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SHORT COMMUNICATION

Respiratory consequences of targeted losses of *Hoxa5* gene function in mice

Kim Landry-Truchon[1,2,*], Stéphanie Fournier[3,4,*], Nicolas Houde[1,2], Jean-Philippe Rousseau[3,4], Lucie Jeannotte[1,2,‡] and Richard Kinkead[3,4,‡]

ABSTRACT

Fetal development of the respiratory tract and diaphragm requires strict coordination between genetically controlled signals and mechanical forces produced by the neural network that generates breathing. HOXA5, which is expressed in the mesenchyme of the trachea, lung and diaphragm, and in phrenic motor neurons, is a key transcription factor regulating lung development and function. Consequently, most *Hoxa5*$^{-/-}$ mutants die at birth from respiratory failure. However, the extensive effect of the null mutation makes it difficult to identify the origins of respiratory dysfunction in newborns. To address the physiological impact of *Hoxa5* tissue-specific roles, we used conditional gene targeting with the *Dermo1*^{Cre} and *Olig2*^{Cre} mouse lines to produce specific *Hoxa5* deletions in the mesenchyme and motor neurons, respectively. *Hoxa5* expression in the mesenchyme is critical for trachea development, whereas its expression in phrenic motor neurons is essential for diaphragm formation. Breathing measurements in adult mice with whole-body plethysmography demonstrated that, at rest, only the motor neuron deletion affects respiration, resulting in higher breathing frequency and decreased tidal volume. But subsequent exposure to a moderate hypoxic challenge ($F_{I_{O_2}}$=0.12; 10 min) revealed that both mutant mice hyperventilate more than controls. *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ mice showed augmented tidal volume while *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice had the largest increase in breathing frequency. No significant differences were observed between medulla–spinal cord preparations from E18.5 control and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mouse embryos that could support a role for *Hoxa5* in fetal inspiratory motor command. According to our data, *Hoxa5* expression in the mesenchyme and phrenic motor neurons controls distinct aspects of respiratory development.

KEY WORDS: Transcription factor, Respiration, Mutant mouse models, Diaphragm

INTRODUCTION

Breathing is a primal behavior that requires functional respiratory and nervous systems to produce rhythmic contractions of the diaphragm to generate airflow within the lungs. Development of the respiratory tract and diaphragm during fetal life must coincide with the onset of the respiratory motor command to ensure proper breathing at birth and throughout life. In mice, respiratory tract development begins around embryonic day (E)9, with evagination and elongation of the foregut endoderm into the adjacent mesenchyme to give rise to trachea and primary bronchi. Lung bud branching then generates a highly arborized airway tree with thousands of terminal saccules that mature postnatally to form alveoli. Throughout development, lung mesenchyme interacts with (and thus instructs) the flanking endoderm to promote airway branching morphogenesis and differentiation of the respiratory epithelium. All these events are tightly controlled by genetic pathways involving transcriptional regulators and signaling molecules (Herriges and Morrisey, 2014; Chang et al., 2013).

Mechanical forces resulting from intrathoracic pressure, amniotic fluid volume and fetal breathing movements are also necessary to promote proliferation and differentiation of lung tissues (Inalou et al., 2005). In mice, rhythmic motor activity produced by the medullary network begins at E15 (Thoby-Brisson et al., 2005; Greer, 2012). This neural activity promotes the formation of neuromuscular junctions, and electrically mediated effects combined with diffusible substances ensure proper development and function of the diaphragm as the major respiratory muscle (Greer et al., 1999; Hall and Sanes, 1993; Wakelam, 1985). Defects that interfere with any aspect of this process can impair lung growth, causing pulmonary hypoplasia, and, in turn, compromise respiratory function and viability (Wigglesworth and Desai, 1979; Liggins et al., 1981; Jay et al., 2007; Greer, 2012).

HOXA5, a transcription factor regulating lung development and function, is expressed in the mesenchyme of the trachea, lung and diaphragm, and in phrenic motor neurons innervating the diaphragm. Consistent with this expression pattern, *Hoxa5*$^{-/-}$ mutant mice display occlusion of the trachea with abnormal cartilage patterning, lung hypoplasia, cell misspecification and defective diaphragm innervation (Aubin et al., 1997; Boucherat et al., 2013). Consequently, 50–70% of *Hoxa5*$^{-/-}$ mutants die at birth from respiratory failure. Survivors display lung airspace enlargement, abnormal elastin deposition and mucus hypersecretion (Mandeville et al., 2006). *Hoxa5*$^{-/-}$ mice compensate for these morphological deficits by increasing breathing frequency and overall minute ventilation (Kinkead et al., 2004). However, the broad impact of the null mutation makes it difficult to understand the origins of respiratory dysfunction in newborns.

