

RESEARCH ARTICLE

Elp1 is required for development of visceral sensory peripheral and central circuitry

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ABSTRACT

Cardiovascular instability and a blunted respiratory drive in hypoxic conditions are hallmark features of the genetic sensory and autonomic neuropathy, familial dysautonomia (FD). FD results from a mutation in the gene *ELP1*, the encoded protein of which is a scaffolding subunit of the six-subunit Elongator complex. In mice, we and others have shown that *Elp1* is essential for the normal development of neural crest-derived dorsal root ganglia sensory neurons. Whether *Elp1* is also required for development of ectodermal placode-derived visceral sensory receptors, which are required for normal baroreception and chemosensory responses, has not been investigated. Using mouse models for FD, we here show that the entire circuitry underlying baroreception and chemoreception is impaired due to a requirement for *Elp1* in the visceral sensory neuron ganglia, as well as for normal peripheral target innervation, and in their central nervous system synaptic partners in the medulla. Thus, *Elp1* is required in both placode- and neural crest-derived sensory neurons, and its reduction aborts the normal development of neuronal circuitry essential for autonomic homeostasis and interoception.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Familial dysautonomia, *Elp1*, Baroreceptors, Chemoreceptors

INTRODUCTION

Familial dysautonomia (FD), also referred to as hereditary sensory and autonomic neuropathy type III, is a devastating rare genetic neuropathy that results from mutations in the gene *ELP1* (previously referred to as *IKBKAP*). Over 99.5% of patients inherit the identical T-C founder mutation that weakens inclusion of exon 20, causing a frame shift and nonsense-mediated decay of the truncated mRNA (Slaugenhaupt et al., 2001; Cuajungco et al., 2003; Ibrahim et al., 2007). The dysfunctional splicing is cell-type specific, with neurons being least capable of splicing the mutated pre-mRNA and hence being the cell type most impaired in FD (Slaugenhaupt et al., 2001; Cuajungco et al., 2003). The *Elp1*

protein is a key scaffolding subunit of the six-subunit Elongator complex, which is essential for tRNA wobble uridine (U₃₄) modifications; in the absence of a functional elongator complex, additions of thiol and methoxycarbonyl-methyl do not occur, which perturbs the translation of mRNAs that preferentially use either AA- or AG-ending codons (Huang et al., 2005; Bauer and Hermand, 2012; Karlsborn et al., 2014; Kojic et al., 2021). Consequently, loss of *Elp1* function leads to considerable changes in the cellular proteome, which can also indirectly perturb the cellular transcriptome, both of which negatively impact pathways that are critical for neurogenesis and survival (George et al., 2013; Chaverra et al., 2017; Ohlen et al., 2017; Goffena et al., 2018; Kojic et al., 2021).

Although both the peripheral nervous system (PNS) and central nervous system (CNS) are affected in FD, the major impaired cell type in this disease is sensory neurons, and in particular, visceral sensory neurons. For example, one of the most debilitating clinical hallmarks suffered by FD patients is cardiovascular instability, which results from a faulty baroreflex and chemoreflex. These reflexes are vital for rapidly adjusting blood pressure, heart rate and respiration to maintain homeostasis. When blood pressure drops in healthy individuals, there is a compensatory increase in heart rate; in contrast, when blood pressure is too high, the homeostatic response is heart rate reduction. This reflex circuit is initiated by changes in activity of visceral sensory afferents that have cell bodies residing in the petrosal ganglion (PG; cranial nerve IX) and nodose ganglion (NG)/jugular ganglion (JG) (cranial nerve X) (Kumada et al., 1990; Chapleau, 2003; Benarroch, 2008; Wehrwein and Joyner, 2013; Min et al., 2019). The axons of the petrosal baroreceptors innervate the carotid sinus, while the aorta is innervated by axons extending from NG neurons. These same primary visceral sensory neurons project axons onto second-order neurons in the nucleus of the solitary tract (NTS) in the medulla, the first central relay for visceral information (Blessing, 1997; Kim et al., 2020). Dorsal to the NTS and also projecting onto it, is the area postrema (AP), a circumventricular organ with neurons in direct contact with the bloodstream and cerebrospinal fluid. In summary, visceral sensory information concerning blood pressure and levels of O₂ and CO₂ are sent into the brainstem via cranial nerve IX and X visceral peripheral afferents, to activate compensatory changes in autonomic output, either through the vagus nerve or sympathetic nerves, to maintain homeostatic heart rate and blood pressure. This circuitry is illustrated in Fig. 1.

In addition to the mechanosensory baroreceptors that innervate the aorta, a second key visceral sensory population is the chemoreceptors, which ensure homeostatic maintenance of ventilatory drive for respiration by detecting partial pressures of O₂, CO₂ and pH (Kameda et al., 2002; Ortega-Sáenz and López-Barneo, 2020). These chemoreceptive cells are located both peripherally in the carotid body, and centrally in the brainstem, in the retrotrapezoid nucleus (RTN) (Souza et al., 2019). The carotid

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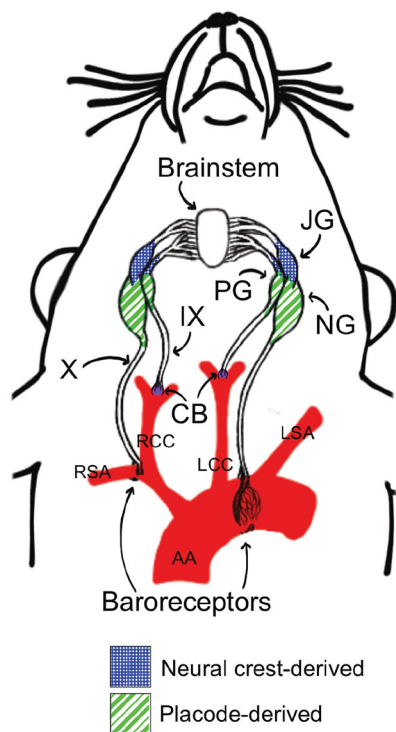


Fig. 1. Cranial nerve IX and X circuits that mediate chemoreception and baroreception. The schematic depicts vagal sensory afferents that sense blood pressure through the aortic depressor nerve branch of the vagus nerve (X). The glomus cells in the carotid body (CB; purple) relay chemosensory information concerning levels of O_2 , CO_2 and pH, in addition to glucose, lactate and more, through the glossopharyngeal nerve (IX) to the brainstem. Baroreceptors sensing blood pressure in the aortic arch (AA) and right subclavian artery (RSA) relay this information to the nucleus of the solitary tract in the brainstem. The jugular ganglion (JG) of the vagus is neural crest derived, whereas the nodose ganglion (NG) and petrosal ganglion (PG) are ectodermal placode derived. These pathways are disrupted in familial dysautonomia (FD). LCC, left common carotid artery; LSA, left subclavian artery; RCC, right common carotid artery.

body sits in the bifurcation of the carotid artery (Fig. 1) and is composed of the sensory glomus cells (in addition to the supporting sustentacular cells), which express tyrosine hydroxylase (TH) and serotonin, and are derived from neuronal precursors in the adjoining sympathetic cervical ganglion (Kameda, 2005). The glomus cells are multimodal sensory cells and relay essential information concerning blood O_2 , CO_2 , acid and glucose levels to the petrosal axonal afferents (Gonzalez et al., 1994; Pardal and López-Barneo, 2002; Dager et al., 2003). Through the glossopharyngeal nerve, these afferents synapse in the NTS in the brainstem, and from there projections are sent to other brainstem nuclei that drive respiration, including the pre-Botzinger complex (Finley and Katz, 1992).

Elegant studies in FD patients have demonstrated that the major point source of failure in their baroreflex is reduced sensory afferent signaling. Moreover, FD patients have a blunted hypoxic ventilatory drive and do not respond normally to low levels of CO_2 (Norcliffe-Kaufmann et al., 2010; Palma et al., 2019; Kaufmann et al., 2020). Both of these deficits underlie the frequent hypotension experienced by FD patients. Furthermore, FD patients suffer from swallowing abnormalities, which trigger aspiration and subsequent lung infections. In addition, they suffer from dysfunction of the gastrointestinal, kidney and liver, all tissues that are heavily innervated by the vagus nerve. In summary, the most life-threatening symptoms FD patients experience result from

severe disruption in vagal and glossopharyngeal cranial nerve function.

The vagus nerve is the major visceral sensory and motor (parasympathetic) nerve, innervating all visceral organs to convey key physiological changes in the body to the CNS. The sensory neuronal cell bodies for this nerve are all located in cranial ganglia X, which include the NG and JG, which in the mouse tend to be fused with the PG (cranial ganglion IX) to form the nodose-petrosal-jugular complex (NPJ). This ganglia complex in mammals derives embryologically from both neural crest and the epibranchial placodes (Harlow et al., 2011; Karpinski et al., 2016). Given the numerous vital functions subserved by vagal sensory neurons and the diversity of targets surveilled by them, several recent studies have transcriptionally profiled NG/PG/JG neurons and cells in the carotid body by single-cell RNA sequencing (Peeters et al., 2006; Kupari et al., 2019; Mazzone et al., 2020; Prescott et al., 2020) to generate an atlas and map of the diversity of cell populations within the NPJ. Depending on the study, 18–37 classes of molecularly distinct NG/PG/JG population subsets were identified due to the considerable heterogeneity and diversity of this population. Neuronal subsets have also been identified and distinguished based on site of target innervation, function and axonal terminal morphology (e.g. Chang et al., 2015; Williams et al., 2016; Hennel et al., 2018; Bai et al., 2019; Min et al., 2019; Prescott et al., 2020).

