

The E3 ubiquitin ligase TRIM11 mediates the degradation of congenital central hypoventilation syndrome-associated polyalanine-expanded PHOX2B

Sara Parodi · Eleonora Di Zanni · Simona Di Lascio · Paola Bocca · Ignazia Prigione · Diego Fornasari · Maria Pennuto · Tiziana Bachetti · Isabella Ceccherini

Received: 18 May 2011 / Revised: 15 December 2011 / Accepted: 10 January 2012 / Published online: 4 February 2012
© Springer-Verlag 2012

Abstract Expansions of a polyalanine (polyA) stretch in the coding region of the *PHOX2B* gene cause congenital central hypoventilation syndrome (CCHS), a neurocristopathy characterized by the absence of adequate control of autonomic breathing. Expansion of polyA in *PHOX2B* leads to protein misfolding and accumulation into inclusions. The mechanisms that regulate mutant protein degradation and turnover have been poorly elucidated. Here, we investigate the regulation of degradation of wild-type and polyA-expanded *PHOX2B*. We show that expanded *PHOX2B* is targeted for degradation through the ubiquitin–proteasome system, resulting in lowered levels of the mutant protein relative to its wild-type counterpart. Moreover, we show that mutant *PHOX2B* forms ubiquitin-positive inclusions, which sequester wild-type *PHOX2B*. This sequestration correlates with reduced transcriptional activity of endogenous wild-type protein in neuroblastoma cells. Finally, we show that the E3 ubiquitin ligase TRIM11 plays a critical role in the clearance of mutant *PHOX2B*

through the proteasome. Importantly, clearance of mutant *PHOX2B* by TRIM11 correlates with a rescue of *PHOX2B* transcriptional activity. We propose that CCHS is partially caused by a dominant-negative effect of expanded *PHOX2B* due to the retention of the wild-type protein in pathogenic aggregates. Our results demonstrate that TRIM11 is a novel modifier of mutant *PHOX2B* toxicity and represents a potential therapeutic target for CCHS.

Keywords *PHOX2B* · Congenital central hypoventilation syndrome · Polyalanine expansion · Insoluble fraction · Aggregation · E3 ubiquitin ligase · Ubiquitin–proteasome system

Introduction

Expansion of trinucleotide repeats in the coding region of a variety of genes results in the expression of proteins containing expanded amino acid repeats, which cause neurogenetic diseases by unknown mechanisms [1]. Expansion of CAG repeats encoding polyglutamine (polyQ) tracts is the genetic basis of at least nine neurodegenerative disorders, known as polyQ diseases [1]. Expansion of GCN repeats encoding polyalanine (polyA) tracts are responsible for at least nine genetic disorders, including synpolydactyly syndrome, hand–foot–genital syndrome, cleidocranial dysplasia, holoprosencephaly, X-linked mental retardation and growth hormone deficit, X-linked mental retardation and abnormal genitalia, oculopharyngeal muscular dystrophy, blepharophimosis–ptosis–epicanthus inversus syndrome, and congenital central hypoventilation syndrome (CCHS) [2–5]. CCHS, also known as Ondine’s curse, is a life-threatening disorder that primarily manifests as sleep-

S. Parodi · E. Di Zanni · T. Bachetti · I. Ceccherini (✉)
Laboratory of Molecular Genetics, G. Gaslini Institute,
16148 Genoa, Italy
e-mail: isa.c@unige.it

S. Parodi · M. Pennuto
Department of Neuroscience and Brain Technologies,
Italian Institute of Technology,
Genoa, Italy

S. Di Lascio · D. Fornasari
Department of Pharmacology, School of Medicine,
University of Milan and CNR—Institute of Neuroscience,
Milan, Italy

P. Bocca · I. Prigione
Laboratory of Oncology, G. Gaslini Institute,
Genoa, Italy

associated respiratory insufficiency and marked impairment of ventilatory responses to hypercarbia and hypoxemia [6].

CCHS is caused by heterozygous in-frame duplications within the gene encoding the paired-like homeobox 2B (PHOX2B) transcription factor. In normal individuals, the polyA tract is 20 alanines in length, whereas in affected individuals it ranges from 25 (+5) to 33 (+13) alanines [7–10]. Similar to polyQ diseases and other repeat expansion disorders, the severity of disease and age of onset correlate with the length of the expanded repeat [9, 10].

PHOX2B is a paired box homeodomain transcription factor that plays a critical role in the development of autonomic nervous system reflex circuits in mice. Mice in which *Phox2b* has been deleted die in utero and are characterized by absent autonomic nervous system circuits due to dysfunction or loss of autonomic nervous system neurons, indicating that PHOX2B is essential during development [11]. Mice heterozygous for *Phox2b* deletion have only mild and transient breathing defects, which only marginally resemble the human disease caused by polyA expansion [12]. Importantly, mice expressing polyA-expanded PHOX2B present with respiratory problems, do not respond to hypercapnia, and die soon after birth from central apnea [13]. The observation that haploinsufficiency or complete loss of PHOX2B does not fully recapitulate the human condition caused by polyA expansion supports the idea that polyA expansion in PHOX2B either confers primarily a toxic gain of function to the mutant protein and/or exerts a dominant-negative effect.

Similar to polyQ diseases, pathogenic polyA expansions lead to protein misfolding, aberrant protein–protein interactions, protein degradation, mislocalization, and aggregation, which represent the cytopathological hallmark of polyA diseases. Expanded polyA leads to decreased transactivation of target genes, impaired DNA binding, extranuclear localization of PHOX2B, and aggregation, and the severity of such alterations correlate with the size of the repeat [14, 15]. Misfolded proteins are recognized by molecular chaperones of the heat shock protein (HSP) family, which bind to and promote protein refolding. When the chaperones' ability to repair misfolded proteins is exceeded, misfolded proteins are targeted for degradation mainly through the ubiquitin–proteasome system (UPS) and lysosome-mediated autophagy. To be recognized by the UPS, protein substrates are covalently modified with ubiquitin in a process that involves the consecutive activities of enzymes designated E1, E2, and E3, the last of which determines the substrate specificity. The E3 ubiquitin ligase tripartite motif-containing protein 11 (TRIM11) was recently shown to interact with wild-type PHOX2B [16]. However, it is unknown whether TRIM11 interacts with polyA-expanded PHOX2B and whether TRIM11 promotes its degradation.

In the present study, we investigate the role of the UPS and TRIM11 in polyA-expanded PHOX2B degradation. We show that the steady state level of polyA PHOX2B is altered compared to that of wild-type protein, and provide evidence that degradation of mutant PHOX2B is mediated by the UPS. Moreover, we demonstrate that expression of mutant protein alters the ability of endogenous wild-type PHOX2B to activate transcription in neuroblastoma cells. In addition, we demonstrate that TRIM11 overexpression leads to clearance of PHOX2B proteins and recovery of the transcriptional activity of expanded PHOX2B.

Materials and methods

Expression plasmids

PHOX2B

The generation of pcDNA3.1TOPO-wild-type PHOX2B, pcDNA3.1TOPO-DUP39 PHOX2B, pcDNA3.1/CT-GFP-TOPO[PHOX2B], pcDNA3.1/CT-GFP-TOPO[DUP39], and myc-tagged PHOX2B, have been described elsewhere [10].

To generate pcDNA3.1/myc-DUP39, a 270-bp region containing the expanded polyA stretch was isolated by PpuMI enzymatic digestion from pcDNA3.1TOPO-DUP39 and cloned into pcDNA3.1/myc-PHOX2B, after removing the corresponding region. All constructs were verified by DNA sequencing. To generate pcDNA3/HA-PHOX2B, the coding region of PHOX2B was cloned into the *EcoRI* site of pcDNA3-HA.

All constructs referred to as WT or DUP39 express wild-type and +13Ala mutant PHOX2B proteins, respectively, either alone or fused to different tags.

