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Noradrenergic neuronal development is impaired by mutation of the proneural *HASH-1* gene in congenital central hypoventilation syndrome (Ondine's curse)

Loïc de Pontual[1,†], Virginie Népote[2,†], Tania Attié-Bitach[1,†], Hassan Al Halabiah[2], Ha Trang[2], Vincent Elghouzzi[1], Béatrice Levacher[2], Karim Benihoud[3], Joëlle Augé[1], Christophe Faure[2], Béatrice Laudier[1], Michel Vekemans[1], Arnold Munnich[1], Michel Perricaudet[3], François Guillemot[4], Claude Gaultier[2], Stanislas Lyonnet[1], Michel Simonneau[2] and Jeanne Amiel[1,*]

[1]Unité de Recherches sur les Handicaps Génétiques de l'Enfant INSERM U-393, and Département de Génétique, Hôpital Necker-Enfants Malades, Paris, France, [2]Service de Physiologie, INSERM E9935, and CIC INSERM 9202, Hôpital Robert Debré, Paris, France, [3]UMR 1582 CNRS, IGR, Villejuif, France and [4]NIMR, Mill Hill, UK

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Congenital central hypoventilation syndrome (CCHS, Ondine's curse) is a rare disorder of the chemical control of breathing. It is frequently associated with a broad spectrum of dysautonomic symptoms, suggesting the involvement of genes widely expressed in the autonomic nervous system. In particular, the HASH-1–PHOX2A–PHOX2B developmental cascade was proposed as a candidate pathway because it controls the development of neurons with a definitive or transient noradrenergic phenotype, upstream from the RET receptor tyrosine kinase and tyrosine hydroxylase. We recently showed that *PHOX2B* is the major CCHS locus, whose mutation accounts for 60% of cases. We also studied the proneural *HASH-1* gene and identified a heterozygous nucleotide substitution in three CCHS patients. To analyze the functional consequences of *HASH-1* mutations, we developed an *in vitro* model of noradrenergic differentiation in neuronal progenitors derived from the mouse vagal neural crest, reproducing *in vitro* the HASH–PHOX–RET pathway. All *HASH-1* mutant alleles impaired noradrenergic neuronal development, when overexpressed from adenoviral constructs. Thus, *HASH-1* mutations may contribute to the CCHS phenotype in rare cases, consistent with the view that the abnormal chemical control of breathing observed in CCHS patients is due to the impairment of noradrenergic neurons during early steps of brainstem development.

INTRODUCTION

Congenital central hypoventilation syndrome (CCHS, Ondine's curse, MIM 209880) is a life-threatening disorder characterized by persistent hypoventilation during sleep, beginning in the neonatal period and requiring life-long ventilatory assistance (1). While the pathophysiologic mechanisms underlying CCHS remain elusive, several lines of evidence suggest that genetic factors are involved (occurrence in siblings, concordant monozygotic twins and rare vertical transmission) (2). Changes in the integration of afferent inputs from central and peripheral chemoreceptors in the brainstem are the most likely disease mechanisms. In addition, most CCHS patients present a broader defect of the autonomic nervous system (3), including Hirschsprung disease (25–30% of cases, Haddad syndrome, MIM 209880) (2), a malformation of the enteric nervous system that has been ascribed to a mutation at the *RET* receptor tyrosine kinase locus. In mice, the Ret signaling pathway, which involves the sequential expression of the *Mash1*, *Phox*, *Ret* and *TH* genes, is responsible for the development of all

*To whom correspondence should be addressed at: Département de Génétique, Hôpital Necker-Enfants Malades, 149, rue de Sèvres, 75743 Paris cedex 15, France. Tel: +33 144495648; Fax: +33 144495150; Email: amiel@necker.fr
†The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

transient or permanent noradrenergic derivatives (4,5). We recently showed that *PHOX2B* is the major disease-causing gene in both CCHS and Haddad syndromes, with an autosomal dominant mode of inheritance and *de novo* mutations at the first generation (2). Cross-regulation of the *Phox2* and *Mash-1* genes has been shown in mice (6,7); moreover, newborn *Mash-1*$^{+/-}$ heterozygous mice show an impaired ventilatory responses to hypercarbia (8). We therefore regarded the human ortholog of *Mash1* (*HASH-1*), which encodes a tissue-specific basic helix–loop–helix transcription factor, as an additional candidate gene for CCHS. We identified a heterozygous nucleotide substitution of the *HASH-1* gene in three patients. The functional consequences of *HASH-1* mutations were studied using a novel *in vitro* model of noradrenergic differentiation based on neuronal progenitors derived from the mouse vagal neural crest and reproducing *in vitro* the HASH–PHOX–RET pathway. Forced expression of each of the mutant *HASH-1* alleles induced the impairment of noradrenergic neuronal development. We conclude that, at least in some cases, heterozygous *HASH-1* mutation may contribute to the CCHS phenotype by modifying noradrenergic neurons during early steps of brainstem development, and that the *HASH-1* gene is tightly involved in the formation of the neuronal network for autonomous control of ventilation in human.

