UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO

Medicina Sperimentale e Biotecnologie Mediche

DIPARTIMENTO

Biotecnologie Mediche e Medicina Traslazionale

CURRICULUM /CORSO DI DOTTORATO

Neuroscienze e Neuropatologia

XXXI Ciclo

TESI DI DOTTORATO DI RICERCA

Newly identified PHOX2B-regulated genes as possible drug targets for the pharmacological intervention in Congenital Central Hypoventilation Syndrome (CCHS)

settore scientifico disciplinare: Bio14

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A.A. 2017/2018

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Abstract

Congenital Central Hypoventilation Syndrome (CCHS, OMIM #209880) is a very rare neonatal neurological disorder characterized by a broad variety of symptoms of autonomic nervous system dysfunction including inadequate control of breathing (Weese-Mayer et al., 2017). It is often associated with Hirschsprung's disease (HSCR) and neural crest-derived tumours (i.e. neuroblastoma). Frameshift mutations (5%) and polyalanine triplet expansions (from 4 to 13 residues) (95%) have been detected in the coding region of *PHOX2B*, a transcription factor required for the development of neurons that regulate the cardiovascular, respiratory and digestive organs, forming the sensory and motor arms of the visceral reflex circuits. Consistent with its role as transcriptional regulator, transcriptional dysregulation might be an important mechanism of CCHS pathogenesis. CCHS is a life-long disorder for which the only treatment option is ventilatory support provided by tracheotomy, nasal mask or diaphragm pacing by phrenic nerve stimulation, as pharmacological respiratory stimulants have proved to be ineffective. A strong limitation to the comprehension of the pathogenesis of CCHS, and the development of new and effective treatment for this disease, is the missing knowledge of target genes regulated by PHOX2B, whose expression may be eventually dysregulated. Very little is known about the genes regulated by PHOX2B. Most of the genes identified so far are regulatory genes that encode for transcription factors and enzymes that control downstream processes involved in the survival and differentiation of specific neural structures, such as *TH, DBH* (Lo et al., 1999; Adachi et al., 2000), *PHOX2A* (Flora et al., 2001), *TLX2* (Borghini et al.,2006), *RET* (Bachetti et al., 2005) *MSX-1* (Revet et al., 2008), SOX10 (Nagashimada et al., 2012), *ALK* (Bachetti et al., 2010) and *PHOX2B* itself (Cargin et al., 2005).

The aim of this thesis was to identify new potential pharmacological targets for the development of drugs in order to improve the respiratory symptoms and the quality of life of CCHS patients.

In the first part of my project we investigated the molecular mechanisms underlying the recovery of chemosensitivity, observed in two CCHS patients, following the administration of the progestinic desogestrel (Straus et al., 2010). In SK-N-BE(2)C cell clones, stably expressing nuclear progesterone receptor isoforms PR-B and PR-A, we demonstrated that 3-KDG treatment, active metabolite of the desogestrel, reduces the expression of PHOX2B, its target

genes as well as PHOX2B +7 alanine expanded protein, by means of a post-transcriptional mechanism. This finding provided the evidence of a direct molecular link between PHOX2B and desogestrel and suggested the possibility that reduction of PHOX2B mutant protein may contribute to the positive effects observed in the two CCHS patients.

In the second part of my project we proposed to identify new PHOX2B target genes that can be deregulated in the pathology, in the future perspective that might be potential pharmacological targets, alternative to PHOX2B. In particular, we demonstrated that transcriptional dysregulation and dysfunctions of K^+ and Na^+ channels activity may contribute to the onset of respiratory problems associated with CCHS. Target directly the de-regulated PHOX2B target genes is an alternative pharmacological strategy to by-pass the effect on PHOX2B in the perspective of rescuing their activity. Since several drugs targeting these proteins are already used in clinics, the potential progress toward a therapeutic intervention to treat CCHS is today more than concrete.

List of abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
3-KDG	3-kedodesogestel
7TMPR	Trans-Membrane Progesterone receptor
ANS	Autonomic Nervous System
ATRA	All trans retinoic acid
BMP	Bone morphogenetic proteins
CB	Carotid Body
CCHS	Congenital Central Hypoventilation Syndrome
ChAT	Choline acetyl transferase
ChIP-seq	Chromatin Immunoprecipitation-sequencing
CNS	Central Nervous System
CPG	Central Pattern Generator
CRISPR	Clustered regularly interspaced short palindromic repeats
DBH	Dopamine-β-hydroxylase
DRG	Dorsal respiratory group
DSB	Double stranded break
ENS	Enteric Nervous System
FS	Frameshift
GA	Geldanamycin
GO	Gene Ontology
gRNA	guide RNA
HD	Homeodomain
HSCR	Hirschsprung's disease
HSP	Heat Shock Protein
KO	Knockout
LC	Locus Coeruleus
LO-CCHS	Late Onset - Congenital Central Hypoventilation Syndrome
MS	Missense

NB	Neuroblastoma
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NMD	Nonsense-mediated RNA decay
NPARMs	Non-Polyalanine repeat mutations
NREM	Non-rapid eye movement
NS	Nonsense
NTS	Nucleus of the Solitary Tract
OMIM	Online Mendelian Inheritance in Men
ORF	Open reding frame
PAM	Proto-spacer adjacent motif
PARMs	Polyalanine repeat mutations
Pg	Progesterone
PGR-A/B	Progesterone nuclear receptor isoform A/B
PGRMC	Progesterone receptor Membrane Component
PHOX2A	Paired like homeobox 2A
PHOX2B	Paired like homeobox 2B
PNS	Peripheral Nervous System
pre-BötC	pre-Bötzinger complex
RADICA	Respiratory and Autonomic Disorder of Infancy, Childhood, and Adulthood
REM	Rapid eye movement
ROHHAD	Rapid-onset Obesity with Hypothalamic dysfunction, Hypoventilation and Autonomic Dysregulation
RTN	retro-trapezoid nucleus
RTN/pFRG	retro-trapezoid/parafacial respiratory group
SOX10	SRY-related HMG-box
TH	Tyrosine hydroxylase
TLX2	T Cell Leukemia Homeobox 2
TSS	Transcription start site
VRG	Ventral respiratory group
WT	Wild type

Introduction

Congenital Central Hypoventilation Syndrome

In 1970 Mellins and colleagues reported the first case of Congenital Central Hypoventilation Syndrome (CCHS) (Mellins et al., 1970). They described about an infant affected by hypoventilation due to a "failure of automatic control of ventilation". CCHS (OMIM #209880) is also known as Ondine's curse, name derived from an ancient German myth. The myth narrates the story of a man cursed by the Ondine nymph who is no longer able to breath autonomously during sleep (Suslo et al., 2015). CCHS is a rare life-long genetic respiratory disorder belonging to a group of other rare disorders with respiratory defects named RADICA (Respiratory and Autonomic Disorder of Infancy, Childhood, and Adulthood) (Patwari et al., 2010; Weese-Mayer et al., 2009). These disorders have a varying presentation and are caused by dysfunction of autonomic nervous system (ANS) and defect in respiratory control. Among these diseases, CCHS and ROHHAD (Rapid-onset Obesity with Hypothalamic dysfunction, Hypoventilation, and Autonomic Dysregulation) exhibit the greatest respiratory control deficits.

This syndrome is characterized by an abnormal ventilatory response to hypoxia and hypercapnia due to deficiency in autonomic respiratory control that normally functions during sleep. Subjects show adequate ventilation during wakefulness while have marked hypoventilation rate during sleep and, in the most severe cases also when they are awake (Weese-Mayer et al., 2010; Carroll et al., 2010). These respiratory symptoms are already present at birth or during early infancy and are more pronounced during non-rapid eye movement (NREM) sleep (Healy and Marcus 2011). However, some cases that present mild respiratory symptoms are diagnosed later in childhood or early adulthood, (late-onset cases LO-CCHS) following episodes such as anaesthesia, sedation or sleep apnea (Patwari et al., 2010; Weese-Mayer et al., 2010). CCHS occurs with an incidence of 1:200,000 born alive in France (Trang et al., 2005) and 1:148,000 in Japan (Shimokaze et al., 2015) but it could be underestimated due to the milder cases of CCHS and LO-CCHS not recognised or misdiagnosed.

An adequate ventilation is guaranteed by the interaction between ventilatory muscle power and central respiratory drive that are able to overcome the respiratory load (Fig.1A) (Kasi et al.,

2016). In CCHS, there is a decrease in central drive that leads to a displacement in favour of respiratory load with respiratory failure (Fig.1B). The reduction of the physiological response to increased CO_2 seems to be due to abnormalities in the integration of chemoreceptor input to central ventilatory controllers.

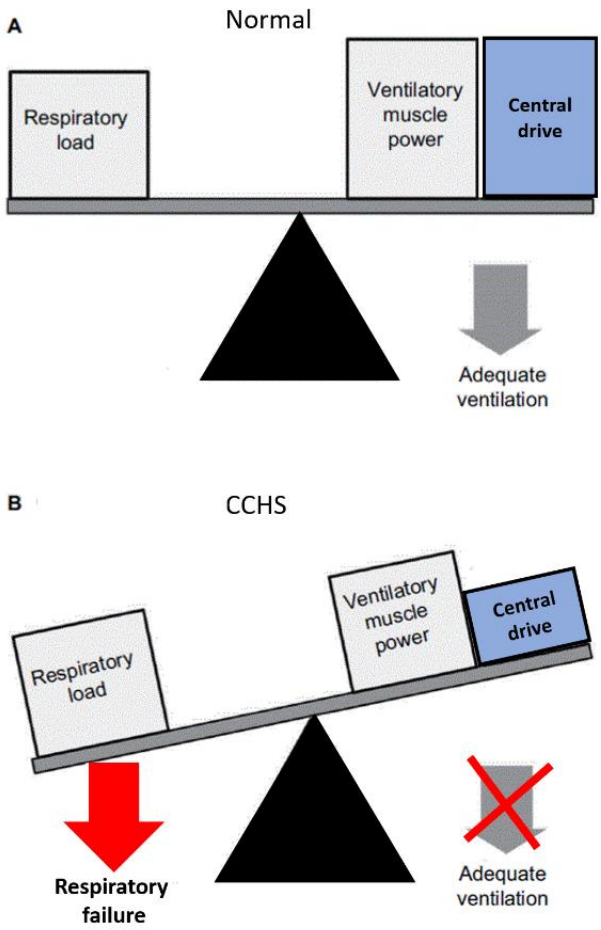

Fig.1 Diagram of respiratory balance. A: in a normal situation, ventilatory muscle power and central respiratory drive (blue box) overcome the respiratory load and guarantee adequate ventilation. B: in CCHS patients, central driver is decreased (blue box); ventilatory muscle power is not sufficient to overcome respiratory load thus leading to a respiratory failure. (From Kasi et al., 2016)

Hypoventilation is the principal feature of this autonomic syndrome but CCHS may also be associated with a more generalised ANS dysregulation, including reduced pupillary response to light, abnormal oesophageal motility (gastroesophageal reflux, constipation), decreased control of the basal body temperature, sporadic and sudden profuse sweating, heart rhythm disturbances, impaired perception of anxiety and pain. CCHS can also cause respiratory arrest

which could not only be responsible for severe neurological damages but also it can be fatal if not adequately controlled (Weese-Mayer et al., 1999).

Moreover, CCHS can occur alone or in association with other conditions including Hirschsprung's disease (HSCR) (Vanderlaan et al., 2004; Trochet et al., 2005; Berry-Kravis et al., 2006) and tumours derived from neural crest precursors such as neuroblastoma (NB), ganglioneuroblastomas, ganglioneuromas (Bourdeaut et al., 2005; Van Limpt et al., 2004).

HSCR (OMIM #142623) is an autonomic dysfunctions of the enteric nervous system (ENS) due to a defect in the development of ENS leading to the absence of formation of ganglia in the lower digestive tract. Aganglionosis results in a functional obstruction and the surgery is the only treatment for HSCR patients in order to remove the part of the gut that is not properly innervated (Heanue and Pachnis 2007). The aganglionic megacolon Hirschsprung's disease can be isolated or associated with other syndromic disease such as CCHS and a variety of genes have been reported to be mutated in different HSCR cases (*SOX10, ECE1, RET, NRTN, GDNF, PHOX2B, EDN3, EDNRB*) (Tam and Garcia-Barceló 2009). The association with CCHS is known as "Haddad syndrome" (OMIM #209880) and occurs in 16% of CCHS patients.

NB (OMIM #256700) is an embryonic neuroblastic tumours stemming from neuronal crest progenitors (Schleiermacher et al., 2014). In 50% of NB familial cases mutations that constitutively activate *ALK* (Anaplastic Lymphoma Kinase) (Mossé et al., 2008) have been reported, whereas *PHOX2B* (Paired Like Homeobox 2B) is found to be over-expressed in a small fraction of patients with sporadic or familiar NB. In addition, NB and CCHS occurs in approximately 20% of CCHS patients (Rohrer et al., 2002; Di Lascio et al., 2018).

So far, there are no pharmacological ventilatory stimulants able to restore the normal ventilation, therefore patients need ventilatory supports to ensure adequate ventilation for life, at least during sleep by means of nasal mask, tracheostomy or diaphragmatic pacemaker implantation that stimulate phrenic nerve. Moreover, central hypoxia with risks of neurological damages and fatal consequence can results by inadequate treatment.

The *PHOX2B* gene and protein

The *PHOX2B* gene codifies for a 314 amino acids long homeodomain-containing transcription factor and it is highly conserved in mammals; the human *PHOX2B* is 100% homologous with the chimpanzee, rat and mouse *Phox2b*. The human gene maps to chromosome 4p13 and consists of three exons (Fig.2).

The PHOX2B protein is member of the Q50 paired-like homeobox domain transcription factor family and has two core domains: a homeodomain (HD) and two polyalanine tracts.

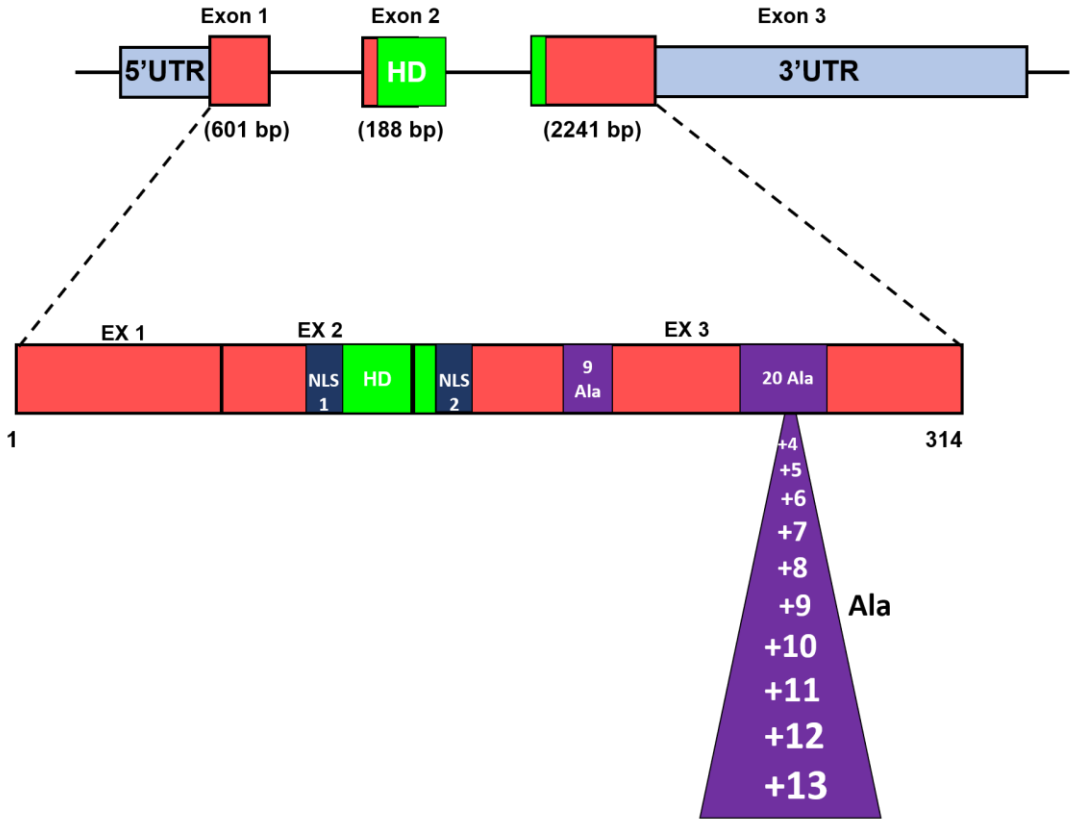

Fig.2 Schematic representation of *PHOX2B* gene and protein structure. Top: *PHOX2B* gene consists of three exons (pink); untranslated regions and introns are shown in light blue and black lines, respectively. The numbers below correspond to the length of *PHOX2B* exons. Bottom: 314 amino acids protein structure. The HD encoded by a region spanning exons 2 and 3 is showed in green. Blue boxes indicate the two nuclear localization signals (NLS 1 and 2); the two poly-alanine tracts of 9 and 20 residues within the C-terminal domain of the proteins are indicated in violet. In the 20-alanine long stretch all the possible polyalanine repeat mutations (PARMs) are shown.

The HD region, spanning exons 2 and 3, is highly conserved among the members of the Q50 paired-like homeodomain transcription factor family. It is a 60 amino acid long domain, forming a helix-loop-helix structure, able to recognize the DNA binding motif "ATTA" in the promoter region of PHOX2B target genes. The HD is flanked by two nuclear localization signals (NLSs) involved in nuclear translocation (Di Lascio et al. 2016). Furthermore, the HD is involved in homo- and hetero-dimerization and in particular, our laboratory provides evidence that PHOX2B protein is able to homodimerize and to strongly heterodimerize with its paralogue gene product PHOX2A (Di Lascio et al., 2016).

The C-terminal portion of PHOX2B protein, encoded by exon 3, contains two short and stable polyalanine repeats of nine and 20 residues encoded by GC(N) triplets (Fig.2). Their functional role is still unknown but there is evidence that they are involved in the correct protein conformation, protein-protein interaction and DNA binding, as recently demonstrated (Di Lascio et al., 2016).

Heterozygous mutations in the coding region of *PHOX2B* gene are responsible for CCHS.

The genetics of CCHS

CCHS is an autosomal dominant disease with variable expressivity and incomplete penetrance, and with no gender preference and most of the *PHOX2B* mutations are *de novo* mutations.

The majority of CCHS patients (95%) present in-frame triplet duplications within the 20-Alanine stretch (PARM; polyalanine repeat mutations), ranging from +4 to +13 alanine residues (Fig.2, bottom) (Amiel et al., 2003; Weese-Mayer et al., 2003). However, the remaining 5% of CCHS patients present non-PARM mutations (NPARM) within exon 1, 2 or 3. These include rare missense (MS), nonsense (NS) and frameshift (FS) mutations and are frequently associated with the onset of NB or HSCR disease (Di Lascio et al., 2018). Between the NPARM mutations, FS mutations are the most frequent, in which the insertion or deletion of a single nucleotide induces a change in the open reading frame (ORF) that leads to an aberrant C-terminal region (reviewed in Weese-Mayer et al., 2017). NS mutations are very rare, and the generation of a premature stop codon implicates the production of an incomplete non-functional protein. Moreover, in a very few subjects with CCHS associated with HSCR disease it has been reported the presence of *PHOX2B* mutation that cause the loss of alanine residues (Di Zanni

et al., 2017) with a contraction of the 20 Alanine stretch. This mutation is also present in the general population with a low percentage (around 1-1.5%) and it may play a role as modifier/predisposing factor in the HSCR pathogenesis (Toyota et al., 2004).

PARMs are almost exclusively identified in isolated CCHS patients that exhibit a respiratory phenotype with a clear correlation between the length of the alanine tract and the severity of the symptoms (Matera 2004; Berry-Krevis et al., 2006; Di Lascio et al., 2018). The +6 and +7 Alanine insertion mutations are the most frequent mutations; the +4, +5 Alanine insertion mutations are associated with a milder phenotype, whereas patients carrying the longest expansion (+13 Alanine) show the most severe phenotype, often associated with other ANS dysregulations like cardiac abnormalities (Weese-Mayer et al., 2010). NPARM mutations are less frequent but, on the contrary, they are associated with more severe respiratory symptoms and with other autonomic disorders (e.g. NB and HSCR disease occur in 50% and in 20% of NPARM patients, respectively) (Berry-Krevis et al., 2006; Di Lascio et al., 2018).

PHOX2B mutations are required to diagnose CCHS disease; nevertheless, in few CCHS patients negative for *PHOX2*B mutation, it has been reported the presence of mutation in some genes under PHOX2B transcriptional control involved in the correct development of neuronal crest cells or with an important role in the neuronal respiratory network (Gallego 2012). The mutations described are located in genes such as: *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) and additionally, mutations in *MYO1H* (Myosin IH) have been recently found in children affected by a rare recessive form of CCHS (Spielmann et al., 2017).

The role of these non-*PHOX2B* mutations remain largely unknown but it possible to speculate that mutations in these genes could cause defect in the respiratory system similar to CCHS or CCHS itself. Jennings et al, reported very few cases with variable CCHS-like phenotypes due to variation in the copy numbers of *PHOX2B* with partial or whole gene deletion (Jennings et al., 2012).

The role of PHOX2B in neurodevelopment

PHOX2B protein, together with its paralogue gene product PHOX2A, are two transcription factors essential for the development of the ANS. The role of PHOX2B during neurodevelopment is well established and most of this information derives from experiments conducted in animal models, whereas the role of PHOX2B in adulthood is still an open question.

Phox2b is expressed in neurons of both the central (CNS) and peripheral (PNS) nervous systems and it is essential for the maintenance of their differentiated stage and neuronal phenotype (Stanke et al., 1999). It has been demonstrated that in the CNS Phox2b is expressed in all noradrenergic neurons, in visceral motor neurons and in the hindbrain (Brunet and Pattyn 2002). Moreover, it is expressed in neurons of the nucleus of the solitary tract (NTS), in the carotid bodies (CB) and in the retro-trapezoid nucleus (RTN), an important CO_2-sensitive structure that fail to form properly in *Phox2b* knockout mice (Véronique Dubreuil et al., 2008). In the PNS, it is expressed by all autonomic ganglia in sympathetic, parasympathetic and enteric nervous system and in ganglia of the VII, IX and X cranial nerves. On the contrary, the expression of Phox2a is limited to parasympathetic and sensory ganglia and neurons of oculomotor and trochlear nuclei. Null Phox2a$^{-/-}$ mice die after birth with selective loss of the locus coeruleus (LC), the main noradrenergic centre in CNS (Morin et al., 1997).

Study in adult rats revealed the expression of Phox2b in important structures in the hindbrain that control orofacial movement, auditory function and eyes. Moreover, it seems to be absent in structures involved in respiratory rhythm and pattern generator, but it is highly expressed by neurons that mediate chemoreflex (Kang et al., 2007). In particular Phox2b was detected in the dorsal vagal complex and in RTN. In neurons of the NTS it is confined to cholinergic efferent neurons (salivary, vestibulocochlear) and, at the level of pons it is present only in the oculomotor complex. The persistence of the expression of Phox2b in the brain of the adult rat may also have an important role in maintaining the function of the noradrenergic neurons as suggested by data by Fan and colleagues; they demonstrated that both Phox2a and Phox2b are able to upregulate the expression of two important protein (DBH and NET) in noradrenergic neurons of rat brain (Fan et al., 2011).

PHOX2B plays a crucial role in determining neuronal identity during neurodevelopment. The

correct development of the autonomic ganglia (enteric and sympathetic) is a crucial step that occurs at the level of neuronal crest cell precursors and that requires mutual negative regulation between *Phox2b* and *Sox10* (SRY-related HMG-box). During normal neurodevelopment, crest-derived precursors of sympathetic and enteric neurons initially express only Sox10. When they migrate to the periaortic or enteric mesenchyme, they start to express Phox2b becoming double positive $Phox2b^{+}/Sox10^{+}$ cells (Fig.3). At this stage the destiny of bipotent progenitors is still undetermined; they can form either neuron or glia. *Sox10* is inactivated in cells that are destined to form neurons, while *Phox2b* is inactivated in cells that give rise to glia. The balance between *Phox2b* and *Sox10* is the result of their mutual and reciprocal suppression and it is required for the correct maintenance and differentiation of progenitors in enteric and sympathetic ganglia (Nagashimada et al., 2012).

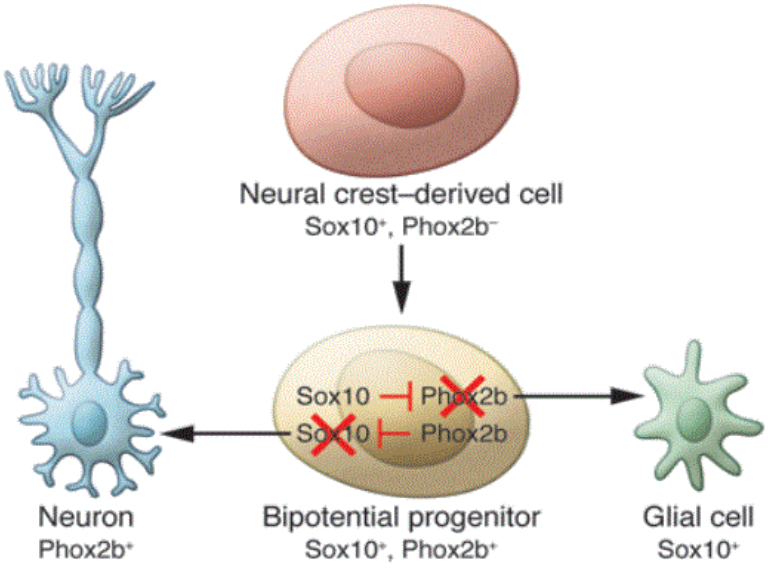

Fig.3 Schematic representation of the balance between *Phox2b* and *Sox10* in glial and neuronal differentiation. During normal development, initially crest-derived precursors are only $Sox10^{+}$. After the migration to the site of ganglion formation they become bipotent progenitors $Phox2b^{+}/Sox10^{+}$. The balance between *Phox2b* and *Sox10* is the result of their mutual and reciprocal suppression; glial cells are $Phox2b^{-}/Sox10^{+}$, while neuron cells are $Phox2b^{+}/Sox10^{-}$ (From Gershon 2012).

In addition to playing a major role in the correct formation of the entire autonomic nervous system, PHOX2B and PHOX2A are essential for the lineage specification toward a noradrenergic phenotype (Tiveron, Hirsch, and Brunet 1996) and maintains neuronal

differentiation by regulating neuronal cell cycle exit (Dubreuil et al., 2000; Paris et al., 2006).

Noradrenergic neurons are generally defined as neurons expressing tyrosine hydroxylase (TH) and DBH (Dopamine-β-hydroxylase), the rate-limiting enzymes required for the biosynthesis of noradrenaline. The specification of neuronal identity results from the interaction between intrinsic (cascades of transcription factors) and extrinsic factors (secreted or membrane associated signals). At embryonic day 10, neuroblast progenitors migrate to the dorsal aorta region, where the secreted bone morphogenetic proteins (BMPs) induce the expression of other factors necessary for the acquisition of a catecholaminergic phenotype. These transcription factors are expressed in a specific temporal order and form a complex regulatory network. Briefly BMPs stimulate the expression of *MASH1* and *PHOX2B*. MASH1 regulates *PHOX2A* and the bHLH transcription factor dHand, while PHOX2B regulates *PHOX2A, dHand* and *GATA3*. In turns, PHOX2B, dHand and GATA3 regulate the expression of *TH* and *DBH* (Fig.4) (Howard 2005).

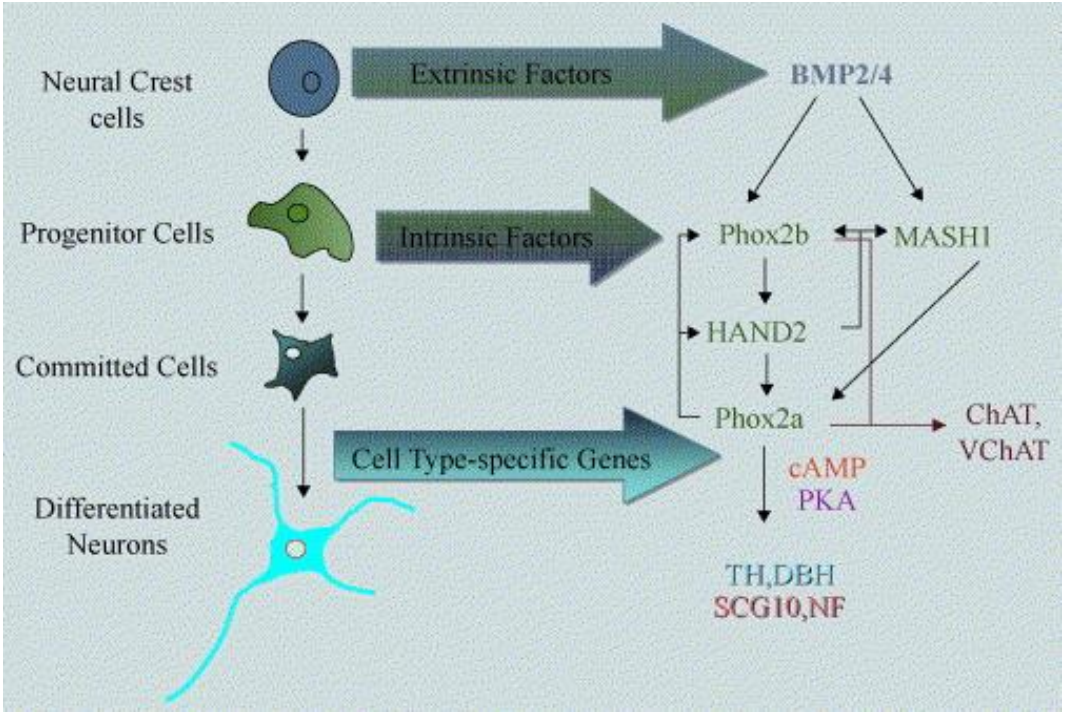

Fig.4 Schematic representation of transcriptional cascade for the noradrenergic differentiation. The migration of progenitor cells to dorsal aorta region activate extrinsic factors (BMP) that activate a complex network of intrinsic factor that lead to the specification of noradrenergic neuronal identity (From: Howard 2005).

