Metabolic functions of the multifunctional protein E4F1 in skin homeostasis
Sevde Berfin Seyran

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Présentée par Sevde Berfin SEYRAN

METABOLIC FUNCTIONS OF THE MULTIFUNCTIONAL PROTEIN E4F1 IN SKIN HOMEOSTASIS

Soutenue le 25 juillet 2017 devant le jury composé de

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M. le Dr Laurent LE CAM
M. le Dr Matthieu LACROIX
M. le Dr Jacques POUSSEGUER
M. le Dr Lionel LARUE
Mme le Dr Chloé FERAL

Président du jury
Directeur de Thèse
Co-directeur de Thèse
Rapporteur
Rapporteur
Examinateur
A mon grand-père Muharrem Kalyoncu et,

Aux scientifiques injustement enfermés, dont mon oncle Ali Riza Sayin...
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Montpellier, June 2017
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**RESUME EN FRANCAIS**

I. **E4F1**

L’étude des petites onco-protéines virales a mené à l’identification de nombreux facteurs cellulaires essentiels à la prolifération et la survie cellulaire. Parmi ces facteurs, la protéine E4F1 fut identifiée comme une cible de l’onco-protéine E1A capable d’induire la transcription du gène E4 lors de l’infection par l’adénovirus de sérotype V.

Le gène *E4F1* est localisé dans la région 13.3 du chromosome 16 (16p13.3) chez l’homme et dans la région A3.3 du chromosome 17 (17A3.3) chez la souris. Le gène contient 14 exons répartis sur 11 kilobases et code une protéine de 120 kDa. L’homologie de séquence entre la protéine de souris et celle de l’homme est de 86%. Elle est présente chez tous les vertèbres. E4F1 est une protéine multifonctionnelle ubiquististe de la famille des protéines à doigts de zinc GLI-KRUPPEL, qui possède une activité transcriptionnelle. Cette propriété est due à la présence de deux domaines à doigts de zinc situés dans l’extrémité N-terminale de la protéine. Le premier gène cible cellulaire identifié d’E4F1 fut le gène de la Cycline A2. Ainsi, la surexpression d’E4F1 conduit à l’arrêt de la prolifération cellulaire par un mécanisme impliquant la répression du promoteur de la Cycline A2. Hormis ce gène, très peu de gènes cibles d’E4F1 étaient connus jusqu’à récemment. En effet, mon laboratoire d’accueil en collaboration avec l’équipe du Dr Claude Sardet a identifié le programme transcriptionnel régulé par E4F1 par des approches à l’échelle du génome entier (“ChIP-sequencing” et “microarrays”) en absence ou en présence du gène *E4f1* dans des cellules ES, des fibroblastes embryonnaires de souris (MEFs) et MEFs transformées. Parmi ces gènes cibles, environ un tiers code des protéines mitochondriales tels que les protéines impliquées dans la biosynthèse des cardiolipines (TAZ, CRLS1), la respiration OxPhos (NDUF55), les translocases transmembranaires (TOMM7, DNAJC19). Un autre groupe de gènes code des protéines impliquées dans la stabilité du génome et la réponse aux dommages à l’ADN (CHK1, PPP5C, RAD52, SENP8 et DDI2).

Au-delà de son activité de facteur de transcription, la protéine E4F1 possède également une activité ubiquitine E3 ligase atypique. Ce domaine ne ressemble pas aux domaines ubiquitine E3 ligase conventionnels tels que les domaines RING, HECT ou U-box. Il présente de fortes similitudes avec deux régions IR1 et IR2, qui sont importantes pour l’activité catalytique de la sumo E3 ligase RANBP2, ce qui suggère qu’E4F1 puisse agir à la fois comme une sumo E3ligase et une ubiquitin E3 ligase. Mon laboratoire d’accueil a identifié le suppresseur de tumeur p53 comme le premier substrat de cette activité d'E4F1. E4F1 est capable d’ubiquityler la protéine p53 sur les résidus lysine K319, K320 et K321. De manière intéressante, cette ubiquitylation de p53 médie par E4F1 n’induit pas la dégradation de cette protéine comme cela avait déjà été montrée pour d’autres E3 ligases telles que Mdm2, mais elle régule les activités transcriptionnelles de p53. Ainsi, les formes ubiquitylées de p53 se retrouvent au niveau de la chromatine et activent un programme
transcriptionnel impliqué dans l’arrêt du cycle cellulaire incluant les gènes Cycline G1, GADD45 et p21.

Afin de mieux comprendre les fonctions in vivo d’E4F1, mon laboratoire d’accueil a développé plusieurs modèles de souris génétiquement modifiés. Grâce à ces modèles, l’équipe a pu montré que l’inactivation constitutive d’E4F1 (E4f1 KO) conduit à la mort des embryons très tôt au cours du développement embryonnaire (au stade E5.5). Afin de contourner cette létalité embryonnaire précoce et identifier les fonctions d’E4F1 chez l’adulte, nous avons alors généré un modèle d’inactivation conditionnelle du gène E4f1 (E4f1 cKO) basé sur la technologie Cre/LoxP. A partir de ces souris E4F1 cKO, nous avons tout d’abord étudié les conséquences de l’inactivation d’E4f1 dans des fibroblastes embryonnaires de souris (Mefs) afin d’étudier les rôles d’E4F1 dans une cellule primaire normale. Ces travaux ont permis de confirmer le rôle majeur d’E4F1 dans la progression à travers le cycle cellulaire au cours de la transition G2/M par un mécanisme dépendant de la voie p53. Par ailleurs, ces résultats ont également permis de démontrer qu’E4F1 est un régulateur important d’un programme transcriptionnel impliqué dans le contrôle de l’activité mitochondriale et du métabolisme cellulaire. En parallèle de cette étude, mon laboratoire d’accueil a également entrepris de caractériser les phénomètes cutanés des souris E4f1 cKO. Pour cela, nous avons croisé ces animaux E4f1 cKO avec des souris exprimant la recombinase Cre dans la couche basale de l’épiderme (keratin 5-Cre, K5Cre), ou les souris RERT qui expriment de façon ubiquistique la recombinase Cre fusionnée à une version mutée du récepteur aux œstrogènes (Cre-ER\(^T2\)) inducible par le 4-hydroxytamoxifen (4-OHT) (Knock-in de la Cre-ER dans le locus de la grande sous-unité de la RNA pol II). Dans le modèle E4f1floX; RERT, l’inactivation d’E4F1 est obtenue par l’application cutanée de 4-OHT sur des souris adultes. Grâce à ces modèles, nous avons pu démontrer que l’inactivation d’E4F1 dans la peau entière ou plus spécifiquement dans le compartiment épidermique, conduit à des phénomètes cutanés complexes dont une partie résulte de la perturbation du pool des cellules souches épidermiques. Ainsi, l’inactivation d’E4f1 conduit à une hyperplasie transitoire de l’épiderme suivie d’une désorganisation complète de la peau aboutissant, in fine, à une perte des cellules de l’épiderme et à une alopecie. Ces lésions sont la conséquence de défauts affectant le pool des cellules souches de l’épiderme qui sortent massivement et définitivement de leur niche. Au niveau moléculaire, nous avons pu montrer que ces défauts résultent de la perturbation d’une voie de signalisation impliquant le supresseur de tumeurs p53 et 2 de ses régulateurs amont: le membre de la famille polycomb Bmi1, et la protéine Arf (« Alternative Reading Frame »). Ces résultats ont ainsi permis d’identifier une nouvelle voie de signalisation impliquant E4F1 et l’axe Bmi1-Arf-p53 dans le maintien de la potentielle souche épidermique.

Cependant, il semble que les fonctions d’E4F1 ne se limitent pas à la régulation de la prolifération et de la survie des cellules normales. Il semble qu’E4F1 soit également indispensable aux cellules transformées. Son interaction avec plusieurs
oncogènes ou suppresseurs de tumeurs (pRb, p53, p14ARF, Bmi1, RASSF1A) et plusieurs oncoprotéines virales (E1A, HBX, Gam1) supportent la notion qu'E4F1 puisse jouer un rôle important au cours de la progression tumorale. Cependant, à ce jour, hormis une mutation du gène E4F1 récemment identifiée dans un échantillon de tumeur de l’ovaire, aucune autre altération génétique d’E4F1 n’a été rapportée dans une tumeur. Il n’est néanmoins pas exclu que des changements d’expression de l’ARN et/ou de la protéine interviennent au cours du processus de transformation cellulaire. Ainsi, il a été montré par le laboratoire que l'expression protéique d’E4F1 augmentait dans des leucémies aigues myéloïdes humaines (LAM).

II. LA PEAU

La peau est un organe essentiel qui constitue la première barrière de protection de l’organisme contre l’environnement extérieur et qui permet de maintenir les liquides corporels en limitant les pertes d’eau. La peau se compose de trois tissus répartis de l’extérieur vers l’intérieur :

- L’épiderme
- Le derme
- L’hypoderme

L’épiderme est un épithélium pluristratifié qui assure l'essentiel de la fonction de barrière de la peau. Cette fonction est rendue possible grâce à la structure particulière de ce tissu composé principalement de kéraninocytes. Ces cellules suivent un programme de différenciation terminale bien spécifique caractérisée par une migration verticale des cellules filles issues de la division des cellules basales accompagnée de changement dans le profil d’expression des kérotines. Au cours de la migration vers la surface de l’épiderme, les kéraninocytes subissent de nombreuses modifications histologiques observables, qui sont spécifiques du niveau de différenciation cellulaire. Ces cellules sont disposées en quatre couches, représentatives des stades successifs de la différenciation des kéraninocytes : les cellules basales (au niveau de la couche la plus profonde de l’épiderme), les cellules épineuses (couche muqueuse), les cellules granuleuses (couche granuleuse) et les cellules de la couche cornée. Ces cellules superficielles desquament à la surface et sont continuellement remplacées. L’épiderme est donc un tissu qui se renouvelle constamment et où le taux de cellules perdues à la surface est contrebalancé par le taux de cellules produites au niveau de la couche basale, unique couche composée de kéraninocytes mitotiquement actifs incluant les cellules souches et les cellules progénitrices.
L’épiderme est également composé d’autres types de cellules qui possèdent des fonctions spécifiques:

- les mélanocytes sont des cellules qui produisent le pigment appelé “mélanine” pour donner à la peau sa couleur et pour la protéger contre les rayonnements ultra-violets.

- les cellules de Langerhans sont des cellules transépithéliales immunocompétentes présentatrices d’antigènes qui sont produites au niveau des organes hématopoïétiques. Elles sont localisées dans la couche granuleuse de l’épiderme. Elles capturent les antigènes par la voie des endosomes et les présentent à leur surface après avoir migrées jusqu’à ganglions lymphatiques.


Le derme est un tissu conjonctif fibro-élastique composé de fibroblastes et de fibres baignant dans une matrice extracellulaire. Cette matrice est constituée de collagènes, d’élastine et de glycoprotéines. De plus, le derme est riche en vaisseaux sanguins et nourrit ainsi l’épiderme. En plus de ses fonctions nutritives, le derme assure la résistance et l’élasticité de la peau. Le derme héberge également les annexes épidermiques tels que les follicules pileux, les glandes sébacées et les glandes sudoripares. Enfin, l’hypoderme est la couche la plus profonde de la peau. Il est constitué d’adipocytes et est importante pour la thermorégulation et l’apport de nutriment pour les cellules de l’épiderme.

A la jonction entre le derme et l’épiderme, se trouve une zone particulière: la membrane basale. Cette jonction permet l’ancrage des kératinocytes dans la couche basale. Elle est composée de protéines de la matrice extracellulaire telles que la laminine, le collagène de type IV, le nidogène et le perlecain.

**Les cellules souches épidermique:**

Le renouvellement de la peau est rendu possible grâce à la présence de cellules souches épidermique. Il existe deux types de cellules souches épidermique différents:

- **Les Cellules souches de l’épithelium interfolliculaire** sont responsables du renouvellement constant de l’épiderme. Elles participent au maintien de l’homéostasie de la peau mais également à la réparation du tissu lors d’une blessure. Ces cellules sont localisées dans la couche basale de l’épiderme. Il n’y a, à ce jour, aucun marqueur spécifique identifié pour ces cellules. Cependant, il est possible de les suivre par une technique appelée “long-term label retaining” basée sur la capacité des cellules souches à maintenir, sur de longues périodes de temps, un intercalant d’ADN tels que du BrdU ou de l’EdU.
- **Cellules souches folliculaires sont** situées au niveau du bulge, une région localisée sous les glandes sébacées du follicule pileux. Ces cellules ne sont pas uniquement responsables du renouvellement des poils mais elles contribuent majoritairement à la réparation de l'épiderme lors d'une blessure. Plusieurs marqueurs de ces cellules souches folliculaires ont été identifiés telles que la kératine 15, la kératine 19 ainsi que l’expression de la protéine membranaire CD34.

Le métabolisme énergétique, autrefois délaissé, est aujourd’hui considéré comme étant au centre de nombreuses voies de signalisation permettant à la cellule de s’adapter à son environnement voire même à dicter le devenir de la cellule. Constitué du métabolisme glucidique, lipidique et mitochondrial, le métabolisme énergétique semble participer à l’homéostasie cutanée.

En effet, de nombreuses études portent sur le rôle plus ou moins direct du métabolisme lipidique sur l’homéostasie cutanée. Il a ainsi été décrit que les acides gras libres, issus de la dégradation des phospholipides, participaient aux fonctions de la couche cornée, tout comme les triacylglycérols. Cette notion selon laquelle le métabolisme des lipides est important pour l’homéostasie épidermique est également renforcée par des modèles de souris génétiquement modifiées. Ainsi, l’inactivation du transporteur des FFAs, *Fatp4* chez la souris, conduit à la mort de ces animaux quelques heures après leur naissance. Ces souris présentent de nombreux défauts cutanés tels qu’une hyperprolifération des kératinocytes, une hyperkératose, des altérations de la barrière ainsi qu’une jonction derme-épiderme anormale. De façon intéressante, l’inactivation de ce transporteur engendre également une perturbation de la composition de l’épiderme en FFA, céramide et lipide. De la même façon, l’inactivation spécifique du gène *PPARY* (un régulateur majeur du métabolisme des lipides) au niveau des cellules souches folliculaires entraîne une alopécie des souris KO rappelant la pathologie humaine. Enfin, d’autres modèles murin génétiquement modifiés pour des enzymes permettant la synthèse de triglycérides tels que *DGAT1 KO* et *DGAT2 KO* (acyl-CoA : diacylglycerol acyl transferase) sont à mettre en relation avec le rôle potentiel du tissu adipeux dans l’homéostasie de la peau. En effet, ces deux modèles murins montrent plusieurs anomalies cutanées tels que, pour les souris *DGAT1 KO*, une perte des follicules pileux, de la thermorégulation, un métabolisme lipidique changé et une déficience en leptine sécrétée par les adipocytes. Les souris *DGAT2 KO* meurt après la naissance due à une quantité insuffisante de substrat pour la production d’énergie (lipopénie). Ces souris présentent également des défauts épidermiques: perte de l’élasticité, barrière cutanée non fonctionnelle, orthohyperkératose, mal formation de la jonction derme-épiderme.

En plus du métabolisme lipidique, il semblerait également que le métabolisme glucidique puisse aussi être impliqué dans l’homéostasie cutanée. Quelques études faites sur des rats montrent qu’une mauvaise métabolisation du glucose puisse entrainer des
problèmes cutanés. En effet, il a été observé chez des rats diabétiques une perturbation du métabolisme du collagène (atrophie et dégénérescence des fibres) ainsi que des défauts de cicatrisation et un épaississement de l’ épiderme. D’autres études montrent également la présence de transporteurs de glucose, Glut1 et 4, dans l’ épiderme notamment au niveau de la couche cornée mais également au niveau du follicule pileux, suggérant un rôle du glucose dans l’ homéostasie des cellules souches épidermique.

Enfin, il a été démontré que la mitochondrie jouait un rôle important dans l’ homéostasie de l’ épiderme. Des modèles murins d’ inactivation de gènes régulant la synthèse d’ ADN mitochondrial (Tfam) ou la structure des mitochondries (Phb2) présentent des défauts dans la structure de l’ épithélium interfolliculaire (hyper-prolifération, inhibition de la différenciation, défaut de la barrière cutanée) ainsi que des défauts de développement du follicule pileux entraînant un alopécie précoce de ces animaux.

Bien que les études portant sur le métabolisme énergétique et son impact sur l’ homéostasie de la peau soient aujourd’hui de plus en plus nombreuses, le rôle du métabolisme dans la régulation des cellules souches épidermiques reste mal connu. Certaines données suggèrent cependant que le contrôle du métabolisme énergétique pourrait avoir un rôle dans le destin cellulaire et notamment dans la régulation de la balance entre quiescence et engagement / mobilisation des cellules souches.

III. MÉLANOCYTES

Le mélanocyte est une cellule qui pigmenté la peau et les poils en produisant de la mélanine chez les vertèbres. Les mélanocytes sont dérivés des cellules de la crête neurale au cours du développement embryonnaire. Les mélanocytes sont localisés principalement dans l’ épiderme et dans le follicule pileux. Au niveau de l’ épiderme, ils sont situés dans la couche basale, où ils interagissent avec les kératinocytes par l’ intermédiaire de leurs dendrites. Cette interaction permet d’ assurer leur fonction de protection contre les dommages induits par les UV en produisant de la mélanine. Les pigments synthétisés par les mélanocytes se situés dans des structures particulières, appelées mélanosomes, qui sont ensuite transférées aux kératinocytes. Au niveau des follicules pileux, les mélanoblastes (cellules souches mélanocytaires) sont répartis en deux populations. La première va donner naissance, dans le bulbe, aux mélanocytes matures produisant la mélanine qui sera ensuite transférée aux kératinocytes constituant le poil, permettant ainsi la coloration de ce dernier. La seconde population colonise le bulbe pour constituer le réservoir de cellules souches mélanocytaires (MSC). Chez la souris, contrairement à l’ homme, les mélanocytes disparaissent rapidement de l’ épiderme interfolliculaire après la naissance et ne persistent qu’ au niveau des follicules pileux. L’ homéostasie des mélanocytes est alors contrôlée par le cycle pileux. A chaque formation d’ un nouveau poil, les MSC entrent en division et une des
deux cellules filles va migrer et se différencier en mélanocyte dans le bulbe du poil, ce qui permet sa pigmentation.

La fonction des mélanocytes différenciés est de produire des pigments de mélanine en réponse aux UV afin de protéger la peau. Les mélanocytes produisent deux types de mélanine, la phéomélanine, dont le pigment est jaune-rouge et l’œumélanine, dont le pigment est brun-noir. L’enzyme clé de la mélanogénèse est la tyrosinase. Elle permet l’hydroxylation de la tyrosine en DOPA, puis l’oxydation de la DOPA en DOPAquinone. La voie de synthèse se sépare alors en deux, afin de produire de l’œumélanine ou de la phéomélanine. La voie de la synthèse de l’œumélanine se poursuit par une conversion de la DOPAquinone en DOPAchrome. La synthèse se termine par l’intervention de deux enzymes, la DCT (DOPAchrome tautomérase) et TYRP1 (Tyrosinase-related protein 1). La production de phéomélanine est moins complexe. La seule enzyme nécessaire à sa production est la Tyrosinase. En présence de cystéine, DOPAquinone est converti en 3- ou 5-cysteïnyl DOPA qui va être oxydé et polymérisé pour générer phéomélanine.

IV. LES OBJECTIFS DE LA THÈSE

L’étude des réseaux protéiques perturbés au cours de l’infection par les petits virus oncogéniques amena, vers la fin des années 80, à la découverte de nombreux régulateurs clés de la division et de la survie cellulaire. Parmi ceux-ci, la protéine E4F1 fut initialement identifiée comme une cible de l’oncoprotéine virale E1A. Originellement identifié comme un facteur de transcription, E4F1 est également une ubiquitine-E3 ligase atypique pour d’autres facteurs de transcription tel que le suppresseur de tumeurs p53. Au travers de ses multiples activités, E4F1 est nécessaire à la prolifération des cellules somatiques et souches, et à la survie des cellules cancéreuses. De plus, les travaux de différents laboratoires dont le mien suggèrent qu’E4F1 se situe au carrefour de plusieurs voies de signalisation qui sont fréquemment altérées au cours de l’oncogénèse, et notamment la voie impliquant le suppresseur de tumeurs p53.

Afin d’étudier les fonctions physiologiques in vivo d’E4f1, mon laboratoire d’accueil a développé plusieurs modèles de souris génétiquement modifiées. Le premier modèle était un modèle d’inactivation constitutive du gène E4f1 (E4f1 knock-out KO). L’étude de ces animaux a permis de montrer que la perte d’E4f1 conduit à la mort des embryons au stade E5.5 du développement. Afin de contourner cette léthalité embryonnaire précoce et étudier les conséquences de l’inactivation d’E4f1 dans les tissus adultes, l’équipe a généré un second modèle d’inactivation conditionnelle basé sur la technologie Cre/LoxP. Ce modèle a alors été croisé avec des souris génétiquement modifiées RERT exprimant une forme inducible au tamoxifène de la recombinase Cre fusionnée au récepteur aux œstrogènes (CreERT2) et introduit dans le locus du gène de l’ARN polymérase 2 pour permettre
l'expression de la Cre dans tout l’animal. De façon surprenante, le premier phénotype observé sur ces animaux après application du tamoxifène était des altérations importantes de la peau. La caractérisation de ce modèle a permis de montrer que l’inactivation d’E4f1 dans la peau entière conduit à une hyperplasie transitoire de l’édérome suivie d’une désorganisation complète de la peau aboutissant, in fine, à une perte des cellules de l’édérome et à une alopécie. Le laboratoire a pu montrer que ces lésions cutanées résultent de la perturbation du pool des cellules souches épidermiques. En utilisant un autre modèle d’inactivation d’E4f1 dans les kératinocytes basaux (K5-Cre; E4f1 cKO), l’équipe a également pu récapituler les défauts observés dans la souris RERT; E4f1 cKO, démontrant que ces altérations étaient spécifiques à l’édérome. Au niveau moléculaire, la caractérisation de ces modèles a permis de démontrer que ces défauts résultaient en partie de la perturbation d’une voie de signalisation impliquant le suppresseur de tumeurs p53 et 2 de ses régulateurs amonts: le membre de la famille polycomb Bmi1 et la protéine Arf.

Les objectifs de ma thèse se sont alors inscrits dans la suite logique de ces résultats précédents obtenus par mon laboratoire d’accueil. Puisque les défauts des cellules souches épidermiques (CSE) médiés par l’inactivation d’E4f1 n’étaient que partiellement dépendant de la voie de signalisation impliquant la protéine p53, l’un des premiers objectifs de ma thèse a été d’identifier et d’étudier d’autres cascades moléculaires régulées par E4F1 et impliquées dans le maintien des CSE et l’homéostasie de la peau. Cet objectif comprenait également une meilleure caractérisation des phénotypes cutanés liés à la perte d’E4f1 dans les différents compartiments de la peau (cellules basales versus cellules suprabasales) en utilisant plusieurs modèles de souris spécifiques de ces compartiments.

Sur la base de ces résultats, le second objectif de mon projet de thèse a été d’étendre notre compréhension des fonctions d’E4f1 dans l’homéostasie de la peau en étudiant ses fonctions dans d’autres types cellulaires de la peau tels que les mélanocytes. Pour cela, j’ai évalué les conséquences de l’inactivation E4f1 dans les mélanocytes. J’ai également déterminé les mécanismes moléculaires impliqués dans les phénotypes liés à la perte d’E4f1 dans ces mélanocytes en portant une attention particulière à l’implication de la voie p53 et/ou des fonctions liées à l’activité PDH (pyruvate déshydratase) dans l’homéostasie des mélanocytes.

V. RESULTATS

Afin de répondre au premier objectif de ma thèse qui était d’identifier et de caractériser de nouveaux mécanismes moléculaires régulés par la protéine E4F1 et impliqués dans l’homéostasie cutanée, nous avons entrepris d’identifier le programme transcriptionnel directement contrôlé par E4F1 en utilisant des approches à l’échelle du génome entier (ChIP-sequencing et Microarray). Nous avons ainsi pu montrer en collaboration avec l’équipe du Dr Claude Sardet (IRCM) qu’E4F1 régule un programme
transcriptionnel impliqué dans la régulation de l’activité mitochondriale et notamment un sous-groupe de gènes codant des composantes et des régulateurs du complexe de la pyruvate déshydrogénase (PDC). Le PDC est un complexe multimérique situé dans la mitochondrie qui catalyse la décarboxylation oxydative du pyruvate (le produit final de la glycolyse) en acétyl coenzyme A (AcCoA). Ce complexe lie ainsi le métabolisme du pyruvate au cycle de Krebs, ce qui en fait un élément essentiel du métabolisme de la cellule. Il se compose de multiples copies de trois composantes catalytiques : la PDHA1/E1, la dihydrolipoamide transacétylase DLAT/E2 et dihydrolipoamide déshydrogénase DLD/E3.

Pour alimenter le PDC, le pyruvate traverse la membrane interne de la mitochondrie au moyen d’un transporteur, appelé MPC1. L’activité de ce complexe nécessite de nombreux cofacteurs tels que le lipoate, le Co-enzyme A (CoA), le FAD+, le NAD+ et la thiamine pyrophosphate, importée dans la mitochondrie par le transporteur SLC25A19. Jusqu’à présent, les données suggèrent que l’activité du PDC est principalement régulée par plusieurs modifications post-traductionnelles intervenant au niveau des différentes sous-unités du complexe, comme des phosphorylations de la PDHA1/E1 modulées par les pyruvate déshydrogénases kinases (PDKs) et phosphatases (PDPs). Parmi les gènes cibles d’E4F1, nous avons identifié Mpc1/Brp441 (transporteur de pyruvate), les composantes catalytiques Dlat/E2, Dld/E3, le transporteur de la thiamine pyrophosphate (co-facteur pour la réaction de PDC) et la composante régulatrice de la PDP, Pdpr.

En accord avec ces résultats, nous avons pu montrer que l’inactivation du gène E4F1 dans les cellules conduit à une diminution de l’expression de ses gènes cibles ainsi qu’à une diminution importante de l’activité de la PDC. Ces altérations moléculaires ont pour conséquence une reprogrammation métabolique de ces cellules caractérisée par une redirection du flux glycolytique vers la production et la sécrétion de lactate. Nous avons alors cherché à déterminer les conséquences de ces défauts in vivo chez la souris dans deux tissus : le muscle et la peau. Ainsi, nous avons pu montrer que l’inactivation d’E4F1 dans les muscles striés conduit à une acidose lactique massive ainsi qu’à une diminution importante de la résistance à l’effort des souris (course sur un tapis roulant) (Publication #1). De façon intéressante, ces défauts récapitulent certains symptômes cliniques de patients atteints du syndrome de Leigh, une maladie neurodégénérative rare caractérisée par une acidose lactique et des problèmes musculaires et pour laquelle une mutation homozygote a récemment été identifiée dans le gène E4F1.

En parallèle, afin de mieux caractériser les conséquences de l’inactivation d’E4F1 dans le compartiment épidermique, j’ai croisé les souris conditionnelles E4F1 cKO avec des animaux transgéniques exprimant la recombinase Cre sous le contrôle du promoteur de la kératine 14 (K14) ou la kératine 10 (K10). Ces protéines s’expriment respectivement dans la couche basale et couche épinière de l’épiderme. Alors que les souris E4F1(K10)cKO ne présentaient aucun phénotype, les animaux E4F1(K14)cKO présentaient à des temps précoces (7 jours après recombinaison de l’allèle E4F1) une hyperplasie de l’épiderme,
associé à une hyperprolifération des cellules basales et une hyperkératose (augmentation de la couche cornée). Ce phénotype était cependant transitoire car suivi à des temps plus tardifs (15 jours après recombinaison) par une aplasie de l’épidermique avec une disparition quasi complète des cellules épithéliales. Ce phénotype était associé à la perte des cellules souches épidermiques et à une alopécie. Sur la base des données de ChIP-seq, j’ai alors vérifié si les gènes cibles d’E4f1 impliqués dans le PDC étaient affectés dans ces kératinocytes. J’ai pu observer que l’inactivation d’E4f1 conduisait à une diminution importante de l’expression transcriptionnelle et protéique de DLAT/E2 et par conséquent à une diminution de l’activité de PDH dans l’épiderme des animaux E4f1(K14)cKO. Comme attendue, j’ai pu montrer que le blocage de l’activité de la PDH insuisait une reprogrammation métabolique de ces cellules qui se traduisait par la sécrétion de lactate dans le sérum et une augmentation de l’expression d’un des transporteurs du lactate, MCT4, dans l’épiderme des souris E4f1(K14)KO. J’ai alors pu démontrer que cette sécrétion induisait une activation des enzymes de la matrice extracellulaire, telles que les metalloprotéases et les cathepsines. L’activation accrue de ces enzymes conduisait à des altérations importantes de la membrane basale de la peau (qui assure entre autre l’adhésion des cellules souches dans leur niche) et à terme à un détachement des cellules souches de leur niche. Cette perte des cellules souches épidermiques aboutissait in fine à une absence de renouvellement de l’épiderme. Cette partie de mes travaux a donc permis d’illustrer pour la première fois l’importance du métabolisme du pyruvate dans l’homéostasie des cellules souches de la peau (Publication #2).

A la suite de ces données, j’entrepris de déterminer les fonctions d’E4f1 dans d’autres types cellulaires de la peau, et plus précisément dans les mélanocytes. Pour cela, j’ai croisé les souris E4f1cKO avec des animaux transgéniques qui expriment une forme inductible par le tamoxifène de la recombinase Cre (CreER) sous la promotrice de Tyrosinase. Les souris E4f1(Tyr)cKO présentent quelques semaines après la perte d’E4f1 un blanchiment important des poils sur la peau du dos et sur la queue ainsi qu’une dépigmentation de l’épiderme. Grâce à un reporteur génétique (Rosa26mTmG), j’ai pu suivre le devenir de ces mélanocytes et démontrer que ces cellules sont toujours vivantes et ne présentent pas de défaut de différenciation mais qu’elles sont incapables de produire de la mélanine. Ex vivo, j’ai pu récapituler certains des défauts de pigmentation dans les cellules de mélanome par des approches de shRNA dirigé contre E4f1 suggérant que ces altérations sont bien propres aux cellules dépourvues d’E4f1. Je suis actuellement en train de déterminer les mécanismes moléculaires responsables des défauts de pigmentation liés à la perte d’E4f1 en me focalisant plus précisément sur la voie de signalisation qui implique le suppresseur de tumeurs p53 mais également sur le lien potentiel avec le métabolisme du pyruvate et l’activité de la PDH.
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ABBREVIATIONS

ACAT1, Acetyl-CoA acetyltransferase
Acetyl CoA, Acetyl CoEnzyme A.
ACLY, ATP citrate lyase
AMP, ADP, ATP, Adenosine mono/di/triphosphate
ALT, Alanine aminotransferase
AML, Acute myeloid leukemia
ANT, Adenosine nucleotide transporter
ARF, Alternative reading frame
BCKDH, Branched-chain α-keto dehydrogenase
BCL2, B cell lymphoma 2
bHLH, basic helix-loop-helix
BMP, Bone morphogenetic protein
C/EBP, CCAAT/enhancer-binding protein
cAMP, Cyclic adenosine monophosphate
CDK, Cyclin dependent kinase
CHIP, Chromatin immunoprecipitation
CHK1, Check point kinase 1
cKO, Conditional knock out
CO2, Carbon dioxide
CoA, Coenzyme A
CRE, cAMP response element
CREB, cAMP response element binding protein
CRLS1, Cardiolipin synthase 1
DCA, Dichloroacetate
DCT, DOPAchrome tautomerase
DDR, DNA damage response
DGAT, Diglyceride acyltransferase
DHI, Dihydroxyindole
DHICA, dihydroxyindole carboxylic acid
DKK1, Dickkopf-related protein
DLAT, Dihydrolipoamide transacetylase
DLD, Dihydrolipoamide dehydrogenase
DNAJC19, DnaJ Heat Shock Protein Family
DOPA, Dihydroxyphenylalanine
E1A, Adenovirus early region 1A
E2F, E2 promoter binding factor
E4F1, E4 promoter binding factor 1
EBF1, Early B cell factor-1
EGFR, Epidermal growth factor receptor
ESC, Epidermal stem cell
ETC, Electron transport chain
FADH2, Flavin adenine dinucleotide
FATP, Fatty acid transporter protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>FCCP</td>
<td>Carbonyl cyanide-p-</td>
</tr>
<tr>
<td>Trifluoromethoxyphenylhydrazone</td>
<td></td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FHL2</td>
<td>Four and a half LIM domains 2</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model pathway</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUD2</td>
<td>Glutamine dehydrogenase 2</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase 3-beta</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin staining</td>
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<td>Human cytomegalovirus</td>
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<tr>
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</tr>
<tr>
<td>HKII</td>
<td>Hexokinase II</td>
</tr>
<tr>
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<td>High mobility group 2</td>
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<tr>
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<tr>
<td>HSP70</td>
<td>Heat-shock protein 70 oxidoreductase subunit S5</td>
</tr>
<tr>
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<td>Thiamine transporter</td>
</tr>
<tr>
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<td>interfollicular epidermis</td>
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<td>Knock out</td>
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<td>Lactate Dehydrogenase</td>
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<td>LEF1</td>
<td>Lymphoid enhancer-binding factor 1</td>
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<td>LGR5</td>
<td>Leucine-rich repeat containing G-protein coupled receptor 5</td>
</tr>
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<td>Mitogen-activated signaling</td>
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<tr>
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<td>Melanocortin receptor 1</td>
</tr>
<tr>
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<td>Monocarboxylate transporter</td>
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<td>Mouse double minute 2</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
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<td>MPC</td>
<td>Mitochondrial pyruvate carrier</td>
</tr>
<tr>
<td>MSC</td>
<td>Melanocyte stem cells</td>
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<tr>
<td>MUFA</td>
<td>Monosaturated fatty acid</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NCC</td>
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<td>NDUFS5</td>
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<td>Oxaloacetate</td>
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<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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**IRF6**, Interferon regulatory factor 6

**PAH**, Phenylalanine hydroxylase

**PAX3**, Paired box transcription factor 3

**PC**, Pyruvate carboxylase

**PCAF**, P300/CBP associated factor

**PDC**, Pyruvate dehydrogenase complex

**PDH**, Pyruvate dehydrogenase

**PDHE1**, pyruvate dehydrogenase E1 subunit

**PDK**, Pyruvate dehydrogenase kinase

**PDP**, Pyruvate dehydrogenase phosphatase Fermentable

**PFK1**, Phosphofructokinase-1

**PGC1α**, Peroxisome proliferator-activated repectator gamma coactivator 1-alpha

**PKA**, protein kinase A

**PKC**, Protein Kinase C

**PKCβ**, Protein kinase C-beta

**PMEL**, Premelanosome protein

**pRB**, Retinoblastoma protein.

**PRC1**, Polycomb repressive complex 1

**PRC2**, Polycomb repressive complex 2

**RASSF1A**, Ras-association domain family 1

**RENT**, Cre-ERT2 recombinase

**RING**, Really interesting new gene

**ROS**, Reactive oxygen species

**RSK**, Ribosomal S6 kinase

**SCD1**, Stearyl-CoA Desaturase-1

**SCF**, Stem cell factor

**SHH**, Sonic hedgehog

**ShRNA**, short hairpin RNA

**SiRNA**, short interfering RNA

**SOX**, Sex determining region Y box proteins

**SUMO**, Small ubiquitin like modifier

**SWI/SNF**, SWitcch/Sucrose Non-

**TAZ**, Tafazzin

**TCA cycle**, Tricarboxylic acid cycle

**TDE**, Tyrosinase distal element

**TFAM**, Mitochondrial transcription factor A

**TFE3**, Transcriptional factor E3

**TFEB**, Transcriptional factor EB

**TGF-β**, Transforming growth factor beta

**TYR**, Tyrosinase

**TYRP1**, Tyrosinase-related protein 1

**TYRP2**, Tyrosinase related protein 2 (DCT)

**UV**, Ultra violet radiation

**VEGFR**, Vascular endothelial growth factor

**WT**, Wild type
**RTK**, Receptor tyrosine kinase

**RUNX2**, Runt-related transcription factor 2 dehydrogenase

**SASP**, Senesence associated secretory phenotype

**α-KGDH**, Alpha-ketoglutarate dehydrogenase

**α-MSH**, Alpha-melanocyte stimulating hormone
INTRODUCTION
CHAPTER I- CELL METABOLISM

I. GLYCOLYSIS

A. Glucose

Glucose is the major energetic source, which is necessary to carry out anabolic and catabolic reactions in all organisms. It is one of the most important substrate for cellular respiration.

B. Glucose Transport

Most mammalian cells uptake glucose through glucose transporters (GLUT)s by an energy-independent bidirectional transfer which is called facilitative diffusion. Glucose transporters GLUTs (SLC2A) belong to the family of Major Facilitator Superfamily (MFS) membrane transporters. They encode approximately 500 amino acid proteins. In human, 14 different glucose transporters have been identified so far (GLUT1 to GLUT14) (Thorens and Mueckler 2009). GLUT1 is expressed in all types of cells whereas GLUT2 is mostly expressed in pancreatic β-cells (Thorens 1992). GLUT3 is the main neuronal glucose transporter (Simpson et al., 2003) and GLUT4 is important for whole body glucose metabolism (Thorens and Mueckler 2009) (Figure 1). Many studies on GLUTs revealed that these transporters have tissue or substrate specific functions (i.e GLUT5 has a high affinity for fructose rather than glucose) and different reaction kinetics. Therefore, each isoform can play a different role in glucose metabolism explaining the existence of numerous GLUT isoform transporters.

![GLUT transporters table]

**Figure 1:** Expression profile of glucose transporters in different tissues.
C. Glycolysis Pathway

Glycolysis is a series of metabolic reactions carried in the cytoplasm that converts glucose into pyruvate. It consists of 10 different steps and each one of them is catalyzed by a specific enzyme. Glycolysis intermediates can branch into different other metabolic biosynthetic pathways.

Step I:

Once glucose is imported into the cell by glucose transporters (GLUT), it is phosphorylated by Hexokinase II (HKII). A phosphate group is transferred to glucose from ATP (Adenosine triphosphate) generating Glucose-6-phosphate. This molecule can be processed toward the Pentose Phosphate Pathway by Glucose-6-Phosphate Dehydrogenase (G6PDH) to fuel nucleotide synthesis and to produce NADPH which is important to maintain the redox status of the cell.

Step II:

Alternatively, Glucose-6-phosphate can be converted into its isomer Fructose-6-phosphate by Glucose-6-phosphate isomerase.

Step III:

The enzyme Phosphofructokinase-1 (PFK-1) uses an ATP molecule to transfer a phosphate group to the Fructose-6-phosphate to generate Fructose-1,6-biphosphate.

Step IV:

The Aldolase enzyme splits Fructose-1,6-biphosphate into 2 isomers: Dihydroxyacetone phosphate and Glyceraldehyde-3-phosphate. Dihydroxyacetone phosphate is important for lipid synthesis and only Glyceraldehyde-3-phosphate is used downstream to pursue the glycolytic reactions.

Step V:

Dihydroxyacetone phosphate is isomerized to generate Glyceraldehyde-3-phosphate by Triose phosphate isomerase. Therefore, 2 molecules of Glyceraldehyde-3-phosphate are present as a result of this step.

Step VI:

Triose phosphate dehydrogenase enzyme transfers a hydrogen (H⁺) atom from Glyceraldehyde-3-phosphate to oxidize nicotinamide adenine dinucleotide (NAD⁺) and form NADH. Then it transfers a Phosphate group to Glyceraldehyde-3-phosphate and converts it to 1,3-biphosphoglycerate. These reactions occur for both 2 molecules of Glyceraldehyde-3-phosphate.
Step VII:

Phosphoglycerokinase enzyme transfer a phosphate group from 1,3-biphosphoglycerate to one ADP molecule and generates ATP. Two molecules of ATP and 2 molecules of 3-phosphoglycerate are formed.

Step VIII:

Phosphoglyceromutase enzyme translocates the phosphate from 3rd to 2nd Carbon and generates 2-phosphoglycerate.

Step IX:

Enolase enzyme removes a molecule of H₂O from 2-phosphoglycerate and converts it to Phosphoenolpyruvate.

Step X:

At the last step of glycolysis Pyruvate Kinase removes a phosphate from Phosphoenolpyruvate and transfers it to ADP (Adenosine diphosphate) generating ATP and the final product Pyruvate.

As a result of glycolysis, 1 molecule of glucose is converted into 2 molecules of pyruvate with the production of 2 molecules of NADH and a net gain of 2 molecules of ATP (Figure 2).
Glucose $\rightarrow$ 2 Pyruvate + 2 NADH + 2 ATP

**Figure 2:** Steps of the glycolysis (red: metabolic intermediates, green: enzymes).

## II. PYRUVATE METABOLISM

### A. Cytosolic pyruvate metabolism

Pyruvate is a critical intermediate used in many anabolic and catabolic reactions such as oxidative metabolism, gluconeogenesis (re-synthesis of glucose), de novo lipid synthesis, cholesterol synthesis and the citric acid (TCA) cycle. Cytosolic pyruvate can be obtained from multiple sources:
- The major source of pyruvate is obtained from the irreversible last reaction of glycolysis in which phosphoenol pyruvate is dephosphorylated into 2 molecules of pyruvate by the pyruvate kinase enzyme (PK). This reaction provides a net gain of two molecules of ATP. Pyruvate Kinase enzyme is particularly important for catalyzing one of the two steps of glycolysis which generates ATP. For instance, this reaction is essential for red blood cells, which don’t have mitochondria and rely on glycolysis for ATP production. Besides, it is also important for skeletal muscle during exercise when the oxidative phosphorylation is not sufficient to sustain muscular contraction.

- Another source of cytosolic pyruvate is the oxidation of lactate by lactate dehydrogenase-B which is the reverse reaction that is catalyzed by lactate dehydrogenase-A. Lactate dehydrogenase is a ubiquitously expressed enzyme that catalyzes the reduction of pyruvate into L-lactate coupled with oxidation of NADH to NAD⁺. This reaction is important for skeletal muscle during exercise when energy demand for muscular contraction exceeds the mitochondrial capacity. Glycolysis requires NAD⁺ for ATP production, however when NAD⁺ is decreased, LDHA replenishes the NAD⁺ pool through pyruvate to lactate conversion (Kopperschläger and Kirchberger 1996). Secreted lactate is mainlyuptaken by the liver to be converted to pyruvate and used in the TCA cycle and further anabolic reactions such as those involved in fatty acid and triglycerides synthesis. This lactate-pyruvate cycle is called the Cori cycle.

LDH is a tetrameric complex with subunits H found in the heart and M in skeletal muscles. There are five tetrameric isozymes depending on the H/M isoform ratio in mammals. Two of the isomers exhibit different functions. LDHA converts pyruvate to lactate whereas LDHB converts lactate to pyruvate. The reaction catalyzed by LDHB is inhibited by the amount of pyruvate, but LDHA activity is not inhibited by lactate (Dawson et al., 1964).