RESULTS

HASH-1 heterozygous nucleotide substitutions

We screened the single coding exon of *HASH-1* for nucleotide variations in a series of 30 CCHS patients (Fig. 1). We obtained an abnormal SSCP pattern in three cases. Direct DNA sequencing showed a C to A transversion at nucleotide 52, resulting in a proline to threonine substitution at amino acid position 18 (P18T), in a female patient with isolated CCHS. Two in-frame deletions of five (111–125del15nt, A37–A41del) and eight (108–131del24nt, A36–A43del) alanine codons in a 13-residue polyalanine tract, were identified in female patients with CCHS and Haddad syndrome respectively (Fig. 1A). Both the P18T and the A37–A41del mutations were inherited from the healthy father (Fig. 1A), while the mother of the patient with the A36–A43del mutation was not available for study. The *HASH-1* mutations are located in regions highly conserved across mammalian *ASH* genes as shown by Clustal W analysis (Fig. 1B). These data suggest that these protein domains are functionally relevant. Accordingly, none of the *HASH-1* gene mutations was detected in a panel of 120 control chromosomes. Interestingly, a *de novo* mutation of *PHOX2B* (polyalanine expansion of seven alanines) was found in the patient harboring the P18T mutation of the *HASH-1* gene, whereas direct sequencing of the three exons of *PHOX2B* showed no variation in the other two cases. Indeed, all CCHS patients described in this series have been tested for the *RET*, *GDNF* and *PHOX2B* genes (2).

HASH-1 expression in early human development

We further investigated the possible involvement of *HASH-1* mutations in the CCHS phenotype by studying the pattern of expression of *HASH-1* in early human development (Fig. 2). Consistent with the wide spectrum of afferent nervous system (ANS) dysfunction observed in CCHS patients and the pattern of expression in mice (9,10), *HASH-1* is expressed in both the central nervous system (mesencephalon, rhombencephalon, spinal cord; Fig. 2A, B and E–H), and the peripheral nervous system (cells lining the dorsal aorta, presumed to be neural crest-derived precursors of the sympathetic ganglia, Fig. 2C and D) from Carnegie stage 14. At Carnegie stage 18, *HASH-1* expression was detected in the presumptive enteric nervous system, from the esophagus to the rectum (Fig. 2E–H) and in the adrenal medulla (Fig. 2I and J).

Functional analysis of HASH-1 mutant alleles

We investigated the effects of the *HASH-1* mutated alleles on their transcriptional activity by means of a luciferase assay. P19 cells were co-transfected with *HASH-1* alleles (+/− E47) and the luciferase gene under the control of the Delta1 promoter. The transcriptional activities of the P18T and A36-A43del alleles were similar to that of the control, regardless of whether the E47 cofactor was present (data not shown, available on request).

We then overexpressed the wild-type and mutant *HASH-1* alleles, and assessed the functional consequences for the differentiation of the noradrenergic lineage, using the PHOX–RET signaling pathway and the tyrosine hydroxylase (TH) phenotype as markers (11,12). We demonstrated that undifferentiated vagal neural crest progenitor cells, isolated from E9 mouse embryos, may be engaged in noradrenergic differentiation, and then sequentially produce Phox2a, Ret, and TH, thereby reproducing the mouse Mash-1 molecular cascade (Fig. 3A). Interestingly, no alternative lineage was obtained, as almost all TH+ cells were derived from *PHOX2A*-expressing neurons. We then showed that it was possible to reproduce the catecholaminergic differentiation pathway, 72 h after the forced expression of *HASH-1* by recombinant adenoviruses (Fig. 3B and C). All the infected cells (100%) were transduced with the *HASH-1* adenoviral constructs (Fig. 4), as shown by GFP production (Fig. 4B). The overexpression of wild-type HASH-1 resulted in the differentiation of $52.3 \pm 3\%$ ($n = 12$) of neural crest cells into postmitotic noradrenergic derivatives, as shown by Phox2a production (data not shown). These values are similar to those previously reported for retroviral forced expression (11). We investigated the functional consequences of *HASH-1* gene mutations for the noradrenergic lineage (Fig. 4B). All mutated forms of *HASH-1* tested led to a significant decrease (20–30%) in the number of neuronal derivatives of the noradrenergic lineage, as shown by Phox2a production (Fig. 4B). In particular, only $34.4 \pm 0.7\%$ ($p < 0.001$) of neural crest progenitors differentiated to give a noradrenergic phenotype using the A37–A41del *HASH-1* mutant construct, whereas $39.3 \pm 0.6\%$ and $38.7 \pm 0.6\%$ of cells produced Phox2a when the P18T and A36–A43del *HASH-1* mutant alleles, respectively, were used ($P < 0.01$; Fig. 4B).

