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Doctoral dissertation

Role of the histone deacetylase SIRT7 in tumor invasion and metastasis through epigenetic regulation of E-cadherin

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Dedicated to don Alvaro and nonno Alfredo

Chapter 1 - INTRODUCTION

1.1. Sirtuins, a unique class of lysine deacetylases

During the last decade, the members of the Sir2 family, also known as sirtuins, have become firmly established as key regulators of the response to stress of various types, from metabolic to genotoxic stress. Sirtuins have been implicated in the most important human diseases such as cancer, cardiovascular diseases, diabetes and other endocrine pathologies, malaria, and neurodegenerative diseases, among others[1; 2].

Sirtuins were originally described as NAD^+-dependent histone deacetylases[3] and were included in the superfamily of histone deacetylase (HDAC) enzymes as class III HDACs. In fact, there are 4 classes of HDACs: class I (HDAC1, 2, 3, and 8), which are closely related to the yeast transcriptional factor RPD3; class II (HDAC4, 5, 6, 7, 9, and 10), which are similar to another yeast deacetylase HDA1; class III, which includes sirtuins; and class IV (HDAC11). Indeed, the process of deacetylation differs markedly between sirtuins and all other HDACs. While class I, II, and IV HDACs transfer the final acetyl group to the aqueous solution and are sensitive to the inhibitor trichostatin A (TSA), sirtuins require NAD+ as an enzymatic co-factor, transfer the acetyl group from the substrate to an ADP-ribose molecule, and are insensitive to TSA[4]. Interestingly, ADP-ribosyltransferase activity is also known in sirtuins, although our knowledge about this is currently very limited[5; 6].

The members of the Sir2 family have been present since they evolved in prokaryotes. They have subsequently undergone considerable functional diversification during the course of evolution in order to adapt to increased complexities. For instance, mammals harbor 7 different sirtuins (SIRT1-SIRT7) that differ in their cellular localization, substrate specificity, and functions[7]. Sirtuins seem to have developed in some types of bacteria as regulators of the metabolic adaptation to energetic fluctuations. Although we do not yet fully understand all the implications of sirtuin functions in prokaryotes, their ability to deacetylate proteins may have first appeared as a mechanism to catabolize acetate before adapting specifically to perform regulatory functions. In this sense, one of the best-conserved sirtuin functions is the regulation of the intermediate metabolism through control of the key enzyme acetyl–CoA synthetase (ACS). The Sir2 family members encompass all the main phylogenetic domains of living organisms, bacteria, archaea and eukaryotes, although not all prokaryotes contain sirtuins. Phylogenetic studies have defined five lineages or classes of sirtuins: classes I to IV and U[8]. The seven mammalian sirtuin genes

include all four classes: SIRT1, SIRT2, and SIRT3 are class I, SIRT4 is class II, SIRT5 is class III, and SIRT6 and SIRT7 are class IV. Prokaryotes and eukaryotes share only classes II and III. Consistent with this, the eukaryotic members of classes II and III show mitochondrial localization and target mitochondrial proteins. Interestingly, the two eukaryote-specific lineages (I and IV) seem to have appeared in early eukaryotes probably at the same time as chromatin.

The number of sirtuins per organism appears to have increased during evolution along with complexity, from the presence of 1 member in prokaryotes to 2 in *Plasmodium*, 4 in *Caenorhabditis elegans*, 6 in *Drosophila*, and 7 in mammals. This probably reflects a constant dynamic acquisition of new functions associated with the response of metabolic homeostasis to different types of stress. Since the evolution of the prokaryotes, sirtuins seem to have been involved in the crosstalk between the genome and environmental changes. The functional diversification of Sir2 homologs during evolution is clearly illustrated by their different cellular locations[9]. Three of them (SIRT1, SIRT6, and SIRT7) are clearly localized in the nuclear compartment; in particular, SIRT7 is mostly restricted to the nucleolar region. However, SIRT1 is known to shuttle to the cytoplasm[10; 11]. Meanwhile, SIRT3 to SIRT5 proteins are mitochondrial proteins with well-known mitochondrial substrates, although full-length SIRT3 is also found in the nucleus under normal conditions[12]. SIRT2 is the only mammalian sirtuin localized mainly in the cytoplasm, although it has been found to translocate to the nucleus during G2/M transition[13] and during bacterial infection[14].

1.1.1. Structure and enzymatic activity

The high degree of conservation among Sir2 family members between bacteria and humans is restricted to their catalytic domain, a region of approximately 250 residues[15]. Eukaryotic sirtuins have developed amino (N)– and carboxy (C)–terminal extensions that are divergent among the members of the family[16] and that have allowed the acquisition of specific new functions and substrates during evolution. This variety of terminal regions has been proposed as explaining the diversity of sirtuin functions, including the regulation, recruitment, and differential activity of each of the family members[17]. The Sir2 family structure is based on an NAD^+ binding Rossmann-fold domain and a Zn^{2+} binding domain. The catalytic site is situated inside a hydrophobic channel

formed between these two binding domains, so that the end of the acetyllysine chain is located close to the nicotinamide ribose of NAD^{+}[18]. As expected, mutations in the conserved residues along the NAD^{+} binding domain disrupt HDAC activity[19]. However, in contrast to other classes of HDACs, Zn^{2+} does not have a catalytic function but a structural role. The mechanistic similarities and significant conservation of the catalytic domain between sirtuins and the superfamily of poly (ADP-ribose) polymerases (PARPs) strongly suggest that sirtuins are directly related to these enzymes.

One of the most important aspects of sirtuin biology is the dual enzymatic nature of the family. Sirtuins harbor two types of related enzymatic activity: deacetylase activity, which in some metabolic contexts can also be defined more generally as a deacylase activity[20; 21], and mono-ADP-ribosyltransferase (ADPRT) activity. Both appear to derive from the general enzymatic reaction of sirtuins, proposed by Sauve *et al*. in 2001[22] (Figure 1). First, the enzyme binds to NAD^{+} in the presence of a substrate. Second, it breaks the NAD^{+} molecule, releasing nicotinamide and retaining the resulting ADP-ribose molecule. Third, in the case of deacetylation, the enzyme transfers the acetyl group from the substrate to the ADP-ribose molecule, releasing *O*-acetyl-ADP-ribose (*O*AADPr). Alternatively, the ADP-ribosyltransferase is active when, similarly to what occurs with PARPs, the sirtuin enzyme transfers the ADP-ribose molecule to another protein. The *O*AADPr molecules generated in the deacetylation reaction are themselves a potential second messenger. The exact molecular functions of *O*AADPr remain elusive, although studies have suggested that *O*AADPr promotes pathways that suppress ROS accumulation.

At present, we do not completely understand the nature of this catalytic duality. Our current knowledge suggests that the preeminence of any of these activities or the existence of both in a given sirtuin may be related to specific differences between lineages. For instance, the best-studied class II sirtuin, SIRT4, appears to be mainly an ADP-ribosyltransferase, while the vast majority of class I sirtuins, such as mammalian SIRT1 to SIRT3 or yeast sirtuins, show robust deacetylase activity. Evidence suggests that class III and IV sirtuins may generally exhibit both activities (SIRT6, pfSir2A, CobB), depending on the substrate and functional context. However, given our currently limited knowledge of sirtuin substrates outside mammals, these conclusions are not definitive and will require periodic reconsideration. For instance, we cannot rule out the possibility that all sirtuins may harbor both enzymatic activities,

using one or the other in different contexts and with the appropriate substrates. Some groups have suggested that this general activity is more likely to be an inefficient side effect associated with the deacetylase activity[6]. However, the fact that an acetylated residue is not required for ADPRT activity[23] and that certain point mutations in the conserved catalytic domain of SIRT6 can stop its deacetylation activity without altering its ADPRT activity and *viceversa*[24], suggests that the two enzymatic activities are different. This matter is still open to debate.

Some recent studies have also revealed a previously unknown enzymatic activity that recall the original direct involvement of sirtuins in metabolism: long-chain deacylation. The mitochondrial SIRT5 was identified as an efficient desuccinylase and demalonylase[20], and the removal of a myrystoil group from the tumor necrosis factor-α (TNF-α) in the endoplasmic reticulum was attributed to SIRT6[21]. Long-chain deacylation was later identified as a general feature of most mammalian sirtuins, ranging from SIRT1 to SIRT6[25]. The activation mechanism is consistent with fatty acid inducing a conformation that binds acetylated H3 with greater affinity. Consistent with this, several biologically relevant free fatty acids, including myristic acid, at physiological concentrations induced up to a 35-fold increase in deacetylation efficiency of SIRT6 on H3K9Ac peptides[25].

Sirtuins were originally identified in 1996, as ADP-ribosyltransferases, when the *Salmonella typhimurium* protein CobB was found to compensate for the absence of CobT in the synthesis of cobalamin (vitamin B12). In 2000, NAD^+-dependent histone deacetylase activity was reported in yeast Sir2p, the founding member of the family, and was shown to be essential for the role of Sir2p in silencing[26]. With the study of the mammalian members of the family, SIRT1 to SIRT7, it soon became clear that sirtuin deacetylase activity was not restricted to histones, encompassing a whole new world of non-histone substrates. The first of these substrates, identified for mammalian SIRT1, was the tumor suppressor p53. Since then, the list of non-histone substrates of the members of the family has grown so long to include metabolic enzymes, chromatin machinery factors, key transcription factors, cytoskeleton subunits, and many others. A puzzling aspect of sirtuin activity is that there does not seem to be a defined consensus sequence in the regions they target, as a group or individually.

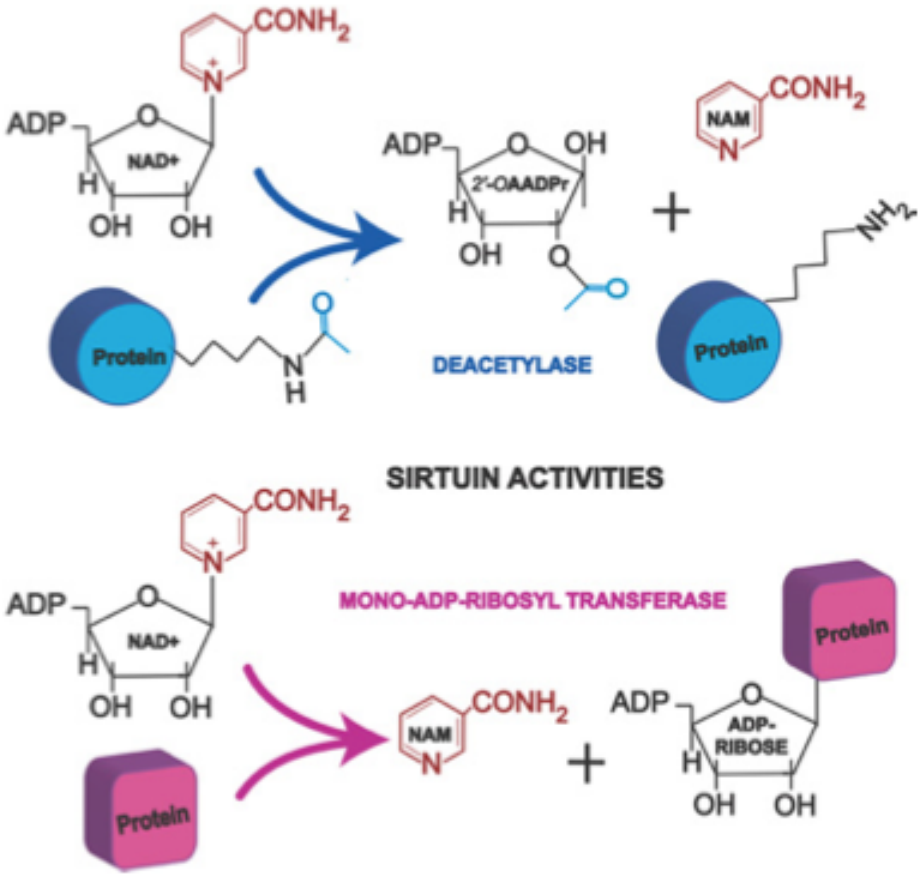

Figure 1. Sirtuin catalytic mechanisms (Michan S and Sinclair D, Biochem J 2007).

1.1.2. Sirtuin chromatin regulators

Despite its prokaryotic origin, the development of chromatin in eukaryotes appears to have been a milestone in sirtuin history, since they underwent major adaptation that enabled them to signal stress conditions to the genome[26]. For this purpose, two new lineages intimately related to chromatin, classes I and IV, seem to have arisen in the early stages of eukaryote evolution. The functions regulated by this "chromatin adaptation" range from the control of metabolism homeostasis and survival upon stress to the protection of genome stability. Sirtuins perform these chromatin functions through three mechanisms.

The main mechanism involves the transcriptional silencing of a particular region, which may encompass a single gene, a defined set of genes, or a whole locus. In most of the cases studied, the silencing established by sirtuins is epigenetic and involves the formation of compacted heterochromatin

structures. Two loci have been found to be consistently epigenetically regulated by sirtuins in early eukaryotes onwards: nucleolar rDNA transcription and subtelomeric regions. Both seem to reflect functional adaptations of sirtuins for regulating different types of stress through chromatin. In the first case, it appears to be linked to the response to metabolic and energetic stress in order to control ribosome expression and thereby protein production. This is a very significant regulatory process since it is an energetically expensive process that is key to regulating proliferation. The second case, the subtelomeric region, is mainly related to genotoxic stress and is directly associated with a second conserved mechanism of sirtuins in chromatin: the regulation of chromatin structure and organization in order to maintain genome stability. The most obvious cases are the conserved regulation of telomere structure by sirtuins from unicellular protozoa and yeast to humans. Sirtuins have also adapted to regulate the other great structural chromosomal region or constitutive heterochromatin, the pericentromeric area. Constitutive heterochromatin refers to the regions that tend to have a structural role and never decompact, such as centromeres and telomeres, in contrast to facultative heterochromatin, which corresponds to regions that can be compacted in response to certain programs or stimuli, such as development, stress response, or differentiation, and that can decompact when required. Thus, in fission yeast *S. pombe* and mammals, spSir2p and SIRT1 are required for the formation and maintenance of pericentromeric heterochromatin structure[27]. Another functional aspect closely associated with the role of sirtuins in protecting genome integrity is their conserved role in signaling DNA damage and DNA repair, as we discuss below. Finally, another interesting aspect of sirtuins in chromatin is associated with the global control of cell cycle progression, as has been shown for mammalian SIRT2.

Sirtuin chromatin-associated functions are largely realized through the modulation of epigenetic information by direct deacetylation of specific histone acetylation marks (Figure 2). In this regard, two modifications have been widely conserved during evolution and are functionally relevant to the function of sirtuins: acetylation of histone H4 in lysine 16 (H4K16Ac) and acetylation of histone H3 in lysine 9 (H3K9Ac).

