MASH-1/RET pathway involvement in development of brain stem control of respiratory frequency in newborn mice

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Transgenic mice have identified several potentially important genes, such as genes encoding transcription factors that regulate brain stem morphogenesis or cell differentiation (28, 46), genes encoding trophic factors (3, 15, 20), and genes involved in neural crest development (5, 8, 9, 30, 31, 43).

The present study focused on the respiratory phenotype in MASH-1 (mammalian achaete-scute homologous gene) mutants. MASH-1 is a basic helix-loop-helix transcription factor at the top of a hierarchy of genes controlling the differentiation and survival of progenitors of peripheral and central nervous system (CNS) neurons (6, 7, 19, 21, 22, 27, 48). Forced expression of MASH-1 in neural crest cells in vitro induces expression of PHOX2a, a homeobox gene encoding a transcription factor, and of c-RET (“rearranged during transfection”), a gene encoding a tyrosine kinase receptor (36). Homozygous c-RET−/−, MASH-1−/−, or PHOX2a−/− mice die soon after birth (21, 22, 37, 41).

Furthermore, respiratory abnormalities have been found at birth in c-RET−/−, c-RET+/− mice and in MASH-1+/− heterozygous mice (5, 9). However, the neural mechanisms underlying these abnormalities have not been studied.

Here, we tested the hypothesis that the MASH-1 mutation may impair c-RET expression in brain stem neurons important for respiratory rhythm modulation.

Breathing; quantitative reverse transcription-polymerase chain reaction; in situ hybridization; plethysmography

Few data are available on the molecular mechanisms underlying the ontogenesis of the brain stem neuronal network involved in the control of breathing, including rhythm generation and modulation, gasping, and apnea (18, 25, 42). Studies of the respiratory phenotype of
Vaginal plugs were examined on the next morning, and this day was counted as embryonic day 0 (E0). We obtained 171 newborn mice: 35 pups died within 20 min following birth, and 136 were tested for respiratory function. Sample size and genotype are indicated in Table 1. Experimental protocols were approved by our institutional review board and met the animal research guidelines issued by the Institut National de la Santé et de la Recherche Médicale (INSERM, the French National Institute for Health and Medical Research).

Genotyping. After ventilatory measurements, each newborn was weighed, then killed by neck section. Leg and tail tissue fragments were taken for MASH-1 and sry genotyping. These samples were subjected to PCR under standard conditions. The PCR primer sequences were 5’-GCCAGCTATTCCTCCACCTCA-3’ (5’ region of the PGKneo gene) and 5’-GCAGTTGTAAGATGACTAC-3’ (3’ region of the MASH-1 gene). The amplification program involved 34 cycles, each of which consisted of 40 s at 94°C, 40 s at 56°C, and 30 s at 72°C. PCR products were analyzed in 1% agarose gel.

The gender of newborn mice was determined by PCR detection of the chromosome Y sry gene. Primer sequences were 5’-GAGAGCTAGGAGGCCAT-3’ and 5’-CCACTCTCTGTGTGACTAC-3’. The amplification program involved 30 cycles, each of which consisted of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. PCR products were analyzed in 2% agarose gel.

In situ hybridization. We used the mesencephalic and medulla oblongata flexures as anatomical boundaries for brain stem microdissection. The tissues were fixed in 4% paraformaldehyde in PBS, equilibrated with 20% sucrose in PBS, embedded in optimal cutting temperature medium (OCT, Miles), and cut into 10-μm thick sections on a cryostat. Digoxigenin-labeled sense or antisense RNA probes were synthesized from a c-RET cDNA clone (41), using T7- or T3-primed in vitro transcription with digoxigenin-UTP (dig-UTP, Boehringer). The probe (1 μg/μl) was hybridized overnight at 65°C to mouse embryo sections. The slides were washed twice with 50% formamide/1×SSC at 65°C, then twice for 30 min each time in a solution of 100 mM maleic acid, 50 mM NaCl, and 0.1% Tween-20 (MABT), at room temperature. The slides were incubated overnight at room temperature with an alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Boehringer). The hybridized probe was then detected by incubation with the BM purple substrate (Boehringer) for 6–12 h. For each in situ hybridization experiment, a sense probe was hybridized as a control. Neuroanatomical structures were identified using a prenatal mouse brain atlas (45).

