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THE ROLE OF HISTONE N-TERMINAL ACETYLTRANSFERASE NAA40 IN GENE EXPRESSION AND COLORECTAL CANCER CELL DEATH

DEMETRIA PAVLOU

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DEMETRIA PAVLOU

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PhD Candidate: Demetria P. Pavlou

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The present Doctoral Dissertation was submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Department of Biological Sciences and was approved on the 15.04.2016 by the members of the Examination Committee.

Examination Committee:

- **Research Supervisor:** Antonis Kirmizis, Assistant Professor
- **Committee Member:** Pantelis Georgiades, Assistant Professor
- **Committee Member:** Katerina Strati, Assistant Professor
- **Committee Member:** Kyriacos Kyriacou, Professor
- **Committee Member:** Panagiotis Papageorgis, Assistant Professor

DEMETRIA PAVLOU

DECLARATION OF DOCTORAL CANDIDATE

The present doctoral dissertation was submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes or any other statements.

________________________________ [Full Name of Doctoral Candidate]

________________________________ [Signature]

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A PhD, is definitely an exhausting journey and in many cases a frustrating one, as well. Your world can be limited to your lab and your bed and ‘‘it may cost you a kidney and a lung’’, as many people state. There are times you lose yourself and many more times that you want to give up in an attempt to get your old self back. But there are also times that you remember the bigger scope and the greatest goals you set. There are times you feel pride about your achievements, your contributions to research and society and times that you realise that your new self is your better version. They say, to survive you must be willing to fail. I say, you need to have the right people by your side, the whole time.

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the title of

Released-Unchained-FREE Pavlou.D.

THE END :)

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ΠΕΡΙΛΗΨΗ

Η αμινο-τελική ακετυλίωση (Ντ-ακετυλίωση) των πρωτεϊνών αποτελεί μία από τις συνηθέστερες τροποποιήσεις που παρουσιάζονται στο 80-90% των κυτταροπλασματικών, ευκαρυωτικών πρωτεϊνών. Αυτή η τροποποίηση έχει αναδειχθεί ως σημαντικός ρυθμιστής των πρωτεϊνών στόχων, επιφέροντας αξιοσημείωτες συνέπειες στη δράση τους. Αλλαγές στη σταθεροποίηση της πρωτεΐνης-στόχου στο κύτταρο, η διευκόλυνση αλληλεπιδράσεων καθώς και η συμμετοχή των πρωτεϊνών-στόχων σε μονοπάτια μεταβολισμού είναι μόνο λίγες από τις πολλαπλές επιπτώσεις που επιφέρει η προσθήκη της ακετυλομάδας στο αμινο-τελικό άκρο των πρωτεϊνών-στόχων. Η Ντ-ακετυλίωση καταλύεται από εξελικτικά συντηρημένες πρωτεΐνες, γνωστές ως αμινο-τελικές ακετυλτρανσφεράσες (NATs). Η απορρύθμιση αυτών των ενζύμων έχει συνδεθεί με την ανάπτυξη καρκινικών όγκων και ως εκ τούτου, έχουν αναδειχθεί ως υποσχόμενοι στόχοι σε διαγνωστικές και θεραπευτικές αντικαρκινικές μεθόδους. Η Ντ-ακετυλτρανσφεράση 4 (Naa40) αποτελεί μία εξαιρετικά εκλεκτική αμινο-τελική ακετυλτρανσφεράση, καθώς ακετυλιώνει μόνο δύο πρωτεΐνες-στόχους, την ιστόνη H4 και την ιστόνη H2A. Στον ζυμομύκητα, ο οποίος αποτελεί χρήσιμο απλουστευμένο μοντέλο οργανισμού, η Naa40 παρουσιάζεται ως σημαντικός αισθητήρας της κυτταρικής ανάπτυξης.

Στην παρούσα μελέτη εξετάζουμε τον βιολογικό και μοριακό ρόλο της Naa40 στην επιβίωση των καρκινικών κυττάρων. Αποδεικύουμε ότι η απουσία έκφρασης και δράσης της Naa40 στις HCT116 και HT-29 ανθρώπινες κυτταροσειρές ορθοκολικού καρκίνου, μειώνει την επιβίωση των κυττάρων ενεργοποιώντας το μιτοχονδριακό αποπτωτικό μονοπάτι, καθώς η ιστόνη H4 παύει να φέρει την αμινοτελική ακετυλίωση. Αντιθέτως, η απουσία της Naa40 σε εμβρυϊκούς, μη καρκινικούς ινοβλάστες ποντικού (STO κύτταρα) δεν επιφέρει καμία αλλαγή στη βιωσιμότητα των κυττάρων. Συγκεκριμένα, η μείωση της Naa40 σε καρκινικά κύτταρα με επεμβατικές RNA αλληλουχίες (siRNAs), οδηγεί στην ενεργοποίηση της μιτοχονδριακής κασπάσης-9, η οποία με τη σειρά της ενεργοποιεί άλλες εκτελεστικές κασπάσες, ούτως ώστε να επέλθει κυτταρικός θάνατος. Η απαραίτητη συμμετοχή της κασπάσης-9 στην επαγωγή της απόπτωσης επιβεβαιώνεται με τη χρήση μη αναστρέψιμου αναστολέα κατά της κασπάσης-9, όπου, παρόλο που η Naa40 απουσιάζει, η απόπτωση παρεμποδίζεται. Σημαντική είναι η ανακάλυψη που αποδεικνύει ότι η ενεργοποίηση του αποπτωτικού μηχανισμού προκαλείται χωρίς τη συμμετοχή της

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ογκοκατασταλτικής πρωτεΐνης p53, η οποία είναι συχνά μεταλλαγμένη και ανενεργή στους πλείστους καρκίνους.

Ο ρόλος της Naa40 στη ρύθμιση της γονιδιακής έκφρασης έχει επίσης διερευνηθεί. Αρχικά, δείξαμε ότι η δράση της Nαα40 είναι απαραίτητη για την έκφραση του ριβοσωμικού RNA, καθώς η απουσία του ενζύμου οδήγησε στη μείωση των επιπέδων του ριβοσωμικού RNA. Αυτή η παρατήρηση συνάδει με την πρόσφατη ανακάλυψη ότι η Naa40 ενεργοποιεί το ριβοσωμικό DNA στον ζυμομύκητα, καταδεικνύοντας την εξελικτικά συντηρημένη δράση της. Επιπλέον, δείξαμε ότι κατά την απώλεια της Naa40, η έκφραση των αντι-αποπτωτικών γονιδίων είναι σημαντικά εξασθενημένη, ενώ αντιθέτως, η έκφραση των προ-αποπτωτικών γονιδίων εντείνεται. Αυτά τα ευρήματα ενισχύουν τον αντι-αποπτωτικό ρόλο της Naa40 και υποδηλώνουν ότι, η αμινοτελική ακετυλομάδα που διακοσμεί την ιστόνη H4, λόγω της δράσης της Naa40, είναι απαραίτητη για τη μεταγραφική ενεργοποίηση γονιδίων που εμπλέκονται στην επιβίωση των κυττάρων. Η μείωση της Naa40 στα κύτταρα επέφερε αλλαγές και σε άλλες επιγενετικές τροποποιήσεις. Σημαντική μείωση παρατηρήθηκε στα επίπεδα της συμμετρικής μεθυλίωσης της αργινίνης 3 στην ιστόνη H4 (H4R3me2s) καθώς και αύξηση της τριπλής μεθυλίωσης στη λυσίνη 4 της ιστόνης H3 (H3K4me3). Οι προαναφερθείσες τροποποιήσεις αντιπροσωπεύουν την καταστολή και την ενεργοποίηση σημάτων της μεταγραφής, αντιστοίχως. Αυτή η παρατήρηση εξηγεί την ενεργοποίηση των προ-αποπτωτικών γονιδίων στην απουσία της Naa40 ογκοπρωτεΐνης. Τέλος, δείξαμε ότι η μεταγραφική ενεργοποίηση της PRMT5 μεθυλτρανσφεράσης, η οποία είναι υπεύθυνη για την κατάλυση της H4R3me2s και η οποία συχνά υπερεκφράζεται σε πολλούς καρκινικούς τύπους, εξαρτάται άμεσα από τη δράση της Naa40. Συνολικά, αυτά τα ευρήματα καταδεικνύουν τον αντι-αποπτωτικό ρόλο της Naa40 και την προτείνουν ως ένα υποσχόμενο θεραπευτικό στόχο στους ορθοκολικούς καρκίνους.

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ABSTRACT

Protein N-alpha terminal acetylation (Nt-acetylation) is an abundant modification occurring on 80-90% of cytoplasmic, eukaryotic proteins. This modification has emerged as an important protein regulator with various and significant implications, including changes in protein stability, protein localisation and metabolism. Nt-acetylation is catalysed by evolutionarily conserved N-terminal acetyltransferases (NATs) whose deregulation has been linked to cancer development and thus, are emerging as useful diagnostic and therapeutic targets. Naa40 is a highly selective NAT that acetylates the amino-termini of histones H4 and H2A and acts as a sensor of cell growth in yeast.

In the present study, we examine the biological and molecular role of Naa40 in cancer cell survival. We first demonstrate that depletion of Naa40 in HCT116 and HT-29 colorectal cancer cells decreases cell survival by enhancing apoptosis, whereas Naa40 reduction in non-cancerous mouse embryonic fibroblasts (STO cells) has no effect in cell viability. Specifically, Naa40 knock-down in colon cancer cells activates the mitochondrial caspase-9-mediated apoptotic cascade. Consistent with this, we show that caspase-9 activation is required for the induced apoptosis because treatment of cells with an irreversible caspase-9 inhibitor impedes apoptosis when Naa40 is depleted. Additionally, the effect of Naa40-depletion on cell-death is mediated through a p53-independent mechanism since p53-null HCT116 cells still undergo apoptosis upon reduction of the acetyltransferase.

Furthermore, we look into the role of Naa40 in the regulation of gene expression. Initially, we show that depletion of Naa40 results in reduced levels of ribosomal RNA transcripts, which is consistent with the recently described function of yeast Naa40. Also, we show that upon Naa40 loss, the expression of anti-apoptotic genes is impaired whereas the expression of pro-apoptotic genes is up-regulated. These findings, together with the aforementioned identified anti-apoptotic role of Naa40 suggest that N-acH4 mediated by Naa40 is necessary for transcriptional activation of genes implicated in cell survival. Moreover, Naa40 knockdown results in a significant reduction of H4R3me2s and a great elevation of H3K4me3, which represent repressive and activation marks of transcription, respectively. Interestingly, we show that transcriptional activation of PRMT5 methyltransferase, which is responsible for catalysing H4R3me2s and which is frequently over-expressed in many cancer cell types, is dependent upon Naa40 activity. Altogether,

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these findings reveal the anti-apoptotic role of Naa40 and exhibit its potential as a therapeutic target in colorectal cancers.

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ABBREVIATIONS

AIF	Apoptosis Inducing Factor
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
bp	base pair
BSA	Bovin Serum Albumin
CI-PCD	Caspase-independent programmed cell death
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chromatin Immunoprecipitation sequencing
CHOP	C/EBP homologous protein
CpG	Cytosin-phosphate-guanine
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
ETS	External Transcribed Spacer
GNAT	GCN5-Related N-acetyltransferases
HDAC	Histone deacetylase
Hmt1	Histone methyltransferase 1
H2A126su	Histone H2A alanine 126 sumoylation
H3R2me2a	Histone H3 arginine 2 asymmetric dimethylation
H3R2me2s	Histone H3 arginine 2 symmetric dimethylation
H3K4ac	Histone H3 lysine acetylation

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H3K4me3	Histone H3 lysine 4 trimethylation
H3K9ac	Histone H3 lysine 9 acetylation
H3K9me3	Histone H3 lysine 9 trimethylation
H3S10ph	Histone H3 serine 10 phosphorylation
H3K27ac	Histone H3 lysine 27 acetylation
H3K27me3	Histone H3 lysine 27 trimethylation
H3K36me3	Histone H3 lysine 36 trimethylation
H4K5, K8, K12	Histone H4 lysine 5,8,12
H4R3me2a	Histone H4 arginine 3 asymmetric dimethylation
H4R3me2s	Histone H4 arginine 3 symmetric dimethylation
H4K20me3	Histone H4 lysine 20 trimethylation
IAP	Inhibitors of Apoptosis Proteins
IP	Immunoprecipitation
IF	Immunofluorescence
ITS	Internal Transcribed Spacer
JMJD6	Jumonji domain-containing 6
KATs	Lysine acetyltransferases
KDAs	Lysine deacetylases
KDMs	Lysine demethylases
KMTs	Lysine methyltransferases
MOMP	Mitochondrial outer membrane permeabilisation
MTT	3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium
Naa40	N(Alpha)-terminal acetyltransferase 40
N-acH4	N-terminal acetylation of histone H4
NAT	N-terminal acetyltransferase
ncRNA	Non-coding RNA

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NTS	Non-transcribed Spacer
PARP-1	Poly(ADP-ribose) polymerase-1
PBS	Phosphate-Buffered-Saline
PCR	Polymerase Chain Reaction
PHD	Plant homeodomain
PI	Propidium iodide
PRMTs	Protein Arginine Methyltransferase
PTM	Post-translational Modification
qRT-PCR	Quantitative RT-PCR
Rb	Retinoblastoma
rDNA	Ribosomal rDNA
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
RNA-seq	RNA sequencing
RT-PCR	Real Time-PCR
SAM	S-Adenosyl-Methionine
SDS	Sodium Dodecyl Sulfate
SWI/SNF	SWItch/Sucrose Non-Fermentable
UPR	Unfolded Protein Response

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CHAPTER 1

INTRODUCTION

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1.1 ‘‘On top of’’ genetics

Ever since researchers sequenced the human and chimp genome, we have known that there is 96% similarity between the DNA of these two organisms (Chimpanzee and Analysis, 2005, Britten, 2002). Yet, the two species differ developmentally, morphologically and behaviourally, in ways that the answer as to why, cannot lie entirely in the make-up of the genome. Therefore, mechanisms other than those within the DNA sequence should exist. In 1942, the developmental biologist Conrad Waddington, coined the term ‘‘*epigenetics*’’, which at first described the interaction of genotype with its environment in order to bring the phenotype into being (Van Speybroeck, 2002, Waddington, 1940, Dolinoy et al., 2007). Over the years, numerous studies challenged and revised the traditional paradigms of Mendelian inheritance and helped to realise that epigenetic regulation also exists on top of or in addition to the mechanisms of classical genetics. In other words, the evolved definition of ‘‘*epigenetics*’’ refers to those heritable changes that do not involve the DNA sequence itself (Holliday, 1987, Berger et al., 2009).

1.2 Epigenetic mechanisms at a glance

Epigenetic processes play a critical role in the differential expression of genes and in cellular heterogeneity. When disrupted, they may lead to altered transcriptional states and adverse health effects, including tumorigenesis (Sharma et al., 2010). The epigenetic landscape encompasses mainly DNA methylation, post-translational modifications of histone proteins and noncoding RNAs (Figure 1) (Goldberg et al., 2007, Kanherkar et al., 2014). Altogether provide tight control of gene expression and fascinating new levels of functional diversity.

DNA methylation, represents the best understood DNA-associated modification. It is catalysed by a specific class of enzymes, known as DNA methyltransferases (DNMTs) which catalyse the transfer of a methyl group (-CH_3) to cytosine nucleotides of DNA (Lai and Shiekhattar, 2014). In mammals, it occurs predominantly in regions that are rich in CpG dinucleotides, known as CpG islands, which are mostly found at the promoter regions of genes (Figure 1A) (Chen and Riggs, 2011, Vavouri and Lehner, 2012). This modification has been linked to transcriptional repression as it locks genes to the ‘‘off’’ state and is an important component of cellular processes including embryonic development and genomic imprinting (Jin et al., 2011). In addition to gene silencing, this

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epigenetic modification can mask many repetitive elements, contributing in this way to the stability of the genome (Robertson, 2001, Meng et al., 2015).

One of the most established hallmarks of epigenetic control, is the post-translational modification (PTM) of histones. With the help of mass spectrometry analysis and proteomic characterisation, a plethora of PTMs has been identified, such as acetylation, methylation and phosphorylation (Zhang et al., 2003) (Arnaudo and Garcia, 2013). These are chemical moieties that decorate amino acid residues of the protruding tails as well of the globular domains of histone core proteins and their variants (Figure 1B). Most of histone PTMS are transient, orchestrating in this way various chromatin-associated processes such as chromatin condensation, transcriptional silencing or activation, DNA repair, replication and recombination (Bannister and Kouzarides, 2011).

Noncoding RNAs (ncRNAs) are widely known as the ‘‘dark matter’’ of the genome as they do not have the blueprint of yielding functional proteins. Nonetheless, they comprise more than 95% of the human genome (St Laurent et al., 2012, Kapranov and St Laurent, 2012). Emerging evidence suggests that ncRNAs represent an integral component of the large epigenetic regulatory network. More specifically, many of them have been found to be functional in modulating gene transcription, through interactions with histone modifying complexes or by modulating DNA methyltransferases, thus suppressing or facilitating DNA methylation (Figure 1C) (Lai and Shiekhattar, 2014, Peschansky and Wahlestedt, 2014, Tsai et al., 2010, Mohammad et al., 2010).

The importance of regulation beyond the genomic sequence is becoming increasingly clear. The integration of all these different epigenetic processes is complex and dynamic and depends on the collaboration of numerous epigenetic modulators (Bartke et al., 2010) (Rothbart and Strahl, 2014). These, will regulate mostly chromatin accessibility and compactness in order to establish heritable states that will define cell identity and fate. Unlike genetic aberrations, epigenetic changes are reversible and can be restored to their primary state. Therefore, manipulation of epigenetic processes represents a promising and therapeutically relevant approach in epigenetic therapies.

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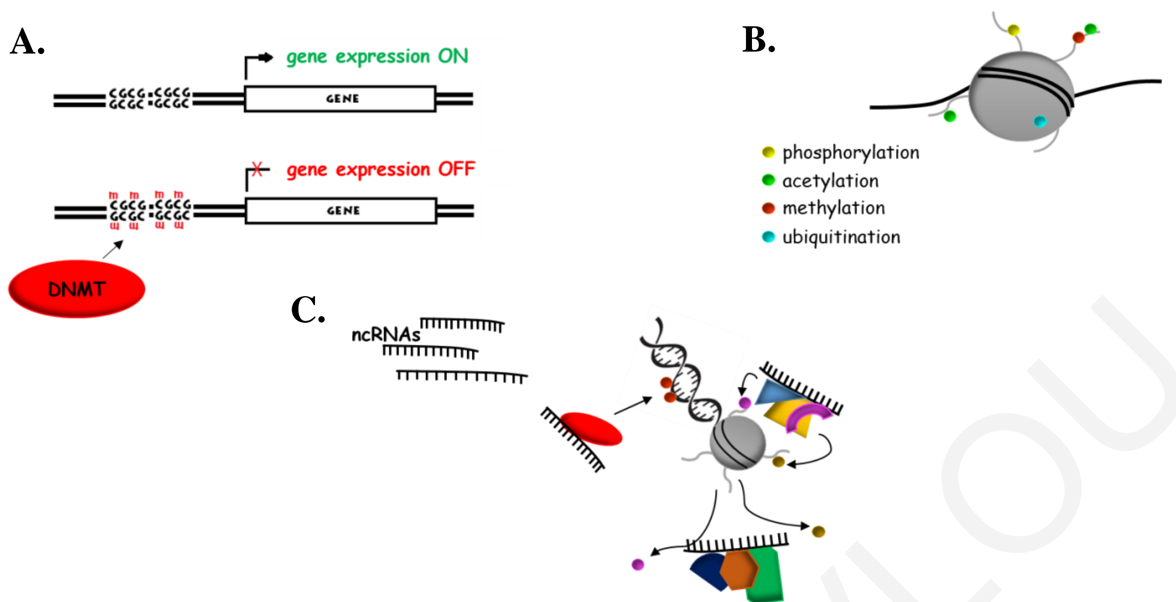

Figure 1. Schematic representation of epigenetic mechanisms. (A) DNA methylation catalysed by DNMTs occurs in regions rich in CpG dinucleotides, often found in promoters of genes. This results in supressed gene transcription. **(B)** Histones build the nucleosome (grey sphere) around which DNA is wrapped. Histone protruding tails or core domains are subjected to post-translational modifications that affect gene expression. **(C)** Noncoding RNAs (ncRNAs) can regulate gene expression by modulating DNMTs or by recruiting histone modifying complexes that will either add or remove post-translational modifications.

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1.3 Chromatin organisation and transcriptional regulation by histone post-translational modifications (PTMs)

The genome holds all the genetic information of an organism. In order to fit into the small volume of the cell nucleus, DNA must densely wrap around nucleosomes that are made up of histone proteins, which all together form the chromatin. Histones were originally thought to be needed only to provide a static scaffold for DNA packaging. Nowadays, it is evident that histones are dynamic proteins essential for regulating the structure of chromatin and histone post-translational modifications (PTMs) play a fundamental role in the chromatin remodelling process.

1.3.1 Chromatin structure

The basic structural repeating unit of chromatin, the nucleosome, was first described by Roger Kornberg, in 1974 (Kornberg, 1974). It consists of two pairs of histone heterodimers H2A/H2B and H3/H4 that form the histone octamer. Each histone has a globular domain forming the nucleosomal core, where 146/147 base pairs (bp) of DNA wrap around, and unstructured tails that extend outwards from the nucleosome (Figure 2) (Luger et al., 1997). One nucleosome is connected to the adjacent nucleosome through linker DNA to form a 'beads-on-a-string' nucleosomal array, with a diameter of 11nm (Li and Reinberg, 2011). Two full turns of DNA are locked on the surface of the histone octamer by linker histone H1, which organises chromatin in a more condensed 30nm chromatin fiber (Li and Reinberg, 2011, Harshman et al., 2013). Further condensation leads to higher-order structures, such as the known mitotic chromosomes (Figure 3).

Recent studies show that chromatin is dynamically remodelled. A transition between euchromatin, a relaxed open-state of chromatin, and heterochromatin, a highly compacted chromatin, is central to the regulation of DNA-coupled activities, including gene expression (Lee et al., 2010b, Suganuma and Workman, 2008, Berger, 2007). Accordingly, euchromatin represents regions of active transcription as it renders the DNA accessible to the transcriptional machinery, whereas heterochromatin typically correlates with gene silencing (Figure 3). Key contributors to chromatin structure and function are the post-translational modifications placed on histone proteins.

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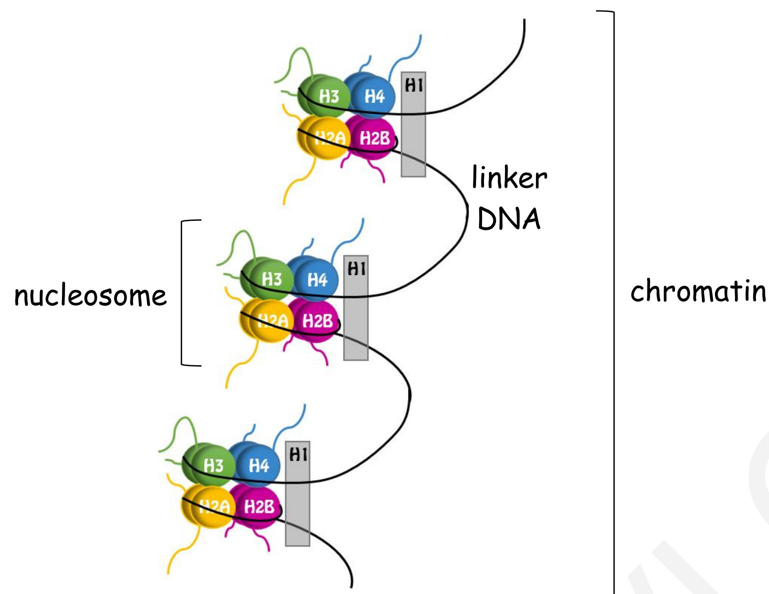

Figure 2. The structure of chromatin. Chromatin is a complex of DNA wound around the nucleosome. Dimers of four core histones (H2A, H2B, H3, H4) build the nucleosome and linker histone H1 seals DNA close to the histone octamer. Two neighbouring nucleosomes are associated with linker DNA, forming a ‘‘beads-on-a-string’’ chromatin model.

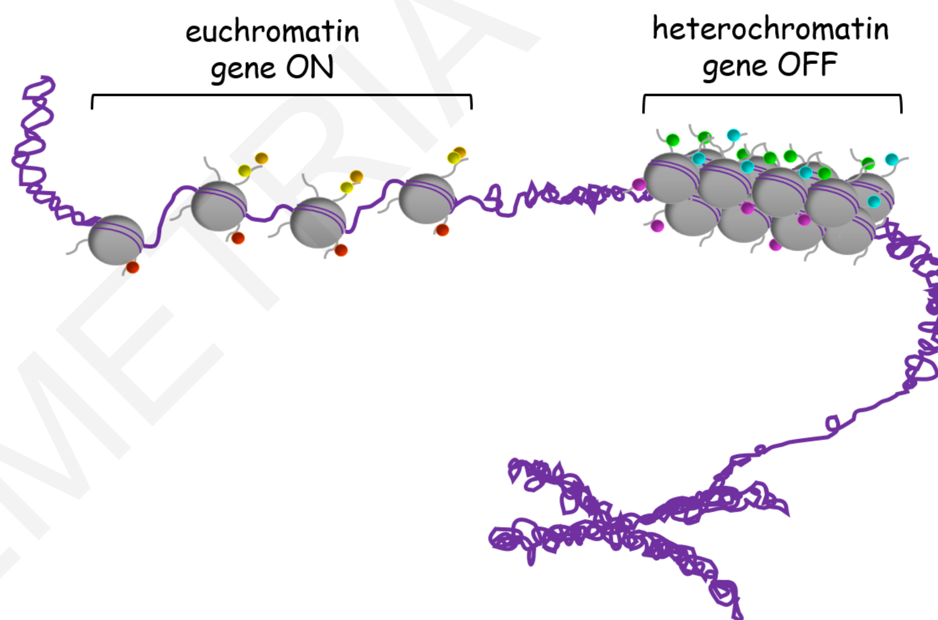

Figure 3. Differential states of chromatin assembly. Chromatin remodeling is dynamic and necessary for the control of DNA-based processes, like transcription. When nucleosomes are spaced far apart (euchromatic region), gene transcription is allowed to occur. When nucleosomes are closely together (heterochromatic region), transcriptional activation is inhibited. Tight compaction leads to the formation of chromosomes during cell division. Post-translational modifications on the protruding histone tails affect chromatin remodeling.

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1.3.2 Histone PTMs and the ''histone code''

The level of chromatin compaction and the associated genomic functions are highly regulated through an astonishing number of histone post-translational modifications (PTMs). These are chemical moieties that decorate mainly the amino acid residues found on the flexible N- and C-terminal ''tails'' that protrude away from nucleosome core. (Kouzarides, 2007). However, histone-histone and histone-DNA interactions have also been reported to be regulated by a significant number of PTMs found on the globular domain of nucleosomes (Cosgrove and Wolberger, 2005, Zhang et al., 2003, Tropberger and Schneider, 2013, Lawrence et al., 2016). Lysine acetylation, lysine and arginine methylation, lysine ubiquitination and serine/threonine/tyrosine phosphorylation are only some examples of histone PTMs found on histone proteins (Table 1) (Kouzarides, 2007, Arnaudo and Garcia, 2013, Bannister and Kouzarides, 2011). Acetylation, methylation and phosphorylation represent the most well-studied histone chemical modifications. They are the most abundant and as so, they were the first to be identified (Allfrey et al., 1964, Gutierrez and Hnilica, 1967). Additionally, a great number of novel PTMs has just recently been revealed, like lysine crotonylation and lysine formylation, and their function in chromatin regulation largely awaits discovery (Arnaudo and Garcia, 2013, Tessarz et al., 2014, Dai et al., 2014).

Table 1. Different PTMs identified on histone amino acid residues (Arnaudo and Garcia, 2013, Tessarz et al., 2014, Dai et al., 2014)

Histone Modification	Amino acid Modified Residue
Acetylation	Lysine (K), Kac
Methylation	Glutamine (Q), Qme; Lysine (K), Kme1, Kme2, Kme3; Arginine (R), Rme1, Rme2a, Rme2s
Phosphorylation	Serine (S), Sph; Threonine (T), Tph; Tyrosine (Y), Yph
Ubiquitination	Lysine (K), Kub
Sumoylation	Lysine (K), Ksu
ADP ribosylation	Glutamic acide (E), Ear
Citrullination (or deimination)	Arginine (R), Citrullin (Cit), R→Cit
Proline isomerisation	Proline (P), P-cis→P-trans
Formylation	Lysine (K), Kfo
Hydroxylation	Tyrosine (Y), Yoh; Lysine (K), 5-Koh
Butyrylation	Lysine (K), Kbu
2-hydroxyisobutyrylation	Lysine (K), Khib
Propionylation	Lysine (K), Kpr
Succinilation	Lysine (K), Ksuc
Malonylation	Lysine (K), Kma
Crotonylation	Lysine (K), Kcr
O-glcNAcylation	Serine (S), Sog; Threonine (T), Tog

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Many years had to pass in order to comprehend the functional significance of PTMs. The role of epigenetic chemical moieties in the regulation of transcription has been disclosed just two decades ago, with the landmark identification of p55/Gcn5 histone acetyltransferase as an activator of gene expression (Georgakopoulos and Thireos, 1992, Brownell et al., 1996) and HDAC/Rpd3 histone deacetylase as a contributor of transcriptional silencing (Taunton et al., 1996). Now, it is profound that specific histone modifications are predictive for certain gene regulation outcomes (Karlic et al., 2010).

Transcriptionally active chromatin regions are generally accompanied by acetyl- or phosphorylation marks (i.e H3K9ac and H3S10ph), added by histone acetyltransferases (HATs) or protein kinases, respectively (Strahl and Allis, 2000, Shen et al., 2015, Li et al., 2013). This is because histone lysine acetylation and phosphorylation neutralize positive charges in histones, weaken histone-DNA interactions and favour chromatin decondensation. Protein methyltransferases (i.e lysine methyltransferases, KMTs; protein arginine methyltransferases, PRMTs) and ubiquitinases are found to be implicated in both transcriptional activation and inhibition, whereas sumoylation is linked to transcriptional repression exclusively (Kouzarides, 2007). The corresponding transcriptional role of some well-characterised PTMs is depicted in Figure 4. Among them, is the trimethylation of lysine 4 of histone H3 (H3K4me3), a modification generally associated with transcriptionally active genes. It is also known that this modification provides a binding site for a variety of factors that include histone-modifiers and chromatin remodelers (Shilatifard, 2006).

Transcriptional Control

H3R2me2s	H3R2me2a
H3K4me3	H3K9me3
H3K4ac	H3K27me3
H3K27ac	H4R3me2s
H3K36me3	H4K20me3
H3S10ph	H2A126su

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Figure 4. Histone post-translational modifications and their role in transcriptional regulation, in humans. Distinct epigenetic modifications are predictive for gene activation or gene silencing (Strahl and Allis, 2000, Balazs et al., 2011) (Mozzetta et al., 2015, Migliori et al., 2012).

The numerous identified histone PTMs may work alone or in concert with other modifications found in *cis* (on the same histone protein) or in *trans* (on different histone protein or different nucleosomes) or in contrast abrogate the deposition of other epigenetic marks, in order to exhibit specific regulatory outcomes (Suganuma and Workman, 2008, Zhang et al., 2015c, Molina-Serrano et al., 2013). This PTM communication formed the concept of a complex histone language, known as the ‘‘histone code’’ (Jenuwein and Allis, 2001, Strahl and Allis, 2000, Lee et al., 2010b). Accordingly, PTMs can synergistically or antagonistically interact to alter the binding of DNA on the nucleosome core by changing the charge of the nucleosome particle and enable or block chromatin-remodeling proteins to dictate transient transitions between euchromatin and heterochromatin states (Jenuwein and Allis, 2001, Molina-Serrano et al., 2013, Bannister and Kouzarides, 2011, Izzo and Schneider, 2010, Ng and Cheung, 2016, Beaver and Waters, 2016, DesJarlais and Tummino, 2016). In turn, they affect the recruitment of non-histone proteins to chromatin. Consequently, PTMs nicely direct chromatin-templated processes, including transcription, DNA replication, DNA-repair, differentiation, development, as well as disease processes (Li et al., 2007, Groth et al., 2007, Cohen et al., 2011, Bhanu et al., 2016). For example, the catalysis of lysine 4 trimethylation of histone H3 (H3K4me3), a well-established activation of enhancers and promoters of genes (Guenther et al., 2007), requires the presence of ubiquitinated lysine K120 on histone H2B (H2BK120ub1) to promote transcription elongation (Nakanishi et al., 2009). On the other hand, H3R2me2a, in yeast, acts as a repressive mark of transcription that antagonises H3K4me3 modification by occluding the methylatransferase Set1 from H3K4 site (Kirmizis et al., 2007, Kirmizis et al., 2009).

Recent advances have shown that the plasticity of epigenetic marks and their distinct functional outcome relies upon a dynamic interplay between histone ‘‘writers’’, ‘‘erasers’’ and ‘‘readers’’ which lead to combinatorial complexity. “Writers” are the proteins that can attach PTMs to histone amino acid residues (i.e lysine acetyltransferases KATs), “erasers” are enzymes that remove the PTMs (i.e lysine deacetylases HDACs) and “readers” are proteins that bind in a ‘‘key-to a lock’’ manner to translate the specific epigenetic modification (Musselman et al., 2012, Falkenberg and Johnstone, 2014, Beaver and Waters, 2016, Andrews et al., 2016, Klein et al., 2016). Importantly, there are ‘‘writers’’ that not only ‘‘write’’ a certain PTM on the histone amino acid residue, but also

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can bind and ‘‘read’’ pre-existing histone marks, establishing and maintaining in this manner chromatin states (Zhang et al., 2015c). Readers, are recruited and bind to certain modifications via evolutionarily conserved domains, like chromodomains that bind to methylated lysine amino acids, bromodomains and PHD domains that bind solely to acetylated residues and Tudor domains that bind to methylarginines (Figure 5) (Kouzarides, 2007, Filippakopoulos et al., 2012, Lachner et al., 2001, Cote and Richard, 2005, Yun et al., 2011). Altogether, the above epigenetics factors highlight the sophisticated, complex and dynamic language by which PTMs ensure proper regulation of chromatin activities. Misreading, miswriting and miserasing of histone marks is now evident that lead to defects in cellular processes and hence to many diseases, including age-related diseases, birth defects and several types of cancers (Chi et al., 2010, Nimura et al., 2010, Pedersen and Helin, 2010, Wang et al., 2014, Brookes and Shi, 2014).

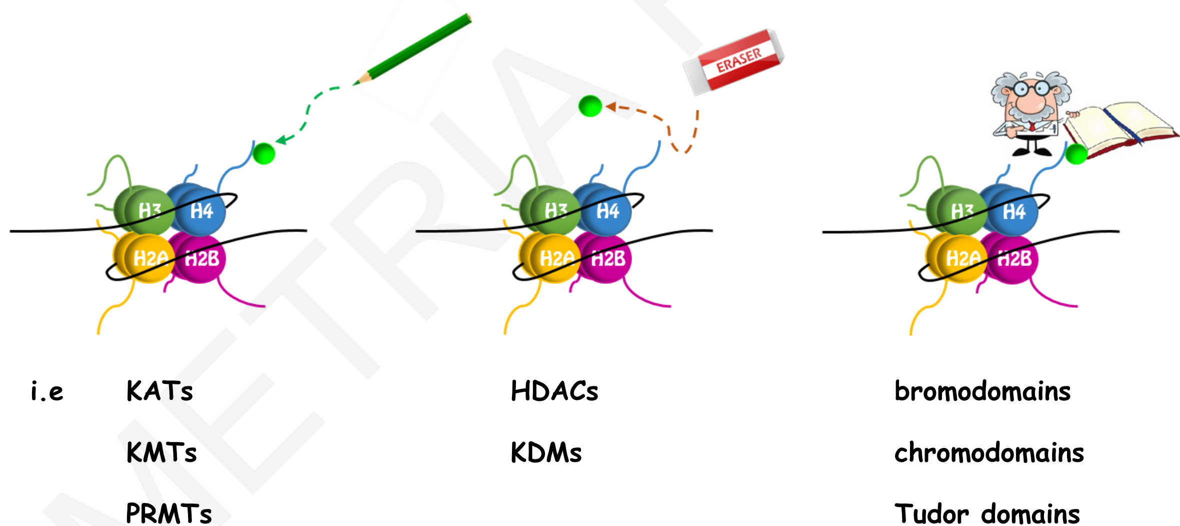

Figure 5. Writers, erasers and readers contribute to the dynamic regulation of chromatin processes. Epigenetic writers such as lysine acetyltransferases (KATs), lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs) lay down epigenetic marks on amino acid residues on histone tails. Protein erasers like histone deacetylases (HDACs) and lysine demethylases (KDMs) are responsible for removing the epigenetic marks. Readers such as proteins containing bromodomains, chromodomains and Tudor domains bind and interpret PTMs, regulating in this way various DNA-dependent processes i.e. transcription and DNA-repair.