TRIM11

Total RNA was extracted from HeLa cells and full-length TRIM11 cDNA was obtained by PCR (primers: 5'-GAATT CATGGCCGCCCCGACCTGTCC-3' and 5'-GAATTCT CACTGGGGAGCCAGGGTGT-3'). The PCR product thus obtained was cloned into the *EcoRI* site of pcDNA3-HA.

Cell cultures and transfection

HeLa and IMR32 cells were grown in minimal essential medium (MEM, Euroclone) and RPMI (Euroclone), respectively, supplemented with 10% fetal bovine serum (FBS) (Gibco, New Zealand), 1% L-glutamine 100×, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO₂. Transfections were performed using FugeneHD Transfection Reagent (Roche).

Pharmacological treatments of transfected cells

Proteasome inhibitors lactacystin (Calbiochem) or MG132 (Sigma) were added 24 h after transfection, 12 h before harvesting. The autophagy inhibitor 3-methyladenine (3-MA; Sigma) was added to cells 4 h after transfection.

Analysis of PHOX2B protein levels

Transient transfections were performed by plating HeLa cells on 60-mm diameter dishes, using 1.5 μg of either pcDNA3.1/CT-GFP-TOPO[PHOX2B] or pcDNA3.1/CT-GFP-TOPO[DUP39] expression constructs. After 48 h, the mean fluorescence intensity (MFI) of 10,000 cells was measured by FACS analysis (FACSCalibur, BD Biosciences, Cell Quest software).

Total cellular lysates, SDS-PAGE, and Western blotting

Cells were washed in ice-cold PBS and lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 1 mM EDTA, 0.1% sodium dodecyl sulfate) plus protease inhibitor cocktail (Roche Diagnostics). The lysates were sonicated and denatured at 100°C in 1 \times Laemmli sample buffer (Bio-Rad).

Equal amounts of cellular lysates were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% non-fat dried skim milk in PBST solution for at least 1 h and incubated with the appropriate primary antibody overnight. Membranes were then rinsed three times with PBST for 10 min, incubated with secondary antibody conjugated with alkaline phosphatase for 1 h and washed three times with PBST. Signals were detected using chemiluminescent ECL Advanced (GE Healthcare). Protein levels in each sample were evaluated by comparison with the housekeeping proteins actin or tubulin. Quantification of band intensities was performed using ImageJ software.

Assessment of the half-life of myc-tagged WT and DUP39 PHOX2B proteins

Twenty-four hours after transfection, HeLa cells were chased with cycloheximide (10 $\mu\text{g}/\text{ml}$; Sigma). Protein levels were determined by collecting cells at different time points and performing immunoblotting as described above. Relative amounts of myc-tagged PHOX2B proteins were quantified using densitometry.

Ubiquitination assay

HeLa cells transiently co-transfected with mammalian expression plasmids for expanded or wild-type PHOX2B-myc

and HA-ubiquitin were treated with MG132 (10 μM , Sigma) 24 h post-transfection. Twelve hours later, cells were lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40) containing protease inhibitors (Roche Diagnostics). Cell lysates were clarified by centrifugation at 500–1,000 $\times g$ for 10 min, thus minimizing the chances of sedimentation of the expanded protein, and supernatants were then collected. We used the ExactaCruz system (Santa Cruz Biotechnology) to immunoprecipitate mutant and WT PHOX2B-myc: 500 μg of cell lysates were mixed with 1 μg of anti-myc antibody (Invitrogen) and incubated at 4°C overnight. Antigen–antibody complexes were collected by centrifugation, washed three times with lysis buffer and then boiled in Laemmli sample buffer (Bio-Rad) for 5 min to elute proteins. Proteins separated by SDS-PAGE were transferred to PVDF membranes (Millipore) and probed with anti-HA antibody (Invitrogen).

Fluorescence microscopy

Fluorescence analysis was performed in HeLa cells transfected with 750 ng of expression plasmid. Forty-eight hours later, cells were washed with PBS, fixed for 3 min with methanol–acetone mix, permeabilized for 15 min with 0.1% Triton-X-100/PBS and blocked for 5 min with 10% FBS/1% BSA/0.1% Tween20/PBS. Plates were then incubated with the appropriate primary antibody for 90 min, rinsed twice with PBS and incubated with TRITC-conjugated and/or FITC-conjugated secondary antibody (Santa Cruz Biotechnology) for 60 min. Plates were analyzed with a Zeiss Axiophot fluorescence microscope. Nuclei were stained with DAPI (Roche). Images were acquired by ACT-U software (Nikon).

The experiment shown in Fig. 3 was carried out using Alexa Fluor 488 anti-rabbit (Invitrogen) and DyLight 549 anti-mouse (Jackson ImmunoResearch) secondary antibodies. Imaging of fluorescent cells in Fig. 3 was performed with an LSM 510 Meta confocal microscope (Carl Zeiss, Inc.) using 40 \times (Nikon) Plan Apochromat lenses (1.4 NA).

Preparation of detergent-soluble and detergent-insoluble fractions

To analyze the distribution of proteins in the soluble and insoluble fractions, cultured cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100) containing protease inhibitors. Lysates were centrifuged at 14,000 $\times g$ for 30 min at 4°C and the resulting supernatants were considered the detergent-soluble fraction. Pellets (referred to as the insoluble fraction) were solubilized with an equal volume of Laemmli sample buffer (Bio-Rad).

Luciferase reporter activity assay

Transfections were performed using 200 ng of expression plasmids or empty vector and 50 ng of the 807-bp *DBH* regulatory region cloned upstream of the luciferase reporter gene.

The plasmid pRL-CMV, expressing the *Renilla* luciferase gene, was used as an internal control for each sample. Forty-eight hours after transfection, cells were assayed for luciferase activity (Dual-Luciferase Reporter Assay System; Promega) using a TD-20/20 luminometer following the manufacturer's instructions.

Results

Expansion of the polyA tract increases the turnover of PHOX2B

Pathogenic expansions of polyA tracts result in protein misfolding and aggregation [14, 15]. Proteins are targeted for degradation by cellular protein degradation machinery. To test whether polyA expansion in PHOX2B leads to increased degradation, we transiently transfected HeLa cells, which do not express endogenous PHOX2B, with vectors expressing either wild-type PHOX2B (WT) or mutant PHOX2B carrying 39 alanine residues (DUP39) tagged with green fluorescent protein (GFP). The levels of PHOX2B were analyzed after 48 h by cytofluorimetric assay. We found that polyA-PHOX2B levels were lower than those of wild-type PHOX2B, indicating that polyA expansion leads to reduced accumulation of PHOX2B (Fig. 1a). We hypothesized that polyA expansion affects the stability of mutant protein. To test this hypothesis, we analyzed the turnover of WT and DUP39 PHOX2B in the presence of the protein synthesis inhibitor cycloheximide. For these experiments, HeLa cells were transfected with myc-tagged WT or mutant PHOX2B, and after 24 h protein synthesis was blocked by incubating the cells with cycloheximide (Fig. 1b). Total lysates were collected at various time points and analyzed by Western blotting. We found that the half-life of WT PHOX2B was 6 h, whereas that of DUP39 was 3 h, indicating that the presence of an expanded polyA tract in PHOX2B increases the turnover rate of the protein.

