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# Functions of LKB1 in neural crest development: The story unfolds

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## Abstract

Neural crest cells (NCCs) are highly motile, multipotent, embryonic cells that delaminate from the dorsal edges of the neural tube. NCCs follow stereotypical long-range migratory pathways to reach target organs during development, where they give rise to multiple derivatives. The identification of reservoirs of neural crest stem cells that persist to adulthood has recently aroused renewed interest in the biology of NCCs. In this context, several recent studies have demonstrated the essential role of the metabolic kinase LKB1 in NCC establishment. This review surveys how LKB1 governs the formation and maintenance of several neural crest derivatives, including facial bones, melanocytes, Schwann cells, and the enteric nervous system. We also detail the underlying molecular mechanisms that involve downstream effectors of LKB1, in particular the contribution of the AMPK-mTOR signaling pathway to both polarity and metabolic processes. Collectively, these recent discoveries open promising perspectives for new therapeutic applications for the treatment of neural crest disorders.

## KEYWORDS

cell polarity, energy metabolism, LKB1 signaling, neural crest disorders, neural crest cells

## 1 | NEURAL CREST CELLS FORM A REMARKABLE STEM CELL POPULATION

Neural crest cells are specific to vertebrates and their contribution to the formation of the head has led to an evolutionary behavioral shift from filter feeding to active predation.<sup>1-3</sup>

NCCs arise from the embryonic ectoderm germ layer and their formation follows a multistep process starting

with their induction.<sup>4-6</sup> Next, NCCs delaminate from the dorsal part of the neural tube. NCCs then migrate through the embryo and proliferate to reach and colonize diverse target organs, where they differentiate.<sup>7,8</sup> After their establishment, they are maintained throughout embryonic, postnatal, and adult stages. The establishment and maintenance of neural crest derivatives is essential for the shaping of a plethora of organs and precise tissue homeostasis and organ activity. The cellular origin and timing of the induction of neural crest cells

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has been the subject of recent studies that have modified the classical vision of these processes but are not the subject of this review and all work on neural crest cells will not be detailed here.

Briefly, four NCC subpopulations have been defined according to their level of origin along the rostrocaudal axis: cranial, vagal (along with cardiac), trunk, and sacral NCCs.<sup>1</sup> These subpopulations exhibit specific gene regulatory networks, migratory behavior, and differentiation potential that is highly conserved among vertebrates. NCCs give rise to a large variety of cell lineages. NCCs form the facial bones, cartilage, and connective structures of the head. They also generate melanocytes (the pigmented cells of the skin), the neurons and glial cells of peripheral nerves, and the enteric nervous system, which controls digestive motility. Transcriptomic studies have established precise maps of the gene regulatory networks involved at each step of NCC formation and in specific NCC derivatives, made possible from recent advances in single-cell RNA sequencing combined with lineage tracing tools. Several recent reviews provide a more exhaustive list of NCC derivatives and details of their complex lineage trees and the master genes involved in neural crest formation (eg, References 4,7,8,10,11 among others). Their directional migration within the embryo relies on cooperation between gradients of chemical and mechanical signals.<sup>12</sup> These data have collectively renewed our knowledge of NCC development, along with the characterization of signaling pathways and microenvironmental cues required for neural crest formation.

Although considered to be a “transient” population of stem cells, neural crest stem cells were recently shown to be abundant at prenatal stages, even persisting after birth.<sup>13</sup> One well-characterized neural crest stem cell population is that of Schwann cell precursors, originally described as progenitors of immature Schwann cells that then mature into myelinating or nonmyelinating Schwann cells in the peripheral nerves. Schwann cell precursors are also able to give rise to many nonglial derivatives, such as endoneurial fibroblasts,<sup>14</sup> melanocytes,<sup>15,16</sup> sympathetic and parasympathetic neurons,<sup>17-19</sup> and enteric neurons,<sup>20,21</sup> as well as a subpopulation of craniofacial and trunk skeletal cells,<sup>22</sup> among other derivatives. Recent analyses based on single-cell RNAseq showed Schwann cell precursors to have similar transcriptional profiles as late migratory NCCs and to thus constitute a common multipotent “hub” state.<sup>23</sup> Several excellent recent reviews highlight the developmental timing of Schwann cell precursors during embryogenesis and discuss their multipotent, evolutionary, and therapeutic properties.<sup>8,9,11,13,24</sup> These discoveries may lead to novel therapeutic approaches for the treatment of neural crest disorders.

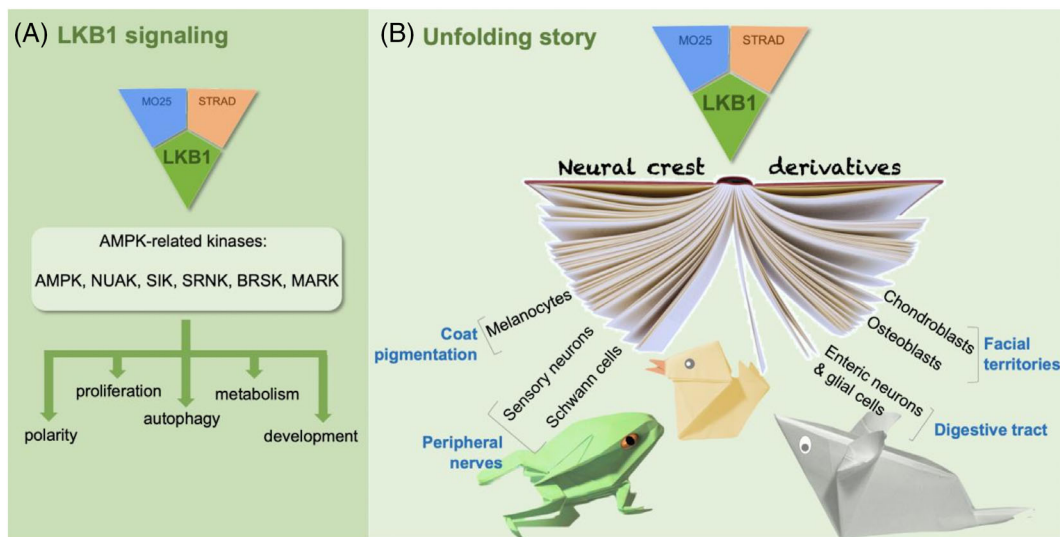
Impaired NCC formation and/or maintenance are responsible for a wide spectrum of human diseases called neurocristopathies.<sup>25,26</sup> These disorders include craniofacial malformations, such as Treacher Collins Syndrome, among others, digestive motility disorders, including Hirschsprung disease, defective heart function, and pigmentation abnormalities and for some patients, peripheral demyelinating neuropathy (subtype 4 of Waardenburg syndrome).

Neurocristopathies are caused by various genetic mutations,<sup>9,25,27</sup> often involving multiple NCC-containing organs (eg, CHARGE or Waardenburg syndromes), that have a severe impact on the quality of life of patients. Current available treatment mainly consists of corrective surgery.<sup>9,13,28</sup> A heterogeneous group of cancers are also derived from NCCs.<sup>29</sup> A better understanding of the underlying molecular mechanisms that lead to defective NCC formation and/or maintenance is needed to design new therapeutic strategies.

## 2 | THE KINASE LKB1 IS ESSENTIAL FOR THE DEVELOPMENT OF NCC LINEAGES

LKB1 forms a heterotrimer complex with the pseudokinase STRAD ( $\alpha$  and  $\beta$ ) and the scaffolding protein MO25, which promote its nuclear export and enhance its kinase activity (Figure 1).<sup>30,31</sup> Several upstream enzymes involved in posttranslational modifications (phosphorylation, acetylation, farnesylation) of LKB1 also modulate LKB1 activity (reviewed in Reference 31). LKB1 phosphorylates and activates 14 related kinases.<sup>32</sup> Among them, the AMP-activated protein kinase (AMPK) is a direct sensor of AMP, ADP, and ATP levels and adapts the cellular energy supply during metabolic stress, notably through the inhibition of the nutrient-sensor target of rapamycin (mTOR).<sup>33</sup> Once activated, LKB1 is involved in a broad array of cellular processes, such as cell survival, proliferation, cell polarity, and the regulation of energy metabolism. These LKB1-mediated biological mechanisms are essential for how LKB1 takes part in NCC ontogeny, as we will review here.