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1.4 Histone arginine methylation (Rme)

Histone arginine methylation is one of the most characterised histone modification to date. This is due to the fact that the responsible enzymes for catalysing this modification, the protein arginine methyltransferases (PRMTs), are highly specific and depict an important role over many crucial biological functions, including transcriptional regulation, DNA-repair and signal transduction (Bedford and Clarke, 2009, Bedford and Richard, 2005).

1.4.1 Protein arginine methyltransferases (PRMTs)

The mammalian family of PRMTs consists of eleven members, in which nine of them catalyse the transfer of methyl groups from S-adenosylmethionine (AdoMet) to the terminal (or ω) guanidino nitrogen atoms of arginine residues of histone and non-histone proteins (Jahan and Davie, 2015). This results in the formation of S-adenosylhomocysteine (AdoHcy) and methylarginine. There are three main types of PRMTs (type I, II and III) which all can catalyse monomethylation of arginine (Rme1). Type I (PRMTs 1, 2, 3, 4, 6, and 8) can further catalyse the formation of asymmetric dimethylarginines (Rme2a) and type II (PRMTs 5, 7 and 9) can catalyse the formation of symmetric dimethylarginines (Rme2s) (Migliori et al., 2012). Although PRMT7 is a type II PRMT, it also exhibits type III enzymatic activity, as it can only catalyse the monomethylation of some of its protein substrates (Figure 6) (Yang et al., 2015, Di Lorenzo and Bedford, 2011, Bedford, 2007). The remaining two PRMTs (PRMT10 and PRMT11) were identified by their homology to PRMT7 and PRMT9, respectively, but their enzymatic activity has not been experimentally supported, yet (Wolf, 2009, Krause et al., 2007). Furthermore, Type IV PRMTs have also been reported but only demonstrated in *Saccharomyces cerevisiae* and *Candida albicans.* This type mediates the monomethylation of the internal guanidino nitrogen atom of arginine (Niewmierzycka and Clarke, 1999, McBride et al., 2007).

Type I PRMT1 and type II PRMT5 arginine methyltransferases are among the most essential PRMTs, as complete loss of either of these enzymes is incompatible with life (Table 2) (Yu et al., 2009b, Tee et al., 2010). PRMT1 is expressed in all embryonic and adult tissues and has broad substrate specificity towards histone and non-histone proteins (Huang et al., 2005, Pahlich et al., 2006, Gary and Clarke, 1998, Lee and Bedford, 2002). PRMT5 is found extensively in heart, muscle and testis, without excluding its expression across other organs and tissues (Pollack et al., 1999).

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Whether arginine demethylases exist to erase the methyl marks on arginine residues is still under investigation. Initial studies were supporting the notion that arginine methylation is a permanent covalent mark and the only way to reverse the effects of this PTM was degradation of the protein and synthesis of a new one from the start. The first evidence of demethylation, was presented a decade ago, when Jumonji domain-containing 6 (JMJD6) enzyme was reported to be capable of demethylating arginine 2 on histone H3 (H3R2me2) and arginine 3 on histone H4 (H4R3me2) (Chang et al., 2007). However, a study just few years later showed that this enzyme catalyses lysine hydroxylation and not demethylation (Webby et al., 2009). In addition, monomethylated arginine is shown to be irreversibly converted to citrulline by deamination, where the guanidinum side chain of arginine is hydrolysed and the methyl group is released (Thompson and Fast, 2006). Although these two processes reverse or annul arginine methylation, they do not provide any evidence towards the existence of a functional arginine demethylase.

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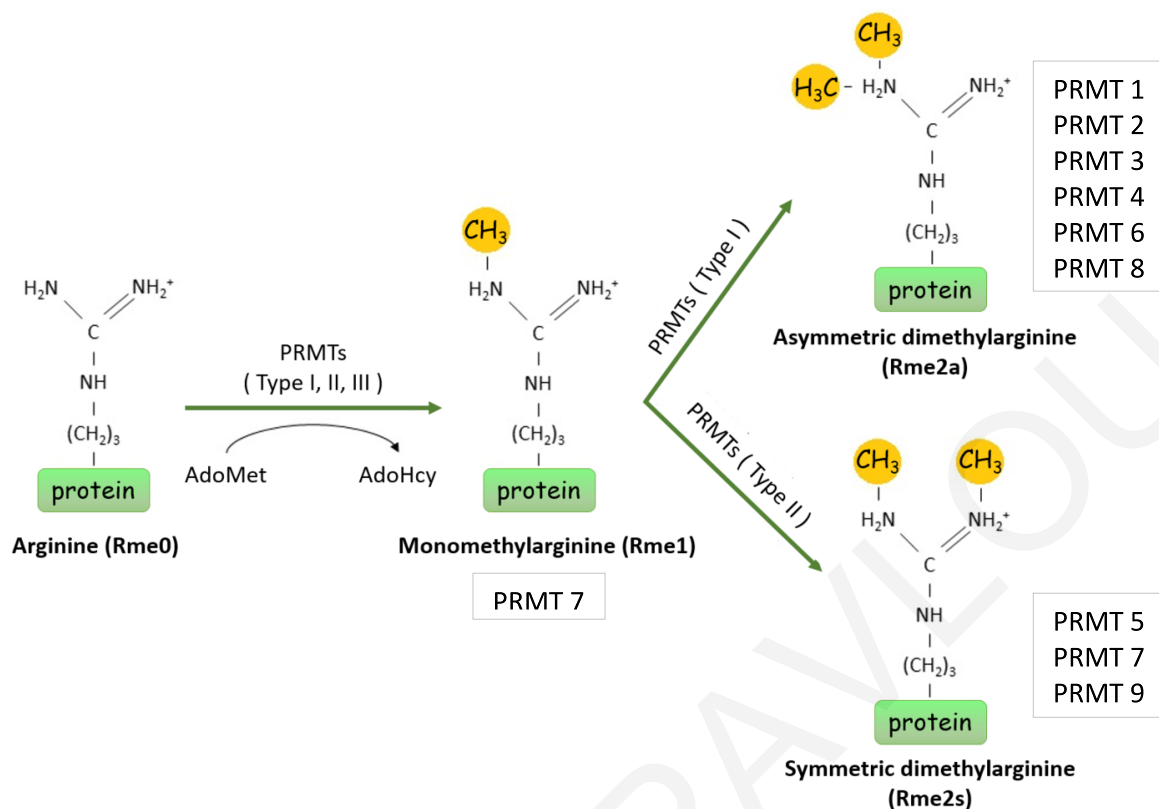

Figure 6. Types of methylation on arginine residues catalysed by PRMTs. PRMTs fall into three types. All three types (type I, II and III) can monomethylate arginine on the terminal guanidine nitrogen atoms of arginine. Type I (PRMTs 1, 2, 3, 4, 5, 6 and 8) can asymmetrically dimethylate arginines (Rme2a) and type II (PRMTs 5, 7 and 9) can catalyse the symmetric dimethylation of arginines (RMe2s).

Table 2. Phenotype of PRMT knockout in mice

PRMT	Phenotype of knockout mice	Reference(s)
PRMT1	Embryonic lethal	(Pawlak et al., 2000, Yu et al., 2009b)
PRMT2	Viable mice. Mouse embryonic fibroblasts (MEFs) are resistant to apoptosis compared to wild type cells	(Ganesh et al., 2006, Yoshimoto et al., 2006)
PRMT3	Viable mice. Mutants that generate hypomorphic allele result in smaller embryos	(Swiercz et al., 2007)
PRMT4/ CARM1	Small new-borns, die shortly after death	(Yadav et al., 2003, Kim et al., 2004)
PRMT5	Early embryonic lethality (die by E6.5). PRMT5 is essential for embryonic epiblast differentiation	(Tee et al., 2010)
PRMT6	Viable mice. MEFs undergo rapid senescence	(Neault et al., 2012)
PRMT7	Viable mice with frequent post-natal death with increased obesity and decreased skeletal muscle mass	(Blanc et al., 2016)
PRMT8	Viable but displayed abnormal mot behaviours	(Kim et al., 2015)

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1.4.2 Asymmetric and symmetric arginine dimethylation exhibit opposing roles in transcriptional regulation

In general, histone arginine asymmetric dimethylation in mammals leads to active gene transcription whereas symmetric dimethylation is linked to gene silencing (Wysocka et al., 2006). The N-terminal tail of histone H3 harbours several arginine residues that are targeted by arginine methyltransferases. These are H3R17 and H3R26 that are methylated by PRMT4, H3R2 methylated by PRMT6 or PRMT5 and PRMT7 and H3R8 methylated by PRMT5. Histone H4 N-terminal tail contains only one arginine residue (H4R3) that is subject to methylation. H4R3 is a major target site for many PRMTs. Asymmetric dimethylation of H4R3 is mediated by PRMT1, PRMT6 and PRMT8, while symmetric dimethylation is catalysed by PRMT5 and PRMT7 (Lee et al., 2005). Thus, depending on the state of dimethylation, H4R3 influences transcriptional control differently either by affecting the deposition of other PTMs or the recruitment of transcriptional effector proteins.

In particular, H4R3me2a catalysed by PRMT1 is known to form a complex with ATP-remodeling factors (SWI/SNF) (Xu et al., 2004), so that when is present on the promoters of genes, it further methylates H3R17 and H3R26 to induce transcription. On the other hand, the presence of H4R3me2s mediated by PRMT5 recruits SUV4–20h1 complex to lay down H4K20me3 and reinforce transcriptional silencing (Rank et al., 2010). In addition, H4R3me2s by PRMT7 blocks the histone methyltransferase MLL4 from trimethylating H3K4 (H3K4me3), inhibiting gene transcription. Conversely, MLL4 binding activity on H3K4me2 is favoured by the presence of unmodified or asymmetrically dimethylated H4R3 (H4R3me0 or H4R3me2a) (Dhar et al., 2012). Notably, the first five residues on the N-terminal tails of histones H4 and H2A are identical, therefore, it is likely that most of activities described for H4R3 may also hold true for H2AR3 (Di Lorenzo and Bedford, 2011).

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1.5 Protein N-terminal acetylation (Nt-ac)

One of the most abundant and conserved protein modifications in eukaryotes, is N-alpha-terminal acetylation (Nα-terminal acetylation or Nt-acetyltation). It is estimated that it occurs in about 50-70% in yeast proteins and in 80–90 % of all soluble human proteins (Arnesen et al., 2009, Brown and Roberts, 1976, Varland et al., 2015). Nt-acetylation, unlike other common modifications, is irreversible and occurs mainly co-translationally, as soon as the first 25–50 amino acid residues of the nascent polypeptide emerge from the ribosomal exit tunnel (Strous et al., 1974, Polevoda et al., 2008).

Key findings over the last decades point to the diverse and important molecular functions of Nt-acetylation (Arnesen, 2011). One of the early proposed roles of this epigenetic mark, was the stabilisation and protection of proteins from degradation (Hershko et al., 1984, Jornvall, 1975). However, new findings contradict this conception, as they show that Nt-acetylation creates specific degradation signals for ubiquitin ligases in order to eliminate unfolded or misfolded proteins (Hwang et al., 2010, Park et al., 2015, Lee et al., 2016). In addition, loss of acetylation was shown to be responsible for the misfolding of protein substrates, suggesting that Nt-acetylation could represent a general factor necessary for proper protein folding (Holmes et al., 2014). Studies have also shown that Nt-acetylation is important for anchoring proteins to membranes, like Golgi, nuclear membrane or lysosomes (Behnia et al., 2004, Behnia et al., 2007, Murthi and Hopper, 2005, Hofmann and Munro, 2006), or for mediating protein-protein interactions (Scott et al., 2011). Other roles of this N-terminus mark include inhibition of protein translocation into the endoplasmic reticulum (ER) (Forte et al., 2011), regulation of normal chromosomal segregation, control of different developmental stages and viability of multicellular organisms (Silva and Martinho, 2015, Tooley and Schaner Tooley, 2014, Van Damme et al., 2011, Ree et al., 2015). Importantly, in 2011, a study by Rope et al., revealed that the X-linked lethal Ogden syndrome is caused by a missense mutation in an N-terminal acetyltransferase gene (NAA10) (Rope et al., 2011). *De novo* missense mutations in the *NAA10* gene have been also identified in two independent cases of global developmental delay (Myklebust et al., 2015). Altogether, these research studies highlight the molecular and physiological importance of protein Nt-acetylation and of the enzymes that catalyze this modification.

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1.5.1 Nt-acetyltransferases (NATs)

Nt-acetylation is specifically catalysed by a group of N-terminal-acetyltranferase enzymes, denoted as NATs. NATs are members of the GNAT protein superfamily (Vetting et al., 2005), which all contain the consensus acetyl-coenzyme A (ac-CoA) binding sequence (Q/R)XXGXX(G/A) (Neuwald and Landsman, 1997). Thus, they are capable of catalysing the transfer of an acetyl group from Ac-CoA to the very N-terminal end of proteins. Unlike other modifications, Nα-terminal acetylation targets the alpha amino group of the first residue of a protein substrate, and not the side chain of the amino acid (Figure 7). Some NATs function as individual enzymes but others constitute multi-component complexes and therefore, the function of these complexes relies on the catalytic subunit which is necessary for the acetyltransferase activity and on one or two auxiliary subunits, which, among other purposes, enable the catalytic component to anchor on ribosomes and modify polypeptides co-translationally (Figure 8) (Gautschi et al., 2003, Polevoda et al., 2008).

Five different NAT enzymes have been identified in eukaryotes, designated NatA–NatE and their substrate specificity seems to be conserved through evolution (Polevoda et al., 2009a, Arnesen et al., 2009, Hole et al., 2011, Bienvenut et al., 2012). A sixth one, designated as NatF, is also present in higher eukaryotes, only (Aksnes et al., 2015b, Van Damme et al., 2011). Each member of the NAT family differs both in subunit composition and substrate specificity. The substrate specificity towards amino acid residues is depicted in Figure 8, where it is obvious that NATs recognise their substrates primarily based on the sequence of the last several N-terminal residues. Some NATs target the initiator methionine (iMet) and others the first amino acid after the excision of iMet. Among all NATs, NatA catalyses ~40% of the proteome targeting proteins beginning with Ser, Ala, Thr, Val Gly or Cys, therefore from that prespective, NatA represents the major NAT in eukaryotes (Starheim et al., 2012, Popp et al., 2015). Subunit composition and substrate specificity of each NAT is depicted in Figure 8 (Varland et al., 2015, Starheim et al., 2012).

Deletion of NATs has been shown to cause different phenotypic outcomes. Deletion of NatA subunits in yeast led to slow growth, failure of sporulation, inability to enter G0 phase and decreased survival rate after exposure to stress stimuli (Polevoda and Sherman, 2003). In human cell lines, NatA deletion resulted in both enhanced and decreased proliferation rate, indicating that Nt-acetylation behaves differently in different

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cellular contexts (Yi et al., 2011, Gromyko et al., 2010). Yeast growth defects were also observed upon *natB* or *natC* deletion (Polevoda and Sherman, 2003, Polevoda and Sherman, 2001), while knockdown of the enzymes in human cell lines induced p53-dependent apoptosis (Ametzazurra et al., 2008, Starheim et al., 2009). Importantly, over the last years, Nt-acetylation has attracted growing attention, as deregulated expression and activity of NAT enzymes has been linked to carcinogenesis (this is further discussed below).

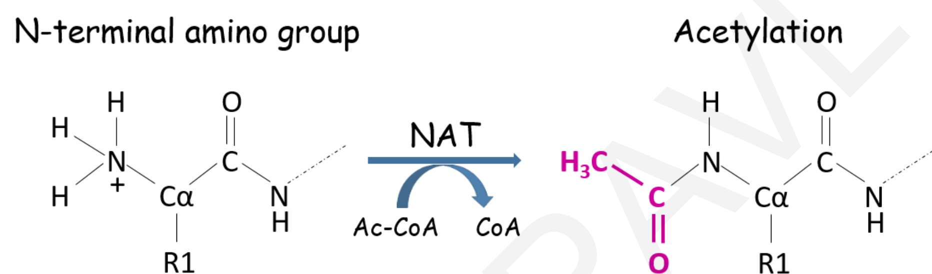

Figure 7. Nt-acetylation catalysed by NATs. A NAT enzyme removes the acetyl moiety (Ac) from Ac-CoA and acetylates the N-terminal α-amino group of a protein or polypeptide substrate. The acetylation neutralizes the positive charge of the free α-amino group, and thereby efficiently blocks it for further ionization and other modifications (acetyl mark shown in magenta colour).

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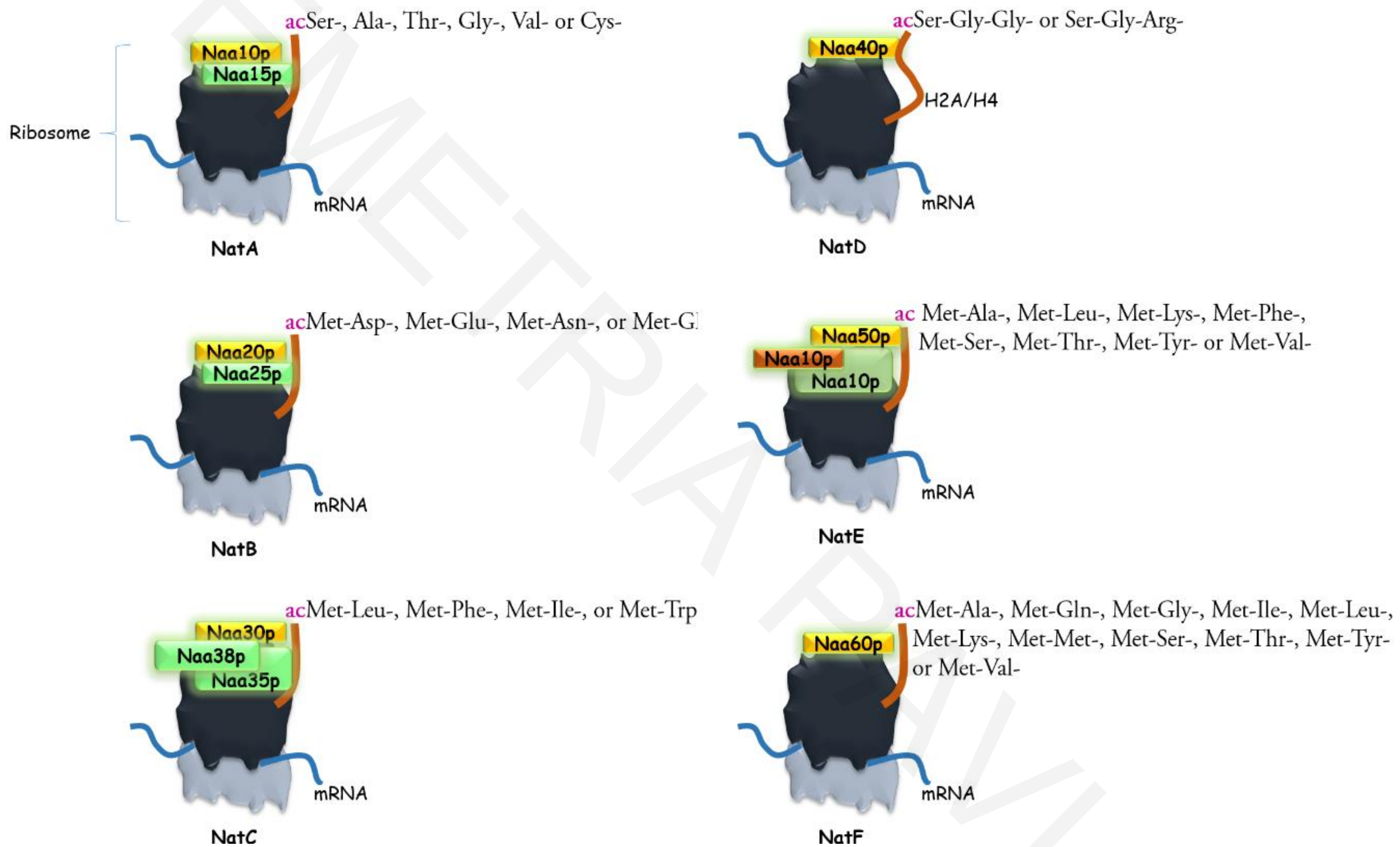

Figure 8. Subunit composition and substrate specificity of each eukaryotic NAT. All NAT enzymes, apart from NATF which is present only in higher eukaryotes, are conserved from yeast to humans. NATs exhibit their function cotranslationally, thus are associated with ribosomes. In humans, they are also found in non-ribosomal forms. Yellow boxes indicate the catalytic subunit of each NAT while green boxes indicate the auxiliary subunit. Figure is modified from Starheim et al., 2012.

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1.5.2 Naa40 in the spotlight

Naa40 (also known as NatD, Nat4, Nat11 or Patt1) is unique among NATs because, unlike the other enzymes that have broad substrate selectivity and acetylate numerous proteins, this NAT protein member is extremely selective. So far, Naa40 has been shown to act as an epigenetic regulator by modifying histones H4 and H2A, only. Both histones contain the same amino acid sequence at the very N-terminal end (Ser-Gly-Arg-Gly) which has been shown to act as the recognition sequence for Naa40 (Starheim et al., 2012, Song et al., 2003, Polevoda et al., 2009b, Magin et al., 2015). Whether additional Naa40 substrates exist remains elusive. However, it was suggested that two other human proteins (H2A.X and SMARCD2) have the proper N-terminal sequence that could be targeted by Naa40 based on its recently determined structure and substrate-specificity (Magin et al., 2015). Additionally, four other yeast proteins (Scl1, Ypi1, Dph1 and Lge1) have been proposed as targets of yeast Naa40 (Van Damme et al., 2014). These proteins are typically substrates of NatA, but in yeast lacking NatA activity these proteins remain partially N-terminally acetylated. Intriguingly, their N-terminus sequence starts with Ser-Gly, which is compatible with the sequence specificity of Naa40 (Hole et al., 2011).

Naa40 was first identified and characterised in the yeast *Saccharomyces cerevisiae* (Song et al., 2003) and it was later demonstrated that its acetyltransferase activity towards histones is evolutionarily conserved in human cells (Hole et al., 2011). This conservation highlights the functional importance of histone N-terminal acetylation. Indeed, work from our lab showed that N-terminal acetylation of H4 in yeast promotes ribosomal RNA (rRNA) expression by inhibiting the deposition of an adjacent histone H4 modification, namely arginine 3 asymmetric dimethylation (H4R3me2a) (Schiza et al., 2013). Consistently, *in vitro* experiments revealed that N-terminally acetylated H4 (N-acH4) impairs the binding and thus activity of Hmt1 (PRMT1 homologue) methyltransferase towards H4R3. While deletion of yeast Naa40 (yNaa40) displays minor phenotypes, including sensitivity towards inhibitors of protein synthesis and mitosis (Polevoda et al., 2009b), combinatorial loss of N-acH4 with other internal lysine acetylation marks (H4K5, H4K8 and H4K12) results to synthetic growth defect (Schiza et al., 2013, Polevoda et al., 2009b). Furthermore, the activity of Naa40 towards histone H4 in the yeast rDNA region is reduced during calorie restriction suggesting that Naa40 may act as a sensor for cell growth (Riesen and Morgan, 2009, Smith et al., 2009). Additionally, a study in mice showed that liver-specific Naa40 knockout males have aberrant lipid metabolism, reduced fat mass and are protected from age-associated hepatic steatosis (Liu et al., 2012). Collectively, these

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findings demonstrate that Naa40 has numerous and vital roles in eukaryotes and stress the necessity for elucidating further the underling biological and molecular mechanisms by which Naa40 employs its function.

1.6 Cancer epigenetics

The genetic origin of cancer, is widely accepted. Mutations in key regulatory genes such as oncogenes (i.e *RAS and MYC*) or tumor suppressors (i.e *P53 and RB*) are responsible for aberrant cell growth and division, which ultimately lead to tumor formation, tumor progression, invasion and metastasis (Hanahan and Weinberg, 2011). Sustained proliferation signals and defective programmed cell death, the so called apoptosis, represent major causative factors in cancer development, as tumor cells in this way promote their growth and survival. Recent advances in the field of cancer research have shown that global changes in the epigenetic landscape also culminate in malignant cellular transformation (Huang and Wen, 2015, Chen et al., 2016, Jones and Baylin, 2002, Jones and Baylin, 2007, Esteller, 2007, Berdasco and Esteller, 2010). Consequently, a comprehensive initiative has emerged to understand the implication of epigenetic regulators in cancer. Histone modifications majorly contribute to various biological processes, thus deregulation of the modifying enzymes actively contributes to the initiation and progression of human cancers, by evading apoptosis and acquiring a proliferative advantage.

1.6.1 PRMTs in cancer

Most of the members of the PRMT family are ubiquitously expressed and thus, it was of no surprise when their deregulation was found to be implicated in the pathogenesis of a number of different diseases, including cancer. Colon cancer, liver, breast and prostate cancers are only some of the malignancies that harbour aberrant expression of PRMTs (Zhang et al., 2015a, Zhang et al., 2015b, Goulet et al., 2007, Majumder et al., 2006). Arginine methyltransferases function in multiple cellular processes, including cell cycle progression, DNA-replication and transcription, which are fundamental for cancer cell survival and proliferation (Yoshimatsu et al., 2011). Abnormal PRMT expression or activity not only results in hypo- or hyper- arginine methylation of protein substrates but

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also to the deregulation of all downstream events, including evasion of apoptosis (Cheung et al., 2007).

Alterations in PRMT1 expression, mostly upregulation, has also been reported in human cancers, including breast, colon, lung, prostate, bladder cancer and leukaemia (Mathioudaki et al., 2011, Mathioudaki et al., 2008, Zou et al., 2012, Seligson et al., 2005, Yoshimatsu et al., 2011). More specifically, PRMT1 in breast cancer cells was shown to mediate the asymmetric dimethylation of H4R3 (H4R3me2a) at the Zinc Finger E-Box Binding Homeobox 1 (ZEB1) promoter. This resulted to its transcriptional activation which in turn induced epithelial-mesenchymal transition (EMT) and promoted migratory and invasive behaviours. In contrast, down-regulation of ZEB1 by PRMT1 knockdown, resulted in cellular senescence (Gao et al., 2016). Particularly, PRMT5 is reported to be up-regulated in lung cancer, leukemia, lymphoma, ovarian cancer, and breast cancer (Gu et al., 2012, Pal et al., 2007, Pal et al., 2004, Powers et al., 2011, Cho et al., 2012, Bao et al., 2013). A new study from Zhang et al., showed that PRMT5 is overexpressed in colorectal carcinoma and in patient-derived primary tumors and is responsible for increased cell growth and reduced patient survival (Zhang et al., 2015b). Interestingly, PRMT5 knockdown reduced H4R3me2s and H3R8me2s levels on the promoter of two oncogenes (Fibroblast Growth Factor Receptor 3, FGFR3 and eukaryotic initiation factor 4E, elF4E) leading to their repression. Their inhibition, in turn, increased Bax/Bcl-2 ratio and induced apoptosis. Collectively, this study supports the anti-apoptotic role of PRMT5 and more importantly, it provides new evidence on PRMT5 implication in transcriptional activation. Two recent studies also reveal the survival dependency of a large number of various cancer cell lines upon PRMT5. More specifically, they show that when methionine metabolism is disordered though MTAP (methylthioadenosine phosphorylase) deletion, which is essential for metabolising MTA (methylthioadenosine) to adenine and methionine, then PRMT5 is inhibited by the accumulation of MTA and cell proliferation and viability are impaired (Mavrakis et al., 2016, Kryukov et al., 2016). The increased metastatic potential of breast carcinoma has been also reported to be attributed to the overexpression of PRMT7 (Yao et al., 2014). This arginine methyltransferase was found to directly inhibit the activation of E-cadherin promoter, by inducing symmetric dimethylation of arginine 3 in histone 4 (H4R3me2s) and inhibiting H3K4me3, H3 acetylation and H4 acetylation. Attenuation of cell migration and invasion was observed after PRMT7 knockdown, indicating that both epithelial markers and arginine methyltransferases could be manipulated in targeted cancer therapeutics against highly invasive cancers. Altogether this evidence indicates that

targeting PRMTs, presents a promising clinical strategy for selectively inducing apoptosis and eliminating tumor cells.

1.6.2 NATs in cancer

Several recent studies have linked the various NAT enzymes to carcinogenesis (Kalvik and Arnesen, 2013, Aksnes et al., 2015a). For example, Naa10 (the catalytic subunit of NatA complex) has been proposed to behave both as an oncoprotein and as a tumor suppressor. In certain types of cancer tissues like breast, colorectal and lung cancers Naa10 is overexpressed (Yu et al., 2009a, Lee et al., 2010a, Ren et al., 2008) and functions as a pro-proliferative and anti-apoptotic factor since its depletion promotes p53-dependent apoptosis. On the other hand, Naa10 expression counter correlates with lung cancer progression and in xenograft experiments Naa10 overexpression suppresses tumor growth and metastasis (Hua et al., 2011). Furthermore, NatB is required for cell-cycle progression and depletion of its subunits in cancer cells results in growth arrest and sensitization of cells to pro-apoptotic stimuli (Ametzazurra et al., 2008, Starheim et al., 2008). Moreover, previous knockdown studies demonstrated that NatC is required for cancer cell survival and proliferation since its depletion induced p53-dependent cell-death in Hela and colon cancer cells (Starheim et al., 2009). NatC catalytic activity has been also correlated with glioblastoma growth and survival, as it was shown to regulate genes involved in the p53 pathway, ribosome assembly, hypoxia response and cell proliferation (Mughal et al., 2015). Naa40 (NatD) deregulation has also been implicated in cancer. In a recent study, Naa40 was shown to be down-regulated in hepatocellular carcinoma whereas its overexpression enhanced drug-induced apoptosis that was dependent on its acetyltransferase activity (Liu et al., 2009). According to the Human Protein Atlas project, Naa40 protein levels vary in different cancer types, with the highest expression observed in colorectal, ovarian and prostate cancers and the lowest in lymphomas, glioma, renal and liver cancers (The Human Atlas, 2016). The collective data highlight the importance of investigating the role of NATs in different cancer tissues. In addition, the data indicate that their manipulation represents an attractive therapeutic strategy for inducing cell-cycle arrest or apoptosis in cancer cells.

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1.7 Apoptosis

Apoptosis or Programmed cell death has been recognized and accepted as the altruistic decision of cells for committing suicide (Hotchkiss et al., 2009, Wyllie, 2010, Portt et al., 2011, Kerr et al., 1972). It represents a tightly regulated physiological process for cell elimination that occurs normally during development, such as the removal of inter-digital cells during limb formation in embryogenesis (Zuzarte-Luis and Hurle, 2002). It is also critically implicated in aging, tissue homeostasis and immune response against damaged or infected cells (Czabotar et al., 2014, Labbe and Saleh, 2008, Elmore, 2007, Vicencio et al., 2008, Norbury and Hickson, 2001). Failure of cells to appropriately induce apoptosis has been reported to majorly contribute to the pathogenicity of many diseases, including malignancies. Evasion of apoptosis has been established as a prominent hallmark of cancer, thus the goal of many therapeutic strategies is to trigger tumor-selective cell apoptosis (Kasibhatla and Tseng, 2003, Hanahan and Weinberg, 2011).

1.7.1 Mechanisms of apoptosis

The mechanisms of apoptosis are highly complex and trigger nicely-orchestrated events in order to promote death and eradication of undesired cells. Apoptosis is mainly induced by two major routes; one that receives signals from the extracellular environment, the so called extrinsic pathway, and another one that is initiated by intracellular stimuli, known as the intrinsic or mitochondrial pathway (Parrish et al., 2013, Ashkenazi and Dixit, 1998). It was believed that these two pathways work independently. Now, it is widely known that both apoptotic processes can crosstalk and that molecules in one pathway can influence the other (Igney and Krammer, 2002). Overall, both extrinsic and intrinsic pathways converge to activate a series of cysteine aspartyl-specific proteases (caspases), which subsequently chop critical proteins to initiate cell dismantling (Koff et al., 2015).

So far, 15 different caspases (Ca2+-independent proteases) have been described in mammalian genomes, however only 11 have been identified in humans (caspase -1 to -10 and caspase -14) (Sadowski-Debbing et al., 2002, Eckhart et al., 2005). All 15 proteases are expressed as inactive pro-enzymes (zymogens) which become activated once their aspartic acid peptide bonds are proteolytically cleaved. Caspases can be divided into two types: the initiator (apical) caspases and the effector (executioner) caspases. The initiator caspases (caspase-1, -2, -4, -5, -8, -9 and -10) activate the caspase-cascade by cleaving

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downstream effector caspases, whereas effector caspases (caspase -3, -6, -7, -14) have a broader spectrum of protein substrates (Lamkanfi, 2011, Taylor et al., 2008).

1.7.1.1 Extrinsic apoptosis

The extrinsic apoptotic pathway connects the extracellular microenvironment to the intracellular compartment. It is initiated through the binding of specific death ligands (i.e. FasL) to their corresponding transmembrane death receptors of the tumor necrosis factor (TNF) receptor superfamily (i.e Fas receptor) (Locksley et al., 2001). These receptors are composed of cysteine-rich extracellular domains and a ''death domain'' that faces the cytoplasm. Upon ligation, death receptors oligomerise and recruit adapter molecules (i.e. FADD) to the ''death domain'', which in turn stimulates the recruitment and dimerization of initiator caspase-8. Adapter molecules and initiator caspase form the multi-protein signalling platform, known as the death-inducing signalling complex (DISC) (Ashkenazi and Dixit, 1998, Fulda and Debatin, 2006). Upon recruitment, caspase-8 becomes activated and initiates a proteolytic cascade by directly cleaving and activating the downstream effector caspases-3, -6 and -7 (McIlwain et al., 2013). Additional death receptors that participate in the extrinsic apoptotic pathways include the TRNFR1-TNFα ligand complex and the DR4/5–Apo2 L/TRAIL ligand complexes (Parrish et al., 2013). The extrinsic pathway is outlined in Figure 9. Active caspase-8 can also engage intrinsic apoptosis (see below), through the proteolytic processing of Bid, a pro-apoptotic protein of the Bcl-2 family. Truncated Bid (tBid) will translocate to mitochondria to initiate local damage and release of pro-apoptotic proteins from the mitochondrial inter-membrane space (Li et al., 1998, Esposti, 2002).