- Pyruvate can also be generated through the catabolism of several amino acids such as serine, threonine and particularly alanine, which is the main gluconeogenic precursor (Terretaz and Jeanrenaud 1990). Pyruvate and Alanine cycle links liver and muscle metabolism. Pyruvate is transaminated to alanine in muscles and liver converts it to pyruvate to use it in the TCA cycle. This cycle occurs in parallel to the Cori cycle. The enzyme responsible for pyruvate to alanine conversion is alanine aminotransferase. There are two different isoforms identified: the ALT1 is mostly present in white and brown adipose tissues whereas ALT2 is expressed in muscles and brain (Gray et al., 2014).

B. Mitochondrial pyruvate metabolism

i. Mitochondrial pyruvate carriers MPC1 and MPC2 (BRP44L and BRP44)

Part of pyruvate metabolism occurs in the mitochondrial matrix and therefore pyruvate has to be transported throughout the outer and inner mitochondrial membranes (OMM and IMM, respectively).
Pyruvate diffuses freely through the OMM by porins. However, the IMM is impermeable to charged molecules. Therefore, pyruvate needs a specific carrier to reach the mitochondrial matrix. The existence of a mitochondrial pyruvate carrier was known for several decades with the discovery of pyruvate carrier inhibitors such as the ω-cyanocinnamate analog UK-5099. However, the genes encoding these carriers remained unknown until recently. Two different labs identified simultaneously a hetero-oligomeric protein complex consisting of MPC1 and MPC2 proteins (previously known as Brain protein 44-like- BRP44L and Brain protein 44- BRP44) as the mitochondrial pyruvate carrier (Bricker et al., 2012; Herzig et al., 2012). MPC1 and MPC2 are 12 kDa and 15 kDa proteins respectively. Together they form a 150 kDa oligomeric structure in the IMM inner mitochondrial membrane, which is required for the stability of the complex. They do not exhibit any sequence homology with other known mitochondrial transporters such as the phosphate carrier (PiC) or the adenosine nucleotide transporter ANT (Hildyard et al., 2003). MPCs are suggested to belong to the PQ-loop/Mtn3/MPC superfamily, involved in diverse functions of different organelles. They contain 7 transmembrane domains and 2 conserved proline glutamine motifs. MPC proteins have 3 of these transmembrane domains and 2 conserved proline glutamine motifs.

Pyruvate is not the only substrate for MPC. Dichloroacetate and other small halogenated monocarboxylates can also be transported by MPC (Halestrap, 1975). Depletion of MPC1 in yeast and Drosophila resulted in accumulation of glycolytic intermediates and decreased abundance of TCA cycle intermediates suggesting that these mutants cannot link cytosolic pyruvate to mitochondrial TCA cycle to carry out cellular respiration (Bricker et al., 2012). Further studies have also shown that RNAi-mediated silencing of MPC1 or MPC2 in mammalian cells decreases glucose-derived Acetyl-CoA formation. Cell survival in MPC-depleted cells was due to the utilization of alternative energetic sources such as glutamine in these experiments (Yang et al., 2012; Vacanti et al., 2014). Moreover, MPC1 or MPC2 knock down causes a decrease in pyruvate-dependent mitochondrial activity in mouse embryonic fibroblasts in basal conditions. However, mitochondrial activity is strongly decreased when the maximal respiration is induced with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Bricker et al., 2012). Constitutive deletion of MPC1 or MPC2 in mice is lethal during embryonic development around E11.5 when the mitochondrial biogenesis is at its maximum rate (Vanderperre et al., 2004; Larsson et al., 1998). Thus, all these findings prove that mitochondrial pyruvate carrier proteins MPC1 and MPC2 are indispensable for proper energy metabolism.

In humans, mutations in MPC1 were identified in three different families so far. The 1st one had a homozygous R97W mutation in MPC1 leading to lactose acidosis, due to severe pyruvate oxidation defects. (Brivet et al., 2003). The second and third patient had a L79H change on MPC1 which also led to decreased pyruvate oxidation and increased lactic acidosis; however a less severe phenotype compared to the first patient (Bricker et al.,
2012). How different mutations impair pyruvate transport remains unclear and the incidence of MPC mutations is still unknown.

ii. Pyruvate Dehydrogenase

Once in the mitochondrial matrix, pyruvate can follow two different routes. Most of the pyruvate is used for Acetyl Coenzyme A (Acetyl-CoA) production by the Pyruvate Dehydrogenase Macro Complex (PDH complex). Acetyl-CoA is then used to fuel the TCA cycle or alternatively used for several anabolic reactions such as fatty acid, cholesterol and acetylcholine synthesis.

1. Mitochondrial gate keeper Pyruvate Dehydrogenase Macro Complex

The PDC links cytosolic pyruvate to the mitochondrial TCA cycle. The main function of this enzyme is to convert pyruvate to Acetyl CoA so that it can be used in TCA cycle to carry out the cellular respiration. The PDC is one of the largest known multiprotein complex in mammals (approximately 9.5MDa). In higher eukaryotes, PDH complex contains multiple copies of 3 catalytic enzymes (E1, E2 and E3), 1 binding (E3BP) and 2 regulatory components (PDK and PDP). All these proteins are encoded by nuclear genes (McFate et al., 2008).

a) Catalytic domains E1, E2 and E3

In mammals including human, the E1 subunit, called PDHE1, has a heterotetrameric structure containing two copies of E1α and E1β subunits (Seifert et al., 2007). Besides, there are two isoforms of alpha subunits encoded by different nuclear genes. In human, X chromosome-linked gene PDHA1 encodes the E1 alpha 1 subunit and is present in all somatic tissues whereas the other isoform E1 alpha 2 is encoded by an autosomal intronless gene PDHA2 and is only expressed in testis (Patel et al., 2014). Beta subunit is encoded by the PDHB gene.

The active site of the E1 domain forms a deep cleft at the E1α and E1β interaction site and it contains a thiamine pyrophosphate cofactor and Mg$^{2+}$ ion (Gray et al., 2014). The dihydrolipoamide acetyltransferase E2 subunit of the PDC (hereinafter referred to as DLAT) contains four domains. At its C\(^\ast\) terminal end, DLAT contains a binding domain that is necessary for the recruitment of the E1 subunit and two lipoyl domains that covalently attach lipoyl groups (the outer lipoyl domain L1 and the inner lipoyl domain L2) (Vijayakrishnan et al., 2011; Brautigam et al., 2009). The inner domain of the DLAT forms the central core structure of the complex together with the E3BP subunit (Figure 3). In the central core structure of the PDC, 48 subunits of E2 are present for 12 subunits of E3BP. Together they form a pentagonal dodecahedron structure and recruit the E1 and E3 catalytic subunits (Patel et al., 2014). The dihydrolipoamide dehydrogenase E3 subunit (hereinafter referred to as DLD) is a homodimer. Six to 12 E3 subunits associate within the core component of the PDC (Smolle et al., 2006).
Pyruvate decarboxylation reaction is initiated by the E1 subunit (PDHE1) in a thiamine diphosphate-dependent manner and is performed by two reactions: the decarboxylation of pyruvate to CO$_2$ by generating C$_2$α-hydroxyethylidene-ThDP (enamine) and reductive acetylation of lipoyl groups attached to the E2 subunit. The E2 subunit (DLAT) transfers an acetyl group from lipoate to form Acetyl CoA and dihydrolipoate. The E3 subunit (DLD) regenerates a lipoate group from the dihydrolipoate that is produced by E2 (DLAT) in the previous step. The electron transferred from dihydrolipoate in this process is used to reduce the bound FAD to FADH$_2$ (Figure 3). Then FADH$_2$ is reoxidized subsequently by NAD$^+$ generating NADH and FAD. The E3 subunit DLD also contributes to similar catalytic functions in the α-ketoglutarate dehydrogenase complex and the branched-chain amino acid complex. However, its affinity for the PDC is stronger than for other complexes (Brautigam et al., 2011; Ciszak et al., 2006) (Figure 4).

b) Binding domain

The E3BP (E3-binding protein) is a structural protein without any catalytic activity. It contains an inner domain, a subunit binding domain and a lipoyl domain (L3) (Smolle et al., 2006; Ciszak et al., 2006). E3BP is important for the formation of the core component of the PDC through interactions with the E2 subunit DLAT and helps the recruitment of the E3 subunit DLD to the complex (Vijayakrishan et al., 2010; Vijayakrishan et al., 2011).

c) Regulatory domains

The PDH enzyme is mainly regulated by reversible phosphorylation/dephosphorylation of the α subunit of PDHE1. In mammals, 3 different phosphorylation sites of PDHE1 have been identified. PDHE1 is phosphorylated on serine residues Ser293, Ser300 and Ser232. Since PDHE1 is a heterotetramer (α2β2), it contains 6 potential phosphorylation sites. The phosphorylation of each residue results in the inactivation of the enzyme. Ser293 is located in the substrate channel and is therefore proposed to lead to conformational changes inhibiting the interaction between the lipoyl domain of E2 (DLAT) and the active
site of the complex (Wynn et al., 2004). Since Ser300 and Ser232 are not located in the active site, they are proposed to be affecting enzymatic activity differently; however the overall mechanism is not yet fully understood (Patel et al., 2006).

![Diagram of metabolic pathways]

**Complete reaction:** \( \text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{Acetyl CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2 \)

**Figure 4:** Reactions catalyzed by the PDC subunits in mitochondria.

i. **Pyruvate Dehydrogenase Kinase (PDKs)**

The decrease of PDH activity is achieved by phosphorylation of the E1α subunit. This regulatory mechanism is performed by the Pyruvate Dehydrogenase Kinases (PDKs). PDKs are serine-specific protein kinases. They are composed of two subunits: a catalytically active α subunit and a regulatory β subunit. The α subunit also has 8 components, which is important for the function of the kinase by regulating the orientation and the binding of the ATP molecule. 4 different isoforms have been identified so far: PDK1, PDK2, PDK3 and PDK4 (Rowles et al., 1996; Gudi et al., 1995). They have slightly different sizes (PDK1 is approximatively 48 kDa whereas the others are 45 kDa) and share 70% homology. All of them are expressed in the heart and in skeletal muscles. PDK2 is also expressed in liver and PDK4 is expressed in kidney, brain and liver (Rowles et al., 1996; Gudi et al., 1995; Wynn et al., 2008; Green et al., 2008; Devedijev et al., 2007; Steussy et al., 2001).
PDKs act as homo- or hetero-dimers depending on the tissue and on the expression of other PDK isoforms (Boulatnikov et al., 2003). The recruitment of PDKs to the PDC is achieved by the inner L2 domain or the outer L1 domain of the E2 subunit and, one or two copies of PDKs are present per PDH complex (Patel et al., 2009). This interaction opens the active site of the enzyme and release ADP (Tuganova et al., 2003; Baker et al., 2000).

The four isoforms differ in the kinetics and their binding affinity for the L2 domain of DLAT. All PDKs can phosphorylate Ser293 and Ser300, but only PDK1 can phosphorylate Ser232 (Korotchkina et al., 2001). PDK3 has the highest activity for Ser300 whereas PDK2 has the strongest activity for Ser293. PDK3 also has the highest affinity for the L2 domain whereas PDK4 has the lowest (Tugana et al., 2002; Baker et al., 2000).

ii. Pyruvate Dehydrogenase Phosphatases (PDPs)

PDPs act in the opposite way to PDKs and remove the phosphate group from the PDHE1 to activate the PDC. Two isoforms have been identified in mammals: PDP1 that is mostly expressed in brain, heart and skeletal muscles and PDP2 that is expressed in liver and adipose tissue (Huang et al., 2003). PDP1 is a heterodimer containing a catalytic subunit (Pdp1c) of 52kDa and a regulatory subunit (Pdp1r) of 96kDa (Saunier et al., 2016). PDP2 has only one catalytic subunit (Pdp2c) (Kato et al., 2010). PDPs are related to the phosphatase 2C family of serine phosphatases (Huang et al., 1998; Vassylev et al., 2007) and reside in the mitochondrial matrix. PDP1 binds to the L2 domain of DLAT. Ca\(^{2+}\) and Mg\(^{2+}\) are two cofactors needed for this binding. PDP2 doesn’t bind to lipoyl domains and it requires only Mg\(^{2+}\) for its catalytic activity. Both PDPs can dephosphorylate the 3 phosphorylation sites at different rates, dephosphorylation of Ser300 being more efficient than that of Ser232 and Ser293 (Karpova et al., 2003; Korotchkina et al., 2004).

2. Regulation of PDH Complex (PDC)

PDH complex (PDC) is a key player in glucose metabolism. Around 50% of caloric intake is processed through PDC. Therefore, PDC is strongly regulated in order to respond to different physiological conditions. Short term regulations are achieved by PDKs and PDPs which turn ON and OFF the activity of the enzyme. These actors are also tightly regulated by many other mechanisms (Figure 5).
**Figure 5:** Control of PDH activity by PDKs and PDPs and their regulations by different substrates.

During the short-term fasting/food intake cycle, acute regulation of PDK depends on the concentrations of pyruvate, ADP, P, and NADH/NAD+ ratio (Roche *et al.*, 2001). PDKs are inhibited by increased pyruvate concentration and low energy state (high ADP, NAD+, CoA and P) to increase the PDH activity, whereas they are stimulated by ATP, NADH and Acetyl CoA (Bao *et al.*, 2004). Each PDK isoform responds differently to these factors. For instance, NADH induces PDK activity by 20% for PDK1 and by 200% for PDK4. High NADH/NAD+ ratio reduces the number of lipoyl groups as a result of the reverse reaction catalyzed by DLD whereas high Acetyl CoA/CoA ratio stimulates the acetylation of the reduced lipoyl groups by DLAT. These processes affect in an allosteric manner the binding of PDK to the lipoyl domain of PDC (Bao *et al.*, 2004).

In addition to PDKs, the binding of PDP to PDC is also regulated. The recruitment of PDP1 to the L2 domain of the E2 subunit is controlled by the concentration of calcium (Turkan *et al.*, 2004). PDP activity increases with Ca\(^{2+}\) in skeletal muscles to enhance oxidative phosphorylation and ATP production during muscular contraction. In contrast, this mechanism was not observed for PDP2 (Karpova *et al.*, 2003). Both PDP1 and PDP2 can be regulated by post translational modifications. In skeletal muscle cells exposed to insulin, PDP1 and PDP2 are phosphorylated by PKC(delta) which in turn increases PDP activity and PDH activity (Caruso *et al.*, 2001).

In different nutritional conditions, PDKs and PDPs levels can be regulated at the transcriptional level. For instance, *Pdp1* and *Pdp2* mRNA levels drop during starvation whereas PDK levels (mostly *Pdk4*) increase consistent with decreased glucose oxidation. As a result, PDH activity decreases to conserve glucose. Finally, their expression levels are also modified in pathological conditions such as diabetes and hyperthyroidism (Huang *et al.*, 2003; Jeoung *et al.*, 2006) (Table 1).
<table>
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<th>Transcriptional Level</th>
<th>PDK1</th>
<th>PDK2</th>
<th>PDK3</th>
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<td></td>
<td>Up-regulated during starvation (kidney,</td>
<td>Up-regulated during starvation (kidney,</td>
<td>Up-regulated during starvation (kidney,</td>
<td>Down-regulated during diabetes (heart,</td>
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<td>muscle) and hyperthyroidism (type II</td>
<td>diabetes (heart and skeletal muscle) and</td>
<td>diabetes (heart and skeletal muscle) and</td>
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<td>Post translational modifications</td>
<td>Activated by NADH and acetyl-CoA, inhibited by pyruvate and ADP.</td>
<td>Activated by NADH and Acetyl CoA, inhibited by pyruvate and ADP.</td>
<td>Inhibited slightly by pyruvate</td>
<td>Activated by NADH and Acetyl CoA, inhibited by pyruvate and ADP.</td>
<td>Activated by Mg²⁺ and Ca²⁺ concentrations.</td>
<td>Activated by spermine concentrations.</td>
</tr>
</tbody>
</table>

**Table 1:** PDK and PDPs can be regulated at the transcriptional level and by post translational modifications in response to different stimuli under physiological and pathological conditions.

3. PDH Complex Associated Pathologies

a) PDH deficiency

Since PDH Complex plays a critical role in cellular metabolism, alterations or mutations in genes encoding component of the complex result in severe metabolic diseases. The PDH deficiency is associated with high levels of systemic lactate and neurological and motor function failures (Patel et al., 2012). The decreased PDH activity impairs mitochondrial oxidation of pyruvate, therefore redirecting the glycolytic flux toward lactate production and/or transamination to alanine. As a result of this metabolic rewiring, Acetyl CoA, NADH and FADH2 levels decrease, leading to cellular energetic failure. Therefore, ATP deprivation, accumulation of free radicals and lactate accumulation are suspected to be the primary causes of the neurological features observed in patients harboring PDH deficiency.

PDH deficiency has been causally linked to mutations in genes that encode the catalytic (PDHA1, PDHB, DLAT, DLD), as well as the binding subunit (E3BP) of the complex. The PDHA1 is the most frequently mutated representing 84% of the cases. Mutations of genes encoding different PDH complex subunits (E1α, E2, E3 or E3BP) results in similar clinical symptoms, including neurological defects and chronic lactate acidemia (Patel et al., 2012).

Of note, no mutation was reported in the regulatory subunits (PDKs) of the complex except 2 mutations in the PDP1 gene: a frame shift mutation causing the deletion of
Leucine-213 in a patient that suffered from lactic acidosis, hypotonia and exercise intolerance (Maj et al., 2005), and a premature stop codon E93X leading to a complete absence of the PDP1 protein in a patient that exhibited lactic acidosis and increased alanine and proline levels (Cameron et al., 2009).

b) Leigh syndrome

The Leigh syndrome is a neurodegenerative disorder characterized by necrotic lesions in the basal ganglia and brain stem together with motor neuron function failure, respiratory difficulties, dystonia and ataxia (Gray et al., 2014). Approximately 25% of the patients that suffer from PDH deficiency are diagnosed with Leigh syndrome but the primary cause of Leigh syndrome is defects of the mitochondrial respiratory chain, which leads to energy failure and lactic acidosis. So far, mutations in more than 75 different nuclear and mitochondrial genes have been causally linked to the Leigh syndrome. The most frequent mutations are found in genes encoding component of the mitochondrial respiratory chain complexes. There are five complexes: Complex I (NADH: Ubiquinone oxidoreductase), Complex II (Succinate dehydrogenase), Complex III (ubiquinol cytochrome c reductase or cytochrome bc1 complex), Complex IV (Cytochrome Oxidase) and Complex V (ATP synthase). Each of them is a large multimeric protein complex made of several subunits encoded by both the nuclear and the mitochondrial genomes. Genes encoding Complex I and IV subunits are the most frequently mutated in Leigh syndrome although mutations in Complex II, III and IV have also been reported with lower frequency (See Electron Transport Chain for more details about respiratory chain complexes) (Gray et al., 2014).

The first Leigh syndrome patient that resulted from PDH deficiency was reported in 1993 (Matthews et al., 1993). Since then, 25% of the patients that have PDH deficiency have been reported to develop clinical symptoms that are characteristic of the Leigh disease (Patel et al., 2012). In these patients, PDH activity can drop up to less than 10% of the normal activity (Finsterer 2008). So far, mutations were observed in PDHA1, PDHB, DLD (E3) and E3BP with the most frequently mutated gene being PDHA1 with 84% of the patients suffering from Leigh syndrome. More than 100 different mutations have been reported in this gene (Patel et al., 2012; Quintana et al., 2008). Apart from mutations in genes encoding proteins of the mitochondrial respiratory chain and the PDC, multiple complex deficiencies related to translation of mtDNA encoded proteins were reported in Leigh syndrome patients. Half of these defects were associated with mtDNA maintenance and replication (Gerards et al., 2016). Finally, alterations in thiamine or vitamin B metabolism were also identified to cause the disease. Thiamine transport is the only mechanism that does not take place in the mitochondria but its deficiency is still associated to the Leigh syndrome. Thiamine (vitamin B) is taken from nutrients and transported to cells by thiamine transporters hTHTR1 or hTHTR2 where it is converted to TPP (active form of thiamine pyrophosphate) by TPK1 enzyme (thiamine pyrophosphokinase) in the cytosol. TPP is an essential cofactor for transketolase enzyme that functions in the pentose phosphate
pathway. It is also transported to the mitochondria by the SLC25A19 transporter and serves as a cofactor for pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and branched-chain α-keto dehydrogenase (BCKDH) enzymes. Mutations in SCL19A3, which encodes hTHTR2, and TPK1 were also reported in Leigh syndrome patients with decreased PDH activity (Banka et al., 2014; Gerards et al., 2013).

c) The Warburg Effect (Aerobic Glycolysis)

Alterations of PDH activity can occur in different pathophysiological conditions and result in a metabolic shift. One such condition is cancer development. Otto Warburg discovered almost 100 years ago that tumor cells uptake high levels of glucose and produce more lactate than normal cells, even when oxygen is available (Warburg 1956). In glycolytic tumor cells, ATP is produced less efficiently but faster than oxidative phosphorylation to sustain high proliferation. Glycolytic intermediates are also used to fuel biosynthetic branches that contribute to biosynthesis of several molecules such as nucleotides, amino acids and NADPH that cancer cells require for proliferation (Lunt and Vander Heiden 2011). Besides, lactate and H⁺ products of glycolysis facilitate tumor invasion (Smallbone et al., 2005).

HIF-1 is one of the important actors of the Warburg effect that switch metabolism from oxidative phosphorylation to glycolysis in cancer cells. Its transcription is regulated by the P13K/ AKT/ mTOR pathway, which is often altered during tumorigenesis. HIF-1 can be activated under hypoxic and non-hypoxic conditions. On the one hand, its activation induces glycolysis-related enzymes such as hexokinase (Mergenthaler et al., 2012) and pyruvate kinase (Palsson et al., 2015) as well as glucose transporters (Starska et al., 2015) to enhance the glycolytic flux. On the other hand, HIF-1 inhibits oxidative phosphorylation by inducing PDK1 and PDK3 expression (Kim et al., 2006; Papandreou et al., 2006; Denko et al., 2003; Lu et al., 2008). Finally, it induces LDHA level to promote lactate conversion from pyruvate, thereby favoring glycolysis over OXPHOS.

PDKs are overexpressed in multiple tumors with decreased PDH activity and promote tumorigenesis (Jha et al., 2013). Several groups have demonstrated that PDK1 is upregulated in different tumors such as myeloma, renal cell carcinogenesis and melanoma, and it is required for tumor progression (Kaplon et al., 2013; McFate et al., 2008). Expression of other PDK isoforms were also reported to be correlated with the poor prognosis (i.e PDK1 in gastric cancer, PDK2 in breast cancer and PDK3 in colon cancer) (Hut et al., 2013; Sutendra et al., 2012; Lu et al., 2011; Blouin et al., 2011). Furthermore, cancer cells are reported to be resistant to detachment-dependent apoptosis (anoikis). This resistance is also linked to upregulation of PDKs (PDK1 and PDK4) (Kamarajugadda et al., 2012).

During cancer progression, it was shown that oncogenes and tumor suppressors are responsible for the modulation of PDH activity. As an illustration of this notion, the
constitutively active BRAF^{V600E} was linked to PDH activity. 
\textit{Braf}^{V600E} mutation, which is often found in melanoma and other cancers, was shown to increase PDK1 transcriptional expression and induce oncogene-induced senescence (Kaplon \textit{et al.}, 2013). In contrast to the regulation by oncogenes such as \textit{BRAF} and \textit{c-MYC}, the tumor suppressor p53 was reported to negatively regulate PDK2 (Contractor \textit{et al.}, 2012), a mechanism that may contribute to its tumor suppressor activities.

Since increased activity of PDKs suppresses PDH activity and favors aerobic glycolysis, inhibition of PDKs could be a useful mechanism to counteract the Warburg effect (Papandreou \textit{et al.}, 2006). Several studies were performed to identify targets inhibiting PDK activity and among them only DCA (dichloroacetate) went to Phase II of a clinical trial for cancer therapy (Bowker-Kinley \textit{et al.}, 1998). DCA binds and induces conformational changes of the nucleotide-binding and the lipoyl-binding domains of PDKs thereby inhibiting its kinase activity (Kato \textit{et al.}, 2007).

Finally, although PDH complex is mainly repressed by PDK-induced phosphorylations, global acetyome analysis revealed that PDH E1α can be acetylated at several lysine residues (K77, K83, K244, K321 and K336) in cancer cells (Fan \textit{et al.}, 2014). ACAT1 acetyl transferase and SIRT3 deacetylase were shown to be involved upstream of this process (Ozden \textit{et al.}, 2014). It was shown that dephosphorylation of tyrosine Y381 residue by PDP1 drives SIRT3 dissociation and ACAT1 recruitment in epidermal growth factor (EGF)-stimulated cells or in different cancer cells. Then ACAT1 acetylates PDP1 at lysine K202 residue and PDH E1α at lysine K321 residue. These acetylations result in dissociation of PDP1 from PDC and recruitment of PDKs to PDC. As a consequence, PDH activity decreases (Fan \textit{et al.}, 2014). A global lysine desuccinylation analysis was also performed in Sirt5 WT and KO mouse liver cells and several succinylation sites were identified in E1α, E1β, E2 and E3 subunits of PDH complex. Desuccinylation by SIRT5 decreased the PDH activity (Park \textit{et al.}, 2013).

4. A novel function for PDH Complex in nucleus

Although PDH complex localized in the mitochondria to link glycolysis to mitochondrial respiration, it was recently demonstrated that PDC also display important functions in the nucleus. Indeed, it is an essential regulator of epigenetic modifications by providing Acetyl CoA, which is the major source of acetyl group necessary for histone acetylation. Acetylation is catalyzed by histone acetyl transferase enzymes that transfer an acetyl group coming from the Acetyl CoA to lysine residue of histone protein by removing the positive charge from the histone. This modification is associated with a transcriptionally active chromatin.

Interestingly, all the subunits of the PDH complex were detected and were found to be fully functional in the nucleus of primary human cells as well as cancer cells. Knock down of nuclear PDH complex decreased de novo Acetyl CoA synthesis and acetylation of lysine residues on histones (Figure 6). PDH complex was translocated from the mitochondria to
the nucleus by heat shock protein 70 (HSP70), which binds to the E1 and E2 subunits. This translocation was induced by different growth factors including epidermal growth factor as well as mitochondrial stress. Expression of PDKs was not detected in the nucleus suggesting that nuclear PDH is constitutively active and/or regulated by other mechanisms. Importantly, nuclear PDH knock-down blocked cells at the G1/S transition suggesting that PDH-dependent epigenetic regulations are important for cell-cycle progression (Sutendra et al., 2014).

![Figure 6: Mitochondrial PDC translocates to nucleus for Acetyl CoA generation (Extracted from Sutendra et al., 2014)](image)

ii. Pyruvate Carboxylase

Mitochondrial pyruvate is not only converted to Acetyl CoA via PDH to be used in the TCA cycle. Another fate for mitochondrial pyruvate is its irreversible carboxylation by pyruvate carboxylase (PC) enzyme to generate oxaloacetate (OAA) (Utter et al., 1960).

In human, there is only one PC isoform located in the mitochondrial matrix (Jitrapakdee et al., 1996; Botger et al., 1969). Active PC is a homotetramer composed of 4 different 120 kDa-subunits with functional quaternary structure, while its monomeric structure does not exhibit any activity. PC has one biotin carboxylase domain, one allosteric regulatory domain, one carboxyl transferase domain and one biotin carboxyl carrier protein domain (Wallace et al., 2010; Xiang et al., 2008; Jitrapakdee et al., 1996). PC is ubiquitously expressed and tightly regulated by Acetyl CoA levels. When the level of Acetyl CoA is high, PC activity increases. The pyruvate carboxylation starts by the producion of carboxy-biotin, which is then transferred to pyruvate to generate OAA (Wallace et al., 2010; Adina-Zada et al., 2011; Jitrapakdee et al., 1996). Then, OAA can be used for gluconeogenesis if the cellular energy is sufficient or to replenish the TCA cycle if there is not enough OAA to drive TCA.
cycle. The coordinated activity of PDH and PC regulates the carbon flux coming from glucose (Gray et al., 2014).

III. OXIDATIVE PHOSPHORYLATION

The oxidative phosphorylation (OxPhos) is a metabolic process that oxidizes the nutrients to produce energy in cells. TCA cycle is the first step of OxPhos, which produces energy precursors through modifying the Acetyl CoA.

B. Acetyl CoA

Acetyl CoA is a key metabolic intermediate at the crossroad of energy production and a number of anabolic reactions necessary for cell survival and many other biological processes (Figure 7). It is composed of an acetyl group (CH$_3$CO) and a coenzyme A (CoA) together with derivative of vitamin B5 and cysteine that are bound by a thioester bond (Shi and Tu 2015). Mitochondrial Acetyl CoA can be derived from three major metabolic pathways.

![Figure 7: Acetyl CoA is a key intermediate in cell metabolism.](image)

One of the major sources is pyruvate decarboxylation through PDH activity (see above). The second major source of Acetyl CoA arises from fatty acid oxidation (β-oxidation). During this process, free fatty acids are catalyzed to generate acyl CoA, which is then condensed with L-carnitine to produce Acyl carnitine and CoA by carnitine

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palmitoyltransferase 1 (CPT1). Acyl carnitine is then imported in the mitochondria through SLC25A20 transporters and is converted into L-carnitine and acyl CoA by carnitine palmitoyltransferase 2 (CPT2) enzyme. Finally, acyl CoA undergoes β-oxidation to generate NADH and Acetyl CoA (Ruffer et al., 2009). The third main source that can produce Acetyl CoA is branched-chain amino acid (BCAA, i.e. valine, leucine, and isoleucine) metabolism (Harries et al., 2005). This reaction depends on mitochondrial deacetylase sirtuin 3 enzyme (SIRT3) and takes place in various tissues such as brain, liver, kidney and skeletal muscles (Dittenhafer-Reed et al., 2015). BCAAs are first transaminated to branched-chain α-ketoacids by branched-chain amino acid transaminase (BCAT1) in the cytoplasm. Then, branched-chain α-ketoacids are imported to the mitochondria and undergo multiple steps of irreversible reactions through branched-chain α-ketoacid dehydrogenase complex (BCKD). As a result of these reactions, NADH, Acetyl CoA and other acyl-thioesters are generated (Harris et al., 2015).

Apart from these three main pathways, there are other tissue-specific pathways for Acetyl CoA production. For instance, neurons use ketone bodies (D-β-hydroxybutyrate) to generate Acetyl CoA (Cahill et al., 2006).

Three different pools of Acetyl CoA have been described in the cells: a mitochondrial, a cytoplasmic and a nuclear pool. Mitochondrial Acetyl CoA can be derived from glycolysis, fatty acid oxidation and amino acid deamination as discussed. Alternatively, glutaminolysis is a source of cytoplasmic Acetyl CoA. The reductive carboxylation of glutamine when glycolysis is blocked under hypoxia, generates Acetyl CoA (Yang et al., 2014; Metallo et al., 2012). Glutamine is converted to glutamate by glutaminase and then imported to mitochondria by the aspartate/glutamate carrier SLC25A13 (solute carrier family 25 member 13). Glutamate is converted to α-ketoglutarate by glutamine dehydrogenase 2 (GLUD2) in mitochondria then its reductive carboxylation generates citrate. At this point, citrate is transported back to the cytosol by the SLC25A1 citrate carrier and converted to OAA and Acetyl CoA by the enzyme ATP citrate lyase ACLY (Zaidi et al., 2012). In addition to these pathways, cytosolic acetate can be taken from the extracellular medium through monocarboxylate transporters (Halestrop and Price 1999). Lastly, it has been reported that there is a nuclear pool of Acetyl CoA generated by PDC directly in the nucleus (Sutendra et al., 2014).

The cytosolic and mitochondrial pools of Acetyl CoA are important for several cellular functions (Figure 8). It balances the anabolic and catabolic reactions of the cell. Mitochondrial Acetyl CoA is important to drive the TCA cycle and to generate ATP through oxidative phosphorylation. Besides, it can be used in ketone body synthesis. Cytosolic Acetyl CoA is used in fatty acid and steroid biosynthesis. Finally, Acetyl CoA can be used as a substrate for protein acetylation reactions. Acetylation of proteins, including histones, plays
a central role in many cellular processes such as metabolism, autophagy mitosis and regulation of the epigenome.

![Diagram](image)

**Figure 8:** Cytosolic and mitochondrial pools of Acetyl CoA are the source of many important reactions in the cell.

C. **Tricarboxylic acid (TCA)/Krebs/ Citric Acid cycle**

Many studies on aerobic metabolism led to the discovery of the Tricarboxylic acid (TCA) cycle, also known as the Citric Acid or the Krebs Cycle. Two-third of carbons in the cells is used by the TCA cycle as a result of multiple reactions that generate CO₂ and provide reductive power through NADH and FADH2. The CO₂ is disposed as a waste product whereas NADHs are transferred to the electron transport chain. TCA cycle does not need O₂ to process however NADH transfers its electrons to oxygen and this step is required to keep the cycle going. The complete process is composed of 8 reactions and forms a cycle because at the end, the starting molecule OAA is regenerated and allows the initiation of a new round of cycle.

During these reactions, carbons of the Acetyl CoA are gradually and completely oxidized and converted to CO₂. First, Acetyl CoA and oxaloacetate is condensed by citrate synthase to generate 6-carbons product citrate. Aconitase catalyzes the conversion of citrate to isocitrate. The isocitrate dehydrogenase enzyme transforms isocitrate into α-ketoglutarate by generating NADH and CO₂. Then, the α-ketoglutarate dehydrogenase catalyzes the transformation of α-ketoglutarate to succinyl CoA, producing another molecule of NADH and CO₂. Succinyl CoA is then converted to succinate by succinyl CoA thiokinase with the production of one molecule of guanosine triphosphate (GTP). Succinate dehydrogenase (SDH) converts succinate to fumarate by generating one molecule of FADH₂. Then fumarase converts fumarate into malate. Finally, malate dehydrogenase regenerates OAA by producing NADH (Figure 9). For each cycle, 3 molecules of NADH, 1 molecule of
FADH$_2$ and 1 molecule of GTP are generated. NADH and FADH2 generated from the TCA cycle will then fuel the respiratory chain.

![Citric Acid Cycle Diagram](image)

**Figure 9:** Steps of the TCA cycle.

**D. Electron Transport Chain (ETC)**

After the TCA cycle, reduced electron carriers NADH and FADH2 transfer their electrons to the electron transport chain (ETC) and to oxygen to produce ATP and H$_2$O. This process is called oxidative phosphorylation (OxPhos).

The ETC is composed of four different respiratory chain complexes located in the inner mitochondrial membrane. These large protein complexes are electron donor and acceptor enzymes responsible for the continued flux of the electrons to generate energy. During this process, the Complex I (NADH dehydrogenase) accepts electrons from NADH generated by the TCA cycle and transfers it to small hydrophobic electron carrier ubiquinone (Q). This reaction is an energy yielding process and causes free energy decrease. Ubiquinone then transfers electrons to Complex III (Cytochrome b-c). Complex III transfers electrons to cytochrome c which is also a mobile electron carrier like ubiquinone and finally Complex IV (Cytochrome c oxidase) accepts the electron from cytochrome c. Complex IV
accepts electrons one by one and it transfers four of them at once to oxygen in order to produce H₂O. The complex II (succinate dehydrogenase) receives electrons from TCA cycle intermediate succinate via FADH₂ and then transfers it to ubiquinone (Q). Here, unlike the NADH to ubiquinone transfer, there is not any decrease of free energy and therefore it is not coupled to ATP synthesis. Only the Complex III and Complex IV electron transfers yield energy. Consequently, energy derived from electron flux through Complex I, Complex III and Complex IV is coupled to ATP synthesis. However, this is different from the ATP production of glycolysis. In contrast, in electron transport chain, energy yield from electron transfer is coupled to generation of proton gradients across the inner membrane of mitochondria. This energy is then used by a fifth protein complex, Complex V (ATP synthase) which couples it to ATP synthesis (Figure 10).

![Figure 10: Simplified representation of the electron transport chain and mitochondrial respiratory complexes.](image)

Although TCA cycle is considered as a part of the oxidative phosphorylation, it does not require oxygen. Only the last step of the electron transport chain needs oxygen to transfer its electrons. During oxidative phosphorylation, 1 molecule of glucose is oxidized generating 30 or 32 molecules of ATP if NADH generated in glycolysis transfer its electrons to Complex III or Complex I respectively.

IV. LACTATE METABOLISM

The cytosolic pyruvate can be taken by the mitochondria through the PDH complex or converted to lactate through LDHA (lactate dehydrogenase A) in the cytoplasm. In human, there are two stereoisomers of Lactate: the D-Lactate and L-Lactate, the latter being the most abundant enantiomer (Connor et al., 1983; Talasniemi et al., 2008). Pyruvate is reduced to L-Lactate by LDHA whereas NADH is oxidized to NAD⁺ in parallel (Le et al., 2010). Lactate is exported from cells via monocarboxylate transporters (MCT) which consists
of 14 family members. They are encoded by genes belonging to the solute carrier family of \textit{SLC16}. They are present in all eukaryote cells and they belong to the major facilitator super family (MFS). The MCTs are reported to have many different substrates. Four of them (MCT1, MCT2, MCT3 and MCT4) are responsible for the proton-linked transport of monocarboxylate metabolites such as L-lactate, pyruvate and ketone bodies (acetoacetate, D-β-hydroxybutyrate) (Figure 10) (Halestrap 2012). The other members can also transport numerous substrates: MCT6 bumetanide, MCT7 ketone bodies, MCT8 thyroid hormones, MCT9 carnitine, MCT10 aromatic amino acids. Even though they are closely related and transport the same substrates, they have different binding affinities for their substrates. For instance, MCT1 has high affinity for pyruvate and lactate compared to MCT4 (Dimmer \textit{et al}., 2000; Manning \textit{et al}., 2000). Therefore, in addition to their different affinities for their respective substrates, they exhibit tissue specific expression patterns (Fishbein \textit{et al}., 2002). Due to low affinity for pyruvate and lactate, MCT4 is mostly expressed in glycolytic tissues such as white skeletal muscle and astrocytes, whereas MCT1 is widely expressed in red skeletal muscle, heart and neurons.

The mechanism of action of MCT1 has been the most widely studied so far. During its activation, a proton (H\textsuperscript{+}) binds to the lysine residue K38 of MCT1 and positively charges the transporter. Then lactate, which binds to the same residue, makes an ionic pair resulting in a conformational change of MCT1, switching it to an open state. Lysine K38 is then deprotonated by transferring H\textsuperscript{+} to aspartic acid residue D302 and lactate to the arginine residue R306 located in the inner surface of the channel. Deprotonated K38 returns to its close state by pushing D302 and R306 residues towards the cytosol. Then lactate and proton are released in the cytosol (Halestrap 2012; Manoharan \textit{et al}., 2006; Wilson \textit{et al}., 2009). Since MCTs are symporters, their activities depend on the intra and extra cellular lactate and proton concentrations. The residues involved in the action of MCT1 are thought to be conserved in other transporters MCT2, MCT3 and MCT4. Lactate is the major substrate for MCT1 and MCT4. The MCT4 has a lower affinity but a higher turn-over rate for lactate. The anchorage, activity and localization of MCTs require the interaction of these transporters with chaperon proteins such as BASIGIN/CD147/EMBIGIN/GP70. In the absence of BASIGIN, MCTs cannot be transferred to the cell surface (Nakai \textit{et al}., 2006; Philip \textit{et al}., 2003). MCT1-4 needs to associate with transmembrane glycoproteins for its folding and transfer to cell surface. Studies demonstrated that MCT1 and MCT4 can interact with CD147/BASIGIN (BSG) (Kirk \textit{et al}., 2000). BASIGIN is a transmembrane glycoprotein which belongs to the immunoglobulin superfamily. It consists of extracellular lg-like domain, a single-membrane spanning segment and a short intracellular cytoplasmic tail (Muramatsu \textit{et al}., 2003). Previous \textit{in vitro} studies demonstrated that MCT and BASAGIN interaction is necessary for expression of MCT at the cell surface. Recently, it has been demonstrated that expression of MCT is also required for surface expression and trafficking of BASAGIN. Inactivation of MCT4 alone or together with MCT1 resulted in accumulation of BASAGIN in the endoplasmic reticulum and proteasomal degradation (Marchiq \textit{et al}., 2015; Le Floch et
al., 2011). Up to date, BASIGIN is the only known chaperone protein for MCTs. However, MCT1 expression was detected in some tissues such as sertoli cells, myoepithelial cells in Basasin−/− mice (Nakai et al., 2006). Expression of MCT1 and MCT4 was also detected in Basasin-inactivated cancer cells suggesting that there may be other proteins involved in the trafficking of MCTs to the cell surface. It was shown that CD44 hyaluronan receptor co-immunoprecipitates with MCT1 and MCT4 together with BASIGIN and all these proteins co-localize at the cell membrane in breast cancer cells. These interactions (MCT-BASIGIN-CD44) contribute to the localization and function of MCTs at the plasma membrane (Slomiany et al., 2009).

Not much is known about the regulation of MCTs. Studies demonstrated that MCT1 and MCT4 can be regulated at the transcriptional level and by post-translational modifications. MCT4 is upregulated in response to HIF-1α activation under hypoxic conditions as an adaptation to increased glycolysis and lactate production (Ullah et al., 2006). There is no-hypoxia responsive element identified in MCT1 promoter. However, it has been reported that it can be controlled by other transcription factors or transcriptional regulators, including c-MYC and PGC1-α (Doherty et al., 2004). MCTs are often upregulated in different cancers (kidney, breast, melanoma, lung, colorectal and prostate). Their expression does not only differ depending on the tumor types, but it also varies within the tumor. On the one hand, MCT4 is mostly expressed in glycolytic tumors and functions in lactate secretion. On the other hand, MCT1 expression is increased in OxPhos-dependent tumors to import lactate and use it as a substrate to fuel mitochondrial activity (Bonuccelli et al., 2010). This process requires increased LDHB activity to convert lactate to pyruvate before entering the mitochondria. Finally, lactate secretion by MCT1 and MCT4 during tumorigenesis increases the extracellular acidification. Lactate/H+ secretion is associated with increased tumor cell migration, angiogenesis and metastasis. Lactate secretion by tumor cells stimulates VEGFR (vascular endothelial growth factor receptor) production by endothelial cells, leading to increased angiogenesis (Ruan et al., 2013), while proton secretion increases the acidification of the micro-environment and enhances the proteolytic activity of matrix metalloproteases (MMPs) and cathepsins (Martinez-Zaguilan et al., 1996; Rothberg et al., 2013). In turn, this leads to the degradation of the extracellular matrix further enhancing tumor invasion and dissemination (Figure 11). It is currently not known if this process is relevant to non-cancerous cells. Our data suggest that it contributes to the normal homeostasis of epithelia, as illustrated in the epidermis (See publication #2).
**Figure 11:** High level of lactate secretion in cancer cells is linked to increased activity of matrix remodeling enzymes and angiogenesis resulting in enhanced tumor invasion (Adapted from Marchiq and Pouysségur 2016).
CHAPTER II- E4F1

I. DISCOVERY

In the late 80ies, numerous studies aiming at understanding how viral oncoproteins hijack the cellular machinery to transcribe and translate their own genome led to the discovery of many cellular factors important for proliferation and cell survival. As an example, E1A (adenovirus early region 1A) was one of the most investigated viral oncoprotein. E1A is expressed within the first hours of adenovirus infection. It is expressed as two major forms: 12S and 13S. These 2 proteins are known to be important for the regulation of transcriptional regulators of several viral and cellular genes. Studies on these targets of E1A enabled the discovery of the first tumor suppressor identified in humans, the retinoblastoma protein pRb (Lee and Green 1987) as well as the acetyl-transferases CBP/300, P/CAF and the chromatin remodelers of the SWI/SNF family members (Gallimore and Turnell 2001). Meanwhile, 2 transcription factors targeted by the E1A oncoprotein that were characterized for their role in the transcriptional regulation of the viral E4 and E2 genes: E2F and E4F (Figure 12). Their names stand for their implications on the transcriptional regulation of the viral E2 and E4 promoters, respectively (Lee and Green, 1987; Raychaudhuri et al., 1987). The identification of the physical interaction between pRb and E2F paved the way for thousands of seminal studies aiming at understanding their role in proliferation and cell survival. These studies showed that "E2F" represents a family of transcription factors that contains 8 members (E2F1 to E2F8), which play a major role in cell cycle progression at the G1/S transition. In contrast, E4F stays the only member of the E4F family and is currently called E4F1.

