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Effects of 3-ketodesogestrel and all-*trans* retinoic acid on PHOX2A and PHOX2B expression: a common strategy as new therapeutic perspective in Congenital Central Hypoventilation Syndrome (CCHS) and Neuroblastoma (NB) treatment.

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NOME DEL DOTTORANDO

Debora Belperio

NOME E COGNOME DEL TUTOR

Prof. Diego Fornasari

NOME E COGNOME DEL COORDINATORE DEL DOTTORATO

Prof. Massimo Locati

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Abbreviations:

3-KDG	*3-Keto-desogestrel*
ANS	Autonomic Nervous System
AP	Area Postrema
ATRA	*all-trans* Retinoic Acid
CB	Carotid Body
CCHS	Congenital Central Hypoventilation Syndrome
cPGR	Classical nuclear Progesterone Receptor
DMH	Dorsomedial hypothalamic nucleus
DRG	Dorsal Respiratory Group
HD	Homeodomain
LC	*Locus Coeruleus*
mPRG	Membrane Progesterone Receptor
NB	Neuroblastoma
NPARMs	Non-Polyalanine repeat mutations
NST	Nucleus of the solitary tract
PARMs	Polyalanine repeat mutations
Pg	Progesterone
PGR	Progesterone Receptor
PR-A/B	Progesterone nuclear receptor isoform A/B
PRG	Pontine Respiratory Group
RU486	Mifepristone
VLPO	Ventrolateral preoptic nucleus
VRG	Ventral Respiratory Group

Abstract

PHOX2B (Paired-like homeobox2B) and PHOX2A (Paired-like homeobox2A), are two paired like homeodomain proteins required for the correct autonomic nervous system development and for the specification of the noradrenergic phenotype. The neuronal development and specification driven by PHOX2 proteins is the result of a fine temporal and spatial control of the two-paralogue proteins. In particular, *PHOX2B* is considered a "master regulatory gene" in the network leading to the cathecolaminergic phenotype specification, and regulate PHOX2A expression (Coppola *et al*., 2005).

As PHOX2 proteins play crucial role in embryonic neuronal differentiation, their mutations or altered regulation are linked to congenital pathologies, such as Congenital Central Hypoventilation Syndrome (CCHS), a neurodevelopmental disorder characterized by a failure in the autonomic control of breathing, and neuroblastic tumours.

The aim of this project was to disclose still unknown mechanisms involved in the pathogenetic mechanisms of PHOX2B-driven disease development, to improve the recognition of new sensitive therapeutic intracellular targets

The results obtained during the three years of my PhD are organized in three parts.

Part 1) In the first part of the project, we better characterized PHOX2B homeodomain-mediated functions, as hetero-/homo-dimerization and nuclear import, also in the perspective of better analysing the possible effect of polyalanine expansion on these functions, as the expansion of the polyalanine tract in PHOX2B results in a protein with altered DNA-binding, reduced transcriptional activity and defect in nuclear localisation (Trochet *et al*., 2005; Bachetti *et al*., 2005; Di Lascio *et al*., 2013). Interestingly our data clearly underlined a strong interaction between PHOX2A and PHOX2B wild type/ mutants proteins, indicating a possible involvement of PHOX2A in the pathogenesis of CCHS.

Part 2) Consistently with PHOX2B role as transcriptional regulator, it is reasonable to suppose that transcriptional dysregulation might be an important mechanism of CCHS pathogenesis and/or tumour development. Recently, studies conducted on NB cell lines have highlighted a correlation between drugs, used in NB treatment or clinical trials (e.g. GA, 17-AAG), and the modulation of *PHOX2B* gene expression. Moreover, NBs are very

sensitive to retinoids, and they were introduced in NB treatment as “additional drugs” in order to induce differentiation in residual cancer cells after NB surgical elimination. In the second part of the project, we showed that “all *trans* retinoic acid” (ATRA), a retinoid drug that is known to suppress growth in cancer, differently regulates both *PHOX2A* and *PHOX2B* expression in SK-N-BE(2)C neuroblastoma cell line, thus suggesting that the molecular mechanism for retinoic-induced cellular differentiation, in NB treatment, is due to a direct regulation of PHOX2 protein expression.

Part 3) An interesting strategy into treatments of CCHS, that is currently under investigation, regards the use of progestinic drugs for ameliorating respiratory symptoms in patients. This strategy stems from the fortuitous observation of Straus and colleagues, that progestin drug Desogestrel can relief some symptoms in CCHS female patients. The molecular mechanism of the unexpected Desogestrel pharmacological effect is at the moment completely unknown, but this observation gave us a useful indication in the perspective of pharmacological interventions in CCHS. As previously data collected in my laboratory, on T47D cell line, suggested that 3-KDG can influence both the activity of PHOX2B and PHOX2A, and that this is mediated by the intracellular receptor PGR, we decided to deeply investigate the Desogestrel mechanism in a cellular background more properly associated with the physiological PHOX2B environment. To this purpose, we generated a neuroblastoma cell line (SK-N-BE(2)C) stably expressing nuclear progesterone receptor isoforms, and analysed the effect of 3-KDG treatment on endogenous protein amount and transcription levels by using western blot and quantitative PCR analyses. Our findings demonstrated that desogestrel treatment is able to alter *PHOX2B* and *PHOX2A* expression, as well as the expression of *DBH* and some other target genes of PHOX2 proteins, particularly reducing their expression.

Taking together these results point out the existence of a specific cross-talk between ATRA and 3-KDG treatments and PHOX2 pathway, suggesting that the regulation of PHOX2 proteins expression could be a common strategy into the treatment of NB and CCHS.

Chapter 1

General introduction

Debora Belperio

1.1 Phox2 proteins in nervous system development

PHOX2B (Paired Like Homeobox 2B) and its paralogue gene *PHOX2A* (Paired Like Homeobox 2A) are members of the Q50 paired-like homeodomain transcription factor family, essential for the autonomic nervous system (ANS) development (Tiveron *et al*., 1996). They also control adrenergic and noradrenergic neuronal differentiation in peripheral and central nervous system (Stanke *et al*., 1999), by coordinately regulating neuron cell cycle exit (Paris *et al*., 2006; Dubreuil *et al*., 2000).

In vivo knockout experiments clearly revealed the Phox2a/b importance in early neurodevelopment and their significance as determinants of the noradrenergic phenotype.

The *Phox2a* knockout mouse models die shortly after birth leading to a selective loss of the main noradrenergic centre of the brain, the *locus coeruleus* (LC)(Morin *et al*., 1997), whereas *Phox2b* null mutants mouse models die before birth because ANS circuits do not form or degenerate as well as brainstem (nor)-adrenergic centres (Pattyn *et al*., 1999).

Different classes of neurons required the expression of Phox2 proteins for their differentiation and maintenance of neuronal phenotype.

In particular, Phox2b is expressed:

- in peripheric nervous system, in all autonomic ganglia (sympathetic, parasympathetic and enteric ganglia), in distal ganglia of the facial (VII), glossopharyngeal (IX), and vagus (X) cranial nerves;
- in central nervous system, in all noradrenergic centres (A1-C1, A2-C2, A5 e A7), in motor cranial nuclei (except abducent and hypoglossal nuclei), in neurons of the nucleus of the solitary tract (NTS) and area postrema (AP) (Brunet and Pattyn, 2002) as in the hindbrain and spinal cord interneurons (Fig. 1).

Phox2a expression during neurodevelopment is far more limited with respect to its paralogue protein, being it confined in principal noradrenergic centres as in the parasympathetic and sensory ganglia and in the aforementioned LC, and in neurons of oculomotor and trochlear nuclei.

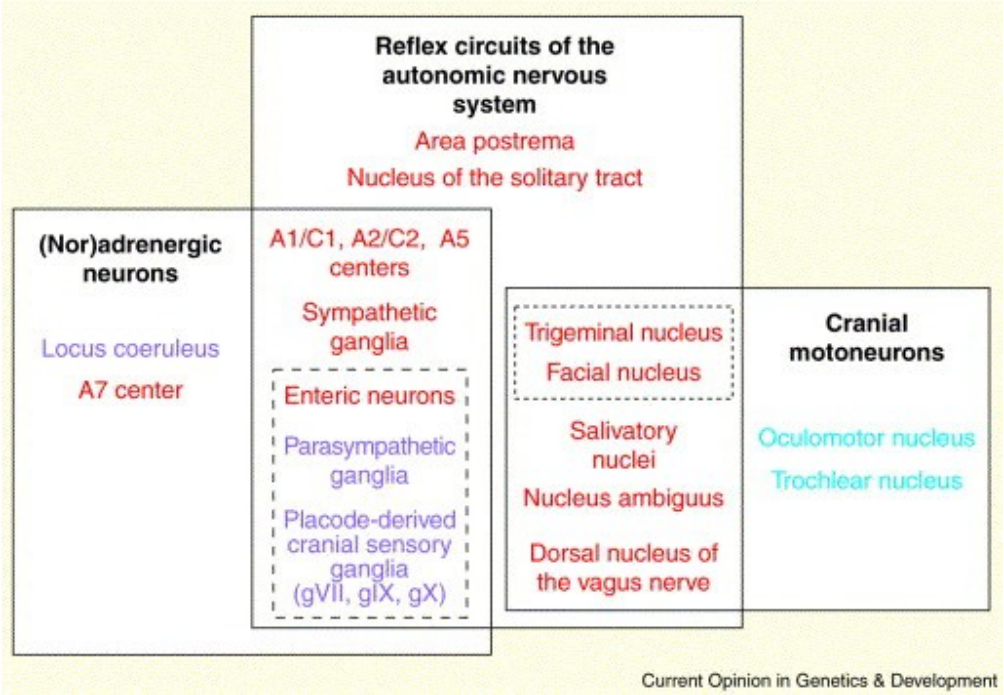

Figure 1 Expression profiles of Phox2 proteins.

Classes of neurons under Phox2a control are represented in blue; in red the ones regulated by Phox2b, in violet classes controlled by both Phox2a and Phox2b expression. The dashed boxes indicate neuronal types and nuclei that are not noradrenergic but transitorily expressing noradrenergic markers, as DBH (Brunet & Pattyn 2002).

The Phox2a/b specification of neurotransmitter identity and neuronal fate starts soon during neurodevelopment, at the level of neuronal crest cells precursor.

Nagashimada *et al* (2012) have identified a reciprocal regulation between Phox2b and Sox10 (SRY-related HMG-box), that is central to development of the autonomic ganglia, both enteric and sympathetic (Fig. 2). Neural crest cells initially express Sox10, but not Phox2b; after their migration to the site of ganglion formation they rapidly begin to express Phox2b. These Phox2b/Sox10 double-positive cells are bipotential progenitors whose future identity (either neurons or glia) is still undetermined. As differentiation proceeds, Phox2b- and Sox10-expressing cell populations are segregated and give raise to neurons and glia, respectively. Sox10 has the ability to suppress Phox2b expression (Kim *et al*., 2003). Phox2b has also the ability to suppress Sox10 expression, acting on a Sox10 enhancer. Together, this reciprocal inhibition between Sox10 and Phox2b must be required to establish a balance between Sox10 and Phox2b expression, which is crucial

for the maintenance and differentiation of bipotential progenitors in the sympathetic and enteric ganglia (Nagashimada *et al*., 2012).

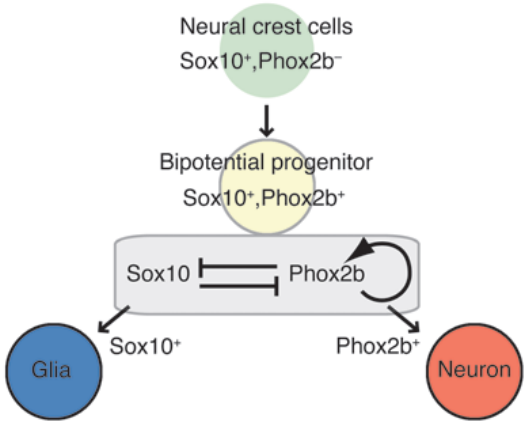

Figure 2 Phox2b-Sox10 reciprocal regulation.

Neural crest cells are Sox10+ Phox2b-. After their migration to the site of ganglion formation they begin to express Phox2b. Regulation of Phox2b and Sox10 expression by reciprocal suppression between these two transcription factors, at bipotential progenitors level, plays an important role in generating appropriate numbers of neurons and glia in the enteric and sympathetic ganglia.

Phox2b expression is therefore the crucial step for the determination of the neuronal cell fate. In particular, neuroblast progenitors migrate from the neural crest and around neural tube to a region immediately lateral to the dorsal aorta, at embryonic day 10. The dorsal aorta secretes bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, which induce the activation of a specific signalling pathway, essential for sympathetic-neuron development. Under normal conditions BMPs induce Phox2b and the helix-loop-helix transcription factor Mash1 (ASCL1 Achaete-scute homolog 1), which in turn induces Phox2a, the zinc finger protein GATA3 (GATA Binding Protein 3), and the basic helix-loop-helix transcription factor dHand (Howard *et al*., 2000). Finally, Phox2b, Mash1 and GATA3 regulate the expression of the tyrosine hydroxylase (TH) and dopamine β hydroxylase (DBH) enzymes, responsible for noradrenaline synthesis (Fig. 3).

Phox2b also regulates MSX1 (Msh homeobox 1) homeobox transcription factor (Revet *et al*., 2008), and ALK (Anaplastic lymphoma kinase) (Bachetti *et al*., 2010) both important for neural crest and peripheral nervous system development.

Furthermore, in our laboratory it has been demonstrated that PHOX2A regulates the expression of the human α3 nAChR subunit gene (Nicotinic acetylcholine receptors, subunit α3), the ligand-binding subunit expressed in every terminally differentiated ganglionic cell, which represents a pan-autonomic gene that participates in the fast synaptic transmission between CNS and ganglia (Benfante *et al*., 2007).

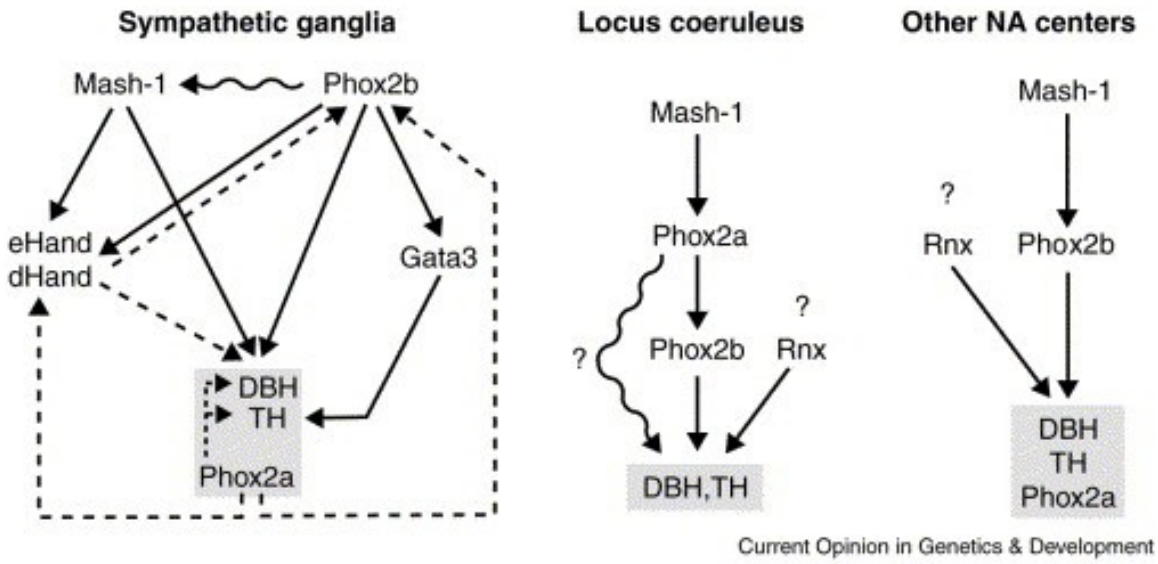

Figure 3 Transcriptional cascades for the control of the noradrenergic neuronal differentiation into different neuronal classes. Diagram of regulatory interactions between transcription factors deduced from the analysis of knock-out mice for individual genes. The wavy arrows indicate a role in maintaining gene expression (putative in the case of LC), while the dashed arrows indicate regulatory interactions derived exclusively from overexpression experiments. (Brunet & Pattyn 2002).

Despite Phox2b and Phox2a are predominantly co-expressed in several noradrenergic regions, their expression is temporally regulated.

Phox2a and *Phox2b* knock-out (KO) murine models clearly identified the temporal/spatial expression of the two transcription factors during noradrenergic differentiation.

Moreover, *in vivo* experiments based on the replacement of *Phox2a* gene into the *Phox2b locus*, and *vice versa,* demonstrated that *Phox2* genes are not functionally equivalent (Coppola *et al*., 2005).

Taking together, these data indicates that in the transcriptional cascades controlling noradrenergic differentiation, Phox2b regulates Phox2a expression.

Indeed, deletion of *Phox2b* leads to failure in the development of most noradrenergic structures, in which Phox2b precedes Phox2a: the sympathetic ganglia and the parasympathetic ganglia of the brain stem, the enteric nervous system, and the hindbrain branchiomotor (bm)/visceralmotor (vm) neurons.

On the other hand, Phox2a inactivation results in lack or atrophy of the oculomotor (nIII) and trochlear (nIV) neurons, the *locus coeruleus* (LC) and the three cranial ganglia as well as parasympathetic ganglia of the head, thus indicating that Phox2a precedes Phox2b in the development of such structures. However, the lack of Phox2b partially affects the correct development of cranial sensory ganglia, parasympathetic ganglia and LC (Pattyn *et al*, 1999, 2000) demonstrating that both factors are required for a proper neuronal development in these areas (Hirsch *et al*., 1998).

Moreover, gene replacement of *Phox2b* in *Phox2a locus* generally rescues the defects exerted by *Phox2a* KO mouse, as in LC and cranial sensory ganglia, thus indicating that probably the main function of Phox2a in these structures is to switch on Phox2b expression. In fact, Coppola *et al* referred to Phox2a as a surrogate promoter of Phox2b in these structures.

On the contrary, *Phox2a* substitution in *Phox2b locus* does not rescue the developmental defects of *Phox2b* KO mouse, indicating the predominant role of its paralogue gene.

1.2 Phox2b and the brainstem respiratory circuit

Numerous neurophysiology and molecular biology studies have identified that a limited number of neuronal types, which express and depend on the Phox2b transcription factor, play a crucial role in the control of breathing. In particular, Phox2b is known to be necessary for the correct development of anatomical structures responsible for hypoxia

and hypercapnia sensitivity: the Carotid Body (CB), Petrosal chemoreceptors (gP), and Nucleus of the Solitary Tract (NTS) (Fig. 4; Dubreuil *et al*., 2009).

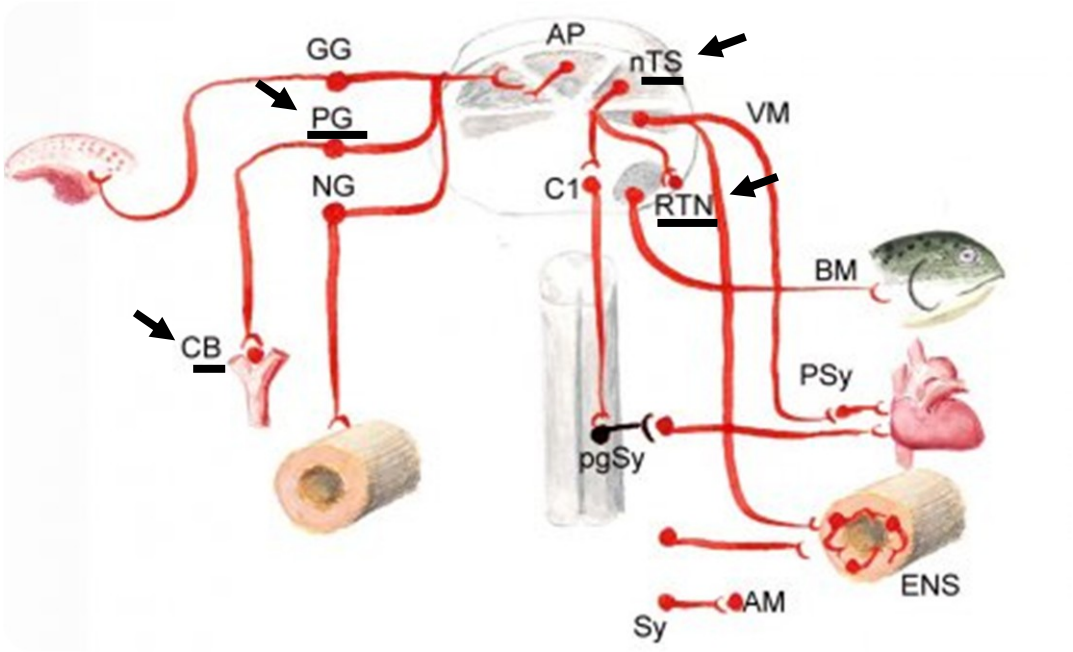

Figure 4 Functional anatomy of the visceral reflex circuits. Neurons represented in red are Phox2b-positive and their differentiation is Phox2b-dependent. Black arrows indicate the principal Phox2B dependent structures involved in chemosensitivity response. AM, adrenal medulla; AP, area postrema; BM, branchiomotor neuron; C1, adrenergic centre; CB, carotid body; ENS, enteric nervous system; GG, geniculate ganglion; NG, nodose ganglion; nTS, nucleus of the solitary tract; PG, petrosal ganglion; RTN, retrotrapezoid nucleus; pgSy, pre-ganglionic sympathetic neuron; PSy, parasympathetic ganglion; Sy, sympathetic ganglion; VM, visceromotor neuron (Dubreuil *et al*., 2009).

The normal breathing is maintained and controlled by a network of neurons localized in the brainstem, responsible for respiratory rhythm generation by providing a regular respiratory input.

Respiratory rhythm production is the result of the interaction of two distinct, but functionally linked, rhythmogenic networks: the retro-trapezoıd/parafacial respiratory group (RTN/pFRG) (Mulkey *et al*., 2004; Onimaru *et al*., 2003) and the pre-Bötzinger complex (pre-BötC), (Smith *et al*., 1991). Each of these oscillators controls different respiratory phases: the pre-BötC drives inspiration, while the RTN/pFRG controls active

expiration and plays an important role in central chemosensitivity (Guyenet *et al*., 2008; Janczewski *et al*., 2006) (Fig. 5, Panel A).

In detail three main neuronal groups (Fig. 5, Panel B), bilaterally located at pons-medulla level, are responsible for the respiratory rhythm generation:

- the dorsal respiratory group (DRG),
- the ventral respiratory group (VRG);
- the pontine respiratory group (PRG).

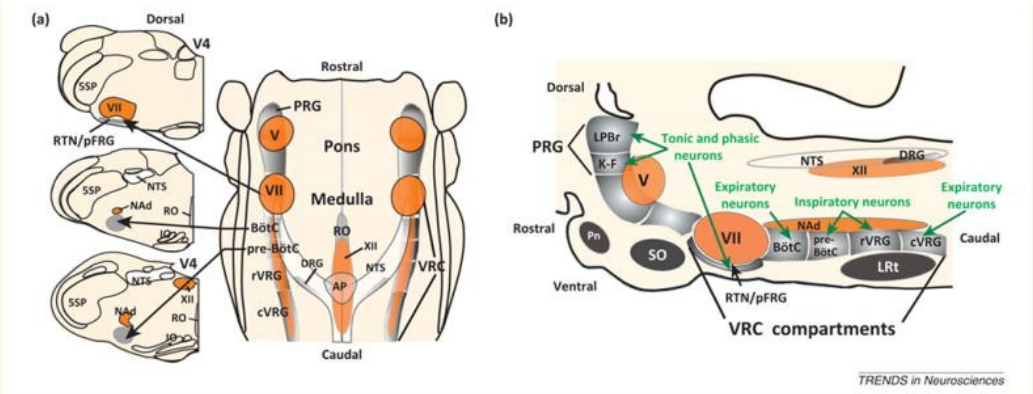

Figure 5. Neuroanatomical representation of brainstem respiratory compartments of the rat. Overview of bilaterally distributed brainstem respiratory compartments of the rat arranged from the rostral pons to the caudal medulla. (a) horizontal (right) and coronal (left) brainstem sections, (b) a parasagittal section of one side of the brainstem. AP, area postrema; LRt, lateral reticular nucleus; NAd, nucleus ambiguus, dorsal division; Pn, ventral pontine nucleus; SO, IO, superior and inferior olivary complexes; SP5, spinal trigeminal nucleus; V, motor nucleus of the trigeminal nerve; V4, fourth ventricle; XII, hypoglossal motor nucleus. (Smith *et al*. 2013).

The dorsal respiratory group (DRG) is localized in the dorsal portion of the bulb and mainly controls the inspiration. DRG consists of inspiratory neurons in the ventrolateral region of the NTS (Jordan, 2001). The NTS includes a series of nuclei forming a vertical column, in the dorsomedial position of the bulb, and is an important centre innervated by visceral afferences. The caudal third of NTS (CNTS) is the termination site of the vagus nerve (cranial nerve X) and glossopharyngeal (IX cranial nerve), that transmit sensory information from the peripheral chemoreceptors, baroreceptors as well as from different

types of receptors in the lungs. Neurons from caudal regions of the NTS and the DRG project to pontine and VRG compartments (Smith *et al*., 2013).

The ventral respiratory group (VRG) is located in the ventrolateral position of the bulb and controls the exhalation.

VRG extends from the facial nucleus (nVII) up to the spinal cord and consists of populations of excitatory and inhibitory interneurons, indicated as respiratory central pattern generator (CPG), which interact with each other and are interconnected with several pontine nuclei.

The VRG output is transmitted through premotor networks to cranial and spinal motor neurons, which respectively control the muscles of the upper airways and diaphragm, and thoracic and abdominal respiratory muscles (Smith *et al*., 2013).

VRG includes four different compartments:

- the Bötzinger complex (BötC), containing glycinergic or GABAergic expiratory neurons (Ezure *et al*., 2003), which inhibit the inspiratory neurons during expiration, providing the inspiratory-expiratory phase alternation;
- the pre-Bötzinger complex (pre- BötC), essential for normal inspiratory activity *in vivo* McKay *et al*., 2005). The pre-BötC cellular composition is very heterogeneous and includes glutamatergic neurons, representing the primary source of the rhythmic inspiratory pulse, as well as populations of inspiratory glycinergic (Winter *et al*., 2009; Morgado-Valle *et al*., 2010) or GABAergic (Kuwana *et al*., 2006) neurons, which inhibit expiratory neurons during inspiration;
- the rostral ventral respiratory group (rVRG) contains the main group of inspiratory premotor bulb-spinal neurons (Alheid & McCrimmon 2008) transmitting the impulse to the spinal phrenic motor neurons that innervate the diaphragm;
- the caudal ventral respiratory group (cVRG) is composed by excitatory bulbospinal expiratory neurons, receiving convergent inputs including those from the RTN/pFRG and BötC.

Finally, the pontine respiratory group (pRG), also known as pneumotaxic center, is located dorsally in the upper portion of the pons, and mainly controls the frequency and depth of breathing (Lindsey *et al*., 2012).

In particular, pRG transmits signals to the inspiratory area regulating the transition from the inspiratory to the expiratory phase (Smith *et al*., 2013), in order to adjust the duration of inspiration in the respiratory cycle, of each breathing process.

Multiple behavioural aspects stimulate the respiratory rate, regulated by the pontine circuits.

In particular, the most potent drives for breathing are inputs generated by peripheral chemoreceptors that constantly monitor CO_2 and O_2 blood pressure.

Inputs sensitive to the level of blood or brain O_2 are originated from peripheral chemoreceptors (e.g. carotid body chemoreceptors), which provide input to the NTS.

The central chemoreception, detection mechanism of CO_2/ pH, is primarily mediated by chemoreceptors of the brainstem in the ventrolateral medulla.

Currently there are two possible structures delegated to carry out these functions:

- medullary serotoninergic neurons (5HT; Corcoran *et al*., 2009, Hodges *et al*., 2010) of the raphe nuclei;
- retro-trapezoid nucleus (RTN; Guyenet *et al*., 2008 and 2009), a group of glutamatergic neurons placed ventral and immediately caudal to the facial nucleus (nVII), defined by the expression of Phox2b and by the absence of markers for catecholaminergic neurons (Goridis *et al*., 2010;Guyenet *et al*., 2013). They are vigorously activated by increases acidification *in vitro*, and by hypercapnia *in vivo* and selectively innervate brain regions containing the respiratory CPG (Mulkey *et al*., 2004; Dubreuil *et al*., 2008; Onimaru *et al*., 2012b; Wang *et al*., 2013).

It was found that injury or inhibition of RTN neurons widely attenuate the chemoreceptor reflex of breathing in adult rats, and their activation increases the respiratory rate, the inspiratory amplitude and active expiration (Guyenet *et al*. 2012).

1.3 PHOX2 proteins in normal condition: structure and function

PHOX2B and *PHOX2A* genes have similar genetic organization, as they are both composed by 3 exons and 2 introns. The *PHOX2B* gene is localized in the short (p) arm of chromosome 4 at position 12 (4p12), and encodes for a 314 amino acids long protein,

whereas the *PHOX2A* gene is localized in the long (q) arm of the chromosome 11 at position 13.4 (11q13.4) and encodes for a protein of 284 amino acids (Adachi *et al*., 2000). PHOX2A and PHOX2B share important sequence homologies, with 56.4% similarity in the region N terminal to the homeodomain, and 100% within the homeodomain (HD) (Fig. 6) (Vallarche *et al*., 1993; Zellmer *et al*., 1995; Pattyn *et al*., 1997; Yokoyama *et al*., 1996, 1999). No homologies have been found in the C- terminal region, downstream the homeodomain.

The HD is a highly conserved 60-amino acids long region that exerts critical protein functions. In particular, the HD is able to bind to specific *ATTA* sequences in target genes promoter, resulting in transactivation of their expression; to mediate nuclear import of the protein; and protein-protein interactions (Homo- and hetero- dimerization with other homeodomain-containing proteins among which PHOX2A itself).

PHOX2B protein is characterized by the presence of two alanine stretches of 9 and 20 residues in the third exon, encoding the C-terminal region of the protein.

The exact function of the polyalanine stretch is not completely clear, but it has been supposed to be a flexible spacer element located between functional domains and therefore implicated in protein conformation, protein-protein interaction and DNA binding.

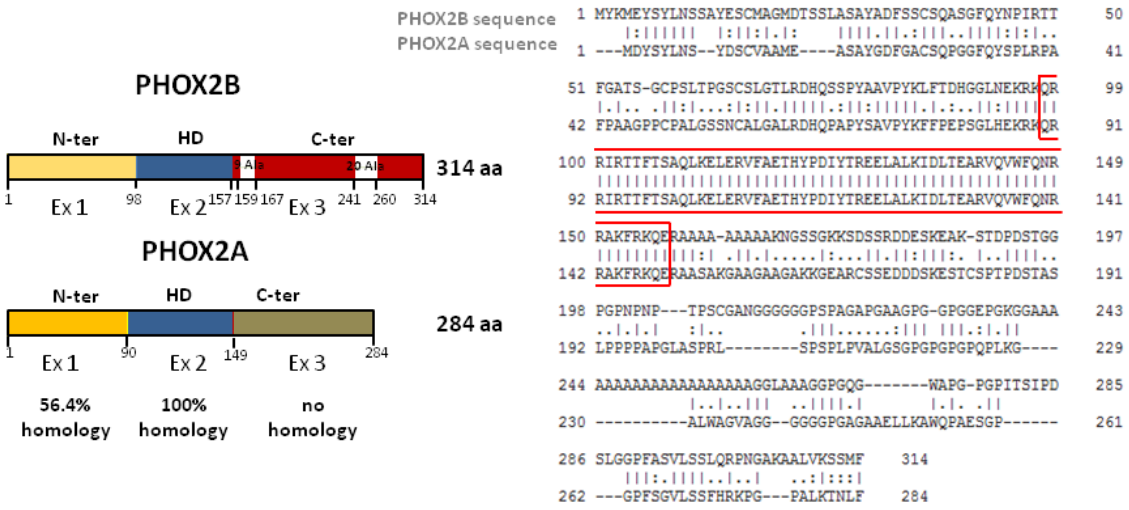

Figure 6 PHOX2B and PHOX2A proteins. Panel A) Schematic representation of domain structure and amino acid sequence homology of PHOX2B and PHOX2A proteins. Numbers refer to the amino acid (aa) residues for each PHOX2 protein. N-ter, N-terminal domain; HD, Homeodomain; C-ter, C-terminal domain.

The percentage of homology is the results of the amino acids sequence comparison between PHOX2B/PHOX2A domains. Panel B) Protein sequence alignment of PHOX2B and PHOX2A aminoacid sequences. Red box indicates the homeodomain.

From a molecular point of view, PHOX2A/PHOX2B transcription factors are able to regulate genes involved in autonomic nervous system development and neuronal specification by binding to specific "*ATTA"* sequence (Homeodomain Binding Site, HBS) in the promoter of target genes.

Biochemical studies demonstrated that PHOX2A and PHOX2B bind with similar affinity to the multiple HD target sequences in the *DBH* promoter; and independently regulate the DBH transcription in a similar extent (Adachi *et al*., 2000; Seo *et al*., 2002).

Interestingly, HBS are also present within *PHOX2A* and *PHOX2B* promoters, indicating that they can also be regulated by homeodomain transcription factors. Indeed, in our laboratory, it has been demonstrated that PHOX2B positively regulate the expression of its paralogue gene *PHOX2A* by binding to HBS present within its promoter (Flora *et al*., 2001), and to modulate its own expression through a self-regulatory mechanism (Cargnin *et al*., 2005).

Moreover, PHOX2B and PHOX2A positively regulate the *TLX2* gene expression (T-Cell Leukemia Homeobox2) (Borghini *et al*., 2006, 2007), and both PHOX2B and TLX2 proteins are found to be downstream targets of BMP signalling (Tang *et al*., 1998; Howard *et al*., 2000; Schneider *et al*., 1999).

Functional analyses indicate that PHOX2A protein, deleted of the region C-terminal to the HD, results in a still functional protein, indicating that the N-terminal region represents the *trans*activation domain of PHOX2A (Adachi *et al*., 2000). Despite the high homology in this region between PHOX2A and PHOX2B proteins suggest that they may also share the same function, PHOX2B deletion analyses indicate that the N-terminal region alone is not sufficient to *trans*activate target genes (unpublished data, our laboratory).

The main condition necessary for the vertebrate neural development is the coordination of cell cycle regulation and cell fate determination (Ohnuma *et al*., 2001).

In the cathecolaminergic CAD cell line, a variant of Cath.a-cell line derived from *locus coeruleus* mice brain tumours, PHOX2A mediates cell-cycle exit and neuronal

differentiation by inducing the cell-cycle regulatory protein p27 ^{Kip1} (Cyclin-dependent kinase inhibitor 1B), in response to the activation of the cyclic AMP (cAMP) pathway (Shin *et al*., 2009). In detail, PHOX2A is maintained in inactive-form by phosphorylation of Ser206/202 and 208, located in the C-terminal repression domain of PHOX2A (Swanson *et al*., 1999). The activation of the cAMP pathway mediates the dephosphorylation of Ser 206, resulting in PHOX2A activation. Active PHOX2A binds to HBS of the p27 ^{Kip1} promoter and induces its transcription (Shin *et al*., 2009). PHOX2A dephosphorylated in phosphoserine cluster is then recognised and phosphorylated by protein kinase A (PKA) on Ser153, which prevents PHOX2A DNA binding and turns off consequent p27Kip1 transcription (Shin *et al*., 2009).

Moreover, Adachi *et al*. (2000) demonstrated that both PHOX2A and PHOX2B are able to interact with PKA pathway and to synergistically increase the transcription of the *DBH* promoter (Adachi *et al*., 2000), suggesting that dephosphorylation/phosphorylation events may also control PHOX2B temporal activation.

Direct proof of the role of PHOX2B in the coordination of cell cycle exit has been obtained by gain and loss of function experiments. In particular, forced PHOX2B expression in embryonic chick spinal cord drives cells to become post-mitotic; conversely, in the absence of PHOX2B, post-mitotic neuron precursors did not form properly (Dubreuil *et al*., 2000 2002).

Despite their central role in embryonic neurodevelopment, so far very little is known about Phox2 proteins functions in adulthood.

1.4 PHOX2 proteins and disease:

Neuroblastoma and Genetic disorders

PHOX2 proteins are found to be correlated with congenital pathologies since homozygous mutations in *PHOX2A* gene result in congenital fibrosis of the extraocular muscles type 2 (CFEOM2, OMIM 602078; Nakano *et al*., 2001), whereas heterozygous mutations in *PHOX2B* gene lead to Congenital Central Hypoventilation Syndrome (CCHS, OMIM 209880; Amiel *et al*., 2003; Sasaki *et al*., 2003), Hirschsprung's disease (HSCR; OMIM

142623)(Trochet *et al*., 2005 ; Berry-Kravis *et al*., 2006), and neuroblastoma (NB, Bourdeaut *et al*., 2005; Van Limpt *et al*, 2004).
Moreover, both PHOX2A and PHOX2B are found to be over-expressed in NB samples (Longo *et al* 2008).

1.4.1 Neuroblastoma

Neuroblastoma (NB; OMIM 256700) is an embryonic neuroblastic tumour stemming from neural crest progenitors (Schleiermacher *et al*., 2014).
NB represents the most common infant tumour (8-10% of pediatric tumours) often occurring in association with other genetic syndromes caused by the aberrant growth, migration and differentiation of neural crest cells.
Neuroblastic tumours are characterized by a high heterogeneity in cellular differentiation stage, ranging from tumours with undifferentiated cells that are usually indicative of a poor prognosis to those with more differentiated cells that generally provide a favourable outcome (Park *et al*., 2008).

Genetic basis of NB

The increasingly frequent association of NB with genetic diseases and the observation of rare familiar cases (1% of NB) have suggested a possible involvement of genetic factors in the NB development.
Evidences of NB genetic predisposition have been recently reported, involving mutations in different genes (Fig. 7):
- constitutive activating *ALK* (Anaplastic Lymphoma Kinase) mutations, identified in more than 50% of familial cases (Janoueix-Lerosey *et al*., 2008; Mossé *et al*., 2008);
- *PHOX2B* mutations, found in a small fraction of patients with sporadic or familial NB (Bourdeaut *et al*., 2005; Van Limpt *et al*, 2004);
- amplification and overexpression of *LIN28B* gene (Lin-28 homolog B) resulting in high MYCN protein expression, an important prognostic factor in NB (Molenaar *et al*., 2012).

In addition, a functional relationship between two of the above-mentioned genes involved in NB has been described, as PHOX2B drives ALK transcription (Bachetti T. *et al*., 2010).

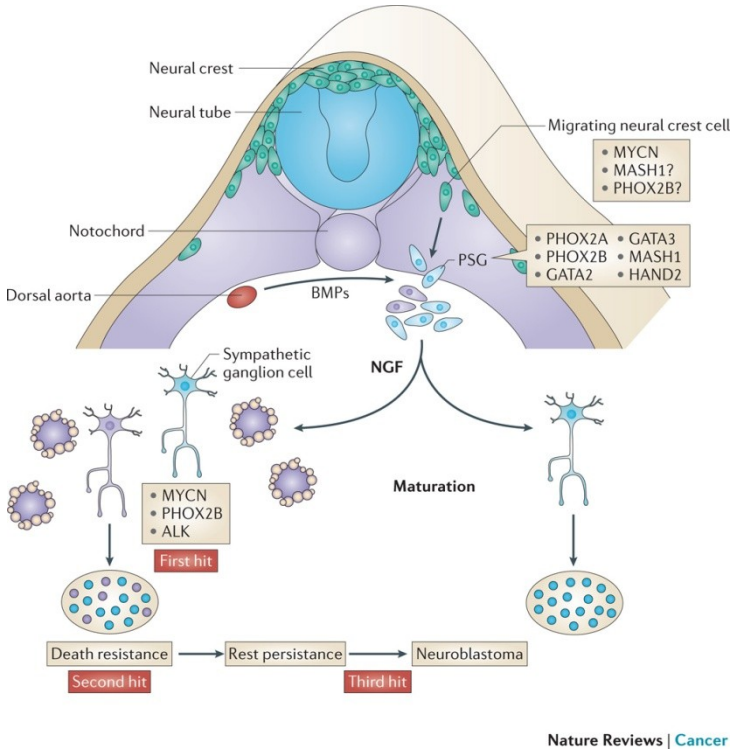

Figure n. 7 Sympathetic ganglionic cell differentiation and neuroblastoma. MYCN together with bone morphogenetic proteins (BMPs) induced neuroblast progenitors to migrate from neural crest up to the region laterally to dorsal aorta, here they become primary sympathetic ganglia (PGS). Later, nerve growth factor (NGF) determines the differentiation of cells into mature sympathetic ganglionic cell (blue) or apoptotic cells, in cases of abnormal precursors. *MYCN*/*PHOX2B*/*ALK* are indicated as 'first hit' in NB manifestation, as mutations/overexpression of these genes are the primary events in tumour development. A 'second' hit is the acquisition of death resistance of neuroblast precancer cells (purple), following by a third change which induces transformation into neuroblastoma cells in early infancy (Marshall *et al*., 2014).

PHOX2B pathogenetic mechanisms in NB

The findings that *PHOX2B* is mutated and overexpressed in NB, as well as the PHOX2B-mediated regulation of genes involved in NB (e.g *PHOX2A*, *ALK*) indicate this transcription factor as an important player in NB development.

However, mechanisms whereby *PHOX2B* mutations or overexpression may be considered pathogenic are still unrevealed.

PHOX2B mutations, linked to NB, are both frameshift and missense mutations, which lead to truncated or malfunctioning proteins. An explanation for the role of these PHOX2B variants in NB predisposition is provided by the observation of increased proliferation and dedifferentiation of sympathetic neuron, as a result of overexpression of these protein variants (Reiff *et al*., 2010). Moreover, in NB patients with mutations in PHOX2B gene, it has been observed a 50% reduction of PHOX2B wild-type level, thus suggesting a dominant-negative mechanism of PHOX2B mutated proteins on its own transcription (Reiff *et al*., 2010).

The pathogenetic function of PHOX2B overexpression opened a deep debate among scientists, as conflicting data have been obtained over the years. In particular, despite some works indicate that PHOX2B exerts an anti-proliferative and pro-differentiating function by regulating neuronal cell cycle-exit (Dubreuil *et al*., 2000; Reiff 2010), additional studies indicate the importance of this transcription factor in the proliferation of immature sympathetic neurons (Coppola *et al*., 2010; Ke *et al*., 2015).

As mentioned before, patients with CCHS have a high predisposing risk to develop tumors of the sympathetic nervous system (Rohrer *et al*.2002); indeed, NB or ganglioneuroma tumours have been diagnosed in approximately 20% of CCHS patients, mainly in patient bearing non poly-alanine repeat mutations (NPARM, described later in Genetic basis of CCHS).

In this regard, a quantitative expression analysis of *PHOX2B* and its paralogue *PHOX2A* was performed by Longo and co-workers in 2008 (Longo *et al*., 2008), using a panel of NB tumour samples and NB cell lines to detect a possible differential expression of the two paralogue genes. This study revealed that both *PHOX2A* and *PHOX2B* are over-expressed in NB cell lines and tumours samples. Particularly, the enhanced expression level of the two genes in NB samples suggests a possible involvement in tumour development through two possible mechanisms: a direct up-regulation or a failure in maintaining their correct transcript level after embryonic development.

Unlike *PHOX2B,* in which both mutations and altered expression levels were found in correlation with NB development, no mutations were found in *PHOX2A* coding and promoter region of tumour samples (Wilzén *et al*., 2009). This observation does not

preclude that PHOX2 pathway deregulation is strictly linked to neuroblastoma onset and development.

Pharmacological research in NB

The Children's Oncology Group (COG) classified NB children into 3 different risk groups (low, intermediate, and high) in order to predict the most effective treatment, in each specific case. The NB treatment varies significantly based on the risk for relapse classification of the disease.

Generally, NB patients with a low risk disease have tumours that are localized to one area, which can be effectively surgically removed. However, sometimes, NB tumours spontaneously remit in part or completely and the potential risks of surgery to remove the tumour can be avoided.

In intermediate risk disease, not always tumours can be easily and completely removed with surgery, they present a high heterogeneity in cellular composition (different differentiation stage), and may create symptoms related to the compression of other organs. In this stage, moderately intensive chemotherapy is given initially to reduce the tumour size and make it easier to surgically remove it .

Finally, NB tumours are classified as high risk disease because of aggressive characteristics of the tumour cells or for the presence of tumours in multiple places. Patients, classified in this risk category, require a strong treatment combining chemotherapy, surgery, stem cell transplant, radiation therapy and immunotherapy.

As high-risk patients often undergo a relapse of tumour, nowadays "additional drugs" aimed to eliminate residual tumours cells have been introduced, after consolidation therapy.

These drugs act by stimulating body's immune system (e.g. antibodies against GD-2, disialoganglioside; and IL-2, interleukin-2; IL-15 interleukin-15)(Yang and Sondel 2010; YU *et al*., 2010; Cappel *et al*., 2016) or stimulating the differentiation of residual immature cancer cells (e.g 13-cis-retinoic acid, isotretinoin).

Neuroblastoma cells are very sensitive to retinoids, which are vitamin A derivatives that include, 13-cis- retinoic acid (RA), 9-cis-retinoic acid, and all trans-retinoic acid (ATRA).

Retinoids are known as differentiating agents because they are thought to inhibit the development and growth of several types of cancer, including gastrointestinal, oral cavity, skin, lung, and breast cancers (Niles *et al*., 2000; Niles *et al*., 2004; Altucci & Gronemeyer 2001; Siddikuzzaman *et al*., 2011; Arrieta *et al*., 2010; Bryan *et al*., 2010).

Since their efficacy in inducing cancer cells to differentiate has been demonstrated (Reynolds & Lemons 2001), retinoids have been introduced in some cancer treatment.

In particular, ATRA is used alone in the treatment of acute promyelocytic leukaemia (APL) as non-chemotherapy drug or in combination with chemotherapy giving more solid long-term results (Degos *et al*., 2001).

Both ATRA and 13-cis-RA, are found to rapidly-drive immature cancer cells differentiation, reverting the malignant neuroblast phenotype (Altucci *et al*., 2001), and 13-cis RA is currently used as "additional drugs" in the treatment of high risk NB, given their advantageous pharmacokinetic properties (Chu *et al*., 2003).

Despite retinoids are now being introduced in NB treatment, the molecular mechanisms by which they regulate different signalling pathways necessary for retinoic-induced cellular differentiation are still largely unknown.

Starting from the knowledge that PHOX2B is mutated and overexpressed in NB, and since it is a direct regulator of ALK and PHOX2A, both linked to NB, a new pharmacological research strategy in treatment of such tumours regards the control of PHOX2B expression.

In particular, in 2015, Di Zanni and co-workers analysed the effect on PHOX2B expression of different molecules and epigenetic modulators, known to be involved in PHOX2B expression regulation and already used in neuroblastoma clinical trials.

In their analysis, different molecules were found to decrease PHOX2B mRNA expression and, in particular, in the case of Curcumin treatment it has been observed also a significant down-regulation of PHOX2B protein.