1.7.1.2 Intrinsic apoptosis

The intrinsic apoptotic pathway, which is often considered as the main barrier to carcinogenesis (Hanahan and Weinberg, 2011), is also referred as the mitochondrial pathway due to the central role of these organelles. Mitochondria are essential for the survival of cells, mainly because they are implicated in biosynthetic processes such as ATP generation. In the mid-1990s, evidence emerged implicating mitochondria to the induction of apoptotic cell death. Specifically, the localisation of the anti-apoptotic Bcl-2 protein to the outer mitochondrial membrane was shown to maintain cell survival. In contrast, the inhibition of the protein resulted in mitochondrial changes and the release of pro-apoptotic

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factors to the cytoplasm, which led to the induction of apoptosis (Hockenbery et al., 1990, Hockenbery et al., 1993, Newmeyer et al., 1994, Kroemer et al., 1995, Zamzami et al., 1996, Kluck et al., 1997). Over the years, accumulating evidence verified the abovementioned and indicated that the intrinsic pathway is initiated by intracellular regulators that belong to the Bcl-2 protein family. This family comprises of anti-apoptotic (like Bcl-2, Bcl-XL, Bcl-w and BAG) and pro-apoptotic (like Bax, Bak, PUMA and NOXA) factors, whose equilibrium determines whether a cell will undergo apoptosis by inducing outer mitochondrial membrane permeabilisation (MOMP) (Oltvai et al., 1993). Anti-apoptotic Bcl-2 proteins, which are constitutively bound to mitochondria, can trap and block the oligomerisation of pro-apoptotic proteins (Youle and Strasser, 2008). If the number of pro-apoptotic proteins exceeds the number of anti-apoptotic ones, then they are able to oligomerise with each other, translocate from cytosol to the outer membrane of the mitochondria, block anti-apoptotic Bcl-2 proteins, form pores and induce MOMP. MOMP initially leads to the release of cytochrome-c from the inter-membrane space of the mitochondrion into the cytosol. Binding of cytochrome-c to the apoptotic protease activating factor 1 (Apaf-1) will form the apoptosome. This will eventually mediate the activation of the initiator caspase-9, which is specific for the intrinsic pathway (Bednarek and Kilianska, 2005, Brentnall et al., 2013). Once caspase-9 activates, it cleaves and activates the effector caspases -3, -6 and -7. These effector caspases subsequently cleave several other substrates, promoting numerous cellular changes that will lead to apoptosis. The regulatory steps of the intrinsic/mitochondrial apoptotic pathway are depicted in Figure 9.

Poly(ADP-ribose) polymerase-1 (PARP-1) is among the several protein substrates of effector caspases, which has a pivotal role in the DNA damage response (Weaver and Yang, 2013). In the absence of DNA damage, the levels of PARP-1 remain low. Upon single- or double-strand breaks, PARP-1 is overexpressed in order to induce the DNA-base-excision-repair mechanism. However, in the presence of excessive DNA damage, cells engage the apoptotic machinery instead of the repairing mechanisms. Inactivation of the 116kDa PARP-1 protein is achieved through cleavage by caspases that will result in the formation of two fragments: the 89kDa C-terminal fragment which possesses minor catalytic activity and the 24kDa N-terminal peptide that is essential for the downstream inactivation of the uncleaved nuclear PARP-1 (Soldani and Scovassi, 2002, Chaitanya et al., 2010).

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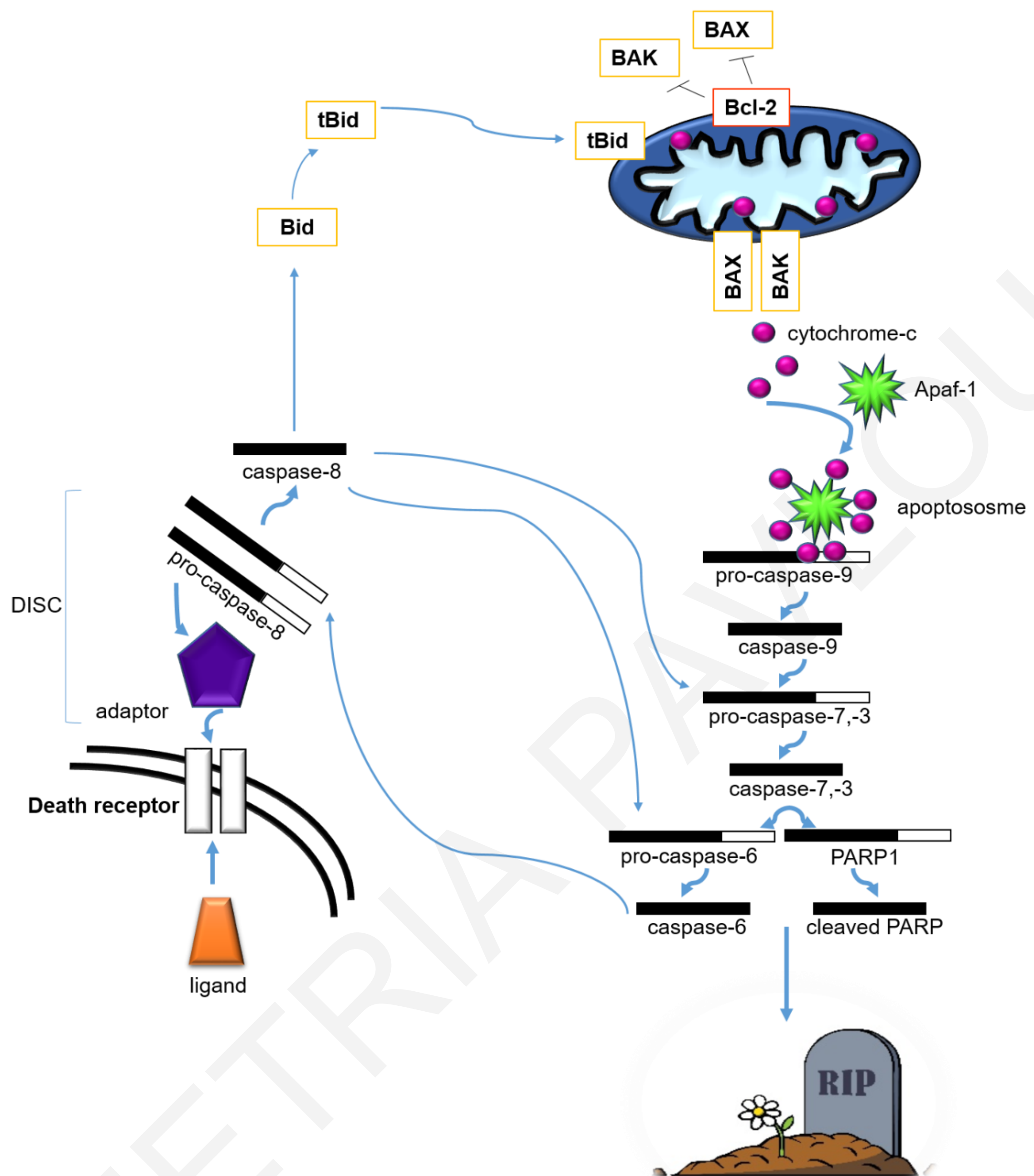

Figure 9. The extrinsic and intrinsic pathways of apoptosis. The apoptotic signalling in the extrinsic pathway is initiated through the binding of a ligand to the corresponding death-receptor. Adaptor proteins are recruited to the receptor and allow the binding of the initiator pro-caspase-8. The DISC complex that is formed eventually leads to the activation of caspase-8 which will activate downstream effector caspases-3, -6 and -7. The intrinsic pathway is initiated once pro-apoptotic proteins of the Bcl-2 family translocate to the outer mitochondrial membrane, causing mitochondrial membrane permeabilisation (MOMP). This leads to the release of cytochrome-c from the inner membrane of mitochondrion to the cytoplasm, the formation of the apoptosome and the activation of the initiator caspase-9. Active caspase-9 cleaves and activates effector caspases-3,-6 and -7 which will further cleave other substrates to carry on and complete apoptosis (caspase-8 may be one of those target substrates). The two pathways may communicate. For instance, caspase -8 can induce mitochondrial apoptosis through cleavage and translocation of tBid pro-apoptotic protein to mitochondrial outer membrane which will form pores and facilitate MOMP events (Parrish et al., 2013, Fan et al., 2005).

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1.7.1.3 Endoplasmic reticulum stress-induced apoptosis

The initiator murine caspase-12 (homologue of human caspase-4), has been shown to mediate apoptosis through the Unfolded Protein Response (UPR) (Martinez et al., 2010). UPR apoptotic pathway is activated upon endoplasmic reticulum (ER) stress, which arises mainly due to the accumulation of unfolded or misfolded proteins in ER lumen and/or the perturbation of calcium ion homeostasis (Fan et al., 2005). For the execution of apoptosis through the UPR machinery, the apoptotic transcription factor C/EBP homologous protein (CHOP; also known as DNA damage inducible transcript-3, DDIT3) is normally induced (Vandewynckel et al., 2013). CHOP protein regulates positively or negatively the transcription of pro-apoptotic (i.e PUMA and Bim) or anti-apoptotic genes (i.e Bcl-2), respectively. Thus, in response to UPR activation, mitochondrial apoptosis can be triggered or inhibited. In addition, it has been reported that caspase-12 can cleave and activate caspase-9, which will ultimately activate the effector caspases -3, -6 and -7 (Morishima et al., 2002). Reversely, mitochondrial apoptosis induction can be responsible for CHOP activation. Active caspase-7 which results through induction of the intrinsic apoptotic cascade can translocate from cytosol to ER membrane and activate caspase-12 (Rao et al., 2001). Altogether emphasise that both UPR and intrinsic apoptosis are interconnected.

1.7.1.4 Caspase-independent apoptosis

Cells, apart from caspase-dependent apoptosis, can also undergo caspase-independent programmed cell death (CI-PCD). CI-PCD is defined as death that ensues when caspases fail to activate. Just like in intrinsic apoptosis, mitochondria play a central role as they should undergo MOMP to release pro-apoptotic factors. Similar important roles play the lysosomes and ER, which release and activate other pro-apoptotic factors, known as cathepsins and calpains (Broker et al., 2005).

During CI-PCD, pro-apoptotic proteins like apoptosis-inducing factor (AIF) and endonuclease G (endoG) are released from mitochondria. These apoptotic mediators exert their function in nucleus, thus their translocation from the cytoplasm is necessary (Sun et al., 2016). Within the nucleus, AIF and endoG induce chromatin condensation and large-scale DNA fragmentation (≥50Kb pieces) (Joza et al., 2001, Li et al., 2001). Since these represent late apoptotic events, CI-PCD through AIF and endoG takes place only when cells have already committed to die (Tait and Green, 2008, Elmore, 2007). Regulated and

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selective lysosomal permeabilisation leads to the release of lysosomal cathepsin-proteases to cytosol. Cytosolic cathepsins can either translocate to nucleus to induce bile-salt induced apoptosis (Roberts et al., 1997) or remain in cytosol to cleave Bid (Cirman et al., 2004) and induce tBid and Bax translocation to mitochondria for subsequent mitochondrial permeabilisation (Bidere et al., 2003). Moreover, cathepsins may cleave and activate caspases, thereby it is suggested that lysosomal destruction is often an early apoptotic event (Vancompernolle et al., 1998). Furthermore, CI-PCD can be triggered by ER stress, which causes intracellular calcium influx. The increased calcium levels activate calpain proteases (Ca2+-activated neutral cysteine) (Wingrave et al., 2003) in the cytoplasm which can either activate Bcl-2 pro-apoptotic protein members for triggering caspase-activation or release lysosomal cathepsins to initiate the abovementioned apoptotic events (Neumar et al., 2003, Yamashima, 2004).

1.7.2 Inhibition of apoptosis

Inhibition of apoptosis is central for cell viability. Whether this is achieved under physiological or pathological conditions, it requires the inhibition of pro-apoptotic proteins and/or the enhanced expression of anti-apoptotic factors. Dysfunctional programmed cell death is a major causative factor in the development and progression of tumorigenesis. Identifying and targeting the mediators that contribute to the blockade of apoptosis could induce this biological process and hence, perturb the viability of cancer cells.

1.7.2.1 Inhibitors of Apoptosis proteins (IAPs)

Inhibitors of Apoptosis Proteins (IAPs), represent an evolutionarily conserved family of proteins that can negatively regulate the activity of initiator and effector caspases and can positively regulate cell migration (Oberoi-Khanuja et al., 2013) (Crook et al., 1993). Their expression and activity in many cancer types has been shown to promote survival and resistance to chemotherapy (Wright and Duckett, 2005, Fulda and Vucic, 2012). Thereby, IAPs are attractive targets in anti-cancer treatments (Hunter et al., 2007). The mammalian IAP family consists of eight protein members (NAIP, XIAP, Survivin, c-IAP1, c-IAP2, Livin, ILP2 and BRUCE/Apollon) that all contain at least one baculoviral IAP repeat (BIR) domain (Oberoi-Khanuja et al., 2013). BIR is essential for the coordination of protein-protein interactions. Most IAPs contain also a RING domain (XIAP, c-IAP1, c-IAP2, Livin, ILP2) which confers to the proteins an E3 ubiquitin ligase

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activity for promoting proteosomal degradation of target proteins (Blankenship et al., 2009). IAPs exist in the cytosol and are frequently found as heterodimers (Rajalingam et al., 2006). An example, is the heterodimeric complex of survivin and XIAP which has been shown to promote increased XIAP stability and induce cell survival through inhibition of caspases -3, -7 and -9 activation (Dohi et al., 2004). A potent inhibitor of IAPs, is the pro-apoptotic Smac/DIABLO protein that is released from mitochondria after MOMP to disrupt IAP's activity against caspases (Salvesen and Duckett, 2002, Tait and Green, 2010). However, XIAP, c-IAP1, and c-IAP2 have been also demonstrated to ubiquitinate Smac/DIABLO and subsequently, block apoptotic cascade (MacFarlane et al., 2002, Hu and Yang, 2003).

1.7.2.2 Epithelial-mesenchymal transition (EMT)

Apoptosis is the main mechanism triggered in cells to eliminate unwanted cells and ensure proper development. Several lines of evidence support that the adhesive phenotypes of cells, are closely interconnected with cell survival or cell death decisions. Huge scientific interest focuses on epithelial-mesenchymal transition (EMT) of cells, which is required for development and physiological responses to injury as well as during human cancer development (Kiesslich et al., 2013, Thiery, 2002, Thiery et al., 2009, Thiery and Sleeman, 2006). EMT represents a series of biochemical cellular changes that enable epithelial, polarised and tightly anchored cells to acquire a mesenchymal phenotype. This phenotype of cells contributes enormously in cancer progression, as it includes enhanced migratory capacity, invasiveness, resistance to apoptosis and elevated production of multiple proteins that will compose the extracellular matrix (ECM) (Radisky, 2005, Kalluri and Weinberg, 2009) (Yao et al., 2011). Therefore, EMT mechanisms represent an integral component of the progression of tumors to malignant states. Over the years, the fundamental role of the extracellular matrix (ECM) in the survival of cells has been clearly demonstrated (Santini et al., 2000). The ECM is composed of a variety of proteins, including collagen, laminins and fibronectin, that provide structural support to cells or tissues. Notably, fibronectin influences a variety of cellular functions apart from cell adhesion, including migration, metastasis, differentiation, and growth and therefore, has been linked to cancer survival and progression (Han and Roman, 2006). In contrast, reduced levels of fibronectin lead to the induction of apoptosis (Wu et al., 2005, Fornaro et al., 2003). Vimentin, an intermediate filament protein of cytoskeleton, is also a marker of mesenchymal cells which is involved in cell adhesion, migration and cell signalling

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(Mendez et al., 2010). Caspase cleavage of vimentin, not only leads to mesenchymal-epithelial transition of the cell, but also promotes mitochondrial apoptosis (Byun et al., 2001). The transmembrane protein E-cadherin on the other hand, has been shown to suppress invasion, metastasis and proliferation. As a result, down-regulation of this epithelial marker is commonly observed in different cancers (Ferreira et al., 2005, Liu and Chu, 2014).

1.7.3 Cell cycle and apoptosis

Cell proliferation and cell death decisions, are tightly related. Both processes share common protein factors, thus manipulating their expression and activity may either prevent or activate specifically one of the two cellular responses (King and Cidlowski, 1995, Saini and Sanyal, 2015). This linkage has been recognized for tumor suppressor genes such as *p53, RB* and *PUMA,* the dominant oncogene, c-*Myc*, several cyclin-dependent kinases (Cdks), cyclins and their regulators, like p21(Pucci et al., 2000). Exploiting the knowledge gained by studying the connection between cell cycle progression and apoptosis enables us to propose novel desirable strategies to circumvent cancer progression.

Cell cycle is responsible for cell proliferation, in other words for cell duplication. Prior to mitosis (M phase), the cell grows (G1 and G2 phases) and duplicates its DNA (S phase). The progression of cell through the different phases is rigorously orchestrated by different serine/threonine kinases, known as Cdks and their activating subunits, known as cyclins (Pucci et al., 2000). The control of cell cycle by different Cdks/cyclins is represented in Figure 10. In conditions where the cell should stop dividing, i.e for DNA repair, p21 protein acts as an inhibitor of Cdks to induce cell cycle arrest. If excessive DNA damage occurs, then the cell does not re-enter cell cycle and instead, apoptosis is induced. The P53 tumor suppressor is another protein with fundamental cellular functions, as it is involved in G1 or G2/M cell cycle transitions, in apoptosis, DNA repair and ensures genomic integrity (Levine, 1997). Mutations on *P53* gene are observed in more than half of all human cancers, leading to the deregulation of all the over-mentioned processes (Joerger and Fersht, 2007).

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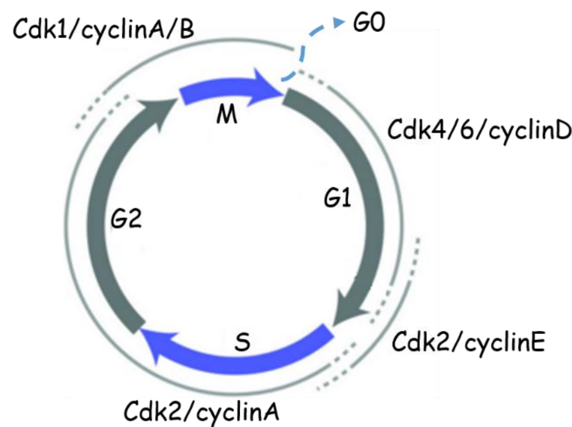

Figure 10. Cell cycle control by cdk/cyclin complexes. Cyclins increase during the phase of the cell cycle in which they are required and decrease during phases in which they are not needed. When cells undergo differentiation, they exit cell cycle ant enter into a quiescent state referred to as G0.

1.7.4 Differentiating apoptosis from necrosis

Apart from the very well-coordinated apoptotic process, cells can be also eliminated by other mechanisms, such as necrosis. Necrosis can occur independently of apoptosis, sequentially or simultaneously (Zeiss, 2003, Hirsch et al., 1997). While apoptosis is often an energy-dependent process that affects individual cells or clusters of cells, necrosis is an energy-independent mode of death that affects many groups of neighbouring cells. In many cases, the decision of cells regarding which two processes to follow depends upon the type of the initial stimulus or the amount of stimulus given. For example, at low doses, stimuli such as radiation, hypoxia or anti-cancerous dugs lead to the induction of precisely regulated apoptosis, whereas higher doses of the same stimuli will result in necrosis (Elmore, 2007).

Apoptosis and necrosis have distinct and peculiar morphological traits (Ziegler and Groscurth, 2004). During early events of the apoptotic process the cell shrinks, the cytoplasm and organelles become tightly packed and pyknosis takes place. Pyknosis, is the most characteristic event of apoptosis and involves the irreversible condensation of chromatin. What follows is the fragmentation of nucleus, known as karyorrhexis, the blebbing of the intact plasma membrane and the formation of apoptotic bodies with ‘‘eat-me’’ or ‘‘find-me’’ signals on their surface, so that will be eventually engulfed and digested by nearby phagocytic cells and lysosomes (Vandenabeele et al., 2010, Elmore, 2007, Czabotar et al., 2014, Martin et al., 2012). In this way, autoimmune reactions,

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inflammation or tissue destructions are prevented. On the other hand, necrosis leads to karyolysis (the nuclear fading due to the action of DNAses and RNAses) and cell swelling (oncosis), which will culminate in the disruption of plasma membrane and the release of cellular content (i.e danger-associated molecular patterns, DAMPs) in the surrounding environment (Martin et al., 2012). Therefore, necrosis is considered to be a cytotoxic process since it can trigger inflammatory responses (Nagata et al., 2010, Galluzzi and Kroemer, 2008, Grivennikov et al., 2010). A shared biochemical network between the two processes is also described, indicating that the apoptosis and necrosis can overlap (Zeiss, 2003). For example, deprivation of intracellular ATP or unavailability of caspases could convert an ongoing apoptotic process to necrotic. In addition, despite the well-defined differences between apoptosis and necrosis, accumulating evidence supports that necrosis should be also considered as the natural outcome of the complete apoptotic program. The so-called secondary necrosis involves the autolytic disintegration of cells that takes place only when apoptosis is fully evolved. When phagocytosis is inefficient, apoptotic bodies progressively lose integrity and their content is released into the extracellular milieu (Silva, 2010). Interestingly, PARP-1 is also processed during necrosis. The necrotic cleavage of PAPR-1 though, results in a fragment of 50 kDa due to the activity of lysosomal proteases (Shah et al., 1996, Gobeil et al., 2001). Figure 11 illustrates the process of apoptosis, secondary necrosis and necrosis and their differential features.

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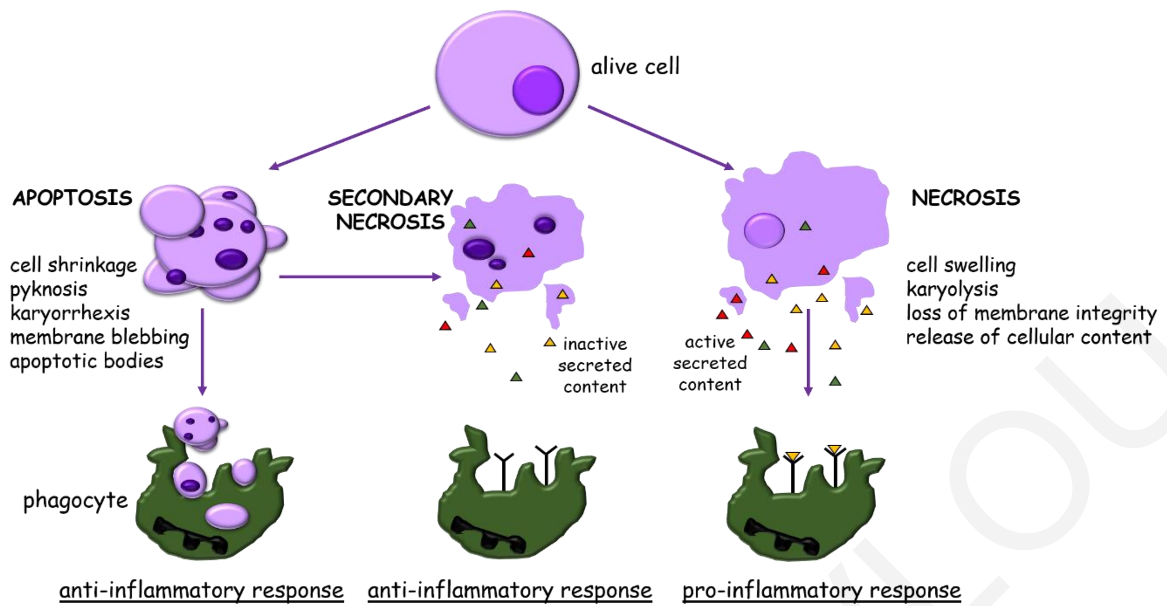

Figure 11. Apoptotic, necrotic and secondary necrotic forms of cell death. Induction of apoptosis leads to the removal of apoptotic cells by phagocytes. Secondary necrosis may follow apoptosis, when phagocytosis is inefficient. Apoptosis and secondary necrosis do not elicit immune responses. In contrast, necrotic cells spill out their contents which exhibit potent inflammatory properties.

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CHAPTER 2

SIGNIFICANCE, HYPOTHESIS AND AIMS

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2.1 Significance

The majority of eukaryotic proteins are subjected to N-terminal acetylation, by N-terminal acetyltransferases (NATs). Over the last years, NATs have attracted growing attention due to the various functional consequences that arise from N-terminally modified proteins. Protein degradation, translocation and complex formation are only some of the specific molecular functions for Nt-acetylation (Starheim et al., 2012). Importantly, NAT deregulated expression and activity has a central role in the development and progression of pathological conditions, including cancers (Kalvik and Arnesen, 2013). The acquired capability of cancer cells to evade programmed cell-death by apoptosis enables them to sustain their proliferative potential that eventually leads to tumor development, invasion and metastasis. Efforts to trigger apoptosis and re-establish this crucial natural barrier to tumorigenesis have also been the focus in several cancer therapy studies (Helin and Dhanak, 2013, Feng et al., 2010, Bhola and Letai, 2016, Karlic et al., 2014). Interestingly, knockdown studies have shown that depletion of NAT enzymes promotes p53-dependent cell death (Yu et al., 2009a, Lee et al., 2010a, Ren et al., 2008, Starheim et al., 2009), suggesting that NATs could represent key targets in cancer therapies. The first specific NAT inhibitors that resemble known substrates of NAT proteins have been developed and is anticipated that in the future, will be exploited as potent therapeutic drugs against NATs, in *vivo* (Foyn et al., 2013). The implementation of such inhibitors should be though, carefully assessed, since NATs have been shown to behave both as oncoproteins and tumor-suppressors in different cancer types (Kalvik and Arnesen, 2013, Lee et al., 2010a, Arnesen et al., 2006, Hua et al., 2011, Yi et al., 2011, Ren et al., 2008, Kuo et al., 2010), suggesting that their regulation is more complex than previously believed and that possibly their role is context-dependent.

Nt-acetyltransferase Naa40, is unique among other NATs because it can target and acetylate only two substrates both in yeast and humans, histones H4 and H2A (Song et al., 2003). Since histone modifications control various DNA-template processes, it is very likely that this acetyltransferase regulates transcriptional outcomes. The only evidence in support of this, comes from a recent study showing that the Naa40 yeast orthologue regulates rRNA expression (Schiza et al., 2013). Therefore, efforts in elucidating whether this role also holds true for the human Naa40 will ultimately lead to a deeper understanding of its function in mammalian cells. If this function of Naa40 is conserved, one could easily hypothesize that inhibition of Naa40 could induce detrimental

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consequences to cancer cells, as it has been shown that overexpression of rRNAs is necessary for cancer cell survival (Uemura et al., 2012, Ghoshal et al., 2004, Drygin et al., 2010, Ruggero and Pandolfi, 2003, White, 2005, Williamson et al., 2006). It is also intriguing to speculate that the activity of Naa40 towards histone proteins could affect the transcriptional activation of pro- or anti-apoptotic genes implicated in various survival pathways. This is supported by the fact that loss of other NATs triggers p53-dependent apoptosis. Furthermore, it has been proposed that Naa40 plays a critical role in cancers, since its expression controls the viability of hepatocellular carcinoma (Liu et al., 2009). According to Human Protein Atlas, the levels of Naa40 vary among different cancer types (The Human Atlas, 2016) and hence, ways to inhibit or induce its activity in tumors should be considered. Recent studies have deciphered the molecular structure of Naa40, shedding light into how Naa40 recognises, targets and binds to its histone substrates (Magin et al., 2015, Jedrzejewski and Kazmierkiewicz, 2013). These findings provide valuable information that could help in the design and synthesis of Naa40 specific inhibitors. Future research studies should focus on exposing the hidden biological functions of Naa40 and its underlying molecular mechanisms, in order to effectively exploit this enzyme in cancer therapy.

Although the Nt-acetylation mark does not change the charge of the targeted amino acid, it can neutralise the positive charge of the free α-amino group, and thereby efficiently block it for further ionization and other modifications. This could suggest that the underlying mechanisms for transcriptional regulation by Naa40 could implicate the exclusion or induction of other modifications, found adjacent to the N-terminus. Indication for such cross-talk was reported to occur in yeast, where N-acH4 and H4R3me2a modifications are mutually exclusive (Schiza et al., 2013). Taking into consideration that epigenetic modifications do not function alone but work synergistically or antagonistically with other chemical marks, an identified cross-talk between Naa40 and some other modifying enzymes could be exploited in the diagnosis of pathological conditions, like cancers. Additionally, the induction or repression of gene expression by manipulating such cross-talk, could be exploited in the development of novel strategies in the treatment of diseases.

Collectively, the current knowledge emphasizes the importance of investigating the biological function of Naa40 and its regulatory mechanisms in depth in an attempt to exploit this unique N-terminal acetyltransferase as a viable strategy in targeted cancer

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therapies. To achieve this goal we addressed the following hypothesis through two specific research aims.

2.2 Hypothesis and Aims

In this project, we sought to explore the link between histone N-terminal acetylation (N-acH4) mediated by Naa40 and colorectal cancer. Our overall hypothesis is that aberrant Naa40 expression and thus histone acetyltransferase activity may be implicated in colorectal carcinogenesis. This hypothesis is supported by the following facts:

a) Naa40 is differentially expressed among various cancer and non-cancerous tissues, suggesting that its function may be implicated in carcinogenesis (The Human Atlas, 2016).
b) Naa40 is down-regulated in hepatocellular carcinoma, whereas its overexpression triggers apoptosis (Liu et al., 2009), pointing out that Naa40 has a tumor-suppressor activity.
c) Colorectal cancer cells depleted for Naa40 exhibit morphological features of cell death.
d) Naa40 acetylates specifically the N-terminus of histones H2A and H4 across eukaryotes (Hole et al., 2011, Song et al., 2003, Polevoda et al., 2009b). Its conservation suggests that Naa40 could control fundamental cellular processes through transcriptional regulation of genes.

To examine our hypothesis, the following aims are proposed:

Specific Aim 1: Investigate the biological role of Naa40 in colorectal carcinoma

Preliminary results showed that Naa40 protein expression is significantly elevated in colon cancer cells compared to liver cancer cells. In addition, colorectal cancer cells depleted for Naa40 exhibit morphological features of cell death such as cellular rounding and detachment. Thus, we hypothesize that Naa40 could behave as an oncoprotein in colorectal carcinoma.

Specific Aim 2: Investigate the molecular role of Naa40 in gene regulation

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We recently showed that in yeast, N-acH4 acts as a transcriptional activation mark, since it promotes rRNA expression by inhibiting the deposition of an adjacent histone H4 modification, namely arginine 3 asymmetric dimethylation (H4R3me2a) (Schiza et al., 2013). Thus, we hypothesize that Naa40 regulates gene expression through its histone catalytic activity, in a similar way as in yeast and we aim to reveal the molecular function of Naa40 towards rRNA expression. Importantly, rRNA silencing in human cells could impact on cell growth and proliferation, as rRNA overexpression has been linked with cancer survival and progression (Uemura et al., 2012, Ghoshal et al., 2004, Drygin et al., 2010, Ruggero and Pandolfi, 2003, White, 2005, Williamson et al., 2006) . Since Naa40 targets histone proteins only, Naa40 activity towards histone proteins could also affect the transcriptional outcome of other genes, as well. Additionally, N-acH4 in yeast, antagonizes H4R3me2a levels to control rRNA expression (Schiza et al., 2013). Therefore, in humans, a cross-talk with other modifications to bring about transcriptional outcomes in mammalian cells will be also explored.

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CHAPTER 3

MATERIALS AND METHODS

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Cell culture and reagents

HCT116 cell lines (HCT116 p53+/+ and HCT116 p53-/-) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University) (Bunz et al., 1998) and were cultured for no more than 15 passages in McCoy's 5a medium supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin/streptomycin). HT-29 and STO cell lines were purchased from ATCC (catalogue no. HTB-38 and CRL-1503, respectively) and were cultured for no more than 10 passages in McCoy's 5a and DMEM media, respectively, supplemented with 10% FBS and 1% pen/strep. The cells were maintained at 37°C under 5% CO_2. McCoy's and DMEM media, FBS, penicillin-streptomycin and trypsin were purchased from Gibco, Invitrogen. All cell-lines used were routinely checked for mycoplasma contamination according to the protocol established by Harasawa and colleagues (Harasawa et al., 1993). The caspase-9 inhibitor z-LEHD-fmk was purchased from BD Pharmingen and DMSO from Gibco, Invitrogen. Target cells were preincubated with 5μM of either z-LEHD-fmk or DMSO, 2h prior siRNA treatment. Fresh inhibitor was reintroduced to cells 6h and 24h post-transfection to ensure the continued presence of active inhibitor.

RNA interference

siRNA against human and mouse Naa40 and negative controls were purchased from GenePharma (Shanghai, China). Human negative control anneals from UUCUCCGAACGUGUCACGUTT and ACGUGACACG UUCGGAGAATT. Human Naa40 siRNA1 and Naa40 siRNA2 sequences were taken from Liu et al. 2009 [49], and anneal from 5'-CUUUCCCAGUGUUCAAGAATT-3' and 5'-UUCUUGAACACUGG GAAAGTT-3' for siRNA1 and 5'-GAAGGUUAUGUUAACAGUATT-3' and 5'-UACUGUUAACAUAACCUUCTT-3; for siRNA2. siRNA sequences against mouse Naa40 anneal from 5'-'-CAUAAUCAUGGCGCCUAUCTT-3' and 5'-GAUAGG CGCCAUGAUUAUGTT-3' for siRNA1 and 5'-CAGAGAGUCCUUUCUUGCUTT-3' and AGCAAGAAAGGACUCUCUGTT for siRNA2. Mouse negative siRNA control anneals from 5'-CAGCUGAUUUCGUUCGUUCTT-3' and 5'-GAACGAACGA AAUCAGCUGTT-3'. All cells were seeded in antibiotic free medium such that they will be ~30% confluent (HCT116 and HT-29 cells) or ~60% confluent (STO cells) at the time of transfection and were transfected with siRNA oligo-nucleotides using Lipofectamin

RNAiMAX (Invitrogen) according to manufacturer instructions, to a final concentration of 7.5 nM (HCT116 and STO cells) or 25nM (HT-29 cells).

Primer Design for RT-PCR and ChIP analysis

DNA or mRNA (CDS) sequences were obtained from the UCSC Genome Browser (https://genome.ucsc.edu/) or the NCBI database (http://www.ncbi.nlm.nih.gov/). The sequence was entered in ‘‘Primer3 Input’’ online software and primer parameters were adjusted as shown: primer length of 18-22 base pairs, primer melting temperatures (Tm) in the range 59-61 °C and a product size of 50-150 bp. Specificity of primers was confirmed through UCSC blast analysis. Primer sequences were provided by Integrated DNA Technologies (IDT).

Gel Electrophoresis

Agarose gel electrophoresis was performed in order to determine the quality of the RNA samples and to check DNA fragmentation during the sonication optimization for ChIP analysis. Gel electrophoresis was performed using 1% agarose gel. 0.4g dry agarose powder (Biorad, #AC003522500) suspended in 40ml 1X TAE buffer (40mM Tris, 20mM glacial acetic acid, 1mM EDTA pH 8.0 and ddH2O) and heated in order to be dissolved. 0.8μl of Ethidium Bromide (Sigma Aldrich, #030M8710) was added. Solution was then poured into a plastic casting tray for 15 minutes to solidify. 1X TAE buffer was poured to cover the agarose gel. Before the loading, samples were mixed with 2μl of 5x Loading buffer (Invitrogen, #10816-015) and 7μl of ddH2O. 4μl of 1Kb DNA ladder (Invitrogen, #10488-072) was used as a molecular weight standard. The electrophoresis was performed in 1x TAE buffer at 100 V/cm for 20 minutes. Bands were visualized under UVP bioimaging system.

Gene Expression analysis

Total RNA was isolated using the RNease Mini kit according to the manufacturer’s instructions (Qiagen). DNAse treatment was followed using the TURBO DNA-free kit. To 10μg of total RNA, 5μl of TURBO DNAse buffer and 1μl of TURBO DNAse were added to a final volume of 50μl and the mixture was incubated at 37°C for 30 minutes. The mixture was re-incubated for 30 more minutes after the re-addition of another 1μl of

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TURBO DNAse. The reaction ended with the addition of 5μl of DNase inactivation reagent and the mixture was left to set for 2 minutes at RT. After centrifugation (2' x 14000rpm (18400rcf)), the supernatant was collected in a fresh tube and RNA quantity and quality was measured with nanodrop. Synthesis of cDNA from total RNA was accomplished using PrimeScript RT reagent Kit (Takara). 0.5μg of total RNA were reversed transcribed in 10μl of reaction mixture, which included 2μl of 5x PrimeScript Buffer, 0.5μl of PrimeScript RT enzyme Mix I, 0.5μl of oligo dT primer (50μM), 0.5μl of random hexamers (100μM) and RNase free water. The reaction mixture was incubated for 15 minutes at 37°C and for 5 seconds at 85°C. cDNA was diluted in DNAse RNase-free water so that the concentration of every sample was 0.5ng/μl. For Real Time PCR, the following mixture was prepared in each well of a multiplate 96-well PCR plate: 5μl KAPA SYBR Green (SYBR Green Fast qPCR Master Mix), 1μl cDNA, 3μl ddH2O and 1μl primer pair mix (40ng of each primer). Reactions were incubated in Biorad CFX96 Real-Time System using the following programme

1. 95°C, 2 min
2. 95°C, 2 sec
3. 60 °C, 20 s
4. 60 °C, 1 s
5. Back to step 2 for 39 more cycles
6. 72°C to 95°C, 5 sec

The PCR products were normalised to those obtained from b-actin mRNA amplification. Primer sequences were designed using Primer 3 and are indicated in Table 3.