Using a conditional gene-targeting approach with *Dermo1*^{Cre} and *Olig2*^{Cre} deleter mouse lines, we have addressed the tissue-specific roles of *Hoxa5* in mesenchyme and motor neurons, respectively (Landry-Truchon et al., 2017). Both mouse lines presented lung hypoplasia. *Hoxa5* ablation in the mesenchyme recapitulated the abnormal patterning of tracheal cartilage and the lung cell misspecification defects of *Hoxa5* null embryos, but did

[1]Centre de recherche sur le cancer de l'Université Laval, CRCHU de Québec, Hôtel-Dieu de Québec, Québec, QC, Canada, G1R 3S3. [2]Department of Molecular Biology, Medical Biochemistry and Pathology, Université Laval, Québec, QC, Canada, G1V 0A6. [3]CRIUCPQ, Québec, QC, Canada, G1V 4G5. [4]Department of Pediatrics, Université Laval, Québec, QC, Canada, G1V 0A6.
*These authors contributed equally to this work

‡Authors for correspondence (lucie.jeannotte@crhdq.ulaval.ca; richard.kinkead@fmed.ulaval.ca)

L.J., 0000-0001-5578-420X; R.K., 0000-0001-9686-2577

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not affect diaphragm formation and did not cause neonatal mortality of mutants. Conversely, deletion of *Hoxa5* in motor neurons resulted in defective diaphragm innervation, fewer neuromuscular contacts and diaphragmatic muscle atrophy, and it reproduced death at birth.

To further our understanding of the genetic regulation of respiratory ontogeny, we investigated the physiological impact of the *Hoxa5* conditional deletion in the mesenchyme or motor neurons. We first determined whether the *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ genotypes affect breathing pattern in adult mice using whole-body plethysmography. We also tested the hypothesis that the *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ deletion reduces phrenic motor output produced by the central respiratory network from mouse embryos at E18.5, using electrophysiological recordings on isolated medulla–spinal cord preparations. Our data reveal that *Hoxa5* expression in the mesenchyme and phrenic motor neurons controls distinct aspects of respiratory development.

MATERIALS AND METHODS

Mice

Hoxa5 null and *Hoxa5*$^{flox/flox}$ mouse lines were described previously (Jeannotte et al., 1993; Tabariès et al., 2007). *Hoxa5*$^{flox/flox}$ mice were used as control for these experiments. *Olig2*^{Cre} and *Dermo1*^{Cre} deleter mice were obtained from Jeremy Dasen (Dessaud et al., 2007) and David Ornitz (Yu et al., 2003), respectively. Mice were maintained in the 129/Sv genetic background. Following mating, the presence of a vaginal plug was considered as E0.5. Experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the Institutional Animal Care Committee.

Respiratory measurements: whole-body plethysmography (*in vivo*)

Ventilatory measurements were performed on 41 adult mice (21 males and 20 females) aged between postnatal day (P)30 and P57 while breathing room air (fraction of inspired O_2, $F_{I_{O_2}}$=0.21) followed by exposure to hypoxia ($F_{I_{O_2}}$=0.12; Kinkead et al., 2004). Airflow through the chamber was set to 200 ml min^{-1}. The system was calibrated by injecting 0.5 ml of air into the chamber. Barometric pressure, chamber temperature and humidity, and mouse body temperature (T_b) were measured in normoxia and at the end of hypoxia to correct the tidal volume (V_T) and minute ventilation ($\dot{V}_I$), and values are expressed in ml BTPS (body temperature, pressure standard) (Drorbough and Fenn, 1955). The composition of gas mixtures flowing into and out of the chamber was analyzed for calculation of oxygen consumption ($\dot{V}_{O_2}$; Mortola and Dotta, 1992).

Normoxia

Rectal temperature was measured before placing the mouse in the plethysmograph. Baseline measurements were made for 30 min when the animal was quiet and breathing room air. Breathing frequency (f_R), V_T and $\dot{V}_I$ were recorded (IOX, EMKA Technologies, Falls Church, VA, USA), and averaged over the 5 min preceding hypoxia.

Hypoxic ventilatory response (HVR)

Mice were exposed to moderate hypoxia by replacing air with a pre-mixed gas (12% O_2, balance N_2) for 10 min. Peak HVR magnitude was assessed by averaging ventilatory variables between the third and fifth minute of hypoxia and results are expressed as the percentage change from baseline values. HVR is bi-phasic (Powell et al., 1998), and therefore this period was chosen to quantify the rapid increase in breathing associated with chemoreceptor activation at the onset of hypoxia. The late phase of the response reflects sustained ventilatory effort and the impact of hypoxia on neuronal networks; this was assessed by averaging values over the last 2 min of hypoxia.

