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Regulation of the tumor suppressor LKB1 by the acetyltransferase GCN5

Maya Ghawitian

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THESIS

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Presented by

« **Maya GHAWITIAN** »

PhD thesis supervised by « **Jean VIALLET** » and co-supervised by « **Marc BILLAUD** »

Prepared in **team 3 of the Albert Bonniot Institute**
at the **Ecole Doctorale Chimie et Sciences du Vivant**

Regulation of the tumor suppressor LKB1 by the acetyltransferase GCN5

Defended in public « **June 18, 2015** »,
In front of the jury composed of:

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Préparée au sein de l'équipe 3 de l'Institut Albert Bonniot
à l'École Doctorale Chimie et Sciences du Vivant

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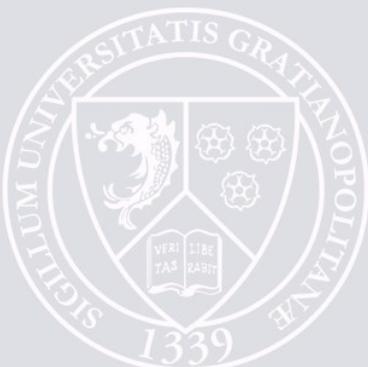
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“Important thing in science is not so much to obtain new facts as to discover new ways of thinking about them”

(Sir William Bragg)

“The secret of life, though, is to fall seven times and to get up eight times.”

(Paulo Coelho)

“The roots of education are bitter, but the fruit is sweet.”

(Aristotle)

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I. Introduction

1 The tumor suppressor LKB1

1.1 The Peutz-Jeghers syndrome

The Peutz-Jeghers syndrome (PJS) is an autosomal inherited disease firstly described by Johannes Peutz in 1921 and further characterized by Harold Jeghers in 1948. This rare disease is characterized by hamartomatous polyposis in the gastrointestinal tract, mucocutaneous hyperpigmentation of oral mucosa, of the lips, of the nose, of fingers and toes (**Figure 1**). The patients have also a high risk to develop malignant tumors affecting various organs such as the digestive tract (Banno et al., 2013), breast, testis and pancreas (Gan & Li, 2014).

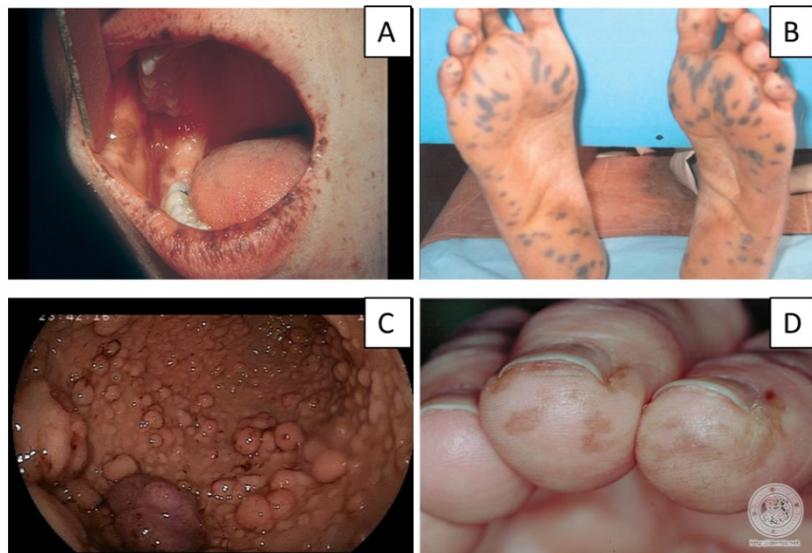


Figure 1: PJS characteristics

The PJS is characterized by hyperpigmentation of oral mucosa and the lips (**A**) (http://www.medicinenet.com/image-collection/peutz-jeghers_syndrome_picture/picture.htm), of toes (**B**) (http://www.ijdvl.com/articles/2008/74/2/images/ijdvl_2008_74_2_154_39705_2.jpg), of fingers (**D**) (<http://www.dermis.net/dermisroot/en/51592/image.htm>) as well as polyposis in the gastrointestinal tract (**C**) (<http://4.bp.blogspot.com/-ULLXZnuvP5k/UdPi0Zi4TJI/AAAAAAAAADPU/B3s7qxY7nzo/s720/18.jpg>).

1.1.1 Germline mutations of LKB1

Genetic linkage analysis led to the localization of a predisposing locus located on chromosome 19p13.3. Positional cloning allowed the identification of mutations in the *LKB1* gene (also called *STK11*) in the germline of PJS patients. These mutations encompass all types of loss of function mutations including deletions of the *LKB1* locus, as well as nonsense and frameshift mutations (Launonen, 2005). Missense mutations are mostly located in the catalytic domain and they disrupt the LKB1 kinase activity. A few mutations have also been observed in the C-terminal tail of LKB1, which impairs the biological activity of LKB1 (Forcet et al., 2005) but no point mutations in the N-terminal non-catalytic region have been identified (Alessi, Sakamoto, & Bayascas, 2006) (**Figure 2**). *LKB1* is the major gene involved in PJS and mutations have been found in more than 80% of the families worldwide. However, linkage with other locus than *LKB1* has been mapped in a few families without *LKB1* mutations. Recently, a germline mutation of the gene coding MYH11 (myosin heavy chain) was found in a PJS patient but the significance of this observation is not clear (Alhopuro et al., 2008). Thus, despite circumstantial evidence of a genetic heterogeneity, no additional genes besides *LKB1* have been ascribed to PJS.

LKB1 is a *bona fide* tumor suppressor since this gene is the target of a double mutational hit that disrupts both *LKB1* alleles. Genetic analysis of PJS cancers has shown that the wild type allele is frequently lost and leads to a loss of heterozygosity (LOH) (Banno et al., 2013). Several reports indicate that additional somatic mutations in other genes such as *β-catenin* and *p53* also contribute to the conversion of hamartomatous polyps into adenomatous and carcinomatous lesions (Miyaki et al., 2000).

1.1.2 *LKB1* mutations in sporadic cancers

LKB1 mutations have also been described in sporadic cancers, more precisely in 4 to 7% of pancreatic cancers, 20% of cervical cancers and 30% of human non-small cell lung cancer (NSCLC) (Banno et al., 2013; Sahin et al., 2003; Hardie & Alessi, 2013). It is now well established that the loss of LKB1 function is critical to pulmonary tumorigenesis, being involved in different stages from tumor initiation to metastasis spreading (Ji et al., 2007).

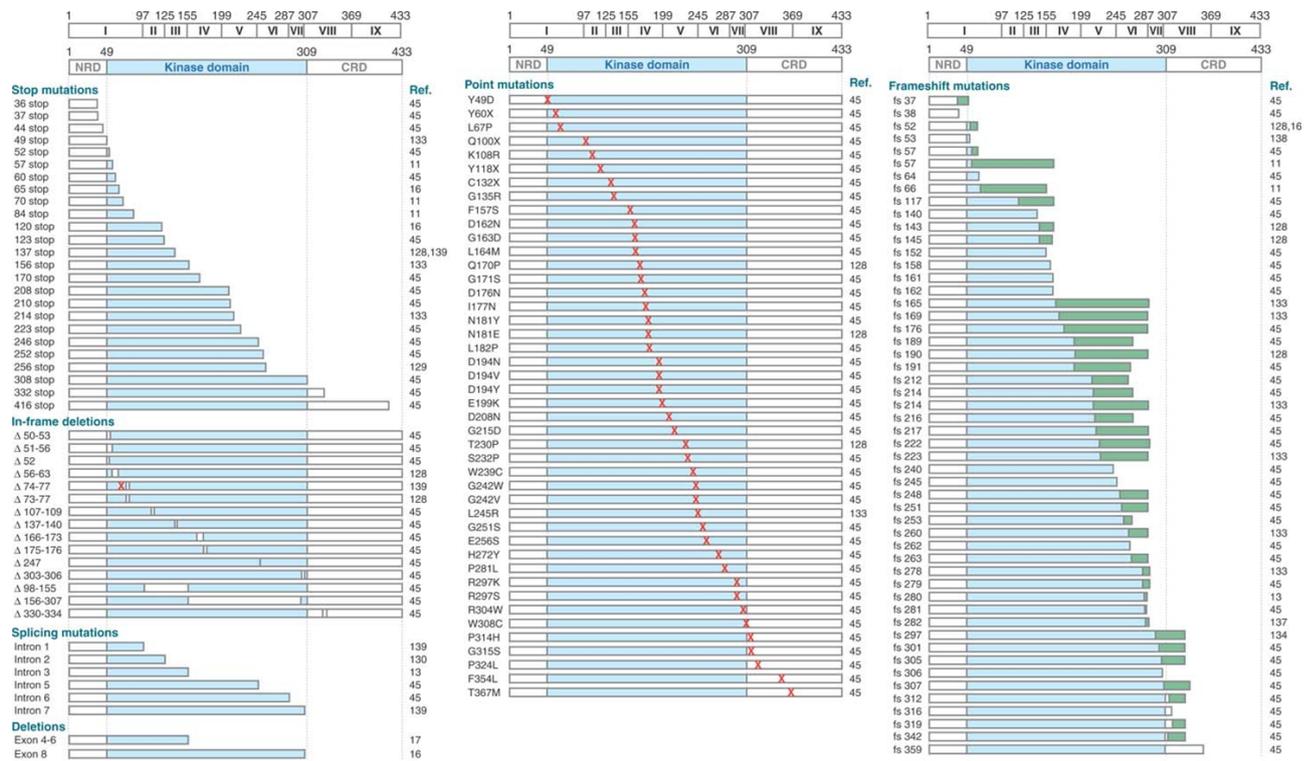


Figure 2: Mutations identified in the human *LKB1* gene in patients with PJS and sporadic cancer.

Schematic representation of the mutations predicted effects on the primary structure of the *LKB1* protein. The genomic organization of the coding sequence of the *LKB1* gene is shown on the top, and the functional domains of the protein are shown below with (a) stop mutations, in-frame deletions, splicing mutations, and deletions; (b) point mutations; (c) frameshift mutations. Abbreviations used: NRD, N-terminal regulatory domain; CRD, C-terminal regulatory domain (*white boxes*); Δ , in-frame deletion; fs, frameshift; Ref, reference; X, point mutation. The protein kinase domain (*blue boxes*) and amino acid sequence introduced by the frameshift (*green boxes*) are also indicated (Alessi et al., 2006).

1.1.3 Murine models of *Lkb1* inactivation

1.1.3.1 Role of LKB1 during embryogenesis and in tissue homeostasis

In order to study the physiological role of LKB1 in mammals, homozygous and heterozygous inactivation of *Lkb1* were conducted in mice. These models have shown that inactivation of *Lkb1* through homologous recombination or 'knock-out' (KO) does not always lead to tumors. This observation is partly due to essential functions of *Lkb1* in development and partly demonstrates the tissue-specificity of *Lkb1* functions.

Following *Lkb1* inactivation in the murine germ line, heterozygous (*Lkb1*^{+/-}) intercrosses resulted in both *Lkb1*^{+/+} and *Lkb1*^{+/-} animals at expected frequencies, whereas *Lkb1*^{-/-} mice died *in utero*. *Lkb1*^{-/-} embryos developed normally up to E8.0. However, macroscopic analysis beyond E8.25 revealed multiple abnormalities including neural tube closure defects and an absence of the first branchial arch which form some facial structures at later stages (Figure 3). The embryos also exhibited defective somitogenesis, excessive mesenchymal cells death and vascular abnormalities associated with increased vascular epidermal growth factor (VEGF) production (Ylikorkala et al., 2001). Altogether, these observations show the essential role of LKB1 in early development for angiogenesis and head formation in mice.

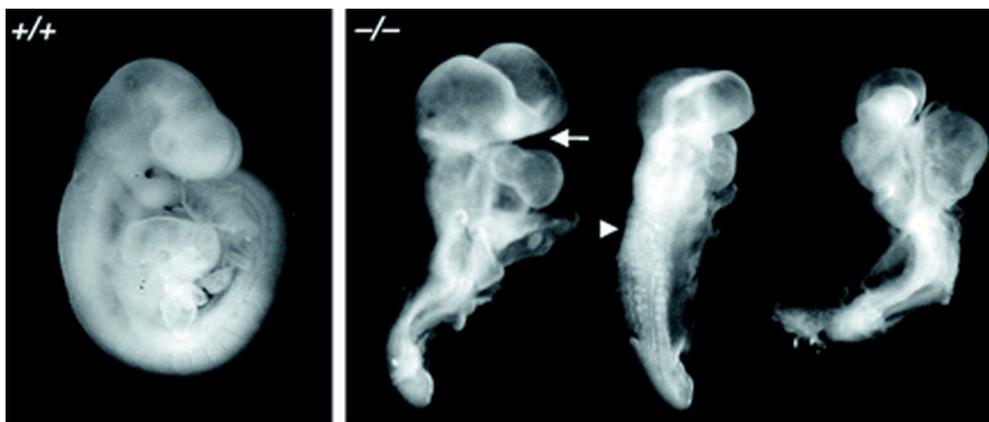


Figure 3: Characterization of developmental arrest in *Lkb1*^{-/-} embryos.

Light microscopy of E9.25 *Lkb1*^{+/+} and *Lkb1*^{-/-} embryos showing unturned embryos with open neural folds, a missing branchial arch (arrow), and aberrant somites (arrowhead). (Ylikorkala et al., 2001).

The role of LKB1 during angiogenesis remains controversial. Deletion of *Lkb1* in vascular endothelial cells using *Tie1-Cre* and analysis of heterozygous *Tie2-Cre;Lkb1^{flox/+}* mice suggest a proangiogenic role of *Lkb1*. *Tie2-Cre;Lkb1^{flox/+}* mice exhibit a normal phenotype, including vasculature. However, they display reduced revascularization after hind-limb ischemia in adult mice (Londesborough et al., 2008; Ohashi, Ouchi, Higuchi, Shaw, & Walsh, 2010). In contrast, during embryonic development, increased VEGF signaling upon *Lkb1* deletion rather suggests an antiangiogenic role for *Lkb1* (Ylikorkala et al., 2001). This antiangiogenic effect is also present in the context of PJS polyps where a loss of *Lkb1* leads to increased vasculature. Thus, the role of *Lkb1* in angiogenesis seems to be context-dependent (Shackelford et al., 2009).

Other studies have reported the role of *Lkb1* in maintaining the homeostasis of other tissues such as liver, skeletal muscle, pancreas and nervous system. Non-tumorigenic mouse phenotypes following *Lkb1* targeting in different tissues are presented in **Figure 4**.

For instance, conditional hepatocyte-specific *Lkb1* deletion using *Adeno-Cre* resulted in impaired glucose metabolism in the mice as demonstrated by elevated blood glucose levels and expression of the PGC-1 α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) gluconeogenic target genes encoding the G6Pase (glucose-6-phosphatase) and PEPCK (phosphoenolpyruvate carboxylase). Thus *Lkb1* is involved in the regulation of hepatic glucose production (Shaw et al., 2005). The increase in the expression of the genes encoding PGC-1 α , G6Pase, and PEPCK was also observed in another study in *Lkb1*-deficient hepatocytes (Foretz et al., 2010). Other reports of liver-targeted deletions of *Lkb1* also demonstrate that *Lkb1* is required for lipid, bile, and cholesterol metabolism (Angela Woods et al., 2011).

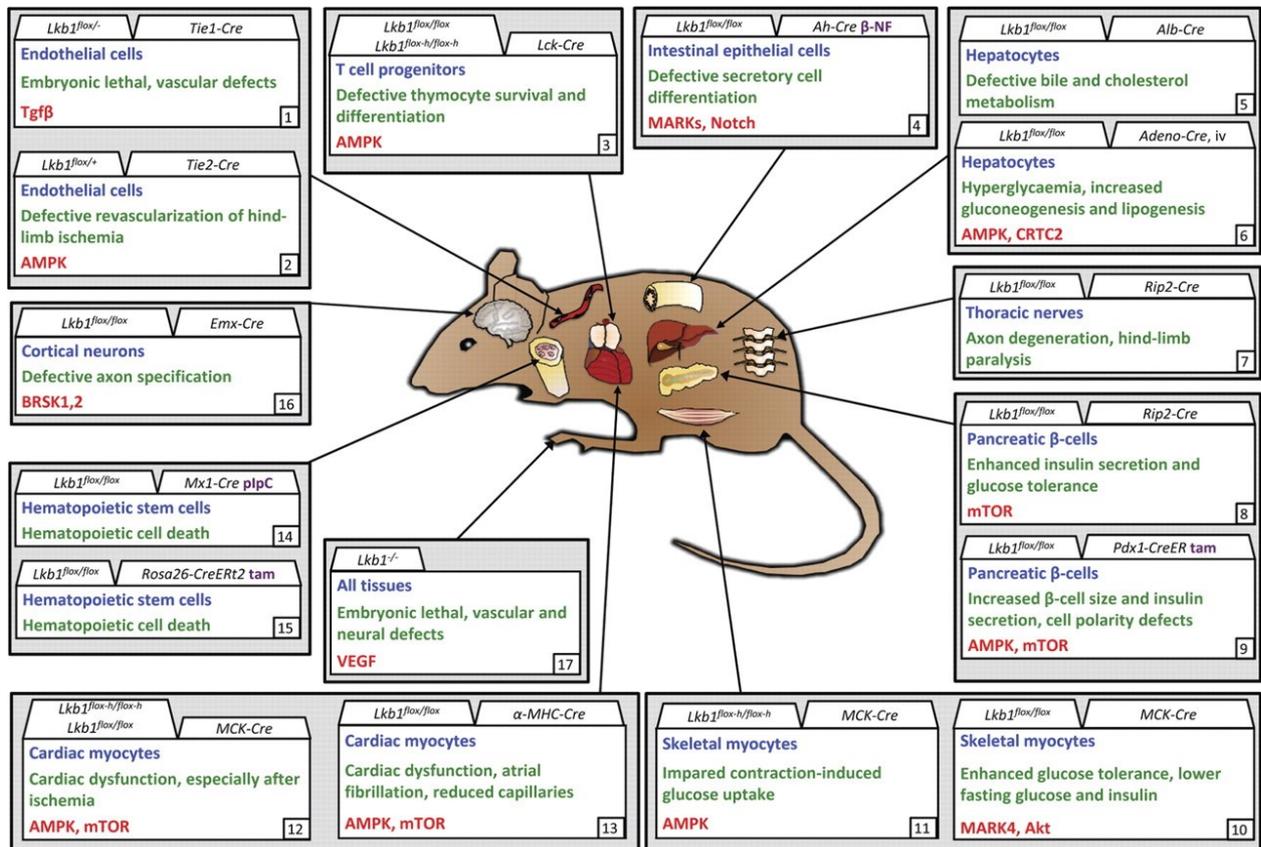


Figure 4: Non-tumorigenic phenotypes following *Lkb1* targeting in mice.

Phenotypes (green) are grouped according to tissue type, cell type affected/analyzed (blue), and alleles used for targeting. When appropriate, activator or deleter is indicated in purple. Noted signaling change(s) indicated in red. Alleles as displayed in original publications except for *Lkb1 flox-h/flox-h* hypomorphic *Lkb1* (Sakamoto et al., 2005). (1) Londesborough et al., 2008; (2) Ohashi et al., 2010; (3) Cao et al., 2010; Tamas et al., 2010; (4) Shorning et al., 2009; (5) Woods et al., 2011; (6) Shaw et al., 2005; (7) Sun et al., 2010a; (8) Sun et al., 2011; (9) Granot et al., 2009; Fu et al., 2009; (10) Koh et al., 2006; (11) Sakamoto et al., 2005; (12) Sakamoto et al., 2006; Jessen, et al., 2010; (13) Ikeda et al. 2009; (14) Gurusurthy et al., 2010; Nakada et al., 2010; (15) Gan et al., 2010; (16) Barnes et al., 2007; (17) Ylikorkala et al., 2001. tam, tamoxifen; β -NF, β -naphthoflavone; plpC, polyinosinic–polycytidylic acid; iv, intravenous. (Ollila & Mäkelä, 2011).

1.1.3.2 Understanding the PJS

Since homozygous inactivation of *Lkb1* has proven to be lethal, heterozygous *Lkb1* mice were generated and have proven to be useful for understanding *Lkb1*-induced polyp formation.

The development of large occluding hamartomatous polyps in the gastrointestinal (GI) tract is a major characteristic in PJS. Different laboratories have established that biallelic *Lkb1* inactivation is not required for polyp development. Indeed, *Lkb1* monoallelic inactivation was sufficient for the development of polyps in mice (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002). The *Lkb1* +/- mice are viable and fertile and show no apparent phenotype until the adult age. Polyps are first detected at around 5 months and are particularly prominent in the pyloric region rather than in the small intestine like in human, in line with recent reports on gastric polyposis in human. They cause premature lethality from 8 months onwards, presumably due to intestinal obstructions (Katajisto et al., 2008; Udd et al., 2004). Polyps from both patients and murine models were shown to retain the *Lkb1* wild-type allele suggesting that haploinsufficiency triggers polyp formation (Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002). Cyclooxygenase 2 (COX2) was shown to be up-regulated in the mouse and PJS patient polyps (Rossi et al., 2002), and COX2 inhibitors have been shown to be efficient suppressors of PJS polyps (Udd et al., 2004).

Another study suggests that LOH is required for polyp development. In this study, 25% of polyps which were isolated from *Lkb1* +/- mice retain the *Lkb1* wild-type allele in the stromal compartment but lose it in the epithelial compartment. However, in half of the polyps which conserved the *Lkb1* wild-type allele, *Lkb1* was not detected at the protein level suggesting epigenetic modifications. The mouse model used in this study exhibits polyps within the gastrointestinal tract in addition to the gut. The differences between this model and the previous ones might be due to the genetic background (Bardeesy et al., 2002).

To date, the implication of a *p53*-deficient background in malignant transformation of murine *Lkb1* +/- polyps remains controversial (Jansen, Ten Klooster, Offerhaus, & Clevers, 2009) vs (Ollila & Mäkelä, 2011). However, inactivating *Lkb1* in the smooth muscle lineage, using a tamoxifen-inducible *SM22-CreERt2* line, is sufficient for GI polyposis (Katajisto et al.,

2008). In this model attenuated TGF β signaling from stromal cells to epithelial cells was associated with increased epithelial proliferation. The endothelium-specific deletion of *Lkb1* resulted in loss of TGF β secretion, followed by loss of supporting smooth muscle cells (Londesborough et al., 2008). Finally, *Lkb1* deficiency in MEFs leads to attenuated TGF β signaling resulting in defects in smooth muscle cell lineage differentiation. These observations suggest that stromal *Lkb1* acts as a 'landscaper' tumor suppressor gene (Vaahtomeri et al., 2008) and that stromal TGF β signaling is an important growth restrictive signal to GI epithelial cells.

Despite these latest reports, the mechanism whereby the cell carcinomas occur in PJS and *Lkb1* +/- mice remains unclear. However, understanding the mechanisms underlying this cellular transformation is worth further investigation in order to better understand the tumor suppressor function of LKB1.

1.1.3.3 Tumor models

In several tissues, *Lkb1* inactivating mutations result in the development of cancer. Targeted inactivation of *Lkb1* in mice, sometimes in combination with other tumorigenic mutations, have led to the development of various tumors in multiple tissues, sometimes modeling human cancers in very useful ways as discussed below (some examples) and summarized in **Figure 5**. Investigating the role of *Lkb1* during malignant tumor progression has firmly established its tumor suppressive activity.

In mice, both homo- and heterozygous loss of *Lkb1*, combined with *Kras* activation, promote lung carcinogenesis and metastasis (Ji et al., 2007).

As for cervical cancer, to date, no targeted *Lkb1* mouse models have been reported. However, Contreras group has generated three mouse models with *Lkb1* deficiency. In the first model, (53%) of surviving *Lkb1*^{-/+} females spontaneously developed endometrial adenocarcinomas by 55 weeks of age and the tumors were highly invasive. In the second model, they injected an *Adeno-Cre* virus into the uterine lumen of female mice homozygous for a floxed *Lkb1* allele (*L*). Again, 65% of the mice developed endometrial adenocarcinomas which were confined to the uterus, but one was diffusely metastatic within the peritoneum. The fact that not all of the mice developed uterine tumors and the focal nature of these

neoplasms suggest that cooperating genetic events are required for *Lkb1*-driven neoplasia. Interestingly, the team has also observed that decreased expression of *Lkb1* correlates with invasiveness in human tumors, presenting the first evidence that *Lkb1* similarly drives human endometrial carcinogenesis (Contreras et al., 2008). Finally, Contreras team has generated a third mouse model allowing conditional inactivation of *Lkb1* only in the epithelial cells of uterine lumen and endometrial glands. These mice died rapidly of invasive endometrial adenocarcinomas. These mouse models developing endometrial adenocarcinomas show that the frequent uterine cancer in PJS patients can be recapitulated in mice, and targeting therapies can be developed to treat *LKB1*-deficient endometrial cancer (Contreras et al., 2010).

One study has reported that 70% of aged *Lkb1* +/- male mice develop hepatocellular carcinoma (HCC) in contrast to 20% of female mice. These neoplasms recapitulated human HCC types and were shown to have lost the wild-type copy of *Lkb1* (Nakau et al., 2002). The tumor development was accelerated upon crossing *Lkb1* +/- mice with *p53* mutant mice, or forced activation of Wnt signaling (Miyoshi et al., 2009; Takeda, Miyoshi, Kojima, Oshima, & Taketo, 2006). However, complete deletion of *Lkb1* in hepatocytes resulted in metabolism impairment without tumor development (Shaw et al., 2005; Angela Woods et al., 2011). This observation may reflect the time-span required for the lesions to occur.

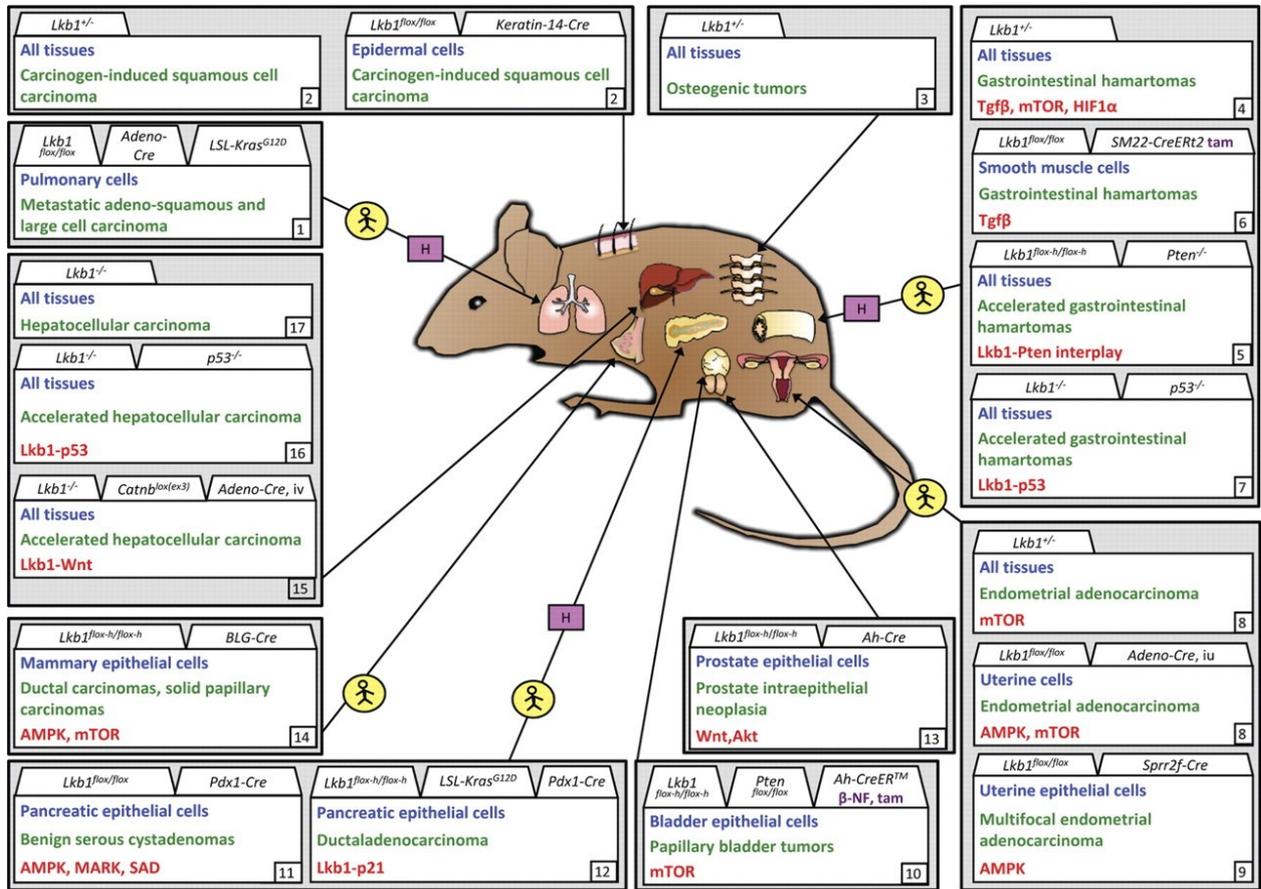


Figure 5: Tumorigenic phenotypes following *Lkb1* targeting in mice.

Tumor types (green) are grouped according to tissue type, cell type affected/analyzed (blue), and alleles used for targeting of *Lkb1* and possible other alleles. When appropriate, activator of deleter is indicated in purple. Noted signaling change(s) indicated in red. Tissues where LKB1 deficiencies noted in human tumors are marked with yellow circle. Tumorigenic phenotypes resulting from *Lkb1* haploinsufficiency indicated with a pink H box. Alleles as displayed in original publications except for *Lkb1 flox-h/flox-h*, hypomorphic *Lkb1* (Sakamoto et al., 2005). (1) Ji et al., 2007; (2) Gurusamy et al., 2008; (3) Robinson et al., 2008; (4) Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Rossi et al., 2002; Katajisto et al., 2008; Shackelford et al., 2009; (5) Huang et al., 2008; (6) Katajisto et al., 2008; (7) Wei et al., 2005; Takeda et al., 2006; (8) Contreras et al., 2008; (9) Contreras et al., 2010; (10) Shorning et al., 2011; (11) Hezel et al., 2008; (12) Morton et al., 2010; (13) Pearson et al., 2008; (14) McCarthy et al., 2009; (15) Miyoshi et al., 2009; (16) Takeda et al., 2006; (17) Nakau et al., 2002. tam, tamoxifen; β-NF, β-naphtoflavone; plpC, polyinosinic–polycytidylic acid; iv, intravenous; iu, intrauterine; LSL, lox-stop-lox. (Ollila & Mäkelä, 2011)

1.2 LKB1: from the gene to the protein

LKB1 is a tumor suppressor gene which is evolutionarily conserved. The human gene spans 23 kb and comprises nine coding exons and one non-coding exon (Mehenni et al., 1998). The murine *Lkb1* gene is located on chromosome 10, also contains 10 exons, and the encoded protein shares more than 90% with the human protein sequence. Besides, murine *Lkb1* has a conserved prenylation motif (Cys433–Lys–Gln–Gln436) at the carboxyl-terminus, directly downstream from a consensus cAMP-dependent protein kinase (PKA) phosphorylation site (Arg428–Arg–Leu–Ser431) (Collins, Reoma, Gamm, & Uhler, 2000).

The *LKB1* gene is ubiquitously expressed in fetal and adult tissues as well as tumors. During embryonic development in mouse, *Lkb1* (mRNA) is mainly expressed in the gastrointestinal tract, lung and testis. Human *LKB1* is predominantly expressed in epithelia and the seminiferous tubules of the testis, with higher levels in fetal than in adult tissues. However, the ortholog of *LKB1* in *Xenopus*, *XEEK1*, seems to be restricted to early embryogenesis while in *C. elegans*, the protein *Par-4* can be found in the gonads, oocytes and early embryos. Finally, some malignant tumors express high levels of *LKB1* while some cancer cells show no expression of *LKB1* which suggests a dual role of *LKB1* at different stages of tumorigenesis (Gan & Li, 2014).

1.2.1 Splice variants of *LKB1*

The 23 kb human *LKB1* gene is transcribed in the telomere-to-centromere direction and the 3 kb mRNA can be alternatively spliced to produce two isoforms: *LKB1_L* (long isoform) and *LKB1_S* (short isoform). The alternative splicing occurs at exon 9; the C-terminal sequence of *LKB1_L* is encoded by exon 9B, while in *LKB1_S* this sequence is encoded by exon 9A of the mRNA leading to the replacement of the last 63 amino-acids (a.a.) of the long isoform by 39 different amino acids in the short isoform (Towler et al., 2008) (**Figure 6**). Thus, the corresponding proteins are distinguishable by their molecular weights which are 50 KDa for *LKB1_L* and 48 KDa for *LKB1_S*. *LKB1_L* is ubiquitously expressed, whereas *LKB1_S* is predominant in the testis where it appears to be involved in spermiogenesis. Indeed, male knockout mice for this isoform are sterile and show a decrease in the number of mature spermatozoa. The major cause of the infertility phenotype is a defect in the release of mature spermatids from

the seminiferous epithelium (spermiation) during spermatozoan development (Denison et al., 2011; Denison, Hiscock, Carling, & Woods, 2009; Reuben J Shaw, 2008).

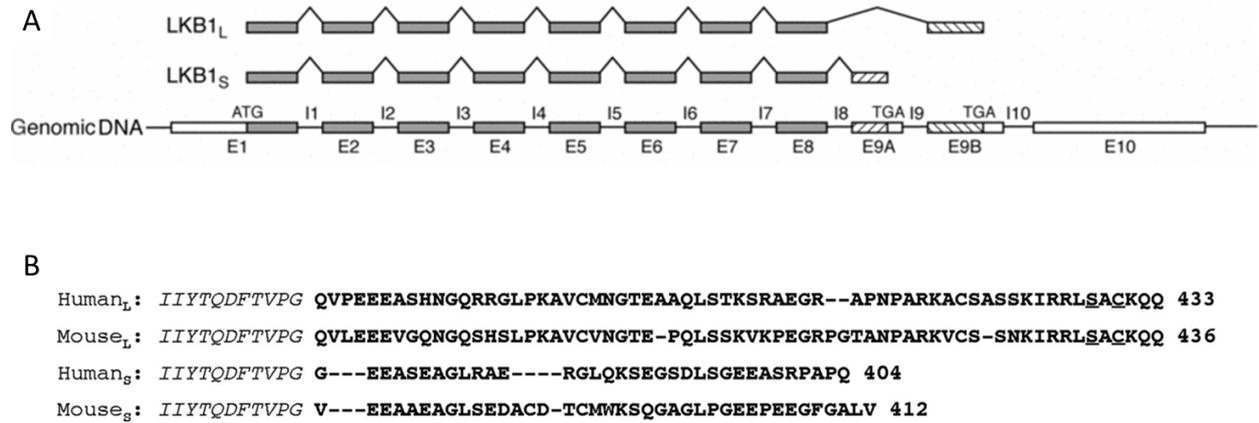


Figure 6: LKB1 splice variants: the long and short isoforms

(A) The arrangement of exons (E1 through E10) and introns (I1 through I10) of the LKB1 gene are shown. Protein coding regions common to both isoforms are shown in grey, while the regions in exons 9A and 9B encoding the unique C-terminal regions of LKB1_S and LKB1_L are shown with forward or backward cross-hatching respectively. **(B)** The deduced amino acid sequences of the C-terminal regions of human and mouse LKB1_L and LKB1_S are shown. A portion of the sequence encoded by exon IIIIV, common to both forms, is represented in *italics*. Serine 428/431 (human/mouse) and cysteine 430/433 in LKB1_L that have been shown to undergo post-translational modification are *underlined*. The number of amino acids in each of the full-length proteins is indicated. Adapted from (Denison et al., 2009; Towler et al., 2008).

Recently, a third isoform of LKB1 was identified by the team of Christine Perret (Institut Cochin, Paris) in collaboration with our team. This isoform of LKB1 is generated by alternate splicing and encodes a protein called Δ N-LKB1. This novel isoform is truncated in its N-terminal domain and results from an alternative splicing in exon 1 and internal initiation of translation of the mRNA in exon 3 (**Figure 7**). The 42 KDa corresponding protein is mainly expressed in the heart and skeletal muscle. It has pro-oncogenic properties and may account for some of the paradoxical effects of LKB1 during tumorigenesis (Dahmani et al., 2014).

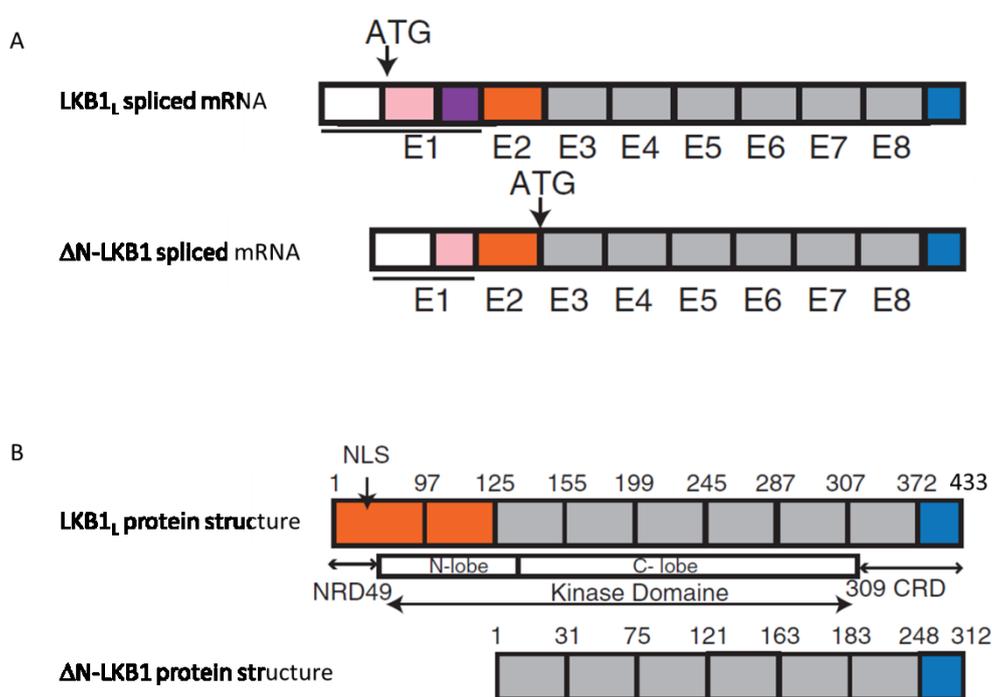


Figure 7: A novel LKB1 isoform generated by alternate splicing encodes a protein called Δ N-LKB1

(A) The initiation of translation of the Δ N-LKB1 isoform starts in exon 3. **(B)** Structure of the human LKB1 isoforms LKB1_l and Δ N-LKB1. The alternative splicing in exon 1 and internal initiation of translation in exon 3 result in a novel isoform called Δ N-LKB1, which is truncated in its N-terminal domain. CRD: C-terminal-regulated domain; NLS: nuclear localization signal; NRD: N-terminal-regulated domain. Adapted from (Dahmani et al., 2014).

1.2.2 The protein LKB1

1.2.2.1 Structure and subcellular localization of LKB1

LKB1 encodes a serine/threonine kinase comprising 433 amino acids (a.a) in human (436 in mouse). Its kinase catalytic domain is localized between a.a. 49 and a.a. 309 (44-309 for murine *Lkb1*). The N-terminal region of *LKB1* contains a nuclear localization signal (NLS) which is localized between a.a. 38 and 43 and which regulates *LKB1* shuttling between the nucleus and the cytoplasm (**Figure 7**). As for the C-terminal region, it contains a prenylation site (Cys433) which is associated to the localization of *LKB1* at the plasma membrane.

Thus, *LKB1* can be localized within the nucleus, the cytoplasm, the plasma membrane in living cells but can translocate into mitochondria during apoptosis (Alessi et al., 2006; Jansen et al., 2009; Karuman et al., 2001).

1.2.2.2 Post-translational modifications

LKB1 undergoes post-translational modifications such as phosphorylation, prenylation, ubiquitination as well as acetylation.

1.2.2.2.1 Phosphorylation

So far, nine *LKB1* Ser/Thr residues were found to be phosphorylated (**Figure 8**). The major autophosphorylation site of *LKB1* is on Thr336. Mutation of this residue to Glu (to mimic phosphorylation), but not Ala (to abolish phosphorylation), prevents *LKB1* from inhibiting the growth of G361 melanoma cells. This observation indicates that this phosphorylation seems to inhibit the tumor suppressor function of *LKB1* (Alessi et al., 2006). *LKB1* is also autophosphorylated on Thr185, Thr189 and Ser404 (murine)/Ser402(human) but no functional role has been attributed to these residues (Alessi et al., 2006; A F Baas et al., 2003; Gopal P Sapkota, Boudeau, et al., 2002).

LKB1 is also phosphorylated by upstream kinases on Ser31, Ser307, Ser325, Thr366 and Ser428 (human)/431(mouse). Mutation of Ser31, Ser325 and Thr366 had no effect on the catalytic activity of subcellular localization of *LKB1* and the autophosphorylation on Ser31 and Ser325 remain controversial (Gopal P Sapkota, Boudeau, et al., 2002). However, the phosphorylation of Thr366 by ATM (ataxia-telangiectasia-mutated), in response to oxidative

stress, induces LKB1-dependant autophagy (Alexander et al., 2010; Gopal P Sapkota, Deak, et al., 2002). LKB1 is also phosphorylated on this residue by DNA-PK (DNA-dependant protein kinase) *in vitro* (Gopal P Sapkota, Deak, et al., 2002).

Ser428 is phosphorylated by PKC- ζ (protein kinase C- ζ), PKA (protein kinase A) and RSK (ribosomal protein S6 kinase). Mutating Ser428 into an Ala resulted in an LKB1 mutant which is retained in the nucleus, indicating that the phosphorylation of this residue regulates the subcellular localization of LKB1 (Song et al., 2008). Indeed, the LKB1 S428A mutant cannot translocate into the cytoplasm following metformin treatment, nor associate with its cytoplasmic substrate AMPK (AMP-activated protein kinase) in HeLa-S3 cells. However, this mutation has no effect on the catalytic activity of LKB1 (Xie, Dong, Scholz, Neumann, & Zou, 2008). Interestingly, the short isoform of LKB1 (LKB1_s) lacks this residue in its C-terminus, but can still activate the LKB1 substrate AMPK, suggesting that the phosphorylation of Ser428 is dispensible for the subcellular distribution of LKB1 and its downstream signaling. Surprisingly, a team of researchers has shown that in this short isoform, PKC- ζ phosphorylates LKB1 in its C-terminus on Ser399, which is also essential for the nucleocytoplasmic export of LKB1_s and subsequent AMPK activation, but has no effect on the catalytic activity of LKB1 (Zhu, Moriasi, Zhang, Zhao, & Zou, 2013).

The phosphorylation of LKB1 on Ser428 is important for the role of LKB1 in axon specification in the developing nervous system in response to BDNF. In *Drosophila*, loss-of-function mutations in the *LKB1* gene cause defects in the oocyte polarity. Although this defect is rescued by low level expression in the germ line of wild-type LKB1, the Ser535 (homologous of Ser428) LKB1 mutant fails to rescue the polarity defects in the oocyte. Thus, the phosphorylation of LKB1 on Ser428 is also important for LKB1 functions *in vivo* (Houde et al., 2014).

Despite the available data on the importance of LKB1 phosphorylation on Ser428 for the translocation of LKB1 into the cytoplasm and subsequent AMPK activation, these data remain controversial. Indeed, a homozygous *Lkb1*^{S431A/S431A} (homologous of Ser428 in human) knockin mouse model was recently generated (Houde et al., 2014). These mice were viable, fertile and displayed no overt phenotypes. As expected, the S428A mutation had no effect on LKB1 kinase activity *in vitro*. However, in contrast to the previous data, AMPK was activated normally in *Lkb1*^{S431A/S431A} tissues and MEFs, raising doubts regarding the importance

of this phosphorylation on LKB1 functions *in vivo*. Despite these intriguing observations, the authors draw our attention to the fact that these mice might actually display a phenotype that they did not notice. They also suggest that the phosphorylation of LKB1 on Ser428 is important for the regulation of oocyte polarity in *Drosophila*, but that mammalian embryonic development is not significantly affected by ablation of this phosphorylation site. Besides, we cannot ignore the possibility of the presence of compensatory mechanisms in mammalian cells *in vivo*. Finally, the residue equivalent to Ser428 is conserved in all species where LKB1 has been reported, even in *C. elegans* where the prenylation motif is not conserved, which strongly suggests that phosphorylation of this residue must indeed have a significant function. Thus, the relevance of this phosphorylation site regarding LKB1 subcellular localization and functions requires further investigation.

LKB1 phosphorylation by PKC- ζ on Ser307 also induces the nucleocytoplasmic export of LKB1 and subsequent AMPK activation, without affecting the catalytic kinase activity of LKB1. This phosphorylation promotes the LKB1-dependant regulation of cell cycle progression, proliferation, angiogenesis and apoptosis (Xie et al., 2009).

The LKB1 Thr336, Thr366, and Ser428 phosphorylation sites and the residues surrounding them are highly conserved in *Drosophila*, *Xenopus*, and mammalian LKB1, but not in *C. elegans* (Gopal P Sapkota, Boudeau, et al., 2002).

1.2.2.2.2 Prenylation

LKB1 is prenylated in cultured cells and invertebrates (Collins et al., 2000; Martin & St Johnston, 2003a; G P Sapkota et al., 2001; J. Watts, Morton, Bestman, & Kempfues, 2000). As previously mentioned, murine Lkb1 has a conserved prenylation motif (Cys433–Lys–Gln–Gln436) at the carboxyl-terminus (**Figure 8**). Cys433 is prenylated *in vivo*, and PKA-mediated LKB1 prenylation targets it to the cellular membrane (Collins et al., 2000). This prenylation occurs by the addition of a farnesyl moiety to Cys433, and mutation of this residue prevents LKB1 prenylation (G P Sapkota et al., 2001). Interestingly, point mutation of the homologous residue to Cys433 in *Drosophila* constitutes an allele with severely reduced rescue activity. These data indicate that the C-terminus of LKB1 is important for its function (Jansen et al., 2009).

More recently, an LKB1^{C433S/C433S} knockin mouse model was generated. These mice were viable, fertile and displayed no overt phenotypes. This study showed that the majority, if not all, of the endogenous LKB1 is prenylated. In these mice, the levels of LKB1 localized at the membrane of the liver cells and fibroblasts were reduced compared with the wild-type mice, confirming that farnesylation plays a role in mediating membrane association. In addition, in all of the examined tissues and cells taken from these mice, LKB1 failed to activate its substrate AMPK. Thus, these data confirm that farnesylation of LKB1 is important for its localization to the cell membrane and present the first evidence that this farnesylation is required for downstream signaling of LKB1 (Houde et al., 2014).

1.2.2.2.3 Ubiquitination

Two groups, including our team, have reported that the molecular chaperone heat shock protein 90 (Hsp90) binds to and stabilizes LKB1. Disrupting the LKB1-Hsp90 complex favors the recruitment of both Hsp/Hsc70 and the U-box dependent E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) that triggers LKB1 degradation by the proteasome. Thus, LKB1 can be ubiquitinated (Nony et al., 2003) (Gaude et al., 2012).

1.2.2.2.4 Acetylation

Several teams have shown that LKB1 can be acetylated (**Figure 9**) and deacetylated (Calamaras et al., 2012; Lan, Cacicedo, Ruderman, & Ido, 2008; M.-J. Lee et al., 2010; Yi Yang et al., 2014; Z. Zheng et al., 2012; Zu et al., 2010). Although the enzymes that acetylate LKB1 remain unknown, a team has shown that Sirtuin 1 (SIRT1) is responsible for deacetylation of LKB1 on lysine 48 (K48) which allows its translocation from the nucleus to the cytoplasm (Lan et al., 2008). This team, as well as ours, has identified lysine residues that undergo acetylation using mass spectrometry (**Figure 9**).

SIRT1 is a conserved NAD⁺ -dependent deacetylase which increases LKB1 phosphorylation on Ser428 and Thr 336 and activation of AMPK in HEK293T cells (Lan et al., 2008). However, in primary porcine aortic endothelial cells, SIRT1 promotes proliferation and prevents senescence through targeting LKB1, thus acting as a negative regulator for LKB1/AMPK signaling (Zu et al., 2010). Finally, in HepG2 cells and mouse liver, over-expression of SIRT1 can stimulate basal AMPK signaling via phosphorylation and activation of LKB1 (Hou et al.,

2008). Although these data are controversial, LKB1 acetylation/deacetylation seems to modulate its kinase activity and subcellular localization.

Our team has identified the acetyltransferase GCN5 (detailed in section 2) as an enzyme which acetylates LKB1. During my PhD, I have focused on studying the role of the acetylation of LKB1 by the acetyltransferase GCN5 on the functions and subcellular localization of LKB1 (**chapter II-project 2**).

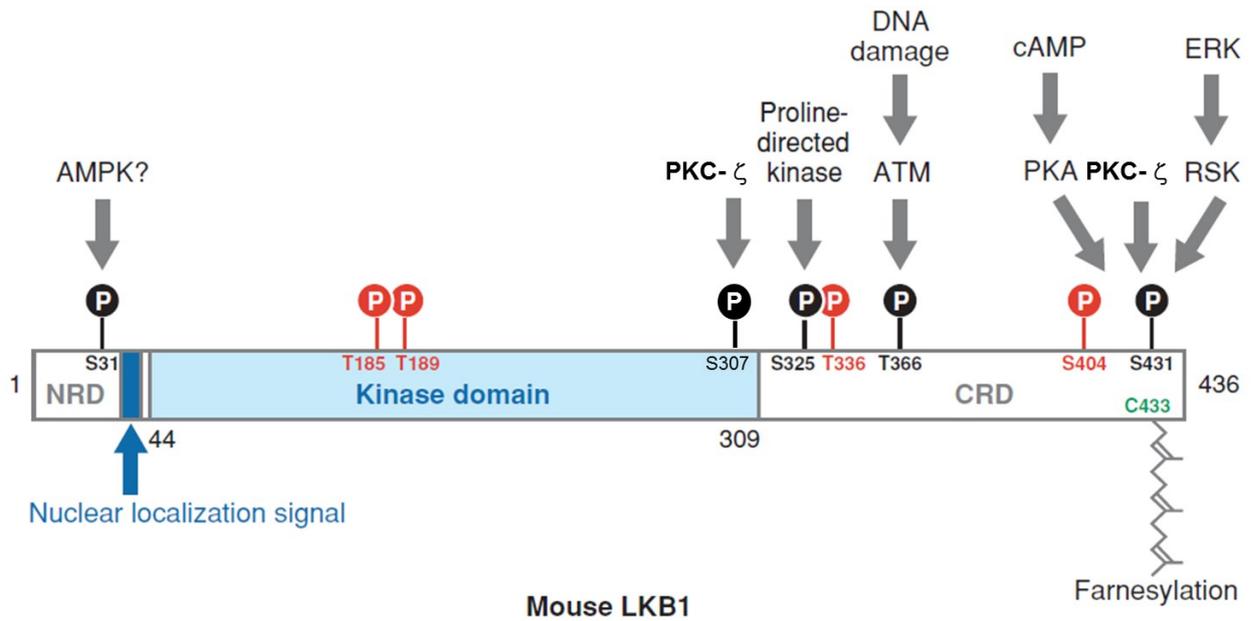


Figure 8: Posttranslational modification sites of the mouse LKB1 protein.

Autophosphorylation sites are depicted in red, and the sites phosphorylated by other kinases are in black. The Cys433 farnesylation site is depicted in green. The agonists and upstream protein kinases postulated to phosphorylate each site are indicated. Residues Thr366, Ser404, Ser431, and Cys433 in the mouse sequence correspond to human LKB1 residues Thr363, Thr402, Ser428, and Cys430, respectively. The noncatalytic domains are in white, and the kinase domain is light blue. Adapted from (Alessi et al., 2006).

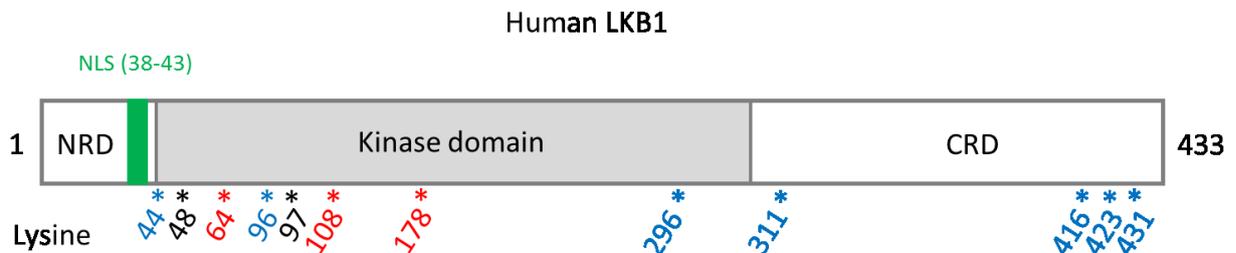


Figure 9: Human LKB1 acetylation sites

Schematic representation of LKB1 acetylated lysine residues identified by mass spectrometry analysis in Lan's team (Lan et al., 2008) as well as our team (unpublished data). Numbers in blue red and black indicate lysine residues identified by Lan's team, our team and those common to both studies respectively.

1.3 LKB1 binding partners

The stability, subcellular localization and kinase activity of LKB1 are modulated by its association with several partners.

1.3.1 The LKB1/STRAD/MO25 complex

Yeast two-hybrid analysis and affinity purification from mammalian cells have shown that LKB1 forms a heterotrimeric complex with two other proteins: the pseudokinase STE20-related adaptor (STRAD) and the scaffolding protein MO25 (CAB39, calcium binding protein 39). The formation of this complex results in the activation and translocation of LKB1 from the nucleus to the cytoplasm (Alessi et al., 2006).

Two isoforms of STRAD (α and β) and MO25 (α and β) were identified and they all interact with LKB1. STRAD α and β possess high sequence similarity to protein kinases and can bind ATP. However, they lack several residues required for catalytic function and are therefore qualified as pseudokinases (Alessi et al., 2006). The kinase domain and the region between a.a. 319 and 343 of LKB1 are essential for its interaction with STRAD (A F Baas et al., 2003).

Homozygous deletion of STRAD α (*LYK5*) is associated with the PMSE syndrome (polyhydramnios, megalencephaly, symptomatic epilepsy) which is a severe human developmental and epileptic disorder. PMSE patients suffer from craniofacial abnormalities, severe mental retardation, gross movement disorders, and childhood mortality (Puffenberger et al., 2007) (**Figure 10**).

MO25 has no identifiable sequence similarity with other proteins. It stabilizes the complex by interacting with the conserved Trp–Glu–Phe sequence (WEF motif) at the C-terminus of STRAD which enhances the binding of STRAD to LKB1 and further stimulates STRAD-induced kinase activity and nucleocytoplasmic transport of LKB1 (Jérôme Boudeau, Baas, et al., 2003).



Figure 10: The PMSE syndrome: dysmorphic features

(A) Young persons with homozygous *LYK5* deletions had a long face, large forehead, peaked eyebrows, broad nose and wide-set eyes. As patients got older (from upper left to lower right), the mouth became enlarged and the lips thickened. (B) Individual craniofacial structure changed considerably with age. Serial pictures of one affected individual show macrocephaly, frontal bossing, hypertelorism and broad nasal bridge shortly after birth (*left panel*), lengthening of the face during early childhood (*middle panel*), and overgrowth of the mandible, enlargement of the mouth and thickening of the lips during adolescence (*right panel*). (Puffenberger et al., 2007).

Recently, the first structure of the STRAD α pseudokinase and its interaction with MO25 α was described. Interestingly, STRAD α could interact with MO25 α through its WEF motif as previously described, but could also form an extensive network of interactions with the highly conserved concave surface of MO25 α . This study has revealed that despite of being catalytically inactive, STRAD α can adopt a closed active-like conformation, with an ordered activation loop similar to active protein kinases, which is stabilized through binding of ATP and/or MO25 α . STRAD α mutants that cannot interact with both ATP and MO25 α are unable to activate LKB1, while STRAD α mutants that retain the ability to bind either ATP or MO25 α still activate LKB1. Thus, it is rather the closed active-like conformation of STRAD α , rather than a catalytic phosphoryltransferase activity, that triggers the activation of the tumor suppressor LKB1 by STRAD α . A model presenting the interaction of STRAD α /MO25 α with LKB1 based on known mutagenesis and structural data is presented in **Figure 11** (Zeqiraj, Filippi, Deak, Alessi, & van Aalten, 2009).

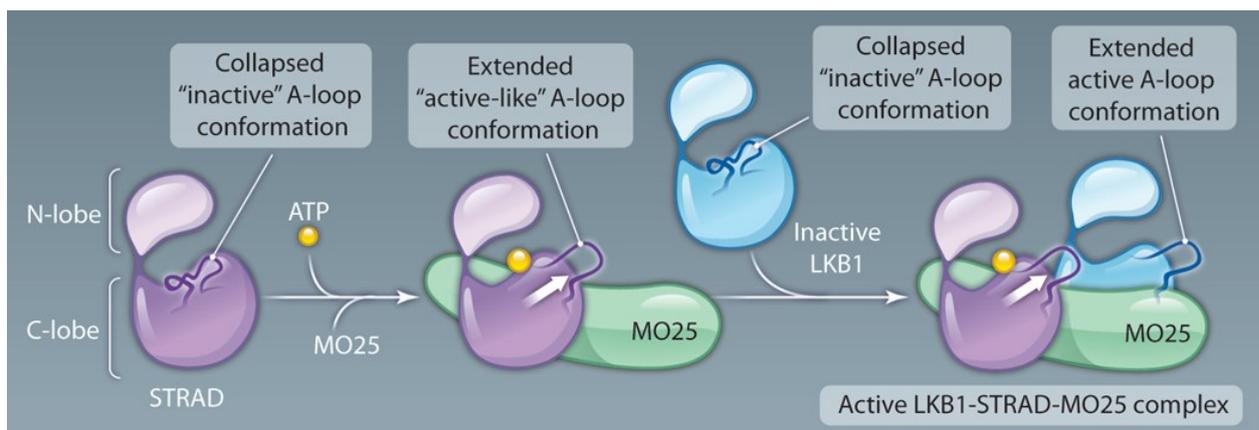


Figure 11: STRAD allosterically activates the kinase domain of LKB1.

The kinase domain of STRAD is presumed to be maintained in an inactive conformation in its isolated state. The binding of ATP and MO25 to STRAD transitions its kinase domain to an active-like kinase conformation that is characterized by the extended conformation of its A-loop. This active-like conformation allows STRAD to bind LKB1 as a pseudosubstrate. STRAD binding allosterically induces the kinase domain of LKB1 to adopt an active kinase conformation, which is further stabilized by the binding of MO25 to the A-loop of LKB1 that positions the loop in an extended conformation (Rajakulendran & Sicheri, 2010).

The PMSE-causing mutation in humans results in the truncation of the pseudokinase STRAD α at residue 251 and consequent loss of the last 180 amino acids (almost half of the C-terminal lobe of the pseudokinase domain). The expression levels of the PMSE-STRAD α (residues 1–251) mutant in 293 cells are significantly lower than full-length STRAD α . Thus, this mutant is unstable and fails to interact with or activate LKB1 *in vitro*. These results indicate that the STRAD α mutation found in PMSE patients represents a loss-of-function mutation that would destabilize STRAD α and prevent it from binding to and activating LKB1 (Zeqiraj et al., 2009).

1.3.2 Regulation of the subcellular localization of LKB1

The cytoplasmic localization of LKB1 has been demonstrated to be essential for its functions. Indeed, mutant forms of LKB1 found in PJS patients localize exclusively in the nucleus and are catalytically inactive and unable to suppress cell growth (J Boudeau et al., 2003). Mutant LKB1 which lacks the nuclear localization signal (NLS) located in its N-terminal non-catalytic region (residues 38–43) (**Figure 9**) is still able to suppress cell growth (Tiainen, Vaahtomeri, Ylikorkala, & Mäkelä, 2002).

LKB1 lacks a nuclear export domain of its own and is retained in the nucleus in the absence of metabolic stress (low glucose, low oxygen). STRAD α and MO25 diffuse passively into the nucleus or are actively imported by importins α and β . Once synthesised, the LKB1 protein is imported to the nucleus by importins α and β . Only STRAD α allows LKB1 to translocate from the nucleus to the cytoplasm by its binding to the nuclear export proteins CRM1 and exportin-7. MO25 stabilizes the heterotrimeric complex. STRAD α also competes with the importins binding site on LKB1 to prevent it from re-entering the nucleus (van Veelen, Korse, van de Laar, & Peppelenbosch, 2011) (**Figure 12**). STRAD β lacks the binding sites to exportin-7 and CRM1 and cannot transport LKB1 to the cytoplasm suggesting unique functions of the LKB1/STRAD β complex in the nucleus (Dorfman & Macara, 2008). Co-expression of LKB1 and STRAD α targets the majority of LKB1 to the cytoplasm, but a significant amount remains nuclear. However, expression of LKB1, STRAD α and MO25 fully localizes LKB1 to the cytoplasm (Alessi et al., 2006). Thus, the formation of LKB1/STRAD α /MO25 complex is essential for LKB1 localization.

The subcellular localization of LKB1 is also modulated by its phosphorylation on several residues. As previously mentioned, LKB1 phosphorylation on Ser428, Ser307 by PKC- ζ

seems to be important for LKB1 nucleocytoplasmic export and consequent AMPK activation. The LKB1 S307A mutant (Ser307 to Ala) exhibits a decreased association with STRAD α and fails to interact with CRM1 (Xie et al., 2009).

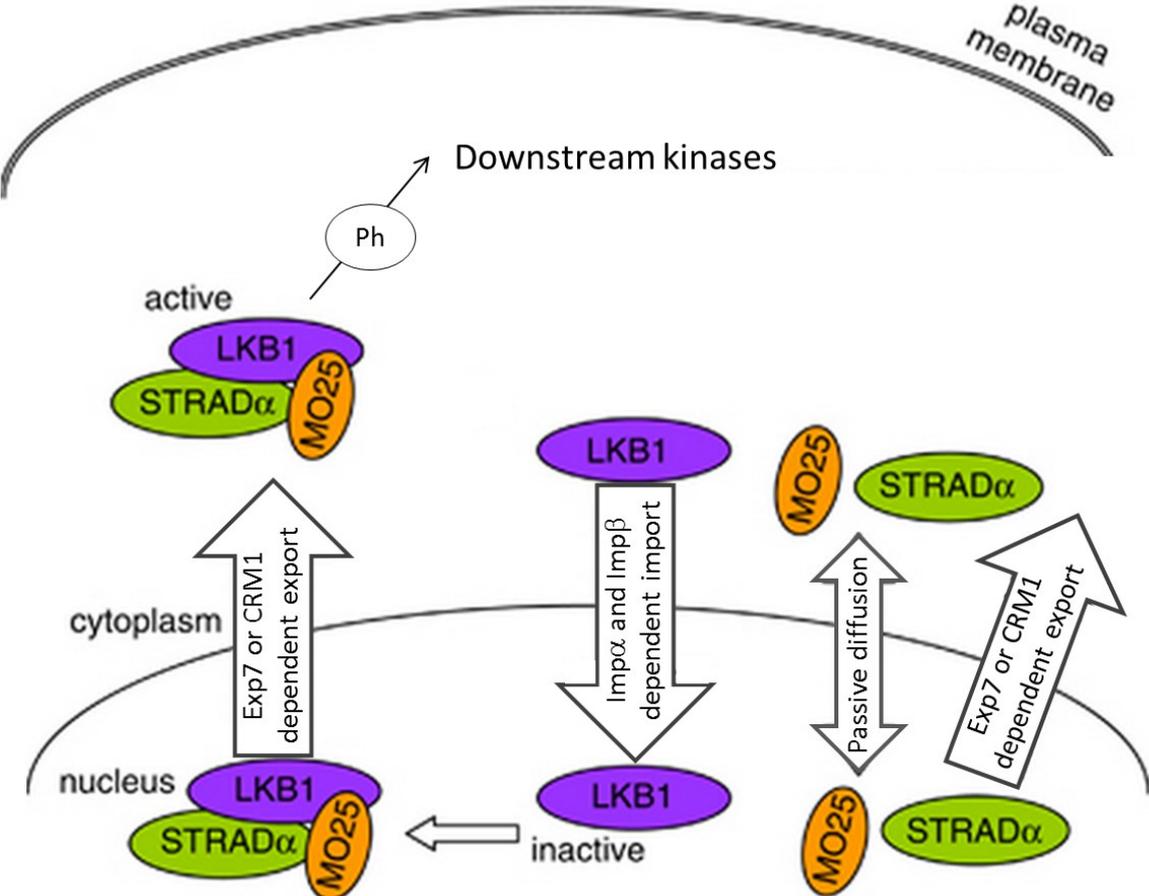


Figure 12: Activation and translocation of LKB1

LKB1 is activated by its translocation from the nucleus to the cytoplasm. Normally, LKB1 remains in the nucleus in an inactive state. Upon activation, LKB1 is bound by STE20-related adaptor protein α (STRAD α) and mouse protein 25 (MO25), proteins that enter the nucleus either by passive diffusion or active import by importins α/β (Imp α , Imp β). The stable LKB1/STRAD α /MO25 complex is actively exported out of the nucleus by exportin-7 and CRM1. In the cytoplasm, LKB1 exerts its serine/threonine kinase activity by phosphorylating and activating downstream kinases. Adapted from (van Veelen et al., 2011).

A screen for novel LKB1-interacting proteins using the yeast two-hybrid system proteins led to the identification of the cytoplasmic scaffolding protein LIP1 (LKB1-interacting protein 1). LIP1 is a leucine-rich repeat containing protein and its overexpression with LKB1 dramatically increases the proportion of LKB1 in the cytoplasm (D. P. Smith et al., 2001). It was recently shown that LKB1 interaction with LIP1 results in the negative control of the TGF β signaling (Morén, Raja, Heldin, & Moustakas, 2011).

Finally, a recent study has shown that LKB1 can bind to and be sequestered by the orphan nuclear receptor Nur77 in the nucleus. Consequently, the activation of LKB1 cytoplasmic downstream targets is attenuated. The chemical compound ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl]acetate (TMPA), interacts with Nur77 with high affinity and results in the release and shuttling of LKB1 to the cytoplasm, thereby antagonizing the Nur77 function (Zhan et al., 2012).

1.3.3 Regulation of LKB1 stability

LKB1 stability is finely tuned by two chaperone complexes with antagonizing activities, HSP90–Cdc37 and HSP/HSC70–CHIP.

