Etude des modifications épigénétiques en fonction de l'agressivité du cancer sporadique du sein : l'implication de l'histone désacétylase SIRT1 dans la progression tumorale

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UNIVERSITÉ Clermont Auvergne

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ – AGRONOMIE – ENVIRONNEMENT

THÈSE

Présentée pour obtenir le grade de

DOCTEUR D'UNIVERSITÉ

Spécialité : Épigénétique, Biologie du Cancer, Physiologie, Nutrition, Santé

Par :

Khaldoun RIFAI

Étude des Modifications Épigénétiques En Fonction de l'Agressivité du Cancer Sporadique du Sein: L'Implication de l'Histone Désacétylase SIRT1 dans la Progression Tumorale

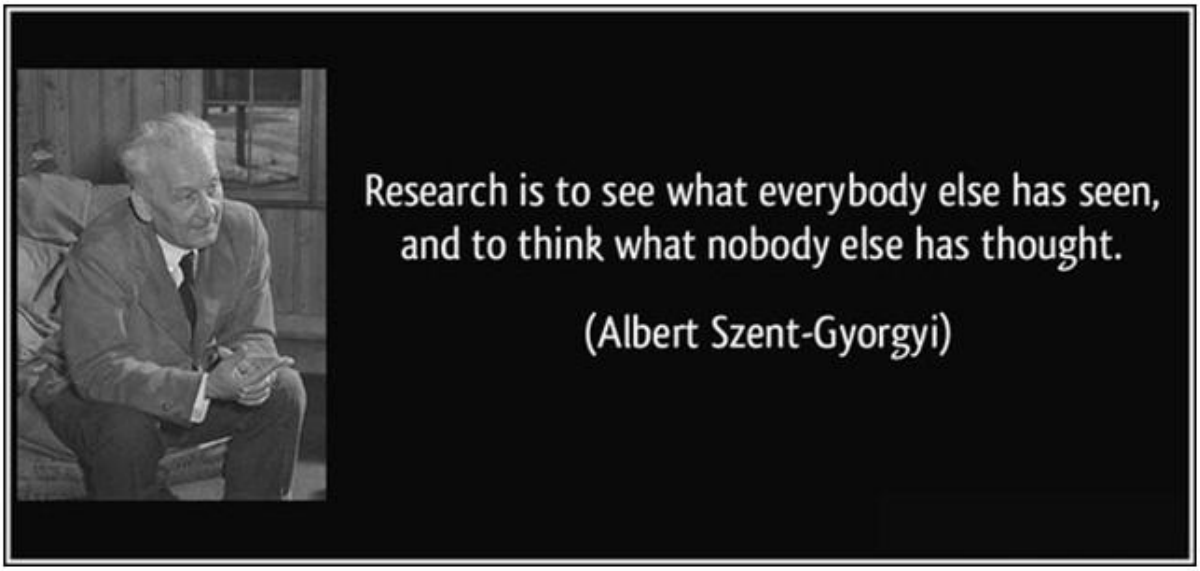
Soutenue publiquement le **29 Novembre 2018**, devant la commission d'examen :

Président
Pr. Yves-Jean Bignon, Centre Jean Perrin, INSERM U1240- IMOST, Clermont-Ferrand

Directeur de thèse
Dr. Dominique Bernard-Gallon, Centre Jean Perrin, INSERM U1240- IMOST, Clermont-Ferrand

Rapporteurs
Pr. Hervé Curé, UFR de Médecine de Grenoble, CHU de Grenoble
Dr. Pascale Rio, INSERM, Délégation Régionale Grand Ouest, Nantes

INSERM U1240 - Département d'Oncogénétique du Centre Jean Perrin


Research is to see what everybody else has seen,
and to think what nobody else has thought.
(Albert Szent-Gyorgyi)

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II. **Rifaï K,** Judes G, Idrissou M, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. SIRT1-dependent epigenetic regulation of H3 and H4 histone acetylation in human breast cancer. **Oncotarget.** 2018; 9: 30661–78.

III. **Rifaï K,** Idrissou M, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. SIRT1 in Colorectal Cancer: A Friend or Foe ? **OMICS J Integr Biol.** 2018; 22: 298–300.

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VI. Judes G, **Rifaï K,** Daures M, Dubois L, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. High-throughput «Omics» technologies: New tools for the study of triple-negative breast cancer. **Cancer Letters**. 2016 Mar 7. pii: S0304-3835(16)30137-9.

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Communications Scientifiques Orales

10ème Journée Scientifique du CRNH Auvergne. 23 Novembre 2017, Campus de Cézeaux, Clermont-Fd. Effet de l'HDAC SIRT1 sur la progression du cancer sporadique du sein.

Fourth International Congress of Translational Research in Human Nutrition – Cancer and Nutrition, 22-23 Juin 2017, Clermont-Fd. The implication of HDAC SIRT1 in Human Breast Carcinomas.

20ème Journée de l'Ecole Doctorale – Session CLARA, 18-19 Mai 2017, Clermont-Fd. Etude de l'histone déacétylase SIRT1 dans le cancer sporadique du sein.

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Communications Scientifiques Affichées

13éme édition du Forum de la Recherche en Cancérologie Auvergne-Rhône-Alpes (CLARA), 3-4 Avril 2018, Lyon. L'Implication de l'Histone Déacétylase SIRT1 dans le Cancer Sporadique du Sein : Un Biomarqueur Pronostique Potentiel.

Fourth International Congress of Translational Research in Human Nutrition – Cancer and Nutrition, 22-23 Juin 2017, Clermont-Fd. The implication of HDAC SIRT1 in Human Breast Carcinomas.

20ème Journée de l'Ecole Doctorale – Session CLARA, 18-19 Mai 2017, Clermont-Fd. Etude de l'histone déacétylase SIRT1 dans le cancer sporadique du sein.

8ème Journée Scientifique du CRNH Auvergne. Clermont-Ferrand, 26 novembre 2015. Mise en évidence de l'interaction TIP60-H3K4ac dans le cancer sporadique du sein.

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LISTE DES ABREVIATIONS

ADN : Acide désoxyribonucléique

ADP : Adenosine diphosphate

AMPK : AMP-activated protein kinase

ANOVA : Analyse de variance

AR : Androgen Receptor

ARNm : Acide ribonucléique messager

ARNnc : ARN non codant

ATM : ATM serine/threonine kinase

BRCA1/2 : Breast Cancer 1/2

C-MYC : V-myc avian myelocytomatosis viral oncogene homolog

CBP : CREB-binding protein

ChIP : Chromatin immunoprecipitation

ChIP re-ChIP : ChIP suivie d'une deuxième ChIP

Co-IP : Co-immunoprécipitation

DNMT : DNA methyltransferase

EMT : Epithelial–mesenchymal transition

EP300: E1A binding protein P300

Epi-marque : Marque épigénétique

ER : Estrogen Receptor

ER-α : Estrogen Receptor-alpha

ERBB2 : Erb-b2 receptor tyrosine kinase 2

ERE : Elément de réponse aux œstrogènes

ESR1/ 2 : gènes codant pour Estrogen Receptor 1/2

EZH2 : Enhancer of zeste homolog 2

E2F1 : E2F transcription factor 1

FOX : Forkhead box

GNAT : Gcn5-related N-acetyltransferase

GPx : Glutathione Peroxidase

GST : Gène Suppresseur de Tumeur

HAT : Histone acetyltransferase

HDAC : Histone deacetylase

HDACi : Histone deacetylase inhibitor

HER2 : Human epidermal growth factor receptor 2

HIF1α :Hypoxia-inducible factor 1-alpha

HMT : Histone methyltransferase

HRBC : Hormone Receptor-positive Breast Cancer

H2BC : HER2 Breast Cancer

ING : Inhibitor of Growth Protein

KAT : Lysine acétyltransférase

LAR : Luminal Androgen Receptor

miRNA : micro ARN

MUC2 : Mucin 2

MYST : MOZ, Ybf2 (Sas3), Sas2, and TTP60.

NAD : Nicotinamide adenine dinucleotide

NF-κB : Nuclear factor kappa-B

NLS : Nuclear localization signal

NOXA : PMAIP1 - Phorbol-12-myristate-13-acetate-induced protein 1

P/CAF : p300/CBP Associated Factor

PGR : Progesterone Receptor

PI3K : Phospho-inositid-3-OH kinase

PPARγ : Peroxisome proliferator activated receptor gamma

PTMs : Modifications Post-Traductionnelles

qPCR : quantitative Polymerase Chain Reaction

ROS : Reactive Oxygen Species

SBR : Scarff, Bloom et Richarson

shRNA : short hairpin RNA

siRNA : small interfering RNA

SOD : Superoxide dismutase

TF: Transcription Factor

TIP60 : Tat interactive protein 60kDa

TLDA : Taqman Low Density Array

TNBC : Triple negative breast cancer

TNM : Tumor Node Metastasis

VEGF : Vascular endothelial growth factor

INTRODUCTION GÉNÉRALE

Le cancer du sein est une maladie génétique multifactorielle qui reste la principale cause de décès par cancer chez les femmes dans les pays les moins développés, et la deuxième cause de décès par cancer chez les femmes dans les pays les plus développés après le cancer du poumon. Dix à 15% des tumeurs du sein ont une origine génétique héréditaire principalement liée à la mutation des gènes *BRCA1* ou *BRCA2*, tandis que 85 à 90% des tumeurs mammaires sont appelées tumeurs sporadiques ou non héréditaires. Ces tumeurs sporadiques ont des origines environnementales variables et elles sont surtout caractérisées par la présence d'anomalies sous-jacentes profondes au niveau de leur épigénome.

En effet, la complexité de la cancérogenèse mammaire ne peut être représentée uniquement par des mutations génétiques, mais implique également des altérations épigénétiques profondes. Ces altérations contribuent au processus de tumorigenèse en régulant l'expression des oncogènes et de gènes suppresseurs de tumeurs (GST). La régulation épigénétique du génome comprend entre autres, les modifications post-traductionnelles (PTMs) des histones H3 et H4. La dérégulation de ces dernières est considérée comme un biomarqueur du pronostique du cancer, ainsi elle s'est avérée prédictive de l'évolution du patient dans divers carcinomes humains. Dans le cancer du sein, l'analyse de tumeurs mammaires humaines a révélé une corrélation très significative entre les profils globaux des marqueurs d'histone et les phénotypes moléculaires de la tumeur, ainsi que les facteurs pronostiques du cancer.

D'autre côté, les histones désacétylases (HDACs) et les histones acétyltransférases (HAT) sont des enzymes épigénétiques modulatrices des PTMs, et des acteurs majeurs de la régulation épigénétique. Elles peuvent réguler l'expression de nombreux gènes liés au cancer et moduler l'activité d'une multitude d'oncoprotéines impliquées dans la carcinogenèse humaine. L'activité désacétylase aberrante des HDACs, aussi que leurs expressions altérées dans les cancers, ont été étroitement liées au développement et à la progression du cancer. À ce titre, une approche innovante dans le traitement du cancer est récemment née : C'est la thérapie épigénétique par des « Épi-drogues » ou les inhibiteurs des enzymes épigénétiques modulatrices.

En se basant sur ces données, l'objectif général de notre équipe a été d'identifier la dérégulation des modifications épigénétiques des histones dans le cancer sporadique du sein, en mettant en évidence leurs rôles dans la progression tumorale du cancer, et d'étudier les enzymes épigénétiques responsables de leur modulation dans les différents sous-types moléculaires du cancer du sein.

Dans ce contexte, nous nous sommes focalisés sur les profils d'acétylation et de désacétylation des histones H3 et H4 dans le cancer sporadique de sein, notamment les 3 marques activatrices de la transcription : H3k4ac, H3k9ac et H4k16ac, qui restent toujours peu étudiées dans le cancer du sein. Nous nous sommes également intéressés à l'histone désacétylase SIRT1 et à l'histone acétyltransférase TIP60, leurs rôles controversés dans le cancer du sein, et l'effet de leur régulation des marques activatrices sur le développement du cancer. Le but de ce travail a été d'étudier les implications de SIRT1 et de TIP60 dans la progression tumorale du cancer en mettant en évidence leurs rôles comme biomarqueurs pronostiques et aussi comme cibles thérapeutiques potentielles dans le cancer sporadique du sein, et d'identifier les enzymes modulatrices de la marque H3K4ac dans le cancer du sein.

Nous terminerons enfin notre propos par l'exposé des conclusions et perspectives majeures découlant de ce travail.

CHAPITRE I. ETAT DE LART

A. Le Cancer du Sein

Malgré leurs différences cliniques et anatomiques, tous les types de cancer se développent suite à une croissance cellulaire incontrôlée. C'est un processus en plusieurs étapes qui évolue généralement d'une lésion précancéreuse à une tumeur maligne. Ces transformations peuvent être facilitées par des agents mutagènes et cancérogènes externes, qui causent des mutations fonctionnelles dans la séquence d'ADN. Ainsi, ces événements aboutissent à la surexpression des oncogènes, ce sont des gènes dont l'expression favorise la survenue d'un cancer, ou à la perte d'expression des gènes suppresseurs de tumeur (GST) (**Figure 1**). Tous ces mécanismes de carcinogenèse sont retrouvés dans le cancer du sein. La survenue d'un cancer du sein est donc, un processus complexe, multifactoriel, régulé par des gènes différents à différentes étapes de la formation des tumeurs mammaires.

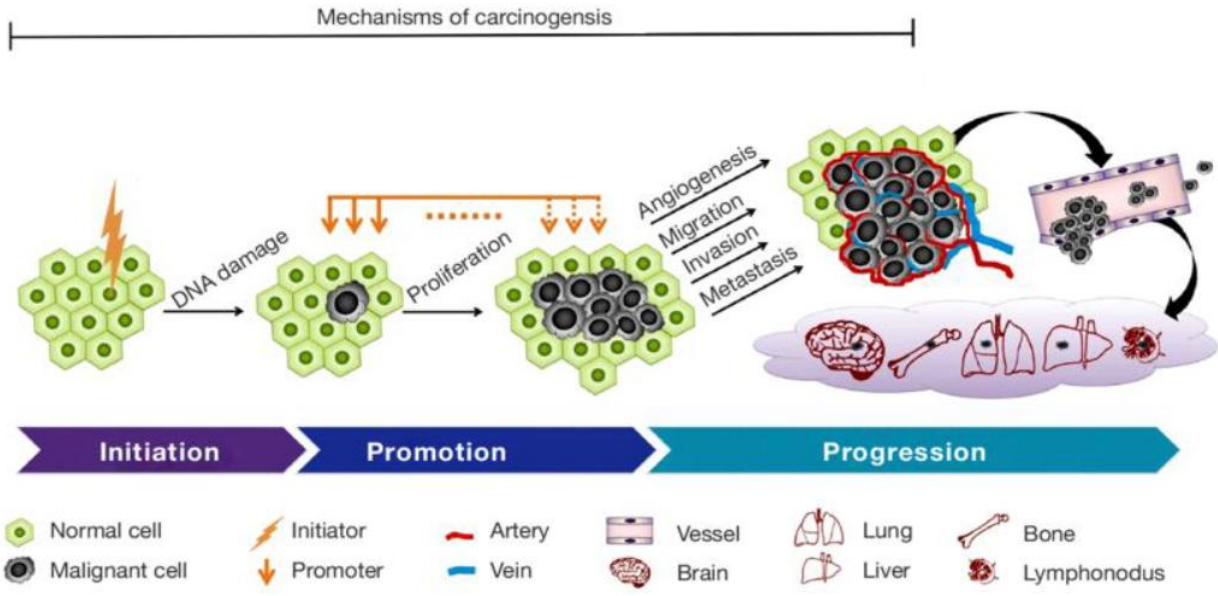

Figure 1. Schéma simplifié représentant le processus mullti-étapes de la carcinogenèse. Adopté et modifié de (Liu et al., 2015).

1. Aspects Épidémiologiques et Facteurs de Risque

Selon l'organisation mondiale de la santé (OMS), le cancer est la deuxième cause de mortalité dans le monde après les maladies cardiovasculaires. À l'échelle mondiale, il y a 17,5 millions de personnes atteintes de cancer en 2015 avec 8,8 millions de décès, dont plus de 571 000 décès sont dus au cancer du sein [1]. En 2018, on estime qu'environ 1 735 350 nouveaux cas de cancer seront diagnostiqués aux États-Unis et 609 640 personnes mourront de cette maladie [2]. Le cancer du sein reste toujours le cancer le plus fréquent chez les femmes dans le monde, il représente environ 25% de l'ensemble des cancers féminins (Kohler et al., 2015). Il est aussi la deuxième cause de décès par cancer chez les femmes dans les pays plus développés après le cancer du poumon (Torre et al., 2015). Aux Etats-Unis, le cancer du sein atteint 1 femme sur 8, soit environ 12.4% de la population féminine [2].

En France métropolitaine, le cancer est la première cause de mortalité devant les maladies cardiovasculaires. En 2017, on estime à 400 000 le nombre de nouveaux cas de cancer, dont 186 000 (46 %) chez la femme. Le pourcentage étonnant de 32% de nouveaux cas chez la femme est attribué au cancer du sein. On estime aussi 66 000 décès par cancer chez la femme en 2017, dont 11 900 (12%) décès sont dus au cancer du sein.

L'incidence du cancer augmente depuis 1980 chez la femme en France, mais cette augmentation tend à ralentir depuis 2005. Entre 2005 et 2012, le taux d'incidence tend à se stabiliser chez les femmes françaises (+ 0,2 %) par an en moyenne, alors que la mortalité par cancer chez les femmes a baissé de 1.4% par an pour la même période (**Figure 2**). Cependant, les taux de mortalité par cancer du sein sont stables ou en baisse depuis les années 90' en Amérique du Nord et dans les pays européens à ressources plus élevées comme la France. Cette baisse est attribuée à la détection précoce par mammographie et à l'amélioration du traitement du cancer (Althuis et al., 2005). Tous les chiffres mentionnés de cancer en France sont tirés du rapport « Les cancers en France – Edition 2017 », publié en juin 2018 par l'institut national français du cancer (INCa) [3].

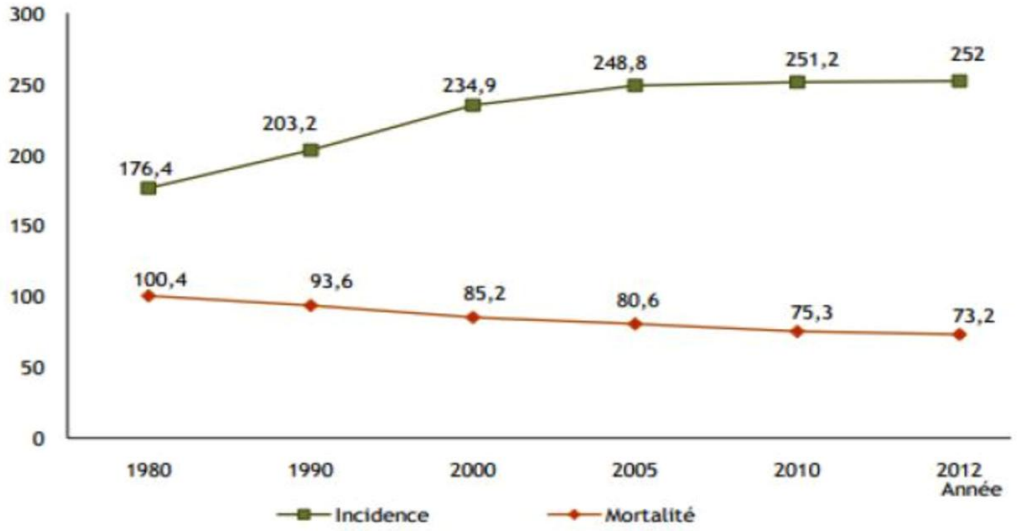

Figure 2. Le taux standardisé d'incidence et mortalité du cancer chez la femme en France entre 1980 et 2012. Tiré du rapport rédigé par Binder-Foucard F et al. 2013 [4].

En effet, différents facteurs de risque favorisent l'apparition d'un cancer du sein (**Figure 3**), l'âge est parmi les facteurs les plus importants, à peu près 75% des cancers du sein se déclarent après 50 ans. On distingue trois grands types de facteurs de risque (Key et al., 2001) :

- Les facteurs génétiques dont la grande partie est liée à la mutation des gènes *BRCA1* ou *BRCA2*. Ces derniers sont les principaux gènes de prédisposition au cancer du sein. Ils sont responsables d'environ 10 à 15% des cas de cancer chez des familles présentant une histoire familiale de cancer sein (Stratton and Rahman, 2008).
- Les facteurs hormonaux et reproductifs, ce sont les facteurs qui augmentent l'exposition aux hormones comme les estrogènes. On distingue les facteurs hormonaux endogènes tels que les cycles menstruels précoces (avant 12 ans), une ménopause tardive (après 50 ans) ou une première grossesse tardive (après 35 ans), aussi que des facteurs hormonaux exogènes tels que l'utilisation des contraceptifs oraux ou un traitement hormonal substitutif (THS) de la ménopause.

- Les facteurs comportementaux et environnementaux. Les facteurs comportementaux sont liés aux habitudes de vie comme l'alimentation, l'activité physique, l'obésité, la consommation de tabac et de l'alcool. Alors que les facteurs environnementaux comprennent entre autres, les radiations ionisantes, l'exposition aux produits chimiques cancérogènes et les champs électromagnétiques.

Finalement, une bonne alimentation équilibrée, l'augmentation de l'activité physique, et la réduction de la consommation d'alcool et de tabac sont parmi les meilleures stratégies disponibles pour réduire le risque de développer un cancer du sein.

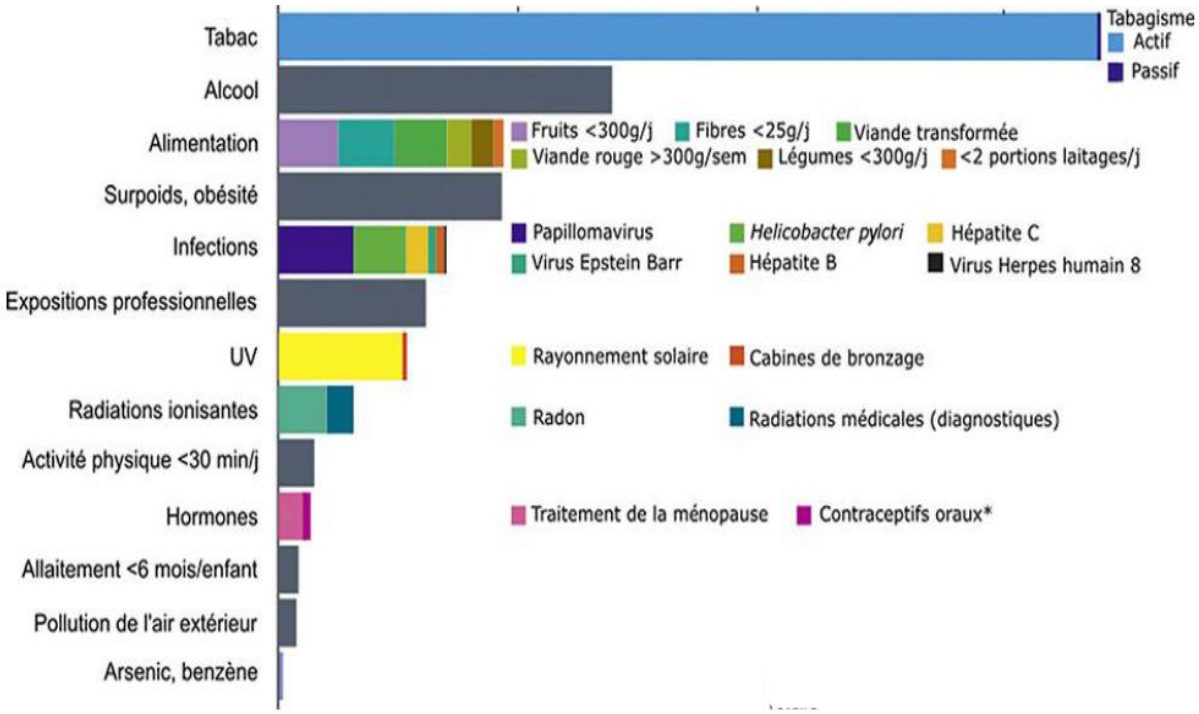

Figure 3. Schéma représentant les différents facteurs de risque du cancer du sein chez la femme. Tiré du Bulletin épidémiologique hebdomadaire n° 21 [5].

2. Classification des Cancers du Sein

Le cancer du sein est un groupe de maladies hétérogènes dont les caractéristiques moléculaires et cliniques varient considérablement. En conséquence, une classification précise du cancer du sein est nécessaire pour fournir des informations pronostiques sur la maladie, et pour bien choisir des thérapies adaptées en fonction du type de la tumeur.

2.1. Les Facteurs Clinico-pathologiques et Biomarqueurs des Tumeurs

Plusieurs facteurs pronostiques ainsi que des biomarqueurs biologiques ont été identifiés, et utilisés par la suite en pratique clinique pour classer les tumeurs du sein. Ces facteurs permettent d'identifier des sous-groupes de patientes pour comparer leur évolution, définir des populations à risque, et proposer des traitements adjuvants les mieux adaptés.

Les classifications des cancers du sein dépendent des critères histologiques et moléculaires, le plus souvent : la taille de la tumeur, son degré d'agressivité, la présence des métastases ganglionnaires, la présence d'emboles, l'expression des récepteurs hormonaux, et l'amplification des oncogènes. On distingue la classification histologique de stade qui suit la classification internationale TNM (Tumor Node Metastasis). Cette classification est établie par The American Joint Committee on Cancer (AJCC), et repose sur l'examen histologique de la taille de la tumeur primaire (T), du statut ganglionnaire régional anatomo-pathologique (N) et l'existence de métastases (M) (Edge and Compton, 2010) (**Tableau 1**).

Un autre type répandu de classification est celle du grade tumoral. Cette classification combine 3 paramètres morphologiques : la formation de tubules, le pléomorphisme nucléaire, et le nombre de mitoses. Le grade histo-pronostique de Scarff Bloom et Richardson (SBR) (Bloom and Richardson, 1957) est le plus utilisé dans la pratique courante pour évaluer le degré d'agressivité de la tumeur. Des modifications ont été apportées sur ce système afin d'améliorer la reproductibilité du grade et sa valeur pronostique, donnant naissance au système Nottingham Prognostic Index (NPI) (Elston and Ellis, 2002) (Rakha et al., 2008). En 2003, l'OMS a classifié le cancer du sein en 20 sous-types histologiques différents (Vajpeyi, 2005).

T = tumeur primitive
TX : aucune information sur la tumeur **T0 :** tumeur non perceptible cliniquement **Tis :** carcinome in situ ou maladie de Paget sans tumeur associée **T1 :** tumeur de 2 cm : - **T1mic :** micro-invasion ≤ 1 mm - **T1a :** tumeur entre 1 et 5 mm - **T1b :** tumeur de 5 à 10 mm - **T1c :** tumeur de 1 à 2 cm **T2 :** tumeur de 2 à 5 cm **T3 :** tumeur de plus de 5 cm **T4 :** décrite de la façon ci-dessous quelle que soit la taille : - **T4a :** extension à la paroi thoracique (à l'exclusion des muscles pectoraux - **T4b :** œdème (inclusion « peau d'orange ») ou ulcération cutanée ou nodules de perméation - **T4c :** T4a + T4b - **T4d :** carcinome inflammatoire
N : nœuds lymphatiques régionaux
NX : aucune information **N0 :** pas d'adénopathie perceptible cliniquement **N1 :** une ou plusieurs adénopathies axillaires homolatérales mobiles et individualisables **N2 :** adénopathies axillaires homolatérales fixées OU adénopathies mammaires internes homolatérales sans adénopathie axillaire clinique : - N2a : adénopathies axillaires homolatérales fixées - N2b : adénopathies mammaires internes homolatérales sans adénopathie axillaire clinique **N 3 :** comme décrit ci-dessous : - N3a : adénopathie homolatérale sous-claviculaire, avec ou sans adénopathie axillaire - N3b : adénopathies homolatérales mammaires internes et axillaires - N3c : adénopathies supraclaviculaires homolatérales
M = métastases
MX : aucune information sur la présence de métastase à distance **M0 :** absence de métastase à distance **M1 :** présence de métastase à distance

Tableau 1. La classification histopathologique TNM dans le cancer du sein, selon l'American Joint Committee on Cancer: AJCC Cancer Staging Manual, 6th ed, New York **[6].**

Outre les critères histo-pathologiques, l'évaluation clinique du cancer du sein implique la caractérisation immunohistochimique de biomarqueurs moléculaires par des anticorps monoclonaux. Les récepteurs hormonaux estrogen receptor alpha (ER-α) et progesterone receptor (PR), et l'oncogène human epidermal growth factor receptor 2 (HER2) sont les 3 marqueurs essentiels à évaluer lors d'un diagnostic et qui permettent d'identifier et de distinguer les sous-types principaux du cancer du sein. Ainsi, leur utilité pronostique et prédictive du cancer guide efficacement la sélection du traitement convenable pour chaque sous-type (Patani et al., 2013).

En effet, le statut hormonal des tumeurs évalué par l'expression des récepteurs hormonaux ER et PR, est utilisé pour prédire la réponse des patientes aux thérapies endocrines (Hammond et al., 2010). Ainsi, les tumeurs ER+ sont corrélées à un bon pronostique clinique (Dunnwald et al., 2007), comme les tumeurs PR+ (Cui et al., 2005). De plus, le statut hormonal des 2 récepteurs peut être prédictif de la réponse à l'hormonothérapie à la fois chez les patientes avec un cancer métastatique (Ravdin et al., 1992) et dans les traitements adjuvants (Bardou et al., 2003). Un gradient de sensibilité à l'hormonothérapie et de la survie en fonction du statut ER/PR a été alors mis en évidence. Cette combinaison a permis de distinguer 4 groupes phénotypiques différents des tumeurs : ER+/PR+, ER+/PR-, ER-/PR+, et ER-/PR- (Grann et al., 2005). La majorité des patientes avec un statut ER+/PR+ répondent favorablement à l'hormonothérapie, tandis que près d'un tiers avec un statut ER+/ PR- et seulement 10 % avec un statut ER-/ PR+ répondent favorablement. Cependant les tumeurs ER-/PR- ne répondent pas à l'hormonothérapie et sont considérées comme de mauvais pronostique (Rusiecki et al., 2005) (Rakha et al., 2010).

Le statut d'expression du récepteur du facteur de croissance épidermique humain 2 (HER2) est aussi indispensable en clinique et est exploré en routine. L'oncogène est codé par le gène *ERBB2* et appartient à la famille des EGFR (Epidermal Growth Factor Receptor) qui comprend 4 membres (HER1/EGFR, HER2, HER3, et HER4). Le statut de HER2 est un marqueur prédictif à la réponse à des thérapies particulières, comme au Trastuzumab (anticorps anti-HER2) (Slamon et al., 2011), ainsi qu'aux anthracyclines (Pritchard et al., 2006) et aux taxanes (Konecny et al., 2004). En fait, l'amplification du gène *ERBB2* et la

surexpression de HER2 est observée dans 15-25% des tumeurs mammaires (Dandachi et al., 2002). Cependant, elle est associée à un mauvais pronostic et à une mauvaise réponse à la chimiothérapie (Ross et al., 2003) (Rakha et al., 2010).

D'autres biomarqueurs peuvent être utiles sur le plan clinique. On distingue l'antigène KI-67 qui est un marqueur de prolifération exprimé dans les phases G1, S, G2 et M du cycle de division cellulaire, mais absent en G0. Il est également utilisé dans la classification moléculaire du cancer sein. Un score élevé pour KI-67 indique une prolifération rapide des cellules cancéreuses, et est considéré comme un facteur pronostique défavorable du cancer du sein (Urruticoechea et al., 2005).

Il y a aussi des marqueurs émergents comme les récepteurs hormonaux estrogen receptor beta (ER-β), androgen receptor (AR), et EGFR, ainsi que les cyclin D1, cyclin E, et les cellules tumorales circulantes (Weigel and Dowsett, 2010) (Patani et al., 2013).

2.2. La Classification Moléculaire des Tumeurs du Sein

Le cancer du sein est une maladie génétique multifactorielle, il est caractérisé par son hétérogénéité moléculaire et clinique avec des variations dans les profils d'expression génique entre les différentes tumeurs mammaires (Sørlie et al., 2001) (Sorlie et al., 2003). En effet, il s'est avéré que l'utilisation des facteurs histologiques seuls pour classer toutes les tumeurs du cancer était insuffisante à cause de l'absence de critères standardisés pour leur diagnostic, ainsi qu'une reproductibilité faible dans certains cas. De ce fait, une classification moléculaire qui tenait compte des variations géniques, devenait indispensable pour éviter les cas de traitements inappropriés ou aussi les sur-traitements. Effectivement, le processus de sous-typage moléculaire du cancer du sein en se basant sur des profils d'expression génique, a permis de clarifier les différences de comportement biologique entre les sous-groupes de la maladie, en permettant un traitement individualisé et un meilleur pronostique pour chaque sous-type intrinsèque (Carey et al., 2006) (Parker et al., 2009) (O'Brien et al., 2010).

La classification moléculaire établie par les consensus internationaux de St Gallen sur le cancer du sein, divise les tumeurs du sein en 4 grands sous-types en fonction de leur agressivité (**Tableau 2**). : Luminal A et Luminal B, ces 2 sous-types sont compris dans le groupe Hormone Receptor-positive Breast Cancer (HRBC), HER2-enriched qui définit le groupe HER2 Breast Cancer (H2BC), et triple-négative breast cancer (TNBC), aussi nommé Basal-like. Cette classification est basée sur l'analyse d'expression par immunohistochimie (IHC) de 4 marqueurs pronostiques dans les tumeurs du sein: les récepteurs hormonaux ER et PR, le statut HER2, et le taux de KI-67. L'expression différentielle de ces biomarqueurs prédictifs définit une classification clinique du cancer du sein (Goldhirsch et al., 2013) (Coates et al., 2015) (Curigliano et al., 2017).

Sous-type moléculaire	Expression des biomarqueurs prédictifs
Luminal A	• ER+ • PR+ • HER2- • KI-67 <14%
Luminal B	○**Luminal B (HER2-)** • ER+ • PR+/− • HER2− • KI-67 ≥14% ○ **Luminal B (HER2+)** • ER+ • PR+/− • HER2+ • KI-67+/−
HER2-enriched	• ER- • PR- • HER2+
TNBC	• ER- • PR- • HER2-

Tableau 2. Répartition des tumeurs du sein en 4 sous-types intrinsèques selon les consensus internationaux de St Gallen.

Les tumeurs Luminal A représentent 50-60% des cancers du sein. Ce sous-type est le plus commun et le moins agressif, il est associé à un pronostic et un taux de survie plus favorables. Il est aussi caractérisé par l'activation des voies de signalisation œstrogène / ER-α qui stimulent la prolifération cellulaire et la croissance tumorale. Le profil d'immunohistochimie luminal est caractérisé par l'expression des récepteurs ER et PGR, et les cytokératine CK8 / 18, aussi que l'absence d'expression de HER2 et d'un faible taux de KI-67 (<14%) (Feeley et al., 2014) (Lam et al., 2014).

Les tumeurs Luminal B sont moins fréquentes et constituent environ 10% à 30% des cancers du sein. Elles ont un phénotype plus agressif et un pronostique moins favorable que les tumeurs Luminal A. Du point de vue immunohistochimique, les tumeurs Luminal B sont divisées en 2 sous-groupes : Luminal B (HER2-) qui est caractérisé par l'expression des récepteurs ER, PGR, l'absence d'expression de HER2, et des taux élevés de KI-67 (≥ 14%). L'autre sous-groupe Luminal B (HER2+) est caractérisé par l'expression des récepteurs ER, PGR, et la surexpression de HER2. Ainsi, les tumeurs Luminal B (HER2+) sont considérées plus agressives et ont un plus mauvais pronostic que les tumeurs Luminal B (HER2-) (Cheang et al., 2009) (Lam et al., 2014).

Le sous-type HER2-enriched représente 15-25% de tous les cancers du sein. Il est caractérisé par une forte expression du gène *ERBB2* et d'autres gènes associés à la voie HER2, il présente aussi une surexpression de gènes liés à la prolifération cellulaire, et un pourcentage élevé des mutations de gène suppresseur de tumeur p53. Les tumeurs HER2-enriched sont aussi caractérisées par un mauvais pronostique et l'absence d'expression des récepteurs ER et PGR (Dandachi et al., 2002) (Ross et al., 2003).

Les cancers du sein triple-négatifs (TNBC), également connus comme Basal-like, constituent 15 à 20% de tous les cancers du sein. Ils sont considérés très agressifs et caractérisés par un très mauvais pronostic, ainsi qu'un taux de mortalité plus élevé par rapport aux autres sous-types moléculaires. Cependant, les TNBCs sont très hétérogènes, plusieurs classifications ont été proposée pour les différencier. Dans la revue suivante, on présente les différentes classifications des TNBCs en utilisant des nouvelles technologies «Omiques», on parle aussi d'application clinique de ces technologies à haut débit.

2.3. L'Utilisation des Technologies «Omiques» dans la Classification des TNBC

Présentation de la publication 1

High-throughput «Omics» technologies: New tools for the study of triple-negative breast cancer

Judes G, **Rifaï K**, Daures M, Dubois L, Bignon YJ, Penault-Llorca F, Bernard-Gallon D.

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Les cancers du sein triple-négatifs (TNBC) affectent plus fréquemment les patients plus jeunes. Ce sont des tumeurs très agressives qui sont caractérisées par l'absence d'expression des récepteurs ER, PR, et HER2, et une surexpression des cytokératines CK5, 6, 14, et 17. Cependant, le traitement des patientes atteintes d'un TNBC est difficile en raison de l'hétérogénéité de la maladie et de l'absence de cibles moléculaires bien définies.

Les techniques «Omiques» visent principalement la détection à grande échelle de gènes (génomique), d'ARNm (transcriptomique), de protéines (protéomique) et de métabolites (métabolomique) dans un échantillon biologique spécifique. En utilisant cette nouvelle technologie à haut débit, les chercheurs peuvent décortiquer la complexité des tumeurs triples négatives, et essayer d'identifier de nouveaux biomarqueurs et cibles thérapeutiques potentiels dans TNBC. Cette revue présente les différentes découvertes sur le TNBC en utilisant les technologies Omiques.

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Mini-review

High-throughput «Omics» technologies: New tools for the study of triple-negative breast cancer

Gaëlle Judes [a,b], Khaldoun Rifaï [a,b], Marine Daures [a,b], Lucas Dubois [a,b], Yves-Jean Bignon [a,b,*], Frédérique Penault-Llorca [b,c], Dominique Bernard-Gallon [a,b]

[a] Department of Oncogenetics, Centre Jean Perrin, CBRV, 28 place Henri-Dunant, 63001, Clermont-Ferrand, France
[b] EA 4677 "ERTICA", University of Auvergne, 63011, Clermont-Ferrand, France
[c] Department of Pathology, Centre Jean Perrin, 63011, Clermont-Ferrand, France

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ABSTRACT

Triple negative breast cancer (TNBC) represents about 15% to 20% of all breast cancers and is typically associated with poorer outcome than other breast cancer subtypes. The heterogeneity of this breast cancer subtype and present lack of clinically established targeted therapies further complicates treatment of patients. The treatment of TNBC emphasizes enhancing health care and developing personalized medicine. To respond to this need, the researchers have turned their attention to a different approach to scientific enquiry: the era of "big biology" and the integrative study of biological systems, also called "Omics" technologies. The term omics comprises different fields of molecular studies and characterizes a global view on biological molecules such as DNA, RNA, proteins, and metabolites. Combined "omics" approach offers a major tool for the understanding of a challenging cancer model, TNBC. This review discusses the different discoveries made using omics technologies concerning the molecular mechanisms underlying TNBC phenotypic heterogeneity, and their potential transfer to clinical applications.

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Introduction

Breast cancer is a heterogeneous disease. Numerous subtypes and biological, histological and molecular characteristics confer its variability and result in different responses to therapy. In particular, triple-negative breast cancer (TNBC), the most heterogeneous group of breast cancers, is a major challenge for effective clinical management. This tumor subtype is defined by the absence of estrogen receptor (ER) and progesterone receptor (PR) expression, and by a lack of human epidermal growth receptor 2 (HER2) amplification. It accounts for some 15–20% of all breast cancers [1,2]. TNBC tumors are generally higher grade with a large size, have lymph node involvement, and are biologically more aggressive than breast tumors in general [3,4]. TNBC patients are generally younger [5] and have a higher risk of recurrence and death within three to five years after treatment than non-TNBC patients [6].

Fifteen years ago, a subtype presented a negative expression of ER, PR and HER2; the subtype was discovered using first-generation cDNA microarrays [1]. This subtype is basal-like and represented approximately 75% of TNBC. Markers used to determine prognosis and therapy in TNBC, such as hormone receptor status, size of tumor or histological grade are limited. Moreover, there is no international standard for the molecular subtyping of cancers, although some guidelines exist for the application of molecular stratification to prognostic or predictive guidance. Hence new biomarkers are needed together with a robust method to stratify TNBC and obtain better and more accurate diagnosis, prognosis and treatment response.

In the last 10 years, major technological discoveries have transformed cancer biology. Various profiling strategies such as DNA methylation, epigenetic alterations, DNA copy number, genome and transcriptome sequencing, and proteome and metabolome analysis are used to study the tumor genome [7–9]. These omics technologies can help us gain a better understanding of carcinogenesis. Approaches are based on a thorough study of complex biological systems considered as a whole. System-wide analyses are used on a large scale for the quantification of genes coding for proteins, regulatory elements and noncoding sequences (genomics), RNA and gene expression (transcriptomics), protein expression

Abbreviations: TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth receptor 2; LOH, loss of heterozygoty; SNP, single nucleotide polymorphism; BRCA1, breast cancer 1; BRCA2, breast cancer 2; GWAS, genome-wide association studies; HRD, homologous recombination deficiency; LST, large-scale state transition; MBD Cap-seq, methyl-CpG-binding domain capture sequencing; B-CIMP, breast-CpG island methylator phenotype; PAM50, prediction analysis of microarrays 50; iTRAQ, isobaric tags for relative quantitation; LC-MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; NHI, national institutes of health; BU, bottom-up; TD, top-down; TCGA, the cancer genome atlas; ICGC, international cancer genome consortium; TNBCDb, triple negative breast cancer database; ASCO, American society of clinical oncology; IOM, institute of medicine; IRB, institutional review board; FDA, food and drug administration.

* Corresponding author. Tel.: +33 473178358; fax: +33 473178359.
E-mail address: yves-jean.bignon@cjp.fr (Y.-J. Bignon).

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(proteomics), metabolites and metabolic pathways (metabolomics). Omics techniques enable the identification of biomarkers such as biologically important genes, pathways disrupted in cancer, and protein expression signatures. These data can be used to predict responses to drugs and the clinical course of disease, and such information can be used to individualize therapy. A personal approach to treatment is defined by a trial with $n = 1$; this approach eliminates biological variability. However, it implies the setting-up and verification of a baseline for comparison. "Omics" technologies can supply multiple datasets and form such a baseline. Given the diversity and complexity of TNBC, omics technologies are indispensable tools to extend our knowledge of TNBC subtypes.

Genomics and epigenomics in TNBC

The fundamental goal of genomics and epigenomics is to understand genome biology and its impact on cancer. Genomics is a set of genetic and molecular biology techniques that enables genetic mapping, and DNA sequencing of genes or global genome. Epigenetics concerns inheritable changes in gene expression that do not involve changes to the underlying DNA sequence. Genomics and epigenomics technologies generate vast amounts of biological data including gene sequences (high-throughput sequencing) and information on gene expression. All these data are then studied by bioinformatics tools to carry out an integrative analysis, and finally visualization of genomics and epigenomics data sets.

Genomics: copy number, LOH, mutations and SNPs

A study of genomic profile identified a variation in number copy between mutated *BRCA1* or *BRCA2* breast tumors and sporadic tumors [10,11]. Mutated *BRCA1* tumors presented a high frequency of copy number alterations compared with sporadic cancer. Analysis by array comparative genomic hybridization detected losses in 5q12.1, 5q11-23 and 12q21 genomic regions and a gain in 8q24 and 17q25.3 genomic regions in TNBC with mutated *BRCA1*. In particular, 17q25.3 gain was identified in 90% of mutated *BRCA1* tumors, and 50% of TNBCs presented a hypermethylated promoter. The analysis of copy number by DNA microarrays in 997 breast tumors found a deletion on chromosome 5 in the basal-like subtype [12]. The correlation between copy number and gene expression showed that this zone was involved in different transcriptional changes concerning cell cycle, DNA damage repair and apoptosis, which are characteristics of basal-like tumors. Different genes were therefore associated with this deletion on chromosome 5 such as *AURKB, BCL2, BUB1, CDCA3, CDCA4, CDC20, CDC45, CHEK1, FOXM1, HDAC2, IGF1R, KIF2C, KIFC1, MTHFD1L, RAD51AP1, TTK* and *UBE2C*. A clustering analysis highlighted a tumor subgroup that presented a high genomic instability typical of basal-like tumors.

Single nucleotide polymorphism (SNP) arrays were used to investigate genome-wide patterns of variation. SNP arrays contain a large number of known SNPs. New cancer-specific aberrations of TNBC were identified, such as TTK protein kinases involved in DNA damage response [13]. Microarray analyses showed an up-regulation of TTK in different cancers, and specifically in breast cancer. TTK was overexpressed in TNBC compared with luminal breast cancer subtype [14]. SNP arrays showed that the copy number of the *TTK* gene was increased in TNBC compared with other breast tumors, and this was correlated with *TTK* mRNA levels [15]. In recent years, genome-wide association studies (GWAS) have highlighted an association between different genetic variants and high risk of developing a cancer such as breast cancer [16,17]. Hicks et al. studied the link between genetic variants and their associated genes in TNBC [18]. This work combined GWAS information with gene expression data (genomics approach) to identify potential biologic signatures, pathways enriched for SNPs and dysregulated in TNBCs, and to stratify this subtype. Thirty-one genes identified in TNBC presented SNPs found in different GWAS. Among these, *PTEN, RB1, BRCA1, BRCA2, ATR, ATM, MAP3K1, CDKN2A, ATR, CHEK1, CCND1, NOTCH2* genes were significantly associated with TNBC and were mutated in breast cancer, and the molecular pathway analysis and networks enriched for SNPs were found to be involved in different mechanisms in TNBC. However, their expression levels differed according to the TNBC subset, normal-like, basal-like and basal subtypes. These studied genes are known to act in several pathways of tumorigenesis, and the analysis of SNPs could help us understand more about the development and the distinctive features of different TNBC phenotypes.

Genome or exome (part of the genome formed by exons) DNA sequencing identified specific mutations allowing a stratification of breast cancer. Compared with other molecular breast cancer subtypes (luminal A, luminal B and HER2$^+$), TNBC presented a higher mutation rate, particularly for the *TP53* gene, which was mutated in 80% of cases, and these mutations were nonsense or frame shift [19]. Basal subtype and non-basal TNBC presented different types of mutations. The major mutation concerned the *BRCA1* or *BRCA2* genes, and patient carriers have 70–80% probability of developing breast cancer, and a higher risk of ovarian cancer (30–50%). Vaca-Paniagua et al., identified different genes with pathogenic mutations on 12 analyzed TNBCs such as *BRCA1* found in 18% of cases, *TP53* (54%), *RB1* (27%), *PTEN* (18%), *KDM6A* (18%) and *SETD2* (18%) [20]. Similarly, another study with 80 cases of TNBCs [21] showed the *p53* gene to be the most frequently mutated gene in basal TNBC (62%); furthermore, other genes presented a high frequency of mutations in TNBC such as *PIK3CA* (10.2%), *USH2A* (9.2%) and *MYO3A* (9.2%). Finally, 104 cases of TNBCs were studied by Affymetrix SNP6.0, which identified different genes presenting copy number alterations more frequently for *PARK2* (6%), *RB1* (5%), *PTEN* (3%) and *EGFR* (5%). However, the distribution of somatic mutation levels in TNBC was not linked to the percentage of the genome altered by copy number alterations.

TNBC presented genomic signatures of homologous recombination deficiency (HRD) [22]. The HRD tumors were the most highly sensitive to therapeutic agents such as platinum salts and PARP inhibitors [23]. These findings prompted the use of the genomic signature of HRD to discriminate breast tumors into two groups (HR-deficient and HR-proficient) [22], and several clinical trials in TNBC highlighted the utility of genomic signatures of HRD in therapeutic response prediction [24,25]. Another genomic signature in the basal-like subtype with *BRCA1* germline mutation was established: large-scale state transitions (LSTs), defined as the number of chromosomal breakpoints [26]. LST genomic signature was tested as a biomarker of HRD in TNBC and validated with high specificity: it allowed a differentiation between HR-deficient and HR-proficient tumors [27]. This signature might be a potential tool to help stratify breast cancer subtypes in clinical trials involving platinum salts and PARP inhibitors.

Among these findings, the five main genes presenting genomic alterations emerged in TNBC tumors are: *TP53, BRCA1, PIK3CA, RB1* and *PTEN*. Different studies showed no correlation between *TP53* mutation and *BRCA1* mutation status in TNBC tumors [28]. The tumor suppressors *PTEN* (phosphatase and tensin homolog) and *TP53* are frequently altered in TNBCs, and the loss of both genes combined occurred in 18% of TNBCs. *PTEN/TP53* deficiency was associated with a poor prognosis in tumors compared with normal levels of these genes. The combination of *PTEN* deficiency and *TP53* mutation might play a role in oncogenesis by increasing proliferation and motility, and alteration of pathways involved in apoptosis and immune response [29]. The *PIK3CA* gene is also frequently mutated in breast cancer, and is often associated with luminal subtype compared with TNBC tumors [30]. However, mutations and increased copy numbers of *PI3K* gene were found in TNBCs, and particularly in non-BRCA1-like tumors [31]. These genomic alterations and the loss of *PTEN*

expression caused an activation of the PI3K pathway [32]. The loss of inositol polyphosphate 4-phospathase type II (INPP4B) and overexpression of the EGF receptor also contributed to an aberrant activation of the PI3K pathway in TNBC [33–35]. In basal-like breast cancer, *Rb1* tumor suppressor gene presented a loss of heterozygosity (LOH) and a decreased expression in 72% of cases [36]. This loss of expression was associated with high expression of p53 and increased $p16^{ink4a}$ expression. This $Rb^{-}/p16^{ink4a+}$/p53 phenotype was correlated with high proliferation in basal-like tumors [37].

All of genomic analyses have thus detected specific genes in TNBCs that could be used in several potential treatment approaches.

Epigenomics: DNA methylation

DNA methylation is an epigenetic modification that assures in normal cells a regulation of gene expression and a stable gene silencing. This process is a critical player in transcriptional regulation and an alteration of this mechanism may lead to various diseases such as breast cancer [38,39]. Aberrant DNA methylation is involved in tumor origin, development and progression. The study of DNA methylation by omics technologies performed to establish distinct DNA methylation patterns according to breast cancer subtypes. A microarray approach of DNA methylation showed a lower methylation in TNBC than in other breast cancer subtypes such as luminal A, luminal B and $HER2^{+}$. *NPY, FGF3, HS3ST2, RASSF1* and *Let-7a* genes exhibited non-methylated promoters in TNBC tumors compared with luminal B $HER2^{+}$ and $HER2^{+}$ subtypes, and discriminated basal-like and $HER2^{+}$ subtypes [40]. The DNA methylation status of homologous recombinant (HR) genes, including *BRCA1, BRCA2, BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, PALB2, RAD50, RAD51, RAD51C, MRE11, NBS1, CtIP* and *ATM*, was not significantly associated with TNBC subtype and chemosensitivity. Only *BRCA1* and *RNF8* had a significantly higher methylation in TNBC compared with luminal breast cancer. Aberrant methylation of BRCA1 was associated with pathological complete response to neoadjuvant chemotherapy and at the opposite; *RNF8* methylation was significantly lower in pathological complete response cases than in poor response cases [41].

Stirzaker et al. looked at the DNA methylation profile of TNBC and the course of the disease [42]. Using a new technique, MBD Cap-seq (affinity capture of methylated DNA with recombinant methyl-CpG binding domain of MBD2 protein followed by next-generation sequencing), they identified specific methylation profiles in TNBC; 822 regions were hypermethylated compared with matched normal samples, and were mainly located in CpG islands. These regions grouped 308 genes with a hypermethylated promoter divided into two groups: one mostly contained transcription factors, and the other contained genes involved in signaling pathways such as DNA binding and homeobox proteins. From these data, TNBC tumors were stratified into three distinct groups according to the methylation level associated with prognosis; 17 differentially methylated regions allowed this stratification, and a better prognosis was associated with the hypomethylated profile [42]. A recent study [43] looked at DNA methylation in two molecular subsets of TNBC, basal-like and claudin-low breast cancers. The analysis of microarray gene expression data showed an association between the aberrant DNA hypermethylation and gene expression for a panel of nine characteristic genes (*CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A* and *TFF3*) in basal-like and claudin-low subtypes. The aberrant promoter CpG hypermethylation predominated in TNBC compared with other breast cancer subtypes. The *BRCA1* promoter is known to be highly hypermethylated in TNBC tumors and correlated with *TP53* mutation. Methylated *BRCA1* tumors had a higher frequency of *TP53* mutation compared with no-methylated *BRCA1* TNBC [44]. Concerning basal-like subtype, *TP53* mutation was found in *BRCA1* mutated as well as sporadic basal-like breast cancer. However, the number of insertion and deletion mutations was more important in BRCA1 basal-like tumors than sporadic tumors [45].

Distinct profiles of DNA methylation were identified according to metastatic status. The ER^{-}/PR^{-} breast tumors presented a negative CpG island methylator phenotype (B-CIMP) compared with ER^{+}/PR^{+} tumors. B-CIMP- tumors were associated with high metastatic risk and death [46], and the mutation of different genes such as *BRCA1*, *PTEN* and *ERBB2* was correlated with increased risk of metastasis [47,48]. The DNA methylation pattern is a powerful predictor of breast cancer survival, and can predict outcomes of the breast cancer molecular subtypes.

At this time, high-throughput technologies are enabling us to study epigenomic status in TNBC and identify new biologic signatures for patient treatment and help TNBC stratification into different prognostic profiles.

Transcriptomic landscape of TNBC

Over the past 10 years, transcriptomic approaches have played an important role in breast cancer research. This technique has allowed the screening of thousands of genes in one operation. Several research groups have carried out gene expression profiling of breast cancer and classified clinically distinct subclasses of tumors and treatment prediction.

Many microarray studies have led to the discovery of several genes associated with breast cancer, and determined a molecular profile of human breast cancer. Fifteen years ago, several studies defined six different subtypes using gene expression level [1,49–51]. They distinguished luminal A, luminal B and luminal C subtypes with ER and luminal epithelial cell gene expression, distinct from negative ER subtypes (basal-like, $HER2^{+}$ and normal-like) that expressed phenotype corresponding to basal epithelial cells. These classifications were applied to different studies of patient outcome [52–54], mutation pattern [55,56] and tumor progression [57,58]. The study of global transcriptome by RNA-sequencing analysis detected 2617 transcripts with a different expression between TNBC, no-TNBC and $HER2^{+}$ subtypes [59]. These transcripts were known to play a role in extracellular matrix remodeling and cell motility in breast cancer. Vaca-Paniagua et al. [20] performed transcriptomic (mRNA and miRNA) profiling of 12 TNBC from Mexican women classified according to PAM50 gene signature. This minimal gene set (PAM50) classified breast cancers in "intrinsic" subtypes (Luminal A, Luminal B, HER2-enriched, Basal-like, and Normal-like) [51]. The hierarchically-clustered gene expression divided up the 12 TNBCs into two groups: 75% of tumors presented basal-like molecular profile, and 25% presented a HER2 profile. This transcriptomic analysis showed different genes overexpressed in TNBC tumors: *MKI67, TOP2A, CCNE1, CCNE2, EGFR, FGFR1, FGFR2, VEGFA, HIF1A, ARNT, FOXM1* and *BRCA1-repressor ID4*. As regards typical markers of TNBC phenotype, *ERS1* and *ERBB2* were down-regulated.

Among the breast cancer molecular subtypes, the TNBC group is especially heterogeneous. Lehmann et al. highlighted different subsets within the TNBC subtype [60]. An analysis of gene expression by cDNA microarray identified seven different TNBC subtypes with a particular gene expression profile. A validation set made up with new patients was used to validate the 7 TNBC subtypes. A specific gene expression profile was established for each TNBC subtype: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR) and unstable (UNS). Among these subtypes, unlike BL1, BL2, M and UNS groups, which expressed basal-like cytokeratins, LAR subtype presented high levels of luminal cytokeratins and other luminal markers such as FOXA1 and XBP1. Moreover, each subtype was characterized by a specific gene expression profile: BL1 and BL2 were characterized by the expression of genes involved in

Table 1
TNBC subtypes and sensitivity to therapeutic agents.

Subtypes	Cytokeratin and claudin expressions	High gene expression	Pathways	Therapies*
Basal-like 1	High level of basal-like cytokeratins	Genes involved in proliferation (*AURKA, AURKB, CENPA, CENPF, BUB1, TTK, CCNA2, PRC1, MYC, NRAS, PLK1 and BIRC5*), Gene expression associated with DNA damage response (*CHEK1, FANCA, FANCG, RAD54BP, RAD51, NBN, EXO1, MSH2, MCM10, RAD21, and MDC1*)	Cell cycle DNA replication reactome G_2 cell-cycle pathway RNA polymerase G_1 to S cell cycle	Antimytotic agents (taxanes combined with neoadjuvant anthracyclin) DNA-damaging agents Cisplatin (high sensitivity)
Basal-like 2	High level of basal-like cytokeratins	Genes in relation with growth factor receptors (*EGFR, MET, EPHA2*) Basal-myoepithelial marker genes (*TP63, and MME*)	Growth factor signaling Glycolysis Gluconeogenesis	Antimytotic agents (taxanes) DNA-damaging agents Cisplatin (high sensitivity)
Immunomodulatory	High level of basal-like cytokeratins	Genes involved in immune cell process pathways (*CTLA4, IL12, NK, TH1/2, IL7, NFKB, TNF, DC, BCR, JAK, STAT, ATR/BRCA*)	Immune cell signaling Antigen processing and presentation Immune signal transduction pathways	–
Mesenchymal-like	High level of basal-like cytokeratins	Genes involved in TGF-β signaling, epithelial-mesenchymal transition associated genes	Cell motility Cell differentiation pathway ECM receptor interaction	Drugs targeting Wnt/β-catenin pathway Abl/src inhibitor dasatinib PI3K/mTOR pathway inhibitor (NVP-BEZ235
Mesenchymal stem-like	Low level of claudins 3, 4, 7 and luminal cytokeratins High level of basal-like cytokeratins	Genes involved in TGF-β signaling, epithelial-mesenchymal transition associated genes Genes involved in angiogenesis Genes associated with stem cell	Cell motility Cellular differentiation Growth pathway	Abl/src inhibitor dasatinib PI3K/mTOR pathway inhibitor (NVP-BEZ235
Luminal androgen receptor	High level of luminal cytokeratins	AR targets and coactivators (*DHCR24, ALCAM, FASN, FKBP5, APOD, PIP, SPDEF, and CLDN8*) Luminal gene expression pattern (*FOXA1, KRT18, and XBP1*)	Steroid synthesis Porphyrin metabolism, androgen/estrogen metabolism	Therapies targeting AR (bicalutamide and 17-DMAG) PI3K/mTOR pathway inhibitor (NVP-BEZ235)

Basal-like cytokeratins (KRT5, KRT6, KRT6B, KRT14, KRT17, KRT23 and KRT81).
Luminal cytokeratins (KRT7, KRT8, KRT18 and KRT19).
* Each TNBC subtype is represented by different breast cancer cell lines that have different sensitivities according to the therapeutic drug. Also, xenograft tumors from basal-like, luminal androgen receptor and mesenchymal-like cell lines showed different sensitivities to therapeutic agents [60]. A retrospective study of 130 TNBC biopsies showed a higher response to neoadjuvant anthracycline and taxane chemotherapy combination in basal-like 1 tumors than in basal-like 2, luminal androgen receptor and mesenchymal-like stem tumors [61].

proliferation and DNA damage response; IM subtypes presented a gene signature connected to immune cell processes; M and MSL subtypes were enriched in components and pathways involved in cell motility and cell differentiation; the most differential subtype was LAR, which exhibited genes with a role in different pathways regulated by hormones and specifically androgen/estrogen metabolism. In addition, the AR expression was strongly expressed in these tumors compared with other subtypes.

