# Distinct pathogenetic mechanisms for *PHOX2B* associated polyalanine expansions and frameshift mutations in congenital central hypoventilation syndrome

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**Congenital central hypoventilation syndrome (CCHS) is a rare neurocristopathy characterized by absence of adequate autonomic control of respiration with decreased sensitivity to hypoxia and hypercapnia. Frameshift mutations and polyalanine triplet expansions in the coding region of *PHOX2B* have been identified in the vast majority of CCHS patients and a correlation between length of the expanded region and severity of CCHS has been reported. In this work, we have undertaken *in vitro* analyses aimed at identifying the pathogenetic mechanisms which underlie the effects of *PHOX2B* mutations in CCHS. According to the known role of this gene, a transcription factor expressed during autonomic nervous system development, we have tested the transcriptional activity of WT and mutant *PHOX2B* expression constructs on the regulatory regions of two target genes, *DβH* and *PHOX2A*. We observed that the two sets of mutations play different roles in the transcriptional regulation of these genes, showing a correlation between the length of polyalanine expansions and the severity of reduced transcriptional activity. In particular, although reduced transactivation due to polyalanine expansions may be caused by retention of the mutated protein in the cytoplasm or in the nuclear aggregates, frameshift mutations did not impair the PHOX2B nuclear income, suggesting a different mechanism through which they would exert the observed effects on target promoters. Moreover, the frameshift due to deletion of a cytosine residue seems to cause sequestration of the corresponding mutant PHOX2B in the nucleolar compartment.**

## INTRODUCTION

Congenital central hypoventilation syndrome (CCHS) is a rare neurocristopathy characterized by absence of adequate autonomic control of breathing, especially during sleep, with decreased sensitivity to hypoxia and hypercapnia (1,2). Autosomal dominant transmission with reduced penetrance has been demonstrated in CCHS families, in association with mostly *de novo* mutations of the *PHOX2B* gene (3–6). *PHOX2B* encodes a highly conserved paired box homeodomain transcription factor, characterized by two poly-alanine repeats of nine and 20 residues in the C-terminal region. Mutation screening of the *PHOX2B* exon 3 in CCHS patients has revealed in-frame duplications within the 20 alanine stretch, leading to expansions from +5 to +13 alanine residues, and frameshift mutations, leading to aberrant C-terminal regions (3–6).

Expression studies have detected the *PHOX2B* transcript in several different districts of the autonomic nervous system, in particular in the developing hindbrain and peripheral nervous

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system as well as in all noradrenergic centres and in specific neuronal groups such as those involved in the medullary control reflexes of autonomic functions (7). In the mouse, by embryonic day 9–9.5, the Phox2b protein is detected in enteric neuroblasts invading the foregut mesenchyme and it is expressed in the oesophagus, small intestine, large intestine and in all undifferentiated neural crest-derived cells in the gut with a rostrocaudal gradient (8,9). These observations are supported by *in vivo* studies showing that in *Phox2b* knocked-out mice, enteric neuronal precursors colonize the foregut but cannot migrate further (10).

Functional studies have shown that PHOX2B is a transcription factor required both for correct expression of tyrosine hydroxylase and for dopamine-β-hydroxylase (DβH), two genes encoding enzymes involved in the catecholamine biosynthesis, and to maintain expression of the mammalian achaete scute homologue-1, indicating that PHOX2B regulates the noradrenergic phenotype in vertebrate neural cells (11,12). Moreover, PHOX2B is also involved in the transcriptional regulation of the related *PHOX2A* gene, whose product is required for the formation of the locus coeruleus and expressed in all neurons that, either permanently or transiently, show the (nor)adrenergic phenotype (13,14). Finally, we have very recently demonstrated that both these two homeoproteins indirectly take part in the transcriptional activation of *c-RET* (15).

CCHS patients often show different and variable series of associated symptoms, mostly concerning dysfunctions of the autonomic nervous system (16). We have already reported that patients carrying the largest alanine expansions (+11 and +13) and 38 bp deletion show an extremely severe phenotype, needing ventilation support during both night and daytime and presenting in addition, in two cases, also HSCR disease. In contrast, CCHS patients carrying the shortest alanine expansions show a less severe phenotype which can sometimes lead to a delay in the diagnosis (late-onset cases) and reduced penetrance, (6). Such a correlation between length of alanine expanded stretches and phenotype severity of CCHS patients suggest that, for these mutations, the onset and severity of the disease can be predicted with a good approximation (5,6). In contrast, whether severe additional symptoms, like Hirschsprung's disease, are more frequently associated with the largest alanine expansions is still debated (6,17).

Polyalanine regions are frequent in transcription factors and in-frame duplications in these regions are emerging as a recurrent cause of human genetic diseases (18,19). In particular, polyalanine expansions have been identified as mutational hotspots in several genes, in association with congenital developmental disorders (19). Although the functional role of polyalanine stretches is still unknown, recent studies have shown that polyalanine expansions can lead to protein intra-cellular aggregation, thus affecting the physiological localization of the wild-type protein (20–22). In addition to these studies, co-transfection assays revealed that transcription factors carrying polyalanine expanded tracts would impair transcriptional regulation of target genes, like in the case of alanine expanded ZIC-2 which induces a decreased transcriptional activity of the *Apo* E regulatory region, when compared with the wild-type protein (23).

In this work, we report a functional study aimed at understanding the molecular pathogenesis of CCHS. To this end, we have investigated the level of transcriptional activity of wild-type and mutant PHOX2B constructs on *DβH* and *PHOX2A* target promoters and checked their sub-cellular localization by using green fluorescent protein (GFP)-vectors. A different pattern of molecular effects could be observed when testing polyalanine expansions compared with the frameshift mutations, thus suggesting distinct mechanisms underlying CCHS development.