The HSP90–Cdc37 complex interacts with the long and short isoforms of LKB1 (LKB1-L and LKB1-S), but not with the novel variant lacking its N-terminal domain (LKB1- Δ N) (**Figure 13**). In addition to the fact that LKB1 possesses no catalytic activity in this complex, the latter does not include STRAD and MO25. Disruption of the LKB1–HSP90 complex results in the recruitment of both HSP/HSC70 and the U-box dependent E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) that triggers LKB1 degradation. Thus, HSP90 and Cdc37 stabilize LKB1 and protect it from proteasomal degradation while the interaction of LKB1 with HSP70 and CHIP has the opposite effect. However, the impact of LKB1 binding to STRAD/MO25 on the ability of LKB1 to interact with HSP/HSC70–CHIP remains unknown (Gaude et al., 2012; Wanping Xu & Neckers, 2012).

1.3.4 Regulation of LKB1 activity

LKB1 activation is mainly dependent on its interaction with STRAD α . The latter binds directly to the kinase domain of LKB1 and induces a conformational change of LKB1, enhancing its catalytic activity over 100-fold and leading to LKB1 autophosphorylation. MO25 stabilizes the complex and assists STRAD in maintaining LKB1 in an active conformation (A F Baas et al., 2003; Zeqiraj et al., 2009).

As previously mentioned, LKB1 is stabilized by forming a complex with HSP90 and Cdc37. However, in this complex, LKB1 is catalytically inactive and incapable of autophosphorylation. A treatment with geldanamycin (HSP90-specific inhibitor) leads HSP90 to dissociate from LKB1 thereby triggering a transitory activation of LKB1 (Gaude et al., 2012).

Thus, while STRAD and MO25 enhance the kinase activity of LKB1, HSP90 and Cdc37 block this activity. A study has revealed that HSP90 and Cdc37 undergo dynamic tyrosine phosphorylation events that lead to the orderly association/dissociation of HSP90, Cdc37 and client kinases. Thus, the involvement of HSP90 and Cdc37 tyrosine phosphorylation in LKB1 activation is worthy of further investigation (Gaude et al., 2012; Wanping Xu & Neckers, 2012).

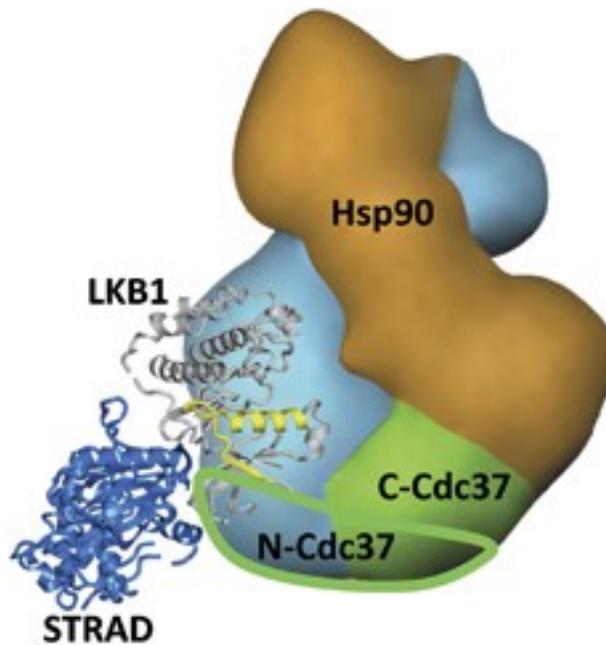


Figure 13: Molecular modeling of the Hsp90–Cdc37–LKB1 complex.

The Hsp90–Cdc37 complex is shown as cyan, orange and green surface representing respectively each subunits of the Hsp90 dimer and Cdc37. LKB1 (gray shading) is represented bound to the Hsp90 cyan subunit and to STRAD (dark blue shading). The LKB1 N-lobe points downward with its ‘ α C- β 4’ strand-loop-helix structure colored in yellow. The N-terminal Cdc37 domain involved in kinase binding lies within the area delimited by a green line where it could bind to the LKB1 α C- β 4 strand and competes with STRAD binding. In this model, the Hsp90–Cdc37 structure was adapted from the Hsp90–Cdc37–Cdk4 structure determined by electron microscopy (Vaughan *et al.*, 2008). The LKB1–STRAD complex was extracted from the LKB1–STRAD–MO25 crystal structure (Zeqirajet *al.*, 2009, and PDB ID code 2WTK) and has been placed onto Hsp90–Cdc37 by manual superimposition of its LKB1 kinase domain onto Cdk4 (Gaude *et al.*, 2012).

1.4 LKB1 regulates downstream kinases

LKB1 is a master kinase of 14 AMPK-related protein kinases (ARKs) (**Figure 14**) and activates them by phosphorylating their T-loop threonine residue (Lizcano et al., 2004). The best characterized LKB1 substrate is the intracellular energy sensor AMPK (AMP-activated protein kinase). Indeed, the serine/threonine kinase AMPK is activated by any metabolic stress leading to ATP (adenosine triphosphate) depletion and consequent increase of AMP (adenosine monophosphate): ATP and ADP (adenosine diphosphate): ATP ratios. In order to restore normal ATP levels, AMPK promotes catabolic processes such as fatty acid oxidation, and switches off anabolism and other ATP-consuming processes such as lipid and protein synthesis.

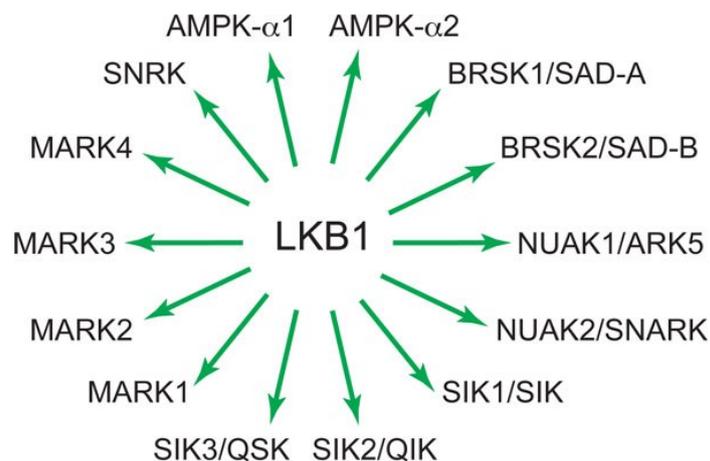


Figure 14: Members of the AMPK and AMPK-related kinase (ARK) family.

All the kinases named in the figure are phosphorylated and activated by LKB1, although what regulates this phosphorylation is known only for AMPK. Alternative names are shown, where applicable. AMP-activated kinase (AMPK), NUAK family SNF1-like kinase 1 (NUAK1), sucrose non-fermenting protein-related kinase (SNRK), brain selective kinase (BRSK), synapses of amphids-deficient kinase (SAD), Salt-inducible kinase (SIK1), microtubule affinity regulating kinase (MARK). (Hardie & Alessi, 2013).

Unlike AMPK, the other ARKs are not regulated by energy stress and ADP:ATP ratios. Although the role and regulation of these other ARKs downstream of LKB1 require further investigation, an optimal substrate motif for LKB1 phosphorylation including the T-loop phosphorylated threonine residue was proposed (**Figure 15**), and their implication in cell polarity regulation (MARK, BRSK/SAD), proliferation (NUAKs) and CREB-regulated gene transcription (SIKs) has been reported (Gan & Li, 2014; Hardie & Alessi, 2013). Some of these functions will be detailed further in this manuscript and are presented in **Table 1**.

Peptide	
NUAK2	LSNLYHQGKFLQ <u>T</u> FCGSPLYRRR
SIK	FGNFYKSGEPLS <u>T</u> WCGSPPYRRR
AMPK	LSNMMSDGEFLR <u>T</u> SCGSPNYRRR
BRSK2	MASLQVGDLSLE <u>T</u> SCGSPHYRRR
MARK3	FSNEFTVGGKLD <u>T</u> FCGSPPYRRR

Figure 15: Optimal substrate motif for LKB1 phosphorylation in ARKs

Kinetic analysis of the phosphorylation of the indicated T-loop by the LKB1:STRAD:MO25 complex was performed. The T-loop Thr residue in each peptide is underlined and is in boldface type. Three Arg residues were added to the C-terminus of each T-loop peptide to enable their capture on phosphocellulose p81 paper (Lizcano et al., 2004).

ARKs	Name	LKB1 target residue in human proteins	Role downstream of LKB1	References
AMPK	AMP-activated protein kinase	Thr 172	-Epithelial cell polarity -Lipid homeostasis -Cell growth/survival -Cell migration	See text for details and references
BRSK1/SAD-A	Brain-specific kinase	Thr 189	Neuronal polarization	(Barnes et al., 2007; Kishi, Pan, Crump, & Sanes, 2005)
BRSK2/SAD-B		Thr 174		
MARK1/PAR-1c	MAP/microtubule affinity-regulating kinase	Thr 215	-Epithelial cell polarity	see (Drewes et al., 1998; Biernat et al., 2002; Suzuki et al., 2004; Chen et al., 2006) in (Shelly & Poo, 2011), (Martin-Belmonte & Perez-Moreno, 2012), (Chan et al., 2014; Goodwin et al., 2014; Kojima et al., 2007)
MARK2/PAR-1b		Thr 208	-Microtubule dynamics	
MARK3/PAR-1a		Thr 211	-Cancer cell migration (inhibition)	
MARK4		?		
NUAK1/ARK5	AMPK-related kinase 5	Thr 211	-Axon branching -Cell adhesion	(Courchet et al., 2013; Hou et al., 2011; Humbert et al., 2010; Zagórska et al., 2010) In (Vaahnotmeri & Mäkelä, 2011)
NUAK2/SNARK	SNF/AMPK-related kinase	Thr 208	-Proliferation -Actin cytoskeleton	
SIK1/SIK	Salt-induced kinase	Thr 182	-p53-dependent anoikis (SIK1)	(Cheng et al., 2009; Eneling et al., 2012; Patel et al., 2014; Selvik et al., 2014; Tang et al., 2013)
SIK2/QIK		Thr 175	-Maintenance of epithelial junctions stability (SIK1)	
SIK3/QSK		Thr 163	-Cancer cell migration (inhibition) (SIK1)	
SNRK	Snf-related serine/threonine kinase	Thr 173	-HTLV1 transcription -Gluconeogenesis ?	

Table 1: Reported role of the ARKs downstream of LKB1

The phosphorylation sites were based UniProt informations (www.uniprot.org). **Anoikis**: a form of programmed cell death that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (ECM). **HTLV-1**: Human T-cell Leukemia Virus Type 1.

1.4.1 The energy sensor AMPK: structure and regulation by upstream kinases

AMPK is a heterotrimeric protein complex containing a catalytic subunit α 1/2 and two regulatory subunits, β 1/2 and γ 1/2/3. It is well established that the α subunit can directly interact with both β and γ subunits, and that the carboxy-terminal domain of the β subunit bridges the carboxy-terminal domain of the α subunit and the γ subunit. The γ subunit contains four tandem sequence repeats known as CBS repeats with four potential adenine nucleotide-binding clefts (Hardie & Alessi, 2013; Benoit Viollet et al., 2010; Wong & Lodish, 2006) (**Figure 16**). While site 2 always appears to be unoccupied, site 4 appears to have permanently bound AMP. However, sites 1 and 3 bind AMP, ADP or ATP in competition; in an unstressed cell (high ATP:ADP/AMP ratios) they are probably largely occupied by ATP, but under metabolic stress (low ATP) they will progressively be occupied by AMP and/or ADP (Hardie & Alessi, 2013). AMPK is phosphorylated by LKB1 on Thr172 within the activation loop of its kinase domain (Shaw et al., 2004). Binding of ADP and/or AMP to the γ subunit promotes AMPK phosphorylation and inhibits AMPK dephosphorylation of Thr172 causing an allosteric activation. This activation is more pronounced when AMP levels are extremely high in response to severe stress (Gan & Li, 2014; Hardie & Alessi, 2013).

Interestingly, *Lkb1* deficiency in murine embryonic fibroblasts (MEFs) results in nearly complete loss of Thr172 phosphorylation and downstream AMPK signaling in response to different AMPK activators. Although genetic loss of *LKB1* in mammalian cells greatly reduces AMPK phosphorylation on Thr172, a residual phosphorylation remains, suggesting that other kinases must also phosphorylate this site (Shaw et al., 2004). Indeed, AMPK is also activated by CaMKK β (Ca²⁺/calmodulin-dependant protein kinase kinase β) and TAK1 (Transforming growth factor beta-activated kinase 1).

An alternative Ca²⁺ -stimulated pathway involves CaMKK β in AMPK activation. This pathway was observed in neurones exposed to membrane depolarization (Hawley et al., 2005), in T cells activated via the antigen receptor (Tamás et al., 2006), and in cells treated with ligands for G protein-coupled receptors that trigger intracellular release of inositol trisphosphate and hence Ca²⁺ (Andersson et al., 2004; Stahmann, Woods, Carling, & Heller, 2006; Yunlei Yang, Atasoy, Su, & Sternson, 2011). In mouse muscles, after 2 minutes of contraction, AMPK phosphorylation was strongly inhibited by CaMKK inhibitors (Corton, Gillespie, & Hardie,

1994). Interestingly, none of the ARKs are phosphorylated by CaMKK β , despite the sequence similarities within the activation loops of AMPK and the ARKs (Sarah Fogarty et al., 2010).

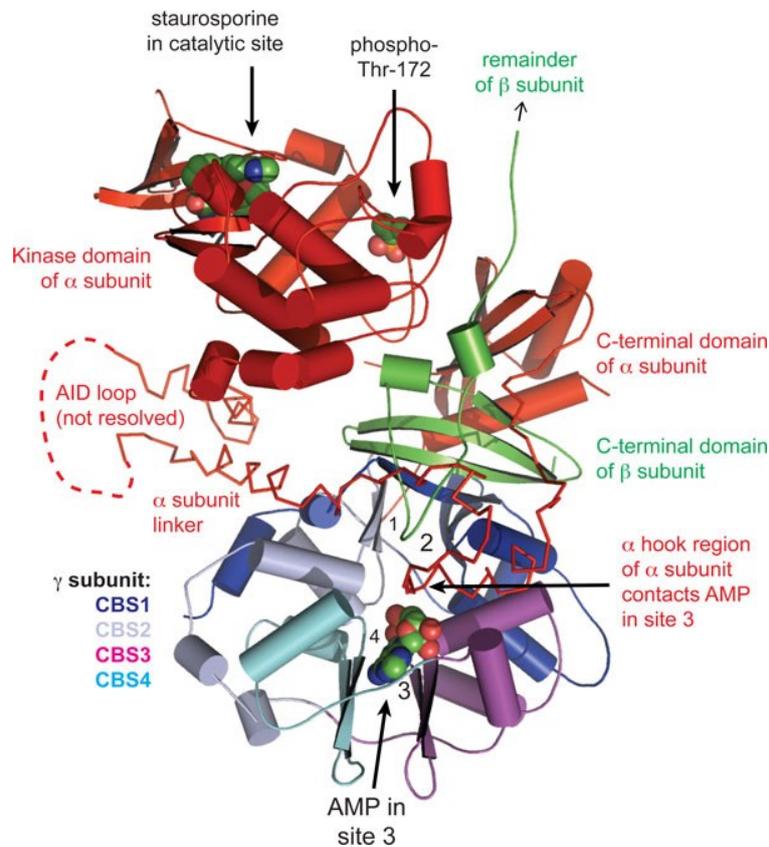


Figure 16: Graphic structure of AMPK.

The α subunit is in red, the β subunit in green, and the four CBS motifs of the γ subunit in various shades of blue, magenta and cyan. For this structure, an AMPK complex phosphorylated on Thr172, lacking most of the β subunit and also a flexible loop from the α subunit, was crystallized in the presence of AMP and the kinase inhibitor staurosporine. For clarity, only the AMP in site 3 is shown, but the approximate location of binding clefts 1, 2 and 4 are also shown; in this view, sites 1 and 4 are at the back of the γ subunit and sites 2 and 3 at the front. The carboxy-terminal domain of the β subunit forms the core of the complex, bridging the carboxy-terminal domain of the α subunit and the γ subunit. Note the extended α subunit linker peptide between the kinase domain and carboxy-terminal domain, with its 'alpha hook' region contacting AMP in site 3. AID: α subunit autoinhibitory domain (this domain is believed to hold the catalytic domain in an inactive conformation in the absence of the regulatory domains). Drawn using MacPyMOL with Protein Database Entry 2Y94. (Hardie & Alessi, 2013).

In yeast, the AMPK homologue Snf1 was shown to be activated by TAK1, a cytokine-activated member of the mitogen-activated protein kinase kinase kinase family (MAPKKK) *in vivo*. TAK1 also activates mammalian AMPK *in vitro* (Momcilovic, Hong, & Carlson, 2006). The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) preferentially triggers apoptosis in cancer cells, sparing normal cells, and TRAIL receptor agonists are potential anti-cancer drugs. In this context, an interesting study has shown that depletion of TAK1 using RNAi abrogated TRAIL-induced AMPK activation. The TRAIL-induced TAK1–AMPK signaling pathway promotes cytoprotective autophagy to protect normal epithelial cells against TRAIL-induced cell death. In MCF10A cells, LKB1 and CaMKK β depletion influenced neither TRAIL-induced AMPK activation nor autophagy (Herrero-Martín et al., 2009).

Finally, phosphorylation of AMPK at Ser485 of the α 1 subunit by Akt, protein kinase A (PKA) as well as its autophosphorylation in various cell types and tissues, such as the heart, adipocytes and vascular smooth muscle cells, reduces AMPK activity (Coughlan, Valentine, Ruderman, & Saha, 2014).

The different regulators of AMPK are presented in **Figure 17**.

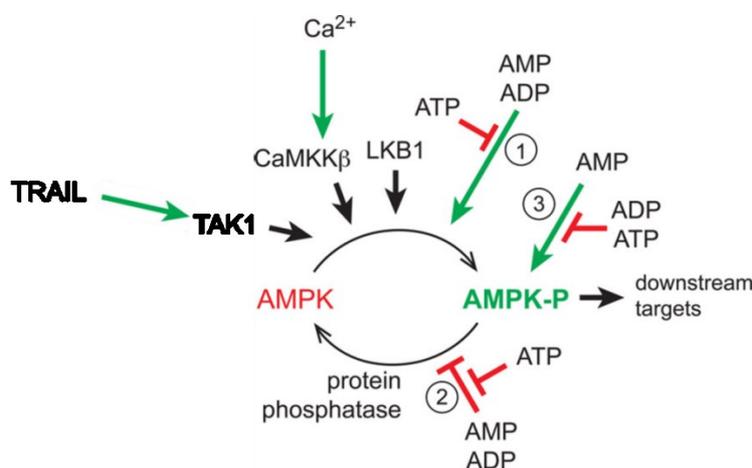


Figure 17: Regulation of AMPK

AMPK can be activated by increases in cellular AMP:ATP or ADP:ATP ratio, or Ca²⁺ concentration. AMPK is activated on conversion from a dephosphorylated form (AMPK) to a form phosphorylated at Thr172 (AMPK-P) catalyzed by at three upstream kinases: LKB1, which appears to be constitutively active, CaMKK β , which is only active when intracellular Ca²⁺ increases, and TAK1, which is activated by TRAIL. Increases in AMP or ADP activate AMPK by three mechanisms: (1) binding of AMP or ADP to AMPK, causing a conformational change that promotes phosphorylation by upstream kinases (usually this will be LKB1, unless [Ca²⁺] is elevated); (2) binding of AMP or ADP, causing a conformational change that inhibits dephosphorylation by protein phosphatases; (3) binding of AMP (and not ADP), causing allosteric activation of AMPK-P. All three effects are antagonized by ATP, allowing AMPK to act as an energy sensor. Adapted from (Hardie & Alessi, 2013; Herrero-Martín et al., 2009).

1.4.2 AMPK regulating drugs

In addition to being activated by upstream kinases and low ATP levels, AMPK is also regulated (activated or inactivated) by hormones (Leptin, adiponectin, interleukin-6), natural compounds (resveratrol, rooibos, berberine, α -lipoic acid), or pharmacological drugs (Coughlan et al., 2014).

AMPK-activating drugs can act directly or indirectly. Direct activating drugs bind to AMPK leading to its allosteric activation, promoting phosphorylation of Thr172 and/or inhibiting dephosphorylation of Thr172. Indirect activation occurs when the molecule does not interact with AMPK but rather increases the cellular AMP:ATP and ADP:ATP ratios, often through inhibiting mitochondrial ATP production. In this case, AMPK is activated by AMP and ADP. Some other drugs activate AMPK through unique pathways that are not as well characterized (Mark Goodman, Zhenling Liu, 2014).

Many of the AMPK-activating drugs have shown promising effects for the treatment of a wide variety of diseases including diabetes, cancer, and cardiovascular pathologies. A selection of these activators will be described in this section and are presented in **Figure 18**.

1.4.2.1 AICAR (5-aminoimidazole-4-carboxamide riboside)

AICAR (5-aminoimidazole-4-carboxamide riboside) was the first molecule reported to activate AMPK in intact cells and *in vivo* (Corton, Gillespie, Hawley, & Hardie, 1995; Sullivan et al., 1994). Upon entering the cell, AICAR is taken up by adenosine transporters and is then converted by adenosine kinase to the monophosphorylated derivative ZMP. The latter mimics the effect of AMP by binding to the γ subunit and inducing the allosteric activation of the kinase while inhibiting its dephosphorylation (Corton et al., 1995; Gadalla et al., 2004).

In mouse and rat models with metabolic defects, AICAR administration improved glucose tolerance, reduced plasma triglyceride and free fatty acid levels, increased whole-body glucose disposal and decreased hepatic glucose output (S Fogarty & Hardie, 2010). In normal mice, AICAR administration also had beneficial effect resulting in the induction of genes linked to oxidative metabolism and in enhanced running endurance (Narkar et al., 2008). However, AICAR can mimic other actions of AMP and exhibits AMPK-independent effects due to interaction of ZMP with other AMP-regulated enzymes. Among these effects, AICAR

inhibits the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) and stimulates the muscle isoform of glycogen phosphorylase. These specificity issues, combined with poor bioavailability and short half-life, make AICAR and related adenosine analogues rather unpromising compounds for drug development (Coughlan et al., 2014; S Fogarty & Hardie, 2010).

1.4.2.2 *The A-769662 compound*

The A-769662 compound is a thienopyridone that was identified by the Abbott Laboratories as the first direct activator (unlike AICAR) of AMPK. The molecule binds AMPK in a cleft between the kinase domain of the α -subunit and the carbohydrate-binding domain of the β -subunit, thereby causing its allosteric activation and preventing its dephosphorylation at Thr172 (Coughlan et al., 2014; Xiao et al., 2013). Treatment of *ob/ob* mice with this compound was found to improve glucose homeostasis and lipid levels (Cool et al., 2006). Unfortunately, due to its poor oral absorption and its reported AMPK-independent effects (inhibition of the 26S proteasome activity and cell cycle progression arrest) (Moreno, Knecht, Viollet, & Sanz, 2008), A-769662 is unlikely to be used to treat human metabolic syndrome, but is however used as a research tool to further study the effects of AMPK activation.

1.4.2.3 *Biguanides*

Biguanides include metformin, phenformin and buformin. Metformin is an anti-hyperglycemic agent which is currently the first-line oral therapy for T2D (Type 2 Diabetes). Although phenformin and buformin are more potent anti-diabetic drugs than metformin, they were withdrawn from the market due to severe side effects, namely lactic acidosis (Rena, Pearson, & Sakamoto, 2013).

Metformin inhibits hepatic gluconeogenesis, thereby reducing blood glucose levels in human subjects (Hundal et al., 2000). Treatment of intact cells with metformin increases AMPK activity, which consequently increases fatty acid oxidation, down-regulates lipogenic genes, decreases hepatic glucose production and stimulates glucose uptake (Zhou et al., 2001). Interestingly, in cell-free assays, metformin does not directly activate AMPK. In this context, both metformin and phenformin have been reported to indirectly activate AMPK through the inhibition of the mitochondrial respiratory chain complex I. As a result, a switch

from aerobic to anaerobic glycolysis occurs, thus increasing the AMP/ATP ratio and promoting AMPK activation (M. R. Owen, Doran, & Halestrap, 2000). Later, it was shown that deletion of *Lkb1* in the liver of adult mice results in a loss of metformin –induced AMPK activity, along with hyperglycemia and increased lipogenic and gluconeogenic gene expression such as the PGC-1 α target genes PEPCK and G6Pase. These data have shown that LKB1 is required for metformin-induced AMPK activation and regulation of blood glucose levels (Shaw et al., 2005).

However, the AMPK-dependent effects of metformin on hepatic glucose production were subject to controversy. Indeed, a recent study has shown that metformin inhibits hepatic gluconeogenesis through a decrease in hepatic energy state, meaning that it's the perturbation of intracellular ATP levels, but not AMPK activation *per se* that mediates the effects of metformin on hepatic glucose production. This mechanism was indeed shown to be independent from LKB1 and AMPK since metformin treatment lowered blood glucose levels in liver-specific *AMPK α 1/ α 2* or *LKB1* knockout mice (Foretz et al. 2010). Another study has suggested another mechanism whereby metformin lowers hepatic glucose output. Indeed, another putative mitochondrial target of metformin, mGPD (mitochondrial glycerophosphate dehydrogenase), was identified. mGPD is inhibited by metformin, which results in the retention of the glycerophosphate shuttling. Consequently, glycerol gluconeogenesis is blocked, and the incorporation of lactate into glucose is impaired (Madiraju et al., 2014).

1.4.2.4 Thiazolidinediones

Thiazolidinediones are also currently used to treat type 2-diabetes. They indirectly activate AMPK by inhibiting complex I of the respiratory chain, thus increasing the cellular AMP: ATP ratio. However, the major effect of thiazolidinediones is believed to be the stimulation of adiponectin through activation of the transcription factor PPAR- γ in adipocytes. Reversing insulin resistance and improving glucose tolerance in high-fat fed rats, following treatment with thiazolidinediones, are greatly reduced in adiponectin knockout mice. Adiponectin now appears to act on its target cells primarily by activating AMPK. The thiazolidinediones may therefore exert their therapeutic benefits in part *via* two independent effects to activate AMPK (Hardie, 2008).

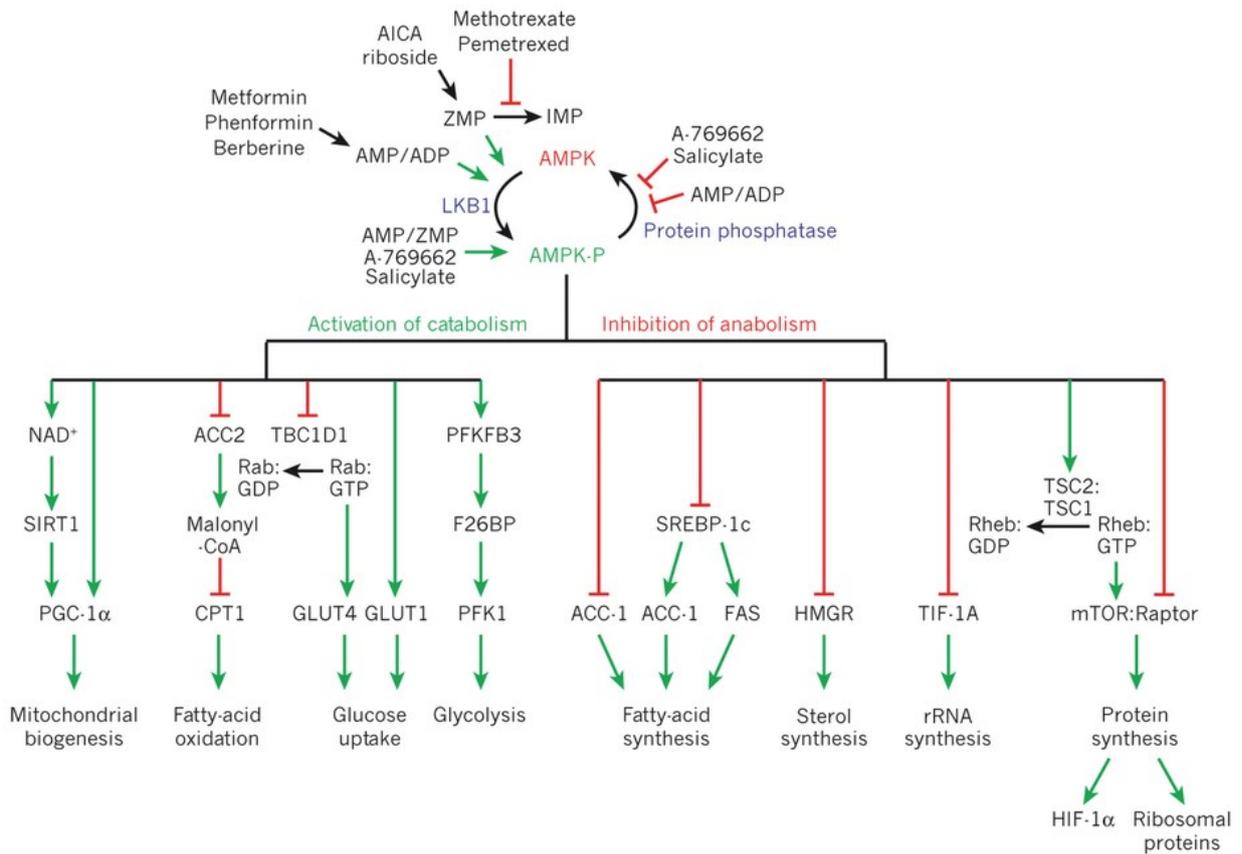


Figure 18: Regulation of AMPK by drugs and the principal metabolic pathways it regulates.

AMPK is activated by phosphorylation by LKB1, and inactivated by dephosphorylation by protein phosphatases. Binding of AMP or ADP (which increase in response to drugs inhibiting mitochondrial function; for example, metformin, phenformin and berberine) or ZMP (which increases in response to drugs modulating purine biosynthesis; for example, AICA riboside, methotrexate and pemetrexed) activates AMPK by promoting phosphorylation, by inhibiting dephosphorylation and by direct allosteric activation. A-769662 and the anti-inflammatory agent salicylate bind to AMPK at a site distinct from the nucleotides, causing allosteric activation and inhibition of dephosphorylation. Once activated, AMPK activates catabolic pathways (left) and inhibits anabolic pathways (right). One mechanism to explain how AMPK activation has an anti-inflammatory effects would be its promotion of mitochondrial biogenesis and fatty-acid oxidation (left), while down-regulating glycolysis by inhibiting mTORC1 (shown here as mTOR:Raptor) and hence expression of HIF-1 α (right). ACC-1, acetyl-coenzyme A carboxylase-1; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; FAS, fatty acid synthase; F26BP, fructose 2,6-bisphosphate; PFK1, phosphofructokinase-1; rRNA, ribosomal RNA; SREBP-1, sterol regulatory element binding protein-1, TIF-1A, transcription intermediary factor-1A; TSC, tuberous sclerosis complex protein. (O'Neill & Hardie, 2013).

1.4.3 AMPK functions downstream of LKB1

AMPK is a key regulator of the cell metabolism, but is also implicated in the regulation of transcription, cell growth, autophagy and polarity (Mihaylova & Shaw, 2011; Mirouse & Billaud, 2011). This regulation occurs through direct phosphorylation of downstream AMPK target but also as a long-term effect mediated by phosphorylation of transcription factors and coactivators. In this bibliographic section, we will focus on some downstream effectors of AMPK and LKB1/AMPK-regulated processes.

1.4.3.1 LKB1/AMPK maintains lipid homeostasis

The functions of AMPK were initially associated to the regulation of lipid metabolism through the inactivation of ACC (acetyl-CoA carboxylase) (Carlson & Kim, 1973) and HMGR (3-hydroxy-3-methylglutaryl (HMG)-CoA reductase) (Beg, Allmann, & Gibson, 1973), the key regulatory enzymes of fatty acid and cholesterol synthesis respectively (**Figure 18**).

HMGR is anchored at the membrane of the endoplasmic reticulum and regulates the mevalonate pathway. The latter is a metabolic pathway that produces cholesterol and other isoprenoids. Following a decrease in cellular ATP levels, AMPK inhibits HMGR through its direct phosphorylation, but can also reduce its expression through the phosphorylation and degradation of FoxO1a (forkhead transcription factor 1a) (Fisslthaler & Fleming, 2009).

Activated AMPK also inhibits fatty acid synthesis in liver and adipose cells while stimulating fatty acid oxidation in heart and muscle cells (**Figure 19**). AMPK inhibits fatty acid synthesis by phosphorylating and inactivating ACC1 (acetyl-CoA carboxylase 1) but can also switch off the expression of lipogenic genes including ACC1 and fatty acid synthase (Corton et al., 1995; A Woods et al., 2000). It stimulates fatty acid oxidation by phosphorylating and inactivating ACC2 (acetyl-CoA carboxylase 2) which decreases malonyl-CoA levels. Consequently fatty acid uptake into mitochondria is increased (Merrill, Kurth, Hardie, & Winder, 1997).

1.4.3.2 LKB1/AMPK regulates glucose homeostasis

AMPK up-regulates glucose uptake in heart and skeletal muscle cells (**Figure 19**). This up-regulation occurs through increased transcription and translocation of GLUT4 (glucose transporter-4) to the plasma membrane (Kurth-Kraczek, Hirshman, Goodyear, & Winder,

1999; D. Zheng et al., 2001) and by activation of GLUT1 at the plasma membrane (K. Barnes et al., 2002) (**Figure 18**). The translocation of GLUT-4 is indirectly induced by AMPK. Indeed, the latter phosphorylates the Rab GTPase-activating protein AS160 (Akt substrate 160) which modulates docking and fusion of GLUT4 vesicles with the plasma membrane (Chavez, Roach, Keller, Lane, & Lienhard, 2008). In various transgenic mouse models where AMPK activation is reduced, AMPK seems to be implicated in the effects of contraction on glucose uptake, although other parallel pathways also appear to be involved (Hardie, 2008).

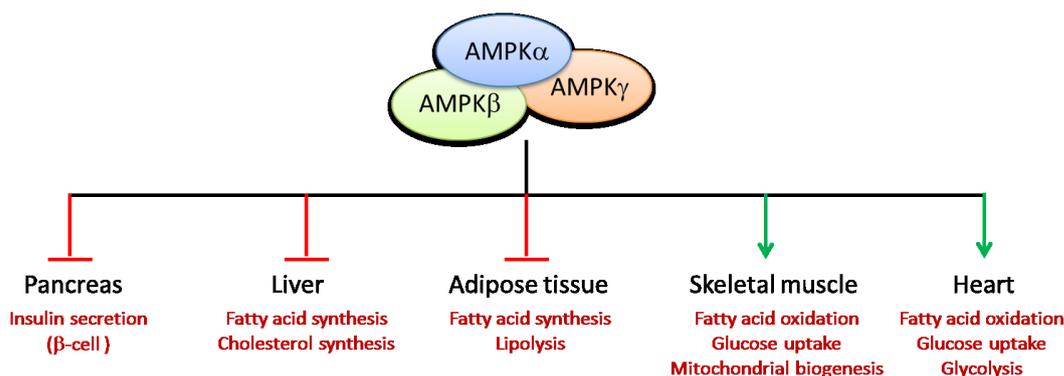


Figure 19: Role of AMPK in the regulation of metabolism in response to events such as nutrient- or exercise-induced stress.

AMPK-activated mechanisms in different tissues are shown. Red lines show inhibition and green arrows show activation. Adapted from

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and

http://www.google.de/imgres?imgurl=http%3A%2F%2Fwww.mtipharm.com%2Fscience%2Fscience.jpg&imgrefurl=http%3A%2F%2Fwww.mtipharm.com%2Fscience%2Ftechnology.htm&h=594&w=776&tbid=DEErISXQJ4wkPM%3A&zoom=1&dclid=g5DOPyQ_g69LpM&ei=wHSIVNLSLab7ygPVqYGgCw&tbm=isch&iact=rc&uact=3&dur=800&page=1&start=0&ndsp=18&ved=0CCYQrQMwAg

In the liver, AMPK is also implicated in the regulation of hepatic gluconeogenesis. CRTC2 is a transcriptional coactivator of CREB (cAMP response element-binding protein) and a critical regulator of gluconeogenesis in mice. It mediates the transcription of PGC1 α and its gluconeogenic targets glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). The phosphorylation of CRTC2 by AMPK leads to its cytoplasmic translocation in primary hepatocytes cultures. In *LKB1*-deficient livers, CRTC2 was dephosphorylated and entered the nucleus, leading to transcriptional activation of PGC-1 α which drives gluconeogenesis. Thus, CRTC2 is a target of LKB1/AMPK in the regulation/inhibition of gluconeogenesis (Shaw et al., 2005) (**Figure 20**). Interestingly, the LKB1/AMPK regulation of PGC-1 α expression, increases mitochondrial biogenesis in muscle cells (Sriwijitkamol et al., 2006).

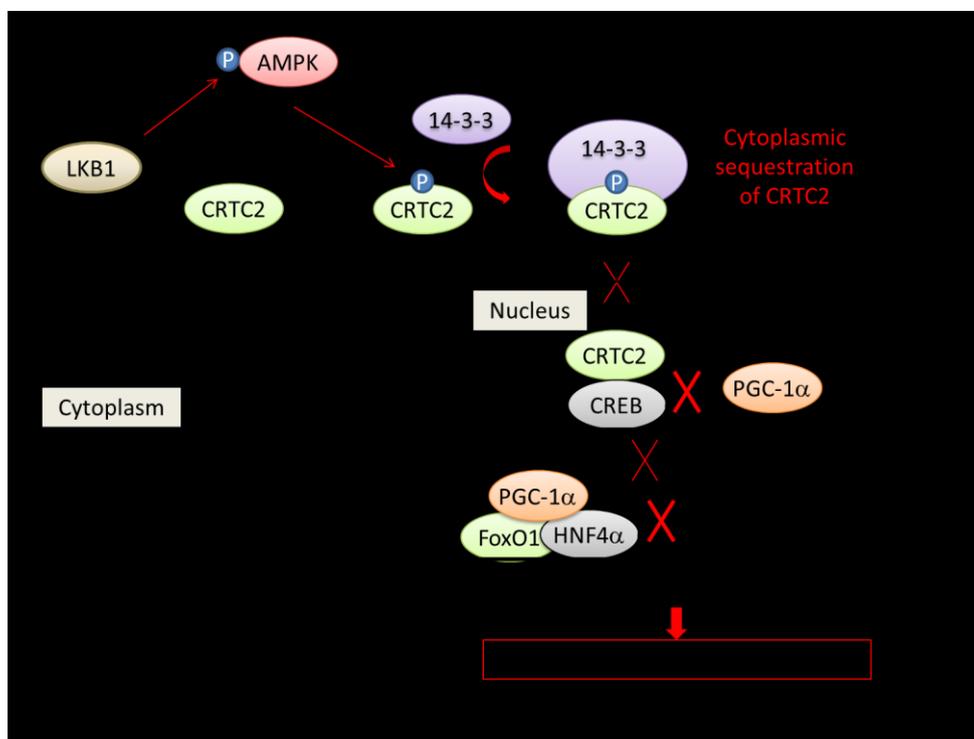


Figure 20: LKB1/AMPK controls gluconeogenic gene expression

In response to metabolic stress, LKB1 phosphorylates AMPK which in turn phosphorylates CRTC2 leading to its cytoplasmic sequestration by 14-3-3. Consequently, CRTC2 cannot translocate into the nucleus and bind to CREB transcription factor. Thereby, the co-activator PGC1 α co-activator expression is restrained. Thus, the LKB1/AMPK pathway prevents PGC-1 α from driving the transcription of key gluconeogenic enzymes such as PEPCK and G6Pase in association with the transcription factor HNF4 α and the forkhead family activator FoxO1. Red arrows and crosses represent the intervention of LKB1/AMPK leading to gluconeogenesis inhibition. Adapted from (B Viollet et al., 2009).

Although I present some of AMPK functions in the regulation of glucose metabolism in the liver in my manuscript, these data need to be evaluated with caution. Indeed, a body of recent data has shown that AMPK is not required for the regulation of hepatic glucose production downstream of LKB1 and that it's the SIKs, rather than AMPK, that regulate glucose production in the liver by keeping the gluconeogenic program repressed (K. Patel et al., 2014) (**Figure 21**). The role of AMPK in regulating glucose uptake in skeletal muscle and heart remain valid.

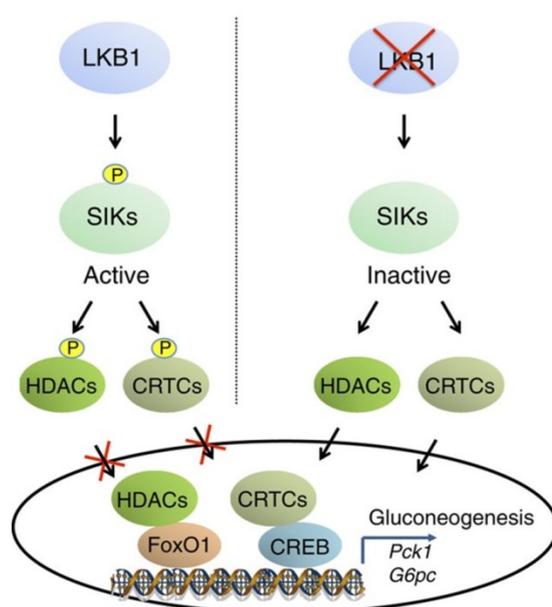


Figure 21: Schematic diagram showing LKB1–SIK pathway in the control of hepatic gluconeogenesis.

Activity status of SIKs influences phosphorylation of transcription co-activators such as HDACs (histone deacetylases) and CRTCs their nuclear-cytoplasm shuttling and gluconeogenic gene expressions in the liver. Modified from (K. Patel et al., 2014).

1.4.3.3 LKB1/AMPK regulates cell growth, autophagy and metastasis

The best understood mechanism whereby LKB1/AMPK limits cell growth is through inhibition of the mTOR pathway.

The mammalian target of rapamycin (mTOR) controls cell growth in all eukaryotes, acting as a central integrator of nutrient and growth factor inputs (Guertin & Sabatini, 2007). mTOR is found in a biochemically and functionally discrete signaling complex called mTORC1 (mTOR complex 1) which includes the raptor (regulatory-associated protein of mTOR) protein. mTORC1 positively regulates cell growth through the phosphorylation of two regulatory proteins called p70S6k (p70 ribosomal S6 protein kinase) and 4E-BP1 (the eukaryotic initiation factor 4E-binding protein-1) which stimulate the translation of cell growth regulators such as cyclin D1, hypoxia inducible factor 1 α (HIF-1 α), and c-myc (Guertin & Sabatini, 2007; Holz, Ballif, Gygi, & Blenis, 2005).

Upstream components of the mTOR signaling pathway include the tumor suppressor complex formed by TSC1 and TSC2 (the tuberous sclerosis complex). TSC2 is a GTPase activating protein that inactivates mTOR through the stimulation of the intrinsic GTPase activity of the G protein Rheb. The latter is thereby converted from its GTP-bound active state, which is responsible for mTOR activation, to a GDP-bound inactive state (J. Huang & Manning, 2008).

AMPK inhibits protein synthesis, an anabolic pathway consuming a significant amount of ATP. One of the main targets of AMPK amongst the regulatory elements implicated in protein synthesis is mTOR. When levels of ATP, glucose or oxygen are low, AMPK directly phosphorylates TSC2 at Thr1227 and Ser1345, which increases the activity of TSC1–TSC2 complex to inhibit mTOR (Inoki, Zhu, & Guan, 2003). Interestingly, in lower eukaryotes and in *TSC2*^{-/-} MEFs, AMPK activation still partially suppress mTORC1, suggesting an alternative mechanism of mTOR inhibition by AMPK. In this context, AMPK was found to phosphorylate raptor at Ser722 and Ser792 which induces the binding to 14-3-3 and results in suppression of mTORC1 kinase activity (Gwinn et al., 2008; Hahn-Windgassen et al., 2005) (**Figure 22**).

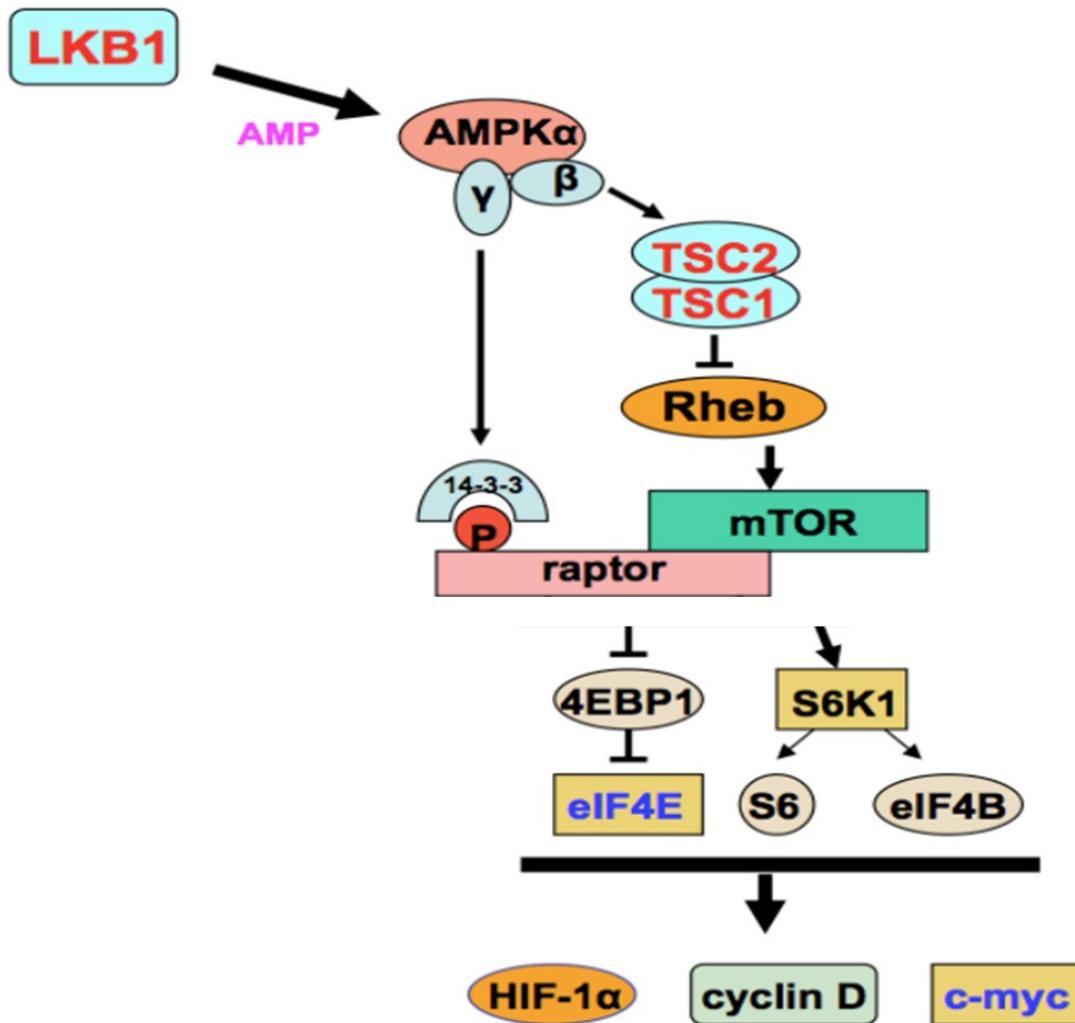


Figure 22: The LKB1/AMPK/mTORC1-dependent regulation of protein translation.

LKB1 activates AMPK which directly phosphorylates both TSC2 and raptor to inhibit mTORC1 activity by a dual mechanism, and mTORC1 controls the translation of a number of cell growth regulators. Once activated, mTOR is able to phosphorylate two regulatory proteins, the p70 ribosomal S6 protein kinase (S6K1 or p70S6K) and the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1). The phosphorylation of 4E-BP1 relieves its inhibitory action on eukaryotic initiation factor 4E (eIF-4E), which can then bind the mRNA cap and stimulate protein synthesis initiation. p70S6K phosphorylates several substrates including the S6 ribosomal protein (S6) and the eukaryotic translation initiation factor 4B (eIF4B). Adapted from (Han, Li, Zhu, Liu, & Li, 2013).

It is now established that mTORC1 is currently the only signaling pathway downstream of LKB1 that has been shown to be deregulated in tumors arising in humans and mouse models of both Peutz-Jeghers syndrome and NSCLC (non-small cell lung carcinoma) (Han et al., 2013). The current data indicate that energy stress results in LKB1-dependent activation of AMPK, leading to the inhibition of mTORC1 activity through TSC2 and raptor, although additional AMPK substrates may contribute to the regulation of mTOR.

Solid tumors are heterogeneous and inner cells are usually less irrigated and oxygenated generating a hypoxic environment. In these conditions, the expression of the transcription factor HIF-1 (hypoxia inducible factor 1) is induced allowing the cells to survive and grow (Powis & Kirkpatrick, 2004). In *LKB1*-deficient tumors *in vivo*, mTORC1 and HIF-1 are up-regulated. Interestingly, it has been well established that mTORC1 up-regulates HIF-1 and that LKB1/AMPK can negatively regulate mTORC1. These results indicate that LKB1-AMPK-mTORC1 signaling may inhibit the expression of HIF-1 thereby suppressing cancer cell growth under hypoxic conditions (Gan & Li, 2014). Interestingly, two recent studies have reported that loss of LKB1 or AMPK induce a pro-growth metabolic program in proliferating cells in a HIF-1-dependent manner. While loss of LKB1 or AMPK in tumor cells increases glucose and glutamine uptake and utilization, silencing HIF-1 α reverses these metabolic advantages and impairs the growth and survival of LKB1- or AMPK- deficient tumor cells under low-nutrient conditions (Faubert et al., 2013a, 2014).

In addition to the regulation of cell growth through the mTORC1 complex, LKB1/AMPK/mTORC1 is also implicated in the regulation of autophagy and metastasis.

ULK1 and ULK2 (unc-51-like kinase) are inducers of autophagy, identified as orthologs of yeast Atg1 in eukaryotes, and have partially redundant functions in starvation-induced autophagy. ULK1/2 forms a complex with 2 other proteins called Atg13 and FIP200. Under normal growth conditions, mTORC1 associates with the ULK1/2 via raptor, allowing mTOR to phosphorylate ULK1/2 and Atg13, thereby suppressing ULK1/2 kinase activity. Under starvation conditions or when mTORC1 activity is inhibited by drugs or upstream kinase such as AMPK, mTORC1 is unable to suppress ULK1/2-induced autophagy. Alternatively, AMPK can also directly phosphorylate ULK1, thereby inducing its kinase activity. Interestingly, AMPK and raptor are also phosphorylated in an ULK1-dependent manner in a negative

feedback loop that restrains autophagy induction (Alers, Löffler, Wesselborg, & Stork, 2012) **(Figure 23)**.

A recent study has reported that nitrosative stress induced by steady-state nitric oxide (NO) caused rapid activation of an ATM damage-response pathway leading to downstream signaling by the LKB1/AMPK/TSC/mTORC1/ULK1 pathway and resulting in increased autophagy. Since cancer cells are particularly sensitive to nitrosative stress, these data could lead to anti-cancer therapies based on the ability of reactive nitrogen species to induce autophagy-mediated cell death (Tripathi et al., 2013).

In MDA–MB-435 breast cancer cells lacking LKB1 expression, over-expression of wild-type LKB1 can significantly inhibit the invasion and metastasis *in vitro* and *in vivo*. This observation is associated with downregulation of matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) as well as vascular endothelial growth factor (VEGF). LKB1/AMPK is also essential for adiponectin-mediated inhibition of migration and invasion of breast cancer cells through inhibition of p70S6 kinase inhibition (Gan & Li, 2014). In a model of ErbB2-mediated breast cancer, the loss of LKB1 promotes tumor initiation and induces a characteristic shift to aerobic glycolysis ('Warburg effect'). In these tumors, mTOR was found to contribute to the metabolic reprogramming and *LKB1*-deficient cells displayed enhanced early tumor growth and increased migratory properties *in vitro* (Dupuy et al., 2013).

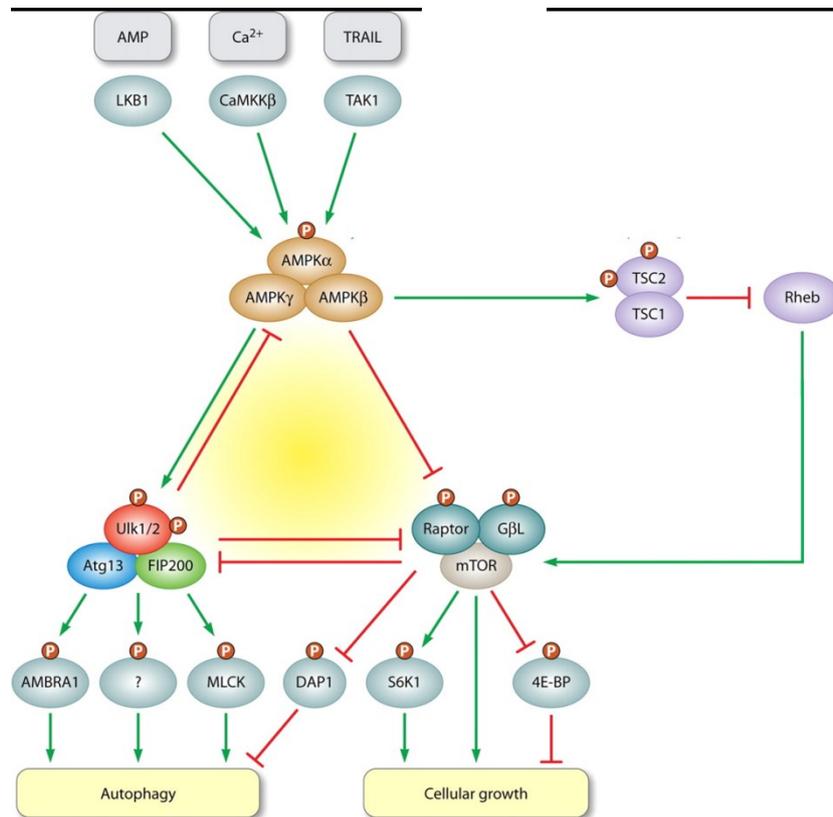


Figure 23: Fine adjustment of autophagy by the AMPK-mTORC1-Ulk1/2 kinase network

The two protein complexes AMPK and mTORC1 are known to oppositely regulate the autophagy inducing complex Ulk1/2-Atg13-FIP200. Under sufficient supply of growth factors and nutrients, the active mTORC1 stimulates growth related processes such as protein translation, e.g., by phosphorylation of S6K1 and 4E-BP, while simultaneously inhibiting self-consuming processes such as autophagy. The catalytic activity of AMPK crucially depends on phosphorylation by upstream kinases, such as the constitutively active LKB1. AMPK activity is further enhanced by decreasing ATP/AMP ratios. In addition, the other two known upstream kinases, CaMKKβ and TAK1, have been implicated in AMPK-mediated autophagy induction by intracellular $[Ca^{2+}]$ and TRAIL treatment, respectively. Under low-energy conditions, AMPK positively regulates autophagy induction through inhibition of mTORC1. This releases the negative regulation of mTORC1 on the Ulk1/2-Atg13-FIP200 complex, especially on Ulk1/2 kinase activity. AMPK inhibits mTORC1 either via the TSC1/2-Rheb pathway or by direct phosphorylation of raptor. However, AMPK is also able to bind, phosphorylate, and directly activate Ulk1/2. Again, this interaction is counteracted by mTORC1. Furthermore, mTORC1 not only inhibits autophagy by suppressing Ulk1/2 kinase activity, it also simultaneously inhibits DAP1, a negative regulator of autophagy. mTORC1 inhibition thus leads to both autophagy induction via Ulk1/2-Atg13-FIP200 and to its restriction via DAP1. Two Ulk1-dependent feedback loops additionally help to fine-tune the autophagic response. Ulk1 has been shown to phosphorylate and inhibit both of its upstream regulators AMPK and mTORC1. While phosphorylation of raptor might help to maintain mTORC1 inhibition when nutrients are limited, the inhibition of AMPK activity by Ulk1 antagonizes this action and restricts the autophagic response. This perplexingly complex network of mutual activation and inhibition will ultimately establish an appropriate response to conflicting demands. Adapted from (Alers et al., 2012).

1.4.3.4 Role of LKB1/AMPK in primary cilia

The primary cilium is a non-motile microtubule-based organelle that projects from the surface of vertebrate cells and functions as a signaling center. Unlike motile cilia, only one primary cilium is present by cell. It resorbs as the cell enters into the cell cycle (S phase) and reassembles by the end of mitosis (G1 phase). Sonic hedgehog (Shh), platelet-derived growth factor (PDGF) and Wnt signaling essentially depends on the primary cilium (Hoerner & Stearns, 2013; Hsiao, Tuz, & Ferland, 2012).

The importance of primary cilia in vertebrate development was first revealed in genetic experiments in mice, where it was shown to be essential and required for the survival and patterning of the mouse embryo. Phenotypic, genetic and biochemical analysis then pinpointed the importance of the primary cilia in Shh signal transduction, which is impaired in cilia mutant embryos (Huangfu et al., 2003).

Defects in primary cilia leads to human disorders called ciliopathies, including Bardet-Biedl syndrome, Joubert syndrome, Meckel-Gruber syndrome, with clinical manifestations including brain malformations, skeletal abnormalities, retinal degeneration, and cystic kidney disease (Hsiao et al., 2012). The mechanisms underlying many of the disorders associated with ciliary dysfunction have yet to be fully elucidated.

Cell size is mainly regulated by the mTOR pathway, where mTORC1 processes the external signals from growth factors and nutrients. Deregulation of the mTOR pathway occurs in polycystic kidney disease (PKD), a cilopathy due to failure of the cilia to sense urine flow because of inherited mutations in ciliary proteins.

Recently, Christopher Boehlke and his colleagues have published a remarkable study showing that *Lkb1* is localized in the cilium-basal body compartment in MDCK epithelial cells (Madin-Darby canine kidney) and that bending of the cilium by flow activates the *Lkb1*/AMPK pathway in the cilia-basal body compartment to inhibit mTORC1 activity and reduce cell size (**Figure 24**). *Lkb1*-deficient cells presented a constitutively active mTOR pathway (phosphorylated S6K), associated with hypertrophy. Although the role of LKB1 in the primary cilia has been unveiled in this study, the molecular mechanisms whereby LKB1 is activated by bent cilia, and is localized in this cellular compartment, require further investigation (Aznar & Billaud, 2010; Boehlke et al., 2010).

Interestingly, cilia-deficient mice have impaired metabolic control after glucose challenge, suggesting a functional link between glucose metabolism and cilia. Since insulin stimuli activate mTORC1 and glucose control, at least in part, through the LKB1/AMPK pathway, these findings raise the possibility that, beyond mechanotransduction, cilia may have a broader role in metabolic control through Lkb1, AMPK and mTOR signaling (Boehlke et al., 2010).

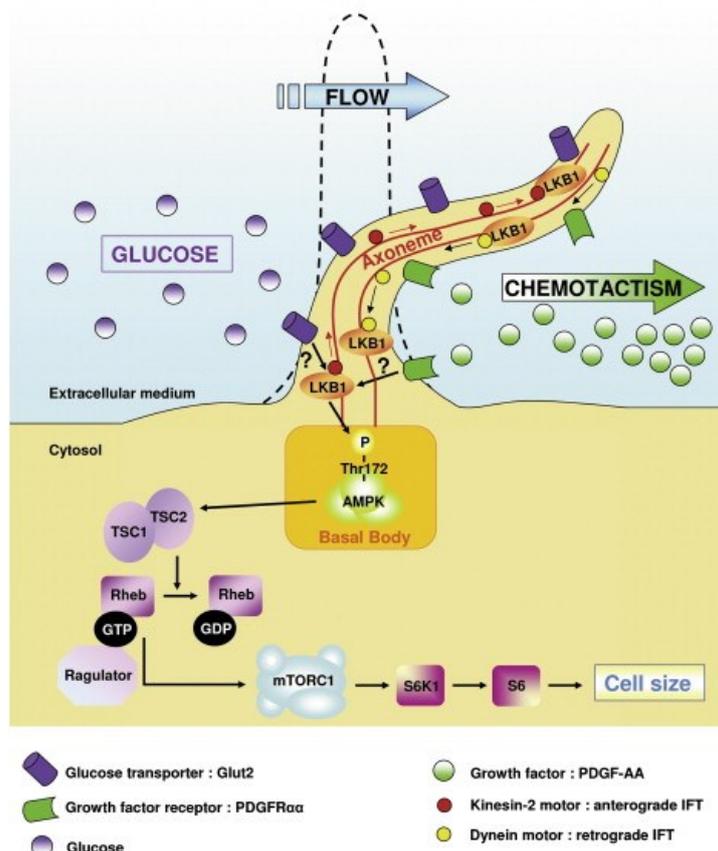


Figure 24: Regulation of Cell Size by the Primary Cilium

The axonemal microtubule structure is depicted in red, and the basal body that anchors the cilium in the plasma membrane is shown in orange. The work of Boehlke and colleagues (2010) shows that the LKB1 tumor suppressor kinase is located in the primary cilium, whereas its substrate, the AMP-dependent protein kinase (AMPK), is found in the basal body. Upon cilium bending by fluid flow, LKB1 is activated and transported into the basal body, where AMPK is phosphorylated (on the threonine-172 of the α catalytic subunit) and activated. Stimulation of the LKB1-AMPK signaling leads to the downregulation of the mTORC1 pathway and subsequent reduction of cell size via the inhibition of protein synthesis. The primary cilium may act as a glucose-sensing antenna (see Boehlke et al., 2010) or may be involved in polarized migration toward a chemotactic source, producing factors such as PDGF. In these two latter situations involving the primary cilium, the LKB1-mTORC1 pathway may also be involved in transducing intracellular signals. (thesis Nicolas Aznar, 2011)

1.5 Role of LKB1 in cellular polarity and migration

LKB1 regulates several cellular processes such as proliferation, cell polarity, migration, transcription and cellular stress and damage responses. In this manuscript, we will mainly focus on the regulation of cell polarity which is related to my PhD project.

Cell polarity is a universal biological process which relies on the asymmetric distribution and organization of cellular components and structures. The establishment and maintenance of cell polarity occurs in uni-and multicellular organisms, during development and in adult tissues. It is regulated by signaling pathways, membrane trafficking events and cytoskeletal rearrangements, which need to be finely-tuned. Indeed, impaired cell polarity can lead to developmental disorders as well as cancer. One of the main functions of LKB1 is the regulation of cellular polarity.

1.5.1 LKB1 regulates polarity in invertebrates

Before the discovery of AMPK as an LKB1 downstream target implicated in the regulation of metabolism, LKB1 functions were prominently linked to the regulation of cell polarization in model organisms: *Caenorhabditis elegans* and *Drosophila melanogaster*.

The *par* genes were identified in genetic screens for regulators of cytoplasmic partitioning in the early embryo of *C. elegans* (Kemphues, Priess, Morton, & Cheng, 1988). Cloning and characterizing the *par* genes resulted in the discovery of six encoded proteins: PAR-1 to 6. Another gene *pkc3* (protein kinase C3), which encodes an atypical protein kinase C (aPKC) was also identified in the screening and is a member of the PAR protein complex. The PAR proteins are required for asymmetric cell division by the worm zygote, are localized asymmetrically (all but PAR-4 and PAR-5), and interact physically and functionally (**Figure 25**). The PAR proteins were found to regulate polarization, not only in *C. elegans*, but also in many different contexts, in diverse models, which highlights their role as a fundamental mechanism for cell polarization across evolution. PAR2 is the only member which is not conserved beyond *C.elegans* (Goldstein & Macara, 2007; Jansen et al., 2009; Macara, 2004).

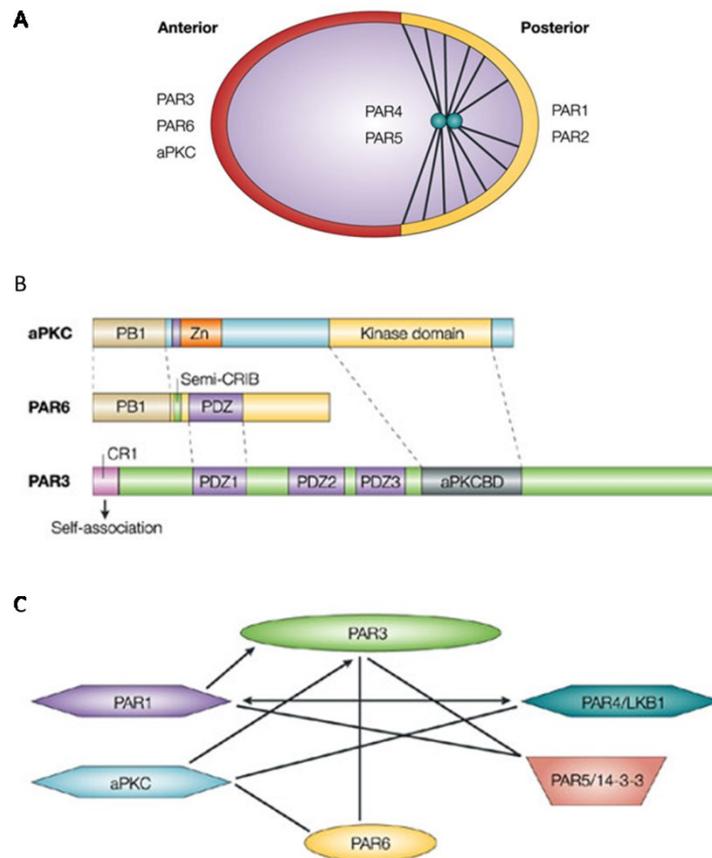


Figure 25: Introducing the PAR family.

A) Distribution of the PAR proteins in the *Caenorhabditis elegans* zygote. The zygote is shown after the pronuclei (small circles) have moved towards one pole of the cell and polarization has been established. Black lines represent the microtubule asters. Location of PAR3, PAR6 and atypical protein kinase C (aPKC) is shown in red; PAR1 and PAR2 in yellow; and PAR4 and PAR5 in purple. **B) and C) The PAR proteins and their interactions.** **B)** Domain organization of atypical protein kinase C (aPKC), PAR6 and PAR3. The CRIB domain of PAR6 is about half the normal length, so is referred to as a 'semi-CRIB' domain. The aPKC also contains a zinc-finger (Zn) motif that functions as a regulatory domain. An aPKC-binding domain (aPKCBD) in PAR3 is phosphorylated by the kinase. The amino-terminal conserved region (CR1) is required for oligomerization of PAR3. Connecting lines indicate regions of the proteins that interact with one another. **C)** Connection map for the PAR proteins. Straight lines show known interactions; arrows show known phosphorylation events. Interactions are based on studies from different organisms, so it is not yet clear whether they all occur in any particular species. There are also many isoforms of several of the PAR proteins in vertebrates (four *PAR6*, two *PAR3* and four *PAR1* genes). CRIB, CDC42/Rac-interactive binding; LKB1, product of gene responsible for Peutz–Jeghers syndrome; PB1, phagocyte oxidase/Bem1; PDZ, (PSD-95, Discs large and Zona occludens-1). Adapted from (Macara, 2004).