![Diagram](image)

**Figure 12**: E4F1 is a target of the viral oncoprotein E1A. It can activate and repress the transcription of viral genes.
Paradoxically, although E2F and E4F were discovered at the same time, the roles of E4F remained poorly investigated (Friend et al., 1986). Ironically, fourteen years later, E4F was also shown to interact with pRb (Fajas et al., 2000), raising a new interest for this protein and promoting studies aiming at understanding its functions in both normal and cancer cells.

II. STRUCTURE AND FUNCTION OF E4F1

E4F1 is a ubiquitously expressed protein that is found in all vertebrates including human, mouse, chicken, xenope and zebra fish. It exhibits 86% sequence homology between human and murine protein (previously known as AP3) (Fernandes and Rooney 1997). However, no E4F1 sequence has been yet documented in invertebrates (C. Elegans, Drosophila melanogaster) suggesting that E4F1 only appeared during late stages of the evolution. E4F1 is localized in the 13.3 region of chromosome 16 (16p13.3) of the human genome (Saccone et al., 1998) and in the A3.3 region of chromosome 17 (17A3.3) in the mouse genome. It contains 14 exons both in human and mouse, and encodes a full length protein of 120kDa (Fernandes and Rooney 1997).

E4F1 belongs to GLI-Kruppel zinc finger family (Fognani et al., 1993). It contains six C2H2 type zinc finger domains, two of which are located in the N-terminus of the protein and are important for its DNA-binding activities. Four additional C2H2 type and 3 C2HC type zinc finger domains and a basic helix-loop-helix (bHLH) domain located in the C-terminus implicated in protein-protein interactions (Fajas et al., 2000). Finally, E4F1 contains an atypical E3 ligase domain located in the N-terminus, allowing the ubiquitylation of its cellular targets among which the tumor suppressor protein p53 (Figure 13) (Le Cam and Linares 2006).

![Figure 13 Structure of the protein E4F1.](image)

A. Transcription Factor

E4F1 was first discovered as a transcription factor that regulates the transcriptional activity of the viral E4 gene during adenoviral infection. It binds the cAMP response element (CRE) site of the E4 promoter on the -ATGACGTCAC- motif (Raychaudhuri et al., 1987) (Lee
and Green 1987). During adenoviral infection, E1A activates the transcriptional activity of E4F1 through phosphorylation (Raychaudhuri et al., 1987). This modification drives the proteolytic cleavage of 120 kDa protein and generates a shorter 50 kDa form which contains the N-terminus part of the protein (Fernandes and Rooney 1997). Even though these two different isoforms have the same DNA binding domain, they have been shown to have different functions: p120E4F1 inhibits the transcription of the E4 gene whereas p50E4F1 activates it. So far, p50E4F1 was only detected in adenovirus-infected cells and not in any other physiological or pathophysiological conditions.

The first cellular target gene of E4F1 was found to be the Cyclin A2 gene (Fajas et al., 2001). E4F1 binds the Cyclin A2 promoter on a CRE motif that resembles that of the viral E4 promoter. Expression of ectopic p120E4F1 drives cell cycle arrest by a mechanism involving Cyclin A2 transcriptional repression. Apart from this finding, not much was known about the cellular targets of E4F1 until recently when the repertoire of E4F1-targets genes was identified using a combination of whole genome chromatin immuno-precipitation coupled to next generation sequencing (ChIP-seq) and gene expression profiling approaches (Rodier et al., 2015). Combined ChIP sequencing and transcriptomic analyses of E4F1-deficient (E4f1 knock out) mouse embryonic fibroblasts, embryonic stem cells and transformed mouse embryonic fibroblasts identified a set of approximately 100 E4F1-direct target genes. This analysis defined a new consensus binding site for E4F1 (CTTTACGGC) that is usually located nearby the transcription start site of these genes. Interestingly, this–E4F1-motif was also found in E4f1 itself, suggesting that E4F1 controls its own expression. Inactivation of E4F1 decreased the expression of most of its target genes suggesting that E4F1 mainly works as a transcriptional activator. However, a small set of E4F1-target genes exhibited increased expression in E4F1-deficient cells, indicating that E4F1 can also mediate repressive effects. Gene ontology analyses of these E4F1 target genes indicated a strong enrichment in genes implicated in mitochondrial activity and mitochondrial homeostasis as well as in DNA damage and cell cycle checkpoints (see "E4F1 and Metabolism" and "Cell cycle check points for more details).

### B. E4F1: An atypical E3 Ligase

In addition to its intrinsic transcriptional activity, E4F1 also displays an atypical E3 ligase activity. E4F1 contains an E3 ligase domain located in the N-terminus part (30-80 aa) of the protein. However, this domain does not exhibit any sequence homology with previously known domains mediating E3 ligase activity such as RING, HECT or U-box domains (Pickart and Eddins 2004). Instead, this domain exhibits strong similarities with two regions (IR1 and IR2) which are important for the catalytic activity of the E3 sumo ligase RANBP2 (Pichler 2004, Tatham 2005), suggesting that E4F1 can act both as an E3 SUMO ligase and/or an E3 ubiquitin ligase. Thus, these data clearly indicate that E4F1 is a multifunctional protein.
My host laboratory identified p53 tumor suppressor as the first cellular target ubiquitylated by E4F1. P53 ubiquitylation was long thought to be associated with its degradation or its cytoplasm-nucleus shuttling. Interestingly, E4F1-mediated ubiquitylation of p53 doesn’t mark this protein for degradation like other E3 ligases such as Mdm2, but instead regulates p53 transcriptional activities. Indeed, the ubiquitylated forms of p53 were found to be tightly associated to chromatin, and gene expression profiling showed that p53 target genes related to cell cycle arrest were activated in response to this ubiquitylation. E4F1 ubiquitylates p53 on specific lysine residues: K319, K320 and K321 to drive its transcription activity toward cell cycle arrest by activating Cyclin G1, GADD45 and p21 genes. In contrast, the PCAF acetyl transferase acetylates p53 on these same residues to trigger the transcription of apoptosis-related genes (Liu et al., 1999). Thus, the competition between E4F1 and PCAF-mediated modifications on p53 determines the cell fate between growth arrest and apoptosis (Figure 14) (Le Cam, Linares et al., 2006). Interestingly, this E3 ligase activity is very unusual for a transcription factor. E4F1 is to my knowledge the first transcription factor to exhibit both activities. Non-published data from my host laboratory demonstrates that p53 can be ubiquitylated during senescence, however further research required to identify other physiological conditions that induces p53 ubiquitylation by E4F1.

Figure 14: E4F1 and PCAF mediated regulation of p53 determines cell fate between cell cycle arrest and apoptosis.
III. PARTNERS OF E4F1

A. E4F1 and Bmi1-Ink4a/Arf-p53 pathway

Converging evidence indicates that E4F1 seems to be a crucial regulator of the Bmi1-Ink4a/Arf-p53 pathway and interplays with several components of this pathway to control normal tissue homeostasis and stem cell function (Figure 15) (Chagraoui et al, 2006; Lacroix and Caramel 2010).

![Diagram of Bmi1/Arf/p53 pathway and E4F1](image)

Figure 15: Schematic representation of Bmi1/Arf/p53 pathway and E4F1.

i. The p53 tumor suppressor

The p53 protein is a transcription factor also known as the gate keeper of the genome that triggers different cellular responses to diverse stress signals including DNA damage, hypoxia, oxidative stress or oncogenic stress. The p53-mediated responses extend from cell cycle arrest to senescence or apoptosis. The molecular mechanisms underlying p53’s ability to induce these very different cell fates are not fully understood. However, it is believed that it differs according to the strength and type of stimuli, the cell of origin, the cofactors and post-translational modifications on p53. Interestingly, growing evidences suggest that in addition to its major role in acute stress response, p53 is also implicated in adaptive response among which is the control of cellular metabolism. For instance, p53 regulates the expression of genes implicated in various metabolic pathways such as glycolysis, oxidative phosphorylation, lipid metabolism and redox status.
P53 interacts with the C-terminal part of E4F1. This interaction is involved in E4F1-dependent anti-proliferative effects in human and mouse cell lines (Sandy et al., 2000). Furthermore, E4F1 can regulate p53-mediated transcriptional activities through its intrinsic E3 ligase activity as described previously (Le Cam and Linares et al., 2006).

In addition, very recent data from my host lab have also shown that independently of its E3 ligase activity, E4F1 also interacts with p53 on the promoters of genes implicated in metabolism. These genes include PGC1α, InsR (Insulin receptor) and Steaoryl-CoA Desaturase-1 (Scd1) and their control by E4F1 and p53 is important for proper adipocyte function both in vitro and in vivo (Lacroix and Linares et al., Nature Communications, under revision) (See Lipid Metabolism for more details).

ii. Ink4a/Arf

The Ink4a/Arf (Cdkn2a) locus encodes 2 distinct proteins, p16INK4a and p19ARF (p14ARF in human), that are generated through the utilization of 2 different reading frames (Quelle et al., 1995). p16INK4a is a cell cycle inhibitor that targets the cell cycle dependent kinases Cdk4 and Cdk6 (Serrano et al., 1993). Genetic inactivation of p16INK4a is often observed in human tumors and p16INK4a knock-out mice develop spontaneous tumors, indicating that p16INK4a also functions as a tumor suppressor (Sharpless et al., 2002). p19ARF also functions as a tumor suppressor. It can induce p53 activation by inhibiting MDM2 activity. It sequesters MDM2 in the nucleolus and blocks its E3 ubiquitin ligase activity, preventing the degradation of p53 (Sherr and Weber 2000). Furthermore, mutated forms of Ras, c-Myc, E1A and E2F1 can stimulate the expression of p19ARF which induce cell cycle arrest in G1 or G2 phase or apoptosis (de Stanchina et al., 1998; Bates et al., 1998; Gil et al., 2006). P19ARF has been shown to sequester E4F1 in the nucleolus, and together with p53 they form a trimeric complex in vivo that induces cell cycle arrest in G2 in human osteosarcoma cells (Rizos et al., 2003).

iii. Bmi1

Bmi1 is a member of the Polycomb family. This family of epigenetic regulators was initially identified as transcriptional repressors of homeotic genes in Drosophila (Moehrle and Paro 1994). There are two polycomb complexes known as PRC1 (Polycomb repressive complex I) and PRC2 (Polycomb repressive complex II). They are co-recruited to chromatin and maintain gene silencing. Bmi1 is a part of a Polycomb repressive complex I (PRC1) which also contains RING1/2, MEL-18, MPH1 and M33 proteins (Valk-Lingbeek et al., 2004). PRC1 stabilizes the chromatin and is recruited to chromatin through a specific epigenetic mark: trimethyl histone H3 on lysine 27 residue. Then, Polycomb repressive complex II (PRC2) which displays histone methyl transferase activity, deposits H3K27me3 marks (Cao et al., 2002). In mammals, one locus that is targeted by polycombs is Ink4a/Arf.
Bmi1 plays an important role in the development of the central nervous system as well as in the hematopoietic system through its role in stem cell functions (Shakhova et al., 2005; Lessard et al., 2003). Whole-body inactivation of Bmi1 in mouse results in aplasia of the hematopoietic system due to hematopoietic stem cell-renewal defects (Park et al., 2003). Bmi1<sup>−/−</sup> hemotopoietic stem cells and primary fibroblasts have decreased proliferation capacity and exhibit premature senescence due to the increased expression of p16<sup>Ink4a</sup> and p19<sup>ARF</sup>. This phenotype is partially restored by activation of p53 or p16<sup>Ink4a</sup> and p19<sup>ARF</sup> proteins (Bruggeman et al., 2005; Oguro et al., 2006). In contrast, overexpression of Bmi1 is correlated with p16<sup>Ink4a</sup> and p19<sup>ARF</sup> protein downregulation in certain types of lymphomas and medullablastomas (Leung et al., 2004), suggesting that Bmi1 is an oncogene.

Bmi1 was found to interact with E4F1 using two-hybrid assays. This interaction takes place in the cytoplasm of hematopoietic stem cells independently of ARF. Besides, shRNA-mediated depletion of E4f1 completely restores the phenotype of Bmi1 KO. Thus, these findings suggest that E4F1 can be a mediator or an effector of Bmi1 functions in hematopoietic stem cells (Chagraoui et al., 2006).

Although E4F1 is involved in diverse cellular functions as a member of the p53 pathway, it is noteworthy that E4F1 functions seem to extend beyond its implication in this pathway. Several data suggest that it is also involved in many other cellular processes independently of p53.

B. Other partners of E4F1

i. pRb

The pocket family members pRb, p107 and p130 are important regulators of cell cycle progression. pRb, also known as the retinoblastoma protein, was the first tumor suppressor identified in humans and it is also reported to be mutated in other types of tumors such as lung, prostate and breast cancer (Goodrich et al., 1993). It is a transcriptional repressor of genes required for the G1/S transition of the cell cycle. It binds to the trans-activating domain of members of the E2F family and represses the expression of their target genes. Besides, it can also repress transcription through its interaction with other proteins which are involved in histone acetylation, deacetylation or methylation (Giacinti and Giordano 2006). In quiescent cells (G0 phase) and post-mitotic cells, pRb is hypo-phosphorylated. Its phosphorylation increases during G1 phase and is maintained until the end of mitosis. The phosphorylation status is controlled by mitogenic signals through the activation of Cyclin D/ Cdk4 and Cdk6 complexes at the beginning of G1 phase and Cyclin E/Cdk2 at the end of G1 phase. Most of the studies done on pRb focused on the E2F/DP family of transcription factors which are implicated in G1/S phase progression. During the G0 and the G1 phases of the cell cycle, the hypo-phosphorylated forms of pRb interact with E2F/DP preventing them to interact with their targets, thereby blocking
cell cycle progression. At the G1 to S phase transition, phosphorylation of pRb induces its dissociation from E2Fs, allowing them to transactivate a series of genes (i.e. DHFR, Pol II, TK, MCMs, CCNE, CDC6) that are implicated in S phase entry (Cobrinik et al., 2005).

Interaction of E4F1 with the hypo-phosphorylated form of pRb was detected in the G0 phase of the cell cycle. This interaction involves both the C and N-terminal domains of E4F1 and the C-terminal part of pRb. This domain of interaction with E4F1 is distinct from the one implicated in the interaction of pRb with E2Fs. pRb increases the affinity of E4F1 for the Cyclin A2 promoter and in turn enhances the repressive activity of E4F1 on this gene to prevent cell cycle progression. Consistent with the role of pRb in E4F1-mediated effects on cell division, this inhibition of proliferation was partially reduced in pRB<sup>−</sup> fibroblasts (Fajas et al., 2001; Fajas et al., 2000).

ii. **RASSF1A**

RASS1FA (Ras-association domain family 1) is one of the Ras effectors that possess tumor suppressor activity. Although Ras signaling is associated with oncogenic activities such as proliferation, some of its effectors exhibit tumor suppression activity. Indeed, several studies demonstrated that RASSF1A inactivation is associated with tumorigenesis. It is often deleted or inactivated by methylation in many cancer types (lung, testis, breast and prostate cancer) (Hesson et al., 2007). Besides, RASSF1A KO animals develop tumors (adenomas or lymphomas) spontaneously, confirming the notion that RASSF1A is a tumor suppressor (Tommasi et al., 2005).

RASSF1A is implicated in several cellular functions; it co-localizes with microtubules and this interaction is required for microtubule stability. RASSFA1 inactivation results in cell-cell adhesion defects. Moreover, it is recruited to the spindle pole and inhibits the anaphase promoting complex (APC) thereby slowing the mitotic progression (Amin et al., 2012). It is also involved in other cellular functions such as cell cycle regulation (through the modulation of CYCLIN D activity) and apoptosis (Shivakumar et al., 2002; Feig et al., 2002).

Interaction of E4F1 with RASSF1A was observed both in vitro and in vivo. Overexpression of RASSF1A increases the anti-proliferative effects of E4F1 (Fenton et al., 2004). RASSF1A can control the expression of Cyclin A2 through its interaction with E4F1. Depletion of RASSF1A by siRNAs decreases the affinity of E4F1 for the Cyclin A2 promoter and induces its expression, promoting cell cycle progression (Ahmed-Choudhury et al., 2005).

iii. **High-mobility group A2 (HMGA2)**

The transcription factors that belong to the high-mobility group A2 family (HMGA2), regulates gene expression through structural changes on chromatin. These modifications on chromatin enable the transcriptional activity of several genes implicated in cell cycle regulation and differentiation (Young and Narita, 2007). HMGA2 interacts with E4F1 in vitro
and in vivo, and ectopic expression of HMGA2 results in the dissociation of E4F1 from the Cyclin A2 promoter, thereby increasing its expression (Tessari et al., 2003).

IV. CELLULAR FUNCTIONS OF E4F1

E4F1 is important for multiple cellular processes such as proliferation, differentiation, senescence and apoptosis. It interacts with several protein partners (described above) to achieve these functions (Figure 16). The different cellular functions of E4F1 are discussed below:

![Diagram showing the interactions of E4F1 with other proteins and processes.]

**Figure 16:** E4F1 is in the center of important cellular functions through regulation of other factors.
A. Cell cycle and proliferation

To assess the cellular functions of E4F1, numerous studies were performed by modulating E4F1 expression (overexpression or inactivation). Initially, it was demonstrated that overexpression of the p120 isoform of E4F1 results in cell cycle arrest at the G1/S transition. This growth arrest is associated with an increased expression of the cyclin-dependent kinase regulator (CDK) inhibitor p21\(^\text{WAF1}\), which reduces Cyclin D-Cdk4/Cdk6 kinase activity and thereby slows down the G1 to S transition during cell cycle progression. Interestingly, this mechanism was linked to the stabilization of p21\(^\text{WAF1}\) protein in a p53-independent manner through a yet poorly defined mechanism (Fernandes et al., 1998). However, E4F1 can still block cell cycle progression in the absence of p21\(^\text{WAF1}\), suggesting that E4F1 also controls cell proliferation through other mechanisms. As an illustration of this notion, Fajas et al. demonstrated that overexpression of E4F1 leads to the transcriptional inhibition of the Cyclin A2 gene whose expression is also required for the G1/S transition. This work was of particular interest since Cyclin A2 was identified as the first cellular target gene of E4F1. It was shown that E4F1 also requires its protein partners to mediate cell cycle arrest. For instance, E4F1 interacts with pRb which increases its affinity for Cyclin A2 promoter. In return, E4F1 represses the transcription of this gene which contributes to G0 phase maintenance (Fajas et al., 2000). RASSF1A protein also contributes the anti-proliferative effects of E4F1 through the same mechanism as described for pRb (Ahmed-Choudhury et al., 2005). Finally, other partner of E4F1, HMGA2 regulates E4F1 association to Cyclin A2 promoter and allows the activation of this gene. Thus, in contrast to pRb and RASSF1A, HMGA2 induces proliferative effects of E4F1 (Tessari et al., 2003).

Moreover, E4F1 and p53 interaction was observed in mouse and human cell lines that expressing ectopic E4F1. This interaction led to growth suppression (Sandy et al., 2000). A trimeric complex implicating ARF, E4F1 and p53 was also detected which enhances a cell cycle arrest in the G2 phase in a p53-dependent manner (Rizos et al., 2003). Besides, E4F1 intrinsic E3 ligase activity also drives growth arrest through ubiquitylation of p53, which in turn activates p53 target genes that are implicated in cell cycle arrest (Le Cam, Linares et al., 2006).

Paradoxically, \(E4f1\) deficiency also results in anti-proliferative effects that are partly mediated by p53. Data obtained in primary mouse embryonic fibroblasts isolated from \(E4f1\) conditional Knock-out mice indicated that inactivation of \(E4f1\) leads to a cell cycle arrest in the G2 phase. Interestingly, genetic inactivation of the p53 checkpoint in these E4F1-deficient cells released their block in G2 and led to mitotic progression defects and induced cell death (Rodier et al., 2015). Thus, these findings suggest that E4F1 drives growth arrest or cell death depending on the p53 status of the cells. These pro-proliferative effects of E4F1 are in apparent contraction with the cell cycle arrest observed upon expression of
ectopic E4F1. Nevertheless, these different studies illustrate the importance of E4F1 in cell cycle progression and its intimate links with the p53 pathway.

B. Cell cycle checkpoint

Interestingly, a subset of E4F1 target genes that were identified by ChiP-seq (Rodier et al., 2015), includes genes that are implicated in cell cycle checkpoints and in the DNA damage response (DDR). Thus, E4F1 regulates the transcriptional of Chk1 and its regulator Ppp5c as well as the DDR related genes Rad52 and Ddi2 (Rodier et al., 2015). Chek1 encodes a Serine and Threonine (Ser/Thr) kinase that controls S phase progression and activates a G2/M checkpoint in response to DNA replication defects. The importance of CHEK1 in the G2 arrest observed in E4F1-deficient cells was further strengthened by the observation that E4F1 also physically interacts with the CHEK1 protein and is responsible for its stability in the hematopoietic system (Grote et al., 2015).

C. Senescence

Cellular senescence occurs in response to different cellular stresses such as aberrant oncogenic stimulation, telomere shortening, DNA damage, or upon oxidative stress. The induction of cellular senescence leads to an irreversible cell cycle arrest and is therefore considered as an early barrier against the malignant transformation of cells. Senescent cells display several phenotypic features including specific epigenetic changes such as increased trimethylation of Histone H3 on Lysine 9 (H3K9me3) that contributes to the heterochromatinization of their genome, increased lysosomal activity leading to enhanced SA-β galactosidase activity, and the induction of a senescence-associated secretory phenotype (SASP) leading to the increased production of pro-inflammatory cytokines, growth factors and chemokines (Campisi 2013).

At the molecular level, senescence is driven by two major pathways: the p53/p21 and the p16ink4a/pRb tumor suppressor pathways. Recent results from our laboratory have highlighted an important role for E4F1 in cellular senescence. Thus, ectopic expression of E4F1 in primary mouse embryonic fibroblasts induces an irreversible cell cycle arrest in the G1 phase that correlated with the induction of several typical features of senescent cells including increased SA-β-galactosidase activity, stabilization of p21 and p16ink4a proteins, and the induction of a SASP, but that was not associated with detectable levels of DNA-damage. Interestingly, E4F1-mediated senescence relied on the one hand on its E3 ligase activity and its ability to physically interact with p53, and on the other hand on its transcriptional activities that control the expression of several metabolic genes, in particular those controlling the activity of the pyruvate dehydrogenase complex (discussed below). These data are consistent with previous data that unraveled the role of the PDH complex in oncogene-induced senescence (Kaplon et al, 2013).
D. Metabolism

i. Mitochondrial Activity

Unexpectedly, the identification of E4F1 target genes also highlighted a role for E4F1 in metabolism and mitochondrial function. Careful genetic analyses demonstrated that E4F1 regulates the transcriptional expression of this metabolic/mitochondrial program independently of p53. These mitochondrial genes included:

- *Taz* (*Tafazzin*) and *Cr1s1* (*Cardiolipin synthase 1*) and *DNAJC19* which gene products are involved in cardiolipin synthesis. Cardiolipins are phospholipids that are specific of the mitochondrial membranes and that are essential for the maintenance of quaternary structures of transmembrane proteins including components of the respiratory chain complexes. Interestingly, genetic mutation of these 3 genes has been causally associated with the Barth Syndrome, a human condition leading to cardiac defects.

- *Ndufs5* that encodes NADH/Ubiquinone Oxidoreductase Subunit S5, a complex I subunit of the respiratory electron transport chain (ETC).

These results indicate that E4F1 is a novel regulator of mitochondrial homeostasis and that its inactivation causes mitochondrial dysfunction, impaired energy production and de novo pyrimidine synthesis defects (Rodier *et al.*, 2015).

ii. Lipid Metabolism

Recent findings from our laboratory also demonstrated that E4F1 is essential for proper lipid metabolism. In mouse, adipocyte-specific inactivation of *E4f1* resulted in lipid metabolism defects leading to a lean phenotype associated with insulin resistance and cold intolerance. Interestingly, these lipid metabolism defects were almost entirely rescued upon concomitant inactivation of p53, indicating that the E4F1-p53 crosstalk plays an essential role in adipocytes. At the molecular level, p53 target genes implicated in de novo lipid synthesis and increased fatty acid oxidation were strongly deregulated in E4F1-deficient adipocytes. These analyses also highlighted the importance of of *Stearyl-CoA Desaturase-1* (*Scd1*), a gene implicated in the synthesis of monounsaturated fatty acids (MUFAs), in the metabolic phenotypes observed in E4F1 cKO adipocytes. Concomitant genetic inactivation of p53 or complementing the diet of *E4f1* cKO animals with oleate (the MUFA under-represented in E4F1-deficient adipocytes) partially restored their adiposity, decreased their insulin resistance and normaized their adaptive thermogenesis. Finally, E4F1 and p53 physically interacted and are both recruited to the same regulatory region on the *Scd1*
promoter to control its proper expression. These data identified the E4F1-p53-Scd1 pathway as a major determinant of normal adipocyte function (Lacroix and Linares et al., Nature Communications, under revision).

These data are consistent with previous results describing the role of E4F1 in lipid metabolism in hepatocyte carcinoma cells. siRNA-mediated depletion of E4F1 in these cells resulted in impaired lipid metabolism. P53 inactivation partially rescued the phenotypes of E4F1-deficient cells, suggesting that E4F1-mediated lipid metabolism was also p53-dependent in this context (Dai et al., 2014).

Altogether these results highlight the complex metabolic network regulated by E4F1 and the complex interplay with p53.

V. IN VIVO FUNCTIONS OF E4F1

A. Embryonic development

To investigate the physiological functions of E4F1 in vivo, the murine E4f1 gene was deleted by homologous recombination in ES cells (see generation of E4f1+/− heterozygote mice for details). While E4f1+/− heterozygote mice were phenotypically normal and undistinguishable from E4f1+/+ (WT) mice, constitutive homozygous deletion of E4f1 resulted embryonic lethality at the peri implantation stage (between E4.5 and E5.5) (Figure 17). Embryonic cells that lacked E4f1 exhibited mitotic progression and chromosome segregation defects characterized by the constitutive activation of the spindle checkpoint, indicating that E4f1 is indispensable for early embryonic development. The cultured blastocysts isolated from E4f1−/− animals exhibited mitotic defects, accumulated in the pro-metaphase stage, and displayed chromosomal segregation alterations resulting in increased cell death. E4F1 was found to be localized at the mitotic spindle during M phase in embryonic cells from WT blastocysts suggesting that it may be directly involved in mitotic progression (Le Cam et al., 2004).

![E4f1 WT and E4f1 KO embryos](image)

**Figure 17:** E4f1−/− embryos die around day E5.5 at peri-implantation stage.

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B. **Adult tissue homeostasis**

To investigate the functions of E4f1 in adult tissues, an E4f1 conditional knock out mouse (E4f1^lox^) based on the Cre-LoxP system was generated by my host laboratory (see E4f1 mouse models for detailed explanations). This E4f1 conditional mouse mutant (E4f1 cKO) was then crossed with several strains expressing the Cre recombinase in different tissues, including in skin that I have been more specifically studying.

i. **Stem Cell Homeostasis**

Several studies from our laboratory and others have illustrated essential roles of E4F1 in stem cell-dependent regulation of normal tissue homeostasis.

1. **Epidermal stem cells**

E4f1 cKO mice were initially crossed with a knock-in (KI) strain expressing the tamoxifen-inducible CRE-ERT2 fusion under the control of the ubiquitously active RNA polymerase II promoter. Interestingly, 10 to 15 days after topical application of tamoxifen, E4f1^floxed^; RERT animals displayed severe skin alterations evidenced by transient hyperplasia of the interfollicular epithelium and alteration of keratinocyte differentiation. This hyperplasia was then followed at later time points (3 weeks) by a complete loss of cellularity in the epidermis and severe skin ulcerations (Figure 18A). In order to confirm that these phenotypes were skin autonomous, we also crossed E4f1^floxed^ animals with the keratin5-Cre transgenic mice that express the Cre recombinase under the control of the Keratin 5 promoter (K5-Cre) which expression is restricted to the basal keratinocytes of the epidermis. These E4f1 (K5) KO newborns recapitulated the key phenotypes observed in the adult E4f1 cKO; RERT mice. These strong skin alterations led to the death of the animals 4 days after birth, likely due to severe dehydration (Figure 18B). In addition, E4f1 deficiency also altered the clonogenic potential of epidermal stem cells (ESCs) ex vivo and ended in the exhaustion of the ESC pool in vivo, indicating that the skin lesions observed in the E4f1 mutant skin resulted, at least in part, from cell-autonomous alterations in ESC maintenance (Figure 18C). At the molecular level, the clonogenic potential of E4f1 KO ESCs was restored by Bmi1 overexpression, Ink4a/Arf inactivation or p53 depletion, identifying for the first time a regulatory axis essential for ESC-dependent skin homeostasis implicating E4F1 and the Bmi1-Arf-p53 pathway.
Figure 18: E4f1 inactivation results in severe skin defects. (A) E4f1 KO skin displays hyperplasia at early time points associated with increased proliferation as shown by ki67 staining, and then the phenotype is followed by complete loss of cellularity. (B) E4f1 inactivation leads to exhaustion of epidermal stem cell pool in vivo (left panel) and in vitro right panel.

2. Hematopoietic stem cells

The implication of E4F1 in the hematopoietic system was investigated after its interaction with the polycomb member BMI1 was discovered in a two-hybrid screen using BMI1 as bait (Chagraoui et al., 2006). BMI1 is essential for hematopoietic stem cell (HSC) maintenance, as shown by the impact of Bmi1 inactivation on the proliferative capacity of...
HSC and the induction of premature senescence that associated with increased expression of p16<sup>ink4a</sup> and p19<sup>arf</sup> proteins. Accordingly, Bmi1 KO mice exhibited aplasia due to self-renewal defects of HSC. Interestingly, depletion of E4f1 by shRNA in BMI-deficient HSC restored the viability of BMI KO animals revealing the importance of E4f1 in HSC maintenance (Chagraoui et al., 2006). Paradoxically, complete genetic inactivation of E4f1 in the hematopoietic system (including in HSC) resulted in acute bone marrow failure that correlated with accumulation of DNA damage, S phase progression and mitotic defects that were linked to decreased CHEK1 protein stability (Grote et al., 2015). Altogether these results demonstrate that E4f1 is important for HSC homeostasis.

ii. Myogenesis and osteogenesis

The bone morphogenetic proteins (BMPs) belong to TGF-β superfamily of proteins. They stimulate bone formation when expressed ectopically in muscle cells and promote myoblast to osteoblast differentiation in vitro. A constitutively active form of SMAD1 protein induces osteoblastic differentiation in cooperation with SMAD4 and RUNX2, and inhibits myogenesis in C2C12 muscle cells. In Smad4-inactivated mouse derived cells, BMP-induced osteoblastic differentiation is inhibited and myogenesis is enhanced. Interestingly, E4f1 was identified as a interactor of SMAD4 protein by two-hybrid assay. Upon BMP signaling, the SMAD-E4f1 complex translocates into the nucleus to control the transcription of inhibitor of differentiation (Id) genes that are implicated in myogenesis. These data suggest that E4f1 might play an important role in myogenesis/osteogenesis fate of the muscle cells (Nojima et al., 2010). However, E4f1 inactivation is differentiated skeletal muscles using the Acta-Cre model did not alter the expression of muscular markers (Publication #1).

VI. E4f1 and CANCER

The multiple roles of E4f1 in cell cycle, cell division, senescence, and cell metabolism as well as its interaction with several oncogenes and tumor suppressors including pRb (Fajas et al., 2000), p53 (Sandy et al., 2000), RASSF1A (Fenton et al., 2004), p19<sup>arf</sup> (Rizos et al., 2003), FHL2 (Paul et al., 2006), Bmi1 (Chagraoui et al., 2006), DRAL, and HMGA2 (Tessari et al., 2003) have suggested an important role during tumorigenesis (Figure 19). However, no recurrent mutations of E4f1 have been described in human tumors so far apart from one mutation was identified in ovarian cancer. Nevertheless, increased expression of E4f1 protein was found in myeloid leukemias (Hatchi et al., 2011) and hepatocarcinomas (Dai et al., 2014). Its role as a survival factor was confirmed in a mouse model of histiocytic sarcoma (a leukemia of macrophage origin) derived from Ink4α/Arf KO mice. In that tumor type, Cre-mediated deletion of E4f1 impaired mitochondrial function leading to increased production of reactive oxygen species (ROS) and autophagic cell death, delaying tumor growth ( Hatchi
et al., 2011). Its role in cell survival of cancer cells was also confirmed in p53-deficient transformed mouse fibroblasts derived from E4f1 cKO mice (Rodier et al., 2015). Furthermore, siRNA-mediated silencing of E4f1 in human hepatocyte carcinoma cells led to abnormal autophagy, mitochondrial defects and alteration of cell metabolism (increased lipid biosynthesis and decreased glutathione/glutamine metabolism). Despite the oxidative stress, these cells underwent cell cycle arrest rather than apoptosis (Dai et al., 2014), indicating that depending of the cancer type E4F1-deficiency leads to cell proliferation or cell death.

![Diagram](image.png)

**Figure 19:** Partners of E4F1 (green: tumor suppressors, pink: oncogenes). E4F1 is a target of different viral proteins such as E1A, HBX and GAM1.

### VII. REGULATION OF E4F1

Although E4F1 is involved in various cellular processes, not much is known about the signaling pathways and the conditions that regulate its expression. Initially, it was shown that E4F1 can be regulated by post-translational modifications such as phosphorylation (Raychaudhuri et al., 1989; Rooney et al., 1998) and sumoylation (Rizos et al., 2005).

Previous studies on cells infected by adenovirus suggested that phosphorylation is an important regulatory mechanism of E4F1. Consistent with this notion, the phosphorylation
of E4F1 was shown to vary during cell cycle (hypo-phosphorylated in G0 and G1) and suggested to control its association with chromatin (Fognani et al., 1993). However, the kinases that are involved in E4F1 phosphorylation still remain to be identified.

In addition to phosphorylation, E4F1 was also shown to be sumoylated following its association with ARF. ARF promoted sumoylation of MDM2, E2F1 and E4F1 by SUMO-1 (small ubiquitin-like proteins). In silico analysis identified a potential sumoylation site on K479 lysine residue of E4F1 (Rizos et al., 2005). The physiological consequence of E4F1 sumoylation is still not known. However, it may regulate its stability, its subcellular localization or its E3 ligase activity. This hypothesis is supported by the fact that Gam1 (an early adenoviral protein), an E4F1-binding protein, controls SUMO-1 sumoylation and E4F1 transcriptional activity (Colombo et al., 2003).

E4F1 level of expression can also be modulated in different patho-physiological conditions. For instance, E4F1 protein level, but not the mRNA level, is increased in acute myeloid leukemia (AML) (Hatchi et al., 2011), as well as in hepatocarcinoma (Dai et al., 2014) suggesting that E4F1 can be regulated by post-translational modifications in certain patho-physiological conditions. On the other hand, both protein and mRNA expression of E4F1 were shown to be upregulated in obese patients compared to lean individuals (Lacroix and Linares et al., Nature Communications, under revision) suggesting that expression of E4F1 can be modulated at the transcriptional level or by post-translational modifications depending on the context.
Chapter III- SKIN

I. FUNCTION

Skin is the essential barrier that protects organism from the environment by keeping the pathogens out and essential body fluids inside. It performs the following functions:

- Protection against UV radiation, sun damage, dehydration, microorganisms, physical damages.
- Sensation through somatic sensory receptors.
- Temperature regulation.
- Immunity (Microorganisms destruction).
- Excretion of waste products released from organisms.
- Endocrine function such as Vitamin D synthesis (Holbrook 1993).

Skin is composed of 3 main tissues: epidermis, dermis and hypodermis. The epidermis is responsible for the barrier function. The dermis contains appendages derived from the epidermis such as hair follicles, sebaceous glands and sweat glands and provides support and nutrition to the epidermis (Figure 20). Hypodermis functions as an energy supply for the skin due to the presence of adipocytes and blood vessels (Festa et al., 2011).

![Figure 20: Structure of the skin (Adapted from the website medicaldermagroup.com).](image-url)
II. DEVELOPMENT OF SKIN

Epidermis is derived from the neuroectoderm during embryogenesis. Epidermal cells first form a single layer of ectodermal cells just after gastrulation at E9.5 that stays until E12.5 in mice. This first layer of epidermis which is called periderm contains multipotent epithelial cells (Fuchs 2007; Blainpain and Fuchs 2009). Mesenchymal cells, which are derived from dermomyotome, colonize below the epidermal layer and this interaction stimulates the stratification of the epidermis. This also initiates hair follicle generation. Together with mesenchymal cells, the inner layer of the epidermis then produces the basement membrane that is rich in growth factors and extracellular matrix proteins and serves as a physiological border between epidermis and dermis. Stratification is completed around E17.5 and at this stage epidermis contains proliferative basal cells and terminally differentiated suprabasal keratinocytes (Blainpain and Fuchs 2009).

Several signaling pathways are implicated during these developmental stages. The neuroectoderm cells can follow two different fates and specialize in the nervous system or the epithelium. WNT signaling is a key determinant in this process. WNT avoids ectoderm to respond to FGF (fibroblast growth factor) signaling and FGF signaling suppresses BMP (bone morphogenetic protein) expression. This drives the specification of epidermal cells. In contrast, in absence of WNT, ectoderm expresses FGF, which blocks BMP signaling and therefore favors nervous system specification. The crosstalk between mesenchymal cells from the dermis and epithelium promotes hair placode formations, which are small invaginations towards dermis. Different studies on chick and mouse embryos demonstrated that FGF signaling and BMP-inhibitory signals from the mesenchymal cells are important for this process. Increased BMP or absence of BMP inhibitors decreases follicle density. In addition to these dermal signals, ectodermal WNT signaling also induces epidermal cell proliferation towards the dermis to generate hair bud together with Shh (Sonic hedgehog) signaling (Fuchs, 2007) (Figure 21).

![Figure 21: Schematic representation of skin development and the driver signaling pathways (Extracted from Fuchs 2007).](image-url)
III. STRUCTURE AND LAYERS

A. Epidermis

The epidermis is a stratified epithelium that covers the outer surface of the skin. It functions as a barrier between organisms and the microenvironment by protecting against exogenous insult (pathogens, UV light) and provides mechanical resistance. Epidermis is mainly composed of keratinocytes as the predominant cell type (90%). The different cell types located in the epidermis are discussed below.

i. Keratinocytes

Keratinocyte's name comes from the fibrous keratin protein they produce which is the key structural material to make up the outer surface of the skin. Molecular structure of keratin is similar to other cytoplasmic intermediate filaments. They contain a central coil-coil α-helical domain surrounded by non-α-helical N-terminal and C-terminal tail (Fuchs and Weber 1994). Keratins are expressed in a differentiation-dependent and site-specific manner (Alam et al., 2011). There are 2 different types of keratins: Type I keratins consist of acidic low molecular weight proteins whereas type II keratins include basic or neutral high molecular weight proteins. These two different types are assembled together to make cytoplasmic intermediate filaments. The different layers of epidermis express different keratin pairs. In human, there are 52 genes encoding functional keratins (Table of Keratin proteins). Only half of them are fully characterized and some of them are specifically found in hair and nails such as Hal-1 and Hbl-7.
<table>
<thead>
<tr>
<th>Type I (Acidic)</th>
<th>Type II (Basic)</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14</td>
<td>K5</td>
<td>Basal layer of stratified epidermis.</td>
</tr>
<tr>
<td>K15</td>
<td></td>
<td>Bulge keratinocytes (Hair follicle)</td>
</tr>
<tr>
<td></td>
<td>K6</td>
<td>Suprabasal keratinocytes, expressed during hyperproliferation.</td>
</tr>
<tr>
<td>K19</td>
<td>K10</td>
<td>Basal layer of epidermis</td>
</tr>
<tr>
<td>K1</td>
<td>K2E</td>
<td>Suprabasal keratinocytes.</td>
</tr>
<tr>
<td></td>
<td>K2E</td>
<td>Keratinocytes of spinous layer.</td>
</tr>
<tr>
<td>K9</td>
<td></td>
<td>Terminally differentiated keratinocytes.</td>
</tr>
<tr>
<td>K3</td>
<td>K12</td>
<td>Cornified layer keratinocytes.</td>
</tr>
<tr>
<td>K13</td>
<td>K4, K2P</td>
<td>Suprabasal keratinocytes, oral mucosa</td>
</tr>
<tr>
<td>K16, K17</td>
<td></td>
<td>Suprabasal keratinocytes, hyperproliferative epithelium.</td>
</tr>
<tr>
<td>K18, K20</td>
<td>K8</td>
<td>Epithelial tissues</td>
</tr>
<tr>
<td>Hal-1</td>
<td>Hbl-7</td>
<td>Hair/ nail (hard) keratins.</td>
</tr>
</tbody>
</table>

Table 2: Tissue and cell specific distribution of keratin proteins.

