Effects of physiological overexpression of SIRT6 in metabolism and cancer

Maciel Ioris, Rafael

UNIVERSITÉ DE GENÈVE

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Thèse de Monsieur Rafael MACIEL IORIS

intitulée :

«Effects of Physiological Overexpression of SIRT6 in Metabolism and Cancer»

La Faculté des sciences, sur le préavis de Monsieur R. COPPARI, professeur ordinaire et directeur de thèse (Faculté de médecine, Département de physiologie cellulaire et métabolisme), Monsieur R. J. LOEWITH, professeur ordinaire et codirecteur de thèse (Département de biologie moléculaire), Monsieur L. FAJAS COLL, professeur ordinaire (Département de physiologie, Center for Integrative Genomics, Université de Lausanne), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 20 novembre 2018

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Le Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Com a certeza de que todos estão e sempre estiveram presentes,

dedico este trabalho a vocês!

Mother and Wife

Father

Aunt Cleusa

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Abstract

Sirtuins are a family of conserved enzymes, from bacteria to human, which have in common a deacetylase domain and a dependency for oxidized nicotinamide adenine dinucleotide (NAD^+) as co-substrate. The first member of Sirtuin family identified, the silent information regulator 2 (Sir2) from yeast *Saccharomyces cerevisiae*, when overexpressed resulted in lifespan extension. This exciting discovery was soon confirmed in others organisms, *Caenorhabditis elegans* and *Drosophila melanogaster*, and generate enormous interest of the scientific community to uncover whether any of the seven mammalian Sirtuins (Sirt1-7) could also extend lifespan. The mammalian Sirtuin family members are link with most of the essential cellular process and have a function in all hallmarks of aging. Several members are able to improve healthspan however, only SIRT6 was proved able to extent lifespan. The mechanisms behind life span extension is unclear but hypothesized to be due to SIRT6 capacity to maintain genomic stability, improve insulin signalling and reduce tumorigenesis. Although there are vast evidence of SIRT6 role in those processes, the absolute majority of the studies used SIRT6 loss-of-function *in vivo* approaches or a supraphysiological overexpression. Those opposite approaches generated conflicting results as, for example, regarding glucose metabolism where both approaches improve it. In order to address these issues we used a mouse model were SIRT6 is physiologically overexpressed. More specifically, we use this model to assess metabolic parameters in different diet context to understand SIRT6 could be beneficial in the context of obesity and type 2 diabetes mellitus (T2DM) and breed with a known mammary cancer mouse model to assess SIRT6 role in tumorigenesis. Our data indicate that SIRT6 improve insulin sensitivity in skeletal muscle and liver protecting against diet-induced

T2DM and, in the cancer context, SIRT6 suppresses cancer stem cells diminishing *in vitro* and *in vivo* tumorigenesis.

Résumé

Les sirtuines appartiennent à une famille d'enzymes hautement conservées de la bactérie à l'homme. Elles ont en commun un domaine de déacétylation et l'utilisation du nicotinamide adénine dinucléotide oxydé (NAD+) comme co-facteur. Le premier membre de cette famille, Sir2, pour silent information regulator 2, a été identifié chez la levure *Saccharomyces cerevisiae*. Lorsque surexprimé chez cet organisme, il entraîne un allongement de l'espérance de vie. Cette importante découverte a rapidement été confirmée chez d'autres organismes uni ou pluricellulaire comme *Caenorhabditis elegans* et *Drosophila melanogaster*, suscitant un énorme intérêt de la part de la communauté scientifique pour déterminer si l'une des sept sirtuines que compte cette famille chez le mammifère (Sirt1-7) pouvait également jouer sur la longévité. Chez ce dernier, les sirtuines sont impliquées dans la plupart des processus cellulaires essentiels, dont ceux associés au vieillissement cellulaire. Plusieurs membres de cette famille semblent capables « d'améliorer » ce processus, alors que seule SIRT6 est associée à l'allongement de l'espérance de vie. Les mécanismes impliqués sont encore mal définis mais les hypothèses avancées sont que SIRT6 permettrait une meilleure stabilité du génome, augmenterait la sensibilité à l'insuline et réduirait l'apparition de tumeurs. La majorité de ces études a cependant employé des approches *in vivo* soit de « perte de fonction », soit de surexpression supra-physiologique. Ces approches opposées ont alors parfois généré des résultats contradictoires comme dans le cas du métabolisme du glucose, où les deux méthodes l'améliorent. Afin de répondre à ces questions, nous avons utilisé un modèle de souris dans lequel SIRT6 est surexprimé de façon physiologique. Nous avons plus spécifiquement évaluer les paramètres métaboliques de ce modèle lorsque soumis à

différents régimes alimentaires afin de déterminer le rôle potentiellement bénéfique de SIRT6 dans un contexte d'obésité et/ou de diabète de type 2. Ces souris ont également été croisées avec un modèle murin développant spontanément des tumeurs mammaires afin de déterminer le rôle de SIRT6 dans la tumorigénèse. Nos résultats montrent que SIRT6 améliore la sensibilité à l'insuline dans les muscles squelettiques et le foie, protégeant ainsi du développement d'un diabète de type 2 induit par l'alimentation, et, dans le contexte du cancer, que SIRT6 supprime les cellules souches cancéreuses, diminuant ainsi la tumorigénèse à la fois *in vivo* et *in vitro*.

ABBREVIATIONS LIST

1. high fat diet (HFD)
2. brown adipose tissue (BAT)
3. fatty-acid oxidation (FAO)
4. long-chain acyl coenzyme A dehydrogenase (LCAD)
5. mouse embryonic fibroblast (MEF)
6. FOXO/ NRF1
7. NAD+ - oxidized nicotinamide adenine dinucleotide
8. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)
9. Peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC-1β)
10. GCN5
11. Histone 4 lysine 16 (H4K16)
12. Histone 3 lysine 9 (H3K9)
13. Forkhead box protein O (FOXO)
14. Forkhead box protein O3A (FOXO3A)
15. Peroxisome proliferator-activated receptor gamma (PPARy)
16. Nuclear factor kappa B (NF-kB)
17. Myc - MYC proto-oncogene, bHLH transcription factor
18. p53
19. CLOCK/Per
20. CR – caloric restriction
21. IL-1b
22. NLRP3
23. hematopoietic stem cells (HSCs)
24. INK4/ARF
25. reactive oxygen species (ROS)mTORC1
26. 5' AMP-activated protein kinase (AMPK)

27. Adenosine triphosphate (ATP)
28. Adenosine diphosphate (ADP)
29. Adenosine monophosphate (AMP)
30. ribosomal S6 protein kinase 1 (S6K1)
31. insulin-like growth factor 1 - IGF-1
32. DAF-16 or dFOXO
33. DNA
34. rDNA
35. H2B and H4
36. miRNAs
37. base excision repair (BER),
38. nucleotide excision repair (NER)
39. global genome NER (GG-NER)
40. transcription-coupled NER (TC-NER)
41. mismatch repair (MMR)
42. AP site (apurinic or apyrimidinic site)
43. gap 1 (G1) phase
44. gap 2 (G2) phase
45. synthesis (S) phase
46. M phase
47. homologous recombination (HR)
48. non-homologous end-joining (NHEJ
49. DNA double-strand breaks (DSBs)
50. Ku proteins (Ku70 and Ku80 heterodimers, encoded by X-ray repair cross complementing 6 and 5, respectively)
51. Radiation sensitive 54 (RAD54)
52. Histone deacetylase 6 (HDAC6)
53. fatty acid oxidation (FAO)

54. malonyl CoA decarboxylase (MCD)

55. glutamate dehydrogenase (GDH)

56. pyruvate dehydrogenase complex (PDH)

57. mammalian target of rapamycin complex 1 (mTORC1)

58. carbamoyl phosphate synthetase 1 (CPS1)

59. acyl-CoA dehydrogenase family proteins (ACAD)

60. Very-long-chain acyl-CoA dehydrogenase, ACADV;

61. long-chain acyl-CoA dehydrogenase, ACADL;

62. medium-chain acyl-CoA dehydrogenase, ACADM; shortchain

63. acyl-CoA dehydrogenase, ACADS;

64. trifunctional enzyme a subunit, ECHA;

65. trifunctional enzyme b subunit, ECHB;

66. 2,4-dienoyl-CoA reductase, DECR; enoyl-CoA delta isomerase 1, ECI1;

67. short-chain enoyl-CoA hydratase, ECHM;

68. short-chain 3-hydroxyacyl-CoA dehydrogenase, HCDH;

69. acetoacetyl-CoA thiolase, THIL;

70. 3-hydroxy-3-methylglutaryl coenzyme A synthase 2, HMCS2;

71. 3-hydroxy-3-methylglutarate-CoA lyase, HMGCL;

72. 3-hydroxybutyrate dehydrogenase, BDH.

73. polymerase-associated factor 53 - PAF53

74. RNA polymerase I (Pol I)

75. endoplasmic reticulum (ER)

76. polymerase beta (Polβ)

77. DNA-PK (DNA-dependent protein kinase)

78. DNA-PK catalytic subunit (DNA-PKcs)

79. neocarzinostatin (NCS)

80. Camptothecin (CPT)

81. CtIP [C-terminal binding protein (CtBP) interacting protein]

82. poly (ADP-ribose) polymerase (Parp1)

83. WRN gene (Werner syndrome ATP-dependent helicase)

84. telomere associated satellite-like (TAS) sequences

85. telomeric position effect (TPE)

86. T-cell factor (*TCF*)

87. Lymphoid enhancer-binding factor 1 (*LEF1*)

88. Mouse embryonic stem cells (mESC)

89. Human embryonic stem cells (hESC)

90. human induced pluripotent stem cells (iPSCs)

91. Sterol regulatory element binding proteins (SREBPs)

92. Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)

93. AKT - Protein kinase B (PKB), also known as Akt

94. Phosphorylated AKT (pAKT)

95. Phosphatase and tensin homolog (PTEN)

96. polyomavirus middle T antigen (PyMT)

97. mouse mammary tumor virus (MMTV)

INTRODUCTION

AGING: causes and consequences

The efficacy in avoiding and treating infectious diseases led to exceptional increases in life expectancy during the first half of the last century. The decrease in microorganisms-related death and major improvements in medical care, food availability, and lifestyle extended human lifespan significantly. Yet, this improvement has been accompanied by a new set of major health problems an age-related diseases as cancer, diabetes and neurodegenerative and cardiovascular pathologies (Fielding 1999). Many cellular and molecular features of aging are conserved from yeast to mammals (Lopez-Otin et al. 2013). Aging definition is complex as it is the aging process per se. In general, aging can be defined as progressive decline in physiological homeostasis leading to cellular damage, susceptibilities and death (Lopez-Otin et al. 2013). More specifically, genomic and epigenomic alteration (Lombard et al. 2005; Calvanese et al. 2009), telomere integrity (Stewart and Weinberg 2006), variations in nutrient sensing (Kapahi et al. 2010; Efeyan, Comb, and Sabatini 2015), deregulated proteostasis (Kaushik and Cuervo 2015; Steffen and Dillin 2016) and mitochondrial function (Kauppila, Kauppila, and Larsson 2017; Sun, Youle, and Finkel 2016), altered intercellular communication, stem cell exhaustion (Oh, Lee, and Wagers 2014; Signer and Morrison 2013) and cellular senescence (Herbig et al. 2006; Childs et al. 2015) are processes identified as hallmarks of aging (Lopez-Otin et al. 2013).

Which one of the hallmarks comes first? Nuclear DNA mutations (genomic alterations) are likely to represent an acute contributor to aging as mutations can alter the function of telomerase, deacetylases, ribosome proteins and others components

related to the Hallmark of aging (Lindahl 1993; Takata et al. 1998; Krokan et al. 2000; Li 2008; Shrivastav, De Haro, and Nickoloff 2008; Robertson et al. 2009; Nemec et al. 2012; Pena-Diaz and Jiricny 2012; Davis and Chen 2013; Krokan and Bjoras 2013; Scharer 2013; Marteijn et al. 2014; Kowalczykowski 2015; Lombard et al. 2005; Lopez-Otin et al. 2013). However, it is important to understand that aging process is a conjunct of errors happening in parallel or in a subsequent manner and in most of the cases point a specific hallmark as founder would be inaccurate (Lombard et al. 2005; Lopez-Otin et al. 2013). For example, epigenetic alteration, telomere shortening and loss of proteostasis can also alter replication and DNA repair mechanisms and thus cause genomic alteration, aging and aging related diseases (Lombard et al. 2005; Stewart and Weinberg 2006; Lopez-Otin et al. 2013; Kaushik and Cuervo 2015; Sun, Youle, and Finkel 2016; DeBalsi, Hoff, and Copeland 2017). For that reason, López-Otín et al. (Lopez-Otin et al. 2013) grouped the Hallmarks of aging into three categories, Primary hallmarks, Antagonistic hallmarks, and Integrative hallmarks. Primary hallmarks, which include Genomic instability, Telomere attrition, Epigenetic alterations and Loss of proteostasis, are negative process that cause cellular damage altering homeostasis. Antagonistic hallmarks, represented by Deregulated nutrient sensing, Mitochondrial dysfunction, and Cellular senescence, are compensatory process that protect the organism from Primary hallmarks (i.e. damage). For instance, cellular senescence induces cell death upon deleterious mutations (Gorgoulis and Halazonetis 2010). Nevertheless, while at low levels Antagonistic hallmarks protect organism homeostasis, at high levels it became pathological as, for example, excessive reactive oxygen species (ROS) leading to cellular damage (Hekimi, Lapointe, and Wen 2011; Sena and Chandel 2012). Lastly, Integrative hallmarks – Stem cell exhaustion and Altered intercellular communication – are the responsible for the organism functional

decline cause by the accumulation of damage (Primary hallmarks) that subvert compensatory mechanisms (Antagonistic hallmarks) leading to further damage.

Figure 1.

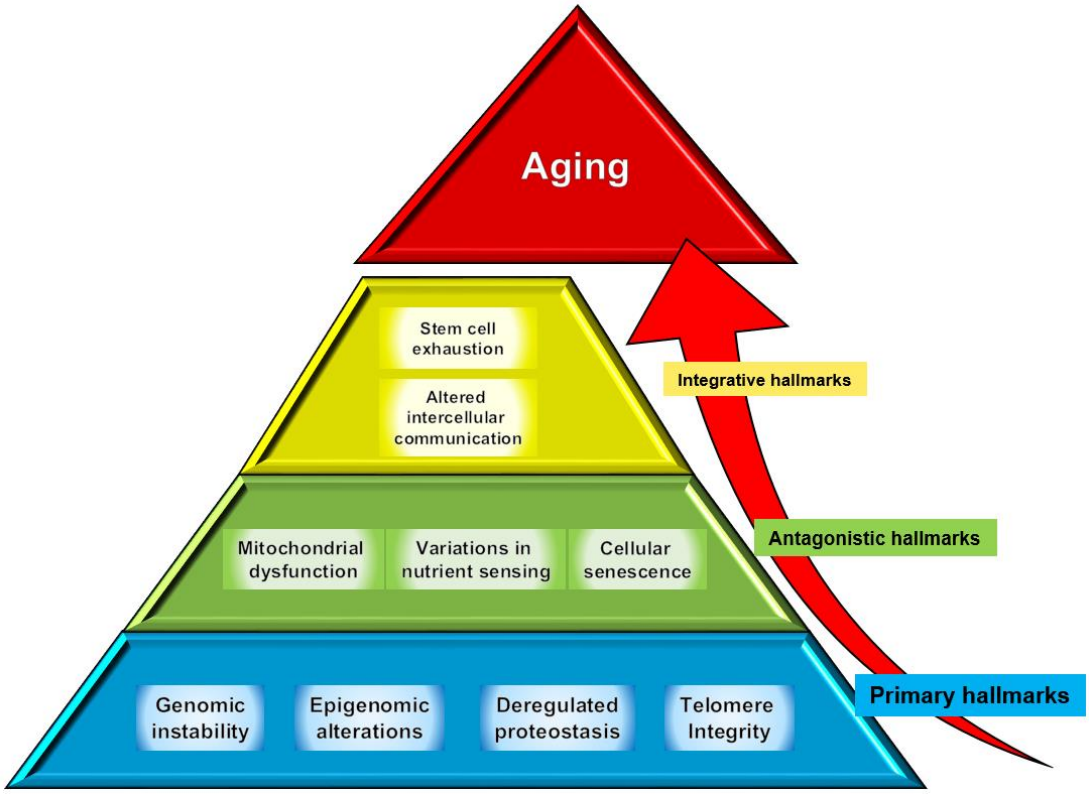

Figure 1. The hallmarks of aging. Primary hallmarks (blue) are the initial cause of damage and loss of homeostasis. Antagonistic hallmarks (green) raise to keep homeostasis as protective mechanisms against primary hallmarks damage, however prolonged activity became pathological increasing cellular damage. Integrative hallmarks (yellow) leads to organismal functional decay, due to accumulation of molecular and cellular damage, culminating in Aging (red).

Genomic Stability

Errors leading to mutations, deletions, insertions and translocations occur every time nuclear DNA is duplicated and segregated. The replication machinery per se is error prone and internals, as ROS, and externals agents, as ultraviolet radiations,

increase this inaccuracy compromising cellular homeostasis and lead to subsequent deleterious processes as the ones cited as hallmarks of aging (Lombard et al. 2005; Stewart and Weinberg 2006; Lopez-Otin et al. 2013; Kaushik and Cuervo 2015; Sun, Youle, and Finkel 2016; DeBalsi, Hoff, and Copeland 2017). For that reason, several DNA repair mechanisms act to correct DNA damage (Lindahl 1993; Takata et al. 1998; Krokan et al. 2000; Li 2008; Shrivastav, De Haro, and Nickoloff 2008; Robertson et al. 2009; Nemec et al. 2012; Pena-Diaz and Jiricny 2012; Davis and Chen 2013; Krokan and Bjoras 2013; Scharer 2013; Marteijn et al. 2014; Kowalczykowski 2015; Lombard et al. 2005).

DNA double-strand breaks (DSBs) are corrected by two main repair pathways, non-homologous end-joining (NHEJ) mediate by Ku proteins (Ku70 and Ku80 heterodimers, encoded by X-ray repair cross complementing 6 and 5, respectively) and homologous recombination (HR) mediate by Rad54 (Takata et al. 1998; Shrivastav, De Haro, and Nickoloff 2008). HR required the availability of a homologous sequence template to repair the damaged DNA and thus believed to be active in cell cycle phases where the sister chromatid is present, i.e. synthesis (S) and gap 2 (G2) phases (Takata et al. 1998; Shrivastav, De Haro, and Nickoloff 2008). NHEJ seems to overlap with HR at first, nonetheless, it acts during gap 1 (G1) early and S phases ligating DNA breaks without homologous template (Takata et al. 1998; Shrivastav, De Haro, and Nickoloff 2008; Davis and Chen 2013).

Three main excision repair pathway are in place to keep DNA integrity, base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). BER pathway is highly conserved throughout evolution and suggested to be a mechanism that counteracts DNA decay caused by environmental factors and aginig (Robertson et al. 2009; Krokan et al. 2000; Lindahl 1993). It is active in nuclei and

mitochondria and responsible for correction of small base alterations that has no effect or slight distort DNA helix structure (Krokan and Bjoras 2013; Krokan et al. 2000; Robertson et al. 2009). Basically, the removal of a damaged DNA base through BER pathway is a 5 steps concerted action of four classes of enzymes (Krokan and Bjoras 2013; Krokan et al. 2000; Robertson et al. 2009). DNA glycosylases recognize and removed the damaged base (short-patch) or bases (long-patch) leaving an AP site (apurinic or apyrimidinic site), which leads to DNA backbone cleavage by AP endonuclease (5` nick) or AP lyase (3` nick) and subsequent action of polymerase and ligase (Krokan and Bjoras 2013; Krokan et al. 2000; Robertson et al. 2009). NER can also be divided in two subpathways, global genome NER (GG-NER) and transcription-coupled NER (TC-NER) (Marteijn et al. 2014; Scharer 2013). Essentially, GG-NER pathway follow the same 5 steps of BER pathway nonetheless, GG-NER correct bulky DNA damage that cause helix distortion (Marteijn et al. 2014; Scharer 2013). TC-NER pathway is initiated when RNA Polymerase II stall during transcript elongation leading for the successive activation of the core NER pathway which removes and correct the DNA (Marteijn et al. 2014; Scharer 2013). The third excision repair pathway, MMR pathway, is well conserved from bacteria to mammals and acts during DNA replication correcting DNA mismatches (Li 2008; Pena-Diaz and Jiricny 2012). The repair process is fundamentally similar to the others excision repair pathway involving error recognition, excision and DNA correction (Li 2008; Pena-Diaz and Jiricny 2012). However, the strand specificity (newly synthesized DNA strand), bi-directionality repair (5`-3` and 3`-5`) and substrate specificity (base-base mismatches and small ID mispairs) are key features that differs from BER and NER (Li 2008; Pena-Diaz and Jiricny 2012).

DNA damage happens every time DNA is replicated and even in health organisms not all damage is corrected (Lombard et al. 2005; Lopez-Otin et al. 2013). During aging process an accumulation of DNA damage is evident and when these damages are artificially imposed it induces accelerated aging (Lindahl 1993; Krokan et al. 2000; Li 2008). Genetic evidences are clearly demonstrating that defects in the machinery that ensures faithful genomic replication lead to aging and aging related diseases (Lombard et al. 2005; Lopez-Otin et al. 2013).

Epigenomic Alteration

Among the Hallmarks of Aging, epigenetic alterations are the lesser comprehended. It is clear that aging processing is accompanied by epigenetic alterations as several age-related changes have been found in comparison between young and old tissues (de Magalhaes, Curado, and Church 2009). In this study, de Magalhães et al. (2009) compared microarrays from mice, rat and human, uncovering age-related transcriptional changes linked to pathophysiological processes as, for example, overexpression of genes related to inflammation and immune response and reduced expression of mitochondrial energy metabolism genes (de Magalhaes, Curado, and Church 2009). Not only coding RNAs were identified for its association with aging process, in *C. elegans* and *D. melanogaster* a class of miRNAs can also modulate lifespan (Boulias and Horvitz 2012; Toledano et al. 2012; Liu, Landreh, et al. 2012).

DNA methylation, histones modification and, consequently, chromatin remodeling were likewise identified as altered in aging (Han and Brunet 2012; Tsurumi and Li 2012). Aging processing is related with a global DNA hypomethylation

(Calvanese et al. 2009) nonetheless, specific regions (including several tumor suppressor and polycomb target genes) are hypermethylated (Maegawa et al. 2010). Hypermethylation is also seem in rDNA in progeroid syndromes mouse model and in physiological aging in rodents (Osorio et al. 2010). The same mouse model also shows global histones H2B and H4 hypoacetylation leading to alteration in the expression of cell proliferation and metabolic process related genes (Osorio et al. 2010). Apart from the most studies histone post-translation modifications (PTMs), methylation and acetylation, several others were identified (Wang et al. 2009; Sabari et al. 2017; Fellows et al. 2018; Rousseaux and Khochbin 2015). Although, β-hydroxybutyrylation, 2-hydroxyisobutyrylation, butyrylation, glutarylation, succinylation, propionylationmalonylation, citrullination and crotonylation can influence gene expression no comprehensive analyses was performed to link these PTMs to aging (Fellows et al. 2018; Rousseaux and Khochbin 2015; Sabari et al. 2017; Wang et al. 2009). Covalent modification of DNA and histones as the PTMs cited above has the potential to remodel chromatin and doing so, can induce others Hallmarks of aging and the aging process *per se*. For example, chromatin modifiers are required for BER DNA repair mechanisms (i.e. DNA stability) (Odell, Wallace, and Pederson 2013; Nakanishi et al. 2007).

Epigenetic alterations are highly dynamic and been so, reverse a pathological epigenetic alteration could be, theoretically, achieved clinically in order to extend life and healthspan (Rando and Chang 2012). For instance, changing the acetylation status of histones has been shown, in mice, to ameliorate aging symptoms and extend lifespan (Peleg et al. 2010; Krishnan et al. 2011). Nevertheless, the “epigenetic code” is highly complex and the relationship among different DNA and histone modifications is essentially unknown.

Telomere integrity

Telomeres are DNA repeat sequences (double-strand with a single-strand tail) bound to a multiprotein complex, called shelterin, working as capping structures at the chromosomal ends (de Lange 2005; Palm and de Lange 2008; Stewart and Weinberg 2006). Shelterin is not just important in the formation of a higher-order structure but also to avoid chromosomal ends to be inappropriately recognized as DNA breaks and thus be processed by the action of DNA repair machinery resulting in chromosomal fusion (de Lange 2005; Palm and de Lange 2008; Stewart and Weinberg 2006). Immediately proximal to the Shelterin complexes are the silenced sequences called telomere associated satellite-like (TAS) sequences. The phenomenon that silencing the genes inserted into the TAS is known as telomeric position effect (TPE) (Palm and de Lange 2008).

The replication of telomeric DNA is executed by telomerases since DNA polymerases fail to replicate terminal ends of linear DNA (Fumagalli et al. 2012; Hewitt et al. 2012). The absence of telomerase in most somatic cells leads to progressive shortening of telomeres sequence limiting proliferative capacity and triggering replicative senescence (Stewart and Weinberg 2006; Zvereva, Shcherbakova, and Dontsova 2010). This mechanism of telomere shortening that limit the number of cell division working thus as an internal clocking culminating in senescence is known as the Hayflick limit (Stewart and Weinberg 2006).

There are strong links between telomere length and aging. For instance, telomere shortening occurs in mammals during normal aging process (Blasco 2007). Moreover, while telomerase deficiency in associated with diseases involving

diminished regenerative capacity (Armanios and Blackburn 2012), telomerase reactivation can revert aging (Jaskelioff et al. 2011; Bernardes de Jesus et al. 2012).

Deregulated Proteostasis

The status when the proteome is functionally stable is define as proteostasis, or protein homeostasis (Kaushik and Cuervo 2015; Steffen and Dillin 2016; Lopez-Otin et al. 2013). The machinery of protein production rely in the coordination of dozens of protein (Hinnebusch and Lorsch 2012) and thousands of ATP molecules (Lane and Martin 2010). To regulate protein synthesis process and ensure proteostasis all cells have evolved several quality control mechanisms responsible for sensing and correct disturbances (for example, chaperones and proteolytic pathway, respectively) (Vilchez, Saez, and Dillin 2014; Hartl, Bracher, and Hayer-Hartl 2011; Mizushima et al. 2008; Kaushik and Cuervo 2015; Lopez-Otin et al. 2013; Steffen and Dillin 2016). Even though an intricate and robust mechanism to keep proteostasis chronic stressors can lead to proteotoxicity development.

Numerous evidences associate aging and loss of proteostasis (Rubinsztein, Marino, and Kroemer 2011). For example, age-related diseases as Alzheimer and Parkinson are characterized by protein aggregation (Powers et al. 2009; Morimoto and Cuervo 2014) and several animal models with altered chaperone content or autophagy-related gene showing divergence in lifespan (Walker and Lithgow 2003; Morrow et al. 2004; Min et al. 2008; Pyo et al. 2013).

Lastly, pharmacologic (rapamycin, metformin, resveratrol and spermidine) and non-pharmacologic (calorie restriction and exercise) interventions known to extend

healthspan and lifespan increase activity of chaperones, proteasome and autophagy pathways (Jamart et al. 2012; Madeo et al. 2015; Ulbricht et al. 2015).

Variations in nutrient sensing

In general, evidences support that food abundance accelerates aging while scarcity without malnutrition extends lifespan (de Cabo et al. 2014; Lopez-Otin et al. 2013; Mitchell et al. 2016). Intra and extracellular levels of glucose, lipids and amino acids are detected by distinct pathways in an integrated and coordinated process (Efeyan, Comb, and Sabatini 2015). In multicellular organisms, the nutrient oscillation trigger hormones release which in turn activate homeostatic and hedonic mechanisms in order to avoid dramatic changes in circulating nutrient availability (Efeyan, Comb, and Sabatini 2015). Nutrient-sensing pathways are deregulated in aging and aging-related diseases (de Cabo et al. 2014; Lopez-Otin et al. 2013; Mitchell et al. 2016).

Insulin signaling (IS) pathway participates in glucose sensing and is one of the main focus of investigation in aging research. Insulin (and also insulin-like growth factor 1 - IGF-1) initiate a cascade signaling that activate AKT which in turn positively induces mTORC1 and negatively regulates FOXO transcription factors. At least in flies and worms, the effect of extended lifespan upon reduction in insulin signaling is suppressed when DAF-16 or dFOXO (worm and fly FOXO transcription factor, respectively) is absent (Slack et al. 2011; Kenyon et al. 1993). In mammals, the role of FOXO transcription factors and its relationship with insulin signaling to increase lifespan are not understood. Nonetheless, studies have correlated FOXO3A gene polymorphisms with increased longevity (Anselmi et al. 2009; Bao et al. 2014; Flachsbart et al. 2009).

In addition to the IS that react to increased glucose concentration, mTOR, another nutrient sensing mechanism, detects increased amino acid levels. AMPK and Sirtuins, other two nutrient sensing mechanisms, act in the opposite direction sensing low-energy states by detecting high AMP and high NAD+ levels, respectively (Houtkooper, Williams, and Auwerx 2010). Increase lifespan can be achieved in mice when levels of mTORC1 and its downstream substrate, ribosomal S6 protein kinase 1 (S6K1), are downregulated (Lamming et al. 2012; Selman et al. 2009). Furthermore, S6K1 downregulation mimics calorie restriction gene expression patterns as it does pharmacological manipulation using rapamycin, a mTORC1 inhibitor, which also increase lifespan (Lamming et al. 2012; Selman et al. 2009; Harrison et al. 2009). All nutrient sensing mechanisms are interconnected, AMPK activation leads to mTORC1 shut off (Alers et al. 2012), SIRT1 and AMPK interact in a positive feedback loop when low-energy state is detected (Price et al. 2012) and SIRT6 has been shown to increase insulin sensitivity (i.e. signalling) (Anderson et al. 2015).

Mitochondrial Dysfunction

Primary hallmarks can lead to mitochondrial dysfunction and thus to aging. Mitochondrial DNA is in an oxidative microenvironment without histones protection and having limited DNA repair mechanisms. This mitochondrial “genomic instability” can contribute to aging as evidenced by DNA polymerase γ deficient mice which exhibit reduced lifespan (Edgar et al. 2009). Telomere shortening can also reduce lifespan through decrease mitochondrial biogenesis as result of PGC-1α and PGC-1β p53-mediated repression in telomerase-deficient mice (Sahin and DePinho 2012). Also, telomerase activation in wild-type aged mice can partially reverse mitochondrial

decline (Bernardes de Jesus et al. 2012). Sirtuins mitochondrial modulation, which includes epigenetic alterations, will be discussed in a subsequent chapter.

ROS levels and its role in aging have been extensively studied, nevertheless, recent results have put the mitochondrial free radical theory of aging under re-evaluation (Hekimi, Lapointe, and Wen 2011). In yeast, increased ROS level does not accelerate aging, in fact it may even prolong lifespan in this organisms (Mesquita et al. 2010). In mammals, more specifically in mice, there are several evidences where oxidative damage caused by increased mitochondrial ROS levels does not reduced lifespan (Zhang et al. 2009). The decrease in ROS level was also tested and manipulations to ameliorate antioxidant defences does not have effect over lifespan (Perez et al. 2009). As cited above, DNA polymerase γ deficient mice which exhibit reduced lifespan with no changes in ROS levels (Edgar et al. 2009).

Results regarding the effect of ROS levels in aging (positive, negative or neutral) may are seemingly conflicting however this lines of evidence place mitochondrial dysfunction as an antagonistic hallmark of aging. ROS levels increase as stress-response leading to proliferation and survival been thus, a compensatory mechanism to maintain homeostasis (Sena and Chandel 2012). On the other hand, if the stress is continuously prolonged the beneficial pro-survival (antagonistic) effect of ROS may be overcome and aggravate age-associate damage (Hekimi, Lapointe, and Wen 2011).

Cellular Senescence

Senescence is a compensatory mechanism aimed to avoid proliferation of damage cells (Gorgoulis and Halazonetis 2010). The accumulation of senescence cells in aging is due to the increased number of damage cells that undergo senescence

and to the deficient mechanism to replace those cells. Therefore, the initially compensatory mechanism becomes deleterious exhausting the regenerative capacity of progenitor cells leading then to aging phenotype. Another characteristic of senescence cells that can lead to aging is the ‘‘senescence-associated secretory phenotype’’ which includes an increased secretion of proinflammatory cytokines and matrix metalloproteinases (Rodier and Campisi 2011).

