The Repo-Man/PP1 complex role in chromatin remodelling, nuclear structure and cancer progression

A thesis submitted for the degree of Doctor of Philosophy

by

Ezgi Gokhan

College of Health and Life Sciences

Brunel University London

December 2016

Decleration

I hereby declare that the work presented in this thesis is my own work, unless otherwise specified, and has not been submitted for any other degree.

Ezgi Gokhan

Acknowledgements

It has probably been the toughest 3 years of my life with all the ups and downs, all the tears and anger as well as those short precious moments of laughter and fun. Having moved from another country all by myself, I faced it alone most of the time but of course there were some people without whom, I would be lost. I know this is not enough to express how grateful I am to have them in my life, but this is probably the most amazing achievement of my life and I want to thank them with all my heart by sharing this with them.

To the best supervisor I could ever have in the whole wide world, Dr. Paola Vagnarelli: Thank you for being there for me whenever I needed. For believing in me even when I did not, and for always showing me the right way. Thanks for being not only my supervisor but my friend and listening to me whenever I needed to talk to someone. You were probably the only person who could understand how I feel by just looking at my face and most importantly you cared about my mental well-being more than you cared about the experiments. Thanks for always pushing me and teaching me not to give up. You have witnessed and helped me grow up in the last 3 years and I am thankful for the relationship we developed over the years. I could not have done this without your guidance and patience. I do not know how many times I failed and did stupid mistakes but you never gave up on me. I am honoured to be your PhD student and I will remember your advice and dedication all my life. Lastly, I am sorry for random breakdowns and tears in your office and I will miss the lab meetings (especially the food), the office parties and seeing you at the corridor asking me for data with excitement. Thank you for everything.

To the two most important people to me in the world, my parents: Thank you for your support not just through my PhD but all my life. You have always encouraged me to

follow my dreams and never complained about the choices I made. When I came to you 3 years ago to say that I wanted to move to the UK, you did not even think twice. I can only imagine how it feels like to send your only daughter to another country and I know it has been very hard both financially and mentally but you kept supporting me no matter what. I know you have given up so many things so that I could do my PhD and for that I will always be grateful. You have taught me to follow my dreams and not be afraid no matter what. I know I have sacrificed my time with family for the last 3 years but I promise this will all be worth it. Your little girl has grown up and from now on she will take care of you.

To the craziest and the weirdest group of people I know: Haroon, Chrissi and Sheila. Guys I don't even know what to say to you other than thanks for making my life even crazier than it was, because I needed that (sarcasm in case you didn't get it!). I will miss the long hours of procrastination called "getting coffee" and making plans that we could never follow. Let's not forget the shisha sessions that were totally awkward thanks to the weirdest place in Uxbridge with the slowest staff in the world. Sheila, your good heart and sense of humour amazes me. You are one powerful woman! Haroon, I would put a nice brown, polished, fresh emoji here (you know what it is) but this is my PhD thesis so better not! Instead, send me a fist bump when you read this and that will do. Chrissi, you are welcome for the advice and wisdom I gave you. Seriously what would you do without me! I love the fact that when you say 5min it's actually 30min and that you are mostly late and that you know way too many people in the department. I will miss that when you say we're going for dinner you actually mean 20 other people because you cannot say no (no I won't miss that). Your heart is so big and so pure that you only see the best in people and that's what I love

about you. You are one amazing bunch of weirdos and I know I have you guys for life. Thanks for making this PhD a bit more bearable.

To my sister from another mother, Muge Erdem: Can you believe it has been 8 years since we met? The moment we met we became inseparable to the point that people thought we were twins and the lecturers mixed our names and even exam papers. It is true that most of the time you drive me crazy because you're one stubborn, crazy person but I wouldn't have it any other way. It has been 4 years that we have been apart, and we have maybe seen each other just a couple of times during this period but I knew the distance could never be a problem for us. I knew that whenever I needed I would find you at the other end of the phone no matter what. I don't know what I would do without our 5hr conversations. Believe me there have been so many times I wanted you to be by my side during the last 3 years. I miss you so much and you know you will always be my best friend and the sister I never had. Thanks for your support and I know you will be there always and forever.

To Sebnem and Zeynep, for being my family away from home and for always welcoming me whenever I needed a change of place. Sebnem, thank you for your advice and for opening your house to me. Zeynep, thank you for being the little sister I never had. I will always be here for you whenever you need.