These omics data allowed a differentiation of TNBC tumors and orientated the treatment of different TNBC subtypes (Table 1).

Proteomic landscape of TNBC

The proteomic term defines the large-scale characterization of the entire protein of a cell line, a tissue, or an organism [62]. This definition groups several areas of protein study including protein modification, protein function, protein–protein interaction and protein localization. New proteomic tools enable us to generate large data sets to detect potential targets in diagnosis, prognosis and therapeutics in cancer. For example, the study of treatment impact on post-translational modifications, translocations within cells and degradation or synthesis of protein can be of clinical utility [63]. Moreover, a transcriptomic approach by measurement of mRNA levels is not necessarily correlated to protein level. In carcinogenesis, proteomic studies are used to compare differences in protein patterns between tumor and normal tissues and to study the tumor microenvironment.

A global proteomic analysis showed that TNBC cell lines presented a strong expression of different pathways involved in metastasis development, cell adhesion and angiogenesis [64]. Different protein signatures were identified in TNBC tumors by omics techniques. Campone et al. [65] studied quantitative global proteome profiling by the iTRAQ-OFFGEL-LC-MS/MS approach in TNBC tumors and highlighted three proteins that could be protein targets for TNBC: tryptophanyl-tRNA synthase (TrpRS), desmoplakin (DP) and thrombospondin-1 (TPS1). TrspRS, which plays a role in different pathways such as angiogenic signaling [66], cytokine activities in inflammation, synthesis and transcription of proteins was considered a good prognostic marker. A high expression of TrspRS protein was associated with better disease-free survival (DFS). Conversely, DP and TPS1 proteins were identified as bad prognosis markers and had an expression that was correlated with a less favorable DFS. In breast cancer cells, TSP1 had a proangiogenic activity [67] and DP was involved in desmosomal junctions; a high expression of this protein was found in luminal breast cells [68]. Another study determined a signature of 11 proteins for TNBC by nanoscale liquid chromatography combined with mass spectrometry [69]. Among these 11 selected proteins, 10 were up-regulated (CMPK1, AIFM1, FTH1, EML4, GANAB, CTNNA1, AP1G1, STX12, AP1M1, and CAPZB), involved in immune response and cell death, and were associated with a good prognosis. The last protein (MTHFD1) was down-regulated, played a role in nucleotides and noncoding RNA metabolic pathways, and was associated with poorer prognosis. Using the signature of the 11 proteins to guide the treatment of TNBC patients, the authors showed that it was useless to give adjuvant chemotherapy for more than 60% of the patients studied compared with St Gallen [70] and NIH criteria [71].

In MDA-MB231 TNBC cells, a HMGA1 (high motility group A1)-linked protein molecular signature was identified by proteomics analyses, and grouped 21 factors that have prognostic value in TNBC [72]. The expression of these factors was associated with a poor prognosis. Inside this signature, three proteins (KIFC, TRIP13 and LRRC59) were of particular interest because their role in cancer was not well-researched. However, in breast cancer, these three proteins were involved in tumor cell motility, and their expression was linked with HMGA1 expression level.

Recently, a complete analysis of proteome in breast cancer subtypes highlighted a specific proteomic profile between these subtypes [73]. TNBC was distinguished by positive markers such as *MCM5, STMN1, RCL1* and *C9ORF114*, characteristic of proliferation cells. This proliferative capacity of TNBC was increased by the depletion of tumor suppressor PTEN, which promoted the oncogenic PI3K pathway. This high cell activity raised consumption of glucose and glutamine. The presence of glycolytic enzyme enolase (ENO1) and GLS therefore showed that TNBC had a specific metabolism comprising metabolic enzymes (glutaminase and hexokinase 2) compared with other breast cancer subtypes.

Two different analyses can be used to explore proteomics: shotgun bottom-up (BU) and top-down (TD). These techniques gave an overview of protein forms as signal peptide cleavage, proteolysis products and sequence variants. A BU study allowed sequencing coupled with mass spectrometry and detection of peptides after protein digestion by protease [74]. This method was sensitive and identified more 5000 protein groups per sample. Reverse-phase liquid chromatography and GELFrEE using tandem liquid chromatography in line with a mass spectrometer were used for TD proteomics [75]. Recently, a study compared these two methods from patient-derived mouse xenograft models of basal (WHIM2) and luminal B (WHIM16) human breast cancer [76]. TD and BU detected a high level of unmodified protein in WHIM2 compared with WHIM16, which presented a high level of phosphorylate forms. However, comparison of BU and TD gave conflicting results, e.g. for protein phosphatase 1 regulatory subunit 1B: BU analysis detected this protein in WHIM2 compared with WHIM16, whereas TD highlighted no significant difference in its intensity between the two groups. These findings illustrate the need for a more rigorous and meaningful analysis of proteomics by combining BU and TD.

The identification of new protein marker is an important issue in TNBC treatment. In fact, a protein quantitative analysis of drug targets and genetic alterations could be necessary to select appropriate adjuvant therapy in TNBC. Proteomics is an indispensable tool to determine drug sensitivity and elucidate the mechanisms of drug resistance in TNBC [64]. In addition, the combination of genomics, transcriptomics and proteomics data with drug sensitivity data could assist the design of predictive models of drug response, and thus be of real clinical utility. Fig. 1 illustrates different analysis steps to identify novel TNBC regulators and subtype-specific biological

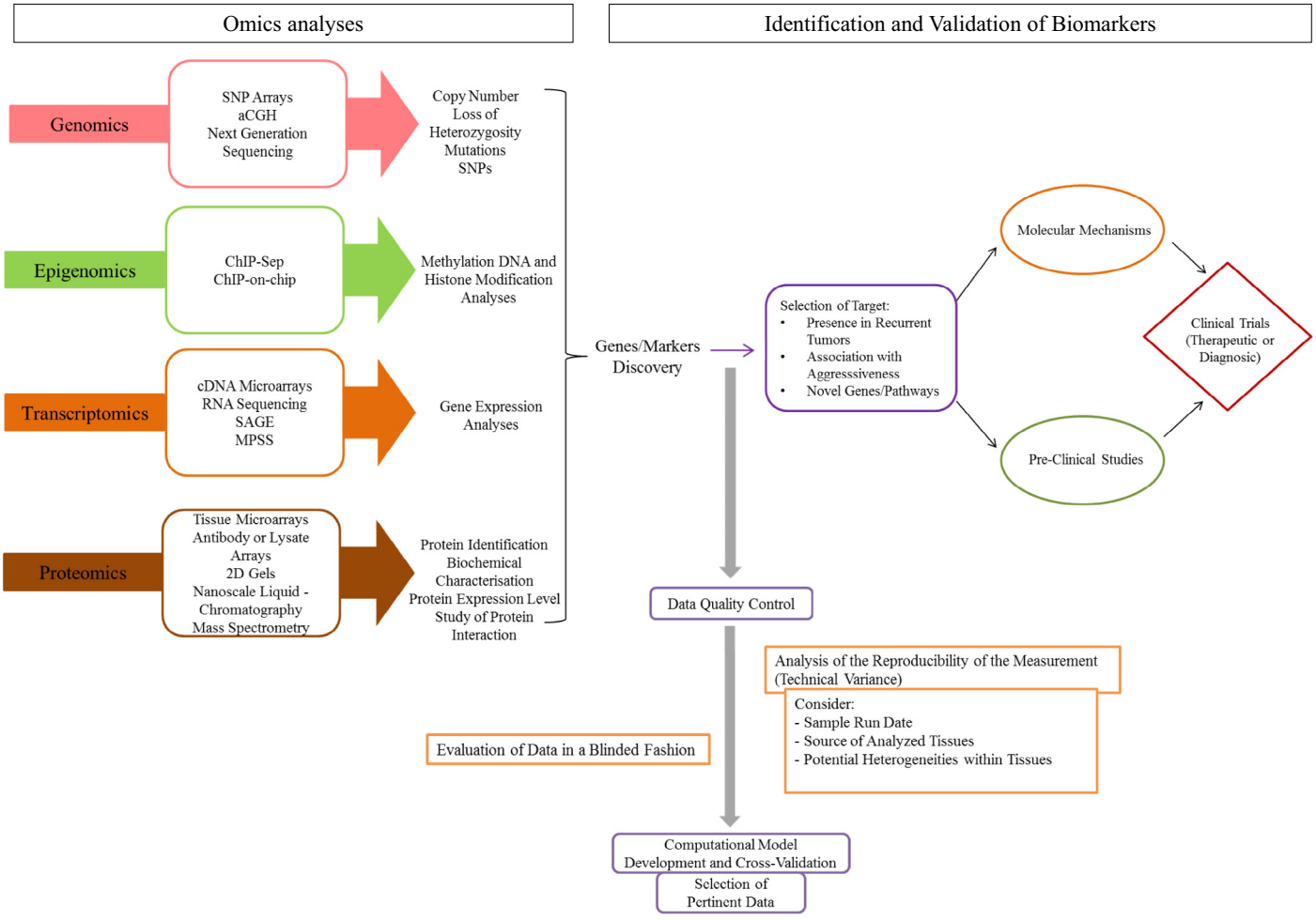

Fig. 1. Strategy of TNBC omics study. Different steps are necessary to identify new biomarkers in TNBC tumors. A large number of omics techniques exist to study global genome, epigenome, transcriptome and proteome of TNBC. All these omics data must be validated by a rigorous process before clinical use. SAGE: Serial analysis of gene expression; MPSS: Massive parallel signature sequencing; aCGH: Array comparative genomic hybridization; ChIP-seq: Chromatin immunoprecipitation-sequencing.

processes and develop predictive TNBC signatures based on omics data, and so lead up to a clinical trial.

Clinical application of Omics technologies

Management and analysis of omics-data

Omics technologies use high-throughput screening to analyze a large numbers of gene sequences, gene expressions and proteins in a single procedure or a succession of procedures. These operations are carried out with high sensitivity and specificity. Massive complex datasets can be generated, up to 10^8 per day. However, the sheer mass and heterogeneity of these data hinder analysis and interpretation of results. At present, omics technologies are routinely used in laboratories and are becoming a basic science application. Accordingly, specific analytical resources and tools such as computational analyses have been developed to offset the difficulty met when interpreting the abundant data. The field of bioinformatics is expanding and is becoming an essential part of omics research.

Data deluge soon led to the development of a new methodology: data integration, defined as "the use of multiple sources of information (or data) to provide a better understanding of a system/situation/association/etc. Hence data integration, as defined here, is an action performed on a daily basis by most individuals, and critical element in research" [77]. Several systems of data integration have been developed to store and analyze omics results. Two important databases have compiled omics data from cancer research: The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) [78–80]. More specifically, Raju et al. [81] have created an omics platform providing genomic, transcriptomic and proteomic data for triple-negative breast cancers. This Triple Negative Breast Cancer Database (TNBCDb) integrates varied data on TNBC biology, and can be an analytical reference for the study of TNBC. A specific TNBC framework, APOLLOH, was set up from omics data and used to identify LOH regions by comparing tumors with normal tissues [82]. This statistical approach was based on heterozygous SNPs in the normal genome, copy number profile of TNBC and allelic count data of identified heterozygous SNPs in tumors. In TNBC, LOH was an important factor involved in allelic imbalance, acting on the transcriptome, and in particular on monoallelic expression. APOLLOH was a useful statistical tool to analyze LOH and more fully understand the TNBC genome.

Omics-based tests

All these data have been used to develop omics-based tests, defined by the committee of Institute Of Medicine (IOM) as "an assay composed or derived from many molecular measurements and interpreted by a fully specified computational model to produce a clinically actionable result", which may bring new information to assist diagnosis and guide treatments in breast cancer management [83]. An omics-based test must be associated with a phenotype of interest, such as a biologic subgroup, clinical outcome or preclinical responsiveness to a new therapy [84]. Several approaches have been used to design these tests, and different genomic assays are currently being used for breast cancer: Oncotype DX (Genomic health) is used to predict the risk of recurrence in patients with ER^+, node-negative disease treated with tamoxifen. This test analyzes the activity of 21 genes by RT-QPCR and then calculates a recurrence score number between 0 and 100; the higher the score, the greater the risk of recurrence. Another genomic assay, Mammaprint (Agendia) used gene expression data obtained by DNA microarrays from a cohort of 78 patients with known clinical outcomes, and identified 70 genes associated with prognosis with no prior biologic assumption. This assay addresses hormone-receptor-positive or hormone-receptor-negative breast cancer, and yields a prognostic score that classifies patients into "good or "poor" risk groups [85].

Considering molecular subtypes of breast cancer, referred to as luminal A, luminal B, HER2-enriched and basal-like, Parker et al. set up a genomic array, a Prediction Analysis of Microarrays (PAM50) [51]: the 50 studied genes were successively validated by RT-QPCR. PAM50 is independent of survival and clinicopathologic variables in breast cancer [44].

A novel therapeutic option in patients with TNBC was presented at the 2015 American Society of Clinical Oncology (ASCO) annual meeting: enzalutamide [86]. This androgen receptor inhibitor is used to treat prostate cancer. However, the Traina TA team hypothesized that enzalutamide could have an activity in advanced androgen receptor positive TNBC. This trial (phase II) was conducted on 118 patients, and showed that 35% (95% CI: 24.46) and 29% (95%, CI: 20.41) experienced clinical benefit at 16 and 24 weeks with a complete or partial response. Data collected in this trial enabled the development of a novel genomic assay, PREDICT AR, which has the ability to identify patients who might benefit from enzalutamide [86].

Currently, the leitmotiv in cancer care is to assign personalized treatment according to the biological characteristics of the tumor. In response to this challenge, treatments are increasingly designed to target specific tumor subtypes, and the diagnostic tests identify and stratify sets of patients who present similarities in treatment response. TNBC therapeutic care is complex due to biological heterogeneity and aggressiveness of these tumors; these new omics tools thus open new perspectives. Genomic tests enable us to improve patient care and avoid, in certain cases, toxicities and high cost of treatment, and to increase quality of life. Even so, progress in applying these omics-based tests to clinical trials is slow and inconclusive. The absence of instructions to determine and validate biomarkers and the lack of standardization for appropriate trial design and analytical methods further add to the difficulties in developing these omics-based tests.

Evaluation of Omics-based tests and their clinical utility

The different problems in overseeing systems using omics methods, and a lack of clearly defined good practices for omics-based test validations have been pointed out by the scientific community, and particularly by statisticians and bioinformatics scientists. In this light, the IOM has set up a committee to clarify and draw up recommendations for the development and evaluation of omics-based tests and the transfer of these data from research laboratories to clinical trials [83] (Fig. 2). The committee defines two major work steps. The first stage includes discovery and validation phases of omics-based tests. In the discovery phase, the omics assays (molecular measurements) identify different biomarkers in a disease or a particular condition. The candidate test must be developed from a training set and specific computational procedures, and the validation of this test is performed with an independent sample set. The test validation phase includes analytical and clinical/biological validation on a blinded sample set. Two institutions can be involved in this step: approval by the Institutional Review Board (IRB) is necessary, and the opinion of the Food and Drug Administration (FDA) can be required. The second stage consists of evaluation for clinical utility, and a use stage test. To determine clinical utility, a prospective randomized clinical trial is best-suited, but different pathways for this evaluation may be feasible in some circumstances. At this time, consultation of the FDA is strictly required before clinical use. These recommendations give decisive directions to develop omics-based tests with sound, reproducible scientific practices, thus enabling a new approach to patient care, particularly in TNBC subtypes.

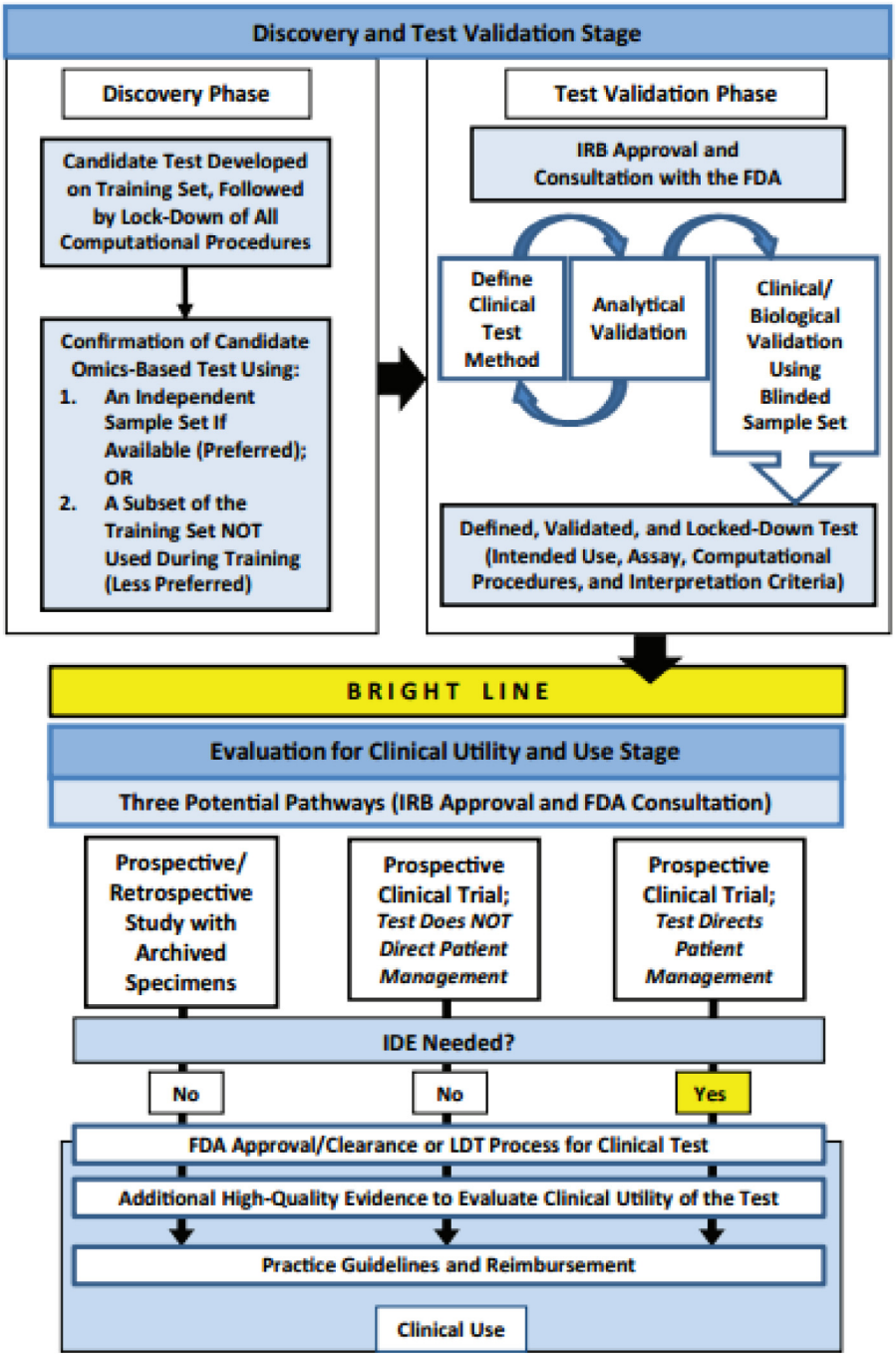

Fig. 2. Omics-based test development process. This figure describes the different phases recommended by the IOM committee for the transition from candidate omics-based test to clinical trial [48].

Conclusions

All these data demonstrate substantial biological heterogeneity between TNBC and other breast cancers and within TNBC subtypes. High-throughput technologies stratify TNBC groups and help predict metastatic risk and patient outcome, and guide patient treatment. However, analysis and integration of these data remain an important challenge owing to the technical complexity and broad diversity of the data produced by omics technologies. The utilization of these tools calls for a multidisciplinary approach to carry out data modeling, data visualization and data exploitation, involving various research areas, such as medicine, biology, biochemistry, bioinformatics and statistics. However, the utilization of these technologies must still be validated, and the application of omics data to clinical trials standardized.

Currently, TNBC treatment is a major issue owing to poor outcome, high proliferation, marked somatic mutations, intrinsic molecular variability and few therapeutic targets. It is urgent to achieve a better understanding of carcinogenesis of TNBC to find the best treatment options to match the biologic features of the different TNBC subtypes. High-throughput « Omics » technologies can address this issue. Meanwhile, translating omics data into clinical practice remains a formidable challenge for improving TNBC treatment.

Conflict of interest

The authors have no conflicts of interest.

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Conclusion de la publication 1

Dans cette revue, on a mis en évidence les avancées faites par les chercheurs sur le TNBC au niveau génomique et épigénomique, transcriptomique, protéomique, ainsi que les applications cliniques en utilisant des techniques Omiques. On a ainsi présenté les différents gènes et marqueurs qui ont été identifiés, et qui pourraient être des outils de pronostic et diagnostic efficaces.

Ces données ont montré une grande hétérogénéité entre les tumeurs TNBC et les tumeurs non-TNBC. En effet, les technologies à haut débit stratifient les groupes TNBC, et aident à identifier des outils de pronostique et à prédire le risque métastatique des tumeurs triples négatives. Cependant, l'analyse et l'intégration des données produites par ces techniques restent un défi important en raison de leur vaste diversité. L'utilisation de ces outils fait alors appel à une approche multidisciplinaire pour analyser, interpréter et modéliser ces données afin de trouver des traitements efficaces contre les TNBCs.

B. NOTIONS D'ÉPIGÉNÉTIQUE

Alors que la génétique correspond à l'étude des gènes, l'épigénétique s'intéresse à une "couche" d'informations complémentaires qui définit comment ces gènes vont être utilisés par une cellule. En d'autres termes, l'épigénétique correspond à l'étude des mécanismes qui modifient l'expression du gène sans altérer la séquence d'ADN primaire, ces mécanismes épigénétiques sont héréditaires et réversibles (Holliday, 2006) (Berger et al., 2009). Ainsi, ces mécanismes changent avec l'âge et sont sensibles aux influences comportementales et environnementales (Fraga and Esteller, 2007).

Au cours des dernières décennies, l'épigénétique s'est développé dans la recherche sur le cancer. Les altérations épigénétiques sont largement décrites comme des acteurs essentiels dans la progression du cancer et ont été associées à tous les stades de la formation de la tumeur. Par conséquent, elles ont été identifiées comme biomarqueurs putatifs du cancer pour la détection précoce, la surveillance de la maladie, le pronostique et l'évaluation des risques (Jones and Baylin, 2002) (Baylin and Ohm, 2006) (Esteller, 2008).

1. Altérations Épigénétiques dans le Cancer du Sein

Il existe 3 mécanismes principaux de la régulation épigénétique : la méthylation des îlots CpG de l'ADN, les modifications post-traductionnelles des histones ainsi que le positionnement des nucléosomes le long de l'ADN, et finalement la régulation de la transcription des gènes par les microARNs non codants **(Figure 4**). La complémentarité et les interactions substantielles entre ces modifications épigénétiques, désignées sous le nom d'épigénome, entrainent et amplifient la diversité cellulaire en régulant les informations génétiques accessibles par la machinerie transcriptionelle. Si les marques épigénétiques héritables ne sont pas correctement entretenues, elles peuvent générer une activation ou une inhibition inappropriée de diverses voies de signalisation et entraîner des pathologies telles que le cancer, y compris le cancer du sein (Esteller, 2008) (Dawson and Kouzarides, 2012).

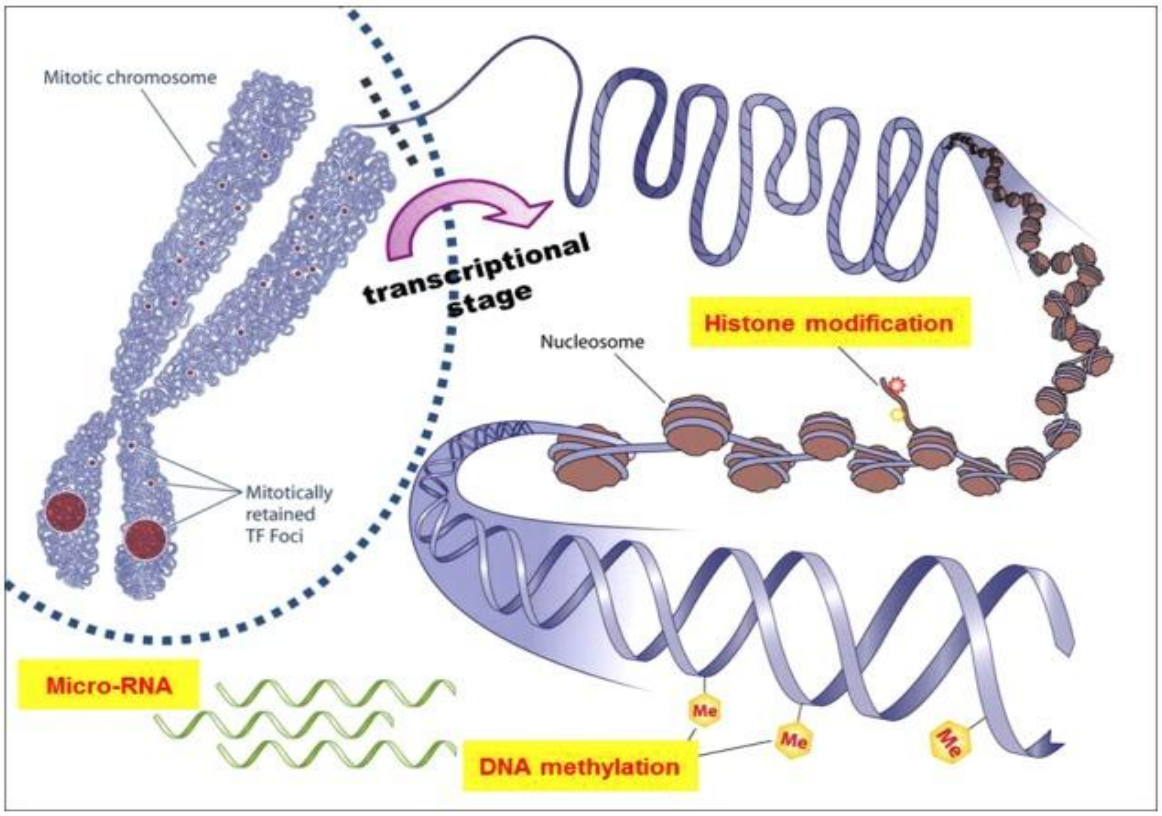

Figure 4. Schéma représentant les 3 mécanismes majeurs de la régulation épigénétique du génome : la méthylation de l'ADN, les modifications des histones, et les micro-ARNs (Kim, 2014).

En fait, les aberrations épigénétiques dans les cellules cancéreuses mammaires, leur confèrent une croissance conduisant à une déficience apoptotique, une prolifération cellulaire non inhibée et éventuellement, la formation de tumeurs. Les altérations épigénétiques contribuent au développement du cancer du sein en régulant l'expression des oncogènes et des gènes suppresseurs de tumeur ainsi que l'activité des oncoprotéines impliqués dans la tumorigènese mammaire (Veeck and Esteller, 2010) **(Figure 5)**. Ainsi, les modifications post-traductionnelles des histones H3 et H4 constituent une grande partie de ces événements épigénétiques qui sont ultérieurement responsables de l'établissement d'un phénotype cellulaire transformé (Fucito et al., 2008) (Davalos et al., 2017). En outre, les modifications aberrantes des histones, combinées avec une hyperméthylation de l'ADN sont fréquemment associées à une répression des GSTs et une instabilité génomique dans le cancer du sein (Veeck and Esteller, 2010) (Huang et al., 2011).

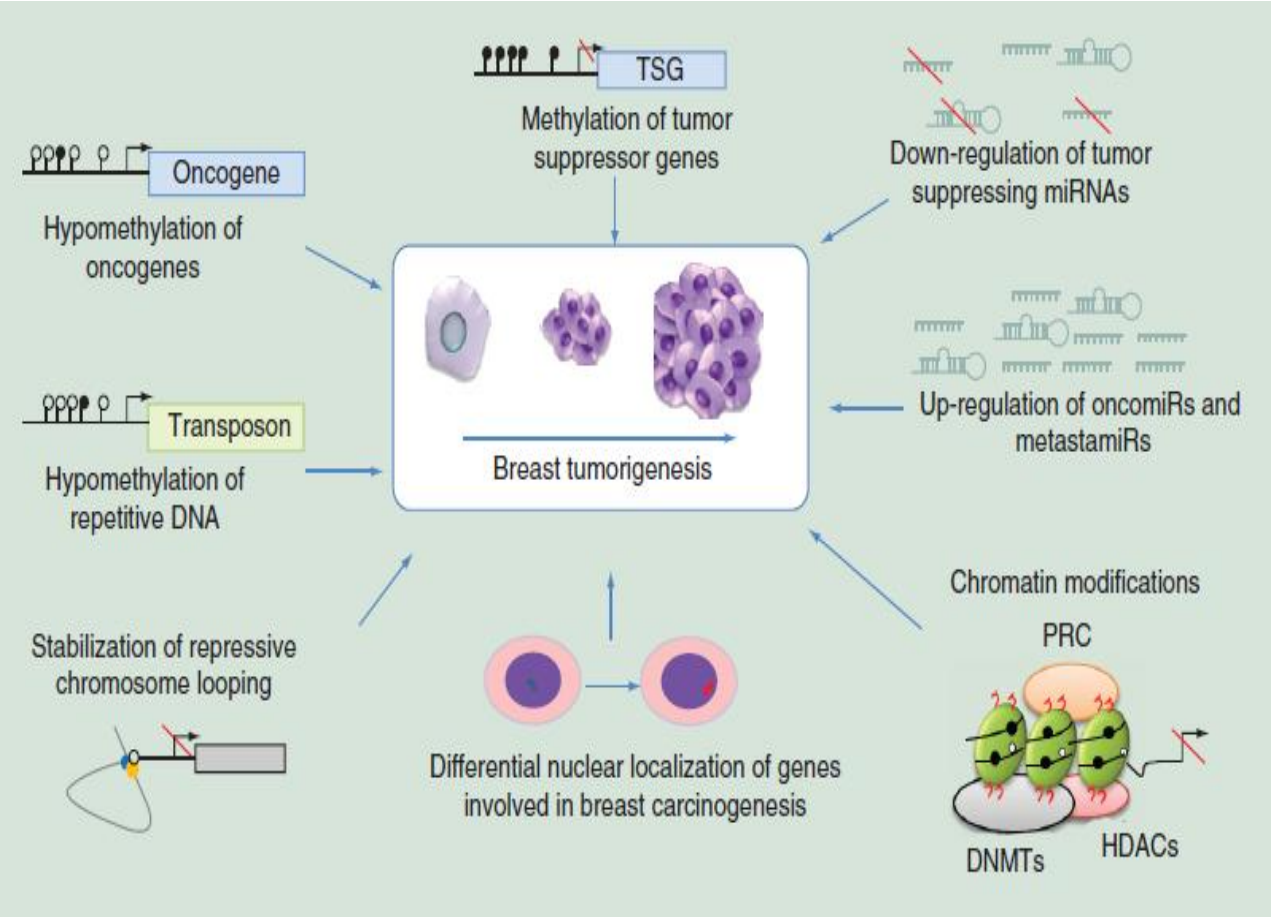

Figure 5. Schéma simplifié représentant les différentes altérations épigénétiques qui aboutissent à la transformation maligne des cellules mammaires. TSG (gène suppresseur de tumeur), PRC (Polycomb repressor complex); DNMTs (DNA methyltransferases), (Toland, 2012).

1.1. Les Modifications Post-Traductionnelles (PTMs) des Histones

À l'intérieur du noyau de la cellule, l'ADN est empaqueté avec des protéines pour constituer la chromatine. Les principales protéines de la chromatine sont les histones qui déterminent la structure de la chromatine et jouent un rôle central dans la régulation de l'expression des gènes. L'unité de base de la chromatine est le nucléosome, il est formé d'un octamère d'histone contenant 2 molécules de chaque histone H2A, H2B, H3 et H4 assemblées en deux hétérodimères [H2A-H2B] et 2 hétérotétramères [H3-H4]. Il est ainsi entouré d'un segment d'ADN d'environ 146pb (Luger et al., 1997) (**Figure 6**). Cette association entre nucléosome et ADN joue un rôle primordial dans la régulation de l'expression des gènes (Mellor, 2006) (Cedar and Bergman, 2009) (Bartke et al., 2010).

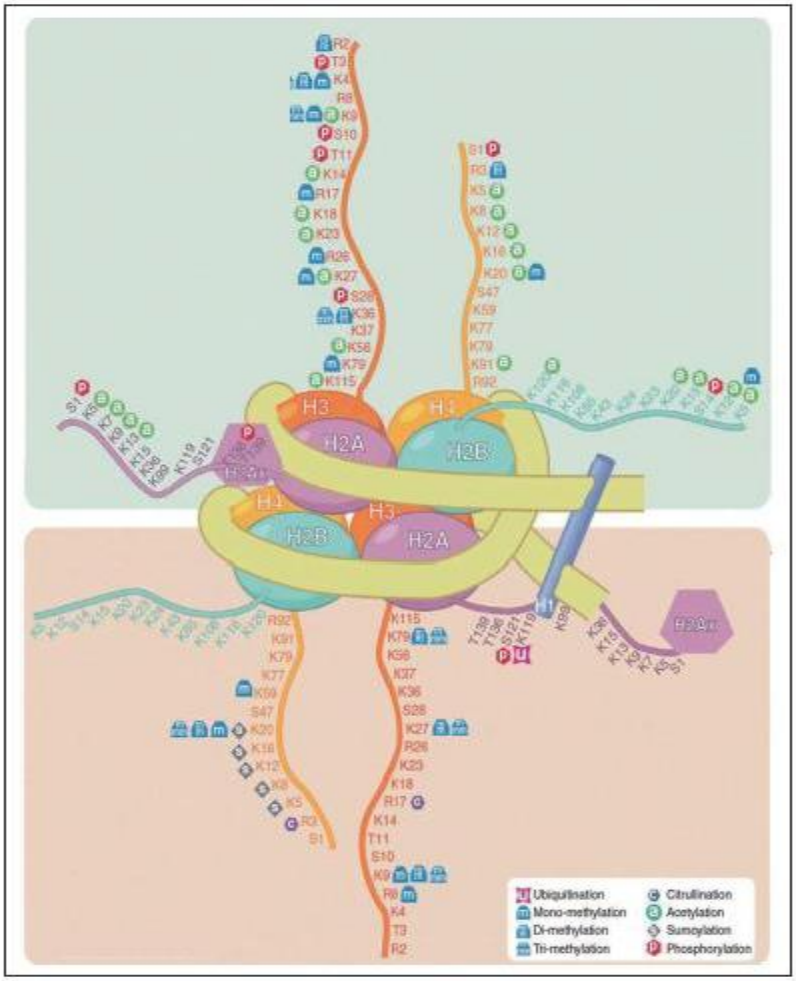

Figure 6. Schéma représentant le structure d'un nucléosome, en montrant les résidus modifiables des queues N-terminales des histones qui forment le nucléosome (Kim, 2014).

Les PTMs des histones s'effectuent sur leurs résidus lysines (K) au niveau de leurs queues N-terminales, désignées par le nom de marqueurs d'histones ou marques épigénétiques (**Figure 6**). Il existe plusieurs types de modifications d'histones dont les plus principales restent toujours l'acétylation et la méthylation. Cependant, ces PTMs semblent agir de manière combinatoire et cohérente dans la régulation des processus cellulaires clés, tels que la transcription, la réplication, et la réparation d'ADN. La complémentarité entre ces modifications est proposée pour stocker la mémoire épigénétique dans une cellule sous la forme d'un «code histone» (**Figure 7**), qui détermine la structure et l'activité des différentes régions de la chromatine (Santos-Rosa and Caldas, 2005) (Kouzarides, 2007a) (Izzo and Schneider, 2010) (Bannister and Kouzarides, 2011).

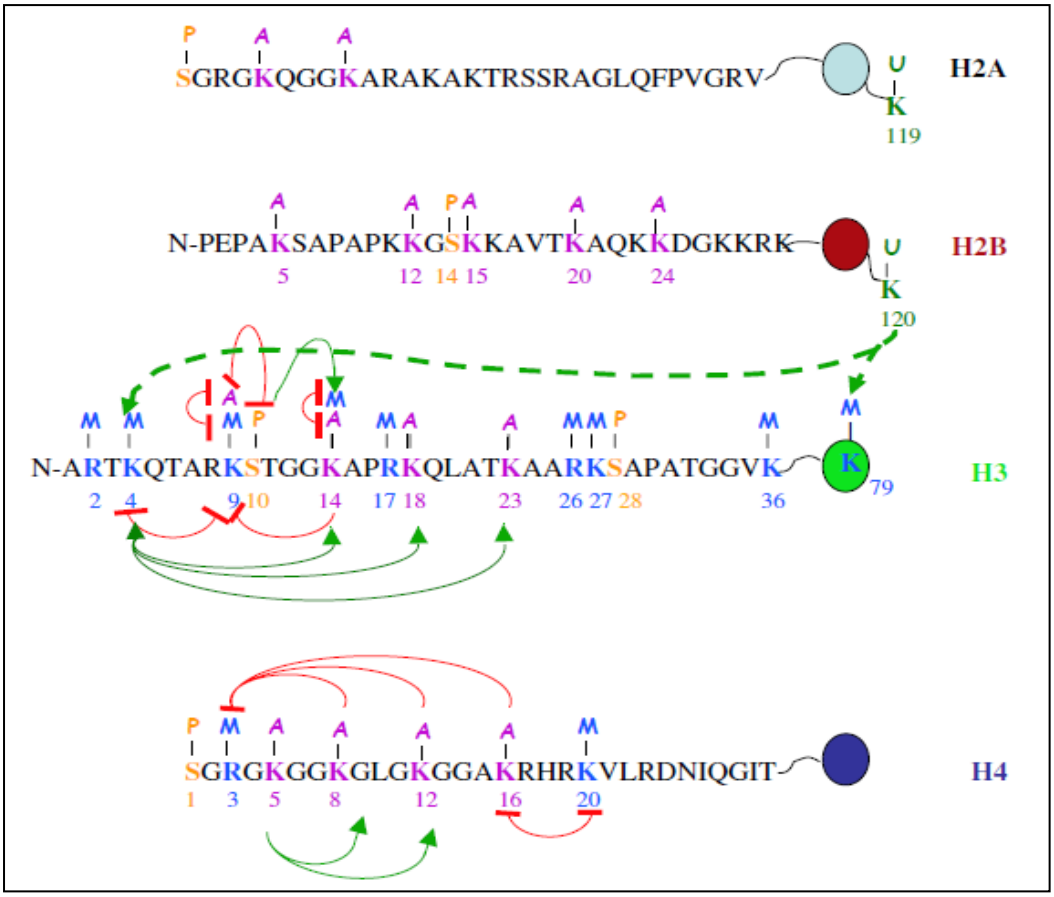

Figure 7. Schéma simplifié représentant les interactions entre les différentes modifications post-traductionnelles des histones. Les flèches vertes correspondent à la coopération entre les différentes PTMs, tandis que les flèches rouges correspondent à l'incompatibilité entre certaines (Santos-Rosa and Caldas, 2005).

Ces modifications régissent la structure de la chromatine et la transcription des gènes, et peuvent conduire à l'activation ou la répression de l'expression des gènes en fonction de la position du résidu modifié et du type de la modification présente. Par exemple, l'acétylation des queues d'histones par les histones acétyltransférases (HATs) entraine l'activation de l'expression génique, tandis que leur désacétylation par les histones désacétylases (HDACs) réprime l'expression des gènes.

À ce titre, les acétylations de l'histone H3 sur la lysine 4 (H3K4), H3K8, H3K9, H3K12, H3k56, et H4 sur la lysine 16 (H4K16), désignées comme des marques activatrices, aboutit à la formation d'euchromatine, c'est-à-dire une structure ouverte de la chromatine permettant la transcription et l'expression génique (Schübeler et al., 2004) (Esteller, 2007), tandis que la désacétylation et la mono-, di- et tri-méthylation des H3K9, H3K27, et H4K20, nommées marques répressives, aboutit à la formation de l'hétérochromatine, une structure compressée et fermée de la chromatine qui réprime et empêche l'expression des gènes (Schotta et al., 2004) (Martin and Zhang, 2005) (Wang et al., 2008c). Les PTMs des histones sont ainsi catalysées par des différentes types d'enzymes épigénétiques: les histones acétyltransférases (HATs), histones désacétylases (HDACs), histones méthyltransférases (HMTs), ou histones déméthylases (HDMs) (**Figure 8**), (Kouzarides, 2007b) (Wang et al., 2009) (Zentner and Henikoff, 2013).

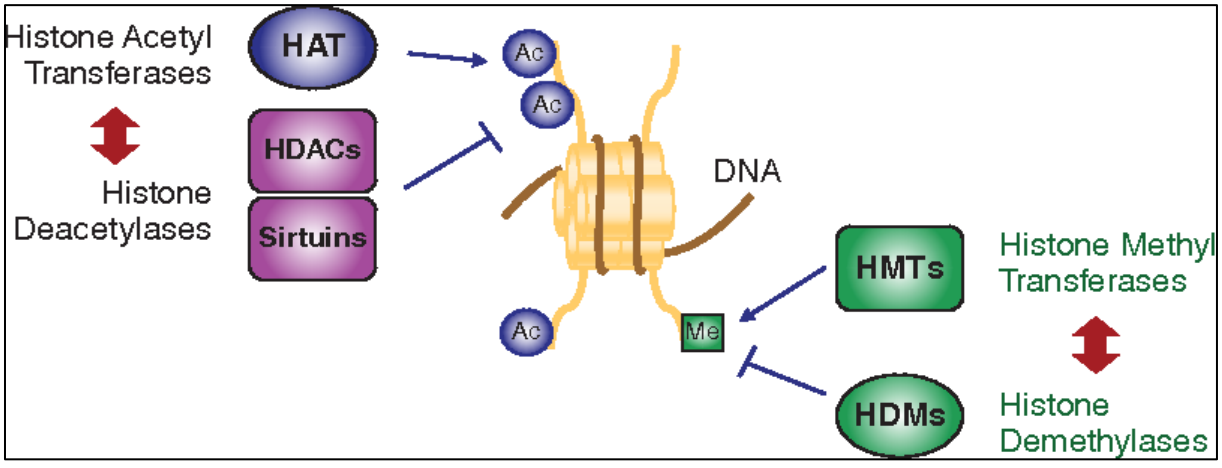

Figure 8. Schéma représentant la modulation de l'acétylation ou de la méthylation des histones par les enzymes épigénétiques HATs, HDACs, HMTs, et HDMs (Gerhauser, 2014).

1.2. Les PTMs des Histones H3 et H4 dans le cancer du sein

Parmi les modifications des 4 types d'histones formant le nucléosome, celles des histones H3 et H4 sont les plus importantes et les plus étudiées dans la recherche sur le cancer. En effet, la dérégulation de l'acétylation et de la méthylation des queues des histones H3 et H4 jouent un rôle dans la prédiction, ainsi que dans la progression du cancer. Elles sont considérées comme des biomarqueurs diagnostiques et prédictifs du développement tumoral, de la réponse thérapeutique, et de l'évolution du patient dans divers carcinomes humains, y compris le cancer du sein (Kurdistani, 2007) (Seligson et al., 2009) (Chervona and Costa, 2012).

À ce titre, les chercheurs ont étudié ces derniers années la valeur pronostique et prédictive des modifications d'histones dans plusieurs types de cancer, tels que le cancer du poumon (Song et al., 2012), cancer de l'œsophage (I et al., 2010), cancer de la prostate (Seligson et al., 2005), cancer du côlon (Benard et al., 2014), cancer colorectal (Tamagawa et al., 2012), cancer de l'estomac (Park et al., 2008) (Xu et al., 2016), cancer de la bouche (Chen et al., 2013b), et le cancer du pancréas (Manuyakorn et al., 2010). Les chercheurs ont suggéré alors une association fondamentale entre les modifications globales des histones et l'agressivité de la tumeur.

Dans le cancer du sein, les études portant sur les variations globales des marqueurs d'histones H3 et H4 dans les tumeurs mammaires à différents grades et types morphologiques, ont montré leur signification biologique et clinique dans le cancer. Il a été montré qu'une corrélation très significative existe entre les profils globaux de ces PTMs et les phénotypes moléculaires des tumeurs, ainsi que les facteurs pronostiques du cancer du sein. Les chercheurs ont suggéré que les changements dans les profils d'acétylation et de méthylation des histones pourraient représenter un signe précoce de cancer du sein (Elsheikh et al., 2009). En outre, des profils distincts de ces marqueurs sont observés en fonction de sous-types moléculaire du cancer du sein. L'altération de ces profils est ainsi corrélée avec des activités transcriptionnelles variantes au niveau des promoteurs des oncogènes spécifiques à chaque sous-type, et par conséquent, l'activation des voies oncogéniques différentes (Li et al., 2014). De plus, l'étude de la dérégulation de ces PTMs dans le cancer du sein fournit une

meilleure compréhension de son hétérogénéité moléculaire, et aide considérablement à la stratification des différents sous-types intrinsèques du cancer (Judes et al., 2016).

Ainsi, il devient de plus en plus essentiel d'étudier les voies épigénétiques régulatrices de l'épigénome des histones dans les différents sous-types du cancer du sein afin de décortiquer les mécanismes moléculaires sous-jacents qui régissent l'hétérogénéité du cancer, et par conséquent, améliorer la prise en charge thérapeutique de cette maladie.

1.3. L'Implication des Marques activatrices H3K4ac, H3K9ac et H4K16ac dans le cancer du sein

Classiquement, l'acétylation des marques épigénétiques H3K9, et H4K16 est associée à l'activation de la transcription génique, ces marques sont particulièrement enrichies sur les promoteurs des gènes actifs (Esteller, 2007) (Wang et al., 2008c). La marque H4K16ac est l'une des premières marques associées au processus de tumorigenèse, sa perte globale est observée dans la plupart des carcinomes humains à des stades précoces de formation de tumeurs, et s'est révélée être un biomarqueur pronostique du cancer chez l'Homme (Fraga et al., 2005). Il a également été démontré que H3K9ac est sous-exprimée dans le cancer du sein, ainsi que dans d'autres cancers, et que sa diminution est liée à la progression tumorale et un mauvais pronostic (Elsheikh et al., 2009). En effet, H3K9ac et H4K16ac sont fortement conservées au cours de l'évolution, et ont des rôles bien définies dans la régulation de la structure de la chromatine. Leur désacétylation par des histones desacétylases (HDACs) entraîne la formation d'hétérochromatine facultative et, par la suite, une répression de la transcription (Vaquero et al., 2004) (Vaquero et al., 2007).

En revanche, peu de rapports ont étudié le rôle de H3K4ac dans le cancer. Cette marque est aussi très peu caractérisée dans le cancer sporadique du sein, mais semble être modifiée. Le rôle de cette marque a été mis en évidence lors d'études effectuées sur la levure *Saccharomyces cerevisiae* (Guillemette et al., 2011). Dans cette étude, il a été montré que le dépôt de la marque H3K4ac sur les promoteurs des gènes favorise leur transcription, donc cette marque joue le rôle d'activateur de la transcription. De plus, la fonction de H3k4ac est souvent liée et confondue avec celle de H3K4me3, car à la fois l'acétylation et la méthylation du résidu lysine (K4) sont associées à une transcription active et à l'expression génique

(Wang et al., 2001) (Guillemette et al., 2011) (Kimura, 2013). Par contre, chez *Schizosaccharomyces pombe*, l'acétylation de H3K4 joue un rôle dans l'assemblement de l'hétérochromatine par le recrutement des protéines qui sont associées à la méthylation de la marque H3K9 (Xhemalce and Kouzarides, 2010).

Les histones acétyltransférases (HATs) responsables de l'acetylation des marques H3K9 et H4K16 sont : GCN5/PCAF pour H3K9 (Jin et al., 2011), et hMOF/TIP60 pour H4K16 (Taipale et al., 2005) (Renaud et al., 2016), tandis que SIRT1 est l'HDAC majeure de ces 2 marques (Imai et al., 2000) (Vaquero et al., 2004). Cependant, les modulateurs de l'acétylation de H3K4 n'ont pas encore été identifiés chez l'Homme. Par ailleurs, l'HAT MST1 et l'HDAC SIR2 sont responsables de l'acétylation et de la désacétylation de H3K4 chez la levure. MST1 et SIR2 sont les orthologues hautement conservés de TIP60 et SIRT1 humains, respectivement, (Imai et al., 2000) (Xhemalce and Kouzarides, 2010). Alors, il est assez logique d'émettre l'hypothèse que chez l'Homme, le dépôt de la marque H3K4ac est imputable à TIP60, et son enlèvement est assuré par SIRT1. Néanmoins, cette possible interaction n'a pas encore pu être mise en évidence jusqu'à présent.

Dans la partie suivante, on va essayer de caractériser les rôles et les fonctions diverses de l'HAT TIP60 et l'HDAC SIRT1, et exposer leurs implications controversées dans le cancer du sein.

2. Acétylation et désacétylation dans la carcinogenèse humaine

L'acétylation et la désacétylation sont parmi les principales modifications épigénétiques post-traductionnelles des protéines dans la cellule, elles sont indispensables pour la majorité de processus cellulaires clés (Santos-Barriopedro et al., 2017). Le groupe acétyle, donné par le métabolite acétylcoenzyme A (Ac-CoA), peut être attaché conjointement ou post-traductionnellement au groupe s-amino des résidus lysine (K). L'incorporation du groupe acétyle neutralise la charge positive de ce résidu, modifie la structure de l'acide aminé et bloque d'autres modifications susceptibles de se produire au même résidu. Ces réactions sont catalysées par deux groupes d'enzymes : les HATs, nommées aussi lysine acétyltransférases (KATs) qui déposent le groupe acétyle, et les HDACs, ou lysines desacétylases (KDACs) qui l'enlèvent (Yang and Seto, 2007) (Sadoul et al., 2008) (Wang et al., 2009).

L'équilibre strict entre les événements d'acétylation et de désacétylation des protéines joue un rôle central dans la régulation de l'expression des gènes et des voies de la signalisation cellulaire, affectant par suite une myriade de mécanismes cellulaires. Des dysfonctionnements dans l'équilibre entre les HATs et les HDACs sont ainsi étroitement associés à diverses maladies, surtout le cancer (**Figure 9)** (Parbin et al., 2014) (Liu et al., 2017). Ainsi, la perte d'agents d'acétylation associée à un gain d'activité des HDACs, est l'une des nombreuses anomalies épigénétiques du cancer (Ropero and Esteller, 2007) (Suzuki et al., 2009) (Barneda-Zahonero and Parra, 2012)

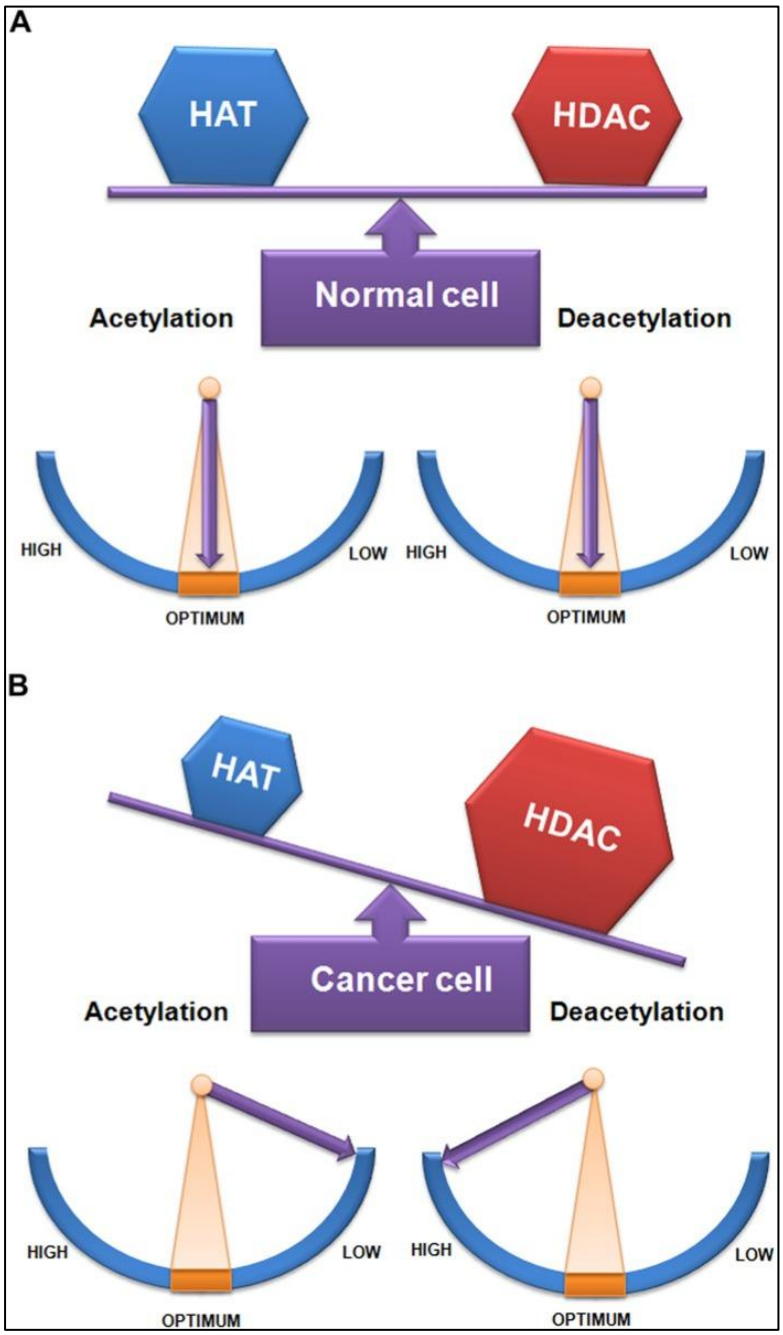

Figure 9. Schéma représentant l'homéostasie d'acétylation entre les HATs et les HDACs. A. Dans des conditions normales, l'activité des HATs et des HDACs est finement équilibrée où elles se neutralisent pour assurer l'homéostasie physiologique. **B.** Une perte d'activité des HATs couplée à un gain d'activité des HDACs perturbe l'homéostasie d'acétylation dans les cellules cancéreuses (Parbin et al., 2014).

2.1. Les Histones Acétyltransférases (HATs) et Cancer

Les HATs peuvent acétyler à la fois des substrats histones et non-histones. La réaction est enzymatiquement réversible via des mécanismes étroitement régulés, ce qui en fait un outil indispensable pour que la cellule active et désactive certaines voies (Turner, 2000) (Eberharter and Becker, 2002) (Choudhary et al., 2009). Ces dernières années, notre compréhension de l'acétylation des protéines a considérablement augmenté grâce à des analyses protéomiques globales et à des études fonctionnelles approfondies (Kim et al., 2006) (Mertins et al., 2013) (Olsen and Mann, 2013).

Parce que l'acétylation cible presque la majorité des processus biologiques, cette modification est associée au cancer. Ainsi, certaines des KATs impliquées dans le contrôle de cette modification et de leurs cibles sont dérégulées dans les cancers humains, et ont été liées au développement tumoral (Arif et al., 2010) (Farria et al., 2015) (Di Martile et al., 2016). De ce fait, de nombreuses molécules ciblant certaines de ces protéines ont été utilisées comme traitements anticancéreux (Dekker and Haisma, 2009) (Hewings et al., 2012) (Kaypee et al., 2016).

En se basant sur des similarités de séquence, les HATs sont composées de 3 familles majeures (**Figure 10)**. La première famille est Gcn5-related N-acetyltransferase (GNAT), y compris KAT2A et KAT2B, ces deux acétyltransférases sont hautement homologues. KAT2A, également connu sous le nom de GCN5, fait partie de deux grands complexes impliqués dans la modification de la chromatine, les complexes SptAda-Gcn5-acétyltransférase (SAGA) et Ada2-containing (ATAC) (Nagy et al., 2010). KAT2B, nommée p300/CBP-associated factor (PCAF), est aussi trouvé dans les complexes SAGA-like ou ATAC. Les deux enzymes partagent certaines fonctions redondantes, telles que l'acétylation de l'histone H3 au niveau du résidu K9 (Jin et al., 2011); toutefois, ils ont aussi des fonctions distinctes (Nagy and Tora, 2007).

La deuxième famille est p300/CREB-binding protein (CBP). Les acétyltransférases p300 et CBP partagent une similarité de séquence de 75%; néanmoins, elles partagent peu d'homologie de séquence avec les autres KATs. Elles sont de grandes protéines multi-domaines qui interagissent avec plus de 400 protéines cellulaires différentes (Bedford et al., 2010).

La troisième est la famille MYST, nommée selon le nom de ses membres fondateurs MOZ, Ybf2 / Sas3, Sas2 et TIP60. Ces acétyltransférases sont considérées comme des coactivateurs transcriptionnels qui jouent un rôle important non seulement dans la régulation de la transcription, mais également dans le processus de réparation des dommages à l'ADN, en particulier dans les cassures double-brin (DSB). KAT5 ou TIP60, est le KAT la plus étudiée de la famille MYST (Utley and Côté, 2003) (Doyon et al., 2004) (Avvakumov and Côté, 2007).

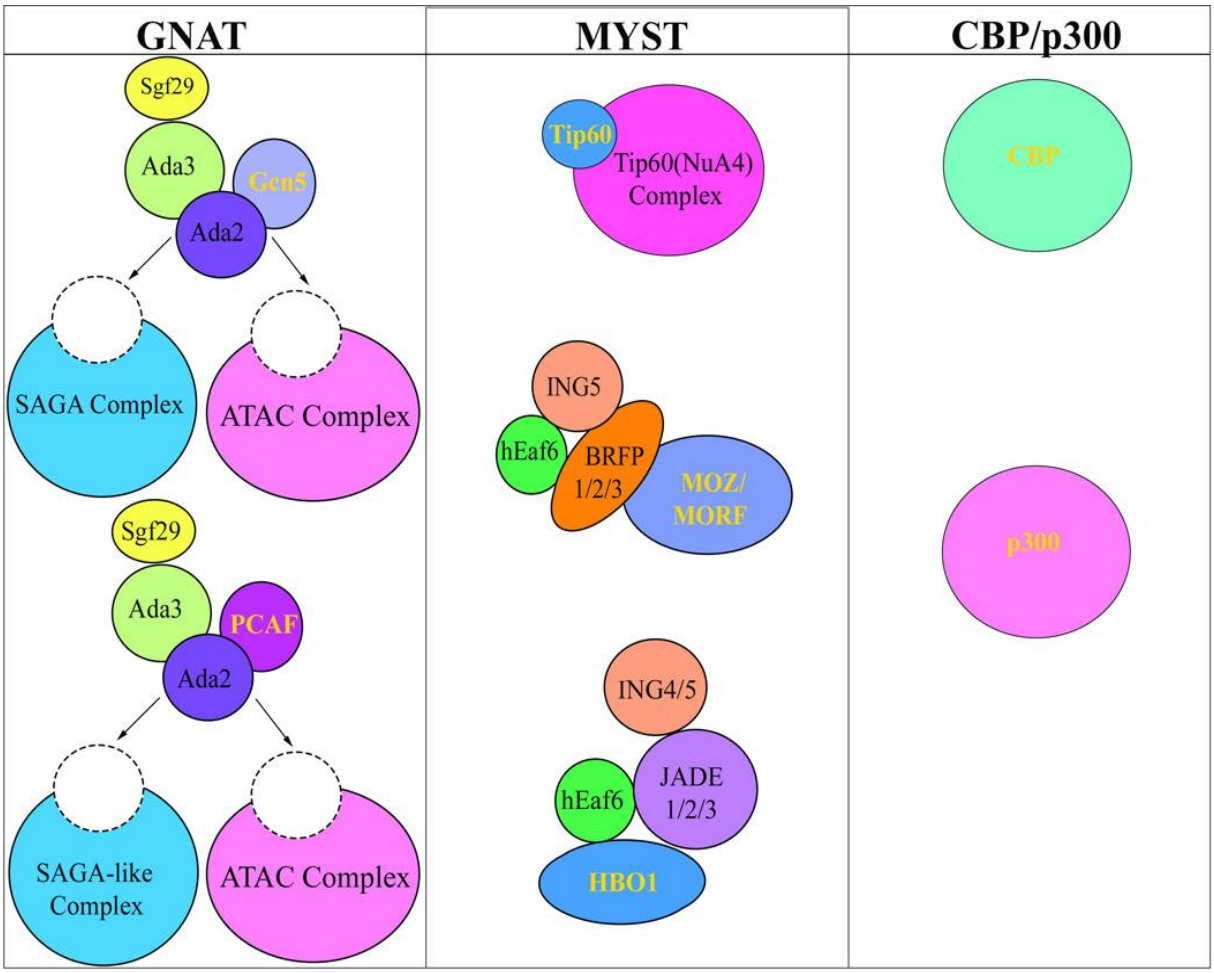

Figure 10. Schéma représentant les 3 familles principales des Histones acétyltransférases (HATs) ou (KATs): Les familles de GNAT, MYST, et CBP/p300 (Farria et al., 2015).