Expanded polyA PHOX2B is degraded by the ubiquitin–proteasome system

The UPS and autophagy, two mechanisms responsible for the maintenance of protein balance in eukaryotic cells, have been previously shown to play a role in the clearance of PHOX2B [17]. To explore their involvement in the

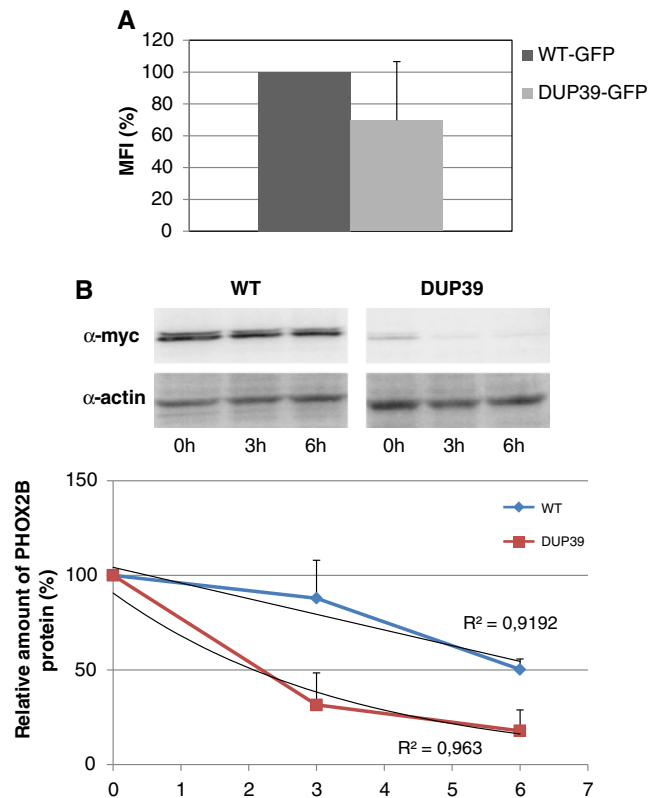


Fig. 1 Degradation rates of wild-type and expanded PHOX2B proteins. **a** PHOX2B protein levels in HeLa cells transfected with WT-PHOX2B-GFP or polyA-expanded-DUP39-GFP. Graph shows mean fluorescence intensity (*MFI*) \pm SD of three independent experiments. DUP39-GFP levels are shown normalized to WT-PHOX2B-GFP levels. **b** HeLa cells transfected with myc-tagged WT-PHOX2B or mutant PHOX2B (DUP39) were chased in the presence of 10 μ g/ml cycloheximide for indicated time periods. PHOX2B levels were determined by immunoblotting with anti-myc antibody. Values were normalized to PHOX2B levels prior to cycloheximide treatment and graphed as means of three independent experiments \pm SD. The best interpolation of the data is achieved by linear and exponential regressions for WT and DUP39, respectively

degradation of expanded PHOX2B in more detail, we expressed myc-tagged WT PHOX2B and DUP39 in HeLa cells and treated the cells with increasing amounts of either the proteasome inhibitor lactacystin or the autophagy inhibitor 3-methyladenine (3-MA). Proteasome inhibition resulted in a dose-dependent accumulation of DUP39, but did not have any effect on the levels of WT PHOX2B (Fig. 2a). Treatment of the cells with 10 mM 3-MA increased the accumulation of DUP39, though the effect was weaker than that observed in the presence of proteasome inhibitor (Fig. 2b). These results indicate that DUP39 is mainly degraded through the UPS.

Proteins targeted for degradation by the UPS are conjugated to a poly-ubiquitin chain. Studies of several polyA-expanded proteins have shown that nuclear inclusions or aggregates are often ubiquitinated [17, 18]. Therefore, we sought to determine whether PHOX2B

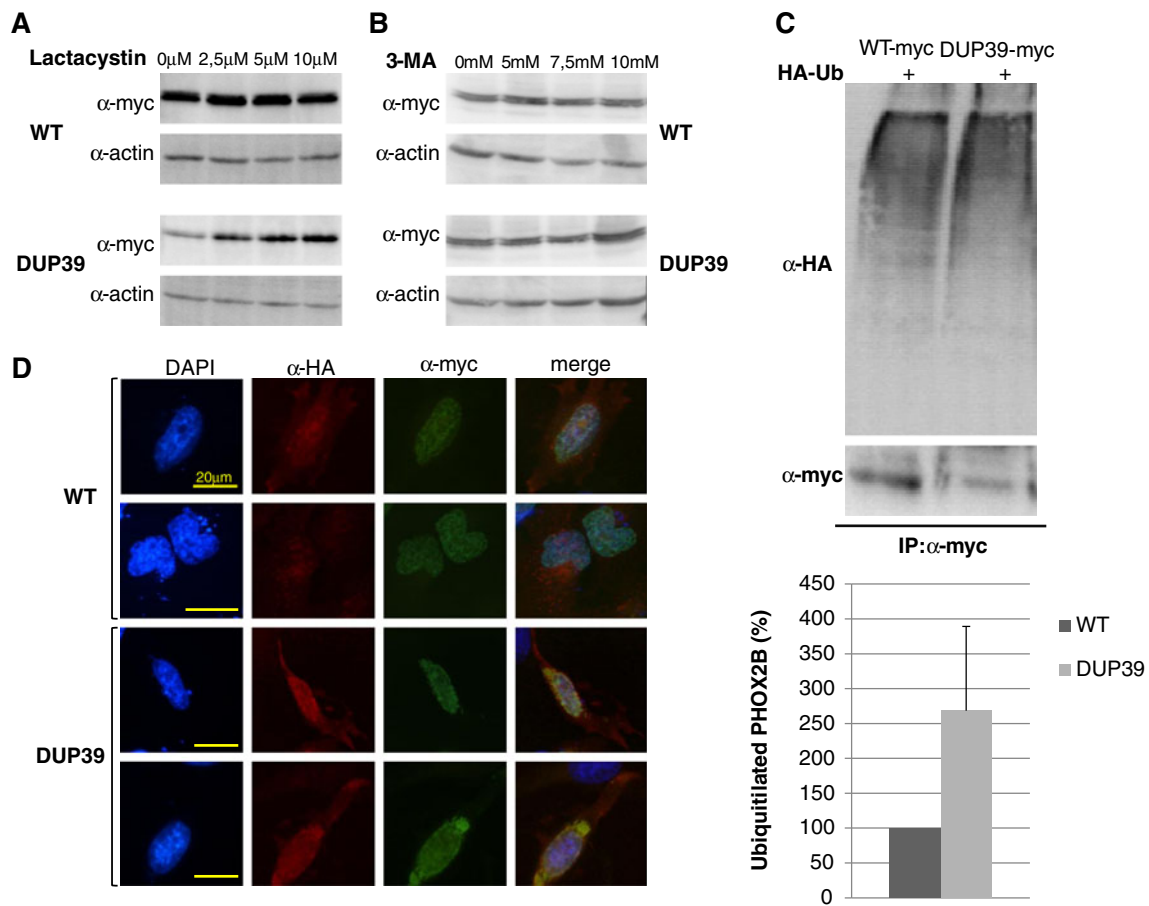


Fig. 2 Role of the ubiquitin–proteasome system and autophagy in the clearance of PHOX2B proteins. **a** Myc-tagged wild-type (*WT*) and mutant (*DUP39*) PHOX2B constructs were transfected in HeLa cells. Twenty-four hours after transfection, cells were treated with lactacystin for 12 h and immunoblotted using anti-myc and anti-actin antibodies. **b** Dose-dependent effect of 3-methyladenine (*3-MA*) on PHOX2B protein levels in HeLa cells transfected with myc-tagged *WT* and expanded (*DUP39*) PHOX2B. Cells were treated with 3-MA 4 h after transfection, collected 44 h later and processed as described in **a**. **c** Ubiquitination

assays were performed in HeLa cells co-transfected with indicated plasmids and immunoprecipitated using anti-myc antibody. Western blot analysis revealed strong smeared bands of ubiquitinated proteins in both lanes. In the lower graph, ubiquitinated PHOX2B proteins are represented as the ratio between the signal from anti-HA and anti-myc antibodies, normalized to levels of ubiquitinated *WT* PHOX2B. Mean \pm SD values from two independent experiments are shown. **d** Co-localization of *WT* and expanded (*DUP39*) PHOX2B proteins with HA-tagged ubiquitin was analyzed by immunocytochemistry 48 h after transfection in HeLa cells

is ubiquitinated, and whether PHOX2B ubiquitination is influenced by expanded polyA. To test this hypothesis, we performed a ubiquitination assay in HeLa cells over-expressing either myc-tagged *WT* PHOX2B or *DUP39* together with HA-tagged ubiquitin (HA-Ub) in the presence of the proteasome inhibitor MG132. Total lysates were processed for immunoprecipitation with an anti-myc antibody. As shown in Fig. 2c, the degree of ubiquitination of *DUP39* was higher than that of wild-type protein. Next, we asked whether *DUP39* forms ubiquitin-positive inclusions in HeLa cells transfected as above. By immunocytochemical analysis, we found that *DUP39* forms ubiquitin-positive inclusions in the nucleus and cytosol of transfected cells (Fig. 2d). In contrast, we did not detect ubiquitin-positive inclusions in cells expressing *WT* PHOX2B. Taken together, these

data indicate that expansion of polyA leads to degradation of mutant protein through the UPS.