Given the profound roles of the vagus nerve in the body, and our incomplete understanding of which neuronal cell populations require *Elp1*, the goal of this study was to determine the fate of key visceral sensory neurons in mouse models for FD to better understand the pathophysiological mechanisms underlying the faulty baroreflex and chemoreflex of FD patients. This impairment could result from reduced neuronal number in the IX and X cranial ganglia, diminished target innervation by sensory axons, and/or reduced neuronal number in the key brainstem nuclei that receive and process visceral afferent input. Our study reveals that *Elp1* is required in the entire circuit – in both the sensory and brainstem neurons – and is required for target innervation.

RESULTS

Mice that are null for *Elp1* are embryonic lethal because they fail to neurulate and form vasculature (Chen et al., 2009; Dietrich et al., 2011). Thus, given the dual embryonic origin of visceral sensory neurons, in order to determine the requirement for *Elp1* in the cranial ganglia IX and X, three different conditional knockout mouse models were analyzed: (1) in which *Elp1* is deleted from neural crest cells but not placodes, using a *Wnt1-cre* (Dickinson and McMahon, 1992; George et al., 2013; Jackson et al., 2014); (2) in which *Elp1* is deleted from placode-derived cells and only a minority of neural crest-derived cells using a *Phox2b-cre* (Rossi et al., 2011); and (3) in which *Elp1* is deleted from nascent neurons throughout the nervous system regardless of embryonic origin using *Tuba1a-cre* (Gloster et al., 1994; Fernández et al., 2008; Ueki et al., 2016; Chaverra et al., 2017). In the new line we generated by crossing the *Phox2b-cre* driver to our floxed *Elp1* mice (George et al., 2013), *Phox2b-cre;Elp1^{LoxP/LoxP}* mice are born at the expected Mendelian ratio of 1:4.

Elp1 expression during visceral sensory neuron development

To determine the temporal requirement for *Elp1*, we first investigated its expression in the developing carotid body and cranial ganglia IX and X using a β -galactosidase (β -gal)/LacZ reporter system (George et al., 2013), as antibodies that detect *Elp1*

expression with high fidelity in these tissues are not available. We found that *Elp1* is prominently expressed during development of both the NG and PG (Fig. 2A) and in the carotid body (Fig. 2B).

To determine the activity of each Cre driver in the developing nerve IX and X ganglia, we crossed each of these lines to a GFP reporter line driven by the *Rosa26* locus, *Rosa^{mt-mG}*, and imaged their expression at the end of embryogenesis, embryonic day (E)18.5 (Fig. 3). As noted by others, depending upon the age examined, it can be difficult to distinguish the boundaries between the NG, PG and JG because they are frequently fused. There is a progression in their segregation as they mature; the nodose-petrosal ganglia (NPG) separate into the NG and PG before postnatal day (P)9. When all three ganglia appear fused, they are referred to as the NPJ.

We found that the *Tuba1a*-cre was expressed in ~40% of the neurons in the NPJ complex (Fig. 3, $41.3 \pm 1.1\%$ of TrkB⁺ neurons were also GFP⁺; $n=3$), whereas the *Wnt1*-cre was expressed in essentially all neurons in the jugular ganglion (JG) but not in the NG (Fig. 3). The *Phox2b*-cre was expressed in essentially all neurons in the nodose-petrosal (NP) complex (Fig. 3; Fig. S1), but only in a few cells in the JG. These findings support previous lineage analyses of cranial ganglia and single-cell RNA-sequencing studies (Karpinski et al., 2016; Kupari et al., 2019). One difference we did note from the description in Rossi et al. (2011) is detection of *Phox2b*-cre expression in some of the TH⁺ glomus cells in the carotid body and in a minority of TH⁺ neurons in the superior cervical ganglion (SCG) (Fig. S1) as well as in a few TH⁺ neurons in the spinal chain of sympathetic ganglia (data not shown). No expression was observed in the dorsal root ganglia (DRG) (data not shown). Other groups (Gautron et al., 2013) have also seen some expression of this *Phox2b*-cre line in a subset of enteric neurons in the myenteric plexus. The robust expression of the *Phox2b*-cre line in the NG enables studies of *Elp1* requirement in the nodose and inferior petrosal neurons in our study. In a previously published analysis of the *Wnt1*-cre;*Elp1* conditional knockout (cKO), we showed that the Cre was active throughout neural crest-derived gangliogenesis (George et al., 2013), but to confirm its activity in the carotid body specifically, here we asked whether the *Wnt1*-cre was expressed in the carotid body. We found robust Cre expression in the developing carotid body, which was to be expected as it is neural crest derived (Fig. 3).

Placode-derived NG/PG neurons require *Elp1* for normal development

We and others have established that neural crest-derived neurons depend on *Elp1* for survival; however, whether placode-derived neurons are also dependent on *Elp1* has not been determined. Because the majority of the *Wnt1*-cre;*Elp1* cKO mice die within 24 h

of birth (George et al., 2013; Jackson et al., 2014), we quantified visceral sensory number just prior to birth, at E18.5. Here, we report that the *Phox2b*-cre;*Elp1* cKO newborn mice also die within a few days of birth, with the longest-living mouse surviving to P18. We also found that the majority of the *Phox2b*-cre;*Elp1* cKO mice were smaller than their non-mutant littermates, weighing ~28% less than controls: at P9, the average weight of the control mice was 4.79 ± 0.33 g (s.e.m.), whereas the average weight of mutants was 3.44 ± 0.26 g ($n=7$ mutants and $n=8$ controls; $P=0.007$). Jackson et al. (2014) also made a *Wnt1*-cre;*Elp1* cKO mouse, which also died postnatally, which they state was due to impaired breathing associated with cleft palate. We checked whether our *Wnt1*-cre;*Elp1* cKO and *Phox2b*-cre;*Elp1* cKO mice also develop cleft palate and found that although the *Wnt1*-cre;*Elp1* cKO mice did show failure in palate fusion, the *Phox2b*-cre;*Elp1* cKO mice did not (data not shown). This finding is perhaps not surprising because *Wnt1*-cre is expressed in the cranial crest whereas *Phox2b*-cre is not.

Baroreceptive neurons have previously been shown to depend on the neurotrophic factors BDNF and NTF4 (hereafter referred to as NTF4) for their survival via TrkB (also known as NTRK2) activation (Erickson et al., 1996; Hellard et al., 2004). In the absence of TrkB signaling, there is a 94% reduction in nodose-petrosal cell number at birth (Erickson et al., 1996). Single-cell RNA-sequencing analysis and reporter-lineage tracking identifies *Trkb* as a cardinal gene expressed by the vast majority of nodose neurons [and, in contrast, is only sparsely expressed in the JG (Kupari et al., 2019; Kim et al., 2020)]. To quantify changes in neuronal number in the ganglia, we used TrkB as a neuronal marker in mice just prior to or after birth (Fig. 4). We found a reduction in the number of TrkB⁺ neurons in the cKOs in all three mouse lines. Because the JG is neural crest derived and does not contain many TrkB neurons, we also quantified total neuronal number using an anti-HuC/D antibody and found a significant reduction in jugular neurons in the absence of *Elp1* (Fig. 4B,C).