## RESULTS

### *PHOX2B* mutations in CCHS cases

Following a previous report of *PHOX2B* mutations in CCHS patients (6), we have proceeded finding, so far, a total of 32 in-frame nucleotide duplications inside the 20 alanine stretch, leading to expansions from +5 to +13 alanine residues, and four frameshift mutations (c.614–618delC, c.862–866insG, c.721–758del38nt and c.930insG), leading to different aberrant C-terminal of the protein. In particular, a change of the WT translational frame (frame 1) starting at progressively distal sites of exon 3 and leading to 358 amino acids elongated proteins (frame 2) is assumed as a result of the latter three mutations, whereas a second aberrant shift of the WT reading frame, resulting in a truncated protein of 307 amino acids (frame 3), can be predicted for the c.614–618delC mutation (Fig. 1). The c.930insG mutation is reported here for the first time.

### *In vitro* transactivation of PHOX2B target promoters

To investigate the role of *PHOX2B* mutations in CCHS pathogenesis, we generated *PHOX2B* expression constructs containing each of the 10 mutations we have detected so far in CCHS patients and analysed their ability to induce activation of the regulatory regions of *DβH* and *PHOX2A*, earlier identified as *PHOX2B* target genes (12,14).

HeLa cells were co-transfected with *PHOX2B* expression plasmids and a *Luciferase* reporter construct containing the *DβH* regulatory region. The Luciferase level induced by each PHOX2B mutant was compared with the value obtained by co-transfecting the *DβH* promoter with the WT *PHOX2B* construct (100%). A significant decrease in *DβH* promoter activation, subsequent to the expression of each mutant PHOX2B, was found. In particular, a strict correlation ($r = 0.987$, $P < 0.0001$) between the reduction of Luciferase activity and increasing lengths of the co-transfected polyalanine tracts could be observed, as shown in Figure 2A (left) where a regression line is also traced. Similarly, values of Luciferase activity obtained by co-transfection of *PHOX2B* frameshift mutations showed a marked decrease with respect to the WT construct (Fig. 2A, right) suggesting impaired activation of *DβH* 5′ flanking region when the C-terminal of the protein is disrupted.

To test the effect of mutant versions of the PHOX2B protein on a further target promoter, each expression construct was also co-transfected with the *PHOX2A* regulatory region sub-cloned upstream of the *Luciferase* reporter gene. Although mutant PHOX2B proteins displayed a reduction of transcriptional activation ability on the *PHOX2A* promoter in comparison
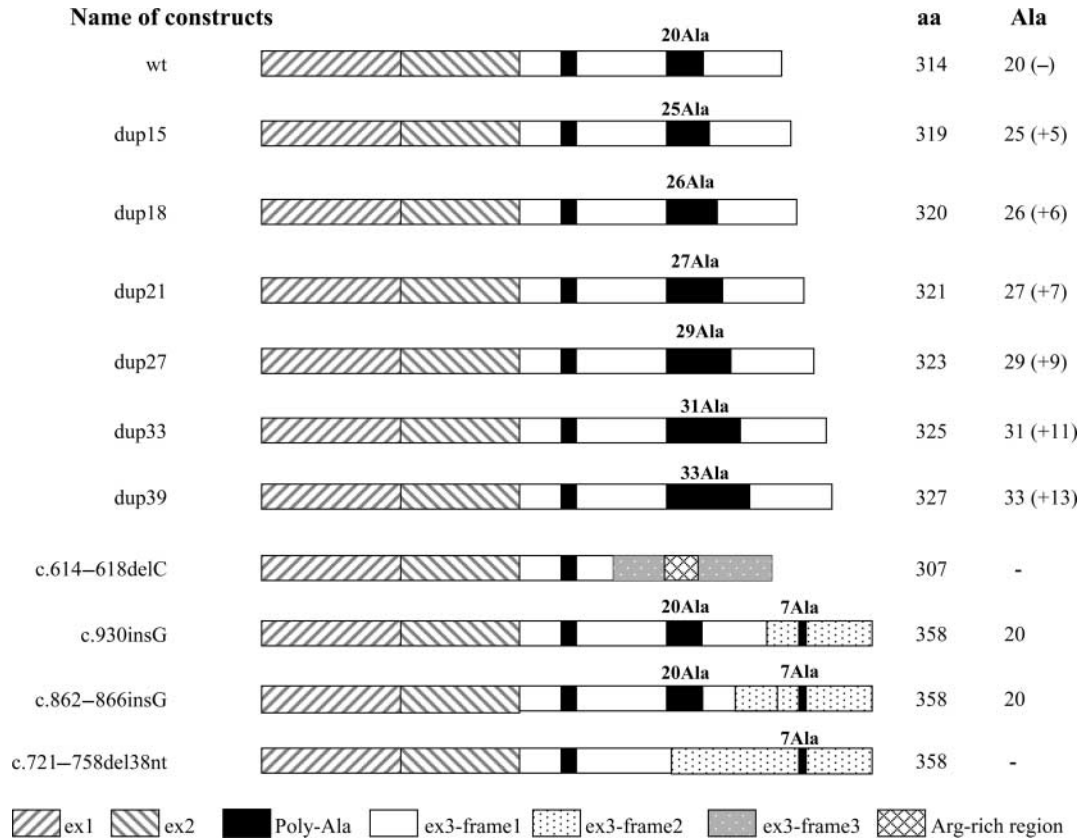
**Figure 1.** Schematic representation of expression constructs carrying *PHOX2B* mutations detected in CCHS patients. For each construct, name, exon 3 structure, number of total amino acid residues and length of the largest polyalanine stretch of the corresponding protein are reported. 'WT': normal PHOX2B construct; from 'dup 15' to 'dup 39': proteins with poly-Ala expansions; last four constructs: proteins derived from the four frameshift mutations and characterized by variable shifted regions of exon 3 amino acidic sequence. Protein fragments and domains, including the two newly determined translational reading frames, are represented by differently stained boxes.

to the *DβH* promoter, a significant correlation ($r = 0.948$, $P = 0.0012$) between decreased reporter gene activity and length of the polyalanine stretch could still be detected (Fig. 2B, left). Surprisingly, all the frameshift mutations conferred a 10–30% increased ability in activating the *PHOX2A* promoter with respect to the WT protein (Fig. 2B, right). Such an effect has resulted statistically significant, following a randomization test, in those cases where the shift resulted in frame two-elongated proteins (c.862–866insG, c.721–758del38nt and c.930insG).

