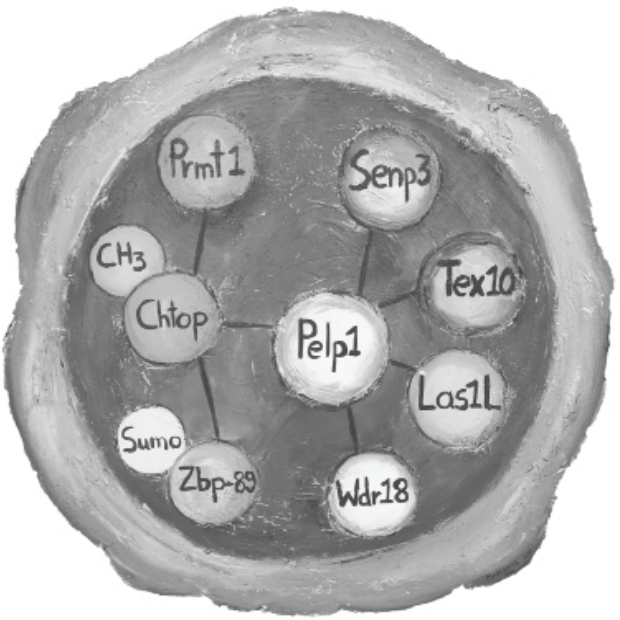
Functional proteomics analysis of transcription factor networks in erythroid cells



Pavlos Fanis

ISBN: 978-90-8570-435-5

The work presented in this thesis was performed at the Department of Cell Biology at the Erasmus MC, Rotterdam, The Netherlands. The department is a member of Medisch Genetisch Centrum Zuid-West Nederland (MGC).

The research has been funded by the Netherlands Genomics Initiative (NGI), the Landsteiner Foundation for Blood Transfusion Research, and the Netherlands Scientific Organization (NWO).

Cover Design: Margarita Fani

Layout: Pavlos Fanis

Printed by Wöhrmann Print Service, Zutphen

Functional Proteomics Analysis of Transcription Factor Networks in Erythroid Cells

Functionele analyse van transcriptiefactor netwerken in erythroïde cellen met behulp van proteomics

Thesis

to obtain the degree of Doctor from the

Erasmus University Rotterdam

by command of the

rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board

The public defense shall be held on

Thursday 20 October 2011 at 13.30 hours

by

Pavlos Fanis

born in Limassol, Cyprus

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To my family and friends

Contents

LIST OF ABBREVIATIONS

5FMC	Five Friends of Methylated Chtop
BirA	Biotin Protein Ligase
CARM1	Coactivator-associated arginine methyltransferase 1
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin Immunpprecipitation
Chtop	Chromatin Target of Prmt1
CoREST	Co-Repressor for RE1-Silencing Transcription factor
DBF	DNA Binding Factor
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase
Fop	Friend of Prmt1
FRAP	Fluorescence Recovery after Photobleaching
FLIP	Fluorescence loss in photobleaching
GAR	Glycine Arginine Rich
GST	Glutathione S-transferase
GTF	General Transcription Factors
HDAC	Histone Deacetylase
HP1	Heterochromatin Protein 1
IgG	Immunoglobulin G
IP	Immunoprecipitation
Jmjc	Jumonji C domain
Las1L	Las1 like
LSD1	Lysine Specific Demethylase 1
MBD	Methyl CpG Binding Domain
MEL	Murine Erythroleukemia
MMA	Monomethylarginine
mRNA	messenger RNA
MS	Mass Spectrometry
NoLS	nucleolar localization signal
NR	Nuclear Receptor
RBP	RNA Binding Protein
NuRD	Nucleosome Remodeling and Deacetylase
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
Pelp1	Proline, Glutamate and Leucine rich protein 1
PIC	Pre-Initiation Complex
PRC	Polycomb Repressive Complex
PRMT	Protein Arginine Methyltransferase
PTM	Post-translational Modification
aDMA	asymmetric arginine dimethylation
RNA Pol II	RNA Polymerase II
RNA	Ribonucleic acid
RNAi	RNA interference
RT-QPCR	Reverse transcription quantitative PCR
sDMA	symmetric arginine dimethylation
SENP	SUMO/sentrin specific peptidase
shRNA	short hairpin RNA
SIM	Sumo Interacting Motif
SRAG	Small protein Rich in Arginine and Glycine
ER(α)	Estrogen Receptor (alpha)
Su(VAR)	Suppressor of variegation
SUMO	Small Ubiquitin – like MOdifier
Tex10	Testis Expressed protein 10
TF	Transcription Factor
Wdr18	WD repeat domain 18
Zbp	Zinc finger binding protein

SCOPE OF THE THESIS

Post translational modifications (PTMs) are critical for regulating the function of many proteins. PTMs are widespread in eukaryotic cells and catalysed by specific enzymes. An important PTM is arginine methylation which is involved in many cellular processes, such as RNA metabolism, DNA damage repair, DNA replication and transcriptional regulation. Arginine methylation is catalysed by a family of enzymes called protein arginine methyltransferases (PRMTs). PRMT1 is ubiquitously expressed and is responsible for the majority of total protein arginine methylation activity in mammalian cells. The focus of the research described in this thesis is the isolation of Prmt1 associating proteins and the subsequent characterization of Chromatin target of Prmt1 (Chtop), one of the identified interactors.

Chapter 1

Introduction

INTRODUCTION

General Introduction

The hereditary information of all living cells consists of DNA which in eukaryotes is located in the nucleus of the cell. In a somatic human cell, the DNA measures ~2m in length and is divided over 46 chromosomes. In order to fit into the small volume of the nucleus, the long DNA molecules are folded and compacted by proteins in a structure called chromatin. The first level of condensation is the formation of a nucleosome: 146 bp of DNA is wrapped twice around a core of histone proteins, composed of two copies of the H2A, H2B, H3 and H4 proteins (Alberts, 2008; Luger et al., 1997). The nucleosomes are further organized into the 30nm fiber, a higher chromatin organization that is shown only by *in vitro* studies (Horn and Peterson, 2002). The next stage of DNA packaging is when the 30nm fiber is compacted into even thicker fibers (Belmont and Bruce, 1994; Horn and Peterson, 2002). The folding of chromosomes at the previously described levels results in highly compacted interphase chromosomes. The different cell types in a multicellular organism differ dramatically in structure and function. The distinct cell types arise as a result of the expression of different sets of genes. Genes are transcribed into messenger RNA (mRNA), which thereupon is translated into proteins. In eukaryotes, the transcription of genes into mRNA is performed by the RNA polymerase II (RNA Pol II) enzyme. The activity of RNA Pol II is regulated by a large protein complex, which consists of the general transcription factors (GTF). Together, RNA Poll II and the GTF form the pre-initiation complex (PIC) (Alberts, 2008; Dynlacht et al., 1991). However, this basal transcription machinery alone is not sufficient to initiate or to direct transcription efficiently. RNA Poll II transcription is directed by cis control elements in the proximal promoter and distal enhancer regions in the genome. DNA binding factors bind to the cis control regions and recruit cofactors (corepressors and/or coactivators) to direct the activity of the basal transcription machinery (Kadonaga, 2004; Ptashne and Gann, 1997). Most transcriptional regulators can

not bind to compacted DNA efficiently. However, some transcription factors are able to bind their target sequence on packaged DNA. Once bound, they can recruit enzymes that target either the histone tails or change the conformation of nucleosomes on the DNA template. This loosens the packaging grade of the DNA, resulting in enhanced accessibility of transcription machinery to the DNA (Lodish, 2003; Seila et al., 2008; Tong et al., 1998).

Epigenetic Regulation – DNA methylation, histone modifications

Regulation of mammalian transcription is a complex and dynamic process. Gene regulation is not achieved only by the binding of transcription factor to specific sequences in the genome, but also by mechanisms that occur by chemically modifying the DNA and nucleosomes at their target regions. These mechanisms, collectively referred to as epigenetic modifications, usually result in changes in chromatin structure and DNA accessibility leading to maintenance or alteration of gene expression in these regions. Epigenetic regulation is achieved by two categories of chemical modifications, methylation of cytosine residues in the primary DNA strand and modification of histones, such as methylation and acetylation, which make up the nucleosomes. In general, epigenetic modifications are maintained during cell division and they may even by passed on to the next generation of the organism (Bernstein et al., 2007).

DNA methylation

DNA methylation is essential for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, suppression of repetitive elements and carcinogenesis. In DNA, methylation usually occurs at cytosine residues in the context of CpG dinucleotides. Unmethylated CpGs are often grouped in clusters called CpG islands, GC-rich regions often associated with the 5' regulatory regions of genes.

In mammals, DNA methylation is carried out by a group of enzymes called DNA

methyltransferases (DNMTs). These enzymes not only determine the DNA methylation patterns during early development, but are also responsible for copying these patterns to the new strands generated from DNA replication (Ioshikhes and Zhang, 2000; Klose and Bird, 2006). Methylation at CpG islands may affect the transcriptional silencing in two ways. First, the methylation of DNA itself physically prevents the binding of transcriptional regulators to the gene, and second, methylated DNA recruits proteins known as methyl CpG binding domain (MBD) proteins. MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins, which deposit repressing histone modifications, thereby forming compact inactive chromatin (Jaenisch and Bird, 2003; Klose and Bird, 2006). The MBD-containing MeCP2 protein targets the Sin3A histone deacetylase, while the MBD3 protein targets components of the Nucleosome Remodelling and histone Deacetylase (NuRD) complex (Bird, 2002; Fuks et al., 2003; Nan et al., 1998; Zhang et al., 1999). The importance of DNA methylation in gene regulation is clearly evident from the enrichment of this modification in promoter regions. Between 60-70% of the human promoters contain CpG islands, of which their methylation is dependent on the tissue, the developmental stage where the gene is expressed and on the function of the gene itself (Bernstein et al., 2007; Fazzari and Greally, 2004; Saxonov et al., 2006; Weber et al., 2007). In many disease processes, such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in transcriptional silencing that can be inherited by daughter cells following cell division. Alterations of DNA methylation have been recognized as an important component of cancer development (Daura-Oller et al., 2009).

Histone modifications

As described in a previous paragraph, the nucleosome is the fundamental unit of chromatin and is composed of an octamer of the four core histones (H3, H4, H2A, and H2B). Histones are small basic proteins consisting of a globular domain and

more flexible and charged N-terminal tails. Specific amino acids of the histone tails can be subjected to different posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation. Modifications have been identified at more than 60 different amino acid positions on histones.

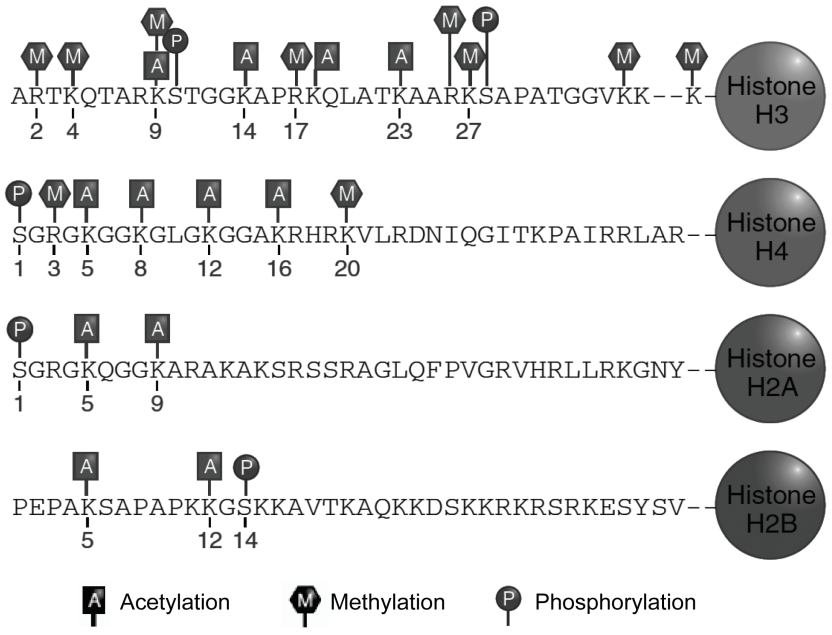

Figure 1. Summary of methylation, acetylation and phosphorylation histone modifications. The amino acid sequences of the core histone tails and amino acid residues that are subject to epigenetic modifications are shown. Of particular significance are mono-, di-, or tri-methylation (M) at arginine or lysine residues; acetylation (A) at lysine residues and phosphorylation (P) at serine residues. Some amino acids, such as lysine 9 of histone H3, can be subjected to either acetylation or methylation, but not both. Adapted and modified from (Dressler, 2008).

Additional complexity comes from different degrees of methylation (mono-, di-, or tri-methylation) for lysines and mono- or di- (symmetric or asymmetric) for arginines on the same residue, each having its own function (Jenuwein and Allis, 2001; Kouzarides, 2007). The majority of amino acid residues that can be modified are identified at the N-terminal tails of histones H3 and H4 (Figure 1). Most of the histone modifications are involved in transcription activation or repression, but some

modifications play role in DNA replication, recombination or repair (Kouzarides, 2007). The large number of possible combinations of modifications present on a nucleosome, has led to the hypothesis of a “histone code” (Jenuwein and Allis, 2001). There are two characterized mechanisms for the function of histone modifications. First, the modifications can affect the higher-order structure of chromatin by disrupting the contacts between nucleosomes and second, they can recruit non-histone proteins. These proteins carry with them enzymatic activities that further modify the chromatin (Kouzarides, 2007). The regulatory histone modifications that have been characterized most extensively, and which are most relevant to the scope of this thesis, are methylation, acetylation and phosphorylation of histone H3.

In the genome there are two major types of chromatin environment, the transcriptionally silent heterochromatin and the transcriptionally active euchromatin. This broad organization of chromatin can be divided in five principal chromatin types proposed by van Steensel in drosophila as the five “colours” of chromatin indicated by the colours YELLOW, RED, BLUE, BLACK and GREEN. In this review, most transcriptionally active genes are marked by YELLOW or RED chromatin. YELLOW chromatin harbours components of basal transcription machinery, some DNA binding factors (DBFs) and enzymes that control histone acetylation and marks ubiquitously expressed housekeeping genes. RED chromatin is also associated with active genes and marks primarily genes that are tissue specific. Regions marked by RED chromatin are bound by enormous diversity of proteins. BLUE chromatin is characterized by the presence of polycomb (PcG) proteins and the histone mark H3K27me3 (Filion et al., 2010). PcG proteins generally repress transcription and exhibit a strong preference to bind genes involved in the regulation of developmental processes. BLACK chromatin covers almost half of the non-repetitive genome, while all genes embedded in BLACK chromatin are transcriptionally silent. Finally, GREEN chromatin occupies large domains in pericentric regions and is specifically marked by Heterochromatin Protein 1 (HP1) and

SU(VAR)3-9, together with several HP1-associated proteins and the histone modifications H3K9me2 and H3K9me3 (Filion et al., 2010; Greil et al., 2007; Riddle et al., 2011). HP1 and SU(VAR)3-9 are known for the ability to repress genes that are integrated into HP1-rich regions (Girton and Johansen, 2008).

Each type of chromatin is associated with a distinct set of histone modifications. Transcriptional repression is usually associated with tri-methylation of histone H3 at lysine 9 (H3K9me3), of histone H3 at lysine 27 (H3K27me3) and of histone H4 at lysine 20 (H4K20me3) that were thought to be permanent modifications (Jenuwein and Allis, 2001; Kouzarides, 2007; Martin and Zhang, 2005). Although, two families of histone demethylases were discovered. The first, was Lysine Specific Demethylase 1 (LSD1) enzyme which can demethylate mono- and di- methylated lysines, specifically H3K4 and H3K9 (Shi et al., 2004). The second, were the Jumonji domain-containing (JmjC) histone demethylases which can demethylate mono-, di- or tri- methylated lysines (Tsukada et al., 2006).

H3K9me3 is implicated in the silencing of genes by recruiting HP1 to the promoter of repressed genes, and in forming silent heterochromatin (Bannister et al., 2001; Kwon and Workman, 2008). In contrast to tri-methylation, mono- and di-methylation marks at H3K9 are mainly enriched at promoters in euchromatic regions (Lachner and Jenuwein, 2002; Martin and Zhang, 2005). The two most important histone lysine methyltransferases involved in depositing H3K9 mono- and dimethylation are G9a and GLP. Deficiency of G9a leads to upregulation of specific genes (Tachibana et al., 2002; Tachibana et al., 2005). Like tri-methylated H3K9, mono- and di-methylated H3K9 also attract HP1 to its sites on the chromatin template (Nielsen et al., 2001; Tachibana et al., 2005). Is not well understood how HP1 silences gene expression, though HP1 has also been found to interact with the DNA methyltransferase DNMT3b, thereby possibly inducing promoter DNA methylation (Lehnertz et al., 2003). H3K27me3 has been implicated in silencing of the inactive X chromosome and silencing of specific genes, such as HOX genes (Martin and Zhang, 2005). Repression by H3K27me3 is associated with the

polycomb repressive complex 2 (PRC2), which tri-methylates H3K27 via its EZH2 subunit (Cao et al., 2002; Kirmizis et al., 2004). Silencing is further induced by PRC1 binding to the H3K27me3 mark, preventing chromatin remodeling (Shao et al., 1999). Moreover EZH2 targets the DNA-methyltransferases DNMT1, DNMT3a and DNMT3b to promoters, leading to simultaneous DNA methylation (Vire et al., 2006). Very little is known about H3K27 mono- and di-methylation. In a recent genome-wide study on human CD4+ T-cells, H3K27me3 was enriched mainly at the promoters of silent genes. H3K27me2 was also mainly enriched at promoters, with less distinction between promoters of active and inactive genes. In contrast, H3K27me1 was exclusively enriched at active promoters (Barski et al., 2007).

While H3K9me3, H3K27me3 and H4K20me3 are strongly associated with transcriptional repression, tri-methylation of H3K4 (H3K4me3) and H3K36 (H3K36me3) are associated with transcriptional activation. For instance, H3K4me3 localized at the promoters and the 5´ end of active genes and is associated with the serine 5 (Ser5) phosphorylated form of RNA Pol II (Li et al., 2007). Rather, H3K4me3 is most abundant at promoters and the 5´ of genes, H3K4me2 is distributed through genes and H3K4me1 is most enriched at the 3´ of genes (Li et al., 2007; Pokholok et al., 2005). Like H3K4me3, histone H3 acetylation is associated with transcription activation. Acetylation of histone H3 occurs at lysine residues in the N-terminal tail which are exposed on the surface of the nucleosome core (Figure 1).

The reaction of acetylation is catalyzed by enzymes with histone acetyltransferases (HAT) activity (Sterner and Berger, 2000). Acetylation of histones brings in a negative charge which neutralizes the positive charge of the histones and subsequently decreases the interaction of the N-termini of histones with the negatively charged DNA. This would then allow nucleosomal DNA to become more generally relaxed, enabling remodeling of nucleosomes or allowing former hidden sites to be accessible by sequence-specific transcription factors or RNA Pol II (Hong et al., 1993; Kouzarides, 2007). In contrast to the quite stable methylation marks of H3K9 and H3K27, histone H3 acetylation marks are highly dynamic. The relaxation of

nucleosomal DNA by histone acetylation can be reversed by enzymes with histone deacetylase (HDAC) activity. HDACs are present in the large complexes such as the NuRD complex and Co-repressor for RE1-silencing Transcription factor (CoREST) complex (Gray and Ekstrom, 2001). Interestingly, NuRD complex binds to MBD3 protein that interacts with methylated DNA. The importance of this cross-talk between HDACs and DNA methylation is shown by the finding that loss of DNA methylation leads to hyperacetylation of histone H3 and hypomethylation of H3K9 in human cells, indicating that DNA methylation is required to maintain the repressive chromatin state (Espada et al., 2004; Fuks, 2005; Zhang et al., 1999).

Phosphorylation of histone H3 at serine10 has dual and opposing roles in interphase and metaphase. During interphase, phosphorylation of H3 at Ser-10 can facilitate transcription of the immediate early genes (Labrador and Corces, 2003; Mahadevan et al., 1991; Strelkov and Davie, 2002), whereas during mitosis, such phosphorylation facilitates chromosome remodeling and condensation (Prigent and Dimitrov, 2003; Wei et al., 1998). Global phosphorylation of histone H3 occurs in a step wise and ordered manner during chromosome assembly (Sauve et al., 1999). In mammalian cells in late G2 phase, phosphorylation is first detected in pericentromeric heterochromatin and, as mitosis proceeds, spreads throughout the whole chromosomes. It is completed in late prophase and maintained through metaphase. Dephosphorylation of histone H3 begins in anaphase and ends at early telophase. A strong correlation between the initial chromatin condensation and H3 phosphorylation was observed (Hans and Dimitrov, 2001; Hendzel et al., 1997; Van Hooser et al., 1998)

Posttranslational modifications - Arginine methylation, Sumoylation

Posttranslational modifications (PTMs) are not restricted to histones, they are crucial for regulating the functions of most, if not all, eukaryotic proteins. Among the prominent PTMs are: (1) serine, threonine, and tyrosine phosphorylation, (2) lysine acetylation, lysine ubiquitination, and lysine sumoylation, and (3) lysine and argin-

ine methylation. In the next paragraphs will be discussed the most relevant to the scope of the thesis PTMs: arginine methylation and sumoylation.

Arginine methylation

A wide variety of methylation reactions occurs at the side chains of lysine and arginine residues. These modifications generate distinct sets of chemical interactions that play roles in a numerous of regulatory pathways (Clarke and Tamanoi, 2006). Modification of arginine residues in proteins can modulate their binding interactions leading to regulation of their physiological functions. In mammalian cells three types of methylated arginine residues occur, (1) the asymmetric arginine dimethylation (aDMA), (2) the symmetric arginine dimethylation (sDMA) and (3) the mono-methylation of arginine (Paik and Kim, 1980).

Protein arginine methyltransferases (PRMTs)

In mammals, arginine methylation is a prevalent posttranslational modification found in proteins in the nucleus, cytoplasm and other organelles (Bedford and Richard, 2005). Proteins that are arginine methylated are involved in a number of different cellular processes, including RNA metabolism, DNA damage repair, DNA replication and transcriptional regulation (Bedford and Richard, 2005). Methylated arginines in proteins are often flanked by one or more glycine residues, but there are exceptions to this general rule (Gary and Clarke, 1998). The methylation of arginine residues is catalyzed by the protein arginine methyltransferase (PRMT) family of enzymes which consist of nine members, PRMT1-9 (Bedford and Clarke, 2009). Most PRMTs methylate glycine and arginine-rich domains (GAR motifs) within their substrates. PRMTs are classified in two major types, type I and type II enzymes. Type I enzymes catalyze the formation of aDMA, whereas type II enzymes catalyze the formation of sDMA (Figure 2) (Bedford and Clarke, 2009).

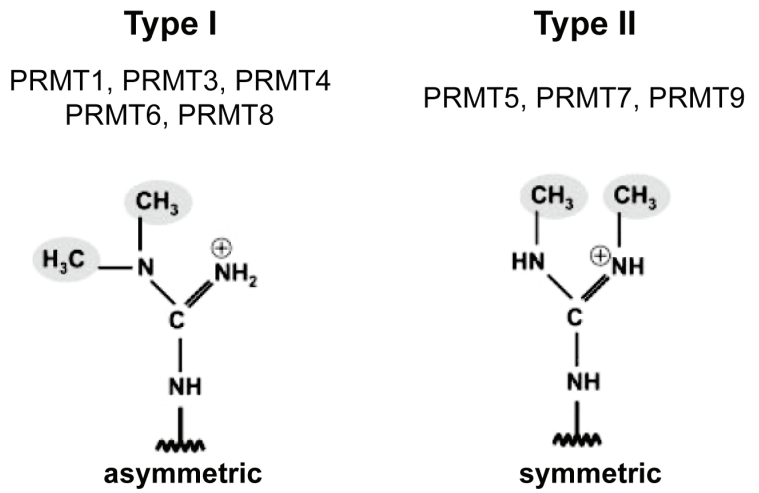

Figure 2. Methylated arginine residues and PRMTs. Type I PRMTs that catalyzed the formation of asymmetrically dimethylated arginine (aDMA) and type II PRMTs that catalyzed the formation of symmetrically dimethylated arginine (sDMA). Adapted and modified from (Bachand, 2007).

To date, three mammalian PRMTs have been found to catalyze histone methylation at their N-terminal tail: the major type I enzymes PRMT1 and PRMT4/CARM1 (co-factor associated arginine methyltransferase 1) and the type II enzyme PRMT5 (Pal et al., 2004; Schurter et al., 2001). PRMT4 methylates histone H3 at arginines 2, 17 and 26, PRMT1 targets histone H4 at arginine 3 for methylation and PRMT5 methylates histone H3 at arginine 8 and histone H4 at arginine 3 (Figure 3) (Chen et al., 2000; Schurter et al., 2001; Strahl et al., 2001; Zhang and Reinberg, 2001). Whether other arginine sites, such as R17, R19, R22 in H4, or any arginine residues within the histone globular domains are methylated, remains to be established (Figure 3). PRMT1 and PRMT4, the major type I enzymes, are both critical for mammalian development. PRMT1 and PRMT4 knock-out mice embryos fail to develop, however PRMT1 knock-out ES cells are viable (O'Brien et al., 2010; Pawlak et al., 2000).

PRMTs and transcription regulation

PRMTs can methylate and regulate transcription factors, other coactivators, and histones. As described above, the ability to methylate histones provides a direct line into the epigenetic regulation of gene expression. However, the arginine methylation of coactivators, such as p300/CBP and SRC3 acetyltransferases, provides an indirect mechanism of regulation of gene expression by regulating the activity of these acetyltransferases. PRMT4 was first shown to coactivate nuclear receptor (NR) – mediated transcription through histone methylation (Chen et al., 1999). Furthermore, PRMT1 was also shown to stimulate histone acetyltransferase activity of p300 and act as a coactivator of NR-mediated transcription (Lee et al., 2002). In transient transfection assays, PRMT4 and PRMT1 cooperate and enhance NR-mediated gene activation (Bedford and Richard, 2005). PRMT1 and PRMT4 were initially identified as coregulators of estrogen and androgen receptors, however, they are also recruited to promoters by a number of transcription factors such as p53, YY1 and NF-κB resulting in the methylation of additional coactivators and histones (Wang et al., 2001; Zhao et al., 2008). Although PRMT1 methylates transcription coactivators, it can also directly methylate transcription factors. For example STAT1 methylation by PRMT1 enhances its transcriptional activity (Mowen et al., 2001). Another target is RUNX1 and its methylation triggers the dissociation of the transcriptional repressor SIN3A, thereby promoting RUNX1 transcriptional activity (Zhao et al., 2008). Methylation of histones by PRMT1 and PRMT4, both type I enzymes, has been linked to transcriptional activation. In contrast, histone methylation by PRMT5, a type II enzyme, has been associated with transcriptional repression (Cosgrove et al., 2004; Schurter et al., 2001). PRMT5 was identified as a component of a large repressor protein complex containing mSin3/HDAC histone deacetylase and BRG1/hBRM chromatin remodeling complex and efficiently methylates H3R8 and H4R3 when histones H3 and H4 are hypoacetylated (Pal et al., 2004; Pal et al., 2003). H4R3 symmetric methylation (H4R3me2s) by PRMT5 is required for subsequent DNA methylation. H4R3me2s serves as a direct binding

target for the DNMT3A which associated with repressed chromatin, linking histone and DNA methylation in gene silencing (Zhao et al., 2009).

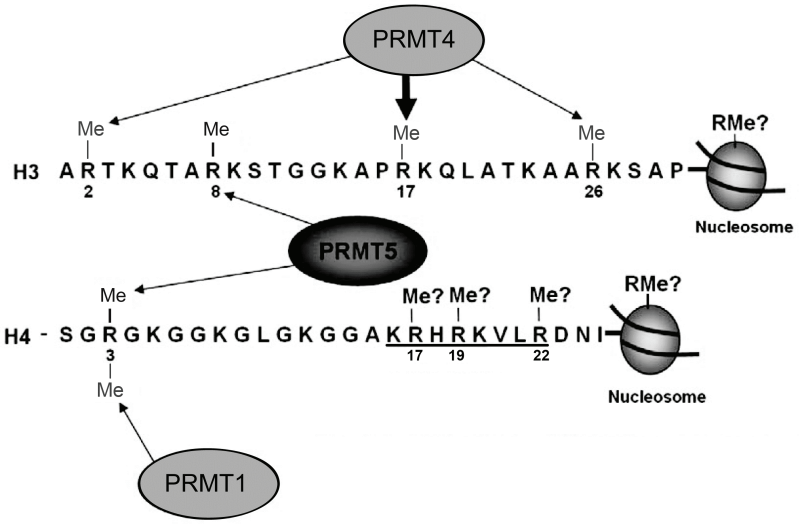

Figure 3 Sites of arginine methylation on histone H3 and H4. PRMT4 methylates arginine residues 2, 17 and 26 on histone H3 (the major PRMT4 target site, H3R17 indicated by the large arrow). PRMT1 methylates arginine 3 on histone H4. PRMT5 methylates arginine 8 on histone H3 and arginine 3 on histone H4. It remains to be determined whether arginine 17, 19 and 22 on histone H4 and possibly other arginine residues within the histone H3 and H4 core peptide sequence are methylated. Adapted and modified from (Wysocka et al., 2006).

PRMTs and protein-protein interactions

The role of arginine methylation in regulating protein-protein interactions is well documented. In yeast, the methylation of the heterogeneous ribonucleoproteins (hnRNPs) Npl3p and Hrp1p and RNA-binding proteins (RBPs) Sam68 and RNA helicase A is critical for their proper cellular localization (Shen et al., 1998; Yu et al., 2004). Arginine methylation facilitates the interaction of GAR motifs with tudor domains. The symmetric dimethylation of SmB by PRMT5 is required for its interaction with the Tudor domains of SMN, SPF30 and TDR3 (Cote and Richard, 2005). The asymmetric dimethylation of CA150 by PRMT4 also provides a docking site for the Tudor domain of SMN (Cheng et al., 2007a). Arginine methylation can

also act as a negative regulator of protein-protein interactions. For example, the methylation of Sam68 can block its binding to SH3, but not WW domains (Bedford et al., 2000). A second example of blocking protein-protein interaction by arginine methylation is the PRMT4-mediated modification of the GRIP1-binding domain of p300 (Lee et al., 2005). Finally, PRMT6-mediated methylation of H3R2 completely blocks the interactions of WDR5, component of MLL1 complex, with H3 (Couture et al., 2006). Methylation of H3R2 prevents the MLL1 complex from methylating H3K4 (Hyllus et al., 2007). H3K4 methylation is responsible for recruiting chromatin remodeling enzymes to establish and maintain a transcriptionally active state (Iberg et al., 2008).

Relatively little is known about the regulation of PRMT activity. Only a few proteins have been described that modulate PRMT function. BTG1 and TIS2/BTG2 bind PRMT1 and stimulate its activity toward selected substrates (Lin et al., 1996). Binding of the tumor suppressor DAL-1 inhibits PRMT3 enzymatic activity (Singh et al., 2004). Evidence that PRMTs themselves are regulated by posttranslational events was recently described (Higashimoto et al., 2007). In this case, PRMT4 can be phosphorylated during mitosis preventing its homodimerization and decreasing its enzymatic activity.

Sumoylation

The posttranslational modification of sumoylation is a major regulator of protein function that has a role in a wide range of cellular processes (Hay, 2005). In mammals there are four SUMO (small ubiquitin-like modifier) paralogs, SUMO-1 to SUMO-4. SUMO-2 and SUMO-3 are 95% identical, while they share only 45% similarity with SUMO-1 (Saitoh and Hinchey, 2000; Su and Li, 2002). The SUMO-4 paralog is very similar (~87%) to SUMO-2 and SUMO-3 (Wei et al., 2008). Sumoylation involves the covalent attachment of a SUMO peptide to specific target proteins via a series of enzymatic steps similar to the ubiquitination pathway. SUMO conjugation occurs through a cascade of reactions that are performed by an E1

activating enzyme, an E2 conjugating enzyme (Ubc9) and a SUMO E3 ligase (Hershko and Ciechanover, 1998; Johnson, 2004). The initially synthesized SUMO precursors are inactive until the C-terminal extensions are cleaved by sentrin-specific proteases (SENPs), which act as isopeptidases to expose the C-terminal diglycine (GG) motif for subsequent conjugation (Figure 4) (Xu et al., 2009). SENPs also act as desumoylation enzymes (see below). The target protein consensus motif

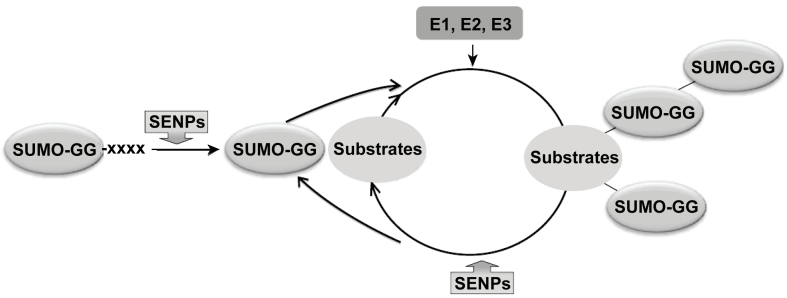

Figure 4. The small ubiquitin-related modifier (SUMO) conjugation pathway. SUMOs target proteins via ATP-dependent enzymatic cascade that requires the activating enzyme (E1) and conjugation enzyme (E2). The initially synthesized SUMO precursors are inactive until the C-terminal extensions (xxxx) are cleaved by sentrin-specific proteases (SENPs), which act as isopeptidases to expose the C-terminal diglycine (GG) motif for subsequent conjugation. The presence of E3 ligases stimulates the efficiency of SUMO conjugation and promotes the formation of poly-SUMO chain. SENPs also act as desumoylation enzymes. Adapted from (Wang, 2011).

comprises ψKxD/E (where ψ is a large hydrophobic residue and x is any amino acid). Approximately 75% of the known SUMO substrates are modified within the consensus motif (Xu et al., 2008). SUMO is also able to associate with proteins in a non-covalent manner through the SUMO interacting motifs (SIMs) present in certain proteins (Kerscher, 2007). SUMO modification modulates many processes such as nuclear transport, protein stability, chromosome segregation, recombination and transcription regulation (Wilkinson and Henley, 2010). For example, the SUMO-1 modification of RanGAP1 (the first identified SUMO substrate) leads to trafficking from cytosol to nuclear pore complex (Mahajan et al., 1997; Matunis et al., 1996).

Histone sumoylation was first shown in mammal cells for histone H4 (Shiio and Eisenman, 2003) and later was shown for all core histones in yeast (Nathan et al., 2006). Sumoylation of core histones in yeast compete with other modifications of the N-terminal tail, such as ubiquitination and acetylation (Nathan et al., 2006). However, in mammal cells sumoylation of H4 is enhanced upon expression of the histone acetyltransferase p300 (Shiio and Eisenman, 2003). SUMO-H4 and SUMO-H2B fusions have similar consequences in repressing transcription. For instance, SUMO-H4 co-immunoprecipitates HDAC1 and HP1γ (Nathan et al., 2006; Shiio and Eisenman, 2003). These results suggest that histone sumoylation might repress transcription, at least partially, through the recruitment of repressor proteins or complexes (will discussed later in this introduction). In case of transcription, SUMO modification of transcriptional regulators correlates with inhibition of transcription as it promotes the recruitment of repressive complexes (see below) (Garcia-Dominguez and Reyes, 2009; Ouyang and Gill, 2009).

Sumoylation can be reversed by the action of specific enzymes, SENPs, that are responsible for SUMO deconjugation. In addition, SENPs are involved in the maturation of pro-SUMO, as described above. In yeast they are called Ubl (ubiquitin-like-protein)-specific proteases (Ulp) (Li and Hochstrasser, 1999). There are six SENPs in mammals, SENP1-3 and SENP5-7, which vary in their subcellular distribution and activities (Yeh, 2009). SENPs are divided into three groups. The first group consists of SENP1 and SENP2, which have broad specificity to SUMO1/2/3 (Gong et al., 2000; Hang and Dasso, 2002). The second group consists of SENP3 and SENP5, which favour SUMO2/3 over SUMO1 (Di Bacco et al., 2006; Nishida et al., 2000) and the third group consists of SENP6 and SENP7 which are not involved in the maturation of SUMO and are specific for removal of SUMO2/3 (Mukhopadhyay et al., 2006; Shen et al., 2009). All mammalian SENPs are present in the nucleoplasm, with SENP3 and SENP5 also localized in the nucleolus (Di Bacco et al., 2006; Nishida et al., 2000). SENP1 and SENP2 are critical for mammalian development (Cheng et al., 2007b; Yeh, 2009), while SENP5

knockdown results in an increase in the number of cells with multiple nuclei and aberrant nuclear morphology, defects in mitosis and/or cytokinesis, and decreased cell proliferation (Di Bacco et al., 2006). This supports the hypothesis that certain cell-cycle components need to be in a sumoylated state, while others have to be desumoylated for proper control of the cell cycle. Little is known about the function of SENPs in transcription regulation, although the number of transcription factors and regulators that are sumoylated is increasing. SENP1 was shown to be a regulator of androgen receptor dependent transcription through desumoylation of HDAC1, leading to reduction of its deacetylase activity allowing transcription to increase (Cheng et al., 2004). In addition, SENP1 also regulates c-Jun transcription by desumoylation of CRD1 (cell cycle regulator domain 1) of p300, thereby releasing the cis-repression of CRD1 on p300. Several transcription factors have implicated in SUMO-dependent repression. The transcription factor Sp3 is a ubiquitously expressed member of the Sp family of transcription factors. Sp3 can act as an activator or a repressor depending on the promoter context (Li et al., 2004; Valin and Gill, 2007) Sp3-mediated repression is dependent on the sumoylation at the carboxy terminus of the protein in mammals (Ross et al., 2002). Sumoylated Sp3 recruits several heterochromatin factors such as HP1α, HP1β, HP1γ, the H3K9-specific HMTase SETDB1 and the H4K20-specific HNTase SUV4-20H (Stielow et al., 2008b). RNAi mediated depletion of the drosophila polycomb protein dSfmbt, the ATPase Mi-2 and the zinc finger protein dMEP-1 which bind to SUMO and sumoylated Sp3, do not alter the level of sumoylation of Sp3 but impair its repression ability (Stielow et al., 2008a). Mammalian orthologs of drosophila dSfmbt, the MBT –domain protein L3MBTL1 and L3MBTL2 are recruited to a target gene by WT but not non-sumoytable Sp3 (Stielow et al., 2008b), suggesting that L3MBTL1 and L3MBTL2 may be involved in SUMO-dependent transcription repression in mammal cells. Since Sp3-SUMO recruits L3MBTL1 and L3MBTL2, which bind mono- and di- methylated H4K20, it is possible that these proteins help to recruit histone methyltransferases such as SUV-20HI and SUV-20H2 to get

H4K20 trimethylation.