H4K16Ac has exclusive properties due to its unique role in regulating chromatin structure[26]. Its presence inhibits the folding of the chromatin fiber *in vitro* and therefore, as has been suggested, also inhibits the formation of

higher orders of chromatin compaction. Acetylation/deacetylation of H4K16 has been associated with epigenetic phenomena throughout evolution, from silencing in *S. cerevisiae*, through X-chromosome dosage compensation in *Drosophila*, to silencing in mammals. H4K16Ac has also been linked to the regulation of cell cycle progression, transcription, DNA repair, and DNA replication. Moreover, hypoacetylation of H4K16 has been proposed as a hallmark of cancer. The functional link between sirtuins and H4K16Ac is mainly restricted to the class I sirtuins, including yeast Sir2p and mammalian SIRT1 to SIRT3[12; 28; 29].

The behavior of the other silencing-related mark, H3K9, is very different from that of H4K16. Deacetylation of H3K9 is a requirement for subsequent methylation in the same residue, H3K9me2/3, a hallmark of higher orders of chromatin compaction or heterochromatin conserved from amoeba to humans. Among mammalian sirtuins, class I SIRT1 and class IV SIRT6 are the most functionally important H3K9Ac deacetylases. SIRT6 H3K9Ac deacetylase activity is important for modulating telomere structure and DNA repair of double-strand breaks (DSBs)[30; 31].

In the case of mammalian SIRT1, deacetylation of H4K16Ac and H3K9Ac is directly associated with the capacity of SIRT1 to coordinate the formation of constitutive and facultative heterochromatin. Mammalian SIRT3 is mainly a mitochondrial protein that acts as the primary mitochondrial lysine deacetylase[32]. However, a small SIRT3 subpopulation localizes in the nucleus, where it participates in the repression of key stress-related genes through deacetylation of their promoters in H3K9Ac and H4K16Ac[12; 33]. It is of particular note that H4K16Ac deacetylation by SIRT2 is related to cell cycle control and not to heterochromatin formation. During G2/M transition, SIRT2 is shuttled to the nucleus, where it deacetylates H4K16Ac globally before entering mitosis[29].

Recently, a newly identified modification involved in transcriptional regulation, H3K18Ac, has been linked to another class IV sirtuin, SIRT7[34]. SIRT7 deacetylation and the consequent silencing of a specific set of genes were shown to be crucial for maintaining the transformed phenotype in cancer cells. Additionally, the enrichment of SIRT7 in nucleoli also underlines the positive function of SIRT7 activity in regulating RNA polymerase I transcription and cell growth. Conversely to SIRT1, which silences rDNA by deacetylating H4K16Ac and H3K9Ac, SIRT7 binds directly to the RNA polymerase I complex[35] and deacetylates the PolI subunit PAF53[36], exerting a positive effect on rDNA

transcription. In addition, H3K18 has been recently shown to be a deacetylation target of SIRT2. During infection with the bacterium Listeria monocytogenes, the host SIRT2 translocates to the nucleus in a manner dependent on the bacterial factor InlB[14]. SIRT2 associates with the transcription start site of a subset of genes repressed during infection and deacetylates H3K18. Thus, SIRT2-mediated H3K18 deacetylation plays a critical role during infection, which reveals an epigenetic mechanism imposed by a pathogenic bacterium to reprogram its host.

Sirtuins have also been linked to DNA damage signaling and DNA repair through the deacetylation of another mark, H3K56Ac. This is involved in DNA damage signaling during S phase and is targeted by mammalian SIRT6[37; 38]. Mammalian SIRT1 and SIRT2 are also believed to deacetylate H3K56Ac upon DNA damage.

Another interesting functional relationship between histones and sirtuins involves the linker histone H1. Mammalian SIRT1, the ortholog of S. cerevisiae Sir2p, has been shown to bind directly and deacetylate the histone H1 isoform H1.4 in lysine 26 (H1K26) during the formation of facultative heterochromatin in mammals.

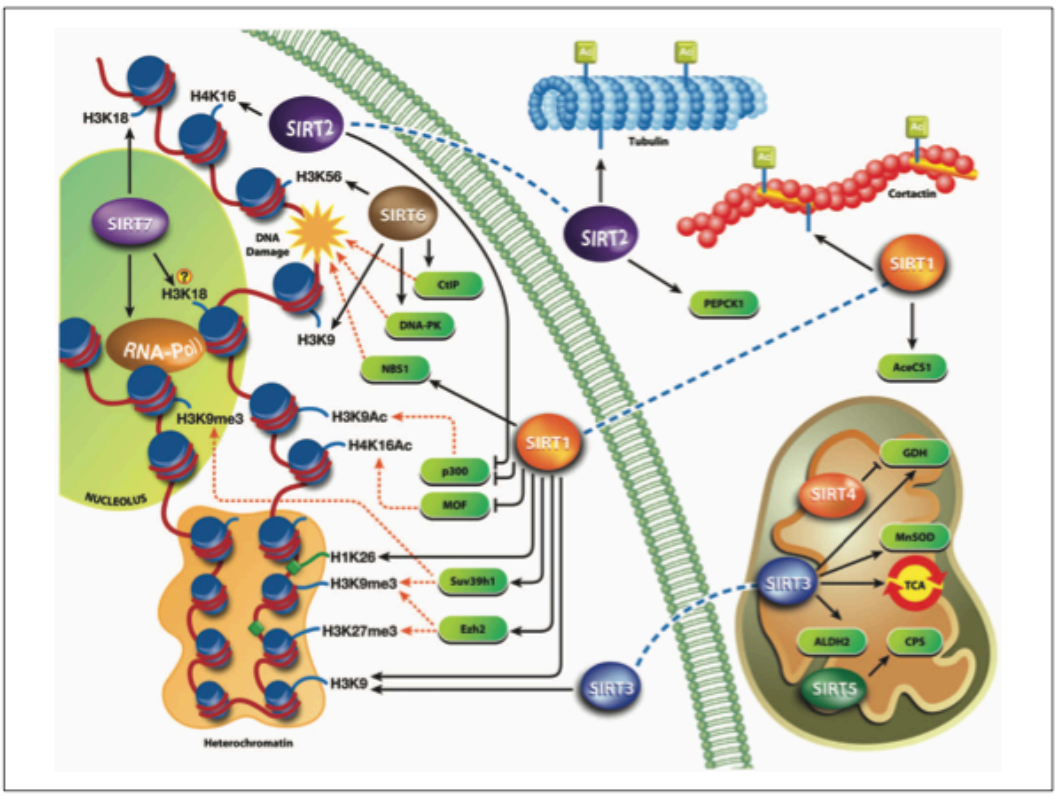

Figure 2. Sirtuin functions and targets in the cell with a focus on sirtuin chromatin–related functions (Martinez-Redondo *et al.* Genes Cancer 2013).

1.2. Emerging roles of SIRT7 in cancer

SIRT7 is possibly the least understood mammalian sirtuin, but has several features that suggest it may have unique cellular functions that are important for human disease, particularly cancer. First, SIRT7 is a nuclear, chromatin-associated lysine deacetylase that selectively removes a specific histone mark, acetylated H3K18 (H3K18Ac), depletion of which is associated with highly malignant cancers and poor patient prognosis[34]. In addition, SIRT7 is enriched in nucleoli[9], sub-nuclear structures that are the sites of ribosome assembly and are increased in size and number in aggressive tumors[39]. Indeed, SIRT7 impacts on ribosome biogenesis through multiple mechanisms and may thereby play a major role in supporting the high biosynthetic demands of cancer cells. Through these and other functions, SIRT7 is a central coordinator of epigenetic and metabolic programs that support cancer progression.

1.2.1. Reprogramming tumor suppressive gene expression by selective H3K18 deacetylation

Cancer cells exhibit epigenetic chromatin alterations in histone marks both at the global genome level and at specific gene regulatory sequences. Clinico-pathological studies have shown that in tumor tissues, low levels of H3K18Ac, a histone mark associated with transcriptional activation, correlate strongly with cancer disease severity. Indeed, global hypoacetylation of H3K18 can be prognostic of aggressive cancer phenotypes, increased risk of cancer recurrence, and poor patient survival[40; 41; 42]. H3K18Ac hypoacetylation is also linked to epigenetic reprogramming during oncogenic transformation by viral oncoproteins[43; 44]. Thus, H3K18 hypoacetylation is a potential biomarker for advanced disease in human cancer, and changes in the genome-wide distribution of H3K18Ac are proposed to control epigenetic gene expression programs that drive cancer progression.

Many lysine deacetylases are relatively promiscuous, and SIRT7 stands out in being highly selective for specific substrates and physiologic contexts. At chromatin, SIRT7 specifically deacetylates H3K18Ac, but not numerous other histone acetylation sites[34]. At present, SIRT7 is the only known H3K18Ac-specific deacetylase enzyme, and it plays an essential role in establishing the genome-wide landscape of H3K18Ac. SIRT7 selectively deacetylates H3K18Ac at

promoters of a network of genes with multiple links to tumor suppression[34]. The spectrum of SIRT7 target genes is defined in part by the interaction of SIRT7 with the sequence-specific ELK4 transcription factor (Figure 3A), which is implicated in prostate and other cancers. When SIRT7 is inactivated in cancer cells, H3K18 hyperacetylation leads to up-regulation of the tumor suppressive gene network and reversal of essential cancer cell phenotypes that are hallmarks of oncogenic transformation. Most strikingly, depletion of SIRT7 is sufficient to reduce the tumorigenicity of cancer cells in mouse xenograft assays *in vivo*[34; 45]. Thus, SIRT7 plays a fundamental role in epigenetic maintenance of the neoplastic state of cancer cells.

Another major category of SIRT7 target genes consists of ribosomal protein genes, which are transcriptional targets of the oncogene Myc[34; 46] (Figure 3A). Dysregulated protein translation and mutations of individual ribosomal proteins are linked to cancer in multiple settings[47]. In a recent study, Shin and colleagues showed that by opposing Myc-dependent expression of ribosomal proteins, SIRT7 plays an adaptive role in the Unfolded Protein Response (UPR) to suppress ER stress[46]. This study focused primarily on ER stress and the UPR in the context of liver pathology, where chronic ER stress in SIRT7-deficient mice leads to fatty liver disease. However, up-regulation of the UPR is also important in many cancers, where rapid cell growth and hypoxic conditions can trigger ER stress. The UPR allows cancer cells to evade ER stress-induced apoptosis, in part by transiently reducing protein synthesis rates[48]. In this context, SIRT7 can prevent ER stress-induced apoptosis of cancer cells in a Myc-dependent manner. Thus SIRT7-mediated repression of ER stress through control of ribosomal protein gene transcription might be an underlying mechanism that promotes cancer cell survival and tumor progression.

It is likely that SIRT7 also influences other cancer-regulatory gene expression pathways, perhaps in specific cancer cell types or tumor conditions. For instance, Myc coordinates a broad transcriptional program that promotes cancer cell proliferation, survival, and metabolism through many targets[49]. SIRT7 might co-repress oncogenic Myc functions in such settings, with potentially tumor suppressive effects. Other *in vitro* evidence suggests that SIRT7 might directly regulate genes that control cancer cell adaptations to hypoxia, by possibly interacting with the hypoxia inducible factors HIF-1a and HIF-2a[50]. Finally, SIRT7 is proposed to inhibit signaling by the tumor suppressor p53 and its target gene p21. Increased p53 acetylation and activity were

observed in SIRT7-deficient mouse cardiomyocytes, although there is conflicting data on whether SIRT7 deacetylates p53 directly[9; 34; 51]. SIRT7 might also repress transcription at p53-dependent promoters by H3K18Ac deacetylation, or inhibit the p53 pathway indirectly. For example, in hepatocellular carcinoma cells, SIRT7 is proposed to inhibit transcriptional activation of p21 through tumor suppressive microRNAs, miR-125a-5p and miR-125b[45]. In addition, up-regulation of rRNA synthesis, which is induced by SIRT7 (see below), can promote proteasomal degradation of p53[52].

In summary, much evidence indicates that SIRT7 plays an important role in the maintenance of epigenetic patterns of H3K18 acetylation in cancer cells, which in turn drive gene expression programs that stabilize the transformed phenotypes of these cells. Future work should uncover additional pathways through which SIRT7 influences cancer cell epigenetics, through histone deacetylation at chromatin or novel mechanisms[53].

1.2.2. Nucleolar guardian of ribosome biogenesis and protein homeostasis

Nucleoli are factories where ribosomal RNA (rRNA) is expressed and assembled with ribosomal proteins into ribosomal complexes. Metabolically active tumor cells show dramatically increased nucleolar size and number, which support the increased protein synthesis requirements of these cells[39]. Indeed, enhanced rRNA synthesis is now proposed to be an essential hallmark of cancer cells[54]. Early studies showed that SIRT7 is enriched in nucleoli, where it associates with PolI and rDNA sequences (Figure 3B). This finding was intriguing, because yeast Sir2p also localizes to rDNA in nucleoli, and one of its central functions is to suppress rDNA transcription by histone deacetylation[55]. Surprising, however, SIRT7 was found to activate, not repress, rDNA transcription[56]. Moreover, reduced rRNA synthesis in SIRT7-depleted cancer cells was associated with decreased cell viability and proliferation[56]. This observation suggested that increased SIRT7 activity in cancer cells might fuel tumor growth by promoting rRNA synthesis and ribosome biogenesis.

The effect of SIRT7 on rRNA synthesis depends on an intact SIRT7 catalytic domain, but the molecular substrate of SIRT7 in this context was not initially clear. Indeed, histone deacetylation by SIRT7 would have the opposite effect on rDNA transcription. Recent findings now reveal that SIRT7 can control transcription of rDNA through deacetylation of a new substrate, the PAF53

subunit of PolI, which facilitates recruitment of PolI to rDNA sequences[36] (Figure 3B). Additional protein interactions of SIRT7 with several nucleolar chromatin remodeling complexes with rDNA regulatory activities (e.g., NoRC, B-WICH) might also impact on rDNA transcription[35].

Recent studies provide direct evidence that SIRT7 is important for efficient protein translation and implicate additional molecular mechanisms (Figure 3B). SIRT7-depleted cells have substantially reduced rates of protein synthesis, and a functional network analysis of the SIRT7 interactome identified several SIRT7-enriched factors with tight links to ribosome dynamics and protein translation[57]. For example, SIRT7 interacts with mTOR and the TFIIIC2 transcription factor, which control PolIII-mediated synthesis of transfer RNA (tRNA). In addition, SIRT7 interacts with multiple ribosomal proteins, which might directly influence ribosome function. The effects of SIRT7 on alleviating ER stress during the UPR, described above, may also contribute to promoting efficient protein translation. Indeed, a reduction in active polysomal ribosomes is characteristic of ER stress[58], and is observed in SIRT7-deficient cells[46].