1. Stratification of epidermis

Epidermal stratification begins during embryonic development and continues throughout the post-natal life. The different layers of epidermis are described below (Figure 22).
Figure 22: Structure of epidermis (Adapted from Radtke and Raj 2003).

a) Basal Layer

The basal layer contains the cubic and cylindrical cells, which are connected to each other through desmosomes and to basal membrane through integrins. This compartment includes the proliferative cells, stem cells and progenitor cells. The keratinocytes located in this region undergo two different types of cell division: asymmetrical and symmetrical. They can either undergo symmetrical division where both daughter cells remain as stem cells in order to replenish stem cell pool or undergo asymmetrical division where one daughter cell remains as stem cell whereas the other one becomes a progenitor cell (Blainpain and Fuchs 2009). Epidermal progenitor cells have a high proliferation capacity and they are called "transit amplifying cells". These cells undergo a couple of rapid cell division before they enter the differentiation process and start migrating toward the upper layers.

b) Spinous Layer

In this layer, keratinocytes are attached to each other through desmosomes, which connects the keratin intermediates of neighboring cells. Keratinization starts in spinous layer to give the epidermis its rigidity. Because of shrinking of microfilaments, their desmosomes are visible as "prickles" under light microscope.
c) **Granular Layer**

Cells migrating from spinous layer become granular cells as they contain keratohyalin granules, which are filled with cysteine and histidine rich proteins. Filaggrin (filament aggregating protein) is important for the barrier function of the skin. It clusters to make a large protein structure known as profilaggrin which is found in keratohyalin structure. Together they form dense cytoplasmic granules, which stimulate aggregation of the cells by crosslinking the keratin fibers.

d) **Cornified Layer**

The cornified layer is the outermost layer of the epidermis and consists of terminally differentiated keratinocytes (corneocytes). The cells in this compartment do not contain any nucleus or organelles. These cells tightly adhere to each other and form cornified envelope that makes the skin rigid and water proof. Cornified envelope is made up of proteins (involucrin, loricrin, filaggrin, trichohyalin, small proline-rich proteins and many others) which are crosslinked by transglutaminases, joined by different lipids (ceramide, cholesterol and fatty acids) at the outside of the structure (Sandilands, Sutherland, Irvine, & McLean, 2009). In a healthy skin, skin renewal is maintained by the balance between proliferation of basal layer cells and desquamation of cornified layer. Desquamation is the process of shedding cornified cells to maintain the integrity of the epidermis. During desquamation, the corneodesmosomes are enzymatically broken down and the cells are shed from the surface. In the absence of water, skin cannot perform this function successfully and becomes rough and dry. In a normal skin homeostasis, there is a balance between corneocyte production and shedding. This process is important for the renewal of the epidermis and the renewal occurs every 2 to 4 weeks.

ii. **Merkel Cells**

Merkel cells are specialized sensory cells (mechanoreceptors) that respond to touch sensation. Although transcriptional profiling of these cells demonstrated that they express several neuronal lineage specific genes such as ion channel subunits and neuron-specific transcription factors, lineage tracing experiments proved that they have epithelial origin. Indeed, these cells are derived from Keratin 14 (K14) expressing epidermal stem cells which are located in the basal layer (Bardot et al., 2013; Morrison et al., 2009; Van Keymeulen et al., 2009; Woo et al., 2010).

iii. **Langerhans Cells**

Langerhans cells are present in all the layers of the epidermis except cornified layer and their function is to protect skin against infections. They are dendritic cells which make a continuous network to sense the foreign antigens that enter the skin. They are antigen
presenting cells which make the skin the first immunological barrier against infection (Merad et al., 2008).

iv. Melanocytes

Melanocytes are the pigment producing cells of the skin. Their primary function is to produce melanin pigments in response to UV exposure. They transfer the melanin to keratinocytes through their dendrites in order to protect the skin from DNA damage. Melanocytes will be discussed in detail at the end of the Skin Chapter.

B. Basement Membrane

The basement membrane is a dermoepidermal junction between the epidermis and the dermis. It is composed of extracellular matrix proteins such as laminin, type IV collagen, nidogen and perlecan (Yurchenco et al., 2010). Basal keratinocytes are attached to the basal membrane by hemidesmosomes and by focal adhesions. Hemidesmosomes include α6β4 integrin transmembrane core, which is connected to intracellular keratin filaments whereas focal adhesions contain α3β1 integrins connected to actin and tubulin network in keratinocytes. Both junctions bind to laminin V which is one of the core components of the basal membrane (Fuchs, 2007). The function of basement membrane is to provide skin resistance against shearing forces and maintain skin integrity by attaching epidermis to dermis. Mutations in genes encoding components of this basement membrane (focal adhesion and hemidesmosome transmembrane components) are associated with skin disorders characterized by decreased adhesion of skin layers, skin fragility and mechanically induced blisters. These disorders are called epidermolysis bullosa (EB)(Bruckner-Tuderman and Has 2014).

C. Dermis

The dermis is the layer between the epidermis and the underlying hypodermis. Its main function is to provide support and nutrients to the epidermis. It contains vascular and lymphatic vessels, nerves, and sensory neural receptors for touch, pressure and temperature. The dermis also contains macrophages and mast cells. It consists of fibroblasts as the main cell type that produces collagen, elastin and proteoglycans. The collagen fibers represent 70% of the dermis and give the dermis its strength and toughness. Elastin gives elasticity and proteoglycans provides hydration and viscosity.

The dermis is divided into two parts: a thin papillary dermis and a thicker reticular dermis. The uppermost layer, so-called papillary dermis, is connected to the epidermis and it contains thin and loosely organized collagen fibers, consisting of type I and mostly type III collagens. The function of this layer is to supply nutrient to the epidermis due to the presence of blood vessels and to regulate temperature. The reticular layer is the underlying
part of the dermis. It is made of tightly organized large collagen fibers, which contains type I and type III collagens. It gives the skin its toughness and elasticity.

**D. Hypodermis**

The hypodermis is the lowermost and thickest layer of the skin. It is attached to the dermis by collagen and elastin fibers. The hypodermis is mainly composed of adipocytes, which store fats to provide an energetic source for the skin. Besides, adipocytes help the thermoregulation of skin. Importantly, it was demonstrated that intradermal adipocytes contribute to skin homeostasis through activation of hair follicle stem cells. The mouse models that lack adipocyte progenitors led to major defects in hair follicle regeneration (See epidermal metabolism) (Festa et al., 2011).

**IV. SKIN TURNOVER CYCLE**

**A. Differentiation of keratinocytes**

The differentiation of keratinocytes is related to the stratification of the epidermis. Each layer of the epidermis is associated with a differentiation step and expression of specific keratin proteins. Proliferating keratinocytes express keratin 14 (K14) and keratin 5 (K5) proteins. These proteins expand from the desmosomes to the nuclear lamina together with microtubules and microfilaments to make up the cytoskeleton of epidermal cells. Keratinocytes start differentiating and change morphology as they move up in the epidermis from one layer to another. In the spinous layer, the keratinocytes express keratin 1 (K1) and keratin 10 (K10) and these proteins replace the K5 and K14 intermediate filaments. Spinous layer contains several layers of prickle cells, which forms tightly bundled microfilaments. In the granular layer, cells produce keratohyalin granules that contain filaggrin, which bundles keratin filaments. This is responsible for the flattened shape of these cells, which are sealed together to make the skin waterproof. In the cornified layer, both filaggrins and keratin intermediate filaments constitute the cornified envelope, an insoluble protein structure that functions as a barrier to protect the organism from its environment. In addition to filaggrins and keratin intermediate filaments, several lipids such as cholesterol and ceramide also join the formation of cornified envelope to complete the barrier. Thus, the cornified layer is made up of flattened cells known as corneocytes. These cells are connected together by corneodesmosomes that are modified desmosome structures. (These structures are are degraded during the desquamation process). The other proteins such as involucrin, loricrin and profilaggrin also participate to the formation of the cornified envelope (Candi et al., 2005).
V. DERIVATIVE STRUCTURES OF SKIN

B. Hair follicle

Hair follicle is the structure that produces hair. The hair keeps the organism dry, warm and protects it from exogenous insult. Hair follicle is under constant renewal during the lifetime of the organisms.

v. Structure

The hair root is the part of the hair follicle under the skin. It consists of 3 parts:

• The dermal papilla is the connective tissue that gathers different types of cells (keratinocytes, melanocytes, fibroblasts). Blood vessels provide nutrients to hair follicle through this dermal papilla.

• The germinal matrix covers dermal papilla. It contains epithelial cells and melanocytes, which give the hair its color.

• The bulb is the bottom of the hair follicle and it contains actively dividing cells to regenerate the hair follicle (Figure 23).

Figure 23: Structure of hair follicle and hair shaft (Adapted from Albert’s molecular biology).
The hair shaft is the visible part of the hair follicle above the epidermis. It is made up of 3 layers: the cuticle is the outermost layer that protects the hair, the medulla is in the middle and the cortex is the innermost layer that is responsible for appearance of the hair (Figure 23). The part of the hair shaft, which is under the skin is surrounded by a channel called the inner root sheath and it supports hair shaft during differentiation.

The arrector pili muscle is a tiny smooth muscle that is connected to the base of the hair follicle on one side and to the dermis on the other side. It is contracted in response to different stress stimuli and causes the erection of hair, an effect known as "goose bump".

The lower permanent part of the hair follicle is called bulge and it is the reservoir for hair follicle stem cells. This compartment contains 3 groups of cells: the stem cells, the transit amplifying cells and the mature cells. Both keratinocyte and melanocyte stem cells are located in the bulge and allow hair renewal. These stem cells have a low proliferation capacity in contrast to transit amplifying cells, which are engaged progenitors derived from the bulge stem cells that undergo multiple rounds of cell division before becoming terminally differentiated (Figure 24). Both transit amplifying cells and mature cells are found in the hair matrix. To renew the existing follicles, hair follicles undergo multiple cycles of anagen (growth), catagen (regression) and telogen (resting).

Figure 24: Follicular stem cells (Adapted from Fuchs, 2007).
vi. **Hair follicle cycle**

2. **Anagen**

Anagen is the growth phase of the hair follicle cycle. During this phase, the stem cells of the bulge start migrating towards the bulb, where TAC rapidly proliferate. Then, these cells terminally differentiate and push one another upwards forming the hair shaft itself (Figure 24). These cells extrude their organelles and become tightly assembled through cysteine-rich hair keratins, which give the hair its strength and flexibility. The inner root sheath also undergoes keratinization to support the hair shaft during differentiation. As the proliferation rate decreases in the bulb of the follicle keratinocytes undergo apoptosis, differentiation of the hair shaft slows down and follicle enters into catagen phase.

Interplay of multiple signaling pathways between epidermal and mesenchymal cells are required to induce anagen phase and hair follicle differentiation. Briefly, WNT signaling is a key pathway in hair follicle regeneration that initiates the anagen phase. Notch signaling is important for stem cell fate and it directs bulge stem cells towards a follicular fate. BMP signaling is required for differentiation and Shh controls hair follicle morphogenesis as well as late differentiation stages of follicular cells (Rishikaysh *et al.*, 2014).

3. **Catagen**

This phase is the regression step of the hair follicle. Epithelial cells and melanocytes in the bulb undergo apoptosis. The hair shafts shrink and disconnect from its blood supply, the hair bulb move upwards detaching from the dermal papilla (Figure 25). Finally, the hair shaft is completely shed from the follicle. Catagen is the shortest phase of hair cycle. The first catagen phase in mice is seen 2 weeks after birth and it takes 3-4 days.

Several different actors were shown to promote catagen phase. Epidermal growth factor receptor (EGFR) is important for catagen entry. In nude mice engrafted with epidermal growth factor receptor (EGFR) null hair follicles, the follicles fail to proceed from anagen to telegon phase (Hansen *et al.*, 1997). Another study showed that *Fgf5*−/− (Fibroblast growth factor 5) null mice exhibit abnormal long hair, suggesting a defect in anagen to catagen phase switch (Hebert *et al.*, 1994). Finally, it was demonstrated that TGF-β1 (Transforming growth factor β1) signaling which is an inhibitor of keratinocyte proliferation is important for hair follicle regression. *Tgf-β1*−/− mice had a delay in hair follicle cycle compared to their WT littermates (early anagen observed in KO whereas WT littermates were already in telogen) and forced expression of TGF-β1 induced premature entry into catagen phase (Foitzik *et al.*, 2000).
4. Telogen

After hair shaft is shed, hair follicles undergo a resting phase to prepare for the next round of hair cycle. The first telogen is quite short in mice and takes 1 to 2 days around P19 to P20. The second telogen lasts more than 2 weeks and it is seen around P42 (Fuchs, 2007).

Figure 25: Hair follicle cycle (Adapted from Fuchs, 2007).
C. Sebaceous gland

These are oil secreting glands of the skin. They are located in the dermis and develop from epithelial cells of hair follicle (from the external root sheath). Sebaceous glands secrete a substance called sebum composed of triglycerides, wax esters, squalene and free fatty acids. Sebum is lubricant and it moisturizes skin by avoiding water evaporation. It prevents drying of the hair. Sebaceous glands also contain some chemicals such as acid that prevent bacterial infection.

D. Sweat glands

These are exogenous glands located in the dermis. They are made up of coiled tubes which secrete “sweat” to regulate temperature and remove the waste off the skin.

VI. EPIDERMAL STEM CELLS

Skin is under constant renewal throughout the lifetime of organisms. This regeneration of this tissue is enabled due to the presence of epidermal stem cells. Stem cell pool remains constant during normal tissue homeostasis. This is mainly explained by the capacity of these stem cells to perform asymmetrical cell division (Figure 26). In mouse, there are two types of asymmetrical cell divisions seen in the epidermis (Blainpain and Fuchs 2009):

- The first type is mostly seen in the mouse skin during embryonic development where stem cell divides positioning its mitotic spindle perpendicular to the basement membrane. In this case the daughter cell in contact with the basement membrane stays as a stem cell whereas the one above moves towards suprabasal layer and starts to terminally differentiate.

- The second type of asymmetrical division is mainly observed in adult tail epidermis (Clayton et al., 2007). Even though the cell divides positioning its mitotic spindle parallel to basement membrane and therefore both daughter cells stay in contact with it, one of them starts differentiating. This is mainly due to differential signaling that plays a role on the destiny of this cell. Integrins are downregulated in this cell and subsequently detaches from the niche moving upwards to differentiate.

Another division that stem cells undergo is symmetrical division in which the daughter cells follow the same fate. This gives rise to two stem cells to replenish the stem cell pool (Blainpain and Fuchs 2009) (Figure 26).
Figure 26: Assymetric and symmetric cell division in epidermis (Extracted from Blainpain and Fuchs, 2009).

A. Interfollicular (IFE) stem cells

These stem cells are responsible for the constant renewal of the epidermis. They function in normal epidermal homeostasis (Hall and Watt 1989) as well as during wound healing process together with follicular stem cells. They are located in the basal layer of epidermis surrounded by transit amplifying cells forming an "epidermal proliferative unit" (Potten and Morris 1988) (Figure 27).

Figure 27: Interfollicular stem cells located in the epidermis.

The initial identification of these cells was possible due to their slow proliferation property, which permitted the retention of nucleotide analogs such as BrdU or EdU during pulse-chase experiments (Bickenbach 1981; Mackenzie and Bickenbach 1985). These cells
are so-called “label retaining cells” since they can retain these nucleotide analogs for a long period of time which allows to follow them in vivo.

The organization of the stem cell niche surrounded by TAC (transit amplifying cells) has been proposed to maintain the “stemness” of stem cells by protecting them from differentiation stimulus. Indeed, whole mount imaging and lineage tracing experiments demonstrated that epidermal stem cells are present at the regions where dermis comes close to the epidermis and they are surrounded by transit amplifying cells (Estrach et al., 2007; Jensen et al., 1999).

B. Bulge Stem Cells (Follicular Stem Cells)

Follicular stem cells have been more characterized than the IFE stem cells, because of the identification of specific markers owing to their easily identifiable and distinct localization. Indeed, follicular stem cells reside in the lower permanent portion of hair follicle called “bulge” in a quiescent state. They are activated during each hair cycle in order to regenerate hair (Taylor and Lehrer 2000) but importantly, they also participate to regeneration of interfollicular epidermis during wound healing process (Oshima and Rochat 2001). Bulge stem cells are slow proliferating cells and based on this property BrdU/EdU incorporation approach was also used to chase them in long term (Cotsarelis et al., 1990).

A number of different studies were performed including in vitro clonogenic growth assays following the isolation of the follicular cells based on their surface markers. These analyses also helped making a distinction between stem cells and transit amplifying cells according to their clonogenic potential, with stem cells having a higher proliferative potential. Importantly, these studies enabled the identification of some of the inter-follicular stem cell markers (Jones and Watt, 1993; Jones et al., 1995; Li et al., 1998; Kaur and Li, 2000; Tani et al., 2000; Fortunel et al., 2003; Legg et al., 2003; Wan et al., 2003). For instance, high expression of the stem cell markers β-integrins and EGF receptor antagonist Lrig1 is correlated with high proliferation capacity in vitro clonogenic assays (Jensen and Watt 2006; Jones and Watt 1993; Jones et al., 1995; Jones et al., 2007). Since then, several different markers were identified for these cells such as keratin 15 (K15) and CD34 which is a cell surface protein important in cell-cell adhesion (Lyle et al., 1998; Morris et al., 2004; Trempus et al., 2003).

Several distinct populations of follicular stem cells were identified in the hair follicle (Figure 28):

- The cells that express CD34 are divided into 2 populations according the integrin α6 expression. The first stem cell population exhibit high integrin α6 expression whereas the other CD34-positive population exhibit low α6 level (Blainpain et al., 2004).
• A third population was also identified located below the bulge and expressing both CD34 and leucine-rich repeat containing G-protein coupled receptor 5 (Lgr5) (Jaks et al., 2008). This population contains actively dividing cells that can reconstitute all epidermal lineages.

• Another population that expresses the epitope MTS24 that was first identified in thymic epithelial progenitor cells (Depreter et al., 2005). MTS24 can bind to membrane-bound antigen which is present during early hair follicle development. This MTS24-positive stem cell population expresses α6 integrin but not Keratin 15 or CD34 (Jensen et al., 2009; Nijhof et al., 2006). They have clonogenic potential in culture like other stem cells however they haven’t yet been tested in reconstitution assays.

• Finally, a population was identified between the infundibulum and the bulge (Jensen et al., 2008), which express MTS24, low integrin α6, but not CD34. They are actively dividing cells like the Lgr5+ stem cell population and they can reconstitute all epidermal lineages (hair follicle, interfollicular epidermis and sebaceous gland) (Jensen et al., 2008; Jensen et al., 2009).

Figure 28: Distribution of the different follicular stem cell pools.

VII. MECHANISMS OF SKIN DIFFERENTIATION

Genetically engineered mouse models allowed the identification of different signaling pathways and transcriptional factors required for proper skin development and
stratification of the epidermis. These pathways include Notch, nuclear factor-kB (NF-κB), mitogen-activated protein kinase (MAPK) and transcriptional regulators AP2 family, CCAAT/enhancer-binding protein (C/EBP), interferon regulatory factor 6 (IRF6) and Kruppel-like factor 4 (KLF4).

Strikingly, p63 (a member of the p53 family) is believed to be a master regulator of stratification process (Blainpain and Fuchs 2007). It is expressed in all layers of the epidermis and in the absence of p63, mouse epidermis fail to stratify suggesting that p63 is required to initiate this stratification process and/or to maintain stem cell renewal (Mills et al., 1999; Yang et al., 1999; Truong et al., 2006).

Furthermore, canonical Notch signaling pathway is also important for basal cells to engage into differentiation. Conditional inactivation of RBPJ, which is the effector of Notch signaling, inhibits keratinocytes differentiation (Blainpain et al., 2006). Consistently, loss of Hes1 another downstream target of Notch altered the fate of spinous cells (Moriyama et al., 2008).

Wnt/β-Catenin signaling is important for hair follicle renewal. β-catenin is an adhesion molecule that drives Wnt signaling from the membrane to the nucleus. In quiescent bulge stem cells, β-catenin expression is very low or absent. β-Catenin stabilization in these quiescent bulge stem cells is sufficient to induce hair growth (DasGupta et al., 1999). In addition, Wnt/β-catenin level is increased in later stages of differentiation in the matrix cells. This high expression level allows a transcriptional reprogramming that leads to differentiation of the hair shaft (Merrill et al., 2001). However, β-catenin expression alone is not sufficient for quiescent cells to become terminally differentiated and other signals are required as well (Fuchs, 2007).

In contrast to Wnt/β-Catenin, BMP signaling is important for stem cells to stay in quiescent state. The balance between BMP and its inhibitors (i.e noggin) is necessary for hair follicle morphogenesis. Extensive analysis in adult mice showed that BMP is expressed in bulge stem cells and is down regulated during telogen phase in order to activate the stem cells for the entry in growth phase (Blainpain et al., 2004; Kobielsak et al., 2007). Once the stem cell is activated, several downstream signals are necessary such as Shh (Silva-Vargas et al., 2005) the proliferative phase and Notch for early steps of differentiation. BMP is also required for the differentiation of hair follicle. For instance, through the transcription factor GATA3, it induces matrix cells to join hair shaft formation in contrast to Wnt signaling that favors the inner rooth sheath formation through lymphoid enhancer-binding factor 1 (LEF1) (Figure 29) (Kobielsak et al., 2003; Andl et al., 2004).

Other factors are involved in skin differentiation such as pH and Calcium. For instance, there is a pH gradient in the epidermis with the microenvironment becoming more acidic from the basal layer to the suprabasal layers. This acidification is essential for several
processes in the skin: it helps protecting skin from pathogens, it keeps it hydrated and it is implicated in activation of some proteases in the cornified layer during the desquamation process (Krien et al., 2000; Brattsand et al., 2005). Calcium is required for proper keratinocyte differentiation. There is a calcium gradient in the epidermis. Concentration of calcium increases towards suprabasal layers and it is required for activation of Protein Kinase C (PKC) signaling pathway, which will initiate a transcriptional program involved in differentiation (Yuspa et al., 1993). Consistently, keratinocytes culture in presence of very low concentration of calcium keep their characteristics of basal cells ex vivo. Increasing concentration of calcium more than 0.1 mM induces the redistribution of desmosomes from the cytoplasm to the membrane promoting the cell-cell contact and differentiation (Braga et al., 1995).

![Diagram](image-url)

**Figure 29:** Signaling pathways involved in hair follicle regeneration and differentiation (Extracted from Fuchs, 2007).

VIII. **EPIDERMAL CELL METABOLISM**

Different studies have demonstrated that carbohydrate, lipid and mitochondrial metabolism contribute to proper skin homeostasis. As an illustration of this notion, it was demonstrated that free fatty acids (FFA) like triacylglycerols join the structure of the cornified layer to maintain the permeability and barrier function of the skin (Radner et al., 2000).
The importance of such lipids was further supported by mouse model lacking fatty acids transport protein *Fatp4*. These mice died soon after birth and displayed several skin defects such as hyperproliferation and hyperkeratosis of keratinocytes, disruption of skin barrier function, basement membrane defects as well as altered composition of free fatty acids in the epidermis (abnormal ceramide and lipid level) (Herrmann et al., 2003). Another study illustrated the close link between adipose tissue and skin homeostasis. Ebf1 (Early B cell factor-1) KO mouse which lacks adipocyte progenitors showed abnormal skin homeostasis (hyperplasia, absence of hair and dysfunction of sebaceous glands) (Festa et al., 2011) suggesting that immature adipocytes are necessary for proper skin homeostasis. This tight connection was further demonstrated with studies showing that inactivation of *DGAT1* (diglyceride acyltransferase 1) and *DGAT2* (diglyceride acyltransferase 2), which catalyzes the formation of triglycerides from Acyl CoA and diacylglycerol, led to severe lipid metabolism defects in adipocytes together with different skin-related alterations such as loss of hair follicles and defective basement membrane (Chen et al., 2002; Stone et al., 2004). Finally, patients suffering from lichen planopilaris display alopecia due to the loss of follicular stem cells with decreased expression of numerous genes implicated in lipid metabolism such as peroxisome proliferator activated receptor γ (PPARγ) (Karnik et al., 2009).

In addition to lipid metabolism, carbohydrate metabolism is also implicated in skin homeostasis. It was shown that altered glucose metabolism can lead to skin homeostasis defects. Consistent with this notion, diabetic rats exhibited dermatological problems related to collagen disturbance (atrophy and degeneration of fibers), altered wound healing and thickening of epidermis. This was due to impaired balance between matrix metalloproteases (MMPs) and their inhibitors TIMPs. In diabetic rats, TIMP activity increased and MMP activity decreased resulting in extracellular matrix defect (Knas et al., 2013).

Lastly, it was shown that mitochondrial activity also plays an important role in skin homeostasis. *Tfam* (Mitochondrial transcription factor A) encodes a transcription factor that is implicated in activation of mitochondrial genes. In mice, *Tfam* inactivation in basal layer of epidermis resulted in complete absence of mitochondrial respiratory chains in basal cells. These *Tfam* KO mice exhibited altered epidermal differentiation and hair follicle regeneration defects and died 2 weeks after birth. This was due to a lack of reactive oxygen species normally produce by mitochondria and required for epidermal differentiation (Hamanaka et al., 2013).

Besides, another mouse model that lacks Prohibitin-2, which is a structural protein in mitochondrial inner membrane, was generated to understand the effect of mitochondrial activity in skin regardless of oxidative phosphorylation. These mice exhibited severe skin defects and died one hour after birth due to dehydration. All together these results strongly
suggest that mitochondrial activity and/or mitochondrial integrity is essential for normal skin homeostasis (Baris et al., 2011).
IX. MELANOCYTES

Melanocytes constitute a rare cell population in the skin. They produce melanin pigment in response to UV exposure to protect the skin cells from the DNA damage.

A. Origin of melanocytes

Melanocytes are derived from neural crest cells (NCC). NCC are pluriopotent cells which differentiate into neural cells, endocrine cells and melanocytes. NCC arise from dorsal neural ectoderm. They undergo epithelial to mesenchymal transition and gain migratory capacity during embryonic development. After reaching their final destination, they can give rise to number of different cell types. The migration of neural crest cells occurs in two waves. The first one is a dorso ventral migration along the neural tube in the anterior part of the somite and these cells give rise to neural and ganglionic cells (Dupin and Le Douarin 2003). The second wave of migration is dorso lateral between the outer ectoderm and the dermomyotome (dorsal part of the somite under the ectoderm). These cells give rise to melanoblasts which are melanocyte precursors (Dupin and Le Douarin 2003). In mice, melanoblasts are still found in the neural crest around £10.5 and then they migrate and proliferate to reach the limb buds at £12.5. Around £13.5 and £14.5, they colonize the epidermis (Mayer 1973).

B. Localization

In human, melanocytes are present in ear, eye and heart (Goding, 2000; Tachibana, 1999; Yajima and Larue, 2008). Within the epidermis, they are located in the basal layer where they interact with the keratinocytes through their dendritic extensions (Figure 30). This melanocyte-keratinocyte interaction ensures the melanin production in melanocytes. In the hair follicle, melanoblasts migrate and generate two different populations. The first population is the hair bulb melanocytes, which will differentiate into mature melanocytes to produce melanin and gives the hair its natural color. The second population migrates towards hair follicle bulge to constitute the melanocyte stem cell (MSC) pool.

In mice, melanocytes rapidly disappear from the inter-follicular epidermis after birth and are only present in the hair follicle of mouse dorsal skin. In this case, the melanocyte homeostasis is controlled by the hair follicle. Importantly, mouse tail skin is more similar to human skin because it contains melanocytes in both inter-follicular epidermis and in the hair follicle (Aoki et al., 2009).
C. Melanogenesis

The function of a melanocyte is to produce pigment which gives the skin, hair and eye their color. Melanocytes produce melanin in specialized lysosome-like organelles called “melanosome”. Two different types of melanin can be synthesized: pheomelanin a reddish yellow pigment and eumelanin a brownish black pigment. The amount of pheomelanin and eumelanin will determine the color of human skin and hair. Interestingly, cultured melanocytes produce more pheomelanin while the opposite is observed in vivo (Hunt et al., 1995).

Melanogenesis happens through a chain of reactions called the Raper-Mason pathway. Melanin is produced from the phenolic amino acid precursor L-tyrosine in vertebrates. It derives from the essential amino acid phenylalanine in the cytosol. The enzyme phenylalanine hydroxylase (PAH) catalyzes the hydroxylation of the aromatic side chain of phenylalanine to generate tyrosine. The rate limiting key enzyme of melanogenesis is Tyrosinase (Tyr), an oxidase copper-containing enzyme found on the membrane of melanosomes that catalyzes different reactions in the melanin synthesis pathway. Tyrosinase contains an internal, transmembrane and cytoplasmic domain. The cytoplasmic domain is important for the transport from the nucleus to the melanosomes. The internal region consists of the 90% of the protein including the catalytic region. Copper ion binds to histidine residues and induces the activation of the enzyme. It is also activated by electron donors such as L-DOPA. Melanogenesis starts with L-Tyrosine hydroxylation to L-3,4-dihydroxyphenylalanine (DOPA) by Tyrosinase. Then, DOPA is rapidly oxidized to DOPA quinone again by Tyrosinase (Fitzpatrick et al., 1967). At this stage, the availability of the substrate determines which type of melanin will be produced. In the presence of cysteine,
3- or 5-cysteinyldOPAs is produced from DOPAquinone which is then oxidized and polymerized to generate pheomelanin (Hearing 2011). In the absence of cysteine, DOPAquinone is converted to DOPAchrome and DOPAchrome loses its carboxyl group forming 5,6-dihydroxyindole (DHI). DHI is then oxidized and polymerized to generate dark-brown-black DHI-melanin. However, in the presence of DOPAchrome tautomerase enzyme (DCT) (also known as Tyrosine-related protein-2, TYRP2), DOPAchrome will be converted to DHI-2-carboxylic acid (DHICA) (Del Marmol et al., 1996). Then, Tyrosinase and another melanogenesis enzyme Tyrosine-related protein 1 (TYRP1) catalyzes the last steps of melanogenesis to generate a light brown colored DHICA-melanin (Figure 31) (Slominski et al., 2004; Simon et al., 2009).

Figure 31: Eumelanin and pheomelanin synthesis in melanosomes.

The assembly and maturation of melanosomes are tightly organized and coupled to melanin synthesis. Stage I is the premelanosome stage where melanosomes contain small rounded vesicles at the periphery of the nuclear. Stage I to II transition is achieved by elongation of these small vesicles and formation of organized and structured fibrillar matrix. The formation of these fibrils depends on the presence of PMEL17 glycoprotein, also known as GP100 and SILV. At this stage, Tyrosinase is present but found in its inactive form. Melanin production starts at Stage III and the pigment is deposited on fibrils, resulting in gradually pigmented internal matrix. Finally, at the stage IV, melanosomes are completely melanized, tyrosinase is inactivated and melanin is ready to be phagocyted by
keratinocytes (Hearing 2011; Schiaffino et al., 2010). Of note, the melanosomes of eumelanin and pheomelanin exhibit different morphologies and pheomelanin melanosomes do not contain any internal fibril structure.

**D. REGULATION OF MELANogenesis**

Pigmentation is regulated by more than 125 different genes (Bennett et al., 2003). These genes drive the essential functions of melanoblasts such as differentiation, survival and development as well as melanosome formation and pigmentation. Some of these main regulators are discussed below.

i. **Mitf**

Mitf (Microphthalmia-associated transcription factor) is a transcription factor of bHLH-Zip (basic helix-loop-helix leucin zipper) family which contains DNA binding and dimerization motif. It is the major determinant of melanocyte development (Goding 2000, Steingrimsson 2004). Mitf regulates the expression of numerous genes that are important for melanocyte proliferation, differentiation, survival and pigment production. Due to these indispensable functions, Mitf is termed as “master regulator” of melanocyte lineage. MITF and two other related members, transcription factor E3 (TFE3) and transcription factor EB (TFEB) constitute the “Mit” family of transcription factors. In combination, they regulate gene expression through homo and hetero dimerization and binding to E-box motifs which contains hexanucleotide sequence CA[C/T]GTG (Hemesath et al., 1994).

Mitf contains 9 promoter-exon units which control its expression. The M promoter is specifically used in melanocytes and is targeted by several transcription factors such as Sox10, Sox9, CREB, Pax3 and even Mitf itself (Levy et al., 2006; Passeron et al., 2007). Mitf is also regulated through post-translational modifications. MAPK, Ribosomal S6 Kinase, Glycogen Synthase Kinase 3β (GSK3β) and p38 phosphorylate Mitf and trigger its transcriptional activity in response to different stimuli (See signaling pathways in melanogenesis). Genome wide approaches of gene expression and MITF binding regions in primary human melanocytes as well as melanoma cell lines enabled the identification of MITF target genes. These genes are enriched in pigmentation, DNA replication, mitosis and metabolism. MITF can induce pigmentation through transcriptional activation of melanogenesis genes. **TYROSINASE** gene contains a specific sequence (CATGTG) on its promoter, which is called TDE (Tyrosinase Distal Element). MITF specifically binds to this region located on the -1861 to -1842 positions and activates the transcription of the **TYROSINASE** gene (Yasumoto 1987). **TYRP1** and **TYRP2** also contain this TDE and regulated by MITF. Besides, MITF activates the expression of other key regulators of pigmentation such as **PMEL17/GP100/SILV** and **MART1/MLANA**. The products of these genes are required for melanosome maturation by forming structural fibrils. In addition to its functions in melanogenesis, MITF was reported to regulate the activity of cell cycle progression genes.
such as CDK2, of stress response genes and apoptotic genes such as BCL2 (McGill et al., 2002; Du et al., 2004). Thus, Mitf regulates a wide range of genes implicated in proliferation, differentiation and apoptosis in melanocyte lineage (Figure 32). Furthermore, Mitf acts in combination with other co-regulators and chromatin remodeling complexes to regulate gene expression. It was demonstrated that MITF recruits p300 and CBP histone acetyltransferases to activate gene expression (Sato et al., 1997; Price et al., 1998). MITF was reported to require SWI/SNF complexes to regulate gene expression in melanoma cell lines. SWI/SNF (SWItch/ Sucrose Non-Fermentable) is chromatin remodeling multi-protein complexes consisting of ATPases (BRG1 or BRG2) and associated factors BAFs. Mitf was shown to interact with BRG1 to regulate pigmentation genes such as TYR in melanoma cell line (de la Serna et al., 2006; Keenen et al., 2010).

![Figure 32: Target genes and functions of MITF in melanocyte lineage (Extracted from Kawakami and Fischer 2016).](image)

In mice, mutations in Mitf results in skin depigmentation, deafness, bone hyperdensity and absence of pigments in eyes. The role of Mitf in melanocyte development was also confirmed by generating Mitf KO mice. Mitf null mice exhibit loss of neural crest derived melanocytes resulting in deafness and retinal pigment epithelium differentiation defect (Moore 1995). Several mutations have been identified in MITF gene in human, some of which are related to the Waardenburg type-2 syndrome. This syndrome is associated with pigmentation abnormalities in skin, hair and eye confirming the importance of Mitf in pigmentation (Tassabehji et al., 1994). Mutations in Pax3 and Sox10 which, are upstream transcription regulators of MITF were reported to be also associated with Waardenburg type-3 and 4 respectively.

ii. α-Melanocyte Stimulating Hormone (α-MSH)

Hormonal signaling is involved in intrinsic and extrinsic (UV-induced) pigmentation. In keratinocytes and melanocytes, UVB exposure induces p53 which upregulates Pomc (Pro-opiomelanocortin) gene. POMC is then cleaved and give rise to peptide hormones such as α-MSH (α-Melanocyte-Stimulating Hormone), ACTH (Adrenocorticotropic hormone) and β-
endorphin. α-MSH binds to MC1R (Melanocortin 1 receptor) receptor in melanocytes to initiate melanogenesis (See Signaling pathways).

iii. **Wnt**

*Wnt* genes are a family of secreted proteins which bind to their receptor Frizzled family. This induces the translocation of β-Catenin into the nucleus and activation of its transcriptional co-activator. β-Catenin together with other transcription factors initiates gene expression implicated in cell fate, development and proliferation. Wnt1 and Wnt3 are expressed in dorsal neural tube and were shown to stimulate neural crest cells to differentiate into melanocytes. As an illustration of this notion, the mice embryos deficient for Wnt1 and Wnt3 lack Dct-positive neural crest derived melanoblasts (Ikeya et al., 1997).

The effect of Wnt signaling was further analyzed in neural crest-derived Dct-positive mouse melanoblasts. Overexpression of Wnt3a or β-catenin in those cells increased the number of melanoblasts in culture. In contrast, these cells did not respond to Wnt1 expression in the same manner and instead, Wnt1 increases the paracrine factor secretion on melanoblast precursors to increase the number of neural crest cells to become melanocytes (Dunn et al., 2005).

iv. **Protein Kinase C**

PKC families of proteins are encoded by 9 different genes split into 3 groups based on their mechanism of actions. They can be activated by Calcium or Diacylglycerol (DAG), or both (Gordon et al., 1989). Melanin synthesis depends more on the activity of Tyrosinase rather than the amount of the enzyme. The activity of Tyrosinase is controlled by phosphorylations on its cytoplasmic domain on Ser505 and Ser509 residues. During UV exposure, PKCB (Protein Kinase C-beta), which is a member of the PKC family translocates from the cytoplasm to the membrane in order to phosphorylate and activate tyrosinase enzyme (Park et al., 2006).

v. **Sox family transcription factors**

The family of Sox proteins consists of 20 transcription factors with SRY high mobility box domains required for the binding activities on specific DNA sequence. There are divided in 9 groups in mammals (SoxA, B1, B2 and C to H). SoxE group contains Sox9 and Sox10 that are essential for melanocyte development. Sox8, Sox9 and Sox10 are expressed in dorsal neural tube and neural crest of most species and are important for cellular specialization and differentiation. During development, Sox10 controls Mitf expression, which activates the melanogenesis genes *Tyr, Tyrp1, Dct* and *Pmel*. Sox10 KO mouse lacks melanocytes. Complementation with Mitf in Sox10-deficient melanocyte precursors partially rescues the survival of melanoblast together with the expression of several melanocytic genes, but not
**Tyrosinase.** Thus, both Sox10 and Mitf are required for proper Tyrosinase expression (Hou et al., 2006).

Sox proteins are not only required for melanocyte development but are also important for melanocyte homeostasis during post-natal life. During melanocyte differentiation, Sox9 expression increases where Sox10 is downregulated. Ectopic expression of Sox9 is sufficient to promote melanocyte differentiation. Sox9 is also involved in melanin synthesis pathway upon UVB exposure. Sox9 does not contain any UV response element itself however it works downstream of the α-MSH/MCR1/cAMP signaling pathway. cAMP-dependent PKA upregulates Sox9, which together with CREB activate Mitf expression that initiates the expression of Tyrp2 and Tyr (Passeron et al., 2007). Sox10 has also important roles in survival and differentiation of adult melanocytes. Both Melanocyte Stem Cells (MSC) and mature melanocytes express Sox10. Inactivation of Sox10 in mouse results in the loss of both stem cells and differentiated melanocytes. Conversely, overexpression of Sox10 leads to premature differentiation and loss of melanocyte stem cells resulting in hair greying. Altogether these findings prove that level of Sox10 is an essential determinant of the maintenance of proper function of both differentiated melanocytes and MSC (Harris et al., 2013).

vi. **Pax3**

**Pax3** belongs to paired box family of genes and is important for development of neural crest cells and its derivatives including melanoblasts (Tassavehji et al., 1992). **Pax3** is expressed in melanoblasts, mature melanocytes and nevi as well as in primary and metastatic melanoma. Sox10 and Pax3 work together to regulate Mitf expression (Bondurand et al., 2000).

E. **SIGNALING PATHWAYS INVOLVED IN MELANIN PRODUCTION**

Melanin synthesis is initiated and regulated by several signaling pathways. Pharmacological and genetic inhibition of MC1R (Melanocortin 1 receptor) receptor showed that MC1R is one of the main signaling pathways important for pigmentation. The MC1R belongs to the family of a subgroup of G-protein coupled receptors, which includes 5 members MC1R to MC5R. Eumelanin synthesis is initiated by MCR1 agonist α-MSH whereas pheomelanin synthesis is initiated by agouti signaling protein ASP (Produced by ASIP gene, inverse agonist of MC1R) (Hida et al., 2009). ASP antogonizes α-MSH effects and decreases Mitf expression, favoring pheomelanin production by suppressing eumelanin (Aberdam et al., 1998). ASP and α-MSH competes to bind to MCR1 receptor and therefore determine pheomelamine versus eumelanin synthesis in melanocytes (Slominski et al., 2004; Hida et al., 2009).

During pigmentation (eumelanin), α-MSH binding to MC1R activates the protein kinase A (PKA), which then phosphorylates CREB (Edelman 1987). Although expression of
Tyrosinase, Tryp1 and Tryp2 is regulated by this mechanism, Tryp1 and Tryp2 promoters do not contain any cAMP response element (CRE) suggesting that CREB controls the expression of Tryp1 and Tryp2 through mediating the activity of Mitf first.

One of the important pathways that regulates melanocyte differentiation is the MAPK pathway. It contains sequentially activated protein kinases, which control cell proliferation, differentiation and survival. It is activated by extracellular ligands which activate receptor tyrosine kinase (RTK). Then RTK activates a cascade of kinases RAS-RAF-MAP2K-MAPK. In term, MAPK (ERK) regulates the activities of several transcription factors. Paracrine factors that are secreted by keratinocytes initiate intracellular signaling pathways in melanocytes (as discussed in the case of α-MSH and MC1R-cAMP-PKA pathway). MAPK pathway can be activated by most of these paracrine factors, which bind and activate receptor tyrosine kinase. In addition, RTK can also be activated by SCF/c-KIT signaling. SCF (Stem cell factor), also known as steel factor, is a cytokine produced by keratinocytes. It binds to c-KIT receptor in melanocytes. As a result of these bindings several signaling pathways are activated including MAPK (Figure 33). Knock-out mouse models for different genes involved in melanogenesis are summarized in Table 4 at the end of this chapter.

![Diagram of signaling pathways involved in pigmentation in melanocytes.](image-url)
MAPK pathway also controls the balance between proliferation and differentiation of melanocytes (pigmentation). For instance, increased cAMP levels which induce melanocyte differentiation can also activate ERK transiently (<60 minutes) (Wellbrock et al., 2002). ERK phosphorylates Mitf on Ser73 residue and increases its transcriptional activites for Tyrosinase stimulating pigmentation (Hemesath et al., 1998). Moreover, when receptor tyrosine kinase (RTK) is activated by SCF, FGF or HGF in a synergistic manner, this leads to a strong sustained ERK activation, which induces melanocyte proliferation (Bohm et al., 1995). In this case, SCF/c-KIT stimulated activation of MAPK results in phosphorylation of Mitf on Ser409 by 90 ribosomal S6 kinase 1 (p90RSK) (Wu et al., 2000). This phosphorylation marks Mitf for proteosomal degradation resulting in a decreased Mitf level consistent with melanocyte proliferation (Wellbrock and Marais 2005). Thus, MAPK pathway is a major regulator of melanocyte proliferation-differentiation balance through modulating Mitf level.

F. Melanocyte Differentiation

Melanoblasts derive from Sox10-positive bipotent glial progenitors of the neural crest cells. Then, specified melanoblasts start expressing Mitf, Tyrp2 and KIT, forming the first embryonic hair follicle. However, some of these cells dedifferentiate, losing Mitf and KIT, to constitute the melanocyte stem cell pool of hair follicle in the bulge region. This pool will allow replenishing of the pigmented melanocyte population. Melanocyte stem cells (MSC) express Tyrp1 and Dct but not Tyrosinase (Mort et al., 2015) (Figure 34).