In addition, the use of the natural spice has been already found to enhance the beneficial effect of epigenetic modifier Trichostatin (TSA) and Suberoylanilide hydroxamic acid (SAHA), both inhibitors of histone deacetylase, already established to have anticancer effects in combination to ATRA (De los Santos *et al*., 2007).

Very interestingly, the combination of Curcumin to TSA or SAHA results in a markedly effect on PHOX2B regulation with respect to the single treatment (Di Zanni *et al*., 2015).

1.4.2 PHOX2 proteins in genetic disorders

Congenital fibrosis of the extraocular muscles type 2 (CFEOM2)

Homozygous loss-of-function mutations in PHOX2A gene are found to be causative for Congenital Fibrosis of the Extraocular Muscles type 2 (CFEOM2) (Nakano *et al*., 2001), a complex strabismus syndrome.

CFEOM2 is inherited in autosomal recessive manner and mutations causing its manifestation, mainly upstream or within the PHOX2A homeodomain, result in a truncated not functional protein (Nakano *et al*., 2001).

As discussed before, PHOX2A and PHOX2B have a primary role in noradrenergic neuronal development and determination. Oculomotor (nIII) and trochlear (nIV) neurons are the only Phox2a structures co-expressing Phox2b, that are missing in Phox2a $^{-/-}$ mouse but they are preserved in Phox2b $^{-/-}$ thus indicating that nIII/nIV are the only neurons Phox2a-dependent that do not need Phox2b for proper development.

This finding supports the idea that CFEOM2 is due to failure in the development of oculomotor and trochlear nerves, with consequent malfunctioning of muscles that they innervate.

Human alterations in these structures result in bilateral severe congenital exotropia and ptosis and small unreactive pupils (Wang *et al*., 1998; Yazdani *et al*., 2003).

Despite the primary role exerted by PHOX2A in neuron development and specification of oculomotor (nIII) and trochlear (nIV) neurons, *locus coeruleus* (LC), the three cranial ganglia and parasympathetic ganglia of the head, only III/IV cranial nuclei and nerves are found to be defective in CFEOM2 (Fig. 8).

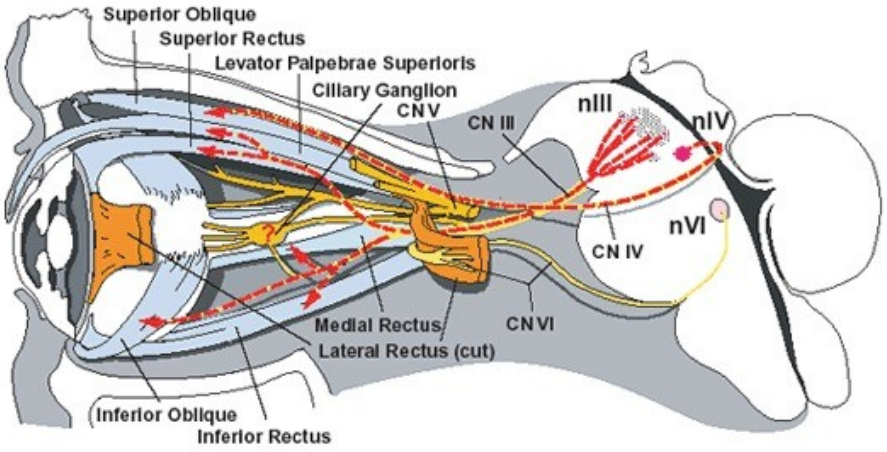

Figure 8: **Schematic lateral representation of the brainstem and left orbit in CFEOM2 affected individual**. nVI, abducens nucleus; CN VI abducens nucleus cranial nerve; CN V, trigeminal cranial nerve; are normal in CFEOM2 as well as muscles that they innervate. nIII, oculomotor and nIV, trochlear cranial nuclei (dotted pink nuclei) as CN III & CN IV, corresponding cranial nerves (dashed red lines), are abnormally developed as well as the muscles innervated by nIII (inferior oblique, medial and inferior recti, superior, levator palpebrae superioris,) and nIV (superior oblique) all represented in light blue. The ciliary ganglion status (question mark) is not clear yet. Modified from Nakano M. *et al*., 2001 Nature Genetic

Neurological examinations in individuals with CFEOM2 did not report any autonomic, cognitive or developmental neurological abnormalities, and neurological anatomical defects involve only the eyes and eyelids (Bosley *et al*., 2006).

As afore described, *Phox2b* gene replacement in *Phox2a locus* is able to rescue almost the totality of anatomical defects manifested by *Phox2a* $^{-/-}$ mouse in neurological regions in which *Phox2a/Phox2b* are co-expressed. This suggest that PHOX2B may compensate for the lack of PHOX2A in CFEOM2 patients, in all the other brain regions regulated by both transcription factors.

Congenital Central Hypoventilation Syndrome (CCHS)

Congenital Central Hypoventilation Syndrome (CCHS), also known as Ondine's course, is a rare neonatal neurological disorder (1: 200.000 birth alive), caused by defective migration of the neuronal precursor from the neuronal crest tube.

CCHS is manifested as a respiratory disorder along with symptoms of autonomic nervous system dysregulation (ANSD), making part of a group of rare disorders defined as RADICA (Respiratory and Autonomic Disorders of Infancy, Childhood, and Adulthood; Weese-Mayer., 2009).

As PHOX2B is important for the development of structures responsible of the control of central respiration, this justifies the finding that mutations in this transcription factor are causative for CCHS.

The principal feature of this autonomic syndrome is the breathing deficit mostly prominent during sleep hours, associated with diffuse autonomic dysregulation (Weese-mayer *et al*., 1999 and Weese-Mayer *et al*., 2001), Hirschsprung's disease (HSCR) (Vanderlaan *et al*., 2004) and neural crest tumours (Haddad *et al*., 1978).

Hirschsprung's disease (HSCR), or congenital aganglionic megacolon, is a developmental disorder characterized by the absence of ganglia in the lower digestive tract, resulting in a functional obstruction (Heanue *et al*., 2007). Aganglionosis is linked to incorrect development of the enteric nervous system (ENS), therefore, ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. Treatment for HSCR consists of surgery to remove the part of the gut that is not innervated and re-connection of the remaining intestine to the anus. HSCR can occur alone or in syndromic disease (e.g. CCHS). Different studies have led to the identification of mutations in different genes (RET, GDNF, NRTN, PHOX2B, EDNRB, EDN3, ECE1, SOX10, ZFHX1B), which lead to isolated HSCR or syndromic HSCR phenotype (reviewed in Tam & Garcia-Barcelò 2009). The association between CCHS and HSCR is configured as Haddad syndrome. Birth incidence of HSCR is approximately 1:5000 newborns, and occurs in 16% of CCHS patients.

The ANSD symptoms of CCHS include altered blood pressure homeostasis (Trang *et al*., 2005), decreased heart rate variability and attenuated heart rate response to exercise (Woo *et al*., 1992; Ogawa *et al*., 1993; Silvestri *et al*., 2000; Trang *et al*., 2005), ocular abnormalities (Weese-Meyer *et al*., 1992), severe constipation and oesophageal dysmotility (Weese-Mayer *et al*., 1992; Faure *et al*., 2002).

Instead, the respiratory symptoms consist in ventilatory arrest during sleep and a reduced or, in severe cases, absent response to hypercapnia (Gozal *et al*., 1998; Chen *et al*., 2004).

The disease typically occurs soon at birth by hypoventilation or apnoea periods during sleep, even though severely affected infants are not able to breathe properly even during wakefulness (Gozal *et al*., 2008). Moreover, there are rare late-onset forms (LO-CCHS) of the disease (Weese-Mayer *et al*., 2005) in which the symptoms reveal during late childhood or even in adulthood (very few cases).

Currently, there is no pharmacological treatment capable of restoring the normal ventilation in patients, therefore the only available support for CCHS affected patients is the mechanical ventilation by means of tracheotomy, nasal masks or phrenic nerve stimulation through diaphragmatic pacemaker implantation.

The absence of adequate treatments can result in central hypoxia with consequent risks of neurological damages, and fatal consequences.

Genetic basis of CCHS

Almost the totality of CCHS patients (98%) presents mutations in the coding region of PHOX2B, and in some patients mutations have been reported in other genes involved in neuronal crest development: RET (REarranged during Transfection), GDNF (Glial cell Derived Neurotrophic Factor, RET ligand), EDN3 (Endothelin 3), BDNF (Brain Derived Neurotrophic Factor), MASH1 (Mammalian Achaete-Scute complex homolog 1), PHOX2A (Paired-like Homeobox 2A), GFRA1 (GDNF Family Receptor Alpha 1), BMP2 (Bone Morphogenetic Protein 2) and ECE1 (Endothelin Converting Enzyme 1) (reviewed in Gallego, 2012).

PHOX2B mutations in CCHS patients are divided in two classes (Weese-Mayer *et al*., 2010) (Fig. 9):

- Polyalanine repeat mutations (PARMs), due to the insertion of +5 to +13 Alanine in the 20-Alanine stretch at the C-terminus of the homeodomain (95% of CCHS patients);
- Non-Polyalanine repeat mutations (NPARMs), including frameshift mutations, in which the insertion/deletion of a single nucleotide induces a change in the reading frame of the protein (<5%), and very rare non-sense mutations in which the generation of a stop codon induces the consequent production of an incomplete non-functional protein.

Generally, patients with PARMs mutations exhibit a respiratory phenotype with a clear correlation between the length of the alanine tract and the severity of the symptoms; starting from the milder phenotype in the +5 alanine insertion mutation, up to the most severe phenotype in the +13 alanine insertion, whereas the +7 alanine insertion represent the most common mutation.

On the other hand, in patients with the less frequent nPARM mutations, respiratory symptoms are more severe and often associated with Hirschsprung's disease, neuroblastoma and serious autonomic dysregulation.

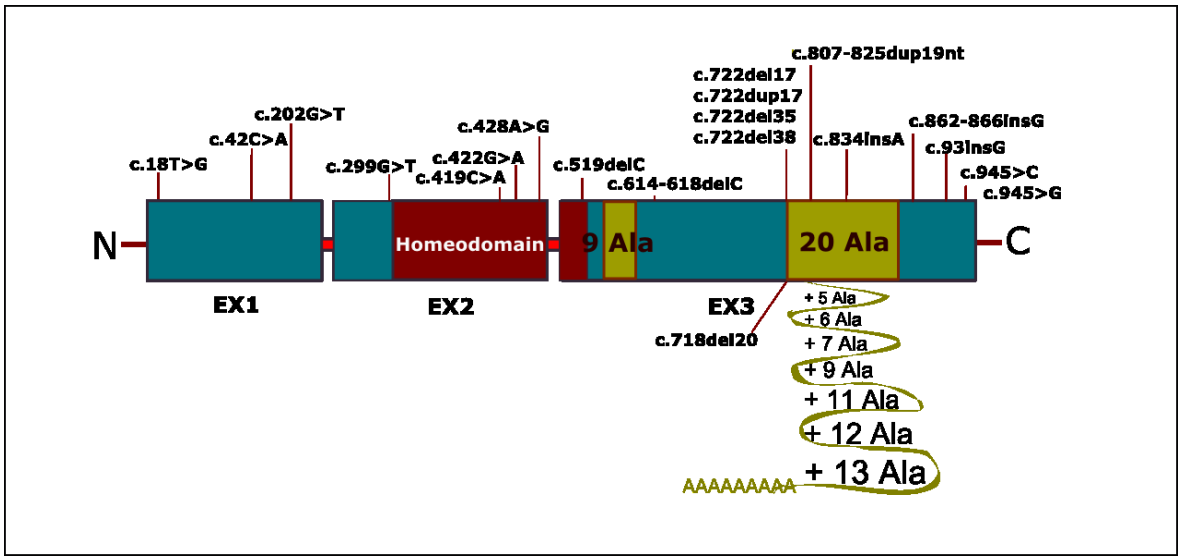

Figure 9 *PHOX2B* mutations in CCHS. Schematic representation of *PHOX2B* mutations associated with CCHS. Red box indicates homeodomain. Yellow boxes represent polyalanine regions. The totality of polyalanine repeat mutations (PARMs) regards the 20-alanine long stretch at the C-terminus of exon 3. Non-polyalanine repeat mutations (NPARMs) are found to be located principally at 3' end of exon 2 or in exon 3.

CCHS is inherited in an autosomal dominant manner. Most of the *PHOX2B* mutations in individuals with CCHS are de novo mutations. However, it is emerging that approximately 10-25% of parents are asymptomatic carriers, responsible for transmission to their offspring (Weese-Mayer *et al*., 2003; Amiel *et al*., 2003, Parodi *et al*.,2008; Trochet *et al*., 2008). Different possibilities are described for asymptomatic carriers, including the presence of minor polyalanine expansion (e.g. 20/23 genotype), and genotypes with

incomplete penetrance, somatic or germinal mosaicism (Loghmanee *et al*., 2009; Weese-Mayer *et al*., 2003; Amiel *et al*., 2003, Parodi *et al*.,2008; Bachetti *et al*., 2014).

Pathogenetic mechanisms in CCHS

The clinical features of patients with CCHS are very variable. Indeed, patients bearing the same *PHOX2B* mutations may present different clinical manifestations and severity of the respiratory phenotype. In addition, NPARMs mutations are associated with increased number of clinical symptoms, compared to PARMs, thus suggesting that it could be associated with different pathogenetic mechanisms (Trochet *et al*., 2004 and 2005).

The divergence in the pathological condition observed in CCHS patients, along with in *vivo and* in *vitro* studies, indicate the cooperation of different mechanisms at the basis of CCHS, including:

1) haploinsufficiency;

2) loss of function;

3) dominant-negative function;

4) gain of function.

1) *In vivo* analysis, conducted on *Phox2b* heterozygous knockout mice (*Phox2b* $^{+/-}$), is suggestive of a haploinsufficiency mechanism in CCHS pathogenesis.

Phox2b $^{+/-}$mice show a respiratory phenotype, limited to the first post-natal period, in association with life-long ANS dysfunctions (Durand *et al*., 2004). This finding indicates that a single copy of *Phox2b* gene leads to a reduction in protein amount that is not sufficient to perform the normal Phox2b function, resulting in aberrant phenotype.

2) Phox2b 27 Ala+/- mutants phenocopy the Phox2b -/- null mutants and die in utero, suggesting that mutant proteins have lost their transactivation ability or the capacity to form homo- and heterodimers and to interact with transcriptional co-activators, impairing the correct developmental programme.

In vitro experiments have demonstrated that mutated PHOX2B protein is less functional compared to the wild-type protein. PARM mutants have a significant reduction in their capability to regulate the *DBH*, *PHOX2A* and *TLX2* gene expression, which represent three

of the known PHOX2B target genes (Trochet *et al*., 2005; Bachetti *et al*., 2005; Di Lascio *et al*., 2013).

Moreover, in our laboratory it has been shown that PHOX2B is able to directly regulate its own expression by means of an auto-regulatory loop (Cargnin *et al*., 2005), and that expanded proteins interfere with this mechanism, inducing an additional critical reduction in the expression of the wild-type protein (Di Lascio *et al*., 2013).

3) Mice bearing the most common polyalanine expansion observed in CCHS (Phox2b $^{+/+7Ala}$), die soon after birth for respiratory failure, phenocopying the most severe CCHS cases (Dubreuil *et al*., 2008). Moreover, they show a selective loss of RTN neurons, but other Phox2b-expressing structures involved in respiratory control are preserved, thus indicating a gain of function of the Phox2b mutant protein in the development of susceptible neuronal structure.

It has been observed, *in vitro*, that PHOX2B mutants, carrying frameshift mutations or the +12 alanine expansion, interfere with the synergistic transcriptional activation of the *DBH* gene mediated by the wild-type protein and the transcriptional co-activator CBP (CREB binding protein) (Wu *et al*., 2009).

Moreover, in cells co-transfected with equal amount of mutant and wild-type protein, resembling the heterozygous pathological condition, PHOX2B +13 Ala protein affects the PHOX2B wild-type mediated expression of *DBH*, *TLX2* and *PHOX2B* itself (Parodi *et al*.,2012; Di lascio *et al*., 2013).

Furthermore, the negative effects of PHOX2B mutant proteins on the transcriptional activity of the wild-type protein are promoter-specific (Trochet *et al*.,2005; Di Lascio *et al*., 2013), but it is not clear if the observed functional effects are the result of direct aberrant interactions between wild-type and mutant proteins and/or with other proteins. These data may explain the different susceptibility of neuronal structures to Phox2b mutations.

4) Available data from literature (Trochet *et al*., 2005; Bachetti *et al*.,2005 and 2007) and from our laboratory (Di Lascio *et al*., 2013) have shown that, *in vitro*, the longest polyalanine expansions (+11, +13 residues) are responsible for the formation of nuclear and cytoplasmic toxic aggregate, and that there is a strong correlation between the length of the polyalanine tract and protein cytoplasmic mislocalization. It has been also described that, *in vitro*, PHOX2B protein carrying the longest polyalanine tract (+13 Ala),

can retain a fraction of wild-type protein in both nuclear and cytoplasmic inclusions (Bachetti *et al*., 2005; Parodi *et al*., 2012).

The contribution of the cellular aggregates to the CCHS pathogenesis is still not completely clear and represents a key issue for the study of pharmacological agents capable of destroying PHOX2B aggregates or eventually of preventing their formation, e.g. activator of the protein degradation pathway, inhibitors of the protein incorrect folding, neuroprotective agents and apoptotic inhibitors.

Furthermore, we cannot exclude that proteins with the shortest polyalanine expansion and NPARM mutated proteins, that correctly localize into the nucleus, are able to properly fold and to adequately interact with co-activators. In this regard Wu *et al*. (2009) have demonstrated that PHOX2B nPARM mutants interact with CBP co-activator (CREB-binding protein) through different domains with respect to wild-type protein.

Therapeutic approach in CCHS treatment:

Aggregates targeting approach

As of today no pharmacological respiratory stimulants have turned out to be effective; the ventilatory supports represent the only option available for CCHS affected children.

Until now, the only pharmacological strategy investigated is targeted to dissolving intra-cytoplasmic aggregates of longest expanded PHOX2B proteins by using pharmacological agents which activate the heat shock protein (HSP) response (Bachetti *et al*., 2007 Di Zanni *et al*., 2012).

This observation starts from the remark that numerous chaperones, member of the HSP family, actively interact with intracellular aggregates of huntingtin protein, reducing their formation and cellular toxicity caused by the expanded polyQ tract (Abu-Baker *et al*., 2003; Bachetti *et al*., 2007; Bao *et al*., 2004; Cummings *et al*., 1998; Hay *et al*., 2004; Jana *et al*., 2000; Wang *et al*., 2005).

In particular, in 2007, Bachetti and co-workers demonstrated that geldanamycin (GA) is able to induce disaggregation of cytoplasmic protein aggregates in cell transfected with

PHOX2B carrying the +13 Ala expansion, thus also rescuing its nuclear localization and transcriptional activity (Bachetti *et al*., 2007). In addition, they observed that GA treatment in HeLa cell lines also induced a high increase in apoptosis (50%) after 5 days of treatment (Bachetti *et al* 2007).

In 2012, the same group tested the effect of other compounds, known to be capable of dissolving protein aggregates or of preventing their formation (Di Zanni *et al*., 2012), on PHOX2B expanded protein. In particular, they analysed the effect on PHOX2B polyalanine protein disaggregation, on the rescue of its nuclear localization/transactivation activity, and the cellular toxicity of the treatment. In this work the authors found that in addition to the afore mentioned GA, also the treatment with its analogue 17-AAG (17-N-allylamino-17-demethoxygeldanamycin), as well as the Curcumin (diferuloylmethane) (Cheng *et al*., 2001; Sharma *et al*., 2004), resulted to be effective.

Indeed, these two drugs successfully eliminate PHOX2B +13 Ala protein aggregates formation inducing a 70-80% rescue of its transcriptional activity.

Moreover, they confirmed that, as already observed for GA, 17-AAG induced HSP70, while Curcumin did not exert any effect on HSP70 expression, thus suggesting that it may use a different refolding mechanism.

Interestingly, 17-AAG, GA or Curcumin induced a faster degradation of wild-type protein with respect to the expanded mutants probably as consequence of its more soluble conformation.

This finding suggests the use of 17-AAG and curcumin as possible pharmacological strategy targeted to ameliorate the toxic cellular consequences linked to the presence of PHOX2B proteins containing expanded polyalanine tract.

Another pharmacological target, studied for CCHS treatment, regards the induction of the Ubiquitine Proteasome Response (UPS) by overexpressing the E3 ubiquitin ligase TRIM11 (Parodi *et al*.,2012). Usually, misfolded proteins are contacted by HSP, which refold them by reducing their toxic effects and restoring their normal phenotype. However, misfolded proteins are also targeted for the degradation by the UPS, and to this end the protein were ubiquitin marked by a process involving E1, E2, and E3 ubiquitin ligase enzymes, which act consequently. The TRIM11 protein is an E3 ubiquitin ligase that determines the substrate specificity, and it has been found to directly interact with PHOX2B wild-type

protein and to increase *DBH* expression (Hong *et al*., 2008). Parodi and co-workers (Parodi *et al*., 2012), in particular, demonstrated that TRIM11 overexpression is capable of inducing wild type and mutant protein degradation by UPS and of rescuing the impairment of transcription from *DBH* promoter associated with the dominant-negative effect exerted by mutant proteins.

These data suggest that the mechanism, which induces TRIM11 up-regulation, may offer a potential target for CCHS treatment.

Whilst these observations propose a possibility in the functional recovery of PHOX2B mutant protein, through dissolution of toxic cellular aggregates, on the other hand the big issue related to these strategies is that aggregation was demonstrated only *in vitro*. Therefore, we cannot exclude that aggregate formation could be the result of an artefact consequence of protein over expression in cells.

In vivo, data obtained by the knock-in mouse carrying the most common heterozygous Phox2b +7 alanine expansion (Phox2b $^{+/+7Ala}$) highlight the lack of RTN neurons development. However, it is not yet clear if the other neuronal regions, involved in the control of respiration, are correctly preserved and functioning.

In humans, studies on the RTN are very difficult. RTN has been tentatively identified in man as a small neuronal region, PHOX2B positive, at the pontomedullary junction ventral to the facial nucleus and lateral to the superior olivary nucleus (Rudzinski & Kapur, 2010). Altered development of the putative hRTN (human RTN) has been identified in human sudden infant death syndrome (SIDS) victims (Lavezzi *et al*., 2012); thus suggesting a possible neuronal anatomical defect in this area, also in CCHS human patients.

Very recently, analyses of post-mortem brain of two CCHS patients, one PARM and one NPARM, have shown the presence of anatomical abnormalities in different neuronal regions.

In particular, *locus coeruleus* atrophy was found in both patients' brain, whereas the NPARM patient presents markedly abnormalities in different brain regions including: decreases in the dorsal motor nucleus of the vagus nerve, absent mesencephalic trigeminal nucleus, and decreased serotonergic median raphe and DBH-positive adrenal medullary neurons (Nobuta *et al*., 2015). These findings indicate the presence of anatomical neurodevelopmental defects in CCHS patients, obviously irreparable with

pharmacological treatments. However, other neural structures involved in breathing control, whose function depends on PHOX2B, may get beneficial effects by the restoration of PHOX2B activity.

An absolute limitation to the comprehension of the molecular mechanism underlying the disease is due to the substantial lack of information about the genes regulated by PHOX2B, and their specific involvement in the different clinical manifestations of the disease, as well as their implication in the regulation of the afore mentioned affected neuronal areas. The importance of better identifying PHOX2B target genes arise from the interesting prospective that some of them may become possible pharmacological targets in the CCHS treatment.

The progestin desogestrel: from bedside to bench side

In 2010 a hopeful indication for the treatment of CCHS patients came from a direct clinic observation.

In particular, it has been observed that two female patients (20/25 and 20/26 genotype), using a progestin drug, Desogestrel, for contraceptive purposes, dramatically ameliorated the clinical symptoms of CCHS, showing chemosensitivity recovery (Straus *et al* 2010). This fortuitous observation suggests a possible link between this drug and the recovery of the CO_2 chemosensitivity, however the molecular mechanism of this unexpected pharmacological effect is completely unknown.

Starting from this observation Straus' group of clinicians from Groupe Hospitalier Pitie-Salpetriere in Asistance Publique-Hopitaux de Paris has started a clinical trial to study the effect of desogestrel on ventilatory response to hypercapnia in a small cohort of female CCHS patients (clinicaltrials.gov. NCT01243697).

The use of progestin drugs for CCHS treatment theoretically represent a strong option, as it is known that hormones can act as stimulants or inhibitors of respiratory activity even in healthy subjects (Bayliss and Millhorn, 1992; Peña and Garcia, 2006).

Among the various classes of hormones involved in the endocrine modulation of breathing, steroid hormones, in particular sex hormones, have a major impact.

Sex hormones (androgens, oestrogens, and progesterone) are synthesized from cholesterol (Stoffel-Wagner, 2001) principally in the gonads, the adrenal gland and the placenta, but they are produced in the CNS (neurosteroids) as well (Nelson & Bulun *et al*., 2001).

Sex hormones have significant effect on the respiratory neuronal network during early development, as the activation of their receptors in embryonic brain induces differences in the number of neurons, organization of synaptic connections, and neurotransmitter expression in the adult brain nuclei (Negri-Cesi *et al*., 2008).

Hormones are able to easily cross blood-brain barrier, and to contact neurons in peripheral and central nervous system. Possible mechanisms, whereby sex hormones can modulate breathing, include direct effects on gene expression in respiratory neurons (by steroid hormones receptors activation) or indirect regulation by neurotransmitter.

Interestingly, sex hormone receptors are expressed in several brain regions involved in respiratory control, including the NTS, hypoglossal and phrenic nuclei, and carotid body (reviewed in Behan & Wenninger 2008).

As different studies have highlighted differences in respiratory parameters between men and women, and in different menstrual cycle phases in women, progesterone (Pg) has been pointed as a key hormone in the control of breathing. Indeed, despite Pg exerts its effect mainly on the uterus and the breast, it also induces numerous effects on other tissues different from its primary targets, as in brain areas (Brinton *et al*., 2008); indeed, its receptors were detected at the level of the trachea, lungs, brain and brainstem (Saaresranta, 2002). As a neurosteroid, Pg regulates neuronal function, primarily via allosteric regulation of some membrane-bound receptors, such as GABA-A (Gamma-aminobutyric acid) receptors. In particular, Pg acts as a potent positive modulator of the GABA-A receptor, in the form of its neuroactive metabolite, allopregnanolone (Porcu *et al*., 2012; Andrade *et al*., 2012). Recently, etonogestrel, the active metabolite of desogestrel, has been shown to positively modulate GABA-A receptor resulting in increased respiratory frequency (Joubert *et al*., 2016).

Progesterone is considered a powerful stimulant of the respiratory activity.

Several studies have shown that the physiological Pg increases ventilation in women during pregnancy (Saaresranta *et al*., 2003), whereas Pg administration reduces apnoea

frequency in adult women (Shahar *et al*., 2003) and it has proposed as drug for the treatment of prematurity apnoea (Finer *et al*., 2006).

In vivo experiments indicate that Pg administration enhanced the respiratory response to hypoxia in newborn rat (Hichri *et al*., 2012; Bairam *et al*., 2013); whereas R5020, a Pg receptor agonist, increased phrenic nerve activity and respiratory frequency, in male and female cats (Bayliss and Millhorn 1992).

Taken together these data support the hypothesis that the use of progestin drugs theoretically represent a good option for CCHS treatment.

Nevertheless, Sritippayawan and colleagues (2002) claimed that hypoventilation symptoms do not ameliorate in CCHS patients during gestation, in spite of the corresponding increase in progesterone level. The effect of Desogestrel on CCHS patients can be due to the greater biological potency of desogestrel with respect to natural progesterone (Wiegratz and Kuhl, 2004), as its affinity for the progesterone receptor is three times greater than that of progesterone (Straus *et al*., 2010; Wiegratz and Kuhl, 2004).

Starting from these observations, it is reasonable to suppose that the potent progesterone receptor agonist desogestrel may be able to induce changes in respiratory control by stimulating autonomic CO_2 chemosensitive regions, affected by *PHOX2B* mutations.

The Desogestrel is a progestin commonly used in hormonal contraception in association with the estradiol.

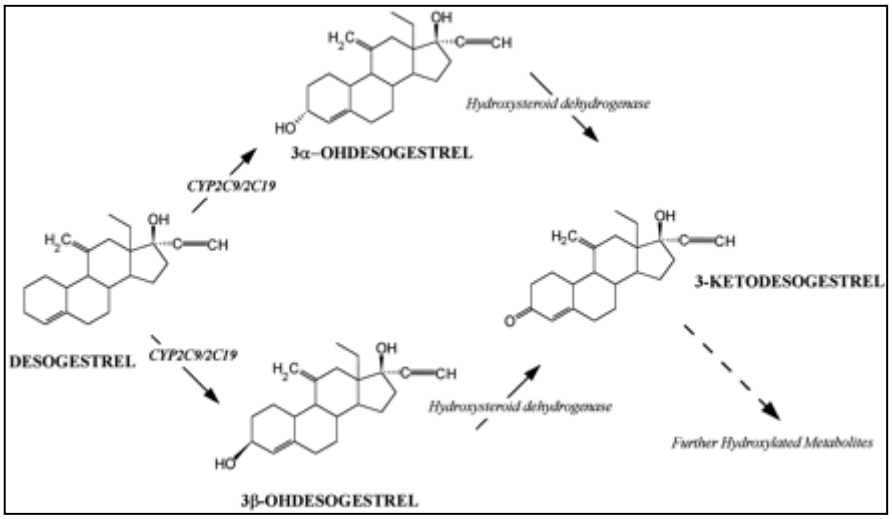

Figure 10 Bioactivation of the contraceptive steroid desogestrel mediated by CYP2C. Modified from Gentile *et al*., 1998.

Desogestrel constitutes the pro-drug of the active metabolite 3-keto-desogestrel (etonogestrel, 3-oxodesogestrel, Org-3236), and this conversion is operated in vivo by cytochromes CYP450 2C9 (CYP2C9) and CYP450 2C19 (CYP2C19) (Fig. 10; Gentile *et al*, 1998).

- Progesterone receptors

From the molecular point of view, the progesterone response is mediated by the binding to different Pg receptors (PGRs):

- classical nuclear progesterone receptors (cPRs);
- membrane progesterone receptors (mPRs).

Usually, the binding of Pg to one of these two receptor types leads to different mechanisms of action, in particular, a rapid non-genomic mechanism mediated by progesterone membrane receptors, or a slower genomic mechanism mediated by nuclear progesterone receptor isoforms (described later).

- Classical nuclear progesterone receptors (cPRs)

Classical nuclear progesterone receptors (cPRs) were described by Sherman *et al* in 1970 (Sherman *et al*., 1970). Despite their principal reproductive functions, cPRs have been soon localized to different CNS regions including the hypothalamus, cortex, hippocampus and cerebellum (Auger *et al*., 2002; Guerra-Araiza *et al*., 2002; Quadros *et al*., 2007; Lopez & Wagner 2009). Over the years, accumulating data are emerging on the neuroprotective and neuroregenerative effects of progesterone in the brain (Callier *et al*., 2001; Guerra-Araiza *et al*., 2002; Murphy *et al*., 2000).

Progesterone exerts its action mainly by binding to classical nuclear progesterone receptor isoforms: the full length B isoform (PR-B, Progesterone Receptor-B) and the N-terminal truncated isoform A (PR-A, Progesterone Receptor-A) (Conneely *et al*., 1987). The two isoforms are encoded by the same gene located on chromosome 11 (11q22) composed by 8 exons, with two alternative translational start sites (Fig. 11, Panel A).

Further, some evidence described that the two PR isoforms may result from alternative transcripts driven by independent promoters (Kastner *et al*., 1990; Gronemeyer *et al*., 1991).

Both isoforms share an inactivation domain (IF, inhibition factor), two activation domains (AF), respectively AF1 in the N-terminal region, and a C-terminal AF2; a highly conserved DNA-binding domain (DBD), followed by a small hinge region (h) and a ligand-binding domain (LBD), which also contains a dimerization domain (DI) and 4 nuclear localization signals (NLS) (Fig. 11, Panel B) (Leonhardt *et al*., 2003).

The short PR-A truncated isoform (94 kDa) lacks 164 amino acids, which encodes in PR-B (120 kDa) an additional activation domain (AF3) that acts in synergy with the two other activation domains (AF1-AF2) (Brinton *et al*., 2008).

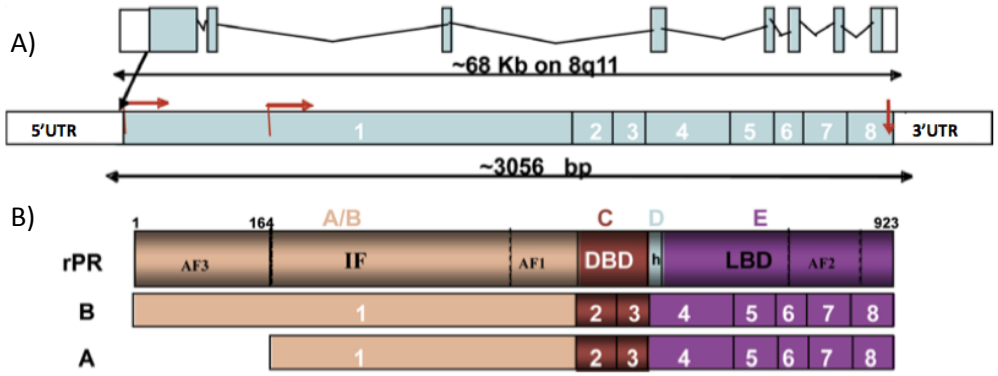

Figure 11 Panel A) PGR gene structure. The classical progesterone receptor is composed of 8 exons with a coding region 3056-bp long and 5' - 3' -untranslated region. Both cPR-B and cPR-A isoforms are transcribed from this gene, but they use alternative initiation codons indicated by red horizontal arrows. **Panel B) Functional domains of classical nuclear progesterone receptors (PR-B, PR-A).** cPR proteins are both composed of an inactivation domain (IF) and an activation domain (AF1) encoded by exon 1, a highly conserved DNA-binding domain (DBD) encoded by exons 2 and 3, a variable hinge region (encoded by part of exon 4), a conserved ligand-binding domain (LBD, encoded by exons 4–8) and a second activation domain (AF2) encoded by part of exon 6 and exon 7. (Brinton *et al*., 2008).

In the absence of ligand, nuclear PRs are maintained in an inactive form in the cytoplasm, complexed with different chaperone molecules, in particular with heat shock proteins (hsp90, hsp70, Hsp40).

The binding of the hormone to the receptor causes a conformational change in the receptor, dissociation from the chaperone proteins, followed by dimerization of two free receptors, and dimer translocation into the nucleus, where it directly interacts with specific progesterone responsive elements (PREs) in the target genes promoter (Fig. 12) (Leonhardt *et al*. 2003).

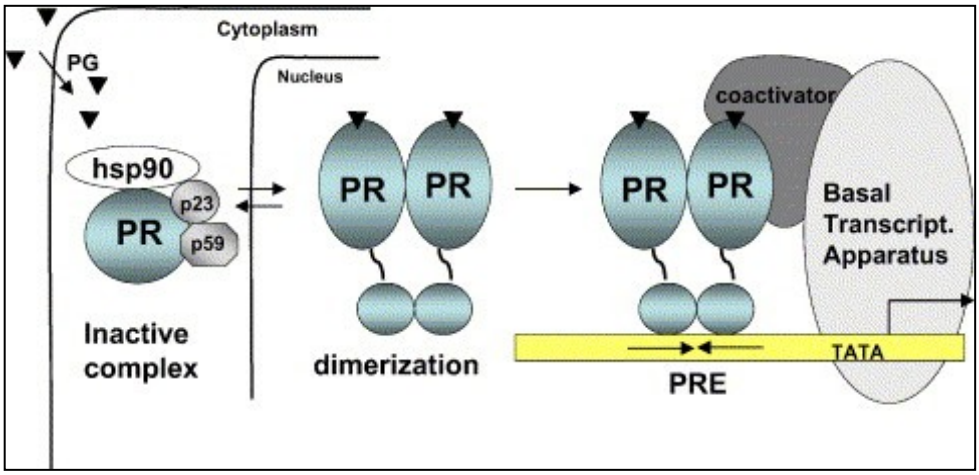

Figure 12 Mechanism of action of nuclear progesterone receptors. Binding of progesterone to inactive nuclear progesterone receptor complex induces a conformational change that allows the dissociation of chaperone molecules, receptor dimerization, binding to PRE sequence on the promoter of target genes, and finally recruitment of co-activators, which act as bridge with the basal transcriptional apparatus (Leonhardt *et al*. 2003).

Although the DNA-binding affinity of the two cPR isoforms is comparable, their transcriptional activity is variable depending on the cell type, as evidence suggests that they adopt distinct conformations within the cell, allowing them to interact with different co-regulators (Bain *et al*., 2000;Bain *et al*., 2001; Tetel *et al*., 1999).

In mouse models, the selective ablation of one isoform has revealed that PR-A and PR-B have different exclusive roles, demonstrating that PR-B is essential for normal mammary gland development (Mulac-Jericevic *et al*., 2003), while PR-A is required for uterine development and reproductive function (Mulac-Jericevic *et al*., 2000).

Moreover, given the differential roles of PR-A and PR-B, these two receptors occasionally play synergic roles in gene expression regulation. For instance, in certain breast cancer cell lines, it has been shown that an imbalance in the PR-A/PR-B ratio induces a remarkable increase in the transcription of genes involved in cell growth (Hopp *et al*., 2004), resulting in a more aggressive phenotype of cancer.

PR-A and PR-B isoforms are not equally expressed in all the cell types (Mote *et al*., 2000).

In general, there are cell types where only a single PR isoform is expressed and in which the PR homodimer is the dominant molecular species, and cell types expressing both

isoforms, where the formation of three different molecular species (PR-B homodimers, PR-A homodimers and PR-A/PR-B heterodimers) can occur.

PR-A and PR-B, affect the target genes in a different manner; PR-B generally represents the stronger transcriptional activator (Vegeto *et al*., 1993; Tung *et al*., 2006), while PR-A is a weaker activator of progesterone-dependent genes and is often inactive. When both the isoforms are activated, PR-A assumes a different conformation, which leads it to become a repressor of the same PR-B and estrogen receptor (hER) (Vegeto *et al*., 1993; Mohamed *et al*., 1994; Sartorius *et al*., 1994; Mulac-Jericevic *et al*., 2000). This conformation may expose sequences bound by transcriptional co-repressors and reactive amino acids that can be modified, resulting in some cellular contexts in repressor activities of PR-A (Giangrande *et al*., 2000).

This difference in cPRs function lies in the fact that hPR-B contains three transcriptional activation domains, and the AF3, which is only present in the hPR-B, appears to be the critical point of the different action of the two isoforms.

The study of the intracellular structure of PRs has shown that activator domains AF1 and AF2 act synergistically and their intermolecular interaction is necessary and essential for the recruitment of additional co-activators (steroid receptor co-activator, SRCs) and for the interaction with other transcription factors (Leo & Chen 2000).

The AF1/AF2 interaction is prevented by the inhibitory domain (ID), which blocks the transcriptional activity as well; the presence of the AF3 domain in PR-B allows activating transcription, acting as suppressor of the inhibitory activity of the ID (Abdel-Hafiz *et al*., 2002; Tung *et al*., 2006).

- Membrane progesterone receptors (mPRs)

In addition to the involvement of the classic nuclear receptors, progesterone can also interact with different membrane receptors (mPRs), including a seven trans-membrane Progesterone Receptor and single transmembrane receptors.

The seven membrane receptor coupled to G proteins, 7TMPR (seven Trans-Membrane Progesterone Receptor), is a receptor with seven transmembrane domains associated with a G inhibitory protein, belonging to the progestins and adipo Q receptor family

(PAQR family) (Thomas & Pang, 2012; Zhu, *et al*., 2003). When bound to progestin, this receptor blocks the activity of the enzyme adenylyl cyclase, thus inhibiting the production of the intracellular second-messenger cAMP (Zhu *et al*., 2003). Three 7TMPR isoforms have been identified: – 7TMPRα, β, and γ. 7TMPR isoforms localize in different human compartments exerting different functions. In particular, 7TMPRα is expressed in reproductive tissues and in the kidney; the β form exclusively localizes in neural tissues including cerebellum, the cerebral cortex, pituitary gland, thalamus, caudate nucleus, and spinal cord; whereas the γ-subtype is present in the kidney, colon, and possibly in the adrenal and lung (Zhu *et al*., 2003).

Pg binds also single transmembrane receptors, including PGRMC1 (Progesterone receptor Membrane Component 1) and PGRMC2 (Progesterone receptor Membrane Component 2) (Gerdes *et al*., 1998). Only little information is available about this class of progesterone receptors. PGRMC1 is expressed predominantly in the liver and kidney, and it was shown to bind several cytochrome P450 enzymes, to play a role in chemotherapy resistance (Hughes *et al*., 2007), and to promote the antiapoptotic effect of Pg (Crudden *et al*., 2006; Peluso *et al*., 2008.) Moreover, it has been demonstrated that progesterone acts through PGRMC1 to increase brain derived neurotrophic factor (BDNF) release (Su *et al*., 2012), promoting survival of neurons (Gonzalez *et al*., 2005).

PGRMC2 is particularly expressed in the placenta (Gerdes *et al*., 1998), and overexpressed in breast cancer (Causey *et al*., 2011). Both PGRM1 and PGRM2 are found to be expressed in different regions of the rat brain, particularly in all hypothalamic nuclei, indicating a possible role in neuroendocrine functions (Intlekofer *et al*., 2011).

Progesterone: genomic and non-genomic mechanism

Besides the known roles as sex hormones in menstrual cycle, pregnancy, and embryogenesis in human, Progesterone exerts important functions also in human brain.

As mentioned before, almost all brain areas express PGRs, although difference of various receptor types in relative amounts and expression in brain regions are observed (Brinton *et al*., 2008; Intlekofer and Petersen, 2011).

Progesterone produces multiple effects in the CNS by means of three main mechanisms: gene regulation, neurotransmitters release modulation, and signalling cascades activation. Although the classical long-term genomic effect of progesterone has been considered for years the main mechanism of action, recent studies have highlighted the existence of short term non-genomic mechanisms.

These short term progesterone-dependent effects appear linked to the activation of several cellular pathways that lead to membrane receptors regulation, ion channels activation, cytoplasmic second messengers increase, regardless of gene expression (Boonyaratanakornkit, *et al*., 2008; Leonhardt *et al*., 2003; Schumacher *et al*., 1999).

Numerous studies have shown that the non-genomic effects of progesterone are the consequence of the activation of various extranuclear signals, such as second messengers (cAMP, cGMP) and kinases (PKA, PKC, CaMKII). The subsequent activation of signalling cascades, associated with these second messengers, finally induces post-translational modifications of transcription factors (TFs), co-activators and co-repressors of nuclear receptors, which in turn modify the activity of other transcription factors.

One of the most characterized pathways activated by progesterone and progestogens, is the MAPK kinase cascade, through Src kinase activation (Fig. 13) (Leonhardt *et al*. 2003). However, the action of progesterone seems to be obtained from the integration of both mechanisms, the rapid non-genomic mechanism and the slowest genomic mechanism, which both provide a sensitive regulation of gene expression.

Furthermore, besides the progesterone receptors-mediated effects, extensive data indicates that an important neuroactive metabolite of Progesterone, the Allopregnanolone (ALLO), could signal through pathways other than cPGR and mPRs. In particular, ALLO acts as a potent endogenous positive allosteric modulator of GABA-A (gamma-amininobutyric acid-a) receptor. Several data indicate that ALLO is the principal mediator of the neuroprotective effects of progesterone, including neurogenesis of cerebellar granule cells (Keller *et al*., 2004), neural progenitor proliferation (Wang *et al*., 2005) and neuronal cell death decrement (Wang and Brinton, 2008), and all of these effects appear to be through GABA-A receptors.

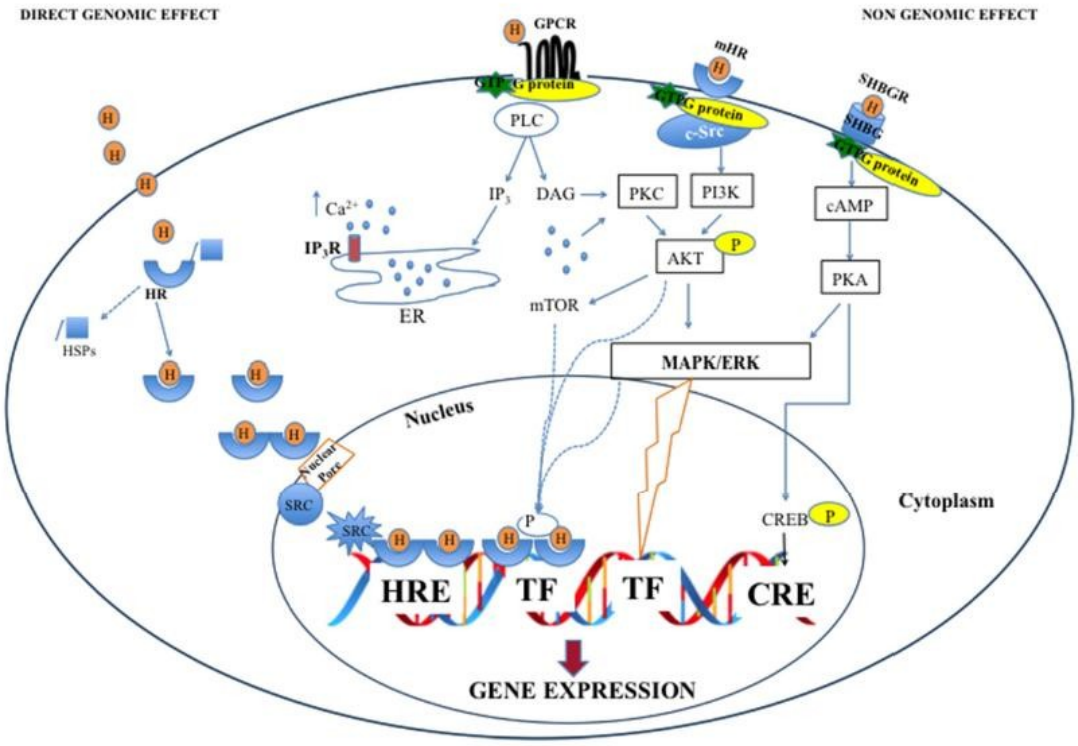

Figure 13: Schematic representation of genomic and non-genomic progesterone mechanisms of action. The classic progesterone mechanism of action is mediated by nuclear receptors PRs, which promote interaction with co-activators and play the predominant role in the response to progesterone (genomic mechanism). Progesterone may mediate its effects also via second messengers (cAMP, cGMP), activation of kinase cascades (PKA, PKC, CaMKII), activation of the MAP kinase cascade (MAPK), the phosphorylation of transcription factors, co-activators and CREB responsive elements (non-genomic mechanisms). All of these mechanisms appear to interact with each other mediating the biological effects of progesterone. (Contrò *et a*l., 2015)

Aim of the thesis:

The paired like homeodomain proteins PHOX2B (Paired-like homeobox2B) and PHOX2A (Paired-like homeobox2A) are deeply involved in autonomic nervous system development, as well as noradrenergic neuronal differentiation (Tiveron *et al*., 1996).