Together, this body of work has identified SIRT7 as a global regulator of diverse aspects of ribosome biogenesis and protein translation. Reprogramming of cellular metabolism and biosynthetic machinery towards anabolic processes is crucial to fuel the unlimited cell growth and division of cancer cells[59]. Increased ribosome biogenesis and rates of protein synthesis are required for the elevated levels of cell proliferation in cancer, and this likely constitutes one general paradigm through which SIRT7 impacts on cancer biology[53].

1.2.3. SIRT7 modulation for epigenetic cancer therapy

Genome-wide loss of epigenetic stability is a common feature of diverse tumors and plays a significant role in cancer development[60], but unlike DNA mutations, which are permanent, epigenetic changes are potentially reversible. Because of its effects on the chromatin landscape and malignant phenotypes of cancer cells, SIRT7 is a promising target for epigenetic cancer therapy. Moreover elevated SIRT7 expression has been observed in multiple human cancer tissues, including prostate, hepatocellular, breast, thyroid, and other carcinomas[34; 45; 61; 62]. Analysis of large HCC patient cohorts revealed that SIRT7 is overexpressed by >1.8 fold ($p<0.0001$) in cancer tissues compared to normal controls[45], and in microarray analyses, relative SIRT7 expression increased 2-,

3.5-, and 4.5- fold in tumors of increasing grade (G1-3), respectively, compared to premalignant samples (p-values <0.05, 0.001, 0.001)[45]. These observations strongly suggest that up-regulation of SIRT7 in cancer cells may contribute to the malignant phenotype of human patient tumors.

On the other hand, it will be important for future translational studies to determine whether pleiotropic and potentially cell type-specific functions of SIRT7 might influence overall cancer incidence and tumor progression in unexpected ways. For example, it is still unclear how SIRT7 impacts on the process of oncogenic transformation itself. Indeed, in early stages of cancer initiation in premalignant cells, it is possible that SIRT7 might have tumor suppressive effects. This could occur through its repression of oncogenic Myc-dependent genes or other SIRT7 gene targets that have not yet been identified. The tools are now available to ask how SIRT7 activity influences both the efficiency of oncogenic transformation of primary human cells, and the chromatin landscape of H3K18Ac and gene expression programs in these cells both prior to and during induction of cellular transformation.

It has been also speculated that SIRT7 might have differential effects depending on the particular genetic elements that underlie neoplastic transformation in specific tumors[53], and this can be modeled in pre-clinical studies using cellular transformation assays in primary human cells and mouse tumor models[63]. For example, given its functional interplay with MYC-, ELK4-, and E1A-dependent epigenetic programs, SIRT7 activity might be particularly important for neoplastic transformation programs and tumors associated with these factors. Finally, the effects of SIRT7 on spontaneous tumor development in mice are not yet known. SIRT7-deficient mice have been reported to die from causes unrelated to cancer at seven months of age[51], precluding analysis of long-term tumor incidence. However, a different SIRT7-mutant mouse strain does not show this premature lethality[46], and these mice should be studied for tumor development and survival.

The possibility of SIRT7 inactivation as a pharmacologic strategy in cancer therapy is complicated by evidence that SIRT7 might have beneficial effects on human health. Knockout mice lacking SIRT7 develop degenerative pathologies associated with aging, such as kyphosis, loss of subcutaneous fat, and degenerative cardiac hypertrophy[51]. Moreover, the increased ER stress in SIRT7-deficient mice leads to fatty liver pathology, which in humans, predisposes to cirrhosis and hepatocellular carcinoma[46]. ER stress is also

implicated in other disease processes, from pancreatic beta cell failure and insulin resistance to neurodegeneration. It will be important to determine whether SIRT7 activity influences the spontaneous or induced development of these or other pathologies in mice, and to ask whether SIRT7 levels or mutations are associated with metabolic or neurodegenerative disease in human patients. In addition, the transcriptional programs that are regulated by SIRT7 in the context of normal human physiology have not yet been examined systematically. An important goal for future work will be to carry out genomic studies of the chromatin landscape of SIRT7 binding in non-cancer cells and mammalian tissues, and the effects of SIRT7 activity on H3K18Ac patterns and gene expression. Finally, it might be possible "dial down" SIRT7 activity pharmacologically to levels that might attenuate cancer progression, without being sufficient to induce the disease processes observed in mice completely lacking SIRT7. Thus, development of compounds that can modulate SIRT7 activity will be instrumental in examining these questions.

Several features of SIRT7 suggest that chemical modulators of SIRT7 activity could be designed to have relatively high levels of biological specificity. First, cross-reactivity of SIRT7 modulators with other mammalian sirtuins might be minimized by taking advantage of unique features of SIRT7's structure and enzymatic mechanism. The conserved catalytic domains of sirtuins are flanked by variable N- and C- terminal extensions. Within the conserved domain, SIRT7 is only ~40% similar to its closest mammalian family member SIRT6, and <30% similar to the others. In addition, the non-conserved N- and C- terminal regions have been shown in other sirtuins to influence catalytic activity and contain sequences that are bound by endogenous and chemical regulators[64; 65; 66]. Development of compounds that target these unique regions of SIRT7 might be a promising strategy. Similarly, SIRT7 gene regulatory activity depends on interactions with specific binding partners (e.g., MYC, ELK4), and such interactions might be selectively targeted pharmacologically to enable pathway-specific modulation of SIRT7 function. The identification of distinct substrates of SIRT7 also suggests the possibility of substrate-selective activity modulation, which has recently been demonstrated for SIRT1-activating compounds (STACs)[67; 68].

Finally, there are indications that SIRT6 and SIRT7 are unusual among Sirtuins in requiring activation by endogenous regulators for efficient catalytic activity in cells, and such regulatory mechanisms might offer additional useful drug

targets. This notion is supported by the observations that purified SIRT6 and SIRT7 proteins have relatively weak deacetylase activity on peptide substrates *in vitro*, despite the clear importance of their enzymatic activities for cellular and whole organism physiology[69]. For SIRT6, the efficiency of histone deacetylation can be stimulated ~35-fold by certain free fatty acids (FFAs)[25]. The sensitivity of SIRT6 to FFA activation results from structural features of SIRT6 that may be shared with SIRT7 but not other mammalian sirtuins. For example, the low intrinsic activity of SIRT6 enzyme is proposed reflect an unusually "splayed" conformation that binds acetylated substrate poorly[69]. Binding of FFAs to SIRT6 can induce a conformational change to a more enzymatically active structure[25]. The unusual conformation of SIRT6 is partly due to its lack of a conserved helix bundle region that forms important structural contacts in most other sirtuins[69]. Although the structure of SIRT7 has not yet been characterized, predictions based on sequence comparisons indicate that SIRT7 also lacks this domain[69]. It remains to be shown whether SIRT7 requires activation by FFAs, but if so, this regulation would provide a link of SIRT7 activity to metabolic conditions that might be targeted through pharmacologic or dietary regimens. Structural and biochemical studies of SIRT7 in this context should provide invaluable insights into how the binding by FFAs might be exploited for therapeutic strategies.

In conclusion, mammalian sirtuins have been the subject of much excitement as potential therapeutic targets for treating aging-related, metabolic, and neurodegenerative disease. A large effort has focused on STACs, which have numerous beneficial health effects in mice. The selectivity of STACs for SIRT1 highlights the structural differences among sirtuins and the possibility of sirtuin-selective targeting[68; 70; 71]. Several chemically diverse small molecule inhibitors of Sirtuins also have therapeutic potential and varying degrees of specificity[70; 71; 72]. By contrast to other Sirtuins, SIRT7 has not been amenable to screens for small molecule modulators, because it does not efficiently deacetylate the substrates used in such screens, and its physiologic substrates were only recently discovered[34; 36]. Thus, a major barrier in the field has finally been overcome, and it should now be possible to design strategies to screen for SIRT7-specific modulating compounds. Thus, these are early days for conceptualizing the translation of SIRT7 biology into clinical applications. Current findings suggest that pharmacologic inhibition of SIRT7 might allow for simultaneous attack on both the epigenetic programming and metabolic

machinery of cancer cells that support tumor progression. However, deeper understanding of SIRT7 biology at the molecular and physiologic levels will be essential for elucidating the potential therapeutic benefits and detrimental side effects of SIRT7 inactivation[53].

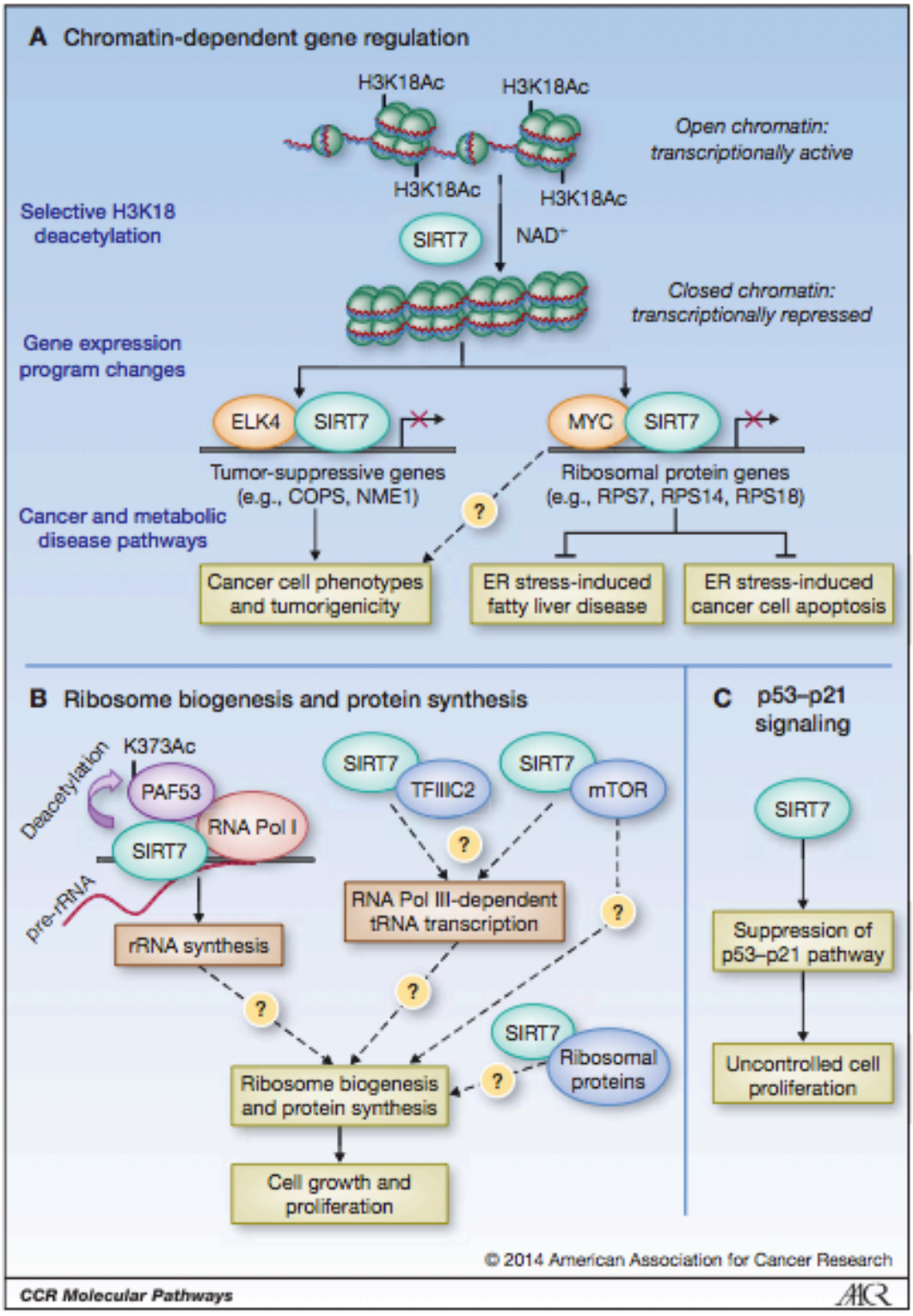

Figure 3. Molecular pathways of SIRT7 and their known or predicted effects on cancer cell

biology. Question marks indicate connections that are predicted but not yet demonstrated experimentally (Paredes S, Villanova L and Chua KF. Clin Cancer Res 2014).

1.3. The tumor metastatic cascade

As the culprit behind most cancer-related deaths, metastasis is the ultimate challenge in our effort to fight cancer as a life-threatening disease. Overt metastasis is the end result of a multistep process that involves dissemination of tumor cells to distant organs and subsequent adaptation to foreign tissue microenvironments. The explosive growth of metastasis research in the past decade has yielded an unprecedented wealth of information, and many traditional notions have been challenged.

The metastatic propensity of a given tumor is influenced by both genetic mutational events and cell of origin. The same target cell hit by different oncogenic driver mutations can give rise to tumors with distinct metastatic potential. However, more recent studies have revealed that the cell of origin also contributes to the malignancy of a tumor. Indeed, the same oncogenic alterations, when occurring in cells of different lineages or at different stages of differentiation, may lead to tumors with distinct metastatic behaviors.

In contrast to the conventional model that metastasis is a late event in tumor progression, increasing evidence suggests that tumor cells can disseminate from the earliest preneoplastic lesions, sometimes even before the formation of overt primary tumors[73]. In patients with localized disease such as ductal carcinoma *in situ*, disseminated tumor cells (DTCs) can be detected in the bone marrow, suggesting early dissemination despite the lack of stromal invasion or even overt primary tumor formation. Whether early and constant shedding of DTCs occurs in all tumors and what the link is between these early DTCs and ultimate metastasis remain unclear.

Metastatic tumor cells can follow different evolution paths. According to a “linear” progression model, the heterogeneity of the primary tumor results in clonal selection, during which the dominant clone expands and dominates over the others, with additional mutational changes occurring within the clonal population, hence resulting in different degrees of tumor heterogeneity. The acquisition of metastatic competence and organ tropism occurs predominantly, if not entirely, within the primary tumor. When disseminated, these

heterogeneous cells seed and colonize different organs. Additional site-specific subclonal changes could occur that endow these DTCs with additional metastatic properties that are needed for the formation of overt metastases. In contrast, a different body of clinical evidence suggests a “parallel” progression model, where dissemination starts early, and different tumor cell clones are seeded in parallel to different organs. These DTCs remain dormant or develop into overt metastasis after considerable genetic evolution that occurs independently of primary tumor progression.

