    | Inherited (mosaicism) | Berry-Kravis et al. (2006); Trochet et al. (2005b); van Limpt et al. (2004) | 3    |
| 24 | 3   | c.722_759del         | p.(A1a241Glyfs*106) | Elongated         | 240                            | 345                         | IN                          | +              | −              | +               | −               | +               | Yes             | HSCR (L) ± NB           | 2    | Denovo/ Inherited | Amiel et al. (2003); Berry-Kravis et al. (2006); Mataras et al. (2004); Trochet et al. (2005b) | 11   |
| 25 | 3   | c.722_759dup         | p.(A1a254Glnfs*68) | Elongated         | 253                            | 320                         | IN                          | +              | 13A1a          | −              | +               | −               | Yes             | HSCR (mosaicism)   | 3    | Inherited (mosaicism) | This report | 1    |
| 26 | 3   | c.807_825dup         | p.(Gly276Trpfs*90) | Elongated         | 275                            | 364                         | DOWN                       | +              | +              | −               | −               | +               | Yes             | NB             | 2                | n.a. (*1)       | This report          | 1    |
| 27 | 3   | c.834_835insA         | p.(Pro279Thrfs*81) | Elongated         | 278                            | 358                         | DOWN                       | +              | +              | −               | −               | +               | Yes             | HSCR (2)               | 2    | Denovo         | Bachetti & Ceccherini (2013); and authors’ unpublished data | 1    |
TABLE 1 (Continued)

| ID | Exon | Mutation description | Mutant protein | Effect on protein | Length of unaltered protein (AA) | Total length of protein (AA) | Mutation-position** | 9 Ala\(^a\) | 20 Ala\(^b\) | G-S rich tract\(^b\) | Q-R rich tract\(^b\) | 7 Poly-Ala\(^b\) | CCHS\(^c\) | Reported associated phenotypes\(^d\) | Frame | Inheritance\(^e\) | References |
|----|------|----------------------|----------------|------------------|-------------------------------|----------------------------|-------------------|---------|---------|----------------|----------------|----------|----------|--------------------------|------|----------|-----------|
| 28 | 3    | c.866dup             | p.Pro290Serfs*70 | Elongated        | 289                          | 358                        | DOWN              | +       | +       | −                | −                | +        | Yes      | HSCR + NB                 | 2    | Denovo   | Matera et al. (2004); Sasaki et al. (2003) |
| 29 | 3    | c.930dup             | p.Ser311Glufs*49 | Elongated        | 310                          | 358                        | DOWN              | +       | +       | −                | −                | +        | Yes      | HSCR (TCA) + NB            | 2    | Denovo   | Bachetti et al. (2005b) and authors' unpublished data |
| 30 | 3    | c.931_935del         | p.Ser311Tyrfs*47 | Elongated        | 310                          | 356                        | DOWN              | +       | +       | −                | −                | +        | Yes      | HSCR + NB                 | 2    | Inherited | Trochet et al. (2005b) |
| 31 | 3    | c.936dup             | p.Met313Tyrfs*47 | Elongated        | 312                          | 358                        | DOWN              | +       | +       | −                | −                | +        | Yes      | HSCR (TCA) + GNB           | 2    | Inherited | Trochet et al. (2005b) |

Mutations identified as part of the current report are indicated in bold

\(^{a}\)UP, upstream the polyAla region; IN, inside the polyAla region; DOWN, downstream the polyAla region.

\(^{b}\)+ = presence; − = absence.

\(^{c}\)CCHS, congenital central hypoventilation syndrome.

\(^{d}\)NB, neuroblastoma; HSCR, Hirschsprung disease; HSCR(L), long segment HSCR; TSNS, tumors of the sympathetic nervous system; TCA, total colonic aganglionosis; GNB, ganglioneuroblastoma; NF1, neurofibromatosis type 1 (MIM# 162200), linked to an inactivating mutation in NF1.

\(^{e}\)n.a., not assessed; n.a. (‘’), only one parent assessed (resulted negative for the mutation).

\(^{f}\)FS mutations numbers 8, 10, 15, 16, 17, 23, 24, 30, and 31 were already reported in the LSDB https://www.lsvl.nl/PHOX2B. The remaining novel and published variants in PHOX2B gene were submitted to the same database.
occurrence could not be verified. Overall, these data lead to a proportion of sporadic cases of 74.5%.

As previously reported (Weese-Mayer et al., 2010), although less frequent, NPARMs, and in particular FS mutations, are more severe than PARMs and more frequently associated with syndromic forms of CCHS (Figure 1B).