Of all the mouse lines, the most dramatic neuronal loss of ~60% occurred in the *Phox2b*-cre;*Elp1* cKO (Fig. 4D,E). In contrast, in the *Wnt1*-cre;*Elp1* cKO, there was a reduction of only 16% of TrkB⁺ neurons, which is consistent with the minor contribution of the neural crest to the NG (Nassenstein et al., 2010; Karpinski et al., 2016) and/or could be due to the small number of TrkB⁺ (neural crest-derived) jugular neurons. We also quantified neuronal numbers in *Phox2b*-cre;*Elp1* cKO mice that were crossed to the *Rosa^{mt-mG}* reporter and found again a ~60% reduction in neurons. In this case, the numbers of (Cre⁺) GFP⁺ cells were counted as we showed previously that the vast majority of them (>90%) are TrkB⁺. This reduction is consistent with the visible decrease in diameter of

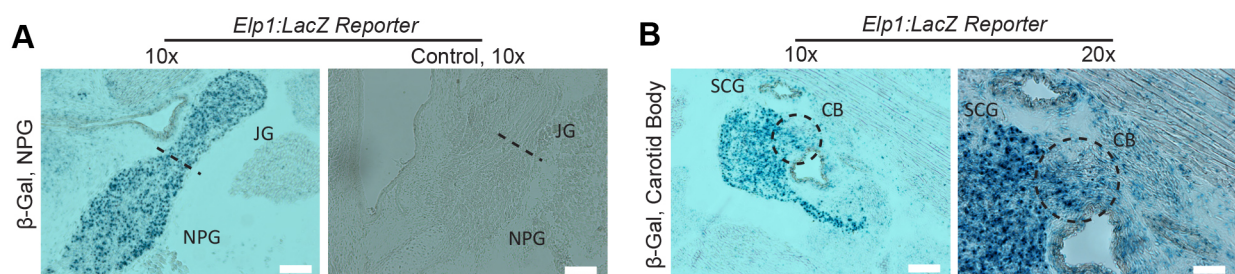


Fig. 2. Endogenous *Elp1* expression in nodose, petrosal and jugular ganglia and in the carotid body. (A,B) The *Elp1*:LacZ reporter mouse reveals β -gal staining in *Elp1*-expressing tissue: the nodose-petrosal-jugular ganglion (NPG) and the jugular ganglion (JG) at E17.5, with a LacZ⁻ littermate shown for comparison (A); in the carotid body (CB) at E17.5 (black dashed line circles surround the CB) (B). The cells that form the CB derive from cells in the superior cervical ganglion (SCG). JG, jugular ganglion; NPG, nodose-petrosal ganglia. Black dashed line in A indicates the border between the jugular ganglion and the nodose petrosal ganglia. Scale bars: 50 μ m.

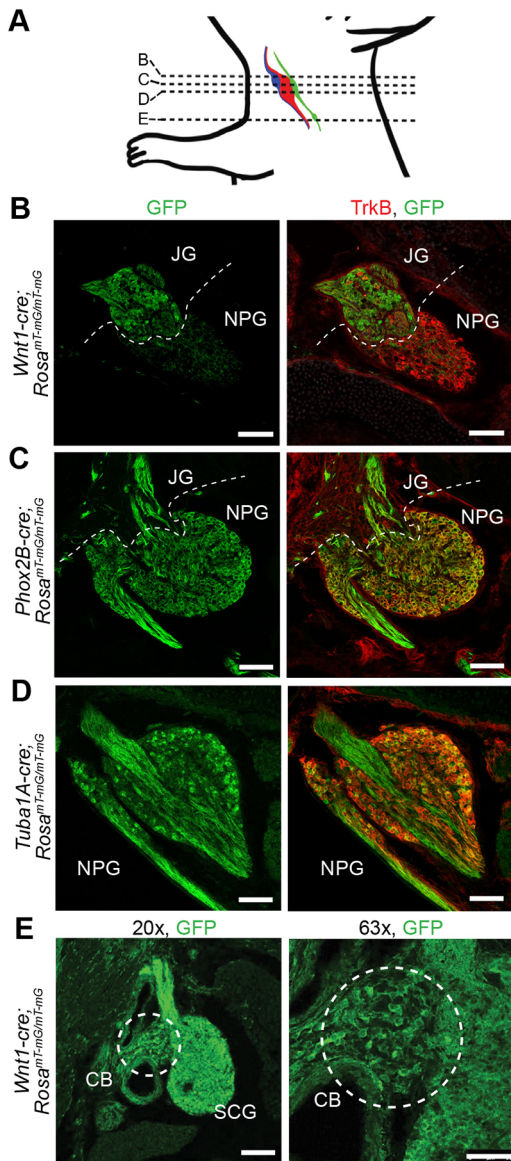


Fig. 3. Cre activity in the NPJ. (A) Schematic showing the locations of the jugular ganglion (JG, blue), of the nodose ganglion (NG, red) and petrosal ganglion (PG, green) that both form the nodose-petrosal ganglia (NPG), and of the carotid body (CB, green). The PG axons form the carotid branch of the glossopharyngeal nerve that innervates the carotid body, i.e. the nodule positioned at the lower end of the PG. Dashed horizontal lines indicate the transverse section locations for the images shown in B-E. *Rosa^{mT-mG}/mT-mG* mice were crossed with respective Cre lines to report expression of the Cre recombinase enzyme in the complex. (B-D) GFP-reported Cre expression (left column) at E17.5 for *Wnt1-cre* (B), P1 for *Phox2b-cre* (C) and E18.5 for *Tuba1a-cre* (D). The NPG is TrkB⁺, whereas the JG is TrkB⁻ (merged images in right column, B-D). (E) Cre expression in the CB of E17.5 *Wnt1-cre* mice. White dashed lines indicate ganglia borders. White dashed line circles enclose the CB. Magnification 200× unless specified. *n*=2-3 embryos/line. Scale bars: 75 μm (B-D,E, left), 50 μm (E, right).

the vagus nerve at P9 in the *Phox2b-cre;Elp1* cKO compared to control (Fig. S2). There were no significant differences between left and right ganglia nor differences between males and females. The number of NPJ neurons in the *Tuba1a-cre* at E18.5 was not significantly reduced in the mutants. Of these lines, only the *Tuba1a-cre;Elp1* cKO mice live into adulthood; therefore, we also quantified TrkB⁺ neurons in their adult NJ complex and found a

~20% reduction. Although the difference was insignificant, it was increased in the adult compared to the E18.5 ganglia, suggestive of a progressive decline. These data are perhaps not surprising given that the *Tuba1a-cre* is only expressed in ~40% of NPJ neurons.

Dopaminergic neurons in the NG depend on *Elp1* for development

We also investigated the requirement for *Elp1* in the dopaminergic neurons located in the NP complex, because they have been shown to innervate the chemosensory glomus cells in the carotid body (Katz et al., 1983; Katz and Black, 1986) and are required for normal respiratory drive (Erickson et al., 1996). These cells are known to give rise to the super laryngeal nerve (Kummer et al., 1993), which detects sensory stimuli for the swallowing reflex, which is also severely impaired in FD patients (Palma et al., 2014) and innervates the esophagus and stomach (Kummer et al., 1993). As shown in Fig. 4, the loss of dopaminergic neurons, as marked by expression of TH, was more dramatic than that of the reduction in TrkB⁺ neurons, being reduced from 16% to 80% depending on the mouse line and age. The biggest reductions occurred in the *Phox2b-cre;Elp1* cKO mouse, suggesting that the majority of the TH⁺ neurons are placode derived. In contrast, only ~2% of TH⁺ neurons were located in the (neural crest-derived) JG in all of the mouse models analyzed. In the *Tuba1a-cre;Elp1* cKO, the reduction in TH⁺ neurons was significant in the older adult mice (44%), but not at E18.5 (16%), consistent with a progressive loss of these neurons as the mice age.

Interestingly, the TH⁺ neurons were typically found in clusters within the NP complex (Fig. 4A and Fig. 5A), which could suggest a clonal origin of these neurons from a common precursor cell(s). TH clusters were present in both mutants and controls in all three mouse lines. Colocalization analysis of *Phox2b-cre;Elp1;Rosa^{mT-mG}* tissue sections stained with anti-TH antibody showed that the TH⁺ neurons in the NPG do express Phox2b and are targeted by *Phox2b-cre* (Fig. 5). Quantification of the number of TH⁺ cells per cluster revealed a significant reduction in the cKO mice compared to control littermates in all three lines (Fig. 5). There is conflicting information on whether TH clusters are localized to the NG or PG specifically (Kummer et al., 1993), but because they were found in postnatal and adult mice at times in which the NG and PG can be clearly distinguished from each other and selectively dissected, we conclude they are clearly present in the NG.

Chemoreceptive glomus cells in the carotid body require *Elp1* for normal development

To determine whether neural crest-derived glomus cells also require *Elp1* for their development, the number of glomus cells – as identified morphologically, by their stereotyped location between the external and internal carotid, and by their expression of TH – was quantified in the *Wnt1-cre;Elp1* cKO mice and control littermates at the end of embryogenesis (E18.5). The cKO carotid body was dramatically reduced in size compared to that of control littermates, and the number of TH⁺ glomus cells was significantly reduced by ~40% in the cKO carotid bodies (Fig. 6A,B) compared to control carotid bodies.