Sumo association with repressor complexes

Literature on the role of sumoylation in transcription regulation is limited, mainly because SUMO modification is removed by isopeptidases and is very unstable on most proteins, but so far this modification has always been found in the context of transcriptional repression. This supports the model that SUMO interaction promotes the recruitment of repressor complexes that regulate chromatin structure leading to repression of genes (Ivanov et al., 2007; Ouyang et al., 2009; Stielow et al., 2008a; Stielow et al., 2008b). Sumoylation of several subunits of a complex is required to direct them to specific nuclear compartments. Furthermore, a structural-based hypothesis propose that sumoylation is required for the assembly of different factors that compose repressive chromatin complexes such as NuRD and CoREST, possibly through SIM-SUMO interfaces. Once they adopt the appropriated position and interact in the complex the SUMO modification is not required any more. SUMO directs diverse chromatin modifying enzymes to specific promoters to regulate transcription and chromatin structure. For instance, histone deacetylases (HDACs) are often found in corepressor complexes, such as CoREST and NuRD, and have been found to contribute to transcriptional repression mediated by SUMO (Ivanov et al., 2007; Ouyang et al., 2009). HDAC1 is shown to bind directly to SUMO (Ahn et al., 2009) but recent studies suggest that SUMO binding by factors that associated with HDACs may bridge SUMO and HDACs. For example, non-covalent binding of CoREST and NuRD complex components to SUMO may contribute to recruitment of HDAC1 and 2 to these complexes (Ivanov et al., 2007; Ouyang et al., 2009).

Moreover, histone demethylase LSD1 has also shown to be an effector of SUMO-dependent transcriptional repression. LSD1 binds non-covalently to Rcor1 which together with HDAC1 and 2 are core components of the CoREST corepressor complex (Ouyang et al., 2009). LSD1 catalyses the removal of methyl groups

from methylated H3K4 (mark of active transcription) promoting transcriptional repression (Shi et al., 2004). Another histone modifying enzyme which correlates with SUMO dependent transcriptional repression is the histone methyltransferase SETDB1. SETDB1 catalyzes tri-methylation of H3K9, which has been correlated

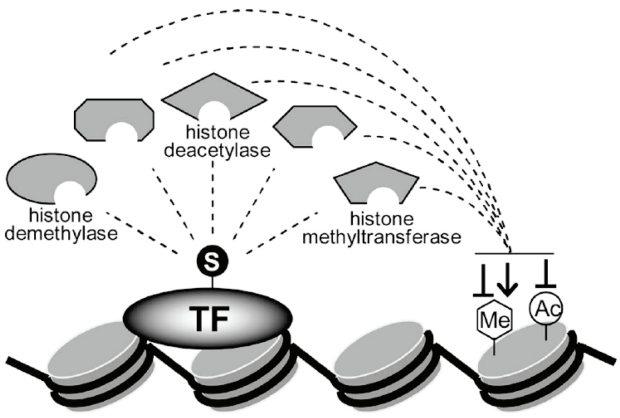

Figure 5. SUMO recruits multiple chromatin modifying activities to regulate transcription. SUMO (S) modification of a transcription factor (TF) recruits and/or coordinates specific chro¬matin modifying enzyme(s), which are components of corepressor complexes. These interactions support SUMO-dependent regulation of histone modifications and chromatin structure at specific promoters to repress gene expression. Adapted from (Ouyang and Gill, 2009).

with transcriptional repression. SUMO is conjugated with KAP-1 (KRAB associated protein 1) and mediates silencing of target genes by recruiting both SETDB1 and CHD3 (Mi-2) of the NuRD complex, leading to tri-methylation of H3K9 and histone deacetylation (Ayyanathan et al., 2003). Both SETDB1 and CHD3 can bind directly to SUMO via a SIM motif and interact with KAP-1 (Ivanov et al., 2007). Finally, a chromatin associated complex that mediates SUMO-dependent repression is the Polycomb repressive complex 1 (PRC1). Pc2 is a component of the PRC1 and binds the H3K27me3 mark, a modification that is carried out by PRC2. Pc2 can function as a SUMO ligase and has at least four SUMO substrates: the kinase HIPK2, the DNA methyltransferase DNMT3a, SIP1/ZEB2 and the transcriptional corepressor CtBP1 (Kagey et al., 2003; Lin et al., 2003; Roscic et al., 2006; Wotton

and Merrill, 2007). Sumoylation of HIPK2 and CtBP1 increases their repression activity (Lin et al., 2003; Roscic et al., 2006). In conclusion, sumoylation plays a critical role in directing of diverse chromatin modifying enzymes to specific promoters to regulate transcription and chromatin structure (Figure 5).

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Chapter 2

Friend of Prmt1, a Novel Chromatin Target of Protein Arginine Methyltransferases

Thamar Bryn van Dijk[1], Nynke Gillemans[1], Claudia Stein[2], Pavlos Fanis[1], Jeroen Demmers[3], Mariëtte van de Corput[1], Jeroen Essers[4], Frank Grosveld[1], Uta-Maria Bauer[2], and Sjaak Philipsen[1]

1. Department of Cell Biology, Erasmus MC, 3015 GE Rotterdam, the Netherlands
2. Institute for Molecular Biology and Tumor Research, Philipps-University Marburg, 35032 Marburg, Germany
3. Netherlands Proteomics Centre, Erasmus MC, 3015 GE Rotterdam, the Netherlands
4. Department of Genetics, Erasmus MC, 3015 GE Rotterdam, the Netherlands

(Mol Cell Biol. 2010 Jan;30(1): 260-272)

ABSTRACT

We describe the isolation and characterization of Friend of Prmt1 (Fop), a novel chromatin target of protein arginine methyltransferases. Human Fop is encoded by C1orf77, a gene of previously unknown function. We show that Fop is tightly associated with chromatin, and that it is modified by both asymmetric- and symmetric arginine methylation *in vivo*. Furthermore, Fop plays an important role in the ligand-dependent activation of estrogen receptor target genes including TFF1 (pS2). Fop depletion results in an almost complete block of estradiol-induced promoter occupancy by the estrogen receptor. Our data indicate that Fop recruitment to the promoter is an early critical event in activation of estradiol-dependent transcription.

INTRODUCTION

Arginine methylation is a widespread posttranslational modification in eukaryotic cells that is catalyzed by a family of enzymes called protein arginine methyltransferases (Prmts). Prmts use S-adenosyl-L-methionine (SAM) as a donor to transfer methyl groups to the side chain nitrogens of arginine residues. To date, nine Prmts have been identified in humans and they have been subdivided in two major classes. Type I enzymes (Prmt1, Prmt3, Prmt4, Prmt6, and Prmt8) promote the formation of asymmetrical ω-N^G, N^G-dimethylated arginines (aDMA), and type II enzymes (Prmt5 and Prmt7) form symmetrical ω-N^G, N'^G-dimethylated arginines (sDMA). ω-N^G-monomethylarginine (MMA) is thought to be an intermediate formed by both enzyme types. So far, methyltransferase activity of Prmt2 and Prmt9 has not been formally demonstrated (Bedford and Clarke, 2009; Pal and Sif, 2007). Although methylation does not change the overall charge of an arginine residue, it modulates intermolecular interactions by increasing steric hindrance and hydrophobicity and decreasing hydrogen bonding capacity. Furthermore, methylation protects the reactive guanidino groups of arginine residues against inappropriate modification by dicarbonyl reagents (Fackelmayer, 2005).

Prmt1 is ubiquitously expressed and is the predominant Prmt activity in mammalian cells. Although Prmt1-deficient embryonic stem cell lines are viable, Prmt1 knockout mice die around the onset of gastrulation, consistent with a fundamental and non-redundant function (Pawlak et al., 2000). The majority of previously identified Prmt1 substrates are nucleic acid binding proteins that play a role in RNA processing, DNA repair, signal transduction, and transcription (Bedford and Clarke, 2009; Pahlich et al., 2006). How Prmt1 recognizes its specific substrates and to what degree this is regulated by additional factors is only partially understood. Prmt1 has a high affinity for glycine-arginine-rich (GAR) regions and the majority of identified methylated arginines are located within such domains (Bedford and Clarke, 2009). The crystal structure of Prmt1 in complex with a GAR peptide reveals three different binding channels for these motifs (Zhang and Cheng, 2003). GAR regions are a common feature of many RNA-binding proteins (RBPs), including the heterogeneous ribonucleoproteins (hnRNPs). These proteins play roles in mRNA processing and transport and contain up to 65% of total nuclear DMA (Boffa et al., 1977; Liu and Dreyfuss, 1995). Although the arginines in these regions have been recognized as key residues in RNA-protein interactions, it remains to be determined whether methylation has a profound effect on protein-RNA interactions. In contrast, a role for arginine methylation in regulating protein-protein interactions is well documented. Methylation of the yeast hnRNPs Npl3p and Hrp1p and the RBPs Sam68 and RNA helicase A is critical for their proper cellular localization (Shen et al., 1998; Yu et al., 2004). Methylation of Sam68 regulates binding to SH3 domain-containing proteins, while binding to WW domains is unaffected (Bedford et al., 2000). Other interactions controlled by DMA include transcription factor complexes, such as the binding of Nip45 to Nfat and the binding of Cbp/p300 to Creb (Mowen et al., 2004; Xu et al., 2001).

Another mechanism of substrate recognition is regulated via controlled recruitment. For example, Prmts are recruited to promoters and other regulatory elements to control gene expression by methylation of histones and components of

the transcription machinery (Pal and Sif, 2007). Recruitment of Prmt1 by nuclear hormone receptors and the transcription factors p53, YY1/Drbp76, and Upstream stimulatory factor 1 (Usf1) results in local methylation of histone H4 at R3 (An et al., 2004; Huang et al., 2007; Rezai-Zadeh et al., 2003; Wang et al., 2001). This modification is critical for subsequent histone acetylation and further activation events (Huang et al., 2005).

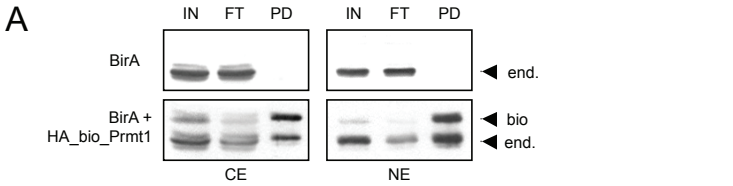
Little is known about the regulation of the enzymatic activity of Prmt1. As most target proteins appear to be entirely methylated at any given time, Prmt1 is considered to be a constitutively active enzyme. Prmt1 activity is abolished when dimerization is prevented and it has been suggested that Prmt1 is catalytically active only in the form of oligomers (Lim et al., 2005; Zhang and Cheng, 2003). In all cell lines tested, Prmt1 is a component of a 250-400 kDa complex, both in the cytoplasm and in the nucleus. It is unclear whether additional proteins are a constitutive component of this complex or whether it reflects a large Prmt1 polymer. Furthermore, only a limited number of Prmt1-interacting proteins have been described to affect Prmt1 activity under certain conditions. CCR4-associated factor 1 (Caf1) and the related proteins B-cell translocation gene 1 (Btg1) and Btg2/Tis21 bind Prmt1 and stimulate its activity toward selected substrates, while Protein phosphatase 2A (Pp2a) has an inhibitory effect (Duong et al., 2005; Lin et al., 1996; Robin-Lespinasse et al., 2007).

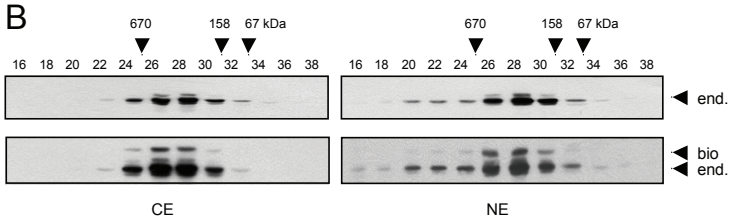
The identification and characterization of Prmt1-interacting proteins is critical for further understanding the role of Prmt1 in different cellular processes, and may answer questions regarding the regulation of Prmt1 activity and specificity. Thus far, Prmt1 substrates and Prmt1-interacting proteins have been identified through candidate approaches, serendipitous discovery, *in vitro* substrate screens, and proteomic strategies that identify proteins with methylated arginines (Boisvert et al., 2003; Ong et al., 2004; Wada et al., 2002). Here we describe single step isolation of Prmt1-associating proteins using a biotinylation-proteomics approach, and the characterization of a novel Prmt1-interacting protein, which we termed Friend of Prmt1 (Fop).

RESULTS

Identification of Prmt1-associated proteins by biotinylation tagging and mass spectrometry

Tagged Prmt1 (HA_bio_Prmt1) was generated by fusing an HA epitope and a short (23 aa) Bio-tag to its N-terminus. The Bio-tag is efficiently biotinylated by the bacterial BirA biotin ligase, which is coexpressed in stably transfected mouse erythroleukemia (MEL) cells (de Boer et al., 2003). HA_bio_Prmt1 was expressed below endogenous levels to reduce the likelihood that non-physiological interactions would be identified. Biotinylated Prmt1 was efficiently recovered from MEL extracts with magnetic streptavidin beads, and associated with endogenous Prmt1 in both the cytoplasm and the nucleus (Fig. 1A). Size fractionation experiments showed that HA_bio_Prmt1 behaved similar to endogenous Prmt1, eluting in fractions with a molecular mass range of 250-400 kDa (Tang et al., 1998) (Fig. 1B). Complexes with a molecular mass of more than 1 MDa were detected exclusively in the nuclear fraction. In contrast, Prmt1 with a C-terminal HA_bio-tag appeared to be monomeric (42 kDa; not shown). These experiments show that HA_bio_Prmt1 is faithfully incorporated into oligomeric complexes. Prmt1-associating proteins were identified by streptavidin pull down followed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) and compared to samples from cells expressing BirA alone. Putative Prmt1-interacting proteins identified in two independent HA_bio_Prmt1 pull downs are listed in Fig. 1C. The candidates are predominantly RNA-binding proteins involved in RNA processing (Rbmxrt/hnRNPG, hnRNPU, and Lsm14a), RNA stability (Serbp1), RNA export (Refbp2), translation (Msy4), and ribosome synthesis (Nol5a). All proteins contain GAR domains, suggesting that these proteins are direct targets for Prmt activity. Indeed, methylation of hnRNPs, including Rbmxrt/hnRNPG and hnRNPU, is well documented, while members of the SM/LSm family are previously identified targets of Prmt5 (Boisvert et al., 2002; Liu and Dreyfuss, 1995). Multiple known Prmt1 targets/-interacting proteins, including additional hnRNPs, U5 snRNP components, Sam68, and





C

		experiment 1		experiment 2	
Description	Gene ID	% cover.	pept. uniq.	% cover.	pept. uniq.
Prmt1	9790109	39,7	14	39,7	14
Rbmxrt	1327760	24,7	9	22,7	8
Msy4	7385223	19,1	6	24,4	8
C1orf77 homolog	54036077	23,2	4	23,2	4
hnRNPU	7949051	4,6	3	7,1	6
Serbp1	20988551	7,9	2	3,9	1
Refbp2	68534069	12,9	2	23,9	3
Nol5a	12805509	4,9	1	7,8	3
Lsm14a	21618722	6	2	6	2

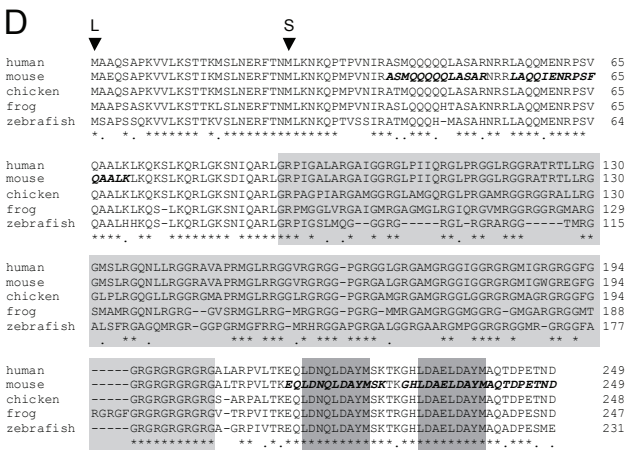


Figure 1. Biotinylation of Prmt1 in MEL cells. (A). Biotinylated Prmt1 (bio) is pulled down from cytoplasmic (CE) and nuclear extracts (NE) of MEL cells expressing the BirA biotin ligase. Since Prmt1 forms oligomers *in vivo*, endogenous (end) Prmt1 co-purifies with HA_bio_Prmt1 in streptavidin pull-downs. IN: input; FT: flow through; PD: pull down. (B). Size-fractionation profiles of Prmt1; the profile of tagged Prmt1 (bio) closely follows that of endogenous Prmt1 (end) in cytoplasmic (CE) and nuclear extracts (NE). Molecular mass markers are indicated on the top. Top panels indicate BirA control cells.

(C). Prmt1-interacting proteins identified by mass spectrometry. Gene ID (NCBI) and number of unique peptides identified are indicated. (D). Alignment of predicted full-length amino acid sequences of vertebrate Fop homologs (Fop_L). Transcripts lacking the first coding exon are found in human and mouse only and start at M26 (Fop_S). The GAR domain (light gray) and C-terminal duplication (dark gray) are indicated. Identified tryptic peptides are indicated in bold/italics.

fibrillarin were also detected, but these abundant proteins were also found in the BirA-only control samples as observed previously (de Boer et al., 2003). A newly identified putative Prmt1-interacting protein is encoded by the homolog of the human C1orf77 gene. The protein has not been characterized previously, and as it interacts with Prmt1 (see also Fig. 3), we named it Friend of Prmt1 (Fop). Fop has an expected molecular weight of 27 kDa and is highly conserved in all vertebrates (Fig. 1D), while no orthologs could be identified in yeast, worms, and flies. The protein has no known conserved domains, but its central sequence consists of a GAR domain that contains 26 RG/GR repeats, while the C-terminus harbors a duplication of the sequence LDXXLDAYM (where “X” is any amino acid). Furthermore, we note that the sequence of the GAR domain shows more variation (70 % conservation) when compared to the N- and C-termini (both 80% conservation).

Intracellular localization and expression pattern of Fop

For further characterization of the protein, monoclonal antibodies were raised against the N- and C-terminus of Fop. Both clone KT59 (specific for the N-terminus) and KT64 (specific for the C-terminus) recognized a protein running at the expected molecular weight of 27 kDa (Fig. 2A) and additional proteins of 23 and 25 kDa. These proteins were not detected in cells expressing an shRNA against Fop, suggesting that they represent full length Fop and smaller isoforms, respectively. It is possible that the 23 or 25 kDa isoform represent Fop_S, an isoform lacking the first 25 amino acids at the N-terminus (see also Fig. 1D). In immunoprecipitation (IP) experiments, the different isoforms of Fop were purified by both KT59 and KT64, although the 25 kDa isoform is masked by the IgG light chain of KT59 (Fig. 2B).

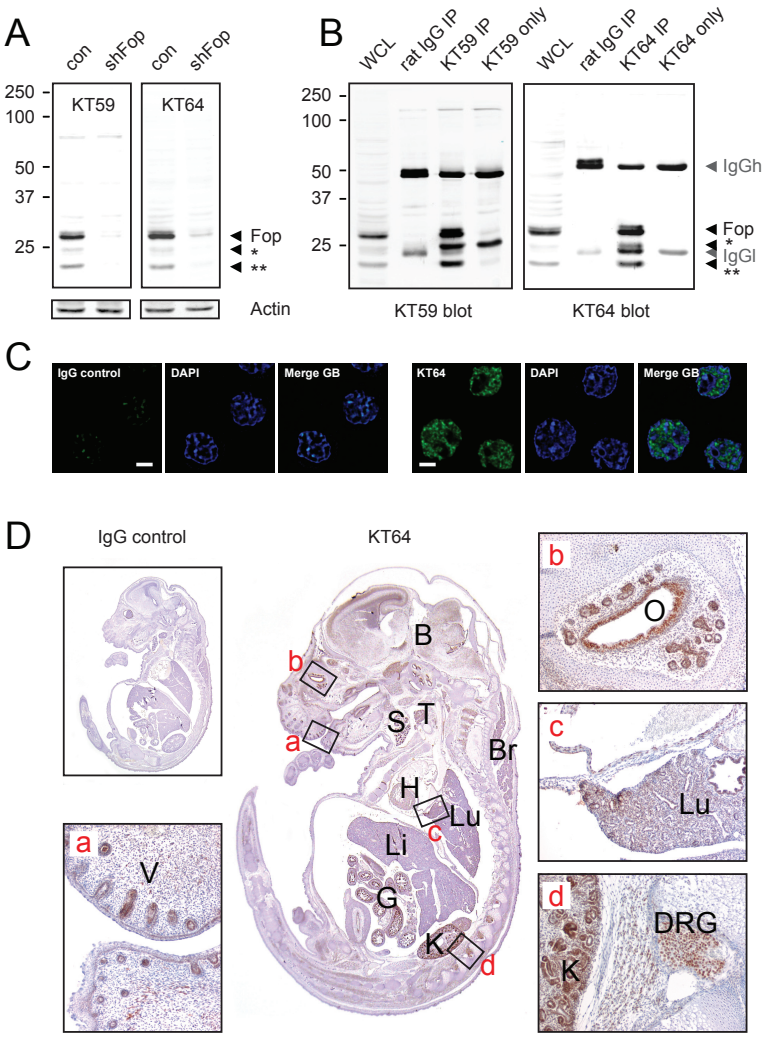


Figure 2. Intracellular localization and expression pattern of Fop. (A). A doublet of ~27 kDa and isoforms of ~25 kDa and ~23 kDa (indicated with '*' and '**', respectively) are recognized (A) and precipitated (B) by KT59, a monoclonal raised against the N-terminus of Fop (aa 1-90), and by KT64, raised against the C-terminus (aa 206-249). Detection of these proteins is sharply diminished in lysates from cells expressing an shRNA against Fop. (C). Confocal images showing that Fop localizes to DAPI-low regions in the nucleus and displays a granulated/speckle-like distribution in MEL cells. (D). Sagital paraffin sections of E16.5 mouse embryos were incubated with rat IgG control and KT64, followed by peroxidase staining. B = brain, Br = brown fat, DRG = dorsal root ganglion, G = gut, H = heart, Li = liver, O = olfactory epithelium, S = submandibular gland, T = thymus, V = follicles of vibrissae.

Full-length Fop appeared as a doublet, indicating that it might be a target for post-

translational modifications. Analysis by confocal microscopy showed that Fop is a nuclear protein localized to DAPI-low regions with a punctuate/speckle-like distribution (Fig. 2C, see also Fig. 5). Next, we determined the expression of Fop in E16.5 mouse embryos. We find that Fop has a wide, but not ubiquitous expression pattern (Fig. 2D). Tissues expressing Fop include the heart (H), lungs (Lu), gut (G), kidney (K), submandibular gland (S), thymus (T), follicles of the vibrissae (V), muscle, brown fat (Br), and neuronal cells, including brain (B), olfactory epithelium (O), and dorsal root ganglia (DRG) (Fig. 2D). Identical results were obtained with KT59 (not shown).

Fop is a novel Prm1-interacting protein

To validate the interaction between Prmt1 and Fop, we transiently co-transfected HA_Fop with wild type Myc_Prmt1 or the enzymatic inactive mutant Myc_Prmt1_E171Q in 293T cells. Wild type and mutant Myc_Prmt1, as well as endogenous Prmt1 are efficiently recovered in HA_Fop IPs, confirming the interaction between Prmt1 and the Fop protein (Fig. 3A). Cotransfection with wild type Prmt1 resulted in a slightly slower migration of HA_Fop, suggesting that Fop is modified by Prmt1 (Fig. 3A). Incubation with an antibody that specifically recognizes asymmetrically methylated arginines (Asym24) shows that Fop is indeed an aDMA-containing protein. Next, the interaction between endogenous proteins was studied using monoclonal antibodies KT59 and KT64. Fig. 3B shows that Prmt1 is detected in Fop purifications (left panel) and that Fop co-immunoprecipitates with Prmt1 (right panel), confirming that the endogenous proteins interact.

To identify the region of Fop that interacts with Prmt1, we generated a panel of Fop deletion mutants fused to the C-terminus of GST. The deletion series included two potential isoforms (Fop_L and Fop_S that lacks the first 25 amino acids, Fig. 1D) and progressive N- and C-terminal deletions (Fig. 3C). The GST_Fop fusions were incubated with whole cell extracts from MEL cells as a source of Prmt1. These results were obtained under stringent washing conditions (Ripa buffer containing

0.1% SDS, 0.5 % DOC, 1% NP40), indicating that the observed interactions are specific. The C-terminal half of the central GAR domain (from R153 to G205) was identified as the major interaction site, in line with previous observations that Prmt1 has a high affinity for GAR sequences (Bedford and Clarke, 2009; Pahlich et al., 2006). A second, weaker binding domain was found within the first 90 amino acids in the N-terminus (Fig. 3C; see also Fig. 4D).

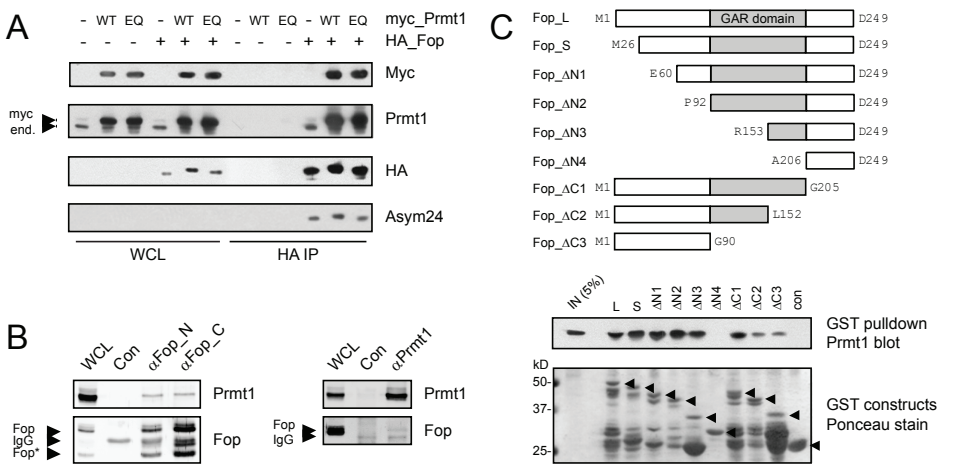


Figure 3. Fop is a Prmt1-associating protein. (A). 293T cells were co-transfected with HA_Fop and wild type Myc_Prmt1 (WT) or enzymatically inactive Myc_Prmt1_E171Q (EQ). HA_Fop was precipitated and blots were stained for Myc, Prmt1, HA, and an antibody recognizing asymmetrically methylated arginines (Asym24). Prmt1 binds and methylates HA_Fop. (B). Monoclonal antibodies specifically recognizing the N- and C-terminal domain of Fop confirm the interaction of endogenous Fop and Prmt1. The smaller isoform of Fop is indicated with *. (C). Upper panel: schematic representation of GST_Fop deletion constructs. Lower panels: GST constructs were incubated with MEL extracts as a source of Prmt1. Western blot analysis identified two regions in Fop that mediate binding to Prmt1: the N-terminal 90aa (Fop_ΔC3) and R153 to A206 (compare Fop_ΔC2 and Fop_ΔN3). Total protein staining served as loading control (lower right panel). Arrowheads indicate full-length protein GST fusion proteins. Fop_L: full length Fop, Fop_S: isoform lacking first 25 aa, con: GST only, IN: input MEL cell extract.

Fop is methylated by Prmt1 and Prmt5 *in vitro* and *in vivo*

To investigate whether Fop is a target of Prmt activity, we performed *in vitro* methylation assays using purified GST_Prmt1, GST_Prmt4, GST_Prmt6, and immunoprecipitated Prmt5, in the presence of methyl-14C-labeled SAM. Core histones served as a positive control (Fig. 4A and B, upper panels). We find that Prmt1 is

the only type I enzyme tested that is able to methylate Fop (Fig. 4A, lower panel). In addition, Prmt5 can use Fop as a substrate (Fig. 4B, lower panel), opening the possibility that Fop contains symmetrically methylated arginines *in vivo*. To investigate this further, we performed lentiviral-mediated knockdown of Prmt1 and Prmt5 in MEL cells. Reduction of the protein level of Prmt1 resulted in a dramatic shift of the migration pattern of Fop (Fig. 4C, upper panel), suggesting that Fop is heavily modified by Prmt1. To test this directly, endogenous Fop was immunoprecipitated from control cells and Prmt1 knockdown cells, and stained with the Asym24 antibody. This revealed that asymmetrical arginine methylation of Fop is severely reduced in the absence of Prmt1 (Fig. 4C, lower panel). This shows that: (1) target arginines of Prmt1 in Fop are methylated *in vivo*, (2) Prmt1 is the major type 1 enzyme that methylates Fop *in vivo*, and (3) the reduced mobility of Fop on SDS-PAGE correlates with the presence of aDMA residues.

Since Prmt5 can methylate Fop *in vitro*, we stained similar blots with antibodies specific for Prmt5 and symmetrically methylated arginines (Sym10). In control cells the interaction between Fop and Prmt5 can be detected (Fig. 4C). However, much more Prmt5 is co-immunoprecipitated with HA-Fop in the absence of Prmt1, indicating that Prmt1 and Prmt5 compete for binding to Fop. In line with the binding of Prmt5 and the *in vitro* methylation experiments, sDMA residues are detected in Fop, both in the presence and absence of Prmt1 (Fig. 4C). Interestingly, the increased binding of Prmt5 to Fop in the absence of Prmt1 does not result in elevated symmetrical methylation of Fop. In contrast, the Sym10 staining is less in the Prmt1 knockdown, suggesting that Prmt1 positively affects symmetric dimethylation of Fop. Partially knocking down Prmt5 did not reduce Sym 10 staining of Fop, although this did reduce the expression level of Fop to ~75% of that observed in control cells. It is unclear whether this is the result of incomplete Prmt5 depletion or that other type II Prmt enzymes can methylate Fop *in vivo*. This could not be tested, as a complete knockdown of Prmt5 resulted in cell death, as has been previously shown for transformed B cells (Pal et al., 2007). We conclude that Fop

contains asymmetric and symmetric DMA residues *in vivo*, and that symmetric methylation partially depends on the presence of Prmt1. To map the arginines

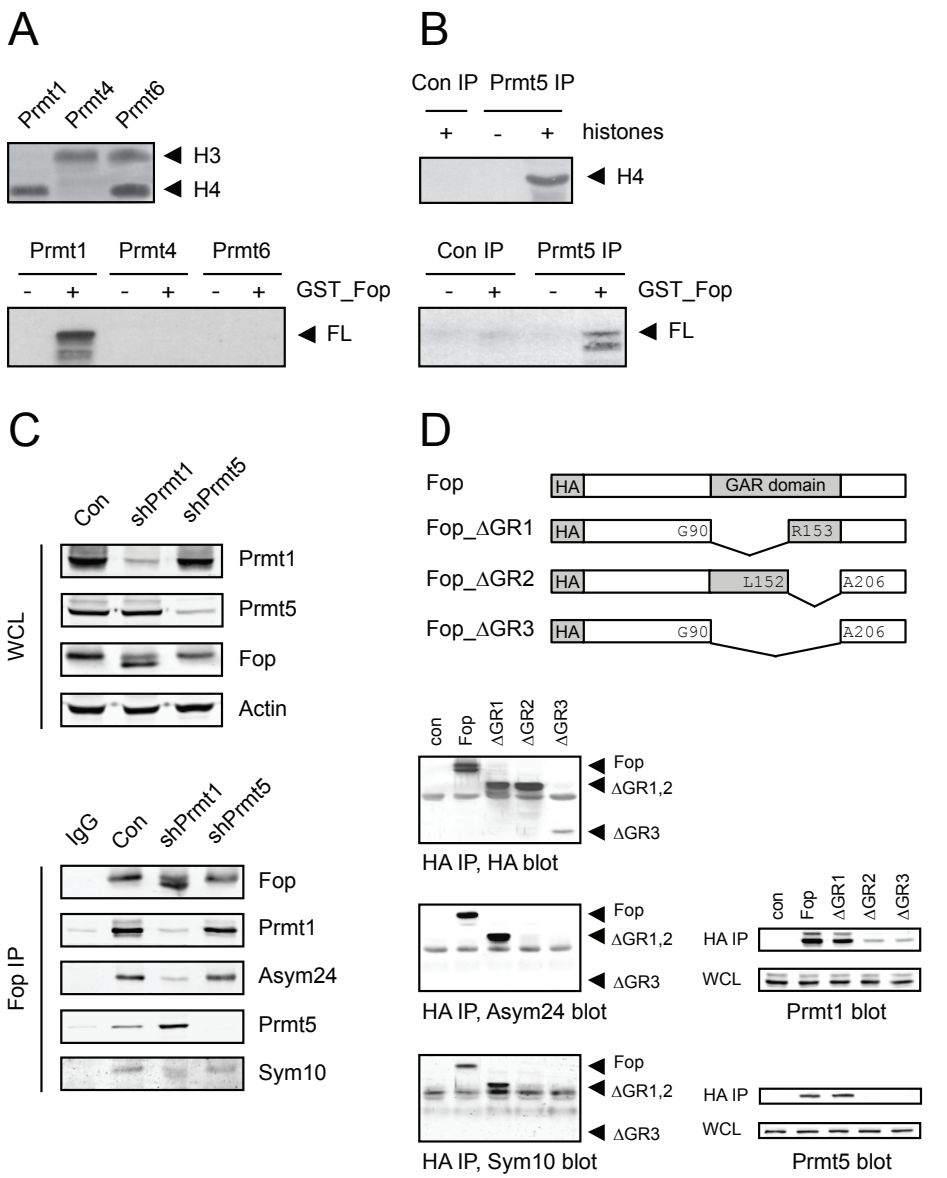


Figure 4. Fop is a target for type I and type II Prmts. (A). GST_Fop_L was used as a substrate in an *in vitro* methylation assay using three type I GST_Prmts. Core histones were used a positive control. Prmt1 is the only type I enzyme that can methylate full-length (FL) protein. (B). GST_Fop_L was used

as a substrate in an *in vitro* methylation assay using immunoprecipitated Prmt5. (C). MEL cells were infected with control lentivirus (Con) or lentivirus expressing an shRNA against Prmt1 (Prmt1 kd). Whole cell lysates (WCL; upper panel) were tested for Prmt1, Prmt5, and Fop. Staining for actin served as a control for equal loading. Fop was precipitated (IP; lower panel) and tested for binding to Prmt1 and Prmt5, and for asymmetric DMA residues (Asym24) and symmetric DMA residues (Sym10). (D). HA_Fop constructs lacking GAR sequences were tested for methylation and Prmt1 and Prmt5 binding.

that are methylated within the GAR, we generated internal deletion constructs that lacked either the N-terminal or the C-terminal half of the GAR, or the entire GAR (ΔGR1, ΔGR2, and ΔGR3, respectively; Fig. 4D). Binding of Prmt1 and Prmt5 (Fig. 4D, right panels) as well as methylation status (Fig. 4D, left panels) are not reduced when the N-terminal half is deleted, indicating that the majority of methylated arginines is not in this region. In contrast, methylation and Prmt binding of Fop ΔGR2 is undetectable (Prmt5) or greatly reduced (Prmt1). Hence, the major Prmt-interaction surface and methylation sites appear to overlap. Consistent with the interaction mapping, Fop ΔGR2 and Fop ΔGR3 are still able to recruit reduced levels of Prmt1 via the N-terminal domain (Fop ΔC3, Fig. 3C).

Fop is a chromatin-associated protein

We next examined Fop and Prmt1 co-localization by confocal microscopy and fractionation experiments. Fig. 5A shows that HA_Fop is a nuclear protein exclusively localized to DAPI-low regions with a punctuate/speckle-like distribution (Fig. 5A; green signal), identical to endogenous Fop (Fig. 2C). Staining for endogenous Prmt1 revealed that, within the nucleus, Prmt1 also localizes to DAPI-low regions, resulting in a high degree of co-localization with Fop (Fig. 5A; red signal). Compared to Fop, the distribution of Prmt1 is more diffuse. The distribution and pattern of Fop localization did not change in Prmt1-depleted cells (Fig. 5A, lower panel). Next, control MEL cells and Prmt1 knockdown cells were fractionated in cytoplasmic, cytoskeletal/nucleoplasmic, chromatin-associated, and nuclear matrix-associated proteins. An antibody directed against the C-terminus of Prmt1 showed

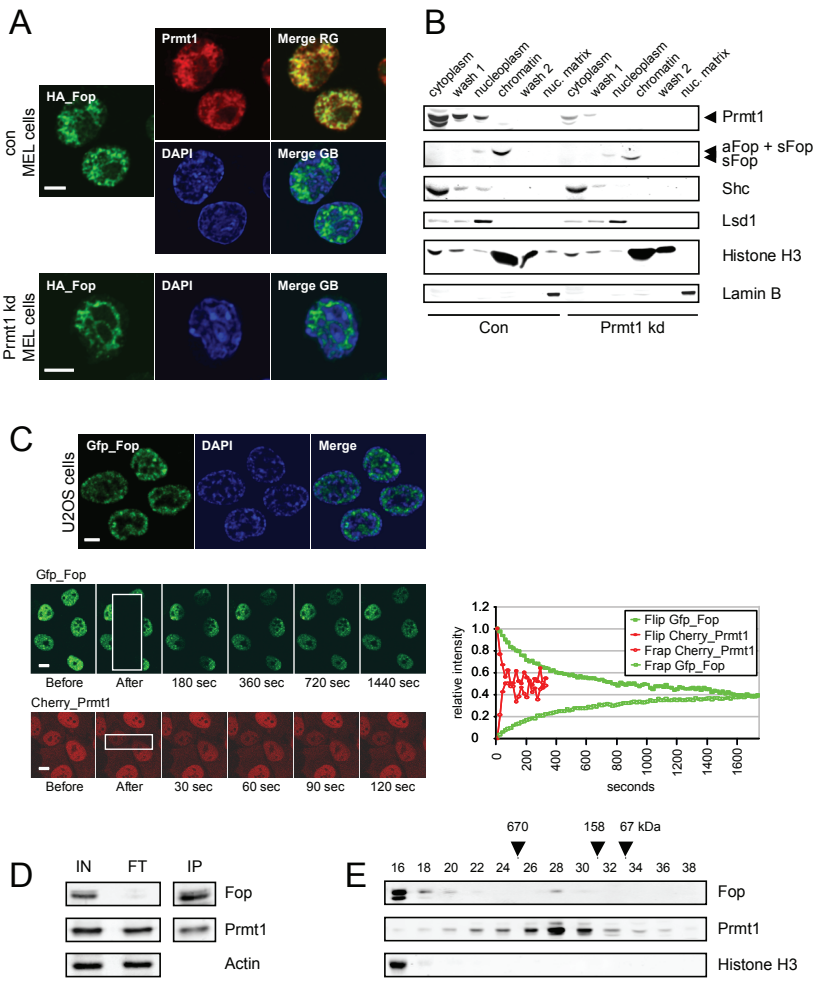


Figure 5. Fop stably interacts with chromatin. (A). Confocal slices indicate that HA_Fop (immunofluorescence; cytospins of MEL cells) and Gfp_Fop (living U2OS cells; (C), upper panel) localize to DAPI-low regions in the nucleus and display a granulated/speckle-like distribution. Nuclear Prmt1 has a more diffuse distribution, but also localizes to DAPI-low / euchromatic regions. The distribution of HA_Fop is not changed in cells with reduced Prmt1 expression (Prmt1 kd). Scale bars indicate 5 µm. Image stacks were deconvolved and corrected for chromatic shift. (B). Cellular fractionation of control (Con) and Prmt1 knockdown (Prmt1 kd) MEL cells expressing HA_Fop. Cytoplasmic, nucleoplasmic, chromatin, and nuclear matrix fractions were tested for Prmt1 and Fop. Prmt1 localizes to cytoplasm and nucleoplasm, while Fop (asymmetrically (aFop) and/or symmetrically methylated (sFop) is associated to chromatin. Shc, LSD1, Histone H3, and Lamin B served as controls for individual fractions. (C). *In vivo* mobility of Fop and Prmt1 was determined by combined FRAP-FLIP. A new equilibrium in the distribution of Cherry_Prmt1 was reached within 30 seconds, consistent with the diffusion characteristics of a macromolecular complex of less than 1 MDa. Gfp_Fop is highly immobile with complete redistribution taking more than 25 minutes. Scale bars indicate 10 µm. (D). The level of Prmt1 was de-

termined in input (IN) and flow-through (FT) of Fop-depleted nuclear extracts of MEL cells (left panel). (E). Size-fractionation profiles of Fop and Prmt1 in MEL nuclear extracts. Histone H3 staining served as a control for chromatin-containing fractions. Molecular mass markers are indicated on the top.

that Prmt1 localized to the cytoplasm and the soluble nuclear fraction (Fig. 5A). In contrast, Fop was found exclusively in the nucleus, with the majority of the protein tightly associated to chromatin. Reducing the level of Prmt1 resulted in hypomethylated Fop but did not change its distribution pattern. This is consistent with the confocal data and shows that the association of Fop to chromatin does not depend on its asymmetrical methylation (Fig. 5A).