3.2 Analysis of PHOX2B FS mutations

We concentrated on FS mutations as they are more frequent than MS or NS mutations, and are associated with a larger range of CCHS phenotypes. To this end, we first collected all the PHOX2B FS mutations identified in patients with isolated or syndromic CCHS, isolated HSCR or NB that have so far been published in the literature or, although unpublished, come from our own CCHS patient panel.

The 31 different FS mutations are shown schematically in Supp. Figure S1 and described in more detail in Table 1, where they are listed on the basis of the starting point of the frame loss from 5′ to 3′ along the gene. During protein translation, these mutations can have different consequences: (1) the phase displacement of the nucleotide triplets can cause the creation of a premature stop codon, thus producing a truncated protein; and (2) the correct stop codon can be surpassed, thus leading to an elongated protein. In this article, the correct reading frame will be referred to as “frame 1”; the insertion of one or more nucleotides will be referred to as “frame 2”; and the insertion of two or more triplets +1 nucleotide and the deletion of two or more triplets +2 nucleotides will be referred to as “frame 3”; and the insertion of two or more triplets +2 nucleotides and the deletion of one or more triplets -1 nucleotide will be referred to as “frame 3″. Almost all of the FS mutations listed in Table 1 (except FS1, FS2, and FS3) arise in exon 3, and frame 2 mutations are more frequent than frame 3 mutations, and more frequently can also cause NB in the absence of CCHS (Supp. Figure S1 and Figure 2A).

As we were interested in the role that PHOX2B FS mutations play in predisposing to syndromic CCHS, we concentrated on the 25 mutations that have so far been identified in CCHS patients; the remaining six (written in gray italics in Supp. Figure S1, and Figure 2A) have never been associated with CCHS, and two have only been found in tumor samples (Table 1).

As shown in Supp. Figure S1 and Figure 2A, (1) all of the frame 2 CCHS-related FS mutations, except FS2, produce elongated proteins due to stop codon loss, and all of the frame 3 mutations, except FS25, cause protein truncation due to the gaining of a premature stop codon; (2) all of the patients with isolated CCHS present frame 3 mutations (in red in Supp. Figure S1, and circles with black dots in Figure 2A); (3) the vast majority of the cases of syndromic CCHS are associated with frame 2 mutations; (4) all of the \"syndromic\" phenotypes associated with frame 3 mutations include HSCR (three alone, two with NB); (5) syndromic CCHS is more frequently accompanied by HSCR than by NB (Supp. Figure S2).

These observations highlight a distortion in the distribution of NB or other TSNS among the mutations. Although HSCR is associated with almost all the FS mutations (alone or together with NB/TSNS, respectively dark and light gray circles in Figure 2A), only two of the five patients with frame 3 mutations present NB (40 %), whereas 10 of the 15 patients with frame 2 mutations present NB/TSNS \pm HSCR (66.6 %), thus suggesting that the predisposition to developing NB/TSNS is greater in the case of frame 2 mutations.

3.3 Consequences of FS start site in relation to the polyAla region

FS mutations of the PHOX2B gene may occur upstream, downstream, or inside the polyAla region (Supp. Figure S3), and their effect on the final gene product depends on both their position and the class of frame shift (Supp. Figures S4 and S5).

In relation to their position, frame 2 mutations are scattered throughout the whole gene: six mutations occur upstream, three inside and six downstream the polyAla region; conversely, almost all frame 3 mutations occur upstream of the polyAla stretch, with only two located within the repeat (Supp. Figure S4). The proportion of frame 2 mutations inside and downstream of the polyAla region associated with both HSCR and NB/TSNS is slightly higher than that of upstream mutations (six out of nine = 66% vs. three out of six = 50%) (Supp. Figure S5). Moreover, the most severe frame 3 mutation (associated with HSCR (TCA) \pm NB) is an insertion of 17 nt (FS22) affecting the polyAla region (Supp. Figure S4).

Interestingly, mutations affecting the polyAla region involve the insertions/deletions of a much larger number of nucleotides than those arising outside the repeat and, unlike the triplet duplications leading to polyAla expansions which occur throughout the polyAla region (Matera et al., 2004), four out of the six FS mutations occurred inside the polyAla region start from the first codon, thus disrupting the entire stretch of 20 Ala residues (FS 20, 21, 23, and 24). In the presence of frame 2 mutations upstream of and inside the polyAla region, the 20 alanine residues domain is enriched in glycine (Gly) and serine (Ser) residues, and an additional seven alanine sequence is produced within the translated portion of the 3′ UTR, whereas in the presence of frame 3 mutations in the same positions, the 20 alanine residues stretch is enriched in arginine (Arg) and Gln residues (Supp. Figure S3).