Respiratory measurements: isolated brainstem preparations (*in vitro*)

Electrophysiological measurements were performed on isolated brainstem–spinal cord preparations from 12 *Hoxa5*$^{flox/flox}$ control and 8 *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ E18.5 fetuses according to standard procedures (Fournier et al., 2013). Following decerebration, the medulla and rostral spinal cord were dissected and put in a recording chamber irrigated with artificial cerebrospinal fluid at 5 ml min^{-1} (129 mmol l^{-1} NaCl, 3.35 mmol l^{-1} KCl, 1.15 mmol l^{-1} $MgCl_2$, 30 mmol l^{-1} D-glucose, 21 mmol l^{-1} $NaHCO_3$, 1.26 mmol l^{-1} $CaCl_2$, 0.58 mmol l^{-1} NaH_2PO_4). The superfusate was maintained at 27°C and equilibrated with a 95% O_2+5% CO_2 gas mixture (pH 7.4±0.1). Bursts of inspiratory (phrenic)-related motor activity were recorded extracellularly from the cervical (C)3 or C4 nerve rootlet using a suction electrode. The signal was amplified, rectified and integrated. Once the preparation produced stable rhythmic activity (~30 min), baseline recording was performed for 5 min.

Histology and morphometric measurements

After physiological experiments, the trachea and diaphragm from control and mutant adults and the diaphragm from E18.5 control and mutant embryos were collected. Five specimens per genotype were embedded in paraffin, sectioned (4 µm) and stained with Alcian Blue/nuclear Fast Red or hematoxylin/eosin.

Leica SCN 400 F SlideScanner and SlidePath Gateway software were used to measure the tracheal luminal surface (at six rostro-caudal locations) and the diaphragm thickness (at eight dorso-ventral locations in the right costal muscle).

Statistical analyses

Student's *t*-tests were performed to evaluate the effect of genotype on lung mass/body mass ratio, tracheal luminal surface and diaphragm thickness. $P<0.05$ was considered statistically significant. For physiological experiments, the effect of genotype, stimulus and time was assessed using analysis of variance (ANOVA; Statview version 5.0, SAS Institute, Cary, NC, USA). A repeated-measures design was used when appropriate. The ANOVA were followed by Fisher's protected least significant difference test ($P<0.05$) whenever a factor (or interaction between factors) was significant and thus justified a *post hoc* test. ANOVA results are reported in the text or figure legends; results from *post hoc* tests are displayed as symbols in figures.

RESULTS AND DISCUSSION

Specific morphological defects of the respiratory system in *Hoxa5* conditional mutants

Breathing movements and upper airway resistance are essential for the maintenance of lung distension (Kotecha, 2000). Tracheal malformations and abnormal diaphragm innervation are fully penetrant in *Hoxa5*$^{-/-}$ embryos (Boucherat et al., 2013). Here, we examined trachea and diaphragm of adult mutants. *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ mice were the only ones to present tracheal anomalies that recapitulated the *Hoxa5*$^{-/-}$ phenotype, including incompletely formed cartilaginous rings, hypertrophy of the lamina propria and decreased tracheal luminal surface (by 37%) (Fig. 1A–C,L). Conversely, the diaphragm was normal in *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ adults. However, like *Hoxa5*$^{-/-}$ animals, the ventral costal diaphragm in *Hoxa5*$^{flox/flox}$;