DISCUSSION

Taking advantage of the well-characterized specification of catecholaminergic neurons derived from the vagal neural crest

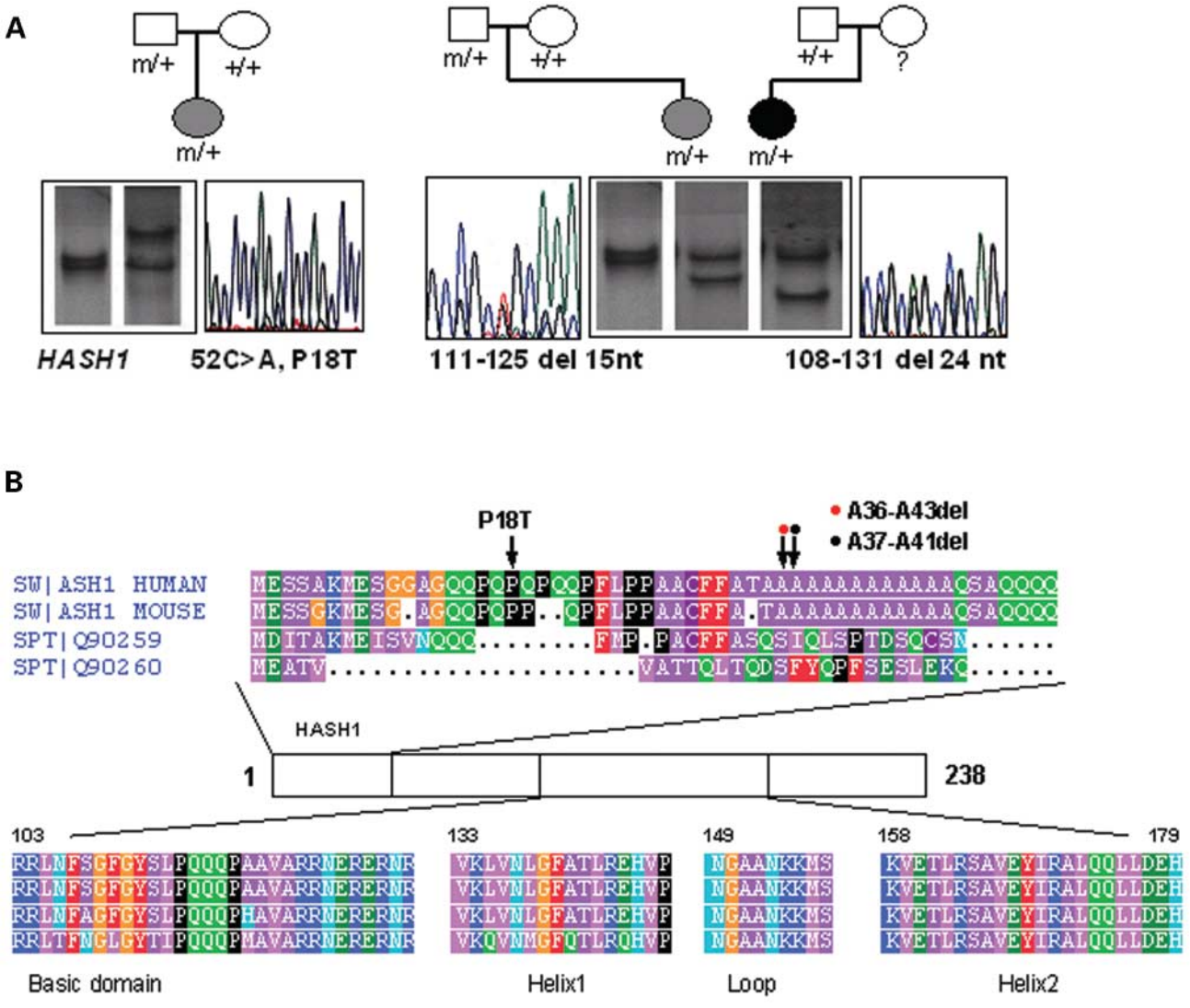

Figure 1. *HASH-1* gene mutations and phylogenetic analysis of HASH-1 proteins. (**A**) *HASH-1* gene mutations. SSCP results and mutated sequences are illustrated for the three individuals whose pedigrees are shown directly above. (**B**) Clustal W alignment of the human HASH-1 protein and its mouse, *Drosophila* and *C. elegans* orthologs. The residues mutated in CCHS patients are indicated by arrows. Note the conservation of the basic, helix 1, loop and helix2 amino acid domains between the four species studied.

(5), we recapitulated *in vitro* the molecular cascade involved in the early steps of noradrenergic determination. In that system, we used a forced expression of wild-type and mutant HASH-1 to test the functional relevance of mutated *HASH-1* alleles identified in CCHS patients. We showed a significant decrease in the number of noradrenergic neurons generated after expression of mutant HASH-1 alleles. However, this is a limited effect considering that patients are heterozygotes for the HASH-1 mutated allele as opposed to our experimental model. Nevertheless, subtle consequences on the noradrenergic neuronal development could be expected with *HASH-1* gene mutations lying outside of the bHLH domain of the protein. Moreover, the HASH-1 mutations were located in highly conserved regions of the protein, although not involved in the basic helix–loop–helix consensus motives (13). Together with the expression pattern of *HASH-1* in the developing human embryo, these results cope well with the phenotype of heterozygous *Mash-1*$^{+/-}$ mice (10). These data also suggest that variant *HASH-1* alleles may contribute to the CCHS phenotype. However, *HASH-1* gene mutations are neither necessary (most patients do not have a *HASH-1* gene variant), nor sufficient for CCHS to occur (carriers have no phenotypic expression). This observation is reminiscent of the findings in isolated Hirschsprung disease in the Mennonite population, where endothelin B receptor (EDNRB) mutations are neither necessary nor sufficient for the disease to occur and act in conjunction with a yet unknown hypomorphic *RET* allele (14). The question as to whether *HASH-1* acts as a modifier of *PHOX2B*, and/or a disease-causing autosomal dominant gene with incomplete penetrance remains unresolved.