The cDNA product was quantified using the following calculations:

- ΔCT = CT1- CT2, where CT1 refers to the gene of interest and CT2 to the reference gene
- ΔΔCT= ΔCT1- ΔCT2, where ΔCT1 refers to the treated cells and ΔCT2 to the untreated control
- Fold change in cDNA levels = $2^{-(\Delta\Delta CT)}$

The relative expression levels were expressed as a percentage of the indicated control.

*Threshold cycle, CT: The cycle number at which the amplified product accumulates to yield a detectable fluorescent signal.

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Table 3. Primers used in qRT-PCR

Gene	Forward 5'→3'	Reverse 5'→3'
Naa40	TGGTGCCTACCAGTTCTTCA	CTCCGGCTCAGGATCTCATA
β-actin	GGCATCCTCACCCTGAAGTA	AGGTGTGGTGCCACATTTTC
Caspase-9	CGACATCTTTGAGCAGTGGG	CCCTTTCACCGAAACAGCAT
Caspase-8	ACTTTGCCAGAGCCTGAGAG	GCATCTGTTTCCCCATGTTT
Caspase-7	GAATGACAGAGTTGCCAGGC	TCCTTGGTGAGCATGGAGAC
Caspase-6	TGGAGCTGACTTCCTCATGT	ACCAGTGTGAGGAGTTCTGT
Caspase-3	ATGCACATTCTTACCCGGGT	GCGTCAAAGGAAAAGGACTCA
PARP-1	GGCAAAACTACCCCTGATCC	CACACCAGATGAAATCCCGG
5.8S rRNA	ACTCGGCTCGTGCGTC	GCGACGCTCAGACAGG
28S rRNA	AGAGGTAAACGGGTGGGGTC	GGGGTCGGGAGGAACGG
18S rRNA	GCAAATTACCCACTCCCGAC	CCCTCCAATGGATCCTCGTT
45S rRNA	GAACGGTGGTGTGTCGTT	GCGTCTCGTCTCGTCTCACT
Vimentin	TGTCCAAATCGATGTGGATGTTTC	TTGTACCATTCTTCTGCCTCCTG
Fibronectin	ATTGCCCAATTGAGTGCTTC	CGGGAATCTTCTCTGTCAGC
P53	CCATCCTCACCATCATCACA	AGCTGTTCCGTCCCAGTAGA
P21	GACTCTCAGGGTCGAAAACG	GGCGTTTGGAGTGGTAGAAA
PUMA	GACTCCTGCCCTTACCCAG	ATGGTGCAGAGAAAGTCCCC
BAX	TCTGACGGCAACTTCAACTG	GGAGGAAGTCCAATGTCCAG
BAK	CCCCAATTGATGCCACTCTC	TCAACCGACGCTATGACTCA
BCL-2	ATGTGTGAGAGCGTCAA	ACAGTTCCACAAAGGCATCC
CYCLIN-D	CCCTCGGTGTCCTACTTCAA	AGGAAGCGGTCCAGGTAGTT
CYCLIN-E	ATCCTCCAAAGTTGCACCAG	AGGGGACTTAAACGCCACTT
CHOP (B)	GCTGGAACCTGAGGAGAGAG	TGCTTTCAGGTGTGGTGATG
CHOP (M)	ACACTACCCACCTTTCCCAG	GAGGGTCACATCATTGGCAC
CHOP (E)	TCTTGACCCTGCTTCTCTGG	TGGTTCTCCCTTGGTCTTCC
XIAP	GACAGGCCATCTGAGACACA	AGGGTTCCTCGGGTATATGG
SURVIVIN	GGACCACCGCATCTCTACAT	CAAGTCTGGCTCGTTCTCAGT
PRMT5	TTGCCGGCTACTTTGAGACT	ACAGATGGTTTGGCCTTCAC

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Chromatin Immunoprecipitation (ChIP) assays

Cells were cultured in 100mm or 150mm plates. After RNAi treatment, protein-DNA complexes were cross-linked using 0.75% formaldehyde and incubated on a shaking device for 10 minutes. The cross-linking process was quenched with the addition of glycine diluted to a final concentration of 125 mM. After incubating cells on a shaking device for another 5 minutes, the media with floating cells was collected. Plates were rinsed twice with 1xPBS and adherent cells were scraped and pooled together with floating cells. All cells were centrifuged for 5 minutes at 2000rpm (400 rcf) at 4˚C , washed once with 1xPBS and pelleted again (at this point, samples can be stored overnight at < -70 °C). Each sample was lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM TrisHCl, adjusted to final volume 500 ml dH2O) containing 1mM PMSF (Sigma) and protease inhibitor tablet (Roche), sonicated on ice to shear DNA to lengths between 200 and 1000bp and centrifuged for 10 minutes at maximum speed (14000rpm; 18400rcf), at 4˚C. In the meantime, protein Sepharose A beads needed for immunoprecipitation, were washed before use with cold IP buffer and after centrifugation at 2000rpm (400rcf) for 2 minutes, they were resuspended in an equal amount of IP buffer so that a 50% slurry was formed. Beads were then blocked with 0.07mg/ml sonicated single stranded salmon sperm DNA (1mg/ml) and with 0.7mg/ml BSA (10mg/ml), at 4˚C, on a rotor, for 1 h. After cell sonication and centrifugation, the supernatant (chromatin) was collected in a clean tube and the pellet was discarded. Chromatin was diluted in Immunoprecipitation buffer (IP buffer: 0,1% SDS, 1,1% Triton X-100, 1,2 mM EDTA, 16,7 mM TrisHCl, 167 mM NaCl, adjusted to final volume 500 ml dH2O) in a ratio 1:10 and the resulting diluted chromatin fraction was incubated with the pre-cleaned/pre-blocked protein Sepharose A beads (Roche) for at least 1.5 hour, at 4˚C , on rotor. Pre-cleaned chromatin fraction was afterwards incubated at 4 °C on a rotor, with primary antibodies against histone H3(Ab1791), histone H4 (Millipore05-858) and against histone modifications H3K4me3(ab8580), H4S1ac, H4R3me2a and H4R3me2s (ab5823). IgG (Biogenesis 5180-2104) was used as control. After 1 hour rotation, 50μl of beads were added in every sample and were incubated on a rotor, at 4 °C overnight. The following day, samples were centrifuged and washed 3 times with TSE-150 washing buffer (1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM TrisHCl, 150 mM NaCl, adjusted to final volume 500 ml dH2O) and 2 times with TSE-500 washing buffer (1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM TrisHCl, 500 mM NaCl, adjusted to final volume 500 ml dH2O) following centrifugation between the intervals, where supernatant was discarded. The immunoprecipitated

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beads/antibody/chromatin samples, were then incubated twice for 15 minutes in 100μl Elution buffer each time (1% SDS and 0.1 M $NaHCO_3$), in a thermomixer at 25°C, 1400rpm. 200μl of eluate was centrifuged and supernatant was collected in new labelled tubes. Finally, samples were reversed-cross linked with the addition of 12μl 5M NaCl and 10μl RNase (0.5μg/μl). DNA purification was performed using Qiagen purification kit and RT-PCR was performed using specific primers (Table 4) for the genes of interest.

Table 4. Primers used in qPCR-ChIP

Gene	FORWARD 5'→3'	Reverse 5'→3'
β-actin	CGACCAGTGTTTGCCTTTTAT	AGATTGGGGACAAAGGAAGC
45S rDNA promoter	CGATGGTGGCGTTTTTGG	CAGGTCGCCAGAGGACAGC
28S rDNA	GCGACCTCAGATCAGACGTGG	CTGTTCACTCGCCGTTACTGAG
PRMT5 promoter	GTCTCTCAACGGCGTTCTTC	GTCGCCTTAACAACCAGAGC
PRMT5	ATAGCTGACACACTAGGGGC	CTAGTCTGCCCTTCTCCGTC

Dot blot analysis

Synthesized peptides with at least 90% purity (Cambridge Peptides, UK) were dissolved in water. Polyvinylidine difluoride (PVDF) membranes were used. Having the membrane ready for use, a grid was drawn by pencil to indicate the region to be blotted. Using a narrow-mouth pipette tip, 50, 10 and 2 ng were deposited onto the membrane at the center of each possition on the grid. The membrane was left to air-dry for 1 hour. The membrane was then submerged in 100% Methanol for 1 minute, water for another minute and then stained with Ponceau S or blocked by soaking in TBS-T blocking buffer 5% BSA 0.1% Tween-20 TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 8). Incubation followed with appropriate antibody and dissolved in BSA/TBS-T for 2 hours on the shaker at low speed at RT. Next, the membranes were washed with TBS-T (three times with 10 min incubation). Incubation with appropriate secondary antibody followed, diluted in blocking buffer for 1h at RT. Membranes were washed three times with TBS-T (three times with 10 min incubation). Incubation with ECL detection reagent (GE Healthcare, Amersham # RPN2209) followed for 1 min and membrane was exposed.

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Western Blotting

Western Blotting was performed for separating and identifying proteins and assessing their protein expression levels. Cells were washed with ice-cold PBS (1X) and were solubilised in Lysis buffer composed of 50 mM Tris-Cl (pH 8), 3 mM EDTA, 100 mM NaCl, 1% Triton-X-100, 10% glycerol, 0.5 mM PMSF and protease inhibitor (Roche). Protein concentration was measured by the Bradford assay. Forty micrograms of total protein were separated by 8%, 12.5% or 15% SDS-PAGE. Tables 5-9 list the recipes for preparing the required buffers for SDS-Page electrophoresis. When proteins were separated on gel, were then transferred to a nitrocellulose membrane, which was blocked with TBS-T/5%BSA. Incubation with primary antibodies of interest was performed overnight, at 4°C. Horseradish peroxidase–conjugated secondary anti-rabbit or anti-mouse antibody was used at a concentration of 1:30000. Washes with TBT-T (3 x 5 mins) were performed after every antibody incubation, in order to wash off any unbound antibody. Immunoreactive bands were visualized with an enhanced chemiluminescent system.

Rabbit Naa40 antibody was kindly provided by Dr. Qiwei Zhai [49]. Rabbit polyclonal antibody was raised against H4R3me2a in collaboration with Eurogentec (Belgium). For the generation of H4R3me2a the animal was immunized with the keyhole limpet hemocyanin (KLH) conjugated synthetic peptide H2N-SGR (AsymDimethyl) GKG GKG LGK C-CONH2. Serum was affinity-purified by the manufacturer in a two-step procedure. Antibodies specific to the modification were captured on the AF-Amino TOYOPEARL 650 M matrix together with the immunization peptide. After elution with 100 mM glycine (pH 2.5), the antibodies recognizing the peptide in the absence of modification were eliminated with a matrix coupled with the unmodified peptide H2N- SGR GKG GKG LGK C- CONH2. After the purification, antibody specificity was determined by enzyme-linked immunosorbent assay (ELISA) using the specific and non-specific peptides as antigens. The purity of the H4R3me2a antibody was >89% as determined by high pressure-liquid chromatography. The rabbit polyclonal N-acH4 antibody was raised against the acNH-SGRGKGGKGLGKC antigen using the Eurogentec ‘Speedy 28-day polyclonal antibody’ service (Belgium). The modification-specific antibody was isolated by affinity purification of total serum through the specific N-terminally acetylated H4 peptide. To eliminate immunoglobulins that recognise the backbone of H4, the bound fraction from the above purification was further cleaned up by passing it through a column consisting of the corresponding ‘unmodified’ version of the peptide. The H4R3me2s and PRMT5 antibodies were purchased from Abcam. The p53, Bcl-2 and β-actin antibodies were purchased from

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Santa Cruz Biotechnology Inc. PARP-1, Bax, caspase-3, -6, -7, -8, and -9 antibodies were purchased from Cell Signalling Technology (Danvers, Massachusetts, USA). H4 antibody was purchased from Millipore and GAPDH and fibronectin antibodies were purchased from

Abcam. Histones purified from calf thymus were purchased from Sigma. Immunoreactive bands were visualized with the enhanced chemiluminescence system. The intensity values from the densitometry analysis were normalised against GAPDH or β-actin using Image J analysis software (NIH). Intensity values were expressed as fold change compared to control.

Table 5. Separating Gel (bottom gel)

For 1mm gel x1	7.50%	10%	12.50%	15%	17%
ddH_2O	5.4ml	4.8ml	4.175ml	4ml	3.5ml
4x Tris separating ph 8.8	2.5ml	2.5ml	2.5ml	2.5	2.5ml
40% acrylamide	1.875ml	2.5ml	3.125ml	3.75ml	4.25ml
10% SDS	50µl	50µl	50µl	50µl	50µl
Temed	15µl	15µl	15µl	15µl	15µl
APS 10% (-20°C)	150µl	150µl	150µl	150µl	150µl
Total	**10ml**	**10ml**	**10ml**	**10ml**	**10ml**

Table 6. Stacking Gel (top gel)

For 1mm gel x1	3.90%
ddH_2O	3.260 ml
4x Tris stacking ph 6.8	1.25 ml
40% acrylamide	0.49 ml
10% SDS	25 µl
Temed	7.5 µl
APS 10% (-20°C)	75 µl
Total	**5ml**

Table 7. 10x Running Buffer

Reagents	0.5L	1L	2L
0.25M Tris	15.14g	30.29g	60.58g
1.9M Glycine	72.07g	144.13g	288.26g
1% SDS (toxic, use mask)	5g	10g	20g
pH should be between 8.3 and 8.8			

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Table 8. 10x Transfer Buffer

Reagents	0.5L	1L	2L
250mM Tris	15.14g	30.26g	60.58g
1.9M Glycine	71.32g	142.63g	285.66g
pH should be between 8.3 and 8.8			

Table 9. 10x TBS

Reagents	1L
NaCl	80g
KCL	2g
Tris	30g

1x TBS-T
900ml H2O
100ml 10x TBS
Addition of 1ml Tween

1x TBS-T / BSA (Blockin
BSA stored at 4°C
400ml from 1x TBS-T
20g BSA

MTT viability assay

The Methyl Thiazol Tetrazolium (MTT) reduction assay is a colorimetric assay used for assessing cell metabolic activity, and therefore determining cell proliferation and viability. The assay measures the activity of mitochondrial enzymes that reduce yellow MTT to a purple formazan crystal product. When cells die, they lose the ability to convert MTT to formazan, thus colour discrepancy serves as a useful and convenient marker of only the viable cells. The formazan product accumulates as an insoluble precipitate inside cells as well as near the cell surface and in the culture medium. Therefore, the formazan, prior recording absorbance readings, must be solubilised in DMSO. The absorbance of this coloured solution can be quantified by measuring at 570 nm by a spectrophotometer. The amount of signal generated is dependent upon several parameters including the concentration of MTT, the incubation period, the number of viable cells and their metabolic activity. All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of product that can be detected above background. When the amount of purple formazan produced by treated cells is compared with the amount of formazan produced by control cells, the effectiveness of the agent that causes cell death can be deduced, through a dose-response curve (Figure 12).

For this research study, cells were seeded at a concentration of $5x10^{3}$ cells per well (HCT116 and HT-29) or $1x10^{4}$ cells per well (STO cells) in a 96-well plate and incubated with 7.5nM of siRNAs (HCT116 and STO cells) or 25nM of siRNAs (HT-29 cell), for

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different time periods (24, 48 or 72 h). MTT dye (1mg/ml) was added to each well at the end of the incubation period and cells were incubated for 4h at 37°C. Formazan product was dissolved in DMSO and the plate was incubated on a plate shaker at 250 rpm for 30min. The absorbance was measured at 570 nm using Perkin Elmer Wallac Victor 1420-002 Multilabel Counter and was proportional to the number of viable cells per well.

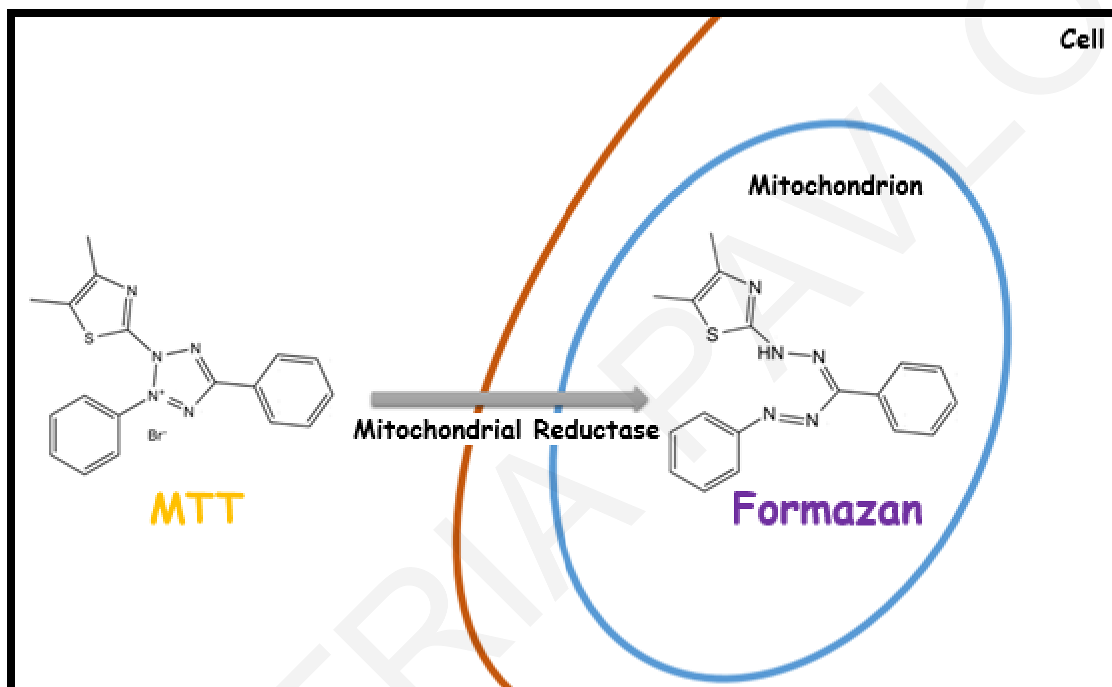

Figure 12. MTT reduction to formazan. Yellow MTT is reduced in live cells by mitochondrial reductase and results in the formation of insoluble purple formazan, which is characterised by high absorptivity at 570nm.

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Cell Cycle Analysis

Cell cycle analysis by flow cytometry, measures the DNA content of a cell population and is based on the ability of Propidium Iodide (PI) dye to stain DNA in a stoichiometric manner (the amount of stain is directly proportional to the amount of cellular DNA). Therefore, this method enables the identification of cell distribution across the cell cycle, since cells do not divide simultaneously. Figure 13 displays the stages of cell cycle. The G1 phase is where cells spend most of their life. Two copies of each chromosome are present in each cell (2N amount of DNA). When the cell prepares for cell division, it should first duplicate its DNA, and thus cells enter the so called S phase. Once the cells have finished DNA duplication, they contain twice as much DNA as the original cells (4N amount of DNA). This stage of the cell cycle is called the G2 phase. Next, the cells go through mitosis (M) which splits the 4N amount of DNA back into 2N and the cells return to G1. The DNA distribution through flow cytometry, is shown through DNA content histograms. As Figure 14 shows, the phases that can be recognised through a histogram are a) the G1 phase, b) the S phase and c) the G2/M phase. The G2- and M phase have an identical DNA content and therefore cannot be discriminated. In addition, cells that cease cell division and fragment their DNA (apoptotic cells) are also visible on the left region of the histogram. On the other hand, polyploid cells are visible on the right side of the histogram.

In this project, cells were seeded in 6-well plates or in 100mm dishes so that they would be ~30% confluent by the time of transfection. After siRNA treatment for 24h, 48 or 72h, cells were harvested, washed in 1x PBS and fixed in ice-cold 70% ethanol. Cells were left at 4°C overnight and the next day were pelleted, washed in 1x PBS and resuspended in PI staining solution (containing 0.02 mg/ml Propidium Iodide and 0.2 mg RNase A). After a 45 minute incubation at 37 °C, the samples were transferred to FACS tubes and analysed with the ''Guava EasyCyteTM'' flow cytometer and the "GuavaSoft" analysis software (Millipore, Watford, UK).

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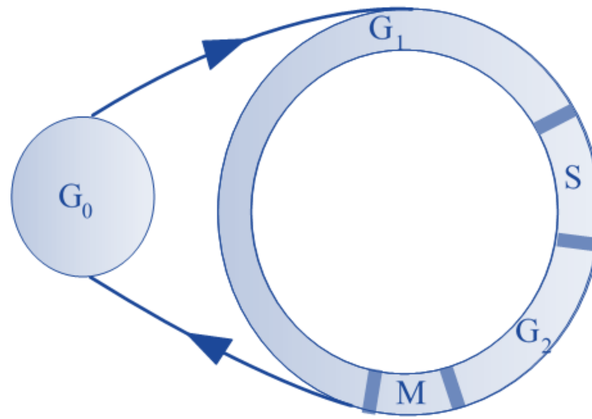

Figure 13. The stages of cell cycle. Actively dividing cells pass through a series of stages known as the cycle: two gap phases (G1 and G2), an S phase (for DNA duplication) and an M phase, in which mitosis partitions the genetic material and the cell divides. Cells that cease cell division enter the G0 phase.

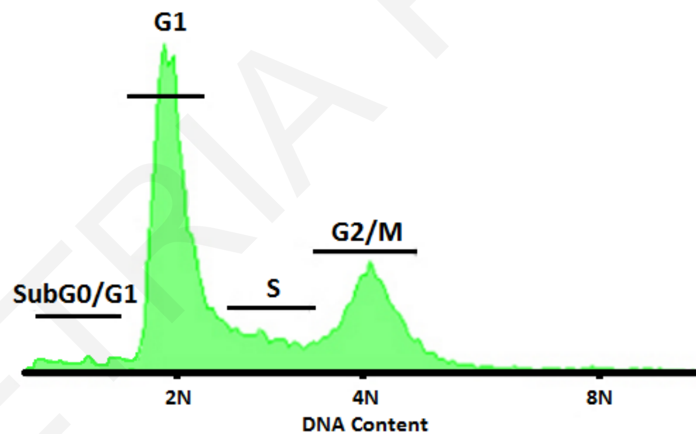

Figure 14. DNA histogram obtained by flow cytometry. Different peaks on the histogram represent different phases of the cell cycle (G1, S and G2/M phases). SubG0/G1 fraction represents the apoptotic population.

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Annexin V/PI staining assay

The Annexin V/PI staining represents one of the most reliable approaches for assessing cell death. The two stains, Annexin V and Propidium Iodide (PI) are conjugated, in order to determine if cells are viable, apoptotic or necrotic through differences in plasma membrane integrity and permeability that occur at different stages of cell death (Vermes et al., 1995, Rieger et al., 2011). Annexin V stain has the capability of binding to phasphatidyl serine (PS) phospholipids, which, in viable cells, are located in the inner leaflet of plasma membrane. One of the very early events of apoptosis, is the translocation of PSs to the outer surface of plasma membrane, which leads to the loss of plasma membrane asymmetry. As a result, the exposed PSs are now accessible to Annexin V dye which display a green fluorescence. PI is a nuclear stain that binds to DNA and therefore represents a great indicator of cell viability, as the dye does not stain live or early apoptotic cells due to the presence of an intact plasma membrane (Darzynkiewicz et al., 1992, Vermes et al., 2000, Vermes et al., 1995). In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases (Denecker et al., 2001, Kroemer et al., 1998), allowing PI to pass through the membranes, intercalate into nucleotides, and display red fluorescence (Figure 15).

The assay works in the following manner: cells that are viable are both Annexin V/PI negative, early apoptotic cells are Annexin V positive and PI negative and cells that are in late apoptosis, or are already dead are both Annexin V/PI positive. The movement of cells through these three stages suggests apoptosis and not necrosis and therefore samples should preferably be analysed at multiple time points, to reveal more information about the process by which the cells underwent.

Annexin V/PI staining was performed using Tali Apoptosis Kit (Invitrogen A10788). After treatment, cells were trypsinised, centrifuged for 15 minutes at 1000rpm (95rcf) and cell pellet was dissolved in 100μl Annexin binding buffer ($5x10^5$-$5x10^6$cells/ml). To each 100μl of samples, 5μl of Annexin V were added and after thorough mixing, samples were incubated at RT for 20 minutes. Cells were then centrifuged for 15 minutes at 1000rpm (95 rcf) and supernatant was discarded. Cell pellet was resuspended in 100μl of Annexin binding buffer and 1μl of Propidium Iodide (PI). After incubating the samples for 5 minutes at RT in the dark, they were transferred in Tali cellular analysis slides. Analysis of apoptosis was performed using Tali image-based cytometer (Invitrogen). The annexin-V positive/PI negative cells were recognized as early apoptotic cells by the cytometer

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software whereas the annexin V positive/PI positive cells were identified as late apoptotic/dead cells. Similarly, the annexin V negative/PI negative cells were identified as viable cells. The baseline apoptosis varied between 5-15% among the various apoptosis related experiments performed.

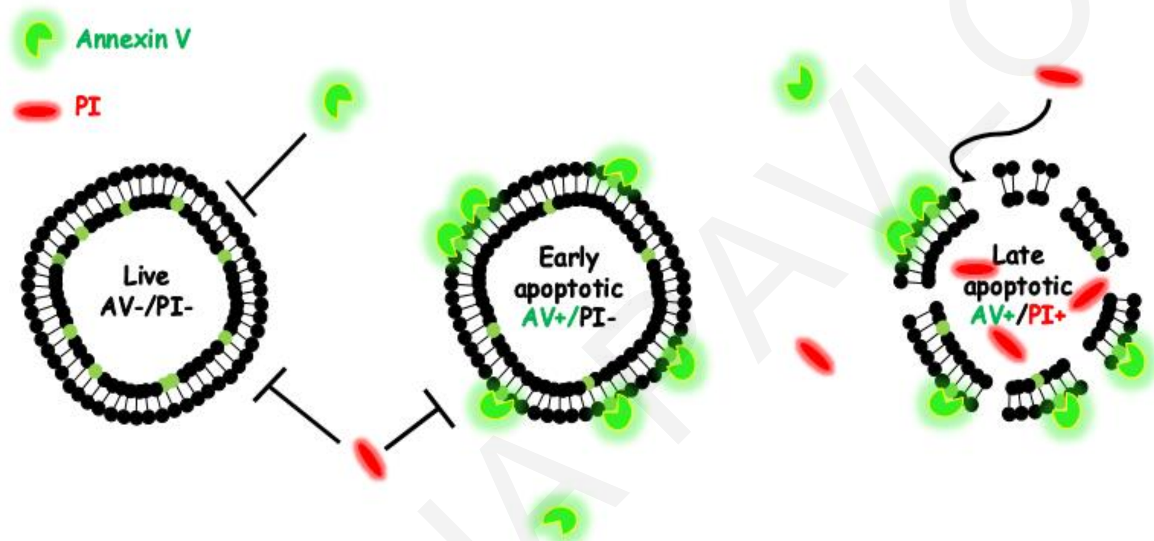

Figure 15. Dual staining with Annexin V and PI. In a live, intact cell, PI cannot enter the cell and label DNA and phosphatidyl serine (PS) phospholipids (green spheres) are maintained exclusively on the inner leaflet of plasma membrane, resulting in negative staining for both dyes. Annexin V labels externalized PSs on the cell surface (green moieties) following the initiation of apoptosis. During the latter stages of apoptosis both PSs are exposed and PI can enter the cell (red moieties) due to the loss of membrane integrity.

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Immunofluorescence

Immunofluorescence (IF) microscopy enables the assessment of both the localisation and endogenous expression levels of a protein (antigen) of interest. IF relies on the use of antibodies to label a specific cellular target antigen with a fluorescent dye (also called fluorophore or fluorochrome) such as fluorescein isothiocyanate (FITC). The fluorophore allows visualization of the target distribution in the sample under a fluorescent microscope.

For this project, cells were grown on poly-L-Lysine coated coverslips, in a 6-well plate. At the end of the incubation, cells were washed 2 times with 1xPBS and then fixed at RT, for 10 min in 4% paraformaldehyde solution in 1x PBS. Cells were washed 2 x 5minutes with 1x PBS and then permeabilized using 0.5% Triton-X solution in PBS for 10 min. After 2 x 5 minutes washes with 1x PBS permeabilised cells were blocked for 45 minutes in PBG (0.2% cold water fish gelatin, 0.5% BSA in 1 x PBS) and then incubated with primary antibody diluted in PBG, for 2 hours, at RT, in the dark. Cells were then washed for 3 x 5 minutes with PBG. Incubation with secondary antibody diluted in PBG followed, for 45 minutes at RT, in the dark. After 2 x 5 minutes washes with PBG, cover slips were placed to microscope slides with mounting media (prolong Gold). Nuclei were stained with Hoechst dye (33342, Invitrogen). Anti-Rabbit FITC antibody was purchased from Jackson ImmunoResearch. All images were acquired using a ZeissAxio Observer.A1 microscope.

Phase contrast microscopy

Images were acquired using ZeissAxio Observer.A1 microscope and ZeissAxioVision 4.8.2 software.

Statistical Analysis

Data represent measurements of three independent experiments and values are expressed as mean ± SD. Statistical analysis was carried out using "Mstat" software (version5.5.3, McArdle Laboratory for Cancer Research,University of Wisconsin – Madison [http://mcardle.oncology.wisc.edu/ mstat/]). Results are compared using a Wilcoxon rank-sum test. For all statistical tests differences are considered significant at $p \leq 0.05$ (*).

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CHAPTER 4

RESULTS OF AIM 1

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4. Unveiling the apoptotic role of histone N-terminal acetyltransferase Naa40 in colorectal cancer

The great importance of N-terminal acetylation in the regulation of cell proliferation and apoptosis has been reported by several studies. The expression of N-terminal acetyltransferases has been shown to be both up-regulated and down-regulated in human cancers compared to non-oncogenic tissues. Thus, N-terminal acetyltransferases have been characterised either as oncogenes or tumor suppressors (Arnesen et al., 2006, Starheim et al., 2009, Kalvik and Arnesen, 2013, Starheim et al., 2012). The fact that they can behave in an opposing manner in different or the same type of cancer, has led to a steep rise in N-terminal acetylation research, in an attempt to develop NAT-selective therapies. The role of Naa40 in human cancers has only recently been described by a study conducted by Liu et al. (Liu et al., 2009), linking hepatocellular carcinoma with low levels of the enzyme. Interestingly, when Naa40 is overexpressed in liver cancer cells it exhibits a pro-apoptotic activity. Since the levels and functions of NATs are shown to vary in different cancers, we sought to determine the unknown role of Naa40 in highly metastatic and aggressive colorectal cancer, which, according to data from the Human Protein Atlas project, they exhibit the highest NAA40 expression (The Human Atlas, 2016). In order to explore this, HCT116 and HT-29 colorectal cancer cells were used, together with the non-cancerous STO mouse embryonic fibroblast cell line.

Taking into consideration the known role of Naa40 in hepatoma cells, we first performed western blot assays to determine the protein levels of Naa40 in HCT116 colorectal cancer cells and compared them to those expressed in HepG2 hepatocellular cancer cell line. As shown by western blot analysis, Naa40 is differentially expressed in the two cancer cell lines, with a significantly higher protein expression observed in HCT116 cells (Figure 16). These results may suggest that Naa40 acetyltransferase behaves in a different way, and possibly in a contradictory manner in the HCT116 opposed to the HepG2 cell line.

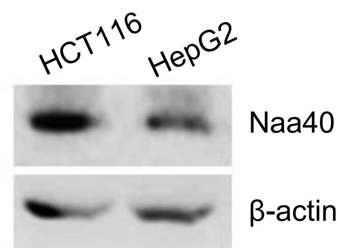

Figure 16. HCT116 colon cancer cells express higher levels of Naa40 compared to HepG2 liver cancer cells. Immunoblot analysis of Naa40 protein levels in whole cell extracts. β-actin is used as a loading control.

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4.1 Depletion of Naa40 in HCT116 colorectal cancer cells decreases N-acH4 while H4 histone total levels remain unaltered

In order to reveal the function of the acetyltransferase Naa40 in HCT116 colorectal cancer cells, the double-stranded RNA-mediated interference (RNAi) approach was exploited. Two different 21bp siRNA duplexes were transfected into cells to achieve a fast and effective knock-down of both the Naa40 mRNA (Figure 17A) and Naa40 protein levels (Figure 17B). The depletion was already evident and robust at 24 hours and sustained until 72 hours after Naa40-siRNA treatment (Figure 17). Cells were also subjected to the transfection procedure in the absence of any siRNA (mock control) and to a negative non-targeting siRNA control (scramble siRNA). These controls enabled us to determine whether the transfection process on its own results in cytotoxicity or to other non-specific effects and to distinguish Naa40-specific silencing from sequence-independent effects that are associated with the delivery of siRNA into the cell. As expected, the scramble-siRNA did not alter the levels of Naa40 compared to mock-treated sample (Figure 17B), indicating that the achieved knockdown using the targeting Naa40 siRNAs was specific and that controls did not result in any observable and unintended off-target effects. Since Naa40 results in the N-terminal acetylation of histones H2A and H4, we also examined the levels of endogenous N-terminal acetylation of histone H4 (N-acH4), using an antibody that we had previously raised (Schiza et al., 2013). The N-acH4 antibody we raised, recognises specifically histone H4 when is acetylated at its N-terminus, as shown by dot-blot and western blot analysis (Figure 18). In agreement with the above knockdown results, the levels of N-acH4 were significantly decreased in cells treated with both Naa40-siRNAs (Figure 19), indicating that the acetyltransferase activity of the enzyme is abolished. As expected, the levels of N-acH4 did not change in the scramble-siRNA treated cells (Figure 19). Moreover, Naa40 depletion did not affect the levels of total H4 (Figure 19) demonstrating that only the N-terminally acetylated fraction is decreased. These findings indicate that RNA interference achieved an efficient and sustained depletion of Naa40 in HCT116 cells that consequently led to reduction of its substrate N-acH4.

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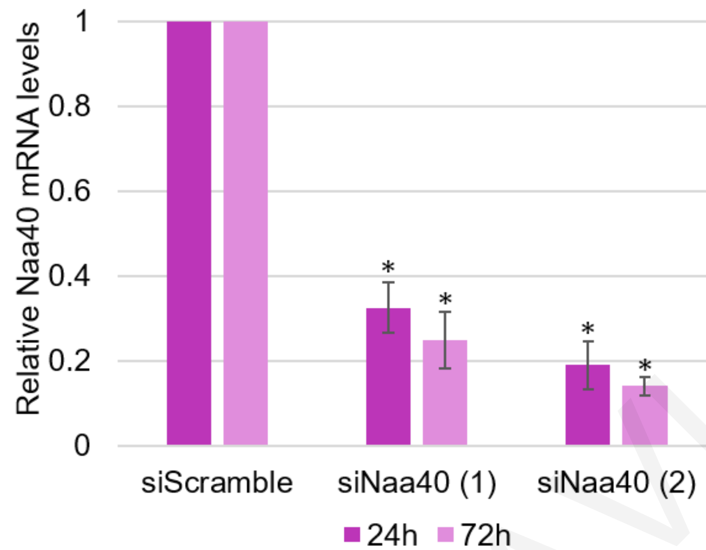

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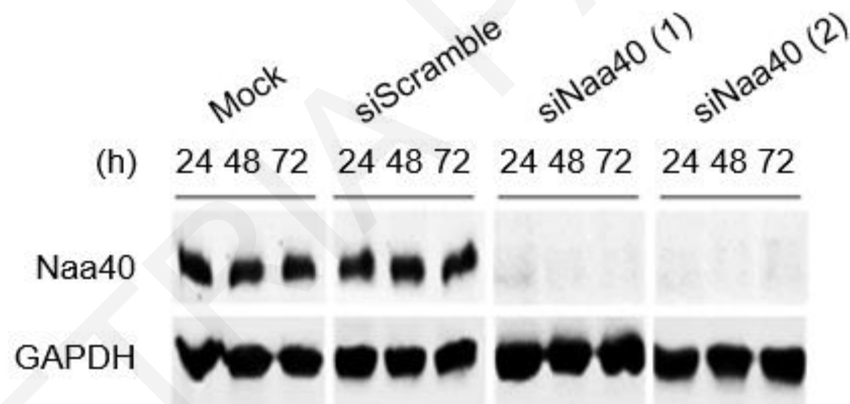

Figure 17. Efficient Naa40 silencing after siRNA treatment. (A) Colorectal cancer HCT116 cells were transfected with 7.5 nm of Naa40 siRNA-1, Naa40 siRNA-2 or scramble siRNA and incubated for 24–72h. **(A)** RT-PCR analysis of whole cell lysates. The levels of Naa40 mRNA were quantified and then normalised to the levels of β-actin mRNA, whose expression remained unchanged. **(B)** Immunoblot analysis to determine Naa40 protein levels in whole cell extracts. Mock represents sample in which cells were subjected to the transfection procedure in the absence of any siRNA. GAPDH levels were monitored and used as a loading control. Western blot image is representative of at least three independent experiments.