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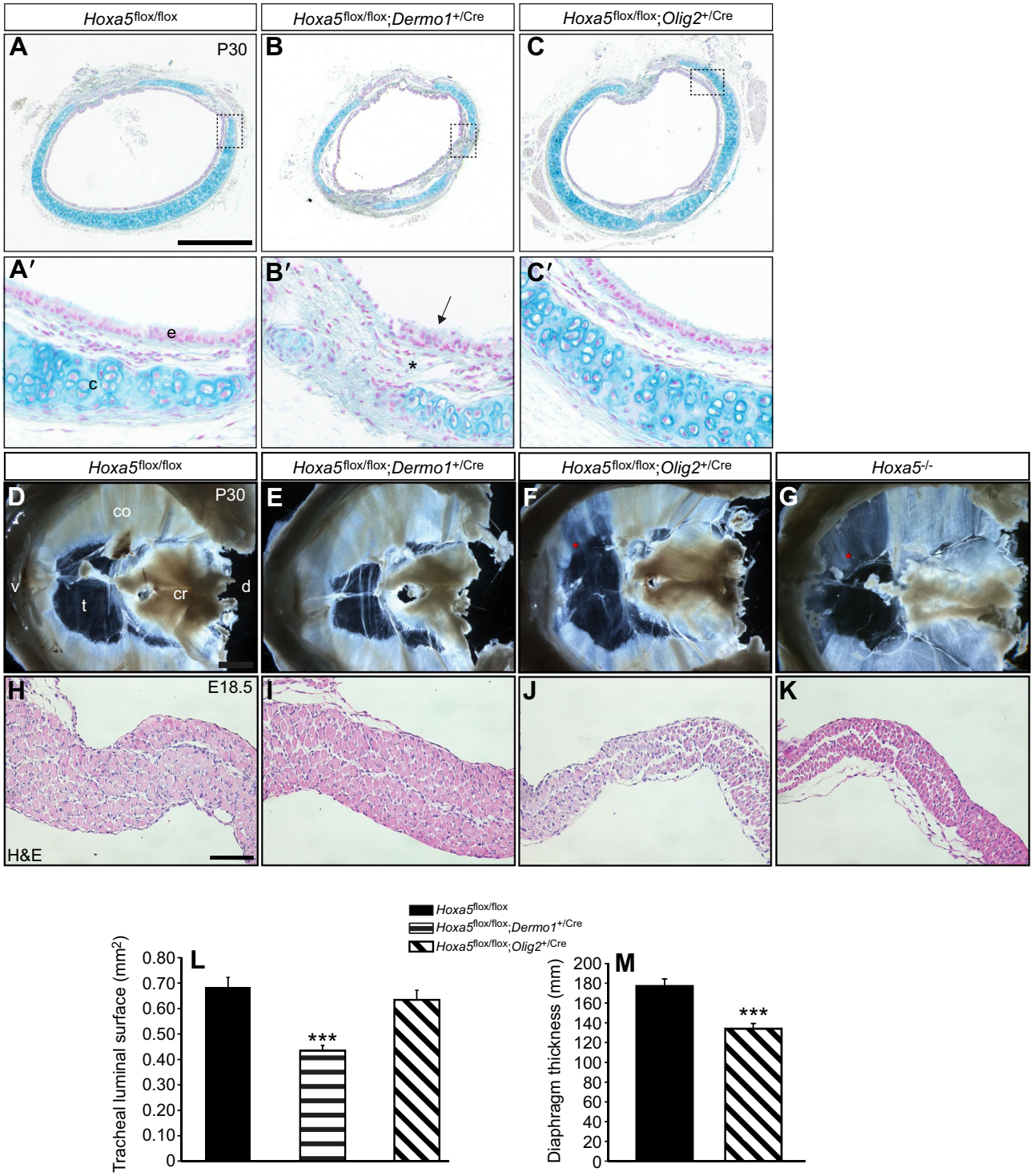

Fig. 1. Trachea and diaphragm muscle are differentially affected by *Hoxa5* conditional mutations. (A–C) Trachea from *Hoxa5*$^{flox/flox}$ (control; A), *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ (B) and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ (C) adult mice. Boxed regions are shown at higher magnification in A′–C′. Alcian Blue staining revealed tracheal cartilage defects, epithelium disorganization (arrow) and thicker submucosa (asterisk) in *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ specimens. c, cartilage; e, epithelium. (D–G) Whole diaphragms from *Hoxa5*$^{flox/flox}$ (D), *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ (E), *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ (F) and *Hoxa5*$^{-/-}$ (G) adult mice. *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ and *Hoxa5*$^{-/-}$ mutant mice presented an irregular distribution of ventral costal muscles (red asterisks). co, costal muscle; cr, crural muscle; d, dorsal; t, tendon; v, ventral. (H–K) Comparative histology of tissue sections stained with hematoxylin/eosin (H&E) showed thinner diaphragms in E18.5 *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ (J) and *Hoxa5*$^{-/-}$ (K) mutant embryos. (L) The tracheal luminal surface was significantly reduced at postnatal day (P)30. (M) Measurements of diaphragm thickness revealed significant differences between genotypes at embryonic day (E)18.5. Values are expressed as means±s.e.m. ***P<0.001. Scale bars: A–C, 500 µm; D–G, 2 mm; H–K, 100 µm.

Olig2$^{+/Cre}$ surviving mice was atypical, showing weaker musculature and resembling the central tendinous region (Fig. 1D–G). Further, diaphragm skeletal muscle was significantly thinner in E18.5 *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ and *Hoxa5*$^{-/-}$ mutant embryos (Fig. 1M). Altogether, these data show that *Hoxa5* expression in the mesenchyme is critical for trachea development, and *Hoxa5* expression in phrenic

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motor neurons is essential for correct formation of the diaphragm muscle.