3.4 Inheritance of PHOX2B FS mutations

As shown in Figure 2B and Table 1, the possible inheritance of the PHOX2B FS mutations that cause CCHS \pm ASN symptoms could be assessed only for 21 mutations: three mutations (FS 10, 21, and 24), accounting for 15 patients, were reported to be both de novo and inherited; seven of the remaining eighteen were de novo, and eleven were inherited from one parent. The frame 2: frame 3 ratio is 5:2 among the de novo mutations, and 5:6 among the inherited mutations; further the percentage of frame 3 inherited mutations is higher than that of frame 2 (six out of a total of eight frame 3 mutations = 75% vs. five out of a total of ten frame 2 mutations = 50%), which is in line with the view that frame 3 mutations are less severe and may be incompletely penetrant.

With respect to their position, we observed that three of the 10 mutations upstream of the polyAla stretch were de novo and seven were inherited; among the three mutations inside the polyAla region, one was de novo and two, identified in four probands, were inherited...
FIGURE 2  ANS symptoms in CCHS patients carrying FS PHOX2B mutations. A: Distribution of ANS symptoms in syndromic CCHS by FS mutation. The hierarchical diagram shows the number of FS mutations (in the circles) divided on the basis of the class of patients (those with and without CCHS), clinical manifestation (isolated or syndromic), and the type of frame (2 or 3). Mutations associated with isolated CCHS (circles with black dots), without any other symptom, are indicated with NONE. The ANS dysfunctions associated with CCHS are indicated for each patient class: HSCR (dark gray) = CCHS and Hirschsprung disease; NB (white) = CCHS and neuroblastoma; HSCR + NB/TSNS (light gray) = CCHS, Hirschsprung disease and neuroblastoma/tumors of the sympathetic nervous system. B: Distribution of ANS symptoms based on the inheritance of FS mutations. Only those 21 FS mutations for which information on inheritance was available are considered. The hierarchical diagram shows the number of FS mutations (in the circles) on the basis of distribution of de novo, de novo/inherited and inherited mutations, and the frequency of frame 2 and frame 3 in the three groups. The position of the mutations is indicated in relation to the polyA tail region (upstream; inside; downstream). Among the inherited variants, the figure shows the constitutive (C) and mosaic (M) mutations of the carrier parents. Mutations associated with isolated CCHS (circles with black dots) are indicated with NONE = no associated symptoms reported (only CCHS). The associated ANS symptoms are indicated below each circle: HSCR/HSCR (TCA)/HSCR (L) (dark gray circles) = CCHS and Hirschsprung disease/total colonic aganglionosis/long segment HSCR; HSCR + NB/GNB (light gray) = CCHS, Hirschsprung disease and neuroblastoma/ganglioneuroblastoma)
(in three cases from mosaic parents); interestingly, only two of the 11 patients carrying FS24, that occurs inside the polyAla, inherited the mutation from one parent. Three of the five mutations downstream of the polyAla region were de novo and two inherited. Thus, the majority of inherited mutations occur upstream the polyAla stretch, in line with the observation that mutations in the terminal part of the coding region are likely more severe and penetrant.

Finally, the ratio between constitutive and mosaic variants among the eleven inherited mutations was 9:2, with both mosaic variants occurred inside the polyAla region.

3.5 PHOX2B proteins carrying c.618del(p.Ser207fs) and c.930dup(p.Ser311fs) mutations

In order to investigate the molecular mechanism underlying the occurrence of isolated or syndromic CCHS in the presence of PHOX2B FS mutations, we characterized the c.618del (p.Ser207Alafs*102) and c.930dup (p.Ser311Glufs*49) variants, which have very different characteristics (Supp. Table S2). To simplify, according to the HGVS nomenclature of variants, the full name of mutant proteins derived from c.618del and c.930dup hereon has been shortened from p.Ser207Alafs*102 to p.Ser207fs and p.Ser311Glufs*49 to p.Ser311fs, respectively.

The c.618del mutation previously reported by Matera et al. (2004) was inherited from the asymptomatic mother and is associated with a mild respiratory phenotype, whereas the clinical phenotype associated with the c.930dup mutation reported by Bachetti et al. (2005b), is quite severe, with apnea during sleep, total colon aganglionosis (TCA), and early onset NB.

The c.618del and c.930dup mutations lead to different aberrant C-terminal regions of the protein: the first causes a shift of the translational frame from amino acid 207 and leads to a truncated protein of 307 amino acids (frame 3, Figure 3A); the second affects the distal part of the protein and the induced shift leads to an elongated protein of 358 amino acids (frame 2, Figure 3A).