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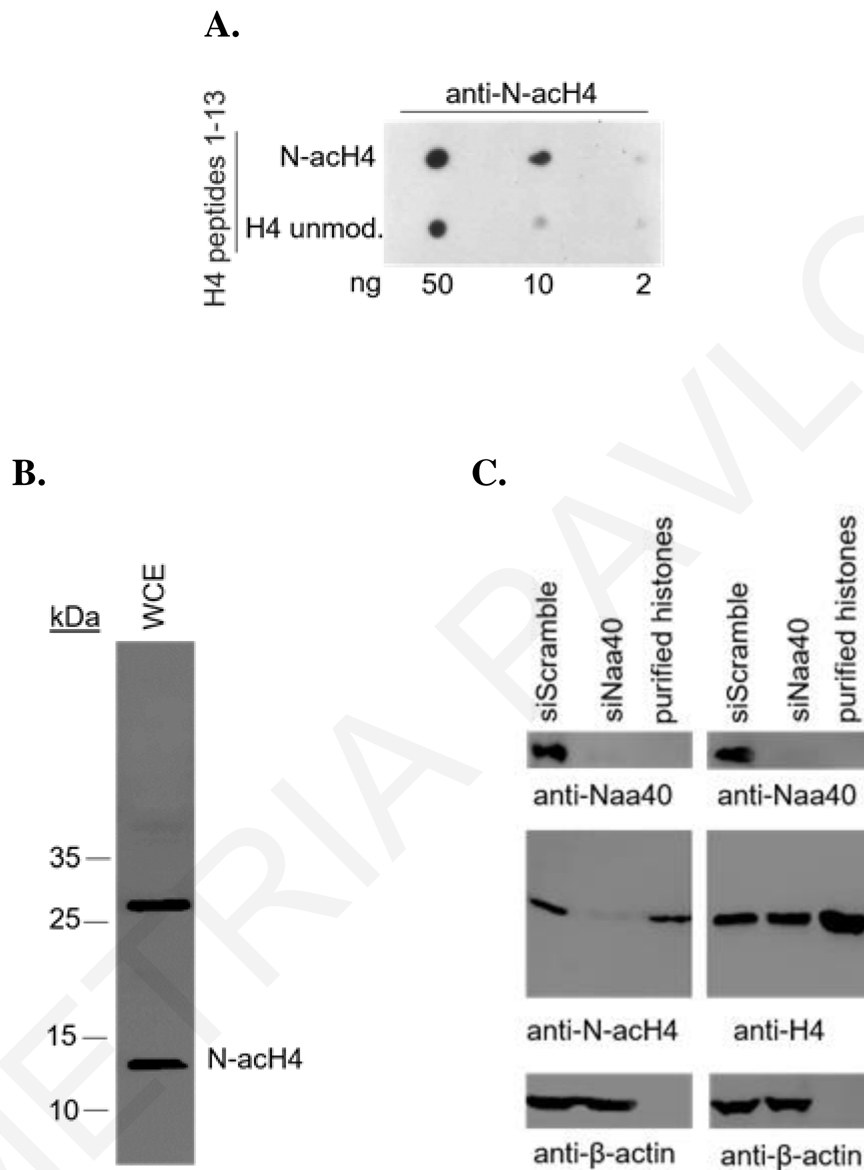

Figure 18. N-acH4 antibody preferentially recognises the N-terminally acetylated version of histone H4. **(A)** Dot blot analysis using the N-acH4 antibody against H4 N-terminally acetylated (N-acH4) and unmodified (H4 unmod) peptides representing the first 13 amino acids of H4 (SGRGKGGKGLGKC). The amount of loaded peptides in nanograms (ng) is shown below the blot. **(B)** Immunoblot analysis of whole cell extracts (WCE) prepared from HCT116 cells using the antibody against N-acH4. **(C)** Immunoblot analysis of siScramble and siNaa40-treated cells as well as purified histones extracted from calf thymus, using the antibodies against Naa40, N-acH4, total H4 and β-actin. Images are representative of at least three experiments.

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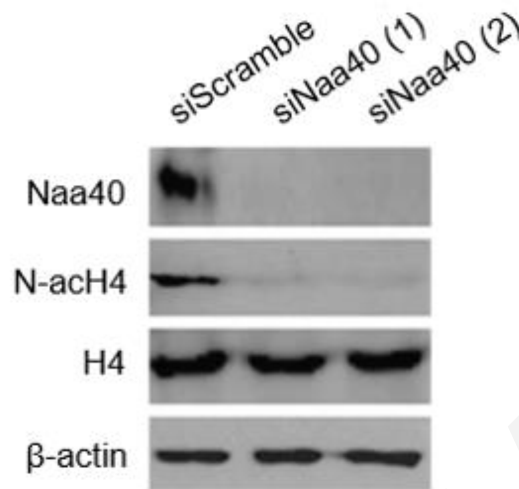

Figure 19. Naa40 knockdown reduces N-acH4 while H4 total levels remain unaltered. Immunoblot analysis using antibodies against Naa40, N-terminal acetylation of histone H4 (N-acH4), total histone H4 and β-actin (72 h). Image is representative of at least three experiments.

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4.2 Naa40 knockdown in HCT116 cancer cells reduces cell viability

Naa40 knockdown elicited a strong and profound phenotypic effect on HCT116 cells that was clearly observable under a phase contrast microscope. An extensive loss of viable cells was evident in the Naa40-siRNA transfected cell population compared to the scramble-siRNA control. This decreased viability was maintained throughout the 72 hr time course of the siRNA treatment. More specifically, the cells treated with the Naa40 siRNAs showed decreased colony formation and classic morphological characteristics of dead cells, including cellular rounding, shrinkage and detachment from plates (Figure 20 and 21A). On the other hand, control-treated cells kept proliferating, keeping their elongated shape. To quantify these observations we first counted adherent and non-adherent cells, representing the viable and dead cells respectively, in several microscopic fields of view. We found at least a 2-fold increase in the number of non-adherent cells upon depletion of Naa40 (Figure 21B), indicative of enhanced cell death. To validate these results, the MTT colorimetric assay was performed to quantify cell viability and proliferation capacity more accurately. As anticipated, cells that did not express Naa40 showed a significant reduction in survival compared to control cells. After 24h of siRNA treatment, Naa40 depleted cells showed a viability loss of 20% compared to control-treated

cells, while a greater reduction of cell survival was evident 72h post-transfection, reaching 40% in the Naa40-silenced cell population (Figure 21C). Overall, these results indicate that Naa40 is essential for HCT116 cell survival and possibly its role is critical for colon cancer progression.

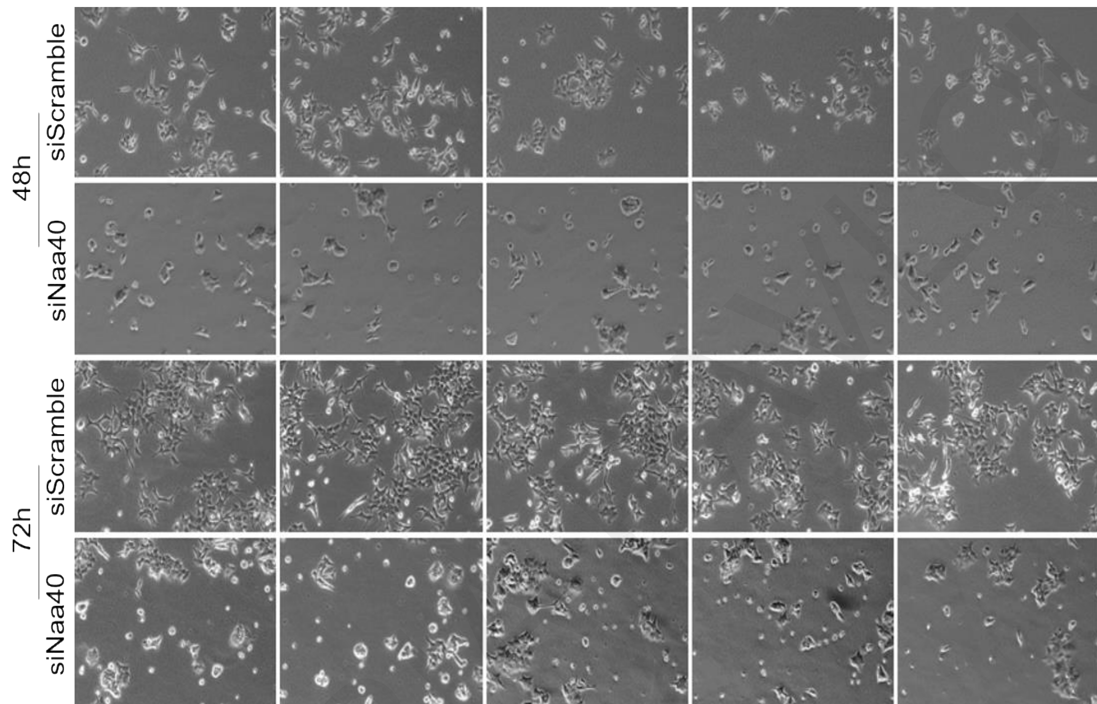

Figure 20. Naa40-kncokdown leads to distinct morphological changes associated with cell death. Phase contrast microscopy images of HCT116 cells shown at different time points (48h and 72h) after treatment with siScramble or siNaa40. The images are representative fields of view from at least three reproducible independent experiments.

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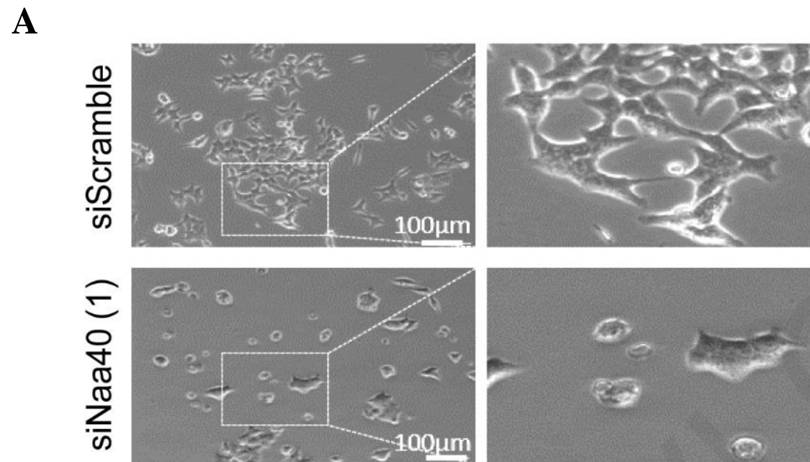

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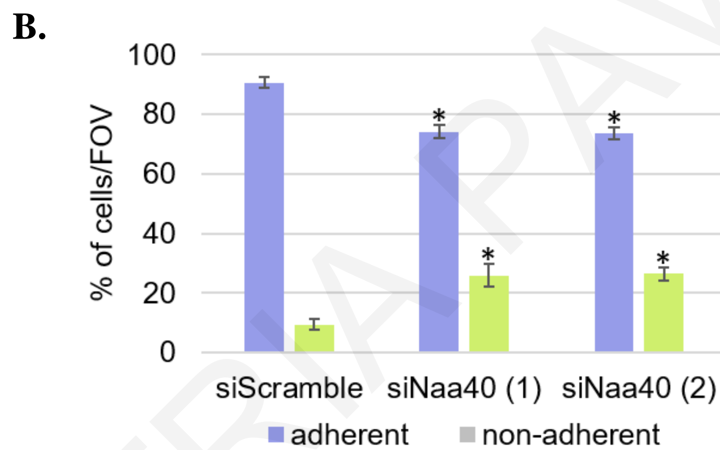

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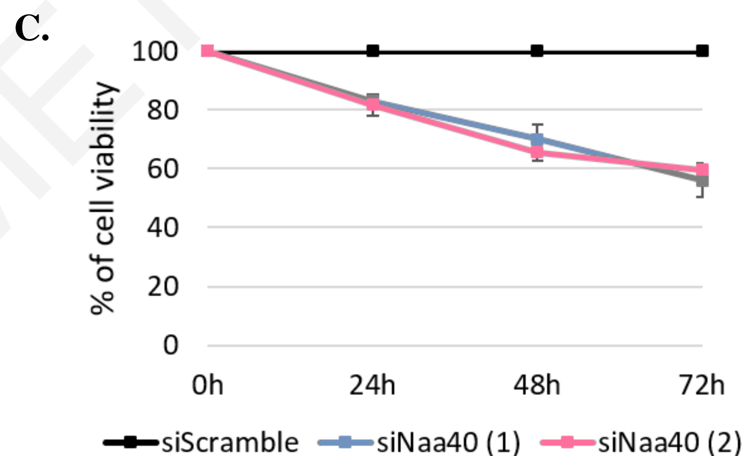

Figure 21. Naa40 depletion reduces cell survival in HCT116 cells. (A) Phase contrast microscopy analysis of scrambled siRNA and Naa40 siRNA treated cells (48 h) (scale bar 100 lm). Dashed rectangles represent the zoomed-in regions that are shown on the right panels. The images are representative fields of view from at least three independent experiments. **(B)** Quantitation of the percent of adherent and non-adherent cells per field of view, in siScramble and siNaa40 conditions (48 h). Data represent the mean number of 15 fields of view taken from three

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independent experiments ±SD (p value<0.05). **(C)** MTT cell viability assay. Cell viability is shown as a percentage relative to the scramble-siRNA control. The data represent the mean of three replicates and are representative of at least three different experiments ±SD (p value<0.02).

4.3 Silencing of Naa40 in HCT116 cells triggers apoptosis

It is widely acknowledged that deregulation of Nt-acetylation by NATs, is implicated in carcinogenesis. As mentioned before, reduced levels of Naa40 were observed in hepatocellular carcinoma, whereas the overexpression of the enzyme triggered apoptosis (Liu et al., 2009). Therefore, the significant decrease of viable cells in the Naa40-silenced cells (Figure 21B and 21C), raised the hypothesis that depletion of Naa40 in HCT116 colorectal cancer cells induces cell death, opposite to the observed phenotype in liver carcinoma. Additionally, this hypothesis is supported by the fact that the morphological changes observed in Figures 20 and 21A comprise a characteristic of apoptotic cells, which often occur due to loss of cytoskeletal and cell-adhesion proteins. Fibronectin is one such molecule that is implicated in cell adhesion and migration and has been shown to promote cell survival and metastasis (Han and Roman, 2006). Importantly, its reduction has been linked to loss of metastatic potential, loss of cell survival and initiation of apoptosis (Wu et al., 2005, Fornaro et al., 2003). Therefore, we explored the expression levels of fibronectin, as a possible indicator of cell death induction. Immunofluorescence assay (Figure 22A) and western-blot analysis (Figure 22B) revealed that fibronectin protein levels are indeed reduced upon Naa40 knockdown, supporting the idea that Naa40 loss initiates apoptosis. In addition, consistent with the reduction of the mesenchymal marker fibronectin and the observed cellular morphological changes, Hoechst staining shows that siNaa40-treated cells are more brightly stained compared to control cells. This staining difference between control and Naa40 depleted cells indicates nuclear degradation and condensation which are key features of apoptotic cells (Figure 22A). So far, the results point towards a Naa40-knockdown mediated apoptosis, as the major reason of cell viability loss.

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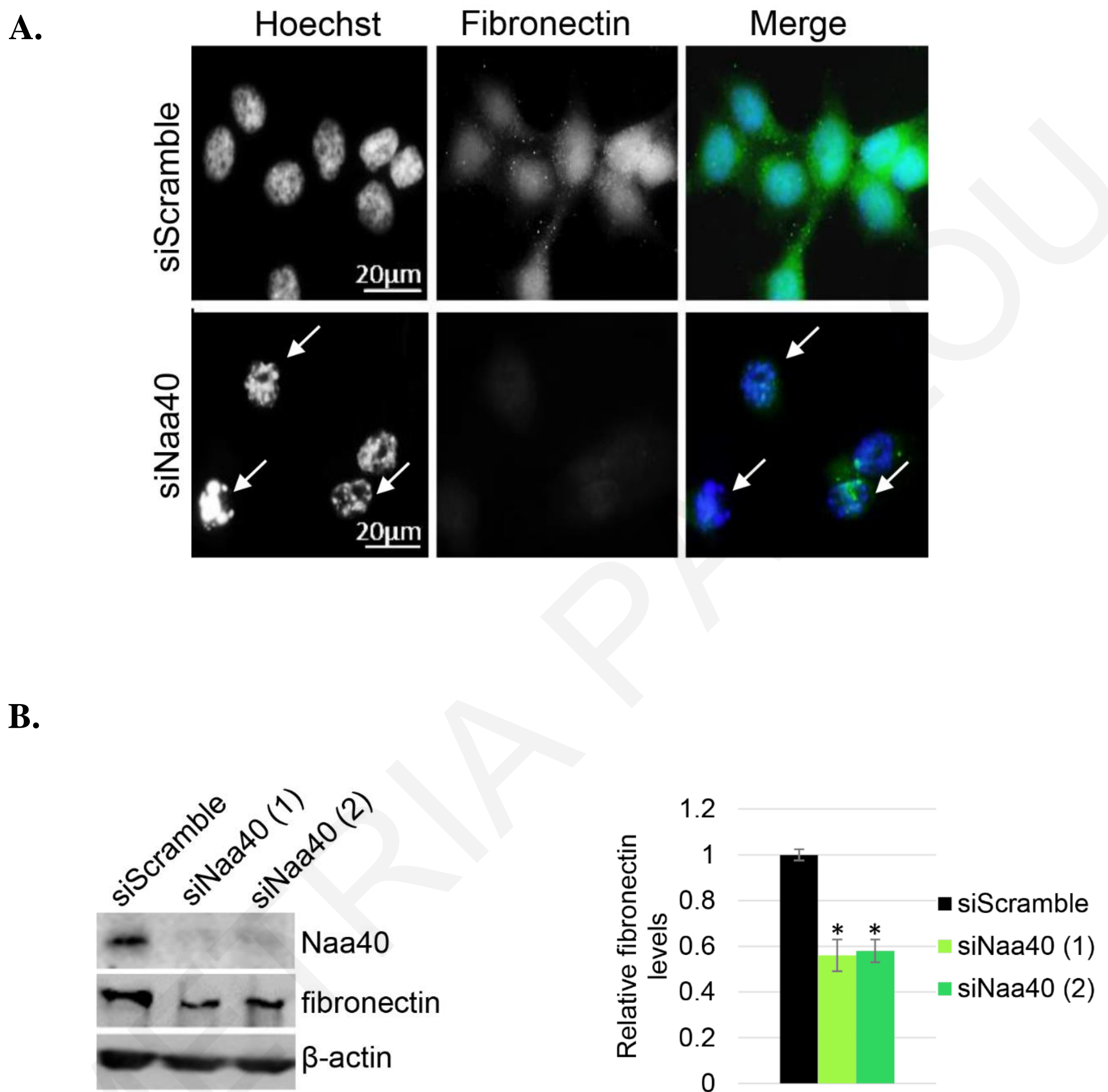

Figure 22. siRNA against Naa40 leads to fibronectin protein loss and nuclear fragmentation. **(A)** Representative immunofluorescence pictures of HCT116 cells transfected with Naa40-siRNA or a scramble-siRNA for 72 h. GreenFITC staining was used against fibronectin and blue Hoechst staining was used to visualise the nuclei. Arrows indicate apoptotic cells (scale bar 20 lm). **(B)** Immunoblot analysis of fibronectin levels in the absence of Naa40 (72 h post-transfection). β-actin was used as a control. Densitometry analysis (right panel) shows the levels of fibronectin in siNaa40 treatments relative to the scramble-siRNA sample. Images are representative of three independent experiments. Statistically significant changes (p value<0.02) are indicated by an asterisk (*). The data in (B) represent the mean of at least three replicates and are representative of at least three different experiments ±SD (p value<0.02)

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To further support our hypothesis that Naa40 knockdown reduces cell survival by triggering apoptosis, we proceeded with analysis of cell cycle progression. To distinguish cell cycle phases, siRNA treatment was first performed and cells were analysed after 24h, 48h and 72h, by flow cytometry. A significant increase of the SubG0/G1 fraction was observed early on, at the 24h timepoint, in the Naa40 treated cells (22.3%), compared to the corresponding fraction (4.7%) observed in the Scramble-treated cells (Figure 23A). This SubG0/G1 phase represents the apoptotic cell population and was progressively increased upon siNaa40 transfection compared to the siScramble control, reaching up to 38.7% of the total cell population, 72h post-transfection (Figure 23A). Finally, to corroborate the results of the cell-cycle analysis we also examined the induction of apoptosis by analysing siRNA transfected cells with dual staining for Annexin V and Propidium Iodide (PI). This approach, provides a more accurate assessment of cell death as its gives values of early apoptosis, late apoptosis or necrosis. At 24hr post siNaa40 transfection, a significant accumulation of early apoptotic cells was detected, while at 48h and 72h post-transfection a progressive increase in the late apoptotic/dead cell population was observed (Figure 23B). This sequential transition from early to late cell death, demonstrates that Naa40 depletion mediates apoptosis and excludes necrosis as the mechanism of cell death. The percentage of early versus late apoptotic cells remained approximately the same in the control sample throughout the time course of the treatment, confirming that apoptosis is specifically induced upon Naa40 knockdown (Figure 23B) and not due to off-target effects. Taken together, our data show that loss of Naa40 acetyltransferase induces programmed cell death through apoptosis in HCT116 colorectal cancer cells.

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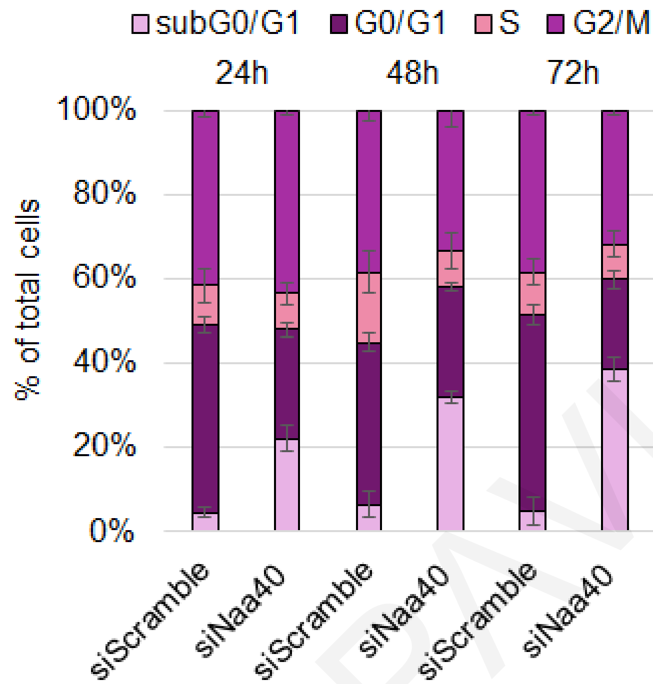

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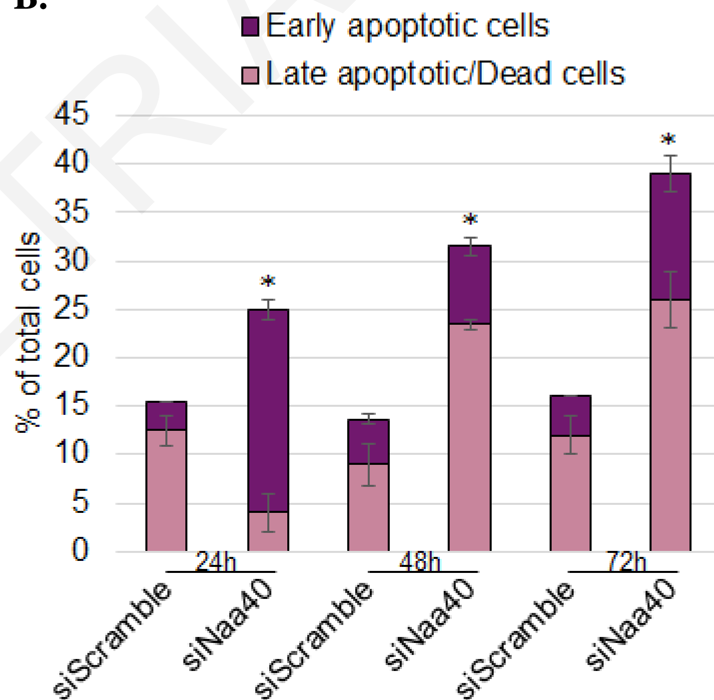

Figure 23. Depletion of Naa40 induces apoptosis in HCT116 cells. (A) Cell-cycle analysis of HCT116 cells after treatment with a scramble-siRNA or Naa40-siRNA for 24, 48 and 72 h. **(B)** Annexin V/PI staining was used to assess the apoptotic cell population. Statistically significant changes (p value<0.02) are indicated by an asterisk (*). The data in (A) and (B) represent the mean

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of at least three replicates and are representative of at least three different experiments ±SD (p value<0.02)

4.4 Naa40-knockdown mediated apoptosis is induced in colorectal cancer cells but not in non-malignant cells

All the above results, reveal that loss of Naa40 protein, provokes an apoptotic trigger in HCT116 cells. To determine whether induction of apoptosis in the absence of Naa40 is specific to HCT116 cells, we also used another highly metastatic, fast growing colorectal cancer cell line, HT-29, and non-cancerous mouse embryonic STO fibroblasts. Similarly to HCT116, Naa40 was depleted in HT-29 cells using the same specific and non-specific siRNA duplexes. As indicated in Figure 24A, the knock-down was again specific and efficient. MTT assay was then performed to assess cell viability. Depletion of Naa40 in HT-29 cells resulted in a significant reduction in cell proliferation and viability, reaching 40% at 72h post-transfection (Figure 24B). In contrast, when Naa40 was depleted in STO mouse embryonic fibroblasts (Figure 24A), no significant change on the percentage of viable cells was observed after 72h post-transfection compared to the control treatment (Figure 24B). To validate the results presented in HT-29 cells, cell cycle analysis was also performed. Naa40 depleted and control cells were collected 72h post-transfection and were subjected to flow cytometry, where cell cycle phases were distinguished. Notably, down-regulation of Naa40 resulted in an almost threefold increase of the subG0/G1 fraction when these cells were treated with the two separate siNaa40-RNAs (34.4% and 30.9% respectively) as compared to the siScramble (11.5%) control (Figure 24C). Altogether, these results suggest that colorectal cancer development or/and progression rely upon Naa40 and when the acetyltransferase is down-regulated, apoptosis can initiate. On the other hand, Naa40 depletion does not affect the viability of non-malignant cells, suggesting that Naa40 could represent a great candidate in targeted cancer therapies.

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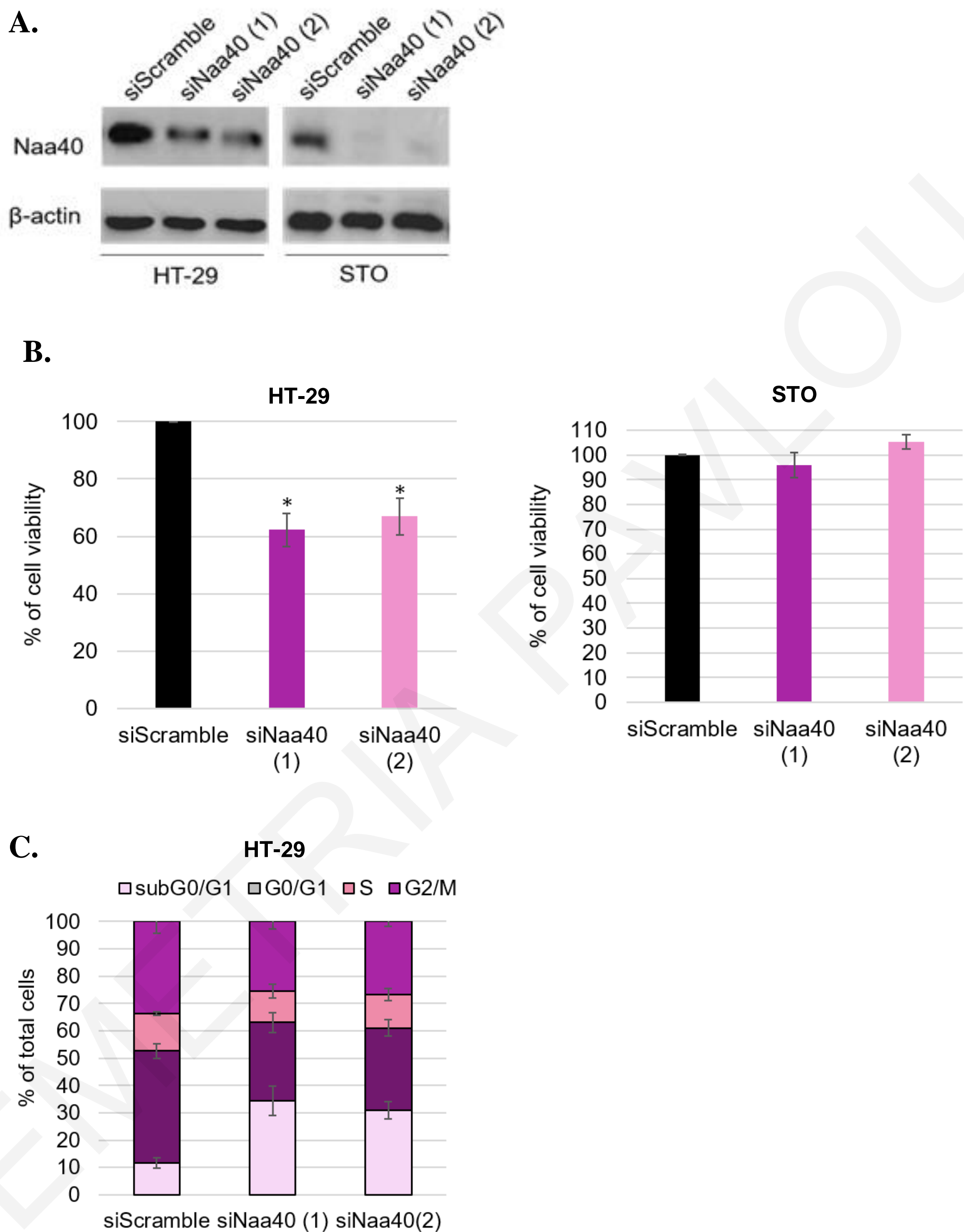

Figure 24. Depletion of Naa40 induces apoptosis in HT-29 colon cancer cells but not in STO mouse embryonic fibroblasts. (A) Immunoblot analysis of HT-29 and STO cells that were transfected with 25nM and 7.5nm, respectively, of Naa40 siRNA-1, Naa40 siRNA-2 or scramble siRNA and incubated 72h. Antibodies against Naa40 and β-actin, as a loading control, were used. **(B)** MTT cell viability assay. Cell viability of HT-29 cells (left panel) and STO cells (right panel) is shown as a percentage relative to the scramble-siRNA control. Statistically significant changes (p with a scramble-siRNA or Naa40-siRNAs, for 72h. **(C)** Cell-cycle analysis of HT-29 cells after treatment with a scramble-siRNA or Naa40-siRNAs, for 72h. The data in (B) and (C) represent the mean of three independent experiments ±S.D (p value <0.02).

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4.5 Naa40 knockdown-mediated apoptosis is caspase-mediated

The conserved molecular machinery that eliminates cells through the suicide mechanism, the so called apoptosis, can be triggered by various stress stimuli and tightly controlled by diverse pathways (Fulda et al., 2010). There are two major apoptotic routes that rely upon the activation of caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases); the central regulators of programmed cell death. The two pathways are the extrinsic or death receptor pathway and the intrinsic mitochondrial pathway, both extensively exploited in anticancer chemotherapies (Pommier et al., 2004). Although well distinguished, the mitochondria may also behave as the cross-talk organelles that can connect the two apoptotic pathways together.

To determine which apoptotic mechanism is induced by the depletion of Naa40 in colon cancer cells, we first investigated its effect on caspase activation. As illustrated in Figure 25A, caspases-8 and -9 represent the initiator caspases for the extrinsic and intrinsic pathway, respectively. Upon pro-apoptotic stimulus, the initiator caspases are activated by cleavage, which in turn cleave and activate common downstream effector caspases (caspase-3, -6, and -7). The cascade triggers at the end apoptosis, by cleaving cellular proteins, like PARP-1 (Elmore, 2007). Hence, the levels of cleaved executioner caspases and PARP-1 were examined in cells transfected with siRNAs during a 6 to 72 hour timecourse. By western blots, we observed the maximum reduction of Naa40 at 12h post-transfection which coincided with the appearance of the cleaved/activated forms of caspase-3 and caspase-7 (Figure 25B). From that timepoint and onwards, the active form of caspases (including caspase-6, which appeared 48h post-treatment), started to accumulate. As expected, the apoptotic PARP-1 fragment (89kDa) was detected soon after the cleavage of the executioner caspases (Figure 25B). On the other hand, cells transfected with the siScramble control did not show significant accumulation for any of the cleaved forms of caspases or PARP-1, suggesting that their activation is specific upon Naa40 depletion (Figure 25B). Hence, these results verify that lack of Naa40 expression in colon cancer cells leads to the activation of apoptosis and reveal the implication of caspases within this process.