Brainstem nuclei that process visceral sensory information depend on *Elp1*

Remarkably, the peripheral visceral sensory neurons and their central targets in the brainstem not only all express Phox2b (Tiveron et al., 1996; Dauger et al., 2003), but Phox2b specifies the entire neural circuitry that mediates both the baroreflex and chemoreflex (Dauger et al., 2003). Deletion of *Phox2b* impairs development of the sensory afferents in the carotid body, the PG and NG, their

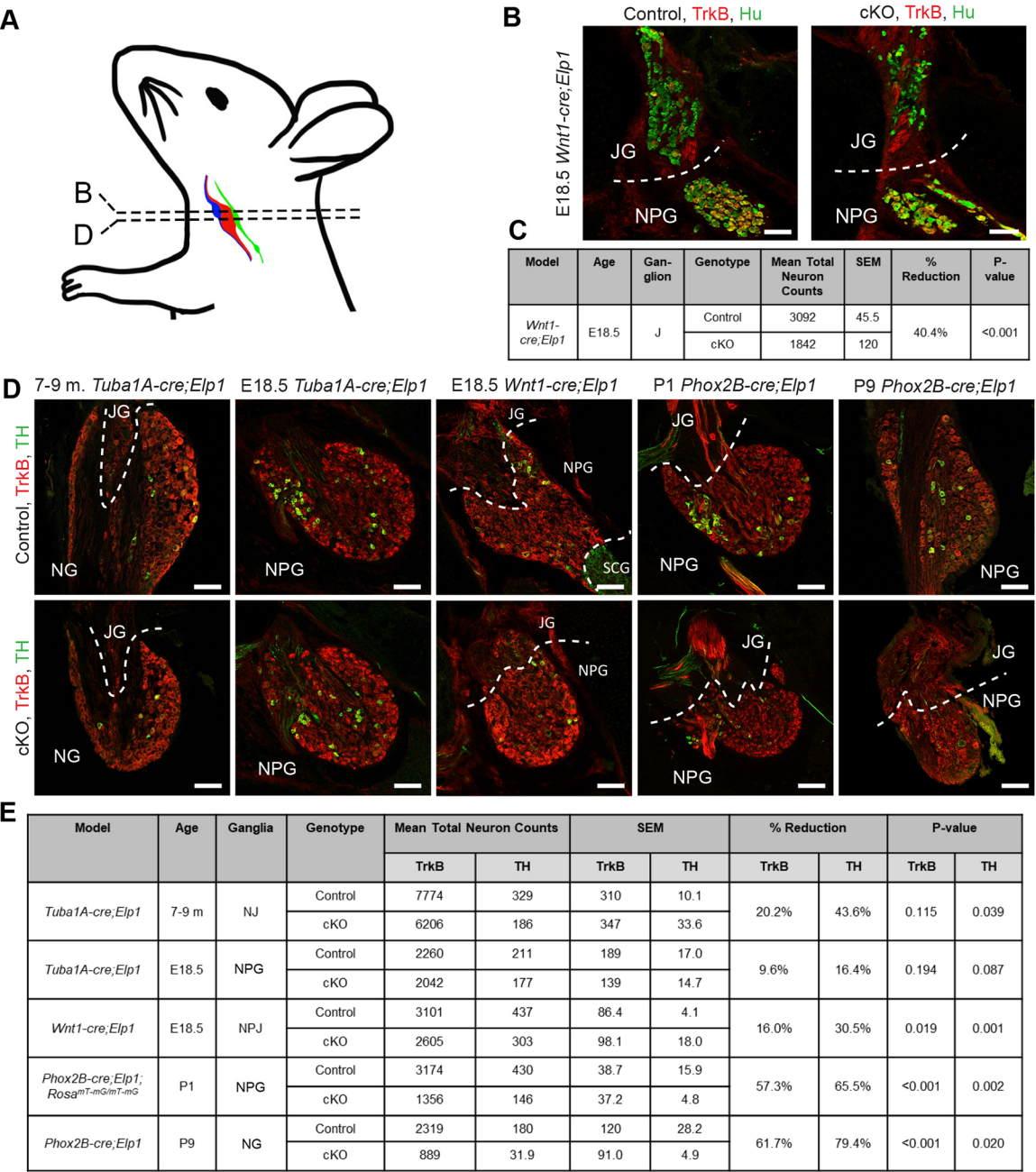


Fig. 4. Visceral sensory neurons are reduced in the absence of *Elp1*. (A) Schematic showing the locations of the jugular ganglion (JG, blue), of the nodose ganglion (NG, red) and petrosal ganglion (PG, green) that both form the nodose-petrosal ganglia (NPG), and of the carotid body (CB, green). The PG axons form the carotid branch of the glossopharyngeal nerve that innervates the carotid body, i.e. the nodule positioned at the lower end of the PG. Dashed horizontal lines indicate the transverse section locations for images shown in B and D. (B,C) Jugular neurons (TrkB⁺, HuC/D⁺; green) were significantly reduced by 40% in the *Wnt1-cre;Elp1* conditional knockout (cKO) mouse line. (D,E) Total ganglia cell counts (TrkB⁺; red) and dopaminergic neurons (TH⁺; green) are reduced in the absence of *Elp1* in mouse models of FD, with the NG and NPG of the *Phox2b-cre;Elp1* cKO model showing the greatest neuronal reduction compared to controls. In E, GFP⁺ cells in P1 *Phox2b-cre;Elp1;Rosa^{mT-mG/mT-mG}* were counted instead of TrkB⁺ cells. *n*=4 mice for each treatment group. *P*-values correspond to an unpaired one-tailed Student's *t*-test. Magnification 200×. White dashed lines indicate ganglia borders. Scale bars: 75 μm.

brainstem targets in the NTS and efferent arms for parasympathetic and enteric circuits. Consequently, we used the *Phox2b-cre;Elp1^{LoxP/LoxP}* mouse line to investigate whether the central components of respiratory and cardiovascular circuits were also dependent on *Elp1* expression.

Brainstems were sectioned from the pons to the medulla and immunolabeled with antibodies against Phox2b (Fig. 7). Reduced numbers of Phox2b⁺ neurons were evident throughout the brainstem of the *Elp1* cKO mouse compared to that of control littermates. The

numbers of Phox2b⁺ neurons in the central targets of the NPJ axons were quantified in the AP, NTS, ventral respiratory group (VRG) and nucleus ambiguus (NA) in the *Phox2b-cre;Elp1* cKO and littermate controls at P0. The intermediate region of the NTS receives input from vagal gastrointestinal afferents, while the caudal NTS receives input from the vagal and glossopharyngeal baroreceptors, vagal chemoreceptors, vagal cardiac receptors and vagal pulmonary receptors (Cutsforth-Gregory and Benarroch, 2017). The VRG and RTN are functionally interconnected in

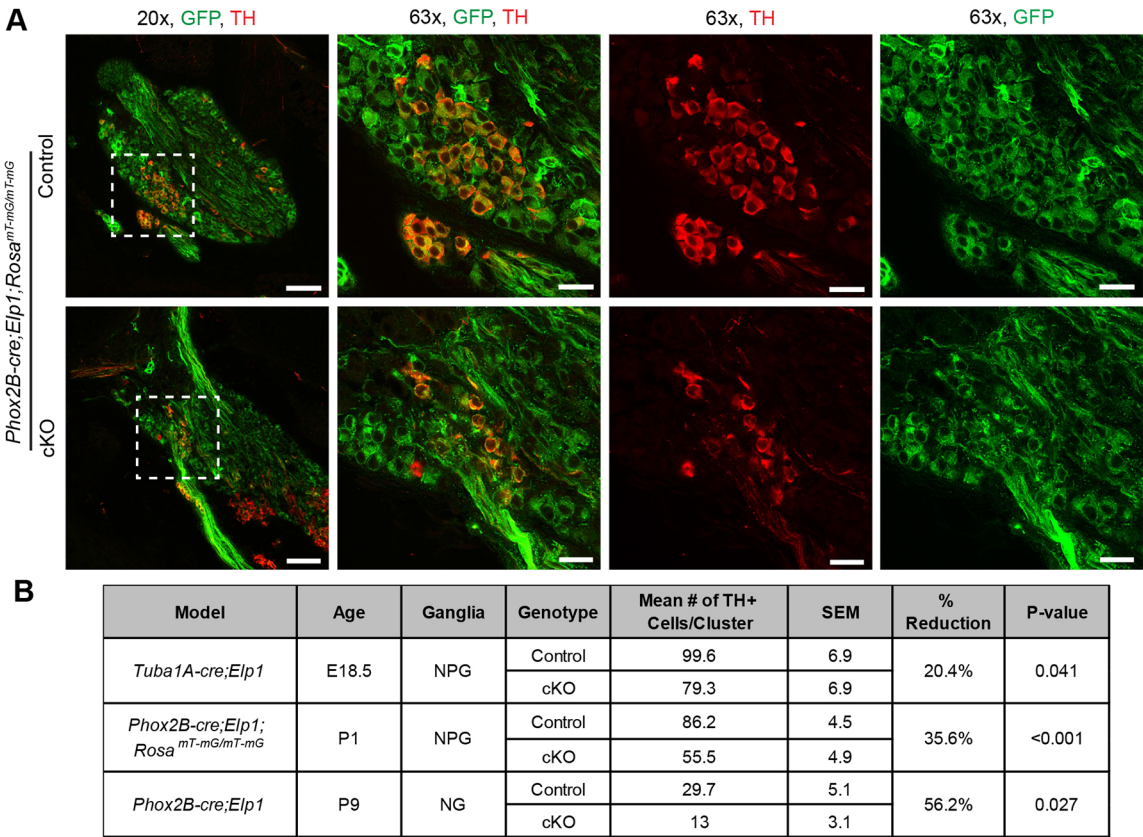


Fig. 5. Tyrosine hydroxylase-positive (TH⁺) cell clusters in the NPG express *Phox2b-cre* and are reduced in number with deletion of *Elp1*. (A) TH⁺ clusters in the NPG are *Phox2b*⁺, as shown by colocalization of GFP and TH in P1 *Phox2b-cre;Elp1;Rosa^{mT-mG/mT-mG}* mice (A, 20× and 63×). White dashed line boxes indicate the location of the 63× image. (B) The numbers of TH⁺ cells in the NPG cluster are significantly reduced in cKO *Phox2b-cre;Elp1^{LoxP/LoxP};Rosa^{mT-mG/mT-mG}* mice compared to control *Phox2b-cre;Elp1^{+/LoxP};Rosa^{mT-mG/mT-mG}* mice at P1. The numbers of TH⁺ cells in the cluster are also reduced in the NG of P9 *Phox2b-cre;Elp1* cKO compared to control and the NPG of E18.5 *Tuba1a-cre;Elp1* cKO compared to control mice. *n*=4 for each group of mice. *P*-values correspond to an unpaired one-tailed Student's *t*-test. Scale bars: 75 μm (20×), 25 μm (63×).