*PHOX2B* mutations resulted in progressive decrease of activation ability, but constructs carrying the most expanded poly-Ala stretches caused a reduction of promoter activity below the non-specific basal level detectable, when the pcDNA3.1 empty vector was co-transfected (Fig. 2, graphs on the left). Such an inhibitory effect was likely due to inactivation of transcription factors, necessary for the basal expression of promoter constructs, by interactions with these poly-Ala expanded proteins.

## Sub-cellular localization of WT and mutant PHOX2B proteins

To verify the hypothesis that poly-Ala expansions may cause a retention of PHOX2B in the cytosol, we first analysed by western blot the level of WT and mutant proteins under various conditions. Total cellular lysates and nuclear extracts were obtained from HeLa cells transfected with wild-type, dup15, dup27 and dup39 expression plasmids. Forty-eight hours after transfection, we observed that the PHOX2B level detected in whole cellular lysates did not show any significant difference when comparing cells transfected with mutant and wild-type *PHOX2B* constructs (Fig. 3A). In contrast, the amount of PHOX2B protein in nuclear extracts of cells expressing alanine expansions was lower than in WT transfected cells, and, in particular, it was inversely correlated to the length of the expanded poly-Ala tract, as shown in Figure 3B where a very low level of PHOX2B protein could be detected in cells transfected with the +39 bp construct.

To determine whether such a reduced amount of nuclear PHOX2B underlay the defective transcriptional regulation induced by mutant versions of PHOX2B on its targets, we produced constructs containing wild-type PHOX2B (20Ala), PHOX2B carrying the shortest Ala expansion (+5Ala), the middle expansion (+9Ala) and the longest Ala expansion (+13Ala), each fused to the GFP protein. We transiently transfected these plasmids in COS-7 cells and analysed the samples 48 h later by fluorescence microscopy. Values were obtained performing at least two independent experiments and counting 100–200 cells by two distinct investigators. The patterns of
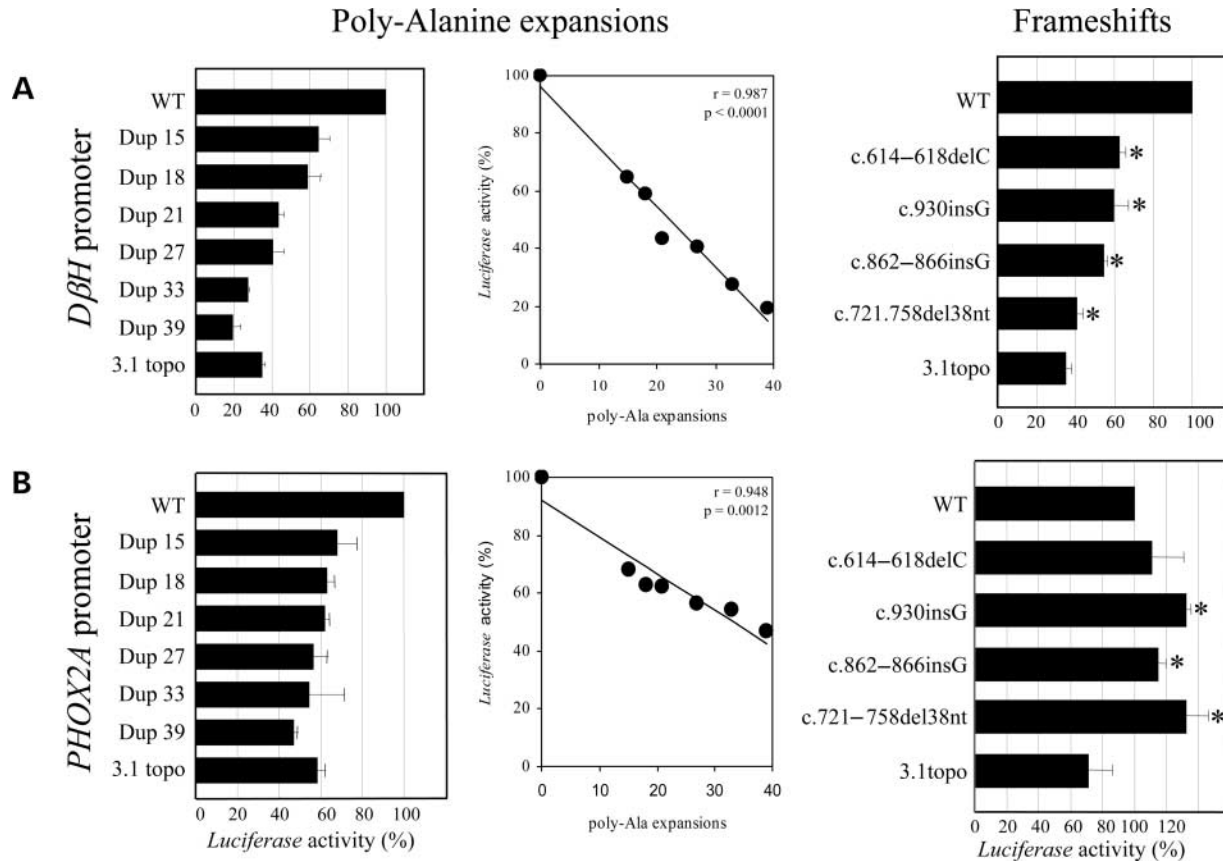
**Figure 2.** Co-transfection of mutant *PHOX2B* constructs with *DβH* and *PHOX2A* regulatory regions. From left to right, *Luciferase* activity obtained by co-transfection of the *DβH* promoter (**A**) and the *PHOX2A* promoter (**B**) constructs with *PHOX2B* expression constructs carrying poly-Ala expanded tracts and frameshift mutations. Mean values of *Luciferase* activation induced by each *PHOX2B* mutation under analysis, and obtained from two independent experiments performed in duplicate, are reported as percentage of the wild-type construct. In the case of poly-Ala expansions, regression lines have been traced (graphs in the middle) and the significance of the correlation thus obtained tested. Significant differences ($P < 0.05$) in the means between the WT and each specific mutant frameshift, tested by permutation–randomization analysis, are reported with asterisks in the graphs in the right.

fluorescence distribution observed in transfected cells (Fig. 4A) allowed to deduce that, while the wild-type protein localized almost exclusively inside the nucleus, a large amount of PHOX2B proteins carrying alanine expansions was retained in the cytoplasm, both in widespread diffuse and in aggregated forms. Moreover, nuclear aggregates were observed in only 4% of the cells expressing the wild-type protein and, in contrast, in approximately half of the cells expressing the +13Ala protein. A correlation between increasing lengths of the alanine repeats and percentage of cells characterized by a complete or partial cytoplasmic localization ($r = 0.974$, $P = 0.0259$) was observed (Fig. 4B), with cells transfected with +9Ala and +13Ala constructs showing, accordingly, a large formation of both cytosolic and punctate intra-nuclear protein aggregates.