In light of their co-purification (Figs. 1, 3, and 4) and co-localization (Fig. 5A), it is surprising that Prmt1 and Fop localize to different cellular compartments after biochemical fractionation (Fig. 5B). To investigate this further we performed live-imaging of Fop and Prmt1, N-terminally tagged with Green Fluorescent Protein (Gfp) and Cherry, respectively. We first confirmed that Gfp_Fop and Cherry_Prmt1 still interact by co-immunoprecipitation experiments (not shown). We then co-expressed the proteins stably in U2OS cells. We selected clones with low expression levels of both proteins. Western blot analysis showed that Cherry_Prmt1 was expressed at approximately 25% of the endogenous Prmt1 level (not shown). In fractionation experiments, Gfp_Fop was distributed similarly to that seen with HA_Fop in MEL cells (not shown). The localization of Gfp_Fop was strictly nuclear with a punctuate/speckle-like distribution in DAPI-low areas similar to endogenous and HA-tagged Fop in MEL cells (Figs. 2C and 5A, respectively). Time-lapse imaging shows that Gfp_Fop is completely released from condensed chromosomes during mitosis and relocates in ~1 hour after cell division (not shown). To determine the mobility of Fop and Prmt1 *in vivo*, we combined fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments. One half of a nucleus was bleached, and the recovery of fluorescence was subsequently monitored in the bleached part of the nucleus during 1800 seconds. At the same time, the fluorescence loss in the unbleached part of the nucleus

was monitored until a new equilibrium in fluorescence distribution was reached between the bleached and the unbleached regions. This experiment revealed that Cherry_Prmt1 behaves as a soluble protein with diffusion characteristics of an oligomeric complex (Fig. 5C), consistent with previous data on Gfp_Prmt1 (Herrmann et al., 2005). In contrast, Gfp_Fop is a highly immobile protein with a diffusion rate more than 50 times slower than Prmt1 (Fig. 5C). Although the N-terminal tags may influence the behavior of both fusion proteins, these experiments indicate that Fop is not a component of a stable Prmt1-holoenzyme complex *in vivo*(Goulet et al., 2007). To determine the fraction of Prmt1 that is associated to Fop, nuclear extracts were immuno-depleted for endogenous Fop. Fig. 5D shows that complete depletion of Fop has a marginal effect on the amount of Prmt1 in the supernatant, indicating that only a small fraction of Prmt1 is bound to Fop. This is further demonstrated by size fractionation experiments: the majority of Fop and Prmt1 do not elute in the same fractions (Fig. 5E).

Fop co-localizes with facultative heterochromatin

The localization of Fop was further characterized by co-immunostaining with antibodies against different histone modifications. First, we used an antibody that was raised against asymmetrically dimethylated R3 of histone H4 (H4R3me2as). This methylation is performed by Prmt1 and is a critical step in subsequent transcription activation events, including histone acetylation (Huang et al., 2005; Strahl et al., 2001). Staining for H4R3me2as, as well as for acetylated H4 (acH4), a mark for active genes, revealed distinctive fluorescent spots (Fig. 6A, B). Although these spots resided within euchromatic (DAPI-low) regions of the nucleus, they showed only minor co-localization with Fop. H3K9me2 has been implicated in heterochromatin formation and gene silencing (Bannister et al., 2001) and marks condensed DNA. As expected, H3K9me2 staining showed an almost complete overlap with these heterochromatic regions (intense DAPI staining, Fig. 6C). Fop was excluded from those regions that are positive for H3K9me2 staining, showing that Fop is not

localizing to condensed DNA, in line with the observation that Gfp_Fop detaches from mitotic chromosomes. Methylation of K27 of histone H3 (H3K27me3) creates binding sites for the Polycomb repressive complex 1 (Min et al., 2003) and is therefore a mark for facultative heterochromatin. Staining with an antibody that specifically recognized H3K27me3 identified bright spots and a more diffuse staining throughout the 'DAPI-low" regions (Fig. 6D). The diffuse signal showed a striking co-localization with Fop, while only a minority of the bright spots was found to be double positive for Fop and H3K27me3. We conclude that at this level of resolution Fop is mainly associated with facultative heterochromatin *in vivo*.

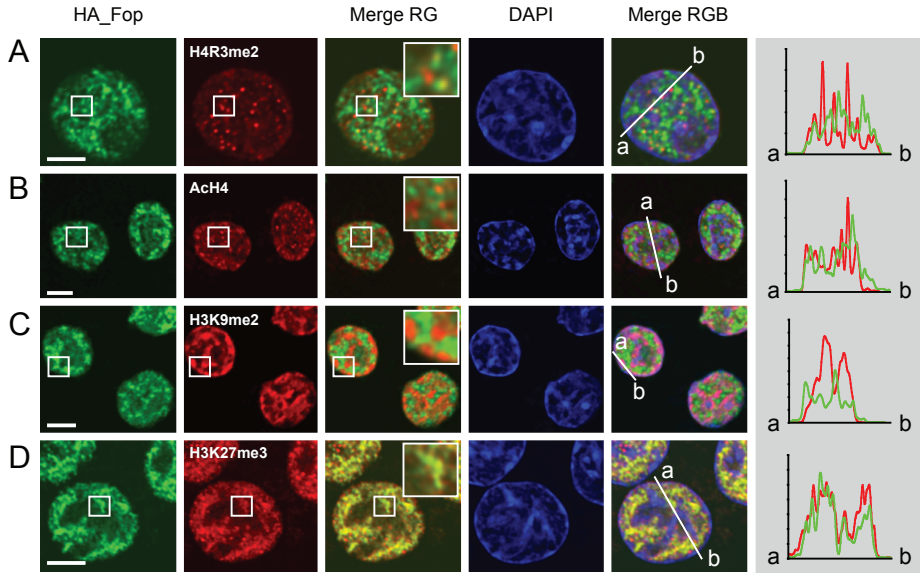


Figure 6. HA_Fop colocalizes partially with H3K27me3. (A-D). Co-localization of HA_Fop was studied in cytospins of MEL cells labeled for immunofluorescence with anti-HA (green) and the antibodies indicated (red). Histograms represent quantified intensity profiles over line a-b (Y-axis = pixel intensity). Partial co-localization is observed with H3K27me3, a mark for facultative heterochromatin. Scale bars indicate 5 µm. Image stacks were deconvolved and corrected for chromatic shift.

Fop is critical for estrogen-dependent gene activation

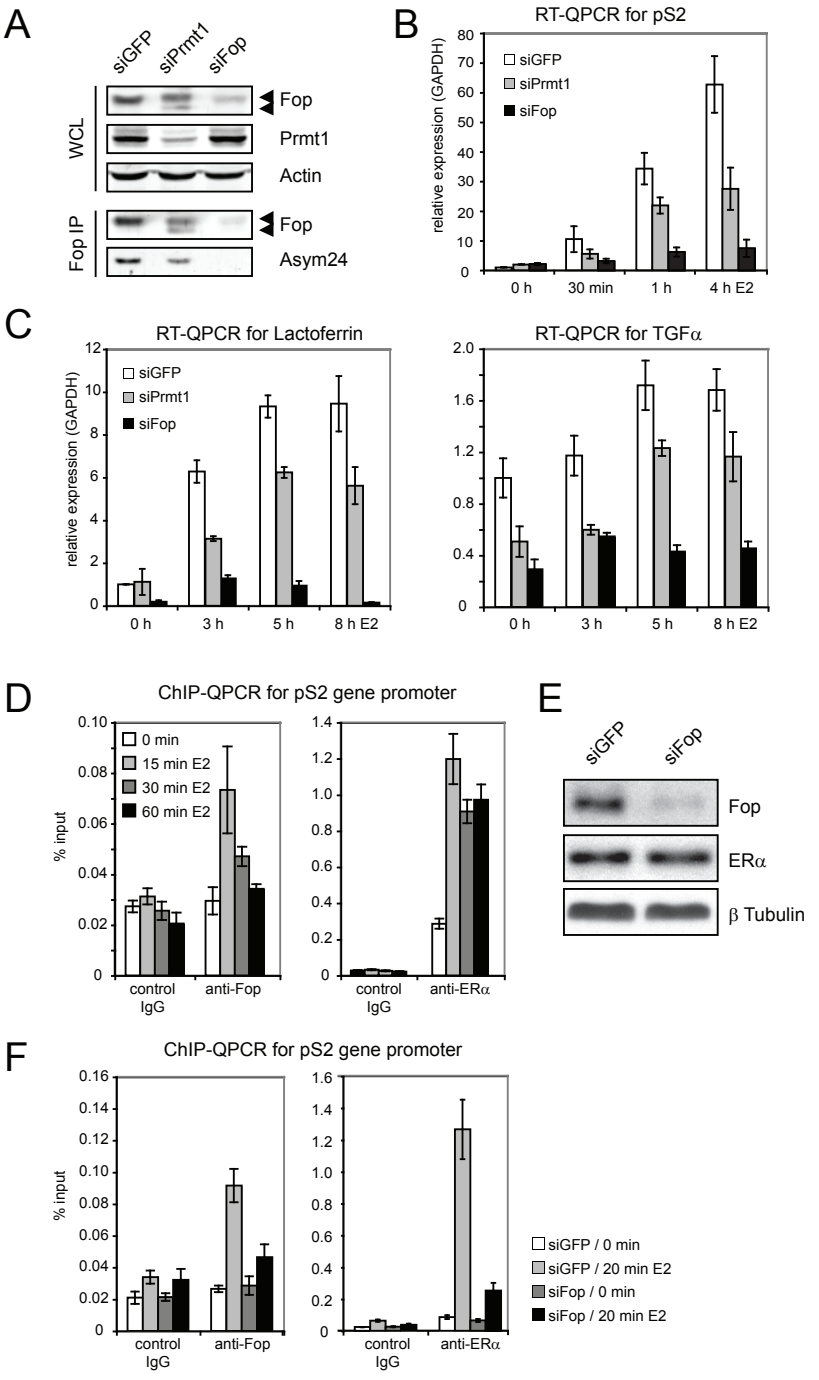
The observations that Fop is tightly bound to chromatin after biochemical fractionation and co-localizes with facultative heterochromatin *in vivo* suggest that it might

be involved in transcriptional regulation. The significance of Prmt1 in transcriptional regulation has been most vividly demonstrated in the model of nuclear hormone signaling. Recruitment of Prmt1 and the subsequent methylation of H4R3 are critical events in estrogen receptor (ER)-regulated activation of the pS2 gene, (TFF1, encoding trefoil factor 1). Since the molecular events leading to activation of this gene have been described in detail (Metivier et al., 2003; Metivier et al., 2006), we studied the functional importance of Fop in estradiol (E2) induction of pS2 expression in MCF7 cells, a human E2-responsive breast cancer cell line. Cells were seeded in hormone-free medium and subsequently transfected with siRNAs against Gfp (siGfp), Prmt1 (siPrmt1), and Fop (siFop), respectively. Western blot analysis showed suppression of endogenous Prmt1 and Fop 48 hours after siRNA treatment (Fig. 7A). Furthermore, reduction of Prmt1 expression resulted in partial hypomethylation of Fop, as demonstrated by the appearance of a faster migrating species of Fop and reduced staining for Asym24 (Fig. 7A). To analyze ER-regulated transcription, cells were induced with E2 for various times and RNA was isolated. As described previously (Wagner et al., 2006), real-time quantitative PCR (RT-QPCR) revealed that the E2-induced transcriptional activity of endogenous pS2 was reduced upon siPrmt1 transfection compared with siGfp transfection (Fig. 7B). Interestingly, reduction of the endogenous Fop level had a more dramatic inhibitory effect on pS2 induction. Similar results were obtained with two siRNAs which target different regions of the Fop mRNA (not shown). Next, we tested the effect of reduced Fop levels on the E2-induced transcription of Lactoferrin and TGFα, two other Prmt1-dependent E2-inducible genes. Consistent with the observations for the pS2 gene, E2-induced transcriptional activity of these genes was diminished upon siPrmt1 transfection and almost absent after siFop transfection (Fig. 7C). Furthermore, reduced Fop levels resulted in lower pre-induction transcript levels of these genes. To investigate whether the transcriptional effect of Fop correlates with binding of Fop to the pS2 promoter, we performed ChIP analysis following E2 induction. Chromatin was precipitated with antibodies against Fop, ERα, and

control IgG, and analyzed by PCR for the presence of pS2 promoter fragments including the ERE (estrogen response element). Promoter occupancy by Fop was not detected in uninduced cells, but in E2-treated cells a transient interaction was observed with a peak at 15 minutes post-induction (Fig. 7D, left panel). Promoter occupancy of ERα also increased 15 minutes after E2 addition and remained constant over the measured period (Fig. 7D, right panel). Next, we tested whether Fop depletion affected the binding of ERα to the pS2 promoter. MCF7 cells were transfected with siGFP and siFop. Two days later, cells were induced with E2 for 20 minutes and analyzed by ChIP using antibodies against Fop, ERα, and control IgG. As expected, reduced Fop levels (Fig. 7E) resulted in reduced Fop binding to the pS2 promoter (Fig. 7F. left panel). Although transfection with siFop did not change the protein level of ERα (Fig. 7E), a dramatic reduction in promoter occupancy by ERα was observed (Fig. 7F, right panel). Together these data show that Fop is required for E2-inducible expression of the ERα target genes investigated, and for binding of ERα to the pS2 promoter region.

DISCUSSION

Here, we identified a novel protein, Fop, in an unbiased proteomics screen for Prmt1-interacting proteins. All identified Prmt1-associated factors, including Fop, contain glycine-arginine-rich regions, a sequence with high affinity for Prmt1 (Zhang and Cheng, 2003). Proteins that have been described to bind to Prmt1 with domains other than GARs, such as Btg1 and Btg2, Nip45, or Usf1 were not identified. There are several possible explanations: the proteins are not expressed in MEL cells or only during a short period of the cell cycle (as is the case for Btg1 and, Btg2 (Bakker et al., 2004; Rouault et al., 1992)), the interactions are transient or unstable, or GAR-containing proteins compete efficiently for Prmt1 binding during the isolation. In this study we show that Fop and Prmt1 are strongly associated in co-IP assays. However, immunodepletion, size fractionation and FRAP/FLIP experiments show that both *in vitro* and *in vivo* the majority of Prmt1 is not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siGFP
siPrmt1
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Prmt1
Actin
Fop IP
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Asym24
B
RT-QPCR for pS2
relative expression (GAPDH)
siGFP
siPrmt1
siFop
0 h
30 min
1 h
4 h E2
C
RT-QPCR for Lactoferrin
RT-QPCR for TGFα
0 h
3 h
5 h
8 h E2
D
ChIP-QPCR for pS2 gene promoter
% input
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30 min E2
60 min E2
control IgG
anti-Fop
anti-ERα
E
siGFP
siFop
Fop
ERα
β Tubulin
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ChIP-QPCR for pS2 gene promoter
siGFP / 0 min
siGFP / 20 min E2
siFop / 0 min
siFop / 20 min E2

Figure 7. Fop is critical for E2-dependent gene activation. (A-F). MCF7 cells were hormone-starved and treated with siRNA as indicated. (A). Whole cell lysates and IPs were stained by western blot with the indicated antibodies. (B-C). MCF7 cells were induced with E2 for the times indicated. Total RNA was analyzed by RT-QPCR using primers for pS2, lactoferrin, and TGFα. (D). MCF7 cells were treated for 0, 15, 30, and 60 minutes with E2. ChIP reactions was performed with indicated antibodies and examined by QPCR for the presence of proximal pS2 promoter fragments. (E). Whole cell lysates were analyzed by western blot with the indicated antibodies. (F). MCF7 cells were treated with siRNAs against Gfp or Fop for 48 hours, followed by E2 induction. ChIP-QPCR was performed as in (D).

associated with Fop. We therefore conclude that Fop is not a part of the Prmt1 holoenzyme complex. The remaining Prmt1-associated factors identified in our screen are RNA-binding proteins and most likely represent Prmt1 targets, rather than structural components of the 250-400 kDa Prmt1 complex. Collectively, our results favor the hypothesis that the Prmt1 holoenzyme is composed of Prmt1 multimers (Lim et al., 2005; Wang et al., 2001; Zhang and Cheng, 2003).

Fop is a novel target of protein arginine methyltransferases

Structurally, Fop can be divided in three regions: a central GAR domain flanked by N- and C-terminal parts that do not contain any known functional motifs. Nevertheless, the fact that Fop is highly conserved in all vertebrates suggests that it is an important protein for this subphylum and that all three regions harbor critical information for Fop function. Possibly, Fop acts as a scaffold protein with three interaction domains.

Type I methylation by Prmt1 and Prmt4 serves as a general marker for active transcription, while type II methylation by Prmt5 and Prmt7 is associated with transcriptional repression. From this perspective, it is highly interesting that Fop is also symmetrically methylated by Prmt5 *in vivo*. In the absence of Prmt1, Fop is no longer asymmetrically methylated. Still, its distribution pattern and chromatin association are not affected. Since arginine methylation is known to regulate protein-protein interactions, this suggests that Fop may recruit proteins to chromatin in a methylation-dependent manner.

Western blot analysis revealed an unprecedented mobility shift of Fop in cells with reduced Prmt1 levels, strongly suggesting that multiple arginines are methy-

lated by this enzyme. However, mapping of the target arginines is compromised by the fact that the GAR domain contains 26 arginines that are potentially methylated, as they are flanked by a glycine residue. Our results indicate that the majority of target arginines are located in the C-terminal half of the GAR domain. However, the number and position of target arginines, as well as the dynamics of their methylation status, is at present unclear. The observation that both the slower migrating and the faster migrating species of Fop lacking aDMA are recognized by the SYM10 antibody opens the possibility that a single Fop molecule contains aDMA and sDMA at the same time.

Fop is associated with chromatin and required for E2-dependent gene expression

Immunofluorescence and Gfp-fusion studies reveal that Fop mainly associates with non-condensed (DAPI - and H3K9me2-negative) chromatin, while it is released from mitotic chromosomes. It is possible that Fop binds directly to DNA, as it shares some characteristics with histones: (1) it is a highly basic protein, with an estimated pI of 12.2, and (2) its association to chromatin is extremely stable. The localization of Fop shows a striking colocalization with H3K27me3, indicating that Fop is associated with facultative heterochromatin. This raises the possibility that Fop is involved in the regulation of genes responding to environmental and developmental cues, such as growth factors and hormones. To test this directly, we analyzed the role of Fop in the induction of the pS2 gene by E2. Ligand-bound ERα induces recruitment of coactivators and specific histone modifications at the pS2 promoter (Metivier et al., 2006). While Prmt1 methylates cytoplasmic ERα to control the extranuclear function of the receptor, recruitment of Prmt1 is a critical event for H4R3 methylation and subsequent histone acetylation and binding of the basal transcription machinery to the pS2 promoter (Le Romancer et al., 2008; Metivier et al., 2006; Wagner et al., 2006). Our ChIP data indicate that Fop is not present at the proximal promoter of the pS2 gene in uninduced MCF7 cells, while

it is recruited rapidly after E2 induction. Fop recruitment peaks after 15 minutes, coinciding with the recruitment of Prmt1 and the initiation of the first transcription cycle of the pS2 gene. Remarkably, reduced Fop levels have a much stronger inhibiting effect on the induction of the pS2, Lactoferrin, and TGFα genes, compared to those observed after Prmt1 depletion (Fig. 7, (Wagner et al., 2006)).

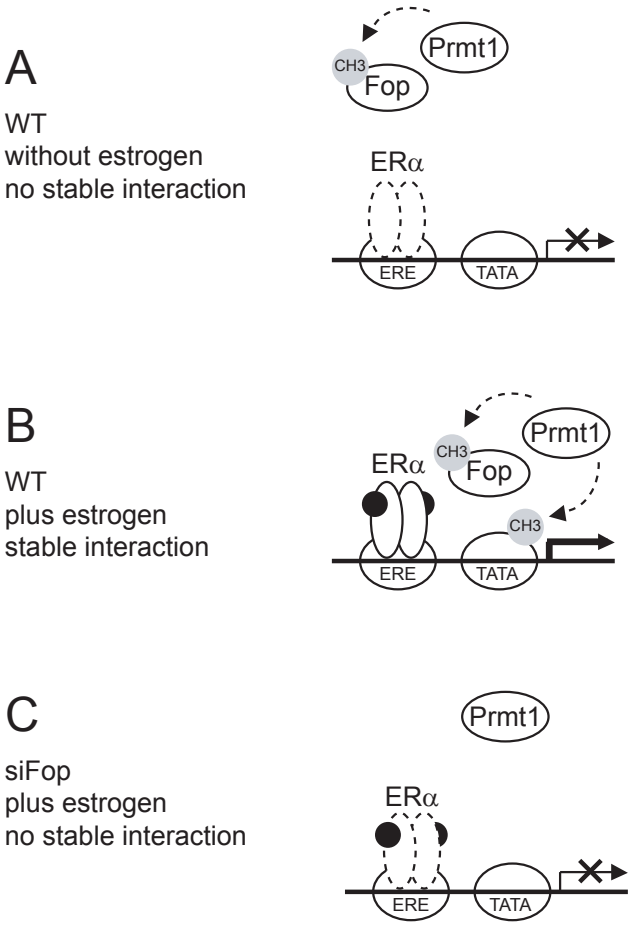

Figure 8. Model for Fop function in ER-regulated gene expression. (A). In the absence of hormone, ERα is weakly bound to the promoter. (B). Upon ligand binding, the interaction between ERα and its targets sequences is stabilized while Fop and Prmt1 are recruited. Prmt1 methylates H4R3 and Fop, although the latter also may take place elsewhere. These events are critical for high transcription levels. (C). Reduced Fop levels impair a stable interaction of ERα and the promoter, resulting in a block of transcription.

Furthermore, Fop depletion results in an almost complete block of E2-induced promoter occupancy by ERα. These results indicate a central role for Fop in stable recruitment of ERα to the pS2 promoter. Since promoter occupancy by ERα in uninduced cells remains very similar after Fop knockdown, it appears that the inefficient binding of unliganded ERα to the ERE of the pS2 promoter is unchanged in the absence of Fop. As a result of the transient nature of the knockdown experiments in MCF7 cells, depletion for Prmt1 resulted only in partial hypomethylation of Fop (Fig. 7A).Therefore, we were not able to study the role of Prmt1-dependent methylation of Fop in this context, as the knockdown level of Prmt1 was not sufficient to obtain non-methylated Fop (Fig. 7B). Since we have no evidence that Fop and ERα interact directly in non-induced or E2-induced MCF7 cells, our data suggest a model where Fop recruitment is an early event critical for a stable interaction between ERα and its target sequences (Fig. 8). Alternatively, Fop could facilitate a chromatin environment that is permissive for ERα-binding in uninduced cells. Future experiments have to clarify the exact role of Fop in transcription regulation, as well as the significance of its methylation status in this process.

MATERIALS AND METHODS

Constructs and cells

The coding sequence of Prmt1 (isoform 1) was amplified from mouse erythroblast cDNA by PCR using Expand (Roche), cloned into pMT2_HA and pMT2_myc (Kaufman et al., 1989) using SalI and NotI, and verified by sequencing. After introduction of the 23-aa biotinylation tag, HA_bio_Prmt1 was subcloned into the erythroid expression vector pEV-neo (Needham et al., 1992), and electroporated into mouse erythroleukemic (MEL) cells expressing the BirA biotin ligase (de Boer et al., 2003). The cDNA of Fop was obtained from RZPD/imaGenes (Berlin, Germany), clone IRAKp961L04114Q2. The first 25-aa were introduced using SalI/HindIII, after subcloning the 930 bp HindIII/EcoRV fragment into pBluescript (Stratagene). Full-length Fop was cloned into pMT2_HA using SalI and EcoRI sites. GST-Fop

fusion constructs were generated by cloning PCR fragments into pGEX3X (Pharmacia). Gfp and Cherry were cloned in frame at the N-terminus of Fop and Prmt1, respectively. MEL, 293T, U2OS, and MCF7 cells were grown in Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% FCS. Before hormone treatment, MCF7 cells were grown for 3-4 days in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped fetal calf serum following addition of 200 nM 17β-estradiol (E2; Sigma) for the indicated times.

Size fractionation

Size fractionation of protein complexes was carried out on an AKTA FPLC apparatus with a Superose 6 10/30 column (Amersham Biosciences). Fractions were precipitated with trichloroacetic acid and analyzed by Western blotting.

Transient transfection, immunoprecipitation, and Western blot analysis

Transfection of 293T cells, immunoprecipitation, GST pull downs, and Western blot analysis were performed as described previously (van Dijk et al., 2000). Membranes were blocked in 0.6% bovine serum albumin (BSA), incubated with appropriate antibodies, and developed with the use of enhanced chemoluminescence (ECL; NEN), or by using the Odyssey Infrared Imaging System (Li-Cor Biosciences). The following primary antibodies were used: Prmt1 (07-404), Prmt5 (07-405), H4R3me2 (07-213), AcH4 (06-598), H3K27me3 (07-449), Asym24 (07-414), and Sym10 (07-412) from Upstate; HA (monoclonal F7, sc-7392), HA (polyclonal Y11, sc-805), Myc (sc-40), Actin (sc-1616), Shc (sc-967), and Lamin B (sc-6216) from Santa Cruz; LSD (ab18036), H3K9me2 (ab1220), and H3 (ab1791) from Abcam. Rat monoclonal antibodies against the N- and C-terminus of Fop (KT59 recognizing aa 1-90 and KT64 recognizing aa 206-249, respectively) were generated by Absea Biotechnology Ltd (Bejing, China). Additionally, polyclonal antibodies were raised in rabbits using the same epitopes. Cellular fractionation was performed as described previously (Pasqualini et al., 2001). Subcellular fractionation (cyto-

plasm, nucleoplasm, chromatin, and nuclear matrix) was performed as described (Nair et al., 2004).

Cellular extracts and mass spectrometry

Procedures involving biotinylated proteins were performed as described previously with minor modifications (de Boer et al., 2003). Cytoplasmic and nuclear extracts were generated using the method of Andrews and Faller from 5 x 108 MEL cells (Andrews and Faller, 1991). Tryptic digestion was performed on paramagnetic streptavidin beads. Mass spectrometry was performed as described previously (Bajpe et al., 2007). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBInr database (release 20090430; total number of sequences: 8,483,808). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 75. Typical contami-

nants, also present in purifications using BirA-only MEL cell extracts, were omitted from the table (de Boer et al., 2003).

Lentiviral mediated knockdown and siRNAs

After subcloning into pSuper (Brummelkamp et al., 2002), the H1 promoter and shRNA coding sequences against Prmt1 (GATTGTCAAAGCCAACAAG) and Fop (GGAGCAGCTGGACAACCAA) were cloned into a modified pRRLsin.sPPT. CMV.GFP.Wpre lentiviral vector (Follenzi et al., 2002). Lentivirus was produced by transient transfection of 293T cells according to standard protocols (Zufferey et al., 1997). Knockdowns in MCF7 were performed using siRNA transfection as described previously (Wagner et al., 2006). The sequences of siFop are: 5'-GGAGCAGCUGGACAACCAA-3', 5'-GUUAGUCAACACAUCUGUAAA-3', and 5'-GACUCUUGUUAGUCAACACAU-3' (sense strand indicated; Genepharma, Shanghai).

Confocal imaging

Cells were spotted on poly-prep slides (Sigma), fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and blocked in 1% BSA / 0.05% Tween 20 in PBS. Primary antibody incubation was performed in blocking solution for 16 hrs at 4˚C. Cells were imaged using the meta 510 confocal laser scanning microscope (meta LSM510, Zeiss) using AIM software provided. Images were recorded as an 8bit image stack of 512x512 with a voxel size of 47x47x250nm of a 4x line average. The point spread function was determined by scanning green fluorescent beads with a diameter of 100nm (Duke Scientific). The chromatic shift was determined by scanning multi-colored fluorescent beads with a diameter of 500nm (500nm TetraSpeck beads, Invitrogen). The empirically obtained PSF was used for deconvolving the image stacks with the classic maximum of likelihood (CMLE) algorithm that is implemented in the Huygens deconvolution, visualization, analysis and archiving software package 3.0 for linux (Scientific Volume Imaging).

After deconvolution, the image stack was corrected for chromatic shift. FRAP/FLIP experiments were performed as described (Essers et al., 2006).

Reverse transcription, ChIP and QPCR

Reverse transcription (RT) and ChIP were performed as previously described (Wagner et al., 2006). For RT-QPCR, GAPDH gene transcription was used as a reference for normalization. The following primers were used: GAPDH, 5'-AGCCACATCGCTCAGACAC-3' (forward), 5'-GCCCAATACGACCAAATCC-3' (reverse); pS2, 5'-GCCTTTGGAGCAGAGAGGA-3' (forward), 5'-TAAAACAGT-GGCTCCTGGCG-3' (reverse); Lactoferrin, 5'-TAAGGTGGAACGCCTGAAAC-3' (forward), 5'-CCATTTCTCCCAAATTTAGCC-3' (reverse); TGFα, 5'-TGCTGC-CACTCAGAAACAGT-3' (forward), 5'-ATCTGCCACAGTCCACCTG-3' (reverse). For ChIP-QPCR immunoprecipitated chromatin was amplified using the following primers: pS2 gene promoter 5'-GTTGTCACGGCCAAGCCTTTT-3' (forward), 5'-AGGATTTGCTGATAGACAGAGACGAC-3' (reverse); GAPDH: gene promoter 5'-CCATCTCAGTCGTTCCCAAAGTCC-3' (forward); 5'-GATGGGAGGTGATCGGT-GCT-3' (reverse). The ChIP results were quantified as recently described (Hyllus et al., 2007).

ACKNOWLEDGEMENTS

We thank Karel Bezstarosti and Silvia Hoeboer for technical assistance. This work was supported by the Landsteiner Foundation for Blood Transfusion Research (LSBR), the Dutch scientific organization (NWO), the Centre for Biomedical Genetics (CBG), the Netherlands Genomics Initiative (NGI), the Deutsche Forschungsgemeinschaft (DFG), and the European Union FP6 EUtracc consortium.

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Chapter 3

Five Friends of Methylated Chtop, a complex linking arginine methylation to desumoylation

Pavlos Fanis[1], Nynke Gillemans[1], Jeroen Demmers[2], Fatemehsadat Esteghamat[1], Farzin Pourfarzad[1], Ali Aghajanirefah[1], Ratna K. Vadlamudi[3], Frank Grosveld[1], Sjaak Philipsen[1], and Thamar B. van Dijk[1]

1. Department of Cell Biology, Erasmus MC, 3000 CA, Rotterdam, The Netherlands
2. Proteomics Center, Erasmus MC, 3000 CA, Rotterdam, The Netherlands
3. Department of Obstetrics and Gynecology, UTHSCSA, 7703 Floyd Curl Drive, San Antonio, TX 78229, Texas, United States of America

(Manuscript in preparation)

ABSTRACT

Chromatin target of Prmt1 (Chtop) is a vertebrate-specific chromatin-bound protein that plays an important role in transcriptional regulation. As its mechanism of action remains unclear, we identified Chtop-interacting proteins using a biotinylation-proteomics approach. Here we describe the identification and initial characterization of Five Friends of Methylated Chtop (5FMC). 5FMC is a nuclear complex that can only be recruited by Chtop when the latter is arginine-methylated by Prmt1. It consists of the co-activator Pelp1, the Sumo-specific protease Senp3, Wdr18, Tex10, and Las1L. Pelp1 functions as the core of 5FMC, as the other components become unstable in the absence of Pelp1. We show that recruitment of 5FMC to Zbp-89, a zinc-finger transcription factor, affects its sumoylation status and transactivation potential. Collectively, our data provide a mechanistic link between arginine methylation and (de)sumoylation in the control of transcriptional activity.

INTRODUCTION

Transcription factor activity is often controlled by post-translational modifications such as acetylation, phosphorylation, methylation and sumoylation. Some modifications are associated with both gene activation and repression, while others appear to be more exclusive: asymmetrical dimethylation of arginine residues is restricted to transcriptional activation, while modification by sumoylation correlates with inhibition of transcription (Verger et al., 2003).

Arginine methylation occurs frequently within glycine-arginine-rich (GAR) regions and is catalyzed by members of the protein arginine methyltransferase (Prmt) family. These enzymes are subdivided in two major classes: type I enzymes catalyze the formation of asymmetrically dimethylated arginines (aDMA), while type II enzymes form symmetrically dimethylated arginines (sDMA) (Bedford and Clarke, 2009). Prmt1 and Prmt4/Carm1 (Coactivator-associated arginine methyltransferase 1) are the major type I enzymes and both are critical for mammalian development (O'Brien et al., 2010; Pawlak et al., 2000). Their substrates include

RNA-binding proteins, nuclear matrix proteins, cytokines and transcriptional regulators (Bedford and Clarke, 2009). Prmt1 methylates transcription factors such as Runx1 and STAT1 thereby promoting their transcriptional activity (Mowen et al., 2001; Zhao et al., 2008). Furthermore, Prmt1 and Prmt4 are recruited by nuclear hormone receptors and other transcription factors including YY1, p53 and NF-κB (Wang et al., 2001; Zhao et al., 2008), resulting in the methylation of additional coactivators and histones. Prmt4 methylates histone H3 at arginine 17 and 26, while Prmt1 targets histone H4 at arginine 3 for methylation promoting subsequent acetylation of histone H3 at lysine 9 and histone H3 at lysine 14 (Li et al., 2010) and further activating events (Cheng et al., 2007).

Small ubiquitin-like modifier (SUMO) has an important regulatory function in several cellular processes, including DNA repair, cell cycle progression, signal transduction, chromatin structure and transcriptional regulation (Hay, 2005). Mammalian cells express four SUMO paralogs (SUMO-1 to SUMO-4). SUMO-1 differs in sequence by about 50% from SUMO-2 and 3, whereas SUMO-2 and SUMO-3 are 97% identical to each other. Conjugation of SUMO to target proteins occurs by a series of reactions conducted by the E1 activating enzyme, E2 conjugating enzyme and an E3 SUMO ligase (Johnson, 2004). The reverse desumoylation process is mediated by the isopeptidase activity of SUMO-specific proteases (Senps). In mammals, six members of Senps have been reported, known as Senp1-3 and Senp5-7. Sumoylation of multiple transcription factors, including Sp3, Sox6, Zeb1, and Zbp-89, has a negative effect on their transactivation potential, as it promotes the recruitment of repressive complexes (Fernandez-Lloris et al., 2006; Stielow et al., 2008; Wang et al., 2007). Many components of the repressor complexes CoREST1, NuRD, PRC1, Setdb1, and MEC themselves are also sumoylated, or have SUMO interacting motifs (SIMs). This suggests that that sumoylation plays an important role in the formation and/or stabilization of these complexes (Garcia-Dominguez and Reyes, 2009).

We recently identified Chromatin target of Prmt1 (Chtop), also known as Friend

of Prmt1 (Fop) or Small protein rich in arginine and glycine (SRAG), encoded by the mouse 2500003M10Rik and human C1orf77 genes, respectively (van Dijk et al., 2010b; Zullo et al., 2009). Chtop is a chromatin-associated protein that plays an important role in the ligand-dependent activation of estrogen target genes such as TFF1 (pS2) in breast cancer cells (van Dijk et al., 2010b). In addition, it is a critical regulator of γ-globin gene expression (van Dijk et al., 2010a). However, little is known about the molecular mechanism of transcriptional control mediated by Chtop.

Chtop contains a central glycine-arginine-rich (GAR) region that is recognized and methylated by Prmt1. Since arginine methylation controls protein-protein interactions, we used a biotinylation-proteomics approach to identify proteins that bind Chtop in the presence and absence of Prmt1. In this study we identified and characterized a novel complex that binds specifically to methylated Chtop. As this nuclear complex consists of five proteins – SUMO1/sentrin/SMT3 specific peptidase 3 (Senp3), proline-glutamate and leucine rich protein 1 (Pelp1), LAS1-like protein (Las1L), Testis expressed 10 protein (Tex10), and WD repeat domain 18 protein (Wdr18) – we call it Five Friends of Methylated Chtop (5FMC). We show that Pelp1 is critical for the integrity of 5FMC and that Chtop and 5FMC are recruited by Zinc finger binding protein-89 (Zbp-89), thereby regulating both (de)sumoylation of, and transactivation by, Zbp-89.