response to chemoreceptor drive, and the RTN neurons are themselves central CO₂ chemoreceptors (Ott et al., 2012; Souza et al., 2019). Interestingly, there are numerous *Phox2b*⁺/GFP[−] neurons in adjoining nuclei that are missing in the cKO brainstem, suggesting that they are lost indirectly. As shown in Fig. 7, the numbers of *Phox2b*⁺ neurons in all of these nuclei were reduced by ~50% in the cKO compared to their littermate controls.

Elp1 is required for normal innervation of baroreceptive peripheral targets

For sensory neurons to function effectively, they must innervate their peripheral targets successfully. Previous studies have established that neural crest-derived sensory neurons that lack *Elp1* fail to normally innervate their peripheral targets (George et al., 2013; Jackson et al., 2014; Ohlen et al., 2017); however, whether placode-derived sensory neurons also require *Elp1* for peripheral target innervation has not been investigated. Baroreceptive neurons in the NG innervate the carotid sinus and aortic arch (Min et al., 2019) by extending decorated ‘claws’ that circumscribe the diameter of the aortic arch. Via activation of mechanoreceptive proteins such as Piezo1 and Piezo2, baroreceptive neurons are stimulated by changes in blood pressure in these vessels (Zeng et al., 2018; Min et al., 2019). Thus, in their absence, changes in blood pressure cannot be detected or relayed to the CNS to appropriately adjust cardiac output. To determine whether the baroreceptive targets in the aortic arch and subclavian

artery were innervated normally in the *Phox2b-cre;Elp1* cKO mice, the *Rosa^{mT-mG}* reporter line was crossed into this line, the aortic arches and subclavian arteries were dissected, and whole-mount staining with GFP antibodies was conducted (Fig. 8; Fig. S3). Z-stacks were collected through the entire aortic arch, from the anterior to the posterior sides of the arch, thus revealing both the ligament and saddle regions of the aortic arch. The left NG innervates the aortic arch and the right NG innervates the right subclavian artery (RSA). Vagal afferents also innervate the RSA at the bifurcation of the brachiocephalic artery. The aortic depressor nerve (ADN) branches from the vagus. Near the aortic arch, the left ADN bifurcates with one branch forming the saddle region on the posterior side of the arch and the other branch forming the ligament region on the anterior side on the arch (Fig. 8A, Tomato shows the aortic arch). The right baroreceptors form a claw around the RSA (Fig. 8B, Tomato). Innervation of the aortic arch (Fig. 8B) in three mutants and controls, and in the RSA in four mutants and controls (Fig. 8C), revealed severe reductions in innervation in the cKO mice compared to littermate controls, with some variation amongst the mice. The data show that the right and the left baroreceptor branching in both the saddle and the ligament are decreased in density in cKO mice compared to their control littermates, with the left ADN appearing thinner than that in controls. Afferents terminated in two types of endings, the flower-sprays and end-nets as described (Cheng et al., 1997; Min et al., 2019). Both types of endings are reduced in the cKO mice (Fig. S3), with the end-net

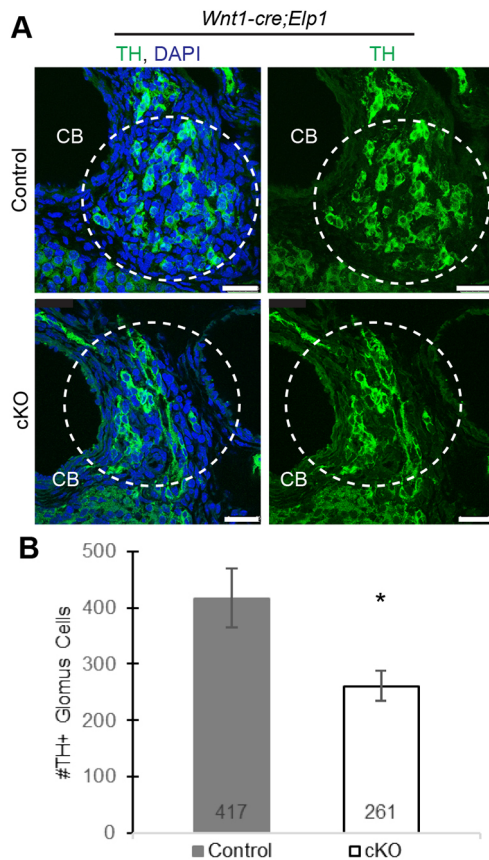


Fig. 6. Glomus cells in the carotid body (CB) require *Elp1* for normal development. (A,B) Embryonic E18.5 *Wnt1-cre;Elp1* transverse sections were stained with antibodies against TH (A), and the number of glomus cells was quantified through each CB (B). $n=5$ mice per condition. * $P<0.026$, unpaired one-tailed Student's *t*-test. Scale bars: 25 μ m.

endings severely reduced in the ligament. Interestingly, many of the axons terminating in end-net endings appear to be misrouted and extend to regions ectopic to their normal target in the ligament (Fig. 8B, white arrowheads). Thus, although the viscerosensory axons that comprise the ADN navigate successfully to and invade the aortic arch and RSA, the final innervation of target tissue is impaired for the majority of the baroreceptive axons in the absence of *Elp1*.

DISCUSSION

Failure in key cardiovascular and respiratory reflexes such as the baroreflex and chemoreflexes are clinical hallmarks of FD, with often fatal consequences. Using mouse models for FD, this study delineates the potential underlying cellular basis for these failed reflexes. Although impaired somatic sensory neuron development has been demonstrated in several FD mouse model studies, here we report the first investigation of visceral sensory neurons, which mediate these critical cardiorespiratory reflexes. Our data indicate that, despite arising from a distinct embryological lineage, visceral sensory neurons, like somatic sensory neurons, also depend on *Elp1* for development. Although the neural crest-derived somatic sensory neurons in the DRG mediate somatic functions such as pain, temperature, pressure and proprioception, the cranial placode-derived visceral sensory neurons located in the vagal and glossopharyngeal ganglia are vital for key autonomic functions such as respiration, cardiovascular regulation and gastrointestinal function. Moreover, this study reveals that *Elp1* is

required in the entire CNS/PNS circuitry underlying baroreception and chemoreception: in both the central brainstem targets of the visceral afferents, and, for proper innervation of their baroreceptive peripheral targets.

The fundamental importance of *Elp1* in development is apparent by the fact that mice that are null for *Elp1* die by E10, owing to failures in neurulation and vasculature formation (Chen et al., 2009; Dietrich et al., 2011). Yet why dependence on *Elp1* varies temporally and by cell type is not understood. This study expands our knowledge of the cell types that require *Elp1* for their development because in spite of being broadly expressed in both the CNS and PNS, not all neuronal populations depend on *Elp1* for their development and survival (George et al., 2013; Ueki et al., 2016). For example, in the DRG, TrkA^+ (also known as NTRK1^+) and TrkB^+ neurons require *Elp1* during development, but TrkC^+ (also known as NTRK3^+) neurons do not. Yet, in adulthood, the TrkC subpopulation, constituted of proprioceptors, does progressively die, causing the degenerative gait ataxia of FD patients and mice (Morini et al., 2016). Similarly, our work has shown that in spite of robust expression of *Elp1* in the retina, retinal neurons do not require *Elp1* for their development; however, within 2 weeks of birth, *Elp1* cKO retinal ganglion cells progressively die, whereas, strikingly, the other retinal cell types can persist and function in the absence of any *Elp1* expression. Here, we add visceral sensory neurons to the list of neurons that do require *Elp1* for their development and survival.

Elp1 functions with the five other elongator subunits, *Elp2-Elp6*, to form the Elongator complex, which modifies tRNA to facilitate the translation of mRNAs that exhibit a biased usage of either AA- or AG-ending codons for lysine, glutamine and glutamic acid (Huang et al., 2005; Bauer and Hermand, 2012; Goffena et al., 2018). It is possible then that the codon usage of a particular cell type's transcriptome could determine to what extent an *Elp1*-deficient cell lives or dies. Moreover, the fact that mutations and/or variants in *ELP2-ELP4*, are associated with distinct neurological disorders (albeit with some overlapping features), ranging from intellectual disability to amyotrophic lateral sclerosis, but excluding the sympathetic, sensory and optic neuropathies exhibited by FD patients, may indicate variation in function of the *Elp* subunits independent of their joint actions as part of the Elongator complex. Although the vast majority of *Elp1* is localized to the cytoplasm, there are reports that the Elongator complex is also involved in transcriptional elongation (Otero et al., 1999; Hawkes et al., 2002; Close et al., 2006; Morini et al., 2021); this effect may be the indirect consequence of changes in the cells' proteome that occur with loss of Elongator function.