PHOX2B target genes

So far, only few PHOX2B target genes have been identified. Some target genes encode proteins that enable neurons to perform their automatic function such as *TH* and *DBH* encoding for enzymes involved in the catecholamine biosynthesis, as mentioned before (Adachi et al., 2000; Lo et al., 1999). Others are regulatory genes that encode transcription factors that control downstream processes involved in the survival and differentiation of specific neuronal structure. For example, *PHOX2A* and *TLX2* (T Cell Leukemia Homeobox 2) are two gene positively regulated by PHOX2B. TLX2 is mainly expressed in tissues derived from neural crest cells at the end of their migration at the level of vesical and enteric nerve ganglia where it plays a crucial role in the development of the ENS (Borghini et al., 2006). Another transcriptional target of PHOX2B that play a crucial role in the correct intestinal innervation is the rearranged during transfection (*RET*) gene; a mutation in its regulatory regions is associated with HSCR disease (Bachetti et al., 2005). Moreover, it has been demonstrated that PHOX2B directly regulates the expression of the gene encoding for the receptor tyrosine kinase *ALK*; mutations in this gene are associated with NB (Bachetti et al., 2010). Furthermore, PHOX2B negatively regulates two important genes essential for the embryonic neuronal crest development: MSH homeobox 1 (*MSX-1)* (Revet et al., 2008) and *SOX10* (Nagashimada et al., 2012).

From a molecular point of view, PHOX2B regulates the expression of these genes by binding to specific "ATTA" sequences present within the promoters of its target genes. The "ATTA" sites are also present within the promoter of *PHOX2B* itself therefore, the same *PHOX2B* has to be considered a target gene because it modulates its own expression through an auto-regulatory mechanism (Cargnin et al., 2005).

This nucleotide sequence is present in the promoter region of *PHOX2A* and it has been demonstrated that PHOX2B positively regulates the expression of its paralogue gene, *PHOX2A* (Flora et al., 2001). As described in the previous chapter, PHOX2B and PHOX2A are co-expressed in several noradrenergic neurons but their expression is temporally and spatially regulated. *In vitro* experiments demonstrated that the *PHOX* genes are not functionally equivalent. In particular, in the sympathetic and parasympathetic ganglia of the brain stem, in the branchiomotor and visceral motor neurons and in the enteric system the expression of

Phox2b is essential for the correct development and precedes the expression of Phox2a. On the contrary, the LC is absent in null *Phox2a*$^{-/-}$ mice suggesting that Phox2a precedes the expression of Phox2b in this structure. However, the LC is also partially affected by the lack of phox2b demonstrating that both factors are required for its proper development (Pattyn et al., 1999)

PHOX2A and PHOX2B together regulate the expression of *DBH, TH, ALK* and *TLX2*; furthermore, PHOX2A regulate the expression of the α3 subunit of the human nicotinic acetylcholine receptor (α3 nAChR) in the post-ganglionic cells of the sympathetic nervous system (Benfante et al., 2007).

The role of Phox2b in respiratory circuit and animal model of CCHS

Breathing is an innate response essential for mammalian survival that ensures constant regulation of oxygen, carbon dioxide and hydrogen levels in the body. A physiological ventilation is guaranteed by the right integration of chemoreceptor input to central ventilatory control. This integration appears to be altered in CCHS patients. Much of our knowledge of the basic mechanisms that lead to a stable respiratory model derives from animal studies, both adults and new-borns. Breathing cycle can be divided into three main phases: inspiration (I); post-inspiration (Post-I or first stage of expiration) and late expiration (E2 or second stage of expiration) and is the result of the phasic contraction of respiratory muscles (thoracic muscles, muscles of the upper airway, diaphragm and expiratory muscles) that are activated by the spinal respiratory motor neurons. The timing of the respiratory cycle is controlled by excitatory and inhibitory interneurons that discharge during specific phases of the respiratory cycle. This complex network of neurons is called the Central Pattern Generator (CPGs) and represents also the centre for the integration of both motor and sensory signals (Smith et al., 2013; Tremoureux et al., 2014). Autonomic control of breathing is anatomically positioned in the brainstem, on the contrary voluntary control is located in the promoter areas, cerebellum and primary motor cortex (Zaidi et al., 2018).

The respiratory rhythm generator in the medulla can be separated in two groups containing respiratory neurons from either the dorsal or ventral respiratory group: the pre-Bötzinger complex (pre-BötC) (Smith et al., 1991) and the retro-trapezoid/parafacial respiratory group

(RTN/pFRG) (Onimaru et al., 2003). The dorsal respiratory group (DRG) is formed by inspiratory neurons that send signal to the motor neurons in the diaphragm, while ventral respiratory group (VRG) consists of both inspiratory and expiratory neurons.

Typically, respiration is initiated by respiratory neurons activated by peripheral chemoreceptor. Chemoreceptors providing information about pO_2, pCO_2/pH influence the respiratory cycle and are present in the aortic arch, in the CB (peripheral chemoreceptors) and in the ventral part of the bulb, near the VRG (central chemoreceptors). Central chemoreceptors are widely distributed in the nervous system, but the two main players are: RTN (Guyenet et al., 2008; Guyenet et al., 2009) and bulbar serotonergic neurons of the raphe nuclei (Corcoran et al., 2009; Hodges and Richerson 2010).

RTN is another vital source of respiratory drive, located in the ventral medullary surface and it is a collection of glutamatergic neurons that express *Phox2b* and the vesicular glutamate transporter *VGltu2*. These neurons receive signals from CB, hypothalamus and neurons located in the NTS which are stimulated by afferent axon in the fasciculus solitarius tract that innervates the phrenic nerve nucleus and the muscles of inspiration as well as afferent chemoreceptor in the CB. RTN is more important during NREM than during REM sleep (Zaidi et al., 2018).

CO_2-sensitive structures such as the LC, area postrema, RTN and the afferent pathways from the CB depend on Phox2b for their development (Dubreuil et al. 2009); moreover, in adult rats, it has been demonstrated that inhibition or injury of RTN neurons widely attenuate the chemoreceptor reflex of breathing.

Today, genetically engineered animals are still an important model to study and understanding better the pathophysiological mechanisms of CCHS. Studies on animal model that try to recapitulate CCHS disease, helped to understand the direct assessment of the role of PHOX2B in the neuronal control of breathing as well as its role in neuronal development and cellular function.

Phox2b knockout mice (*Phox2b*$^{-/-}$) are not vital and die in utero at E14 stage for complete agenesis of the ANS underlying the essential role of *Phox2b* in ANS development (Pattyn et al., 2000; Pattyn et al., 1999). Mice bearing only one invalidated allele (*Phox2b*$^{+/-}$), survive, are fertile but do not recapitulate the CCHS phenotype (Dauger 2003). In 2-day-old mice it was reported

an impaired ventilatory response to hypercapnia however resolved 10-days post birth. In comparison to adults, neonatal respiratory control is immature and the ventilatory response to CO_2 is influenced by environment and body temperature as well as age. Ramanantosoa and colleagues demonstrate that *Phox2b* mutation affects the interaction of breathing with thermoregulation control (Ramanantsoa et al., 2007). The neuroanatomical bases of this dysfunction are not clear because thermoregulation is mediated by the hypothalamus that does not express *Phox2b*. Serotonergic neurons that are involved in both thermoregulation and in respiration could be involved.

More information concerning the link between *Phox*2b expression and brainstem respiratory circuits derived from study of the first mouse model of CCHS in 2008. This model was obtained using a knock-in approach to insert the most frequent polyalanine expansion (+7) into the 20-alanine tract (*Phox2b*$^{27Ala/+}$) (Dubreuil et al., 2008). The heterozygous mice die within a few hours after birth because of central apnea, blunted response to hypercapnia and abnormal breathing patterns in normal air; symptoms that recapitulate symptoms of human neonates with CCHS. Neuroanatomical studies showed loss of RTN/pFRG neurons and precursors population at embryonic day 12.5, whereas no abnormalities in other structures involved in controlling breathing were revealed. Conditional mice (*Egr2*^{cre}*; Phox2b*$^{lox/lox}$ and *Lbx1cre; Phox2b*$^{lox/lox}$) were generated to further investigate the link between RTN and chemosensitivity; both mutant mice showed massive and selective depletion of RTN neurons, no response to hypercapnia, and died at birth due to respiratory failure (Dubreuil et al., 2009), thus suggesting that loss of RTN/pFRG disrupts respiratory rhythmogenesis and chemosensitivity during early development.

However, mice in which the selective ablation of the RTN was caused by the expression of the Phox2b +7 Alanine mutant only in neurons descending from the Egr2 lineage *(Egr2*^{cre}*; P2b27*^{Alacki}) survived to adulthood (Ramanantsoa et al., 2011), although they still showed blunted response to hypercapnia up to 4 months, that recovered in the adult stage. Taken together, these results support the pivotal role of RTN in CO_2 sensitivity, especially during NREM sleep but this is not explaining the respiratory defects that these same patients have also during wakefulness. Moreover, since conditional mice survived despite the deletion of RTN, this suggests that the neurological bases of CCHS are not confined to RTN, and other structures can be involved. Furthermore, compensatory mechanisms could have an important

role in partially restoring CO_2 sensitivity.

Studies to identify the presence of RTN-like structure in humans are very difficult and are based on the identification of PHOX2B positive regions by immunohistochemistry analysis.

Analyses of post-mortem brain of two CCHS patients with confirmed PARM and NPARM (*PHOX2BΔ8*) mutation revealed important defects of the LC. In particular, patients presented abnormalities in dorsal motor neurons of the vagus nerve, decrease serotoninergic median raphe and absence of mesencephalic trigeminal nucleus (Nobuta et al., 2015). These findings were confirmed in a mouse model (*Hprt-cre /PHOX2B*$^{20/E3\text{-}cKi\text{-}\Delta8\text{-}ires\text{-}GFP}$) carrying the same NPARM mutation detected in patients, expressed at early embryonic stages (<E10.5). These mice died at delivery and showed intestinal aganglionosis and loss of RTN and facial nerve nucleus due to the failure of precursors migration. Mice also showed abnormal LC due to a wrong precursor differentiation with altered expression of catecholamine synthetizing enzymes (DBH and TH), indicating noradrenergic and LC dysfunction. In contrast, the expression of this NPARM mutation after neurogenesis, at E11.05 (*Blbp-cre/ PHOX2B*$^{20/E3\text{-}cKi\text{-}\Delta8\text{-}ires\text{-}GFP}$), and limited to Phox2b+ neurons, did not affect LC development. Despite the failure of RTN development, the perinatal respiratory lethality was rescued (Nobuta et al., 2015).

This data suggests that LC and RTN neurons can both contribute to the respiratory abnormalities of CCHS patients.

Molecular pathogenetic mechanisms

It is well known that CCHS is due to mutations in PHOX2B gene resulting in aberrant proteins but what is the exact pathogenetic role of the PHOX2B mutant protein at the molecular level is still unclear. Data derived from *in vivo* and *in vitro* studies suggest that the combination of at least three different mechanisms is responsible for the different manifestation of the clinical phenotypes in CCHS patients; loss-of-function mechanism, dominant-negative effect and toxic gain of function (Di Lascio et al., 2013; Trochet, Hong, et al., 2005; Bachetti et al., 2005; Durand et al., 2005; Goridis et al., 2010; Dubreuil et al., 2008) (Fig.5). The respiratory problems observed in the first post-natal period, in engineered mice expressing only one copy of *Phox2b* gene (*Phox2b*$^{+/-}$) is suggestive of a haploinsufficiency mechanism in CCHS. The

presence of only one functional *Phox2b* allele although it is sufficient to prevent CCHS manifestation, on the other hand it contributes to the mild autonomic dysfunction of the post-natal period. Haploinsufficiency is often caused by a loss-of-function mutation indeed mice bearing only the mutant allele +7 Ala (*Phox2b* $^{-/+27\,Ala}$) die in utero providing the evidence that mutation in *Phox2b* lead to loss-of-function. Finally, the presence of a toxic-gain of function mechanism in CCHS due to mutation in *Phox2b* gene is suggested by the lack of development of Phox2b positive neurons (RTN neurons) in heterozygous mice (*Phox2b*$^{+27Ala/+}$).

In order to better understand the role of the mutant protein in the onset of CCHS symptoms, experiments with PHOX2B mutant proteins bearing different polyalanine expansion and/or with frameshift mutations have been carried out in cellular models. Alterations in the cellular localisation and transcriptional activity were reported with both mutant proteins (PARM and NPARM) in contrast to wild type (WT) protein (Bachetti et al., 2005; Trochet et al., 2005; Trochet, de Pontual, et al., 2008; Di Lascio et al., 2018; Bachetti et al., 2007).

More precisely, *in vitro*, all the mutant proteins show a tendency to oligomerize probably due to protein misfolding, however the loss of nuclear localization and the formation of cytoplasmic aggregation is especially evident in the mutant protein with the longest polyalanine stretch expansion (loss-of-function mechanism) (Bachetti et al., 2005; Trochet et al., 2005; Trochet, de Pontual, et al., 2008), whereas FS mutations preserve the nuclear localization with formation of nuclear inclusion (Di Lascio et al., 2018; Bachetti et al., 2005). The overexpression of PHOX2B +13 Ala in cellular model induces not only cytoplasmic aggregation but also cellular death by apoptotic mechanism (toxic gain of function) (Bachetti et al., 2007). It is important to underline that, until now, there is no evidence concerning aggregation of PHOX2B *in vivo*, so further investigations are necessary to understand the role of this mechanisms in CCHS.

Moreover, studies on the transcriptional activity of PHOX2B mutant proteins reported a dominant-negative mechanism in combination with a loss-of-function mechanism (Di Lascio et al., 2013). In particular, mutant proteins with longer expansion retain PHOX2B WT proteins in the cytoplasm and interfere with the PHOX2B WT autoregulatory loop, thus leading to the reduction of the expression of the normal protein (Di Lascio et al., 2013; Parodi et al., 2012). In addition, the presence of the alanine expansion in the C-terminal portion of the protein decreases PHOX2B capacity to regulate the expression of important target genes such as DBH,

PHOX2A and TLX2 (Di Lascio et al., 2013; Bachetti et al., 2005; Trochet et al., 2005). Elongation of the alanine stretch in PHOX2B alters also the ability to form homo-dimers important for the transcriptional activity. PHOX2B proteins with long expansions and with NPARM mutation show a reduction in protein affinity to DNA and weakly interact with the WT protein, while mutant proteins with short expansions maintain this ability (Di Lascio et al., 2016; Trochet et al., 2005; Trochet et al., 2008).

Moreover, PHOX2B WT protein forms hetero-dimers with PHOX2A. Data from our laboratory demonstrated that the mutant protein retains the ability to interact with PHOX2A without interfering with its nuclear localisation and above all, with its transcriptional activity (Di Lascio et al., 2016).

In this perspective, the dominant-negative effect observed in the presence of the mutant protein is not probably due to an aberrant interaction with the WT protein but conversely to a mechanism of squelching of co-activators or co-repressors that are usually recruited by WT homo-dimer in a physiological situation. In line with this idea, it has been demonstrated that CREB-binding protein is a PHOX2B co-activator that mediate synergistic activation (Wu et al., 2009). The two proteins interact by means of specific domains. In the presence of the mutant protein, the interaction domains are different than those used with the normal protein thus leading to an impaired synergistic activation, a mechanism that can contribute to the pathogenesis of CCHS (Wu et al., 2009). Moreover, PHOX2B protein bearing FS mutations can regulate PHOX2B target genes in a different way. For instance, *SOX10* that is negatively regulated by PHOX2B during neuronal differentiation is transactivated by PHOX2B mutant protein (Nagashimada et al., 2012).

CCHS occurs with a wide variety of symptoms and patients with the same mutation show different severity of respiratory phenotype. As *in vitro* studies have shown that PARM and NPARM mutant proteins share some of the molecular mechanism leading to impaired transcription of PHOX2B and its target genes, it is reasonable to suggest that different mutation may induce different defects by means of a combination of the three molecular mechanisms described, depending on the genetic background of the patient (Trochet at al., 2005).

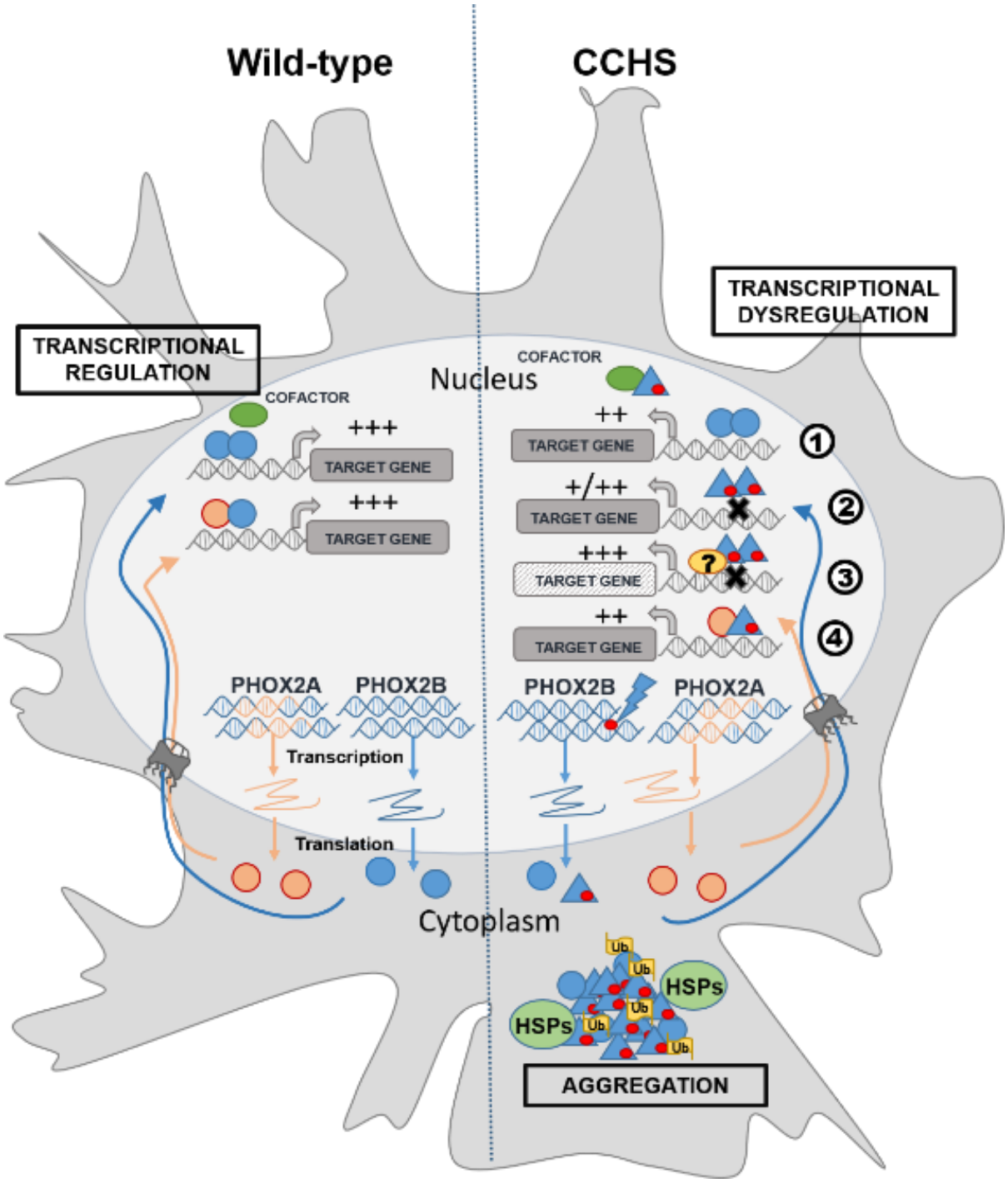

Fig.5 Schematic representation of molecular pathogenesis of CCHS. Left: wild-type condition. PHOX2B (blue) and PHOX2A (red) proteins are translated into the cytoplasm and then migrate to the cell nucleus where they form homo- and hetero-dimers, bind specific DNA sequences, recruit co-activators (green), and induce the transcription of target genes. Right: In CCHS, one of the two *PHOX2B* alleles is mutated (red dot). As in the case of the WT, both copies of the gene are transcribed into mRNA molecules and then translated into proteins; half normal (blue circle) and half mutated (blue triangle). Mutated proteins are misfolded, aggregate in the cytoplasm and fail to enter into the nucleus. Aggregates activate heat shock proteins (HSPs) that try to refold mutant proteins or ubiquitin ligase enzymes (Ub) for the proteolytic degradation by the proteasome. In cell models of CCHS, the WT protein can be recruited into protein aggregates, which may lead to a dominant negative effect. A fraction of the mutated proteins can enter to the nucleus and induce transcriptional dysregulation by means of various molecular mechanisms:1) aberrant interactions with the cofactors important for transcriptional activity; 2) decreased transcriptional activity due to a decreased DNA binding; 3) induction of the expression of aberrant target genes; and 4) a decreased ability to interact with PHOX2A. (From Di Lascio et al., 2018)

Therapeutic approaches in CCHS

The limited knowledge regarding the genes regulated by PHOX2B and their involvement in the different clinical manifestations of the disease prevent the development of pharmacological treatments able to improve the disease-related respiratory defects and the quality of life of patients. So far, no pharmacological treatment has been developed and the ventilatory support is the only option available.

In vivo experiments show the tendency of mutant PHOX2B protein to form aggregates. Therefore, a current pharmacological strategy consists in counteracting the toxic effect of aggregation activating the heat shock protein (HSP) response pathway (Bachetti et al., 2007; Parodi et al., 2012). Physiologically, the presence of misfolded protein in the cytoplasm or in the nucleus is recognised by the cell quality control mechanisms that activate chaperon proteins in order to refold the protein or facilitate the activation of the ubiquitin/proteasome pathway, which degrades misfolded proteins. The formation of aggregates is a pathological mechanism that usually occurs when the production of misfolded proteins exceeds the proteolytic capacity of the proteasome or the activity of chaperones proteins. In line with this idea, Bachetti and colleagues demonstrated that geldanamycin (GA), a benzoquinone ansamycin antibiotic that activates the heat shock response, is able to induce cytoplasmic disaggregation. In particular, GA rescues the nuclear localisation and transcriptional activity of PHOX2B mutant protein bearing the +13 Alanine expansion (Bachetti et al., 2007).

This strategy was then expanded to curcumin (diferuloylmethane) and to 17-allylamino-17-demethoxygeldanamycin (17-AAG), an anticancer drug. Both compounds resulted to be effective in refolding and rescuing PHOX2B nuclear localization and transactivation activity and promoting the clearance of mutant PHOX2B protein aggregates. While GA and 17-AAG induce the expression of molecular chaperones HSP70, curcumin does not induce activation of HSP, thus suggesting the presence of an alternative pathway (Di Zanni et al., 2012).

Other possible pharmacological targets are ubiquitin ligase enzymes (E1, E2 and E3) that are necessary to target misfolded protein to the degradation by proteasome proteolytic activity. In particular, TRIM11, an E3 ubiquitin ligase, has been found to induce *DBH* expression by directly interacting with *PHOX2B*. The overexpression of *TRIM11* induces both mutant and WT

protein degradation and counteracts the dominant-negative effect of mutant protein on the transcription activity of DBH promoter. Mechanisms, which upregulate the expression of *TRIM11*, could be an alternative target for treatment of CCHS patients (Parodi et al., 2012).

All systems involved in protein quality control are possible pharmacological targets, but it is important to underline that the presence of aggregates was demonstrated only by *in vitro* experiments with the overexpression of mutant protein. In addition, these drugs affect also WT PHOX2B protein, although it has been demonstrated that the presence of WT protein has a protective role in preventing the formation of aggregates in cell models (Di Lascio et al., 2013). However, PHOX2B mutant proteins mislocalization is not the only mechanism suggested in the pathogenesis of CCHS; as already described, the mutant protein exerts a dominant-negative effect on WT protein activity and the refolding of aggregates may improve the normal protein function.

Indeed, a PHOX2B mutant protein is not only a misfolded protein as it maintains the ability to interact with co-activators (Wu et al., 2009) and with *PHOX2*A (Di Lascio et al., 2016), although with different results; the binding with the co-activator CBP interferes with the activation mediated by the PHOX2B WT/CBP complex, whereas the heterodimerization with PHOX2A partially prevents transcription defect of PHOX2B mutant protein. These data suggest that the functional effect of the mutant protein depends on the interactor proteins and they could represent an important pharmacological target, alternative to *PHOX2B*.

Recently, the use of progesterone has been investigated as strategy to improve respiratory symptoms. The idea that progesterone therapy could be useful arise from the fortuitous observation that two female patients, with +5 and +6 Alanine expansions, experienced a partial CO_2-chemosensory recovery and increased ventilation under treatment with desogestrel for contraceptive purposes (Straus et al., 2010). It is well known that progesterone (Pg) and synthetic progesterone derivatives are respiratory stimulant in adult humans and have been used to treat adults with obesity hypoventilation disorders and are potential candidate for the treatment of adult apnoea (Boukari et al., 2017; Shahar et al., 2003). Pg is a steroid mainly synthesized in the gonads and in the placenta and as a neurosteroid, in the peripheral and central nervous system (Nelson and Bulun 2001). Sex hormones can modulate breathing through a direct effect on gene expression in respiratory neurons (by steroid hormones receptors activation) or indirectly (Behan and Wenninger

2008). Pg can bind membrane receptor or nuclear receptor expressed in several brain regions and activates different response: a rapid non-genomic mechanism and a slower genomic mechanism, respectively (Contrò et al.,2015).

The classical nuclear progesterone receptor (PGR) is present in two isoforms, resulting from an alternative transcription driven by independent promoter of the same gene: the full-length B isoform (progesterone receptor B, PGR-B) and the N-terminal truncated isoform A (progesterone receptor A, PGR-A) (Conneely et al., 1987). The two isoforms have distinct role: PGR-B is essential for normal mammary gland development, while PGR-A is required for uterine development and reproductive function (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). However, in gene expression regulation they can also have a synergic role (Hopp et al., 2004). From a transcriptional point of view, PGR-A and PGR-B display different transcriptional regulatory activities: PGR-B is a strong activator of transcription of several PGR-dependent promoters whereas PGR-A is associated to a repressor activity (Mulac-Jericevic et al., 2000).

PGR has been localized in different region of CNS including cortex, hypothalamus, hippocampus, cerebellum and NTS (Bayliss and Millhorn 1992) where it plays an important role as modulator of chemoreflex sensitivity and respiratory control, especially during sleep (Bayliss et al., 1987). In addition, experiments conducted on nuclear progesterone receptor knockout mice (*PRKO* mice) demonstrated that the absence of PGR increases apnea frequency during NREM sleep and reduces chemoreflex responses to hypercapnia after Pg treatment (Marcouiller et al., 2014).

Pg binds also to membrane receptors such as the seven Trans-Membrane Progesterone receptor (7TMPR) and the single transmembrane receptors Progesterone receptor Membrane Component 1 and 2 (PGRMC1 and PGRMC2). The 7TMPR isoform α is expressed in reproductive tissues and kidney, the 7TMPR isoform β is localized in neuronal tissues (cerebral cortex, cerebellum, thalamus, caudate nucleus, spinal cord and pituitary gland), whereas the 7TMPR isoform γ is present in colon and kidney (Zhu et al., 2003). PGRMC1 and PGRMC2 are expressed in liver, kidney and placenta, moreover they are found in hypothalamic nuclei of rat brain (Gerdes et al., 1998).

Women affected by CCHS do not recovery CO_2 sensitivity during gestation, thus suggesting that physiological progesterone is not able to improve hypercapnia response per sé (Sritippayawan

et al., 2002). Consequently, the effect of desogestrel is exclusively mediated by this progestin. The fact that desogestrel has a stronger affinity for progesterone receptor than progesterone itself (three times greater) could justify this effect (Grandi et al., 2014). However, the exact mechanism through which desogestrel could ameliorate respiratory symptoms in CCHS patients remains still unknown.

Very recent findings indicate that multiple neuronal circuits, such as those involving medullary and supramedullary neurons, are involved in the effect mediated by desogestrel (Joubert et al. 2016; Loiseau et al., 2014). In particular, experiments conducted in mice or in *ex vivo* murine medullary-spinal cord preparations, demonstrated an increase in respiratory frequency after exposition to etonogestrel (active metabolite of desogestrel), mediated by the activation of the medullary respiratory neurons, in particular serotoninergic neurons of the raphe pallidus and nucleus raphe obscurus (Joubert et al., 2016). In addition, other experiments on *ex vivo* preparations of CNS (medullary–spinal cord and diencephalon–brainstem–spinal cord preparation) of new-born rats, demonstrated that acute exposure to etonogestrel involves supramedullary structures located in pontine, mesencephalic or diencephalic regions (Loiseau et al., 2014). It is worth noting that the same regions activated by desogestrel express *Phox2b* in adulthood rodent brain (Kang et al., 2007). All together these data indicate that more than one neuronal circuit should be considered in the chemosensitivity recovery observed in the two CCHS patients after desogestrel treatment.