As for mitochondrial dysfunction, cellular senescence is also induced by Primary hallmarks of aging. Apart from Telomere integrity induction of cellular senescence (discussed above), other stimuli independently of telomeric shortening can trigger stable cell cycle arrest, characteristic of cellular senescence. DNA damage (“genomic stability”) and epigenetic control of the INK4/ARF locus, observed in aging, can also induce senescence (Collado, Blasco, and Serrano 2007; Jeck, Siebold, and Sharpless 2012).

Stem Cell Exhaustion

The decline of stem cell self-renewing and proliferation direct impacts organismal regenerative capacity culminating in aging and aging-associated diseases. Anemia and myeloid malignancies are more frequent in old organisms due to hematopoiesis decay and consequent reduced differentiation towards adaptive immune cells (Shaw et al. 2010). Comparing with hematopoietic stem cells (HSCs) from young mice, HSCs from aged mice have reduced cell-cycle activity and endure fewer divisions, and it is correlated with DNA damage accumulation (Rossi et al. 2007). The failure in maintain telomere structure also contribute to stem cell decline in aging (Flores, Cayuela, and Blasco 2005; Sharpless and DePinho 2007).

Not only decline of stem cell functions is detrimental for the organism, also excessive proliferation can lead stem cells to exhaustion, bringing a higher degree of complexity to therapeutics development (Kippin, Martens, and van der Kooy 2005; Cheng et al. 2000). Nonetheless, transplanting muscle stem cells from young mice into old mice decrease degeneration of several tissues, even where donor cells cannot be detected and extent lifespan (Lavasani et al. 2012). Furthermore, systemic factors from young mice can restore muscle and neural stem cell function (Villeda et al. 2011; Conboy et al. 2005). Those results are indicating that secreted factors derived from young stem cells or young mice circulation are the responsible for the observed therapeutic benefit.

Altered Intercellular Communication

Parabiosis experiments cited above (Conboy et al. 2005; Villeda et al. 2011) demonstrate that further than cell-autonomous alterations, intercellular communication changes likewise are involved in aging (Laplante and Sabatini 2012; Zhang et al. 2013). Inflammation is one of the most prominent aging-associated process that alter intercellular communication. The decline in tissue regeneration (Stem Cell Exhaustion) concomitant with reduced immune system capacity and autophagy response lead to an accumulation of senescence cells and increase secretion of proinflammatory cytokines (Salminen, Kaarniranta, and Kauppinen 2012). Those events bring about NLRP3 inflammasome activation, IL-1b, tumor necrosis factor and interferons production (Salminen, Kaarniranta, and Kauppinen 2012).

Dietary restriction approaches are one of the interventions that can restore defective intercellular communication and extend healthy lifespan (Piper et al. 2011;

Sanchez-Roman et al. 2012). Moreover, pharmacological approach as the long-term administration of aspirin, an anti-inflammatory agent, is correlated with increase longevity and healthy aging (Rothwell et al. 2011; Strong et al. 2008).

SIRTUINS

Silent information regulator 2 (Sir2) and its homologs are a class of highly conserved enzymes, from bacteria to human, which have in common a deacetylase domain and a dependency for oxidized nicotinamide adenine dinucleotide (NAD^+) as co-substrate (Guarente 2013; Landry et al. 2000; Imai et al. 2000). Sir2 gene and its function were first identified in 1987 in *Saccharomyces cerevisiae*, where it represses mating type interconversion (Rine and Herskowitz 1987). In the following years, Sir2 was attributed having transcriptional repression functions at the telomeres (Gottschling et al. 1990) and ribosomal DNA loci (Smith and Boeke 1997), been involved in cell-cycle progression, chromosomal stability and repair of double-strand DNA breaks (Brachmann et al. 1995; Martin et al. 1999).

The starling discovery was the Sir2 involvement in life-span extension in *S. cerevisiae (Kaeberlein, McVey, and Guarente 1999)*. Lack of Sir2 reduce life-spam while overexpression extends (Lin, Defossez, and Guarente 2000; Kaeberlein, McVey, and Guarente 1999). This life-span extension due to increased Sir2 gene dosage (or its homologs) was confirmed using other organisms as, for example, *Caenorhabditis elegans* (Tissenbaum and Guarente 2001), *Drosophila melanogaster* (Rogina and Helfand 2004) and mammals (Kanfi et al. 2012). The primary instinct of survival thrives the way for aging research, not only live well (healthspan) but also live longer (lifespan), and the possibility that Sir2 and its mammals' homologs could extend lifespan drove

great interest from the research community. Important to mention that later studies have pointed that the lifespan extension upon Sir2 (*Caenorhabditis elegans*) or dSir2 (*Drosophila melanogaster*) overexpression was strain dependent and could not be reproduced in other strains (Burnett et al. 2011).

The observation that Sir2 use NAD+ as co-substrate for its deacetylase activity was the crucial and spontaneously link between caloric restriction (which increases NAD+ levels), epigenetics modifications and aging (which decrease NAD+ levels)(Landry et al. 2000; Imai et al. 2000). Sir2 was also show to be required for increase life-span in *S. cerevisiae* upon caloric restriction (Lin, Defossez, and Guarente 2000). These results were confirmed using another organism, *Caenorhabditis elegans*, where increased dosage of a sir-2 gene extends life-span by 50% (Tissenbaum and Guarente 2001).

Calorie restriction (CR) is a non-genetic intervention that prolongs lifespan and healthspan and decreases age-associate diseases (Finkel and Holbrook 2000). Not only a reduction in calorie intake but also alteration in specific macronutrients or different feeding regimens can bring beneficial effects (Fontana and Partridge 2015; Solon-Biet et al. 2015). The evidences that CR are overwhelming, nonetheless, it depends of the degree of calorie reduction, gender and strain (Mitchell et al. 2016). Therefore, the factors impacting the response to CR and the exact underlying molecular mechanism(s) leading to its beneficial effects remain only partially understood.

The difficulties to underlie a mechanism is because CR brings about several changes at cellular and molecular levels. Some features are commonly observed in organisms submitted to caloric restriction, including reduction of insulin/IGF-1 signalling (Baur et al. 2006), oxidative stress (Sohal and Weindruch 1996; Finkel and

Holbrook 2000), body temperature (Rikke et al. 2003) and reproductive investment(Mitchell et al. 2015), and protection against carcinogenesis (Pietrocola et al. 2016)

Complementing the favorable effect of CR on health and lifespan, evidences show that CR counteract all Hallmarks of aging - genomic instability (Kisby et al. 2010; Moore et al. 2007), telomere integrity (Vera et al. 2013; Kark et al. 2012), epigenetic alteration (Li, Daniel, and Tollefsbol 2011; Lee et al. 1999; Mercken et al. 2013), deregulated proteostasis (Leprivier et al. 2013), variations in nutrient sensing (Wu et al. 2013; Stenesen et al. 2013), mitochondrial dysfunction (Nisoli et al. 2005), cellular senescence (Messaoudi et al. 2006), stem cells exhaustion (Cerletti et al. 2012) and altered intercellular communication (Yilmaz et al. 2012).

Mammalian Sirtuins

Mammalian Sirtuin family is compose of seven members displaying widespread subcellular distributions and enzymatic activity (Yanagisawa et al. 2018; Jablonska et al. 2016; Jiang et al. 2013; Michishita et al. 2005). Sirtuins were classified in five phylogenetic branches based in sequence alignment analysis, Class U, I-IV, and each Class has its own subclasses (Frye 2000). Class I has the highest similarity with yeast Sir2 and includes mammalian SIRT1 (class Ia), SIRT2 and SIRT3 (both class Ib); SIRT4 is a member of Class II and SIRT5 of Class III; SIRT6 and SIRT7 are classified as Class IV, been SIRT6 Class IVa and SIRT7 Class IVb. Class IV is the only class to not have prokaryotes and Class U is composed only by prokaryotes (Frye 2000).

Figure 2.

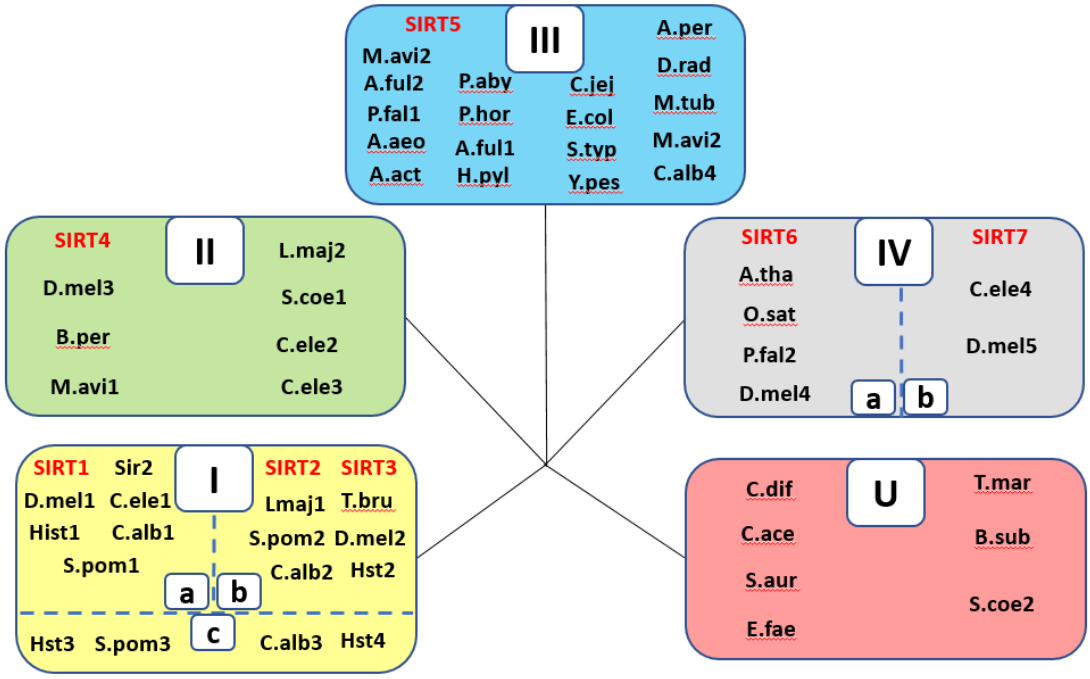

Figure 2. The five classes of Sirtuins. Mammalian Sirtuins (red) divided in four classes (I-IV). Class U is represented exclusively by prokaryotes. A.act - Actinobacillus actinomycetemcomitans; A.aeo - Aquifex aeolicus; A.ful - Archaeoglobus fulgidus; A.per - Aeropyrum pernix; A.tha - Arabidopsis thaliana; B.per - Bordetella pertussis; B.sub - Bacillus subtilis ; C.ace - Clostridium acetabutylicum; C.alb - Candida albicans; C.dif - Clostridium difficile; C.ele - Caenorhabditis elegans; C.jej - Campylobacter jejuni; D.mel - Drosophila melanogaster; D.rad - Deinococcus radiodurans; E.col - Escherichia coli; E.fae - Enterococcus faecalis; Hst - Saccharomyces cerevisiae; H.pyl - Helicobacter pylori; L.maj - Leishmania major; M.avi - Mycobacterium avium; M.tub - Mycobacterium tuberculosis; O.sat - Oryza sativa; P.aby - Pyrococcus abyssi; P.fal - Plasmodium falciparum; P.hor - Pyrococcus horikoshii; S.aur - Staphylococcus aureus; S.coe - Streptomyces coelicolor ; S.pom - Schizosaccharomyces pombe; S.typ - Salmonella typhimurium; Sir2 - Saccharomyces cerevisiae; SIRT - Homo sapiens; T.bru - Trypanosoma brucei; T.mar - Thermotoga maritima; Y.pes - Yersinia pestis.

Initially, the enzymatic activities of the Sir2 family were analysed as if all have the same activities, not taking in consideration the individuality of each of the members of this family (Frye 1999; Tanny et al. 1999; Landry et al. 2000; Smith et al. 2000; Tanner et al. 2000). Mammalian Sirtuin family is classified as Class III Histones Deacetylases (HDACs) due to its sequence homology, having the dependency of NAD+ for its enzymatic activities and a conserved deacetylase domain as common

feature of all Sirtuins (Landry et al. 2000). Although present in all Sirtuins, the deacetylase catalytic efficiencies are variable and others enzymatic activities were detected as, for example, lipoamidase, desuccinylates, demalonylates, mono-ADP-ribosyl-transferase, demyristoylase, glutarylation, decrotonylase among others (Jiang et al. 2013; Jin et al. 2016; Liszt et al. 2005; Michishita et al. 2008; Michishita et al. 2009; Yang et al. 2009; Anderson et al. 2017; Mao et al. 2011; Mathias et al. 2014; Tao et al. 2010; Teng et al. 2015; Nishida et al. 2015; Rardin et al. 2013; Zhou, Wang, et al. 2016; Tan et al. 2014; Feldman, Baeza, and Denu 2013).

CR, Sirtuins and aging are also connected in higher organisms. CR increases lifespan in mice and induces expression of a subset of the Sirtuins – Sirt6 (Zhang, Li, et al. 2016; Kanfi et al. 2008), Sirt5 (Nakagawa et al. 2009), Sirt3 (Someya et al. 2010; Hebert et al. 2013), Sirt2 (Wang et al. 2007) and Sirt1 (Cohen et al. 2004). This CR-activation can vary depending on degree of restriction, gender, age, strain and tissue analysed (Mitchell et al. 2016). Among the Sirtuins activated by caloric restriction, enhanced SIRT6 expression leads to increase lifespan in mice (Kanfi et al. 2012). SIRT1 was also showed to increase lifespan when overexpressed in specific brain regions (Satoh et al. 2013), nonetheless the authors could not reproduce lifespan extension using another line of the same mouse model.

There are evidences of a relationship between the mammalian Sirtuin family members. SIRT1 was shown to form a complex with FOXO/ NRF1 and thus regulate Sirt6 expression (Kim, Xiao, et al. 2010). Which is in odd with the results showing that SIRT1 and SIRT6 have opposite function in the regulation of PGC-1α (Dominy et al. 2012). SIRT1 is also involved in at least other two mechanisms with other Sirtuins, on with SIRT7 impairing SIRT1 autodeacetylation and thus its activation (Fang et al. 2017)

and the other where SIRT1 and SIRT4 have opposite related to insulin secretion (Haigis et al. 2006; Moynihan et al. 2005).

Another degree of complexity among mammalian Sirtuin family is related to the target specificity. Sirtuins appears to have overlapping functions and differently regulate share target proteins as, for example, the regulation of FOXO family (Kim, Xiao, et al. 2010; Wang et al. 2007; Sundaresan et al. 2009), PGC-1α (Nemoto, Fergusson, and Finkel 2005; Dominy et al. 2012), NF-κB (Yeung et al. 2004; Rothgiesser et al. 2010; Zhang, Li, et al. 2016), Myc (Menssen et al. 2012; Jing et al. 2016) and HIF1α (Krishnan et al. 2012; Finley et al. 2011).

SIRT1

SIRT1 is the closest homolog of Sir2 and one of the most studied mammalian Sirtuins (Frye 2000). It has a strong deacetylase activity that removes acetyl groups from lysine in target proteins (Landry et al. 2000). Even lacking a DNA-binding domain, SIRT1 can target gene promoter regions, through the recruitment of transcriptional machinery, leading to changes in transcriptional levels of numerous genes. It can also alter transcriptional levels through is deacetylase activity in histones (H4K16 and H3K9) (Hajji et al. 2010; Ryall et al. 2015).

Several non-histones proteins are SIRT1 targets, including transcription factors (several members of FOXO family (Kim, Xiao, et al. 2010), PGC-1α (Nemoto, Fergusson, and Finkel 2005), PPARγ (Qiang et al. 2012), NF-κB (Yeung et al. 2004), among others(Gomes et al. 2016)), oncogenes and tumour suppressors (Myc (Menssen et al. 2012) and p53 (Cheng et al. 2003), respectively) and members of the circadian clock machinery (CLOCK/Per (Asher et al. 2008; Nakahata et al. 2009)).

Sirt1 overexpression improves healthspan acting over several hallmarks of aging, nevertheless does not increase lifespan in health animals (Herranz et al. 2010). Through its deacetylase activity, Sirt1 promotes Epigenetic Alterations leading to Genomic Stability (Wang et al. 2008; Oberdoerffer et al. 2008), improved Stem cell (Ryall et al. 2015; Liu, Ghosh, et al. 2012) and Mitochondrial function (Nemoto, Fergusson, and Finkel 2005), respond to Variations in Nutrient Sensing(Price et al. 2012; Ramadori et al. 2011; Ramadori et al. 2008), helps to keep Proteostasis (Lee et al. 2008) and its manipulation can alter Intercellular Communication (Ramadori et al. 2011; Ramadori et al. 2008; Yeung et al. 2004; Satoh et al. 2013).

SIRT2

SIRT2, as SIRT1, also has strong deacetylase activity. It's mainly localized in the cytoplasm were it deacetylases α-tubulin when in a complex with another deacetylase, HDAC6 (North et al. 2003). During G2/M phase of cell cycle, SIRT2 accumulate in the nucleus were it co-localize with chromatin and correlates with H4K16 deacetylation (Vaquero et al. 2006). In mouse embryonic fibroblast (MEF) SIRT2, and not SIRT1, is also able to regulate NF-κB deacetylation and NF-κB-dependent gene expression leading to increase apoptosis upon TNF-α induction (Rothgiesser et al. 2010).

The connection of SIRT2 and cell cycle is also observed by the SIRT2 function regulation by cyclin-dependent kinases (Pandithage et al. 2008), in the control of mitotic exit (Dryden et al. 2003) and induction of checkpoint kinase BubR1 (North et al. 2014). SIRT2 has an important function in white adipose tissue (WAT) where its expression is upregulated and, through FoxO1 deacetylation, it inhibits adipocytes

differentiation (Jing, Gesta, and Kahn 2007), although SIRT2 KO mice have normal body weight (Kim et al. 2011).

Caloric restriction is able to increase SIRT2 protein levels in WAT and Kidney (but not liver and brain) leading to FoxO3a deacetylation, increase FoxO3a DNA binding and target gene activation (Wang et al. 2007). This cascade lead to decrease ROS levels or cell death activation under severe stress (Wang et al. 2007). On the other hand, while caloric restriction increase SIRT2 levels, diet induced obesity decrease it by HIF1α activation (Krishnan et al. 2012).

As discussed above, SIRT2 expression, protein level and activity can vary depending on energy state, cell cycle phase, cell type and cell localization (cytoplasmic or nuclear). In the cancer field SIRT2 has been shown to be a tumor suppressor (Hiratsuka et al. 2003; Kim et al. 2011) and an oncogene (Jing et al. 2016; Zhou, Ni, et al. 2016). For instance, absence of SIRT2 lead MEFs to spontaneous malignant transformation and tumor formation in female (mainly breast) and male mice (mainly liver and lung) (Kim et al. 2011). In contrast, SIRT2 is frequently overexpressed in basal-like breast cancer (BLBC) and linked with SLUG scape from proteasome degradation and downstream expression profile activation (including basal differentiation, stemness and invasion) (Zhou, Ni, et al. 2016).

Although SIRT2 is able to ameliorate several hallmarks of aging (Mitochondrial Function (Wang et al. 2007), Cell Senescence (Wang et al. 2007; Rothgiesser et al. 2010), Genomic Stability (Kim et al. 2011), Stem Cell exhaustion (Jing, Gesta, and Kahn 2007)) it does increase longevity only in a mouse model that display shorter lifespan but not in wild type mice (North et al. 2014).

SIRT3

SIRT3 is mitochondrial-localized (Cooper and Spelbrink 2008; Hallows, Albaugh, and Denu 2008; Kim, Patel, et al. 2010) and responsible for the global regulation of lysine acetylation in this organelle (Hebert et al. 2013; Lombard et al. 2007). The conclusion is based in comparison of mitochondrial protein acetylation status in liver of SIRT4 KO and SIRT5 KO mice, where no hiperacetylation was observed (Lombard et al. 2007). In contrast, SIRT3 KO mice showed marked increase in mitochondrial protein acetylation (Lombard et al. 2007). This suggest no functional redundancy between SIRT3 and the other two mitochondrial Sirtuins (Lombard et al. 2007).

SIRT3 is highly expressed in important metabolic tissues as brain, liver, brown adipose tissue (BAT) and heart, indicating a significant role for this Sirtuin in metabolism homeostasis (Lombard et al. 2007). Nonetheless, SIRT3 KO mice display normal body composition, energy expenditure and food consumption in normal (chow diet *ad libitum*) or in fasting conditions (Lombard et al. 2007). Although no differences were found in normal or fasting conditions, mice submitted to a high fat diet (HFD) have reduced SIRT3 expression and protein level in liver correlating to increased steatosis (Bao et al. 2010), and SIRT3 KO cells are more susceptible to lipotoxicity upon palmitate treatment (Bao et al. 2010). While Bao et al. (Bao et al. 2010) could only correlate decrease SIRT3 content with steatosis in mice fed on HFD, Hirschey et al. (Hirschey et al. 2010) found a possible the link. SIRT3 deacetylates and activate long-chain acyl coenzyme A dehydrogenase (LCAD), a key enzyme involved in long-chain fatty-acid oxidation (FAO) (Hirschey et al. 2010). In the absence of SIRT3, LCAD is

hiperacetylated and several FAO intermediates products and triglycerides accumulate into the liver (Hirschey et al. 2010).

SIRT3, as SIRT2, is also considered tumor suppressor or linked with tumor progression, depending on the tissue affected and even which subtype of cancer within a given tissue (Bell et al. 2011; Cui et al. 2015; Finley et al. 2011; Kim, Patel, et al. 2010; Yang et al. 2017). SIRT3 KO mice develop mammary tumors spontaneously and SIRT3 levels were found to be reduced in several Human tumors comparing with adjacent health tissue (Kim, Patel, et al. 2010). The effect of SIRT3 as a tumor suppressor is direct linked with its capacity to reduce oxidative stress through increase manganese superoxide dismutase activity leading to reduction in ROS levels and HIF1α destabilization and insuring mitochondria integrity (Bell et al. 2011; Finley et al. 2011; Kim, Patel, et al. 2010).

Caloric restriction can activate SIRT3 (Someya et al. 2010) and SIRT3 mediate several CR benefits (Hebert et al. 2013; Qiu et al. 2010; Someya et al. 2010). Through a widespread reprogramming of mitochondrial protein acetylation, including superoxide dismutase 2 and mitochondrial isocitrate dehydrogenase 2 activation, SIRT3 maintain mitochondrial functions coordinating diverse metabolic pathways and promoting oxidative stress resistance (Hebert et al. 2013; Qiu et al. 2010; Someya et al. 2010)

In relation with aging, SIRT3 is able to delay or modulate some hallmarks of aging promoting epigenomic alterations (Hebert et al. 2013; Someya et al. 2010), maintaining mitochondrial integrity (Bell et al. 2011), keeping genomic stability (reducing ROS levels) (Tao et al. 2010), cellular senescence (Fan et al. 2014) and stem cell exhaustion (Brown et al. 2013). Although SIRT3 mediate important beneficial

effects of CR and oppose several hallmarks of aging there is no evidence that it prolongs lifespan.

SIRT4

Although SIRT3 was pointed to be the main deacetylase into mitochondria, this activity and importance of the other two mitochondrial Sirtuins cannot be neglected. SIRT4 has first been shown to have no deacetylase activity but ADP-ribosytransferase instead (Haigis et al. 2006; Ahuja et al. 2007). Nonetheless, SIRT4 does has deacetylase activity but it seems to be restricted to few targets (Laurent et al. 2013). SIRT4 is able to supress fatty acid oxidation (FAO) through deacetylation of malonyl CoA decarboxylase (MCD) and, consequently, to accumulation of malonyl CoA (Laurent et al. 2013). Such activity could not be confirmed in vitro using recombinant SIRT4 against differentially-modified synthetic MCD K471 Acetyl peptides (Mathias et al. 2014).

SIRT4 has other two enzymatic activities, ADP-ribosytransferase and lipoamidase, which regulates key metabolic steps (Haigis et al. 2006; Mathias et al. 2014). SIRT4 is able to downregulate glutamate dehydrogenase (GDH) activity, by adding an ADP-ribosyl group to its structure, leading to a reduction in glutamate metabolism, ATP generation and amino acid-stimulated insulin secretion (Haigis et al. 2006). The third key metabolic step that SIRT4 regulates is the conversion of pyruvate to acetyl-CoA through downregulation of pyruvate dehydrogenase complex (PDH) (Mathias et al. 2014). The enzymatic activity responsible for this control, lipoamidase, is conserved in bacteria (Sirtuin homolog CobB) and SIRT4 was shown to be able to insert lipoyl- and biotyl-lysine modifications (Rowland et al. 2017).

The role of SIRT4 in control FAO, glycolysis and glutamine metabolism was readily linked to the possibility that SIRT4 can suppress tumorigenesis (Csibi et al. 2013; Jeong et al. 2013; Miyo et al. 2015; Fu et al. 2017). Sirt4 expression is reduced in human cancers and mammalian target of rapamycin complex 1 (mTORC1) induce glutamine metabolism and cell proliferation by reducing SIRT4 levels (Csibi et al. 2013). Opposing this effect, Sirt4 overexpression in MEF lacking a negative regulator of mTORC1, tuberous sclerosis 2 (TSC2), lead to reduce colony formation, tumor onset and volume in xenograft mouse (Csibi et al. 2013). SIRT4 KO mice develop tumor spontaneously due to increase stress-induced genomic instability and glutamine-dependent proliferation (Jeong et al. 2013). The mechanism uncovered links SIRT4 induction upon DNA damage, leading to GDH inactivation and anaplerotic metabolism shut down allowing cells to arrest and DNA to be repaired (Jeong et al. 2013).

CR does not induces SIRT4 (at least in liver) (Haigis et al. 2006) and overnight fasting reduces its level in muscle and white adipose tissue (Laurent et al. 2013). Moreover, SIRT4 KO mice does not display any gross phenotype and SIRT4 overexpression was not shown to prolong lifespan in mammals, only in flies (*Drosophila melanogaster)* (Wood et al. 2018).

SIRT5

SIRT5, the third mitochondrial Sirtuin, is localized more specifically at mitochondrial matrix and is responsible for regulating key metabolic networks (Nakagawa et al. 2009; Ogura et al. 2010; Rardin et al. 2013; Nishida et al. 2015; Zhou, Wang, et al. 2016). Similarly to SIRT4, SIRT5 has restricted capacity to remove acetyl

groups from lysines of its target proteins, however, it has others identified enzymatic activities responsible for the removal of succinyl, malonyl, and glutaryl groups (Nakagawa et al. 2009; Ogura et al. 2010; Du et al. 2011; Rardin et al. 2013; Tan et al. 2014; Nishida et al. 2015; Zhou, Wang, et al. 2016; Wang et al. 2017).

Prolonged fasting, caloric restriction or high protein diet increase the use of amino acid as energy source leading to ammonia production. The urea cycle counteract the hiperammonemia converting the toxic ammonia to carbamoyl phosphate. SIRT5 was first reported to regulate ammonia detoxification deacetylating and activating carbamoyl phosphate synthetase 1 (CPS1), the first enzyme in the urea cycle (Nakagawa et al. 2009; Ogura et al. 2010). SIRT5 indeed interact and activates CPS1 nonetheless, apart from deacetylase, all the other enzymatic activity seems to control CPS1 activity, desuccinylase, demalonylase and deglutarylase (Du et al. 2011; Tan et al. 2014; Nishida et al. 2015).

SIRT5 control over amino acid metabolism goes beyond urea cycle and link FAO to ketogenesis, where it target several FAO (Very-long-chain acyl-CoA dehydrogenase, ACADV; long-chain acyl-CoA dehydrogenase, ACADL; medium-chain acyl-CoA dehydrogenase, ACADM; shortchain acyl-CoA dehydrogenase, ACADS; trifunctional enzyme a subunit, ECHA; trifunctional enzyme b subunit, ECHB; 2,4-dienoyl-CoA reductase, DECR) and ketogenesis enzymes (enoyl-CoA delta isomerase 1, ECI1; short-chain enoyl-CoA hydratase, ECHM; short-chain 3-hydroxyacyl-CoA dehydrogenase, HCDH; acetoacetyl-CoA thiolase, THIL; 3-hydroxy-3-methylglutaryl coenzyme A synthase 2, HMCS2; 3-hydroxy-3-methylglutarate-CoA lyase, HMGCL; 3-hydroxybutyrate dehydrogenase, BDH). SIRT5 activates FAO increasing acetyl-coA production which then is utilized to ketogenesis (Rardin et al. 2013).

At least 430 proteins contain malonyllysine sites and 120 of those proteins have increased malonylation in SIRT5 KO animals (Nishida et al. 2015). Gluconeogenesis and glycolysis are the top pathways regulated by SIRT5 demalonylation activity and primary hepatocytes from SIRT5 KO mice have diminished glycolytic flux compared with control animals (Nishida et al. 2015). Glycolysis was also showed to be controlled by SIRT5 in a process involving pyruvate kinase muscle isozyme M2 (PKM2) desuccinylation and subsequent translocation into nucleus where it prevent inflammation induced by macrophage interleukin-1β (Wang et al. 2017).

SIRT5 protein levels does not change upon caloric restriction or fasting(Nakagawa et al. 2009). SIRT5-deficient mice are fertile and healthy until at least 18 months of age (Lombard et al. 2007). There is no evidence showing that SIRT5 overexpression leads to increase lifespan.

SIRT7

SIRT7 is the last and least studied Sirtuin. It is nucleolar localized where from it can be released upon stress (Ford et al. 2006; Michishita et al. 2005). SIRT7 was shown to activate RNA polymerase I (Pol I) transcription (Chen et al. 2013; Ford et al. 2006). SIRT7 depletion blocks cell proliferation and leads to apoptosis (Ford et al. 2006). Only a decade after the mechanism between SIRT7 and Pol I was uncovered, and it is linked with SIRT7 deacetylase activity acting over a subunit of Pol I (polymerase-associated factor 53 - PAF53) increasing Pol I occupancy at rDNA and its transcription activation (Chen et al. 2013). Other two studies are in line with the role of SIRT7 in regulating ribosome biogenesis (Chen et al. 2016; Shin et al. 2013).

Lipid metabolism is the main metabolic pathway controlled by SIRT7 (Shin et al. 2013; Yoshizawa et al. 2014; Cioffi et al. 2015; Fang et al. 2017), nevertheless, conflicting results have been published. SIRT7-deficient mice have increased expression of genes related to lipogenesis, inflammation and consequently accumulation of triglycerides in the liver (Shin et al. 2013). The same study showed that SIRT7 overexpression prevent the development of fatty liver disease (Shin et al. 2013). Mechanistically, SIRT7 is induced by endoplasmic reticulum (ER) stress and in a Myc-dependent manner restores hepatic metabolic homeostasis (Shin et al. 2013). On the other hand, Yoshizawa et al. (Yoshizawa et al. 2014) showed complete opposite results with SIRT7 KO mice displaying resistance to high fat diet-induced obesity and fatty liver. Furthermore, liver-specific SIRT7 KO led to reduce hepatic triglycerides accumulation (Yoshizawa et al. 2014). The mechanism uncovered link SIRT7 regulation over ubiquitin-proteasome pathway, inhibiting the degradation of a nuclear receptor involved in fatty acid uptake and triglycerides synthesis (Yoshizawa et al. 2014). In agreement, SIRT7 was shown to induce differentiation and maturation of premature adipocyte having thus a major role in *in vivo* adipogenesis (Cioffi et al. 2015; Fang et al. 2017).

SIRT7 has histone deacetylase activity linked to maintenance of oncogenic transformation repressing tumor suppressor genes when deacetylate H3K18 (Barber et al. 2012). Indeed, SIRT7 prevents cell death attenuating ER stress in a Myc-dependent manner (Shin et al. 2013), and depletion of SIRT7 impairs DNA-damage response and sensitizes cells to genotoxic stresses (Li et al. 2016). In another context, SIRT7 depletion led to opposite results relating to sensitive to genotoxic stress, increasing survival of breast cancer cells results (Yu et al. 2017).