Among the PAR protein members, the serine threonine kinase PAR-4 is the ortholog of *Xenopus*, *Drosophila* and mammalian LKB1. *par-4* mutations in *C.elegans* lead to defective asymmetric cell division of the fertilized worm zygote, and absence of intestinal cells. Thus, PAR4 is essential for establishing cell polarity during asymmetric cleavage patterns of blastomeres and specification of the intestinal lineage (J. L. Watts, Morton, Bestman, & Kemphues, 2000). A host of functional studies revealed a complex epistatic regulation among the PAR family members. PAR4 mutations are epistatic to all other PAR family mutants. For instance, the subcellular localization of PAR-4 was not affected by other PAR proteins, whereas mutations in PAR-4 results in a disturbed localization of PAR3 and PAR6, which act in a complex (Hung & Kemphues, 1999). These observations suggest that PAR-4 may reside at the top of a cascade regulating the asymmetric segregation of PAR family proteins.

Likewise, in *Drosophila*, *dLkb1* mutants show defective anterior-posterior oocyte axis formation. Thus, dLKB1 is required for the early A–P (anterior-posterior) polarity of the oocyte, and for the repolarization of the oocyte cytoskeleton that defines the embryonic A–P axis (Martin & St Johnston, 2003a). More recently, a study has revealed the role of dLKB1 in regulating asymmetric division in neuroblasts, neural stem cells whose division generates most neuronal and glial cells of the central nervous system in *Drosophila* (**Figure 26**). Indeed, *dlkb1* mutations suppress unequal cytokinesis and abrogate proper localization of Bazooka, Par-6, DaPKC and Miranda. Besides, dLKB1 mutants show reduced microtubules density and an absence of formation of astral microtubules which are essential for a proper mitosis. These observations suggest a growth or stability defect of microtubules and show that dLKB1 is essential for the formation of the mitotic spindle (Bonaccorsi et al., 2007). These studies remain some of the strongest evidence linking LKB1 to cellular polarization.

Another serine threonine kinase, PAR-1, is present among the PAR family members. Interestingly, in *C.elegans*, the phenotype of *par-4* mutants highly resembles that of *par-1* mutants, suggesting that both kinases are components of a signaling pathway implicated in the regulation of polarity (Pellettieri & Seydoux, 2002; J. L. Watts et al., 2000). Proteomic analysis has shown that LKB1 is capable of interacting with MARK4/PAR-1 and activating it by phosphorylation *in vitro*. This observation has been recently confirmed in *C.elegans*, where PAR-1 was phosphorylated by PAR-4 (Narbonne, Hyenne, Li, Labbé, & Roy, 2010). In

contrast, this wasn't the case in *Drosophila*; although dLKB1 and PAR-1 are both required for the A-P axis polarity, LKB1 rather acts as a PAR-1 substrate. Indeed, the N-terminus of LKB1 is phosphorylated by PAR-1 *in vitro* and is probably implicated in PAR-1 induced cytoskeleton organization in the *Drosophila* oocyte (Martin & St Johnston, 2003a).

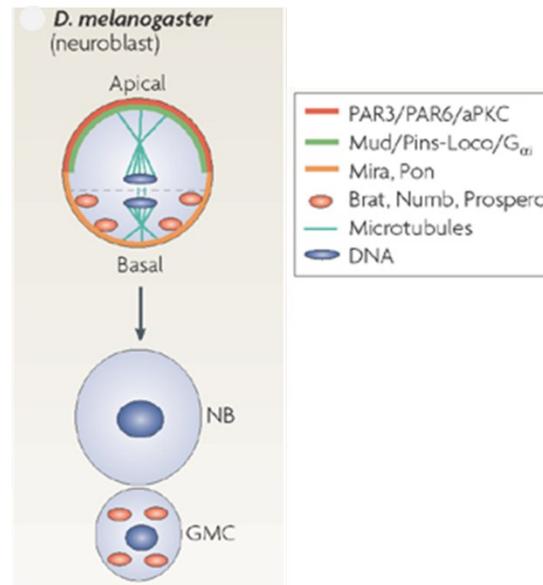


Figure 26: Asymmetric division of *Drosophila* neuroblast

Schematic representation of *Drosophila melanogaster* larval neuroblast. The polarized mother cell during anaphase is shown at the top, whereas the daughter cells that are produced by asymmetric division are shown at the bottom. For simplicity, centrosomes and microtubules are depicted only in the top part. The distribution of several components that are important for polarity establishment, spindle positioning and cell-fate determination is illustrated for the mother cell, and the distribution of cell-fate determinants is shown for daughter cells immediately after mitosis. GMC, ganglion mother cell; Mud, mushroom body defectice; NB, neuroblast. Adapted from (Gönczy, 2008).

Finally, dLKB1 and PAR-1 are both localized in the oocyte follicular cells. The phosphorylation of LKB1 by PKA, as well as its prenylation in the C-terminal region which induces its cortical localization, are both required for the LKB1-dependent regulation of polarity in the *Drosophila* oocyte (Martin & St Johnston, 2003a).

Thus LKB1 is essential for the regulation of polarity in invertebrates but was also shown to have important implication in polarity regulation in mammals.

1.5.2 LKB1 regulates epithelial and neuronal cell polarity

Epithelial tissues are characterized by the formation of cellular sheets displaying apicobasal polarity and tight intracellular junctions. Evidence on the role of LKB1 in epithelial polarization was provided in the human intestinal epithelial cell line LS174T-W4 in which the induced expression of STRAD by doxycyclin leads to the stabilization, activation and translocation of LKB1 into the cytoplasm. Upon LKB1 activation, the cells rapidly remodel the actin cytoskeleton to form an apical brush border around which several junctional proteins such as ZO-1 (zonula occludens) redistribute in a dotted circle. The activation of LKB1 in these cells is accompanied by the relocalization of CD66 (carcinoembryonic antigen/CEA), CD13 (dipeptidyl peptidase IV), and CD26 (amino peptidase-N) to the apical membrane whereas the basolateral protein CD71 was completely excluded from the brush border domain (Annette F Baas et al., 2004). These data establish the importance of LKB1 in epithelial cell polarity in mammals (**Figure 27**).

Likewise, in the Madin-Darby Canine Kidney Epithelial Cell line (MDCK), when co-expressed with STRAD, LKB1 relocates to the cytoplasm and plasma membrane and partially colocalizes with PAR3 at tight junctions (Brajnovic, Joberty, Küster, Bouwmeester, & Drewes, 2004) and with E-cadherin at adherent junctions (Sebbagh, Santoni, Hall, Borg, & Schwartz, 2009).

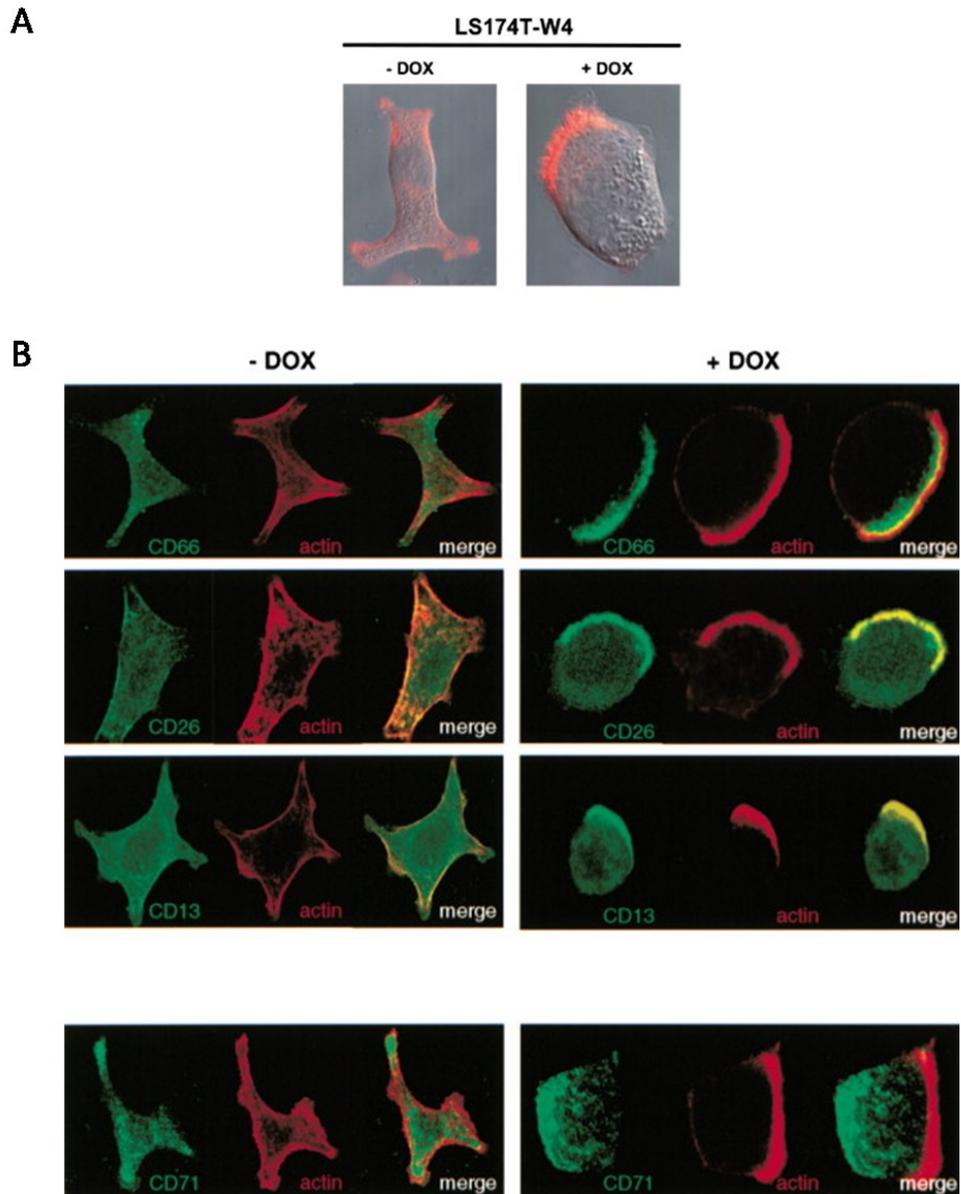


Figure 27: Role of LKB1 in intestinal cell polarity

A) Remodeling of the Actin Cytoskeleton upon Activation of the LKB1/STRAD Complex. DIC image of the LS174T-W4 clone superimposed on a phalloidin-TRITC staining shows retraction of cell extensions and actin cytoskeleton reorganization upon 24 hr doxycyclin treatment. Cell organelles redistribute in a polarized fashion. **B)** Apical and Basolateral Markers Sort to Their Respective Plasma Domains in Induced LS174T-W4 Cells. Apical and basolateral proteins rearrange to their respective plasma domains in 24 hr doxycyclin-treated LS174T-W4 cells. This is visualized in compilations of individual focal planes of double staining for actin and the apical markers CD66/CD26/CD13 (top) and of double staining for actin and the basolateral marker CD71/transferrin receptor (bottom). Adapted from (Annette F Baas et al., 2004).

Several studies have already pinpointed the link between cell polarity and tumor development. An interesting study using a 3D culture procedure (Matrigel) presents a link between the human epithelial organization in controlling normal and oncogene-driven cell proliferation. When non-transformed mammary epithelial cells are cultured in Matrigel, they proliferate and eventually form organized acini-like structures. When the oncogene c-myc is conditionally induced in the developing 3D mammary structures, it induces hyperproliferation and spontaneous apoptosis. However, c-myc fails to exert these functions in the established, organized epithelial structure. Interestingly, silencing *LKB1* in these acini-like structures disrupts their integrity and polarity, which restores c-myc-induced proliferation and apoptosis. Thus, LKB1 is essential for mammary epithelium organization and suppresses c-myc oncogenic properties in mammary epithelial cells. In a complementary study, the same team shows that loss of *Lkb1* facilitates oncogenic proliferation by releasing epithelial cells from structural basement membrane boundaries (Partanen et al., 2012; Partanen, Nieminen, Mäkelä, & Klefstrom, 2007).

PJS patients are highly predisposed to developing pancreatic cancer. In this context, *in vivo* conditional deletion of *Lkb1* in the pancreatic epithelium of the mouse results in defective acinar cell polarity, an abnormal cytoskeletal organization, a loss of tight junctions, and an inactivation of the AMPK/MARK/SAD family kinases. Starting with rapid and progressive postnatal acinar cell degeneration and acinar-to-ductal metaplasia, the mice later develop pancreatic insufficiency culminating with the development of pancreatic serous cystadenomas, a tumor type associated with PJS. *Lkb1* deficiency also impacts the endocrine pancreas where the Langerhans islets become smaller and scattered and display transient alterations in glucose control. Thus, *Lkb1* is essential for the establishment of epithelial cell polarity that is vital for pancreatic acinar cell function and viability *in vivo* and for the suppression of neoplasia (Hezel et al., 2008).

Polarization of hepatocytes is manifested by bile canalicular network formation. Bile canaliculi are fine tubular channels forming a three-dimensional network within the parenchyma of the liver. They collect the bile which is secreted by hepatocytes. They are formed by grooves on some of the lateral faces of connected hepatocytes, and join to form the bile ductules and eventually the hepatic duct.

A recent study reports that liver-specific deletion of *Lkb1* in mice leads to defective canaliculi and bile duct formation, resulting in impaired bile acid clearance and subsequent accumulation of bile acids in serum and liver (Angela Woods et al., 2011). The major mammalian bile acid, taurocholate, accelerates canalicular network formation and concomitantly increased cAMP in rat hepatocyte sandwich cultures. In this context, taurochlorate was found to signal through a cAMP-Epac-MEK-Rap1-LKB1-AMPK pathway, thereby accelerating polarity establishment in hepatocytes (Fu, Lippincott-Schwartz, & Arias, 2011; Fu, Wakabayashi, Lippincott-Schwartz, & Arias, 2011). The same team has more recently found that, in addition to the regulatory role for LKB1 in hepatocyte polarization, and canalicular network formation, the kinase is also required for microtubule-dependent trafficking of the canalicular bile acid transporter ABCB11 to the canalicular membrane (Homolya et al., 2014).

The role of LKB1 in neuronal polarization has been well documented and additional downstream substrates were found and will be discussed in the next part of this section. The conditional knock-out of *Lkb1* in telencephalic neuronal progenitors impairs axon extension in mice (A. P. Barnes et al., 2007). An approach using *in vivo* electroporation of RNAi constructs targeting *Lkb1* transcripts in neonatal mouse cortex led to impaired neuronal migration and axonal polarity. This observation was associated with a malpositioning of the centrosome in migrating and differentiating neurons (Asada, Sanada, & Fukada, 2007).

1.5.2.1 ARKs mediate LKB1 function in polarity establishment

As previously discussed, AMPK mediates LKB1 functions in regulating cell growth and metabolism, but is also implicated in LKB1 regulation of polarity.

Our team has observed that mutations of *LKB1* in PJS patients and in sporadic tumors which map in the C-terminal domain of LKB1 do not disturb the catalytic or the anti-proliferative activity of LKB1 but rather alters its capacity to activate AMPK. As a consequence, LKB1 loses its ability to establish and maintain polarity of both intestinal epithelial cells and migrating astrocytes (Forcet et al., 2005).

AMPK is essential for normal development of *Drosophila* since all *ampk*-null mutant flies die before the mid-pupal stage and fail to enter adulthood, even in the presence of sufficient nutrients. The embryos show severe abnormalities in cell polarity and mitosis, similar to

those of *lkb1*-null mutants. Constitutive activation of AMPK restored many of the phenotypes of *lkb1*-null mutants, suggesting that AMPK is a downstream mediator of LKB1, controlling mitosis and cell polarity (J. H. Lee et al., 2007).

LKB1 controls cell polarity through the activation of the actin motor myosin II. It is the phosphorylation of the Myosin II Regulatory Light Chain (MRLC), a downstream target of AMPK, which regulates myosin II activity and stability in humans. The expression of a phosphomimetic mutant of MRLC rescues the polarity defects of *ampk* and *lkb1*-null mutants in *Drosophila* embryos, and induces polarization of LS174T cells. It has been suggested that MRLC is a direct substrate of AMPK (J. H. Lee et al., 2007), but purified AMPK did not efficiently phosphorylate MRLCII *in vitro* (Bultot et al., 2009). Thus, it has been suggested that other kinases could mediate the phosphorylation of MRLC by AMPK (Mirouse & Billaud, 2011). The Rock kinase (ROCK) for instance is activated by AMPK and phosphorylates MRLC through MLC kinase (Miranda et al., 2010). Another member of the AMPK family, NUA1, can indirectly inactivate the MLC phosphatase protein thereby enhancing the phosphorylation of MRLC (Zagórska et al., 2010). Thus, ROCK and NUA1 are potential mediators of the phosphorylation of MRLC by LKB1. Interestingly, our team has recently shown that in cranial neural crest cells (CNCCs), LKB1 can activate AMPK which in turn activates ROCK leading to the phosphorylation of MRLC. This signaling pathway is important for the directional migration of CNCCs and head development in chick embryos. This work to which I have contributed during my PhD will be presented in **chapter II-project 1**. Thus, the LKB1/AMPK signaling regulates cell polarity and is conserved among species from invertebrates to human.

Genetic analyses of *D. melanogaster* have suggested that dLKB1/PAR-1 (MARK ortholog) regulate microtubule dynamics and cell polarity (Martin and St Johnston 2003; Doerflinger 2003; Shulman, Benton, and St Johnston 2000). Likewise, genetic studies in *C. elegans* have revealed that PAR-1 and LKB1 are essential for cell polarization (Kemphues et al., 1988). In mammalian cells, MARK isoforms (MARK1-MARK4) phosphorylate microtubule-associated proteins (MAPs) thereby modulating the organization of the microtubule skeleton and centrosome repositioning (Jansen et al., 2009). In this context, Kojima and his colleagues have investigated the role of LKB1 in microtubule dynamics. They show that in *Lkb1*-deficient MEF cells, microtubule dynamics are disturbed and microtubule polymerization is increased.

LKB1, together with STRAD α and MO25 α subunits, phosphorylates the activation loop of protein kinase MARK2 at threonine 208. MARK2 then phosphorylates the microtubule-associated protein (MAP) Tau at serine 262 triggering its degradation, thereby suppressing microtubule polymerization and microtubule regrowth. Thus, LKB1 is involved in the regulation of microtubule dynamics through the activation of MARK2 (Kojima et al., 2007).

MARK2 knockout mice do not reveal obvious defects in cellular polarization. This observation might be due to redundancy among the MARK isoforms. However, *MARK2* (also called Par-1b) knockout mice are hypoinsulinemic and display a metabolic phenotype (Hurov et al., 2007). This indicates that MARK family members may function in the regulation of metabolic homeostasis in addition to cellular polarization, and that LKB1 might orchestrate this regulation.

The conditional knock-out of *Lkb1* in telencephalic neuronal progenitors impairs axon extension in mice. In this study, the authors show that *Lkb1* is required for axon initiation during neuronal polarization in the embryonic cortex *in vivo* and *in vitro*. Interestingly, the role of *Lkb1* in neuronal polarization was enhanced by its phosphorylation at Ser428, the substrate of protein kinase A (PKA) and p90 Ribosomal S6 Kinase (p90RSK) as previously discussed. PKA and p90RSK mediate effects of extracellular cues that promote axon growth. When activated *Lkb1* would phosphorylate SAD-A and B, resulting in the phosphorylation of microtubule-associated proteins such as Tau (involved in the growth and differentiation of axons and dendrites) and leading to neuronal polarization (A. P. Barnes et al., 2007) (**Figure 28**).

Thus, the role of LKB1 in the regulation of epithelial and neuronal polarity has been investigated *in vitro* and *in vivo* and is mediated by ARKs such as AMPK, MARKs and SADs. Further investigation might lead to the identification of downstream targets of these pathways as well as novel ARKs mediating LKB1 function in cell polarity.

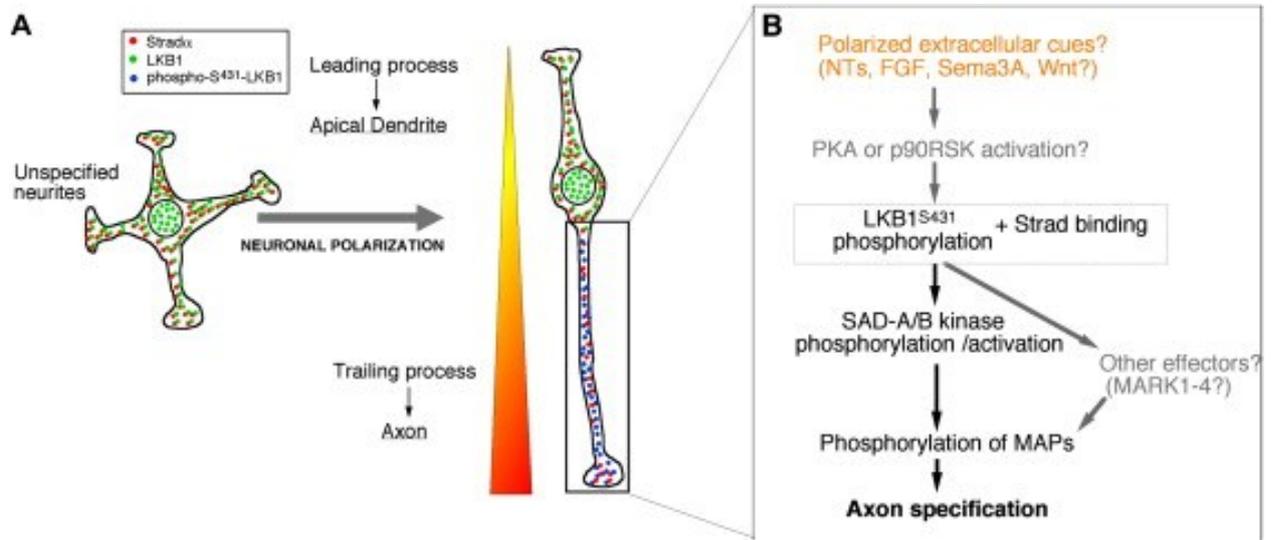


Figure 28: Model of the kinase pathway identified in this study

The authors propose that specific extracellular signals control axonal specification and polarization in cortical neurons by activating LKB1 and SAD kinases in the neurite becoming the axon (A. P. Barnes et al., 2007).

1.5.3 LKB1 regulates polarized cell migration

Cellular migration is a fundamental mechanism in embryonic development but also in adults where it contributes to the recruitment of immune cells to infection sites and wounds. Tumor cells also have the capacity to migrate and metastasize. Understanding the molecular mechanisms allowing tumor cells to detach from the epithelium and migrate *via* epithelial-to-mesenchymal transition (EMT) is important in order to counteract tumor spreading.

Our team has shown that mutations of *LKB1* in its catalytic or C-terminal domain (found in PJS patients and sporadic cancers) lead to impaired polarized migration of astrocytes (Forcet et al., 2005). LKB1 is also important for neuronal migration and centrosome positioning. Knock-down of *Lkb1* in migrating immature neurons impairs neuronal migration, and alters the centrosomal positioning, the centrosome position being a spatial indicator for the cell's migrational direction. In addition, impairment of *Lkb1* in differentiating neurons within the cortical plate induces malpositioning of the centrosome at the basal side of the nucleus, instead of the normal apical positioning (Asada, Sanada, & Fukada, 2007b). The same team has later shown that *Lkb1* mediates Ser9 phosphorylation of GSK3 β (glycogen synthase kinase 3 beta) to inactivate it at the leading process tip of migrating neurons in the developing neocortex. The inactivation of GSK3 β enables APC (the microtubule plus-end binding protein adenomatous polyposis coli) to localize at the distal ends of microtubules in the tip, thereby stabilizing microtubules near the leading edge. These steps are required for forward movement of the centrosome, and neuronal migration (Asada & Sanada, 2010) **(Figure 29)**.

Whether AMPK mediates LKB1-induced neuronal migration remains unclear, although several data point in this direction. Indeed, in HepG2 cells, the activation of AMPK by AICAR increases the phosphorylation of GSK3 β at Ser9 (Horike et al., 2008). AMPK also phosphorylates CLIP-170 directly at Ser311, enabling its localization closer to the distal end of microtubules than non-phosphorylated CLIP-170. Depleting AMPK with siRNA decreases CLIP-170 phosphorylation and shifts the dissociation pattern of CLIP-170 at the distal end of the microtubules, resulting in a disturbance of cell polarity (Nakano & Takashima, 2012). Thus, AMPK might be a downstream target of LKB1 and mediate the LKB1-induced migrational polarity.

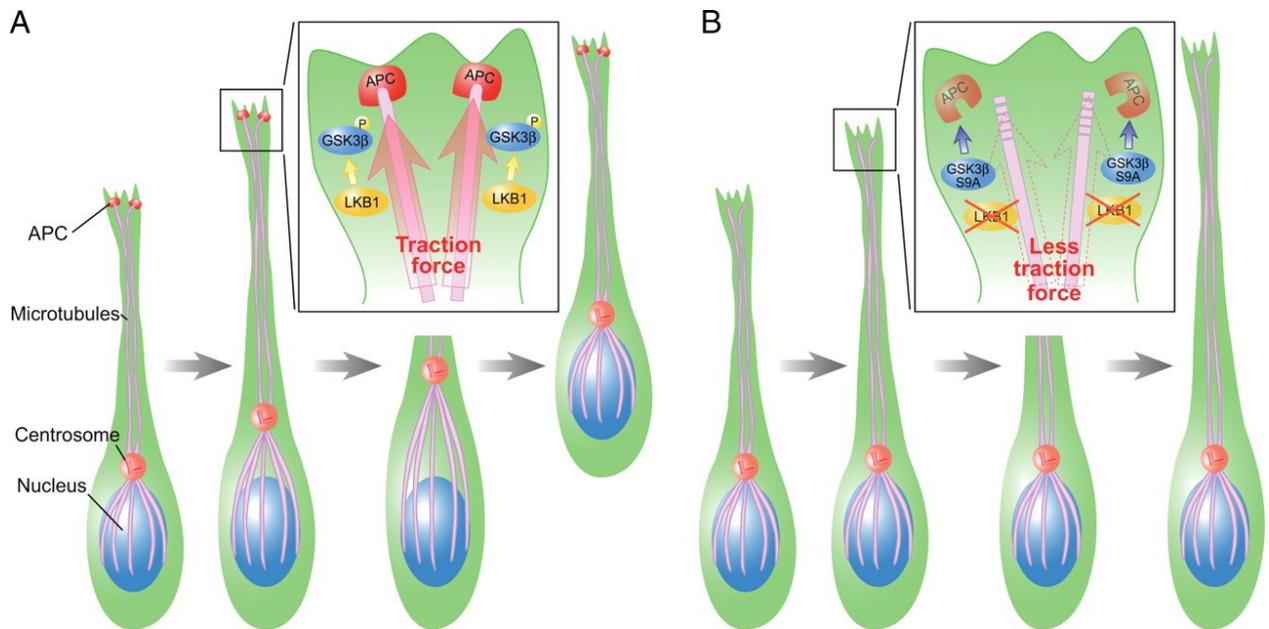


Figure 29: A model for a role of LKB1-GSK3β-APC pathway in centrosomal forward movement.

A) In migrating neurons, LKB1 mediates Ser9 phosphorylation and inactivation of GSK3β in the leading process tip, and this enables APC, a microtubule plus-end protein, to bind to the microtubule ends, anchor them to the leading edge cortex, and stabilize them. Certain motor proteins such as dynein/dynactin (not shown) at the cell cortex provide a traction force on the microtubules and pull the centrosome up in the leading process. **B)** When either Ser9 phosphorylation of GSK3β or APC binding to the microtubule ends is disrupted, microtubules in the leading process tip is destabilized, probably due to impairment of microtubule capture at the cell cortex. This may lead to less traction force provided on the microtubules and results in impairment of centrosomal upward movement and elongation of the leading process. (Asada & Sanada, 2010).

In NSCLC lung cancer cells, *LKB1* depletion using RNAi leads to impaired formation of lamellipodia and aberrant centrosome and Golgi positioning. In these cells, LKB1 colocalizes with the small rho GTPase Cdc42 and its downstream binding partner p21-activated kinase (PAK) at the cellular leading edge during migration. Indeed, LKB1 was shown to be required for Cdc42 polarization to the leading edge by interacting with its active form, maintaining active Cdc42 levels, and downstream PAK phosphorylation. Thus, LKB1/Cdc-42-GTP mediates NSCLC polarity and migration through the LKB1-Cdc42-PAK pathway (Zhang et al., 2008). Interestingly, The *LKB1* depletion enhances cell migration and PAK1(p21-activated Kinase-1) activity in human colon cancer HCT116 cells, whereas overexpression of LKB1 in *Lkb1*-null mouse embryonic fibroblasts (MEFs) suppresses PAK1 activity and PAK1-mediated cell migration (Deguchi et al., 2010). Thus, LKB1 regulates cell migration in a cell type-dependent manner.

A recent study has described the role of LKB1 in metastatic progression of lung cancer, in a signaling pathway which is independent from AMPK, but is rather dependent on MARK1 and MARK4. In this study, the authors propose a model whereby LKB1 phosphorylates the downstream kinases MARK1 and MARK4, which positively regulates the poorly studied scaffold protein DIXDC1 to promote focal adhesion maturation and suppress cell migration, invasion, and metastatic potential. Loss of LKB1-dependent phosphorylation, focal adhesions become more dynamic, and resident kinases FAK/Src activate a signaling cascade through ERK. Consequently, the expression of the EMT transcription factor Snail1 is induced. Snail1 was uniquely upregulated upon *LKB1* deletion across cell types. Expression of Snail drives genes associated with invasion and migration (Goodwin et al., 2014). A more recent study has also demonstrated the role of the LKB1/MARK axis in melanoma invasion. Inhibition of the LKB1–MARK pathway facilitated invasive motility, suggesting that loss of the ability to sense inhibitory signals from the ECM (extracellular matrix) may promote melanoma invasion (Chan et al., 2014) (**Figure 30**).

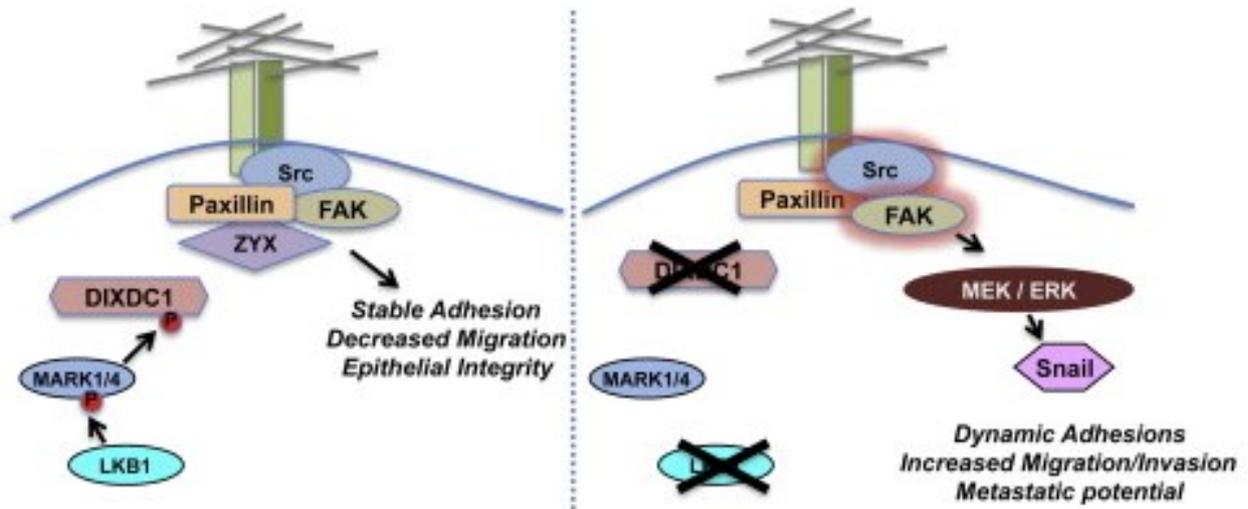


Figure 30: Model presenting the role of LKB1 in regulating cell invasion

LKB1, functioning through the downstream kinases MARK1 and MARK4, positively regulates DIXDC1 to promote focal adhesion maturation and suppress cell migration, invasion, and metastatic potential. Upon loss of LKB1-dependent phosphorylation, or upon downregulation or deletion of the *DIXDC1* gene as occurs in human cancer, focal adhesions become more dynamic, and resident kinases FAK/Src activate a signaling cascade through ERK to induce the EMT transcription factor Snail. Expression of Snail drives genes associated with invasion and migration (Goodwin et al., 2014).

1.6 Role of LKB1 in cancer

We have discussed the role of LKB1 in regulating metabolism, limiting cell growth and establishing polarity, the latter providing tissues with resistance to oncogene actions and tumor development. Here we will discuss the link between metabolism and cancer as well as the dual role of LKB1 in tumorigenesis.

1.6.1 LKB1/AMPK in cancer and metabolism

The Warburg effect is used by tumor cells to switch from oxidative metabolism towards rapid glucose uptake, glycolysis and lactate output in a poorly oxygenated and irrigated environment. The Warburg effect allows tumor cells to quickly increase macromolecular biosynthesis in order to proliferate (Vander Heiden, Cantley, & Thompson, 2009).

Under metabolic stress, LKB1/AMPK limits cell growth through inhibiting glycolysis among other processes, thereby acting as a tumor suppressor. The hypoxia-inducible factor-1 α (HIF-1 α) is required for rapid cell growth in low oxygen environments. By suppressing mTORC1 activity, LKB1/AMPK decreases the expression levels of HIF-1 α . The latter is a transcription factor that promotes the expression of enzymes and transporters required for the Warburg effect. They include most glycolytic enzymes, as well as the transporters GLUT1 and MCT4, which are required for glucose uptake and lactate output, respectively (Shackelford et al., 2009). Thus, LKB1/AMPK exerts an anti-Warburg effect through inhibiting the mTOR pathway. This idea is supported by the fact that, in *Lkb1*-null or *AMPK- α 1 - α 2* double null mouse embryo fibroblasts (MEFs), the expression of HIF-1 α and downstream glycolytic genes are up-regulated. In this context, AMPK-activating drugs should be efficient in the treatment of cancer. Indeed, T2D patients treated with metformin display reduced incidence of cancer development, making metformin a promising anti-cancer drug (Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005; Faubert et al., 2013). Although the effect of metformin on the LKB1/AMPK pathway remains uncertain in liver cells, it might be more relevant in other cell types. Other studies suggest that metformin might delay the initial development of tumors. In a mouse model which is tumor-prone due to heterozygous loss of *PTEN*, combined with reduced expression of LKB1, tumor development (mostly lymphomas) can be delayed by treating the mice from the time of weaning with metformin or phenformin, or by another AMPK activator, A-769662 (Xu Huang et al., 2008). The activation of AMPK by

metformin would exert a cytostatic effect on pre-neoplastic lesions through its ability to inhibit cell growth, thereby delaying the onset of tumorigenesis.

Biguanides could alternatively lead to the suppression of tumor growth through inhibiting the mitochondrial respiratory chain, in *LKB1*-deficient cells. Indeed, cells with a defective LKB1-AMPK pathway are less able to restore ATP levels in response to metabolic stress. Therefore, tumor cells with a down-regulated LKB1/AMPK pathway may be less able to adapt to mitochondrial inhibition by biguanides, and become more susceptible to cell death (Foretz et al., 2010; Hawley et al., 2010; M. R. Owen et al., 2000; Russell et al., 2004; Sakamoto et al., 2005). This mechanism of action has been experimentally supported in a mouse xenograft study of MC38 colon carcinoma cells, in which treatment with metformin reduced the rate of tumor growth in insulin-resistant mice, but also in insulin-sensitive mice where *Lkb1* had been first knocked-down in the tumor cells using RNA interference (Algire et al., 2011). In another NSCLC mouse model, phenformin (more potent inhibitor of the respiratory chain than metformin) prolonged survival of the mice when tumors were induced by activation of mutant K-Ras combined with loss of LKB1 (Shackelford et al., 2013). In this way, biguanides can target *LKB1*-deficient tumor cells which become more sensitive to their ATP-depleting effects, without affecting the surrounding normal cells in which LKB1/AMPK is functional.

1.6.2 A dual role of LKB1 in tumor progression

LKB1 has been well investigated as a tumor suppressor. However, recent studies also report that under specific conditions, LKB1 can be a cancer promoter.

Indeed, a novel isoform of LKB1 has been recently identified. This isoform, Δ N-LKB1, lacking the N-terminal domain is catalytically inactive. It is solely expressed in the lung cancer cell line NCI-H460, and its silencing decreases the survival of the cells and inhibits their tumorigenicity when engrafted in nude mice. Thus, Δ N-LKB1 is rather a promoter of tumorigenesis (Dahmani et al., 2014).

In established tumors that retain the function of the LKB1-AMPK pathway, AMPK would rather protect tumor cells from metabolic stress and allow them to survive. Indeed, in *LKB1*-deficient lung adenocarcinoma (A549) cells, reexpressing LKB1 in cells which are subjected to

glucose starvation, protects them against cell death, apparently through inhibition of fatty acid synthesis by AMPK and consequent sparing of NADPH, which could be utilized to provide protection against the oxidative stress induced by glucose deprivation (Jeon, Chandel, & Hay, 2012). Another study had also pinpointed a pro-oncogenic role of AMPK, whereby the latter is activated in hypoxic regions of tumors and promotes cell survival in metabolically defective cells (Laderoute et al., 2006).

In a recent study, the subcellular localization of LKB1 was analyzed in tumor cells using a PLA derived technique, the “Single Detection method” and FFPE analysis. LKB1 was mainly detected in the cytoplasm of MCF-7 cells, but its localization was mainly nuclear in ZR75-1 cells. This dual localization was correlated with clinical features, whereby cytoplasmic LKB1 expression was associated with bad prognostic markers, whereas nuclear LKB1 was associated with good prognostic markers. Interestingly, the activation of 4E-BP1 correlated with cytoplasmic LKB1 expression comforting the hypothesis whereby LKB1 is sequestered by the cytosolic complex metER α /Src/PI3K and is functionally inactivated in primary sporadic breast carcinomas. LKB1 is therefore unable to down-regulate the mTOR pathway in order to inhibit cell growth (Bouchekioua-Bouzaghrou et al., 2014).

Thus, LKB1 signaling can also promote tumorigenesis and care should be taken when targeting LKB1 signaling for cancer treatment.

1.7 Conclusion

The tumor suppressor gene LKB1 encodes a serine/threonine kinase which regulates several downstream targets such as AMPK, BRSKs, SADs and MARKs and many cellular processes such as polarity and metabolism. In order to better LKB1 tumor-suppressive functions and to better apprehend its intriguing pro-oncogenic activity, it is important to gain insights into upstream regulatory mechanisms. So far, the regulation of LKB1 by upstream kinases and post-translational modifications has been described, but no evidence of the physiological effects of this regulation has been clearly established and requires further investigation.

2 The acetyltransferase GCN5

2.1 Acetylation: a post-translational modification

Cellular functions are regulated by a signaling network which allows the cell to adapt and respond to internal and external cues. These networks are regulated by reversible protein post-translational modifications (PTMs) such as phosphorylation, acetylation, ubiquitination among others. Although protein phosphorylation has been extensively described, protein acetylation studies were more complicated due to technical challenges. However, acetylation studies were propelled forwards during the last decade thanks to advances in proteomic analysis and a large number of acetylated proteins and acetylation sites have been identified (Choudhary, Weinert, Nishida, Verdin, & Mann, 2014).

Protein acetylation is conserved from bacteria to humans and is involved in diverse cellular processes such as chromatin remodeling, cell cycle progression, cellular metabolism, splicing, nuclear transport and actin nucleation (Choudhary et al., 2009) as well as cell migration, aging, and inflammation (Waluk, Sucharski, Sipos, Silberring, & Hunt, 2012). It typically occurs on lysine residues and the reaction relies on acetyl-coenzyme A (Acetyl CoA) as the acetyl group (CH₃ CO) donor. Histone proteins are acetylated by histone acetyltransferases (HATs) on lysine residues in the N-terminal tail as part of gene regulation. This reaction is reversible and the deacetylation is catalyzed by histone deacetylases (HDACs) (**Figure 31**). While histone acetylation is associated with transcriptional activation, histone deacetylation is associated with transcriptional repression. Although the first studies on acetylation were mainly focused on the acetylation of histones, it was later discovered that non-histone proteins, such as p53 and tubulin, can also be acetylated and deacetylated. Lysine acetylation can favor or interfere with protein-protein interactions and can also affect protein stability, activity and localization (Sadoul, Boyault, Pabion, & Khochbin, 2008)(Choudhary et al., 2014).

2.1.1 Acetylation regulates protein stability

The stability of Smad7 is regulated by the balance between acetylation and deacetylation. While the acetylation of Smad7 by the acetyltransferase p300 protects it from TGFbeta-

induced degradation, the HDAC1-mediated deacetylation of Smad7 decreases its stability by enhancing its ubiquitination (Simonsson, Heldin, Ericsson, & Grönroos, 2005). Many other studies have linked protein acetylation to protein stability in the measure where acetylation prevents the protein from being ubiquitinated and undergoing proteasomal degradation. It is the case for p53 (Li, Luo, Brooks, & Gu, 2002) and HIF1 α (Geng et al., 2012). Protein acetylation was also shown to promote lysosome-dependant degradation. For instance, acetylation of PKM2 at K305 is stimulated by high glucose concentrations, which increases PKM2 interaction with HSC70, and its uptake by lysosomes (Lv et al., 2011).

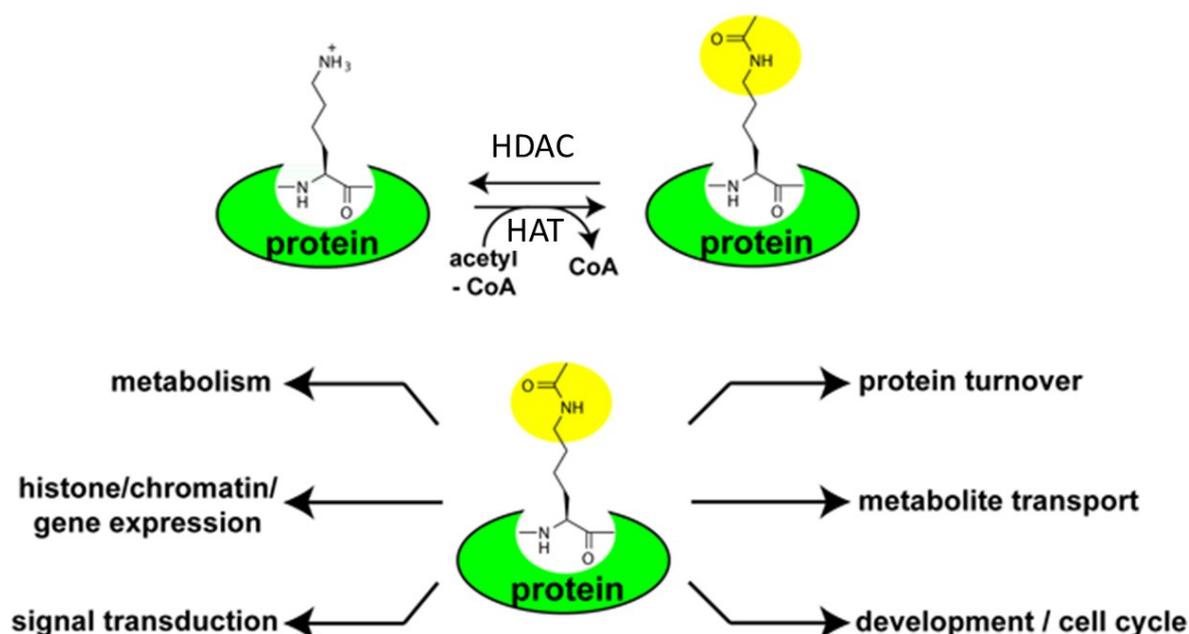


Figure 31: Lysine acetylation is involved in diverse cellular processes

Lysine acetylation is a reversible post-translational modification of proteins catalysed by lysine acetyltransferases (KATs) and deacetylases (KDACs). Protein acetylation is involved in the regulation of diverse cellular processes in different organisms (modified from Finkemeier and Schwarzer, Biospektrum, 2013).

2.1.2 Acetylation regulates protein localization

Acetylation can also have an impact on the subcellular localization of proteins. While some proteins will translocate into the cytoplasm upon acetylation, others will translocate or be retained in the nucleus. The regulation of cellular localization can occur either by the alteration of an interaction with a binding partner leading to localization in a particular compartment (e.g., p53-Mdm2 interaction), or by interactions with nuclear import/export factors. For example, acetylation of the adenovirus-transforming protein (E1A) within its C-terminal nuclear localization signal (NLS) disrupts its interaction with importin-alpha, thereby enhancing its localization in the cytoplasm (Madison, Yaciuk, Kwok, & Lundblad, 2002). Conversely, acetylation of hepatocyte nuclear factor-4 (HNF-4) within its NLS retains it in the nucleus, thus preventing its export to the cytoplasm via the CRM-1 pathway (Soutoglou, Katrakili, & Talianidis, 2000). Recently, proteomic analysis of lysine acetylation sites in different rat tissues revealed an interesting subcellular pattern. The majority of lysine-acetylated proteins were localized in the cytoplasm (30%) and the nucleus (30%). The remaining acetylated proteins were found in mitochondria (15%), plasma membrane (15%), endoplasmic reticulum or Golgi apparatus (5%), and extracellular region (5%). However, it is important to note that the subcellular distribution of lysine-acetylated proteins is tissue-dependent (Lundby et al., 2012) and is probably modified upon stress-induction.

2.1.3 Acetylation regulates protein activity

Recent advances in mass spectrometry technologies suggest that acetylation is one of the most abundant chemical modifications in nature and appears to modulate the activity of proteins in diverse biological processes. For example, acetylation of PGAM1 (Mammalian phosphoglycerate mutase 1) stimulates its enzymatic activity by 30–40%. Following glucose deprivation, this activity is decreased, presumably by the increase of SIRT1 deacetylase, which can deacetylate PGAM1 *in vitro* (Hallows, Yu, & Denu, 2012). Another example is that the autoacetylation of lysine residues within the activation loop motif of p300 enhances its acetyltransferase activity, whereas its deacetylation by SIRT2 decreases its activity (Choudhary et al., 2014).

2.2 Histone acetyltransferases

Histone acetyltransferases (HATs) are catalytic subunits of large HAT complexes that acetylate the ϵ -amino group of lysine residues of N-terminal histone tails, thus promoting transcriptional activation. HATs can also acetylate non-histone proteins. They are involved in cellular processes such as cell cycle progression, chromosome dynamics, DNA repair and apoptosis (Roth, Denu, & Allis, 2001).

The first HATs were isolated and cloned from the yeast, *Saccharomyces cerevisiae*, and were called GCN5 and Hat1. Interestingly, comparing GCN5 from yeast and protozoans provided the first evidence linking histone acetylation to transcriptional regulation (Brownell et al., 1996). Two years later, the first HAT/GCN5-containing complexes called ADA and SAGA were identified (Grant et al., 1997).

Over the years, the advances in molecular and biochemical analysis led to the isolation of other HATs from various organisms and were shown to be evolutionarily conserved from yeast to human. They were then categorized in groups regarding their catalytic domains. Thereby, we distinguish 3 HAT family groups called GNAT, MYST and the “orphan group” presented in **Table 2**. According to their subcellular localization, HATs belong to two separate classes. Type A HATs are nuclear, contain a bromodomain and regulate gene expression through acetylation of lysine residues on nucleosomal histones. Type B HATs are cytoplasmic, lack the bromodomain and recognize newly synthesized core histones which are not acetylated. However, due to different subcellular localization and function according to their association with different partners in different complexes, some HATs cannot fit in any class (Roth et al., 2001; Sterner & Berger, 2000).

2.2.1 The GNAT family

GNAT stands for Gcn5-related *N*-acetyltransferases since GCN5 is the founding member of this group. The other members are PCAF, Elp3, Hat1, Hpa2, ATF-2 and Nut-1. GCN5 is a type A HAT while Hat1 is a type B HAT.

Family	Organism	Associated complexes	Substrate specificity	Structural features
GNAT				
Gcn5	<i>S. cerevisiae</i>	SAGA, SLIK (SALSA), ADA, HAT-A2	H2B, H3, (H4)	Bromodomain
GCN5	<i>D. melanogaster</i>	SAGA, ATAC	H3, H4	Bromodomain
GCN5	<i>H. sapiens</i>	STAGA, TFTC	H3, (H4, H2B)	Bromodomain
PCAF	<i>H. sapiens</i>	PCAF	H3, H4	Bromodomain
Hat1	<i>S. cerevisiae - H. sapiens</i>	HAT-B, NuB4, HAT-A3	H4, (H2A)	
Elp3	<i>S. cerevisiae</i>	Elongator	H3, H4, (H2A, H2B)	
Hpa2	<i>S. cerevisiae</i>	HAT-B	H3, H4	
Hpa3	<i>S. cerevisiae</i>		H3, H4	
ATF-2	<i>S. cerevisiae - H. sapiens</i>		H2B, H4	
Nut1	<i>S. cerevisiae</i>	Mediator	H3, H4	
MYST				
Esa1	<i>S. cerevisiae</i>	NuA4, piccolo NuA4	H2A, H4, (H2B, H3)	Chromodomain
Sas2	<i>S. cerevisiae</i>	SAS, NuA4	H4, (H2A, H3)	
Sas3 (Ybf2)	<i>S. cerevisiae</i>	NuA3	H3, (H4, H2A)	
Tip80	<i>H. sapiens</i>	Tip80, NuA4	H2A, H4, (H3)	Chromodomain
MOF	<i>D. melanogaster</i>	MSL	H4, (H2A, H3)	Chromodomain
MOZ	<i>H. sapiens</i>	MSL	H3, H4	
MORF	<i>H. sapiens</i>	MSL	H3, H4	
HBO1	<i>H. sapiens</i>	ORC	H3, H4	
p300/CBP				
p300	<i>H. sapiens</i>		H2A, H2B, H3, H4	Bromodomain
CBP	<i>H. sapiens</i>		H2A, H2B, H3, H4	Bromodomain
SRC (nuclear receptor coactivators)				
SRC-1	<i>H. sapiens</i>	ACTR/SRC-1	H3, H4	
ACTR (RAC3, AIB1, TRAM-1)	<i>H. sapiens</i>	ACTR/SRC-1	H3, H4	
TIF-2 (GRIP1)	<i>H. sapiens</i>		H3, H4	
SRC-3	<i>H. sapiens</i>			
Other				
TAF _{II} 250 (TAF1)	<i>S. cerevisiae - H. sapiens</i>	TFIID	H3, H4, (H2A)	Bromodomain
TFIIIC (p220, p110, p90)	<i>H. sapiens</i>	TFIIIC	H2A, H3, H4	
Rtt109	<i>S. cerevisiae</i>	Histone chaperones	H3	
CLOCK	<i>H. sapiens</i>		H3, H4	

Orphan family

Table 2: HAT family groups and their histone substrates

The HAT family members are categorized in three groups called GNAT, MYST and the “orphan” group. Most of these enzymes are often included in multiprotein complexes. Each HAT targets specific histones.

Although the sequence identity between the members of this family does not exceed 23%, their basic fold structure is remarkably conserved. The most highly conserved sequence is the motif A, found within the HAT domain, and contains an Arg/Gln-X-X-Gly-X-Gly/Ala sequence which recognizes and binds to Acetyl CoA (Roth et al., 2001).

2.2.2 The MYST family

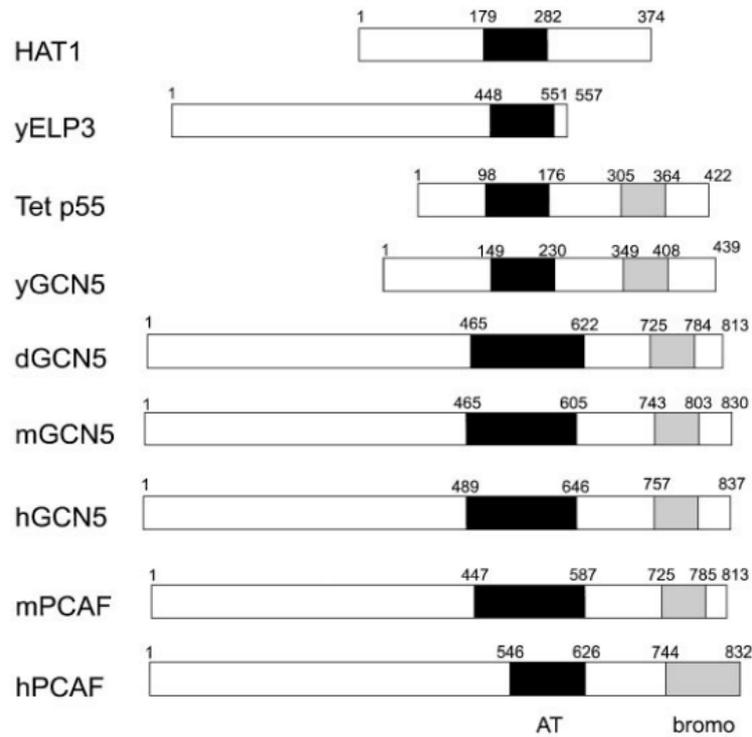
MYST stands for the founding members of this family: Morf, Ybf2, Sas2 and Tip60. The other members are Esa1, MOF, MORF and HBO1. The MYST family proteins contain the highly conserved motif A found in the GNATs required for acetyl CoA binding, but they also contain zinc fingers as well as chromodomains (Roth et al., 2001). The region containing motif A and a zinc finger form the highly conserved MYST domain (Avvakumov & Côté, 2007). Recently, the circadian rhythm protein CLOCK was found to share homology with acetyl-coenzyme A binding motifs within the MYST family of HATs (Doi, Hirayama, & Sassone-Corsi, 2006).

MYST family members are involved in cellular processes such as gene transcription, DNA repair and DNA replication. Alterations of the MYST family proteins activity have already been linked to a number of human diseases including cancer (Avvakumov & Côté, 2007).

2.2.3 The “orphan” family

Among HATs, the GNAT and MYST are the predominant families (**Figure 32**). Another group of HATs has been shown to possess intrinsic HAT activity but do not contain true consensus HAT domains. This group was thereby classified as the “orphan” family. It includes p300/CBP (CRB-binding protein), nuclear receptor coactivators (K. K. Lee & Workman, 2007) as well as other proteins such as CLOCK, the circadian rhythm protein that was recently found to share homology with acetyl-coenzyme A binding motifs within the MYST family of HATs (Doi et al., 2006). We will mainly focus on p300/CBP, two of the most widely studied HATs in transcriptional regulation.

A. GNAT family.



B. MYST family.

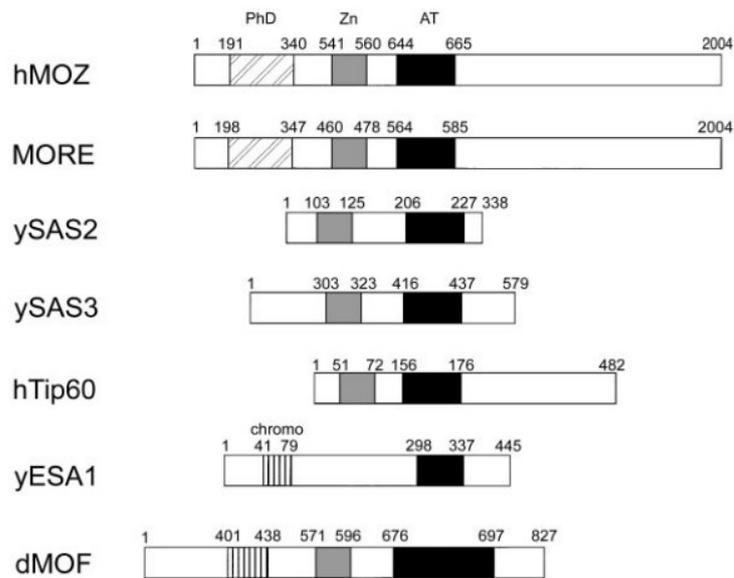


Figure 32: Structure of the GNAT and MYST HAT families

The relative sizes and locations of conserved motifs for the GNAT and MYST families of HATs are indicated. AT, acetyltransferase domain; bromo, bromodomains; PhD, plant homeo domains; Zn, zinc finger domains; chromo, chromodomains (Roth et al., 2001).

p300 and CBP are highly related and present interchangeable functions in cultured cells. So unless wanting to pinpoint distinct characteristics of one or the other, they are often referred to as p300/CBP. They contain zinc finger domains, a bromodomain, an HAT domain and at least two independent regions that interact with transcription factors and they serve as transcriptional coactivators. They share similarities with the GNAT family such as the motif A and the bromodomain and interact with the GNAT family member PCAF. Finally, p300/CBP regulate cellular processes such as cell growth, transformation and development.

All these HATs share a common core in which motif A is responsible for the recognition and binding of Acetyl CoA, the substrate that is transferred by HATs onto specific lysine residues (Roth et al., 2001) (**Figure 33**). The N- and C-terminal segments assist in binding histone substrates (Marmorstein, 2001). HATs are mainly considered as transcriptional activators. Indeed, histone acetylation is considered as an activation marker allowing chromatin decompaction which generates binding sites for transcription factors and coactivators. However, it is important to note that recently, acetylation was also associated with protein degradation (Hwang, Shemorry, & Varshavsky, 2010).

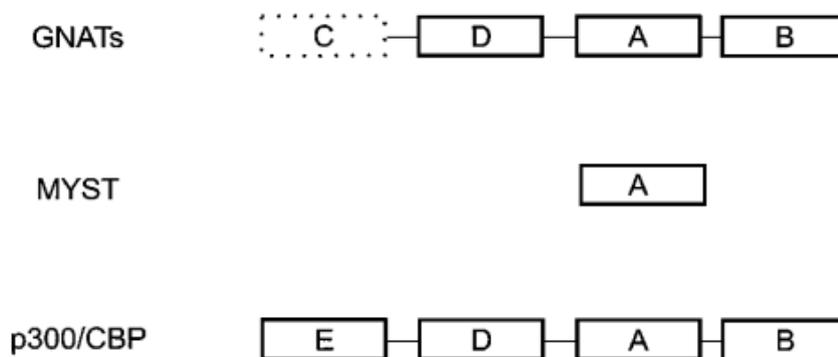


Figure 33: HAT domain motifs

The relative positions of conserved sequence motifs in the three HAT families GNAT, MYST, and p300/CBP are indicated. Motif A contains the highly conserved acetyl-CoA binding site (Roth et al., 2001).

2.3 GCN5 from the gene to the protein

2.3.1 Identification of GCN5 and its conservation across evolution

GCN5 was the first transcription-related histone acetyltransferase to be described. Using an activity gel assay in 1995, Brownell and Allis have identified a 55 kDa polypeptide bearing a histone acetyltransferase activity that they called p55. This histone acetyltransferase (HAT) was isolated from *Tetrahymena thermophila*, a ciliated protozoan, and was shown to have a high acetyltransferase activity on histone H3 (**Figure 34**) (Brownell & Allis, 1995).

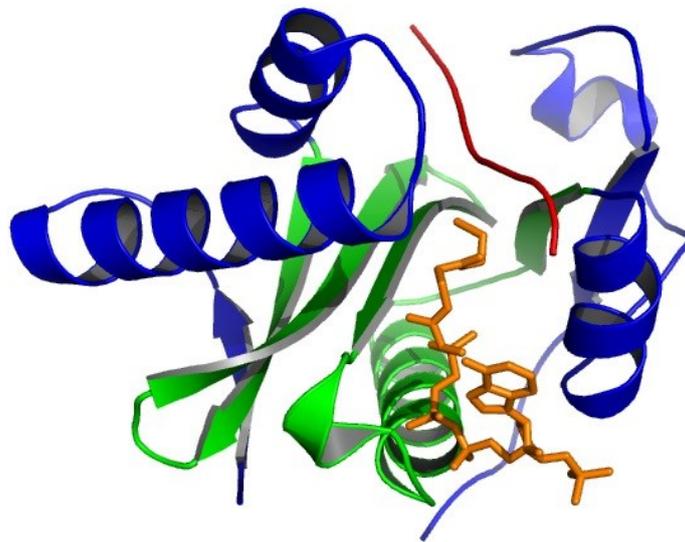


Figure 34: Crystal structure of *Tetrahymena* Gcn5 with bound coenzyme A and histone H3 peptide.

The crystal structure of the histone acetyltransferase Gcn5 from *Tetrahymena* with bound coenzyme A and an 11-residue histone H3 peptide (PDB 1QSN) is shown. The central core is shown in green, the flanking N- and C-terminal segments are shown in blue, coenzyme A is shown in orange, and the histone peptide is shown in red. This image was generated using PyMOL.

After cloning and sequencing p55, they found sequence similarities with Gcn5p (General control non repressed), a yeast homolog previously described as a transcriptional regulator by Georgakopoulos and his coworkers in 1992 (Brownell et al., 1996; Georgakopoulos & Thireos, 1992). As expected, they found that recombinant Gcn5p also bears a HAT activity, thus providing the first mechanistic evidence linking histone acetylation to transcriptional regulation (Brownell et al., 1996). Further in this manuscript, I will refer to the yeast Gcn5p as yGCN5.

GCN5 is conserved across the evolution. Indeed, in addition to protozoans and yeast, database analyses also led to the identification of GCN5 homologs in other species such as drosophila (E. R. Smith et al., 1998), mouse (W Xu, Edmondson, & Roth, 1998) and human (Candau et al., 1996).

These homologs present structural as well as functional similarities (Atanassov et al., 2009). However, an extra N-terminal region is found in metazoans in contrast to protozoans and yeast forms of GCN5 (**Figure 35**).

2.3.2 Structure of GCN5

In metazoans, GCN5 is composed of three conserved domains: a bromodomain in the C-terminal region, an acetyltransferase (AT) central domain and a so-called PCAF homology domain (PCAF-HD) in the N-terminal region, which is lacking in yeast and protozoans.

2.3.2.1 The AT domain

This domain bears the catalytic activity of GCN5 allowing it to transfer acetyl groups onto specific lysine residues. Although the central core region of the AT domain was shown to mediate Acetyl CoA binding and catalysis, the C- and N- terminal sides of this domain seem to be responsible of the histone substrate specificity (Marmorstein, 2001). We will discuss GCN5 substrates and acetylated lysine residues further in this manuscript.

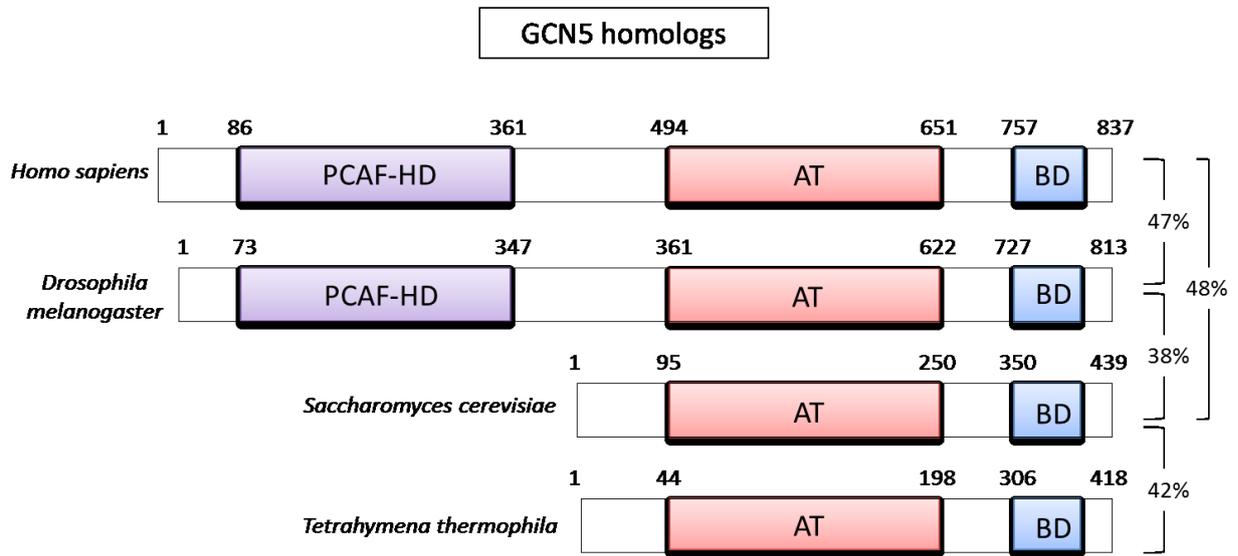


Figure 35: Schematic representation of the structure of GCN5 homologs

A schematic representation of the GCN5 homologs is shown, along with the percentage of identical amino acid residues shared between members. All family members share regions of significant similarity, including the region required for full catalytic activity of γ Gcn5p and the bromodomain. The metazoan members of the family share an N-terminal domain not found in the yeast or *Tetrahymena* proteins (PCAF homology domain, PCAF-HD). Adapted from (E. R. Smith et al., 1998) and (Z Nagy & Tora, 2007).

2.3.2.2 The Bromodomain

Bromodomains are mainly found in nuclear HATs but not in cytoplasmic HATs. They recognize specific lysine residues on histone tails thus promoting transcription-related acetylation of these histones (Z Nagy & Tora, 2007). For instance, yGCN5 bromodomain binds to histone H4 tail lysine 16 (H4K16) (D. J. Owen et al., 2000). Interestingly, bromodomains sometimes bind to acetylated residues, probably in order to protect them from deacetylation and/or to maintain their interaction with the HAT, the recruitment of adaptor proteins and acetylation of other nearby residues (Hudson, Martinez-Yamout, Dyson, & Wright, 2000).

However, the importance of the bromodomain for GCN5 functions seems to be controversial and species-dependent. For instance, it has been shown that the interaction of hGCN5 bromodomain with the DNA-PK holoenzyme results in the phosphorylation of GCN5 *in vitro* and *in vivo* thus inhibiting its HAT activity (Barlev et al., 1998). In contrast, *in vivo* analysis of *Drosophila* dGCN5 mutant proteins has shown that while the PCAF-HD and the AT domains are essential for dGCN5 functions, the Bromodomain is dispensable, suggesting that it is not the only region that is responsible for GCN5 anchoring to its substrates (Carré, Szymczak, Pidoux, & Antoniewski, 2005).