So far CCHS has been considered a neurodevelopmental disorder resulting from total disruption of central chemosensory integration. However, data from heterozygous animal models carrying the Phox2b +7 Alanine mutation (Trochet et al., 2008) suggest the possibility of residual chemosensory function in the human disease. In fact, these animals showed a reduction by 70% of the crucial RTN chemoreceptor function. The hypothesis of a residual chemosensitivity response was confirmed recently in CCHS patients by Carroll and colleagues (Carroll et al., 2014). In a large cohort of CCHS patients and in particular in young patients, they reported a detectable, although still physiologically inadequate, ventilatory response to chemosensory challenges. This residual cardiorespiratory response suggests a partial preservation of CNS networks; this could provide a pivot for potential pharmacological interventions capable to take advantage of this function to reduce mortality and morbidity while enhancing the overall quality of life in patients with CCHS.

Aim of the thesis

For many years, it was thought that CCHS was an irreversible disease for two main reasons: first of all, it is a neurodevelopmental disorder and second because CCHS mice model show missing formation of RTN, a crucial structure responsible for hypoxia and hypercapnia sensitivity, thus frustrating any effort to restore CO_2 chemosensitivity in CCHS patients (Carroll et al., 2014). However, recent studies reported the presence of a residual cardiorespiratory response in CCHS patients (Trochet et al., 2008). This finding opens up the possibility to search for potential pharmacological interventions capable to take advantage of this residual function to reduce mortality and morbidity while enhancing the overall quality of life in patients with CCHS.

In 2010 a hopeful indication for the treatment of CCHS patients derived from a direct clinic observation (Straus et al., 2010). 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. This fortuitous observation suggests a possible link between this drug and the recovery of the CO_2 chemosensitivity. Additionally, recent findings indicate that multiple structures in rodent brain (including some structures Phox2b positive in adulthood) are activated by desogestrel suggesting their hypothetical role in the recovery observed in the two CCHS patients (Joubert et al., 2016; Loiseau et al., 2014). However, the molecular mechanism of desogestrel effect was so far completely unknown.

In the first part of my PhD thesis I present the results obtained by investigating the mechanism underlying this effect. These results have been published in the paper “DESOGESTREL DOWN-REGULATES PHOX2B AND ITS TARGET GENES IN PROGESTERONE RESPONSIVE NEUROBLASTOMA CELLS” in Experimental Cell Research (Cardani et al., 2018), and provide the evidence of a direct molecular link between PHOX2B and desogestrel. This finding suggests the possibility that this mechanism may contribute to the positive effects observed in the two CCHS patients.

A strong limitation to our comprehension of the molecular mechanisms underlying CCHS and the development of new and effective treatment for this disease is due to the substantial lack of knowledge of all target genes regulated by PHOX2B and their specific involvement in the

different clinical manifestations of CCHS and the availability of CCHS cellular models. Specific genes are affected differently by distinct *PHOX2B* mutation (Di Lascio et al., 2016), thus leading to different severity of respiratory symptoms, organ involvement, request of medical assistance, quality of life, and probably different life expectancy in CCHS patients. Since PHOX2B is an important transcriptional regulator, it is very likely to hypothesize that a general transcriptional dysregulation might be an important mechanism of CCHS pathogenesis. So far, very little is known about the genes regulated by PHOX2B; most of them are regulatory genes encoding for transcription factors and enzymes that control downstream processes involved in the survival and differentiation of specific neural structures (Lo et al., 1999; Adachi et al., 2000; Flora et al., 2001; Borghini et al., 2006; Bachetti et al. 2005; Revet et al., 2008; Nagashimada et al., 2012; Bachetti et al., 2010; Cargin et al., 2005).

The possibility of improving ventilatory and sensory responses to hypercapnia upon progestinic treatment emphasizes the importance of identifying new PHOX2B target genes that can be deregulated in the pathology. Finding additional PHOX2B target genes is important not only for explaining the pathogenetic mechanisms underlying the dysfunctions of specific neural structure, but especially because they might be potential targets, alternative to PHOX2B, for pharmacological interventions in order to develop alternative molecules, without contraceptive effect, that can be also chronically administered to mail patients.

For this reason, in the second part of my PhD project I will show results on the identification of some of the relevant PHOX2B target genes, whose deregulation is eventually responsible for the disease and the constellation of clinical symptoms, in the perspective of rescuing their normal activity and ameliorate breathing and dys-autonomic CCHS symptoms, also by-passing *PHOX2B* mutations.

Previous studies, conducted in our laboratory, by means of ChIP-seq experiment identified many PHOX2B target gene candidates (around 3000 genes) consistent with PHOX2B role during ANS development and maintenance that need to be validated. Interesting, among these predictive genes, there were several genes encoding for K^+ and Na^+ channels. Several findings demonstrated that many of them are involved in the regulation of the excitability of autonomic neurons and in the modulation of respiratory activity *in vivo* (Mulkey et al., 2015; Torrecilla et al., 2013; Bond et al., 2000) suggesting that their expression could be deregulated in CCHS patient, and they represent potential therapeutic target for the

treatment of respiratory dysfunction. I focused my attention on this promising category.

By means of stable IMR32 cell clones, where the expression of PHOX2B has been knocked-down by means of CRISPR-Cas9 editing technology, I will show that the expression of three K^+ channels (*KCNN3, KCNMA1* and *KCNQ5*) and four Na^+ channels (*SCN2A, SCN3A, SCN8A* and *SCN9A*) is up-regulated in the absence of PHOX2B, thus suggesting a negative modulation of their expression by PHOX2B. Moreover, transient expression of PHOX2B +7 and +13 Alanine mutant proteins showed an up-regulation of the expression of *KCNN3* gene, thus suggesting that the mutant proteins acts as inducer of this gene (gain of function mechanism).

Since ion channels are the primary determinants of neuronal cell excitability and in PHOX2B knockout (KO) IMR32 clone their expression was up-regulated, in collaboration with Flavia Antonucci (University of Milan) I investigated whether this deregulation impairs the electrophysiological properties of the cells. We tested ion channels activity *in PHOX2B KO and* control cells by measuring total ion currents induced by injection of hyper-to-depolarizing currents. Preliminary data demonstrated that *PHOX2B* KO cells are more polarized than control, thus leading us to hypothesize that these cells might be less prone to respond to activating stimuli.

These data suggest that de-regulation of the expression of genes important for the correct response of neuronal cells to stimulatory signals, such as response to increase CO_2 levels, might be one of the mechanisms underlying CCHS pathogenesis. Consequently, these genes represent suitable candidates for pharmacological intervention in CCHS that aims to restore their correct expression to ensure a correct function of the neurons in the central and peripheral chemoreceptors.

Part 1

Desogestrel down-regulates PHOX2B and its target genes in progesterone responsive neuroblastoma cells

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Experimental Cell Research. 2018. Volume 370. Issue 2. Page 671-679

Publisher: Elsevier Inc. doi: 10.1016/j.yexcr.2018.07.032.

Inserted as Appendix 1

CCHS is considered a life-long disorder. Until now, no pharmacological treatment has yet provided to be capable of restoring the normal ventilation in patients; for this reason, the only available support for CCHS affected patients is the mechanical ventilation by means of nasal masks, tracheotomy, 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.

The aim of current CCHS treatment research is to counteract the toxic effect of PHOX2B mutant protein; drugs promoting the refolding and/or clearance of mutant protein aggregates have provided to be effective in rescuing the nuclear localisation and transactivation activity of mutant proteins *in vitro* (Di Zanni et al., 2012; Bachetti et al., 2007; Trochet et al., 2005).

Recently, a new pharmacological perspective arose from the observation that two female patients with +5 and +6 Alanine expansion showed a partial recovery of the ventilatory response to CO_2 and increased ventilation after treatment with the oral contraceptive desogestrel (Straus et al., 2010).

The molecular mechanism underlying this effect is unknown. Recent data suggests that multiple pathways are involved in the ventilatory effect mediated by desogestrel (Loiseau et al., 2014; Joubert et al., 2016) and, in addition, experiments on adult rodent brain have revealed that some regions, including some phox2b$^+$, are activated by desogestrel, thus suggesting a possible link between PHOX2B and desogestrel.

Since it was reported that neurons, that play a central role in CO_2 chemosensitivity, co-express PHOX2B and PGR (Kang et al., 2007; Quadros et al., 2008) we investigated whether there was a relationship between Pg and PHOX2B activity and in particular, whether desogestrel was able to affect the expression of PHOX2B and its target genes.

Preliminary data collected in my laboratory on T47D human breast adenocarcinoma cell line, provided us the first evidence that 3-ketodesogestrel (3-KDG), the active metabolite of desogestrel, is able to influence the activity of PHOX2B, by the activation of the intracellular PGR.

In order to further investigate the desogestrel mechanism in a cellular background more properly associated with the physiological PHOX2B environment, we used SK-N-BE(2)C neuroblastoma cell line that express high level of *PHOX2B* but low level of *PGR*.

In these cells, transiently transfected with *PGR* and treated with 3-KDG, we showed a reduction in PHOX2B protein level and a decrease activity of two known PHOX2B target genes (*TLX2* and *DBH*) thus confirming the existence of a relationship between PHOX2B activity and desogestrel.

To further investigate this relationship, we generated SK-N-BE(2)C cell clones, stably expressing PRG isoforms *B* and *A*. Among the generated clones, we chose two clones that express differently the two PGR isoforms and in this cellular model we analysed the effect of 3-KDG treatments on endogenous PHOX2B protein amount and transcription levels by using western blot and qRT-PCR analyses.

Our findings demonstrated the existence of a direct molecular link between desogestrel and the expression of PHOX2B and in particular, our data showed that:

- 3-KDG down-regulates endogenous PHOX2B protein;
- 3-KDG reduces the protein level of some direct PHOX2B target gene (DBH and TFP2A) whereas genes not regulated by PHOX2B are not affected (*SP1, CREB* and *c-JUN*)
- 3-KDG partially affects *PHOX2B* mRNA degradation but does not affect PHOX2B protein stability;
- 3-KDG down-regulates mutant PHOX2B (+7 Ala) protein

Our data provide the evidence that the biologically active metabolite of desogestrel, 3-KDG, directly affects *PHOX2B* gene expression and its target genes, as well as mutant protein by reducing their expression. This specific effect on the PHOX2B pathway is mediated through PGR-B by a post-transcriptional mechanism. Our results open up the possibility that the down-regulation of PHOX2B mutant proteins may contribute to the recovery of CO_2 sensitivity observed in the two CCHS patients. However, further research is necessary to evaluate the benefit of pharmacological approaches that reduces the expression of both the mutant and the WT proteins in CCHS patients.

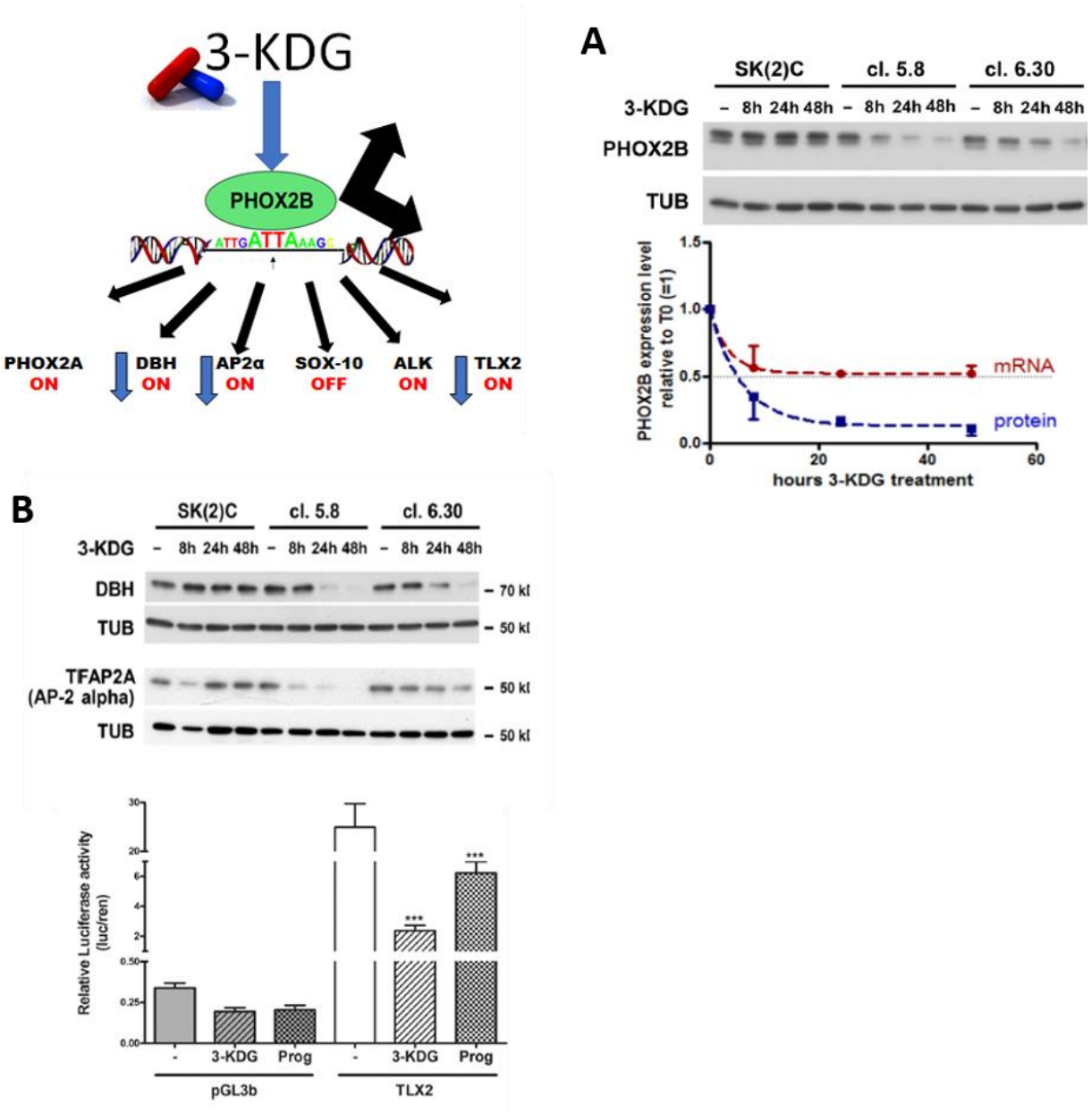


Fig.6 3-KDG has a specific effect on the PHOX2B pathway. Schematic representation of 3-KDG effects on endogenous protein and transcription levels. A) 3-KDG treatment down-regulates endogenous PHOX2B protein level up to 20% whereas reduces *PHOX2B* mRNA by no more than 50%. B) 3-KDG reduces the expression of some of PHOX2B target genes (*DBH* and *AP2α*) with two different kinetics in the two clones, that express different level of PGR-B, and the promoter activity of TLX2 in SK-N-BE(2)C cells transiently transfected with *PGR*.

Part 2

Preliminary data

In order to identify new PHOX2B target genes we took advantage of a genome-wide approach. Cross-linked chromatin from IMR32 was immunoprecipitated by means of PHOX2B antibody (Cargnin et al. 2005) followed by massive parallel sequencing of the co-immunoprecipitated genomic DNA fragments (ChIP-seq). We used, as starting material, human neuroblastoma cell line IMR32, which is a universally accepted model of neural crest derivative. Moreover, IMR32 cell line shows most of the molecular features of a sympathetic ganglionic cell, a kind of structure heavily affected in knockout mice, with the advantage of unlimited amounts of homogeneous material. Immune-precipitated chromatin and input DNAs were sequenced by means of the 5500 W Series Genetic Analysis Systems, SOLiD® (Applied Biosystems), in collaboration with Genomnia, s.r.l. The sequencing generated a data set of approximately 60 million mapped reads for both samples. MACS software has been applied to identify PHOX2B-binding enriched regions. To determine genomic location, a total of 8512 peaks corresponding to approximately 3000 genes, were detected and were mapped to RefSeq genes (UCSC Genome Browser (GRCh37/h19, February 2009). The distance of each peak to the nearest transcription start site (TSS) was analysed and plotted, revealing a significant enrichment of PHOX2B-binding sites around genes TSS (Fig.7A). The analysis of peaks distribution demonstrates that 48.9% of PHOX2B-binding sites were located 5' of its regulated genes, 34% were mapped to the intergenic regions downstream to a known gene and the remaining binding sites (16.86%) were found internal to known genes (introns or exons) (Fig.7B). The search for enriched motifs within the PHOX2B-binding sites revealed, in more than 50% of the peaks, a sequence element containing an inverted arrangement of two consensus motifs for homeodomain proteins (ATTA sequence). This is compatible with the binding properties shown by other paired-type homeodomain transcription factors, which have exhibited cooperative interactions between two closely spaced binding sites (Fig.7C).

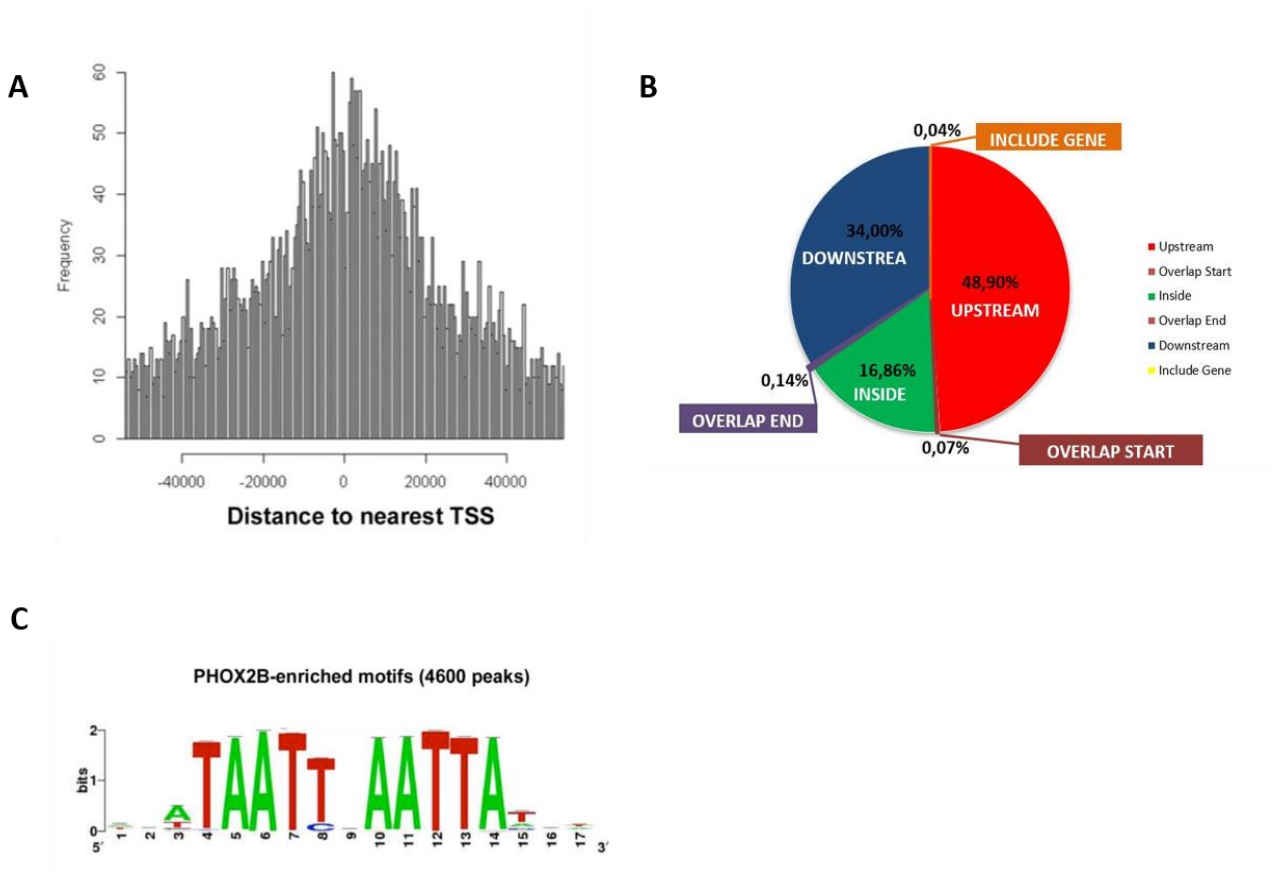


Fig.7 Genome-wide distribution of PHOX2B binding sites in human neuroblastoma IMR32 cell line. A: the graph shows the distribution of peaks obtained by ChIP-seq analysis relative to TSS. B: genomic distribution of PHOX2B-binding sites relative to human RefSeq genes (GRCh37/hg19, February 2009). Location analysis of peaks for PHOX2B relative to TSS shows that most PHOX2B-binding regions are enriched upstream its regulated target genes. C) PHOX2B recognition motif predicted using data from ChIP-seq experiments.

Gene Ontology (GO) analysis of the set of peak-associated genes have identified several enriched terms consistent with PHOX2B role during ANS development and maintenance, such as synaptic transmission, regulation of embryonic development, cell-cell signalling, axonogenesis, and neuron development. A further functional annotation clustering by Reactome (Fabregat et al., 2016; Milacic et al., 2012) revealed pathways that were associated with axon guidance, signal transduction, neuronal system and neuronal transmission (Tab.1), among which potassium ion channels.

Pathway name	#Entities found	#Entities total	Entities ratio	Entities pValue	Entities FDR
Axon guidance	93	598	0.046983	2.24E-07	3.99E-04
Signalling by NGF	81	520	0.040855	1.31E-06	1.16E-03
NGF signalling via TRKA from the plasma membrane	67	426	0.03347	8.04E-06	3.99E-03
Signaling by Interleukins	68	436	0.034255	8.98E-06	3.99E-03
DAP12 signaling	61	392	0.030798	2.75E-05	9.76E-03
Signaling by EGFR	61	402	0.031584	5.53E-05	1.47E-02
Signaling by PDGF	63	420	0.032998	5.80E-05	1.47E-02
DAP12 interactions	61	409	0.032134	8.82E-05	1.89E-02
Signaling by SCF-KIT	56	367	0.028834	9.58E-05	1.89E-02
Downstream signal transduction	58	389	0.030563	1.31E-04	2.31E-02
Signaling by ERBB4	56	373	0.029305	1.44E-04	2.31E-02
Neuronal System	60	410	0.032212	1.65E-04	2.43E-02
Signaling by VEGF	56	381	0.029934	2.42E-04	3.29E-02
Cell-Cell communication	27	144	0.011314	3.27E-04	4.12E-02
Adherens junctions interactions	11	35	0.00275	3.84E-04	4.53E-02
VEGFA-VEGFR2 Pathway	54	372	0.029227	4.13E-04	4.58E-02
Sodium/Calcium exchangers	7	15	0.001179	4.62E-04	4.80E-02
Potassium Channels	21	103	0.008092	5.11E-04	5.00E-02
MAPK family signaling cascades	48	324	0.025456	5.53E-04	5.14E-02
Cardiac conduction	27	151	0.011864	6.61E-04	5.82E-02

Tab.1 Functional annotation clustering performed by Reactome revealed clusters associated with the indicated terms. The first 20 significant pathways are listed. Included genes have been previously sorted by GO analysis (Biological Process).

Material and methods

Cell cultures

The human neuroblastoma cell line IMR32 was grown in RPMI 1640 medium, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (LONZA), supplemented with 10% foetal bovine serum (FBS, Euroclone). The IMR32 6.4 stable clone was maintained under selection by adding Puromycin dihydrocloride (SIGMA-ALDRICH) at 0.06 µg/mL final concentration, with selective medium changing every second day. All cell lines grow in adhesion incubated at 37°C in the presence of 5% CO_2.

CRISPR/Cas9 knock-out IMR32 cells generation.

Stable *PHOX2B* KO in IMR32 cells were generated by CRISPR/Cas9 technology. Three different gRNAs were designed to target the exon 1 of *PHOX2B* (PHOX2B-A: 3' GCAGGAACTGAAGTCAGCAT 5', PHOX2B-B 3' GCTGCAGGATCCCFGGCGTGA 5' and PHOX2B-C 3' CATACAGGACTCGTAGGCAG 5') and cloned in transEDIT gRNA plus Cas9 (pCLIP-All) vector (transOMIC) implemented with a puromycin resistance gene. The transEDIT pCLIP-All negative control was used as empty vector containing a gRNA with no target sequence (SCR: 3' GGAGCGCACCATCTTCTTCA 5').

Cleavage detection assay was performed by means of GeneArt™ Genomic Cleavage Detection Kit (Life technologies) after transfection of gRNAs. In particular, $1x10^6$ IMR32 cell line were plated in 60 cm plate and transfected with 1 µg of PHOX2B gRNA plasmid and 3 µL of Lipofectamine 3000/plate (Invitrogen). Cells were also transfected with the empty vector as a control. Seventy-two hours post transfection, transfected cells were selected by using 0.250 µg/ml puromycin for five days. Cells were harvested, and genomic DNA was extracted (Cell Lysis buffer/Protein Degrader, Invitrogen). A region of exon 1 of the *PHOX2B* gene was amplified with specific primers (forward primer: 5' GCTCGGTGCGTATGGTGTGGTA 3'; reverse primer: 5' TCAGAAAGTTGACCCAACTC 3'). Homoduplex PCR products were denatured and rehybridized using step down annealing conditions to generate homo- and hetero-duplex. The mixture of duplexes was treated with the Detection Enzyme for 1 hour at 37°C (Life technologies) and

the samples were then analysed on 2% agarose gel and stained in ethidium bromide solution (0.5 µg/mL). For each sample was included a no enzyme control. The relative proportion of cleaved DNA contained in each band was determined using UVITEC Cambridge transilluminator and UVI-1D analysis software. The cleavage efficiency was calculated as the percentage of cleaved bands with respect to total amplificated DNA (parental plus cleaved bans).

Before stable transfection, pCLIP-All vectors containing gRNA -A, -B and SCR were linearized by incubation with restriction enzyme SmaI (New England BioLabs). 30 units of enzyme were used to cleave 10 µg of DNA in 25°C for 2 hours. The cut DNA was analyzed by 0.8% agarose gel and stained in ethidium bromide solution (0.5 µg/mL).

One day before transfection $1x10^6$ IMR32 cells were plated in 100 mm dishes in RPMI 1640 medium devoid of antibiotics and transfected with 5 µg of transOMIC vector in a ratio of 1 DNA: 3 Lipofectamine™ 3000 (invitrogen). 48 hours post-transfection cells were diluted to a final concentration of $1X10^6$ IMR32 cells/dish and selected by the addition of puromycin 0.250 µg/mL to the culture medium. Selective medium was changed every second day and after around twenty days individual colonies were picked up and transferred to multi-well plates for further propagation. Selected clones were screened by immunoblotting with PHOX2B antibodies and hypothetical knockout clones were analysed by sanger sequence.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation assay was performed as those presented in Preliminary data.

The day before fixation and immunoprecipitation, IMR32 cells were plated in 10 cm dishes in order to reach a density of 1×10^7 cells/petri dishes, and the chromatin immunoprecipitation performed as described below.

The day after plating, IMR32 cells were cross-linked with 1% final formaldeyde solution (SIGMA-ALDRICH) for 10 minutes at room temperature and neutralized with 0.125 M glycine for 5 minutes. The cells were then washed twice with cold PBS 1X and collected by centrifugation at 3000 rpm at 4°C for 2 minutes. 2×10^7 IMR32 cells were then washed twice by repeated resuspension in 1 mL of Cell Lysis Buffer (Hepes 5mM, KCl 85 mM and Triton X-100 5%) added

with 1 mM PMSF and protease inhibitor 100X and, kept 5 minutes on ice, followed by centrifugation at 3000 rpm at 4°C for 2 minutes. The pellet was then resuspended in 500 µL of Nuclei Lysis Buffer (Tris HCl pH 8 50 mM, EDTA 10 mM, SDS 1%) added with 1 mM PMSF and protease inhibitor 100X and the lysate was sonicated in order to obtain chromatin fragments of about 200 nucleotides.

100 µL of sonicated lysate were then diluted 1:5 in 400 µL Dilution Buffer (SDS 0.01%, Triton X-100 1.1%, EDTA 1.2 mM, Tris HCl pH 8 16.7 mM, NaCl 167 mM) and 5 µL of the chromatin was removed and saved as "Input 1%." Before to add Novex rProtein G Agarose beads (Life Technologies) to sonicated chromatin, 200 µL of Novex rProtein G Agarose beads were pre-cleared three times in PBS 1X and centrifuged at 3000 rpm at 4°C for 30 seconds and then resuspended in 400 µL ChIP Dilution Buffer. The beads were then added to the sonicated chromatin, diluted with 1.2 mL of ChIP Dilution Buffer, and pre-cleared for 1 hours at 4°C on rotation wheel. 500 µL of the pre-cleared chromatin was added in 1.5 mL tubes together with 5 µg of appropriate antibodies: anti-hPHOX2A and anti-hPHOX2B antibodies (Davids Biotechnologie, Ragensburg, Germany) (Cargnin et al., 2005) and anti-PHOX2B (B-11) (Santa Cruz, Oregon, USA) or with non-specific Rabbit IgG (Santa Cruz, Oregon, USA) or Chicken IgY (Davids Biotechnologie, Ragensburg, Germany) as control. The chromatin and the antibodies were then incubated over night at 4°C on rotation wheel.