To the craziest colleagues I could possibly have, Ines and Lorena. Thank you for making long hours in the lab a lot more bearable. It was nice to know I wasn't the only crazy person around. I will miss our transformation from quiet scientists to crazy ones after 5pm. Thanks for all your help. Lorena, I will miss taking the night bus at 4am with you. I will miss all the crazy adventures we had. We should definitely have more of those. If one day you

have a bakery, I am willing to work as a waitress, entertainer or whatever you need (just keep in mind). I will miss your amazing cakes and for your next birthday I'm thinking of buying you your very own, personal tissue culture hood, so that you can work from home!

To Sarah, for being an amazing office buddy and listening to my problems. I know we are going to be very good friends and imagine what we can do when we finish our PhDs. By the way I'm sorry to remind you that you're next but I can't wait to graduate together. Don't forget, I have to be the first person to call when you need a babysitter in the future (you know ginger babies are my specialty). As always, have a bunny day and thank you for all your support.

To Becky, Sheba and Piyali, thanks for the craziest nights. I feel like another one is approaching soon. It is always so much fun making plans and ending up somewhere entirely different and not remembering what happened (I still have the picture, you know which one!). Most importantly, thanks for always supporting and guiding me. You are real friends and I am lucky to have met all of you. Cocktails for life!

To the members of the "Turkish gang" Samet and Ural (aka Mehmet): Thanks for being there for me whenver I needed. It is always nice to have people around from your home country that you can speak the same language with in case of emergencies. We will definitely continue having our lovely dinners and night outs.

Publications

Kumar, G. S., Gokhan, E., De Munter, S., Bollen, M., Vagnarelli, P., Peti, W., Page, R., 2016. The Ki-67 and RepoMan mitotic phosphatases assemble via an identical, yet novel mechanism. *Elife. doi: 10.7554/eLife.16539.*

de Castro, I.J., Gokhan, E. & Vagnarelli, P., 2016. Resetting a functional G1 nucleus after mitosis. *Chromosoma*. Available at: http://link.springer.com/10.1007/s00412-015-0561-6.

de Castro, I. J., Budzak, J., Di Gacinto, M. L., Ligammari, L., Gokhan, E., Spanos, C., Moralli, D., Richardson, C., de las Heras, J. I., Salatino, S., Schrimer, E. C., Ullman, K., Bickmore, W., Green, C., Rappsilber, J., Lamble, S., Goldberg, M. W., Vinciotti, V., & Vagnarelli, P., 2016. Repo-Man/PP1 regulates heterochromatin formation in interphase. *Nature Communications.*

List of figures

Figure 1.1: Cell cycle and checkpoints explained……6

Figure 1.2: Structure of PP1……15

Figure 1.3: Structure of PP2A……18

Figure 1.4: The PP1-PP2A phosphatase relay at mitosis……23

Figure 1.5: The components and mechanism of SAC……27

Figure 1.6: CPC components and assembly mechanism……30

Figure 1.7: CPC-Haspin-H3T3ph feedback loop……33

Figure 1.8: Repo-Man localisation throughout the cell cycle……39

Figure 1.9: A diagram showing the regulation of Repo-Man/PP1 complex throughout mitosis……40

Figure 1.10 A diagram showing Repo-Man binding domains……43

Figure 1.11: : Structure and layers of the nuclear envelope……46

Figure 3.1: Repo-Man does not dimerize……88

Figure 3.2: First crystal structure of Repo-Man/PP1 complex and identification of a novel interaction site……90

Figure 3.3: Oligo resistant Repo-Man is still expressed in the presence of Repo-Man siRNA……93

Figure 3.4: Phospho-deficient mutations on newly identified sites cause pre-mature localisation of Repo-Man on chromosomes……94

Figure 3.5: Remaining H3T3 phosphorylation overlap with the centromeres. HeLa cells……95

Figure 3.6: PP1 binding is important for Repo-Man localisation on chromosomes……98

Figure 3.7: Phospho-mimetic mutations on Repo-Man, inactivate the complex by abolishing PP1 binding…….100

Figure 3.8: Phospho-mimetic mutants do not affect localisation or H3T3 phosphorylation……………………………………………………………………………………………………..101

Figure 3.9: Phosphorylation of T394 by Aurora B abolishes the binding between Repo-Man and PP1……103

Figure 3.10: Phospho-deficient mutations on Plk1 sites do not affect localisation but makes a difference with H3T3 phosphorylation………………………………………………………………………..106