The visceral sensory neurons in the NG are generated and specified in cranial placodes and, upon becoming postmitotic, they delaminate as specified, differentiating neurons and migrate to their targets (Graham et al., 2007; Blentic et al., 2011; Moody and LaMantia, 2015). Neurogenesis does not occur in the ganglion (Blentic et al., 2011). This is in contrast to how neural crest cells give rise to neurons: neural crest cells migrate to multiple locations in the head and trunk, and only upon stopping to colonize their targets do they either differentiate into neurons directly or give rise to progenitor cells that then differentiate into neurons (Maro et al., 2004; George et al., 2007, 2010; Ventéo et al., 2019). For example, in the DRG, TrkA^+ neurons derive from intermediate Pax3^+ progenitors (George et al., 2010, 2013) that are especially sensitive to reductions in *Elp1*. Although here we report that *Elp1* is expressed in both the NP ganglia and in the carotid body during development, we did not determine at what developmental stage the

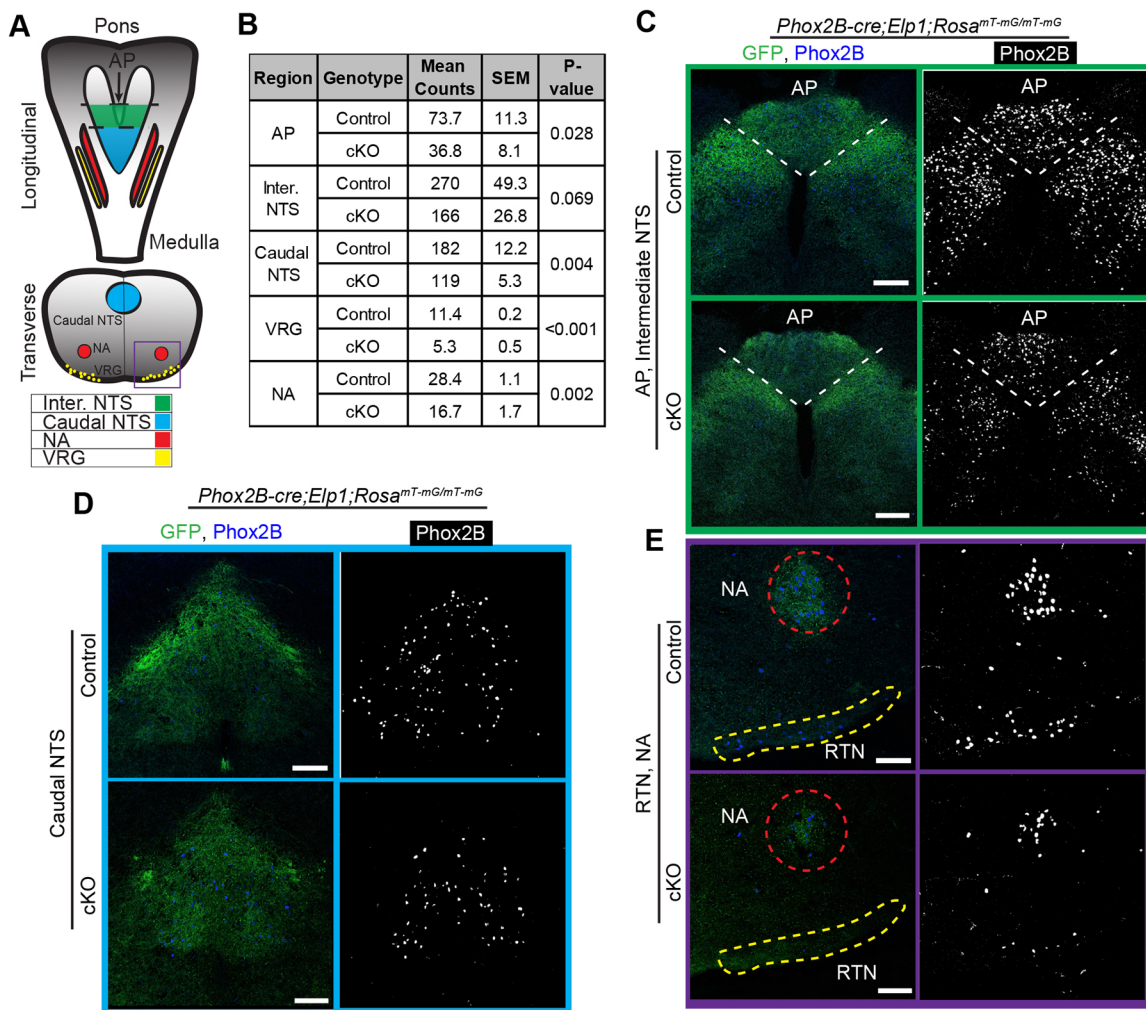


Fig. 7. Neurons in the brainstem respiratory circuitry are reduced in the absence of *Elp1*. (A) Schematic of the brainstem regions. (B) Phox2b⁺ neurons in the brainstem respiratory nuclei were visualized with an anti-Phox2b antibody, and shown to be significantly reduced in the AP, NTS, VRG and NA of cKO *Phox2b; Elp1^{LoxP/LoxP}; Rosa^{mT-mG/mT-mG}* mice compared to control *Phox2b; Elp1^{+/LoxP}; Rosa^{mT-mG/mT-mG}* mice at E18.5 or P1. (C-E) The intensity of GFP staining, marking Phox2b-cre-expressing cells, is also reduced in each region. *n*=3 mice for each analysis. AP, area postrema; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; RTN, retrotrapezoid nucleus; VRG, ventral respiratory group. Scale bars: 75 μm.

reduction in the glomus cells and NP neurons in the cKO mice occurred. If, in fact, *Elp1* is required in nodose neurons while they are still in their placode, this study would have missed that developmental window, because *Phox2b* is first expressed when neurons are differentiating in the NG.

What our data do reveal is that *Elp1* is required to acquire a full complement of TrkB⁺ neurons by birth. NP neurons, in particular those that mediate the afferent pathway driving respiration and baroreception, have been demonstrated to depend on TrkB activation via BDNF and NT4 for their survival (Erickson et al., 1996). Mice in which *Bdnf* was deleted have dysfunctional respiratory behavior including a reduction in chemosensory drive. At a cellular level, 94% of NP neurons are missing in TrkB knockouts, versus a ~50% loss if either BDNF or NT4 is deleted (Erickson et al., 1996). Here in the *Phox2b-Elp1* cKO, we lost ~60% of the TrkB⁺ neurons, potentially consistent with an overlap in the TrkB signaling pathway. Interestingly, patients with Rett syndrome have impaired autonomic respiratory function (Katz et al., 2009), which in mouse models is marked by reductions in nodose neurons and can be ameliorated with TrkB agonists (Schmid et al., 2012; Kron et al., 2014). Given the dependency of NG neurons on

BDNF signaling, and studies showing that *Elp1* cKO neurons have impaired NGF retrograde transport and TrkA signaling (Abashidze et al., 2014; Naftelberg et al., 2016; Li et al., 2020), it will be interesting to investigate in future studies whether nodose neurons in FD die due to faulty TrkB retrograde signaling.

To investigate the dependency of the visceral sensory neurons on *Elp1*, we used three different cKO lines. As described previously, because the NPJ includes cells that derive from both the neural crest and from cranial placodes, to ensure that each cell population was probed, we used a *Wnt1-cre* to target the neural crest cell derivatives, a *Phox2b-cre* to target the nodose and inferior petrosal neurons, and a *Tuba1a-cre*, which targets ~40% of neurons throughout the NPJ. In all lines, the *cre* is expressed during development (Fig. 3), and in all three lines, neuronal number was reduced in the NG, NP or NPJ. The largest deficit was in the *Phox2b-cre;Elp1^{LoxP/LoxP}* line, which could be due to the high fidelity and robust expression of the *cre* (Fig. 3) in the NG. Interestingly though, in spite of its ubiquitous expression in TrkB⁺ neurons, deletion of *Elp1* does not result in the loss of all TrkB⁺ or of all TH⁺ neurons by birth. Moreover, we here show that loss of *Elp1* reduced glomus cell number and jugular neuronal number by 40%. Similar results have been discovered in