100 µL of pre-coated beads were added to the chromatin samples and incubated for 4 hours at 4°C on rotation wheel. The following steps consisted in four 10 minutes washes at 4°C with ChIP Dilution Buffer, Dialysis Buffer (EDTA 2 mM, Tris HCl pH8 50 mM, N-lauryl sarkosyl 0.2%), TSE-500 (SDS 0,1%, EDTA 2 mM, Triton X-100 1%, Tris HCl pH8 20 mM, NaCl 500 mM) and LiCL Buffer (LiCl 500 mM, Deoxycholic Na 1%, Triton X-100 1%, Tris HCl pH8 100 mM) and two 5 minutes washes with TE (Tris HCl pH8 10 mM, EDTA 1mM). The beads were then eluted twice in 50 µL of Elution Buffer ($NaHCO_3$ 50mM, SDS 1%) and incubated for 15 minutes on rotation wheel; 6.6 µL of NaCl 5M was added and DNA de-crosslinked by over-night incubation at 65°C.

The DNA was then purified using the Chromatin IP DNA Purification kit (Active Motif) and the quantification of the precipitated DNA was done using SYBR-Green based qRT-PCR assay using the ABI Prism Thermocycler (QuantStudio 5, Applied Biosystems, CA). The DNA sample were heated to 50°C for 2 minutes and then to 95°C for 10 minutes, followed by 40 cycles of

heating to 95°C for 15 seconds, and combined annealing/extension at 60°C for 1 minute.

ChIP specific primers used in the studies are provided in table 2. Primers for the *PHOX2B* promoter region (Cargnin et al., 2005) were used as positive control for the ChIP.

Name	Sequence
PHOX2B promoter ChiP NEW FW	5’-GCT CGG TGC GTA ATG GTG TGG TA-3’
PHOX2B promoter ChiP NEW REV2	5’-GGT TGG TCT TAT TGC TGG CGC TT-3’
KCNJ16 peak 6100 FW	5’-TTT GGG GAG CAG CAA CAC T -3’
KCNJ16 peak 6100 REV	5’-ATG ACA CAC TGT TTG TTC CTA CT-3’
KCNJ16 peak 6101 FW	5’-CAC ATA GCT AGC TTA CCT CCA GAA-3’
KCNJ16 peak 6101 REV	5’-ACT AGA AAT GTG ACT CCC CCA AT-3’
KCNJ3 peak 727 FW	5’-AGG AAT ATT CAG GCA ACA TCA CT -3’
KCNJ3 peak 727 FW	5’-GAC ACC ACC GGC TGT AGA AA -3’
KCNMA1 peak 2512 FW	5’-TGC TGG AGC ATT TAA ATC CGT TG-3’
KCNMA1 peak 2512 REV	5’-TCC TTT ACC AGA AAC CTC AAA AAC A-3’
KCNK13 peak 4859 FW	5’-CCT TGG ACA CTT GCT TAC CCT AT-3’
KCNK13 peak 4859 REV	5’-TTC CCA CCT AAA TTT GCC AAT GC-3’
KCNAB1 peak 9029 FW	5’-GGT GCT CTG AAG TCC GTA ACT AA-3’
KCNAB1 peak 9029 REV	5’-TCT TCA GTA AAC CAC ACC TAA ACT-3’
KCNN3 peak 1205 FW	5’-GGC AGG TTA TAC CAT TGT AGG CT-3’
KCNN3 peak 1205 REV	5’- TCC TGT CTT CTG TTC GAT CAC TG-3’
KCNJ6 peak 8184 FW	5’-GCT GAA GTC CTA ATC TCC GGT ATT T-3’
KCNJ6 peak 8184 REV	5’-GGA CTA GGG CCC ACC AGT AT-3’
KCND2 peak 12364 FW	5’-TCC ACT GCA GAT GTA ATA AGA ACA A-3’
KCND2 peak 12364 REV	5’-TCA ACT ACA TTG CAT TCA CAA AGG A-3’
KCNQ5 peak 11287 FW	5’-TGA TGA AAT GAG ATG ATG CCA GAT-3’
KCNQ5 peak 11287 REV	5’-TTA GTT ACT AGG GAC ACA GTG CT-3’
KCNK2 peak 2003 FW	5’-AGA AGT TGT GGT TGC TTG GC -3’
KCNK2 peak 2003 REV	5’-GGA TTT CAC TTT TTA CTG CAT GGT -3’
KCNK2 peak 2005 FW	5’-AGG CAA TTG TCC TGA GCA AAT TAA A-3’
KCNK2 peak 2005 REV	5’-GTT CCC ATG GAA TCT GTT TCT TCA -3’
KCNB2 peak 12800 FW	5’-GCT GTG ATG GAA TAT GGA GCC TA -3’
KCNB2 peak 12800 REV	5’-TCA ATC TTT CCC CAT TGT GAT CCT -3’

Tab.2 Sequences of the primers used in the conventional ChIP followed by SYBR-Green based qRT-PCR

Transient Transfections

IMR32 cells and IMR32 6.4 stable clone were transiently transfected by lipofection method using FuGENE HD Transfection Reagent (Promega) or Lipofectamine® 3000 Transfection Reagents (Life Technologies). Briefly, the day before transfection $6x10^5$ cells were plated on 6-well plate in 2 mL RPMI 1640 medium without antibiotics. The day of transfection 2.5 µg of plasmid DNA were incubated with 7.5 µL FuGene HD in a 1:3 ratio (DNA/ FuGene HD) in plain DMEM medium to a final volume of 200 µL. After incubation for 15 minutes at room temperature the mixtures are added drop-wise to the cells (200 µL/well). For the transfection with Lipo3000 the day after plating, 7.5 µL Lipofectamine® 3000 Reagents were diluted in Opti-MEM Medium (Life Technologies) to a final volume of 125 µL. In a second master mix, 2.5 µg of plasmid DNA were incubated with 5 µL P3000 Reagents (2µl/µg DNA) and Opti-MEM Medium to a final volume of 125 µL. The diluted DNA mix was added to diluted Lipofectamine® 3000 Reagents (1:1 ratio) and incubated for 5 minutes at room temperature and added to cells (250 µL/ well). The final ratio between DNA and transfection reagent was 1:3.

The plasmid constructs used were:

- PHOX2B WT and +7Ala Myc-His: the Myc-tagged PHOX2B wild-type and +7 alanine mutant plasmid have been described previously (Di Lascio et al., 2018; Di Lascio et al., 2013);
- PHOX2B WT and +7Ala V5-His: the V5 tagged PHOX2B wild-type and +7 alanine mutant plasmid have been described previously (Bachetti et al., 2005);
- PHOX2A pCMV Myc-His: the Myc-tagged PHOX2A expression vector was obtained by cloning human PHOX2A cDNA into the EcoRI site of pCMV-Myc (Clontech) (Benfante et al., 2007);
- pCMV-Myc (Clontech) and pcDNA3.1TOPO/V5-His (Invitrogen): empty vectors used as control

All the constructs were checked by means of restriction analysis and partial sequencing

Total RNA Extraction and Reverse Transcription

Total RNA, from cultured cells, was extracted using *the RNeasy Mini kit* and accompanying QIAshredder (Qiagen), according to information provided by the company.

Briefly, about $1x10^7$ cells, grown in 100 mm dishes, were washed twice with PBS 1X (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 5 minutes centrifugation at 5000 rpm at 4 °C in an Eppendorf microcentrifuge. The cells were lysed with 350 µL of buffer RLT added with β-mercaptoethanol (10 µL/mL RLT buffer). The lysate was homogenized by means of QIAshredder column centrifuged for 2 minutes at maximum speed.

To avoid DNA contamination, samples were incubated on-column with DNase I (2.73 U/uL) for 15 minutes at room temperature and RNA eluted with 30 µL of RNase-free water. The amount of eluted total RNA was determined by spectrophotometer at 260 nm (1 OD_{260} = 40 µg/ mL) and its purity was evaluated using the 260/280 nm ratio (≥ 2).

500 ng of total RNA per sample were reverse transcribed using the Go Script™ Reverse Transcriptase kit (Promega), in accordance with the manufacturer's instructions.

Briefly, total RNA was firstly incubated at 70°C for 5 minutes with Random primers (Hexamers) 0.5µg/ reaction and Nuclease-free water to a volume of 5 µL, and then chilled on ice for 5 minutes. After that, each sample was added with 15 µL of a second reaction mix composed by Nuclease-Free Water, GoScript™ 5X Reaction Buffer, $MgCl_2$ (final concentration 4mM), PCR Nucleotide Mix (final concentration 0.5mM each dNTP), Recombinant RNasin®Ribonuclease Inhibitor 20 units and 200 units GoScript™ Reverse Transcriptase. The reaction is continued at 25 °C for 5 minutes to allow the annealing of the primers, 42 °C for 1 hour for the extension and 70 °C for 15 minutes for the inactivation of Reverse Transcriptase. The cDNAs of interest were then amplified and analysed 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 Technologies, Inc.) used in the studies are provided in table 3. Each sample was run in triplicate, and the $2^{-\Delta CT}$ and the $2^{-\Delta\Delta CT}$ method were used to calculate the results, thus allowing the normalization of each sample to the endogenous control GAPDH, and comparison with the calibrator for each experiment (set to a value of 1) as indicated in the figure legends. Results are shown as the mean values ± standard deviation and the statistical analyses were made by means of one-way ANOVA, Tukey's test or an unpaired two-tail student's T-test using GraphPad Prism 5 Software (GraphPad Software, Inc.). P values <0.05 were considered significant.

TaqMan® assay	
human *PHOX2B* (Paired like homeobox 2B)	ID #Hs00243679_m1
human *PHOX2A* (Paired like homeobox 2A)	ID #Hs00605931_m1
human *DBH* (Dopamine-β-hydroxylase)	ID #Hs00168025_m1
human *TLX2* (T Cell Leukemia Homeobox 2)	ID #Hs00740145_m1
human *ChAT* (choline acetyl transferase)	ID #Hs00252848_m1
human *SOX10* (SRY-related HMG-box)	ID #Hs00366918_m1
human *KCNMA1* (Potassium Calcium-Activated Channel Subfamily M Alpha 1)	ID #Hs01119498_m1
human *KCNN3* (Potassium Calcium-Activated Channel Subfamily N Member 3)	ID #Hs01546821_m1
human *KCNQ5* (Potassium Voltage-Gate Channel Subfamily Q Member 5)	ID #Hs01068536_m1
human *SCN2A* (Sodium Voltage-Gate Channel Alpha Subunit 2)	ID #Hs00221379_m1
human *SCN3A* (Sodium Voltage-Gate Channel Alpha Subunit 3)	ID #Hs00366902_m1
human *SCN8A* (Sodium Voltage-Gate Channel Alpha Subunit 8)	ID #Hs00274075_m1
human *SCN9A* (Sodium Voltage-Gate Channel Alpha Subunit 9)	ID #Hs01076699_m1
human *GADPH* (glyceraldehyde-3 phosphate dehydrogenase)	ID #Hs99999905_m1

Tab.3 TaqMan assays used in qRT-PCR

Total protein extraction

Total protein extraction was prepared from sub-confluent cells grown in 100 mm Petri dishes using the freeze and thaw method.

Briefly, IMR32 cells were washed twice with PBS 1X, mechanically harvested and collected by 5 minutes centrifugation at 5000 rpm at 4°C in an Eppendorf centrifuge. The pellet was resuspended in an appropriate volume of isotonic solution (PBS 1X, PMSF 0.2 mM, DTT 0.5 mM, phosphatase inhibitors (Cocktail I, Sigma) and protease inhibitors (Cocktail II, Sigma)). The cells were kept in solution for 10 minutes on ice and lysed by three cycles of 5 minutes in liquid nitrogen and 5 minutes in a thermal bath at 37 ° C, to obtain complete cell lysis.

NaCl 420 mM was added drop-wise to the lysate in order to extract the nuclear proteins. After 30 minutes of incubation at 4°C on a slowly turning rotating wheel, the extract was clarified by centrifugation at 13000 rpm for 30 minutes at 4°C.

Finally, the supernatant was aliquoted, quickly frozen in liquid nitrogen and stored at -80°C; the concentration was evaluated using the Bradford method (Bradford, 1976).

SDS-PAGE and Western blot

20 µg proteins extract from native IMR32 cells and *PHOX2B* KO clone were analysed by means of Western blot as previously described (Benfante et al. 2008). Protein extract were separated on an 8%, 10% and 12% denaturing polyacrylamide gel, depending on the dimension of protein to be analysed, in the presence of 0.1% SDS extract (sodium dodecyl sulphate).

Before loading, each sample was added with Laemmli loading solution (Tris-HCl pH 8.5 20Mm, SDS 2%, β-mercaptoethanol 3%, glycerol 8%, bromophenol blue 0.03%) and denatured at 100°C for 5 minutes. A standard molecular weight (PageRuler^{TM} Broad Range Unstained Protein Ladder, Thermo-Fisher Scientific) was loaded in parallel.

After gel separation at 25 mA in Tris-glycine (Trizma base 25 mM, Glycine 192 mM, SDS 0.1%), the proteins were transferred to nitrocellulose membranes (Amersham) by electroblotting at 30V over-night in transfer buffer (Trizma base 25 mM, Glycine 192 mM, MetOH 20%). To control

the transfer, the membrane was then stained with Ponceau S, washed with distilled H_2O and saturated with blocking buffer (non-fat milk 5%, Tris-HCl pH 7.5 20 mM, NaCl 150 mM, Tween-20 0.1%) for at least 1 hour.

After blocking, appropriate primary antibody diluted in blocking buffer was added to the membrane and incubated for 2 hours, or over-night, depending on the manufacturer's instructions. Excess of primary antibodies was removed with three washes with blocking buffer (5 minutes each), and the membrane was incubated in the presence of the secondary antibody conjugated with horseradish peroxidase, diluted in blocking solution for 1 hour. The antibodies used in Western blot experiments are reported in table 4.

The excess of antibody was then removed by different washes for 5 minutes each: two washes with blocking buffer; three washes in TS 1X (Tris-HCl pH 7.520 mM, NaCl 150 mM) + Tween-20 0,1%; three washes in TS 1X + Tween-20 0.3 % and three final washes in TS 1X.

After washes, the bands were revealed using Super Signal west Dura (Thermo Fisher Scientific) and the signal detected by Hyperfilm ECL film (GE Healthcare) or by ChemiDoc™ Imaging System (Bio-Rad) at different time exposures.

Densitometric analysis of the obtained signals was carried out using NIH ImageJ 1.61/ fat, or ImageLab software 6.0 and the results are shown as the mean values ± standard deviation.

The statistical analyses were made by means of one-way ANOVA, Tukey's test or an unpaired two-tail student's t-test using GraphPad Prism 5 Software (GraphPad Software, Inc.) as indicated in the figure legends. P values <0.05 were considered significant.

Primary antibody	dilution
PHOX2B B-11: sc-376997 mouse mAb (Santa Cruz Biotechnology) N-terminal	1:10000
anti-hPHOX2B antibodies chicken (Davids Biotechnologie) * C-terminal	1:400
PHOX2A Ab chicken (Davids Biotechnologie) *	1:1000
β-Tubulin (D3U1W) mouse mAb (Cell Signalling Technology)	1:1000
Monoclonal anti-actin Clone AC-40 (Sigma-Aldrich)	1:1000
Sox10 (D5V9L) Rabbit mAb (Cell Signaling)	1:1000
β3 tubulin (D71G9) XP Rabbit mAb (Cell Signaling) *	1:1000
Dopamine-β-Hydroxilase (DBH) (N-Terminal) sheep mAb (Sigma-Aldrich)	1:500
Goat anti-choline acetyltransferase (ChAT) Affinity purified polyclonal antibody (Chemicon International) *	1:500
Synaptotagmin 1 105 011 Monoclonal mouse purified IgG (Synaptic Systems)	1:1000
Monoclonal anti-syntaxin clone HPC-1 Mouse Ascites Fluid (Sigma-Aldrich)	1:1000

Secondary antibody	dilution
Stabilized Goat anti-mouse HRP-conjugated (Pierce)	1:10000
Stabilized Goat anti-rabbit HRP-conjugated (Pierce)	1:10000
Rabbit Ab against chicken IgG-HRP (Davids Biotechnologie)	1:100000
Donkey Ab against sheep IgG-HRP (Abcam)	1:5000
Anti-goat IgG (WHOLE MOLECULE) peroxidase conjugate (Sigma-Aldrich)	1:10000

Tab.4 Primary and secondary antibodies used in Western blot with the appropriate dilutions. *indicates antibodies incubated over-night according to manufacturer instruction.

Validation of the binding of PHOX2B to the region corresponding to the peaks associated with genes that encode for potassium channels.

The ChIP-seq experiment carried out previously in the laboratory, has allowed us to identify many PHOX2B target gene candidates that need to be validated by means of biochemical and functional approaches. Interestingly, among the predicted genes (approximately 3000 genes), there were several encodings for ionic channels (Tab.5), including seventeen selective for K^+, belonging to four different families, and four selective for Na^+ ions (two families).

A

Voltage-gated K^+ (K_v) channels

Gene	Channel
KCNB2	K_v2.2
KCNC2	K_v3.2
KCND2	K_v4.2
KCNQ5	K_v7.5
KCNH1	K_v10.1
KCNH5	K_v10.2
KCNH7	K_v11.3
KCNH8	K_v12.1
KCNAB1	Auxiliary β subunit

Ca^{2+}-activated K^+ (KCa) channels

Gene	Channel
KCNMA1	K_{Ca}1.1 (Slo1, MaxiK, BK)
KCNN3	K_{Ca}2.1 (SK_{Ca}3, SK3)
KCNMB2	Auxiliary β subunit

Two-pore K^+ (K2P) channels

Gene	Channel
KCNK2	K_{2p}2.1 (TREK-1)
KCNK13	K_{2p}13.1 (THIK-1)

Inwardly-rectifying K^+ (Kir) channels

Gene	Channel
KCNJ3	Kir3.1 (GIRK1,KGA)
KCNJ6	Kir3.2 (GIRK2)
KCNJ16	Kir5.1 (BIR9)

B

Voltage-gated Na^+ channels

Gene	Channel
SCN2A	Sodium channel protein type 2 subunit alpha isoform 1
SCN3A	Sodium channel protein type 3 subunit alpha isoform 2
SCN8A	Sodium channel protein type 8 subunit alpha isoform 2
SCN9A	Sodium channel protein type 9 subunit alpha isoform X1

Tab.5 List of genes obtained from ChIP-seq analysis. A: genes encoding potassium channels are divided in four different families. B: genes encoding sodium channels. In red, genes expressed in IMR32 cell lines are highlighted.

Since several findings suggest that many ion channels are involved in the regulation of the excitability of autonomic neurons and in the modulation of respiratory activity *in vivo* (Bond at al., 2000; Torrecilla et al., 2013; Mulkey et al., 2015) I focused my study on this category and started the validation by verifying the binding of PHOX2B to those regions corresponding to the peaks associated with these genes.

Specific oligonucleotides have been designed and conventional ChIP assay followed by SYBR-Green based qRT-PCR has been carried out. IMR32 cells were fixed with 1% formaldehyde solution in order to generate a reversible protein-DNA cross-link that "preserves" the protein-DNA interactions occurring in the cell. Cells were then lysed, and chromatin was fragmented by means of sonication to obtain fragments of about 200 nucleotides. The chromatin fragments were then immunoprecipitated using the appropriate PHOX2B antibody already used in ChIP-seq analysis (Cargnin et al., 2005) or with no specific antibody. After immunoprecipitation, the protein-DNA cross-links were reversed by over-night incubation at 65°C and the DNA was purified by means of Chromatin IP DNA Purification kit (Active Motif). SYBR-Green based qRT-PCR with specific oligonucleotides for region corresponding to potassium channels (Tab.2), was used to measure the abundance of these specific DNA sequences enriched by a protein-specific immunoprecipitation versus immunoprecipitation with a non-specific antibody control (Fig. 8 black bars vs white bars). A ChIP positive control corresponding to *PHOX2B* promoter was included (Fig. 8, CTRL region). Figure 8 shows the quantification of the level of enrichment of the specific PHOX2B-binding regions (black bars) and the non-specific control (white bars) relative to the total amount of input DNA (percentage of input). The antibody directed versus PHOX2B is capable of immunoprecipitating the regions corresponding to potassium channels while no amplification products were observed using no specific antibodies. 100% of the analysed regions (14 in total) were found to display abundant occupancy of *PHOX2B* thus confirming ChIP-seq results.

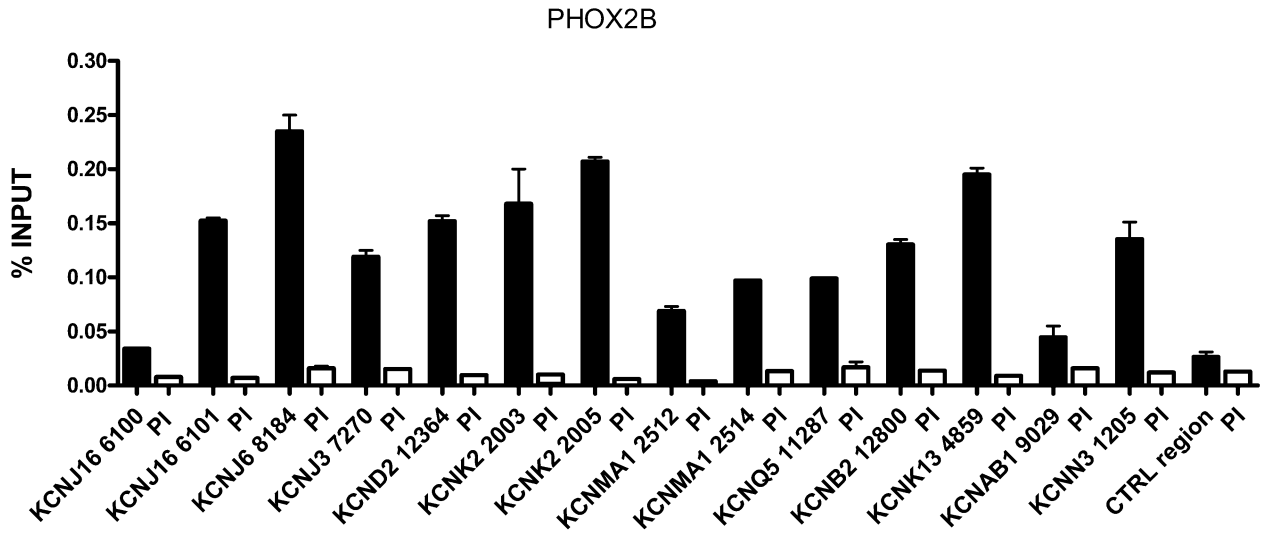


Fig. 8 Validation of ChIP-seq binding sites by single gene ChIP-PCR. Conventional ChIP experiments were performed using PHOX2B (black bars) and IgY (PI, white bars) antibodies followed by qRT-PCR using oligonucleotides specific for the peaks associated with the indicated gene. 14 out of 17 sites have been validated. A positive control region corresponding to *PHOX2B* promoter region was included. The chromatin was amplified by means of SYBR-Green chemistry and expressed as a percentage of the input DNA ± SEM.

As PHOX2A, the *PHOX2B* paralogue gene product, has in common with PHOX2B two known target genes (*DBH* and *TLX2*), I investigated whether it was able also to bind to the identified peaks and therefore regulate potassium channels. The experiment was performed as previously described with a specific antibody directed versus PHOX2A. I investigated six out of fourteen peak-associated potassium channel genes. A positive control region was included (Fig. 9, CTRL region). Remarkably, the antibodies directed against PHOX2A were also able to immunoprecipitate the same regions (Fig. 9 black bars), thus suggesting that this transcription factor might also participate in the transcriptional regulation of potassium channels.

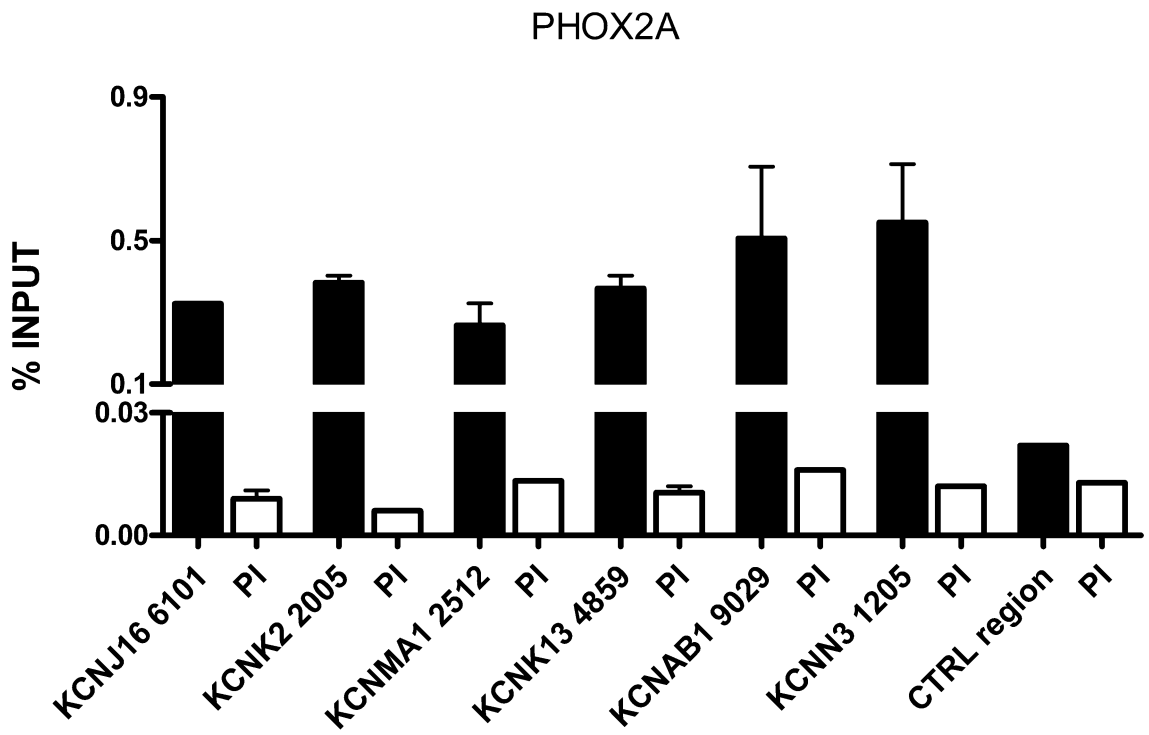


Fig.9 PHOX2A binds region corresponding to genes encoding for potassium channels. Conventional ChIP experiments were performed using PHOX2A (black bars) and IgY (PI, white bars) antibodies followed by qRT-PCR, using oligonucleotides specific for the peaks associated with the indicated gene. Six peaks have been validated. A positive control region (*PHOX2B* promoter) was included. The chromatin was amplified by means of SYBR-Green chemistry and expressed as a percentage of the input DNA ± SEM.

To further validate ChIP-seq results, another single gene ChIP-PCR was performed. In two parallel experiments, chromatin fragments were immunoprecipitated with a new PHOX2B antibody (Phox2b (B-11) mouse mAb) and with anti-PHOX2A antibody as control (Fig. 10 black and white bars, respectively). As ChIP positive control, the amplification of the region corresponding to *PHOX2B* promoter was included (Fig. 10, CTRL region). Quantification of the precipitated DNA, expressed as percentage relative to the INPUT showed that specific amplification was observed using PHOX2B and PHOX2A antibodies in 100% of the analysed peaks, while no amplification was detected using IgY (white bars), thus confirming previous results.

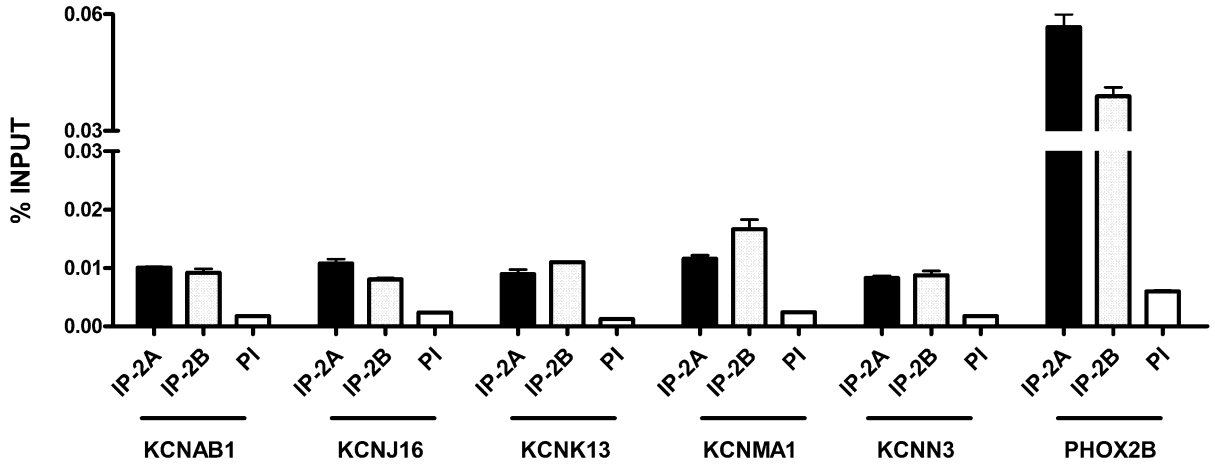


Fig.10 PHOX2B and PHOX2A bind region corresponding to encoding gene for potassium channels. Conventional ChIP experiments were performed using PHOX2A (black bars), PHOX2B (grey bars) and IgY (PI, white bars) antibodies followed by qRT-PCR using oligonucleotides specific for the peaks associated with the indicated gene. Six sites have been validated. A positive control region (*PHOX2B* promoter) was included. The chromatin was amplified by means of SYBR-Green chemistry and expressed as a percentage of the input DNA.