2.3.2.3 The PCAF-HD

This domain is only found in metazoans in the N-terminal region of GCN5 and allows it to bind to the transcriptional coactivators p300 and CBP (PCAF stand for p300/CBP Associated Factor) (Wanting Xu, Edmondson, & Roth, 1998). The absence of the PCAF-HD in protozoans, yeast and even plants suggests that this domain was present in the ancestral metazoan GCN5 and is unique to animals (E. R. Smith et al., 1998).

2.3.3 GCN5 isoforms and homolog

2.3.3.1 2 isoforms of GCN5 in metazoans

The cloning and characterization of GCN5 in *Drosophila* led to the identification of two polypeptides of 55 KDa and 98 KDa, which were also found in human. The 55 KDa isoform was called GCN5-S and corresponds to the short isoform of GCN5 found in yeast. The longer isoform has an additional N-terminal region that we previously presented as the PCAF-HD (Martinez, Kundu, Fu, & Roeder, 1998; E. R. Smith et al., 1998) (**Figure 36**).

Those isoforms result from an alternative splicing leading to a second translational initiation site. It is all about a 191 base element present in the intron 6 of human as well as mouse *GCN5* genes (W Xu et al., 1998). Indeed, in human cells, the 840 base intron 6 has two 5' splice sites located at nt 1 and nt 192. If nt 1 is used as the 5' splice site, the whole intron is removed to generate a fully spliced product corresponding to the long isoform of GCN5, hGCN5-L. If nt 192 is used as the 5' splice site, only the last 649 bases of the intron are removed. This will lead to premature termination of translation of the hGCN5-L protein due to a stop codon that is present within the 191 base element, and the translation of the short isoform hGCN5-S will start at a new initiation codon present in exon 7 (E. R. Smith et al., 1998) (**Figure 37**).

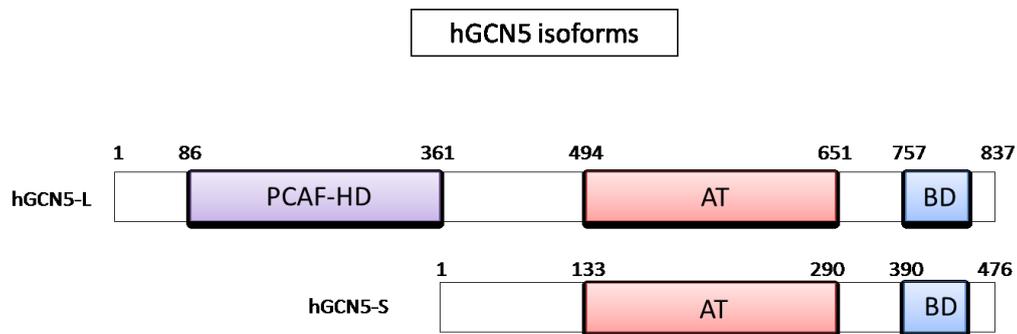


Figure 36: Schematic representation of hGCN5 isoforms

The *hGCN5* gene can encode a long (hGCN5-L) and short (hGCN5-S) protein. Amino acid one of hGCN5-S corresponds to amino acid 362 of hGCN5-L. Adapted from (E. R. Smith et al., 1998).

However, it is important to note that in mouse cell extracts, only the long isoform of GCN5 was observed. This and the observation that the long isoform is predominant in HeLa cell extracts suggest a differential regulation of GCN5 isoforms and functions in different species and/or developmental stages (W Xu et al., 1998).

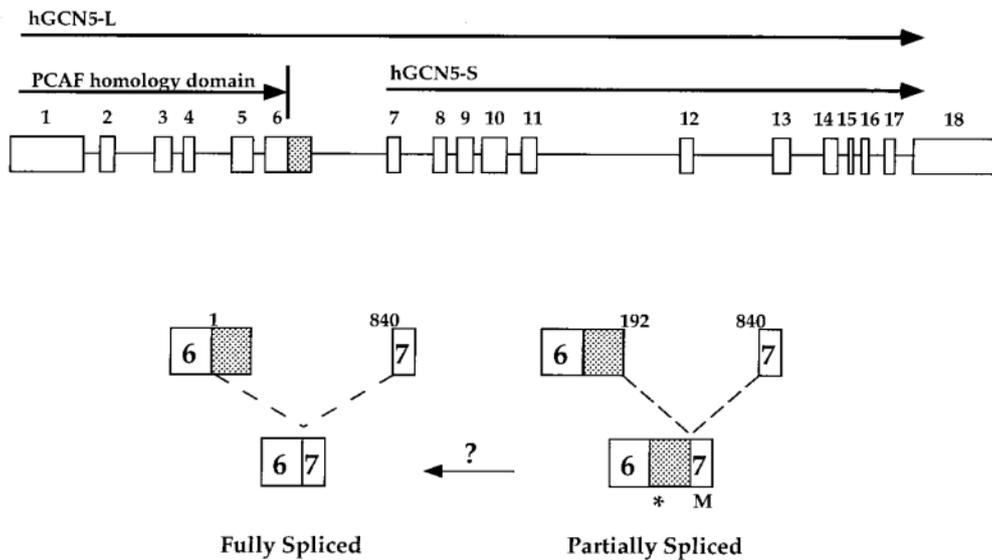


Figure 37: The hGCN5 mRNA is alternatively spliced

(A) Schematic representation of the intron–exon boundaries of the hGCN5 gene. Numbered boxes represent exons. Arrows show which exons encode hGCN5-L, hGCN5-S and the PCAF homology domain. The shaded box adjacent to exon 6 represents a 191 base portion of intron 6 that is not removed in some cDNA clones. (B) A schematic representation of intron 6 alternative splicing. Two 5' splice sites are located at nt 1 and nt 192 of the 840 base intron. If the 5' splice site at nt 1 is used, the whole 840 base intron is removed to generate a fully spliced product. If the 5' splice site at nt 192 is used, only the last 649 bases of the intron are removed, to make a partially spliced product. The 191 base segment from intron 6 that remains in the partially spliced message may serve as an alternative exon for hGCN5 translational control. An asterisk marks a stop codon within this 191 base element that would cause premature termination of translation of the hGCN5-L protein, while the M below exon 7 represents a proposed start site of translation for hGCN5-S. An interesting feature of the 191 base segment is that it begins with a 5' splice site and ends with a potential 3' splice site, allowing for the possibility that intron 6 is removed in two steps (arrow with question mark) (E. R. Smith et al., 1998).

Finally, in contrast to yGCN5 and hGCN5-S that only acetylate free core histones, interesting observations have shown that hGCN5-L and mGCN5 can also acetylate nucleosomal substrates. This shows that the additional N-terminal region provides GCN5 with additional features such as chromatin components recognition and gene regulation (Gamper, Kim, & Roeder, 2009; W Xu et al., 1998).

Further in the manuscript, I will focus on the longer isoform of GCN5 (GCN5-L).

2.3.3.2 *The GCN5 homolog: PCAF*

Since the identification and characterization of GCN5 in *Tetrahymena thermophila* and the yeast *Saccharomyces cerevisiae*, homologs of GCN5 were cloned in other species based on sequence similarities from cDNA databases. In 1996, a larger GCN5-related protein with unique sequences in its N-terminal region was identified. This protein was called PCAF (p300/CBP Associated Factor) since it interacts with the transcriptional coactivators and histone acetyltransferases p300 and CBP (CREB Binding Protein) (Wanting Xu et al., 1998; X. J. Yang, Ogryzko, Nishikawa, Howard, & Nakatani, 1996). PCAF was then identified in other metazoans (**Figure 38**).

2.3.3.2.1 GCN5 and PCAF different features

Despite their homology, GCN5 and PCAF are not only located on different chromosomes but are also structurally and functionally different.

Indeed, PCAF bears an intrinsic ubiquitination activity within the PCAF-HD in the N-terminal region of the protein, in addition to its acetyltransferase activity. In HeLa and U2OS cells, PCAF is involved in the ubiquitination of Hdm2(also called MDM2) leading to its degradation and the regulation of p53 stability and functions (Linares et al., 2007). Interestingly, PCAF has been shown to be ubiquitinated by Hdm2 resulting in the inhibition of PCAF's acetyltransferase activity towards p53 (Y. Jin, Zeng, Dai, Yang, & Lu, 2002; Y. Jin, Zeng, Lee, & Lu, 2004). Together these data suggest that a regulatory crosstalk exists between PCAF and Hdm2 and they also highlight functional connections between acetylation and ubiquitination (Linares et al., 2007).

A further difference between GCN5 and PCAF is their tissue specific expression. In mouse tissue for instance, both proteins are expressed in inverse ratios suggesting that they have

tissue-specific functions (Wanting Xu et al., 1998). Studying the expression profile of GCN5 and PCAF in various human tissues shows that both mRNAs are ubiquitously expressed but while PCAF is most abundant in the heart, GCN5 is highly expressed in the pancreas (X. J. Yang et al., 1996).

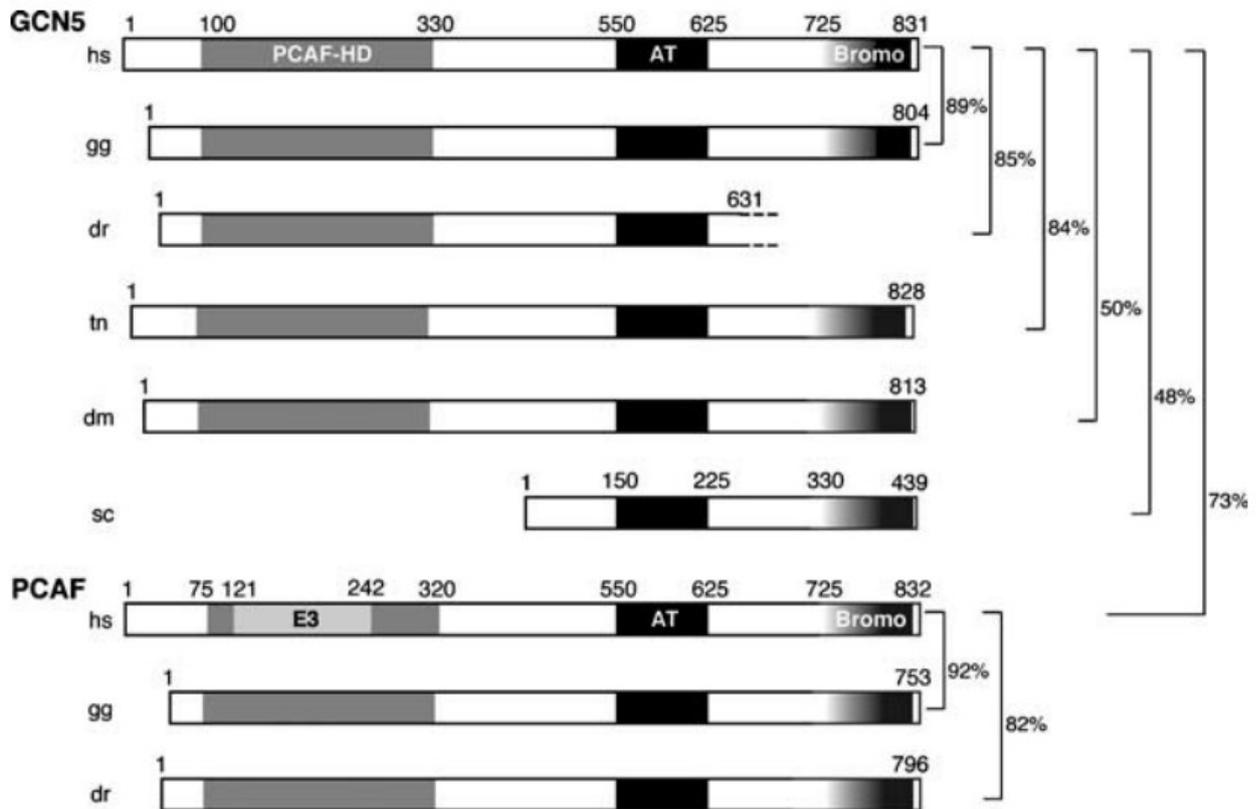


Figure 38: The overall structure of the GCN5 and PCAFenzymes in vertebrates, Drosophila and yeast.

Schematic representation and domain organization of the GCN5 and PCAF proteins from human (hs; Homo sapiens), chicken (gg; Gallus gallus), zebrafish (dr; Danio rerio), pufferfish (tn; Tetraodon nigroviridis), Drosophila melanogaster (dm) and yeast (sc; Saccharomyces cerevisiae) are shown. The PCAFhomology domain (PCAF-HD) is shown in grey, the AT domain is shown in black and the bromo domain (Bromo) is shaded. The recently described ubiquitin E3 ligase domain (E3) of PCAF is also indicated. The numbers over the boxes indicate amino-acid positions. The identity between the different factors is indicated in % on the right of the horizontal lines, representing the pair wise comparisons. AT, acetyl transferase (Z Nagy & Tora, 2007).

2.3.3.2.2 GCN5 and PCAF shared features

hPCAF is highly similar to hGCN5 (75% identity) (X. J. Yang et al., 1996) and both proteins share some structural as well as some functional features.

Indeed, like GCN5, PCAF is also composed of 3 domains: the bromodomain in the N-terminal region binds to lysine residues, the acetyltransferase (AT) central domain and the C-terminal region that bears a sequence of interaction with p300 and CBP (**Figure 38**).

The fact that both proteins bind to CBP and p300 suggest that in addition to their structural similarities, GCN5 and PCAF could share some functional similarities. Indeed, both proteins acetylate free histones as well as nucleosomes primarily on H3K14 and more weakly on H4K8 (Sterner & Berger, 2000). In mouse embryonic fibroblasts (MEFs), GCN5 and PCAF show a redundant function in the acetylation of H3K9. Double knock-out of these HATs eliminates histone H3 acetylation on K9 (Q. Jin et al., 2011). In addition, both proteins were found to acetylate non-histone proteins such as p53 *in vitro* and this acetylation is increased *in vivo* in response to DNA damage (Bu, Evrard, Lozano, & Dent, 2007). Finally, in human cells, GCN5 and PCAF are included in similar related multiprotein complexes that we will discuss further (Ogryzko et al., 1998).

Finally, in order to study the *in vivo* functions of those HATs in mammals, Bu and his coworkers have performed knock-downs of *GCN5* and *PCAF* in mouse embryos. Deletion of *GCN5* in mice led to embryonic death after gastrulation, whereas *PCAF* null mice are viable with no obvious abnormalities. These results show that GCN5 is essential for embryonic development in mice unlike PCAF. Interestingly, the double knock-out of GCN5 and PCAF together leads to much earlier death of the embryos than with *GCN5* knock-out alone, suggesting that PCAF does actually contribute to the early development of the mice at some level, but is not as essential as GCN5 (Bu et al., 2007).

2.4 GCN5-containing multiprotein complexes

As previously mentioned, GCN5, as well as PCAF, are components of multiprotein complexes that we will describe in this part. The subunits in these complexes are well conserved in the yeast *S. cerevisiae*, flies and mammalian cells and are presented in **Table 3**.

2.4.1 The SAGA complex

Yeast SAGA (Spt-Ada-GCN5-acetyltransferase) is the best characterized HAT containing complex among all. It is a large complex of 1.8-2 MDa containing 18 to 20 subunits that can be separated into five different groups of previously described transcription-related proteins (K. K. Lee et al., 2011; Weake & Workman, 2012) (**Figure 39**). The first module is composed by Gcn5, Ada2, Ada3 and Sgf29 and is called the HAT module which is involved in the acetylation of H3 on several lysine residues by GCN5. The second module is a deubiquitination module called DUB and is composed of the ubiquitin specific protease Ubp8 in association with Sgf11, Sgf73 and Sus1. This association activates Ubp8 leading to the deubiquitination of substrates such as histone H2B. As for the third module, it is composed of several TATA-binding protein (TBP)-associated factor (TAF) proteins that are shared with the general transcription factor TFIID (TAF 5, 6, 9, 10 and 12) and forms a structural core with Ada1. Finally, the fourth module of SAGA is composed of the transcription activator-binding protein Tra1, together with several Spt proteins such as Spt3 and Spt8. Thus the yeast SAGA complex is involved in histone acetylation as well as transcriptional activation (Weake & Workman, 2012). However, while yGCN5 is not essential for the SAGA complex integrity as shown by subunit deletion experiments (K. K. Lee et al., 2011), it is essential for SAGA's interaction with the chromatin, which is maintained by its own HAT activity.

Complex	2 MDa complexes					700 kDa complexes		
	<i>ySAGA</i>	<i>dSAGA</i> <i>TFTC</i>	<i>hTFTC</i>	<i>hSTAGA</i>	<i>hPCAF</i>	<i>ADA</i>	<i>ATAC</i>	<i>ATAC</i>
Organism	<i>Saccharomyces cerevisiae</i>	<i>Drosophila melanogaster</i>	<i>Homo sapiens</i>	<i>H. sapiens</i>	<i>H. sapiens</i>	<i>S. cerevisiae</i>	<i>D. melanogaster</i>	<i>H. sapiens</i>
HAT subunit	<i>yGcn5</i>	<i>dGcn5</i>	<i>hGcn5</i>	<i>hGcn5</i>	<i>hPCAF</i>	<i>yGcn5</i>	<i>dGcn5</i>	<i>hGcn5</i>
	<i>yAda1</i>	<i>dADA1</i>	<i>hADA1*</i>	<i>hSTAF42</i>	?	—	—	
	<i>yAda2</i>	<i>dADA2b</i>	<i>hADA2b*</i>	<i>hADA2b</i>	?	—	—	
	—	—	—	—	<i>hADA2a</i>	<i>yAda2</i>	<i>dADA2a</i>	<i>hADA2a</i>
	<i>yAda3</i>	<i>dADA3?</i>	<i>hADA3</i>	<i>STAF54</i>	<i>hADA3</i>	<i>yAda3</i>	<i>dADA3</i>	?
	<i>yAda5/Spt20</i>	?	?	?	?	—	—	
	<i>ySpt3</i>	<i>dSPT3</i>	<i>hSPT3</i>	<i>hSPT3</i>	<i>hSPT3</i>	—	—	
	<i>ySpt7</i>	?	<i>hSPT7L</i>	<i>STAF65g</i>	?	—	—	
	<i>ySpt8</i>	?	?	?	?	—	—	
			<i>hTAF2'</i>					
			<i>hTAF4'</i>					
	<i>yTAF5</i>	—	<i>hTAF5'</i>	—	—	—	—	
		<i>dTAF5L/</i>	<i>hTAF5L</i>	<i>hTAF5L</i>	<i>hTAF5L</i>	—	—	
		<i>WDA</i>						
	<i>yTAF6</i>	?	<i>hTAF6'</i>	—	—	—	—	
			<i>hTAF6L</i>	<i>hTAF6L</i>	<i>hTAF6L</i>	—	—	
	<i>yTAF9</i>	<i>dTAF9</i>	<i>hTAF9</i>	<i>hTAF9</i>	<i>hTAF9</i>	—	—	
			<i>hTAF9b</i>			—	—	
	<i>yTAF10</i>	<i>dTAF10</i>	<i>hTAF10</i>	<i>hTAF10</i>	<i>hTAF10</i>	—	—	
	<i>yTAF12</i>	?	<i>hTAF12</i>	<i>hTAF12</i>	<i>hTAF12</i>	—	—	
	<i>Tra1</i>	<i>dTra1/</i>	<i>hTRRAP</i>	<i>hTRRAP</i>	<i>hPAF400</i>	—	—	
		<i>dTRRAP</i>						
	<i>ySgf11</i>	?	?	<i>hSTAF46?</i>	?	—	—	
	<i>ySgf29</i>	?	<i>hSGF29*</i>	<i>STAF36</i>	?	—	—	
	<i>ySgf73</i>	?	<i>HATXN7</i>	<i>hATXN7</i>	?	—	—	
	<i>yUbp8</i>	?	?	<i>hSTAF60?</i>	?	—	—	
	<i>ySus1</i>	<i>dE(y)2</i>	?	?	?	—	—	
			<i>hSTAF55</i>					
	—	?	<i>hSAP130</i>	<i>hSAP130</i>	?			
	—	—	—	—	—	<i>yAhc1</i>	—	?
							<i>dATAC</i>	?
							<i>dHCF</i>	?

Table 3: Composition of the 2 MDa and 700 kDa GCN5- and PCAF-containing multiprotein complexes

The factors, described in the different complexes are represented on a horizontal line as homologues from different species. Different names on a horizontal line mean that these homologues are known under different names in different species. The novel TAF nomenclature has been used. '?' means that cDNAs encoding homologue factors to the yeast proteins in the given organism have been identified, but the presence of the factor in the corresponding complex has not yet been demonstrated. '—' means that in the given complex, the corresponding factor is absent. Factors shown with '#' have been recently identified in TFTC by mass spectrometry. Factors in TFTC shown with '*' copurify with TFTC, but seem to be present in a separate complex. STAFs in green were positioned as homologues of the yeast proteins, when cDNAs encoding putative human proteins with approximately the STAF size were found (Z Nagy & Tora, 2007).

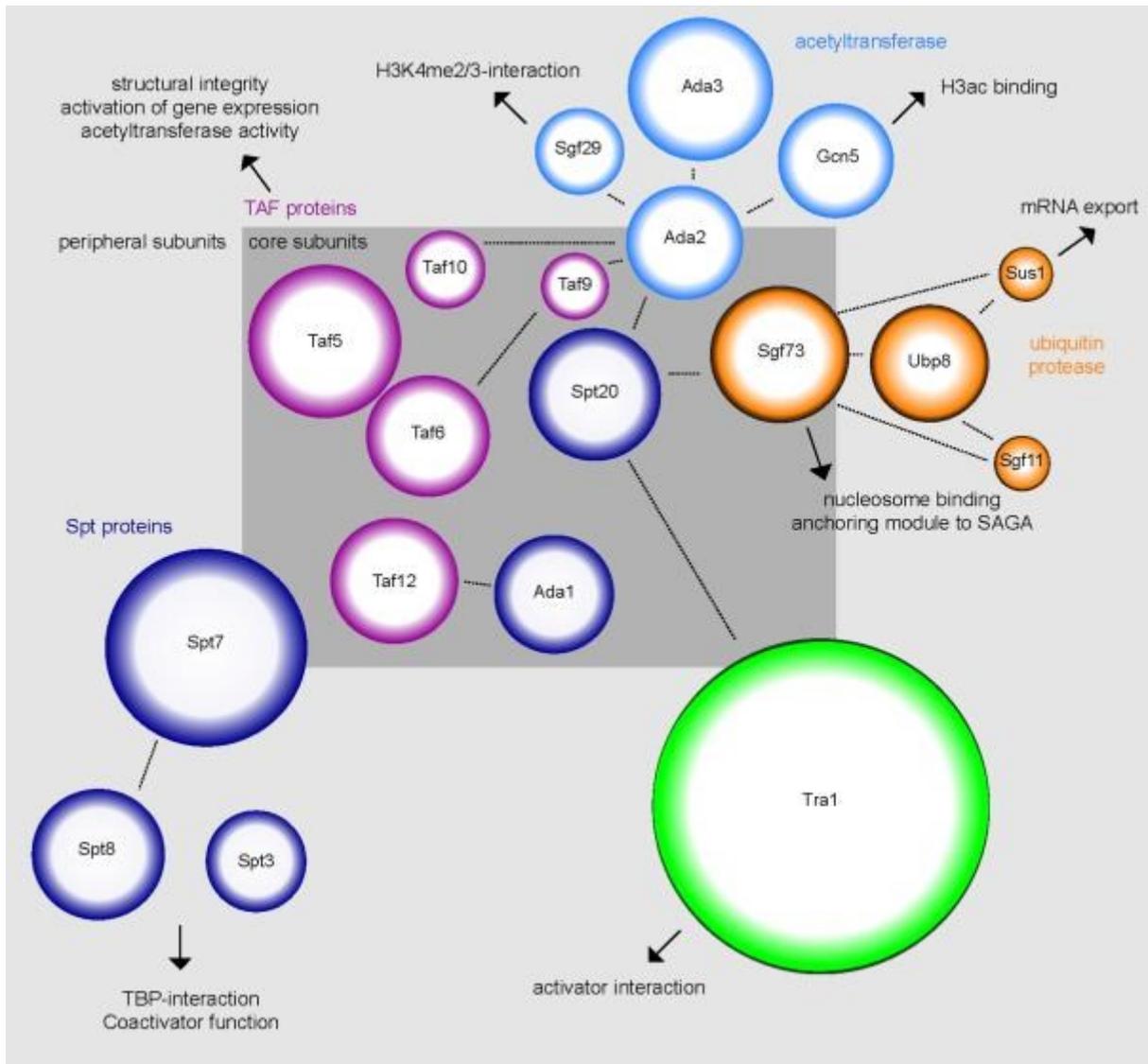


Figure 39: The modular nature of ySAGA supports multiple activities

Subunits within the different modules of *S. cerevisiae* SAGA are indicated by different colors: acetyltransferase (blue), ubiquitin protease (orange), TAF (pink), Spt (purple) and Tra1 (green). The relative molecular weight of each subunit is indicated by the area of the circle. Subunits that are more central to the complex are shown within the inner box, and peripheral subunits are shown inside the outer box. Different functions of individual subunits are indicated by the arrows and text. Probable physical connections between subunits are indicated by dotted lines (Weake & Workman, 2012).

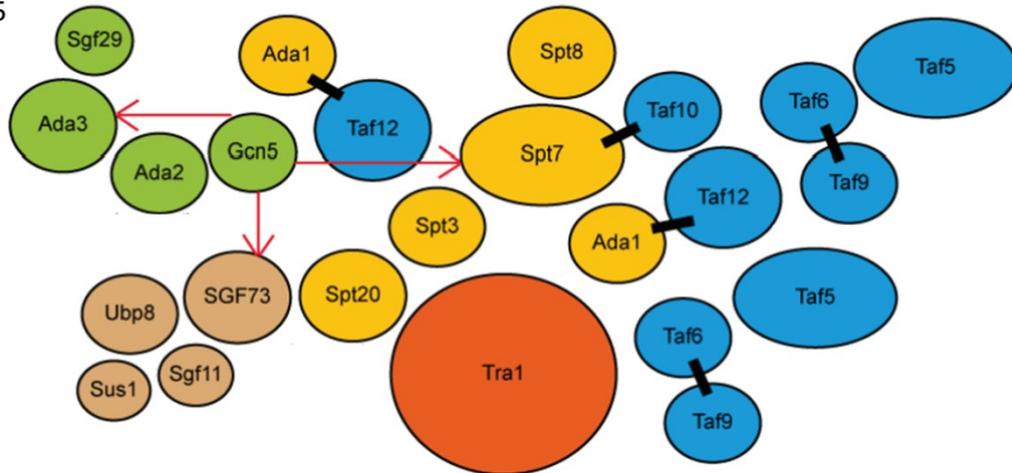
In *Drosophila melanogaster*, the dSAGA complex is homologous to the SAGA complex found in yeast and includes most of its components orthologs ADA, TAFs and SPT (Guelman et al., 2006; Kusch, Guelman, Abmayr, & Workman, 2003; Muratoglu et al., 2003; Weake et al., 2009). dGCN5 acetylates histone H3 *in vivo* and is required for *Drosophila* metamorphosis (Carré et al., 2005).

Two variants of Ada2 were identified in *Drosophila*: Ada2a and Ada2b. Only Ada2b is a component of the yeast SAGA-like complex in *Drosophila* (Carré et al., 2005). More recently, two isoforms of Ada2b differing in their C-terminal region were described, a long and a short isoforms called Ada2bL and Ada2bS respectively. The identification of those isoforms suggests more complex functional characteristics of the dSAGA complex (Pankotai et al., 2013).

hSAGA was the first GCN5-containing complex purified from human cells (Candau et al., 1996) and was found to be similar in its composition to the 2MDa ySAGA complex (Z Nagy & Tora, 2007). This complex contains a catalytic HAT activity beared by either GCN5 or PCAF as well as a deubiquitination module (DUB) that acts through hUSP22, the human homolog of yUbp8, a ubiquitin-specific protease component (Z Nagy & Tora, 2007) (**Figure 40**). As mentioned previously, the two ADA2 isoforms, Ada2a and Ada2b were also found in mice and humans. Like in dSAGA, only Ada2b is present in the hSAGA complex but no short nor long variants were described (Z Nagy & Tora, 2007).

A

yGCN5



B

hGCN5

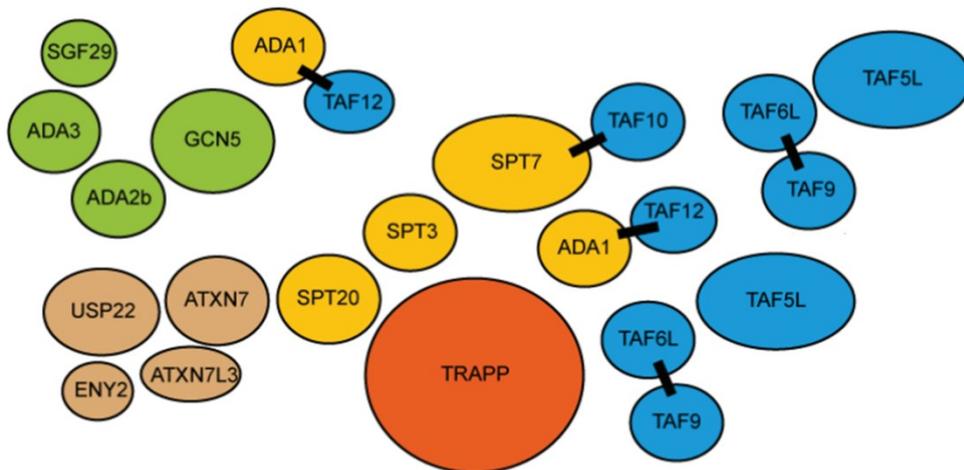


Figure 40: Schematic representation of yeast and human SAGA

The subunit association of human and yeast SAGA complexes is represented. The size of the circles is relatively proportionate to the size of the proteins. The modules are represented with different colors: the HAT module in green, the DUB module in brown, Tra1 in orange, the TAFs in blue, the Spts and Ada1 in yellow. The red arrows indicated the known targets of GCN5 within the complex. The black lines between the circles represent the dimers formed between the proteins. Adapted from (Riss, 2012).

Two other complexes called TFTC (TBP-free-TAF-containing) (Wieczorek, Brand, Jacq, & Tora, 1998), and STAGA (SPT3-TAF9-GCN5 acetyltransferase) (Martinez et al., 1998) were described. Surprisingly, they were demonstrated to share much more conserved subunits than originally thought, and are now referred to as hSAGA and preferentially acetylate free as well as nucleosomal histone H3 (Z Nagy & Tora, 2007) like the yeast and *Drosophila* SAGA complexes. While free GCN5 acetylates mainly H3K14 as previously stated, once incorporated into the hSAGA complex its specificity changes towards K9, K14, K18, K23 of histone H3 and to a lesser extent H2B and H4 (Brand, Leurent, Mallouh, Tora, & Schultz, 1999; Guelman et al., 2006). It is important to note that both Ada2b and ADA3 are required for efficient acetylation of nucleosomes by GCN5 but not for free core histones acetylation (Gamper et al., 2009) (**Figure 41**).

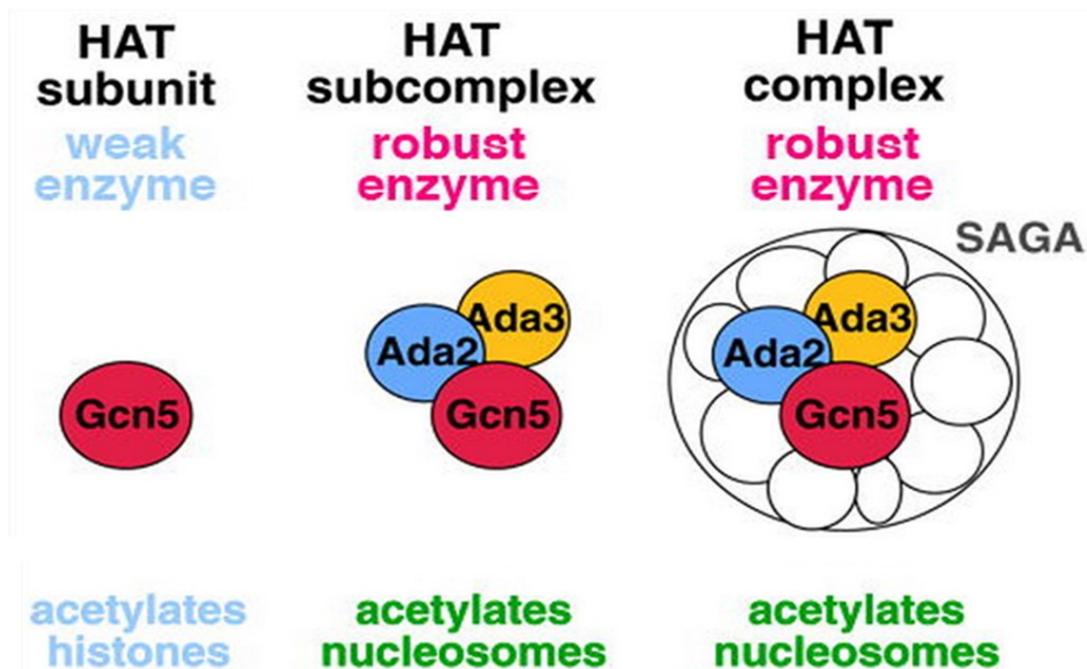


Figure 41: GCN5 acetylates nucleosomes when in complex with Ada2 and Ada3

Gcn5 is the catalytic HAT subunit of SAGA, but acetylates only histone tails and weakly, whereas the Ada2/Ada3/Gcn5 is the sufficient subcomplex with similar robust HAT activity and histone H3 and H2B specificity for nucleosomal histones as the full SAGA complex (Barrios et al., 2007).

To date, almost all of the ySAGA components were found to have homologs in *Drosophila* and human showing that this is a well-conserved GCN5-containing complex across the evolution. Three-dimensional structures of ySAGA and hSAGA were obtained and overlapped showing 5 main modules. Domain I contains Tra1. Domains II, III and IV contain several histone fold-containing TAFs and TAF5, which might play an architectural role in these complexes. In domain III the two bromodomain-containing subunits, Gcn5 and Spt7, were detected and the HAT activity can be beared by GCN5 or PCAF. Thus, domain III is a central architectural domain, but also harbours the HAT activity of the complex. Finally, domain V contains Spt3, Spt20 and probably also Spt8 and defines the TBP interaction module (Z Nagy & Tora, 2007) (**Figure 42**).

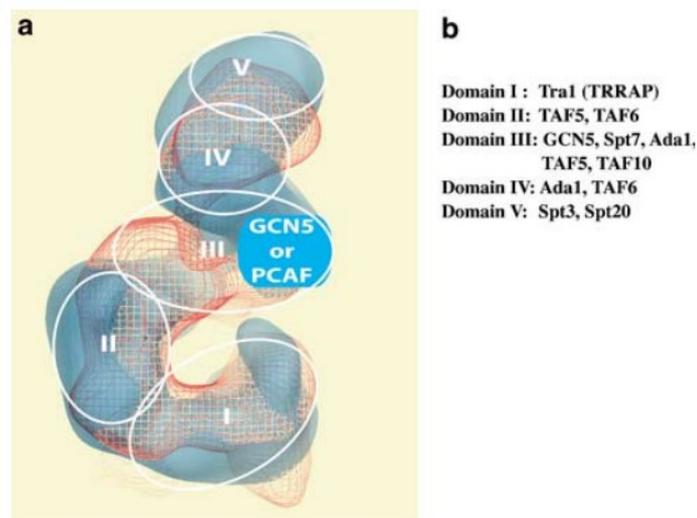


Figure 42: The overall three-dimensional structure of the yeast and human SAGA complexes are evolutionarily conserved.

(a) The low-resolution three-dimensional structure of the yeast and human SAGA complexes was elucidated via EM methods (Brand et al., 1999a; Wu et al., 2004). Image reconstruction yielded a three-dimensional model at B30 Å resolution, which revealed that both SAGA complexes have an evolutionarily well-conserved structure. Alignment and superposition of the SAGA model (blue) with that of human SAGA (red) is shown and the five modular domains of the complexes as defined by Wu et al. (2004) are indicated with white circles. The theoretical position of GCN5 (or PCAF) in the superposition is indicated. (b) The different subunits of SAGA, which were identified in the distinct domains (Wu et al., 2004), are summarized (Z Nagy & Tora, 2007).

2.4.2 The ADA complex

This complex was only described in yeast. Indeed, GCN5 is also a catalytic subunit in a smaller macromolecular complex of 0.8 MDa. This complex is called ADA and is composed of Gcn5 in association with Ada2 and Ada3 (Grant et al., 1997), and other ADA-specific subunits such as Ahc1, Ahc2 and Sgf29 (Eberharter et al., 1999) (K. K. Lee et al., 2011) (**Figure 43**).

SAGA and ADA present overlapping yet distinct acetylation profiles. While ADA acetylates H3K14 and H3K18, SAGA can also acetylate H3K9 and H3K23 (Grant et al., 1999).

2.4.3 The SLIK complex

In 2002, Pray-Grant and his coworkers have fractionated two yGCN5-containing complexes; the first one was the SAGA complex and the second was similar in size but showed some divergent features. This new complex was referred to as SLIK, for SAGA-LIKE (also called SALSA for SAGA altered, Spt8 absent) (Pray-Grant et al., 2002).

GCN5 is essential for SLIK's structure and functions. Indeed, deletion of *GCN5* disrupts SLIK integrity. In addition, deletion of *GCN5* also suppresses the expression of the retrograde response diagnostic gene *CIT2* and has an effect on genome stability (S. Kim, Ohkuni, Couplan, & Jazwinski, 2004).

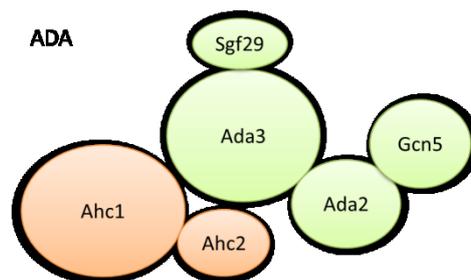


Figure 43: Schematic representation of the ADA complex

The ADA complex is composed of Gcn5 in association with Ada2, Ada3, Ahc1, and the two novel subunits called Ahc2 and Sgf29. Adapted from (K. K. Lee et al., 2011).

2.4.4 The ATAC complex

The observation that Ada2a and Ada2b were bound to GCN5 in fractions with two different molecular masses, one of 0.8 MDa and another of 2 MDa, suggested that those variants were part of distinct macromolecular GCN5-containing complexes (Muratoglu et al., 2003). Interestingly, it was shown that only Ada2b is a component of the *Drosophila* SAGA complex (Kusch et al., 2003) while Ada2a is a component of a novel distinct complex from SAGA called ATAC (Ada two A containing). This difference in the composition of the two complexes highly suggests unique functional characteristics of those GCN5-containing macromolecules.

Thus, the dATAC complex is composed by Gcn5, Ada2a and Ada3, together with ATAC-specific subunits presented in **Table 4**. Interestingly, dATAC2 also bears an acetyltransferase domain showing that dATAC contains a double HAT activity (Suganuma et al., 2008).

The dATAC HAT activity is thereby afforded by dGCN5 and dATAC2 which allows the acetylation of histones H3 and H4 (Suganuma et al., 2008).

A 700KDa ATAC complex was also described in human, following the identification of two *Drosophila Ada2* genes encoding ADA2a and ADA2b proteins (Muratoglu et al., 2003; Z Nagy & Tora, 2007). Like in *Drosophila*, hATAC also contains hATAC2, another HAT. However, hATAC2 doesn't seem to be efficiently acetylate histones (Zita Nagy et al., 2010).

In mammals, hATAC and hSAGA mainly acetylate histone H3. The interesting question concerned the biological relevance of having those two distinct GCN5-containing complexes. The answer mainly manifests in the *in vivo* functions of the two complexes. Indeed, in *Drosophila* salivary glands and human cells both complexes respond to different stimuli and thus regulate the transcription of distinct sets of target genes. ATAC is also involved in the regulation of global histone H3 Ser10 phosphorylation suggesting a cross-talk between histone acetylation and phosphorylation on histone H3 tails (Zita Nagy et al., 2010).

	ySAGA <i>Saccharomyces cerevisiae</i>	dSAGA <i>Drosophila melanogaster</i>	hSAGA <i>Homo sapiens</i>	dATAC <i>Drosophila melanogaster</i>	hATAC <i>Homo sapiens</i>
HAT	yGcn5	dGCN5	hGCN5/hPCAF	dGCN5	hGCN5/hPCAF
	yAda2	dADA2b	hADA2b	-	-
		-	-	dADA2a	hADA2a
	yADA3	dADA3	hADA3	dADA3	hADA3
	ySgf29	dSGF29	hSGF29	dSGF29	hSGF29
DUB	yUbp8	Nonstop	USP22	-	-
	ySgf73	CG9866	ATXN7	-	-
	ySgf11	dATXN7L3	hATXN7L3	-	-
	ySus1	dE(y)2	ENY2	-	-
Others	Tra1	dTra1	TRRAP		
	ySpt3	dSPT3	hSPT3	-	-
	ySpt8	-	-	-	-
	yTAF5	dTAF5L/WDA	hTAF5L	-	-
	yTAF6	SAF6	dTAF6L	-	-
	yTAF9	dTAF9	dTAF9	-	-
	yTAF10	dTAF10	dTAF10	-	-
	yTAF12	?	dTAF12	-	-
	yAda1	dADA1	hADA1	-	-
	ySpt7	?	hSPT7	-	-
	ySpt20	dSPT20	hSPT20	-	-
	-	-	-	dATAC1	hZZZ3
	-	-	-	dATAC2	hATAC2
	-	-	-	dATAC3	-
	-	-	-	HCF	-
	-	-	-	WDS	WDR5
	-	-	-	D12	YEATS2
-	-	-	CHRAC14	-	
-	-	-	NC2 β	NC2 β	
-	-	-	CG10238	MBIP	

Table 4: Composition of GCN5 and PCAF SAGA and ATAC complexes

ATAC and SAGA are two multiprotein complexes containing GCN5 or PCAF (red) in their HAT module (green). Both complexes share Sgf29 and Ada3 but can also possess specific subunits such as Ada2a or Ada2b.

?: presence of the subunit was not demonstrated

-: absence of the protein in the complex

Adapted from (Riss, 2012).

Thus, GCN5 can perform its HAT activity alone, in complex with ADA2 and ADA3 or in macromolecular complexes and its functions seem to be affected by its association with different partners in different complexes as well as by the *in vitro* or the *in vivo* context. But what exactly are these functions?

2.5 GCN5 functions

2.5.1 GCN5 acetylates histone proteins

In vitro, the primary site of acetylation by yeast and human GCN5 is H3K14 and to a lesser extent histone H4 (Marmorstein, 2001). However, other lysine residues can also be acetylated such as H3K9, H3K18, H3K23, and H3K27 (Kuo & Andrews, 2013). Interestingly, using a recombinant form of hGCN5, Kuo and his coworkers could quantify the specificity and selectivity of GCN5-mediated acetylation of histone H3 as follows: K14>K9≈K23>K18>K27≈K36 (Kuo & Andrews, 2013). In *Drosophila*, GCN5 is involved in the acetylation of larval polytene chromosomes at positions K9 and K14 of histone H3, and surprisingly also acetylates H4K5 and H4K12 (Z Nagy & Tora, 2007).

This specificity and selectivity in lysine acetylation is highly dependant on the incorporation of GCN5 in multiprotein complexes. For instance, when incorporated into the hSAGA complex, GCN5 preferentially acetylates H3K14 but will also target K9 and K18 (Zita Nagy et al., 2010). In the context of yeast ADA and SAGA complexes, histone H2B also becomes a substrate (Marmorstein, 2001). Interestingly, while the recombinant short-form hGCN5 only acetylates free histones, the full-length forms of human and mouse GCN5 can also acetylate nucleosomal histones, implicating the N-terminal region in chromatin substrate recognition (Sternner & Berger, 2000).

2.5.2 GCN5 acetylates non-histone proteins

GCN5 was initially described as a histone acetyltransferase triggering chromatin decompaction and transcriptional activation. Later on, the implication of GCN5 in the acetylation of non-histone proteins started emerging and several non-histone substrates were identified. We will only refer to some examples in this part of the bibliographic section and discuss the role of GCN5 in several cellular processes.

Chromatin remodeling is accomplished by the action of two general classes of multiprotein complexes: HATs, such as Gcn5 in the SAGA complex, acetylate nucleosomal histones whereas ATPases, such as Swi2 in the SWI/SNF complex, provide the energy for nucleosome remodeling. The bromodomain of Gcn5 allows the Swi2-dependent nucleosome remodeling

and consequent transcriptional activation by stabilizing the SWI/SNF complex on a promoter (Syntichaki, Topalidou, & Thireos, 2000). However, it was recently shown that GCN5 is also responsible for the detachment of the SWI/SNF complex from chromatin after remodeling nucleosomes at promoters. Indeed, GCN5 acetylates the Swi2 subunit of the SWI/SNF complex at two lysine residues thereby inhibiting the interaction between its bromodomain and acetylated histones (J.-H. Kim, Saraf, Florens, Washburn, & Workman, 2010) (**Figure 44**). Thus, Gcn5 can modulate the retention of the SWI/SNF complex on chromatin positively through histone acetylation but also negatively through acetylation of the Snf2 subunit.

GCN5 is implicated in cell cycle progression. Indeed, in mammalian cells, the ATAC complex localizes at the mitotic spindle and is tightly associated with the microtubule network which is implicated in chromosome segregation. The degradation of Cyclin A at the onset of mitosis is essential for proper mitotic progression. In the ATAC complex, GCN5 mediates Cyclin A acetylation, thereby triggering its degradation and regulating the CyclinA/Cdk2 kinase activity. Consequently, the SIRT2 deacetylase activity is upregulated (phosphorylation of SIRT2 on S331 by the CyclinA/Cdk2 complex inhibits its activity) leading to H4K16 and α -tubulin deacetylation. and proper cell division (Orpinell et al., 2010).

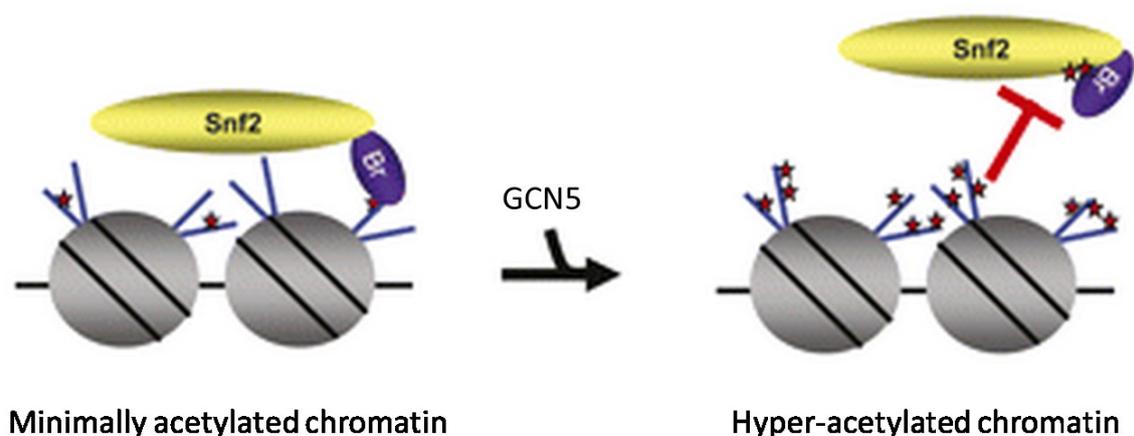


Figure 44: A model illustrating the role of Snf2 acetylation by GCN5 *in vivo*

The acetylation of Snf2 by GCN5 leads to the dissociation of its bromodomain from histones acetylated lysine residues. Red stars represent acetylated lysines (J.-H. Kim et al., 2010).

In late G1 and early S phase, GCN5 is found in a complex including Cyclin A/Cdk2 as well as CDC6 (cell-division cycle 6) which is implicated in cell cycle regulation. GCN5 acetylates CDC6 at three lysine residues (K92, K105 and K109) flanking its cyclin-docking motif thereby triggering its phosphorylation by Cdk2 on Ser106. This phosphorylation leads to CDC6 translocation into the cytoplasm and its degradation by the non-mitotic form of the anaphase-promoting complex cyclosome (APC/C) E3 ubiquitin-ligase after entering the G1 phase of the cell cycle (**Figure 45**). Thus, GCN5 is implicated in the CDC6-mediated S phase progression (Paolinelli, Mendoza-Maldonado, Cereseto, & Giacca, 2009).

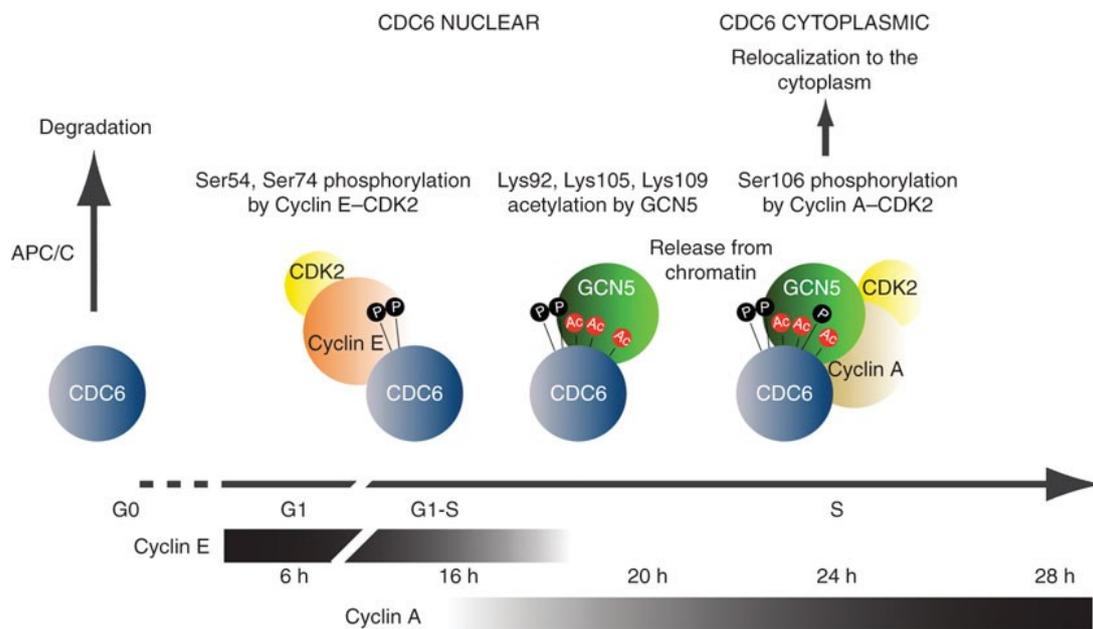


Figure 45: Model showing the regulation of CDC6 by sequential modification (acetylation and phosphorylation) in early S phase

In G0 cells, CDC6 is not phosphorylated and is continuously degraded by the APC/C. Upon entry into the cell cycle, phosphorylation of CDC6 on Ser54 by Cyclin E-CDKs opens a 'window of opportunity', during which degradation of the protein is prevented and assembly of the pre-RC is thus allowed. Upon S-phase entry, CDC6 is specifically acetylated by GCN5; this modification determines the release of the protein from chromatin and permits its further phosphorylation on Ser106, a modification that is carried out by Cyclin A-CDKs. CDC6, GCN5, Cyclin A and CDK2 indeed interact in early S-phase cells. Phosphorylation of CDC6 on Ser106 determines the relocalization of the protein to the cytoplasm, followed by its degradation (Paolinelli et al., 2009).

GCN5 is also implicated in the regulation of glucose metabolism. The regulation of hepatic gluconeogenesis mainly occurs through the regulation of the transcriptional coactivator PGC-1 α (peroxisome proliferator-activated receptor-gamma coactivator-1 alpha). Liver specific *PGC-1 α* knockout mice exhibit abnormal hepatic production of glucose and decreased blood glucose levels (Handschin et al., 2005). PGC-1 α was found in a multiprotein complex containing the acetyltransferase GCN5. The direct acetylation of PGC-1 α by GCN5 results in a transcriptionally inactive protein that relocalizes from promoter regions to nuclear foci. Ectopic expression of GCN5 represses the activation of gluconeogenic enzymes and the decrease of hepatic glucose production in cultured hepatocytes and in mouse liver (Lerin et al., 2006).

Interestingly, the same team has recently shown that this signaling pathway is controlled by the deacetylase SIRT6 (Sirtuin 6, a class III HDAC) which deacetylates GCN5 on K549 inducing its phosphorylation on Ser307 and Thr735. Consequently, GCN5 is activated and acetylates PGC-1 α leading to decreased gluconeogenesis (Dominy et al., 2012) (**Figure 46**). This observation is also interesting in the measure where it highlights a cross-talk between HATs and HDACs.

The c-myc oncoprotein is overexpressed in several human cancers and is implicated in transcriptional regulation of genes involved in cellular proliferation and apoptosis. mGCN5 acetylates c-myc on two major lysine residues mapped within the NLS (nuclear localization signal) and LZ (leucine zipper) motifs, thereby increasing its stability (J. H. Patel et al., 2004). Previous studies had also shown that the c-myc-dependent cellular transformation involves the association of c-myc with hGCN5 and that GCN5 is recruited by c-myc during transcription activation (X. Liu, Tesfai, Evrard, Dent, & Martinez, 2003). Thus GCN5 is also involved in cellular transformation.

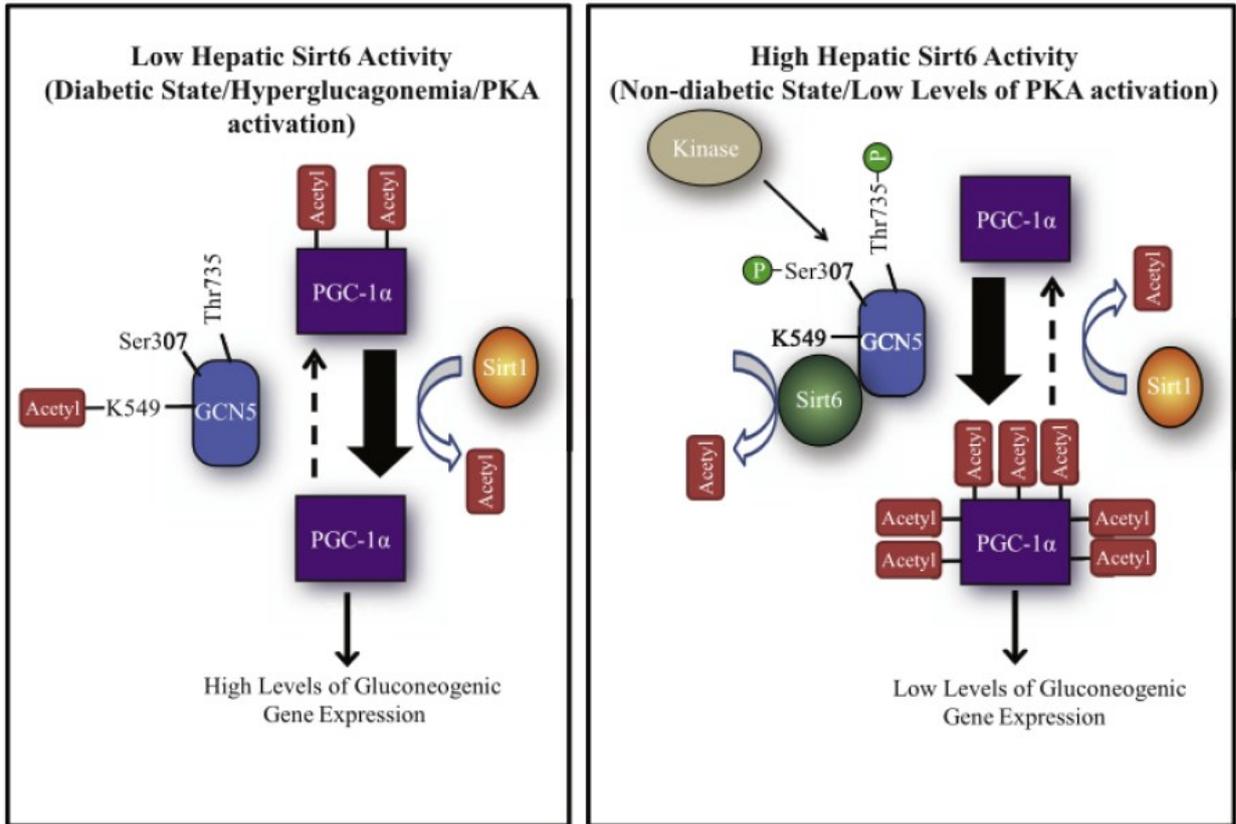


Figure 46: GCN5-mediated regulation of hepatic gluconeogenesis

A model for how Sirt6 is able to regulate the acetylation state of PGC-1 α and the gluconeogenic program of hepatocytes through GCN5 acetylation. Left: when hepatic Sirt6 activity is low, GCN5 is acetylated at K549 and residues Ser307 and Thr735 are unphosphorylated. In this state, GCN5 activity is low, PGC-1 α acetylation level is low, PGC-1 α activity is high, and gluconeogenic gene expression is activated. Right: when hepatic Sirt6 activity is high, GCN5 is deacetylated at K549 and residues Ser307 and Thr735 are phosphorylated. In this modified state, GCN5 activity is enhanced, resulting in high levels of PGC-1 α acetylation, reduced levels of PGC-1 α activity, and a decrease in gluconeogenic gene expression (Dominy et al., 2012).

2.5.3 Role of GCN5 in growth and development

As we mentioned previously, GCN5 is conserved from yeast to human. The first GCN5 mutants in yeast were shown to cause slow growth and temperature sensitivity (Marcus, Silverman, Berger, Horiuchi, & Guarente, 1994). What about the role of GCN5 in more complex organisms?

In *Drosophila*, GCN5 is essential for histone H3 acetylation on K9 and K14 and plays a key role in the control of morphogenesis during larva-to-adult metamorphosis. Indeed, Gcn5 mutants die at the end of the larval period thereby failing to undergo metamorphosis (Carré et al., 2005).

In mice, deletion of *GCN5* leads to early embryonic lethality with increased apoptosis in mesodermal lineages. Interestingly, *GCN5*^{-/-} *p53*^{-/-} embryos survive longer but still die in midgestation. The mouse embryos that survive even longer are homozygous for point mutations in the GCN5 catalytic domain (*GCN5*^{hat/hat}); they do not exhibit increased apoptosis but do exhibit severe cranial neural tube closure defects and exencephaly (**Figure 47**). Thus, GCN5 acetyltransferase activity is required for cranial neural tube closure in mice. However, the fact that the *GCN5*^{hat/hat} embryos survive longer than the two other models indicates that GCN5 has important developmental functions that are independent of its acetyltransferase activity.

It is important to note that *PCAF* null mice are viable with no obvious abnormalities. Interestingly, in chick cells as well as in mice, it was observed that when GCN5 levels decreased, PCAF levels were increased and it works in both ways. However, although some observations have suggested redundant or compensatory roles between GCN5 and PCAF, they were also shown to be expressed in different tissues and at different developmental stages, thus highlighting distinct functions of those proteins especially during early development (Bu et al., 2007).

In human, amniotic fluid and serum cells (AFSCs) women in the second trimester of pregnancy were isolated, cultured and analyzed. These women were pregnant with either normal fetuses or with fetuses displaying craniofacial abnormalities (myelomeningocele, anencephaly, holoprosencephaly, encephalocele).

The AFSCs from the woman pregnant with a fetus affected by myelomeningocele had decreased levels of H3K9ac, H3K18ac, and GCN5 compared to the AFSCs from the women with healthy fetuses. In AFSCs from the woman carrying an anencephalic fetus, the levels of H3K9Ac, H3K18ac, and Gcn5 were increased. These abnormalities are due to neural tube defects (NTD) and these results suggest an important role of GCN5 and histone H3 acetylation in human fetal development and neural tube closure during early development (Tsurubuchi et al., 2013).



Figure 47: $GCN5^{hat/hat}$ embryos exhibit defects in neural tube closure and exencephaly.

Lateral view of embryos at E13.5.

The embryo on the left is a wild-type or $GCN5^{hat/+}$ embryo, and the embryo on the right is a $GCN5^{hat/hat}$ embryo.

2.6 Conclusion

Thus the acetyltransferase GCN5 is conserved from yeast to human. It is implicated in the acetylation of histone proteins, mainly histone H3, as well as non-histone proteins such as tubulin, PGC-1 α and c-myc and regulates several cellular processes such as proliferation, chromatin remodeling and metabolism.

GCN5 has also important roles in development. The alteration of GCN5 levels or activity leads to neural tube closure defects and craniofacial abnormalities. In the previous part of this manuscript, we have also described the role of the tumor suppressor LKB1 in head formation. These observations suggest an *in vivo* interaction between GCN5 and LKB1 leading to the formation of craniofacial structures. But how are these structures formed during development and at which levels could GCN5 and LKB1 be involved?

3 The neural crest

Neural crest cells (NCCs) are a pluripotent and migratory cell population which arises during early development. The neural crest (NC) is uniquely found in vertebrate embryos, although it appears that the protochordates had the beginnings of a genetic program for neural crest formation (Holland & Holland, n.d.; Ivashkin & Adameyko, 2013). It has played a major role in the development of the vertebrate head and in the remarkable diversification and radiation of this group of animals (Dupin & Le Douarin, 2014).

3.1 Neural crest cells

The neural crest is a transitory structure which is induced and originates after gastrulation from the lateral margins of the neural primordium, to end up in the dorsal neural tube region. NC induction requires the competence of the ectoderm to respond to neural inducers from the mesoderm and the interaction of the neural plate with the non-neural ectoderm. After neural tube closure, NCCs arise at the margin between the ectoderm and the neural ectoderm. They undergo an epithelial to mesenchymal transition (EMT), detach from the neural primordium (delamination) and start migrating throughout the embryo, either ventrally close to the neural tube or dorso-laterally in proximity to the somatic ectoderm (future skin) (**Figure 48**). The cranial, trunk, vagal (from the neck region) and sacral (from the tail region) NCCs subpopulations are defined according to their axial level and their migrational path. Once they reach their final destination, NCCs inhibit their migration and differentiate in response to environmental factors and specific gene expression within NCCs. They give rise to multiple cell types such as neurons and glial cells of the peripheral neural system, melanocytes of the skin, craniofacial cartilage, the dentin, dental pulp, and alveolar bone of the head (**Figure 49**). The wealth of NCCs derivatives found in almost all tissues of the body has led to the conception that the NC is a potential “fourth germ layer” in addition to the ectoderm, mesoderm and endoderm.

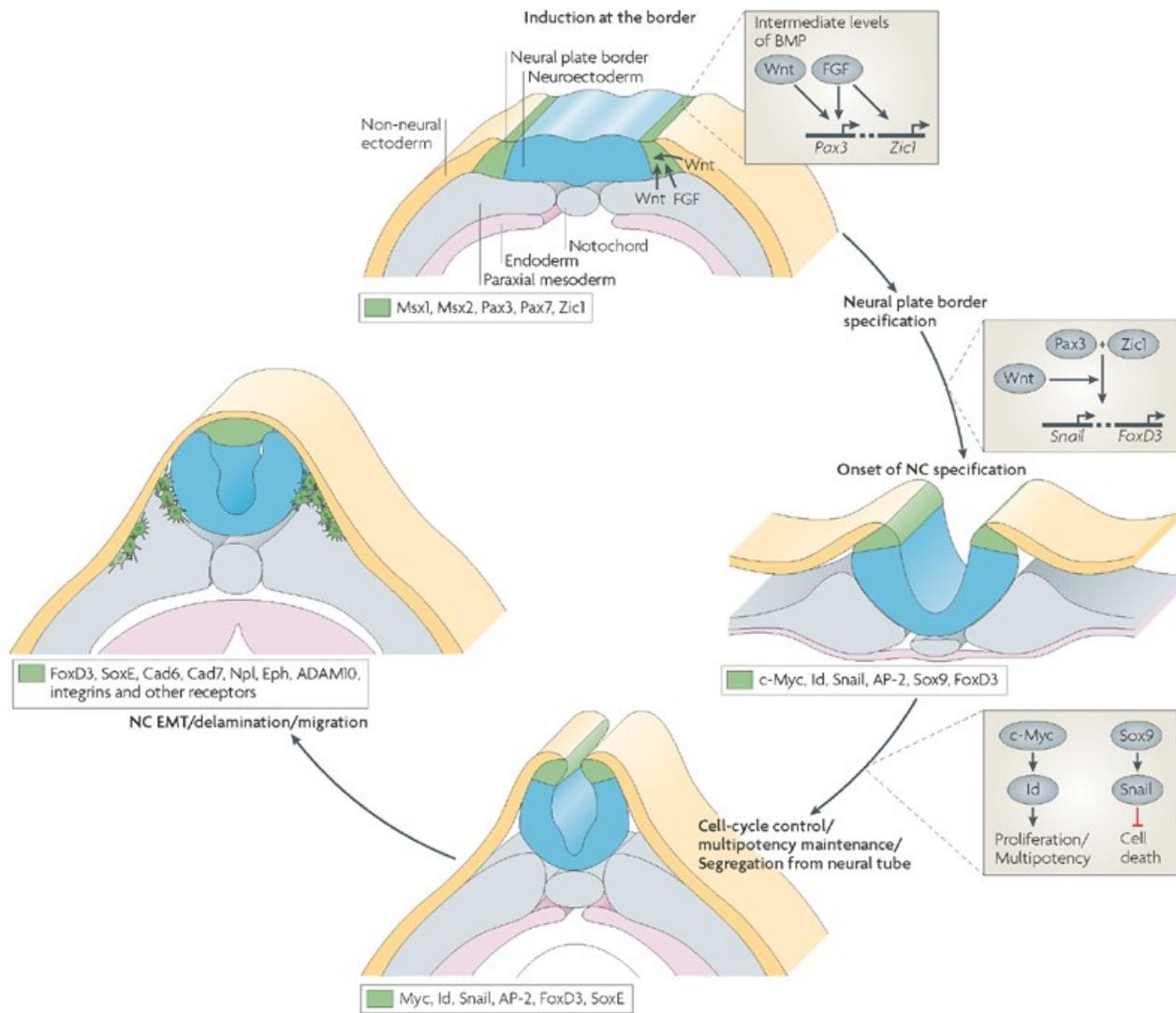


Figure 48: A gene regulatory network orchestrates neural crest formation

Induction initiates at the neural plate border and is mediated by signals including fibroblast growth factor (FGF) from the underlying mesoderm as well as Wnts from mesoderm and adjacent non-neural ectoderm. One or both of these signals independently induce the expression of individual neural plate border specifiers, such as *Pax3* and *Zic1*, in a manner that is dependent on intermediate levels of bone morphogenetic protein (BMP). *Pax3* and *Zic1* in turn act synergistically, in a Wnt-dependent manner, to up-regulate neural crest (NC) specifiers such as *Snail* and *FoxD3* in the neural folds and/or dorsal neural tube. These interactions have been primarily derived from work in *Xenopus laevis* and as such may not uniformly apply to other organisms. The *c-Myc*–*Id* cassette is a network switch that mediates cell-fate decisions by controlling the cell cycle, but also may maintain the NC progenitor pool in a multipotent state. *Sox9* confers survival properties to trunk NC precursors through up-regulation of *Snail*, an anti-apoptotic factor. Expression of these early NC specifiers in the NC progenitor population segregates them from the dorsal neuroepithelium, as these genes control the events of cell proliferation, delamination and the onset of the epithelial to mesenchymal transition (EMT). Other NC specifiers, such as *FoxD3* and *Sox10*, persist in delaminating and migrating NC cells, where they control expression of downstream effector genes such as type II cadherin, cadherin-7 (*Cad7*), matrix metalloproteases (MMPs; including a disintegrin and metalloprotease-10 (*ADAM10*)), integrins, neuropilins (*Npl*), Eph and other transmembrane receptors. (Sauka-Spengler & Bronner-Fraser, 2008).