![Figure 34: Expression of different melanocyte markers during development and differentiation.](image)

Several actors and signaling pathways are involved in melanocyte homeostasis. TGF-β signaling is activated in MSC in vivo when they enter quiescent state after hair follicle cycle and this requires Bcl2 expression for the survival of these stem cells. TGF-β type-II receptor
deficiency in melanocyte lineage results in stem cell immaturity and hair graying (Nishimura et al., 2010). Notch signaling is also important for melanocyte survival and maintenance through the activity of its downstream transcription factor Hes1 protecting the MSC from differentiation. Genetic inactivation of Notch also results in premature hair graying in mice (Moriyama et al., 2006). Wnt ligands are responsible for melanocyte stem cell activation. Wnt signaling targets Pax3, Sox10 and Mitf which are also implicated in MSC maintenance. More precisely, Pax3 prevents terminal differentiation of MSC to melanocytes, a process antagonized by β-catenin (Kubic zadet al., 2008). MSC express Wnt ligands inhibitors such as DKK5 and Dab2 in order to keep their quiescent state (Osawa et al., 2005).

G. Keratinocytes-Melanocytes crosstalk

Once melanoblasts migrate to the epidermis, they tightly attach to the basement membrane. Each melanocyte in the basal layer is in contact with 30 to 40 keratinocytes by its dendritic extensions. Their attachment, growth and migration are strongly regulated by the neighboring keratinocytes. Keratinocytes can control melanocyte homeostasis either by secreting paracrine factors or by direct cell-cell attachments. For instance, TGF-β excreted by keratinocytes inhibits melanocyte proliferation by repressing the expression of Pax3 (Yang et al., 2008). In response to UV, upregulated p53 inhibits TGF-β signaling and induces SCF and α-MSH secretion by keratinocytes, therefore enabling melanocyte proliferation and pigmentation (Cui et al., 2007). Different paracrine factors secreted by keratinocytes and their effects in melanocytes are reported in Table 3.

<table>
<thead>
<tr>
<th>The factors derived from keratinocytes</th>
<th>The effects on melanocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>↑ Proliferation, ↓ melanogenesis</td>
</tr>
<tr>
<td>ET-1</td>
<td>↓ Proliferation, ↑ dendricity, ↓ survival</td>
</tr>
<tr>
<td>IL-1α/ILβ</td>
<td>↑ Proliferation, ↑ melanogenesis, ↑ survival</td>
</tr>
<tr>
<td>ACTH</td>
<td>↑ Proliferation, ↑ melanogenesis, ↑ survival</td>
</tr>
<tr>
<td>α-MSH</td>
<td>↑ Dendricity, ↑ melanogenesis, ↑ melanosomal transfer</td>
</tr>
<tr>
<td>PGE_2/PGF_β</td>
<td>↑ Proliferation, ↑ melanogenesis</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>↑ Melanogenesis</td>
</tr>
<tr>
<td>NO</td>
<td>↓ Melanogenesis</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↑ Dendricity, ↑ survival</td>
</tr>
<tr>
<td>NGF</td>
<td>↑ Melanogenesis</td>
</tr>
<tr>
<td>BMP-4</td>
<td>↑ Proliferation, ↑ dendricity, ↑ melanogenesis</td>
</tr>
</tbody>
</table>

Table 3: Paracrine factors secreted by keratinocytes and their effects on melanocytes (Extracted from Cichorek et al., 2013).
In addition to these secretory mechanisms, keratinocytes regulate melanocyte homeostasis through direct cell-cell adhesions (Haass et al., 2005). Keratinocytes are required for in vitro melanocyte proliferation (Halaban et al., 1988; Sviderskaya et al., 1995). Even in presence of keratinocyte-derived growth factors such as SCF or FGF2, melanocytes barely proliferate unless they are co-cultured with keratinocytes (Yonetani et al., 2008). Together, these observations suggest that melanocytes strongly rely on cell-cell contact to proliferate. This keratinocyte-melanocyte crosstalk is also important for melanocyte stem cell homeostasis in the hair follicle. As an illustration of this notion, TGF-β secreted by keratinocyte stem cells has been shown to regulate melanocyte stem cell homeostasis (Tumbar et al., 2004).

H. SUNTAN RESPONSE AND HYPERPIGMENTATION

Tanning is the natural process of skin pigmentation in response to sunlight or ultraviolet radiation. Melanin pigment absorbs free radicals that are generated by UV light and protects the skin from its dangerous effects. P53 was shown to be important in UV-induced tanning response. On one hand, p53 directly binds Tyrosinase and Tyr1 promoter and activates their expression in vivo. On the other hand, p53 is activated in response to UV-mediated DNA damage and binds to the promoter of POMC in order to induce its transcription (Cui et al., 2007). α-MSH, the product of POMC cleavage, is then secreted by both keratinocytes and melanocytes (Schauer et al., 1994; Chakroarty et al., 1996). It binds to MC1R receptor in melanocytes and induces cAMP signaling pathway to initiate melanogenesis. Taken together with p53−/− mice that fail to tan in response to UVs, these data strongly suggest that p53 is a major actor of the tanning response.

Finally, transcriptional co-activators PGC1-α and PGC1-β were recently reported to be important regulators in the tanning response. They are stabilized in response to α-MSH signaling and activate Mitf which then induces the expression of melanogenesis genes and pigmentation (Shoag et al., 2013) (Figure 35).
Figure 35: Tanning response in melanocytes.

i. Follicular Melanocytes

ii. Matrix Melanocytes

Hair follicle pigmentation is coupled to anagen phase. In the bulb, one melanocyte is present for every 5 keratinocytes (Tobin and Paus 2001). Bulbar melanocytes are larger than epidermal melanocytes as they have more Golgi and Endoplasmic Reticulum. These melanocytes also contain more dendritic extensions than the epidermal ones. After having proliferated in the bulb, melanocytes form their dendritic extensions and start producing pigment. They eventually transfer the melanin to cortical and medulla keratinocytes, which gives the hair shaft its color. Melanin production is switched off during catagen and is completely absent during telogen phase. Terminally differentiated melanocytes undergo apoptosis during catagen (Tobin et al., 1998).

ii. Melanocyte Stem Cells (Amelanotic Cells)

Melanocytes in the IFE epidermis barely proliferate. They produce melanin in response to UV exposure to protect the skin. In contrast, melanocytes of hair follicles repeatedly proliferate and differentiate during each hair cycle in order to pigment the hair. Existence of a melanocyte stem cell population was first observed by Montagna and Chase. They detected a cell population in the outer root sheath of human hair follicle which exhibited a distinct morphology than keratinocytes (Montagne and Chase 1956). It was shown that these cells are amelanotic and negative for DOPA reaction. However, they can be activated by UV or in response to wounds (Cui et al., 1991) and migrate to the epidermis to re-pigment it (Starrico et al., 1963). An amelanotic and morphologically distinct cell
population was also detected in mice in the secondary hair germ during telogen (Silver et al., 1968). It was suggested at that time that these differentiated hair matrix melanocytes de-differentiate to melanoblasts during telogen phase (Sugiyama 1979).

Several lines of evidence have proved the existence of melanocyte stem cell population. Treatment of neonatal mice with a KIT antibody resulted in hair pigment loss by inhibiting melanocyte proliferation and differentiation. However, new hair came up repigmented in the next hair cycle suggesting that another KIT-negative melanocyte population could give rise to mature melanocytes. They also identified another melanoblast population in the bulge, which expresses Dct and Tryp2 (Botchkareva et al., 2001). The formal proof of the existence of the melanocyte stem cell population came from a study using Dct-lacZ reporter transgenic mice treated with KIT antibody in which, they followed the fate of these Dct+ cells that normally reside in the bulge. They demonstrated that this cell population replaces the mature matrix melanocytes. Moreover, pulse-chase experiments with BrdU showed that these cells were very slow proliferating cells and this labelling was only possible at the beginning of the anagen phase of the hair cycle, indicating that melanocyte stem cells only proliferate during anagen (Nishimura et al., 2002).

So far, no specific marker for MSC has been identified. The only criterion to distinguish these MSCs from differentiated melanocytes is their localization (bulge and sub-bulge area) and the absence of melanin. MSCs are small, oval-shaped cells and they exhibit high nucleus/cytoplasm ratio. At the onset of anagen, they upregulate melanin genes, form dendritic extensions and divide. By mid-anagen, they downregulate melanin genes, lose their extensions and become quiescent (Nishimura et al., 2005; Nishimura et al., 2007).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Function</th>
<th>Phenotype</th>
<th>Associated human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fgfr2</em></td>
<td>Fibroblast growth factor receptor 2</td>
<td>Growth factor receptor</td>
<td>Lighter skin</td>
<td>Crouzon syndrome, Apert syndrome, Pfeiffer syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vitiligo-associated multiple autoimmune disease associated 2, Susceptibility</td>
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<td></td>
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<td></td>
<td>to autoimmune disease 1</td>
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<td><em>Foxd3</em></td>
<td>Forkhead box D3</td>
<td>Transcription factor</td>
<td>Mutant mice are embryonic</td>
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<td></td>
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<td>lethal, Essential for neural</td>
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<td></td>
<td>crest cells and melanoblasts</td>
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<td></td>
<td>formation</td>
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<td><em>Itgb1</em></td>
<td>integrin beta 1</td>
<td>cell attachment, migration</td>
<td>transient patchy hypopigmentation, crest migration defect</td>
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<td><em>Kit</em></td>
<td>Kit oncogene</td>
<td>Receptor for Kit ligand SCF; required for</td>
<td>White spotting, anemia and</td>
<td>Piebaldism</td>
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<td></td>
<td></td>
<td>melanoblast survival</td>
<td>germ-cell deficiency</td>
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<td>(SCF/SI) Steel factor (SI), Stem cell</td>
<td>Stem cell factor (SCF) (Kit ligand);</td>
<td>White spotting, anemia and</td>
<td>Unknown</td>
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<td>factor (SCF)</td>
<td>melanoblast growth and differentiation factor</td>
<td>germ-cell deficiency</td>
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<td><em>Krt1</em></td>
<td>Keratin 1</td>
<td>cytoskeleton</td>
<td>dark skin, Primary action in</td>
<td>epidermolytic hyperkeratosis</td>
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<td></td>
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<td>clustered melanin granules</td>
<td>multiplex</td>
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100
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<tr>
<th>Lef1</th>
<th>lymphoid enhancer binding factor 1</th>
<th>transcription factor, Wnt/β-catenin mediator, Mutations result in impaired binding to β-catenin</th>
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<th>Sebaceous adenomas</th>
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<td>microphthalmia (mi)</td>
<td>transcription factor, master regulator of melanocyte lineage</td>
<td>melanocyte differentiation, White spotting and small or absent eyes</td>
<td>Waardenburg syndrome type 2</td>
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<td>Myc</td>
<td>myelocytomatosis oncogene (when KO targeted by Wnt1 promoter-Cre)</td>
<td>Myc proto-oncogene protein (c-myc), Transcription factor, regulator of cell proliferation</td>
<td>Pigmentary spotting, not head</td>
<td>unknown</td>
</tr>
<tr>
<td>Notch1</td>
<td>Notch gene homolog 1 (Drosophila)</td>
<td>Receptor for ligands in Delta and Jagged families</td>
<td>Scattered grey hairs, when KO targeted to melanocytes (Tyr-Cre)</td>
<td>unknown</td>
</tr>
<tr>
<td>Notch2</td>
<td>Notch gene homolog 2 (Drosophila)</td>
<td>Receptor for ligands in Delta and Jagged families</td>
<td>Scattered grey hairs, when KO targeted to melanocytes (Tyr-Cre). All grey with Notch1 KO, eventually white</td>
<td>Alagille Syndrome 2</td>
</tr>
<tr>
<td>Pax3</td>
<td>Paired box 3</td>
<td>transcription factor</td>
<td>neural tube development</td>
<td>Waardenburg syndrome type 1 Waardenburg syndrome type 3</td>
</tr>
<tr>
<td>Sox10</td>
<td>SRY-box containing gene 10,</td>
<td>Transcription factor</td>
<td>White spotting, megacolon and neural crest defects</td>
<td>Waardenburg-Shah Syndrome</td>
</tr>
<tr>
<td>Gene</td>
<td>Full name</td>
<td>Function</td>
<td>Phenotype</td>
<td>Associated human disease</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------</td>
<td>-----------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>Wnt1</td>
<td>wingless-related MMTV integration site 1</td>
<td>Growth factor/morphogen</td>
<td>Defects of neural crest including melanoblasts in mice lacking both Wnt1 and Wnt3a</td>
<td>Unknown</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>wingless-related MMTV integration site 3A</td>
<td>Growth factor/morphogen</td>
<td>Defects of neural crest including melanoblasts in mice lacking both Wnt1 and Wnt3a</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

### Melanogenesis genes and pathway components

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Function</th>
<th>Phenotype</th>
<th>Associated human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>tyrosinase</td>
<td>melanogenic enzyme</td>
<td>No pigment in null mice (multiple allelic variants)</td>
<td>oculocutaneous albinism type 1 (OCA1)</td>
</tr>
<tr>
<td>Tyrp1</td>
<td>tyrosinase related protein 1 (TRP1)</td>
<td>melanosomal enzyme/stabilizing factor</td>
<td>Brown eumelanin. Allele iso can contribute to glaucoma</td>
<td>oculocutaneous albinism type 3 (OCA3) Rufous albinism</td>
</tr>
<tr>
<td>Dct</td>
<td>tyrosinase related protein 2 (TRP2), slaty (slt)</td>
<td>DOPAchrome tautomerase, melanosomal enzyme</td>
<td>Dilution of eumelanin color</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pme1</td>
<td>gp100/gp87/silver protein, Pme17, silver (si)</td>
<td>premelanosome protein, melanosomal matrix protein, trapping of melanin intermediates.</td>
<td>Silvering with postnatal melanocyte loss in eumelanic animals (varying with strain background) in Pme1&lt;sup&gt;b&lt;/sup&gt;. Mild effect on visible pigmentation, substantial reduction in eumelanin content in hair and spherical melanosomes in Pme1&lt;sup&gt;biss&lt;/sup&gt;</td>
<td>Unknown</td>
</tr>
<tr>
<td>Mc1r</td>
<td>Melanocortin 1 receptor</td>
<td>Eumelanin to pheomelanin switch. Yellow mouse</td>
<td>Hair color/skin type</td>
<td></td>
</tr>
<tr>
<td>ASIP</td>
<td>agouti signal protein (ASIP)</td>
<td>Induces pheomelanin synthesis</td>
<td>Absence of pheomelanin. Black mouse</td>
<td>hair color/skin type</td>
</tr>
<tr>
<td>-----------------</td>
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<td>------------------------------</td>
<td>-------------------------------------</td>
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</table>

**Table 4:** Knock-out mouse model descriptions for melanocyte genes implicated in development and pigmentation (Adapted from the website http://www.espcr.org/micemut).
OBJECTIVES AND EXPERIMENTAL MOUSE MODELS
Objectives of the Thesis

Studies on the protein network that is altered upon infection by oncoviral proteins in the late 80ies, led to discovery of many key factors implicated in cell division and survival. Among these, the E4F1 protein was initially identified as a target of the viral oncoprotein E1A. Originally identified as a transcription factor, E4F1 is a multifunctional protein that also exhibits an atypical ubiquitin-E3 ligase activity. The first cellular target identified to be ubiquitylated by E4F1 was the tumor suppressor p53. Since then, my host lab and other have demonstrated that through its interactions with a number of partners, E4F1 is necessary for early embryonic development, proliferation of somatic cells as well as survival of cancer cells. To determine the physiological in vivo functions of E4f1 in adult tissues, my host laboratory generated different mouse models based on the Cre-LoxP system.

The first mouse model generated by our laboratory was a whole-body inactivation of E4f1 gene. This mouse strain was obtained by crossing E4f1 conditional knockout mouse (discussed below) with a transgenic animal model (RERT) that expresses the Cre recombinase under the promoter of the ubiquitously expressed RNA polymerase II gene. Using this mouse model, my host laboratory showed that inactivation of E4f1 led to severe skin defects characterized by a transient hyperplasia of the epidermis at early time point, strong alterations of the keratinocytes differentiation and a complete loss of cellularity at later time points. Using another animal model of constitutive deletion of E4f1 in basal keratinocytes (E4f1; K5CRE), my host lab was able to recapitulate the skin defects observed in RERT; E4f1−/− mutant mice, demonstrating that these defects are skin-specific. However, these animals died 3 days after birth due to severe dehydration. Besides, the defects observed were due to the exhaustion of epidermal stem cells in vivo. Consistently, E4f1 inactivation completely altered the clonogenic potential of keratinocyte stem cells ex vivo. At the molecular level, the clonogenic potential of epidermal stem cells was partially restored by genetically modifying the one specific branch of p53 pathway (such as p53 inactivation, Bmi1 overexpression) that includes Bmi1-p53-Ink4a/Arf axis. Thus these results suggest that E4F1 partially impinges of this specific p53 pathway to control epidermal stem cell maintenance.

My PhD objectives are the logical follow up of these previous findings obtained by my host lab. Since inactivation of the p53 pathway only partially rescues the epidermal stem cell defects mediated by E4f1 depletion, one of the first aims of my PhD was to investigate and identify other molecular pathways regulated by E4f1 that was involved in ESC maintenance and skin homeostasis. This objective also included a better characterization of E4f1 mediated-skin defects in the different skin compartments (basal cells versus suprabasal cells) using skin specific mouse models. Based on the results obtained from the first objective, the second aim of my PhD was then to extend our understanding of E4f1 in skin homeostasis by investigating E4f1 function in other cell types of the skin such as melanocytes. To this purpose, I evaluated the consequences on E4f1 inactivation in
melanocytes and characterized the implication of the p53 pathway and/or the PDH related functions in the melanocyte homeostasis.

**Mouse Models**

**PART I**

**I. E4f1 mouse models**

**A. E4f1+/- heterozygote mouse**

The exons 3 to 14 of the murine E4f1 (encoding amino acids 104 to 784) were replaced by a phosphoglycerokinase (PGK)-puromycin poly(A) resistance cassette (**Figure 36**). This construct was electroporated into J1 embryonic stem cells (ES) and selected with puromycin. The resistant clones were screened for homologous recombination by Southern blot. The ES cells were then injected into C57Bl/6 blastocysts to generate a germ line transmitting chimeric mice. These animals were then crossed with wild type CB7BL/6 mice to obtain E4f1+/- heterozygote mice (Le Cam *et al*., 2004).

![Diagram of E4f1 gene and targeting vector](image)

**Figure 36:** Generation of E4f1 knock-out (E4f1-/-) mutant (KO). Structure of E4f1 WT allele, targeting vector and modified allele after homologous recombination. Extracted from Le Cam *et al*., 2004.

**B. E4f1 conditional knock-out mutant (cKO)**

The exons 4 to 14 of the E4f1 gene were flanked by loxP sequences, which will be recognized and excised by the Cre recombinase. A selection cassette (hygromycin-TK)
flanked by two FRT sequences was also inserted next to the poly-adenylation site of E4f1. The target vector was then electroporated into embryonic stem cells from a 129SV/1 background and the cells were selected with hygromycin B. The clones of interests were electroporated with a vector containing FLP recombinase in order to excise hygro-TK selection cassette, and were selected by gancyclovir (Figure 2A). The E4f1+/- clones having a normal karyotype were injected into the blastocysts of C57Bl/6 background. The E4f1+/- heterozygote mice were backcrossed in order to generate E4f1+/- mice.

For all the in vivo studies, experimental groups were generated by crossing E4f1+/- mice with E4f1+/-;Cre compound animals which contained tissue-specific Cre recombinase allele. The offspring contained E4f1+/- and E4f1+/- mice which gave rise to E4f1+/- (WT) and E4f1+/- (KO) mice with Cre-mediated excision of flox allele (Figure 37).

A.

![Diagram](image)

B.

![Diagram](image)

**Figure 37:** Generation and recombination of E4f1 conditional KO allele (cKO). (A) Structure of E4f1 WT allele, targeting vector and recombinated allele after homologous recombination. Extracted from Lacroix et al., 2010. (B) Cre-mediated recombination of E4f1 flox allele.
RESULTS
PUBLICATION #1
II. Muscle specific mouse model

A. Acta1-Cre transgenic mouse

To inactivate $E4f1$ in skeletal muscle, we crossed $E4f1$ cKO animals with Acta1-Cre transgenic mouse, which express the Cre recombinase under the promoter of human $\alpha$-skeletal actin (Figure 38) (Miniou et al., 1999).

Figure 38: Skeletal muscle specific inactivation of $E4f1$. $E4f1$ flox mice were crossed with Acta1-Cre transgenic mouse which express Cre recombinase under the control of the skeletal $\alpha$-actin promoter.
SUMMARY

In this study, we have found that multifunctional protein E4F1 directly regulates the transcriptional expression of a group of genes encoding components (Dlat, Dld, Brp44L/Mpc1) and regulators (Pdpr, Slc25a19) of the pyruvate dehydrogenase complex (PDC). PDC catalyses the pyruvate to Acetyl CoA conversion which links pyruvate metabolism to TCA cycle. Inactivation of E4f1 in primary mouse embryonic fibroblasts (MEFs) resulted in down-regulation of these components and decreased PDH activity. To investigate the in vivo consequences of impaired PDH activity, we have generated an engineered mouse model in which we have inactivated E4f1 specifically in skeletal muscle. These mice exhibited decreased PDH activity, exercise intolerance and systemic lactic acidosis, which recapitulate some of the symptoms observed in patients suffering from PDH-deficiency. Thus we identified for the first time E4F1 as a critical regulator of PDC and pyruvate metabolism.
E4F1 controls a transcriptional program essential for pyruvate dehydrogenase activity

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Edited by Steven L. McKnight, The University of Texas Southwestern Medical Center, Dallas, TX, and approved August 9, 2016 (received for review February 18, 2016)

The mitochondrial pyruvate dehydrogenase (PDH) complex (PDC) acts as a central metabolic node that mediates pyruvate oxidation and fuels the tricarboxylic acid cycle to meet energy demand. Here, we reveal another level of regulation of the pyruvate oxidation pathway in mammals implicating the E4 transcription factor 1 (E4F1). E4F1 controls a set of four genes [dihydrolipoamide acetyltransferase (Dlat), dihydrolipoyl dehydrogenase (Dld), mitochondrial pyruvate carrier 1 (Mpc1), and solute carrier family 25 member 19 (Slc25a19)] involved in pyruvate oxidation and reported to be individually mutated in human metabolic syndromes. E4F1 dysfunction results in 80% decrease of PDH activity and alterations of pyruvate metabolism. Genetic inactivation of murine E4F1 in striated muscles results in viable animals that show low muscle PDH activity, severe endurance defects, and chronic lactic acidemia, recapitulating some clinical symptoms described in PDC-deficient patients. These phenotypes were attenuated by pharmacological stimulation of PDH or by a ketogenic diet, two treatments used for PDH deficiencies. Taken together, these data identify E4F1 as a master regulator of the PDC.

E4F1 | PDH | pyruvate | muscle | endurance

The pyruvate dehydrogenase (PDH) complex (PDC) is a mitochondrial multimeric complex that catalyzes the oxidative decarboxylation of pyruvate into acetyl-CoA (AcCoA), thus linking pyruvate metabolism to the tricarboxylic acid (TCA) cycle. Localized in the mitochondrial matrix, the core PDC is composed of multiple copies of three catalytic enzymes: PDHA1/E1, dihydrolipoamide transacetylase (DLAT)/E2, and dihydrolipoyl dehydrogenase (DLD)/E3 (1). To fuel the PDC, pyruvate translocates across the inner mitochondrial membrane through the heterodimeric pyruvate transporter mitochondrial pyruvate carrier 1 (MPC1)/MPC2 (2, 3). The activity of the PDC depends on several cofactors, including lipoate, CoenzymeA (CoA), FAD4+, NAD+, and thiamine pyrophosphate, the latter being imported in the mitochondria by the SLC25A19 transporter (4). So far, fine-tuning of PDC activity has been mainly attributed to post-translational modifications of its subunits (5, 6), including the extensively studied phosphorylation of PDHA1/E1 modulated by PDH kinases (PDK1–4) and phosphatases (PDP1–2). However, in lower organisms, such as Escherichia coli and Candida albicans, PDC is also controlled at the transcriptional level by the coordinated regulation of genes encoding its components and regulators (7, 8). The importance of such transcriptional regulation of the PDC in mammals remains elusive. Physiological regulation of PDC plays a pivotal role in metabolic flexibility to adjust energetic metabolism and biosynthesis to nutrient availability and energy demand (9), such as in skeletal muscles during exercise (10). PDH activity is altered in several human metabolic syndromes associated with chronic lactate acidosis, progressive neurological degeneration, and muscular atonia (11). Genetic mitochondrial disorders associated with PDH deficiency mainly result from hypomorphic mutations in genes encoding subunits or regulators of the PDC, including in PDHA1, DLAT, DLD, PDP1, MPCI, and SLC25A19 (3, 11–13). The diverse clinical manifestations of PDC-deficient patients are significantly, but only partly, improved by ketogenic diets that provide alternative energetic substrates or by treatment with PDK inhibitors, such as dichloroacetate (DCA). Thiamine/lipoic acid supplements that favor optimal PDH activity, or bicatecarnate treatment that buffers lactate acidosis, have also been tested, although with moderate efficiency (14, 15). The design of new and more efficient therapeutic approaches will require a better understanding of PDH regulation and the development of clinically relevant animal models. Here we reveal another level of regulation of the pyruvate oxidation pathway in mammals that implicates the E4 transcription factor.

Significance

Pyruvate dehydrogenase (PDH) deficiency is the cause of several human metabolic diseases. In mammals, the transcriptional control of PDH complex components and its impact on pathophysiology remain poorly understood. We show that E4 transcription factor 1 (E4F1) controls a transcriptional program essential for PDH activity that involves genes linked to human metabolic syndromes. Genetic inactivation of murine E4F1 results in a strong decrease of PDH activity and severe perturbations of pyruvate metabolism. In concordance with the work of Legati et al., we show that striated muscle-specific E4F1 KO animals display phenotypes that recapitulate these clinical symptoms, providing an exciting clinical perspective to the present work.


The authors declare no conflict of interest.

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2J.H. and H.D. contributed equally to this work.

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1 (E4F1). Initially identified as a cellular target of the E1A viral oncoprotein (16), E4F1 was then described as a physical interactor of several tumor suppressors that gate cell division and survival in proliferating cells, including pRB, RASSFP1A, p14ARF, and p53 (17–21). E4F1 is essential for early embryonic mouse development (22), and for either proliferation or survival of actively dividing mammalian cells (23–25). In proliferating cells, we have recently shown that E4F1 controls genes implicated in cell-cycle checkpoints and genome surveillance, but also unexpectedly, a transcriptional program involved in mitochondria functions (24, 26). Here we further characterized this mitochondria-associated program and found that E4F1 coordinates the transcription of a set of genes involved in PDH-mediated pyruvate oxidation. Accordingly, tissue-specific inactivation of murine E4F1 in the postmitotic and differentiated compartments of striated muscles resulted in a strong reduction of muscular PDH activity. Surprisingly, this constitutively low PDH activity did not compromise animal viability, although these animals displayed chronic lactic acidemia and endurance defects that recapitulate some clinical symptoms described in PDC-deficient patients.

Results

E4F1 Controls Genes Involved in PDH Activity. We recently identified by ChIP, combined with deep sequencing (ChIP-seq), the repertoire of endogenous target DNA sites bound by E4F1 in primary and Ha-Ras<sup>G12D</sup> transformed mouse embryonic fibroblasts (MEFs) (24, 26). We completed this gene list by performing additional E4F1 ChIP-seq analyses in murine embryonic stem (ES) cells and defined a common set of promoter regions that were bound by E4F1 in these two cell types (Fig. 1A) (GSE57222 and GSE57228). Genomic footprinting analysis of E4F1 target genes revealed an unexpected enrichment for nuclear genes encoding mitochondrial proteins (24). Surprisingly, a closer analysis of this subprogram identified four genes, located on distinct chromosomes, which are directly involved in PDH function. These genes encode the E2 and E3 subunits of the PDH core enzyme (Dlat, Dld), the mitochondrial pyruvate transporter MFC1 (Brop44l), and the mitochondrial transporter of the PDH cofactor thiamine pyrophosphate (Slc25a19/DNC). A fifth gene, encoding the negative regulator of the PDH phosphatases (Pdpr) (27) was also identified as an E4F1 target gene by ChIP-seq in transformed fibroblasts, but not in ES cells (data not shown).

Fig. 2. Impaired PDH activity and deregulation of the pyruvate pathway in E4F1<sup>−/−</sup> cells. (A) Protein levels of E4F1, DLAT, DLD, lipoylated proteins (DLAT and DLD), PDHE1α, MPC1/BRPF4L, and Tubα11 (loading control) determined by immunoblotting of total cell extracts prepared from E4F1<sup>−/−</sup> and control (CTRL) MEFs. (B) PDH enzymatic activity measured in E4F1<sup>−/−</sup> and CTRL MEFs. (C) Schematic representation of the pyruvate-AcCoA pathway. (D) Relative levels of several metabolites linked to the pyruvate pathway in E4F1<sup>−/−</sup> and CTRL MEFs, measured by LC-MS (n = 6). (E) Extracellular lactate level in the medium of E4F1<sup>−/−</sup> and CTRL MEFs. Histobars represent the mean value ± SEM (n = 5). (F) Relative abundance of M+2 isomers of AcCoA and citrate that derive from pyruvate oxidation as determined by LC-MS in E4F1<sup>−/−</sup> and match CTRL MEFs cultured in α-[U-<sup>13</sup>C]glucose for 30 min or 6 h (mean ± SD, experiment performed in triplicate). (G) Relative levels of FAO reporter gene expression as determined by qPCR in MEFs with 10 μM palmitate (mean value ± SEM, n = 3). **P < 0.01; *P < 0.05; ns, not significant.
Sequence analyses revealed that these genes contained one or two bona fide E4F1 binding sites nearby their transcription start site (TSS) (Fig. 1A). These E4F1 direct target genes were further validated by ChIP-quantitative PCR (qPCR) experiments performed upon Cre-mediated inactivation of E4f1 in E4f1KO mice (hereafter referred to as E4f1KO) (Fig. 1B and C). Consistent with a role for E4F1 as a bona fide transcriptional activator for these PDH-related genes, the mRNA levels of Dlat, Bp44Lmpc, Dld, Slc25a19, and Pdpn decreased in E4f1KO cells, although to various extents (Fig. 1D). In contrast, the transcript levels of another PDH core component, Pdh1a, and of the mitochondrial enzyme citrate synthase (CS), which were not identified as E4F1 direct target genes, did not vary upon acute E4F1 inactivation (Fig. 1D). At the protein level, a strong down-regulation of DLAT and BRP44LMPC1, and a moderate decrease of DLD were observed in E4f1KO cells (Fig. 2A). Of note, siRNA-mediated depletion of E4F1 in fibroblasts also resulted in the down-regulation of DLAT, BRP44LMPC1 and DLD proteins (Fig. S1A), confirming the role of E4F1 in the control of these genes. Taken together, our data highlight a previously undescribed function of E4F1 in the transcriptional control of genes involved in PDH activity in mammals.

**E4f1 Inactivation Results in Reduced PDH Activity and Metabolic Reprogramming.** As a direct consequence of decreased expression of PDC subunits, E4f1KO fibroblasts and E4f1 siRNA-treated cells exhibited a marked decrease of PDH enzymatic activity (Fig. 2B and Fig. S1B). In E4F1-deficient cells, this reduced PDH activity should impact on pyruvate-derived mitochondrial AcCoA production, lead to accumulation of glycolytic intermediates, and induce the redirection of the glycolytic flux. We addressed this notion by performing comparative nontargeted gas chromatography/liquid chromatography-mass spectrometry (GC/LC-MS) metabolic analyses in control and E4f1KO fibroblasts. As predicted, these analyses showed an accumulation of intracellular pyruvate and of its upstream precursor 2-3-phosphoglycerate (2/3PG) in E4f1KO cells as well as lower levels of citrate and succinate, two intermediates of the TCA cycle (Fig. 2C and D). E4F1-deficient cells also exhibited increased level of extracellular lactate in their culture medium (Fig. 2E). To further assess the PDH-dependent pyruvate oxidation pathway, we next performed stable isotope tracing experiments in control and E4f1KO fibroblasts cultured in presence of uniformly labeled [U-13C]glucose. Comparative LC-MS analyses of intracellular metabolites clearly showed a strong decrease of 13C incorporation into AcCoA (M4-2 isotopomer) and in its downstream metabolite, citrate (M4-2 isotopomer), in E4f1KO cells (Fig. 2F and Fig. S2A). Of note, the relative 13C enrichment in the first glycolytic intermediates (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, 2/3-PG) was unaffected, suggesting that both control and E4f1KO fibroblasts display comparable glycolytic fluxes. Taken together, these analyses indicate that E4F1-deficiency impairs PDH activity with impacts on the pyruvate oxidation pathway (Fig. S2A).

Of note, we also assessed mitochondrial protein lipoylation as both a direct readout of DLAT expression and of an indirect readout of defective AcCoA production by PDH in E4f1KO cells (2, 28). Indeed, the precursor of lipoic acid, octanoic acid, is synthesized from mitochondrial AcCoA through fatty acid biosynthesis. Lipoic acid is then covalently attached to few proteins, for which it serves as a cofactor. At first glance, total protein lipoylation seemed to be strongly reduced in E4f1KO cells, as revealed by immunofluorescence (Fig. S2B) using an antibody that recognizes all lipoylated proteins. Although this strong decrease of total protein lipoylation likely reflects mainly DLAT protein down-regulation (the most abundant lipoylated protein), we also observed a moderate down-regulation of the lipoylation of dihydroxyoacid-S-succinyl transferase (DLST) by immunoblotting (Fig. 2A and Fig. S2C), suggesting that the AcCoA-dependent lipoylation pathway is also partly affected in E4f1KO cells, and could also contribute to the phenotype of E4f1KO. Finally, despite their low PDH activity, E4f1KO cells showed a moderate, but significant, decrease of intracellular ATP, as described in our previous report (see also ref. 24) (Fig. S2D). This moderate alteration of ATP levels suggests that alternative energetic pathways were activated in E4f1KO cells. Indeed, E4f1KO cells show signs of adaptive metabolic responses, as illustrated by increased fatty acid oxidation (FAO) (Fig. 2G). Accordingly, these cells were highly sensitive to the FAO inhibitor Bt2omix (Fig. S2E). Taken together, these data show that PDH activity and the mitochondrial pyruvate pathway are impaired in E4f1KO cells.

**E4f1 KO in Striated Skeletal Muscles Results in PDH Dysfunction.** We next assessed the in vivo relevance of E4f1-mediated control of this PDC transcriptional program in striated muscle, a tissue where physiological function relies on high PDH activity during exercise (10). First, we confirmed by ChIP-qPCR the recruitment of E4FI on Dlat, Dld, Slc25a19, and Bp44Lmpc promoters in adult tibialis and
gastrocnemius muscles of resting mice, indicating that E4f1–PDH program also exists in adult muscle cells (Fig. 3 A and Fig. S3A).

Then, we inactivated E4f1 in vivo in striated muscles by crossing E4f1−/− mice with Acta1-Cre transgenic (Tg) mice that express the Cre recombinase under the control of the skeletal α-actin promoter [hereafter referred to as Tg(Acta1-Cre)] (Fig. 3B) (29). We verified the efficiency and the tissular specificity of Cre-driven recombination of the E4f1 allele in E4f1−/−, Tg(Acta1-Cre) and E4f1KoA(Acta) mice, examine the ectopic expression of E4f1KoA(Acta) alleles was largely restricted to skeletal muscles, as shown by the strong reduction of E4f1 mRNA and protein levels in gastrocnemius of adult E4f1KoA(Acta) mice (Fig. 3 D and E).

Although limited Cre-mediated recombination (20% efficiency) was also detected in heart (Fig. 3C), this did not impair significantly the cardiac mRNA level of E4f1 when assessed at the whole tissue level (Fig. S3B). E4f1KoA(Acta) mice were healthy and viable, and detailed anato-pathological analyses of skeletal muscles at 16 wk of age revealed neither major histological alterations nor significant differences in the number and size of muscle fibers compared with control littermates (Fig. S4A, B, and D). Accordingly, mRNA levels of muscular differentiation markers and inducers such as MyoD and Msx1, Myf5, and Myogenin were similar in adult striated muscles of 16-wk-old E4f1KoA(Acta) and control mice (Fig. S4E).

However, alterations resembling degenerative to necrotizing and diffuse myopathy were gradually detected in older animals. Thus, H&E staining of striated muscle sections prepared from 18-mo-old animals showed that E4f1−/− led to myophagocytosis, hypercontracted fibers, centralized regenerative fibers, immune cell infiltration, and the presence of adipocytes (Fig. S4F). These data suggest that in the long term, E4f1 deficiency results in skeletal muscle disorganization and histological alterations.

Next, we evaluated the consequences of E4f1 inactivation in vivo on this PDH transcriptional program. In skeletal, but not in cardiac E4f1−/− muscles, mRNA, and protein levels of Dlat were strongly altered, whereas the mRNA level of Pdhfa1, used as control, remained unchanged (Fig. 3 A and B, Fig. S3B). Expression of Bap34, Sli25a19, and Dlat was also slightly decreased at the mRNA level (Fig. 3D), although to a lesser extent than Dlat. As in E4f1−/− fibroblasts (tMEFs) in culture, protein lipoylase was also markedly decreased in E4f1KoA(Acta) muscles, as shown by immunoblotting on proteins extracts and immunostaining of tissue sections (Fig. 3 E and F).

Impaired expression of these PDC components in E4f1−/− muscles resulted in adult 80–90% reduction of PDH enzymatic activity in gastrocnemius, as measured by two independent methods (Fig. 4 A and B and Fig. S3D). E4f1KoA(Acta) mice also exhibited increased level of circulating ketone bodies, suggesting that E4f1−/− muscles activated FAO as in E4f1−/− MEFS (Fig. 4C). Of note, Dlat expression and PDH activity were also strongly down-regulated in extensor digitorum longus and soleus striated muscles isolated from E4f1−/− mice, indicating that both red and white muscle fibers are equally affected by E4f1-deficiency (Fig. S5 A and B). Consistent with the absence of deletion of E4f1 mRNA in cardiac tissue in this animal model (Fig. S3B), no significant difference in PDH activity was detected in the heart of E4f1KoA(Acta) mice (Fig. S3C). These data show that the E4f1–PDH connection is critical for the pyruvate-ACCoA metabolic pathway in adult striated skeletal muscles, confirming its biological relevance in vivo.

E4f1 Inactivation in Skeletal Muscles Results in Lactate Acidosis and Muscular Endurance Defects. Although E4f1−/− mice did not show spontaneous locomotor deficiency in normal housing conditions, as quantified by infrared light beam interruption in cages (Fig. S4C). This surprising result indicates that a low muscular PDH activity (10–20% of normal levels) (Fig. 4 A and B) is sufficient to sustain basal locomotor activity and viability. PDH activity has been documented to increase in skeletal muscles during high-intensity exercise and to contribute to muscular endurance (10).

Therefore, we hypothesized that the residual PDH activity in muscles of E4f1KoA(Acta) mice might not be sufficient to support the energetic demand that occurs during an acute and high exercise workload. Locomotor activity of control and E4f1KoA(Acta) adult mice was assessed upon forced treadmill running (Fig. 4D). Although PDH activity increased in all animals in this experimental setting, it remained much lower in E4f1KoA(Acta) mice relative to control littermates (Fig. 4G). Accordingly, E4f1KoA(Acta) animals displayed a marked decrease of their physical endurance, as documented by a twofold reduction of their running performance [total running distance (Fig. 4E) and time to exhaustion (Fig. S6A)].

Because PDH deficiency results in chronic lactic acidemia in patients, we measured lactate levels in the serum of E4f1−/− and control mice. E4f1KoA(Acta) mice showed a remarkable decrease of lactate levels relative to controls under normal housing conditions and other Chow diet (Fig. S6B). Lactic acidemia was further exacerbated upon acute exercise (Fig. 4F). Importantly, 16-wk-old E4f1KoA(Acta) mice showed no apparent alterations of glucose homeostasis, as assessed by insulin- and glucose-tolerance tests (Fig. S6 C and D), glucose uptake, and expression of the glucose transporter GLUT1 (Fig. S6, E and F). Collectively, our data show that E4f1−/− mice display phenotypes that recapitulate some clinical symptoms observed in PDC-deficient patients, including lactic acidemia and exercise intolerance (11, 30).
Gal4p transcriptional regulator controls the expression of the five main components of the PDC complex, including the Pda1/R1, Pdh1/E1, Dlat1/R2, Lpd1/R3, and Pdx1 subunits (8). Our data reveal that such a coordinated transcriptional program, important for PDH-mediated pyruvate oxidation, also exists in mammals. Composed of at least four genes—*Dlat/E2, Dld/E3, Byp44/MPC1*, and Sk2a19—this program is controlled by E4F1, a sequence-specific transcription factor bound nearby the TSS of these genes.

This E4F1-controlled transcription program is a main contributor of the total PDH activity, as demonstrated by the impact of conditional gene targeting of E4F1 in proliferating cells and in postmitotic differentiated muscular cells that resulted in a 80–90% reduction of the basal PDH activity. Surprisingly, we show that, despite their weak muscular PDH activity, animals lacking E4F1 in their striated muscles were viable and displayed normal basal locomotor activity, at least in normal housing conditions. Nevertheless, these animals exhibited lactic acidemia and severe exercise intolerance that were partly rescued by the pharmacological reactivation of the remaining pool of PDH by DCA, or by shunting the need for PDH activity by promoting FAO using a ketogenic diet. Our histological analyses indicate that although E4F1 inactivation did not result in major disorganization of this tissue in young animals, long-term PDH deficiency led to a degenerative muscular myopathy in older animals. So far, such clinical symptoms have not been described in PDH patients, likely because most of these patients do not live long enough to develop myopathies. On the other hand, it is commonly described that these patients often exhibit epileptic seizures and microcephaly. These symptoms were not observed in our muscle-specific E4F1KO mice, despite these animals exhibited chronic lactic acidemia. This finding questions the origin of the neurological manifestations observed in PDH patients and suggests that the latter symptoms do not result solely from chronic systemic lactic acidemia, but could also arise from multiple brain-specific metabolic alterations. Tissue-specific inactivation of E4F1 in the central nervous system may provide a definitive answer to this clinically relevant question.

Strikingly, recent genetic studies designed to identify new mutated genes involved in unsolved cases of primary mitochondrial disorders, led to the identification of a homoygous nonsynonymous mutation in the E4F1 gene of a patient showing reduced PDH complex activity, muscular defects, and lactic acidemia (36). This first indication that the E4F1-controlled program could be deregulated in a pathological situation provides an exciting clinical perspective for the future. Indeed, our E4F1KO mice should display phenotypes that recapitulate some clinical symptoms observed in this PDH-deficient patient. Thus, these animals could represent potential models for preclinical studies aiming at testing new therapeutic strategies to improve the consequences of PDH deficiency.

Furthemore, it should be noted that misense mutations in the E4F1-target genes *Dlat, Dld, Byp44*, and *Sk2a19* have been identified in several congenital metabolic disorders associated with reduced PDH activity and alteration of the pyruvate oxidation pathway. These disorders include PDH deficiency, lipoamid dehydrogenase deficiency, or Amish lethal microcephaly syndromes (3,11,13). It is also worth noting that complete KO mouse models for *Pdh1, Dld, Sk2a19*, as well as for *E4F1*, all show severe developmental defects and lethality during early embryonic development (4,22,37–39). This finding raises interesting questions about the importance of the E4F1-controlled PDH-program during embryogenesis and beyond, about the poorly characterized metabolic rewiring of the pyruvate pathway that may occur during development.