Several studies have recently found that LKB1 is a master regulator of neural crest stem cell formation and differentiation (Figure 1, Table 1). A first hint concerning the contribution of LKB1 to NCC development was given by germline inactivation of *Lkb1* in mice, which is lethal around embryonic day 10.5 (E10.5) and triggers various embryonic defects, including the absence of the first branchial arch, a structure highly enriched in NCCs that later contributes to formation of the face.<sup>34</sup> These data suggested that *Lkb1* ablation disrupts the development of



**FIGURE 1** Recent data concerning LKB1 & neural crest cells: an unfolding story. (A) Schematic representation of the active LKB1 complex (top), downstream kinase substrates (middle) and nonexhaustive cellular functions (bottom). (B) LKB1-mediated regulation of neural crest derivatives of amphibian, avian, and murine animal models. Only neural crest derivatives for which the contribution of LKB1 was established are represented here.

a cranial NCC subpopulation. Since then, several studies have accumulated evidence of a major contribution of LKB1 signaling during NCC differentiation and maintenance in various vertebrate species and NCC subpopulations (Table 1, see associated references). In the mouse, a comprehensive understanding of the role of LKB1 in NCC throughout embryonic development could not be obtained upon germline ablation of *Lkb1* due to early embryonic lethality.<sup>34</sup> Further studies therefore required the development of genetically engineered mouse models with spatio-temporal conditional ablation of *Lkb1* in NCCs. These models allow *Lkb1* inactivation at different times during NCC formation in different NCC subpopulations (Table 1). The effects of *Lkb1* inactivation have also been studied in *Xenopus* and chick models (Table 1). In-depth phenotyping of these vertebrate models collectively identified LKB1 as an important regulator during the formation and maintenance of various NCC-derived lineages (Figure 2).

## 2.1 | LKB1 is required to establish chondrocytic and osteocytic lineages

Cranial NCCs emerge from the posterior forebrain, as well as from the midbrain and hindbrain areas, and migrate to populate either the facial mesenchyme, fronto-nasal process, or pharyngeal arches. Cranial NCCs form the bones, cartilage, and connective tissues of the face, cranial sensory ganglia, and nerves,<sup>35</sup> as well as dental pulp mesenchymal stem cells, which give rise to

odontoblasts in teeth.<sup>36</sup> The development of the tongue is initiated by NCCs and is tightly associated with mandible formation.<sup>37</sup> A number of cranial sutures, as well as the meninges of the forebrain, are also derived from cranial NCCs and are essential for fontanelle closure. Formation of the craniofacial structures and correct brain development are intimately linked and patients with craniofacial dysmorphism often exhibit associated developmental delay.<sup>38,39</sup>

*Lkb1* inactivation in post-delaminating NCCs (from E8.5) has been obtained in mice using the human tissue plasminogen activator (HtPA) promoter to drive the expression of the Cre recombinase (*HtPA-Cre* driver).<sup>40</sup> *Lkb1* ablation in this model caused perinatal lethality and mutants exhibited craniofacial malformations, including a shortened nose and micrognathia. Cranial bones derived from NCCs, such as the maxillaries and mandibles, were greatly reduced, whereas mesodermal-derived occipital bones were unaffected by *Lkb1* loss. However, animals presented defects of the bones of the skull vault, an open fontanelle, and a cleft palate. Both shape and/or bone structure were observed to be altered in the absence of *Lkb1*, leading to severe impairment of the skeletogenic properties of cranial NCCs upon *Lkb1* inactivation.<sup>40</sup>

The critical function of LKB1 in cranial NCCs was confirmed using the avian embryo model, which allows *in ovo* *Lkb1* knockdown in cranial NCCs using small interfering RNA (siRNA) targeting chick *Lkb1* (Table 1). *Lkb1* silencing in cranial NCCs impaired their delamination, migration, survival, and differentiation. A marked reduction in the number of NCCs colonizing the

TABLE 1 Vertebrate models of *Lkb1* conditional ablation targeted to NCCs

Specie	Targeted NCCs	Ablation or inhibition approach	Ablation or inhibition timing	Survival and global phenotype	Description	References
Xenopus	Migrating cranial NCCs	Morpholino microinjection	8-cell stage	Decreased migration of cranial NCCs	Impaired polarized migration of cranial NCCs due to defective nucleocytoplasmic transport of LKB1 upon <i>RanBPI</i> knockdown	Barriga, Alasaadi et al., 2022 Dev Biol
Chick	Pre-delaminating NCCs	RNAi using dsRNA targeting <i>Lkb1</i> mRNA	HH8 (~29h of development)	Most probably not viable Craniofacial malformations	Defective delamination, migration of NCC during early steps of development Defective chondrocytes and osteocytes forming craniofacial structures	Creuzet, Viallet et al., 2016 Dev Biol
Mouse	Pre-delaminating NCCs	<i>Lkb1</i> <sup>F/F</sup> × <i>Wnt1-Cre</i>	From ~E8	Perinatal lethality	Impaired axonal growth and branching of sensory neurons	Ulisse et al., 2020 Life Sc. Alliance
	Post-delaminating NCCs	<i>Lkb1</i> <sup>F/F</sup> × <i>HtPA-Cre</i>	From ~E8	Perinatal lethality Craniofacial malformations	Defective differentiation of chondrocytes and osteocytes forming craniofacial structures	Creuzet, Viallet et al., 2016 Dev Biol
	Post-migrating NCCs	<i>Lkb1</i> <sup>F/F</sup> × <i>Tyr-Cre</i>	From ~E10.5	Lethality around weaning age - Hind limb paralysis - Intestinal pseudo-obstruction - Coat depigmentation	- Hypomyelination due to defective glial specification for both myelinating and nonmyelinating Schwann cells - Impaired enteric nervous system maintenance at postnatal stages - Defective melanoblasts survival	Radu, Torch et al., 2019 Sc. Adv.
	Schwann cell lineage	<i>Lkb1</i> <sup>F/F</sup> × <i>CNP-Cre</i>	From ~E12	Premature death at P30 50% animals required euthanasia by 4 months Survival not described	- Peripheral neuropathy - Hypomyelination - Nonmyelinating Schwann cells are also impaired	Pooya et al., 2014 Nature Com Shen et al., 2014 Nature Com
	Melanocytes	<i>Lkb1</i> <sup>F/F</sup> × <i>P0-Cre</i> <i>Lkb1</i> <sup>F/F</sup> × <i>PLP-Cre</i> <sup>ERT</sup> From P30 (daily injections by i.p.)	From ~E13.5	No early lethality Progressive axonopathy	- Nonmyelinated Schwann cells are most impacted - Defective axonal maintenance	Beirowski et al., 2014 Nature Neurosc.
		<i>Lkb1</i> <sup>F/F</sup> × <i>Tyr-Cre</i> <sup>ERT2</sup> +/− <i>KRAS</i> <sup>G12D</sup>	Newborn pups (topical applications)	Increased melanogenesis and melanoma growth in oncogenic context	- No impact on melanogenesis when <i>Lkb1</i> only is ablated - Increased melanogenesis and melanoma growth when <i>Lkb1</i> ablation is combined to oncogenic <i>KRAS</i> mutation	Liu et al., 2012 Cancer Cell

nasofrontal process was observed upon *Lkb1* knockdown, while later during embryonic development, chondrogenic differentiation of the nasal process was reduced and nose calcification was absent.<sup>40</sup> Consistent with coordinated brain and craniofacial development,<sup>39,41,42</sup> *Lkb1* mutants also exhibited defective brain formation, associated with the abrogated expression of morphogens that pattern the prosencephalon.<sup>40</sup>

In *Xenopus*, *Lkb1* knockdown in cranial NCCs also affects their polarized migration. Nucleocytoplasmic transport of LKB1 is essential for NCC polarity, as over-expressed LKB1 was shown to rescue developmental defects due to the knockdown of the nucleocytoplasmic shuttling protein RanBP1.<sup>43</sup>

Overall, these data show that LKB1 orchestrates several steps of cranial NCC formation essential for the craniofacial bones and cartilage development that pattern the vertebrate head.