<table>
<thead>
<tr>
<th>Table 1. MASH-1 population</th>
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<tr>
<td>171 MASH-1 Pups (85 males and 86 females)</td>
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<tr>
<td>136 Survivors</td>
</tr>
<tr>
<td>+/+</td>
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<tr>
<td>Sample size 20 min after birth</td>
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<tr>
<td>RT-PCR</td>
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<td>Trop/RT-PCR</td>
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Mortality was assessed before ventilatory measurements. Numbers of gasping pups are indicated in parentheses. All pups were killed for genotyping after ventilatory measurements. RT-PCR was performed in two of the 35 dead pups and in 11 of the 136 survivors (10 pups were used for the covariation study). Trop, breath duration.

Quantitative RT-PCR. The brain stems of 13 pups (4 MASH-1 +/+, 5 MASH-1 +/−, and 4 MASH-1 −/−) were microdissected as for in situ hybridization, then frozen in liquid nitrogen. The RNAs were extracted using Trizol reagent (GIBCO-BRL) as recommended by the manufacturer. Aliquots (2 μg each) of total RNA were reverse-transcribed into first-strand cDNA using 200 U Superscript II Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo(dT) primer (Life Technologies) according to the manufacturer’s instructions. Primer Express software (Perkin-Elmer Applied Biosystems) was used to design probes that amplify a single region of c-RET or cyclophilin. Primers (from Genset) were as follows: c-RET R5, TAC TGA CCA TGG GTG ACC TC; c-RET R3, ACT GAA CCT GAC CGT ACA AGC CCA AAG TCG GAA ATC T; MASH-1 M5, ACT TGA ACT CTA TGG CGG GT; MASH-1 M3, ACT GAA CCT GAC CGT ACA GCT TCC AAA GTC CAT TCC C; Cyclo forward, GGT CTT TGG GAA GGT GAA AG; Cyclo reverse, ACT GAA CCT GAC CGT ACA GGT TTG ATG GAA ATG CC.

All PCR reactions were performed using the AmpliTaq Universal Amplification and Detection System (Intergen, NY). Briefly, each amplification mix (25 μl) contained about 200 ng of sample DNA, 1.25 U of Platinum Taq (GIBCO), 1× buffer, 1.5 mM MgCl2, 250 μM dNTPs, 500 μM Uniprim, 500 nM forward primer, and 50 nM reverse primer (Z sequence added). Amplifications were carried out on an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised denaturation at 94°C for 4 min followed by 50 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 40 s.

For MASH-1 +/+, MASH-1 +/−, and MASH-1 −/− brain stem samples, target gene (c-RET), standard gene (cyclophilin), and no-template control were run in triplicate. A standard curve was constructed from plasmid serial dilutions by plotting the cycle threshold (Ct) vs. the known numbers of standard gene copies. Using this standard curve, all Ct samples were converted to copy numbers. The c-RET transcript levels in MASH-1 +/− and MASH-1 −/− mice were divided by cyclophilin transcript levels, and this ratio was expressed as the percentage of levels in MASH-1 +/+ mice.

Respiratory phenotype. Respiratory phenotype was assessed in 136 surviving pups based on mortality rates, neonatal adaptation scores, and plethysmographic measurements of baseline breathing within 2 h after birth. Embryos were delivered by caesarean section to control for gestational age (9, 30). Pregnant mice were killed by dislocation of the neck on day 18.5 postcoitum (30), i.e., 1 day before normal littering (E19.5) for this background. All pups were stimulated by pinching the thorax and were placed immediately after birth in a thermoregulated box for 20 min at 32°C (the temperature in normal litters) to minimize the after-effects of caesarean section. The time from death of the dam to extraction of the last pup was 255 ± 64 s.