Altogether, our data highlight the role of E4F1 in PDH-dependent metabolic homeostasis and pave the way for new studies on the physiological rewiring of the pyruvate pathway. This work should also stimulate new research aiming at exploring the role of nuclear transcription factors in unsolved cases of mitochondrial diseases.

**Experimental Procedures**

**Accession Numbers.** The full series of data, including expression arrays and ChIP-Seq data reported in this paper were deposited on the Gene Expression Omnibus (GEO) database repository (see GEO Series SuperSeries GSE57572 and GSE57272) (24, 26). E4F1 binding regions were defined by combining bioinformatic toolboxes.
provided by CuGenome and Quest software systems. Detailed protocols, bio-
informatic tools and primers used for Chip-seq and Chip-qPCR validations were as
previously described (24, 26) and detailed in SI Experimental Procedures.

Mouse Models and Experimental Treatment. Eif1a−/−, Eif1a−/*, and Eif1a+/+ mice
(22, 40) were intercrossed with ActaCre mice to obtain Eif1a−/*; ActaCrea
Cre+/−, ActaCreaCre−/−, and ActaCreaCre−/− mice. ActaCreaCre−/− mice were
mated under chow diet (A01, Safe, i.e., 22 kcal% protein, 65 kcal% carbohydrate, and 13 kcal% fat) in the presence
or absence of DCA in the drinking water (added to the drinking water for 2 wk at a
final concentration of 2 g/L) or were fed with a ketogenic diet (F3666, Bio-Serv; 5 kcal% protein, 3 kcal% carbohydrate, and 93 kcal% fat) for 2 wk before assessing
their physical performances, as detailed in SI Experimental Procedures.

PDH Activity and Lactate Metabolism, Metabolomics. Two different pro-
tocols were used in parallel to measure PDH activity in protein extracts
prepared from cells or muscles (gastrocnemius and heart). PDH activity
DipStick-Assay Kit (dianova, L2552, Abcam) was used on 1 (25 µg) or muscle
(5 µg) extracts prepared according to the manufacturer’s protocol, and
quantified by ImageJ. PDH enzymatic activity was also assayed by mea-
suring the release of 14CO2 after incubation of protein extracts (1 mg of
protein per milliliter) with [1-14C] pyruvate, as previously described and
detailed in SI Experimental Procedures (41). To determine the concentra-
tion of glucose, 2-DG, pyruvate, citrate, and succinate in Eif1a WT and KO
TMES, extracts were prepared from 2 × 106 cells and analyzed by GC/MS
and LC/MS/MS platforms (Metabolon). Eight independent samples were
analyzed for each cell line. Lactate production by cells was measured in
culture medium using a l-Lactate Assay Kit (Eton Bioscience). Lactate and
ketone bodies concentration in blood were measured using a lactometer
(KEF Diagnostic) and β-ketone strips (Optiwork, Abbott), respectively.

Statistical Analysis. Unpaired Student’s t-test was used in all analyses. Statistical
significance was expressed as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

Supplemental Materials and Methods. Experimental procedures relative to
genotyping of animals, mice, gavage of cells, RT-qPCR analyses, SirNA-mediated
Eif1a-depletion, immunoblotting, immunohistochemistry, and immu-
nofluorescence assays, measurement of FAO, and stable isotope tracing,
insulin- and glucose-tolerance tests, and in vivo glucose uptake, are described
in SI Experimental Procedures.

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Supporting Information

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SI Experimental Procedures

Genotyping of Mouse Models. E4f1K0(Acta) and CTL(Acta) mice were genotyped by PCR on tail genomic DNA using Red-N extract kit (Sigma), with the following primers: E4f1 WT (E4f1W) and conditional KO flox alleles (E4f1flox): Fwd, 5′-CCTTGAGACGAGGAAGGACG-3′ and Rev, 5′-GCC-TGACCTGGTCTGGATC-3′.

E4f1 null allele (E4f1−/−): Fwd, 5′-CAGCCTGTGAGGAC-GTTG-3′ and Rev, 5′-CCTGTCCTAGCAGAATTG-3′. Acta-1 Cre allele: Fwd, 5′-GGCGTTCGTGACGTAAGC-3′ and Rev, 5′-GTGAAAGAGATGCTGCTAC-3′.

Endurance Test. Normal activity (average daily distance) was gauged for 24 h in cages equipped with infrared beams in the x and y dimensions. For the running test, mice were first trained to run on a treadmill (Columbus Instruments) for 10 min at slow speed (10 m/min). Seventy-two hours later, their running capacity was measured by gradually increasing speed (every 5 min) from 5 m/min to 25 m/min until exhaustion.

Insulin and Glucose Tolerance Tests. For insulin resistance (ITT) and glucose tolerance (GTT) tests, 16-week-old males were fasted 4 h or overnight, and injected intraperitoneally with 0.75 U/kg of insulin or 2 g/kg of glucose, respectively. Glucose concentration was measured with a blood glucometer (Accu-Chek, Roche).

Generation of E4f1K0 Cells and siRNA-Mediated Depletion. HaRasV12-transformed E4f1flox and E4f1flox MEFs were generated and grown as previously described (24). Conditional inactivation of the E4f1flox allele was achieved by transduction of tMEFs with retroviral particles encoding a self-excising Cre recombinase (42) produced in Platinum-E retroviral packaging cell lines (Cell Biologs). For siRNA-mediated depletion of E4f1, MEFs were transfected with 50 nM of siRNA (Sigma Mission siRNA 00060727 and 00060728) using Lipofectamine RNAmax according to the manufacturer’s recommendations.

Quantitative ChIP Assays. Detailed protocols used for ChIP, as well as Bioinformatic tools and parameters used to treat ChIP-seq data and annotate E4f1 binding regions were previously described (24, 26). Briefly, t_flash inhibitor complexes (3 × 105) or gastrocnemius muscle (100 mg) were cross-linked (1% formaldehyde), nuclei were isolated, and chromatin was extracted and sonicated (vibraCell, bioblock). E4f1 ChIPs were carried out with affinity-purified rabbit anti-E4f1 polyclonal antibody (21) and pulled down by Dynabeads coupled to protein G. Input and immunoprecipitated DNA were decross-linked, treated with RNAase A and proteinase K, and purified by phenol-chloroform-isomylalcohol extraction/precipitation and then chromatography (nucleosipin extract II columns, Macherey-Nagel). Immunoprecipitated DNAs were analyzed by qPCR (MXPro Stratagene and SYBRGreen mix) with promoter-specific primers (see below) or processed for deep-sequencing (Hi-Seq, 2000, Illumina) analyzed using the mm9 mouse genome sequence. ChIP-qPCR were performed as previously described (24) using a validated anti-E4f1 rabbit polyclonal antibody (21) and the following primers:

- Dbl: Fwd, 5′-ACACAGCGGCCACACTCTTGCACGTC-3′ and Rev, 5′-GTTCTGTGGAGAGAATGCTAC-3′.
- Dbl: Fwd, 5′-ACACAGCGGCCACACTCTTGCACGTC-3′ and Rev, 5′-ATGAACTCTTACCCGAGGGACCCGTC-3′.
- Bp4: Fwd, 5′-ACACAGCGGCCACACTCTTGCACGTC-3′ and Rev, 5′-ACAGAAGAGGGGATGATCTCGGAAA-

- Pdpr: Fwd, 5′-TCTGAGGCTCAGTGAACAATGCT and Rev, 5′-TAAAGGCCTTATCAGTGTCGGTGC-3′.
- Sk2a: Fwd, 5′-TGAAGCTCTGCACAATGTAATCAGTGAATA and Rev, 5′-TAATACGACCATATGACGTCAC-3′.
- NCI (gene-poor non coding region of chromosome 8): Fwd, 5′-AAGGGGCTCCTGTTTTTAAA and Rev, 5′-AGAGCTCCA-TGCCAGGTT-3′.
- Pdh1a: Fwd, 5′-AGGAAAGATGTGCGCCGCTTCTTA and Rev, 5′-TTCACACTCCTCCGTGGTC-3′.

14C-Pyruvate Assay to Assess PDH Activity in Protein Extract. Briefly, we measured the release of 14C02 after incubation of protein extracts [1 mg of protein per milliliter, prepared by sonication in ice-cold homogenizing buffer (100 mM phosphate buffer, 2 mM EDTA, 1 mM DTT)] with 0.2 mM [1-14C] pyruvate in the presence of 20 mM MgCl2, 0.5 mM CaCl2, 0.03 mM cytochrome C, 480 U/L cytochrome C reductase, 5 mM 3-meratinine. CS activity was measured in parallel in the same extracts and used as a reference to normalize PDH activities (41).

RNA Extraction and RT-qPCR. mRNA expression was evaluated in tMEFs and muscles by RT-qPCR. Cells or muscles were lysed in TRIZOL reagent (Invitrogen), and total RNAs were isolated according to the manufacturer’s recommendations and loaded on eukaryote total RNA 6000 nano chips (Agilent) to verify quality. cDNAs were synthesized from 1 μg of total RNA using random hexamers and SuperScript III Reverse transcription (Invitrogen). Real-time qPCR was performed on a LightCycler 480SW 1.5 apparatus (Roche) with Platinum Taq DNA polymerase (Invitrogen) and an SYBR Green mix containing 3 mM MgCl2 and dNTPs 30 μM each: 45 cycles of 95 °C for 4 s, 65 °C for 10 s, and 72 °C for 30 s. Rpl31a transcripts were used for normalization. Primers sequences were as follows:

- E4f1: Fwd, 5′-CTCAAGGCCCCACATGTTGAA and Rev, 5′-CACA-CCTGCCCCATTCAGAGGATCC and Rev, 5′-TACCTCCTCTCGTGGTGAAG.
- Dila: Fwd, 5′-TTGCTTTCGCTGAAAGTTCC and Rev, 5′-TTACCTCCTCTCGTGGTGAAG.
- Dlc: Fwd, 5′-TGAGATGCGTGTTGACCCGTTCCCTT and Rev, 5′-GGACTTGAAGAATGCCTCAGAACTGGCCGCA-3′.
- Sk2a: Fwd, 5′-TGACGTTGAGATTTGTCACCCTGT and Rev, 5′-AGAAGTTCTTGGCTTCCTCCCTTCTT.
- Bp4: Fwd, 5′-TCCAGAGATTATCATGGTGCCGAGT and Rev, 5′-GCCATTTGTTGTCCTCGCTTCTT.
- Pdpr: Fwd, 5′-GAACAAAGAAACAGGGAATCCAAAC and Rev, 5′-CTCGTTAGATGACGCTCAGAG.
- Pdh1a: Fwd, 5′-GCTGGTGGTCGCTCGTGAAT and Rev, 5′-TGATCCTGGACCTAACCCACTCTTCTTCCT.
- CS: Fwd, 5′-TCTGACCTGGAACGAGAC and Rev, 5′-TGOAGGACAAGATGCTGCGGCAT.

Protein Extraction, Immunoprecipitation, and Western Blotting. Total protein extracts were prepared by lysing cells or muscles extracts in Triton X-100 lysis buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM Na orthovanadate, 10−6 M PMSE, 10−6 M leupeptin, 10−6 M pepstatin A, and 1% Triton X-100). Protein extracts were separated by SDS/PAGE and transferred to nitrocellulose membranes, blocked in TBS containing 5% (wt/vol) nonfat milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. The following antibodies were used: anti-E4f1 (2), DLAT (sc32925, Santa Cruz), DLD (sc135027, Santa Cruz), BRP44L/MPCI (HPA045119, Sigma), and Lipid drop (ab58724, Abcam).

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and 437695, Calbiochem), VDAC1 (ab14734, Abcam), Phospho (Ser300) PDHE1-A type I (ABS 204, Millipore), HSP70 (MA3-028, thermo Fisher Scientific), DLST (ab95946, Abcam), and Tubulin (T9026, Sigma).

**Immunostainings and Muscle Fiber Size Distribution.** Muscle biopsies were fixed in 4% (vol/vol) neutral-buffered formalin (VWR Chemicals) for 24 h and paraffin-embedded tissues were sectioned (4 μm) and processed for immunostainings or for H&E staining. Immunostainings were performed on muscle sections and tMEFs using anti-lipidic acid (437695, Calbiochem), anti-DLAT (H-160, Santa Cruz), and glucose transporter GLUT1 (ab15309, Abcam) antibodies in PBS supplemented with 1.5% (wt/vol) BSA (Roche). Alexa488-conjugated secondary anti-rabbit IgG antibody (ThermoFisher) was incubated for 2 h at room temperature. Cover glasses were mounted with Mowiol (Biovalley). To measure the size of muscle fibers, gastrocnemius muscles were frozen in Tissue-Tek OCT (Sakura), cryosections (10 μm) were stained with Azurin dye, and fiber size and banding were analyzed and quantified (ImageJ software) by high-resolution light microscopy, as previously described (43). For immunofluorescence analyses, tMEFs were fixed with methanol (Sigma) at −20 °C for 5 min.

**FAO.** FAO was measured in triplicates by quantifying the production of H2O2 from [9,10-3H]palmitate. Briefly, the cells were trypsinized, counted, plated at 5 × 104 cells per well in 12-well culture plates, and allowed to grow for 2 d. Cultured cells were washed three times with Dulbecco’s PBS. Then, 200 μL of [9,10(n)-3H]palmitic acid (60 Ci/mmol; NEN) bound to fatty-acid-free albumin (final concentration 125 μM) containing 1 mM carnitine was added per well. After 2-h incubation at 37 °C, the mixture was removed and added to a tube containing 200 μL of cold 10% (vol/vol) TCA. The tubes were then centrifuged for 10 min at 2,200 × g at 4 °C, and aliquots of supernatants (350 μL) were removed, mixed with 55 μL of 6 M NaOH, and applied to ion-exchange resin (Dowex). The columns were washed twice with 750 μL of water, and the eluates were counted. The results of FAO were normalized according to the total protein content that was determined by the BCA method (Pierce).

**Annexin Staining.** tMEFs were incubated with the FAO-inhibitor etomoxir (100 μM final, 24 h) before staining with Annexin-V-FITC (Invitrogen) and propidium iodide and then analyzed by flow-cytometry (FACS Calibur; BD).

**In Vivo Glucose Uptake.** Glucose uptake was assessed using the Glucose uptake-Glo assay kit (Promega). Briefly, E4f1KO(Acta) and control littermates were injected intraperitoneally with 100 mg/kg of unlabeled 2-deoxyglucose (2DG) and then subjected to an acute exercise (10 min). Skeletal muscles (gastrocnemius) of these animals were harvested as fast as possible and snap frozen in liquid nitrogen. Tissues were lysed in PBS/0.2 N NaOH/0.5% DTAB and 2DG-GloP was measured according to the manufacturer’s instructions.

**Stable Isotope Tracing Experiments.** E4f1KO tMEFs and match control cells were incubated in DMEM without glucose (01-056-01, Clinsciences) supplemented with 4.5 g/L 13C6-p-glucose (CLM-1393-1, Euriso-Top) for 30 min, 6 or 24 h. Intracellular metabolites were sampled according to Martano et al. (44). Central metabolites were extracted at −20 °C with 8 mL of acetonitrile/methanol/water-0.1% of formic acid (2:2:1), except for AcCoA that was extracted at −20 °C with 12 mL of a solution containing 125 mM formic acid in 80% (vol/vol) methanol at pH 2.9. Samples were evaporated in a Rotavap RII (Buchi), and resuspended in 100 μL of water for analysis of central metabolites, or in 100 μL of a solution of 25 mM ammonium formate with 2% (vol/vol) of methanol for analysis of AcCoA. Debris were removed by centrifugation at 10,000 × g for 5 min and samples were then analyzed by LC-MS. Carbon isotopolog distributions were expressed relative to the sum of all analyzed isotopologs.

![Fig. S1. Impact of E4f1 depletion on PDC. (A) Protein levels of E4f1, DLAT, DLD, MPC1, and of the loading control Tubulin, determined by immunoblotting of total protein extracts prepared from tMEFs, 3 d after transfection of control or E4f1-siRNAs. (Right) Quantification of this representative experiment using image. Data were normalized according to Tubulin levels. (B) PDH activity measured by DipStick assay in protein extracts prepared from tMEFs, 3 d after transfection of control or E4f1-siRNAs. Histograms represent the mean value ± SEM (n = 3). **P < 0.01.](image-url)
Fig. S2. Metabolic consequences of PDH deficiency in E4F1KO cells. (A) The mass isotope distribution (without correction for natural isotope abundance) for AcCoA, citrate, glucose-6 phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F16BP), 2,3-bisphosphoglycerate (2/3PG) was determined by LC-MS in E4F1KO tMEFs and match control cells cultured with Na1/[U-13C]glucose for 30 min, 6 or 24 h, as indicated (mean ± SD). (B) Immunofluorescence staining of lipoylated proteins in E4F1KO and CTR tMEFs. Scale bar, 50 μm. (C) Protein levels of DLST and of the loading control Tubulin, determined by immunoblotting of total protein extracts prepared from E4F1KO tMEFs and match control cells. (D) Total ATP level (Left) and the AMP/ATP ratio (Right) were determined in E4F1KO and control (CTR) tMEFs. Histobars represent the mean value ± SEM of n = 3 independent experiments. (E) FACS analysis of apoptotic cells in E4F1KO and control (CTR) tMEFs, 24 h after incubation with the FAO-inhibitor etomoxir. Histobars represent the mean value of annexin-V cells ± SEM (n = 5 independent experiments). All analyses were performed in E4F1KO tMEFs and match control cells, 5 d after transduction with a self-excision Cre-encoding retrovirus. *P < 0.05.
Fig. S3. E4F1 regulates the PDC in skeletal muscles. (A) ChIP-qPCR experiments performed with an anti-E4F1 antibody on the promoter region of Dilat and Dld in murine striated skeletal muscles of E4F1\(^{KO/ACTA}\) mice and CTL\(^{ACTA}\) littersates. A gene-poor noncoding region of chromosome 8 (NC1) and the Pdha1 promoter region (TSS) were used as controls. Enrichments are represented as percentages of input (mean value ± SEM of \(n = 3\) independent experiments). (B) mRNA levels of E4F1, Dilat, Dld, Slic25a19, Brp4aIImpc1, Pdha1, and of two control genes (CS and Pdha1) determined by RT-qPCR analysis in the heart of E4F1\(^{KO/ACTA}\) mice and CTL\(^{ACTA}\) littersates. Histobars represent the mean value ± SEM of \(n = 6\) animals per experimental group. (C) PDH activity (arbitrary unit) measured in protein extracts prepared from heart of resting 16 wk-old E4F1\(^{KO/ACTA}\) and CTL\(^{ACTA}\) animals. Histobars represent the mean value ± SEM of \(n = 6\) animals per experimental group. (D) CS activity (arbitrary unit) measured in protein extracts prepared from gastrocnemius of resting 16 wk-old E4F1\(^{KO/ACTA}\) and CTL\(^{ACTA}\) animals. Histobars represent the mean value ± SEM of \(n = 2\) animals. *\(P < 0.05\); ns, not significant.
Fig. 5A. E4f1 deficiency does not induce morphological alterations of skeletal muscles in young E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> mice but results in progressive skeletal muscle disorganization and histological alterations during aging. (A) H&E staining of striated muscle sagittal sections prepared from 16-wk-old (Left) or 18-mo-old (Right) E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> males and control littermates. Note that old E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> animals display myophagocytosis, hypercontracted fibers, centralized regenerative fibers, and evidence of replacement of muscular cells by adipocytes. (Scale bars, 200 µm.) (B) Fiber size distribution (cross-sectional area) in skeletal muscles of 16-wk-old E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> and control animals. Histobars represent the mean value ± SEM of cross sectional area of muscular fibers (arbitrary unit) (n = 5 males per experimental group). (C) Spontaneous locomotor activity of 16-wk-old E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> males and control littermates was determined using an automated system measuring infrared beam break. Histobars represent the number of times the animals broke the beam by spontaneous locomotor activity in the cage during 24 h (mean value ± SEM, n = 5 animals per group). (D, Left) Body weight of E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> males and control littermates was measured over a period of 4 mo. (Right) Histobars represent the food intake of 16-wk-old E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> males and control littermates, measured over 96 h (mean ± SEM, n = 6 animals per group). (E) mRNA levels of the muscular differentiation markers and inducers Mif6, Mef2c, MyoD, and Myogenin determined by RT-qPCR analysis in adult striated muscles of 16-wk-old E4f1<sup>C<sub>KO</sub>A<sub>C</sub></sup> and control mice. Histobars represent the mean value ± SEM (n = 5 animals per experimental group). ns, not significant.
Fig. S5. E4F1 controls PDC in red and white muscle fibers. (A) PDH activity (arbitrary unit) measured in protein extracts prepared from extensor digitorum longus (EDL) and soleus striated muscles of resting 16 wk-old E4F1<sup>+/+Acta</sup> and Acta<sup>−/−</sup> animals, as indicated. Histobars represent the mean value ± SEM of n = 5 animals per experimental group. (B) Protein levels of DLAT in EDL and soleus muscles was determined by immunoblotting of total protein extracts prepared from two independent E4F1<sup>+/+Acta</sup> and Acta<sup>−/−</sup> animals. Red ponceau staining of the same membranes was performed to ensure equal loading (Lower). *P < 0.05.
Fig. S6. Phenotypic characterization of E4fryKO/ACTA animals. (A) Locomotor performances (running time before exhaustion) of E4fryKO/ACTA and CTL/ACTA males under chow or ketogenic (KETO) diets, or under chow diet in presence of DCA, were evaluated using forced treadmill running. Three independent measurements were performed 1 d apart for each animal. Histograms represent the mean value ± SEM of n = 8 males for each experimental group. (B) Lactate level in the serum of resting 16 wk-old E4fryKO/ACTA and CTL/ACTA animals. Histograms represent the mean value ± SEM of n = 6 animals per experimental group. (C and D) Intraperitoneal glucose tolerance test (IPGTT) (C) and ITT (D), performed on E4fryKO/ACTA and control littermates (mean ± SEM, n = 5 males per group). (E) Relative glucose uptake was measured in gastrocnemius of E4fryKO/ACTA and CTL/ACTA males after an acute exercise workload. Histograms represent the mean value ± SEM of n = 4 males for each experimental group. (F) GLUT1 immunofluorescence performed on striated muscle (gastrocnemius) tissue sections prepared from E4fryKO/ACTA and CTL/ACTA animals. Sections were counterstained with DAPI. (Scale bar, 200 μm.) **P < 0.01; *P < 0.05; ns, not significant.
PUBLICATION #2
III. Keratinocyte specific mouse models

A. K14CreERT2 transgenic mouse

To investigate the function of E4f1 in adult skin, I crossed E4f1 conditional knockout mice (E4f1 cKO) with K14CreERT2 transgenic animals. This mouse strain expresses a tamoxifen-inducible version of the Cre recombinase fused to the estrogen receptor (ER) under the control of the Keratin 14 (K14) promoter (Vasioukhin et al., 1999). In this model, Cre-mediated recombination occurs specifically in the basal layer of the epidermis, which contains epidermal stem cells and progenitor cells (Figure 39).

![Figure 39: Basal layer specific inactivation of E4f1. E4f1 flox mice were crossed with K14CreERT2 transgenic mouse expressing CreER recombinase under the promoter of Keratin 14 that is expressed in basal layer of epidermis. Cre-mediated recombination of E4f1 allele was obtained by topical Tamoxifen application.](image)

B. K10CreERT2 transgenic mouse

I also crossed E4f1 cKO animals with K10CreERT2 mice in order to inactivate E4f1 in differentiated keratinocytes. This mouse strain expresses the tamoxifen-inducible CreER72 recombinase under the control of the Keratin 10 (K10) promoter. This drives the recombination specifically in the suprabasal layer of the epidermis, which contains differentiated keratinocytes (Figure 40).

![Figure 40: Suprabasal layer specific inactivation of E4f1. E4f1 flox mice were crossed with K10CreERT2 transgenic mouse expressing CreER recombinase under the promoter of Keratin 10 that is expressed in suprabasal layer of epidermis. Cre-mediated recombination of E4f1 allele was obtained by topical Tamoxifen application.](image)
SUMMARY

The multifunctional protein E4F1 is an essential regulator of normal skin homeostasis. E4F1 inactivation in embryonic or adult skin results in stem cell autonomous defects causing exhaustion of the epidermal stem cell (ESC) pool from their niche. At the molecular level, we showed that E4F1 controls ESC maintenance through the transcriptional regulation of components or major regulators of the pyruvate dehydrogenase (PDH) macro complex, which metabolizes pyruvate into acetyl-coA in order to link glycolysis to the Krebs cycle. Our data demonstrates that defective PDH activity in E4F1KO keratinocytes results in the redirection of glycolytic flux towards lactate secretion both in vivo and in vitro. This metabolic reprogramming correlates with alterations of the microenvironment and affects ESC adhesion in their niche. Thus, our data highlights for the first time how pyruvate metabolism impacts on ESC maintenance and skin homeostasis.
E4F1-mediated control of pyruvate dehydrogenase activity is essential for skin homeostasis

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The multifunctional protein E4 transcription factor 1 (E4F1) is an essential regulator of epidermal stem cell (ESC) maintenance. Here, we found that E4F1 transcriptionally regulates a metabolic program involved in pyruvate metabolism that is required to maintain skin homeostasis. E4F1 deficiency in basal keratinocytes resulted in deregulated expression of dihydroxyacetone acetyltransferase (D4AT), a gene encoding the E2 subunit of the mitochondrial pyruvate dehydrogenase (PDH) complex. Accordingly, E4F1 knock-out (KO) keratinocytes exhibited impaired PDH activity and a redirection of the glycolytic flux toward lactate production. The metabolic reprogramming of E4F1 KO keratinocytes associated with remodeling of their microenvironment and alterations of the basement membrane, led to ESC mislocalization and exhaustion of the ESC pool. SHRNA-mediated depletion of D4AT in primary keratinocytes recapitulated defects observed upon E4F1 inactivation, including increased lactate secretion, enhanced activity of extracellular matrix remodeling, increased migratory potential, and impaired clonogenic potential. Altogether, our data reveal a central role for D4AT in the metabolic program regulated by E4F1 in basal keratinocytes and illustrate the importance of PDH activity in skin homeostasis.

E4F1 | PDH | pyruvate | skin | stem cell

Renewal and wound healing of the epidermis rely on a pool of epidermal stem cells (ESC) located in the basal layer of the interfollicular epithelium (IFE) and in the bulge region of the hair follicle (HF). In the IFE, these long-lived ESC give rise to progenitors with increased proliferative capacities that differentiate into keratinocytes as they migrate upward into suprabasal layers. Numerous studies have addressed the role of several key signaling pathways, such as those implicating bone morphogenetic proteins, TGF-β, Notch, Sonic Hedgehog, or Wnt in skin homeostasis, and how they control ESC maintenance (1–3). The role of these pathways in regulating stemness has been attributed to the regulation of cell proliferation, cell death, cellular senescence, cell adhesion, or differentiation. Although previous data indicate that some of these stem cell regulators also control energy metabolism in the hematopoietic or neuronal lineages (4), few studies have yet addressed their metabolic functions in keratinocytes. In addition, the potential role of specific metabolic regulators in the control of skin homeostasis remains poorly documented. Nevertheless, previous observations indicate that deregulation of the nutrient-sensing mammalian target of rapamycin pathway in basal keratinocytes occurs as a consequence of prolonged Wnt signaling, leading to the progressive exhaustion of HF bulge stem cells (5). Recent data also indicate that genetic inactivation in mouse epidermis of mitochondrial transcription factor A (Tfam), a gene involved in mitochondrial DNA replication and transcription, impinges on keratinocyte differentiation but does not impair maintenance of basal keratinocytes (6). Although these results suggest that basal keratinocytes display a metabolic status that is different from their differentiated counterparts, further studies are warranted to decipher the poorly understood role of metabolism in the regulation of epidermal cell fate.

We previously identified the multifunctional protein E4 transcription factor 1 (E4F1) as an essential regulator of skin homeostasis and ESC maintenance (7). E4F1 was originally identified as a cellular target of the E1A viral oncoprotein (8, 9). Since then, several laboratories have shown that E4F1 directly interacts with several oncopgenes and tumor suppressors, including p53, BMI1, RB, RASSF1A, SMAD4, or HMG2A proteins (10–16). Consistent with its implication in different oncogenic pathways, E4F1 acts as a survival factor in cancer cells (17, 18). Moreover, characterization of E4F1 knock-out (KO) mice showed that E4F1 is an essential gene in embryonic stem cells and during early embryogenesis (19). Using E4F1 conditional KO mice, we previously reported that E4F1

Significance

We found that the multifunctional protein E4 transcription factor 1 (E4F1) transcriptionally regulates a metabolic program involved in pyruvate metabolism that is required to maintain skin homeostasis. E4F1 deficiency in basal keratinocytes resulted in deregulated expression of dihydroxyacetone acetyltransferase (D4AT), a gene encoding the E2 subunit of the mitochondrial pyruvate dehydrogenase (PDH) complex. Accordingly, E4F1 knock-out (KO) keratinocytes exhibited impaired PDH activity and a metabolic reprogramming associated with remodeling of their microenvironment and alterations of the basement membrane, leading to epidermal stem cell mislocalization and exhaustion of the epidermal stem cell pool. Our data reveal a central role for D4AT in the metabolic program regulated by E4F1 in skin and illustrate the importance of PDH activity in skin homeostasis.


The authors declare no conflict of interest.

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inactivation in the epidermis results in ESC defects through a mechanism that involves, at least partly, the deregulation of the Bmi1–Arf–p53 pathway (7). Here, we show evidence supporting a major role for E4F1 in pyruvate metabolism that governs ESC maintenance and skin homeostasis.

Results
E4F1 Inactivation in Basal but Not Suprabasal Adult Keratinocytes Leads to Epidermal Defects and Exhaustion of the ESC Pool. Using E4f1 whole-body conditional KO mice (E4f1KO, RERT), we previously identified an essential role for E4f1 in adult skin homeostasis (7). In this genetically engineered mouse model, E4f1 inactivation was achieved in the entire skin or, in the case of compound knockouts, the entire skin or, in the case of compound knockouts, including the dermal compartment. To assess the cell of origin of these skin defects, we generated new mouse strains by crossing E4f1 conditional KO mice to transgenic animals expressing the tamoxifen (tam)-inducible CreER recombinase under the control of the keratin 14 (K14) or keratin 10 (K10) promoters [hereafter referred to as E4f1(K14)KO and E4f1(K10)KO strains], allowing acute inactivation of E4f1 in adult keratinocytes of the basal or spinous layers, respectively (20).

Molecular and histological analyses of adult back skin of 8- to 12-week-old E4f1(K10)KO animals confirmed that topical skin applications of tam activated the Cre recombinase in suprabasal but not in basal keratinocytes (Fig. S1). Neither histological alterations nor differences in the expression pattern of the basal-cell specific K14 marker and of the differentiation markers K10 and involucrin were identified in skin samples harvested up to 4 mo upon regular tam administration (Fig. S2). In sharp contrast, inactivation of E4f1 in adult basal keratinocytes of E4f1(K14)KO mice resulted in skin phenotypes that recapitulated those originally observed in tam-treated E4f1-KO, RERT adult mice. Thus, 2 wk after tam administration, E4f1(K14)KO mice displayed epidermal hyperplasia (acanthosis, thickened dermis), and a thinner and parakeratotic cornified layer (Fig. L4 and Fig. S3). Aberrant hyperproliferation and mislocalisation of basal keratinocytes was evidenced in E4f1-deficient epidermis by increased Ki67 staining, the presence of K14+ cells in suprabasal layers, and the expression of keratin 6 (K6) in the IFE (Fig. S4). At later time points (4–5 wk after tam administration), E4f1-deficient epidermis then became hypocellular, whereas the hyperkeratosis remained evident (Fig. L4). In addition, impaired keratinocyte differentiation was illustrated by aberrant expression of K10 and involucrin in K14–/– skin (Fig. S4).

Consistent with our previous observations, ablation of E4f1 in adult basal keratinocytes altered ESC function and resulted in the definitive exhaustion of the ESC pool. Indeed, tam administration to E4f1(K14)KO mice led to the loss of expression of the bulge HF stem cell marker keratin 15 (K15) (Fig. L8). Flow cytometry analysis of HF stem cells identified by the coexpression of CD34 and high levels of α6-integrin (CD34+/α6(int)) confirmed that tam-treated E4f1(K14)KO adult epidermis contained fewer HF stem cells compared with control epidermis (0.65% ± 0.15% vs. 2.25% ± 0.4% (Fig. L4). We also tracked ESCs in the IFE by analyzing the number of label retaining cells (LRCs) using an adaptation of an in vivo labeling protocol of multipotent ESC based on the utilization of the nucleotide analog ethynyl-2′-deoxyribose (EdU) (21). These analyses showed that E4f1 inactivation resulted in a significant decrease in the number of LRCs 5 wk after tam administration, indicating that E4f1 deficiency led to the definitive loss of ESC in vivo (Fig. L1D). Finally, E4f1/KO ESC defects were also illustrated ex vivo by their impaired clonogenic potential (Fig. L5).

Thus, these data demonstrate that the epidermal hyperplasia, hyperkeratosis, differentiation defects, and exhaustion of the ESC pool of E4f1-deficient epidermis originate from alterations in basal rather than suprabasal keratinocytes.

Fig. 1. E4f1 deficiency in basal keratinocytes leads to skin defects and exhaustion of the ESC pool. (A) Microphotographs of (HES-stained) skin sections prepared from E4f1(K14)KO mice or E4f1(K14)CTR littermates, 1, 2, or 5 wk after tam administration. Dashed lines indicate the separation between the epidermis and the dermis. (Scale bars, 100 μm). (B) Whole mounts of tail epidermis prepared from adult E4f1(K14)KO and CTR mice, 5 wk after tam application, stained with K15 antibody and DAPI. Brackets: bulge area (BG) of the HF. (Scale bar, 100 μm.) (C) Number of follicular stem cells (FSC) in back epidermis prepared from the same mice as in B; FACS analysis of CD34+ CD24+CD45- FSC in back skin epidermis prepared from the same mice as in B (mean ± SEM; n = 10). (D) Number of label-retaining (EdU+1) interfollicular stem cells (LSCs) detected by immunofluorescence (IF) on back-skin sections prepared from adult E4f1(K14)KO mice or E4f1(K14)CTR littermates, 5 wk after tam application. Histograms represent the mean value ± SEM of EdU+ cells per millimeter of epidermis (n = 5 animals per group). Each clonogenic assays performed with E4f1KO and CTRL primary murine keratinocytes cultured in presence or absence of 40HT, as indicated (n = 5). Histograms represent the total number of clones per well relative to control cells (expressed as percentages) determined after rhodamine B staining. ***P < 0.001; **P < 0.01; ns, not significant.

E4F1 Controls Pyruvate Metabolism in Keratinocytes Through Transcriptional Regulation of the E2 Subunit of the Pyruvate Dehydrogenase Complex Diast. Using a pan-genome ChIP approach combined with next-generation sequencing (ChIP-seq), we identified E4f1 binding sites at the whole-genome level in primary mouse embryonic fibroblasts and embryonic stem cells (18, 12). Functional annotation of E4f1 direct target genes indicated a significant enrichment in genes implicated in metabolism, including a set of five genes encoding core components or regulators of the mitochondrial pyruvate dehydrogenase (PDH) complex (PDC), a multicompartment complex that converts pyruvate into Acetyl-CoA (AcCoA). In embryonic stem cells, this set of E4f1-controlled genes includes the E2 and E3 subunits of the PDC, dihydroxyacetone dehydrogenase (Dld), the regulatory subunit of the PDC phosphatase complex (Pdpr), the pyruvate transporter of the inner mitochondrial membrane (Bhp44/Mpc1) (23, 24), and the mitochondrial transporter Slc25a19 that transports the PDH cofactor thiamine pyrophosphate (25). These results prompted us to evaluate whether E4f1 also controlled this set of PDH-related genes in primary keratinocytes. First, we confirmed by quantitative ChIP that endogenous E4f1 was recruited to the promoter of Dld, Dld, Slc25a19, and Bhp44 in cultured primary murine keratinocytes (Fig. 2A). Similarly to E4f1KO fibroblasts and muscle cells, E4f1-deficient keratinocytes displayed a marked decrease of Dld mRNA level. However, the expression of Dld, Pdpr, Slc25a19, and Bhp44/Mpc1 remained unchanged upon E4f1 inactivation, suggesting that other E4f1-independent mechanisms contribute to their expression in keratinocytes (Fig. 2B). The mRNA levels of other PDH-related genes, including Pdhb1, which encodes the E1
subunit of the PDC, the PDH -kinases 1/4 (Pdk1, Pdk4) and -phosphatases 1/2 (Pdp1, Pdp2) remained unchanged in E4f1-deficient keratinocytes (Fig. S5). Decreased Dlat expression was confirmed at the protein level, as shown by immunostaining of skin samples prepared from E4f1(K14)KO animals, 1 wk after tam-administration. This decrease was further confirmed by immunoblotting both in tam-treated E4f1(K14)KO epidermis and in cultured E4f1KO primary keratinocytes (Fig. 2 C-E). Consistent with DLAT deregulation, altered PDH enzymatic activity was detected in these cells (Fig. 2 F and G and Fig. S6D). Taken together, these data indicate that the E2 subunit of the PDC Dlat is a major direct transcriptional target of E4f1 in basal keratinocytes.

**E4f1KO Results in Metabolic Reprogramming of Keratinocytes.** Next, we characterized the metabolic consequences of impaired PDH activity in E4f1-deficient keratinocytes. We postulated that decreased PDH activity in E4f1-deficient keratinocytes triggered a decrease of glucose-derived AcCoA production and the redirection of the glycolytic flux toward lactate production (Fig. 3A). Consistent with this hypothesis and the role of AcCoA as a donor substrate for acetylation reactions, E4f1KO keratinocytes exhibited decreased histone H4 acetylation, as shown by immunoblotting using an anti-pan-acetyl lysine histone H4 antibody (Fig. 3B). Moreover, in line with increased pyruvate metabolism by the NADH-dependent lactate dehydrogenase (LDH), E4f1KO keratinocytes displayed an increased NAD+/NADH ratio (Fig. 3C). Increased expression of the glucose transporter GLUT1 suggested that glucose uptake increased upon E4f1 inactivation in keratinocytes (Fig. S6B). These cells also exhibited increased expression of the monocarboxylate transporter MCT4 that favors the efflux of lactate outside the cell (Fig. 3 D and E). Accordingly, increased lactate secretion by E4f1KO keratinocytes was evidenced by a change in their extracellular acidification rate (ECAR) (Fig. 3F). Other metabolic changes were observed in E4f1-deficient keratinocytes, as illustrated by increased fatty acid oxidation (FAO) (Fig. 3G). This adaptive metabolic response was likely sufficient to sustain mitochondrial respiration because no significant difference was observed in oxygen consumption upon E4f1 inactivation in keratinocytes cultured in complete medium (Fig. S6D). Analyses of tam-treated E4f1(K14)KO mice and control littermates confirmed that E4f1-deficient keratinocytes underwent the same metabolic
reprogramming in vivo. Thus, immunohistochemistry (IHC) analysis of skin samples prepared from these animals indicated that E4f1 inactivation in basal keratinocytes resulted in the increased expression of GLUT1, MCT4, and of CD147/BASIGIN, a chaperone required for MCT4 relocalization at the cytoplasmic membrane (Fig. 3H and Fig. S6C). Strikingly, E4f1(K14)KO mice exhibited lactic acidemia and increased level of circulating ketone bodies, a by-product of FAO (Fig. 3 I and J). Moreover, the clonogenic potential of E4f1KO keratinocytes was partly rescued by addition of exogenous acetate that can replenish AcCoA pools (Fig. 3K), confirming that the profound metabolic reprogramming of E4f1-deficient keratinocytes impinged on their biological functions.

**Metabolic Reprogramming of E4f1-Deficient Keratinocytes Associates with Remodeling of the Microenvironment and Loss of Adhesion of the ESC with the Basement Membrane.** In many tumors, increased lactate secretion has been linked to the remodeling of the extracellular matrix (ECM) and degradation of the basement membrane (BM) by ECM-remodeling enzymes (26). To further characterize the consequences of the metabolic reprogramming of E4f1-deficient keratinocytes, we performed histological analyses of E4f1(K14)KO skin. Electron microscopy analyses indicated that E4f1 inactivation in basal keratinocytes resulted in disorganization of the BM, which appeared either diffused with thinner lamina densa or focally disrupted (Fig. S7A). Alterations of the BM in E4f1KO skin was confirmed upon staining of skin sections by the Gomori reticulin method, which stains the argyrophilic (silver staining) fibrous structures present in the BM (Fig. S7B). Immunostaining of skin samples prepared from E4f1(K14)KO mice with anti-lamin V antibody showed that the expression pattern of this essential component of the BM was diffused and focally discontinuous in E4f1KO skin sections compared with its defined and continuous pattern in control samples (Fig. 3A). This defect also correlated with an abnormal expression pattern of integrin pα4 (Itgb4). In areas showing broad disruption of the BM, Itgb4 expression was not restricted to the basal pole of keratinocytes but was also detected at the apical or lateral sides of both basal and suprabasal keratinocytes (Fig. 4A). Remodeling of the ECM within the dermal compartment was also evidenced by picro-Sirius red staining of collagen fibers on skin sections prepared from tam-treated E4f1(K14)KO mice (Fig. S7C). These results led us to investigate whether the massive remodeling of the ECM and alterations of the BM observed upon E4f1 inactivation resulted from increased activity of ECM-remodeling enzymes. Increased matrix metalloproteinase 9 (MMP9) and cathepsin activities were detected by gelatin-zymography in protein extracts prepared from total skin samples of tam-treated E4f1(K14)KO mice (Fig. 4B and Fig. S7D). Moreover, increased MMP2, MMP9, and cathepsin activities were also evidenced in the culture medium of E4f1KO primary keratinocytes (Fig. 4 C and D). Addition of the LDH-inhibitor oxamate in the culture medium decreased cathepsin activities, confirming that their induction resulted from the metabolic reprogramming of these cells (Fig. 4D). Moreover, stable expression of ectopic TIMP1, a broad MMP inhibitor, in feeder cells partly rescued the clonogenic potential of E4f1KO ESC (Fig. 4E). Improved clonogenicity of E4f1-deficient ESC was also observed upon incubation with GM6001, a pharmacological MMP inhibitor with broad spectrum (Fig. 4F). Taken together, these data indicate that the induction of ECM remodeling enzymes in E4f1-deficient keratinocytes is a consequence of their metabolic reprogramming and impinges on their clonogenic potential.