DUP39 sequesters *WT* PHOX2B into inclusions

The observation that *DUP39* forms inclusions prompted us to investigate whether *DUP39* sequesters *WT* PHOX2B into such species. To test this idea, we co-expressed HA-tagged PHOX2B (*WT*-HA) and expanded myc-tagged PHOX2B (*DUP39*-myc) in HeLa cells and analyzed the subcellular localization of the proteins by immunocytochemistry. We found that *DUP39* sequesters *WT* PHOX2B into nuclear and cytoplasmic inclusions (Fig. 3a, arrows). To rule out the possibility that overexpression of *WT* PHOX2B leads to inclusion formation in HeLa cells, we transfected the cells with vectors expressing HA-tagged *WT* PHOX2B or myc-

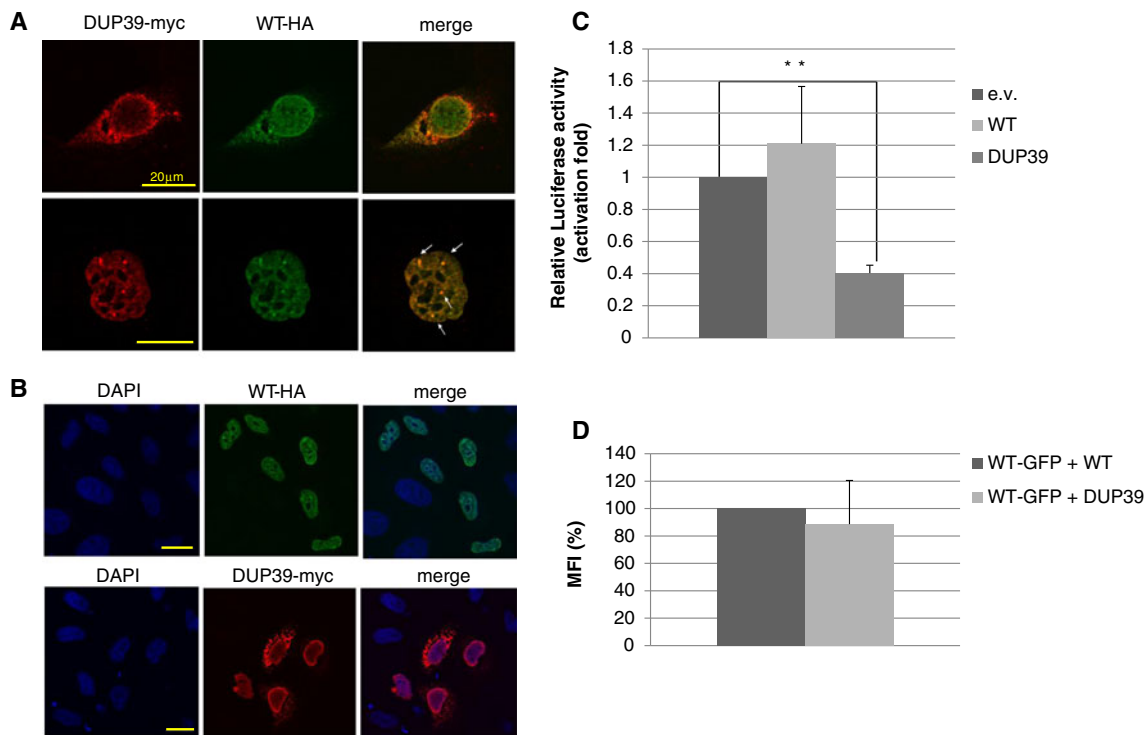


Fig. 3 Effect of polyA-expanded PHOX2B on wild-type PHOX2B. **a** HeLa cells were co-transfected with HA-tagged PHOX2B (WT-HA) and expanded myc-tagged PHOX2B (DUP39-myc). Arrows show colocalization of WT and expanded PHOX2B into nuclear and cytoplasmic aggregates. **b** HeLa cells were transfected with equivalent amounts of WT-HA and DUP39-myc. **c** Luciferase levels obtained after co-transfecting IMR32 cells with a DBH promoter construct and 200 ng of empty vector, WT, or expanded (DUP39) PHOX2B. Mean values were

obtained from three independent experiments performed in duplicate and are reported as fold of activation shown by the empty vector. $*P < 0.0001$, Student's *t*-test. **d** WT PHOX2B protein levels expressed as mean fluorescence intensity (MFI) in HeLa cells co-transfected with equivalent amounts of GFP-tagged wild-type (WT-GFP) and untagged plasmids expressing either WT or expanded PHOX2B. Values shown represent mean \pm SD of three independent experiments

tagged DUP39 (Fig. 3b). When overexpressed alone, HA-tagged WT PHOX2B localized exclusively to the nucleus and did not form inclusions. In contrast, overexpressed myc-tagged DUP39 localized to both the nucleus and cytoplasm and formed extensive inclusions. These results indicate that mutant PHOX2B sequesters its wild-type counterpart into inclusions.

Sequestration of proteins into inclusions may hamper their function, resulting in partial dominant-negative effects on the sequestered protein. PHOX2B is a transcription factor that regulates the expression of several genes, including dopamine- β -hydroxylase (DBH) [19]. To test whether localization of WT PHOX2B into inclusions is associated with a reduction of protein function, we used IMR32 neuroblastoma cells, which express endogenous PHOX2B [20]. IMR32 cells were transfected with vectors expressing either WT PHOX2B or DUP39, together with a luciferase reporter construct containing four copies of domain II of the DBH promoter. We found that overexpression of WT PHOX2B did not affect reporter gene expression (Fig. 3c). However, overexpression of DUP39 significantly reduced transactivation, suggesting that mutant protein hampers the function of endogenous wild-type protein.

Given that the degradation rate of DUP39 is faster than that of wild-type protein (Fig. 1b), we asked whether the colocalization of mutant and wild-type proteins into inclusions leads to degradation of the wild-type protein. We tested this idea in HeLa cells co-transfected with an equivalent amount of GFP-tagged WT PHOX2B and a vector for expression of either untagged WT PHOX2B or DUP39. The levels of GFP-tagged WT PHOX2B were analyzed after 48 h by cytofluorimetric assay (Fig. 3d). The levels of WT PHOX2B were not affected by co-expression of DUP39. These results indicate that sequestration of WT PHOX2B into DUP39-positive inclusions does not promote clearance of WT protein. These data support the idea that the expanded form of PHOX2B exerts a partial dominant-negative effect on the wild-type protein, which is likely due to sequestration into inclusions.