Adaptation to extra-uterine life was assessed just before ventilatory measurements, based on a score similar to the Apgar score used for human neonates. This score was obtained by assigning a value on a three-point scale (0 to 2) to the four following characteristics: color (marked cyanosis, mild cyanosis, or pink), breathing (apneas, irregular breathing, or regular breathing), spontaneous motor activity (no movements, weak movements, or vigorous movements), and reactivity to tail pinching (no response, movements, or movements and cries). These four values were added to yield a global score that could range from 0 (bad adaptation) to 8 (good adaptation).
Breathing variables were measured noninvasively using whole body barometric plethysmography, based on the principle of Drorbaugh and Penn (11, 13, 14, 38, 40). According to this principle, when an animal breathes in a nondistensible chamber, the pressure in the chamber increases during inspiration owing to addition of water vapor to the inspired gas and to warming of the inspired gas from the temperature in the chamber to that in alveoli. Conversely, pressure decreases during expiration owing to condensation of water vapor and cooling of expired gas. In practice, pressure is measured with respect to an identical reference chamber connected to the animal chamber through a slow leak, thus isolating measurements from disturbances in ambient pressure. This differential pressure is used to calculate breath duration (TTOT, s), tidal volume (VT, μl), and ventilation (VE, calculated as VT/TTOT, and expressed in μl/s). VT and VE are divided by body weight and expressed in microliters per gram and microliters per second per gram, respectively. The differential pressure is also used to detect gasping, which is an autosusceptuation mechanism consisting of low-frequency, brief, maximal inspiratory efforts responsible for rapid reoxygenation. Gasping differs from eunpnea in that the increase in inspiratory activity and amplitude of inspirations are greater. In addition, gasping is accompanied by a stereotyped motor pattern characterized by mouth opening and by neck and forelimb extension.

The plethysmograph used for the newborn mice has been described previously (9, 43). Briefly, the system was composed of two 30-ml syringes serving as animal and reference chambers, respectively, connected to each other by a catheter (time constant, 2 s). The syringes were immersed in a thermoregulated water bath that maintained the temperature inside the syringes between 28 and 30°C. These temperatures were measured continuously. To avoid having to restrain the animals, body temperature was not recorded and was assumed to be stable at 32°C (the temperature measured in several litters at the same age). The differential pressure between the animal and the reference chambers (EPPA pressure transducer, range ± 0.1 mb; Asnières, France) was filtered (bandwidth, 0.05–15 Hz at –3 dB), converted to a digital signal (MacAdios A/D 12-bits converter; GW Instruments, Somerville, MA) at a sample rate of 100 Hz, and processed by custom-written software (Software Superscope II, GW Instruments) run on a Powerwave 604/150 computer (Mac OS 8.0) to calculate TTOT (s), VT (μl/g), and VE (μl/s•1•g⁻¹). Calibration was done before each test by injecting 2 μl of air into the animal chamber from a Hamilton syringe. The pressure rise induced by this injection was of similar magnitude to that induced by the VT of a newborn mouse. After calibration, the animal was placed in the plethysmograph for 1 min of familiarization. Then, baseline ventilation was recorded for 90 s.

Among survivors, the three genotype groups (MASH-1 +/+ , MASH-1 +/−, and MASH-1 −/−) used for the study of breathing variables were not significantly different in terms of age at breathing variable measurements, body weight, or temperature after breathing variable measurements (Table 2).

Statistics. The adaptation score, breathing variables (TTOT, VT, and VE), and gasping were determined from ventilatory traces before genotype determination. Mortality and occurrence of gasping were compared among genotype groups using Fisher exact probability test. We used ANOVAs to compare adaptation scores, breathing variables, and c-RET transcript levels among groups (Superanova Software; Abacus Concepts, Berkeley, CA). Differences were considered significant at a confidence level >95% (P < 0.05).