Based on these results, we hypothesized that the observed disruption of the BM impacted on the maintenance of ESC within their normal microenvironment, leading to the definitive exhaustion of the ESC pool. To test this hypothesis, we analyzed EdU+ LRCs on skin sections prepared from E4f1(K14)KO mice or control littermates 2 wk after tam administration and evaluated their localization within the epidermis. The same skin sections were also processed to assess MCT4 expression as a surrogate marker of the metabolic reprogramming of E4f1KO

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**Fig. 4.** E4f1 inactivation in basal keratinocytes results in alterations of the BM, remodeling of the ECM and ESC mislocalization. (A) IP analysis of Lamin V (Lam V, red) and Int4 (green) expression in skin sections prepared from E4f1(K14)KO and CTR mice, 2 wk after tam administration. Sections were counterstained with DAPI (blue). (Scale bars, 50 μm.) (B) Representative zymogram analysis of MMP activities in protein extracts prepared from skin samples of E4f1(K14)KO and CTR mice, 2 wk after tam administration. (C) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (D, E) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (F) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (G) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (H) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (I) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (J) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (K) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (L) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (M) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (N) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (O) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (P) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (Q) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (R) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (S) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (T) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (U) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (V) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (W) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (X) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (Y) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (Z) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (AA) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (BB) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (CC) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (DD) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (EE) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (FF) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (GG) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (HH) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (II) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (JJ) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (KK) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (LL) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (MM) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (NN) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (OO) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (PP) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (QQ) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (RR) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (SS) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (TT) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (UU) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (VV) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (WW) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (XX) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (YY) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (ZZ) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group). (AAA) Representative zymogram analysis of MMP activities (mean ± SEM, n = 5 animals per group).
keratinocytes. Two weeks after tam administration, E4f1 KO epidermis displayed approximately the same number of EdU+ LRCs than control epidermis (0.25 ± 0.05 vs. 0.23 ± 0.02 per millimeter, respectively). However, the number of E4f1 KO LRCs in a suprabasal position was significantly increased compared with LRCs of control epidermis that remained, as expected, in the basal layer (suprabasal: 0.1 ± 0.04 vs. 0.01 ± 0.01; basal: 0.14 ± 0.03 vs. 0.21 ± 0.003 per millimeter, respectively) (Fig. 4G).

Interestingly, this atypical feature of E4f1 KO LRCs was particularly evident within focal epidermal lesions exhibiting MCT4 positivity, whereas LRCs remained in contact with the BM in the adjacent MCT4+ areas of the same epidermis (Fig. 4G). Five weeks after tam administration, the number of LRC diminished in E4f1 KO epidermis, confirming that E4f1 inactivation finally ended in the exhaustion of the ESC pool (Fig. 1D). Thus, these data show that the metabolic reprogramming triggered by E4f1 inactivation in basal keratinocytes associates with the remodeling of their microenvironment and alterations of the BM, leading to the loss of attachment of the ESC within their normal niche and their definitive loss.

**DLAT Is a Central Component of the E4f1-Regulated Metabolic Program in Basal Keratinocytes.** Because Dlat appeared as one of the most deregulated E4f1 target genes in the epidermis of tam-treated E4f1 (K14)KO animals, we evaluated the consequences of shRNA-mediated depletion of Dlat in primary keratinocytes. Lentiviral-mediated delivery of shRNAs targeting Dlat in populations of primary murine keratinocytes led to an expected decrease of DLAT protein level and PDH activity (Fig. S4 and Fig. S8A) and induced phenotypes that were reminiscent of those observed in E4f1 KO keratinocytes. Thus, DLAT depletion in primary keratinocytes resulted in their metabolic reprogramming, as illustrated by increased MCT4 expression, and increased MMP2, MMP9, and cathepsin activities (Fig. 5B and C and Fig. S8 B and C). Furthermore, similarly to E4f1-deficient ESC, DLAT-depleted keratinocytes also displayed an alteration of their clonogenic potential (Fig. 5D). Taken together, these data strengthen the role of DLAT as a central component of E4f1-regulated metabolic program in primary keratinocytes.

**Discussion**

Our analyses performed in different mouse models where E4f1 was genetically inactivated in the basal or the spinous layers of the epidermis show that the complex skin phenotypes observed upon E4f1 inactivation originate from defects in basal keratinocytes. Our results indicate that E4f1 deficiency in these cells leads to a metabolic reprogramming of keratinocytes that affects skin homeostasis and ended in the definitive exhaustion of the ESC pool. We found that this metabolic shift, which includes the redirection of the glycolytic flux toward lactate production, is a direct consequence of PDH deficiency. Moreover, our data identify DLAT, the E2 subunit of the PDC, as an essential component of this metabolic program regulated by E4f1 in keratinocytes.

Whether E4f1-mediated control of the PDC in keratinocytes is clinically relevant remains to be determined. It is worth noting, however, that a homologous nonsynonymous mutation in the coding region of the E4f1 gene has been recently identified in a patient presenting clinical symptoms resembling those of Leigh syndrome patients (27). Although skin abnormalities have been reported only in some Leigh syndrome patients (28), they are part of the broad spectrum of clinical manifestations that are commonly observed in several mitochondrial disorders (29). Further investigations are necessary to evaluate whether E4f1-mediated control of mitochondrial activities, which likely extend beyond the control of the PDC, contribute to the skin manifestations observed in these patients.

Another pathological situation that has been associated with changes in PDH activity is cancer. Interestingly, the metabolic rewiring of E4f1KO keratinocytes is reminiscent of the one observed in many cancer cells that display increased aerobic glycolysis, even in high oxygen conditions, an effect known as the Warburg effect. It is well established that PDH deregulation in cancer cells can result from posttranslational modifications of PDC subunits by inhibitory kinases (PDKs), activating phosphatases (FDPs), or the lipomamide dehydrogenase (LID) (30, 31). We failed to detect deregulation of Pdk2 and Pdp2 mRNA levels in E4f1 KO keratinocytes, and our data rather support the notion that transcriptional control of Dlat is the main mechanism by which E4f1 controls PDH activity in normal epidermal cells. It remains to be seen whether E4f1-mediated control of Dlat is an alternative regulatory mechanism of the PDC in skin cancer cells. Nevertheless, our data clearly show that the control of PDH activity by E4f1 in basal keratinocytes is essential for normal skin homeostasis.

Interestingly, as with cancer cells, we show that the metabolic reprogramming of E4f1KO basal keratinocytes results in increased activity of ECM-remodeling enzymes, including MMPs and cathepsins. The exact molecular mechanism by which increased glycolysis activates MMP activity in cancer cells remains controversial. Previous studies have suggested that the MCT-chaperone CD147/BASIGIN increases MMP activity through a yet undefined mechanism (32). However, recent data contradict this working model (33). Whatever the mechanism, the high glycolytic profile and increased activity of tissue-remodeling enzymes of fully transformed cells have been associated with their increased migratory and invasive properties that contribute to metastatic dissemination. Our data show that the metabolic reprogramming of normal E4f1KO keratinocytes recapitulates some features of cancer cells, including their ability to induce the focal degradation of the basement membrane and to remodel their microenvironment. Here, we show that these alterations impact on ESC maintenance within their niche, leading to their mechanical elimination and ending in the complete exhaustion of the ESC pool. Interestingly, we previously reported that the ability of E4f1 to control the Bmi1-ARF-p53 pathway partly contributes to ESC self-renewal (7). These data raise interesting questions regarding the connection between the metabolic reprogramming of E4f1-deficient keratinocytes and the deregulation of the p53 pathway in these cells. The potential cross-talk between PDH activity and the control of the p53 pathway is a promising hypothesis that warrants further investigation.
It was recently proposed that basal keratinocytes rely more on glycolysis to sustain their energetic demand than their differentiated progeny in which mitochondrial-reactive oxygen species trigger epidermal differentiation through Notch and β-catenin signaling (6). Our data do not necessarily contradict this model, but provide clear evidence that when glycolysis is further increased in basal keratinocytes, such as in E4F1-deficient cells, this profoundly alters epidermal homeostasis and ESC maintenance. Our results also question the mechanisms leading to the inhibition of keratinocyte differentiation observed in E4F1 KO cells.

Altogether our results identify E4F1 as an essential regulator of the metabolic status of basal keratinocytes and stress the importance of a tight control of the PDH activity for epidermal homeostasis.

Materials and Methods

Generation of Mouse Models and Experimental Treatment. Generation of E4F1 KO and E4F1 XCO mice was previously described (7, 19). These mice were intercrossed with K14CreER+ (20) or K10CreERT2 mice to generate experimental groups E4F1;K14CreER, E4F1+;K14CreER, E4F1−;K14CreER, and E4F1−;K14CreERT2 (referred to as E4F1[K14CTR], E4F1[14K14DTR], E4F1[K14DTR], and E4F1[14K14DTR], respectively). Compound mice were maintained on a mixed genetic background (129sv/c57Bl/6) and housed in a pathogen-free barrier facility. Cre-mediated recombination of the E4F1+ allele was induced by topical applications of tamoxifen (Sigma; diluted in ethanol). 2 mg for 4 consecutive days) on shaved back or tail skin of 8- to 12-wk-old animals. Animals were approved by the regional ethics committee for animal welfare (Comité d’éthique pour l’utilisation des animaux du Languedoc Roussillon, protocol 12068). Oligonucleotides used for genotyping these animals are provided as in SI Materials and Methods.

Histology, IHC, and Immunoblotting of Skin Sections. IHC and immunoblotting of skin sections were performed as previously described (7) using the following antibodies: anti-DLAT (sc-32925 Santa Cruz), MCT4 (sc-50229 Santa Cruz), BASIGIN (G-19 sc-9757, Santa Cruz), Laminin V (generous gift from C. Feral’s laboratory, University of Nice, Nice, France), Int5α (553745 BD Pharmingen), K14 (PRB-155P Covance), K10 (PRB-153P Covance), involucrin (sc-15230 Santa Cruz).

Culture of Primary Keratinocytes. Murine primary keratinocytes were isolated from newborn skin as previously described (7) and grown in calcium-free Eagle’s MEM (Bio-Whittaker; Lonza) supplemented with 8% (v/v) calf serum (RBS), Sigma) and supplemented with 2% (v/v) B27. Cre-mediated recombination was achieved by adding 4-hydroxy Tamoxifen (4OH T, Sigma, 1 μM final) to the culture medium.

Lactate, Ketone Bodies, and PDH Activity Measurement. Lactate and ketone bodies concentration were measured from tail blood samples using a lactate meter (EKF Diagnostics) and β-ketone strips (Optimum, Abbott). PDH activity was measured with PDH Enzyme Activity Dipstick Assay Kit (Abcam) and PDH Activity Colorimetric Assay Kit (Biovision) according to the manufacturers’ recommendations.

Statistic Analyses. The unpaired Student’s t test was used for all analyses. Statistical significance was expressed as: *P < 0.05, **P < 0.01, ***P < 0.001.

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Supporting Information

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SI Materials and Methods

Generation of K10CreERT2 Mice and Genotyping of Animal Models. The Tg(Krt10-creERT2)29.28.ICS model expressing the CreERT2 in the spinous layer was generated by pronuclear injection in FVB/N embryos of the modified BAC RP23-336D20 that express a tam-dependent Cre recombinase under the control of the Krt10 promoter. The line was backcrossed into the C57Bl/6J genetic background for 10 generations before being intercrossed with E4f1 foam cells. To assess Cre-mediated recombination in suprabasal keratinocytes, these mice were crossed with a Cre-dependent β-galactosidase reporter strain Gt(Rosa)26Sortm1Bset (MGI:1869392).

E4f1(K14)KO and E4f1(K10)KO animals were genotyped using the following primers:

E4f1 KO allele: Fwd: 5′-CACTGGCTTGAGGACCTTTG-3′; Rev: 5′-CCTGCTCCACATACTTACCTTC-3′.
E4f1 KO allele: Fwd: 5′-CCCCAGAGCCTTACAAGTTCC-3′; Rev: 5′-GGCTGTGCGTGATTTCC-3′.

K14CreEr allele: Fwd: 5′-GCAAGGGAATAGGAGGTGCC-3′; Rev: 5′-CTGGTTGCACTAGGCGGTA-3′.
K10CreEr allele: Fwd: 5′-CCTGCTTGAGGACCTTTG-3′; Rev: 5′-CCTGCTCCACATACTTACCTTC-3′.

Recombination of the E4f1 allele in E4f1(K10)KO mice was determined by semiquantitative PCR on genomic DNA prepared from total skin and the following primers:

E4f1 recombinant allele (floxed allele): Fwd: 5′-CCTGGTGTCAGATGGATC-3′; Rev: 5′-GCTAGTAGGTTAGGAGGCTGTTC-3′.

Internal control (NC2): Fwd: 5′-ACTGGGATCTCCACATCCTTGGAG-3′; Rev: 5′-GATGTTGGGCGACTGGCTACCTAC-3′.

Clonogenic Assays. For clonogenic assays, primary keratinocytes were seeded in collagen-I-coated plates (BD Biosciences; 50 μg/mL) on a feeder layer of Mitomycin C-treated 3T3-J1 fibroblasts, as previously described (7). Briefly, cells were cultured in DMEM/HamF12 (3:1; Invitrogen) medium supplemented with 10% (vol/vol) calcium-free FBS (Sigma), 4 mM l-glutamine (Gibco), 110 μg/mL sodium pyruvate (Gibco), 0.16 ng/mL cholera toxin (Sigma), 0.4 μg/mL hydrocortisone (Sigma), 5 μg/mL insulin (Sigma), and 10 μg/mL EGF (Roche). Cells were grown for 15 d at 32 °C in 8% CO2 and fixed with 3.7% (vol/vol) PFA (Electron Microscopy Sciences) for 10 min at room temperature, then stained with 1% Rhodamine B. For rescue experiments, Na-Acetate (0.25 mM; Sigma) and GM6001 MMP inhibitor (0.5 μg/mL, MedChem Express) were added in the culture medium and maintained until the end of the assay. Fresh medium was replaced every other day. For TIMP1-based rescue experiments, ESCs were seeded on 3T3-J1 fibroblasts that were previously transduced with empty or TIMP1-encoding retroviruses.

Identification of Label-Retaining ESC. Interfollicular ESC were identified on skin sections by IHC using an adaptation of an in vivo labeling protocol of multipotent ESC (21) based on the utilization of the nucleotide precursor EdU. Briefly, 10- or 11-day-old mice were injected every 12 h intraperitoneally for a total of four injections with 5 mg/mL EdU (CarboSynth) diluted in 7 mM NaOH. After a 2 mo-chase, EdU-ε ESC were identified on skin sections using a detection method of EdU following the manufacturer's instructions (Life Technologies). Follicular bulge stem cells were identified in tail or back-skin whole mounts upon staining with an anti-keratin 15 (LHK15, Vector Laboratories) antibody, or by FACS analysis upon staining with anti-CD34 (BD Biosciences) and anti-integrin α6 (BD Biosciences) antibodies, as previously described (7).

FACS Analysis. For analysis of GLUT1 expression, cultured primary keratinocytes were resuspended in PBA buffer (PBS containing 1 mM EDTA, 2% (vol/vol) FBS (Sigma)) and were incubated at 37 °C for 30 min with the GFP-lagged receptor binding domain of the human T-cell leukemia virus that binds to the extracellular domain of the GLUT1 receptor (34). Cells were then washed with PBA buffer and incubated with 5 μg/mL of propidium iodide to discriminate dead cells before analysis on a FACSCALIBUR flow cytometer (BD Biosciences). For FACS analysis of ESC, freshly isolated primary keratinocytes from adult skin were stained for 30 min on ice with PE-conjugated anti-α6-integrin (BD Pharmingen) and anti-CD34 biotinylated (RAM34; BD Biosciences) antibodies and then with Alexa 647-conjugated streptavidin (BD Pharmingen). FACS-analysis was performed on a FC500 (Beckman Coulter) and data were analyzed with FlowJo software.

Immunofluorescence Analysis of Cultured Primary Keratinocytes. Cells were fixed with methanol (Sigma) at −20 °C for 5 min before overnight incubation at 4 °C with an anti-MCT4 rabbit polyclonal antibody (sc-50329, Santa Cruz). Revealing was performed using an Alexa 488-conjugated anti rabbit IgG antibody (ThermoFischer) for 2 h at room temperature. Cover glasses were mounted with Mowiol (Biovalley) before analysis on a Zeiss apotome.

Lentiviral Particle Production and Transduction. Lentiviral particles were produced in 293T packaging cells by transient transfection using Jet-PEI reagent (Ozyme) of pLKO vectors encoding either a control ShRNA (Sigma, SHC002) or ShRNAs targeting mouse Dlat (Sigma-Mission, TRCN0000041608 and TRCN0000041609). Seventy-two hours after transfection, viral supernatants were harvested and added on primary keratinocytes overnight in presence of polybrene (5 μg/mL, Sigma). Antibiotic selection of transduced keratinocytes was performed 48 h after transduction with puromycin (1.17 μg/mL, HyClone).

Transmission Electron Microscopy and Ultrastructural Evaluation. Adult skin samples were fixed in 3.5% (wt/vol) glutaraldehyde in 0.1 M Sorensen’s phosphate buffer, pH 7.4, overnight at 4 °C. The tissues were then rinsed in Sorensen’s buffer and postfixed in 1% osmic acid for 2 h in the dark at room temperature. After two rinses, the tissues were dehydrated in a graded series of ethanol solutions (30–100%) and embedded in EMBed 812 resin. Sections (60-nm thickness; Leica-Reichert Ultracut E) were counterstained with uranyl acetate and observed using a Hitachi 7100 transmission electron microscope (Centre de Ressources en Imagerie Cellulaire de Montpellier, France).

Immunoblotting. Total cell extracts were lysed in 0.08 M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 12% (wt/vol) sucrose, 2% β-mercaptoethanol, bromophenol blue traces and separated with 10% (wt/vol) SDS/PAGE, transferred on a nitrocellulose membrane before probing with the following antibodies recognizing E4f1 (18), DLAT (sc-271534, Santa Cruz), MCT4 (sc-90, sc-503329, Santa Cruz), PDHA1 (459400, Life technologies), BASIGIN (Santa Cruz), panacetylated lysines of histone H4 (Cell signaling) and ACTIN (A3854, Sigma).

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Quantitative RT-PCR. Primary keratinocytes were lysed in TRIZOL reagent (Invitrogen), and total RNAs were isolated according to the manufacturer’s recommendations. CDNAs were synthesized from 500 ng of total RNA using random hexamers and SuperScript III Reverse transcriptase (Invitrogen). Quantitative real-time PCR (RT-qPCR) was performed on a LightCycler 480 SW 1.5 apparatus (Roche). 18S transcripts were used for normalization. Primers sequences were as follows:

**E4F1:** Fwd: 5’-CCAAAGCCCTACTGCTCAAG-3’; Rev: 5’-CTGGCACTTCTGGTTTGTG-3’. **Dlat:** Fwd: 5’-TCTCCGTCGCCAGAAGGTTG-3’; Rev: 5’-CCACCTGAAACATTCTG-3’.

**Dfd:** Fwd: 5’-CCITGTAGACCTAGGGCTCAG-3’; Rev: 5’-CCACATGACCCAAAAATTC-3’. **Bpp44/46:** Fwd: 5’-TCAGAGATATATCTAGGGGAGAT-3’; Rev: 5’-GCCATTTCCAGGTTACCTGT-3’. **Pdp4:** Fwd: 5’-AAGACAGGAGGACCCCCAACG-3’; Rev: 5’-GATAGCCACGAGTACCC-3’. **Pdh1:** Fwd: 5’-GCTGTTGTCCTCCGCTGAAAT-3’; Rev: 5’-TAAGTATGACGGCAGCTCC-3’.

18S: Fwd: 5’-GTAACCCCGT TGAACCCATT-3’; Rev: 5’-CCATCAGGCTGATAGCC-3’.

Zymology. MMP activity was determined in murine keratinocytes lacking E4F1 or on DLAT depletion. Two days after tam administration or viral transduction, cells were washed twice with PBS and incubated for an additional 48 h in the same serum-free medium as the one used for clonogenic assays. Conditioned media from equal number of cells were collected and mixed with sample buffer [0.25 M Tris pH 6.8, 8% (wt/vol) SDS, 40% (vol/vol) glycerol, 0.096 mg/mL Bromophenol blue]. Samples were separated by electrophoresis in TG-SDS buffer (Euromedex) in a 10% (wt/vol) polyacrylamide gel containing 1 mg/mL gelatin (Sigma) at 4°C. The gel was treated with 2.5% (vol/vol) Triton X-100 (Sigma) for 1 h at 4°C and was then incubated with activity buffer (50 mM Tris pH 7.6, 5 mM CaCl2, 0.02% (vol/vol) Triton) for 24 h at 37°C. The gel was stained with 40% (vol/vol) methanol, 10% (vol/vol) acetic acid, 0.1% Coomassie blue. For catharsis zymology, gels were washed in renaturing buffer [65 mM Tris pH 7.4, 20% (vol/vol) glycerol] three times for 10 min and then incubated with activity buffer (0.1 M NaP buffer pH 6, 2 mM DTT, 1 mM EDTA) for 30 min at room temperature and then overnight at 37°C, as described previously (35). Samples were treated with the Lactate dehydrogenase inhibitor sodium-oxamate (0.5 mM; Sigma) for 4 d. For normalization, β-Actin expression was analyzed by immunoblotting in protein extracts prepared from the same cells.

ChIP. To perform CTRL and E4F1 ChIP qPCR, primary keratinocytes from 18 to 20 newborn skins were pooled and cultured for 2 d. Cells were then fixed with PFA 1% for 8 min, then washed with cold PBS-Glycine 125 mM and incubated 5 min on ice with PBS-glycine 125 mM. Cells were then scraped in PBS and washed extensively. Cells were lysed for 1 h on ice using lysis buffer (10 mM Tris pH 8, 140 mM NaCl, 0.1 SDS, 0.5% (vol/vol) Triton X-100, 0.05% NaDoc, 1 mM EDTA, 0.5 mM EGTa, 1 mM pepstatinA, 1 mM aprotonin, and 0.1 mM PMSF) Chromatin sonication was performed with an EpiSure Sonicator (Active Motif). An aliquot was decross-linked and deproteinized for DNA fragmentation size control. E4F1 ChIP was performed with 1 μL of rabbit polyclonal antibody raised against full-length E4F1 protein incubated in presence of 25 μg of keratinocytes chromatin and 20 μL of Dynabeads protein G. After 16-h incubation, immunoprecipitates are washed with the five following buffers: W1: Tris pH 8 10 mM, KCl 150 mM, Nonidet P-40 0.5%, EDTA 1 mM. W2: Tris pH 8 10 mM, NaCl 100 mM, NaDoc 0.1%, Triton X-100 0.5%. W3a: Tris pH 8 10 mM, NaCl 400 mM, NaDoc 0.1%, Triton X-100 0.5%. W3b: Tris pH 8 10 mM, NaCl 500 mM, NaDoc 0.1%, Triton X-100 0.5%. W4: Tris pH 8 10 mM, LiCl 250 mM, NaDoc 0.5%, Nonidet P-40 0.5%, EDTA 1 mM. W5: Tris pH 8 10 mM, EDTA 1 mM. Input and immunoprecipitated DNA were decross-linked overnight at 65°C, diluted in TE and incubated first with RNAaseA (37°C, 45 min) and then Proteinase K (55°C, 45 min). Proteins were removed with phenol-chloroform-isomylalcohol and DNA was recovered by chromatography (nucleosipin extract II columns, Macherey-Nagel). Immunoprecipitated DNAs were analyzed by QPCR (Roche LC480 and SYBRGreen mix) with the following primer-specific primers:

**Dlat:** 5’-ACAGACGCGCCACATTACCTG-3’ and 5’-GCTGCTTCTCGGAGGTCAC’.

**Dfd:** 5’-TACAACAGACTCCAGCTCTGCAAT-3’ and 5’-ATAAACGTCTACAGGCGTG-3’.

**Bpp44/46:** 5’-ACACTTGTGAGCACTGATGCTC-3’ and 5’-AAGACAGGAGGAGCCAGCAC-3’.

**Pdp4:** 5’-CTCTGAGGCTCAAGTCAATGCT-3’ and 5’-TAAAGCCCTTCAGTTGCTTC-3’.

**Pdh1:** 5’-AGGAACATGTGGCCGGTCATCCAT-3’ and 5’-TATTACGCGCCTTCCCCAC-3’.

**NC2:** (gene-poor noncoding region of chromosome 6) 5’-CCCTTGTAAGCACTCCTG-3’ and 5’-TAAGGCCTTCAGTTGCTTC-3’.

**Pdh1:** 5’-AGGAACATGTGGCCGGTCATCCAT-3’ and 5’-TACTCTTCTGTGTCCTGTG.

ECAR and Oxygen Consumption Rate. ECAR and oxygen consumption rate (OCR) were measured using a XF24 Extracellular Flux Bioanalyzer (Seahorse Bioscience). Briefly, 25 × 10⁶ primary keratinocytes per well were seeded on a XF24 V7 cell culture microplate. Measurement was done in XF Assay medium (Seahorse Bioscience) pH 7.4 supplemented with 100 μM Sodium Pyruvate (Gibco). For measurements of ECAR, glucose (25 mM final, Sigma) was injected in the culture medium during the analysis. After the analysis, cells were lysed in Laemmli buffer [0.08 M Tris-HCl (pH = 6.8), 2% (vol/vol) SDS, 12% (vol/vol) sucrose] and doped by Bradford protein assay.

For measurements of OCR, cells were incubated for 1 h at 37°C in XF Assay medium containing Glutamax (Seahorse Bioscience) pH 7.4 supplemented with 4.5 g/L glucose, 1 mM pyruvate, 0.2 μM BSA-conjugated palmitate, and 0.5 mM carnitine. OCR was measured over 4 min in three measurement intervals to assess basal metabolic rate, oligomycin C (1 μg/mL),-sensitive OCR associated to ATP production, and maximal respiratory capacity [upon FCCP (0.6 μM final) followed by rotenone (0.1 μM final) administration]. The ECAR and OCR data were normalized by the amount of protein for each sample.

NAD⁺/NADH Ratio. The NAD⁺/NADH ratio was determined using NAD/NADH-Glo Assay kit (Promega) following the manufacturer’s recommendations.

FAO. FAO was measured in triplicates by quantifying the production of ³H₂O from [9,10⁻²H]palmitate. Briefly, cells were plated at 5 × 10⁶ cells per well in 12-well culture plates, and treated for 4 d with
40HT. Cultured cells were washed three times with Dulbecco’s PBS and incubated with 200 μL of [9,10-3H]palmitic acid (60 Ci/mmol, NEN) bound to fatty-acid-free albumin (final concentration 125 μM) containing 1 mM carnitine. After 2-h incubation at 37 °C, the mixture was removed and added to a tube containing 200 μL of cold 10% (wt/vol) TCA. The tubes were centrifuged for 10 min at 2,200 × g at 4 °C, and aliquots of supernatants (350 μL) were removed, mixed with 55 μL of 6 M NaOH, and applied to ion-exchange resin (DOWEX). The columns were washed twice with 750 μL of water, and the eluates were counted. Palmitate oxidation rates were expressed as counts per minute per cell.

Fig. 51. Characterization of E4F1(K10)KO model. (A) β-Galactosidase staining of skin section prepared from K10CreERT2 animals crossed with Cre-dependent β-Gal reporter mice. (Magnification: 20x.) (B) mRNA level of the Cre recombinase in different tissues prepared from K10CreERT2 mice determined by RT-qPCR. (C, Upper) schematic representation of the E4F1flx allele before/after Cre-mediated recombination. Recombination of the E4F1flx allele in E4F1(K10)KO was verified by semiquantitative PCR performed on genomic DNA prepared from total skin samples (containing epidermis, dermis, and hypodermis) using the indicated primers. E4F1KO mouse embryonic fibroblasts (MEFs) were used as a positive control.
Fig. S2. E4f1 inactivation in suprabasal keratinocytes. Histological analyses of skin sections prepared from E4f1(K10)KO mice or E4f1(K10)CTR control littermates, 6 wk after tam administration. Shown are representative microphotographs of H&E-stained skin sections or IHC analyses of cytokeratin 14 (K14), cytokeratin 10 (K10), or involucrin expression, as indicated. (Scale bars, 100 μm.)
Fig. S3. (A) Microphotographs of H&E-stained skin sections prepared from E4f1(K14)KO mice or E4f1(K14)CTR control littermates, 2 wk after tam administration. Note the increased thickness of the epidermis (= epidermal hyperplasia or acanthosis) and increased thickness of the cornified layer (= hyperkeratosis). Epi, epidermis; SC, stratum corneum or cornified layer. The dashed line indicates the separation between the epidermis and the dermis. (B) Microphotographs at higher magnification of skin sections prepared from the same animals as in A showing the presence of nuclei in the cornified layer (parakeratosis) and abnormal keratinocytes (dyskeratosis). (Scale bars, 100 µm.)
Fig. S4. IHC analyses of K14, K10, involucrin, cytokeratin 6 (K6), and K67 expression in skin sections prepared from representative E4f1(K14)KO mice or E4f1(K14)CTR control littermates, 2 wk after tam administration. (Scale bars, 100 μm.)
Fig. S5.  (A) mRNA levels of E4f1, Dlat, Dld, Brp44l, Pdp2, Slc25a19 in E4f1KO or CTR cultured primary keratinocytes, 4 d after 4OHT addition to the culture medium. Histograms represent the mean value ± SEM (n = 4) determined by RT-qPCR. (B) mRNA levels of Pdha1, Pdk1, Pdk4, Pdp1, Pdp2 in the epidermis of E4f1(K14)KO mice or E4f1(K14)CTR control littermates, 1 wk after tam administration. Histograms represent the mean value ± SEM (n = 5 animals per group) determined by RT-qPCR. (C) mRNA levels of Pdha1, Pdk1, Pdk4, Pdp1, Pdp2 in E4f1KO or CTR cultured primary keratinocytes, 4 d after 4OHT addition to the culture medium. Histograms represent the mean value ± SEM (n = 4) determined by RT-qPCR. *P < 0.05; **P < 0.01; ns, not significant.
Fig. S6. E4F1 depletion leads to decreased PDH activity. (A) PDH activity in E4F1KO or CTR cultured primary keratinocytes, using a different method, DipStick Assay Kit (ab109882, Abcam), than the one used in Fig. 2. (B) Histograms represent the mean value ± SEM (n = 5). (B) GLUT1 levels determined by flow cytometry in E4F1KO or CTR cultured primary keratinocytes. (C) IHC analysis of BASIGIN (Upper) and of the glucose transporter GLUT1 (Lower) expression in skin sections prepared from E4F1(K14)KO mice and control litters, 2 wk after 4OHT administration. (Scale bar, 50 μm.) (D) OCR measured using a Seahorse Bioanalyzer in E4F1KO or CTR cultured primary keratinocytes. All analyses in cultured primary keratinocytes were performed after 4 d of culture in presence of 4OHT. **P < 0.01.
Fig. 57. E4F1 deficiency results in remodeling of the extracellular matrix and alterations of the basement membrane. (A) Transmission electron microscopy analysis of skin samples prepared from E4f1(K14)KO mice and control littermates, 1 wk after tam administration. (B and C) Staining of skin sections prepared from E4f1(K14)KO mice and E4f1(K14)CTR littermates with reticulin (B) and with Sirius red (C). Arrows indicate structural alterations of the basement membrane. (D) Cathepsins activities measured by zymography in total skin extracts prepared from E4f1(K14)KO mice and E4f1(K14)CTR control littermates. *P < 0.05. (Scale bars, 50 μm.)
**Fig. S8.** DLAT depletion leads to decreased PDH activity, induction of MCT4 expression, and increased cathepsin activities. (A) PDH activity was measured in cultured primary keratinocytes transduced with lentiviruses encoding control or DLAT shRNAs. Histobars represent the mean value ± SEM ($n = 3$). (B) MCT4 protein expression in DLAT-depleted primary keratinocytes analyzed by immunoblotting. (C) Cathepsin activities measured by zymogel in conditioned media from primary keratinocytes expressing control or DLAT shRNAs. **P < 0.01.**
UNPUBLISHED RESULTS
IV. Melanocyte specific mouse model

A. Tyr::CreER transgenic mouse

Tyr::CreER is a transgenic mouse that expresses the tamoxifen-inducible CreER recombinase under the promoter of Tyrosinase gene (Figure 41). This drives the recombination specifically in melanocyte-lineage (Delmas et al., 2003). The expression of the construct is only restricted to mature melanocytes and melanocyte stem cells do not express Tyr::CreER.

![Figure 41: Melanocyte specific inactivation of E4f1. E4f1 flox mice were crossed with Tyr::CreER transgenic mouse which express Cre recombinase under the control of the Tyrosinase promoter. Cre-mediated excision of E4f1 was obtained upon topical Tamoxifen application.](image)

V. Genetic reporter

B. Rosa26<sup>mt/mg</sup> knock-in mouse

Mt/mg is a membrane targeted two-color reporter that was introduced in the Rosa26 locus by knock-in. Rosa26 is a constitutively and ubiquitously expressed locus in mice. Prior to tamoxifen administration and Cre-mediated recombination, the cells of the whole body express tdTomato (mT) fluorescence. Upon Cre activation, the tdTomato (mt) sequence is excised allowing the expression of EGFP (mG) fluorescence (Muzumdar et al., 2007). This knock-in model was crossed with my Tyr::CreER; E4f1<sup>flox</sup> mice in order to follow the recombined cells (Figure 42).

![Figure 42: mT/mG genetic reporter. Tyr::CreER; E4f1<sup>flox</sup> mice were crossed with Rosa26<sup>mt/mg</sup> knock-in mice. Rosa26 locus is ubiquitously expressed in mice therefore all the cells express tdTomato (red fluorescent protein) prior to Tamoxifen application. Tyr::CreER mediated recombination switched on GFP (green fluorescent protein) expression in melanocytes.](image)
RESULTS

I. \textit{E4f1} inactivation in melanocytes leads to hair greying and skin depigmentation.

In order to determine \textit{E4f1} functions in melanocytes, I crossed \textit{E4f1}\textsuperscript{\textit{fl}} cKO animals with mice expressing Tamoxifen-inducible Cre recombinase (CreER) under the promoter of \textit{Tyrosinase}. I applied tamoxifen on the tail and the back skin of 6-week old adult \textit{E4f1}\textsuperscript{\textit{fl}}, \textit{Tyr::CreER}; \textit{Rosa26}\textsuperscript{\textit{m7/mG}} mice during 2 months to induce \textit{E4f1} inactivation and GFP expression in the melanocytes (Figure 1A). I first characterized the expression pattern of GFP positive cells in \textit{Tyr::CreER}; \textit{E4f1WT}; \textit{Rosa26}\textsuperscript{\textit{m7/mG}} mice in order to determine the efficiency of recombination in our model. Using whole mounts of tail epidermis from those mice, I clearly demonstrated that GFP expressing melanocytes are present in the hair follicle bulb and inter follicular epidermis 2 months after the first tamoxifen application (Figure 1). In order to study the consequences of \textit{E4f1} inactivation in melanocyte homeostasis, I next analysed \textit{Tyr::Cre}; \textit{E4f1KO}; \textit{Rosa26}\textsuperscript{\textit{m7/mG}} mice after topical application of tamoxifen on tail and on a shaved area of back skin. 2 months after the first tamoxifen administration, \textit{E4f1} inactivation resulted in hair greying of the back skin and a complete depigmentation of tail skin compared to control littersmates (Figure 2A, left and middle panel). Epidermis separated from those tail skins also exhibited a complete depigmentation (Figure 2A, right panel). Consistent with the hair greying phenotype, microscopic analysis of plugged hair follicles exhibited an complete absence of melanin pigment in the \textit{E4f1} KO hair shaft whereas the melanin was properly distributed along the WT hair shaft (Figure 2B). Together, these results demonstrated that \textit{E4f1} inactivation leads to hair graying and skin pigmentation defects.

II. \textit{E4f1}-deficient melanocytes are viable and differentiate properly.

Next, I wondered if the greying of the hair and the pigmentation defects were due to the loss of \textit{E4f1}-deficient melanocytes. In order to test this hypothesis, I took advantage of the genetic reporter to follow several weeks after \textit{E4f1} inactivation the recombined melanocytes. Immunohistochemistry analysis of GFP expression performed on the tail skin sections clearly demonstrated that melanocytes were still present in the inter-follicular epidermis (Figure 3A) and in the hair bulb of \textit{E4f1} cKO mice (Figure 3B) 2 months after recombination suggesting that \textit{E4f1} depletion did not lead to the death of melanocytes. Then, I checked if \textit{E4f1}-mediated depigmentation was due to abnormal differentiation of melanocytes by analysing Sox10 expression. Immunostaining performed on whole mounts of tail epidermis demonstrated that \textit{Tyr::Cre}; \textit{E4f1}\textsuperscript{\textit{\textit{?}f}} (\textit{E4f1} KO) melanocytes expressed Sox10 (Figure 4A). Consistently, Sox10 and GFP co-expressing melanocytes were detected in \textit{Tyr::Cre}; \textit{E4f1}\textsuperscript{\textit{\textit{?}f}}; \textit{Rosa26}\textsuperscript{\textit{m7/mG}} (\textit{E4f1} KO) mouse tail epidermis sections 2 months after the recombination. All together, these results demonstrate that \textit{E4f1} inactivation does not affect the viability nor the differentiation process of melanocytes.
III. **E4f1** inactivation in early post-natal development results in hair greying and skin pigmentation defects.

The transgenic *Tyr::CreER* model has been described to allow recombination in mature melanocytes but not in melanocyte stem cells of adult animals. This experimental issue was due to the fact that *Tyrosinase* promoter is progressively turned off in melanocyte stem cells during post-natal development. To bypass this issue and in order to evaluate the impact of E4f1 inactivation in melanocyte stem cells (MSC), I applied tamoxifen on pregnant mothers which will give birth to the experimental group (*Tyr::Cre; E4f1^+/−; Rosa26^mt/mG* (KO) and *Tyr::Cre; E4f1^+/−; Rosa26^mt/mG* (WT) mice) to induce E4f1 inactivation as early as possible and potentially in MSC where the *Tyrosinase* promoter is still active (Figure 5A). Using this experimental approach, the pregnant mothers will deliver tamoxifen through milk feeding. Surprisingly, 2 months after their birth, E4f1 KO mice displayed hair graying on the dorsal and ventral side with a depigmentation of the tail skin (Figure 5B). These results were strongly reminiscent to those observed upon E4f1 inactivation in adult animals confirming the important role of E4f1 in melanocyte homeostasis. However it still remains to be determined whether these phenotypes results from defects in the MSCs or in mature melanocytes like in the previous experimental protocol.

IV. **E4f1** inactivation in hyper-pigmented mouse model results in hair greying and skin depigmentation.

In parallel, in order to challenge the hypothesis that E4f1 is essential for proper skin pigmentation, I used another mouse model exhibiting hyper-pigmentation in response to *BRAF^V600E* activation. I crossed E4f1^flox; Tyr::CreER animals with a mouse strain expressing a tamoxifen-inducible active form of the mutated BRAF oncogene which induces hyper-proliferation and hyper-pigmentation of melanocytes through the constant activation of MAPK signaling pathway (Figure 6A). In this model, tamoxifen application will induce simultaneously E4f1 inactivation and BRAF activation. Where *Tyr::CreER; BRAF^V600E*, E4f1 WT animals displayed strong hyper-pigmentation on the back skin, *Tyr::CreER; BRAF^V600E*; E4f1 KO mice surprisingly exhibited hair graying on the back skin and depigmentation of the tail skin, 2 months after the first tamoxifen application (Figure 6B). Eventhough, I still need to further characterize the underlying cellular and molecular mechanisms, these data illustrate the strong impact of E4f1 inactivation on melanocyte pigmentation and homeostasis.

V. **Ex vivo** inactivation of E4f1 recapitulates **in vivo** pigmentation defect.

At the molecular level, in order to characterize the pigmentation defects associated to E4f1 inactivation, I used a human melanoma cell line isolated from metastatic melanoma tumor in which I depleted E4f1 using a lentivirus encoding shRNA. I first checked the efficiency of E4f1 depletion by western blotting and showed that E4f1 protein expression was significantly decreased 5 days after lentiviral infection compared to ShCtrl cells (Figure 7A).
Consistently, E4F1-depleted cell also displayed a strong downregulation of DLAT expression, the E2 subunit of the PDH complex recently identified as a direct target gene of E4F1 (Figure 7A). I then investigated the consequences of E4F1 depletion on pigmentation. To this purpose, I first checked the expression of MITF, which is the master regulator of melanin production. I showed that MITF protein level was downregulated in ShE4F1 infected cells compared to control cells (Figure 7B). Since MITF regulates the expression of several genes involved in pigmentation, I then checked the expression levels of two of its downstream targets: TYROSINASE, an enzyme responsible for melanin production and PMEL (GP100), a structural protein of melanosomes. Consistent with MITF downregulation, TYROSINASE and GP100 were also downregulated as illustrated by immunoblotting (Figure 7B) and by immunofluorescence (Figure 7C). Taken together these results suggest that E4F1 regulates pigmentation ex vivo in a cell-autonomous manner and that the pigmentation defect mediated by E4F1 depletion is at least partially due to the deregulation of MITF.

VI. Depletion of E4F1 in melanoma cells induces p53 stabilization.

p53 was previously shown to play a important role in melanin production in response to UVs through the transcriptional activation of melanogenic genes such as Tyr and Tyrp1, and Pомc which activates pigmentation pathway via MC1R receptor (Nylander et al., 2000; Cui et al., 2007). In addition, my host lab previously showed that E4F1-mediated regulation of ESC maintenance was also implicating the p53 pathway (with a stabilization of p53 protein upon E4f1 inactivation). In order to test the involvement of p53 in E4F1-associated melanocyte defects, I checked its expression in E4F1 inactivated cells. I showed by immunoblot analysis that p53 was stabilized in cells infected with ShRNA directed against E4F1 compared to control cells (Figure 7D), suggesting the induction of a p53 response in E4F1-depleted melanocytes that could be involved in melanin production defects.

VII. E4F1-dependent melanogenesis defect is PDH dependent.

I recently demonstrated that PDH activity was essential for proper ESC maintenance and skin homeostasis. At the molecular level, I showed that DLAT was a key mediator of E4F1-associated skin phenotypes since Dlat depletion recapitulated key features observed in E4f1 KO keratinocytes. Based on these results, I checked if E4F1 effects on melanogenesis were a consequence of PDH deregulation. I infected the melanoma cell line with lentivirus encoding control or three different DLAT ShRNAs. Western blot analysis confirmed the efficient depletion of DLAT protein with the three ShRNAs (Figure 8). Interestingly, the DLAT-depleted cells also exhibited decreased TYROSINASE and GP100 expression (Figure 8) recapitulating the results obtained in E4F1 depleted cells and illustrating that E4F1-mediated depigmentation phenotype could occur in a DLAT-dependent manner. Taken altogether, these findings suggest that E4F1 controls melanogenesis through mechanisms that include the p53 pathway and PDH activity.
Figure 1

A.

Adult mouse (1.5 months) → 1st week → 2nd week → 3rd week → Adult mouse (4 months)

Tamoxifen application

B.

*Tyr::CreER; Rosa26*<sub>MT/ME</sub>*

![GFP dapi](image)

Hair bulb

Scale bar: 50 μm
Figure 2

A. 