Basal metabolism and breathing pattern in *Hoxa5* conditional mutant adult mice

All surviving adult mice appeared healthy, and body mass, lung mass/body mass ratio, body temperature and O_2 consumption did not differ across genotypes (Table 1 and Fig. 2C). This was consistent with observations that *Hoxa5*$^{-/-}$ null mice show heighted lung proliferation, allowing significant functional recovery (Mandeville et al., 2006). These results also suggest that gas exchange was adequate under normal conditions. Respiratory variables obtained in *Hoxa5*$^{flox/flox}$ compared favorably with those reported in wild-type mice measured under similar experimental conditions (Kinkead et al., 2004; Marcouiller et al., 2014).

While $\dot{V}_I$ was similar for each genotype, differences in breathing pattern (f_R versus V_T) were observed between conditional *Hoxa5* mutant lines, providing valuable insight into the compensatory strategies that mutants employ to maintain adequate gas exchange in response to morphological defects. As indicated by the representative recordings, spirograms and population data (Fig. 2A, B and C, respectively), V_T in *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice was, on average, 14% lower than in controls and *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ mutants. This difference was due to reduced inspiratory flow (V_T/t_I) and shorter inspiratory and expiratory times (t_I and t_E, respectively; genotype effect: P=0.03 and 0.01, respectively). The shorter breathing cycle of *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice resulted in a f_R that was 9% faster than in controls (Fig. 2C). This breathing pattern was shallower than that previously reported for *Hoxa5*$^{-/-}$ mice (Kinkead et al., 2004) but made it possible to maintain adequate lung ventilation under conditions of a significant reduction in diaphragm thickness, which, in *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice, likely attenuated contraction strength and, in turn, contributed to the reduced V_T and V_T/t_I. The fact that V_T was unaltered in *Hoxa5*$^{-/-}$ mutants despite similar diaphragm thinning is difficult to reconcile but suggests that the concomitant reduction in tracheal surface elicited compensatory mechanisms from other inspiratory muscles.

The reduced tracheal luminal surface in *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ adults predicted augmented V_T/t_I in these mice as well, but plethysmography data showed no functional consequence at rest. Consistent with this, tracheal malformations do not cause lethality of *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ mutant newborns.

We reasoned that a hypoxic challenge could reveal an effect of the *Hoxa5* mesenchymal deletion. As meeting the organism's O_2 demands is more difficult under such conditions, breathing is reflexively stimulated. Both mesenchyme and motor neuron *Hoxa5* deletions led to lung hypoplasia (Landry-Truchon et al., 2017) and indeed we found that, at the onset of hypoxia, HVR of both lines was greater relative to that of controls. However, their reactions to hypoxia differed. Specifically, *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice showed the largest increase in f_R (genotype: P=0.004; Fig. 2D,E). This tachypnea was combined with an increase in V_T/t_I (genotype: P=0.002), slightly augmented V_T (genotype: P=0.006) and a 30% increase in $\dot{V}_I$ over controls (genotype: P=0.007). In contrast, the increase in f_R observed in *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ did not differ from that of controls, but their HVR was 18% larger owing to augmentations of V_T/t_I and V_T (Fig. 2E).

As hypoxia progressed, the differences subsided and were insignificant by the end of the protocol (P>0.05 for all variables; Fig. 2F). Reductions in T_b and $\dot{V}_{O_2}$ measured at the end of hypoxia (hypoxia: P<0.0001 and P=0.003, respectively) did not differ between groups (genotype×hypoxia: P=0.77 and P=0.80, respectively; data not shown).

Typically, V_T contributes marginally (if at all) to HVR but, interestingly, in *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ and *Hoxa5*$^{-/-}$ mutants, this parameter was augmented (Kinkead et al., 2004). While the impact of this strategy was not tested here, it is generally more effective than augmenting f_R. The fact that *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice opted for an augmented tachypnea is consistent with an inability to generate forceful inspirations as a result of diaphragm defects.

Impact of *Hoxa5* on the embryonic inspiratory motor command

Hoxa5 expression in phrenic motor neurons is essential for proper diaphragm innervation (Boucherat et al., 2013; Landry-Truchon et al., 2017). In *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ and *Hoxa5*$^{-/-}$ mutants, axons failed to reach the ventral part of the costal muscle and produced fewer branches in the dorsal region. Combined with the abnormal ventral diaphragm musculature, this suggests that axon projections in the dorsal diaphragm, although sparse, were sufficient to produce neuromuscular junctions and allow muscle recovery.