These data, obtained by using two different antibodies directed versus PHOX2B, also confirmed the specificity of the interaction of PHOX2B with the regions corresponding to the peaks identified by ChIP-seq analysis in potassium channels genes, and again we demonstrated that some of potassium channels encoding genes might be co-regulated by PHOX2B and PHOX2A.

Generation of *PHOX2B* knockout cell lines

To study the role of PHOX2B on ion channels encoding-genes expression, I generated a stable *PHOX2B* KO cell model, by means of CRISPR-Cas9 editing technology. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system is an RNA-programmable host defence mechanism in bacteria and archaea that degrade foreign nucleic acid to protect them against invading virus and bacteriophages (Hsu et al., 2014). The type II CRISPR system, found in *S. pyogenes*, has been engineered to edit genes in mammalian cells (Jinek et al., 2013) by fusing the essential non-coding RNA components of the CRISPR locus into a single guide RNA (gRNA). The 5'-end of gRNA could be programmed with a 20-bp guide sequence that will bind to its complementary genomic target through Watson-Crick base pairing while the 3'-end is

associated with the Cas9 enzyme. The target sequence is always followed by a proto-spacer adjacent motif (PAM) that corresponds to a trinucleotide sequence “NGG”, where N can be any nucleotide. At the level of its target site the Cas9 nuclease generates a double stranded break (DSB) near the PAM that induces DNA repair mechanisms such as non-homologous end joining (NHEJ), leading to formation of nucleotide insertion or deletion “indels” (Fig.11) causing frameshift mutation of the ORF, with the possibility to create a stop codon with the premature termination of protein synthesis (Ran et al. 2013).

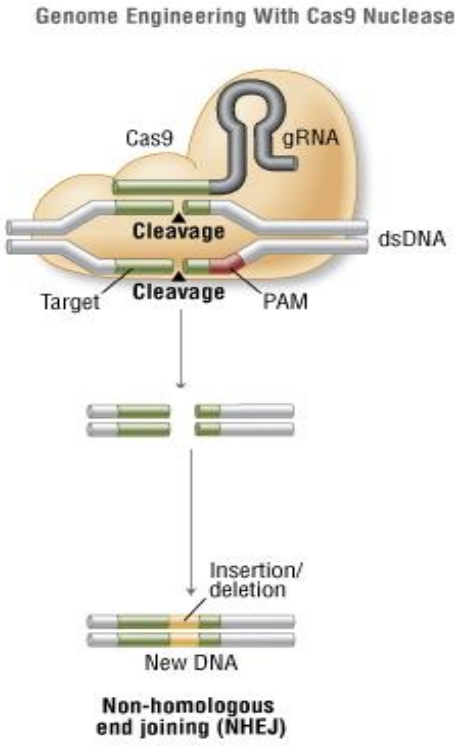


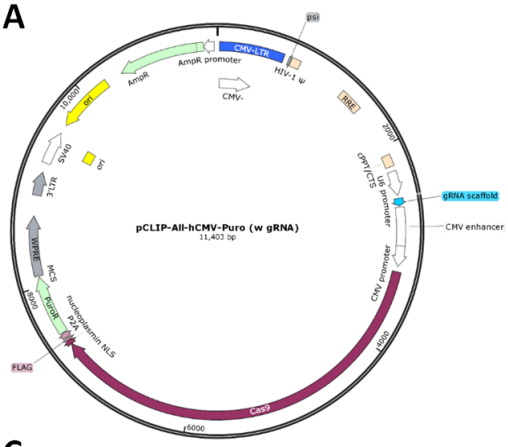
Fig.11 Schematic representation of CRISPR-Cas9 system. The Cas9 nuclease from S. pyogenes (brown) is targeted to genomic DNA by a gRNA consisting of a 20-nt guide sequence (green) and a scaffold (grey). The guide sequence pairs with the DNA target (green sequence on dsDNA), directly upstream of a requisite 5’-NGG adjacent motif (PAM; red). Cas9 mediates a DSB ~3 bp upstream of the PAM (black triangle) that can be repaired in the error-prone NHEJ pathway with the generation of random insertion/deletion (indel; yellow) mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frameshifts and the creation of a premature stop codon, resulting in gene knockout. Modify from NEW ENGLAND BioLabs.inc

To selectively knock out *PHOX2B* expression from cells that natively expresses it (IMR32 neuroblastoma cell lines) I first had to identify an appropriate target site in *PHOX2B* encoding sequence for gene editing. In order to avoid off target effects, the site was selected outside

the HD encoding region, which show a very high homology with other homeobox protein (Yokoyama et al., 1999). Despite *PHOX2B* and *PHOX2A* show 56.4% homology in exon1 encoding sequence, I focused on this exon as a putative target site, because NHEJ-mediated frameshift within this region could potentially terminate translation upstream the HD, that contain the DNA-binding site for target gene promoter, thus eliminating PHOX2B transcriptional activity (Fig.12C).

Three different candidates 20-bp gRNAs, named PHOX2B-A, PHOX2B-B and PHOX2B-C positioned immediately 5' to the trinucleotide PAM sequence on the minus strand of the *PHOX2B* gene (Fig.12B) have been chosen. They were cloned into transEDIT gRNA plus Cas9 (pCLIP-All) vector (transOMIC) that contains in the same vector the Cas9 gene and the human cytomegalovirus promoter hCMV implemented with a puromycin resistance gene (Fig.12A) that allows a single transfection to generate stable CRISPR clones. Moreover, as a control, we used a transEDIT pCLIP-All negative control empty vector containing a gRNA lacking the *PHOX2B* guide (Fig.12B; SCR).

A



B

Gene	Clone id	Guide sequence 3'-5'
PHOX2B	PHOX2B-A	TACGACTGAAGTCAAGGACG
PHOX2B	PHOX2B-B	AGTGCGGCCCTAGGACGTCG
PHOX2B	PHOX2B-C	GACGGATGCTCAGGACATAC
TELA1015	Non targeting control (SCR)	GGAGCGCACCATCTTCTTCA

C

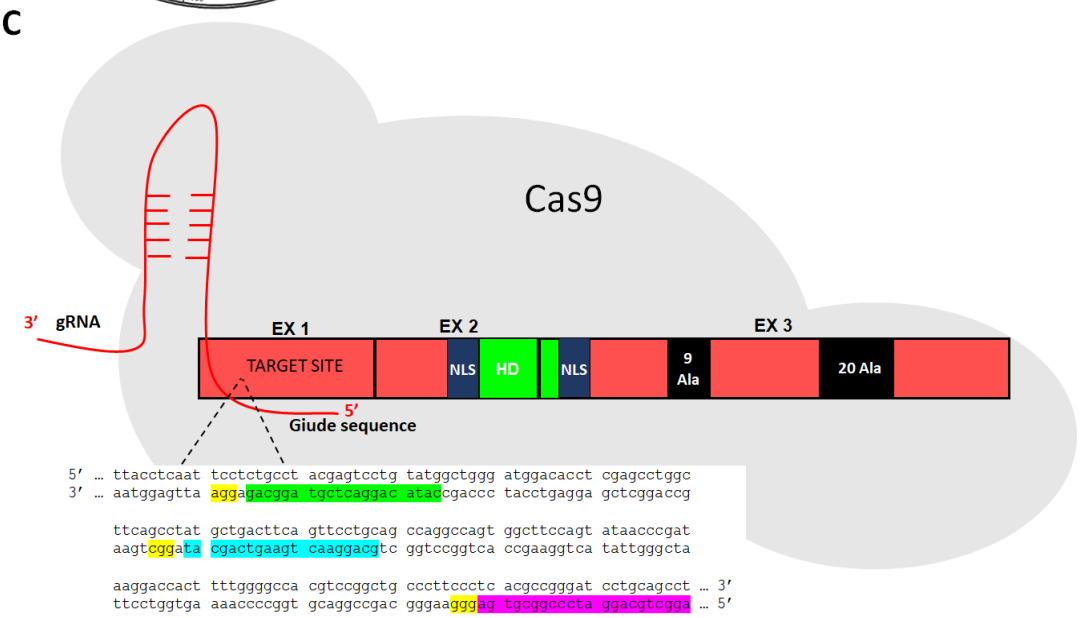


Fig.12 The three *PHOX2B* guides RNAs and target site. A: Detailed map of transEDIT gRNA plus Cas9 (pCLIP-All) vector (transOMIC). The all-in-one vector contains a programmable single gRNA (light blue) and a Cas9 construct (in purple) in the same vector implemented with a puromycin resistance gene (in green). B: Three different sequences of the gRNAs directed versus exon1 of *PHOX2B* and a non-targeting control lacking *PHOX2B* guide (SCR). C top: Schematic representation of the *PHOX2B* gene and the CRISPR/Cas9 machinery. Domain structure of full-length PHOX2B. The location of HD (green), the NSL (blue) and the two stretches of polyalanine sequence (9 Ala and 20 Ala in black) are shown. The gRNA (in red) target site is located at the beginning of exon1 (target side in pink). Bottom: close-up representation of the three-gRNAs target sites. Immediately 3' to the target sequence (PHOX2B-A in light blue, PHOX2B-B in green and PHOX2B-C in purple respectively) is the trinucleotide spacer adjacent motif (PAM in yellow). The Cas9 enzyme introduces a DSB near the PAM, triggering imperfect NHEJ.

To test the effectiveness and the specificity of the three PHOX2B gRNAs at triggering Cas9-mediated gene editing at the target site, IMR32 cells were transiently transfected with pCLIP-All-hCMV-Puro vector targeting different regions of the human PHOX2B locus or with the transEDIT pCLIP-All Negative control, resulting in the generation of a mixed population of edited and non-edited cells. Since IMR32 cell line shows a very low efficiency of transfection and we were not able to detect any cleavage efficiency on the heterogeneous pool of cells, seventy-two hours post transfection transfected cells were selected by the addition of 0.250 µg/ml puromycin to the culture medium. After five days of selection, pool of cells was lysed, and the genomic DNA was extracted and the region containing the target site was amplified by PCR (primer forward: 5' GCTCGGTGCGTAATGGTGTGGTATTA 3', primer reverse: 5' CTTCGGAACTAACGTAAACTTTT 3'). The PCR amplicons were then denatured and reannealed in a thermal-cycler in order to generate mismatched heteroduplex, with strands with an indel reannealed to strands with no indel or a different indel. These mismatches were then selectively detected and digested by the Detection Enzyme T7E1 (Cho et al., 2013) and the resultant bands were then analysed by gel electrophoresis and band densitometry. CRISPR/Cas9 complex trigger site generates specific DNA fragments in each sample transfected with the gRNAs (Fig.13 shown by a bracket) while no bands were detected in cells transfected with the negative control (SCR) as expected. The cleavage efficiency, reported in figure 13 as formation of indel, was calculated as difference between undigested band and the sum of the cleaved bands, expressed in percentage. Densitometry analyses showed a cleavage efficiency of 12.96%, 34.04% and 5.01% for the three gRNAs A, B and C, respectively. Since the GeneArt Genomic Cleveage Detection Kit (Life Technologies) suggested that pCLIP-All vector containing the PHOX2B-A and PHOX2B-B gRNAs were functional and give the highest cleavage efficiency, we next chose them to generate clonal *PHOX2B* KO stable cell lines.

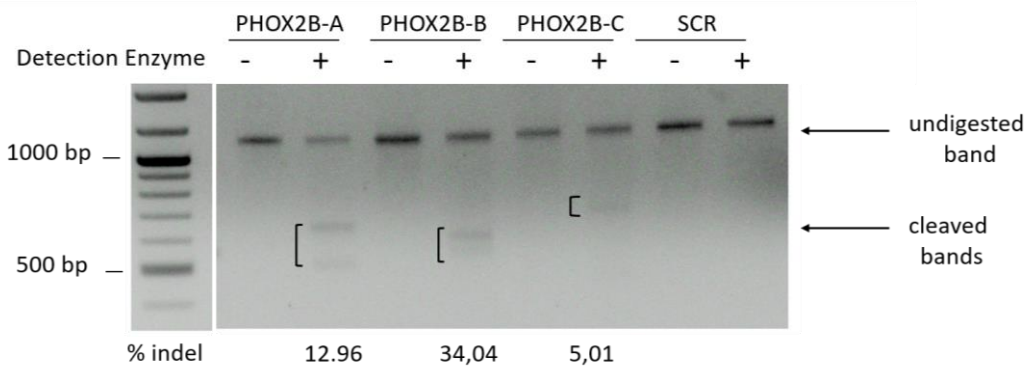


Fig.13 CRISPR-Cas9 mediated cleavage efficiency. Gel image of a cleavage assay using the GeneArt® Genomic Cleavage Detection Kit (Life Technologies). IMR32 cells were transfected with GeneArt® CRISPR All-in-one vectors targeting different regions of the exon1 of human *PHOX2B* locus (PHOX2B-A, -B and -C). A negative control sample for gene modification was also used (SCR). The samples were PCR amplified using the same set of primers flacking the region of cleavage. After re-annealing, samples were treated with (+) or without (-) Detection Enzyme and run on a 2% agarose gel. Treatment with the nuclease results in the generation of specific cleaved bands, shown by a bracket in samples transfected with All-in-one vectors while no bans were observed in cells transfected with the empty vector. The cleavage efficiency, expressed as formation of indel, was calculated for each sample as a difference between undigested band and the sum of the cleaved bans.

In order to favour genomic integration of the gRNAs containing constructs in IMR32 cells, the two pCLIP-All plasmids and the negative control were linearized using the restriction enzyme SmaI, which recognizes a single site in the plasmid backbone without destroy any relevant sequence within construct, and transfection performed as described in Materials and methods section. Around 100 clones transfected with gRNA A and B and 30 clones with gRNA SCR were obtained and probed for PHOX2B protein expression by Western blotting analysis. To exclude any false negative results, two different antibodies, directed to the extreme N- and C- terminal ends of the protein, were used for clones obtained with gRNA-A and gRNA-B (Fig.14A). From this initial screening, four clones showed a complete absence of PHOX2B protein expression compared with the native IMR32 (Fig.14A bottom: asterisk shows clone A6.4 as an example), while any differences were reported between SCR clones and native IMR32 cells (Fig.14B). The majority of clones A and B presented specific bands, recognized by both antibodies, with higher or lower molecular weight than control (Fig.14A), suggesting the formation of insertions or deletions at the cleavage site that generate truncated or longer protein.

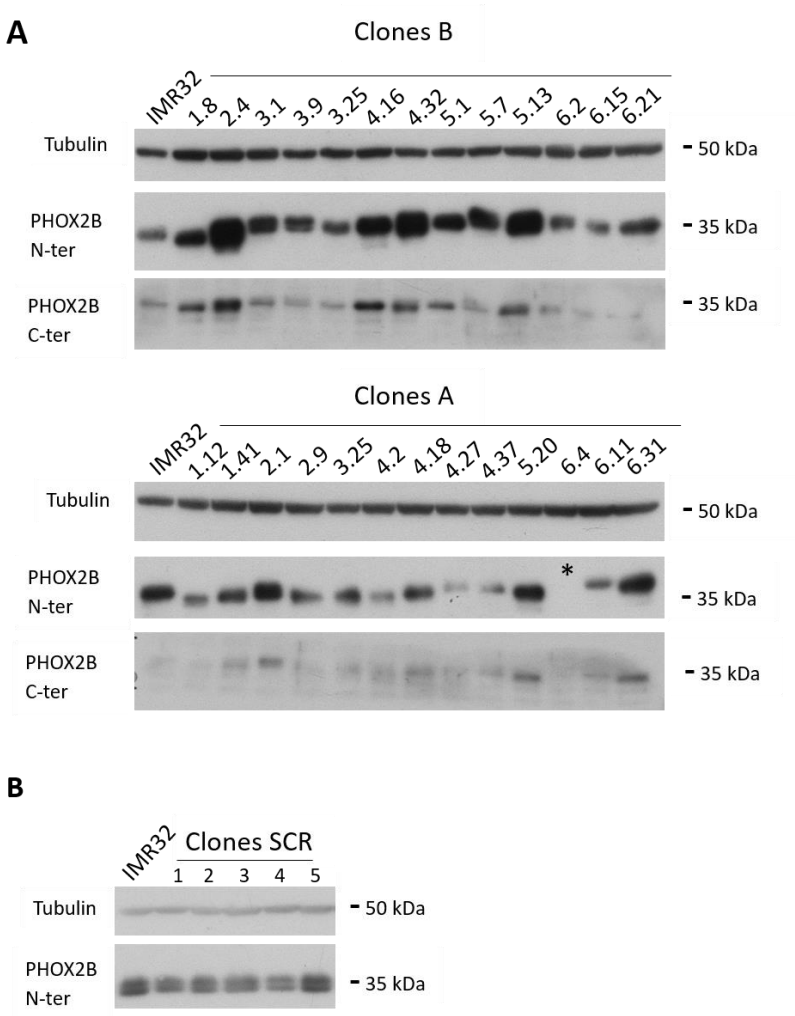


Fig.14. Generation of *PHOX2B* KO cell lines. A representative Western blot of total cell lysates from IMR32 clones selected with puromycin containing medium. 20 µl of cellular extract obtained from 1/3 of confluent 100 mm dish were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Each numbered lane represents one sample clone. Star indicates clone A6.4 that does not express PHOX2B protein. Extract of the IMR32 cell line was used as positive control of PHOX2B. β-Tubulin was used as normalizer (upper boxes). A: Anti-PHOX2B directed to the extreme N- and C- terminal ends of the protein were used to detect the presence of the protein (middle and lower boxes). Top and bottom: clones generated by the transfection of All-in-one vectors containing the gRNA PHOX2B-B and gRNA PHOX2B-A, respectively. B: clones generated by the transfection of All-in-one negative control (SCR).

We decided to initially characterize one of the four obtained clones (A 6.4, Fig.15), by qPCR and western blotting analysis. Clone A6.4 and native IMR32 cells, used as *PHOX2B* positive control, showed comparable levels of *PHOX2B* mRNA (Fig.15A white bar vs black bar), whereas the expression of PHOX2B endogenous protein in the clone was completely absent (Fig.15B), as expected.

In order to characterize the nature of gene editing in the IMR32 6.4 clone, we PCR amplified exon 1 from genomic DNA and the PCR product was then analysed by Sanger sequencing. Cas 9 cleavage has generated a 10-bp deletion in clone A6.4 (Fig.15C, black arrows) that cause a frameshift in the ORF with consequent generation of a new amino acid sequence (10 aa, Fig.15C in red) and a premature stop codon (Fig.15C, showed by asterisk), resulting in no protein synthesis (Fig.15B). The deletion has occurred in both alleles as we were unable to detect any intact wild-type allele. The formation of premature stop codon and the complete lack of detectable PHOX2B protein expression by Western blotting with multiple PHOX2B-specific antibodies confirmed the generation of one *PHOX2B* KO cell line using the CRISPR/Cas9 system.

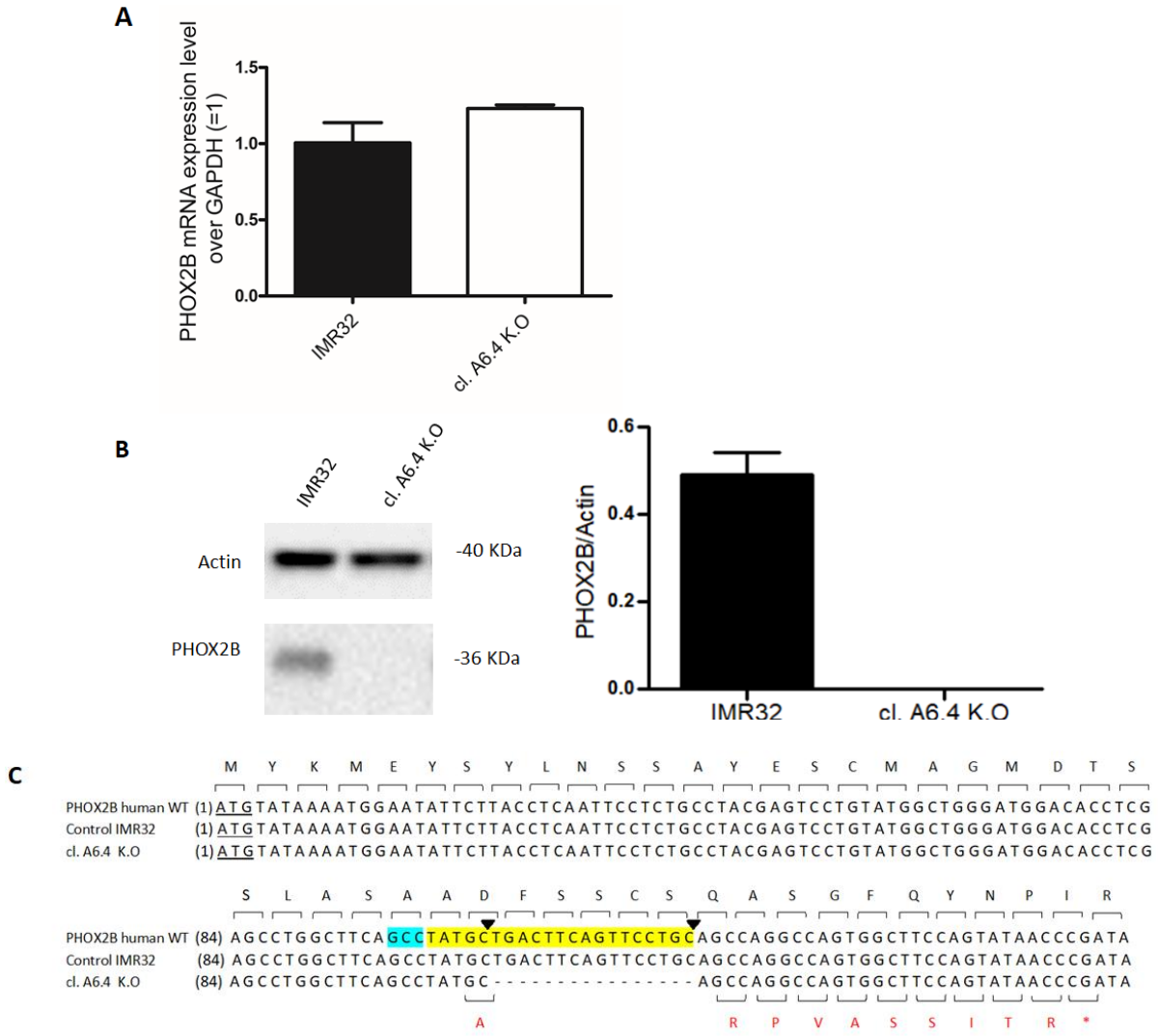


Fig.15 Validation of *PHOX2B* KO clone A6.4. A: qPCR analysis of endogenous *PHOX2B* mRNA expression normalised to that of the GAPDH gene in native IMR32 (black bar) and clone A6.4 *PHOX2B* KO (white bar). The bars are the mean values ± SD (error bars) of at least three independent experiments, performed in triplicate, and are expressed as fold relative to IMR32 native cells (=1). The $2^{-\Delta\Delta CT}$ method was used to calculate the results. B: Left: representative western blot of total cell lysates from IMR32 *PHOX2B* KO clone A6.4 and native IMR32 as positive control of PHOX2B. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Actin was used as normalizer (upper boxes). Right: the bar chart shows the relative quantification of PHOX2B protein in clone A6.4 and IMR32 native cells (black bars) expressed as ratio of PHOX2B to actin. C: Sequence alignment of WT exon 1 region (RefSeqGene human *PHOX2B* sequence WT) with sequences obtained from native (Control IMR32) and edited (cl. A6.4 K.O.) IMR32 cell lines. Clone A 6.4 lacks 10-bp at the level of cleavage site (limited by arrows). The putative transcription start codon ATG is underlined. The complementary plus strand sequence corresponding to the 20-nt target and 3-nt PAM is highlighted in yellow and blue, respectively. The WT amino acid sequence is shown above the nucleotide sequence and variable amino acid residues in clone A 6.4 are shown below the sequence and are highlighted in red. The formation of a premature stop codon is indicated by asterisk.

Molecular and cellular characterization of IMR32 *PHOX2B* KO clone A6.4

In literature there are controversial data regarding the role of PHOX2B; some works indicated that PHOX2B is essential for cellular differentiation by regulating neuronal cell cycle-exit and has an anti-proliferative function (Dubreuil et al., 2000; Reiff et al., 2010). On the contrary, other studies reported PHOX2B to be an important factor for the proliferation of immature sympathetic neurons (Coppola et al., 2010). For these reasons, before studying PHOX2B role on the transcription of ion channels encoding genes, we characterized the cellular phenotype of the clone, to understand whether the absence of PHOX2B has changed it.

Cell differentiation is an essential process in developmental biology and it is the result of different processes (differential gene expression, paused proliferation, epigenetic modifications and morphological changes) that convert the precursor cells to specialized ones. The differentiation process is ceased in certain cancer cells, but this process could be resumed using differentiation inducing agents; all trans retinoic acid (ATRA), for example, is one of the most important agents that help the cancer cell to acquire a terminally differentiated state (Shastry et al., 2001).

NB is the most common extra-cranial solid tumours derived from neuronal crest cells which migrate and give rise to PNS, the ENS, pigment cells, Schwannian-like cells, adrenal medullary cells, and cells of the craniofacial skeleton. A block in differentiation program of neuronal crest progenitor cells causes the development of NB.

The neuronal crest cells are multipotent, and the cell lines derived from NB, such as the IMR32 cell line, maintain this ability to transdifferentiate when treated with differentiation inducing agents. For example, Chaudhari et al. demonstrated that CDDO, a PPARγ antagonist, in combination with ATRA is able to induce IMR32 NB differentiation (Chaudhari et al., 2017). As mentioned in the introduction, it is well known that at the level of bipotential progenitors ($Sox10^+$, $Phox2b^+$) downregulation of *Phox2b* is a prerequisite for glial lineage differentiation ($Sox10^+$, $Phox2b^-$), whereas downregulation of *Sox10* expression is necessary for neuronal lineage differentiation ($Sox10^-$, $Phox2b^+$) (see Fig.3). Moreover, it has been demonstrated that mutations in *Phox2b* gene altered the balance between the two genes in favour of *Sox10* (Nagashimada et al., 2012). For this reason, we investigated whether the absence of PHOX2B

in clone A6.4 would be able to cause a drastic changing in the cellular phenotype with a differentiation of IMR32 cells toward the glial fate ($Sox10^{+,}$ $Phox2b^{-}$), detrimental to neuronal lineage differentiation. It is worth noting that during neuronal development, the down-regulation of *PHOX2B* expression, at the level of neuronal precursor, is functional and essential to restrict cell division and allows the cells to reach a mature functional and specific neuronal phenotype.

The differentiation of NB cells *in vitro* can be studied by changing in morphological features, as well as by functional criteria based on the differential expression of glial, neuronal and maturation markers, detected by using Western blotting and qRT-PCR analyses. *PHOX2B* K.O A6.4 stable clone was analysed at different cell passages (7, 10 and 15 days in colture) to detect whether the messenger and protein level expression of genes under investigation varied over multiple cell passages, thus suggesting a progressive differentiation to a specific cellular phenotype.

Morphological differentiation of neuronal cells is defined by the appearance of one (or more) neuritic extension. Phase contrast images of clone A6.4 (Fig.16) showed that the complete absence of PHOX2B protein expression caused a changing in the cell morphology, grossly distinguishable from controls: clone A6.4 showed increase in neuritis length and in cellular body and a strong adherence to the flask surface, phenotypic changes typical of a more differentiated cell.

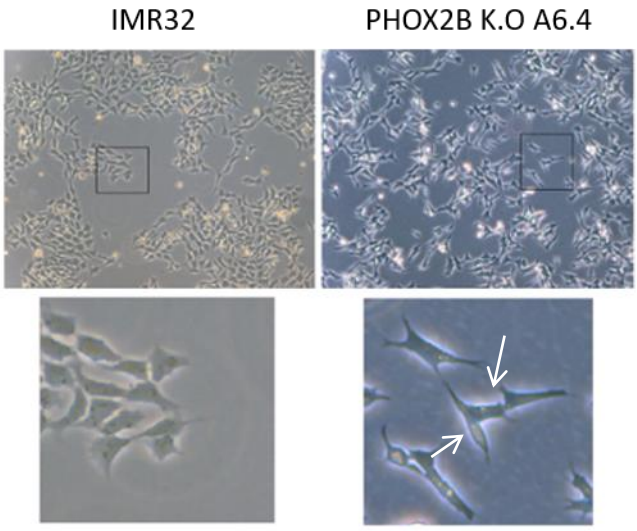


Fig.16 Morphological study. Cells were analysed under phase contrast microscope to study the morphological features of IMR32 in the absence of PHOX2B protein (right boxes) in contrast with IMR32 native cells (left boxes). The images were captured on a microscope equipped with a monochrome camera. High-magnification images of areas bounded by black boxes are shown below the corresponding low-power image. Arrows indicate increase in neurites length in cl A6.4.