SIRT6

In vitro and in vivo characterization

The first comprehensive study on SIRT6 was in 2005 by Liszt et al, identifying SIRT6 as preferentially nuclear protein expressed at embryonic stage (peak at day E11) and broadly detected in adult tissues (Liszt et al. 2005). In agreement with a previous report showing that SIRT6 had low deacetylase activity on H4 peptides (10x lower than SIRT1, 2 and 3) (North et al. 2003), Liszt et al also reported low to undetectable *in vitro* SIRT6 HDAC activity in histones in comparison with SIRT1 (Liszt et al. 2005). Instead, SIRT6 was identified having ADP-ribosyltransferase activity, more specifically, auto-ADP-ribosyltransferase activity (Liszt et al. 2005). Nonetheless, subsequent works showed H3K9Ac and H3K59Ac as in vitro substrates for SIRT6 deacetylase activity (Michishita et al. 2008; Yang et al. 2009). In the following years deacetylase activity was confirmed in vivo, as well as, others activities, as deacylase, were described (Dominy et al. 2012; Feldman, Baeza, and Denu 2013; Jiang et al. 2013; Ferrer et al. 2018; Zhang, Khan, et al. 2016; Zhang et al. 2018).

In 2006, Mostoslavsky et al.(Mostoslavsky et al. 2006), showed that SIRT6 is essential for mice development. SIRT6-deficient mice die before they reach the fourth week of life displaying several metabolic defects, loss of subcutaneous fat, reduced lymphocyte in the blood and lordokyphosis, and severe hypoglycaemia (Mostoslavsky et al. 2006).

Recent reports have showed the essentiality of SIRT6 in primates and human development (Ferrer et al. 2018; Zhang et al. 2018). SIRT6-null cynomolgus monkey (Macaca fascicularis) displays neuronal and muscular differentiation retard, loss of subcutaneous fat and lower bone density among others abnormalities (Zhang et al.

2018). The result are smaller animals that phenocopied 2-4 months fetuses from wild type animals (normal gestational period is 5 months) (Zhang et al. 2018). Mechanistically SIRT6-null monkeys display H3K56 hyperacetylation leading to upregulation of a developmental repressor, the long non-coding RNA H19 (Zhang et al. 2018). In humans, Mostoslavsky group identified a germline homozygous mutation (amino acid change at Asp63 to histidine – D63H) in SIRT6 that results in perinatal lethality (Ferrer et al. 2018). Mimicking such mutation in vitro, the researches could link the mutation to inactivation of deacetylase and demyristoylase activities and loss of pluripotent gene expression repression capacity (Ferrer et al. 2018). Due to this failure to repress pluripotent genes, mouse embryonic stem cells and human induced pluripotent stem cells derived from D63H homozygous fetuses were not able to differentiate into embryoid bodies, functional cardiomyocytes or neuronal progenitor cells (Ferrer et al. 2018).

Lifespan

SIRT6 loss-of-function clearly result in lifespan shortening in mammals (Zhang et al. 2018; Ferrer et al. 2018; Mostoslavsky et al. 2006). And the opposite is also true, enhanced SIRT6 extends lifespan (at least in male mice) (Kanfi et al. 2012). While the lifespan shortening is linked with reduced stem cell differentiation capacity (Ferrer et al. 2018; Zhang et al. 2018), the increase in lifespan was linked with improved metabolic aspects as alteration in insulin/IGF1 signalling (Kanfi et al. 2012).

Apart from direct influence in shortening or extending lifespan, SIRT6 is linked to several aging-related mechanisms as DNA stability (Mostoslavsky et al. 2006; McCord et al. 2009; Yang et al. 2009; Kaidi et al. 2010; Mao et al. 2011), telomere

maintenance (Michishita et al. 2008; Michishita et al. 2009), nutrient sensing (Kanfi et al. 2008; Feldman, Baeza, and Denu 2013), intercellular communication (Kawahara et al. 2009; Jiang et al. 2013; Santos-Barriopedro et al. 2018) and stem cell homeostasis (Wang et al. 2016). Moreover, SIRT6 acts against age-associated diseases as metabolic diseases (Kanfi et al. 2010; Kim, Xiao, et al. 2010; Dominy et al. 2012; Anderson et al. 2015; Gertman et al. 2018) and cancer (Min et al. 2012; Sebastian et al. 2012; Kugel et al. 2016; Ioris et al. 2017; Liu et al. 2018; Strub et al. 2018; Tian and Yuan 2018).

DNA repair pathways

Mostoslavsky et al. showed that SIRT6 is chromatin-associated and its deficiency increases DNA damage leading to genomic instability. Mouse cells display decreased survival upon challenging with methyl-methane sulphonate, γ irradiated or hydrogen peroxide and this hypersensitivity can be rescue with polymerase beta (Polβ) overexpression, leading the authors to conclude that SIRT6 affect BER pathway(Mostoslavsky et al. 2006). Although there evidences of SIRT6 affecting BER pathway, it is still not know precisely how it does so as no physical association was detect between SIRT6 and BER factors(Mostoslavsky et al. 2006).

Whether SIRT6 action over BER pathway is still to be mechanistically demonstrated, DSB repair pathway is regulated by SIRT6 via several different mechanisms (McCord et al. 2009; Kaidi et al. 2010; Mao et al. 2011; Toiber et al. 2013).

First, SIRT6 associate with nucleosomes (basic unit of chromatin) and with DSB repair factor DNA-PK (DNA-dependent protein kinase) catalytic subunit (DNA-PKcs), and DNA-PKcs associate with chromatin. Those associations are dynamic, increasing

in response to radiomimetic DNA DSB agent neocarzinostatin (NCS), and SIRT6 is required for the association between DNA-PKcs and chromatin as SIRT6 deficient cells maintain the same level of DNA-PKcs at chromatin. Furthermore, SIRT6 and DNA-PKcs are present near DNA DSBs sites and required for efficient repair, and this mechanisms seems to be dependent on the SIRT6 deacetylase activity since H3K9Ac levels increase in response to DNA DSB agent in SIRT6 deficient cells and a SIRT6 deacetylase dead mutant is not able to mobilize DNA-PKcs to chromatin and to efficiently repair DSBs(McCord et al. 2009).

Second, Kaidi et al.(Kaidi et al. 2010), identified the first non-histone SIRT6 target protein, CtIP [C-terminal binding protein (CtBP) interacting protein], and showed that SIRT6-dependent CtIP deacetylation promotes DSB repair by HR. The authors revealed that a CtIP mutant that cannot be acetylate is able to rescue the hypersensitivity of SIRT6 depleted cells in response to DSB agents (in this case the agent was Camptothecin - CPT, a topoisomerase I inhibitor that induces replication-dependent DSBs).

Third, Mao et al.(Mao et al. 2011), confirmed the SIRT6 function in induce HR and identified also a stimulation of NHEJ under oxidative stress. SIRT6 levels increase in response to oxidative stress leading to SIRT6 recruitment to DSB sites and stimulation of DSB repair. Additionally, the authors identified a second non-histone SIRT6 target protein, poly (ADP-ribose) polymerase (Parp1), which is substrate of SIRT6 mono-ADP-ribosylation instead of deacetylation. SIRT6 and PARP1 form a complex which amount increase upon DNA damage, and PARP1 depletion or PARP1 inhibitors blocked SIRT6-mediated HR and NHEJ repair.

Fourth, Toiber et al. (Toiber et al. 2013), identified a mechanism that is also dependent on SIRT6 deacetylation activity and consequent recruitment of a secondary

player. SIRT6 is recruited to DSB sites, deacetylate H3K56 and recruits the chromatin remodeler SNF2H.

Telomere maintenance

Anomalous telomere structures is a common feature in SIRT6 depleted cells resembling defects observed in a premature aging disorder, Werner syndrome, which has as factor a mutation in the WRN gene (coding Werner syndrome ATP-dependent helicase) (Michishita et al. 2008). The telomere dysfunction is characterized by end-to-end chromosomal fusions and consequent premature cellular senescence (Michishita et al. 2008). Mechanistically, SIRT6 deacetylates H3K9 at telomeric chromatin allowing the association and stabilization of WRN and then maintenance of proper telomere function (Michishita et al. 2008). SIRT6 was also linked to H3K56 deacetylation at telomeric chromatin during cell cycle S phase, nonetheless the relevancy of such activity was not determined (Michishita et al. 2009).

Another piece of evidence that SIRT6 acts to maintain an intact telomere structure is the SIRT6 silencing effect over telomere associated satellite-like (TAS) sequences (Tennen et al. 2011). RNAi-mediated Sirt6 downregulation affect the telomeric position effect (TPE) as suggested by the increased expression of telomere-proximal genes (Tennen et al. 2011).

Nutrient availability

As discussed above, caloric restriction is a non-pharmacological intervention able to regulate lifespan and Sirtuins sense the raise in NAD+ levels upon nutrient

deprivation and are believed to be the link for the lifespan extension. SIRT6 levels increase upon nutrient deprivation (glucose and serum) in cultured cells or fasting and caloric restrict regimens in mice and rat (Kanfi et al. 2008; Kim, Xiao, et al. 2010). In mice, SIRT6 mRNA levels increase upon 24 hours of starvation but promptly lower to basal levels when mice are re-fed (Kanfi et al. 2008; Kim, Xiao, et al. 2010). Protein levels also raise in several metabolic active tissues as brain, heart, WAT and kidney, and in liver it reach a peak after 18 hours of starvation (Kim, Xiao, et al. 2010). Nonetheless, the increase in SIRT6 levels upon nutrient deprivation seems to be more complicate than NAD+ sensing as Kim et al. (Kim, Xiao, et al. 2010) demonstrated that this mechanism is dependent on SIRT1. Deletion of SIRT1 impairs SIRT6 increasing upon starvation (Kim, Xiao, et al. 2010). The proposed mechanism involve SIRT1 raise upon nutrient deprivation, forming a complex with FOXO3a and NRF1 and leading to SIRT6 expression and consequent changes in metabolic pathways (Kim, Xiao, et al. 2010).

Furthermore, not only SIRT6 levels can increase in response to change in nutrients, but also SIRT6 deacetylase catalytic efficiency can rise up to 35-fold in response to physiological concentrations of free fatty acids (Feldman, Baeza, and Denu 2013).

Intercellular communication

As discussed above, Inflammation alter intercellular communication. SIRT6 deacetylate H3K9 at NF-κB target gene promoters attenuating expression, this repression required SIRT6 interaction with RELA, a NF-κB subunit (Kawahara et al. 2009; Kawahara et al. 2011). NF-κB target genes are increased in SIRT6-deficient

tissues and cell lines and this increase was proposed to be one of the cause of early lethality of SIRT6-deficient mice since haploinsufficiency of RelA partially rescue this phenotype (Kawahara et al. 2009; Kawahara et al. 2011).

Stem cell homeostasis

SIRT6 definitely regulates in stem cell function however, while SIRT6 deficiency leads to pluripotency maintenance in mESCs, hESCs and cynomolgus monkey neuronal and muscular stem cells (discussed above) it does the opposite in HSCs (Ferrer et al. 2018; Zhang et al. 2018; Wang et al. 2016).

SIRT6-deficient HSCs display aberrant activation of Wnt/β-catenin signalling (a critical pathway for homeostasis maintenance in this cells) impairing self-renewal, promoting proliferation and culminating in HSCs exhaustion (Wang et al. 2016). Mechanistically, Wang et al. showed that SIRT6 interact with T-cell factor (TCF) and Lymphoid enhancer-binding factor 1 (LEF1), deacetylates H3K56 and repress transcription of Wnt target genes. Another evidence that SIRT6 deficiency leads to Wnt activation is the reserve of aberrant HSC proliferation upon pharmacological inhibition of Wnt pathway (Wang et al. 2016).

SIRT6 role in Metabolism and Cancer

Metabolism: Obesity and Type II Diabetes

Obesity is a complex disease determined by the interaction between genetic, environmental, and psychosocial factors resulting in a state in which body fat

accumulates in excess (Spiegelman and Flier 2001; Gesta, Tseng, and Kahn 2007).

This adipose tissue hypertrophy may lead to adverse metabolic effects on blood pressure, cholesterol and triglyceride levels and can result in a number of obesity-related disorders, including non-alcoholic fatty liver disease, hypertension, cardiovascular pathology, and type II diabetes (Angulo 2002; Poirier and Eckel 2002; Haslam and James 2005; Hossain, Kawar, and El Nahas 2007). The prevalence of obesity and type 2 diabetes mellitus (T2DM) has risen significantly in the last decades. According to the World Health Organization T2DM and/or obesity are a global epidemic with more than 1 billion adults overweight [body mass index (BMI) of 25.0–29.9 kgm^2] or obese (BMI >30 kgm^2).

Unfortunately, current treatments are still suboptimal due to their side effects and the fact that long-lasting and debilitating morbidities (e.g.: heart disease, neuropathy, and hypertension) are still too often associated (Steffes et al. 2003; Bluestone, Herold, and Eisenbarth 2010; Maahs et al. 2010; Vianna and Coppari 2011; Coppari and Bjorbaek 2012). Thus, development of more effective anti-obesity and -T2DM treatments is urgently needed. To these ends, the identification of novel molecular target(s) that exert protective effects against dietary obesity and/or T2DM is of paramount medical significance.

SIRT6 in metabolism and metabolic-related diseases

SIRT6-deficient mice display several hypoglycaemia indicating SIRT6 as a regulator of glucose metabolism (Mostoslavsky et al. 2006). Indeed, SIRT6 deficiency leads to upregulation of glycolysis sustained by increase glucose uptake (Zhong et al. 2010). This phenomenon is explained by the fact that SIRT6 deficiency increase the

expression of several glycolytic genes and activity of Hif1a, evidenced by reduced mitochondrial respiration and increased lactate production (Zhong et al. 2010).

During periods of fasting and consequent lowering of blood glucose levels the liver produce and release glucose into the bloodstream in order to maintains its levels (Spiegelman and Flier 2001). However, this beneficial mechanisms become a problem in the context of insulin resistance where it contribute even further to the hyperglycaemia and means to reduce gluconeogenesis could be used to treat patient with T2DM for example (Spiegelman and Flier 2001). The hepatic glucose production, or more specifically gluconeogenesis, is controlled by a signalling/transcriptional network where PGC-1a is a key mediator (Nemoto, Fergusson, and Finkel 2005). PGC-1a, as a transcription factor, activate gluconeogenic gene expression in a SIRT1-dependent manner (Nemoto, Fergusson, and Finkel 2005). GCN5 acetylates PGC-1a and thus inactivate it while SIRT1 deacetylase and activates PGC-1a. SIRT6 overexpression reduces hepatic glucose production (Dominy et al. 2012; Anderson et al. 2015). It increase the complexity of PGC-1a regulation acting as an indirect repressor of PGC-1a and therefore gluconeogenesis (Dominy et al. 2012). Not only PGC-1a can be activated upon removal of an acetyl group but also GCN5, and SIRT6 is the deacetylase enzyme responsible for GCN5 activation (Dominy et al. 2012).

As discussed above, SIRT1 form a complex with FOXO3a and NRF1 and thus increase SIRT6 expression leading to a SIRT6-dependent deacetylation of H3K9 and repression of glycolytic and fat metabolism pathways genes (Kim, Xiao, et al. 2010). The opposite is also true, liver-specific knockout of SIRT6 leads to increase glycolysis and triglyceride synthesis, and repression of β-oxidation which culminate in fat accumulation into liver (Kim, Xiao, et al. 2010). In human with fatty liver disease exhibited reduced levels of SIRT6 comparing with control, pointing for a possibility of

development of SIRT6 agonists for therapeutic therapeutics purposes (Kim, Xiao, et al. 2010).

SIRT6 not only control glucose and lipid metabolism but it does in a circadian fashion (Masri et al. 2014). SIRT6 interacts with core circadian clock machinery, CLOCK and BMAL1, controlling their chromatin recruitment and therefore circadian gene expression as, for example, genes involved in fatty acid and cholesterol metabolism (Masri et al. 2014).

Cancer

There are more than 100 different type of cancer classified by organ, tissue, cell type or mutation that originates it (https://www.cancer.gov/types). Solid tumors are complex tissues including heterotypic interactions between multiple distinct cancer and normal cell types that enable growth and progression. The so called “tumor microenvironment” is composed by cancer cells and CSC, cancer-associated fibroblasts, immune cells, endothelial cells and pericytes (Hanahan and Weinberg 2000, 2011).

Basically, cancer cells are specialized in growth, been able to acquire nutrients from different sources to keep their viability and produce biomass for cell division (Hanahan and Weinberg 2000, 2011; Pavlova and Thompson 2016). Beyond uncontrolled growth, normal cells acquire a number of others molecular, biochemical and cellular traits in the evolution process to become cancer cells (Hanahan and Weinberg 2000, 2011). Genomic instability foster the generation of genetic diversity and selection of favourable changes in a Darwinian way leading normal cells into cancer cells (Hanahan and Weinberg 2000, 2011). Growth advantage can be due to a

state mimicking constant proliferative signal (as activate PI3K and KRAS pathways) or due to loss of growth suppressors (as loss of retinoblastoma gene/protein) (Hanahan and Weinberg 2000, 2011). Others common acquired characteristics are present in almost all tumors, including apoptosis resistance, replicative immortality, angiogenesis activation, ability to migrate to distant sites, immune surveillance evasion and, the most ancient discovery, energy metabolism flexibility (Warburg 1956; Hanahan and Weinberg 2000, 2011; Warburg, Wind, and Negelein 1927)

At cellular and molecular levels, cancer cells are well linked with metabolic adaptation. Cancer cells are characterized by the ability to sustain uncontrolled proliferation, which demand a metabolic shift in order to empower cell growth and division (Hanahan and Weinberg 2000, 2011; Lunt and Vander Heiden 2011). Cancer cells reprogram their glucose metabolism, even in the presence of oxygen, to convert pyruvate into lactate, and use glycolytic intermediates as building blocks or macromolecular synthesis (Vander Heiden, Cantley, and Thompson 2009; Warburg, Wind, and Negelein 1927; Warburg 1956). To sustain that inefficient conversion of glucose into ATP, cancer cells exhibit increase glucose uptake and enhanced expression and activity of glycolytic enzymes (Vander Heiden, Cantley, and Thompson 2009). In a recent review, Natalya N. Pavlova and Craig B. Thompson (Pavlova and Thompson 2016), enumerate six hallmarks of cancer metabolism: “(1) deregulated uptake of glucose and amino acids, (2) use of opportunistic modes of nutrient acquisition, (3) use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, (4) increased demand for nitrogen, (5) alterations in metabolite-driven gene regulation, and (6) metabolic interactions with the microenvironment”.

SIRT6 and Cancer

The first evidence that SIRT6 could be a tumor suppressor come from the work of Sebastian et al. (Sebastian et al. 2012) where was showed that loss of SIRT6, and no other additional mutation, was enough to transform cells. Furthermore, this study revealed that metabolic alteration is sufficient to underlie oncogenic transformation and not a consequence of it (Sebastian et al. 2012). One of the mechanism behind was already known but not linked with tumorigenesis, to increased aerobic glycolysis upon SIRT6 loss. This SIRT6 loss-dependent transformation is impair by inhibition of pyruvate dehydrogenase kinase (PDK)-1, a glycolytic enzyme, or pharmacological inhibition of aerobic glycolysis, supporting the oncogenic capacity of altered metabolism (Sebastian et al. 2012). The second mechanisms described is related with well-known oncogene Myc, where SIRT6 loss leads to activation of a subset of Myc target genes (but not Myc levels per se) increasing ribosome biogenesis and glutamine metabolism. In this context, Myc deletion decrease tumorigenic capacity of SIRT6 deficient cells (Sebastian et al. 2012).

Sirt6 is frequently found to be downregulated or mutated in human cancers and reduce Sirt6 expression inversely correlate with survival rates (Sebastian et al. 2012; Kugel et al. 2015; Tian and Yuan 2018). SIRT6 point-mutation that occurs naturally in patient-derived cancers significantly altered SIRT6 catalytic activity, stability or cellular localization and failed to decrease tumorigenic potential of SIRT6 deficient cells (Kugel et al. 2015).

SIRT6 was shown to suppress breast (Ioris et al. 2017) and pancreatic (Kugel et al. 2016) cancer in mouse models as well as, suppress proliferation and

tumorsphere formation capacity in a wide range of cancer cell lines from different tissues (Kugel et al. 2016; Ioris et al. 2017; Liu et al. 2018; Tian and Yuan 2018).

CSCs, a subpopulation of cancer cells with self-renew capacity and resistant to conventional chemotherapies, are affected by SIRT6 physiological overexpression (expression mimicking SIRT6 increase upon CR) (Ioris et al. 2017). We showed that SIRT6 overexpression reduces tumorsphere formation potential, delay onset of tumors in xenograft models and maintain mouse mammary tumors more differentiated, as visualized by reduce area of carcinoma within the tumor and increased expression of genes expressed in normal mammary gland, than controls. SIRT6 acts at chromatin level impairing expression of genes normally activate by PI3K signalling, including several genes related to lipid metabolism, and, the anti-stemness function is independent of SIRT6 deacetylase activity (Ioris et al. 2017).

OBJECTIVE

To address the role of SIRT6 overexpression we have generated a mouse model using a Bacterial Artificial Chromosome (BAC) strategy. The BAC houses the whole SIRT6 gene sequence and its endogenous promoter, allowing a mild and physiological regulated overexpression of SIRT6 that mimics caloric restriction-like increase in SIRT6 protein levels.

The first goal of this study was to clarify the role of SIRT6 in regulating metabolism, aiming to combat obesity-associated metabolic diseases, particularly Diabetes Mellitus type II. This was accomplished by comparing several metabolic parameters, in normal and high fat diet, between our mouse model that overexpress SIRT6 physiologically (SIRT6BAC) and its littermate wild type control.

The second goal was to determine the effect of SIRT6 in tumorigenesis. We aimed at testing the relevance of enhanced SIRT6 in cancer and to this end, we crossed SIRT6BAC mice with a well-establish mammary cancer mouse model. Additionally, we assessed SIRT6 role on tumor biology using a range of *in vivo*, xenograft, and *in vitro* assays.

RESULTS

1. Enhanced insulin sensitivity in skeletal muscle and liver by physiological overexpression of SIRT6

Here we generate a mouse model overexpressing SIRT6 (SIRT6BAC) at levels comparable to SIRT6 increase upon caloric restriction. Our results, based in several metabolic analyses, including hyperinsulinemic-euglycemic clamp assays, point to SIRT6-driven glucose metabolism improvement in skeletal muscle and liver. Therefore, supporting the potentiality of SIRT6 as a putative molecular target for the treatment of obesity and type II diabetes mellitus.

Author contributions

I contributed directly to Figure 1 B and C (reviewer request to include additional tissues), Figure 4 A-D, Figure 5 (performed by Dr. Christelle Veyrat-Durebex at the glucose/insulin clamp platform at University of Geneva), Figure 6, Supplemental Figure 1 and 2.

Original article

MOLECULAR METABOLISM

Enhanced insulin sensitivity in skeletal muscle and liver by physiological overexpression of SIRT6*

Jason G. Anderson [1,7], Giorgio Ramadori [1,2,3,7], Rafael M. Ioris [2,3,7], Mirco Galiè [2], Eric D. Berglund [4,5], Katie C. Coate [5], Teppei Fujikawa [1], Stefania Pucciarelli [6], Benedetta Moreschini [6], Augusto Amici [6], Cristina Andreani [6], Roberto Coppari [1,2,3,*]

ABSTRACT

Objective: Available treatment for obesity and type 2 diabetes mellitus (T2DM) is suboptimal. Thus, identifying novel molecular target(s) exerting protective effects against these metabolic imbalances is of enormous medical significance. *Sirt6* loss- and gain-of-function studies have generated confounding data regarding the role of this sirtuin on energy and glucose homeostasis, leaving unclear whether activation or inhibition of SIRT6 may be beneficial for the treatment of obesity and/or T2DM.

Methods: To address these issues, we developed and studied a novel mouse model designed to produce eutopic and physiological overexpression of SIRT6 (Sirt6BAC mice). These mutants and their controls underwent several metabolic analyses. These include whole-blood reverse phase high-performance liquid chromatography assay, glucose and pyruvate tolerance tests, hyperinsulinemic-euglycemic clamp assays, and assessment of basal and insulin-induced level of phosphorylated AKT (p-AKT)/AKT in gastrocnemius muscle.

Results: Sirt6BAC mice physiologically overexpress functionally competent SIRT6 protein. While Sirt6BAC mice have normal body weight and adiposity, they are protected from developing high-caloric-diet (HCD)-induced hyperglycemia and glucose intolerance. Also, Sirt6BAC mice display increased circulating level of the polyamine spermidine. The ability of insulin to suppress endogenous glucose production was significantly enhanced in Sirt6BAC mice compared to wild-type controls. Insulin-stimulated glucose uptake was increased in Sirt6BAC mice in both gastrocnemius and soleus muscle, but not in brain, interscapular brown adipose, or epididymal adipose tissue. Insulin-induced p-AKT/AKT ratio was increased in gastrocnemius muscle of Sirt6BAC mice compared to wild-type controls.

Conclusions: Our data indicate that moderate, physiological overexpression of SIRT6 enhances insulin sensitivity in skeletal muscle and liver, engendering protective actions against diet-induced T2DM. Hence, the present study provides support for the anti-T2DM effect of SIRT6 and suggests SIRT6 as a putative molecular target for anti-T2DM treatment.

Keywords SIRT6 overexpression; Sirtuin; Insulin sensitivity; Glucose homeostasis; Diabetes

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[1]Department of Internal Medicine, Division of Hypothalamic Research, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA [2]Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland [3]Diabetes Center of Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland [4]Advanced Imaging Research Center, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA [5]Department of Pharmacology, Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA [6]School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy

[7] Co-first author.

*Corresponding author. Department of Cell Physiology and Metabolism, and Diabetes Center of the Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland. Tel.: +41 (0)22 3795539; fax: +41 (0)22 3795260. E-mail: roberto.coppari@unige.ch (R. Coppari).

Abbreviations: BAC, bacterial artificial chromosome; T2DM, type 2 diabetes mellitus; HCD, high-caloric diet; IPGTT, intraperitoneal glucose tolerance test; IPPTT, intraperitoneal pyruvate tolerance test; R_g, tissue-specific glucose uptake rate; GIR, glucose infusion rate; $EndoR_a$, endogenous glucose appearance rate; Rd, glucose disposal rate

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1. INTRODUCTION

The prevalence of obesity and T2DM has risen significantly in the last decades. According to the World Health Organization T2DM and/or obesity are estimated to affect hundreds of millions worldwide (http://www.who.int/mediacentre/factsheets/fs311/en/ and http://www.who.int/mediacentre/factsheets/fs312/en/). Significant contributors to the rapid increase in the incidence of obesity and T2DM include recent lifestyle changes such as chronic consumption of hyper-caloric diets [1]. Unfortunately, current anti-obesity and -T2DM treatments are still suboptimal due to their side effects and the fact that long-lasting and debilitating morbidities (e.g.: heart disease, neuropathy, and hypertension) are still too often associated with these maladies [1–5]. Thus, development of more effective anti-obesity and -T2DM treatments is urgently needed. To these ends, the identification of novel molecular target(s) exerting protective effects against dietary obesity and/or T2DM is of paramount medical significance.

Recent genetic and pharmacological studies have identified sirtuins, which are nicotinamide adenine dinucleotide-dependent enzymes exerting post-translational modifications of their target proteins, as potential therapeutic targets for improving HCD-induced metabolic imbalances [6–15]. SIRT6, one of the seven mammalian sirtuins, is a lysine-deacetylase and mono-ADP-ribosyl-transferase with pleiotropic effects [16]. However, the role of SIRT6 in metabolism is controversial. For example, *Sirt6* knockout mice exhibit reduced adipose tissue mass and hypoglycemia [17]. SIRT6 deficiency also leads to attenuation of SIRT6-dependent transcriptional silencing resulting in increased expression of genes involved in glycolysis and glucose transport [18,19]. Of note, secretion of tumor necrosis factor-alpha (TNF-α), which is known to exert detrimental actions on energy homeostasis and insulin sensitivity [20], is diminished following knock-down of SIRT6 [21]. Therefore, according to these observations, systemic delivery of SIRT6 inhibitors should diminish adiposity, increase insulin sensitivity, glucose uptake and utilization, and consequently improve obesity and T2DM. However, in contrast to this notion, ubiquitous and supra-physiological overexpression of SIRT6 also leads to reduced adiposity and improved glucose metabolism in mice fed on a HCD [22]. Furthermore, adenoviral-mediated overexpression of SIRT6 in liver of diabetic mice suppresses hepatic glucose production and improves hyperglycemia [23]. Hence, these latter results suggest that systemic delivery of SIRT6 activators should bring about beneficial effects in the context of obesity and T2DM.

Based on the aforementioned data, it is unclear whether means to inhibit or enhance SIRT6 protein activity should be sought in order to treat obesity and/or T2DM. Also, the tissues underlying the effect of SIRT6 on whole-body glucose homeostasis are unknown. In order to address these issues, we developed and studied a novel mouse model designed to produce eutopic and physiological overexpression of SIRT6 (Sirt6BAC mice).

2. MATERIAL AND METHODS

2.1. Animals

Mice were housed with chow diet and water available *ad libitum* in light (12-hour light/12-hour dark cycles) and temperature (20–22 °C) controlled environments. Male mice were used for all experiments. Mice in HCD cohorts were fed a 58 kcal% fat w/sucrose diet (Open Source Diet Product #D12331 Research Diets Inc.) beginning at 8 weeks of age. Care of mice was within the Institutional Animal Care and Use Committee (IACUC) guidelines, and procedures were approved by the University of Texas Southwestern Medical Center IACUC and the ethical commission of the Canton of Geneva, Switzerland.

2.2. Generation of Sirt6BAC mice

A bacterial artificial chromosome (BAC) possessing 185.7 kb (70.7 kb upstream and 109.3 kb downstream) of unmodified mouse genomic DNA sequences flanking the *Sirt6* gene (BAC clone RP23-352G18, BACPAC RESOURCES, CHILDREN'S HOSPITAL Oakland, CA, USA) was purified as previously described [18] and used as template sequence for PCR reactions with the primer sets: 5′GACTGGGACCACACCAGAGT and 5′GTGAGAGCGGGAAGAGTACG; 5′AGGTGCCTGTGGACACTACC and 5′CAGGGGACACACTGGTTTCT; 5′CTGTCCACCTGTTGGAAGGT and 5′CTTCTGGGTCACCACAAGGT; 5′CATGAATGCTGTTTGGTTGG and 5′ATGCTGTAGGGTGGGAAGTG; 5′CCTTTGGAAAAGCAGTCAGC and 5′GAACTCCTGGCAAGTCGAAG; 5′CCACTGGGTCAGTCACACAC and 5′AGGACTCCACCTGGATTGTG. Upon observing the expected size of each amplicons, the purified BAC DNA was then electroporated into EL250 bacteria rendered electro-competent as previously described [24]. loxP sequences contained in the pBACe3.6 backbone of RP23-352G18 BAC DNA were replaced via homologous recombination [25] by an ampicillin resistance gene cassette generated via PCR amplification of a PGEM-T-Easy vector template with the primer sets: 5′GATAAACTACCGCATTAAAGCTTATCGATGATAAGCTGTCAAACATGAG-AATTGATCCGGATATATGAGTAAACTTGGTCTGAC and 5′GTTAACCGGGCTGCATCCGATGCAAGTGTGTCGCTGTCGACGGTGACC-CTATAGTCGAGGCGGTATTTTCTCCTTACGCATC. The modified RP23-352G18 BAC DNA was then purified and microinjected in its circular state into pronuclei of fertilized embryos of C57Bl/6J mice using standard methods [24]. By genotyping of tail biopsies using the following primer sets 5′TTGCTCTTGCGGGAAGCCCG and 5′GATACCGAGGGCGCCGTTCG (specific to the pBACe3.6 backbone sequence of RP23-352G18); 5′TTGCTGCATCAGGAGGGCGC and 5′TCCCACAATGCCCCGCTTCG (specific to the wild-type *Sirt6* allele) three mice were identified having germ-line incorporation of RP23-352G18 BAC DNA. These three Sirt6BAC mice were bred to wild-type C57Bl/6J mice establishing three distinct Sirt6BAC lines. Data shown here were obtained from the study of the line displaying two to four fold expression of SIRT6 compared to wild-type littermates.