RESULTS

Methylation dependent and independent interactions of Chtop

In order to identify interaction partners of Chtop, an N-terminal double-tagged version of Chtop protein (Bio_HA_Chtop) was expressed in mouse erythroleukemia (MEL) cells. These cells also stably expressed BirA, a bacterial biotin ligase which efficiently biotinylates the Bio-tag (de Boer et al., 2003). Protein complexes from nuclear lysates were recovered by streptavidin pull down followed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) and were

A

Strept. PD of Bio_HA_Chtop		
Protein	uniq. pept.	coverage (%)
Prmt1	13	39,9
Chtop	5	28,8
Set	3	20,5
Prmt5	3	8,6
40-2-3	1	5,4
Pc2	1	3,1
Ring1B	1	3,3
Phc2	1	2,5
Las1L	7	12,5
Pelp1	7	10
Senp3	6	14,8
Wdr18	6	20,9
Tex10	5	10,2

B

Strept. PD of Bio_HA_Chtop shControl			Strept. PD of Bio_HA_Chtop shPrmt1		
Protein	uniq. pept.	coverage (%)	Protein	uniq. pept.	coverage (%)
Prmt1	12	34	Prmt1	4	13,6
Chtop	3	24,3	Chtop	4	30,7
Prmt5	7	17,4	Prmt5	6	12,5
Set	4	18,3	Set	2	11,3
40-2-3	2	8,5	40-2-3	3	12,6
Pc2	2	5,6	Pc2	1	3,1
Ring1B	1	5,1	Ring1B	2	9,3
Phc2	1	3,4	Phc2	3	12,4
Las1L	11	18,4	not detected	x	x
Pelp1	10	14,2	not detected	x	x
Senp3	6	16,5	Senp3	1	2,5
Wdr18	7	23,2	Wdr18	1	4,2
Tex10	10	15,7	Tex10	1	2,3

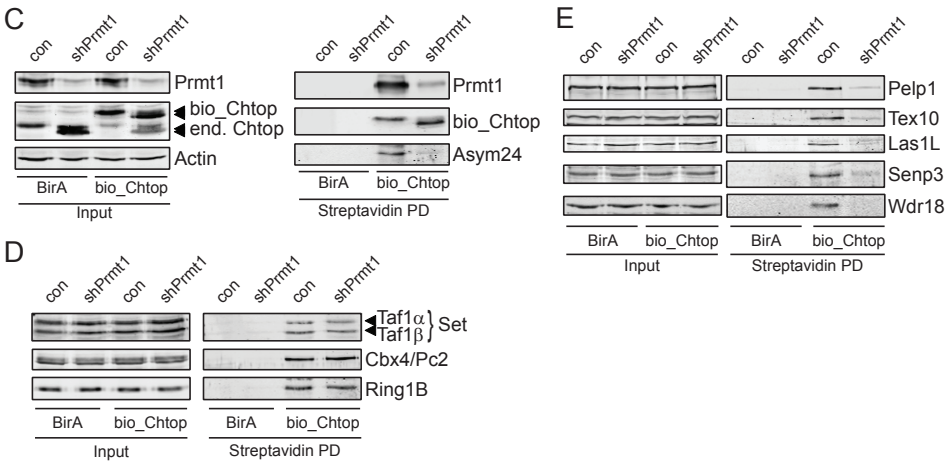

Figure 1. Identification of Chtop-interacting proteins. (A) List of Chtop interacting proteins indentified by mass spectrometry (MS). Protein complexes from nuclear lysates of MEL_BirA cells ectopically expressing Bio_HA_Chtop were recovered by MS. Proteins, unique peptides (uniq. pept.) and percent coverage (coverage %) are indicated. (B) List of Chtop interacting proteins in MEL_BirA cells treated with control lentivirus (shControl) and lentivirus expressing shRNA against Prmt1 (shPrmt1) identified by MS. Interactions with major differences between shControl and shPrmt1 treated cells are shown in a white box. (C) Chtop is hypomethylated in the absence of Prmt1. Whole cell lysates (Input) and streptavidin pull downs (Streptavidin PD) were analyzed for Prmt1, Chtop and asymmetric dimethyl arginine (Asym24) residues. Actin staining serves as a loading control. Ectopically expressed (bio_Chtop) and endogenous Chtop (end. Chtop) are indicated. (D) Chtop methylation-independent interactions. Whole cell lysates (Input) and streptavidin pull-downs (Streptavidin PD) were analyzed with the antibodies indicated. (E) Chtop methylation-dependent interactions. Whole cell lysates (Input) and streptavidin pull-downs (Streptavidin PD) were analyzed with the antibodies indicated.

compared to samples from cells expressing BirA alone (Figure 1A, Table S2). This confirmed the association of Chtop with Prmt1 and Prmt5, factors that we have

previously identified as Chtop binding proteins (van Dijk et al., 2010). MS analysis also revealed the binding of three members of the polycomb repressor complex 1 (PRC1; Pc2, Ring1B, and Phc2) (Alkema et al., 1997; Levine et al., 2002; Shao et al., 1999), the SET nuclear oncoprotein (Nagata et al., 1995), and the mRNA export protein 40-2-3 (Hautbergue et al., 2009). Furthermore, high MASCOT scores were obtained for the proteins Pelp1, Las1L, Tex10, Senp3 and Wdr18, four of which were previously co-purified with the MLL complex and were recently described as regulators of ribosome biogenesis (Dou et al., 2005; Finkbeiner et al., 2011). Pelp1 is a coactivator involved in nuclear hormone signaling (Vadlamudi and Kumar, 2007), while Senp3 is a SUMO-specific protease (Nishida et al., 2000). The WD-repeat protein Wdr18, Tex10, and Las1L had not been characterized previously. To investigate whether arginine methylation of Chtop played a role in these interactions, we performed lentiviral-mediated knockdown of Prmt1 in Bio_HA_Chtop expressing MEL_BirA cells and used a similar purification approach. When compared to cells transduced with a control lentivirus, no major differences were observed in the binding of Chtop to Prmt5, Pc2, Ring1B, Phc2, 40-2-3 and Set (Figure 1B, Table S2). Interestingly, copurification of Las1L, Pelp1, Tex10, Senp3 and Wdr18 was strongly reduced or absent when Chtop was hypomethylated. Next, we confirmed the methylation (in)dependent interactions by streptavidin affinity purification, followed by Western blot analysis. Chtop is hypomethylated in the absence of Prmt1, as indicated by its faster migration pattern and by staining with an antibody that specifically recognizes aDMA (Asym24; Figure 1C). Endogenous Chtop interactors were efficiently recovered in Bio_HA_Chtop pull downs, while no background staining was observed in MEL_BirA cells (Figure 1D-E). In addition, no association was observed with Wdr18 in the absence of Prmt1, while the interactions with Pelp1, Las1L, Tex10 and Senp3 were strongly reduced. Together, these results validate the interactions identified by MS and show that methylation of Chtop is required for the recruitment of Pelp1, Las1L, Tex10, Senp3 and Wdr18.

5FMC is a novel nuclear complex

We have previously shown that the majority of Chtop is bound to chromatin (van Dijk et al., 2010b). To elucidate where the newly identified methylation-specific partners of Chtop localize in the cell, we performed biochemical fractionation of MEL cells. This revealed that all five proteins were mainly found in the nucleoplasm, while low levels were also detected in the cytoplasmic and chromatin fractions (Figure 2A). To examine whether the five proteins form a complex, we first performed size-exclusion chromatography of MEL nuclear extracts. The elution patterns of Pelp1, Las1L, Tex10, Senp3 and Wdr18, as well as Chtop, overlapped substantially (Figure 2B). The molecular mass of the positive fractions was >1MDa, indicating that the factors were present in a high molecular weight protein complex or were bound to chromatin. Similar experiments in human 293T cells revealed comparable results, although larger proportions of LAS1L, TEX10, and WDR18 were detected in fractions corresponding to lower molecular mass (Figure S1). Next, doubled tagged (Bio_HA) Pelp1, Las1L, Senp3 and Wdr18 were stably expressed in MEL_BirA cells. Of note, we were not able to exogenously express the Tex10 protein, probably due to protein stability issues. Associated proteins were identified by streptavidin pull down in nuclear lysates followed by nanoLC-MS/MS and were compared to samples from MEL_BirA only cells. In all four experiments, the associated proteins with the highest MASCOT score were Pelp1, Las1L, Tex10, Senp3 and Wdr18 (Figure 2C (Bio_HA_Senp3) and Figure S3 (Pelp1, Wdr18, and Las1L)). Moreover, in the MS analysis of Bio_HA_Senp3, Bio_HA_Wdr18 and Bio_HA_Pelp1 we observed an association with the Nol9 protein. The Nol9 ortholog in S. pombe (Grc3) was recently shown to associate with Las1 and the yeast IPI complex that consist of Rix1, Ipi1 and Crb3 (Kitano et al., 2011). These proteins share homologous regions with Pelp1, Tex10 and Wdr18, respectively. Chtop was only detected in the Bio_HA_Senp3 purification (Figure

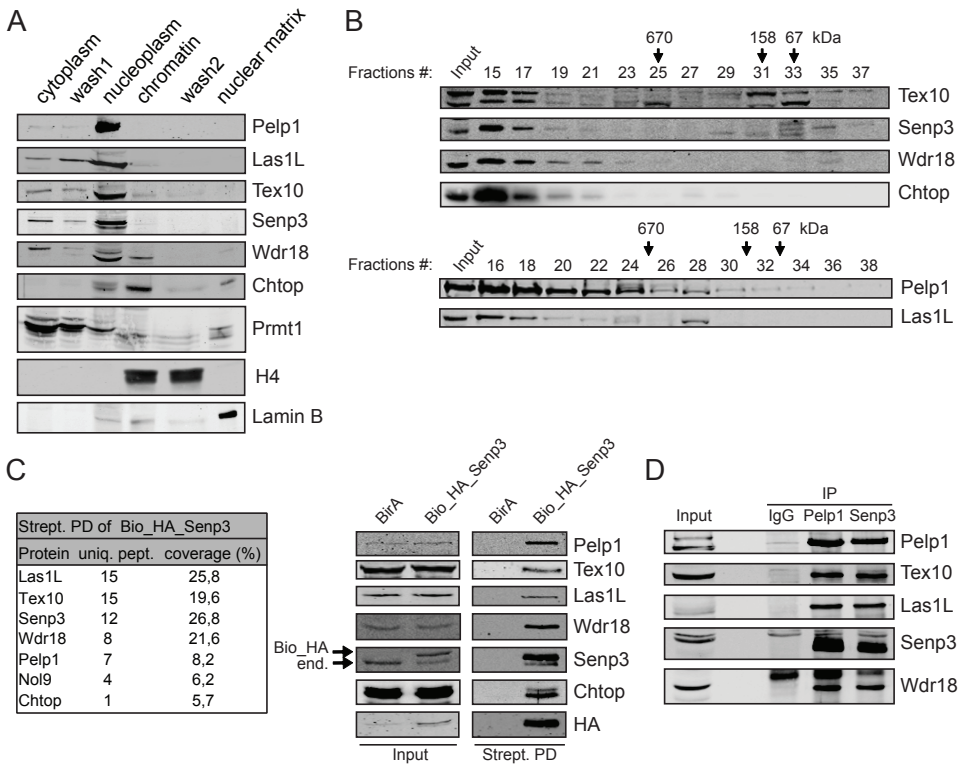

Figure 2. 5FMC is a novel nuclear complex. (A) Chtop methylation dependent interaction proteins are localized mainly in the nucleoplasm. MEL_BirA cells were biochemical fractionated as described in Experimental Procedures. Cytoplasmic, nucleoplasmic, chromatin and nuclear matrix were tested using Pelp1, Las1L, Tex10, Senp3, Wdr18 antibodies against endogenous proteins. Chtop, Prmt1, H4, and Lamin B served as controls for individual fractions. (B) MEL_BirA cell nuclear extracts were analyzed by sized-exclusion chromatography on a Superose 6 column. Proteins eluted from the indicated fractions were blotted with the indicated antibodies. Molecular mass markers are indicated at the top. (C) Senp3 interactions in MEL cells. Whole cell lysates (Input) and streptavidin pull downs (Strept. PD) from MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Senp3 (Bio_HA_Senp3) were analyzed by MS (table) and western blotting. Immunoblot probed with the indicated antibodies. Arrows indicate endogenous (end.) and biotinylated (Bio_HA) Senp3. (D) 5FMC is a novel nuclear complex. Endogenous association between the 5FMC components. MEL_BirA cell nuclear lysates were analyzed by immunoprecipitation (IP) and western blotting with the antibodies indicated.

2C, left panel). The MS results were confirmed by immunoblot analysis of the streptavidin pull downs of tagged proteins (Figure 2C, right panel and Figure S3), and immunoprecipitations of endogenous proteins from both mouse and human cells (Figures 2D and S2, respectively). Taken together, these results show that

Pelp1, Las1L, Tex10, Senp3 and Wdr18 form a novel nuclear multi-protein complex. As this complex binds selectively to methylated Chtop, we named it Five Friend of Methylated Chtop, or 5FMC.

Pelp1 is the core subunit and critical for 5FMC stability

To further study the composition of the 5FMC complex, we transiently co-transfected tagged 5FMC components in 293T cells, followed by co-immunoprecipitation (co-IP). We found that T7_Pelp1 is efficiently recovered in HA_Wdr18 IPs, while Myc_Senp3 interacts with HA_Wdr18 (Figure 3A). In contrast, Myc_Senp3 co-purifies with HA_Wdr18 only when T7_Pelp1 is co-transfected (Figure 3A, lanes 3 and 4). In addition, we observed that cotransfection of T7_Pelp1 resulted in higher protein levels of HA_Wdr18 and Myc_Senp3 (Figure 3A, lanes 2 and 4). Identical results were obtained when Myc_Senp3 was immunoprecipitated (data not shown). No conclusive results could be obtained with ectopically expressed Tex10 and Las1L, probably due to stability issues. These results show that Pelp1 is required for the interaction between Wdr18 and Senp3 and that Pelp1 might be required for the stability of these proteins. To further study the potential central role of Pelp1 within the 5FMC complex, we depleted endogenous Pelp1 in MEL_BirA cells by lentiviral-mediated knockdown using two different shRNAs. Interestingly, the protein levels of Senp3 and Las1L were dramatically decreased, while Wdr18 and Tex10 could not be detected in the absence of endogenous Pelp1 (Figure 3B, left panel). Quantitative RT-PCR showed that the reduced protein levels were due to protein stability rather than reduced mRNA levels (Figure 3B, right panel). The individual knockdown of Las1L, Senp3 or Wdr18 had no significant effect on the protein levels of the other 5FMC subunits. These results indicated that Pelp1 is the central component of the 5FMC complex and that the integrity of 5FMC is essential for the stability of its components. Analysis of the primary sequence of Pelp1 showed that Pelp1 contains a cysteine-rich region, two proline-rich regions and a C-terminal glutamine-rich region (Vadlamudi and Kumar, 2007). To map the

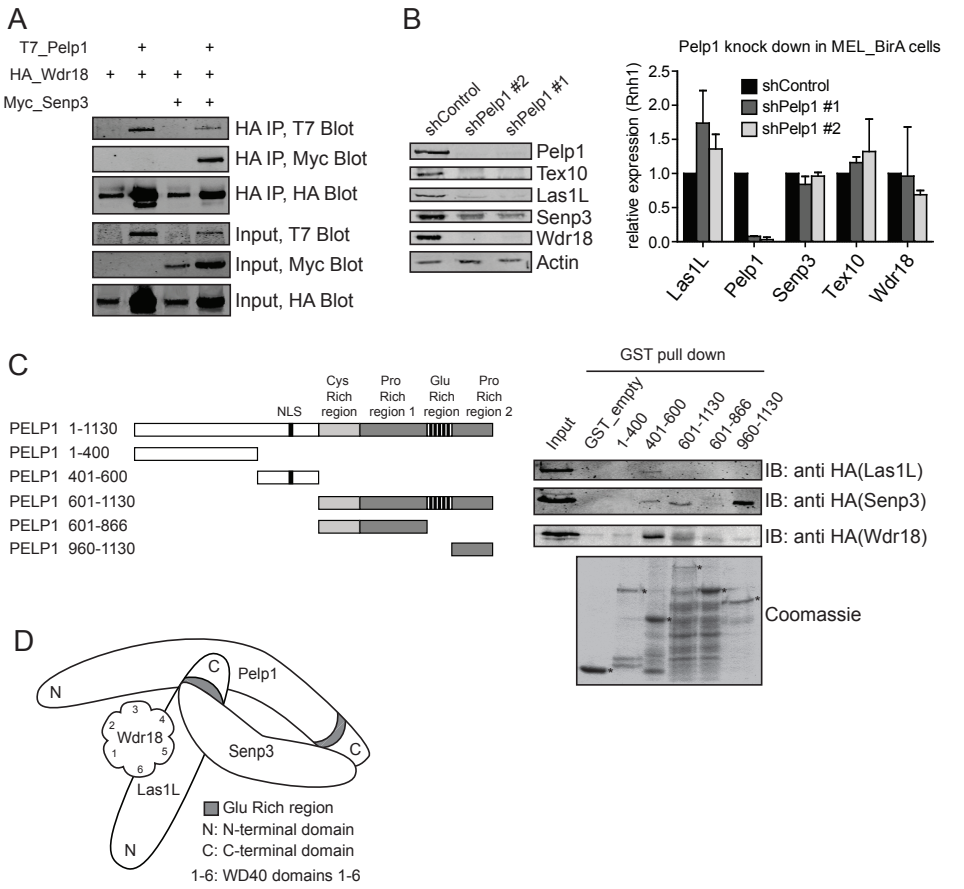

Figure 3. Pelp1 is the core component of the 5FMC complex. (A) Pelp1 is required for interaction between Senp3 and Wdr18. 293T cells were transiently transfected with expression vectors encoding T7_Pelp1, HA_Wdr18 and Myc_Senp3. Cell lysates were analyzed by IP and western blotting with the antibodies indicated. (B) Pelp1 is needed for the stability of the 5FMC complex. MEL_BirA cells were treated with the indicated shRNAs. Nuclear lysates were analyzed by western blotting with the indicated antibodies. Actin staining serves as a loading control. Total RNA was extracted from MEL_BirA cells transduced with the indicated shRNA and analyzed by RT-QPCR for Las1L, Pelp1, Senp3, Tex10 and Wdr18. Error bars: SD of triplicate experiment. (C) Mapping the interaction regions of Pelp1. Schematic representation of Pelp1 deletion constructs. GST fused Pelp1 domains and GST alone (GST_empty) were immobilized onto glutathione beads (lower panel stained with coomassie) and used to pull down nuclear cell lysates from 293T cells expressing HA_Las1L, HA_Senp3 and HA_Wdr18. Asterisks indicate GST fusion proteins. (D) Schematic representation of interaction between Pelp1, Las1L, Senp3 and Wdr18 protein domains.

interactions between Pelp1 and other 5FMC components, we tested the ability of HA-tagged Las1L, Wdr18 and Senp3 proteins to bind to various domains of

Pelp1 fused to GST. HA_Las1L and HA_Wdr18 interacted with the GST_Pelp1 fusion containing amino acids (aa) 401-600, while HA_Senp3 mainly interacted with domain 960-1130. Interactions were further studied using a series of deletion mutants of Las1L. These experiments showed that the C-terminal part of Las1L (aa 552-734) mediated the interaction with Pelp1, while the central domain (aa 370-552) mediated the interaction with Wdr18. The binding to Senp3 could not be mapped in detail: any deletion between aa 188-734 disrupted the interaction (Figure S4A). Similar experiments with Wdr18 deletion constructs revealed that the region containing WD40 domains 4-6 were required for binding to Las1L, while deletion of any WD40 domain disrupted the binding to Pelp1 (Figure S4B). These initial domain-mapping experiments suggest complex multi-intermolecular interactions and are in line with the proposed model shown in Figure 3D.

Chtop recruits 5FMC to Zbp-89

MS analysis of the Zbp-89 interactome revealed Chtop and several Chtop-associated factors as potential interaction partners of Zbp-89 in MEL cells (manuscript in preparation). To further explore the possibility that 5FMC interacts with Zbp-89, we performed bio_Zbp-89 pull downs in MEL_BirA cells followed by immunoblotting. We observed that Chtop, Prmt1 and 5FMC components associated with Zbp-89 (Figure S5). These interactions were DNA independent, as degradation of DNA by Benzonase treatment did not affect the efficiency of co-purification. To investigate whether Chtop is required for the interaction between Zbp-89 and 5FMC, we examined the association of Zbp-89 and 5FMC complex components in Chtop knockdown cells. Bio_Zbp-89 was precipitated more efficiently when Chtop protein levels were reduced, suggesting that bio_Zbp-89 was more accessible in the absence of Chtop (Figure 4A). Indeed, co-purification of 5FMC complex components was reduced to ~30% of control samples (Figures 4A and 4B). These results indicate that Chtop is required for the association of Zbp-89 with the 5FMC complex.

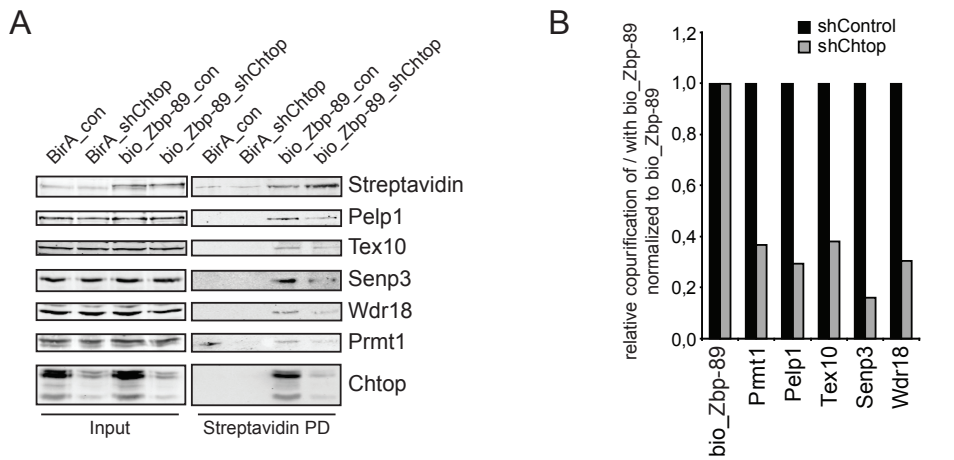

Figure 4. Chtop recruits 5FMC to Zbp-89. (A) Zbp-89 is associated with Chtop and 5FMC complex. Nuclear cell lysates (Input) of MEL_BirA cells (BirA) and MEL_BirA cells expressing biotinylated Zbp-89 (bio_Zbp-89) treated with lentivirus expressing shRNA against Chtop (shChtop) and control lentivirus (shControl) analyzed by streptavidin pull down (Streptavidin PD) and western blotting with the antibodies indicated. (B) Quantification of protein levels using the Odyssey Infrared Imaging System.

Senp3 regulates the sumoylation-status of Zbp-89

It has been reported that Zbp-89 can be post-translationally modified by SUMO in transient overexpression experiments (Chupreta et al., 2007). To investigate whether endogenous Zbp-89 could be sumoylated, we first performed streptavidin pull downs from bio_HA_SUMO-2 expressing MEL_BirA nuclear extracts. Staining with an antibody recognizing Zbp-89 detects multiple sumoylated Zbp-89 species (Figure 5A, lower panel), in line with the observation that Zbp-89 contains at least 2 domains that can be sumoylated (Chupreta et al., 2007). Knockdown of Senp3 in these cells led to a significant increase of SUMO-2 detection in whole cell lysates, as well as of sumoylated Zbp-89. It should be noted that the reduction of Senp3 expression affected cell growth and survival, thereby limiting the effect of the knockdown. Next, we performed similar experiments in MEL cells expressing bio_Zbp-89. Streptavidin pull downs probed with an anti-SUMO 2/3 antibody showed that Senp3 depletion resulted in an increase of the levels of SUMO modi-

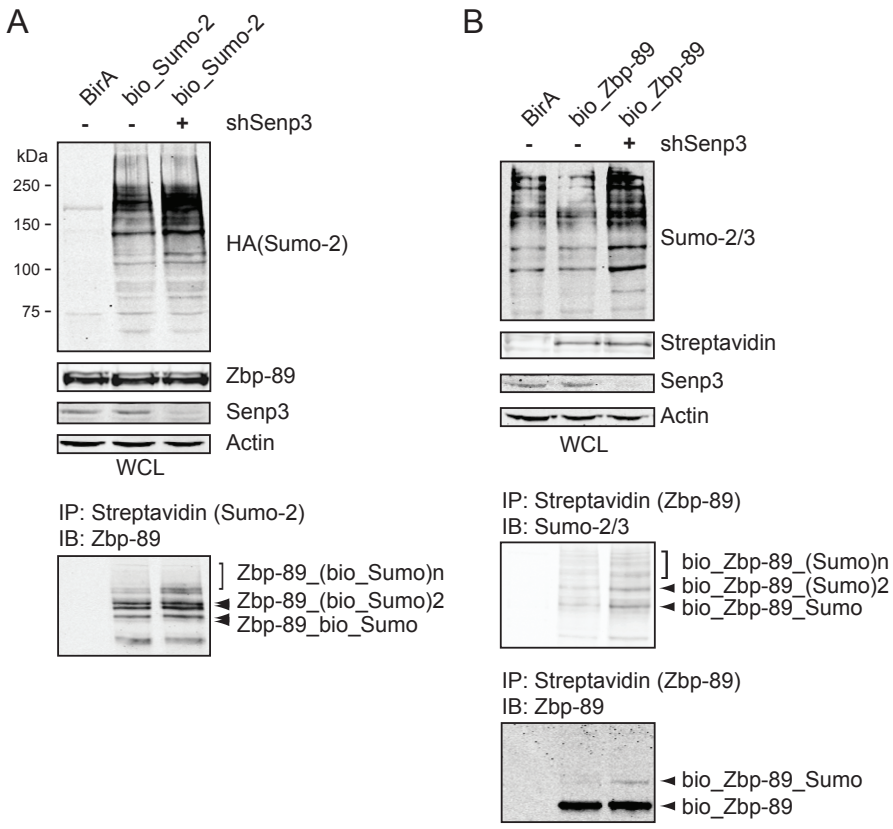

Figure 5. Senp3 regulates Zbp-89 sumoylation. (A-B) Senp3 plays a role in Zbp-89 sumoylation. (A) MEL_BirA (BirA) and MEL_BirA cells expressing bio_HA_Sumo2 (bio_Sumo2) were treated with shRNA against Senp3 (shSenp3). Whole cell lysates (WCL) analyzed by western blotting with the indicated antibodies. Actin staining serves as a loading control. Nuclear extracts were pull down using magnetic streptavidin beads and analyzed by western blotting with anti-Zbp-89 antibody. (B) MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Zbp-89 (bio_Zbp-89) were treated with shRNA against Senp3 (shSenp3). Whole cell lysates (WCL) analyzed by western blotting with the antibodies indicated. Actin staining serves as a loading control. Nuclear extracts were pulled down using magnetic streptavidin beads and analyzed by western blotting with anti-Zbp-89 and anti Sumo-2/3 antibodies.

fied bio_Zbp-89 and the appearance of a slower mobility form of bio_Zbp-89 when probed with anti-Zbp-89 antibody (Figure 5B). The upper band is consistent with SUMO modification. Taken together, these results show that Zbp-89 is sumoylated *in vivo*, and that Senp3 plays a role in this process.

5FMC is critical for Zbp-89 dependent gene regulation

The observations that 5FMC is a desumoylating complex that is recruited by Zbp-89 suggest that it is involved in transcriptional regulation. To examine whether 5FMC is recruited to Zbp-89 target genes we performed chromatin immunoprecipitation (ChIP) experiments for Pelp1, the core component of the complex. We used MEL_BirA cells that ectopically expressed bio_Pelp1, as this increased sensitivity (not shown). After chromatin precipitation, the promoter or coding regions of four Zbp-89 target genes (Dusp6, Tubb1, Zbp-89, Atf5), that were identified by ChIP-sequencing as binding sites of Zbp-89 (manuscript in preparation), were analyzed with the corresponding primers. Occupancy by Pelp1 was indeed observed for these Zbp-89 target genes (Figure 6A). To further investigate the potential role of 5FMC in transcription regulation, we depleted Pelp1, Senp3 and Chtop in MEL_BirA cells using shRNA (Figure 6B) and performed ChIP using an antibody against RNA polymerase II (Pol II). As expected, reduced levels of Pelp1, Senp3 and Chtop resulted in reduced Pol II occupancy at the promoter or coding regions of Dusp6, Tubb1, Zbp-89 and Atf5 when compared to cells treated with scrambled control shRNA (Figure 6C). To exclude that the observed differences in occupancy by Pol II were not due to altered occupancy by Zbp-89 we performed ChIP using an antibody against Zbp-89. Binding of Zbp-89 to the promoter or coding region of its target genes was unaffected upon depletion of Senp3 and Pelp1, while binding of Zbp-89 was reduced when Chtop was depleted (Figure 6D). The reduction of Zbp-89 binding upon depletion of Chtop was due to reduced protein levels of Zbp-89 (Figure 6B, right panel). The same regions were also tested for changes in the histone modifications H3K4 and H3K27, but no changes were detected (not shown). Collectively, our data indicate that 5FMC is recruited to Zbp-89 target genes and that it is involved in their transcriptional activation by Zbp-89. This most likely involves desumoylation of Zbp-89 and possibly of other factors.

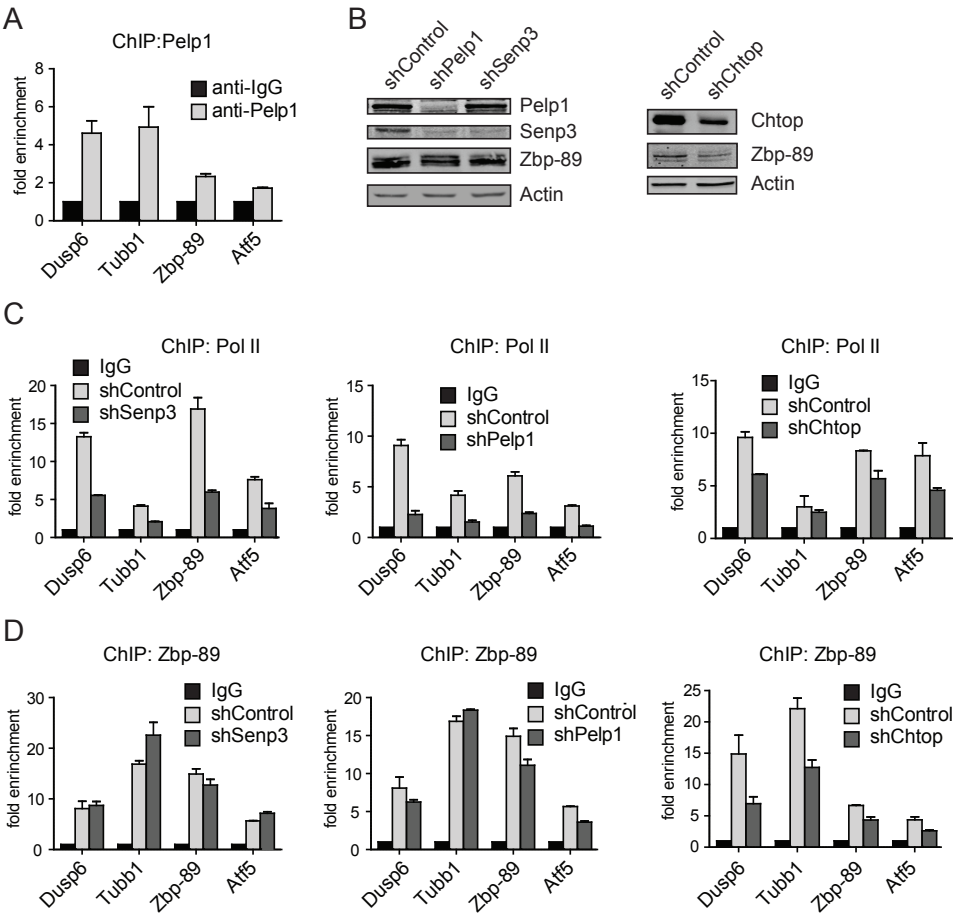

Figure 6. 5FMC is involved in the regulation of Zbp-89 target genes. (A) Pelp1 is recruited at the promoter or coding regions of Dusp6, Tubb1, Zbp-89 and Atf5. MEL_BirA cells that ectopically expressed bio_Pelp1 analysed by ChIP using Pelp1 antibody for the indicated gene promoters or coding regions. Error bars: SD of triplicate experiment. (B) Knockdown of Pelp1, Senp3 and Chtop in MEL_BirA cells. MEL_BirA cells were treated with the indicated shRNAs. Cell lysates were analyzed by western blotting with the indicated antibodies. Actin staining serves as a loading control. (C) Pelp1, Senp3 and Chtop knockdowns reduced RNA polymerase II (Pol II) occupancy at the promoter or coding regions of Dusp6, Tubb1, Zbp-89 and Atf5. MEL_BirA cells were treated as in (B). ChIP analysis at the indicated regions was performed using PolI II antibody. Error bars: SD of triplicate experiment. (D) Pelp1, Senp3 and Chtop knockdowns do not affect Zbp-89 binding at the promoter or coding regions of Dusp6, Tubb1, Zbp-89 and Atf5. MEL_BirA cells were treated as in (B). ChIP analysis at the indicated regions was performed using Zbp-89 antibody. Error bars: SD of triplicate experiment.

DISCUSSION

In the present study, we have identified 5FMC, a desumoylating protein complex that exclusively binds to arginine-methylated Chtop. It acts as a key regulator of Zbp-89 sumoylation and is required for full transcriptional activation of Zbp-89 dependent genes. To our knowledge, this is the first description of a mechanism that adds specificity to desumoylation processes.

The 5FMC complex is composed of five proteins: Pelp1, Las1L, Tex10, Senp3, and Wdr18. These factors are amongst the most abundant proteins present in Chtop purifications, but only when Chtop is methylated by Prmt1. Little is known about most of the components of the 5FMC complex. Pelp1 is a coactivator involved in nuclear hormone signaling (Vadlamudi and Kumar, 2007), Senp3 is a SUMO-specific protease (Nishida et al., 2000), while Wdr18, Las1L, and Tex10 have not been characterized yet. In yeast, the proteins Rix1, Ipi1 and Ipi3 (S. cerevisiae) / Crb3 (S. pombe) share conserved regions with Pelp1, Tex10 and Wdr18, respectively. They form the IPI complex and have been shown to function in ribosomal RNA processing (Krogan et al., 2004). It was recently shown that Las1, the yeast ortholog of Las1L, is also associated with the IPI complex (Kitano et al., 2011). Furthermore, PELP1, TEX10, LAS1L, SENP3, and WDR18 were recently linked to ribosome biogenesis in human cells (Finkbeiner et al., 2011). These data indicate an evolutionary conserved complex with a role in ribosomal RNA processing.

Additionally, several studies suggested a role for 5FMC components in transcriptional regulation and (de)sumoylation events, although this had not been explored further. Doseff and Arndt proposed in their initial identification of Las1 in S. cerevisiae that it functions as a transcription factor (Doseff and Arndt, 1995), while it was recently shown to localize to heterochromatic regions (Kitano et al., 2011). In human cells, components of the 5FMC complex were first detected in the MS analysis of the MLL1-WDR5 complex, a complex that regulates transcription activation by H3K4 methylation (Dou et al., 2005). PELP1, LAS1L, TEX10 and SENP3

were also detected together with components of the CoREST1/HDAC1 co-repressor complex in a MS study for proteins that are modified by SUMO-2 (Ouyang et al., 2009). Sumoylation is important for stability and recruitment of repressive complexes such as CoREST, NuRD, and SetDB1 (Garcia-Dominguez and Reyes, 2009), indicating that desumoylation of transcription factors and corepressors is required for derepression.

Our results indicate that Pelp1 is the core component of 5FMC. Pelp1 has the ability to interact with nuclear receptors (NRs) and enhances transcription of their target genes (Vadlamudi et al., 2001). Pelp1 has been shown to interact with the acetyltransferases CBP and p300 (Nair et al., 2004), KDM1, a member of the CoREST1 repressor complex (Nair et al., 2010), and deacetylases, including components of the NuRD repressor complex (Mishra et al., 2004). Interestingly, Pelp1 was also identified in a blind screen for SUMO-2 interacting proteins (Rosendorff et al., 2006). This opens the possibility that Pelp1 acts, in addition to its scaffold function within 5FMC, as a Sumo-2 sensor to detect Senp3 substrates.

As Chtop is strongly associated with chromatin (van Dijk et al., 2010b), while 5FMC mainly resides in the nucleoplasm (this paper), the interactions between Chtop and 5FMC are most likely transient and highly dynamic. Possibly, Chtop recruits 5FMC complex, in order to desumoylate its substrates, in a “hit-and-run” manner. In addition, 5FMC may very well act as a desumoylation complex outside the chromatin environment and independent of Chtop, e.g. in ribosome biogenesis (Finkbeiner et al., 2011).

In line with our previous observation that Chtop colocalizes to H3K27me3 (van Dijk et al., 2010b), we found that Chtop interacts with the Prc1 complex. In contrast to 5FMC, this association does not depend on the methylation status of Chtop. Intriguingly, the Pc2 component of Prc1 (also known as Cbx4) has been identified as a SUMO-ligase for several transcriptional regulators (Kagey et al., 2003; Wotton and Merrill, 2007). Although these studies mainly focused on SUMO-1 modification, Chtop may recruit 5FMC as an antagonist of Pc2/Cbx4.

We have shown that Chtop recruits 5FMC to Zbp-89 and that Zbp-89 is subsequently desumoylated by Senp3, resulting in higher Pol II levels on Zbp-89 target genes. We anticipated that depletion for Senp3 and Pelp1 would also affect H3K4 and/or H3K27 methylation of these regions, as 5FMC is might be connected with the MLL1-WDR5 (Dou et al., 2005) and Prc1 complexes (this paper). However, no changes were observed, which is in line with the observation that overexpression of catalytically inactive Senp3 does not affect diMeH3K4 of specific promoters (Ouyang et al., 2009).

Collectively, these observations suggest a model where methylated Chtop recruits the 5FMC complex to factors like Zbp-89. Subsequently, the Senp3 protease desumoylates Zbp-89 and possibly additional components of repressor complexes, resulting in the stimulation of transcription of target genes (Figure 7).

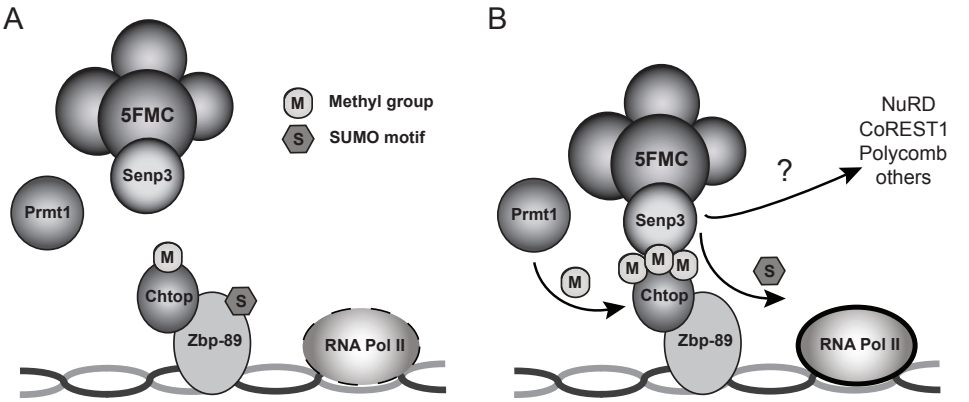

Figure 7. 5FMC complex recruitment to Zbp-89 stimulates transcription of target genes. (A-B) Model illustrating 5FMC complex function in Zbp-89 dependent gene expression. Methylated Chtop recruits the 5FMC complex to transcription factor Zbp-89. Upon 5FMC binding, Senp3 protease desumoylates Zbp-89 and possibly additional components of repressor complexes, resulting in the stimulation of transcription of target genes.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Cells

The following plasmids have been described previously: GST-PELP1 deletions (Nair et al., 2004), T7-Pelp1 (Vadlamudi et al., 2001) and pMT2_HA_Chtop (van Dijk et al., 2010b). The cDNA of human SUMO-2 (hSMT3b) was kindly provided by Dr. Guntram Suske (Philipps-Universität Marburg, Germany). The cDNA of full-length LAS1L was obtained from Open Biosystems (Clone ID 3140243; Huntsville, AL, USA), full-length cDNA of Wdr18 was obtained from RZPD/imaGenes (clone IRAVp968G04150D6; Berlin, Germany) and full-length cDNA of Senp3 was obtained from RZPD/imaGenes (clone IRAVp968B0184D6; Berlin, Germany). The cDNA of PELP1 was subcloned from T7-PELP1. After the introduction of the 23-amino acid (aa) biotinylation tag into the pMT2_HA (Kaufman et al., 1989) and pMT2_HA_Chtop, LAS1L, Senp3 and PELP1 were cloned to pMT2_bio_HA using SalI and NotI, Wdr18 was cloned using SalI and SUMO-2 was cloned using SalI and EcoRI. Bio_HA_LAS1L, Bio_HA_Senp3, Bio_HA_PELP1, Bio_HA_Wdr18, Bio_HA_SUMO-2 and Bio_HA_Chtop were subcloned into the erythroid expression vector pEV-neo (Needham et al., 1992) and electroporated into mouse erythroleukemic (MEL) cells expressing the BirA biotin ligase (de Boer et al., 2003). To make the Gateway pSG513_myc destination plasmid, the Attr1-CmR-ccdb-Attr2 fragment was subcloned from pDEST17 (Invitrogen) to a modified pSG5 (Stratagene) using HindIII, downstream the myc-tag sequence that was introduced to the pSG513 plasmid using EcoRI and BamHI. cDNAs for Senp3, Wdr18 and LAS1L were cloned into pDONR221 (Invitrogen), from which they were cloned by Gateway LR reaction to pSG513_myc. Internal deletion mutants of LAS1L and Wdr18 were generated using the QuikChange site-directed mutagenesis kit (Stratagene). MEL and 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (FCS).