To verify whether retention in the cytosol and formation of cytoplasmic and nuclear aggregates could be common to all *PHOX2B* mutations, we transfected COS-7 cells with GFP fusion constructs expressing the frameshift mutations c.930insG and c.614–618delC, each exemplifying the effects of a different aberrant reading frame. In both cases, an almost exclusively nuclear localization, as observed for wild-type PHOX2B, could be demonstrated (Fig. 4C). Nevertheless,

~84% of the cells expressing c.614–618delC and showing nuclear localization were characterized by segregation of the mutant protein into sub-nuclear round compartments resembling nucleoli, as shown in Figure 5A where the peculiar localization of this mutant is visualized under optical (o), fluorescence (f) and both (o + f) modalities. In Figure 5B, a COS-7 cell transfected with c.614–618delC-GFP and stained for nucleolar regions is shown in optical mode (o) to visualize the dark stained nucleoli and in fluorescent mode (f) to confirm the nucleolar localization of this mutant. This observation is in accordance with the presence of an Arg-rich region at the C-terminal region of the protein, encoded by frame 3 as a consequence of the cytosine deletion (Fig. 5C), confirming that basic amino acids, and in particular arginine residues, are required for nucleolar localization (24).

## Interaction between the +13Ala mutant and WT proteins

Interaction between wild-type and mutant proteins, leading to dominant negative effects, has already been reported for poly-alanine expanded proteins in human disease (20–23). For this reason, we undertook experiments to verify whether such pathogenetic mechanism could play a role also in CCHS
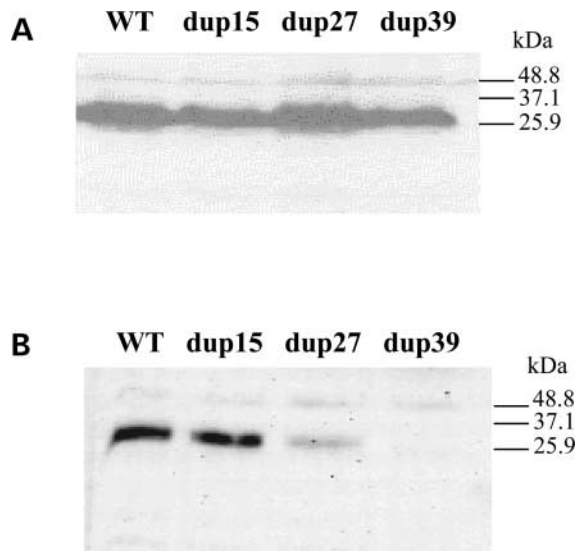
**Figure 3.** Nuclear translocation of wild-type, +5Ala, +9Ala and +13Ala PHOX2B proteins. Western blotting assays were performed with equal amounts of total lysate (**A**) and nuclear extract (**B**) proteins from HeLa cells transfected with 'WT', 'dup15', 'dup27' and 'dup39' constructs, expressing WT PHOX2B, +5Ala, +9Ala and +13Ala PHOX2B, respectively.

associated *PHOX2B* mutations. First, we co-expressed equal amounts of wild-type PHOX2B-GFP and mutant +13Ala-GFP in COS-7 cells without observing any retention of the total fluorescent proteins in a specific cellular compartment. This suggests that the mutant protein does not induce mislocation of the wild-type PHOX2B into the cytoplasm (Fig. 6A).

Subsequently, we co-transfected a fixed amount of PHOX2B-GFP ('WT') with increasing amount of the expression constructs 'WT' and 'dup39' (+13Ala) without GFP. The analysis of the nuclear localization of the fluorescent signal conjugated to the wild-type protein demonstrated that WT PHOX2B was present in nuclear aggregates to a higher extent when co-transfected with the construct carrying +13Ala than with the wild-type construct (10−15% versus 4%) and that the effect was dose-dependent (Fig. 6B). This result indicates that the mutant protein is able to interact with a fraction of wild-type PHOX2B and sequester it in nuclear aggregates, although the percentage of cells with nuclear aggregates is far from 50% observed after transfecting the +13Ala GFP construct only.

## DISCUSSION

In this work, we report a functional study of *PHOX2B* mutations detected in CCHS patients. PHOX2B is a nuclear protein involved in the transcriptional regulation of several genes expressed during development and maintainance of the autonomic nervous system. In particular, co-transfections and gel shift assays have shown that the transcription of *DβH*, a gene involved in cathecolamine synthesis, and *PHOX2A*, a gene crucial in the specification of subsets of noradrenergic neurons, is regulated to some extent by PHOX2B, through direct binding to homeodomain binding sites identified in their 5′ regulatory regions (12−14). Therefore, such an information has provided a straightforward mean to investigate the effect of CCHS associated mutations on PHOX2B activity.

### Polyalanine triplet expansions

A genotype−phenotype correlation between extension of the PHOX2B poly-Ala tract and clinical severity of CCHS, in terms of both respiratory phenotype and associated symptoms, especially intestinal dysfunction, has been reported by Matera *et al.* (6) and others (5). Accordingly, in our *in vitro* transfection experiments, a strictly correlated progressive decrease in *DβH*- and *PHOX2A*-promoters mediated transcriptional activation of the *Luciferase* reporter gene has been observed with the lengthening of the alanine expanded tract, thus suggesting a more pronounced impairment of PHOX2B cellular effects in largest mutants. In contrast, rare polymorphic *PHOX2B* alleles with contractions in the poly-Ala tract were shown to decrease weakly the *DβH* transcription (25), suggesting that a polyalanine stretch of 20 residues is the most appropriate to provide high PHOX2B transcriptional efficiency.