In both Phox2a and Phox2b KO mice brain the principal noradrenergic centre *locus coeruleus* does not form (Morin *et al*., 1997; Pattyn *et al*., 1999), and confirming their central role in the specification of noradrenergic identity, both transcription factors directly regulate the expression of Dopamine Beta Hydroxylase (*DBH*), a key enzyme in noradrenaline synthesis (Adachi *et al*., 2000; Seo *et al*., 2002).

The neuronal development and specification driven by PHOX2 proteins is the result of a fine temporal and spatial control of the two-paralogue proteins. In particular, PHOX2B is considered a "master regulatory gene" in the network leading to the cathecolaminergic phenotype specification, and regulate PHOX2A expression (Coppola *et al*., 2005).

As PHOX2 proteins play crucial role in embryonic neuronal differentiation, their mutations or altered regulation are linked to congenital pathologies, such as CCHS, and neuroblastic tumours (Nakano *et al*., 2001; Amiel *et al* 2003, Weese-Mayer *et al* 2003 Longo *et al*., 2008). Consistently with its role as transcriptional regulator, it is reasonable to suppose that transcriptional dysregulation might be an important mechanism of CCHS pathogenesis and/or tumour development. Current research into treatments of CCHS and neuroblastoma is on the strategy aimed at counteracting the toxic effects of the mutated PHOX2B protein and/or to down-regulate PHOX2B expression, respectively. In particular, drugs promoting the refolding and/or the clearance of mutant protein aggregates have been turned out to be effective *in vitro* in rescuing the nuclear localization and transactivation activity of mutants (Bachetti *et al*.,2007; Di Zanni *et al*., 2012). Moreover, screening of a FDA approved library led to the identification of molecules capable of down-regulating PHOX2B expression (Di Zanni *et al*., 2015), that may induce differentiation of neuroblastoma cells. Another strategy that is currently under investigation regards the use of progestinic drugs for ameliorating respiratory symptoms in CCHS patients. The molecular mechanism of this unexpected pharmacological effect is completely unknown, but this observation has a strong proof of concept value, in the

perspective of a pharmacological intervention in CCHS, at least for ameliorating respiratory symptoms.

Absolute limitations to the comprehension of the pathogenesis of CCHS, and to develop new and effective treatments for this disease, regard i) the missing knowledge of target genes regulated by PHOX2B (whose expression may be eventually dys-regulated in the disease); and ii) substantial lack of information on the structure-function relationships in PHOX2B wild-type and mutant proteins.

The aim of this thesis was to disclose still unknown mechanisms involved in the pathogenetic mechanisms of PHOX2B-driven disease development, to improve the recognition of new sensitive therapeutic intracellular targets. The results obtained during the three years of my PhD thesis are organized in three chapters, two of which (chapter 2 and 3) report results that are summarized in published papers, here enclosed, whereas the third chapter concerns with the molecular mechanism of Desogestrel effect and are still unpublished results.

In summary, the first paper **"Alanine Expansions Associated with Congenital Central Hypoventilation Syndrome Impair PHOX2B Homeodomain-mediated Dimerization and Nuclear Import"** by Di Lascio, Belperio *et al*. (Appendix 1), deals with a better characterization of PHOX2B functions, including homo-and hetero dimerization and nuclear import, also in the perspective of better analysing the possible effect of polyalanine expansion on these functions, as the expansion of the polyalanine tract in PHOX2B results in a protein with altered DNA-binding, reduced transcriptional activity and defect in nuclear localisation (Trochet *et al*., 2005; Bachetti *et al*., 2005; Di Lascio *et al*., 2013). Interestingly our data clearly underlined a strong interaction between PHOX2A and PHOX2B wild type/ mutants proteins, indicating a possible involvement of PHOX2A in the pathogenesis of CCHS.

Recently, studies conducted on NB cell lines have highlighted a correlation between drugs, used in NB treatment or clinical trials (e.g. GA, 17-AAG), and the modulation of PHOX2B gene expression (Bachetti *et al*., 2007, Di Zanni *et al*., 2015).

NBs are very sensitive to retinoids, and they were introduced in NB treatment as "additional drugs" (Reynolds & Lemons 2001; Altucci *et al*., 2001; Chu *et al*., 2003) in order to induce differentiation in residual cancer cells after NB surgical elimination.

In the second paper **"PHOX2A and PHOX2B are differentially regulated during retinoic acid-driven differentiation of SK-N-BE(2)C neuroblastoma cell line"** by Di Lascio *et al*., 2016 (Appendix 2), we tested the hypothesis of a direct regulatory link between "all *trans* retinoic acid" (ATRA), a retinoid drug that is known to suppress growth in cancer, and the expression/activity of the candidate tumour suppressor gene *PHOX2A* and *PHOX2B* in the SK-N-BE(2)C neuroblastoma cell line. We showed that ATRA differently regulates both *PHOX2A* and *PHOX2B* expression, thus suggesting that they may be useful biomarkers for NB staging, prognosis and treatment decision making.

Following the observation (Similowski and Straus, 2010) that desogestrel assumption ameliorates ventilatory symptoms in CCHS patients, we decided to investigate the mechanism underlying this effect (**Chapter 3**).

Previous studies, conducted in our laboratory, on T47D cell line suggested us that 3-KDG can influence both the activity of PHOX2B and PHOX2A, and that this is mediated by the intracellular receptor PGR. We do not know at the moment the relative contribution of the two isoforms PR-A and PR-B. Although T47D cell line has been an invaluable tool to generate these very interesting and promising data, it presents limits due to a cellular background not properly associated with the physiological PHOX2B environment.

To further exploit Desogestrel mechanism , we generated a neuroblastoma cell line (SK-N-BE(2)C) stably expressing nuclear progesterone receptor isoforms. In order to analyse the possible effect of the progestin drug on the PHOX2B pathway we treated SK-N-BE(2)C stable clones with the active metabolite of desogestrel (3-keto-desogestrel) and evaluated alteration in endogenous protein amount and transcription levels by using western blot and quantitative PCR analyses. Our findings demonstrated that desogestrel treatment is able to alter PHOX2B and PHOX2A expression, as well as the expression of DBH and some other target genes of PHOX2 proteins, particularly reducing their expression.

Chapter 2

Alanine Expansions Associated with Congenital Central Hypoventilation Syndrome Impair PHOX2B Homeodomain-mediated Dimerization and Nuclear Import

Simona Di Lascio, Debora Belperio, Roberta Benfante, and Diego Fornasari

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 25, pp. 13375–13393, June 17, 2016

Inserted as Appendix 1

The most frequent PHOX2B mutations, leading to Congenital Central Hypoventilation Syndrome, include expansion repeat mutations in the second polyalanine stretch of the protein, and point mutations in different protein regions including the homeodomain (HD) (Weese-Mayer *et al*., 2010).

Evidences collected so far indicate that the elongation in the PHOX2B polyalanine stretch leads to a protein with reduced functionality. In particular, nuclear import defects and cytoplasmic aggregate formation have been observed in the longer expanded mutants, whereas impairment of DNA-binding and transcriptional activity are also detectable in shorter expansions (Bachetti *et al*., 2005; Di Lascio *et al*., 2013).

Furthermore, data collected hitherto support the idea that CCHS is not due to a pure loss of function mechanism, but also involves a dominant-negative effect and toxic gain of function of PHOX2B mutants.

A recent study conducted in my laboratory (Di Lascio *et al*., 2013) provided novel *in vitro* experimental evidence of a transcriptional dominant-negative effect of PHOX2B polyalanine mutant proteins on wild-type protein functions, as they interfere with the PHOX2B mediated regulation of *TLX2* gene.

However, it was not clear if the observed functional effects of PHOX2B mutant variants were the result of direct aberrant interactions between wild-type and mutant proteins and/or with other proteins (e.g. transcription factors or co-activator).

As we found that PHOX2B forms heterodimers with PHOX2A, in the first part of my project we tested the hypothesis that the dominant negative effects of the mutated proteins were due to non-functional/aberrant interactions with the wild-type protein or PHOX2A.

Moreover, as PHOX2B function-structure relationships has not been fully elucidated so far, and knowing how PHOX2B protein is structured (i.e., domains involved in protein-protein interaction; transactivation domain) may help in understanding how mutations can affect its function, we also focused on characterizing PHOX2B protein domains associated with the different functions, also in a view to better understand the molecular mechanism at the basis of CCHS.

From the molecular point of view, the HD has different important functions for the protein, including DNA binding, target genes transactivation, homo-/hetero-dimerization, and cellular localizations.

On the other hand, the function of the polyalanine stretch is still unknown, although it has been supposed to be flexible spacer element located between protein domains and therefore it is likely to be involved in correct protein folding, DNA-binding and protein-protein interaction.

Starting from these findings, we better characterized PHOX2B homeodomain-mediated functions, as hetero-/homo-dimerization and nuclear import, and the effects of the alanine tract expansion on these activities.

In particular, our findings showed that:

- PHOX2B protein is able to homodimerize and to strongly heterodimerize with its paralogue gene, PHOX2A;
- PHOX2B polyalanine-expanded proteins weakly interact with wild-type protein but retain a partial ability to interact with PHOX2A;
- PHOX2B polyalanine-expanded proteins do not interfere with PHOX2A-mediated transactivation of the DBH promoter, on the contrary they act synergistically;
- the PHOX2B dimerization domain comprises the homeodomain and the C-terminal region and does not involve the alanine stretch;
- the expansion of the polyalanine tract inhibits homeodomain-mediated PHOX2B nuclear import, interfering with PHOX2B nuclear localization signals (NLSs), that we have identified at the opposite ends of the HD.

Our data exclude the possibility that the formation of non-functional heterodimers between PHOX2B wild-type protein and mutants may play a major role in CCHS pathogenesis.

However, we cannot exclude that the formation of PHOX2A/PHOX2B mutants heterodimers could have some implication in the pathogenesis of the disease, as they could interact with different proteins (i.e. co-activators, co-repressors) to those usually recruited by PHOX2A/PHOX2B wild type heterodimers. Moreover, a deeper knowledge of

genes regulated by PHOX2B and PHOX2A is required, in order to better identify which genes are differentially regulated in the presence of PHOX2B mutant protein.

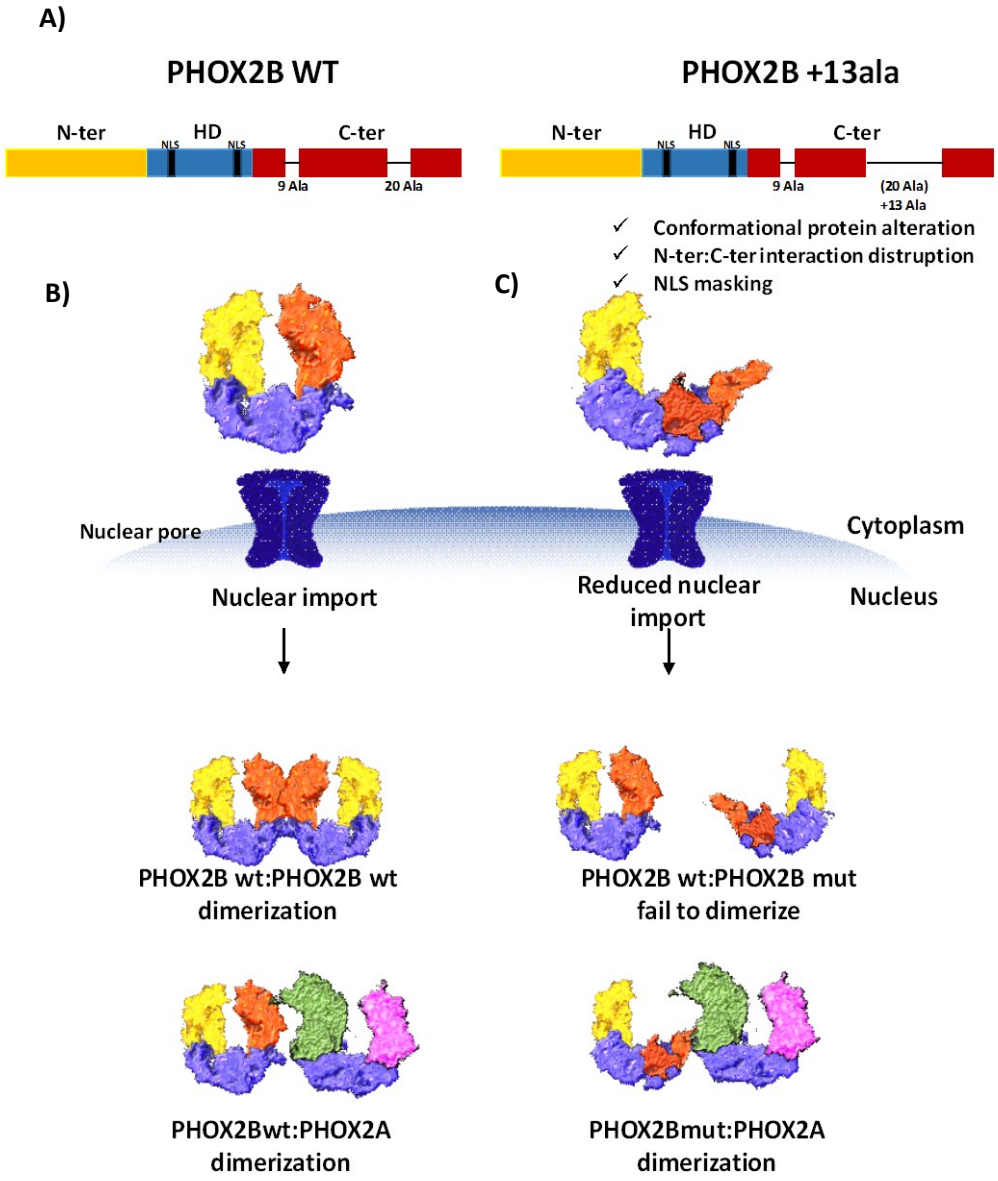

Figure 14 PHOX2B:PHOX2A dimerization.

A) Schematic representation of PHOX2B wild type and PHOX2B expanded mutant protein (i.e. +13 Ala). B) PHOX2B wt protein is transported into the nucleus by means of nuclear localization signals (NLSs)/nuclear pore interaction. In the nucleus compartment, PHOX2B wt protein is able to form homodimers and heterodimers with PHOX2A protein. C) The expansion of the 20 polyalanine tract induces a conformational change of the PHOX2B protein; the expansion interferes with PHOX2B NLSs function, by inhibiting its complete transport into the nucleus. In the nucleus, PHOX2B expanded mutant protein loses the ability to dimerize with its wild type counterpart, but it retains the ability to dimerize with PHOX2A protein.

Chapter 3

PHOX2A and PHOX2B are differentially regulated during retinoic acid driven differentiation of SK-N-BE(2)C neuroblastoma cell line

Simona Di Lascio, Elena Saba, Debora Belperio, Andrea Raimondi, Helen Lucchetti, Diego Fornasari, Roberta Benfante

Experimental Cell Research 342 (2016) 62–71. Elsevier.

Inserted as Appendix 2

PHOX2B, and its paralogue gene, *PHOX2A,* have been associated with the pathogenesis of neuroblastoma (NB), as they are found to be overexpressed in different NB cell lines and tumour samples (Longo *et al*., 2008).

Unlike *PHOX2B,* in which both mutations and altered expression levels were found in correlation with NB development, no mutations were found in *PHOX2A*.

As these findings clearly indicate that PHOX2 pathway deregulation is strictly linked to neuroblastoma onset and development, recently NB therapies aimed to reduce PHOX2B expression are emerging (Bachetti *et al*., 2007; Di Zanni *et al*., 2015).

In view of the fact that retinoids have been introduced in NB treatment, as differentiating agents of cancer cells (Reynolds & Lemons 2001), in the second part of this project, we tested the hypothesis of a direct regulatory link between the retinoic drug "*all-trans* retinoic acid" (ATRA), and *PHOX2* genes regulation.

Retinoic acid (RA) has an important role in differentiation of neuronal crest-derived cells, supporting proliferation and survival of neuronal crest cells in early stage, and regulating the expression of receptors for neurotrophic factors in later stage of development (Thang *et al*., 2000; Wyatt *et al*., 1999).

Retinoids play a fundamental role in embryonic development by interacting with two classes of nuclear receptors (NRs): the retinoic acid receptors (the multiple isoforms of the RAR α, β, and γ isotypes), and retinoid x receptors (the multiple isoforms of the RXR α, β, and γ isotypes).

RXR and RAR receptors are capable to form heterodimers, which mediate Retinoids signals transduction during developmental processes. In particular, multiple RXR/RAR heterodimers regulate gene transcription by binding to retinoic acid responsive elements (RAREs) in the promoter regions of numerous sets of RA target genes (Mark M. *et al*., 2006)

Despite retinoids are now being introduced in NB treatment, the molecular mechanisms by which they induce cellular differentiation in cancer cells, are still largely unknown.

In this part of the project it has been found that there is a regulatory link between ATRA, the candidate tumour suppressor gene *PHOX2A* and *PHOX2B* expression, in the SK-N-BE(2)C NB cell line.

In detail, our findings demonstrated that:

- PHOX2A expression is finely controlled during retinoic acid differentiation at transcriptional level by up-regulating *PHOX2A*-mRNA;
- extended ATRA treatment selectively degrades PHOX2A protein, while the corresponding mRNA remains up-regulated;
- ATRA treatment down-regulates *PHOX2B* expression.

Previously, in my laboratory, it has been demonstrated that PHOX2B regulates the transcription of the *PHOX2A* gene by directly binding and *trans*activating its promoter (Flora *et al*., 2001). It has been further defined that PHOX2B is able to regulate its own expression by directly binding to its promoter (Cargnin *et al*., 2005). Since we demonstrated that PHOX2A is capable of binding to the ATTA sequences on the *PHOX2B* promoter, negatively modulating its expression (Di Lascio *et al*., 2016), we investigated whether PHOX2B down-regulation, induced by ATRA treatment, was at least partially due to the increase in PHOX2A expression, by blocking the ATRA-induced PHOX2A up-regulation using a shRNA against PHOX2A.

However, our findings excluded that PHOX2A-mediated regulation of PHOX2B was involved in ATRA-induced PHOX2B down-regulation.

On the other hand, PHOX2A mRNA up-regulation, followed by its protein degradation, together with PHOX2B down-regulation suggested that they might be useful biomarkers for NB staging, prognosis and treatment decision-making.

In addition, as it is known that PHOX2B is down regulated in later stage of differentiation, we could speculate that the molecular mechanism for retinoic-induced cellular differentiation, in NB treatment, is due to a direct regulation of PHOX2 protein expression.

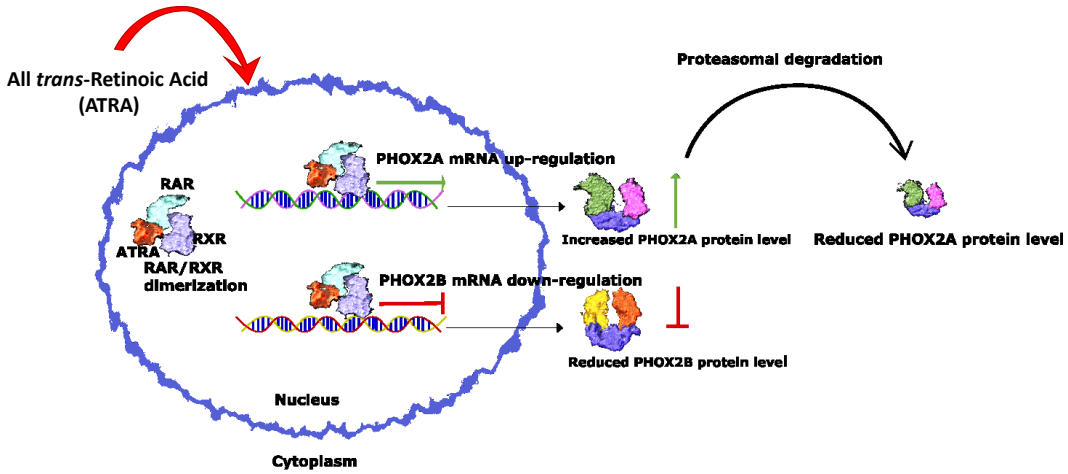

Figure n. 15 ATRA regulation of *PHOX2A* and *PHOX2B*

SK-N-BE(2)C neuroblastoma cell line was treated with all-*trans*-retinoic acid (ATRA). ATRA binds RXR-RAR receptor heterodimer that binds DNA by recognizing the specific retinoic acid responsive elements (RAREs) in the promoter of target genes. The binding of this active complex to *PHOX2A* promoter is able to up-regulate *PHOX2A*-mRNA enhancing gene transcription; extended ATRA treatment selectively degrades PHOX2A protein, by proteasomal degradation, while the corresponding mRNA remains up-regulated. ATRA treatment down-regulates *PHOX2B*-mRNA resulting in reduced PHOX2B protein level.

Chapter 4

3-ketodesogestrel effects in SK-N-BE(2)C neuroblastoma cell lines stably expressing human progesterone receptors (hPGR)

In preparation

4.1 Preliminary data

So far, a possible pharmacological treatment to ameliorate symptoms in CCHS aimed to counteract the toxic effects of the mutated PHOX2B protein; in particular, drugs promoting the refolding and/or the clearance of mutant protein aggregates have proved to be effective in rescuing the nuclear localization and transactivation activity of mutants *in vivo* (Trochet *et al*., 2005; Bachetti *et al*., 2007; Di Zanni *et al*., 2012).

The fortuitous observation of Straus and colleagues, that progestin drug Desogestrel can relief some symptoms in CCHS female patients (Straus *et al*., 2010), represents a great proof of concept to provide new therapeutic strategies in the treatment of the disease.

The molecular mechanism of the unexpected Desogestrel pharmacological effect is at the moment completely unknown, but this observation gave us a useful indication in the perspective of pharmacological interventions in CCHS, at least for ameliorating respiratory symptoms.

Our first working hypothesis concerned the possibility that the progestin could act on the expression of PHOX2B or some PHOX2B target genes.

Previous experiments conducted in our laboratory using breast cancer T47D cell line, clearly highlighted a correlation between the progestin treatment, PHOX2B expression, and the regulation of one of the known PHOX2B target gene, *TLX2*. As T47D cells have the advantage to express all the types of progesterone receptors (both membrane and intracellular receptors), but do not express PHOX2B protein, they represent a helpful and clean model in order to investigate the progestin mechanism (unpublished data).

Data collected hitherto showed that, in this cellular contest, the 3-keto-desogestrel (3-KDG) treatment enhanced PHOX2B transcriptional activity on *TLX2* promoter, already at low concentration (50 ng of plasmid DNA; Fig 16, Panel A).

Dose-response curve with increasing concentration of 3-keto-desogestrel (1 nM - 10 nM - 100 nM - 1 mM) for 48 hours, on T47D cells co-transfected with 50 ng of PHOX2B cDNA and the TLX2-luc promoter construct, showed that cells were fully responsive already at lower dose (1 nM) of 3-KDG (Fig. 16, Panel B).

These treatments were performed by treating cells for 48 hours, as we assume that Desogestrel is administered, as contraceptive drug, in a chronic manner; however, recent studies have highlighted the existence of non-genomic mechanisms of action of

progesterone. We therefore investigated whether the effect of the 3-KDG on TLX2 promoter activity, could already be measured at shorter time point (8-24 hours).

8-24 hours treatment did not induce significant changes in the transcriptional activity of the reporter construct compared to untreated cells, leading us to exclude a rapid activation of PHOX2B target genes and to hypothesize that the action of the progestin involves a long term mechanism (Fig. 16, Panel C).

Mutations in PHOX2B protein, particularly those due to the expansion of the 20 alanines stretch in the C-terminus region of the protein, are the principal cause for the onset of CCHS, causing a drastic reduction in PHOX2B protein transactivation activity correlating with the length of the expanded stretch (Di Lascio *et al*., 2013).

1nM 3KDG treatment induced a partial rescue of the activity of +7 or +13 PHOX2B expanded mutant proteins, resulting in increased transactivation of *TLX2* promoter (Fig16, Panel D, blue and green hatched bars), as they have lost their capability to transactivate the reporter gene (Fig. 16, Panel D, blue and green full bars). Moreover, we analysed the 3-KDG effects on another well known PHOX2B target gene, *Dopamine β-hydroxylase* gene (*DBH*). Unexpectedly, this promoter was not responsive to the 3-KDG treatment (Fig. 16, Panel E, black hatched vs full bar), and no recovery was observed in the presence of mutant proteins (Fig. 16, Panel E, blue and green hatched bars), as well.

These data suggested us that the progestin 3-KDG enhances the activity of some PHOX2B target genes in a promoter specific manner.

This promoter specificity was previously observed also on the effects of the mutant proteins on gene regulation (Di Lascio *et al*., 2013) suggesting that a class of target genes more sensitive to PHOX2B amount and/or functional alteration exists.

Given that T47D cell line express all kind of progesterone receptors, we used the selective antagonist for PgR nuclear receptors, Mifepristone (RU486) to verify which receptor mediate the effect of the progestin desogestrel.

The co-treatment with 1 μM of RU486 together with 1 nM 3-KDG of T47D cells transfected with the TLX2 promoter reporter construct, abolished the progestin activity on reporter construct (Fig. 16, Panel F, orange hatched bar).

These data suggested that the Desogestrel increases PHOX2B transcriptional ability through a mechanism mediated by the Progesterone intracellular receptors (PR-A/PR-B).

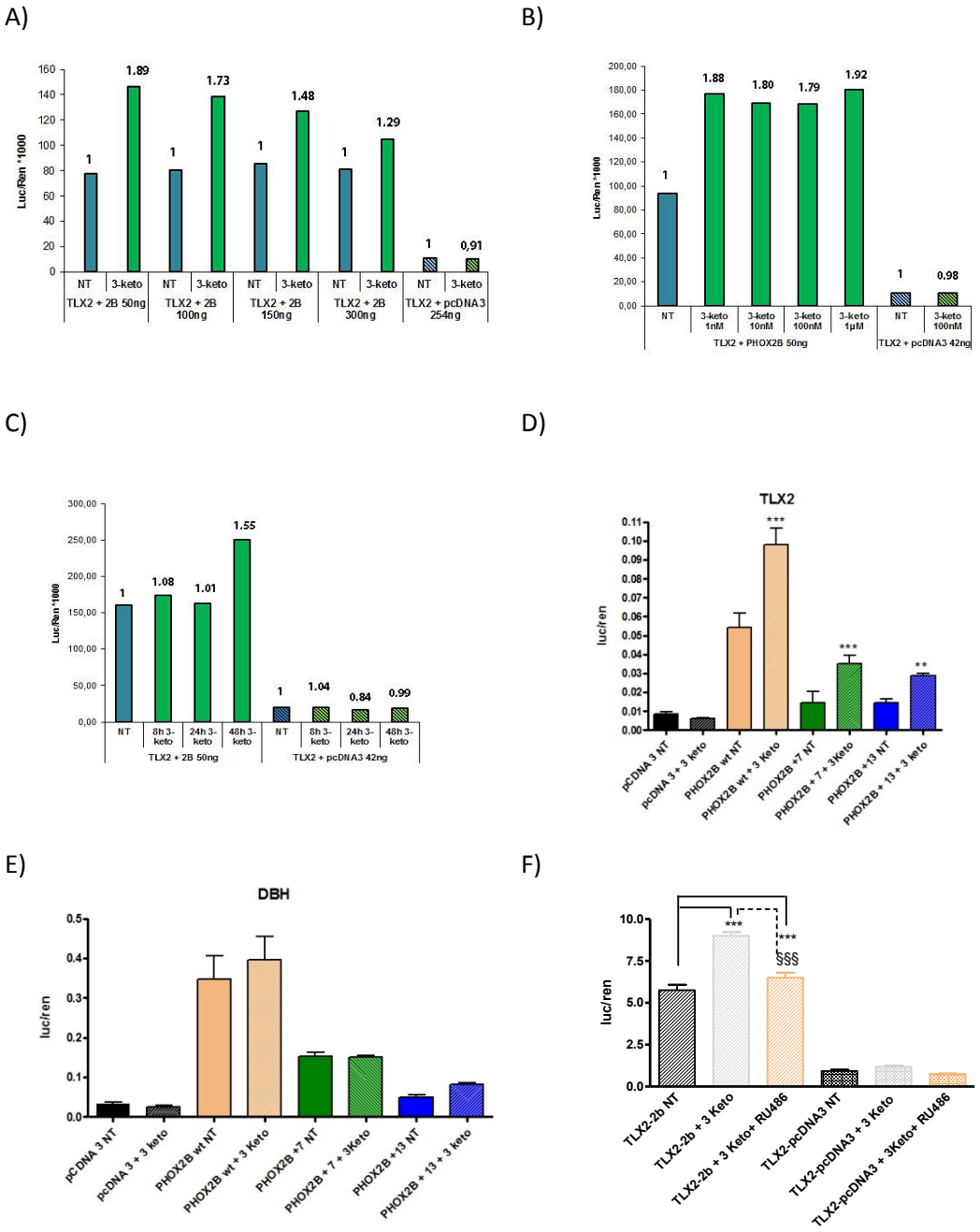

Figure 16 Panel A) Luciferase assay of the activity of the reporter construct TLX2-luc in T47D cells, in the presence of increasing amount of PHOX2B expression vector, treated or not (green bars vs blue bars), with 100nM 3-keto-desogestrel for 48h. The values are expressed as luc/ ren ratio. In black fold-induction compared to untreated control (NT) is indicated. **Panel B)** Luciferase assay of the activity of the reporter construct TLX2-luc in T47D cells, in the presence of 50ng of PHOX2B expression vector and increasing concentrations of the progestin 3-keto-desogestrel (1 nM, 10 nM, 100 nM; 1 µM, green bars). NT = not treated. The values are expressed as luc/ ren ratio (arbitrary units). In black fold-induction compared to their untreated control (blue bars; NT) is indicated. **Panel C)** Luciferase assay of the activity of the reporter

construct *TLX2*-luc in T47D cells, treated for increasing time with 1nM 3-keto-desogestrel (8h, 24h, 48h, green bars). T47D cells were co-transfected with 50 ng of *PHOX2B* (full bars) or the empty vector pcDNA3 myc-his (hatched bars) and the *TLX2*-luc reporter construct. Values are expressed as luc/ ren ratio (arbitrary units). In black fold-induction (green bars) compared to their untreated control (blue bars; NT = not treated) is indicated. **Panel D)** Luciferase assay. The bars indicate the transcriptional activity of *TLX2* constructs after co-transfection of the cDNA encoding the PHOX2B wild-type protein (orange bars) or mutant protein with the +7 alanine (green bars) or +13 alanine (blue bars) expansion, treated with 1 nM 3-keto-desogestrel, for 48h. The empty expression vector was used as negative control (black bars). Results are expressed as mean ± SD (error bars) of the transcriptional activity of the constructs tested in at least three independent experiments performed in triplicate *** p <0.001 (one-way ANOVA Tukey's test). **Panel E)** Luciferase assay. The bars indicate the transcriptional activity of the reporter constructs under the control of the *DBH* promoter after co-transfection of the cDNA encoding the PHOX2B wild-type protein (orange bars) or PHOX2B mutant protein bearing +7 (green bars) or +13 (blue bars) alanine expansion, treated with 1 nM 3-keto-desogestrel (hatched bars) for 48 hours. The empty expression vector was used as negative control (black bars). Results are expressed as mean ± SD (error bars) of the transcriptional activity of the constructs tested in at least three independent experiments performed in triplicate *** p <0.001 (one-way ANOVA, Tukey's test). **Panel F)** Effect of RU486 on the activity of the luciferase reporter construct TLX2-luc. Levels of the transcriptional activity of the reporter construct in untreated cells (black hatched line), in cells treated with 1 nM 3-KDG (hatched grey bar) and co-treated with 3-KDG (1 nM) and RU486 (1 mM) (hatched orange bar) are compared. The activity was measured 48 hours after treatment. The empty expression vector was used as negative control (cross-hatched bars). Results are expressed as mean ± SD (error bars) of the transcriptional activity of the construct tested in at least three independent experiments performed in triplicate. Significance was measured using ANOVA (§§§ and *** p <0.001).

4.2 Material and methods

Cell cultures

All mammalian cell lines (SK-N-BE(2)C, SK-N-BE, IMR32, CAD and T47D) are grown in RPMI 1640 medium (LONZA) containing 10% fetal bovine serum (FBS, LONZA), with penicillin (100U/ml, Lonza), streptomycin (0.1 mg/ml, Lonza),and L-glutamine (2 mM, Lonza).

The T47D breast cancer cell line is maintained in medium added with Insulin (human insulin, 100 mg/ml in 25mM Hepes, pH 8.2, Sigma) to a final concentration of 5µg/ml.

SK-N-BE(2)C 5.8 and 6.30 stable clones are maintained under selection by adding Geneticin (G-418 Sulfate, Gibco) at 0.4 mg/mL final concentration, with selective medium changing every second day.

All cell lines grow in adhesion in 75 cm^2 and 175 cm^2 culture flasks (Primo, Euroclone) incubated at 37°C with 5% CO_2.

Transient Transfections and Luciferase Assays

Cells are transiently transfected by lipofection method using FuGENE HD Transfection Reagent (Promega), as described in Flora *et al* (2001).

Briefly, the day before transfection, cells are plated on 6-well plate at different density depending on the cell line used (1.7 x 10^5 SK-N-BE(2)C; 1.6 x 10^5 SK-N-BE; 2.0 x 10^5 IMR32; 3.0 x 10^5CAD; 2.0 x 10^5 T47D) in 2 ml RPMI 1640 supplemented with fetal bovine serum and L-Glutamine, without antibiotics.

For the co-transfection experiments in T47D cells, different DNA mixtures are prepared.

Each mixture contains 72 femtomoles of the RL-TK reporter construct, used to normalize the data obtained according to differences in transfection efficiency, mixed with equimolar amounts of TLX2 promoter reporter constructs and 11.6 femtomoles of PHOX2B wild type expression vector (PHOX2B/pcDNA3.1 myc-his) or equimolar amount of pcDNA3.1 myc-his empty vector, used as empty control vector.

The mixture of plasmid DNA is incubated with Fugene HD in a 3: 1 ratio (3 µl FuGene HD, 1 µg DNA), in plain DMEM medium. After incubation for 15 minutes at room temperature the mixtures are added dropwise to the cells (100 µL / well).

For the co-transfection experiments in neuroblastoma cell line, 74 femtomoles phRGB reporter construct (or RL-TK in IMR32), used to normalize data, are mixed with equimolar

amounts of TLX2 promoter reporter constructs or pGL3b empty control vector along with 54 femtomoles of the hPGR-pcDNA 3.1 myc-His. Cells are transfected with Fugene HD in 6: 1 (SK-N-BE(2)C, SK-N-BE, CAD) or 4:1 ratio (IMR32 cells).

In all co-transfection experiments, cells are treated, 24 hours after transfection, with 1nM of the active metabolite of progestin Desogestrel, 3-keto-desogestrel (3-KDG also indicated as Etonogestrel, 3-oxodesogestrel, Org-3236; Implanon), alone or in combination with 1 μM of the nuclear progesterone receptors antagonist, RU486 (Mifepristone, Sigma), as indicated in the figure legends. Luciferase activity or western blot analysis was determined after 48 hours of treatment

The luciferase assay is carried out using the Dual-Luciferase reporter assay system (Promega) as described previously (Flora *et al* 2001, Battaglioli *et al* 1998). All of the transfections are performed in triplicate, and each construct is tested in at least three independent experiments using different batches of plasmid preparation. The number of independent transfection experiments is indicated in the figure legends.

Plasmids used:

Expression plasmids:

- hPGR-pcDNA 3.1 myc-His: expression vector containing the human intracellular progesterone receptor coding sequence in pcDNA 3.1 myc-His plasmid (Invitrogen) bearing neomycin resistance used for the generation of stable clones (described in Erika Di Biase master degree thesis).

- PHOX2B-pcDNA 3.1 Myc-His: expression vector containing the coding sequence of the human transcription factor PHOX2B in pcDNA 3.1 Myc-His (Di Lascio *et al*., 2013).

- pcDNA 3.1 Myc-His: (Invitrogen) expression vector in eukaryotic cells containing the promoter of the cytomegalovirus (CMV), Myc epitope and has a poly-histidine tag.

Reporter constructs:

- TLX2-luc: reporter vector containing the promoter sequence (-1885 bp) of TLX2 gene, cloned upstream of the Firefly Luciferase gene in pGL3b (Borghini *et al*., 2006).

- TLX2 mut HBS -luc: reporter vector containing the promoter sequence of the TLX2 gene (-1885 bp / -36 bp) with two mutated ATTA sites, cloned upstream of Firefly Luciferase gene in pGL3b (Borghini *et al*., 2006).

- TLX2 3X mut-luc: reporter vector containing the promoter sequence of the TLX2 gene (-1885 / -36) with the three ATTA consensus sequences mutagenized, cloned upstream of the Firefly Luciferase gene in pGL3b.

- TLX2 -1455 bp: reporter vector containing the promoter sequence of the TLX2 gene(-1455 pb / -36 bp) cloned upstream of the Firefly Luciferase gene in pGL3b.

- pRL-TK: (Promega) vector containing the minimum promoter of thymidine kinase of the herpes simplex virus (HSV-TK) upstream of the reporter gene encoding the Renilla luciferase. Used to normalize transfection efficiency.

- phRG-B:(Promega) promoter-less mammalian vector encoding Renilla luciferase. Used to normalize transfection efficiency.

Total protein extraction

Proteins were extracted from subconfluent cells grown in 10 cm Petri dishes. The cells are washed with 1 ml of 1X PBS, mechanically harvested and collected by centrifugation for 5 minutes at 5000 rpm at 4°C in an Eppendorf centrifuge. The pellet is then resuspended in an appropriate volume of 1X PBS containing 0.2 mM PMSF, 0.5 mM DTT phosphatase inhibitors (Cocktail I, Sigma) and protease inhibitors (Cocktail II, Sigma). The homogenate is subjected to three freeze / thaw cycles in liquid nitrogen and 37°C, to obtain complete cell lysis. Then an appropriate volume of NaCl to a final concentration of 420 mM, is added to extract the nuclear proteins. After 30 minutes incubation at 4°C on a slowly turning rotating wheel, the extract is clarified by centrifugation at 13000 rpm for 30 minutes at 4°C. The supernatant is aliquoted, quickly frozen in liquid nitrogen and stored at -80°C; the concentration is evaluated using the Bradford method (Bradford, 1976).

SDS-PAGE and Western blot

20 µg protein extract from T47D transfected cells, native SK-N-BE(2)C and over-expressing PGR stable clones 5.8 and 6.30 treated with 3-KDG at different time point have been analyzed using western blot. 10 µg proteins from T47D and 7.5 µg nuclear extract from IMR32 are used as positive control. Protein extract are separated on a 10% denaturing polyacrylamide gel in the presence of 0.1% SDS extract (sodium dodecyl sulphate). Before loading, each sample is added with 3X Laemmli loading solution (final concentration 1X) and denatured at 100°C for 5 minutes.

After gel separation at 25 mA in Tris-glycine (25 mM Trizma base, 192 mM Glycine, 0.1% SDS) for about 2 hours, the proteins are transferred to nitrocellulose membrane (Schleicher and Schuell) by electroblotting at 30 V over-night in transfer buffer (25 mM Trizma base, 192 mM Glycine, 20% MetOH). The membrane is then stained with Ponceau S to control the transfer, washed with distilled H_2O and saturated with blocking buffer (5% non-fat milk, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). After one hour blocking, primary antibodies diluted in blocking solution are added and incubated for 2 hours, or over-night, depending on the manufacture's instructions. At the end of incubation excess of primary antibodies is removed with three 5 minutes washes with blocking buffer, and the membrane incubated in the presence of the appropriate secondary antibody conjugated to horseradish peroxidase (HRP), diluted in blocking buffer, for 1 hour. The excess antibody is then removed by two washes with blocking buffer for 5 minutes, followed by three washes in 1X TS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) + 0,1% Tween-20, three washes in 1X TS + 0.3 % Tween-20 and three final washes in 1X TS.

The membrane is then incubated for 5 minutes with the peroxidase substrate, Super Signal West Dura (Pierce) and the signal detected by Hyperfilm ECL films (Amersham) at different time exposures.

The antibodies used in Western blot experiments are the following:

Primary Antibody	Diluition
Progesterone receptor A/B (D8Q2J) XP Rabbit mAb (Cell Signalling Technology)	1:1000 O.N
Phox2b (B-11):sc-376997 mouse mAb (Santa Cruz Biotechnology)	1:10000
Phox2a chicken Ab (Davids Biotechnologie)	1:1000
Dopamine-β-Hydroxylase (N-terminal) sheep mAb (Sigma)	1:500
CREB (48H2) Rabbit mAb (Cell Signalling Technology)	1:1000 O.N.
Sp1 (PEP2):sc-59 rabbit Ab (Santa Cruz Biotechnology)	1:10000
c-Jun (N):sc-45 rabbit Ab (Santa Cruz Biotechnology, INC.)	1:500
Ap-2α (C-18):sc-184 rabbit mAb (Santa Cruz Biotechnology, INC.)	1:500
GATA3 (D13C9) XP rabbit mAb (Cell Signalling Technology)	1:1000 O.N.
β-Tubulin (D3U1W) mouse mAb (Cell Signalling Technology)	1:1000 O.N.

Secondary Antibody	Diluition
Stabilized Goat anti-rabbit HRP-coniugated (PIERCE)	1:10000
Stabilized Goat anti-mouse HRP-coniugated (PIERCE)	1:10000
Rabbit Ab against chicken IgG-HRP (Davids Biotechnologie)	1:10000
Donkey Ab against sheep IgG-HRP (Abcam)	1:5000

RNA extraction

Total RNA, from cultured cells, was extracted by means of the kit "RNeasy Mini" (Qiagen,), according to the instructions provided by the company.

Briefly, about 10^7 cells, grown in 10 cm Petri dishes, are washed twice with 10 ml of 1X PBS (0.14 M NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), detached mechanically and collected by centrifugation for 5 minutes 5000 rpm at 4 °C in an Eppendorf microcentrifuge.

The pellet is homogenized in 350 µL of RTL buffer previously added with β-mercaptoethanol (10 µl / ml RTL) and the viscosity reduced by passage through the column "Qiashredder" (Qiagen). After the addition of a volume of 70% ethanol, the eluate containing precipitated RNA is loaded onto a column and centrifuged for 30 seconds at 8000 rpm. The matrix of the column, to which RNA is bound, is treated with DNase I (2.73 U / uL) for 15 minutes at room temperature. After subsequent washing, the RNA is eluted with 60 µL of double-distilled RNAse-free water.

RNA concentration is determined by a spectrophotometer reading at 260 nm (1 OD260 = 40 µg / ml) and its purity assessed by the ratio of the readings at 260 nm and 280 nm (≥ 2). To analyze transcript stability, cells were treated with 75µM DRB (Sigma), and RNA was extracted using "RNA plus micro kit" (Qiagen, Hilden, Germany) at time points indicated in figure legend.

Retrotranscription

1 µg of total RNA was subjected to reverse transcription using the kit GoScript™ Reverse Transcriptase (Promega), according to information provided by the company.

Briefly, total RNA is incubated at 70 ° C for 5 minutes in the following reaction mixture:

RNA	1 µg	
Random primers (Hexamers)	50 µM	
Nuclease-free water		to a volume of 5 µl

After 5 minute on ice, each sample are added with 15 µl of a mixture composed by:

GoScript™ 5X Reaction Buffer	4 µl
$MgCl_2$ (final concentration4mM)	3.2 µl
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)	1 µl
Recombinant RNasin®Ribonuclease Inhibitor	20units
RNase OUT Recombinant RNase Inhibitor (40 U/µl)	1 µl
GoScript™ Reverse Transcriptase	1 µl
Nuclease-Free Water	to a final volume of 15 µl

The reaction is continued at 25 ° C for 5 minutes, 42 ° C for 1 hour, 70 ° C for 15 minutes. The cDNA of interest are then amplified and analyzed quantitatively by Real-Time PCR.

Quantitative Real-Time PCR

Gene expression analysis was performed by quantitative Real-Time PCR assay using the ABI Prism Thermocycler (QuantStudio 5, Applied Biosystems, CA). The target sequences were amplified from 50 ng of cDNA in the presence of TaqMan® Gene expression master mix (Life Technologies, Inc.).

The TaqMan® primer and probe assays (Life Techologies, Inc.) used were human PHOX2B (ID #Hs00243679_m1), human PHOX2A (ID #Hs00605931_m1) human PGR (ID #Hs01556702_m1), human DBH (ID #Hs00168025_m1), human TLX2 (ID #Hs00740145_g1) and the endogenous control glyceraldehyde-3 phosphate dehydrogenase GADPH (ID #Hs99999905_m1). Each sample was run in triplicate, and the results were calculated using the $2^{-\Delta CT}$ and the $2^{-\Delta\Delta CT}$ method, thus allowing the normalization of each sample to the endogenous control, and comparison with the calibrator for each experiment (set to a value of 1).

4.3 Results

4.3.1 PHOX2B binding to "ATTA" consensus sequences on *TLX2* promoter is necessary for the 3-KDG effects

Preliminary data, obtained in our laboratory, have shown that the progestin Desogestrel induces an increase in PHOX2B transcriptional activity on the T-Cell Leukemia homeobox gene 2 (*TLX2*), a key regulator of neural crest cells differentiation in autonomic neurons.

The regulation of the *TLX2* promoter by PHOX2B transcription factor has been previously well studied and characterized in the SK-N-BE(2)C neuroblastoma cell line (Borghini *et al*., 2006).

The regulatory region of *TLX2* gene contains two highly conserved regions, between humans and mice, localized in human in position -1517 / -1187 and -801 / -638 (Fig. 17, gray boxes).

The -1711/ -1396 region is important for the transcriptional tissue specific regulation of the *TLX2* gene. The deletion of this region results in a strong decrease in the activity of the promoter in some neuronal cell lines, particularly in human neuroblastoma cell lines (Borghini *et al*., 2006).

In this region, different binding sites for transcription factors including three "TAAT / ATTA" sites repeated in tandem, also known as HBSs (Homeodomain Binding Sequences), have been identified (Fig.17, black mark).

PHOX2B transactivates *TLX2* promoter, by binding to two "TAAT/ ATTA" sequences located in the -1489 / -1480 region. Indeed, the site-specific mutation of these two sequences results in a dramatic reduction in *TLX2* promoter transactivation by PHOX2B (Borghini *et al*., 2006).

Figure 17 Regulatory region of the human *TLX2* gene. Grey boxes indicate the highly conserved region between humans and mice. In black the "TAAT/ATTA" homeodomain binding sites are marked. With *del* the deletion start points for the generation of the *TLX2* promoter deletion constructs, tested in IMR32 and SK-N-BE neuroblastoma cell lines, are indicated (Borghini *et al*., 2006).

Given the importance of the two sequences in *TLX2* promoter, we wondered if the PHOX2B binding to "TAAT/ ATTA" sites was necessary to mediate the effect of Desogestrel.