## 2.2 | LKB1 contributes to establish the melanocytic lineage

NCCs produce melanocytes of the dermis and hair follicles, oral mucosa, heart, and ears.<sup>44-47</sup> Melanoblasts arise from NCCs delaminating from all axial levels and migrate along a dorsolateral path between the ectoderm (future epidermis) and dorsal surface of the somites. Alternatively, another pool of melanoblasts originate from Schwann cell precursors, an intermediate state of the Schwann cell lineage.

In a study conducted by our laboratory, we inactivated *Lkb1* in the subpopulation of NCCs that expresses tyrosinase, an enzyme that participates in melanin synthesis.<sup>48</sup> The *Tyr-Cre* driver is active after NCC migration from E10.5 (Table 1).<sup>49</sup> This model allows the targeting of several NCC subsets, including migrating melanoblasts, as well as neural and glial precursors of the peripheral nervous system.<sup>48</sup> Mice with conditional disruption of *Lkb1* were born with the expected Mendelian ratio. However, most of the mutants failed to thrive and died within a few weeks after birth. *Lkb1* mutants showed a complex phenotype that associated an array of defects involving several neural crest derivatives. They notably showed a wide range of coat depigmentation patterns. *Lkb1* loss did not impair melanoblast migration but triggered a marked decrease in melanocytes in the hair follicles, thus uncovering the essential role of LKB1 in melanocyte development. However, inducible *Lkb1* ablation in skin melanocytes of newborn pups (*Tyr-Cre<sup>ERT2</sup>* driver) did not trigger any skin or coat color defects,<sup>50</sup> suggesting that LKB1 activity is not required for the maintenance of postnatal melanocytes.

## 2.3 | LKB1 functions in the peripheral nervous system

### 2.3.1 | LKB1 triggers axonal growth and the branching of sensory neurons

In the peripheral nervous system, bipotent neural crest progenitors give rise to sensory neurons, in which the cell bodies are located in the dorsal root ganglia of the spinal cord. *Lkb1* ablation from E8-E9 using the *Wnt1-Cre* driver causes perinatal lethality (Table 1).<sup>51</sup> *Lkb1* loss in this model did not impair axonal polarity but was associated with reduced axonal growth and branching of sensory neurons. These results suggest a function for LKB1 in neuronal maturation in neural crest-derived neurons. In contrast to the *HtPA-Cre* transgene, which is exclusively expressed in neural crest cells, the *Wnt1-Cre* transgene is expressed in neural crest derivatives, as well as in the midbrain and dorsal neural tube of the central nervous system.<sup>52</sup> Given that LKB1 is also essential for neurons of the central nervous system, it cannot be excluded that communication between central and peripheral neurons contributes to the phenotype in this model.

### 2.3.2 | LKB1 is essential for the establishment of the Schwann cell lineage

Aside from sensory neurons, bipotent neural crest progenitors also give rise to glial cells, such as satellite glial cells in the dorsal root ganglia and mature Schwann cells in peripheral nerves. Several reports recently elucidated the involvement of LKB1 in the Schwann cell lineage (Figure 2, Table 1). Schwann cells are essential for correct saltatory conduction of electric signals along axons, notably through myelin production, as well as for trophic support of neurons.<sup>53</sup> Schwann cell fate specification and maturation is a multistep process that occurs at embryonic and postnatal stages.<sup>54</sup> In mice, NCCs first develop into Schwann cell precursors around E12/E13. Next, they differentiate into immature Schwann cells at E15/16 and persist until birth. Immature Schwann cells then become either pro-myelinating and myelinating Schwann cells, which surround motor and sensory axons at a 1:1 ratio, or nonmyelinating Schwann cells, which wrap several sensory axons together. Myelination then starts around postnatal days 4 to 6 (P4-6).<sup>55</sup>

Conditional ablation of *Lkb1* in Schwann cell precursors impairs the formation of myelinating and nonmyelinating Schwann cells and consequently triggers peripheral axonopathies and neuropathies. These peripheral nerve defects upon *Lkb1* loss have been observed in three different mouse models generated by four independent laboratories.

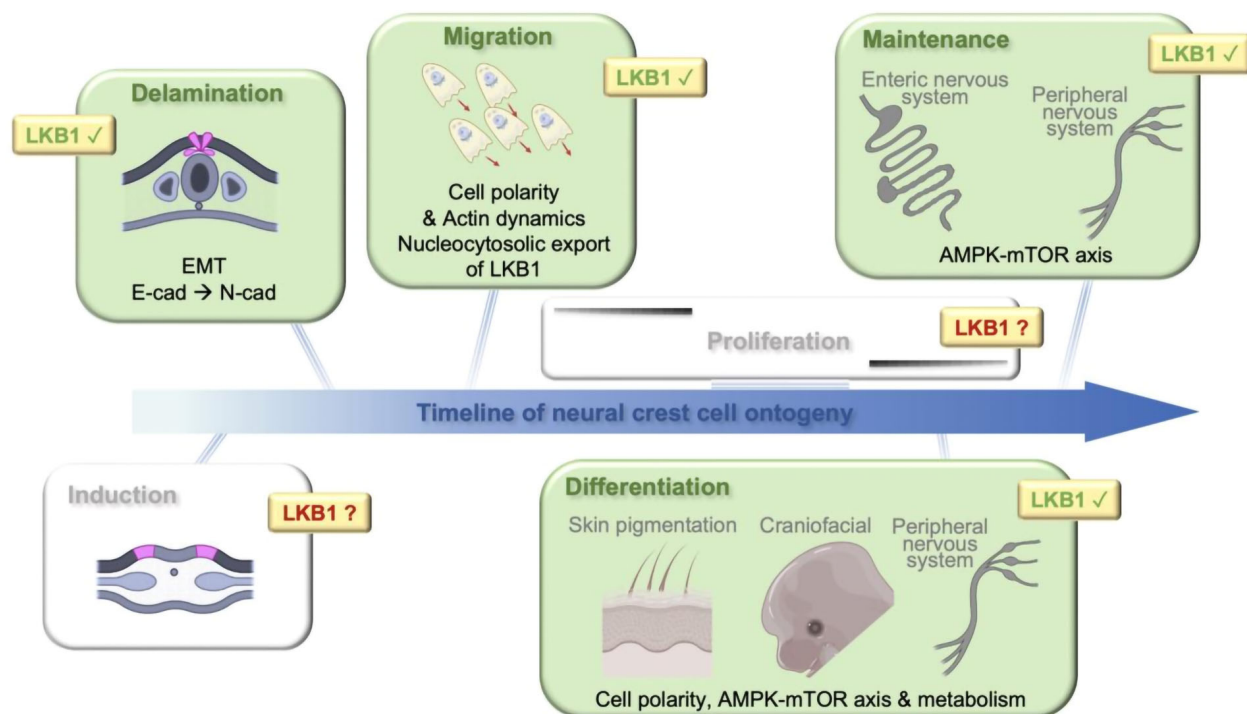


FIGURE 2 LKB1 orchestrates the multistep process of neural crest cell formation by exerting pleiotropic roles.

*Lkb1* inactivation using the *Tyr-Cre* driver (Table 1) has been shown to trigger progressive peripheral neuropathy.<sup>48</sup> At 3 weeks after birth (P21), mutant animals exhibited an abnormal gait and reflexive contraction of the hindlimbs. Macroscopic observations showed that their sciatic nerves were thinner than those of wildtype mice and exhibited myelination defects. Imaging of the nerve ultrastructure revealed abnormal differentiation of myelinating and nonmyelinating Schwann cells. Degrading axons were observed and the number of myelinated neurons was substantially reduced. Furthermore, the increased g-ratio (the ratio of the inner axonal diameter to the outer myelinated axon radius) confirmed the hypomyelination phenotype of the mutant nerves.<sup>48</sup> Moreover, *Lkb1* loss was associated with an increase in the neural crest progenitor population (Sox2- and p75<sup>NTR</sup>-positive cells) in sciatic nerves.