The emerging concept of tumor “self-seeding” has also extended our understanding of the pathological impact of tumor dissemination. The conventional unidirectional model of metastasis poses that tumor cells leave primary sites to seed metastasis in distant sites. In contrast, the emerging self-seeding hypothesis proposes that circulating and disseminated tumor cells can return to the primary tumor. In fact, the supportive stroma that arises in a primary tumor and contributes to its acquisition of malignant traits may intrinsically provide a hospitable site for reseeding and colonization by circulating cancer cells emanating from metastatic lesions. This model suggests that tumor cells may recirculate from a distant site (i.e. bone marrow) back to the primary site to give rise to recurrent tumors[74].

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade. This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed ‘‘colonization’’[59] (Figure 4).

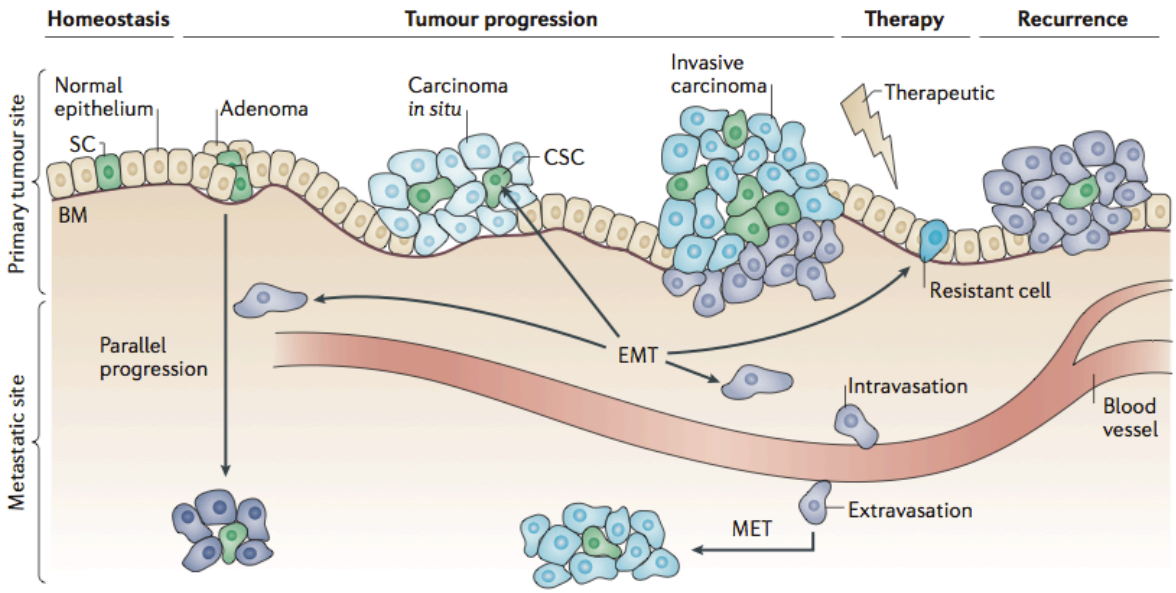

Figure 4. The invasion-metastasis cascade (De Craene B *et al.* Nature Rev Cancer 2013).

1.3.1. Tumor-intrinsic pathways and tumor-stroma interactions

This multistep process is governed by both tumor-intrinsic programs and tumor-stroma crosstalk.

Cell-autonomous mechanisms include secretion of matrix metalloproteases (MMPs), that enable the tumor to invade into the stroma (local invasion) by breaking down the basement membrane and the extracellular matrix. In addition, MMPs can release cell-surface and matrix-bound latent growth factors and cytokines, such as epidermal growth factor (EGF) family ligands, tumor necrosis factor-a (TNF-a), vascular endothelial growth factor (VEGF) and RANKL, that can act in an autocrine or paracrine manner to influence cell growth, survival and inflammation[75]. Additional molecular changes enable carcinoma cells to enter the blood circulation (intravasation), including expression of adhesion molecules that mediate the interaction with endothelial cells (integrin β1), and secretion of factors that increase the permeability of the vasculature (TGF-β, epiregulin, COX2, MMP1, MMP2, MMP3, ANGPT2, MMP10 and VEGF). Activation of the PI3K-Akt pathway and the expression of surface molecules such as CD47 promote the survival of circulating tumor cells (CTCs) by inhibiting anoikis and evading macrophage phagocytosis respectively.

Cancer cells corrupt resident tissue cells and recruited stromal cells to form a supporting tumor microenvironment (TME)[76; 77] in which stromal cells coevolve with tumor cells[78] to influence the initial dissemination and subsequent metastatic traits of cancer cells at the primary tumor site, and create a permissive niche at the metastatic site. The TME in metastatic lesions differs markedly from that of a primary tumor, and emerging evidence suggests that the formation of a receptive microenvironment preceding the arrival of disseminated tumor cells contributes to metastasis efficiency, echoing the "seed and soil" hypothesis proposed by Stephen Paget more than a century ago[79]. The stromal cells increase tumor dissemination through paracrine signals that induce the mobility, invasiveness and survival of cancer cells, such as TGF-β, which is often secreted by cancer-associated fibroblasts (CAFs). The TME can exert a major role also in the later stage of colonization at metastatic sites. Most DTCs land in the metastatic site as solitary tumor cells and have to resume growth to spawn a new colony and establish metastases or otherwise die or enter dormancy. Laboratory studies have led to two proposed dormant states: "cellular dormancy", defined as the growth arrest of solitary tumor cells, and "micrometastasis dormancy", which is achieved by a balance of proliferation and apoptosis due to an inability to recruit a vascular bed or overcome immunosurveillance. Growth arrest might result from maladaptation of solitary DTCs to the surrounding stroma (which includes normal resident tissue cells), as they are deprived of the appropriate adhesive and signaling interactions that are found in the primary tumor. Either at primary sites or in the circulation, tumor cells can release soluble factors or microvesicles (i.e. exosomes) to convert incipient metastatic sites into compatible "premetastatic niches", which are formed by bone-marrow-derived cells (BMDCs) before the arrival of DTCs. Alternatively, DTCs may occupy pre-existing physiological niches, such as in the bone, where DTCs may compete with quiescent hematopoietic stem cells (HSCs) for their niche, to stay in a stem-like, dormant state before resuming expansion into overt metastasis through interaction with bone stromal cells (osteoblasts and osteoclasts). Engagement of bone stromal cells by cancer cells often leads to abnormal bone degradation or bone building, which further promotes metastatic tumor growth through factors released from bone stromal cells or the bone matrix.

1.3.2. Epithelial plasticity in the invasion-metastasis cascade: EMT and MET

As carcinomas arising from epithelial tissues progressed to higher pathological grades of malignancy, reflected in local invasion and distant metastasis, the associated cancer cells typically developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM). The best characterized alteration involved the loss by carcinoma cells of E-cadherin, a key cell-to-cell adhesion molecule. By forming adherens junctions with adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and maintain the polarity and quiescence of the cells within these sheets. Increased expression of E-cadherin was well established as an antagonist of invasion and metastasis, whereas reduction of its expression was known to potentiate these phenotypes. The frequently observed downregulation and occasional mutational inactivation of E-cadherin in human carcinomas provided strong support for its role as a key suppressor of this hallmark capability[80; 81].

Additionally, expression of genes encoding other cell-to-cell and cell-to-ECM adhesion molecules is demonstrably altered in some highly aggressive carcinomas, with those favoring cytostasis typically being downregulated. Conversely, adhesion molecules normally associated with the cell migrations that occur during embryogenesis and inflammation are often upregulated. For example, N-cadherin, which is normally expressed in migrating neurons and mesenchymal cells during organogenesis, is upregulated in many invasive carcinoma cells.

A developmental regulatory program, referred to as the ‘‘epithelial-mesenchymal transition’’ (EMT), has become prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate[59]. By co-opting a process involved in various steps of embryonic morphogenesis and wound healing, carcinoma cells can concomitantly acquire multiple attributes that enable invasion and metastasis. This multifaceted EMT program can be activated transiently or stably, and to differing degrees, by carcinoma cells during the course of invasion and metastasis.

Contextual cues from the tumor-associated stroma, such as conditions of hypoxia and inflammation, are likely to act as the initial trigger of EMT. Indeed, cancer cells at the invasive front of certain carcinomas often undergo EMT, suggesting that these cancer cells are subject to microenvironmental stimuli distinct from those received by cancer cells located in the core of the tumor.

These cells are more exposed to the extracellular ligands supplied by fibroblasts, immune cells and mesenchymal stem cells populating the stroma. TGF-beta, WNT proteins, platelet-derived growth factors (PDGFs) and interleukin-6 (IL-6) activate and maintain the EMT program in the tumor cells. Being redirected away from suppressing cell proliferation, TGF-beta signaling is found instead to be a major inducer of EMT during cancer progression[82].

The EMT program underlies all the steps of tumor dissemination, from local invasion to extravasation (Figure 5), whereas the colonization requires the reverse process, called mesenchymal-to-epithelial transition (MET). During this process, motile undifferentiated mesenchymal cells are converted into polarized epithelial cells with increased E-cadherin levels. In fact, the mesenchymal state is associated with growth arrest and disseminated cancer cells have to exit the mesenchymal state to resume proliferation at the metastatic sites. Therefore, at incipient metastatic sites, MET of metastatic tumor cells could occur because of either the absence of EMT-inducing signals or the presence of MET-inducing signals, provided by bone marrow-derived monocytes or normal resident cells. It has to be clarified whether MET occurs right after extravasation, during cellular dormancy or perhaps after reactivation from dormancy. However, the notion that cancer cells routinely pass through a complete EMT program is likely to be simplistic; instead, in many cases, cancer cells may enter into an EMT program only partially, thereby acquiring new mesenchymal traits while continuing to express residual epithelial traits.

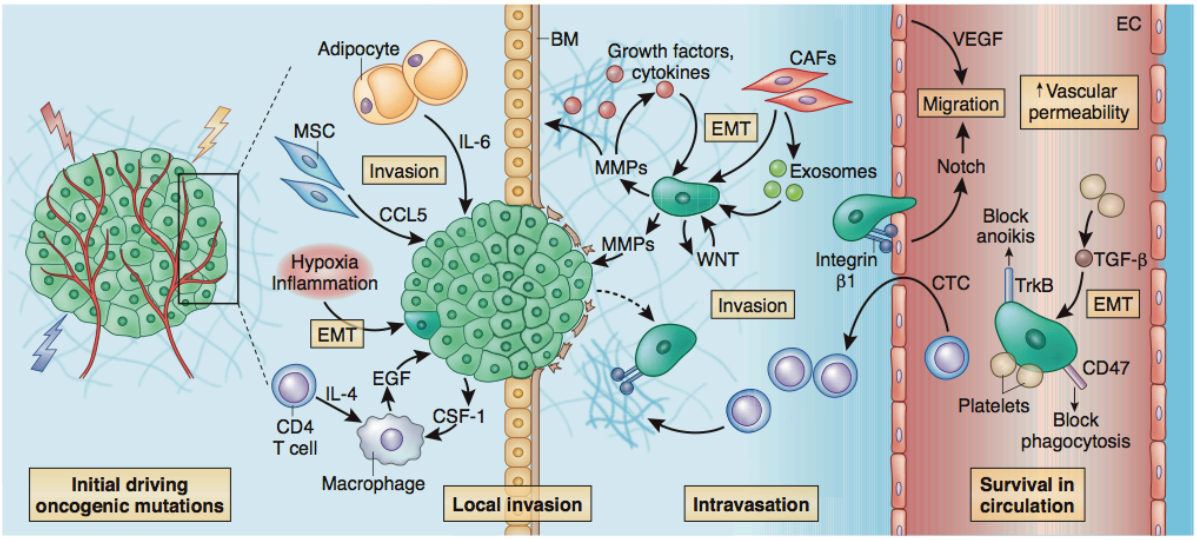

Figure 5. Role of EMT in all the stages of tumor dissemination (Wan L *et al.* Nat Med 2013).

1.3.3. Regulatory networks defining EMT

EMT is a reversible reprogramming of the cell, defined by a plethora of changes that are initiated and maintained by several regulatory circuits. So far, five different layers of regulation have been identified.

Transcriptional control. A set of pleiotropically acting transcriptional factors, including Snail, Slug, Twist, and Zeb1/2, orchestrate the EMT and related migratory processes during embryogenesis. These transcriptional regulators have been shown in experimental models of carcinoma formation to be causally important for programming invasion; some have been found to elicit metastasis when ectopically overexpressed[59]. Included among the cell-biological traits evoked by such transcription factors are: loss of adherens junctions and associated conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix-degrading enzymes, increased motility, and heightened resistance to apoptosis—all traits implicated in the processes of invasion and metastasis. Several of these transcription factors (Snail, Slug, ZEB1, ZEB2, E47, Brachyury) can repress E-cadherin gene expression by direct binding to the *CDH1* promoter, thereby depriving neoplastic epithelial cells of this key suppressor of motility and invasiveness.

Epigenetic regulation. The term "epigenetics" describes the mechanisms that impose cellular phenotypes without concomitant changes in the genome of a cell, meaning without changes in its nucleotide sequences. More recently, however, it has become apparent that epigenetic regulation is achieved in large part by the covalent modification of DNA, specifically the methylation of certain cytosine residues (DNA methylation), as well as by the covalent modifications of the histone proteins that form DNA-associated nucleosomes[83]. The reversibility of epigenetic modifications is likely to contribute to EMT plasticity, allowing cancer cells to switch back to the epithelial state on colonization at a secondary site. DNA methylation on CpG dinucleotides in regulatory sequences is a typical mechanism for silencing tumor suppressors in cancer cells and plays a major role in repressing the *CDH1* promoter in invasive breast carcinomas[84; 85; 86]. Other repressive epigenetic modifications include methylation and deacetylation of histone tails. The polycomb group (PcG) proteins constitute a group of epigenetic regulators that have a key role in regulating the expression of E-cadherin. They function as transcription repressors by directing lineage choices during early development and stem cell

differentiation, ensuring that progenitor or stem cells remain in an undifferentiated state. The PcG proteins assemble with other scaffolding proteins to form multi-subunit polycomb repressive complexes (PRCs), which silence transcription by histone methylation and recruitment of a variety of additional repressors. Two distinct classes of polycomb complexes, PRC1 and PRC2, participate in promoting EMT. PRC2 is made up of the subunits EZH2 (Enhancer of Zeste homolog 2) and SUZ12 (Suppressor of Zeste 12 homolog) and is recruited by Snail to the *CDH1* promoter, where PRC2 catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3), thereby silencing *CDH1* transcription. Deacetylation of lysine histone residues is an additional mechanism of epigenetic repression of the *CDH1* promoter. Indeed, the histone deacetylase HDAC1 and HDAC2, which function as components of NuRD, are recruited to the *CDH1* promoter by Snail and Twist. Similarly, ZEB1 has been shown to recruit the sirtuin deacetylase SIRT1 to repress *CDH1* transcription[87]. The final outcome of epigenetic modifications depends also on the crosstalk between different histone marks on the same promoter, as exemplified by the case of "bivalent" modifications. Coexistence of both a repressive and an activating histone mark on the same promoter region indicates an inactive but poised state. Genes in a "bivalent" state are not being actively transcribed but are poised to become readily activated in response to a signaling cue that leads to the removal of the repressive histone mark. Therefore, bivalent genes are not stably repressed but instead remain responsive to dynamic regulation by certain physiologic signals. The bivalent configuration of *CDH1*, *ZEB1* and *TWIST* promoters contributes to the epithelial plasticity of cancer cells, allowing for prompt adaptation to signals from the tumor microenvironment[88].