Recent evidences have shown that expanded poly-Ala tracts cause intracellular aggregation of several transcription factors such as HOXD13 (20), ARX (21) and FOXL2 (22). Therefore, we investigated whether this mechanism could be responsible also for CCHS pathogenesis and could explain the apparent loss-of-function displayed to different extent by poly-Ala expanded proteins on the three target promoters tested. To this end, after excluding, by western blot assay in total cell lysates, defects in proteins expression, we could demonstrate a correlation between reduced amounts of transcription factor in nuclear extracts and length of the expanded region. This observation has been confirmed by expressing GFP fused to WT and mutant PHOX2B carrying five, nine and 13 extra alanine residues. In particular, similar to what already reported for other polyalanine containing disease genes (20−22), we have observed that expansions in the poly-Ala tract resulted in a length dependent cytoplasmic retention with aggregates, both inside the nucleus and in the cytosol.

In addition, recent studies have shown that some transcription factors carrying the expanded poly-Ala region can interact with the wild-type protein, resulting in its sequestration in cytoplasmic or in nuclear aggregates (20−22). Analysis of the sub-cellular localization of co-expressed wild-type and +13Ala-PHOX2B showed that the protein carrying the largest alanine expanded region could not retain wild-type PHOX2B in the cytoplasmic compartment. Nevertheless, the +13Ala protein resulted to interact with the wild-type transcription factor in the nucleus, with limited but significant formation of nuclear aggregates. Therefore, in addition to cause functional haploinsufficiency, PHOX2B poly-Ala expanded tracts exert a partial dominant negative effect, thus resembling the pathogenetic mechanism suggested for HOXD13, FOXL2 and ZIC-2 poly-Ala expanded factors (20,22,23).

At present, there is no clear evidence on the role of the poly-Ala domain in the PHOX2B transcription factor, a circumstance which does not exclude additional pathogenetic
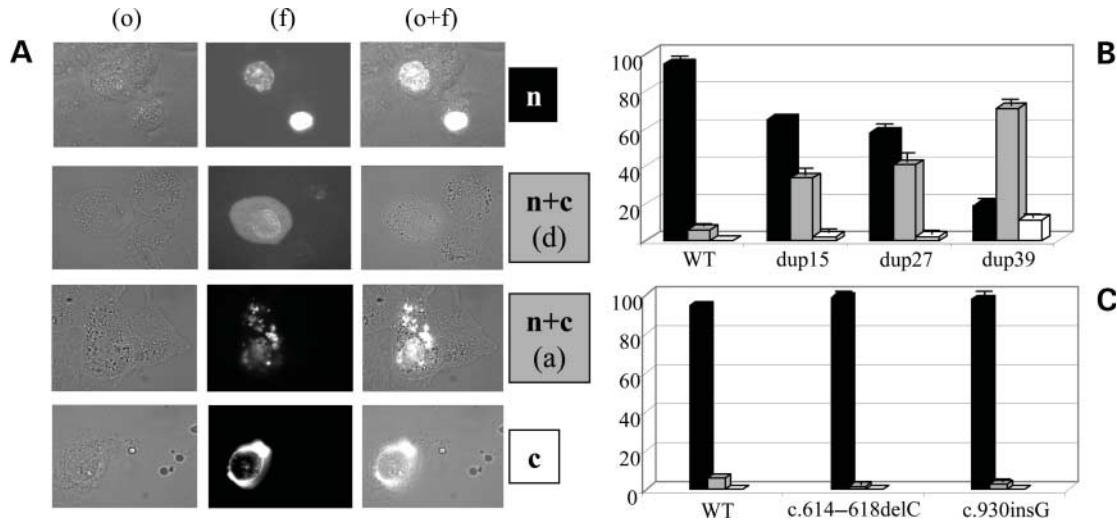
**Figure 4.** Expansions of the poly-Ala region lead to retention of PHOX2B in the cytoplasmic compartment. (**A**) Four patterns of PHOX2B cellular localization visualized with optic (o), fluorescence (f) and both optic and fluorescence (o + f) modalities. n, nuclear localization only; [n + c(d)], both nuclear and cytoplasmic diffuse localization; [n + c(a)], both nuclear and cytoplasmic localization, with formation of nuclear and/or cytoplasmic aggregates; c, cytoplasmic localization only. (**B**) Distribution of cellular localization of transfected PHOX2B-GFP, +5Ala-GFP, +9Ala-GFP and +13Ala-GFP. (**C**) Distribution of cellular localization of transfected PHOX2B-GFP, c.930insG-GFP and c.614−618delC-GFP. In (B and C), black bars indicate the percentage of cells showing exclusive nuclear localization, grey bars represent the sum of nuclear and cytoplasmic localization, both with formation of nuclear and/or cytoplasmic aggregates [n + c(a)] and with wide and diffused fluorescence [n + c(d)], white bars represent the percentage amount of exclusive cytoplasmic localization. Results are the mean ± SD of at least two independent experiments performed in duplicate.

mechanisms of its length expansions in CCHS. However, aggregation of misfolded proteins is emerging as a common feature of a wide range of human diseases (26), and it has become clear that an increase of Ala repeat length can result in aggregation of the misfolded protein thus compromising the folding of other proteins and possibly influencing more general cellular functions (20,22). In this light, we suggest that, also in the case of poly-Ala expanded PHOX2B proteins, altered folding is ultimately responsible for haploinsufficiency of the transcription factor in the nucleus and for WT protein sequestration with dominant negative effects.