Interestingly, the elongated protein differs from the wild-type protein in terms of the loss of the last four amino acids and the novel 44 amino acid C-terminal that includes an additional polyAla stretch of seven residues from residue 320 to residue 326 (boxed in Figure 3A).

The secondary structure of the PHOX2B protein, which was predicted using the PSIREN server (https://bioinf.cs.ucl.ac.uk/psirend/) (Buchan et al., 2013; Jones, 1999), indicates the presence of α-helices in the HD as previously reported for other HD proteins (data not shown), and in the C-terminal region: one long helix in the 20 polyAla stretch and one shorter helix in the terminal part of the protein from residue 304 to residue 310 (Figure 3B). In the p.Ser311fs (c.930dup) mutant the C-terminal helix is substituted by two close helices separated by a short protein sequence in a random coil conformation, the second of which corresponds to the additional polyAla stretch of seven residues (residues 306–316 and 320–326; Figure 3B). The p.Ser311fs mutant is also characterized by the presence of an additional helix from residues 344 to 355, corresponding to the terminal part of the novel elongated tract.

The p.Ser207fs (c.618del) mutant is characterized by the replacement of the polyAla tract by an Arg/Gln-rich stretch generating two α-helices instead of one, which are separated by a short protein sequence in a random coil conformation (residues 239–244 and 253–266; Figure 3B).

3.6 Effect of p.Ser207fs and p.Ser311fs mutant proteins on PHOX2B expression

An auto-regulatory mechanism based on PHOX2B binding its own promoter allows PHOX2B to control and sustain its own expression (Cargnin et al., 2005). As most FS mutations show a decrease in transactivating regulatory regions of different PHOX2B target genes (i.e., DBH, PHOX2A, and TLX2) (Bachetti et al., 2005b; Borghini et al., 2006; Nagashima et al., 2012; Trochet et al., 2005a, 2009), we investigated the ability of the p.Ser207fs and p.Ser311fs mutants to regulate PHOX2B expression in terms of the transactivation of the PHOX2B promoter.

To this end, we co-transfected HeLa cells with the PHOX2B promoter reporter construct and each of the two PHOX2B expression constructs. As shown in Figure 4A, the forced over-expression of wild-type PHOX2B induced a fivefold increase in reporter gene expression in comparison with the empty pcDNA3.1 Myc-His vector, whereas the two mutants showed significantly less activity, with that of the p.Ser311fs mutant being almost reduced to basal level (given the unspecific transactivation shown when co-transfecting with the empty pHRG-B reporter vector).

In order to test the ability of the PHOX2B mutants to bind the PHOX2B promoter, we used EMSAs and radiolabelled oligonucleotides containing the ATTA 2 and ATTA 3–4 sites, which have the highest affinity for the protein expressed in vitro (Cargnin et al., 2005). Incubation of the wild-type protein with each radio-labeled oligonucleotide caused the appearance of specific retarded bands (complex I in Figure 4B, and I and II in Figure 4C) that could be competed with a molar excess of cold oligonucleotide and were supershifted by the anti-Myc antibody (Figure 4B and C, lanes 1–3).

The two frameshift mutations almost completely abolished DNA binding, an effect that was stronger than that shown by the longest expansion (p.Ala241[13]; +13 alanine) (Figure 4B and C, lanes 4–12). As in the case of the +13 alanine mutant, the presence of the p.Ser311fs mutant led to the appearance of a band at the top of the gel, corresponding to the well (Figure 4B and C, lanes 10–12), thus confirming that this protein has a tendency to oligomerize. Incubation of the p.Ser207fs mutant protein with each radio-labeled oligonucleotide caused the appearance of a very weak and faster retarded band (complex II, Figure 4B, lane 7, and complex III, Figure 4C, lane 7) that could be competed with a molar excess of cold oligonucleotide, but was not super-shifted by the anti-Myc antibody, thus suggesting abnormal protein conformation.

Gel filtration analyses have shown that frameshift mutants (regardless of the frame) have a strong tendency to oligomerize in vitro; however, cytoplasmic aggregation has not been reported for the frameshift mutants, including the p.Ser207fs and p.Ser311fs mutants (Bachetti et al., 2005b; Raabe et al., 2008; Reiff, Tarsovina, Majdazari, Schmidt,
Sequences and predicted secondary structures of PHOX2B WT and PHOX2B p.Ser207fs and p.Ser311fs mutants. The full name of mutant proteins derived from c.618del (p.Ser207fs) and c.930dup (p.Ser311fs) has been shortened in p.Ser207fs and p.Ser311fs.