Pour assurer l'interaction avec d'autres protéines et substrats, les KATs possèdent également d'autres domaines en plus du domaine catalytique, y compris le bromodomaine (domaine qui reconnaît le résidu de lysine acétylé), et d'autres domaines de reconnaissance de la modification (Marmorstein and Zhou, 2014). Bien que les fonctions biologiques et les substrats de ces enzymes ne soient pas tous caractérisés, diverses KATs ont été impliquées dans le développement du cancer, y compris TIP60 (**Tableau 3)**.

KATs	Cancer Type	Alteration	Oncogene or tumor suppressor
p300/CBP	Lung, colon, breast and ovarian cancers	mutation	tumor suppressor
	Hepato, colorectal, oral, breast, ovarian, gastric cancers and glioblastomas	loss of heterozygosity or deletion	tumor suppressor
	Prostate cancers	overexpression	oncogene
	Haematological malignancies	chromosome translocations	oncogene
GCN5	Glioma, colon and lung cancers	overexpression	oncogene
PCAF	Hepato, ovarian, gastric and esophageal cancers	deletion	tumor suppressor
Tip60	Head and neck, breast cancers and lymphomas	mutation	tumor suppressor
	Prostate cancer	overexpression	oncogene
MOF	Breast, renal cell colorectal, gastric, ovarian, and hepato cancers, medulloblastoma	deletion	tumor suppressor
	Lung cancer	overexpression	oncogene
MOZ/MORF	Haematological malignancy	chromosome translocations	oncogene
	Acute leukemia	chromosome translocations	oncogene
MORF	Lung cancer	deletion	tumor suppressor

Tableau 3. Les implications des lysines acétyltransférases (KATs) dans le cancer. Les différentes KATs sont impliquées dans la tumorigenèse de plusieurs types de cancer humain et peuvent avoir des rôles oncogènes ou suppresseurs de tumeur en fonction du type de cancer (Di Martile et al., 2016).

2.2. Les Rôles Bivalents de l'HAT TIP60 dans les cancers

Présentation de la publication 2

Review
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EPIGENOMICS, VOL. 7, NO. 8 | REVIEW

A bivalent role of TIP60 histone acetyl transferase in human cancer

Judes G, **Rifaï K**, Ngollo M, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D.

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Les acétyltransférases de la famille MYST sont impliquées dans de nombreux processus biologiques clés telles que les modifications post-traductionnelles des histones, le remodelage de la chromatine, la régulation de l'expression des gènes et la réparation des dommages de l'ADN. L'une des protéines MYST la mieux caractérisée est la protéine TIP60.

TIP60 est une histone acétyltransférase conservée de la levure *S.cerevisiae* à l'Homme, elle fait partie intégrante du complexe humain NuA4. Ce complexe est composé d'au moins 16 autres protéines parmi elles: TRRAP, p400, RUVbl1/2 et YL-1, qui confèrent d'autres propriétés à TIP60 que son activité HAT. Le complexe protéique TIP60 fonctionne par au moins 2 façons: (a) comme un facteur de remodelage de chromatine, en contrôlant la structure de la chromatine et la transcription par son activité HAT, et (b) il régule l'activité des autres protéines non-histones par l'acétylation protéique directe.

Dans cette revue, nous avons exploré les différents rôles de TIP60 dans le cancer en mettant en évidence son rôle d'oncogène dans certains cancers et son rôle suppresseur de tumeur dans d'autres. On a également présenté les différents inhibiteurs de TIP60 et leurs modes d'action.

Review

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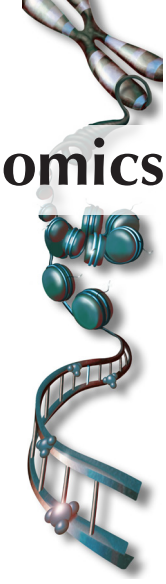

A bivalent role of TIP60 histone acetyl transferase in human cancer

Acetylation is a major modification that is required for gene regulation, genome maintenance and metabolism. A dysfunctional acetylation plays an important role in several diseases, including cancer. A group of enzymes-lysine acetyltransferases are responsible for this modification and act in regulation of transcription as cofactors and by acetylation of histones and other proteins. Tip60, a member of MYST family, is expressed ubiquitously and is the acetyltransferase catalytic subunit of human NuA4 complex. This HAT has a well-characterized involvement in many processes, such as cellular signaling, DNA damage repair, transcriptional and cellular cycle. Aberrant lysine acetyltransferase functions promote or suppress tumorigenesis in different cancers such as colon, breast and prostate tumors. Therefore, Tip60 might be a potential and important therapeutic target in the cancer treatment; new histone acetyl transferase inhibitors were identified and are more selective inhibitors of Tip60.

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Keywords: acetyltransferase • cancer • HAT inhibitor • oncogene • suppressor of tumor • Tip60

Gaëlle Judes[*,1,2], Khaldoun Rifaï[**,1,2], Marjolaine Ngollo[***,1,2], Marine Daures[****,1,2], Yves-Jean Bignon[*****,1,2], Frédérique Penault-Llorca[******,2,3] & Dominique Bernard-Gallon[*******,1,2]

[1]Department of Oncogenetics, Centre Jean Perrin, CBRV, 28 place Henri Dunant, 63001 Clermont-Ferrand, France

[2]EA 4677 'ERTICA', University of Auvergne, 63011 Clermont-Ferrand, France

[3]Centre Jean Perrin, Laboratory of Biopathology, 63011 Clermont-Ferrand, France

*Author for correspondence: judes.gaelle@wanadoo.fr

**Author for correspondence: k.rifaii@live.com

***Author for correspondence: nmarjolaine@yahoo.fr

****Author for correspondence: marine.daures63@gmail.com

*****Author for correspondence: yves-jean.bignon@cjp.fr

******Author for correspondence: frederique.penault-llorca@cjp.fr

*******Author for correspondence: dominique.bernard-gallon@cjp.fr

Human Tip60 or nucleosome acetyltransferase of histone H4 (NuA4) complex is the fusion of two yeast histone acetyl transferase (HAT) complexes, NuA4 and SWR1 [1]. The components of these complexes correspond to all human subunits. The studies performed knockdown and/or mutation of human Tip60 (NuA4) complex to determine functions of the different subunits of NuA4. This complex is involved in transcriptional regulation, DNA repair, chromatin structure alteration, cell migration and invasion, mitosis and genomic instability [2]. Recently, *Tip60* has proved to be an essential gene. In fact, homozygous ablation of the *Tip60* gene in mice prompted embryo lethality near the blastocyst stage of development [3]. Tip60 is a vital protein and its function cannot be compensated by other members of the MYST family.

The human NuA4 complex includes Tip60, which is the catalytic subunit, acetyltransferase [4]. Lysine acetyltransferases, enzymes that catalyze the transfer of acetyl groups from acetyl coenzyme A to the e-amino group of internal lysine residues, are categorized as several categories including the MYST family composed by different members: MOZ, Ybf2/Sas3, Sas2 and Tip60. MYST family members have a major effect on chromatin structure in the eukaryotic nucleus by playing a key role in post-translational modification of histones. MYST HATs are involved in a wide range of nuclear biological processes, such as gene regulation and DNA damage repair [5]. Moreover, Tip60 is distinguished by its membership in the family of KATs, lysine acetyltransferase. Two subgroups of this family have been characterized according to their structure: Gcn5, PCAF and P300/CBP, MYST family including Tip60 (KAT5). Tip60 acetyltransferase acetylates different histones on

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their lysine [4,6], and influences gene transcription and DNA-damage response (DDR). In addition, Tip60 can also acetylate nonhistone proteins involved also in transcription control and activation of DNA damage checkpoint pathways [7]. Indeed, Tip60 coregulates several transcription factors that either promote or suppress tumorigenesis, such as c-Myc and p53 [8]. Tip60 also modulates DDR signaling, and a DDR caused by oncogenes can limit tumor development [9]. The loss of Tip60 leads to an increase of dsDNA breaks and has been linked to an important number of cancer. The alteration of Tip60 functions has been associated with several human diseases including cancer. This review, in a first part, will focus on the Tip60 acetyltransferase, its role and its link with tumorigenesis; then in a second part, we will describe different therapeutic inhibitors of Tip60 and their potential roles in the cancer treatment.

Human Tip60 complex: NuA4

Tip60 was originally isolated as an HIV-1 Tat interactive protein [10]. Human Tip60 complex is a multiprotein complex that consists of at least 16 subunits (Figure 1). Human Tip60 complex is derived from the fusion of two yeast HAT complexes, NuA4 and SWR [1]. These two yeast HAT complexes include four components (Eaf2, Arp4, ACT1 and Yaf9); these parts correspond to all human subunits.

The transformation/transcription domain-associated protein (TRRAP), central to the Tip60 stable complex, has an FATC (FRAP, ATM, TRRAP C-terminal) and a PI3K-like domain. However, the kinase domain lacks the conserved amino acids required for binding the ATP and does not appear to be catalytically inactive. Murr *et al.* [12] reported that TRRAP facilitates the multiprotein assemblies and the recruitment of different regulatory factors and complexes to chromatin. Moreover, several studies have linked the adaptor TRRAP protein to human cancer and to oncogenic factors [12].

Another essential component of the complex is hDomino (also known as p400, EP400, E1A binding protein p400) is RNA-dependent ATPase. P400 can intervene in oncogenesis by the destabilization of histone–DNA interactions in an ATP-dependent manner and by binding to oncogene c-myc with other protein [13,14].

The bromodomain containing protein 8 can bind to acetylated histones, suggesting its role in regulation of chromatin remodeling by histones and gene transcription [15,16]. Brd8 protein is overexpressed in rats presenting a colon cancer [17] and a Brd8 inhibition by using a shRNA induced a growth reduction of all proliferation [18].

BAF53a (53 kDa BRG-1/human BRM-associated factor) and actin are also present in the TIP60 complex. They have an ATPase activity and formed BAF chromatin–remodeling complex [4,6,19]. BAF53a is also named actin-like 6a (ACTL6a) owing to similarity with β-actin. BAF53a is a target for HIV-1 proviral gene silencing and activation [20]. In yeast, BAF53a ortholog is required for DNA–double-strand break repair mechanisms [21].

The complex contains inhibitor of growth 3 (ING3), a protein with a C-terminal plant homeodomain-finger motif which is found in proteins involved in chromatin remodeling and is a sequence-specific histone recognition protein module [16,22–23]. ING3 is also involved in DNA damage-responsive p53 transcription and its expression or loss of heterozygosity (LOH) are noticed in tumors [24,25]. YL1 was identified as a subunit of Tip60 complex and is a nuclear protein, which plays multiple roles in chromatin modifications and remodeling cells. In addition, another complex, *S. cerevisiae* SWR1, contains this protein and includes the SNF2-related helicase SWI2/SNF2-related CBP activator protein that contributes to incorporation of the histone variant Htz1 (H2AZ) into nucleosomes [26].

The RuvBL1 and RuvBL2 helicases belong to the family of AAA + ATPase (ATPase associated with various cellular activities) present a homology with the bacterial DNA repair RuvB protein and are also subunits of the NuA4 complex [27]. However, the NuA4 complex has helicase activity, which is not attributable to RuvBL1/2 [13]. An overexpression of these two helicases was found in different types of cancer [28].

The Tip60 complex contains two further chromodomain-containing proteins, mortality factor 4 related gene 15 (*Mrg15*) and Mrg binding protein. They are involved in regulation of cell proliferation, viability and senescence. Mrg15 has helix–loop–helix and leucine zipper domains, common in transcriptional regulators and a chromodomain, which enables a protein–protein interaction in chromatin remodeling factors [6].

Enhancer of polycomb 1 (EPC1) is an essential subunit in the chromatin regulatory complex, NuA4. In mammalian biology, EPC1 is involved in regulation of skeletal muscle differentiation [29] and seems to have a critical role in leukemic hematopoiesis over and above regulation of oncogene C-Myc. EPC1 prevented accumulation of C-Myc and acute myeloid leukemia cell apoptosis [30].

DNA methyltransferase associated protein 1 (DMAP1) is one of the common components of the NuA4 complex and interacted with DNA methyltransferase 1. The DMAP1/DNA methyltransferase 1 complex localized at pericentric heterochromatin contributes to heterochromatin formation by DNA methy-

lation and histone modifications such as histone desacetylation and methylation of histone H3 at lysine 9 [31]. Moreover, a knockdown of DMAP1 in mammalian cells led to chromosomal instability and tumorigenesis [32].

Glioma amplified sequence 41 (GAS41) is a part of the human Tip60, but also SCRAP complexes. Park *et al.*, [33] reported that GAS41 was involved in pathway of p53. Indeed, GAS41 repressed the $p14^{ARF}$ and *p21* genes that are two principal tumor suppressor genes. This activity is independent of Tip60 HAT and could contribute to development of tumor, such as human glioma [34]. In brain tumor, an overexpression of GAS41 was found and a common regulation existed between GAS41 and both c-Myc and n-Myc [35].

A subunit of the NuA4 complex is primordial for the complex function: the acetyltransferase Tip60. Although Tip60 is a previously identified histone acetylase, the Tip60 monomer is unable to acetylate chromatin [36]. In a stable multiprotein complex, Tip60 acetylates core histones H2A (Lys5), H3 (Lys14) and H4 (Lys5, Lys8, Lys12 and Lys16 *in vitro*) [37]. In addition to histone, Tip60 can acetylate and activate the kinase ataxia telangiectasia mutated (ATM); a protein kinase that regulates the cell response to DNA damage through the phosphorylation of protein involved in cell-cycle checkpoints and DNA repair [38]. This HAT is also involved in transcription regulation as coactivator for nuclear hormone receptors, such as androgen or estrogen receptor [39–41], nuclear factor-κB (NF-κB) [42], c-Myc oncoprotein [43] and p53. All these proteins play a key cellular role in the development of different pathologies and especially in human cancer.

Tip60 paradox in human cancer

Tip60 histone acetyltransferase has been shown to be underexpressed or overexpressed in many human cancers [44,45]. Many studies have shown the involvement of Tip60 in oncogenesis, in resistance to cancer or potential antioncogenic activities; therefore Tip60 represents a new paradox in cancer.

Tip60: oncogene

HAT Tip60 establishes several connections with other subunits of NuA4 complex and these links seem to play a role in oncogenesis.

p400 is an ATPase [46] in a human NuA4 complex and has a role in incorporation of histone H2A.Z variant in mammals [47] and H2A.Z can be acetylate by different HAT including Tip60. Moreover, p400 is an inhibitor of some Tip60-dependent pathways and an increase in the activity of these Tip60-dependent pathways such as p53 pathway enables to reduce the effects of p400 knockdown. The p400/Tip60 ratio is

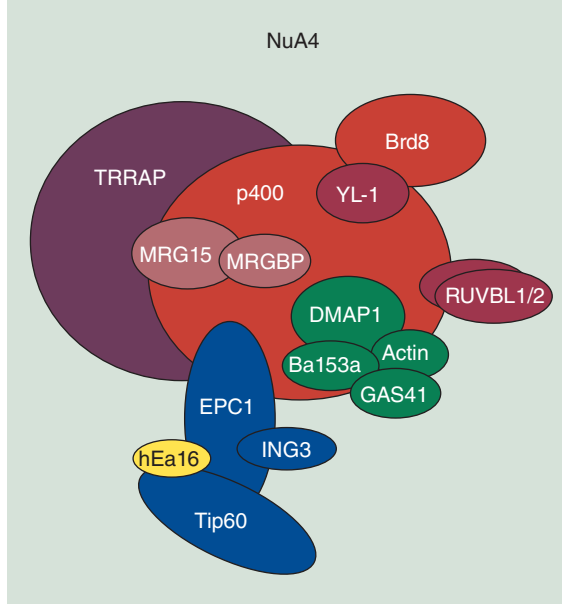


Figure 1. Human NuA4 complex. Multisubunit protein formed a complex that possessed acetyltranseferase activity.
Reproduced with permission from [11].

affected in colorectal cancer and leads to an increase of cell proliferation through DDR pathways. However, Tip60 function cannot sustain a normal response to DNA damages and induces cancer progression. Reversing the p400/Tip60 imbalance by the use of siRNAs resulted in increased apoptosis and decreased proliferation of colon cancer cells, suggesting that the imbalance of this ratio is important for cancer progression [48]. Chan *et al.* [34] showed that p400 plays a role in inhibition of p53 → p21 transcription and cellular senescence program. p53 leads to overexpression of p21 in normal cells and is blocking cell cycle in G1 phase. Furthermore two oncogenes, E1A and c-Myc, upregulate p400 expression and suppress cellular senescence and this step is prerequisite for the tumor development [35].

Tip60 interacts with another subunit of NuA4 complex: BRD8. In fact, hormone-receptor complexes such as thyroid hormone receptor-ß and/or retinoid X receptor interacted with BRD8, and activated Tip60 complex that regulates chromatin remodeling and transcription [49,50]. Brd8 protein is overexpressed in human metastatic colorectal cancer cell lines and colon adenocarcinoma in rats. SiRNA-mediated Brd8 knockdown induced cell death or reduced a cellular growth. This inhibition also increased the effect of spindle poisons and the proteasome inhibitor. BRD8 and the interacting proteins including NuA4–HAT complex may be involved in colorectal tumor cell survival and drug resistance [51,52].

Other evidence linking Tip60 to cancer involves connections between Tip60 and other proteins; indeed

Tip60 possess physical or functional interactions with other cellular proteins and particularly steroid hormone receptors. The androgen receptor (AR) is a steroid hormone receptor and AR signaling is crucial in prostate cancer progression and developments that are associated with increase of AR expression level suggesting that downregulation of AR expression could decrease tumor growth. Numerous post-translational modifications on the AR modulate its activity by regulation of protein stability, interaction with other proteins and structure of the receptor itself. One of these translational modifications is the acetylation. Indeed, AR can be acetylated on lysine 630 by Tip60 and this acetylation event is essential for Tip60-dependent AR coactivation [40]. AR interacts with Tip60 via its hinge region and is a substrate for Tip60. In addition, Tip60 was shown to be overexpressed in several cases of prostate cancer [53]. Shiota *et al.* [54] determined the different functions of Tip60 protein in AR localization and cell growth in prostate cancer. Tip60 overexpression in castration-resistant LNCaP induces an increase of the AR acetylated form and AR translocation in the nucleus even without androgen. Moreover, Tip60 silencing contributes to stop cell cycle at G1 phase and causes the decrease of growth of AR-expressing PCa cells, similar to inhibition of androgen/AR signaling. Furthermore, Tip60 knockdown suppressed the cell growth of CxR cells. The translocation of AR in nucleus induces contact with target gene promoter and transcriptional activation of AR gene. Tip60 regulated transcriptions of AR target genes androgen independently [55]. Modulation of Tip60 expression or function may be a useful strategy for developing novel therapeutics for prostate cancer, even castration-resistant LNCaP.

Consequently, Tip60 acts as a ligand-dependent coactivator for the AR but also progesterone receptor and estrogen receptor (ER) [39]. Tip60 is necessary to induce the efficient hormonal of some endogenous ER-α target genes and interaction of ER-α with Tip60 is important for E2-induced occupancy by Tip60 on ER-α target genes. On these gene promoters, H3K4me3, which is an activated transcription mark, contributes to Tip60 recruitment and acetylation by Tip60 of histone H2AK5 [56]. Concerning the other estrogen receptor, ER-β also interacts with Tip60 both in the absence or the presence of estrogen. Tip60 modulates ER-β action in different *cis*-regulatory elements; it can enhance or reduce ER-β transactivation at the AP-1 or ERE sites. In prostate cancer cells, Tip60 interacts with ER-β to regulate endogenous gene expression such as *CXCL12* involved in tumor progression, angiogenesis and metastasis [57] and *cyclin D2* playing a critical role in cell-cycle progression and tumorogenesis in several cancers [58]. After a Tip60 knockdown, expression of *CXCL12* was upregulated contrary to *cyclin D2* for which the expression was decreased [59]. In different cancers such as lymphomas, breast carcinomas and head and neck tumors, Tip60 expression is reduced and conversely, increased in other tumor types such as prostate cancer. These variations of Tip60 expression may cause a modification of the expression of the Tip60-dependent subset of genes regulated by steroid hormones. The modulation of these genes and steroid hormone responses could lead to formation and development of cancer.

The nuclear factor-κB (NF-κB) family is composed by five members, ReA/p65, c-Rel, RelB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) [60]. NF-κB is expressed ubiquitously and is involved in the expressions of numerous genes, and in diverse biological processes such as apoptosis, cell proliferation, inflammatory responses and oncogenesis [61]. The association of NF-κB with various cofactors such as nuclear receptor coactivators SRC3/Rac3 and SRC1/N-CoA1 [62,63] or HAT p300/CBP [64] and especially Tip60, induces transcriptional activation of NF-κB. Kim *et al.* [65] reported that Tip60 interacts with the NF-κB RelA/p65 subunit, contributes to maintain lysine 310 acetylation of NF-κB RelA/p65 and increases its transcriptional activity through physical interaction. Therefore, Tip60 acts in NF-κB pathway by expression regulation of 4 NF-κB target genes, *IL-6, IL-8, C-IAP1* and *XIAP*, which all have a role in oncogenesis [65].

C-Myc is also a substrate of the acetyltransferase Tip60. C-Myc is involved in 20% of all human cancers and is an oncogene overexpressed in cancer [66]. C-Myc regulates transcription through several mechanisms, including recruitment of chromatin modulating proteins, basal transcriptional factors, DNA methyltransferase and histone acetyl-transferase [67–70]. C-Myc recruits Tip60 and other subunits of Tip60 complex such as TRRAP, RuvBL1, RuvBL2 and p400 on Myc-target genes and contributes to histone acetylation of these genes [43]. Moreover, acetylation of C-Myc by Tip60 and mGCN5/PCAF acetyltransferases increased the protein stability and C-Myc level in cells [71]. All these data suggested that Tip60 regulates C-Myc functions and participates to events which lead to C-Myc oncogene amplification in various human cancers.

Tip60: tumor suppressor

Other evidences involve Tip60 functions in mechanisms of cancer protection. The most important non-histone target of Tip60 acetyltransferase activity is p53, a tumor suppressor and transcriptional factor that

is regulated by numerous post-translational modifications. A large-scale inhibitory RNA (RNAi) screen identified Tip60 as a component of the p53 pathway. The inhibition of Tip60 contributed to stop the p53 response to DNA damage in human cells [72]. Tip60 directly acetylates p53 at lysine 120 (K120) within the DNA binding domain [73–76]. Consequently, acetylated p53 activated expression of apoptotic genes and led to cell apoptosis. When acetylation at K120 was eliminated by mutation of K120 to arginine, a nonacetylatable residue, DNA damage-induced p53-dependent cell-cycle arrest, rather than p53-dependent apoptosis. However, the process by which acetylation of p53 at K120 is regulated after DNA damages is still unclear [75]. The p53 K120 acetylation by Tip60 is one of the examples where a post-translational modification of p53 has been mapped to a residue sometimes mutated in cancer. This K120 acetylation is crucial for p53 to activate the transcription of PUMA [76,77]. Indeed, in response to DNA damage, p53 modified binds to promoters of its specific target genes, such as *p21, PUMA, BAX, GADD45 and NOXA*, to activate expression of these genes, which are involved in either cell-cycle arrest or apoptosis [78]. It has been reported that PUMA is regarded as the main inducer of the p53-dependent apoptotic response [79,80]. Another protein, $p14^{ARF}$, is a tumor suppressor [81] and interacts with p53 and retinoblastoma (Rb) tumor suppressor pathways. $P14^{ARF}$ interacts with Tip60 to induce a p53-independent DNA damage checkpoint in response to genotoxic stress [82,83]. Tip60 acetylates Rb and induces its proteasome degradation. However, $p14^{ARF}$ inhibits Rb acetylation mediated by Tip60 and induces hypoacetylated Rb accumulation [84].

Moreover, the Tip60 complex also contains the tumor suppressor ING3, a component of the p53 pathway and is involved in cell-cycle regulation and apoptosis [6,19,22]. A decrease of ING3 expression or LOH is found in different cancers. Decrease or no expression of ING3 mRNA has been observed in primary head and neck squamous cell carcinomas, tongue tumors, larynx tumors and melanoma [16,85]. In the ING family, another member, ING5, of this protein family interacts with Tip60 and is considered to a potential tumor suppressor. ING5 is a subunit of histone H3-specific HAT complex that includes MOZ/MORF leukemic proteins [19]. In human head and neck squamous cell carcinoma [86], colorectal [87] and gastric carcinoma [88], a decrease of ING5 expression is observed. Besides, ING5 modifies p53 acetylation at K120 via its interaction with Tip60. Liu *et al.* [74] showed that an inhibition of ING5 by shRNA led to decrease p53 acetylation. Consequently, the knockdown of ING5 brings about reduction of apoptosis of DNA-damaged cells.

Previous studies showed that acetylation by p300/CBP and PCAF acetyltransferases of E2F1 transcription factor is an important modification in DDR. Van Den Broeck *et al.* [89] demonstrated that Tip60 acetylated the lysine residues 120/125 of E2F1 and stabilized also E2F1. After DNA damages, accumulation of E2F1 is bound to Tip60 activity. Acetylation and accumulation of E2F1 by Tip60 never play a role in an E2F1-dependent apoptotic program. However, E2F1/Tip60 complex induces an accumulation of the ERCC1 protein after cisplatin exposure [89]. This ERCC1 (enzyme excision repair crosscomplementing group 1) protein acts in the repair of platinum-DNA adducts and its accumulation is associated with the repair cisplatin-damaged DNA and clinical resistance to platinum chemotherapy [90,91]. There is a direct correlation between cisplatin resistance and the expression of E2F1, Tip60 and ERCC1 in various cancer cell lines. By the regulation of diverse suppressors of tumor, Tip60 is a key element in protection mechanisms in human cancer. Moreover, its action is not limited to these proteins; Tip60 is involved in the inhibition of many oncoproteins. Particularly, in human leukemia, histone acetyltransferase Tip60 interacted with c-Myb oncoprotein, which plays a major role in G1/S transition in cycling hematopoietic cells and coactive major cellular genes, for example *c-Myc, cdc-2 and Bcl-2* [92]. This interaction with Tip60 led to the decrease of c-Myb transcriptional activity by recruiting histone desacetylase HDAC1 and HDAC2. Consistent with this finding, it is also reported that Tip60 decreased STAT3 oncoprotein expression [93]; in human cancer dysregulation of STAT3 activity contributed to tumor development. However, other HAT such as CBP/p300 and NCoA/SRC1a interacted with STAT3 and enhance its transcriptional activity [94]. Therefore, an underexpression of Tip60 such as in breast cancer might be involved in abnormal activation of STAT3 and at the opposite; an overexpression of Tip60 regulated the STAT3 expression.

Gorrini *et al.* [45] highlighted that Tip60 KO mice were not viable and this characterized by early embryonic death. In E_{μ}-myc Tip60 heterozygous mice, they demonstrated that Tip60 is a haplo-insufficient tumor suppressor required for an oncogene-induced DDR and was p53-independent manner in mice and human being. Loss of nuclear Tip60 staining was found in mammary carcinomas. This study indicates that reduced Tip60 expression correlates with tumor development. However, the link between Tip60 expression and survival of cancer patients has not been examined. Tang *et al.* [95] showed that the acetylation by Tip60 regulated the balance between breast cancer (early onset) 1 (BRCA1) and p53 bind-

ing protein 1 (53BP1) at DNA double-strand breaks (DSBs) in cancer cells. BRCA1 and 53BP1 are tumor suppressors underexpressed in triple-negative breast cancer [96]. Tip60 knockdown consistently increased 53BP1 and decreased both H4ac and BRCA1 association with hyperacetylated regions on chromosome 16p, whereas HDAC inhibition induced the opposite effects [95]. Moreover, the TRRAP subunit of Nu4A is also a direct interactant of BRCA1 [97], mutations of this gene predispose women to breast and ovarian cancers. BRCA1 mutations found in breast cancer patients abrogate interaction with TRRAP and impair transactivation function of BRCA1 [97], suggesting that the loss of interaction between TRRAP and BRCA1 may inhibits normal function of the tumor suppressor leading to an increased susceptibility to cancer.

Tip60 established different connections with cell proteins or the other NuA4 subunits. These relations can contribute to oncogenesis with two different actions: antioncogenic or pro-oncogenic (Figure 2). Therefore, Tip60 may be a future therapeutic target.

Inhibitors of histone acetyltransferase Tip60

Acetyltransferases are known to perturb post-translational modifications of histones and other proteins in tumors. Drugs able to regulate these acetyltransferases are therefore a potential tools in the treatment against cancer. To treat cancer, different agents are used to produce DNA damage and induce death of replicating cancer cells. Tip60 plays a major role in induction of DDR and might be a potential target in the treatment against cancer. In fact, several studies have reported some Tip60 inhibitor molecules (natural or synthetic molecules) (Table 1).

Three natural molecules have been described as inhibitor of the global HAT activity; curcumin (diferuloylmethane) derived from the plant *Curcuma longa* [98], garcinol (plyisoprenylated benzophenone) coming from *Garcinia indica* fruit [99] and anacardic acid (AA) (6-pentadecylsalicylic acid), which is a compound from *Anacardiumoccidentale*is [100]. Curcumin and garcinol inhibited CBP/p300 HAT activity *in vitro*, however PCAF HAT appeared only sensitive to garcinol [98,99]. These compounds have different effects on breast cancer MCF7 cell line, garcinol stopped cell proliferation and upregulated the DNA damage markers such as p53, whereas curcumin treatment had a weak effect on DNA damage markers [101]. Anacardic Acid, a noncompetitive lysine acetyltransferase is derived from traditional medicinal plants, such as cashew nuts, and has been linked to anticancer, anti-inflammatory and radio sensitization activities [102]. More specifically, AA inhibits the acetylation activity of Tip60 and activation of ATM kinase in HeLa cells and sensitize tumor cells to ionizing radiation [100]. These three inhibitors are not specific to Tip60 inhibition, therefore different investigations worked toward to identify new Tip60-specific inhibitors.

Recently, AA analogs were synthesized and inhibited the enzyme Tip60 selectively compared with PCAF and p300 HAT and in particular, an analog that was specifically competitive with Ac-CoA and noncompetitive with histone subtrats [103]. The bisubstrate strategy was used to design a Tip60/Esa1 inhibitor. First, bisubstrate HAT inhibitors were synthesized such as lysine-CoA for p300/CBP and histone H3 peptide-CoA analogs for PCAF-Gcn5 [105–107]. Based on this technique, Wu *et al.* [104] showed that the bisubstrate H4K16CoA inhibited the activities of Esa1 and Tip60 with a much higher potency than the previously reported with other HAT inhibitors. Especially, H4K16CoA inhibits Tip60 with 20-fold more efficacious than with AA. However, these inhibitor compounds have low permeability. Further to these studies, Yang *et al.* [108] designed a second generation of Tip60 bisubstrate inhibitors which had K4me1 and/or K9me3 modifications integrated on peptide region of the H3 peptide-CoA. Indeed, the H3K4me1 and H3K9me3 promoted the Tip60 binding on these histone trails [56]. These methyl marks improved the inhibition of Tip60 by bisubtrate inhibitor.

Other molecules can be used in HAT inhibition such as pentamidine (PNT). This compound is an antiparasite treatment that has been used against trypanosomiasis, leishmananiasis and pneumocystis carinii. PNT had the ability to bind to the minor groove of doublestrand DNA and inhibited protein synthesis, DNA synthesis in human tumors [109]. PNT treatment decreased also the activity of human endoexonuclease *in vitro* and induced cell death in several tumor cells, therefore PNT could be used as a new anti-tumor drug [110]. More recently, PNT has been reported to reduce the acetylation of histone H2A mediated by Tip60 and to inhibit the Tip60 activity *in vitro* [111].

Considering this new information, another recent study developed a novel Tip60 inhibitor, TH1834, designed from the scaffold of pentamidine and acetyl-CoA [112]. The Tip60 inhibition by TH1834 increased the effect of ionizing radiation in breast cancer cell line (MCF7) with low AR expression and prostate cancer cell lines (PC-3 and DU-145) with low AR expression, induced apoptosis and increased unrepaired DNA damage in cancer cells. TH1834 is a specific inhibitor to Tip60 HAT; indeed, the acetylation level of H4K16 and H4K8 induced by hMOF HAT was not reduced by TH1834 treatment [112]. This Tip60 inhibitor seems to play a major role in

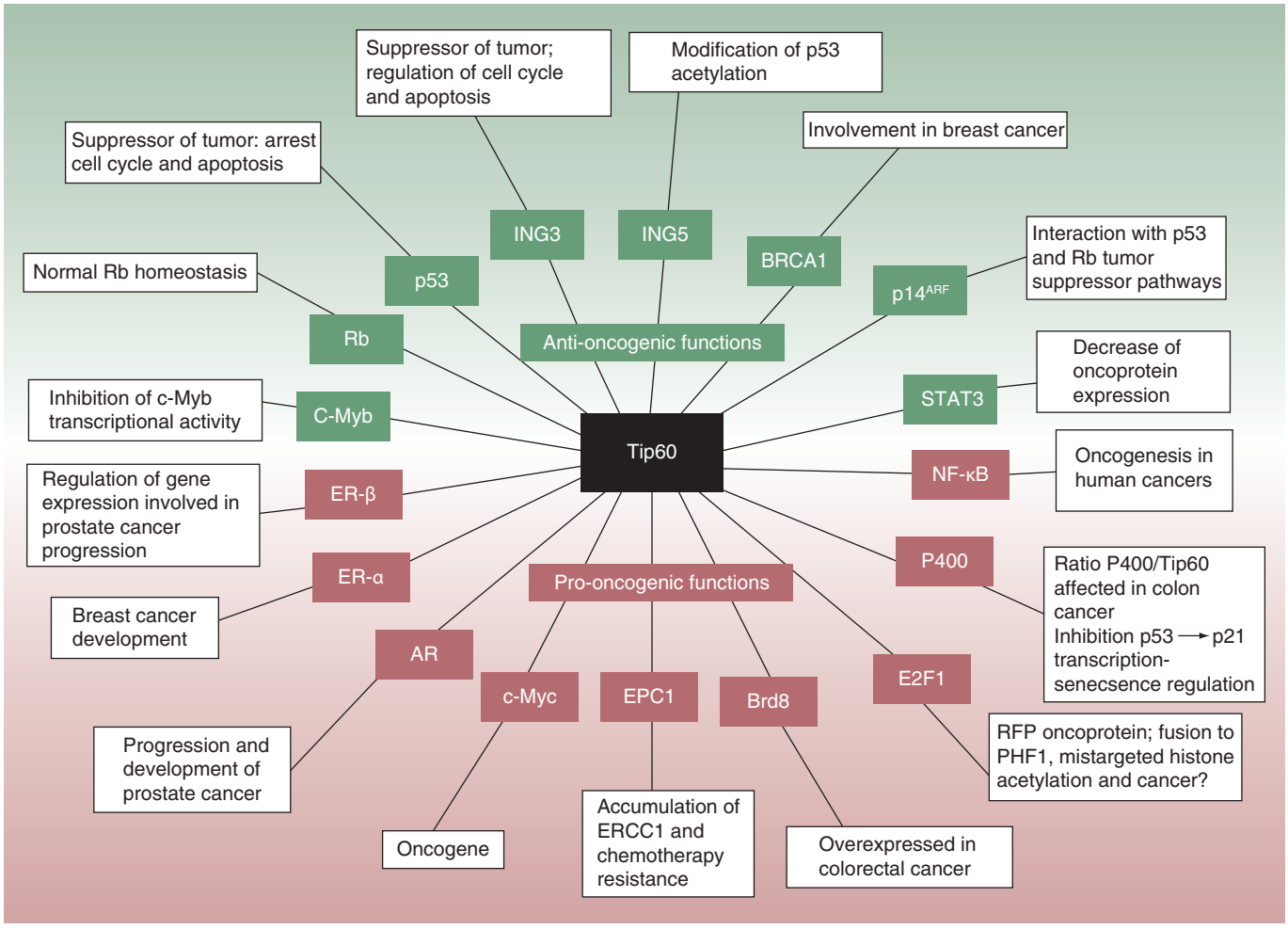


Figure 2. Tat-interactive protein 60kDa, a new paradox in cancer. Different connections established between HAT Tip60 and cell proteins or other subunits of Nu4A complex. These connections might contribute to tumor formation or progression (pro-oncogenic) and at the opposite, they might have an antioncogenic action.
HAT: Histone acetyl transferase; Tip60: Tat-interactive protein 60kDa.
Adapted with permission from [11].

breast and prostate cancer and possess a potential therapeutic effect.

Another team, Coffey *et al.* [113] characterized a new Tip60-specific inhibitor, NU9056 (1,2-bis[isothiazol-5-yl]disulfane) identified by high-throughput screening analysis. This compound is an isothiazolone that covalently binds to the thiol HAT-activated site and inhibits their activity. Isothiazolones have been previously identified as inhibitor of the acetylase activity of many HAT including P300 and PCAF. This compound has been tested in prostate cancer cell lines (LNCaP). In these cells, the Tip60 expression is aberrant and lead to increase of AR transcriptional activity by acetylation [40]. Coffey *et al.* [113] showed that NU9056 treatment induced a decrease of AR, prostate-specific antigen, p21 and p53 levels in LNCaP cells. This might explain the increase of apoptosis and the decrease of proliferation of prostate cancer cells. NU9056 inhibitor can be a substitutive treatment to an androgen deprivation therapy in prostate cancer with castrate resistant.

The development of Tip60 inhibitors could play an important role in treatment of cancers. The study of Tip60 expression level could enable to identify tumors that can be sensitive to these treatments. Furthermore, Tip60 acetylation is a signal to activate ATM kinases and to phosphorylate H2AX (γH2AX) following DNA double-strand breaks. The status of γH2AX foci can be used as a reporter of Tip60 activity *in vivo*. However, Tip60 inhibitors require further investigation in other cell lines and *in vivo* models to clarify the mechanism of inhibition and to prevent harmful effects in humans.

Conclusion & future perspective

Cancer is a major health problem in the world. Nowadays, the understanding of cancer disease and the

Table 1. Tip60 inhibitors.

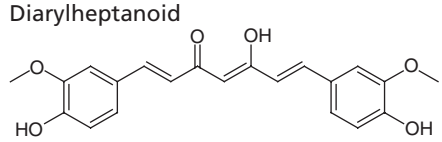
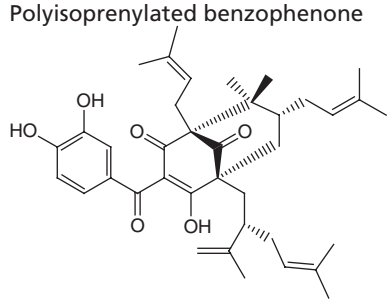
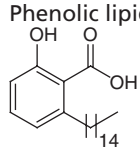
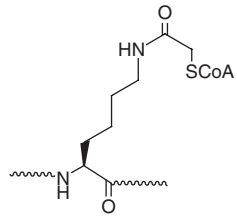
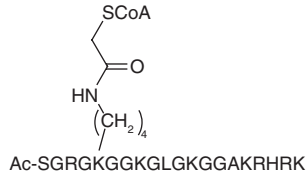
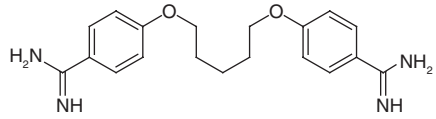
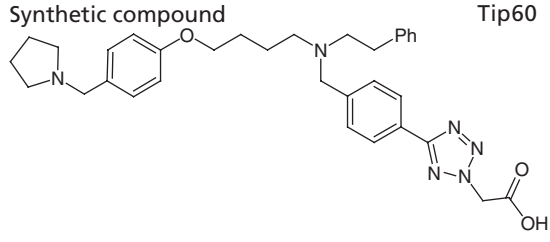
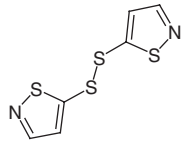
Names	Chemical structures	Targets	Tested cells
Natural molecules			
Curcumin	Diarylheptanoid 	P300/CBP, Tip60	HeLa cells
Garcinol	Polyisoprenylated benzophenone 	P300, PCAF, Tip60	Lung cancer cells, HeLa cells, breast cancer cells, pancreatic cancer cells, hepatocellular carcinoma cells and head, neck squamous cell carcinoma cells
Anacardic acid	Phenolic lipid 	P300, PCAF, Tip60	Myeloid KBM-5 cells, lung adenocarcinoma H1299 cells, prostate cancer DU145, cells squamous cell carcinoma SQ20B and SCC35 cells, HeLa cells
Synthetic molecules			
Bisubstrate analogs General structure		P300, PCAF, Tip60, GCN5	NA
Example of bisubstrate analog: H4K16CoA	 Ac-SGRGKGGKGLGKGGAKRHRK	Tip60	HeLa cells
Pentamidine	Bisbenzamidine derivative 	Tip60	HeLa cells
TH1834	Synthetic compound 	Tip60	Breast cancer cells (MCF7) Prostate cancer cells (DU145, PC-3)

Table 1. Tip60 inhibitors (cont.).

Names	Chemical structures	Targets	Tested cells
Synthetic molecules (cont.)			
NU9056	**Isothiazolones derivative** 	Tip60	Prostate cancer cells (LNCaP)

design of future treatment has become a primordial issue. To predict aggressiveness and development of cancer, several investigations have led to the detection of a class of enzymes involved in tumorigenesis, the histone acetyltransferases. In fact, these HATs are recruited to gene promoters and facilitate gene transcription. Tip60 in NuA4 human complex is a protein with multiple roles in cancer; it affects major functions including DNA repair and transcriptional regulators by direct or indirect protein interactions. However, Tip60 role seems to be different depending on the kind of cancer, indeed it can act as a tumor suppressor or as an oncogene. Different Tip60 inhibitors were designed and showed effectiveness in prostate cancer cells. However, few Tip60 inhibitors have been reported and new inhibitors are not under clinical investigation at present. In conclusion, Tip60 has a central role in cancer pathogenesis and is an attractive therapeutic target; currently, all the functions of this multifaceted protein and its regulation have not been totally elucidated. Further investigations could find new therapeutic molecules targeting Tip60 activity.

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Executive summary

Histone acetyltransferase

- Histone acetyl transferases (HATs) are enzymes that catalyze the transfer of acetyl group from acetyl coenzyme to the e-amino group of a substrate lysine residue.

NuA4 human complex

- The fusion of two yeast HAT complexes, Eaf1 and Swr1, recreates Human Tip60 complex, which possesses 16 subunits similar to yeast composition. This complex contains a catalytic site represented by acetyltransferase Tip60.
- Tip60 is an HAT of the MYST family, highly conserved in eukaryotes; this enzyme is involved in several key processes including post-translational modification of histone transcription regulation, DNA repair and interacts directly or indirectly with different major factors relevant to tumorigenesis.

Tip60 in cancer

- Tip60 expression is altered in several cancers; in breast, colon and lung carcinomas, a significant downregulation of Tip60 expression is found and at the opposite Tip60 is overexpressed in prostate cancer. Tip60 might be involved in oncogenesis or in resistance to cancer.
- Tip60 is involved in cancer in two different ways; the first promotes the development of cancer by the interaction of Tip60 with oncogenes, such as C-Myc and NF-κB. However, in the second way, Tip60 seems to stimulate different tumor suppressor pathways especially p53, ING family and retinoblastoma.

Tip60: a therapeutic target

- Tip60 could have a high therapeutic potential in the cancer treatment and the design of drug with a chemotherapeutic action is necessary. However currently, only a small number of Tip60 inhibitor has been identified until now.

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•• **The authors designed and synthesized a selective inhibitor of Tip60 using pentamidine and acetyl-CoA, then tested it in breast and prostate cancer cell lines.**

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Conclusion de la publication 2

TIP60 est une histone acétyltransférase qui est impliquée dans le processus de la tumorigenèse, et semble avoir un rôle bivalent dans les cancers humains. En effet, les fonctions opposantes de TIP60 dépendent principalement du type de cancer, ainsi que de ses taux d'expression.

Dans cette revue, on a montré un rôle oncogène de TIP60 via l'activation des oncoprotéines tels que NF-κB, c-Myc, et le récepteur d'androgène (AR) impliqué dans le développement du cancer de la prostate. En revanche, TIP60 peut jouer le rôle d'un oncosuppresseur en activant les GSTs BRCA1, p53, et ING1.

Le développement d'inhibiteurs de TIP60 pourrait jouer un rôle important dans le traitement des cancers où TIP60 est surexprimée. Par conséquent, différents inhibiteurs de TIP60 ont été conçus et ont montré une efficacité dans les cellules cancéreuses de la prostate par exemple.

En conclusion, les études ont montré le rôle important que TIP60 pourrait jouer dans le processus de la tumorigenèse humaine.

C. Histone Deacétylase SIRT1 : Une Protéine Multi-Facettes

1. Familles des Histones Désacétylases (HDACs)

A l'heure actuelle, le génome humain code pour 18 HDACs qui appartiennent à deux familles distinctes avec des mécanismes catalytiques différents: les histones désacétylases 'classiques' dépendantes du Zn2 + (HDAC1-11) et les histones désacétylases dépendantes de NAD + (SIRT1-7). Les désacétylases dépendantes de Zn2 + sont principalement exprimées à la fois dans le noyau et dans le cytoplasme, alors que les sirtuines sont également présentes dans les mitochondries. Les 18 HDACs peuvent ainsi être classifiées en 4 groupes distincts selon leur homologie avec les HDACs de la levure (Yang and Seto, 2008).

1.1. Classification et caractérisation des HDACs classiques

Les 11 HDACs humaines classiques sont réparties en 3 classes. La classe I est composée de 4 membres (HDAC1, 2, 3 et 8), et ceux-ci présentent une grande similitude avec la protéine de levure RPD3. HDAC1, 2 et 3 ont été décrites en tant que composantes des complexes multi-protéines, alors que HDAC8 n'en fait pas partie (Haberland et al., 2009a). Les HDAC1, 2 et 8 sont généralement situées dans le noyau, alors que HDAC3 possède également un signal d'exportation nucléaire et peut se déplacer entre le noyau et le cytoplasme (Moser et al., 2014).

Les HDACs de classe II sont ensuite divisées en classe IIa (HDAC4, 5, 7, 9) et classe IIb (HDAC6 et 10). Les deux sous-classes sont étroitement liées à la levure HDA1. Les HDACs de classe IIa ont été principalement impliquées dans la différenciation et le développement, et agissent comme des répresseurs transcriptionnels dans de nombreux tissus, ainsi que dans les systèmes immunitaire et vasculaire. Cependant, elles présentent significativement moins d'activité de désacétylase que les classes I, IIb et IV, qui ont été attribuées à la présence d'un résidu d'histidine au lieu de la tyrosine dans le site catalytique (Lahm et al., 2007) (Di Giorgio et al., 2015). Les HDACs de classe IIb (HDAC6 et 10) se trouvent principalement dans le cytoplasme ou à la fois dans le cytoplasme et le noyau dans le cas de HDAC10. Le membre unique des HDAC de classe IV est l'HDAC11, une protéine essentiellement

nucléaire, homologue des HDACs de classe I et de classe II (Gao et al., 2002). Les HDACs classiques ainsi que leurs homologues de levure, sont représentées schématiquement dans la **Figure 11**.

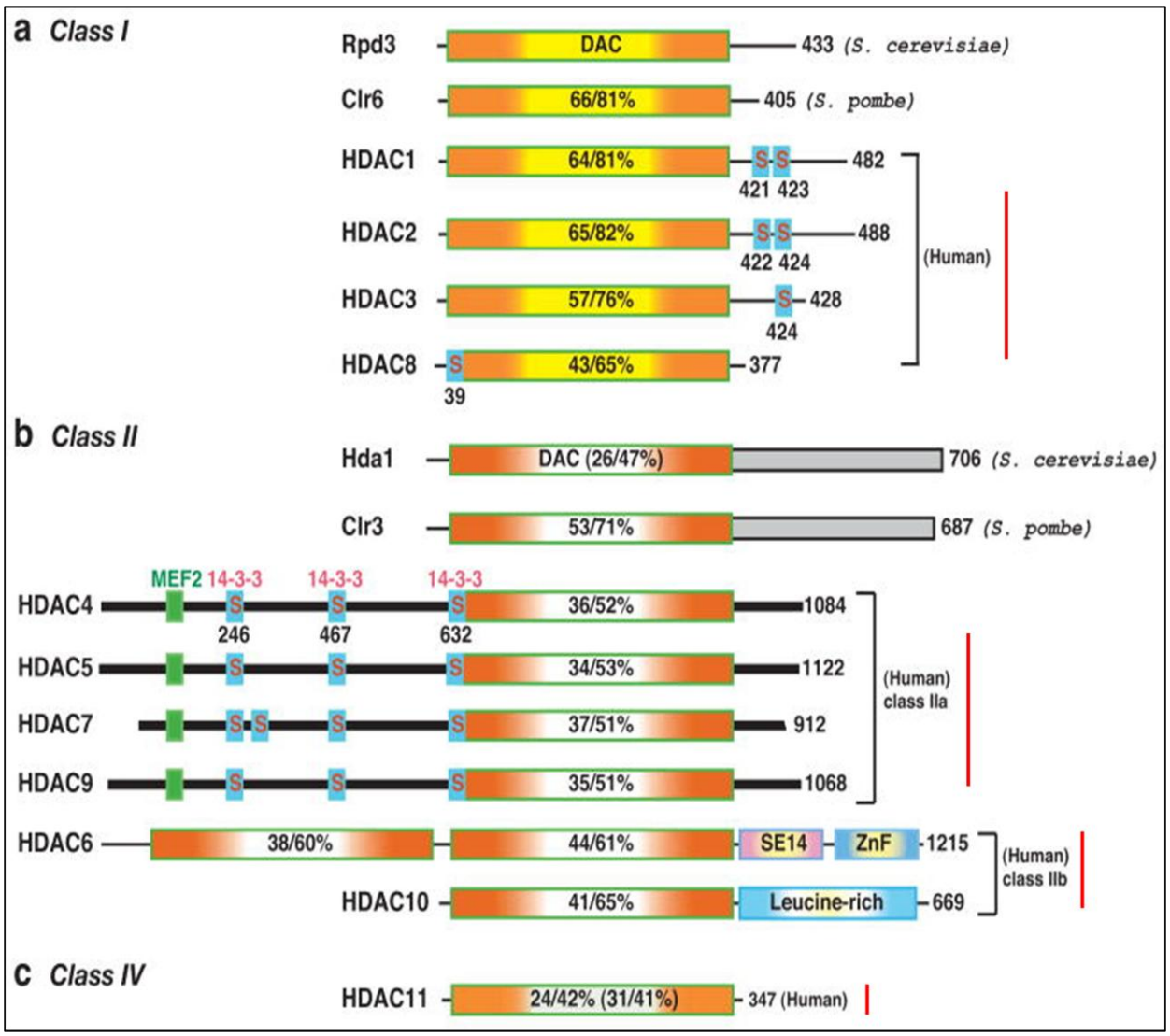

Figure 11. Schéma représentant la classification des HDACs humaines classiques et leur homologie avec les HDACs de levure (Yang and Seto, 2008).

1.2. Classification et caractérisation des Sirtuines

Les HDACs de classe III NAD + dépendantes, nommées aussi sirtuines, comprennent un groupe de 7 protéines (SIRT1-7) homologues de la famille de protéines de levure SIR2. SIRT1 est la plus similaire à SIR2, possède l'activité l'histone désacétylase la plus robuste, et a fait l'objet des études les plus approfondies. Comme les HDAC de classe I, II et IV, les sirtuines ont aussi des substrats non histones. Cependant, elles présentent plusieurs caractéristiques particulières par rapport aux autres HDACs classiques. En effet, elles ont deux activités enzymatiques différentes : la lysine desacétylase et la mono-ADP-ribosyltransférase. Une autre caractéristique intéressante des sirtuines est leur localisation. SIRT1 et SIRT2 se trouvent dans le noyau et le cytoplasme, SIRT3 est située dans le noyau et les mitochondries, SIRT4 et SIRT5 sont exclusivement situées dans les mitochondries, SIRT6 ne se trouve que dans le noyau, et SIRT7 dans le nucléole (**Figure 12**) (Seto and Yoshida, 2014).

Les sirtuines sont omniprésentes dans la cellule et jouent un rôle essentiel dans le maintien de l'intégrité génomique. Elles ciblent des marques d'histones différentes, surtout les marques activatrices H3K9ac et H4K16ac, mais ciblent également des facteurs de transcription, des protéines structurelles, des récepteurs nucléaires, des HATs, ainsi que des composants non histones de la chromatine (Martínez-Redondo and Vaquero, 2013) (Bosch-Presegué and Vaquero, 2015). Par conséquent, elles interviennent dans la régulation d'un large éventail de processus cellulaires, y compris l'apoptose, la prolifération et la différenciation cellulaire, la régulation de l'expression des gènes, le remodelage de la chromatine, la réparation de l'ADN, la régulation du métabolisme, la réponse au stress, et autres (Dali-Youcef et al., 2007) (Verdin et al., 2010) (Haigis and Sinclair, 2010) (Houtkooper et al., 2012) (Mei et al., 2016). En raison du grand nombre de fonctions biologiques dans lesquelles les sirtuines sont impliquées via leur activité desacétylase, elles ont été associées à plusieurs maladies, notamment le diabète (Martinez-Pastor and Mostoslavsky, 2012) (Turkmen et al., 2014), les maladies neurologiques et cardiovasculaires (Donmez, 2012) (Hall et al., 2013) (Matsushima and Sadoshima, 2015), et surtout le cancer (Voelter-Mahlknecht and Mahlknecht, 2010) (McGuinness et al., 2011) (Yuan et al., 2013) (Roth and Chen, 2014) (O'Callaghan and Vassilopoulos, 2017), mais la nature de leurs fonctions dans le cancer reste très controversée (Bosch-Presegué and Vaquero, 2011) (Chalkiadaki and Guarente, 2015), en particulier les rôles fonctionnelles de SIRT1 (Liu

et al., 2009) (Fang and Nicholl, 2011) (Stünkel and Campbell, 2011) (Song and Surh, 2012). Les implications controversées de SIRT1, et les autres sirtuines, dans le développement et la progression du cancer seront exposées dans les parties suivantes.

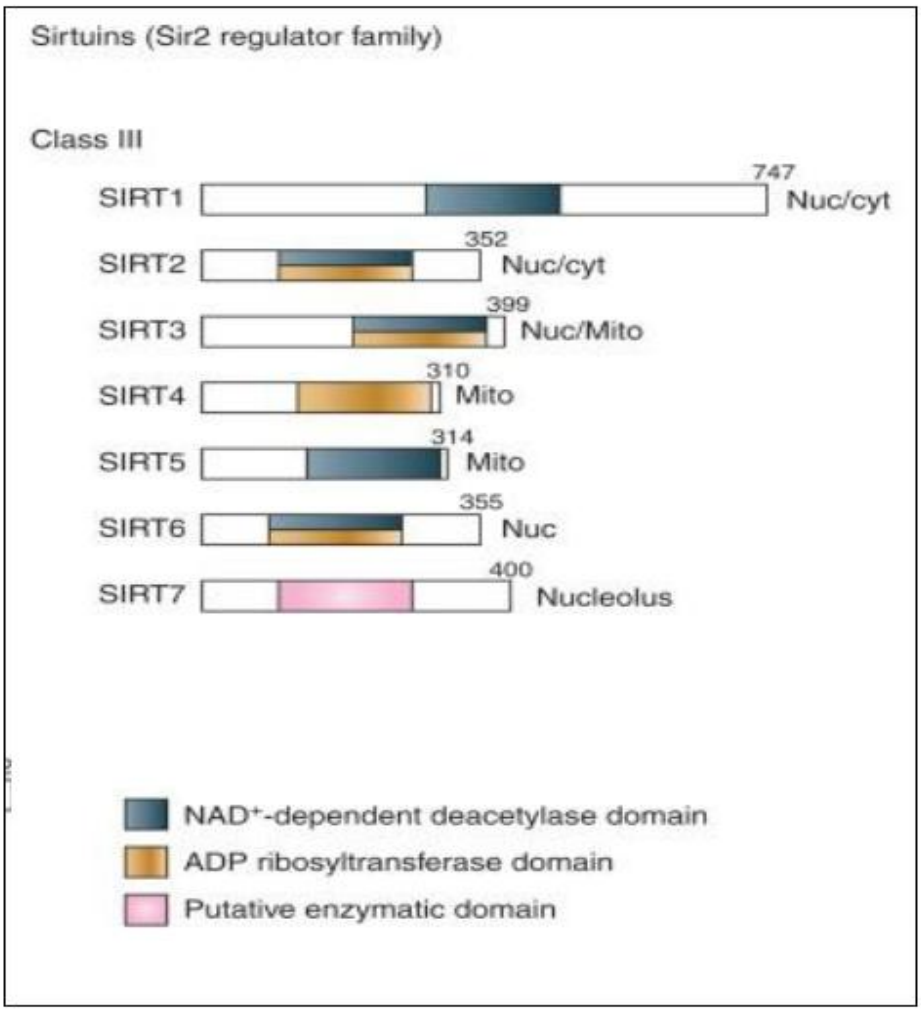

Figure 12. Schéma représentant les domaines d'activité enzymatique des 7 sirtuines et leur localisation dans la cellule. Nuc : noyau, cyt : cytoplasme, Mito : mitochondrie, Nucleolus : nucléole, (Seto and Yoshida, 2014).

2. HDACs et Cancer

Les histones désacétylases (HDACs) sont parmi les acteurs essentiels de la régulation épigénétique de l'expression des gènes et l'activité des protéines dans la cellule, elles sont impliquées dans la régulation d'une multitude des processus biologiques clés. À ce titre, les HDACs ont été associées au cancer, et plus particulièrement en raison de leur répression de l'expression des oncogènes ou des gènes suppresseurs de tumeurs, ainsi pour leur activation ou répression de l'activité des oncoprotéines impliquées dans la carcinogenèse. L'activité désacétylase aberrante des HDACs, ainsi que leurs expressions altérées, ont été mises en évidence dans la majorité des cancers humains (Weichert, 2009) (Wang et al., 2017), y compris le cancer du sein (Suzuki et al., 2009) (Müller et al., 2013) (Derr et al., 2014) (Cao et al., 2015). Elles ont ainsi été étroitement liées à l'initiation et à la progression du cancer (Marks et al., 2001) (Glozak and Seto, 2007) (Haberland et al., 2009b) (Barneda-Zahonero and Parra, 2012).

En fait, une expression et une fonction alternée des HDACs perturbent l'homéostasie d'acétylation finement réglée dans les protéines cibles histones et non-histones. Cela entraîne des altérations de l'expression des gènes impliqués dans la régulation de la prolifération, de la différenciation, de l'apoptose et d'autres processus cellulaires caractéristiques du cancer (**Figure 13**), (Haberland et al., 2009a) (Hagelkruys et al., 2011) (Parbin et al., 2014).