The adult respiratory phenotypes described above could be caused by an insufficient motor command during fetal life. Therefore, we assessed functionality of the embryonic respiratory motor command using isolated medulla–spinal cord preparations from E18.5 embryos. Unlike *in vivo* results obtained in adults, the burst frequency recorded from *Hoxa5*$^{flox/flox}$ embryos was slower and more variable than those measured in wild-type mice (Pagliardini et al., 2008; Viemari et al., 2003). While this raises questions about the impact of the genetic manipulations on functionality of these fetuses, these mice are the proper reference group for comparisons. Based on the similarity between controls and *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ mice, the latter was excluded from the analysis. Recordings of inspiratory (phrenic) activity under basal conditions from control and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice showed no differences in burst frequency, duration or amplitude (Fig. 3; genotype effect: P=0.19, 0.92 and 0.69, respectively). The coefficient of variation of burst frequency did not differ between groups (genotype effect: P=0.35; data not shown). Under hypoxia, an increased burst frequency (but not amplitude) was observed in both groups (hypoxia effect: P=0.004 and 0.12, respectively; data not shown), with no effect of genotype (genotype effect: P>0.05 for both variables; data not shown), thereby suggesting the

Table 1. Physiological variables according to genotype

	Hoxa5$^{flox/flox}$ (n=15)	*Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ (n=13)	*Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ (n=13)	Genotype effect
Age (days)	46±1.6	47±1.4	43±1.4	P=0.35
Body mass (g)	19.4±0.6	18.3±0.6	19.2±0.5	P=0.38
Lung mass/body mass ratio	0.0097±0.0003	0.0103±0.0004	0.0094±0.0004	P=0.18
T_b (°C)	36.4±0.2	37.0±0.1	36.2±0.2	P=0.14

Comparison of physiological variables between genotypes under normoxia. Values are expressed as means±s.e.m.

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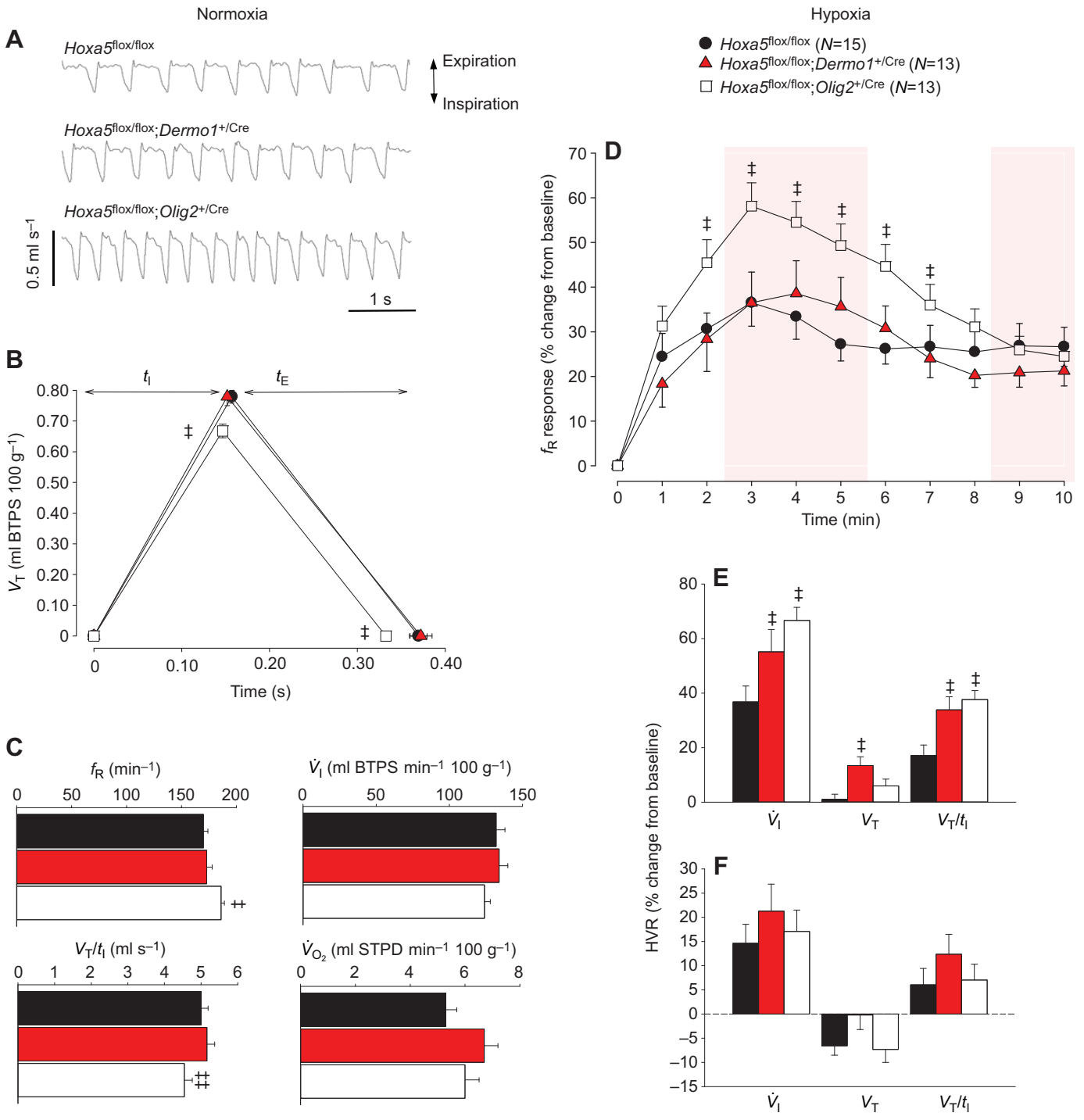