To this aim we tested the effect of the 3-KDG on the *TLX2* promoter construct in which the two “TAAT/ATTA” in position -1489/-1480 were mutagenized (indicated as *TLX2* mut HBS), on a construct in which all the three HBSs were mutagenized (*TLX2* 3Xmut) and on a deletion construct of the promoter characterized by the absence of all the HBS sequences (*TLX2* -1455) (Fig. 18, Panel A).

The *TLX2* mut HBS construct appears to be still responsive to PHOX2B, even if at a lesser extent compared to wild-type promoter. The mutation and the deletion of all the homeodomain binding sites result in constructs not responsive to PHOX2B transactivation (Fig. 18 Panel B, compare black bars).

Treatment with 3-KDG induces a 1.7-fold statistically significant increase, compared to the respective untreated construct, in the activity of both luciferase reporter constructs containing the *TLX2* wild-type promoter or the promoter with the two mutagenized sequences "TAAT / ATTA", in the presence of PHOX2B (Fig.18, Panel B, black vs striped bars).

The -1455 deletion, which removes all three "TAAT / ATTA" sites, as well as the mutation of the three HBS sites, destroy the responsiveness of the constructs to the 3-KDG both in the absence and in the presence of PHOX2B (Fig.18, Panel B, TLX2 3X mut and TLX2 -1455).

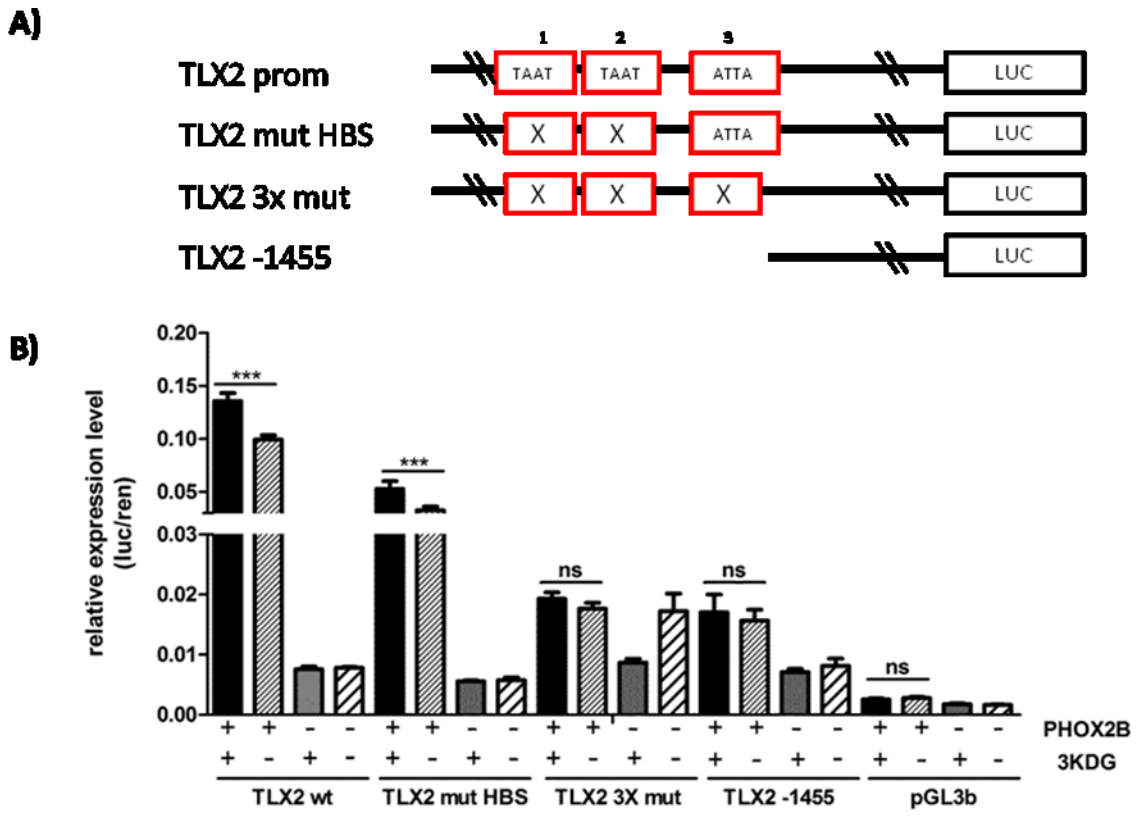

Figure 18 Luciferase assay activity of *TLX2* reporter constructs after 48h treatment with 3-KDG. The bars indicate the transcriptional activity of the reporter constructs under *TLX2* promoter control after co-transfection of the cDNA encoding the PHOX2B wild-type protein (black and hatched bars) or empty expression vector (grey and black and white stripes bars) treated with 1 nM 3-KDG (black and grey bars) for 48 hours. The data of each construct are expressed as fold-induction compared to untreated sample transfected with PHOX2B (black bars). The results are expressed as mean ± SD (error bars) of the transcriptional activity of the constructs tested in at least three independent experiments performed in triplicate *** p <0.001 (one-way ANOVA, Tukey's test)

These data suggest that the PHOX2B binding to at least one of the "ATTA" sequence in the region -1490 / -1458 is sufficient and necessary to mediate PHOX2B dependent effect of 3-KDG.

4.3.2 3-KDG treatment increases the PHOX2B protein amount in T47D cell line

Figure 16 shows that, in T47D cells, 3-KDG treatment is able to increase PHOX2B transcriptional activity on *TLX2* target gene, and that this effect is mediated by nuclear progesterone receptors, since the co-treatment with specific inhibitors of nuclear receptors (RU486) completely abolished the drug effect on *TLX2* promoter.

As T47D cells do not express PHOX2B, and we cannot study a possible direct effect on PHOX2B gene expression, we asked whether 3-KDG had a direct effect on protein level. We then transfected T47D cell line with *PHOX2B*-Myc tagged expression vector and treated cells with 1 nM 3-KDG alone (Fig. 19, lane 4) or in combination with 1 µM RU486 (Fig. 19, lane 6), for 48 hours.

The 3-KDG treatment alone results in a visible increment of PHOX2B protein amount with respect to untreated sample (Fig. 19, compare lane 4 vs lane 3), whereas in sample co-treated with both 3-KDG and RU486 no changes in PHOX2B protein level were observed (Fig 19 compare lane 6 vs lane 5).

These data suggest that the enhancement in *TLX2* promoter activity, obtained by the progestin treatment, could be mediated by increasing level of PHOX2B protein, maybe due to post-translational modifications that stabilize the protein, or inhibition of protein degradation mechanisms.

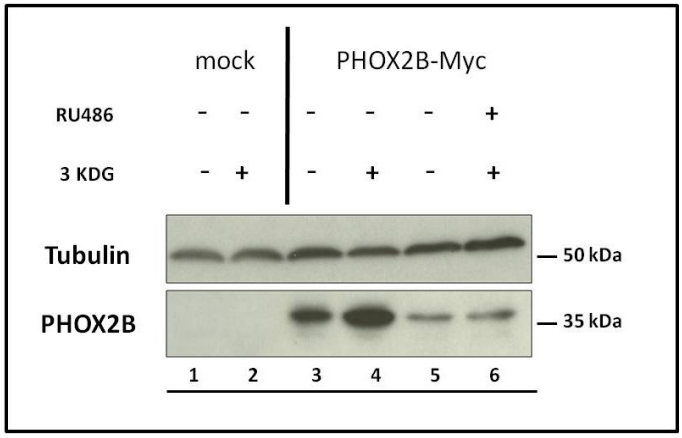

Figure 19. Western blot analysis of PHOX2B protein after 48 hours treatment with 3-KDG alone, or in combination with RU486, in T47D cell line. Representative immunoblotting image showed the expression of PHOX2B Myc-tagged protein (lanes 3-4-5-6) in T47D samples after 48 hours treatment with 1 nM 3-KDG alone (lanes 2-4) or in combination with 1 µM RU486 (lane 6). T47D not transfected samples are used as control (lanes 1-2). The whole cell lysate was immunoblotted with Myc-antibody. The same blot was reprobed with β-tubulin to compare the loading of each lane.

4.3.3 Analyses of the endogenous nuclear progesterone receptor level in neuroblastoma cell lines

Data collected so far, related to the 3-KDG effects on PHOX2B expression and activity, were obtained using T47D as cellular model.

The presence of all kind of progesterone receptors make T47D cell line a useful model of study in our project. However, the identification of the nuclear receptors, as mediators of the 3-KDG effects, gave us the opportunity of moving to a more physiologic cellular context (i.e. neuronal cell lines).

Being the CCHS a neurodevelopmental disorder, we started to investigate the 3-KDG effects in a neuroblastoma cellular model.

Using quantitative Real-time PCR analysis, we measured the PGR amount in different neuronal cellular models, comparing PGR levels detected with those of T47D, and MCF-7 (Fig. 20, yellow and green bars, respectively), a breast cancer cell line also expressing high levels of PGR.

The graph in figure 20 shows the PGR expression level measured in the different cell lines relative to SK-N-BE(2)C, in particular:

- SH-SY5Y, a human derived cell line subcloned from SK-N-SH cell line, isolated from a bone marrow biopsy taken from a four-year-old female with neuroblastoma, turned out to be devoid of PGR;

- SK-N-BE(2)C (used to compare the PGR level of the other neuroblastoma cell lines analyzed, set =1, grey bar), a clonal subline of the SK-N-BE(2) human neuroblastoma cell line, shows a very low PGR expression level than T47D cell line (Fig. 20,compare grey bar *vs* green bar, 200.000 fold less);

- IMR32, a human derived cell line established by an abdominal mass of a 13-month-old male patient with neuroblastoma, shows higher PGR amount compared to SK-N-BE(2)C about 60 fold higher compared to SK-N-BE(2)C (Fig. 20, compare blue *vs* yellow bar).

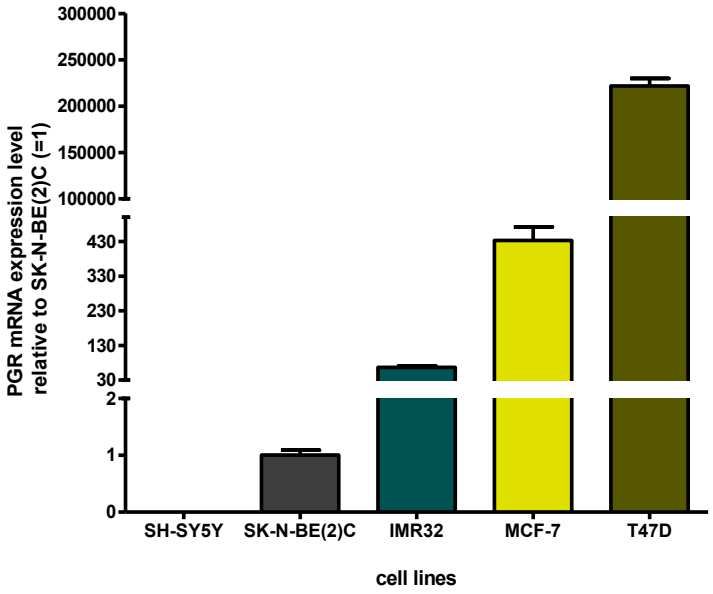

Figure 20 Real-time quantitative PCR of intracellular Progesterone Receptor (PGR) mRNA expression in SH-SY5Y, SK-N-BE(2)C, IMR32, MCF-7 and T47D cell lines.

1 µg of total RNA was reverse transcribed and analysed by Real Time PCR, using a specific TaqMan assay for the human transcript PGR. The level of messenger RNA is expressed as relative to the cell line SK-N-BE (2) C, (set to a value of 1). The $2^{-\Delta\Delta CT}$ method was used to calculate the results.

4.3.4 3-KDG treatment alters the *TLX2* promoter activity in neuroblastoma cell lines

Given that PHOX2B is a neuro-specific transcription factor and its ability to activate *TLX2* gene transcription has already been studied in the human SK-N-BE neuroblastoma cell line (Borghini *et al* 2006), we assessed whether the 3-KDG effect is also expressed in neuroblastoma cell lines, by measuring the *TLX2* promoter activity using luciferase assay.

As qPCR analysis conducted in neuroblastoma cell lines revealed low level of nuclear progesterone receptors in these cell lines with respect to the breast cancer cells (T47D and MCF7) used as positive control, we decided to transfect different neuroblastoma cell lines with an expression vector containing the coding sequence of human *PGR* gene, allowing cells to be responsive to 3-KDG treatment.

Transfection of IMR32 and SK-N-BE(2)C with *TLX2* promoter along with human *PGR* gene expression vector, results in a strong basal activity of the promoter, higher in IMR32 (950-fold respect to pGL3b; Fig. 21, Panel B compare black vs white bar) compared to SK-N-BE(2)C (73-fold respect to pGL3b; Fig. 21, Panel A compare black vs white bar) probably due to the presence of endogenous PHOX2B / PHOX2A transcription factors. In these cell lines, 1nM 3-KDG treatment for 48 hours induced a strong reduction in *TLX2*-promoter transactivation, with a more drastic effect in IMR32 with respect to SK-N-BE(2)C cells (Fig 21, compare black striped bars, panel B vs panel A).

The same result was obtained with the CAD cell line (Cath.a-differentiated), a CNS cathecolaminergic cell line derived from Cath.a cell line originated from *locus coeruleus* brain tumours of transgenic mice expressing the simian virus 40 T antigen under the control of the *TH* promoter (Suri *et al*., 1993). TH and DBH expression, along with catecholamines synthesis, characterize this cell line, and it is known that CAD cell differentiation depends on *PHOX2A* expression and activation mediated by cAMP pathway (Chen *et al*., 2005). In this cellular model the *TLX2*-luc promoter activity was drastically reduced by 3-KDG treatment (331-fold respect to pGL3b; Fig. 21, Panel C compare black vs white bar).

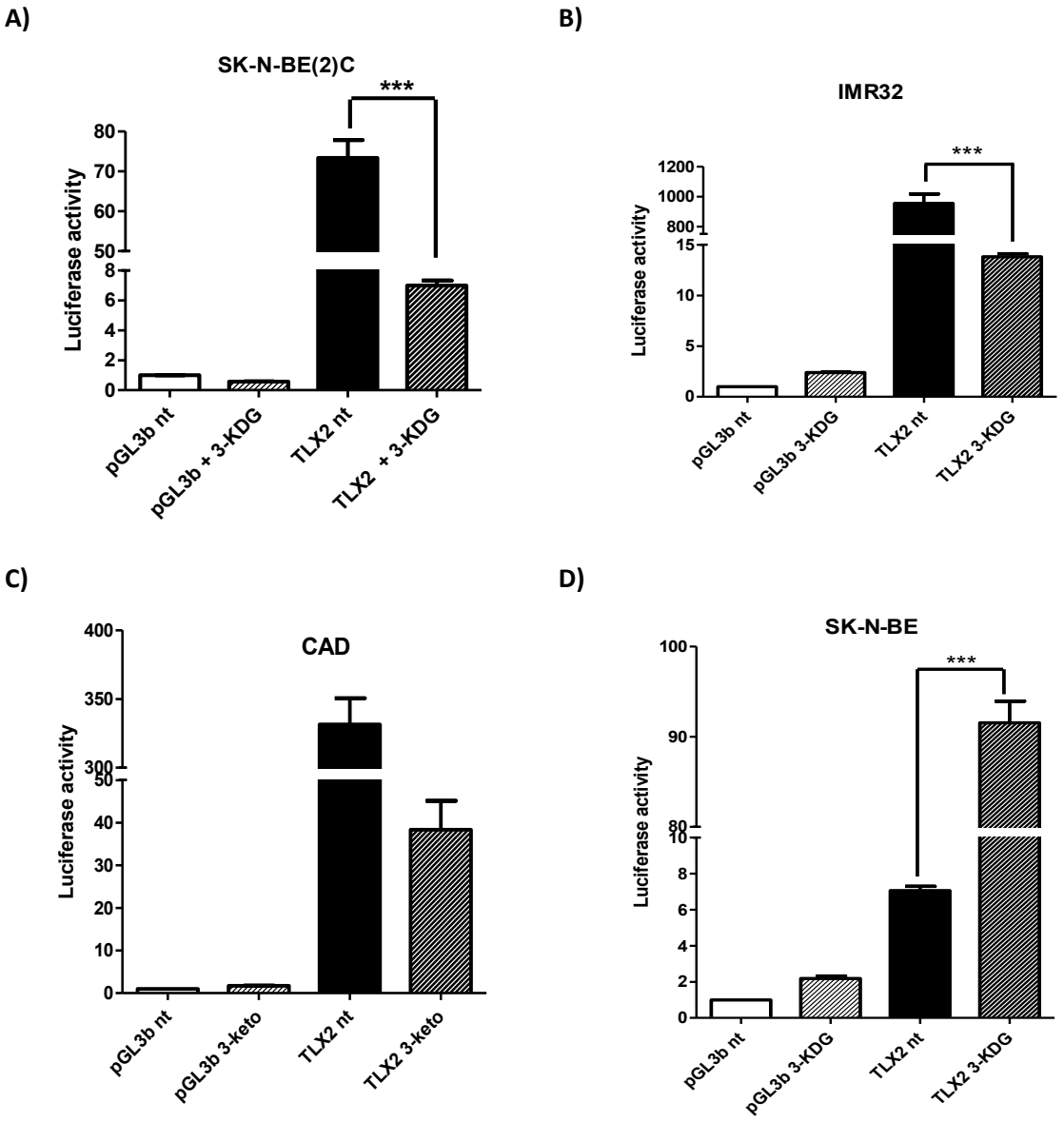

Figure 21 Luciferase assay activity of *TLX2* reporter constructs in neuroblastoma cell lines after 48 hours treatment with 1nM 3-KDG. The bars indicate the transcriptional activity of the reporter constructs under *TLX2* promoter control after co-transfection of the *TLX2*-luc promoter reporter construct (black bars) or pGL3b empty vector (white bars) treated with 1 nM 3-KDG (white and black hatched bars) for 48 hours. The data are expressed as fold-induction compared to untreated sample transfected with *TLX2*-luc construct (black bars). The results obtained in SK-N-BE(2)C, IMR32 and SK-N-BE cells are expressed as mean ± SD (error bars) of the transcriptional activity of the constructs tested in at least three independent experiments performed in triplicate (n=3), *** $p < 0.001$ (one-way ANOVA, Tukey's test). The graph obtained in CAD cells is to consider as representative (n=1).

On the contrary, in the SK-N-BE neuroblastoma cell line, that lacks *PHOX2B* expression (Borghini *et al* 2006) whereas it expresses *PHOX2A*, *TLX2* -luc basal activity appears to be lower than other neuroblastoma cell lines (7 -fold respect to pGL3b; Fig 21, Panel D compare black vs white bar). This may be due to the lack of *PHOX2B* expression in this cell line. Unexpectedly the 3-KDG treatment induces an increase in *TLX2* promoter activity (13-fold, Fig. 21, Panel D compare black striped vs black bar), similarly to the situation observed in T47D cell line (see preliminary data). The opposite results observed in the *TLX2*-luc promoter activity, between SK-N-BE(2)C, T47D and the other neuronal cell lines tested, could be due to the recruitment of different co-factors (i.e. co--activators and/or co-repressors), mediated by 3-KDG, in the different cellular contexts.

4.3.5 3-KDG treatment does not alter *PHOX2B* / *PHOX2A* mRNA levels in SK-N-BE(2)C cell line transiently overexpressing human PGR.

Results obtained measuring the luciferase activity of *TLX2* reporter constructs in neuroblastoma cell lines after 48 hours treatment with 1nM 3-KDG indicated that, interestingly, the progestin treatment in these cell lines induces the opposite effect on TLX2 activity with respect to T47D breast cancer cell line, with the exception of SK-N-BE cell line.

Given the predominant decrement in *TLX2* activity after 3-KDG treatment in neuronal context, we wondered if a possible mechanism of action of desogestrel, in reducing *TLX2* expression, could be explained by a direct effect of the progestin on PHOX2B.

To answer this question, we decided to treat SK-N-BE(2)C cells with 3-KDG at increasing time point in order to analyse its possible effect on *PHOX2B* transcript level by means of qPCR.

Figure 22 shows the quantification of *PGR* mRNA expression level in four independent experiments of PGR construct transfection in SK-N-BE(2)C cell line, which results in a highly variable transfection efficiency of progesterone receptor in different experiments.

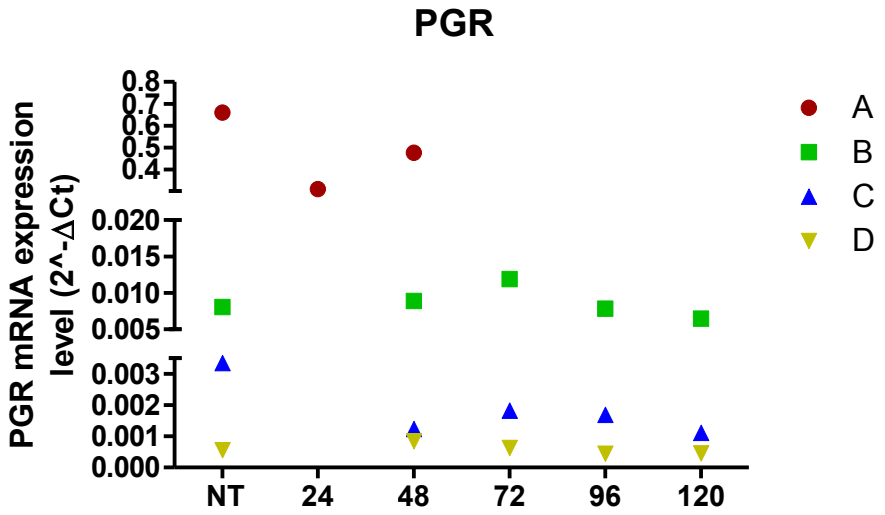

Figure 22 mRNA expression level of transfected PGR in SK-N-BE(2)C cell line under 3-KDG treatment at different time point.

Symbols indicate the mRNA levels of transfected PGR in SK-N-BE(2)C in four different transfected samples (A, B, C and D) treated with 1nM 3-KDG for different time (24h, 48h, 72h, 96h, 120h). The level of messenger RNA is expressed as relative expression normalized to that o GAPDH endogenous gene and expressed according to the 2−ΔCT method.

The analysis of PHOX2B mRNA (Fig. 23) extract from cells treated at different time (24h, 48h, 72h, 96h and 120h), in the four independent experiments, did not reveal significant alteration with respect to untreated sample (Fig. 23, compare hatched bars vs black bar), even in the experiment with the higher level of PGR expression (red dots in Fig, 22).

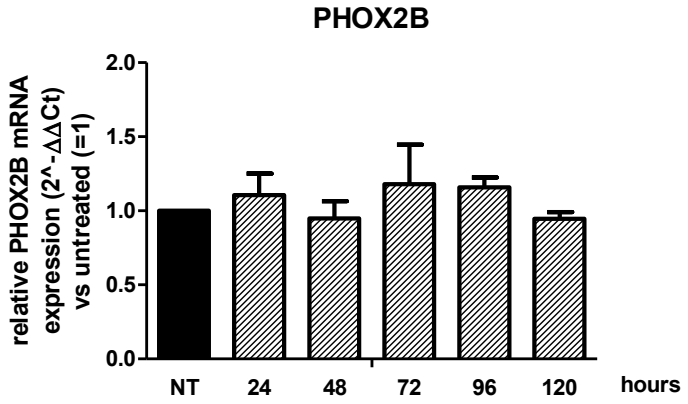

Figure 23 qPCR analysis of *PHOX2B* expression level in SK-N-BE(2)C cell line transfected with PGR expression vector treated with 3-KDG at different time point.

Total RNA was extracted from cells treated for different times with 1 nM 3-KDG (24h, 48h, 72h, 96h, 120h; hatched bars) and the level of PHOX2B determined by qPCR.. The bars are the mean values ± SD (error bars) of the PHOX2B mRNA expression level detected in four independent experiments performed in triplicate, and are expressed as fold relative to the untreated cells (NT=1, black bar).

Given that *TLX2* gene is also a transcriptional target of PHOX2A (Borghini *et al* 2007), along with the fact that we observed a 3-KDG effect also in neuroblastoma cell lines, which express PHOX2A but not PHOX2B (SK-N-BE cell line), we considered the hypothesis that 3-KDG effect could be mediated by PHOX2A.

To test this hypothesis, we analysed also *PHOX2A* mRNA in the same cell samples used for *PHOX2B* mRNA analysis.

However, the analysis of the *PHOX2A* mRNA revealed a not statistically significant increase in samples treated at different time point (Fig. 24; compare hatched bars vs black bar), suggesting that the action of the progestin is not directed on regulating *PHOX2B/2A* gene expression.

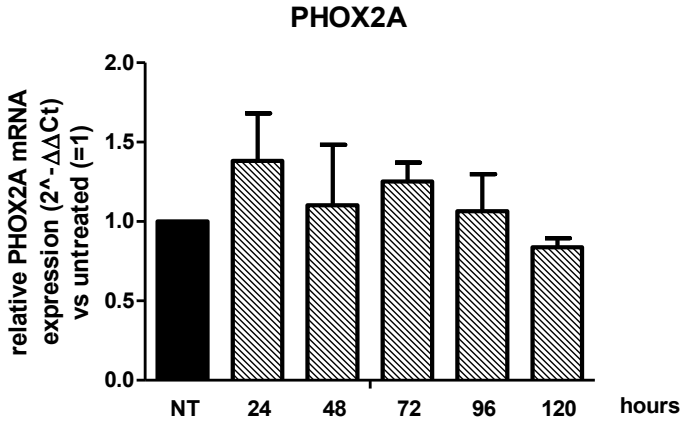

Figure 24 qPCR analysis of *PHOX2A* expression level in SK-N-BE(2)C cell line transfected with PGR expression vector treated with 3-KDG at different time point.

Total RNA was extracted from cells treated for different times with 1 nM 3-KDG (24h, 48h, 72h, 96h, 120h; hatched bars) and the level of *PHOX2A* determined by qPCR. The bars are the mean values ± SD (error bars) of the *PHOX2A* mRNA expression level detected in four independent experiments performed in triplicate, and are expressed as fold relative to the untreated cells (NT=1, black bar).

However, we cannot rule out whether the considerable difference in the levels of PGR in the different samples could result in an underestimation of the 3-KDG effect in this cell line.

4.3.6 3-KDG treatment induces a decrement in PHOX2B protein amount in SK-N-BE(2)C cell line transiently overexpressing human PGR.

Data obtained in T47D cell line have shown that 3-KDG treatment induces an augment in PHOX2B ability to transactivate one of its target gene, *TLX2*, probably due to an increase in transfected PHOX2B protein level.

Given the known PHOX2B-mediated regulation of *TLX2* promoter in neuroblastoma cell lines, we asked whether the consistent alteration in *TLX2* promoter luciferase activity, observed upon 3-KDG treatment in SK-N-BE(2)C cell lines, is related to a change in endogenous PHOX2B protein amount. To test this hypothesis we evaluated endogenous

PHOX2B protein level in SK-N-BE(2)C cells transfected with human PGR cDNA expression vector (hPGR in pCDNA3.1 Myc-Hys), treated with 1nM 3-KDG for 48 hours.

Western blot analysis (Fig. 25, Panel A) of total extract from SK-N-BE(2)C cell line transfected with PGR expression vector (Fig. 25, Panel A, lanes 1 and 2), and treated with 3-KDG (Fig. 25, panel A, lanes 2 and 4), has shown a 3-fold reduction in PHOX2B protein (Fig. 25, Panel B), thus suggesting that 3-KDG has a direct effect on PHOX2B protein also in this cell line, although contrary to what we have observed in T47D cells, and that the reduced activity of *TLX2* promoter could be due to decrease PHOX2B protein availability.

The 3-KDG treatment induces an increase also in the progesterone receptors amount (Fig. 25 PR-A/PR-B, compare 5 vs 4 lanes) while no changes were observed in PGRs mRNA (Fig. 22), indicating that an increment in the stability of the protein or of the ligand/receptor complex after progestin treatment can occur.

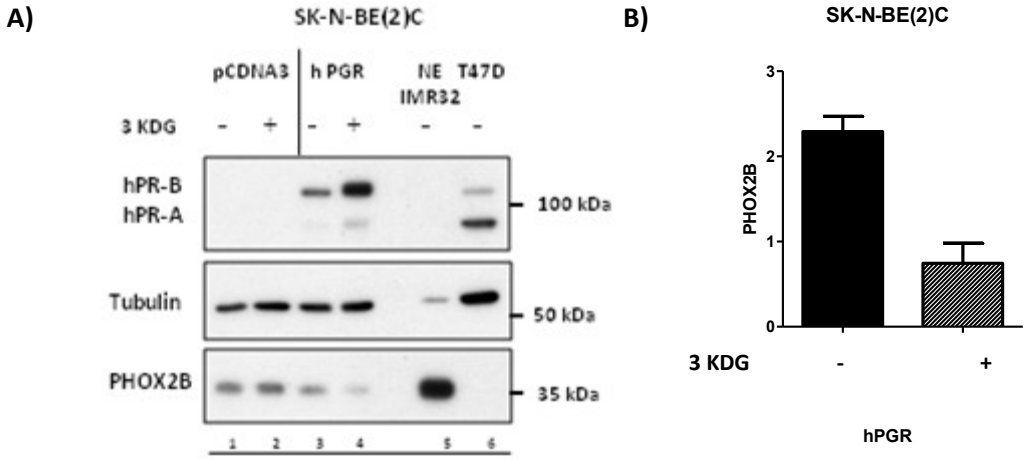

Figure 25 Western blot analysis of PHOX2B protein amount after 48 hours 3-KDG treatment in SK-N-BE(2)C cell line transiently expressing hPGR. **Panel A)** Representative immunoblotting image showed the expression of PHOX2B endogenous protein in SK-N-BE(2) C cells transfected with hPGR expression vector (lanes 3-4) or equimolar amount of empty vector, used as negative control (lanes 1-2) after 48 hrs treatment with 1 nM 3-KDG (lanes 2-4). IMR32 nuclear extract are used as PHOX2B positive control (IMR32 NE, lane 5); T47D are used as hPGR positive control (lane 6). The nitrocellulose membrane was immunoblotted with PGR antibody (upper panel) to detect PGR isoforms and PHOX2B antibody (lower panel). The same blot was reprobed with α-tubulin to compare the loading of each lane (middle panel). **Panel B)** Quantification of PHOX2B endogenous protein in SK-N-BE(2)C cells transfected with hPGR vector after 48h 3-KDG treatment. Value are expressed as mean ± SEM (n=2) of PHOX2B values normalized to that of tubulin.

To study the mechanism underlying this reduction, and in order to avoid the limited step of PGR transfection, SK-N-BE(2)C stably expressing hPGR were generated. The details about the generation of SK-N-BE(2)C stably expressing hPGR are described in the master degree thesis by Erika Di Biase (our lab).

The hPRG expression vector used for transient transfection and for the generation of stable clones encodes for both PR-A and PR-B isoforms. Of the screened clones, we obtained 16 out of 95 positive clones. All clones express PR-B isoform, whereas PR-A expression was present only in a small percentage of clones. Taking into account that 3-KDG effect on neuroblastoma cells may vary depending on different amount of PR-B and PR-A expressed, we started our analysis on the effect of 3-KDG by focusing on two SK-N-BE(2)C positive clones (5.8 and 6.30), that differ for the relative expression level of the two isoforms, PR-B and PR-A (Fig. 26, blue and red bars, respectively). The graph in figure 26 shows the protein level of the two receptor isoforms in the two selected clones, expressed as percentage compared to that measured in T47D cells (set=1). Clone 5.8 expresses comparable amount of PGR-B as in T47D and 10% of PGR-A, whereas in clone 6.30, PR-B expression is 50% compared to that of T47D (Fig. 26); in this clone no PGR-A expression was detected.

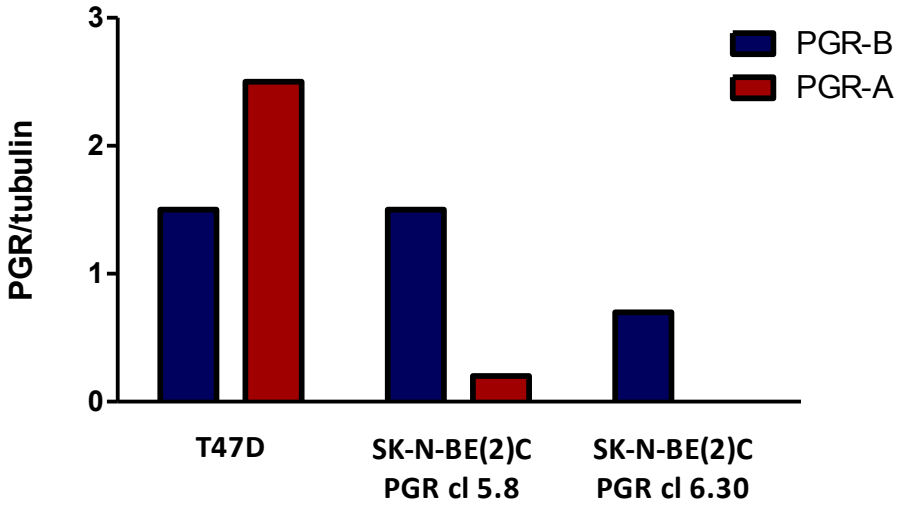

Figure 26 Western blot quantification of PR-A and PR-B protein amount in SK-N-BE(2)C-PGR stable clones. Relative quantification of intracellular progesterone receptor isoforms PR-A (red bars) and PR-B (blue bars) in SK-N-BE(2)C clones 5.8 and 6.30 and T47D cell line, expressed as ratio of PR-A or PR-B to β-Tubulin.

4.3.7 Effect of 3-KDG in SK-N-BE(2)C cell lines stably over-expressing human progesterone receptors.

The SK-N-BE(2)C cell lines, stably over-expressing human PGR receptors, provide suitable cell models for studying the effects of 3-KDG in a neuronal background.

In order to analyse the 3-KDG effect on the endogenous level of PHOX2 transcription factors and of some known PHOX2 target genes, clones 5.8 and 6.30 and native SK-N-BE(2)C used as control cell line (mock), have been treated with 1nM 3-KDG for 8, 24 and 48 hours (Fig. 27).

Western blot analysis and quantification (Fig 27 and Fig 28, respectively) revealed that in both clones the level of all the proteins known to be regulated by PHOX2A/B transcription factors (PHOX2A, PHOX2B, DBH and AP2α (Activator protein 2 alpha), with the exception of GATA3) are significantly reduced upon treatment at each time point, compared to untreated clones (Fig. 27, Panel A), with different kinetics between clones. Indeed, in clone 5.8, PHOX2B, PHOX2A and DBH proteins decrease with a faster kinetic than in clone 6.30 (Fig 27, Panel A, compare lanes 5-8 *vs* lanes 9-12).

In detail, at 8 hours treatment, PHOX2B was reduced by 60% in clone 5.8 vs 30% in clone 6.30 (Fig. 28, Panel A, compare black vs white bar), PHOX2A reduced by 90% in clone 5.8 vs 20% in clone 6.30 (Fig. 28, Panel B, compare black vs white bar); at 48 hours, DBH was undetectable in clone 5.8 vs 90% reduction in clone 6.30 (Fig. 28, Panel C, compare black vs white bar), Ap2α was undetectable in clone 5.8 vs 50% reduction in clone 6.30 (Fig. 28, Panel D, compare black vs white bar).

As a control, we analysed the expression level of proteins that are not direct PHOX2B target genes (Fig. 27; Panel B), such as Sp1 (Specificity Protein 1), CREB (CAMP Responsive Element Binding Protein) and c-Jun (c-Jun proto-oncogene). No significant changes in the expression of these proteins were detected in the clones, as well as in the control cells (Fig 28, Panels F, G H), thus correlating the clinical observation with a direct effect on PHOX2B activity. Whether PHOX2B has to be down- or up-regulated to allow the recovery of chemosensitivity observed in the two French CCHS patients, is an important topic that needs to be further investigated.

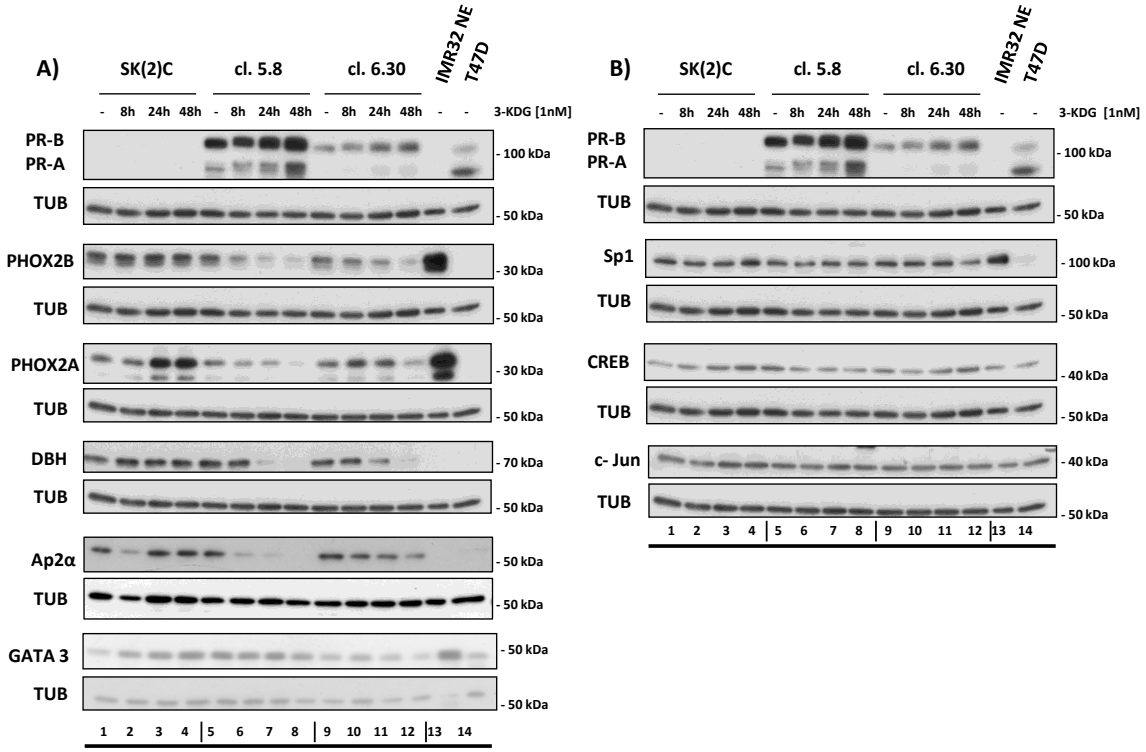

Figure 27 Representative western blot image of endogenous proteins in SK-N-BE(2)C cell lines stably over-expressing hPGR and SK-N-BE(2)C not transfected cells, after 3-KDG treatment.

SK-N-BE(2)C not expressing hPGR (lanes 1-4), SK-N-BE(2)C clone 5.8 expressing both PR-B and PR-A isoforms (lanes 5-8), and SK-N-BE(2)C clone 6.30 expressing only PR-B isoform (lanes 9-12) were treated with 1 nM 3-KDG at different time point: 8 hours (8h; lanes 2,6, 10); 24 hours (24h; lanes 3, 7, 11); 48 hours (48h; lanes 4, 8, 12); or not treated (-; lanes 1, 5, 9). IMR32 nuclear extract (IMR32 NE) is used as PHOX2A/B positive control (lane 13). T47D total extract is used as progesterone receptor isoforms positive control (lane 14). 20 µg of total lysate for each sample were separated by 10 % SDS-PAGE. PR-A, PR-B, PHOX2B, PHOX2A, DBH, Ap2α, Sp1, GATA3, CREB, c-Jun were detected with specific antibodies. All blots were reprobed with β-tubulin (TUB) to compare the loading of each lane.

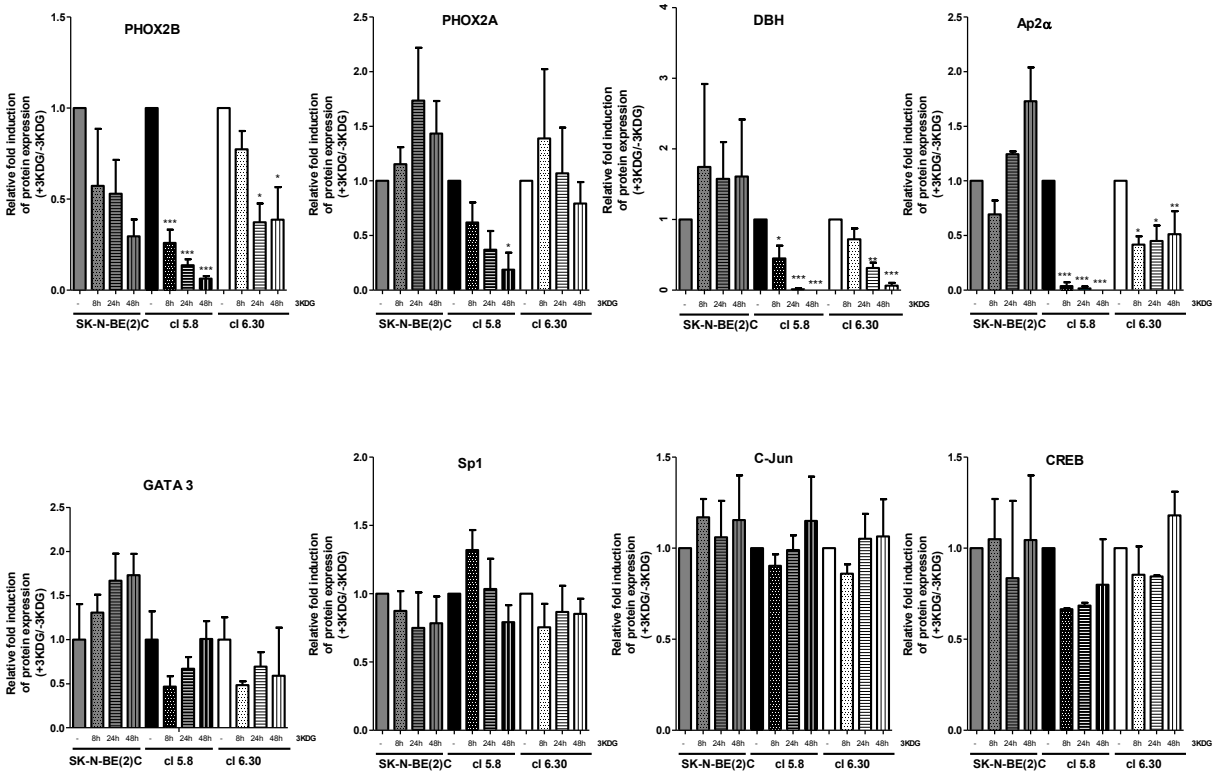

Figure 28 Quantification of endogenous proteins amount in SK-N-BE(2)C cell lines stably over-expressing hPGR and SK-N-BE(2)C not transfected cells, after 3-KDG treatment.

Quantification of PHOX2B, PHOX2A, DBH, Ap2α, GATA3, Sp1, C-Jun, CREB endogenous protein amount in SK-N-BE(2)C not expressing hPGR (grey bars), SK-N-BE(2)C clone 5.8 expressing both PR-B and PR-A isoforms (black bars), and SK-N-BE(2)C clone 6.30 expressing only PR-B isoform (white bars). Intensities of immunoreactive bands on western blots were quantified by densitometry analysis (ImageJ). Data are represented as relative fold induction of treated samples (8h, 24, 48h) with respect to untreated samples(-). The results obtained in SK-N-BE(2)C, SK-N-BE(2)C clone 5.8 and SK-N-BE(2)C clone 6.30 are expressed as mean ± SD (error bars) of the densitometry intensity of each lane normalized vs respective tubulin in at least three independent experiments (n=3). *** p <0.001, **p <0.01, * p <0.05 (one-way ANOVA, Tukey's test).

4.3.8 Effect of 3-KDG on endogenous *PHOX2A*, *PHOX2B TLX2* and *DBH* transcript levels in SK-N-BE(2)C cell line stably over-expressing PR-B and PR-A progesterone receptors.

The 3-KDG treatment, in SK-N-BE(2)C cell line stably expressing hPGR, resulted in a strong reduction in the level of all the proteins known to be regulated by PHOX2A/B transcription factors, with the exception of GATA3. On the contrary, the level of all PHOX2B non-target proteins, here analysed, seemed not to change between treated and untreated clones, thus indicating a specific effect of 3-KDG treatment on PHOX2B pathway.

Moreover, we previously observed a strong decrement in *TLX2* promoter activity in neuronal cell lines transiently expressing hPGR, with the exception of SK-N-BE, after 3-KDG treatment.

To study the mechanism underlying these effects, we performed a quantitative PCR analysis, in order to test whether the decrease in protein level is due to a reduced transcription or mechanism acting at post-transcriptional level.

Quantitative PCR analysis of *PHOX2B*, *PHOX2A*, *DBH* and *TLX2* mRNA (Fig. 29), extract from SK-N-BE(2)C clones 5.8 and 6.30 and native SK-N-BE(2)C cell samples, treated with 1nM 3-KDG for 8, 24 and 48 hours, shows a significant reduction in their expression level, in 5.8 clone (Fig. 29, black bars), and to a much less extent in clone 6.30 (Fig. 29, white bars), similarly to the decrement observed in protein levels (Fig. 27 and 28). However, the reduction is more pronounced for *PHOX2A* and *DBH* gene than *PHOX2B* and *TLX2* that, after 48 hours treatment, decreased by 50% and 60%, respectively, in 5.8 clone (Fig. 29, Panel A and C, compare black hatched bars vs black bar) and by 40% and 20%, respectively, in clone 6.30 (Fig. 29, Panel A and C, compare white hatched bars vs white bar). On the contrary, *PHOX2A* and *DBH* transcripts decreased up to 80% and 90%, respectively, in clone 5.8 (Fig. 29, Panel B and D, compare black hatched bars vs black bar) and by 20% and 30%, respectively, in clone 6.30 (Fig. 29, Panel B and D, compare white hatched bars vs white bar). In native SK-N-BE(2)C cells, no changes in the level of mRNA have been observed for all the genes, but *PHOX2A* expression is again down-regulated,

although we have never observed such a reduction in SK-N-BE(2)C cells transient transfected with PGR (Fig. 24).