In order to understand the cellular phenotype of clone A6.4, we evaluated the expression of neuronal crest derived glial-cell lineage marker, Sox10 and the expression of neuron specific marker, like β3-tubulin, by qRT-PCR and by Western blot. Sox10 expression, an immature neural crest marker also expressed in glia cells, was not detected by RT-PCR and Western blotting analyses (data not shown) in both IMR32 native and in clone A6.4, thus excluding any change in the differentiation state of the clone towards the glial lineage. This data suggests that IMR32 cells, although blocked in their differentiation to sympathetic neurons, are already committed along neuronal lineage ($Sox10^-$, $Phox2b^+$) and the absence of PHOX2B at this stage is not sufficient to revert this process, to a more glial-differentiated stage ($Sox10^+$; $Phox2b^-$). Moreover, the expression of beta β3-tubulin, a neuron associated tubulin isotype whose expression is up-regulated during neuronal differentiation, was increased in clone A6.4 compared to IMR32 native cells (Fig.17), thus confirming that the absence of PHOX2B in cl. A6.4 does not cause a change in the lineage specification, but on the contrary, it induces the cells to differentiate along the neuronal lineage and to acquire a more mature phenotype.

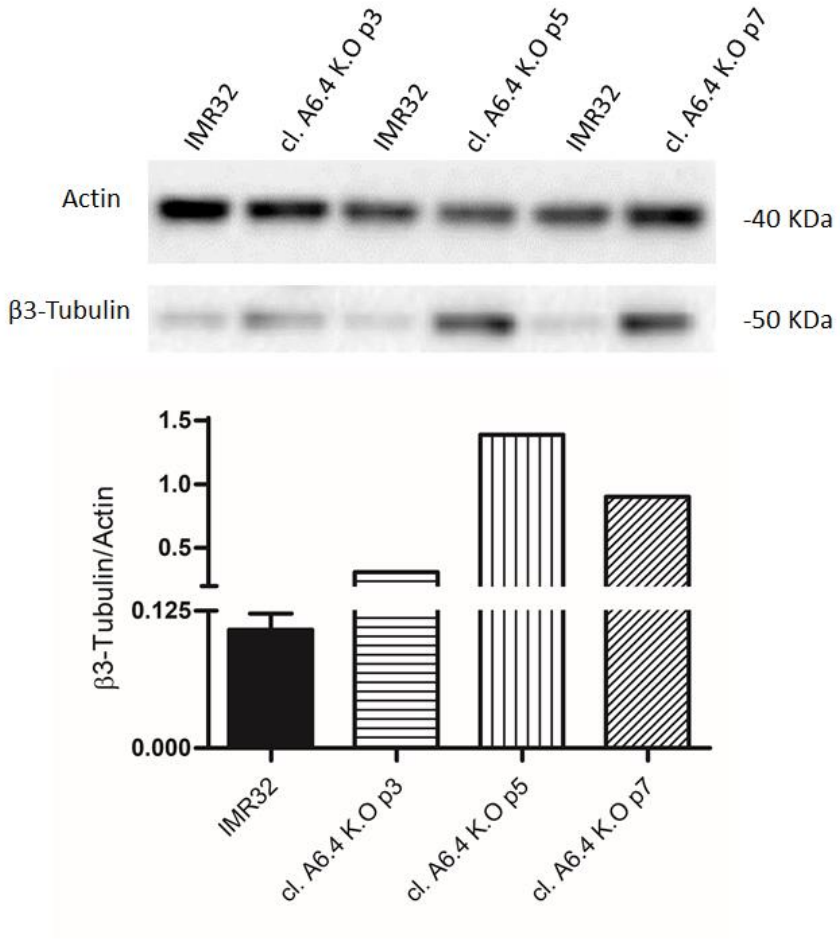


Fig.17 Expression of the neuron specific marker β3-tubulin. Top: a representative Western blot showed the expression of β3-tubulin (lower box) in total cell lysates from IMR32 and clones A6.4 K.O at three different cell passages. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Extract of the IMR32 cell line was used as positive control of PHOX2B. Actin was used as normalizer (upper box). Bottom: relative quantification of β3-tubulin protein levels in cl. A6.4 K.O at different cell passages (white hatched bars) and IMR32 native cells (black bars) expressed as ratio of β3-tubulin to actin.

During differentiation of the sympatho-adrenal lineage, cells undergo a cholinergic-catecholaminergic switch, that give raise respectively to neurons of the parasympathetic or sympathetic division of the ANS, depending on the target tissues they will innervate. Neuroblastoma cells, being blocked at earlier stage of differentiation are capable of expressing enzymes of both phenotypes, such as TH and DBH, adrenergic markers, and choline acetyl transferase (ChAT), cholinergic marker; the increase or reduction in the expression of these enzymes is usually used to characterize neuronal specification. Recently, Boeva et al. demonstrated that neuroblastoma cell lines have three types of identity and, in

particular, the sympathetic noradrenergic identity is further defined by the expression of PHOX2B, HAND2 and GATA3 (Boeva et al. 2017). In order to evaluate whether the absence of PHOX2B in KO clone affected the noradrenergic identity in IMR32 cell line, we studied *PHOX2A, DBH* and *TLX2* mRNA and protein expression levels, three important noradrenergic markers.

PHOX2A is positively regulated by PHOX2B (Flora et al., 2001) and together with PHOX2B cooperates to determine the noradrenergic phenotype through the induction of *DBH*, the rate-limiting enzyme in the pathway of noradrenaline synthesis (Adachi et al., 2000), and *TLX2* (Borghini et al., 2006) gene expression, a transcription factor involved in ENS development and mainly expressed in tissues derived from neural crest cells at the end of their migration. Unexpectedly, we found that the absence of PHOX2B strongly induced an increase of *PHOX2A* transcript levels by 73% (Fig.18A) in IMR32 cl. A6.4 cell line. Moreover, Western blot analysis showed a progressive increase in the PHOX2A protein level over multiple cell passages that reaches a steady-state level after seven days of culture in the KO clone compared to the WT (Fig.18B). As mentioned before, *PHOX2A* is positively regulated by PHOX2B and its unexpected increase in protein expression in clone A6.4 might be explained as a compensatory mechanism of the cells in order to maintain the noradrenergic phenotype.

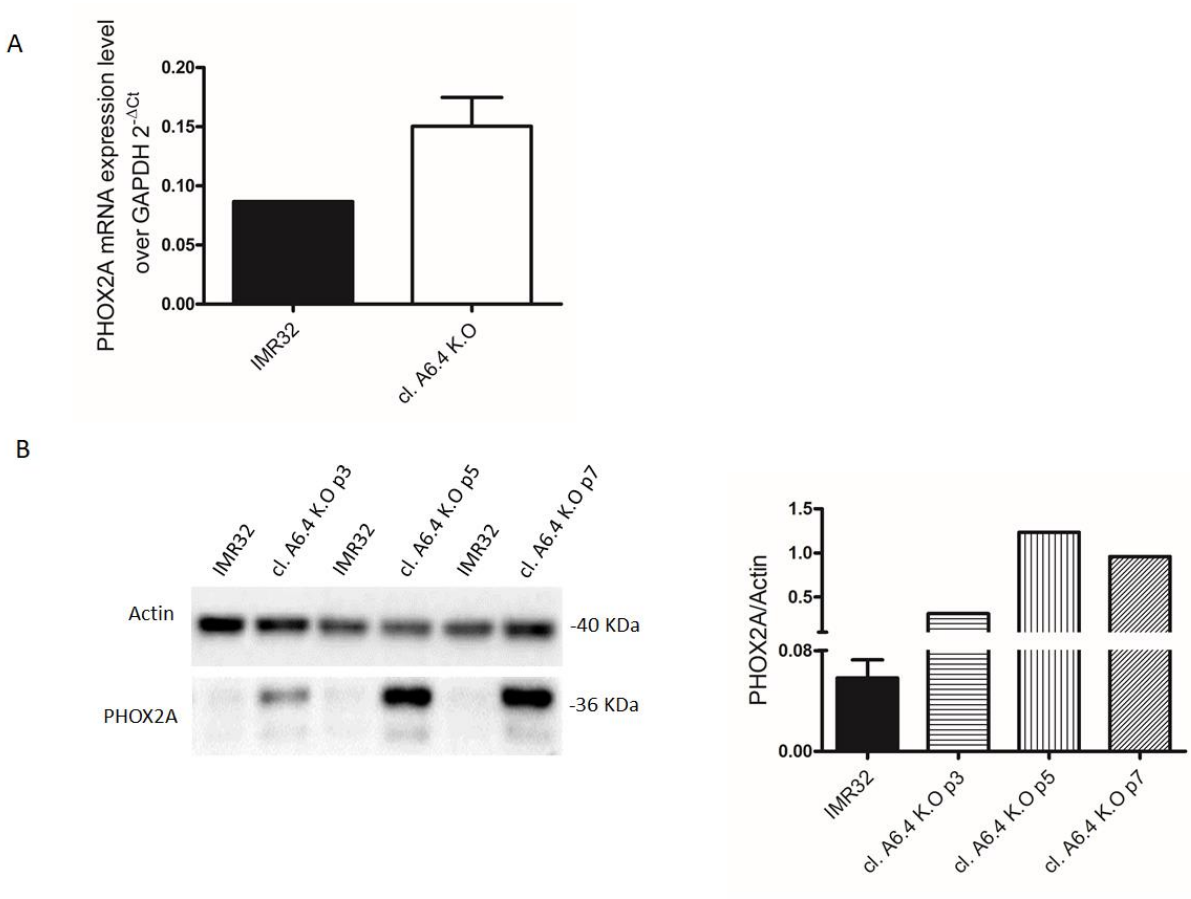


Fig.18 Expression of *PHOX2B* paralogue gene, *PHOX2A*. A: qPCR analysis of endogenous *PHOX2A* mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (black bar) and *PHOX2*B K.O clone A6.4 (white bar). The bars are the mean values ± SD (error bars) of at least three independent experiments, performed in triplicate. The $2^{-\Delta CT}$ method was used to calculate the results. B Left: a representative Western blot showed the expression of PHOX2A (lower box) in total cell lysates from IMR32 and clone A6.4 at different cell passages. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Actin was used as normalizer (upper box). Right: relative quantification of PHOX2A protein level in cl. A6.4 at different cell passages (white hatched bars) and IMR32 native cells (black bars) expressed as ratio of PHOX2A to actin.

PHOX2A co-regulates *DBH* and *TLX2* genes; we therefore evaluated how the deletion of PHOX2B and the increased of PHOX2A proteins in cells affects the abundance of these two target genes. No changes were observed at both RNA and protein level of DBH (Fig.19A and B respectively) and at *TLX2* mRNA level (Fig.19C) in the clone compared to native IMR32. These results are in agreement with what has been previously reported by Boeva et al. that the silencing of *PHOX2B* expression is not sufficient to change the noradrenergic identity in CLB-GA and SH-SY5Y cell lines; indeed *PHOX2A, HAND2, GATA3, TH* and *DBH* are only slightly affected (Boeva et al. 2017). However, unlike the *PHOX2B* silenced cells that showed a slight

decrease in PHOX2A protein level, these data suggest that the maintenance of the noradrenergic identity in clone A6.4 might be mediated by the up-regulation of *PHOX2A* expression, with no changes in the expression of *DBH* and *TLX2*.

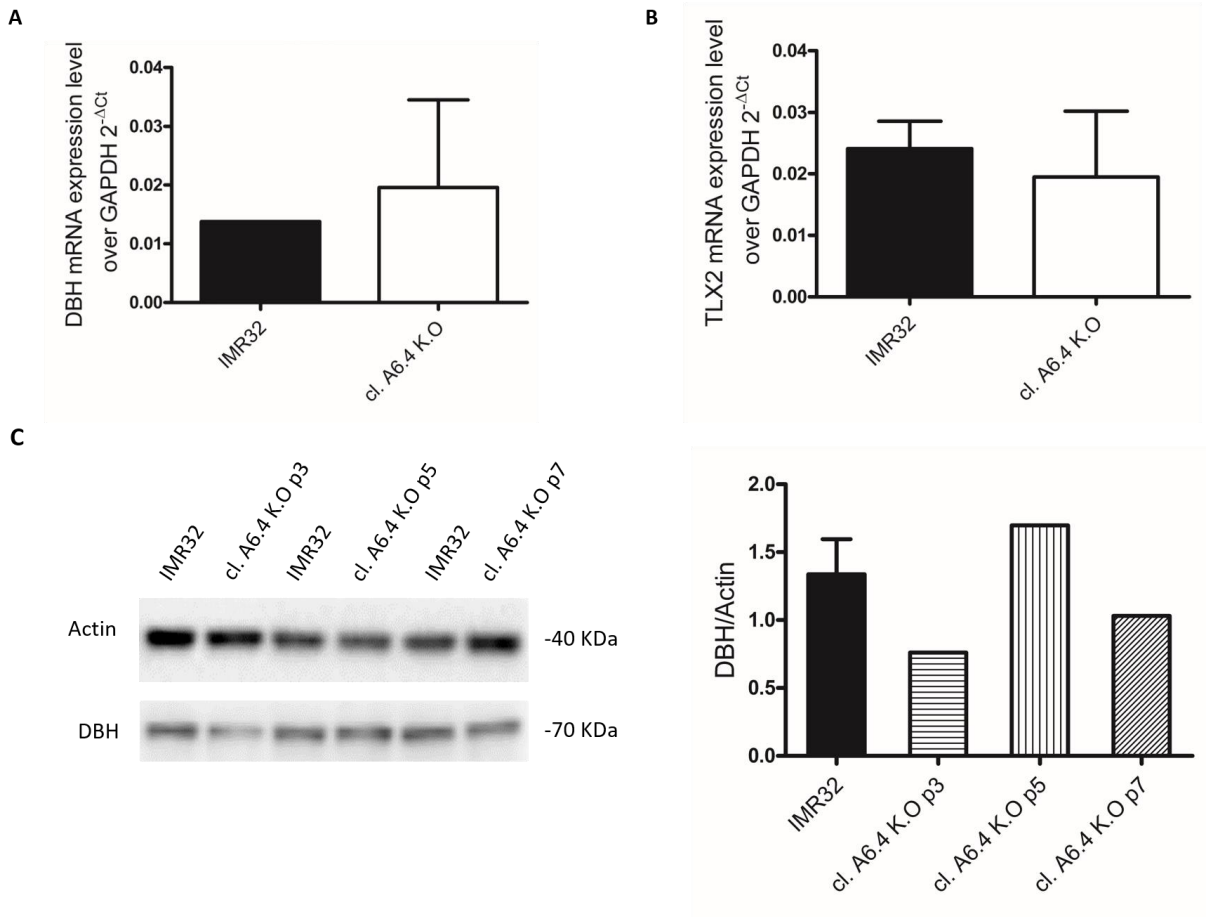


Fig.19 Expression of the adrenergic markers DBH and TLX2. A and B: qPCR analysis of endogenous *DBH* and *TLX2* mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (black bar) and *PHOX2B* K.O clone A6.4 (white bar). The bars are the mean values ± SD (error bars) of at least three independent experiments, performed in triplicate. The $2^{-\Delta CT}$ method was used to calculate the results. C Left: a representative Western blot showed the expression of DBH (lower box) in total cell lysates from IMR32 and clones A6.4 K.O at different cell passages. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Actin was used as normalizer (upper box). Right: relative quantification of DBH protein level in cl. A6.4 at different cell passages (white hatched bars) and IMR32 native cells (black bars) expressed as ratio of DBH to actin.

The increase in *PHOX2A* and the maintenance of *DBH* expression level led us to investigate whether the absence of PHOX2B induced any differences in the expression of ChAT an enzyme responsible for the synthesis of the neurotransmitter acetylcholine in

parasympathetic nervous system. Figure 20 shows a reduction only at protein level in clone A6.4 compared to control cells (Fig.20A white hatched bars vs black bar) but did not affect mRNA (Fig.20B). These results suggested that *PHOX2B* KO cell line maintains a heterogeneous identity leading us to hypothsize that the cholinergic-catecholaminergic switch is not completed yet. However, the maintenance of the expression of *DBH* and *TLX2,* the reduction of the expression of *ChAT* and the increase in expression of *PHOX2A* suggests that the clone A6.4 defective for PHOX2B expression has a tendency to differentiate into a more mature adrenergic neuronal lineage phenotype compared to IMR32 native cells.

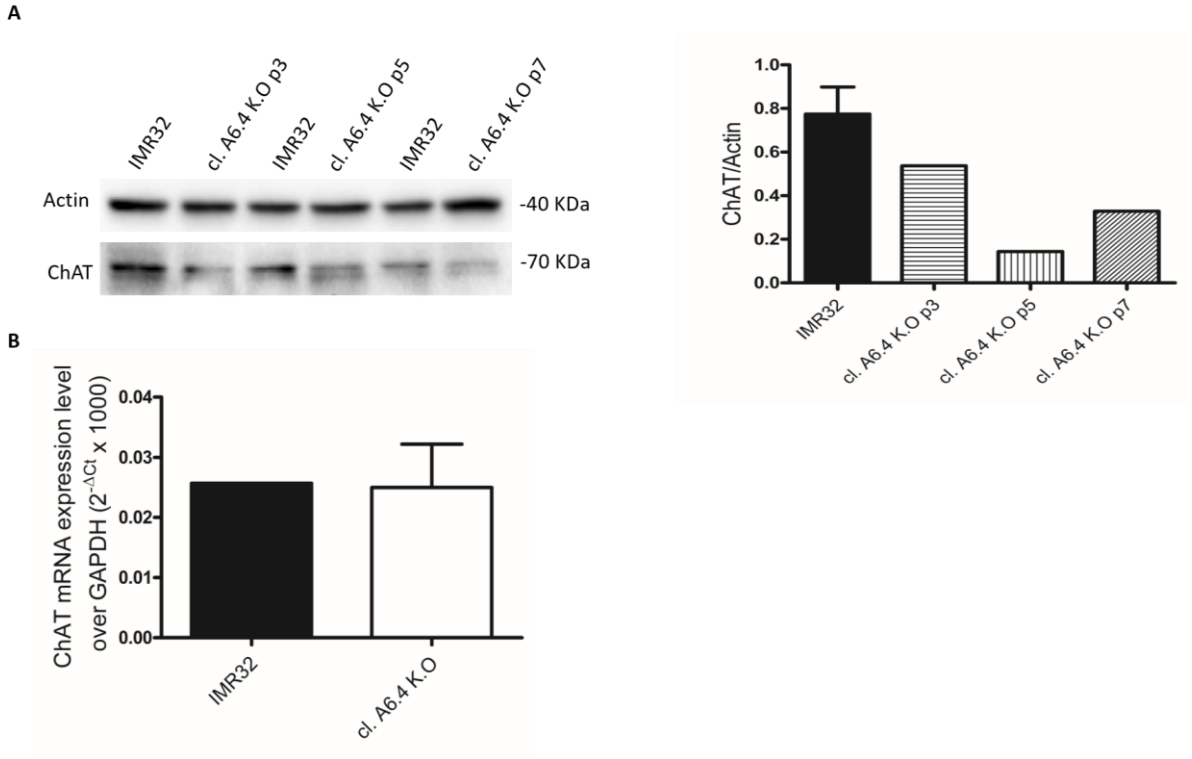


Fig.20 Expression of the cholinergic specific enzyme Choline acetyltransferase (ChAT). A Left: a representative Western blot showed the expression of ChAT (lower box) in total cell lysates from IMR32 and clone A6.4 K.O at different cell passages. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Actin was used as normalizer (upper box). Right: relative quantification of ChAT protein levels in cl. A6.4 at different cell passages (white hatched bars) and IMR32 native cells (black bars) expressed as ratio of ChAT to actin. B: qPCR analysis of endogenous *ChAT* mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (black bar) and PHOX2B K.O clone A6.4 (white bar) and expressed according to the $2^{-\Delta Ct}$ method.

To further investigate the morphological changes induced by PHOX2B depletion, we investigated the protein levels of synaptotagmin and syntaxin, two vesicular markers that play an

important role during docking and priming vesicular process. Both markers were upregulated in clones A6.4 in contrast to control, suggesting a greater level of neuronal maturation (Fig.21).

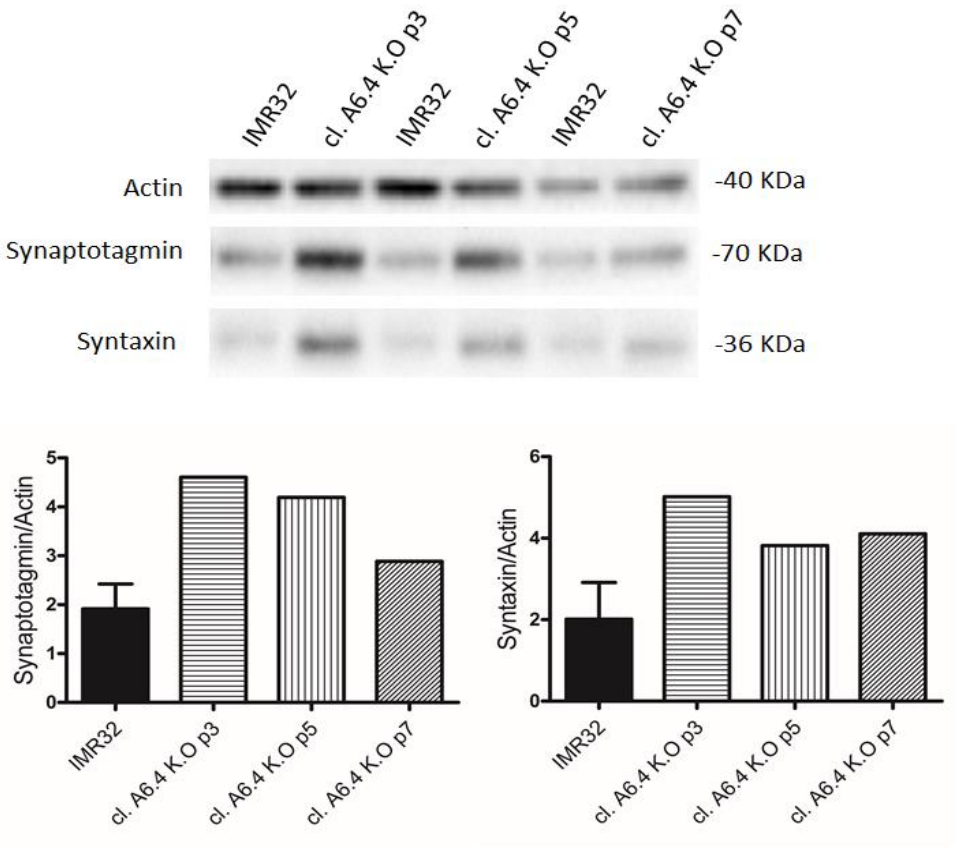


Fig.21 Expression of vesicular markers. Top: a representative Western blot showed the expression of synaptotagmin (middle box) and syntaxin (lower box) in total cell lysates from IMR32 and clones A6.4 at different cell passages. 20 µl of cellular extract were separated by means of 10% SDS-PAGE and transferred to a nitrocellulose membrane. Actin was used as normalizer (upper box). Bottom: relative quantification of synaptotagmin (right) and syntaxin (left) protein levels in cl. A6.4 at different cell passages (white hatched bars) and IMR32 native cells (black bars) expressed as ratio of synaptotagmin or syntaxin to actin.

In conclusion, our results demonstrated that the morphological changes observed in *PHOX2B* KO cells were associated with an increase expression of neuronal markers β3-tubulin (Fig.17), synaptotagmin and syntaxin (Fig.21), suggesting a changing from immature neuronal phenotype into a mature and functional one. Moreover, the differentiation was accompanied by a decrease in the expression of *ChAT* enzyme (Fig.20) and the upregulation of *PHOX2A* (Fig.18) sufficient to maintain the noradrenergic identity (Fig.19).

Ion channels are new PHOX2B target genes

Gene expression analysis of ion channels in *PHOX2B* knockout cell lines

Since the absence of PHOX2B did not affect dramatically the cellular phenotype of clone A6.4 and the cells still maintained their adrenergic identity, we studied the transcriptional role of PHOX2B on ion channels by comparing their expression between this clone and IMR32 native cells. We chose three potassium channels identified by ChIP-seq: two belong to the Ca^{2+}-activated k^{+} channels family (KCNN3 and KCNMA1) and one belongs to the voltage gated k^{+} channel family (KCNQ5). Recently, the KCNQ family of k^{+} channels have been described to have a determinant role in chemoreceptor function in RTN (Mulkey et al., 2015); moreover, it was reported that the overexpression of *KCNN3* channel cause cardiac and respiratory problems (Mahida et al., 2014), suggesting that they are potential therapeutic targets for the treatment of respiratory dysfunction.

qRT-PCR analyses (Fig.22) showed that PHOX2B depletion strongly increased by 2-fold *KCNQ5* mRNA level in KO clone compared to native IMR32 and scramble transfected cells (Fig.22A, black bar vs white and grey bars). *KCNN3* mRNA expression was also significantly increased, although to a lesser degree (Fig.22B, black bars vs white and grey bars). No difference in the *KCNMA1* mRNA expression level was reported between *PHOX2B* knockout cells and control cells (Fig.22C, black bar vs white and grey bars).

Collectively, these data confirm the previous observation that potassium channels are PHOX2B target genes and moreover, surprisingly, they suggest that PHOX2B modulates negatively the expression of these genes.

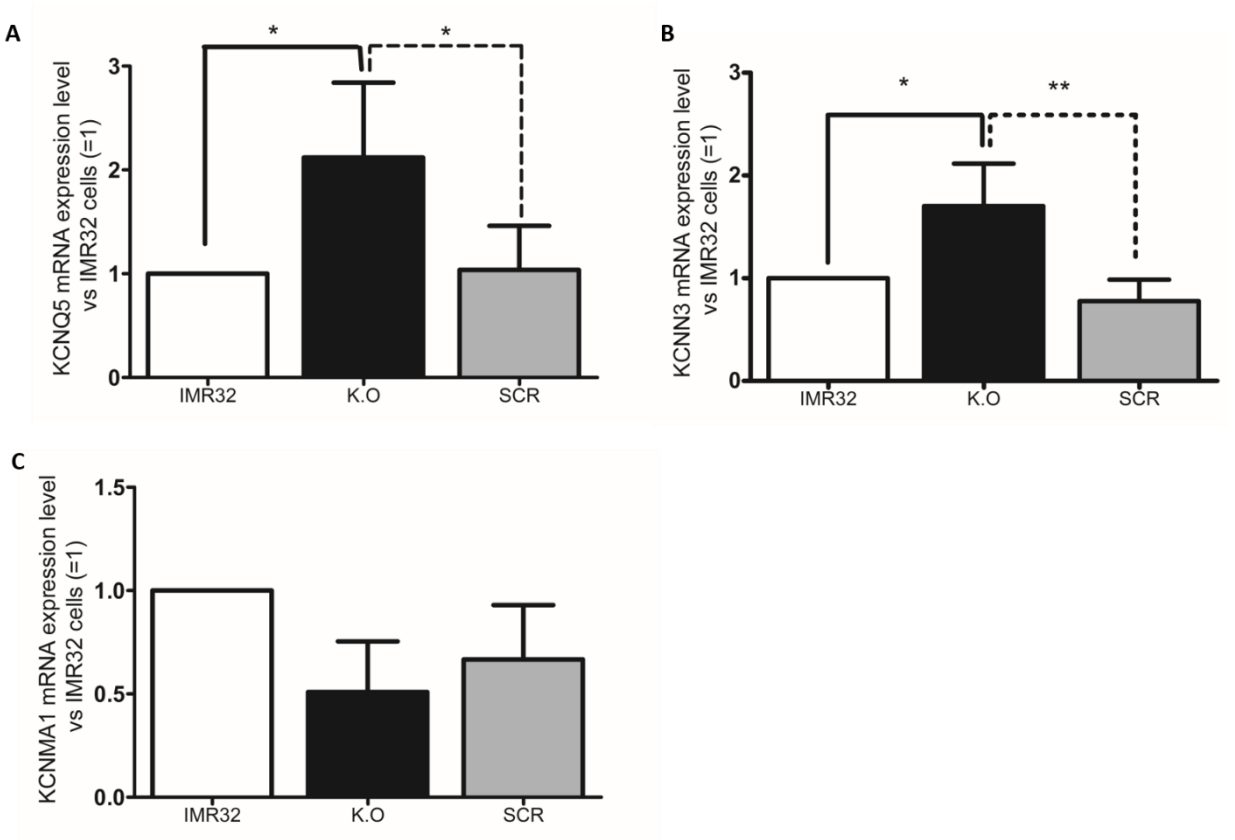


Fig.22 Potassium channels expression. qPCR analysis of endogenous *KCNQ5* (A), KCNN3 (B) and *KCNMA1* (C) mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (white bar), *PHOX2B* K.O clone A6.4 (black bar) and SCR (grey bar). The bars indicate the relative fold induction in *PHOX2B* K.O clone A6.4 and SCR with respect to the native IMR32 (=1) calculated using the $2^{-\Delta\Delta Ct}$ method and are expressed as the mean values ± SD (error bars) of at least three independent experiments, performed in triplicate. A: *p < 0.05, statistically significant differences in *KCNQ5* mRNA levels between K.O clone and IMR32 and SCR, respectively (one-way ANOVA, Tukey's test). B: **p < 0.01 and *p< 0.05, statistically significant differences in *KCNN3* mRNA levels between K.O clone and IMR32 and SCR, respectively (one-way ANOVA, Tukey's test).

We then investigated the role of PHOX2B on sodium channels expression. A significant up-regulation of *SCN2A* and *SCN3A* mRNA by 2- and 3-fold, respectively, was observed in clone A6.4 compared to IMR32 and scramble transfected cells (Fig.23A and B, black bar vs white and grey bars). *SCN8A* channels encoding gene expression also increased in the absence of PHOX2B but to a lesser degree (Fig.23C black bars vs white and grey bars). In contrast to *SCN2A, SCN3A* and *SCN8A*, we observed a not specific decrease in *SCN9A* mRNA levels, as its level detected in the A6.4 clone parallels that in the scramble transfected cells (Fig.23D, black and grey bars vs white bar).