2.3. Generation of Sirt6BAC; *Sirt6*$^{-/-}$ mice

Sirt6BAC mice were bred to mice heterozygous for the *Sirt6* null allele (*Sirt6*$^{+/-}$) [17]. F1 offspring were mated (*Sirt6*$^{+/-}$ × *Sirt6*$^{+/-}$; Sirt6BAC) to generate F2 breeder mice. From these F2 breeding pairs (*Sirt6*$^{+/-}$ × *Sirt6*$^{+/-}$; Sirt6BAC), F3 cohorts were obtained for experimental study including wild-types, mice harboring the Sirt6BAC (Sirt6BAC), or *Sirt6* knockout mice harboring Sirt6BAC (Sirt6BAC; *Sirt6*$^{-/-}$). To determine whether a mouse contained none, one, or two endogenous wild-type *Sirt6* sequences, we utilized a multiplex Taq-Man qPCR Copy Number genotyping analysis in which the commercially available Transferrin Receptor (*Tfrc*) (VIC dye-labeled probe) from Applied Biosystems (Foster City, CA, USA) was used as endogenous reference copy-number and the commercially available *Escherichia coli* β-galactosidase Mr00529369_cn (FAM dye-labeled probe) from Applied Biosystems (Foster City, CA, USA) was used to quantify the endogenous wild-type *Sirt6* allele copy number. Of note, the endogenous wild-type *Sirt6* sequences were swapped with the β-galactosidase sequences in *Sirt6*$^{+/-}$ mice [17]. Thus, mice bearing two of the endogenous wild-type *Sirt6* alleles were found to have zero copies of the β-galactosidase allele, whereas mice bearing one of the

endogenous wild-type *Sirt6* alleles were found to have one copy of the β-galactosidase allele, and mice lacking both of the endogenous wild-type *Sirt6* alleles were found to have two copies of the β-galactosidase allele.

2.4. Western blot

Tissues were lysed with RIPA buffer (R0278 Sigma Aldrich®) with 1:100 dilution of protease inhibitor cocktail (P8340 Sigma Aldrich®). Equal amounts of protein lysates (20 μg) were separated via SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Subsequently, the membranes were blocked using LI-COR® Odyssey® blocking buffer (927−40000) for 2 h at room temperature, then placed in primary antibody incubation (rabbit polyclonal anti-SIRT6 [Abcam® ab62739] 1:3000 dilution and mouse monoclonal anti-βActin [Sigma Aldrich® A5316] in LI-COR® Odyssey® blocking buffer w/0.1% Tween-20, overnight at 4 °C. After washing the membranes 3 × 10 min in PBS w/0.1% Tween-20, they were incubated at room temperature in darkness for 2 h with fluorescently conjugated secondary antibodies (goat anti-rabbit LI-COR® IRDye® 800CW [926−32211] 1:5,000 and goat anti-mouse LI-COR® IRDye® 680 [926−32220] 1:10,000) in Odyssey® blocking buffer w/0.1% Tween-20, 0.01%SDS. The membranes were imaged with a LI-COR® Odyssey® infrared imager. SIRT6 specific bands (~37kD) were quantified relative to housekeeping protein level using LI-COR® Odyssey® Image Studio software. p-AKT/AKT ratio was assessed as previously described [10].

2.5. Quantitative real-time PCR (qPCR)

Tissues were lysed in TRIzol® reagent in order to extract total RNA. cDNA was reverse transcribed from these purified RNA samples with Invitrogen SuperScript® III reverse transcriptase. Genes present on RP23-352G18 BAC were assayed via qPCR gene expression analysis to determine the levels of mRNA transcript expression. qPCR gene expression analysis was performed using inventoried TaqMan gene expression assays (Applied Biosystems®). These genes included: *AES* (Mm01148854_g1), *Ankrd24* (Mm01147213_m1), *BC025920* (Mm02763635_s1), *Gna11* (Mm01172792_m1), *Gna15* (Mm00494669_m1), *Tle2* (Mm00498094_m1), *Tle6* (Mm00475103_m1), and *Sirt6* (Mm01149042_m1). Expression levels were measured with an Applied Biosystems® 7900HT Sequence Detection System with SDS2.1 software. Baseline values of amplification plots were set automatically and threshold values were kept constant. The mRNA levels were expressed as arbitrary units and were obtained by dividing the averaged sample values (in triplicate) for each gene by that of the control housekeeper 18S rRNA (Mm03928990_g1).

2.6. Body weight and body composition

Mice were weighed every other week beginning at 8 weeks of age. Body composition was determined monthly beginning at 8 weeks of age using the EchoMRI-100™ quantitative nuclear magnetic resonance system providing precise measurements of whole body fat and lean mass.

2.7. Blood chemistry

Mice were singly housed in the morning (9am−12 pm) in cages with fresh bedding and access to water, but without food during this time period to ensure that the experimental measurements were not affected by postprandial effects. Fasting levels were gathered from blood samples obtained from mice fasted over-night. Glycemia measurements were taken from tail blood samples with OneTouch® Ultra® 2 glucometer with OneTouch® Ultra® Blue Test Strips. Immediately following, 50 μL of blood was collected in tubes at room temperature for five minutes before placing into ice. The blood samples were then centrifuged at 2,000 × g for 10 min at 4 °C. The blood serum supernatant was then pipetted into a new tube for storage at −80 °C. Blood chemistry was assessed as previously described [9,10,26].

2.8. Intraperitoneal glucose and pyruvate tolerance tests

Intraperitoneal glucose tolerance tests (IPGTT) and intraperitoneal pyruvate tolerance tests (IPPTT) were performed in male, age-matched mouse cohorts with similar body weights. Mice were singly housed and fasted overnight (6pm−10 am) in cages with fresh bedding and access to water, but without food during this time period to ensure that the experimental measurements were under fasting conditions. An hour before the *in vivo* experiment, mice were weighed to determine the dosage of glucose (1.5 g glucose per kg bodyweight) or pyruvate (2 g pyruvate per kg bodyweight) to be administered. Just prior to injecting the glucose (or pyruvate) bolus, fasting glycemia was measured from tail blood samples with an AlphaTRAK blood glucometer. The glucose (0.15 g glucose/mL 0.9% saline) or pyruvate (0.2 g sodium pyruvate/mL 0.9% saline) solution was then injected into the intraperitoneal cavity of each mouse. Blood glucose measurements were taken from tail blood samples at times indicated in the figures.

2.9. Hyperinsulinemic-euglycemic clamp

Clamp was performed in male, standard chow-fed, age-matched mice with similar body weights. 5 days prior to the experiment, mice were anesthetized using isoflurane and a catheter was surgically implanted in the right jugular vein and exteriorized above the neck as previously described [27]. Hyperinsulinemic-euglycemic clamps were then performed in these 14−16 week old, conscious, unrestrained catheterized mice. Mice were fasted 5 h prior to the start of the experiment (t = 0 min). At t = −120 min, an infusion of [3-^{3}H]glucose (0.05 μCi/min) was initiated. At t = −15 and −5 min, blood samples were collected from the tail vein to measure basal blood glucose and plasma insulin as well as to calculate the rate of endogenous glucose appearance (EndoR_a) and glucose disposal (R_d). At t = 0 min, a continuous insulin infusion (4 mU/kg bodyweight/min.) was used to induce hyperinsulinemia and the infusion of [3-^{3}H]glucose was increased to 0.1 μCi/min. Blood samples were then taken every 10 min to measure blood glucose, and 50% dextrose was infused as needed to maintain target euglycemia (120 mg/dL). This target was chosen because it was the average basal glycemia of the two groups. Additional blood samples were taken every 10 min from t = 100−120 min (steady state clamp) to determine plasma insulin and calculate glucose turnover. After the blood sample was taken at t = 120 min, a 13 μCi bolus of 2[^{14}C]deoxyglucose tracer was administered for the measurement of tissue-specific glucose uptake (R_g). Blood samples were obtained at t = 122, 130, 137, 145 min to assess blood glucose and 2[^{14}C]deoxyglucose specific activity. Mice were then anesthetized using chloral hydrate. Brain, interscapular brown adipose tissue, liver, epididymal white adipose tissue, gastrocnemius, and soleus muscle were flash-frozen in liquid nitrogen for storage at −80 °C for further analysis. Plasma concentrations of [3-^{3}H]glucose were determined following deproteinization of plasma samples with zinc sulfate and barium hydroxide. Basal glucose turnover and insulin-stimulated R_d was determined as the ratio of the [3-^{3}H]glucose infusion rate to the specific activity of plasma [3-^{3}H]glucose at the end of basal period and during clamp steady state, respectively. EndoR_a during the clamp was determined by subtracting steady state GIR from R_d. R_g was determined by measuring the accumulation of phosphorylated 2[^{14}C]

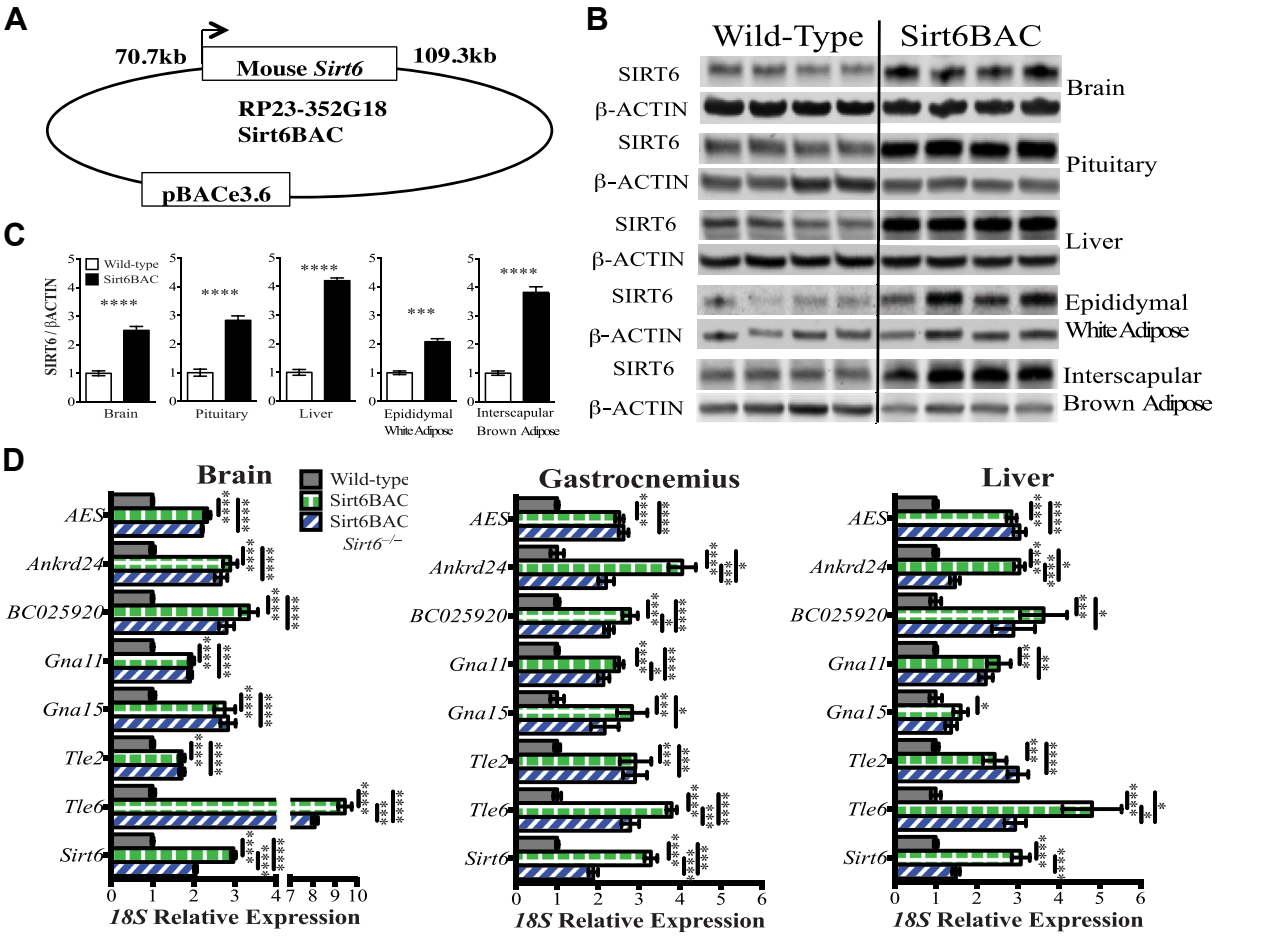

Figure 1: Sirt6BAC mice eutopically overexpress mouse SIRT6. **(A)** Schematic representation of the RP23-352G18 (BACPAC Resources, Children's Hospital Oakland Research Institute) BAC DNA construct containing the mouse *Sirt6* gene used to generate Sirt6BAC mice. **(B)** Anti-SIRT6 Western blots of tissues from Sirt6BAC and wild-type standard-diet-fed mice on a pure C57Bl/6 genetic background. (Each lane represents tissue from a single mouse). For each tissue, data were gathered from the same membrane. The same membrane was digitally separated to improve clarity. **(C)** Quantification of the relative SIRT6/βACTIN expression shown in panel B (n = 4 per group). **(D)** qPCR measurements of brain, gastrocnemius and liver gene transcripts present in wild-type, Sirt6BAC, and Sirt6BAC; *Sirt6*$^{-/-}$ standard-diet-fed mice on a C57Bl/6; 129SvJ mixed genetic background (n = 6–8 per group). Values are mean ± S.E.M. Statistics were analyzed using unpaired two-tailed t-test when 2 groups were compared and one-way ANOVA with Tukey correction for multiple comparisons when 3 or more groups were compared (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

deoxyglucose in dissected tissues and the disappearance of 2[^{14}C] deoxyglucose from blood.

2.10. Reverse phase high-performance liquid chromatography (RP-HPLC)

Blood was collected from 2-month-old male standard-diet-fed mice and then treated with 10 μL of 1% EDTA. In order to detect polyamines through RP-HPLC, blood samples underwent a dansylation procedure. 100 μl of blood (previously treated with 5% TCA to allow protein precipitation) and 1 ml of standard polyamines stock solutions (1 mg/ml) were subjected to a derivatization process with dansyl chloride according to Seiler [28]. After incubation at 40 °C for 45 min with dansyl chloride, mixtures were filtered through a 0.45 μm RC-membrane (ALBET-LabScience, Dassel/Relliehausen, Germany) and injected into a HPLC apparatus (Agilent 1100) for analysis. The separation was executed on a Supelcosil LC 18 RP column (5 μm; 4,6 × 250 mm) maintained at 35 °C. The mobile phase was composed of water and methanol with the gradient elution system at a flow rate of 1.0 ml/min. The initial mobile phase contained 70% methanol for 7 min. The gradient volume of methanol was 70–75% at 7–10 min, 75–90% at 10–20 min and 90–100% at 20–25 min. The signal detection was through a Diode Array Detector (DAD) and the chromatograms were recorded at a wavelength of 253 nm. Polyamines calibration curves were obtained using dansylated polyamine standard solutions (concentrations ranging between 0.001 mg/ml and 0.1 mg/ml). Polyamines concentration was normalized to total protein concentration obtained with Bradford assay and expressed as nmol/mg.

2.11. Statistical analyses

Data are reported as mean ± SEM. All statistical analyses were performed using Graphpad Prism® 6.0c software. Unpaired two-tailed t-tests were employed when 2 groups were compared, and one-way ANOVA with Tukey correction for multiple comparisons were employed when 3 or more groups were compared, and repeated measures two-way ANOVA with Tukey correction for multiple comparisons were used when 3 or more groups were compared over a time-course.

3. RESULTS

3.1. Generation and validation of Sirt6BAC mice

To determine the role of SIRT6 on energy and glucose homeostasis, we generated genetically-engineered mice harboring bacterial artificial chromosome (BAC) DNA containing 70.7 kb upstream and 109.3 kb downstream sequences flanking the mouse *Sirt6* gene (Sirt6BAC mice) (Figure 1A). In several tissues of Sirt6BAC mice, SIRT6 protein level was found to be two to four times greater than wild-type controls (Figures 1B,C and S1), suggesting that DNA sequences contained in the BAC include the crucial transcriptional regulatory elements of the endogenous *Sirt6* gene. Of note, this magnitude of SIRT6 overexpression found in Sirt6BAC mice is physiological as it approximates that which is observed in calorically restricted rodents [29].

Virtually all BAC-based transgenic approaches suffer from two major confounders that may alter gene expression and hence cause phenotypes: 1) the presence of additional coding and/or non-coding sequences contained in the large BAC DNA sequences and 2) DNA construct insertion site into the genome. As the genomic fragment used to generate Sirt6BAC mice bears additional known mouse genes (i.e.: *AES, Ankrd24, BC025920, Gna11, Gna15, Tle2,* and *Tle6*), we assessed their mRNA contents and found them to be increased in Sirt6BAC mice compared to wild-types (Figure 1D). Hence, to disassociate the effect of SIRT6 overexpression from the potential effect of non-SIRT6 BAC-born products and/or BAC insertion site(s) on a given phenotype, we generated an additional control genotype. This was accomplished by breeding the Sirt6BAC allele to the *Sirt6* null (*Sirt6*$^{-}$) allele [17]. This breeding scheme produced three experimental genotypes that were used in this study: 1) wild-type, 2) Sirt6BAC, and 3) Sirt6BAC; *Sirt6*$^{-/-}$ mice. Of note, quantitative PCR profiling of brain, gastrocnemius and liver indicated that while *AES, Ankrd24, BC025920, Gna11, Gna15, Tle2,* and *Tle6* mRNA levels are similarly increased in Sirt6BAC and Sirt6BAC; *Sirt6*$^{-/-}$ mice the latter showed reduced *Sirt6* mRNA level compared to the former genotype (Figure 1D). Also, while SIRT6 protein level is significantly increased in several tissues of Sirt6BAC mice compared to wild-type controls (Figures 1B,C and S1), it is similar between gastrocnemius of Sirt6BAC; *Sirt6*$^{-/-}$ mice and wild-type controls (Figure S1).

To validate the functional competence of BAC-DNA-derived SIRT6 protein, the ability of Sirt6BAC allele to rescue the phenotypes displayed by *Sirt6*$^{-/-}$ mice was assessed. Among other defects, *Sirt6*$^{-/-}$ mice have increased early-postnatal mortality and reduced body length and weigh compared to wild-type mice [17]. These aberrancies were all rescued by introduction of Sirt6BAC allele in the *Sirt6* null background. Indeed, while viability, body length and weight were all reduced in *Sirt6*$^{-/-}$ mice (as predicted) all these parameters were found to be normal in Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 2A–C). Collectively, these data demonstrate that Sirt6BAC mice physiologically overexpress functionally competent SIRT6 protein.

3.2. Normal energy balance in Sirt6BAC mice

To determine the effect of physiological overexpression of SIRT6 on energy homeostasis, we measured body weight and composition of Sirt6BAC mice, and Sirt6BAC; *Sirt6*$^{-/-}$ and wild-type controls either fed on a standard chow or on a HCD. In both feeding contexts, body weight, fat and lean mass were all similar between Sirt6BAC, Sirt6-BAC; *Sirt6*$^{-/-}$, and wild-type mice (Figure 3A–F). Together, these data demonstrate that physiological overexpression of SIRT6 neither affects energy homeostasis nor protects from, or predisposes to, developing diet-induced obesity.

3.3. Improved glucose homeostasis in Sirt6BAC mice

To determine the impact of physiological overexpression of SIRT6 on glucose homeostasis, we measured several parameters of glucose metabolism in Sirt6BAC mice and controls. Data shown in Figure 4A indicate that in standard chow and HCD feeding conditions, 16-week-old Sirt6BAC mice have reduced circulating glucose levels compared to controls indicating that these mice are protected from developing diet-induced T2DM. Of note, circulating glucose levels were not different between Sirt6BAC; *Sirt6*$^{-/-}$ and wild-type HCD-fed mice (Figure 4A) hence suggesting that the glycemia phenotype shown by Sirt6BAC mice is very likely the result of SIRT6 overexpression. The improved hyperglycemia concomitant with unchanged circulating fed (Figure 4B) and fasting (ng/mL; mean $\pm$ SEM: wild-types $=$ 0.53 $\pm$ 0.058; Sirt6BAC mice $=$ 0.51 $\pm$ 0.02; Sirt6BAC; *Sirt6*$^{-/-}$ mice $=$ 0.53 $\pm$ 0.060; n $=$ 9–15 per group, $p > 0.05$) insulin level hinted that Sirt6BAC mice may exhibit enhanced insulin sensitivity.

To further investigate the effects of SIRT6 overexpression on glucose metabolism, Sirt6BAC mice and controls were assessed for pyruvate and glucose tolerance. In both feeding conditions, Sirt6BAC mice exhibited reduced glycemic excursions during these tests (Figure 4C–F). Of note, the improved pyruvate and glucose handling were more pronounced in the HCD feeding context. These phenotypes of Sirt6BAC mice are very likely the result of SIRT6 overexpression because Sirt6BAC; *Sirt6*$^{-/-}$ mice displayed intermediate degrees of glycemic excursions during these tests (Figure 4C–F). Collectively, our data indicate that physiological overexpression of SIRT6 reduces glycemia and improves the glucose imbalance brought on HCD feeding.

A

Genotype	n	Mendelian Ratio Observed	Mendelian Ratio Expected	Observed / Expected
Wild-type	36	0.138	0.125	110.8%
Sirt6BAC	35	0.135	0.125	107.7%
Sirt6BAC; *Sirt6*$^{-/-}$	37	0.142	0.125	113.8%
Sirt6$^{-/-}$	9	0.035	0.125	27.7%

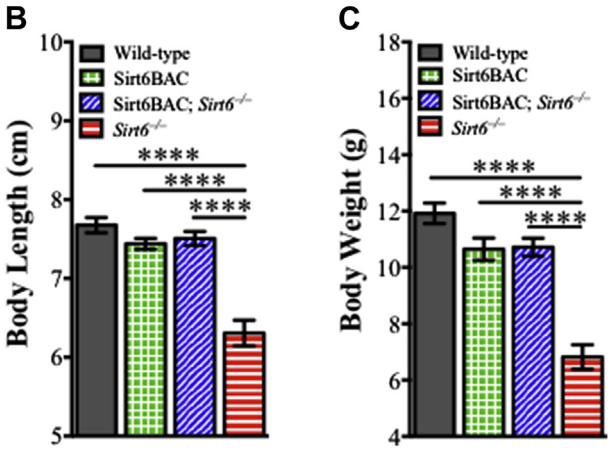

Figure 2: SIRT6 generated from Sirt6BAC is functionally competent. (A) Frequency of viable mice per genotype observed at 4-week of age, shown as a percentage of the expected Mendelian ratio. Mice were fed on a standard diet. **(B)** Body length (n $=$ 9–13 per group) and **(C)** body weight (n $=$ 9–13 per group) of 3-week-old mice fed on a standard diet. Values are mean $\pm$ S.E.M. Statistics were analyzed using one-way ANOVA with Tukey correction for multiple comparisons (****$p < 0.0001$).

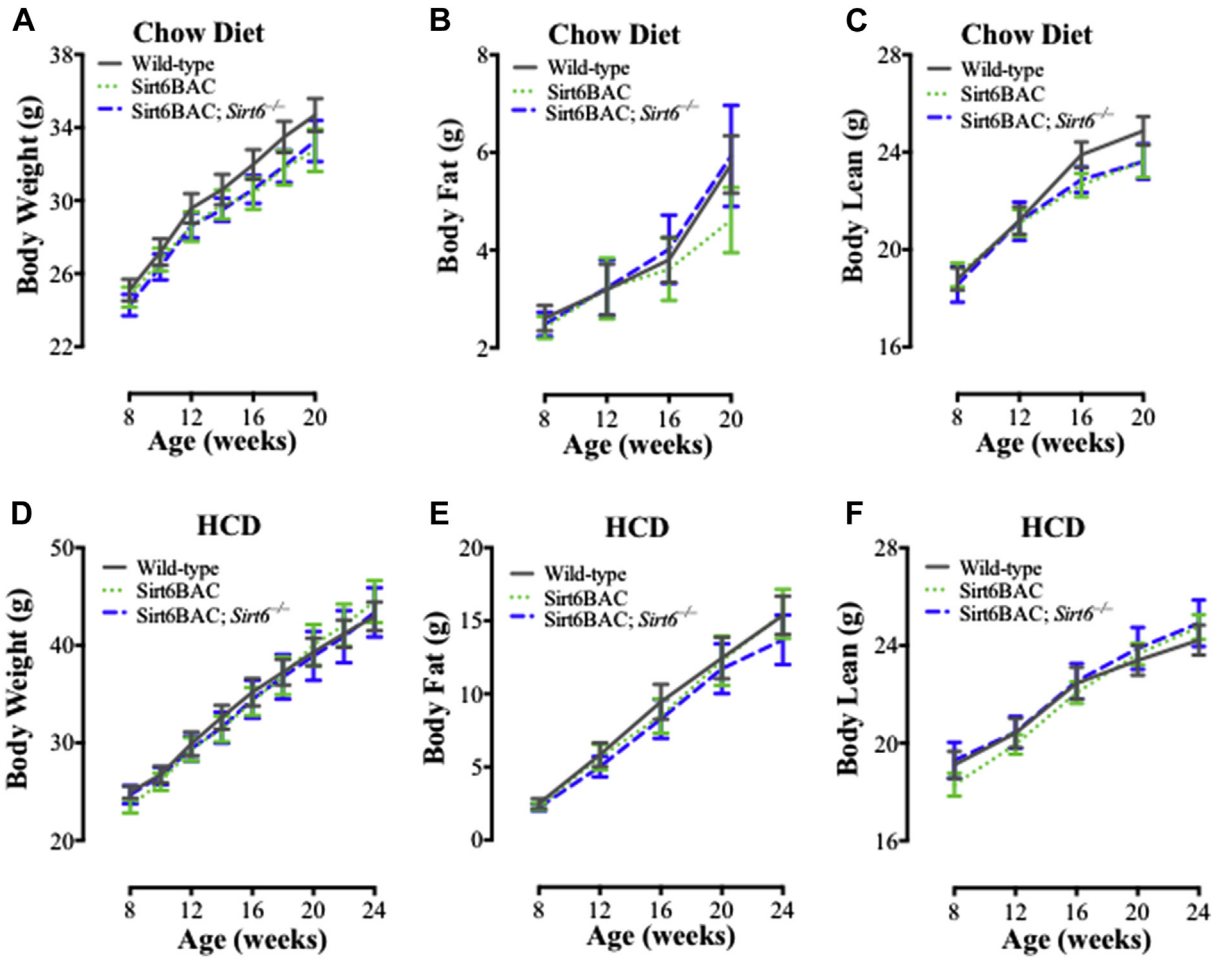

Figure 3: Sirt6BAC mice display normal body weight, fat mass and lean mass. **(A)** Body weight, **(B)** body fat weight, and **(C)** body lean weight of mice in chow diet context (n = 14–21 per group). **(D)** Body weight, **(E)** body fat weight, and **(F)** body lean weight of mice in HCD context (n = 10–14 per group). Values are mean ± S.E.M. Statistics were analyzed using a repeated measures two-way ANOVA with Tukey correction for multiple comparisons.

3.4. Enhanced insulin sensitivity in liver and skeletal muscle of Sirt6BAC mice

One possible explanation for the improved glucose homeostasis displayed by Sirt6BAC mice is enhanced insulin sensitivity. To directly test this hypothesis, hyperinsulinemic-euglycemic clamp assays were performed. During the clamp, insulin was infused to achieve hyperinsulinemia while D-glucose was infused through the same intravenous line at modulated rates to maintain euglycemia. In agreement with the aforementioned data suggestive of increased insulin sensitivity of Sirt6BAC mice, the glucose infusion rate (GIR) (Figure 5A) needed to clamp euglycemia (Figure 5B) was greatly increased in chow-fed Sirt6BAC mice compared to wild-type littermates. Basal endogenous glucose appearance rate (EndoR_a) (Figure 5C) and basal glucose disposal rate (R_d) (Figure 5D) were not significantly different between Sirt6BAC mice and wild-type controls. During the clamp however, the ability of insulin to suppress endogenous glucose production was significantly enhanced in Sirt6BAC mice compared to wild-type controls (Figure 5C). Insulin-stimulated glucose disposal was also increased in Sirt6BAC mice compared to wild-type controls (Figure 5D).

To determine the relative contributions of certain tissues to glucose disposal, the amounts of exogenously administered radiolabeled glucose analog 2[^{14}C]deoxyglucose were measured in several tissues. As shown in Figure 5E, insulin-stimulated glucose uptake was enhanced in Sirt6BAC mice in both gastrocnemius and soleus muscle, but not in brain, interscapular brown adipose, or epididymal adipose tissue. The whole-body glycolytic rate as percent of glucose disposal (Figure 5F) was not found to be significantly different between genotypes. Overall, these data demonstrate that insulin sensitivity is selectively augmented in liver and skeletal muscle of Sirt6BAC mice.

To independently assess *in vivo* insulin sensitivity, we used a well-established biochemical approach. The level of phosphorylated AKT (p-AKT) following a bolus of insulin has been used as measure of the ability of the hormone to activate its receptor [10]. Intraperitoneal insulin administration enhanced p-AKT/AKT ratio in gastrocnemius of Sirt6BAC, Sirt6BAC; *Sirt6*$^{-/-}$, and wild-type mice (Figure 6A,B). Of note, while the ability of insulin to induce phosphorylation of AKT was significantly increased in gastrocnemius of Sirt6BAC compared to wild-type mice (Figure 6A), it was not different between Sirt6BAC; *Sirt6*$^{-/-}$ and wild-type controls (Figure 6B). These results are in keeping with data shown in Figure 5E and further support the idea that SIRT6 overexpression drives enhanced insulin sensitivity in Sirt6BAC mice.