TRIM11 promotes WT PHOX2B and DUP39 degradation

Recent studies have demonstrated that the E3 ligase murine TRIM11 interacts with wild-type PHOX2B [16]; therefore, we investigated whether TRIM11 promotes the degradation of DUP39. To test this idea, we overexpressed myc-tagged

WT PHOX2B or DUP39 alone or together with increasing amounts of HA-tagged TRIM11 in HeLa cells, and analyzed protein levels by Western blotting (Fig. 4a). Overexpression of TRIM11 resulted in reduced accumulation of both WT PHOX2B and DUP39. The effect of TRIM11 on protein accumulation was dose-dependent. Moreover, we analyzed soluble and insoluble lysates of exogenous WT PHOX2B and DUP39. We observed that the majority of WT PHOX2B is contained in the insoluble fraction, whereas DUP39 is present exclusively in the insoluble fraction. Nonetheless, overexpression of TRIM11 decreased the levels of both proteins. We tested whether TRIM11 degrades PHOX2B also in IMR32 cells [20]. In the presence of TRIM11, the levels of both wild-type and expanded PHOX2B were remarkably decreased in total lysates (Fig. 4b).

In IMR32 cells, levels of insoluble PHOX2B proteins were strongly affected by TRIM11, whereas the soluble fraction of PHOX2B proteins seemed to remain unchanged.

To assess whether TRIM11 promotes PHOX2B turnover, HeLa cells were transiently transfected with myc-tagged WT PHOX2B or DUP39 in the presence or absence of HA-TRIM11. The cells were treated with cycloheximide, and PHOX2B levels were analyzed by Western blotting at various time points (Fig. 5a). Overexpression of TRIM11 increased the rate of degradation of both WT PHOX2B and DUP39. Notably, the half-life of WT PHOX2B was 6 h in the absence of TRIM11 and decreased to 3 h in the presence of TRIM11; for DUP39, the half-life decreased from 4.5 to 2 h in the presence of TRIM11.

To determine whether the degradation of PHOX2B induced by TRIM11 occurs through the UPS, we compared the levels of myc-tagged WT PHOX2B and DUP39 after co-transfection with HA-TRIM11 in the presence or absence of MG132. Inhibition of the proteasome restored PHOX2B levels, particularly in the case of DUP39 (Fig. 5b). Together, our results indicate that the E3 ubiquitin ligase TRIM11 promotes the degradation of PHOX2B and DUP39 through the UPS in cultured cells.

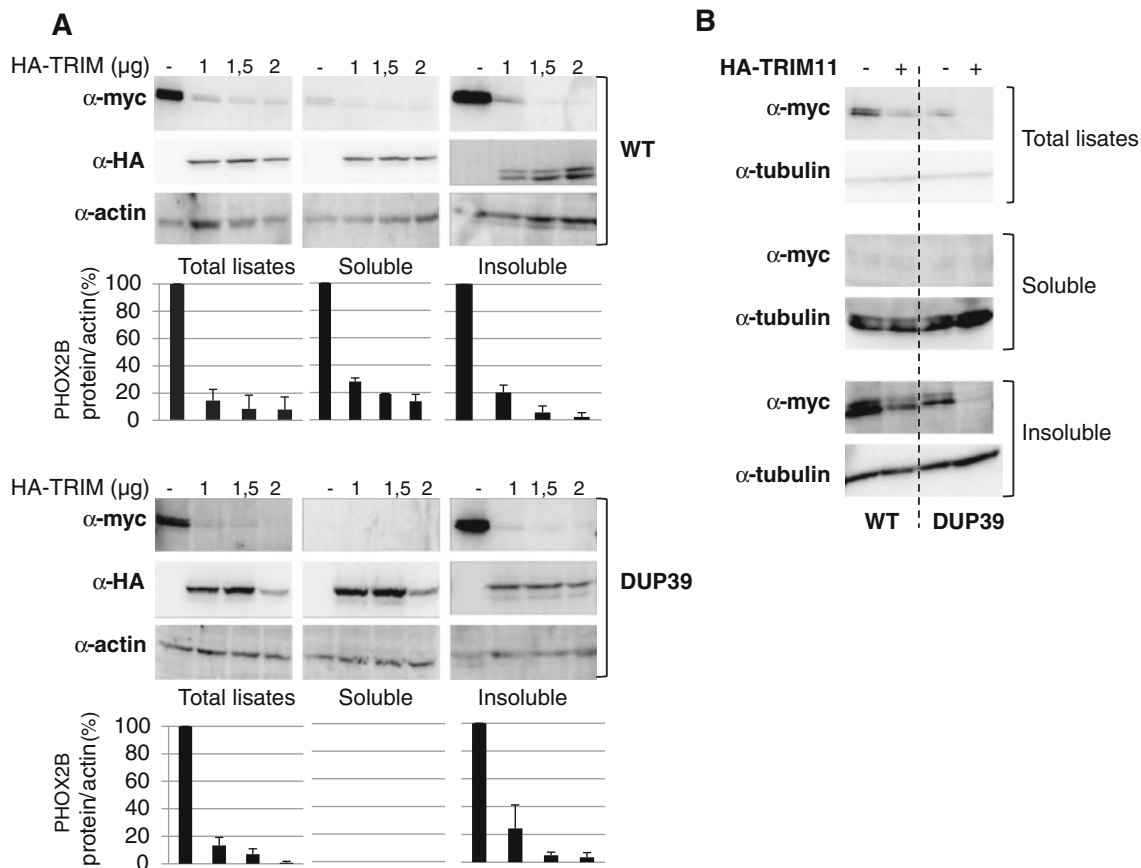


Fig. 4 Effect of TRIM11 on stability of wild-type and expanded PHOX2B in HeLa and IMR32 cells. **a** Dose-dependent effect of TRIM11 on WT and expanded (DUP39) PHOX2B stability as shown by Western blotting in total, soluble, and insoluble lysates. HeLa cells were co-transfected with varying amounts of HA-TRIM11 along with myc-tagged PHOX2B constructs. In each experiment, GFP vector was added so that the total amount of transfected DNA summed to 3 μg. The TRIM11 dose-dependent degradation of PHOX2B is represented as the

ratio between signals detected with anti-myc and anti-actin antibodies. Data were normalized to PHOX2B levels in the absence of TRIM11. **b** Effect of TRIM11 on WT and expanded (DUP39) PHOX2B stability as revealed by Western blotting in total, soluble and insoluble lysates. In these experiments, IMR32 cells were co-transfected with a constant amount of HA-tagged TRIM11 along with myc-tagged PHOX2B constructs. In each experiment, GFP vector was added so that the total amount of transfected DNA summed to 3 μg