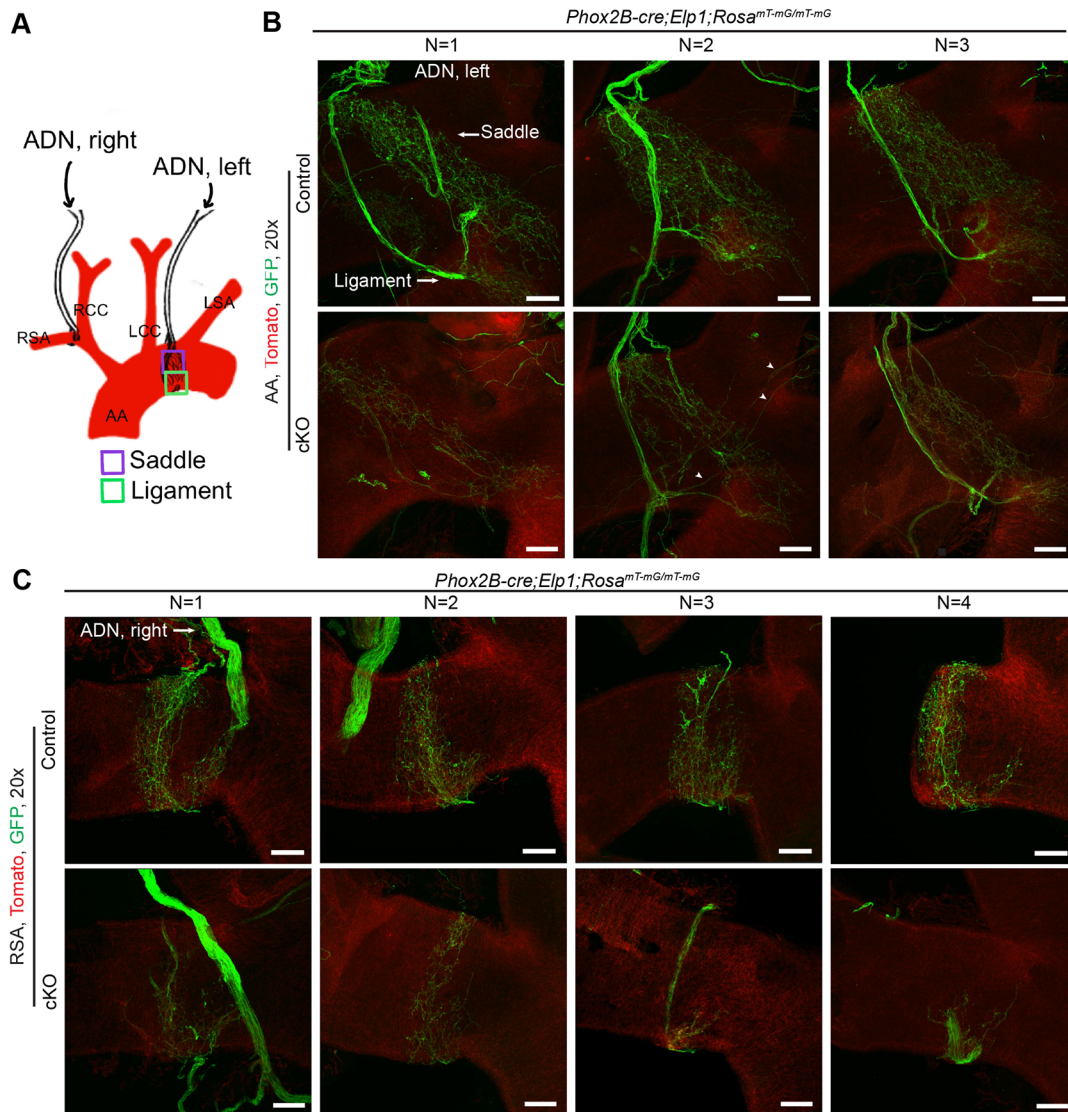


Fig. 8. *Elp1* is required for normal innervation of the aortic arch. (A–C) Schematic (A) depicting visceral sensory neurons innervation of the saddle and ligament regions of the aortic arch (B) and the right subclavian artery (C), at the bifurcation of the brachiocephalic artery. Right and baroreceptive endings in both the saddle and the ligament are decreased in density and branching in cKO *Phox2b;Elp1^{LoxP/LoxP};Rosa^{mT-mG/mT-mG}* mice compared to control *Phox2b;Elp1^{+/LoxP};Rosa^{mT-mG/mT-mG}* mice. $n=3$ –4 mice per condition. White arrowheads in B point to ligament axons that, rather than staying within the ligament, grow in an incorrect direction to traverse through regions of the aortic arch they do not normally grow into. AA, aortic arch; ADN, aortic depressor nerve; LCC, left common carotid artery; RCC, right common carotid artery; RSA, right subclavian artery. Scale bars: 100 μ m (B), 75 μ m (C).

other studies: deletion of *Elp1* in the neural crest causes the loss of 40% of DRG and of ~70% of sympathetic neurons (George et al., 2013; Jackson et al., 2014). This is a clinically promising finding because, if upheld in humans, therapeutic interventions that protect the remaining neurons could be feasible in FD infants.

Within the NP complex, one of the most functionally important cell populations is the dopaminergic neurons, which innervate the carotid body and transmit key sensory information on levels of O_2 , CO_2 and pH, i.e. chemoreceptive information, to the brainstem (Erickson et al., 2001). We found that this subpopulation is particularly dependent on *Elp1* expression and was reduced in number from 30% to 65% depending on the mouse line. Kummer et al. (1993) found that TH^+ NG neurons project axons to the esophagus and stomach, while TH^+ PG neurons project to the carotid body (Katz and Black, 1986; Kummer et al., 1993). Many of the TH^+ neurons appear in clusters that reside in the NG near the exit of the superior laryngeal nerve (Kummer et al., 1993) and were retrogradely

labeled by injections of tracers into the esophagus and stomach. A reduction in this cell population most likely contributes to the severe swallowing impairment suffered by FD patients in addition to the impaired response to hypoxia and hypercapnea in FD patients.

Here, we show that deletion of *Elp1* in the neural crest lineage resulted in a ~40% reduction in glomus cells by E17.5 (Fig. 6), and the carotid body itself was reduced in size in these cKOs. The carotid glomus cells derive from the SCG (Kameda, 2005; Kameda et al., 2008), and we and others have shown that at the ages we examined (E17.5–E18.5), neuronal number in the SCG is reduced by ~70% (George et al., 2013; Jackson et al., 2014) in *Elp1* cKO mice; hence, it is not surprising that loss of *Elp1* would consequentially reduce the number of glomus cells in the carotid body. This finding of reduced glomus cell number may explain the blunted chemoreflex responses to hypoxic conditions in FD patients (Palma et al., 2019). Hence, loss of *Elp1* could interfere with this sensory arm, both autonomously in the glomus cells and indirectly

via reduced innervation of glomus cells by the dopaminergic afferents in the NP complex (Conover et al., 1995). Interestingly, recent work has expanded our understanding of the chemosensory role of this organ: in addition to detecting changes in oxygen, CO₂ and pH, glomus cells are also responsive to changes in blood levels of glucose, lactate, insulin and leptin (Ortega-Sáenz and López-Barneo, 2020). The glial sustentacular cells can act as stem cells and produce new glomus cells under chronic hypoxemic conditions (Pardal et al., 2007). It would be interesting to know whether they are triggered to generate new glomus cells over the lifespan of FD patients.

Given that previous studies (Abashidze et al., 2014; Jackson et al., 2014; Ohlen et al., 2017) revealed failures in peripheral target innervation by *Elp1* cKO sensory neurons, we investigated the integrity of innervation of baroreceptive targets. Here, we show that although many vagal sensory axons do reach the aortic arch and RSA, they do not elaborate the normal complex array of endings in the saddle or along the ligament (Fig. 8). This incomplete target invasion could be the result of impaired retrograde transport of neurotrophins from their targets (Naftelberg et al., 2016). Alternatively, axonal branching has been shown to be dependent on spatial and temporal localization of mitochondria at branch points in order for tubulin to invade the branches (Smith and Gallo, 2018), a process that is impaired in *Elp1* cKO neurons (Ohlen et al., 2017). Misrouted ligament axons were also detected, suggesting that some axonal guidance mechanisms in the aorta also depend on *Elp1* signaling. Taken together, these findings indicate that impaired arterial innervation can significantly negatively impact the normal sensory response to elevated or decreased arterial pressure.