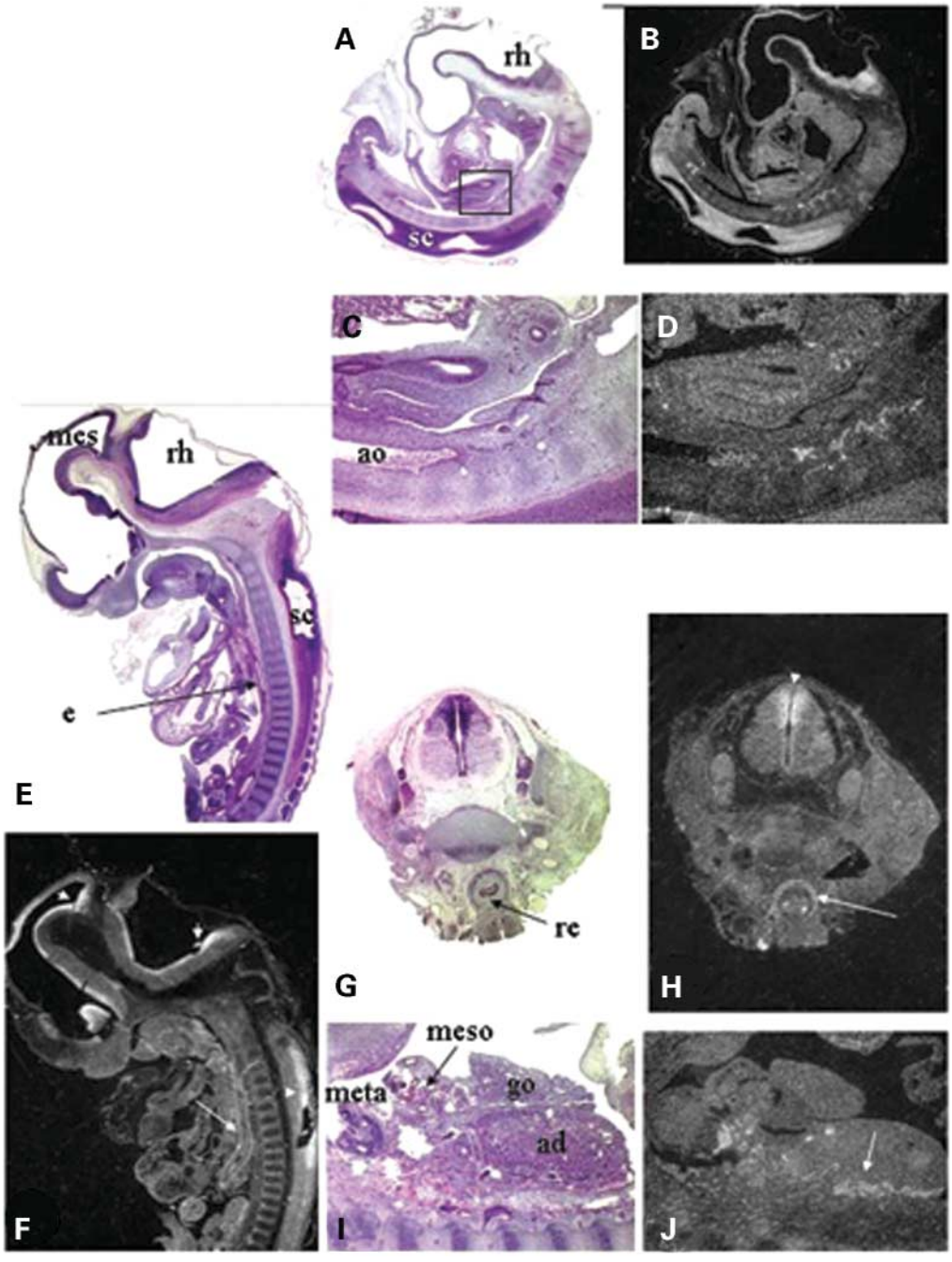

Figure 2. *HASH-1* gene expression in developing human embryos. Slides stained with hematoxylin-eosin (**A**, **C**, **E**, **G**, **I**) and dark-field illumination of the hybridized adjacent sections (**B**, **D**, **F**, **H**, **J**) on day 32 (Carnegie 14, A–D), and day 44 (Carnegie 18, E–J). Parasagittal sections showing weak *HASH-1* expression at Carnegie stage 14 and strong expression of this gene at Carnegie stage 18 in mesencephalon (mes), rhombencephalon (rh), and the dorsal area of the spinal cord (sc) (arrowheads, A, B and E–H, respectively). From Carnegie stage 14, expression was detected in cells to the side of the dorsal aorta (arrow, D). *HASH-1* expression was also detected in the intestine, from the foregut (arrow, F) to the hindgut (arrow, H), and in the adrenal medulla (arrow, J) at Carnegie stage 18. ad, Adrenal gland; ao, aorta; go, gonad; meta, metanephros; mes, mesencephalon; meso, mesonephros; e, esophagus; re, rectum; rh, rhombencephalon; sc, spinal cord.

Polyalanine repeats are common in transcription factors but their normal function is largely unknown. Expansions of polyalanine tracts in transcription factors, including *de novo* *PHOX2B* polyalanine expansions in CCHS, have been shown to be responsible for disease in several malformation syndromes in humans (15,16), whereas contractions have not been reported thus far. Polyalanine contractions may therefore act as hypomorphic alleles.

As the CCHS phenotypes of patients with *PHOX2B* and *HASH-1* mutations are undistinguishable, our data suggest that any mutation affecting the HASH-1–PHOX developmental pathway is likely to impair the development of neurons of the