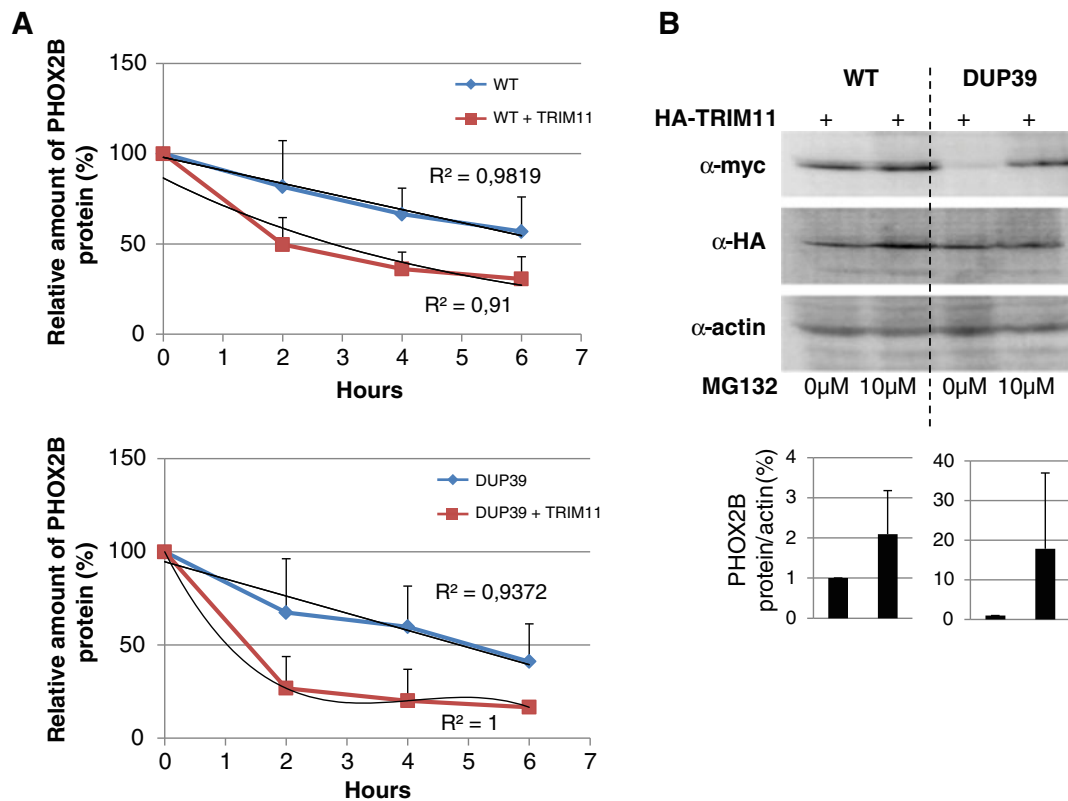


Fig. 5 TRIM11 promotes degradation of wild-type and expanded PHOX2B through the UPS. **a**) Cycloheximide chase assays were used to assess the effect of TRIM11 on the steady-state levels of WT and expanded (DUP39) PHOX2B proteins. Graphs show relative levels of PHOX2B normalized against actin, quantified from Western blotting performed at different time points following cycloheximide treatment. Values were normalized to PHOX2B levels prior to CHX treatment and graphed as means of at least three independent experiments \pm SD.

Linear regression was used to interpolate data from experiments without transfection of TRIM11, whereas exponential and binomial regressions fit data from experiments with transfected TRIM11 in the top and bottom panels, respectively. **b**) HeLa cells were transfected with HA-TRIM11 and myc-PHOX2B (WT or DUP39) plasmids and treated with MG132 as indicated. Relative levels of PHOX2B proteins are shown in the graph below

Association of TRIM11 with DUP39-positive inclusions

Components of the heat shock response pathway and members of the proteasome machinery have been shown to co-localize with DUP39-positive inclusions [15, 17]. We sought to determine whether TRIM11 localizes to DUP39-positive inclusions in HeLa cells. We expressed either myc-tagged DUP39 or myc-tagged WT with HA-tagged TRIM11 in HeLa cells. Cells were treated with MG132 to reduce the degradative effect of TRIM11. Immunocytochemical analysis revealed that TRIM11 co-localizes with PHOX2B and DUP39 in the nucleus of HeLa cells (Fig. 6). Notably, TRIM11 was recruited to nuclear and cytosolic inclusions in cells expressing DUP39 (see arrows).

Effect of TRIM11 on in vitro transactivation induced by PHOX2B

To investigate whether the effect of TRIM11 on PHOX2B degradation affects PHOX2B transactivation, HeLa cells

were co-transfected with a luciferase reporter construct containing the regulatory PHOX2B binding region of the *DBH* gene together with expression vectors encoding PHOX2B or HA-tagged TRIM11. Consistent with previous reports [14, 15], overexpression of WT PHOX2B induced a significant increase of reporter gene expression compared to cells transfected with the empty vector (Fig. 7a). Overexpression of DUP39 resulted in reduced transactivation compared to cells overexpressing the wild-type protein. Co-expression of TRIM11 and PHOX2B proteins dramatically prevented the PHOX2B effect on luciferase activity. Notably, the presence of TRIM11 caused a reduction in promoter activity driven by the pcDNA3.1 empty vector, to below the basal level. Such an inhibitory effect was likely due to the degradation of factors necessary for the basal expression of the promoter construct. Therefore, we decided to test the effect of TRIM11 on the activation induced by PHOX2B in IMR32 cells (Fig. 7b). In this case, TRIM11 did not affect the transactivation induced by the wild-type protein, but completely rescued the impairment of DBH promoter activity

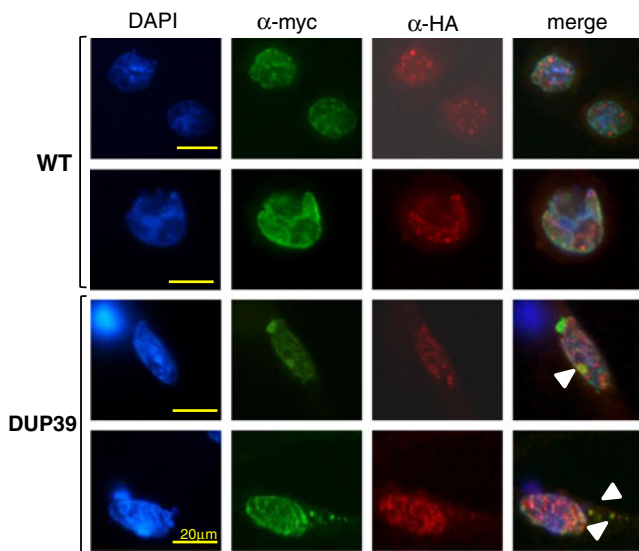


Fig. 6 Subcellular localization of TRIM11 and PHOX2B proteins in HeLa cells. The distribution pattern of exogenous HA-TRIM11 is shown in HeLa cells co-transfected with WT or mutant (DUP39) myc-tagged PHOX2B. Arrows show co-localization of TRIM11 with nuclear and cytoplasmic aggregates

associated with the presence of expanded PHOX2B. These findings are consistent with the effect of TRIM11 on PHOX2B degradation, a circumstance which could suppress the dominant-negative effect of DUP39 on the wild-type protein.

Discussion

In this study, we demonstrate that expansion of the polyA tract in PHOX2B leads to protein degradation through the UPS. We also provide evidence that DUP39 overexpression results in inclusion formation in cultured cells. These inclusions are ubiquitin-positive and, importantly, sequester WT PHOX2B. Localization of WT PHOX2B into inclusions correlates with a reduction in protein function, suggesting that this mechanism contributes to disease pathogenesis through a dominant-negative effect. Finally, we show that DUP39, as well as WT PHOX2B, is a target of the E3 ubiquitin ligase TRIM11, and that overexpression of TRIM11 reduces the accumulation of the disease protein and restores the function of its wild-type counterpart. Our findings highlight TRIM11 as a novel modifier of DUP39 toxicity.

PolyA tracts are present in several transcription factors, and in-frame duplications in these sequences are responsible for several human genetic diseases [2–5]. In vivo experiments have shown that mice expressing a mutant PHOX2B with 27 alanine residues show a more severe phenotype than mice heterozygous for a targeted *Phox2b* deletion [12, 13].