In addition to identifying the potential cellular basis for the failure in the sensory arm of the baroreflex in FD, we also demonstrate here that the central circuitry that integrates this sensory input is marked by significant reductions in cell number. An elegant series of studies by Brunet, Goridis and their colleagues demonstrated that *Phox2b* remarkably delineates the entire cardiorespiratory reflex pathway (Pattyn et al., 1999; Dager et al., 2003). By immunolabeling the brainstems of *Phox2b-cre;Elp1* cKO mice with antibodies against *Phox2b*, we found that the target nuclei for the NP afferents in both the intermediate and caudal regions of the NTS were significantly reduced by ~40% in the *Elp1* cKO mice by E18.5 (Fig. 7). Neurons in the NTS then transmit this sensory input information to neurons in the NA, which were reduced by ~50% in the *Elp1* cKO. Similar reductions were found in the ventrally located medullary relay nuclei including the RTN, which conveys information regarding CO₂ levels as part of the central respiratory chemoreflex (Souza et al., 2019). While examining the *Phox2b*⁺ neurons in the brainstem, it became evident that there were also fewer neurons located in the AP; upon quantification, a significant reduction in ~50% of the AP neurons was detected. Chemoreceptor afferents that innervate the carotid body have also been shown to project to the AP in addition to their major projection to the NTS (Finley and Katz, 1992). Finding reductions in specific brainstem nuclei that mediate cardiorespiratory reflexes may help explain the cellular basis for the major cause of death of FD patients, sudden death during sleep (Palma et al., 2017, 2019). The impaired ventilatory responses of FD patients to hypoxia and hypercapnia can be particularly dangerous while patients sleep and underlies the sleep-disordered breathing experienced by 95% of patients (Palma et al., 2019). We show here both reduced numbers of the chemoreceptors in the carotid body and also reduced cell numbers in the NP ganglia, in addition to reduced neuronal number in the brainstem respiratory circuitry. In a previous analysis we conducted on the brainstem in the *Tuba1a-cre;Elp1* cKO mice, we not only found widespread

expression of *Elp1* throughout the brainstem, but also discovered a reduced number of cholinergic neurons in the dorsal motor nucleus of the vagus (Chaverra et al., 2017). These findings also are supported by autopsy studies that found brainstem atrophy in FD patients at death and dysfunctional brainstem reflexes in patients (Cohen and Solomon, 1955; Brown et al., 1964; Gutiérrez et al., 2015). Collectively, our data indicate the cellular basis for the patient chemoreflex failure that underlies significant patient morbidities.

In summary, the data reported here indicate that *Elp1* is essential both in placode- and neural crest-derived neurons, and that it exerts comparable effects, including on survival, axonal morphology and target innervation, in both lineages. Given the important surveillance role of vagal sensory neurons in maintaining homeostasis, and their diversity in terms of function and gene expression, future experiments should include single-cell RNA-sequencing studies to identify the resilient neurons versus those that are lost in the *Elp1* cKO vagal ganglia. Such an approach would not only help us understand differential cell dependence on *Elp1*, but also inform strategies for therapeutic interventions.

MATERIALS AND METHODS

Mice

All experiments with animals were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Montana State University Institutional Animal Care and Use Committee. The *Rosa^{mT-mG}* [The Jackson Laboratory, Strain #007576; *Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J*]; *Elp1^{LoxP/LoxP}*; *Tuba1-cre1Tes* [kind gift from Dr Lino Tessarollo, National Cancer Institute, Frederick, MD, USA (Coppola et al., 2004)]; *Elp1^{LoxP/LoxP}*; *Wnt1-Cre* [The Jackson Laboratory, Strain #003829; *H2az2^{Tg}(Wnt1-cre)^{11Rth}* *Tg(Wnt1-GAL4)11Rth/J*]; *Elp1^{LoxP/LoxP}* (International Knockout Mouse Consortium) and *Elp1^{BetaGal}* [*Elp1^{tm1c(KOMP)Wtsi}*; Mouse Genome Informatics] mice have all been previously described by our laboratory (George et al., 2013; Chaverra et al., 2017). *Phox2b-Cre;Elp1^{+/LoxP}* mice were generated in our laboratory using a *Phox2b-cre* line purchased from The Jackson Laboratory [B6(Cg)-Tg(*Phox2b-cre*)3Jke/J] and crossed to our *Elp1^{LoxP/LoxP}* mice to generate *Phox2b-cre;Elp1^{LoxP/LoxP}* mice, in a 1:4 ratio, and have *Elp1* deleted from *Phox2b*⁺ cells. All genotyping was performed via PCR.

LacZ staining

Mice that contained a *LacZ* *Ikbk* reporter were obtained from the International Knockout Mouse Consortium [*Elp1^{tm1c(KOMP)Wtsi}*]. This allele contains an FRT-flanked β-gal reporter cassette (*LacZ*) that disrupts the expression of *Elp1*, but heterozygotes are used; these mice have no mutant phenotype (George et al., 2013). Tissue-specific expression of the *Elp1*: *LacZ* reporter can be visualized by β-gal staining. Embryos were fixed (1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS) for 30 min at room temperature. Standard LacZ staining was performed at 37°C overnight. Embryos were then cryoprotected in 30% sucrose/PBS and embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA, USA) for cryostat sectioning. Sections were analyzed with a light microscope.

Immunohistochemistry

Aortic arch whole-mount immunolabeling

Upper bodies of E18.5 or P1 mice were fixed in 4% paraformaldehyde (PFA) at 4°C for 2.5 h. Following three rinses in 1× PBS, aortic arches and surrounding cardiovascular tissue, including the bifurcation of the brachiocephalic artery into the RSA and right common carotid artery, were dissected. Tissue was blocked overnight in 1× animal-free blocker (AFB; Vector Laboratories, #SP-5030) with 0.5% Tween-20, 10% dimethyl sulfoxide and 2% glycine, followed by incubation in primary antibody for three nights. Tissue was then washed 3×15 min in block followed by

overnight incubation in secondary antibody and 3×15 min washes in 1× PBS. Tissue was then mounted in Vectashield (Vector Laboratories, #H-1000-10) or ProLong Gold Antifade Mountant (ThermoFisher Scientific, #P36934).

Embryos were fixed in 4% PFA for 2 h at 4°C. After PBS washes, embryos were cryoprotected in 30% sucrose overnight at 4°C and embedded in OCT. Tissue was then sectioned at 14 µm. For immunohistochemistry, sections were blocked in AFB and 0.5% Triton X-100 for 1 h at room temperature, then primary antibodies were applied and incubated at 4°C overnight. Primary antibodies used included anti-TH rabbit (1:1000; Millipore, #152; RRID AB_390204), anti-GFP (1:2000; Abcam, ab13970; RRID AB_300798), anti-TrkB (1:200; R&D Systems, AF1494; RRID AB_2155264), anti-Phox2b (1:200; kind gift from Dr Christo Goridis, IBENS, Paris, France) and anti-HuCD (1:20,000; kind gift from Dr Vanda Lennon, Mayo Clinic, Rochester, MN, USA). Sections were washed three times with PBS and incubated with secondary antibodies donkey anti-goat Alexa Fluor 568 (Thermo Fisher Scientific, #A-11057, 1:2000), donkey anti-rabbit Alexa Fluor 488 (SouthernBiotech, #6441-30, 1:2000), donkey anti-rabbit Alexa Fluor 647 (Thermo Fisher Scientific, #A31573, 1:2000), donkey anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch #703-545-155, 1:1000), donkey anti-human DyLight 488 (Thermo Fisher Scientific, #SA5-10126, 1:2000 and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature. Sections were mounted in Prolong Gold and imaged by confocal microscopy.

Cell quantification

Ganglion cell quantification

Ganglia were dissected from P9 to 10-month-old mice; to analyze ganglia in neonates, the upper bodies were embedded. Dissected ganglia from adults were fixed for 1.5 h; neonate tissue was fixed for 2 h in 4% PFA at 4°C, then embedded in 100% OCT after a series of sucrose infusions. Tissue was cryosectioned at 12 µm for ganglion analysis and 14 µm for brainstem analysis. All tissue was imaged using a Leica TCS SP8 confocal microscope, and Z-stack images were obtained using Leica Application Suite Advanced Fluorescence software. All sections containing ganglia were imaged. The numbers of TrkB⁺ and TH⁺ cells in each section were counted blind as to genotype, and the total number of cells composing the entire nodose-jugular ganglia (NJG), NPG, JG or NG was determined. The total number of cells per ganglion, or average of left and right total number of cells if both were able to be counted, was compared between mutant and control groups using an unpaired one-tailed Student's *t*-test.

NTS cell quantification

The NTS was divided into the AP, intermediate and caudal regions. To count Phox2b⁺ neurons, at least six equivalent planes from each region were compared using ImageJ. The total number of cells in each region was compared between mutants and controls using an unpaired one-tailed Student's *t*-test. All quantifications were done blind as to genotype.

Carotid body glomus cell quantification

Wnt1-cre;Elp1 upper bodies were fixed for 2 h at 4°C and embedded in OCT as described (George et al., 2013). Consecutive sections were collected at 16 µm. Slides were immunolabeled with antibody against TH. The carotid body is located in the bifurcation of the carotid artery and can be distinguished by the adjacent SCG. Images were taken at 63× in a single focal plane, and image parameters were identical for all slides. All TH⁺ neurons in the carotid bodies were counted (*n*=10 embryos; five mutants and five controls). All quantifications were done blind as to genotype.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.G., F.L.; Methodology: M.C., Z.T.; Validation: Z.T., M.C., L.G.; Formal analysis: M.C., Z.T.; Investigation: Z.T., M.C., L.G., F.L.; Resources: F.L.; Data curation: Z.T., M.C., L.G.; Writing - original draft: F.L.; Writing - review & editing: L.G., F.L.; Visualization: Z.T., F.L.; Supervision: F.L.; Project administration: F.L.; Funding acquisition: F.L.

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