The NC is described as a transitory structure since at this stage (after NCCs migration and differentiation), it is exhausted and replaced by the roof plate (Basch & Bronner-Fraser, 2006; Dupin & Le Douarin, 2014; Kuriyama & Mayor, 2008; Nicole M Le Douarin, Creuzet, Couly, & Dupin, 2004; Noisa & Raivio, 2014).

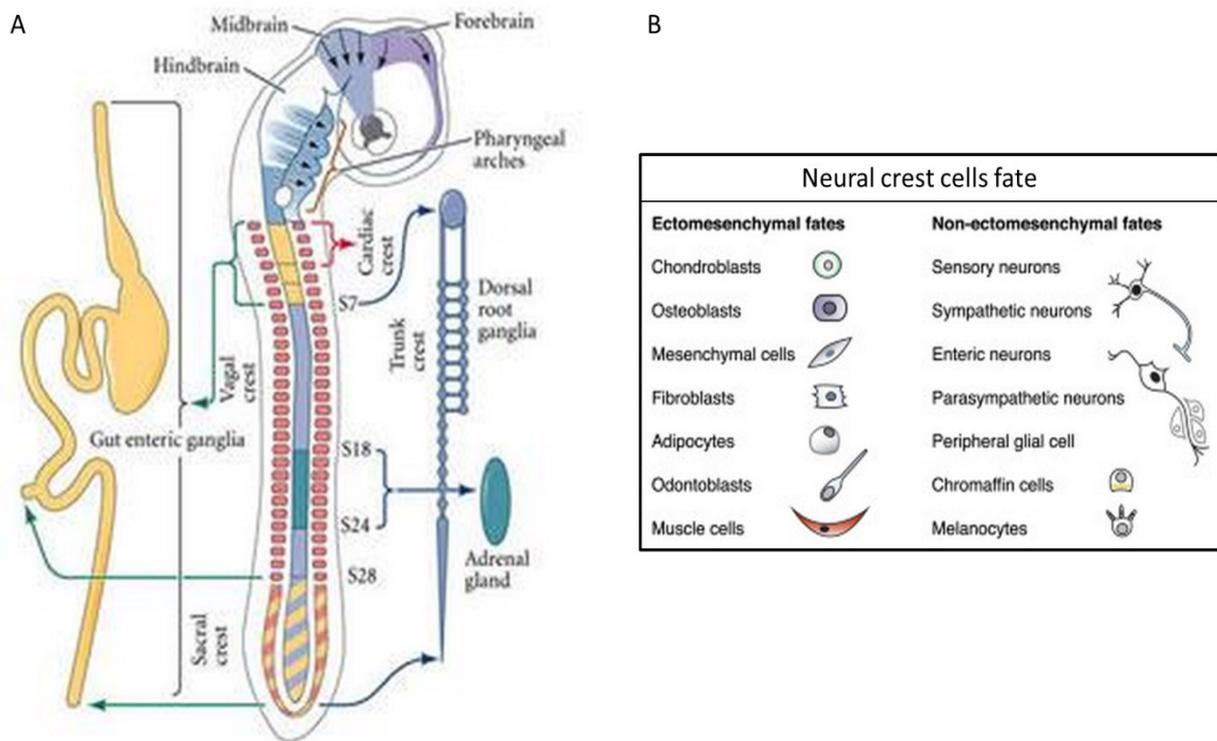


Figure 49: Neural crest cells subpopulations and derivatives

A) Regions of the neural crest. The cranial neural crest migrates into the branchial arches and the face to form the bones and cartilage of the face and neck. It also produces pigment and cranial nerves. The vagal neural crest (near somites 1-7) and the sacral neural crest (posterior to somite 28) form the intrinsic neurons of the gut. The cardiac neural crest cells arise from the neural crest near somites 1-3; they are critical in making the division between the aorta and the pulmonary artery. Neural crest cells of the trunk (about somite 6 through the tail) make the sympathetic neurons, and a subset of these (at the level of somites 18-24) form the medullary portion of the adrenal gland. (After Le Douarin 1982.) (Developmental Biology, 6th edition, S. Gilbert)/(Extracted from the lecture of Dr. Taube P. Rothman). **B) Neural crest cells derivatives.** Neural crest cells are embryonic pluripotent stem cells giving rise to a broad range of ectomesenchymal and non-ectomesenchymal fates. Adapted from (Ivashkin & Adameyko, 2013).

3.1.1 Neural crest cells subpopulations

Neural crest cells can be divided into four subpopulations according to their origin along the anterior-posterior axis and the location to which they migrate and settle. Thereby, the cranial (cephalic), trunk, vagal (and sacral) and cardiac neural crest cells are classically distinguished.

3.1.1.1 Cranial (cephalic) neural crest cells

Cranial neural crest cells (CNCCs) originate from the presumptive brain region and migrate dorsolaterally to produce the craniofacial mesenchyme. The latter differentiates into cartilage or bone, cranial neurons as well as glia (Taneyhill, 2008). The rostral CNCCs form the fronto-nasal cartilage and cranial bones. The more posterior CNCCs enter the pharyngeal arches and pouches and give rise to odontoblasts of the tooth primordia, the bones of middle ear and jaw, as well as hyoid and thyroid cartilages, parafollicular C cells of the thyroid, and contribute to thymic development (Dudek & Fix, 2005; Gilbert, 2000; Minoux & Rijli, 2010)(**Figure 50**). The fate and importance of CNCCs in head formation will be detailed further in this manuscript.

3.1.1.2 Trunk neural crest cells

Depending on their fate, the trunk neural crest cells (TNCCs) (somite 7 to the tail) take three different routes. The cells which are destined to become melanocytes migrate dorsolaterally into the ectoderm and continue towards the ventral midline of the belly. The second group of TNCCs follows a ventrolateral migratory pathway and reaches the anterior half of sclerotomes, which are blocks of mesodermal cells derived from somites. These sclerotomes differentiate into the vertebral cartilage of the spine. The TNCCs that remain near the sclerotome form the dorsal root ganglia containing the sensory neurons while those that continue more ventrally form Schwann cells, sympathetic ganglia, the adrenal medulla, and the nerve clusters surrounding the aorta (Gilbert, 2000). The third group of TNCCs also migrates in a ventrolateral way and reaches the neural tube surface where they give rise to boundary cap cells. These cells form clusters at the surface of the neural tube, at entry and exit points of peripheral nerve roots (Maro et al., 2004) (**Figure 51**).

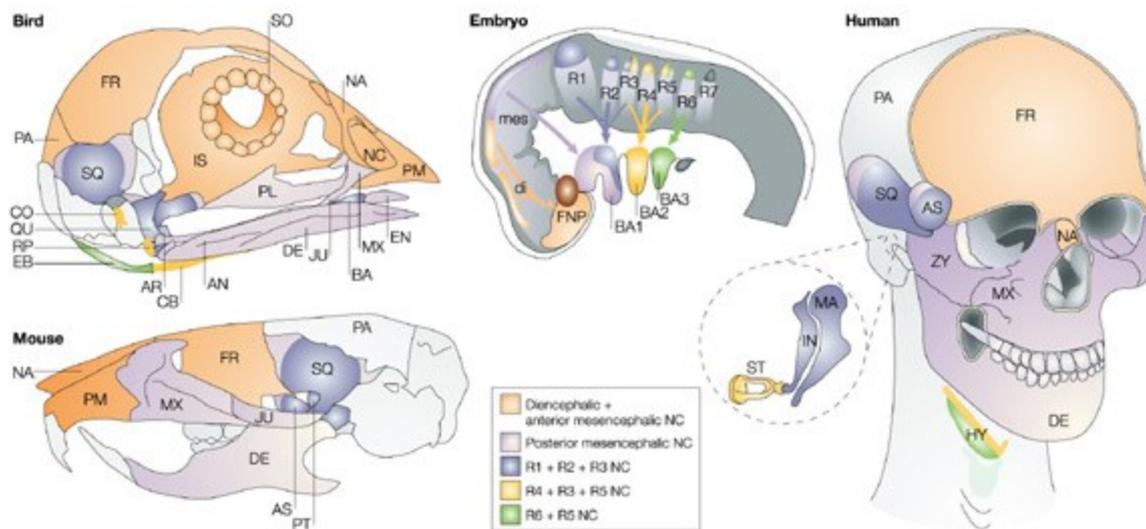


Figure 50: Skeletal fate of cranial neural crest cells in vertebrates.

The embryo figure shows colonization of the head and pharyngeal arches by diencephalic, anterior and posterior mesencephalic, and rhombencephalic neural crest cells (NCCs), as indicated by the colour code. The diagram is representative of chick, mouse, and human embryos, although the NCC migratory pathways might differ slightly in different species. The skull drawings show comparative contributions of NCC populations to cranial skeletal elements of humans, mice and birds. Drawings are based on NCC fate-mapping studies and on extrapolation of avian and mouse data to known homologues in the human. Some bones, including the squamosal (SQ), alisphenoid (AS), and pterygoid (PT), are shown with mixed contribution from different NCC populations. Note that in mammals the frontal (FR) and parietal (PA) bones have been reported to be of neural crest and mesodermal origin, respectively. In birds, the frontal and parietal bones have been reported to be either entirely derived from NCCs, as shown in the figure, or derived from a dual neural crest/mesodermal origin. AN, angular bone; AR, articular bone; BA, basihyal; BA1–BA3, pharyngeal arches 1–3; CB, ceratobranchial; CO, columella; DE, dentary bone; di, diencephalon; EB, epibranchial; EN, entoglossum; FNP, frontonasal process; HY, hyoid bone; IN, incus; IS, interorbital septum; JU, jugal bone; MA, malleus; mes, mesencephalon; MX, maxillary bone; NA, nasal bone; NC, nasal capsule; PL, palatine bone; PM, premaxillary bone; QU, quadrate; RP, retroarticular process; R1–R7, rhombomeres 1–7; SO, scleral ossicles; ST, stapes; ZY, zygomatic bone (Santagati & Rijli, 2003).

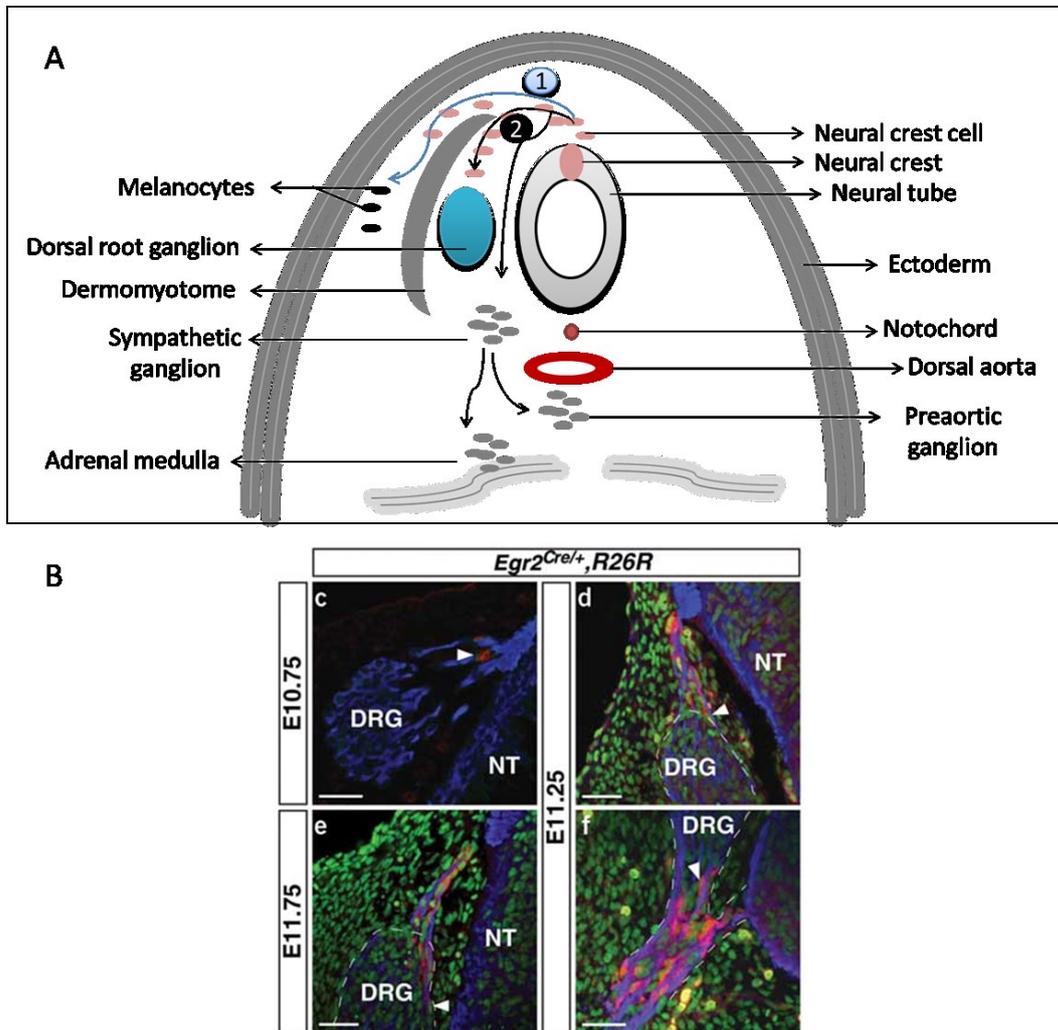


Figure 51: Trunk neural crest cells progression and fate

A) Schematic representation of the trunk NCCs migration routes and derivatives in the corresponding region. Trunk NCCs delaminate from the dorsal neural tube and migrate along a dorsolateral pathway (1) to become melanocytes, or follow a ventrolateral migratory pathway (2) forming the dorsal root ganglia containing the sensory neurons, but can also form, more ventrally, Schwann cells, sympathetic ganglia, the adrenal medulla, and the nerve clusters surrounding the aorta. Adapted from

[http://web.uni-plovdiv.bg/stu1104541018/docs/res/skandalakis%20surgical%20anatomy%20-%202004/Chapter%2027_%20Adrenal%20\(Suprarenal\)%20Glands.htm](http://web.uni-plovdiv.bg/stu1104541018/docs/res/skandalakis%20surgical%20anatomy%20-%202004/Chapter%2027_%20Adrenal%20(Suprarenal)%20Glands.htm)

B) Fate tracing of Boundary cap cells. (a,b) Transverse sections of E13.5 *Egr2^{lacZ/+}* and *Egr2^{Cre/+} R26R* embryos, stained by X-gal. (a) In the *Egr2^{lacZ/+}* embryo, *lacZ*-expressing cells were located at the boundary cap (BC) and along the proximal part of the root (arrow). (b) In the *Egr2^{Cre/+} R26R* embryo, in which the progeny of *Egr2*-expressing cells were labeled, positive cells were found along the entire root and within the DRG. (c-f) *Egr2^{Cre/+} R26R* embryo sections from trunk levels immunostained with antibodies specific to β -galactosidase (red) and β -III-tubulin (Tuj1, blue), and counterstained with a nuclear marker (green) at the indicated stages. (c) At E10.75, β -galactosidase-positive cells were located adjacent or very near to the spinal cord (SC), reflecting *Egr2* expression at the BC. (d,f) At E11.25, more cells were labeled and cover the dorsal and ventral roots. (e) At E11.75, β -galactosidase-positive cells had reached the DRG. The arrowheads indicate the most ventral β -galactosidase-positive cells in the dorsal root. NT, neural tube. Scale bars (c-f), 50 μ m. (Maro et al., 2004)

3.1.1.3 Vagal and sacral neural crest cells

The vagal (between somites 1 and 7 in chick) and sacral (posterior to somite 28) neural crest cells give rise to the enteric nervous system (ENS) of the digestive tract including the esophagus, the stomach and the entire length of the gut (N M Le Douarin & Teillet, 1973; Pomeranz, Rothman, & Gershon, 1991) (Anderson RB, Newgreen DF, 2000). Studies in chick embryos have revealed that sacral neural crest-derived precursors colonise the gut 4 days after vagal-derived cells had completed their migration along the entire gut. Although interdependence between both subpopulations was suggested, sacral neural crest cells had rather a predetermined profile (Burns, Champeval, & Le Douarin, 2000). In mouse embryos, organotypic cultures and time-lapse imaging led to the observation whereby sacral and vagal NCCs display different capabilities of entering the hindgut, implying differences in their intrinsic migratory properties (Wang, Chan, Sham, Burns, & Chan, 2011). When NCCs fail to migrate from these regions to the colon, peristaltic movement in the bowels are defective due to the absence of enteric ganglia (Gilbert, 2000). This anomaly is mainly observed in the Hirshsprung's disease.

3.1.1.4 Cardiac neural crest cells

The cardiac neural crest cells originate between the cranial and trunk neural crests, postotically from the otocyst to somite 3 corresponding to rhombomeres 6, 7 and 8 in the neural tube (Kirby & Hutson, 2010). They differentiate into melanocytes, neurons, cartilage, and connective tissue (of the third, fourth, and sixth pharyngeal arches (the fifth degenerates in humans)). They also generate the entire musculoconnective tissue wall of the large arteries as they arise from the heart, and contribute to the septum that separates the pulmonary circulation from the aorta (Gilbert, 2000). The cardiac neural crest is unique and is already determined to generate cardiac cells. It cannot be substituted since its replacement by cranial or trunk neural crest leads to cardiac abnormalities (notably the failure of the truncus arteriosus to separate into the aortic and pulmonary arteries) (Gilbert, 2000).

3.2 Neural crest cells ontogenesis: from induction to migration

3.2.1 Neural crest induction and specification networks

The induction of neurulation leading to neural tube closure and NCCs delamination and migration is orchestrated by a cascade of gene regulation, molecular signaling and morphogenetic mechanisms. NCCs are characterized by their pluripotency, giving rise to a wide range of cell types, and their invasive potential. Understanding the molecular networks that govern NCCs ontogenesis is essential for better apprehending the NC defects-associated pathologies.

3.2.1.1 Neural Crest induction

At least three major signaling pathways are involved NC induction, including BMPs (Bone morphogenetic proteins), Wnt family members, and FGFs (fibroblast growth factors). An array of transcription factors ensures the signals conversion into precise temporal, spatial and quantitative responses and promotes the differentiation of non-neural ectoderm into neural ectoderm. This phenomenon is called neural induction (Sauka-Spengler & Bronner-Fraser, 2008) .

BMPs are secreted proteins of the TGF β (transforming growth factor- β) superfamily and are required for dorsoventral patterning during early embryonic development. According to the classical gradient model, it was proposed that a BMP gradient is necessary for NC induction and is established by the activity of BMP antagonists, such as chordin, noggin or follistatin. These antagonists are found in the underlying paraxial mesoderm and induce a response of the ectoderm to intermediate levels of BMPs. Thus, the cells expressing high levels of BMP differentiate into epidermal cells while cells expressing low BMP levels form the neural plate and cells expressing intermediate BMP levels become NCCs (Xiao Huang & Saint-Jeannet, 2004; Sauka-Spengler & Bronner-Fraser, 2008) (**Figure 52**).

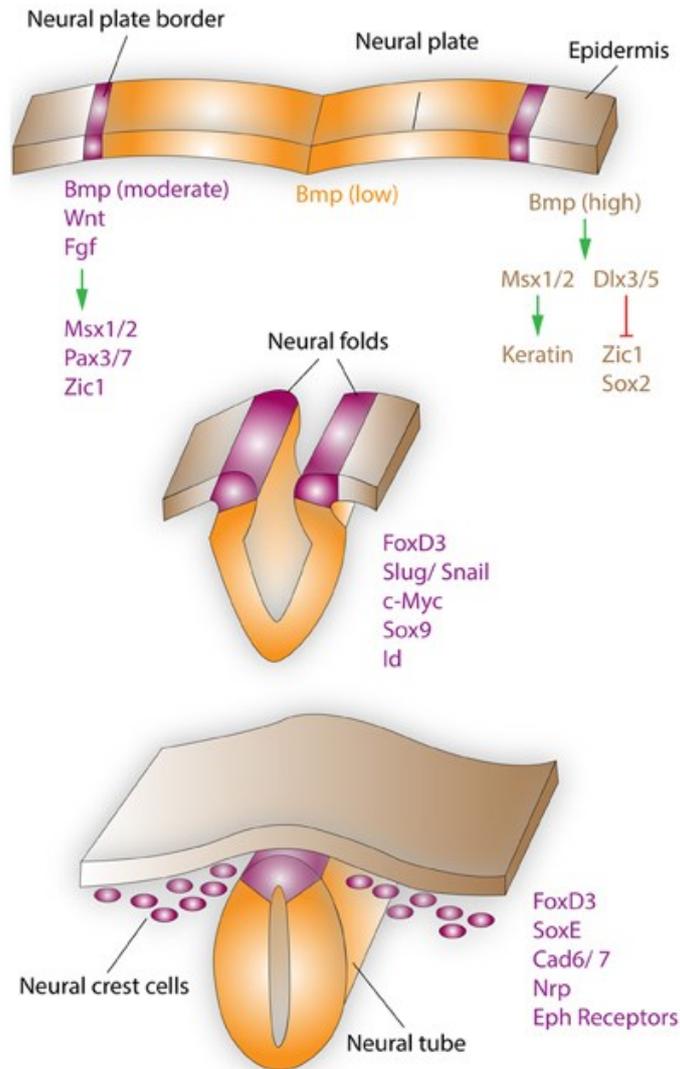


Figure 52: Neural crest formation and migration during development.

Neural crest regionalization (top) at the boundary of the neural plate and epidermis is a multi-step process. First, the border of the neural plate is set via secretion of neural plate inductive signals (Fgf, Bmp, and Wnt) from the ventral ectoderm and paraxial mesoderm (not shown). Anteriorly, the timing of Bmp and Wnt signaling contributes toward setting the boundaries between epidermis, prospective neural crest, and neural plate. In the narrow band, where Wnt signaling induces Bmp signaling and Wnt signaling is not subsequently turned off, NCCs are formed. Bmp, Wnt, and Fgf, which are secreted by the prospective neural crest, induce the expression of border regionalization genes such as *Msx1/2*, *Pax3/7*, and *Zic1*. In contrast, in the epidermis high concentrations of Bmp induce the expression *Msx1/2*, which promote keratin expression and *Dlx3/5*, which induce *Zic1* and *Sox2* expression. Neural crest specification (middle) starts with the expression of *FoxD3*, *Slug/Snail*, *c-Myc*, *Sox9*, and *Id* by the border cells, which prevents this region from becoming either neural plate or epidermal tissue. EMT, delamination, and migration of NCCs (bottom), is primarily induced by *FoxD3*, *Snail*, and *Sox9*. These factors are also capable of inducing a cranial neural crest fate for cells of the lateral neural tube, when ectopically expressed in this region. After delamination NCCs migrate to their respective destinations, regulated by the expression of proteins such as *FoxD3*, *SoxE*, *Cad6/7*, *Nrp*, and *Eph* receptors. Specifically, the head and facial structures are largely products of the cranial neural crest, which is a mixed population of cells, with about 10% of these cells being multipotent progenitor cells (Ross & Zerbali, 2014).

It is now well established that BMP levels alone cannot account for NC induction and that other signaling pathways are also involved. For instance, in *Xenopus laevis* animal cap assays, the combination between fibroblast growth factor-2 (Fgf2) and BMP antagonists leads to the up-regulation of NC markers such as *Snail2* and induces NC formation. Moreover, Fgf8 secreted from the paraxial mesoderm is necessary and sufficient to transiently induce the NC. In this context, intermediate levels of BMP might have a role in the maintenance of the newly induced cell population (Sauka-Spengler & Bronner-Fraser, 2008).

Finally, the Wnt signaling operates through a 'canonical' β -catenin-dependent pathway or via the planar cell polarity (PCP) pathway and the protein kinase C (PKC)–Ca²⁺ pathway. The role of Wnts in NC formation is still ambiguous. In the frog, chick and zebrafish, both gain-of-function and loss-of-function experiments show that the canonical Wnt pathway is sufficient and necessary for NC induction. However, analysis of mouse mutants with targeted inactivation of Wnts or downstream components reveals severe defects in NC derivatives and indicates that Wnts are involved in NC lineage specification, rather than in induction (Sauka-Spengler & Bronner-Fraser, 2008). *Wnt1/Wnt3a* double mutant mice exhibit skeletal defects and a marked reduction in other neural crest derivatives such as cranial and spinal sensory neurons and melanocytes (Ikeya, Lee, Johnson, McMahon, & Takada, 1997). Likewise, targeted inactivation of downstream components of the canonical Wnt signaling pathway (β -catenin or APC), in the dorsal neural tube of mouse embryos, results in severe defects in cranial neural crest derivatives including the cranial and dorsal root ganglia and the craniofacial skeletal elements (Brault et al., 2001; Hari et al., 2002; Hasegawa et al., 2002).

3.2.1.2 *The neural plate border specifiers*

The neural plate border specifiers are transcription factors induced by signals from surrounding tissues and mediate BMPs, Wnts and FGFs signaling at the neural plate border. They synergize to induce bona fide NC cells by up-regulating neural crest-specifier genes such as *Snail2* and *FoxD3*. Their combinatorial expression is thought to uniquely define this territory. These transcription factors include *Zic1*, *Msx1*, *Msx2*, *Dlx3*, *Dlx5*, *Pax3* and *Pax7* genes. It was also suggested that *Snail1* and AP-2 may also function at the border just after the traditional border specifiers (**Figure 48**, **Figure 53**).

Although the three major signaling pathways FGF, Wnt and BMP can act independently to regulate neural plate border specifiers, recent evidence also suggest that these pathways may converge at the level of a common downstream effector, Smad1.

3.2.1.3 The neural crest specifier genes

Once the competence of the neural plate border territory is established, the prospective NC cells will start the process of specification into the bona fide NC. Another group of transcription factors, termed NC specifiers (previously referred to as NC markers) such as Snail1, Snail2, Sox8, Sox9, Sox10, FoxD3, AP-2, Twist, c-Myc and Id family members are up-regulated. They induce changes in the adhesive properties, shape, motility and signaling of NCCs precursors. As a consequence, NCCs segregate and delaminate from the neuroepithelium, then migrate and differentiate in their distant territories. Thus, the NC specifiers control EMT, population size (which consists of compensatory mechanisms that ensure an additional increase in the number of progenitors if a portion of NC cells is lost), proliferation and differentiation of NCCs (Sauka-Spengler & Bronner-Fraser, 2008) (**Figure 48, Figure 53**).

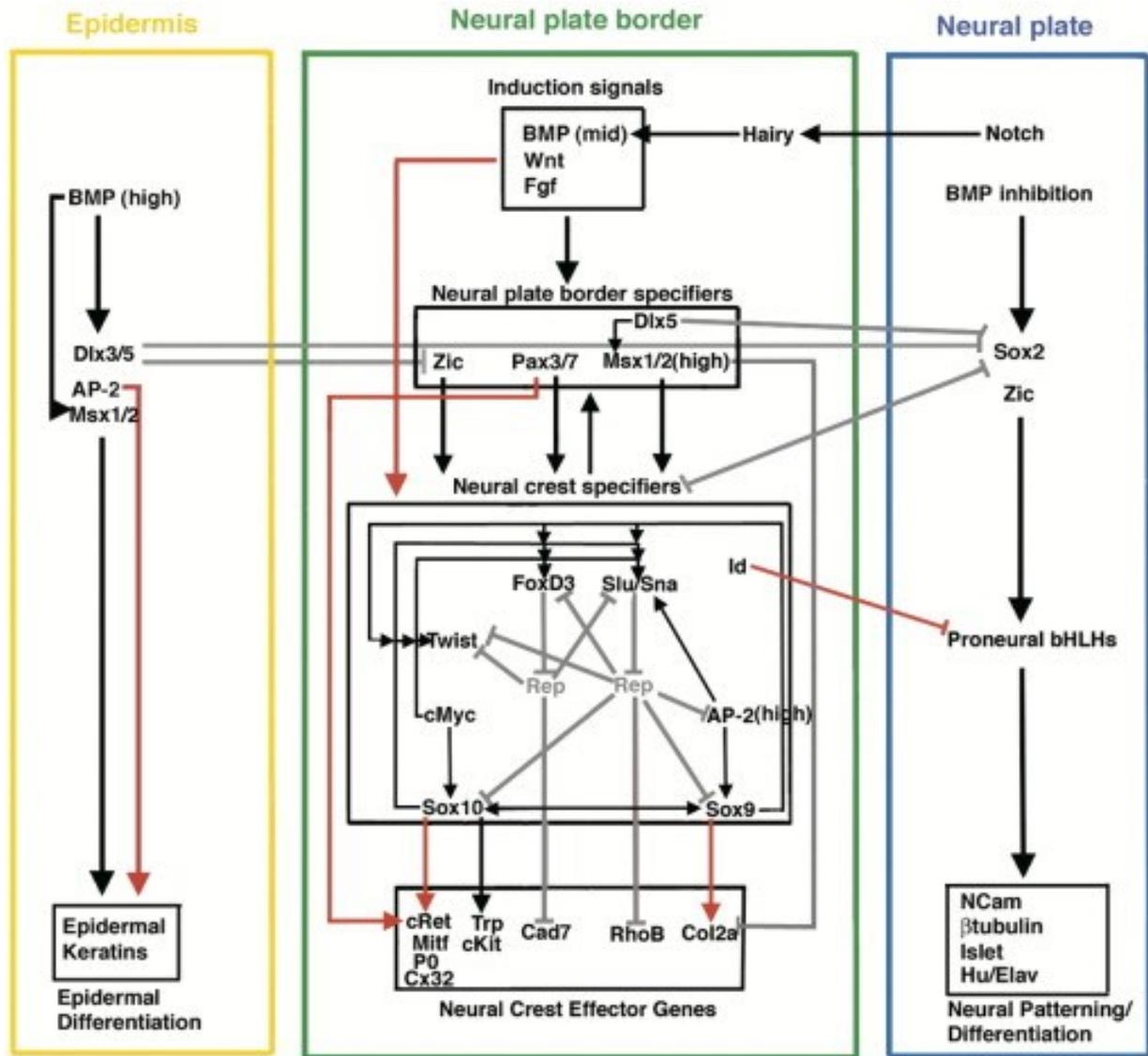


Figure 53: Putative gene-regulatory and signaling interactions at the neural plate border of vertebrates

Red arrows indicate proven direct regulatory interactions. Black arrows are genetic interactions suggested by gain- and loss-of-function analyses largely in *Xenopus*. Gray lines indicate repression. In vertebrates, dorsal ectoderm is segregated into presumptive epidermal, neural crest, and neural plate domains by distinct but interacting genetic cascades. The epidermal fate is specified early by high levels of BMP signaling which act through a battery of transcription factors to turn on epidermis-specific effector genes such as keratin. In the neural plate, BMP inhibition, as well as inductive signals from underlying mesoderm, leads to the expression of *Zic* and *Sox1,2,3* (group B) genes, proneural bHLH transcription factors, and neural-specific effectors. At the neural plate border, Wnt and Fgf signals, as well as intermediate levels of BMPs, induce expression of neural plate border and neural crest specifiers. Gene-regulatory crosstalk between neural crest genes maintains their expression until migration and differentiation, when neural crest effector genes are expressed.

3.2.1.4 Effector genes

The specifier genes control NCCs pluripotency and migration through the induction of effector genes such as Rho GTPases and cadherins which are involved in cellular shape, cytoskeleton organization and adhesion properties. In addition, the transcription factors Sox9 and Sox10 can regulate NCCs differentiation by activating the response of cell-specific effectors such as Mitf (Microphthalmia-associated transcription factor) and Trp (Tyrosinase-related protein) (Meulemans & Bronner-Fraser, 2004) (**Figure 48, Figure 53**).

3.2.2 Neural crest cells survival

When the cells in the elevating neural folds and/or dorsal neural tube start expressing the NC markers, they become specified to a NC fate. The expression of these early pre-migratory NC markers segregates NCCs from neuroepithelial cells and prepares them for EMT, delamination and migration. At the beginning, NC precursors maintain their pluripotency, delaminate and expand away from the neural epithelium avoiding massive proliferation. In *X. laevis*, it was shown that the maintenance of a pluripotent cell-pool of NC progenitors is provided by the small helix-loop-helix protein Id3 which mediates the decision between proliferation and apoptosis, acting as a cell-cycle-control switch. Since Id3 is a downstream target of the proto-oncogene c-Myc, it was suggested the latter maintains NC precursors pluripotency by mediating cell-fate decisions in the embryonic ectoderm. In this context, depletion of Id3 or c-Myc in *X. laevis* led to the loss of NC progenitors and excess formation of central nervous system progenitors (Sauka-Spengler & Bronner-Fraser, 2008).

Another NC specifier, Sox9, is implicated in cell-cycle regulation, and has also been shown to confer survival properties to trunk NC precursors. Depletion of Sox9 in the pre-migratory NCCs induces massive apoptosis in mouse embryos. It was suggested that this depletion is associated with the down-regulation of another anti-apoptotic factor, *Snail1*, in these mutants. Similarly, in zebrafish, Sox9 depletion triggers massive apoptosis of NCCs within the branchial arch (Sauka-Spengler & Bronner-Fraser, 2008).

3.2.3 Delamination

Delamination defines the segregation of NCCs from their surrounding tissues including the neural ectoderm. However, this process does not occur at the same time along the antero-posterior axis of the embryo and is specie-dependent.

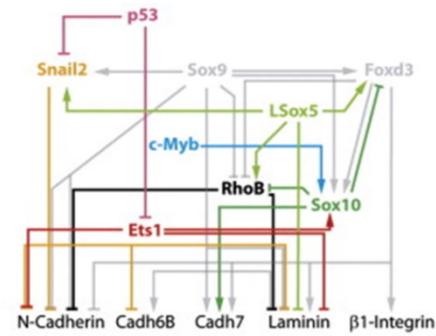
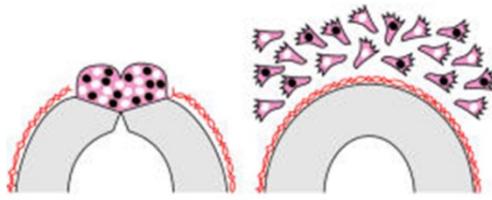
In mouse and *Xenopus* embryos, cranial NCCs delaminate all at once when the neural plate is still wide open, while in birds delamination coincides with the fusion of the neural folds. In all animal models, the trunk NC cells delaminate from the neuroepithelium one by one in a dripping fashion, after neural tube closure. However, time variations exist between the end of neurulation and NC departure along the AP axis. In the chick embryo, while rostral trunk NCCs delaminate a few hours after neural tube closure, caudal-most NC cells emigrate one day after completion of neurulation.

In chick embryos, delamination is triggered by a BMP/canonical Wnt cascade. This signaling involves *Bmp4*, *Wnt1*, *Msx1* and *c-Myb* and promotes EMT via activation of *Snail2*, *Foxd3* and members of the SoxE family such as *Sox9* and *Sox10* (Theveneau & Mayor, 2012) (**Figure 54**).

3.2.4 Cell cycle control

Delaminating NCCs are synchronized in S-phase and the G1/S transition of NC precursors is ensured by a BMP4/Wnt1 cascade (**Figure 54**). No synchronization prior to delamination is observed. Inhibiting the G1/S transition in chick rostral trunk NCCs blocks delamination. However, the entry in S-phase is uncoupled from delamination and is not sufficient to promote delamination since the latter can be blocked without affecting the cell cycle (Theveneau & Mayor, 2012).

A) Cranial delamination



B) Trunk delamination

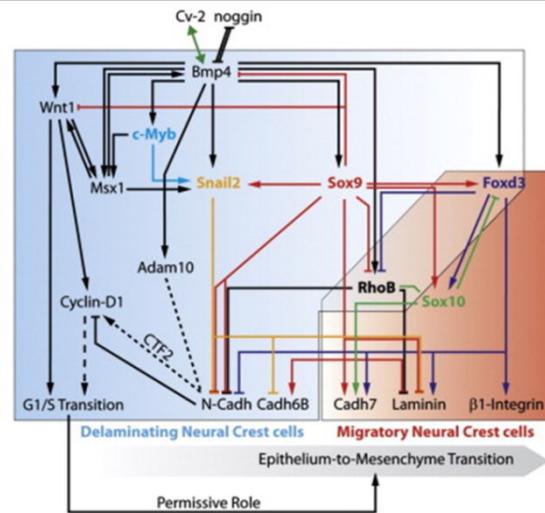
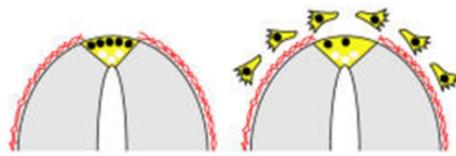


Figure 54: Cranial and trunk NCCs delamination.

A) Collective migration of cranial NCCs and the molecular control of cephalic delamination. The tumour suppressor p53 is expressed in the dorsal neural tube before delamination and inhibits Snail2 and Ets1 expression. p53 disappearance allows Snail2 and Ets1 expression levels to go up and triggers the delamination. Alongside Snail2 and Ets1 the cascade involves several transcription factors such as LSox5, Sox9 and Foxd3, but the relationship between these factors at cephalic levels is poorly understood. Putative roles for Sox9 and Foxd3 are based on their known functions at trunk levels. Networks at both trunk and cephalic levels are based on gain- and loss-of-function experiments performed *in vivo* in the chick embryo, see main text for references. Arrows and lines are color-coded in register with the genes they originate from. Single lines/arrows mean that one gene is sufficient to activate/inhibit a specific downstream effector. Double lines/arrows mean that a co-expression is required to activate/inhibit a specific downstream effector. For example, Snail2 alone can inhibit Cadherin-6B expression but Snail2 and Ets1 co-expression is required to block N-Cadherin expression. **B)** Trunk NCCs delamination from the neuroepithelium in a dripping fashion (one by one) and the molecular cascade controlling their delamination. Premigratory NCCs express Bmp4 and the Bmp inhibitor Noggin. Noggin expression is progressively lost while Cv2, a Bmp carrier/enhancer, expression goes up. This change unleashes Bmp4 signaling, which triggers a Wnt1-dependent cascade. The Bmp4–Wnt1 axis activates a set of transcription factors (including but not restricted to Snail2, Sox9 and Foxd3) that control the EMT by modifying cell–cell and cell–matrix adhesion molecules. In parallel, Wnt1 promotes cell cycle progression through Cyclin-D1 (left part of the panel). Finally, the metalloprotease ADAM10 is activated downstream of Bmp4 and degrades N-Cadherin. Cleavage of N-Cadherin contributes to the loss of cell–cell adhesion and promotes cell cycle progression through Cyclin-D1 activation. Adapted from (Théveneau, Duband, & Altabef, 2007; Théveneau & Mayor, 2012).

3.2.5 Epithelial-to-mesenchymal transition

The delamination involves a partial or complete epithelial-to-mesenchymal transition (EMT). Although all NCCs undergo EMT, the timing and completion of EMT is not always concomitant with the delamination phase. Therefore, it is important to carefully use those terms in their context (Theveneau & Mayor, 2012).

After specification, NCCs undergo EMT through sequential molecular events orchestrating changes in cell junctions and adhesion properties, loss of apical polarity, and the acquisition of a mesenchymal and migratory phenotype (**Figure 55**).

3.2.5.1 *Tight-to-gap junctions transition*

Premigratory NCCs form an epithelium with a typical apical–basal polarity and intercellular tight junctions. The dissolution of these junctions is one of the first steps of EMT. 5 hours before the onset of migration, occludin and claudin, major components of tight junctions, are downregulated in the neural tube. The tight junction components are then progressively replaced by gap junctions such as Connexin 43 (**Figure 55**). Connexin-43 α 1 (Cxn-43 α 1) is expressed in mouse NCCs and its knockout produces defects in NC derivatives, including heart morphogenesis. This tight-to-gap junctions transition is associated with changes in cell–cell interactions which are dependent on cadherin expression patterns (Kuriyama & Mayor, 2008; Sauka-Spengler & Bronner-Fraser, 2008).

3.2.5.2 *Changes in cadherins expression*

One of the first indicators of NCCs EMT is a switch from type I cadherin expression which characterizes stable cell assemblies such as epithelial cells, to type II cadherins which are expressed in less cohesive mesenchymal cells and are correlated with the acquisition of cell motility (**Figure 55**). Cadherins are transmembrane proteins which bind cells together by forming adherens junctions and their functions are calcium-dependent, hence their name. Type I cadherins include E (epithelial), N (neural), P (placental), and R (retinal) cadherin, which share the basic structure of the cadherin family, with a conserved HAV tripeptide motif in the most distal EC (EC1). Type II cadherins include human cadherin- 5, 6, 8, 11, and 12 as well as other type II cadherins found in mouse, rat, chicken, and *Xenopus* (Halbleib & Nelson, 2006; Shibata, Shimoyama, Gotoh, & Hirohashi, 1997).

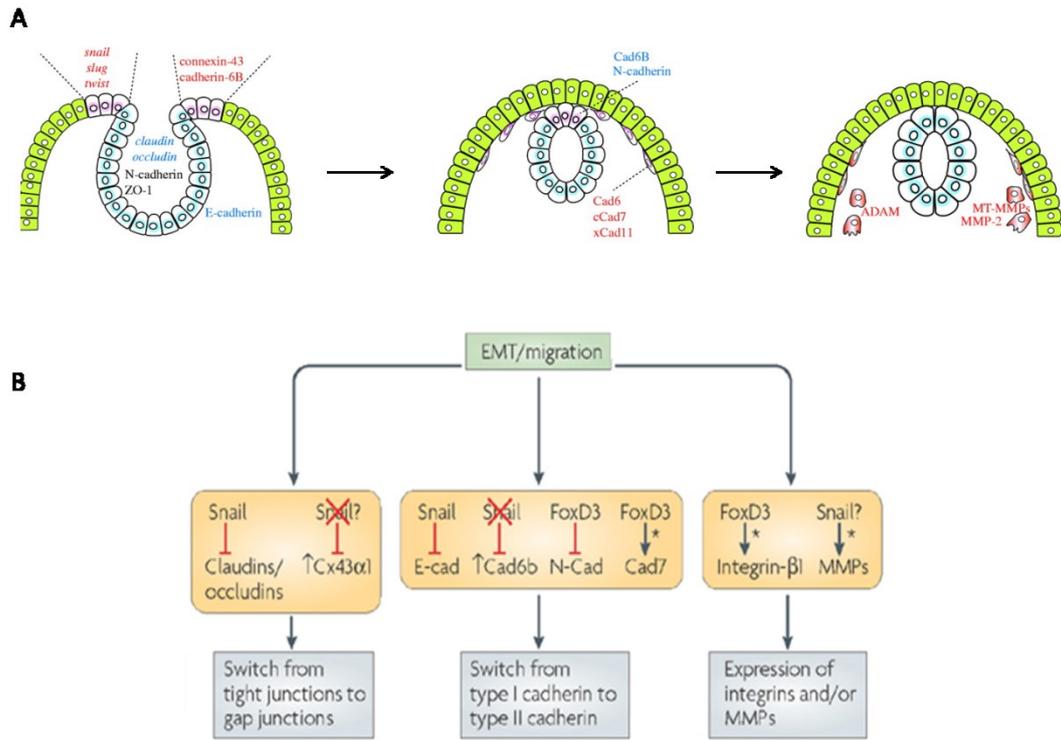


Figure 55: Neural crest cells epithelial-to-mesenchymal transition (EMT)

A) EMT steps. NCCs lose their apical-basal polarity, undergo changes in cell adhesion and cytoskeleton, and acquire a mesenchymal phenotype. Red letters represent upregulated factors, blue letters represent downregulated factors. Purple cells: NCCs; green cells: ectoderm; blue cells: neural tube. **B) Schematic representation of the regulation of EMT.** Transcriptional repressors, Snail and FoxD3, down-regulate expression of molecules that are associated with epithelial and/or stable cell populations, such as type I cadherins, E-cadherin (E-cad) and N-cadherin (N-cad), respectively. At later stages, removal of these specifiers allows de-repression and/or expression of effectors that are associated with mesenchymal and/or migratory populations. Such is the case of cadherin-6b (Cad6b), a type II cadherin that is up-regulated following the endogenous down-regulation of Snail (crossed out in red). Similarly, up-regulation of the gap junction protein connexin-43 α 1 (Cx43 α 1) may also depend on decreased Snail expression (crossed out in red). Gene batteries in which the repressors Snail or FoxD3 up-regulate the expression of matrix metalloprotease-2 (MMP2), integrin- β 1 or cadherin-7 (Cad7) represent indirect regulatory interactions, possibly mediated by another repressor. Snail–Cx43 α 1 and Snail–MMP interactions were inferred from cancer studies (question marks). Adapted from (Kuriyama & Mayor, 2008; Sauka-Spengler & Bronner-Fraser, 2008).

E-cadherin characterizes epithelial cells. Downregulation of E-cadherin and the loss of cell polarity are essential steps in EMT initiation. Downregulation of E-cadherin is controlled by Snail1 (formerly Snail) and Snail2 (formerly Slug) as well as SIP-1. N-cadherin (N-cad) is then transiently expressed in the neural epithelium and is downregulated in NCCs (dorsal neural tube) before EMT. However, N-cad expression is maintained in the lateral and ventral neural tube cells. Cadherin 6B (Cad6B) is expressed in premigratory NCCs before EMT, and then for a short time after delamination. Cad6B is later downregulated in migratory NCCs by Snail-2 mediated activity. Thus, during EMT, both N-cad and Cad6B are downregulated and replaced by cadherin-7 in avian NCCs and cadherin-11 in *X. laevis* NCCs.

However, the loss of N-cad- and Cad6b-mediated adhesion is not sufficient for EMT initiation. Indeed, in the avian trunk, BMP signaling initiates EMT. During this process, BMP signaling results in the downregulation of N-cad expression in premigratory NCCs. However, BMP also stimulates the proteolytic cleavage of N-cad, releasing a cytoplasmic fragment of N-cad. This fragment enters the nucleus and stimulates expression of other EMT-promoting genes. This could explain why the addition of exogenous addition of N-cadherin in breast cancer cells can induce cell migration, invasion and metastasis. Thus N-cad is bifunctional according to its structure; while its transmembrane form is important for maintaining cell adhesion and tissue integrity, its cytoplasmic fragment is a promotor of cell migration.

Finally, like N-cad, proteolytic cleavage of the extracellular domain of Cadherin 11 eliminates its adhesive functions and promotes cell migration. Indeed, Cadherin 11 knockdown in *Xenopus* cranial NCCs results in impaired migration whereby NCCs fail to form lamellipodia and lose their migratory ability. These defects were rescued by expression of the membrane anchored cytosolic fragment of Cadherin 11.

Together, these data show that cadherins are multi-functional molecules with diverse roles in controlling cell adhesion as well as NCCs migration. Thus, the balance between adhesion and motility is finely tuned by cadherin turnover and cleavage, the levels of one cadherin relative to another and the timing of their expression (Clay & Halloran, 2011; Kuriyama & Mayor, 2008; Park & Gumbiner, 2012; Sauka-Spengler & Bronner-Fraser, 2008).

3.2.6 Extracellular matrix remodeling

As NC cells undergo delamination and active migration, they respond to environmental signals, penetrate basement membranes and invade extracellular matrices in a controlled manner. This environment consists in mesenchymal tissue containing intricate connective tissue barriers that are comprised of collagens, fibronectin, laminins, vitronectin and proteoglycans.

The migrating NCCs need to navigate without delaying their movement. For this purpose, they modify the extracellular matrix (ECM) using proteolytic activity of metalloproteases (MMPs), which are involved in the invasive behaviour of metastatic cells. Cardiac NCCs migration relies on MMP2, which is secreted by the surrounding mesoderm or expressed later by non-neurogenic NC-derived cranial and pharyngeal mesenchyme. Another member of ADAM family of metalloproteases, ADAM10, forms a complex with CD44, a major cell-surface receptor for hyaluronic acid, and cleaves the ectodomain of CD44. This cleavage results in CD44-mediated cell–matrix adhesion alteration in the developing cornea, thus facilitating the migration of NC-derived corneal precursors. ADAM10 is also responsible for the cleavage of N-cad resulting in a cytoplasmic fragment which allows NCCs to detach from the neuroepithelium as previously mentioned. ADAM metalloproteases are cell-surface bound glycoproteins which mediate cell–cell adhesion. They also act as 'shedases', which are membrane-bound enzymes that cleave (or shed) extracellular portions of transmembrane proteins, releasing soluble domains from the cell surface. In *X. laevis*, ADAM13, decreases the adhesion of premigratory NCCs to the ECM and allows their detachment from the neuroepithelium (**Figure 55**).

NCCs directional migration following EMT depends on interactions between expressed integrins and the ECM. Integrin receptors link the matrix to the cell's actin cytoskeleton by localizing to focal contact sites. It is the dynamic assembly and disassembly of focal junctions which conducts directional migration of NCCs (Sauka-Spengler & Bronner-Fraser, 2008; Theveneau & Mayor, 2012).

3.2.7 Neural crest cells migration

NCCs migrate collectively, moving in streams of high directionality, and they interact with each other while adapting to their environment. While loose interaction are observed in chains of trunk neural crest, tighter interactions allow the cephalic neural crest cells to migrate as compact clusters. Despite the differences between neural crest subpopulations according to their position along the anterior–posterior axis, and among different species, there are some general rules followed by all NCCs during their migration (Mayor & Theveneau, 2014).

3.2.7.1 Streams and cell-free zones

The path and direction of migrating NCCs is orchestrated by the extracellular matrices (ECM) surrounding the neural tube. While a set of molecules promotes migration, another set impedes it thereby providing the specificity for cellular movements. The migration promoting set of proteins includes fibronectin, laminin, tenascin, various collagen molecules, and proteoglycans (Gilbert, 2000). Another group of positive regulators includes members of the VEGF, FGF and PDGF families of growth factors, as well as the chemokine stromal cell-derived factor 1 (Sdf1 or CXCL12) which plays a role in the homing of NCCs, NC development and chemotaxis (Theveneau & Mayor, 2012). In addition to physical barriers such as the otic vesicle, repressive proteins are involved in the restriction of neural crest cell migration. The main described proteins are the ephrins, semaphorins which interact with their respective receptors, Eph, neuropilin/plexin as well as the Robo/Slit family (Sauka-Spengler & Bronner-Fraser, 2008). These repellent signals allow the NCCs to migrate following defined streams and delimit cell-free zones (**Figure 56**).

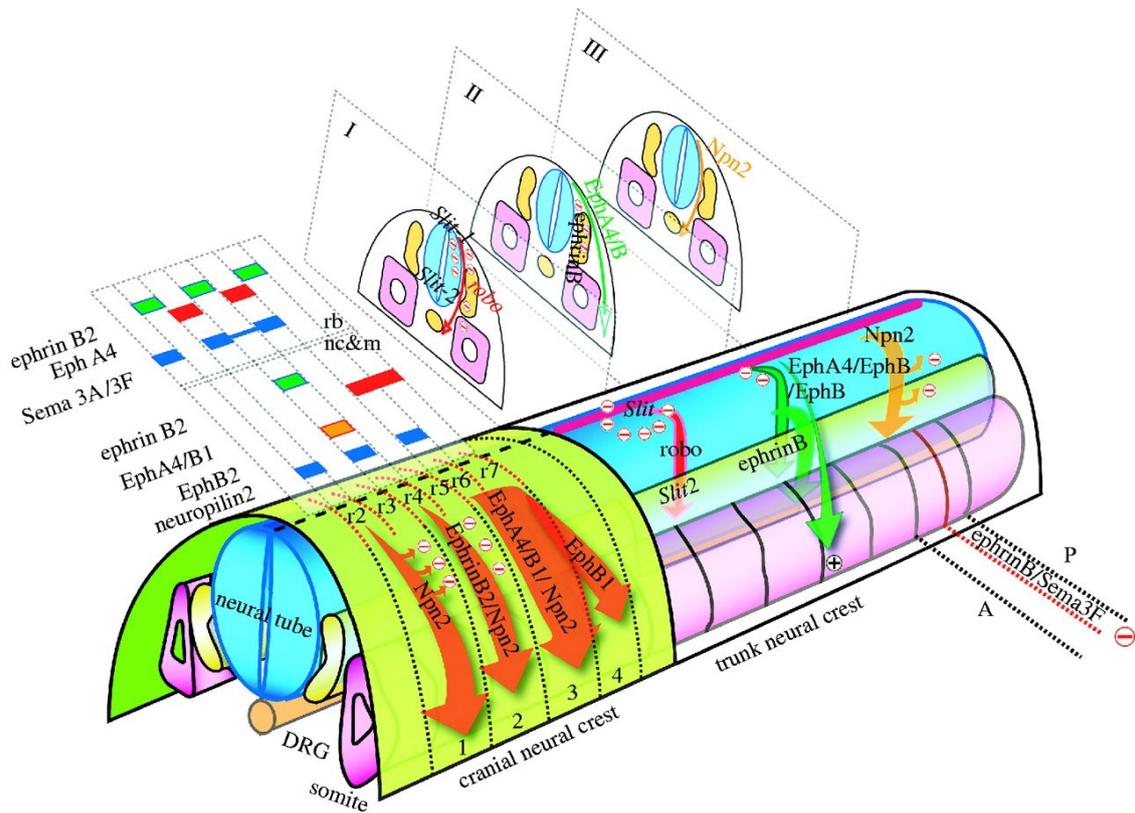


Figure 56: Migration of cranial and trunk NC.

Different factors that control the migration in the cranial and trunk NC are shown. Arrows represent streams of migrating NC. Cranial NC: migration in the head is controlled by Eph/eprins and semaphorins/neuropilins. The expression of the different factors is shown in the rhombomeres (rb) and in the NC and mesoderm (nc&m). Trunk NC: migration in the trunk: I, ventromedial migration controlled by Slit/Robo; II, dorsolateral migration controlled by Eph/eprins; III, ventromedial migration controlled by semaphorins/neuropilins. In addition, the anterior-posterior (A-P) patterning of NC migration is controlled by Eph/eprins and semaphorins/neuropilins. (Kuriyama & Mayor, 2008)

Ephrins and their Eph receptors as well as class3-semaphorins and their neuropilin/plexin receptors are expressed according to a code. The latter prevents NCCs with different ephrin/Eph and semaphorin/neuropilin profiles to share the same migratory stream and forbids entry into areas where the surrounding tissues exhibit another code, which avoids cell mixing among the streams. Indeed, impaired semaphorin or ephrin signaling results in the invasion of NC-free zones, which are normally present in between the streams, by NCCs. For instance, in mouse, chick and rat, migratory NCCs from the trunk region are restricted to the anterior sclerotome and express EphA/B receptors while the posterior sclerotome expresses ephrin-B ligands. When the ephrin signaling is inhibited, it leads to ectopic migration of NCCs through the posterior sclerotome (Theveneau & Mayor, 2012).

3.2.7.2 Contact inhibition of locomotion (CIL) and Planar cell polarity (PCP)

Directional migration of NCCs requires collective migration and Contact Inhibition of Locomotion (CIL). During this process, when a cell collides with another, it ceases migrating, retracts its protrusions, repolarizes, and moves away (Theveneau & Mayor, 2012). This anciently described phenomenon was suggested earlier but was recently demonstrated *in vivo* using *Xenopus* and zebrafish cephalic NC cells (Carmona-Fontaine et al., 2008).

While migrating, NCCs abandon their apico-basal polarity to acquire a front-back polarity. The latter is characteristic of planar cell polarity (PCP) which was first identified in *Drosophila* and then shown to be involved in axis elongation, inner ear patterning, neural tube closure, ciliary beating, left/right patterning, wound healing and directional cell migration in vertebrates (Mayor & Theveneau, 2014).

PCP of migrating NCCs involves the non-canonical Wnt pathway, which is independent from β -catenin-gene regulation but rather regulates cellular polarity as a consequence of cytoskeletal structure rearrangements. PCP signalling controls CIL between migrating NCCs by localizing different PCP proteins at the site of cell contact and locally regulating the activity of Rho GTPases such as RhoA (ras homologue family member A) and Rac1 (Mayor & Theveneau, 2014). Rac proteins regulate actin polymerization during lamellipodial extension while Rho proteins induce stress-fibre formation and activate ROCK (Rho-associated kinase), which stimulates actomyosin-based contractility. The mutual antagonism between Rac and Rho proteins contributes to cell polarization; while Rac is active at the front, Rho is mainly

active at the back of the cell (Mayor & Theveneau, 2014). In a polarized migrating cell, active protrusions such as lamellipodia, filopodia, blebs and invadopodia are usually formed at the front of the cell, whereas a retraction area is formed at the back.

The mechanism proposed for the role of PCP on NCCs migration links CIL with the asymmetric distribution of RhoA and Rac small GTPases. Different PCP proteins localize at the cell–cell contact upon collision between NCCs, leading to a localized inhibition of Rac and activation of Rho at the region of cell interaction. Thereby, the inhibition of Rac and the activation of ROCK at the collision area between the cells, allow the cell to retract its protrusions and change its migrational direction. In this context, ROCK inhibition supresses CIL (Mayor & Theveneau, 2014) (**Figure 57**).

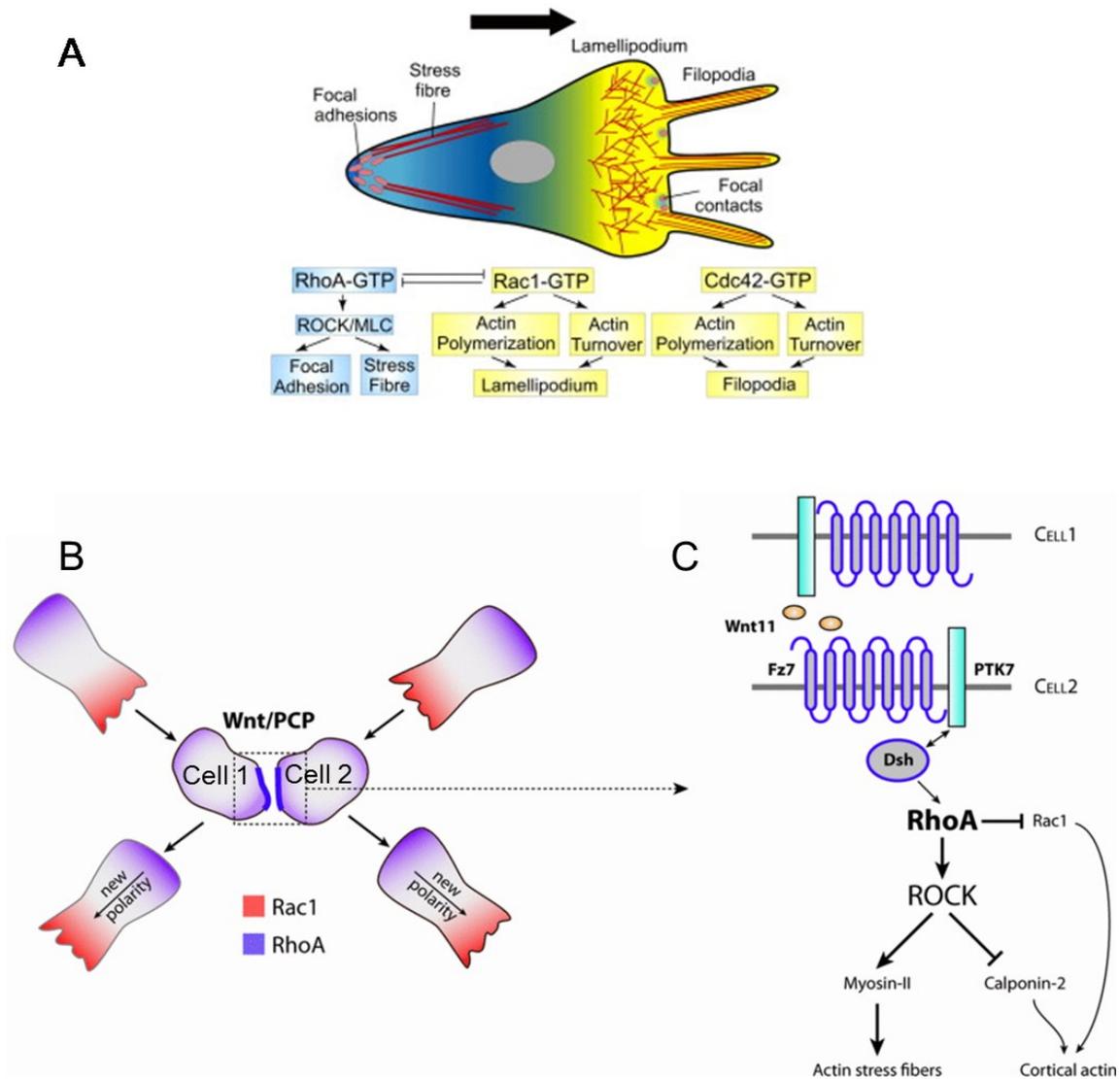


Figure 57: NCCs migration: planar cell polarity and contact inhibition of locomotion

A) Rho GTPases and cell protrusion control. During the migration of a polarized cell, Rac1 is mainly active at the front rear and regulate actin polymerization during lamellipodial extension. RhoA is mainly active at the back where it induces stress-fibre formation and activates ROCK (Rho-associated kinase), which stimulates actomyosin-based contractility. The mutual antagonism between Rac and Rho proteins contributes to cell polarization. **B), C) Wnt–PCP signalling during neural crest migration.** **(B)** Collision between two neural crest cells. Rho and Rac activities are initially polarized. This polarity is lost upon cell–cell interaction owing to accumulation of Wnt–PCP components such as Fz and Dsh at the region of contact which specifically activate RhoA. **(C)** Wnt–PCP signalling cascade in neural crest cells involves activation of Rho/ROCK signalling. Rho/ROCK inhibits Rac1 and calponin 2 and promotes actomyosin contractility favouring both the collapse of cell protrusions and retraction of the cell body away from the contact. Adapted from (Mayor & Carmona-Fontaine, 2010; Mayor & Theveneau, 2014).

3.3 Neurocristopathies

The term neurocristopathies was coined by Robert Bolande in 1974 (BOLANDE, 1974) and refers to a large number of congenital malformations and tumor syndromes resulting from NC developmental defects. These neurocristopathies may arise from functional deficits at different stages of NC ontogenesis, such as NC induction, delamination, migration, proliferation or maintenance of the pluripotent property of NC stem cell (M. E. Barnes, 2014). Furthermore, the clinical presentation of the neurocristopathies varies according to the subset of NCC that is affected and consequently depends on the rostro-caudal level of the neural tube from which they originate (Trainor, 2013). For example, aberrant development of cephalic NCC gives rise to craniofacial abnormalities and cardiac defects while maldevelopment of vagal NCC that colonize the embryonic intestine, the ultimobranchial body and the superior cervical ganglion leads to various pathologies including Hirschsprung disease (aganglionosis of the enteric nervous system) (**Table 5**). Even if the majority of these pathological conditions are rare and belong to the category of orphan diseases, it is believed that the dysfunction of NC development is a major cause of embryopathies since one third of congenital malformations are craniofacial abnormalities that often implicate a failure of cephalic NCCs formation (Dixon et al., 2006).

A broad array of genes that are causally responsible, when mutated, of neurocristopathies has been identified over the last thirty years. These genes encode proteins belonging to different class of effectors, from receptors (eg RET receptor tyrosine kinase in Hirschsprung disease and Multiple endocrine neoplasia type 2, Endothelin-B receptor in Waardenburg syndrome), to cytokines (ET3, the ligand of the Endothelin-B receptor in Waardenburg syndrome) (Amiel, 2001; Pingault et al., 2010) to signaling transducers (components of the Ras pathway in Neurofibromatosis type 1, Costello, Noonan, Leopard syndromes and other related diseases) (Rauen, 2013) to transcription factors (eg Sox10 and Pax3 in Waardenburg syndrome) (Pingault et al., 2010) and chromatin remodelers (eg CHD7 in the CHARGE syndrome) (Vuorela et al., 2007).

With the advent of the Next Generation Sequencing, one can foresee that the genetic underpinning of the 7000 monogenic disorders will be characterized in a near future and

that we will have at that time a complete picture of the genes involved in the majority of neurocristopathies.

Disease	Description
DiGeorge syndrome	Defects in heart, palate, thymus, facial features; learning disabilities
Treacher-Collins syndrome	Hypoplasia of facial bones, defects in ear development and eye function, cleft palate
CHARGE syndrome	Defects in CNS, heart, hearing; growth and/or developmental retardation; genital and urinary tract defect
Waardenburg syndrome	Hearing loss; eye and hair pigment abnormality
Hirschsprung's disease	Intestinal aganglionosis
Congenital heart disease	Outflow tract defects are common
Familial dysautonomia	Problems with formation and function of parasympathetic and sympathetic neurons
Pediatric and adult cancer	Examples include neuroblastoma and melanoma
Piebaldism	Pigment defects as a result of problems with melanocyte formation
Axenfeld-Rieger syndrome	Glaucoma; malformations of eye, teeth and skeleton
Goldenhar syndrome	Malformation of palate, ear, nose, lip and jaw

Table 5: Neural crest-associated diseases (Menendez et al., 2013)

3.4 Cranial neural crest cells

3.4.1 Formation of cranio-facial structures: fate of cephalic neural crest cells

The origin of craniofacial structures was studied using fate mapping experiments and the quail-chick chimeras approach which was pioneered by Nicole Le Douarin. More precisely, experiments in the avian embryo have led to the notion that the facial and hypobranchial skeletons are derived from cranial neural crest cells (CNCCs) migrating from the mid-diencephalon down to rhombomere r7 (Couly, Coltey, & Le Douarin, 1993; Le Lièvre & Le Douarin, 1975). Besides, surgical ablation of the cranial neural crest (CNC) leads to neural tube closure defects, exencephaly and absence of the facial skeleton (Creuzet, Martinez, & Le Douarin, 2006) (Figure 58). Altogether, these data show the importance of CNCCs in craniofacial morphogenesis.