Transient transfection, Immunoprecipitation, GST pull-down assay and Western blot analysis

Transient transfections in 293T cells, immunoprecipitations and western blot analysis were performed as described previously (van Dijk et al., 2000). For immunoprecipitations combined with Benzonase (Novagen) incubation, 250 units of Benzonase were used followed by 3 hours incubation at 4oC. Nitrocellulose membranes were blocked in 1% bovine serum albumin (BSA), probed with the appropriate primary antibodies and analyzed using the Odyssey Infrared Imaging System (Li-Cor Biosciences). GST pull-down assays were performed as described previously (Nair et al., 2004). Western blots were probed with the following primary antibodies: Prmt1 (07-404), Asym24 (07-414), and Cbx4 (09-029) were from Upstate; Actin (clone I-19; sc-1616), Taf1β (clone H-120; sc-25564), Lamin B (sc-6216), HA (monoclonal F7; sc-7392), HA (polyclonal Y11; sc-805), Myc (monoclonal 9E10; sc-40), Myc (polyclonal A-14; sc-789) and Pol II (polyclonal N-20; sc-899) were from Santa Cruz Biotechnology; Tex10 (17372-1-AP), Las1L (16010-1-AP), Senp3 (17659-1-AP) and Wdr18 (15165-1-AP) were from Proteintech Group; Zbp-84 (ab69933) was from Abcam, SUMO 2/3 (clone 1E7; M114-3) was from MBL; Pelp1 (A300-876A) was from Bethyl Laboratories; T7 (69522-3) was from Novagen; Taf1α (B100-56353) was from Novus Biologicals and Chtop (KT64) was from Absea Biotechnology. Ring1B antibody was kindly provided from Dr. Miguel Vidal (Madrid, Spain).

Cell lysates and Mass Spectrometry (MS)

Preparation of nuclear and whole cell extracts from MEL and 293T cells were carried out as described previously (van Dijk et al., 2000). Purification of biotinylated proteins, tryptic digestion of paramagnetic streptavidin beads and LC-MS/MS were performed as described previously (de Boer et al., 2003; van Dijk et al., 2010b). The MASCOT score cut-off value for positive protein hits was set to ….

Lentivirus Mediated Knockdown

The Chtop and Prmt1 shRNA lentiviral vectors were described previously (van Dijk et al., 2010a; van Dijk et al., 2010b). For Pelp1 and Senp3, clones from the TRC Mission shRNA library ((Moffat et al., 2006); Sigma Aldrich, St. Louis, MO, USA) were used for knockdown experiments in MEL cells, including a non-targeting shRNA control virus (SHC002). Lentivirus was produced by transient transfection of 293T cells as described before (Zufferey et al., 1997). The following clones were used from the TRC shRNA library: TRCN0000177043 (shPelp1 #1), TRCN0000178252 (shPelp1 #2) and TRCN0000031016 (shSenp3).

Size-Exclusion Chromatography and Subcellular Fractionation

Nuclear extracts from MEL cells expressing BirA biotin ligase enzyme, were chromatographed over a Superose 6 column (Amersham Biosciences) using an AKTA fast-performance liquid chromatography apparatus. Fractions were collected and precipitated with trichloroacetic acid and analyzed by western blotting. Subcellular fractionation was performed as described previously (van Dijk et al., 2010b).

RT, QPCR and ChIP assay

Reverse transcription (RT), RT-quantitative PCR (RT-QPCR) and ChIP were performed as described previously (Esteghamat et al., 2011). Primers used for RT-QPCR and ChIP-QPCR are summarized in Table S1.

ACKNOWLEDGEMENTS

We thank Karel Bezstarosti and Erikjan Rijkers for technical assistance. This work was supported by the Netherlands Genomics Initiative (93518009 and 93511036), the Landsteiner Foundation for Blood Transfusion Research (1040), the Netherlands Scientific Organization (NWO DN 82-301 and ZonMW 912-07-019 and 40-00812-98-08032) and the Centre for Biomedical Genetics.

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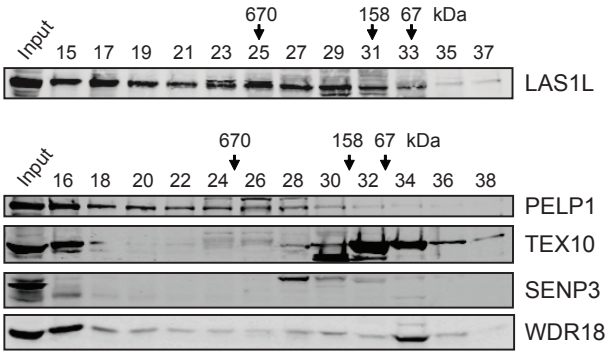
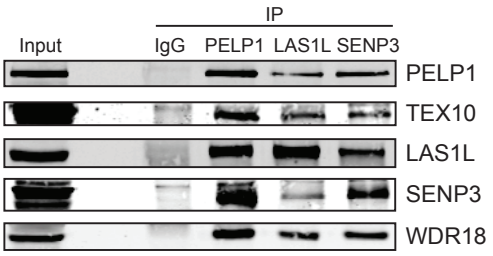
SUPPLEMENTAL FIGURES



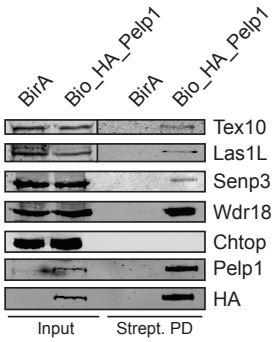
Supplemental Figure 1. 5FMC elution patterns in 293T cells. 293T cell nuclear extracts were analyzed by sized-exclusion chromatography on a Superose 6 column. Proteins eluted from the indicated fractions were blotted with the indicated antibodies. Molecular mass markers are indicated at the top.



Supplemental Figure 2. 5FMC is a novel nuclear complex. Endogenous association between the 5FMC components. 293T cell nuclear lysates were analyzed by immunoprecipitation (IP) and western blotting with the antibodies indicated.

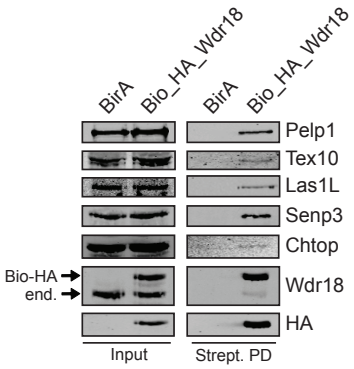
A

Strept. PD of Bio_HA_Pelp1		
Protein	uniq. pept.	coverage (%)
Las1L	19	26,3
Wdr18	14	39,7
Tex10	16	19,2
Pelp1	11	11,4
Senp3	8	16,9
Nol9	4	6,4



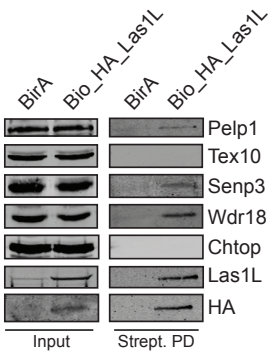
B

Strept. PD of Bio_HA_Wdr18		
Protein	uniq. pept.	coverage (%)
Las1L	20	31,3
Wdr18	15	41,3
Tex10	16	21
Pelp1	15	18,1
Senp3	15	30,1
Nol9	9	10,9

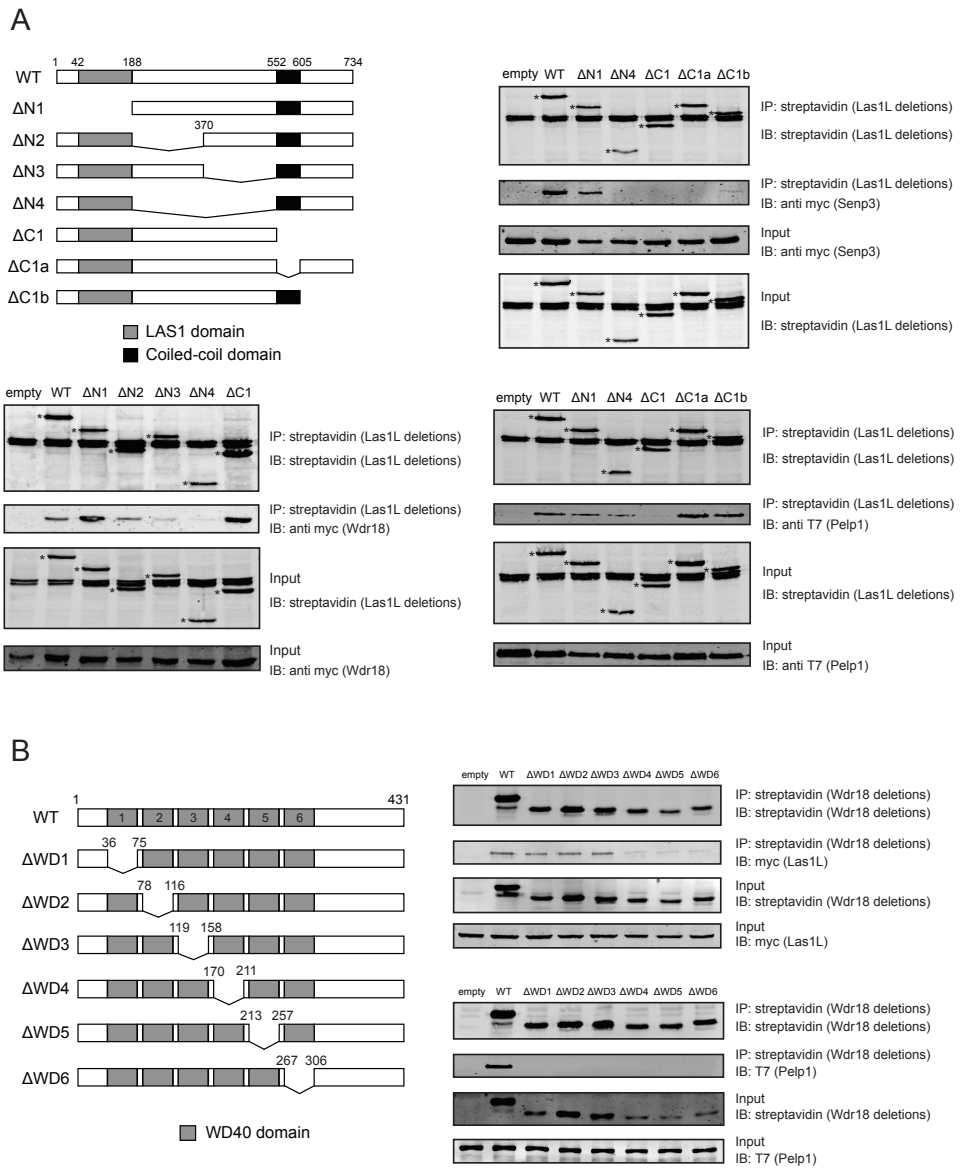


C

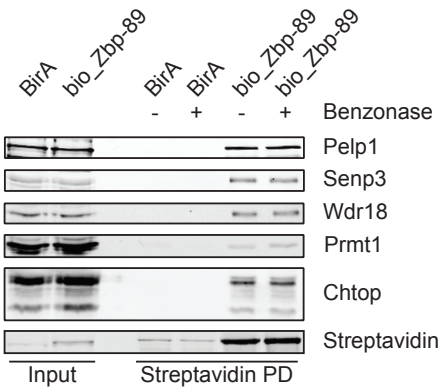
Strept. PD of Bio_HA_Las1L		
Protein	uniq. pept.	coverage (%)
Pelp1	9	9,7
Wdr18	9	23
Tex10	4	4,5
Las1L	4	5,9
Senp3	4	7,9



Supplemental Figure 3. 5FMC nuclear interactions. (A) Pelp1 interactions in MEL cells. Whole cell lysates (Input) and streptavidin pull downs (Strept. PD) from MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Pelp1 (Bio_HA_Pelp1) were analyzed by MS (table) and western blotting. Immunoblot probed with the antibodies indicated. (B) Wdr18 interactions in MEL cells. Whole cell lysates (Input) and streptavidin pull downs (Strept. PD) from MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Wdr18 (Bio_HA_Wdr18) were analyzed by MS (table) and western blotting. Immunoblot probed with the antibodies indicated. Arrows indicate endogenous (end.) and biotinylated (Bio_HA) Wdr18. (C) Las1L interactions in MEL cells. Whole cell lysates (Input) and streptavidin pull downs (Strept. PD) from MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Las1L (Bio_HA_Las1L) were analyzed by MS (table) and western blotting. Immunoblot probed with the antibodies indicated.



Supplemental Figure 4. Mapping the interaction regions of Las1L and Wdr18. (A) Bio_HA-tagged wild-type Las1L and its deletion mutants were expressed in 293T cells. Cell lysates were immunoprecipitated (IP) and blotted (IB) with the indicated antibodies. Whole cell lysates (Input) were blotted and ectopically expressed wild-type Wdr18 and its deletion mutants were visualized using a streptavidin antibody. Asterisks indicate wild-type Las1L and its deletion mutants. (B) Bio_HA-tagged wild-type Wdr18 and its deletion mutants were expressed in 293T cells. Cell lysates were immunoprecipitated (IP) and blotted (IB) with the antibodies indicated. Whole cell lysates (Input) were blotted and ectopically expressed wild-type Wdr18 and its deletion mutants were visualized using a streptavidin antibody.



Supplemental Figure 5. Chtop, Prmt1 and 5FMC complex are associated with Zbp-89. Streptavidin pull downs (Strept. PD) from MEL_BirA (BirA) and MEL_BirA cells expressing biotinylated Zbp-89 (Bio_Zbp-89) were treated with Benzonase. Whole cell lysates (Input) and streptavidin pull downs were analyzed by western blotting. Immunoblot probed with the antibodies indicated.

SUPPLEMENTAL TABLES

Supplemental Table 1. Oligonucleotides used in this study

Name	Sequence (5´-3´)
QPCR	
Rnh1_Fwd	TCCAGTGTGAGCAGCTGAG
Rnh1_Rev	TGCAGGCACTGACTGAAGCACCA
Senp3_Fwd	GAGAGGGTCTCCACCAGTGCC
Senp3_Rev	CTGCGCTCCCCTTCAGGACCA
Wdr18_Fwd	CTGGCCTGGAGCCTCTGCAGT
Wdr18_Rev	CCAATGAAGCGGTGGCCACCC
Las1L_Fwd	CGAGTGCCAGGGGTATCGTCG
Las1L_Rev	GCCAGGGGCAGTTCGTTGCCT
Tex10_Fwd	GCTGCTTGGAGTCTCTTGGAGG
Tex10_Rev	GCCAGCCAACGGGAGAGCACT
Pelp1_Fwd	GCTGGGCCTCAGACCAGAGTG
Pelp1_Rev	CTCGCAGGCCAGCTGTTGGAG
ChIP	
Amylase_Fwd	CTCCTTGTACGGGTTGGT
Amylase_Rev	AATGATGTGCACAGCTGAA
Dusp6_Fwd	ACACACGATCTAAAGGAGGAC
Dusp6_Rev	CAATTAGCAAGCACAAAAGC
Tubb1_Fwd	GGATGTACAAGTGTCTCTGAGC
Tubb1_Rev	TATCTTTCCGCTCATTTCC
Zbp-89_Fwd	CTGGGAGGAGGAAGAGAAG
Zbp-89_Rev	GAGAGAACTTTTGCTGTGGC
Atf5_Fwd	GGTTCCTCACTTCGTCTCC
Atf5_Rev	TTCACTCTCCGCTCACACC

Supplemental Table 2. Mass spectrometry results (unique peptides).

		Unique peptides																		
		exp. #1		exp. #2		exp. #3		exp. #4	exp. #5		exp. #6					exp. #7		exp. #8		
IPI (International Protein Index)	UniProt Accession	MEL_BirA	MEL_Bio-Chtop	MEL_BirA	MEL_Bio-Chtop	MEL_Bio-Chtop_shControl	MEL_Bio-Chtop_shPrmt1	MEL_BirA	MEL_Bio-Senp3	MEL_BirA	MEL_Bio-Wdr18	MEL_BirA	MEL_Bio-Pelp1	MEL_Bio-Las1L	MEL_Bio-Wdr18	MEL_BirA	MEL_Bio-Las1L	MEL_BirA	MEL_Bio-Pelp1	Symbol
IPI00848443	Q5SWU9	74	68	81	69	63	64	58	72	79	73	88	66	90	87	125	76	75	74	Acaca
IPI00124959	E9PVX6	3	36	5	37	22	14	5	41	17	12	48	9	51	50	5		12	6	Mki67
IPI00331361	Q7TPV4	7	21	11	26	20	19	3	20	20	23	14	29	19	27	10		9	34	Mybbp1a
IPI00652987	Q9JIK5	1	19	5	21	14	21	2	14	14	13	21		21	20	4		3		Ddx21
IPI00120495	**Q9JIF0**		**13**		**10**	**12**	**4**													**Prmt1**
IPI00227140	Q61781	22	12	8	5		8			4	3									Krt14
IPI00468696	Q6IFX2	22	12	7	4	3	6				2	3		3						Krt42
IPI00230365	Q9QWL7	22	12	5	3	3	6	1						4						Krt17
IPI00139301	Q922U2	17	12	4	3	7	8			6	6									Krt5
IPI00131368	P50446	17	12	4	6	7	8			5				2						Krt6a
IPI00221797	Q8BGZ7	17	12	4		6	7			5	5									Krt75
IPI00625729	P04104	17	12	3	2	2	4	3	2		3	3	2	2	2	2	1	3	2	Krt1
IPI00222228	Q148Q7		12			3	5													4732456N10Rik
IPI00114710	Q8BP54	8	10	16	8	5	6	1		7	9	22	6	21	10	11	5	26	29	Pcx
IPI00551454	Q6NS46		9	2	25	8	18		3		2	4		10	5					Pdcd11
IPI00153660	Q8BMF4	9	8	11	8	9	10			4	3	5		4		7		8	3	Dlat
IPI00785240	Q8BTI8	7	8	7	14	6	7		3	12	11	15		14	3	19	3	11		Srrm2
IPI00396797	Q501J6	2	8	5	13	6	8		3	8	8	8		12	8		1		2	Ddx17
IPI00648763	Q61656	2	8		10	6	8	1	3	11	13									Ddx5
IPI00111412	Q9D8E6	1	8	1	7	6	6	1	2	2	3	11		11	10	5				Rpl4
IPI00133708	P16381		8		3					2	2			4	1					D1Pas1
IPI00119581	P35550		8		10	7	8		2	1	2	7		10	5	2				Fbl
IPI00311453	Q922K7		8		11	7	7		2	2	3	10		10	9					Nop2
IPI00553419	E9Q557	11	7	2	2		5													Dsp
IPI00318048	Q9D6Z1	1	7	5	15	8	10	1	6	2	3	8		11	8	2		2		Nop56
IPI00113635	Q9ESX5		7	3	11	8	9		4	1	1	4		7	4					Dkc1
IPI00128441	Q8BL32		7		1	2	2													Hnrnpr
IPI00462502	**A2BE28**		**7**		**3**	**11**			**15**		**14**		**19**	**4**	**20**		**1**		**16**	**Las1l**
IPI00321597	**Q9DBD5**		**7**		**8**	**10**			**7**		**7**		**11**	**9**	**15**		**1**		**16**	**Pelp1**
IPI00136252	**Q4VBE8**		**6**		**4**	**7**	**1**		**8**		**10**		**14**	**9**	**15**		**1**		**12**	**Wdr18**
IPI00461416	Q8BJ05		6		5	4	1													Zc3h14
IPI00109326	**Q9EP97**		**6**		**5**	**6**	**1**		**12**		**8**		**8**	**4**	**15**		**1**		**13**	**Senp3**
IPI00134599	P62908		6		7	8	8			3	3	6		9	9	3		2		Rps3
IPI00459381	Q8K363		6		12	5	8		3	1	2	3		6	3					Ddx18
IPI00463468	Q6DFW4		6		14	7	7		4	1	1	4		8	5					Nop58
IPI00407339	P62806	4	5	5	4	5	4	4	4	3	4	4		5	4	9		4	5	Hist1h4a
IPI00719871	Q3UI16	1	5	4	9	5	5	3	8	4	3	10		15	14	1				Nolc1
IPI00109764	Q04750		5	5	14	9	10		7	2	2	12		16	12	7			8	Top1
IPI00469107	**Q9CY57**		**5**	**3**	**4**	**3**	**4**		**2**			**1**		**2**	**1**	**1**		**2**		**Fop**
IPI00458057	**Q3URQ0**		**5**		**6**	**10**	**1**		**15**		**12**		**16**	**4**	**16**				**23**	**Tex10**
IPI00113232	Q08288		5		6	2	5		6	4	6	7		7	5					Lyar
IPI00122011	Q921M3	6	4	4	6	2	1				1	2		3				5		Sf3b3
IPI00480507	Q99KP6	3	4	1	4	1	4	1			3	3		3						Prpf19
IPI00755309	P27659	1	4	3	5	4	3		1	3	2	8		9	8	4				Rpl3
IPI00224505	P47963	1	4	2	5	3	3		5	3	3	5		5	5	2		5	4	Rpl13
IPI00330363	P12970	1	4	1	5	5	5	1	4	4	3	8	1	9	10	4				Rpl7a
IPI00314950	P14869		4	2	3	4	5	2	3	2	2	2		3		1				Rplp0
IPI00122421	P61358		4	2	4	4	4		3	5	5	3	5	4	6			3	4	Rpl27
IPI00420726	Q6ZWN5		4	2	5	5	5	1	5	3	5	4		7	7	2		2	2	Rps9
IPI00313222	P47911		4	1	5	4	5		3	4	4	8		9	9	4		2		Rpl6
IPI00626366	E9Q070		4		3	4	5	2			2	2		3		1				Gm8730
IPI00122413	P51410		4		4	2	3		1			2		3	3	2				Rpl9
IPI00139795	P99027		4		5	4	4	1	4	2	3	3		3	2	1		1		Rplp2

Supplemental Table 2. (Continued)

IPI00466820	P62242		4		5	5	4		4	2	2	8		8	7	4		3	2	Rps8
IPI00664886	B1AZI6		4		7	1	3											3		Thoc2
IPI00134097	D3YX54		4			3	3									2				Rpl13-ps3
IPI00755181	P02535	22	3	5	3	4	5			7	7	6	4	5	5	5		5	4	Krt10
IPI00127763	Q8VH51	3	3	3	5	3	5			2	2					3		2	2	Rbm39
IPI00453768	Q9CPR4	2	3	3	5	4	4		4	2	1	2		3	3	1				Rpl17
IPI00169477	Q8K019	2	3	2	11	4	3		5	2	3	7		6	4	4	1	2		Bclaf1
IPI00606508	D3YWA0	2	3	1	3	3	2	1	5	2		2	3	5	5	5	1	2	7	Rps2
IPI00121136	Q9JIX8	1	3	2	2	1	2			2	4	8		8		2		3		Acin1
IPI00311236	P14148	1	3	2	3	3	2	3	6	2	5	8		10	9			2		Rpl7
IPI00556768	Q569Z6	1	3	2	9	3	4	2	4	5	5	7		12	9	7		3		Thrap3
IPI00127415	Q61937	1	3	1	3	3	3		2	2	5		1			1				Npm1
IPI00273803	Q9CZM2		3	2	4	3	3		3	1		5		5	6	2		2		Rpl15
IPI00315548	Q9CQM8		3	1	3	2	2		1			2		3	3			2		Rpl21
IPI00115660	O08784		3	1	8	3	3		1	4	2	7		8	9	5			2	Tcof1
IPI00849793	P35979		3		3	2	2		2	1	2	5		3	5			3		Rpl12
IPI00849927	Q3U561		3		3	2	3					3		2						Rpl10a
IPI00625464	**Q3TZX8**		**3**		**3**	**4**			**4**		**5**		**4**	**3**	**9**				**10**	**Nol9**
IPI00111821	Q922P9		3		4	2	1													Glyr1
IPI00308706	P47962		3		4	5	5		2			2		3	3	2				Rpl5
IPI00226149	Q8BVY0		3		6	1	3													Rsl1d1
IPI00111560	**Q9EQU5**		**3**			**4**	**2**													**Set**
IPI00229845	**Q8CIG8**		**3**			**7**	**6**													**Prmt5**
IPI00131209	Q9EQD7	22	2	3	1	1	3				3									Krt16
IPI00890234	P53395	4	2	7	4	3	3	4	8	13	12	13	2	13	10	8	2	11	11	Dbt
IPI00121596	Q99PV0	3	2	4	13	5	9	3	1	3	1	7		4		3		4		Prpf8
IPI00222767	Q8BKZ9	3	2	3			1					2				2		2	2	Pdhx
IPI00229475	Q02257	3	2				7													Jup
IPI00348328	Q6IFT3	2	2	2	1	2	2	1	1	1	1	1	1	1	1	1	1		1	Krt78
IPI00114642	P10853	2	2	2	2	3	2	1	2		3			4	2	4			2	Hist1h2b
IPI00761713	Q8CGP2	2	2	2	2	3	2	1	2		3			4	2	4			2	Hist1h2bp
IPI00876549	Q8CBB6	2	2	2	2	3	2	1	2		3			4	2	4			2	Gm13646
IPI00122227	Q62150	2	2		4		2					3		3		2				Rnps1
IPI00469260	O08810	1	2	6	9	3	7				1	7		5				3		Eftud2
IPI00153400	Q8R1M2	1	2	2	2	3	3	3	3	2	3			2	2	3			3	Hist1h2a
IPI00402981	P62849	1	2	1	2	3	4		1		1				2					Rps24
IPI00555113	P35980		2	3	3	2	2		4	3	4	4		5	5	2		4	2	Rpl18
IPI00622240	Q3TTY5		2	2	2	3	4		1	4	4	2		3	1	2		3	2	Krt2
IPI00377441	P62855		2	2	2			1	2		1				1	1		2		Rps26
IPI00263048	Q80Y35		2	1	2	1	2								2					Numa1
IPI00133185	Q9CR57		2	1	2	2	2		1	1	1				4					Rpl14
IPI00474407	D3Z6J9		2	1	2	1		1	2			3		3	3	3			1	Rpl27a
IPI00463297	D3Z3R1		2	1	3	2	1	1	2			1		3	2					Rpl36
IPI00331461	Q9CXW4		2	1	3	2	2		2	2	2	4		4	4	1		2		Rpl11
IPI00322562	P62264		2	1	3	4	4	1	1	2	3	1		2	2	1				Rps14
IPI00340036	E9PZB3		2	1		2	2		2	2	2	4		4	4	1		2		Gm5093
IPI00648513	Q0P678		2		1	1	2													Zc3h18
IPI00896020	Q3TEA8		2		2	1	1					2	2	3	2			4	7	Hp1bp3
IPI00110931	Q9CY66		2		2	3	1		2		1	2		5	2					Gar1
IPI00120162	Q60737		2		2	4	1		2		3								3	Csnk2a1
IPI00474637	Q6ZWV3		2		2	1	2		1			3		4	3	4				Rpl10
IPI00108454	D3Z6N6		2		2	2	2		3	2	3	4		4	4	1				Rps6
IPI00139259	P62996		2		2	2	2				1									Tra2b
IPI00465880	P63276		2		2	2	2													Rps17
IPI00461456	P62751		2		2	2	3		3	1	1	3		4	3	1				Rpl23a
IPI00469918	P14131		2		2	3	3	1	1		2			3	3	2				Rps16
IPI00323806	Q3UW40		2		2	3	3	1	2		1	2		5	2	2		2	2	Rpl24
IPI00222546	P67984		2		2	1			2	2	2			2	1					Rpl22
IPI00162790	P62717		2		2	2						5		6	4			3		Rpl18a
IPI00123949	Q5U4D9		2		2									1						Thoc6
IPI00153778	Q8R3N6		2		3	1	3	1	1		2									Thoc1
IPI00331345	P97351		2		3	2	4		2	1	1	4		4	5	3		3	3	Rps3a
IPI00663587	Q91VM5		2		3	3			4	2	2	2				4	1	5		Rbmxl1
IPI00125901	P62301		2		4	4	3	2	3	3	4	4		4	4			3		Rps13
IPI00132456	Q9CQE8		2		5		1													
IPI00339468	O70133		2		8	4	2	3			4	8		7	4					Dhx9

Supplemental Table 2. (Continued)

IPI00114819	Q99J09		2			2	4												Wdr77
IPI00124979	Q9WV02		2						4	2	2	2					1				Rbmx
IPI00420807	Q6PDM2		2							1	2										Srsf1
IPI00119959	O54962		2																		Banf1
IPI00129323	P84104	3	1	1	2	1	1			2	2					1					Srsf3
IPI00153743	Q8BL97	3	1	1		1	1		2	3	3			3							Srsf7
IPI00117687	P59708	2	1	2	1	2	2														Sf3b14
IPI00115831	O55128	2	1	2	1											1					Sap18
IPI00331121	Q9JJI8	2	1					1						2		2		2			Rpl38
IPI00317794	P09405	2	1						3	6	9	4		3		9	2	4	3		Ncl
IPI00124499	Q8VED5	1	1	2	2		4			3						2					Krt79
IPI00117348	P05213	1	1	2	3	5	2		3	3	2			1	3	1			3		Tuba1b
IPI00403810	P68373	1	1	2	3	5	2		3	3	2			1	3	1					Tuba1c
IPI00128818	O35286	1	1	2	4	1	3	1		2	2	4		6		5		2			Dhx15
IPI00420329	Q69ZZ3	1	1	2	14	2	6	3	3	4		12		11		1		8			Snrnp200
IPI00131695	P07724	1	1	1	1	1	1	1	1	2	2	2	2	3	3	4	4	1	3		Alb
IPI00123007	P62900	1	1	1	1	1	1		2		1	1		1	1				1		Rpl31
IPI00130391	B9EJ35	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1		Gm10334
IPI00117831	Q61147	1	1	1	1	1	2	1	2	3	2			3	3	2			2		Cp
IPI00406870	Q2VIS4	1	1	1	1																Flg2
IPI00112448	P63325	1	1	1	2	2	3			1											Rps10
IPI00223217	P19253	1	1	1	4	2	3		2	2	2	5		5	4	1			1		Rpl13a
IPI00395100	Q7M754	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1		Try10
IPI00133008	Q9CQS2	1	1	1		1	1														Nop10
IPI00462204	D3YY61	1	1	1												1			1		Rpl13a-ps1
IPI00139780	P62830	1	1		2	2	2		1	1	1	4		3	3	3	2	2			Rpl23
IPI00331092	P62702	1	1		2	3	3	3	3	2	2	6		6	8	7		4	2		Rps4x
IPI00117350	P68368	1	1													1					Tuba4a
IPI00311175	Q9JJZ2	1	1													1					Tuba8
IPI00130409	Q91YR7		1	4	4	1					1	2									Prpf6
IPI00228616	P43275		1	2	1	1	1		4			2	4	2		4		3	4		Hist1h1a
IPI00132460	P61255		1	2	2	2	2			1		2		4	5	1					Rpl26
IPI00131357	Q9CZI5		1	1	1	1	1	1	1	2	2	2		5	4	2		2	2		Rps23
IPI00621272	Q9D0T1		1	1	1	1	1		1	1	1	2		2	2			1			Nhp2l1
IPI00122426	P84099		1	1	1	1	1		1			2		1	1	1					Rpl19
IPI00223714	P43274		1	1	1	1	1		3	4	3	3	5	4	4	6			6		Hist1h1e
IPI00113377	P47955		1	1	1	1	1														Rplp1
IPI00222550	P61514		1	1	1	1	1														Rpl37a
IPI00137787	P62918		1	1	1	2	2	2	3		2	5		6	7	3		1			Rpl8
IPI00230660	P62245		1	1	4	2	3		3		1	3		4	3	2		3			Rps15a
IPI00114407	O08583		1	1	4	2	4		1							1					Thoc4
IPI00114593	P68033		1	1	6	7	5		5								1	1			Actc1
IPI00110827	P68134		1	1	6	7	5										1	1			Acta1
IPI00649438	A2A4X6		1	1		1	1									1					Gm12355
IPI00113394	D3YXU0		1	1		2	3		3		1	3			3	2		3			Gm7263
IPI00377350	Q9JJW6		1	1		2			1							1					Refbp2
IPI00117043	P62737		1	1					5								1	1			Acta2
IPI00404804	P63268		1	1													1	1			Actg2
IPI00311948	Q923D4		1	1														2			Sf3b5
IPI00133550	Q9CRB2		1		1	1	1		1					1	1						Nhp2
IPI00230623	P62911		1		1	1	1					3		4	4						Rpl32
IPI00116041	**O55187**		**1**		**1**	**2**	**1**														**Cbx4**
IPI00410804	Q9CYI4		1		1		1			3	4								2		Luc7l
IPI00125521	P97461		1		1		1								1						Rps5
IPI00117771	Q99MJ9		1		1		1														Ddx50
IPI00319231	P62843		1		1		1														Rps15
IPI00133880	**Q9CQJ4**		**1**		**1**		**2**														**Rnf2**
IPI00136169	Q8CCS6		1		1	1															Pabpn1
IPI00128108	Q9R0T7		1		1				1					1							Try5
IPI00989590	E9Q015		1		1				1												Try4
IPI00130591	Q9Z1X4		1		1				2	3	2										Ilf3
IPI00116423	O70237		1		1																Gfi1b
IPI00318561	Q9D7Z3		1		1																Nol7
IPI00410967	Q9ER69		1		1																Wtap
IPI00317590	P62270		1		2	1	1		2	2		3		9	6	5		3	2		Rps18
IPI00120886	P62960		1		2	1	1				1										Ybx1

Supplemental Table 2. (Continued)

IPI00340815	Q66JP8		1		2	1	1				2								Rbm15
IPI00111248	D3YWV7		1		2	1	1												Gm5321
IPI00133917	**Q9QWH1**		**1**		**2**	**1**	**3**												**Phc2**
IPI00222687	Q8BKT7		1		2		3												Thoc5
IPI00115992	D3YXG3		1		2	2	4		3	2	3			2		1			1	Rps25
IPI00128256	Q91WM3		1		2															Rrp9
IPI00311761	Q8VDQ9		1		3	1	2													Kri1
IPI00222461	Q8CI11		1		3	2	3													Gnl3
IPI00348445	A2AIV2		1		3															Kiaa1429
IPI00462979	**Q91Z49**		**1**		**4**	**2**	**3**							**2**						**Fyttd1**
IPI00132443	Q9D0E1		1		5	4	2		2		5	2		3				5		Hnrnpm
IPI00111343	Q9QZH3		1				1													Ppie
IPI00126762	P67871		1				1													Csnk2b
IPI00134809	Q9D2G2		1			4	5		3	3	5	2							3	Dlst
IPI00118166	D3Z3Q9		1							1										Rpl26-ps2
IPI00314709	O35326		1							2	3							2		Srsf5
IPI00310880	Q921K3		1							3	3	4		4	3			2		Srsf6
IPI00114162	Q05816		1																	Fabp5
IPI00117232	Q9R0U0		1																	Srsf10
IPI00132479	Q6NSQ7		1																	Ltv1
IPI00139364	Q8VE97		1																	Srsf4
IPI00620887	Q8CF51		1																	Fusip1
IPI00672962	Q8CEN5		1																	6330405D24Rik
IPI00845808	Q4FK66		1																	Prpf38a
IPI00112947	P19001	22						1												Krt19
IPI00347110	Q6NXH9	17		2	2	2				3	3			2			1			Krt73
IPI00420312	P07744	17																		Krt4
IPI00330523	Q91ZA3	7		10				1	1	15	8	19	3	15	11	15	2	14	14	Pcca
IPI00623284	Q99NB9	4		5	2	3	1		2	1	1	5		2		6		7	4	Sf3b1
IPI00349401	Q3UAI4	4		2	5	2	3					2		4		3		8	2	Sf3b2
IPI00468896	Q569Z5	4		2						3	2	7		6		7		5	12	Ddx46
IPI00309068	Q8BN78	4		1						4		8		7		7		3	5	Zbtb33
IPI00330483	Q80W00	3		3					2	4	4	4		7	2	7		5	10	Ppp1r10
IPI00129032	P97762	2		2			1	2			2					3		4	3	rp9
IPI00875992	F6Z0H0	2		2			1	2			2					3		4	3	
IPI00467507	Q6NV83	2		1	1					3								4	4	U2surp
IPI00154082	Q8QZY9	2		1	1									1				1		Sf3b4
IPI00110850	P60710	2		1	7	10	7	2	7			4		5	3	6	1	1		Actb
IPI00652436	Q3TSB7	2		1	7			2	7			4		5	3	6	1	1		Actg1
IPI00170008	P57784	2		1														5		Snrpa1
IPI00170394	Q8JZX4	2									1			2				2		Rbm17
IPI00230395	P10107	2																		Anxa1
IPI00320011	Q99K43	2																		Prc1
IPI00132042	Q9D051	1		4																Pdhb
IPI00117352	P99024	1		2		10	4			1	2				3					Tubb5
IPI00468653	Q9CT71	1		2														3	4	Pccb
IPI00461621	Q6PE01	1		1	2						1									Snrnp40
IPI00131674	Q9D7Y7	1		1		1	1													2210010C04Rik
IPI00331597	P43277	1		1			2		4	5	5	4	5	5		6	3	2	6	Hist1h1d
IPI00462006	F6U2H0	1		1				1	4	4	3		1			4				
IPI00223713	P15864	1		1				4				4	5	5	6	6	3	2	6	Hist1h1c
IPI00129215	Q9Z0K8	1		1							2	1				1		1	1	Vnn1
IPI00461356	Q8CDE3	1		1									1							Gm5766
IPI00317891	O35691	1			1			2	1			7		8		2				Pnn
IPI00284213	Q9R1C7	1					1													Prpf40a
IPI00652831	Q3UL36	1					2			3	3			2		3	2			Arglu1
IPI00458337	F6ZA13	1					2											2		
IPI00109061	Q9CWF2	1					4			1	2									Tubb2b
IPI00338039	Q7TMM9	1					4			1	2									Tubb2a
IPI00347096	Q6IME9	1				2														Krt72
IPI00169463	P68372	1				8				1										Tubb2c
IPI00311873	P62141	1						2	2			2		4						Ppp1cb
IPI00130185	P62137	1						2		1		2		5		4		1		Ppp1ca
IPI00123862	P63087	1						2								2				Ppp1cc
IPI00408796	Q8K4Z5	1								1		2		5		3		8		Sf3a1
IPI00121251	Q8BU11	1								2		3		5	2	3		2	5	Tox4

Supplemental Table 2. (Continued)

IPI00229604	Q8CGZ0	1													1				Cherp
IPI00673513	D3Z6N4	1															2		Gm5045
IPI00113746	P26369	1																	U2af2
IPI00124111	P97350	1																	Pkp1
IPI00468203	P07356	1																	Anxa2
IPI00850843	E9PYB0	1																	Ahnak2
IPI00756257	A2ASS6			12	4		7	3	5	7	5				2				Ttn
IPI00337893	P35486			3													1		Pdha1
IPI00345676	A2AJK6			3															Chd7
IPI00110753	P68369			2		5	2		3	3	2			1	3	1			Tuba1a
IPI00331734	P0C0S6			2		2	3		3	2		2		2	2	3		3	3 H2afz
IPI00919098	Q3THW5			2		2	3		3	2				2	2	3			3 H2afv
IPI00880868	B7ZWH2			2			3												Hfm1
IPI00357734	Q5SS00			2															Zdbf2
IPI00378446	F6XLV1			2															E030010N08Rik
IPI00453654	Q6DFV5			2															Helz
IPI00757897	D3YVY0			2															
IPI00758084	D3Z7Q9			2															Prdm15
IPI00988262	E9Q7W2			2															Invs
IPI00126716	Q91VC3			1	1							4		5					Eif4a3
IPI00109860	Q9CWZ3			1	2							1							Rbm8a
IPI00378437	D3Z1Q1			1		2			1			2		3	3			2	Gm5528
IPI00606615	D3YVN8			1		2			1			2		3	3			2	Gm8054
IPI00664929	D3Z422			1		2			1			2		3	3			2	Rpl21-ps7
IPI00457852	E9PUX4			1		4			3	4	4					4		2	Gm5428
IPI00112407	D3Z1P6			1				1	1	2		1				1			Gm5805
IPI00320850	Q99MR8			1				1		3	4	8				5	1	7	7 Mccc1
IPI00114472	D3YZN6			1				1											1 Rpl27a-ps1
IPI00421119	B2RY56			1						1								2	Rbm25
IPI00875177	D3Z536			1						2							1		Gm8225
IPI00918622	D3YU49			1								4		5					Gm8994
IPI00123762	Q6PFB2			1												2			Rcc1
IPI00129264	Q9R1Z8			1															Sorbs3
IPI00130348	D3Z4H4			1															Rpl21-ps14
IPI00221951	Q8BHS3			1															Rbm22
IPI00230394	P14733			1															Lmnb1
IPI00330263	Q8CFQ3			1															Aqr
IPI00338745	P18608			1															Hmgn1
IPI00408194	Q9R0M5			1															Tpk1
IPI00468196	D3YWQ7			1															Gm16415
IPI00624454	D3Z3K3			1															Gm10155
IPI00755959	D3YX71			1															Gm8973
IPI00137736	P62858				1	1	1			1									Rps28
IPI00755623	F7CZ57				1	1	1							3					Gm10343
IPI00226073	Q9Z2X1				1	1	1												Hnrnpf
IPI00379029	D3Z2H8				1	1	1												Rpl30-ps6
IPI00421196	Q3TLT4				1	1	1												Ddx31
IPI00990575	P62889				1	1	1												Rpl30
IPI00399483	P62862				1		1	1								1			Fau
IPI00311904	Q8VE80				1		1		1	2	1								Thoc3
IPI00130510	Q91YU8				1	1	2												Ppan
IPI00458704	Q9CPT5				1	1	2												Nop16
IPI00323357	P63017				1	3	3		4	3	6				2	2			Hspa8
IPI00118899	P57780				1	1													Actn4
IPI00380436	Q7TPR4				1	1													Actn1
IPI00263879	Q6ZWV7				1				1			1		1	1	1			Rpl35
IPI00109203	Q9CWK3				1				1										Cd2bp2
IPI00319992	P20029				1					1									Hspa5
IPI00173160	Q6ZWU9				1									2	2	3			Rps27
IPI00331546	P17156				1										2				Hspa2
IPI00928040	D3YYB0				1										2				Rps27l
IPI00114052	P27048				1													2	2 Snrpb
IPI00118172	O70422				1														Gtf2h4
IPI00121159	Q9JJ80				1														Rpf2
IPI00126176	Q9WVM1				1														Racgap1
IPI00126389	Q6P5B5				1														Fxr2

Supplemental Table 2. Mass spectrometry results (MASCOT scores).