Fig. 2. Breathing pattern in *Hoxa5* conditional adult mutant mice during normoxia and hypoxia. (A) Original plethysmography recordings comparing the breathing pattern of *Hoxa5*$^{flox/flox}$ (control), *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ adults at rest (normoxia). (B) Representative spirograms with tidal volume (V_T) and duration of inspiratory (t_I) and expiratory phases (t_E) determining an average breath for control (*Hoxa5*$^{flox/flox}$; black circles), *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ (red triangles) and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ (white squares) adults. (C) Comparison of mean values of selected respiratory variables between genotypes under normoxia: f_R, breathing frequency; $\dot{V}_I$, minute ventilation; V_T/t_I, inspiratory flow; $\dot{V}_{O_2}$, oxygen consumption. (D) Comparative time course of the increase in breathing frequency (f_R) during hypoxia illustrating the bi-phasic nature of the response between controls (*Hoxa5*$^{flox/flox}$) and *Hoxa5*$^{flox/flox}$;*Dermo1*$^{+/Cre}$ and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ mice. The red shaded areas represent the periods analyzed at peak (left) and late (right) phases of the response. (E,F) Minute ventilation, tidal volume and inspiratory flow are shown for peak (3–5 min; E) and late (9–10 min; F) phase hypoxic ventilatory response (HVR). Data are reported as means±s.e.m. Following a significant ANOVA, a Fisher's protected least significant difference *post hoc* test was performed; ‡ and ‡‡ indicate values significantly different from controls at $P<0.05$ and $P<0.08$, respectively.

functionality of the respiratory network in *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ embryos. While it is tempting to propose that lack of *Hoxa5* expression in phrenic motor neurons during fetal development contributes to the high mortality rate of this group as a result of defective respiratory motor command, the present data do not support this hypothesis.