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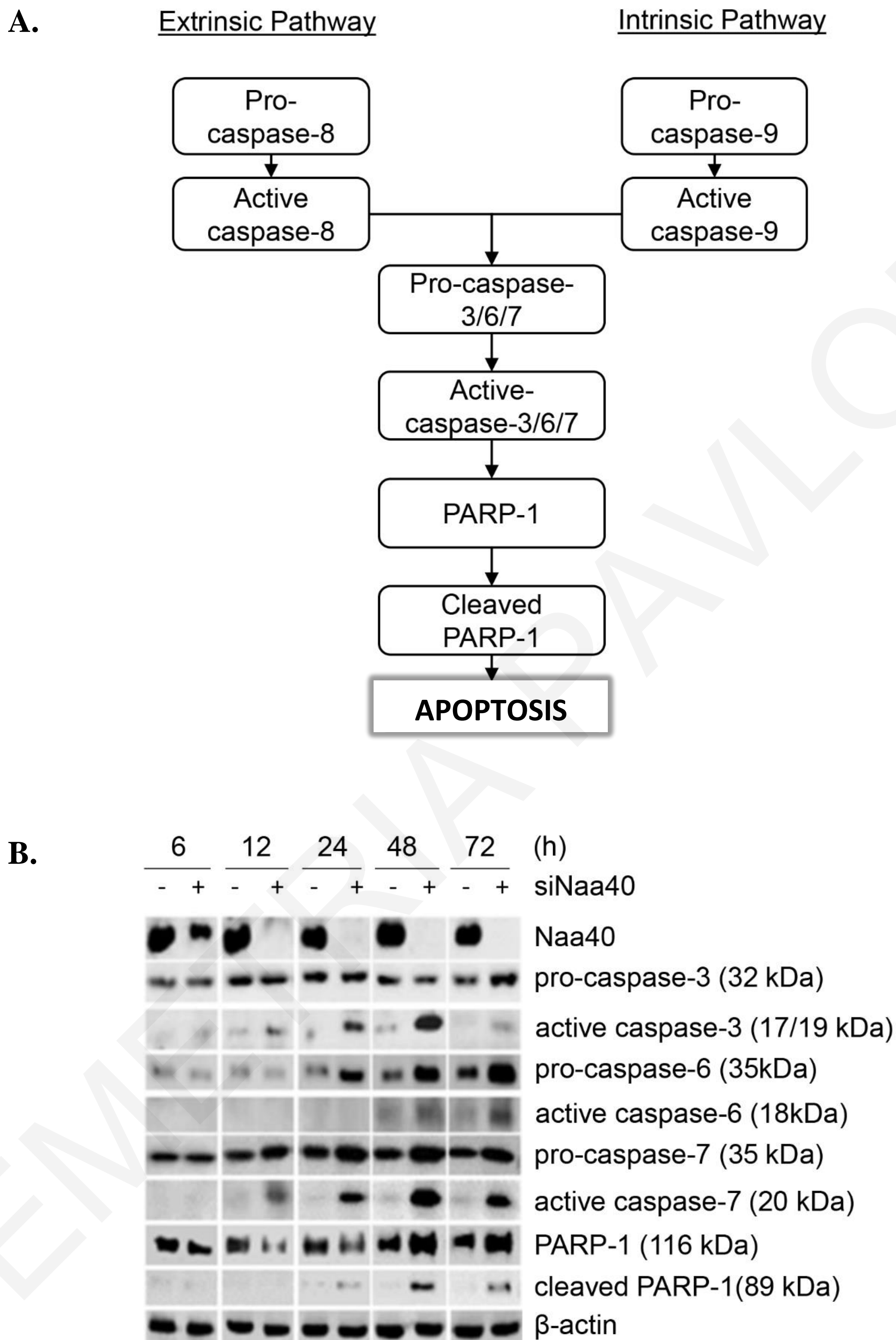

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Figure 25. Depletion of Naa40 stimulates apoptosis through a caspase-dependent mechanism. **(A)** Simplified schematic illustrating caspase-dependent apoptotic pathways. The signalling cascade could be induced by either the extrinsic or intrinsic apoptotic pathway through cleavage of the initiator caspase-8 or casapse-9, respectively. In turn, these caspases activate common executioner caspases (caspases-3/6/7) that subsequently cleave PARP-1 and lead to apoptosis. **(B)** Immunoblot analysis of scramble-siRNA (-) or Naa40-siRNA (+) transfected HCT116 cells during a timecourse of 6–72 h. Antibodies were used against Naa40, caspase-3, -6, -7, PARP-1 and β-

actin, which served as a loading control. The image is representative of at least three independent experiments.

4.6 Induction of apoptosis in Naa40-depleted cells requires activation of the mitochondrial/intrinsic pathway

Since the effector caspases and PARP-1 are found downstream of both the intrinsic and extrinsic apoptotic pathways (Figure 25A), we next sought to determine which pathway is specifically activated when Naa40 is depleted. Upon death stimuli, it is known that the pro-apoptotic Bax protein translocates from the cytosol to the mitochondrial membrane where it homodimerises. This leads to the formation of pores in the outer mitochondrial membrane through which pro-apoptotic molecules escape (Oltvai et al., 1993, Youle and Strasser, 2008). Antagonistically, Bcl-2 anti-apoptotic protein, inhibits the dimerization of Bax and thus, the activation of mitochondrial pathway is prohibited. Therefore, the Bax/Bcl-2 ratio determines the susceptibility of cells to mediate mitochondrial apoptosis. Considering the abovementioned, we examined the protein levels of Bax and Bcl-2 in siRNA transfected cells. In Naa40 depleted cells, we detected an increase in the levels of the pro-apoptotic factor Bax and a decrease in the levels of the anti-apoptotic protein Bcl-2 (Figure 26A). Additionally, the ratio between Bax/Bcl-2 levels was gradually increasing until 24hr after siNaa40 transfection and remained more than double for 72h (Figure 26B), implicating the mitochondrial pathway in the induction of apoptosis.

Induction of the mitochondrial pathway does not necessarily exclude the activation of the extrinsic apoptotic route. Therefore, to determine whether the mitochondrial pathway is the only pathway activated upon Naa40 down-regulation, we next examined the cleavage of the initiator caspases-8 and -9 in HCT116 cells, which, respectively, stimulate specifically the extrinsic and intrinsic apoptotic pathways (Figure 25A). After siRNA treatment, whole cell extracts were subjected to Immunoblot analysis. As Figure 26C shows, caspase-8 cleavage was not detected at any timepoint throughout the siRNA treatment, indicating that the extrinsic pathway was not induced upon Naa40 knockdown. In contrast, caspase-9 cleavage was detected in Naa40 depleted cells but not in the control samples. Furthermore, the active caspase-9 was primarily observed 12h post-transfection, coinciding with the cleavage of the effector caspases (compare Figure 25B and 26C). Importantly, caspase-9 activation was also observed in HT-29 cells after Naa40 depletion (Figure 26D). Altogether, these results suggest that the mitochondrial pathway could be the main route through which Naa40 knockdown promotes programmed cell death.

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To conclusively demonstrate the requirement of the mitochondrial pathway for the induction of apoptosis by Naa40 knockdown, we monitored apoptosis of Naa40-depleted HCT116 cells in the presence of the irreversible caspase-9 inhibitor, z-LEHD-fmk. As western blot results indicate, incubation of HCT116 cells with z-LEHD-fmk prevented the cleavage of caspase-9 after depletion of Naa40 (Figure 27A), as expected. Notably, cells depleted of Naa40 and siScramble transfected cells showed similar morphology and growth under the microscope in the presence of the caspase-9 inhibitor, suggesting that apoptosis induced by Naa40 knockdown was prevented (Figure 27B and 27C). Quantification of cells from different microscopic fields of view also showed that Naa40 depletion in the presence of the caspase-9 inhibitor does not increase the number of non-adherent cells (7.2%) relative to the siScramble control (8%), but a significant increase is observed, as expected, in the siNaa40 only treatment (21%) (Figure 27D). The lack of siNaa40-induced apoptosis in the presence of the caspase-9 inhibitor was further verified by cell cycle analysis. Treatment with the caspase-9 inhibitor reduced significantly the apoptotic subG0/G1 cell population (from 25.95% to 12.8%) and restored the G1-phase fraction in the Naa40 depleted cells (from 25% to 35%) (Figure 27E). Taken together, these results demonstrate that activation of caspase-9 and thus, the mitochondrial pathway are essential for the induction of apoptosis in colon cancer cells depleted of Naa40.

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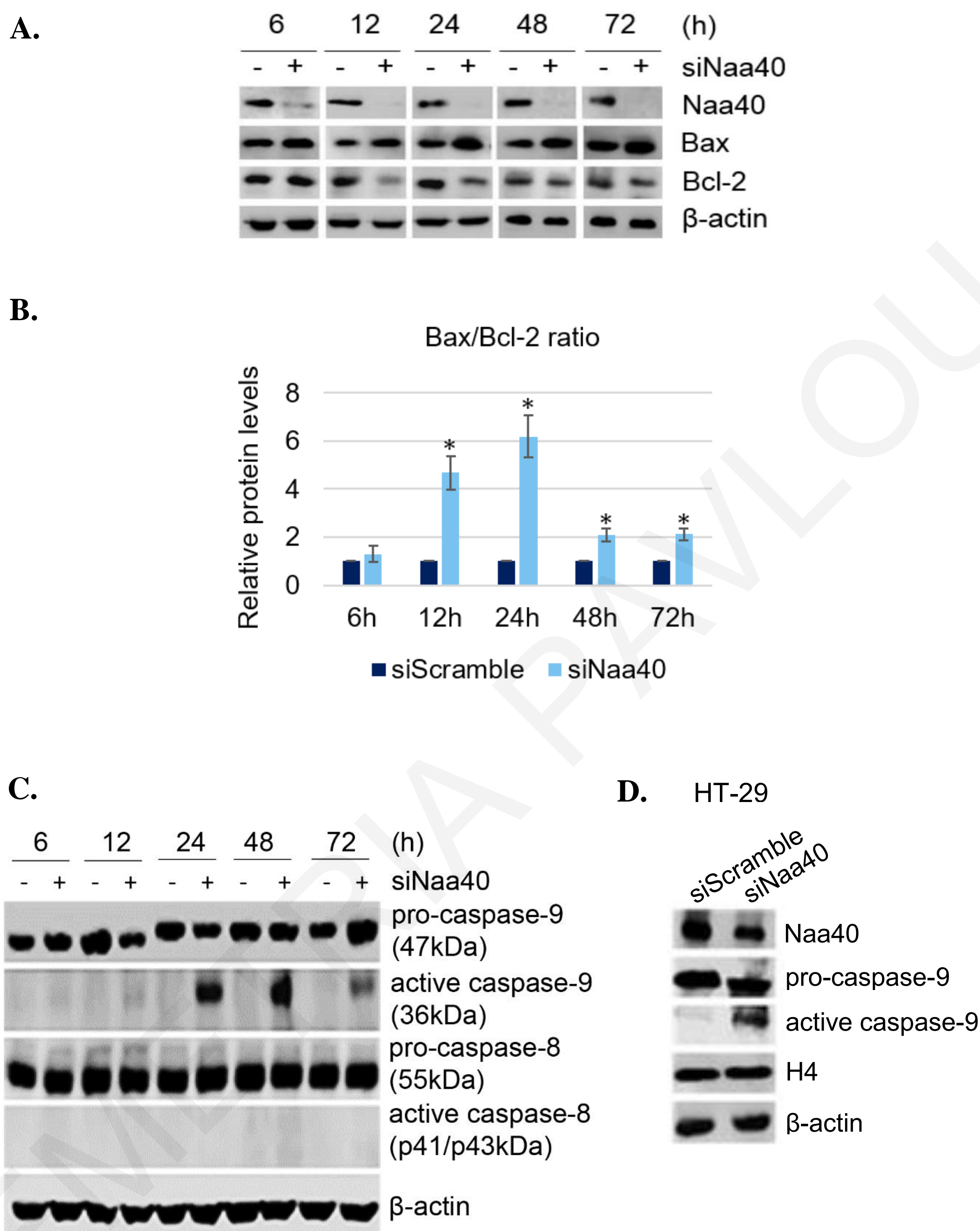

Figure 26. Naa40-knockdown induces the mitochondrial apoptotic pathway through activation of caspase-9. (A) Immunoblot analysis of untransfected (-) or Naa40-siRNA (+) transfected HCT116 cells during a timecourse of 6–72 h using antibodies against Naa40, Bax, Bcl-2 and β-actin, as a loading control. **(B)** Quantitative densitometry showing the ratio between Bax and Bcl-2 protein levels according to western blots in (A). Values represent the mean of three independent experiments and the asterisk (*) indicates statistically significant changes (p value <0.02). **(C)** Immunoblot analysis as in (A) using antibodies against caspase-9, -8 and β-actin. **(D)** Immunoblot analysis of siScramble and siNaa40-treated cells (72h) using antibodies against Naa40, caspase-9, total H4 and β-actin that was used as a loading control. Western blot images in (A), (C) and (D) are representative of three independent reproducible experiments.

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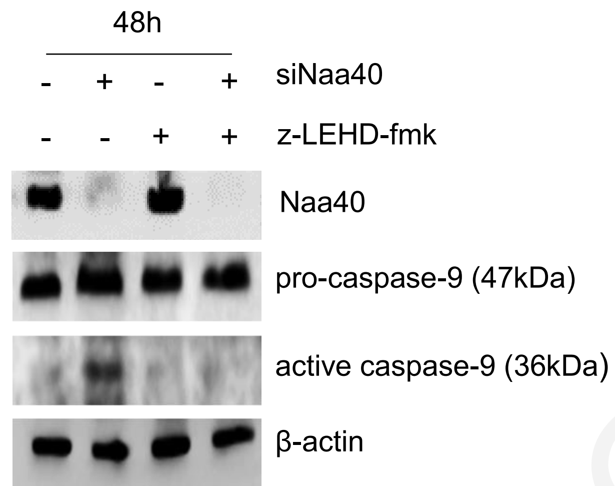

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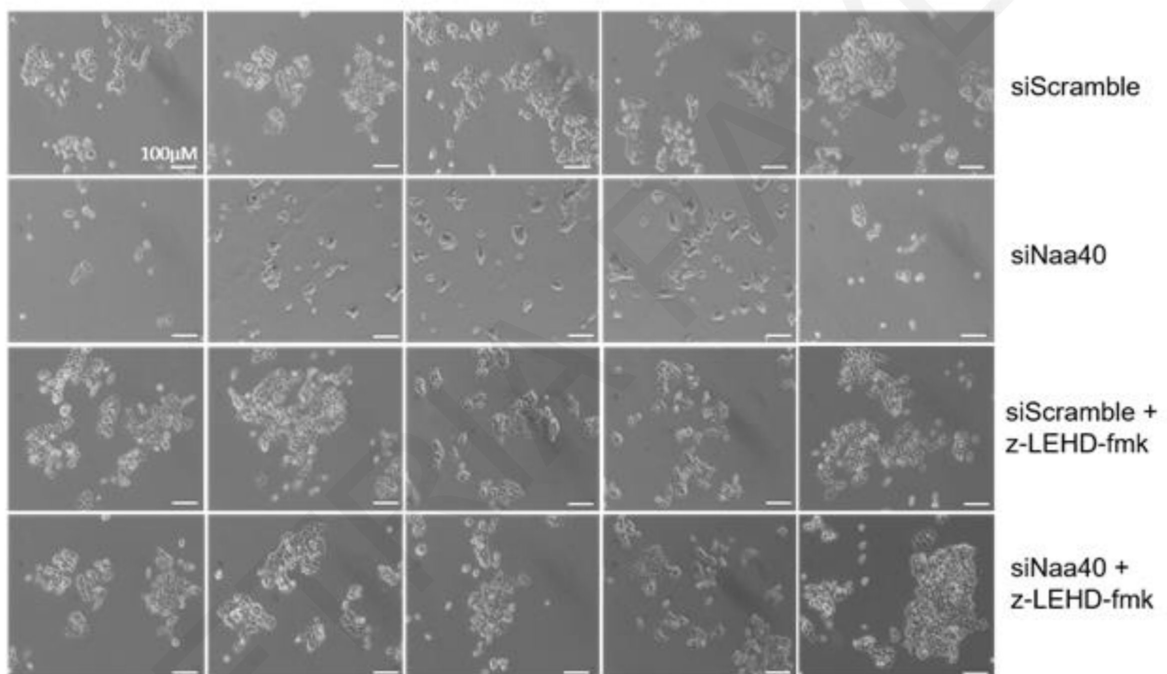

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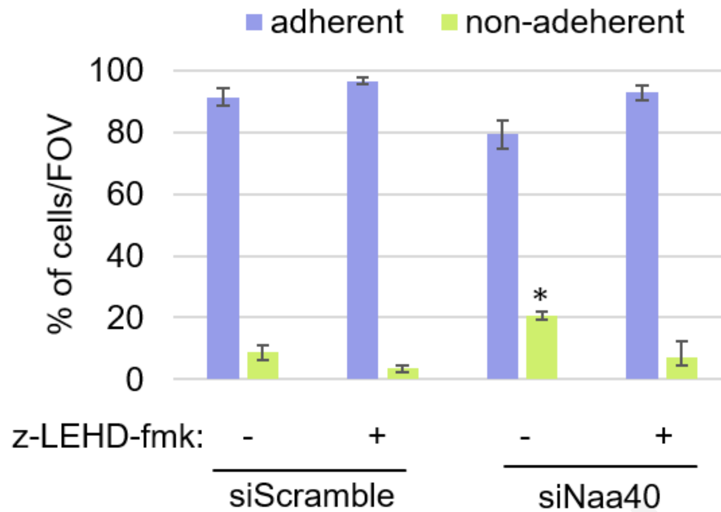

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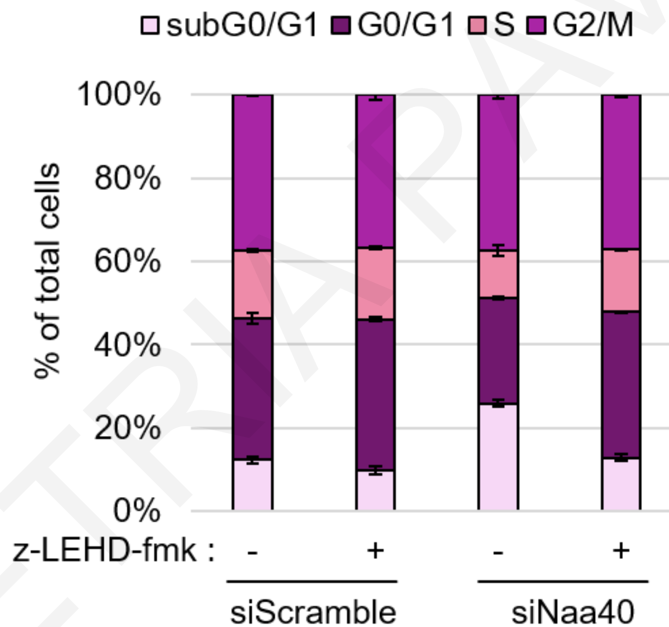

Figure 27. Caspase-9 inhibitor prevents loss of cell viability in Naa40-depleted HCT116 cells. (A) Immunoblot analysis of HCT116 cells transfected with siScramble (-) or siNaa40 (+) in the presence (+) or absence (-) of caspase-9 inhibitor z-LEHD-fmk. Whole cell lysates were collected 48 h post-transfection and assayed using antibodies against caspase-9 and β-actin. Western blot image is representative of three independent reproducible experiments. **(B)** Phase contrast microscopy images, 48h post-siRNA treatment. HCT116 cells are treated as in (A). The images are representative fields of view from at least three reproducible independent experiments (scale bar 100 μm). **(C)** Representative phase contrast images from at least three independent experiments showing HCT116 cells treated as in (A) for 48 h (scale bar 100 μm). Dashed rectangles represent zoomed-in regions that are shown on the right panels. **(D)** Quantitation of adherent and non-adherent cells per field of view, in the conditions mentioned in (A). Data represent the mean number of 15 fields of view taken from three independent experiments ±S.D (p value <0.02). **(E)** Cell cycle analysis of cells treated as in (A) for 48 h. Values represent the mean of three independent experiments ±SD (p value<0.02).

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4.7 Depletion of Naa40 promotes apoptosis in a p53-independent manner

The p53 tumor suppressor holds distinction as the most inactivated gene in human cancers, including colon cancer (Rivlin et al., 2011, Liu and Bodmer, 2006). With a significant and complex implication in the maintenance of cellular genomic integrity by inducing repairing mechanisms or by triggering cell death, p53 is of great biological and clinical importance for almost four decades now (Levine and Oren, 2009). p53 kills cells predominantly via the mitochondrial apoptotic pathway (Johnstone et al., 2002, Mihara et al., 2003) since it transcriptionally activates proapoptotic BH3 genes, like Bax and Puma. In addition, p53 induces mitochondrial apoptosis by transcription-independent signalling, where it can directly induce permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-XL and Bcl-2 proteins, resulting in cytochrome c release (Mihara et al., 2003). In conditions where active p53 is not required, MDM2 ubiquitin ligase targets the protein for proteosomal degradation (Shi and Gu, 2012). Therefore, having p53 as the major suspect for the induction of apoptosis in the Naa40 depleted cells, we compared the p53 protein levels in cells transfected with siNaa40 and siScramble control. Surprisingly, we found that Naa40 depletion in HCT116 cells had no effect on p53 protein levels, as the signal obtained in western blots was the same between the siNaa40 and siScramble treatments (Figure 28A). This result raised the possibility that p53 is not involved in the apoptotic pathway induced by Naa40 knockdown.

To look into this possibility, we utilised the HCT116 p53 null isogenic cell line (HCT116 p53-/-), in which the *p53* gene has been deleted by homologous recombination (Bunz et al., 1998) (Figure 28B). RNAi against Naa40 was performed and cell proliferation/viability and apoptosis induction were examined (Figure 28C). We observed that lack of p53 in HCT116 cells did not impede the effects of Naa40 depletion. After 72h of siNaa40 treatment using two distinct siRNAs there was about a 40% reduction of viable cells (Figure 28C) and approximately 35% increase of the subG0/G1 cellular fraction, representing the apoptotic population (Figure 28D). Notably, the percentage of cells that undergo apoptosis in HCT116 p53-/- cells upon Naa40 depletion is similar to the apoptotic fraction observed in p53+/+ HCT116 cells (compare Figure 21C with 28C and Figure 23A with 28D), suggesting that the Naa40-knockdown apoptotic induction is irrelevant of the p53 status. The above result is also supported by the fact that depletion of Naa40 elicits a significant induction of apoptosis in HT-29 highly metastatic colon cancer cell line (Figure 24B, 24C and 26D), which harbours a defective p53 protein due to a missense mutation in

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the DNA-binding domain of the *p53* gene (He et al., 2015). Altogether, our data show that p53 function is not required for the apoptosis induced by Naa40 knockdown.

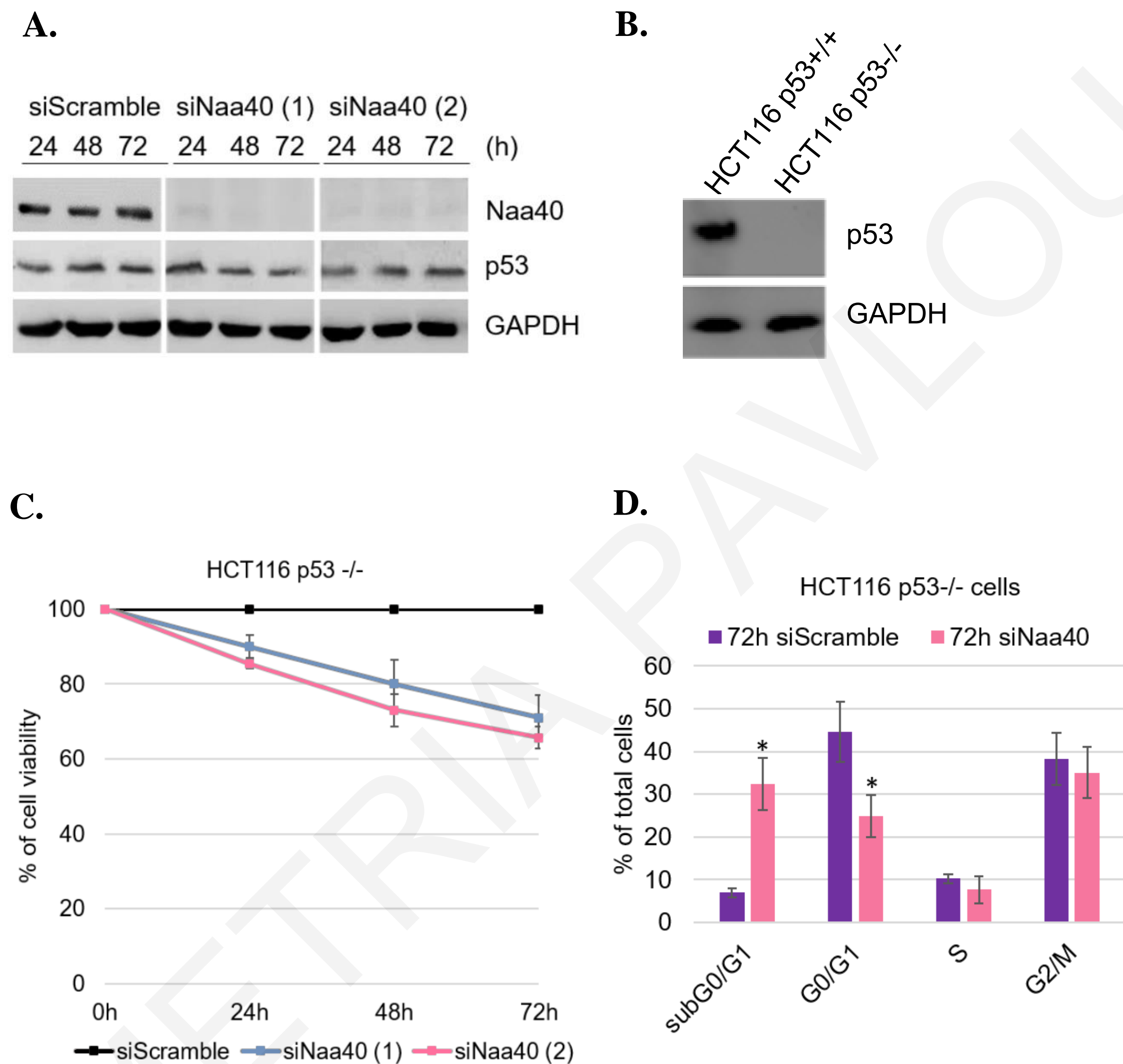

Figure 28. Naa40-knockdown induces apoptosis in HCT116 cells independently of p53 status. (A) Immunoblot analysis of whole cell lysates prepared from HCT116 cells transfected with scramble-siRNA or Naa40-siRNAs for 24, 48 and 72 h using antibodies against p53, Naa40 and GAPDH, as a loading control. Representative western blot is shown from three independent experiments. **(B)** Immunoblot analysis of HCT116 p53+/+ and HCT116 p53-/- whole cell extracts against p53 protein. GAPDH was used as a loading control. **(C)** MTT cell viability assay was performed in p53 null cells (HCT116 p53-/-) transfected with scramble-siRNA or Naa40-siRNAs for 24, 48 and 72 h. Cell viability is shown as a percentage relative to the siScramble control. **(D)** Cell-cycle analysis of HCT116 p53-/- cells treated with a scramble-siRNA or Naa40-siRNA for 72 h. Error bars indicate standard deviation of triplicate experiments ±SD (p value<0.02). Statistically significant changes (p value<0.02) are indicated by an asterisk (*).

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CHAPTER 5

RESULTS OF AIM 2

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5. Unveiling the role of histone N-terminal acetyltransferase Naa40 in gene regulation

Protein N-terminal acetylation is a prevalent modification of eukaryotic proteins and is specifically catalysed by Nt-acetyltransferases (NATs). N-terminal acetyltransferase Naa40, in contrast to other NATs that have multiple protein substrates, has been shown to catalyse the acetylation of histones H4 and H2A, only. This suggests that Naa40 has a distinct and specialised role in the control of chromatin structure and thus, gene expression. So far, the molecular mechanism by which N-terminal histone acetyltransferase Naa40 impacts on gene regulation remains unknown. In yeast, the role of the yNaa40 in the control of heterochromatic rDNA region has just recently been disclosed (Schiza et al., 2013), throwing for the first time light in the way through which Naa40 and histone N-terminal acetylation regulates gene expression. Therefore, I was interested to investigate the function of Naa40 in human cells, in an attempt to provide insights into the mechanisms by which histone N-terminal acetylation mediated by Naa40 regulates gene expression.

5.1 Knockdown of the human Naa40 reduces rRNA expression

Work from our lab exposed the novel role of histone H4 N-terminal acetylation (N-acH4) and its associated acetyltransferase Naa40 (yNaa40) in the control of ribosomal RNA (rRNA) expression, in *Saccharomyces cerevisiae*. More specifically, rDNA expression was shown to be dependent on yNaa40 activity towards histone H4, as deletion of the yNaa40 enzyme, resulted in enhanced rRNA silencing. In order to investigate whether Naa40 also regulates the expression of the ribosomal RNAs in human cells, the RNAi technique against the enzyme was employed in HCT116 colorectal cancer cells.

Ribosomal DNA is one of the most well-characterized repetitive arrays and is made up of genes that are transcribed into the building components of the ribosome (Long and Dawid, 1980). The human rDNA is organised in tandem repeats each being 43kb long and containing a promoter region, distributed on chromosomes 13p12, 14p12, 15p12, 21p12, and 22p12 (Figure 29) (Henderson et al., 1972). Transcription by RNA polymerase I gives rise to the 45S primary transcript, which is then processed to give the mature 18S, 5.8S and 28S rRNAs (Stults et al., 2008, Paule and White, 2000). To explore the effect of Naa40 towards rRNA expression, the levels of 45S precursor-rRNA, 18S, 5.8S and 28S rRNA in

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the two cell populations, siScramble and siNaa40, were analysed by quantitative RT-PCR, at 24h and 72h post-transfection. Depletion of Naa40 resulted in a great decrease in the expression levels of all rRNA transcripts compared to control treated cells and this reduction was consistent in both Naa40-siRNA treatments (Figure 30). Since the newly forming 45S transcript is rapidly processed to yield the mature 18S, 5.8S and 28S rRNAs, the observed changes in the 45S levels most likely reflect a decrease in transcription. These results suggest that human Naa40, just like its yeast homolog, is important for the regulation of ribosomal RNA expression, since loss of the enzyme leads to rDNA silencing.

To explore whether Naa40 affects directly the expression of the rRNA, ChIP assays were performed 72h post-siRNA transfection, using primers that anneal either at the 45S rDNA promoter locus or at the 28S rDNA (Figure 31). Loss of Naa40 resulted in a significant reduction of N-acH4 levels at the nucleosomes residing at both loci, indicating that rDNA array is a direct target of this histone N-terminal acetyltransferase. In addition, the results show that H4 N-terminal acetylation is necessary for rDNA transcriptional activation. The higher acetylation levels observed in the enhancer/promoter region of the rDNA locus, may suggest that this mark mainly accumulates there to enhance and initiate transcription. Therefore, these findings propose that attenuation of Naa40 inhibits the expression of the pre-45S, which in turn affects the abundance of the downstream mature rRNAs.

Altogether, our results reveal the conserved function of human Naa40 in the control of the rDNA locus, throughout eukaryotic evolution. It is worth mentioning that tumor cells are known to markedly overexpress rRNA species (Uemura et al., 2012, Ghoshal et al., 2004, Drygin et al., 2010, Ruggero and Pandolfi, 2003, White, 2005, Williamson et al., 2006). Taking this fact into consideration, together with the newly identified anti-apoptotic activity of Naa40 in colon cancer cells (Chapter 3), it can be deduced that this role of Naa40 upon rRNA expression could be responsible for the loss of cancer cell proliferative potential and viability.

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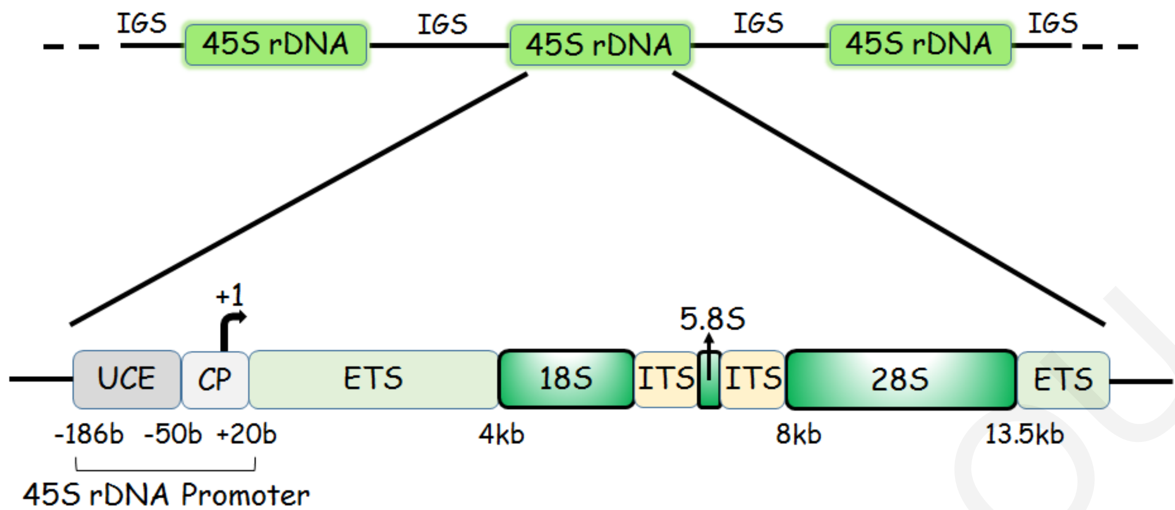

Figure 29. Schematic diagram of the human rDNA locus. The precursor-rRNA genes (45S) are arranged in long tandem arrays separated by regions of non-transcribed intergenic spacers (IGS). The 45S rDNA promoter region consists of the upstream control element (UCE) and core promoter (CP). Transcription start site is indicated by a horizontal arrow and 18S, 5.8S and 28S rRNAs are produced from the RNA Pol I precursor transcript. The mature sequences for 18S, 5.8S and 28S are embedded in noncoding 5′ and 3′ external transcribed spacers (ETS) and internal transcribed spacers (ITS).

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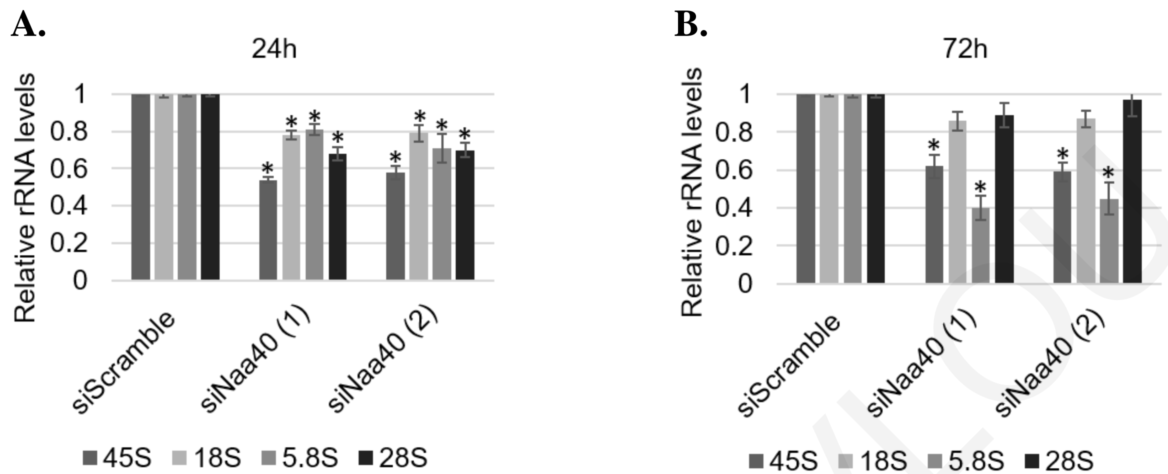

Figure 30. Depletion of Naa40 reduces rRNA expression in HCT116 cells. Expression levels of rRNAs 45S, 18S, 5.8S and 28S were analyzed by qRT-PCR using total RNA extracted from siScramble and siNaa40-treated samples, **(A)** 24h and **(B)** 72h post-transfection. The levels of rRNA were quantified and then normalised to the levels of β-actin mRNA, whose expression remained unchanged. The data represent the mean of three replicates and are representative of three independent experiments ±S.D (p value <0.02).

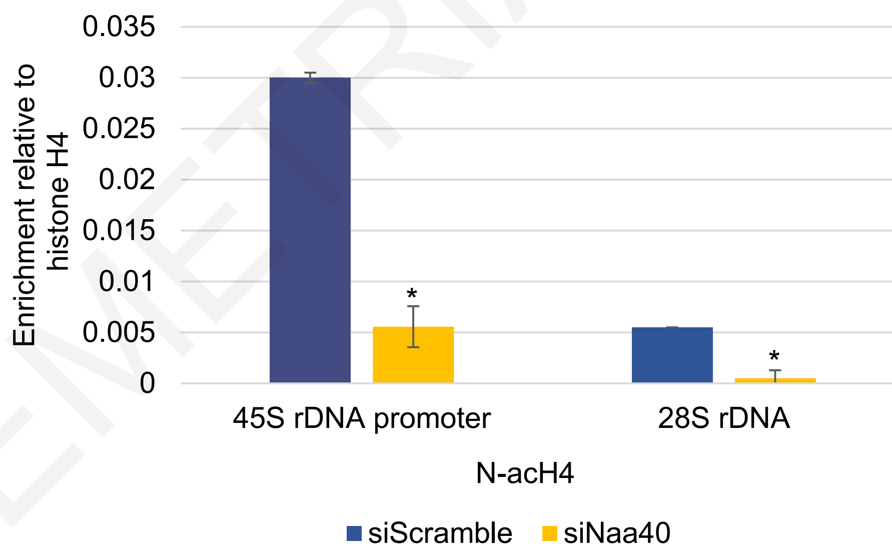

Figure 31. Naa40 knockdown down-regulates the deposition of arginine methyl-marks, in *cis*. ChIP assays performed in siScramble and siNaa40 HCT116 samples, using antibody against N-acH4 (H4S1ac). The immunoprecipitated chromatin was analysed by RT-PCR using primers against 45S rDNA promoter and 28S rDNA, 72h post-treatment. The enrichment was normalized to the levels of histone H4. Values represent the mean of duplicate experiments and the asterisk (*) indicates statistically significant changes (p value <0.02).