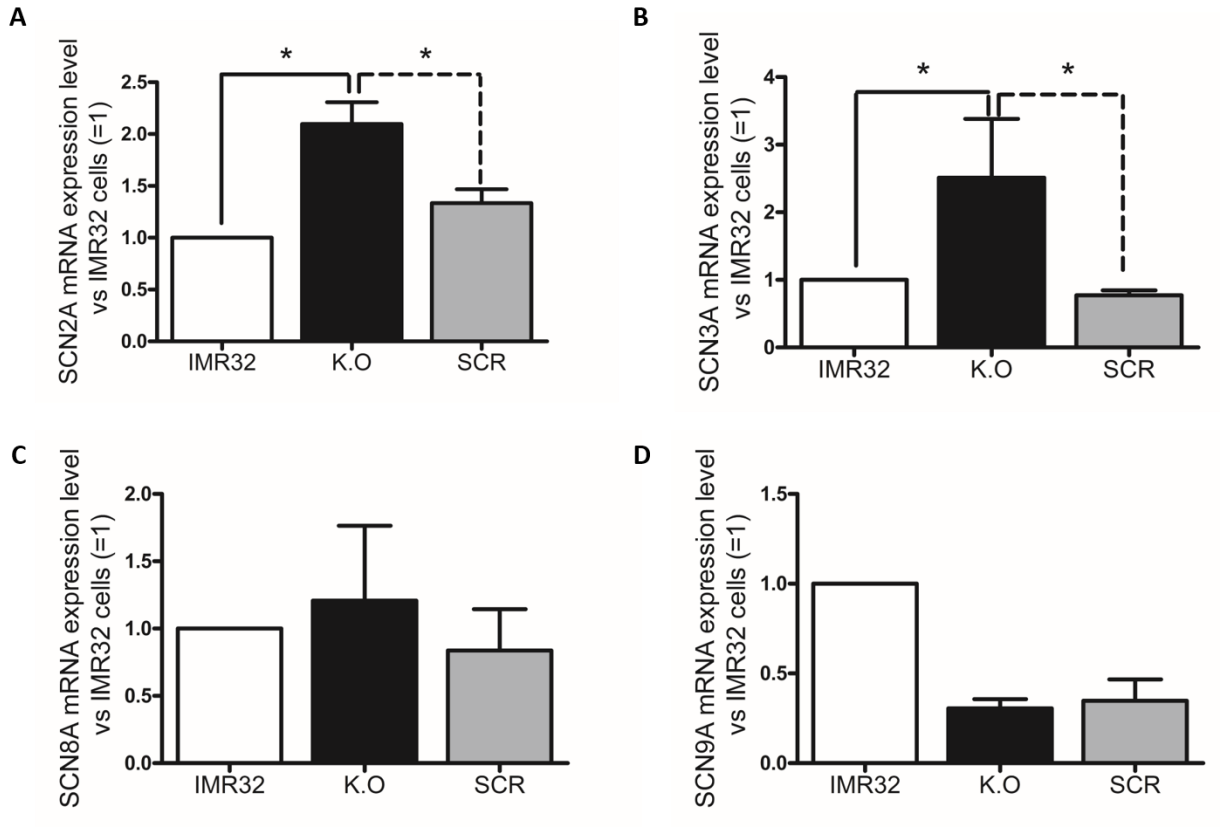


Fig.23 Sodium channels expression. qPCR analysis of endogenous *SCN2A* (A), *SCN3A* (B), *SCN8A* (C) and *SCN9A* (D) mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (white bar), *PHOX2B* K.O clone A6.4 (black bar) and SCR (grey bar). The bars indicate the relative fold induction in *PHOX2B* K.O clone A6.4 and SCR with respect to the native IMR32 cells (=1) calculated using the $2^{-\Delta\Delta Ct}$ method and are expressed as the mean values ± SD (error bars) of at least two independent experiments, performed in triplicate. A and B: * $p < 0.05$, statistically significant differences in *SCN2A* and *SCN3A* mRNA levels between K.O clone and IMR32 and SCR, respectively (one-way ANOVA, Tukey's test).

These data demonstrated that also sodium channels are PHOX2B target genes and confirmed that PHOX2B, usually considered as a positive regulator of transcription is also able to negative regulate the expression of ion channels. So far, only two genes have been identified that are negatively regulated by PHOX2B: *MSX-1* (Revet et al., 2008) and *SOX10* (Nagashimada et al., 2012), important for the correct development of the ANS. The reciprocal negative regulation between *Phox2b* and *Sox10* plays a crucial role in generating appropriate numbers of neurons and ganglia in the ANS (Fig.3). Processes of vital importance for the neuron, such as its differentiation or electrical activity must be finely regulated. Therefore, we can hypothesize that PHOX2B could play a repressive role on transcription of its target genes that have to be fine-tuned for a correct function of the neuron.

The main potential pitfall with the CRISPR-Cas9 system is the risk of cross-reactivity of gRNA with target sites in different genes that shear a similar sequence. To rule out the possibility that the increase in *KCNN3* and *KCNQ5* mRNA levels observed in *PHOX2B* knockout cells was due to an off-target effect, we performed a "rescue" experiment with the expression of WT PHOX2B protein.

Expression of WT PHOX2B protein in clone A6.4 knockout cells (Fig.24 black bar) was not however capable of inducing any change in the *KCNN3* and *KCNQ5* mRNA expression levels with respect to mock-transfected cell (Fig.24A and B, black bars vs white bars). As control A6.4 clone has been transfected with empty vector (Fig.24A and B, black bars vs grey bars).

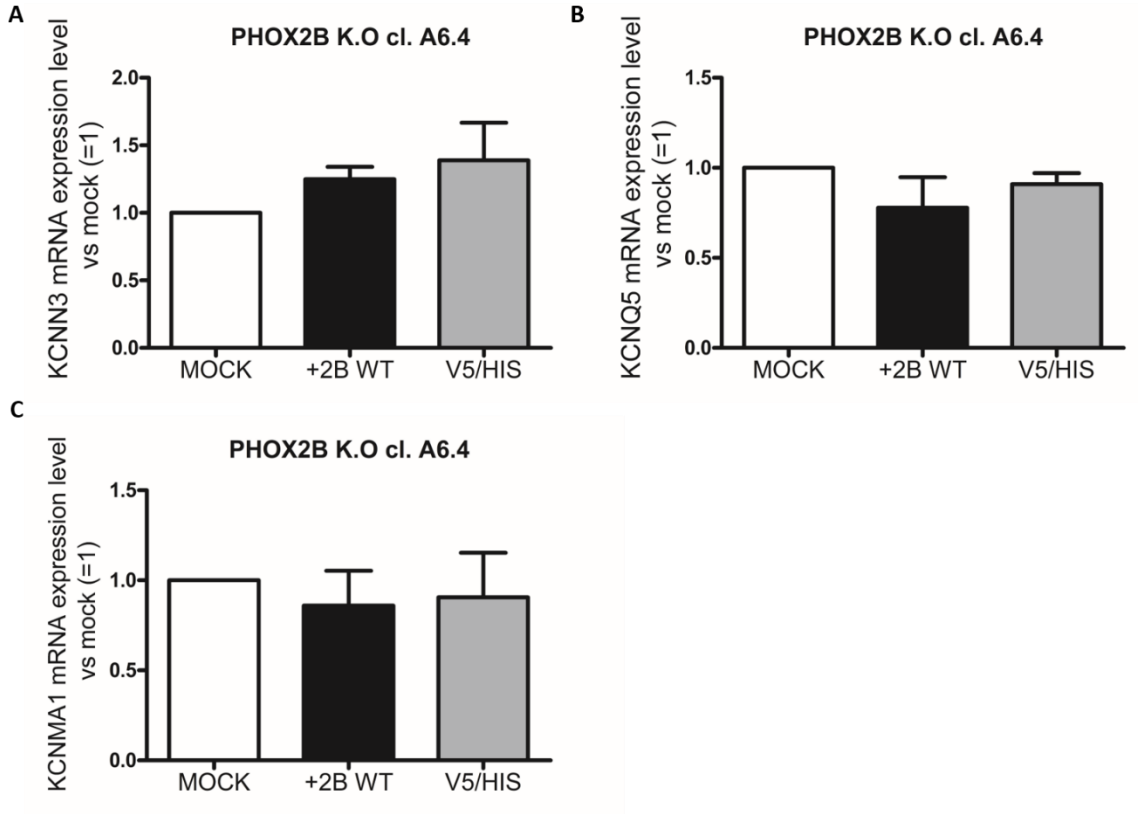


Fig.24 Expression of *PHOX2B* does not rescue potassium channels expression in *PHOX2B* K.O cells. qPCR analysis of endogenous *KCNN3* (A), *KCNQ5* (B) and *KCNMA1* (C) mRNA expression normalised to that of the *GAPDH* gene in clone A6.4 (white bar) transfected with *PHOX2B* WT V5/HIS (black bar) and with empty vector V5/His as control (grey bar). The bars indicate the relative fold induction in cells transfected in relation to the not transfected cells (MOCK =1) calculated using the $2^{-\Delta\Delta Ct}$ method and are expressed as the mean values ± SD (error bars) of at least two independent experiments, performed in triplicate.

Although these results may suggest that the effects so far described are not due to PHOX2B depletion, but otherwise to an off-target effect, we cannot rule out that the missed down-regulation, as we expected, of ion channels by WT PHOX2B was due to a not sufficient expression of the WT protein in these cells. It is well known that neuroblastoma cell lines are difficult-to-transfect cells.

In order to avoid this problem, we tested different transfection parameters, by changing the DNA plasmid construct (*PHOX2B* WT V5/His and *PHOX2B* WT Mic/His encoding plasmids) and the transfection reagent (Fugene HD vs LIPOFECTAMINE 3000). Figure 25 shows the quantification of *PHOX2B* mRNA expression after *PHOX2B* construct transfection in clone A6.4, which results in a highly variable transfection efficiency in different experiments. However, cells expressed very low level of *PHOX2B* and no significant alteration was observed in the expression of potassium channels, even in the experiment with the higher level (3-fold) of *PHOX2B* expression (Fig.25 red triangle) (data not shown).

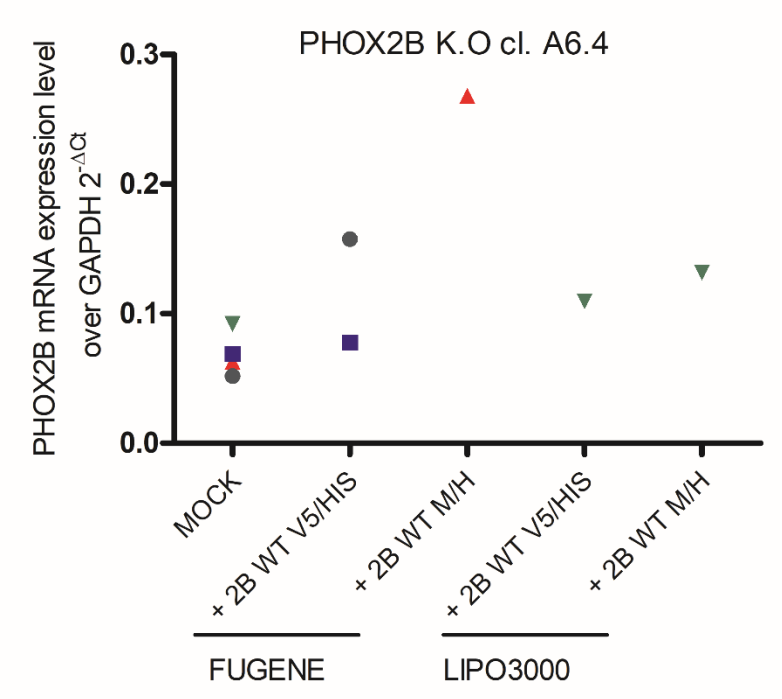


Fig.25 mRNA expression level of transfected *PHOX2B* WT in clone A6.4 with two different transfection reagents. Symbols indicate the mRNA levels of transfected *PHOX2B* knockout cell with two different constructs encoding for P*HOX2B* WT with two transfection reagents in order to optimize transfection efficiency. The level of mRNA is expressed as relative expression normalised to that of the *GAPDH* gene and expressed according the $2^{-\Delta Ct}$ method.

Given that *PHOX2B* knockout cell clone still expresses endonuclease Cas9 that can recognize the PAM sequence presents in the *PHOX2B* WT transfected DNA, we considered the hypothesis that the low expression of *PHOX2B* in transfected cells was due to the cleavage activity of this enzyme. To test this hypothesis, we transfected A6.4 cells with two constructs encoding for PHOX2B +7 and +13 Alanine mutant protein to study also the effect of mutant protein in the expression of ion channels genes. The empty vector V5/HIS was used as control. Unexpectedly, the expression of +7 and +13 Alanine mutant proteins significantly increase *KCNN3* mRNA expression in A6.4 cells compared to control (Fig.26A hatched bars vs white bars), whereas no significant changes were observed in *KCNQ5* and *KCNMA1* channel mRNA levels (Fig.25B and C).

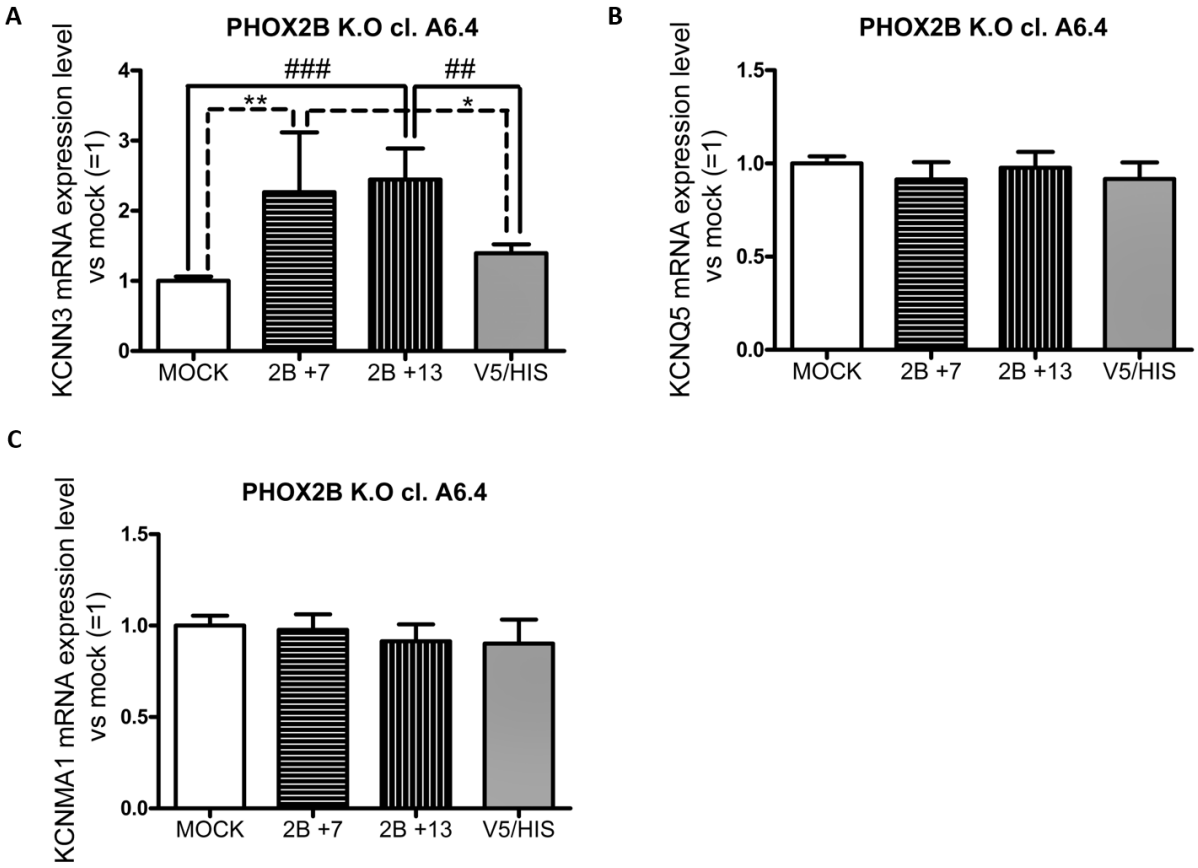


Fig.26 Mutant proteins up-regulate *KCNN3* channel expression. qPCR analysis of endogenous *KCNN3* (A), *KCNQ5* (B) and *KCNMA1* (C) mRNA expression normalised to that of the *GAPDH* gene in clone A6.4 (white bar) transfected with *PHOX2B* +7 and +13 V5/HIS (black hatched bar) and with empty vector V5/HIS as control (grey bar). The bars indicate the relative fold induction in cells transfected with respect to the mock-transfected cells (=1) calculated using the $2^{-\Delta\Delta Ct}$ method and they are expressed as the mean values ± SD (error bars) of at least two independent experiments, performed in triplicate. A: **p < 0.01 and *p < 0.05, significant differences in *KCNN3* mRNA level between cl. A6.4 cells transfected with PHOX2B +7 vector and cells not transfected or transfected with empty vector, respectively. $^{###}$ p < 0.001 and $^{##}$ p < 0.01, significant differences in *KCNN3* mRNA level between cl. A6.4 cells transfected with PHOX2B +13 vector and cells not transfected or transfected with empty vector, respectively (one-way ANOVA, Tukey's test).

The expression of the mutant proteins does not seem to be affected by the presence of endonuclease Cas9, so we excluded the possibility that this mechanism could be responsible for the low expression of PHOX2B WT protein. *PHOX2B* KO cells appeared to be not prone to *PHOX2B* overexpression. Furthermore, the effect of *PHOX2B* mutation on the expression of *KCNN3* was only detectable when cells have been efficiently transfected (at least 10 times over basal *PHOX2B* mRNA level; data not shown) confirming the difficulty in modulating *PHOX2B* levels in this cellular system. However, this aspect deserves further investigation.

In conclusion, the data obtained in clone A6.4 confirmed that

- ion channels encoding genes are indeed PHOX2B target genes
- PHOX2B negatively modulates the expression of *KCNN3, KCNQ5* potassium channels genes and *SCN2A, SCN3A, SCN8A* Na^+ channels genes.
- The over expression of +7 and +13 Alanine mutant proteins exacerbates the up-regulation of *KCNN3*, thus confirming that PHOX2B mutant proteins affect the expression of its target genes in a gene-dependent manner
- PHOX2B mutant proteins have acquired a gain of function activity in the expression of *KCNN3* gene.

Study of the role of the PHOX2B mutant protein on the expression of ion channels in the native IMR32 cell line

CCHS is due to heterozygous mutations of PHOX2B. The gain of function acquired by the mutant proteins in the expression of the *KCNN3* gene has been detected in a system depleted of WT PHOX2B. In order to confirm that PHOX2B mutant proteins lead to a de-regulation of *KCNN3* expression also in the presence of WT PHOX2B protein, we studied how the mutant protein (+7 Alanine) affects the expression of ion channel genes in native IMR32 cell line. As with the A6.4 clone, IMR32 native cells were difficult to transfect with the WT PHOX2B protein

Analysis of mRNA (Fig.27) extracted from IMR32 cells with higher level of expression of transfected WT P*HOX2B* did not reveal any significant alteration in the expression of potassium channel genes compared to mock-transfected sample and cells transfected with empty vector (Fig.27 horizontal hatched bars VS white and grey bars). On the contrary, the overexpression of mutant protein bearing the +7 Alanine mutation increased *KCCN3* mRNA expression (Fig.27 A vertical hatched bar VS white and grey bars) confirming the results obtained in clone A6.4. Likely to clone A6.4, *KCNQ5* and *KCNMA1* channels gene expression (Fig.27 B and C) was not affected, as no differences were measured between transfected and not transfected sample.

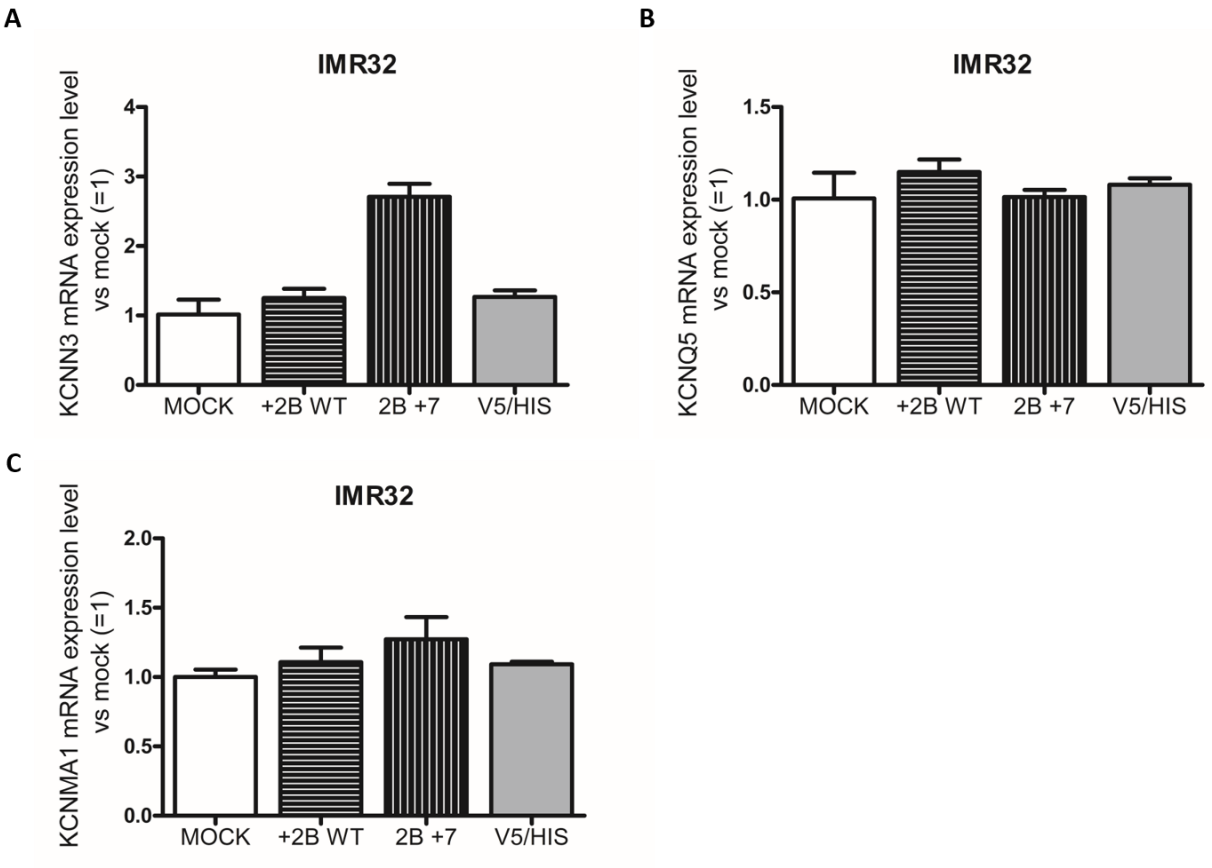


Fig.27 Mutant protein effect on ion channels genes expression in IMR32 cells. qPCR analysis of endogenous *KCNN3* (A), *KCNQ5* (B) and *KCNMA1* (C) mRNA expression normalised to that of the *GAPDH* gene in native IMR32 (white bar) transfected with *PHOX2B* WT and +7 V5/HIS (black hatched bar) and with empty vector V5/HIS as control (grey bar). The bars indicate the relative fold induction in cells transfected with respect to the not transfected cells (MOCK =1) calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as the mean values ± SD (error bars) of at least one experiment, performed in triplicate.

We recently published that PHOX2A can interact with mutated version of PHOX2B (Di Lascio et al. 2016), rescuing the impaired transcriptional activity of mutant proteins. Although additional study will be needed to further evaluate the significance of this finding, we can speculate that the effect of mutant proteins on the expression of ion channels genes might be mediated by the interaction between the two proteins. Furthermore, we showed that potassium channels genes are co-regulated by PHOX2B and PHOX2A, and that *PHOX2A* is up-regulated in *PHOX2B* A6.4 K.O cells. All together these findings led us to hypothesize that PHOX2A may play a role in the up-regulation of ion channels genes. We therefore investigated the effect of PHOX2A on ion channel expression in IMR32 cell line transiently transfected with *PHOX2A* expression

vector (Benfante et al., 2007) and with an empty vector (pCMV-myc) as control. *PHOX2A* overexpression affects the expression of four ion channels: *KCNQ5* and *SCN9A* expression level is significantly reduced, whereas *SCN3A* and *KCCN3* genes are significantly up-regulated, with respect to cells transfected with the empty vector (Fig.28). In particular, *KCNN3* expression level is 1.6-fold significantly higher than in control cells (FIG.26 white bar).

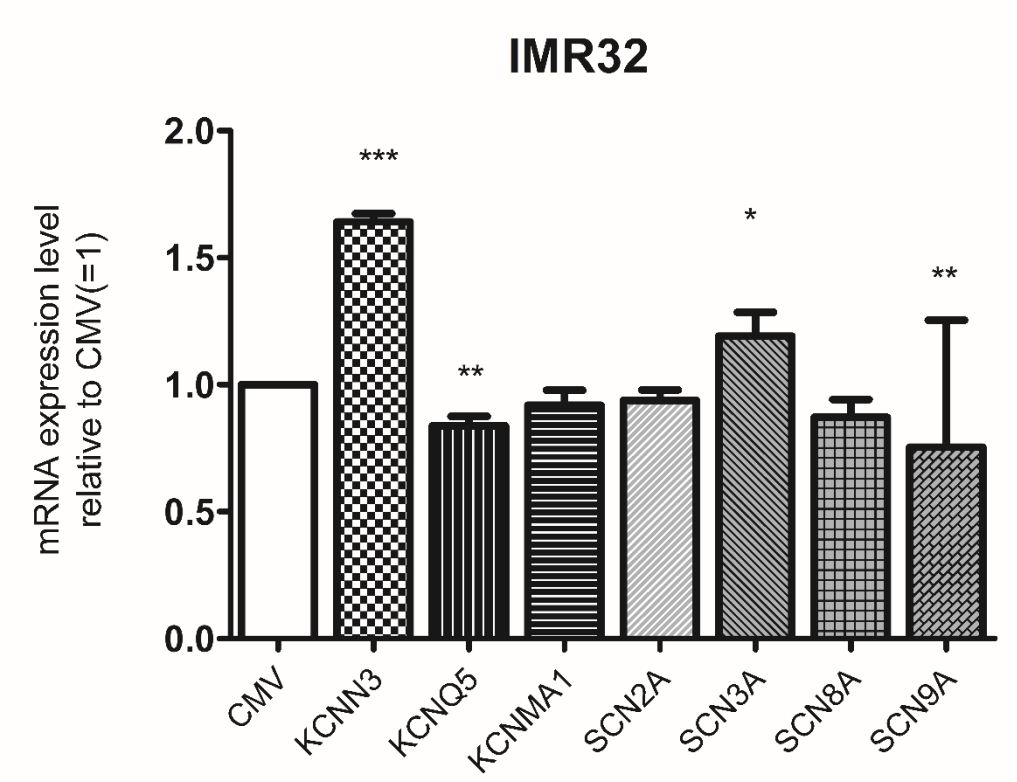


Fig.28 *PHOX2A* over-expression affects ion channels genes expression. qPCR analysis of endogenous ion channels mRNA expression normalised to that of the *GAPDH* gene in native IMR32 transfected with *PHOX2A* expressing vector and with empty vector pCMV-myc as control (white bar). The bars indicate the relative fold induction in cells transfected in relation to cells transfected with the empty vector (CMV=1) calculated using the $2^{-\Delta\Delta Ct}$ method and are expressed as the mean values ± SD (error bars) of at least three experiments, performed in triplicate. ***p < 0.001, **p < 0.01 and *p < 0.05, significant differences in KCNN3, KCNQ5, SCN3A and SCN9A mRNA levels between IMR32 cells transfected with PHOX2A expressing vector and control (one-way ANOVA, Tukey's test).

In conclusion, our data demonstrate that some of the ion channels we investigated are PHOX2B target genes, which negatively modulates their expression. In particular, we showed that the *KCNN3* channel gene expression is highly affected by the presence of mutant proteins (both in native IMR32 cell line and in *PHOX2B* K.O cells) and by the up-regulation of *PHOX2A* in native

cells, suggesting that it is a potential therapeutic target for the treatment of respiratory dysfunction. Further investigations are however mandatory in order to answer the following questions:

- Are the different expression levels of ion channel genes in clones A6.4 due to the absence of PHOX2B and not to morphological differentiation-associated changes?
- Is the effect of the mutant proteins on the expression of *KCNN3* due to the formation of hetero-dimer between PHOX2A and PHOX2B mutant protein?

To answer these questions the same experiments will be repeated in the other PHOX2B depleted clones and in IMR32 cells transiently silenced for *PHOX2B*. Furthermore, silencing of *PHOX2A* in the A6.4 clone may give some insights in the mechanism leading to the up-regulated expression of *KCNN3* by the mutant proteins**.**

Electrophysiology study on *PHOX2B* knockout cells

Ion channels and in particular, potassium channels are the primary determinants of neuronal cell excitability and several findings suggest that are involved in neuronal development and the modulation of respiratory activity *in vivo* (Bond at al., 2000; Torrecilla et al., 2013; Mulkey et al., 2015).