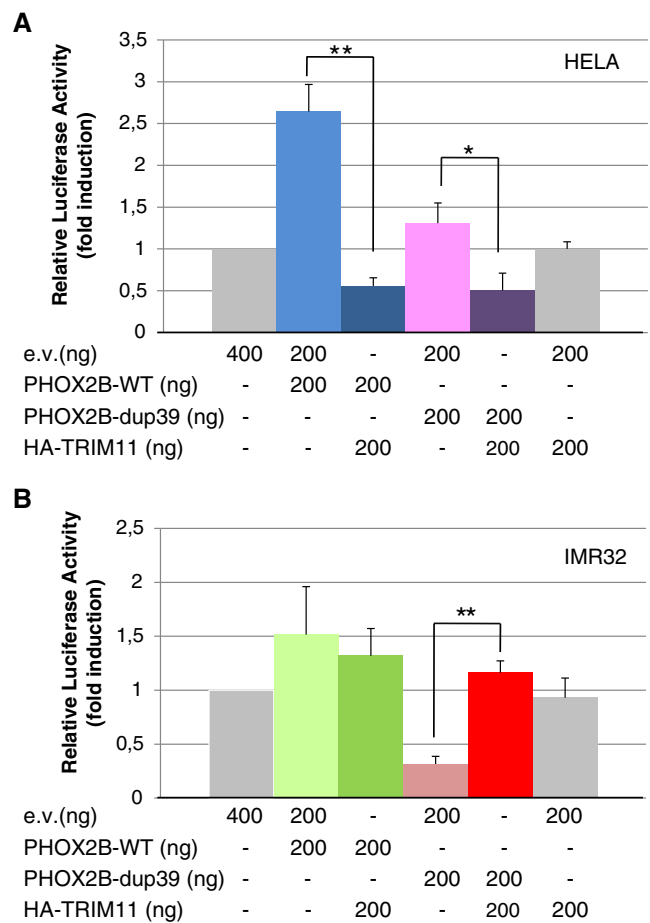


Fig. 7 Effect of TRIM11 on in vitro transactivation induced by PHOX2B in HeLa and IMR32 cells. The transcriptional activity of WT and expanded (DUP39) PHOX2B was measured in **a** HeLa cells and **b** IMR32 cells co-transfected with DBH promoter-based reporter and indicated amounts of expression plasmids. Mean values were obtained from at least three independent experiments performed in duplicate and are reported as percentage of activation shown by the empty vector. **P* < 0.05, ***P* < 0.005, Student’s *t*-test

These results support a pathogenic mechanism in which expansion of polyA in PHOX2B confers a toxic gain of function or a dominant-negative effect to the mutant protein. The increased apoptosis and formation of polyA aggregates associated with expression of DUP39 [17] support the toxic gain of function model as a major pathogenic mechanism in CCHS. Our present data demonstrate that expanded PHOX2B retains wild-type PHOX2B in inclusions and causes a strong reduction of basal DBH promoter activity in a neuroblastoma cell line. Altogether, our observations are consistent with the hypothesis that a dominant-negative effect of the expanded PHOX2B also contributes to CCHS pathogenesis. A partial dominant-negative effect of PHOX2B with polyA expansions has been reported [14, 15], and we have previously demonstrated that DUP39 interacts with and sequesters a fraction of WT PHOX2B in nuclear aggregates [14]. In addition, Trochet et al. [15]

observed a significant impairment of target transactivation after co-transfection of the wild-type protein with a 10-fold excess of DUP39 in HeLa cells.

Similar to expanded polyQ tracts, expanded polyA tracts lead to protein misfolding and aggregation in a repeat length-dependent manner [14, 21–23]. Proteins that do not fold properly are normally recognized by protein quality control systems and are rapidly and efficiently degraded. In some cases, however, the misfolded protein forms aggregates or causes excessive activation of the degradation machinery, resulting in profound cellular dysfunction and disease [4, 5]. Several studies have reported that the presence of large trinucleotide repeat expansions causes a reduction in the levels of these mutant proteins [21, 24]. In particular, Klein and colleagues [24] observed that the relative decrease in the level of the polyA-expanded protein poly(A) binding protein nuclear 1 (PABPN1) is likely due to enhanced clearance of the protein through autophagy. Our data demonstrate that expanded-polyA PHOX2B is targeted for degradation through the UPS, resulting in lower protein expression levels.

Regulation of the steady state levels of expanded polyA proteins and, more generally, of expanded trinucleotide repeat proteins, represents a crucial component in the pathogenesis of human disorders. To deepen our understanding of cellular clearance of such expanded misfolded proteins, we tested the effect of the E3 ubiquitin ligase TRIM11, which is already known to interact with PHOX2B in mouse [16], in the clearance of wild-type and expanded PHOX2B proteins. E3 ubiquitin ligases have been implicated in the biology of neurodegenerative disorders that involve protein misfolding, including polyQ diseases [25–31]. In particular, CHIP and E6-AP have neuroprotective effects, in that they promote the proteasome-mediated degradation of disease proteins and reduce polyQ protein-induced cell death [27–29]. These observations are consistent with the fact that E3 ubiquitin ligases are involved in the ubiquitination and degradation of misfolded proteins through the proteasome. TRIM11 is already known to destabilize several interacting proteins via the UPS [32–34]. TRIM11 also transports ubiquitinated proteins, such as expanded polyQ and polyA proteins, to the proteasome, playing a role in their clearance [34]. Therefore, TRIM11 has dual functions: it works as a RING E3 ligase degrading specific substrates, such as Humanin, ARC105 and Pax6 [32–34], as well as a “ubiquitin receptor” transporting ubiquitinated, misfolded proteins to the proteasome [34]. Our data demonstrate the role of TRIM11 in regulating the proteasome-mediated degradation of both wild-type and expanded PHOX2B. Importantly, cycloheximide chase experiments revealed that TRIM11 has a greater effect on the degradation of DUP39 compared to WT PHOX2B, indicating that DUP39 is efficiently targeted for degradation.

In agreement with our observation that TRIM11 overexpression reduces the insoluble form of expanded PHOX2B in IMR32 cells, we also demonstrated a significant TRIM11-mediated rescue of DBH promoter activity, which is decreased in the presence of mutant PHOX2B. Our results support a model in which overexpression of TRIM11 counteracts the dominant-negative effect of DUP39 by enhancing the degradation of the disease protein. We have already reported that activation of the heat shock response by geldanamycin and additional molecules is efficient both in preventing formation and in inducing clearance of PHOX2B polyA aggregates [17, 35]. Consistent with these reports, our work demonstrates that processes that lead to protein degradation represent an additional cell response to the presence of expanded-polyA PHOX2B. Our results suggest that mechanisms up-regulating intracellular TRIM11 may offer potential targets for therapeutic intervention in CCHS.

Acknowledgements We are grateful to Dr. B. Eggen (Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, Haren, The Netherlands) for his kind gift of the pcDNA3-HA plasmid. We are also thankful to the Associazione Italiana per la Sindrome da Ipoventilazione Centrale Congenita and to all CCHS patients and their families. We gratefully acknowledge the financial support of Fondazione Mariani (grant 08/69 to I.C.), Ministry of Health (Progetti Finalizzato 2006 and Strategico 2009 to I.C.), and Fondazione Cariplo (grant 2010- 0688 to D.F.). S.P. was supported by a Travel Grant award from the Boehringer Ingelheim Fonds and the Fondazione Aiuti per la Ricerca sulle Malattie Rare A.R.M.R. SDL is the recipient of an “Assegno di ricerca - Dote Ricerca” of the University of Milan, co-funded by FSE, Regione Lombardia. This work was also supported by funds from a Marie Curie Reintegration grant (FP7-256448 to M.P.), Telethon-Italy (GGP10037 to M.P.), and the Muscular Dystrophy Association (196646 to M.P.).

Conflicts of interest The authors declare that they have no commercial or other associations that might pose a conflict of interest in connection with submitted material.