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5.2 The effect of Naa40-knockdown towards the expression of genes implicated in cell survival

The current research project has linked Naa40 down-regulation with the induction of p53-independent apoptosis in colon cancer cells. As shown in Chapter 3, activation of the initiator caspase-9 upon Naa40 knockdown resulted in a downstream initiation of a cascade of events, including the activation of the executioner caspases-3, -6 and -7, and the cleavage of PARP-1 substrate (Figure25B and 26C). Caspase-8 cleavage was not observed (Figure 26C) suggesting that cell death was induced through the intrinsic/mitochondrial apoptotic pathway. Consistent with these results, the use of a caspase-9 inhibitor abolished apoptosis (Figure 27), revealing that the activation of the mitochondrial apoptotic pathway is essential for the induction of programmed cell death, in Naa40 depleted cells.

5.2.1 Pro-caspase expression is not controlled by Naa40 activity

We have observed that in addition to the accumulation of the active cleaved form of caspases (Figure 25B and 26C), Naa40 depletion also enhances the cellular levels of pro-caspases. Overexpression of pro-caspases has been previously reported to represent a common molecular mechanism for stimulating apoptosis. Specifically, it was suggested that up-regulation of pro-caspases results in sufficient amounts of these precursors within the cell which are required to precede and further complete the apoptotic process (Sabbagh et al., 2004, Droin et al., 1998, Druskovic et al., 2006). We have also observed an upregulation of full-length PARP-1 at the later time-points (48h and 72h) of the siNaa40 treatment (Fig. 25B). Interestingly, expression of full-length PARP-1 has been linked with induction of cell death as a response to excessive DNA damage (Ethier et al., 2007, Sousa et al., 2012), which is probably taking place within HCT116 apoptotic cells after 48h and 72h of siNaa40 treatment.

The observed increase in the levels of pro-caspases and PARP-1 raised the possibility that depletion of Naa40 and perhaps its associated N-acH4 modification might regulate their expression at the transcriptional level. To test this possibility, RT-PCR analysis was performed 24h and 72h post-transfection to quantify the mRNA levels of all pro-caspases and PARP-1. As shown in Figure 32, the mRNA levels of all these proteins, with the exception of pro-caspase-7, did not change in the absence of Naa40 when compared to the siScramble control treatment. Consequently, it is inferred that depletion of

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Naa40 and thus, loss of histone N-terminal acetylation do not directly affect the promoter of these genes and point towards post-transcriptional or post-translational rather than transcriptional regulation. Moreover, the upregulation of caspase-7 could also represent an indirect effect of Naa40 knockdown, as histone N-terminal acetylation was previously shown to be linked with the activation of genes (Schiza et al., 2013) and therefore, loss of this modification most possibly would have led to the down-regulation of caspase-7 gene expression.

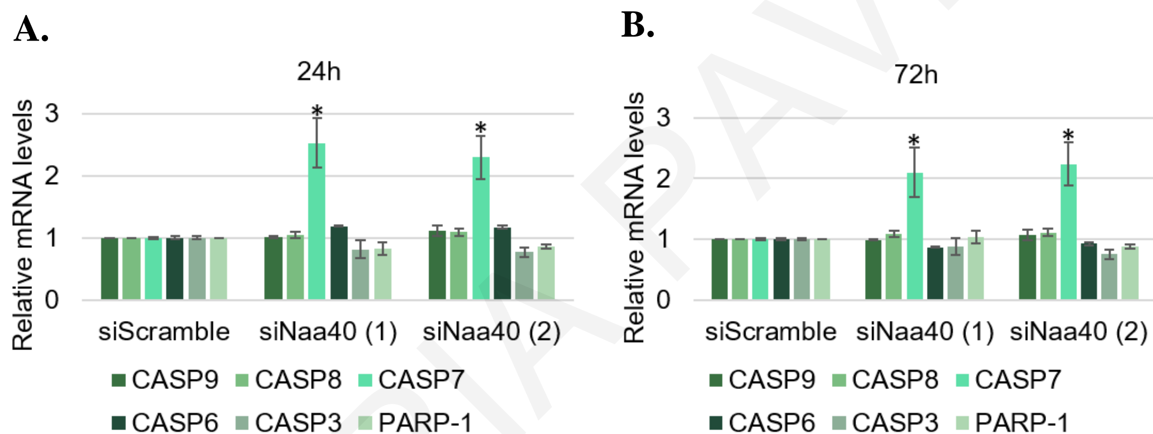

Figure 32. The effect of Naa40 depletion on the mRNA levels of pro-caspases and PARP-1. RT-PCR analysis of the mRNA levels of caspase-9, -8, -7, -6, -3 and PARP-1, **(A)** 24h and **(B)** 72h post-transfection. The results are normalised to the levels of β-actin mRNA. The data represent the mean of three replicates and are representative of three independent experiments ±S.D (p value <0.02).

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5.2.2 Naa40 depletion leads to fibronectin and vimentin transcriptional down-regulation

Our previous results show that fibronectin protein levels are down-regulated in the apoptotic siNaa40 treated cell population (Figure 22). Therefore, in order to examine whether Naa40 depletion affects fibronectin at the transcriptional level, we performed RT-PCR in cells that were treated either with the Scramble- or the Naa40-siRNAs, 24h and 72h post-treatment (Figure 33). The mRNA levels of vimentin were also analysed, as its expression has been linked to epithelial-mesenchymal transition of cells, induced cell growth, migration and metastasis whereas its down-regulation promotes mitochondrial apoptosis (Byun et al., 2001, Mendez et al., 2010, Nodale et al., 2012). The mRNA levels of both proteins were significantly decreased 24h post-siRNA transfection and were further down-regulated as treatment progressed (48h and 72h). These results support that N-acH4 by Naa40 could play a direct role in the transcriptional regulation of fibronectin and vimentin. More specifically, it can be assumed that N-acH4 of the two genes leads to the decompaction of chromatin which will allow the transcriptional machinery to take place and induce their expression. Furthermore, our data exclude protein degradation as the main mechanism for their down-regulation during siNaa40-induced apoptosis.

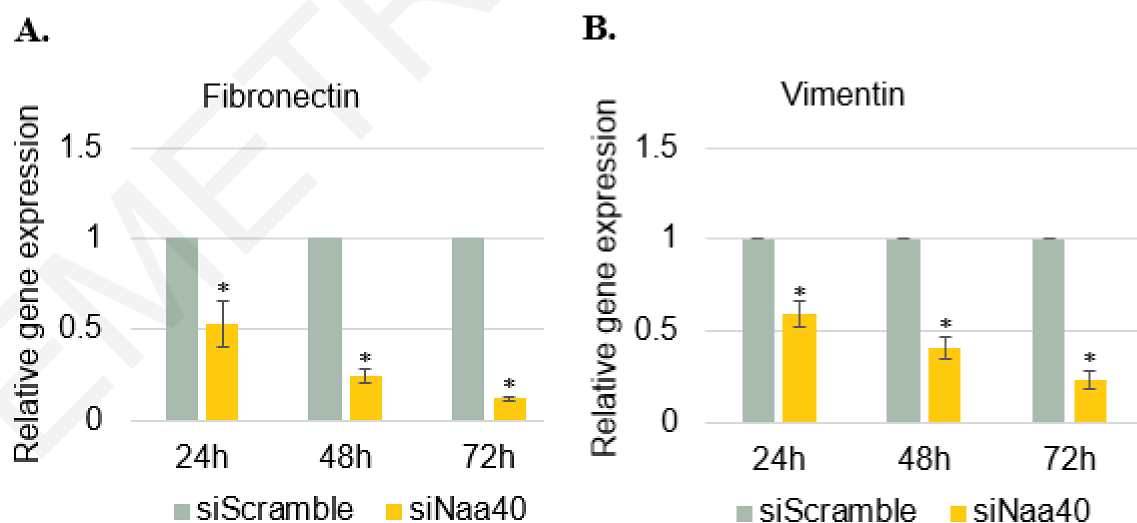

Figure 33. Naa40 silencing reduces the mRNA levels of mesenchymal protein markers. Gene expression levels of (A) fibronectin and (B) vimentin, 24h, 48h and 72h post-siRNA transfection in HCT116 cells. The levels of mRNA were quantified by RT-PCR and normalised to the levels of β-actin mRNA, whose expression remained unchanged. The data represent the mean of three replicates and are representative of three independent experiments ±S.D (p value <0.02).

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5.2.3 Naa40 activity affects the expression of genes involved in cell cycle progression and viability

Since down-regulation of Naa40 results in apoptosis (Chapter 3), we also monitored the mRNA levels of well-known genes implicated in cell death and cell cycle regulation (Figure 34). Tested genes included p21that is involved both in cell cycle arrest and apoptosis, members of the Bcl-2 family such as the pro-apoptotic PUMA, BAX and BAK and anti-apoptotic Bcl-2, cyclins D and E that are necessary for G1-to-S phase transition and inhibitors of apoptosis (IAP) genes (XIAP and Survivin) which are implicated in many cancers by favouring cell survival and by inducing cell proliferation. In addition, p53 mRNA levels were analysed by RT-PCR, as a control, since it was already known that Naa40-knockdown does not affect p53 protein levels (Figure 34). The mRNA levels of the pro-apoptotic proteins p21, Bax, Bak and PUMA were up-regulated 24h after the siRNA treatment in the Naa40 silenced cells, while at 72h post-treatment, the levels dropped and were comparable to those of control treated cells. If we take into account the newly identified role of N-acH4 in transcriptional activation (Schiza et al., 2013), these results suggest that these pro-apoptotic genes are not directly regulated by the activity of the Naa40 acetyltransferase and point towards the implication of other chromatin regulators for their transcriptional activation. In addition, the data indicate that the upregulated Bax protein that was previously monitored in western blots could be an outcome of post-transcriptional regulation, as well (Figure 26A and Figure 34). Contrary to the above results, the expression levels of anti-apoptotic genes, Bcl-2, XIAP and Survivin, and of genes necessary for G1/S cell cycle progression, cyclin D and cyclin E, were significantly reduced in the Naa40 depleted cells. Altogether, these results are consistent with the anti-apoptotic role of Naa40 in colorectal cancer cells and raise the possibility that Naa40 controls directly the transcription of genes involved in cell survival and cell cycle progression.

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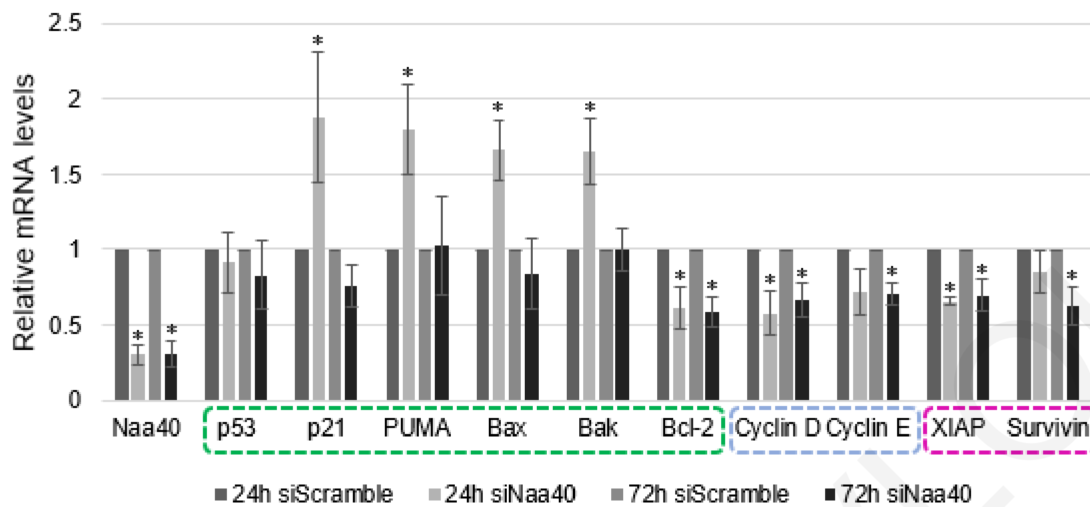

Figure 34. The effects of Naa40-knockdown on the mRNA levels of genes implicated in apoptosis and cell cycle. Gene expression levels were examined 24h and 72h post-siRNA treatment. The levels of mRNA were quantified by RT-PCR and normalised to the levels of β-actin mRNA, whose expression remained unchanged. Genes in green-dashed rectangle represent pro- and anti-apoptotic genes, genes in blue-dashed rectangle are needed for G1/S phase transitions and genes in violet-dashed rectangle are necessary for cell survival and apoptosis inhibition. The data represent the mean of at least three replicates and are representative of three independent experiments ±S.D (p value <0.05).

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Stress signals may also lead to mitochondrial apoptosis through the Unfolded Protein Response (UPR), due to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) (Oyadomari and Mori, 2004). Although the UPR represents primarily a survival cellular response, if ER stress persists, cells induce the apoptotic transcription factor CHOP (also known as DDIT3) to promote the apoptotic cell death pathway (Vandewynckel, Laukens et al. 2013, Mollereau 2013, Ma, Hendershot 2004). CHOP protein regulates positively or negatively the transcription of apoptotic (i.e. PUMA) or anti-apoptotic genes (i.e. Bcl-2), respectively, which are implicated in mitochondrial apoptosis initiation or inhibition. Since loss of N-terminal acetylation by Naa40 triggers mitochondrial apoptosis (Chapter 3), and since N-terminal acetylation has been shown to promote global protein misfolding (Holmes et al., 2014), it was obligatory to look into the expression of CHOP gene, as a possible target of Naa40. If so, CHOP may also represent an additional regulator of the induction of apoptosis in the Naa40 depleted cells. Hence, HCT116 cells were treated against the Naa40- or the Scramble-siRNA and RT-PCR was performed after 24h and 72h. As Figure 35 shows, the mRNA levels of CHOP gene dropped significantly 24h post-treatment, suggesting that apoptotic CHOP could represent one of the direct gene-targets of Naa40 and that depletion of the enzyme could have resulted in its down-regulation. However, this contradicts the anti-apoptotic role of Naa40 in colon cancer cells, proposing that CHOP expression may be regulated by a different mechanism. Interestingly, after 72h, a substantial upregulation of the CHOP gene was recorded. This enhances the idea that CHOP regulation is not directly regulated by Naa40 acetyltransferase activity and implicates an interplay between different modifiers in the control of CHOP transcription. Naa40 depletion triggers stress signals in the cell to induce the mitochondrial pathway. It is known, that upon mild ER stress, CHOP is induced, though it is rapidly degraded with a limited half-life resulting in low expression levels. In severe ER stress though CHOP is overexpressed through transcriptional and post-transcriptional regulation, it is stabilised and induces cell death by various mechanisms, including inhibition of the anti-apoptotic Bcl-2 (McCullough et al., 2001, Rutkowski et al., 2006, Oyadomari and Mori, 2004). The differential expression of CHOP observed at 24h and 72h post-Naa40 silencing could be the result of the aforementioned conditions. It is therefore suggested that prolonged Naa40 loss leads to severe cellular damages which in turn engage other additional apoptotic mechanisms, like CHOP activation, to augment mitochondrial apoptosis.

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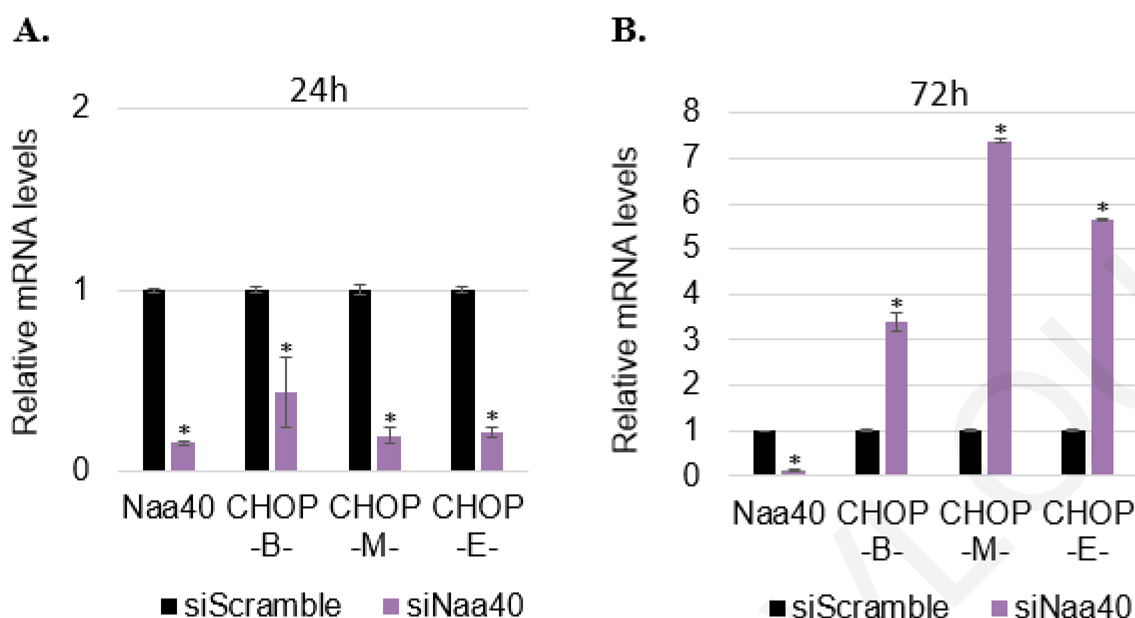

Figure 35. The effects of Naa40-knockdown on the mRNA levels of CHOP gene. Primers were annealing at the beginning (B), middle (M) and the end (E) of CHOP gene. Gene expression levels were examined 24h and 72h post-siRNA treatment. The levels of mRNA were quantified by RT-PCR and normalised to the levels of β-actin control mRNA. The data represent the mean of three replicates and are representative of three independent experiments ±S.D (p value <0.02).

The altered mRNA levels of genes upon Naa40 knockdown, suggest a Naa40-dependent transcriptional regulation. Table 10 summarises the transcriptional outcome of all the genes examined after Naa40 depletion in HCT116 cells. The fact that anti-apoptotic genes are down-regulated when Naa40 is abolished, might suggest that histone Nt-acetylation is directly involved in their transcriptional activation, raising the intriguing idea that Naa40 behaves as an oncoprotein in colon carcinoma. On the other hand, the genes that are upregulated (i.e. p21, PUMA, Bax, Bak) upon Naa40 depletion most likely represent indirect effects of histone Nt-acetylation, possibly, due to direct down-regulation of their transcriptional repressors.

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Table 10. Differential gene expression after Naa40 knockdown

Gene	Function	Changes in expression
Naa40	histone N-terminal acetyltransferase of histones H2A and H4	↓
rDNA	codes for ribosomal RNA components of ribosomes for protein synthesis	↓
caspases-3	effector caspase in the cascade for apoptosis induction, cleaves downstream substrates like PARP-1	-
caspase-6	effector caspase in the cascade for apoptosis induction, cleaves downstream substrates like PARP-1	-
caspase-7	effector caspase in the cascade for apoptosis induction, cleaves downstream substrates like PARP-1	↑
caspase-8	initiator caspase of the extrinsic apoptotic pathway	-
caspase-9	initiator caspase of the intrinsic/mitochondrial apoptotic pathway	-
PARP-1	modifies nuclear proteins by poly(ADP-ribosyl)ation, implicated in proliferation, DNA repair, differentiation and tumor transformation, inactivated by effector caspases	-
fibronectin	glycoprotein of cytoplasm, cell surface and extracellular matrix, involved in cell adhesion and migration/metastasis	↓
Vimentin	intermediate filament of the cytoskeleton, involved in cell attachment, migration/metastasis and signalling	↓
p53	tumor suppressor, transcriptional regulator of DNA repair, cell cycle arrest and apoptosis	-
p21	cyclin dependent kinase inhibitor, activates G1/G2 cell cycle arrest	24h ↑ 72h ↓
PUMA	pro-apoptotic protein, induces mitochondrial apoptosis and caspase-activation	24h ↑ 72h -
BAX	pro-apoptotic protein, translocates to mitohondria leading to cytochrome-c release and activation of apoptosis	24h ↑ 72h -
BAK	pro-apoptotic protein, translocates to mitohondria leading to cytochrome-c release and activation of apoptosis	24h ↑ 72h -
BCL-2	anti-apoptotic protein, inhibits BAX translocation to mitochondrion and blocks the release of cytochrome-c from the mitochondria	24h - 72h ↓
cyclin D	cyclin family member for G1/S cell cycle transition	↓
cyclin E	cyclin family member for G1/S cell cycle transition	↓
XIAP	protein member of Inhibitor of Apoptosis (IAP) family, direct caspase-inhibitor	↓
Survivin	protein member of Inhibitor of Apoptosis (IAP) family, direct caspase-inhibitor	24h - 72h ↓
CHOP	transcription factor, mediator of cell cycle arrest/apoptosis in response to ER stress trough the UPR pathway	24h ↓ 72h ↑

- unchanged ↓down-regulated ↑ up-regulated

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5.3 Interplay between histone Nt-acetylation and methylation

Histone modifications, just like all the epigenetic marks, do not function alone, but rather communicate and cross-regulate each other in a way that diversifies their functional states (Molina-Serrano et al., 2013). Recently, work from our lab revealed a cross-talk between Nt-acetylation by Naa40 and arginine methylation during the regulation of rRNA expression (Schiza et al., 2013). More specifically, during transcriptional activation of the yeast rDNA region, Naa40 catalyzes N-acH4 in order to block the deposition of the adjacent asymmetric dimethylation of arginine 3 on histone H4 (H4R3me2a), a modification which is associated with silenced chromatin in yeast. Interestingly, ectopic expression of the human Naa40 in mutant yeast strain lacking the endogenous yNaa40 gene can also activate this cross-regulation between H4R3me2a and N-acH4 at the ribosomal DNA locus (Schiza et al., 2013), highlighting the functional conservation of yeast and human Naa40. Hence, the conserved role of Naa40 in rDNA expression (Figures 30 and 31) prompted us to investigate whether Naa40-mediated histone Nt-acetylation in human cells also interplays with other modifications to regulate gene transcription.

5.3.1 Loss of Naa40 leads to reduced H4R3me2s reduction and enhanced H3K4me3 levels

To investigate whether Naa40 activity affects arginine methylation, as reported in yeast (Schiza et al., 2013), we performed Naa40 knockdown in HCT116 colon cancer cells (Figure 21A) and whole cell lysates were subjected to western blot analysis against H4R3me2a (Figure 36B). As expected a global loss of N-acH4 was observed (36A). Although a decrease of the H4R3me2a modification was recorded in the absence of Naa40, it was not of statistical significance, therefore, we deduced that Naa40 does not have a robust effect towards H4R3me2a and that a communication between N-acH4 and H4R3me2a is weak, at least at a global level (Figure 36B).

In the mammalian genome and contradictive to yeast, the presence of H4R3me2a signifies gene activation instead of repression. In contrast, the symmetrically dimethylated state of arginine 3 (H4R3me2s) is strongly associated with silenced heterochromatic regions in human cells (Saha et al., 2016, Di Lorenzo and Bedford, 2011, Wang et al., 2008). Thus, we wanted to explore whether N-acH4 has an effect on this modification, instead. Indeed, western blots revealed a significant global decrease of H4R3me2s in the

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Naa40 depleted sample compared to siScramble control, while the total levels of H4 histone remained unaffected (Figure 36A and 36C). This reduction was observed early on (24h) and persisted throughout the course of treatment (48h and 72h), suggesting that H4R3me2s works in concert with N-acH4. Similarly to yeast observations, human Naa40 strongly affects the global levels of the repressive mark (H4R3me2a in yeast, H4R3me2s in human cells).

Since N-acH4 loss led to such a great reduction of H4R3me2s silencing mark, we hypothesised that Naa40 could also cross-talk with a modification that designates transcriptional activation, other than H4R3me2a. Trimethylation of lysine 4 in histone H3 (H3K4me3) is widely known to enrich promoters and enhancer regions of actively transcribed genes (Guenther et al., 2007). Thus, it was intriguing to monitor the levels of H3K4me3 in the absence of Naa40. Interestingly, a great induction of H3K4me3 was observed from 24h post-siRNA treatment and persisted throughout the siRNA treatment (72h) (Figure 36D).

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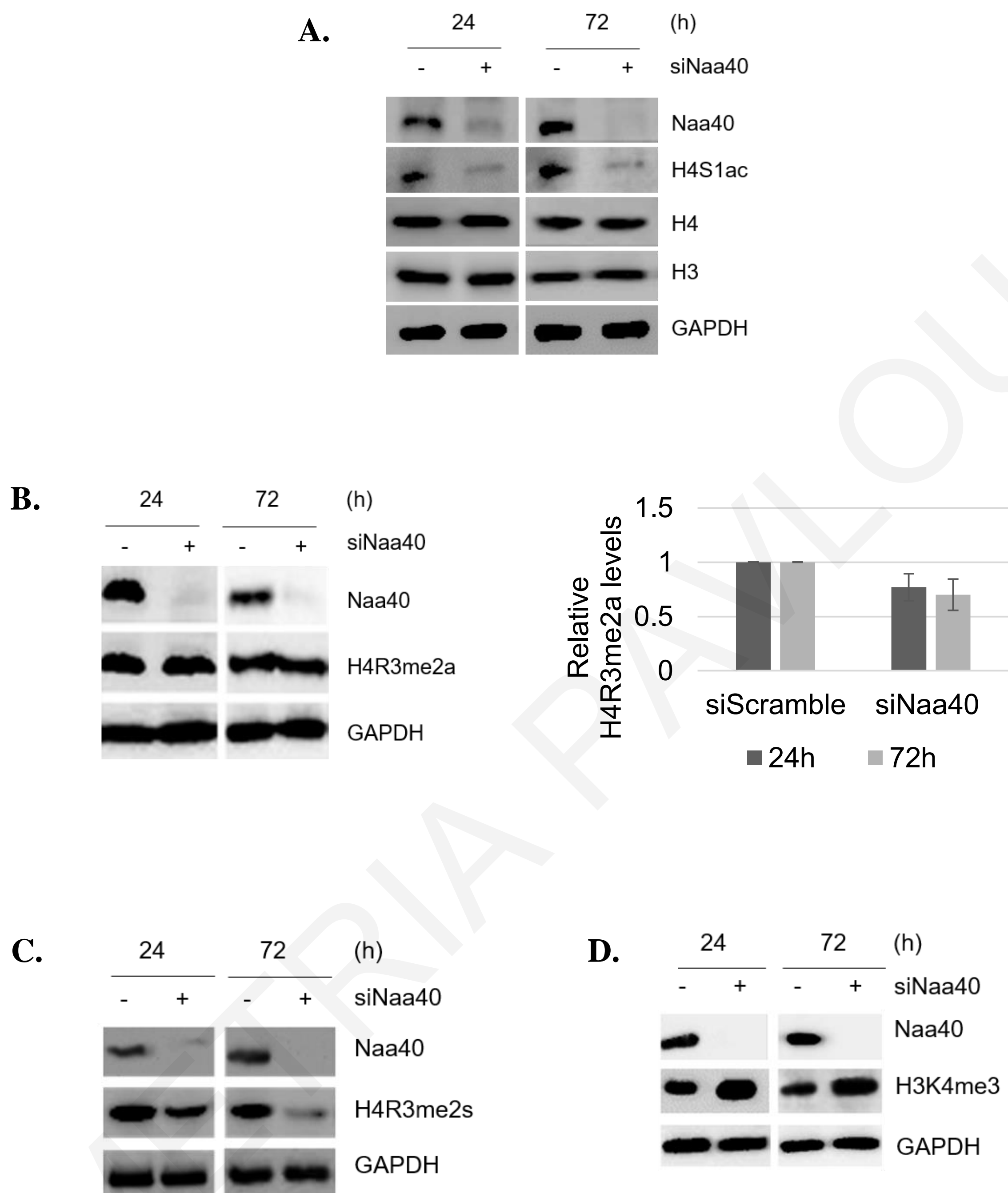

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Figure 36. Loss of Naa40 reduces H4R3me2s global levels, while H4R3me2a levels remain unaltered. **(A)** Immunoblot analysis of HCT116 cells that were transfected with 7.5 nm of Naa40 siRNA or scramble siRNA and incubated for 24h and 72h. Antibodies were used against Naa40, N-acH4, H3, H4 and GAPDH, which was used as a loading control. **(B)** H4R3me2a levels, **(C)** H4R3me2s and **(D)** H3K4me3 were determined in same conditions as (A). Western blot images are representative of three independent experiments. Images are representative of three independent experiments. Densitometry analysis (B, right panel) shows the levels of H43me2a in siNaa40 treatments relative to the scramble-siRNA sample.

5.3.2 PRMT5 expression is regulated by Naa40 function

Considering that Nt-acetylation (N-acH4) and H4R3me2s have opposing roles in gene transcription, the simultaneous global presence or loss of these modifications (Figure 36A and 36C) perhaps could be explained by a complex cross-talk that involves additional histone modifications and epigenetic factors. An alternative scenario is that N-acH4 simply regulates the expression of the gene that encodes for the H4R3me2s 'writer' which is the protein methyltransferase PRMT5. To explore the latter hypothesis, we first performed RT-PCR analysis 24h and 72h post-siRNA transfection (Figure 37A) and western blotting 72h post-treatment (Figure 37B), to determine the mRNA and protein levels of PRMT5, respectively, in the presence or absence of Naa40. Notably, PRMT5 mRNA and protein levels were significantly reduced in the absence of Naa40 (Figure 37A and 22B). Once again, we confirmed the reduction of H4R3me2s after Naa40 depletion which coincided with the decrease of its writer PRMT5 (Fig 37B). These findings suggested that Naa40 and its associated N-acH4 might regulate the activity of the PRMT5 gene promoter.

In order to reveal whether Naa40 activity affects the transcription and therefore activation of PRMT5, we quantified histone modifications on chromatin flanking the promoter region of the PRMT5 gene using ChIP assays. We thus examined chromatin with an antibody against N-terminally acetylated H4 (N-acH4) and an antibody against trimethylated H3K4 (H3K4me3), which is an epigenetic signature of active promoters (Guenther et al., 2007). In the absence of Naa40, the levels of N-acH4 and H3K4me3 significantly dropped, signifying the switch of PRMT5 from a transcriptionally active to a transcriptionally inactive state.

Altogether, these results provide an explanation for the global decrease of H4R3me2s observed in western blots (Figure 37C). N-acH4 modification at the promoter/enhancer region of PRMT5 is essential for its transcriptional activation, so it can thereafter symmetrically dimethylate its substrate genes. Reversely, loss of the N-terminal modification inhibits PRMT5 expression and thus H4R3me2 is reduced. Unlike the findings reported in yeast, where N-acH4 blocks the responsible methyltrnsferase Hmt1 from methylating its substrate H4R3 on chromatin (Schiza et al., 2013), we reveal that PRMT5 expression is controlled by Naa40 at the transcriptional level. Moreover, the contradicting H3K4me3 results obtained from western blots (Figure 36D) and from ChIP against PRMT5 gene (Figure 37C) emphasise the necessity of approaching Naa40 molecular function in the gene level, rather than the global one. Collectively, the findings

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implicate N-acH4 by Naa40 in the control of gene promoters, either directly or indirectly, through cross-talk with other modifications. Interestingly, loss of PRMT5, which catalyses the repressive H4R3me2s mark, could provide an explanation for the activation of pro-apoptotic genes in the absence of Naa40.

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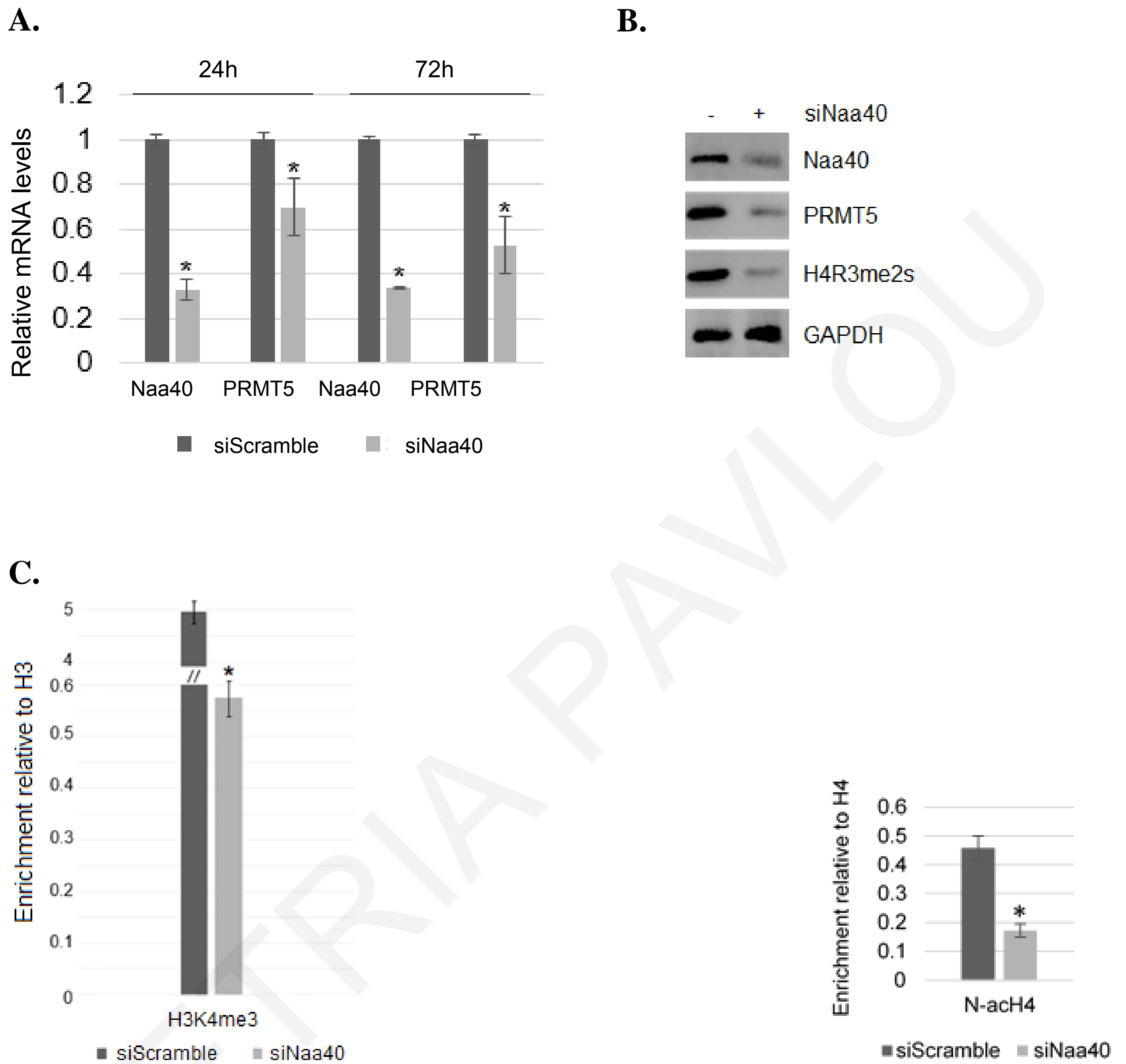

Figure 37. Naa40 acetyltransferase activity is necessary for PRMT5 transcriptional activation. **(A)** RT-PCR analysis of HCT116 transcfected with siScramble or siNaa40 nucleotides, for 24h or 72h. The levels of Naa40 and PRMT5 mRNA were quantified and then normalised to the levels of β-actin control mRNA, whose expression remained unchanged. **(B)** Immunoblot analysis to determine PRMT5 and H4R3me2s levels in the absence of Naa40, 72h post-transfection. GAPDH levels were monitored and used as a loading control. (C) ChIP analysis against H3K4me3 (left chart) and N-H4ac (right chart) epigenetic modifications at the enhancer/promoter region of PRMT5 gene, 72h post Naa40 attenuation. The enrichment from each antibody was normalized to the levels of histone H3 or H4, respectively. Values represent the mean of duplicate experiments and the asterisk (*) indicates statistically significant changes (p value <0.02).