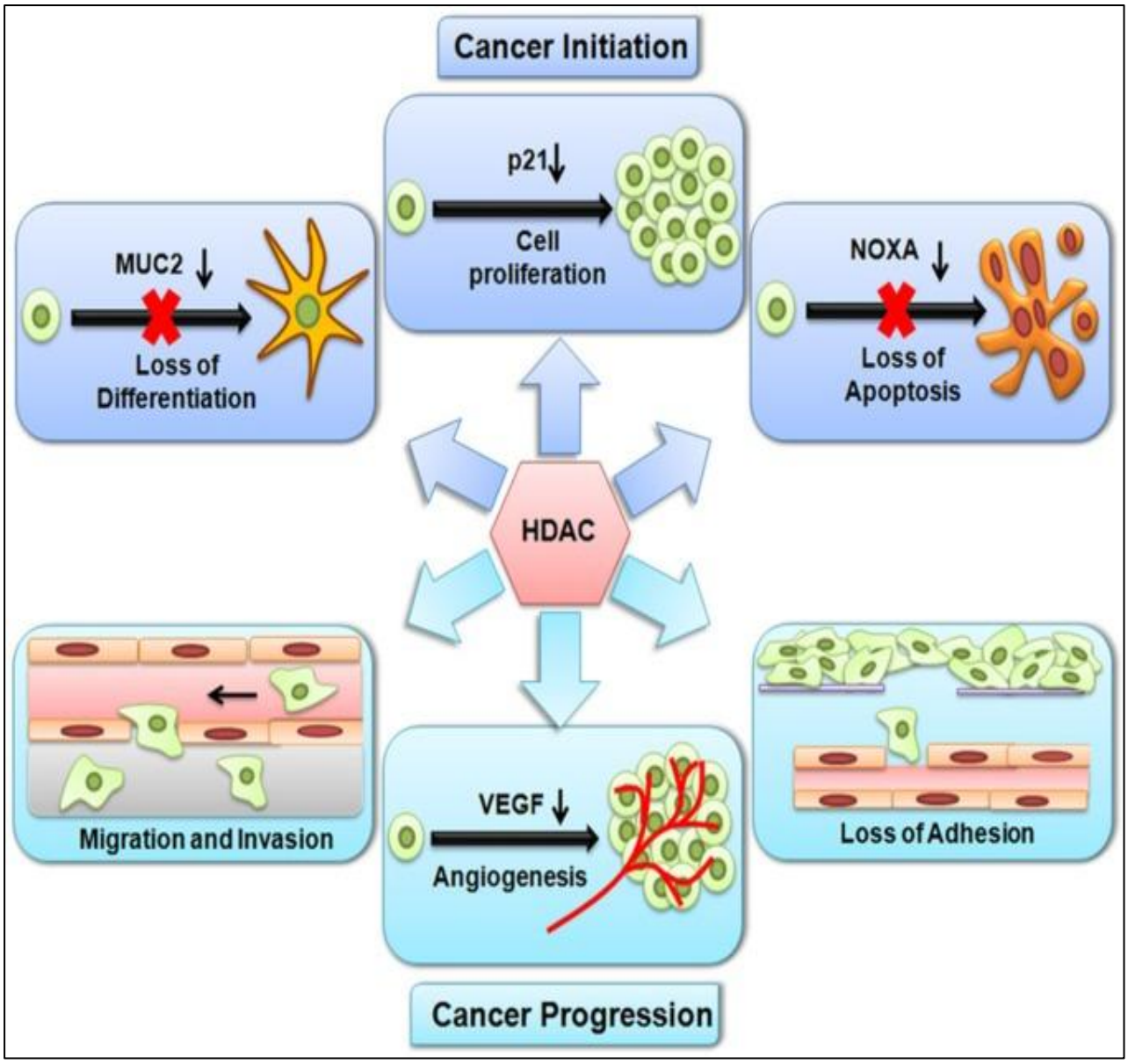

Figure 13. Schéma représentant les implications des HDACs dans l'initiation et la progression du cancer. La modulation de l'expression des gènes par les HDACs stimule la prolifération cellulaire et provoque l'inhibition de l'apoptose et la différenciation cellulaire. Ces 3 caractéristiques cellulaires sont typiques dans la phase d'initiation du cancer. Les HDACs suscitent aussi la progression du cancer en stimulant les processus de l'angiogenèse, la perte d'adhésion et la migration cellulaire (Parbin et al., 2014).

2.1. Les Sirtuines, SIRT1, et Cancer

Toutes les sirtuines, à l'exception de SIRT5, se sont avérées impliquées dans la tumorigenèse, et ont fait l'objet d'un examen minutieux en raison de leurs rôles contradictoires dans le cancer. En effet le rôle des sirtuines dans le cancer sont complexes, et peuvent contribuer à la promotion ou à la suppression des tumeurs en fonction du contexte cellulaire et moléculaire.

Le rôle de SIRT1 est très controversé dans le cancer. SIRT1 peut fonctionner à la fois comme un suppresseur de tumeur ou comme un promoteur de tumeur. Ainsi, ces 2 fonctions opposantes ont été observées dans le même cancer, comme c'est le cas du cancer du sein. Cette dualité de fonctions dépend de plusieurs facteurs tels que son taux d'expression, sa localisation cellulaire, la localisation de ses régulateurs et de ses substrats, le contexte cellulaire et tissulaire, le type et le stade du cancer.

D'une part, l'expression de SIRT1 est réduite dans certains cancers. Une surexpression ectopique de SIRT1 dans ces cancers favorise la stabilité génomique, limite le développement et le métabolisme du cancer, et inhibe la signalisation proinflammatoire amplifiée de manière aberrante pendant la promotion et la progression de la carcinogenèse. SIRT1 peut ainsi empêcher l'apparition d'un cancer ou le ralentissement d'un cancer à un stade précoce. D'autre part, une surexpression de SIRT1 est détectée dans plusieurs types de tumeurs. Cette forte expression de SIRT1 confère des avantages de survie aux cellules cancéreuses en inhibant les suppresseurs de tumeurs comme p53, et en activant des oncogènes comme les Myc oncoprotéines. Ainsi, SIRT1 bloque l'apoptose, la sénescence et la différenciation cellulaire, en favorisant la survie, la croissance cellulaire, et l'angiogenèse (Stünkel and Campbell, 2011) (Song and Surh, 2012) (Yuan et al., 2013) (Chalkiadaki and Guarente, 2015). Une partie des fonctions connues de SIRT1 dans les processus de la carcinogenèse humaine, est résumée dans la (**Figure 14**).

Par conséquent, il existe jusqu'à présent beaucoup de contradictions dans la littérature concernant l'expression et le rôle de SIRT1 dans les cancers humains, y compris le cancer du sein. L'implication et les rôles opposés de SIRT1 dans le cancer du sein ont fait l'objet d'une revue présentée dans la partie suivante.

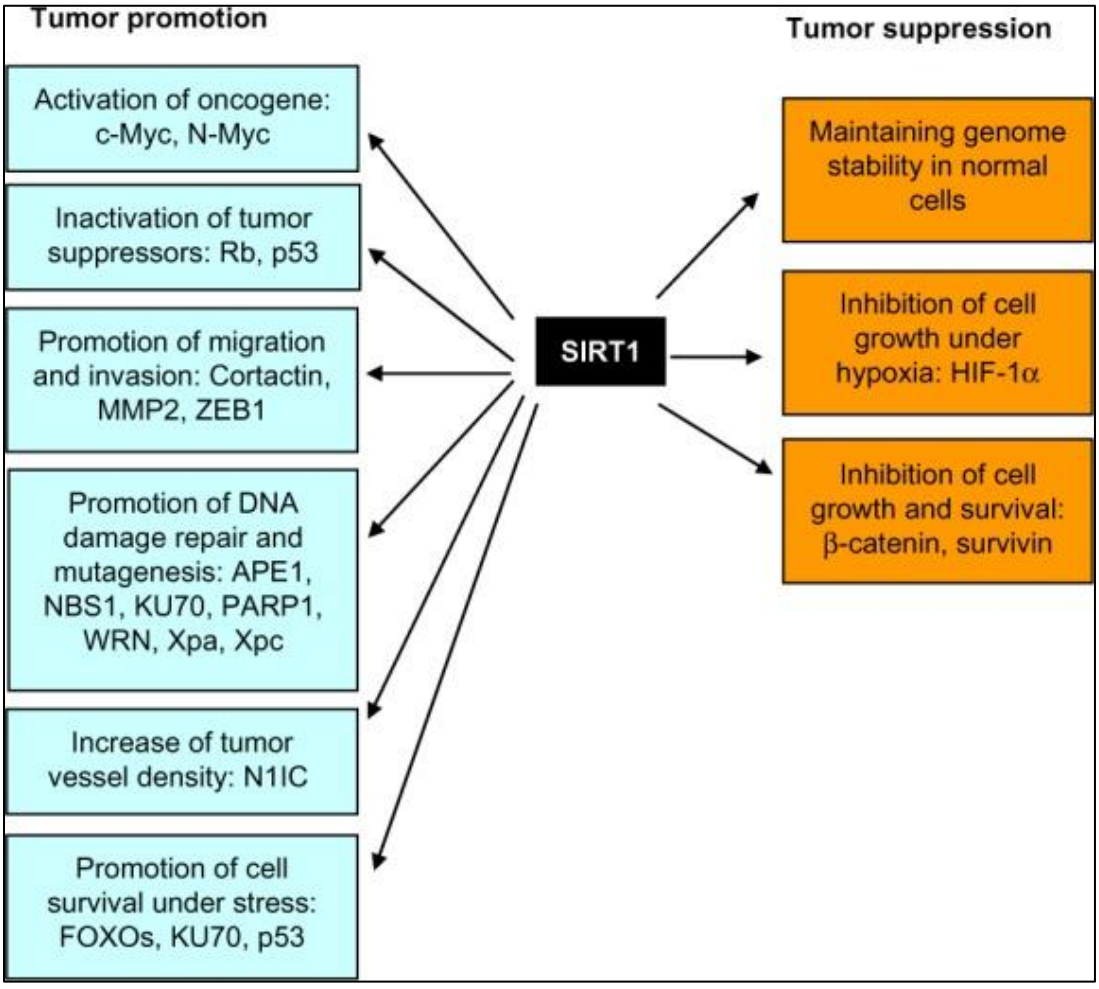

Figure 14. Schéma représentant les fonctions opposantes du SIRT1 dans la promotion versus la suppression des tumeurs. SIRT1 peut jouer le rôle d'un promoteur de tumeur en activant des oncogènes, désactivant des GSTs, et promettant la migration et la survie des cellules cancéreuses. SIRT1 peut agir également en tant que suppresseur de tumeur en maintenant la stabilité génomique, et en inhibant la croissance et la survie cellulaire, (Yuan et al., 2013).

Bien que la majorité des études portant sur les sirtuines soit concentrée sur l'implication de SIRT1 dans le cancer, le rôle des autres sirtuines a aussi été caractérisé.

SIRT2 peut fonctionner en tant que suppresseur de tumeur en maintenant l'intégrité mitotique dans la cellule (Hiratsuka et al., 2003). Il a été démontré que l'expression de SIRT2 est réduite dans les cancers des carcinomes cervicaux (Lai et al., 2013), des gliomes et les carcinomes gastriques (Inoue et al., 2007), et dans les mélanomes, SIRT2 possède une mutation dans le domaine catalytique qui élimine son activité enzymatique (Lennerz et al., 2005). Ainsi, il a été montré que les souris SIRT2 -/- sont plus susceptibles de développer des tumeurs (Serrano et al., 2013). Cependant, d'autres études suggèrent que SIRT2 pourrait également avoir des caractéristiques de promoteur de tumeur. Il a été montré que SIRT2 est surexprimée dans les leucémies aiguës myéloïdes (Dan et al., 2012), les cellules de neuroblastome et les cellules cancéreuses du pancréas (Liu et al., 2013), et les carcinomes hépatocellulaires (Chen et al., 2013a). Elle est aussi régulée positivement par c-MYC dans les cellules cancéreuses du pancréas, et par N-MYC dans les cellules de neuroblastome. SIRT2 stabilise les protéines N-MYC et c-MYC par une régulation négative de l'expression de NEDD4, une ligase d'ubiquitination (Liu et al., 2013).

SIRT3 a été proposée en tant que suppresseur de tumeur en raison de son rôle dans la réduction des complexes ROS (reactive oxygen species) produits par la mitochondrie. SIRT3 a été décrite comme la principale désacétylase mitochondriale, alors que l'acétylation des enzymes mitochondriales est en grande partie inhibitrice, l'activité déacétylase de SIRT3 était donc liée à la préservation de la fonction mitochondriale (Finley and Haigis, 2012) (Weinert et al., 2015). De plus, SIRT3 inhibe la tumorigenèse en désacétylant et en inactivant l'oncogène SKP2, une sous-unité de l'ubiquitine kinase E3 importante dans la phase S du cycle cellulaire, (Inuzuka et al., 2012).

Le rôle de SIRT4 n'a pas encore été élucidé, mais des études ont montré qu'elle pouvait jouer un rôle suppresseur de tumeur. SIRT4 est sous-exprimée dans les cancers de l'estomac, de l'ovaire, de la vessie et du sein, par rapport aux tissus normaux. Toutefois, une surexpression de SIRT4 réduit la transformation et la prolifération cellulaire (Csibi et al., 2013). En outre, les souris knock-out SIRT4 sont plus susceptibles de développer des tumeurs spontanées du poumon que les souris de type sauvage (Jeong et al., 2013).

On en sait moins sur le rôle des SIRT6 et 7 dans le développement ou la progression du cancer. Certaines études ont montré que SIRT6 peut avoir une fonction de suppresseur de tumeur en réduisant l'activité transcriptionnelle des oncogènes Myc et HIF1α (Sebastián et al., 2012). SIRT7 peut être un promoteur de tumeur en activant les voies oncogènes des cellules cancéreuses, telle que la croissance indépendante de l'ancrage, par contre, l'absence de SIRT 7 réduit le potentiel tumoral des cellules cancéreuses (Kim et al., 2013).

En conclusion, les sirtuines jouent des rôles bivalents dans l'initiation et la progression du cancer en régulant de nombreuses voies et cibles différentes. Des études plus approfondies seront nécessaires pour définir leurs rôles fonctionnels dans la carcinogenèse.

2.2. Les Implications Controversées de SIRT1 dans le Cancer Du Sein

Présentation de la publication 3

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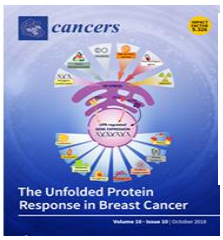
Review

Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer

Rifaï K, Idrissou M, Penault-Llorca F, Bignon YJ, Bernard-Gallon D.



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Les actions contradictoires de SIRT1 dans le processus de cancérogenèse humaine, et son implication dans la biologie du cancer restent toujours une question ouverte. Malgré les études de la dernière décennie, le rôle de SIRT1 dans le cancer du sein reste controversé. Les études ont montré que SIRT1 peut exercer à la fois des fonctions de promotion ou de suppression de la tumeur mammaire. En effet, SIRT1 fonctionne comme un promoteur de tumeur en régulant négativement l'activité des gènes suppresseurs de tumeur comme p53, E2F1 et les facteurs de transcription FOXO, et en activant des oncogènes comme c-Myc and N-Myc. En revanche, elle peut agir également comme un suppresseur de tumeur en supprimant l'activité des voies de signalisation oncogènes Wnt/β-catenin et NF-kappaB et en optimisant l'activité des enzymes de réparation des dommages à l'ADN, telles que Ku70, APE1, et WRN.

Ainsi, le taux d'expression de SIRT1 est altéré dans la majorité des cancers. Par exemple, SIRT1 est surexprimée dans les cancers de la peau, du foie, de l'estomac et de prostate, et sous-exprimée dans les cancers du colôn. La variation d'expression de SIRT1 selon le type de cancer suggère son activité bivalente dans la carcinogenèse humaine.

Dans cette revue, on donne un aperçu général sur les implications de SIRT1 dans la tumorigenèse du sein et on explore également les mécanismes sous-jacents qui contribuent aux fonctions opposées de SIRT1 dans la cancérogenèse du sein.

cancers

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Review

Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer

Khaldoun Rifaï [1,2], Mouhamed Idrissou [1,2], Frédérique Penault-Llorca [2,3], Yves-Jean Bignon [1,2] and Dominique Bernard-Gallon [1,2,*]

1 Department of Oncogenetics, Centre Jean Perrin, CBRV, 28 place Henri-Dunant, 63001 Clermont-Ferrand, France; k.rifaii@live.com (K.R.); mouhamed.idrissou@etu.uca.fr (M.I.); Yves-Jean.BIGNON@clermont.unicancer.fr (Y.-J.B.)
2 INSERM—UMR 1240—Imagerie Moléculaire et Stratégies Théranostiques (IMoST), 58 Rue Montalembert, 63005 Clermont-Ferrand, France; Frederique.PENAULT-LLORCA@clermont.unicancer.fr
3 Department of Biopathology, Centre Jean Perrin, 58 Rue Montalembert, 63011 Clermont-Ferrand, France
* Correspondence: dominique.gallon-bernard@clermont.unicancer.fr; Tel.: +33-(0)4-73-17-83-58

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Abstract: Breast cancer (BC) is the most common type of cancer in women worldwide; it is a multifactorial genetic disease. Acetylation and deacetylation are major post-translational protein modifications that regulate gene expression and the activity of a myriad of oncoproteins. Aberrant deacetylase activity can promote or suppress tumorigenesis and cancer metastasis in different types of human cancers, including breast cancer. Sirtuin-1 (SIRT1) is a class-III histone deacetylase (HDAC) that deacetylates both histone and non-histone targets. The often-described 'regulator of regulators' is deeply implicated in apoptosis, gene regulation, genome maintenance, DNA repair, aging, and cancer development. However, despite the accumulated studies over the past decade, the role of SIRT1 in human breast cancer remains a subject of debate and controversy. The ambiguity surrounding the implications of SIRT1 in breast tumorigenesis stems from the discrepancy between studies, which have shown both tumor-suppressive and promoting functions of SIRT1. Furthermore, studies have shown that SIRT1 deficiency promotes or suppresses tumors in breast cancer, making it an attractive therapeutic target in cancer treatment. This review provides a comprehensive examination of the various implications of SIRT1 in breast cancer development and metastasis. We will also discuss the mechanisms underlying the conflicting roles of SIRT1, as well as its selective modulators, in breast carcinogenesis.

Keywords: breast cancer; SIRT1; deacetylation; epigenetic silencing; tumor promoter; tumor suppressor; SIRT1 modulators

1. Introduction

Breast cancer is a genetically heterogeneous disease that remains the most commonly diagnosed malignancy amongst women worldwide. It is also the second leading cause of cancer death among females in developed countries after lung cancer [1]. Epigenetic alterations of proteins, histones, and chromatin play a fundamental role in gene expression regulation and ultimately, cancer formation. Reversible protein acetylation and deacetylation are amongst those alterations [2]. Histone deacetylases (HDACs) are major actors in gene expression regulation. By removal of acetyl groups from N-terminus tails of histones, HDACs repress the expression of genes implicated in the carcinogenesis process, such as oncogenes and tumor suppressor genes (TSGs). In addition to histones, HDACs regulate the expression and activity of a myriad of proteins involved in both cancer initiation and progression.

Furthermore, aberrant expression of HDACs in various human cancers, and consequently their dysfunctional deacetylase activity, is deeply involved in the carcinogenesis process [3].

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD+)-dependent class-III HDACs or lysine deacetylases (KDACs) that belong to the silent information regulator 2 (SIR2) family. The seven mammalian sirtuins (SIRT1–7) are key regulators in major biological processes, including cell death and survival, regulation of genomic stability, cellular senescence, metabolic regulation and inflammation [4,5]. Therefore, sirtuins have gained tremendous attention in the past decade in cancer research and numerous studies have demonstrated their direct implication in the carcinogenesis process of multiple human cancers [6]. Silent mating type information regulation 2 homolog 1 (Sirtuin-1) is the founding member of the sirtuin family and the most extensively studied. SIRT1 is expressed ubiquitously and is mainly found in the nucleus, but can shuttle between the nucleus and cytoplasm using its two nuclear localization signals and two nuclear export signals [7]. Due to its deacetylase activity, SIRT1 regulates a wide variety of fundamental cellular processes including apoptosis, DNA damage response and repair, cell differentiation and proliferation, chromatin remodeling and gene expression, endocrine signaling, aging, metabolism, stress response, and cancer development and metastasis [4,5,8–10]. Similar to most HDACs, aberrant SIRT1 expression is identified in numerous human malignancies and is directly linked to the tumorigenesis process and metastasis.

The implications of SIRT1 in breast cancer occurrence and development have been reported and largely studied over recent years, but its exact role in breast cancer remains very controversial and paradoxical so far. In fact, bifurcated SIRT1 can act as either a tumor suppressor or promoter in cancer cells. This highly context-specific role of SIRT1 in breast carcinoma seems to depend mainly on its upstream regulators or downstream substrates, as well as on its spatial distribution, cellular and molecular context, and tumor types. In this review, we summarize available data and give a general overview of the multiple implications of SIRT1 in breast tumorigenesis. We also explore the mechanisms underlying SIRT1 opposite functions in breast carcinogenesis.

2. The Multifaceted Functions of HDAC SIRT1 in Cancer Biology

Other than histone deacetylation, the functional roles of SIRT1 are fulfilled by directly interacting with and deacetylating a wide range of downstream non-histone substrates, resulting in activation or repression of their catalytic activity. SIRT1 deacetylase activity regulates:

1. Tumor suppressors, including p53 [11], p73 [12], Forkhead transcription factors (FoXO) [13], E2F1 [14], and Rb (Retinoblastoma) [15].
2. Tumor promoters, including c-Myc [16], N-Myc [17], cortactin (CTTN) [18], NF-κB [19], β-catenin [20], and HIF-1α [21].
3. Chromatin-related enzymes, including p300 [22], hMOF and TIP60 [23], PCAF [24], HDAC1 [25], DNMT1 [26], SUV39H1 [27], and EZH2 (enhancer of Zest 2) [28].
4. Nuclear receptors and related factors, including estrogen receptor-alpha (ER-α) [29], androgen receptor (AR) [30], liver X receptor (LXR) [31], PPARγ [32], and PPARγ coactivator 1α (PGC-1α) [33].
5. DNA damage repair enzymes, such as Ku70 [34], XPC [35], XPA [36], APE1 [37], and WRN [38].

As a result of its diverse biological functions, multifaceted SIRT1 is critically implicated in the occurrence and progression of numerous human malignancies. Yet researchers have long been baffled by SIRT1 contradictory actions in the carcinogenesis process, and its involvement in cancer biology remains an open question.

3. SIRT1-Dependent Epigenetic Silencing via Histone Modification in Breast Carcinogenesis

SIRT1 lysine deacetylase activity regulates chromatin structure and transcription through epigenetic mechanisms [9]. Lysine acetylation of histones H3 and H4 is classically associated with transcriptional activation and increased gene expression. On the contrary, their deacetylation is

generally associated with inactive chromatin and repression of gene expression [39]. In carcinogenesis, histone deacetylation leads to epigenetic silencing of various cancer-related genes; thus, HDACs could exert either tumor-promoting or tumor-suppressive roles depending on whether the repression happens in the genomic region of a tumor suppressor or a tumor promoter respectively. HDAC SIRT1 embodies these properties, and orchestrates the regulation of multiple cancer-related genes through histone deacetylation. Indeed, SIRT1 contributes to the epigenetic silencing by deacetylating H3 and H4 acetylated markers such as histones H3 lysine 4 (H3K4) [40], lysine 56 (H3K56) [41], lysine 9 (H3K9), lysine 14 (H3K14), and histone H4 lysine 16 (H4K16) [42,43]. In breast cancer, SIRT1-dependent epigenetic silencing of both oncogenes and TSGs is reported.

Pruitt et al. demonstrated that SIRT1 deficiency re-activates aberrantly silenced TSGs by increasing the acetylation of H3K9 and H4K16 epigenetic markers at their promoters in two breast cancer (BC) cell lines, indicating SIRT1-mediated epigenetic repression of TSGs through histone modifications in BC [44]. In contrast, Wang et al. revealed a tumor suppressor role of SIRT1 in BC. They reported that SIRT1 inhibits tumor growth in vivo by suppressing the expression of survivin, a member of the inhibitor of apoptosis (IAP) family that drives cell proliferation and viability [45]. SIRT1-mediated epigenetic silencing of survivin occurs through deacetylating the H3K9 marker on the survivin promoter, consequently suppressing its transcription in mammary tumors [46]. To address the confusion regarding SIRT1-dependent epigenetic regulation in BC pathogenesis, we characterized in recent studies an aspect of SIRT1 epigenetic behavior in human breast carcinoma. We showed that the opposite functions of SIRT1 in breast cancer are closely related to the molecular subtype. By modulating the acetylation status of key H3 and H4 epigenetic markers in a subtype-specific fashion, SIRT1 is more likely to exert an oncogenic role in luminal molecular subtypes and a tumor suppressor role in the triple-negative subtype (TNBC), also known as basal-like, both in vitro and ex vivo. Furthermore, we revealed that SIRT1 deficiency is associated with substantial induction of acetylated markers on six breast cancer-related gene promoters: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2*, and *EP300*, suggesting an active role of SIRT1 in regulating the expression of these genes in BC. We concluded that SIRT1 differential epigenetic regulation in breast cancer is predominantly governed by gene type and molecular subtype [40,47]. Other than BC, the duality of SIRT1 epigenetic regulation was also highlighted in colorectal cancer [48].

In fact, SIRT1-mediated epigenetic regulation extends to histone acetyltransferases (HATs), and other histone modifiers involved in transcription repression. SIRT1 fine-tuning of gene expression regulation is partly manifested through the repression of acetyltransferase activity of major HATs. Remarkably, these HATs acetylate the same histone targets as SIRT1. For instance, SIRT1 downregulates and blocks the activity of p300 of the p300/CBP family [22], hMOF and TIP60 of the MYST family [23], and PCAF of the GNAT family [24]. It stabilizes and stimulates the activity of HDACs, e.g., HDAC1 [25], DNA methyltransferases, e.g., DNMT1 deacetylated at Lys1349 and Lys1415 [26], and histone methyltransferases (HMTs), e.g., SUV39H1, deacetylated at Lys266 [27]. Furthermore, SIRT1 interacts and has a close functional relationship with EZH2, an essential HMT that constitutes the core catalytic subunit of polycomb repressive complex 2 (PRC2). SIRT1 and EZH2 form part of the PRC4 complex, along with other polycomb group proteins which are found overexpressed in breast cancer tumors. The two enzymes recruit SUV39H1 and DNMT1 to promote transcriptional repression at targeted genes [27,28,49,50]. Collectively, the data indicated that SIRT1 epigenetic regulation of gene expression is implemented by the means of regulation both, histone markers and their epigenetic 'writers' and 'erasers'.

4. SIRT1 Assuming the Role of Tumor Promoter in Breast Carcinogenesis

The 'guardian of the genome' p53, a vital TSG that is frequently mutated in human tumors, was one of the first identified non-histone substrate of SIRT1 and the first evidence of SIRT1 implication in tumorigenesis. SIRT1 deacetylation of p53 at its Lys382 residue (p53K382) results in repression of p53-dependent apoptosis in response to DNA damage and promotes cell survival [11]. Upon DNA

damage stimuli, SIRT1-mediated deacetylation of p53 is optimized by breast cancer metastasis suppressor 1 (BRMS1) [51]. BRMS1 potentiates SIRT1 activity through physically interacting with deleted in breast cancer 1 (DBC1), a negative regulator of SIRT1 [52]. SIRT1 also deacetylates and represses the activity of other damage-response enzymes, the mammalian forkhead transcription factors FoXO3a and FoXO4, inhibiting forkhead-dependent cell death [13,53]. In addition, SIRT1 binds to and inhibits the activity of E2F1, a tumor suppressor and apoptosis regulator, impairing its apoptotic functions [14]. SIRT1 also downregulates the activity of the tumor-suppressing retinoblastoma protein (Rb). Deacetylation of Rb by SIRT1 formed a domain similar to the SIRT1-targeted domain of p53, resulting in inhibiting Rb-dependent apoptosis [15]. Furthermore, SIRT1 overexpression in tumors is associated with upregulation of various oncoproteins. For example, SIRT1-dependent deacetylation of prototypic Myc oncogenes, c-Myc and N-Myc, enhances their stability and transcriptional activity, resulting in cancer cell survival and proliferation [16,17], respectively.

On the premise that SIRT1 is upregulated in various human cancers, SIRT1 could act as a tumor promoter. Since an abundance of SIRT1 expression is observed in breast tumors, many studies assert an oncogenic role of SIRT1 in breast carcinogenesis, and clinical studies demonstrated SIRT1 as a prognostic factor that significantly correlates with unfavorable clinicopathological factors. Actually, SIRT1 overexpression in breast tumors and mammary BC cell lines is significantly associated with lymph node metastasis, advanced TNM stage, low grade as per the modified Bloom–Richardson system, lymphovascular invasion, shorter disease-free survival (DFS), and overall survival (OS), luminal subtype, ER and PR expression, and is marginally associated with p53 loss [54–56]. Hence, SIRT1 upregulation is strongly correlated with breast tumorigenesis.

Xu et al. reported an upregulation of SIRT1 in breast tumors and (ER+) luminal BC cell line MCF-7. The authors revealed that SIRT1 upregulation promotes the proliferation, migration, and invasion of MCF-7 cells, whereas SIRT1 knockdown inhibits those effects. They showed that SIRT1 overexpression positively correlates with decreased expression of p53 and increased expression of DNA polymerase delta1 (*POLD1*) gene, an oncogene involved in genomic instability and cell proliferation; whilst the result of SIRT1 silencing is opposite. They concluded that SIRT1 is involved in breast carcinogenesis by inhibiting p53 and activating *POLD1* [57]. This was in line with a study by Jin et al. who revealed that SIRT1 upregulation significantly promotes breast cancer growth both in vitro and in vivo, whereas SIRT1 deficiency inhibits cancer cell proliferation. The authors showed that SIRT1 has effects on breast cancer cell growth through promoting the activity of oncogenic PI3K/Akt signaling pathway in vitro, and that SIRT1 is positively correlated with the expression of P-Akt in vivo [56]. SIRT1 is also involved in breast cancer progression and metastasis. Ota et al. demonstrated that SIRT1 inhibition by Sirtinol, a selective SIRT1 inhibitor, induces a senescence-like growth arrest in luminal cell line. The cellular senescence induced by SIRT1 inhibition co-occurs with impaired activation of oncogenic Ras–MAP kinase signaling pathways, implicated in cell growth and proliferation. These findings suggest an active role of SIRT1 in driving cell proliferation through Ras-MAP kinase signaling pathways [58]. Meanwhile, Zhang et al. found that SIRT1 and Cortactin; an oncogene associated with breast cancer metastasis), are more abundant in breast tumors than in their normal adjacent tissues. They showed that SIRT1-mediated deacetylation of cortactin promotes cell migration and breast tumorigenesis [18].

Meanwhile, SIRT1 oncogenic activity in BC is downregulated by different subclasses of miRNAs [59]. MiRNAs are small non-coding microRNAs that regulate the expression of many cancer-related genes. A recent study by Zou et al. reported that SIRT1 is negatively regulated by miR-22, a subclass of miRNAs, in the ER+ MCF-7 cell line. The authors showed that an ectopic expression of miR-22 reduces the proliferation, migration and invasion of MCF-7 cells, whereas SIRT1 overexpression eliminates the suppressive effects of miR-22. They concluded that miR-22 inhibitory effects are partly fulfilled by downregulating SIRT1 expression in vitro [60]. A similar study by Zhang et al. confirmed SIRT1 as a direct target of miR-22 in both (ER+) and (ER−) cell lines. The authors showed that SIRT1 knockdown induces apoptosis, inhibits tumorigenesis, and enhances radiosensitivity of breast cancer cells. In addition, miR-22 overexpression suppresses tumorigenesis

and improves radiosensitivity of breast cancer cells by targeting SIRT1 in vitro. They concluded on the same note as Zou et al. [61].

SIRT1 is a confirmed target of another subclass of miRNAs. MiR-34a represses SIRT1 expression through a miR-34a-binding site within the 3′UTR of SIRT1. MiR-34a-mediated inhibition of SIRT1 leads to an increase of acetylated p53 and consequently, increased expression of pro-apoptotic genes *p21* and *PUMA* in colon cancer cells [62]. In breast cancer, an ectopic expression of miR-34a inhibits the growth of breast cancer cells by inducing apoptosis and suppressing cell migration in both ER+ and ER− cell lines. It was revealed that miR-34a tumor-suppressive role is partly implemented by the means of suppressing SIRT1 expression in vitro [63]. Another study showed that SIRT1 downregulation or miR-34a upregulation inhibits cell proliferation and colony formation ability in the MCF-7 cell line, as well as in CD44+/CD24− breast cancer stem cells (BCSCs). SIRT1 knockdown in BCSCs positively correlates with decreased expression of BCSCs markers: ALDH1, BMI1, and NANOG. In addition, a stable expression of miR-34a or silencing of SIRT1 reduces tumor growth in nude mice xenografts. SIRT1 downregulation also positively correlates with decreased ALDH1 in vivo. It is postulated then, that miR-34a upregulation suppresses the proliferative potential of BCSCs in vitro and in vivo by partially downregulating SIRT1 [64]. The diverse tumor-promoting properties of SIRT1 in breast cancer are resumed in Table 1.

Table 1. Mechanisms of action of SIRT1 tumor-promoting functions in breast carcinogenesis. SIRT1: sirtuin-1; TSG: tumor suppressor gene; POLD1: DNA polymerase delta1; BC: breast cancer; BCSC: breast cancer stem cell.

Mechanism of Action	References
SIRT1 represses TSG expression through epigenetic silencing	[44]
SIRT1 upregulation positively correlates with p53 downregulation and POLD1 upregulation	[57]
SIRT1 stimulates the activation of PI3K/Akt signaling pathway	[56]
SIRT1 downregulation co-occurred with impaired activation of Ras-MAPK signaling pathway	[58]
SIRT1-mediated deacetylation of cortactin promotes cell migration	[18]
SIRT1 upregulation eliminates the tumor-suppressive effects of miR-22	[60]
SIRT1 downregulation induces apoptosis and enhances radiosensitivity of BC cells	[61]
SIRT1 downregulation by miR-34a suppresses proliferation and migration of BC cells	[63]
SIRT1 downregulation in BCSCs positively correlates with decreased expression of BCSCs markers and reduces tumor growth in nude mice xenografts	[64]

5. SIRT1 Assuming the Role of Tumor Suppressors in Breast Carcinogenesis

Alternatively, there is much convincing evidence supporting a tumor suppressive role of SIRT1 in carcinogenesis, considering its implication in maintaining genome integrity via chromatin regulation and DNA damage response. Following DNA damage, SIRT1 regulates and optimizes DNA repair pathways, and is required for efficient single-strand and double-strand DNA breaks (DSB) repair [65]. SIRT1 stabilizes and upregulates the activity of DNA damage repair enzymes including Ku70 [34], XPC [35], XPA [36], APE1 [37] and WRN [38]. Aside from regulating genome stability, SIRT1 represses the expression of oncogenes through epigenetic silencing, and downregulates the activity of oncoproteins through direct deacetylation. For example, SIRT1 downregulates the transcriptional activity of the NF-kappaB-dependent cell survival pathway through physically interacting and deacetylating the RelA/p65 subunit of NF-kappaB at lysine 310 (NF-κB K310) [19]. SIRT1 also impairs the oncogenic activity of the Wnt/β-catenin signaling pathway. Aberrant activation of this pathway in various cancers promotes the transcription of many oncogenes through the transcriptional activity of β-catenin. SIRT1-mediated deacetylation of β-catenin suppresses its ability to activate transcription and drive cell proliferation [20].

In breast cancer, Wang et al. asserted a tumor suppressor role of SIRT1 through its implication in DNA damage response and genome integrity. The authors revealed that SIRT1 haploinsufficiency in SIRT1$^{+/-}$ p53$^{+/-}$ mice facilitates tumorigenesis, whereas SIRT1 activation by resveratrol, a bona fide activator of SIRT1 [66], reduces tumorigenesis in vivo. Moreover, by mutating the *SIRT1*

gene, they found that SIRT1-null mice embryos die during embryonic development and that SIRT1 deficiency causes genetic instability and impaired DNA damage repair. The authors also found an increased expression of anti-apoptotic oncoproteins Bcl-2 and survivin in SIRT1-null embryos [67]. To investigate this observation, Wang et al. conducted another study on human BRCA1-associated breast cancers. The authors noticed that lack of BRCA1 in BRCA1-mutant breast tumors is associated with reduced expression of SIRT1 and high levels of survivin, and showed BRCA1 to positively regulate SIRT1 expression in vitro. They also demonstrated that SIRT1 activation by Resveratrol blocks cell proliferation and antagonizes tumor growth through downregulating survivin expression in vivo [46]. Paradoxically, survivin is also repressed by wild-type p53 [45], the latter being a certified target of SIRT1 [11].

The interplay between SIRT1 and BRCA1 in BC is uncovered in another study. Zhang et al. revealed that BRCA1 induction suppresses AR-dependent tumor growth through SIRT1 activation in both (ER^+) and (ER^-) cell lines. They showed that resveratrol inhibits AR–stimulated proliferation by activating SIRT1 in vitro, and that SIRT1 overexpression in xenograft model BALB/c mice represses tumor growth in vivo. They concluded on the note that SIRT1 inhibits breast cancer development through diverse cellular processes [68], further establishing SIRT1 tumor-suppressive properties in breast cancer. In fact, the direct functional link of SIRT1 with AR was previously characterized by Fu et al. who revealed that SIRT1 binds to and downregulates AR activity in vitro. They showed that SIRT1-mediated repression of AR activity inhibits androgen-induced cell proliferation in prostate cancer [30]. A recent study by Yu et al. showed that an ectopic expression of SIRT1 in mesenchymal stem cells (MSCs) effectively suppresses breast tumor growth by inhibiting proliferation and inducing apoptosis in vivo. The authors found that SIRT1-induced antitumor activity in MSCs is achieved by increasing CXCL10 expression, a chemotactic factor necessary for the recruitment of the antitumor natural killer (NK) cells. They showed that breast tumor suppression is carried out through the actions of CXCL10-recruited NK cells [69].

In addition, SIRT1 reduces drug-resistance in breast cancer. A well-structured study by Shi et al. reported that SIRT1 deficiency induces chemo-resistance to paclitaxel (PTX), a chemotherapy drug used to treat BC, by disrupting the SIRT1-PRRX1-KLF4 axis which regulates chemo-resistance. The authors found that SIRT1 depletion destabilizes PRRX1 and leads to KLF4 upregulation, a core stemness factor that promotes carcinogenesis. KLF4 subsequently promotes transcription of ALDH1, which induces BCSCs, confers cellular resistance to chemotherapy, and promotes distant metastasis [70]. SIRT1 was also shown to reduce drug-resistance to tamoxifen (TAM), a widely used drug in the treatment of luminal BC. Li et al. revealed that SIRT1 silencing leads to TAM-resistance in luminal MCF-7 cell line (TAMR-MCF-7 cells), whereas SIRT1 restoration compromised brachyury-mediated TAM-resistance. The authors demonstrated that the overexpression of brachyury, a molecular mediator of resistance to tamoxifen, enhances TAM-resistance by increasing cell viability, reducing cell apoptosis, and downregulating SIRT1 expression in vitro. They concluded that brachyury mediates TAM-resistance by downregulating SIRT1 expression [71]. However, a study by Choi et al. postulated that SIRT1 overexpression contributes to TAM-resistance in MCF-7 cells by activating FoxO1 (Forkhead box-containing protein, O subfamily1), which in turn upregulates the expression of MRP2 (multidrug resistance protein 2) in TAMR-MCF-7 cells [72]. The diverse tumor-suppressive properties of SIRT1 in breast cancer are resumed in Table 2.

Table 2. Mechanisms of action of SIRT1 tumor-suppressive functions in breast carcinogenesis. AR: androgen receptor; MSC: mesenchymal stem cell; NK: natural killer.

Mechanism of Action	References
SIRT1 upregulation by resveratrol reduces breast tumorigenesis in vivo SIRT1 loss causes genetic instability and impaired DNA damage repair SIRT1 loss positively correlates with an increased expression of oncoproteins Bcl-2 and survivin	[67]
SIRT1 upregulation antagonizes tumor growth by downregulating survivin expression in vivo SIRT1 represses survivin expression through epigenetic silencing	[46]
SIRT1 upregulation inhibits AR–stimulated proliferation in vitro SIRT1 upregulation represses tumor growth in xenograft BALB/c mice	[68]
SIRT1 upregulation in MSCs suppresses tumor growth in vivo through CXCL10-recruited NK cells	[69]
SIRT1 downregulation causes chemo-resistance by impairing SIRT1-PRRX1-KLF4 axis	[70]
SIRT1 downregulation induces brachyury-mediated tamoxifen-resistance in the luminal cell line	[71]

6. The Functional Duality of SIRT1 in Breast Cancer

Conflicting studies concerning SIRT1 ambiguous involvement in breast cancer extend to many aspects of the disease.

6.1. SIRT1 Role in ER-α-Positive Luminal BC Molecular Subtypes

The oncogenic estrogen/ER-α-mediated signaling pathways stimulate cell proliferation and tumor growth in luminal hormone-dependent subtypes, through the activation of estrogen-responsive genes by ER-α transcriptional activity. Yu et al. revealed that SIRT1 binds to and inhibits the transcriptional activity of ER-α by regulating its acetylation status. They showed that SIRT1 represses the co-activator synergy between DBC1 and CCAR1, ER-α co-activators that enhance its transcriptional activity. They asserted SIRT1 as a major regulator of ER-α activity and co-activator synergy [29]. Meanwhile, Moore et al. reported that SIRT1 inhibits tumor cell reaction to estrogen in vitro. The authors showed that SIRT1 represses basal and inducible expression of estrogen-responsive genes, while inhibition of SIRT1 activity results in transcriptional activation of estrogen-responsive genes and consequently, cancer cell proliferation. They demonstrated that SIRT1-mediated repression of the proliferative response to estrogens is ER-α-dependent. They concluded that SIRT1 downregulates the ER-mediated signaling pathway in BC cells [73]. A more recent study by Xu et al. showed that SIRT1-mediated deacetylation of ER-α represses the transactivation of ER-α and consequently, inhibits the proliferation of BC cells in vitro. The authors showed that checkpoint suppressor 1 (CHES1) interacts with ER-α and enhances the recruitment of SIRT1, thus enabling SIRT1-mediated repression of ER-α transactivation and impairing ER-α transcriptional activity [74]. Furthermore, the SIRT1 activator resveratrol has been reported to suppress estrogen-dependent growth of luminal BC cells [75]. These studies demonstrated an anti-tumor role of SIRT1 in luminal subtypes through impairing ER-mediated signaling pathways (Figure 1).

On the other hand, alternative studies reported an oncogenic role of SIRT1 in luminal breast tumors. Elangovan et al. revealed that SIRT1 is activated and upregulated by ER-α in response to estrogens. They showed that ER-α physically binds to and functionally cooperates with SIRT1 toward the stimulation of breast tumor cells. In addition, SIRT1 inactivation eliminates estrogen/ER-α-induced cell growth and tumor development, triggering apoptosis and cell growth arrest. The authors concluded that SIRT1 is required for estrogen-induced breast cancer growth [76]. Another study by Yao et al. demonstrated that SIRT1 deficiency suppresses ER-α expression and leads to inhibition of estrogen-responsive gene expression in vitro. They showed that SIRT1 deficiency downregulates ER-α-mediated estrogen response genes in vivo, impairing ER-α-mediated signaling pathways in breast tumors. They postulated that SIRT1 may be a co-activator of ER-α in breast cancer [77]. In accordance with these findings, Santolla et al. investigated the expression and function of SIRT1 by

estrogens in ER-negative BC cells and cancer-associated fibroblasts (CAFs). The authors showed that estrogens upregulate SIRT1 expression through GPER (G protein-coupled ER) along with subsequent activation of the oncogenic EGFR/ERK/c-fos/AP-1 transduction pathway in vitro. They demonstrated that SIRT1 and GPER promote tumor growth both in vitro and in vivo. The authors then asserted a pro-survival role of SIRT1 and its implication in the prevention of apoptosis and cell cycle arrest [78].

6.2. SIRT1 Role in Non-Hormone-Dependant Triple-Negative Subtype (TNBC)

There are also contrasting studies concerning SIRT1 biological role in the TNBC subtype. Yi et al. reported that SIRT1 activation by a SIRT1 specific activator YK-3-237, induces the deacetylation of mt-p53, the oncogenic mutant form of p53, Deacetylation of mt-p53 upregulates the expression of wild-type p53-targets the *PUMA* and *NOXA* pro-apoptotic genes, suppressing cell proliferation and arresting cell growth of TNBC cell lines [79]. On the other hand, Wu et al. asserted an oncogenic role of SIRT1 in TNBC subtype. They revealed that an increased expression of SIRT1 is associated with poor prognosis, shorter DFS and OS, and distant metastasis in both TNBC and non-TNBC subtypes [55]. These findings are in agreement with those of Chung et al. who reported that SIRT1 upregulation positively correlates with tumor invasion and lymph node metastasis. They also showed that *SIRT1* gene silencing with SIRT1-siRNA significantly reduces the invasion ability of transfected versus non-transfected TNBC cell lines. The authors suggested the potential role of SIRT1 as a prognostic indicator, as well as a novel therapeutic target in triple negative BC [80]. Interestingly, a recent study by Urra et al. showed that SIRT1-mediated activation of AMPK selectively inhibits fibronectin-dependent migration of TNBC cells. However, the activation of SIRT1/AMPK axis has a cyto-protective effect in TNBC cells, promoting cell survival and proliferation but suppressing their ability to migrate. The authors demonstrated that SIRT1/AMPK activation impairs cell migration by reducing β1-integrin, a key protein involved in fibronectin-stimulated cell migration, on the cell surface and in turn, reduces cellular adhesion to the extracellular matrix [81].

6.3. SIRT1 Implication in the Epithelial-to-Mesenchymal Transition (EMT) Process, and Breast Cancer Invasion and Metastasis

The EMT process refers to the transformation of an epithelial cell to a mesenchymal cell; the process results in repressed E-cadherin expression and loss of cell-adhesive properties of epithelial cells. It also prevents apoptosis, and is critically implicated in cancer invasion and metastasis [82]. Using a xenograft mouse model, Simic et al. analyzed the metastatic potential of BC cells with or without SIRT1 in vivo. They found that SIRT1 upregulation suppresses cancer metastasis by reducing EMT, consequently maintaining E-cadherin expression; whereas SIRT1 repression promotes metastasis of breast epithelial cells in an orthotopic model of breast cancer. The authors also demonstrated that SIRT1 restrains the transforming-growth-factor (TGF)-β-signaling pathway that drives EMT. They postulated that SIRT1 suppression leads to E-cadherin degradation from the cell surface, thereby releasing β-catenin from the cadherin junctions to the nucleus, which is the characteristic of mesenchymal cells [83], thus asserting SIRT1 tumor-suppressive properties in the EMT process of BC. In contrast, Eades et al. reported that SIRT1 is overexpressed upon EMT-like transformation of human mammary cells in vitro, and that TGF-β-induced EMT leads to SIRT1 overexpression in epithelial cells. They also observed an increased SIRT1 recruitment to the E-cadherin promoter, resulting in SIRT1-mediated epigenetic silencing of E-cadherin, while SIRT1 knockdown restores E-cadherin expression. The authors also showed that SIRT1 deficiency prevents transformation of mammary epithelial cells by decreasing anchorage-independent growth and cell migration in vitro, hence indicating SIRT1 role in maintaining EMT-like transformation of the mammary epithelium [84]. Another study by Jin et al. revealed that SIRT1 expression is significantly correlated with increased expression of EMT-related proteins, vimentin and snail-1, and reduced expression of E-cadherin in triple-negative breast tumors; whereas inhibition of SIRT1 has opposite effects in vitro. They showed that SIRT1 inhibition also reduces the

invasion ability of TNBC cell lines in vitro. The authors then suggested an oncogenic role of SIRT1 in association with EMT in tumor invasion of TNBC subtype [85].

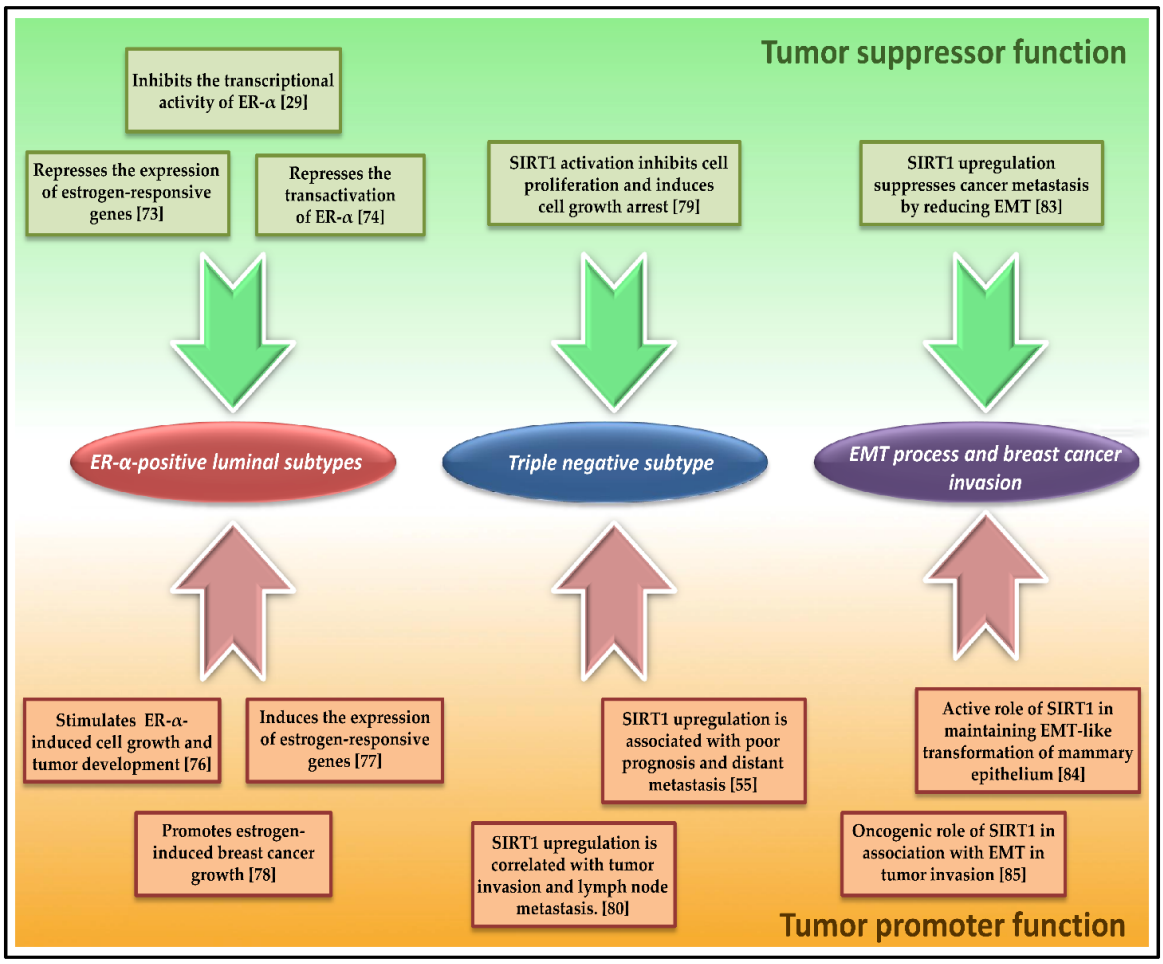

Figure 1. Bifurcated functions of SIRT1 in breast carcinogenesis. ER: estrogen receptor; EMT: epithelial-to-mesenchymal transition.

7. SIRT1 Modulators towards Breast Cancer Treatment

Being a key regulator of numerous cancer-associated processes, SIRT1 has been the subject of intense research in recent years. As a consequence, countless studies investigated/reviewed the therapeutic potential of SIRT1 in cancer treatment, and a plethora of small chemical compounds that modulate SIRT1 activity were discovered and patented [86–89]. These modulators (i.e., activators/inhibitors) not only enabled researchers to have a greater understanding of SIRT1 biological function and regulatory mechanisms, but also showed promising therapeutic applications in clinical trials for various human diseases, such as metabolic disorders, cardiovascular and neurodegenerative diseases, endothelial dysfunctions, inflammation, and cancer [90–92]. Although SIRT1 modulators have proven their efficiency in cancer cells by reducing cell viability and inducing apoptosis, their therapeutic functions remain utterly related to the role and expression rate of SIRT1 in a specific cancer, which in turn may vary drastically as we previously described.

While SIRT1 activators were initially favored as calorie restriction mimetics, researchers demonstrated their beneficial effects in delaying age-related decline in heart function and neuronal loss, also in preventing tumorigenesis. Resveratrol, a polyphenol described as an anti-aging drug and calorie restriction mimetic, was amongst the first characterized activators of SIRT1 [66,93]. In breast cancer, we previously showed that SIRT1 activation by resveratrol in SIRT1$^{+/-}$ p53$^{+/-}$ mice reduces tumorigenesis in vivo [67], as well as AR–stimulated proliferation [68]. Also, resveratrol was

shown to repress estrogen-dependent growth by impairing ER-α-mediated signaling pathways [75]. Due to shortage in resveratrol bioavailability, synthetic compounds that are structurally unrelated to resveratrol but 1000-fold more potent were synthesized and collectively named SIRT1-activating compounds (STAC) [94]. These STACs are currently being used as SIRT1 activators in breast cancer studies; they include among others SRT1460, SRT1720, SRT2104, and SRT2183 [86,87,91].

SIRT1 inhibitors have shown their therapeutic potentials in the treatment of various pathologies such as immunodeficiency virus infections, parasitic diseases, Parkinson's disease, and cancer therapy. Since SIRT1 is upregulated in multiple types of cancer, anticancer studies were more focused on SIRT1 inhibitors compared to SIRT1 activators [87,92]. As a result, a wide range of pharmacological inhibitory molecules were designed and tested such as sirtinol, salermide, splitomicin, cambinol, suramin, tenovin, nicotinamide, indole derivatives, and their structurally similar analogs. In breast cancer, in vitro and in vivo studies on ER^+ and ER^- cell lines showed that SIRT1 inhibition by these molecules suppresses cancer cell proliferation and induces p53-mediated apoptosis through increasing the acetylation of its Lys382 (p53K382), or in some cases, induces p53-independent apoptosis by reactivating proapoptotic genes (such as *CASP* genes that encode for caspase-3/8/9) that were epigenetically repressed by SIRT1 [95–100], thus proving the antitumor activity of SIRT1 inhibitors in BC.

8. Conclusions and Future Directions

In conclusion, regardless of whether SIRT1 has a pro-survival role by repressing TSGs, upregulating the expression of oncogenes, and activating oncogeneic signaling pathways such as PI3K/Akt and Ras-MAP kinase, or whether it has a proapoptotic role by reducing tumorigenesis and AR-mediated proliferation, downregulating the expression of oncogenes, and participating in ER-α-mediated signaling pathways and the EMT process, there is no doubt as to its significant role in breast carcinogenesis. Studies showed that SIRT1 plays different roles according to different BC molecular subtypes. Since BC is characterized by its molecular and clinical heterogeneity, with variations in gene expression profiles compared to intrinsic subtypes, one might argue that researchers should take into account the molecular classification of used human mammary tumors and cell lines in their future studies. Further investigations should also include a statistically sufficient sample size, and use of multiple cell lines in the same study. Nonetheless, considerable progress has been made in this research area in the last 10 years. SIRT1 modulators have been discovered or designed, and clinical studies investigating the therapeutic potential of SIRT1 in cancer treatment hold promising results. Thus, this research field should be prioritized and more large-scale studies are needed in order to decipher the code of the enzymatic duality of SIRT1 in breast carcinogenesis.

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Conclusion de la publication 3

Dans cette revue, on a trié les fonctions multiples de SIRT1 dans la pathogenèse du cancer du sein. On a vu que SIRT1 peut exercer des fonctions anti-tumorales ainsi que pro-tumorales dans les tissus mammaires.

En effet, SIRT1 est étroitement impliquée dans le processus de tumorigenèse des tumeurs luminales hormono-dépendantes, ainsi que les tumeurs triples négatives non hormono-dépendantes. En outre, SIRT1 est impliquée dans la promotion ou l'inhibition de processus de la transition epithelio-mesenschymal (EMT) qui conduit vers la métastase du cancer du sein.

On a aussi mis en évidence la régulation épigénétique des modifications d'histones par SIRT1, cette régulation dépendante de SIRT1 de l'expression génique s'applique à la fois à travers les marqueurs histones et leurs enzymes épigénétiques modulateurs.

Ainsi, on a montré que SIRT1 est impliquée dans la réduction de la résistance aux chimiothérapies, et que SIRT1 est régulée par des miRNAs, cette régulation peut avoir des applications thérapeutiques dans le traitement du cancer du sein.

En conclusion, SIRT1 est profondément impliquée dans la cancérogenèse mammaire, et a un rôle essentiel dans le développement et la progression tumoral. Cependant, vu les contradictions énorme dans les fonctions de SIRT1 dans le cancer du sein, des études à plus grande échelle seront nécessaires pour déchiffrer le code de la dualité enzymatique de SIRT1 dans la tumorigenèse du sein.

3. Inhibiteurs des HDACs Comme des Cibles Thérapeutiques: Les Épi-drogues dans le Cancer du Sein

La nature réversible de la modulation épigénétique par les HDACs en fait une cible intéressante pour le traitement du cancer par des inhibiteurs pharmacologiques (HDACi), nommées aussi Épi-drogues. Ces composés peuvent rétablir l'homéostasie d'acétylation trouvée dans les cellules non transformées, et par conséquent, annuler les événements qui sont induits par les HDACs et qui conduisent vers le développement et la progression du cancer (Shankar and Srivastava, 2008) (Delcuve et al., 2012) (Li and Zhu, 2014) (Eckschlager et al., 2017). Effectivement, dans les dernières années, un nombre croissant d'études a démontré l'activité anti-tumorale considérable des HDACi, et a consolidé leur rôle potentiel de médicaments épigénétiques dans le traitement des cancers (Balakin et al., 2007) (Marson, 2009) (Arrowsmith et al., 2012), y compris le cancer du sein (Tate et al., 2012) (Kai et al., 2015) (Damaskos et al., 2017) (Garmpis et al., 2017) (Fedele et al., 2017). Les HDACi ont aussi été testées dans des essais cliniques en combinaison avec des agents déméthylants de l'ADN, des médicaments chimiothérapeutiques classiques et en immunothérapie, et ont montré des résultats prometteurs. Ainsi, il y a déjà quelques Épi-drogues qui ont été approuvées par le US Food and Drug Administration (FDA) ces derniers années (**Figure 15**), et qui sont utilisées actuellement dans le traitement du cancer (Thaler, 2012) (De Souza and Chatterji, 2015) (Ma et al., 2016).

Ainsi, en tant que régulatrice clé de nombreux processus associés au cancer, SIRT1 a fait l'objet des recherches approfondies qui ont examiné son potentiel thérapeutique dans le traitement du cancer, par la suite, une pléthore de petits composés chimiques modulant l'activité de SIRT1 ont été découverts et brevetés (Mahajan et al., 2011) (Mellini et al., 2015) (Bai et al., 2018). Ces modulateurs spécifiques de SIRT1 n'ont seulement permis aux chercheurs de mieux comprendre la fonction biologique et les mécanismes de régulation de SIRT1, mais ont également montré des applications thérapeutiques prometteuses dans le cadre d'essais cliniques portant sur diverses maladies humaines, telles que les troubles métaboliques, les maladies cardiovasculaires et neurodégénératives, les dysfonctions endothéliales, l'inflammation, et le cancer, y compris le cancer du sein (Milne and Denu, 2008) (Morris, 2013) (Mellini et al., 2013) (Hu et al., 2014) (Kozako et al., 2014) (Yoon et al., 2014a) (Yoon et al., 2014b).