A: Alignment of the amino acid sequences of PHOX2B wild-type protein and the PHOX2B proteins encoded by the c.618del (p.Ser207fs) and c.930dup (p.Ser311fs) mutant genes. The regions of the three proteins that show homology are in black, and the region of homology between the PHOX2B wild-type and p.Ser311fs mutated proteins are in gray. The alanine-rich regions are boxed.

B: The secondary structures of the PHOX2B wild-type and mutated isoforms were predicted using the PSIPRED server. Only the tail sequences were used as identity is maintained up to amino acid 206. The program predicts the possibility of a helix (box) or a coil (straight line) for the target amino acid sequences. GenBank accession numbers: NM_003924.3 was used as the reference sequence for the cDNA and NP_003915.2 for the protein.

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3.7 Effects of PHOX2B p.Ser207fs and p.Ser311fs mutant proteins on the expression of CCHS-related genes

To investigate the molecular mechanisms underlying the occurrence of HSCR and NB in CCHS patients, we tested the effects of the two FS mutations on the promoter regions of genes that play a role in the clinical manifestations of these diseases. In particular, in order to account for the development of HSCR, we investigated their effect on the promoter of the RET gene, which has previously been identified as a PHOX2B target gene (Bachetti et al., 2005a) and whose loss-of-expressing cells showed the formation of nuclear inclusions (Figure 4D, panels g–l).
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**FIGURE 4** Functional effects of PHOX2B wild-type and mutant proteins on the PHOX2B promoter. The full name of mutant proteins derived from c.618del (p.Ser207Alafs*102) and c.930dup (p.Ser311Glufs*49) has been shortened in p.Ser207fs and p.Ser311fs.

**A:** Luciferase assay. The bars indicate the transcriptional activity of the PHOX2B reporter construct upon co-transfection in HeLa cells with PHOX2B expression vectors containing the cDNA of wild-type protein or carrying the frameshift mutations (black bars). pcDNA3.1Myc-His indicates the empty vector used as a negative control. The white bars show the transcriptional activity of the corresponding empty reporter construct (phRG-B) upon co-transfection with the PHOX2B expression vectors. The results are the mean values ± SD (error bars) of the transcriptional activity of the constructs in at least three experiments performed in triplicate (= 3), and are expressed as percentages of the activity of the reporter plasmid co-transfected with the WT protein (= 1). *****Significant differences between the luciferase activity of the reporter plasmid co-transfected with PHOX2B wild-type-encoding vector and the tested mutants (ANOVA, Tukey’s test, *P* < 0.001).

**B and C:** Gel shift assays using oligonucleotide probes corresponding to the regions of the PHOX2B promoter containing ATTA core motifs that have been shown to bind PHOX2B. The labeled probes were incubated with in vitro expressed Myc-tagged PHOX2B wild-type protein (lanes 1–3), or mutated PHOX2B proteins (lanes 4–12). The in vitro translated pcDNA 3.1 Myc-His empty vector was used as a control to exclude non-specific interactions (lanes 13). The competition experiments were carried out by adding a molar excess of unlabeled oligonucleotide (lanes 2, 5, 8, and 11). Supershift experiments were performed by pre-incubating the in vitro expressed proteins with anti-Myc antibody (lanes 3, 6, 9, and 12). The roman numbers on the left indicate the specific retarded complexes obtained using the in vitro expressed PHOX2B proteins; the asterisks indicate the supershifted complexes containing PHOX2B. The free probes are visible at the bottom of the gels. **D:** Representative immunofluorescence images of the localization of the PHOX2B wild-type and the mutated p.Ser207fs and p.Ser311fs proteins. HeLa cells were transfected with the Myc-tagged proteins and analyzed 48 hr after transfection by means of immunofluorescence using anti-Myc antibody (b, e, and h); the nuclei were visualized using DAPI (a, d, and g), and merged with the proteins detected by the anti-Myc antibody (c, f, and i). Scale bars: 10 μm.

function (LOF) mutations are responsible for 15%–50% of the cases of HSCR cases (most of the others are caused by gene downregulation) (Griseri, Bachetti, Puppo, Lantieri, Ravazzolo, Devoto, & Ceccherini, 2005).

As shown in Figure 5A, the transfection of SK-N-BE cells with the two PHOX2B constructs and the RET promoter reporter plasmid showed that the p.Ser207fs mutant (which is not known to be associated with HSCR) transactivated the RET promoter to an extent that was comparable with that of the wild-type protein, whereas the activity of the p.Ser311fs mutant was significantly less.