Later inactivation of *Lkb1* in Schwann cell precursors from E12 (Table 1) based on the expression of CNP (2', 3'-cyclic nucleotide 3' phosphodiesterase) was performed using the *CNP-Cre* driver.<sup>56,57</sup> *Lkb1* mutant mice were viable at birth but half of the population became moribund from P14. Their phenotype was characterized by growth retardation and hindlimb muscle atrophy as a consequence of hypomyelination of the peripheral nerves due to a deficit of the myelination process.<sup>57</sup>

*Lkb1* ablation in Schwann cell precursors around E13.5 was obtained with the myelin protein zero (P0, also

called MPZ) promoter using the *P0-Cre driver*.<sup>58</sup> In this model, the features of peripheral neuropathy stochastically appeared at around 3 months of age and progressively worsened until ambulatory impairment that affected all mice. The sciatic nerves were characterized by a reduced number of axons, among which the unmyelinated axons were the most affected. These models have been reviewed in depth.<sup>59</sup>

All studies reported a delay in axonal sorting and myelination upon *Lkb1* loss during the early steps of myelination (from birth to P21). However, compensation of these defects was observed only in the *P0-Cre* model with later *Lkb1* inactivation, with a normal number of myelinating Schwann cells recovered at P30.<sup>58</sup> This recovery may be driven by the accumulation of Sox2- and Oct6-positive progenitors at P21.

Overall, these data highlight the crucial contribution of LKB1 during Schwann cell specification and maturation. During development, axons guide and coordinate the proliferation and differentiation of Schwann cell precursors, which are in contact with them through a juxtacrine effect. Schwann cells are thus not essential for axonal development.<sup>54</sup> In return, mature myelinating and nonmyelinating Schwann cells support neuronal viability and activity.<sup>54</sup>

Collectively, these three mouse models (*Tyr-Cre*, *Cnp-Cre*, and *P0-Cre*) show that the deletion of *Lkb1* leads to axonal degeneration and neuropathy. However, the

knockout of *Lkb1* within a few hours/days of each other gave rise to subtly different phenotypes, with earlier ablation having more drastic effects on myelination and nerve development.

It cannot be excluded that the observed phenotypic discrepancy could be due to specificities of each genetic model, such as the efficiency of Cre recombination. Although all models specifically target *Lkb1* ablation in myelinating and nonmyelinating Schwann cells but not sensory neurons,<sup>48,57,60</sup> *Cnp-Cre* also drives Cre expression in oligodendrocytes of the dorsal spinal cord<sup>61</sup> and *Lkb1* ablation in these cells could thus potentially account for the observed motor defects.

### 2.3.3 | LKB1 maintains mature Schwann cells

The role of LKB1 in the maintenance of mature Schwann cells has also been explored. Inducible inactivation of *Lkb1* in the Schwann cell lineage was performed by tamoxifen injection of *PLP-Cre<sup>ERT</sup>* mice starting at P30 (at the beginning of adulthood), leading to reduced *Lkb1* protein levels in the nerves. After such treatment, 12-month-old mutant mice exhibited an abnormal gait and signs of neuropathy.<sup>58</sup> This phenotype was associated with collapsed myelinating fibers, abnormal unmyelinated axon tracts, and a decrease in the number of myelinated and unmyelinated fibers. However, these degenerative events were not preceded by marked defective myelin structure in intact axons and nerve conduction velocity was not altered up to 10 months of age. These data show that *Lkb1* is essential for axonal maintenance in adult nerves but is dispensable for myelin maintenance, at least in the *PLP-Cre<sup>ERT</sup>* model.

### 2.3.4 | LKB1 is required for the maintenance of the enteric nervous system

Digestive tract motility is ensured by the enteric nervous system (ENS), an extensive network embedded within the lining of the gastrointestinal tract.<sup>62</sup> The ENS makes up the largest portion of the peripheral nervous system and is comprised of millions of enteric neurons and glial cells organized into two interconnected layers of ganglia, the submucosal and myenteric plexuses. The ENS governs bowel motility, as well as digestive secretion and local blood flow. Essential for nutrient absorption, the ENS also interacts with the immune system and gut microbiota while contributing to the gut-brain axis.<sup>62,63</sup>

The ENS is composed of several cell populations of mixed origin: (i) vagal and sacral neural crest

subpopulations and (ii) neural crest-derived cells associated with the vagal nerve or sympathetic fibers innervating the gut through the mesentery, mainly described as Schwann cell precursors.<sup>63,64</sup> In mice, vagal enteric progenitors reach the foregut by E9.5 and then migrate following the rostro-caudal axis to colonize the entire digestive tract, whereas sacral progenitors join the distal part of the hindgut by E13.5.<sup>63</sup> Around E11/12, a subpopulation of vagal NCCs bypass the caecum to follow the so-called “transmesenteric” route and migrate through the mesentery from the midgut toward the hindgut.<sup>63</sup> A recent study suggested that vagal NCCs actually separate into two populations: one adopting the classic rostro-caudal migration route along the gut and one migrating first through the mesentery surrounding the foregut to later colonize the gut and complementarily contribute to the ENS.<sup>65</sup> Along with the historically named “vagal” neural crest, Schwann cell precursors (or nerve-associated bi-potent progenitors) covering the vagal nerve invade the esophagus and stomach around E11.5 and give rise to approximately half of the neurons in the esophagus.<sup>21</sup> At later embryonic and postnatal stages, Schwann cell precursors from the sympathetic efferents innervating the gut through the mesentery contribute to neurogenesis for a small proportion of the submucosal ganglia in the distal part of the small intestine, whereas in the large intestine (colon), they contribute to both myenteric and submucosal ganglia for approximately 20% of neurons, mainly forming calretinin-expressing neurons.<sup>20,66</sup>

NCCs at the migratory front are highly proliferative, whereas NCCs behind the migratory wavefront differentiate into neurons and glial cells and gradually organize the enteric nervous system.<sup>63,67</sup> By E15.5, the digestive tract is completely colonized and proliferates to adjust to the increase in body size until birth. In addition, the proliferation of enteric progenitors and enteric neurogenesis in mice have also been shown to coincide with global body growth after birth.<sup>20,68</sup>

Defective development or maintenance of the ENS triggers enteric neuropathies in humans that substantially affect the quality of life and for which the main therapy consists of surgical resection. Recent studies based on enteric stem cell transplantation support the hypothesis that postnatal reorganization of the ENS is possible, thus opening new therapeutic perspectives.<sup>13</sup> These discoveries emphasize the importance of understanding the biological mechanisms responsible for the formation of the ENS, in particular, given that enteric neuropathies in humans are also associated with neurodegenerative disorders of the central nervous system, inflammatory conditions, diabetes, and as a side effect of chemotherapy for cancer patients.<sup>69,70</sup>

In this context and as mentioned above, *Lkb1* inactivation using the *Tyr-Cre* driver triggers a broad range of defects, including progressive intestinal pseudo-obstruction<sup>48</sup> (Figure 2). In this mouse line, *Lkb1* inactivation occurred at E10.5, when NCCs had already reached the midgut. Although formation of the ENS was not affected by E15.5, the loss of *Lkb1* led to progressive postnatal reduction in the number of enteric ganglia in the distal intestine and colon (Table 1). The remaining ganglia were smaller and displayed a punctiform morphology. At adulthood, *Lkb1* mutants showed atrophy of the stomach, dilation of the small intestine, hypertrophy of the cecum, and constriction of the colon, most likely due to longtime dysfunction of intestinal motricity. These results show that *Lkb1* inactivation is associated with progressive postnatal degeneration, leading to enteric hypoganglionosis, and highlight, for the first time, the importance of LKB1 signaling in the maintenance of the ENS.<sup>48</sup>

Interestingly, animals with conditional inactivation of *Lkb1* using the *HtPA-Cre* driver, which drives earlier recombination in NCCs (by E8.5, Table 1), also exhibited signs of digestive dysfunction and enteric hypoganglionosis but with a more drastic phenotype than that of *Tyr-Cre* mutants (Lucas A., Torch S., Billaud M., Thibert C., personal comm.). These data suggest that as in the Schwann cell lineage, LKB1 may also have pleiotropic roles in enteric lineages. Further studies are required to better delineate the roles of LKB1 in enteric neurogenesis, as well in the indirect trophic effect on the ENS.

### 3 | LKB1-DEPENDENT MOLECULAR MECHANISMS IN NCC

A number of studies have started to shed light on the molecular basis of LKB1-dependent regulation in NCCs, as discussed below.