3.5. Increased spermidine content in blood of Sirt6BAC mice

To gather insights on potential mechanisms by which SIRT6 overexpression boosts insulin sensitivity we surveyed hepatic gene

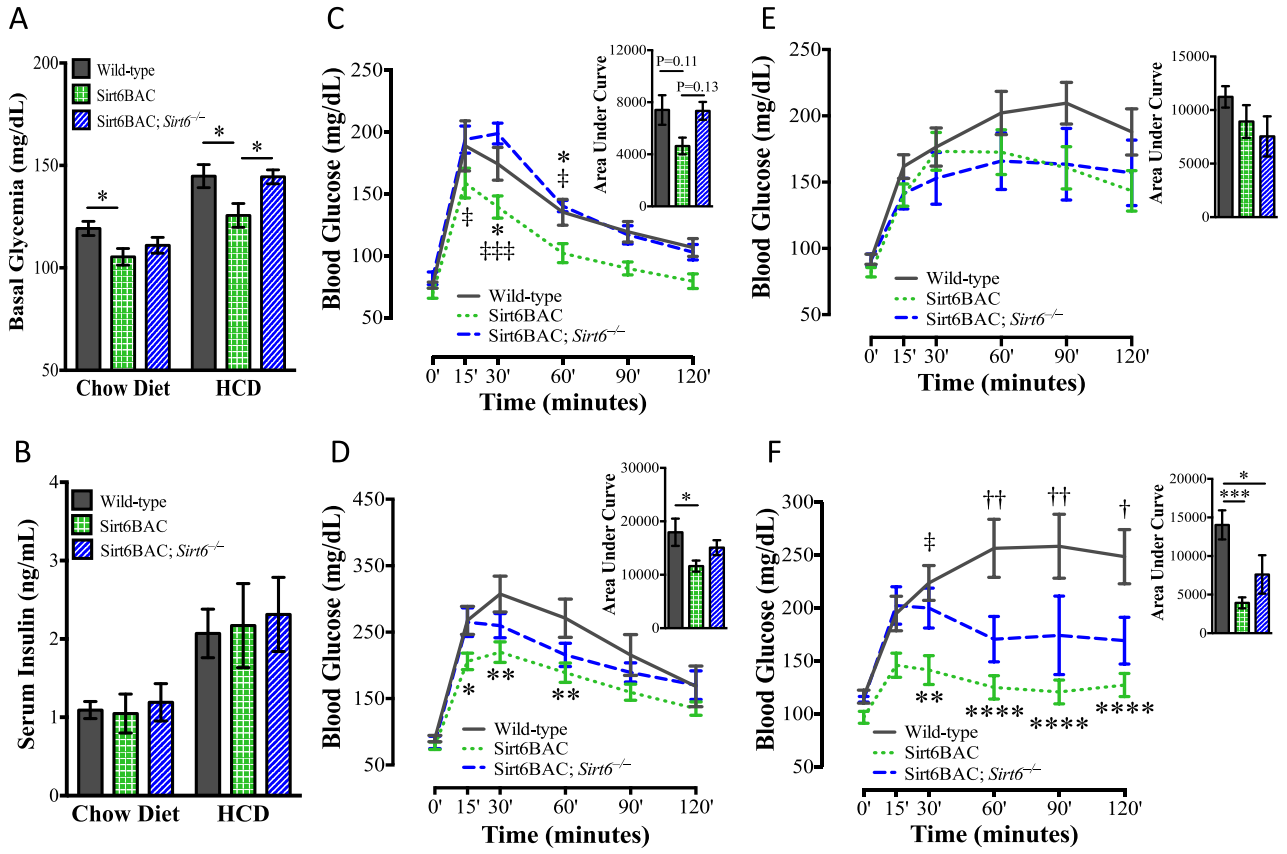

Figure 4: Sirt6BAC mice exhibit improved glucose homeostasis. **(A)** Basal (non-postprandial) glycemia of 16-week-old mice fed on a chow diet (n = 17–24 per group) and on a HCD (n = 9–12 per group). **(B)** Basal (non-postprandial) serum insulin levels of 16-week-old mice fed on a chow diet (n = 9–15 per group) and on a HCD (n = 8–10 per group). **(C)** Intraperitoneal glucose (1.5 g/kg bodyweight) tolerance test and area under curve of mice at 11–13 weeks of age in the chow diet context (n = 6–8 per group). **(D)** Intraperitoneal glucose (1.5 g/kg bodyweight) tolerance test and area under curve of mice at 18–20 weeks of age in the HCD context (n = 5–10 per group). **(E)** Intraperitoneal pyruvate (2 g/kg bodyweight) tolerance test and area under curve of mice at 25–26 weeks of age in the chow diet context (n = 5–8 per group). **(F)** Intraperitoneal pyruvate (2 g/kg bodyweight) tolerance test and area under curve of mice at 26–28 weeks of age in the HCD context (n = 5–10 per group). Values are mean ± S.E.M. Panel A, B and C-F (area under curve): Statistics were analyzed using one-way ANOVA with Tukey correction for multiple comparisons (*$p < 0.05$). Panel C–F: Statistics were analyzed using repeated measures two-way ANOVA with Tukey correction for multiple comparisons (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ Wild-type *vs.* Sirt6BAC), (†$p < 0.05$, ††$p < 0.01$ Wild-type vs. Sirt6BAC; *Sirt6*$^{-/-}$), (‡$p < 0.05$, ‡‡‡$p < 0.001$ Sirt6BAC *vs.* Sirt6BAC; *Sirt6*$^{-/-}$).

expression and whole-blood polyamine content by qPCR and RP-HPLC, respectively. Our results indicate increased mRNA content of the glycolytic enzymes ATP-dependent 6-phosphofructokinase (PFKL) and liver-type pyruvate kinase (LPK) in liver of Sirt6BAC mice compared to wild-types (Figure S2). Furthermore, our data indicate increased mRNA content of Elovl6, Acetyl-CoA carboxylase-b (ACCb), and Fatty acid transport protein 5 (FATP5) in liver of Sirt6BAC mice compared to wild-types (Figure S2). However, we found no difference in serum triglyceride level between 16 and 20-week-old Sirt6BAC and wild-type mice fed on a standard diet (ng/dL; mean ± SEM: wild-types = 146 ± 12; Sirt6BAC mice = 139 ± 16; serum was collected from mice that were fasted over-night, n = 6 per group, $p > 0.05$).

Due to important role of polyamines on energy metabolism [30], we also assessed their level and found that total polyamines and spermidine contents are significantly higher in blood of Sirt6BAC mice compared to wild-types (Figure 7A,B). Collectively, these results suggest that SIRT6 overexpression improves glucose/insulin homeostasis in part by enhancing circulating spermidine contents and hepatic glucose and lipid metabolism.

4. DISCUSSION

Due to the staggering number of people suffering from defects in energy and glucose homeostasis and the shortcomings of current therapies, there is a pressing need for developing better approaches for the treatment of obesity and T2DM [1]. Several genetic studies have recently provided support for the idea that activation of SIRT1 (one of three mammalian nuclear sirtuin members) leads to improved metabolic homeostasis in the context of hyper-caloric feeding [8–11,31–34]. However, the role of another nuclear-localized sirtuin, namely SIRT6, in metabolism is controversial. Indeed, *Sirt6* loss- and gain-of-function studies have brought about counterintuitive and outwardly incongruent results, rendering it difficult to determine whether activation or inhibition of SIRT6 would result in beneficial metabolic outcomes [17–19,21–23,35]. These apparently paradoxical findings may be the result of the following non-mutually exclusive possibilities: i) reduced glycemia and body adiposity displayed by *Sirt6* null mice may not be direct consequence of SIRT6 deficiency, but secondary effects due to other serious abnormalities (e.g.: colitis, lymphopenia, etc.) caused by this mutation [17], ii) reduced glycemia and body

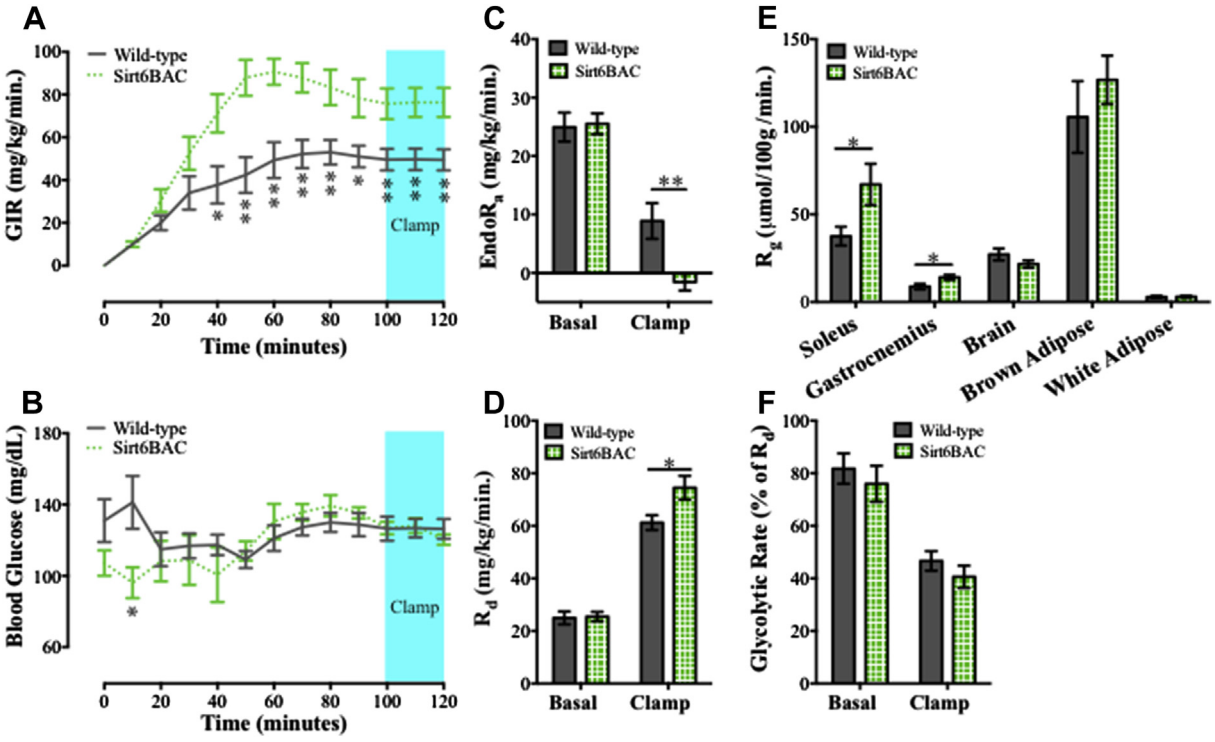

Figure 5: Sirt6BAC mice exhibit enhanced insulin sensitivity. **(A)** Glucose infusion rate (GIR) (mg glucose/kg bodyweight/minute) **(B)** Blood glucose (mg/dL) **(C)** Endogenous glucose appearance ($EndoR_a$) (mg/kg bodyweight/minute) **(D)** Glucose disposal (R_d) (mg/kg bodyweight/minute) **(E)** Tissue glucose uptake (R_g) (μmol/100 g tissue/minute) **(F)** Glycolytic rate (% of R_d). n = 6–8 per group. All mice were fed on a standard diet. Values are mean ± S.E.M. Panels A&B: Statistics were analyzed using repeated measures one-way ANOVA with Tukey correction for multiple comparisons (*p < 0.05, **p < 0.01). Panels C, D, E&F: Statistics were analyzed using unpaired two-tailed t-test (*p < 0.05, **p < 0.01).

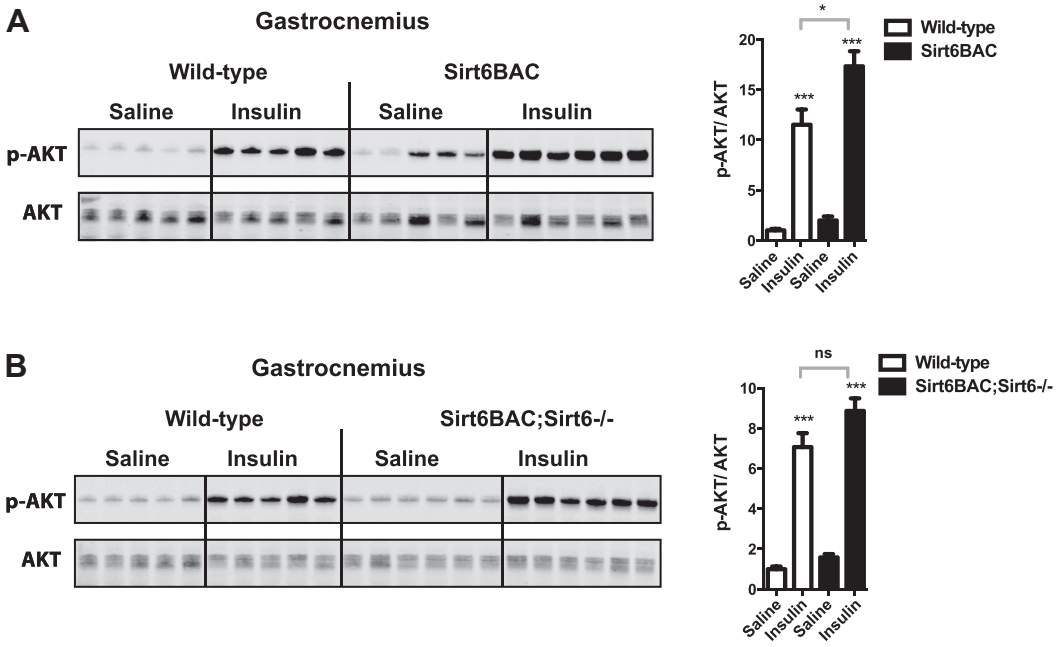

Figure 6: Sirt6BAC mice exhibit enhanced insulin-induced pAKT in gastrocnemius. Immunoblot and quantification of Akt (Thr^{308}) phosphorylation status relative to total AKT (AKT) in gastrocnemius muscle 10 min after an intraperitoneal bolus of insulin (5 U/kg) or saline in **(A)** wild-type *vs.* Sirt6BAC mice and **(B)** wild-type vs. Sirt6BAC; *Sirt6*$^{-/-}$ mice. All mice were fasted 5 h before insulin or saline injection. All mice were age-matched and fed on a standard diet. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test. *P < 0.05; ***P < 0.001.

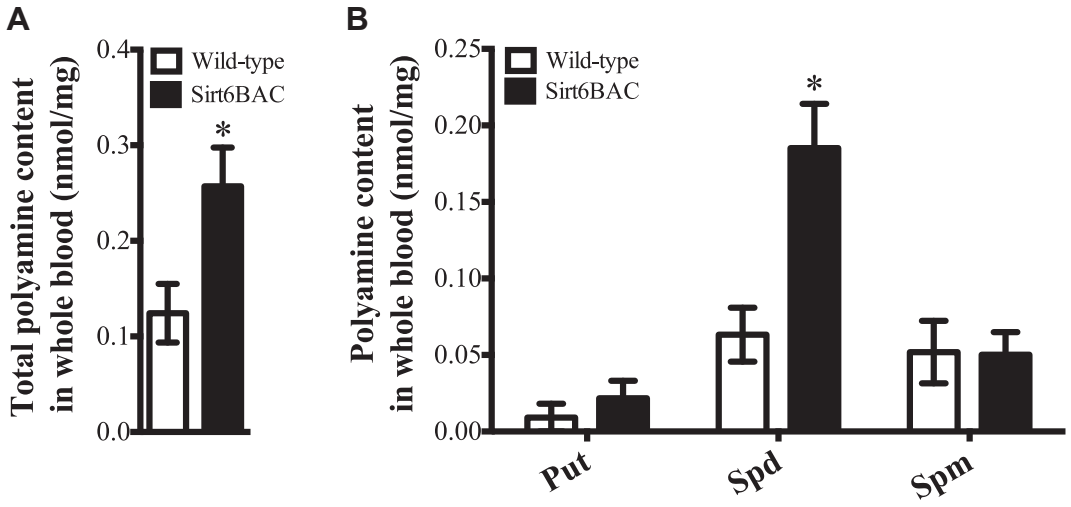

Figure 7: Sirt6BAC mice exhibit enhanced spermidine level in blood. (A) Total polyamine content and **(B)** polyamine profile of putrescine (Put), spermidine (Spd), and spermine (Spm) in whole blood of 2-month-old wild-type (n = 12) and Sirt6BAC (n = 7) mice fed on a standard diet. Error bars represent s.e.m. Statistical analyses were done using two-tailed unpaired Student's t test. *P < 0.05.

adiposity displayed by SIRT6-overexpressor mice may be due to inherent confounding effects of the unnatural chicken β*Actin* promoter/CMV enhancer-driven regulation of *Sirt6* expression in these mutants [22].

To gather insights about the effects of systemic and moderate activation of SIRT6, we genetically-engineered mice that eutopically and physiologically overexpress SIRT6. As the exogenously introduced *Sirt6* gene was under the control of its own natural promoter, SIRT6 overexpression in these Sirt6BAC mice mimicked SIRT6 expression in calorically restricted rodents (Figures 1 and S1) [29]. Importantly, the approach we used is strikingly different from the one Kanfi and colleagues employed. In fact, in the Kanfi and colleagues' model, the artificial chicken β*Actin* promoter/CMV enhancer drives *Sirt6* expression at non-physiological levels and probably in cell-types that would not normally express SIRT6 [22]. These shortcomings cast doubt on the interpretations of results gathered from this mutant. It is also important to note the limitations of our Sirt6BAC mouse model. The major caveat of this method is the presence of large genomic sequences flanking the mouse *Sirt6* gene (Figure 1A). While these large sequences are needed for *Sirt6* transcriptional fidelity, they also contain other known genes whose expression was also found to be increased in Sirt6BAC mice (Figure 1D). Hence, overexpression of these other genes could have contributed to the phenotypes displayed by Sirt6BAC mice. Also, BAC DNA construct insertion site(s) into the genome may have altered endogenous gene expression and hence caused phenotypes in Sirt6BAC mice. While the generation and study of another SIRT6 BAC line could be useful to address the latter this approach cannot address the former issue. In fact, the likelihood that two different BAC transgenic lines bear DNA construct cloned into the same genomic site(s) is virtually zero. Therefore, as expression of genes contained in the BAC likely depends on DNA construct insertion site(s) it is virtually impossible to assure equal expression of SIRT6 and also non-SIRT6 BAC-born products in different tissues of two different lines. Another approach would be to disrupt *Sirt6* sequences in BAC DNA construct and use this modified BAC DNA lacking the possibility to overexpress SIRT6 as a negative control line (hereafter referred to as Sirt6minusBAC mice). Again, while this approach is suitable to address potential insertion site effects, it cannot guarantee equal tissue-specific overexpression of BAC-born products between Sirt6minusBAC and Sirt6BAC mice. Hence, the Sirt6minusBAC mice cannot serve as controls to Sirt6BAC mice. On the other hand, BAC DNA insertion site is identical between Sirt6BAC; *Sirt6*$^{-/-}$ and Sirt6BAC mice. The only appreciable difference between these two lines is SIRT6 expression which in Sirt6BAC mice is enhanced while in Sirt6BAC; *Sirt6*$^{-/-}$ mice is almost normal (Figures 1B,C,D and S1). Hence, if a given phenotype were to be observed in Sirt6BAC mice and displayed at an intermediate degree or not displayed by Sirt6BAC; *Sirt6*$^{-/-}$ mice, then the given phenotype is very likely the consequence of *Sirt6* overexpression. Thus, to dissociate potential effects of BAC DNA insertion site and/or overexpression of non-SIRT6 BAC-born products to SIRT6 overexpression we used Sirt6BAC; *Sirt6*$^{-/-}$ mice as controls. Our study indicates that SIRT6 overexpression underlies the improved glucose/insulin profiles displayed by Sirt6BAC mice. This conclusion mainly rests on the following five observations: i) glycemia is significantly decreased in Sirt6BAC compared to wild-type HCD-fed mice; however, this phenotype is not displayed by Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 4A), ii) following a bolus of glucose, the glycemic level over time is significantly decreased in Sirt6BAC compared to wild-type mice; however, this phenotype is not displayed by Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 4C), iii) following a bolus of glucose, the glycemic level over time is significantly decreased in Sirt6BAC compared to wild-type HCD-fed mice; however, this phenotype is displayed at an intermediate degree by Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 4D); iv) following a bolus of pyruvate, the glycemic level over time is significantly decreased in Sirt6BAC compared to wild-type HCD-fed mice; however, this phenotype is displayed at an intermediate degree by Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 4F), v) the ability of insulin to induce phosphorylation of AKT is significantly increased in gastrocnemius of Sirt6BAC mice compared to wild-type controls; however, this phenotype is not displayed by Sirt6BAC; *Sirt6*$^{-/-}$ mice (Figure 6).

It is worth noting that some of our data are somewhat unexpected. For example, data shown in Figure 5D,E indicate that insulin-stimulated glucose disposal is enhanced in Sirt6BAC mice relative to wild-type controls. This is unexpected considering that loss of SIRT6 causes

enhanced glucose transporter-1 and -4 (GLUT1 and GLUT4) membrane localization and cell-autonomous glucose uptake [18,36]. The fact that our Sirt6BAC mice do not display changes in body adiposity (Figure 3) is not in keeping with Kanfi and colleagues' results as their *Sirt6* overexpressor model displays changes in this parameter [22]. While future studies may be needed to explain these differences, we suggest that these are the results of the different level and/or tissue distribution of *Sirt6* overexpression between our and Kanfi and colleagues' model [22].

In this study, we also attempted to shed light on potential mechanisms underlying the beneficial effect of SIRT6 on glucose homeostasis. Our results indicate that these pathways include increased circulating spermidine level and enhanced insulin sensitivity in liver and skeletal muscle (Figures 5–7). Of note, the improved hepatic insulin sensitivity of Sirt6BAC mice is in keeping with data indicating that SIRT6 inhibits the activity of the gluconeogenic co-transcription factor peroxisome proliferator-activated receptor-γ coactivator 1-α and with the fact that SIRT6 overexpression in liver improves hyperglycemia in diabetic mice [23]. SIRT6 has recently been shown to interact with proteins of the circadian core complex (e.g.: CLOCK and BMAL1) and to regulate expression of several clock-controlled genes [35]. Considering the important role of the circadian clock on metabolic homeostasis [37], it is formally possible that changes in circadian clock function in liver and skeletal muscle underlie the improved insulin sensitivity shown by Sirt6BAC mice. To fully determine the role of SIRT6 gain-of-function in specific tissues, future studies in tissue-restricted SIRT6 overexpressor mice are warranted.

5. CONCLUSIONS

In summary, our data indicate that moderate, physiological overexpression of SIRT6 enhances insulin sensitivity in skeletal muscle and liver, engendering protective actions against diet-induced T2DM. Hence, the present study provides support for the anti-T2DM effect of SIRT6 and may pave the way for the development of SIRT6 agonists aimed at treating T2DM.

DISCLOSURE SUMMARY

Authors have nothing to declare.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.09.003.

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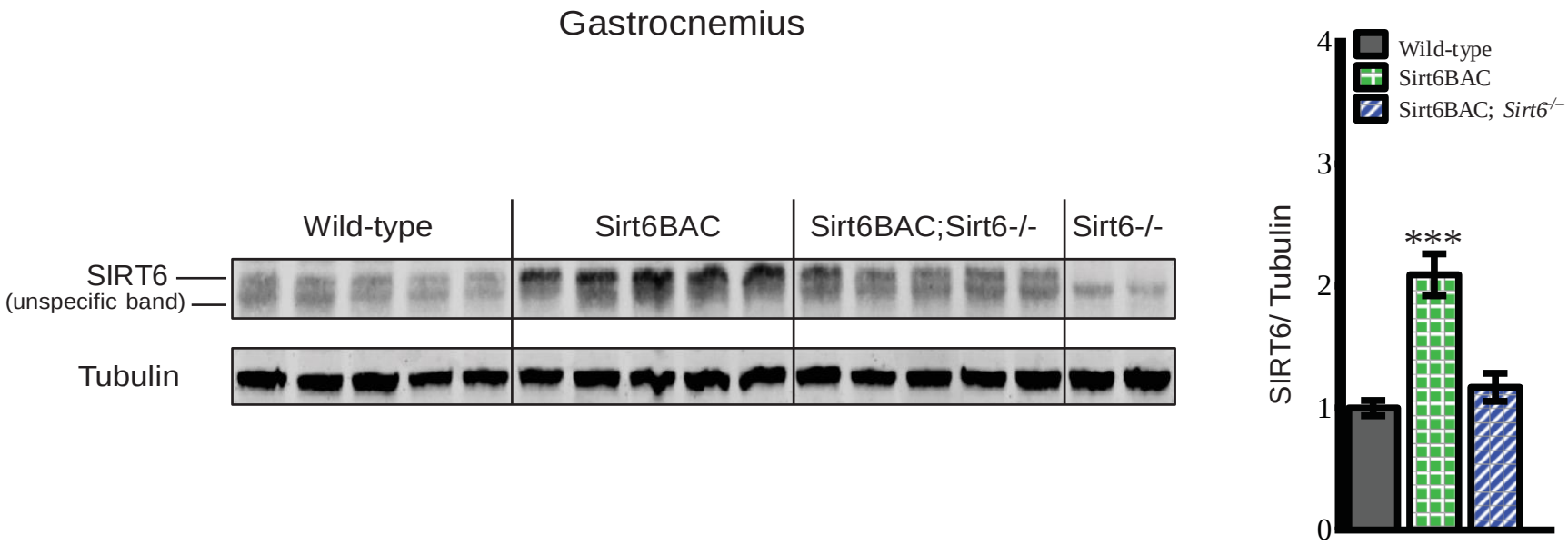
Supplemental Figure 1



Supplemental Figure 2

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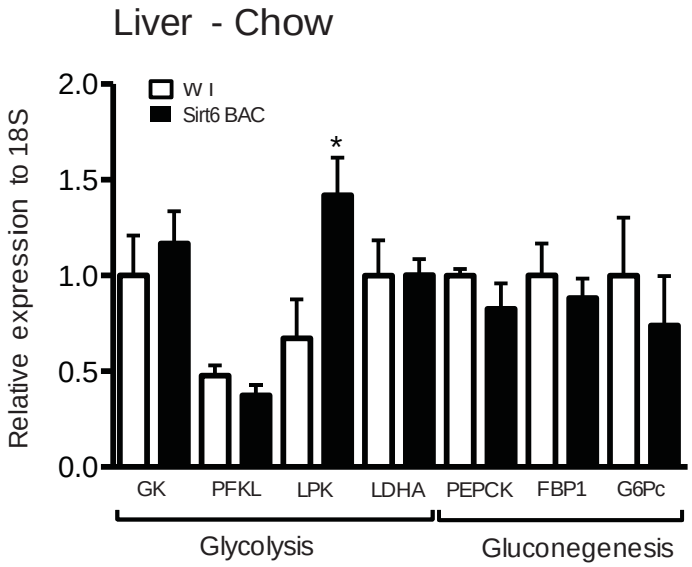

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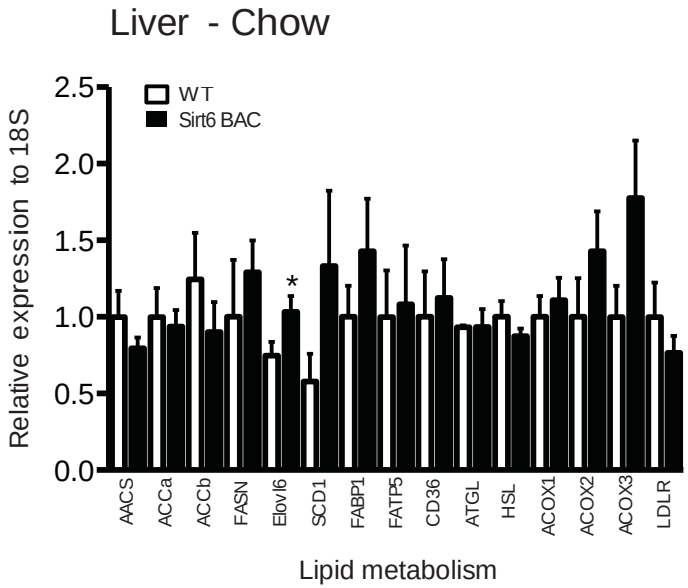

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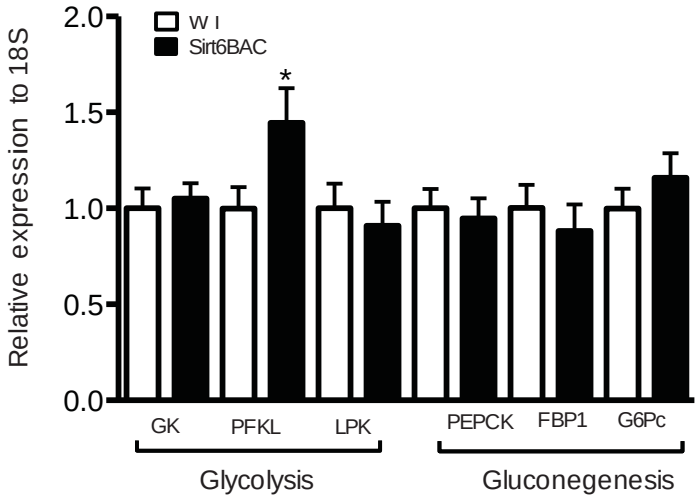

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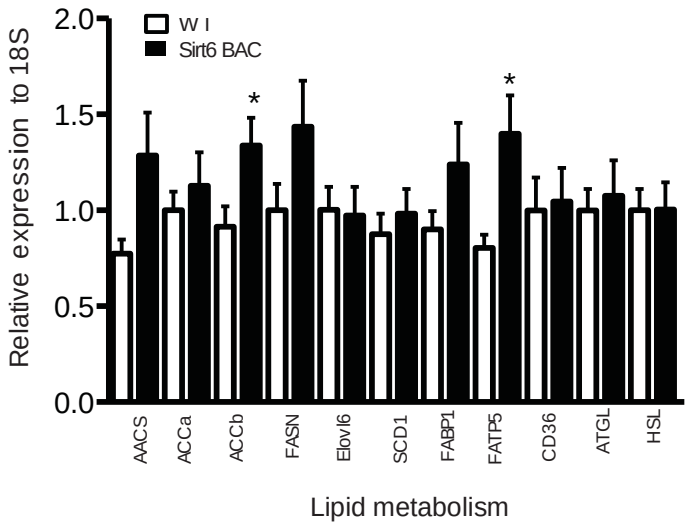


2. SIRT6 Suppresses Cancer Stem-like Capacity in Tumors with PI3K Activation Independently of Its Deacetylase Activity

Here, based in *in vitro* and *in vivo* assessments, we provide evidences that physiological overexpression of SIRT6 suppresses stemness and rearranges metabolism of several human cancer cell lines and a mammary cancer mouse model. Interestingly, this effect was pronounced in a context where PI3K signalling is constitutively active and was independent of SIRT6 deacetylase activity.

Author contributions

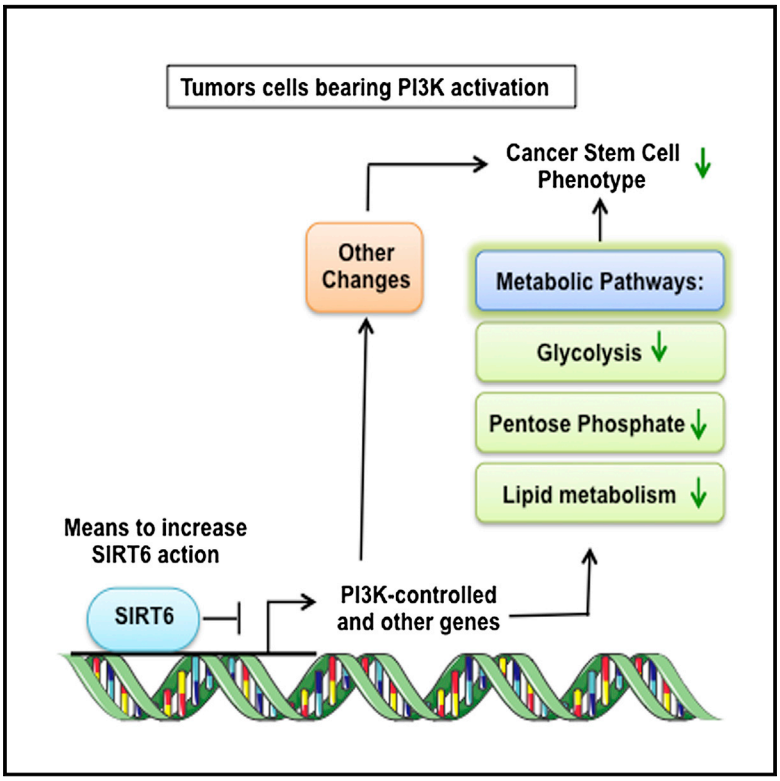
I was direct involved in the experiments that generated Figure 1, Figure 2 A-F, Figure 3 D, Figure 4, Figure S1, Figure S2 A-D and G, Figure S3 C and Figure S4 (with exception to Figure S4 E that was generated in collaboration with Prof. Dr. Denis Martinvalet).

Cell Reports

Report

SIRT6 Suppresses Cancer Stem-like Capacity in Tumors with PI3K Activation Independently of Its Deacetylase Activity

Graphical Abstract



Highlights

- Enhanced SIRT6 hinders stemness of human cancer cells with PI3K activation
- Enhanced SIRT6 rearranges metabolism of cancer cells with PI3K activation
- Enhanced SIRT6 reduces grade and progression of murine tumors with PI3K activation
- Anti-cancer-stemness action is independent of SIRT6 histone deacetylase activity

Authors

Rafael M. Ioris, Mirco Galié, Giorgio Ramadori, ..., Raul Mostoslavsky, Pierre Baldi, Roberto Coppari

Correspondence

roberto.coppari@unige.ch

In Brief

Ioris et al. provide in vitro and in vivo evidence that enhanced SIRT6 suppresses cancer progression and stemness in the context of constitutively active PI3K signaling. This effect is, at least in part, through suppression of the PI3K pathway at the transcriptional level and independent of SIRT6's histone deacetylase activity.