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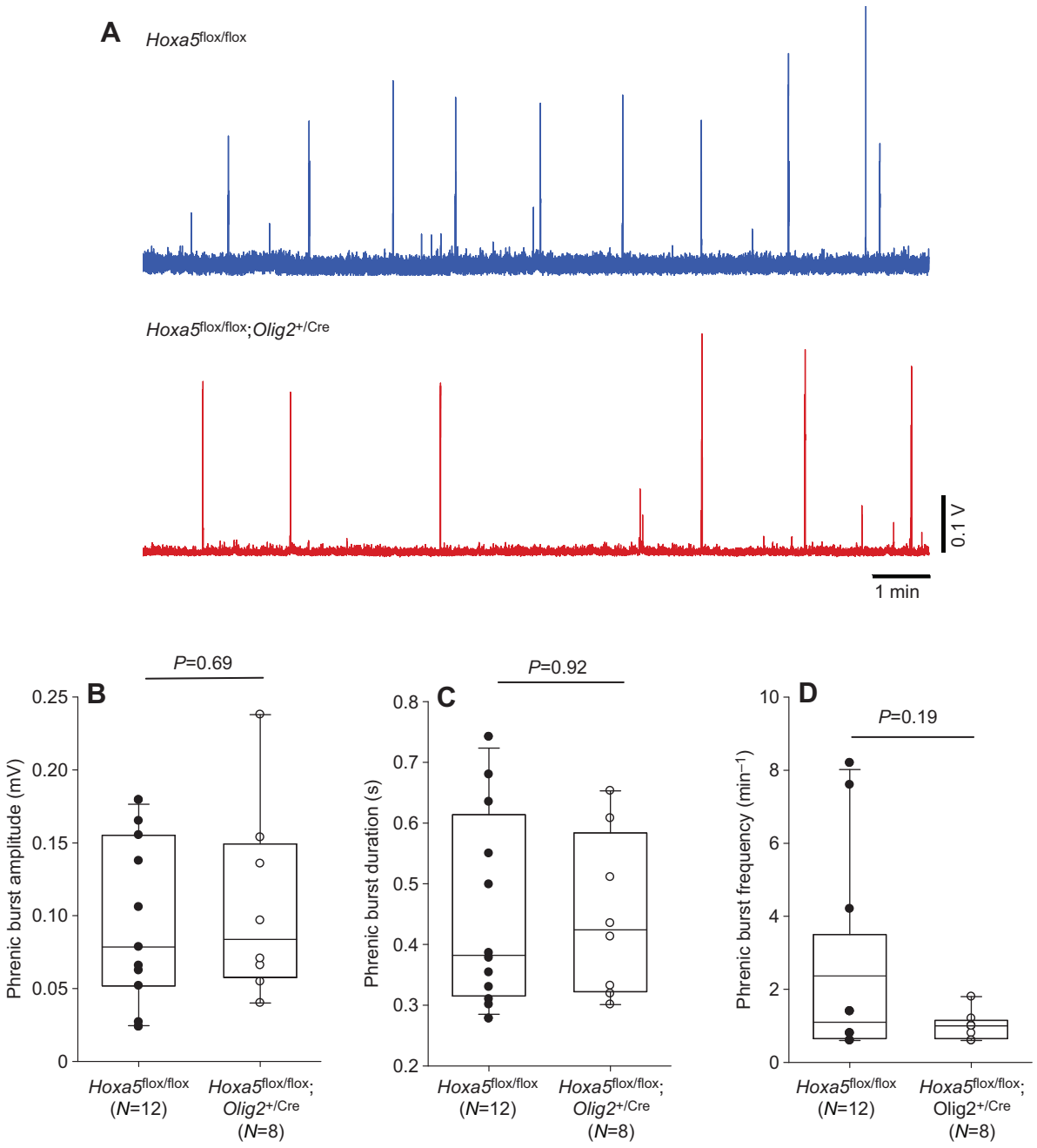

Fig. 3. Effect of *Hoxa5* on the fictive breathing frequency recorded from isolated embryonic brainstem–spinal cord preparations. (A) Phrenic nerve recordings comparing inspiratory (phrenic) activity between preparations from E18.5 *Hoxa5*$^{flox/flox}$ and *Hoxa5*$^{flox/flox}$;*Olig2*$^{+/Cre}$ embryos. (B–D) Box plots reporting population data for phrenic burst (B) amplitude, (C) duration and (D) frequency. Box boundaries correspond to the 25th and 75th percentiles (top and bottom, respectively); the line within the box indicates the median. Bars above and below show the 90th and 10th percentiles, respectively.

That experiments could only be performed in mice that survived the *Hoxa5* mutation biases our sampling and therefore imposes an important limitation on the present study. In surviving pups, the stress resulting from respiratory dysfunction at birth likely influenced the developmental trajectory and makes it difficult to ascribe changes observed at adulthood to genetic defects exclusively. Clearly, understanding the factors responsible for phenotypic heterogeneity (survival versus death) would be valuable but such work is beyond the scope of the present study. Nevertheless, we can conclude that by using *Hoxa5* conditional mutations, we established that *Hoxa5* expression in phrenic motor neurons is important for breathing patterns both at rest and under hypoxic conditions; however, inadequate motor command during fetal life does not contribute significantly to the phenotype. Conversely, while *Hoxa5* deletion in the mesenchyme severely perturbed tracheal morphology, this did not affect the breathing pattern at rest, although it did perturb the breathing response under hypoxic challenge.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.L.-T., N.H., J.R., L.J., R.K.; Methodology: K.L.-T., S.F., N.H., J.R., L.J., R.K.; Validation: K.L.-T., S.F., N.H., J.R., L.J., R.K.; Formal analysis: K.L.-T., S.F., N.H., J.R., R.K.; Investigation: K.L.-T., S.F., J.R.; Resources: R.K.; Data curation: R.K.; Writing - original draft: K.L.-T., L.J., R.K.; Writing - review & editing: K.L.-T., N.H., J.R., L.J., R.K.; Supervision: L.J., R.K.; Project administration: N.H., L.J., R.K.; Funding acquisition: L.J., R.K.

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