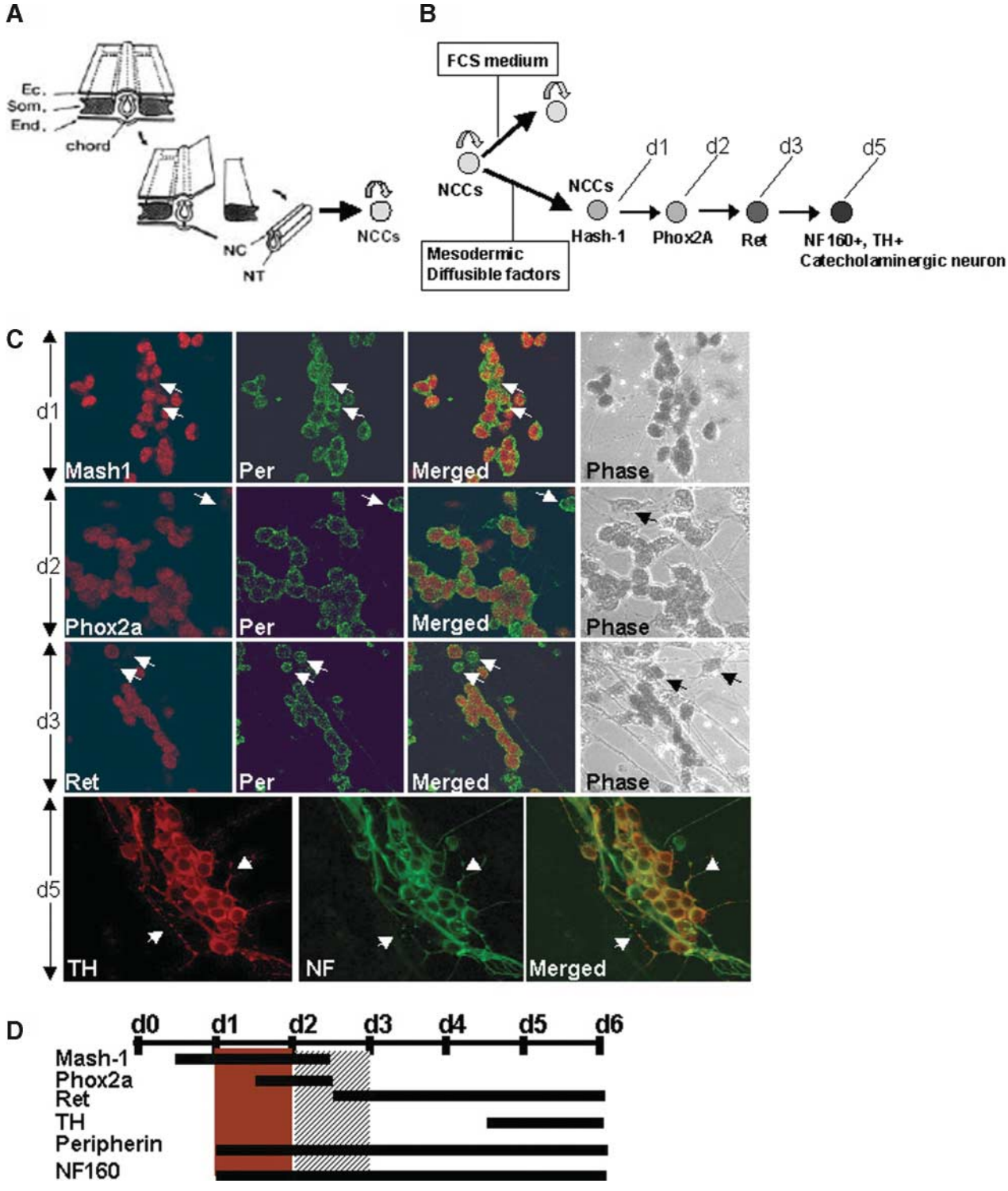

Figure 3. *In vitro* culture model of mouse vagal neural crest cells to analyze the HASH-1–PHOX2B/PHOX2A cascade controlling. (**A**) Experimental design; NCSCs were isolated from E9.5 mouse embryo neural tubes (NT). Ec, ectoderm; Som, somite; End, endoderm; NC, neural crest. (**B**) Transcription-factor cascade controlling the RET and tyrosine hydroxylase (TH) lineage of neural crest cells (NCSCs). Cells cultured in the presence of diffusible embryonic mesodermic factors retain the capacity to generate catecholaminergic derivatives, as shown by neurofilament (NF160) and TH production, recapitulating the MASH1–PHOX2B–PHOX2A–RET pathway as *in vivo* (lower arrow). Conversely, NCSCs cultured in medium containing serum (FCS medium) remain multipotent and retain their proliferative abilities (upper arrow). (**C**) Sequential expression of the *Mash1–Phox2a–Ret–TH* gene pathway, studied in cultured NCSCs by immunohistochemistry (first row), peripherin staining (Per, second row), merged immunohistochemistry and peripherin staining (merged, third row) and *in situ* hybridization (phase, fourth row), from day 1 to day 5 (d1–d5). The Mash1 (d1), Phox2a (d2), Ret (d3) and TH (d5) are shown in red fluorescence; the peripherin (d1–d3) and neurofilament (NF160, NF, d5) are shown in green fluorescence. (**D**) Temporal expression of the *Mash1–Phox2a–Ret–TH* gene pathway during NCSCs culture, from day 0 (d0) to day 6 (d6). The expression timing of Mash1, Phox2a, Ret and TH are indicated by solid bars. Peripherin and NF (NF160) are consistently expressed from d1 to d6.