Accession Numbers

GSE93837

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Report

SIRT6 Suppresses Cancer Stem-like Capacity in Tumors with PI3K Activation Independently of Its Deacetylase Activity

Rafael M. Ioris,[1,2,11] Mirco Galié,[1,3,11] Giorgio Ramadori,[1,2,11] Jason G. Anderson,[4] Anne Charollais,[1,2] Georgia Konstantinidou,[5] Xavier Brenachot,[1,2] Ebru Aras,[1,2] Algera Goga,[1,2] Nicholas Ceglia,[6,7] Carlos Sebastián,[8,9] Denis Martinvalet,[1] Raul Mostoslavsky,[8,9,10] Pierre Baldi,[6,7] and Roberto Coppari[1,2,12,*]

[1]Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, 1211 Geneva 4, Switzerland
[2]Diabetes Center of the Faculty of Medicine, University of Geneva, 1211 Geneva 4, Switzerland
[3]Department of Neurosciences, Biomedicine and Movement, University of Verona, Verona 37134, Italy
[4]Department of Internal Medicine, Division of Hypothalamic Research, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[5]Institute of Pharmacology, University of Bern, 3010 Bern, Switzerland
[6]Department of Computer Science University of California Irvine, Irvine, CA 92697, USA
[7]Institute for Genomics and Bioinformatics, University of California Irvine, Irvine, CA 92697, USA
[8]The Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA 02114, USA
[9]The MGH Center for Regenerative Medicine, Harvard Medical School, Boston, MA 02114, USA
[10]The Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA
[11]Co-first author
[12]Lead Contact
*Correspondence: roberto.coppari@unige.ch
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SUMMARY

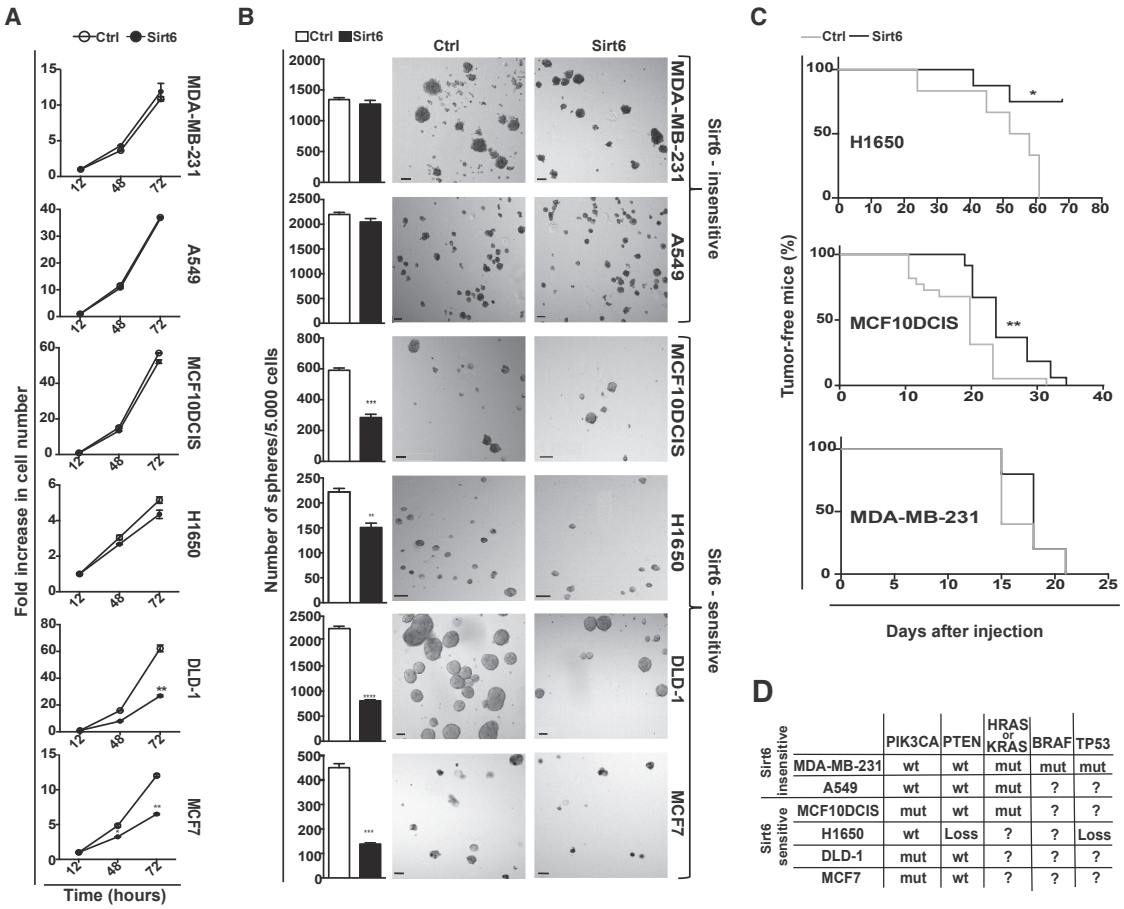
Cancer stem cells (CSCs) have high tumorigenic capacity. Here, we show that stem-like traits of specific human cancer cells are reduced by overexpression of the histone deacetylase sirtuin 6 (SIRT6). SIRT6-sensitive cancer cells bear mutations that activate phosphatidylinositol-3-kinase (PI3K) signaling, and overexpression of SIRT6 reduces growth, progression, and grade of breast cancer in a mouse model with PI3K activation. Tumor metabolomic and transcriptomic analyses reveal that SIRT6 overexpression dampens PI3K signaling and stem-like characteristics and causes metabolic rearrangements in this cancer model. Ablation of a PI3K activating mutation in otherwise isogenic cancer cells is sufficient to convert SIRT6-sensitive into SIRT6-insensitive cells. SIRT6 overexpression suppresses PI3K signaling at the transcriptional level and antagonizes tumor sphere formation independent of its histone deacetylase activity. Our data identify SIRT6 as a putative molecular target that hinders stemness of tumors with PI3K activation.

INTRODUCTION

Cancer kills approximately 8 million people annually (World Health Organization fact sheet number 297). Although anti-cancer therapy is rapidly improving, further therapeutic development is urgently needed. The idea that subpopulations of cells within the tumor mass, cancer stem cells (CSCs) (or tumor-initiating cells), have high tumorigenic and self-renewal ability was proposed more than 20 years ago (Lapidot et al., 1994; Pattabiraman and Weinberg, 2014; Wang and Dick, 2005). Since then, CSCs have been identified in several human tumors (e.g., leukemia, breast, brain, prostate, colon, and pancreatic cancers), and the notion of eradicating cancer by eroding the CSC pool has started to show potential in humans (Lapidot et al., 1994; Pattabiraman and Weinberg, 2014; Prost et al., 2015). Despite the fact that significant efforts aimed at identifying molecular targets to hinder cancer stemness have been made, these anti-CSCs targets remain poorly understood.

The roles of epigenetic changes (e.g., histone modifications) on cancer behavior and stemness are appreciated. For example, it has recently been shown that protein kinase A indirectly affects histone methylation and expression of epithelial genes, an effect that hinders CSC activity and promotes tumor differentiation, a feature associated with better prognosis (Pattabiraman et al., 2016). The histone deacetylase SIRT6 has been suggested to affect tumor behavior as (1) *Sirt6* loss of function facilitates progression of cancer in mice (Kugel et al., 2016; Sebastián et al., 2012), (2) *Sirt6* loss-of-function mutations have been found in human cancers (Kugel et al., 2015), and (3) SIRT6 expression in tumor lesions positively correlates with survival of cancer patients (Sebastián et al., 2012; Thirumurthi et al., 2014). Although these loss-of-function results suggest that SIRT6 is a tumor suppressor, others have shown opposite outcomes (Ming et al., 2014). Whether enhanced SIRT6 function obstructs tumor progression and/or CSCs is unclear.

In this study, we assessed the outcomes of enhanced SIRT6 action in different human and murine cancers. Surprisingly, we found that mutations leading to PI3K activation predict



		PIK3CA	PTEN	HRAS or KRAS	BRAF	TP53
Sirt6 insensitive	MDA-MB-231	wt	wt	mut	mut	mut
	A549	wt	wt	mut	?	?
Sirt6 sensitive	MCF10DCIS	mut	wt	mut	?	?
	H1650	wt	Loss	?	?	Loss
	DLD-1	mut	wt	?	?	?
	MCF7	mut	wt	?	?	?

Figure 1. SIRT6 Thwarts Stemness and Tumorigenic Capacity of Human Cancer Cells with PI3K Activation

(A and B) Proliferation (A) and tumorsphere-forming capacity (B) of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6).

(C) Kaplan-Meier curves comparing percentage of tumor-free mice at different times after subcutaneous injection of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6).

(D) Known mutational status of indicated cancer cells.

In (A) and (B), *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed unpaired Student's t test). Error bars represent SEM. In (C), *p < 0.05, **p < 0.01 (long-rank test). See also Figure S1.

responsiveness to the anti-cancer-stemness action of SIRT6 overexpression.

RESULTS

Differential Responsiveness of Human Cancer Cells to Enhanced SIRT6 Action

To determine the effect of enhanced SIRT6 expression on tumor biology, six different cancer cells obtained from breast (MDA-MB-231, MCF7, and MCF10DCIS), lung (H1650 and A549), and colorectal (DLD-1) human tumors were transduced with either a control vector or a vector expressing SIRT6. As expected, the latter showed increased SIRT6 protein content compared to controls (Figure S1A). Surprisingly only DLD-1 and MCF7 cells displayed reduced proliferation upon SIRT6 overexpression (Figure 1A). Because SIRT6 overexpression has been shown to cause apoptosis in cancer cells (including MDA-MB-231 cells) (Van Meter et al., 2011), we investigated whether changes in apoptosis could underlie the anti-proliferative action of SIRT6 overexpression. Although enhanced SIRT6 expression caused variegate effects on the cell-cycle phases, it was not accompanied by induction of apoptosis because (1) the percentage of the $SubG_0$ population and (2) the level of apoptosis markers cleaved caspase 3 and PARP1 were not significantly different between cells overexpressing SIRT6 and their controls (Figures S1B and S1C). We suggest that the discrepancy between our results and the ones reported by Van Meter and colleagues is due to the different approaches (transient transfection [Van Meter et al., 2011] versus stable transfection [this study]) used to induce SIRT6 overexpression.

When the ability to form tumorsphere in three-dimensional cultures, which is an established readout of CSCs (Dontu et al., 2003; Ponti et al., 2005; Rasheed et al., 2010), was assessed, we found that SIRT6 overexpression drastically decreases tumorsphere-forming capacity of MCF10DCIS, MCF7, H1650,

and DLD-1 cells, while it causes no changes in MDA-MB-231 or A549 cells (Figure 1B). To independently assess cancer stemness, the size of the cell population with high aldehyde dehydrogenase (ALDH) activity was measured (Carpentino et al., 2009; Cheung et al., 2007; Ginestier et al., 2007). This parameter was found to be reduced in H1650 and MCF10DCIS cells (Figure S1D). To directly test whether cancer cells with high ALDH activity ($ALDH^{high}$) represented in Figure S1D are enriched in CSCs, we compared their tumorsphere-forming capacity with the one of cells with low ALDH activity ($ALDH^{low}$). In agreement with previous reports (Ginestier et al., 2007; Liu et al., 2011; Yang et al., 2010), $ALDH^{high}$ cells gave rise to a higher number of tumorspheres compared to $ALDH^{low}$ cells (Figure S1E), hence supporting that $ALDH^{high}$ cells are enriched in CSCs. Collectively, our data indicate that cancer cells could be categorized into two groups: SIRT6-sensitive cells, which show significant reduction in their stem-like trait (MCF10DCIS, MCF7, H1650, and DLD-1) and SIRT6-insensitive cells (MDA-MB-231 and A549) whose stem-like trait is unaffected by SIRT6 overexpression.

Reduced number of CSCs should lead to reduced tumorigenic capacity in vivo. To test this possibility, SIRT6-sensitive (H1650 and MCF10DCIS) and SIRT6-insensitive (MDA-MB-231) cells were injected into the flank of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and their growth was monitored over time. Results shown in Figure 1C demonstrate that while SIRT6 overexpression does not affect appearance of SIRT6-insensitive MDA-MB-231 xenografts it significantly delays this parameter of SIRT6-sensitive H1650 and MCF10DCIS xenografts. Hence, our data suggest that enhanced SIRT6 expression hinders stemness and tumorigenic capacity of specific human cancer cells.

By surveying the mutations characterizing SIRT6-sensitive and -insensitive cells, we found that SIRT6-sensitive cells bear mutations in genes regulating PI3K signaling while SIRT6-insensitive cells do not. Specifically, MCF10DCIS and MCF7 and DLD-1 cells bear an activating mutation (H1047R and E545K, respectively) in *PIK3CA* gene (Kalaany and Sabatini, 2009; Samuels et al., 2005; Su et al., 2015). Also, H1650 cells are characterized by *PTEN* (phosphate and tensin homolog deleted on chromosome 10) loss (Sos et al., 2009) (Figure 1D). On the other hand, MDA-MB-231 and A549 cells are not known to have PI3K activating mutations (Kalaany and Sabatini, 2009; Ramadori et al., 2015) (Figure 1D). In keeping with their genetic types, phosphorylation status of serine 473 of AKT (P-S473-AKT), which is an established marker of PI3K signaling, was enhanced in SIRT6-sensitive cells; yet, SIRT6 overexpression did not affect, or only marginally affected (e.g., DLD-1 cells), AKT phosphorylation suggesting that it does not influence PI3K signaling at the AKT level (Figure S1A). In summary, our data indicate that the anti-tumor action of SIRT6 overexpression is favored in the context of PI3K activation.

Enhanced SIRT6 Hinders Progression of Breast Cancer with PI3K Activation in Mice

Suppression of the CSCs pool is predicted to hinder progression toward high-grade lesions and promote tumor differentiation, a characteristic associated with better prognosis (Pattabiraman et al., 2016). The transgenic mouse expressing polyomavirus middle T oncogene (PyMT) under the mouse mammary tumor virus promoter is an established animal model mimicking progression of human breast cancers originating from hyperplastic lesions to high-grade carcinomas; also, these tumors display PI3K activation (Guy et al., 1992; Lin et al., 2003) (Figures S2A and S2B). Thus, to assess whether enhanced SIRT6 affects stemness and progression of a breast tumor with PI3K activation we crossed genetically engineered mice overexpressing functionally competent SIRT6 protein (Sirt6BAC mice) (Anderson et al., 2015) to PyMT mice. Tumor lesions of mice carrying both PyMT and Sirt6BAC alleles (PyMT/Sirt6 mice) were assessed for SIRT6 expression. Data shown in Figures 2A and 2B indicate that tumors from PyMT/Sirt6 mice have enhanced SIRT6 expression compared to their controls bearing only the PyMT allele. Of note, Sirt6BAC allele had no effect on expression of the oncogene driving tumorigenesis in this cancer model as PyMT mRNA level is similar between groups (Figure 2C). Due to its histone deacetylase activity, SIRT6 overexpression is expected to dampen expression of its target genes. Indeed, gene set enrichment analysis (Subramanian et al., 2005) of whole-genome microarray data showed that mRNA levels of several SIRT6's target genes (Kawahara et al., 2011) are reduced in tumors of PyMT/Sirt6 mice compared to controls (Figure 2D). Noteworthy, a number of mRNA changes identified by microarray assay were confirmed by real-time qPCR analysis (Figures S2C and S2D). Collectively, these results demonstrate that the Sirt6BAC allele brings about enhanced SIRT6 expression and activity in PyMT-driven breast tumors in mice.

To test the consequence of increased SIRT6 function on this cancer model, we analyzed several tumor parameters. PyMT/Sirt6 mice displayed a delay in tumor appearance (Figure S2E). At 12 weeks of age, tumor volume, weight, and number were all found to be reduced in PyMT/Sirt6 compared to controls (Figure 2E). Tumor growth rate, assessed by tumor diameter evolution after its detection, was also found to be significantly reduced in PyMT/Sirt6 mice (Figure 2F). To better understand this anti-tumor action of SIRT6 overexpression, we performed histological analyses. In keeping with our in vitro results shown in Figures S1B and S1C, TUNEL assay indicated that altered apoptotic rate is unlikely to be involved because the portion of TUNEL-positive cells in tumor samples was similar between groups (Figure S2F). Notably, while the percentage of necrotic, hyperplastic, or adenomatous-like area (low-grade tumor) was unchanged, the carcinomatous area, which represents the more aggressive/high-grade tumor lesion, was reduced nearly by a factor of two in tumors of PyMT/Sirt6 mice compared to controls (Figure 2G). These results indicate that enhanced SIRT6 expression/activity keeps this breast cancer model more differentiated, a characteristic associated with better prognosis (Pattabiraman et al., 2016). In line with this notion, several genes typically expressed by differentiated mammary gland epithelia were found to be overexpressed in tumors of PyMT/Sirt6 mice compared to controls (Figures 2H and S2G). Together, our data demonstrate that enhanced SIRT6 function inhibits growth and promotes differentiation of a breast cancer model with PI3K activation.

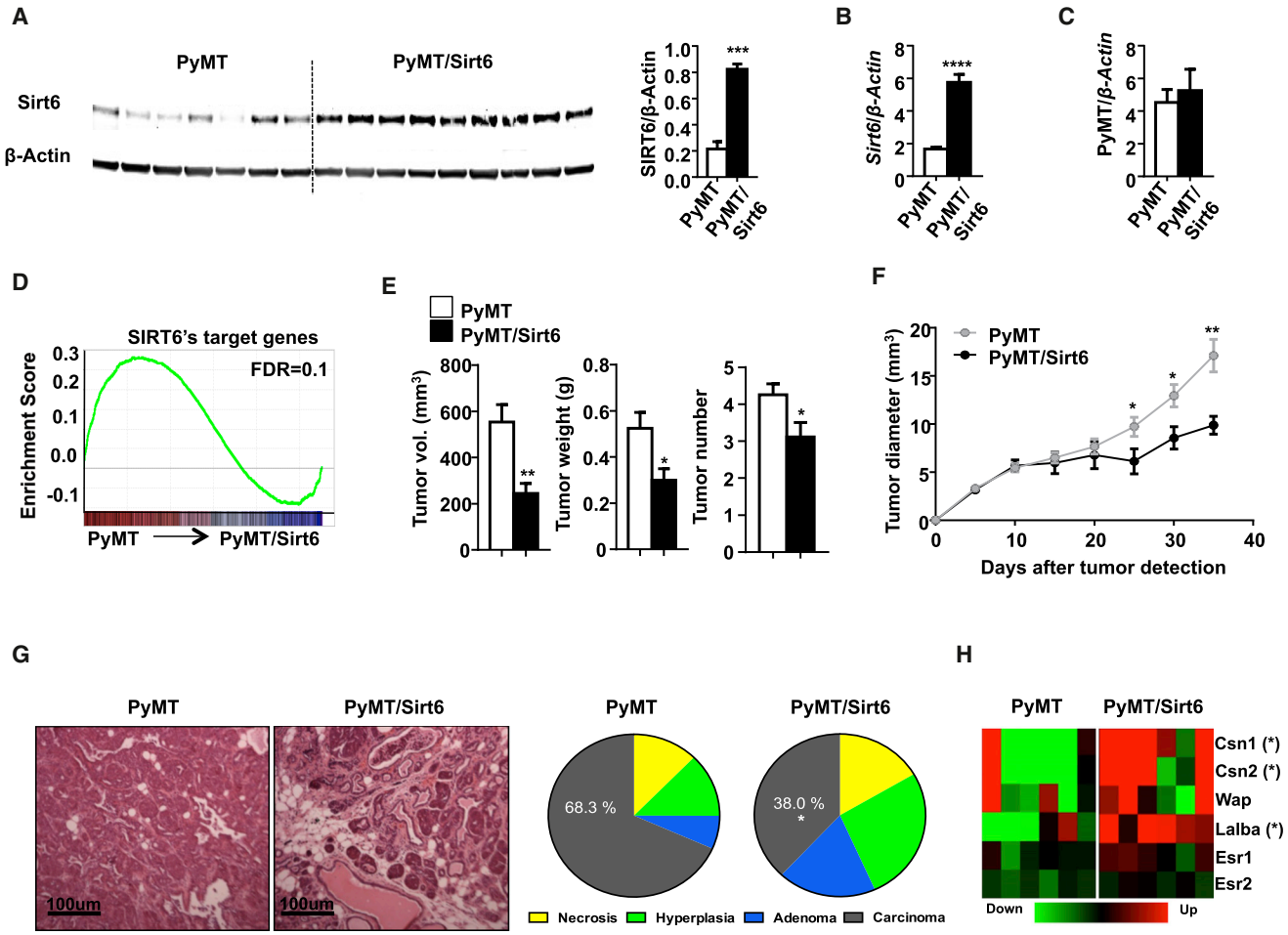


Figure 2. SIRT6 Overexpression Hinders Progression of PyMT-Driven Breast Cancer in Mice

(A) Immunoblot image and quantification of SIRT6 level normalized to β-Actin content in tumors of 12- to 14-week-old PyMT and PyMT/Sirt6 mice.

(B and C) mRNA levels of *Sirt6* (B) and of PyMT (C) normalized to *β-Actin* mRNA content in tumors of 12- to 14-week-old PyMT (n = 7) and PyMT/Sirt6 (n = 8) mice.

(D) Gene set enrichment analysis (GSEA) plot showing the enrichment score (ES) of SIRT6's target genes as indicated in table S2 of Kawahara and colleagues (Kawahara et al., 2011). Gene microarray was performed using RNA extracted from tumors of 12- to 14-week-old PyMT (n = 6) and PyMT/Sirt6 (n = 6) mice.

(E) Tumor volume, weight, and number per mouse in 12-week-old PyMT (n = 18) and PyMT/Sirt6 (n = 18) mice.

(F) Diameter evolution over time of tumors in PyMT and PyMT/Sirt6 mice.

(G) Representative images of tumors stained with H&E (scale bar, 100 μm) and percentage of area of necrosis, adenoma, hyperplasia, and carcinoma in similar-size tumors from PyMT and PyMT/Sirt6 mice (n = 9–10 per group). Scoring of necrosis, adenoma, hyperplasia, and carcinoma was performed on whole tumor area of H&E-stained tissues.

(H) Expression profile of normal mammary epithelial markers using microarray data as in (D).

In (A)–(H), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-tailed unpaired Student's t test). Error bars represent SEM. In (D), FDR, false discovery rate q value. See also Figure S2.

Enhanced SIRT6 Rearranges Metabolism and Suppresses PI3K Signaling and Stem-like Traits in Breast Tumors

To understand the mechanisms underlying the anti-tumor action of SIRT6 overexpression, we performed metabolomic and transcriptomic analyses. By comparing our transcriptomic data with 246 publicly available microarray datasets including embryonic stem cells (ESCs), adult stem cells (ASCs), induced pluripotent stem (iPS) cells, and terminally differentiated tissues (TDTs) (Barger et al., 2008; Mikkelsen et al., 2007, 2008; Sampath et al., 2008; Seale et al., 2007; Thorrez et al., 2008; Ulloa-Montoya et al., 2007), we found that genes downregulated in tumors of PyMT/Sirt6 mice (Figure S3A; Table S1) are enriched in stem cells (ESC, iPS cells, and ASCs) (Figure 3A). Also, we analyzed the enrichment of genes on microarray datasets of human breast cancers (van de Vijver et al., 2002). These samples were classified in normal-like, basal-like, HER2, Claudin-low, and luminal subtypes according to previous reports (Sorlie et al., 2003). Our results indicated that the genes downregulated in tumors of PyMT/Sirt6 mice are enriched in basal-like/Claudin-low tumors (Figure 3B), which represent the most stem-like/aggressive breast cancer types (Ben-Porath et al., 2008; Sorlie et al., 2003). To independently

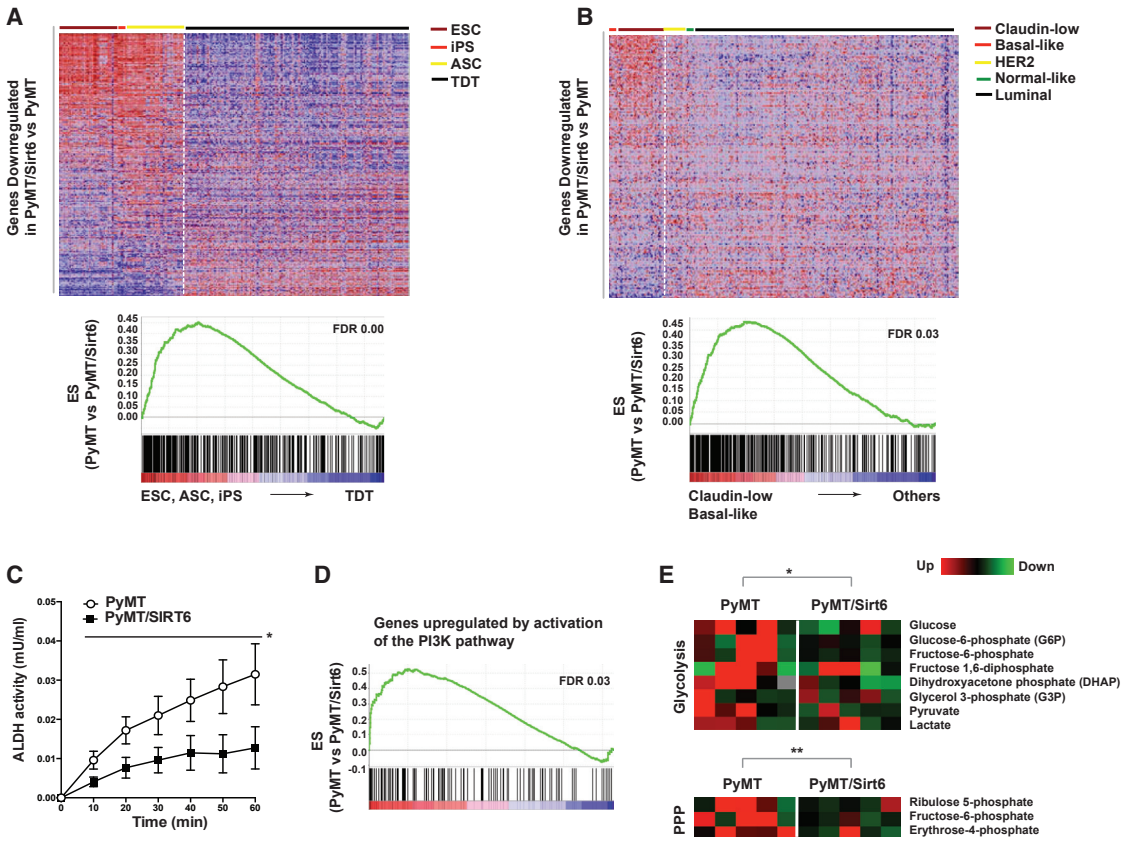


Figure 3. SIRT6 Hinders Stemness of PyMT-Driven Breast Tumors in Mice

(A and B) Enrichment of genes downregulated in PyMT/Sirt6 compared to PyMT tumors were tested in a cohort of public available whole-genome microarrays.

(C) ALDH activity was measured in tumors from 12- to 14-week-old PyMT (n = 6) and PyMT/Sirt6 (n = 6) mice.

(D) Gene set enrichment analysis (GSEA) plot showing the enrichment score (ES) of PI3K's target genes. Gene microarray was performed using RNA extracted from tumors of 12- to 14-week-old PyMT (n = 6) and PyMT/Sirt6 (n = 6) mice.

(E) Pathway enrichment analysis of differentially accumulated metabolites in PyMT/Sirt6 compared to PyMT tumors (n = 6/group). Statistical analysis was done using paired t test between the average values across the samples of each experimental group (PyMT versus PyMT/Sirt6). *$p < 0.05$, **$p < 0.01$.

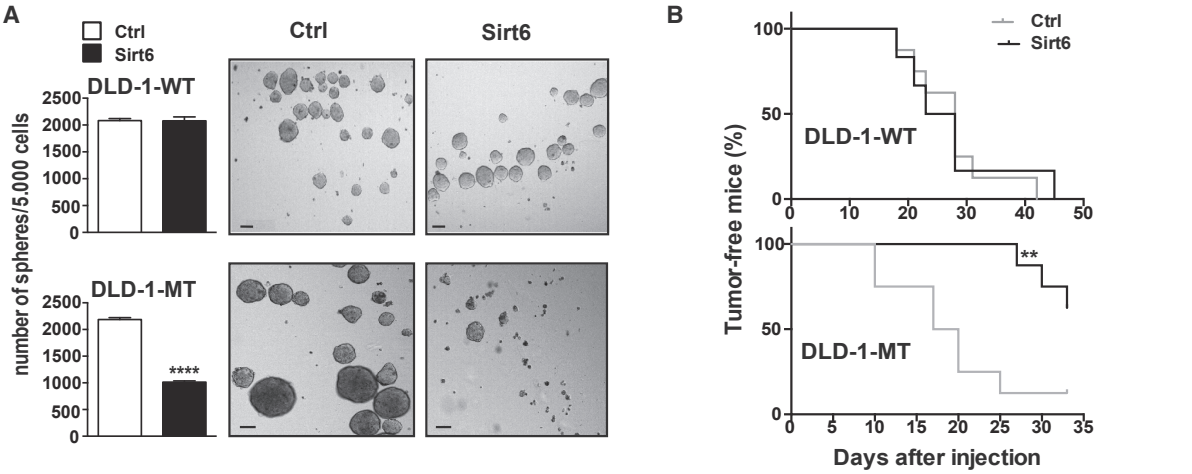
In (A), (B), and (D), FDR represents false discovery rate q value. In (C), Error bars represent SEM. *$p < 0.05$ (two-tailed unpaired Student's t test). See also Figure S3 and Tables S1 and S2.

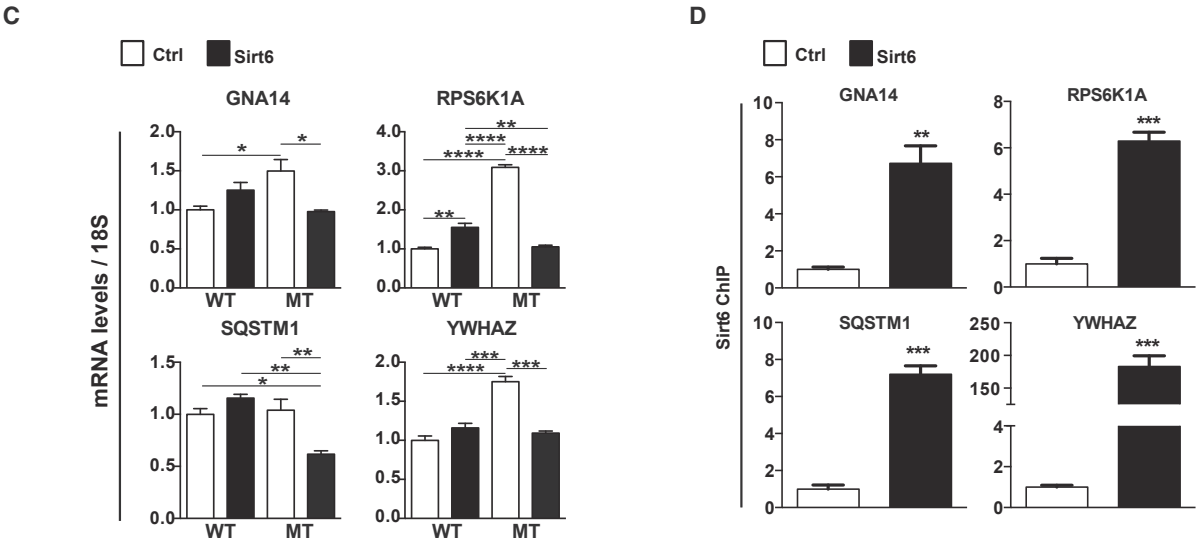
test whether SIRT6 overexpression hinder stemness of PyMT-driven breast tumors, we measured ALDH expression and enzymatic activity and found these parameters to be lowered in tumors of PyMT/Sirt6 mice (Figures 3C, S3B, and S3C). We also found that expression of several genes induced by PI3K signaling is downregulated in tumors of PyMT/Sirt6 mice (Figures 3D and S3D). However SIRT6 overexpression did not affect PI3K signaling at the AKT level as the status of AKT phosphorylation was similar in tumors from PyMT/Sirt6 mice and their controls (Figure S2B). These results are in keeping with our in vitro results shown in Figure S1A and suggest that the effect of SIRT6 overexpression on PI3K signaling is downstream of AKT phosphorylation.