		MASCOT score																			
		exp. #1		exp. #2		exp. #3		exp. #4		exp. #5		exp. #6				exp. #7		exp. #8			
IPI (International Protein Index)	UniProt Accession	MEL_BirA	MEL_Bio-Chtop	MEL_BirA	MEL_Bio-Chtop	MEL_Bio-Chtop_shCon	MEL_Bio-Chtop_shPrm	MEL_BirA	MEL_Bio-Senp3	MEL_BirA	MEL_Bio-Wdr18	MEL_BirA	MEL_Bio-Pelp1	MEL_Bio-Las1L	MEL_Bio-Wdr18	MEL_BirA	MEL_Bio-Las1L	MEL_BirA	MEL_Bio-Pelp1	Symbol
IPI00848443	Q5SWU9	6318	5555	6355	5418	5173	4975	3947	4546	5597	5379	5332	3007	5403	5301	8281	4640	4503	4355	Acaca
IPI00124959	E9PVX6	222	3201	434	3116	1728	938	113	2955	1073	799	1874	300	2161	2032	211		445	209	Mki67
IPI00331361	Q7TPV4	556	1618	835	1998	1421	1555	151	1407	1461	1617	553	1489	831	1152	520		363	1620	Mybbp1a
IPI00652987	Q9JIK5	60	1346	289	1606	1067	1629	132	789	861	770	903		911	780	178		137		Ddx21
IPI00120495	**Q9JIF0**		**960**		**728**	**869**	**247**													**Prmt1**
IPI00227140	Q61781	715	696	715	266		645			201	179									Krt14
IPI00468696	Q6IFX2	594	549	572	265	213	539				116	131		158						Krt42
IPI00230365	Q9QWL7	550	483	366	181	239	469	58						190						Krt17
IPI00139301	Q922U2	438	348	312	286	508	546			386	403									Krt5
IPI00131368	P50446	504	499	251	413	498	619			338				62						Krt6a
IPI00221797	Q8BGZ7	378	388	229		388	488			292	318									Krt75
IPI00625729	P04104	166	164	193	164	163	222	207	165		160	163	139	172	133	116	53	141	120	Krt1
IPI00222228	Q148Q7		161			184	284													4732456N10Rik
IPI00114710	Q8BP54	690	778	1360	610	454	355	61		312	482	929	161	809	315	591	186	1204	1479	Pcx
IPI00551454	Q6NS46		777	133	1808	629	1254		117		67	116		343	134					Pdcd11
IPI00153660	Q8BMF4	750	704	837	721	771	779			267	196	167		136		377		289	130	Dlat
IPI00785240	Q8BTI8	446	691	405	1126	541	541		100	648	477	488		417	83	824	96	443		Srrm2
IPI00396797	Q501J6	152	352	350	767	433	574		207	440	406	292		476	294		77		91	Ddx17
IPI00648763	Q61656	152	184		657	406	556	43	187	644	696									Ddx5
IPI00111412	Q9D8E6	47	497	62	577	373	451	51	71	126	191	466		608	449	207				Rpl4
IPI00133708	P16381		105		241					114	95			150	42					D1Pas1
IPI00119581	P35550		616		817	492	532		84	61	107	242		371	212	50				Fbl
IPI00311453	Q922K7		646		936	584	584		82	94	167	440		395	384					Nop2
IPI00553419	E9Q557	849	559	143	92		404													Dsp
IPI00318048	Q9D6Z1	133	712	468	1323	676	814	50	497	121	190	381		531	449	145		72		Nop56
IPI00113635	Q9ESX5		588	149	906	588	703		333	60	59	143		245	186					Dkc1
IPI00128441	Q8BL32		413		99	166	150													Hnrnpr
IPI00462502	**A2BE28**		**524**		**190**	**821**			**1126**		**1056**		**900**	**178**	**1046**		**66**		**925**	**Las1l**
IPI00321597	**Q9DBD5**		**488**		**611**	**690**			**519**		**463**		**679**	**423**	**788**		**57**		**822**	**Pelp1**
IPI00136252	**Q4VBE8**		**443**		**294**	**506**	**118**		**526**		**839**		**899**	**327**	**948**		**63**		**787**	**Wdr18**
IPI00461416	Q8BJ05		475		388	240	41													Zc3h14
IPI00109326	**Q9EP97**		**447**		**408**	**495**	**112**		**754**		**508**		**326**	**146**	**650**		**47**		**574**	**Senp3**
IPI00134599	P62908		322		493	438	515			205	143	189		327	331	148		64		Rps3
IPI00459381	Q8K363		396		842	335	481		113	50	105	98		217	86					Ddx18
IPI00463468	Q6DFW4		453		1039	492	529		267	53	58	151		280	157					Nop58
IPI00407339	P62806	274	390	359	310	375	316	291	283	233	266	190		267	203	640		233	324	Hist1h4a
IPI00719871	Q3UI16	49	436	184	713	276	259	180	441	132	90	490		698	572	80				Nolc1
IPI00109764	Q04750		313	310	940	568	584		402	118	89	403		630	301	193			249	Top1
IPI00469107	**Q9CY57**		**424**	**131**	**340**	**272**	**352**		**117**			**41**		**109**	**43**	**65**		**85**		**Fop**
IPI00458057	**Q3URQ0**		**406**		**442**	**870**	**99**		**902**		**733**		**747**	**214**	**807**				**1089**	**Tex10**
IPI00113232	Q08288		405		480	151	276		318	215	294	285		271	169					Lyar
IPI00122011	Q921M3	557	349	368	472	213	98				77	97		123				170		Sf3b3
IPI00480507	Q99KP6	133	198	62	192	53	155	57			173	87		111						Prpf19
IPI00755309	P27659	63	348	203	453	323	303		59	158	87	323		337	348	159				Rpl3
IPI00224505	P47963	53	221	136	343	168	182		291	146	175	284		254	251	78		161	141	Rpl13
IPI00330363	P12970	91	318	91	363	354	326	45	275	220	165	353	43	421	406	167				Rpl7a
IPI00314950	P14869		242	139	299	330	393	143	190	132	111	88		102		41				Rplp0
IPI00122421	P61358		236	73	222	238	235		134	233	239	117	194	201	234			92	143	Rpl27
IPI00420726	Q6ZWN5		191	74	288	295	296	44	256	141	238	161		306	299	75		54	62	Rps9
IPI00313222	P47911		322	112	444	333	393		105	182	161	362		412	387	161		53		Rpl6
IPI00626366	E9Q070		250		305	359	418	143			111	88		102		41				Gm8730
IPI00122413	P51410		302		329	109	187		43			125		130	124	82				Rpl9
IPI00139795	P99027		461		439	401	448	127	329	189	280	150		168	123	40		50		Rplp2
IPI00466820	P62242		241		371	381	308		304	136	154	457		471	438	237		108	75	Rps8
IPI00664886	B1AZI6		187		345	73	216											77		Thoc2
IPI00134097	D3YX54		221			168	182									78				Rpl13-ps3
IPI00755181	P02535	225	260	384	165	319	357			440	474	288	176	245	210	292		224	153	Krt10
IPI00127763	Q8VH51	258	271	266	428	163	349			140	96					178		83	51	Rbm39
IPI00453768	Q9CPR4	112	243	177	365	342	329		154	84	57	81		160	123	43				Rpl17
IPI00169477	Q8K019	120	275	133	619	247	113		187	46	79	216		220	115	153	44	61		Bclaf1
IPI00606508	D3YWA0	103	205	83	236	172	146	40	218	93		86	138	189	227	169	42	46	282	Rps2
IPI00121136	Q9JIX8	71	209	85	133	74	124			113	134	275		255		116		89		Acin1
IPI00311236	P14148	46	201	129	196	180	86	133	295	117	193	330		386	381			51		Rpl7
IPI00556768	Q569Z6	83	218	161	513	194	229	111	255	219	232	272		483	314	291		85		Thrap3
IPI00127415	Q61937	68	295	68	270	251	364		68	118	332		40			43				Npm1
IPI00273803	Q9CZM2		193	115	231	174	203		189	64		169		179	193	93		45		Rpl15
IPI00315548	Q9CQM8		240	71	236	190	159		50			88		122	111			78		Rpl21
IPI00115660	O08784		284	87	749	175	290		66	152	81	295		373	376	166			56	Tcof1
IPI00849793	P35979		292		268	241	256		143	62	120	225		98	188			99		Rpl12
IPI00849927	Q3U561		188		238	66	171					71		74						Rpl10a
IPI00625464	**Q3TZX8**		**262**		**297**	**390**			**245**		**347**		**142**	**93**	**306**				**374**	**Nol9**

Supplemental Table 2. (Continued)

IPI00111821	Q922P9		230		450	166	102														Glyr1
IPI00308706	P47962		237		338	387	391		69			52		102	103	46					Rpl5
IPI00226149	Q8BVY0		235		473	69	232														Rsl1d1
IPI00111560	**Q9EQU5**		**218**			**295**	**152**														**Set**
IPI00229845	**Q8CIG8**		**172**			**416**	**483**														**Prmt5**
IPI00131209	Q9EQD7	273	146	261	132	132	199				174										Krt16
IPI00890234	P53395	229	71	516	171	126	162	230	340	793	676	564	74	529	383	358	96	423	476		Dbt
IPI00121596	Q99PV0	181	160	295	950	349	584	90	44	154	43	267		118		90		110			Prpf8
IPI00222767	Q8BKZ9	201	163	239			61					79				109		99	58		Pdhx
IPI00229475	Q02257	199	137				420														Jup
IPI00348328	Q6IFT3	119	124	109	86	171	139	86	94	86	82	76	74	74	71	41	49		55		Krt78
IPI00114642	P10853	155	159	178	127	141	147	41	179		189			170	77	275			76		Hist1h2b
IPI00761713	Q8CGP2	155	159	178	127	141	147	41	179		189			170	77	275			76		Hist1h2bp
IPI00876549	Q8CBB6	155	159	178	127	141	147	41	179		189			170	77	275			76		Gm13646
IPI00122227	Q62150	84	150		190		103					117		112		81					Rnps1
IPI00469260	O08810	72	188	330	849	324	549				41	263		161				79			Eftud2
IPI00153400	Q8R1M2	60	181	140	127	257	273	193	193	100	122			93	90	182			135		Hist1h2a
IPI00402981	P62849	71	180	78	172	207	248		67		50				52						Rps24
IPI00555113	P35980		191	217	262	189	202		312	243	200	242		288	319	112		147	112		Rpl18
IPI00622240	Q3TTY5		168	121	151	180	276		63	260	269	61		110	67	70		97	111		Krt2
IPI00377441	P62855		155	71	113			54	75		46				51	54		66			Rps26
IPI00263048	Q80Y35		143	84	164	114	123								78						Numa1
IPI00133185	Q9CR57		149	93	145	159	144		95	86	91				213						Rpl14
IPI00474407	D3Z6J9		145	56	142	83		65	138			150		176	154	129			42		Rpl27a
IPI00463297	D3Z3R1		143	52	196	143	94	61	108			46		105	68						Rpl36
IPI00331461	Q9CXW4		142	91	205	151	156		137	138	137	185		185	201	50		116			Rpl11
IPI00322562	P62264		139	68	281	363	361	59	72	144	212	50		73	81	61					Rps14
IPI00340036	E9PZB3		142	91		151	156		137	138	137	185		185	201	50		116			Gm5093
IPI00648513	Q0P678		103		92	52	129														Zc3h18
IPI00896020	Q3TEA8		183		168	103	86					58	63	97	77			105	220		Hp1bp3
IPI00110931	Q9CY66		87		100	197	74		90		83	75		177	72						Gar1
IPI00120162	Q60737		190		155	219	70		109		133								95		Csnk2a1
IPI00474637	Q6ZWV3		167		164	98	193		97			112		162	150	169					Rpl10
IPI00108454	D3Z6N6		118		181	181	204		270	108	140	217		225	215	47					Rps6
IPI00139259	P62996		180		153	153	190				41										Tra2b
IPI00465880	P63276		263		240	233	241														Rps17
IPI00461456	P62751		149		140	145	178		186	73	74	102		156	72	50					Rpl23a
IPI00469918	P14131		115		148	179	192	50	70		106			105	93	114					Rps16
IPI00323806	Q3UW40		112		88	217	185	92	124		86	82		228	116	60		93	71		Rpl24
IPI00222546	P67984		108		156	81			155	128	147			60	48						Rpl22
IPI00162790	P62717		109		89	110						205		258	230			72			Rpl18a
IPI00123949	Q5U4D9		102		92									46							Thoc6
IPI00153778	Q8R3N6		165		191	77	128	60	40		60										Thoc1
IPI00331345	P97351		144		256	185	255		88	50	56	127		158	157	130		74	73		Rps3a
IPI00663587	Q91VM5		193		240	237			156	44	98	73				167	52	151			Rbmxl1
IPI00125901	P62301		148		322	258	191	104	175	161	201	159		214	134			95			Rps13
IPI00132456	Q9CQE8		96		346		83														
IPI00339468	O70133		136		506	325	98	131			207	265		263	157						Dhx9
IPI00114819	Q99J09		120			192	299														Wdr77
IPI00124979	Q9WV02		193						156	44	98	73					52				Rbmx
IPI00420807	Q6PDM2		86							46	103										Srsf1
IPI00119959	O54962		124																		Banf1
IPI00129323	P84104	132	84	90	140	84	86			129	134					44					Srsf3
IPI00153743	Q8BL97	137	84	90		84	86		169	216	229			101							Srsf7
IPI00117687	P59708	159	81	161	72	147	169														Sf3b14
IPI00115831	O55128	101	95	195	73											62					Sap18
IPI00331121	Q9JII8	91	58					46						62		108		80			Rpl38
IPI00317794	P09405	103	56						182	355	565	143		88		431	80	112	86		Ncl
IPI00124499	Q8VED5	93	93	122	124		234			199						45					Krt79
IPI00117348	P05213	72	95	195	245	405	176		225	153	110			41	70	41			119		Tuba1b
IPI00403810	P68373	72	95	195	245	405	176		225	153	110			41	70	41					Tuba1c
IPI00128818	O35286	71	76	80	257	43	143	52		72	98	138		242		239		71			Dhx15
IPI00420329	Q69ZZ3	72	73	173	1103	125	304	137	145	136		424		388		41		238			Snrnp200
IPI00131695	P07724	102	91	80	88	89	87	89	89	114	103	163	169	178	187	276	222	97	156		Alb
IPI00123007	P62900	72	94	95	82	97	66		134		63	66		68	66				40		Rpl31
IPI00130391	B9EJ35	64	64	47	42	99	61	54	68	56	67	67	45	72	44	51	61	66	44		Gm10334
IPI00117831	Q61147	66	62	68	53	65	92	65	87	167	95			108	112	79			70		Cp
IPI00406870	Q2VIS4	89	80	74	67																Flg2
IPI00112448	P63325	47	50	54	91	122	168			65											Rps10
IPI00223217	P19253	56	70	93	260	118	145		103	101	86	209		222	163	51			40		Rpl13a
IPI00395100	Q7M754	59	82	74		82	74	73	79	65	71	68	59	72	50	68	73	49	69		Try10
IPI00133008	Q9CQS2	89	98	93		93	99														Nop10
IPI00462204	D3YY61	56	70	91												51			40		Rpl13a-ps1
IPI00139780	P62830	96	113		131	126	153		89	80	66	126		117	93	170	68	90			Rpl23
IPI00331092	P62702	59	129		200	260	252	113	145	64	95	272		247	335	310		147	53		Rps4x
IPI00117350	P68368	72	95													41					Tuba4a
IPI00311175	Q9JJZ2	72	95													41					Tuba8
IPI00130409	Q91YR7		61	330	245	62					45	69									Prpf6
IPI00228616	P43275		98	104	144	91	106		156			132	252	127		218		145	268		Hist1h1a
IPI00132460	P61255		47	78	107	91	68			48		63		146	206	48					Rpl26
IPI00131357	Q9CZI5		91	88	91	76	83	91	44	129	134	115		194	173	123		62	85		Rps23
IPI00621272	Q9D0T1		88	77	75	88	84		89	54	89	89		118	88			43			Nhp2l1
IPI00122426	P84099		101	127	133	137	146		133			72		45	43	64					Rpl19

Supplemental Table 2. (Continued)

IPI00223714	P43274		97	69	72	92	82		234	235	184	205	350	249	234	410			348	Hist1h1e
IPI00113377	P47955		89	81	97	89	78													Rplp1
IPI00222550	P61514		95	96	76	93	111													Rpl37a
IPI00137787	P62918		52	46	78	114	107	70	108		56	202		242	277	128		51		Rpl8
IPI00230660	P62245		106	80	305	165	235		177		58	122		168	148	84		96		Rps15a
IPI00114407	O08583		69	74	343	196	384		67							65				Thoc4
IPI00114593	P68033		58	49	306	403	312		231								51	56		Actc1
IPI00110827	P68134		58	49	306	403	312										51	56		Acta1
IPI00649438	A2A4X6		84	90		84	86									44				Gm12355
IPI00113394	D3YXU0		106	80		165	238		177		58	122			148	84		96		Gm7263
IPI00377350	Q9JJW6		69	74		196			70							65				Refbp2
IPI00117043	P62737		58	49					231								51	56		Acta2
IPI00404804	P63268		58	49													51	56		Actg2
IPI00311948	Q923D4		80	84														66		Sf3b5
IPI00133550	Q9CRB2		83		95	115	115		117					78	52					Nhp2
IPI00230623	P62911		67		72	81	72					108		209	153					Rpl32
IPI00116041	**O55187**		**106**		**51**	**104**	**50**													**Cbx4**
IPI00410804	Q9CYI4		101		113		47			201	268								67	Luc7l
IPI00125521	P97461		104		112		104								51					Rps5
IPI00117771	Q99MJ9		62		54		44													Ddx50
IPI00319231	P62843		76		94		73													Rps15
IPI00133880	**Q9CQJ4**		**77**		**76**		**145**													**Rnf2**
IPI00136169	Q8CCS6		79		79	92														Pabpn1
IPI00128108	Q9R0T7		46		45				50					44						Try5
IPI00989590	E9Q015		46		45				50											Try4
IPI00130591	Q9Z1X4		124		96				72	143	101									Ilf3
IPI00116423	O70237		58		51															Gfi1b
IPI00318561	Q9D7Z3		90		75															Nol7
IPI00410967	Q9ER69		68		113															Wtap
IPI00317590	P62270		47		128	56	53		128	113		87		311	184	228		114	55	Rps18
IPI00120886	P62960		105		216	72	109				70									Ybx1
IPI00340815	Q66JP8		86		120	76	80				41									Rbm15
IPI00111248	D3YWV7		47		128	56	53													Gm5321
IPI00133917	**Q9QWH1**		**76**		**190**	**81**	**218**													**Phc2**
IPI00222687	Q8BKT7		60		156		78													Thoc5
IPI00115992	D3YXG3		76		107	110	229		114	118	119			56		65			50	Rps25
IPI00128256	Q91WM3		63		130															Rrp9
IPI00311761	Q8VDQ9		92		335	98	132													Kri1
IPI00222461	Q8CI11		60		199	108	170													Gnl3
IPI00348445	A2AIV2		50		173															Kiaa1429
IPI00462979	**Q91Z49**		**155**		**372**	**176**	**255**							**50**						**Fyttd1**
IPI00132443	Q9D0E1		41		256	249	136		88		356	82		100				153		Hnrnpm
IPI00111343	Q9QZH3		45				40													Ppie
IPI00126762	P67871		54				49													Csnk2b
IPI00134809	Q9D2G2		97			350	436		193	174	268	102							94	Dlst
IPI00118166	D3Z3Q9		47							48										Rpl26-ps2
IPI00314709	O35326		47							108	134							43		Srsf5
IPI00310880	Q921K3		47							148	174	147		173	104			46		Srsf6
IPI00114162	Q05816		41																	Fabp5
IPI00117232	Q9R0U0		66																	Srsf10
IPI00132479	Q6NSQ7		135																	Ltv1
IPI00139364	Q8VE97		47																	Srsf4
IPI00620887	Q8CF51		66																	Fusip1
IPI00672962	Q8CEN5		41																	6330405D24Rik
IPI00845808	Q4FK66		77																	Prpf38a
IPI00112947	P19001	195						58												Krt19
IPI00347110	Q6NXH9	288		183	170	181				222	218			138			53			Krt73
IPI00420312	P07744	200																		Krt4
IPI00330523	Q91ZA3	504		717				58	51	904	472	831	83	506	393	687	60	575	620	Pcca
IPI00623284	Q99NB9	189		282	150	179	57		105	54	71	171		107		278		263	138	Sf3b1
IPI00349401	Q3UAI4	297		152	317	69	95					91		115		107		294	64	Sf3b2
IPI00468896	Q569Z5	340		182						200	51	252		188		332		187	444	Ddx46
IPI00309068	Q8BN78	273		84						170		282		252		280		109	206	Zbtb33
IPI00330483	Q80W00	235		206					106	210	191	145		246	94	345		166	316	Ppp1r10
IPI00129032	P97762	88		72			44	42			52					113		154	73	rp9
IPI00875992	F6Z0H0	88		72			44	42			52					113		154	73	
IPI00467507	Q6NV83	115		82	47					93								125	94	U2surp
IPI00154082	Q8QZY9	169		94	97									53				58		Sf3b4
IPI00110850	P60710	79		49	461	648	514	116	344			94		154	74	381	51	56		Actb
IPI00652436	Q3TSB7	79		49	461			119	347			94		154	74	381	51	56		Actg1
IPI00170008	P57784	92		46														170		Snrpa1
IPI00170394	Q8JZX4	53									41			69				71		Rbm17
IPI00230395	P10107	183																		Anxa1
IPI00320011	Q99K43	53																		Prc1
IPI00132042	Q9D051	78		296																Pdhb
IPI00117352	P99024	46		90		757	242			61	76				103					Tubb5
IPI00468653	Q9CT71	102		157														108	126	Pccb
IPI00461621	Q6PE01	56		56	130						41									Snrnp40
IPI00131674	Q9D7Y7	90		90		40	75													2210010C04Rik
IPI00331597	P43277	58		85			110		277	297	343	284	421	338		448	118	140	401	Hist1h1d
IPI00462006	F6U2H0	91		91				45	275	220	165		43			167				
IPI00223713	P15864	58		82				253				284	421	338	350	448	118	140	401	Hist1h1c
IPI00129215	Q9Z0K8	103		99							100	42				71		56	50	Vnn1

Supplemental Table 2. (Continued)

IPI00461356	Q8CDE3	91		91									43							Gm5766
IPI00317891	O35691	66			52			63	46			236		241		62				Pnn
IPI00284213	Q9R1C7	40					68													Prpf40a
IPI00652831	Q3UL36	47					92			97	100			62		105	96			Arglu1
IPI00458337	F6ZA13	46					86											51		
IPI00109061	Q9CWF2	46					242			61	76									Tubb2b
IPI00338039	Q7TMM9	46					242			61	76									Tubb2a
IPI00347096	Q6IME9	79				57														Krt72
IPI00169463	P68372	46				640				61										Tubb2c
IPI00311873	P62141	81						94	83			82		147						Ppp1cb
IPI00130185	P62137	81						94		70		59		190		178		67		Ppp1ca
IPI00123862	P63087	81						94								99				Ppp1cc
IPI00408796	Q8K4Z5	60								53		54		123		86		305		Sf3a1
IPI00121251	Q8BU11	62								180		101		209	76	231		86	216	Tox4
IPI00229604	Q8CGZ0	63														58				Cherp
IPI00673513	D3Z6N4	46																51		Gm5045
IPI00113746	P26369	46																		U2af2
IPI00124111	P97350	98																		Pkp1
IPI00468203	P07356	60																		Anxa2
IPI00850843	E9PYB0	86																		Ahnak2
IPI00756257	A2ASS6			136	57		92	45	66	72	68				43					Ttn
IPI00337893	P35486			216													60			Pdha1
IPI00345676	A2AJK6			65																Chd7
IPI00110753	P68369			195		405	176		225	153	110			41	70	41				Tuba1a
IPI00331734	P0C0S6			91		159	183		148	102		75		93	90	155		100	120	H2afz
IPI00919098	Q3THW5			91		159	183		148	102				93	90	155			120	H2afv
IPI00880868	B7ZWH2			43			70													Hfm1
IPI00357734	Q5SS00			51																Zdbf2
IPI00378446	F6XLV1			45																E030010N08Rik
IPI00453654	Q6DFV5			44																Helz
IPI00757897	D3YVY0			40																
IPI00758084	D3Z7Q9			46																Prdm15
IPI00988262	E9Q7W2			43																Invs
IPI00126716	Q91VC3			62	112							97		129						Eif4a3
IPI00109860	Q9CWZ3			55	173							45								Rbm8a
IPI00378437	D3Z1Q1			67		190			47			88		122	111			78		Gm5528
IPI00606615	D3YVN8			71		190			50			88		122	111			78		Gm8054
IPI00664929	D3Z422			67		190			50			88		122	111			78		Rpl21-ps7
IPI00457852	E9PUX4			112		333			102	182	161					161		53		Gm5428
IPI00112407	D3Z1P6			68				59	72	144		50				61				Gm5805
IPI00320850	Q99MR8			67				62		150	249	310				256	45	245	247	Mccc1
IPI00114472	D3YZN6			56				65											42	Rpl27a-ps1
IPI00421119	B2RY56			51						45								48		Rbm25
IPI00875177	D3Z536			83						93							42			Gm8225
IPI00918622	D3YU49			62								97		129						Gm8994
IPI00123762	Q6PFB2			55												80				Rcc1
IPI00129264	Q9R1Z8			43																Sorbs3
IPI00130348	D3Z4H4			71																Rpl21-ps14
IPI00221951	Q8BHS3			40																Rbm22
IPI00230394	P14733			60																Lmnb1
IPI00330263	Q8CFQ3			92																Aqr
IPI00338745	P18608			130																Hmgn1
IPI00408194	Q9R0M5			43																Tpk1
IPI00468196	D3YWQ7			71																Gm16415
IPI00624454	D3Z3K3			68																Gm10155
IPI00755959	D3YX71			52																Gm8973
IPI00137736	P62858				86	83	82			78										Rps28
IPI00755623	F7CZ57				65	56	86							76						Gm10343
IPI00226073	Q9Z2X1				115	107	120													Hnrnpf
IPI00379029	D3Z2H8				63	56	83													Rpl30-ps6
IPI00421196	Q3TLT4				94	74	48													Ddx31
IPI00990575	P62889				65	56	86													Rpl30
IPI00399483	P62862				53		40	54								52				Fau
IPI00311904	Q8VE80				66		75		68	50	46									Thoc3
IPI00130510	Q91YU8				94	44	181													Ppan
IPI00458704	Q9CPT5				64	48	59													Nop16
IPI00323357	P63017				108	131	195		293	178	419				75	112				Hspa8
IPI00118899	P57780				58	61														Actn4
IPI00380436	Q7TPR4				58	61														Actn1
IPI00263879	Q6ZWV7				55				79			48		75	42	48				Rpl35
IPI00109203	Q9CWK3				64				54											Cd2bp2
IPI00319992	P20029				104					49										Hspa5
IPI00173160	Q6ZWU9				88									81	47	137				Rps27
IPI00331546	P17156				108										75					Hspa2
IPI00928040	D3YYB0				88										50					Rps27l
IPI00114052	P27048				75													49	52	Snrpb
IPI00118172	O70422				59															Gtf2h4
IPI00121159	Q9JJ80				51															Rpf2
IPI00126176	Q9WVM1				69															Racgap1
IPI00126389	Q6P5B5				51															Fxr2
IPI00127947	Q9Z1M8				95															Ik
IPI00132418	P16254				108															Srp14
IPI00133208	P16627				108															Hspa1l

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IPI00133955	P62305				70															Snrpe
IPI00134300	P32067				107															Ssb
IPI00162940	Q8R5K4				46															Nol6
IPI00169700	Q76KJ5				130															Cd3eap
IPI00227005	P35922				51															Fmr1
IPI00314963	O35492				49															Clk3
IPI00323674	Q9Z315				41															Sart1
IPI00336965	Q8K301				109															Ddx52
IPI00408781	Q6NZL1				62															Dhx37
IPI00421162	Q6NZF1				122															Zc3h11a
IPI00453860	Q3UYW7				101															Bms1
IPI00457815	Q3TIX9				40															Usp39
IPI00458074	Q8CHD7				51															Dnajc13
IPI00458908	Q9DAW6				66															Prpf4
IPI00468360	Q9D180				41															Wdr65
IPI00515687	Q5SFM8				70															Rbm27
IPI00985620	E9QAB4				88															Gm17241
IPI00458765	Q60749				108	51	73			76	119									Khdrbs1
IPI00457756	Q3TJZ6				198		131													Fam98a
IPI00850690	Q6ZQ06				56					49			50							Kiaa1009
IPI00118438	A2A8V9				86							112								Srrm1
IPI00316184	Q9D287				147														42	Bcas2
IPI00111829	Q9D903				109															Ebna1bp2
IPI00112769	P70700				89															Polr1b
IPI00113475	Q9ESQ1				43															Col4a6
IPI00122202	B2RQA7				49															Ncapg
IPI00123709	Q9WTQ5				42															Akap12
IPI00130436	Q9DCA5				126															Brix1
IPI00136984	P62082				136															Rps7
IPI00137195	Q6P208				48															Rgs17
IPI00223570	Q8BGA5				129															Krr1
IPI00226378	Q8BX07				46															Ctdsp2
IPI00276866	Q8K224				90															Nat10
IPI00318725	Q9CYH6				158															Rrs1
IPI00323144	P52432				127															Polr1c
IPI00330356	F6XCV8				136															Kif23
IPI00453800	Q8K327				43															Znf828
IPI00464066	Q3UR76				46															D030046N08Rik
IPI00469799	P55937				42															Golga3
IPI00470007	Q504N7				49															
IPI00742441	Q2EG98				44															Pkd1l3
IPI00761446	Q504N7				49															
IPI00830307	D3YUI1				43															Zfp828
IPI00850934	F6SVV1				136															
IPI00896597	E9QMR2				45															Dock9
IPI00918040	Q3TDW8				43															Arhgef4
IPI00918111	E9QL87				49															BC094916
IPI00120981	Q8K202				270		58													Polr1e
IPI00114869	P25976				119		54					62		50						Ubtf
IPI00133555	Q9CXK8				231		49													Nip7
IPI00752710	P53569				153		200		50		51			104						Cebpz
IPI00117642	Q99ME9				166		150			50										Gtpbp4
IPI00130343	Q3U6P5				268				144		182				56					Hnrnpc
IPI00119632	Q9DBE9				236															Ftsj3
IPI00125855	P28867				83															Prkcd
IPI00129141	Q9Z0H1				130															Wdr46
IPI00229791	Q497V5				145															Srbd1
IPI00316815	Q3TFK5				238															Gpatch4
IPI00320690	Q61136				205															Prpf4b
IPI00284444	Q6A068				305		71					86								Cdc5l
IPI00409462	Q9Z1N5				266	40	66													Ddx39b
IPI00113241	Q9CZX8				219	115	144				68			46		40				Rps19
IPI00330406	D3Z0M9				245		104					89		73						Ddx23
IPI00123878	Q8VDW0				159	40														Ddx39a
IPI00313726	O35134				373															Polr1a
IPI00652660	Q3UFM5				388															Nom1
IPI00127172	Q91VR5				373		102							54						Ddx1
IPI00116850	Q99LF4				373	73	154													D10Wsu52e
IPI00115902	O55142					71	68		65	41	49	132		153	152					Rpl35a
IPI00113713	D3Z7M8						94	61												Gm5614
IPI00230730	P84228						47	95	149	74	88			161	83	324			85	Hist1h3b
IPI00553538	P68433						47	95	149	74	88			161	83				85	Hist1h3a
IPI00785343	P84244						50	95	149	74	88			161	83				85	H3f3a
IPI00607069	E9PV77						50	95	152	74	88				83					Gm10257
IPI00551412	P61327						60	92		41				40						Magoh
IPI00323819	P60867						48		110	85										Rps20
IPI00458583	Q8VEK3						123			263	523					118				Hnrnpu
IPI00317298	Q9CSN1						88					48							76	Snw1
IPI00117063	P56959						55													Fus
IPI00170228	Q8K4K0						46													Adam34
IPI00224644	Q99MR6						75													Srrt
IPI00264652	Q3TLE4						55													Taf15
IPI00331260	Q91WE2						42													Fam192a

Chapter 4

LAS1L localization in human and mouse cells and its role in cell cycle progression

Pavlos Fanis[1], Nynke Gillemans[1], Mariëtte van de Corput[1], Sjaak Philipsen[1], and Thamar B. van Dijk[1]

1. Department of Cell Biology, Erasmus MC, 3000 CA, Rotterdam, The Netherlands

(Work in progress)

ABSTRACT

LAS1L is a recently identified nuclear protein of which the biological function is not fully understood. It was shown to localize to the nucleolus and to play a role in the biogenesis of the 60S ribosomal subunit. Here we describe by immunofluorescent and biochemical studies that, in contrast to human cells, LAS1L does not localize to the nucleolus in mouse cells. Furthermore, LAS1L shows localization at the mid-body region, indicating its role in late stages of cell cycle in both species. Depletion of LAS1L causes inhibition of cell proliferation by a late mitosis cell cycle phase arrest, while overexpression of LAS1L promotes cell proliferation. Moreover, we demonstrate that depletion of components of the 5FMC (Five Friends of Methylated Chtop) complex, of which LAS1L is a member, results in similar cell cycle defects. Taken together, our data demonstrate that LAS1L, possibly as part of the 5FMC complex, is essential for cell cycle progression.