MicroRNAs. The number of microRNAs associated with EMT is becoming as extensive as the list of EMT-related transcription factors. Among the small non-coding RNAs that promote epithelial differentiation, the miR-200 and the miR-34 families appear two major players. Major targets of these microRNAs are the EMT-inducing transcription factors. Interestingly, both miR-200 and miR-34 have E-boxes in their promoters that are bound by Snail and ZEB, revealing reciprocal feedback loops between epithelium-related microRNAs and EMT-inducing transcription factors[88]. In addition, tumor-suppressive microRNAs are often epigenetically repressed in cancers by methylation of their promoters. There is also evidence for microRNAs promoting the transition to a mesenchymal phenotype, such as miR-9 and miR-92a, which target *CDH1*

directly.

Differential splicing. Alternative pre-mRNA splicing provides an additional post-transcriptional mechanism of regulation of EMT. *Trans*- and *cis*-acting elements controlling both epithelium-specific and mesenchyme-specific splicing have been identified. These RNA-binding proteins recognize splicing-enhancer and splicing-inhibitor sequences in the pre-mRNA. Exon inclusion or skipping is determined by the location of these sequence elements relative to the alternatively spliced exons[88]. For instance, the epithelium-specific splicing of *CTNND1* (which encodes p120 catenin) results in a shorter isoform that promotes cell-cell adhesion by stabilizing E-cadherin at the plasma membrane, whereas the longer mesenchymal isoform induced during EMT binds RHOA and attenuates its activity, resulting in enhanced cellular invasion. Importantly, the *trans*-acting elements that mediate epithelium-specific splicing are directly downregulated by the EMT-inducing transcription factors Snail, ZEB1 and ZEB2 and their expression is often low in EMT-like cancer cell lines.

Translational and post-translational regulation. The expression of EMT-associated transcription factors can be controlled at multiple steps of the translation process. Internal ribosome entry sites (IRES) located in the mRNAs of *SNAI1*, *ZEB2* and *TWIST1* can drive cap-independent translation initiation. TGF-beta signalling can re-activate the translational elongation of EMT-associated transcripts. In addition, EMT-inducing transcription factors, such as Snail and ZEB2, undergo a broad range of post-translational modifications, including phosphorylation, lysine oxidation, ubiquitylation and sumoylation[88].

1.3.4. The invasion-suppressor gene E-cadherin

Most human cancers originate from epithelial tissue. E-cadherin, the prototype member of the “classical” cadherin family, is the key player in inducing cell polarity and organizing an epithelium. In most, if not all, cancers of epithelial origin, E-cadherin-mediated cell–cell adhesion is lost concomitantly with progression towards tumor malignancy. Although E-cadherin expression can still be found in differentiated tumors in patients, there is an inverse correlation between E-cadherin levels, tumor grade and patient mortality[81]. The loss of E-cadherin function during tumor progression can be caused by multiple mechanisms. Predisposition to diffuse gastric cancer and lobular

breast cancer in patients carrying germline mutations in the E-cadherin gene[89; 90] identify E-cadherin as a tumor suppressor gene. The E-cadherin gene fulfills the criteria of classical tumour suppressor genes, inactivated by two genetic hits (Knudson, 1985). In fact, a major cause of E-cadherin inactivation is loss of heterozigosity (LOH) of 16q22.1, a chromosomic region containing the E-cadherin locus. The remaining allele is often inactivated by mutations scattered throughout the coding region or by epigenetic silencing owing to DNA hypermethylation of the promoter[91]. A major level of E-cadherin regulation consists in the binding of the *CDH1* promoter by EMT-inducing transcription factors, as discussed above in the text. Additional mechanisms that can compromise E-cadherin functionality include post-translational modifications and proteolytic degradation by matrix metalloproteases (MMPs)[81].

Multiple evidence has shown that loss of E-cadherin-mediated cell–cell adhesion is a prerequisite for tumour-cell invasion and metastasis formation. Several groups demonstrated that expression of exogenous E-cadherin in invasive tumor cell lines resulted in a reduced invasion ability *in vitro*[92; 93]. Accordingly, tumorigenic cell lines expressing exogenous E-cadherin produced partially differentiated tumors upon subcutaneous injection in SCID mice, compared to the undifferentiated tumors produced by the control transfectants[93]. Further *in vivo* evidence demonstrated the causal role of E-cadherin loss in tumor progression. The *Rip1Tag2* mouse model of pancreatic β-cell carcinogenesis was intercrossed with another transgenic mouse line constitutively expressing E-cadherin in pancreatic β-cells[94]. Whereas the single-transgenic *Rip1Tag2* mice developed tumors in a multi-stage tumorigenesis process, starting with islet hyperplasia, benign adenoma, and finally invasive carcinoma, the tumorigenesis in the double-transgenic mice was arrested at the stage of adenoma. In addition, intercrossing *Rip1Tag2* mice with transgenic mice expressing a dominant-negative form of E-cadherin induced early invasion and metastasis[94]. These findings showed that loss of E-cadherin is one rate-limiting step in the progression from adenoma to carcinoma and the subsequent formation of tumor metastases.

Further studies provided insight into the molecular mechanisms underlying the invasion-suppressive role of E-cadherin. Changes in the expression or function of E-cadherin can contribute to tumor progression not only by altering the adhesion status of the cell, but also by affecting cell signalling. In fact, whereas the extracellular portion of the E-cadherin molecule mediates cell-to-cell

homophylic interaction, the intracellular portion interacts with the Cytoplasmic Cell-adhesion Complex (CCC), which links E-cadherin to the actin cytoskeleton. The CCC consists of α-catenin, β-catenin, γ-catenin and p120 catenin. Upon loss of E-cadherin and disassembly of the CCC, catenins are released and accumulate in the cytoplasm, where they exert signaling functions (Figure 6). Non-sequestered β-catenin can be phosphorylated by GSK-3β and degraded by the ubiquitin-proteasome pathway. However, in case of activated Wnt signaling, the GSK-3β activity is blocked and β-catenin accumulates at high levels in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to members of the TCF/LEF1 family of transcription factors and modulates the expression of target genes, including c-*MYC*, cyclin D1, fibronectin, *MMP7*, *ID2*, *CD44*, *NrCAM*, axin-2 (conductin), *TCF1* and others, which are mostly genes implicated in cell proliferation and tumor progression. Another signal that is elicited by loss of E-cadherin function might involve changes in the organization of the cytoskeleton that are orchestrated by members of the RHO family of small GTPases. Once it is not sequestered any more by E-cadherin, cytosolic p120-catenin is able to activate small GTPases, ultimately leading to formation of filopodia (actin-rich spikes important in defining the directionality of movement) and lamellipodia (actin-rich membrane ruffles at the leading edge of migrating cells).

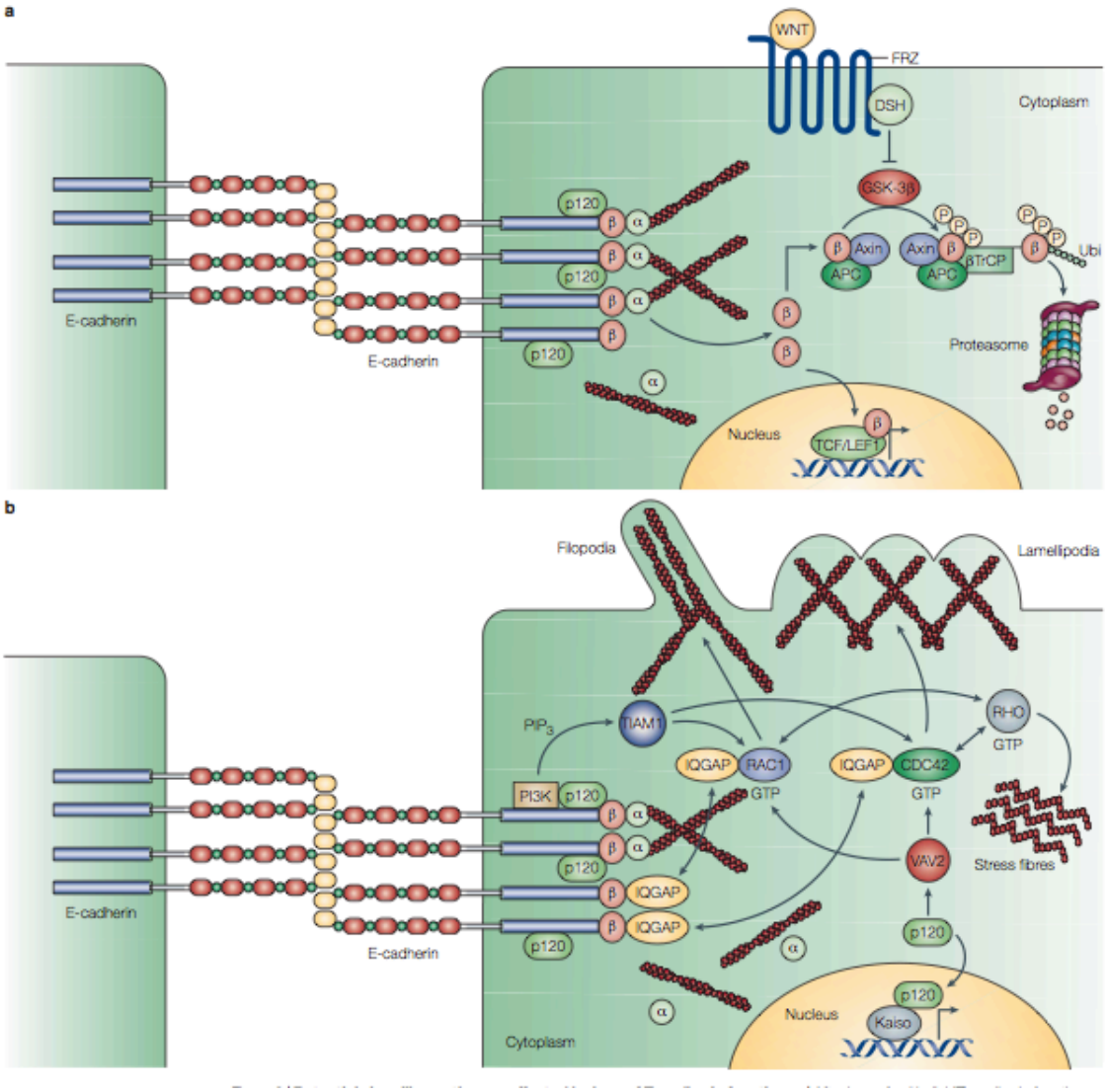

Figure 6. Potential signalling pathways affected by loss of E-cadherin function (Cavallaro U and Christofori G, Nature Rev Cancer 2004).

1.3.5. SIRT1, a sirtuin player in EMT

SIRT1 belongs to the sirtuin family of NAD-dependent lysine deacetylases. SIRT1 shuttles from the nucleus to the cytoplasm, and its targets include both histone marks, such as H3K9Ac, H4K16Ac and H1K26Ac, and non-histone proteins, such as p53, c-Myc, FOXO, p300, Ku70 and many others. SIRT1 up-regulation has been observed in most solid tumors, including breast, prostate, lung, colon, thyroid, gastric, liver, pancreatic, ovarian and cervical cancer[95]. Current evidence shows that SIRT1 can function as both a tumor promoter and

tumor suppressor, suggesting that SIRT1 might play a dual role in different tissue contexts, depending on the temporal and spatial distribution of upstream and downstream factors[96]. SIRT1 silencing induced growth arrest and apoptosis in human epithelial cancer cells[97]. Furthermore, reduced SIRT1 expression decreased the chemoresistance of cancer cells to cisplatin treatment[98]. SIRT1 can deacetylate p53 and FOXO and thereby inhibit P53- and FOXO-mediated apoptosis[99; 100]. However, a different body of evidence suggests that SIRT1 can also function as a tumor suppressor. No tumorigenesis has been reported in several transgenic mouse stains designed to overexpress SIRT1[101; 102]. SIRT1 was found to inhibit by deacetylation the function of NF-kB[103] and c-Myc[104], both major players in tumor progression.

Recent studies have revealed a controversial role for SIRT1 in the epithelial-mesenchymal transition (EMT). SIRT1 depletion led to the increase of E-cadherin and other epithelial markers and decrease of mesenchymal markers in prostate cancer cell lines, ultimately leading to reduced cell migration *in vitro* and metastatic ability *in vivo*[87]. This study pointed out SIRT1 as a negative regulator of E-cadherin transcription, being recruited to the *CDH1* promoter via the EMT-inducing transcription factor ZEB1[87]. In fact, ZEB1-depleted cells showed less recruitment of SIRT1 and RNA PolII to the *CDH1* promoter, together with decreased acetylation of lysine 9 of histone H3 (H3K9). However, more direct evidence is needed to clarify whether the decrease in H3K9Ac, a histone mark of transcriptionally active chromatin, is dependent on SIRT1 deacetylase activity. The EMT-promoting role of SIRT1 has been supported also by the finding that SIRT1 is a target of miR-200a, and SIRT1 expression is upregulated upon activation of TGF-beta signaling in breast cancer cells[105]. Intriguingly, a different body of evidence unveiled an EMT-suppressive role for SIRT1 in breast cancer metastasis and organ fibrosis[106]. SIRT1 depletion was found to decrease E-cadherin protein levels in primary human mammary cells, but a different mechanism of SIRT1-mediated E-cadherin regulation was proposed. According to this model, SIRT1 inhibits Smad4-dependent upregulation of MMP7, induced by TGF-beta signaling. Since MMP7 cleaves E-cadherin from the cell surface, SIRT1 preserves the integrity of the Cytoplasmic Cell-adhesion Complex that sequesters β-catenin, preventing its translocation to the nucleus and thus the activation of the EMT-induced transcription program[106]. Similarly, a suppressive role for SIRT1 in cancer metastasis was suggested by the finding that miR-520c and miR-373 repressed SIRT1

translation, ultimately leading to increased expression of MMP9 and enhanced cell migration of fibrosarcoma cells[107].
In conclusion, SIRT1 seems to have a dual role in EMT, regulating E-cadherin expression and function through multiple mechanisms.