RESULTS

Lack of c-RET expression in MASH-1 −/− brain stem nuclei. The comparison of brain stem c-RET expression in MASH-1 +/+ and MASH-1 −/− newborns is summarized in Table 3.

In MASH-1 +/+ mice, the noradrenergic nuclei A5 and A6 did not express c-RET (Fig. 2). Neither was c-RET expression found in A2 and A7 (not shown). Decreased c-RET expression was found in scattered cells of the rVLM in MASH-1 −/− mice (Fig. 2).

In contrast, c-RET expression was present in the motor nuclei of the IIIrd, Vth, VIIth, Xth, and XIIth cranial nerves and in the midbrain dopaminergic neurons (A9 and A10) of MASH-1 −/− mice (not shown).

Table 3. Distribution of c-RET mRNA in the brainstem in MASH-1 +/+ and MASH-1 −/− mice

<table>
<thead>
<tr>
<th>Regions expressing c-RET</th>
<th>MASH-1 +/+</th>
<th>MASH-1 −/−</th>
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<tbody>
<tr>
<td>Noradrenergic nuclei</td>
<td>++</td>
<td>–</td>
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<tr>
<td>A2</td>
<td>++</td>
<td>–</td>
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<tr>
<td>A5</td>
<td>++</td>
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<td>A6</td>
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<td>A7</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Dopaminergic nuclei</td>
<td>++</td>
<td>++</td>
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<tr>
<td>A9</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>A10</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cranial nerve motor nuclei</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>III</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>VII</td>
<td>++</td>
<td>++</td>
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<tr>
<td>X</td>
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<tr>
<td>XII</td>
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<tr>
<td>NTS</td>
<td>++</td>
<td>++</td>
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<tr>
<td>rVLM</td>
<td>++</td>
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Hybridization signal intensity was evaluated as weak (+), moderate (++), or strong (+++). The minus sign (−) indicates an inability to detect a signal above the background noise. NTS, nucleus tractus solitarius; rVLM, rostral ventrolateral medulla.
This suggests that the development of these structures may be driven entirely by a MASH-1-independent mechanism.

MASH-1 mutation impairs respiratory phenotype. Mortality 20 min after birth was higher in MASH-1 −/− than in MASH-1 +/− and MASH-1 +/+ mice (main effect for group, P = 0.003; comparison of MASH-1 −/− vs. MASH-1 +/+ and MASH-1 +/−, P = 0.001; Fig. 3). Scores at birth in MASH-1 −/− mice showed wide variability in terms of variance (0.8, 0.8, and 5.5 in MASH-1 +/+, MASH-1 +/−, and MASH-1 −/− mice, respectively) and range (2–8, 4–8, and 0–8).

Gasing occurred more frequently in MASH-1 −/− than in MASH-1 +/− and MASH-1 +/+ mice (21.4%, 2.8%, and 2.0%, respectively; Fisher exact probability test, P = 0.015; Fig. 3). This indicated severe impairment of oxygenation in MASH-1 −/− mice, probably respiratory in origin, and demonstrated that the neuronal mechanisms needed for gasing were not abolished by MASH-1 deletion. Two of the three MASH-1 −/− newborns with gasing failed to return to eupnoea and eventually died. The three remaining pups with gasing (1 MASH-1 +/+ and 2 MASH-1 +/−) promptly returned to eupnoea.