Figure 3.11: Plk1 phosphorylates Repo-Man and maintains H3T3 phosphorylation at the centromeres……..107

Figure 3.12: Alignment of PP1γ and PP1α protein sequences…………………………………………110

Figure 3.13: R20Q mutation on PP1γ abolishes the localisation and prevents Repo-Man from recruiting PP1………111

Figure 3.14: Ki67 does not recruit mutant PP1γ……………………………………………………………..112

Figure 3.15: Working model………………………………………………………………………………………..116

Figure 4.1: Repo-Man knock-down affects cell circularity through Lamin A/C…………………121

Figure 4.2: Phospho-Lamin A/C localisation throughout cell cycle…………………………………124

Figure 4.3: Repo-Man knock-down causes increased phospho-Lamin A/C levels at interphase………..125

Figure 4.4: Repo-Man sumo mutant IRCE shows wild type-like localisation…………..............127

Figure 4.5: IRCE mutant cannot rescue phospho-LaminA/C levels at interphase……………129

Figure 4.6: Repo-Man sumoylation cannot be detected in-vitro……………………………………130

Figure 4.7: Repo-Man interacts with phospho-Lamin A/C at interphase and the sumo mutant IRCE decreases this interaction……………………………………………………………………………………133

Figure 4.8: Working model…………………………………………………………………………………………..137

Figure 5.1: Repo-Man is overexpressed in high grade aggressive breast cancer cell lines and decreases survival rates of breast cancer patients………………………………………………………..144

Figure 5.2: Repo-Man is overexpressed in triple negative breast cancer………………………..145

Figure 5.3: Repo-Man knockdown decreases CA1h and HCC1143 cells motility………………149

Figure 5.4: Repo-Man affects Aurora A levels through its inhibitor AURKAIP1…………………152

Figure 5.5: Repo-Man controls C-Myc levels in malignant and triple negative breast cancer cells……….154

Figure 6.1: Overall schematic review of Repo-Man regulation along with Haspin and Aurora B kinases………..166

Figure 6.2: The overall working model……………………………………………………………………………..173

List of tables

Table 1.1: Important histone marks and their significance……………………………………………….35

Table 2.1: Components of the PCR mix…………………………………………………………………………….57

Table 2.2: Parameters for PCR……………………………………………………………………………………………58

Table 2.3: Components of the recombination reaction mix……………………………………………….58

Table 2.4: List of the mutations performed……………………………………………………………………….59

Table 2.5: List of oligonucleotide sequences used for mutation analyses…………………………….60

Table 2.6: List of siRNA sequences used in transfection experiments………………………………….61

Table 2.7: List of antibodies used for immunostaining experiments…………………………………….65

Table 2.8: List of drugs used for immunostaining experiments…………………………………………..65

Table 2.9: PCR ingredients and concentrations…………………………………………………………………66

Table 2.10: PCR parameters for cloning experiments……………………………………………………….67

Table 2.11: List of oligonucleotides used for cloning experiments……………………………………..67

Table 2.12: Reagents for pGEM®-T Easy vector system ligation reaction……………………………69

Table 5.1: List of cell lines used for western blot experiments to detect Repo-Man levels………143