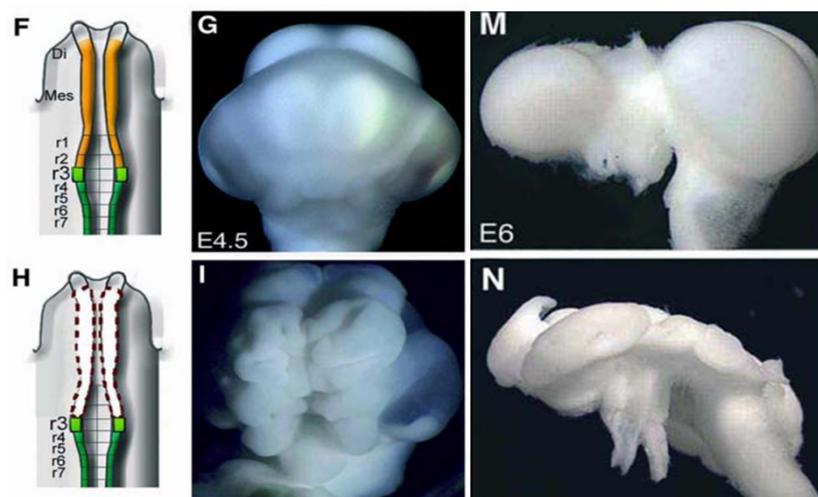


Figure 58: Absence of cephalic NC entails abnormal craniofacial development.

Gross anatomy of E4.5 control (**F** and **G**) or FSNC-deprived (**H** and **I**) embryos. Shown is an extended exencephaly in the operated embryo (**I**), compared with the stage-matched control (**G**). Whole-mount brain preparations dissected out from control (**M**) or FSNC-deprived (**N**). In the absence of cephalic NCC, brain is exencephalic and partitions into telencephalon, diencephalon, and mesencephalon are no longer recognizable (**N**). (Creuzet et al., 2006).

The fate of cranial neural crest cells is determined by the origin and time of their migration. Early-migrating CNCCs form the ectomesenchymal derivatives, while late-migrating CNCCs form the cranial ganglia close to the neural tube. Differentiation of CNCCs that migrate from the diencephalon, the posterior part of the forebrain, in concert with the mesoderm, results in the formation of frontal and parietal bones (Jiang, Iseki, Maxson, Sucov, & Morriss-Kay, 2002).

CNCCs arising from the mesencephalon and rhombomeres r1 and r2 migrate between the eyes to form the palatal shelves and the upper jaw. They also colonize the first pharyngeal arch, also called mandibular arch, which will give rise to the lower jaw and the neurons of the trigeminal ganglion. The second arch, also called hyoid arch, is colonized by CNCCs arising from rhombomere 4. They give rise to the hyoid skeleton and the neurons of the facial nerve. The posterior arches are colonized by cells migrating from rhombomeres r6 and r7. They generate skeletal elements like thymus, thyroid and its parafollicular cells (C cells), and epiglottic cartilages (Lumsden, Sprawson, & Graham, 1991; Schilling & Kimmel, 1994) (**Figure 50**). CNCCs migrating from rhombomeres r3 and r5 deviate rostrally or caudally to join the migrating streams of the even numbered rhombomeres (Sechrist, Serbedzija, Scherson, Fraser, & Bronner-Fraser, 1993).

In addition to mesenchymal derivatives, cranial nerves whose sensory part is built up by the respective NCCs are also formed by pharyngeal arches. The sensory part of the trigeminal nerve (V), the second to the facial nerve (VII) and the third to the glossopharyngeal nerve (IX) are generated by the first arch.

Finally, the cranial neural crest is delimited by the more caudal CNCCs (from rhombomere 7 to somite 3) which actually correspond to the cardiac neural crest cells.

3.4.2 Molecular mechanisms of CNCCs positional identity and migration during craniofacial morphogenesis

3.4.2.1 CNCCs positional identity is orchestrated by transcriptional programs

The positional identity of NCCs subpopulations is governed by a combinatorial set of homeodomain (HD) transcription factors. Here we are going to briefly present some of the molecular mechanisms involved in craniofacial and pharyngeal morphogenesis.

In mouse, *Otx2* is expressed in forebrain NCCs that colonize the frontonasal region and in midbrain NCCs that colonize the distal, mandibular, region of PA1. *Otx2* homozygous mouse mutants lack head structures showing that *Otx2* expression in CNCCs is required for head development. However, this expression needs to be finely tuned and limited to a specific time window (between E8.5 and E10.5 in the mouse). Indeed, upregulation of *Otx2* in CNCCs partially induces severe skull vault defects (Haberland, Mokalled, Montgomery, & Olson, 2009; Kimura et al., 1997; Kuratani, Matsuo, & Aizawa, 1997; Matsuo, Kuratani, Kimura, Takeda, & Aizawa, 1995).

NCCs anterior-posterior (AP) positional identity in pre-migratory progenitors is mediated by the Hox (homeobox) family of transcription factors. Later, the maintenance of Hox gene expression in migrating NCCs and rhombomeres depends on the differential intervention of *Hox* gene enhancers. Consequently, different Hox genes are specifically expressed in different NCCs subpopulations and their rhombomere of origin. For instance, while the anterior limit of the *Hoxa2* expression domain is at the boundary between rhombomeres r1 and r2, NCCs derived from r2 and migrating into PA1 (pharyngeal arch) are devoid of *Hox* gene expression. Another combination of *Hox* genes is expressed in PA2. Interestingly, targeted inactivation of mouse *Hoxa2* resulted in the homeotic transformation of PA2 into PA1-like skeletal elements showing the role of *Hox* combinatorial sets in establishing the AP positional identity of CNCCs (Minoux & Rijli, 2010) (**Figure 59**).

DV patterning of hindbrain NCCs is established through the Dlx (distal-less homeobox) code. In each PA, combinations of *Dlx* transcripts overlap distally but display offset proximal expression limits, which is represented in **Figure 59**. In PA1 for example, *Dlx1* and *Dlx2* are expressed in both the maxillary (upper jaw) and mandibular (lower jaw) processes, *Dlx3* and *Dlx4* expression domains are limited to the distal-most end of the mandibular process, while

Dlx5 and *Dlx6* are expressed only in the mandibular process. Interestingly, when *Dlx1* or *Dlx2* are inactivated on *Dlx5*- or *Dlx6*-deficient backgrounds, the mandibular process derivatives are reduced in size and/or transformed into maxillary-like structures, which reveals synergistic interactions between Dlx paralogue groups (Depew, Simpson, Morasso, & Rubenstein, 2005; Jeong et al., 2008).

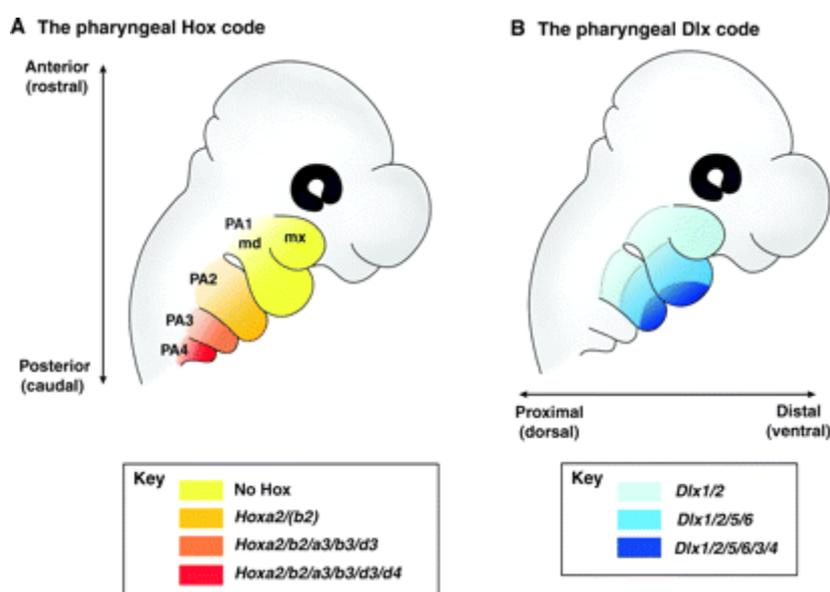


Figure 59: Intrinsic transcriptional programs underlying cranial NCC positional identity.

Schematics of a developing head and pharyngeal regions of a mouse embryo at E10.5. **(A)** The homeobox (Hox) code provides spatial identity (inter-arch identity) along the AP (anteroposterior, rostrocaudal) axis to cranial neural crest cells (NCCs) colonizing the pharyngeal arches (PAs). Each PA is represented by a different colour (see Key) representing its specific Hox expression code. PA1 is devoid of Hox gene expression. The Hox-free molecular program of the PA1 mandibular (md) process represents the PA ground (default) patterning program. In mouse, *Hoxb2* is downregulated in PA2 post-migratory NCCs, and *Hoxb3* and *Hoxd3* are only weakly expressed in PA3. **(B)** The Dlx code provides spatial identity (intra-arch identity) to cranial NCCs along the DV (dorsoventral, proximodistal) axis of PAs. Key: DV-nested expression patterns of Dlx genes in NCCs. Abbreviations: md, mandibular process of PA1; mx, maxillary process of PA1. (Minoux & Rijli, 2010).

3.4.2.2 Environmental signals involved in craniofacial development

3.4.2.2.1 FGF and BMP crosstalk

The spatiotemporal identity of CNCCs is established and maintained by a combination of signaling molecules that control transcriptional programs, leading to the formation of craniofacial structures with the appropriate shape, size and orientation. Among these molecules we are going to mention BMPs (bone morphogenetic proteins from the TGF β family), FGFs (Fibroblast growth factors) and Shh (Sonic hedgehog).

FGFs and BMPs are involved in multiple steps during craniofacial and pharyngeal skeletal morphogenesis. CNCCs potentiate the expression of *Fgf8* in the prosencephalic organizer called ANR (anterior neural ridge) (Creuzet et al., 2006). Besides, FGFs ensure NCCs survival. Indeed, conditional inactivation of *fgf8* in PA1 ectoderm leads to massive NCC apoptosis which results in the absence of most PA1 skeletal elements (Trumpp, Depew, Rubenstein, Bishop, & Martin, 1999). Likewise, in zebrafish, early downregulation of endodermal *fgf3* leads to PA3 and PA4 NCCs apoptosis (David, Saint-Etienne, Tsang, Schilling, & Rosa, 2002; Nissen, 2003).

FGFs are also involved in CNCCs migration and act as chemoattractants. When expressed in the mesoderm and hindbrain, *fgf8* and *fgf3* promote lateral migration of endodermal cells by acting as chemoattractants. This step is important for the correct patterning of NCC-derived skeletal elements (Crump, Maves, Lawson, Weinstein, & Kimmel, 2004; Graham, 2008). In mouse embryos, FGF2 directs mesencephalic NCCs migration and local differences in FGF2 distribution during mesencephalic NCCs migration were observed *in vivo*. Indeed, anti-FGF2 neutralizing antibodies block NCCs migration in NC explants migration assays. Besides, FGF8 promotes the expression of FGF2, and the spatial distribution of FGF8 in the ectoderm during mesencephalic NCCs migration can generate differential FGF2 distribution in the mandibular mesenchyme which is colonized by NCCs *in vivo* (Kubota & Ito, 2000).

Finally, FGFs are involved in the specification of NCCs spatial identity and in establishing the AP and DV polarity of pharyngeal arches. They control the differential expression of transcription factors by diffusing into surrounding tissues and cooperate with BMPs.

In this context, at early developmental stages, the presumptive maxillo-mandibular region and the premandibular domain are segregated by *Fgf8* and *Bmp4*. Before mesencephalic

NCCs arrive at their destination, *Fgf8* which is expressed in the ectoderm, prefigures the prospective oral cavity. This *Fgf8* expression domain is induced by sonic hedgehog (shh) signalling from the endoderm. This domain is also delimited by *Bmp4* expression on both sides of the adjacent *Fgf8*-expressing ectoderm. Thereby, ectodermal *Fgf8* and *Bmp4* control the regionalization of incoming NCCs by activating specific patterning genes in the underlying mesenchyme: *Fgf8* induces the expression of *Dlx1* and *Barx1*, while *Bmp4* induces *Msx1* (muscle segment homeobox like 1) expression. These interactions between the ectoderm and the mesenchyme are crucial for specifying the identity of the pre-mandibular and maxillo-mandibular regions. In addition to its role in establishing AP and DV PA identity, *Fgf8* controls left-right symmetry of the craniofacial skeleton (Minoux & Rijli, 2010).

3.4.2.2.2 Sonic hedgehog signaling in craniofacial development

Severe head skeleton malformations, including holoprosencephaly and cyclopia, were observed in chick, mouse and zebrafish following Shh inhibition. These abnormalities result from defects in NCCs survival, proliferation and patterning. Shh is expressed in the foregut endoderm, the neuroepithelium and the facial ectoderm. Patterning signals from the foregut endoderm provide NCCs with information about the size, shape and orientation of the skeletal elements that are generated in the first and more posterior PAs. Loss of Shh expression in the ventral foregut endoderm leads to massive NCCs apoptosis which prevents the development of Meckel's cartilage and associated PA1 structures. In addition to its role in foregut endoderm, Shh signaling arising from the ventral brain primordium is required in zebrafish for the formation of the anterior neurocranium and upper jaw cartilages. Shh signaling from the facial ectoderm is required for NCCs spatial identity specification. The frontonasal ectodermal zone (FEZ) is a signalling center in the ectoderm overlying the FNP (frontonasal process). It is defined by the juxtaposition of *Fgf8* and Shh expression domains and controls the growth and DV polarity of the upper beak in birds. Ectopically grafted FEZ can reprogram the developmental fate of the underlying NCCs. Consequently, the upper beak is duplicated with a DV polarity that reflects the orientation of the grafted tissue. Interestingly, when the mandibular NCCs come in contact with the FEZ, they form an additional lower (and not upper) beak. Thus, the epithelial-mediated patterning instruction is interpreted by the NCCs according to their relative AP positional identity (Minoux & Rijli, 2010).

3.4.2.3 Cranial neural crest cells migrational cues

Cranial NCCs migrate along stereotypical pathways that are conserved among vertebrates. NCCs that originate from the diencephalon and anterior mesencephalon colonize the frontonasal process (FNP). More caudal CNCCs from the posterior mesencephalon and hindbrain, which is transiently subdivided into rhombomeres (r), colonize the PAs.

While migrating into PAs, CNCCs from different rhombomeres remain spatially segregated through their streams, and contribute to skeletal elements. In most vertebrates, crest-free regions lateral to r3 and r5 separate hindbrain NCCs migratory streams. Surrounding signals control each stream into well-defined pathways. Among these signals, repulsive interactions between the Ephrin tyrosine kinase receptors (Eph) and their ephrin ligands (eph), and between the transmembrane neuropilin (Nrp) receptors and their secreted semaphorin (Sema) ligands are involved in delimiting the NCC migratory streams (Minoux & Rijli, 2010) (**Figure 60**). For example, in zebrafish, NCCs destined to colonize PA1-3 express *nrp2a* and *nrp2b*. They avoid cells expressing *sema3f* and *sema3g* located in the NCC-free zones lateral to r3 and r5 (Yu & Moens, 2005). Directional guidance of CNCCs to their final destination is regulated by transcription factors such as Twist (expressed in the pharyngeal mesenchyme) and Tbx1 (T-box 1) (expressed in the mesodermal core of the PAs and in the endodermal pouches). In addition, both Twist and Tbx-1 are required for the proper segregation of PA1 and PA2 NCCs streams (Moraes, Nóvoa, Jerome-Majewska, Papaioannou, & Mallo, 2005; Soo et al., 2002; Vitelli, 2002).

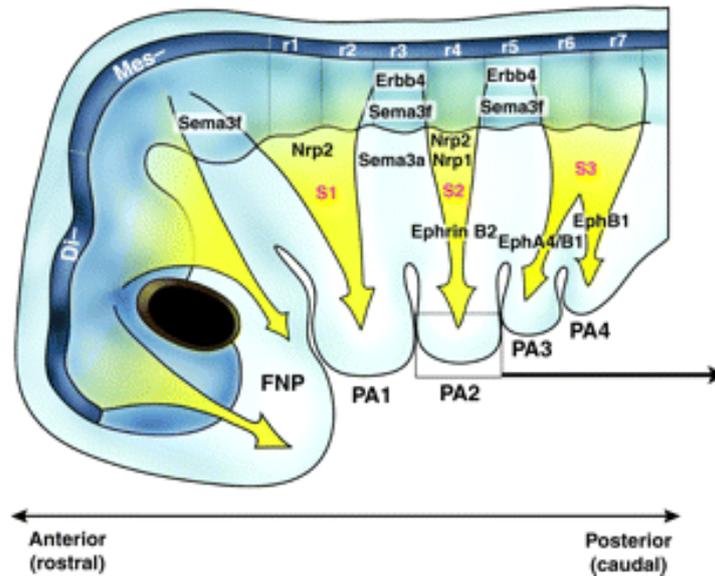


Figure 60: Segmental and directional migration of cranial neural crest cells.

Segmental migration of cranial neural crest cells (NCCs) in a representative vertebrate embryo. The yellow arrows represent the patterns of migration of diencephalic (di-), anterior and posterior mesencephalic (mes-), and rhombencephalic NCCs into the frontonasal process (FNP) and pharyngeal arches 1-4 (PA1-4). The NCCs migrate in three individual streams: S1, S2 and S3. NCCs from the posterior mesencephalon, rhombomere 1 (r1) and r2 fill the first pharyngeal arch (PA1), whereas NCCs from r4 fill the second pharyngeal arch (PA2). In the post-otic hindbrain, NCCs from the r6-r8 region colonize indifferently PA3-6, with PA3 being mainly contributed by r6 NCCs. Some of the molecular mechanisms involved in establishing and maintaining the migration of segmentally restricted NCC streams into the PAs are also shown. The spatial expression patterns of ErbB4 (v-erb-a erythroblastic leukemia viral oncogene homologue 4), neuropilin/semaphorin 3 (Nrp/Sema3) and ephrin B2/EphA4 (Eph receptor A4)/EphB1 in the neural tube, the NCCs and their surrounding mesenchyme correspond to those described in chick, mouse and *Xenopus*. (Minoux & Rijli, 2010).

3.5 Conclusion

In summary, NCCs are essential for the head development in vertebrates. They arise at the margin between the ectoderm and the neural ectoderm, delaminate, undergo EMT and migrate into different regions of the embryo. They then differentiate into a wide range of cell types. Their appropriate positioning in the AP axis defines their fate. NCC subpopulations are guided by complex sets of cues to which they respond locally along their ontogenesis. Understanding the molecular mechanisms underlying NCCs ontogenesis is essential to better understand and apprehend neurocristopathies as well as metastatic processes.

II. Results: Project 1

Role of the LKB1 signaling pathway in vertebrates head development

Scientific context and results summary

As presented in the introduction section, surgical ablation of the cranial neural crest (CNC) in the avian embryo leads to neural tube closure defects, exencephaly and absence of the facial skeleton (Creuzet et al., 2006). Although the molecular mechanisms governing CNC formation and induction have been extensively studied, little is known regarding cranial neural crest cells (CNCCs) migratory properties. Interestingly, homozygous inactivation of *Lkb1* in mice is lethal and leads to neural tube closure defects, absence of the first branchial arch and facial reduction (Ylikorkala et al., 2001), a phenotype that virtually mimics CNC dysgenesis in the avian embryo. Together, these data suggest a role of the LKB1 signaling pathway in CNCCs ontogenesis. During my PhD, I have contributed to this project and our results are reviewed at PlosOne.

Using the tractable avian system that allows us to combine microsurgery procedures of chick embryos to *in ovo* modification of CNCCs by electroporation, we found that LKB1 is essential for head development. *LKB1* silencing by RNA interference of CNCCs disturbed their polarized migration and subsequently compromised their survival thereby leading to craniofacial defects. The malformations include a severe reduction of the telencephalic vesicle and alteration of the development of naso-frontal and maxillo-mandibular structures. These observations were confirmed through the development of a mouse model in which genetic ablation of *Lkb1* in CNCCs, at the onset of their emigration from the neural tube, also led to craniofacial abnormalities. We also showed that the LKB1 signaling pathway, which is involved in CNCCs for these morphogenetic events, requires the sequential activation of AMPK, the ROCK kinase and the actin molecular motor Myosin-II.

Understanding the molecular mechanisms underlying CNCCs development is of particular interest in order to better understand the underlying mechanisms responsible for craniofacial abnormalities which represent one-third of all congenital birth defects (Dixon et

al., 2006). In this setting, our study sheds light on the essential role of the LKB1 signaling pathway in CNCCs ontogenesis and on the molecular processes that shape the formation of the head in vertebrates.

Rôle de la voie de signalisation de LKB1 dans le développement de la tête chez les vertébrés

Contexte scientifique et résumé des résultats

Comme présenté dans l'introduction, l'ablation chirurgicale de la crête neurale céphalique (CNC) chez l'embryon aviaire conduit à des défauts de fermeture du tube neural, une exencéphalie ainsi qu'une absence du squelette facial (Creuzet et al., 2006). Bien que les mécanismes moléculaires régissant la formation de la CNC et son induction aient été largement étudiés, nous disposons de peu de données concernant les propriétés migratoires des cellules de la crête neurale céphalique (CCNC). De manière intéressante, l'inactivation homozygote de *Lkb1* est létale chez la souris et conduit à des défauts de fermeture du tube neural, à l'absence du premier arc branchial ainsi qu'à une réduction de la face (Ylikorkala et al., 2001), un phénotype mimant virtuellement la dysgénésie de la CNC observée chez l'embryon aviaire. Ces données suggèrent donc un rôle de la voie de signalisation de LKB1 dans l'ontogénèse des CCNC. Au cours de ma thèse, j'ai contribué à ce projet et nos résultats sont en révision à PlosOne.

Nous avons basé notre étude sur le système aviaire afin de profiter des techniques de microchirurgie utilisables avec l'embryon de poulet et de la possibilité de modifier les CCNC *in ovo* en les ciblant par électroporation. Avec cette approche combinant ARN interférence et transfection de vecteurs d'expression, nous avons établi que LKB1 est essentiel pour le développement de la tête. L'inactivation de *LKB1* dans les CCNC altère leur migration polarisée et entraîne leur mort, conduisant ainsi à des malformations crânio-faciales. Ces anomalies développementales comprennent une réduction de la vésicule télencéphalique et des défauts de formation des structures naso-frontales et maxillo-mandibulaires. Ces observations ont été confirmées via la génération d'un modèle de souris dans lequel l'inactivation conditionnelle de *Lkb1* dans les CCNC, juste avant leur migration, aboutit à des défauts de formation de la tête similaires à celles observées chez le poulet. Finalement, nous avons montré que la voie de signalisation LKB1, qui est essentielle dans les CCNC pour ces

processus morphogénétiques, requiert l'activation séquentielle de l'AMPK, la kinase ROCK et le moteur moléculaire de l'actine, Myosine II.

La compréhension des mécanismes moléculaires gouvernant le développement des CCNC est un enjeu médical important puisque plus d'un tiers des pathologies congénitales impliquent des malformations crânio-faciales (Dixon et al., 2006). Dans ce contexte, nos travaux ont permis d'identifier une nouvelle voie de signalisation contrôlée par le suppresseur de tumeur LKB1 dont la fonction est essentielle pour la formation de la tête chez les vertébrés.

**LKB1 signaling in cephalic neural crest cells
is essential for vertebrate head development**

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Abstract

Development of the head in vertebrates is a developmental process that involves extensive migration of cephalic neural crest cells (CNCC) that colonize cephalic structures to give rise to most of the craniofacial skeleton. Here, we report that the silencing of the *LKB1* tumor suppressor affects the polarized migration and the survival of CNCC, thus resulting in severe craniofacial defects. We further show that the LKB1-mediated effects in CNCC involve the sequential activation of the AMP-activated protein kinase (AMPK), the Rho-dependent kinase (ROCK) and the actin-based motor protein myosin II. Collectively, these results establish that the morphogenetic processes governing head formation critically depends on the LKB1 signaling network in CNCC.

Introduction

The neural crest (NC) is a transient embryonic structure that arises at the dorsal lips of the folding neural tube [1]. Cephalic NC cells (CNCC) constitute a population of invasive multipotent cells that originate from mid- and hindbrain levels, and give rise to a large part of the head skeleton as well as to musculo-connective derivatives [2]. These embryological observations coupled with paleontological arguments suggested the emergence of the NC as an evolutionarily novelty – one that was key to the development of the “new head” that epitomizes vertebrates [3, 4]. Once specified, CNCC undergo an epithelium to mesenchymal transition (EMT), delaminate from the neuroepithelium and migrate into three main streams that colonize the frontonasal bud, the branchial arches and the heart [5]. Although the persistent directionality and polarized morphology of migrating CNCC have been recognized for a long time, it is only recently that a series of studies have conclusively shown that CNCC migrate collectively rather than individually [5]. Furthermore, CNCC exhibit contact inhibition of locomotion (CIL), a phenomenon by which cells change their direction of migration after contact with another cell [6]. Interestingly, CIL contributes to the polarized migration of the cells by confining the extension of cell protrusions to the free edge, thereby reinforcing their ability to respond to chemoattractants [6]. Yet, our knowledge of the signaling networks that govern the migratory properties of NCC is still limited.

Inactivating germ line mutations of the *LKB1* tumor suppressor gene are responsible for an inherited-cancer condition called Peutz-Jeghers syndrome [7]. *LKB1* codes for a serine/threonine kinase that regulates cell polarization and acts as a metabolic sensor by phosphorylating and activating the AMP-activated protein kinase (AMPK) [8-10]. In addition, *LKB1* phosphorylates 12 additional AMPK-related kinases that are involved in distinct biological processes including the regulation of hepatic gluconeogenesis as well as the polarization and axon branching of cortical neurons [8-10]. *LKB1* associates with the pseudokinase STRAD (STRAD α or STRAD β) and the scaffolding molecule MO25 to form the *LKB1* holoenzyme complex [8-10]. Homozygous disruption of *LKB1* in mice causes a defect in neural tube closure and an absence of the first branchial arch [11-12], a phenotype compatible with a dysgenesis of CNCC. These data suggest that the *LKB1* pathway may be involved in the ontogenesis of CNCC.

To gain insight into this question, we used the tractable system provided by the avian embryo. With this model, we found that the *LKB1* signaling pathway controls both the polarized migration and the survival of CNCC. As a consequence of *LKB1* inactivation and defective CNCC migration, expression of morphogens that pattern the prosencephalic region was abrogated in the anterior neuroepithelium and inhibited forebrain development. Consistent with these observations, we found that genetic ablation of *lkb1* in mouse neural crest cells at the time of their emigration from the neural primordium led to severe craniofacial defects. Finally, delineation of the *LKB1* pathway active in CNCC revealed that the signal converges on myosin II via AMPK and ROCK kinases. Collectively, our results establish that the *LKB1* network orchestrates several aspects of CNCC development that are crucially required during cephalogenesis.

Results

For the purpose of this study, we cloned and sequenced the chicken homologue of *LKB1* cDNA. Sequence analysis revealed that the human and chicken *LKB1* proteins are 90% identical at the amino acid level, and all of the phosphorylation sites and post-translational motifs that have been mapped in human *LKB1* are conserved in the chick homologue. To determine *LKB1* gene expression patterns at neurula stages, we performed a series of in situ hybridization analyses. The *LKB1* transcript was detected at 6 somite stage (ss) when the neural folds elevate before the neural tube closure. The accumulation of *LKB1* transcripts intensified at later stages and was detected throughout the neural primordium before the egress of CNCC from the neural tube at 5-8ss (Figures 1A-C). From this stage on, *LKB1* expression was detected in the migrating CNCC, which progressed along the cephalic vesicles and populated the naso-frontal and maxillo-mandibular regions, as well as the more caudal branchial arches (Figures S1A-D).

To selectively inhibit *LKB1* expression in CNCC, we used the RNA interference approach to bilaterally electroporate the cephalic neural folds with *dsRNA* molecules before the emigration of CNCC out of the neural tube [13, 14]. Using rhodamine-dextran to label transfected cells, we verified that this electroporation procedure selectively transfected the rostral dorsal region of the neural folds and consequently specifically targeted the delaminating CNCC (Figures 1D,E). Next, *dsRNA* targeting *LKB1* were electroporated in embryos and forty-eight hours post-transfection (at E5 stage) we observed that the nasofrontal and maxillo-mandibular processes were dramatically underdeveloped (Figures 1F,G). Fifty-six embryos were analyzed in 12 independent experiments and the phenotypic effects observed were statistically robust with little variability from embryo to embryo. Micrographs of transmission electron microscopy (TEM) of embryos at E5 revealed that the silencing of *LKB1* in CNCC compromised the development of nasofrontal structures which appeared distorted and eventually failed to fuse with the maxillary processes (Figures 1H, I). To visualize the effects of the loss of *LKB1* on the formation and the migration of CNCC, we stained embryos with the monoclonal antibody HNK1 that recognizes a glycolipid epitope expressed on chicken pre-migratory and migratory NCC [15]. As shown in Figure 1K, a marked reduction of the CNCC population that colonizes the frontonasal region as well as the trigeminal ganglion was observed upon

LKB1 knockdown at 30ss. To address further the role of *LKB1* in head skeletogenesis, we used Alcian blue and Alizarin red to label cartilage and ossified bone, respectively. At E11, the chondrogenic differentiation of the nasal septum and capsule and Meckel's cartilages of E11 *LKB1*-deprived embryos were significantly reduced and the osteogenic differentiation was totally hampered (Figures 1 L,M). Finally, to ascertain that the phenotypic effects observed were specific to the silencing of *LKB1*, we coelectroporated *dsRNA-LKB1* together with a vector expressing the human *LKB1* insensitive to the *dsRNA* targeting its chicken homologue. Using this strategy, we observed a complete rescue with the human *LKB1* wild type, whereas the kinase- dead *LKB1* failed to restore proper cephalic development (Figures 1N,O and Figures S2A-C). Cumulatively, these results indicate that *LKB1* activity is required for the development of CNCC.

Next, we set up experiments to reproduce the anencephalic phenotype reminiscent of either ablation of CNCC in birds [14, 16] or *LKB1* null-mutation in mice [11]. To do so, *LKB1-dsRNA* was bilaterally transfected at 1ss at the neural plate border (Figures S2D, E). When performed in early neurula, the inhibition of *LKB1* generated consistent neural tube defects and resulted in anencephalic embryos. These observations indicated that the temporal perturbations in *LKB1* activity elicited the spectrum of dysmorphology recorded in *LKB1* null-mice.

To explore whether the phenotypic effects observed in avians could be confirmed in another experimental model, we deleted the two murine *lkb1* alleles [12] with a transgene containing the human tissue plasminogen activator (*Ht-PA*) promoter that drives the expression of the *Cre* recombinase (Figure S3A). The *Ht-PA::Cre* transgene targets CNCC at the onset of their migration from the neural tube and is expressed in all CNCC-derivatives [17]. Mutant neonates were unable to feed normally and died during the first day after birth. These animals showed a reduced stature and craniofacial abnormalities including a shortened nose and a micrognathia (Figure S3B). Staining with Alizarin red and Alcian blue to respectively mark ossified bones and cartilages provided evidence that the skull was smaller and the length of the maxillary and mandibular bones that are CNCC-derived was shorter in mice deleted for *lkb1*. Furthermore, the anterior fontanel was wide open, accounting for the lack of mesotopic, coronal and sagittal sutures, while the frontal bones that also derive from CNCC were fused together in control animals (Figures S3C and

S3D). Thus, these findings confirm that in absence of *lkb1*, the skeletogenic properties of mouse CNCC are severely affected.

To determine how *LKB1* silencing specifically affects the ontogenic capacity of CNCC, we focused on the earliest stage at which *LKB1* silencing altered the behavior of these cells. As early as 8 hours after *LKB1-dsRNA* transfection, HNK1 labeling revealed a striking reduction in the population of CNCC that have emigrated from the neural tube (Fig. 2A, B). To further examine the consequences of *LKB1* silencing on CNCC development, we studied the expression pattern of both the transcription factors AP2 and the homophilic adhesion molecule N-cadherin on neural tube sections at 10 ss. AP2 α and AP2 β are expressed in premigratory and migratory CNCC and the former is required to maintain the specification of CNCC [18, 19]. N-cadherin is located at the apicolateral junctions of the neuroepithelium and expression of this adherens junction protein is downregulated in the neural folds prior to the emigration of CNCC, thereby contributing to the delamination of these cells from the neural primordium [20, 21]. As shown in Figure 2 (Figures 2 C-H), labeling with the anti-AP2 antibody showed that *LKB1* depletion led to a marked reduction of the CNCC population migrating underneath the surface ectoderm (Figure 2D). Concomitantly, the expression of N-cadherin in the dorsal neural folds was sustained upon *LKB1* silencing. Collectively, these data indicate that *LKB1* is required for the delamination process.

Since *LKB1* is a regulator of cell polarity, we further examined whether the loss of this molecule impacted on the directional migration of CNCC. The reorientation of the Golgi apparatus together with the centrosome is known to occur during cell polarization [22]. Moreover, *LKB1* controls the morphology and the asymmetric distribution of the Golgi at the base of the dendrites of hippocampal neurons, thereby governing polarized dendritogenesis [23]. Using an antibody labeling the Golgi matrix protein GM130 and considering the dorso-ventral direction of CNCC migration, we found that the Golgi complex was consistently located behind the nucleus, at the rear of the cells with respect to the front edge of the migrating CNCC. Strikingly, *LKB1* silencing resulted in a randomized positioning of the Golgi (Figures 2I,J). Further quantification of this effect confirmed that the polarized positioning of the Golgi was significantly disrupted upon *LKB1* knockdown (Figure 2K). Altogether, these results indicate that *LKB1* acts on the recruitment of CNCC

from the neural primordium, promotes their delamination and, once these cells have detached from the neural tube, controls their front-rear polarity.

The defects of CNCC polarization led us to explore the viability of *LKB1*-deprived CNCC. Using LysoTracker to stain apoptotic cells, we found that CNCC egress from the neural tube is rarely accompanied by cell death events which are mostly restrained to the anterior mesencephalic region (Figure S4A). In stage-matched experimental embryos, *LKB1* silencing at 5ss triggered expansion of cell death at the expense of the entire pre-otic neural folds at 7ss, and simultaneously affected the diencephalic, mesencephalic, and anterior rhombencephalic CNCC (Figure S4B). CNCC are known to potentiate *FGF8* expression in the anterior neural ridge (ANR) [16], considered as the prosencephalic-organizing center [24]. Reciprocally, *FGF8* exerts a trophic and chemotactic effect on CNCC [16, 25, 26]. Concomitant to the massive CNCC death in *LKB1*-dsRNA-treated neurula, *FGF8* expression was drastically reduced in the ANR (Figures S4C,D). Change in *FGF8* expression upon *LKB1* silencing was confirmed by qRT-PCR (Figure S4E). Furthermore, expression of three additional genes coding for morphogenetic molecules (Sonic hedgehog (SHH), *Wnt1* and *Wnt8b*) whose activity in the brain is associated with the migration of CNCC [14] was also perturbed. Quantification of these results showed that *LKB1* silencing significantly affected the expression of *FGF8*, *WNT1*, *WNT8b* and *SHH* (Figure S4E), a condition reminiscent of what has been previously described when CNCC are ablated [14, 16].

LKB1 is a master kinase that phosphorylates and activates AMPK and 12 related members of the AMPK family. Although each of these AMPK kinases, either singly or in combination, may relay the *LKB1* signal in CNCC, previous studies have provided evidence that AMPK regulates the assembly of tight junctions, the dynamics of actin cytoskeleton and participates in the mechanisms of hepatocyte and neuronal polarization [27-31]. These findings prompted us to explore the role of AMPK in CNCC ontogeny. To this end, we cloned the cDNAs coding for the two chicken *AMPK* α -subunits, termed $\alpha1$ and $\alpha2$. *In situ* hybridization revealed that *AMPK* $\alpha1$ and $\alpha2$ have an overlapping pattern of expression during neurulation with a strong labeling of the rostral neural folds at 5ss stage (Figure S5). *DsRNAs* targeting $\alpha1$ and $\alpha2$ in CNCC were then electroporated either alone or in combination. No phenotype was observed upon silencing of each *AMPK* α subunit but the combination of both *dsRNAs* led to a marked disorganization of the cephalic region

with an extensive lack of facial structures (Figures 3A, B). These results demonstrate that AMPK exerts a crucial role during head morphogenesis in chicken and suggest that this kinase transduces the LKB1 signal in CNCC. To examine this question, we assessed whether electroporation of two distinct constitutively active forms of AMPK (AMPK- α 2 CA; AMPK- γ 1 CA) [32, 33] in CNCC was able to rescue the *LKB1-dsRNA* phenotype. As shown in Figure 3, expression of each AMPK-CA fully restores normal cephalogenesis in *LKB1*-silenced embryos (Figures 3C-G). Furthermore, expression AMPK- γ 1 CA in *LKB1* knockdown interfered with CNCC apoptosis (Figures 3H-J) and rescued *Fgf8* expression in the ANR (Figures 3K-M), along with other morphogens (Figure S4E). Overall, these findings confirmed that LKB1 effects on morphogenetic processes governing head formation is conveyed in CNCC through the activation of AMPK.

LKB1 is known to control cell polarity through the activation of the actin molecular motor myosin II [34, 35]. The activity of myosin II and the stability of myosin II filaments are upregulated by phosphorylation of the Myosin II Regulatory Light Chain (MRLC) at two sites corresponding to Thr-18/Ser-19 in humans [36]. In *Drosophila*, expression of a phosphomimetic mutant form of MRLC rescues cell polarity defects generated by the deletion of either *LKB1* or *AMPK* genes [34]. Furthermore, AMPK has been found to regulate MRLC phosphorylation through its opposing effect on the phosphatase PP1CB-PPP1R12C complex and the p21-activated protein kinase 2 (PAK2) [36]. These results prompted us to investigate the role of MRLC in CNCC development. Embryos subjected to *LKB1* silencing were co-electroporated with vectors encoding mutant forms of MRLC in which both Thr-18 and Ser-19 were replaced by phosphomimetic aspartate (MRLC-DD) [37]. Expression of MRLC-DD resulted in a complete restoration of brain development and facial morphogenesis in *LKB1* knockdown embryos (Figures 4A-C). Since MRLC is not a direct AMPK substrate [38], we focused our attention on the Rho-associated protein kinase (ROCK). This kinase is implicated in CNCC delamination [39-41], regulates the phosphorylation of MRLC and has been proposed as an AMPK effector [38]. In a first approach, we inhibited ROCK pharmacologically with the Y-27632 compound [42]. Microinjection of this molecule into the neural tube at 5ss led to a reduction of facial and cephalic structures that virtually reproduced the *LKB1* loss of function phenotype (Figure 4D). In a second approach, co-electroporation of *LKB1-dsRNA* together with a constitutively active form of the ROCK1 isoform restored the cephalic development of

LKB1-depleted embryos whereas a ROCK1 kinase-dead mutant failed to rescue the cephalic phenotype (Figures 4E, F, I). Finally, to determine the epistatic position of ROCK within the LKB1-AMPK-myosin II pathway, we carried out a series of rescue experiments. We first observed that the cephalic defect obtained upon *dsRNA* targeting *AMPK α 1* and *AMPK α 2* was rescued when the constitutively active form of ROCK1 was co-electroporated (Figure 4I). Expression of the constitutively active form of AMPK (AMPK- γ 1 CA) did not restore proper cephalic development in embryos treated with Y-27632, whereas MRLC-DD was largely able to correct the shortening of the prosencephalic region induced by this compound (Figures 4G, H, J). Thus, taken together these data place ROCK activity downstream of AMPK and upstream of myosin II in the LKB1 signaling cascade operating in CNCC.

Discussion

By combining experimental approaches in chick and mouse, we have established that the *LKB1* network controls the fate of CNCC by acting at different stages of their development. *LKB1* directs forebrain formation via its cumulated effects on the delamination of pre-migratory CNCC, on their directional migration and their survival. As a consequence of *LKB1* silencing, the morphogenesis of cranial skeleton structures that derive from CNCC is severely impaired. Consistent with this observation, expression of several diffusible molecules that are required for the patterning of the prosencephalic region and play a key role in the vertebrate head ontogenesis, such as *FGF8*, *Wnt1* and *Wnt8*, is abrogated in the anterior neuroepithelium. Finally, we show that the developmental signal transduced downstream of *LKB1* in CNCC involves the sequential triggering of *AMPK*, *ROCK* and *myosin-II*.

Disruption of *LKB1* before the onset of CNCC emigration from the neural tube results in CNCC hypoplasia as attested by the marked diminution of the cell population expressing the markers *HNK1* and *AP2*. Furthermore, upon *LKB1* depletion, a persistent expression of *N-cadherin* at 10ss was observed in the neuroepithelium. These findings are consistent with a defect in the inductive molecular events that contribute to the delamination process. The switch of cadherin expression, from type I cadherin (*N-cadherin*) associated with stable intercellular contacts to type II cadherins with weaker adhesiveness (*Cadherin-6/7/11*) is assumed to prevent the mixing between NCC and non NCC in pre-migratory NC territory and to favor NCC delamination [5, 43]. Although the *LKB1* pathway participates to the molecular mechanisms controlling delamination, we also observed that a substantial proportion of CNCC egressed from the neural tube even in the absence of *LKB1*. Thus, alternative pathways may supersede the lack of *LKB1* and could involve the web of transcription factors including *Ets1*, *Id2*, *Lsox5* and *p53*, known to control CNCC delamination [5].

Using the direction of the Golgi-nucleus axis as a marker of cell polarity, we found that the *LKB1-AMPK* pathway controls CNCC polarity. We also recorded a marked apoptosis of these cells upon *LKB1* silencing, an observation consistent with the cell death observed in various physiological settings when *LKB1* is ablated [44, 45]. We posit that by

ensuring the directional guidance of CNCC, the LKB1 polarizing signal promotes the survival of CNCC that become exposed during their long-range migration to the chemotactic and trophic effects of FGF8 [16]. Reciprocally, it is anticipated that the LKB1 pathway regulates the prosencephalic development via its effect on the directional migration and survival of CNCC that interact with the ANR and controls the territory of FGF8 expression as already demonstrated in a different experimental setting [25]. Although this remains speculative, the demonstration that the genetic ablation of *lkb1* in mice after the delamination of CNCC results in severe craniofacial abnormalities, provides evidence that the contribution of LKB1 in both the directional migration and the survival of CNCC is critical for the morphogenesis of the head.

We show here that AMPK relays the signal downstream of LKB1 in CNCC. AMPK is activated by metabolic stresses that decrease the ATP:AMP intracellular ratio [9]. It is known that low-oxygen tension that triggers AMPK activation characterizes the permissive microenvironment of migrating CNCC [46]. Accordingly, the hypoxia-inducible factor 1 α (HIF-1 α) is expressed in the cephalic mesenchyme, and CNCC do not colonize branchial arches in *HIF1 α* -null mouse embryos [47]. Thus, the LKB1-AMPK signaling node may sense environmental cues and convert this information into morphogenetic outputs. Although, our study establishes a role for AMPK downstream of LKB1 in CNCC, we cannot rule out the contribution of other AMPK-related kinases, such as NUA1 and NUA2 whose double inactivation in mice leads to exencephaly and facial clefting [48].

We also report in this study that LKB1 regulates the actomyosin II dynamics in CNCC through the activation of ROCK and the subsequent phosphorylation of MRLC. These data are in agreement with previous reports that uncovered a role for RhoA and ROCK downstream of LKB1 in actin filament assembly and in actomyosin II contractility [35, 49]. It is also established that the *Caenorhabditis elegans* orthologue of LKB1 controls cell polarization and cytokinesis during the early stage of the worm embryonic development via its effect on annilin and non-muscle myosin [50]. In addition, the Rho-ROCK signaling is required for the apical detachment of truncal NCC [41]. Here, we show that LKB1 controls actomyosin II in CNCC through an AMPK-dependent activation of ROCK. Yet, it remains unclear how ROCK is activated via LKB1 in CNCC and what is the respective contribution of ROCK1 and ROCK2 isoforms [51] downstream of LKB1. As already shown in

epithelial cells, it is possible that LKB1 induces the exchange of GDP for GTP on Rho GTPases via the Dbl RhoGEF [49]. Our rescue indicate show that AMPK exerts a specific action upstream of ROCK and may thus directly modulate the activity or localization of Rho exchange factors expressed in CNCC [52]. If AMPK is known to regulate MRLC phosphorylation through its opposing effect on the phosphatase PPP1R12C and the kinase PAK2 [36], our data establish that ROCK acts downstream of AMPK and controls MRLC phosphorylation, possibly through the activation of the MLC kinase or through the inhibition of the myosin phosphatase [53].

In conclusion, our study shows that the formation of the head in vertebrates critically depends on the activation of the LKB1 network in CNCC. Bi-allelic deletion of the *STRAD α* gene is responsible for an inherited disease called polyhydramnios, megalencephaly, and symptomatic epilepsy (PMSE) [55]. All PMSE patients present craniofacial abnormalities [54], a genetic trait evoking a defect in CNCC development. Thus, our findings raise the idea that a decrease of LKB1 signaling in CNCC owing to the loss of *STRAD α* is the causative origin of the dysmorphic traits of PMSE patients and further suggest that perturbations of this pathway might be implicated in the etiology of other human NC disorders.

Methods

Chick embryos were used as a model throughout this study and were operated according to techniques previously described [25].

Gene silencing in avian embryos. Double-strand RNA (dsRNA) were synthesized from cDNA encoding the targeted genes: *LKB1*, *AMPK α 1*, *AMPK α 2*. Delivery of RNAi molecules was achieved by *in ovo* electroporation using a triple electrode system. For each series, dsRNA was directed towards CNC by a series of 5x27V square pulses (T830 BTX, Genetronics, CA). Solutions of non-annealed sense and anti-sense RNA strands were used for control series and transfected according to the same paradigm. When needed, electroporations were performed at 1ss to target the neural plate border. For the characterization of each phenotype the brain growth was evaluated on 6 embryos. The head of the embryo were half-cut embryos and we measure the distance between: the optic stalk and the dorsal telencephalon (Tel); the optic stalk and theepiphysis (Di); the optic stalk and the mesencephalon (Mes).

Constructs: Chick *LKB1*, *AMPK α 1*, and *α 2* sequences were amplified from E8.5 embryo cDNA using primers:

LKB1 (For-ctcgagttaaggaacttaatccaggatg; Rev-ggatcctgctcccggctcactg), *AMPK 1* (For-gcagtgaagatcctgaatcgac; Rev-tagagaataaccccactgctcc) and *AMPK α 2* (For-tcggcaccttcggcaaagtc; Rev-agggtgggcacatgctcatc) designed based on GenBank sequences NM_001012918, NM_001039603.1, and NM_001039605.1 and cloned to pGEM[®]-T Easy vector (Promega) according to the manufacturer's instructions.

Quantitative real-time PCR analysis of gene expression. Total RNA from 14HH stage embryonic head was extracted using RNeasy Plus Mini kit (Macherey-Nagel). Quantifications were performed on 4 controls, 3 *dsLKB1*-treated and 4 rescued embryos. Two micrograms of total RNA were transcribed with oligo-dT primers using MMLV reverse transcriptase (Promega). *Fgf8* (For-gtacgagggctggtacatgg; Rev – cggttgaaggggtagttgag), *Wnt1* (For-aaatgggcactgggtgtct; Rev-cctcagggtcatctacgg), *Wnt3a* (For-ggagatcatgccagcgtag; Rev-gcggattccctgtagcttt), *Wnt8b* (For- gaactgcagcctgggagatt; Rev-tctccaggcatccacaac) and *Shh* (For-gctgcaaggacaagctgaa; Rev-ggccagcattccgtacttg) primers

were customized to be specific for each mRNA species and synthesized by Invitrogen. Quantitative PCR reactions were performed using LightCycler® carousel-based system (Roche).

Each biological sample was subjected to the assay in triplicates per gene. Ct values were obtained by using Promega software (v.2.0.4). Fold changes were calculated using the relative quantification ($\Delta\Delta Ct$) and normalized on GAPDH gene activity.

Statistical analyses were performed with the GraphPad Prism5.0 software assuming a confidence interval of 95%. Data collected for all the independent observations were compared using the non-parametric significance test of Mann–Whitney U.

Rescue experiments. To investigate the functionality of *LKB1* pathway in CNC cells, we challenged rescue of *LKB1* hypomorphic phenotypes by co-electroporating *LKB1- dsRNA* with the following constructs: p-AMPK γ 1, p-AMPK- α 2 (gifts from Benoît Viollet), p-Un-phospho-MLC, p-Pseudo-phospho-MLC [37], p-Rock-KD, p-Rock-CA (gifts from Pierre Roux).

Pharmacological treatment. A solution of Rock inhibitor Y27632 (50 μ M in PBS)(Calbiochem) was blown in the cephalic neural groove of 3 to 5ss embryos *in ovo*. Control embryos were injected with PBS only. As for nucleotide injection, the solutions were contrasted with Fast Green FCF (Sigma) to visualize the injection site.

Embryo processing. Control and experimental embryos were collected at 7ss, 10ss and 25ss (i.e. 8 or 24 hours post-transfection) and subjected to whole-mount immunocytochemistry or Immunostaining on section with HNK1 (Santa-Cruz Biotechnology) AP2 (3B5, DSHB) and N-Cam (3B5, DSHB) (6B3; DSHB) monoclonal antibodies or *in situ* hybridizations with *LKB1*, *Fgf8*, *AMPK α 1*, and *AMPK α 2* probes. Cell death detection was performed as described.

Scanning electron microscopy. Embryos were dissected in PBS and fixed in formaldehyde 4% overnight at 4°C, briefly washed in PBS, and then transferred in

7% sucrose solution in PBS for 48h at 4°C for cryo-protection. Specimens were then mounted onto aluminum stubs and photographed using a HITACHI S-3000N.

Mice conditional mutant.

Floxed *Lkb1* mice were obtained from RA. DePinho (Boston, USA) and *Ht-PA::Cre* mice from S. Dufour (Paris, France). Heterozygous (*Lkb1*^{+/*F*}) and homozygous (*Lkb1*^{*F*/*F*}) mice were crossed with *Lkb1*^{+/*F*} *Ht-PA::Cre*^{+/*o*} mice to generate *Lkb1* homozygous conditional knockout in neural crest-derived tissues (referred to as cKO in the text) and wild-type littermate animals. Mice were genotyped by PCR using DNA extracted from tails with Cre primers (For-cctggaaaatgcttctgtccgtttgcc; Rev-gagttgatagctggctggtgggagatg) and *Lkb1* primers (Lkb1-55-tctaacaatgcgctcatcgtcatcctcggc; Lkb1-36-gggcttcacctggtgccagcctgt and Lkb1-39-gagatgggtaccaggagttggggct). For timed pregnancies, mice were bred and the time of plug identification was counted as day 0.5. Mice were maintained under standard housing conditions and maintained on a mixed genetic background.

Cartilage and bone staining.

For whole-mount skeletal analysis, newborn mice heads were skinned, fixed overnight in pure ethanol and stained with Alcian Blue and Alizarin Red for 48 hours. After staining, samples were incubated in 20% glycerol in a 1% KOH solution to remove soft tissue. To improve transparency, specimens were cleared in 50% glycerol in a 0.5% KOH solution at 37°C for 2 days, which was then replaced with 80% glycerol solution for completion of the reaction.

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Legends to Figures

Figure 1: LKB1 expression in CNC is crucial for head development. Detection of *LKB1* transcripts in the CNC in 6ss chick embryo (**A**) and in the migrating CNCC (8ss stage, **B**). On section, note the high level of transcript accumulation in CNCC (*, **C**). *In ovo* CNC transfection: nucleic acid solution is blown in the neural groove at 5ss and bilaterally electroporated to the CNC by iterative pulses delivered by a triple electrode system. Co-electroporation with Rhodamin Dextran enables the targeted CNCC to be visualized. The migrated and delaminated CNCC are specifically electroporated **as shown on** the dorsal view (**D**) and on section of 7ss embryo (**E**). Morphology of E5 control (**F**) and *LKB1*-dsRNA-treated (**G**) embryos: animals subjected to *LKB1* silencing in CNC exhibit forebrain hypoplasia (star in G) when compared to control (arrowheads; F). Scanning electro-micrograph of E5 control (**H**) and *LKB1*-deprived (**I**) specimen. Cephalic gangliogenesis at 30ss, when compared to control (**J**), is perturbed in knocked-down *LKB1* embryos (**K**): trigeminal ganglion exhibits a symmetrical structure and the maxillary branch is missing. *LKB1* inactivation alters the maxillo-facial development. Whole-mount preparation of control (**L**) and experimental (**M**) skeletons at E11: Alcian blue staining shows reduced formation and Alizarin red staining reveals the absence of osteogenic differentiation after blockade of *LKB1* activity. Brain growth measurements on half-cut embryos: Tel, optic stalk-dorsal telencephalon; Di, optic stalk-epiphysis; Mes, optic stalk-optic tectum (**N, O**). When compared to control, *LKB1* silencing leads to microcephaly (***, $p < 0.001$); rescuing LKB1 pathway with exogenous human LKB1 wt enables brain growth to be restored ($p > 0.5$), while the supplementation with a kinase-dead form of exogenous LKB1 (Lkb1-KDT) recapitulates forebrain defects (**O**). ol: olfactory placode; op, optic vesicle; md, mandibular bud; mx, maxillary process; nl, nasolateral bud; nfb, nasofrontal bud. Scale bars, 20 μ m.

Figure 2: LKB1 silencing impacts CNCC delamination, and directional migration. HNK1 labeling at 7ss shows the emigration of CNCC from the neural primordium in control (**A**) and *LKB1*-deprived (**B**) embryos. Migration of *AP2* expressing CNCC at 10ss (**C, D**); *LKB1* silencing prevents CNCC EMT and migration (**D**). N-cadherin expression is repressed at the dorsal part of the neural tube in control embryos (**E, G**) whereas its activity is maintained upon *LKB1* silencing (**F, H**). The directional migration of the CNCC is altered in *LKB1*

knocked-down embryos (**I- K**). The Golgi matrix protein GM130, which is accumulated opposite to the front edge of migrating CNCC in controls (**I**), whereas *LKB1* silencing resulted in a randomized positioning (**J**). Centered distribution of individual cell polarity given by the “GM130- nucleus” axis (arrow) in controls and *shRNA* transfected embryos, respective to the dorso-ventral axis of the neural tube. Scale bars, **C-H** : 40 μ m; L, J : 10 μ m.

Figure 3: AMPK transduces LKB1 morphogenetic signal in CNCC.

Cephalic phenotype of chicken embryos at E4 stage subjected to the silencing for AMPK α 1 catalytic subunit (**A**), both AMPK α 1 and α 2 catalytic subunits (**B**), and *LKB1* (**C**). **D-G**: Restored cephalic development of *LKB1* hypomorphic embryos expressing constitutively active forms of AMPK (AMPK-CA). Control embryo (**C**), embryo treated with dsRNA *LKB1* (**D**), embryos coelectroporated with dsRNA *LKB1* and either AMPK α 2-CA (**E**) or AMPK γ 1-CA (**F**). Quantification of microcephalic versus normal embryos following AMPK α 1 and/or α 2 silencing, and AMPK γ 1-CA rescue of *LKB1* knock down (**G**). **H-J**: Expression of AMPK-CA rescues CNCC apoptosis induced by *LKB1* RNAi. Apoptotic cells are evidenced by LTR staining in control (**H**), *LKB1*-dsRNA-treated (**I**), *LKB1*-dsRNA and AMPK γ 1-CA co-electroporated embryos (**J**). **K-M**: expression of a constitutively active form of AMPK restores *FGF8* expression in the ANR. *FGF8* expression in controls (**K**), *LKB1*- dsRNA-treated (**L**), *LKB1*-dsRNA and AMPK γ 1-CA co-electroporated (**M**) embryos. Di, diencephalon; ey, eye; Me, mesencephalon; Te, telencephalon.

Figure 4: ROCK and MRLC are polarity effectors of the LKB1-AMPK pathway in CNCC.

(A, C): Cephalic development, in control (**A**), *MRLC-DD*-treated (**B**) and *LKB1*- silenced (**C**) embryos at E4. **(D)** Microcephaly resulting from treatment with a ROCK inhibitor, Y-27632. **(E)** *LKB1* silencing when combined to a constitutively active form of ROCK (ROCK-CA) results in rescued phenotype, **(F)** while in combination with a kinase-dead mutant (ROCK-KD), it leads to microcephaly. **(G)** In Y-27632-treated embryos, the constitutively active form of AMPK- γ 1 fails to restore the microcephaly, **(H)** while expression of *MRLC-DD* restores the cephalic development of *dsRNA LKB1* electroporated embryos. **(I, J)** Quantification of microcephalic *versus* normal embryos in experiments **(I)** exploring the effects of ROCK, **(J)** of ROCK inhibitor Y-27632 and the respective roles of AMPK γ 1-CA and *MRLC-DD*.

Supplementary informations

Supplementary information 1: *LKB1* gene expression pattern in chick embryos.

As soon as the neural tube closes, *LKB1* expression is maintained in delaminating and migrating CNCC at 11ss **(A)**, but gains in intensity at 15ss **(B)**. From 20ss on, *LKB1* expression is strongly accumulated in the maxillo-mandibular and the nasofrontal CNCC as well as in BA2 and BA3 and to a lesser extend in BA4-6 as shown at E3 **(C)**. Absence of *LKB1* transcript detection in 10ss following transfection with *LKB1-dsRNA* at 5ss **(D)**.

Supplementary Information 2: Cephalic defects in *LKB1*-deprived embryos.

(A, B) Cotransfection of *LKB1-dsRNA* with a vector producing *hLKB1*. The human *LKB1*-KD cannot rescue cephalic development **(A)**, while a vector producing human *LKB1*-KD do so **(B)**. **(C)** Quantification of microcephalic *versus* normal phenotypes in control, *LKB1-dsRNA* series, and after the co-transfection of *LKB1-dsRNA* with either *hLKB1*-WT or *hLKB1-KD*. **(D-E)** When performed in early neurula (1ss), inhibition of *LKB1* generates consistent neural tube defects and results in anencephalic embryos **(E)**.

Supplementary information 3: Craniofacial abnormalities in *LKB1*-deficient mice in CNCC-derivatives.

(A) Genotyping of *lkb1*^{+/*F*} *Ht-PA::Cre*⁻, *lkb1*^{F/*F*} *Ht-PA::Cre*⁺ and *lkb1*^{+/*+*} *Ht-PA::Cre*⁻ mice by PCR. **(B)** Images of a *Lkb1*-deficient newborn mouse (right) and a wild-type littermate (left). *Lkb1* invalidated animals have a reduced stature and and died at P1. **(C, D)** Head skeleton of a *Lkb1*-invalidated mouse **(D)** and a wild-type littermate **(C)** stained with Alizarin red and Alcian blue at P0 showing the lack of metopic, coronal sagittal sutures, (star) and the reduced size of maxillary and mandibullar bones (arrow head in circle).

Supplementary information 4: *LKB1* silencing impacts CNCC survival, and morphogen expression.

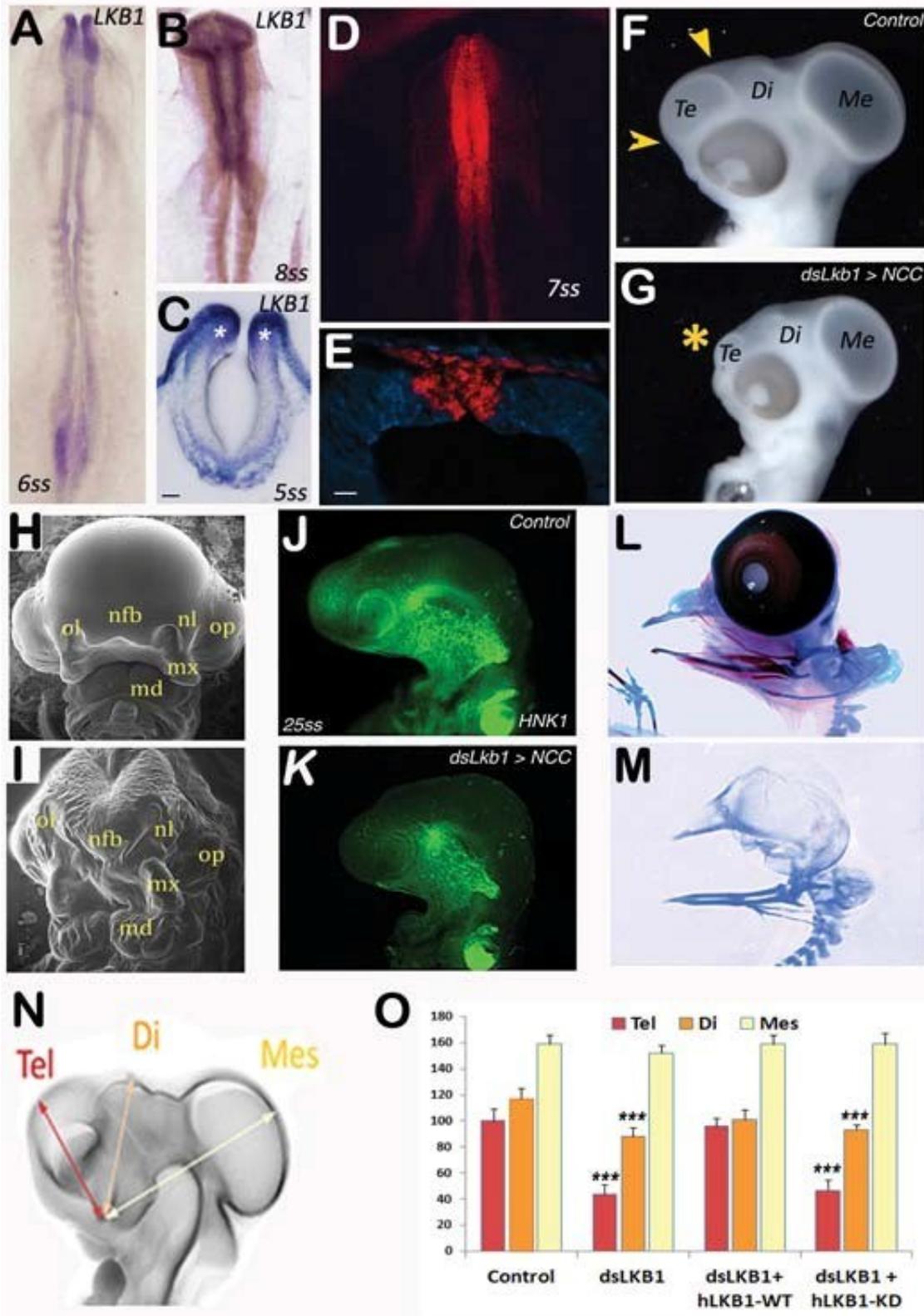
When compared to control **(A)**, LTR staining of 7ss embryos reveals expanded cell death **(B)**. *FGF8*, which is strongly expressed in ANR **(C)**, is down-regulated by *LKB1*-silencing in CNCC **(D)**. **(E)** Quantification of gene expression by qRT-PCR showed that *LKB1* silencing

significantly affected the mRNA levels of *Fgf8* (*Fgf8* Δ = 60%), *Wnt1* (*Wnt1* Δ = 40%), and *Wnt8b* (*Wnt8b* Δ =30%). By contrast, *Shh* expression was up-regulated (1.5 fold).

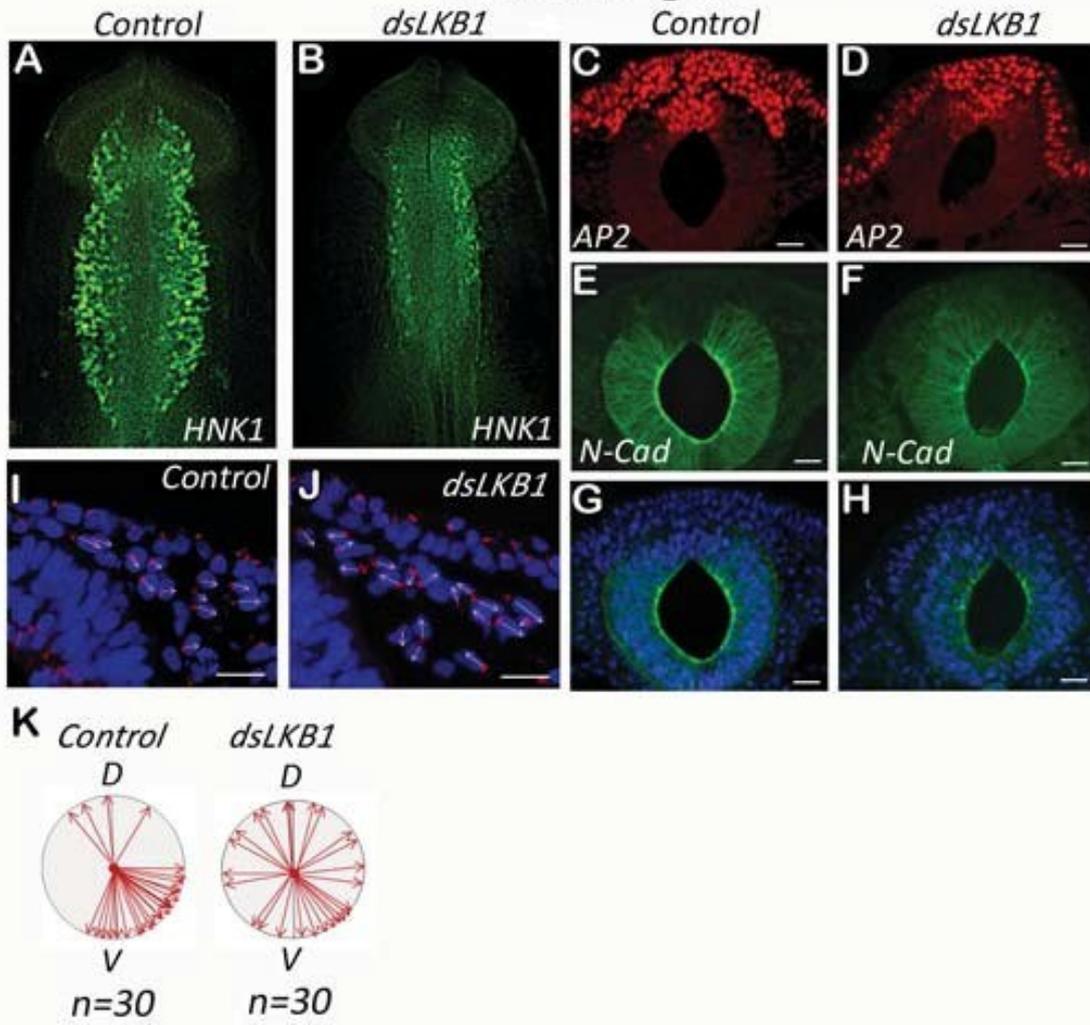
Supplementary information 5: Gene expression pattern of AMPK α 1 and AMPK α 2 in chick embryos.