#### 3.1 | The LKB1-AMPK-mTOR axis controls several NCC fates

##### 3.1.1 | A direct downstream substrate of LKB1: AMPK signaling

AMPK is a heterotrimer composed of one catalytic subunit (AMPK $\alpha$ ) and two regulatory subunits (AMPK $\beta$  and AMPK $\gamma$ ). AMPK regulates cellular processes by promoting catabolic and inhibiting anabolic pathways. Upstream kinases shown to regulate AMPK activity are LKB1, Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase 2 (CaMKK2), and in certain specific settings, transforming growth factor  $\beta$ -activated kinase 1 (TAK1).<sup>33,71</sup> Both

LKB1 and CAMKK2 activate AMPK by its phosphorylation of the Thr172 residue,<sup>33,71</sup> whereas direct phosphorylation of AMPK by TAK1 is still under debate.<sup>71</sup> Although AMPK activity was first observed at the cell membrane and in the cytosol and nucleus, recent studies have also identified AMPK in mitochondria, lysosomes, and the endoplasmic reticulum.<sup>72</sup> Such subcellular pools of AMPK may coordinately regulate different metabolic pathways and underpin the important role of AMPK in cellular and systemic homeostasis. The contribution of LKB1 to NCC biology through AMPK activity is still not completely clear and conflicting explanations have been put forward, as discussed below.

In cranial NCCs, we showed that LKB1 kinase activity is required for the formation of the face, as craniofacial development of chick embryos co-electroporated with *Lkb1*-targeting double-stranded RNA and a plasmid encoding a kinase-dead LKB1 was not rescued whereas it was by co-electroporation with wildtype LKB1.<sup>40</sup> Furthermore, gain-of-function and loss-of-function experiments conducted in chick embryos showed that AMPK activity is also required for LKB1-dependent head morphogenesis. Combined siRNA-based knockdown of the AMPK $\alpha$ 1 and 2 subunits triggered a lack of facial structures reminiscent of the malformations observed upon LKB1 loss.<sup>40</sup> In addition, electroporation of constitutively active forms of AMPK $\alpha$ 2 or  $\gamma$ 1 restored cephalogenesis in *Lkb1*-silenced embryos, thus demonstrating that AMPK transduces LKB1-dependent morphogenetic processes governing head formation.

In sensory neurons from dorsal root ganglia, *Lkb1* ablation triggered reduced AMPK and ACC phosphorylation, suggesting that LKB1 is the upstream activator of AMPK in these cells.<sup>51</sup> Combined biochemical and transcriptomic analyses showed reduced axonal ATP levels upon *Lkb1* loss due to downregulation of the mitochondrial Ca<sup>2+</sup>-binding protein Efh1.

In the Schwann cell lineage, *Lkb1* inactivation using the *P0-Cre* driver led to an increase in AMPK phosphorylation<sup>58</sup> (Table 2). The authors identified high levels of TAK1 activity in *Lkb1* mutant nerves, whereas CAMKK2 activity did not change. These data suggest that TAK1-dependent AMPK phosphorylation could contribute to the peripheral neuropathy caused by *Lkb1* loss.<sup>58</sup> However, conditional ablation of AMPK subunits  $\alpha$ 1/ $\alpha$ 2 or  $\beta$ 1/ $\beta$ 2 in the Schwann cell lineage did not lead to progressive axonal loss but triggered hypomyelination in aging animals.<sup>58,73</sup> Overall, these data indicate a role for AMPK in myelin maintenance, whereas it is not required for axonal maintenance.

Total and phosphorylated AMPK levels were shown to decrease during the process of myelin biogenesis (analyzed between E18 and P9) and AMPK was suggested to

TABLE 2 Metabolic consequences of conditional *Lkb1* ablation in Schwann cells

			General information
<i>Lkb1</i> <sup>F/F</sup> - <i>Tyr-Cre</i> ~ E10.5 Peripheral neuropathy Defective Schwann cell specification and myelination	<i>Lkb1</i> <sup>F/F</sup> - <i>CNP-Cre</i> ~E12 Peripheral neuropathy Myelination delay	<i>Lkb1</i> <sup>F/F</sup> - <i>P0-Cre</i> ~E13.5 Progressive peripheral neuropathy Unmyelinated sensory axons most affected Only transient hypomyelination	Model features <ul style="list-style-type: none"> <li>Genetic model</li> <li>Defloxing stage</li> <li>Nerve phenotype</li> </ul>
N.D. ↘ AMPK activity ↗ mTOR activity	Phospho-Lkb1 at Ser431 by PKA N.D.	N.D. ↘ Phospho-AMPK (TAK1-dependent) No rescue upon mTOR inhibition	Signaling pathways <ul style="list-style-type: none"> <li>LKB1 PTM</li> <li>AMPK activity</li> <li>mTOR activity</li> </ul>
↘ Glucose levels (NMR) ↘ pH acidification (Seahorse) No change in lactate ↗ pyruvate-alanine cycling (NMR) ALAT inhibition (with Cl-ala) => rescue of glial specification	No impact on glycolysis in proliferating Schwann cells ↗ Glycolysis in differentiating Schwann cells (lactate/glucose ratio)	↗ pH acidification (Seahorse) ↗ lactate release Glycolysis inhibition (2DG) => increased axon loss	Cell metabolism <ul style="list-style-type: none"> <li>Glycolysis</li> </ul>
↘ OXPHOS (Seahorse) but not required for glial specification	↘ OXPHOS (Seahorse) ↘ Citrate Overexpression of Citrate synthase in Schwann cells => partial rescue of myelination	↗ TFAM, VDAC mitochondrial proteins ↗ Total mitochondria number ↗ Mitochondrial membrane potential ↗ Cytochrome oxidase ↗ Mitochondrial ROS	• OXPHOS
↘ Lipids with aliphatic chains acyl chain groups (L4 to L6) (NMR)	↘ Myelin lipids (MS lipidomics): phosphatidylcholine, cerebroside, sulphatide, cholesterol	↘ Myelin lipids (MS lipidomics): phosphatidylethanolamine, cerebroside, cholesterol	• Lipid metabolism
↘ ATP production (Seahorse) Supplementation with the AMP precursor AICAR => partial rescue of the peripheral motricity	N.D.	Progressive ↘ ATP ↘ NAD <sup>+</sup> /NADH due to increased NADH levels	• Global energy
Radu, Torch et al., 2019	Pooya et al., 2014 Shen et al., 2014	Beirowski et al., 2014	References

be a negative regulator of myelin biogenesis, although confirmatory studies using more specific and in vivo approaches should be performed to further validate this hypothesis.<sup>74</sup> Consistent with the possible contribution of AMPK at earlier steps of Schwann cell formation, *Lkb1* inactivation in younger Schwann cell progenitors using the *Tyr-Cre* driver triggered a marked decrease in AMPK phosphorylation of Thr172. These observations suggest that the main upstream activator of AMPK in this model is LKB1. Later contribution of AMPK to myelin maintenance in adult animals has also been reported,<sup>58</sup>

suggesting the fine-tuning of AMPK activity in NCCs over time. The regulation of AMPK by LKB1 and/or potentially other upstream kinases could thus be essential for nerve development and function. Such hypotheses merit further exploration using more time-specific conditional inactivation models.

Finally, in enteric ganglia, *Lkb1* ablation in NCCs using the *Tyr-Cre* driver was associated with decreased AMPK phosphorylation and triggered an increase in the neural crest progenitor population (Sox2- and p75<sup>NTR</sup>-positive cells).<sup>48</sup> Furthermore, low levels of AMPK activity

may be important for enteric neuron survival. Indeed, AMPK inhibition using low doses of Compound C in primary cell cultures of myenteric neurons favored neuron survival, whereas higher doses triggered neuronal death.<sup>75</sup>

### 3.1.2 | Recruitment of mTOR

AMPK adjusts the energy available to cells under conditions of stress, partially through the control of mTOR activity. AMPK and mTOR control two intertwined metabolic pathways, both involved in sensing nutrient and energy levels but with counteracting activities: AMPK is activated when ATP levels are low and limits energy-demanding processes, whereas mTOR is activated when nutrients and energy are abundant, acting mainly as an amino-acid sensor.<sup>33</sup> Although numerous studies have characterized how AMPK negatively regulates mTORC1 activity in detail, emerging data suggest that mTORC1 regulates AMPK activity.<sup>33</sup> As LKB1/AMPK have been shown to be involved in the formation and/or maintenance of NCCs, investigating the contribution of mTOR to NCCs with defective LKB1/AMPK activity would be relevant.