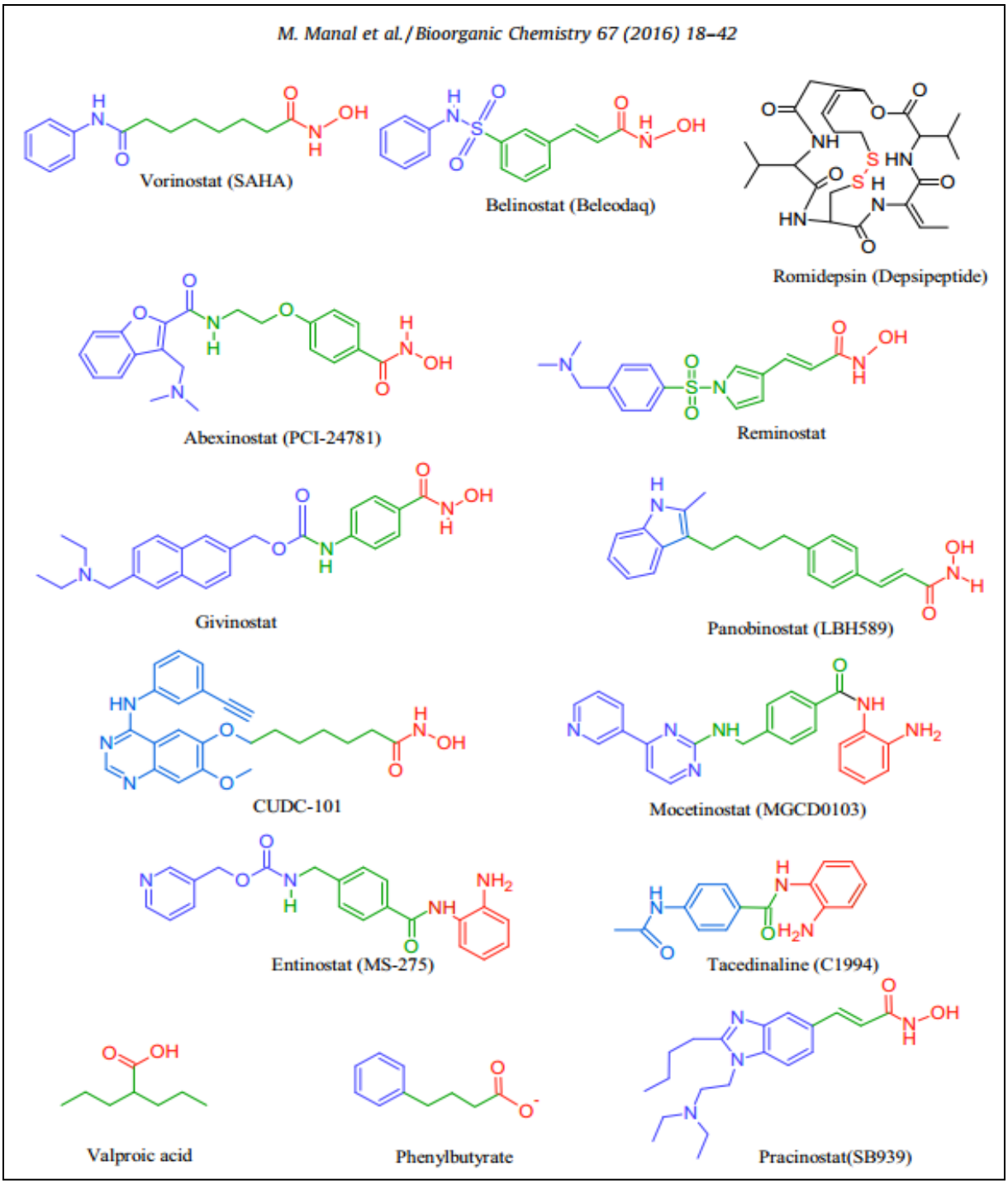


Figure 15. Schéma représentant les structures chimiques des HDACi approuvées dans des essais cliniques (Manal et al., 2016).

CHAPITRE II. OBJECTIFS DU PROJET DE RECHERCHE

D'après les données de la littérature précédemment décrites, l'étude de la dérégulation des PTMs des histones H3 et H4 constitue un outil puissant dans la recherche sur le cancer du sein. Cependant l'implication des marques activatrices H3K4ac, H3K9ac et H4K16ac est encore peu étudiée dans le cancer du sein, surtout la marque H3K4ac dont les enzymes modulatrices ne sont pas encore identifiées.

On a remarqué ainsi que le déséquilibre entre l'acétylation et la désacétylation des protéines histones et non-histones, est l'une des nombreuses anomalies épigénétiques aboutissant à l'initiation et à la progression du cancer, et que l'altération de l'expression et de l'activité enzymatique de l'HAT TIP60 et de l'HDAC SIRT1, est étroitement liée au processus de la carcinogenèse mammaire.

En basant sur ces données, on s'est donc posé les questions suivantes :

- Quel est le rôle des marques activatrices H3K4ac, H3K9ac, et H4K16ac dans la progression du cancer sporadique du sein ?
- SIRT1 est-elle sous-exprimée ou surexprimée dans le cancer du sein ?
- Comment SIRT1 régule ses cibles d'histones dans le cancer, et comment cette régulation épigénétique affecte le développement du cancer du sein ?
- Quel est le rôle de TIP60 et SIRT1 dans le développement du cancer du sein : Est-ce que ce sont des oncogènes ou des suppresseurs de tumeur ?
- TIP60 et SIRT1 ciblent-elles l'acétylation et la désacétylation de la lysine 4 de l'histone 3 (H3K4) ?

Pour répondre à ces questions, ce travail a été centré autour de 4 axes :

1. Evaluer les niveaux d'expression de SIRT1 dans les tumeurs du sein classées selon la classification moléculaire de St Gallen.
2. Caractériser le rôle épigénétique de SIRT1 vis-à-vis de ses cibles d'histones dans le cancer du sein.
3. Déterminer les enzymes modulatrices de la marque H3k4ac dans le cancer du sein.
4. Préciser les rôles de l'HAT TIP60 et de l'HDAC SIRT1 dans le développement du cancer du sein.

CHAPITRE III. RESULTATS

A. Mise En Évidence du Rôle Ambivalent de SIRT1 dans le Cancer Sporadique du Sein

1. SIRT1 : Un Biomarqueur Pronostique Potentiel du Cancer du Sein

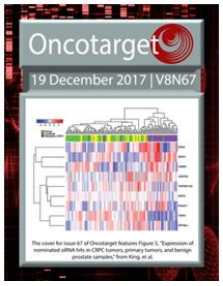
Présentation de la publication 4

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 67), pp: 110922-110930

Research Paper

Dual SIRT1 expression patterns strongly suggests its bivalent role in human breast cancer

Rifaï K, Judes G, Idrissou M, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D.



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La signification clinique des sirtuines dans divers cancers humains a principalement été évaluée sur la base de leurs profils d'expression dans les tissus tumoraux versus les tissus sains. En fait, une surexpression d'une protéine dans les tumeurs indique son rôle oncogène, tandis que son expression réduite indique son rôle suppresseur des tumeurs.

L'expression de SIRT1 dans le cancer du sein a fait l'objet de plusieurs études au cours des dernières années, cependant, ces rapports sont contradictoires. Beaucoup d'entre eux ont montré une surexpression de SIRT1 dans les cellules cancéreuses, et par conséquent, ont suggéré son rôle promoteur de tumeur dans le cancer du sein. Cependant d'autres, ont mis en

évidence une sous-expression de SIRT1 et ont suggéré un rôle plutôt suppresseur de tumeur. Cette contradiction dans la littérature concernant l'expression de SIRT1 dans le cancer du sein, nous a conduit à mener cette étude.

En utilisant une cohorte de 50 tumeurs mammaires humaines et leurs tissus normaux appariés, nous avons étudié les niveaux d'expressions transcriptionnelles et traductionnelles de SIRT1 dans les 5 sous-types moléculaires du cancer du sein [Luminal A, Luminal B-, Luminal B+, HER2-enriched, et TNBC].

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Research Paper

Dual SIRT1 expression patterns strongly suggests its bivalent role in human breast cancer

Khaldoun Rifaï[1,2], Gaëlle Judes[1,2], Mouhamed Idrissou[1,2], Marine Daures[1,2], Yves-Jean Bignon[1,2], Frédérique Penault-Llorca[2,3] and Dominique Bernard-Gallon[1,2]

[1]Centre Jean Perrin, Department of Oncogenetics–CBRV, 63001 Clermont-Ferrand, France

[2]INSERM U 1240–IMoST, 63005 Clermont-Ferrand, France

[3]Centre Jean Perrin, Department of Biopathology, 63011 Clermont-Ferrand, France

***Correspondence to**: Dominique Bernard-Gallon, **email:** dominique.gallon-bernard@clermont.unicancer.fr*

***Keywords:** breast cancer; molecular subtypes; SIRT1; expression levels; statistical analysis*

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ABSTRACT

Breast cancer is the most common cancer in women, and the leading cause of cancer death in women worldwide. SIRT1 (silent mating type information regulation 2 homolog) 1 is a class-III histone deacetylase involved in apoptosis regulation, DNA repair and tumorigenesis. However, its role in breast carcinoma remains controversial, as both tumor-suppressive and tumor-promoting functions have been reported. Also, there are very few reports available where expression of SIRT1 is comprehensively analyzed in breast tumors classified by molecular subtype. Here, using a cohort of 50 human breast tumors and their matched normal tissues, we investigated SIRT1 expression levels in the 5 molecular subtypes of breast cancer according to the St Gallen classification (2013). Tumors and their corresponding normal tissue samples were collected from all patients, and SIRT1 mRNA and protein expression levels were then examined by real-time quantitative polymerase chain reaction and immunoblotting, respectively. After statistical analysis, the results showed a dual expression profile of SIRT1 in human breast carcinoma, with significant overexpression in luminal and HER2-enriched subtypes and significantly reduced expression in the triple-negative subtype. We also found an inverse correlation between SIRT1 expression and breast cancer aggressivity. These novel findings suggest that SIRT1 plays a dual role in breast tumors depending on its expression rate and the molecular subtype of the cancer. Our data also point to a potential role for SIRT1 as a prognostic biomarker in breast cancer.

INTRODUCTION

Breast cancer is the most common cancer in women, and the leading cause of cancer death in women worldwide [1]. It is a multifactorial genetic disease with different prognoses for different subtypes. According to the St Gallen breast cancer classification [2], there are five distinct molecular subtypes of breast cancer classified in ascending order of tumor aggressiveness, from luminal A, relatively the least aggressive with the most favorable prognosis and survival rate [3], to luminal B (HER2-) and luminal B (HER2+), these 3 subtypes are included in the Hormone Receptor-positive Breast Cancer (HRBC), HER2-enriched or HER2 Breast Cancer (H2BC), and finally triple-negative breast cancer (TNBC), also known as basal-like, which is characterized as very aggressive and associated with poor prognosis and a higher death rate compared to the other molecular subtypes [3]. The process of subtyping breast cancer based on gene expression patterns has clarified differences in biological behavior between subgroups, allowing individualized treatment and better prognosis for each subtype [4].

Sirtuins (SIRT) are NAD+-dependent class-III histone deacetylases, a highly-conserved gene family

from yeast to mammals that have drawn increasing attention in recent years due to their action in various pathophysiological processes. In mammals, there are seven known SIRT homologs that localize to different subcellular compartments, and they primarily possess histone deacetylase activity (SIRT1, SIRT2, SIRT3 and SIRT5) or monoribosyltransferase activity (SIRT4 and SIRT6). These sirtuin isoforms can alter a wide variety of substrates involved in cell differentiation, viability, senescence, inflammation, and cellular survival, and thus control diverse key functions ranging from cellular survival to chromatin remodeling. Sirtuins are also closely involved in aging process, lifespan, and various pathologies including cancer, inflammation, immune dysfunction, cardiovascular disorders and neurodegeneration [5, 6].

Silent mating type information regulation 2 homolog 1 (SIRT1), the mammalian counterpart of yeast silent information regulator 2 (Sir2), is the most extensively studied protein in the SIRT family. SIRT1 is involved in key cellular processes such as apoptosis, DNA repair, chromatin remodeling and cancer development [7, 8], but its role in carcinogenesis is controversial, as it can have both tumor-suppressive and tumor-promoting functions, mainly depending on cancer type [9]. For instance, SIRT1-mediated deacetylation of the tumor suppressors p53 [10] and p73 [11] inactivates them, preventing cellular growth arrest, senescence and apoptosis, hence exerting oncogenic functions. On the other hand, SIRT1 is also reported to mediate BRCA1 signaling and inhibit tumor growth through downregulation of oncogenes or by repressing the activity of oncoproteins such as β-catenin [12] and survivin [13]. Furthermore, knockout mice models of SIRT1 are prone to tumor development, which points to a tumor-suppressive SIRT1 action [13]. These seemingly opposite functions might reflect a highly context-specific role of SIRT1 as a tumor-suppressor versus tumor-promotor.

The clinical significance of sirtuins in various human cancers has mostly been evaluated based on sirtuin expression patterns in tumors and non-tumor samples. Generally, overexpression of a protein in tumors indicates its oncogenic properties, whereas reduced expression of a protein indicates its tumor-suppressive properties. Studies using this approach report that SIRT1 is upregulated in a spectrum of cancers including, but not limited to, liver cancer [14], acute myeloid leukemia [15], bone cancer [16], thyroid cancer [17] and skin cancer [18], but downregulated in other cancers including colon cancer [12], oral squamous cell carcinoma [19], glioblastoma and ovarian cancer [20]. Studies in breast cancer have confirmed that SIRT1 is involved in tumorigenesis, metastasis [21] and chemoresistance [22]. However, there have been relatively few studies investigating SIRT1 expression levels to identify its function, and the results are contradictory. A limitation of these studies is that they did not take into account the heterogeneity of various intrinsic breast cancer subtypes, and most of them did not use tissue samples from breast cancer patients but relied on breast cancer cell lines instead. Here, we evaluated both the mRNA and protein expression patterns of SIRT1 using human breast tumors and their corresponding normal breast tissues, in all 5 molecular subtypes of breast cancer. This research brings key insight to the ongoing controversy of SIRT1 behavior in breast cancer carcinoma.

RESULTS

Study population characteristics

The breast cancer molecular subtypes studied here spanned luminal A (n = 10, 20%), luminal B (HER2-) (n = 10, 20%), luminal B (HER2+) (n = 10, 20%), HER2-enriched (n = 10, 20%) and triple-negative (n = 10, 20%). All patients were females aged 45 to 82 years (mean 63.8 ± SD 7.1). Tumor size ranged from 0.5 to 7 cm (2.3 ± 0.5). All tumors were graded according to the modified Scarff-Bloom-Richardson grading system (SBR) as grade 1 (n = 3), grade 2 (n = 25) and grade 3 (n = 22). Samples were ER-, PR- and HER2-positive in n = 30 (60%), n = 16 (32%) and n = 20 (40%) patients, respectively. Table 1 gives the clinico-pathological variables of the 50 breast cancer patients.

SIRT1 is upregulated in (HRBC) and (H2BC) subtypes and downregulated in (TNBC) subtype

To assess SIRT1 expression at transcriptional/post-transcriptional level, SIRT1 messenger RNA (mRNA) was extracted from N = 50 tumors and their matched normal tissues (n = 10 for each of the 5 molecular subtypes), reverse-transcribed into complementary DNA (cDNA), then quantified by real-time quantitative PCR (RT-qPCR). Compared to matched normal tissues, relative SIRT1 mRNA expression was significantly higher in luminal A (mean 7.8 ± SD 2.5, $p < 0.001$; Figure 1A), luminal B (HER2−) (5.7 ± 1.7, $p < 0.001$; Figure 1B), luminal B (HER2+) (6.5 ± 2.1, $p < 0.001$; Figure 1C) and HER2− enriched (2.7 ± 1, $p < 0.001$; Figure 1D), but significantly lower in the triple-negative subtype (0.35 ± 0.2, $p < 0.001$; Figure 1E).

Positive correlation between SIRT1 expression and the St Gallen molecular classification

The differences between SIRT1 mRNA expression levels among the 5 molecular subtypes were further investigated using multi-way analysis of variance (ANOVA) followed by post-hoc analysis. Tukey's range test was then used for multiple comparisons among mean SIRT1 mRNA expression levels. The statistical procedures distinguished 3 distinct patterns of SIRT1

Table 1: Clinico-pathological characteristics of the breast cancer patients included in this study

	Total	Luminal A	Luminal B (HER2−)	Luminal B (HER2+)	HER2-enriched	Triple-negative	*P* value
Patients, *n* (%)	*N* = 50 (100%)	*n* = 10 (20%)	*n* = 10 (20%)	*n* = 10 (20%)	*n* = 10 (20%)	*n* = 10 (20%)	
Age							0.809
45–65	25 (50)	6 (60)	4 (40)	5 (50)	6 (60)	4 (40)	
>65	25 (50)	4 (40)	6 (60)	5 (50)	4 (40)	6 (60)	
SBR grade							**0.001**
I	3 (6)	3 (30)	0	0	0	0	
II	25 (50)	7 (70)	8 (80)	4 (40)	3 (30)	3 (30)	
III	22 (44)	0	2 (20)	6 (60)	7 (70)	7 (70)	
Size (cm)							0.265
<1.5	10 (20)	2 (20)	3 (30)	2 (20)	1 (10)	2 (20)	
1.5–2.5	21 (42)	7 (70)	4 (40)	5 (50)	2 (20)	3 (30)	
>2.5	19 (38)	1 (10)	3 (30)	3 (30)	7 (70)	5 (50)	
ER							**0.0001**
Positive	30 (60)	10 (100)	10 (100)	10 (100)	0	0	
Negative	20 (40)	0	0	0	10 (100)	10 (100)	
PR							**0.0001**
0%–50%	5 (10)	1 (10)	2 (20)	2 (20)	0	0	
51%–100%	11 (22)	9 (90)	2 (20)	0	0	0	
Negative	34 (68)	0	6 (60)	8 (80)	10 (100)	10 (100)	
HER2							**0.0001**
Positive	20 (40)	0	0	10 (100)	10 (100)	0	
Negative	30 (60)	10 (100)	10 (100)	0	0	10 (100)	
Ki-67							**0.0001**
≤20%	19 (38)	10 (100)	2 (20)	3 (30)	2 (20)	2 (20)	
>20%	31 (62)	0	8 (80)	7 (70)	8 (80)	8 (80)	

ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal growth factor Receptor 2, Ki-67: cellular marker for proliferation.

expression in human breast cancer tumors that correspond to the 3 molecular subtypes: overexpression in (HRBC) subtypes, slight overexpression in the (H2BC) subtype, and underexpression in the (TNBC) subtype (Figure 2).

Consistency between SIRT1 mRNA and protein expression patterns

In order to determine whether SIRT1 transcription levels are equally translated into functional proteins, SIRT1 protein levels were assessed in breast tumors and their matched normal tissue samples using immunoblot analysis. We found that SIRT1 protein expression pattern differs amongst the 5 molecular subtypes, as shown in (Figure 3A). In comparison with normal breast tissue, SIRT1 protein expression was significantly higher in (HRBC) subtypes and in the (H2BC) subtype, but significantly reduced in the (TNBC) subtype (Figure 3B). These results are consistent with the mRNA expression level data.

DISCUSSION

In order to clarify the biological behavior of SIRT1 and evaluate its role in breast carcinoma, we evaluated SIRT1 expression patterns at the transcriptional and translational levels in human breast tumors and their corresponding normal breast tissues, according to St Gallen molecular subtype class. Although some studies have investigated SIRT1 expression in breast cancer, however, this is the first study to extensively examine SIRT1 mRNA and protein expression levels according to intrinsic subtypes with a sample size that satisfies statistical power requirements.

SIRT1 is a class-III histone deacetylase critically involved in the occurrence and development of a multitude of tumors, and reported to be involved in regulating a multitude of biological processes including apoptosis, cell survival, proliferation and stress response. SIRT1 expression levels have been extensively investigated in many malignancies in order to assess its

role. SIRT1 expression and function are found to vary drastically depending on cell and tumor types, making it a multifaceted enzyme with contradictory functions depending on its upstream regulators and downstream targets [23]. SIRT1 overexpression has been reported in several human cancers, it was generally associated with poor prognosis and poor overall survival [24], whereas reduced SIRT1 expression was consistent with a tumor-suppressor role [12, 20].

Several studies have investigated SIRT1 expression in breast cancer, but while some studies found upregulated SIRT1 expression, others did not concur. There are multiple reasons that could explain this discrepancy between studies: the fact that SIRT1 expression was evaluated only at transcriptional level [25], or using only breast cancer cell lines [26, 27], and/or using human breast tissue samples but without accounting for the various molecular subtypes [25, 28–30] or without having a statistically sufficient sample size [28, 31]. This unclear picture promoted us to conduct the study here. The results found here revealed different SIRT1 expression patterns among different breast cancer molecular subtypes. We report significant overexpression of SIRT1 mRNA and protein levels in HRBC and H2BC subtypes, and a significant underexpression in the TNBC subtype. This dual expression pattern of SIRT1 in tumors points to a differential role of SIRT1 in human breast cancer. Based on its expression patterns, SIRT1 most probably has an oncogenic role in the HRBC and H2BC subtypes, in line with Elangovan *et al*. [32] and Ma *et al*. [33], who reported that SIRT1 overexpression in luminal breast cancer subtypes is correlated with an oncogenic behavior. In contrast, SIRT1 may play a tumor-suppressor role in the TNBC subtype, in line with Yi *et al*. [34] who reported that the activation of SIRT1 by a SIRT1-specific activator YK-3-237 induced deacetylation of the mutant form of p53 (mtp53), suppressing the proliferation and arresting the cell growth of triple-negative breast cancer cell lines. Furthermore, Simic *et al*. [35] showed that ectopic expression of SIRT1 suppresses cancer metastasis and tumor cell invasion. Moreover, our findings showed a positive correlation between SIRT1 expression and St

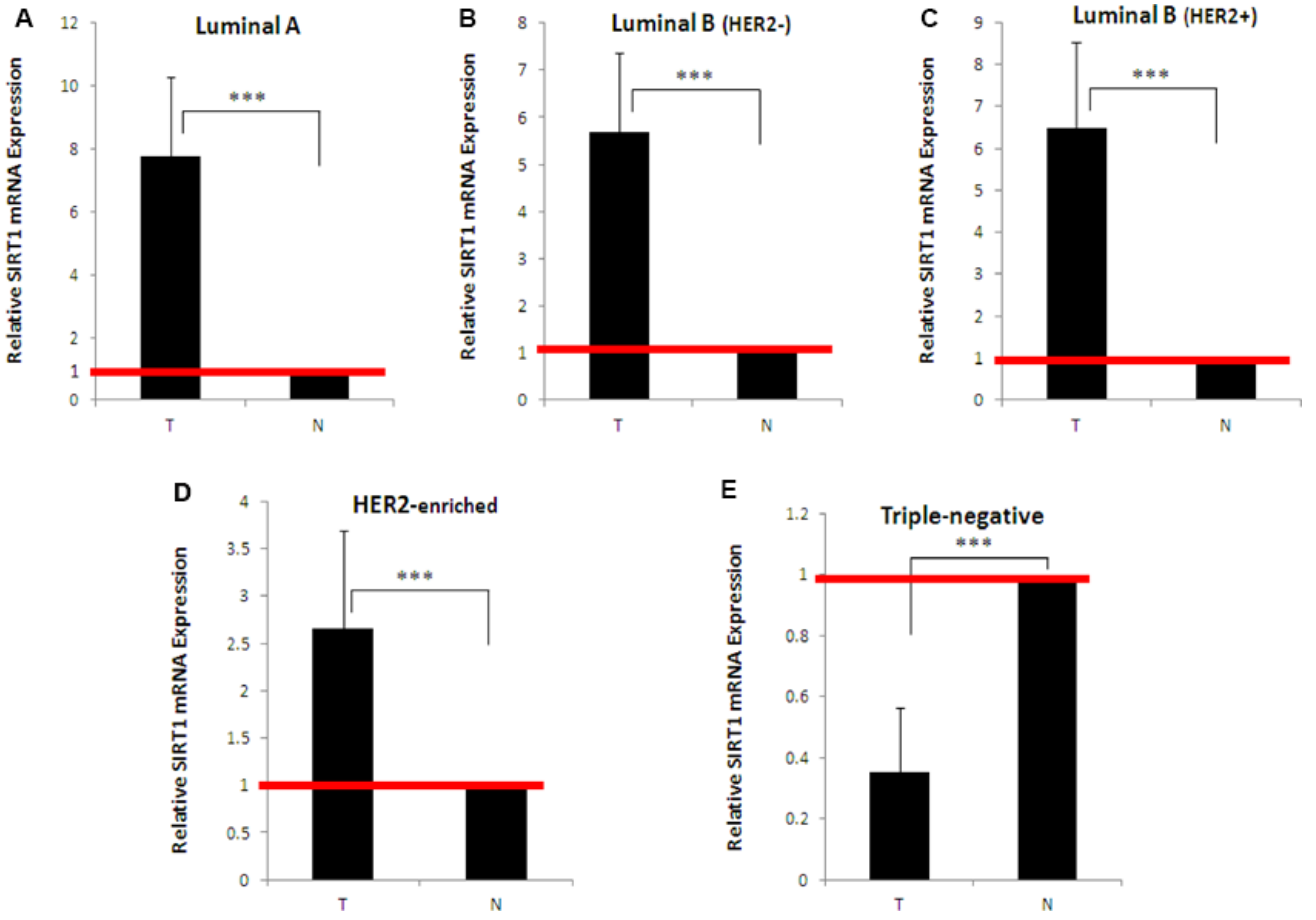

Figure 1: Quantitative expression levels of SIRT1 in different breast tumor subtypes and their matched normal tissue samples. SIRT1 expression levels were quantified by real-time quantitative PCR using mRNA extracted from (**A**) n = 10 luminal A, (**B**) n = 10 luminal B (HER2−), (**C**) n = 10 luminal B (HER2+), (**D**) n = 10 HER2-enriched, (**E**) n = 10 triple-negative breast tumors, and their adjacent normal tissues. SIRT1 mRNA expression was normalized against 18S rRNA levels. SIRT1 expression in breast tumors was expressed as fold-change compared to normal breast tissues (defined as 1). Each real-time PCR reaction was performed in triplicate, the results are expressed as mean ± SD, P values were two-tailed and $^{***}P < 0.001$ was considered statistically significant. T: Tumor, N: Normal.

Gallen molecular subtype classification. After classifying the breast tumors used in ascending order of aggressivity, decreased SIRT1 expression was found to correlate with increased breast cancer aggressivity and poor prognosis. We conclude that SIRT1 may serve as a prognostic biomarker in breast cancer carcinomas.

In conclusion, this study demonstrated for the first time a differential pattern of SIRT1 expression in breast cancer at both transcriptional and protein level using human breast tumors and their uninvolved benign counterparts, it also established an association between SIRT1 expression and St Gallen classification. Taken together, these results suggest that SIRT1 plays a bivalent subtype-dependent role in breast carcinoma, and that SIRT1 could also be a potential prognostic marker in breast cancer. Given that SIRT1 regulates a wide range of substrates directly involved in the tumorigenesis process, it could make a novel and potentially promising anticancer therapeutic target, especially if results from clinical trials currently testing specific SIRT1 inhibitors are deemed good.

MATERIALS AND METHODS

Study population selection and collection of tissue samples

This study included a total of 50 patients admitted to the Centre Jean Perrin from October 2012 to September 2016 for cancer treatment, and diagnosed with breast cancer carcinoma. Patients were informed about the study and gave informed consent prior to inclusion. All 50 tumors and their adjacent normal breast tissues came from the Centre Jean Perrin Biological Resource Center, where they were put in cryotubes and stored in liquid nitrogen at −196°C. Patients who received chemotherapy, hormonal therapy and/or radiotherapy for cancer in other parts of the body were excluded from the study, as were patients with predisposition to breast cancer and/or family members with breast cancer.

Intrinsic breast cancer subtype classification

The breast carcinomas were classified into 5 molecular subtypes according to St Gallen breast cancer conference guidelines [2] based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 proliferative index, as follows:

Luminal A: [ER- and/or PR-positive, HER2-negative, and Ki-67 <14%]

Luminal B (HER2-): [ER- and/or PR positive, HER2-negative and Ki-67 ≥14%]

Luminal B (HER2+): [ER- and/or PR-positive, HER2-positive, and any Ki-67]; these 3 subtypes are included in the hormone receptor-positive breast cancer (HRBC) group.

HER2-enriched/HER2 breast cancer (H2BC): [ER- and/or PR-negative, HER2 overexpressed]

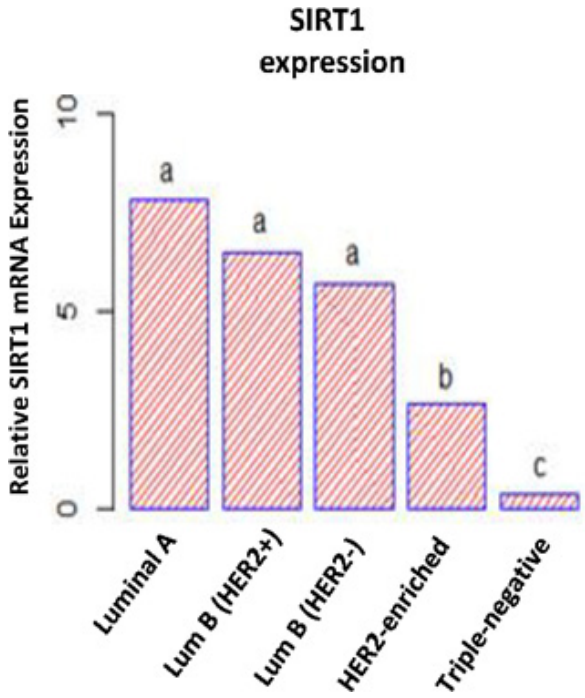

Figure 2: Differential SIRT1 mRNA expression patterns in breast tumors. ANOVA followed by Tukey's multiple comparison test performed on SIRT1 mRNA expression levels. This statistical analysis discerned 3 different SIRT1 expression patterns. The letters 'a', 'b' and 'c' indicated statistical significance between groups.

Triple-negative breast cancer (TNBC): [ER-, PR-, and HER2-negative].

Total RNA isolation from tissues and reverse transcription (RT)

Tumoral and non-tumoral tissue samples were cut into pieces and homogenized with TissueRuptor® (Qiagen, Hilden, Germany). Total RNA was isolated using TRIzol Reagent (Ambion, Life Technologies, CA) then extracted using a PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, CA). RNA samples purity was verified using NanoDrop ND-8000 spectrophotometer. cDNA was then obtained using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

RT-qPCR methods and data analysis

Synthesized cDNA was amplified using TaqMan Gene expression PCR Master Mix (AB Applied Biosystems) as per the manufacturer's protocol. Each duplex PCR was assembled using 96-well MicroAmp Optical plates (AB Applied Biosystems) with 25 ng of

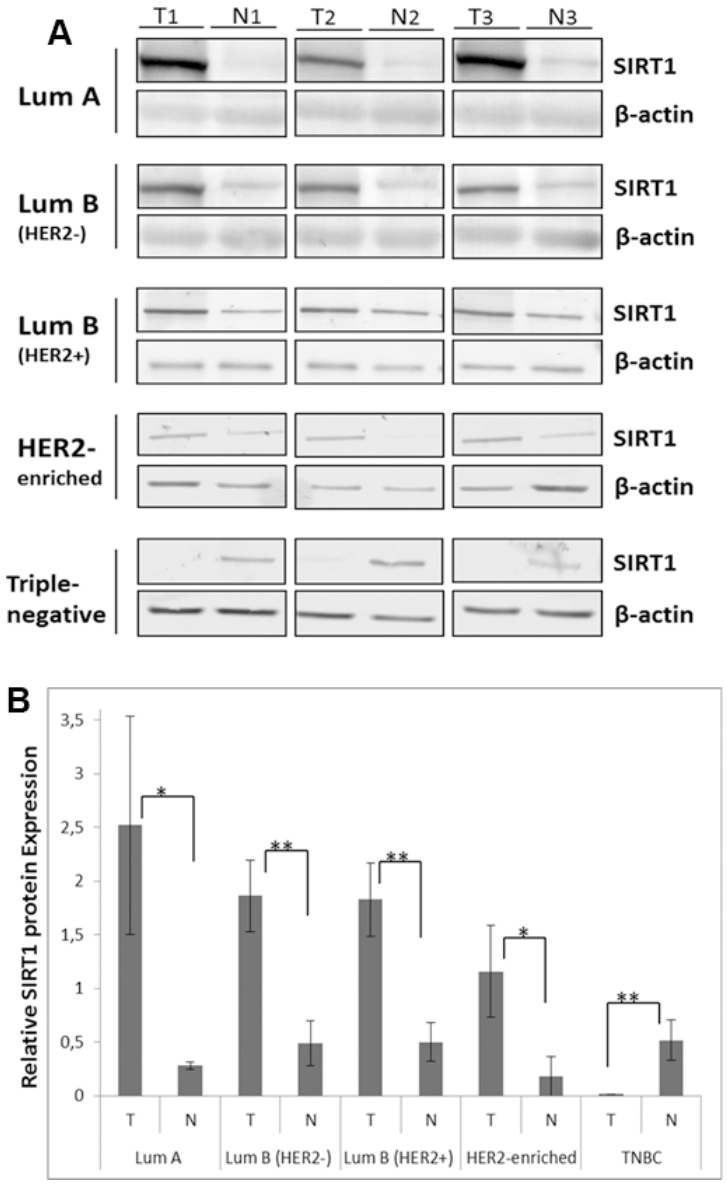

Figure 3: Differential SIRT1 protein expression patterns in breast tumors. (**A**) Representative immunoblots of 3 independent experiments showing SIRT1 protein expression in the 5 molecular subtypes of breast cancer. Equal amounts of proteins were immunoblotted with SIRT1 antibody (110 kDa). β-actin (42 kDa) served as loading control. (**B**) Relative SIRT1 protein expression was evaluated using Quantity One software with SIRT1 expression normalized against β-actin as loading control. Each bar represents the mean ± SD of 3 replicate experiments. For the statistical analysis, *P*-values were two tailed, $^{*}P < 0.05$ and $^{*}P < 0.01$ were considered statistically significant. T: Tumor, N: Normal.

template cDNA in a total volume of 25 µL containing 12.5 µL TaqMan Gene Expression Master Mix (2X), 1.25 µL TaqMan Gene Expression assay-on-demand SIRT1 [Hs01009006_m1] (200 nM), 0.25 µL endogenous control 18S rRNA primers (10 µM) and 0.25 µL 18S rRNA probe (5 µM). Primer sets for specific reverse transcription of SIRT1 and endogenous control 18S rRNA were all obtained from (AB Applied Biosystems), and are as follows: SIRT1 forward 5-CCTGTGAAAGTGATGAGGAGGATAG-3; reverse 5-TTGGATTCCCGCAACCTG-3. 18S forward: 5′-CGG CTA CCA CAT CCA AGG AA-3′, reverse: 5′-GCT GGA ATT ACC GCG GCT-3′, probe: 5′-TGCTGG CAC CAG ACT TGC CCT C-3′. The thermal reaction cycles used were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The signal was collected at the endpoint of each cycle using an AB Prism 7900 Sequence Detector System (AB Applied Biosystems). Relative gene expression was determined by normalizing to reference gene *18S* and according to the relative quantitative (ΔΔCt) method. Fold change in SIRT1 expression was then calculated using the (2−ΔΔCt) method. SIRT1 mRNA expression in breast tumors was calculated relative to the matched normal breast tissues. All experiments were done in triplicate, and results were expressed as means ± SD.

Protein extraction and immunoblot analysis

Frozen tissues were homogenized before being lysed using T-PER™ Tissue Protein Extraction Reagent (ThermoFisher Scientific) containing protease inhibitor cocktail (Sigma Aldrich). Whole protein extracts were resolved by electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), then electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P, PVDF, 0.45 µm, Merck Millipore) in transfer buffer (25 mM Tris-HCL (pH 7.6), 192 mM glycine, 10% methanol). The membranes were blocked with 5% non-fat milk in 0.1% TBS-tween and later immunoblotted with monoclonal anti-SIRT1 antibody (1/500, MAb-063-050, Diagenode) or monoclonal anti-β-actin antibody (1/5000, CP01, Merck Millipore). Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibody anti-mouse IgG (1/2000, S3721, Promega). Immunolabeling was detected using Western Blue® Stabilized substrate for Alkaline Phosphatase (Promega) at room temperature.

Statistical analysis

Correlation between the clinical parameters of our study groups were examined by chi-square test (χ^2 test) using SPSS statistics software (SPSS Inc., Chicago, IL). Multiple-group comparisons were performed by ANOVA using R software (version 3.0.3). Post-hoc comparison of the means was performed using Tukey's multiple comparison test when the F-test was significant ($p < 0.05$). Relative expression levels of SIRT1 protein assayed by immunoblotting were assessed numerically using Quantity One software (Bio-Rad, CA). Groups were compared using a two-tailed unpaired Student's *t*-test carried out after a Fisher's exact test. All experiments were done in triplicate and the results were expressed as mean ± SD. In all cases, statistical significance was set at the following *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Conclusion de la publication 4

Afin de clarifier la controverse entourant l'expression de SIRT1 dans le cancer du sein, et par conséquent, son rôle dans la maladie, nous avons évalué les profils d'expression de SIRT1 au niveau ARNm et protéine dans les tumeurs mammaires humaines et leurs tissus normaux correspondants.

Les conclusions majeures de cette étude sont que :

- L'expression de SIRT1 est largement corrélée à la classification moléculaire de St-Gallen des tumeurs mammaires.
- L'expression de SIRT1 diminue inversement au fur et à mesure de l'agressivité du cancer du sein.
- SIRT1 est surexprimée dans les tumeurs luminales et HER2-enriched, ce qui suggère un rôle oncogène de SIRT1 dans ces sous-types.
- SIRT1 est sous-exprimée dans les tumeurs triple-négatives, ce qui suggère son rôle suppresseur de tumeur dans les TNBCs.

Les résultats de cette étude suggèrent que SIRT1 joue un rôle ambivalent dans les carcinomes du sein, et que SIRT1 pourrait également être un marqueur pronostique potentiel dans le cancer du sein.

2. Rôle Bivalent de SIRT1 dans un Autre Cancer Humain : Le Cancer Colorectal

Présentation de la publication 5

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SIRT1 in Colorectal Cancer: A Friend or Foe?

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Après avoir montré un double rôle de SIRT1 dans le cancer du sein, nous avons voulu voir si cette dualité de fonctions de SIRT1 se retrouve dans d'autres cancers.

Dans la littérature, on a retrouvé beaucoup de contradictions sur le rôle de SIRT1 dans le développement et la métastase du cancer colorectal (CCR). En fait, il existe une grande divergence entre les études concernant le taux d'expression de SIRT1, et par conséquent son rôle exact dans le cancer. L'incohérence entre ces études provient du fait que SIRT1 pourrait aussi jouer des rôles opposés dans la carcinogenèse colorectale, comme c'est le cas pour le cancer du sein.

Ce qui nous a incité à rédiger cette lettre à l'éditeur, en expliquant ce paradoxe et en mettant en évidence les fonctions opposées de SIRT1 dans la carcinogenèse colorectale.

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Letter to the Editor

SIRT1 in Colorectal Cancer: A Friend or Foe?

Khaldoun Rifaï,[1,2] Mouhamed Idrissou,[1,2] Marine Daures,[1,2] Yves-Jean Bignon,[1,2] Frédérique Penault-Llorca,[2,3] and Dominique Bernard-Gallon[1,2]

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It has been well established that SIRT1 plays a key role in the regulation of various crucial biological processes such as apoptosis, DNA damage response, cell senescence, and metabolism. However, its role in carcinogenesis is still subject to debate. SIRT1 is the human ortholog of yeast Sir2 and the protomember of sirtuins family, it is a NAD+ dependent class III deacetylase that can deacetylate both histone and nonhistone proteins. On one hand, studies report that SIRT1 activation reduces tumorigenesis in various human cancers. SIRT1 was also associated with the downregulation and inactivation of several oncogenes or oncoproteins such as NF-κB and β-catenin. Thus, SIRT1 can serve as a tumor suppressor.

On the other hand, it has been demonstrated that SIRT1 activates various oncogenes such as c-MYC and RAS oncoproteins. SIRT1 has also been shown to contribute to the tumorigenesis process by inducing inhibition and epigenetic silencing of tumor suppressor genes such as p53 and members of the FoxO family. Hence, SIRT1 can exert tumor promoter properties as well. Therefore, depending on its regulation of a multitude of substrates, SIRT1 may play different roles in different types of cancer.

Regulated by various different genes, the pathogenesis of colorectal cancer (CRC) is a multifactorial process that involves the deacetylation capability of SIRT1. Numerous studies have revealed the clinical relevance of SIRT1 expression and its role in the tumor formation, prognosis, and overall survival (OS) of CRC. However, there is a wide discrepancy between these studies concerning SIRT1 expression rate and subsequently its exact role in the disease. The inconsistency between these studies comes from the fact that SIRT1 may play opposing roles in colorectal carcinogenesis.

Consistent with the hypothesis that SIRT1 can act as a tumor suppressor, some studies have shown a reduced SIRT1 expression in human colorectal adenocarcinoma in comparison with normal colorectal tissues. These studies also evaluated the association between SIRT1 expression and clinicopathological parameters of CRC patients, they concluded that decreased SIRT1 expression was associated with colorectal tumor progression and frequent regional lymph node metastasis. Accordingly, SIRT1 expression was associated with better prognosis and OS of CRC patients.

Sun et al. (2017) gave a likely explanation for the decreased SIRT1 expression in CRC patients with advanced stages. They reported that SIRT1 activation by a selective SIRT1 activator SRT1720 reduced the invasiveness of CRC cells *in vitro*. Although the inhibition of SIRT1 by a selective SIRT1 inhibitor Ex-527 promoted their migration, they postulated that SIRT1 suppresses the invasion and migration of CRC cells. The authors also focused on the impact of SIRT1 on CRC metastasis. They uncovered a functional role of the SIRT1/miR-15b-5p/ACOX1 axis in suppressing CRC metastasis *in vitro*, using human CRC continuous cell lines, as well as *in vivo* using BALB/c nude mice.

They stated that SIRT1 negatively regulates miR-15b-5p transcription through deacetylation of AP-1, a transcriptional activator, and consequently impairing its ability to bind to the miR-15b-5p promoter (Fig. 1A). MicroRNAs are small noncoding RNAs that can modulate protein expression. It has been shown that miR-15b-5p regulation of certain targets in tumorigenesis leads to an increased proliferation and migration of cancer cells. In their study, the authors highlighted that miR-15b-5p can directly target and downregulate ACOX1 expression in CRC, an enzyme of the fatty acid oxidation pathway. The authors showed that ACOX1-impaired expression by miR-15b-5p promotes CRC metastasis. The authors also revealed that SIRT1 overexpression in CRC cells significantly suppressed miR-15b-5p expression and increased ACOX1 expression. When tested *in vivo*, CRC cells with stable SIRT1 expression inhibited the expression of miR-15b-5p and enhanced ACOX1 expression, resulting in reduced CRC metastasis. Hence, the authors provide evidence of a suppressive role of SIRT1/miR-15b-5p/ACOX1 axis in CRC metastasis (Sun et al., 2017).

In contrast, many studies asserted an oncogenic role of SIRT1 in CRC. They have reported an abundance of SIRT1 expression in human CRC tissues, especially in advanced-stage tumors and tumors with lymph node (Lv et al., 2014). That overexpression was strongly correlated with metastasis and tumor invasion depth. It was also associated with poor prognosis, shorter OS, and disease-free survival of CRC patients. Chen et al. (2014) reported an overexpression of

[1]Department of Oncogenetics, Centre Jean Perrin, CBRV, Clermont-Ferrand, France.
[2]INSERM - U1240 - IMoST Imagerie Moléculaire et Stratégies Théranostiques, Clermont-Ferrand, France.
[3]Department of Biopathology, Centre Jean Perrin, Clermont-Ferrand, France.

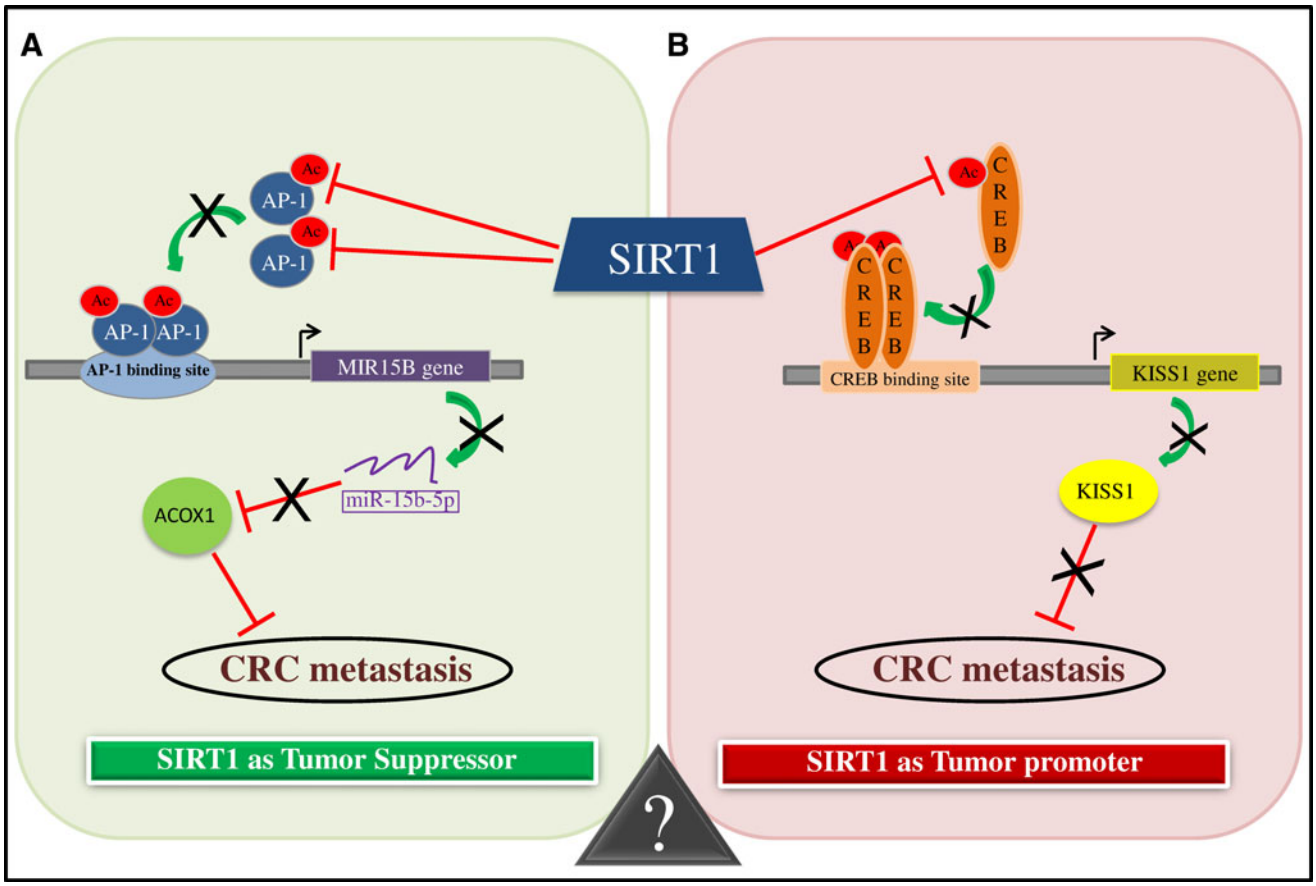

FIG. 1. Contradictory functions of SIRT1 in human colorectal tumorigenesis process. **(A)** SIRT1 deacetylates and inactivates AP-1, unactivated AP-1 is incapable of binding and promoting miR-15b-5p transcription, which, in turn, prevents miR-15b-5p from binding and inactivating ACOX1, that leads to CRC metastasis inhibition (Sun et al., 2017). **(B)** SIRT1 deacetylates and inactivates KISS1 transcriptional activator CREB, which prevents the expression of the tumor metastasis suppressor KISS1 and subsequently promoting CRC metastasis (Shen et al., 2016). CRC, colorectal cancer.

SIRT1 in cancer stem-like cells of CRC, cancer stem cells (CSCs) that have the ability of self-renewal and differentiation. The authors also highlighted an underlying association between SIRT1 and cancer stem-like cells. They found that SIRT1 inhibition in CRC cells led to an increased p53 expression. SIRT1 deficiency also led to a decrease in CD133+ percentage, a common stem cell marker that characterizes colorectal CSCs. Furthermore, SIRT1 inhibition led to decreased expression of several stemness-associated genes such as *Oct4*, *Nanog*, *Cripto*, *Tert*, and *Lin28*. Moreover, the authors observed a significant reduction in colorectal tumorigenicity by knocking down SIRT1 expression using SIRT1 small hairpin RNA *in vivo*. They postulated that SIRT1 plays a prosurvival role in CRC by keeping the stemness of cancer stem-like cells, as well maintaining the ability of colony and sphere formation in CRC cells (Chen et al., 2014).

Whereas Shen et al. (2016) were interested in the role of SIRT1 in distant metastasis, the authors revealed an SIRT1/CREB/KISS1 signaling pathway. They showed that SIRT1 knockdown decelerated the progress of CRC metastasis by functionally reducing the invasion and migration of CRC cells both *in vivo* and *in vitro*. SIRT1 knockdown also enhanced the chemosensitivity of CRC cells to 5-fluorouracil and oxaliplatin, two chemotherapeutic drugs used for clinical treatment of CRC. Furthermore, the authors demonstrated that SIRT1 upregulates the metastasis of CRC by downregulating KISS1 expression, a major tumor metastasis suppressor. KISS1 downregulation was realized through deacetylating and subsequently inactivating CREB transcriptional activity, a direct SIRT1 target that binds and triggers KISS1 transcription (Fig. 1B). Therefore, the authors concluded that SIRT1 actively contributes to the distant metastasis of CRC through SIRT1/CREB/KISS1 signaling pathway (Shen et al., 2016).

In conclusion, it appears that the controversy surrounding SIRT1 role in colorectal tumorigenesis has not been completely resolved. Consistent with recent findings, SIRT1 seems to have a bivalent role in the pathogenesis of breast cancer as well as CRC (Rifaï et al., 2017). Therefore, more in-depth studies are needed to be done to pin down the functional role of SIRT1 in the pathogenesis of CRC.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Address correspondence to:
Dominique Bernard-Gallon, PhD
Department of Oncogenetics
Centre Jean Perrin, CBRV
28 Place Henri Dunant
Clermont-Ferrand 63001
France

E-mail: dominique.gallon-bernard@clermont.unicancer.fr

Abbreviations Used

ACOX1 = peroxisomal acyl-coenzyme A oxidase 1
AP-1 = activator protein 1
CRC = colorectal cancer
CREB = cAMP response element binding
CSCs = cancer stem cells
KISS1 = kisspeptin-1
OS = overall survival
SIRT1 = silent mating type information regulation 2 homolog 1

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Conclusion de la publication 5

Dans cette lettre, on a mis en évidence les fonctions contradictoires de SIRT1 dans le processus de tumorigenèse du CCR humain.

Beaucoup d'études ont détecté une réduction d'expression de SIRT1 dans CCR, et ont démontré son rôle dans l'inhibition du développement du cancer. Cependant, d'autres études ont montré une abondance d'expression de SIRT1 dans les tumeurs colorectales, et ont prouvé le rôle actif de SIRT1 dans la prolifération et la migration des cellules cancéreuses.

Par exemple, SIRT1 réprime la transcription de miR-15b-5p en desacétylant et inactivant le coactivateur transcriptionnel AP-1, ce qui entraine l'inhibition de la métastase du CCR. En revanche, SIRT1 réprime la transcription de KISS1, qui est un suppresseur de métastase tumorale, ce qui favorise par la suite la métastase du CCR.

En conclusion, il semble que la controverse entourant le rôle de SIRT1 dans la tumorigenèse du CCR n'ait pas été complètement résolue. Par conséquent, plus d'études en profondeur seront nécessaires pour déterminer le rôle fonctionnel de SIRT1 dans la pathogenèse du CCR.

B. Identification des Enzymes Épigénétiques Modulatrices de la Marque H3K4ac dans le Cancer du Sein.

1. Caractérisation du Rôle Épigénétique de SIRT1 dans le Cancer du Sein, Son Implication dans la Progression Tumorale et la Désacétylation de H3K4ac

Présentation de la publication 6

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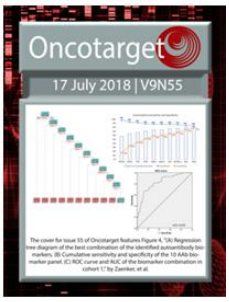
Research Paper

SIRT1-dependent epigenetic regulation of H3 and H4 histone acetylation in human breast cancer

Rifaï K, Judes G, Idrissou M, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D..



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L'implication de SIRT1 dans l'apparition et la progression de la pathogenèse du cancer du sein humain a été identifiée et étudiée de manière approfondie au cours des dernières années. Cependant, la régulation épigénétique des cibles d'histones H3 et H4 dépendante de SIRT1, et par conséquent de l'expression génique dans le cancer du sein, n'a pas encore été étudiée.

Dans cette étude, nous avons élucidé le rôle épigénétique de SIRT1 dans la carcinogenèse mammaire, et investigué les mécanismes épigénétiques par lesquels SIRT1 régule les profils d'acétylation des histones H3 et H4 des histones dans le cancer sporadique du sein.

En utilisant une cohorte de 135 tumeurs mammaires humaines et leurs tissus normaux appariés, ainsi que 5 lignées cellulaires mammaires représentatives des sous-types moléculaires, nous avons examiné la relation fonctionnelle entre SIRT1 et la marque H3K4ac, et étudié la corrélation entre SIRT1 et les trois épi-marques H3K4ac, H3K9ac et H4K16ac dans tous les sous-types intrinsèques de la maladie.

Nous avons également étudié l'interaction entre SIRT1 et les marques d'histones H3 sur les promoteurs de 6 gènes cibles fortement impliqués dans la tumorigenèse mammaire:

- Les gènes *AR, ERS1, ERS2*, qui codent pour les récepteurs hormonaux AR, ER-α, et ER-β, respectivement.
- *BRCA1*, qui code pour le suppresseur de tumeur BRCA1.
- *EZH2* et *EP300* qui codent pour les enzymes modifiant d'histones : les oncogènes EZH2 et p300.

En outre, une inhibition de SIRT1 par des siRNAs spécifiques nous a permis d'observer et de visualiser la régulation épigénétique différentielle dépendante de SIRT1 des marqueurs d'histones H3, et par conséquent, la dérégulation de l'expression des 6 gènes cibles dans le cancer du sein.

Le présent rapport éclaircit la controverse en cours concernant le comportement de SIRT1 dans le cancer du sein.

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Research Paper

SIRT1-dependent epigenetic regulation of H3 and H4 histone acetylation in human breast cancer

Khaldoun Rifaï[1,2], Gaëlle Judes[1,2], Mouhamed Idrissou[1,2], Marine Daures[1,2], Yves-Jean Bignon[1,2], Frédérique Penault-Llorca[2,3] and Dominique Bernard-Gallon[1,2]

[1]Department of Oncogenetics, Centre Jean Perrin, CBRV, Clermont-Ferrand 63001, France

[2]INSERM, UMR 1240, IMoST Imagerie Moléculaire et Stratégies Théranostiques, Clermont-Ferrand 63005, France

[3]Department of Biopathology, Centre Jean Perrin, Clermont-Ferrand 63011, France

Correspondence to: *Dominique Bernard-Gallon,* ***email:*** *dominique.gallon-bernard@clermont.unicancer.fr*

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ABSTRACT

Breast cancer is the most frequently diagnosed malignancy in women worldwide. It is well established that the complexity of carcinogenesis involves profound epigenetic deregulations that contribute to the tumorigenesis process. Deregulated H3 and H4 acetylated histone marks are amongst those alterations. Sirtuin-1 (SIRT1) is a class-III histone deacetylase deeply involved in apoptosis, genomic stability, gene expression regulation and breast tumorigenesis. However, the underlying molecular mechanism by which SIRT1 regulates H3 and H4 acetylated marks, and consequently cancer-related gene expression in breast cancer, remains uncharacterized. In this study, we elucidated SIRT1 epigenetic role and analyzed the link between the latter and histones H3 and H4 epigenetic marks in all 5 molecular subtypes of breast cancer. Using a cohort of 135 human breast tumors and their matched normal tissues, as well as 5 human-derived cell lines, we identified H3k4ac as a new prime target of SIRT1 in breast cancer. We also uncovered an inverse correlation between SIRT1 and the 3 epigenetic marks H3k4ac, H3k9ac and H4k16ac expression patterns. We showed that SIRT1 modulates the acetylation patterns of histones H3 and H4 in breast cancer. Moreover, SIRT1 regulates its H3 acetylated targets in a subtype-specific manner. Furthermore, SIRT1 siRNA-mediated knockdown increases histone acetylation levels at 6 breast cancer-related gene promoters: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2* and *EP300*. In summary, this report characterizes for the first time the epigenetic behavior of SIRT1 in human breast carcinoma. These novel findings point to a potential use of SIRT1 as an epigenetic therapeutic target in breast cancer.

INTRODUCTION

Breast cancer remains the leading cause of cancer death among females in less developed countries, and second leading cause of cancer death in more developed countries after lung cancer [1]. The occurrence of breast cancer is a complex, multifactorial process that is regulated by a number of different genes at different tumor formation stages [2]. Breast cancer is also characterized by its molecular and clinical heterogeneity with variations in gene expression profiles among women [3]. The St. Gallen molecular classification divides breast tumors into 5 distinct subtypes in ascending order of tumor aggressiveness [4]. Luminal A, luminal B (HER2-) and luminal B (HER2+), these 3 subtypes are included in the Hormone Receptor-positive Breast Cancer (HRBC). HER2-enriched or HER2 Breast Cancer (H2BC). And finally triple-negative breast cancer (TNBC), also known as basal-like, which is characterized as very aggressive compared to the other molecular subtypes [5]. 85 to 90%

of breast tumors are called sporadic or non-hereditary tumors that can spawn due to many environmental risk factors. Sporadic breast tumors are especially characterized by the presence of underlying abnormalities in their epigenome [6].

The complexity of carcinogenesis cannot be represented by genetic mutations alone, but also involves profound epigenetic alterations. The epigenetic regulation of the genome includes among others, histone post-translational modifications (PTMs) [7]. Deregulated histone PTMs or histone marks are considered as biomarkers of cancer prognosis and were shown to predict patient outcome in various human carcinomas [8, 9]. In breast cancer, analysis of human breast tumors revealed a highly significant correlation between global histone marks patterns and tumor molecular phenotypes, prognostic factors, and clinical outcome [10, 11]. Lysine acetylation at the N-terminus tails of histones H3 and H4 is classically associated with increased gene expression. The epigenetic marks (epi-marks) H3 lysine 4 (H3K4ac), lysine 9 (H3K9ac) and H4 lysine 16 (H4K16ac) are well-characterized acetylated marks that are particularly enriched at transcriptionally active gene promoters [12] [13]. H3K9ac and H4K16ac have well-defined roles in regulating chromatin structure. Their deacetylation causes the formation of higher-order chromatin compaction and subsequently transcription repression, as neatly described by Vaquero *et al*. [14, 15]. Global loss of H4K16ac has been shown to be a hallmark of human cancer and associated with early tumor formation stages [16]. Also, H3K9ac has been shown to be underexpressed in breast cancer, as well as other cancers, and its decrease was correlated with tumor progression and poor clinical outcome [10]. On the other hand, few reports studied the role of H3K4ac in cancer. The function of H3K4ac was often related to that of H3K4me3, since both acetylation and methylation of lysine (K4) residue are associated with active transcription [17]. In addition, the epigenetic acyl-lysine 'eraser' of H3K4ac histone marker has not been yet identified in humans. In a recent study, Messier *et al.* explored the dynamics of H3K4ac in 2 breast cancer cell lines. They demonstrated the latter as an indicator of deregulated cancer-related pathways. They also uncovered a role of H3K4ac in predicting epigenetic changes associated with early stages of transformation [18].