1.4. Aim of the work

SIRT7 is a chromatin regulatory factor belonging to the sirtuin family of NAD^+-dependent enzymes, whose members play pivotal roles in metabolic and age-related diseases, including cancer. Until few years ago, SIRT7 was the most puzzling sirtuin, being its enzymatic activity and molecular targets still unknown. Exploring SIRT7 molecular function and its impact in aging-related pathologies through chromatin regulation has been one major focus of Chua's lab. Recently, the Chua's lab discovered that SIRT7 is a highly selective H3K18 deacetylase, which stabilizes the transformed phenotype of cancer cells and promotes tumor growth *in vivo*. These findings paved the way for subsequent studies that further supported the tumor-promoting role of SIRT7. However, no evidence has pointed out yet a role for SIRT7 in the regulation of cancer cell invasiveness and tumor metastasis.
In this study, we sought to determine whether SIRT7 has a role in promoting the migration, invasiveness, and metastatic potential of cancer cells. We identified E-cadherin, a key suppressor of tumor progression, as a novel SIRT7 target. We also unveiled a novel crosstalk between two chromatin regulators of the sirtuin family, which promotes the invasive and metastatic properties of cancer cells. Finally, we showed that reduction of SIRT7 dramatically impairs the metastastic potential of cancer cells *in vivo*.

Chapter 2 – MATERIALS AND METHODS

2.1. Cell Culture

Cells were grown in a humidified tissue culture incubator, at 37C, 5% CO_2 atmosphere. LNCaP and PC3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine (Gibco, Invitrogen). HT1080 cells were grown in Advanced DMEM (Gibco, Invitrogen) in the presence of 10% FBS and 1% penicillin-streptomycin. All cells were obtained from the American Type Culture Collection (Manassas, Virginia).

2.2. Plasmids

Lentiviral plasmids pSicoR-puro encoding shRNAs targeting SIRT7 mRNA were generated as previously described[34]. Target sequences were as follows: S7KD1, 5′-CACCTTTCTGTGAGAACGGAA-3′; S7KD2, 5′-TAGCCATTTGTCCTTGAGGAA-3′. As a control for transduction we used the pSicoR-puro empty vector. The human SIRT7 H187Y expression retroviral vector pBabe-puro and the 3XFlag-tagged-SIRT1 WT and H355Y mutant expression vectors were generated as previously described[9]. As a control for transduction we used the pBabe-puro and the 3XFlag-puro empty vectors.

2.3. Transwell invasion assay

The assay was performed according to BD BioCoat™ manufacturer's instructions. Briefly, medium containing 10% FBS as a chemoattractant was added to the wells of a 24-well plate. The Matrigel invasion chambers were transferred to the wells containing the chemoattractant using sterile forceps. A suspension of 10^4 HT1080 and 5*10^4 PC3 cells in serum-free media was loaded into the chambers. Cells were incubated for 22-24hr in a humidified tissue culture incubator, at 37C, 5% CO_2 atmosphere. The non-invading cells were removed by scrubbing with a cotton tipped swab and the invading cells were fixed with methanol for 2 minutes and stained with crystal violet for 10 minutes. The inserts were dried and the membrane was photographed through the microscope at 10x magnification.

2.4. Wound healing assay

Cells were grown to confluent monolayers. A scratch was then made using 200uL tip on cell monolayers. Cells were washed once each with PBS and warm media. Cells were imaged immediately after creating the wound using a time lapse microscope every 15 minutes for 12 hr for HT1080 cells and 20 hr for PC3 cells.

2.5. 3D Matrigel assay

LDEV free Matrigel basement matrix (BD Biosciences, now Corning) was spread evenly on the bottom of chamber slides and allowed to solidify at 37°C for 30 minutes. 500 PC3 control and SIRT7 KD cells were suspended in 2% matrigel and spread over the matrix. Cells were fed every 3 days with fresh 2% matrigel. Images were taken every 2 days.

2.6. Phalloidin staining

Cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% with Triton-X for 5 minutes. Cells were stained with 1:100 dilution of the F-actin probe Alexa Fluor 488 Phalloidin (Life Technologies). Images were acquired on a fluorescence microscope using a 20x objective.

2.7. Co-immunoprecipitation and Immunoblot

Cell lysates were prepared as described previously. 1 mg of total protein extract was used for immunoprecipitation with SIRT7 antibody overnight. The immunoprecipitated complex was incubated with Protein A/G beads (Sigma-Aldrich) for 1 hour, followed by washing 6 times with a 250 mM NaCl-containing buffer. The beads were incubated at 37°C for 20 minutes and the supernatant was used for western blot analysis. Flag-co-immunoprecipitation was carried out by using Anti-Flag M2 affinity gel (A2220-10ML, Sigma-Aldrich).

2.8. Antibodies

E-cadherin antibody (610181) was purchased from BD Biosciences. SIRT1 (07-131) and β-tubulin (05-661) antibody were purchased from Millipore. Vimentin antibody (V5255) was purchased from Sigma. The rabbit polyclonal antibody specifically recognizing SIRT7 was raised against the following synthetic peptide GWFGRGCTKRTKRKKVT and the affinity-purified antibody was used in this study (ref. Michishita E, 2005). Acetylated lysine 18 of histone H3 (H3K18Ac) antibody (ab1191) was purchased from Abcam and Lamin B antibody (C20) from Santa Cruz Biotechnology.

2.9. RT-qPCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen) and reverse transcribed using SuperScript III (Invitrogen) and oligo(dT) primers, according to the manufacturers' instructions. Quantitative real-time PCR analysis was performed on a Roche LightCycler 480 using the manufacturer's SYBR Green system. PCR primers used are: *E-cadherin*: Forward 5'-GGTCTGTCAT-*GGAAGGTGCT*-3'; Reverse 5'-GATGGCGGCATTGTAGGT-3'. *DAB2IP*: Forward 5'-TGGACGATGTGCTCTATGCC-3'; Reverse 5'-GGATGGTGATGGTTTGGTAG-3'. *Slug*: Forward 5'-TGTTGCAGTGAGGGCAAGAA-3'; Reverse 5'-GACCCTGGTTG CTTCAAGGA-3'. *Gapdh*: Forward 5'-AGCCACATCGCTCAGACAC-3'; Reverse 5'-GCCCAATACGACCAAATCC-3'.

2.10. Tail vein injection

$1x10^6$ control and SIRT7-KD HT1080 cells were intravenously inoculated into SCID mice. Lungs were harvested, weighted and imaged 28 days post-injection.

2.11. Subcutaneous injection

The subcutaneous injection of SIRT7-knockdown HT1080 cells in SCID mice was performed as previously described[34] and tumour growth was monitored using calipers.

Chapter 3 – RESULTS

3.1. SIRT7 promotes cancer cell migration and invasion

Current evidence established a role for SIRT7 in the epigenetic maintenance of transformed phenotypes of cancer cells[34]. To investigate whether SIRT7 plays a role in the regulation of cancer cell phenotypes in more advanced stages of tumor progression, and in particular in the invasion-metastasis cascade, we examined the effects of SIRT7 depletion by RNA interference on the directional migration of cancer cells, by wound-healing assay. A wound was created in cell monolayers and a time-lapse microscope was used to observe cell migration until the wound was healed. SIRT7 depletion impaired the migration ability in two different cancer cell lines (prostate carcinoma PC3 cells and fibrosarcoma HT1080 cells), resulting in a smaller healing percentage as compared to control cells (Figure 1). Notably, no significant changes in proliferation were observed under the time conditions of these experiments.

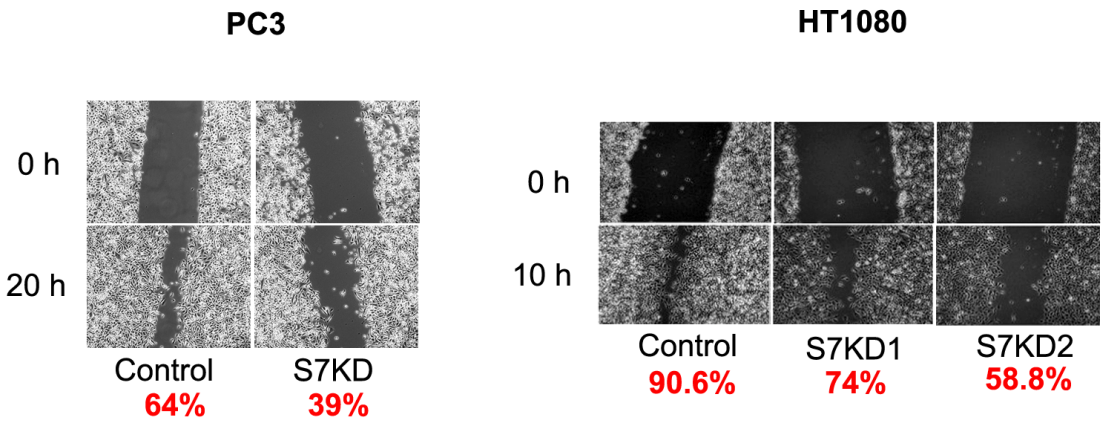

Figure 1. SIRT7 depletion impairs migration of cancer cells in wound-healing assay.

We next asked whether SIRT7 depletion could affect cancer cell invasiveness. To address this question, we performed a matrigel-invasion assay, that allows to assess cell invasion *in vitro* throughout an ECM-like structure that mimics the tissue basement membrane. The assay scores for number of cells invading from a top chamber through the membrane (Matrigel BD BiocoatTM) towards a chemoattractant (e.g. serum). SIRT7-deficient PC3 cells showed a significant reduction in the number of cells invading through the membrane matrix as

compared to control cells (Figure 2, left panel). SIRT7 depletion also inhibited the invasive growth pattern of PC3 cells when grown in three-dimensional basement membrane matrix-like Matrigel (Figure 2, right panel).

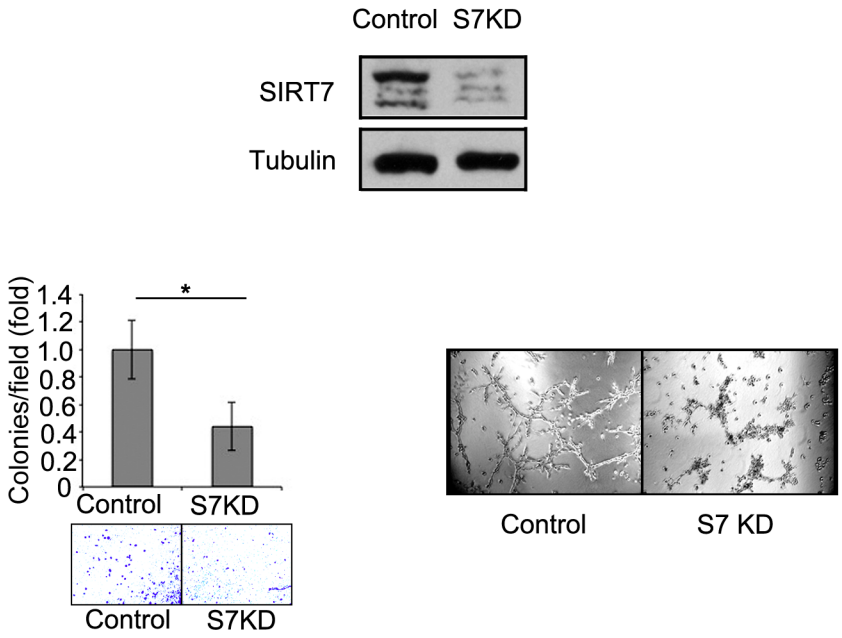

Figure 2. SIRT7 depletion reduces invasiveness of PC3 cells. SIRT7-deficient PC3 cells show reduced invasion ability in Transwell assay (left panel) and impaired growth in 3D culture (right panel). Images were taken 3 days after cell seeding.

A similar phenotype was observed in SIRT7-depleted HT1080 cells (Figure 3A). Moreover, overexpression of a catalytically inactive SIRT7 mutant protein (H187Y) also reduced invasiveness, consistent with a dominant negative effect (Figure 3B). Together, these results suggest that SIRT7 plays an important role in promoting cancer cell migration and invasion.

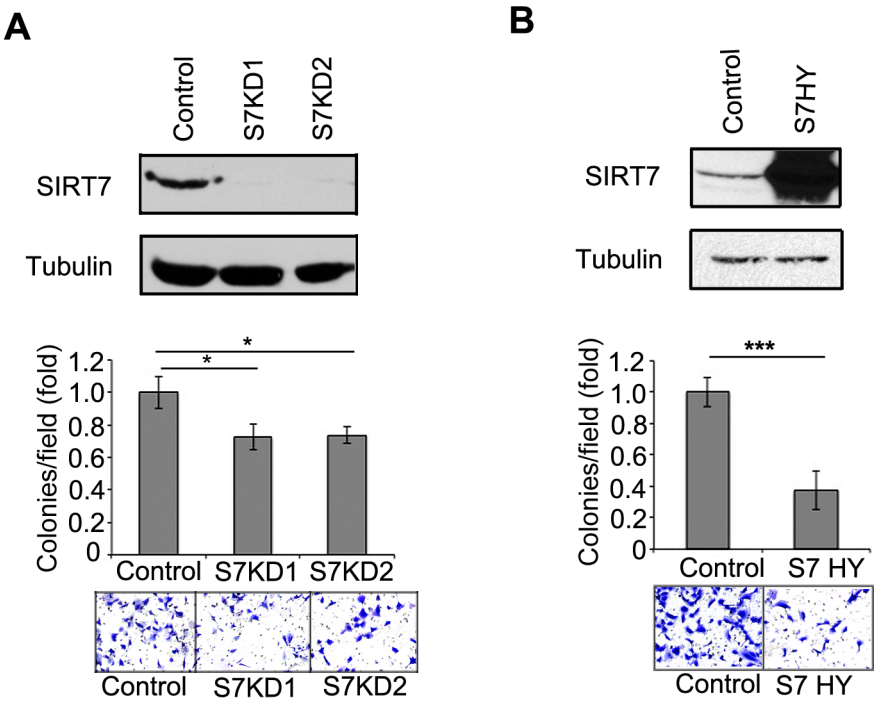

Figure 3. SIRT7 depletion (A) or overexpression of H187Y-SIRT7 catalytic mutant (B) impairs invasion in HT1080 cells.