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CHAPTER 6

DISCUSSION

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6. Unveiling the biological and molecular role of Nt-acetyltransferase Naa40 in colorectal carcinoma

The majority of eukaryotic proteins undergo co-translational and post-translational modifications to ensure correct transcriptional programme and protein function, which will result in normal cellular growth and development (Helin and Dhanak, 2013, Sharma et al., 2010, Holliday, 1987, Duan and Walther, 2015, Tooley and Schaner Tooley, 2014). Over the last years, Nα-terminal acetylation has attracted growing attention, as it is one of the most abundant covalent protein modification marks found across eukaryotes (Arnesen et al., 2009, Brown and Roberts, 1976, Varland et al., 2015).

In this scientific work, we uncover for the first time the link between Naa40 and colorectal carcinoma. We reveal the molecular pathway that is triggered upon Naa40 depletion and we explore the role of Naa40 towards gene regulation, in cells. Additionally, our findings expose the conserved role of human Naa40 in the control of rRNA expression linking this epigenetic enzyme and its associated histone modification with the transcriptional regulation of pro- and anti-apoptotic genes. We also identify a correlation between N-acH4 and histone methylation (H4R3me2s and H3K4me3). Such communication could be essential for switching ''on'' or ''off'' certain genes and could be responsible for changing a benign cell into a cancerous one. In summary, the present study identifies the anti-apoptotic role of Naa40 and therefore, proposes that Naa40 inhibition could serve as a potential epigenetic strategy for battling colon cancer.

6.1 Naa40 depletion induces p53-independent mitochondrial apoptosis in cancer cells without affecting the viability of non-cancerous cells

According to our findings, Naa40 behaves as an oncoprotein as its activity is essential for colon cancer cell survival. Knockdown of Naa40 in colon cancer cells triggers a strong apoptotic signal, characterized by cellular morphological changes (Figure20 and 21A) and reduced viable population. This decrease is evident through the MTT assay (Figure 21C and 24B) and the significant increase of the subG0/G1 apoptotic cell population observed by flow cytometry (Figures 23A and 24C). The transition of cells from early to late apoptosis determined by Annexin V/PI staining shows clearly that Naa40 depletion induces apoptosis and not necrosis (Figure 23B). The mitochondrion has a central role in the induction of Naa40-knockdown mediated apoptosis. This is confirmed

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by altered levels of Bcl-2 protein members (Figure 26A and 26B), the specific activation of the initiator caspase-9 but not of caspase-8 (Figure 26C) and the cleavage of the downstream executioner caspases (Figure 25B). Consistent with this, treatment of cells with an irreversible caspase-9 inhibitor impedes apoptosis in the absence of Naa40 (Figure 27). Collectively, these data demonstrate that the mitochondrial, or intrinsic apoptotic pathway is exclusively responsible for the Naa40-knockdown mediated programmed cell-death. It is known that tumor cells acquire proliferative advantage and resist to treatments though inhibition of apoptotic pathways. Since mitochondrial apoptosis irreversibly locks cells in the cell-death pathway and enhances cells' sensitivity towards stress signals, their stimulation is considered an attractive approach for tumor cell elimination (Lopez and Tait, 2015, Gogvadze, 2011, Hassan et al., 2014). Therefore, manipulating the expression levels of Naa40 in those cancers could be considered a key therapeutic strategy. Importantly, the link of Naa40 knockdown to apoptosis appears to be specific to cancer cells, since depletion of Naa40 in non-malignant embryonic fibroblasts does not stimulate cell death (Figure 24A and 24B). This difference in the survival response proposes Naa40 as a novel candidate in effective targeted treatments which will protect normal cells from generating side-effects throughout therapeutic procedures. Moreover, the effect of Naa40 depletion on cell-death is mediated through a p53-independent mechanism, since p53-null HCT116 cells still undergo apoptosis upon reduction of the acetyltransferase (Figure 28). The transcription factor and tumor suppressor p53 normally acts as a brake to halt damaged cells from propagating their genetic aberrations to progeny cells. However, more than 50% of cancers are afflicted with transcriptional or post-transcriptional errors that lead to abolished function of p53. As a result, cells lose their tumor-protecting capacities and acquire a growth, proliferative and survival advantage (Haupt et al., 2016). The fact that that Naa40-mediated apoptosis in colon cancer cells does not require p53 activity, highlights the potential exploitation of Naa40 as a therapeutic target against cancers, regardless their p53 status (wild-type or mutant).

Collectively, our findings so far place Naa40 upstream the mitochondrial pathway (Figure 38) and suggest that its activity could induce the expression of anti-apoptotic genes or block pro-apoptotic factors involved in the intrinsic apoptotic cascade. Accordingly, Naa40 depletion induces mitochondrial apoptosis. Transcriptional profiles of genes implicated in this process shed light into the underlying molecular mechanisms driven by Naa40 (results are discussed below).

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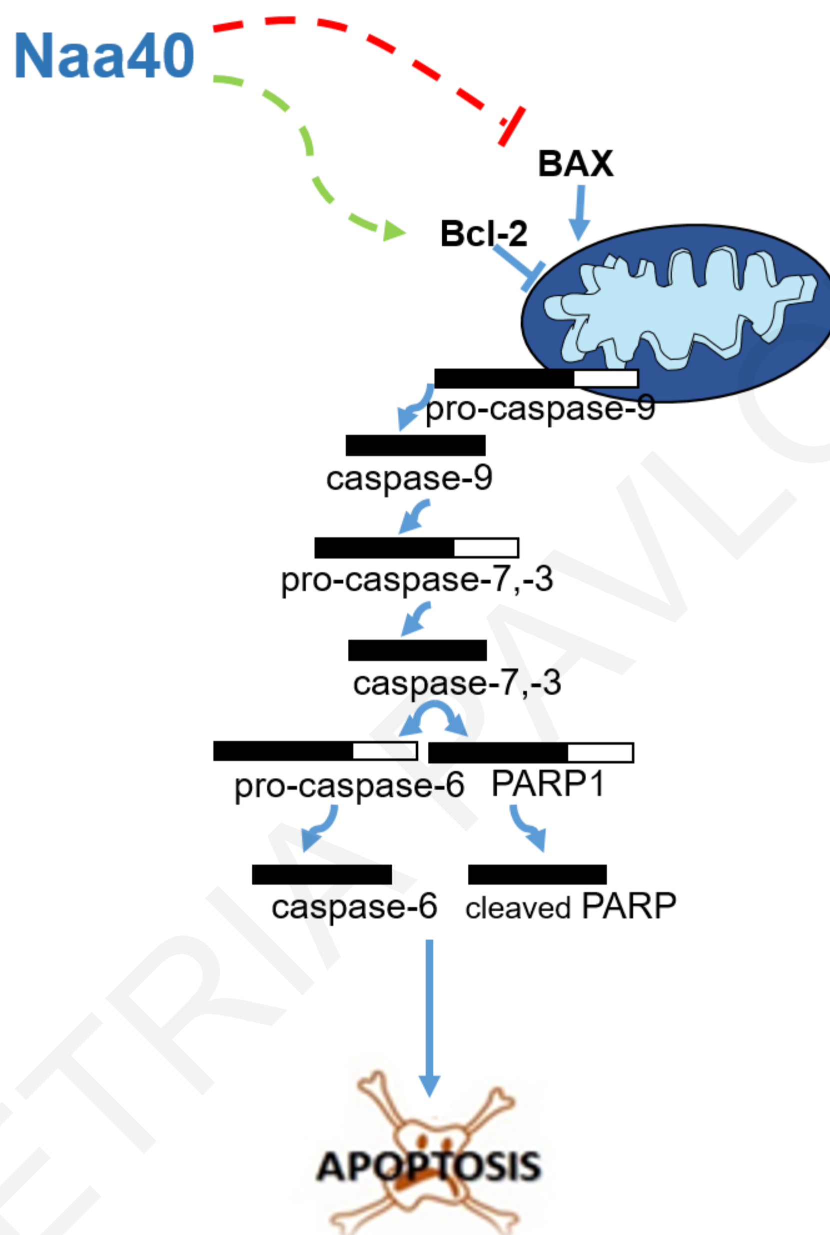

Figure 38. Naa40 regulates mitochondrial apoptosis. Mitochondrial outer membrane permeabilisation (MOMP) relies upon the expression and translocation of pro-apoptotic proteins (i.e Bax) and the inhibition of anti-apoptotic factors (i.e Bcl-2). Naa40 functions upstream the mitochondrial apoptotic pathway, possibly affecting the transcription of genes necessary for mitochondrial apoptosis induction or inhibition.

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6.2 The role of Naa40 in the control of rRNA expression is conserved from yeast to humans

Despite the fact that the enzymatic activity of Naa40 towards histones was identified in yeast more than a decade ago (Song et al., 2003, Polevoda et al., 2009b), evidence for the molecular role of histone Nt-acetylation was only recently described. Specifically, a study in our lab supported a role for this modification in transcriptional activation by showing that N-acH4 mediated by Naa40 was required for rRNA expression in yeast (Schiza et al., 2013). Thus, it was intriguing to investigate whether human Naa40 behaves in a similar manner by controlling the transcription of the rRNAs in human cells. Upon depletion of Naa40 in colon cancer cells, we observed a significant decrease in the expression of different ribosomal RNA transcripts (28S, 18S, 5.8S as well as their precursor 45S) (Figure 30), supporting the above hypothesis. In favour of this, ChIP experiments revealed that the ribosomal DNA loci carry N-terminally acetylated H4 histones (Figure 31). This means that the nucleosomes found at the rDNA regions are direct targets of Naa40. Loss of the enzyme results in a significant reduction of N-acH4, which correlates with the down-regulation of rRNA expression. The higher acetylation levels observed in the enhancer/promoter of the 45S rDNA locus (Figure 31), may suggest that Naa40 modifies mainly nucleosomes found at these regions to enhance and initiate transcription.

Cellular ageing has been previously linked with increased rRNA expression and rDNA instability whereas down-regulation of rRNA transcription has been correlated with extended lifespan (Ganley and Kobayashi, 2014, Kobayashi, 2011, Kobayashi, 2014). Nutrient deprivation has been described to cause down-regulation of rDNA genes. This leads to reduced energy expenditure since rDNA encodes rRNAs which account for approximately 80% of the total RNA in the cell. Consequently, cells save energy and live longer. However, this is not the case in cancer cells. It is generally accepted that tumor cells highly overexpress RNA species to induce cancer cell proliferation. Tumor progression is dependent on a concomitant increase in protein synthesis due to increased cell division rates and anabolic activities, where expression and synthesis of new biomolecules rely upon RNA polymerase I and ribosomes (Nguyen le et al., 2015, Uemura et al., 2012, Ghoshal et al., 2004, Drygin et al., 2010, Ruggero and Pandolfi, 2003, White, 2005, Williamson et al., 2006, Shenoy et al., 2012). Therefore, reduction of RNA content acts as a stress sensor, which activates pathways for cell cycle arrest or p53-independent

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apoptosis. For the over-mentioned reasons, inhibition of rDNA transcription is recognised as a therapeutic strategy against cancers (Kumazawa et al., 2015, Bywater et al., 2012). Taking all these into consideration, we postulate that loss of the anti-apoptotic Naa40 in colon cancer directly impairs rRNA expression providing in this way the initial signal for diminishing the proliferative potential and cell viability of HCT116 and HT-29 colon cancer cells. On the other hand, absence of N-acH4 and possible reduction of rRNAs in STO cells does not influence their viability nor growth rate. According to recent data, deletion of yeast Naa40 orthologue extends replicative life span of cells to similar levels as observed under caloric restriction (Molina-Serrano et al., 2016,unpublished data). It is now accepted that caloric restriction retards the ageing process, prolongs the duration of youthfulness, postpones the onset of age-associated pathologies, and extends longevity of organisms (Sohal and Forster, 2014, Fontana et al., 2010). If the role of yeast Naa40 in cellular longevity is maintained in humans, this implies that Naa40 targeting in therapeutics will not only be valuable for the elimination of malignant cells, but also for the delay of physiological declines that characterise ageing process, in non-cancerous cells. In support of this, recent findings in mice showed that Naa40 knockout offers protection from an age-associated disease, known as hepatic steatosis (Liu et al., 2012).

6.3 Naa40 depletion reduces the expression of anti-apoptotic genes and induces the expression of pro-apoptotic factors

Acetyl-marks that decorate the amino acid residues within the histone tails facilitate access of the transcriptional machinery to DNA, as they ‘open-up’ chromatin by stimulating nucleosome disassembly. Therefore, histone acetylation is linked with transcriptional activation, while deacetylation leads to transcriptional silencing (Kouzarides, 2007, Lu et al., 2015, Venkatesh and Workman, 2015). However, whether histone Nt-acetylation catalysed by NATs plays a similar role in transcription is still not totally clear. Previous work in yeast cells has suggested that N-acH4 by yeast Naa40 contributes to transcriptional activation of the rRNA genes (Schiza et al., 2013). Through this study, further evidence in favour of the notion that N-acH4 by Naa40 acts as an activation mark is provided. Consistent with the proposed Naa40 anti-apoptotic role, we suggest that N-acH4 in colorectal cancer is necessary for the activation of anti-apoptotic genes because depletion of Naa40 results in the down-regulation of these genes (Figure 34).

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In more detail, we showed that the mesenchymal markers fibronectin and vimentin, which promote tumor aggressiveness (Han and Roman, 2006, Mendez et al., 2010), are transcriptionally reduced upon Naa40 loss. These results support the hypothesis that Naa40 plays a direct role in their transcriptional regulation through which N-acH4 could lead to the decompaction of chromatin to allow the transcriptional machinery to land on DNA. Also, our findings exclude protein degradation as the main mechanism for their down-regulation during siNaa40-induced apoptosis (Figures 22 and 33). Additionally, the expression levels of anti-apoptotic genes, Bcl-2, XIAP and Survivin, and of genes necessary for G1/S cell cycle progression, cyclin D and cyclin E, were significantly reduced in the Naa40 depleted cells (Figure 34). These results are in consonance with the anti-apoptotic role of Naa40 in colorectal cancer cells where upon Naa40 depletion G0/G1 phase of cells is decreased and apoptotic population (subG0/G1) is increased (Figure 23A). The data enhance the possibility that Naa40 controls directly the transcription of genes involved in cell survival and cell cycle progression to promote tumorigenesis. However, this should be conclusively resolved through ChIP experiments performed in the presence and absence of Naa40, in order to determine the localisation of N-acH4 histone modification.

On the contrary, pro-apoptotic proteins p21, Bax, Bak, PUMA and CHOP were up-regulated in Naa40-knockdown cells. CHOP activation implicates UPR pathway for the induction of apoptosis, in addition to the intrinsic apoptotic pathway. Naa40 loss seems to stimulate a strong stress signal in cells that triggers apoptosis. Mild ER stress, caused by misfolded or unfolded proteins, is known to lead to the transcription of CHOP, which though is rapidly degraded leading to reduced CHOP levels. However, severe stress, leads to significant increase and stabilisation of CHOP (McCullough et al., 2001, Rutkowski et al., 2006, Oyadomari and Mori, 2004). This could explain our observations of CHOP reduced levels after 24h post-siRNA transfection and its significant induction after 72h of treatment, due to prolonged cellular changes (Figure 35). This scenario is also supported by the elevated levels of the uncleaved PARP-1 (Figure 25B), which according to previous published data, is induced upon severe DNA-damage (McCullough et al., 2001, Rutkowski et al., 2006, Oyadomari and Mori, 2004). Thus, we assume, that upon Naa40 depletion, other apoptotic mechanisms in addition to the mitochondrial pathway are engaged to eliminate damaged cancer cells.

Taking into consideration the evidence pointing towards a role of N-acH4 in transcriptional activation (Schiza et al., 2013), the above results suggest that these pro-

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apoptotic genes are not directly repressed by the activity of the Naa40 acetyltransferase on histones and point towards the implication of other chromatin regulators for their transcriptional activation. One possibility is that their direct transcriptional repressor is itself transcriptionally down-regulated in the absence of Naa40 and N-acH4 (discussed in section 6.4). Another alternative is that Naa40 acetylates the repressor protein itself and loss of this acetylation in Naa40 knockdown cells inhibits its function. However, this scenario is less likely to hold true, considering the fact that Naa40 acetyltransferase activity is directed only towards histone proteins H4 and H2A. So far, no other human proteins have been identified as Naa40 substrates, though H2A.X and SMARCD2 have also been considered as putative Naa40 targets, since they also possess the proper recognition N-terminal sequence (Magin et al., 2015).

The data so far show that Naa40 has a very narrow and specific set of protein targets. Furthermore, the yeast Naa40 orthologue was shown to have a transcriptional role towards rRNA expression (Schiza et al., 2013). Therefore, we postulate that Naa40 influences cell survival by regulating gene expression epigenetically and not through direct N-terminal acetylation of key regulators of cell growth and/or apoptosis. Additionally, the transcriptional down-regulation of an inhibitor may not only directly affect the transcription of pro-apoptotic genes, but could influence other modifications or chromatin factors that in turn will affect those pro-apoptotic genes (Figure 39). Collectively, this study suggests that Naa40 is responsible for maintaining the balance between cell survival and cell-death, through transcriptional regulation of target genes.

6.4 Loss of Naa40 and N-acH4 associates with repression of PRMT5

In this project, we show that loss of N-acH4 due to Naa40 knockdown results in reduced H4R3me2s global levels (Figures 36C and 37B). Interestingly, we found that the overall H4R3me2s reduction is due to the down-regulation of PRMT5, which is the responsible methyltransferase. Our data show that N-acH4 is localised at the promoter/enhancer region of PRMT5 and loss of this mark upon Naa40 depletion correlates with repression of PRMT5 (Figure 37), indicating that PRMT5 is a direct target of Naa40. Unlike the findings reported in yeast, where N-acH4 blocks directly the Hmt1 methyltransferase from methylating its substrate H4R3 on chromatin (Schiza et al., 2013), we show that Naa40 and N-acH4 regulate PRMT5 at the transcriptional level.

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The expression levels and activity of PRMT5 is frequently associated with tumorigenesis, as the enzyme directly inhibits genes important for cell cycle arrest or apoptosis (Chung et al., 2013, Wang et al., 2008, Zhang et al., 2015a, Gu et al., 2012, Pal et al., 2007, Pal et al., 2004, Powers et al., 2011, Cho et al., 2012, Bao et al., 2013, Zhang et al., 2015d). Therefore, we hypothesise that Naa40 directly induces anti-apoptotic genes and in addition activates PRMT5, which subsequently deposits H4R3me2s to suppress pro-apoptotic genes. This is consisted with the already known oncogenic/anti-apoptotic role of PRMT5 exhibited in cancers (Zhang et al., 2015d, Pal et al., 2007, Pal et al., 2004).

Interestingly, upon loss of Naa40 we also observe enhanced H3K4me3 levels (Figure 36D). Based on previous findings that support Naa40 transcriptional activation role (Schiza et al., 2013), we similarly assume that the up-regulated genes observed in the absence of Naa40, are most likely controlled through an indirect regulatory way. Genome wide profiling for H3K4me3 has revealed that this mark is present in most transcriptionally active promoters or enhancers (Kim et al., 2005, Bernstein et al., 2005, Mikkelsen et al., 2007, Bernstein et al., 2006, Wei et al., 2009, Cui et al., 2009, Ke et al., 2009). The inhibition of H3K4me3 activation mark by arginine methyltransferases has been described very recently. PRMT7 methyltransferase, which just like PRMT5 catalyses the symmetric dimethylation of arginine 3 in histone H4 (H4R3me2) was shown to inhibit MLL4 (also known as MLL2 or ALR) from catalysing the trimethylation of lysine 4 in histone H3 (H3K4me3). MLL4 lysine methyltransferase recognises H4R3me2a (also an activation mark) through its homeodomain (which is also required for lysine methyltransferase activity) and thereafter catalyses the methylation of H3K4me3 in order to activate differentiation-specific genes (HOX genes). Induction of H4R3me2s levels by PRMT7 inhibited the expression of MLL4 target-genes, whereas knock-down of PRMT7 raised MLL4-catalysed H3K4me3 levels. The authors propose that H4R3me2s interferes with the homeodomain of MLL4, which can no longer bind to H3K4 to catalyse its methylation (Dhar et al., 2012). In a similar way, we assume that PRMT5-mediated H4R3me2s could block the deposition of H3K4me3. Hence, in the absence of Naa40 activity and subsequent down-regulation of PRMT5, H3K4me3 levels are enhanced. Although this could represent a global effect, the decreased H3K4me3 levels at the PRMT5 promoter region in the absence of Naa40 suggest that the changes on H3K4me3 levels are gene specific. Therefore, in future studies it would be very informative if we approached Naa40 effect towards gene expression at a genome wide level. This will allow us to determine the exact genomic locations where H3K4me3 changes occur upon Naa40 loss, identify the molecular

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pathways the affected genes are implicated in and expose the functional consequences of Naa40 activity.

6.5 Proposed molecular mechanism through which Naa40 controls gene expression

Considering the oncogenic role of Naa40 in colon cancer cells, we propose the following mechanistic model (Figure 39): Naa40 in colon cancer cells is responsible for the Nt-acetylation of nucleosomes found at the rDNA region, the PRMT5 gene and at anti-apoptotic genes required for cell-cycle progression (Bcl-2, cyclin-D. cyclin-E), survival (XIAP and survivin) and metastasis (fibronectin and vimentin). This N-acH4 modification possibly results in the decompaction of chromatin and therefore the transcriptional activation of the target loci. The expression of pro-apoptotic genes (p21, BAX, BAK, caspase-7 and CHOP) can be either suppressed by PRMT5, as it is already known for p21 (Zhang et al., 2015d) and for ST7 and Rb tumor suppressor proteins (Pal et al., 2007, Pal et al., 2004) or by some other chromatin repressors that await to be identified and associate with Naa40 activity. Since H3K4me3 levels are significantly elevated in Naa40 knockdown conditions, it is assumed that this transcriptionally active mark could be inhibited by the Naa40-induced PRMT5 activity, which deposits the H4R3me2s antagonistic repressive mark. Epigenetic marks are dynamic and differentially arise at specific sets of genes, cells, tissues and times. Thus, we assume, that depending on the biological context, the proposed mechanism may be different. Additionally, N-acH4 may work synergistically or antagonistically with other modifications on the same nucleosome, in the same or different histone proteins, to bring about specific transcriptional outcomes. Important tasks for future studies will be to investigate the regulatory mechanisms of Naa40 to answer questions as to how transcriptional activation or repression is controlled through its molecular activity and which or how other genes and modifications are modulated by its function.

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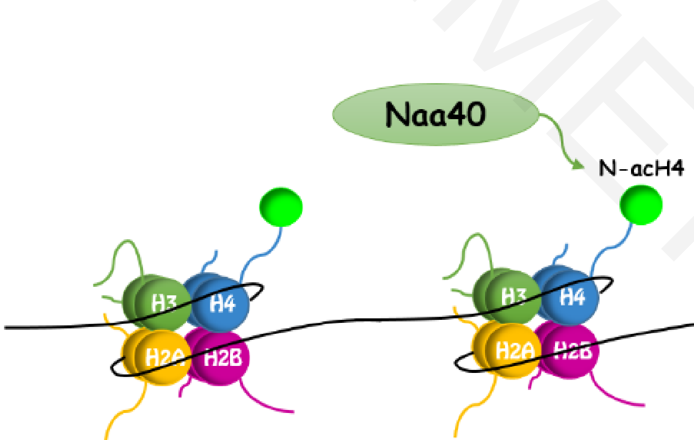

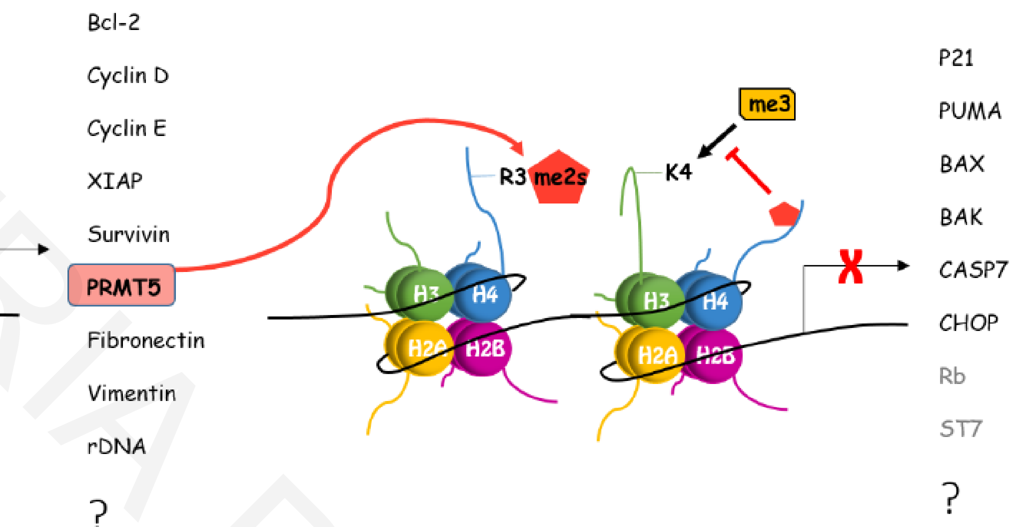

Figure 39. Proposed Naa40 regulatory mechanism for gene regulation. Naa40 in colorectal carcinoma acetylates nucleosomes of anti-apoptotic genes, rDNA and PRMT5. Silencing of pro-apoptotic genes is enhanced through PRMT5 which catalyses the H4R3me2s mark on gene promoters. H4R3me2s blocks the deposition of H3K4me3 activation mark, which in the absence of Naa40 and hence, loss of PRMT5 activity, is enhanced. Gene silencing can be also indirectly induced by Naa40, through a mechanism that still remains elusive.

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6.6 The role of Naa40 in cancer development depends on the specific biological and pathological context

The anti-apoptotic role of Naa40 in colon cancer contradicts the previously reported pro-apoptotic role of Naa40 in hepatocellular carcinoma. In liver cancer cells, siRNA mediated knockdown of Naa40 protected cells from drug-induced apoptosis, whereas overexpression of the enzyme induced programmed cell death (Liu et al., 2009). A bifunctional role of Nt-acetylation has been previously described for N-terminal acetyltransferase NatA (also known as Naa10), which exhibits both an anti- and a pro-apoptotic role. For example, depletion of Naa10 in HCT116 colon cancer cells and Hela cervical adenocarcinoma cells caused caspase-dependent apoptosis (Gromyko et al., 2010, Arnesen et al., 2006), while knockdown of Naa10 in other cell lines, including U2OS osteosarcoma and HT1080 fibrosarcoma cells, conferred resistance to DNA-damage induced apoptosis. It has been proposed that these opposing roles are due to the fact that Naa10 has numerous protein substrates (Polevoda et al., 1999) and employs different mechanisms to exert its functions, which can vary among cell-types and pathological contexts (Kalvik and Arnesen, 2013, Starheim et al., 2012). An analogous rational could perhaps explain the contrasting roles of Naa40 in apoptosis. With more than 90% of histone H4 proteins N-terminally acetylated within cells (Tweedie-Cullen et al., 2012), it is likely that Naa40 impacts chromatin structure and transcription of various genomic loci. Indeed, our results indicate that N-acH4 plays a key role in the regulation of various genes (Table 10). In colon cancer cells, where Naa40 behaves as an oncoprotein, it directly enhances the transcriptional activation of rDNA, PRMT5 and of anti-apoptotic genes whereas it indirectly leads to the transcriptional silencing of pro-apoptotic genes (Table 10). Consequently, knock-down of Naa40 could affect different sets of genes in different cell types and thus exhibiting diverse effects on cell viability. Unfortunately, the previous study did not examine gene expression in hepatocellular carcinomas upon depletion of Naa40. Therefore, it would be interesting to determine if Naa40 targets different genes in colon versus liver cancer cells. Moreover, according to the differential expression of Naa40 in different cancer types (The Human Atlas, 2016), it is possible that Naa40 targets distinct sets of genes to exhibit its oncogenic versus its tumor-suppressive function. Future molecular studies in various biological contexts, pathological or not, will provide new insights for resolving the exact role of Naa40 in apoptosis and carcinogenesis.

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6.7 Naa40 emerges as a promising target in cancer therapy

Resisting cell-death is one of the hallmarks of cancers (Hanahan and Weinberg, 2011). The acquired capability of cancer cells to evade programmed cell-death by apoptosis enables them to sustain their proliferative potential that eventually leads to tumor development, invasion and metastasis. Efforts to trigger apoptosis and re-establish this crucial natural barrier to tumorigenesis have been the focus in several cancer therapy studies. One key regulator of apoptosis is the p53 tumor suppressor, which is inactivated in more than 50% of all tumors, including colon cancer (Liu and Bodmer, 2006, Joerger and Fersht, 2007).

The findings within the current study suggest that the inhibition of Naa40 should be considered as a therapeutic approach because of the following main reasons. Firstly, Naa40 knockdown in colon cancer cells induces a strong apoptotic effect which is specific because depletion of Naa40 in non-malignant mouse embryonic fibroblasts does not affect cell viability. Secondly, the siNaa40-mediated induction of apoptosis in HCT116 cancer cells is irrespective of the p53 status. Since the gene encoding p53 is mutated in 40-50% of colorectal cancers (Liu and Bodmer, 2006), a comprehensive therapeutic regimen should avoid the requirement of having intact endogenous p53 function. Thirdly, the data suggest a direct regulatory effect of Naa40 upon transcriptional activation of proteins implicated in the survival of cells (Bcl-2, cyclins, survivin, XIAP and of mesenchymal markers), which are down-regulated in Naa40 depleted cancer cells. As a result, cell growth, cell-cycle progression and cell-survival are diminished. Fourthly, Naa40 knock-down leads to the transcriptional induction of pro-apoptotic factors that are implicated in intrinsic apoptosis (Bax, Bak, p21, PUMA, caspase-7 and CHOP). The effect of Naa40 loss is completely driven by the mitochondrial pathway, since the caspase-9 inhibitor (z-LEHD-fmk) blocked apoptosis in siNaa40-treated cells. Notably, the mitochondrial apoptotic pathway has a prominent role in chemotherapy effectiveness, since MOMP represents the defining event that irrevocably commits a cell to die (Lopez and Tait, 2015) (Sarosiek et al., 2013). Fifthly, down-regulation of Naa40 diminishes the expression of PRMT5 methyltransferase, whose expression in malignancies, including colorectal cancers, is correlated with induced cell-growth and metastasis (Zhang et al., 2015a, Zhang et al., 2015b, Scoumanne et al., 2009, Cho et al., 2012, Bao et al., 2013, Kryukov et al., 2016, Mavrakis et al., 2016). On the other hand, down-regulation of PRMT5 was shown to induce G1 arrest, Bax activation and thus mitochondrial apoptosis (Scoumanne et al., 2009, Lim et al., 2014, Koh et al., 2015). Therefore, loss of Naa40 could be exploited in cancers that are either PRMT5-

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dependent or -independent, where in the former case, a combination of detrimental effects could be elicited from the targeted cell, due to simultaneous loss of two potent oncogenes.

Similarly to other NATs (Kalvik and Arnesen, 2013), Naa40 has emerged through this study as a potential therapeutic target because its inhibition could induce apoptosis in cancer cells and hence hinder carcinogenesis. Therefore, NAT specific inhibitors could be valuable epigenetic anticancer therapeutics. Specifically, a first generation of NAT compounds has been developed that can selectively target the NatA complex, its catalytic subunit Naa10 or the enzyme NatE/Naa50 (Foyn et al., 2013). Epigenetic therapies for cancer treatment hold great promise and future efforts may add Naa40 to the repertoire of epigenetic regulators that are currently being targeted by small-molecule inhibitors in preclinical and clinical studies. The recent defined structure of Naa40 showed that although the overall fold is similar to other NATs, it also contains unique features, such as in the loops forming the substrate-binding site (Magin et al., 2015), which can be exploited to develop specific Naa40 inhibitors.

6.8 Future Directions

Naa40 acetyltransferase targets specifically the N-terminus of histones H4 and H2A only (Song et al., 2003, Polevoda et al., 2009b). In this study we have specifically looked at the connection between N-acH4 and Naa40. However, future studies should also determine if Nt-acetylation of histone H2A is involved in this same processes and whether N-acH2A affects separate target genes or the same genes as N-acH4. In mammalian cells the N-terminal sequence (Ser-Gly-Arg-Gly) of these two histones is exactly the same so it is possible that Naa40 modifies both histones at the same exact locations and there won't be a functional difference between the two. Creating tools that can study both these modifications separately, such as raising a specific N-acH2A antibody, would be essential to dissect the molecular mechanisms and function of Nat4.

So far, our results reflect the role of Naa40 in cell lines. Monitoring the development and progression of colorectal carcinoma in nude mice after the knockdown of Naa40, will help to deduce whether our findings in the culture system can be extrapolated at the organismal level and whether they could portray human biology. In addition, inducing different tumors in different organs, will allow the generation of valuable information regarding the context-dependent function of Naa40 and will direct future studies into how Naa40 can be successfully utilised in targeted anti-cancer therapies.

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Overexpression experiments will further confirm its biological role towards cell survival or apoptosis.

We can take advantage of high-throughput techniques, like RNA sequencing, for the identification of all the genes whose expression is deregulated in the absence of Naa40. Mapping and grouping all genes that are affected by N-acH4, the cellular processes and pathways controlled by Naa40 will be revealed. The link between Naa40 activity and metabolism is also profound. Under caloric restriction, the activity of Naa40 towards histone H4 in the yeast rDNA region is reduced (Schiza et al., 2013) suggesting that Naa40 may act as a sensor for cell growth (Riesen and Morgan, 2009, Smith et al., 2009). Furthermore, a study in mice showed that liver-specific Naa40 knockout males have aberrant lipid metabolism, reduced fat mass and are protected from age-associated hepatic steatosis (Liu et al., 2012) whereas overexpression of Naa40 in hepatocellular carcinoma leads to induced apoptosis (Liu et al., 2009). Knowing that cancers rely upon a series of biosynthetic and catabolic reactions to generate the energy required to support their rapid proliferation, mostly through mitochondrial processes (Zong et al., 2016, Leithner et al., 2014, Otto, 2016, Khan and Chakrabarti, 2015), a distortion of those metabolic pathways via changes in Naa40 expression and activity could lead to cell death. Thus, identifying through high throughput approaches, like proteomic studies and metabolomics, alterations of metabolite patterns in various tissues, in the presence or absence of Naa40, will enable us to reveal where and how Naa40 is implicated in those biochemical processes (Buescher and Driggers, 2016).

Although Nt-acetylation by Naa40 is an abundant modification, we still do not know where it is localised. ChIP sequencing will be valuable for exposing the localisation of N-acH4 across the whole genome. In this way, all the direct targets of Naa40 will be identified. Immunoprecipitation experiments against other modifications (i.e H4R3me2s and H3K4me3) could also show whether a deregulation/differential positioning of epigenetic marks takes place in the absence of Naa40. Thus, synergistic or antagonistic cross-talks at certain genomic loci will be exposed (Molina-Serrano et al., 2013). To have a homogeneous cellular Naa40 depletion and to facilitate global analysis, the generation of stable knock-down cell-lines are required. The elucidation of the molecular mechanisms that lead to the deposition of Nt-acetylation by Naa40 is crucial for therapeutic advances, where novel putative drug-inducers or inhibitors are anticipated to be developed in order to manipulate the activity of this N-terminal acetyltransferase in cancers.

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Collectively, the current research project reveals for the first time the anti-apoptotic cellular role of Naa40 and emphasises that its function and regulatory effects are more complex than previously believed. The results highlight the need for future in-depth studies which will assess the biological and molecular role of Naa40, in different cancer and non-pathological contexts and will clarify how Naa40 can be utilised in the diagnosis or treatment of cancers.

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CHAPTER 7

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