Histone deacetylases (HDACs) are major actors of epigenetic regulation. Dysfunctional HDACs have been found to be closely related to the tumorigenesis process and cancer metastasis [19]. Due to their deacetylase activity of a broad spectrum of substrates, Sirtuins are considered to be master regulators of several basic cellular mechanisms [20]. Silent mating type information regulation 2 homolog 1 (SIRT1) is a NAD+-dependent class III HDAC. The founding member of the Sirtuins family is tightly implicated in the regulation of numerous key cellular processes including apoptosis and cell survival, DNA damage repair, chromatin remodeling, gene expression regulation, and cancer development and metastasis [20, 21]. It has been shown that SIRT1 regulates genome stability in part through deacetylation of N-terminus tails of acetylated histones: H1K26ac, H3K56ac, H2A variant H2A.Z, in addition to H3K9ac and H4K16ac [14, 15, 22]. SIRT1 also regulates the catalytic activity of a plethora of downstream non-histone targets. For example, SIRT1 can deacetylate and downregulate the activity of tumor suppressor enzymes such as p53 [23], p73 [24], E2F1 [25], and Forkhead box proteins FOXO transcription factors [26], but also oncogenes such as NF-kappaB [27], STAT3 [28], Survivin [29] and β-Catenin [30]. On the contrary, SIRT1 can upregulate the activity of other oncogenes like c-Myc [31] and HIF-1α [32]. As a result, the critical role of multifaceted SIRT1 in human carcinogenesis remains very controversial due to its contradictory functional roles [33, 34]. In breast cancer, both tumor-suppressive and tumor-promoting functions of SIRT1 have been reported and the controversy regarding SIRT1 role in the disease continues still.

SIRT1 implication in the occurrence and progression of breast cancer pathogenesis have been identified and extensively investigated over recent years. However, SIRT1-dependent epigenetic regulation of H3 and H4 acetylated histone marks, and consequently cancer-related gene expression in human breast cancer, has not been investigated yet. In this study, we examined for the first time the epigenetic mechanisms by which SIRT1 regulates the acetylation patterns of histones H3 and H4 epigenetic marks in sporadic breast cancer, we also investigated the link between SIRT1 and the 3 epi-marks H3K4ac, H3K9ac and H4K16ac in all 5 intrinsic subtypes of the disease. The present report adds a layer of clarity on the ongoing controversy of SIRT1 behavior in human breast carcinoma.

RESULTS

Description of the study cohort characteristics

The breast cancer molecular subtypes studied here spanned luminal A (n = 36, 26.7%), luminal B (HER2-) (n = 34, 25.2%), luminal B (HER2+) (n = 25, 18.5%), HER2- enriched (n = 15, 11.1%) and triple-negative breast cancer (TNBC) (n = 25, 18.5%) (Table 1). All patients were females aged 40 to 84 years (mean 64.6 ± SD 5.3). All tumors were graded according to the modified Scarff-Bloom-Richardson grading system (SBR) as grade 1 (n = 17), grade 2 (n = 69) and grade 3 (n = 49). Tumor size ranged from 0.4 to 7.8 cm (3.1 ± 0.7). Samples were ER-, PR- and HER2-positive in n = 95 (70.3%), n = 58 (42.9%) and n = 40 (29.6%) patients, respectively. We found an insignificant correlation between all intrinsic subtypes and age of patients (p = 0.643) and tumor size (p = 0.079). Luminal A and B (HER2-) subtypes presented a significant correlation with low SBR grade tumors, whereas luminal B (HER2+), HER2- enriched and triple-negative subtypes

Table 1: Clinico-pathological characteristics of the breast cancer patients included in this study

	Total	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2-enriched	Triple-negative	*P* value†
Patients, *n* (%)	*N* = 135 (100%)	*n* = 36 (26.7%)	*n* = 34 (25.2%)	*n* = 25 (18.5%)	*n* = 15 (11.1%)	*n* = 25 (18.5%)	
Age at diagnosis							0.643
45–65	66 (48.8)	21 (58.3)	15 (44.1)	12 (48)	8 (53.3)	10 (40)	
> 65	69 (51.2)	15 (41.6)	19 (55.8)	13 (52)	7 (46.6)	15 (60)	
SBR grade							**0.0001**
I	17 (12.5)	13 (36.1)	4 (11.7)	0	0	0	
II	69 (51.1)	21 (58.3)	24 (70.5)	11 (44)	6 (40)	7 (28)	
III	49 (36.4)	2 (5.5)	6 (17.6)	14 (56)	9 (60)	18 (72)	
Size of tumor (cm)							0.079
< 1.5	31 (22.9)	11 (30.5)	9 (26.4)	5 (20)	2 (13.3)	4 (16)	
1.5–2.5	61 (45.1)	19 (52.7)	18 (52.9)	11 (44)	5 (33.3)	8 (32)	
> 2.5	43 (31.8)	6 (16.6)	7 (20.5)	9 (36)	8 (53.3)	13 (52)	
ER status (%)							**0.0001**
Positive	95 (70.3)	36 (100)	34 (100)	25 (100)	0	0	
Negative	40 (29.6)	0	0	0	15 (100)	25 (100)	
PR status (%)							**0.0001**
0%–50%	20 (14.8)	7 (19.4)	8 (23.5)	5 (20)	0	0	
51%–100%	38 (28.1)	29 (80.5)	7 (20.5)	2 (8)	0	0	
Negative	77 (57)	0	19 (55.8)	18 (78)	15 (100)	25 (100)	
HER2 status (%)							**0.0001**
Positive	40 (29.6)	0	0	25 (100)	15 (100)	0	
Negative	95 (70.3)	36 (100)	34 (100)	0	0	25 (100)	
Ki67 status (%)							**0.0001**
≤ 20%	53 (39.2)	29 (80.5)	8 (23.5)	7 (28)	4 (26.6)	5 (20)	
> 20%	82 (60.7)	7 (19.4)	26 (76.4)	18 (72)	11 (73.3)	20 (80)	

Abbreviations: ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal growth factor Receptor 2, Ki-67: cellular marker for proliferation.
†Pearson's Chi-square test.

exhibited high SBR grade tumors ($p < 0.001$). As for the hormonal receptors status, a clear distinction between the 5 molecular subtypes can be noted as per the molecular classification of St. Gallen. The clinico-pathological variables of the 135 breast cancer patients are presented in (Table 1).

Inverse correlation between SIRT1 and H3k4ac, H3k9ac and H4k16ac global expression patterns in breast tumors versus matched normal tissues

In order to investigate the epigenetic role of the histone deacetylase SIRT1 in sporadic breast cancer, we began our studies in *ex-vivo* by assessing the relative expression levels of SIRT1 and the 3 epigenetic marks H3k4ac, H3k9ac and H4k16ac in all 5 molecular subtypes of breast tumors and their matched normal tissue samples using immunoblot analysis (Figure 1A). The blots showed a significant upregulation of SIRT1 expression levels in luminal and HER2-enriched subtypes and significant downregulation in TNBC subtype, in comparison with their matched normal tissues (Figure 1B). The differential expression pattern of SIRT1 across the 5 molecular subtypes was characterized in our earlier study [35]. In contrast, the expression levels of H3k4ac, H3k9ac, and H4k16ac were significantly reduced in luminal and HER2-enriched subtypes and relatively upregulated in TNBC subtype, all compared to their matched normal tissues

(Figure 1B). This inverse correlation provides a causal link between the expression patterns of SIRT1 and the 3 epi-marks in human breast cancer.

SIRT1 simultaneously colocalizes and physically interacts with histone H3 acetylated marks in human breast cancer

In order to determine whether HDAC SIRT1 interacts with histone H3 acetylated epi-marks in human breast cancer, we began by performing chromatin immunoprecipitation (ChIP) assays of SIRT1 on 50 breast tumors and their 50 matched normal tissues (n = 10 tumors for each of the 5 molecular subtypes). The assays were then analyzed with real-time quantitative PCR (q-PCR) targeting the promoters of a gene panel consisted of 6 genes. The targeted genes are strongly deregulated and directly implicated in the pathogenesis of breast cancer, as follows: *ERS1, ERS2* and *AR* genes that code for the Estrogen receptors (ER-α), (ER-β) and the Androgen

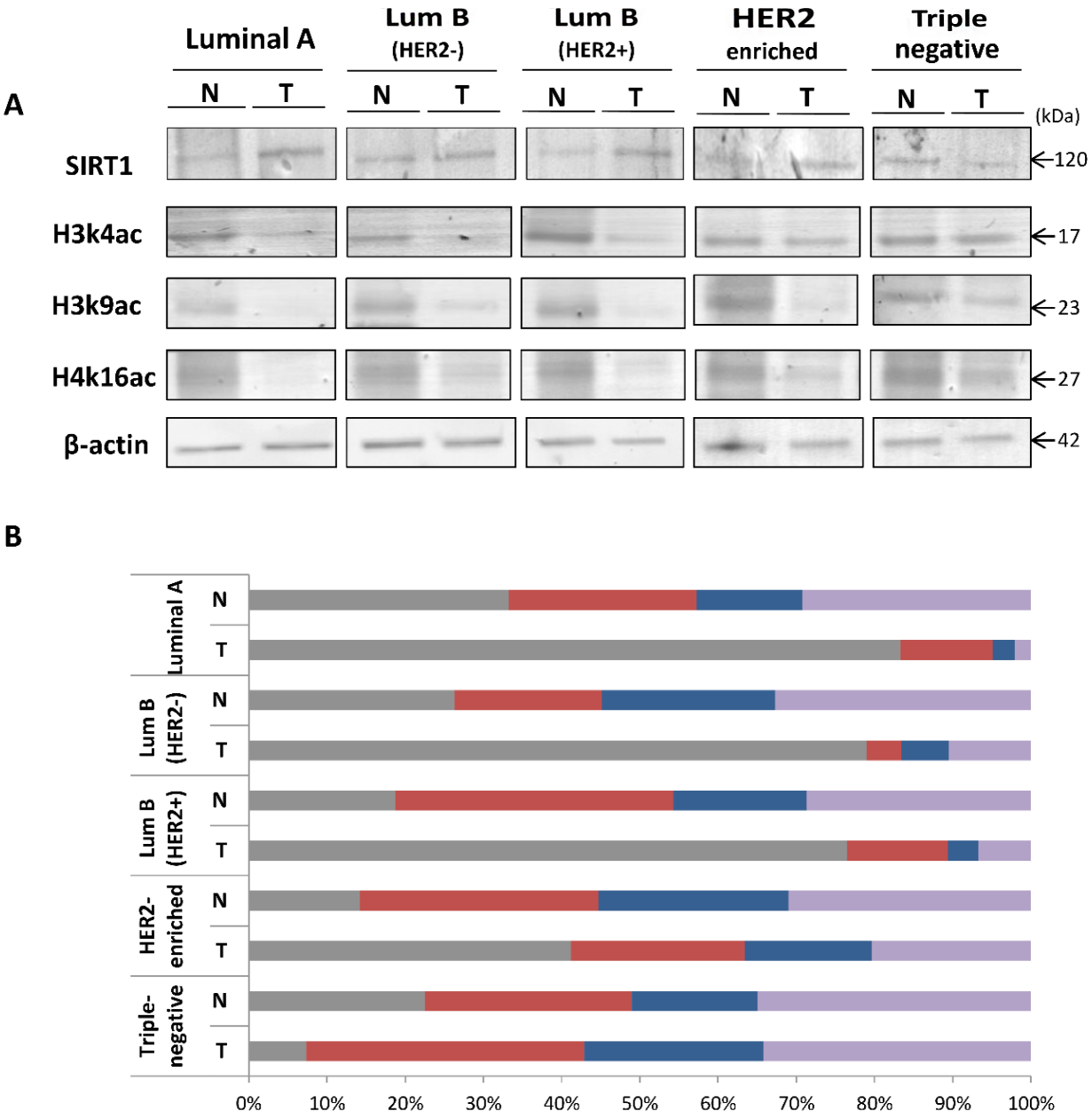

Figure 1: Differential expression patterns of SIRT1, H3k4ac, H3k9ac and H4k16ac in the 5 molecular breast tumor subtypes compared to matched normal tissues. (**A**) Equal amounts of proteins were immunoblotted with anti-SIRT1 Ab (120 kDa), anti-H3k4ac Ab (17 kDa), anti-H3k9ac Ab (23 kDa) and anti-H4k16ac Ab (27 kDa). β-actin (42 kDa) served as an internal loading control. (**B**) Relative expression levels were evaluated using Quantity One software and normalized against the internal control β-actin. Each bar represents the percentage contribution of each of the 4 proteins compared to the total set as (100%). All experiments were performed in triplicate fashion. N: Normal, T: Tumor.

receptor (AR) respectively, the tumor suppressor gene *BRCA1,* and *EZH2* and *EP300* genes coding for histone modifying enzymes (EZH2) and (p300) respectively. The results of ChIP assays showed a significant increase of SIRT1 enrichment on promoters of targeted genes in HRBC and H2BC subtypes, and less significantly in TNBC subtype in comparison to matched normal tissues. The data evoke the possibility that SIRT1 plays a role in the epigenetic regulation of these genes expression in breast cancer (Figure 2). A multi-way analysis of variance (ANOVA) test showed a significant difference of SIRT1 enrichment patterns on gene promoters across all subtypes. Tukey's range test was then used for multiple comparisons to identify sample means that are significantly different from each other. Two factors were taken into account when performing the statistical procedures: breast cancer molecular subtype (Group effect) and targeted gene type (Gene effect). The post-hoc analysis distinguished 3 distinct patterns of SIRT1 enrichment depending on human breast tumors subtypes, SIRT1 was found to be most enriched on target gene promoters in luminal B subtypes, then luminal A and HER2-enriched subtypes and finally, least enriched in TNBC subtype (Figure 3A). However, there was no significant discrimination of SIRT1 enrichment in relation to different types of genes (Figure 3B).

After confirming the presence of SIRT1 on target gene promoters, we proceeded to investigate whether SIRT1 specifically interacts with histone H3 acetylated marks on those promoters by performing ChIP followed by re-ChIP assays on 110 breast tumors from all 5 molecular subtypes and their 110 matched normal tissues (n = 26 luminal A, n = 24 luminal B (HER2-), n = 20 luminal B (HER2+), n = 15 HER2-enriched and n = 25 triple-negative). Breast tissues were first assayed by ChIP using anti-H3k4ac or anti-H3k9ac Abs. The obtained samples were then re-immunoprecipitated a second time with anti-SIRT1 Ab. Finally, the immunoprecipitates were analyzed by real-time q-PCR targeting the promoters of the breast cancer-related gene panel previously described. The results showed a simultaneous co-occupancy of SIRT1 with H3k4ac and with H3k9ac on all 6 gene promoters across all 5 subtypes in comparison to matched normal tissues (Figure 4), suggesting that SIRT1 could affect the expression of our targeted genes through epigenetic modification of histone H3 lysine 4 (K4) and lysine 9 (K9) on their promoters. The results also showed a great discrepancy of SIRT1-H3k4ac/H3k9ac colocalization profiles that seem to differ depending

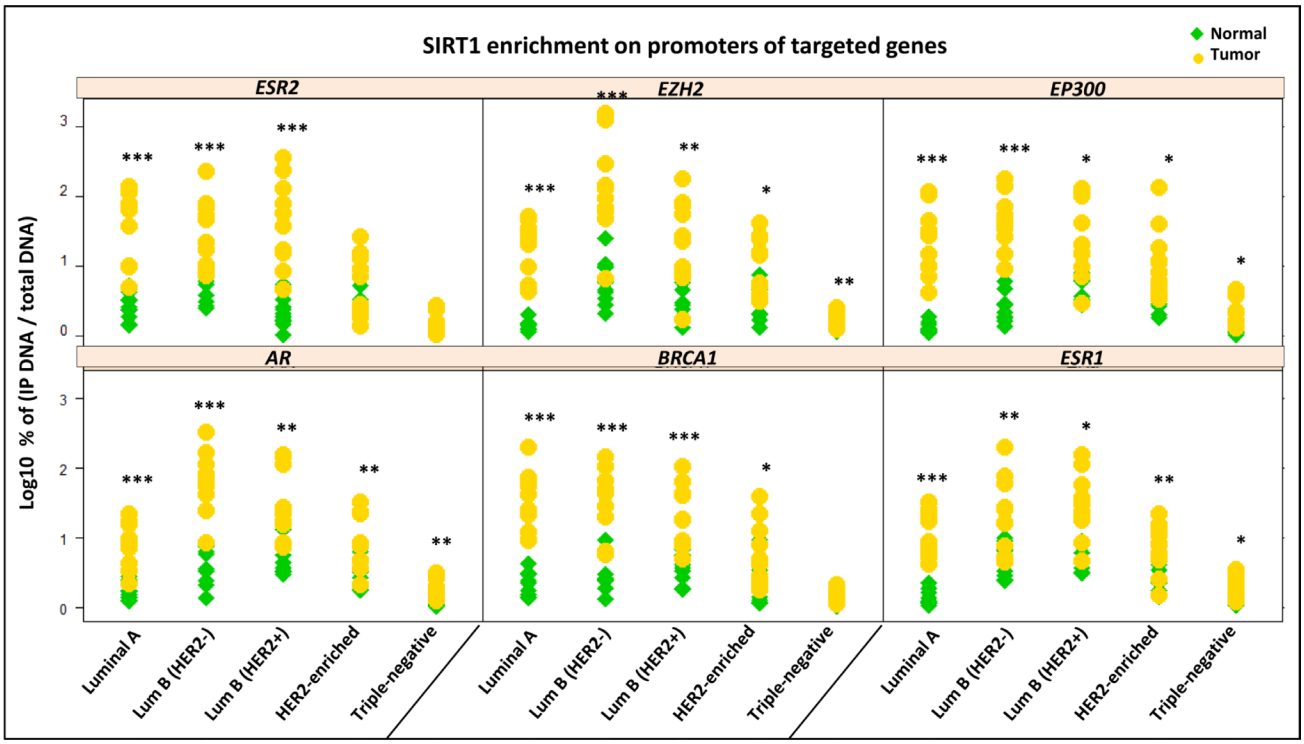

Figure 2: SIRT1 enrichment on promoters of 6 breast cancer-related genes in the 5 molecular breast tumor subtypes versus matched normal tissues. Column scatter plot showing the results of ChIP assays using anti-SIRT1 Ab on 50 breast tumors and their 50 matched normal tissues: n = 10 tumors for each of the 5 molecular subtypes. The efficiency of ChIP was calculated by real time quantitative PCR using the primers and probes of 6 targeted genes: *AR, BRCA1, ERS1, ERS2, EZH2* and *EP300.* The y-axis represents the log expression percent of (IP DNA/Total DNA) on target genes promoters. Statistically significant difference of SIRT1 enrichment in tumors versus normal tissues was analyzed by Student's t-test. P values were two-tailed, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

on molecular subtype, targeted gene type and studied epi-mark, suggesting that SIRT1- epigenetic regulation depends on multiple factors in different molecular subtypes. To further clarify this observation, multiple-group comparisons ANOVA test followed by Tukey's range test were carried out with three factors taken into account: molecular subtype (Group effect), gene type (Gene effect) and targeted epi-mark (Mark effect). The

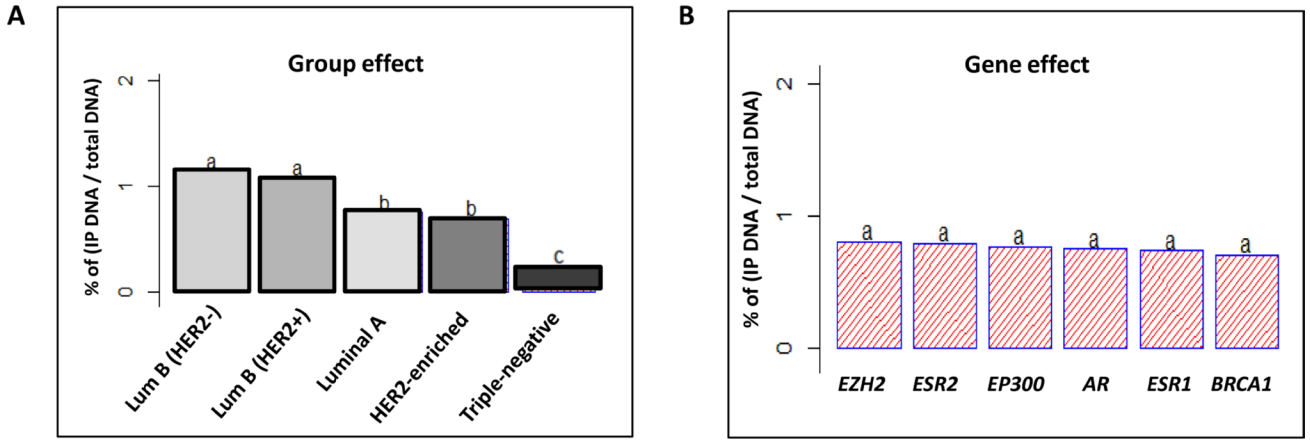

Figure 3: Tukey's post-hoc comparison of the means analyzing Group and Gene effects. ANOVA test followed by Tukey's multiple comparison test were performed on the results of 50 ChIP assays analyzed by q-PCR (**A**) The statistical analysis discerned 3 different SIRT1 enrichment patterns depending on tumor molecular subtype (Group effect). (**B**) Insignificant discrimination of SIRT1 enrichment in relation to variable gene types (Gene effect). The letters 'a', 'b' and 'c' indicate statistical significance between groups.

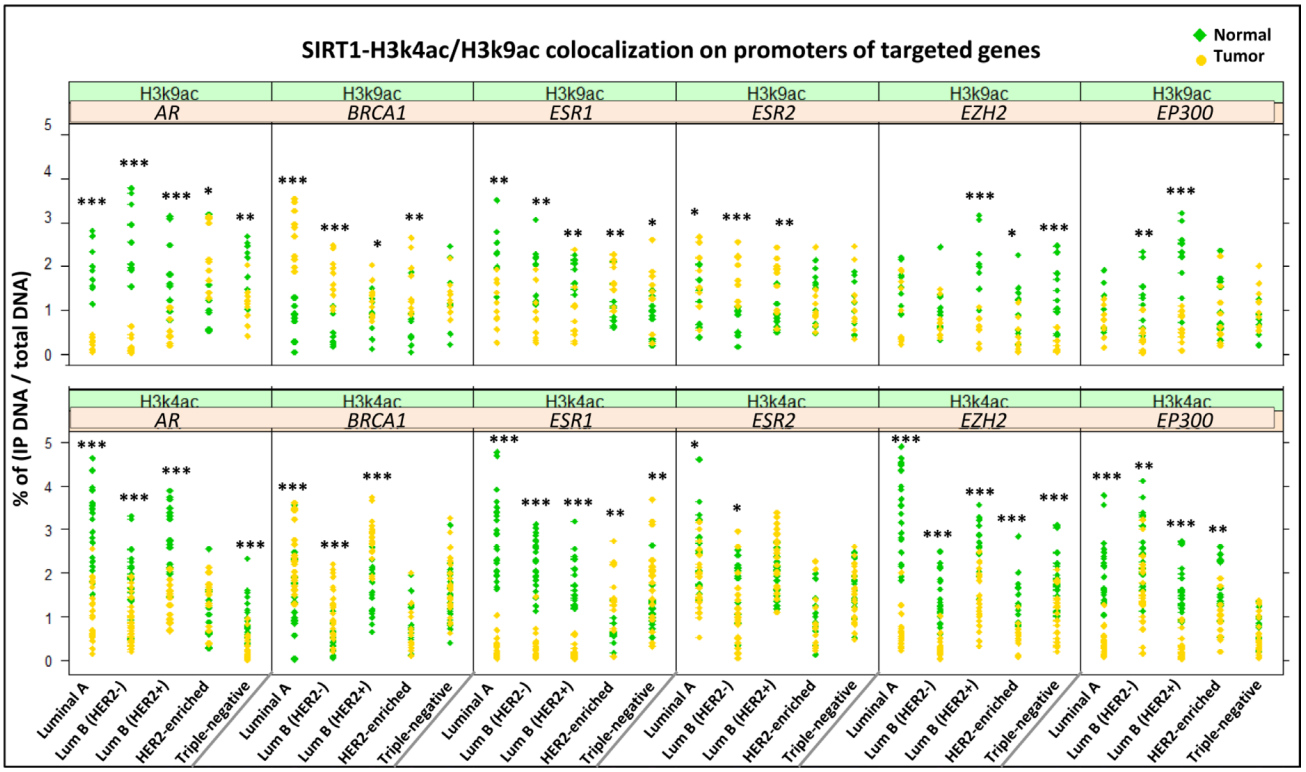

Figure 4: Simultaneous colocalization of SIRT1-H3k4ac and SIRT1-H3k9ac on targeted gene panel promoters across all 5 molecular subtypes versus matched normal tissues. Column scatter plot showing the results of ChIP followed by re-ChIP assays using anti-SIRT1, anti-H3k4ac and anti-H3k9ac Abs on 110 breast tumors and their 110 matched normal tissues. The breast tumors were divided as follows: $n = 26$ luminal A, $n = 24$ luminal B (HER2-), $n = 20$ luminal B (HER2+), $n = 15$ HER2-enriched and $n = 25$ triple-negative breast tumors. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR, BRCA1, ERS1, ERS2, EZH2* and *EP300.* The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Statistically significant difference of SIRT1 colocalization patterns in tumors versus normal tissues was analyzed by Student's *t*-test. *P* values were two-tailed, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

post-hoc analysis discerned 2 different SIRT1-H3k4ac/ H3k9ac colocalization profiles depending on tumor subtype (Figure 5A) and 3 different colocalization profiles depending on gene type (Figure 5B). Intriguingly, the statistical analysis showed that SIRT1 significantly colocalizes with H3k4ac over H3k9ac on targeted genes across all tumor subtypes (Figure 5C). Afterward, we proceeded to examine whether there is an actual direct interaction between HDAC SIRT1 and histone H3 epi-marks in breast cancer. To do so, we conducted several co-immunoprecipitation assays. Proteins were extracted from breast tumors from each of the 5 molecular subtypes and their matched normal tissues. Extracted proteins were at first immunoprecipitated with anti-H3k4ac or anti-H3k9ac Abs, the immunoprecipitates were then immunoblotted with anti-SIRT1 Ab. The co-immunoprecipitation assays highlighted a global physical interaction between SIRT1 and H3k4ac as well as H3k9ac across all molecular subtypes, implying that SIRT1 could directly deacetylate H3k4ac and H3k9ac in breast cancer. Additionally, the direct interaction between SIRT1 and both epi-marks is significantly increased in breast tumors compared to matched normal tissues (Figure 6).

Active role of SIRT1 in the deacetylation of H3k4 acetylated mark (H3k4ac) in human breast cancer

Unlike H3k9ac and H4k16ac, H3k4ac is not a known histone target of human histone deacetylase SIRT1. However, Silent Information Regulator 2 (SIR2), the highly conserved orthologue of mammalian SIRT1 in yeast, is the major HDAC of H3k4ac [36]. After uncovering an inverse correlation between SIRT1 and H3k4ac expression patterns, a simultaneous co-occupancy on the same genomic locus and a direct physical interaction between the two across all breast tumors subtypes, we hypothesized that HDAC SIRT1 could play an active role in the deacetylation of H3k4ac in human breast cancer.

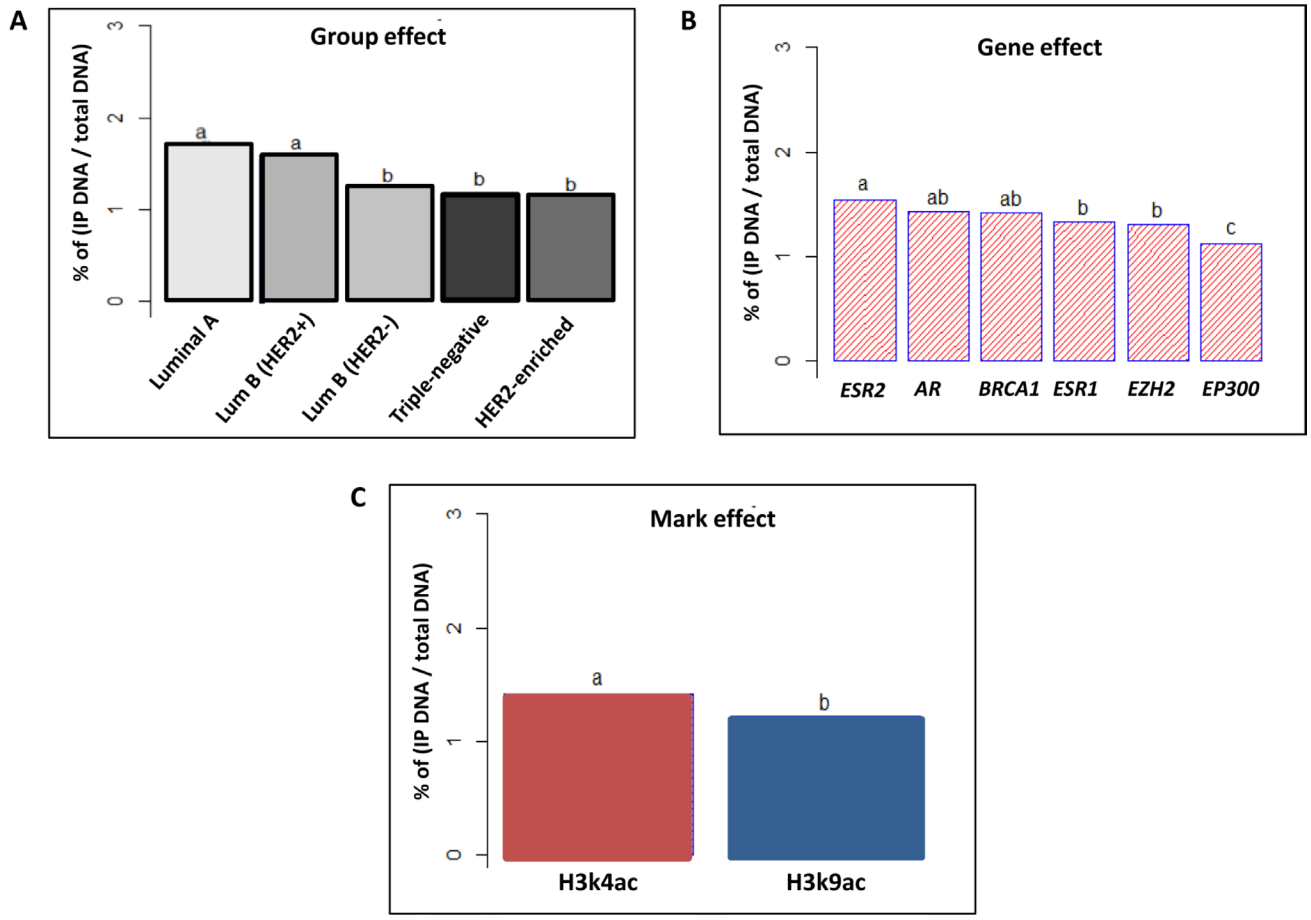

Figure 5: Tukey's post-hoc comparison of the means analyzing Group, Gene and Mark effects. ANOVA test followed by Tukey's multiple comparison test were performed on the results of 110 ChIP assays analyzed by q-PCR (**A**) The statistical analysis distinguished 2 distinct patterns of SIRT1-H3k4ac/H3k9ac collocation on target promoters depending on tumor molecular subtype (Group effect). (**B**) 3 distinct patterns of SIRT1-H3k4ac/ H3k9ac collocation depending on gene type (Gene effect). (**C**) Significant collocation of SIRT1 with H3k4ac over H3k9ac across all gene types and tumor subtypes (Mark effect)**.** The letters 'a', 'b' and 'c' indicate statistical significance between groups.

Inverse correlation between SIRT1 and H3k4ac expression patterns in 5 intrinsic subtype breast cancer cell lines

We began our *in-vitro* experiments by assessing the relative expression levels of SIRT1 and H3k4ac in 5 intrinsic subtype breast cancer cell lines using immunoblot analysis. (ER+) breast cancer cell lines: MCF-7 and T-47D were used as representatives of the luminal subtype, whereas (ER-) breast cancer cell lines: MDA-MB 453, MDA-MB 231 and MDA-MB 468 were used as representatives of the triple-negative subtype. The normal breast cell line MCF10A was used as a control. We observed significantly high expression of SIRT1 in MCF-7, T-47D, MDA-MB 453 and MDA-MB 231 cell lines, and relatively lower expression in MDA-MB 468, all compared to MCF10A cell line. At the opposite, significantly low H3k4ac expression levels were observed in all 5 intrinsic cell lines in comparison with the control cell line (Figure 7).

SIRT1-siRNA suppresses SIRT1 expression and induces a global increase in H3k4ac, as well as H3k9ac and H4k16ac expression levels in breast cancer cell lines

To gain insight into the mechanism responsible for the deacetylation of H3k4 acetylated mark (H3k4ac), we silenced SIRT1 expression with SIRT1-siRNA (small interfering RNA) in the 5 human-derived mammary cell lines previously described. We were interested in determining whether SIRT1 depletion could alter the relative expression patterns of H3k4ac, as well as H3k9ac and H4k16ac in breast cancer. After 48 hours of transfection, extracted proteins were subjected to immunoblot analysis (Figure 8A). The results showed a significant decrease of SIRT1 expression levels in all MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 transfected cell lines compared to non-transfected control cell lines (Figure 8B). More importantly, a significant increase of H3k4ac, as well as H3k9ac and H4k16ac expression levels, were observed in all 5 transfected cell lines compared to control cell lines (Figure 8B). SIRT1 depletion has led to increased H3k4 acetylation in 5 intrinsic subtype breast cancer cell lines, thus, the deacetylation of H3k4ac seems to be mainly dependent on SIRT1 histone deacetylase activity in breast cancer. Furthermore, the inverse correlation between SIRT1 and the 3 epi-marks expression patterns in transfected versus non-transfected cell lines is similar to that found in breast tumors compared to matched normal tissues. Therefore, SIRT1 seems to be directly responsible for the modulation of H3k4ac, as well as H3k9ac and H4k16ac expression patterns, obviously through direct deacetylation, in breast cancer.

SIRT1 knockdown modulates histone acetylation at targeted gene panel promoters in a subtype-specific manner

To further elucidate SIRT1 epigenetic role in human breast cancer, we conducted direct H3k4ac and

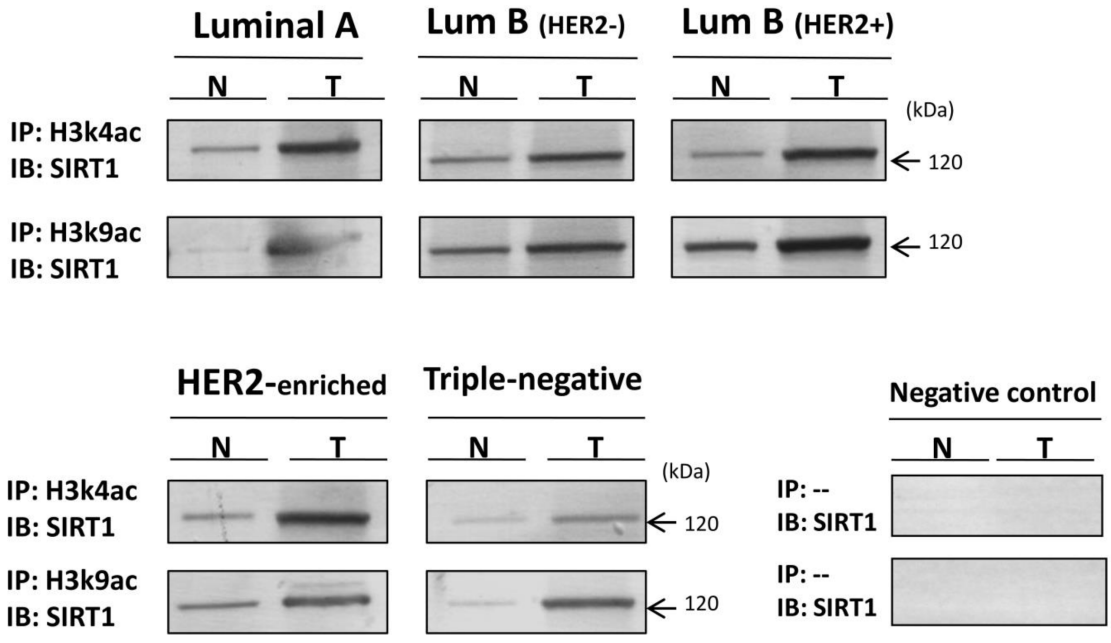

Figure 6: Global physical interaction between SIRT1 and H3k4ac/H3k9ac epi-marks across all molecular breast tumor subtypes compared to matched normal tissues. 100 to 500 μg of extracted proteins were at first immunoprecipitated using anti-H3k4ac or anti-H3k9ac Abs (IP), or immunoprecipitated without Ab (IP:--) that served as negative control. The immunoprecipitates were then immunoblotted (IB) with anti-SIRT1 Ab. All experiments were performed in triplicate fashion. N: Normal, T: Tumor.

H3k9ac ChIP assays on transfected cell lines. We wanted to investigate whether SIRT1 gene silencing could specifically alter the enrichment patterns of histone H3 acetylated epi-marks on the promoters of our target genes, and consequently, impact the targeted genes expression patterns in breast cancer. To explore this possibility, transfected and non-transfected MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cell lines were subjected to ChIP assays using anti-H3k4ac or anti-H3k9ac Abs. The samples were analyzed by real-time q-PCR targeting the promoters of the breast cancer-related gene panel: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2,* and *EP300*. The results showed a significant increase of both targeted epi-marks H3k4ac and H3k9ac on all 6 gene promoters across all 5 transfected cell lines in comparison to non-transfected control lines (Figures 9 and 10). Interestingly, 2 distinct patterns of H3k4ac and H3k9ac enrichment can be observed following SIRT1 knockdown, the patterns seem to be predominantly dependent on breast cancer cell line intrinsic subtype. The 2 epi-marks were found to be particularly enriched on *BRCA1* and *ESR2* gene promoters in luminal (ER+) subtype cell lines: MCF-7 (Figure 9A) and T-47D (Figure 9B). In contrast, both epi-marks were especially enriched on *AR*, *EZH2* and *EP300* promoters in triple-negative (ER-) subtype cell lines: MDA-MB 453 (Figure 10A), MDA-MB 231 (Figure 10B) and MDA-MB 468 (Figure 10C), implying that SIRT1 regulates its H3 histone targets principally depending on molecular subtype. In conclusion, SIRT1 siRNA-mediated knockdown has significantly increased the acetylation levels of H3k4ac and H3k9ac at the breast cancer-related gene panel promoters; thus, SIRT1 mediates the deacetylation of histone marks H3k4ac, as well as H3k9ac, in breast cancer. The results also revealed SIRT1 differential regulation of H3 acetylated epi-marks in a subtype-specific manner.

DISCUSSION

Breast cancer is the most commonly diagnosed cancer in women worldwide; it is a multifactorial genetic disease. Sporadic breast tumors represent 85 to 90% of all breast tumors and are especially characterized by an altered epigenome. Deregulated histone epigenome along with other epigenetic alterations play a crucial role in the initiation and progression of breast cancer [9, 37]. Sirtuin-1 is a class III histone deacetylase that can deacetylate both histone and non-histone targets. The mammalian counterpart of yeast SIR2 is deeply implicated in breast cancer development and metastasis. The contradictory functional roles of SIRT1 in breast cancer have been extensively studied over recent years. However, the underlying molecular mechanism by which HDAC SIRT1 regulates its acetylated histone targets, and consequently cancer-related gene expression in breast cancer, is still unknown. In this study, we identified a new prime target of SIRT1 in breast cancer, the acetylated H3k4 histone mark (H3k4ac). We also highlighted a SIRT1-dependent modulation of histones H3 and H4 acetylation patterns in breast cancer. Moreover, we revealed that SIRT1 regulation of its H3 acetylated targets depends greatly on gene type and molecular subtype. Furthermore, we showed that SIRT1 depletion increases histone H3 acetylation levels in a subtype-specific manner

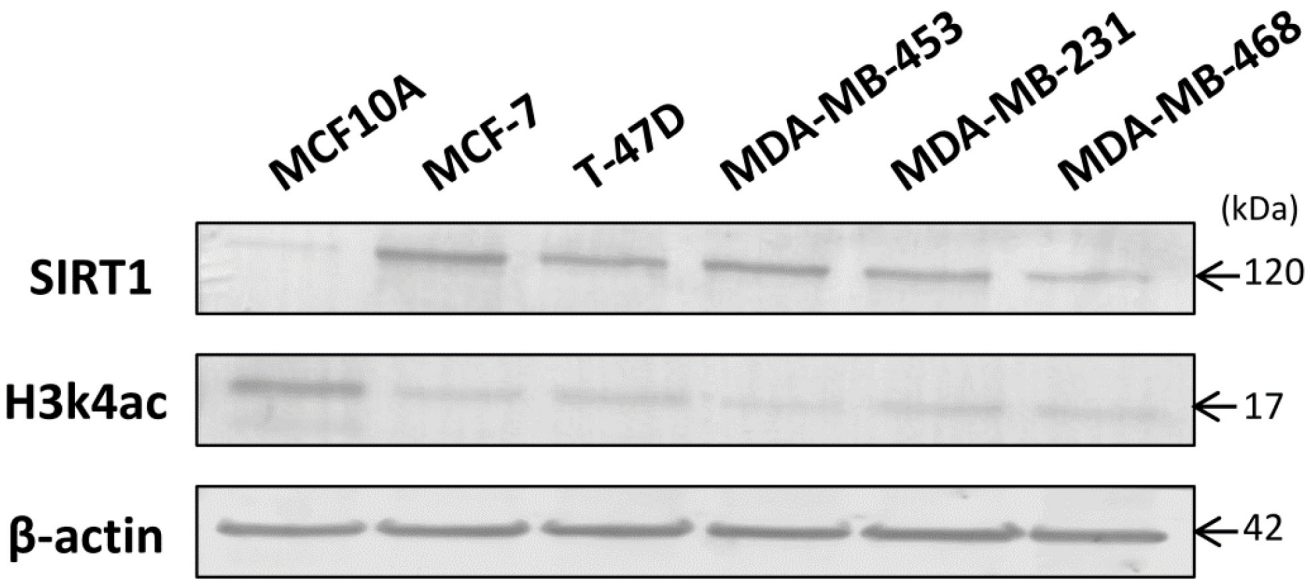

Figure 7: Inverse correlation between SIRT1 and H3k4ac expression levels in 5 intrinsic breast cancer cell lines compared to normal breast cell line. Proteins were extracted from MCF10A, MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cell lines. Equal amounts of extracted proteins were immunoblotted using anti-SIRT1 Ab (120 kDa) and anti-H3k4ac Ab (17 kDa). β-actin (42 kDa) served as an internal loading control.

at 6 breast cancer-related gene promoters: *AR, BRCA1, ERS1, ERS2, EZH2,* and *EP300,* suggesting that SIRT1 could play an active role in regulating their expression in breast cancer pathogenesis. This is the first report that characterizes the epigenetic behavior of SIRT1 in breast cancer and establishes its status as an epigenetic eraser in human breast carcinoma.

Alteration of histone epigenome is one of the earliest steps in oncogenic transformation. Since histone marks have a direct effect on cancer-related gene expression [7, 38], and since different breast cancer subtypes present distinct gene expression profiles [3] [39], it becomes essential to study the mechanisms of histone epigenome regulation in different subtypes of breast cancer pathogenesis. SIRT1 plays a major role in maintaining genome integrity, largely through regulation of epigenetic mechanisms. SIRT1 epigenetic regulation is realized through direct deacetylation of specific histone markers and controlling the activity of chromatin-modifying enzymes [22]. Histone marks H3k4ac, H3k9ac,

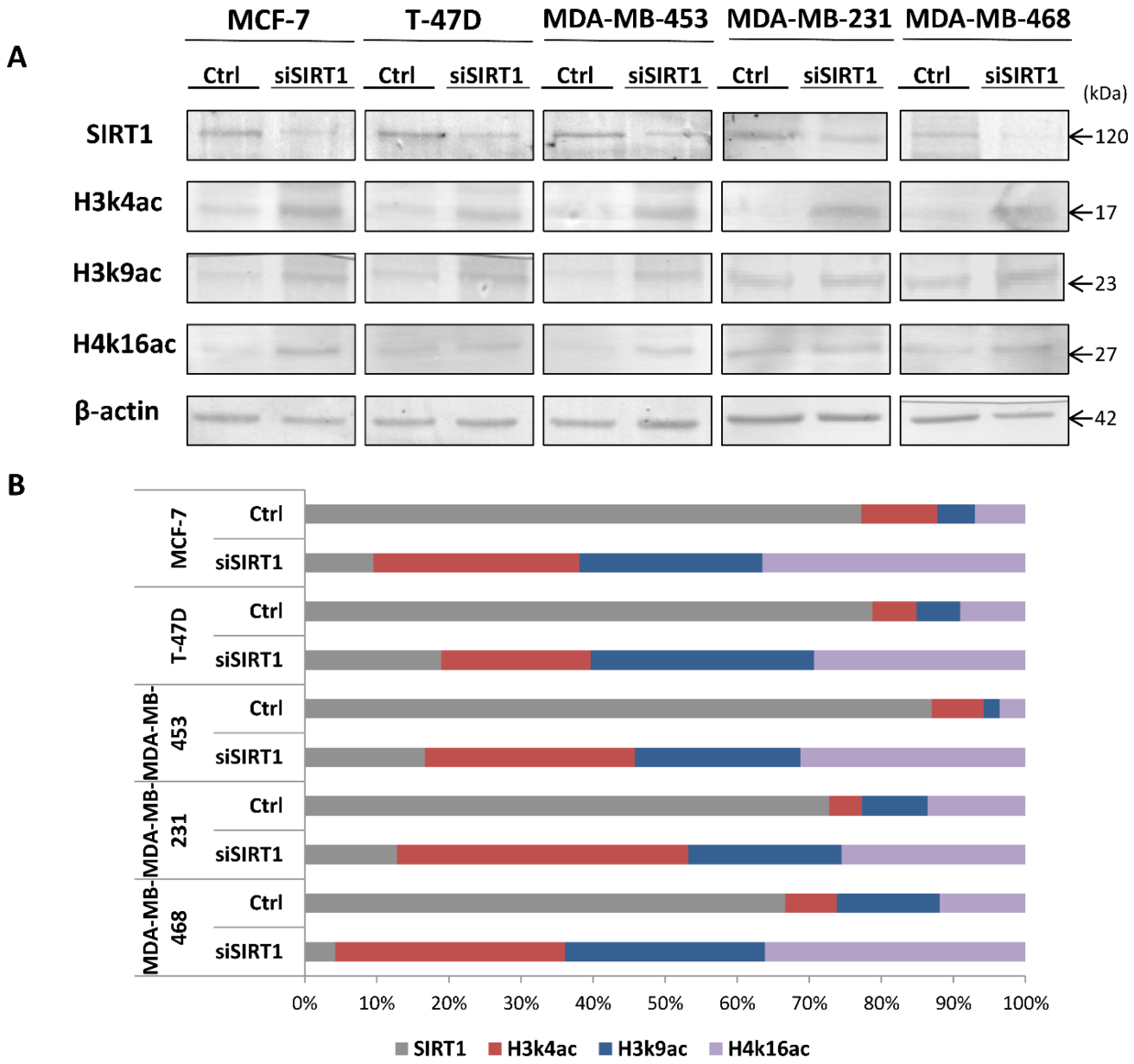

Figure 8: Control of SIRT1 gene silencing with SIRT1-siRNA and its impact on the expression patterns of targeted epi-marks H3k4ac, H3k9ac and H4k16ac *in-vitro.* (**A**) MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cells were transfected with SIRT1-siRNA (siSIRT1) or negative control siRNA (Ctrl). After 48 hours of transfection, equal amounts of proteins were immunoblotted with anti-SIRT1 Ab (120 kDa), anti-H3k4ac Ab (17 kDa), anti-H3k9ac Ab (23 kDa) and anti-H4k16ac Ab (27 kDa). β-actin (42 kDa) served as an internal loading control. (**B**) Relative expression levels were evaluated using Quantity One software and normalized against the internal control β-actin. Each bar represents the percentage contribution of each of the 4 proteins compared to the total set as (100%). All experiments were performed in triplicate fashion.

and H4k16ac are well-established epigenetic markers of active transcription and actively participate in gene expression [12, 13]. In this study, we showed that the 3 epi-marks relative expression patterns were significantly reduced in breast tumors compared to normal tissues, especially in HRBC and H2BC subtypes. Interestingly, SIRT1 is significantly upregulated in those particular subtypes, previously described in our earlier study [35]. This observation prompted us to suggest that SIRT1 is directly or indirectly responsible for the modulation of the 3 targeted marks in breast cancer. To validate this observation, we silenced SIRT1 expression *in-vitro* via small interfering RNA (siRNA). We opted to use 5 human mammary cell lines that represent the 2 main molecular subtypes of breast cancers: luminal (ER+) and triple-negative (ER-) subtypes. We chose MCF-7 and T-47D cell lines that are classically used as representatives of luminal subtype. Whereas (ER-) cell lines MDA-MB 453, MDA-

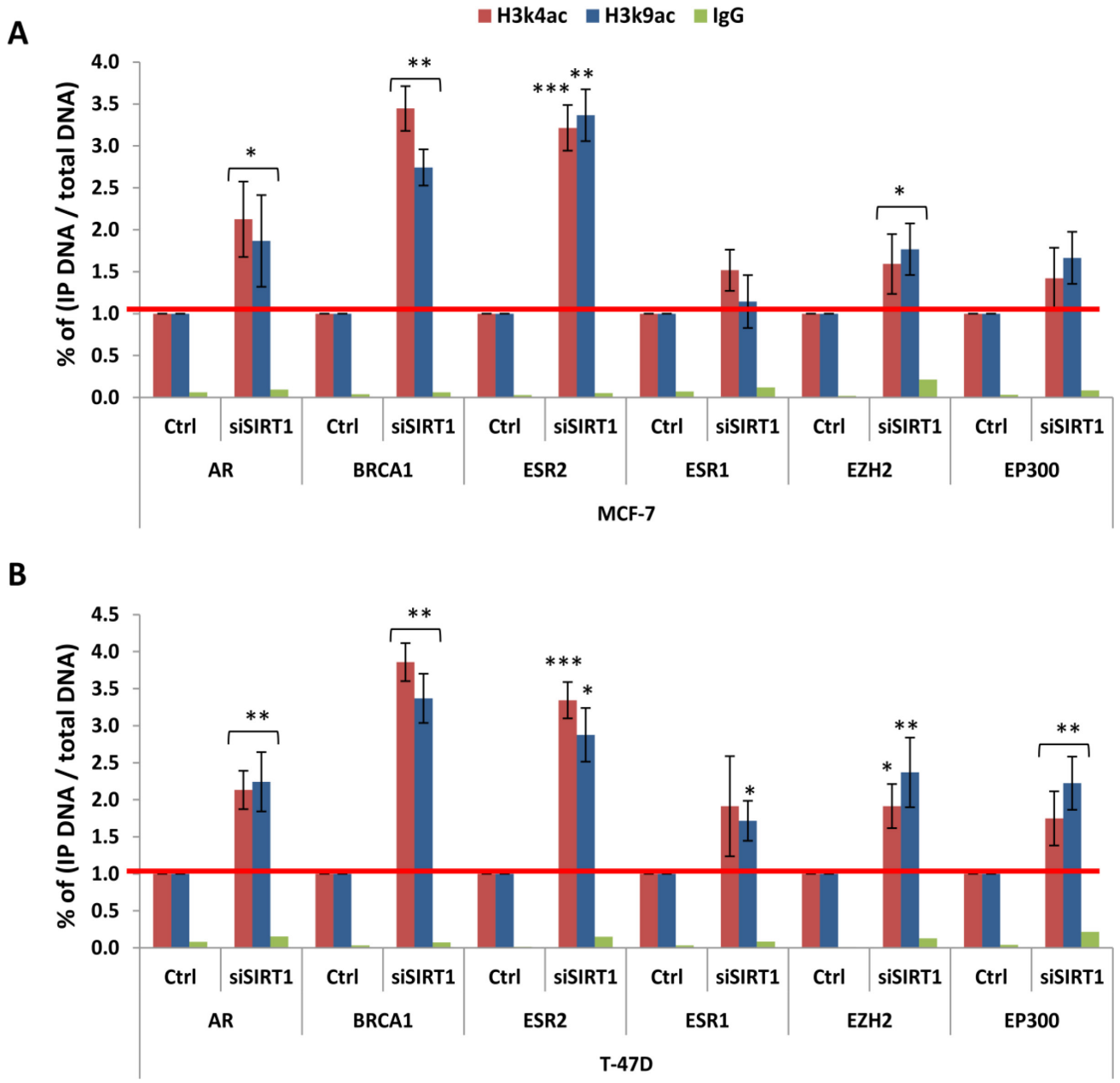

Figure 9: Impact of SIRT1 knockdown on the enrichment of H3k4ac and H3k9ac at targeted gene panel promoters in luminal subtype cell lines. Transfected (siSIRT1) and non-transfected (Ctrl) MCF-7 (**A**) and T-47D (**B**) cell lines were subjected to direct ChIP assays using anti-H3k4ac Ab, anti-H3k9ac Ab and non-immune IgG Ab serving as negative control. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2* and *EP300*. All data are presented as fold enrichment of transfected over control cell lines (set as 1). The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Each column represents the mean ± SD of 3 replicate experiments. *P* values were two-tailed, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

MB 231 and MDA-MB 468 were used as representatives of the 3 main molecular subtypes of triple-negative breast cancers: Luminal Androgen Receptor (LAR), Mesenchymal-like and Basal-like subtypes respectively, as elegantly characterized by Lehman *et al*. [40]. SIRT1 gene silencing has caused a significant increase of global

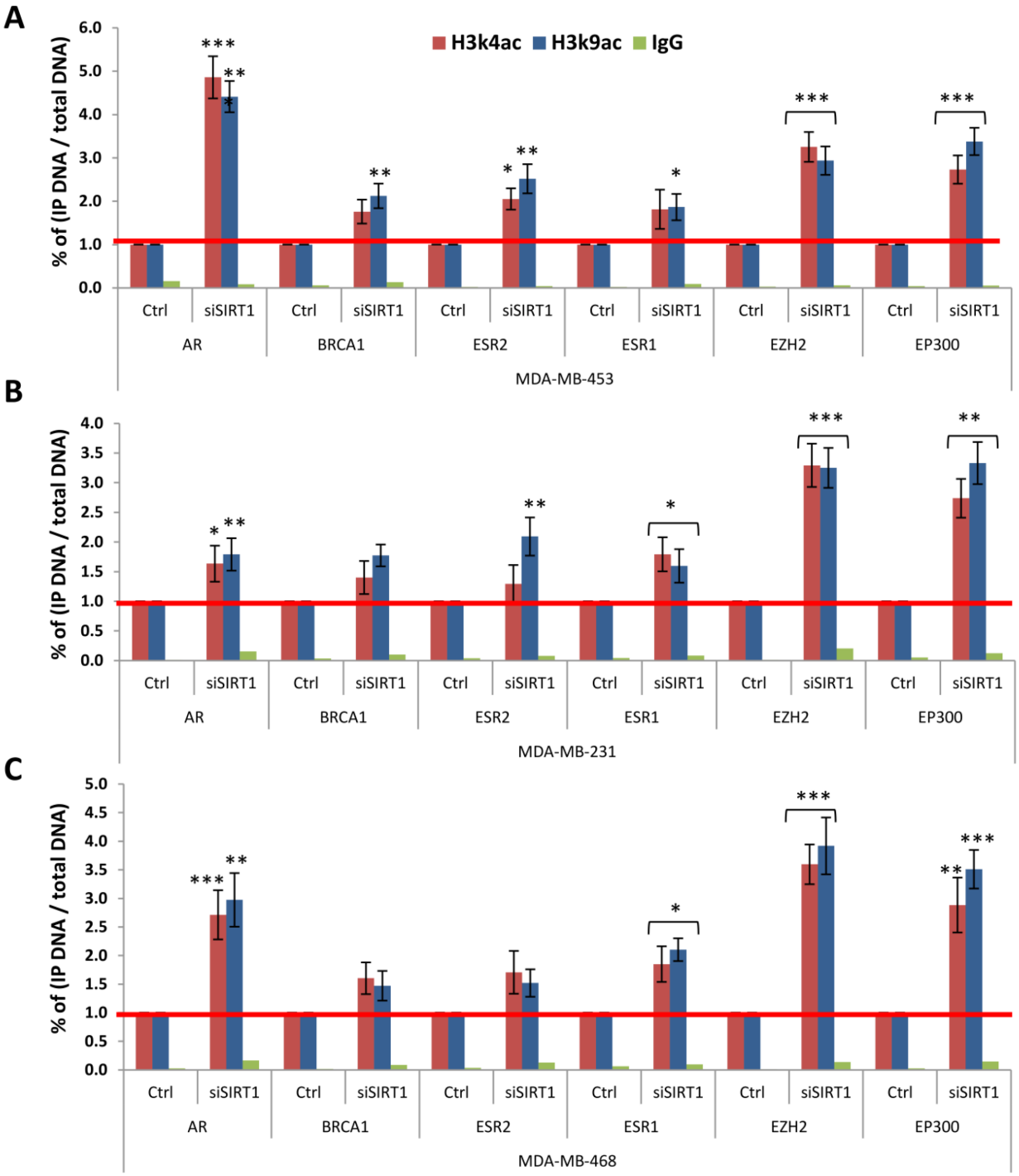

Figure 10: Impact of SIRT1 knockdown on the enrichment of H3k4ac and H3k9ac at targeted gene panel promoters in TNBC subtype cell lines. Transfected (siSIRT1) and non-transfected (Ctrl) MDA-MB 453 (**A**), MDA-MB 231 (**B**) and MDA-MB 468 (**C**) cell lines were subjected to direct ChIP assays using anti-H3k4ac Ab, anti-H3k9ac Ab and non-immune IgG Ab serving as negative control. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2* and *EP300*. All data are presented as fold enrichment of transfected over control cell lines (set as 1). The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Each column represents the mean ± SD of 3 replicate experiments. *P* values were two-tailed, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

expression levels of the 3 targeted epi-marks in all transfected lines versus non-transfected control lines, we thus concluded that SIRT1 is actively responsible for the modulation of H3k4ac, H3k9ac, and H4k16ac in luminal and triple-negative molecular subtypes of breast cancer.

To further analyze SIRT1 epigenetic role in sporadic breast cancer, we opted to study SIRT1 interaction with its H3 acetylated targets by carrying out ChIP and re-ChIP assays on 6 breast-cancer related genes: *AR, BRCA1, ERS1, ERS2, EZH2* and *EP300*. The targeted genes play major roles in breast cancer carcinogenesis, either by stimulating breast tumors development and tumor progression, such is the case for oncogenes *AR* [41], *ERS1* [42], *EZH2* [43] and *EP300* [44], or having anti-proliferative properties such as tumor suppressor genes *BRCA1* [45] and *ERS2* [46]. SIRT1 ChIP data implies that the latter could have an active role in regulating the expression of these genes in breast cancer either by directly modulating the epigenetic histone markers on their promoters and/or recruiting other chromatin-modifying complexes to that genomic area. However, SIRT1 less significant binding in TNBC subtype could be explained by its reduced expression in it. ChIP, re-ChIP as well as co-immunoprecipitation assays confirmed that SIRT1 physically interacts with and regulates its H3 histone targets H3k4ac and H3k9ac across the 5 molecular subtypes. However, SIRT1 epigenetic regulation is significantly discriminated by gene type and molecular subtype. These results are in line with the findings of Li *et al*. [11] who demonstrated widespread subtype-specific histone modifications in different molecular subtypes. In fact, SIRT1 also negatively regulates the activity of epigenetic 'writers' that deposit the histone markers [22]. It has been shown that SIRT1 interacts with and impairs the activity of histone acetyltransferases (HATs) p300 and MOF that are responsible for H3k9 and H4k16 acetylation, [47, 48] respectively. Therefore, SIRT differential epigenetic regulation in breast cancer seems to extend to both histone markers and their epigenetic 'writers'.