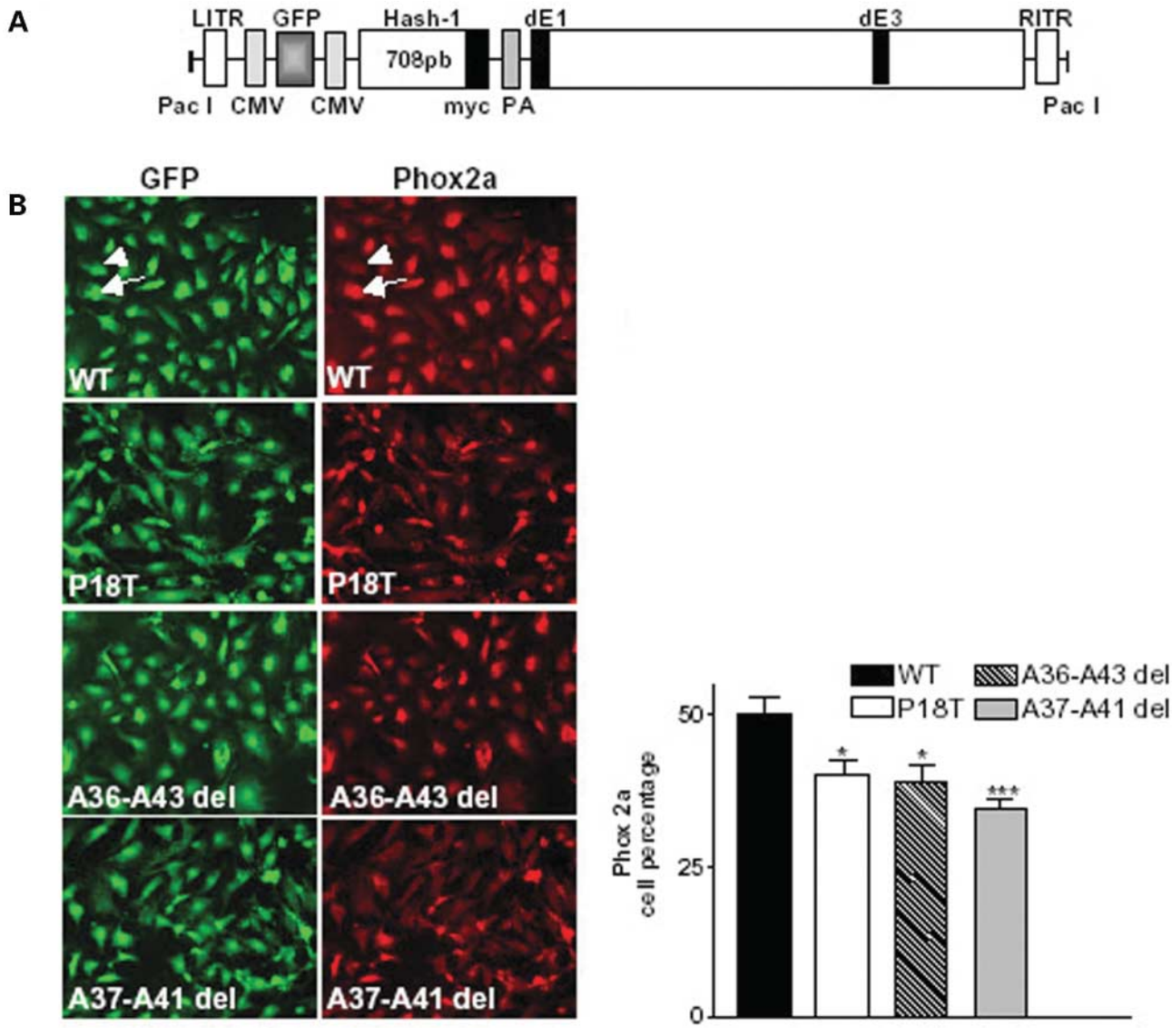

Figure 4. Functional analysis, indicating a decrease in the number of noradrenergic neuronal derivatives obtained from NCSCs after forced expression of the three mutated forms of *HASH-1*, with respect to the wild-type. (**A**) HASH-1 adenovirus constructs for the production of both green fluorescent protein (GFP) and wild-type or mutated forms of HASH1 under cytomegalovirus (CMV) promoter control. (**B**) Left panel: representative samples of infected NCSCs 24 h after infection with adenoviruses producing the wild-type (WT) and mutant (P18T, A36–A43del, A37–A41del) forms of HASH-1. GFP was detected by its green fluorescence and Phox2a was detected by staining with Fast Red. A typical Phox2a-positive cell is indicated by a white arrow; a typical Phox2a-negative cell for is indicated by a white arrowhead. Right panel: percentage of Phox2a positive cells, illustrating the decrease in the number of noradrenergic derivatives obtained from NCSCs after infection with the mutant *HASH-1* retroviral constructs (P18T, A36–A43del and A37–A41del).

noradrenergic lineage and the resulting defect may modify the respiratory network in the brainstem and periphery.