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Tail epidermis

15 μm

60 μm
Figure 3

A. Tail skin

E4f1 WT

E4f1 KO

e

d
20 μm

B. Hair follicle

E4f1 WT

E4f1 KO

bulb

5 μm

5 μm
Figure 4

A.

E4f1 WT  Sox10 dapi  E4f1 KO

B.

E4f1 WT  GFP Sox10 dapi  E4f1 KO
Figure 5

A.

TyrCreER \times E4f1^{floxed}

Pregnant mouse

Birth

1st week

2nd week

3rd week

Adult (litters)

Lactation

Tamoxifen application

B.

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Figure 6

A.

BRAF<sup>V600E</sup> inactivation

Tamoxifen treatment

Hyper-pigmentation

B.  

\begin{array}{c|c|c}
E4f1 & WT & KO \\
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\end{array}

\begin{array}{c|c|c}
E4f1 & WT & KO \\
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Figure 7

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C.

ShSCR

ShE4F1

GP100  dapi
Figure 7

D.

- **Figure 8**

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Figure 1: Validation of Tyr::CreER model. (A) Experimental procedure of tamoxifen administration. 8 weeks-old-adult Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> and Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> animals were shaved and treated with topical applications of 2 mg of tamoxifen on the dorsal skin and tail (5 applications on the first week and 2 additional applications per week during 2 months) to induce Cre-mediated deletion of E4f1<sup>flo</sup>x allele. (B) Three-dimensional microscopic image of whole mounts prepared from tail epidermis of Tyr::CreER; Rosa26<sup>mT/mG</sup> mice showing GFP-positive (green) recombined melanocytes in the hair bulb, 2 months after tamoxifen administration. Samples were counterstained with DAPI (blue).

Figure 2: E4f1 inactivation in melanocytes results in hair greying and skin pigmentation defects. (A) Macroscopic photographs of Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> and Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> animals, 2 months after the first tamoxifen administration. Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> mouse exhibits hair greying on the back skin fur (left panel) and tail skin depigmentation (middle panel) compared to control littermates. Epidermis isolated from the tail of Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> also shows depigmentation (right panel). (B) Microscopic images of plugged hair follicles from Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> animals that exhibit melanin in the hair shaft in contrast to Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> that display hair shaft completely lacking melanin.

Figure 3: E4f1 cKO melanocytes are viable. (A-B). Immunohistochemistry analysis of GFP expression in sections prepared from tail skin of Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> and Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> animals showing the presence of GFP-positive melanocytes in the interfollicular epidermis (A) and in the bulb of hair follicle (B) 2 months after tamoxifen application. Arrows indicate GFP-positive cells.

Figure 4: E4f1 inactivation does not impair melanocyte differentiation. (A) Microscopic three-dimensional image of whole mounts of tail epidermis prepared from Tyr::CreER; E4f1 WT and Tyr::CreER; E4f1 KO mice, 2 months after tamoxifen administration. Samples were stained with Sox10 (green) and DAPI (blue). (B) Immunostaining of GFP (green) and Sox10 (red) on skin sections showing follicular melanocytes in the hair bulb in tail sections from Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> and Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> mice 2 months after Tamoxifen administration. Sections were counterstained with DAPI (blue).

Figure 5: Experimental protocol of tamoxifen administration. (A) E4f1<sup>flo</sup>x/flo, Rosa26<sup>mT/mG</sup> pregnant mothers, that will give rise to Tyr::CreER; E4f1 WT; Rosa26<sup>mT/mG</sup> and Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> mice, were treated by topical applications with 2 mg of tamoxifen on the last day of pregnancy and several days after delivery (5 times during the first week and 2 applications per week for the following 2 weeks until weaning time). Littermates were then analyzed at 8 weeks. (B) Tyr::CreER; E4f1 KO; Rosa26<sup>mT/mG</sup> mice exhibit hair graying on the
ventral and dorsal side and skin depigmentation in the tail 2 months after birth compared to control littermates.

**Figure 6: E4f1 inactivation in Braf-induced hyper-pigmented mice results in hair graying and skin depigmentation.** (A) Schematic representation of Braf^{H600E} inducible knock-in system that was crossed with E4f1^{floxed/lox}, Tyr::CreER. Compound mice were shaved and tamoxifen was applied topically to induce Braf^{H600E} together with E4f1 inactivation. Tyr::CreER; E4f1 WT; Braf^{H600E} control mice displayed increased pigmentation on the back skin 1 month after the application. (B) Macroscopic photographs of Tyr::CreER; E4f1 WT and Tyr::CreER; E4f1 KO animals, 2 months after the first tamoxifen administration. Tyr::CreER; E4f1 KO mice exhibit hair greying on the back skin fur (left panel) and tail skin depigmentation (right panel) compared to Tyr::CreER; E4f1 WT mice.

**Figure 7: E4f1 depletion results in decreased pigmentation of human melanoma cell line.** (A) Western blot showing depletion of E4f1 and downregulation of its direct target DLAT in total protein extracts prepared from human melanoma cell line transduced with lentiviruses encoding control or E4f1 ShRNA. Samples were normalized by ACTIN. (B) Protein levels of MITF, GP100, TYROSINASE and loading control TUBULIN determined by immunoblotting in total protein extracts prepared from human melanoma cell line transduced with lentiviruses encoding control or E4f1 ShRNA. Expression of MITF, GP100 and TYROSINASE decreased in E4f1-depleted cells. (C) GP100 expression analyzed by immunofluorescence in human melanoma cell line expressing control or E4f1 ShRNA. Arrows indicate cells that lack GP100 expression. (D) E4f1 depletion induced p53 induction. P53 and (loading control) ACTIN expression determined by immunoblotting in total protein extracts prepared from human melanoma cell line transduced with lentiviruses encoding control or E4f1 ShRNA.

**Figure 8: DLAT depletion induces decreased pigmentation in human melanoma cell line.** DLAT, MITF, TYROSINASE, GP100 and (loading control) ACTIN expression analyzed by immunoblotting in total protein extracts prepared from human melanoma cell line transduced with lentiviruses encoding control or DLAT ShRNA. MITF, TYROSINASE AND GP100 expression decreased in DLAT-depleted cells.
MATERIALS AND METHODS
- **Genotyping**

  \(E4F1^{\text{flo}x}, \text{Tyr::CreER}\) mice were genotyped by PCR on tail genomic DNA using the following primers:

  E4f1 KO allele: Fwd: 5'-CACTGCCTTGAGGACTTTG-3';
  Rev: 5'-CCCTGCTACATCCAGGCC-3'.

  E4f1 flox allele: Fwd: 5'-CCCAAGAAGCAGATCC-3';
  Rev: 5'-GGCTGCTGGATGTTC-3'

E4f1 recombinant allele (floxed allele): Fwd: 5'-CCTGGTGAGTTGAATGATC-3';
Rev: 5'-GCTAGGTTAGGTAGGCTTC-3'

\(\text{Tyr::CreER}\) allele: Fwd: 5'-GAAGCACTCAGTGATG-3';
Rev: 5'-TGAAGGATGTCTGGATCA-3'.

- **Immunohistochemistry and immunolabelling of skin sections**

IHC of GFP staining was performed by IRCM histology facility.

  o **Paraffin embedded sections**

Skin samples were fixed in 4% neutral-buffered formalin (VWR Chemicals) for 24 hours. They were embedded in paraffin wax and sectioned at 3 µm. For immunostaining, slides were deparaffinized in xylene and then rehydrated by washing with ethanol and water. For antigen unmasking, the slides were treated with citrate buffer pH=6 (Diapath) at 95°C for 30 minutes and they were permeabilized with TBST (0.25 M Tris base Sigma, 0.4% NaCl, 0.1% Tween 20). The slides were blocked with 3% BSA in PBS during 3 hours at room temperature. Primary antibodies (Sox10 N20 Santa cruz, GFP A10263 Invitrogen) were diluted in PBS containing 1% BSA and incubated overnight at 4°C. Slides were washed with PBS containing Tween 20 0.05%. Fluorescent staining was performed using secondary antibodies conjugated to Alexa 488 anti chicken (Sigma), Alexa 594 anti goat (Thermofischer) in PBST (PBS+ 0.05% Tween 20) during 45 minutes at room temperature.
• **Whole mount**

Mice tail skins were peeled and cut into small pieces. Skin samples were incubated in 5 mM EDTA- PBS during 4 hours at 37°C. Tail epidermis was separated from dermis and fixed in 4% Formaldehyde (Sigma) for 2 hours at room temperature. For immunostaining, samples were blocked and permeabilized in PB buffer containing 0.5% milk, 0.25% gelatin, 0.5% Triton X-100 in TBS (0.9% NaCl, 20 mM Hepes) for 30 minutes at room temperature, then they were incubated with Sox10 (Santa Cruz) antibody in PB buffer overnight at 4°C. Samples were washed with 0.2% Tween 20 0.2% in PBS and incubated with Alexa 488 anti goat (Thermofisher) and dapi in PB buffer overnight at 4°C and they were mounted on coverglass with mowiol (Biovalley).

• **Western Blot**

Total cell extracts were lysed in 0.08 M Tris-HCl (pH:6.8), 2% SDS, 12% sucrose, 2% β-mercaptoethanol and bromophenol blue traces then blotted against E4F1 (produced by our laboratory), PDC-E2 (Diat) (Santa Cruz), MITF (HPA003259-Sigma), GP100 (ab137078-Abcam), TYROSINASE (T311-Santa Cruz) and p53 (DO7-Santa Cruz). β-Actin monoclonal peroxydase (Sigma) was used as loading control.

• **Culture conditions**

Human melanoma cell line MM031 was provided by Chris Marine’s laboratory. Cells were cultured in Ham’s F10 nutrient mix (Invitrogen) supplemented with 25 mM Hepes (Invitrogen), GlutaMax (Sigma) and foetal calf serum (eurobio).

• **Lentiviral Particle Production and Infections**

Lentiviral particles were produced in 293T cells by transient transfection using Jet-PEI reagent (Ozyme) of gag/pol, env-VSV-G and indicated viral constructs pLKO vector encoding either control (Sigma, SHC002), anti Diat human shRNAs (Sigma, TRCN0000296579, TRCN0000290473, TRCN000035920) and anti E4F1 human shRNA (Sigma, TRCN000013823). At 48 h after transfection, viral supernatants were harvested and added to melanoma cell line overnight withpolybrene 8 μg/mL (Sigma-Aldrich). Antibiotic selection of transduced melanoma cell line was performed 48 hours later with puromycin (1.25 ng/mL; pLKO).
• Immunofluorescence

Cells were fixed with methanol (Sigma) at -20°C for 5 minutes and incubated with GP100 (ab137078-Abcam) antibody overnight at +4°C. Fluorescent staining was performed using secondary antibody conjugated to Alexa 488 anti rabbit (Thermofischer) for 2 hours at room temperature. Cover glasses were mounted with Mowiol (Biovalley).
DISCUSSION AND PERSPECTIVES
I. E4F1 and METABOLISM RELATED FUNCTIONS

A. Cell Cycle

Growing evidence suggests that cell division is directly linked to cell metabolism. Dividing cells need to double their biomass rapidly before splitting this biological material in the daughter cells and have therefore to adapt to their important anabolic demands. Consistent with this notion, quiescent (resting) cells and proliferating cells have different metabolic status.

How the cell cycle machinery connects to metabolism? For instance, it was shown that in absence of glucose, yeast can’t initiate cell division and become quiescent. When glucose is supplied, they enter cell cycle and start proliferating. In eukaryotic cells, uptake of nutrients is regulated by mitogenic signals (Lloyd et al., 2013). In the presence of mitogenic signals, cells will uptake the nutrients that are necessary to increase their biomass. On the other hand, in absence of proliferative signals, cells won’t undergo cell division even if nutrients are abundant (Rathmell et al., 2000). The reverse is also true: proliferative signals in the absence of nutrient will not induce cell cycle entry. Thus, mitogenic (proliferative) signals and nutrients are both required for cell proliferation.

At the molecular level, several cell cycle regulators have been linked to metabolism. Thus, several studies have linked Cyclins, Cdk5, pRb and E2Fs to lipid synthesis, glucose metabolism and insulin signalling (Fajas et al., 2002; Sarruf et al., 2005; Iankova et al., 2006). These data suggest that these cell cycle regulators are not only responsible to drive cell-cycle progression but are also needed to sense nutrient availability (Figure 43). Interestingly, these studies have also shown that the metabolic functions of these cell cycle regulators are important in non-proliferative differentiated cells. Their genetic inactivation in differentiated cells leads to various in vivo metabolic phenotypes such as obesity and diabetes (Aguilar and Fajas 2010; Saxena et al., 2007; Scott et al., 2007), raising important questions regarding the ancestral function of these genes. Previous works on E4F1 clearly demonstrated that it is an important cell cycle regulator (Fajas et al., 2001; Rizos et al., 2003; Le Cam et al., 2006). Our data showing that E4F1 is also a key player of pyruvate metabolism strengthen the notion that cell cycle has to be highly coordinated with metabolism.
Figure 43: Cell cycle regulators are required for both metabolic adaptations and cell cycle progression (Adapted from Fajas 2013).

B. Viral Replication

Many eukaryotic viruses have evolved to hijack multiple cellular pathways of their host cells including metabolism to provide the optimum conditions for their replication and further dissemination. As an illustration of this notion, several viruses such as dengue virus (Fontaine et al., 2015), human cytomegalovirus (HCMV) (Munger et al., 2008), Hepatitis C (HCV) (Ripoli et al., 2010) and adenovirus (Thai et al., 2014) have been reported to increase aerobic glycolysis.

For instance, it was demonstrated that the E1A adenoviral oncoprotein impairs the metabolic status of myoblasts and stimulates glycolysis through deregulation of the Rb/E2F pathway. In that context, E2F1 was shown to activate the transcription of PDK4, which results in decreased PDH activity, the redirection of the glycolytic flux towards lactate production (Hsieh et al., 2008). Interestingly, E4F1 is also a cellular target of E1A as already discussed. However, if E1A is targeting cellular factors that favor glycolysis, then why would it increase E4F1 activities which are for oxidative metabolism? One has to keep in mind that E1A was shown to increase the binding of E4F1 to the CRE element on the E4 promoter that significantly differs from the consensus E4F1-binding site defined by ChIP-seq approaches (Rodier et al., 2015). Moreover, during adenoviral infection, E1A activates E4F1 by phosphorylation which leads to the generation of a proteolytic cleavage product, pS0E4F1. Thus, it is possible that E1A hijacks E4F1 for the activation of viral genes but diminishes its activity towards its own target genes such as DLAT. Further experiments will be needed to explore this hypothesis. Finally, the data showing that E4F1 is also the target of other viral proteins such as GAM1 (Colombo et al., 2003), and the hepatitis B protein HBX (Rui et al., 2006) raise interesting questions regarding the role of pyruvate metabolism during these viral cycles.
II. E4F1 and p53 CROSSTALK IN METABOLISM

As discussed previously, the aim of my project was to investigate the p53-independent molecular pathways regulated by E4F1 that impact on epidermal stem cell function. I was able to show that in addition to its role in the Bmi1-ARF-p53 pathway, E4F1 controls a metabolic program involved in PDH activity that is important for the maintenance of epidermal stem cells and normal skin homeostasis. The ability of E4F1 to regulate PDH-mediated transcriptional activity was independent of p53 as shown by our results obtained in p53-deficient cells. These results raise important questions regarding the relative contribution of p53 dependent and p53 independent mechanisms in epidermal stem cell maintenance. Of note, activation of p53 was observed in E4F1-deficient epidermis (Figure 44A). Interestingly, although we detected DNA damage in E4f1 KO skin, this is unlikely the molecular trigger of p53 activation in basal cells since histological analyses indicate that P53 and γH2AX (a DNA damage marker) stainings do not colocalize. Thus, whereas p53 stabilization was mainly detected in basal cells, γH2AX staining was restricted to the most upper cells, suggesting that DNA damage occurs as cells undergo successive rounds of cell replication. We currently suspect that the metabolic challenge E4f1-deficient keratinocytes face triggers an adaptive response driven by p53. This hypothesis is supported by some of preliminary results showing that shRNA-mediated depletion of Dlat leads to p53 stabilization (Figure 44B). Although growing evidence suggests that p53 is implicated in cell metabolism in addition to its acute stress responses, such crosstalk between impaired PDH activity and p53 regulation has not been reported yet. We have not yet extensively characterized the p53-regulated transcriptional program triggered in E4F1-deficient keratinocytes. However, based on data obtained in other E4F1-deficient primary cell types, we have started to investigate the role of several p53 target genes implicated in metabolism, including PGC1α, Fasn, Tigar and Glut4 (Figure 44C).
Figure 44: p53 and E4F1 crosstalk. (A) p53 is activated in E4f1 KO basal keratinocytes. Arrows indicate p53-positive cells. DNA damage is observed in suprabasal keratinocytes. Dash arrows indicate γH2Ax-positive cells (e: epidermis, d: dermis). (B) p53 is induced in Dlat-deficient keratinocytes. (C) Metabolic target genes of p53 (Glut1, Pgc1α) are downregulated upon E4f1 inactivation in keratinocytes.

My preliminary data suggest that the induction of p53 triggered by E4F1 deficiency is not restricted to primary keratinocytes and also occurs in melanoma cells. In the latter cells, I was able to show that both E4f1 deficiency and Dlat depletion induce pigment defects, suggesting that PDH activity is implicated in melanocyte pigmentation. However, it remains to be determined whether PDH activity and or p53 activation are implicated in this...
pigmentation defect. Interestingly, previous reports have shown that UV-induced activation of p53 in keratinocytes impairs pigmentation through the control of MITF (Cui et al., 2007). Ongoing genetic and biochemical analyses should clarify the role of p53 in the pigmentation defects observed in E4F1-deficient melanocytes.

III. **E4F1 and PYRUVATE METABOLISM**

A. **E4F1 as an essential regulator of PDH complex**

One of the important outcomes of my PhD is the implication of mitochondrial PDH activity in epidermal stem cell function. The transcriptional control of important PDC subunits by E4F1 highlights a new level of regulation of this important metabolic enzyme. Indeed, until now, studies on PDH regulation have mostly focused on post-translational modifications driven by PDKs and PDPs that allow a fast and acute regulation of the PDC. Although transcriptional regulation of the PDC has been described in more primitive organisms, this is the first example of a transcriptional program implicating several genes controlling PDH in mammals. In *E. coli*, the aceE/E1, aceF/E2, IpDA/E3 genes are organized in a unique operon that is regulated by the transcriptional regulator PdhR (Ogasawara et al., 2007). In *C. albicans*, the transcriptional regulator Gal4p controls the expression of five main components of the PDH complex: Pda1/E1, Pdb1/E1, Dlat/E2, Lpd1/E3 and Pdx1 (Askew et al., 2009). Thus, in mammals, E4F1 target genes included two core components of the PDC (Dlat/E2 and Dld/E3 subunits), the mitochondrial pyruvate carrier (Brp44L/Mpc1), the mitochondrial thiamine pyrophosphate transporter (Slc25a19) that provides an essential cofactor for the PDC, and a regulatory subunit of the phosphatase that controls the complex at the post-translational level (Pdp1r). Although we detected E4F1 on the promoter of these genes in mouse embryonic fibroblasts (Mefs), in muscle cells and in keratinocytes, E4F1 inactivation impacted the expression of these PDH subunits differently depending on the cell type. As an illustration of this notion, in E4F1 KO Mefs, all these target genes were significantly downregulated whereas in primary keratinocytes Dlat was the most affected gene upon E4f1 inactivation. Our results point to a central role of Dlat in E4F1-mediated control of the PDC. These results also suggest that in addition to E4F1, other yet unidentified transcription factors significantly contribute to the control of these PDH-related genes in a tissue specific manner.

A recent study on the PDC revealed that it does not only function in mitochondria to link pyruvate to oxidative phosphorylation, but it could also have nuclear functions. For instance, the PDC complex was also shown to localize to the nucleus to generate locally a pool of Acetyl CoA to favor histone acetylation and regulate gene expression (Sutendra et al., 2014). Importantly, as one potential consequence of impaired PDH activity, we showed that E4f1-inactivated keratinocytes exhibit decreased histone H4 acetylation. These results
highlight a previously unsuspected link between E4F1-associated metabolic functions and the regulation of the epigenome. It will be interesting to address whether such epigenetic modifications influence the expression of the epidermal differentiation cluster, a group of genes involved in keratinocyte differentiation, thereby providing a potential molecular explanation for the block of keratinocyte differentiation that was observed in E4f1cKO; K5/K14Cre animals. In addition, our data also suggest that although Acetyl CoA can be generated from other sources such as fatty acids or acetate, glucose-derived Acetyl CoA production plays an important role for histone acetylation in keratinocytes. We currently do not rule out that defective glucose-derived Acetyl CoA production in E4F1-deficient cells impinge on the acetylation of other non-histone proteins that can play an important role in keratinocyte function. One ongoing project in the host laboratory aims at identifying by proteomics how E4f1 inactivation and/or PDH deficiency affect acetylation of histones and/or non-histone proteins and whether these effects lead to epigenetic changes and modifications of the genome organization.

B. E4F1 and PDH deficiency

Considering the crucial functions of the PDC in mitochondria, it is not surprising that PDH deficiency is associated with severe human metabolic syndromes. PDH-deficiency has been causally linked to hypomorphic mutations in genes encoding different subunits of the PDC complex including the PDHE1/E1, DLAT/E2, DLD/E3 catalytic subunits and the E3BP binding subunit. Patients with PDH-deficiency exhibit systemic lactic-acidosis and a broad range of clinical symptoms among which are common neurological and motor-neuron defects that often start within the first year of life. One such disease is the Leigh syndrome, a genetically heterogeneous disease that results from mutations in more than 60 different genes encoded either by the nuclear or the mitochondrial genome that are part of the complex I mitochondrial respiratory chain or subunits of the PDC complex (Gerards et al., 2016). Leigh syndrome patients suffer from epileptic seizures, microcephalies and have a shorten lifespan. Importantly, a homozygous non-synonymous missense mutation has been recently identified in the E4F1 gene in two patients exhibiting PDH deficiency and neurological retardation. The patients were siblings and the parents were heterozygote for this mutation. These patients presented clinical symptoms that resembled those of Leigh syndrome patients, including lactic acidosis, hypotonia, microcephaly and neurological lesions. Consistent with our results, fibroblasts derived from those patients exhibited decreased DLAT and PDPR mRNA levels. Taken together, these new findings strengthen our data demonstrating that E4F1 is a major regulator of the PDH complex and illustrate that PDH regulation by E4F1 is also relevant in the context of human disorders.

One important perspective of my recent data relate to our understanding of skin alterations observed in patients with these metabolic disorders. It is worth noting that
ten per cent of patients with primary mitochondrial disorders present skin manifestations that can be categorized into hair abnormalities, rashes, pigmentation abnormalities and acrocyanosis (Bodemer et al., 1999). Much less attention has been paid to these skin phenotypes as the severity of symptoms in other organs (in particular those occurring in the central nervous system) overcomes these less life-threatening clinical features. However, my data indicates that impairment of PDH activity in E4f1-deficient epidermis, a situation mimicking that of Leigh Syndrome patients (Legati et al., 2016) leads to severe skin phenotypes raise important biological and clinical questions regarding the direct implication of such metabolic perturbations in the skin manifestations observed in some patients affected by mitochondriopathies. Conversely, it is also currently unknown whether some poorly understood skin diseases are related to metabolic perturbations. For most of these skin diseases, it is very difficult to discriminate between intrinsic metabolic alterations that directly contribute to skin manifestations and secondary metabolic adaptive responses that result from perturbed skin homeostasis. Taken together, these data support the fact that we need to improve our knowledge in skin metabolism and understand how this one is driving skin defects to propose a better diagnosis of these skin diseases and better signs of treatment to fight these disorders. I expect that my results will provide answers to these important questions.

IV. EPIDERMAL METABOLISM

A. Keratinocytes Homeostasis

My data show for the first time that E4f1-mediated control of DLAT is an important determinant of pyruvate metabolism in basal keratinocytes. Using different genetically modified mouse models, we found that E4f1 inactivation impacts on epidermal stem cell maintenance and normal skin homeostasis. However, one question remains unanswered in this study due to technical limitations of the K14CreERT2 model we used to inactivate E4f1 in basal keratinocytes. Indeed, with this transgenic strain, Cre-mediated recombination of E4f1 occurred in all basal keratinocytes that include both undifferentiated progenitors and epidermal stem cells. Thus, although I clearly showed that E4f1-inactivation impacted on epidermal stem cell homeostasis, I was unable to demonstrate that the phenotypes observed upon E4f1 inactivation occurs in a stem cell-autonomous manner. To answer this question, we have generated a follicular stem cell-specific E4f1 conditional KO model based on the Keratin15CrePR transgenic mice. In these animals, the expression of a RU486-inducible version of the Cre recombinase is under the control of the Keratin 15 promoter, whose expression is restricted to epidermal stem cells that are located in the bulge region of hair follicles. With this model, we hope to inactivate E4f1 specifically in follicular bulge stem cells and see whether PDH-deficiency in these cells also leads to the exhaustion of the stem cell pool.
One of the goals of my PhD project was to study the poorly investigated metabolic status of epidermal cells. My study suggests that basal keratinocytes display high PDH activity that is necessary for normal skin homeostasis. Measurement of PDH activity in protein extracts prepared from isolated murine keratinocytes or total epidermis indicates that the epidermis contains cells with high PDH activity which are likely basal keratinocytes given the relative abundance of basal and differentiated keratinocytes in these samples. This activity is significantly higher (5 to 10 times higher) than that detected in primary mouse embryonic fibroblasts (Figure 45). This finding was quite unexpected since these basal cells that contain highly proliferative progenitors are proposed to rely mainly on glycolysis to support their energetic and anabolic demands. However, in a manner reminiscent of highly proliferative cancer cells, this notion does not exclude the possibility that oxidative phosphorylation contributes significantly to the metabolic status of basal keratinocytes, including to support aspartate production and nucleotide biosynthesis (Birsoy et al., 2015). This notion is supported by our results showing that the metabolic reprogramming of E4f1 cKO basal keratinocytes that display a strong glycolytic profile and consume less oxygen, affects skin homeostasis. These results are consistent with those of the Chandel laboratory showing that mice in which inactivation of Tfam has been triggered in basal keratinocytes display phenotypes that resemble those observed in E4f1 cKO mice (Hamanaka et al., 2013), including increased proliferation within the basal layer and reduced expression of differentiation-specific genes.

![Figure 45: Keratinocytes exhibit high PDH activity compared to MEFs.](image-url)
Based on their work showing that mitochondria-derived ROS induce keratinocyte differentiation through the regulation of Notch and β-catenin signaling, we currently suspect that impaired pyruvate oxidation in E4f1 KO keratinocytes may result in defective ROS production and alteration of epidermal differentiation. However, the relative contribution of glycolysis and oxidative phosphorylation in skin biology still remain to be clarified since others have reported that complete ablation of the ETC before birth (also obtained by Tfam inactivation using the same K14Cre model) does not affect the differentiation of interfollicular basal keratinocytes. The reason of these discrepancies remains to be understood. Nevertheless, my data indicate that E4F1-mediated regulation of pyruvate metabolism is important for basal keratinocyte function.

B. Epidermal stem cell homeostasis

Based on studies in other highly self-renewing tissues (in particular the hematopoietic system), it is believed that adult stem cells are mainly quiescent and glycolytic. It was proposed that this metabolic status protects them from mitochondria-derived ROS-induced damages in order to maintain, on the long term, their self-renewal capacities (Rossi et al., 2008; Suda et al., 2011). Is this state dictated by their metabolic status or by their microenvironment? Their localization in a hypoxic niche is believed to contribute to their metabolic status through the induction of HIF, a key transcription factor of the hypoxic response that favors glycolysis and limits oxidative phosphorylation through the PDK-dependent phosphorylation of the PDC (Takubo et al., 2012). The dependence of hematopoietic stem cells on glycolysis limits ROS production that was shown to induce differentiation or apoptosis in these cells (Tothova et al., 2007). The same mechanism was also demonstrated for neural stem cells in brain (Renault et al., 2009). These two examples illustrate the notion that adult stem cells are not only glycolytic as an adaptation to their microenvironments but also to maintain their quiescent state (Shyh-Chang et al., 2013). Taken together, these examples illustrate that adult stem cells rely on glycolysis to stay quiescent and increased ROS level originated from mitochondrial oxidative metabolism induce their differentiation (Shyh-Chang et al., 2013). However, there is currently no real experimental demonstration that this dogma applies to epidermal stem cells.

My recent work showed that basal keratinocytes (a population of cells that contain both epidermal stem cells and proliferative progenitors) exhibit high PDH activity and high mitochondrial activity raises interesting questions. E4f1 inactivation stimulated a metabolic switch towards glycolysis and this forced these stem cells to exit their normal niches. It remains to be confirmed if this phenotype occurs in a stem cell autonomous manner. However, if this was the case, such data would challenge the established dogma that quiescent stem cells are glycolytic. These data may also highlight the notion that the metabolic status of adult stem cells is more heterogeneous than originally thought and that
epidermal stem cells display a stronger dependence on oxidative metabolism than other types of adult stem cells.

One other important aspect of my findings relates to the impact of metabolism on the microenvironment of these basal keratinocytes, and in particular on the organization and integrity of the basement membrane to which they are attached through hemidesmosomes. However, the molecular determinants of the epidermal “stem cell niche” remain to be more precisely defined. How metabolic changes affecting basal keratinocytes can impact this niche is yet a poorly investigated area. Here my data show that as a consequence of impaired PDH activity, the redirection of the glycolytic flux, the increased lactate/H⁺ secretion and the acidification of the microenvironment results in the induction of extra-cellular matrix remodelling enzymes that disrupt the basal membrane and lead to an exit of epidermal stem cells out of their niche. One important future direction will be to investigate whether this mechanism occurs locally and in a stem cell autonomous manner during the normal self-renewal of the epidermis.

Besides normal tissue renewal, improving our knowledge on the metabolic status of normal epidermal stem cells might also have important perspectives for skin cancer therapies. One of the difficulties of current chemotherapies that result in relapse is the failure of these genotoxic drugs to target efficiently quiescent stem cells. My results showing that the control of pyruvate metabolism impacts on the mobilization of epidermal stem cells may lead to new strategies that could help targeting quiescent cancer initiating cells.

C. Melanocyte Homeostasis

In order to further extend our knowledge about E4F1 functions in skin, I started to evaluate E4F1 roles in another cell type such as melanocytes. My data demonstrated that melanocyte specific inactivation of E4f1 leads to hair graying and skin pigmentation defects. Interestingly, both in vitro and in vivo findings indicate that these pigmentation defects could be the consequence of a cell-autonomous impaired melanin production rather than the cell death nor alteration in melanocyte differentiation. Surprisingly, the impaired melanin production seems to be linked to PDH activity since depletion of the core component of the PDC, Dlat, recapitulated the E4f1-dependent defects. Although further investigations will be needed to determine how PDC is linked to melanogenesis, previous data from the literature could already explain the implication of PDC in pigment production and could be interesting hypothesis to challenge. For instance, one could argue that nuclear function of PDC could be necessary for melanogenesis. It was demonstrated that Mitf can work in cooperation with p300 and CBP histone acetyltransferases to regulate gene expression (Sato et al., 1997; Price et al., 1998). Since nuclear PDC is one of the main source of Acetyl CoA for histone acetylation reactions, one could imagine that Mitf requires PDH activity in order to activate its target genes implicated in pigmentation. Further experiments
will be needed to understand the close link between pyruvate metabolism and melanin production.

V. E4F1 and CELLULAR TRANSFORMATION

A. PDH and Senescence

Recent evidences indicate that oncogene-induced senescence (OIS) depends on PDH activity. This notion was initially described in BRAF and NRAS-mediated senescence. Braf and NRas are proto-oncogenes that play important roles in signal transduction of the mitogen-activated protein kinase (MAPK) signaling pathway which is necessary for cell division, proliferation and differentiation (Figure 46). Braf is often mutated in different types of cancer including melanoma which is one of the deadliest skin cancers. It was shown that in cells expressing Braf^{V600E} (a constitutively active form of BRAF), OIS correlated with decreased PDK1 and increased PDP2 expression, leading to increased PDH activity and oxidative stress. Importantly, shRNA mediated depletion PDP or expression of ectopic PDK was sufficient to bypass OIS, confirming the importance of this key metabolic node in OIS (Kaplon et al., 2013). Strikingly, preliminary data from my host laboratory shows that E4F1-mediated cellular senescence shares some common features with Braf-oncogene induced senescence (Figure 47). In E4F1 overexpressing cells, PDH activity is enhanced which is accompanied by decreased PDK and increased PDP expression. Together, these data provide very important clues about E4F1 and its potential role during early steps of tumorigenesis and raise important questions.

![MAPK signaling cascade in melanoma](image_url)

**Figure 46:** MAPK signaling cascade in melanoma development.
First of all, since Braf and E4f1-induced senescence occurs through the same molecular mechanisms, it questions the potential implication of E4F1 in the MAPK signaling pathway. I am currently challenging this hypothesis in vivo in the context of melanocytes expressing oncogenic forms of NRAS and BRAF. In these GEMMs, constitutive activation of the MAPK pathway in melanocytes leads to the generation of naevi, a skin lesion that was previously shown to be composed, at least partly, of senescent cells (Michaloglou et al., 2005). Using these animal models, I’m evaluating whether E4F1 deficiency is sufficient to bypass OIS in vivo and to promote melanomagenesis. Interestingly, my preliminary results indicate that E4f1 inactivation in Braf\(^{600E}\) expressing melanocytes results in their depigmentation. I will investigate whether these mice display impaired melanin production or if E4F1 deficiency leads to the complete loss of melanocytes. So far, these E4f1 cKO; Braf\(^{600E}\) mice did not display increased melanoma incidence. However, the experimental groups were not kept long enough (2 months after E4f1 inactivation) to exclude any impact on melanomagenesis. Using these animal models, I will answer the following questions:

- Does the hypopigmentation observed in E4f1-inactivated melanocytes a consequence of increased cell death or an escape from senescence?
- Is this sufficient to initiate tumorigenesis or increase melanoma development?
- From the molecular point of view, does E4F1 inactivation abrogate MAPK signaling pathway? Does Braf/NRas activation change E4F1 expression or activity? Is E4F1 one of the effector of the MAPK pathway? Does Braf-induced senescence depend on E4F1-mediated control of Dlat, Dld, or Mpc1? How E4F1-E3 ligase activity on p53 contributes to OIS?
B. Cancer

Although no recurrent mutations of E4F1 have been so far identified through whole genome sequencing of various cancer types, there are some findings that suggest that E4F1 can be implicated in carcinogenesis. What we know about E4F1 that its level of expression can be modulated in healthy and transformed cells. For instance, E4F1 protein level is increased in acute myeloid leukemia (Hatchi et al., 2011) and in hepatocarcinoma (Dai et al., 2014). Besides E4F1 interacts with number of oncogenes such as Bmi1 (Chagraoui et al., 2006), DRAL, HMGA2 (Tessari et al., 2003) and tumor suppressors such as pRb (Fajas et al., 2000), p53 (Sandy et al., 2000), RASSF1A (Fenton et al., 2004) and p19ARF (Rizos et al., 2003). Finally it also interacts with viral oncoproteins such as E1A13S (Lee and Green, 1987; Raychaudhuri et al., 1987), GAM1 (Colombo et al., 2003), and hepatitis B protein HBX (Rui et al., 2006). Altogether these results strengthen the idea that E4F1 is involved in tumorigenesis, and that its roles in metabolism influences tumor progression.

Metabolic reprogramming is now considered a hallmark of cancer cells. These metabolic changes are necessary to provide metabolic intermediates that are used for the biosynthesis of macromolecules and the production of biomass. One of the metabolic pathways favorable for these anabolic processes is glycolysis, and most cancer cells switch their metabolism from OxPhos to glycolysis even in the presence of oxygen in order to sustain rapid proliferation. This metabolic rewiring of cancer cells was initially described by Otto Warburg. Here my data demonstrate that E4f1 inactivation recapitulates some aspects of this metabolic switch and that this occurs even in primary cells such as primary keratinocytes. In cancer cells, this phenomenon is usually associated with remodeling of the microenvironment, facilitating the invasion of cancer cells and metastatic dissemination. Consistent with this notion, my data also showed that the metabolic reprogramming of E4F1 KO cells correlates with remodeling of the microenvironment through the activity of extracellular matrix proteases such as MMPs and cathepsins. The mechanism of activation of these metalloproteases in E4F1-deficient cells is still not known. It was initially proposed that BASIGIN/CD147, a molecular chaperone that is necessary for the expression of the monocarboxylate transporters MCT1 and MCT4 at the plasmic membrane, can increase MMP activity through an unknown mechanism (Biswas et al., 1995). However, this hypothesis was then disproved (Marchi et al., 2015). On the other hand, low pH was shown to be involved in the activation of cysteine cathepsins (Robey et al., 2009; Rozhin et al., 1994). Acidic pH induces spreading of lysosomes and endosomes at the cell surface and therefore leads to increased secretion of cathepsins (Glunde et al., 2003; Steffan et al., 2009) and resulted in the remodeling of the microenvironment.

Based on these results, it will be worth investigating the role of E4f1 in cancer progression and dissemination. Further studies based on the inactivation of the gene in an established tumor in vivo would provide interesting findings. For instance, E4f1 inactivation

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can enhance cancer cell proliferation by favoring glycolysis over oxidative phosphorylation which provides essential intermediates for rapid proliferation. Besides, redirection of glycolytic flux towards lactate can further enhance invasion of cancer cells. Of note, loss of E4f1 phenotype is associated with cancer-like feature, however in some cancer models E4F1 protein level was reported to increase such as acute myeloid leukemia and hepatocarcinoma. Therefore it is worth investigating in the context of carcinogenesis how E4F1 affects cell metabolism, and whether E4F1-mediated control of pyruvate metabolism is a suitable target for the design of new anti-cancer therapies.

CONCLUSION

The data obtained during my PhD allowed me to further understand the role of E4f1 in skin homeostasis. Using skin specific mouse models, I was able to show that E4f1 is essential for keratinocyte and melanocyte homeostasis through the regulation of a complex metabolic network. Interestingly, the notion and concept identified in skin could also be applicable in other tissues and could probably help improving our understanding of E4F1 functions in vivo. As an example, implication of E4F1 in cell cycle regulation was largely documented; however these results seem to be the tip of the iceberg. As in the light of my recent findings, I suggest that E4F1 is a crucial regulator of cell metabolism and that these E4F1-associated metabolic functions could impact several of these different processes. As another illustration of this notion, PDH-derived Acetyl CoA provides acetyl group for histone acetylation reactions, which activates gene expression to drive cell proliferation and differentiation. Finally, metabolic reprogramming such as increased glycolysis could have important consequences on the microenvironment of cells and affect cell mobilization and cell differentiation. All together, my recent findings support the fact that E4F1, through the regulation of metabolism, is impacting various cellular functions and thus is indispensable for normal tissue homeostasis (Figure 48).
Figure 48: E4F1-mediated control of cell metabolism and its impact on different cellular processes and in tissue homeostasis.
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Résumé:

L’étude des réseaux protéiques perturbés au cours de l’infection par les petits virus oncogéniques amena, vers la fin des années 80, à la découverte de nombreux régulateurs clés de la division et de la survie cellulaire. Parmi ceux-ci, la protéine E4F1 fut initialement identifiée comme une cible de l’oncoprotéine virale E1A. Originellement identifié comme un facteur de transcription, E4F1 est également une ubiquitine-E3 ligase atypique pour d’autres facteurs de transcription tel que le suppresseur de tumeurs p53. Au travers de ses multiples activités, E4F1 est nécessaire à la prolifération des cellules somatiques et souches, et à la survie des cellules cancéreuses. De plus, les travaux de différents laboratoires dont le mien suggèrent qu’E4F1 se situe au carrefour de plusieurs voies de signalisation qui sont fréquemment altérées au cours de l’oncogénèse, et notamment la voie impliquant le suppresseur de tumeurs p53. Afin d’étudier les fonctions physiologiques in vivo d’E4F1, mon laboratoire d’accueil a développé plusieurs modèles de souris génétiquement modifiées. La caractérisation de ces modèles a permis de mettre en évidence un rôle majeur d’E4F1 dans l’homéostasie de la peau. Plus précisément, E4F1 régule le pool de cellules souches de l’ épiderme au travers de son rôle dans une voie de signalisation qui implique la protéine p53 et deux de ces régulateurs en amont: Arf et Bmi1.

Cependant, il semble que les effets d’E4F1 dans le contrôle du maintien des cellules souches s’étendent au delà de son rôle sur cette voie de signalisation. En effet, j’ai récemment pu démontrer qu’E4F1, au travers de ces fonctions transcriptionnelles, régule directement l’expression d’un sous-groupe de gènes impliqués dans la régulation de l’activité de la pyruvate déshydrégénase (PDH). La PDH est un complexe multienzymatique situé dans la mitochondrie qui catalyse la décarboxylation du pyruvate (le produit final de la glycolyse) en acétyl coenzyme A (AcCoA), liant ainsi le métabolisme du pyruvate au cycle de Krebs. J’ai pu montrer que l’inactivation d’E4F1 spécifiquement dans l’ épiderme conduisait à une diminution importante de l’activité de PDH et à une reprogrammation métabolique de ces cellules. Cette reprogrammation a pour conséquence d’altérer le micro-environnement des cellules souches qui conduit à leur détachement de leur niche et aboutit in fine à une absence du renouvellement de l’ épiderme. Cette partie de mes travaux a donc permis d’illustrer pour la première fois l’importance du métabolisme du pyruvate dans l’homéostasie des cellules souches de la peau. Sur la base de ces résultats, je poursuis l’analyse des fonctions d’E4F1 dans l’homéostasie de la peau en étudiant son rôle dans d’autres types cellulaires tels que les mélanocytes.

Abstract:

The multifunctional protein E4F1 is an essential regulator of normal skin homeostasis. During my PhD, I demonstrated that E4F1 inactivation in adult skin results in stem cell autonomous defects causing exhaustion of the epidermal stem cell (ESC) pool. At the molecular level, I identified E4F1 as a new regulator of the pyruvate dehydrogenase complex (PDC) in keratinocytes, an essential mitochondrial complex that converts pyruvate into Acetyl-CoEnzyme A. Using genetically engineered mouse models; I showed that E4F1-mediated control of PDH activity is required to maintain normal skin homeostasis. Consistently, E4F1 deficiency in basal keratinocytes resulted in deregulated expression of dihydriopoidamide acetyltransferase (Dlat), a gene encoding the E2 subunit of the PDC, and impaired PDH activity. The metabolic reprogramming of E4F1 KO keratinocytes associated with the redirection of the glycolytic flux towards lactate production and increased lactate secretion in their microenvironment, leading to enhanced activity of extra-cellular-matrix remodelking proteases. Finally, these defects ended in alterations of the basement membrane,ESC mislocalization and the exhaustion of the ESC pool. In the second part of my thesis, I have evaluated the role of E4F1-mediated control of the PDC in melanocytes and showed that the metabolic activities of E4F1 are important for melanocyte function. Consistently, mice with E4f1-deficient melanocytes exhibited hair graying and skin pigmentation defects. Altogether, my data demonstrate the importance of E4f1-mediated control of pyruvate metabolism for normal skin homeostasis.