SIRT1 widespread regulation of multiple cancer-related enzymes often leads to its multifaceted functions in various cancers. In consequence, the contradictory roles of SIRT1 were demonstrated in various human malignancies. In colorectal cancer (CRC), both confirmed tumor-suppressive and tumor-promoting functions of SIRT1 have been reported [49]. In a previous study, we suggested a bivalent role of SIRT1 in breast cancer based on its differential expression patterns in human breast tumors. We suggested that SIRT1 most probably has an oncogenic role in HRBC subtypes and a tumor-suppressor role in TNBC subtype [35]. To further explore SIRT1 differential epigenetic regulation in breast cancer, we performed *in-vitro* ChIP analysis with H3k4ac and H3k9ac on SIRT1-siRNA transfected cell lines previously described. SIRT1 knockdown has generated 2 distinct profiles of both epi-marks enrichment on targeted gene promoters that corresponds to the 2 main molecular breast cancer subtypes. The results showed an increase of H3k4ac and H3k9ac expression by 3 to 4-fold on *BRCA1* and *ESR2* genes promoters in both (ER+) cell lines, indicating that SIRT1 contributes to their repression through epigenetic chromatin modification; hence exerting oncogenic properties in breast cancer luminal subtypes. These results are consistent with the findings of Elangovan *et al.* [50] and Ma *et al.* [51] who reported that SIRT1 overexpression in luminal breast cancer subtypes is positively correlated with an oncogenic behavior. At the opposite, in (ER-) cell lines MDA-MB 453, MDA-MB 231 and MDA-MB 468, SIRT1 deficiency induces a 2.5 to 4-fold increase of H3k4ac and H3k9ac expression on *EZH2* and *EP300* promoters, indicating that SIRT1 contributes to the 2 oncogenes repression; hence exerting tumor-suppressive properties in breast cancer triple-negative subtypes. However, a slight increase of H3k4ac and H3k9ac expression on *EZH2* promoter was also observed in the in (ER+) cell lines, implying that *EZH2* expression could be regulated in part by SIRT1 in different subtypes of breast cancer. We also noticed a dramatic increase of H3k4ac and H3k9ac expression by 4.5 to 5-fold on *AR* promoter in MDA-MB 453 cell line representative of the LAR subtype. In fact, LAR subtype or apocrine breast carcinoma is characterized by the expression of *AR* oncogene that contributes to breast tumorigenesis [40]. Therefore, SIRT1 seems to exert tumor-suppressive properties in apocrine breast cancer as well, through epigenetic repression of the *AR* oncogene. These findings are in line with the studies of Yi *et al.* [52] and Simic *et al.* [53] who reported that SIRT1 overexpression suppressed cancer metastasis and tumor cell invasion in triple-negative breast cancer. Based on the above knowledge, we suggest that SIRT1 selectively regulates its histone targets, and consequently gene expression and that SIRT1 epigenetic regulation in breast cancer seems to be predominantly governed by gene type and molecular subtype.

In conclusion, we analyzed an aspect of SIRT1 epigenetic control in breast tumors and established SIRT1 status as an epigenetic eraser in breast cancer. After *ex-vivo* studies on paired breast tumor/normal samples across all molecular subtypes, as well as *in-vitro* experiments on human mammary cell lines, we report that SIRT1 mediates the deacetylation of H3k4ac histone marker in breast cancer. SIRT1 also modulates histones H3 and H4 acetylated marks in different subtypes of breast cancer. In addition, SIRT1 physically interacts with and regulates its H3 acetylated targets in a subtype-specific fashion. Moreover, SIRT1 deficiency is associated with substantial induction of acetylated H3k4 and H3k9 epigenetic marks on 6 breast cancer-related gene promoters: *AR, BRCA1, ERS1, ERS2, EZH2*, and *EP300*. We postulate that SIRT1 plays a differential role in breast cancer development depending on molecular subtype, in part through its epigenetic action. This study thus further consolidates the

potential use of SIRT1 as a druggable epigenetic target in human breast cancer.

MATERIALS AND METHODS

Patients' selection and collection of tissue samples

This study included a total of 135 patients admitted to the Centre Jean Perrin from June 2010 to December 2016 for cancer treatment, and diagnosed with breast cancer carcinoma. Patients were informed about the study and gave informed consent prior to inclusion. All 135 tumors and their adjacent normal breast tissues came from the Centre Jean Perrin tumor bank, Biological Resource Center (CRB), accredited under No.BB-0033-00075, where they were stored in liquid nitrogen at −196° C. Patients who received chemotherapy, hormonal therapy and/or radiotherapy for cancer in other parts of the body were excluded from the study, as were patients with predisposition to breast cancer and/or family members with breast cancer.

Molecular breast cancer subtype classification

Based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 proliferative index; breast tumors were classified into 5 intrinsic subtypes according to the St. Gallen Consensus Conference guidelines [4] as follows: Hormone Receptor-positive Breast Cancer (HRBC) comprising luminal A [ER+, PR+, HER2- and Ki-67 <14%], luminal B (HER2−) [ER+, PR+/−, HER2− and Ki-67 ≥14%] and luminal B (HER2+) [ER+, PR-, HER2+ and any Ki-67]. HER2 Breast Cancer (H2BC): [ER−, PR− and HER2 overexpressed], and lastly Triple-Negative Breast Cancer (TNBC): [ER−, PR−, and HER2−].

Breast cancer cell lines and cell culture

All 6 human cell lines used in this study were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). (ER+) breast cancer cell lines: MCF-7 and T-47D were used as representatives of the luminal subtype. (ER-) breast cancer cell lines: MDA-MB 453, MDA-MB 231 and MDA-MB 468 were used as representatives of the triple-negative subtype. MCF10A, a normal breast cell line, was included as a control. MCF-7 and T-47D cells were cultured in RPMI-1640 medium (Gibco Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (Invitrogen, Carlsbad, CA, USA), 0.1% gentamycin (Panpharma, Luitré, France) and insulin (1–4 mg/ml, Novo Nordisk, Denmark) in a humidified atmosphere at 37° C containing 5% CO2. MDA-MB 453, MDA-MB 231 and MDA-MB 468 cells were cultured in Leibovitz's L-15 medium (Gibco) containing 10% fetal bovine serum, 1% L-glutamine and 0.1% gentamycin in a 37° C humidified atmosphere without CO2. MCF10A cells were grown in DMEM/F-12 meduim (Gibco) containing 10% fetal bovine serum, 1% L-glutamine, 0.1% gentamycin and completed with insulin (10 µg/ml), cholera toxin (100 ng/ml), epidermal growth factor (20 ng/ml), hydrocortisone (500 ng/ml) (Sigma) in a humidified atmosphere at 37° C containing 5% CO2.

SIRT1-siRNA transfection

Breast cancer cell lines were transfected with human SIRT1 Silencer® Pre-designed and Validated siRNAs and Silencer® Negative Control #1 siRNA (Ambion, Life technologies). The sense and antisense RNA sequences are as follows: 5′-GCUGUACGAGGAGAUAUUUtt-3′ and 5′-AAAUAUCUCCUCGUACAGCtt-3′, respectively. According to the manufacturer's instructions, cells were transfected at 80% confluence level with 30–60 nM of SIRT1-siRNA or negative control siRNA using the Lipofectamine® RNAiMAX transfection reagent (Invitrogen, Life technologies), that was diluted in Opti-MEM™ Medium (Gibco). SIRT1 knock-down was verified 48 hours after transfection by immunoblotting.

Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP assays were performed on chromatin extracted from tumors and their matched normal tissues, as well as from transfected cell lines using the Auto iDeal ChIP-seq kit for Histones (C01010171, Diagenode, Seraing, Belgium) according to manufacturer's instructions. The extracted chromatin was later sonicated for 30 min (30 cycles, 30 s ON/ 30 s OFF) at 4° C with Bioruptor™ sonicator (Diagenode). 3 µg of the following antibodies (Abs) were used: anti-H3k4ac Ab (C15410322), anti-H3k9ac Ab (C15410004) (Diagenode) and non-immune rabbit IgG (Kch-504-250, Diagenode) serving as a negative control, The ChIP was carried out by SX-8X® IP-Star® Compact Automated System (Diagenode). The samples were incubated for 3 h for antibody coating with protein A-coated magnetic beads, then for 10 h at 4° C for immunoprecipitation reaction. Later on, 4 µl of elution buffer iE2 was added to the samples and the input was prepared with 2 µl of extracted chromatin in 100 µl of elution buffer iE1/iE2. The reverse cross-linking was performed for 45 min at 65° C. For Re-ChIP assays, the immunoprecipitated DNA from the first ChIP assay was eluted with elution buffer iE1 containing 10 mM dithiothreitol (DTT) for 30 min at 37° C. The second ChIP assay (re-ChIP) was then carried out using 3 µg of anti-SIRT1 Ab (C15200063, Diagenode). At the end, Immunoprecipitated DNA (IP DNA) and total DNA (input) from both ChIP and Re-ChIP assays were purified by MicroChIP DiaPure Columns (C03040001, Diagenode) and analyzed by real-time qPCR. The quality control and

efficacy of all Chip assays performed in this study were verified using positive and negative controls provided in the manufacturer's kit and according to their instructions (Diagenode). Control of ChIP analysis was performed prior to direct SIRT1 ChIP assays (Supplementary Figure 1) and prior to SIRT1 and H3k4ac/H3k9ac ChIP and re-ChIP assays (Supplementary Figure 2).

Quantitative real-time PCR method and data analysis

5 µl of IP DNA or total DNA were amplified by real-time qPCR using Taqman Universal PCR Master Mix as per the manufacturer's protocol using the ABI Prism 7900HT real-time PCR system (AB Applied Biosystems, ThermoFisher Scientific). Real-time PCR was performed in triplicate using 96-well MicroAmp Optical plates (AB Applied Biosystems) with optical adhesive film, at a final reaction volume of 25 µl containing 1X TaqMan Universal PCR Master Mix, 250 nM of probe (AB Applied Biosystems) and 400 nM for each of the forward and reverse primers (Sigma-Aldrich). Primer and probe sequences for *AR, BRCA1, ERS2, ERS1, EZH2,* and *EP300* genes were selected with the help of Primer Express software (ABI), and are as follows:

AR gene, forward primer: 5′-TGCGCCAGCACTTGTTTC-3′; reverse primer: 5′-CACCGCGCGCTAACG-3′; probe: 5′-6FAM-CCAAAGCCACTAGGCAG-MGB-3′; *BRCA1* gene, forward primer: 5′-CCCCGTCCAGGAAGTCTCA-3′; reverse primer: 5′-GCGCGGGAATTACAGATAAATT-3′; probe: 5′-6FAM-CGAGCTCACGCCGCGCAG-TAMRA-3′; *ESR2* gene, forward primer: 5′-GAGAGGCTTTGGGTTTGTCAAAT-3′; reverse primer: 5′-CCTCTAGTCCACGGCTTTGC-3′; probe: 5′-6FAM-CAGCAAACGTAACCTCGGGCCCTG-TAMRA-3′; *ESR1* gene, forward primer: 5′-CCCTACATTGGCTTAAACATCA-3′; reverse primer: 5′-TCTTTGGATCGCTCCAAAT-3′; probe: 5′-6FAM-TCCAGGCACAACTC-MGB -3′; *EZH2* gene, forward primer: 5′-CCCTCCAGAAACACAATCAATAGA-3′; reverse primer: 5′-CCGCCTGGTCTGGCTTTAT-3′; probe: 5′-6FAM-CAGAGCAGCTCGACTCT TCCCTCAAACTT-TAMRA-3′; EP300 gene, forward primer: 5′-CGATGGCACAGGTTAGTTTCG-3′; reverse primer: 5′-GCGCACCGAGTAGAAAAGATTAA-3′; probe: 5′-6FAM-CAGCCCCGGCCTTCCACGTT-TAMRA-3′.

The thermal reaction cycles used were 50° C for 2 min, 95° C for 10 min, then 40 cycles of 95° C for 15 sec and 60° C for 1 min. The signal was collected at the endpoint of each cycle (Ct) using an AB Prism 7900 Sequence Detector System (AB Applied Biosystems). ChIP efficiency was calculated and reported as a percentage using the formula: % (IP DNA/Total DNA) = 2^ [(Ct(X% total DNA) – log(X %) /log2) – Ct (IP DNA)] × 100%. 2 is the amplification efficiency, Ct (input) and Ct (ChIP) are threshold values obtained from exponential phase of qPCR for the immunoprecipited DNA sample and input sample respectively, and log(X %) /log2 accounted for the dilution 1/X of the input (Diagenode).

Protein extraction and immunoblot analysis

Whole protein extracts from frozen tissues and cultured cells were obtained using T-PER™ Tissue Protein Extraction Reagent and RIPA buffer (ThermoFisher Scientific) respectively, containing protease and phosphatase inhibitors cocktail (Sigma Aldrich). 25–40 µg of extracted proteins were resolved by electrophoresis on 8–15% SDS-PAGE sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, USA), then electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P, PVDF, 0.45 µm, Merck Millipore) in transfer buffer (25 mM Tris-HCL (pH 7.6), 192 mM glycine, 10% methanol). The membranes were blocked with 5% non-fat milk in 0.1% TBS-tween and later immunoblotted with the following primary Abs: anti-SIRT1 Ab (1/500, C15200063), anti-H3k4ac Ab (1/750, C15410322), anti-H3k9ac Ab (1/1000, C15410004), anti-H4k16ac Ab (1/500, C15200219), all purchased from Diagenode and anti-β-actin Ab (1/5000, CP01, Merck Millipore). Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary Abs: anti-mouse IgG (1/2000, S3721) and anti-rabbit IgG (1/2000, S3738) (Promega, Madison, USA)). Immunolabeling was detected using Western Blue® Stabilized substrate for Alkaline Phosphatase (Promega) at room temperature.

Co-immunoprecipitation assays

Total proteins were extracted from tumors and matched normal tissues using digestion buffer containing protease and phosphatase inhibitors cocktail (Nuclear complex Co-IP kit, Active Motif, CA, USA) according to the manufacturer's instructions. 100 to 500 µg of protein lysates were incubated in 500 µl of IP Incubation Buffer overnight at 4° C with 5 µg of the following primary Abs: anti-H3K4ac Ab (C15410322) and anti-H3k9ac Ab (C15410004) (Diagenode). Ab/Extract mixture was then incubated with Ab-binding agarose beads for 1 h at 4° C (Protein G Agarose Columns, Active Motif). Afterwards, the Ab/bead complexes were washed with 500 µl of IP Wash Buffer solution supplemented with or w/o BSA, before being eluted with 25 µl of Reducing Buffer. The immunoprecipitates were then immunoblotted with anti-SIRT1 Ab (1/500, C15200063, Diagenode) as previously described.

Statistical analysis

Correlation between the clinical parameters of our study groups were examined by chi-square test (χ^2 test) using SPSS statistics software (SPSS Inc., Chicago, IL). Relative expression levels of SIRT1 protein assayed by

immunoblotting were assessed numerically using Quantity One software (Bio-Rad, CA). Multiple-group comparisons were performed by ANOVA using R software (version 3.0.3). Post-hoc comparison of the means was performed using Tukey's multiple comparison test when the F-test was significant ($p < 0.05$). Groups were compared using two-tailed Student's t-test carried out after Fisher's exact test. All experiments were done at least in triplicate and the results were expressed as mean ± SD. In all cases, statistical significance was set at the following P-values: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

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CONFLICTS OF INTEREST

The authors declare no existing conflicts of interest.

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Conclusion de la publication 6

Afin de clarifier le comportement biologique de SIRT1 et d'évaluer son rôle épigénétique vis-vis de la régulation de ses cibles histones dans le cancer du sein, on a mené cette étude.

Les conclusions majeures de cette étude sont :

- La désacétylation de la marque H3K4ac est dépendante de SIRT1.
- SIRT1 module les profils d'acétylation des histones H3 et H4 dans les différents sous-types de cancer du sein.
- SIRT1 colocalise et interagit physiquement avec les marques d'histones H3 sur le panel des gènes cibles, cette régulation par SIRT1 de ses cibles acétylées dépend étroitement du type de gène et du sous-type moléculaire.
- La déplétion de SIRT1 *in vitro* dans des lignées (ER+) et (ER-), est associée à une augmentation différentielle importante de marques épigénétiques H3k4 et H3k9 acétylées sur les promoteurs du panel de gènes cibles, ce qui suggère que SIRT1 pourrait jouer un rôle actif dans la régulation de l'expression de ces gènes dans la pathogenèse du cancer du sein.

Les résultats de cette étude démontrent donc, que SIRT1 joue un rôle différentiel dans le développement du cancer du sein en fonction du sous-type moléculaire, en partie par son action épigénétique sur les gènes cibles. Ainsi, SIRT1 pourrait être une cible épigénétique potentielle dans le traitement de cancer du sein.

2. TIP60 : Un Acteur Majeur dans l'Acétylation de H3K4ac et le Développement Tumoral du Cancer du Sein

Présentation de la publication 7

Research Article

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Epigenomics

TIP60: an actor in acetylation of H3K4 and tumor development in breast cancer

Judes G, Dubois L, **Rifaï K**, Pajon A, Mishellany F, Besse S, Daures M, Degoul F, Bignon YJ ,Penault-Llorca F, Bernard-Gallon D.

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Comme SIRT1, TIP60 joue un rôle bivalent dans les cancers humains, cependant son rôle exact est peu caractérisé dans le cancer du sein.

En utilisant des tumeurs mammaires humaines et leurs tissus sains correspondants, on a essayé d'analyser la relation entre TIP60 et H3K4ac, et de déterminer si TIP60 cible la marque activatrice H3K4ac dans le cancer du sein.

Pour investiguer le rôle de TIP60 dans le développement du cancer du sein *in vivo*, on a inhibé l'expression de TIP60 chez des souris immunodéprimées Balb-c par des shRNAs dans deux lignées cellulaires de cancer du sein MCF-7 (ER+), et MDA-MB-231 (ER-). On a par la suite identifié des différents effets de la déplétion de TIP60 selon les lignées cellulaires présentant un statut hormonal opposé.

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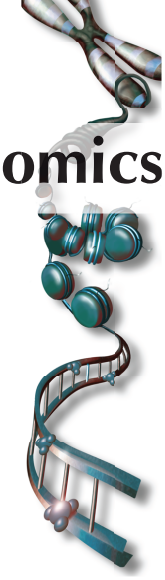

TIP60: an actor in acetylation of H3K4 and tumor development in breast cancer

Gaëlle Judes[1,2], Lucas Dubois[1,2], Khaldoun Rifaï[1,2], Mouhamed Idrissou[1,2], Florence Mishellany[2,3], Amaury Pajon[1,2], Sophie Besse[2], Marine Daures[1,2], Françoise Degoul[2], Yves-Jean Bignon[1,2], Frédérique Penault-Llorca[2,3] & Dominique Bernard-Gallon*[,1,2]

[1]Department of Oncogenetics, Centre Jean Perrin, CBRV, 28 Place Henri-Dunant, 63001, Clermont-Ferrand, France

[2]INSERM U 1240, IMOST, University Clermont Auvergne, 58 rue Montalembert-BP184, 63005 Clermont-Ferrand, France

[3]Department of Biopathology, Centre Jean Perrin, 58 rue Montalembert, 63011, Clermont-Ferrand, France

*Author for correspondence: dominique.gallon-bernard@clermont.unicancer.fr

Aim: The acetyltransferase TIP60 is reported to be downregulated in several cancers, in particular breast cancer, but the molecular mechanisms resulting from its alteration are still unclear. **Materials & methods:** In breast tumors, H3K4ac enrichment and its link with TIP60 were evaluated by chromatin immunoprecipitation-qPCR and re-chromatin immunoprecipitation techniques. To assess the biological roles of TIP60 in breast cancer, two cell lines of breast cancer, MDA-MB-231 (ER-) and MCF-7 (ER+) were transfected with shRNA specifically targeting TIP60 and injected to athymic Balb-c mice. **Results:** We identified a potential target of TIP60, H3K4. We show that an underexpression of TIP60 could contribute to a reduction of H3K4 acetylation in breast cancer. An increase in tumor development was noted in sh-TIP60 MDA-MB-231 xenografts and a slowdown of tumor growth in sh-TIP60 MCF-7 xenografts. **Conclusion:** This is evidence that the underexpression of TIP60 observed in breast cancer can promote the tumorigenesis of ER-negative tumors.

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Keywords: acetyltransferase TIP60 • breast cancer • H3K4ac

The histone acetyltransferases (HATs) fall into three main groups, the largest and most diverse of which, namely the MYST family, includes MOZ, YBF2, MOF and TIP60 [1]. TIP60 (also known as KAT5) belongs to a multiprotein complex called human NuA4 complex, composed of up to 16 subunits. The acetyltransferase TIP60 acetylates not only histones H2A, H4 and H3 *in vitro*, but also nohistone proteins such as p53, c-Myc and ATM [2] and is involved in diverse biological processes, such as DNA damage response signaling, apoptosis, cell cycle progression and transcriptional regulation [3–8]. TIP60 is also a known nuclear receptor coactivator. It binds to the ligand-binding domain of the androgen receptor and enhances hormone-dependent activation of genes by several steroid hormone receptors, including ERα [9]. Because of its pleiotropic functions, the role of TIP60 in tumorigenesis mechanisms is complex and it can act as an oncogene or tumor suppressor according to cancer type [10].

The histone acetyltransferase TIP60 has been shown to be underexpressed in many human cancers of ranging origins, including breast cancer [11–15]. Gorrini *et al.* have shown that TIP60 functions as a haploinsufficient tumor suppressor in a model of tumor induction in mice (Eμ-myc transgenic mice), providing a causal link between its underexpression and tumorigenesis [13]. In breast carcinoma, the frequency of TIP60 loss of heterozygosity was higher in the samples with p53 mutations. Thus, downregulation of TIP60 was associated with p53 mutant and TIP60 acted mainly through the p53 pathway. However, the molecular mechanism of TIP60 downregulation and its involvement in breast cancer development remains unclear.

TIP60 and its associated co-factors contribute to transcriptional control by its acetyl-transferase function and activate target gene promoters. However, some studies have shown TIP60 to be involved in the mediation of transcriptional repression [16,17]. Histone modifications are involved in regulating chromatin compaction or chromatin-decondensing. Classically, acetylation has been defined as a modification involved in active transcription and early studies revealed the association of hyperacetylated histones with actively transcribed genes [18]. However, in *Shi-*

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zosaccharomyces pombe, the acetylation of H3K4 by the Mst1, a homolog of the human TIP60, is involved in heterochromatin formation, where it acts as a switch for recruitment between Clr4 and Swi6 [18,19].

In a previous study, we showed a marked variation in the acetylation of H3K4ac in breast tumors compared with normal breast tissues and the proportion of this histone modification differs according to breast cancer subtypes [20–22]. The function of H3K4ac has received little attention compared with other histone modifications and H3K4 is not known in mammals to be a target of TIP60 *in vivo*.

In this study, we analyzed the link between TIP60 and H3K4ac in breast cancer. We found that H3K4 acetylation was dependent on TIP60 expression, suggesting that TIP60 participates in this acetylation in breast cancer and more surprisingly in heterochromatin formation. To analyze the involvement of TIP60 underexpression in breast cancer development *in vivo*, we induced a knockdown of TIP60 in athymic Balb-c mice by shRNA in two breast cancer cell lines (MDA-MB-231 and MCF-7). We identified different effects of TIP60 depletion according to cell lines presenting opposite hormonal status. Sh-TIP60 MDA-MB-231 had a high growth in tumor development compared with sh-TIP60 MCF-7.

Materials & methods

Patients

The chromatin immunoprecipitation (Chip) and re-Chip studies included a cohort of 76 breast tumors with 76 matched normal tissues, diagnosed from 1998 to 2014 in the Centre Jean Perrin. Patients gave their informed written consent to give a sample for research. Tissues were stored in nitrogen in the Centre Jean Perrin Biological Resource Center (CRB No. BB-0033-00075). Patients with neoadjuvant chemotherapy, hormonotherapy, radiotherapy and with family background of cancer were excluded from the study.

Co-immunoprecipitation

Tumors and matched normal tissues were lyzed in digestion buffer containing protease inhibitor mixture (Nuclear complex Co-IP kit, Active Motif, CA, USA). Protein concentration was determined by the Bradford method: 100 µg of proteins was incubated with 5 µg of H3K4ac antibody (pAb-165-050, Diagenode) at 4°C overnight and antibody/bead complexes were then incubated for 1 h at 4°C on a rotator (Protein G Agarose Columns, Active Motif). The immunoprecipitates underwent western blot analysis with TIP60 antibodies (1:100, goat PoAb, sc-5725 X, Santa Cruz, TX, USA).

Chromatin immunoprecipitation, re-chromatin immunoprecipitation assays & qPCR

ChIP was performed using 3 µg of antibodies (Diagenode): H3K4ac (pAb-165-050), H3K9ac (pAb-103-050), H3K9me3 (pAb-056-050), H3K27me3 (pAb-069-050) and also nonimmune rabbit IgG (negative control; kch-504-250). The ChIP was carried out by SX-8X® Automated System (Diagenode, Seraing, Belgium) and performed as described previously [23].

In re-ChIP assays, DNA-containing magnetic beads and H3K4ac antibodies were incubated in elution buffer H (Auto Histone ChIP-seq Kit, Diagenode) with 10 mM dithiothreitol at 37°C for 30 min to elute the immunoprecipitated DNA after the first ChIP assay. The second ChIP assay was performed with the purified DNA by the second antibody (TIP60, goat PoAb, sc-5725 X, Santa Cruz, TX, USA). The ChIP DNA was amplified by qPCR with the ABI 7900 real-time PCR system.

DNA (5 µl) was then analyzed by qPCR (ABI PRISM 7900HT, Applied Biosystems, CA, USA) in triplicate in a 25 µl reaction volume containing 1× Taqman Universal PCR Master Mix with the forward and reverse primers (400 nM) and probes (250 nM): *ERS1, ERS2, EZH2, BRCA1, P300, SRC3* as described in Dagdemir *et al.* [24] and *PGR* [23]. The efficiency of ChIP of particular locus was calculated by qPCR and reported as a percentage of starting material: %(ChIP/Total Input)= 2∧9(Ct[x% input] – log[x% /log2] – Ct[ChIP]) × 100%.

Cell-culture conditions

MCF-7 and MCF10-A cells were grown in RPMI 1640 and DMEM Ham's F12 medium (Gibco, Thermo Fisher Scientific, CA, USA), respectively, supplemented with 10% fetal bovine serum, 1% L-glutamine and specifically to MCF7, 5 µg/ml of insulin was added. The MCF10-A growth medium was completed with hydrocortisone (0.5 µg/ml), cholera toxin (100 ng/ml) and EGF (20 ng/ml; Sigma). MDA-MB-231 cells were grown in L15 Leibovitz's medium supplemented with 15% fetal bovine serum (ATCC, VA, USA) and 1% L-glutamine. All cells

were grown in 500 µl of gentamicin. Cells were grown at 37°C and 5% CO_2 (MCF-7, MCF-10A) or without CO_2 (MDA-MB-231).

TIP60 silencing

Mission short hairpin RNA (shRNA)-expressing plasmids were purchased from Santa Cruz Biotechnology (TX, USA). TIP60-specific shRNA constructs in a plasmid (ref: sc-37967-SH, shRNA plasmids consist of a mixture of three to five lentiviral vector plasmids. Each individual plasmid encodes shRNA specific target of 19–25 nt) and control shRNA plasmids (ref: sc-108060 C, sc-108066 C) were individually transfected into MDA-MB-231 and MCF-7 cells to a 50–70% confluency. The transfection mixture included 2 µg shRNA plasmid DNA and 1 µl of shRNA plasmid transfection reagent. Cells were incubated for 7 h at 37°C. Culture medium was changed after 24 h. For selection of stably transfected cells, a puromycin selection (3.5 µg/ml) was performed 48 h after transfection. The culture medium was changed and the transduced cells were cultured in fresh medium.

RT-qPCR

RNAs were extracted from tumor and adjacent normal tissues or from breast cell lines and were promptly homogenized using an Ambion extraction kit. Total RNA was extracted according to the manufacturer's instruction (QIAGEN SA, Courtaboeuf, France). The quality of total RNA was assessed with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Massy, France) and 1–2 µg of total RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA). All the genes and control genes studied were amplified in triplicate using the TaqMan Gene expression PCR Master Mix (Applied Biosystems). Data were collected using an ABI PRISM 7900HT sequence detection system. Relative gene expression was determined using the comparative C_T method. Normalization was performed with 18s RNA. The level of each mRNA gene of each tumor was normalized to mRNA of normal tissues. Fold changes were determined using the $2(^{-\Delta\Delta CT})$ method [25].

Immunoblotting

Immunoblotting to detect specific proteins in cell extracts prepared in RIPA buffer (Thermo Fisher Scientific, Illkirch, France) was performed according to standard procedures; 25–50 µg of proteins was studied on SDS-PAGE gel, electrotransferred onto nitrocellulose membrane and respectively probed with primary antibody. The following antibodies (Abs) were used: TIP60, Santa Cruz, goat PoAb, sc-5725 X, 1: 100; H3K4ac, Diagenode, ref: pAb-165-050, 1:100; Actin, Millipore, ref:CP01, 1:4000), alkaline phosphatase conjugated secondary Abs (antirabbit IgG, 1:2000, S3738 or Donkey anti-Goat IgG, 1:2000, V115A, Promega). Bands were detected using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega).

Immunofluorescence

Cultured cells were treated with KCl (0.07 M at 37°C). Cells were centrifuged for 20 min at 770 × *g*, and fixed with acetic acid and methanol for 20 min at RT; 250 µl of nuclei per chamber was deposited on the chamber slide system. The slides were irradiated by UV at 30 cm for 3 h. Nuclei were then rinsed in phosphate-buffered saline (PBS; 1×) and incubated with 200 µl of PBS with 5% bovine serum albumin (BSA) for 1 h at RT. The slides were blocked overnight at 4°C with PBS containing 2% BSA and primary antibody recognizing TIP60 (Santa Cruz Biotechnology, TX, USA; ref: 5725, 1:200) or H3K4ac (Diagenode, ref: pAb-165-050, 1:100). After three washes with PBS for 5 min at RT, samples were incubated for 30 min with a 1:500 dilution (in PBS 1×/BSA 2%) of antigoat IgG conjugated to the fluorescent dye (Jackson Immunosearch, ref: 711-095-152) or with antirabbit IgG conjugated to the fluorescent dye (Jackson Immunosearch, Cambridgeshire, UK, ref: 705-166-147). Slides were washed three-times with PBS 1× and counterstained with Hoechst (1/500; Thermo Fisher Scientific, CA, USA). Images were taken on a Leica SPE confocal microscope (Nanterre, France).

Xenograft studies

Six-week-old female athymic mice (nu/nu genotype, BALB/c background) [26] were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed under aseptic conditions. All protocols in this study were approved by the ethics committee (No. 20160105184547091). To test the effect of TIP60 shRNA *in vivo*, 5×10^6 tumor cells transfected with shRNA TIP60 or control shRNA in 50 µl of cell culture media and 50 µl of BD Matrigel (BD Biosciences) were injected in the right flank of mice. Tumor growth and response to therapy were determined once a week by measuring with calipers. Mice were sacrificed at 33 days postinoculation for the

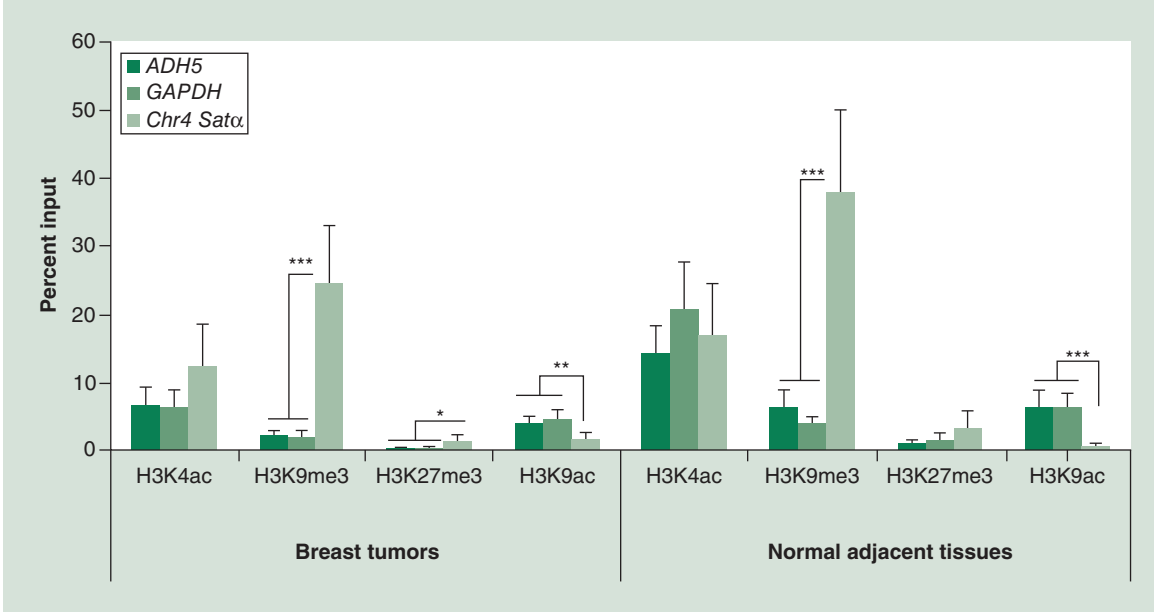

Figure 1. H3K4ac in centromeric heterochromatin and euchromatin in breast cancer. Chromatin immunoprecipitation and qPCR were used to study histone H3 patterns (H3K4ac, H3K9me3, H3K27me3 and H3K9ac) on specific regions of heterochromatin (*Chr4 SATα*) and euchromatin (*ADH5* and *GAPDH*) in breast tumors (n = 22) and their adjacent normal tissues (n = 22); y-axis corresponds to percent input (chromatin immunoprecipitation/total input) on gene promoters of studied epigenetic marks. Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by Student's t-test.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

MDA-MB-231 cell line and at 48 days postinoculation for the MCF-7 cell line and tumors of these mice were removed and placed in 4% alcohol/formaldehyde/acetic acid. Tumor volumes (mm^3) were measured with calipers and calculated using the formula $V = \pi/6(L \times S^2)/2$, where L and S are the largest and smallest diameters in millimeters.

Immunohistochemistry

Tissues were fixed in alcohol formalin acetic acid and embedded in paraffin; 4 µm sections were mounted on silanized glass slides (Starfrost®, Klinipath, Leuven, B) and dried overnight at 37°C. An Automated Benchmark XT immunohistochemistry instrument (Roche, Bâle, CH) was used to process the slides, which were dewaxed and rehydrated using EZ Prep (Roche). Heat-induced antigen retrieval was performed for 30 min with CC1 (pH 8; Roche). The slides were then incubated at 37°C with primary antibodies: anti-Ki67 (1:25, Dako) for 2 h, an UltraView universal DAB detection kit (Ventana) was used for visualization with a horseradish peroxidase secondary antibody and the signal was amplified using the Ventana amplification kit. Slides were then counterstained with hematoxylin, washed in water and coverslipped with an aqueous Faramount mounting media DAKO (Agilent, CA, USA). For negative control, the primary antibody was replaced by PBS.

Results

Lysine 4 acetylation of histone 3 is found in centromeric heterochromatin in breast cancer

No involvement of H3K4ac in cancer development is reported. In breast cancer, a low enrichment of H3K4ac was found on different gene promoters overexpressed in tumors [20]. This mark could be an actor in heterochromatin formation in breast cancer. To test this hypothesis, we investigated the enrichment of H3K4ac in different regions of heterochromatin (*Chr4 satα*) and euchromatin (*ADH5* and *GAPDH*) by ChIP-qPCR in breast tumors and normal breast tissues (Figure 1). We studied H3K9me3 and H3K27me3 as control marks of heterochromatin and H3K9ac as a control mark of euchromatin. Surprisingly, a similar enrichment of H3K4ac was found in heterochromatin and euchromatin regions. For the control mark enrichments, we achieved the expected results, namely a significant enrichment of H3K9me3 and H3K27me3 in the heterochromatin region compared with

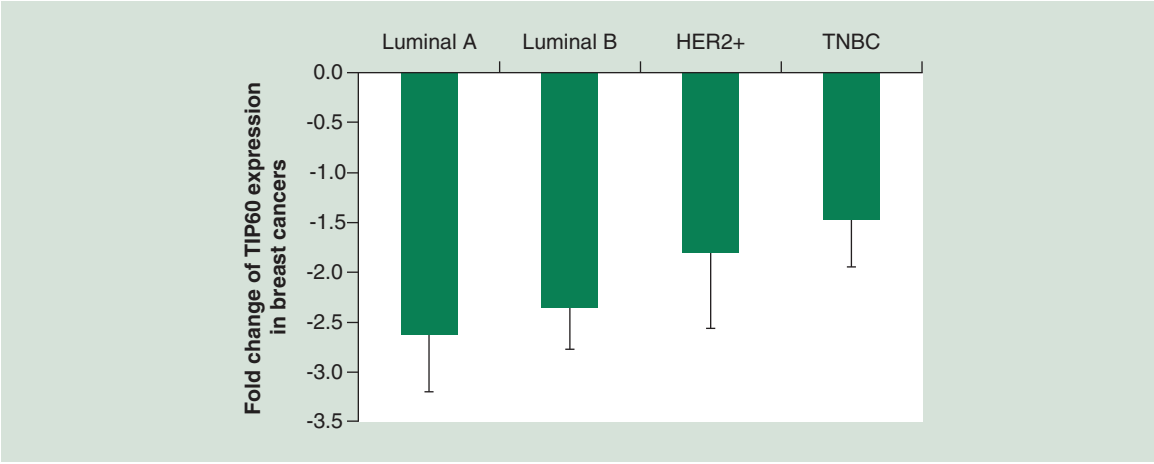

Figure 2. Underexpression of acetyltransferase TIP60 in breast cancer. Graphs showed fold changes ($2^{-\Delta\Delta Ct}$) of gene expression normalized to normal tissue gene expression in breast cancer subtypes (luminal A [n = 7]; luminal B [n = 7]; HER2+ [n = 6]; TNBC [n = 7]); x-axis represents the gene expression fold-change ratios for TIP60 mRNAs. Fold change was used with reference set to 0. Data are expressed as means for each group and error bars indicate the 95% confidence interval of the mean value.
TNBC: Triple-negative breast cancer.

euchromatin and conversely for the H3K9ac level, which was increased in euchromatin. In breast tissues, H3K4ac seems to play a direct role not only in the centromeric heterochromatin, but also in the euchromatin.

TIP60 gene expression is downregulated in human breast cancer

The similar enrichment of H3K4ac in heterochromatin and euchromatin raises the question of the role of H3K4ac in regulating gene transcription in breast cancer. These results led us on to study the actor responsible for H3K4 acetylation in breast tissues. In the yeast *S. pombe*, Mst1, a homolog of the human TIP60 acetyltransferase, is responsible for H3K4 acetylation in pericentromeric regions in late S phase and participates in the recompaction of heterochromatin [19]. However, H3K4ac is not known to be a target of TIP60 in humans. Given the importance of TIP60 in cancer, we analyzed the expression of TIP60 in breast carcinoma. By qPCR analysis and $2^{-\Delta\Delta Ct}$ method, we found that *TIP60* mRNA was underexpressed in tumors compared with their adjacent normal tissues (Figure 2). The mRNA of TIP60 presented a 2.36-fold decrease in luminal A, 2.35-fold decrease in luminal B, 1.81-fold decrease in HER2+ and 1.47-fold decrease in triple-negative breast cancer (TNBC) tumors. No significant difference of TIP60 expression was found between breast cancer subtypes. TIP60 underexpression was observed irrespective of breast cancer subtype, suggesting that the decreased TIP60 expression modification occurs early in tumorigenesis.

Co-occupancy of TIP60 & H3K4ac

We opted to focus on luminal breast cancer subtypes because these represent the majority of breast cancers and present a marked underexpression of TIP60. To confirm that TIP60 and H3K4ac can simultaneously co-occupy the same chromatin location, re-ChIP experiments followed by qPCR were performed on regions of a specific gene panel (*BRCA1, PGR, ESR1, ESR2, P300* and *EZH2*) involved in breast cancer development. From 54 luminal breast tumors and adjacent normal tissues, a first ChIP was performed with H3K4ac antibody, followed by a second ChIP with TIP60 antibody or IgG as a negative control (Figure 3A). Results confirmed that H3K4ac and TIP60 simultaneously co-occupy the same genomic locations in both tissues. However, this co-occupancy was significantly decreased in breast tumors compared with normal tissues for all the target promoter genes. Using co-immunoprecipitation, we observed a direct interaction between H3K4ac and TIP60 (Figure 3B), suggesting that TIP60 could play a role in acetylation of lysine 4 of histone 3. A physical interaction between TIP60 and H3K4ac exists in breast cancer and seems to be decreased compared with normal tissue.

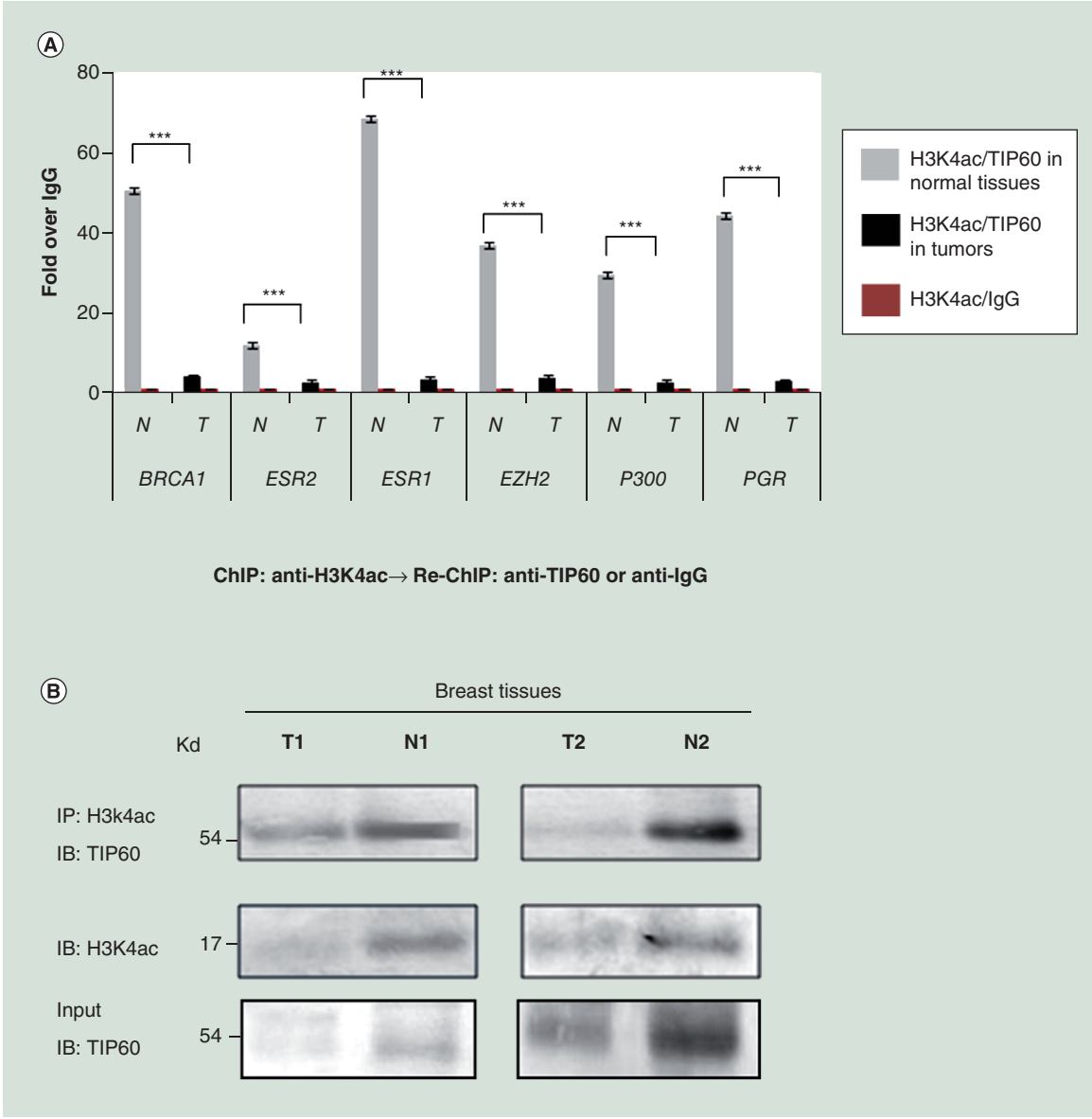

Figure 3. TIP60 shows co-operative binding with H3K4 acetylated in breast cancer. (A) Re-chromatin immunoprecipitation (ChIP) showed a co-occupancy of TIP60 and H3K4ac in promoter regions of target genes where both proteins bind. Re-ChIP assay was performed with H3K4ac antibody followed by the second immunoprecipitation with TIP60 antibody. The ChIP DNA was amplified by qPCR for the target regions of *BRCA1, ESR2, ESR1, EZH2, P300* and *PGR* genes. The fold enrichment of recruitment of H3K4ac and TIP60 at the target regions is relative to respective IgG control. The results are the averages of independent experiments performed on 54 tumors (T) and 54 adjacent normal breast tissues (N). All data are represented as means ± standard deviation and analyzed by paired Student's t-test. The statistical significance of the difference in the recruitment of H3K4ac and TIP60 between tumors and normal tissues is shown as follows: ***p <0.001. **(B)** H3K4ac-TIP60 interaction was confirmed by co-immunoprecipitation. Lysates of tissues were immunoprecipitated with H3K4ac antibody on columns (two independent experiments). An immunoprecipitation without antibody was used as negative control. The immunoprecipitates were immunoblotted with TIP60 antibody.

TIP60 acetylates H3K4 in breast cancer

Because TIP60 interacts physically with H3K4, TIP60 could directly acetylate H3K4. Following this hypothesis, we studied the TIP60 activity in breast cancer *in vitro*. First, we checked two breast cancer cell lines for TIP60 expression by RT-qPCR ($2^{-\Delta\Delta Ct}$ method) and western blot (Figure 4A and B). We opted to focus on MCF-7 and MDA-MB-231 cell lines, because these had different hormonal profiles and represented two subtypes of breast

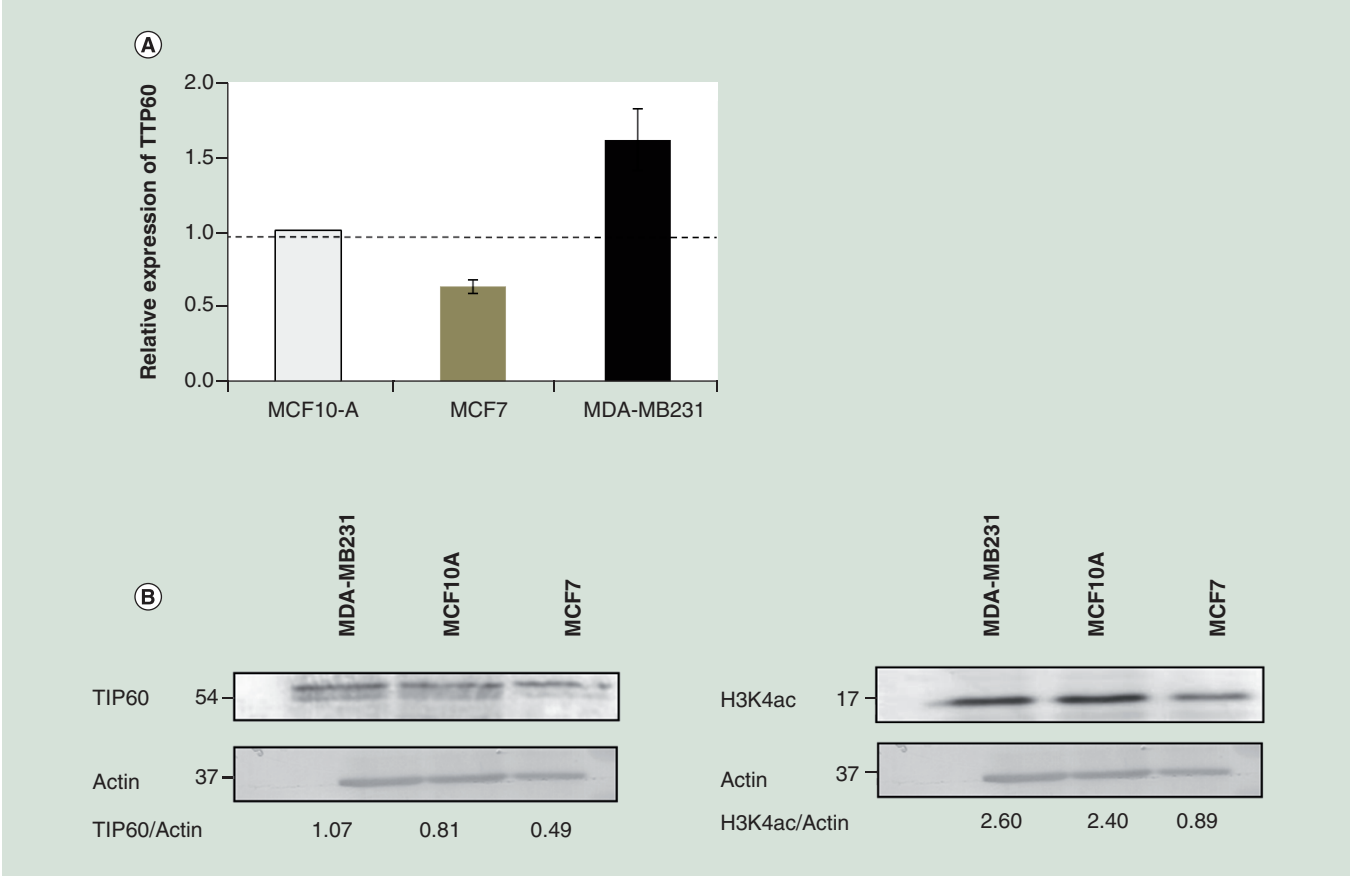

Figure 4. Control of TIP60 expression level in breast cancer cell lines. (A) Analysis of TIP60 mRNA relative quantification ($2^{-\Delta\Delta Ct}$) by RT-qPCR in MDA-MB-231 (TNBC), MCF-7 (luminal) and MCF10-A (control cells) breast cancer cell lines. Graphs showed fold changes of TIP60 expression normalized in relation to TIP60 expression in MCF10-A; x-axis represents the relative fold changes. Fold change was used with reference set to 1. Data were expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. **(B)** Immunoblots to detect TIP60 and H3K4ac in breast cancer cell lines (MDA-MB-231, MCF10-A and MCF-7). Representative quantification was expressed as relative fold change in protein expression of TIP60 and H3K4ac after normalization to actin density. TNBC: Triple-negative breast cancer.

cancer: luminal (ER+) and TNBC (ER-) respectively. The MCF10-A cell line was taken as a control cell line to study TIP60 expression. We identified two different profiles of mRNA TIP60 expression between MCF-7 and MDA-MB-231 cell lines. A high TIP60 expression was found in MDA-MB-231 compared with MCF10-A, and conversely for MCF7. These results were confirmed by the protein level. These cell lines presented a difference in TIP60 expression, and represent suitable models for investigating the role of TIP60 and consequently its depletion. To investigate the mechanism responsible for the acetylation of H3K4, we transfected MCF-7 and MDA-MB-231 cell lines with scrambled shRNA (sh-control) or shRNA, which specifically targets TIP60 sequences (sh-TIP60) to obtain cells stably underexpressed. TIP60 transfection of cell lines with a sh-TIP60 decreased *TIP60* mRNA level approximately twofold (Figure 5A), and significantly inhibited TIP60 protein expression (Figure 5B) in the two breast cancer cell lines. Importantly, a decrease in acetylation H3K4 staining was found in nuclei after TIP60 inhibition (Figure 6). This result thus showed that the acetylation of H3K4ac is dependent on TIP60 acetyltransferase activity in luminal (MCF-7) and TNBC (MDA-MB-231) breast cancers.

TIP60 contributes to breast tumor development

To investigate effects of TIP60 on breast tumor development *in vivo*, we administered sh-TIP60 transfected to MCF-7 and MDA-MB-231 cells in athymic mice. The two principal subtypes of breast cancer were represented by the MCF-7 cell line for the luminal subtype and the MDA-MB-231 cell line for the TNBC subtype. In TNBC tumors, a TIP60 depletion showed a significant growth-increasing activity compared with cells transfected with

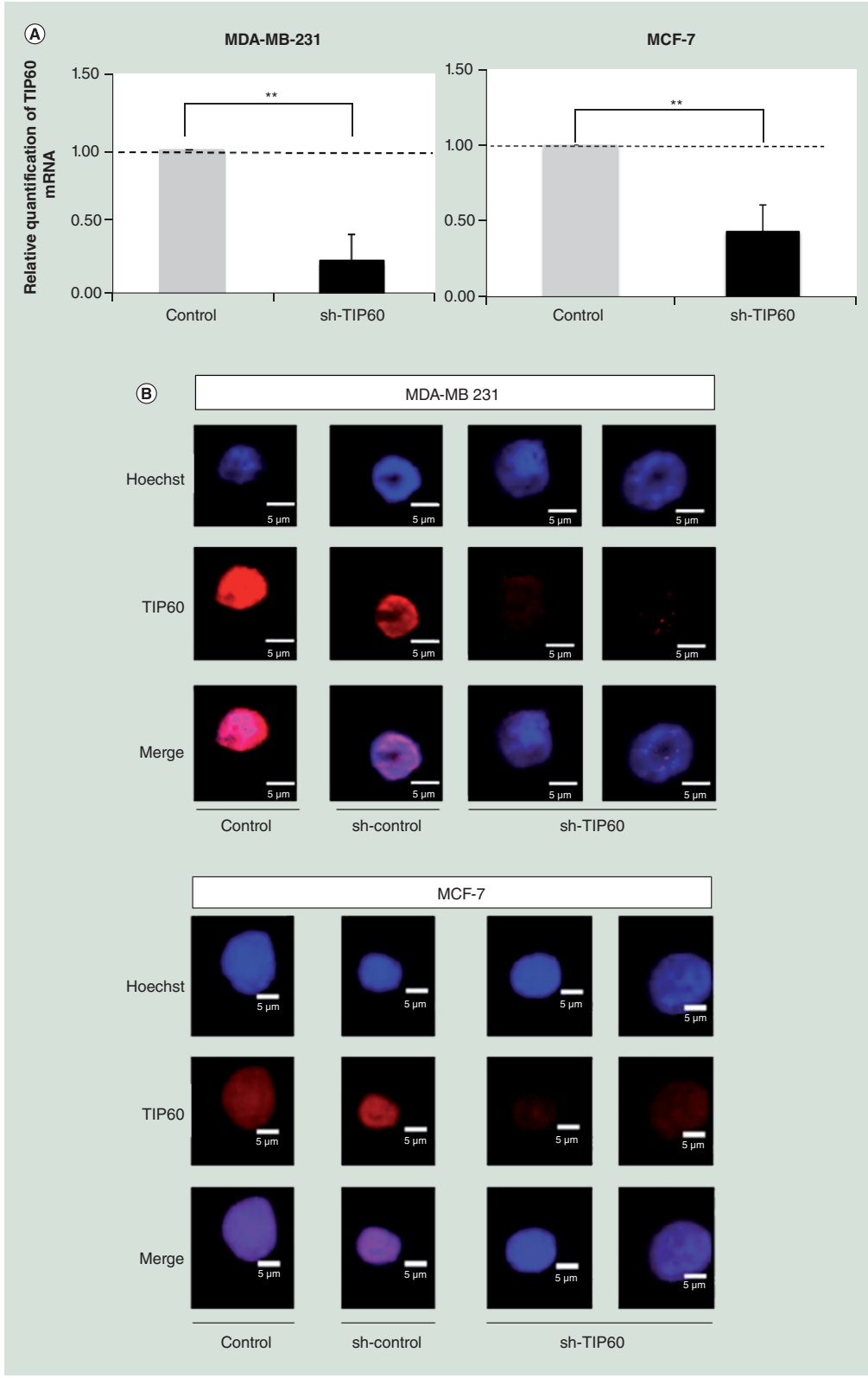

Figure 5. Control of sh-RNA transfection. (A) Analysis of TIP60 mRNA relative quantification ($2^{-\Delta\Delta Ct}$) by RT-qPCR in MDA-MB-231 and MCF-7 cell lines transfected by sh-TIP60 and sh-control. Graphs showed fold changes of TIP60 expression in sh-TIP60 transfected cells normalized TIP60 expression in ShRNA control transfected cells; x-axis represents the relative fold changes in scale. Fold change was used with reference set to 1. Data were expressed as means for each group and error bars indicate the 95% confidence interval of the mean value; data were analyzed by Student's t-test (**$p < 0.01$). **(B)** Representative confocal microscopy images of transfected MDA-MB-231 and MCF-7 cell lines. Endogenous TIP60 (red) was visualized with TIP60-specific antibodies and Hoechst was used to visualize the nuclei (blue). Scale bars indicate 5 µm.

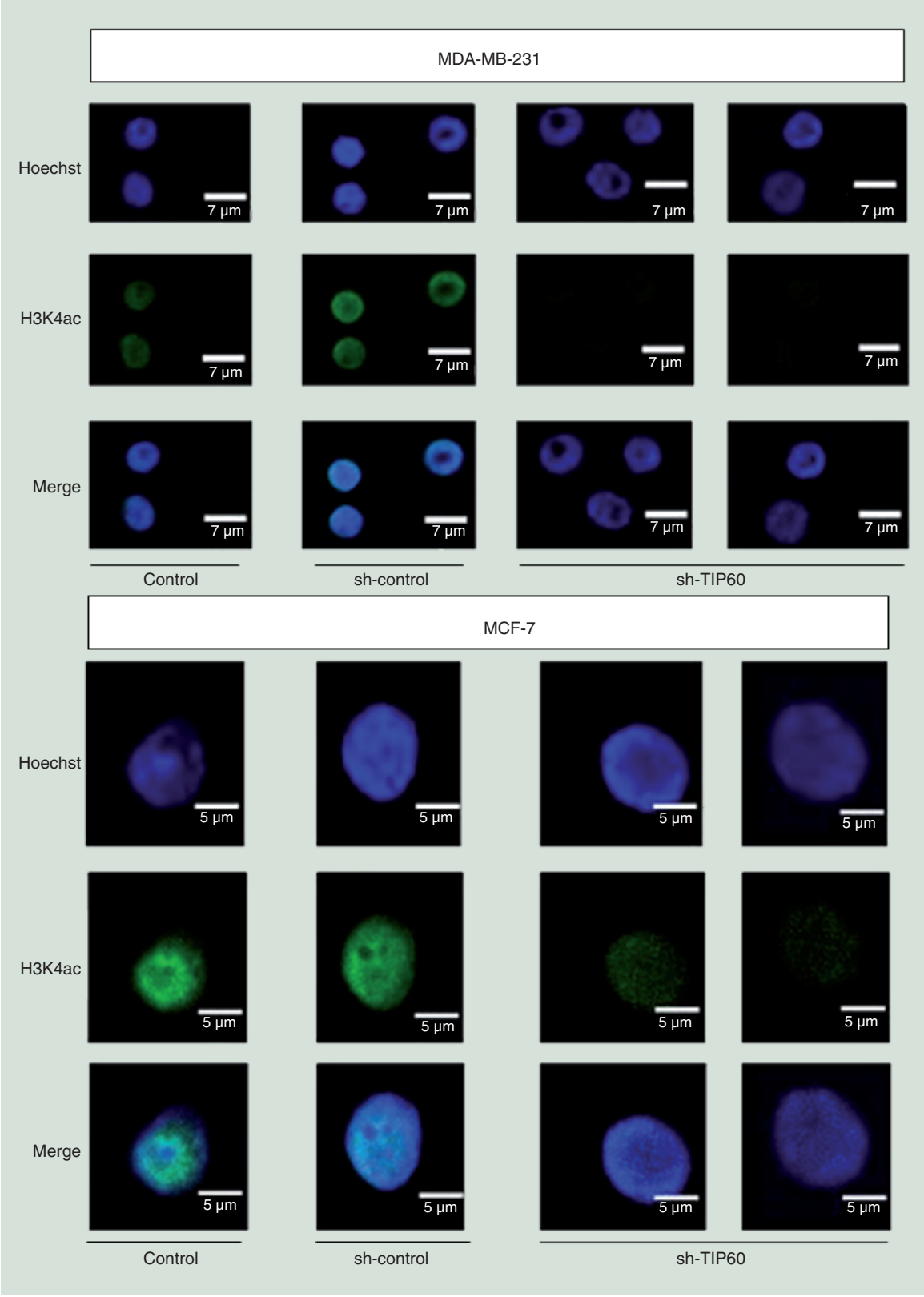

Figure 6. TIP60 inhibition reduces the acetylation of H3K4. Representative confocal microscopy images of transfected MDA-MB-231 and MCF-7 cell lines. H3K4ac modification (green) was visualized using specific antibodies and Hoechst was used to visualize the nuclei (blue). Scale bars indicate 5 and 7 μm.