### Frameshift mutations

Co-transfections of *PHOX2B* constructs carrying frameshift mutations with the *DβH* regulatory region resulted in a 40−60% decrease of transcriptional activation. The c.614−618delC mutation induced the lowest reduction, whereas stronger impairments were due to c.930insG, c.721−758del38nt and c.862−866insG mutations, characterized by a progressive increase of the length of the novel, shifted C-terminal, respectively (Fig. 1). A clear effect of this latter proteic fragment in disrupting the PHOX2B function can be therefore proposed, although a contemporary gain of function due to the creation of a novel seven alanine stretch in the reading frame 2 (C-terminal of the last three frameshift mutations, as shown in Fig. 1) cannot be excluded. In particular, the altered PHOX2B C-terminal region could modify the folding of the protein leading to either defective binding to target regulatory regions or altered binding to factors co-operating in PHOX2B transcriptional activity. Such an altered transcriptional function is in accordance with results reported for holoprosencephaly-associated *ZIC-2* mutations

and showing that both 25 extra-alanine residues and mutations leading to a disrupted C-terminal of the protein impair the transcriptional activation of the *Apo* E regulatory region (23).

Opposite to the impaired *DβH* transcriptional activation, we observed a 10−30% increased ability of frameshift mutations in activating the *PHOX2A* promoter with respect to WT PHOX2B. Such an effect might be related to the specific and similar roles PHOX2B and PHOX2A share in the specification and differentiation of noradrenergic neurons, and to possible relationship of reciprocal expression regulation, as also demonstrated by wide overlaps in their expression pattern.

The recent detection of *PHOX2B* frameshift mutations in specific sets of patients affected with neuroblastoma (NB), like in the familial and multifocal cases (27−29), although not in others (28,30), raises interesting hypotheses on the oncogenic role exerted by PHOX2B. This is confirmed by the observation that *PHOX2B* frameshift mutations seem to predispose CCHS patients to develop tumours of the sympathetic nervous system (17). In this light, and based on our present results, we propose that the PHOX2B oncogenic role on NB development may be mediated by the effect of its frameshift mutations on, among others, the *PHOX2A* promoter.

Differently from PHOX2B proteins carrying expansions in the poly-Ala region, the presence of frameshift mutations seems associated with a different pathogenetic mechanism. Despite impaired expression observed for most frameshift mutants, following western blot assay of total lysates from transfected HeLa cells (data not shown), we have excluded the hypothesis of cytoplasmic retention for both c.930insG and c.614−618delC. Surprisingly, whereas c.930insG showed an exclusively diffuse nuclear localization, PHOX2B
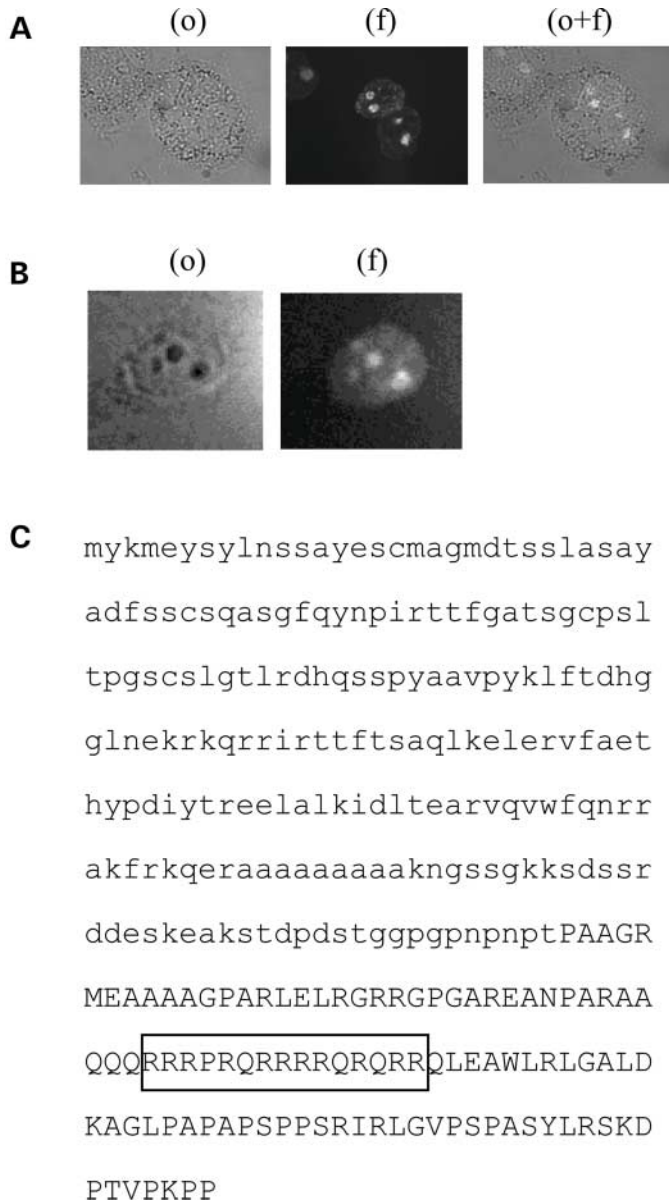
**A** (o) (f) (o+f)

**B** (o) (f)

**C**
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mykmeysylnssayescmagmdtsslasay
adfsscsqasgfqynpirttfgatsgcpsl
tpgscslgtlrdhqsspyaavpyklftdhg
glnekrkqrrirttftsaqlkelervfaet
hypdiytreelalkidltearvqvwfqnrr
akfrkqeraaaaaaaaakngssgkksdssr
ddeskeakstdpdstggpgpnpnptPAAGR
MEAAAAGPARLELRGRRGPGAREANPARAA
QQQRRRPRQRRRQRQRRQLEAWLRLGALD
KAGLPAPAPSPPSRIRLGVPSPASYLRSKD
PTVPKPP
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**Figure 5.** Frameshift mutation c.614−618delC leads to PHOX2B nucleolar localization. (**A**) Localization of c.614−618delC-GFP is visualized with optic modality (o), fluorescence (f) and both optic and fluorescence (o + f). (**B**) Silver staining of nucleoli in a cell transfected with c.614−618delC-GFP: in the left panel (optical modality), dark nucleoli are visible; in the right panel, GFP fluorescence is localized in the same compartment. (**C**) PHOX2B protein encoded by the c.614−618delC mutant gene; capital letters indicate the C-terminal amino acids produced by the translational reading frame 3, whereas the arginine-rich region is boxed.

carrying c.614−618delC resulted mainly accumulated in round nuclear sub-compartments resembling the nucleoli.