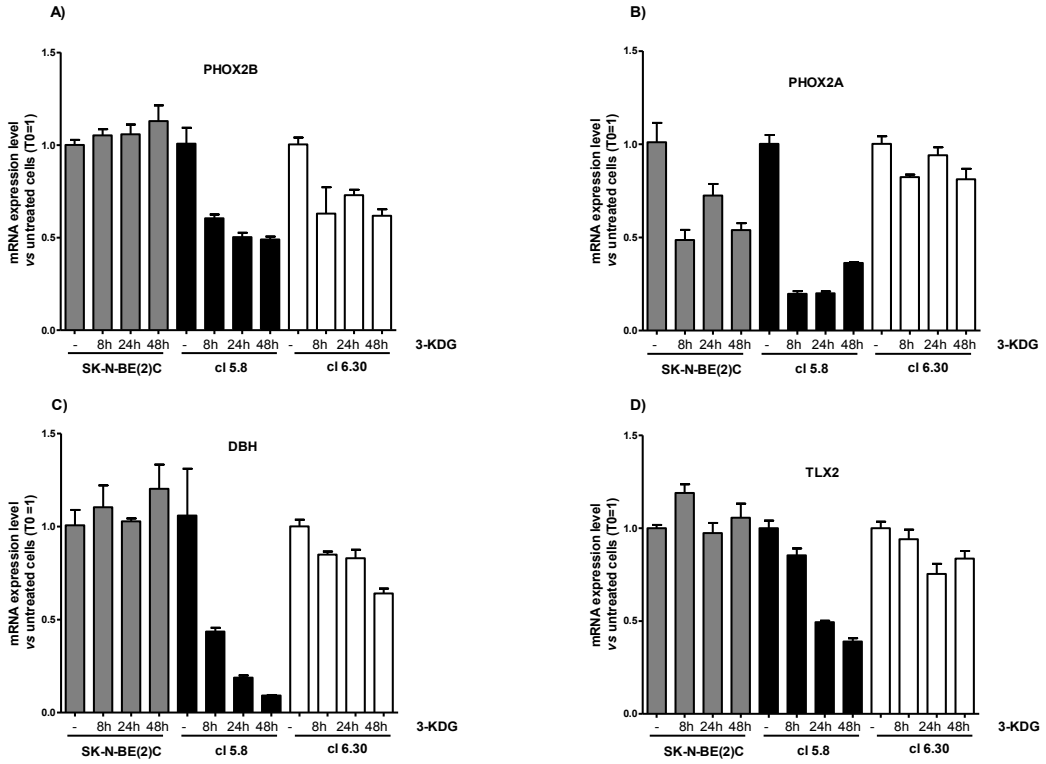

Figure 29 qPCR analysis of endogenous *PHOX2A*, *PHOX2B*, *DBH* and *TLX2* mRNA expression in SK-N-BE(2)C cell lines stably over-expressing hPGR and SK-N-BE(2)C not transfected cells, after 3-KDG treatment at different time point.

The bars indicate the mRNA levels in SK-N-BE(2)C samples (grey bars), SK-N-BE(2)C clone 5.8 (black bars) and SK-N-BE(2)C (white bars) treated with 1nM 3-KDG at different time points (0h, 8h, 24h, 48h). The bars are the mean values ± SD (error bars) of the *PHOX2B, PHOX2A, DBH and TLX2* mRNA expression level detected in a single representative experiment, performed in triplicate, and are expressed as fold relative to the untreated cells (=1). The $2^{-\Delta\Delta CT}$ method was used to calculate the results.

These data support the hypothesis that the reduction in protein levels observed for PHOX2B and its target genes, is in part due to a reduction of mRNA level, that can occur because of reduced transcription or reduced transcript stability.

To deeply investigate the transcriptional mechanism involved in 3-KDG treatment, we choose to analyse the stability of *PHOX2B* mRNA in clone 5.8, which showed the most rapid kinetic reduction in PHOX2B target proteins.

SK-N-BE(2)C clone 5.8 cells were then pre-treated with 1nM 3-KDG, and, after 24 hours, the transcriptional *inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole* (DRB) was added and the cells lysed and analysed at the times indicated in figure 30.

As shown in Figure 30, in not treated cells (black dots) PHOX2B mRNA is stable, as its level remains constantly over 50%; 3-KDG drastically affects *PHOX2B* mRNA stability, by decreasing its half-life to 60 minutes (Fig. 30, red dots) thus indicating an increased degradation of the *PHOX2B* transcript upon treatment.

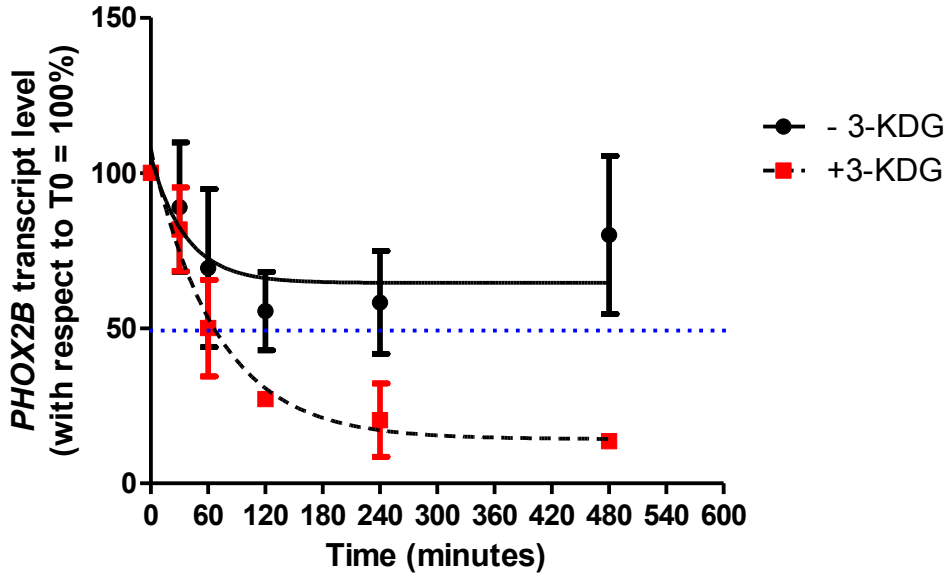

Figure 30 Effect of 3-KDG treatment on *PHOX2B* transcript stability.

SK-N-BE(2)C clone 5.8 was treated with 1nM 3-KDG . After 24h of treatment, cells were incubated with 0.75 µM DRB for 30, 60, 120, 240 or 480 minutes (red dots). SK-N-BE(2)C clone 5.8 not treated cells, incubated with DRB at the same time points, were used as control (black dots). The values are mean ± SD of three independent experiments, performed in triplicate, and are normalized to the endogenous gene GAPDH, and are expressed as percentage relative to the time 0 (=100%), calculated by means of the $2^{-\Delta\Delta CT}$ method.

However, the reduction of PHOX2B mRNA level, measured in the experiment in Fig. 29, does not totally justify the reduction in protein levels, observed in clone 5.8 upon 3-KDG treatment (Fig. 28). Other mechanisms, which impair stability and/or protein translation, due for example to post-translational modifications (i.e. phosphorylation/de-phosphorylation), can be involved in mediating the effect of 3-KDG. We do not know at the moment if the reduced stability of PHOX2B mRNA can be a general mechanism by which 3-KDG down-regulates the expression of PHOX2B target genes. This topic is still under investigation.

Chapter 5

General discussion

Debora Belperio

General discussion

PHOX2 proteins are homeodomain transcription factors deeply involved in ANS development and specification of the noradrenergic phenotype in embryonic neurodevelopment. Phox2b and Phox2a not only have a crucial role in developing brain, but are also found to be widely expressed in mouse post-natal brain, even though in different neuronal populations (Kang *et al.,* 2007 and Card *et al.,* 2010). However, contrary to the important role in neurodevelopment and neuronal specification in early embryogenesis, the role of PHOX2B expression in adult is still unclear. Interestingly, *Phox2b* expression in adult mouse brain is far more limited than that of *Phox2a*, and they show divergence in the function of their gene products in adult brain. In particular, immunostaining experiments revealed consistent divergence within the A1, A5, A6, *Locus Coeruleus* (LC), and A7 noradrenergic cell groups, which densely stain for Phox2a (Card *et al*. 2010) but do not express Phox2b in the adult rat brain (Kang *et al*., 2007).

The evident decrement of Phox2b concentration between embryonic and adult noradrenergic neurons, indicates that, despite the pioneer role of Phox2b in noradrenergic development, this transcription factor is not necessary for the (nor)adrenergic phenotype maintenance in the adult brainstem, such function is probably more controlled by Phox2a protein given its highest expression in noradrenergic adult circuits.

Despite the considerably differences in Phox2 proteins expression patterns in adult brain, there is a substantial overlap in their distribution in several brain regions (summarized in the table n. 1).

Taking together that Phox2 proteins expression is dynamically regulated between neurodevelopmental and adult mouse brain, and that their overexpression is linked with neuroblastoma (NB) manifestation, we can assert that a tight control of their expression is necessary in order to avoid pathologies and neurological abnormalities.

As proof of this observation, we found that treatment with the differentiating agent all-*trans* retinoic acid (ATRA) finely control PHOX2 proteins, we therefore hypotesize that

PHOX2 proteins decrement could be linked with the consequent beneficial effect in tumours treatment (see appendix 2).

In particular, our data indicates that PHOX2A protein product increases during the first 24 hours of ATRA treatment and disappears after 48 hours, by proteasome degradation, while the expression of *PHOX2A* mRNA remains constantly up-regulated. PHOX2B protein instead appears to be immediately down-regulated following ATRA treatment.

Recently, other molecules, which have already reported to have anticancer activity in NB or other tumours, (Curcumin, Tricostatin-TSA and Suberoylanilide hydroxamic acid-SAHA) are found to have a marked effect on *PHOX2B* down-regulation (Di Zanni *et al*., 2015).

This data, together with the observation that *PHOX2B* is down-regulated during final neuronal differentiation (Alam *et al*., 2009,) suggest that the dysregulation of PHOX2 pathway induces a strong alteration in the differentiation process and that ATRA-dependent PHOX2B down-regulation, may possibly drive the differentiation of NB cells.

Besides being involved in neuroblastoma manifestation and progression, and given their relevant role in neurodevelopment, mutations in PHOX2 transcription factors are found to be correlated with congenital pathologies.

In particular, homozygous mutations in *PHOX2A* gene result in congenital fibrosis of the extraocular muscles type 2 (CFEOM2) whereas heterozygous mutations in *PHOX2B* gene lead to Congenital Central Hypoventilation Syndrome (CCHS).

The pioneer role of Phox2b in brain embryo development could explain the reason why mutations in *PHOX2B* lead to more complex pathologies, involving neurodevelopmental and neurocognitive disorders and autonomic impairment/dysfunction.

At the molecular level, the pathological mutations in PHOX2B protein, principally due to the expansion in the 20-alanine stretch downstream the homeodomain, lead to a misfolded protein that has a reduced activity but shows an increase tendency to form aggregate and to interfere with wild type activity (Bachetti *et al*., 2005; Trochet *et al*., 2005; Di lascio *et al*., 2013).

The dissolving of “pathogenetic” PHOX2B aggregates, by means of heat shock protein activation shown by Di Zanni *et al*. (2012) and Bachetti *et al*. (2005), is based on the most common consideration that they are toxic for the cells. However, there is no evidence of

their *in vivo* existence, as they were observed only in overexpression experiments even if in different cell lines. Therefore, we cannot exclude that the strong interaction between expanded mutant proteins could be a simple over-expression artefact due to the high mutant proteins amount, respect to physiological conditions, or even a protective mechanism in order to avoid toxic effects of mutant PHOX2B. Further, Di Zanni *et al*. (2012) also observed that treatments with Curcumin and Geldanamycin, drugs able to dissolve the aggregates, are actually toxic for the cells, since they reduce cellular viability after 48hours of treatment, supporting the protective aggregation theory.

In CCHS the anatomical abnormalities were found in Retrotrapezoid Nucleus (Dubreuil et al., 2008), an anatomical structure that is absent in Phox2b $^{+/+7Ala}$ mouse model. Recently, the analysis of post-mortem brain of two CCHS patients unraveled anatomical defects in the dorsal motor nucleus of the vagus nerve, in the mesencephalic trigeminal nucleus, in the serotonergic median raphe and adrenal medullary neurons and also LC neuronal loss (Nobuta *et al*., 2015). These anatomical neurodevelopmental defects are obviously irreparable by pharmacological treatments. However, other neural structures involved in breathing control, whose function depends on PHOX2B, may get beneficial effects by the restoration of PHOX2B activity.

In 2010 the serendipitous observation of a marked chemosensitivity recovery in two CCHS female patients assuming Desogestrel, exclusively for a contraceptive purpose, gave us an interesting therapeutic perspective for the disease (Similowski and Straus, 2011).

It is well known that progesterone has stimulant effect on respiration (Dempsey *et al.,* 1986; Joseph *et al.,* 2012) and that its administration is able to reduce apnoea during sleep in adult women (Young *et al.,* 2003).

However, the mechanism through which Desogestrel could ameliorate respiratory symptoms in CCHS patients is largely undisclosed. It is also noterworthy that the effect of Desogestrel is exclusively mediated by this progestin and not by physiological progesterone, since women affected by CCHS did not improve hypercapnia response during gestation. This could be a consequence of the stronger affinity of Desogestrel for the progesterone receptor; three times greater than that of progesterone (Straus *et al*., 2010; Wiegratz and Kuhl, 2004).

Our first experiments performed in T47D cell line, highlighted a clear association between 3-KDG and PHOX2B activity, since we observed an enhancement of *TLX2* promoter transactivation in the presence of PHOX2B, together with an increase amount of PHOX2B protein upon cell treatment, in both cases this effect is abolished in the presence of nuclear progesterone antagonist Mifepristone (RU486).

Several functional studies support the role of nuclear progesterone receptor in the control of respiration, as pre-treatment with RU486 abolished positive effect of progesterone injection on apnoeic episodes during sleep in adult rat (Yamazaki *et al*., 2005), and the enhancement of phrenic nerve activity in cats (Bayliss *et al*., 1987). Furthermore, *in vivo* data indicate that nuclear progesterone receptor effects on respiration are mainly mediated by the hypothalamus (Bayliss *et al*., 1990) and the nucleus of the solitary tract (Bayliss *et al*., 1987). In addition, experiments conducted on nuclear progesterone receptor KO mice (PRKO) and WT mice clearly indicate that progesterone treatment is able to increase ventilation in adult under hypercapnic condition, and that this effect is mediated by nuclear PR (Marcouiller *et al.,* 2014). Interestingly, in these models, progesterone reduces frequency of sighs and post-sighs apnoeas during sleep (Marcouiller e*t al* 2014). Sights are part of normal breathing (Bendixen *et al*., 1964) and are generated by central respiratory generator that receives afferents from NTS, which in turn intercepts input from pulmonary stretch receptor (Larrabee *et al*., 1946) and peripheral chemoreceptors (Glogowska *et al*., 1972).

The data obtained in neuroblastoma cell lines revealed a strong reduction in *TLX2* promoter activity after progestin treatment in almost all the cell lines tested, with the exception of SK-N-BE cell line, which exclusively expresses PHOX2A , and that responded in a similar manner to that of T47D cells. This intriguingly result suggests that the cellular background, including the presence of different co-activators and co-repressors, is a crucial determinant of the 3-KDG effect on PHOX2B and PHOX2A pathway.

In addition, experiments carried out in SK-N-BE(2)C cell line, stably expressing human PGR, have shown that the reduction in PHOX2B, PHOX2A and the protein level of other target genes is particularly marked in stable clone expressing high level of progesterone receptor B isoform together with the A isoform (SK-N-BE(2)C clone 5.8). This reduction

occurs, in part, also at mRNA levels, due to a reduced PHOX2B mRNA half-life. However, the reduced level of PHOX2B mRNA does not justify the high decrement in PHOX2 and PHOX2 targets protein levels, thus suggesting that 3-KDG may act also by reducing protein stability. This aspect is still under investigation.

A recent study, on two postmortem CCHS cases (one PARM and one NPARM; Nabuta et al., 2015), reported neuronal loss within the LC, the major noradrenergic nucleus of the brain. Since PHOX2A gene is required for LC development, and we demonstrated that it interacts with both wild type and mutant PHOX2B, we tested the hypothesis that PHOX2B mutations may cause a dominant-negative effect on PHOX2A activity. Our results indicate that PHOX2B mutants do not interfere with PHOX2A transcriptional activity; on the contrary we measured an unexpected synergistic effect of PHOX2A and mutant PHOX2B heterodimers. However, we cannot exclude the possibility of new "toxic" interactions, due to altered recruitment of promoter-specific co-factors by mutant heterodimers , which could be limited by lowering also PHOX2A expression.

The divergent data of the 3-KDG effect in different cell lines, suggested that in order to take Desogestrel in consideration as a possible therapeutic drug, a better understanding of brain areas expressing PGR alone or along with PHOX2B and PHOX2A is needed.

Data available from studies obtained in young mice, and summarized in table 1, showed that PGR is expressed in different CNS structures (Quadros *et al*., 2008), but only in few of those PGR turned out to be co-expressed with PHOX2 proteins.

Interestingly, the nuclus of the solitary tract (NTS) is the only structure which co-expresses Phox2a, Phox2b and PGR (Quadros *et al*., 2008, Card *et al*., 2010, Kang *et al*., 2007). The NTS neurons, located in the dorsal medulla, are known to be indispensable for the integration of sympathetic and respiratory responses to hypoxia, representing the first synaptic centre of the cardio-respiratory afferent inputs, including peripheral chemoreceptors, baroreceptors and pulmonary stretch receptors (Zoccal *et al*., 2014).

Of particular interest are nuclei of the reticular formation, such as the Periaqueductal Gray and the Parabrachial nucleus, known to be involved in the autonomic control of respiration (Subramanian *et al*. 2008, Chamberlin and Saper, 1994), and in which PGR is express together with Phox2a (Card *et al*, 2010). Other Phox2a / PGR positive structures

have been identified in hypothalamus, in particular the Dorsomedial hypothalamic nucleus (DMH) in caudal hypothalamus and Ventrolateral preoptic nucleus (VLPO) in rostral hypothalamus, both found to be important in the control of behavioral state (for reviews see Saper *et al*., 2005; Siegel, 2006; Fuller *et al*., 2007). In particular, VLPO is involved in wake-sleep cycle regulation (Saper *et al*., 2005), whereas chemical stimulation of DMH has been associated to increased phrenic nerve stimulation (Tanaka and McAllen, 2008).

CCHS patients, in addition to the respiratory symptoms, also show symptoms of a generalized impairment of sympathetic functions. We do not know at the moment whether the progestin may be beneficial to restore autonomic functions adversely affected in the disease.

Another important issue in CCHS treatment regards that PHOX2B is known to be necessary for the correct development of anatomical structures responsible for hypoxia and hypercapnia sensitivity, in particular the Carotid body (CB), NTS, petrosal chemoreceptor and RTN.

As the mouse model carrying the heterozygous Phox2b +7ala mutation do not develop the RTN neurons, it is reasonable to expect that also in humans this structure is not properly developed.

The observation of the chemosensitivity recovery in CCHS patients after 3-KDG assumption could therefore suggest that the ameliorated symptoms may perhaps be also linked to respiratory pathways apparently unrelated to PHOX2B.

Very recently Joubert and co-workers (Joubert *et al*., 2016) observed an increase in respiratory frequency in *ex vivo* mouse medullary-spinal cord preparations or *in vivo* mice exposed to Etonogestrel (3-KDG), indicating that other pathways, not involving PHOX2B, should also be considered in 3-KDG effects.

These data, together with our findings concerning the 3-KDG effect on PHOX2B and its targets and jointly with the well known positive respiratory stimulations of progestin drugs, indicate that 3-KDG may have a more complex effect on general ventilation in CCHS patients acting on different respiratory networks.

Adult rat:	Phox2a	Phox2b	PGR
Dorsal motor vagal complex (DVC)[medulla**]:**	+	+	
Nucleus of the solitary tract (NTS)	+	+	+
Area postrema (AP) Dorsal motor vagal nucleus (DMV)	+ +	+ +	
Reticular formation:	+		+/-
Inferior salivatory nucleus	+		-*
Parabrachial nucleus, lateral Parabrachial nucleus, medial	+ +	-(rare) - (rare)	++ +/-
Periaqueductal gray	+	-*	+++(not ventral)
Gigantocellular reticular region			+/-
Raphe pontis nucleus	-		+(+)
Pedunculopontine tegmental Nucleus			+++
Medulla Spinal nucleus of the fifth cranial nerve	+	+(dorsomedial subdivision)	-
Subjacent to the fourth ventricle	+		+/-(Vestibular nucleus)
Ventrolateral brainstem:			
Ventrolateral medulla Rostroventral medulla (RVLM) And pons (Trochlear and oculomotor nuclei) Retrotrapezoid nucleus (RTN) Superior salivary nucleus (SSN)	+ + + + +	+ + + + +	 -
Nucleus ambiguus	-(only embryogenesis)	+	
Rostral-dorsal brainstem(pons)			
Locus coeruleus (LC)	+	-	
Midbrain:			
Substantia nigra, compact Ventral tegmental Area Interfascicular nucleus (VTA) Paranigral region (VTA) Retrorubral field (RRF) Inferior colliculus Anterior pretectal nucleus		-	+/- ++(+) +/- +/- +/- +/- +
A7 cell group (midb. And pons)	+	-	
Diencephalon:			
Dorsomedial hypothalamic nucleus(DMH) in caudal hypothalamus	+	-	+
Ventrolateral preoptic nucleus (VLPO) in rostral hypothalamus	+	-	+(preoptic area)
Retrochiasmatic area and periventricular nucleus near suprachiasmatic nuclei	+ Scattered neurons		

Table n. 1

Conclusion

Our results on ATRA and 3-KDG treatment suggest that a fine control of the PHOX2 pathway is critical in order to ameliorate clinical condition in NB and CCHS patients.

The reducing PHOX2B expression, as observed in 3-KDG treated SK-N-BE(2)C cells overexpressing PGR, could reasonably result also in the decrease of mutants expression level, thus limiting the pathological effects of expanded mutants. However, the divergent data obtained between different cell lines also indicate that these effects strictly depend on physiological background, including the expression of correct receptor isoforms, and co-repressors or co-activators. The NTS seems to be the CNS structure more compelling to mediate 3-KGD effect since PHOX2A, PHOX2B and nuclear progesterone receptors are co-expressed in this region.

Taking together our results point out the existence of a specific cross-talk between ATRA and 3-KDG treatments and PHOX2 pathway, suggesting that the regulation of PHOX2 proteins expression could be a common strategy into the treatment ofboth NB and CCHS.

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Appendix 1

Alanine Expansions Associated with Congenital Central Hypoventilation Syndrome Impair PHOX2B Homeodomain-mediated Dimerization and Nuclear Import

Simona Di Lascio, Debora Belperio, Roberta Benfante, and Diego Fornasari

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Alanine Expansions Associated with Congenital Central Hypoventilation Syndrome Impair PHOX2B Homeodomain-mediated Dimerization and Nuclear Import*

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Simona Di Lascio[‡1], **Debora Belperio**[‡1], **Roberta Benfante**[‡§], **and Diego Fornasari**[‡§2]

From the [‡]*Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, 20129 Milan, Italy and the* [§]*National Research Council (CNR) Neuroscience Institute, 20129 Milan, Italy*

Heterozygous mutations of the human *PHOX2B* gene, a key regulator of autonomic nervous system development, lead to congenital central hypoventilation syndrome (CCHS), a neurodevelopmental disorder characterized by a failure in the autonomic control of breathing. Polyalanine expansions in the 20-residues region of the C terminus of PHOX2B are the major mutations responsible for CCHS. Elongation of the alanine stretch in PHOX2B leads to a protein with altered DNA binding, transcriptional activity, and nuclear localization and the possible formation of cytoplasmic aggregates; furthermore, the findings of various studies support the idea that CCHS is not due to a pure loss of function mechanism but also involves a dominant negative effect and/or toxic gain of function for PHOX2B mutations. Because PHOX2B forms homodimers and heterodimers with its paralogue PHOX2A *in vitro*, we tested the hypothesis that the dominant negative effects of the mutated proteins are due to non-functional interactions with the wild-type protein or PHOX2A using a co-immunoprecipitation assay and the mammalian two-hybrid system. Our findings show that PHOX2B forms homodimers and heterodimerizes weakly with mutated proteins, exclude the direct involvement of the polyalanine tract in dimer formation, and indicate that mutated proteins retain partial ability to form heterodimers with PHOX2A. Moreover, in this study, we investigated the effects of the longest polyalanine expansions on the homeodomain-mediated nuclear import, and our data clearly show that the expanded C terminus interferes with this process. These results provide novel insights into the effects of the alanine tract expansion on PHOX2B folding and activity.

Heterozygous mutations in the *PHOX2B* gene lead to congenital central hypoventilation syndrome (CCHS[3]; OMIM ID: 209880), which is characterized by a failure in the autonomic control of breathing and an abnormal ventilatory response to hypoxia and hypercapnia (1).

CCHS patients have a greater predisposition to Hirschsprung disease and neuroblastoma (2, 3) as well as the symptoms of general autonomic nervous system dysfunction (4).

The transcription factor PHOX2B (paired-like homeobox 2b, also known as PMX2B and NBPhox) is a master regulator of autonomic nervous system development (5), and its human orthologue is a 314-amino acid protein that harbors a homeodomain and two polyalanine stretches of 9 and 20 residues, respectively, within the C-terminal domain (6, 7).

The large majority of CCHS patients carry mutations that cause an expansion of the longer polyalanine repeat (polyalanine repeat expansion mutations) (2, 8) ranging from +5 to +13 alanine residues, and it has been reported that there is a correlation between the length of the polyalanine tract and the severity of the respiratory phenotype and autonomic dysfunction (8, 9). Non-polyalanine repeat mutations (*i.e.* missense, nonsense, and frameshift mutations) are less frequent, but they correlate with more severe respiratory symptoms, Hirschsprung disease, and neuroblastoma.

From a functional point of view, it is well established that the homeodomain of PHOX2B is a highly conserved 60-residue region that contains the DNA-binding motif; furthermore, in line with what has been observed in other homeodomain proteins, the PHOX2B homeodomain may also contain nuclear localization signals, be responsible for the formation of homo- and heterodimers (with other homeoproteins, including its paralogue PHOX2A), and establish protein-protein interactions (10). On the contrary, the exact molecular functions of the polyalanine tracts remain largely unknown.

Polyalanine and, more generally, homopolymeric tracts (single amino acid repeats) are common features of eukaryotic proteins and are especially abundant in transcription factors (11, 12). Increasing experimental data show that they can modulate transcription factor activity by acting as flexible spacer elements located between functional protein domains and therefore play a role in protein conformation, protein-protein interactions, and/or DNA binding (13–15). The coding triplet repeat instability that leads to the expansion of these stretches causes a number of human diseases (16, 17), all of which are characterized by protein misfolding that leads to intracellular aggregation, which may be an intrinsic tendency because, beyond a

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Medical Biotechnology and Translational Medicine, University of Milan, Via Vanvitelli 32, 20129 Milano, Italy. Tel.: 39-02-503 -6960; Fax: 39-02-503-17132; E-mail: diego.fornasari@unimi.it.

[3] The abbreviations used are: CCHS, congenital central hypoventilation syndrome; BD, DNA binding domain; AD, activation domain; NLS, nuclear localization sequence; ANOVA, analysis of variance; HD, homeodomain; Cter, C-terminal; Nter, N-terminal.

TABLE 1

Nucleotide sequences of the synthetic primers used in the PCR amplifications to generate the deleted and mutant variants of PHOX2B

The enzyme restriction sites used for cloning are underlined.

Construct name	Forward primer (5′–3′)	Reverse primer (5′-3′)
PHOX2B Nter	CGGAATTCCAATGTATAAAATGGAATATTC	GCGAATTCTCACTTGCGCTTCTCGTTG
PHOX2B Nter + HD	CGGAATTCCAATGTATAAAATGGAATATTC	GCGAATTCTCACTCCTGCTTGCGAAAC
PHOX2B HD	CGGAATTCAGCAGCGGCGCATCC	GCGAATTCTCACTCCTGCTTGCGAAAC
PHOX2B HD + Cter	CGGAATTCAGCAGCGGCGCATCC	ATGAATTCGGCTTCCGCCGCAGG
PHOX2B Cter	CGGAATTCTTCGCAAGCAGGAGCGC	ATGAATTCGGCTTCCGCCGCAGG
PHOX2B ΔNLS1	CTCAACGAGACCACTTTCACCAGTGCCCAG	AAAGTGGTCTCGTTGAGGCCGCCGTG
PHOX2B ΔNLS2	GTTCCAGCAGGAGCGCGCAGCG	TCCTGCTGGAACCACACCTGGACTCGC
PHOX2B Δ106–147	ACCACTTTCAACCGCCGCGCCAAG	CGGTTGAAAGTGGTGCGGATGCG
External primers (for ΔNLS1, ΔNLS2, Δ NLS1-2 and Δ106–147)	CACAAGCTTGCTGCGGAATTGTACC	CTCCATTCGCCCCGCAGCTG
GAL4 BD-/ VP16 AD-PHOX2B WT	GGGGTACCATGTATAAAATGGAATATTC	CCGGTACCGGCTTCCGCCGCAGG
GAL4 BD-/ VP16 AD-Nter	GGGGTACCATGTATAAAATGGAATATTC	CCGGTACCTCACTTGCGCTTCTCGTTG
GAL4 BD-/ VP16 AD-Nter + HD	GGGGTACCATGTATAAAATGGAATATTC	CCGGTACCTCACTTGCGCTTCTCGTTG
GAL4 BD-/ VP16 AD-HD	GGGGTACCCAGCGGCGCATCC	CCGGTACCTCACTTGCGCTTCTCGTTG
GAL4 BD-/ VP16 AD-HD + Cter	GGGGTACCCAGCGGCGCATCC	CCGGTACCGGCTTCCGCCGCAGG
GAL4 BD-/ VP16 AD-Cter	GGGGTACCCGCAAGCAGGAGCGC	CCGGTACCGGCTTCCGCCGCAGG
GAL4 BD-/ VP16 AD-PHOX2A	GCGGCCGCCGGGCCGATGGACTACTCCTACC	TTGCGGCCGCCTAGAAGAGATTGGTCTTCAGGGC

certain threshold, the polyalanine tracts spontaneously form β-sheets *in vitro* (18).

Increasingly long polyalanine tracts also lead to an increased tendency for protein aggregation and possible toxic effects in the case of PHOX2B (19, 20). Nuclear import defects and cytoplasmic aggregation are detectable only in the case of proteins with longer expansions, whereas other defects, such as decreased DNA binding and transcriptional activity, also characterize shorter expansions (19–21). In addition to loss-of-function defects, it has been reported that the mutant protein with the longest expansion (+13 alanines) has a dominant negative effect on the DNA binding and subcellular localization of the wild-type protein (19, 21, 22). Furthermore, the negative effects of PHOX2B mutant proteins on the transcriptional activity of the wild-type protein are promoter-specific (20, 21), but it is not clear if the observed functional effects are the result of direct aberrant interactions between wild-type and mutant proteins and/or with other proteins. It should be noted that the absence of co-aggregation of the wild-type protein with mutants with the shorter expansions, revealed by immunofluorescence, does not exclude the possibility of interactions between the non-aggregated proteins at the molecular level. Because wild-type PHOX2B forms homodimers *in vitro* as well as an important fraction of the mutants with shorter expansions (7, 20), and our previous *in vitro* results suggest the possible formation of non-functional heterodimers (21), we decided to test this hypothesis using a co-immunoprecipitation assay and mammalian two-hybrid system. Furthermore, using the same approaches, we assess the ability of mutated proteins to form heterodimers with PHOX2A. Moreover, given the central role of the homeodomain in DNA binding, nuclear import, and dimerization, we also exploit the effects of the alanine tract expansion on PHOX2B nuclear import process.

Materials and Methods

Plasmid Construction—All of the oligonucleotides used to generate the constructs are listed in Table 1. The PCR amplifications were performed using the GC-rich PCR system (Roche Applied Science), and all of the DNA fragments obtained by PCR were sequenced on both strands. All of the enzymes used for cloning were purchased from New England Biolabs.

Expression Plasmids—The MYC-tagged PHOX2B wild-type, +7 alanine, and +13 alanine mutant plasmids have been described previously (19, 21, 22).

The generation of the expression plasmid HA-tagged PHOX2B wild type (HA-PHOX2B WT) has been described elsewhere (21). The PHOX2B deletion constructs (Nter, Nter + HD, HD, HD + Cter, and Cter) were obtained by amplifying specific regions of PHOX2B cDNA using HA-PHOX2B WT as the PCR template. The primers used contain the EcoRI restriction site, and after enzymatic digestion with EcoRI, the PCR products were inserted into the HA-PHOX2B WT vector after the same enzyme had been used to remove PHOX2B cDNA. The HD + Cter +13Ala and the Cter +13Ala constructs were obtained by digesting the corresponding wild-type plasmids containing the normal alanine tract with the PpuMI restriction enzyme. The resulting 270-bp region, which encompasses the alanine tract, was replaced by the 309-bp region obtained using the same enzyme to digest the MYC-tagged PHOX2B +13Ala plasmid (19, 22).

The HA-PHOX2B ΔNLS1, HA-PHOX2B ΔNLS2, and HA-PHOX2B Δ106–147 constructs were made using overlap extension PCR (23) with the HA-PHOX2B WT plasmid as the template. Two chimeric primers were used for each construct, each of which consisted of an annealing fragment derived from one flanking region of the deletion and an anchor fragment derived from the flanking region on the other side of the deletion. The external primers were used to insert the deletions in all of the PCR experiments. The first PCR was performed using 40 ng of supercoiled plasmid containing PHOX2B cDNA to amplify two partially overlapping DNA fragments carrying the deletion. The two purified fragments were then annealed and used as the template (50 ng/each) for a second PCR using the external primers to obtain a single product. The PCR products were cloned after double enzymatic digestion with HindIII/PpuMI in the HA-PHOX2B WT plasmid, and the 270-bp region, which was removed by means of PpuMI enzymatic digestion, was subsequently inserted into the resulting plasmid after digestion with the same enzyme. The HA-PHOX2B ΔNLS1-2 plasmid was obtained by means of the same strategy using

the HA-PHOX2B ΔNLS2 plasmid as the template and the PHOX2B ΔNLS1 forward and reverse primers.

The HA-PHOX2B +13Ala, HA-PHOX2B ΔNLS1 +13Ala, HA-PHOX2B ΔNLS2 +13Ala, and HA-PHOX2B ΔNLS1-2 +13Ala constructs were generated by means of enzymatic digestion with PpuMI and the replacement of the region encompassing the two PpuMI restriction sites containing the normal alanine stretch with the expanded stretch.

The MYC- and HA-tagged PHOX2A expression vectors were obtained by cloning human PHOX2A cDNA (24) into the EcoRI site of pCMV-MYC or pCMV-HA (Clontech).

The Mammalian Two-hybrid System Plasmids—The CheckMate mammalian two-hybrid system kit (Promega) was used to provide the pBIND vector containing the yeast GAL4 DNA binding domain (BD) and *Renilla reniformis* luciferase under the control of the SV40 promoter, the pACT vector containing the herpes simplex virus VP16 activation domain (AD), and the pG5LUC plasmid containing five GAL4 binding sites upstream of a minimal TATA box and the firefly luciferase gene.

The plasmids encoding GAL4 BD-PHOX2B, VP16 AD-PHOX2B, VP16 AD-Nter, VP16 AD-Nter + HD, VP16 AD-HD, VP16 AD-HD + Cter, and VP16 AD-Cter were generated by means of PCR using primers containing the KpnI restriction site. The HA-PHOX2B WT plasmid was used as the PCR template, and the PCR products were cloned in the pBIND and pACT vectors after KpnI enzymatic digestion.

The GAL4 BD-PHOX2B +7Ala/+13Ala/ΔAla and the VP16 AD-PHOX2B +7Ala/+13Ala/ΔAla mutant plasmids were obtained by means of PpuMI enzymatic digestion of the corresponding wild-type plasmids. The 270-bp fragment between the two PpuMI restriction sites was replaced by the corresponding regions containing the expanded or deleted alanine stretches, which were isolated from the previously described expression plasmids HA-PHOX2B 0Ala, PHOX2B +7Ala (21), and PHOX2B +13Ala (19, 22) by means of enzymatic digestion using the same enzyme.

The plasmids encoding GAL4 BD-PHOX2A and VP16 AD-PHOX2A were generated by means of PCR using primers containing the NotI restriction site. The PHOX2A in pCDNA3 plasmid was used as the PCR template (24), and the PCR products were cloned in the pBIND and pACT vectors after NotI enzymatic digestion.

Reporter Plasmids—The *DBH* promoter reporter plasmid construct was obtained by cloning a 993-bp *DBH* regulatory region into pGL4 basic vector (Promega), as described previously (21).

Cell Cultures, Transient Transfections, and Luciferase Assays—The HeLa cells were grown in Dulbecco's modified Eagle's medium (Lonza), and the SK-N-BE(2)C cells were maintained in RPMI 1640 without L-glutamine (Lonza). Each medium was supplemented with 10% fetal calf serum (PAA Laboratories), 100 units/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza), and 2 mM L-glutamine (Lonza).

The cells were transiently transfected or co-transfected by means of lipofection (FUGENE HD, Promega) as described previously (21, 25, 26) using 1.5×10^5 SK-N-BE(2)C or 5×10^4 HeLa cells.

In mammalian two-hybrid experiments, 80 fmol of each expression vector were combined with 160 fmol of pG5LUC plasmid. The luciferase assay was carried out using the Dual-Luciferase reporter assay system (Promega) as described previously (26, 27). 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. The numbers of independent transfection experiments are indicated in the figure legends.

Immunofluorescence—Immunofluorescence was performed as described previously (25). HeLa cells plated on 1.7×1.7-cm^2 glass coverslips were grown to 50% confluence and transfected with the expression plasmid of interest (160 fmol of DNA). The HA-tagged PHOX2B proteins were detected by means of primary rabbit anti-HA antibody (1:50; Sigma, catalog no. H6908) and the secondary Alexa Fluor 488 anti-rabbit antibody (1:400; Invitrogen, catalog no. A11034).

The GAL4 BD and VP16 AD fusion proteins (except for VP16 AD-Nter, VP16 AD-Nter + HD, and VP16 AD-HD) were analyzed using primary chicken anti-PHOX2B antibody (1:100 (25)) and the secondary DyLight 549-conjugated donkey anti-chicken antibody (1:200; Jackson ImmunoResearch, Inc. (West Grove, PA), catalog no. 703-505-155). The VP16 AD-Nter, VP16 AD-Nter + HD, and VP16 AD-HD proteins were analyzed using mouse VP16 antibody (Santa Cruz Biotechnology, Inc., catalog no. sc-7546) and the secondary DyLight 549-conjugated goat anti-mouse antibody (1:400; Jackson ImmunoResearch, catalog no. 115-505-146). The nuclei were stained with DAPI, and the images were acquired using an LSM 510 Meta confocal microscope (Carl Zeiss, Inc.) with ×63 Nikon Apochromat lenses (1.5 numerical aperture).

All of the immunofluorescence analyses were replicated three times, representing independent transfections, and representative images are shown in Figs. 1, 2, 3, 6, 7, 8, and 9.

EMSAs—The EMSAs were performed as described previously (25, 28). The *in vitro* expression of PHOX2A, wild-type PHOX2B, and the deletion and mutant variants was obtained using a commercially available rabbit reticulocyte lysate system (TNT Quick-coupled Transcription/Translation System, Promega), as described previously (25). The oligonucleotides bearing the ATTA 2 and ATTA 3-4 sites of the *PHOX2B* promoter have been described previously (25). The homeodomain binding site oligonucleotide corresponding to the PHOX2B binding site in the *PHOX2A* promoter and the oligonucleotides PRS1 and PBD2 have been described previously (26, 29, 30). All of the oligonucleotides were purchased from Sigma-Aldrich. The antibodies used in EMSA experiments are the rabbit anti-HA antibody (Sigma, catalog no. H6908) and the chicken anti-PHOX2A and PHOX2B antibodies, previously validated and characterized (24, 25). The EMSA experiments were replicated at least two times, using different batches of rabbit reticulocyte lysate.

Co-immunoprecipitation Assays and Western Blotting Analyses—HeLa cells (8×10^5) were plated in 100-mm Petri dishes and transiently transfected by means of lipofection (FUGENE HD, Promega) with 700 fmol of each expression vector and harvested after 24 h, when the total extracts were prepared for immunoprecipitation. Proteins were extracted in lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM $MgCl_2$, 0.2 mM phenyl-

methylsulfonyl fluoride, Sigma protease inhibitors mixture, and 250 units/ml Pierce universal nuclease for cell lysis (Thermo Fisher Scientific)). Lysates were clarified by 30 min of centrifugation at 16,000 × *g* and 4 °C to remove cell debris and afterward precleared using 20 μl of protein G-agarose bead slurry (Invitrogen) for 1 h under constant rotation. The precleared extracts were incubated overnight at 4 °C with 5 μg of each primary antibody (monoclonal mouse anti-MYC antibody (Sigma-Aldrich, catalog no. M5546), polyclonal rabbit anti-HA antibody (Sigma-Aldrich, catalog no. H6908), and preimmune mouse or rabbit IgG (Santa Cruz Biotechnology, catalog nos. sc-2025 and sc-2027)), and the immunocomplexes were captured by protein G-agarose bead slurry for 4 h at 4 °C with rotation.

The beads were collected by centrifugation and gently washed and resuspended in sample loading buffer. The immunocomplexes were dissociated from the beads by boiling the samples and then separated by SDS-PAGE and transferred onto nitrocellulose membrane. Western blotting was performed as described previously (24) using the primary anti-MYC (1:1000; Sigma-Aldrich, catalog no. M5546) and anti-HA (1:500; Sigma-Aldrich, catalog no. H6908) antibodies. All of the co-immunoprecipitation experiments were replicated three times, representing independent transfections, and representative immunoblotting images are shown in Figs. 1–4.

Results

PHOX2B Forms Homodimers—GST pull-down and gel filtration chromatography experiments have previously shown that wild-type PHOX2B protein forms homodimers *in vitro* (7, 20, 31). To verify these observations in a more physiological context that takes into account the possible influences of the cell environmental factors, such as interactors and post-translational modifications, we tested the ability of PHOX2B to dimerize by co-immunoprecipitation.

To this end, we transiently transfected HeLa cells, which do not endogenously express PHOX2B, with MYC- and HA-tagged PHOX2B encoding plasmids, and when the proteins were immunoprecipitated with the anti-MYC antibody, the precipitated complex also contained HA-PHOX2B variant (Fig. 1*A*, *lane 2*), indicating PHOX2B homodimer formation in mammalian cells. As a control, protein G-agarose beads coated with a preimmune antibody did not show HA signal after immunoprecipitation (Fig. 1*A*, *lane 3*). This positive interaction was confirmed by means of the anti-HA antibody immunoprecipitation (Fig. 2*A*, *lane 2*).

Next, we extended our analysis by examining PHOX2B self-interactions using a mammalian two-hybrid system. Thus, we generated PHOX2B fusion proteins respectively with the yeast GAL4 DNA binding domain (GAL4 BD) and herpes simplex virus VP16 activation domain (VP16 AD).

We first tested their expression and subcellular localization by transfecting GAL4 BD-PHOX2B- and VP16 AD-PHOX2B-encoding plasmids into HeLa cells and analyzed the transfected cells by means of confocal microscopy upon immunohistochemistry with an antibody directed against the PHOX2B C terminus; as shown in Fig. 1*B*, both the GAL4 BD-PHOX2B (*a*, *c*, and *e*) and VP16 AD-PHOX2B (*b*, *d*, and *f*) proteins localized diffusely in the nucleus.

The two fusion constructs were then co-transfected, alone or in combination, together with a reporter construct containing five GAL4 binding sites upstream of a minimal TATA box controlling the firefly luciferase reporter gene (pG5LUC) into neuroblastoma SK-N-BE(2)C (Fig. 1*C*) and HeLa cells (Fig. 1*D*).

When VP16 AD-PHOX2B was co-transfected with the GAL4 DNA binding domain (GAL4 BD) or the VP16 activation domain (VP16 AD) in SK-N-BE(2)C and HeLa cells, no significant increase in luciferase activity was measured in comparison with the empty vectors (GAL4 BD and VP16 AD) (Fig. 1, *C* and *D*, *gray bars versus white bars*), excluding nonspecific positive interactions between PHOX2B and VP16 AD or GAL4 BD.

Also, GAL4 BD-PHOX2B did not show nonspecific interactions with the VP16 activation domain (VP16 AD) because there was little if any increase (in SK-N-BE(2)C cells) or a significant decrease (HeLa cells) in luciferase activity in comparison with the empty vectors (GAL4 BD and VP16 AD) (Fig. 1, *C* and *D*, *hatched versus white bars*). This also indicated that PHOX2B does not autonomously activate transcription by the reporter construct. A decrease in luciferase activity has previously been observed in the case of other paired-type homeoproteins, thus suggesting that direct DNA binding by the homeodomain is required for transactivation activity (32–34).

On the contrary, when both fusion constructs (GAL4 BD-PHOX2B and VP16 AD-PHOX2B) were co-transfected in SK-N-BE(2)C (Fig. 1*C*, *black bar*) and HeLa cells (Fig. 1*D*, *black bar*), there was a 3- and 8-fold increase, respectively, in luciferase activity, and the lower luciferase activity measured in SK-N-BE(2)C cells was probably due to the lower transfection efficiency, in comparison with HeLa cells. Our results thus indicate that PHOX2B homodimers can be efficiently detected by using both a co-immunoprecipitation assay and mammalian two-hybrid system.

PHOX2B Polyalanine-expanded Proteins Interact Weakly with Wild-type Protein—Mutant proteins can interfere with the activity of the wild-type protein by forming functionally impaired heterodimers and thus have a dominant negative effect. Much evidence has shown that PHOX2B polyalanine-expanded proteins can easily form aggregates, and this has been reasonably interpreted as a consequence of an increased tendency of PHOX2B to self-interact, as has been observed previously in the case of long polyalanine stretches and homopolymeric tracts in general (35). Previous gel filtration experiments have also shown that an important fraction of the mutants with shorter expansions retains the ability to form dimers, whereas there is virtually no formation of species corresponding to the wild-type dimers in the presence of proteins with increasingly long polyalanine tracts (20), and both *in vitro* and *in vivo* experiments suggest the existence of interactions between wild-type and mutant proteins (20–22).