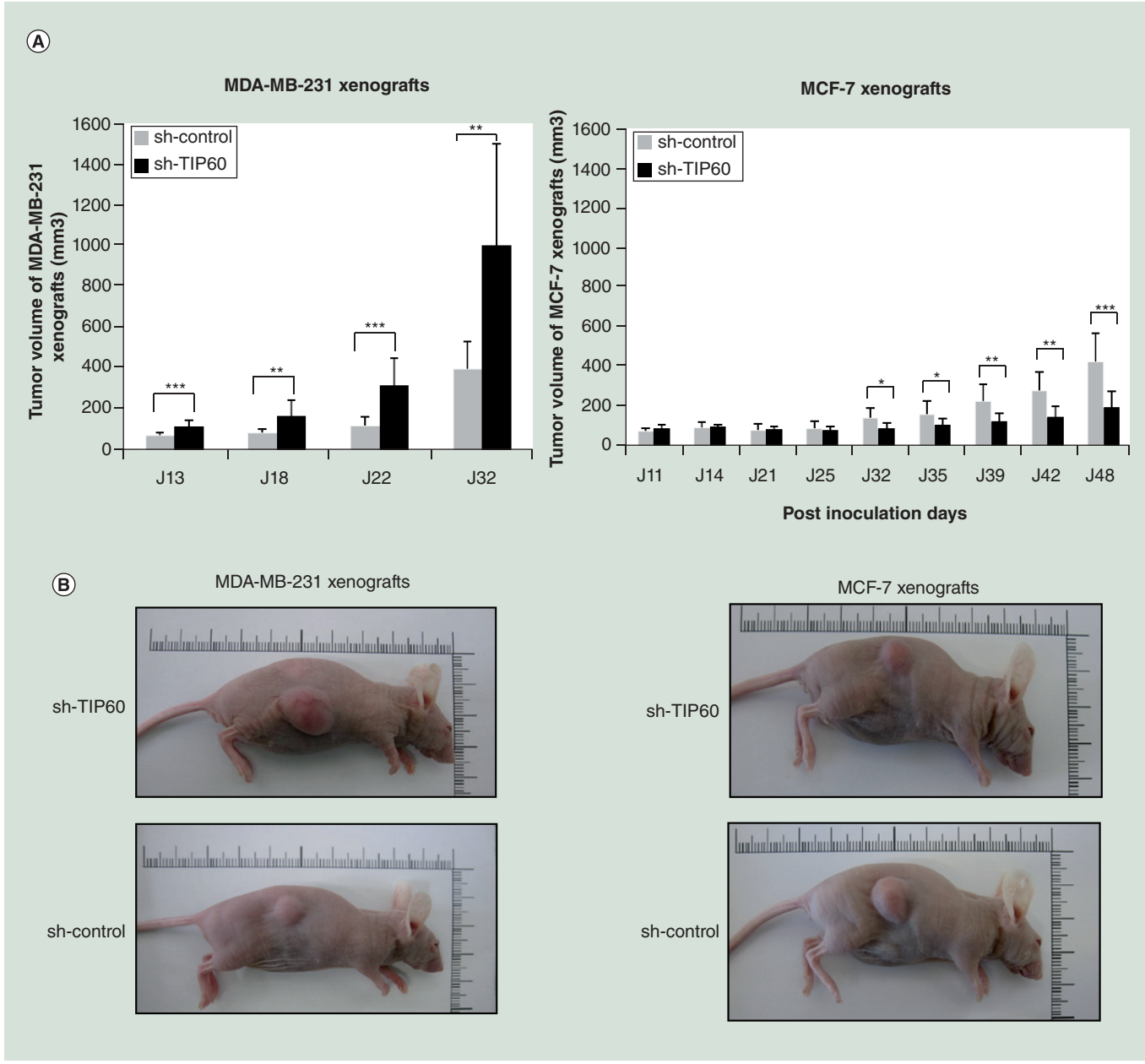

Figure 7. TIP60 depletion in breast cancer xenograft models. (A) Measure of tumor volume in mice after inoculation of MDA-MB-231 cells transfected by sh-TIP60 (n = 11) and sh-control (n = 10), and MCF-7 cells transfected by sh-TIP60 (n = 10) and sh-control (n = 10). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01, ***p < 0.001). TIP60 contributes to development of (MDA-MB-231) triple-negative breast cancer breast xenografts *in vivo*. Conversely, TIP60 depletion reduces development of (MCF-7) luminal breast xenografts *in vivo*. **(B)** Tumor growth in MDA-MB-231 and MCF-7 (sh-TIP60 and sh-control) xenograft models. **(C)** Immunostaining to quantify Ki67 protein and hematoxylin phloxine saffron counterstained showing mitosis (arrows) and cytonuclear atypia (arrowhead) of sh-TIP60 and sh-control MDA-MB-231 and MCF-7 xenografts. Scale bars indicate 100 μm, and images taken at ×20 magnification. **(D)** hematoxylin phloxine saffron section of sh-TIP60 MCF-7 xenografts showing fibrosis. Scale bars indicate 100 μm and images taken at ×10 magnification. **(E)** sh-TIP60 and sh-control MDA-MB-231, and MCF-7 xenografts were subjected to ChIP assays using anti-H3K4ac antibodies and IgG antibodies as a control for gene promoters (*BRCA1, ERS1, ERS2, EZH2, P300, PGR, SRC3*). Quantification was performed by qPCR in the immunoprecipitates versus inputs. Values are expressed as fold enrichment over the sh-control samples (standardized relative to 1 for sh-control tumors). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01).

sh-control (Figure 7A and B). Histopathological analysis showed that TNBC tumors arising in sh-TIP60 and sh-

control mice were high-grade mammary adenocarcinomas (grade 3) as determined by the Nottingham histological scoring system and presented a high level of proliferation (Ki-67 = 80%) (Figure 7C). Thus the analysis of cell proliferation in sh-control and sh-TIP60 TNBC tumors showed no significant difference of Ki-67 expression between the two groups. Because TIP60 is required for H3K4 acetylation as previously demonstrated, we examined H3K4ac status on gene panel promoters in sh-TIP60 TNBC tumors (Figure 7E). ChIP studies found a significant decrease in H3K4ac enrichment at the promoter of *ERS1, ERS2, EZH2, P300* and *PGR* in sh-TIP60 TNBC tumors. Thus a depletion of TIP60 *in vivo* had an effect on the H3K4 acetylation patterns of different genes involved in breast cancer development.

Interestingly, we obtained a specific effect of TIP60 depletion in MCF-7 xenografts: the TIP60 depletion showed significant growth delay relative to growth of MCF-7 cells transfected with sh-control (Figure 7A and B), sh-control and sh-TIP60 tumors presented no histological differences. These tumors were high-grade and with 90% of Ki67 expression (Figure 7C). Histopathology nevertheless revealed marked fibrosis in the sh-TIP60 tumors (Figure 7D). Unlike TNBC tumors, the H3K4ac analysis on gene promoters did not show any difference in H3K4ac enrichment between sh-TIP60 and sh-control luminal tumors (Figure 7E).

Discussion

In this study, we identified an important actor, the acetyltransferase TIP60, in breast cancer development and particularly in the acetylation of histone H3. Acetylation of H3K4 had previously been identified by mass spectrometry in mice and humans by Garcia *et al.* [27] and then detected in *S. cerevisiae* [28] and *S. pombe* [19]. This histone modification has been correlated with the activation of transcription in humans by its presence at promoters of active genes [29,30]. In *S. cerevisiae*, Guillemette *et al.* drew the same conclusion; H3K4ac is present on such promoters, and large-scale transcriptional expression analysis in mutated strains for this lysine or different enzymes confirmed this finding [28]. It is noteworthy that the methylation of H3K4 is largely involved in the transcriptional activation in all these models. However, it is surprising that the acetylation and methylation of this same residue both have a transcriptional activator effect. However, in *S. cerevisiae*, as in humans, H3K4ac is found upstream of H3K4me3 at promoters [28]. Thus, the relationship between these two histone marks is unclear. In the yeast *S. pombe*, Xhemalce *et al.* found H3K4ac at the heterochromatin of pericentromere at late S phase and suggested that H3K4ac facilitated eviction of Fld1 and therefore the complex RITS, by affecting its binding to H3K9me2 residues. This eviction would facilitate the recruitment of proteins Swi6 and Fld2 when heterochromatin is re-formed [19]. These results suggest that H3K4ac could participate in transcriptional repression in mammals. Few data exist on H3K4ac in humans and particularly in cancer. In a previous study, we analyzed this histone modification on various gene promoters (*ERS1, ERS2, BRCA1, P300, PGR* and *EZH2*) in different subtypes of breast cancer and specific H3K4ac enrichments were found in normal tissues and breast tumors [20]. After taking all these factors into consideration, we studied the presence of H3K4ac in constitutive heterochromatin, which formed in centromeric regions. These gene-poor areas are usually made of tandem repetitions, also named satellites, such as Sat-α in Chr4 [31]. We observed an enrichment of H3K4ac not only in the centromeric region, but also in the reference locus of euchromatin (*GAPDH* and *ADH5*). Moreover, the H3K4ac enrichment was more marked in normal breast tissue than in breast tumors. This finding shows that acetylation of H3K4 is decreased in breast cancer. However, no known mechanism explains this depletion of H3K4ac. Accordingly, we focused our research on the acetyltransferase TIP60, whose homologous enzyme MST1 in *S. pombe* is involved in the acetylation of H3K4 [19]. Interestingly, we observed a decrease in TIP60 expression in different subtypes of breast cancer compared with adjacent normal tissues and similar results have already been reported [13,32]. The low enrichment of H3K4ac in breast cancer could be explained by this underexpression of TIP60 acetyltransferase: in breast tumors, a co-localization of TIP60 and H3K4ac was found in the same gene promoters (*ERS1, ERS2, BRCA1, P300, PGR* and *EZH2*), which presented a low enrichment of H3K4ac and a physical interaction confirmed the link between these two proteins. To test the involvement of TIP60 in this acetylation, we used a TIP60 shRNA in the breast cancer cell lines and the depletion of TIP60 led to an overall decrease in H3K4 acetylation level. These results suggest that TIP60 participates in the acetylation of H3K4 in breast cancer cells. H3K4ac offers a research approach to explore the mechanism of TIP60 in heterochromatin formation in breast cancer. Interestingly, TIP60 is recruited to pericentric heterochromatin and mediates acetylation of histone H4K12 by the recruitment of BRD2 [16,33]. The underexpression of TIP60 leads to derepression of satellite transcription and decompaction of pericentric heterochromatin. This acetyltransferase TIP60 can play a role in acetylation of histone, such as H4K12, and in heterochromatin formation. We suggest that

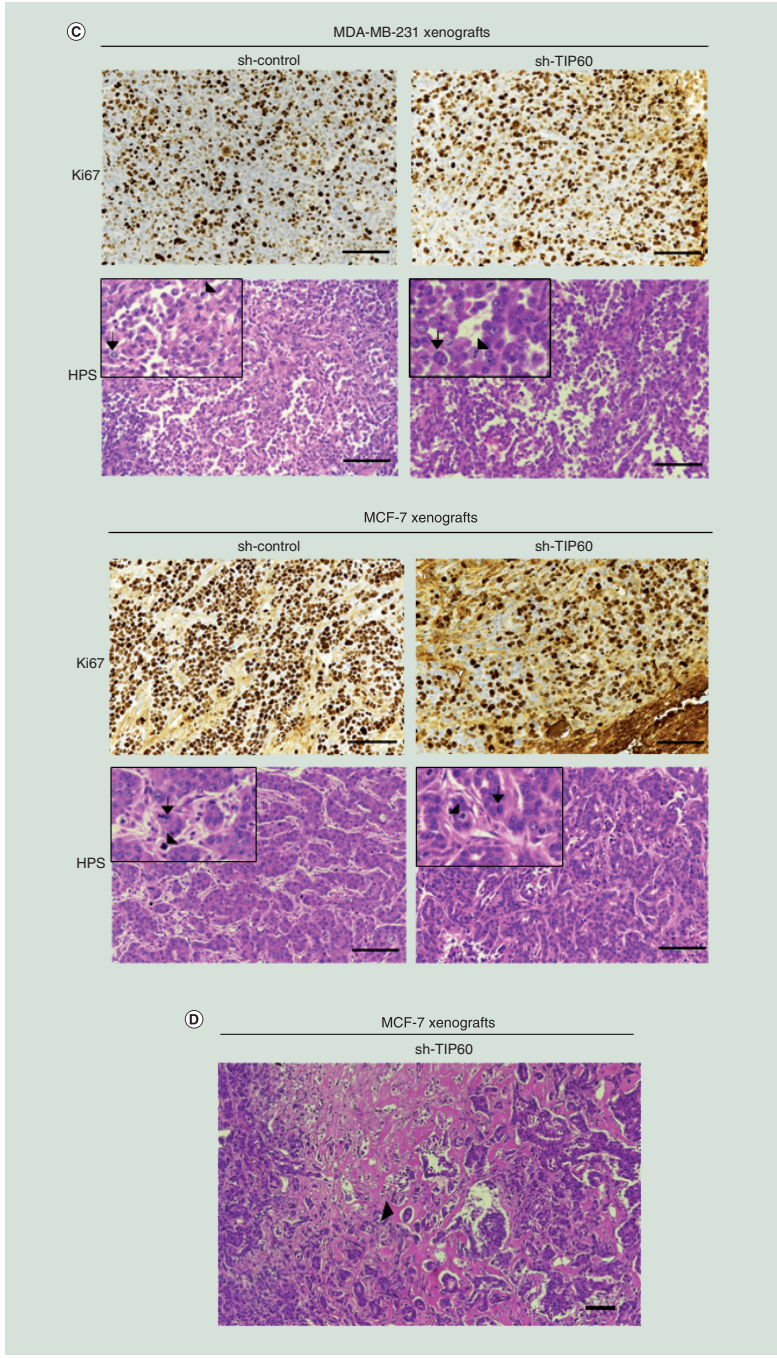

Figure 7. TIP60 depletion in breast cancer xenograft models (cont.). (A) Measure of tumor volume in mice after inoculation of MDA-MB-231 cells transfected by sh-TIP60 (n = 11) and sh-control (n = 10), and MCF-7 cells transfected by sh-TIP60 (n = 10) and sh-control (n = 10). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$). TIP60 contributes to development of (MDA-MB-231) triple-negative breast cancer breast xenografts *in vivo*. Conversely, TIP60 depletion reduces development of (MCF-7) luminal breast xenografts *in vivo*. **(B)** Tumor growth in MDA-MB-231 and MCF-7 (sh-TIP60 and sh-control) xenograft models. **(C)** Immunostaining to quantify Ki67 protein and hematoxylin phloxine saffron counterstained showing mitosis (arrows) and cytonuclear atypia (arrowhead) of sh-TIP60 and sh-control MDA-MB-231 and MCF-7 xenografts. Scale bars indicate 100 μm, and images taken at ×20 magnification. **(D)** hematoxylin phloxine saffron section of sh-TIP60 MCF-7 xenografts showing fibrosis. Scale bars indicate 100 μm and images taken at ×10 magnification. **(E)** sh-TIP60 and sh-control MDA-MB-231, and MCF-7 xenografts were subjected to ChIP assays using anti-H3K4ac antibodies and IgG antibodies as a control for gene promoters (*BRCA1, ERS1, ERS2, EZH2, P300, PGR, SRC3*). Quantification was performed by qPCR in the immunoprecipitates versus inputs. Values are expressed as fold enrichment over the sh-control samples (standardized relative to 1 for sh-control tumors). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*$p < 0.05$; **$p < 0.01$).

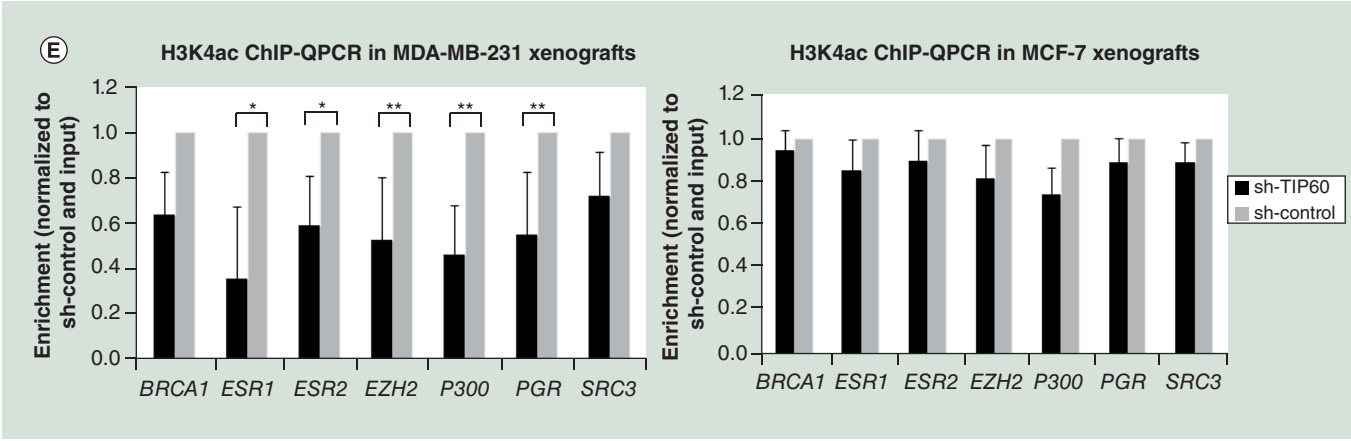

Figure 7. TIP60 depletion in breast cancer xenograft models (cont.). (A) Measure of tumor volume in mice after inoculation of MDA-MB-231 cells transfected by sh-TIP60 (n = 11) and sh-control (n = 10), and MCF-7 cells transfected by sh-TIP60 (n = 10) and sh-control (n = 10). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$). TIP60 contributes to development of (MDA-MB-231) triple-negative breast cancer breast xenografts *in vivo*. Conversely, TIP60 depletion reduces development of (MCF-7) luminal breast xenografts *in vivo*. **(B)** Tumor growth in MDA-MB-231 and MCF-7 (sh-TIP60 and sh-control) xenograft models. **(C)** Immunostaining to quantify Ki67 protein and hematoxylin phloxine saffron counterstained showing mitosis (arrows) and cytonuclear atypia (arrowhead) of sh-TIP60 and sh-control MDA-MB-231 and MCF-7 xenografts. Scale bars indicate 100 μm, and images taken at ×20 magnification. **(D)** hematoxylin phloxine saffron section of sh-TIP60 MCF-7 xenografts showing fibrosis. Scale bars indicate 100 μm and images taken at ×10 magnification. **(E)** sh-TIP60 and sh-control MDA-MB-231, and MCF-7 xenografts were subjected to ChIP assays using anti-H3K4ac antibodies and IgG antibodies as a control for gene promoters (*BRCA1, ERS1, ERS2, EZH2, P300, PGR, SRC3*). Quantification was performed by qPCR in the immunoprecipitates versus inputs. Values are expressed as fold enrichment over the sh-control samples (standardized relative to 1 for sh-control tumors). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*$p < 0.05$; **$p < 0.01$).

the same mechanism could explain our results; H3K4ac could be directly involved in heterochromatin formation through TIP60.

Other questions raised by our findings concern the involvement of the TIP60 depletion in breast cancer development. TIP60 is a tumor suppressor often underexpressed in human cancers and is required for an oncogene-induced DNA damage response [13]. A decrease in TIP60 expression blocked tumor suppressor pathways such as the DDR and p53 pathways [34–36]. These results indicate that decreased TIP60 expression correlates with tumor development. However, the molecular mechanism of TIP60 downregulation was not clarified. We therefore investigated the TIP60 depletion effect *in vivo* by transfection of sh-TIP60 to breast cancer cell lines (MDA-MB-231 and MCF-7) then injected to athymic Balb-c mice. Our results showed that MDA-MB-231 being triple negative (ER-, PR- and HER2-), the mesenchymal cell line presented a significant increase in tumor development with metastasis when expression TIP60 was decreased. Moreover, this depletion led to a marked decrease in H3K4ac enrichment on gene promoters and could contribute to dysregulation of gene expression in breast cancer. A recent study showed miR-22 involvement in regulation of TIP60 in breast cancer [37]. The MDA-MB-231 cell line has an elevated level of miR-22 that is required to maintain the metastasis levels by targeting TIP60 and an inhibition of miR-22 expression leads to a reduction of the metastatic phenotype of MDA-MB-231, as well as an elevation of the expression of TIP60. These results confirm the tumor suppressor role of TIP60 in TNBC breast cancer and show that miR-22 could be a potential target to increase the TIP60 level in breast cancer [38].

We found an opposite effect of TIP60 depletion in the MCF-7 cell line, which is ER+ and an epithelial cell line: sh-TIP60 slowed down tumor development. It has been shown that ER is the defining and driving transcription factor in luminal breast cancers and its target genes regulate cell growth and endocrine response [39–41]. Acetyltransferase TIP60 is required for estrogen-induced transcription of a subset of ER-α target genes in human cells. TIP60 is recruited by estrogen and interacts with ER-α and this interaction leads to increased recruitment of histone methyltransferase MLL1 and increased monomethylation of histone H3 at lysine 4 and acetylation of histone H2A at lysine 5 [9]. This modification of histone pattern activates the transcription of target genes involved in the development of breast cancer. In this light, we hypothesize that TIP60 depletion in MCF-7 cell line provides mediation of estrogen-induced transcription of target genes involved in tumorigenesis.

Conclusion & future perspective

We have identified a target of acetyltransferase TIP60 in breast cancer, the lysine 4 of histone H3, which is found in centromeric heterochromatin and euchromatin. TIP60 could thus be recruited to centromeric heterochromatin, where it mediates acetylation of histone H3K4. We conclude that the underexpression of TIP60 observed in breast cancer may promote tumorigenesis in TNBC tumors. However, a comprehensive study is necessary to know mechanisms and pathways involving the acetyltransferase TIP60 in breast cancer development. The acetyltransferase TIP60 could then be used as a new target and a prognostic marker in breast cancer.

Summary points

- TIP60 expression is altered in different breast cancer subtypes. This alteration modifies acetylation status of proteins such as histones.
- A physical interaction exists between TIP60 and H3K4ac and these two proteins are localized on the same promoter region of specific genes in breast cancer. The acetylation of H3K4 seems to be depending to the presence of TIP60.
- In ER-negative tumors (triple-negative breast cancer xenografts), the TIP60 depletion promotes tumor development. An opposite effect is observed in ER-positive tumors (luminal xenografts).
- The reduced expression of TIP60 observed in breast cancer might alter the regulation of steroid hormone dependent-genes and could promote tumor development or slow it down.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

For *in vivo* xenograft models, all protocols were conducted in accordance with the 2010/63/UE Directive after approval by the institutional review board C2E2A from Auvergne Region (approval number: APAFIS#3399-201601 1 109302187 vS). The samples were obtained from a Biological Resources Center (BB-0033-00075) and a prior signed informed consent was obtained from each patient. It was done in accordance with the Council of Europe's Recommendation on Research on Biological Materials of Human Origin (Rec [2006]) from 2006.

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Conclusion de la publication 7

Dans cette étude, nous avons identifié un acteur important, l'acétyltransférase TIP60, dans le développement du cancer du sein, et en particulier dans l'acétylation de l'histone H3 en lysine 4.

Les conclusions majeures de cette étude sont :

- TIP60 participe activement à l'acétylation de la marque H3K4ac dans le cancer du sein.
- TIP60 est sous-exprimée dans les différents sous-types moléculaires du cancer, et plus particulièrement les sous-types luminales A et B.
- TIP60 colocalise et interagit physiquement avec H3K4ac sur le panel des gènes cibles *BRCA1, ERS2, ERS1, PGR, EZH2,* et *EP300* dans les tumeurs du sein versus leurs tissus sains adjacents.
- La déplétion de TIP60 avec des shRNAs dans la lignée (ER+) *in vivo* dans des souris xénogreffes, entraine une diminution significative de la taille de tumeur par rapport aux souris contrôles. En revanche, la déplétion de TIP60 dans la lignée (ER-) entraine une augmentation significative de la taille de tumeur par rapport aux souris contrôles.

Les résultats de cette étude suggèrent que TIP60, comme SIRT1, joue un rôle différentiel dans le développement du cancer du sein en fonction du sous-type moléculaire.

CHAPITRE III. DISCUSSION ET PERSPECTIVES

Avec 59 000 nouveaux cas en 2017, le cancer du sein est le cancer le plus fréquemment diagnostiqué chez les femmes françaises, et pose un réel problème de santé publique en France, mais aussi au niveau mondial. Cependant, les avancées en diagnostic et en thérapie ont permis d'améliorer le taux de survie des patientes atteintes de cancer du sein dans les pays industrialisés. Le cancer du sein est caractérisé par son hétérogénéité moléculaire (Sørlie et al., 2001). Ainsi, le processus de sous-typage moléculaire du cancer en se basant sur des profils d'expression génique, a amélioré drastiquement le pronostic et la prise en charge thérapeutique du cancer (O'Brien et al., 2010). Les tumeurs du sein sont classées en 5 sous-types moléculaires : luminal A, luminal B (HER2-), luminal B (HER2+), HER2-enriched, et TNBC (Goldhirsch et al., 2013).

Le développement des tumeurs mammaires est un processus complexe multifactoriel. Elles sont caractérisées par des anomalies profondes dans leur génome et leur épigénome. Ces anomalies comprennent des modifications génétiques (mutations, réarrangements chromosomiques) et des altérations épigénétiques comme la méthylation de l'ADN et les modifications post-traductionnelles des histones. Ces modifications génétiques et épigénétiques aberrantes sont censées être déclenchées par l'exposition à des facteurs environnementaux et le style de vie. Les altérations épigénétiques peuvent survenir à différents stades de la tumorigenèse et contribuer ainsi au développement et à la progression du cancer (Veeck and Esteller, 2010) (Huang et al., 2011).

En effet, des anomalies épigénétiques activant des oncogènes ou inhibant des gènes suppresseurs de tumeurs, ainsi que des altérations affectant des gènes codant pour les enzymes responsables des marquages épigénétiques ont été identifiées dans les tumeurs du sein. L'existence de relation causale donc, entre ces altérations épigénétiques et la survenue du cancer du sein ne fait plus aucun doute. Par conséquent, en plus de l'analyse génétique, un profilage épigénétique exhaustif du génome des cancers mammaires est essentiel pour identifier les changements responsables, impliqués dans le développement tumoral et pour améliorer nos capacités à traiter avec succès et prévenir les tumeurs.

En outre, l'intérêt d'étudier l'épigénétique dans le cancer, est fortement augmenté par la notion récente que ces changements épigénétiques peuvent être exploités comme un outil puissant dans la clinique et comme une nouvelle approche dans le traitement du cancer : ce sont «les thérapies épigénétiques» (Campbell and Tummino, 2014) (Benedetti et al., 2015) (Biswas and Rao, 2017).

L'altération de l'épigénome des histones est l'une des premières étapes de la transformation oncogénique. Les modifications post-traductionnelles des histones sont avérées comme marqueurs du pronostic du cancer du sein. Ainsi, des profils distincts de ces marqueurs sont observés en fonction de sous-types moléculaires du cancer du sein. En fait, les marques d'histones ont un effet direct sur l'expression des gènes liés à la tumorigenèse mammaire, et la dérégulation de ces marqueurs aboutit à l'activation des voies oncogéniques différentes spécifiques à chaque sous-type moléculaire du cancer (Elsheikh et al., 2009) (Li et al., 2014) (Judes et al., 2016). Il devient primordial donc, d'étudier les altérations de l'épigénome des histones et de caractériser le rôle de ces modifications épigénétiques, ainsi que le rôle des enzymes responsables des marquages épigénétiques dans le développement du cancer du sein.

Cette thèse s'inscrit dans ce contexte et vise à poursuivre l'identification des altérations épigénétiques au niveau de l'acétylation des histones H3 et H4, et caractériser le rôle de leurs enzymes modulatrices dans les différents sous-types intrinsèques du cancer du sein.

Une perte d'activité des HATs associée à une surexpression et une fonction aberrante des HDACs perturbent l'homéostasie d'acétylation des protéines histones et non-histones, et aboutissent à l'initiation et la progression du cancer (Glozak and Seto, 2007) (Barneda-Zahonero and Parra, 2012) (Parbin et al., 2014). SIRT1 est une HDAC de classe III profondément impliqué dans la régulation de plusieurs processus cellulaires clés tels que l'apoptose, la stabilité génomique, la réparation de l'ADN, la régulation de l'expression génique, et le processus de la carcinogenèse. L'expression altérée de SIRT1, et par conséquent son rôle dans les cancers humains ont fait l'objet d'une multitude d'études au cours ces dernières années. En effet, SIRT1 joue un double rôle dans la promotion et la suppression de cancer, en fonction du son taux d'expression dans un cancer, le contexte tissulaire et de la distribution temporelle et spatiale de ses régulateurs et ses substrats.

Une abondance d'expression de SIRT1, ainsi que son rôle promoteur de tumeur ont été mis en évidence dans de nombreux cancers, y compris les cancers de la prostate (Huffman et al., 2007), du poumon (Noh et al., 2013) (Chen et al., 2017), du foie (Jiang et al., 2017), de thyroïde (Herranz et al., 2013), de la peau non-mélanome (Hida et al., 2007) et mélanome (Ohanna et al., 2014), cancer colorectal (Lv et al., 2014) (Jiang et al., 2014), et cancer gastro-intestinal (Wu et al., 2017). En revanche, une expression réduite et un rôle suppresseur de tumeur de SIRT1 ont été mis en évidence dans les cancers du côlon (Firestein et al., 2008), du pancréas (Cho et al., 2012), de la bouche (Chen et al., 2014), glioblastome et cancer de l'ovaire (Wang et al., 2008a). Dans le cancer du sein, des études contradictoires montrant à la fois une surexpression ou une sous-expression de SIRT1 ont été rapportées. En fait, plusieurs raisons peuvent expliquer cette divergence entre les études: le fait que l'expression de SIRT1 n'ait été évaluée qu'au niveau transcriptionnel (Igci et al., 2016), ou en utilisant uniquement des lignées cellulaires du cancer du sein, et / ou en utilisant des tumeurs mammaires mais sans tenir compte de leur classification moléculaire (Lee et al., 2011) (Kuo et al., 2013) (Derr et al., 2014) (Igci et al., 2016), et sans avoir un nombre d'échantillons statistiquement suffisantes (Sung et al., 2010) (Wang et al., 2008a).

L'objectif du premier travail de cette thèse a été donc, d'évaluer les niveaux d'expressions transcriptionnelles et traductionnelles de SIRT1 dans N=50 tumeurs du sein et leurs tissus sains adjacents selon la classification moléculaire de St Gallen, et avoir une idée concrète quant au rôle de SIRT1 dans le cancer. Effectivement, on a trouvé que SIRT1 joue un rôle ambivalent dans le cancer du sein en montrant un double profil d'expression de SIRT1 dans les tumeurs mammaires. SIRT1 est significativement surexprimée dans les sous-types hormono-dépendants HRBCs et H2BCs, et est significativement sous-exprimée dans les tumeurs non hormono-dépendantes triple-négatives (TNBCs). L'expression de SIRT1 est donc, largement corrélée à la classification moléculaire de St-Gallen, et diminue inversement au fur et à mesure de l'agressivité du cancer du sein. Ces résultats impliquent que SIRT1 a un rôle oncogène dans les sous-types hormono-dépendants et un rôle suppresseur de tumeur dans les sous-types non hormono-dépendants. En outre, le fait que les taux d'expressions transcriptionnelles et traductionnelles de SIRT1 se distinguent significativement parmi les sous-types intrinsèques du cancer, montre l'utilité de cette dernière en tant que marqueur pronostique dans le cancer du sein.

Ces résultats suggèrent que l'activation de SIRT1 et la régulation positive du gène *SIRT1* peuvent être dépendantes du récepteur aux œstrogènes ER-α dans les HRBCs et H2BCs. Ces 2 sous-types hormono-dépendants sont caractérise par une surexpression des récepteurs hormonaux, surtout l'oncogène ER-α, alors que dans les TNBCs, l'expression d'ER-α est absente ou réprimée. SIRT1 et ER-α pourraient donc, former une boucle de rétroaction positive dans le cancer du sein. En effet, les voies oncogènes de signalisation médiées par les œstrogènes/ER-α stimulent la prolifération cellulaire et la croissance de la tumeur dans les sous-types hormono-dépendants en activant les gènes cibles d'ER-α par l'activité transcriptionnelle de ce dernier.

Nos résultats sont en accord avec les travaux d'Elangovan *et al.*, et Yao *et al.*, qui ont montré que SIRT1 est activée et régulée positivement par ER-α en réponse aux œstrogènes. Ils ont montré qu'ER-α et SIRT1 se lient physiquement et coopèrent fonctionnellement dans le but de stimuler la croissance des cellules tumorales du sein, ils ont ainsi proposé un mécanisme de la coopération entre SIRT1 et ER-α dans la régulation du développement tumoral (**Figure 16**).

En outre, l'inactivation de SIRT1 supprime l'expression d'ER-α et élimine la croissance cellulaire induite par l'œstrogène/ER-α en inhibant l'expression des gènes cibles d'ER-α, et en provoquant l'apoptose et l'arrêt de la croissance cellulaire *in vitro* et *in vivo*. Les auteurs ont conclu que SIRT1 est un co-activateur d'ER-α, et est nécessaire pour la croissance du cancer du sein hormono-dépendant (Elangovan et al., 2011) (Yao et al., 2010).

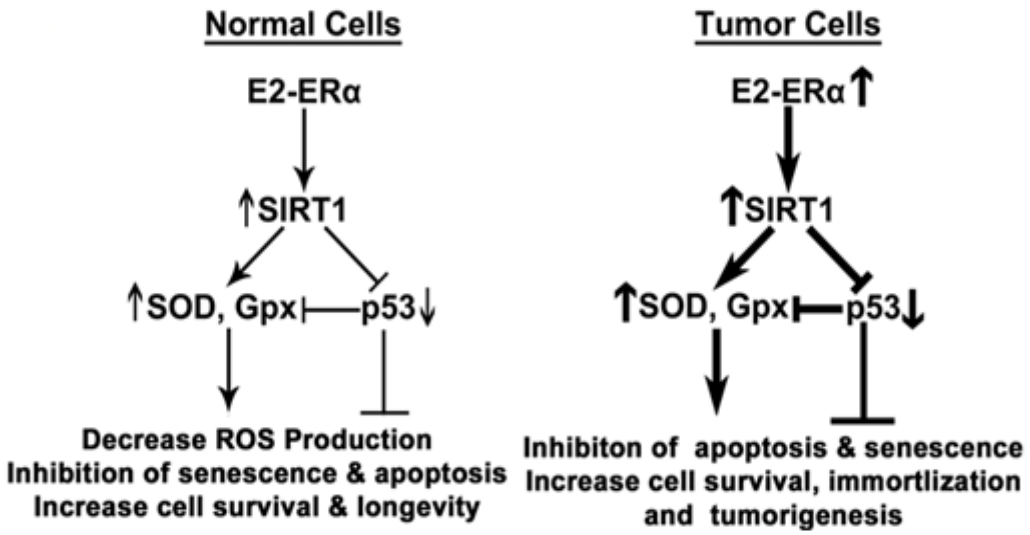

Figure 16. Schéma représentant le mécanisme moléculaire proposé du complexe SIRT1-ER-α dans la régulation de l'immortalisation des cellules tumorales et la promotion des cancers hormono-dépendants. Dans les cellules normales, le récepteur ER-α activé par l'œstrogène E2 forme un complexe avec SIRT1. Le complexe SIRT1-ER-α intervient dans plusieurs fonctions bénéfiques, telles que la protection des cellules contre les dommages oxydatifs d'ADN induits par les complexes ROS, l'inhibition de la signalisation apoptotique et la sénescence cellulaire, et l'augmentation de la survie cellulaire et de la longévité. Les fonctions de ce complexe sont assurées en induisant l'activité des antioxydants SOD et Gpx et réprimant l'activité de p53. Cependant, les mêmes fonctions favorisent la survie et la croissance des cellules tumorales dans les cancers hormono-dépendants (Elangovan et al., 2011).

La deuxième partie de nos travaux de thèse s'est intéressée aux mécanismes de l'acétylation et la désacétylation des histones H3 et H4, aux enzymes épigénétiques responsables de leur modulation dans le cancer, et le rôle de ces enzymes dans le processus de la carcinogenèse mammaire. Dans ce contexte et compte tenu des rôles clés de SIRT1 et de TIP60 dans la progression tumorale, l'objectif principal de cette partie des travaux a été d'étudier l'implication de ses 2 enzymes épigénétiques dans le développement du cancer du sein. Deux études ont été donc effectuées : une concernant l'histone desacétylase SIRT1 et l'autre l'acétyltransférase TIP60.

Nous nous sommes particulièrement intéressées aux 3 épi-marques activatrices H3K4ac, H3K9ac et H4K16ac décrites précédemment. On a vu que H3K9ac et H4K16ac ont des rôles bien définies dans la régulation de la structure de la chromatine, et que leur désacétylation entraîne l'inactivation de la transcription et la répression de l'expression génique (Vaquero et al., 2004) (Vaquero et al., 2007). Ainsi ces 2 marques sont sous-exprimées dans le cancer du sein (Elsheikh et al., 2009), cependant la variation des profils globaux de leur acétylation selon les différents sous-types moléculaires du cancer n'est pas encore été élucidé. Dans notre étude, on a montré une hypoacétylation globale significative des marques H3K4, H3K9, et H4K16 dans les tumeurs luminales A et B versus les tissus sains appariés. Alors que le taux d'acetylation de ces 3 marques tend à augmenter dans les sous-types HER2-enriched et TNBC. En plus, on a détecté une corrélation inverse entre les profils d'expression de ces 3 marques et ceux de SIRT1 dans les différentes tumeurs du sein. Cette corrélation inverse fournit un lien de causalité entre SIRT1 et ces 3 marqueurs et suggère que SIRT1 peut moduler l'expression de ces marques dans le cancer du sein. Pour confirmer cette hypothèse, on a inhibé l'expression de SIRT1 *in vitro* par des siARNs spécifiques. Nous avons opté pour l'utilisation de 5 lignées cellulaires du cancer du sein qui représentent les 2 principaux groupes moléculaires du cancer. Les lignées MCF-7 et T-47D qui sont classiquement utilisées comme représentants des sous-types luminals (ER+), et les lignées MDA-MB 453 (LAR), MDA-MB 231 (Mésenchymal), et MDA-MB 468 (Basal) qui représentent les 3 principaux sous-types triple-négatifs (ER-) (Lehmann et al., 2011). L'inhibition de SIRT1 a engendré une hyperacétylation globale significative des marques H3K4, H3K9, et H4K16 dans les 5 lignées transfectées versus les lignées non transfectées, on a donc conclu que SIRT1 est activement responsable de la modulation des marques activatrices H3k4ac, H3k9ac et H4k16ac à travers les différents sous-types moléculaires de cancer du sein.

En effet, peu de données existent sur la régulation de la marque H3K4ac dans le cancer du sein. Les études antérieures du laboratoire ont mis en évidence une diminution significative de H3K4ac sur les promoteurs de 7 gènes fortement impliqués dans la progression et le développement du cancer du sein: *BRCA1, ERS1, ERS2, PGR, EZH2, EP300,* et *SRC3*. En outre, la diminution de taux de H3K4ac coïncide avec une dérégulation de l'expression de ces gènes dans les tumeurs du sein (Judes et al., 2016). Cette étude corrèle avec les travaux sur le cancer de la bouche qui montrent une diminution de taux de H3K4ac dans les stades avancés de cancer, ainsi la valeur pronostique de H3K4ac dans le cancer a été mise en évidence (Chen et al., 2013b).

Dans une étude récente, Messier *et al.,* ont exploré les dynamiques de l'acétylation et la méthylation de H3K4 *in vitro* dans 2 lignées cellulaires de cancer du sein. Ils ont mis en évidence une association entre H3K4ac et des voies oncogénétiques dérégulées associées au cancer du sein. Ils ont également démontré le rôle de H3K4ac dans la prédiction des changements épigénétiques associés aux premiers stades de la transformation maligne (Messier et al., 2016). Nous nous sommes alors intéressés aux mécanismes de la régulation de cette marque dans le cancer sporadique du sein. Cependant, les enzymes modulatrices de cette marque n'ont pas encore été identifiées chez les humains, or l'HAT MST1 et l'HDAC SIR2 qui sont les orthologues hautement conservés de TIP60 et SIRT1 humains respectivement, sont responsables de l'acétylation et de la désacétylation de H3K4 chez la levure. (Imai et al., 2000) (Xhemalce and Kouzarides, 2010).

Par ailleurs, l'identification des changements épigénétiques dans le profil des modifications des histones à l'intérieur des cellules cancéreuses se fonde sur la disponibilité de techniques pour étudier ces changements. La spectrométrie de masse et les anticorps spécifiques dirigés contre différentes modifications des histones, sont parmi les outils les plus fréquemment utilisés de nos jours. Ainsi, les anticorps permettent non seulement la détermination des changements globaux, mais aussi, dans le contexte d'immunoprécipitation de la chromatine (ChIP), permettent l'identification des changements spécifiques sur des séquences définies, ainsi que mettent en évidence les interactions entre les protéines cibles et ces séquences (Mundade et al., 2014).

On a donc étudié l'enrichissement de la marque H3K4ac par ChIP-qPCR sur les promoteurs du panel de gènes associés cancer du sein. Ensuite les techniques de ChIP re-ChIP et de co-immunoprécipitation (co-IP) nous ont permis de mettre en évidence une colocalisation et une interaction physique entre la marque H3K4ac et les enzymes SIRT1 ou TIP60 sur les promoteurs du panel des gènes ciblés. Dans un deuxième temps, l'inhibition de SIRT1 ou de TIP60 dans les lignées mammaires cancéreuses (ER+) et (ER-), a confirmé que la désacétylation et l'acétylation de H3K4 dans le cancer du sein est dépendante de SIRT1 et de TIP60, respectivement.

SIRT1 joue un rôle majeur dans le maintien de l'intégrité du génome, principalement par la régulation des mécanismes épigénétiques qui régissent la structure de la chromatine. La régulation épigénétique dépendante de SIRT1 est réalisée par la désacétylation directe de ses marqueurs d'histones cibles, y compris les épi-marques H3K9ac et H4K16ac, ainsi que la régulation de l'activité des enzymes modifiant la chromatine. Beaucoup d'études ont examiné l'implication de SIRT1 dans la tumorigenèse mammaire médiée par son activité desacétylase envers ses cibles non-histones, cependant il y a peu d'études qui ont investigué la régulation épigénétique des cibles d'histones H3 et H4 dépendante de SIRT1, et l'effet de cette régulation sur l'expression génique dans le cancer du sein.

Wang *et al.,* ont révélé que SIRT1 inhibe la croissance tumorale *in vivo* par la suppression de l'expression de Survivin, qui est un membre de la famille des inhibiteurs de l'apoptose (IAP). La répression épigénétique de Survivin dépendante de SIRT1 se produit par la désacétylation de la marque H3K9ac sur le promoteur de Survivin et par conséquent, l'inactivation de sa transcription dans les tumeurs mammaires. Ils ont montré donc, un rôle suppresseur de tumeur de SIRT1 dans le cancer du sein (Wang et al., 2008b). En revanche, Pruitt *et al.,* ont démontré que la déplétion de SIRT1 *in vitro* réactive les gènes suppresseurs de tumeurs réprimés en augmentant les taux d'acétylation des marques H3K9 et H4K16 au niveau de leurs promoteurs, indiquant donc une répression épigénétique de ces gènes dépendante de SIRT1 par le biais de modifications d'histones dans le cancer du sein (Pruitt et al., 2006).

En utilisant une cohorte de 110 tumeurs mammaires classées selon la classification moléculaire de St.Gallen et leurs tissus sains appariés, on a étudié la régulation épigénétique des 2 marques activatrices H3K4ac et H3K9ac dépendante de SIRT1 par ChIP-qPCR sur les promoteurs des gènes cibles *AR, BRCA1, ERS1, ERS2, EZH2,* et *EP300* dans le cancer du sein. Certains de ces gènes jouent un rôle promoteur de tumeur en favorisant la prolifération et la croissance tumorale, tels que *AR* (Feng et al., 2017), *ERS1* (Deroo and Korach, 2006), *PGR* (Giulianelli et al., 2012), *EZH2* (Yoo and Hennighausen, 2012), et *EP300* (Fan et al., 2002). D'autres se comportent comme des gènes suppresseurs de tumeur tels que *BRCA1* (Fan et al., 1999) et *ERS2* (Skliris et al., 2003).

Tout d'abord, on a mis en évidence un enrichissement de SIRT1 par ChIP-qPCR sur les promoteurs de nos gènes cibles à travers les 5 sous-types moléculaires, ce qui suggère que SIRT1 pourrait jouer un rôle dans la régulation épigénétique de l'expression de ces gènes dans le cancer du sein. Ensuite, on a pu démontrer une régulation épigénétique différentielle de SIRT1 envers ses cibles d'histones H3 sur les promoteurs des gènes cibles en effectuant des ChIP reChIP-qPCR. En fait, les analyses statistiques ont montré que cette régulation dépend étroitement du type de gène et du sous-type moléculaire.

En outre, l'inhibition de SIRT1 *in vitro* par des siARNs dans les 5 lignées (ER+) et (ER-) cancéreuses, a révélée une induction différentielle significative des marques H3K4 et H3K9 acétylées à travers les promoteurs du panel de gènes cibles. En effet, on a repéré 2 profils distincts d'enrichissement de ces marques qui correspondent aux 2 principaux sous-types moléculaires de cancer du sein. Les 2 marques se sont avérées particulièrement enrichies sur les promoteurs des GST *BRCA1* et *ESR2* dans les lignées luminales (ER +), tandis que elles ont été spécialement enrichies sur les promoteurs des oncogènes *AR, EZH2* et *EP300* dans les lignées triple-négatives (ER-). Ces résultats indiquent que SIRT1 régule l'acétylation des marques activatrices d'une manière différentielle en fonction du sous-type moléculaire, et par conséquent, joue un rôle actif dans la régulation épigénétique de l'expression des gènes impliqués dans la pathogenèse du cancer du sein.

Cette régulation différentielle des gènes associés au cancer du sein implique aussi que SIRT1 joue un double rôle dans le cancer, et confirme l'hypothèse émise dans notre étude précédente. En fait, l'augmentation du taux d'acétylation des 2 marques sur les régions promotrices des GST *BRCA1* et *ESR2* dans les lignées luminales, indique un rôle oncogène de SIRT1 dans ces sous-types en réprimant l'expression de ces gènes par le biais des modifications d'histones. À l'opposé, l'inhibition de SIRT1 dans les 3 lignées triple-négatives induit une augmentation de l'expression de H3K4ac et H3K9ac sur les promoteurs des oncogènes *EZH2* et *EP300*, indiquant un rôle suppresseur de tumeur de SIRT1 dans les sous-types triple-négatives. Ces résultats sont en accord avec les travaux de Yi *et al.*, qui ont montré que SIRT1 induit la désacétylation de mt-p53, la forme mutante oncogène de p53, ce qui aboutit à la régulation positive de l'expression des gènes pro-apoptotiques *PUMA* et *NOXA* et à la suppression de la prolifération et de la croissance cellulaire des lignées triple-négatives *in vitro* (Yi et al., 2013). Ainsi que les travaux de Simic *et al.*, qui ont revelé que l'activation de SIRT1 supprime la migration des cellules cancéreuses *in vivo* en réduisant la transition épithélo-mésenchymateuse (Simic et al., 2013). Cependant, une étude récente a montré que l'activation de l'AMPK médiée par SIRT1 inhibe sélectivement la capacité de l'invasion et la migration des cellules triple-négatives, mais favorise en même temps la survie et la prolifération des cellules (Urra et al., 2018).

On a aussi remarqué une augmentation exceptionnelle de l'expression de H3K4ac et H3K9ac sur le promoteur du gène *AR* dans la lignée MDA-MB 453 représentative du sous-type triple-négatif LAR. En fait, le sous-type LAR ou encore nommé le carcinome apocrine du sein est une forme rare de carcinome mammaire avec un pronostic controversé. Il est ainsi caractérisé par la surexpression du récepteur des androgènes (AR) qui contribue à la tumorigénicité des carcinomes apocrines (Lehmann et al., 2011). Ainsi, SIRT1 semble également exercer des propriétés suppressives de tumeur dans le cancer apocrine du sein par la répression épigénétique de l'oncogène AR. Cette conclusion se corrèle avec les travaux de Zhang W *et al.*, qui ont révélé que la surexpression de BRCA1 supprime la croissance tumorale dépendante de l'AR par l'activation de SIRT1 *in vitro*, et qu'une surexpression de SIRT1 inhibe la prolifération des cellules cancéreuses stimulée par AR (Zhang et al., 2016). Une autre étude par Fu *et al.*, a démontré que SIRT1 se lie et régule négativement l'activité de l'AR *in vitro*, et que la répression de l'activité AR par SIRT1 inhibe la prolifération des cellules induites par l'androgène (Fu et al., 2006).

On a caractérisé dans cette étude, un aspect de la régulation épigénétique de SIRT1 vis-à-vis de ses cibles d'histones H3 et H4 dans le cancer du sein. On a ainsi mis en évidence le rôle différentiel de SIRT1 dans le développement du cancer du sein en fonction du sous-type moléculaire. En perspective, il serait intéressant d'inhiber l'activité desacétylase de SIRT1 *in vitro* dans les lignées (ER+) et (ER-) par des inhibiteurs chimiques spécifiques qui n'affectent pas l'activité des HDACs de classe I/II, et qui sont déjà considérés comme des agents anticancéreux tels que les HDACi : Sirtinol et EX527 (Selesistat) (Villalba and Alcaín, 2012) (Hu et al., 2014) (Kozako et al., 2014). On pourrait étudier par la suite l'effet de cette inhibition sur la dynamique des profils d'acétylation des marques H3K4, H3K9, et H4K16 au sein des promoteurs du panel des gènes cibles, et explorer la corrélation entre les altérations de ces marques et la dérégulation de nos gènes cibles en évaluant leurs taux d'expressions transcriptionnelles et traductionnelles dans les cellules transfectées versus les cellules contrôles. Cela nous permet de vérifier l'impact de SIRT1 sur la régulation de nos gènes cibles en comparant leurs profils d'expression dans les lignées (ER+) versus les lignées (ER-).

Puisque SIRT1 est sous-exprimée dans les tumeurs triple-négatives, il serait intéressant de l'activer *in vitro* en traitant les lignées cancéreuses triple-négatives (ER-) avec des activateurs spécifiques de SIRT1 comme le Resveratrol (Borra et al., 2005) et SRT1720 (Mitchell et al., 2014). On pourrait essayer ainsi de surexprimer SIRT1 dans ces lignées en les transfectant avec des vecteurs d'expression ectopique de SIRT1 (Recombinant Lentiviral Vectors LV5-SIRT1). On pourrait alors évaluer dans un deuxième temps l'effet de la surexpression de SIRT1 sur le profil d'expression de récepteurs nucléaires impliqué dans la carcinogenèse mammaire à une plus grande échelle par analyse transcriptomique (TaqMan Low Density Array).

Afin d'étudier l'implication de SIRT1 dans le développement tumoral du cancer du sein *in vivo*, il serait intéressant d'établir plusieurs modèles de souris xénogreffes de cancer du sein en injectant des souris immunodéprimées avec des lignées cellulaires représentant les différents sous-types luminales et triple-négatives. On pourrait ainsi étudier l'effet de la surexpression ou l'inhibition de SIRT1 sur le développement de tumeur *in vivo* en inhibant SIRT1 avec les HDACi dans les lignées luminales ou en surexprimant SIRT1 par des vecteurs d'expression ectopique dans les lignées triple-négatives. Ainsi, les tumeurs avec des profils moléculaires différents provenant de ces souris pourraient être examinées afin d'étudier les paramètres précédemment décrits.

TIP60 est une lysine acétyltransférase impliquée dans des processus cellulaires clés, y compris le développement tumoral. Cependant, TIP60 semble avoir un rôle bivalent dans les cancers humains. Afin d'étudier l'implication de TIP60 dans le cancer du sein, on a établi 2 modèles de souris xénogreffes de cancer du sein en injectant les souris immunodéprimées avec des cellules cancéreuses MCF-7 (ER+) et MDA-MB-231 (ER-) transfectées par des sh-TIP60. L'inhibition de l'expression de TIP60 par des shARNs nous a permet d'étudier l'impact de celui-ci sur le développement des tumeurs *in vivo*.

L'inhibition de TIP60 dans le modèle MCF-7 sh-TIP60 entraine un ralentissement de la croissance tumorale indiquant un rôle oncogène de TIP60 dans les tumeurs (ER+), tandis que dans le modèle MDA-MB-231 sh-TIP60, l'inhibition de TIP60 conduit à une augmentation de la croissance tumorale, indiquant alors un rôle suppresseur de tumeur de TIP60 dans les tumeurs (ER-). En perspective, il serait intéressant d'établir plusieurs modèles xénogreffes tels que MDA-MB436, MDA-MB453, et MDA-MB468 qui correspondent aux différents sous-types du cancer du sein triple-négatif. Ensuite, on pourrait induire une inhibition chimique de TIP60 par des inhibiteurs spécifiques comme le TH1834 (Gao et al., 2014) et NU9056 (Coffey et al., 2012), ou induire une surexpression de TIP60 par des vecteurs de transfection dans ces modèles. Cela va nous permettre d'observer l'impact de l'inhibition ou de la surexpression de TIP60 sur la croissance tumorale des lignées triples-négatives *in vivo*.

On a montré ainsi que TIP60 est sous-exprimée dans les tumeurs mammaires à travers les différents sous-types moléculaires du cancer du sein, avec une sous-expression plus importante dans les sous-types luminales. On a remarqué ainsi que le profil d'expression de TIP60 dans le cancer du sein est inversement corrélé avec celui de SIRT1 à travers les sous-types moléculaires, ce qui suggère un lien de causalité entre l'expression des 2 enzymes.

En effet, outre les modifications d'histones, la régulation des mécanismes épigénétiques dépendante de SIRT1 s'étend vers d'autres enzymes épigénétiques impliquées dans le remodelage de la chromatine telles que des HATs et des HMTs (Bosch-Presegué and Vaquero, 2015). Ainsi, SIRT1 peut réprimer l'activation excessive de la réponse aux dommages de l'ADN en régulant négativement l'activité de TIP60 et en stimulant la dégradation de TIP60 dépendante du protéasome *in vivo* (Yamagata and Kitabayashi, 2009)

(Wang and Chen, 2010) (Peng et al., 2012). Il serait intéressant alors d'élucider les mécanismes de la régulation de TIP60 dépendante de SIRT1 et explorer la dynamique de leur relation dans les différents sous-types du cancer du sein.

Finalement, Il serait intéressant également de surexprimer TIP60 et d'inhiber l'activité de SIRT1 en même temps dans les lignées luminales (ER+), et à l'opposé, d'inhiber l'expression de TIP60 et d'activer SIRT1 dans les lignées triple-négatives (ER-). Dans un deuxième temps, on pourrait étudier l'impact de ces traitements combinés sur les profils d'acetylation des marques activatrices ainsi que sur l'expression des gènes cibles associés au cancer du sein, et ultimement sur la croissance tumorale *in vivo.*

En conclusion, les travaux de cette thèse ont permis d'examiner la variation des profils globaux des marques activatrices d'histones H3 et H4 dans les différents sous-types de cancer du sein. Nous avons ainsi étudié l'implication de l'histone desacétylase SIRT1 et de l'acétyltransférase TIP60 dans la régulation de leurs cibles histones mais ainsi dans le développement du cancer du sein. Ces travaux mettent en relief SIRT1 et TIP60 comme des cibles thérapeutiques potentielles du cancer sporadique du sein.

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RESUMÉ

Avec 59 000 nouveaux cas en 2017, le cancer du sein est le cancer le plus fréquemment diagnostiqué chez les femmes françaises, et pose un réel problème de santé publique en France, mais aussi au niveau mondial. Il est bien établi que la complexité de la carcinogenèse implique des modifications épigénétiques profondes qui contribuent au processus du développement tumoral. La dérégulation des marques d'histones acétylées H3 et H4 font partie de ces modifications. L'acétylation et la désacétylation des protéines sont des modifications post-traductionnelles majeures qui régulent l'expression des gènes liés au cancer et à l'activité d'une myriade d'oncoprotéines. Ainsi, une activité désacétylase aberrante peut alors favoriser ou supprimer la tumorigenèse dans différents types de cancers humains, y compris le cancer du sein. La désacétylase SIRT1 et l'acétyltransférase TIP60 sont 2 enzymes épigénétiques antagonistes qui sont impliquées dans l'apoptose, la régulation des gènes, la stabilité génomique, la réparation de l'ADN, et le développement du cancer. Dans le cadre de cette thèse, nous avons étudié la dérégulation des profils d'acétylation des histones H3 et H4 dans les différents sous-types moléculaires du cancer du sein, et investigué l'implication de SIRT1 et de TIP60 dans la progression tumorale de cancer du sein. Tout d'abord, nous avons signalé les rôles de SIRT1 et de TIP60 comme des biomarqueurs pronostiques potentiels en révélant leurs expressions différentielles en fonction de l'agressivité du cancer. Ensuite, nous avons montré leur régulation épigénétique différentielle des cibles histones en fonction du sous-type moléculaire, ainsi que leur modulation de la marque activatrice H3K4ac. En outre, l'inhibition de ces 2 enzymes par des Épi-drogues s'est avérée comme une stratégie efficace dans le traitement du cancer. Ces travaux mettent en relief alors, SIRT1 et TIP60 comme des cibles thérapeutiques potentielles du cancer sporadique du sein.

Mots clés : **Cancer du Sein**, **Épigénétique**, **SIRT1**, **TIP60**, **H3K4ac**, **Épi-drogues**

ABSTRACT

With 59,000 new cases in 2017, breast cancer is the most frequently diagnosed cancer among French women, and poses a real public health problem in France, but also worldwide. It is well established that the complexity of carcinogenesis involves profound epigenetic deregulations that contribute to the tumorigenesis process. Deregulated H3 and H4 acetylated histone marks are amongst those alterations. Acetylation and deacetylation are major post-translational protein modifications that regulate gene expression and the activity of a myriad of oncoproteins. Aberrant deacetylase activity can promote or suppress tumorigenesis in different types of human cancers, including breast cancer. The deacetylase SIRT1 and the acetyltransferase TIP60 are 2 antagonistic epigenetic enzymes that are well implicated in apoptosis, gene regulation, genomic stability, DNA repair, and cancer development. In this manuscript, we identified the dysregulation of the histones H3 and H4 acetylation profiles in different molecular subtypes of sporadic breast cancer, and investigated the involvement of SIRT1 and TIP60 in breast tumorigenesis. First, we highlighted the roles of SIRT1 and TIP60 as potential prognostic biomarkers by revealing their differential expression patterns depending on breast cancer aggressiveness. Then, we demonstrated their differential epigenetic regulation of histone targets according to molecular subtype, and revealed their modulation of the H3K4ac epigenetic marker. Moreover, Epi-drugs mediated inhibition of these 2 enzymes has proven to be an effective strategy in the treatment of cancer. Thus, this work highlights the potential use of SIRT1 and TIP60 as epigenetic therapeutic targets for sporadic breast cancer.

Keywords: **Breast cancer**, **Epigenetics**, **SIRT1**, **TIP60**, **H3K4ac**, **Epi-drugs**