We identified ion channels as PHOX2B target genes and showed that the presence of mutations in PHOX2B may lead to the de-regulation of their expression. Due to the role of the K^+ and Na^+ ions in the action potential generation and in the restoring of the resting membrane potential, we asked whether deregulated ion channels expression might impair the electrophysiological properties of neuronal cells. In collaboration with Dr. Flavia Antonucci (University of Milan) we measured total ion currents induced by injection of hyper-to-depolarizing currents in *PHOX2B* knockout cells (clone A6.4) and IMR32 native cells.

Whole-cell current clamp experimental paradigm was used, and repeated pulses of current starting from the resting membrane potential of the cell were injected. Preliminary data showed important differences between the two cell types when a series of hyperpolarizing currents ranging from 120 pA to 240 pA (20 pA/step, 30 sweep) were injected (Fig.29).

Indeed, the degree of hyperpolarization, in the presence of comparable current injection, appears larger in clone with respect to the control (point 1-green), thus suggesting unbalanced expression of Na^+ and K^+ channels at the membrane. Accordingly, the occurrence of a more prominent voltage sag (point 2-light blue) in CRISPR cells suggest an altered presence of hyperpolarization-activated cation channels (I_h), which are a subset of voltage-gated channels permeable to both Na^+ and K^+ ions known to be crucial for the determination of intrinsic excitability. Moreover, after hyperpolarizing current pulse only rebound depolarization can be found in *PHOX2B* KO cells in contrast to the action potential recorded in normal cultures (point 3-red) indicating that the membrane potential of A6.4 clone cells is more hyperpolarized than control cells thus limiting their response stimulation.

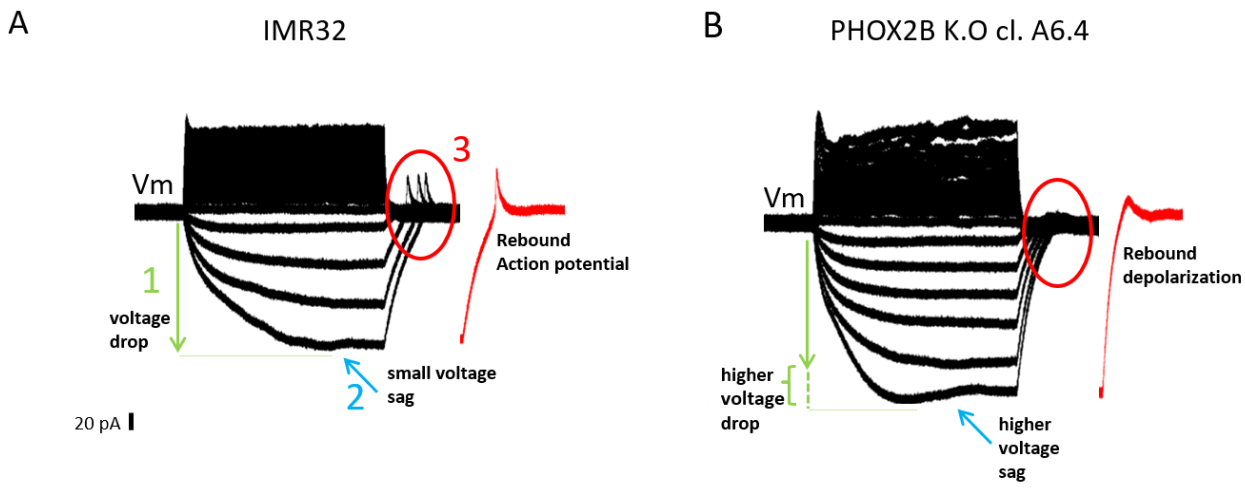


Fig.29 Total ion current in IMR32 cells and *PHOX2B* K.O cells. Whole-cell current clamp was used to determine electrophysiological properties of IMR32 and CRISPR cells by repeatedly injecting pulse of current starting from the resting membrane potential of the cell. *PHOX2B* K.O cells reported only rebounded depolarization (point red), higher voltage drop (green arrow) and higher voltage sag (blue arrow) in comparison with IMR32 cells

Although additional study will be needed to further evaluate the significance of this electrophysiological changes, we can speculate that changing in ion channels mRNA expression induce aberrant electrophysiological property. It will be interesting to study how the mutated protein alters the electrical properties of the cell and by using selective blockers for sodium and potassium currents we will be able to understand which of the two currents are most dysregulated.

Discussion

CCHS is a rare neonatal lifelong and life-threatening disorder. It is characterized by hypoventilation and ANS dysregulation. The hallmark of CCHS is reduced or short and shallow breathing due to dysregulation of the respiratory drive especially evident during NREM sleep. The inability of CCHS patients to control breathing results in the need for life-long ventilatory support during sleep; in the most severe cases, also in the daytime.

In 2003 *PHOX2B* gene was identified as the disease-causing gene; *PHOX2B* gene encodes for a transcriptional factor required for the development of neurons that regulate the cardiovascular, respiratory and digestive organs, forming the sensory and motor arms of visceral reflex circuits (Amiel et al., 2003).

The laboratory, in which I carried out the doctoral thesis, has been studying for years the transcription factor PHOX2B, with particular attention to the molecular mechanisms that link the mutations of this gene to the pathogenesis of CCHS with the perspective to identify a possible pharmacological treatment. Indeed, up today, there is no cure for CCHS.

The aim of this thesis was to identify potential pharmacological targets for the development of molecules that can be chronically administered to CCHS patients in order to improve their respiratory symptoms and the quality of life. We have addressed this problem with two different experimental approaches:

- starting from a clinical report (Straus et al., 2010) we investigated the molecular mechanisms underlying the recovery of chemosensitivity, observed in two CCHS patients, following the administration of the progestinic desogestrel, to identify potential pharmacological targets for the development of alternative molecules lacking contraceptive activity that can be administered to both female and male patients;

- Using a genome-wide approach, (ChIP-seq) we identified 3000 candidate PHOX2B target genes. We discovered that the expression of some of them is dysregulated when PHOX2B is mutated and they could be responsible for the clinical symptoms of the disease. These findings open up the possibility that these new PHOX2B target genes could be new pharmacological targets in the perspective of rescuing their normal activity.

I discuss these two parts of my thesis in separate sections.

The progestinic desogestrel down-regulates PHOX2B: new therapeutic strategy in CCHS

In 2010, a new pharmacological perspective emerged from the observation of two clinical cases of particular interest. Straus and collaborators published a work in which they reported of two female CCHS patients, of different ages, carrying different poly-alanine expansions in the *PHOX2B* gene (+6 and +5, respectively), which showed a marked recovery of the hypercapnia response following chronic progestogen intake desogestrel, for contraceptive purposes.

The interest in the possibility of a pharmacological intervention able to improve the clinical condition of CCHS patients has increased especially after the emerging evidence that sites, responsible of central chemoreception in central nervous system, may be partially preserved in CCHS patients. This observation, in accordance with the known ability of the progestins to stimulate the ventilation (Boukari et al., 2017; Shahar et al., 2003), has led to the hypothesis that there was a link between the intake of the progestin and the recovery of chemo sensitivity in these patients (Straus et al. 2010).

Recent experiments conducted in adult mice claimed that several neurones (such as those located in medullary and supramedullary areas of the CNS) are involved in the effect mediated by desogestrel (Joubert et al., 2016; Loiseau et al., 2014).

Our initial working hypothesis was that the progestin desogestrel could act directly on the transcription factor PHOX2B influencing its expression and consequently the expression of its target genes. The effect of desogestrel on the expression of *PHOX2B* was tested *in vitro* in the human neuroblastoma SK-N-BE(2)C cell line. As described in our published work “Desogestrel down-regulates PHOX2B and its target genes in progesterone responsive neuroblastoma cells” (Cardani et al., 2018), we demonstrated the existence of a direct link between the progestin desogestrel and PHOX2B, as 3-KDG, active metabolite of the desogestrel, reduces the expression of PHOX2B and its target genes, by means of a post-transcriptional mechanism. Moreover, 3-KDG treatment was also able to reduce the level of PHOX2B +7 Alanine expanded protein, that is one of the most common mutations found in CCHS patients.

The polyalanine expanded PHOX2B proteins show a reduced ability to transactivate both its own promoter and that of target genes, in a promoter-specific manner (Di Lascio et al., 2013). The

length of the expanded stretch is inversely proportional to the function of the protein. Furthermore, mutant proteins reduce the activity of WT protein, by a dominant-negative mechanism, and moreover, they acquire a toxic function with the formation of both nuclear and cytoplasmic aggregates, that partially sequester the normal protein, limiting its physiological activity (Di Zanni et al., 2012). Other mechanisms involving squelching of co-factors, PHOX2B decreased binding affinity for its consensus sequences, or defect in protein folding have been hypothesized (Di Lascio et al., 2013; Trochet et al., 2005; Bachetti et al., 2005; Durand et al., 2005; Goridis et al., 2010; Dubreuil et al., 2008). These data led to investigate the pathway linked to aggregates removal and /or protein refolding as possible druggable targets) (Bachetti et al., 2007; Parodi et al., 2012).

Since CCHS is a pathology that occurs in heterozygosis, the search for a therapy can follow two major approaches: by developing drugs that can reduce the pathological effect of the mutated forms of PHOX2B or by identifying molecules capable of significantly enhancing the physiological activity of the WT protein in order to compensate the toxic effect of mutated forms. So far, most of the published studies aimed to the identification of molecules able to rescue the activity of the mutant protein or to eliminate it, without considering the effects that these treatments have on the WT protein (Di Zanni et al., 2012; Parodi et al., 2012; Bachetti et al., 2007).

Our data support the hypothesis that beneficial treatment of breathing defects has to go through the reduction of the mutant protein, although this will affect also the WT protein. The exact role of PHOX2B in adults has not been clarified yet; however, it has been shown to be important for maintaining the functionality of noradrenergic neurons and control orofacial movement, auditory and eyes function (Fan et al., 2011; Kang et al., 2007).

We are aware that the SK-N-BE(2)C cell model used in this study has important limitations as it does not properly represent the human pathophysiology. The difference in the kinetics of PHOX2B reduction in the two SK-N-BE(2)C over-expressing hPGR clones may be due to the different levels of expression of PGR-B compared to PGR-A isoform. The exact contribution of the two isoforms of progesterone receptor, PGR-B and PGR-A, in mediating the mechanism of action of the 3-KDG is still unknown and further investigation will be needed to better understand it. Moreover, we do not expect that *in vivo* the progestin desogestrel reduces the expression of PHOX2B in such a drastic manner; we can hypothesize that in CCHS patients the

reduction of WT and mutant PHOX2B protein is mild but could be sufficient to counteract the toxic effects of the mutant protein contributing to the recovered chemosensitivity and increased ventilation observed in the two CCHS patients, without impairing totally the function of the WT protein. The effect of desogestrel on PHOX2B target genes corroborate the importance of this finding as we can hypothesize that other genes, whose expression is deregulated because of the toxic effect of the mutant protein, may now be expressed at level compatible with a quasi-normal activity of neurons affected by PHOX2B mutations, in particular in those patients carrying mutant PHOX2B with milder phenotype (+5 to +7 Alanine).

This is in agreement with the two patients reported in the clinical study by Straus and colleagues carrying PHOX2B +5 and +6 Alanine mutations, that showed a mild phenotype. Recently the same authors reported of another patient not responding to desogestrel (Loiseau et al. 2017). Therefore, we can speculate that any possible difference in clinical response to pharmacological treatment might be related to the presence of different mutations. In the presence of mutant proteins with shorter expansion (e.g +5 and +6 Alanine) the damages, at the level of $PHOX2B^{+}$ neurons, could be less serious than the damages that occurred in the presence of mutant proteins with longer expansion (e.g. +13 Alanine). In this last case, the reduction of the protein level could be not sufficient to restore, at least partially, the function of those neuronal structures affected by mutation and involved in breathing control.

We can speculate that the clinical observation made by Straus and co. is given by a general effect of desogestrel that can act through both $PHOX2B^{+}$ and $PHOX2B^{-}$ expressing neuronal circuits. Our data, by showing for the first time the presence of a direct link between desogestrel and PHOX2B, have a proof of concept value that, although CCHS is a neurodevelopmental disorder, by modifying the activity of PHOX2B and its mutant counterparts, it is possible to conceive new pharmacological interventions with benefits for CCHS patients and their families and thus leading to a better quality of life. Studies have been conducted with the aim to find out molecules capable of reducing *PHOX2B* expression in neuroblastoma cell lines (Di Zanni et al., 2015).

In this contest, the identification of new and sensitive intracellular therapeutic targets has to take into an account the role of PHOX2B target genes, whose de-regulation may explain the molecular pathogenic mechanisms underlying CCHS, but they can also represent druggable

targets to by-pass PHOX2B mutations.

Ion channels are PHOX2B target genes: new possible pharmacological targets

The hallmark of CCHS is the presence of hypoventilation. However, as PHOX2B is important for the development of the ANS, CCHS patients present a more generalized autonomic dysfunction (reviewed in Weese-Mayer, 2017). The cardiovascular system is affected; CCHS patients with PARM mutation reported also cardiac asystoles and some of them need cardiac pacemaker implantation, even at a young age. In addition, other cardiovascular symptoms can include altered temperature regulation, altered blood pressure regulation, altered heart rate variability and poor circulation. Gastrointestinal manifestations are common. The symptoms are present in both PARM and NPARM CCHS patients and can vary from a mild reflux and poor upper gastrointestinal motility to a more severe HSCR that need surgical treatment. In some cases, CCHS patients present ophthalmological problems like strabismus, abnormal pupil dilation, Marcus Gunn jaw-winking syndrome and absent or reduced depth perception. PHOX2B mutations can also affect the endocrine system with growth hormone deficiency and congenital hyperinsulinemia. Other patients are affected by neurological disorders such as neurocognitive impairment and poor school performance, epilepsy episodes and moreover, they can develop tumours of neural crest origin, such as ganglioneuromas, ganglioneuroblastomas, and NB.

We hypothesized that CCHS is the results of the abnormal expression of PHOX2B target genes due to the presence of PHOX2B mutation that lead a general transcription dysregulation. This transcriptional defect could be due to a reduction or lack of expression of target genes positively regulated, or to an increase in the expression of those genes negatively regulated by PHOX2B. Besides the difference in the behaviour and function of different PHOX2B mutant proteins, the severity of the disease could be linked to the importance and number of dysregulated target genes, as we have demonstrated that different mutations affect the expression of target genes in a promoter-specific manner (Di Lascio et al., 2013). In this perspective, the identification of the PHOX2B target genes is crucial for a future therapeutic intervention in CCHS, capable of restoring the correct level of expression of these targets by-

passing PHOX2B mutations.

ChIP using anti-PHOX2B antibodies, previously generated and characterized in our laboratory, followed by a genome wide sequence, has allowed us to identify more than 3000 genes, possible candidate PHOX2B target genes. Recently, two independent groups published the results of ChIP-seq analyses carried out with PHOX2B antibodies in different neuroblastoma cell lines (Boeva et al., 2017; Durbin et al., 2018). The results match with and reinforced our findings.

Very interesting, among the newly identify PHOX2B target genes, we found genes encoding ion channels, including those selective for potassium and sodium channels. Potassium channels are a huge and ubiquitous family of membrane proteins that play important roles in vital cellular signalling processes and are expressed in both excitable and non-excitable cells. They are involved in processes that regulate neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction. Moreover, it has been reported their involvement in the maintenance of respiratory rhythm and central chemosensitivity in isolated brainstem-spinal cord preparations of rat neonates (Okada et al., 2005). Mutations in K^+ channels and or altered regulation of their function are associated with various disorders, such as diseases of the heart, kidney, pancreas, and CNS. On the other hand, Na^+ channels are responsible for the generation and propagation of action potentials and they have been shown to play a central role in the genesis of epilepsy.

Since CCHS patients presented respiratory problem, cardiac alteration and epilepsy and, both potassium and sodium channels are involved in these symptoms, we investigated whether and how they could be de-regulated in CCHS. An altered expression could be responsible and explain an important part of the symptoms manifested by CCHS patients. In particular, KCNN3, KCNQ5, KCNMA1 and SCN2A, 3A, 8A and 9A channels are of particular interest since they have been demonstrated to be involved in respiratory and cardiac problem and in the onset of epileptic episodes (Mulkey et al., 2015; Mahida et al., 2014; Catterall 2012).

To demonstrate that the genes, identified for the property of their genome sequences to be bound by PHOX2B, were also regulated by PHOX2B, we took the advantage of an IMR32 cell clone (A6.4) in which we knocked-out *PHOX2B* gene expression by CRISPR/Cas9 editing technology. It is really interesting underlying that this clone expresses the *PHOX2B* transcript at normal level, whereas the protein is completely absent. Usually mRNAs encoding

premature termination variants, like clone A6.4, are degraded by nonsense-mediated RNA decay (NMD) mechanism. NMD is a post-transcriptional quality control that targets mRNAs harbouring a premature termination codon to the degradation pathway thus preventing the formation of truncated protein with potentially toxic effect (Juffrey and Wilkinson 2018). Moreover, this mechanism regulates a substantial number of protein-coding transcripts directly and indirectly and recently it has been reported as an important modulator of neural development and disease (Mendell et al., 2004; Juffrey and Wilkinson 2018). We can hypothesise that the PHOX2B mRNA is able to escape degradation by the NMD pathway. Indeed, there are no reports indicating that *PHOX2B* mRNA is targeted by NMD; moreover, it has been already demonstrated that mRNAs encoding PHOX2B bearing nonsense mutations are capable of escaping NMD thus supporting our hypothesis (Cain et al., 2017; Trochet et al., 2009). On the other hand, we have recently demonstrated that the kinetics of *PHOX2B* mRNA degradation is biphasic (Cardani et al., 2018), similar to the process described for those mRNAs regulated by NMD (Trcek et al., 2013) thus suggesting that only a portion of *PHOX2B* mRNA may be degraded by NMD. This aspect deserves further investigation.

Clone A6.4, although missing *PHOX2B* expression, still maintains the features of noradrenergic neurons, but with the characteristics of a more mature stage of neuronal differentiation. This has been demonstrated by the increased expression in the neuronal marker β3 tubulin and the vesicular secretory markers synaptotagmin and syntaxin, by the lack of expression of *SOX10*, a marker of the glial lineage, and by the reduction in ChAT protein expression. This is a marker of the cholinergic lineage that can be co-expressed during development of the sympatho-adrenal neurons until the colinergic-cathecolaminergic switch has been completed. This data is also confirmed by the increase in *PHOX2A* expression level compared to native IMR32 cells and the maintenance of DBH. The up-regulation of *PHOX2A* was unexpected since *PHOX2A* is positively regulated by PHOX2B. Both *DBH* and *TLX2* are PHOX2B and PHOX2A target genes and their expression was not affected by the absence of PHOX2B protein. Murine models in which the two genes were alternatively expressed in the each other locus, demonstrated that *Phox2b* and *Phox2a* genes are not functionally equivalent and Phox2a is not able to compensate for the absence of Phox2b (Coppola et al., 2005). However, from our data we can suppose that in the A6.4 clone the overexpression of *PHOX2A* is due to a compensatory mechanism, leading to the maintenance of *DBH* and *TLX2* expression level. Our data are in line with those recently published by Boeva et al. (2017), showing that the

silencing of *PHOX2B* does not affect the expression of catecholaminergic marker such as *DBH* (Boeva et al., 2017).

The observed changings suggest that clone A6.4 recapitulates the differentiation process observed during development. It has been demonstrated that the reduction of expression of *PHOX2B* is necessary in a specific time window to allow the exit from the cell cycle of the neuronal precursors, and their terminally differentiation to sympathetic neurons (Paris et al., 2006; Dubreuil et al., 2000). Thus, we cannot exclude that any variation observed in the expression levels of the putative target genes is due to the lack of PHOX2B alone or to the differentiation process. However, due to the maintenance of its general noradrenergic phenotype, the A6.4 clone still represents a good model for an initial validation screening of the newly identified PHOX2B target genes.

Using *PHOX2B* KO stable clone A6.4, we found that PHOX2B negatively modulates the expression of K^+ and Na^+ channels, as, in the absence of PHOX2B protein expression, the mRNA level of *KCNN3, KCNQ5* and *SCN2A* is increased. Moreover, re-expression of PHOX2B mutant protein bearing +7 and +13 Alanine expansion has demonstrated, for the first time, that PARM mutations can confer new enhanced PHOX2B mutant protein activity on the transcriptional regulation of *KCNN3* channel, as its expression is further increased in the presence of the mutant proteins. In addition, when tested in a cellular model expressing PHOX2B WT protein, the gain of function activity acquired by the mutant protein has a dominant-negative effect on the transcriptional activity of the wild-type protein, thus maintaining the expression of *KCNN3* higher compared to not transfected cells.

We recently published (Di Lascio et al., 2016) that the dominant-negative effect of mutant proteins on PHOX2B WT activity is not due to direct interaction of the mutant proteins with their WT counterpart, but other mechanisms involving squelching of co-activators or aberrant interactions with other regulatory proteins can be supposed. PHOX2A heterodimerizes with PHOX2B and several genes are co-regulated by the two proteins. Our findings (Di Lascio et al., 2016) demonstrated that PHOX2A is capable of interacting with PHOX2B mutant proteins, thus rescuing their impaired transcriptional activity. Moreover, preliminary data show that the overexpression of *PHOX2A* in neuroblastoma IMR32 cell line induces the expression of *KCNN3*. These results support the idea that PHOX2A may be involved in the up-regulation of *KCNN3* expression by the PHOX2B mutant proteins, a mechanism that deserves further

investigation.

The increased expression of ion channels is associated with an altered electrical activity of the cell clone. Data from the literature (Mulkey et al., 2015; Mahida et al., 2014; Catterall 2012) support the hypothesis that CCHS may be due to de-regulation of ion channels expression and/or activity, a mechanism that could explain some respiratory and non-respiratory symptoms. The basic rhythm of breathing is generated within the pre-BötC and the RTN/pFRG. This brainstem respiratory network receives information about the arterial levels of pO_2, pCO_2/pH, and pH from chemoreceptors and adjusts respiratory motor output, ensuring appropriate ventilation of the lungs in various conditions. In mammals, respiratory inputs originate primarily from the receptors in the CB and from the central chemoreceptors in the brainstem especially from neurons of RTN and bulbar serotonergic neurons of the raphe nuclei (Guyenet et al., 2008; Corcoran et al., 2009). These two structures are critically important for maintaining breathing and it has been demonstrated that some of the ion channels regulated by PHOX2B are expressed in populations of neurons in these structures.

Recent evidence showed that some voltage gated K^+ channel of the KCNQ family are expressed in the RTN and they had key roles in the determination of RTN chemoreceptor function (Mulkey et al., 2015). The authors reported that they are essential determinants of spontaneous activity in RTN, and downstream effectors for serotonergic modulation of breathing, in a murine model. Moreover, loss of function mutations in *KCNQ* channels can cause epilepsy.

The small-conductance Ca^{2+}-activated potassium channel, KCNN3 or SK3, is responsible for the slow after-hyperpolarization that usually follows an action potential. This protein is widely expressed in the murine brain (Bond et al., 2000), including hippocampal formation, striatum, in subsets of neocortical neurons, thalamus, cerebellum, brain stem and, very interesting, in neuronal populations of the medulla oblongata, which are involved in the processing of respiratory signals. SK3 is also express in skeletal muscle and in cardiomyocytes. Recently it has been demonstrated that overexpression of the SK3 channel is associated with high incidence of sudden death and increases susceptibility to cardiac arrhythmic syndromes, including heart block and bradyarrhythmias, slowed ventricular conduction, and respiratory problems (Mahida et al., 2014).

Voltage-gated Na^+ channels (SCN1A, 2A, 3A and 8A) are the primary sodium channels in the CNS. In mice, NaV1.2 channel is primary localized in unmyelinated or pre-myelinated axons and

dendrites and participates in the generation of both axonal and somatodendritic action potential. It is well established that mutations that cause alteration in sodium channels activity are responsible for genetic epilepsy syndromes with a wide range of severity (Catterall 2012).

All these finding support our hypothesis that transcriptional dysregulation and dysfunctions of K^+ and Na^+ channels activity may contribute to the onset of respiratory and cardiac problems and epilepsy episodes associated with CCHS and suggest that these proteins are promising potential target for a therapeutic intervention in CCHS. Due to the important role that these ion channels have on neuronal physiology, restoring their expression and/ or activity to the normal physiological level can by-pass PHOX2B mutant effects, leading to amelioration of CCHS symptoms. Moreover, several drugs targeting these proteins are already used in clinics, thus prompting the idea that the potential progress toward a therapeutic intervention to treat CCHS is today more than concrete.

Limitation

The results presented in this thesis are subject to a number of limitations.

The validation of PHOX2B target genes was performed only in one stable clone. Due to observed progression of the clonal cells towards a more differentiated phenotype, the obtained data will be confirmed in the other *PHOX2B* knocked-out clones. A further confirmation will be obtained by transient silencing the expression of *PHOX2B* by siRNA oligonucleotides, in IMR32 cells.

One of the biases of the CRISPR/Cas9 editing technology is the off-targeting effect, a mechanism by which the gRNA induces the Cas9 to cut in a not specific manner at the level of other sequences different from those of the gene that has to be edited. This is why it will be crucial to rescue the expression of *PHOX2B* in the clones to assess whether the de-regulated expression is indeed due to the missed expression of *PHOX2B*.

The *PHOX2B* knocked-out cell model and the *PGR* overexpressing cells provide several new information about molecular defect induced by the mutated protein on newly identified PHOX2B target genes, and the possible mechanisms sustaining the pharmacological response

to desogestrel. However, they provide a limited representation of human pathophysiology, being neuroblastoma cell lines, where the expression of *PGR* has been forced or the expression of *PHOX2B* completely ablated, thus not resembling the *in vivo* situation. To limit this bias, in the future, we will validate our data from this *in vitro* model in dissected rat hindbrains that have been sham-treated or treated with etonogestrel.

Conclusion and perspectives

Taken together, the results of my thesis support the hypothesis that CCHS is caused by a general transcription dysregulation. For the first time we provide the evidence that ion channels are PHOX2B and PHOX2A target genes and their expression may be dysregulated by the presence of PHOX2B mutant protein. I*n vivo*, more than one mechanism is involved in CCHS pathogenesis, including loss-of-function and gain of function mechanisms. We reported the evidence that toxic functions acquired by mutant proteins could contribute to inducing aberrant expression of ion channels that can impair the restoring of the basal membrane potential after action potential, thus making neurons in the structure involved in breathing control less responsive to stimuli, such as the increased level of pCO_2. In this light, it will important to study and better characterize the molecular mechanism underlying the new functions gained by the mutant proteins, in the perspective to develop pharmacological molecules able to counteract their toxic effect and rescue the normal activity of PHOX2B target genes. Our recent data on the progestin desogestrel strongly supported the idea that reduction of PHOX2B mutant protein can ameliorate CCHS symptoms. However, it is worth noting that drugs that act on mutant proteins also affect the expression of PHOX2B WT protein. An alternative pharmacological strategy, to by-pass the effect on PHOX2B, will be to target directly the de-regulated PHOX2B regulated genes, in the perspective of rescuing their activity.

Our data open up a number of questions that need to be addressed.

First, to evaluate whether deregulated ion channels, because of PHOX2B mutations, impaired the electrophysiological properties of neuronal cells, their activity will be tested in control and *PHOX2B* +7 Alanine expressing IMR32 cells and measure the total ionic currents in the

presence or not of voltage-gated Na^+ and K^+ blockers. An *in vitro* screening of compounds able to restore the expression and/or activity of deregulated ion channels will be performed.

Second, we will explore the possibility that the desogestrel effect observed in two CCHS patients is mediated by the modulation of ion channels expression. Decreasing both mutant and WT PHOX2B may result in the restoration of a balanced expression of otherwise de-regulated PHOX2B target genes.

Third, very little is known about the role of PHOX2A in CCHS pathogenesis, and its possible contribution to the toxic effect of mutant proteins on the expression of PHOX2B target genes. Studies on the transcriptional regulation of *PHOX2B* by the overexpressed PHOX2A/ PHOX2B +7 Alanine hetero-dimer in IMR32 cells and/or silencing of *PHOX2A* in the A6.4 clone will be performed. These findings might suggest novel strategies, targeting PHOX2A, for therapeutic intervention for this complex disease.

Four, as the CCHS murine model die at born, and the neuroblastoma cell lines so far used do not recapitulate all the possible defects of neurons affected by PHOX2B mutations, we are setting up a new cellular model of CCHS based on induced pluripotent stem cells (iPS) technology, obtained by reprogramming fibroblast from CCHS patients and control skin biopsy to generate neurons and neuronal crest cells derivatives. The iPS will be differentiated into different types of neurons, to reveal possible defects during development, induced by the presence of PHOX2B mutations. The CCHS- and control-derived neurons will be used to further validate PHOX2B target genes, including the identified ion channels, and to screen molecules that can revert the possible developmental defects.

Acknowledgment

I am grateful to all the people that contribute to the present work.

In the first place, I would like to thank my tutor Prof. Diego Fornasari and my co-tutor Dr. Roberta Benfante for giving me the opportunity to do my PhD thesis within their group. This gave me the possibility to work on a topic of great interest to me.

I would like to express my gratitude to Dr. Simona Di Lascio for guidance and support during my PhD period.

I would also acknowledge the contribution of the past laboratory members who have worked on the project, Dr. Debora Belperio, Dr. Valeria Corlianò, Dr. Erika Di Biase.

Finally, I would like to thank Dr. Patrizia Rosa and Flavia Antonucci for their precious collaboration.

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