Abbreviations

ACA – Anti-centromeric antibody

AKAP149 – A kinase anchoring protein 149

APC – Anaphase promoting complex

APS – Ammonium persulfate

Arg/R – Arginine

arpp19 - cAMP-regulated phosphoprotein 19

ATM – Ataxia-telangiectasia mutated

ATP – Adenosine triphosphate

BAF - Barrier to autointegration factor

Bcl-2 - B-cell lymphoma 2

BSA – Bovine serum albumin

CaCl2 – Calcium chloride

CDK – Cyclin dependent kinase

CENP-A/E/B – Centromeric protein A/B/E

CPC – Chromosome passenger complex

DARPP32 - dopamine- and cAMP-regulated neuronal phosphoprotein 32

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleotide

E.Coli - Escherichia coli

ECL - Enhanced chemiluminescence

ensa - Alpha-endosulfine

ER – Oestrogen receptor

G1/G2/G0 – Gap 1/2/0

GADD34 - Growth arrest and DNA damage-inducible protein

GFP – Green fluorescent proteinMYPT1 - Myosin phosphatase target subunit 1

GST - Glutathione S-transferas

GTP – Guanosine triphosphate

HER2/neu - Human epidermal growth factor receptor 2

HP1 – Heterochromatin protein 1

I-2 – Inhibitor 2

IDPs/IUPs – Intrinsicly disordered/unstable proteins

INM – Inner nuclear membrane

IPTG - Isopropyl β-D-1-thiogalactopyranoside

KAc – Potassium acetate

KCl – Potassium chloride

KIF14 - Kinesin Family Member 14

KNL1 - kinetochore null protein 1

LAP1/2 – Lamina associated protein 1/2

LB – Luria bertani

LBR – Lamin binding receptor

Lys/K – Lysine

M – Mitosis

MAPK – Mitogen activated protein kinase

MASTL - Serine/threonine-protein kinase greatwall

MCC – Mitotic checkpoint complex

MEN – Mitotic exit network

Met/M – Methionine

$MgCl_2$ – Magnesium chloride

$MgSO_4$ – Magnesium sulfate

MIS12 - Mis-segregation 12

$MnCl_2$ – Manganese chloride

MOPS - 3-(N-morpholino)propanesulfonic acid

mTOR - Mechanistic target of rapamycin

MYPT1 - Myosin phosphatase target subunit 1

NaCl – Sodium chloride

NDC80 - Nuclear division cycle 80

NE – Nuclear envelope

NEBD – Nuclear envelope breakdown

NMR - Nuclear magnetic resonance

NPC – Nuclear pore complex

OD – Optimal density

ONM – Outer nuclear membrane

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

Phe/F – Phenylalanine

PI3K – Phosphatidylinositol 3 kinase

PLA – Proximity ligation assay

Plk1 – Polo-like kinase 1

PNUTS - Serine/threonine-protein phosphatase 1 regulatory subunit 10

PP1 – Protein phosphatase 1

PP2A – Protein phosphatase 2A

PPMT – Protein phoshatase methyltransferase

PR – Progesterone receptor

Pro/P – Proline

PTPs – Protein tyrosine phosphatases

PVDF - Polyvinylidene Fluoride

R5/PTG - Glycogen targeting subunit

RanGAP1 - Ran GTPase Activating Protein 1

Rb – Retinoblastoma

RbCl – Rubidium chloride

RCA – Regulator of chromatin environment

Repo-Man (CDCA2) – Recruits PP1 onto mitotic chromatin (Cell division cycle associated 2)