Several reviews have already thoroughly described the role of mTOR signaling during **Schwann cell** formation and maturation.<sup>59,76</sup> Under normal conditions, mTORC1 activity is associated with the onset of myelination, whereas its activity decreases when myelination is initiated, thus showing dynamic regulation of mTORC1 to be linked to the differentiation status of myelinating Schwann cells.<sup>76</sup> Conditional mutants of several effectors of the mTOR signaling pathway have been generated in developing or mature Schwann cells. Briefly, these data collectively highlight the essential role of mTOR during the onset and maintenance of myelination, as well as in myelin clearance and remyelination upon nerve injury.<sup>59,73,76-79</sup>

In the context of *Lkb1* ablation in Schwann cells using the *P0-Cre* driver, inhibiting mTOR by rapamycin treatment was shown to not rescue the phenotype.<sup>58</sup> Furthermore, mTORC1 and mTORC2 inhibition in Schwann cells using the *P0-Cre* driver to knock out *mTor* led to hypomyelination and aberrant nonmyelinating Schwann cells but did not result in axonal degeneration.<sup>58</sup> As similar results were obtained when AMPK was inactivated, these data suggest that the loss of axon maintenance in *Lkb1*-deficient Schwann cells is largely independent of AMPK and mTOR activity.

In stark contrast, Radu et al. showed that mTOR activity markedly increased upon *Lkb1* loss, consistent with the decrease in AMPK phosphorylation observed in the *Tyr-Cre* model.<sup>48</sup> Such hyperactivation of mTOR was also observed in vitro using a neural crest stem cell line

committed toward the glial lineage. Consistent with this observation, exposure of cells to the mTOR inhibitor Torin1 rescued the absence of glial differentiation triggered by *Lkb1* knockdown in vitro.<sup>48</sup>

Although several studies have delineated the contribution of mTOR activity during Schwann cell development, few have undertaken the exploration of AMPK/mTOR function in the formation of the enteric nervous system.<sup>48,75</sup> However, there is strong evidence that enteric glial cells provide trophic support to enteric neurons that likely involves metabolic mechanisms. Thus, there is a rationale to explore the role of the AMPK/mTOR pathway in this process. In this context, *Lkb1* ablation in NCCs from E8.5 using the *HtPA-Cre* driver triggered massive intestinal hypoganglionosis associated with upregulated mTOR activity (Lucas A., Torch S., and Thibert C., personal communication).

Earlier conditional disruption of mTOR activity in neural crest stem cells using the *Wnt1-Cre* driver (allowing inactivation from E7.5, before NCC delamination) has shown mTOR activity to be essential.<sup>80</sup> Conditional inactivation of both mTORC1 and mTORC2 signaling (*mTOR<sup>F/F</sup>*) is embryonic lethal, with most animals dying at E12.5 and very few surviving until E14.5. Interestingly, mTORC1 inactivation (*Rptor<sup>F/F</sup>*) triggered E12.5 lethality with similar defects as those found in mTORC1/2 mutants. However, specific *mTORC2* inactivation (*Rictor<sup>F/F</sup>*) triggered perinatal death and the mutants exhibited an elongated nose, suggestive of (i) differential recruitment of mTORC1 and 2 activity during development and (ii) critical mTOR activity in cranial NCCs. These models also allowed studies on NCC-derived cells that form the cardiac outflow upon mTOR inactivation and showed mTORC1 activity to be crucial for heart development, whereas mTORC2 activity was dispensable.<sup>81</sup>

Finally, mTOR also contributes to melanocyte function, as reported in several articles, although its role is still not clearly understood. Deficient mTOR activity has been clearly shown to be associated with pigmentary mosaicism in human patients, often along with defects of other neural crest derivatives (craniofacial dysmorphism, Waardenburg syndrome, Hirschsprung disease, etc.) or other neurodevelopmental disorders, such as developmental delay or epilepsy.<sup>47,82,83</sup> However, the possible deregulation of mTOR by AMPK in this context has not been established.

More generally, the close relationship between LKB1/AMPK and mTOR activity in NCC subpopulations other than Schwann cells or craniofacial territories has not been extensively addressed thus far and further studies remain to be conducted. Furthermore, the contribution of other downstream kinases for LKB1 of the AMPK family or upstream kinases that regulate AMPK has been little investigated and will also require further studies.

In conclusion, accumulating evidence highlights critical recruitment of the LKB1/AMPK/mTOR pathway during the formation of various neural crest derivatives, namely craniofacial cells, sensory neurons, and Schwann cells. These findings suggest that cellular processes regulated by LKB1, such as the control of cell polarity, energy metabolism, and oxidative stress could be essential for NCC differentiation and survival. Certain results support this hypothesis, as described below, and already available evidence along this line will be discussed in the following sections.

### 3.2 | LKB1 controls NCC polarity and cytoskeleton dynamics

The control of cell polarity is essential throughout the multistep process of NCC formation. Following their delamination from the neural tube, NCCs lose their cell-cell adhesion properties and apico-basal polarity to generate a leading edge essential for directional migration through the embryo. Such polarized migration relies on remodeling of cytoskeleton topology and dynamics, notably through the activity of the small GTPases of the Rho family.<sup>84</sup> Polarity of neural crest derivatives in their target organs is also essential for their maturation and function. LKB1 was first identified as a member of the family of polarity proteins PAR (partitioning defective), a highly conserved network of scaffolding proteins, adaptors, and enzymes involved in the control of cell polarity. Since then, numerous studies have established the crucial regulation exerted by the LKB1 signaling pathway in various physiological processes through the control of cell polarity.<sup>31,32,85</sup>

Lkb1 silencing by RNA interference in chick cranial NCCs disturbed their polarized migration. The distribution of the Golgi matrix protein GM130, which normally accumulates in the rear edge of migrating cranial NCCs, showed randomized positioning upon Lkb1 silencing, which subsequently compromised their survival and led to craniofacial defects.<sup>40</sup> Disturbed cell polarity due to the absence of Lkb1 was also observed in sciatic nerves, as shown by immunostaining of the Schwann cell markers GFAP and E-cadherin.<sup>48</sup> Lkb1 was found to be specifically enriched at the Schwann cell/axon interface in Schwann cell and neuron co-cultures, which allow Schwann cell maturation and myelin sheath formation in vitro.<sup>56</sup> Polarized LKB1 distribution is dependent on its phosphorylation at Serine 431 by the upstream kinase PKA, a post-translational modification that is essential for Schwann cell maturation. Indeed, Lkb1 phosphorylation in sciatic nerves has been shown to be associated with increased expression of myelin proteins, such as P0

and MBP, and decreased expression of progenitor factors, such as Oct6.<sup>56</sup> Overall, these data indicate that the control of cell polarity by LKB1 is required in the formation of several NCC subpopulations.

Cell polarity during NCC migration, survival, and specification is also controlled through the modulation of cytoskeleton dynamics involving the activity of critical effectors, such as the Rho-dependent kinase (ROCK).<sup>86-88</sup> In chick embryos, pharmacological inhibition of ROCK1 led to reduced craniofacial development and mimicked the Lkb1 loss-of-function phenotype.<sup>40</sup> Interestingly, expressing a constitutively active form of the ROCK1 isoform restored the cephalic development of embryos depleted of Lkb1 or AMPK $\alpha$ 1/2. One ROCK1 phosphorylation substrate is the myosin II regulatory light chain (MRLC). Once phosphorylated on sites corresponding to Thr18/Ser19 in humans, the MRLC upregulates the stability of the myosin II filament. Expression of a phosphomimetic form of the MRLC restored proper cephalic development in Lkb1-depleted embryos.<sup>40</sup> However, expression of a constitutively active form of AMPK $\gamma$ 1 did not rescue facial morphogenesis of embryos treated with the ROCK1 inhibitor. Overall, these data show that craniofacial development requires the sequential activation of LKB1, AMPK, ROCK kinase, and the actin molecular motor myosin-II in cephalic NCCs.

In Schwann cells, LKB1 is distributed at the Schwann cell/axon interface and colocalizes with the polarity protein Par3.<sup>56</sup> Par3 is a scaffolding protein that was reported to inhibit upstream regulators of RhoA in NCCs and promote microtubule destabilization at cell-cell contacts.<sup>89</sup> Par3 was also shown to be essential for the initiation of myelination.<sup>90,91</sup> Upon Lkb1 ablation in Schwann cells or the restriction of Lkb1 phosphorylation of S431, asymmetric localization of Par3 and myelination were impaired.<sup>56</sup> Further exploration of the contribution of the Par3-Rho-ROCK pathway in the molecular circuits underlying LKB1-mediated regulation of cell polarity during Schwann cell maturation should provide new insights.