3.2. SIRT7 regulates the expression of E-cadherin and other EMT-related genes

The EMT program plays a major role in the acquisition of a motile behavior by epithelial cancer cells. As mentioned above, EMT induces reorganization of actin cytoskeleton, a process that can be detected by staining for F-actin. Fluorescent staining of F-actin (Phalloidin staining) in SIRT7-depleted PC3 cells revealed a more collapsed cytoskeleton (Figure 4A), consistent with the impaired migration and invasion ability of these cells. Thus, we reasoned that SIRT7 might affect the motile properties of cancer cells through regulation of the EMT pathway. To test this hypothesis, we assessed the expression of EMT-related genes in SIRT7-depleted cells by RT-qPCR analysis.

A strong and well-established network of cell-to-cell contacts is essential for the polarity and functionality of normal epithelium and greatly limits the ability of epithelial cells to move or migrate. E-cadherin plays a major role in the establishment of these homotypic adhesion junctions, and its transcriptional downregulation is one of the leading events in the epithelial dedifferentiation process occurring during EMT[108]. Indeed, during embryonic development, E-cadherin expression is under strict spatiotemporal control and its repression is essential for certain morphogenetic movements within the embryo, many of which involve EMTs. The EMT occurring in tumors of epithelial origin during the acquisition of the invasive phenotype recapitulates the same molecular events that occur in the embryo, including loss of E-cadherin-mediated cell-to-cell junctions. Notably, SIRT7-deficient PC3 cells showed an increase in the mRNA level of E-cadherin (*CDH1*) (Figure 4B).

DAB2IP (DAB2 interacting protein) is a tumor suppressor gene whose loss promotes epithelial-mesenchymal transition and metastasis in prostate cancer[109; 110]. *DAB2IP* promoter is epigenetically inactivated in human prostate[110; 111] and breast[112] cancer and its expression inversely correlates with tumor grade and predicts prognosis in prostate cancer[110]. Interestingly, we found a significant increase in *DAB2IP* expression in SIRT7-depleted cells (Figure 4B).

SIRT7 reduction also led to decreased expression of Slug (*SNAI2*), a transcription factor of the Snail family that represents one of the master regulators of the EMT program[113] (Figure 4B). In addition to repressing E-cadherin by direct binding to the *CDH1* gene promoter[114], Slug orchestrates the

expression of several EMT-related genes[113,115].

The matrix metalloproteinase MMP16 also showed reduced expression in SIRT7-depleted cells (Figure 4B), consistent with the reduced invasive ability of these cells. In fact, the upregulation of MMPs, normally expressed in activated leukocytes, endows tumor cells with the ability to breakdown the extracellular matrix, allowing tumor invasion upon disruption of the physiological tissue barrier represented by the basement membrane. Western blot analyses further confirmed upregulation of E-cadherin in SIRT7-depleted PC3 cells (Figure 4C) and showed a reduction in the mesenchymal marker Vimentin, an intermediate filament commonly found in mesenchymal cells (Figure 4C).

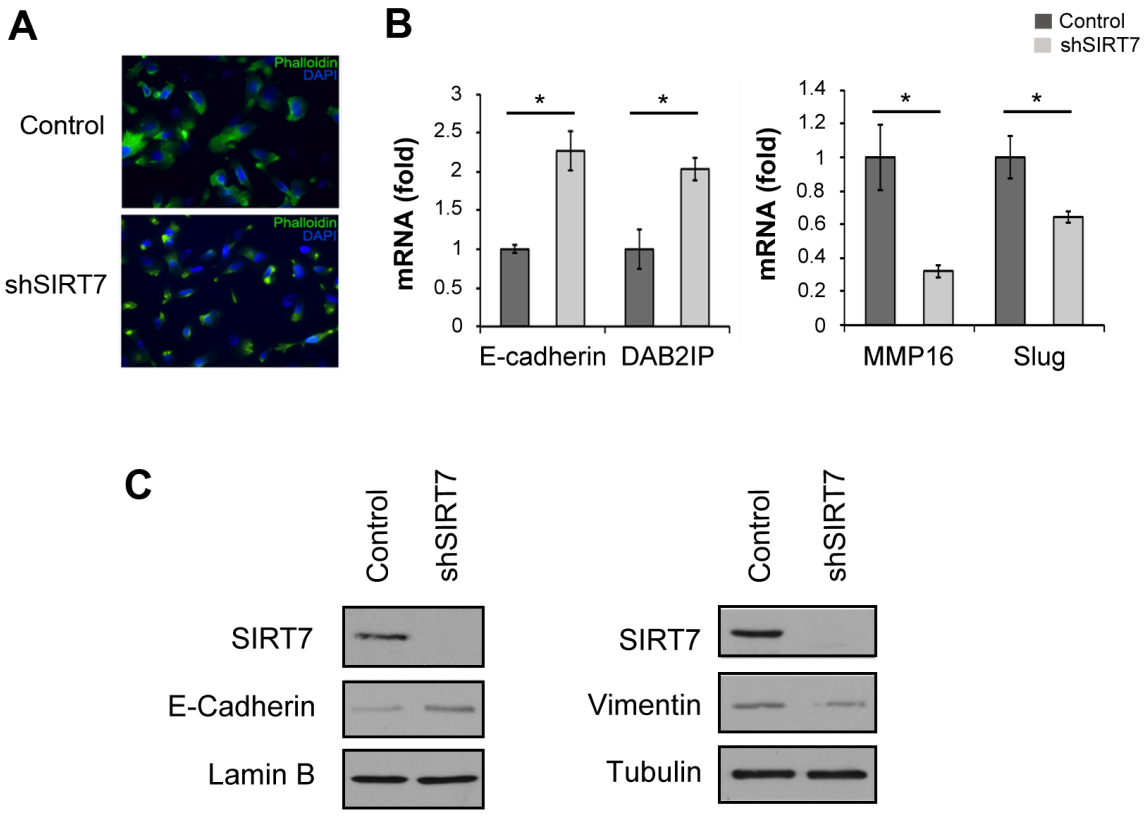

Figure 4. SIRT7 regulates invasion- and EMT-related genes in PC3 cells.

Functional perturbations of E-cadherin have been associated with the dedifferentiation/aggressiveness of tumors[92; 116] and even implicated in the transition from adenomas to invasive carcinomas[94]. Therefore, E-cadherin is thought to be an invasion-suppressor gene[93; 117; 118] and its loss is considered to be diagnostic of a poor clinical prognosis. We asked whether such SIRT7-mediated repression of E-cadherin could correlate with more aggressive tumor stages. To this purpose, we looked at the levels of SIRT7 and E-cadherin in the more aggressive prostate cancer cell line PC3 (derived from a bone metastasis of prostate cancer) compared to the more epithelial counterpart LNCaP (isolated from a lymph node metastasis). Western blot analysis showed enhanced SIRT7 expression and concomitant reduced E-cadherin levels in PC3 compared to LNCaP cells (Figure 5A). It has been previously shown that global hypoacetylation levels of H3K18 decrease in PC3 compared to LNCaP[40]. The concomitant increase in SIRT7 expression suggests that SIRT7 can be responsible for alteration in the global levels of this histone mark at more aggressive tumor stages.

To investigate the possible clinical relevance of the inverse correlation between SIRT7 and E-cadherin expression, we analyzed several metastatic prostate tumors in the Oncomine human cancer gene expression database. Surprisingly, we found that most of the tumors in 4 different datasets analyzed harbored a negative correlation between SIRT7 and E-cadherin expression (Figure 5B).

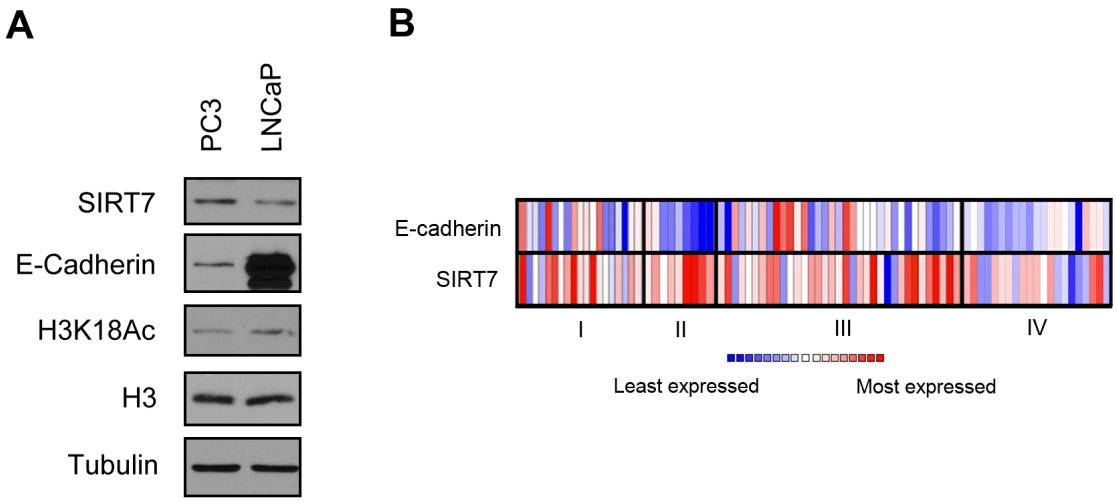

Figure 5. Inverse correlation between SIRT7 and E-cadherin expression in metastatic prostate cancer.

3.3. SIRT7 cooperates with SIRT1 to repress E-cadherin expression

SIRT1, another histone deacetylase member of the Sirtuin family, has been shown to repress E-cadherin transcription in prostate cancer cells, being recruited to the E-cadherin promoter via the EMT-inducing transcription factor ZEB1[87]. Intriguingly, our co-immunoprecipitation assay showed specific interaction *in vivo* between Flag-tagged SIRT7 and endogenous SIRT1 and viceversa (Figure 6A-B). This result was further confirmed by co-immunoprecipitation of the endogenous proteins (Figure 6C).

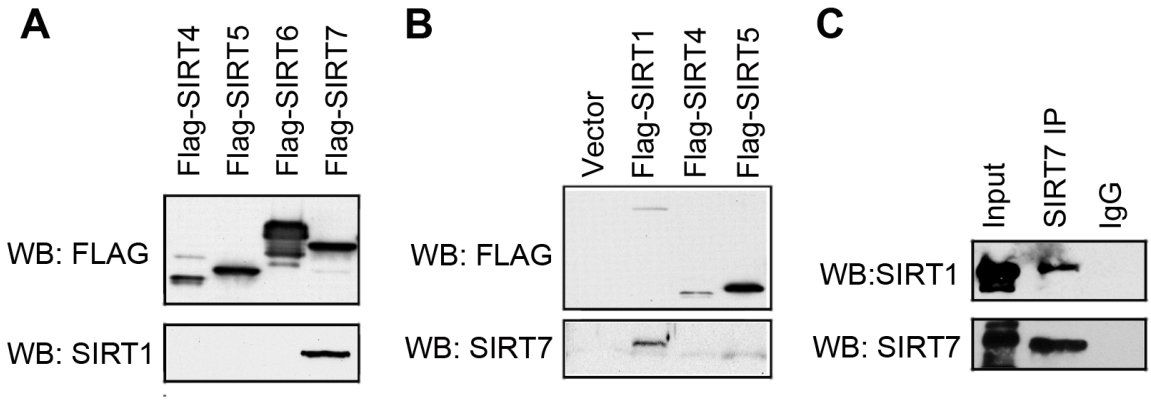

Figure 6. SIRT7 interacts with SIRT1 *in vivo*.

In order to investigate a potential interplay involving both Sirtuins on E-cadherin regulation, we overexpressed SIRT1 in SIRT7-depleted cells and looked at E-cadherin RNA and protein levels. Consistent with previous findings, E-cadherin levels were reduced in SIRT1-overexpressing cells (Figure 7A-B). Interestingly, such decrease was observed also upon overexpression of SIRT1 catalytic mutant (SIRT1 point mutation H355Y), suggesting that E-cadherin regulation by SIRT1 is, at least in part, independent of SIRT1 deacetylase activity (Figure 7A-B). Moreover, SIRT1-induced E-cadherin downregulation was blunted in SIRT7-deficient cells (Figure 7A-B), suggesting that SIRT7 is required to mediate the effect of SIRT1 on E-cadherin regulation. Overall, these findings suggest that SIRT1 represses E-cadherin expression, at least partially, via SIRT7.

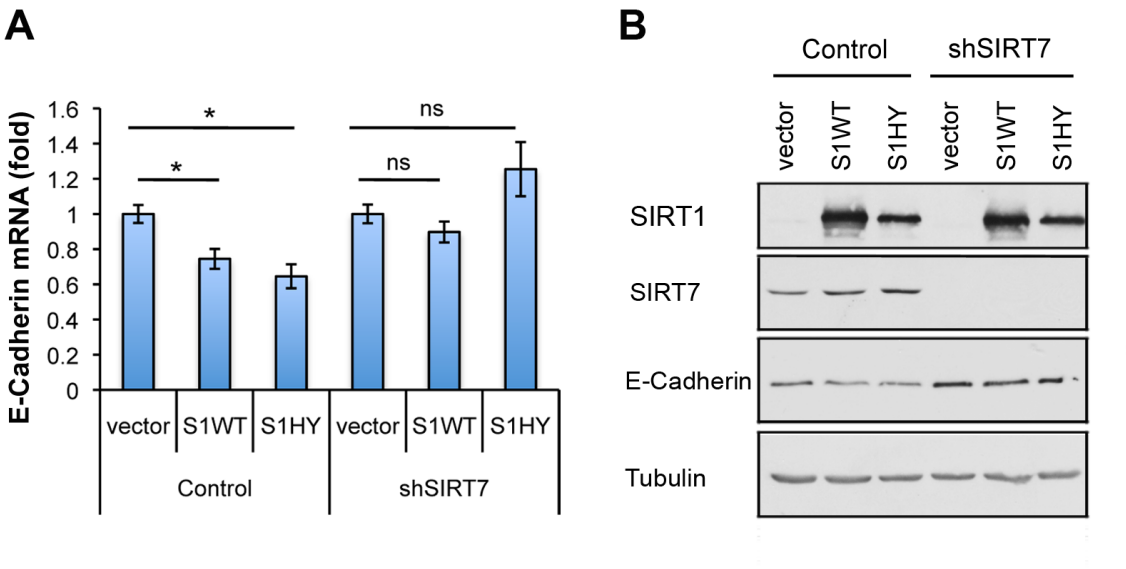

Figure 7. SIRT7 depletion blunts SIRT1-induced E-cadherin downregulation at both the mRNA **(A)** and protein levels **(B).**

3.4. SIRT7 amplification or overexpression correlates with metastasis in cancer patients

We previously demonstrated that SIRT7 is overexpressed in several patient-matched tumor samples[34]. Analysis of publicly available datasets from cBioPortal for Cancer Genomics (www.cbioportal.org) revealed the occurrence of amplifications and mutations at SIRT7 locus in multiple human cancers (Figure 8A). One study conducted on 61 prostate cancer patients[119] showed amplification of SIRT7 locus exclusively in tumors at the metastatic stage associated with low survival status (Figure 8B). In addition, SIRT7 was significantly overexpressed in the tumor metastatic site compared to the primary site in a prostate cancer dataset from Oncomine (Figure 8C). These findings prompted us to investigate whether SIRT7 promotes metastasis *in vivo*.