To test the ability of mutant proteins to heterodimerize, we transiently transfected HeLa cells with HA-tagged PHOX2B WT plasmid in combination with MYC-tagged PHOX2B variants carrying the most frequently identified polyalanine expansion in CCHS patients (+7 alanine) or the longest expansion

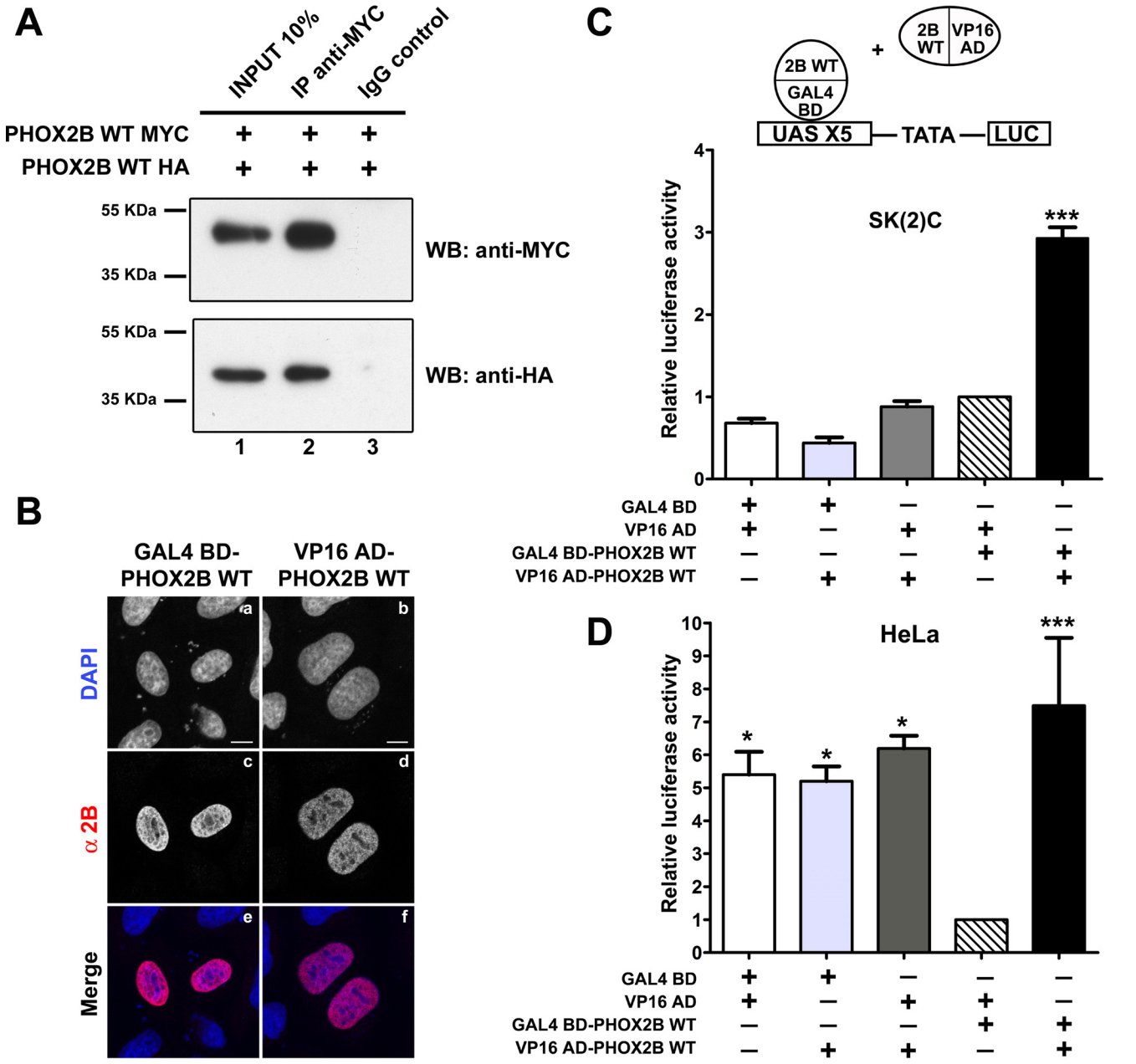

FIGURE 1. **Homodimerization of PHOX2B wild-type protein.** *A*, representative immunoblotting images of co-immunoprecipitation of MYC- and HA-tagged PHOX2B proteins in transfected HeLa cells. Cell extracts were immunoprecipitated with anti-MYC antibody or control immunoglobulin (IgG) and immunoblotted with anti-MYC (*top*) and anti-HA antibodies (*bottom*). *B*, representative immunofluorescence images of the localization of the GAL4 BD- and VP16 AD-tagged PHOX2B WT fusion proteins in transfected HeLa cells stained using anti-PHOX2B antibody (*c* and *d*). The nuclei were visualized using DAPI (*a* and *b*) and merged with the proteins detected by the anti-PHOX2B antibody (*e* and *f*). *Scale bars*, 10 μm. *C* and *D*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in neuroblastoma SK-N-BE(2)C (*C*) or HeLa cells (*D*) with a combination of equimolar amounts of a vector containing the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT) and the empty vector containing VP16 AD (*hatched bars*) or with a combination of equimolar amounts of both GAL4 BD and VP16 wild-type fusion proteins (*black bars*). Nonspecific interactions were excluded upon co-transfection of PHOX2B fused to VP16 AD with VP16 AD or GAL4 BD (*light* and *dark gray bars*). The background level of firefly luciferase expression from the pG5LUC vector was determined upon co-transfection with empty vectors containing GAL4 BD and VP16 AD (*white bars*). The results are the means ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate (*C* and *D*, $n = 5$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B WT protein (= 1). *, significant differences from the activity of the wild-type protein fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.05$); ***, significant differences from the activity of the wild-type protein fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.001$).

(+13 alanine) and examined their binding potential by co-immunoprecipitation assays.

Our results revealed that mutant proteins immunoprecipitated with PHOX2B wild-type protein and that there was a progressively weaker interaction of the mutant proteins as a function of the length of the expansion (Fig. 2*A*, *lanes 5* and *8 versus lane 2*). Moreover, we observed a reduced amount of mutant proteins in total cell lysates in comparison with wild-type protein (Fig. 2*A*, *lanes 4* and *7 versus lane 1*), due to their decreased solubility after detergent extraction, as confirmed by the higher proportion of mutant proteins in the insoluble fraction (data not shown and previously reported (22)). To exclude

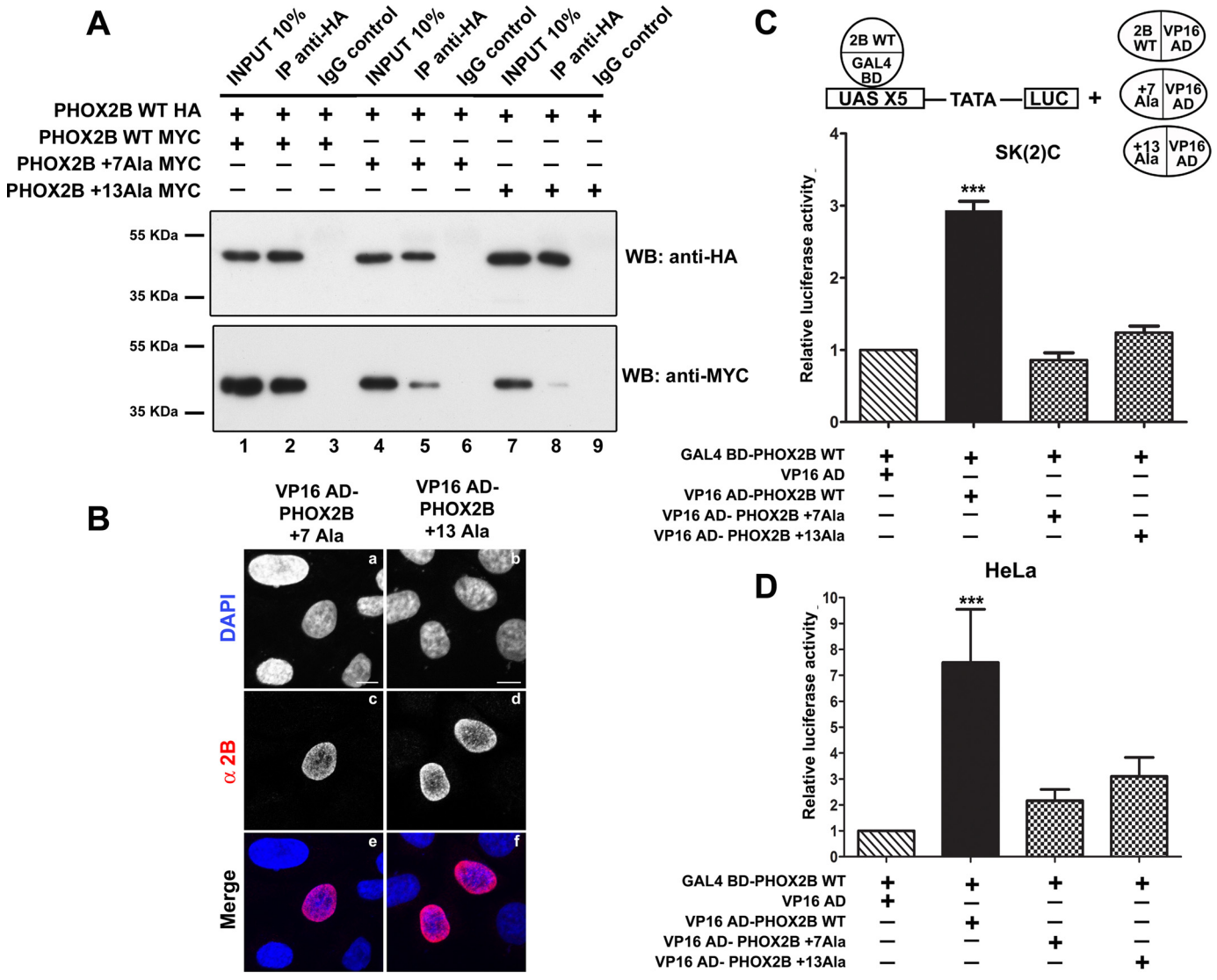

FIGURE 2. **Heterodimerization of PHOX2B polyalanine-expanded mutants with wild-type protein.** *A*, representative immunoblotting images of co-immunoprecipitation of HA-tagged PHOX2B protein along with MYC-tagged PHOX2B polyalanine-expanded mutants in transfected HeLa cells. Cell extracts were immunoprecipitated with anti-HA antibody or control immunoglobulin (IgG) and immunoblotted with anti-HA (*top*) and anti-MYC antibodies (*bottom*). *B*, representative immunofluorescence images of the localization of the VP16 AD-PHOX2B +7Ala and VP16 AD-PHOX2B +13Ala fusion proteins in transfected HeLa cells stained using anti-PHOX2B antibody (*c* and *d*). The nuclei were visualized using DAPI (*a* and *b*) and merged to the proteins detected by the anti-PHOX2B antibody (*e* and *f*). *Scale bars*, 10 μm. *C* and *D*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in neuroblastoma SK-N-BE(2)C (*C*) or HeLa cells (*D*) with a vector containing the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT) in combination with the empty vector containing VP16 AD (*hatched bars*), the VP16 wild-type fusion protein (*black bars*), or the VP16-PHOX2B fusion protein carrying polyalanine expansions (*cross-hatched bars*). The results are the means ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate (*C* and *D*, $n = 5$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B WT protein (= 1). ***, significant differences from the activity of the wild-type protein fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.001$).

the possibility that the lower signals obtained with the mutants in co-immunoprecipitation experiments were due to the decreased protein solubility and to confirm our data, we extended our analysis by using the mammalian two-hybrid system. Co-transfection experiments of VP16 AD-PHOX2B +7Ala or VP16 AD-PHOX2B +13Ala with GAL4 BD-PHOX2B WT showed no functional interactions between the mutants and wild-type protein in SK-N-BE(2)C cells (Fig. 2*C*, *cross-hatched bars versus hatched bar*) and only slight and not statistically significant interactions in HeLa cells, with respect to PHOX2B WT and VP16 AD (Fig. 2*D*, *cross-hatched bars versus hatched bar*).

Immunofluorescence analysis of HeLa cells transiently transfected with VP16 AD-PHOX2B +7Ala and VP16 AD-PHOX2B +13Ala showed that both mutants localized completely in the nucleus (Fig. 2*B*, *a–f*), thus suggesting that the insertion of the nuclear localization signal encoded by the pACT vector is sufficient to force the mutant proteins into the nucleus, particularly the +13 alanine mutants, which usually have a partial cytoplasmic localization (19–21), and excluding the possibility that the reduced capability of the mutant proteins to interact with the wild-type protein may be due to their improper localization.

To evaluate the ability of the mutants to form homodimers, we fused GAL4 BD to PHOX2B carrying +7 or +13 alanine expansions. Unlike the VP16 fusion proteins, the GAL4 BD fusion proteins were not completely detected in the nucleus, thus confirming their tendency to mislocalize in the cytoplasm

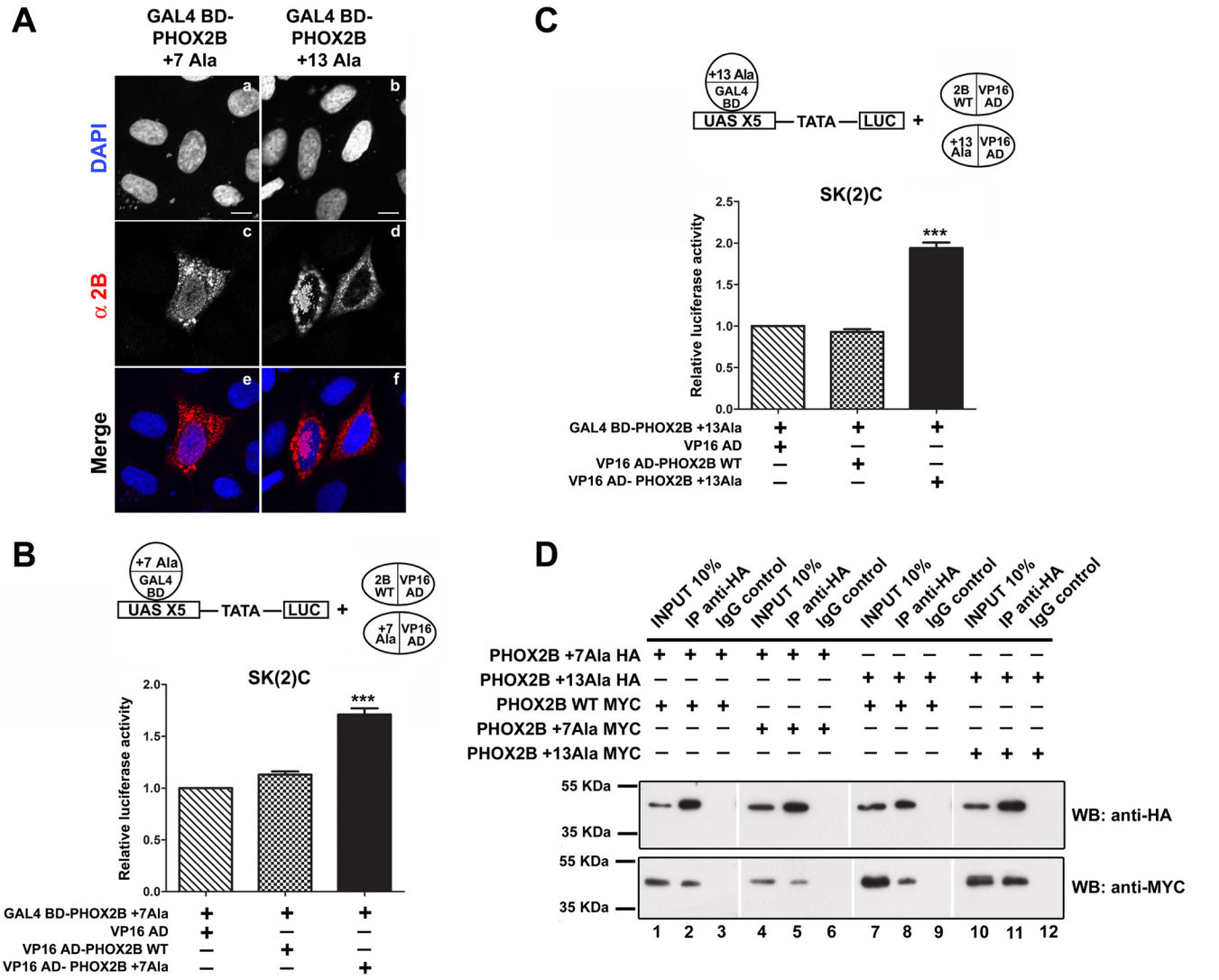

FIGURE 3. **Homodimerization of PHOX2B polyalanine-expanded mutants.** *A*, representative immunofluorescence images of the localization of the GAL4 BD-PHOX2B +7Ala and GAL4 BD-PHOX2B +13Ala fusion proteins in transfected HeLa cells stained using anti-PHOX2B antibody (*c* and *d*). The nuclei were visualized using DAPI (*a* and *b*) and merged with the proteins detected by the anti-PHOX2B antibody (*e* and *f*). *Scale bars*, 10 μm. *B* and *C*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in neuroblastoma SK-N-BE(2)C cells with a vector containing the cDNA of PHOX2B +7Ala fused to GAL4 BD (GAL4 BD-PHOX2B +7Ala in *B*) or the cDNA of PHOX2B +13Ala fused to GAL4 BD (GAL4 BD-PHOX2B +13Ala in *C*), in combination with the empty vector containing VP16 AD (*hatched bars*), VP16 wild-type fusion protein (*cross-hatched bars*), or VP16-PHOX2B fusion protein carrying +7 (Fig. 3*B*) or +13 (Fig. 3*C*) alanine expansions (*black bars*). The results are the means ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate (*B* and *C*, $n = 4$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B +7Ala protein or the GAL4 BD-PHOX2B +13Ala protein (= 1). ***, significant differences from the activity of the PHOX2B protein +7 or +13 alanine fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.001$). *D*, representative immunoblotting images of co-immunoprecipitation of HA-tagged PHOX2B polyalanine-expanded mutants along with MYC-tagged PHOX2B wild-type and mutant variants in transfected HeLa cells. Cell extracts were immunoprecipitated with anti-HA antibody or control immunoglobulin (IgG) and immunoblotted with anti-HA (*top*) and anti-MYC antibodies (*bottom*).

and indicating that the nuclear localization signal encoded by pBIND vector is weaker and unable to counteract their nuclear import defects (Fig. 3*A*, *a–f*).

In SK-N-BE(2)C cells co-transfection experiments along with pG5LUC plasmid, we found a significant increase in luciferase activity, but the strength of the interactions in mutants forming homodimers was weaker than those observed in wild-type homodimers, probably due to the partial mislocalization of the GAL4 BD fusion proteins in the cytoplasm: 1.7- and 2-fold increases in the case of the +7 alanine and +13 alanine mutant homodimers, respectively (Fig. 3, *B* and *C*, compare *black bars* with *black bar* in Fig. 1*C*). Further, it should be noted that our data concerning interactions among mutants may not allow us to distinguish the formation of dimers and oligomers, but the measured luciferase activities apparently correlate with the increasing propensity of the expanded protein to aggregate as a function of the length of the polyalanine tract. Interactions among mutants were confirmed by co-immunoprecipitation (Fig. 3*D*, *lanes 5* and *11*), and the signal obtained with the +13 alanine mutant, compared with its respective input signal, was stronger than that shown by the +7 alanine mutant, thus suggesting, again, a correlation between the increasing formation of oligomers and the length of the polyalanine tract (Fig. 3*D*, *lane 5 versus lane 4* and *lane 11 versus lane 10*).

Because protein-protein interactions can be direction-dependent, we also assessed the dimerization of the mutants and

wild-type protein in the opposite orientation, but once again, we observed no functional interactions with the wild-type protein (Fig. 3, *B* and *C*, *cross-hatched bars*). However, in accordance with our previous co-immunoprecipitation experiments and those performed in HeLa cells using the mammalian two-hybrid system, a small proportion of the wild-type protein was immunoprecipitated with both mutants (Fig. 3*D*, *lanes 2* and *8*). These data indicate that the mutant proteins partially form homodimers and interact very weakly with the wild-type protein and that this was unlikely to be due to the incorrect localization of the mutants. However, the partial discrepancy between biochemical and luciferase data did not exclude the possibility that heterodimers are unable to reconstitute a functional transcription factor and thus stimulate transcription from the reporter gene.

PHOX2B Polyalanine-expanded Proteins Retain a Partial Ability to Interact with PHOX2A—GST pull-down and EMSA experiments have previously shown that PHOX2B protein and its paralogue PHOX2A form heterodimers *in vitro* (7, 20, 31). Overexpression of PHOX2A with the PHOX2B +13 alanine mutant did not show co-aggregation or trapping of PHOX2A in the cytoplasm (20). We decided to test the ability of mutant proteins to heterodimerize with PHOX2A by both co-immunoprecipitation and the mammalian two-hybrid system.

When the MYC-tagged version of PHOX2A together with HA-tagged PHOX2A or PHOX2B WT were overexpressed in HeLa cells, immunoprecipitation with the anti-MYC antibody showed interaction with both HA-PHOX2A and HA-PHOX2B (Fig. 4*A*, *lanes 2* and *5*), thus confirming the formation of PHOX2A homodimers and PHOX2A-PHOX2B heterodimers in mammalian cells. When we tested the ability of PHOX2B mutant proteins to interact with PHOX2A, similarly to our observations on PHOX2B-mutant heterodimer formation, the polyalanine-expanded tract reduced the solubility of PHOX2B mutants in comparison with wild-type protein and even more compared with PHOX2A (Fig. 4, *A* (*lanes 1* and *4*) and *B* (*lanes 1*, *4*, and *7*)) and also their binding to PHOX2A (Fig. 4*B*, *lanes 5* and *8 versus lane 2*). To exclude an effect of the protein extraction procedure on the reduced interaction between PHOX2A and mutant PHOX2B proteins, we tested homo- and heterodimer formation by the mammalian two-hybrid system. To this end, we fused GAL4 BD and VP16 AD to PHOX2A and first measured the ability of PHOX2A to form homodimers and heterodimers with PHOX2B. As shown in Fig. 4*C*, the luciferase activity measured when the GAL4 BD-PHOX2A protein was co-transfected with the VP16-counterpart was >2-fold greater than that obtained using PHOX2B fusion constructs (Fig. 4*C*, compare *white bar* with *black bar*), thus suggesting that PHOX2A forms homodimers more efficiently than PHOX2B. When we assessed the heterodimerization of PHOX2A with PHOX2B, we observed that their interaction is stronger than that measured in PHOX2B homodimers (Fig. 4*C*, *cross-hatched* and *gray bars versus black bar*) and direction-dependent (Fig. 4*C*, *cross-hatched versus gray bar*).

Co-transfection experiments of GAL4 BD-PHOX2A along with VP16-PHOX2B mutant fusion proteins (VP16 AD-PHOX2B +7Ala and VP16 AD-PHOX2B +13Ala) showed that the polyalanine expansions severely affect the ability of PHOX2B to form heterodimers with PHOX2A (Fig. 4*C*, *hatched bars versus gray bar*). Nevertheless, the strength of the interactions measured between PHOX2A and the PHOX2B mutants was comparable with or slightly weaker than that of PHOX2A homodimers (Fig. 4*C*, *hatched bars versus white bar*) and even higher than that of PHOX2B homodimers (Fig. 4*C*, *hatched bars versus black bar*). The above experiments indicate that PHOX2B mutants retain a partial ability to heterodimerize with PHOX2A and, therefore, suggest the possible formation of heterodimers (PHOX2A-PHOX2B mutants) with an efficiency comparable with that of PHOX2A homodimers. To investigate whether the relative strong binding of mutants to PHOX2A might compete with normal PHOX2A homodimerization, we performed competition experiments by co-transfecting equimolar amounts of PHOX2B WT or mutants encoding plasmids together with GAL4 BD- and VP16 AD-tagged PHOX2A. Whereas PHOX2B wild-type protein increased luciferase activity >2-fold (Fig. 4*D*, *black bar versus white bar*), both mutants showed a slight but not significant increase of luciferase activity (Fig. 4*D*, *hatched bars versus white bar*), thus suggesting that PHOX2A homodimer formation is not affected by the presence of PHOX2B mutants. A possible interpretation of the increased luciferase activity in the presence of the wild-type protein is that PHOX2B might stabilize PHOX2A homodimer formation and eventually promote transcriptional complex assembly. Remarkably, a similar increase in PHOX2B homodimer activity occurred in the presence of the wild-type protein (Fig. 4*E*, *black bar versus white bar*), whereas a slight reduction was measured in the presence of PHOX2B mutants (Fig. 4*E*, *hatched bars versus white bar*). Our data thus indicate that PHOX2B mutants do not significantly interfere with either PHOX2B or PHOX2A homodimerization.

PHOX2B Polyalanine-expanded Proteins Do Not Interfere with PHOX2A-mediated Transactivation of the DBH Promoter and, Conversely, Interact Synergistically—Given that PHOX2B mutants retain a partial ability to heterodimerize with PHOX2A, we tested whether PHOX2B mutants might alter PHOX2A-mediated transactivation of the *DBH* (dopamine-β-hydroxylase) promoter, a well characterized PHOX2A and PHOX2B target gene (7, 29, 30). As reported previously (19–21), the mutant proteins showed a marked reduction in their ability to induce the correct activation of the *DBH* reporter construct (Fig. 5*A*, *bars 3* and *4 versus bar 2*), and co-transfection of the polyalanine-expanded proteins with the wild-type protein led to luciferase activity similar to that of the normal PHOX2B alone (Fig. 5*A*, *bars 5* and *6 versus bar 2*). When the *DBH* reporter construct was co-transfected together with PHOX2A, we measured a greater induction by PHOX2B WT protein in comparison with PHOX2A (Fig. 5*A*, *bar 2 versus bar 8*), different from that previously reported (7, 29, 30); this discrepancy could possibly be due to the use of a different regulatory region (993 bp *versus* 232 bp long) and/or reporter plasmid (pGL4 *versus* pGL3 vector). Moreover, the combination of PHOX2A and PHOX2B increased *DBH*-driven luciferase activity to a slightly (but not significant) lower extent than that obtained with PHOX2B alone (Fig. 5*A*, *bar 7 versus bar 2*), confirming that the two factors act independently on the *DBH* promoter (7).

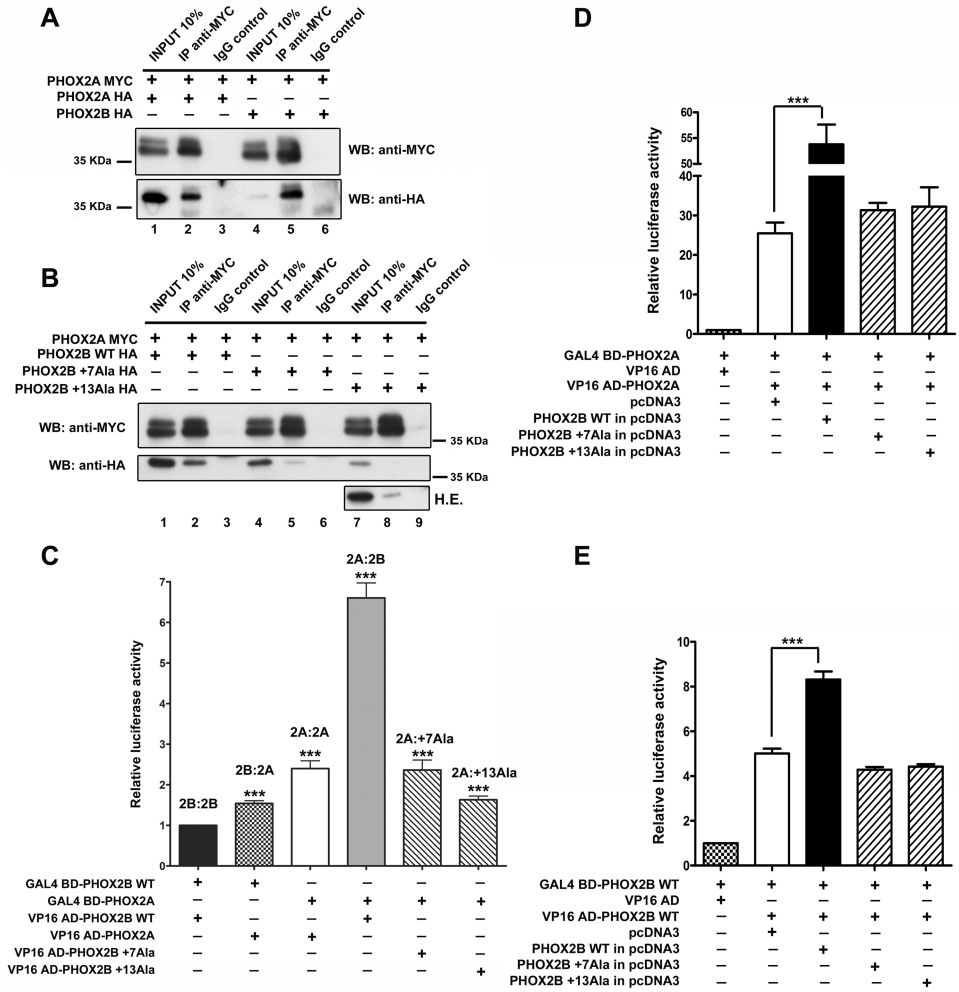

FIGURE 4. **Homodimerization of PHOX2A protein and its heterodimerization with PHOX2B wild-type protein and with polyalanine-expanded mutants.** *A* and *B*, representative immunoblotting images of co-immunoprecipitation of MYC-tagged PHOX2A along with HA-tagged PHOX2A, PHOX2B (*A*), and PHOX2B polyalanine-expanded mutants (*B*) in transfected HeLa cells. Cell extracts were immunoprecipitated with anti-MYC antibody or control immunoglobulin (IgG) and immunoblotted with anti-MYC (*top*) and anti-HA antibodies (*bottom*). *H.E.*, higher exposure. *C*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in HeLa cells with a vector containing the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT) in combination with the VP16 wild-type fusion protein (*black bar*) or the VP16-PHOX2A (*cross-hatched bar*); *white*, *gray*, and *hatched bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in HeLa cells with a vector containing the cDNA of PHOX2A fused to GAL4 BD (GAL4 BD-PHOX2A) in combination with the VP16-PHOX2A fusion protein (*white bar*), VP16-PHOX2B wild-type fusion protein (*gray bar*), or the VP16-PHOX2B fusion proteins carrying polyalanine expansions (*hatched bars*). The results are the means ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate ($n = 4$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B WT protein in combination with the VP16-PHOX2B wild-type fusion protein (= 1). ***, significant differences from the luciferase activity due to the wild-type protein homodimerization (ANOVA, Tukey's test, $p < 0.001$). *D* and *E*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct upon co-transfection in HeLa cells with a vector containing the cDNA of PHOX2A fused to GAL4 BD (GAL4 BD-PHOX2A) in combination with the VP16-PHOX2A fusion protein (*D*, *white bar*) or the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT) in combination with the VP16 wild-type fusion protein (*E*, *white bar*). *Black* and *hatched bars* indicate the transcriptional activity of the pG5LUC reporter upon the co-transfection of the above plasmids with equimolar amounts of a vector encoding PHOX2B WT (*black bars*) or PHOX2B mutants (*hatched bars*). The results are the mean values ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate (*D* and *E*, $n = 3$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2A protein (= 1; *D*) or the GAL4 BD-PHOX2B WT protein (= 1; *E*). ***, significant differences from the luciferase activity due to PHOX2B or PHOX2A homodimerization (ANOVA, Tukey's test, $p < 0.001$).

Interestingly, the luciferase activity measured when PHOX2A was co-transfected with the +7 alanine mutant protein was lower but more comparable with that obtained using the wild-type counterpart (23- and 32-fold over the empty vector, respectively), suggesting a (novel) synergistic interaction between the two proteins (Fig. 5*A*, *bar 9 versus bar 7*). A lower synergistic interaction was observed by combining the +13 alanine mutant with PHOX2A (Fig. 5*A*, *bar 10*) although not statistically significant.

Because PHOX2B mutants showed diminished DNA binding (20, 21), we tested by EMSAs whether the observed transcriptional effects correlated with alterations in their DNA binding properties in the presence of PHOX2A; we used the oligonucleotide corresponding to the site in the *DBH* promoter to which PHOX2A/2B bind as dimers (30). As previously reported with other PHOX2B binding sites (20, 21), the expansion of the polyalanine tract progressively reduced DNA binding (Fig. 5*B*, *lanes 9* and *12 versus lane 6*), with the DNA-+13 alanine mutant complex severely affected and hardly detectable.

The retarded band obtained with the *in vitro* expressed PHOX2A protein was undetectable in this set of experiments

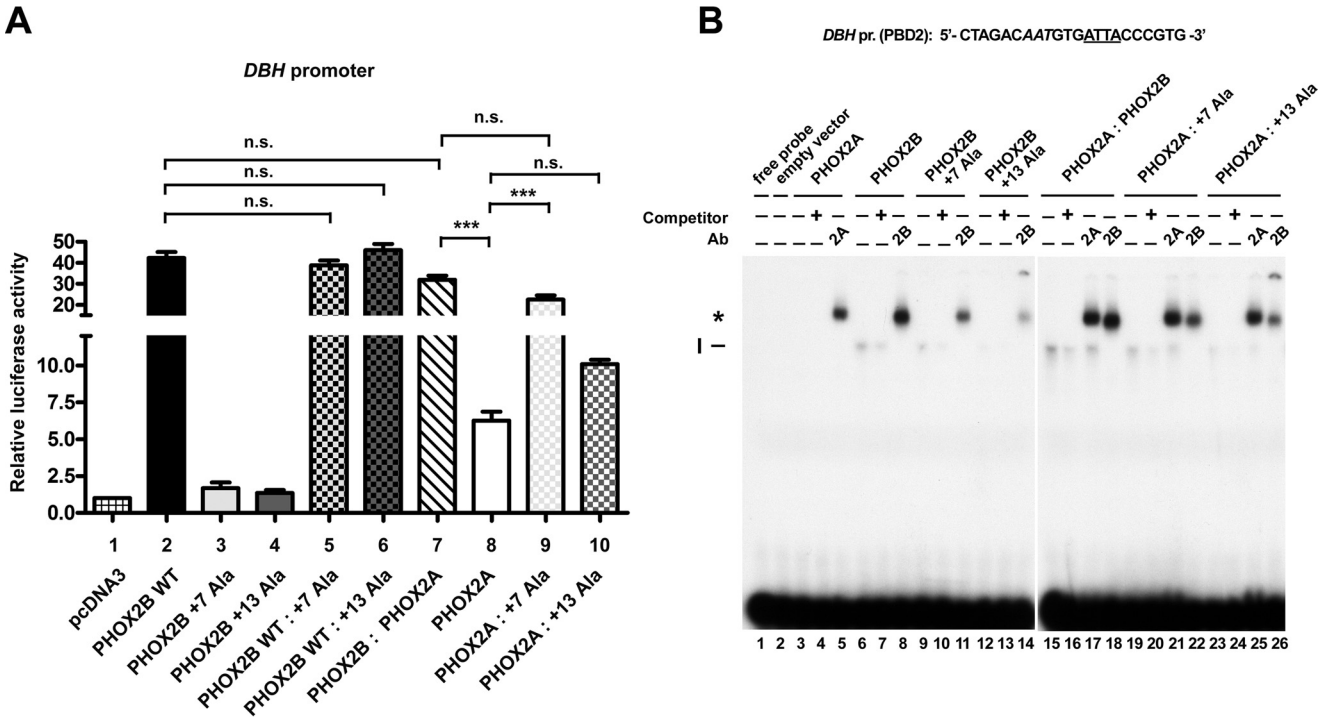

FIGURE 5. **Molecular effects of co-expressing the polyalanine-expanded mutants and PHOX2A on their transcriptional activity and DNA binding properties.** *A*, luciferase assays. The *bars* indicate the transcriptional activity of *DBH* promoter reporter construct upon co-transfection in HeLa cells with expression vectors containing the cDNA of PHOX2B WT protein (*bar 2*) or of PHOX2A (*bar 8*) or carrying the expanded polyalanine tracts (*bars 3* and *4*); *bars 5*, *6*, *7*, *9*, and *10* indicate the transcriptional activity of *DBH* promoter reporter construct upon co-transfection in HeLa cells with a combination of equimolar amounts of the indicated expression vectors. pcDNA3 indicates the empty vector used as negative control (*bar 1*). The results are mean values ± S.D. (*error bars*) of the transcriptional activity of the constructs of at least three experiments performed in triplicate ($n = 3$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the empty vector (= 1). ***, significant differences from the luciferase activity of the reporter plasmid co-transfected with PHOX2A-encoding vector (ANOVA, Tukey's test); *n.s.*, not significant (ANOVA, Tukey's test). *B*, gel shift assays using the oligonucleotide probe corresponding to a region of the *DBH* promoter containing the ATTA core motif known to bind PHOX2A and PHOX2B. *Top*, sequence of the oligonucleotide used as probe; the ATTA core motif is *underlined*, and the incomplete motif is in *italic type*. The labeled probe was incubated with *in vitro* expressed PHOX2A (*lanes 3–5*), PHOX2B wild-type protein (*lanes 6–8*), PHOX2B +7Ala (*lanes 9–11*), PHOX2B +13Ala proteins (*lanes 12–14*), or a combination of equimolar amounts of the indicated proteins (*lanes 15–26*). The *in vitro* translated pcDNA3 empty vector was used as a control to exclude nonspecific interactions (*lane 2*). The competitions were carried out by adding a molar excess of unlabeled oligonucleotide (*lanes 4*, *7*, *10*, *13*, *16*, *20*, and *24*). Supershift experiments were performed by preincubating the *in vitro* expressed proteins with anti-PHOX2A (*lanes 5*, *17*, *21*, and *25*) or anti-PHOX2B antibodies (*lanes 8*, *11*, *14*, *18*, *22*, and *26*). The *Roman numeral* and the *asterisk* on the *left* indicate the specific retarded complexes obtained and the supershifted complexes containing PHOX2A or PHOX2B. The free probes are shown at the *bottom* of the gels.

(Fig. 5*B*, *lane 3*), probably reflecting the relative low affinity of this consensus site and/or the lower binding ability of the *in vitro* expressed protein compared with the PHOX2A-containing nuclear lysates (24, 30, 36, 37); however, a strong ultraretarded band could be obtained in the presence of the anti-PHOX2A antibody (Fig. 5*B*, *lane 5*).

This effect was not due to the interaction of the antibody with other proteins contained in the reticulocyte lysate, because we had previously shown that both PHOX2A and PHOX2B antibodies did not recognize any protein in the lysate (24, 25), but it is in line with previous evidence showing that the antibody may stabilize the interactions between PHOX2A and PHOX2B and their cognate DNA binding sites (21, 24, 25, 36). No specific band was detectable in the presence of reticulocyte lysate programmed with the empty vector pcDNA3 (Fig. 5*B*, *lane 2*), thus excluding nonspecific interactions of the lysate with this probe.

When PHOX2A was combined with PHOX2B, a more intense retarded band than that with the PHOX2B wild-type protein alone was obtained (Fig. 5*B*, *lane 15 versus lane 6*), and the antibody directed against PHOX2A supershifted a more intense band than that observed using the PHOX2A protein alone (Fig. 5*B*, *lane 17 versus lane 5*), suggesting that heterodimers formed of both proteins have a higher DNA binding affinity. Interestingly, a band is still visible in the presence of the antibody against PHOX2A, indicating that only a fraction of the PHOX2B protein forms heterodimers with PHOX2A (Fig. 5*B*, *lane 17*). Similar to the band obtained with the wild-type protein, the retarded bands observed when the +7 and the +13 alanine mutants were mixed with PHOX2A were more intense than those detected with the expanded proteins alone (Fig. 5*B*, *lane 19 versus lane 9* and *lane 23 versus lane 12*). Notably, more intense supershifted bands were observed using the antibody against the PHOX2B mutants, indicating a partial rescue of the DNA binding of the mutants in the presence of PHOX2A (Fig. 5*B*, *lane 22 versus lane 11* and *lane 26 versus lane 14*).

The Dimerization Domain Encompasses the Homeodomain and the C-terminal Region of PHOX2B and Does Not Involve the Alanine Stretch—Despite the predictable central role of the homeodomain in PHOX2B dimerization, because our data from mammalian two-hybrid system experiments indicated that PHOX2B carrying polyalanine-expanded stretches was unable to interact with wild-type protein but at least partially conserved an ability to homodimerize, we estimated the involvement of the alanine tract in dimer formation by gener-

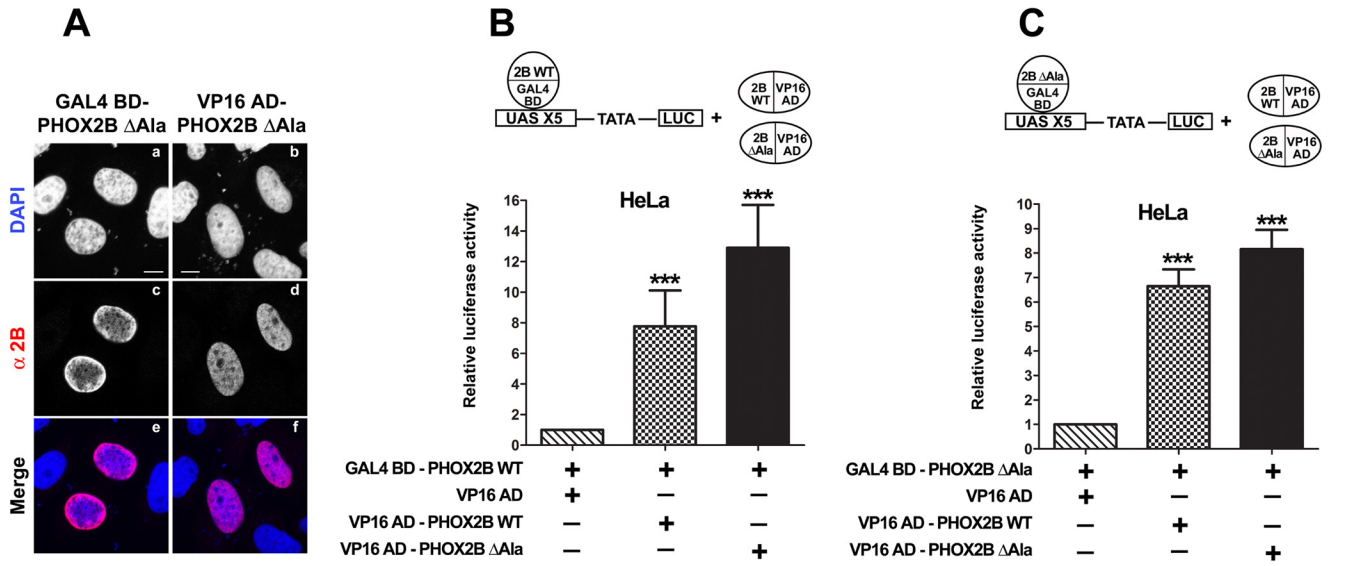

FIGURE 6. **Homodimerization of PHOX2B protein lacking the polyalanine tract and its heterodimerization with wild-type protein.** *A*, representative immunofluorescence images of the localization of the GAL4 BD- and VP16 AD-PHOX2B ΔAla fusion proteins in transfected HeLa cells stained using anti-PHOX2B antibody (*c* and *d*). The nuclei were visualized using DAPI (*a* and *b*) and merged with the proteins detected by the anti-PHOX2B antibody (*e* and *f*). *Scale bars*, 10 μm. *B* and *C*, luciferase assays of heterodimerization with the WT protein (*B*) or homodimerization (*C*) of PHOX2B ΔAla protein. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct in HeLa cells upon co-transfection with a vector containing the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT; *B*) or a vector containing the cDNA of the deleted protein fused to GAL4 BD (GAL4 BD-PHOX2B ΔAla; *C*) in combination with the empty vector containing VP16 AD (*hatched bars*), VP16 wild-type fusion protein (*cross-hatched bars*), or VP16-PHOX2B fusion protein deleted of the polyalanine stretch (*black bars*). The results are the means ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate ($n = 5$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B WT protein (*B*) or GAL4 BD-PHOX2B ΔAla protein (*C*) (= 1). ***, significant differences from the activity of the wild-type protein fused to GAL4 BD (*B*) or the GAL4 BD-PHOX2B ΔAla (*C*) (ANOVA, Tukey's test, $p < 0.001$).

ating two constructs expressing a polyalanine-deleted PHOX2B protein fused to the GAL4 BD or VP16 AD domain (GAL4 BD-PHOX2B ΔAla and VP16-PHOX2B ΔAla). As shown in Fig. 6*A*, the deletion of the polyalanine tract did not affect the intracellular localization of the proteins, as previously reported by Di Lascio *et al.* (21). The luciferase activity measured when the GAL4 BD-PHOX2B WT protein was co-transfected with the VP16-polyalanine-deleted protein was comparable with or even slightly greater than that obtained using the wild-type counterpart, thus suggesting that the deleted alanine tract does not affect the ability of PHOX2B to dimerize (Fig. 6*B*, compare *black bar* with *cross-hatched bar*). We likewise also detected strong homodimerization with the GAL4 BD-PHOX2B ΔAla and VP16-PHOX2B ΔAla fusion proteins, thus confirming the small contribution of the alanine stretch to the dimerization properties of PHOX2B (Fig. 6*C*, *black bar*).

To map the protein dimerization domain, a series of VP16 fusion constructs containing fragments of PHOX2B protein were generated on the basis of its homeodomain boundaries (Fig. 7*B*). We first used immunofluorescence to check the expression and localization of the deleted proteins. The VP16-PHOX2B 1–157 containing the homeodomain and the N-terminal region (Fig. 7*A*, *d–f*; Nter + HD), VP16-PHOX2B 98–157 corresponding to the homeodomain (Fig. 7*A*, *g–i*; HD), VP16-PHOX2B 98–314 including the homeodomain and the C-terminal region (Fig. 7*A*, *l–n*; HD + Cter), and the VP16-PHOX2B 155–314 construct containing the C-terminal region (Fig. 7*A*, *o–q*; Cter) all predominantly localized in the nucleus. Unexpectedly, the proteins containing the N-terminal region (*i.e.* Nter and Nter + HD) showed a very strong tendency to aggregate in the cytoplasm (Fig. 7*A*, *a–c*) and nucleus (Fig. 7*A*, *d–f*), respectively.

We therefore co-transfected the deleted proteins (excluding the construct containing the N-terminal region because it is localized in the cytoplasm) with the GAL4 BD-PHOX2B WT protein. A significant increase in luciferase activity was observed only with the fragment corresponding to the C-terminal region and homeodomain (2B 98–314), but it was less than that obtained using the full-length protein (Fig. 7*B*, compare *cross-hatched bars* with *black bar*); luciferase activity was comparable with that of the background in the case of the C-terminal fragment alone (2B 155–314) and only 2-fold above the background in the case of the fragment containing the N-terminal region and homeodomain (2B 1–157).