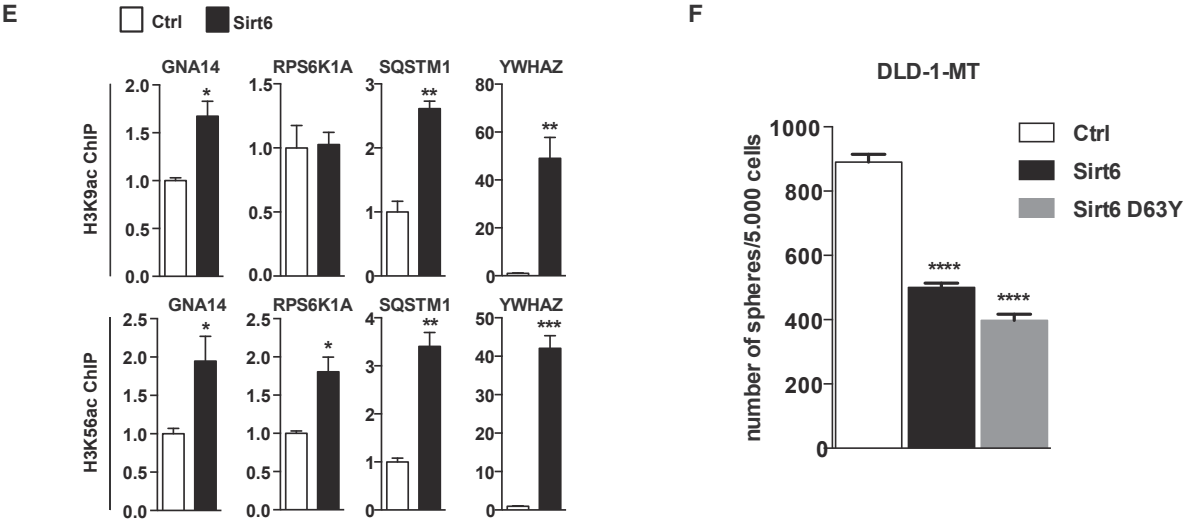
Next, we performed a metabolomic assay and found that, while only eight out of 313 biochemicals analyzed display significant changes between groups (Figure S3E; Table S2), pathway analysis indicated that the contents of several intermediate metabolites of glycolysis and the pentose phosphate (PPP) pathways, both of which are boosted by PI3K signaling (Makinoshima et al., 2015; Sun et al., 2011), are lowered in tumors of PyMT/Sirt6 mice compared to controls (Figure 3E). Collectively, our data suggest that enhanced SIRT6 expression exerts anti-tumor action by rearranging metabolism, suppressing PI3K signaling downstream of AKT, and by hindering cancer stem-like attributes of tumors with PI3K activation.

PI3K Activation Is Required for Anti-cancer Stemness Action of SIRT6 Overexpression

To further determine the mechanisms underlying anti-tumor action of SIRT6 overexpression, we focused on PI3K activation as it correlates with responsiveness to SIRT6 overexpression. To directly test whether PI3K activation is required for the anti-tumor action of SIRT6 overexpression, two cell lines derived from DLD-1 cells were transduced with either a control vector or a vector expressing SIRT6. As expected, the latter displayed increased SIRT6 compared to controls (Figure S4A). These genetically modified clones are isogenic except that one bears a wild-type allele (DLD-1-WT), while the other bears the E545K *PIK3CA* allele (DLD-1-MT) (Samuels et al., 2005). In keeping







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with their genetic types, P-S473-AKT/AKT ratio was enhanced in DLD-1-MT compared to DLD-1-WT cells (Figure S4A). In keeping with data shown in Figures S1A and S2A, SIRT6 overexpression did not suppress P-S473-AKT/AKT ratio also in DLD-1-WT and DLD-1-MT cells (Figure S4A). Similar to the effects observed in parental DLD-1 cells, SIRT6 overexpression significantly diminished tumorsphere-forming capacity of DLD-1-MT cells and appearance of DLD-1-MT xenografts (Figures 4A and 4B). However, SIRT6 overexpression was not able to affect these parameters in DLD-1-WT cells and xenografts (Figures 4A and 4B). Together, these results demonstrate that the presence of a PI3K activating mutation is required for the anti-cancer-stemness and -tumor-forming ability of SIRT6 overexpression.

By surveying our transcriptomic and metabolomic data, we noticed that contents of genes and intermediates of the PPP and lipid metabolism (e.g., 1-palmitoylglycerophosphoinositol) pathways were lowered in tumors of PyMT/Sirt6 mice (Figures 3E, S3E, S4B, and S4C). Because PPP is an important source of NADPH for glutathione regeneration and reactive oxygen species (ROS) management, we assessed glutathione and ROS levels in cells with and without SIRT6 overexpression and found no differences between groups (Figures S4D and S4E). Next, we asked whether expression of SIRT6-suppressed lipid metabolism genes is induced by PI3K signaling. Of note, expression of several of these genes was significantly lower in DLD-1-WT compared to DLD-1-MT cells (Figure S4F), hence indicating that removal of the constitutive active PI3K mutation in otherwise isogenic cells suppresses expression of several lipid metabolism genes. Interestingly, a similar effect was observed by SIRT6 overexpression as mRNA content of these lipid metabolism genes was indistinguishable between DLD-1-MT cells overexpressing SIRT6 and DLD-1-WT cells (Figure S4F). Hence, these data suggest that suppression of lipid metabolism genes could be part of the mechanisms by which SIRT6 overexpression dampens CSCs. As many of these genes are implicated in fatty acid oxidation (FAO), we tested the effect of treatment with etomoxir (a clinically tested, specific FAO inhibitor) (Holubarsch et al., 2007). Interestingly, we found that treatment with etomoxir mimics the effect of SIRT6 overexpression as it minimally changed tumorsphere-forming capacity of SIRT6-insensitive cells while strongly reduced tumorsphere-forming capacity of SIRT6-sensitive cells (Figure S4G). Also, combining etomoxir treatment with SIRT6 overexpression had sub-additional effect on tumorsphere-forming capacity of SIRT6-sensitive cells (Figures S4G). Combined with data indicating that SIRT6 overexpression increases ATP content (Figure S4H), our results indicate that FAO inhibition is unlikely to mediate the anti-CSCs action of SIRT6 overexpression; yet, our data suggest that similarly to enhanced SIRT6 action FAO inhibition could be used to dampen stemness of tumors bearing PI3K activation.

Our in vitro and in vivo data shown in Figures S1A, S2A, and S4A strongly indicate that SIRT6 overexpression does not affect AKT phosphorylation; yet, it dampens expression of several PI3K-controlled genes and metabolites (Figures 3D, 3E, S3D, and S4C). Hence, to further explore the mechanism by which SIRT6 suppresses PI3K signaling, we investigated whether SIRT6 overexpression affects the PI3K signaling further downstream of AKT, that is at the transcriptional level. First, we focused on *GNA14*, *RPSK6A1*, *SQSTM1*, and *YWHAZ* because they are known PI3K-controlled genes (Figure S3D). Our data shown in Figure 4C strongly indicate that PI3K signaling induces their expression as *GNA14*, *RPSK6A1*, *SQSTM1*, and *YWHAZ* mRNA level was higher in DLD-1-MT compared to DLD-1-WT cells (Figure 4C). Of note, as it was the case for several PI3K-controlled genes involved in lipid metabolism (Figure S4E), SIRT6 overexpression reduced expression of these genes in DLD-1-MT cells to level similar to DLD-1-WT cells while did not alter (or modestly increased) their expression in DLD-1-WT cells (Figure 4C). Next, we performed chromatin immunoprecipitation (ChIP) for SIRT6 in cancer cells overexpressing SIRT6 and their controls. Our results indicate that indeed SIRT6 binds to promoters of these PI3K-controlled genes and that SIRT6 overexpression increases SIRT6 binding to these promoters (Figure 4D). However, our ChIP assays for acetylated H3K9 and H3K56 (H3K9ac and H3K56ac) indicated that occupancy of H3K9ac and H3K56ac at these promoters was mainly increased (and not decreased as expected) by SIRT6 overexpression (Figure 4E) suggesting that the effect of SIRT6 overexpression on PI3K-controlled genes and potentially on cancer stemness is independent to SIRT6 histone deacetylase activity. To directly test this idea, we overexpressed a deacetylase inactive mutant of SIRT6 bearing a tyrosine instead of an aspartic acid at position 63 (D63Y) (Kugel et al., 2015) in SIRT6-sensitive cells. Because overexpression of SIRT6 or SIRT6 D63Y mutant exerted similar action on tumorsphere-forming capacity of these cells (Figure 4F), we concluded that SIRT6 overexpression suppresses PI3K signaling at the transcriptional level and exerts anti-tumorsphere-forming action via a mechanism independent to its histone deacetylase activity.

DISCUSSION

SIRT6 expression (Kugel et al., 2015; Sebastián et al., 2012; Thirumurthi et al., 2014) and cancer progression and stemness (Pattabiraman et al., 2016; Pattabiraman and Weinberg, 2014; Prost

Figure 4. PI3K Activation Is Required for Anti-cancer Stemness Action of SIRT6 Overexpression

(A) Tumorsphere-forming capacity of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6).

(B) Kaplan-Meier curves comparing percentage of tumor-free mice at different times after subcutaneous injection of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6).

(C) mRNAs levels of PI3K-controlled genes in DLD-1 WT and MT cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6).

(D and E) ChIP analysis of SIRT6 (D) and of H3K9ac and H3K56ac (E)on gene promoters in DLD-1 MT cells (in D and E, the data are expressed relative to Ctrl).

(F) Tumorsphere-forming capacity of DLD-1 MT cells harboring empty vector (Ctrl), a vector overexpressing human SIRT6 (Sirt6) or a vector overexpressing a deacetylase inactive mutant of SIRT6 (Sirt6-D63Y). Error bars represent SEM.

In (A), (D), and (E), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (two-tailed unpaired Student's t test). In (B), $^{**}p < 0.01$, long-rank test. In (C) and (F), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ (one-way ANOVA). See also Figure S4.

et al., 2015) are clinically relevant; yet, whether one affects the other is unknown. Here, we provide both in vivo and in vitro evidence that enhanced SIRT6 suppresses cancer progression and stemness. Unexpectedly, this effect is not universal to all cancer types and appears to be independent of the histone deacetylase activity of SIRT6. Indeed, our data indicate that the status of PI3K activation is crucial for determining responsiveness to SIRT6 overexpression. Our transcriptomic and metabolomic results from PyMT-driven breast cancer in mice, as well as both in vitro and in vivo functional assays in human cancer cells with different levels of PI3K signaling, and our ChIP assays strongly indicate that SIRT6 thwarts cancer stemness, at least in part, by suppressing the PI3K pathway at the transcriptional level. While our findings are in keeping with previously published results indicating that SIRT6 negatively regulates PI3K signaling, they indicate that this effect is not dependent on the ability of SIRT6 to deacetylate histone 3 at lysine 9 and/or 56 at promoter regions of PI3K effector genes as previously suggested (Sundaresan et al., 2012). A deacetylase-independent action is not at odds with the fact that others have shown very minor histone deacetylase ability of SIRT6 (Jiang et al., 2013; Liszt et al., 2005). As activation of PI3K signaling promotes survival of CSCs (Hambardzumyan et al., 2008; Jiang et al., 2015), our data support a model whereby enhanced SIRT6 dampens PI3K signaling at the transcriptional level, an effect that leads to several changes, including altered glucose and lipid metabolism and reduced cancer stemness. Because SIRT6 overexpression dampens expression of a large number of genes, we suggest that its anti-cancer-stemness action is brought about by a concerted rather than a single mechanistic change. It is important to keep in mind that in addition to PI3K activation our results do not exclude the possibility that SIRT6 overexpression may influence behavior of tumors bearing other types of mutations. Hence, further analysis aimed at testing the effect of SIRT6 overexpression in a large panel of tumors bearing a wide range of different mutations is warranted.

Augmented SIRT6 function has been shown to bring about beneficial effects in the context of metabolism (Anderson et al., 2015) and aging (Kanfi et al., 2012); thus, combined with results reported herein these data provide rationale for developing SIRT6 agonists as therapeutics for age-related diseases (e.g., cancer and diabetes). Furthermore, our findings are relevant in a personalized medicine context as they could be used to stratify patients bearing tumors with PI3K activation into likely responders to means aimed at increasing SIRT6 function.

EXPERIMENTAL PROCEDURES

Mouse Generation and Studies

MMTV-PyMT mice were generated as previously described (Guy et al., 1992). Sirt6BAC mice were generated by BAC transgenesis as described previously (Anderson et al., 2015) and are available from The Jackson Laboratory (JAX#028361). Mice were housed in groups of four or five with food and water available ad libitum in light- and temperature-controlled environments. Care of mice was within the procedures approved by animal care and experimentation authorities of the Canton of Geneva, Switzerland.

SIRT6 Overexpression in Cancer Cells

We produced recombinant retroviruses by transfecting Phoenix-Ampho cells (ATCC), using TransIT-293 transfection reagent (Mirus) and pBABE retroviral constructs. Cells were transfected with pBABE vectors expressing human-SIRT6, or deacetylase-dead human-SIRT6 bearing a tyrosine instead of an aspartic acid at position 63 (D63Y) (Kugel et al., 2015), or pBABE empty vector and selected for puromycin resistance.

In Vitro Assessment of Cell Proliferation

Cells were fixed with formalin and stained with Crystal Violet (CV). Then, CV-stained cells were dissolved with 5% acetic acid for 30 min and 200 μL of this solution was transferred to a 96-well plate and read in a plate reader at 570 nm. T1 and T2 values were normalized to T0. Proliferation curves were compared between groups and experiments were repeated at least three times.

Tumorsphere Assays

Tumorsphere formation was induced in ultralow-adherent 6-well plates. Cells were plated at a density of 5,000 cells per well in triplicate in a 6-well plate in a 1% methylcellulose containing media (MammoCult supplemented with MethoCult media, STEMCELL Technologies). Tumorsphere formation was quantified 7 days after plating. Spheres with a diameter equal or higher than 50 μm were deemed tumorspheres. Etomoxir (Sigma) was added at the specified concentrations to the media. Experiments were repeated at least three times.

Mouse Xenograft Assay

Cells were trypsinized, suspended in PBS, and injected subcutaneously into the flank of NOD/SCID mice. Cells were injected at the following numbers: 1×10^6 and 1×10^4 MCF10DCIS, 1×10^6 MDA-MB-231, 1×10^5 H1650, 1×10^5 DLD-1 MT, and 1×10^5 DLD-1 WT cells. Mice were checked twice a week for tumor appearance and tumor diameter measurements.

Tumor Grade Assessment

Mice bearing at least one tumor with 10 mm of major diameter (as measured manually with a caliper) were sacrificed and all their detectable tumors collected. Tumors of comparable size were used for histologic analysis. Areas of different histological types (adenoma, hyperplasia, necrosis, and carcinoma) were assessed by surveying the whole tumor area as previously described (Santidrian et al., 2013).

mRNA and Protein Contents

Mice were sacrificed, and tissues were quickly removed and snap-frozen in liquid nitrogen and subsequently stored at – 80°C. RNAs were extracted using TRIzol reagent (Invitrogen). Complementary DNA was generated by Superscript II (Invitrogen) and used with SYBR Green PCR master mix (Applied Biosystems) for real-time qPCR analysis. mRNA contents were normalized to *β-actin* and/or *36B4* mRNA levels. All assays were performed using an Applied Biosystems QuantStudio 5 Real-Time PCR System. For each mRNA assessment, real-time qPCR analyses were repeated at least three times. Proteins were extracted by homogenizing samples in lysis buffer (Tris 20 mM, EDTA 5 mM, NP40 1% [v/v], protease inhibitors [P2714-1BTL from Sigma]) and then resolved by SDS-PAGE and finally transferred to a nitrocellulose membrane by electroblotting. Proteins were detected using commercially available antisera as previously described (Ramadori et al., 2011, 2015).

ALDH Activity

ALDH activity was assessed using the ALDH Activity Colorimetric Assay Kit from Biovision (K731-100) and following the manufacturer's instructions. Briefly, samples were homogenized with 200 μL of ice-cold ALDH assay buffer, and 3 μL of the homogenate was used in the assay. Absorbance was measured at 450 nm every 5 min over 1 hr, and the oxidation of Acetaldehyde to NADH was calculated according to the manufacturer's instructions.

FACS

ALDH activity was measured in cancer cells using the Aldefluor kit following the manufacturer protocol. 5×10^5 of H1650 or MCF10DCIS cells were incubated with Aldefluor reagent and N,N-diethylaminobenzaldehyde (DEAB; represented in blue) or Aldefluor reagent only (represented in red). After a 40-min incubation period, cells were analyzed by flow cytometry, and results were generated using FlowJo software. Values mentioned along with the graphs indicate percentage of cells with high ALDH activity.

Microarray and Metabolomic Assays

Mice were sacrificed, and tissues were quickly removed, frozen in liquid nitrogen, and subsequently stored at –80°C. RNAs were extracted by QIAGEN mRNA extract kits (RNeasy plus). Microarray analyses were performed by University of Texas Southwestern Medical Center at Dallas microarray Core facility (https://microarray.swmed.edu/) using Illumina Chip Mouse WG-6 v.2.0 (Illumina). Metabolites contents were measured by Metabolon. The differential analysis of the transcriptomic and metabolomic data were performed using CyberT (Baldi and Long, 2001; Kayala and Baldi, 2012). Briefly, the average signal intensity was transformed using a log base 2 normalization, and a regularized t test was then performed using Cyber-T with a window size of 51 and a Bayesian confidence value of 5. p values of 0.001 and 0.01 were considered significant for microarray and metabolomics analyses, respectively. Pathway and functional enrichment was carried out using DAVID (Huang et al., 2009).

Chromatin Immunoprecipitation Assays

ChIP assays were performed as previously described (Kugel et al., 2016; Sebastián et al., 2012). Antibodies used are 5 μL anti-SIRT6 (Abcam; ab62739), 5 μL anti-H3K9Ac (Millipore 07-352), and 5 μL anti-H3K56Ac (ab76307). Data were normalized to values obtained with unspecific immunoglobulin Gs (IgGs) (Abcam).

Determination of the Cellular Glutathione Content

Cells were seeded in 96-well plate at a density of 10^4 per well and incubated (protected from the light) with 20 μM monobromobimane (mBBr) for 10 min at 37°C. The conjugation of glutathione with monobromobimane was followed by measuring the fluorescent product at Ex/Em 394/490 nm using SpectraMax Paradigm microplate reader (Molecular Devices). The results are expressed as a percentage of the glutathione content in control cells.

Determination of Mitochondrial ROS Content

5×10^4 cells in Hank's balanced salt solution (HBSS), 10 mM HEPES (pH 7.4), 17.5 mM glucose, 1.55 mM $CaCl_2$, and 10% FBS were incubated with 5 μM MitoSox (Thermo Fisher Scientific) for exactly 10 min at room temperature before analysis on a CyAn flow cytometer (Beckman Coulter).

ATP Determination in Tumorspheres

Cells were lysed with 10% trichloroacetic acid × 30 min in ice. Next, the lysate was centrifuged at 13,000 rpm for 5 min at 4 degrees and a 1/16 dilution of the supernatant (in distillated ultrapure water) was used for ATP determination using a commercial kit (Molecular Probe #A22066) according to the manufacturer's instructions. Values were normalized to lysate protein contents.

Dataset Compilation

Datasets of stem cells (ESCs, ASCs, and iPS cells) and terminally differentiated tissues (TDTs) was generated by compiling 246 whole-genome microarray datasets downloaded form ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/). In order to avoid inter-platform biases, only data generated using the same microarray platform (Affimetrix 430 2.0) were selected. Microarrays were downloaded as raw files, concatenated in a unique dataset and normalized according to the Robust Multichip Average (RMA) algorithm.

Statistical Analyses

Data are reported as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0c software. Unpaired two-tailed t tests were employed when two groups were compared, and one-way ANOVA with Tukey correction for multiple comparisons was used when three or more groups were compared unless otherwise specified.

ACCESSION NUMBERS

The accession number for the microarray data from tumor tissues of PyMT and PyMT/Sirt6 mice reported in this paper is NCBI Gene Expression Omnibus: GSE93837.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.065.

AUTHOR CONTRIBUTIONS

Conceptualization, R.C., M.G., R.M.I., and G.R.; Investigation, M.G., R.M.I., G.R., J.G.A., A.C., G.K., X.B., E.A., A.G., C.S., R.M., D.M., and N.C.; Writing – Original Draft, R.C.; Writing – Review & Editing, R.C., M.G., R.M.I., G.R., and P.B.; Funding Acquisition, R.M.I., R.C., and P.B.; Data Curation, R.C., M.G., R.M.I., P.B., N.C., and G.R.; Supervision, R.C. and G.R.

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Cell Reports, Volume *18*

Supplemental Information

SIRT6 Suppresses Cancer Stem-like Capacity in Tumors with PI3K Activation Independently of Its Deacetylase Activity

Rafael M. Ioris, Mirco Galié, Giorgio Ramadori, Jason G. Anderson, Anne Charollais, Georgia Konstantinidou, Xavier Brenachot, Ebru Aras, Algera Goga, Nicholas Ceglia, Carlos Sebastián, Denis Martinvalet, Raul Mostoslavsky, Pierre Baldi, and Roberto Coppari

Supplemental figures

Figure S1

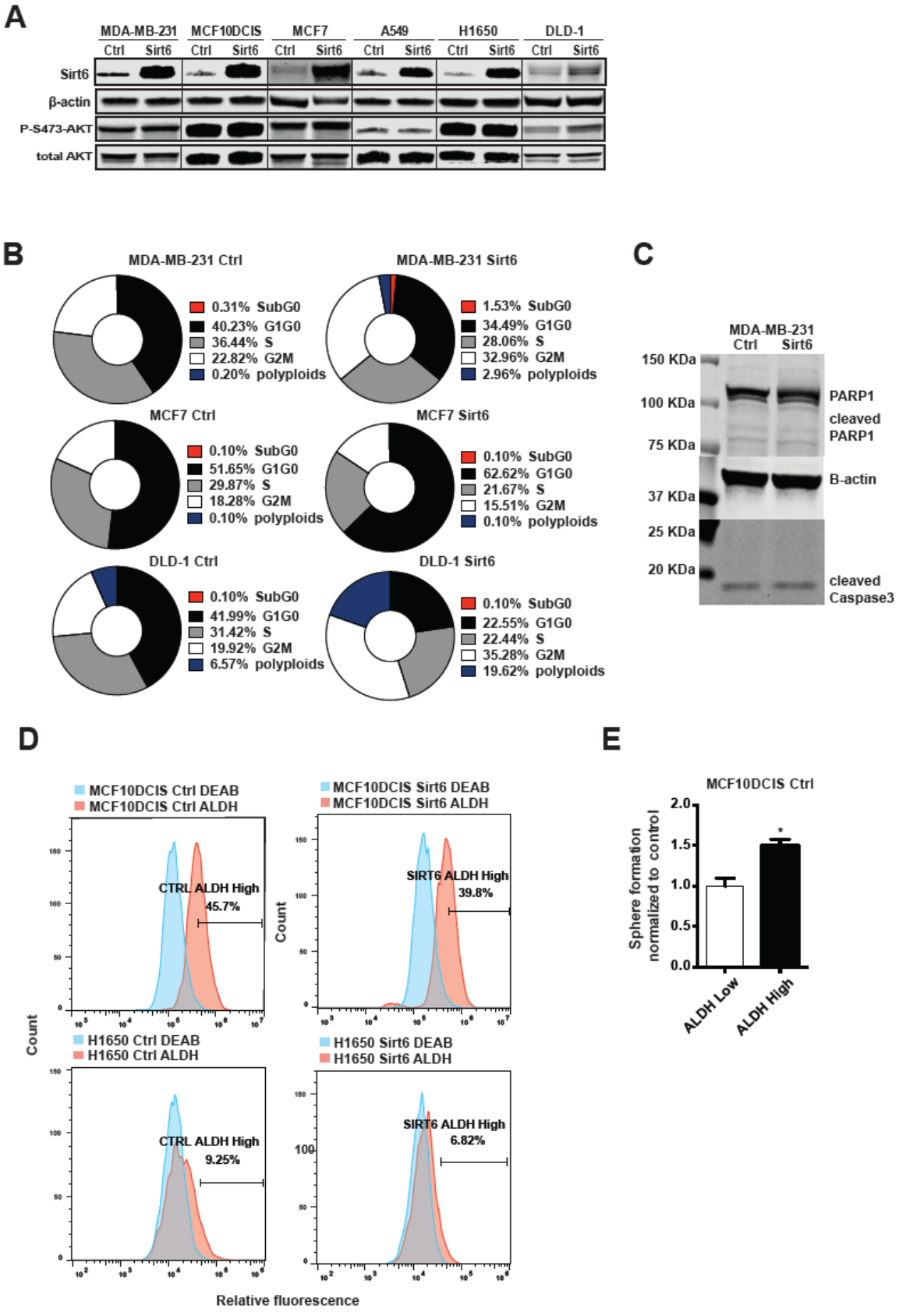

Figure S1. Related to Figure 1. SIRT6 overexpression reduces the size of the cell population with high ALDH activity in human cancer cells with PI3K activation. (A) Immunoblot images of indicated proteins, (**B**) pie-charts representing percentage of cells in distinct cell cycle phases, (**C**) immunoblot images of apoptosis markers, and **(D)** ALDH activity of indicated human cancer cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6) [Aldehyde dehydrogenase (ALDH); N,N-diethylaminobenzaldehyde (DEAB)]. (**E**) Tumorsphere-forming capacity of indicated cells showing low or high ALDH activity. Error bars represent SEM. Statistical analyses were done using two-tailed unpaired Student's t test, *$P < 0.05$.

Figure S2

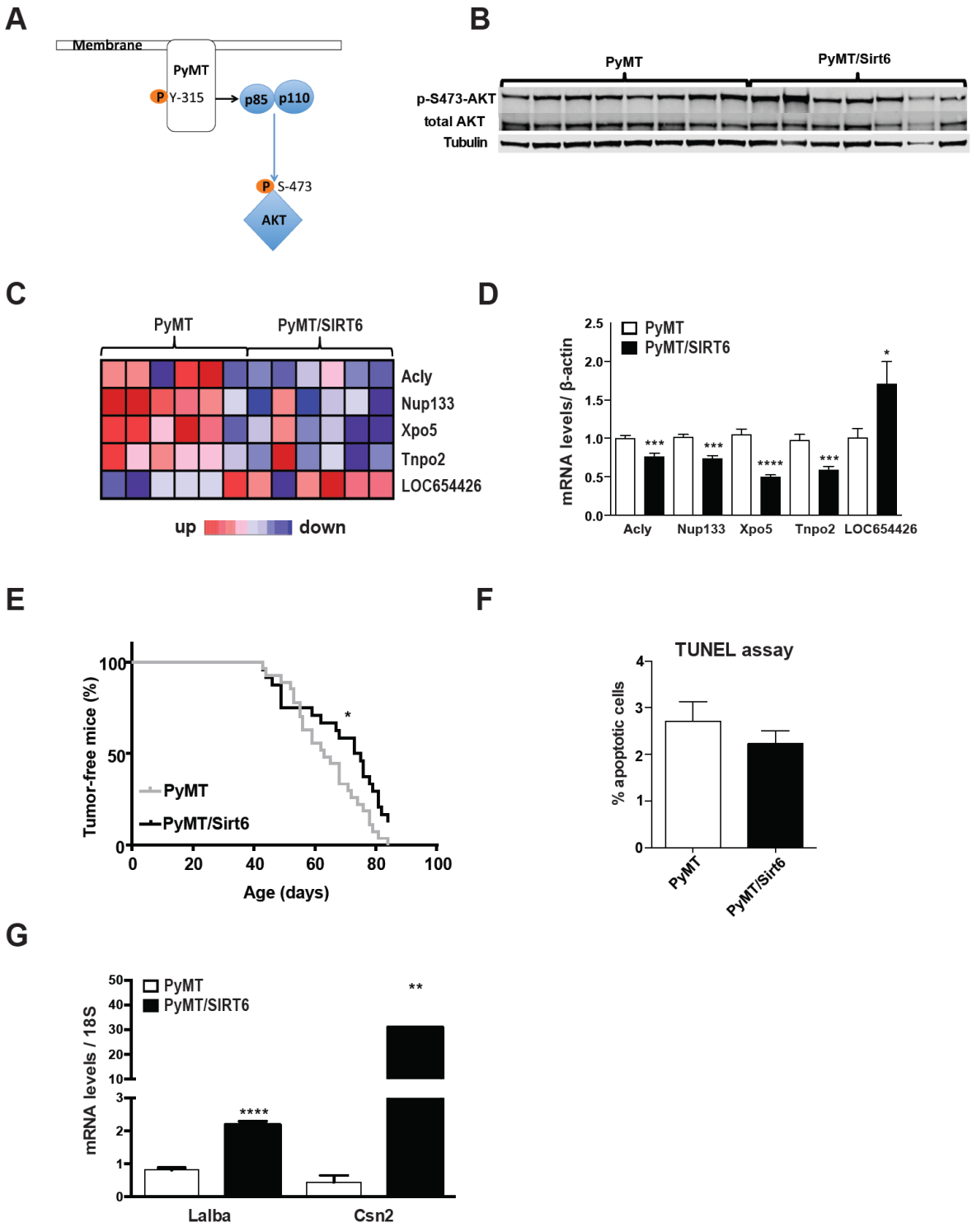

Figure S2. Related to Figure 2. SIRT6 overexpression delays onset and hinders progression of PyMT-driven breast cancer in mice. (A) A diagrammatic scheme of PyMT-mediated PI3K signaling and (**B**) immunoblot images of indicated proteins in tumors of 12-14-week-old PyMT (n=8) and PyMT/Sirt6 (n=7) mice. **(C)** Heat map depicting mRNA levels of several genes assessed by DNA microarray assay and (**D**) mRNA levels of same genes as

in (C) assessed by Q-PCR analysis in tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. **(E)** Kaplan-Meier curves comparing percentage of tumor-free PyMT (n=27) and PyMT/Sirt6 (n=21) mice at different ages. *P = 0.0334, long-rank test. (**F**) Percentage of TUNEL-positive cells in tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. **(G)** Q-PCR analysis of mRNA levels of genes expressed in normal mammary epithelial tissue in tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. Error bars represent SEM. Statistical analyses were done using two-tailed unpaired Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S3

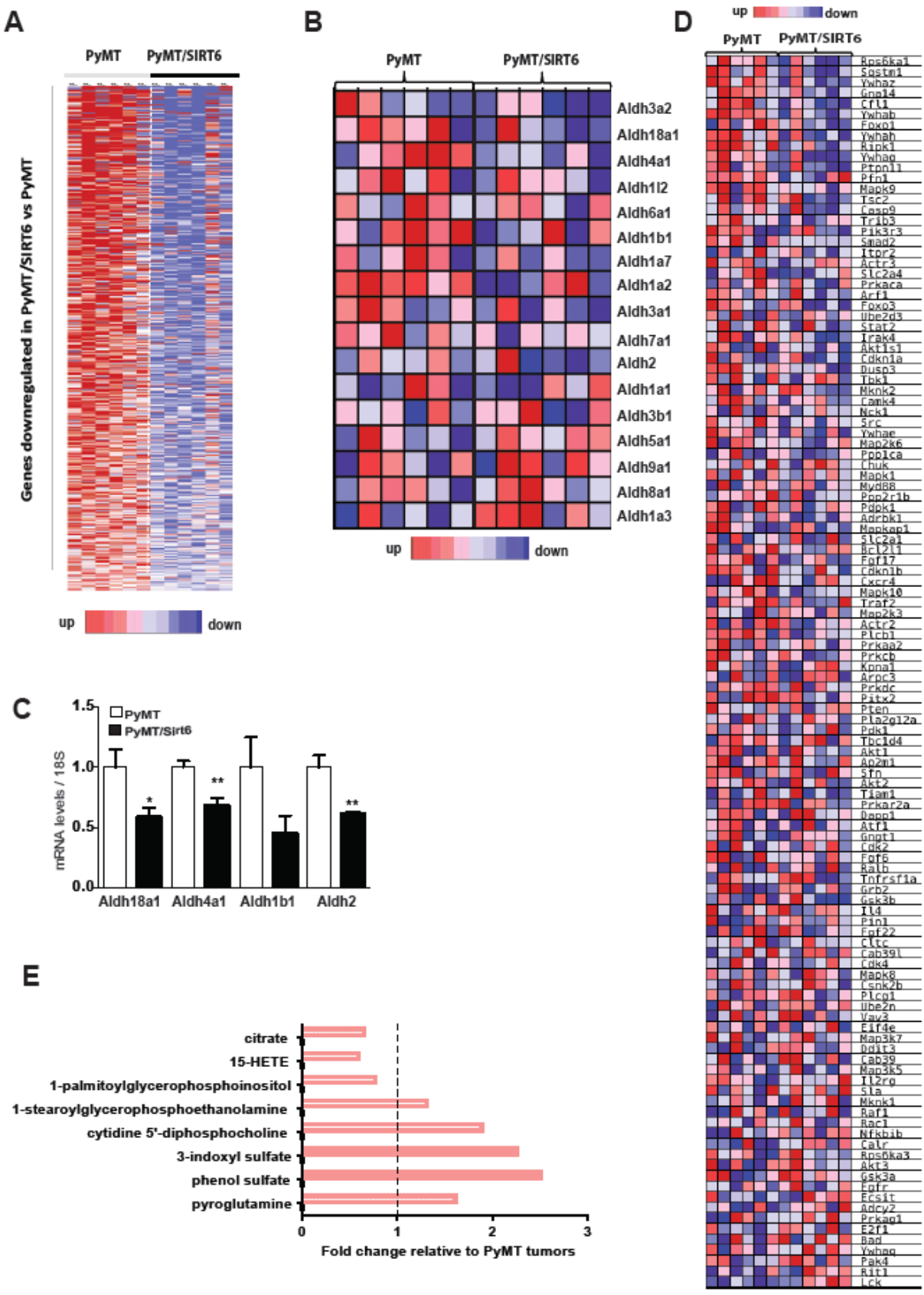
A
PyMT
PyMT/SIRT6
Genes downregulated in PyMT/SIRT6 vs PyMT
up
down
B
PyMT
PyMT/SIRT6
Aldh3a2
Aldh18a1
Aldh4a1
Aldh1l2
Aldh6a1
Aldh1b1
Aldh1a7
Aldh1a2
Aldh3a1
Aldh7a1
Aldh2
Aldh1a1
Aldh3b1
Aldh5a1
Aldh9a1
Aldh8a1
Aldh1a3
up
down
C
PyMT
PyMT/Sirt6
mRNA levels / 18S
1.5
1.0
0.5
0.0
*
**
**
Aldh18a1
Aldh4a1
Aldh1b1
Aldh2
D
up
down
PyMT
PyMT/SIRT6
Rps6ka1
Sqstm1
Ywhaz
Gnq14
Cfl1
Ywhab
Foxo1
Ywhah
Ripk1
Ywhag
Ptpn11
Pfn1
Mapk9
Tsc2
Casp9
Trib3
Pik3r3
Smad2
Itpr2
Actr3
Slc2a4
Prkaca
Arf1
Foxo3
Ube2d3
Stat2
Irak4
Akt1s1
Cdkn1a
Dusp3
Tbk1
Mknk2
Camk4
Nck1
Src
Ywhae
Map2k6
Ppp1ca
Chuk
Mapk1
MyD88
Ppp2r1b
Pdpk1
Adrbk1
Mapkap1
Slc2a1
Bcl2l1
Fgf17
Cdkn1b
Cxcr4
Mapk10
Traf2
Map2k3
Actr2
Plcb1
Prkaa2
Prkcb
Kpna1
Arpc3
Prkdc
Pitx2
Pten
Pla2g12a
Pdk1
Tbc1d4
Akt1
Ap2m1
Sfn
Akt2
Tiam1
Prkar2a
Dapp1
Atf1
Gnpt1
Cdk2
Fgf6
Ralb
Tnfrsf1a
Grb2
Gsk3b
Il4
Pin1
Fgf22
Cltc
Cab39l
Cdk4
Mapk8
Csnk2b
Plcg1
Ube2n
Vav3
Eif4e
Map3k7
Ddit3
Cab39
Map3k5
Il2rg
Sla
Mknk1
Raf1
Rac1
Nfkbib
Calr
Rps6ka3
Akt3
Gsk3a
Egfr
Ecsit
Adcy2
Prkag1
E2f1
Bad
Ywhag
Pak4
Rit1
Lck
E
citrate
15-HETE
1-palmitoylglycerophosphoinositol
1-stearoylglycerophosphoethanolamine
cytidine 5'-diphosphocholine
3-indoxyl sulfate
phenol sulfate
pyroglutamine
0
1
2
3
Fold change relative to PyMT tumors

Figure S3. Related to Figure 3. Transcriptomic and metabolomic changes caused by SIRT6 overexpression in PyMT-driven breast tumors in mice. (**A**) Heat-map of genes downregulated in tumors of PyMT/SIRT6 *vs* PyMT 12-14-week-old mice (for list of genes see Table S1). **(B)** Heat map depicting mRNA levels of several genes encoding for different Aldh isoforms (assessed by DNA microarray assay) and (**C**) mRNA levels assessed by Q-PCR in tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. **(D)** Differential expression of several genes regulated by PI3K signaling in tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice (http://software.broadinstitute.org/gsea/msigdb; combined genes set: systematic names M249, M5942). **(E)** Fold change in metabolite levels in tumors from of 12-14-week-old PyMT/Sirt6 mice (n=5) relative to tumors from PyMT mice (n=5). Welch's two sample t-Test: p<0.0.5. Out of 313 metabolites, only the contents of the ones displayed in this figure were found to be significantly different between groups. Error bars represent SEM. Statistical analyses were done using two-tailed unpaired Student's t test. *P < 0.05, **P < 0.01.