It has previously been reported that, in patients with NB, PHOX2B FS mutations cause developing cells to switch incorrectly toward neuron differentiation, thus leaving them in undefined state than can be detected by double staining for markers of neurons and glia (Nagashimada et al., 2012). We therefore tested the effect of the PHOX2B mutants on a reporter construct containing the entire promoter region of the glial acidic fibrillary protein (GFAP) gene, a known astrocyte marker. As shown in Figure 5B, neither the wild-type protein nor the p.Ser207fs mutant (which is known not to be associated with NBs) transactivated the GFAP promoter, whereas the p.Ser311fs...
FIGURE 5  Transactivation of the RET and GFAP promoters by PHOX2B wild-type and mutant proteins. The full name of mutant proteins derived from c.618del (p.Ser207Alafs*102) and c.930dup (p.Ser311Gufs*49) has been shortened to p.Ser207fs and p.Ser311fs. The bars indicate the transcriptional activity of the RET promoter reporter construct upon co-transfection with PHOX2B expression vectors containing the cDNA of WT protein (white bars) or mutated proteins (black bars) in SK-N-BE cells (A) and the GFAP promoter reporter construct upon co-transfection with PHOX2B expression vectors containing the cDNA of WT protein (white bars) or mutated proteins (black bars) in U251-MG cells (B). pcDNA3.1 Myc-His indicates the empty vector used as a negative control (hatched bars). The results are the mean values ± SD (error bars) of the transcriptional activity of the constructs in at least three experiments performed in triplicate, and are expressed as fold induction over the activity of the reporter plasmid co-transfected with the empty vector. *Significant differences in comparison with the activity of the wild-type protein (Student t test, P < 0.05)

When we co-transfected both wild-type and mutant proteins at a ratio of 1:1, the reporter activity of the PHOX2B, DBH, and TLX2 genes induced by the wild-type protein was significantly reduced in the presence of either mutant (Figure 6, cross-hatched bars), the inhibitory effect of which was much greater in the case of p.Ser311fs mutant. On the contrary, when both FS mutants were tested on the RET reporter, transcriptional activity significantly increased (Figure 6, cross-hatched bars) and, remarkably, was comparable with the reporter activity obtained when testing a double amount of wild-type PHOX2B, thus suggesting that the p.Ser311fs mutant, whose ability to transactivate the RET promoter was reduced, does not interfere with the transcriptional activity of wild-type PHOX2B.

Taken together, our in vitro functional studies show that FS mutations have effects depending on the target promoter. When clustering the promoters in gene categories (±HSCR and NB), considering transcription down-regulation as a LOF effect and acquired upregulation as a gain-of-function (GOF) effect, the c.618del (p.Ser207fs) mutation has a slight LOF effect only on the promoters of the genes predisposing to CCHS, whereas the c.930dup (p.Ser311fs) mutation has a more marked LOF effect on all of the promoters other than the GFAP promoter, on which it has GOF effects (Figure 7).

4 | DISCUSSION

This article provides a detailed description of all of the FS mutations identified so far in CCHS patients and a classification of the associated symptoms in terms of neurocristopathies (mainly HSCR and NB); it also describes the results of functional studies aimed at
investigating the molecular mechanisms underlying isolated and syndromic CCHS.

The graphic representation of the mutant proteins offers information that is useful for molecular diagnosis and genetic counseling as the position of the mutation, and therefore the start of the translational frame, is critical for predicting its structural and clinical consequences. Frame 2 mutations are more frequently associated with syndromic CCHS than frame 3 mutations, and mutations starting upstream of the polyAla region are generally less severe than those occurring within and downstream of the repeated stretch. It is possible to postulate that polyAla region plays a role in FS mutation-mediated pathogenesis. Although the long PHOX2B insertions/deletions span the polyAla stretch starting from the first triplet and lead to the aberrant translation of the polyAla region, it is not clear whether an intact polyAla stretch or its disruption is more deleterious. Mutations in the proximity of the 3′ end of the coding region (c.866dup, c.930dup, c.931_935del, c.936dup) and characterized by an intact polyAla region are very severe. This is somehow unexpected as these distal mutations share the same aberrant C-terminal sequence with other frame 2 mutations that are associated with HSCR alone (c.608dup, c.617_618insT, and c.693_700del) and, except for a few amino acids, the wild-type protein sequence is completely preserved. This observation does not support...
**FIGURE 7** Summary of the effects of PHOX2B on target gene promoters. The full name of mutant proteins derived from c.618del(p.Ser207fs) and c.930dup(p.Ser311fs) has been shortened in p.Ser207fs and p.Ser311fs. The summary of the effects of PHOX2B WT and the two mutants on PHOX2B, DBH, TLX2, RET, and GFAP promoters is shown. In the two columns on left, the constructs are indicated with the corresponding associated clinical phenotype: CCHS = congenital central hypoventilation syndrome; NB = neuroblastoma; HSCR = Hirschsprung disease.