The findings of previous studies of other homeoproteins suggest that the region at the end of the homeodomain (helix III) might be critical for dimer formation (34, 38). Bearing in mind the spotlike distribution of the protein lacking the C-terminal region (Nter + HD) that could interfere with this process, we generated a deletion construct missing the region at the end of the homeodomain (encompassing amino acids 148–155). The obtained protein did not interact with the wild-type protein but, once again, aggregated in the nucleus (data not shown). Furthermore, the luciferase activity of the VP16 AD-HD construct (corresponding to the homeodomain) was comparable with that of the background and that obtained using the C-terminal fragment alone (Fig. 7*B*, 2B 98–157), thus suggesting that the presence and integrity of both domains are required for PHOX2B dimerization. We also found slightly lower luciferase activity in the absence of the N-terminal domain, although not

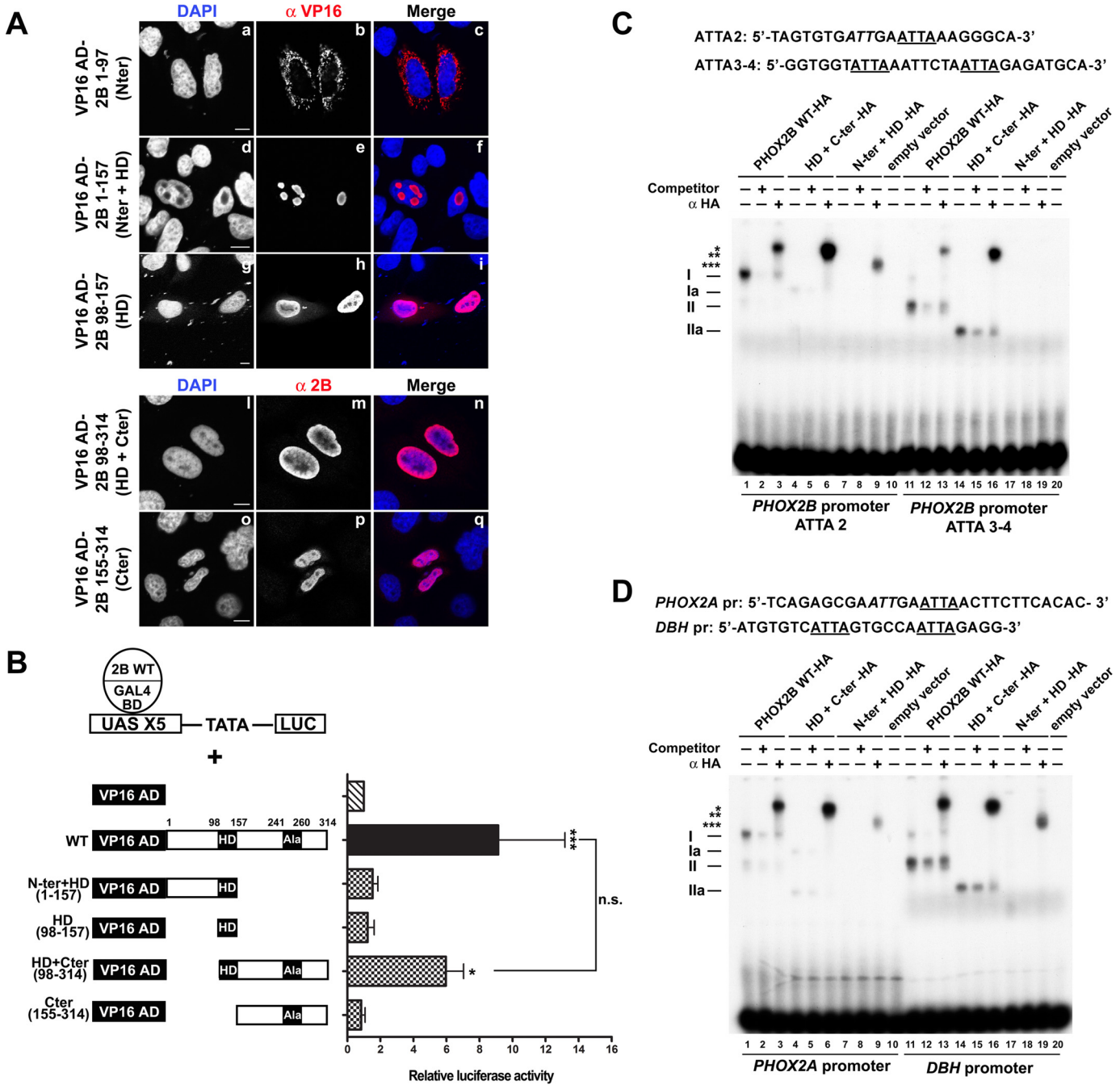

FIGURE 7. **Mapping of the PHOX2B dimerization domain and contribution of the N- and C-terminal domains to dimerization and DNA binding of PHOX2B.** *A*, representative immunofluorescence images of the localization of the VP16 AD-PHOX2B deleted fusion proteins (VP16 AD-Nter, VP16 AD-Nter + HD, VP16 AD-HD, VP16 AD-HD + Cter, VP16 AD-Cter) in transfected HeLa cells stained using anti-VP16 (*b*, *e*, and *h*) or anti-PHOX2B antibody (*m* and *p*). The nuclei were visualized using DAPI (*a*, *d*, *g*, *l*, and *o*) and merged with the proteins detected by the anti-VP16 and anti-PHOX2B antibodies (*c*, *f*, *i*, *n*, and *q*). *Scale bars*, 10 μm. *B*, luciferase assays. The *bars* indicate the transcriptional activity of the pG5LUC reporter construct in HeLa cells upon co-transfection with a vector containing the cDNA of wild-type protein fused to GAL4 BD (GAL4 BD-PHOX2B WT) in combination with the empty vector containing VP16 AD (*hatched bar*), VP16 wild-type fusion protein (*black bar*), or VP16-PHOX2B deleted fusion protein shown on the *left* (*cross-hatched bars*). The results are the mean values ± S.D. (*error bars*) of the transcriptional activity of the constructs detected in at least three experiments performed in triplicate ($n = 4$) and are expressed as -fold increases over the activity of the reporter plasmid co-transfected with the GAL4 BD-PHOX2B WT protein (= 1; *hatched bar*). *, significant differences from the activity of the wild-type protein fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.05$); ***, significant differences from the activity of the wild-type protein fused to GAL4 BD (ANOVA, Tukey's test, $p < 0.001$); *n.s.*, not significant (ANOVA, Tukey's test). *C* and *D*, gel shift assays using oligonucleotide probes corresponding to two regions of the *PHOX2B* promoter (*C*) or a region of the *PHOX2A* and *DBH* promoters (*D*) containing the ATTA core motifs known to bind PHOX2B. At the *top*, the sequences of the oligonucleotides used as probes; the ATTA core motif is *underlined*, and the incomplete motif is in *italic type*. The labeled probes were incubated with *in vitro* expressed HA-tagged PHOX2B wild-type protein (*lanes 1–3* and *11–13*), PHOX2B HD + Cter (*lanes 4–6* and *14–16*), or PHOX2B Nter + HD proteins (*lanes 7–9* and *17–19*). The *in vitro* translated pcDNA3 empty vector was used as a control to exclude nonspecific interactions (*lanes 10* and *20*). The competitions were carried out by adding a molar excess of unlabeled oligonucleotide (*lanes 2*, *5*, *8*, *12*, *15*, and *18*). Supershift experiments were performed by preincubating the *in vitro* expressed proteins with anti-HA antibody (*lanes 3*, *6*, *9*, *13*, *16*, and *19*). The *Roman numerals* on the *left* indicate the specific retarded complexes obtained using *in vitro* expressed PHOX2B wild-type protein (*I* and *II*) or PHOX2B HD + Cter (*Ia* and *IIa*); the *asterisks* indicate the supershifted complexes containing PHOX2B. The free probes are shown at the *bottom* of the gels.

statistically significant compared with that obtained with the full-length protein, thus not excluding the possibility that the N-terminal domain may also play a role in the dimerization process.

We also evaluated the role of the C-terminal and N-terminal domains in dimer formation and DNA binding using EMSAs and radiolabeled oligonucleotides corresponding to the *PHOX2B* promoter region containing ATTA core motifs known to bind PHOX2B (ATTA 2 and ATTA 3-4 motifs) (21, 25), the probe corresponding to the PHOX2B binding site in the *PHOX2A* promoter (26, 39), and the PRS1 oligonucleotide corresponding to a region within domain IV of the *DBH* promoter (29).

Incubation of the wild-type protein with each radiolabeled oligonucleotide caused the appearance of specific retarded bands (complexes I and II) that could be competed by a molar excess of cold oligonucleotide and supershifted by the anti-HA antibody (Fig. 7, *C* and *D*, *lanes 1–3* and *11–13*, *). Conversely, DNA binding of the C terminus-deleted protein (Nter + HD) to all of the probes was severely affected (Fig. 7, *C* and *D*, *lanes 7–9* and *17–19*), although a weak complex could be detected in the presence of the HA antibodies (Fig. 7*C*, *lanes 9* and *19*, ***) with the exception of the ATTA 3-4 motif, which suggests that the antibody may stabilize interactions between the truncated protein and DNA. The behavior of the N terminus-deleted protein (HD + Cter) was different; the migration of complexes I and II was faster because of the smaller size of the protein (complexes Ia and IIa), and the deleted protein retained a partial ability to bind DNA (Fig. 7, *C* and *D*, *lanes 4–6* and *14–16*), with complex Ia being the most affected. The same oligonucleotide shown in Fig. 7*D* (corresponding to a region of the *DBH* promoter and containing two homeodomain binding sites) has previously been used with PHOX2A protein in EMSA experiments (30), and it is worth noting that two DNA-protein complexes were observed: one formed when PHOX2A binds a single site as a monomer and another more retarded complex when two PHOX2A molecules simultaneously bind both sites (probably as monomers). We obtained a similar pattern (complex I and II) using the *PHOX2B* promoter (ATTA 3-4 motifs) and the *DBH* promoter probes, both of which contain two ATTA motifs in tandem position with seven and six intervening bases, respectively (*underlined* in Fig. 7, *C* and *D*), thus suggesting that PHOX2B binds to those sites in the *DBH* and *PHOX2B* promoters mainly as a monomer. Moreover, the authors also showed that PHOX2A binds two other probes as a dimer containing a single motif and a second potential incomplete motif with three intervening bases. Because comparison of the nucleotide sequences of the probes containing the ATTA 2 site of the *PHOX2B* promoter and the homeodomain binding site of the *PHOX2A* promoter revealed the presence of one conserved ATTA motif close to a second incomplete motif (in *italic type* in Fig. 7, *C* and *D*), we can reasonably speculate that the more intense retarded band observed in our experiments was probably formed by the binding of PHOX2B as a dimer (Fig. 7, *C* and *D*, *lane 1*, complex I). This suggests that the HD + Cter protein retained its ability to bind DNA as a monomer (complex IIa) but only partially as a dimer (complex Ia). Interestingly, the antibody against the HA tag fused to the HD + Cter protein supershifted a more intense band than that obtained using the PHOX2B WT-HA protein, thus suggesting that the deletion of the N-terminal domain affected the dimer DNA binding affinity but not its formation (Fig. 7, *C* and *D*, *lane 6*, **). Notably, faint bands were also observed using the Nter + HD protein, thus suggesting that it might form dimers *in vitro* (Fig. 7, *C* and *D*, *lane 9*, ***).

The Polyalanine-expanded Tract Interferes with Each Localization Signal and Blocks PHOX2B Homeodomain-mediated Import—Our results show that dimerization is impaired when the polyalanine stretch is elongated. Given that previous experiments have shown that nuclear import and DNA binding are also progressively reduced by polyalanine tract expansion (19–21) and that the homeodomain presumably plays a central role in all PHOX2B functions (*i.e.* nuclear import, DNA binding, and dimerization), we further characterized the role of the C-terminal region in modulating homeodomain activity, focusing our attention on nuclear import process.

Nuclear localization sequences (NLSs) are quite variable but generally consist of basic residues (40), and in the case of homeodomain proteins, an NLS is often found within or adjacent to the homeodomain itself (41–45). The PHOX2B protein contains two stretches of highly basic amino acids at both ends of the homeodomain (encompassing amino acids 95–102 and 148–155, respectively; Fig. 9*A*). To clarify further the nuclear import of PHOX2B, we identified and characterized PHOX2B NLSs. To confirm the role of the homeodomain in nuclear import of the protein, we cloned the same fragments of the PHOX2B protein (downstream of the smaller HA tag) as those used in the mammalian two-hybrid experiments (Fig. 8*A*) to exclude the possible effects of the heterologous nuclear signal of the VP16 AD tag on protein import and analyzed their subcellular localization by means of immunohistochemistry. We detected cells transfected with all of the truncated constructs except for Nter (corresponding to the N-terminal region), which was only detectable when a MYC tag was cloned downstream of the Nter region (Fig. 8*B*, *panels d-f*), probably because it makes the protein more stable. This confirms that, as previously reported by Wu *et al.* (46), the N-terminal domain of PHOX2B is extremely unstable.

Nuclear staining was only observed when the expressed truncated proteins were those containing the homeodomain and lacking the C-terminal (Nter + HD) or N-terminal region (HD + Cter) (Fig. 8*B*, *g–i* and *o–q*), thus confirming that the HD is required for nuclear localization (47). Moreover, the HD *per se* localized in the nucleus, whereas the Nter and Cter regions were excluded from it, thus indicating that the HD is both required and sufficient for nuclear import (Fig. 8*B*, compare *l–n* (HD) with *d–f* (Nter) and *r–t* (Cter)). It is worth noting that all of the cells transfected with one of the two constructs containing the N-terminal domain (Nter and Nter + HD, Fig. 8*B*, *d–i*) showed very strong cytoplasmic and nuclear aggregation, respectively, thus suggesting that the C-terminal portion of PHOX2B plays a role in keeping the protein in a soluble state, as in the case of the wild-type protein (Fig. 8*B*, *a–c*) and the truncated constructs containing the C terminus (Fig. 8*B*, *o–t*). Remarkably, the peculiar dotlike pattern of the Nter + HD protein showed that the truncated protein is localized in specific

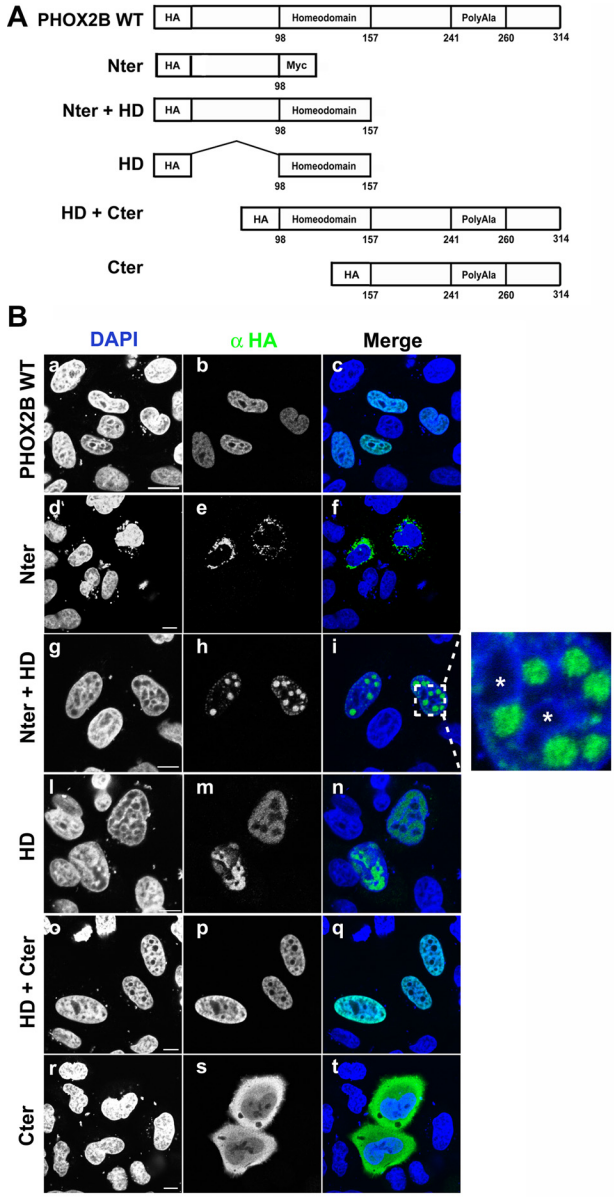

FIGURE 8. **Contribution of the N- and C-terminal domains to nuclear import of PHOX2B.** *A*, schematic representation of wild-type PHOX2B protein and its truncated constructs. All constructs were fused N-terminally to an HA epitope tag. *Numbers* correspond to the amino acids residues of PHOX2B. *B*, representative immunofluorescence images of the localization of HA-PHOX2B truncated fusion proteins. HeLa cells were transfected with the HA-tagged proteins and analyzed 48 h after transfection by means of immunofluorescence using anti-HA antibody (*b*, *e*, *h*, *m*, *p*, and *s*); the nuclei were visualized using DAPI (*a*, *d*, *g*, *l*, *o*, and *r*) and merged with the proteins detected by the anti-HA antibody (*c*, *f*, *i*, *n*, *q*, and *t*). On the *right* of *i*, an *enlarged view* of the indicated area is shown; the *asterisks* indicate the nucleoli.

foci, corresponding to nuclear regions with weak DAPI staining, which seem to be distinct from the nucleoli surrounded by a characteristic ring of DAPI-positive chromatin (marked with *asterisks* in Fig. 8*B*, *enlarged view* of the indicated area in *i*).

To characterize the role of the two stretches of basic residues at both ends of the homeodomain, we first generated a construct encoding a protein carrying the deletion of the entire HD except for the two putative NLSs (PHOX2B Δ106–147, Fig. 9*B*). As shown in Fig. 9*B* (*a–c*), the deletion did not affect nuclear localization, thus suggesting that the two regions (or at least one) are required for PHOX2B nuclear import. To examine the functional role of each NLS motif in PHOX2B nuclear localization, we introduced deletions into the PHOX2B expression vector that eliminated one or both stretches.

Deletion of the proximal NLS (PHOX2B ΔNLS1) led to nuclear staining of the fusion protein with substantial cytoplasmic fluorescence (Fig. 9*B*, *d–f*), whereas deletion of the NLS2 region (PHOX2B ΔNLS2) led to a more marked cytoplasmic localization (Fig. 9*B*, *g–i*). However, in both cases, the DAPI staining was distorted, and we observed nuclear inclusions corresponding to DAPI-negative regions that resembled the findings obtained when the Nter + HD deletion construct was overexpressed (compare Fig. 9*B*, *d–i*, with Fig. 8*B*, *g–i*).

PHOX2B carrying deletions of both NLS motifs (PHOX2B ΔNLS1–2) accumulated in the cytoplasm around the nucleus (Fig. 9*B*, *l–n*), thus confirming that the concerted action of both regions is required for the nuclear localization of the protein.

To assess the effects of the polyalanine expansions on the homeodomain-mediated nuclear import, we inserted the longest polyalanine expansion (+13) into the constructs carrying the single or double NLS deletion. As shown previously (19–21), the cells transfected with the +13 alanine mutant showed cytoplasmic staining and aggregation as well as nuclear staining (Fig. 9*C*, *a–c*). Unlike the wild type, the combined presence of the expanded polyalanine tract and the deletion of just one NLS (PHOX2B +13Ala ΔNLS1 and PHOX2B +13Ala ΔNLS2) is almost sufficient to completely block protein import (Fig. 9, compare *C* (*d–i*) with *B* (*d–i*)).

Moreover, after deletion of the N-terminal domain, the presence of the C-terminal portion carrying the expanded polyalanine tract directed the homeodomain to a subcellular localization that was different from that of its normal counterpart, which was localized exclusively inside the nucleus (Fig. 8*B*, *o–q*); a fraction of the truncated protein was in the cytoplasm and formed aggregates (Fig. 9*D*, *d–f*), thus suggesting that the expanded C terminus actively interferes with the correct folding of the homeodomain, leading to aggregation. Interestingly, the expanded C-terminal domain localized diffusely in the cytoplasm, like the C-terminal carrying the normal polyalanine tract (Fig. 8*B*, *r–t*), thus indicating that the polyalanine tract *per se* does not massively aggregate (Fig. 9*D*, *a–c*). The above experiments indicate that the nuclear import of the protein is regulated by the homeodomain and that the expanded C terminus interferes with this process.

Discussion

In the first part of this study, we analyzed the dimerization properties of PHOX2B wild-type and mutant proteins and their possible interactions in a cell model using co-immunoprecipitation and a mammalian two-hybrid system. The analyses confirmed the formation of wild-type homodimers, as already shown *in vitro* (7, 20), and the formation of mutant homodimers/oligomers (although the interactions observed by measuring luciferase activity in mammalian two-hybrid experiments were weaker than those observed in the homodimers of wild-type protein, probably due to the partial mislocalization of the GAL4 BD-tagged expanded proteins) and indicated that

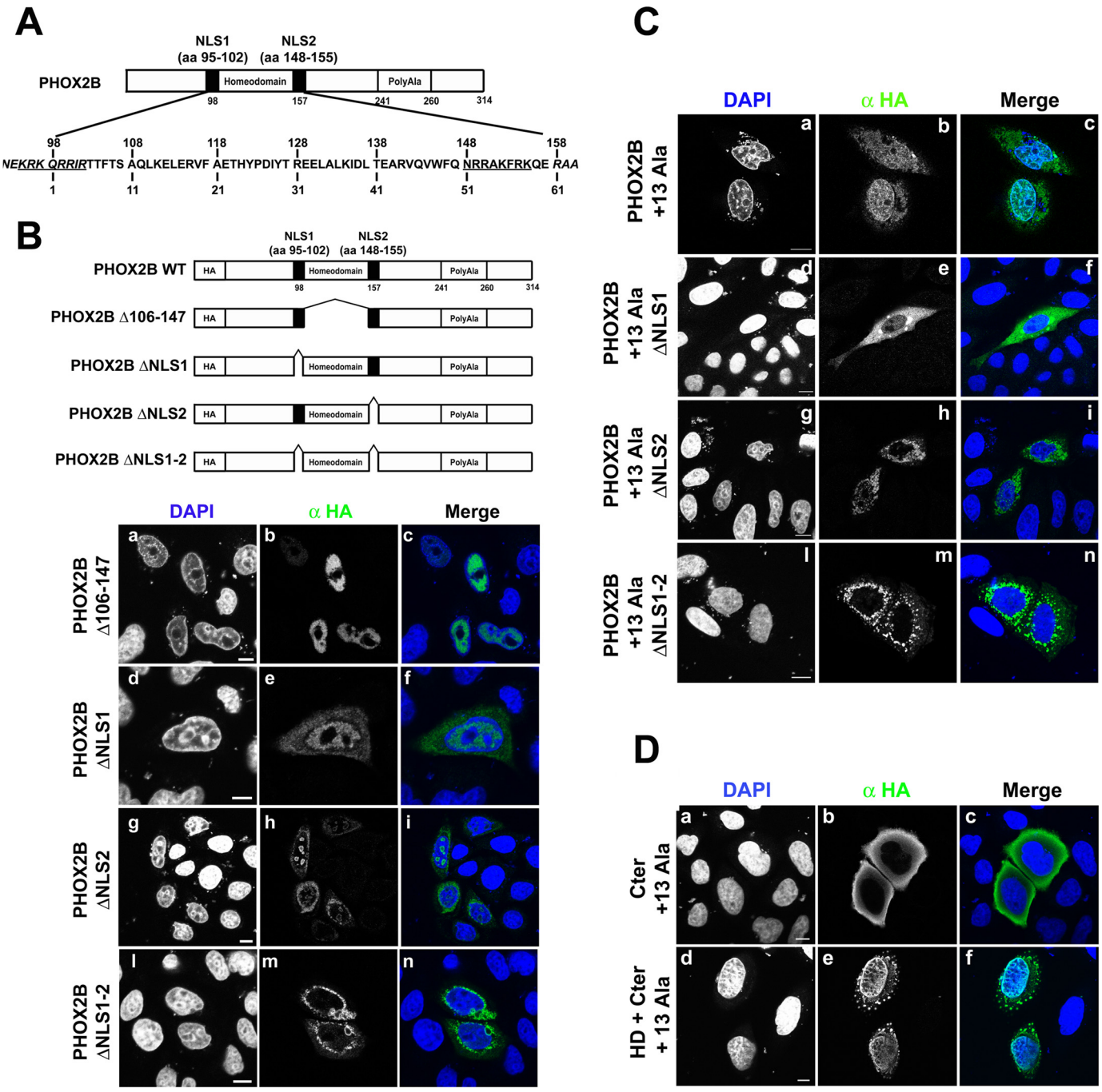

FIGURE 9. **Identification of PHOX2B NLSs and effects of the polyalanine-expanded tract on PHOX2B nuclear import.** *A*, schematic representation of the PHOX2B protein showing the sequence of the homeodomain and the two putative NLSs (*underlined*). *B*, *top*, schematic representation of PHOX2B carrying deletions of the proximal NLS (ΔNLS1), distal NLS (ΔNLS2), both (ΔNLS1-2), or the entire HD except for the NLSs (Δ106–147). *Black boxes*, NLSs. *Bottom*, representative immunofluorescence images of the subcellular localization of HA-tagged PHOX2B deletion proteins in transfected HeLa cells stained by anti-HA antibody (*b*, *e*, *h*, and *m*); the nuclei were visualized using DAPI (*a*, *d*, *g*, and *l*) and merged with the HA-PHOX2B deleted proteins detected by anti-HA antibody (*c*, *f*, *i*, and *n*). *Scale bars*, 10 μm. *C*, representative immunofluorescence images of the subcellular localization of HA-tagged PHOX2B carrying +13 alanine expansions (PHOX2B +13Ala) and the expanded deletion proteins (PHOX2B +13Ala ΔNLS1, PHOX2B +13Ala ΔNLS2, and PHOX2B +13Ala ΔNLS1-2) in transfected HeLa cells stained by anti-HA antibody (*b*, *e*, *h*, and *m*). The nuclei were visualized using DAPI (*a*, *d*, *g*, and *l*) and merged with the HA-PHOX2B deleted proteins detected by anti-HA antibody (*c*, *f*, *i*, and *n*). *Scale bars*, 10 μm. *D*, representative immunofluorescence images of the localization of the expanded HA-PHOX2B truncated fusion proteins. HeLa cells were transfected with the HA-tagged proteins and analyzed 48 h after transfection by means of immunofluorescence using anti HA antibody (*b* and *e*); the nuclei were visualized using DAPI (*a* and *d*) and merged with the proteins detected by the anti-HA antibody (*c* and *f*). *Scale bars*, 10 μm.

expanded PHOX2B mutants interact weakly with wild-type protein.

As in the case of wild type-mutant heterodimers, we detected functional weak interactions only in HeLa cells by the mammalian two-hybrid system, and taking into account that this experimental approach forces proteins into the nucleus and converts an interaction into a defined transcriptional readout, we also considered the possibility that the tendency to aggregation

shown by the expanded proteins could interfere with the correct folding of the VP16-tagged expanded proteins or that the formation of heterodimers do not reconstitute a functional transcription factor. However, we reasonably excluded both hypotheses because we were able to detect the formation of mutant homodimers and heterodimers with PHOX2A, although we cannot rule out the possibility that oligomers are formed, because two-hybrid systems have been successfully used to study the oligomerization of aggregation-prone proteins (particularly polyglutamine- and polyalanine-expanded proteins), thus suggesting that this assay is not intrinsically impaired by protein aggregation (35, 48). Interestingly, our data indicate that also the mutant with short (+7) alanine expansion, although still able to partially form homodimers *in vitro* (20), dramatically reduces its ability to dimerize with its wild-type counterpart. Moreover, the same defects have been observed with the mutant carrying the longest expansion (+13 alanine), and the slightly higher luciferase activity measured may correlate with its partial ability to sequester the wild-type protein in the nuclear and cytoplasmic aggregates (19, 21, 22). Therefore, our data exclude the idea that polyalanine-expanded mutants can form strong dimers with wild-type protein and that the formation of non-functional heterodimers may play a major role in CCHS pathogenesis.

In this study, we also analyzed the dimerization properties of PHOX2A and its ability to form heterodimers with PHOX2B wild-type and mutant proteins. Co-immunoprecipitation experiments showed similar interactions among PHOX2B mutated proteins and the wild-type counterpart or PHOX2A, but conversely, the mammalian two-hybrid system showed that PHOX2B and PHOX2A appear to have different interaction properties. Although the homeodomains of PHOX2B and PHOX2A are identical, we measured significantly different strengths of the interactions in the respective homodimers, and interestingly, our data indicate that the formation of PHOX2A-PHOX2B heterodimers is direction-dependent, suggesting a role for other domains of the proteins in modulating these interactions. Several lines of evidence *in vivo* showed that the two proteins are not functionally equivalent, and given that their C-terminal domains are very different and the role of the C-terminal domain of PHOX2B in homodimerization, it is reasonable to suppose that the formation of homo- or heterodimers may be responsible for the creation of different interfaces for differential binding of cofactors. Moreover, the partial ability of PHOX2B mutated proteins to form heterodimers with PHOX2A, with a comparable affinity with PHOX2A homodimers (at least for shorter expansions), suggests the possible presence of species of dimers that differ from conventional homo- and heterodimers.

The hypothesis that PHOX2B mutants may inhibit PHOX2A function in a dominant negative manner was first suggested by Trochet *et al.* (20); however, in that study, PHOX2A nuclear localization was not affected by the overexpression of +13 alanine mutant. Moreover, the recent data reporting specific defects in locus coeruleus development in two human cases of CCHS (one of which carried the +7 alanine mutation) (49) and the finding that PHOX2A is required for LC differentiation (50) have favored, once again, the hypothesis that PHOX2B mutant proteins may exert dominant negative effects on PHOX2A function. Our results with the *DBH* promoter suggest that PHOX2B mutants do not interfere with the transcriptional activity of PHOX2A, but, conversely, PHOX2A is able to synergize with PHOX2B mutants (and this effect is clearly evident at least with the +7 alanine mutant). This observation was unexpected and uncovers additional interesting characteristics of PHOX2B mutants that open the possibility that they may not be simply misfolded and non-functional molecules. Our data indicate first that, despite the reduced transcriptional activity, probably due to the diminished DNA binding, they have a (partial) conserved ability to interact with other components of the transcriptional complex and transcriptional activators and, second, that their defects can be partially counteracted by the interaction with molecules (such as PHOX2A) able to tether them to the promoter of target genes. However, because the nature of the interactors could vary according to the promoter, and we cannot exclude the possibility of new "toxic" interactions, the possible protective role of PHOX2A needs to be further elucidated and investigated. Although we are probably underestimating the interactions among mutants, because of the partial mislocalization of the GAL4 BD-tagged expanded proteins, our findings indicate that the mutants are able to form homodimers, and both the homeodomain and the C terminus are required; therefore, we can reasonably hypothesize that the expansion causes a conformational change in the C-terminal domain that partially blocks interactions between mutant and wild type but not those between mutants (or between mutants and PHOX2A) and that a strong structural constraint on the length of the polyalanine tract is necessary to impose the correct spatial distance and orientation between the homeodomain and the C-terminal region.

Our findings on PHOX2B nuclear import also support the idea of cross-talk between the homeodomain and the C terminus insofar as they show that PHOX2B has two strong functional cooperative NLSs in the homeodomain (a weaker NLS1 in the N-terminal arm and a stronger NLS2 in helix III) and that the polyalanine expansion alters their functionality. In line with this, we found that the polyalanine-expanded tract *per se* does not lead to visible intracellular aggregation because the cytoplasmic signal of the C terminus with the expanded polyalanine tract was diffuse and comparable with that of the wild-type counterpart, and the addition of the homeodomain to the expanded C terminus not only strongly shifts the protein into the nucleus, but also causes its partial aggregation in the cytoplasm. Because the homeodomain itself apparently does not aggregate, this suggests that the expanded C terminus actively interferes with its correct folding. Nuclear transport is a highly regulated process, and the proteins to be transported into or out of the nucleus are bound by transport receptors that recognize the NLSs in the cargo protein. One mechanism regulating protein nuclear import is to modulate the binding affinity of the transport receptor for the NLS cargo, which may occur in various ways: (i) the intermolecular masking of the NLS by a second macromolecule; (ii) the intramolecular masking of the NLS as a result of a post-translational modification within the NLS; and (iii) the intramolecular/interdomain masking of the NLS due to the protein taking on an inhibitory conformation. We

favor the idea that the normal C terminus adopts an "open" conformation that allows the homeodomain to function correctly and is then masked by the expanded C terminus in such a way as to prevent dimerization, DNA binding, and nuclear localization. A similar mechanism has been proposed for another homeoprotein containing an alanine stretch (extradenticle, the *Drosophila* homologue of PBX1A), and interestingly, the authors suggest that the stretch itself might be important for maintaining the correct conformation for protein nuclear localization (51). Another possible mechanism is the decreased release in the nucleus of the mutated cargo by transport receptors, as already reported for another homeoprotein (ARX) (52).

Unexpectedly, our findings indicate that, although unstable, the N-terminal region of the protein massively aggregates. Furthermore, the addition of the homeodomain to the N-terminal portion of PHOX2B is sufficient to stabilize it (thus forcing the protein into the nucleus) but apparently does not block the aggregation process. In agreement with our data, it has been shown that the K155X mutant (found in a CCHS patient), which lacks the entire C terminus, aggregates in both the nucleus and the cytoplasm. This is probably because of the absence of the last three residues of the homeodomain, which, as has been previously shown, are important for the NLS2 activity (31). Alternatively, as the truncated proteins accumulate in nuclear regions with weak DAPI staining, which seem to be distinct from the nucleoli surrounded by a characteristic ring of DAPI-positive chromatin, we cannot rule out the possibility that, instead of aggregating, the truncated protein is localized in specific foci. The different pattern observed with the HD + Cter protein, which was indistinguishable from that of the full-length protein, suggests that the C-terminal domain plays a major role in protein solubility and, possibly, protein targeting to the proper subnuclear regions. This is probably a result of greater DNA binding affinity, as supported by the findings of our *in vitro* gel shift experiments showing that the deletion of the C-terminal domain greatly decreases protein DNA binding affinity. In line with this idea is the fact that the two other mutants we tested, which completely lacked DNA binding (*i.e.* PHOX2B ΔNLS1 and PHOX2B ΔNLS2), showed defective subnuclear localization. It has been shown that DNA plays a role in regulating the nuclear distribution of other transcription factors, including members of families of proteins bearing a homeodomain. An intriguing possibility is that, as the DNA binding of polyalanine-expanded proteins progressively decreases as a function of the length of the polyalanine tract, the subnuclear localization of mutants might be slightly different, although by immunofluorescence, the nuclear pattern of proteins with shorter polyalanine expansions is virtually identical to that of wild-type protein. Nuclear import defects and cytoplasmic aggregation are detectable only in the case of proteins with longer expansions, and our previous data indicated that the formation of aggregates is dependent on protein abundance (21, 53). Much evidence regarding other polyalanine proteins, suggests that massive overexpression of the protein might trigger the aggregation process and prevent nuclear import *per se*. Moreover, there are no data on *in vivo* aggregation of PHOX2B protein available, and the debate concerning the involvement of aggregates and their toxicity in CCHS is still open. Nevertheless, our data indicate that the expansion of the polyalanine tract diminishes the efficiency of the homeodomain-mediated protein nuclear import, in comparison with wild-type protein, and together with previous data, our findings indicate that the C-terminal domain is an important modulator of DNA binding, homeodomain-mediated dimerization, and solubility of the protein and that the length of the polyalanine tract is critical to drive the folding of the C-terminal domain, which would in turn influence the spatial orientation of the homeodomain and all of its functions.

Moreover, our data exclude the possibility that the formation of non-functional heterodimers between the wild-type protein and mutants with both short and long expansions may play a major role in CCHS pathogenesis. On the other hand, our findings suggest that PHOX2B mutants may form heterodimers with PHOX2A, with biochemical properties possibly different from those of the homodimers.

Author Contributions—S. D. L. and D. B. designed, performed, and analyzed the experiments and wrote the paper. R. B. designed and analyzed the experiments. D. F. conceived and coordinated the study. R. B. and D. F. revised the paper critically for important intellectual content. All authors reviewed the results and approved the final version of the manuscript.

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Appendix 2

PHOX2A and PHOX2B are differentially regulated during retinoic acid driven differentiation of SK-N-BE(2)C neuroblastoma cell line

Simona Di Lascio, Elena Saba, Debora Belperio, Andrea Raimondi, Helen Lucchetti, Diego Fornasari, Roberta Benfante

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Research Article

PHOX2A and PHOX2B are differentially regulated during retinoic acid-driven differentiation of SK-N-BE(2)C neuroblastoma cell line

Simona Di Lascio [a], Elena Saba [b], Debora Belperio [a], Andrea Raimondi [c], Helen Lucchetti [a], Diego Fornasari [a,b,1], Roberta Benfante [a,b,*,1]

[a] Department of Medical Biotechnology and Translational Medicine (BIOMETRA), Università degli Studi di Milano, Milan, Italy
[b] CNR – Neuroscience Institute, Milan, Italy
[c] San Raffaele Scientific Institute, Imaging Research Centre, Milan, Italy

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ABSTRACT

PHOX2B and its paralogue gene PHOX2A are two homeodomain proteins in the network regulating the development of autonomic ganglia that have been associated with the pathogenesis of neuroblastoma (NB), because of their over-expression in different NB cell lines and tumour samples. We used the SK-N-BE(2)C cell line to show that all-*trans* retinoic acid (ATRA), a drug that is widely used to inhibit growth and induce differentiation in NBs, regulates both PHOX2A and PHOX2B expression, albeit by means of different mechanisms: it up-regulates PHOX2A and down-regulates PHOX2B. Both mechanisms act at transcriptional level, but prolonged ATRA treatment selectively degrades the PHOX2A protein, whereas the corresponding mRNA remains up-regulated. Further, we show that PHOX2A is capable of modulating PHOX2B expression, but this mechanism is not involved in the PHOX2B down-regulation induced by retinoic acid. Our findings demonstrate that PHOX2A expression is finely controlled during retinoic acid differentiation and this, together with PHOX2B down-regulation, reinforces the idea that they may be useful biomarkers for NB staging, prognosis and treatment decision making.

1. Introduction

A neuroblastoma, one of the most frequent tumours of childhood, is caused by the arrested differentiation of neural crest sympatho-adrenal progenitor cells [1]. PHOX2B, and its paralogue PHOX2A, are two homeodomain transcription factors that play a pivotal role in the development of the autonomic nervous system and specification of the neurotransmitter phenotype by controlling the expression of the two enzymes responsible for noradrenaline biosynthesis (tyrosine hydroxylase [*TH*] and dopamine-β-hydroxylase [*DβH*]), and thus directing neurons towards their terminal noradrenergic differentiation [2,3]. PHOX2B also modulates its own expression by means of an auto-regulatory mechanism [4] and the expression of *PHOX2A* [5,6], whereas PHOX2A regulates the expression of the human *α3* nAChR subunit gene [7]. Both therefore play a primary role in controlling a number of the molecular determinants of autonomic neurons.

PHOX2A and PHOX2B are also involved in coordinating cell cycle exit and the differentiation of neural progenitors during sympathetic neuronal differentiation [8] as a result of their ability to induce the transcription of $p27^{Kip1}$ [9–11], a cyclin-dependent kinase inhibitor (CKI) whose expression is also regulated by retinoic acid (RA) at post-translational level [12], followed by PHOX2B down-regulation during final neuronal differentiation [13].

Recently, the *PHOX2A* gene has been localised to near the deletion breakpoint of a number of 11q-deleted NB specimens [14], and microarray expression analysis has shown that it is one of nine noradrenaline biosynthesis pathway genes whose expression is reduced in unfavourable NB tumours [14]. However, the possible contribution of PHOX2A to the pathogenesis of NB is not univocal as it is over-expressed in a number of NB tumours and cell lines [15]. As no mutations have been observed in the *PHOX2A* regulatory or coding regions of tumour samples [14,16], it is likely that this gene is involved in the pathogenesis of NB when its

Abbreviations: α3 nAChR, alpha 3 nicotinic Acetylcholine Receptor; ATRA, *all-trans* Retinoic Acid; BMP-2, Bone morphogenetic protein-2; CCHS, Congenital Central Hypoventilation Syndrome; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CKI, Cdk inhibitor; DβH, dopamine-β-hydroxylase; DR, directed repeat; GDNF, glial derived neurotrophic factor; HSCR, Hirschprung's disease; NB, neuroblastoma; NT3, neurotrophin 3; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; RXR, retinoid X receptor; TH, tyrosine hydroxylase; TH-MYCN, tyrosine hydroxylase-*v*-myc avian myelocytomatosis viral oncogene

* Corresponding author at: CNR – Neuroscience Institute, Via Vanvitelli 32, 20129 Milan, Italy.
E-mail address: r.benfante@in.cnr.it (R. Benfante).
[1] These authors share senior authorship.

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expression is deregulated in either direction.

The pathogenetic role of PHOX2B in NB is supported by the presence of heterozygous mutations in familial, sporadic and syndromic cases of NB, and its over-expression in tumour samples and NB cell lines, sometimes associated with other neurochristopathies such as Congenital Central Hypoventilation Syndrome (CCHS) and Hirschprung's disease (HSCR) [16–23], but the underlying mechanisms are still largely unknown. *In vitro* and *in vivo* studies have linked the PHOX2B mutations associated with NB with the impaired differentiation of immature sympathetic neurons that can proliferate, and aberrant differentiation towards the glial lineage [10,24]. PHOX2B over-expression leads to contradictory results as some studies indicate that it inhibits the proliferation of motoneuron progenitors and of immature sympathetic neurons [8,10,16] and promotes the differentiation of human NB cells after treatment with RA [16], whereas conditional Phox2b knockout studies have revealed that Phox2b is required for the proliferation of immature sympathetic neurons [25], and Alam et al. [13], and Ke et al. [23] have shown that a high level of PHOX2B promotes neuroblastoma cell proliferation and xenograft tumour growth in the TH-MYCN murine model, and that this correlates with a high level of MYCN expression. Furthermore, the presence of aberrant Phox2b expression in a zebrafish model has shown that the correct amount of the *Phox2b* gene is important for the differentiation of sympathetic neurons [26].

Vitamin A (retinol) profoundly affects various biological processes during development and adulthood. Most of its actions are mediated by its metabolic product, retinoic acid, which binds to specific nuclear receptors: heterodimers of retinoic acid receptors (RARs) α, β and γ, and retinoid X receptors (RXRs) α, β and γ. These ligand-activated receptors regulate gene transcription by binding to retinoic acid responsive elements (RAREs) in the promoter regions of responsive genes [27]. At embryological level, retinoids control the proliferation, migration and differentiation of neural crest-derived progenitors and, in developing sympathetic neurons, RA cooperates with Bone morphogenetic protein-2 (BMP-2) to make cells responsive to neurotrophic factors such as glial derived neurotrophic factor (GDNF) and neurotrophin 3 (NT3) [28,29]. The pleiotropic effects of RA on the regulatory network governing sympathetic neuron differentiation are well known, but very little is known about its effect on the transcription factors (such as MASH1, PHOX2A and PHOX2B) that play a fundamental role in this process. *In vitro*, retinoids arrest cell growth in the G1 phase of the cell cycle, and induce differentiation in human NB cell lines [30,31] along neuronal- or glial-like lineages depending on the cell line [32] by regulating, for example, the expression of $p27^{Kip1}$, a target gene of PHOX2A and PHOX2B that has major functions in controlling the cell cycle.

As the target genes mediating retinoid-induced differentiation are largely unknown, and the molecular mechanisms by which RA regulates the different signalling pathways necessary for retinoid-induced cellular differentiation in various tissues and at different times are poorly understood, we tested the hypothesis that there may be a direct regulatory link between RA and PHOX2A and PHOX2B expression/activity in the SK-N-BE(2)C NB cell line. The findings show that the retinoic-acid induced differentiation of SK-N-BE(2)C cells is accompanied by a differential regulation of *PHOX2A* and *PHOX2B* expression, with up-regulation of *PHOX2A* mRNA followed by the disappearance of PHOX2A protein (the mRNA remains stably expressed), and a marked decrease in the expression of *PHOX2B* mRNA and protein, thus suggesting that their expression must be finely controlled during RA-induced differentiation, reinforcing the idea that they may be useful biomarkers for NB staging, prognosis and treatment decision making.

2. Material and methods

2.1. Cell lines and cultures

The SK-N-BE(2)C and IMR32 human neuroblastoma cell lines were grown in RPMI 1640, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Lonza). All-*trans* retinoic acid (ATRA; Sigma-Aldrich, St.Louis, Missouri, USA), dissolved in 100% EtOH, was added at a final concentration of 10 μM for the times described in the and the medium was changed every day. Each treatment was carried out in duplicate and repeated at least three times in independent experiments using different batches of ATRA. Cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 10 μg/ml before or after ATRA for the times described in the The proteasome inhibitor MG-132 (8 μM; Calbiochem, Darmstadt, Germany) was added for eight and 24 h after initial treatment with ATRA for 24 and 48 h.

2.2. Total RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted and reverse transcribed, and gene expression was quantitatively analysed as described by Benfante et al. [33] with minimal modifications. The TaqMan® primer and probe assays (Life Technologies, Inc., Carlsbad, CA, USA) were *PHOX2A* (ID #Hs00605931_mH) and *PHOX2B* (ID #Hs00243679_m1), and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*; ID# Hs99999905_m1) was used as an endogenous controls after its compatible with the other assays had been confirmed. The results were calculated using the $2^{-\Delta CT}$ and the $2^{-\Delta\Delta CT}$ methods in order to allow the normalisation of each sample to the endogenous control, and comparison with the calibrator of each experiment (set to a value of 1) as described in the figure legends.

2.3. Nuclear run-on

Nuclear run-on transcription was performed in accordance with the protocol described by Patrone et al. [34]. The nuclei (5×10^7) were prepared from SK-N-BE(2)C cells treated for 24 h with ATRA or vehicle. RNA was synthesised *in vitro* by adding an equal volume of transcription buffer containing 0.4 mM biotin-16-UTP (Roche Diagnostics SpA, Monza, Milan, Italy), and the biotin-labelled RNA was isolated by means of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway). Gene expression was quantitatively analysed by reverse transcribing 8 μl of the nuclear RNA sample and 1 μg of the total RNA sample and using real-time PCR.

2.4. Chromatin immunoprecipitation and qPCR

Chromatin immunoprecipitation was carried out as previously described [4]. Chromatin was incubated overnight at 4 °C with 5 μg of anti-PHOX2A antibody (Davids Biotechnologie, Regensburg, Germany), and chicken pre-immune IgY (Davids Biotechnologie), and the immunocomplexes were collected on monoclonal anti-chicken IgY-agarose beads or protein G/agarose bead slurry (InVitrogen, Carlsbad, CA, USA) pre-adsorbed with 20 μg/μl tRNA and 10 μg/μl salmon sperm DNA (Sigma-Aldrich). After washes and elution, the cross-linking was reversed by heating to 65 °C overnight, and the samples were purified on columns (High Pure PCR product purification kit, Roche Diagnostics SpA, Italy). For the PCR detection of the immunoprecipitated chromatin, 5% of the purified DNA was used as a template to amplify the PHOX2B promoter using the primers ChIP[2 bprom] UP, 5′-CAA GCT TAT TTC CAA GTA GTG TGA TTG AAT-3′, and ChIP[2bprom] LOW, 5′-GCC TCC TAT

GAG ATG CCT TGT CTG A-3′. The DNA samples were heated to 95 °C for 2 min, followed by 47 cycles of heating at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s. For quantitative analysis, the immunoprecipitated chromatin was amplified by means of SYBR-Green chemistry (Life Technologies, Inc.) using the primers #UP, 5′-GCT CGG TGC GTA ATG GTG TGG TA-3′ and #LOW, 5′-GGT TGG TCT TAT TGC TGG CGC TT-3′, and quantitatively analysed using the ABI Prism™ 7000 Sequence Detection System (Applied Biosystems, CA, USA) and SDS software, version 1.2.3.

2.5. shRNA

PHOX2A expression was silenced by means of the transient transfection of a plasmid (MISSION® shRNA, Cod. SHCLNG-NM_005169, TRCN0000013543, **Clone ID:**NM_005169.2-1260s1c1) containing an shRNA targeting the 3′-UTR region of *PHOX2A* (*Sequence:* CCG GCC TTC TAG CTT GGC CTT CTT TCT CGA GAA AGA AGG CCA AGC TAG AAG GTT TTT) and the results were analysed as described in the SHC002 MISSION® pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA (Sigma-Aldrich) was used as the control.

2.6. Electrophoretic mobility shift assays (EMSAs)

The EMSAs were performed as described by Terzano et al. [35] and Cargnin et al. [4]. The oligonucleotides spanning the ATTA sites in the *PHOX2B* promoter have been described by Cargnin et al. [4] and all of the oligonucleotides were purchased from Sigma Aldrich.