MATERIALS AND METHODS

Patients

A series of 30 patients fulfilled the inclusion criteria for diagnosis of CCHS namely: (i) hypoventilation, hypoxemia and hypercarbia during quiet sleep on polygraphic respiratory recording; with (ii) no cardiac, pulmonary, neuromuscular, EEG or cerebral MRI abnormality (17). Haddad syndrome was diagnosed in 11 cases (36%, five long segment HSCR, two short segment HSCR, four unknown). The histological criteria for HSCR were: (i) absence of enteric plexuses with histological evaluation of the aganglionic tract; and (ii) strong histochemical staining of acetylcholinesterase in nerve fibers. Blood samples were obtained with informed consent and DNA was extracted according to standard protocols.

We screened the coding sequence of the *HASH-1* gene by single-strand conformation polymorphism (SSCP) analysis and/or direct DNA sequencing. The PCR products were heated for 10 min at 95°C, loaded onto a Hydrolink MDE gel

(Bioprobe), and subjected to electrophoresis at 4 W. The gel was then dried and placed against a X-ray film for 48 h. If an abnormal SSCP pattern was observed, direct DNA sequencing was performed by the fluorometric method, for both strands (Big DyeTerminator Cycle Sequencing kit, Applied Biosystems, Warrington, UK).

In situ hybridization in human embryos

Human embryos and fetal tissues were collected from legally terminated pregnancies in agreement with French law and ethics committee recommendations. Tissues were prepared as previously described (18). A 222 bp PCR fragment corresponding to *HASH-1* exon 1 was amplified from human genomic DNA with a T7 extension (TAATACGACTCACTAGGGAGA) added to both primers to generate the sense and antisense probes. Probes were labeled with [^{35}S]UTP and purified as previously described (2). Tissues on slides were dehydrated, placed against BIOMAX MR X-ray film (Amersham) for 3 days, and immersed in Kodak NTB2 emulsion for 3 weeks at +4°C. Developed and toluidine blue-counterstained slides were analyzed with dark- and bright-field illumination. No hybridization signal was detected with the sense probe (data not shown).

Construction of adenoviral plasmids

Adenoviral plasmids were constructed using the Adeasy system (19). Polyalanine tract contractions and the control *HASH-1* coding sequence were amplified by polymerase chain reaction with the 5′ primer, TCTAGACGCATGGAAAGCTCTGCCAA, and the 3′ primer, GTCGACTCAGAACCAGTTGGTGAAGTCGA, using genomic DNA from controls or patients. PCR-derived fragments were checked by sequencing and subcloned into the pAd-track CMV vector, using the *Xba*I–*Sal*I restriction sites. PAd-track CMV vectors were used to produce viruses that could be tracked by following GFP fluorescence. Adenoviral plasmids were generated by homologous recombination in electrocompetent *E. coli* BJ5183, according to the manufacturer's instructions (Stratagene). Adenoviral plasmids were then cut with *Pac*I and viral stocks were produced in QBI-293A cells.

Mouse neural crest culture

Neural crest cells were cultured as previously described (20). Neural tubes were microdissected from Swiss mouse (Iffa Credo) embryos on day 9 of gestation. Somites and surrounding tissues were removed to obtain neural tube segments corresponding to the first seven somites of the vagal region. These segments were then plated on fibronectin-coated glass coverslips (10 µg/ml, Boehringer Roche) and cultured for 2 days in DMEM (Life Technologies) supplemented with 10% fetal calf serum (AbCys) and 100 IU/ml penicillin/streptomycin (Life Technologies), at 37°C, in a humidified atmosphere containing 5% CO_2.

Infection of primary cultures of neural crest cells

We tested various levels of multiplicity of infection (MOI) and decided to use 2000 plaque-forming unit (pfu), which gave 100% infected neural crest cells. Neural crest cells (NCCs) were cultured for 2 days, infected on day 2 and fixed 24 h later.

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