Accumulation of AMPK α 1 (**A-C**) and AMPK α 2 (**D-F**) transcripts at 5ss (**A, D**), 10ss (**B, E**), 12-13ss (**C, F**). At neurula stages, at which electroporation experiments are performed AMPK α 1 and AMPK α 2 are expressed in the diencephalic and mesencephalic CNC (5ss; **A, D**). The expression of these two genes is maintained in migratory CNC cells once they have started to migrate, according to a similar pattern at 10ss (**B,E**) and 12-13 ss (**C-F**). (**G**) Loss of AMPK α 2 expression at 12ss in an embryo that have been electroporated with a AMPK α 2-dsRNA at 5ss.

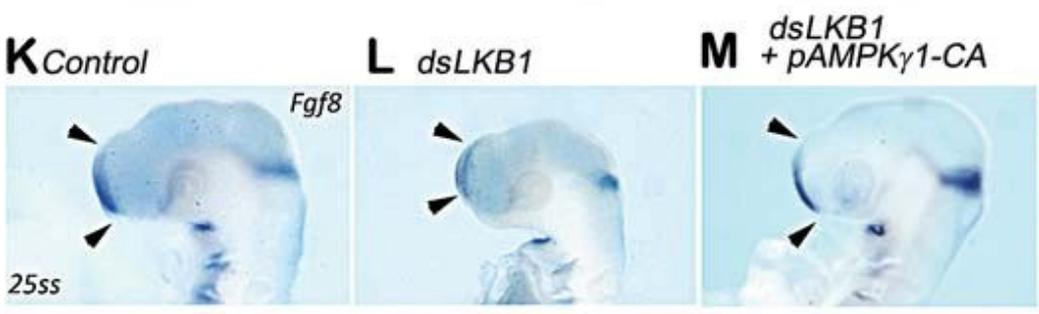
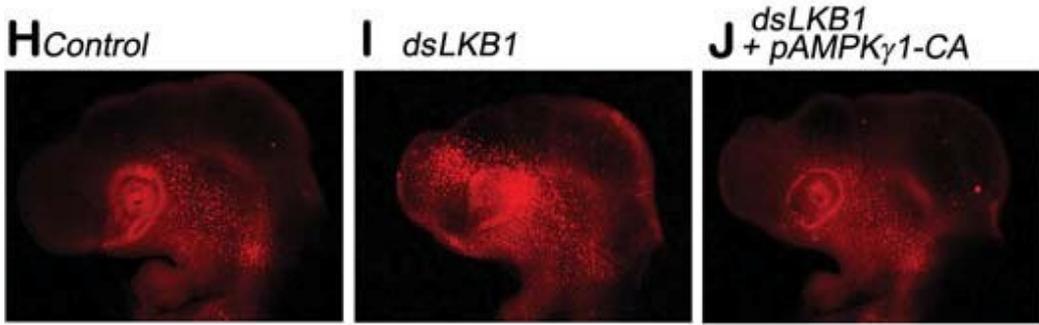
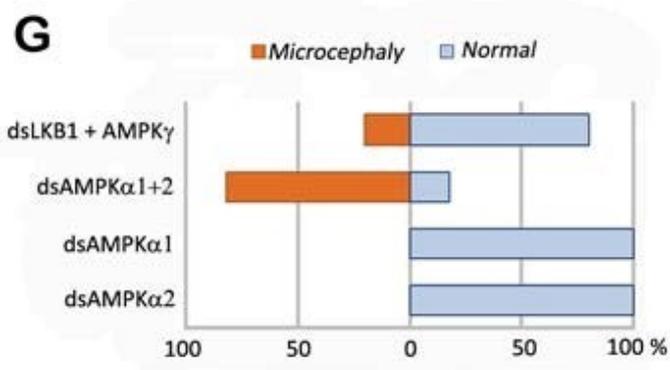
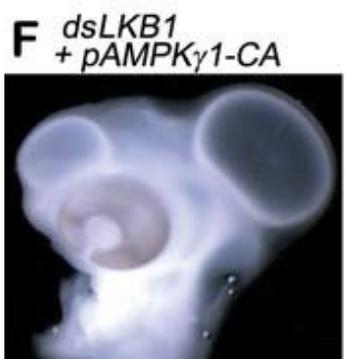
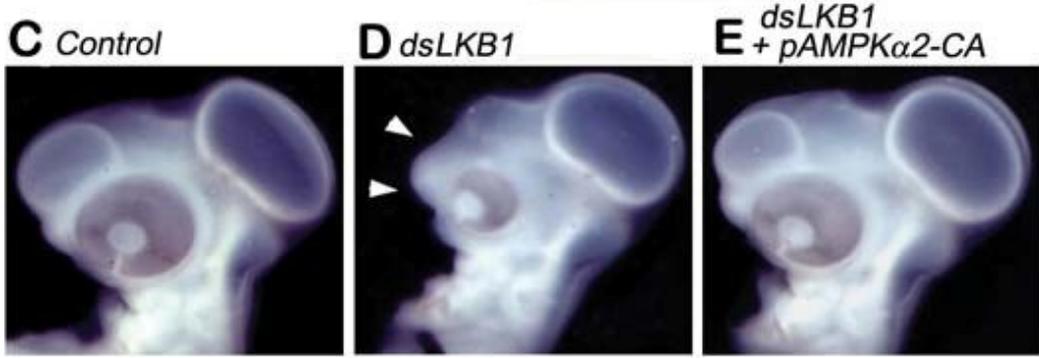
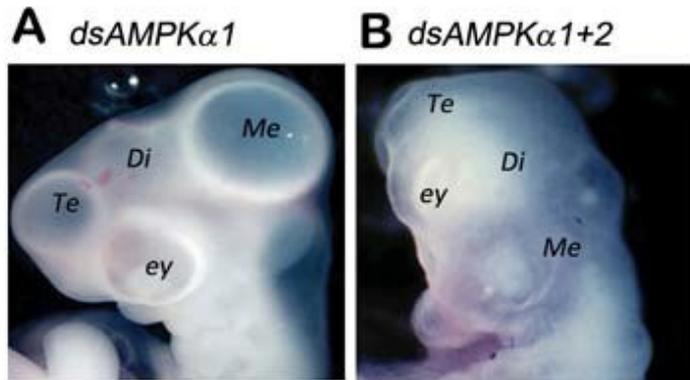
Creuzet Fig.1



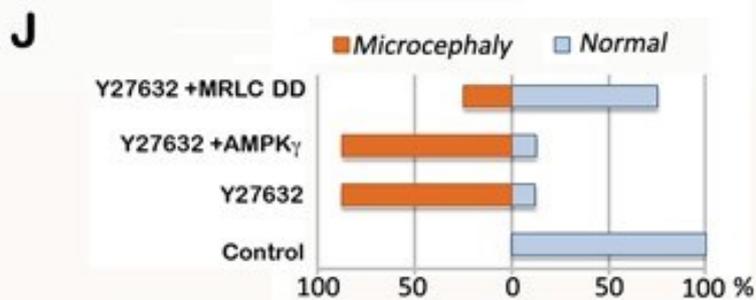
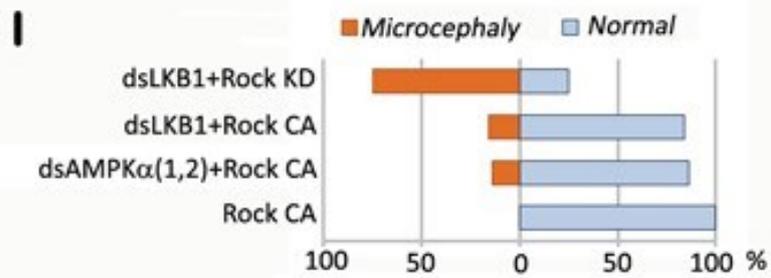
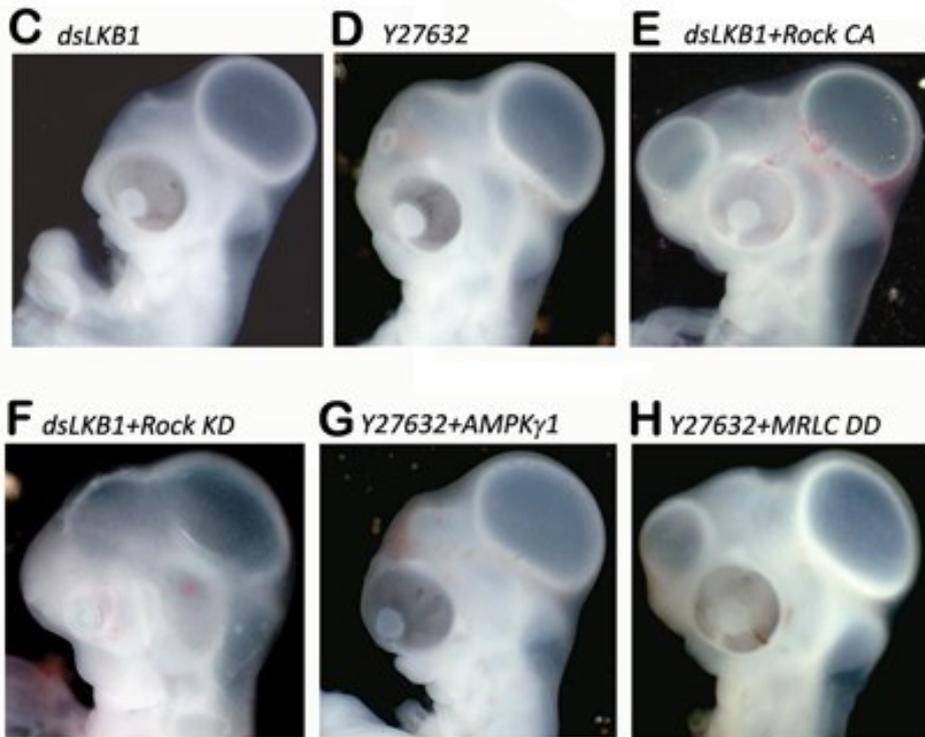
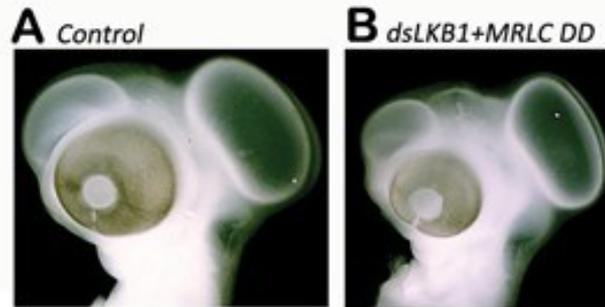
Creuzet Fig. 2



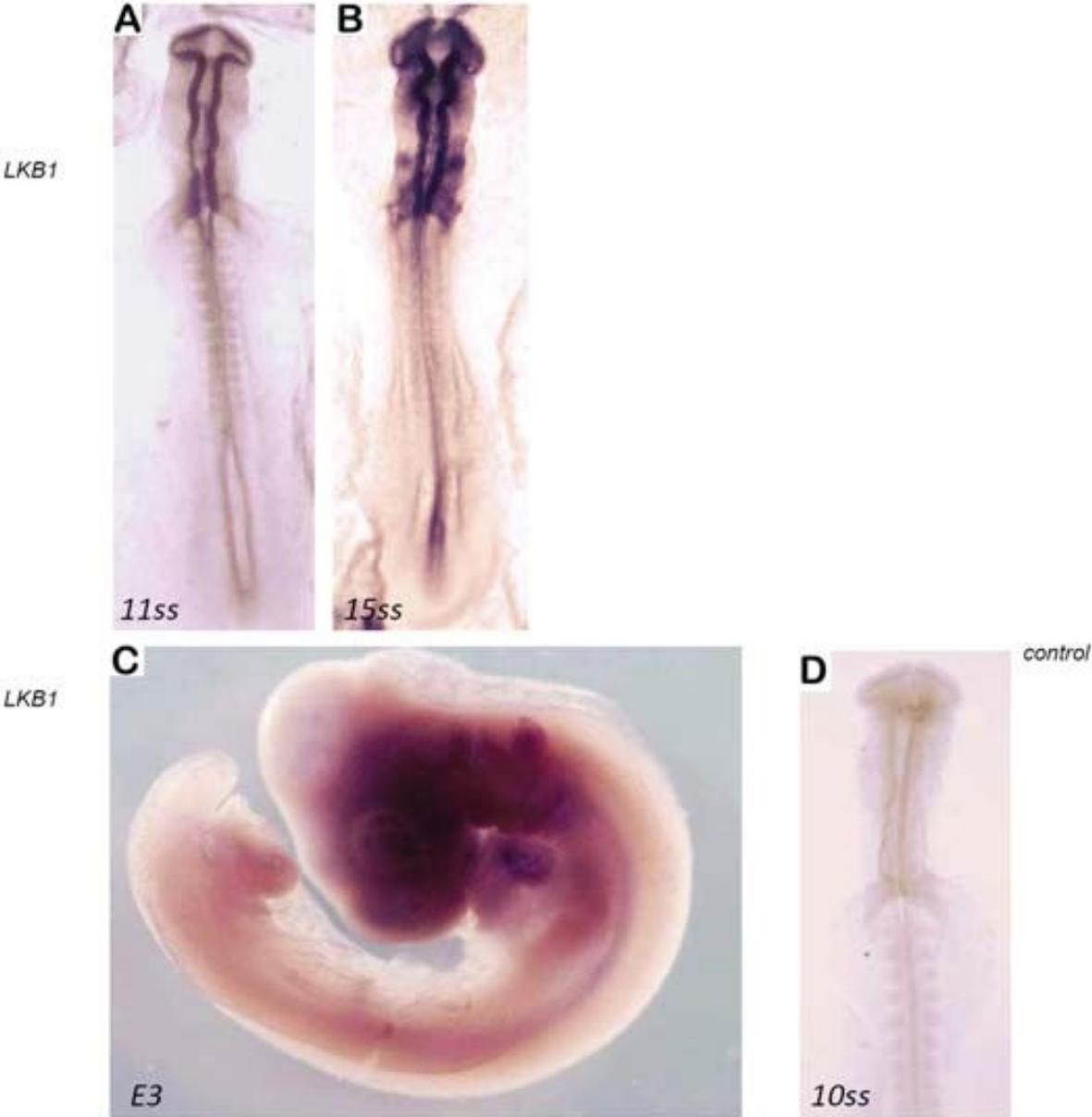
Creuzet Fig.3



Creuzet Fig.4

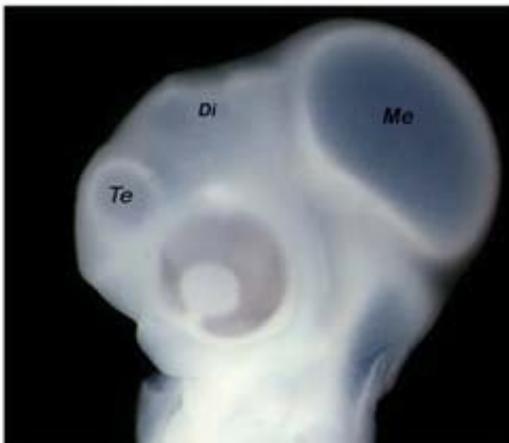


Creuzet Supplement 1

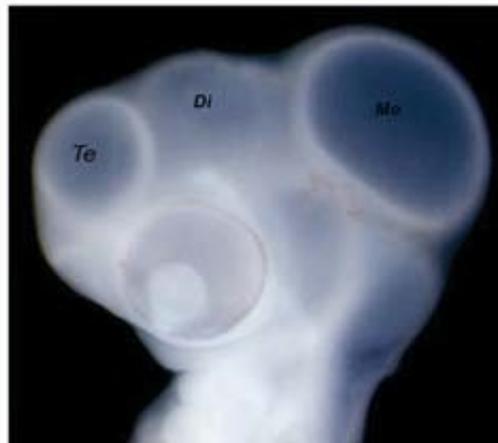


Creuzet Supplement 2

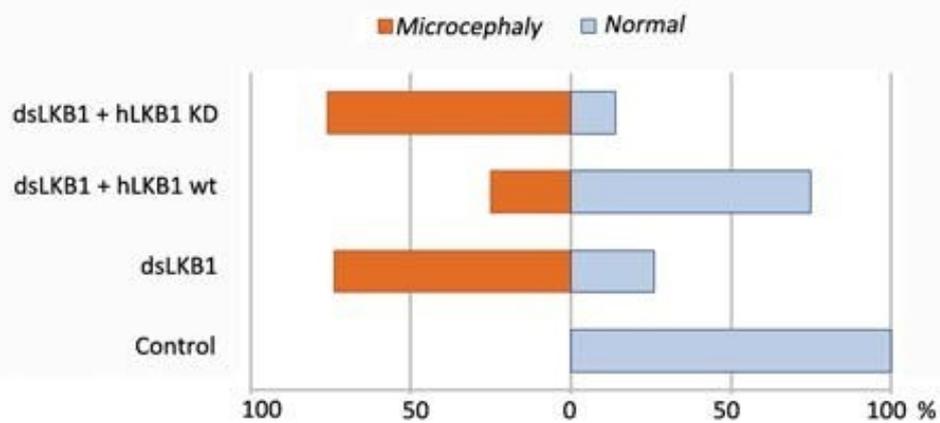
A *dsLKB1+LKB KD*



B *dsLKB1+LKB1 wt*



C



D *Control*

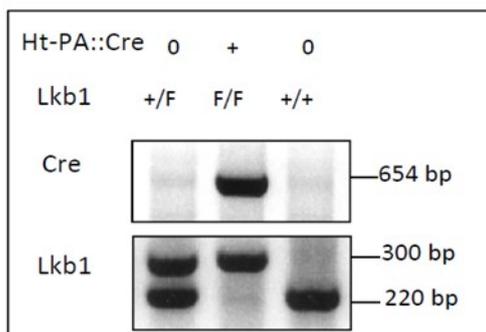


E *dsLKB1*



Creuzet Supplement 3

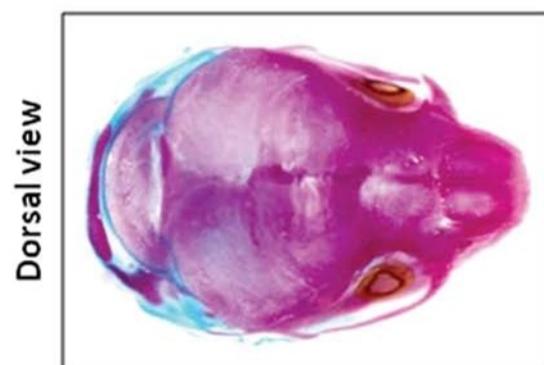
A



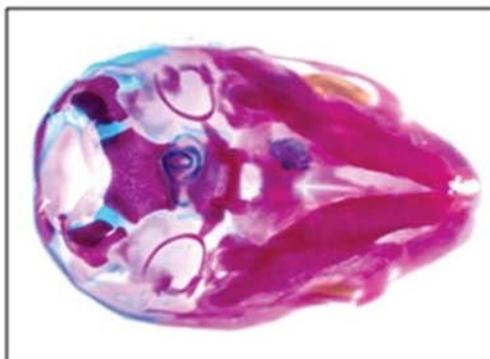
B



C Lkb1^{+/+} Ht-PA::Cre⁻



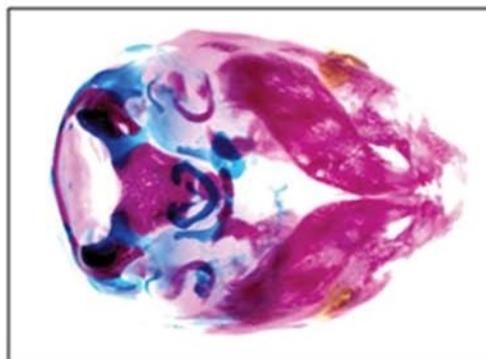
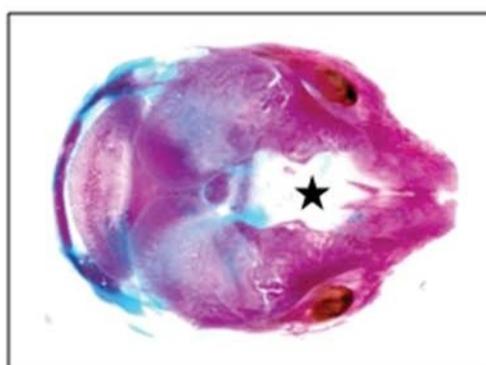
Ventral view



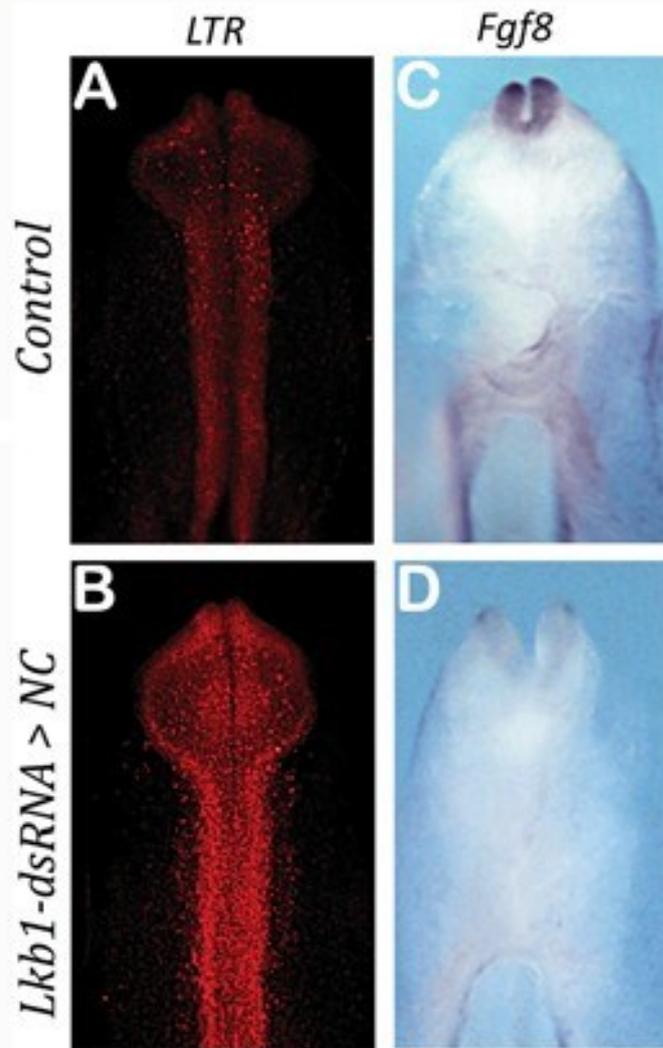
Lateral view



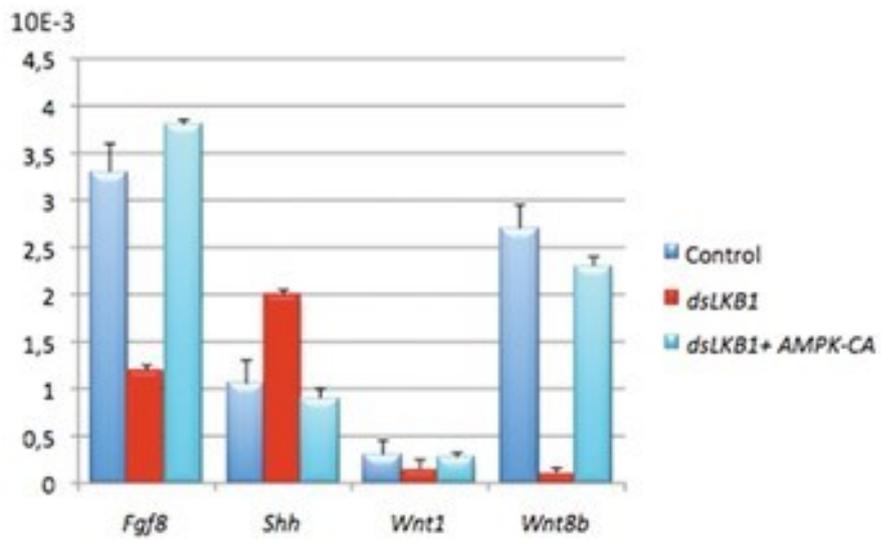
D Lkb1^{F/F} Ht-PA::Cre⁺



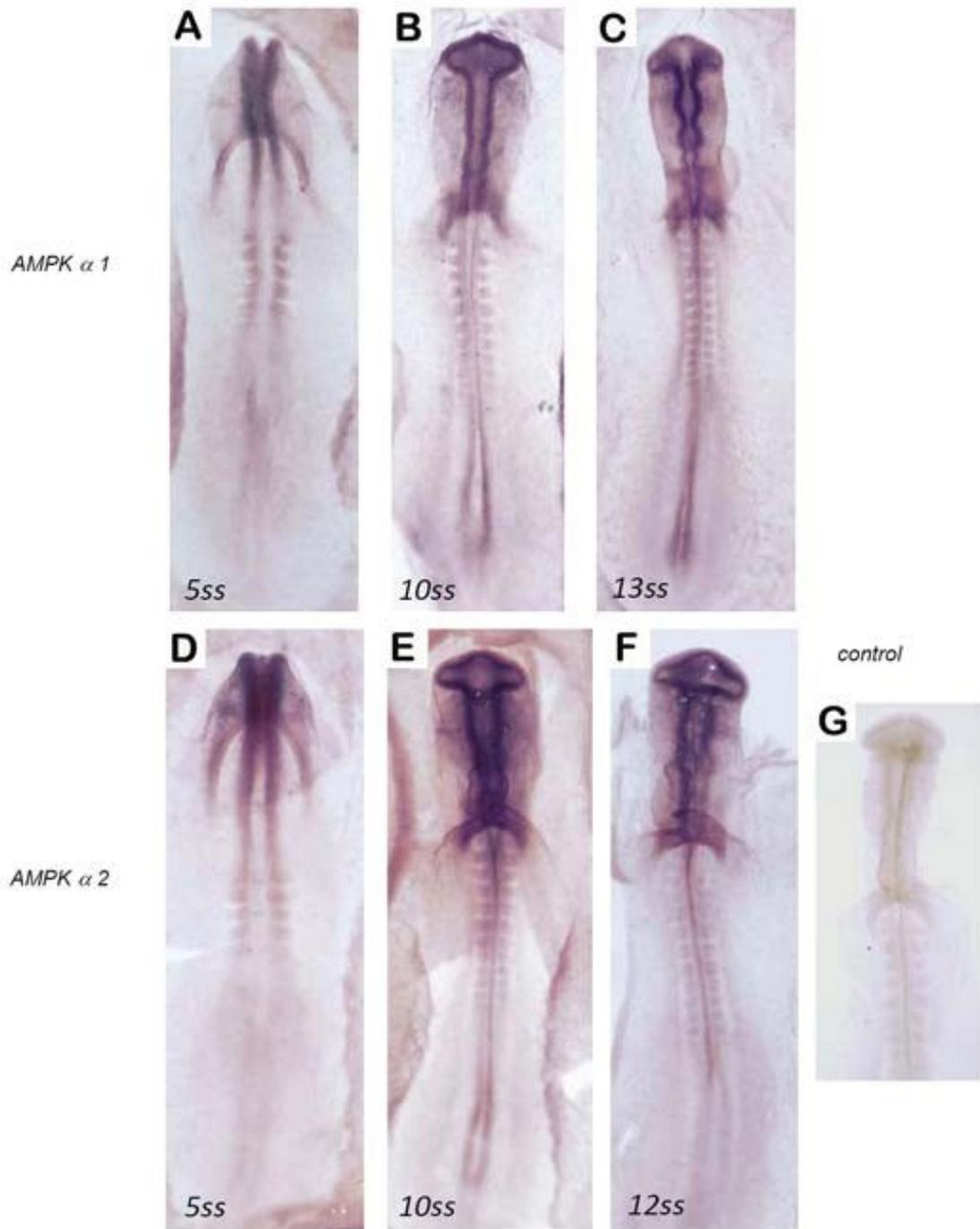
Creuzet Supplement 4



E



Creuzet Supplement 5



II. Results: Project 2

Regulation of the tumor suppressor LKB1 by the acetyltransferase GCN5

Scientific context and results summary

After studying and unveiling the role of the LKB1/AMPK signaling pathway in head formation in vertebrates, we sought to investigate the regulation of LKB1 by upstream factors. As presented in the introduction section, LKB1 undergoes post-translational modifications (PTM) such as phosphorylation, prenylation and ubiquitination. Several groups, including ours, showed that LKB1 is acetylated, but the functional consequences of this PTM have not been clearly established.

Before my arrival to the lab, our team had successfully identified five acetylated LKB1 lysine residues by mass spectrometry analysis (Nicolas Aznar thesis). In another study, the lysine 48 (K48) was shown to be deacetylated by SIRT1, leading to the modulation of both LKB1 subcellular localization and catalytic activity (Lan et al., 2008). Finally, in *Drosophila*, LKB1 is essential for proper oocyte polarity (Martin & St Johnston, 2003). Our collaborator Vincent Mirouse (GReD, UMR 6247, Clermont-Ferrand), observed that while the transgenic expression of a human wild-type form of LKB1 restores the follicular epithelial cells polarity, the non-acetylatable K48R-mutated form of hLKB1, fails to rescue the loss of polarity phenotype observed in follicular epithelial cells of a *Drosophila* strain, in which the LKB1 ortholog was inactivated by mutation. Altogether, these data indicated that the acetylation of LKB1 at K48 modulates its functions.

Our team identified the acetyltransferase GCN5 as an enzyme that is responsible for LKB1 acetylation. Interestingly, mutating *GCN5* in its HAT (histone acetyltransferase) catalytic domain was shown to lead to neural tube closure defects in mice (Bu et al., 2007). This phenotype was reminiscent of that observed following *LKB1* silencing in cranial neural crest cells (CNCCs) in chick embryos, suggesting a functional interaction between GCN5 and LKB1 in CNCCs ontogenesis.

In this context, the second part of my PhD consisted in studying the acetylation of LKB1 by GCN5 and the consequences of this PTM on LKB1 subcellular localization and activity *in vitro* and *in vivo*.

I showed that LKB1 is specifically acetylated by GCN5 at K48 and that GCN5 modulates the subcellular localization of LKB1 in an HAT-dependent and –independent manner. I also observed a differential subcellular localization of LKB1 according to its acetylation state and that the acetylation of LKB1 at K48 is required for its functions in regulating the mTOR pathway *in vitro*. The acetylation of LKB1 at K48 was also required *in vivo* downstream of GCN5, where both proteins were implicated in the formation of craniofacial structures in chick embryos. Finally, I showed that GCN5 regulates LKB1 RNA levels.

Understanding the regulation of LKB1 is essential for apprehending its tumor suppressive functions. Although most LKB1 described functions correlate to its cytoplasmic localization, our results shed the light on a new regulatory mode of LKB1 nuclear localization and open new perspectives regarding its nuclear functions.

Régulation du suppresseur de tumeur LKB1 par l'acétyltransférase GCN5

Contexte scientifique et résumé des résultats

Après avoir étudié le rôle de la voie de signalisation de LKB1 dans la formation de la tête chez les vertébrés, nous nous sommes intéressés à la régulation de LKB1 par des facteurs en amont. Comme présenté dans la section d'introduction, LKB1 est sujet à des modifications post-traductionnelles (MPT) telles que la phosphorylation, la prénylation et l'ubiquitination. Plusieurs groupes, y compris le nôtre, ont montré que LKB1 est acétylé, mais les conséquences fonctionnelles de cette MPT n'ont pas été clairement établies.

Avant mon arrivée au laboratoire, notre équipe avait identifié cinq résidus lysine acétylés sur LKB1 par spectrométrie de masse (thèse Nicolas Aznar). Dans une autre étude, la lysine 48 (K48) s'est révélée être déacétylée par SIRT1, ce qui conduit à la modulation de la localisation subcellulaire et de l'activité kinase de LKB1 (Lan, 2008 et al.). Enfin, chez la drosophile, LKB1 est essentiel à la polarisation de l'ovocyte (Martin & St Johnston, 2003). Notre collaborateur Vincent Mirouse (GReD, UMR 6247, Clermont-Ferrand) a observé que l'expression transgénique d'une forme humaine sauvage de LKB1 restaure la polarité des cellules épithéliales folliculaires, tandis que l'expression de la forme mutée non-acétylable de hLKB1 (K48R), ne parvient pas à sauver le phénotype de perte de polarité observé dans les cellules épithéliales folliculaires d'une souche de drosophile, chez laquelle l'orthologue de LKB1 a été inactivée par mutation. Ces données indiquent donc que l'acétylation de LKB1 au niveau de la K48 est requise pour ses fonctions.

Notre équipe a identifié l'acétyltransférase GCN5 comme étant une enzyme susceptible d'acétyler LKB1. De manière intéressante, la mutation de *GCN5* dans son domaine HAT (histone acétyltransférase) conduit à des défauts de fermeture du tube neural chez la souris (Bu et al., 2007). Ce phénotype rappelle celui observé suivant l'inhibition de l'expression de LKB1 dans les cellules de la crête neurale céphalique (CCNC) chez l'embryon de poulet,

suggérant une interaction fonctionnelle entre GCN5 et LKB1 au cours de l'ontogénèse des CCNC.

Dans ce contexte, la deuxième partie de ma thèse a consisté à étudier l'acétylation de LKB1 par GCN5 et les conséquences de cette MPT sur la localisation subcellulaire et l'activité de LKB1 *in vitro* et *in vivo*.

J'ai pu montrer que LKB1 est spécifiquement acétylé par GCN5 au niveau de la K48 et que GCN5 module la localisation subcellulaire de LKB1 de manière HAT-dépendante et -indépendante. J'ai également observé une localisation subcellulaire différentielle de LKB1 selon son état d'acétylation et que l'acétylation de LKB1 au niveau de la K48 est nécessaire pour ses fonctions dans la régulation de la voie mTOR *in vitro*. L'acétylation de la K48 de LKB1 s'est également avérée être requise *in vivo* en aval de GCN5, où les deux protéines sont impliquées dans la formation de structures crâniofaciales chez l'embryon de poulet. Enfin, j'ai montré que GCN5 régule les niveaux d'ARN de LKB1.

Comprendre la régulation de LKB1 est essentielle afin d'appréhender ses fonctions onco-suppressives. Bien que la plupart des fonctions décrites de LKB1 soient associées à sa localisation cytoplasmique, nos résultats présentent un nouveau mode de régulation de la localisation subcellulaire de LKB1 et ouvrent de nouvelles perspectives en ce qui concerne ses fonctions nucléaires.

Results

Regulation of the tumor suppressor LKB1 by the acetyltransferase GCN5

1 LKB1 is a substrate of the acetyltransferase GCN5

1.1 Validation of a home-made antibody

In order to study the acetylation of LKB1 on K48, our collaborator Vincent Mirouse (GReD, UMR 6247, Clermont-Ferrand) has kindly provided us with a home-made antibody generated in rabbit. This antibody recognizes the sequence of human LKB1 when it is acetylated on K48.

In order to test the specificity of this antibody, Bosc cells (human renal epithelial cell line) were transfected with a vector containing a FLAG-GFP-tagged wild-type (WT) form of LKB1 or a mutated-tagged form of LKB1 which cannot be acetylated on K48, due to a mutation changing the lysine into an arginine (K48R). A mock vector was used as a control and cells were treated with either DMSO or with trichostatin A (TSA) which inhibits class I and II HDACs (histone deacetylases). LKB1 was then immunoprecipitated using an anti-GFP antibody and the acetylation of LKB1 at K48 was analysed by Western Blot using the anti-K48 antibody.

The analysis revealed that the TSA treatment increases the acetylation of the WT form of LKB1 at K48. As for the mutated form of LKB1, we were expecting the detection of a signal which would be comparable to the untreated condition. Instead, we detected a mild signal suggesting that the antibody is reliable but might detect, to a lesser extent, surrounding non-mutated acetylated lysine residues as well. A basal signal was also detected in DMSO treated conditions and was comparable between WT-LKB1 and mutated LKB1 suggesting that the antibody detects basal LKB1 levels (**FIG.1A**) or maybe low acetylation levels due to endogenous GCN5.

We then sought to observe the acetylation of endogenous LKB1 at K48. For this purpose, Bosc cells were treated with either DMSO or TSA and lysed. Endogenous LKB1 was immunoprecipitated (Santa-Cruz, LKB1 Ley 37D/G6) and its acetylation was detected by western blot using the anti-K48 antibody. We detected a basal signal in DMSO treated conditions with an increase in LKB1 acetylation following TSA treatment (**FIG.1B**). Thus, we show, for the first time, that endogenous LKB1 is acetylated at K48 using a specific antibody.

Altogether, these observations show that the antibody is reliable and that the signal detecting the specific acetylation of LKB1 on K48 is strong enough for conducting our study.

1.2 LKB1 is acetylated by GCN5 on lysine 48

Previous experiments in our lab led to the identification of the acetyltransferase hGCN5 as a potential regulator of LKB1 acetylation *in vitro*. In this context, we investigated whether the K48 of LKB1 is specifically acetylated by hGCN5. For this purpose, Bosc cells were transfected with a tagged WT-LKB1 expressing vector alone or together with a vector expressing a WT (WT-GCN5) or a mutated form (Y621A/F622A) of hGCN5-L lacking its acetyltransferase activity (MUT-GCN5). Mock vectors were used as a control. LKB1 was then immunoprecipitated using an anti-GFP antibody and the acetylation of LKB1 at K48 was analysed by Western Blot using the anti-K48 antibody.

We observed that while the WT form of hGCN5 increased the acetylation of LKB1 at K48, the mutated form of hGCN5 failed to acetylate LKB1 (**FIG.1C**). This result shows that LKB1 is acetylated by hGCN5 at K48 and that this acetylation depends on the acetyltransferase activity of hGCN5.

1.3 LKB1 interacts with GCN5

After showing that LKB1 is acetylated by hGCN5 at K48, we wanted to know whether this acetylation requires the interaction of both proteins.

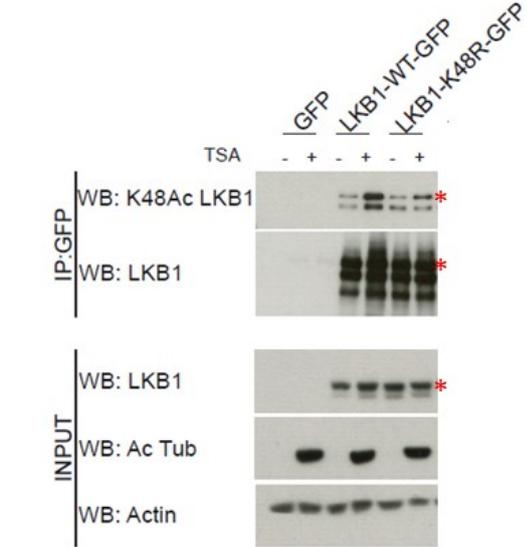
For this purpose, WT-LKB1 was expressed in Bosc cells alone or with WT- or MUT-GCN5. Mock vectors were used as a control. LKB1 or GCN5 were then immunoprecipitated and their interaction was analysed by Western Blot.

We observed that WT-GCN5 coimmunoprecipitates with LKB1 and *vice versa* (**FIG.1D**). Interestingly, MUT-GCN5 seemed to interact with LKB1 with less affinity, suggesting that the acetylation of LKB1 by GCN5 could be important for stabilizing this interaction. However, the non-acetylatable form of LKB1 (K48R) still interacts with GCN5 (**Suppl. data A**). In addition, the same experiment was performed with the short isoform of GCN5, hGCN5-S, lacking the N-terminal extension compared to the long isoform. We observed that hGCN5-S also interacts with LKB1 (**FIG.1E**). This observation indicates that the N-terminus of hGCN5 is dispensable for its interaction with LKB1, and shows that both GCN5 isoforms interact with LKB1.

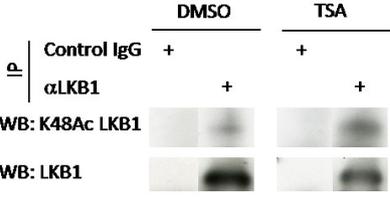
In order to validate our observation, we tested the interaction between endogenous LKB1 and GCN5 proteins. Bosc cells were transfected with GCN5 siRNA for 48h in order to silence *GCN5* expression. Non-targeting siRNAs were used as control. Cells were then lysed and endogenous LKB1 was immunoprecipitated. The interaction between LKB1 and GCN5 was then analyzed by western blot. As shown in **FIG.1F**, endogenous LKB1 and GCN5 proteins also interact, and the specificity of endogenous GCN5 detection was confirmed following GCN5 silencing.

Thus, LKB1 physically interacts with GCN5 and the N-terminus of GCN5 is not required for this interaction.

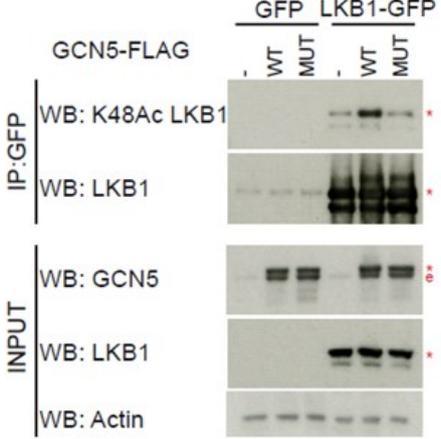
FIG.1



A

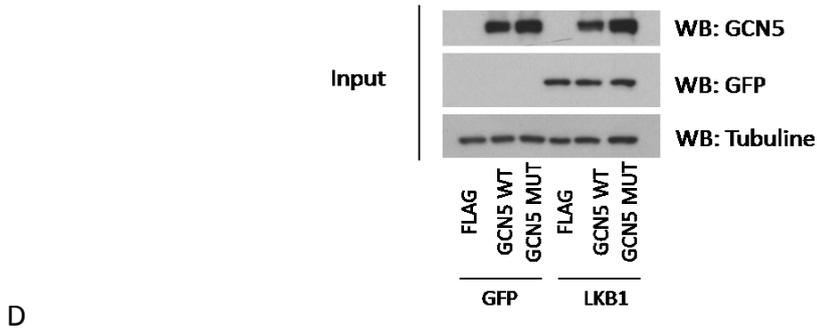
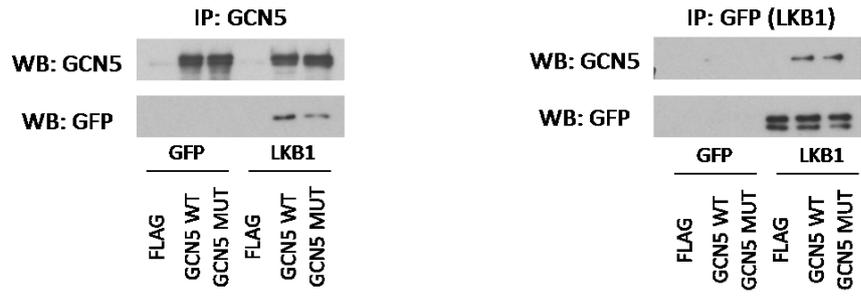


B

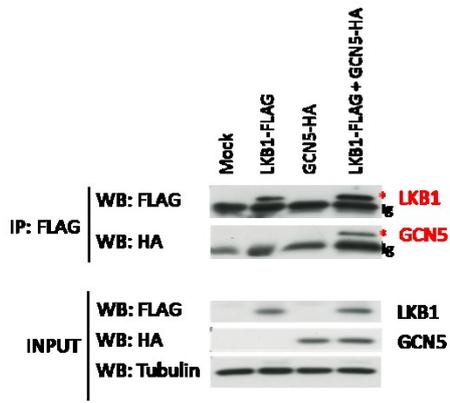


C

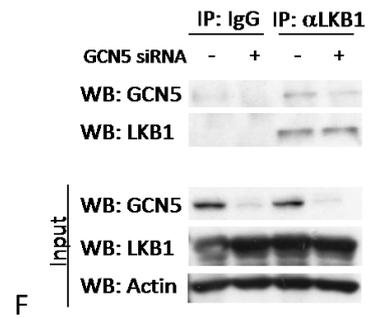
FIG.1



D



E



F

FIG.1: LKB1 is a substrate of the acetyltransferase GCN5

- A) Validation of the anti-K48Ac LKB1 antibody.** Bosc cells were transfected with plasmids expressing a WT-or a mutated (K48R) -FLAG-GFP-LKB1 protein. Cells were treated for 4h with 1 μ M trichostatin A (TSA). LKB1 was immunoprecipitated 48h post-transfection with an anti-GFP antibody and the acetylation of LKB1 at lysine 48 (K48) was detected by western blot with the corresponding home-made antibody. Red (*) = specific band corresponding to LKB1. (+)= with TSA. (-)= with DMSO. Ac Tub= acetylated tubulin.
- B) Endogenous LKB1 is acetylated at K48.** Bosc cells were treated for 4h with 1 μ M trichostatin A (TSA). Endogenous LKB1 was immunoprecipitated using the Santa-Cruz anti-LKB1 Ley 37D/G6 antibody and its acetylation was detected by western blot using the anti-K48 antibody.
- C) GCN5 acetylates LKB1 at lysine 48 (k48).** Bosc cells were transfected with plasmids expressing a WT-FLAG-GFP-LKB1 protein and/or WT-GCN5-FLAG or MUT-GCN5-FLAG .LKB1 was immunoprecipitated 48h post-transfection with an anti-GFP antibody and the acetylation of LKB1 at lysine 48 (K48) was detected by western blot with the corresponding home-made antibody. Red (*) = specific band corresponding to LKB1 or GCN5. Red (e)= endogenous.
- D) E) GCN5 interacts with LKB1.** Bosc cells were transfected with plasmids expressing a WT-FLAG-GFP-LKB1 protein and/or WT-GCN5-FLAG (long isoform) in (D) or WT-GCN5-HA (short isoform) in (E). LKB1 or GCN5 were immunoprecipitated 48h post-transfection with an anti-GFP, or an anti-GCN5, or an anti-FLAG antibody. LKB1 and GCN5 were then detected by western blot with the corresponding antibodies. Red (*) = specific band corresponding to LKB1 or GCN5. Ig= IgG /immunoglobulin heavy chain.

F) Endogenous interaction between GCN5 and LKB1. Bosc cells were transfected with GCN5 siRNA for 48h. Non-targeting siRNAs were used as control. Cells were then lysed and endogenous LKB1 was immunoprecipitated using the Santa-Cruz anti-LKB1 Ley37D/G6 antibody. The interaction between LKB1 and GCN5 was then analyzed by western blot.

2 GCN5 modulates the subcellular localization of LKB1

2.1 The acetylated form of LKB1 on K48 is mainly nuclear

We showed that GCN5 acetylates LKB1 at lysine 48. In order to better apprehend the consequence of this acetylation on LKB1's functions, we sought to know in which cellular compartment was this acetylated form expressed. For this purpose, Bosc cells were transfected with a tagged WT-LKB1 expressing vector alone or together with a vector expressing WT-GCN5. Mock vectors were used as a control. We then performed a cellular fractionation in order to separate the nuclear fraction from the cytoplasmic fraction. LKB1 was then immunoprecipitated using an anti-GFP antibody and the acetylation of LKB1 at K48 was analysed by Western Blot using the anti-K48 antibody.

As LKB1 was present in both fractions, the acetylated form of LKB1 was only present in the nuclear fraction (**FIG.2A**). The residual band observed in the cytoplasmic fraction most probably corresponds to basal levels of LKB1 detected by the anti-K48 antibody, as we suggested previously. This result shows that the acetylated fraction of LKB1 at lysine 48 is retained in the nucleus while the cytoplasmic fraction of LKB1 is not acetylated at lysine 48.

2.2 The non-acetylated form of LKB1 is present in both the nucleus and the cytoplasm

Most of LKB1's associated functions are correlated with its cytoplasmic localization. In this context, we wanted to know whether GCN5 modulates the subcellular localization of LKB1. For this purpose, WT-Flag-LKB1 was expressed in Bosc cells, alone or with a pool of siRNAs targeting the endogenous hGCN5 mRNA. A mock vector and a non-targeting siRNA pool were used as controls. The ectopically expressed Flag-tagged LKB1 and endogenous GCN5 were detected by immunofluorescence and analyzed 16h post-transfection. We observed that, in the presence of endogenous GCN5, WT-LKB1 was detected in the nucleus, the cytoplasm and the plasma membrane as expected. However, after silencing GCN5, LKB1 was mainly localized in the nucleus. Interestingly, the non-acetylatable form of LKB1 (K48R) was

expressed in both the nucleus and the cytoplasm, thereby complementing our previous result. However, after silencing GCN5, the LKB1-K48R was also exclusively nuclear (**FIG.2B**).

These results show that in the absence of GCN5, LKB1 is mainly nuclear and that the non-acetylated fraction is present in both the nucleus and the cytoplasm. They also indicate that GCN5 is required for the cytoplasmic localization of LKB1.

2.3 GCN5 leads to the translocation of LKB1 into the cytoplasm, independently from its acetyltransferase activity

The previous result suggested that the ectopic expression of GCN5 would trigger the translocation of LKB1 from the nucleus into the cytoplasm. In order to test this hypothesis, we expressed the WT form of LKB1 alone or together with WT-GCN5 or MUT-GCN5. Bosc cells were analyzed 48h post-transfection and the subcellular localization of LKB1 was detected by immunofluorescence. This time we used a GFP-tagged form of LKB1 which seems to be mainly expressed in the nucleus when expressed alone, but a small fraction is also detected within the cytoplasm. However, when we ectopically expressed WT-GCN5, we observed a striking increase of LKB1's localization in the cytoplasm, while another fraction remained in the nucleus (**FIG.2C**). This result shows that the ectopic expression of GCN5 triggers the translocation of a fraction of LKB1 from the nucleus into the cytoplasm. Interestingly, the expression of the mutated form of GCN5 in its HAT domain, MUT-GCN5, also led to an increase of LKB1's expression in the cytoplasm (**FIG.2C**). Together these results indicate that GCN5 leads to the translocation of a fraction of LKB1 into the cytoplasm, independently from its acetyltransferase activity.

FIG.2

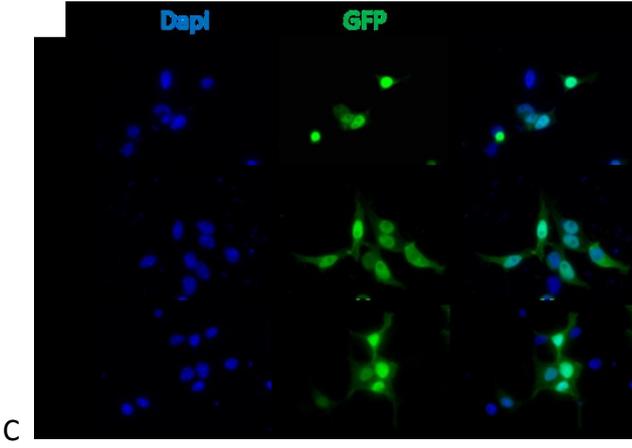
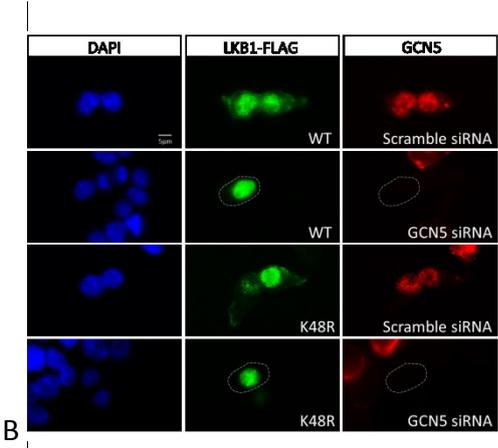
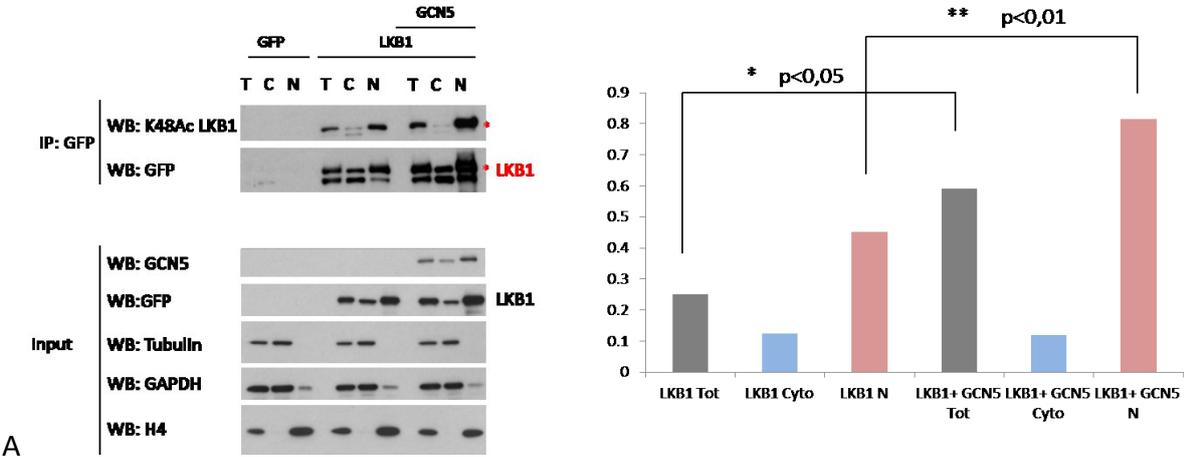


FIG.2: GCN5 modulates the subcellular localization of LKB1

A) The acetylated fraction of LKB1 is nuclear. Left panel: Bosc cells were transfected with plasmids expressing a WT-FLAG-GFP-LKB1 protein with or without WT-GCN5-FLAG. Cellular fractionation was performed 48h post-transfection to separate the nuclear fraction from the cytoplasmic fraction. LKB1 was then immunoprecipitated with an anti-GFP antibody and the acetylation of LKB1 at lysine 48 (K48) was detected by western blot with the corresponding home-made antibody. Red (*) = specific band corresponding to LKB1. T= Total fraction. C= Cytoplasmic fraction. N= Nuclear fraction. **Right panel:** Graphic representation of LKB1's acetylation levels in the different cellular fraction. Tot= Total fraction. Cyto= Cytoplasmic fraction. N= Nuclear fraction. . The experiment was performed 3 times and the statistical significance was calculated with a T-test.

B) Immunolocalization of ectopically expressed LKB1 in the presence or absence of endogenous GCN5. Bosc cells were transfected with a plasmid expressing a WT-LKB1-FLAG or a mutated non-acetylatable form of LKB1 (K48R-LKB1-FLAG) protein and with a pool of siRNAs targeting GCN5. Scramble siRNAs were used as control. LKB1 (in green) and GCN5 (in red) were immunolabelled 16h post-transfection with anti-GCN5 or anti-FLAG antibodies. Nuclei were labeled with DAPI (in blue). The dotted line outlines the cell.

C) Immunolocalization of ectopically expressed LKB1 in the presence or absence of ectopically expressed GCN5. Bosc cells were transfected with a plasmid expressing a WT-FLAG-GFP-LKB1 protein and WT-GCN5-FLAG or MUT-GCN5-FLAG. Nuclei were labeled with DAPI (in blue). GFP-LKB1 is shown in green.

3 The acetylation of LKB1 at K48 is required for the regulation of the mTOR signaling pathway

In order to test whether LKB1 acetylation at K48 is required for its functions, we examined the consequences of LKB1 mutation at K48 on the mTOR pathway. Bosc cells were transfected with a WT- or K48R-LKB1 for 48 h. A mock vector was used as control. Cells were lysed and the phosphorylation of S6RP (protein kinase p70 ribosomal protein S6 kinase 1) was analyzed by western with the corresponding antibody. As expected, we observed that the expression of a WT form of LKB1 decreases the phosphorylation of S6RP while the expression of the K48R form increases it (**FIG.3**). Thus, the acetylation of LKB1 at K48 seems to be required for inhibiting the mTOR pathway.

FIG.3

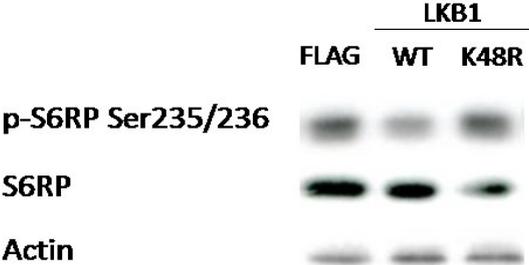


FIG.3: LKB1 acetylation is required for the regulation of the mTOR pathway

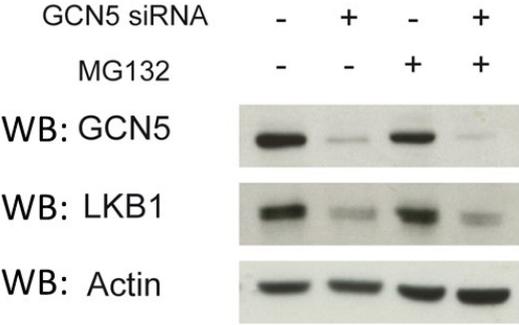
Bosc cells were transfected with WT- or K48R-LKB1. A mock vector was used as control. The cells were lysed 48h post-transfection. The phosphorylation of S6RP (protein kinase p70 ribosomal protein S6 kinase 1) at Ser235/236 was analyzed by western using the corresponding antibody.

4 GCN5 regulates the RNA levels of LKB1

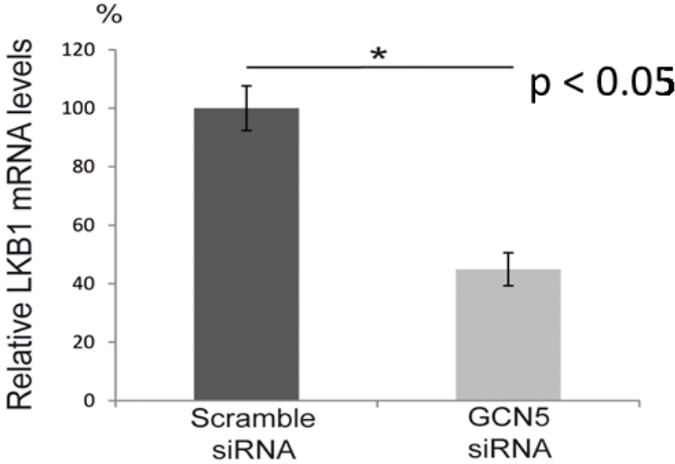
Surprisingly, when we silenced GCN5, we observed an unexpected decrease in endogenous LKB1 protein levels. Based on this observation, we wanted to investigate whether GCN5 modulates LKB1 stability. For this purpose, we silenced the expression of GCN5 using RNA interference. Bosc cells were transfected with a pool of four GCN5 siRNAs for 72h, and the expression levels of the LKB1 protein were analyzed by western blot. A control pool of siRNAs was used for comparison. Silencing GCN5 led to a severe decrease in LKB1 protein levels as expected. However, after treating the cells with the proteasome inhibitor MG132, the levels of LKB1 remained low (**FIG.4A**). The efficiency of the MG132 treatment was verified by detecting the accumulation of ubiquitinated proteins (**Suppl. data B**). Thus GCN5 does not regulate LKB1 stability and protein levels, but could be important for the regulation of the RNA levels of LKB1.

Indeed, GCN5 is known to be implicated in chromatin remodeling and gene expression regulation. In order to test our hypothesis, we performed quantitative RT-PCR, allowing us to measure RNA levels of LKB1 after silencing GCN5 for 72h. Interestingly, we observed a decrease in LKB1 mRNA levels by 60% (**FIG.4B**), showing that GCN5 regulates the mRNA levels of LKB1.

FIG.4



A



B

FIG.4: GCN5 regulates the mRNA levels of LKB1

- A) Silencing GCN5 decreases LKB1 protein levels.** Bosc cells were transfected with a pool of siRNAs targeting the mRNA of GCN5 or with control siRNAs for 72h. Cells were then treated with 25 μ M MG132 for 4h, and the expression levels of endogenous LKB1 were analyzed by western blot.
- B) Silencing GCN5 decreases LKB1's mRNA levels.** Bosc cells were transfected with a pool of siRNAs targeting the mRNA of GCN5 or with control siRNAs for 72h. Following RNA extraction, quantitative RT-PCR was performed and the mRNA levels of LKB1 were quantified. The experiment was performed 3 times and all levels were normalized to the levels of ATP50. The statistical significance was calculated with a T-test.

5 The GCN5/LKB1 signaling is essential for head formation

5.1 GCN5 is expressed in cephalic neural crest cells

After showing the functional and physical interaction between GCN5 and LKB1 in human cells, we sought to investigate their functional interaction *in vivo*, and based our study on the chick embryo model system. In our previous study, we showed that LKB1 is expressed in cephalic neural crest cells (CNCCs) and that LKB1 is essential for head formation. In this context, we first wondered whether GCN5 is also expressed in CNCCs.

In order to answer this question, we performed whole-mount *in situ* hybridization allowing us to establish the gene expression pattern of *GCN5* in chick embryos. For this purpose, we first cloned and sequenced the chicken homologue of GCN5 cDNA. Sequence analysis revealed that the chicken GCN5 amino acid sequence is 89% identical to the human long isoform of GCN5 (GCN5-L) sequence, and the three main domains of GCN5 are conserved in both species. Digoxigenin-labeled “antisense” RNA probes targeting the *GCN5* mRNA were then synthesized. Digoxigenin-labeled “sense” RNA probes that cannot get hybridized with the *GCN5* mRNA were used as control. Whole-mount *in situ* hybridization was then performed on fixed embryos at different developmental stages. The mRNA was detected by a color reaction.

The *GCN5* transcripts were detected at 4 somite stage (ss) in the neural folds, just before neural tube closure. The accumulation of *GCN5* transcripts intensified at 7ss in the neural folds and was detected in migrating CNCCs. At 11ss, *GCN5* expression was detected in the migrating CNCCs, which progressed along the cephalic vesicles. No mRNA was detected with the control probe and at the primitive streak stage (**FIG.5A**). Thus, GCN5 is expressed in CNCCs during chick embryos development.

5.2 Functional interaction between GCN5 and LKB1 *in vivo*

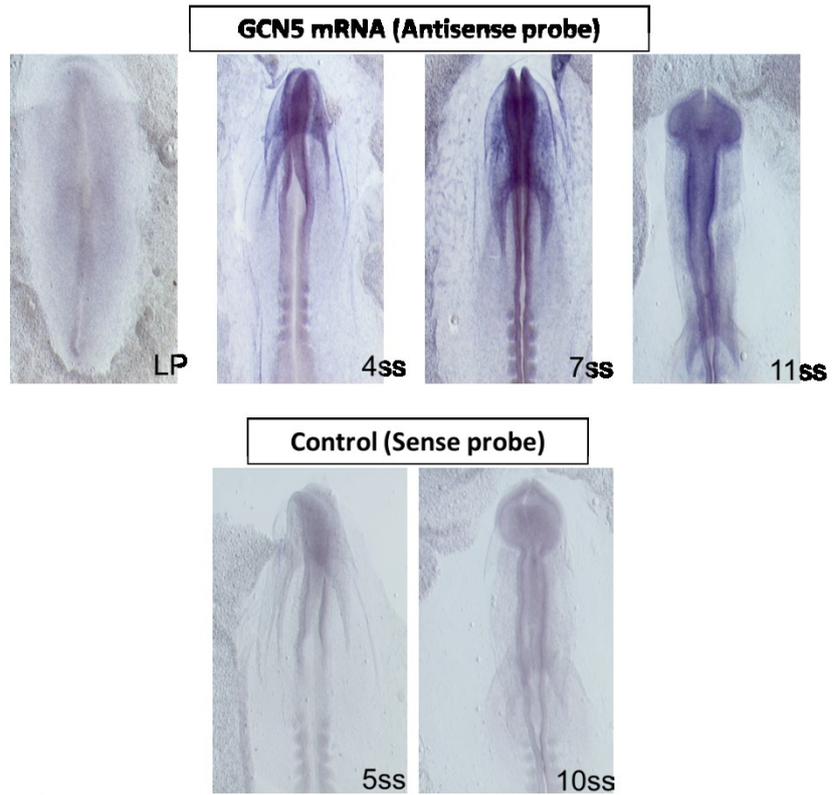
Since both LKB1 and GCN5 are expressed in CNCCs, we first wanted to know whether GCN5 is required for head development. To selectively inhibit *GCN5* expression in CNCCs, we used the RNA interference approach to bilaterally electroporate the cephalic neural folds with *dsRNA* molecules. For this purpose, *dsRNA* targeting *GCN5* mRNA were synthesized and 5ss embryos CNCCs were electroporated, before their emigration from the neural tube. The embryos were fixed 72h post-transfection and their phenotype was analyzed and compared with control electroporated embryos.

As shown in **FIG.5B**, silencing *GCN5* in CNCCs leads to drastic craniofacial malformations in chick embryos. These malformations include a severe reduction of the telencephalon and of the naso-frontal and the maxillo-mandibular structures. Thus, *GCN5* is essential for the formation of craniofacial structures in chick embryos.