2.7. Plasmid construction

The β-RARE luc construct was obtained by cloning the consensus RARE from the β2 RA receptor subunit promoter [36] upstream of the SV40 promoter (Promega Madison, WI, USA). The oligonucleotide sequence was 5′-TCG AGT AAG GGT TCA CCG AAA GTT CAC TCG CAC-3′, in which the RARE sequence is underlined.

Some of the reporter constructs containing regions of the *PHOX2A* promoter have been previously described by Flora et al. [5]. However, as these regions were cloned into the pGL3basic vector backbone and this was unspecifically transactivated by ATRA treatment, we re-cloned fragments of the human *PHOX2A* 5′-flanking region into the ATRA-unresponsive pGL4basic plasmid (Promega) upstream of the *Firefly* luciferase gene. Details concerning the plasmid construction are available in the Supplementary Material. The *PHOX2A* expression vector was obtained by cloning human *PHOX2A* cDNA [7] into the EcoRI site of pCMV-myc (Clontech Laboratories Inc., Mountain View, CA, USA). All of the constructs were checked by means of restriction analysis and partial sequencing.

2.8. Transfections and luciferase assays

The transfection experiments were performed by means of lipofection as described by Flora et al. [37] using 1.6×10^5 SK-N-BE (2)C cells. Luciferase was assayed using the Dual Luciferase Reporter Assay System as previously described [5,38].

2.9. Protein preparation, immunoprecipitation and western blot analysis

The total protein extracts were obtained from sub-confluent cells using the freeze and thaw method as described by Benfante et al. [33] and the nuclear extracts were prepared as previously described [35].

For immunoprecipitation, SK-N-BE(2)C cells were harvested after being treated with ATRA and MG-132 as described in the by means of centrifugation at 7000 rpm for 5 min. The pellet was resuspended in 150 μl lysis buffer containing non-ionic detergent (1% Triton X-100, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and the Sigma-Aldrich protease inhibitors cocktail), and incubated for one hour at 4 °C on a rotating wheel. The extracts were then clarified by means of 30 min centrifugation at 13,200 rpm/4 °C, and pre-cleared using protein G/agarose bead slurry (InVitrogen) and chicken pre-immune IgY (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). Three milligrams of the pre-cleared extracts were incubated overnight at 4 °C with 5 μg polyclonal chicken anti-PHOX2A antibody (Davids Biotechnologie), or pre-immune chicken IgY (Santa Cruz Biotechnology) and the immunocomplexes were captured by protein G/agarose bead slurry (InVitrogen). Because of the poor binding of chicken antibodies to protein G, a bridging antibody (rabbit anti-chicken IgG, Upstate, Lake Placid, NY, USA) was added to enhance the capture of the immunocomplexes. The beads were collected by means of centrifugation, gently washed, and resuspended in sample loading buffer, and the immunocomplexes were dissociated from the beads by boiling the samples.

The proteins were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany), and Western blotting was carried out as previously described [7] using the chicken anti-PHOX2A [7], mouse monoclonal anti-PHOX2B (B-11), rabbit anti-Sp1 (Santa Cruz Biotechnology), mouse anti-β-tubulin (Sigma-Aldrich) and mouse anti-Ubiquitin (BIOMOL GmbH, Hamburg, Germany) as primary antibodies; the secondary antibodies (rabbit anti-chicken, Davids Biotechnologie; goat anti-rabbit and anti-mouse, Pierce Biotechnology Inc., Rockford, ME, USA) were conjugated with horseradish peroxidase. The bands were revealed using Super Signal West Dura (Pierce Biotechnology Inc.), with standard molecular weights (New England Biolabs Inc., Beverly, MA USA) being loaded in parallel.

2.10. Data analysis

NIH Image 1.61/fat software was used for the densitometric analysis of the signals obtained from the Western blots. The results are given as the mean values of at least three independent experiments, and standard deviation (SD) or standard error (SEM) as indicated in the The data were analysed by means of a paired two-tailed Student's *t* test or one-way ANOVA using GraphPad Prism 5 Software (GraphPad Software, Inc.) as described in the; *p* values of < 0.05 were considered significant.

3. Results

3.1. Effects of ATRA on PHOX2A and PHOX2B expression

The effect of ATRA on the expression of *PHOX2A* and *PHOX2B* was studied using the SK-N-BE(2)C NB cell line, a very well-characterised model of RA-induced neuronal differentiation [39]. Its differentiation potential was tested by treating the cells with 10 μM ATRA for different periods of time, evaluating their morphology and the differential expression of some target genes, and measuring the responsiveness of a reporter construct in which transcription was driven by the RARE. Phase-contrast microscopy showed that the morphology of the SK-N-BE(2)C cells started changing after 24 h of ATRA exposure (Fig. S1A, panel b), and neurite outgrowth was clearly evident after 72 h (Fig. S1A, panel c); exposure to the vehicle alone did not affect their morphology at any time (Fig. S1A, panels d and e), leaving them indistinguishable

from the control (Fig. S1A, panel a).

At molecular level, ATRA-induced SK-N-BE(2)C cells differentiation is characterised by changes in the expression of some target genes, including *HASH1* [40,41], and Northern blot analysis of RNA from the cells treated with ATRA for different times revealed a unique transcript of approximately 3 Kb encoding HASH1 (Fig. S1B, lanes 1, 2 and 4), which was observed in all of the tested NB cells, whereas no signal was observed in the HeLa cells (Fig. S1B, lane 3). After 24 h exposure to ATRA, the expression of the transcript became barely detectable (Fig. S1B, lane 5) and remained very low for up to 96 h (Fig. S1B, lanes 6–8). This is in line with the findings of Söderholm et al. [41] and, together with the morphological data, indicates that the most relevant biological responses of neuroblastoma cells to ATRA exposure were completely reproducible under our experimental conditions.

The responsiveness of SK-N-BE(2)C cells to ATRA was also tested by means of the transient transfection of a reporter construct in which the transcription was driven by the RARE identified in the promoter of the *β2* subunit of retinoic acid receptor (β-RARE luc), cloned upstream of SV40 promoter. Fig. S1C shows that the SK-N-BE(2)C cells were fully responsive to ATRA treatment as the expression of the luciferase reporter gene increased 40-fold over that of untreated cells (compare lanes 1 and 2). Treatment with vehicle alone had no effect on the luciferase (Fig. S1C, lane 3). All of these data further confirm that the most relevant biological responses of SK-N-BE(2)C cells to ATRA exposure were completely reproducible under our experimental conditions.

Fig. 1A shows that the *PHOX2A* transcript [5] was considerably induced after 24 h exposure to ATRA, and this level of expression increased for up to 72 h (Fig. 1A, hatched *vs* grey bars), whereas exposure to the vehicle alone did not affect *PHOX2A* expression at any time (Fig. 1A, grey bars *vs* black bar). The induction of *PHOX2A* mRNA by RA was reproducible and statistically significant. On the contrary, *PHOX2B* transcript expression decreased by 70% after 24 h exposure, and by up to 90% after 72 h (Fig. 1B, white *vs* grey bars).

Western blot analysis was used to evaluate whether the induction of *PHOX2A* mRNA and reduction in *PHOX2B* mRNA expression was paralleled by an adequate increase/decrease in the corresponding protein. Fig. 1C shows that, after 24 h treatment with ATRA, there was a substantial and statistically significant increase in PHOX2A protein levels (Fig. 1C, lanes 3 and 4 *vs* control lanes 1 and 2); however, surprisingly, no trace of the protein was found after 48 h and for up to 96 h (Fig. 1C, lanes 5–10). On the contrary, PHOX2B protein was not detectable after no more than 24 h ATRA treatment (Fig. 1D, lanes 3, 5 and 7 *vs* lanes 2, 4 and 6).

3.2. ATRA acts at transcriptional level

We used nuclear run-on experiments to investigate whether ATRA acts directly at transcriptional level. Fig. 2A and B (TOT RNA) confirmed that 24 h ATRA exposure induced a three-fold increase in *PHOX2A* and three-fold decrease in *PHOX2B* mRNA expression, and the quantitative run-on experiments showed that this changes were due to the new induction or repression of transcription caused by the drug (Fig. 2A and B, RUN-ON RNA). In brief, ATRA increased *PHOX2A* and decreased *PHOX2B* mRNA levels by acting on their transcription.

3.3. Mapping the retinoic acid responsive elements (RAREs)

The nuclear run-on analyses (Fig. 2) showed that the changes in *PHOX2A* and *PHOX2B* relative expression was mainly due to a

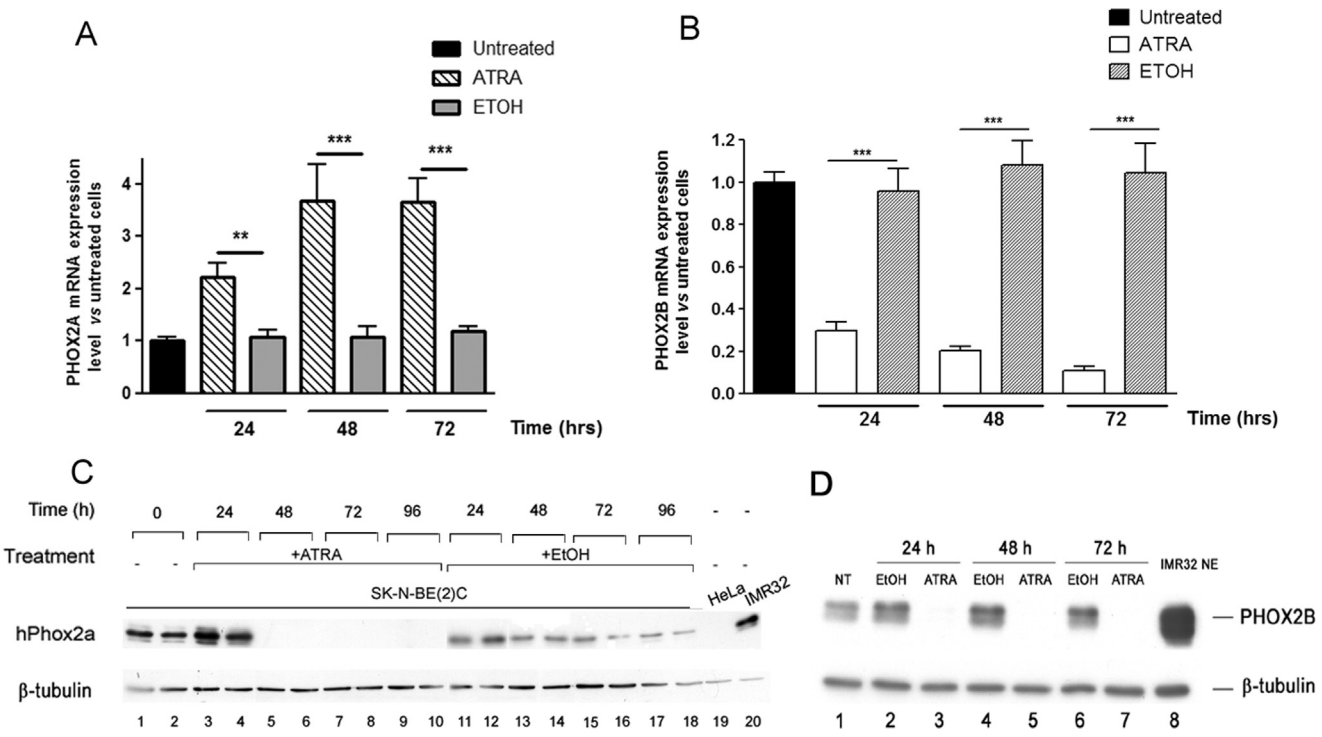

Fig. 1. Effects of ATRA on PHOX2A and PHOX2B expression in SK-N-BE(2)C cells. (A and B) qPCR analyses of PHOX2A (panel A) and *PHOX2B* expression (panel B). SK-N-BE(2)C cells were treated with ATRA for the indicated times, and gene expression was determined by means of real-time PCR using the *GAPDH* gene as an internal control. The bars represent the mean values ± SD of three independent experiments expressed as fold-induction in comparison with the untreated sample (black bars). Hatched (panel A) and empty (panel B) bars = treated cells; grey bars = vehicle-treated samples. $^{**}p < 0.01$; and $^{***}p < 0.001$ indicate statistically significant differences in *PHOX2A* and PHOX2B mRNA expression between the vehicle treated (grey bars) and the cells treated with ATRA for different periods of time (one-way ANOVA, post-Tukey's test). (C and D) Western blots of total protein extracts obtained from SK-N-BE(2)C cells treated with ATRA 10 μM (lanes 3–10 in panel C, and lanes 3, 5 and 7 in panel D) or ethanol (lanes 11–18 in panel C and 2, 4 and 6 in panel D) for different periods of time. The filter was probed with the anti-PHOX2A (panel C) or anti-PHOX2B antibody (panel D). The membranes were probed with an antibody against β-tubulin for normalisation purposes. The negative and positive controls were protein extracts of HeLa (panel C, lane 19) and IMR32 cells (panel C, lane 20, and panel D, lane 8). Lanes 1 and 2 (panel C), and lane 1 (panel D): untreated cells.

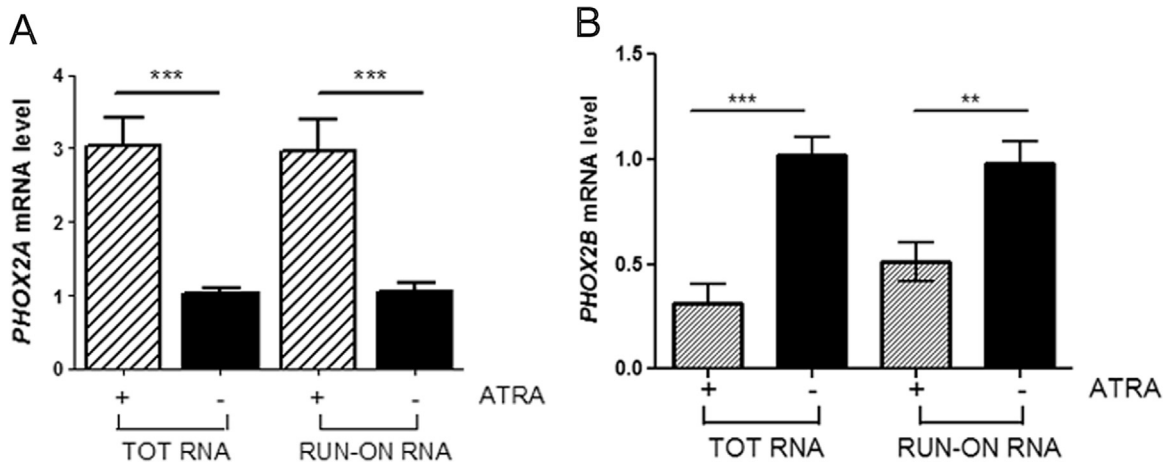

Fig. 2. Effects of ATRA on the transcription of *PHOX2A* and *PHOX2B* mRNA. (A and B) Run-on analysis. The SK-N-BE(2)C cells were treated with ATRA for 24 h, and *PHOX2A* (panel A) and *PHOX2B* gene expression (panel B) was determined by means of real-time PCR using the *GAPDH* gene as an internal control. The bars represent the mean values ± SD of three independent experiments using total and nuclear run-on RNA in the treated cells (striped bars), expressed as fold-induction over the vehicle-treated samples (black bars). $^{**}p < 0.01$ and $^{***}p < 0.001$ (one-way ANOVA, post-Tukey's test).

transcriptional mechanism. A computer-assisted analysis of 10 Kb of the *PHOX2A* promoter sequence using MatInspector software (www.genomatix.de) revealed the presence of putative RAREs, which may explain the increased *PHOX2A* expression induced in the SK-N-BE(2)C cell line by ATRA treatment.

Ten putative RAREs were identified, two of which (#2 and #3) partially overlapped. Most of the RAREs were directed repeats separated by one nucleotide (DR1), two were DR2 (#6 and #8) and only one was a DR5 (#7), although none of them perfectly matched the consensus (Fig. 3A). In order to test whether these sites were functionally responsive to RA, we generated a series of constructs spanning the *PHOX2A* promoter from position −10.53 Kb to position −0.35 Kb, and performed transient transfections. The KpnI-NcoI fragment (Fig. 3B, −10.8/pGL4), which contains all ten RAREs, and the SphI-NcoI fragment (Fig. 3B, −6.6/pGL4), which contains RAREs 1–6, responded to ATRA treatment with an almost five-fold induction of construct activity in comparison with the vehicle-treated cells. The SacI-NcoI fragment (Fig. 3B, −4.5/pGL4), which spans 5.2 Kb of the promoter upstream of the transcriptional start site and contains RAREs 1–4, was also responsive, although to a lesser extent (Fig. 3B). However, further deletion of RARE 4 (construct −1.5/pGL4) did not affect the activity of the *PHOX2A* promoter, which remained fully responsive to ATRA. The −1.2/pGL4 construct containing only the RARE 1 sequence also remained responsive, and showed a statistically significant two-fold increase in promoter activity in comparison with the vehicle-treated cells, whereas the deletion of RARE 1 (Fig. 3B, −0.35/pGL4) completely abolished the ability of ATRA to induce *PHOX2A* promoter activity.

These data indicate that the responsiveness to ATRA is a result of the contribution of the three RARE sequences located in the first 1.5 Kb of the *PHOX2A* promoter.

On the other hand, *in silico* analysis of the *PHOX2B* promoter did not reveal the presence of canonical RARE elements, thus suggesting that the down-regulation of *PHOX2B* expression is not mediated by the direct binding of RA receptors to the promoter, and that other transcription factors mediate the effect of RA on *PHOX2B* gene expression.

3.4. Down-regulation of PHOX2B expression is not due to a direct effect of PHOX2A

Control of the temporal and spatial expression of *PHOX2A* and *PHOX2B* is fundamental during the specification of neuronal identity, and many studies have tried to elucidate the exact molecular mechanisms involved in regulating their expression over the last few years. We have previously demonstrated that PHOX2B regulates the transcription of the *PHOX2A* gene by directly binding and transactivating its promoter [5]. We have also characterised the *PHOX2B* promoter, and demonstrated by means of biochemical and functional assays that most of its transcriptional activity is sustained by auto-regulatory mechanisms involving PHOX2B binding and transactivation [4].

Chromatin immunoprecipitation (ChIP) assays show that PHOX2A also participates in the transcriptional complex assembled on the *PHOX2B* promoter [4] (Fig. 2S, panels A and B). The *PHOX2B* promoter has five binding sites for homeodomain proteins (Fig. S2D), and EMSA analysis (Fig. 2S, panel C) showed that PHOX2A is also capable of binding the ATTA2 (Fig. 2S panel C, lanes 7–10) and ATTA3 (Fig. 2S panel C, lanes 12–15) sites in the *PHOX2B* promoter; moreover, unlike PHOX2B, PHOX2A, binds also the ATTA1 and ATTA5 sites (Fig. 2S, panel C, lanes 2–5 and 22–25), although with lower affinity.

We then asked whether the down-regulated expression of *PHOX2B* was related to, and mediated by the increased expression of PHOX2A induced by ATRA treatment. The over-expression of PHOX2A in the SK(2)C NB cell lines showed a statistically significant 40% reduction in the expression of endogenous *PHOX2B* (Fig. 4A, hatched *vs* black bar), thus suggesting that PHOX2A negatively modulates *PHOX2B* expression, although the reduction was not as great as that observed after an ATRA challenge. These data were confirmed by silencing PHOX2A expression for 24 and 48 h (Fig. 4B, lanes 2 and 4 *vs* 1 and 3), and an approximately 50% reduction in PHOX2A expression (Fig. 4C, black bars) corresponded to a slight, but statistically significant increase in *PHOX2B* expression (Fig. 4C, grey bars). In order to investigate whether the down-regulation of *PHOX2B* induced by ATRA treatment was at least partially due to an increase in PHOX2A expression, we counteracted the ATRA-induced increase in *PHOX2A* expression in SK(2)C cells transfected with *PHOX2A* shRNA, and measured the corresponding level of *PHOX2B* mRNA. Fig. 4D (left panel) shows that the presence of *PHOX2A* shRNA blocked the ATRA-induced *PHOX2A* expression (grey *vs* hatched bars) for up to 72 h to an extent that was not statistically different from that observed in the samples transfected with a scrambled shRNA (shCTRL) and not challenged to ATRA treatment (grey *vs* black bars). However, the reduction in *PHOX2B* expression was not affected by the presence of the *PHOX2A* shRNA (Fig. 4D, right panel; grey *vs* hatched bars) at

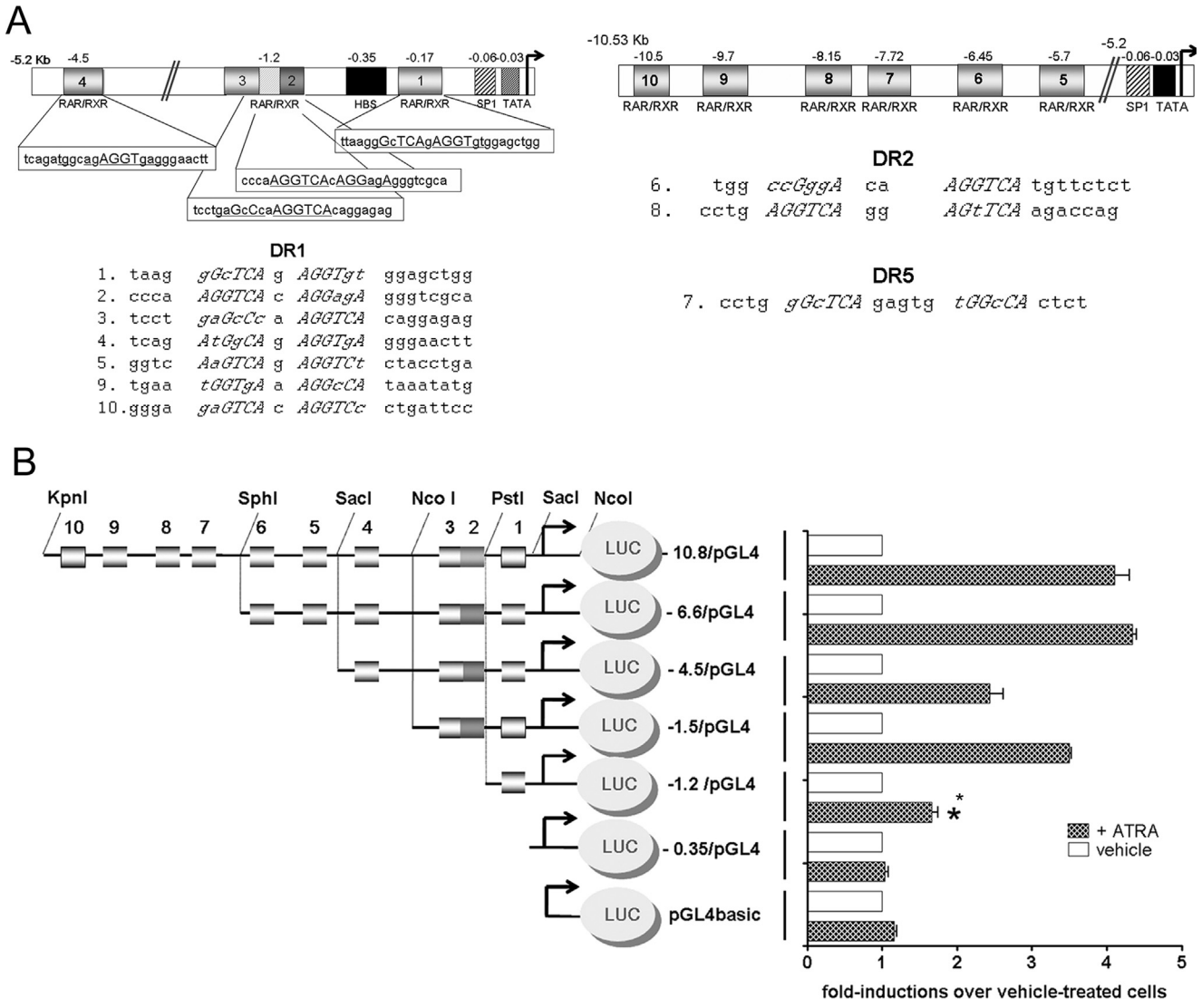

Fig. 3. Functional mapping of *PHOX2A* retinoic acid responsive elements. (A) Schematic illustration of the putative RAREs identified in the *PHOX2A* promoter region. *Left:* The region spanning 5 Kb upstream of the *PHOX2A* transcriptional start site. *Right:* The region spanning nucleotides −10,530 to −5200 of the *PHOX2A* 5′-flanking region. The consensus RARE is also indicated. (B) Left: Schematic illustration of the constructs. The boxes represent the putative RARE sequences #1-#10, as assessed by means of computer-assisted analysis (Genomatix, MatInspector), and the arrows the transcription start site. The figure shows the restriction sites used to clone the different parts of the *PHOX2A* 5′-flanking region; the grey oval represent the *Firefly* luciferase reporter gene (luc). Right: Luciferase assays. SK-N-BE(2)C cells were transiently transfected with the constructs shown on the left, and treated with ATRA for 24 h before luciferase assay. The bars show the transcriptional activity of the constructs expressed as fold-inductions over vehicle-treated cells (mean values ± SD of at least three independent experiments performed in triplicate). The asterisk indicates a statistically significant difference between the cells treated with ATRA or vehicle alone for the same period of time (Student's *t* test. $^{*}p < 0.05$).

any time. These data suggest that PHOX2A and PHOX2B cross-regulate their own expression, but this mechanism is not involved in regulating ATRA-induced PHOX2B down-regulation.

3.5. *ATRA reduces PHOX2A protein half-life by means of proteasomal degradation*

As shown in Fig. 1A, 24 h ATRA treatment led to a substantial and statistically significant increase in PHOX2A expression; however, surprisingly, no trace of the protein was found after 48 h exposure and for up to 96 h (Fig. 1A). In order to investigate whether the delayed effects of ATRA on PHOX2A protein expression correlated with decreased protein stability, we evaluated PHOX2A protein half-life by treating SK-N-BE(2)C cells with ATRA or ethanol for 24 h, followed by 10 μg/ml cycloheximide for different time periods (Fig. 5A). The half-life of the protein was significantly reduced (50%) in comparison with the levels observed in the cells exposed to the vehicle alone (Fig. 5A, lanes 2–5 *vs* lanes 7–10) after no more than 90 min (Fig. 5B).

In order to confirm that the effects of ATRA on protein stability were PHOX2A specific, we studied the expression of the transcription factor Sp1 after 300 min exposure to RA in the same samples as those used in Fig. 5A: Fig. 5C shows that the presence of RA had no effect on Sp1 expression.

In order to investigate whether the reduced stability of PHOX2A protein was due to increased proteasome-mediated degradation, SK-N-BE(2)C cells were treated with MG-132, an inhibitor of proteasomal activity. The cells were exposed to MG-132 for the last eight hours of a 32-h exposure period, and this increased the amount of PHOX2A protein in comparison with the amount induced by ATRA alone (Fig. 5D, lane 5 *vs* lane 4). The simultaneous presence of the inhibitor for up to 24 h (a total of 48 h of ATRA treatment; Fig. 5D, lane 7), or for the last eight hours of a 56 h exposure to ATRA (Fig. 5D, lane 9) rescued PHOX2A protein expression from degradation (compare lanes 7 with 6 and lanes 9 with 8). Furthermore, PHOX2A immunoprecipitation and

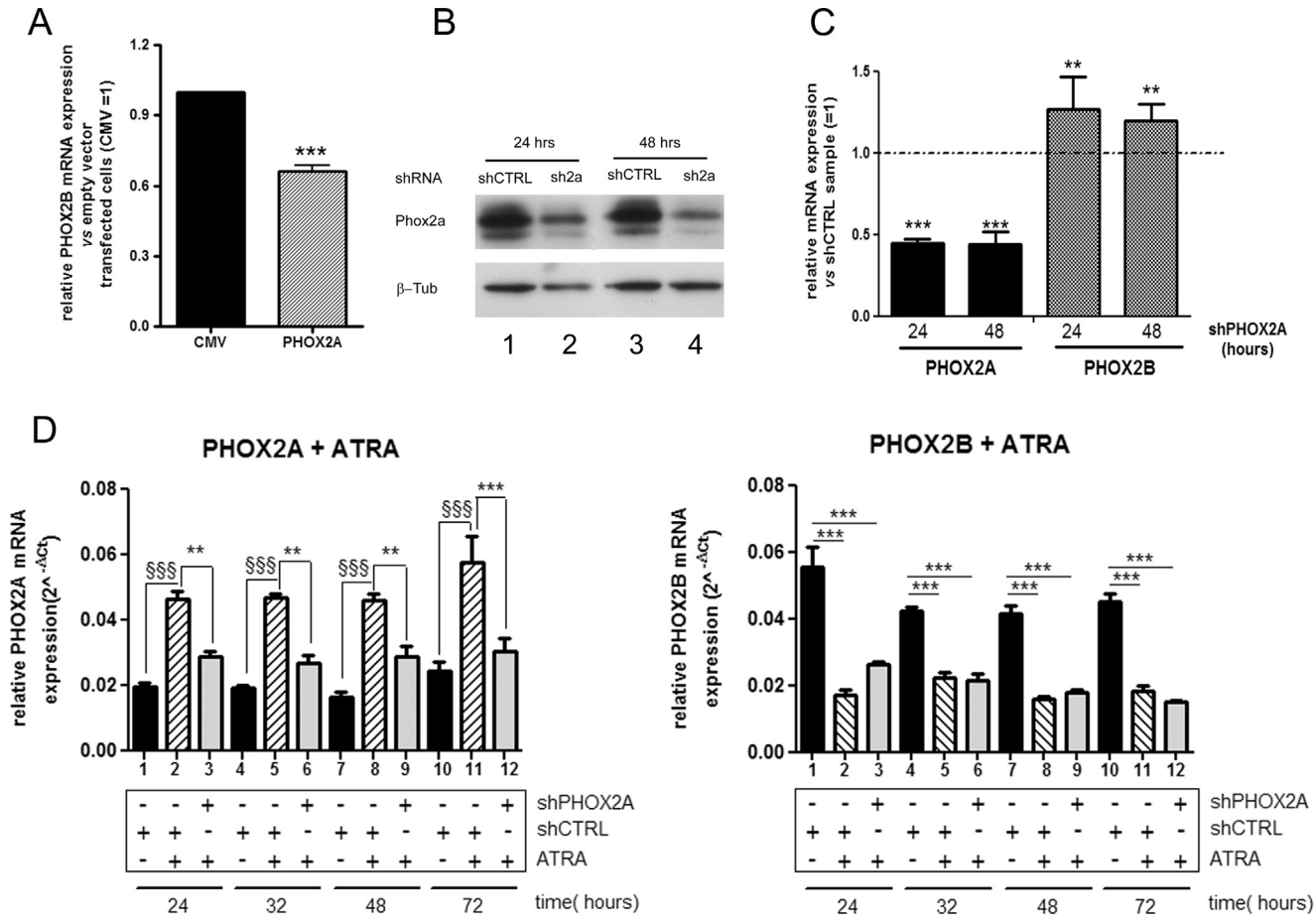

Fig. 4. PHOX2A induction does not mediate the ATRA-induced down-regulation of *PHOX2B* expression. (A) qPCR analysis of *PHOX2B* expression following PHOX2A over-expression. SK-N-BE(2)C cells were transfected with PHOX2A cDNA expression vector (striped bar), and *PHOX2B* gene expression was determined by means of real-time PCR using the *GAPDH* gene as an internal control. The bars represent the mean values ± SD of three independent experiments expressed as fold-inductions in comparison with the cells transfected with empty vector (black bar). $^{***}p < 0.001$ indicates statistically significant differences in *PHOX2B* mRNA expression (Student's *t* test). (B) Western blots of PHOX2A silencing. SK-N-BE(2)C cells were transfected with the shRNA construct targeting the 3′-UTR of *PHOX2A* (sh2a, lanes 2 and 4) or scrambled shRNA (shCTRL, lanes 1 and 3). PHOX2A protein levels were determined 24 (lanes 1 and 2) and 48 h after transfection (lanes 3 and 4), using an anti-PHOX2A antibody. The membrane was probed with an antibody against β-tubulin for normalisation purposes. (C) qPCR analysis of *PHOX2A* (black bars) and *PHOX2B* expression (grey bars) upon PHOX2A silencing. SK-N-BE (2)C cells were transfected with the shRNA construct targeting the 3′-UTR of *PHOX2A* (shPHOX2A) and gene expression was determined 24 and 48 h after transfection by means of real-time PCR using the *GAPDH* gene as an internal control. The bars represent the mean values ± SD of at least three independent experiments, expressed as fold differences in comparison with the cells transfected with scrambled shRNA (shCTRL=1). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant differences in gene expression relative to scramble transfected cells. (D) *PHOX2A* and *PHOX2B* mRNA expression upon ATRA treatment after PHOX2A silencing. SK-N-BE(2)C cells were transfected with *PHOX2A* shRNA (shPHOX2A, grey bars) or scrambled shRNA constructs (shCTRL, striped and black bars), and treated with 10 μM ATRA (grey and striped bars) for the indicated times. *PHOX2A* (left panel) and *PHOX2B* (right panel) gene expression was determined by means of real-time PCR using the *GAPDH* gene as an internal control. The bars represent the mean values ± SD of three independent experiments, expressed as relative mRNA levels calculated using the $2^{-\Delta Ct}$ method. Left panel: $^{**}p < 0.01$ and $^{§§§}p < 0.001$ (one-way ANOVA, post-Tukey's test) indicate statistically significant differences in *PHOX2A* mRNA expression between the cells transfected with scrambled shRNA (striped bars) and the cells transfected with shPHOX2A (grey bars) treated with ATRA or between the cells transfected with scrambled shRNA treated with ATRA (striped bars) or vehicle (black bars). There was no statistically significant difference between the vehicle- and ATRA-treated cells respectively transfected with shCTRL (black bars) and with shPHOX2A (grey bars). Right panel: $^{***}p < 0.001$ indicate statistically significant differences in *PHOX2B* mRNA expression between the ATRA- and vehicle-treated cells transfected with shCTRL (bars 2, 5, 8 and 11 *vs* 1, 4, 7 and 10) or shPHOX2A (bars 3, 6, 9 and 12 *vs* 1, 4, 7 and 10). There was no statistically significant difference between the ATRA-treated cells transfected with shPHOX2A (grey bars) and those transfected with shCTRL (striped bars).

Ubiquitin Western blotting revealed that PHOX2A-Ubiquitin conjugates are increased by treatment with ATRA combined with MG-132 than treatment by ATRA alone (Fig. 5E, lane 8 *vs* lane 5), thus confirming that the disappearance of PHOX2A protein is due to selective proteasomal degradation, whereas the mRNA remains stably expressed.

4. Discussion

We investigated the effects of ATRA on the expression of *PHOX2A*, a candidate tumour suppressor gene [14], using the undifferentiated SK-N-BE(2)C human NB cell line as a model, and found that they were apparently opposite: it initially acted as a positive regulator of gene expression, but later triggered a process that culminated in the complete disappearance of the transcription factor. The positive effects of ATRA were mainly due to direct stimulation of gene transcription by means of the contribution of three RARE sequences located in the first 1.5 Kb of the *PHOX2A* promoter, but the identity of the RA nuclear receptor isoforms involved in this regulation is still unknown.

The increase in PHOX2A protein product during the first 24 h of treatment, followed by its down-regulation after 48 h even though the expression of *PHOX2A* mRNA remained constantly up-regulated, led us to hypothesise that ATRA might promote the disappearance of PHOX2A protein by means of two different molecular mechanisms: translation inhibition and/or increased protein degradation. Our data strongly suggest that the proteasome plays a pivotal role, and raise the question as to which pathways link ATRA exposure to PHOX2A degradation.

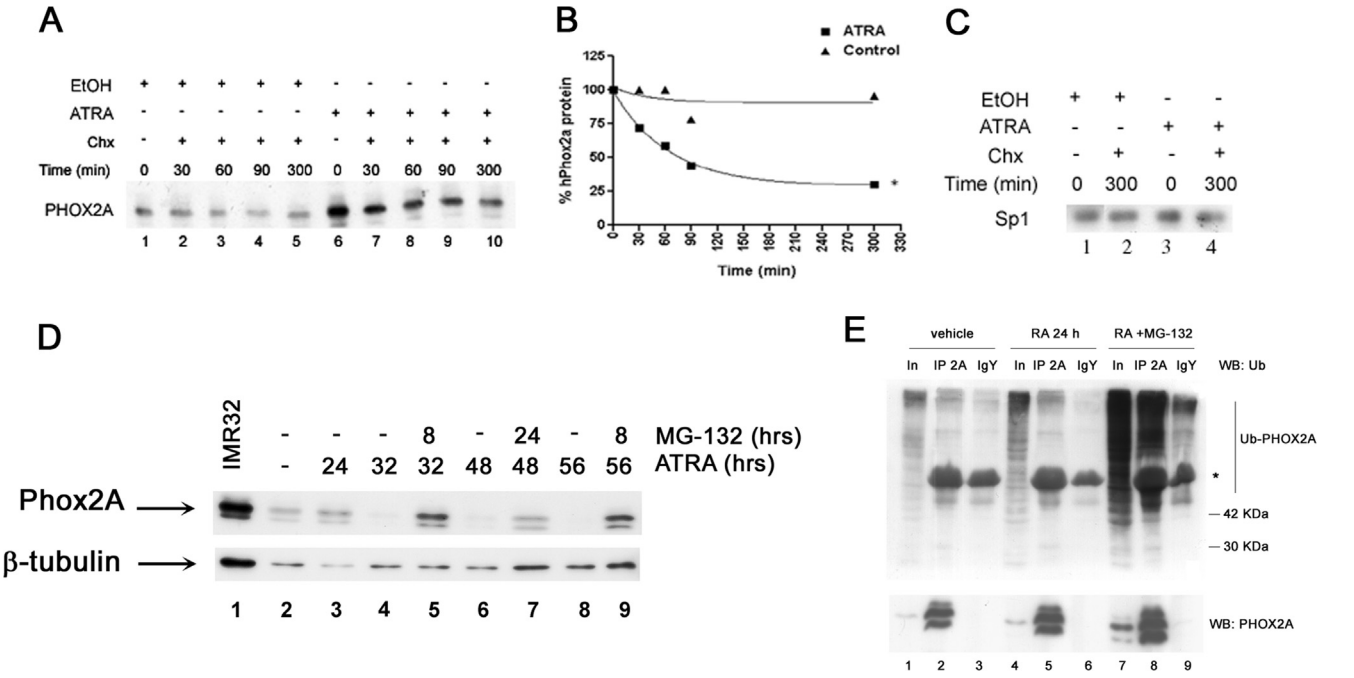

Fig. 5. ATRA induces the proteasome-mediated degradation of PHOX2A protein. (A) SK-N-BE(2)C cells exposed to ethanol (lanes 1–5) or 10 μM ATRA (lanes 6–10) for 24 h were treated with cycloheximide at a final concentration of 10 μg/ml. The total protein extracts were obtained after treatment with cycloheximide for the indicated periods of time, and analysed by means of Western blotting. (B) Densitometric signal quantification expressed as the percentage of PHOX2A expression after 24 h exposure to ethanol (triangles) or ATRA (squares). The asterisk indicates a statistically significant difference between the control and ATRA-treated cells after 300 min (Student's *t* test $p < 0.05$). *C*) The same protein extracts as those used in the experiment shown in panel A (lanes 1, 5, 6 and 10) were analysed for the expression of the Sp1 transcription factor by means of Western blotting. (D) Western blots of extracts from SK-N-BE(2)C cells treated with ATRA for 24 h (lane 3), 32 h (lane 4), 48 h (lanes 6), and 56 h (lane 8), alone or in combination with the simultaneous treatment with the proteasome inhibitors MG-132 for eight (lanes 5 and 9) and 24 h (lane 7). The positive controls were untreated SK-N-BE(2)C cells at time 0 (lane 2), and 10 μg of nuclear extract from IMR32 cells (lane 1) *E*) Total extracts from SK-N-BE(2)C cells treated with EtOH (vehicle, lanes 1–3) or 10^{-5} ATRA for 24 h (lanes 4–6), followed by simultaneous treatment with MG-132 for eight hours (lanes 7–9), were immunoprecipitated with anti-PHOX2A antibodies (IP2A, lanes 2, 5, 8), and the level of ubiquitination was evaluated by means of Western blotting using an anti-ubiquitin (Ub) antibody (upper panel). Immunoprecipitation with chicken pre-immune IgY was used as a control, (IgY, lanes 3, 6 and 9). Lanes 1, 4 and 7 show pre-immunoprecipitation total extract [10% input (In)]. The same membranes were stripped and re-probed with anti-PHOX2A antibody (lower panel). The asterisk indicates an specific band due to IgY heavy chain. Ub-PHOX2A: Ubiquitin-PHOX2A conjugates.

RA down-regulates cell proliferation and promotes neurogenesis by arresting cells in the G1 phase of the cell cycle [31]. Although the underlying mechanism is still unclear, it is known that RARβ is a necessary component of the inhibitory effects of ATRA on the growth of NB cells [42]; furthermore, RA induces cell-cycle arrest in G1 by decreasing the cyclin-dependent kinase (Cdk) activity required for the G1/S transition and the accumulation of the $p27^{Kip1}$Cdk inhibitor (CKI) [43,44] that impair growth and activate the differentiation programme. The stability of $p27^{Kip1}$ is regulated by the E3 ubiquitin ligase SKP2, and RA stabilises $p27^{Kip1}$ by enhancing the proteasome-mediated degradation of SKP2 in a number of cancer cell lines. Like PHOX2A, SKP2 protein disappears, but its mRNA persists during the RA treatment of SH-SY5Y cells, thus suggesting that RA post-transcriptionally regulates SKP2 [45]. Ballas et al. and Singh et al. [46,47] have shown that REST, a key regulator of neuronal genes during neuronal differentiation, is regulated by ATRA in a manner that is similar to the regulation of PHOX2A, and suggested that the degradation of REST may facilitate a rapid transition to terminal differentiation; given that REST is mainly a negative regulator, this permits the differential expression of a subset of genes with lower-affinity REST binding sites. These findings suggest that regulation of proteasome-mediated degradation of the proteins involved in different aspects of cell metabolism may be a common mechanism by means of which RA controls the order of the signalling events necessary for a cell's response to retinoid-induced differentiation.

Like *PHOX2A, PHOX2B*, is overexpressed in a number of tumours and NB cell lines. However, there are conflicting hypotheses concerning the significance of this in the pathogenesis of NBs. Raabe et al. [16] suggest that *PHOX2B* up-regulation is simply a marker of tumour lineage and not a contributor to malignant phenotype, given that neuroblastoma arise at a time when *PHOX2B* is normally expressed during neurodevelopment, and its forced over-expression decreases proliferation. Furthermore, they rule out PHOX2A involvement in the pathogenesis of NBs because no mutations have been found in the PHOX2A coding region [14,16]. As the expression of PHOX2B precedes that of PHOX2A during development, and *in vitro* experiments have shown that the forced over-expression of PHOX2B regulates *PHOX2A* [5,6], it is possible to speculate that *PHOX2A* up-regulation may be due to a high level of PHOX2B expression, because silencing *PHOX2B* in NB cell lines leads to the down-regulation of *PHOX2A* [22].

However, in contrast to data described in Bachetti et al. [22], our data suggest a new mechanism cross-regulating PHOX2A and PHOX2B as they show that PHOX2A negatively modulates *PHOX2B* expression. This discrepancy may be due to differences of cell system (SK-N-BE(2)C cells as opposed to SH-SY5Y and IMR32 cell lines), and so the up-regulated expression of *PHOX2A* and *PHOX2B* in NBs might be the result of cross-regulation.

Conversely, the observation that the proliferation of undifferentiated $PHOX2B^{+}$ neuronal progenitors promotes NB cell proliferation and stemness indicates that PHOX2B is a critical regulator in the pathogenesis of NBs [23]. It is not known what decides whether PHOX2B is pro- or anti-proliferative, but it can be hypothesised that the effects of *PHOX2B* over-expression depend on cell context.

As we did not map any RARE in the *PHOX2B* promoter, we wondered whether the *PHOX2B* down-regulation we observed

during RA-driven differentiation was due to the up-regulation of PHOX2A, which down-regulates the expression of *PHOX2B*. However, by silencing endogenous *PHOX2A* expression, we found that *PHOX2B* regulation by PHOX2A is not the mechanism underlying the effect of RA. The association between MYCN amplification (an important prognostic factor in NB) and *PHOX2B* expression in human NB cell lines [23], the down-regulation of MYCN expression after an RA challenge [48,49], the up-regulation of PHOX2B associated with MYCN over-expression, and the down-regulation of PHOX2B and Mash1 when siRNA is used to inhibit MYCN [23], all support the hypothesis that a high *PHOX2B* expression level is due to the direct regulation of *PHOX2B* by MYCN, and we are currently investigating whether the down-regulation of *PHOX2B* is related to a reduction in MYCN expression.

In any case, our findings highlight the importance of the regulation of gene dosage in the processes driving final cells differentiation. The action of ATRA on PHOX2A and PHOX2B expression may not only determine a cell's fate, but also stabilise it by maintaining lineage-specific expression patterns, as confirmed by the observation that PHOX2B is down-regulated during final neuronal differentiation [13]. The dysregulation of one of these pathways (perhaps leading to the accumulation of PHOX2B$^+$ progenitor cells) may be one of the major mechanisms involved in the pathogenesis of NBs.

The down-regulation of *PHOX2B* expression induced by ATRA supports the idea that *PHOX2B* up-regulation is due to a block in the differentiation process, and so its down-regulation may force the differentiation of NB cells. Experiments aimed at identifying drugs capable of reducing *PHOX2B* expression in the IMR32 cell line [50] have not found that ATRA has any effect, and this is in line with our unpublished findings showing that *PHOX2A* and *PHOX2B* are not regulated by ATRA in IMR32 cells, although the cells are ATRA responsive as measured on the basis of RARE reporter vector activation (data not shown). However, the SH-SY5Y NB cell line responds to ATRA in a similar manner ([33] and data not shown), albeit with different kinetics, thus confirming that the effects of ATRA on *PHOX2A* and *PHOX2B* expression are not peculiar to the SK-N-BE(2)C cell line but may reflect a general mechanism that could be important in retinoid-induced neuronal differentiation.

These observations reinforce the idea that PHOX2A and PHOX2B may be useful biomarkers for NB staging, prognosis and treatment decision making [13].

These findings and other published data [51,52] shed new light on the pathways driving undifferentiated and proliferating cells to activate a differentiation programme by regulating the activation/deactivation of transcription factors such as PHOX2A and PHOX2B, which control cell-specific genes such as the *DβH* and cell cycle regulators such as $p27^{kip1}$. In particular, the work of Andrisani's lab. [52] has shown that PHOX2A is intrinsically programmed to be active for a defined period of time, although in our case it is not the activity but the presence of PHOX2A that is temporally regulated by ATRA. A better understanding of the molecular mechanisms underlying this control may provide new opportunities for the development of new drugs that can be used in cancer therapy.

5. Conclusions

In conclusions, our findings demonstrate that PHOX2A expression is finely controlled during retinoic acid differentiation and suggest that, together with PHOX2B down-regulation, they may be useful biomarkers for NB staging, prognosis and treatment decision making.

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The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2016.02.014.

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