INTRODUCTION

The nucleolus was one of the first intracellular structures to be described (Montgomery, 1898). It occupies a considerable portion of the nucleus, but its size varies greatly depending on species, cell type and physiological state (Gerbi, 1997). The nucleolus is a subnuclear structure in eukaryotes where ribosomal RNAs are transcribed, processed, modified and finally assembled into ribosomes (Pederson, 1998). Although more than 700 human nucleolar proteins have been identified, only ~30% of these have a known function related to the production of ribosomes (Andersen et al., 2002). Additional nucleolar functions include storage of nuclear factors and regulation of tumour suppressor and oncogene activities. The nucleolus is involved in processing of spliceosomal small nuclear U6 RNA, telomerase RNA, signal recognition particle RNA, cell cycle regulation and specific aspects of mitosis (Carmo-Fonseca et al., 2000; Hernandez-Verdun and Roussel, 2003; Lam et al., 2005; Olson et al., 2002)

At the final stage of mitosis cytokinesis occurs and completes cell division. Mi-

tosis is characterized by dramatic morphological changes which occur in a strictly sequential order and include cytoskeletal disassembly, breakdown of the nuclear envelope, chromosome condensation, segregation and, finally, daughter cell separation. In the last stage of cytokinesis actin, myosin and other cytoplasmic molecules are redistributed into the cleavage furrow which forms midway between the segregated chromosomes in early anaphase (Cao and Wang, 1990). Cytokinesis is accomplished by contraction of this actin ring and leads to separation of two daughter cells at the midbody (Fishkind and Wang, 1995). Examples of nucleolar proteins that play a role in cell cycle and growth regulation are Cdc14, Mdm2 and Pch2 which are cell cycle regulators (San-Segundo and Roeder, 1999; Straight et al., 1999; Visintin et al., 1998), TRF2 which recruits to the telomeres during the cell cycle (Zhang et al., 2004), and LYAR which is involved in cell growth regulation (Su et al., 1993).

LAS1 (Lethal in the Absence of SSD1-u) is an essential nuclear protein that is involved in cell surface growth and morphogenesis in Saccharomyces cerevisiae. Inactivation of LAS1 results in unbudded cells and large budded cells that accumulate vesicles at the mother-daughter neck. Overexpression of LAS1 causes extra cell surface projections (Doseff and Arndt, 1995). LAS1-Like (LAS1L) protein, the human homologue of LAS1, was identified within nucleoli in human cells and it was recently shown that it is essential for ribosome biogenesis (Andersen et al., 2002; Castle et al., 2010). Moreover we have characterized Las1L as a component of the 5FMC (Five Friends of Methylated Chtop) complex, which acts as a key regulator of Zbp-89 desumoylation and is required for full transcriptional activation of Zbp-89 dependent genes (Chapter 3 of this thesis). In addition, LAS1L has been characterized as a component of the MLL1 complex, that plays a role in transcriptional activation through modification of histones (Dou et al., 2005).

The identification and characterization of proteins contained within nucleoli is critical for further understanding the role of the nucleolus in mitosis and cell cycle progression. Here, by using immunofluorescence and biochemical approaches,

we describe the subcellular localization of endogenous LAS1L in different human and mouse cell types. Depletion of LAS1L leads to G2/M cell cycle phase accumulation, while overexpression promotes cell proliferation, indicating that LAS1L is important for cell cycle progression.

RESULTS

LAS1L is localized in the nucleoli of human cells

To investigate the subcellular localization of LAS1L we performed immunofluorescence studies with a rabbit polyclonal antibody against the LAS1L protein. Analysis by confocal microscopy revealed that at interphase LAS1L was predominantly localized in the nucleoli and present at a lower concentration throughout the nucleoplasm and cytoplasm of Human Embryonic Kidney 293 (293T) cells (Figure 1A, upper panel). To check whether the subcellular localization of LAS1L is independent of cell type, we performed immunofluorescence with the same antibody in human cell lines derived from different tissues. Predominant nucleolar localization was also observed in primary Human Erythroid Progenitor (HEP) cells (Figure 1A, lower panel) and in a human osteosarcoma (U2OS) cell line (Figure 1B, upper panel), consistent with previous reports on the localization of LAS1L (Andersen et al., 2002; Castle et al., 2010). Interestingly, as the cells progressed to telophase and cytokinesis, we observed that LAS1L became sharply concentrated at the midbody region in U2OS cells (Figure 1B, upper panel). Together, this indicated that LAS1L is mainly localized in the nucleolus of human cells and might have an additional role in cytokinesis.

LAS1L is not localized in the nucleoli of mouse cells

Previous investigations of Las1L localization upon biochemical fractionation in MEL cells showed that it is localized in the nucleoplasm fraction (Chapter 3 of this thesis). To examine whether Las1L is also localized at the nucleoli of mouse cells we performed immunofluorescence staining in different mouse cell lines (Figure 2A).

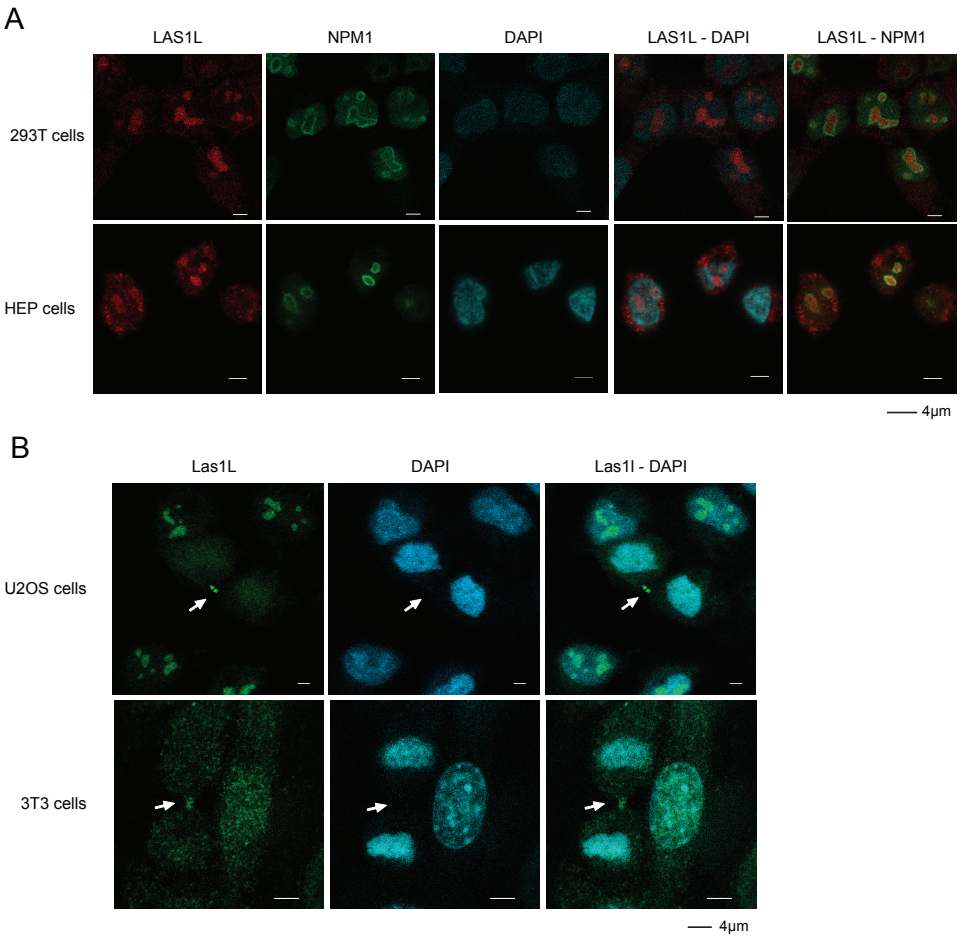


Figure 1. LAS1L is localized in the nucleolus and midbody of human cells. (A) Immunofluorescent staining of 293T (human embryonic kidney) and HEP (human erythroid progenitor) cells with a-LAS1L and a-NPM1 antibodies. Specific staining is seen for LAS1L (red) in the nucleoli of cells as indicate by NPM1 (Nucleophosmin) staining (green). The overlay of LAS1L and DAPI (blue) shows the nuclear localization of LAS1L. Scale bars, 4μm. (B) LAS1L is localized in midbody region in both human and mouse cells. Immunofluorescent staining of U2OS (human osteosarcoma) and 3T3 (mouse embryonic fibroblast) cells with a LAS1L antibody. Specific staining is seen for LAS1L (green) in the midbody region of late mitotic cells. The overlay of LAS1L and DAPI (blue) shows the nuclear localization of LAS1L. Scale bars, 4μm. Arrows indicate the midbody region.

However, using the same antibody we were not able to visualize LAS1L in the nucleolus of murine erythroleukemia (MEL) cells (Figure 2A, upper panel), mouse embryonic fibroblast (3T3) cells (Figure 2A, lower panel) and fetal liver-derived

erythroblasts (not shown). LAS1L was localized to nucleoplasm in interphase cells (Figure 2A), while in late mitosis in 3T3 cells the localization of LAS1L was also concentrated within the intracellular bridge at either side of the midbody (Figure 1B, lower panel). Human and mouse LAS1L are 734 and 776 amino acid proteins respectively, which are expressed in all cell types tested (Figure S1A). In our immunoblot studies in mouse cell lysates we observed a low molecular mass band of ~70 kDa which shows the existence of a different Las1L isoform (Las1L_S; Figure S1A and S1B). When we performed immunoprecipitation with the same antibody, we were not able to immunoprecipitate the expected molecular mass protein of ~90 kDa (Las1L_L), but we purified the low molecular mass protein (Figure S1B). Interestingly, when we performed immunoprecipitations for the other components of 5FMC complex followed by staining for Las1L, we only exclusively observed the high molecular mass protein of ~90 kDa (Chapter 3 of this thesis). Although there is no evidence from the different sequence databases for the existence of a Las1L isoform that can explain the ~70 kDa protein, we hypothesized that an isoform of Las1L exists that is expressed at relative high levels in mouse cells. However, we were not able to amplify any new isoform of Las1L mRNA by PCR from MEL cells cDNA. Further examination of human and mouse LAS1L amino acid sequences revealed that the major difference between the two proteins is that the acidic domain of human LAS1L is shorter compared to the acidic domain of mouse Las1L (Figure S1C). To investigate whether the different nucleolar localization between human and mouse cells is due to the differences in the amino acid sequence, we ectopically expressed human LAS1L fused to GFP (GFP-hLAS1L) in mouse 3T3 and human 293T cell lines. As expected, GFP-hLAS1L was localized in the nucleoli of all 293T cells (Figure 2B, right panel). However, GFP-hLAS1L nucleolar localization in 3T3 cells was observed in only ~20% of the cells (Figure 2B, left panel), suggesting that the cellular context plays a major role in LAS1L localization.

To confirm the localization of LAS1L in the nucleoli of human and mouse cells, we also performed nucleolar isolations in 293T and MEL cells. LAS1L protein was observed

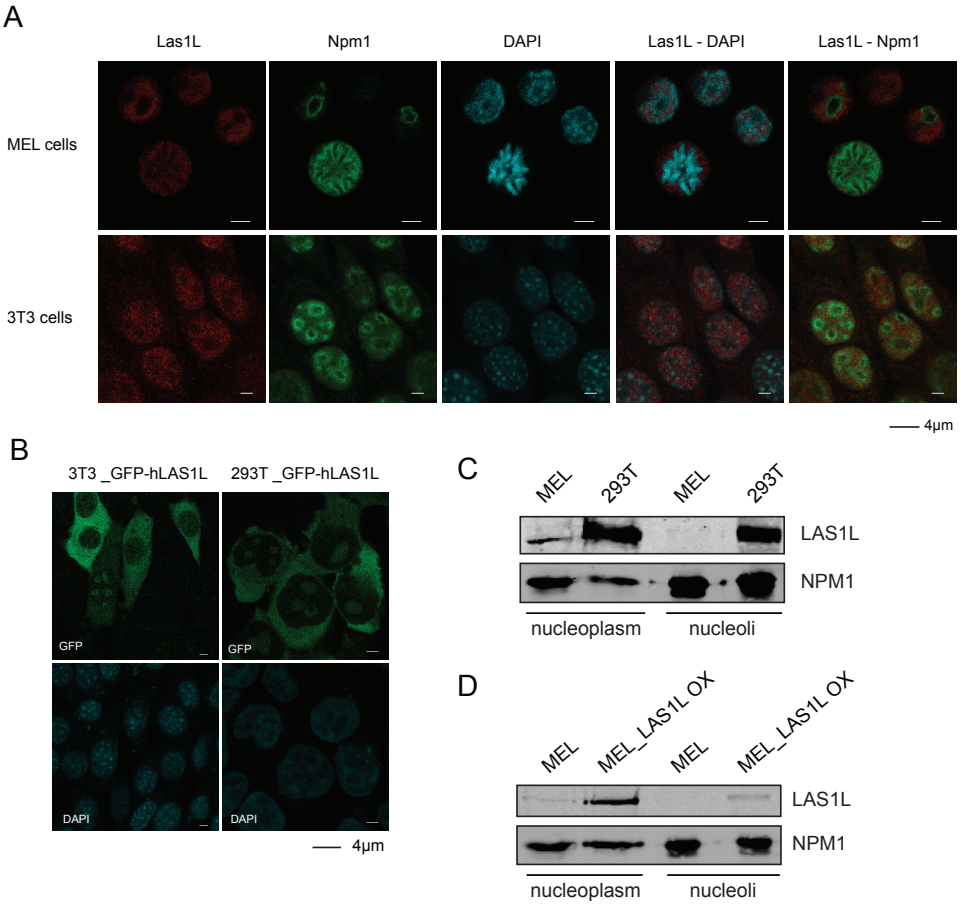


Figure 2. LAS1L localization in mouse cells. (A) Immunofluorescent staining of MEL (murine erythroleukemia) and 3T3 (mouse embryonic fibroblast) cells with LAS1L and NPM1 antibodies. Specific staining is seen for LAS1L (red) in the nucleus of cells as indicate by DAPI (blue) but not in the nucleoli as indicate by NPM1 (Nucleophosmin) staining (green). Scale bars, 4µm. (B) Ectopically expressed human LAS1L fused to GFP (GFP-hLAS1L) in 3T3 and 293T cells. GFP-hLAS1L is localized in the nucleoli of all transfected 293T cells, while in mouse 3T3 cells this is only observed in ~20% of the transfected cells. Scale bars, 4µm. (C) Nucleolar isolation of 293T and MEL cells analyzed by Western blotting and tested for LAS1L. NPM1 served as a control for nucleolar detection. (D) Nucleolar isolation of MEL cells and MEL cells that ectopically expressed human LAS1L (MEL_LAS1L OX) analyzed by Western blotting and tested for LAS1L. NPM1 served as a control for nucleolar detection.

in the nucleolar fraction in 293T cells but not in MEL cells, while it was detected in the nucleoplasm fraction of both cell lines (Figure 2C) consistent with the immunofluorescent results. Moreover, to confirm our observations of ectopically expressed

human LAS1L in mouse cells, we performed nucleolar isolation in MEL cells that ectopically expressed human LAS1L (MEL_LAS1L OX). We were able to observe small amounts of ectopically expressed LAS1L protein in the nucleolar fraction when compared to the amounts of ectopically expressed LAS1L in the nucleoplasm fraction (Figure 2D), confirming our immunofluorescent observations in 3T3 cells. The observed small amounts of human LAS1L in mouse cells (3T3 and MEL) could be an artifact due to the overexpression of the protein. To test the fidelity of the nucleolar fractionation we probed for nucleophosmin (NPM1), a protein known to be concentrated in the nucleoli. Collectively, these data show that the bulk of LAS1L is not localized in the nucleolus of mouse cells. In contrast, the midbody localization of LAS1L is observed in both human and mouse cells, indicating that it might be involved in the process of cytokinesis.

Depletion of LAS1L results in G2/M cell cycle phase arrest and cell death

To study the role of LAS1L protein, we used short hairpin RNAs (shRNAs) and performed lentiviral mediated knockdown of LAS1L in 293T cells. Of the five shRNAs tested, shRNAs 2 and 4 decreased LAS1L protein levels efficiently (Figure 3A). LAS1L-depleted cells displayed a rounded shape and were loosely attached to the tissue culture plate, in contrast to cells treated with scramble shRNA (Figure 3B). To test whether this was due to a cell cycle defect, we performed cell cycle analysis in LAS1L-depleted 293T cells, using propidium iodide staining analyzed by FACS. Compared to cells treated with the scramble shRNA, a significant increase in the G2/M, apoptotic and multinucleated cell populations was observed in LAS1L depleted cells (Figure 3C). The G2/M cell population increase was further confirmed by staining with an antibody recognizing phosphorylation of histone H3 at serine 10, a modification associated with chromosome condensation during mitosis. Immunofluorescence staining with this antibody showed elevated numbers of mitotic phase cells when LAS1L was depleted supporting the FACS analysis studies (Figure S2A). We next used western blotting to determine whether LAS1L

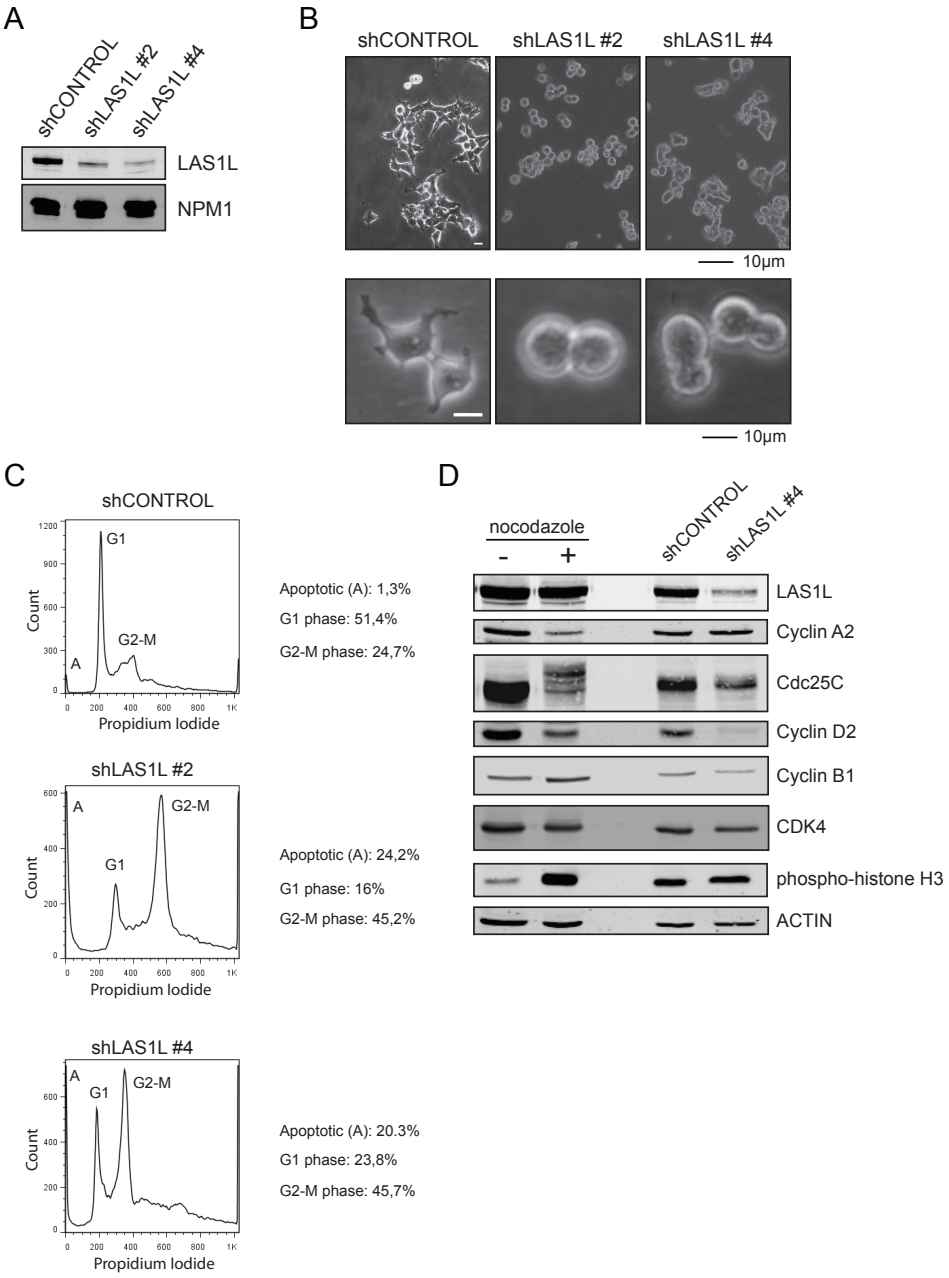


Figure 3. Depletion of LAS1L results in G2/M arrest and apoptosis. (A) 293T cells transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNAs against LAS1L (shLAS1L). Cell lysates were tested for LAS1L. Staining for NPM1 (nucleophosmin) served as a control for equal load-

ing. (B) Light microscopy images of 293T cells transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNAs against LAS1L (shLAS1L). Scale bars are indicated. (C) 293T cells transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNAs against LAS1L (shLAS1L) were stained with propidium iodide (PI), and analyzed by FACS. Percentages of apoptotic cells and cells in G1 and G2/M cell cycle phase are indicated. (D) 293T cell lysates transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNA against LAS1L (shLAS1L#4) were analyzed with the antibodies indicated. Nocodazole treatment of 293T cells serves as a control for cells arrested in G2/M cell cycle phase.

depletion resulted in changes in protein expression of key cell cycle regulators, which might explain the observed G2/M cell cycle phase arrest. For comparison, we used lysates from 293T cells treated with nocodazole, an agent that arrest cells in early in mitosis (the pro-metaphase). In the absence of LAS1L the protein levels of cyclin B1, cyclin D2, cdc25C, and cdc2 were reduced, while the levels of phosphorylated histone H3 were elevated (Figure 3D and Figure S2B). The strong reduction of cyclin D2 indicates that the majority of cells were in the mitotic phase. Cyclin B1, cdc2 and cdc25C levels are elevated when the cell entry the mitosis while they are reduced at the later anaphase stage (Morgan, 2007). The observation of lower levels of Cyclin B1, cdc2 and cdc25C show that the cells accumulate at late anaphase and/or telophase. We conclude that LAS1L knockdown causes an arrest late in mitosis, around anaphase-telophase, followed by nuclear division without cytokinesis and/or cell death.

Overexpression of LAS1L promotes cell proliferation

To determine the effects of overexpression of LAS1L we analyzed three different MEL cell clones that stably over-expressed LAS1L (bio_HA_LAS1L) (Figure 4A). Cell counts revealed that LAS1L over-expressing cells proliferated at higher rates compared to control cells (Figure 4B). Similar results were obtained when we treated the cells with CFSE. This fluorescence dye can be used to monitor cell proliferation by FACS analysis, due to the halving of fluorescence within the daughter cells upon each division. Following the CFSE-treated cells for four days we observed that the LAS1L-overexpressing cells divided at a higher rate (Figure 4C).

The division time of non LAS1L-overexpressing cells was every 12 hours while the division time of LAS1L-overexpressing cells was every 9-10 hours. Our results indicate that LAS1L overexpression promotes or accelerates cell proliferation.

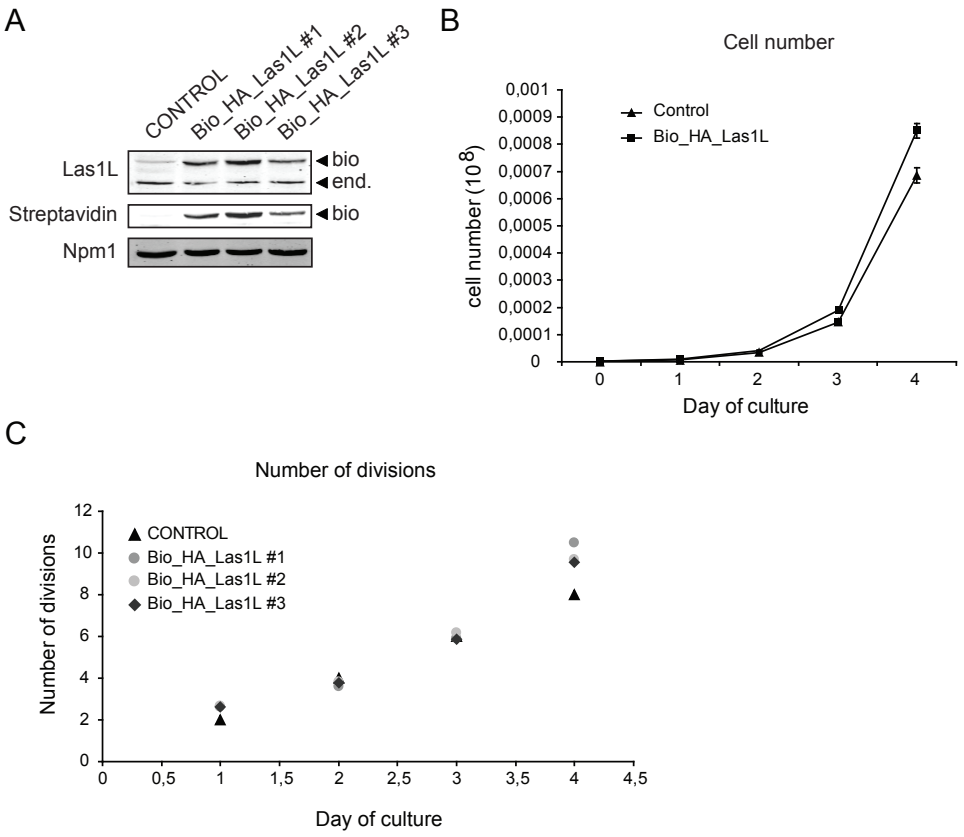


Figure 4. Overexpression of LAS1L promotes cell proliferation. (A) MEL cells ectopically expressed LAS1L (Bio_HA_Las1L) were analyzed by Western blotting and tested for LAS1L and Streptavidin (end. indicates endogenous LAS1L and bio indicates overexpressed LAS1L). Npm1 served as a loading control. (B) Proliferation rate between wild type (Control) and overexpressed MEL cells (Bio_HA_Las1L) for 4 days. Error bars: SD of triplicate experiment. (C) Proliferation rate of wild type (CONTROL) and overexpressed (Bio_HA_Las1L #1, #2 and #3) MEL cells determined by CFSE dilution during 4 days in culture.

Depletion of 5FMC complex components leads to G2/M cell cycle phase accumulation and cell death

We have previously shown that Las1L is a component of the 5FMC complex (Chapter 3), a desumoylating protein complex that specifically binds to arginine-methylated Chtop, a small chromatin-associated protein (van Dijk et al., 2010). To evaluate whether the other components of the 5FMC complex are also required for cell proliferation, we depleted Senp3, Wdr18 and Pelp1 in MEL cells by lentiviral-mediated knockdown (Figure 5A). Cell cycle analysis of Senp3 depleted cells showed a three-fold increased in the population of cells with more than 4N DNA content and an increase in apoptotic cells (figure 5B, right panel). Haematoxylin staining of the Senp3 depleted cells revealed a strong increase of multi-nucleated cells (Figure 5B, left panel), confirming the cell cycle analysis observations. Comparable results (decreased G1, increased apoptosis and multinucleated cells) were also obtained in cells depleted for Wdr18 or Pelp1 (Figures 5C and 5D). Nevertheless, the cell cycle profiles are not completely identical: Wdr18 depletion has a dramatic effect on cell survival, while depletion for Pelp1 has a more prominent effect on the G2/M phase of the cell cycle. Collectively these results show that the components of the 5FMC complex are important for cell cycle progression.

Wdr18 is essential for embryogenesis

To examine the role of Wdr18 *in vivo*, we obtained an embryonic stem cell clone carrying a gene trap insertion in the first intron of the Wdr18 gene (Figure 6A) and a mutant mouse line was established. Heterozygous mutant mice (Wdr18+/-) were phenotypically normal and fertile. Crosses between heterozygotes failed to yield any homozygous mutant animals (Wdr18-/-) among the offspring, indicating that the gene trap insertion caused an embryonic lethal loss-of-function mutation. To determine the time of the embryonic lethality, embryos of heterozygous crosses were genotyped. Between E8.5 and E12.5, all embryos investigated were either wild-type or heterozygotes (Figure 6B). Interestingly, we observed an increase

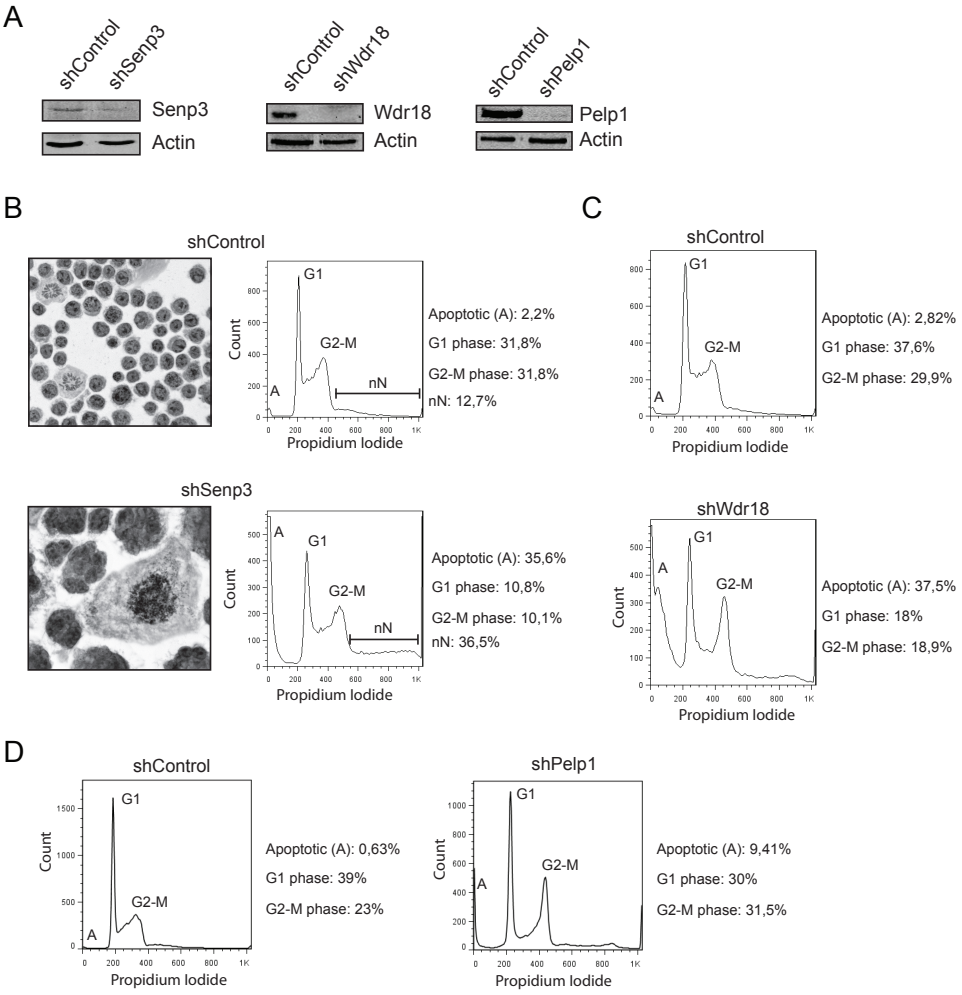
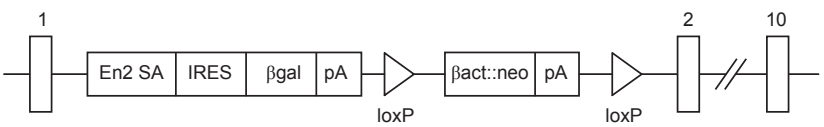


Figure 5. Depletion of 5FMC components leads to G2/M cell cycle phase arrest and apoptosis. (A) MEL cells transduced with control lentivirus (shControl) or lentivirus expressing shRNAs against Senp3 (shSenp3), Wdr18 (shWdr18) and Pelp1 (shPelp1). Cell lysates were tested for Senp3, Wdr18 and Pelp1. Staining for Actin served as a control for equal loading. (B) Cytospins of MEL cells transduced with control lentivirus (shControl) or lentivirus expressing shRNA against Senp3 (shSenp3) stained with haematoxylin. Identical cells were stained with propidium iodide (PI), followed by FACS analysis. Percentages of apoptotic cells, cells in G1 and G2/M cell cycle phase, and cells with more than 4N DNA content are indicated. (C, D) MEL cells transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNAs against Wdr18 (shWdr18; (C)) or Pelp1 (shPelp1 (D)) were stained with propidium iodide (PI), and analyzed by FACS. Percentages of apoptotic cells and cells in G1 and G2/M cell cycle phase are indicated.

number of resorption sites (39 out of 109) which might be representing knockout embryos if all knockout embryos would initiate implantation. This suggested that Wdr18 is already essential during the peri-implantation period. To correlate the homozygous mutant genotype with a late pre-implantation phenotype, 31 blastocyst-stage embryos (E3.5) were collected from heterozygous intercrosses and cultured in ES-medium. At this stage, 21 ES cell lines were initiated of which 11 were unable to grow. These could not be genotyped due to the small amounts of DNA recovered. From the remaining 10 ES cell lines, all cell lines were either wild-type or Wdr18+/-. Taken together, these results show that Wdr18 is essential for normal embryogenesis.

A



B

Embryonic Age (Days)	Resorption sites	Wdr18$^{+/+}$	Wdr18$^{+/-}$	Wdr18$^{-/-}$	total
12.5	10	1	6	0	17
10.5	8	4	8	0	20
9.5	10	3	5	0	18
8.5	11	16	27	0	54
3.5	–	4	6	0	10
total	39	28	52	0	119

Figure 6. Wdr18 is essential for embryogenesis. (A) Schematic representation of the 'trapped' Wdr18 gene. The gene trap was inserted in the first intron of the Wdr18 gene. 1-10: exons; En2 SA: En2 splice acceptor; IRES: Internal Ribosome Entry Site; βgal: β-galactosidase; pA: polyadenylation site; βact: β-actin; neo: neomycin. (B) Genotype analysis of Wdr18+/- intercrosses at different developmental stages. Wdr18+/+: wild type mice; Wdr18+/-: heterozygous mutant mice; Wdr18-/-: homozygous mutant mice.

DISCUSSION

In this study, we demonstrated that Las1L is absent from nucleoli in mouse cells, in contrast to previous observations in human cells. Moreover, we showed that LAS1L is concentrated at the midbody region at the last stage of cytokinesis and that it is essential for normal progression through mitosis. As LAS1L is a member of the 5FMC complex, we further demonstrated the role for other 5FMC components in cell cycle progression.

In line with previous experiments (Castle et al., 2010), our immunofluorescence studies in the human 293T and U2OS cell lines, as well as primary human erythroid progenitors, revealed that LAS1L is a nuclear protein with a relative high concentration at the nucleoli (Figure 1 and Figure S1). Using biochemical fractionation of 293T cells we observed an identical result: LAS1L protein is present in higher concentration in the nucleolus than nucleoplasm (Figure 2C). In mouse cells, Las1L localizes to the nucleus and the midbody, while it can not be detected in the nucleoli (Figure 2A, 2C and Figure S1). It remains to be determined whether this apparent difference in subcellular localization implies a difference in the function of LAS1L in mouse and human cells. For instance, species-specific nucleolar localization comparable to that of LAS1L has also been observed for the Werner syndrome helicase (WRN), which was found in the nucleolus of human cells but not of mouse cells (Gray et al., 1997; Marciniak et al., 1998; von Kobbe and Bohr, 2002). In this case, the inability of mouse WRN to migrate into the nucleolus was due to a difference of a sequence in the region corresponding to the nucleolar localization signal (NoLS) of human WRN (Shiratori et al., 1999; Suzuki et al., 2001). To test whether the difference in localization of human and mouse LAS1L is due to a difference in the amino acid sequences we compared the sequences of both human and mouse LAS1L. A comparison of the human and mouse amino acid sequences of LAS1L showed one major structural difference: the acidic domain in human LAS1L is much shorter compared to the mouse protein (~45 aa and ~95 aa, respectively; Figure S1C). This sequence variation is possibly respon-

sible for the different nucleolar localization, although acidic regions have not been linked with nucleolar localization before. Alternatively, factors may be expressed in human cells and not in mouse cells that targets LAS1L to the nucleolus. The observation that human LAS1L is only present in the nucleoli of ~20% of transfected mouse cells, points into this direction. The reverse experiment has not been performed, due to the absence of full length Las1L mRNA. Another possibility is that the ~70 kDa mouse Las1L protein represents an unknown isoform lacking the nucleolar-targetting sequence. We were not able to clone such isoforms by PCR. Alternatively, the observed low molecular mass protein could be the product after post-translational cleavage by a protease.

In yeast, the LAS1L homologue, LAS1, is also localized in the nucleus (Doseff and Arndt, 1995). In this study it is hypothesized that both the localization of LAS1L in the nucleus and the effects of LAS1/Las1L on cell surface growth/morphogenesis are due to transcription regulation of some components that function more directly in cell morphogenesis. However, it is currently unknown which genes are directly regulated by LAS1/LAS1L.

The effects caused by the over- or under-expression of LAS1L show that LAS1L is involved in the cell cycle progression. LAS1L depletion caused significant accumulation of cells in G2/M phase, specifically in anaphase-telophase of mitosis. This resulted in a failure of cytokinesis, eventually leading to cell death (Figure 3B-D and Figure S3A-B). In yeast, inactivation of LAS1 results in unbudded cells and large budded cells that accumulate vesicles at the mother-daughter neck (Doseff and Arndt, 1995). These findings in yeast are similar to our findings in mammalian cells and strongly indicate that LAS1L may be required for the onset of cytokinesis. This hypothesis is also supported by our finding that LAS1L is localized to the midbody region (Figure S1). Overexpression of LAS1L protein promotes cell proliferation. Interestingly, a similar finding has also been observed with the yeast homologue LAS1. In yeast, overexpression of LAS1 causes extra cell surface projections (Doseff and Arndt, 1995). However, the effector pathway

implicated in LAS1L mediated control of cell cycle progression remains to be identified. Las1L is a member of 5FMC (Five Friend of Methylated Chtop) complex that is composed of five proteins: Pelp1, Senp3, Tex10, Wdr18 and Las1L (Chapter 3 of this thesis). It has been shown that members of the 5FMC complex are also localized within the nucleolus in human cells (Andersen et al., 2002; Finkbeiner et al., 2011; Haindl et al., 2008). To investigate the role of 5FMC complex components in cell cycle progression, we depleted Pelp1, Senp3 and Wdr18. Depletion of 5FMC complex components caused comparable effects as those observed upon depletion of Las1L: an increase of the G2/M cell cycle phase population and an increase of cell death (Figure 5). This suggests that the 5FMC complex is required for cell cycle progression. This may explain why we were unable to recover Wdr18-/- ES cell lines from blastocysts obtained from Wdr18+/- crossings (Figure 6).

In summary, our data provide evidence that LAS1L is localized in the nucleolus of human but not of mouse cells, and that LAS1L is essential for cell cycle progression. Moreover, our results suggest a role for this protein as part of 5FMC complex in coordinating cytokinesis. However, at present little is known about the molecular events upstream or downstream of LAS1L at the last stage of cell division and how the nucleolar localization is associated with this stage.

EXPERIMENTAL PROCEDURES

Constructs and cells

The cDNA of full-length LAS1L was obtained from Open Biosystems (Clone ID 3140243; Huntsville, AL, USA). After the introduction of the 23-amino acid (aa) biotinylation tag into the pMT2_HA, LAS1L was cloned to pMT2_bio_HA using SalI and NotI. Bio_HA_LAS1L was subcloned into the erythroid expression vector pEV-neo (Needham et al., 1992) and electroporated into mouse erythroleukemic (MEL) cells expressing the BirA biotin ligase (de Boer et al., 2003). Green fluorescent protein (GFP) was cloned in frame with the N terminus of LAS1L. MEL, 293T, 3T3 and U2OS cells were grown in Dulbecco's modified Eagle medium (DMEM; Life

Technologies) supplemented with 10% fetal calf serum (FCS). Human erythroid progenitors were cultured from buffy coats in serum-free medium as described previously (Leberbauer et al., 2005).