*In silico* analysis showed that c.614−618delC leads to an out of frame mutant protein characterized by an arginine-rich stretch in the C-terminal region. It has already been reported that basic amino acid sequences are required for nucleolar localization and that sequestration in the nucleolus prevents proteins from reaching their targets in other cellular compartments (24). Silver staining of cells transfected with
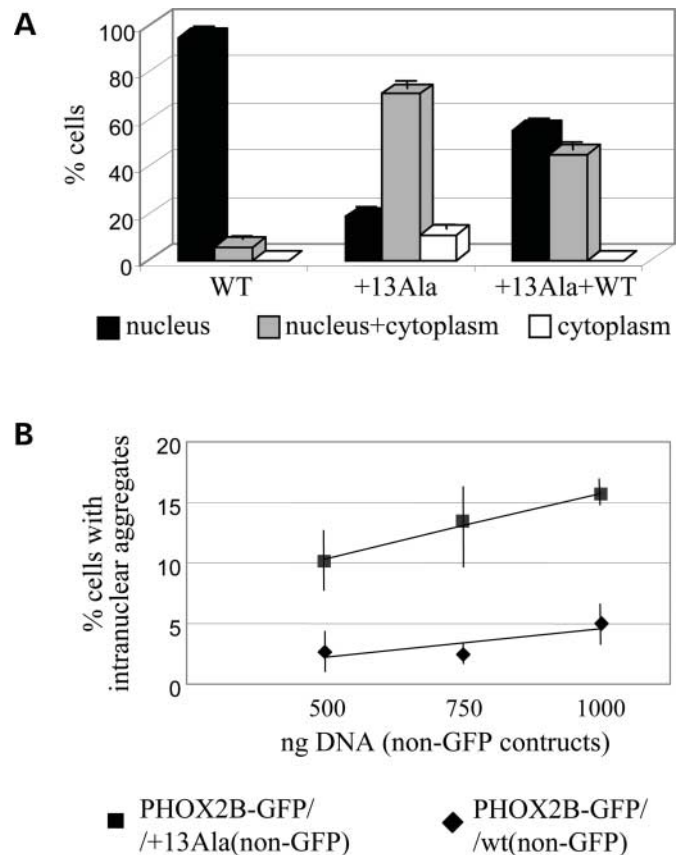


**A**

**B**

**Figure 6.** Interaction of co-transfected wild-type and mutated PHOX2B in nuclear aggregates (**A**) PHOX2B localization in cells expressing equal amounts of both constructs +13Ala-GFP and WT-GFP is compared with values observed in cells expressing wild-type PHOX2B-GFP (WT) and +13Ala PHOX2B-GFP only. (**B**) COS-7 cells were co-transfected with 500 ng/plate of PHOX2B-GFP and increasing amount (500−750−1000 ng DNA) of PHOX2B non-GFP and of +13Ala non-GFP ('WT' and 'dup39' constructs). The standard deviations of two independent experiments and the regression lines are represented.

the c.614−618delC-GFP construct showed a marked nucleolar painting, suggesting that the effective mechanism of this frameshift mutation might mostly rely on such a nuclear mislocalization.

Therefore, modifications in the C-terminal region of PHOX2B could be probably critical for its transcriptional action and, because none of the frameshift mutations tested directly affected the homeodomain, observed loss-of-function effects could likely be due to either sequestration in sub-nuclear compartments (as seen for c.614−618delC) or altered DNA−protein or protein−protein interactions caused by a change in PHOX2B folding and tri-dimensional conformation.

Our findings demonstrate that all the tested *PHOX2B* mutations could induce transcriptional dysregulation of *DβH* and *PHOX2A* genes. In particular, whereas poly-Ala tract expansions appeared to affect to some extent the expression of each target gene, frameshift mutations caused impaired transcription of *DβH* and, conversely, a weak *PHOX2A* transcriptional up-regulation.

In conclusion, despite the distinct effects of *PHOX2B* associated polyalanine expansions and frameshift mutations,

the role that protein folding exerts in regulating biological activity and targeting proteins to different cellular locations suggests that inability to assume correct tri-dimensional protein structure might represent the common molecular basis to explain the PHOX2B associated CCHS pathogenesis.

## MATERIALS AND METHODS

### Mutational screening of the *PHOX2B* gene in CCHS patients

The coding region of the *PHOX2B* gene was screened in our CCHS patients according to a protocol we have already reported (6). PCR fragments were purified with enzymatic mix *Sap* I–*Exo* III by incubation at 37°C for 40 min and 80°C for 15 min and analysed for mutations by direct DNA sequencing using the Big Dye Terminator cycle sequencing kit (Applied Biosystem) on an ABI 3100 DNA automated Sequencer.

### Expression plasmids construction

*Human wild-type PHOX2B expression plasmid.* Total RNA was extracted from IMR32 NB cell line. The full-length *PHOX2B* cDNA was obtained with primers 5′UTR2F (5′-AGCCACCTTCTCCATATCC-3′) and 7921R (5′-TGTGTGCCTTTTCCTTGCTCG-3′) using One Step RT–PCR Kit (Qiagen) and following the manufacturer's instruction. The 2120 bp PCR product thus obtained was cloned in pcDNA3.1TOPO/V5-His expression vector (Invitrogen) and completely sequenced using Big Dye Terminator cycle sequencing kit (Applied Biosystems) on an ABI 3100 DNA automated Sequencer.

*Mutant PHOX2B expression plasmids.* A 270 bp region corresponding to the expanded polyalanine stretch was isolated by *Ppu*MI enzymatic digestion from the *PHOX2B* exon 3 previously amplified from CCHS patients (6) and cloned in the pcR2.1TOPO vector (Invitrogen). Each of the fragments containing the +5, +6, +7, +9, +11 and +13 alanine expansions was inserted in the WT *PHOX2B* expression construct after removing the corresponding 270 bp region. Resultant mutant plasmids were named dup15, dup18, dup21, dup27, dup33 and dup39.