Plethysmographic measurements showed that the group of MASH-1 +/+ mice had longer breath durations than the group combining MASH-1 +/− and MASH-1 +/+ mice (main effect for group, P = 0.026; partial comparison MASH-1 +/− vs. MASH-1 +/+ and MASH-1 +/− mice, P = 0.022; Fig. 4). VT levels were not significantly different in MASH-1 +/+, MASH-1 +/−, and MASH-1 −/− mice (2.7 ± 0.5 /l, 2.6 ± 1.1, and 2.8 ± 1.7 µl/g, respectively). The group difference in Ve levels was not statistically significant (4.1 ± 1.5, 4.9 ± 2.0, and 5.0 ± 2.7 µl/s·g−1, respectively; main effect for group, P = 0.057). The number of apneas, duration of apneas, and apnea frequency per minute were not significantly different across groups (not shown).
Breath duration correlates with c-RET transcript levels in brain stem. The c-RET transcript levels covaried with MASH-1 genotype. The c-RET levels in MASH-1 \(+/--\) and in MASH-1 \(++/--\) mice were lower than in MASH-1 \(++/+\) mice \((P = 0.001\) and \(P = 0.003\), respectively; Fig. 5). There was no significant difference between c-RET transcript levels in MASH-1 \(++/--\) and MASH-1 \(++/+\) mice. The c-RET transcript levels in MASH-1 \(++/--\) mice \((-58\%\) of the value in MASH-1 \(++/+\) mice) confirmed the in situ hybridization result, indicating that c-RET expression occurred in the absence of MASH-1.

A positive correlation, weak but significant, was found between c-RET transcript levels and breath duration \((P = 0.032\), Fig. 6\), suggesting that the lower c-RET transcript levels in the brain stem of MASH-1 \(++/--\) mice may contribute to the abnormal respiratory frequency in these animals.

**DISCUSSION**

The aim of this study was to test the hypothesis that the MASH-1 mutation may impair c-RET expression in brain stem neurons involved in the control of breath-
MASH-1 mutation impaired c-RET expression in the brain stem noradrenergic neurons involved in respiratory control. Quantitative RT-PCR showed that the MASH-1 mutation impaired c-RET expression in the brain stem: both MASH-1 +/- and MASH-1 +/- mice had lower c-RET levels than MASH-1 +/+ mice. These results indicate that MASH-1 is upstream of c-RET in the development of brain stem neurons. Moreover, in situ hybridization showed that the MASH-1 mutation altered c-RET expression in several brain stem neuronal groups identified as noradrenergic nuclei A2, A5, A6, and A7 based on their anatomical location. Most of these noradrenergic nuclei with impaired c-RET expression are important for breathing control (1, 10, 16, 25, 26). Evidence exists that A6 may also contribute to respiratory rhythmogenesis in fetal and neonatal rodents (10, 17, 29, 42, 47). This region overlaps the noradrenergic A1 and adrenergic C1 neurons. Some A1 neurons are intermingled with neurons of the rostral ventral respiratory group, and some C1 neurons are intermingled with those of the Bötzing complex (12), thus suggesting a contribution of these neurons to the modulation of respiratory activity (2, 12). Our data do not allow characterization of the c-RET expressing cells in the rVLM. However, this limitation does not detract from our evidence that the MASH-1 mutation impairs c-RET expression in brain stem regions involved in breathing control.

MASH-1 mutation impairs respiratory phenotype. Loss of a single MASH-1 allele was sufficient to shorten breath duration: MASH-1 +/- and MASH-1 +/+ newborns showed similar breath durations, which were significantly shorter than those of MASH-1 +/-.
newborns, thus leading to increased breathing frequency. A positive correlation, weak but significant, was found between c-RET transcript levels and breath duration, further supporting regulation by MASH-1 of c-RET expression in brain stem structures important in respiratory rhythmicity. This increase in breathing frequency may be ascribable to withdrawal of inhibitory noradrenergic influences. Thus the noradrenergic abnormalities in MASH-1 −/− mice may have altered the perinatal maturation and/or the control of the respiratory network. This interpretation is supported by ongoing in vitro experiments in neonatal MASH-1 −/− mice (Viemari JC and Hilaire G, unpublished data).