### A. Promoter transactivation

| PHOX2B    | Disease | PHOX2B | DBH | TLX-2 | RET | GFAP |
|------------|---------|--------|-----|-------|-----|------|
| WT         | -       | +++    | +++ | +++   | ++  | -    |
| c.618del   | CCHS    | ++     | +   | +++   | ++  | -    |
| (p.Ser207fs) |         |       |     |       |     |      |
| c.930dup   | CCHS    | +      | +   | +     | +   | ++   |
| (p.Ser311fs) |         |       |     |       |     |      |

### B. Dominant negative effect

| PHOX2B    | Disease | PHOX2B | DBH | TLX-2 | RET | GFAP |
|------------|---------|--------|-----|-------|-----|------|
| c.618del   | yes     | yes    | yes | no    | n.a.|
| (p.Ser207fs) |         |       |     |       |     |      |
| c.930dup   | yes     | yes    | yes | no    | n.a.|
| (p.Ser311fs) |         |       |     |       |     |      |

### C. Overall mutation effect

| PHOX2B    | Disease | PHOX2B | DBH | TLX-2 | RET | GFAP |
|------------|---------|--------|-----|-------|-----|------|
| c.618del   | LOF     | LOF    | -   | -     | -   | -    |
| (p.Ser207fs) |         |       |     |       |     |      |
| c.930dup   | LOF     | LOF    | LOF | LOF   | GOF |
| (p.Ser311fs) |         |       |     |       |     |      |

The hypothesis that the association of frame 2 mutations with a predisposition to CCHS-related ANS disorders is solely due to the aberrant amino acid tail.

Another factor that could play a role is the generation of novel domains enriched in particular amino acids. When frame 3 mutations begin upstream of the polyAla stretch, the frame change leads to a protein sequence rich in basic amino acids (Gln–Arg), which have been suggested to be the cause of the localization of the p.Ser207fs protein in the nucleolar compartment (Bachetti et al., 2005b). Arg methylation is a frequent post-translational modification involved in a number of cell processes (Blanc & Richard, 2017), and may confer anomalous properties to an already mutant PHOX2B protein. The Gly–Ser-rich domain, generated in the polyAla region when frame 2 mutations occur upstream of or at the beginning of the polyAla region, could be a target of phosphorylation or a spacer between protein domains, making the protein structure more relaxed (Reddy Chichili, Kumar, & Sivaraman, 2013). Finally, the 7-residue polyAla region aberrantly translated from the 3′UTR when frame 2 mutations occur may take on a pathogenic role as a spacer between domains or in protein–protein interactions.

In order to investigate further the molecular basis of the differences in the pathogenicity of frame 2 and frame 3 mutations, we tested the functional effects of the expression of two mutants representative of different CCHS-associated clinical phenotypes on a number of PHOX2B target genes. It is worth noting that both of the mutants under analysis showed severe defects in DNA binding but retained some transcriptional activity. In the case of p.Ser207fs, this is consistent with our recent reports concerning the role of the C-terminal domain as an important modulator of HD-mediated DNA binding (Di Lascio et al., 2016), but DNA binding defects are unexpected in the case of the c.930dup mutant as it has been reported that
proteins carrying mutations in the same region (i.e. c.931_935del and c.936dup) retain some ability to bind DNA, albeit partially misfolded (Trochet et al., 2005a). Our data indicate that changes in the C-terminal domain affect HD folding and/or functions, but do not completely abolish the transactivation capacity of the mutants, which leads us to speculate that these aberrant sequences can still recruit co-transactivators. This is reasonable in the case of the RET promoter whose expression is likely regulated by PHOX2B through an indirect mechanism (Bachetti et al., 2005a), but it is intriguing in the case of the TLX2 and GFAP promoters. GFAP promoter transactivation by p.Ser311fs mutant may be due to a novel direct interaction or, alternatively, the new C-terminal region may recruit novel co-transactivators that do not normally interact with the wild-type protein. In any case, the induction of GFAP expression reflects the occurrence of GOF effects of the c.930dup mutation.

In vivo studies have provided evidence of functional differences between PARM and NPARM mutations. Mouse models of CCHS expressing PARM or NPARM mutations of Phox2b show respiratory distress with the selective loss of the retro-trapezoid nucleus and an abnormal development of locus coeruleus, (Dubreuil et al., 2008; Nagashimada et al., 2012; Nobuta et al., 2015). In addition, mice with frame 2 FS mutations show defects in a much broader range of neuronal populations, including the autonomic ganglia (Nagashimada et al., 2012). This can be at least partially explained by differences in the transcriptional properties of the PHOX2B mutants as PARM mutants showed no dominant-negative effects on the activity of wild-type protein. In any case, the induction of GFAP expression reflects the occurrence of GOF effects of the c.930dup mutation.