Constructs carrying c.614–618delC and c.721–758del38nt frameshift mutations were also obtained following the earlier mentioned procedure. Two additional frameshift mutations (c.862–866insG and c.930insG) were generated through site-specific mutagenesis starting from the WT cDNA plasmid as a template. Primers 5′UTR2F + INSG(R) (5′-CGAAGGGACCCCCCAAGCGAATC-3′) and INSG(F) (5′-GATTCGCTTGGGGGGGTCCCTTCG-3′) + 7921R were used for c.862–866insG, whereas primers 5′UTR2F + INSG-II(R) (5′-TACTGCTCCTTCACTAAGGCG-3′) and INSG-II(F) (5′-CGCCTTAGTGAAGGAGCAGTA-3′) + 7921R were used for c.930insG. In particular, the entire coding region was amplified in a total volume of 25 µl containing 10 ng of plasmid template DNA, 400 nM primers, 1 × GC-RICH PCR buffer (Roche), 200 mM of each dNTP and 2 U of GC-RICH PCR enzyme mix (Roche) and run for 35 cycles at

95°C (30 s), 58°C (45 s), and 72°C (2 min 30 s). PCR products were cloned in pcDNA3.1TOPO/V5-His and sequenced with Big Dye Terminator cycle sequencing kit (Applied Biosystems) on an ABI 3100 automated Sequencer. Plasmids DNA preparations were obtained using Genopure Plasmid Maxi Kit (Roche).

### Cell cultures, transient transfections and *Luciferase* reporter activity assay

HeLa cells were grown in Minimal Essential Medium supplemented with 10% fetal bovine serum (Gibco, New Zealand), 1% L-glutamine 100×, 1% non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% $CO_2$.

The day before transfection, $8.5 \times 10^4$ cells were plated in 35 mm diameter dishes. Transfections were performed using Fugene6 Transfection Reagent (Roche) with 150 fmol of expression plasmids and 40 fmol of each of two promoter–reporter plasmids. The *DβH* and *PHOX2A* promoter constructs, both kind gift of Dr Fornasari, are characterized by regulatory regions of 807 bp and 4.5 kb, respectively, containing PHOX2B target sequences, sub-cloned in the pGL3 vector upstream of the *Luciferase* reporter gene (12,14 for construction of plasmids). The plasmid pRL-CMV, expressing the *Renilla Luciferase* gene, was used as an internal control of each sample. Forty-eight hours after transfection, cells were assayed for Luciferase activity (Dual-Luciferase Reporter Assay System, Promega) using a TD-20/20 Luminometer following manufacturer's instructions.

### Total cellular lysates, nuclear extracts and western blot

HeLa cells were cultured in 60 mm dishes and transfected with 3 µg of each expression construct (Fugene6 Transfection Reagent, Roche). To obtain total cellular lysates, 48 h after transfection, cells were washed with PBS, centrifuged and lysed with boiling Laemmli Sample Buffer (Bio-Rad); nuclear extracts were prepared in accordance to a protocol already published (31). Equal amounts of total lysates and nuclear extracts were electrophoresed on 15% SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Proteins were identified by probing the membrane with the goat polyclonal PHOX2B antibody (Santa Cruz), specifically addressed against the protein N-terminal region and then with the mouse anti-goat IgG-HRPP (Santa Cruz). Signals were detected using the chemiluminescence reagent ECL plus (Amersham).

### Construction of PHOX2B-GFP fusion proteins

Wild-type and mutant human *PHOX2B* cDNAs, carrying +5Ala, +9Ala, +13Ala expansions and the two frameshift mutations c.930insG and c.614–618delC, were amplified starting from the previously described expression plasmids, using the forward primer 5′UTR2F (5′-AGCCACCTTCTCC ATATCC-3′) with the following reverse primers: PHOX2B-GFP (CGAACATACTGCTCTTCACTAAGG) for WT and polyalanine expanded constructs, insG-GFP (5′-C GGCCCTCAATGAAAAAGCCATG-3′) for the c.930insG

construct and deltaC-GFP (5′-CAGGCGGCTTTGGCACCG-3′) for c.614−618delC. PCR products, lacking the stop codon, were cloned upstream of the GFP cDNA in pcDNA3.1/CT-GFP-TOPO vector (Invitrogen) thus allowing for production of wild-type and mutated PHOX2B-GFP fusion proteins.

### Fluorescence microscopy

COS-7 cells were plated at 60% confluence in 35 mm diameter dishes and the day after transfected with 500 ng of pcDNA3.1/CT-GFP-TOPO[PHOX2B], pcDNA3.1/CT-GFP-TOPO[dup15], pcDNA3.1/CT-GFP-TOPO[dup27], pcDNA3.1/CT-GFP-TOPO[dup39], pcDNA3.1/CT-GFP-TOPO[c.930insG] and pcDNA3.1/CT-GFP-TOPO[c.614−618delC] using Fugene6 Transfection Reagent (Roche).

In co-transfection experiments, 500 ng of pcDNA3.1/CT-GFP-TOPO[PHOX2B] were transfected with 500 ng to 1 μg of wild-type and dup39 PHOX2B non-GFP expression constructs. Forty-eight hours later, cells were washed with PBS and the samples were examined with a Zeiss Axiophot fluorescence microscope.

### Silver staining for nucleoli

Forty-eight hours after transfection with pcDNA3.1/CT-GFP-TOPO[c.614−618delC], COS-7 cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde, permeabilized for 15 min with PBS/0.1%Triton-X/10%FCS and blocked with 5% non-fat milk in PBS/0.1%Tween20. Plates were then incubated for 20 min in 0.01 M $Na_2B_4O_7$ (pH = 9.22), rinsed with distilled water, air-dried, added with 1 g/ml freshly made and filtered silver nitrate solution. Cells were covered with a clean nylon membrane (Hybond) and incubated for 3 h at 37°C. After washing with distilled water and air-drying, cells were examined under a Zeiss Axiophot fluorescence microscope.

### Statistical analysis

Statistical associations between length of poly-Ala expanded tracts and either target promoter activity or extention of cytoplasmic localization of mutated PHOX2B proteins (independent variables) were determined by the Spearman rank test for correlation. Permutation−randomization test was used to assay differences between the means between the WT and each frameshift mutation under analysis (http:/www.bioss.ac.uk/smart).

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