Transient transfection, immunoprecipitation, western blotting and antibodies

Western blot analysis was performed as described previously (van Dijk et al., 2000). Nitrocellulose membranes were blocked in 1% bovine serum albumin (BSA) probed with the appropriate primary antibodies and analyzed using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Western blots were probed with the following primary antibodies: ACTIN (clone I-19; sc-1616), NPM1_B23 (clone FC-8791; sc-32256), Cyclin A2 (clone C-19; sc-596), Cyclin D2 (clone C-17; sc-181), Cyclin B1 (clone GNS1; sc-245), Cdc25C (clone C-20; sc-327), Cdc2_p34 (clone 17; sc-54) and CDK4 (clone C-22; sc-260) were from Santa Cruz Biotechnology; Anti-phospho-Histone H3_Ser10 (06-570) was from Upstate; α-Tubulin (T5168) was from Sigma; LAS1L (16010-1-AP), SENP3 (17659-1-AP) and WDR18 (15165-1-AP) were from Proteintech Group and PELP1 (A300-876A) was from Bethyl Laboratories.

Cellular extracts and isolation of nucleoli

Preparation whole cell extracts from various cell lines and tissues were carried out as described previously (van Dijk et al., 2000). Extracts enriched in nucleoli were prepared from MEL and 293T cells as previously described (http://www.lamondlab.com/f5nucleolarprotocol.htm). For western analysis, samples were resuspended in RIPA buffer (50mM Tris, pH 8.0, 0.14M NaCl, 0.2% SDS, 1% Triton X-100, and 0.5% sodium deoxycholate).

Lentivirus-mediated knockdowns

For Pelp1, Senp3 and Wdr18 clones from the TRC Mission shRNA library ((Moffat et al., 2006); Sigma Aldrich, St. Louis, MO, USA) were used for knockdown

experiments in MEL and 293T cells, including a non-targeting shRNA control virus (SHC002). Lentivirus was produced by transient transfection of 293T cells as described before (Zufferey et al., 1997). The following clones were used from the TRC shRNA library: TRCN0000121835 (shLAS1L #2), TRCN0000142144 (shLAS1L #4), TRCN0000178252 (shPelp1), TRCN0000031016 (shSenp3) and TRCN0000200120 (shWdr18). 293T cells were exposed to lentivirus for 16 h. After infection, cells were cultured in the presence of puromycin (2 µg/ml; Invitrogen) for 5-7 days. Senp3 knockdown MEL cells were fixed in acetone and stained with haematoxylin.

Confocal imaging

For confocal imaging, cells were spotted on poly-prep slides (Sigma) or cultured on cover slips, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100, and blocked in 1% BSA–0.05% Tween 20 in PBS. Primary antibody incubation was performed in blocking solution for 16 h at 4°C. Images were made using a Confocal Laser Microscope LSM 510 (Zeiss), equipped with a 488 nm Argon laser for green fluorescence and 543 nm laser for red fluorescence. The following secondary antibodies were used: Alexa Fluor-594, -488 labeled goat-anti-mouse and goat-anti-rabbit (Invitrogen).

5-Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) labeling and proliferation Assay

To analyze cell cycle profiles, 293T cells were subsequently trypsinized, washed once in cold PBS, and fixed in 70% ethanol at 4 °C overnight. Fixed cells were pelleted and stained in a solution of 50 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA), 50µg/ml RNase A and 0.1% Triton X-100 (Sigma) for 30 min at room temperature prior to flow cytometric quantification of DNA by a FACScan (Becton Dickinson). For labeling with the fluorochrome CFSE (Molecular Probes, Eugene, OR, USA), MEL cells were washed and resuspended in PBS at a con-

centration 1 x 106 cells per ml. Cells were incubated with CFSE at a final concentration of 1 μM for 15 min at 37°C. At the end of the incubation period, the cells were re-pelleted and resuspended in fresh prewarmed medium and incubated for another 30 min. At the end of the incubation cells were counted and cultured as described above. Cells were analysed by FACScan every 24 hours for a period of four days.

Generation of mice and genotyping

The E070C08 ES cell clone with the gene trap vector in the Wdr18 gene was obtained from the German Gene Trap Consortium, now part of the International Gene Trap Consortium (http://www.genetrap.org). The ES cell-derived chimeras were generated by injecting C57BL/6 blastocysts. The resulting male chimeras were bred to C57BL/6 females, and agouti offspring were tested for transgene transmission. Animals heterozygous for the gene trap insertion were back-crossed to C57BL/6 mice. Genotyping by PCR was done with DNA isolated from tail or yolk sac tissue. The following primers were used: Wdr18_Trap_F1: 5' – GCAGGGCAAGAACTACATCTGC – 3'; Wdr18_Trap_R1: 5' – GACACATGATTTTCTGTTGGAGC - 3'; reverse lacz: 5' – CAAGGCGATTAAGTTGGGTAACG - 3'. To establish ES cell lines, blastocyst stage embryos (E3.5) were isolated and seeded onto irradiated mouse embryonic fibroblasts (MEFs) in DMEM supplemented with 15% heat inactivated fetal calf serum, 100U/ml penicillin, 100mg/ml streptomycin, non-essential amino acids (NEAA), 5000U/ml leukemia inhibitory factor (LIF), 0,1 mM β-mercaptoethanol and 50μM MEK1 Inhibitor (Cell signaling; PD98059). ES cells were grown on a layer of mouse embryonic fibroblast (MEF) feeder cells.

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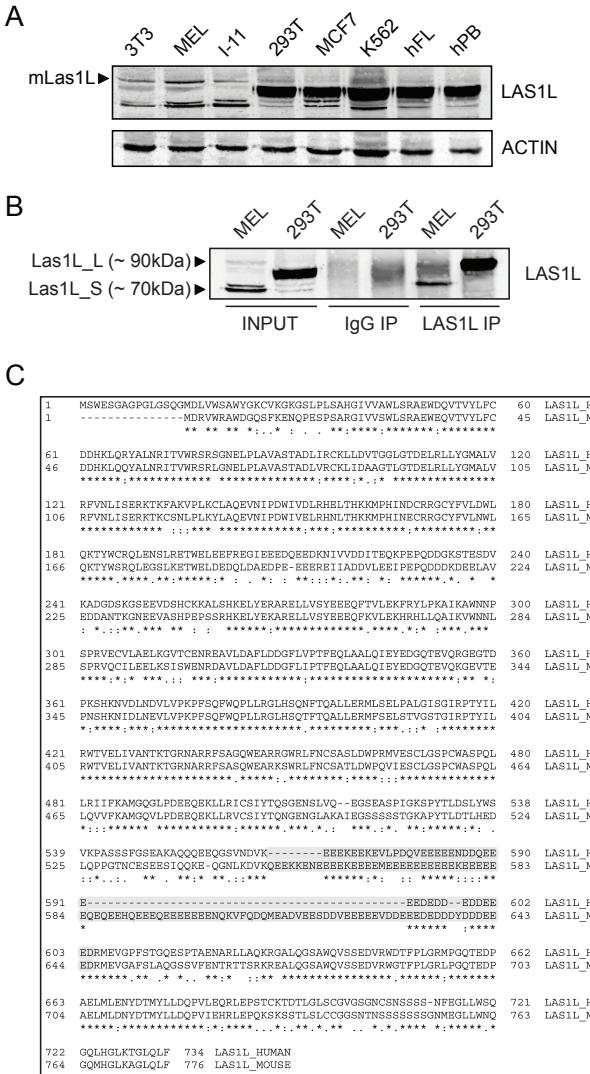
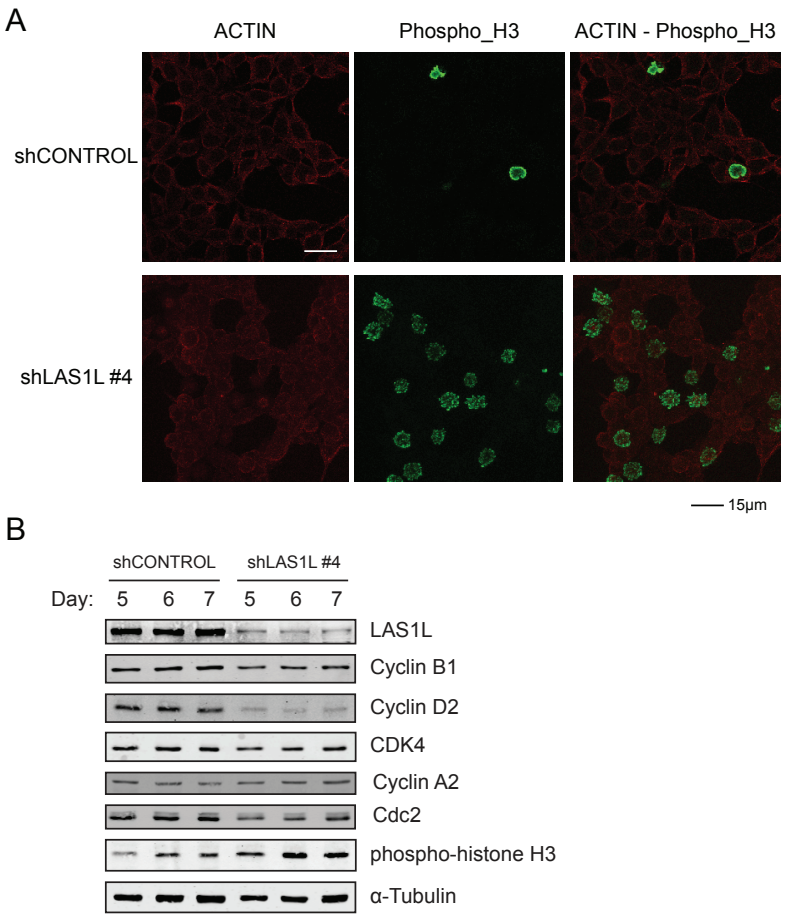
SUPPLEMENTAL FIGURES



Supplemental Figure 1. LAS1L is expressed in a wide array of tissues. (A) Cell lysates from 3T3 (mouse embryonic fibroblast), MEL (murine erythroleukemia), I-11 (primary mouse fetal liver), 293T (human embryonic kidney), MCF7 (human breast cancer cell line), K562 (human erythromyeloblastoid leukemia cell line), hFL (human fetal liver), hPB (human peripheral blood) cells were tested for LAS1L. Staining for ACTIN served as a control for loading. (B) Cell lysates were analyzed by immunoprecipitation and western blotting with the antibodies indicated. Molecular mass is indicated. Las1L_L and Las1L_S indicate the 2 different “species” of Las1L in mouse cells. (C) Alignment of human and mouse full-length amino acid sequences of LAS1L orthologs. The acidic domain is indicated in light gray.



Supplemental Figure 2. LAS1L depletion leads to G2/M cell cycle phase arrest. (A) Immunofluorescent staining with a-phospho-histone H3 (Phospho_H3) and a-ACTIN of 293T cells transduced with control lentivirus (shCONTROL) or lentivirus expressing shRNA against LAS1L (shLAS1L #4). Scale bars, 15µm. (B) 293T cell lysates were analysed for days 5, 6 and 7 after transduction with control lentivirus (shCONTROL) or lentivirus expressing shRNA against LAS1L (shLAS1L #4) with the antibodies indicated.

Chapter 5

Discussion

GENERAL DISCUSSION

Arginine methylation is a prevalent post-translational modification found on both nuclear and cytoplasmic proteins. It provides an important regulatory step in the control of protein-protein interactions. Whether arginine methylation also plays a significant role in protein-nucleic acid interactions is still unclear. Prmt1, the predominant Prmt enzyme, is ubiquitously expressed in mammalian cells (Bedford and Clarke, 2009), therefore it is perhaps not surprising that Prmt1 knockout mice die around embryonic day 6.5 (Pawlak et al., 2000). Prmt1 has a preference for methylating arginine residues that are flanked by glycine residues in so-called glycine-arginine-rich regions (GAR regions). GAR regions are common in many RNA-binding proteins (RBPs) and heterogeneous ribonucleoproteins (hnRNPs) which play roles in the processing and transport of mRNA (Bedford and Clarke, 2009). Furthermore, Prmt1 methylates and regulates transcription factors, histones and other co-activators. It acts as a co-activator in nuclear receptor-mediated transcription (Chen et al., 1999) and can also be recruited to promoters by transcription factors such as p53, YY1 and NF-κB. This results in the methylation of histone H4 at arginine 3 (H4R3me), a mark that is important for subsequent histone acetylation and further activating events (Huang et al., 2005). Little is known about the regulation of the enzymatic activity of Prmt1. It is catalytically active only in the form of dimers, or even oligomers (Lim et al., 2005; Zhang and Cheng, 2003). Gel filtration experiments show that Prmt1, a 42 kDa protein, is a component of a 250-400 kDa complex. It is unclear whether this reflects a large Prmt1 polymer, or whether additional proteins are a constitutive component. The idea that the identification of novel Prmt1-interacting partners is essential to get better insight into the different cellular processes regulated by Prmt1, and possibly the regulation of Prmt1 itself, is the starting point of the studies described in this thesis.

In Chapter 2 we described the isolation of Prmt1-interacting proteins by a single round of protein purification from murine erythroleukemia (MEL) cells using a biotinylation-proteomics approach. Mass-spectrometry analysis revealed candi-

date proteins such as RBPs involved in RNA processing, RNA export, ribosome synthesis and RNA stability. All identified Prmt1-interacting proteins contain one or more glycine-arginine-rich regions, suggesting that those proteins are targets of Prmt enzyme activity. Apart from the RBPs and known Prmt1-associated proteins, a new Prmt1-interacting protein was identified which is encoded by the mouse 2500003M10Rik and human C1orf77 genes, respectively. In view of the fact that it had not been characterized previously, we named it Friend of Prmt1 (Fop). It is also known by the name of Small protein Rich in Arginine and Glycine (SRAG) (Zullo et al., 2009), but the official (human) name is now Chromatin target of Prmt1 (Chtop). The results presented in Chapter 2 describe the first characterization of Chtop. Chtop is highly conserved in all vertebrates with no known protein domains. Its central region consists of 26 arginine residues that are flanked by a glycine, the GAR domain. Chtop is a nuclear protein that is stably associated with facultative heterochromatin. However, transient overexpression studies in HeLa cells demonstrated that apart from the nuclear localization, Chtop is also found in the nucleolus in 25-35% of the cells (Zullo et al., 2009). From our immunofluorescence staining of endogenous Chtop in different cell lines and mouse embryonic tissues, as well as live-imaging of U2OS cell stably expressing GFP_Chtop we were not able to observe any nucleolar localization. In addition, Chtop was not detected in several independent large scale characterizations of the human nucleolar proteome (Andersen et al., 2002; Lam et al., 2007; Scherl et al., 2002). We therefore propose that the presence of Chtop in the nucleolus is an artifact due to the transient overexpression of the protein in HeLa cells.

Chtop is not part of the Prmt1 holoenzyme complex, as it is not detected in the 250-400 kDa fractions in gel filtration experiments. The C-terminal half of its GAR domain is targeted by Prmt1, however it is also symmetrically methylated by Prmt5, indicating that it possibly contains asymmetrically dimethylated arginines (aDMA) and symmetrically dimethylated arginines (sDMA) at the same time. The fact that Chtop is associated with chromatin and that type I methylation by Prmt1

serves as a general marker of active transcription (Bedford and Clarke, 2009) raises the possibility that Chtop is involved in transcription regulation. To test this, we analyzed the role of Chtop in the induction by β-estradiol (E2) of the TFF1 (pS2) gene in breast cancer cells. Ligand-activated ERα causes the recruitment of co-activators and specific histone modifications at the pS2 promoter. Prmt1 recruitment is critical for H4R3 methylation and subsequent histone acetylation and binding of the basal transcription machinery to the pS2 promoter. Indeed, Chtop is required for the binding of E2-activated ERα to the pS2 promoter region and for the E2-inducible expression of ERα target genes. In another study, the role of Chtop in transcription regulation was investigated in erythroid cells. In this study depletion of Chtop resulted in the increase of γ-globin expression (van Dijk et al., 2010). However, it is unknown whether the γ-globin gene(s) or other globin genes are a direct target of Chtop.

What is the function of the methylation of Chtop by Prmt1? How does Chtop regulate its target genes? Since arginine methylation is known to regulate protein-protein interactions (see Chapter 1), it is possible that Chtop recruits proteins in a methylation dependent manner. In Chapter 3 we describe the identification and characterization of a novel nuclear complex that binds specifically to arginine-methylated Chtop. We show that Chtop and the complex are recruited by Zinc finger binding protein-89 (Zbp-89), thereby regulating both the desumoylation of Zbp-89 and transactivation by Zbp-89.

The novel complex is composed of five proteins: Proline-glutamate and leucine rich protein 1 (Pelp1), SUMO1/sentrin/SMT3 specific peptidase 3 (Senp3), Las1 like protein (Las1L), Testis expressed 10 protein (Tex10) and WD repeat domain 18 (Wdr18); which we termed Five Friends of Methylated Chtop (5FMC). These proteins were not present in Chtop purifications when Prmt1 was depleted, indicating that Chtop recruits 5FMC in a methylation-dependent process.

Not much is known about most of the components of the 5FMC complex. The best studied component is Pelp1, a co-regulator involved especially in nuclear

hormone signalling. Pelp1 contains multiple nuclear receptor (NR)-interacting motifs (LXXLL) and interacts with several NRs (Vadlamudi and Kumar, 2007). For example, Pelp1 acts as a co-activator for both ERα and ERβ receptors, androgen receptors, glucocorticoid receptors and progesterone receptors in a ligand dependent manner (Vadlamudi et al., 2001; Wong et al., 2002). Chromatin immunoprecipitation (ChIP) studies showed that, after ligand addition, Pelp1 is recruited to ERα target genes such as pS2, PR and IGF (Nair et al., 2004). As Chtop is also involved in the induction of ERα target genes, this raises the possibility that Chtop, when methylated, recruits Pelp1 (as a component of the 5FMC complex) to the promoters of these genes, promoting their transcription. Pelp1 also interacts with the transcription factor signal transducer and activator of transcription 3 (STAT3), thereby enhancing STAT3 transactivation functions (Manavathi et al., 2005). Furthermore, Pelp1 interacts with histones and several components of chromatin modifying complexes, such as the histone acetyltransferases p300 and CRE-binding protein (CBP), the metastasis associated protein 1(MTA1), histone deacetylase 2 (HDAC2) which are components of the NuRD complex, and the Lysine-specific histone demethylase 1 (LSD1) which is a component of the CoREST complex (Mishra et al., 2003; Nair et al., 2004; Nair et al., 2010). Possibly, Pelp1 (and the 5FMC complex) functions as a platform that affects chromatin remodelling complexes, thereby regulating gene expression.

Little is known about Senp3, an enzyme with isopeptidase activity that catalyzes the removal of SUMO groups from other proteins (see Chapter 1). Senp3 is a nuclear protein that also localizes in the nucleolus where it catalyzes the desumoylation of Nucleophosmin 1 (NPM1) and is crucial for 32S RNA processing (Haindl et al., 2008). Moreover, the desumoylating activity of SENP3 is required for Reactive Oxygen Species (ROS)-induced increase of hypoxia inducible factor (HIF)-1 transactivation. In this example the true substrate of SENP3 is p300, the co-activator of HIF-1 alpha, rather than HIF-1 alpha itself. Removing SUMO2/3 from p300 enhances its binding to HIF-1 alpha resulting in increase of its transcrip-

tional activity (Huang et al., 2009).

The other components of the 5FMC complex, Las1L, Wdr18 and Tex10, have been poorly or not at all characterized yet. Las1, the yeast ortholog of Las1L, was proposed to function as a transcription factor (Doseff and Arndt, 1995) and was recently shown to localize at heterochromatic regions (Kitano et al., 2011). Moreover, in human cells, Las1L was shown to localize in the nucleolus and to be essential for cell proliferation and ribosome biogenesis (Castle et al., 2010).

Interestingly, in yeast the proteins Rix1, Ipi1 and Ipi3 (S. cerevisiae) / Crb3 (S. pombe) share conserved regions with Pelp1, Tex10 and Wdr18, respectively. They are components of the IPI complex which is involved in ribosomal RNA processing (Krogan et al., 2004). In a recent study it was shown that the Las1 and Grc3 proteins, the yeast orthologs of Las1L and Nol9 respectively, were associated with the IPI complex (Kitano et al., 2011). The Nol9 protein was one of the most abundant proteins in our mass spectrometry analysis for the 5FMC complex components (Chapter 3) and was recently shown to localize in the nucleolus of human cells and to function in RNA processing (Heindl and Martinez, 2010). Moreover, all 5FMC components were recently linked to ribosome biogenesis in human cells (Finkbeiner et al., 2011). These data suggest an evolutionary conserved complex with a role in RNA processing. In contrast to the nucleolar localization and RNA processing function, 5FMC components (as mention previously in this chapter) were also detected in complexes which are important in transcription regulation. Components of the complex were initially identified in the mass spectrometry analysis of the WDR5-MLL1 complex, which regulates transcription activation through methylation of H3K4 (Dou et al., 2005). PELP1, TEX10, LAS1L and WDR18 where found in a mass spectrometry study of GT335, an antibody which specifically detect post-translational modified polyglutamate side chains. In this study it was proposed that polyglutamylation of Pelp1 could have a significant influence on its affinity for histone H3 and subsequently affects the acetylation level of histone H3. The glutamate-rich region of Pelp1 was shown to bind to the hypoacetylated histone H3

and protect it to be processed as a substrate for histone acetyltransferases (Choi et al., 2004; Kashiwaya et al., 2010). Finally, in a mass spectrometry analysis for proteins that are modified by SUMO-2, PELP1, SENP3, LAS1L and TEX10 were also detected. As mention in the introduction (Chapter 1), sumoylation is important for stabilization and recruitment of repressive complexes such as CoREST, SetDB1 and NuRD (Garcia-Dominguez and Reyes, 2009) indicating desumoylation of co-repressors and transcription factors is important for gene activation. Indeed, in Chapter 3 we report that the 5FMC complex acts as a desumoylating complex recruited by Chtop, needed for subsequent derepression.

In Chapter 2 we demonstrate that Chtop strongly associates with chromatin, while in Chapter 3 we show that the 5FMC complex mainly localizes in the nucleoplasm. The interaction of Chtop and 5FMC complex may be transient in order for the 5FMC complex to desumoylate Chtop's targets. Moreover, it is very likely the possibility that the 5FMC complex can act outside the chromatin and/or independent of Chtop, for example in ribosome biogenesis.

In Chapter 4 we studied the role of Las1L and the other components of 5FMC complex in cell cycle progression. Las1L shows a different subnuclear localization between human and mouse cells. In human cells it is mainly localized in the nucleolus, as also shown by other studies (Andersen et al., 2002; Castle et al., 2010; Finkbeiner et al., 2011). However, in mouse cells it localizes to the nucleoplasm, while it is almost undetectable in the nucleolus. This might be due to differences in the amino acid sequence of the proteins of the two species, although they are quite homologous. When GFP-fusions of human LAS1L were expressed in mouse cells, LAS1L was only partially transported to the nucleolus in a minority of the cells. This may be due to overexpression of the protein. Alternatively, the differences in localization may be due to different protein-protein interactions between human and mouse LAS1L or that the nucleolar trafficking system may be different between human and mouse cells.

Knock down of different 5FMC components resulted in similar phenotypes.

The cells are arrested in the G2/M cell cycle phase, which subsequently leads to apoptosis. Further investigation of LAS1L knockdown cells for different cell cycle markers revealed that the cells are specifically arrested in anaphase. This observation is in agreement with the midbody localization of LAS1L at the cytokinesis phase (Chapter 4). Also the inactivation of LAS1 in yeast results in metaphase defect with large cells that accumulate vesicles in the mother-daughter neck (Doseff and Arndt, 1995). Moreover, SENP3 was identified in a siRNA screen as a potential component of the spindle assembly checkpoint, indicating that it might be involved in the control of mitotic processes (Stegmeier et al., 2007). Taken together, these data strongly suggest that the 5FMC complex is required for proper mitosis/cytokinesis. We hypothesize that desumoylation of one or more substrates is essential for proper mitosis. In such a scenario, Pelp1 may guide the 5FMC complex to such substrates, as it has a high affinity for Sumo2/3 (Rosendorff et al., 2006) followed by desumoylation by Senp3. Additional studies will be important to understand the mechanisms underlying the involvement of the 5FMC complex in transcriptional and cell growth regulation.

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Summary
Samenvatting

SUMMARY

Post-translational modification of proteins is an important mechanism to regulate several cellular processes such as DNA repair and replication, RNA metabolism, cell cycle progression, nuclear-cytoplasmic trafficking and transcriptional regulation. One of the most common modifications is the methylation of arginine residues which is catalyzed by members of the protein arginine methyltransferase (Prmt) family. Prmt1, the major Prmt enzyme, is ubiquitously expressed in mammalian cells and plays a role in many cellular processes, especially in RNA processing, DNA repair, and transcriptional regulation. Little is known the regulation of its enzymatic activity and its substrate specificity. The identification of new Prmt1-interacting proteins can give further insight into the molecular mechanisms that control Prmt1 and/or are controlled by Prmt1.

In **Chapter 2**, we demonstrate the single-step isolation of Prmt1-interacting proteins using a biotinylation-proteomics approach. The identified candidates were predominantly RNA-binding proteins (RBPs) involved in RNA processing, RNA stability, RNA export and ribosome synthesis. Apart from the RBPs we identified Chromatin target of Prmt1 (Chtop), referred as Friend of Prmt1 (Fop), a newly Prmt1-interacting protein which is encoded by the mouse 2500003M10Rik and human C1orf77 genes, respectively. Chtop is a 27 kDa protein and highly conserved in all vertebrates. It has no known domains other than a central glycine-arginine-rich (GAR) domain that contains 26 RG/GR repeats. We show that Chtop is methylated both asymmetrically by Prmt1 and symmetrically by Prmt5. We demonstrate that Chtop is not a component the Prmt1 holoenzyme complex. Instead, we found that Chtop is a chromatin associated protein and show that this association does not depend on its asymmetrical methylation. We further demonstrate that Chtop is required for the estrogen-induced expression of ERα target genes and for the binding of ERα to the promoter of the pS2 gene.

Next, we studied the function of Chtop methylation. Since arginine methylation often regulates protein-protein interactions, we looked for Chtop-interacting

proteins in the absence and presence of Prmt1 using the biotinylation-proteomics approach. In **Chapter 3**, we describe the isolation and characterization of the Five Friends of Methylated Chtop (5FMC) complex. 5FMC is a novel nuclear complex that is composed of Pelp1, Senp3, Las1L, Tex10 and Wdr18 and is recruited only to Chtop when Chtop is methylated by Prmt1. Pelp1, a known transcriptional co-activator, serves as the scaffold for the other 5FMC components and is essential for the stability of the complex. We show that 5FMC complex is recruited to the transcription factor Zbp-89 and that Chtop is required for this association. It was already known that Zbp-89 can be post-translationally modified by SUMO, thereby negatively regulating its transcriptional potential. We show that recruitment of the 5FMC complex results in higher Pol II levels on the promoters of Zbp-89 target genes, most likely as a result of the desumoylation of Zbp-89 by Senp3.

In **Chapter 4**, we further studied the function(s) of 5FMC components. We describe the localization of Las1L in human and mouse cells, as well as its role in cell cycle control. In human cells, Las1L shows nuclear and nucleolar localization while in mouse cells is localized only to nucleus. Moreover, Las1L is localized in the midbody region at the last stage of cytokinesis in both mouse and human cells. Depletion of Las1L results in a G2/M cell cycle phase arrest. Cells accumulate in late anaphase, resulting in multinucleation followed by increased cell death. Depletion of other components of the 5FMC complex (Pelp1, Senp3 and Wdr18) has similar effects. The critical role for these factors was further demonstrated by the fact that we could not obtain mice homozygous for a ‘trapped’ Wdr18 gene.

SAMENVATTING

De posttranscriptionele modificatie van eiwitten is een belangrijk mechanisme om cellulaire processen, zoals DNA repair, replicatie, RNA metabolisme, de celcyclus, nucleair-cytoplasmatisch transport en transcriptie regulatie, te reguleren. Een veel voorkomende modificatie is de methylering van arginine residuen. Deze reactie wordt gekatalyseerd door leden van de Protein aRginine MethylTransferse (Prmt) familie. Prmt1, het “belangrijkste” lid van de familie, komt tot expressie in alle zoogdiercellen en speelt een rol in verschillende cellulaire processen, in het bijzonder in de verwerking van RNA, DNA repair en transcriptie regulatie. Er is weinig bekend over de regulatie van de enzymactiviteit en substraatspecificiteit van Prmt1. Het identificeren van nieuwe Prmt1-bindende eiwitten verschaft hoogstwaarschijnlijk meer inzicht in de moleculaire mechanismen die Prmt1 reguleren en/of door Prmt1 gereguleerd worden.

In **hoofdstuk 2** beschrijven we de isolatie van Prmt1-bindende eiwitten. Door gebruik te maken van de biotinylerings-proteomics techniek konden deze eiwitten in één stap worden gezuiverd. De geïdentificeerde kandidaten waren vooral RNA-bindende eiwitten die betrokken zijn bij de verwerking, stabiliteit en export van RNA en de aanmaak van ribosomen. Naast deze RNA-bindende eiwitten vonden we ook Friend of Prmt1 (Fop), een nieuwe Prmt1 partner waarvan recent de naam is veranderd naar Chromatin target of Prmt1 (Chtop). Het gen voor Chtop in de muis heet 2500003M10Rik, het humane gen is C1orf77. Chtop is ~27 kDa en geconserveerd in alle vertebraten. Het heeft geen bekende eiwit domeinen, behalve een centraal gelegen “glycine-arginine-rich” domein dat 26 RG/GR motieven bevat. We laten zien dat Chtop wordt gemethyleerd door Prmt1 (asymmetrisch) en door Prmt5 (symmetrisch), maar Chtop is geen onderdeel van het Prmt1 holoenzyme complex. Daarentegen bindt Chtop sterk aan chromatine en deze interactie is onafhankelijk van de methylering door Prmt1. Verder demonstreren we dat Chtop nodig is voor estrogen-geïnduceerde expressie van ERα target genen en voor de binding van ERα aan de promoter van het pS2 gen.

Vervolgens hebben we de rol van de methylering van Chtop bestudeerd. Omdat methylering van arginine residuen vaak een rol speelt in de regulatie van eiwit-eiwit interacties, hebben we gekeken welke eiwitten aan Chtop kunnen binden, zowel in de aan- als afwezigheid van Prmt1. In **hoofdstuk 3** beschrijven we de isolatie en karakterisering van het Five Friends of Methylated Chtop (5FMC) complex. 5FMC is een nucleair complex dat bestaat uit de Sumo-protease Senp3 en de eiwitten Pelp1, Las1L, Tex10 en Wdr18. Dit complex bindt alleen aan Chtop als Chtop is gemethyleerd door Prmt1. Pelp1, eerder beschreven als transcriptionele coactivator, is essentieel voor de stabiliteit van het complex. We laten zien dat 5FMC wordt gerekruteerd naar Zbp-89, een zinc-finger transcriptie factor, en dat Chtop belangrijk is voor deze interactie. Het was al bekend dat Zbp-89 geSUMOyleerd kan worden, een posttranslationele modificatie met een remmende werking op de transcriptionele activiteit van Zbp-89. Wij laten zien dat het rekruteren van 5FMC resulteert in de deSUMOylering van Zbp-89, resulterend in hogere Pol II binding aan Zbp-89 target genen.

In **hoofdstuk 4** gaan we verder met het bestuderen van een aantal 5FMC eiwitten. We beschrijven de lokalisatie van Las1L in humane – en muizencellen en de rol van Las1L in de cel cyclus. In humane cellen is Las1L gelokaliseerd in de kern, met name in de nucleolus, terwijl in muizencellen Las1L wel in de kern, maar juist niet in de nucleoli aanwezig is. Tijdens cytokinese, de laatste fase van de celdeling, bevindt Las1L zich in de midbody, zowel in humane – als muizencellen. Verminderde expressie van Las1L leidt tot een sterke vertraging van de cel cyclus. De cellen hebben moeite om door de anphase/telophase heen te komen, wat resulteert in meerdere kernen per cel en/of celdood. Als het eiwit niveau van andere 5FMC componenten (Pelp1, Senp3 and Wdr18) wordt geremd, worden vergelijkbare effecten op de cel cyclus waargenomen. Een essentiële rol voor deze eiwitten wordt verder onderstreept door het feit dat we niet aan staat zijn om muizen/embryo's/cellijnen te verkrijgen die homozygoot zijn voor een "knockout" allel van het Wdr18 gen.

Curriculum Vitae

Personal details

Name:	Pavlos Fanis
Birth date:	19-01-1984
Birth place:	Limassol, Cyprus

Education and research

2007-2011	Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands (Prof. dr. J.N.J Philipsen and Dr. T.B. van Dijk). PhD student: 'Functional proteomics analysis of transcription factor networks in erythroid cells'.
2003-2007	Democritus University of Thrace, Department of Molecular Biology and Genetics, Alexandroupolis, Greece. BSc in Molecular Biology and Genetics.
2007	Molecular Genetics Thalassaemia Department, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus. Diploma Thesis title: 'Development of non-invasive prenatal method for detection of the Y chromosome from free fetal DNA found in maternal plasma'.
1998-2001	"Archbishop Makarios III" Lyceum, Paphos, Cyprus. High school education.

List of publications

Esteghamat, F., van Dijk, T.B., Braun, H., Dekker, S., van der Linden, R., Hou, J., **Fanis, P.**, Demmers, J., van Ijcken, W., Ozgur, Z., et al. (2011). The DNA binding factor Hmg20b is a repressor of erythroid differentiation. Haematologica 96, 1252-1260.

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Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Pavlos Fanis Erasmus MC Department: Cell Biology Research School: MGC graduate school	PhD period: October 2007 – October 2011 Promotors: Prof.dr. Sjaak Philipsen Prof.dr. Frank Grosveld Copromotor: Dr. Thamar B. van Dijk

1. PhD training

General courses	**Year**	**Workload (Hours)**
- Reading and discussing literature course	2008	56
- Course on laboratory animal science (Article 9)	2008	108
- Experimental Approach to Molecular and Cell Biology	2008	168
- Safety working in the laboratory	2009	8
- Epigenetic regulation	2010	16
- EuTRACC proteomics Course	2010	18
- Biomedical English writing and Communication	2010	84
- From Development to Disease	2011	32
Specific courses		
- 7th Winter School of the International Graduiertenkolleg GRK767 "Transcriptional Control in Developmental Processes" Kleinwalsertal, Germany (oral presentation)	2009	72
- 1st Winter School of the Collaborative Research Centre TRR81, "Chromatin Changes in Differentiation and Malignancies" Kleinwalsertal, Germany (oral presentation)	2011	72
Seminars and workshops		
- MGC PhD Workshop (poster presentation)	2009	32
- The 19th MGC-Symposium, Rotterdam, the Netherlands	2009	12
- Browsing genes and genomes with Ensemble workshop	2010	16
- The 20th MGC-Symposium, Leiden, the Netherlands	2010	12
- Workshop on InDesign CS5	2011	4
- The 21th MGC-Symposium, Leiden, the Netherlands	2011	12
- Erasmus lectures in cell biology and development	2007-2011	60
- Erasmus lectures on stem cell and regenerative medicine	2007-2011	45
- Monday Morning Meetings	2007-2011	176

Presentations		
- Monday Morning Meetings - Work discussion	2007-2011 2007-2011	320 240
International conferences		
- EUrythron annual meeting, Groningen, the Netherlands	2008	24
- Hemoglobin Switching meeting, Asilomar, CA, USA (poster presentation)	2008	80
- EUrythron annual meeting, Lisbon, Portugal (oral presentation)	2009	64
- Hemoglobin switching meeting, Oxford, UK (poster presentation)	2010	80
- Chromatin changes in differentiation and malignancies, Giessen, Germany (poster presentation)	2011	56
2. Teaching		
Supervising practicals and excursions, Tutoring		
- High school students	2008	40
- Master thesis student	2009	120

ACKNOWLEDGEMENTS

During these years as a Phd student in Erasmus MC I have had a great time, have learnt a lot, scientifically and personally, and have met a lot of special people that I would like to thank.

First, I would like to thank Prof. dr. Sjaak Philipsen. Sjaak thank you for your trust and for your continuous support. I am glad that I was lucky to perform research in your lab. I have learnt a lot and really appreciated all the remarks that you have made about my work throughout these four years.

Big thanks to Prof. dr. Frank Grosveld giving me the opportunity to study in an inspiring and challenging environment, supporting my work and always be available for scientific discussions.

Thamar my copromotor and daily lab supervisor it will be very tough to summarize these four years being your Phd student. You have trusted me from the first day to the very last. You were always supportive and available when I needed you. Thank you also for been so helpful in the last stages of the thesis writing. I have learnt an incredible amount of things from you. Wish you a brilliant scientific career and personal life with your family.

Prof.dr. Peter Verrijzer, Dr. Joost Gribnau, Dr.Ruud Delwel, my small committee thank you for reading my thesis, your understanding and your comments.

I must also thank the members of the plenary doctoral committee. Prof. dr. Dies Mejers and Prof. Dr. Uta-Maria Bauer (thank you Uta for coming from Marburg for my defence).

I am thankful to people of my lab. Nynke, Teus. Sylvia. Sahar, Rejane, Jun, Divine, Anna, Maria and Ileana it has been a wonderful time being with you in lab 716. Everybody was very helpful and each one so special. Nynke, Sylvia I appreciate your help throughout all these years. Sahar your presence in the lab create a cheerful atmosphere. Rejane good luck with your thesis writing. Jun, Divine, Anna I enjoyed our discussions. Maria thank you for helping me with the cover layout and for re-

discovering Holland.
Marike, Jasperina, Bep, Melle, Arthur and the “computer guys” thank you for all the great assistance and cooperation.
Jeroen and Karel thank you for analyzing all the time my mass-spec samples and giving me a lot of interesting results.
Thanks to all the colleagues at Erasmus MC that made my life here more pleasant: Dubi (good luck with your Greek lessons), Rick, Alex, Ali (Imam), Rien, Mariette (many thanks for helping me with the confocal microscope), Raymond, Umut, Eric, Debbie, Tiago, Ernie, Sofia (good luck and good start for your Phd in Leiden), Robert-Jan, Eric S., Charlotte, Xiao, Andrea, Mary, Ruud, Anita, Ralph, Farzin, Ali, Petros (enjoyed our conversations about many things), Catherine, Reinier, Gerben, Alvin, Parissa, Dorota, Michaela, Parham, Kris, Nesrin, Jean-Charles, Thomas, Niels, Jeffrey, Jessica, Frank S., Kerstin. For the people that are not mentioned here and they are many, thank you all for the good times we had together in and out of the lab.
Big thanks to my two paranimfs Polynikis and Alireza who helped me to complete many of my thesis tasks but most importantly I thank them for being my friends.
And last, but not least, I would like to thank my parents, my brothers and my sister. Thank you for everything, without you I would not be able to do anything. Thank you for all your love, support, encouragement and believing in me during all my life.

Pavlos