In our study, both mutants showed a reduced ability to induce the correct transactivation of the PHOX2B promoter, and had similar inhibitory effects on the wild-type protein. Given the milder clinical phenotype shown by the patient carrying the c.618del mutation in comparison with that associated with the c.930dup mutant, its negative effects are somehow unexpected. Furthermore, its dominant-negative effects were milder than those of the c.930dup mutant, which suggests that other mechanisms may underlie the clinical manifestations.

Results obtained in vivo argue against the idea that CCHS is due to a pure LOF mechanism (Dubreuil et al., 2008; Durand et al., 2005; Ramanantoa et al., 2006, 2011) and suggest that the ANS symptoms of the enteric and NB phenotypes seem to be associated with PHOX2B gene dosage (Goridis, Dubreuil, Thoby-Brisson, Fortin, & Brunet, 2010; Trochet et al., 2009). Our previous findings indicate that proteins carrying the shortest alanine expansions do not apparently alter PHOX2B auto-regulation (Di Lascio et al., 2013) and, it can be hypothesized that, when the auto-regulatory loop is only marginally involved (as in the case of the shortest polyAla expansions), the phenotype is less severe and limited to respiratory function. On the contrary, when the auto-regulatory loop becomes affected, PHOX2B expression can drop below a critical threshold, thus increasing the severity of the respiratory phenotype and the appearance of more general autonomic deregulation. A dose reduction to <50% (sub-haploinsufficiency) may be required as mice that are haploinsufficient for Phox2b do not develop tumors. As human phenotypes varying from mild to severe have been associated with deletions in the PHOX2B gene, including one case with CCHS and a neural crest tumor (Jennings et al., 2012), it is necessary to consider alternative mechanism(s) that may induce a predisposition to NB.

PHOX2B haploinsufficiency also plays a role in HSCR by acting as a predisposing factor for intestinal developmental and functional defects (Fernández et al., 2013). We have also recently reported that polyAla contractions may predispose to HSCR by down-regulating RET transcription, albeit to a lesser extent than polyAla expansions (Di Zanni et al., 2017). In line with the marked correlation between frame 2 mutations and syndromic forms of CCHS, our functional data show that the transactivation of genes involved in enteric nervous system development (TLX2 and RET) is much more impaired by frame 2 than by frame 3 mutations. Furthermore, the p.Ser311fs mutant gained the ability to transactivate the promoter of the GFAP gene, a marker of glial cells, which is in line with in vivo evidence that two frame 2 NPARMs induce the differentiation of progenitors to glial cells fate (Nagashimada et al., 2012).

As previously reported in the case of PARM mutations, our data indicate that the dominant-negative effects of PHOX2B NPARM proteins are promoter specific and probably due to aberrant protein-protein interactions. Further characterization and the identification of novel molecular interactions and targets are required to clarify further the relationship between NPARM mutants and the molecular pathogenesis of the CCHS-HSCR-NB association. In particular, we suggest that the risk of finding an aganglionic colon in patients with frame 3 mutations depends on the specific mutation itself as only 50% of such mutations cause HSCR (the others are neutral and only cause CCHS). Moreover, HSCR is congenital and its diagnosis is certain, whereas the risk of developing NB in the first years of life is more difficult to estimate: it is therefore crucial to follow up CCHS patients carefully in order to be able to define genotype-phenotype correlations in more details.

In conclusion, the findings of this study show that NPARM mutant proteins have a combination of dominant-negative, LOF and GOF effects that depend on the analyzed promoter. Our data indicate that DBH, TLX2, RET, and PHOX2B are target genes susceptible to the reduced activity of NPARM proteins, and that the GFAP gene is a new aberrant molecular target. Finally, they show that some of the functional differences among the mutations belonging to different frame subgroups are the underlying cause of the ANS disorders associated with CCHS.

ACKNOWLEDGMENTS

We are grateful to the Associazione Italiana per la Sindrome da Ipoventilazione Centrale Congenita (A.I.S.I.C.C.) and to all CCHS patients and their families. We would like to thank Kevin Smart for his help in preparing the manuscript. We would also like to thank Monzino Foundation (Milan, Italy) for its generous gift of the Zeiss LSM 510 Meta confocal microscope.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.
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SUPPORTING INFORMATION

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How to cite this article: Di Lascio S, Benfante R, Di Zanni E et al. Structural and functional differences in PHOX2B frameshift mutations underlie isolated or syndromic congenital central hypventilation syndrome. Human Mutation. 2018;39:219–236. https://doi.org/10.1002/humu.23365