It is not clear from our data what impairment could explain the mortality in MASH-1 −/− mice. The MASH-1 mutation did not abolish gasping, showing that c-RET expression in brain stem noradrenergic neurons is not important for the development of gasping networks (35, 49). Ventilation was not decreased in MASH-1 −/− mice compared with MASH-1 +/+ and MASH-1 +/+ mice. MASH-1 −/− newborns had short breath durations similar to those of MASH-1 −/− mice, but had a low mortality rate similar to that in MASH-1 +/+ mice. This suggests that the mortality in MASH-1 −/− mice was perhaps not primarily related to breathing. However, mortality may have been caused by respiratory factors not detected by the present analysis. First, the early mortality in MASH-1 −/− mice may be related to impairment of A6 neurons, which mediate a strong arousal response at birth, accompanied with a marked increase in noradrenergic activity (32, 34). This activation is important for the forebrain drive to breathing (34). Second, another possible explanation to the mortality in MASH-1 −/− mice may be impaired chemosensitivity to hypercapnia, as previously described in c-RET −/− (5) and MASH-1 +/+ mice (9). The impaired c-RET expression in brain stem noradrenergic neurons, which are normally activated by hypercapnia (4, 24), may account for this impairment.

Finally, the variability of adaptation to extra-uterine life in MASH-1 −/− mice, with half the MASH-1 −/− mice dying within a few minutes, can be ascribed to background-modifying genes. The hybrid background used in the present study induces allelic variability, which may have a strong impact on phenotype (39, 44). Preliminary results from our laboratory showed that the effects of the MASH-1 mutation on noradrenergic enteric neuron development differed among 129SvEv, C57BL6/J, BALB/c, and C3H embryos. Background-modifying genes may similarly affect the noradrenergic respiratory brain stem network, thus accounting for

Fig. 5. c-RET transcript levels in brain stems of MASH-1 +/+ (n = 4), MASH-1 +/− (n = 5), and MASH-1 −/− (n = 4) mice measured by quantitative PCR. A: schematic representation of the brain stem microdissection used for quantitative PCR analysis. Di, diencephalon; Cer, cerebellum; Mes, mesencephalon; Met, metencephalon; Mye, myelencephalon; and 4°V, fourth ventricle. B: c-RET transcript levels in MASH-1 +/− and MASH-1 −/− mice were divided by cyclophilin transcript levels, and this ratio was expressed as the percentage of levels in MASH-1 +/+ mice. The c-RET levels were lower in the MASH-1 +/− and the MASH-1 +/+ mice than in the MASH-1 −/− mice (P = 0.001 and P = 0.003, respectively). Values are means ± SE. NS, not significant.

Fig. 6. Correlation between breath durations and c-RET/cyclophilin transcript ratio in surviving nongasping MASH-1 −/− (n = 3), MASH-1 +/− (n = 4), and MASH-1 +/+ (n = 3, P = 0.031) mice. Same mice as in Fig. 5, except for one MASH-1 +/+ mouse and one MASH-1 −/− mouse that died before being subjected to respiratory testing, as well as one gasping MASH-1 +/+ mouse. The c-RET transcript levels in MASH-1 +/+ and MASH-1 −/− mice were divided by cyclophilin transcript levels, and this ratio was expressed as the percentage of levels in MASH-1 +/+ mice.

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the wide variability in mortality in MASH-1+/− newborns.

Conclusion and perspectives. In conclusion, this study provides evidence that MASH-1 is upstream of c-RET in noradrenergic brain stem neurons important for breathing control and, more specifically, for respiratory rhythm modulation. Studies of the MASH-1 mutation in mice with a pure genetic background may identify modifier genes that affect the respiratory phenotype. Combined analysis of respiratory phenotypes and of genomes in neonatal mice with the MASH-1 mutation in a pure genetic background may provide insight into the molecular mechanisms of respiratory rhythm modulation.

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