### 3.3 | Metabolism-dependent regulation by LKB1 in NCCs

Little is known about the metabolic requirements during NCC formation, although energy metabolism is a central driver of stem cell fate.<sup>92</sup> Measurements of oxygen consumption by trunk explants from amphibians showed that oxygen uptake by NCCs increases when cells begin to differentiate into melanocytes,<sup>93</sup> suggesting that metabolic pathways contribute to the early steps of neural crest formation. After this early study conducted in the

1940s, metabolic studies were long neglected and only recently has there once again been renewed interest in the metabolic contribution to neural crest cell fate. The glycolytic modulator PFKBP2 has been shown to play an important role in the control of delamination and migration of NCCs in *Xenopus* and glycolysis is essential for head formation.<sup>94</sup> Furthermore, the glycolytic status of chick cranial NCCs during early delamination/migration was recently established.<sup>95</sup>

In this context, it is expected that the contribution of LKB1 to NCC formation also relies on its regulation of metabolic functions. However, the metabolic roles of LKB1 have been established thus far mainly in Schwann cells of the peripheral nerves.

We observed a significant decrease in glucose levels using proton nuclear magnetic resonance (NMR) spectroscopy in peripheral nerves of mutants with *Lkb1* ablation from E10.5 (*Tyr-Cre* driver) (Table 2). However, lactate levels were not modified, whereas increased alanine and glutamate levels were detected and confirmed using a neural crest stem cell line that can be differentiated into glial cells. As pyruvate can be transformed into alanine by alanine aminotransferase (ALAT), these data suggest that LKB1 may modify ALAT activity. Indeed, we found that inhibition of ALAT rescued glial specification upon *Lkb1* loss, thus demonstrating that LKB1 governs glial specification through pyruvate-alanine cycling coupled to mTOR signaling without substantially modifying the glycolytic rate.<sup>48</sup> Alanine regulation by LKB1 mainly relies on AMPK activity among the LKB1 downstream kinases. Although oxidative phosphorylation was impaired upon *Lkb1* loss in the *Tyr-Cre* model, the restoration of glial specification by inhibiting ALAT did not rescue mitochondrial activity, indicating that reduced oxygen consumption in this system is independent of glial differentiation. Strikingly, although LKB1 is also required for neuronal specification from NCCs, it does not regulate alanine levels during this process, suggesting tissue specificity in the regulation of pyruvate-alanine cycling (Thibert C., Torch S. and Radu A., unpublished results).

At a later developmental stage, *Lkb1* inactivation in the Schwann cell lineage using the *Cnp-Cre* driver (from E12) did not modify the glycolytic rate of proliferative Schwann cells (Table 2). However, *Lkb1* loss in differentiated Schwann cells prevented the shift from glycolysis to oxidative phosphorylation that typically occurs during the transition from proliferation to differentiation.<sup>57</sup> In addition, *Lkb1* knockout triggered a marked reduction in citrate synthase activity and Schwann cell myelination was partially restored in vitro through the overexpression of this enzyme.

*Lkb1* loss in Schwann cell precursors using the *P0-Cre* driver (from E13.5) triggered upregulation of glycolysis and, thus, increased lactate release.<sup>58</sup> Inhibition of glycolysis and, therefore, normalization of lactate levels in *Lkb1* mutants using 2-deoxy-glucose (2-DG) treatment amplified axonal loss, thus supporting the proposed protective role of lactate observed in this model and confirming the inhibitory action of LKB1 on glycolysis in differentiated cells.<sup>58</sup>

Reduced lipid levels, notably those of major myelin lipids, were observed in all three models, explaining the hypomyelination phenotype. Given the crucial role of these lipids for both glial and neuronal function, such as membrane formation at pre- and post-synaptic sites or energy storage in lipid droplets,<sup>96</sup> the observed decrease in lipid levels upon *Lkb1* loss likely contributes to peripheral neuropathy. Accordingly, citrate synthase overexpression led to the rescue of de novo lipogenesis of Schwann cells in the *CNP-Cre* model, thus strengthening the link between global nerve homeostasis and lipid production by glial cells.<sup>57</sup>

Finally, *Lkb1* ablation with both *Tyr-Cre* and *P0-Cre* drivers triggered an energetically deficient status, as shown by reduced ATP levels (Table 2). Treatment of newborn *Lkb1* mutants of the *Tyr-Cre* model with the AMP precursor AICAR to replenish the ATP pool partially prevented motor defects in the peripheral nerves and the loss of enteric ganglia.<sup>48</sup>

Overall, these data reveal the LKB1-mediated metabolic functions involved in sensory neuron and Schwann cell differentiation and/or maturation. Future studies should more systematically address the metabolic consequences of LKB1 loss during craniofacial formation and enteric neurogenesis.

## 4 | POTENTIAL CONTRIBUTION OF LKB1 SIGNALING TO NEURAL CREST DISORDERS

### 4.1 | Neural crest syndromes

Human germline mutations of the *LKB1*-encoding gene, also named *STK11*, are responsible for Peutz-Jeghers syndrome. This rare autosomal dominant inherited disorder is characterized by the association of benign hamartomatous polyps of the gastrointestinal tract, a high risk of developing various cancers, and hyperpigmented macules that are mainly localized on the lips and buccal mucosa.<sup>31</sup> However, the contribution of defective melanocytes in this syndrome has not been formally established.

TABLE 3 Mutations of LKB1 pathway in human tumors of neural crest origin.

	LKB1 complex				Downstream kinases							Number of Patients
	LKB1	STRAD	MO25	AMPK	NUAK1	NUAK2	SIK1	SIK2	SIK3	BRSK1	BRSK2	
Melanoma	1.7%	2.1%	0.8%	16.7%	3.1%	3.6%	3.5%	1.1%	3.5%	3.9%	1.9%	2494
Neuroblastoma		0.4%	0.4%	1.6%	1.8%		0.4%					1472
Pheochromocytoma				0.5%								31
Paraganglioma												184

Note: Frequencies of genetic alterations were obtained using the cBioPortal database (data from mid-October 2022), including all melanoma studies available on the website except the Pancancer studies and TCGA-PanCancer Atlas to avoid redundancy. Genetic alterations of *AMPK $\alpha$ 2*,  *$\beta$ 2* and  *$\gamma$ 2* account for most of all *AMPK* mutations.

No *LKB1* mutations have been described in human pathologies associated with NCC-based malformations. One likely reason is that this would involve the biallelic loss of *LKB1* function, which is likely embryonic lethal, as shown in mice.<sup>34</sup> However, hypomorphic *LKB1* signaling could be involved in disorders associated with the defective formation of NCCs. This merits exploration. In this context, homozygous mutations of *STRAD $\alpha$* , the pseudo kinase that assembles in the nucleus with the scaffolding protein MO25 and *LKB1*, are responsible for a rare neurodevelopmental disease called PMSE syndrome. PMSE syndrome combines craniofacial malformations, epilepsy, polyhydramnios, and megalencephaly.<sup>97-99</sup> *STRAD $\alpha$*  deficiency in both patients and a mouse model of *STRAD $\alpha$*  knockdown have been shown to be associated with abnormal *LKB1* retention in the nucleus and mTOR hyperactivation in the cortex.<sup>100</sup> Accordingly, inhibition of mTOR using sirolimus (rapamycin) in a pilot study on PMSE patients reduced seizure frequency.<sup>101</sup> It is also conceivable that somatic *LKB1* mosaicism could explain *LKB1* signaling deficiencies in clinical settings. This has been reported for *mTOR* mutations associated with pigmentation mosaicism.<sup>82,83</sup> Interestingly, somatic mosaicism for *LKB1* mutations have been recently observed in Peutz-Jeghers patients.<sup>102,103</sup> These new results support the possible causative contributions of *LKB1* deregulation in neurocristopathies.