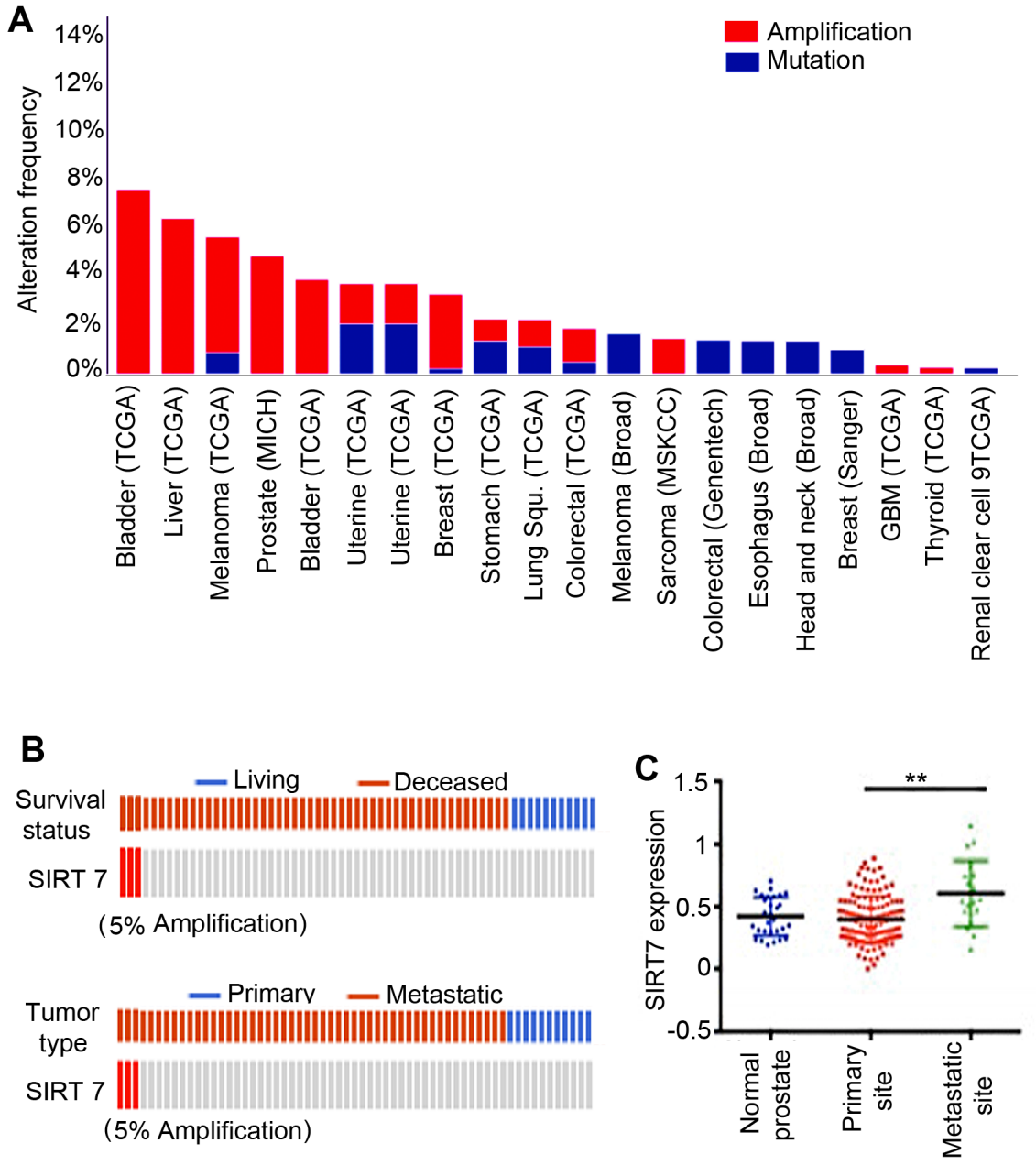

Figure 8. (A) Analysis of several datasets from cBioPortal for Cancer Genomics database. **(B)** Analysis of a reported mutational landscape of metastatic prostate cancer (Grasso CS *et al.*) shows exclusive amplification of SIRT7 in metastatic prostate tumor patients with low survival status. **(C)** Meta-analysis of SIRT7 expression in a prostate cancer dataset from Oncomine database.

3.5. SIRT7 promotes metastasis *in vivo*

We previously showed that SIRT7 depletion impaired tumor growth formation in a mouse xenograft assay[34]. Here we asked whether SIRT7 depletion could reduce the formation of macroscopic metastatic lesions. To look specifically at metastasis without complications from tumor growth changes, we generated HT1080 cells with only partial depletion of SIRT7 (Figure 9A). As expected, we observed only a subtle effect of SIRT7 reduction on primary tumor growth in a mouse xenograft experiment (Figure 9B). Next, tail vein injection experiments were performed with the same cells. Mice were sacrificed 28 days post-tail vein injection and their lungs were examined for macro-metastasis formation. Figure 9C shows significant reduction in the lung tumor burden in mice injected with SIRT7-deficient cells compared to control cells. Strikingly, the effect of SIRT7 reduction on metastasis formation is much more dramatic than on tumor growth. These findings demonstrate, for the first time, that SIRT7 depletion impairs metastasis formation *in vivo*.

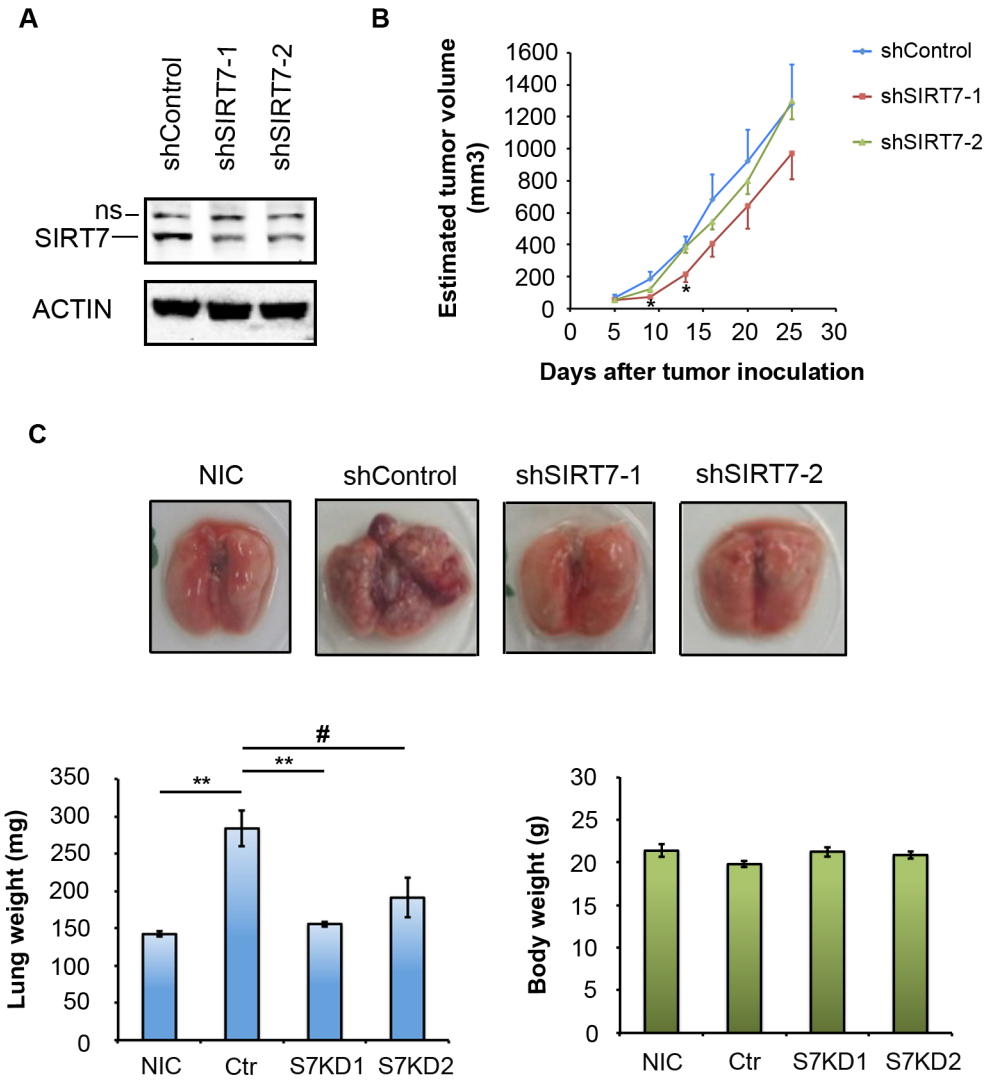

Figure 9. 1x10^6 HT1080 cells stably expressing control shRNA and SIRT7 shRNA were intravenously inoculated into SCID mice. NIC, "no injection control". ** indicates significant differences between the sh-control group by Welch's T test. # indicates significant differences between the sh-control group by Student's T test. (** : P<0.005, # : P<0.05 vs the sh-control group).

Together, our current findings expanded SIRT7 role in tumor progression, uncovering a new function for SIRT7 in promoting cancer cell invasiveness and metastatic ability through regulation of E-cadherin expression.

Chapter 4 – DISCUSSION

We have previously shown that SIRT7 promotes oncogenic transformation and stabilizes transformed phenotypes of cancer cells through deacetylation of the H3K18Ac histone mark at specific promoters. Here, we demonstrated that SIRT7 depletion reduces migration and invasion of cancer cells *in vitro* and metastasis *in vivo*. We showed that SIRT7 is an important regulator of the invasion-EMT circuitry and identified the invasion-suppressor gene E-cadherin as a novel SIRT7 target.

Elevated SIRT7 expression was detected in human biopsies of hepatocellular carcinoma (HCC)[45], thyroid[62; 120] and breast cancers[61] compared to their normal counterparts, and correlated with malignant progression. In fact, relative SIRT7 expression increased in HCCs of increasing grade (G1-3), compared to premalignant samples[45]. Similarly, SIRT7 upregulation was greater in thyroid carcinomas and lymph node-positive breast cancers, which have higher recurrence and poorer prognosis[61; 62; 120]. This evidence has hinted at SIRT7 involvement in tumor progression, suggesting that SIRT7 may prove to be a good marker of disease progression and tumor behavior. However, the molecular mechanisms through which SIRT7 may impact on tumor progression are still largely unknown. Our current work has uncovered a regulatory role for SIRT7 in the expression of EMT-related genes and the epithelial marker E-cadherin, a tumor suppressor gene whose loss has been associated with enhanced invasion ability and metastatic properties in multiple cancers.

Interestingly, H3K18 hypoacetylation is associated with poor prognosis in prostate cancer[40; 41]. We found enhanced expression of SIRT7 and a concomitant decrease in global H3K18Ac levels in the more invasive PC3 prostate cancer cells compared to the less invasive counterpart LNCaP. Therefore, SIRT7-mediated H3K18 hypoacetylation may be an indicator of poor prognosis in prostate cancer. This hypothesis is further supported by our analysis of human patient samples from Oncomine database, showing SIRT7 overexpression in the metastatic sites of prostate tumors compared to the primary site. To further explore the clinical implications of our findings, I'm planning to detect by immunohistochemistry SIRT7 expression at the invading front of the tumor, to test whether SIRT7 is upregulated *in vivo* in those tumor cells that lead the local invasion. Next, I would like to assess whether SIRT7 upregulation is induced in these cells in response to cytokines released from the tumor microenvironment, such as TGF-beta.

Furthermore, our study has unveiled a novel interplay between two sirtuin histone deacetylases, SIRT7 and SIRT1, in the regulation of E-cadherin expression. Analysis of four prostate metastatic datasets from Oncomine revealed an inverse correlation between E-cadherin and SIRT7 expression. We further looked into these datasets of E-cadherin-negative tumors for tumors that showed SIRT1 overexpression. While the number of metastatic tumors overexpressing SIRT1 was less as compared to SIRT7, we found that every single SIRT1-overexpressing tumor also overexpressed SIRT7. This observation further supports the hypothesis that SIRT7 and SIRT1 may have overlapping roles in regulation of metastasis. SIRT7-SIRT1 interaction could potentially represent a conserved mechanism of epigenetic regulation on other target promoters. Our observation that SIRT7 interacts to the same extent with both the WT form and the catalytic mutant of SIRT1, and that overexpression of SIRT1 catalytic mutant is able to repress E-cadherin expression, suggests that SIRT1 catalytic activity is dispensable for *CDH1* repression. We hypothesize that SIRT1 might function as a scaffold protein in recruiting SIRT7 to the *CDH1* promoter, and that SIRT7-mediated deacetylation of H3K18 is necessary for the repressive effect. However, currently we cannot rule out the possibility of a crosstalk between the two histone marks that are targets of the two sirtuins, H3K18Ac and H3K9Ac respectively. By carrying out chromatin immunoprecipitation experiments, my future work will aim at dissecting the molecular mechanism underlying SIRT7-SIRT1 crosstalk at the *CDH1* promoter.

Given the overexpression of SIRT7 in several aggressive forms of cancer, SIRT7 is a promising target for epigenetic therapy. Many HDAC inhibitors (HDACi) are being tested while two have been FDA approved for anti-cancer therapy. In this setting, SIRT7 modulators could be developed as a part of combinatorial therapy with SIRT1 inhibitors and other HDACi. Our study prompts future investigations into the role of SIRT7 in the epigenetic regulation of tumor progression, in order to exploit its use as a potential therapeutic target in advanced cancer stages.

DECLARATION

This is to certify that this thesis comprises only my original work towards the PhD except where indicated:

a) The wound-healing assay in fig. 1 right panel, the transwell invasion assay in fig. 3, the Phalloidin staining in fig. 4A, the co-immunoprecipitation in fig. 6C and the Oncomine database analyses in fig. 5B and fig. 8 were performed by Dr. Shivani Malik.

b) The mouse experiments in fig. 9 were conducted by Dr. Eriko Michischita and colleagues.

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