References

- Orr HT, Zoghbi HY (2007) Trinucleotide repeat disorders. *Annu Rev Neurosci* 30:575–621
- Brown LY, Brown SA (2004) Alanine tracts: the expanding story of human illness and trinucleotide repeats. *Trends Genet* 20:51–58
- Amiel J, Trochet D, Clement-Ziza M, Munnich A, Lyonnet S (2004) Polyalanine expansions in human. *Hum Mol Genet* 13: R235–R243
- Albrecht A, Mundlos S (2005) The other trinucleotide repeat: polyalanine expansion disorders. *Curr Opin Genet Dev* 15:285–293
- Messaed C, Rouleau GA (2009) Molecular mechanism underlying polyalanine diseases. *Neurobiol Dis* 34:397–405
- Weese-Mayer DE, Berry-Kravis EM, Ceccherini I, Keens TG, Loghmanee DA, Trang H (2010) An official ATS clinical policy statement: congenital central hypoventilation syndrome: genetic basis, diagnosis, and management. *Am J Respir Crit Care Med* 181:626–644

7. Amiel J, Laudier B, Attie-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simmoneau M et al (2003) Polyalanine expansion and frameshift mutations of the paired-like homeobox gene *PHOX2B* in congenital central hypoventilation syndrome. *Nat Genet* 33:459–461
8. Sasaki A, Kanai M, Kijima K, Akaba K, Hashimoto M, Hasegawa H, Otaki S, Koizumi T, Kusuda S, Ogawa Y et al (2003) Molecular analysis of congenital central hypoventilation syndrome. *Hum Genet* 114:22–26
9. Weese-Mayer DE, Berry-Kravis EM, Zhou L, Maher BS, Silvestri JM, Curran ME, Marazita ML (2003) Idiopathic congenital central hypoventilation: analysis of genes pertinent to early autonomic nervous system embryologic development and identification of mutation in *PHOX2b*. *Am J Med Genet* 123A:267–278
10. Matera I, Bachetti T, Puppo F, Di Duca M, Morandi F, Casiraghi GM, Cilio MR, Hennekam R, Hofstra R, Schöber JG et al (2004) *PHOX2B* mutations and polyalanine expansions correlate with the severity of the respiratory phenotype and associated symptoms in both congenital and late onset Central Hypoventilation syndrome. *J Med Genet* 41:373–380
11. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. *Nature* 399:366–370
12. Durand E, Dauger S, Pattyn A, Gaultier C, Goridis C, Gallego J (2004) Sleep-disordered breathing in newborn mice heterozygous for the transcription factor *Phox2b*. *Am J Respir Crit Care Med* 172:238–243
13. Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet JF, Goridis C (2008) A human mutation in *Phox2b* causes lack of CO₂ chemosensitivity, fatal central apnea, and specific loss of parafacial neurons. *Proc Natl Acad Sci USA* 105:1067–1072
14. Bachetti T, Matera I, Borghini S, Di Duca M, Ravazzolo R, Ceccherini I (2005) Distinct pathogenetic mechanism for *PHOX2B* associated polyalanine expansions and frameshift mutations in congenital central hypoventilation syndrome. *Hum Mol Genet* 14:1815–1824
15. Trochet D, Hong SJ, Lim JK, Brunet JF, Munnich A, Kim KS, Lyonnet S, Goridis C, Amiel J (2005) Molecular consequences of *PHOX2B* missense, frameshift and alanine mutations leading to autonomic dysfunction. *Hum Mol Genet* 14:3697–3708
16. Hong SJ, Chae H, Lardaro T, Hong S, Kim KS (2008) *Trim11* increases expression of dopamine beta-hydroxylase gene by interacting with *Phox2b*. *Biochem Biophys Res Commun* 368:650–655
17. Bachetti T, Bocca P, Borghini S, Matera I, Prigione I, Ravazzolo R, Ceccherini I (2007) Geldanamycin promotes nuclear localisation and clearance of *PHOX2B* misfolded proteins containing polyalanine expansions. *Int J Biochem Cell Biol* 39:327–339
18. Abu-Baker A, Messaed C, Laganieri J, Gaspar C, Brais B, Rouleau GA (2003) Involvement of the ubiquitin proteasome system and molecular chaperones in oculopharyngeal muscular dystrophy. *Hum Mol Genet* 12:2609–2023
19. Benfante R, Flora A, Di Lascio S, Cargnin F, Longhi R, Colombo S, Clementi F, Fornasari D (2007) Transcription factor *PHOX2A* regulates the human alpha3 nicotinic receptor subunit gene promoter. *J Biol Chem* 282:13290–13302
20. Longo L, Borghini S, Schena F, Parodi S, Albino D, Bachetti T, Da Prato L, Truini M, Gambini C, Tonini GP et al (2008) *PHOX2A* and *PHOX2B* genes are highly co-expressed in human neuroblastoma. *Int J Oncol* 33:985–991
21. Albrecht AN, Kornak U, Böddrich A, Süring K, Robinson PN, Stiege AC, Lurz R, Stricker S, Wanker EE, Mundlos S (2004) A molecular pathogenesis for transcription factor associated polyalanine tract expansions. *Hum Mol Genet* 13:2351–2359
22. Caburet S, Demarez A, Moumné L, Fellous M, De Baere E, Veitia RA (2004) A recurrent polyalanine expansion in the transcription factor *FOXL2* induces extensive nuclear and cytoplasmic protein aggregation. *J Med Genet* 41:932–936
23. Moumné L, Dipietromaria A, Batista F, Kocer A, Fellous M, Pailhoux E, Veitia RA (2008) Differential aggregation and functional impairment induced by polyalanine expansions in *FOXL2*, a transcription factor involved in cranio-facial and ovarian development. *Hum Mol Genet* 17:1010–1019
24. Klein AF, Ebihara M, Alexander C, Dicaire MJ, Sasseville AM, Langelier Y, Rouleau GA, Brais B (2008) *PABPN1* polyalanine tract deletion and long expansions modify its aggregation pattern and expression. *Exp Cell Res* 314:1652–1566
25. Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K et al (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* 25:302–305
26. Niwa J, Ishigaki S, Hishikawa N, Yamamoto M, Doyu M, Murata S, Tanaka K, Taniguchi N, Sobue G (2002) *Dorfin* ubiquitylates mutant *SOD1* and prevents mutant *SOD1*-mediated neurotoxicity. *J Biol Chem* 277:36793–36798
27. Jana NR, Dikshit P, Goswami A, Kotliarova S, Murata S, Tanaka K, Nukina N (2005) Co-chaperone *CHIP* associates with expanded polyglutamine protein and promotes their degradation by proteasomes. *J Biol Chem* 280:11635–11640
28. Miller VM, Nelson RF, Gouvion CM, Williams A, Rodriguez-Lebron E, Harper SQ, Davidson BL, Rebagliati MR, Paulson HL (2005) *CHIP* suppresses polyglutamine aggregation and toxicity in vitro and in vivo. *J Neurosci* 25:9152–9161
29. Mishra A, Dikshit P, Purkayastha S, Sharma J, Nukina N, Jana NR (2008) *E6-AP* promotes misfolded polyglutamine proteins for proteasomal degradation and suppresses polyglutamine protein aggregation and toxicity. *J Biol Chem* 283:7648–7656
30. Iwata A, Nagashima Y, Matsumoto L, Suzuki T, Yamanaka T, Date H, Deoka K, Nukina N, Tsuji S (2009) Intracellular degradation of polyglutamine aggregates by the ubiquitin proteasome system. *J Biol Chem* 284:9796–9803
31. Sroka K, Voigt A, Deeg S, Reed JC, Schulz JB, Bähr M, Kermer P (2009) *BAG1* modulates huntingtin toxicity, aggregation, degradation, and subcellular distribution. *J Neurochem* 111:801–807
32. Niikura T, Hashimoto Y, Tajima H, Ishizaka M, Yamagishi Y, Kawasumi M, Nawa M, Terashita K, Aiso S, Nishimoto I (2003) A tripartite motif protein *TRIM11* binds and destabilizes Humanin, a neuroprotective peptide against Alzheimer's disease-relevant insults. *Eur J Neurosci* 17:1150–1158
33. Ishikawa H, Tachikawa H, Miura Y, Takahashi N (2006) *TRIM11* binds to and destabilizes a key component of the activator-mediated cofactor complex (*ARC105*) through the ubiquitin proteasome system. *FEBS Lett* 580:4784–4792
34. Tuoc TC, Stoykova A (2008) *Trim11* modulates the function of neurogenic transcription factor *Pax6* through ubiquitin-proteasome system. *Genes Dev* 22:1972–1986
35. Di Zanni E, Bachetti T, Parodi S, Bocca P, Prigione I, Di Lascio S, Fornasari D, Ravazzolo R, Ceccherini A (2012) In vitro drug treatments reduce the deleterious effects of aggregates containing polyAla expanded *PHOX2B* proteins. *Neurobiol Dis* 45:508–518