## 4.2 | Neural crest-derived cancers

Several tumors originate from neural crest derivatives and the reemergence of a neural crest stem cell like state has been described in certain neural crest-derived cancers.<sup>104</sup> One of the most frequent cancers derived from NCCs is neuroblastoma. This pediatric malignancy develops from the sympatho-adrenal lineage (ventrally migrating NCCs originating between somites 18-24) and represents 15% of childhood mortality induced by cancer.<sup>29,105</sup> The sympatho-adrenal lineage can also give rise to pheochromocytomas and paragangliomas. Other cancers are also derived from neural crest derivatives, including malignant peripheral nerve sheath tumors (MPNSTs, a type of sarcoma) and Schwannomas, ganglioneuromas, and melanomas.<sup>29,104,106</sup>

Somatic mutations of *LKB1* in neural crest-derived tumors are found almost exclusively in melanomas. Analyses of cBioPortal data of melanoma patients show that 1.7% of patients with melanoma exhibit *LKB1* mutations (Table 3). *LKB1* mutations are mainly associated with cutaneous and metastatic melanoma subtypes. Moreover, mutations of *LKB1* partners (MO25 and *STRAD $\alpha$* ) and *LKB1* downstream substrates (*AMPK*, *NUAK1* and

2, SIK1 to 3, and BRSK1 and 2) are also observed in melanoma patients. *AMPK* mutations are found in more than 16% of patients with melanoma (Table 3).<sup>107</sup> Of note, although *LKB1* haploinsufficient Peutz-Jeghers patients show an increased risk to develop cancer, it does not particularly concern tumors derived from NCCs.

Overall, there is thus far little evidence that *LKB1* mutations are associated with neural crest-derived tumors. However, this question merits further exploration, as subtle signaling defects of the pathway (low phospho-AMPK levels for example) rather than gene mutations per se, could be causally involved, as recently found for the *LKB1* pathway in lung adenocarcinoma.<sup>108</sup>

## 5 | CONCLUSIONS AND PERSPECTIVES

Overall, these studies formally demonstrate the pleiotropic role of *LKB1* in the establishment and maintenance of multiple neural crest lineages. It is likely that the known NCC subpopulations in which *LKB1* function is required will expand in the future as additional studies are conducted. Furthermore, the cranial peripheral nervous system and sense organs are the result of coordinated movement and coalescence of neural crest and placode cells, a population of cells that is adjacent to NCCs during development.<sup>109,110</sup> Given the massive head reduction observed in *Lkb1* null mutants,<sup>34</sup> the exploration of any contribution of *LKB1* signaling in placode formation should be informative. In light of the early onset neural crest phenotype of *LKB1* null mutants,<sup>34</sup> the role of *LKB1* in the early steps of cranial NCC formation are also worth exploring, in particular, as cranial NCCs have recently been shown to uniquely retain pluripotent stem cell programming.<sup>111,112</sup> Furthermore, wider use of inducible genetic mouse models to ablate *Lkb1* should help discriminate between the early and late roles of this kinase in neural crest derivatives.

The identification of novel molecular processes involving *LKB1* in NCC ontogenesis opens new perspectives that merit further mechanistic exploration. Future studies will most likely identify common *LKB1*-mediated metabolic mechanisms involved in these various functions in NCCs. However, thus far, there is only a smattering of data, which does not easily allow such analysis.

In particular, delineating the contribution of other *LKB1* downstream kinases, such as *NUAK* or *SIK*, may identify new regulatory mechanisms. In this context, *NUAK* knockout mice exhibit exencephaly and facial clefting, suggesting potential involvement in face formation<sup>113</sup> through the transcriptional coactivators *Yap* and *Taz* of the Hippo pathway.<sup>114</sup> Conversely, more

systematically exploration of how other upstream regulators of *AMPK*, such as *CAMKK2* and *TAK1*, contribute to NCC formation would also be of interest.

More generally, the interplay between *LKB1* signaling and important regulators of NCCs, such as transcription factors or morphogens, still requires further studies. *LKB1* physically interacts with *BRG1*,<sup>115</sup> a transcription factor and chromatin remodeler that controls critical steps during the specification of neural crest derivatives,<sup>116</sup> such as melanocytes<sup>117</sup> and Schwann cells.<sup>118</sup> Furthermore, *LKB1* modulates *p53* transcriptional activity,<sup>119</sup> while *p53* hyperactivation has been observed both in mouse genetic models of neurocristopathies and patients with neurocristopathy.<sup>120,121</sup> Addressing how *LKB1* intersects with regulators of NCC development, for which defects are associated with neurocristopathies, should provide new avenues for therapeutic intervention.

An improved understanding of *LKB1*-dependent metabolic regulation of NCCs will also be a major issue in the near future. As a consequence of metabolic regulation already described in Schwann cells, adaptation to the stress induced by reactive oxygen species is of great interest during NCC formation. Cranial NCCs arise in a context of low oxygen tension, whereas vagal and trunk neural crest cells form after aerobic conditions have begun and thus develop in a context of higher oxygen availability.<sup>122,123</sup> Hypoxia-inducible factor 1 $\alpha$  (*HIF1 $\alpha$* ) is critical for the colonization of branchial arches by cranial NCCs and *HIF2* is required for the development of trunk NCCs.<sup>124,125</sup> In addition, enzymes involved in the capture of free radicals, such as superoxide dismutase (*SOD*), catalase, and glutathione peroxidase (*GPx*), are expressed only from E8.5 and their activity is even maintained at low levels until birth.<sup>126</sup> Therefore, embryonic cells, especially NCCs, may not be well equipped to adapt to abnormal oxidative stress at early developmental stages, possibly explaining why cranial NCCs are very sensitive to high oxidative stress.<sup>127,128</sup> Given that *LKB1* and/or *AMPK* pathways contribute to the antioxidant response by preventing oxidative stress through various mechanisms during development,<sup>129-131</sup> in-depth exploration of the oxidative stress response upon *LKB1* loss are of interest. Indeed, increased oxidative stress was observed in sciatic nerves upon *Lkb1* ablation in the *P0-Cre* model,<sup>58</sup> thus supporting this hypothesis.

As *LKB1* regulates cell polarity and metabolism, which are both essential for NCC formation, the possible interplay between these two cellular processes would be worth exploring in NCCs. In this context, the *LKB1/AMPK* axis has been shown to modulate cell-cell junctions under energetic stress, notably through direct phosphorylation of *GIV/Girdin*.<sup>132,133</sup> In addition,

spatiotemporal resolution of AMPK activity in various subcellular compartments has been recently proven to be a critical feature of its coordinated regulation of cell polarity and metabolism involved in cell fate choices.<sup>72</sup> As an example, the multiprotein complex Ragulator, located at the lysosomal surface, activates LKB1 and AMPK in conditions of energy deprivation, whereas the same proteins switch to mTOR activation in energy-rich conditions.<sup>72</sup> Further evaluation of the subcellular control exerted by LKB1 and AMPK will be essential to better understand how the interconnected regulation of cell polarity and metabolism governs NCC fates.

Massive transcriptomics data, especially single cell RNAseq, has collectively deepened our understanding of the multipotency mechanisms of neural crest lineages. Strikingly, these data have recently revealed that Schwann cell precursors and late migrating NCCs share a transient “hub” state.<sup>23</sup> These findings led to the recent proposal by Adameyko et al. of new conceptual models of the molecular events that establish neural crest lineages.<sup>11</sup> How metabolic signals, in particular controlled by LKB1 signaling, intervene in this context are essential questions that require future studies.

Finally, knowledge of the contribution of LKB1 to human disorders involving NCCs is still limited and future collaborative studies should be conducted to explore potential deficiencies of LKB1 activity in neuro-cristopathies. The combined increase in our knowledge concerning both the molecular mechanisms linking LKB1 to the development of NCCs and the involvement of LKB1 defects in neural crest disorders should lead to the development of new therapeutic strategies that may relieve the symptoms of patients affected by these frequently devastating diseases.

## AUTHOR CONTRIBUTIONS

**Chantal Thibert:** Conceptualization (equal); funding acquisition (lead); supervision (lead); visualization (equal); writing – original draft (equal); writing – review and editing (lead). **Anthony Lucas:** Conceptualization (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Marc Billaud:** Conceptualization (equal); writing – original draft (equal); writing – review and editing (lead). **Sakina Torch:** Visualization (supporting); writing – review and editing (equal). **Marie Mével-Aliset:** Visualization (supporting); writing – review and editing (supporting). **Jordan Allard:** Visualization (supporting); writing – review and editing (supporting).

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