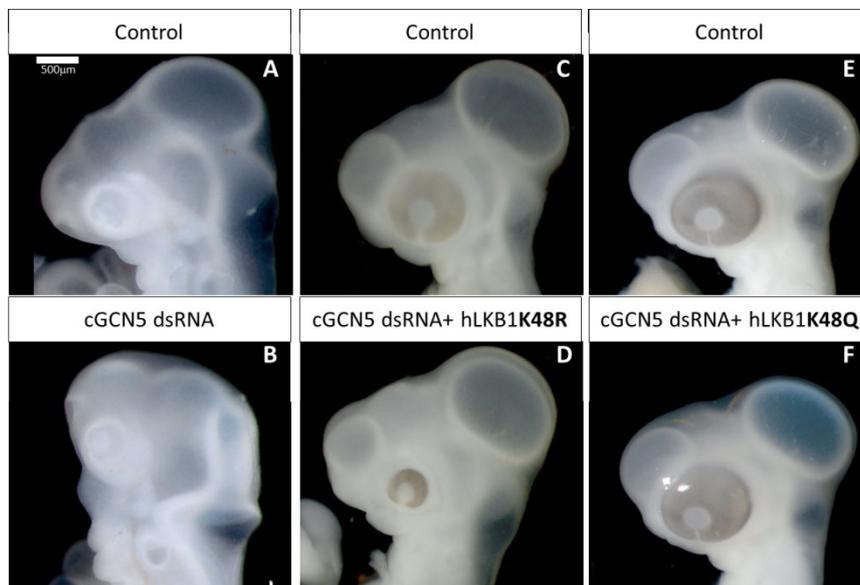
It was of particular interest to know whether the acetylation of LKB1 at K48 downstream of *GCN5* is required for head development. For this purpose, CNCCs were electroporated with *GCN5*-*dsRNAs* together with LKB1-K48R or with an acetylmimetic form of human LKB1 (LKB1-K48Q). Interestingly, while the K48Q form of LKB1 completely rescued the phenotype generated by *GCN5* silencing, the K48R only partially rescued the phenotype. Thus, the acetylation of LKB1 at K48 is required for a proper *GCN5/LKB1* signaling in CNCCs, but the expression of LKB1, even when it is not acetylatable, seems to mediate *GCN5* functions in CNCCs.

Together, these data indicate that LKB1 is a downstream target of *GCN5 in vivo* and that the acetylation of LKB1 at K48 is required for proper *GCN5/LKB1* signaling.

FIG.5



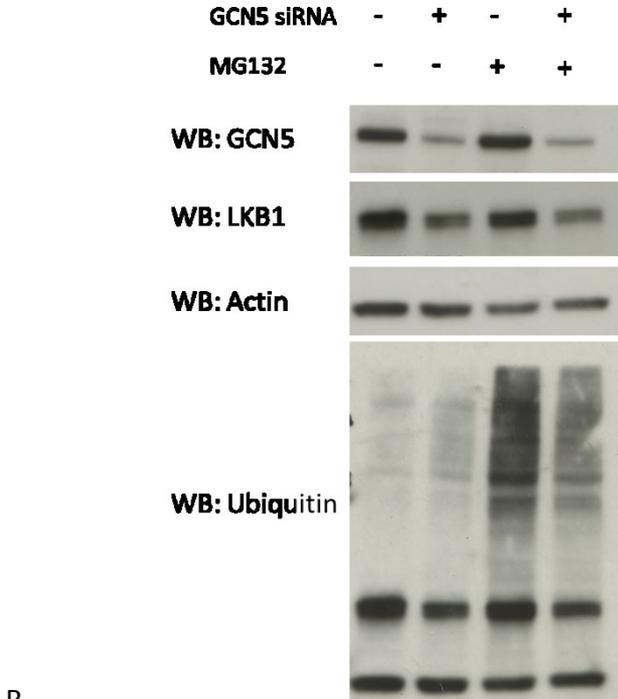
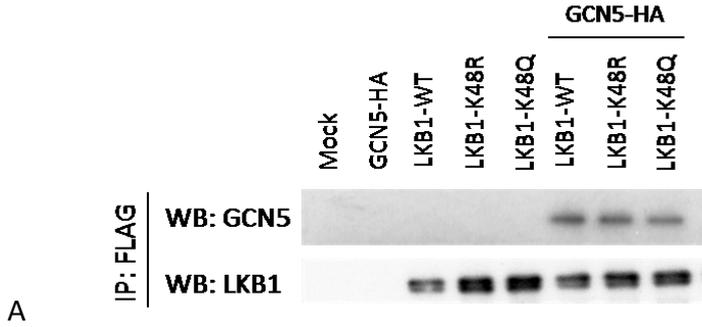
A



B

FIG.5: Functional interaction between GCN5 and LKB1 *in vivo*

- A)** Expression pattern of GCN5 in chick embryos. Chick embryos were fixed in 4% PFA at different stages. Digoxigenin-labelled antisense probes targeting the mRNA of GCN5 were synthesized and used to perform whole-mount *in situ* hybridization on chick embryos. The violet coloration indicates GCN5 transcripts. Sense probes were used as control. ss= somite stage.
- B)** Cephalic phenotype of chicken embryos at E4 stage. **(A, C, E)** Control embryos. **(B)** Embryos subjected to the silencing for cGCN5. **(D, F)** Embryos coelectroporated with cGCN5 dsRNA together with hLKB1-K48R or hLKB1-K48Q respectively. **(D)** hLKB1-K48R partially restores the cephalic development of *GCN5* hypomorphic embryos. **(F)** hLKB1-K48Q completely restores the cephalic development of *GCN5* hypomorphic embryos.



Supplemental data

- A) **GCN5 interacts with WT- and mutated K48-forms of LKB1.** Bosc cells were transfected with plasmids expressing a WT-FLAG-LKB1 protein or mutated K48-forms of LKB1: non-acetylatable (K48R) or acetyl-mimetic (K48Q) and/or with WT-GCN5-HA (long isoform). LKB1 was immunoprecipitated 48h post-transfection with an anti-FLAG antibody. LKB1 and GCN5 were then detected by western blot with the corresponding antibodies.
- B) **Silencing GCN5 decreases LKB1 protein levels.** Bosc cells were transfected with a pool of siRNAs targeting the mRNA of GCN5 or with control siRNAs for 72h. Cells were then treated with 25 μ M MG132 for 4h, and the expression levels of endogenous LKB1 were analyzed by western blot. The efficiency of MG132 treatment was verified by detecting the accumulation of ubiquitinated proteins.

III. Discussion and perspectives

During my PhD, I showed that the acetyltransferase GCN5 regulates the acetylation state, the localization, the functions as well as the RNA levels of the tumor suppressor LKB1. The functional interaction between GCN5 and LKB1 was also observed *in vivo*, where both proteins are implicated in the formation of craniofacial structures of chick embryos.

In this section, we will discuss our observations and highlight their importance in understanding the regulation of LKB1 and its implication in cellular mechanisms.

1 The acetylation state of LKB1 correlates with its subcellular localization and consequent activity

To date, thanks to our collaborator Vincent Mirouse, we are the first team to dispose of a tool that allows us to study the specific acetylation of LKB1 at K48 and its regulation. Thus, after identifying the acetyltransferase GCN5 as an upstream regulator of LKB1 acetylation, we showed that this acetylation occurs at lysine 48 and depends on the acetyltransferase (HAT) activity of GCN5. These findings raised two important questions: **1)** what are the consequences of this acetylation on LKB1 subcellular localization and activity? **2)** what is the relevance of this acetylation in physiological processes?

Indeed, the cellular distribution of LKB1 mostly determines the factors with which it can interact and the substrates it can regulate, consequently modulating its activity and functions. In other words, LKB1 is more likely to interact with cytoplasmic factors and to regulate cytoplasmic proteins within the cytoplasm, and is more likely to interact with nuclear factors and substrates within the nucleus. Thus, it is important to better understand the molecular mechanisms responsible for LKB1 shuttling and the consequences of its localization on the regulation of cellular processes.

1.1 Two GCN5-dependent modes of LKB1 localization modulation

1.1.1 Summary of the results

Regarding LKB1 cellular distribution, we observed that LKB1 is expressed in both the nucleus and the cytoplasm. Acetylated LKB1 is expressed in the nucleus, in line with Lan's observation using the acetyl-mimetic form of LKB1 K48Q (Lan et al., 2008). The non-acetylated form of LKB1 is expressed in both the nucleus and the cytoplasm, consistent with Lan's observation using the K48R mutant. However, the cytoplasmic localization of non-acetylated LKB1 depends on GCN5 expression but is independent of GCN5 HAT activity. These results are summarized in **Figure 61**.

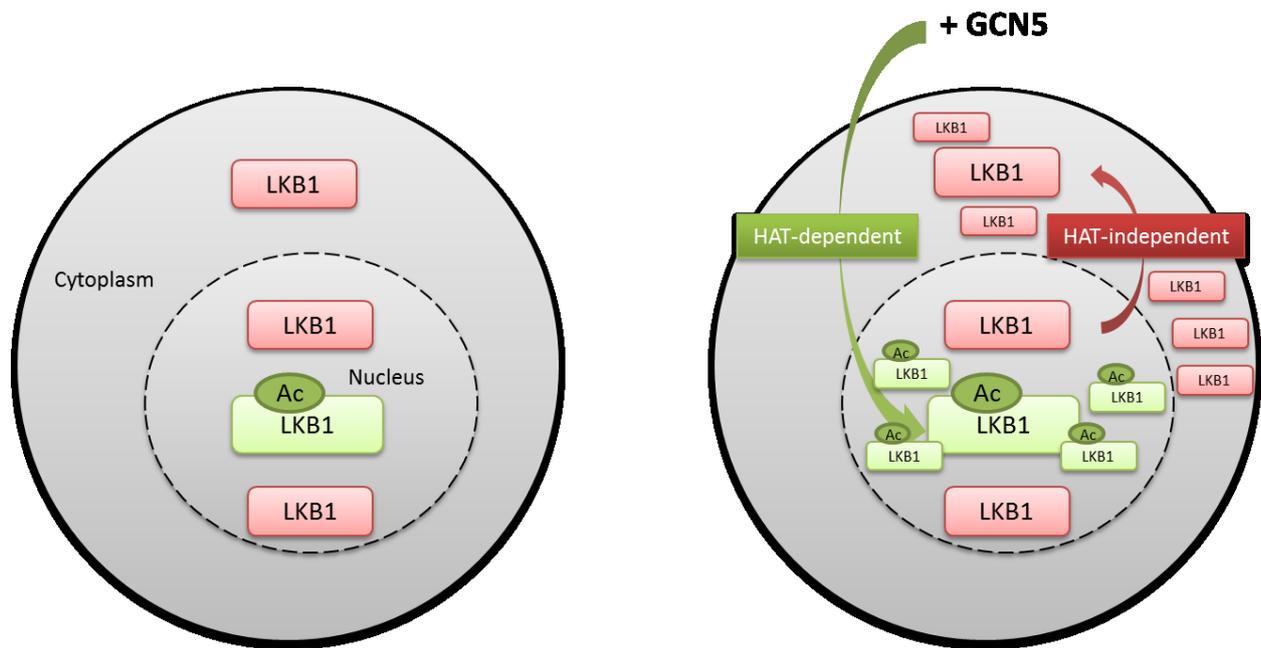


Figure 61: Regulation of LKB1 acetylation and localization by GCN5

On the left: In cells expressing endogenous GCN5, LKB1 is localized in both the nucleus and the cytoplasm. We expect that a small amount of LKB1 is acetylated and localized in the nucleus. **On the right:** GCN5 ectopic expression leads to an increase in acetylated LKB1. This fraction remains in the nucleus. GCN5 expression also results in the translocation of a fraction of LKB1 into the cytoplasm independently from its acetyltransferase (HAT) activity. Another fraction remains in the nucleus.

1.1.2 The HAT activity of GCN5 is dispensable for LKB1 shuttling into the cytoplasm

The observation whereby the HAT activity of GCN5 is dispensable for LKB1 shuttling into the cytoplasm was unexpected. We could not exclude the possibility that the loss of GCN5 acetyltransferase activity would be compensated by the acetyltransferase activity of other members of the GCN5-containing complexes, such as the GCN5 homologue PCAF or the acetyltransferase hATAC2, which were introduced in the first section of this manuscript. However, if it was the case, we would not have observed a complete nuclear localization of LKB1 in the nucleus following GCN5 silencing. Another possibility was that the expression of either forms of GCN5 (WT ou MUT) would promote the increase of endogenous GCN5 expression, thereby showing the same effect on LKB1 localization. In this case, endogenous GCN5 also needed to be silenced. But here again, preliminary results show that when we silence endogenous GCN5 and ectopically express WT- ou MUT-GCN5, we still observe the translocation of a fraction of LKB1 into the cytoplasm (data not shown). Thus these observations tend to confirm that the HAT activity of GCN5 is indeed not required for LKB1 nucleo-cytoplasmic translocation.

GCN5 has already been shown to act independently from its catalytic activity. This was first suggested by Bu and his collaborators, who have shown that the acetyltransferase activity of GCN5 is essential for proper cranial neural tube closure, while this HAT activity seemed dispensable for other developmental processes that they did not investigate (Bu et al., 2007). The latter case was first observed *in vitro* in 2009 (for more details: ((Mao et al., 2009)) and was recently brought to light in an interesting publication, which is more related to the immune response than to developmental processes, but in which GCN5 (and PCAF) is shown to repress IFN- β (interferon β) production and innate antiviral immune response in a HAT-independent and non-transcriptional manner. GCN5 rather promotes the phosphorylation of TBK-1 (TANK-binding kinase 1) at Ser172 thereby inhibiting its kinase activity and signaling in the cytoplasm. Consequently, the TBK-1 downstream target and transcription factor IRF 3/7 (interferon regulatory factors 3 and 7) is not phosphorylated and cannot activate IFN- β transcription (Q. Jin et al., 2014) (**Figure 62**).

These data compel us to further investigate the mechanism whereby GCN5 promotes the translocation of LKB1 from the nucleus into the cytoplasm. In line with our results and the available information in the literature, we can propose the following regulatory elements.

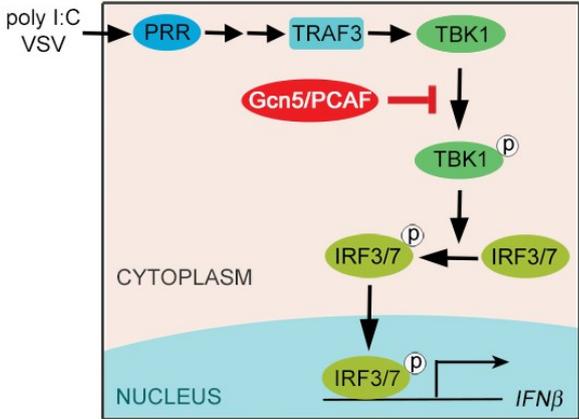


Figure 62: GCN5 inhibits IFN-β production in an HAT-independent manner

Schematic representation of GCN5-dependent inhibition of TBK1 leading to inhibition of IFN-β transcriptional activation and consequent repression of anti-viral immune response. Adapted from (Q. Jin et al., 2014).

1.1.3 GCN5-dependent translocation of LKB1 into the cytoplasm

As presented in the introduction section, the phosphorylation of LKB1 at Ser428 was shown to induce the translocation of LKB1 from the nucleus into the cytoplasm *in vitro* although this observation remains controversial. As previously described, GCN5 indirectly regulates the phosphorylation of proteins such as TBK1, thereby affecting their activity. In this context, we tested whether the expression of GCN5 could enhance the phosphorylation of LKB1 at Ser428. Surprisingly, the expression of both WT-GCN5 and MUT-GCN5 increased the phosphorylation of LKB1 at Ser428 (**Figure 63**). Although this result is preliminary, the phosphorylation of LKB1 at Ser428 is induced following GCN5 expression, but the relevance of this phosphorylation requires further investigation. GCN5 could be responsible for recruiting kinases such as PKC- ζ (protein kinase C- ζ), PKA (protein kinase A) and RSK (ribosomal protein S6 kinase) which are known to phosphorylate LKB1 at this site.

One of the obvious possibilities is that GCN5 would enhance the interaction of the non-acetylated form of LKB1 with STRAD α and/or LIP1 and/or CRM1 (**Figure 64**). This hypothesis can be partly tested by immunoprecipitating GCN5, and testing whether these known LKB1-interacting partners are present in the complex. Using immunofluorescence experiments, we can silence each or a combination of the cited proteins and test whether LKB1 can still be translocated into the cytoplasm following GCN5 ectopic expression.

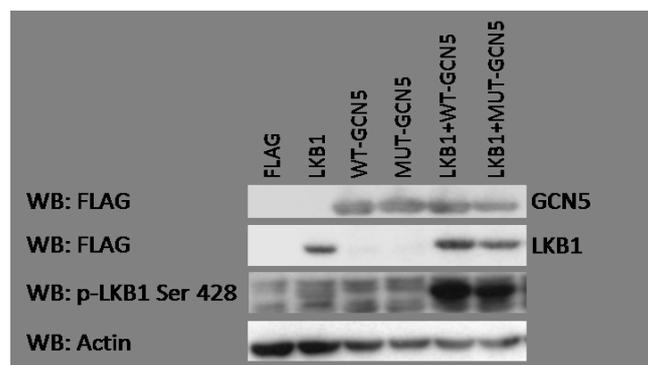


Figure 63: GCN5 induces the phosphorylation of LKB1 at Ser 428.

Bosc cells were transfected with LKB1, WT-GCN5 or MUT-GCN5 or with LKB1 together with WT-GCN5 or MUT-GCN5. Mock vectors were used as control. The phosphorylation of LKB1 at Ser428 was detected by western blot with the corresponding antibody. Actin was used as control.

Finally, we can expect the presence of a balance between GCN5 and SIRT1 in the cell, and K48 could represent a competitive site for both enzymes. The expression of GCN5 might activate the competition with SIRT1 at K48. Both enzymes would interact with LKB1; the fraction that interacts with GCN5 is acetylated and remains nuclear, while the SIRT1-interacting fraction is deacetylated and translocated into the cytoplasm (**Figure 64**). Silencing SIRT1 in the experiments would confirm or reject this possibility.

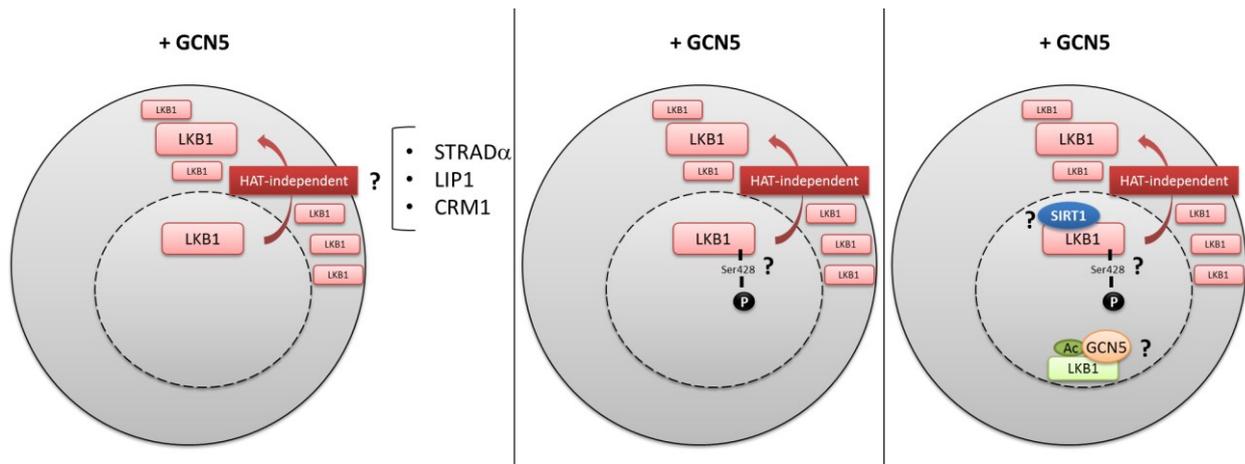


Figure 64: GCN5 modulates LKB1 localization independently from its HAT activity (details in text)

1.1.4 Retention of acetylated LKB1 in the nucleus

One novelty of our study is the observation that the acetylated fraction of LKB1 at K48 is strictly nuclear. But why is this acetylated form retained in the nucleus?

STRAD α binds to LKB1 in its kinase domain (a.a. 49-309) (A F Baas et al., 2003). We still do not know whether the acetylation/deacetylation of LKB1 at K48, which is just next to K49 where the kinase domain of LKB1 starts, could induce structural changes in LKB1 conformation. The latter is determinant in allowing the access of STRAD α to the kinase domain of LKB1 or restraining it. Thus, the folding of LKB1 in its acetylated (or acetyl-mimetic) and non-acetylated (or non-acetylatable) form should be investigated, using the LKB1 mutants K48R and K48Q respectively, in comparison with the WT form that was subjected to WT or MUT-GCN5 expression. This aspect is being currently studied in collaboration with the

Structural Biology Institute (IBS) in Grenoble, whereby the molecular modelization of LKB1 can provide us with predictive structural models. A further step would be analyzing the structural conformation of LKB1 in this context using crystallography. In parallel, the interaction of STRAD α with LKB1 in both its acetylated and non-acetylated state should be investigated.

According to the literature, LKB1 might also be sequestered in the nucleus by two partners: STRAD β and/or Nur77. The STRAD β -LKB1 complex was suggested to possess unique functions in the nucleus. STRAD α and STRAD β share functional similarities; they both bind LKB1 and MO25 and induce autophosphorylation and activation of LKB1. They also inhibit the importin-dependent entry of LKB1 into the nucleus. However, both STRAD isoforms differ in their structure; STRAD β lacks the N-terminal and C-terminal domains that allow STRAD α to bind CRM1 and exportin-7 and to promote LKB1 nuclear export (Dorfman & Macara, 2008). Thus, the acetylated form of LKB1 might bind more avidely to STRAD β rather than STRAD α , which would block the CRM-1-dependent export of LKB1 from the nucleus into the cytoplasm, leading to the retention of acetylated LKB1 in the nucleus.

Nur77 was also shown to bind to and to sequester LKB1 in the nucleus and this interaction is disrupted following treatment with TMPA (ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl]acetate). The latter is a chemical compound that binds to Nur77 with high affinity leading to the release and shuttle of LKB1 into the nucleus (Zhan et al., 2012). Thus, silencing STRAD β in combination with a TMPA treatment might lead to a complete relocalization of LKB1 in the cytoplasm.

1.2 LKB1 acetylation at K48 is required for its functions

1.2.1 Summary of the results

Regarding LKB1 activity, we observed that the non-acetylatable form of LKB1, K48R, does not completely rescue the formation of craniofacial structures in chick embryos following GCN5 silencing. In support of our data, the team of Vincent Mirouse (GReD, UMR 6247, Clermont-Ferrand), with whom we collaborate on this project, observed that in contrast to the wild-type human form of LKB1, the expression of the non-acetylatable K48R mutated form of hLKB1, fails to rescue the loss of polarity phenotype observed in follicular epithelial cells of a *Drosophila* strain, in which the LKB1 ortholog was inactivated by mutation. Finally, we observed that the K48R mutant fails to repress the mTOR pathway as indicated by S6RP phosphorylation, a key enzyme in the regulation of cell growth and proliferation. Together, these data indicate that the acetylation of LKB1 at K48 is required for its functions. They also suggest that both the acetylated and non-acetylated forms of LKB1 may be required or may work synergistically in the regulation of certain processes.

1.2.2 Complementary experiments

In order to better understand the importance of LKB1 acetylation for its activity, we first need to perform a kinase assay, whereby the activity of WT-, K48R- and K48Q-LKB1 are measured in comparison with the activity of WT-LKB1 following the expression of WT-or MUT-GCN5. We also need to further investigate the role of LKB1 acetylation in the regulation of the mTOR pathway, through studying other downstream mTOR targets such as S6K1 and 4-EBP1. The consequences of LKB1 acetylation on AMPK activation will be discussed further. We are currently trying to rescue the GCN5 phenotype in chick embryos with a WT-LKB1 in comparison with a KD-LKB1 (kinase-dead) in order to conclude regarding the role of LKB1 acetylation *in vivo* downstream of GCN5. We will then assess the role of LKB1 acetylation in the growth, polarity and migration of CNCCs and whether this acetylation is required for the sequential activation of AMPK, ROCK and MRLC as we've previously described.

1.2.3 Role of acetylated LKB1 in the nucleus

The cytoplasmic localization of LKB1 is mainly associated with its role in the regulation of cellular polarity, metabolism but most importantly with its tumor suppressing functions whereby it inhibits cell growth (Jérôme Boudeau, Sapkota, & Alessi, 2003; Corradetti, Inoki, Bardeesy, DePinho, & Guan, 2004; Shackelford & Shaw, 2009). These functions of LKB1 in the cytoplasm have already been thoroughly investigated and detailed in the introductory section.

What mostly attracts our curiosity and the most perspective-opening part of our study is the role of LKB1 in the nucleus, which has been poorly investigated. LKB1 was shown to bind to and to activate Brg1 (brahma-related gene 1), an essential component of chromatin remodeling complexes, thereby inducing cell cycle arrest and senescence (Marignani, Kanai, & Carpenter, 2001). LKB1 can also physically bind to p53 in the nucleus and directly or indirectly phosphorylate it at Ser15 (previously shown to be phosphorylated by AMP-dependent kinase) and Ser392. These two p53 residues are required for LKB1-dependent cell cycle arrest. LKB1 is recruited to the *p21/WAF1* promoter in a p53-dependent manner and has a direct role in activating *p21/WAF1* gene transcription (Zeng & Berger, 2006). More recently, LKB1 was shown to regulate the Yes-associated protein (Yap), which has emerged as a transcriptional co-activator that modulates tissue homeostasis in response to cell-cell contact. LKB1 promotes Yap phosphorylation, nuclear exclusion and proteasomal degradation. Phosphorylation-defective Yap mutants fail to rescue LKB1 phenotypes, such as reduced cell proliferation and cell size, suggesting that Yap inhibition contributes to LKB1 tumor suppressing functions. Of note is that LKB1 inhibits Yap independently of either AMPK or mTOR activation (Nguyen, Babcock, Wells, & Quilliam, 2013). Finally, the SL26 LKB1 mutant, found in PJS, was shown to be exclusively nuclear but still retained its catalytic activity (A F Baas et al., 2003). Together, these data indicate that LKB1 functions are not exclusively cytoplasmic, and that nuclear LKB1 is active.

In this context, it would be interesting to know whether the acetylation of LKB1 is required for these functions and to determine potential LKB1 nuclear targets and substrates aside from the ones cited above. This will allow us to better understand how nuclear LKB1 is

implicated in the regulation of physiological processes such as cancer, metabolism and response to stress.

1.2.3.1 Identification of a new nuclear target

Sox-9 and Sox-10 (Sry-related HMG box) are transcription factors that are implicated in NCCs ontogenesis. Both are expressed in cranial and trunk NCCs. However, the role of Sox-9 is predominant in CNCCs, while Sox-10 is essential for trunk NCCs formation, survival, migration and differentiation in mouse, zebrafish, chick and xenopus models.

In human, *sox-10* mutations are associated with the Waardenburg-Hirschprung disease, a neurocristopathy which is characterized by abnormalities such as intestinal aganglionosis (Honoré, Aybar, & Mayor, 2003; Kelsh, 2006) (**Figure 65**). Based on published data, our team is currently investigating the functional interaction between LKB1 and the transcription factor Sox-10 in the nucleus. The expression of the WT form of LKB1 induced the transcriptional activation of Sox-10 target genes while the LKB1 mutant lacking the nuclear localization signal (NLS) failed to promote this activation. These data support the role of nuclear LKB1 in the regulation of gene expression.

Mutations in *sox-9* are associated with Campomyelic Dysplasia in human, a congenital birth defect which is characterized by skeletal defects and distinctive craniofacial features including a small chin and a flat face (Gordon et al., 2014; Kelsh, 2006) (**Figure 65**). It would be interesting to also test whether LKB1 also regulates the expression of *sox-9 in vitro*.

The next step would be to test whether the acetylation of LKB1 is required for this transcriptional regulation by expressing the non-acetylatable mutant form of LKB1, K48R. It would then be of particular interest to test whether the regulation of *sox-9* and *sox-10* expression by LKB1 also occurs *in vivo* in cranial and trunk NCCs and to further investigate the role of this regulation in the formation of craniofacial structures and the enteric nervous system respectively. Indeed, impaired regulation of *sox-9* expression by nuclear acetylated LKB1 might also account for the craniofacial abnormalities observed following LKB1 or GCN5 deletion in CNCCs in chick embryos.

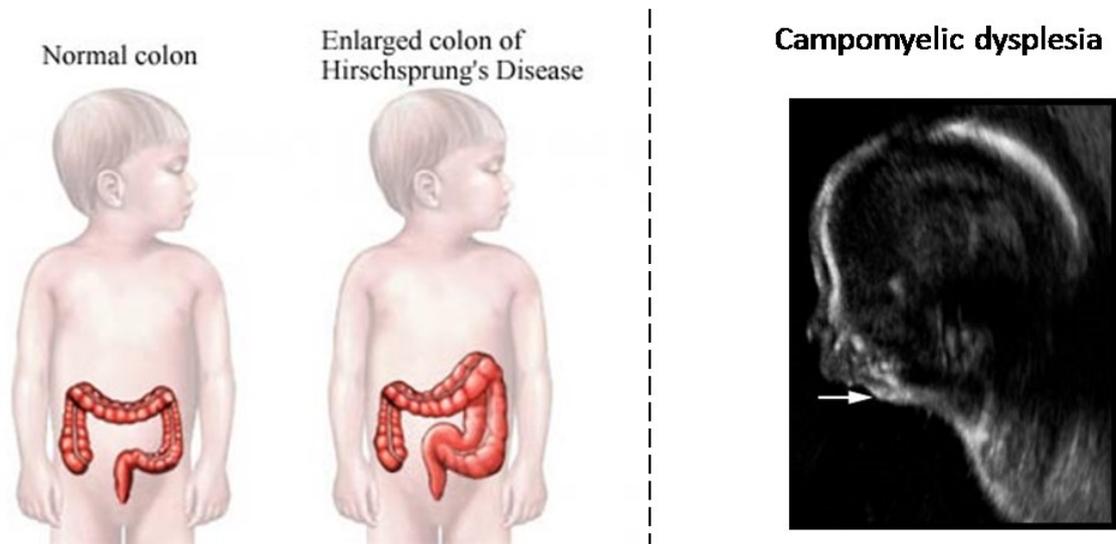


Figure 65: Sox-9 and Sox-10 associated abnormalities

To the left: Enlarged colon of Hirschsprung's disease due to intestine aganglionosis (Nursing Crib © 2015.). **To the right:** Sagittal ultrasound images of the fetal profile showing Campomelic dysplasia at 22 weeks and demonstrating markedly flat facies and retrognathia (arrow) (©2011 UpToDate®).

1.2.3.2 Regulation of lipid homeostasis

GCN5 was shown to be deacetylated by SIRT6 on K549, which induces its phosphorylation on Ser307 and Thr735 and its activation in liver cells (Dominy et al., 2012). In this context, we tested whether SIRT6 also induces acetylation of LKB1 at K48, and confirmed this hypothesis. Indeed, SIRT6 increased LKB1 acetylation compared to LKB1 alone (Error! Reference source not found..A). This effect seems to be mediated by GCN5 (data not shown). Finally, in collaboration with Chantal Thibert and Anca Iordache in our team, we observed that SIRT6, like GCN5, promotes LKB1 phosphorylation at Ser 428 (Error! Reference source not found..B) and that endogenous SIRT6 and LKB1 physically interact (Error! Reference source not found..C).

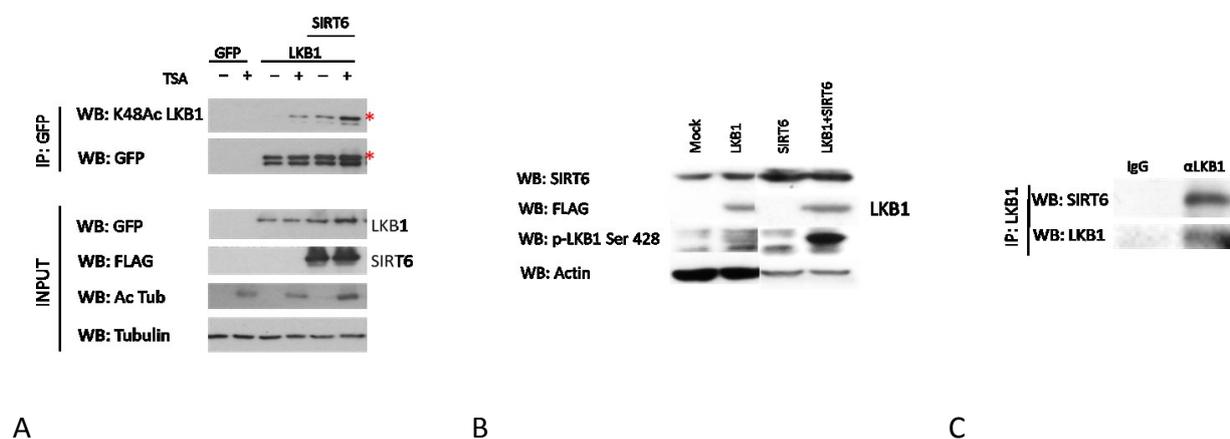


Figure 66: SIRT6 interacts with LKB1 and promotes its acetylation and phosphorylation

A) SIRT6 induces LKB1 acetylation. Bosc cells were transfected by LKB1 alone or together with SIRT6. Mock vectors were used as control. Cells were treated with DMSO or with 1 μ M trichostatin A (TSA) for 4h prior to immunoprecipitation. LKB1 was immunoprecipitated 48h post-transfection with an anti-GFP antibody and the acetylation of LKB1 at lysine 48 (K48) was detected by western blot with the corresponding home-made antibody. **B) SIRT6 induces LKB1 phosphorylation.** Bosc cells were transfected by LKB1 alone or together with SIRT6. Mock vectors were used as control. Cells were lysed and the phosphorylation of LKB1 at Ser 428 was detected by western blot using the corresponding antibody. **C) Endogenous physical interaction between LKB1 and SIRT6.** Bosc cells were lysed and endogenous LKB1 was immunoprecipitated with the corresponding antibody in comparison with control IgG. Endogenous LKB1 and SIRT6 were detected by western blot.

SREBP1 and SREBP2 (Sterol regulatory element binding protein) are lipogenic transcription factors that are regulated by cholesterol, insulin, and glucose. While, SREBP1 mainly regulates lipogenic processes (activation of genes involved in fatty acid and triglyceride biosynthesis), SREBP2 mostly activates genes involved in cholesterol synthesis. When intracellular lipid levels are high, SREBPs are present in the ER (endoplasmic reticulum), which is in contact with the external nuclear envelope, as inactive precursors. Upon low lipid levels, SREBPs are moved to the Golgi where they are activated following a proteolytic processing. A transcriptionally active N-terminal basic helix-loop-helix (bHLH) zip domain is released. The mature form translocates to the nucleus where it promotes a lipogenic program in the liver (**Figure 67**). Dysregulation of SREBPs was shown to be involved in type-2 diabetes, dyslipidemia, and hepatic steatosis (Elhanati et al., 2013; Horton, Goldstein, & Brown, 2002; Raghov, Yellaturu, Deng, Park, & Elam, 2008).

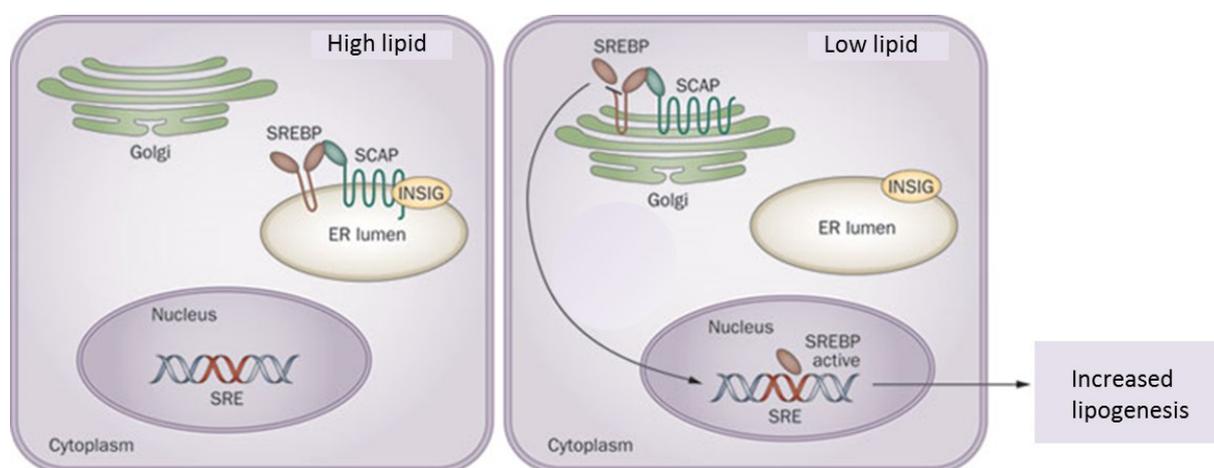


Figure 67: SREBPs regulate hepatic lipogenesis

When cellular cholesterol is high, the SREBP–SCAP complex is retained in the ER membrane by the INSIG proteins, leading to inhibition of sterol synthesis. Under low-lipid conditions, INSIG dissociates from the SREBP–SCAP complex, which is then transported to the Golgi membrane. The active form of SREBP is cleaved and translocates into the nucleus, where it activates the expression of genes containing the SRE (sterol regulatory element) in their promoter region, including genes involved in cholesterol synthesis for example. Abbreviations: ER, endoplasmic reticulum; INSIG, insulin-induced gene; SCAP, SREBP cleavage-activating protein; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein. Modified from (Karasinska & Hayden, 2011)

Interestingly, when intracellular lipid levels are high, SIRT6 was shown to induce AMPK activation by increasing the AMP/ATP ratio, but also by activating LKB1 and inducing its phosphorylation at Ser 428. AMPK would then phosphorylates and inactivate SREBPs, retaining them from entering the nucleus. As a result, the transcription of lipogenic genes and the resulting hepatic lipogenesis are inhibited (Elhanati et al., 2013) (**Figure 68**).

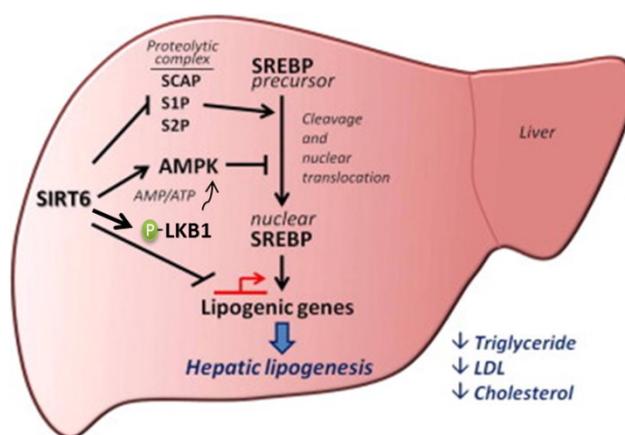


Figure 68: Model of the regulation of hepatic lipogenesis by SIRT6

In high lipid conditions, SIRT6 activates AMPK by increasing the AMP/ATP ratio and by activating LKB1 and phosphorylating it at Ser 428. AMPK then inhibits SREBP proteolytic processing which retains it from activating lipogenic genes expression. SIRT6 also inhibits hepatic lipogenesis by inhibiting the proteolytic complex responsible for SREBP cleavage or by directly inhibiting lipogenic genes expression activation. Modified from (Elhanati et al., 2013).

Finally, AMPK shuttles between the nucleus and the cytoplasm (Kodiha, Rassi, Brown, & Stochaj, 2007) and is a sensor of the ER stress (Xi, Barredo, Merchan, & Lampidis, 2013). If we show that acetylated nuclear LKB1 can phosphorylate nuclear AMPK, we can imagine that in liver cells under lipogenic stress (high levels), SIRT6 could sequentially activate GCN5, LKB1 and AMPK, resulting in the retention of SREBPs in the ER and leading to the inhibition of lipogenic gene expression/hepatic lipogenesis (**Figure 69**).

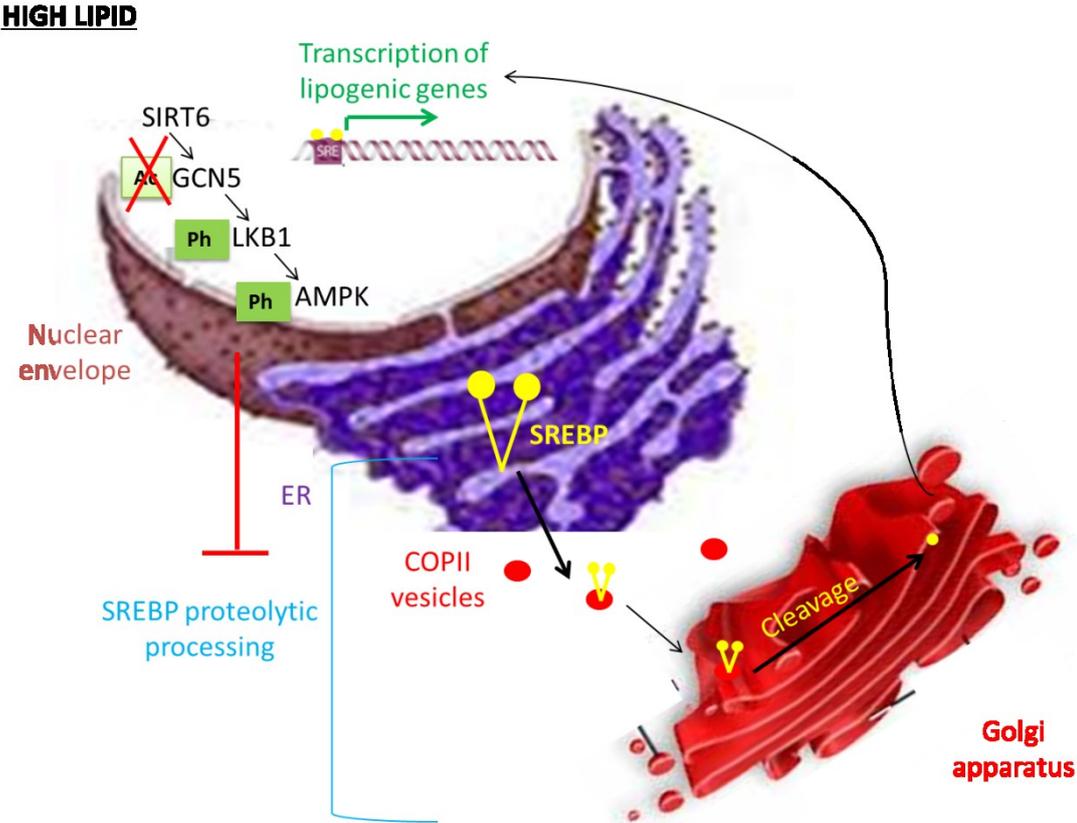


Figure 69: Hypothetic model of the SIRT6/GCN5/LKB1/AMPK signaling in hepatic lipogenesis regulation

(See details in text)

1.2.3.3 Potential role of nuclear LKB1 in response to cellular stress

In response to cellular stress such as the glucose analog 2-DG (2-Deoxy-D-glucose) and UV light, LKB1 and AMPK α 2 physically bind to the chromatin in an interdependent and p53-dependent fashion to regulate gene transcription. AMPK α 2 was also shown to specifically phosphorylate histone H2B at Ser36 in response to stress such as 2-DG treatment, but also in response to well-characterized AMPK activators such as AICAR and phenformin. Interestingly, in *Lkb1*^{-/-} MEFs, H2B was not phosphorylated at Ser36, but the phosphorylation was restored when LKB1 was reintroduced into the cells. These data suggested that in response to metabolic stresses *in vivo*, LKB1/AMPK α 2 are implicated in the phosphorylation of H2B at Ser36 and the regulation of target genes such as *p21*, thereby tuning specific transcriptional responses, regulating cellular metabolism, and promoting cell survival (Bungard et al., 2010). It would be interesting to further investigate the role of LKB1 acetylation in this response to stress and to test whether chromatin remodeling complexes such as GCN5-containing complexes are required for the transcriptional activation of the target genes. But first, the acetylation state of LKB1 should be studied in response to cellular stress such as glucose (using 2-DG) and serum deprivation.

1.2.3.4 Role of nuclear LKB1 in cancer

In addition to understanding the role of the LKB1 signaling pathway in craniofacial structures formation, the NCCs represent for us a valuable tool that allows us to better understand tumor progression. By electroporating the CNCCs with GFP-tagged WT and mutated LKB1 constructs (K48R and K48Q) we can study the role of LKB1 acetylation at K48 in modulating its subcellular localization *in vivo*. As described in the introduction section, the cellular distribution of LKB1 is a prognostic marker in cancer cells. By monitoring the role of this acetylation in the survival, polarity and migration of CNCCs, we would unveil a novel regulatory mode in cancer cells growth and metastasis, thereby providing potential targets for cancer therapies.

One particular somatic LKB1 mutation was observed in PJS patients in sporadic malignant melanomas: the missense mutation Y49D (Rowan et al., 1999). This mutation has never been further investigated. However, this mutation occurs at the first amino acid of the kinase domain of LKB1, and now we know that it is placed next to the K48 residue which can be

acetylated. Thus, it is possible than in these patients, the mutation of Y49 would prevent the acetylation of LKB1 at K48 thereby affecting LKB1 subcellular localization and its consequent activity in melanoma cells. Therefore, it would be interesting to study the kinase activity, the subcellular localization and the acetylation state of this Y49D at K48 in a single mutation context but also in a double mutation context when K48 cannot be acetylated. This will allow us to better understand the consequences of this mutation in the PJS.

2 A new regulatory mode of LKB1 expression levels

Since the identification of the tumor suppressor LKB1, its implication in the regulation of several downstream targets and cellular processes has been thoroughly investigated. However, little is known about the regulation of *LKB1* gene expression and the turnover of its mRNA has never been documented. So far, four cis-regulating elements, corresponding to the transcription factors Sp1 (specificity protein 1), NF-Y (nuclear factor Y) and two FOXO (forkhead box O) family members FOXO3 and FOXO4, have been reported as transcriptional activators of *LKB1* (Lützner, De-Castro Arce, & Rösl, 2012). Androgens and estrogens such as testosterone and ER α were also shown to regulate *LKB1* gene expression through transcriptional regulation, in MCF-7 human breast cancer cells and adipocytes respectively (Linher-Melville, Zantinge, & Singh, 2012; McInnes, Brown, Hunger, & Simpson, 2012). This regulatory mode could be affected in a variety of cases in which *LKB1* was inactivated without having mutations within the coding sequence or independently from DNA methylation.

One of the surprising results of our project was the regulation of the mRNA levels of *LKB1* by the acetyltransferase GCN5. Whether GCN5 is implicated in the transcriptional regulation of *LKB1* or in the regulation of its mRNA stability remains unknown, and definitely requires further investigation. Indeed, impaired LKB1 expression has been linked with various cancers and may be a potential prognostic marker. For instance, loss of LKB1 in NSCLC is predictive of greater resistance to PI3K/Akt and MEK inhibitors, and is a negative prognostic marker in the context of mutant KRAS (W. Liu et al., 2012). Low LKB1 expression was also reported in sporadic breast cancer cases, and the investigation of LKB1 levels in 116 cases of human breast cancer samples has demonstrated that low LKB1 levels correlate with higher histological grade, larger tumor size, presence of lymph node metastasis, and shorter survival (Shen, Wen, Lan, Shen, & Shao, 2002). More recently, it has been shown that in colorectal cancer, where loss of LKB1 rarely occurs by gene mutation, loss of heterozygosity, and promoter methylation, impaired transcriptional activation of LKB1 promotes cell invasion, consequently resulting in poor outcome in colorectal cancer patients (He, Tsai, Huang, Chou, & Lee, 2014). Thus, LKB1 levels are a prognostic marker in cancer, and our findings provide an important step towards a better understanding of the complex

molecular events that govern tumor progression in a *LKB1*-deficient context. The following reasoning suggests that enhancing the transcriptional activation of *LKB1* in these cases would reestablish normal *LKB1* levels thereby promoting tumor regression and decreasing tumor resistance to drugs.

In order to further investigate the regulation of *LKB1* expression by *GCN5*, it would be relevant to start by defining whether this regulation occurs at a transcriptional level or whether it is the mRNA stability that is implicated in this process. In the latter case, we have to inhibit the degradation of the RNA by the exosome and the decapping complexes. As for transcriptional regulation, reporter assays (luciferase assay for instance) would allow us to know whether *GCN5* enhances the promoter activity of *LKB1*. It would be of particular interest to know whether the acetyltransferase activity of *GCN5* is required for this regulation, by using the HAT-mutant *GCN5* (MUT-*GCN5*) and testing the promoter activity of *LKB1* in this case. Afterwards, it would be relevant to know whether *GCN5* binds to this promoter region using an electrophoretic mobility shift assay (EMSA) complemented with chromatin immunoprecipitation experiments (ChIP). *GCN5* is known to be implicated in chromatin remodeling when it is in macromolecular complexes such as SAGA or ADA. In this context, disrupting these *GCN5*-containing complexes might impair the *GCN5*-dependent transcriptional activation of *LKB1*. Finally, since the short isoform of *GCN5* (*GCN5-S*) lacks the p300/CBP binding domain in comparison with the long isoform (*GCN5-L*), it is important to distinguish which isoform is implicated in the regulation of *LKB1* transcription.

IV. Conclusion

Our research allowed us to establish the role of LKB1 in vertebrates head formation and to identify the signaling pathway that is implicated, downstream of LKB1, in this developmental process. Based on experimental approaches in both chick and mouse embryos, we showed that LKB1 is required for cranial neural crest cells (CNCCs) survival, polarity and migration and that the LKB1/AMPK pathway orchestrates morphogenetic events that require the sequential activation of the ROCK kinase and the actin molecular motor Myosin-II. In addition to highlighting the role of LKB1 in craniofacial structures shaping, our study provides new perspectives for apprehending the onco-suppressive functions of LKB1 and its role in tumor development and metastasis in correlation with the pluripotency and highly invasive characteristics of CNCCs.

We have also shed light on the role of post-translational modifications (PTM), and more particularly the role of acetylation, in modulating the spatial distribution of LKB1 and its activity and identified a new upstream regulator of the LKB1 signaling pathway. We showed that the acetyltransferase GCN5 regulates LKB1 acetylation and expression levels, but also modulates its subcellular distribution in an HAT-dependent and -independent manner. While the non-acetylated fraction of LKB1 at K48 is both cytoplasmic and nuclear, the acetylated fraction is retained in the nucleus. This novel finding brings forward the nuclear functions of LKB1, which are to date poorly investigated. We suggest that both the acetylated and non-acetylated forms of LKB1 would act synergistically conducting to an efficient response of LKB1 to physiological changes. Nuclear LKB1 would be implicated in gene expression and cell cycle regulation through p21 translational activation.

Altogether, our data provide new insights into the importance of LKB1 for craniofacial structures formation and pinpoint the role of LKB1 acetylation in modulating its functions. We also propose a new regulatory mode of LKB1 expression mediated by the acetyltransferase GCN5. These novel findings may constitute a step forward towards apprehending congenital malformations, cancer and metabolic diseases.

V. Materials and methods

Cell culture

Bosc cells (human kidney epithelial cells) were cultured in DMEM with 10% of heat-inactivated fetal bovine serum (FBS) and 4.5 g/l of glucose, supplemented with 50 U/ml of streptomycin, and incubated at 37°C in 5% CO₂.

Vectors

The pSG5-FLAG eukaryotic expression vector was derived from the pSG5 plasmid (Stratagene; La Jolla, CA, USA). The pSG5 construct encoding the human wild-type LKB1 was previously described (Nony et al, 2003). The peGFP-C1/FLAG-LKB1 vector encodes the human FLAG-LKB1 fused N-terminally to the eGFP coding sequence as previously described (Forcet et al., 2005). The non-acetylatable mutant (K48R) and the acetyl-mimetic mutant (K48Q) were obtained by site-directed mutagenesis using the PFU DNA polymerase (Promega). The following primers were used:

For K48R:

Forward: 5' AAGCGGGCCAAGCTCATCGGCAGGTACCTGATGGGGGACCT 3'

Reverse: 5' AGGTCCCCATCAGGTACCTGCCGATGAGCTTGGCCCGCTT 3'

For K48Q:

Forward: 5' AAGCGGGCCAAGCTCATCGGCCAGTACCTGATGGGGGACCT 3'

Reverse: 5' AGGTCCCCATCAGGTACTGGCCGATGAGCTTGGCCCGCTT 3'

The pEBB-FLAG and pEBB-FLAG-GCN5 WT vectors were kindly provided by Pr. Ezra Burstein (UT Southwestern medical center, Dallas, Texas). The HAT mutant (Y621A/F622A) was obtained by site-directed mutagenesis according to manufacturer's instructions using the PFU DNA polymerase (Promega). The following primers were used:

Forward: 5' GCCATCGGCGCCGCCAAAAAGCAG 3'

Reverse: 5' CTGCTTTTTGGCGGCGCCGATGGC 3'

The pEBB-HA-GCN5 vectors were obtained by replacing the FLAG-tag with an HA-tag in the pEBB-FLAG-GCN5 vector by deletion/addition mutagenesis according to the manufacturer's instructions (NEB #E0552S). The following primers were used:

Forward: 5' ATGTATCCGTATGATGTGCCGATTATGCAGCGGAACCTTCCCAGGCC 3'

Reverse: 5' GAATTCGGTGAGATCCAGCACAATGGATC 3'

The short pCDNA3-GCN5-HA isoform was kindly provided by Dr. Saadi Khochbin.

Antibodies

The following antibodies were purchased from Sigma-Aldrich: anti-FLAG-M2. The following antibodies were purchased from Santa Cruz: anti-GFP, anti-GCN5, anti-LKB1 Ley37D/G6. The anti-Tubulin antibody was kindly provided by Dr. Laurence Lafanechère. The anti-K48Ac LKB1 antibody was kindly provided by Dr. Vincent Mirouse. The anti-GAPDH was purchased from Life technologies (Invitrogen), the anti-histone H4 from Abcam, the anti-ph-LKB1 Ser428 from Tebu-bio, the anti-actin from Millipore, the anti-HA from Covance and another anti-GCN5 was purchased from Ozyme (cell signalling).

Transfection and gene silencing

Cells were seeded in 10 cm culture dishes 24 hours prior to transfection. 4µg of DNA were transfected using the Jet Pei transfection reagent (Ozyme/Polyplus). The medium was changed 24h later and the cells were lysed 48 hours post-transfection.

The Jet Prime transfection reagent (Ozyme/Polyplus) was used in order to transfect DNA and RNA together. For RNA transfection alone, the RNAi Max reagent (Invitrogen/Life technologies) was used. SMARTpool ON-TARGETplus KAT2A (GCN5) human siRNA (ref. L-009722-02-0005) and ON-TARGETplus Non-targeting Pool (control siRNA) (ref. D-001810-10-05) were purchased from Fermentas /Thermo Scientific, Germany.

Treatment with drugs

Cells were treated for 4h before lysis with 1 μ M TSA (Trichostatin A) (Sigma-Aldrich) at 37°C. Cells were treated with 25 μ M MG132 (Calbiochem) for 4h at 37°C.

Cell lysis and western blotting

Bosc cells were lysed 48h post-transfection in a NP-40 lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% NP-40, supplemented with proteases inhibitors (protease inhibitor cocktail/ref. P8340, SIGMA), phosphatase inhibitors (phosphatase inhibitor cocktail 2/ref. P5726, SIGMA) and PMSF (Phenylmethanesulfonyl fluoride/ref. P7626, SIGMA). After a 15 mins incubation in ice, lysates were clarified by centrifugation at 13 200 rpm for 10 min at 4°C. Protein concentration was determined in supernatants by a modified Bradford assay (Bio-Rad). Clarified lysates were then used for immunoprecipitation experiments or directly boiled in 6X Laemmli buffer [20 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 20 μ g of bromophenol blue/ml] in the presence of 2- mercaptoethanol (10%), 5 mins at 95°C. Proteins were separated on an SDS-PAGE polyacrylamide gel, and transferred to polyvinylidenedifluoride (PVDF) membranes. Membranes were incubated with the appropriate primary (2h at room temperature or overnight at 4°C) and secondary (1h at room temperature) antibodies. Proteins were detected by ECL western blotting reagents (Dutscher).

Immunoprecipitation

After lysis and protein quantification, 500 μ g of lysate (for overexpressed proteins) or 1mg of lysates (for endogenous proteins) were completed to 1ml with lysis buffer. They were incubated for 1h at 4°C on a wheel with protein A-coupled sepharose beads (Dutscher) for pre-clearing. After 5 mins of centrifugation at 4°C and 2000 rpm, lysates were clarified and incubated for 2h or overnight with the corresponding antibody (0.5 μ g or 4 μ g respectively) on a wheel at 4°C. They have undergone 1 supplementary hour of incubation with Protein A-coupled sepharose beads. The beads were then recovered by centrifugation, washed 4 times with the lysis buffer and boiled in 6X Laemmli buffer, 5 mins at 95°C. They were then stored at -20°C or directly analysed by western blotting.

Cellular fractionation

For cellular fractionation, Bosc cells were seeded in 2 10cm culture dishes per condition. Cells were first scraped in PBS and harvested following 5 mins of centrifugation at 1200 rpm at 4°C. They were then washed once with the lysis buffer containing 10mM Tris-HCl, pH 7.5 and 2 mM EDTA. The pellet was then resuspended in 400 µl lysis buffer and 4µl of protease inhibitors were added to the mix. The lysates were incubated for 1h in ice and vortexed every 15 mins. After 1 hours of incubation, the lysate were passed 6 times in 26g syringes. 300µl of lysates were then placed on a sucrose gradient formed by a 60% sucrose solution (500µl) at the bottom above which was gently added a 10% sucrose solution. This solution contains 20mM Tris-HCl, pH 7.5 and 0.5 mM EDTA and 100mM NaCl with the respective sucrose quantity. The lysates were then centrifuged in a Beckmann coulter, Optima LE-80K Ultracentrifuge (Type: CO-LE 80K, Serie: COL00G01). The fractions were then recovered in clean 1.5ml tubes. The proteins were quantified using the Bradford reagent and boiled in the 6X Laemmli buffer for 5 mins at 95°C. The lysates were stored at -20°C or directly analysed by western blotting.

Immunofluorescence

Bosc cells were plated on glass coverslips and were transiently transfected as described. After 16 to 48 h, cells were fixed 20 min in 4% paraformaldehyde, washed in 1X PBS (Euromedex), permeabilized for 5 min in 0.5% Triton X-100, 0.3% BSA (diluted in 1X PBS) and blocked 20 min in PBS containing BSA 0.3%. Cells were then incubated overnight with primary antibody diluted in PBS–BSA 0.3% at 4°C and were incubated 1h with fluorescent-labeled secondary antibodies. Cells were mounted in Moviol/DAPI mounting medium and were observed by epifluorescence microscopy.

Quantitative RT-PCR

Total RNA was extracted from Bosc cells using the NucleoSpin RNA II kit (Macherey- Nagel) according to the manufacturer's instructions. RNA (1 µg) was reversely transcribed using the SuperScript VILO kit (Life Technologies). Quantitative real-timePCR was performed in triplicates with GoTaqR QPCR Master Mix (Promega) in a 25-µl reaction on a thermal cycler (C-1000 Touch; Bio-Rad Laboratories). Ct values were determined with the same software,

and normalization was done with the house keeping genes GAPDH, RELA, or ATP50, yielding very similar results. Expression levels were finally normalized to ATP50 expression. The following PCR primers (Roche) were used:

Forward: 5' ACCGGTCCTTGGTGTCTG 3'

Reverse: 5' ACCGGCCAAGAGGTTCTC 3'

Chick embryos and *in ovo* electroporation

Chick eggs were purchased from EARL MORIZEAU and HAAS. They were stored at 17°C and incubated at 37°C for the required time prior to use. *In ovo* electroporation was performed as described in our submitted publication. Double-strand RNAs were obtained by *in vitro* transcription of the cGCN5 cDNA that was cloned in a pGEMT-easy vector. The cDNA was obtained by RT-PCR using extracted total chick RNA as a matrix and the following primers:

Forward: 5' AAGCACAAGACCTTGGCAC 3'

Reverse: 5' TGACGTAGTAGCGGTTCTTC 3'

For rescue experiments, *GCN5* dsRNAs were co-electroporated with LKB1 expressing vectors.

In situ hybridization

a- Embryo collection and preparation

Fertile chicken eggs were incubated in a humidified incubator at 37.5°C for 1–2 days, depending on the desired stages. Embryos were collected into 1X PBS, removed from the vitelline membrane, and cleaned of yolk. They were fixed overnight at 4°C in fresh 4% paraformaldehyde.

Embryos were rinsed in PBS, then in PBT (1X PBS + 0.1% Tween-20), and dehydrated in increasing concentrations of Methanol/PBT (25%, 50%, 75%, twice in 100%). They were then stored at –20°C overnight (or up to 12 months). Embryos were rehydrated in decreasing concentrations of Methanol/PBT and were rinsed twice with PBT. They were then bleached in 6% Hydrogen peroxide (H₂O₂) in PBT for 1h and then washed 3 times in PBT. Embryos were then treated with 10 µg/mL proteinase K for 10 to 30 min according to the stage. They

were then washed with a fresh solution of 2mg/ml glycine in PBT and refixed in a fresh solution of 0.2% glutaraldehyde/4% PFA in PBT for 20 mins and washed twice in PBT. Embryos were then transferred to prehybridization solution (50% formamide, 5× SSC pH 4.5, 50 µg/mL yeast tRNA, 50 µg/mL heparin, and 1% SDS) 1:1 in PBT, then to prehybridization mix and were finally incubated overnight at 70°C with pre-warmed hybridization mix containing 1µg/ml of digoxigenin-labelled RNA probe.

b- Probe design

cGCN5 cDNA was obtained by RT-PCR using chick total RNA extracts and the following primers:

Forward: 5' ATGTTCTGCTCTGCCTCAAC 3'

Reverse: 5' TGCTGCTGAGCTTCTTGCTG 3'

The cDNA was cloned in pGEMT-easy. Probes were obtained by *in vitro* transcription (enzymes were purchased from Roche) in the presence of DIG RNA labeling mix and Ribonuclease inhibitor.

c- Post-hybridization steps

Following hybridization, embryos were washed in solution 1 (see below), 30 mins at 70°C, then with 1:1 solution1: solution 2 (see below) for 10 mins at 70°C, then three times in solution 2. They were then washed twice with 100µg/ml RNase A in solution 2 for 30 mins at 37°C, then twice in solution 2 and twice in solution 3 (see below). This step was followed by two washes in solution 3 for 30 mins at 65°C and three washes in TBST (1X TBS + 1% Tween - 20). Embryos were then incubated in heat-treated (30 mins at 70°C before use) 10% sheep serum TBST for 60-90 mins. Embryos were then incubated with preadsorbed anti-DIG antibody (Roche) overnight at 4°C.

Solution1: 50% Formamide, 5X SSC pH 4.5, 1% SDS.

Solution 2: 0.5M NaCl, 10mM Tris-HCl pH 7.5, 0.1% Tween-20.

Solution 3: 50% Formamide, 2X SSC pH 4.5.

Following antibody incubation, embryos were washed three times then 1h with TBST. Embryos were then washed twice 10 min in NTMT (100 mM NaCl, 100 mM Tris at pH 9.5, 50 mM MgCl₂, 0.1% Tween-20). Color reactions were carried out in NTMT containing 4.5 μL of NBT and 3.5 μL of BCIP. Staining reactions were carried out for 1–6 h at room temperature until signal or background became visible, followed by five washes in PBT. Embryos were then dehydrated through a graded methanol series to remove background and enhance signal and then rehydrated and stored in PBS plus 0.1% sodium azide.

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RÉSUMÉ

Le gène suppresseur de tumeur *LKB1* code une protéine sérine/thréonine kinase qui régule le métabolisme et la polarité cellulaires. *LKB1* exerce une partie de ses fonctions biologiques en phosphorylant et en activant les 14 kinases appartenant à la famille des protéines kinases activées par l'AMP (AMPK). Le membre éponyme de cette famille, AMPK, agit comme un senseur nutritionnel essentiel dans la cellule. La recherche que j'ai conduite au cours de ma thèse a porté sur le mode de régulation de *LKB1*. L'holoenzyme *LKB1*, un hétérotrimère comprenant deux autres protéines appelées STRAD et MO25, est dotée d'une activité catalytique constitutive. Mon travail a permis de montrer que la lysine 48 de *LKB1* est acétylée par l'acétyltransférase GCN5. Par des approches biochimiques et des techniques d'imagerie, j'ai montré que l'acétylation de *LKB1* par GCN5 favorise sa localisation nucléaire, la fraction non-acétylée étant localisée à la fois dans le cytoplasme et le noyau. GCN5 promeut également l'export cytoplasmique de *LKB1* de manière HAT-indépendente et régule son niveau d'expression. Afin de préciser la contribution de cette acétylation à la fonction *in vivo* de *LKB1*, j'ai utilisé le modèle expérimental de la crête neurale (CN) chez le poulet. En effet, j'ai été impliquée au cours de ma thèse dans une étude issue du laboratoire, qui a établi que l'activité de *LKB1* est requise pour la délamination, la migration polarisée et la survie des cellules de la CN céphalique. Ces dernières contribuent à la formation de la majorité du squelette cranio-facial des vertébrés. Le signal *LKB1* dans ces cellules est relayé par l'AMPK et la kinase ROCK et converge sur le moteur moléculaire dépendant de l'actine, la Myosine II. A l'aide du même modèle expérimental, j'ai montré que GCN5 est exprimé dans les cellules de la CN au cours de l'embryogenèse et que l'interaction fonctionnelle entre *LKB1* et GCN5 est nécessaire à l'activité de *LKB1* au cours de l'ontogénie des cellules de la CN céphalique et donc de la formation de la tête.

SUMMARY

The tumor suppressor gene *LKB1* encodes a serine/threonine kinase which regulates the cellular metabolism and polarity. Its biological activity is partly exerted through the phosphorylation and activation of 14 kinases which belong to the AMP-activated protein kinases (AMPK). The eponym member of this family acts as an essential nutritional sensor in the cell. The research that I conducted during my PhD focused on the regulation of *LKB1*. The *LKB1* holoenzyme is a constitutively active heterotrimer comprising two other proteins called STRAD and MO25. My PhD project shows that *LKB1* is acetylated on the lysine 48 residue by the acetyltransferase GCN5. Using biochemical approaches and cell imaging, I showed that the acetylation of *LKB1* by GCN5 favors its nuclear localization, while the non-acetylated fraction is localized in both the nucleus and the cytoplasm. GCN5 also promotes the cytoplasmic export of *LKB1* in an HAT-independent manner and regulates its expression levels. In order to investigate the contribution of this acetylation to the functions of *LKB1* *in vivo*, I used the experimental model of the neural crest (NC) in chick embryos. Indeed, during my PhD, I have contributed to a study, initiated by my host laboratory, in which we show that *LKB1* is required for the delamination, polarized migration and survival of neural crest cells (NCCs) which contribute to the formation of most craniofacial structures in vertebrates. *LKB1* signaling is mediated by AMPK and the ROCK kinase and converges towards the actin-dependent molecular motor, Myosin II. Using the same experimental model, I showed that GCN5 is expressed in NCCs during embryogenesis and that the functional interaction between GCN5 and *LKB1* is essential for the activity of *LKB1* in the cephalic NCCs ontogenesis and head formation.