Figure S4

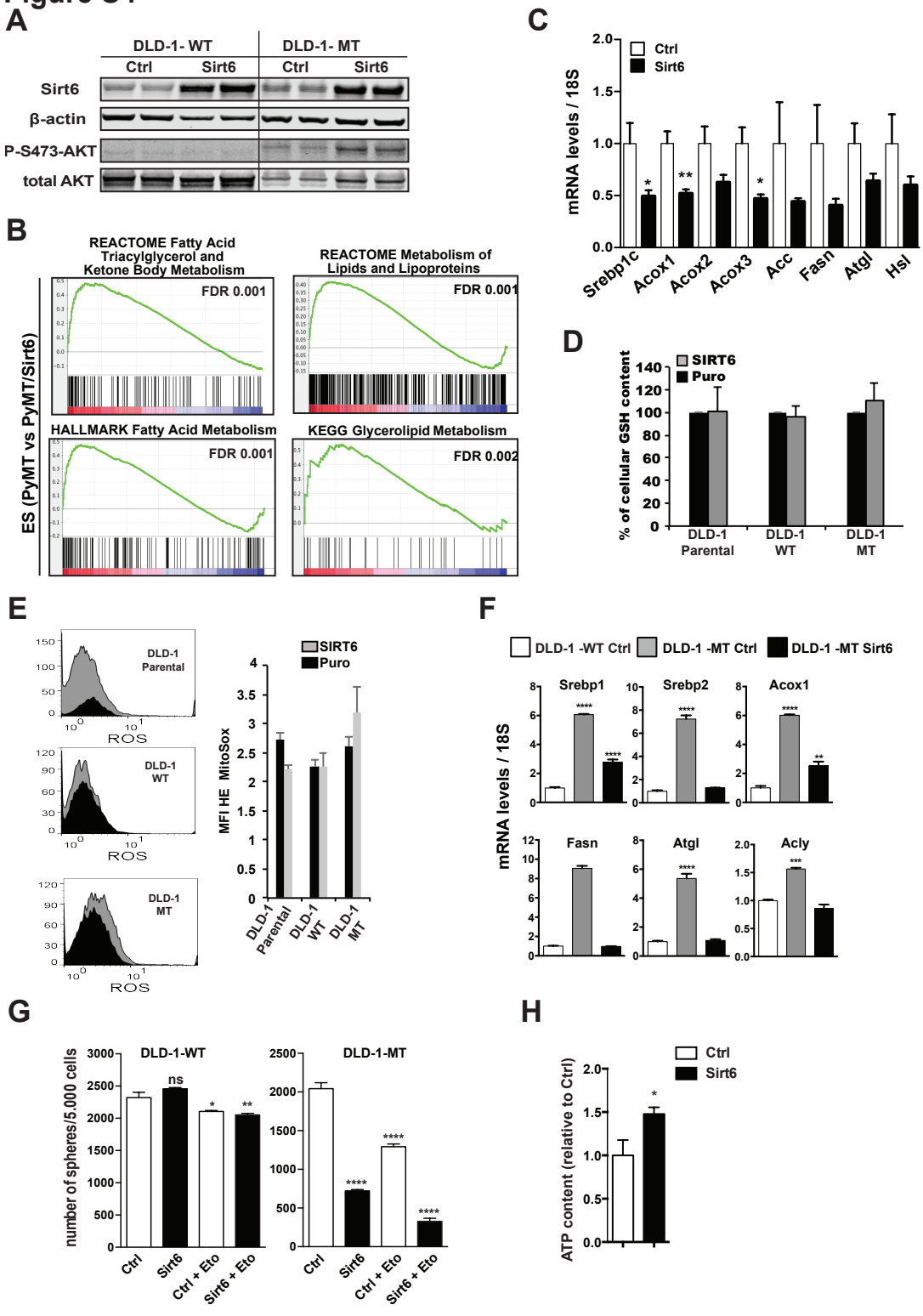

Figure S4. Related to Figure 4. PI3K activation is required for anti-cancer-stemness action of SIRT6 overexpression. (**A**) Immunoblot images of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6). (**B**) Gene set enrichment analysis (GSEA) plot showing the enrichment score (ES) of genes regulating lipid metabolism. Gene microarray was performed using RNA extracted from tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. FDR, False Discovery Rate q-value. (**C**) mRNA expression of genes related to lipid metabolism in extracted from tumors of 12-14-week-old PyMT (n=6) and PyMT/Sirt6 (n=6) mice. (**D**) Steady state cellular glutathione

content. $1x10^4$ cells per well were let to adhere for about 4 hours and the cellular glutathione content were assess by mBBR staining. Data are mean +/- SD of four independent experiments expressed as a percent of the respective control. (**E**) Steady state mitochondrial ROS. $5x10^4$ cells were incubated with MitoSox for 10 minutes and ROS production were followed by FACS analysis. FACS histogram is representative of three independent experiments. Mean +/- SD of three independent experiments (right panel). (**F**) mRNA levels of PI3K controlled genes; **(G)** tumorsphere-forming capacity and (**H**) ATP content of indicated cells harboring empty vector (Ctrl) or a vector overexpressing human SIRT6 (Sirt6). Error bars represent SEM. Statistical analyses were done using two-tailed unpaired Student's t test or one-way-Anova. *P < 0.05, **P < 0.01, ***P < 0.01, ****P < 0.001.

Supplemental Tables

Table S1. Genes downregulated in PyMT/Sirt6 compared to PyMT tumors. Related to Figure 3.

Table S2. Heat map of biochemicals profiled in this study (comparison between PyMT/Sirt6 and PyMT tumors). Related to Figure 3.

DISCUSSION

More than 2 billion people were overweight or obese in 2016, including 381 million children and adolescents (World Health Organization, 16 February, 2018). Obesity is a risk factor for the development of cardiovascular diseases (the leading cause of death), diabetes (it causes, directly or indirectly, almost 4 million death/year) and cancer (it is estimated that it causes 9.6 million deaths in 2018) (World Health Organization, 16 February, 2018) (Mathers and Loncar 2006). Those shocking numbers are letting clear the need for development of new approaches for the treatment of obesity, T2DM, and cancer.

Calorie restriction (CR), a feeding regimen that restricts calorie intake to a level lower than normal without provoking malnutrition, reduces the incidence and risk of several types of malignancy in rodents, including diabetes and cancer (Kritchevsky 1997; Lee and Longo 2011; Mattison et al. 2012). SIRT6 is a histone modifier known to affect expression of thousands of genes and whose amount increases following CR (Kanfi et al. 2008; Kawahara et al. 2011). Hence, SIRT6 is perfectly placed to link changes in energy intake to cellular function and improved health.

Physiological overexpression of SIRT6 in metabolism

The role of SIRT6 in glucose metabolism is controversial. Loss-of-function based studies pointed SIRT6 as a master regulator of glucose homeostasis where SIRT6 deficiency leads to attenuation of SIRT6-dependent transcriptional silencing resulting in increased expression of genes involved in glycolysis and glucose transport (Mostoslavsky et al. 2006; Zhong et al. 2010; Sundaresan et al. 2012). As consequence, SIRT6 knockout mice exhibit increase glycolytic rate which leads to loss

of adipose tissue mass and fatal hypoglycaemia during developmental stage (Mostoslavsky et al. 2006). Nevertheless, in contrast to loss-of-function approach, ubiquitous and supraphysiological overexpression of SIRT6 in mice fed on HCD also leads to reduced adiposity and improved glucose metabolism (Kanfi et al. 2010). Thus, based on these confounding data, it is unclear whether means to inhibit or enhance SIRT6 protein activity should be sought after in order to treat diet-induced obesity and/or T2DM.

SIRT6 controls expression of high number of genes and consequently a wide range of cellular processes (Kawahara et al. 2011) and been so, the metabolic effects observed in deletion or supraphysiological methods may be secondary and not direct consequence of SIRT6 abundancy. Aiming to identify SIRT6 as novel target against metabolic diseases, as T2DM, and cancer, and in order to translate in a clinical perspective the preclinical findings, we used an animal model overexpressing SIRT6 physiologically and assessed its effect in diet-induced T2DM (Anderson et al. 2015). We showed that SIRT6 mild overexpression is able to improve glucose metabolism by increasing insulin sensitivity in liver and skeletal muscle (Anderson et al. 2015).

The model used in our study (SIRT6BAC) drives SIRT6 expression using its own natural promoter, resulting in an overexpression that mimics CR induction of SIRT6 (Anderson et al. 2015; Ioris et al. 2017). Moreover, our approach using SIRT6 endogenous promoter more likely expresses SIRT6 eutopically. On the other hand, Kanfi et al. (Kanfi et al. 2008) used an artificial system where chicken β-actin promoter and CMV enhancer drive SIRT6 overexpression at supra-physiological level and ectopically (i.e. without cell-type discrimination) including cells that normally would not express SIRT6.

Although SIRT6BAC model display a mild SIRT6 expression it also has some limitations. The bacterial artificial chromosome (BAC) used to generate this mouse model houses a bigger genomic region than only SIRT6 gene and its endogenous promoter (Figure 1A), including several genes that consequently are equally overexpressed (Figure 1D) (Anderson et al. 2015). The BAC insertion site and the genes inserted into it could contribute, or be responsible in a SIRT6-independent way, to the phenotype observed in SIRT6BAC mice. In order to address this issues, a SIRT6BAC in a SIRT6 homozygous background was generated which normalize SIRT6 levels. For this, SIRT6BAC mouse was breed with mice that were heterozygous for the *Sirt6* null allele (SIRT6BAC/SIRT6$^{+/+}$ x SIRT6$^{+/-}$) and subsequently SIRT6Bac in a SIRT6 heterozygous background was breed with SIRT6 heterozygous (SIRT6BAC/SIRT6$^{+/-}$ x SIRT6$^{+/-}$). This breeding scheme, SIRT6BAC/SIRT6$^{+/-}$ x SIRT6$^{+/-}$, results in an offspring including the three experimental genotypes used in our study, 1) SIRT6$^{+/+}$ (wild type - WT), 2) SIRT6BAC/SIRT6$^{+/+}$ (overexpression, call hereafter as SIRT6BAC) and 3) SIRT6BAC/SIRT6$^{-/-}$ (control to assess the contribution of the genes present in the genomic region flacking SIRT6 sequence into the BAC) (Figure 2A) (Anderson et al. 2015). SIRT6BAC and SIRT6BAC/SIRT6$^{-/-}$ have the same BAC insertion site, same BAC-present non-SIRT6 genes expression, but differ on SIRT6 levels (Figure 1D) (Anderson et al. 2015). Thus, if SIRT6BAC mice display a given phenotype over WT and SIRT6BAC/SIRT6$^{-/-}$ it is likely to be SIRT6 overexpression consequence.

Another concern, which is not exclusive for our animal model, is if the overexpressed protein is functional. SIRT6 knockout mice (SIRT6$^{-/-}$) at 3 weeks of age are smaller compared to littermates, suffering from several hypoglycaemia and loss of subcutaneous fat which culminate in death before reach adulthood (Mostoslavsky et

al. 2006). SIRT6BAC rescues this phenotype, normalizing body weight and body length at 4 weeks of age, when introduced in SIRT6 knockout background (SIRT6BAC/SIRT6$^{-/-}$) (Figure 2B and C) (Anderson et al. 2015). Additionally, SIRT6BAC/SIRT6$^{-/-}$ mice develop normally, displaying similar body composition (weight, fat and lean mass) compared with WT animals, until at least 24 weeks of age (Figure 3) (Anderson et al. 2015). Furthermore, in our cancer study where we crossed SIRT6BAC mice with an established breast cancer mouse model (Guy, Cardiff, and Muller 1992) and performed microarray in tumors with and without SIRT6 overexpression we showed that several SIRT6 target genes (Kawahara et al. 2011) have mRNA levels reduced when SIRT6 is overexpressed (Figure 2D) (Ioris et al. 2017), demonstrating enhanced SIRT6 activity in SIRT6BAC mice.

In agreement with a previous report (Kanfi et al. 2010), our results show that SIRT6 mild overexpression does not alter body composition (weight, fat and lean mass) in chow or high caloric diet (Figure 3) (Anderson et al. 2015). Regarding glucose profile, in our study, while all three genotypes displayed similar levels of serum insulin, SIRT6BAC mice exhibited lower basal glycaemia in chow diet comparing with WT animals and in high caloric diet comparing with both genotypes controls (Figure 4A and B) (Anderson et al. 2015). Of note, basal glycaemia displayed by SIRT6BAC/SIRT6$^{-/-}$ was intermediated between WT and SIRT6BAC animals in chow diet and at the same level as WT in high caloric diet. To further assess the outcome of SIRT6 overexpression over glucose metabolism we performed glucose and pyruvate tolerance test in all three genotypes and in both diet condition. Once more SIRT6BAC mice displayed improved glucose homeostasis, more pronounced in animals under high caloric diet, and once more SIRT6BAC/SIRT6$^{-/-}$ had an intermediate phenotype (Figure 4C-F) (Anderson et al. 2015). Those results are again in agreement with Kanfi

et al. (Kanfi et al. 2010), with a slight difference regarding basal glycaemia. Thus, two studies using mice with different genetic background (C57BL/6J and BALB/cOlaHsd in Kanfi et al.; C57BL/6J, FVB and BALB/c in our study), different age (at least for glucose tolerance test – 24 weeks in Kanfi et al.; 11-13 weeks for chow diet and 18-20 weeks for high caloric diet in our study) and different level of SIRT6 expression (non-physiologic in Kanfi et al.; physiologic in our study) gave us even more confidence to sustain that our results are due to SIRT6 overexpression and that this overexpression leads to improved glucose profile.

To add more evidence that SIRT6 mild overexpression improved glucose profile by increasing insulin sensitivity a hyperinsulinemic-euglycemic clamp assay was performed. Therefore, we can directly test insulin sensitivity and glucose uptake by specific tissues. First, in this assay insulin is infused in order to achieve hyperinsulinemia, with concomitantly infusion of glucose to maintain euglycemia. The glucose infusion rate needed to achieve euglycemia is a direct measurement of insulin sensitivity thus, the higher the amount of glucose infused the higher is the insulin sensitivity. Second, infusing a radiolabeled glucose analog the glucose disposal was determined for specific tissues. During clamp, SIRT6BAC mice displayed a significantly higher glucose infusion rate comparing with WT control animals (Figure 5A), a reduction in endogenous glucose production (Figure 5C) and increased glucose disposal rate (Figure 5D). Reduction in endogenous glucose production is due to increase liver insulin sensitivity and consequent blocking of gluconeogenesis. With the analysis of the radiolabelled glucose analog uptake we could determine soleus and gastrocnemius skeletal muscles as the responsibles for the increase glucose disposal rate (Anderson et al. 2015). Additionally, to independently assess insulin sensitivity *in vivo* we used another readout of insulin sensitivity, pAKT level upon insulin

administration. Figure 6A and B are showing that SIRT6BAC enhanced pAKT levels (pAKT/AKT ratio) in gastrocnemius skeletal muscle after 10 minutes of intraperitoneal insulin injection comparing with WT controls animals. Of note, SIRT6BAC/SIRT6$^{-/-}$ showed no significant difference when comparing with WT animals (Anderson et al. 2015). Basal pAKT levels in gastrocnemius are not different between all three genotypes, in agreement with previous publication overexpression SIRT6 (Kanfi et al. 2012), although the authors did not specify which muscle was assessed.

Calorie restriction is known to bring about beneficial effects improving glucose and lipid metabolism and extending lifespan (Guarente 2013; Zhang, Li, et al. 2016). Spermidine, a naturally occurring polyamine found in daily human nutrition which content declines in aging, is a caloric restriction mimetics able to improve health and delay aging (Eisenberg et al. 2016; Madeo, Carmona-Gutierrez, et al. 2018; Madeo, Eisenberg, et al. 2018; Eisenberg et al. 2009). Noteworthy, spermidine levels are increased 3 fold in SIRT6BAC mice comparing with WT control (Figure 7) (Anderson et al. 2015). However, additional experiments are warranted to determine whether the improved glucose homeostasis upon SIRT6 gain-of-function is dependent on change in spermidine levels.

Supplementary to glucose metabolism, we showed a change in expression profile of several genes involved in lipid metabolism (in chow and high caloric diet) upon SIRT6 enhancement (Figure S2B and D) (Anderson et al. 2015). Those results are contradicting a previous study, based in loss-of-function, where several genes that are upregulated when SIRT6 is overexpressed are also upregulated in a SIRT6 hepatic-specific deletion context (Kim, Xiao, et al. 2010). Once more indicating that many of the effects observed in experiments using SIRT6 loss-of-function can be also seen in SIRT6 gain-of-function approaches.

Physiological overexpression of SIRT6 in cancer

SIRT6 deficiency is able to transform mouse embryonic fibroblasts, promoting tumor growth *in vitro* and tumor formation in xenograft *via* a mechanism that involves increased aerobic glycolysis (Sebastian et al. 2012). Remarkably, inhibition of glycolysis ablates their tumorigenic potential, suggesting that enhanced glycolysis is one of the driving forces for tumorigenesis in these cells (Sebastian et al. 2012). Furthermore, in order to rule out that SIRT6 deletion could leads to irreversible genomic mutations and this would tumorigenesis driver in those cells while increase glycolysis was a secondary effect, Sebastian et al. overexpressed SIRT6 in those SIRT6 depleted cells and suppressed the tumorigenic potential arguing that genomic stability is not a contributing factor (Sebastian et al. 2012). Although several evidences point SIRT6 as a tumor suppressor candidate (Mostoslavsky et al. 2006; Kaidi et al. 2010; Mao et al. 2011; Sebastian et al. 2012), is not clear if tumorigenesis is affected in the same fashion in SIRT6 overexpression context as it is in SIRT6 deletion because a comprehensive analysis relating SIRT6 overexpression and tumorigenesis is missing.

In order to assess SIRT6 physiological overexpression and its potential to be used in clinical settings as anti-cancer therapy we crossed SIRT6BAC mouse model with a mammary cancer mouse models (Guy, Cardiff, and Muller 1992). In this breast cancer mouse model the polyomavirus middle T antigen (PyMT) is expressed under the control of the promoter/enhancer mouse mammary tumor virus (MMTV) resulting in mammary epithelium transformation and production of mammary adenocarcinoma (Guy, Cardiff, and Muller 1992). Combining our *in vivo* data with several *in vitro* and xenograft assays we could demonstrate that enhanced SIRT6 activity delays cancer progression by suppressing stemness (Ioris et al. 2017). Additionally, SIRT6 anti-

cancer action is not universal, affecting mainly tumors presenting activation of PI3K signalling (i.e. PIK3CA mutation or PTEN deletion), and this effect seems to be independent of its deacetylase activity (Ioris et al. 2017).

Cancer stem cells, a subpopulation of cells with high tumorigenic capacity and self-renew ability, and PI3K signalling are clinically relevant as one is believed to be the responsible for tumor recurrence and metastasis (Batlle and Clevers 2017) and the other is found in 40% of solid tumors (Samuels et al. 2004; Millis et al. 2016; Zhang et al. 2017). Although other studies have demonstrated the requirement of SIRT6 to HSC homeostasis (Wang et al. 2016), transcriptional repression of Lin28b, a gene with critical role in stem cell pluripotency (Kugel et al. 2016) and embryonic stem cells differentiation (Ferrer et al. 2018; Zhang et al. 2018) (all based in SIRT6 loss-of-function), our study is the first to link SIRT6 and cancer stem cells repression. We demonstrated that SIRT6 suppress stem-like phenotype *in vivo*, xenograft and *in vitro* assays. Histological analyses in tumor samples revealed that SIRT6 overexpression kept those tumors more differentiated (Figure 2G) and indeed, genes that are expressed in a normal mammary gland are upregulated in tumors with enhanced SIRT6 (Figures 2H and S2G) (Ioris et al. 2017). Furthermore, comparing our transcriptomic data with public available microarray datasets, including several stem cells subtypes and terminally differentiated tissues, were genes enriched in stem cells datasets were downregulated in PyMT/SIRT6 tumors (Figure 3A). Another characteristic of cancer stem cells, high aldehyde dehydrogenase (ALDH) activity (Marcato et al. 2011; Rodriguez-Torres and Allan 2016), was also found reduced in tumor expressing SIRT6 (Figure 3C) as it was the gene expression of several ALDH isoforms (Figure S3B and C). The ALDH activity was also reduced in human cancer cell lines (Figure S1D), supporting the *in vivo* data. Additionally, using an alternative

CSC readout, the tumorsphere formation assay, we identified two subset of cells regarding responsiveness to SIRT6 overexpression, which we called SIRT6-insensitive and SIRT6-sensitive cells (Figures 1B and 4A). SIRT6-sensitive cells have reduce ability to form tumorspheres and, when injected subcutaneously in NOD/SCID animals, a reduced number of mice developed tumors and the ones that developed presented a delay in tumor onset comparing with controls, while the SIRT6-insensitive did not show difference (Figures 1C and 4B). In common, all SIRT6-sensitive cell lines have mutation or deletion leading to PI3K signalling over activation (Figure 1D) (Ioris et al. 2017).

The relationship between SIRT6 and PI3K signalling appears to be context dependent, where it can vary depending on sex, SIRT6 protein level and tissue analysed. While SIRT6 mild overexpression slight increase pAKT levels in male skeletal muscle, in a context of supraphysiological overexpression or KO there is no change (Kanfi et al. 2012; Sundaresan et al. 2012; Anderson et al. 2015). Kanfi et al., 2012, has also shown differences in pAKT levels among tissues (liver, pWAT and muscle) and between male and female animals (Kanfi et al. 2012). In our cancer study, *in vivo* (mouse mammary tumors) and *in vitro* (human breast, lung and colorectal cancer cell lines) settings showed that SIRT6 overexpression did not change the phosphorylation level of AKT (Figures S1A, S2B and S4A) (Ioris et al. 2017).

At gene expression level we found contrasting results relating lipid metabolism in liver (Figure S2B and D) (Anderson et al. 2015), mammary tumors and human cancer cell lines (Figure S4C and F) (Ioris et al. 2017). In the cancer context that we studied, performing transcriptomic (*in vivo*) and *in vitro* qPCR and chromatin immune precipitation assay using isogenic cell lines, where one cell line has only the PIK3CA wild type allele and the other has only the mutated allele (i.e. PI3K signalling activated),

we uncover a SIRT6 transcriptional suppression of a set of genes known to be activated by PI3K signalling pathway (Figures 3D, 4C, 4D, S4B, S4C and S4F) (Ioris et al. 2017). Among the genes suppressed are the Sterol regulatory element binding proteins (SREBPs), transcription factors that regulates genes expression involved in lipid metabolism, and several of its targets (Figure S4C), which is in agreement with previous report (Elhanati et al. 2013). Furthermore, we could show that PI3K indeed activate those genes (Figure S4F, compare white and grey columns) and SIRT6 overexpression brought their expression back to basal levels (Figure S4F, black columns) (Ioris et al. 2017).

Chromatin are highly dynamics during the progression of mammalian development and stem cells lineage specification (Dixon et al. 2015). In general terms, an open chromatin state (euchromatin) is a hallmark of stem cells and throughout differentiation heterochromatin progressively became predominant and pluripotency is lost (Gaspar-Maia et al. 2009; Moussaieff et al. 2015; Ugarte et al. 2015). The maintenance of euchromatin and pluripotency in embryonic stem cells is, at least in part, due to histone acetylation promoted by glycolytic production of acetyl-CoA (Moussaieff et al. 2015). Glycolysis inhibition or reduction in acetyl-CoA levels causes loss of histone acetylation and differentiation of pluripotent cells (Moussaieff et al. 2015). Is tempting to speculate that SIRT6 can induce CSC differentiation through a similar mechanism since it is a deacetylase that suppress glycolysis. Furthermore, the SIRT6 suppression of stem-like capacity of tumors by inhibiting PI3K signalling is not at odds. PI3K signalling, including its downstream targets AKT and mTOR, is well documented by its contribution in maintaining the self-renew ability of stem cells and cancer stem cells and, consequently, loss of differentiation capacity (Paling et al. 2004; Hambardzumyan et al. 2008; Zhou et al. 2009; Singh et al. 2012; Huang et al. 2015;

Yu and Cui 2016). Two recent studies have shown that SIRT6 inactivation is embryonic lethal in human and monkeys die soon after birth (Ferrer et al. 2018; Zhang et al. 2018) and in both case the lethality was due to the inability of stem cells to differentiate. Our results support the idea that SIRT6 gain-of-function leads cancer stem cells to differentiate.

Surprisingly, contrary to several studies showing SIRT6 action over different target is through its deacetylase activity, in our study the anti-stemness action of SIRT6 is independent of this activity. In fact, in ours settings we found that SIRT6 overexpression lead to increasing in H3K9ac and H3K56ac. While SIRT6 is clearly occupying the promoter region of genes controlled by PI3K signalling (Figure 4D) the acetylation status of H3K9 and K56 at the same genes is increased (or at least not decreased as expected) (Figure 4E) (Ioris et al. 2017). In addition, a SIRT6 deacetylase-dead mutant bearing a tyrosine instead of an aspartic acid at position 63 (D63Y) (Kugel et al. 2015) lead to the same phenotype than SIRT6 WT regarding the tumorsphere-formation capacity (Figure 4F) (Ioris et al. 2017). SIRT6 has other two enzymatic activities, mono-ADP-ribosyl-transferase and defattyacylase, which were neglected and thus poorly understood. SIRT6 enzymatic activity is known to be NAD+ dependent, as all the other Sirtuins, nonetheless nuclear NAD+ concentration is around 100µM and SIRT6 Km for NAD+ is around 13µM, been thus fluctuation levels of NAD+ unlike to modify SIRT6 activity (Canto, Menzies, and Auwerx 2015). Knowing that SIRT6 deacetylase activity is very low when purified SIRT6 is assayed with acetylate histone peptide and NAD+ but increase significantly when long-chain fatty acid are added into the assay (Feldman, Baeza, and Denu 2013), one can speculate that SIRT6 senses metabolic fluctuations independently of NAD+/NADH ratio switch.

CONCLUSIONS

Here we show that mimicking physiological overexpression of SIRT6 without CR increases insulin sensitivity in liver and skeletal muscle, protects the organism against diet-induced diabetes mellitus type II and reduces tumorigenic potential by suppressing cancer stem cells capacity. Our data provide evidences supporting the development of SIRT6 agonist to treat metabolic disease and cancer. Into a clinical perspective, means aiming to increase SIRT6 activity cannot be predicted to bring about side effects since its supraphysiological overexpression extend lifespan in mice (Kanfi et al. 2012).

The Sirtuin family is highly complex and our understanding of it does not even scratch the surface of it. There are much more open questions than certainties as, for examples, the interrelationship between mammalian family members. Some insights of this interrelationship was already uncovered. SIRT1 was shown to form a complex with FOXO/ NRF1 and thus regulate Sirt6 expression (Kim, Xiao, et al. 2010). Which is in odd with the results showing that SIRT1 and SIRT6 have opposite function in the regulation of PGC-1α (Dominy et al. 2012). SIRT1 is also involved in at least other two mechanisms with other Sirtuins, on with SIRT7 impairing SIRT1 autodeacetylation and thus its activation (Fang et al. 2017) and the other where SIRT1 and SIRT4 have opposite related to insulin secretion (Haigis et al. 2006; Moynihan et al. 2005). Another degree of complexity among mammalian Sirtuin family is related to the target specificity. Sirtuins appears to have overlapping functions and differently regulate share target proteins as, for example, the regulation of FOXO family (Kim, Xiao, et al. 2010; Wang et al. 2007; Sundaresan et al. 2009), PGC-1α (Nemoto, Fergusson, and Finkel 2005; Dominy et al. 2012), NF-κB (Yeung et al. 2004; Rothgiesser et al. 2010;

Zhang, Li, et al. 2016), Myc (Menssen et al. 2012; Jing et al. 2016) and HIF1α (Krishnan et al. 2012; Finley et al. 2011).

SIRT6, as discussed above, also has its own degree of complexity. Apart from the one cited above, another conundrum involving SIRT6 is the fact that it increases chemotherapy resistance in breast cancer (Khongkow et al. 2013) but decreases it in melanoma (Strub et al. 2018). Are those opposite roles governed by different enzymatic activity? Is SIRT6 able to read differently cues depending to the context? What are the SIRT6 defatty-acylation and mono-ADP-ribosylation physiological targets?

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