RNA – Ribonucleic acid

RNAi – RNA interference

ROI - Region of interest

S – Synthesis

SAC – Spindle assembly checkpoint

SDS22 - Protein phosphatase 1 regulatory subunit SDS22

SDS-PAGE - Polyacrylamide gel electrophoresis

Ser/S – Serine

SIM – SUMO interacting motif

siRNA – Small interfereing RNA

SOC – Super optimal broth

SS – Synovial sarcoma

SUMO – Small ubiquitin-like modifiers

TEMED – Tetramethylethylenediamine

Thr/T – Threonine

TNBC – Triple negative breast cancer

Try/Y – Tyrosine

Contents

Abstract 1

1. INTRODUCTION 2

1.1 Cell Cycle 23

1.1.1 Role of kinases and phosphatases 27

1.1.2 From mitosis to G1 30

1.2 Ser/Thr phosphatases 32

1.2.1 Structure and function of PP1 33

1.2.2 Structure and function of PP2A 37

1.3 Important players of mitotic exit 40

1.3.1 PP1 and PP2A at mitotic exit 40

1.3.2 Spindle assembly checkpoint (SAC) 44

1.3.3 The chromosome passenger complex (CPC) 48

1.4 Histone modifications 54

1.5 Function and regulation of Repo-Man 57

1.5.1 Repo-Man as a PP1 targeting subunit 57

1.5.2 Regulation of Repo-Man throughout mitosis 61

1.6 Nuclear envelope dynamics during mitosis 44

1.6.1 Nuclear envelope proteins 44

1.6.2 Repo-Man and nuclear envelope 47

1.6.3 Regulation of Lamin A/C throughout the cell cycle 49

1.7 Repo-Man and cancer 52

2. MATERIALS AND METHODS 55

2.1 Tissue Culture 56

2.2 Site Directed Mutagenesis 57

2.3 Transfections 61

2.3.1 Transfections using jetPRIME® reagent 61

2.3.2 Electroporation 62

2.3.3 Generating stable cell lines 63

2.4 Immunofluorescence 64

2.5 Cloning 66

2.5.1 Primer design and PCR 66

2.5.2 Cloning into pGEM®-T Easy vector system 68

2.5.3 Cloning from pGEM®-T Easy vector system into another plasmid 70

2.6 Western Blotting 71

2.6.1 Solutions and reagents 71

2.6.2 SDS-PAGE gel electrophoresis, transfer onto membrane and visualization 73

2.7 In-vitro sumoylation assay 74

2.7.1 Protein expression and purification from bacteria 74

2.7.2 In-vitro sumoylation reaction 76

2.8 Proximity ligation assay 78

2.9 Fluorescence microscopy and analysis 79

2.10 Preperation of competent E.Coli cells 80

2.10.1 Solutions and media 80

2.10.2 Preperation 81

2.11 Other solutions 82

3. STRUCTURE AND REGULATION OF REPO-MAN/PP1 COMPLEX 83

3.1 Introduction 84

3.2 Results 86

3.2.1 Identification of novel sites on Repo-Man 86

3.2.2 Mutations on identified sites affect Repo-Man localisation on chromosomes 91

3.2.3 PP1 binding is important for Repo-Man localisation on chromosomes 96

3.2.4 Phosphorylation of the newly identifies Repo-Man SLIM motif regulates PP1 binding. 99

3.2.5 Phosphorylation of the RVTF motif abolishes the binding between Repo-Man and PP1 103

3.2.6 Is Plk1 involved in the local regulation of Repo-Man/PP1 complex in early mitosis? 104

3.2.7 Repo-Man specifically binds to PP1γ and not PP1α 108

3.3 Discussion 113

4. REPO-MAN REGULATES LAMIN A/C DE-PHOSPHORYLATION 117

4.1 Introduction 118

4.2 Results 119

4.2.1 Repo-Man knockdown causes abnormal nuclear lamina that lack stiffness 119

4.2.2 Repo-Man knockdown affects Lamin A/C phosphorylation. 122

4.2.3 Sumoylation of Repo-Man is essential for Lamin A/C de-phopshorylation. 126

4.2.4 Sumoylation of Repo-Man is important for the interaction with Lamin A/C 131

4.3 Discussion 154

5. REPO-MAN/PP1 COMPLEX AS A THERAPEUTIC TARGET FOR TRIPLE NEGATIVE BREAST CANCER 158

5.1 Introduction 159

5.2 Results 161

5.2.1 Repo-Man is overexpressed in triple negative breast cancer 161

5.2.2 Repo-Man knock-down blocks wound healing ability in CA1h and HCC1143 166

5.2.3 Repo-Man knock-down decreases Aurora A levels in CA1h and HCC1143 cell lines 170

5.2.4 Repo-Man controls C-Myc levels in CA1h and HCC1143 cells 173

5.3 Discussion 175

6. DISCUSSION 178

6.1 Repo-Man binds to PP1 via a novel interaction motif: RepoSLIM 179

6.2 Repo-Man is regulated by multiple kinases via the novel RepoSLIM 182

6.3 Repo-Man selectively binds to PP1γ.. 187

6.4 Lamin A/C is a substrate of Repo-Man at interphase 189

6.5 Repo-Man could represent a therapeutic target for the treatment of triple negative breast cancer 171

Abstract

Repo-Man is a chromatin-associated PP1 targeting subunit that coordinates chromosome re-organisation and nuclear envelope reassembly during mitotic exit. At the onset of mitosis, Repo-Man association with the chromosomes is very dynamic; at anaphase, Repo-Man targets to the chromatin in a stable manner and recruits PP1 to de-phosphorylate histone H3 at Thr3, Ser10 and Ser28. Previous studies have suggested that CDK1 and AuroraB are the kinases responsible for the inactivation of the complex and for its dispersal at the onset of mitosis respectively. We have previously shown that the binding of Repo-Man to PP1 is decreased in mitosis and we have identified a region adjacent to the RVTF motif that contains multiple mitotic phosphosites (RepoSLIM). This region is conserved only in another PP1 targeting subunit: Ki-67. In order to understand the importance of this region for the complex formation and stability, we have conducted mutational analyses on several residues, and addressed their contribution towards Repo-Man chromosome targeting and PP1 binding in vivo. We have identified new sites in Repo-Man that, when phosphorylated, contribute to the weakening of the binding between Repo-Man and PP1. Interestingly, our results also indicate that several kinases are involved in the mitotic regulation of the complex. We have also identified Lamin A/C as a Repo-Man substrate and introduced a new model for Lamin A/C regulation at interphase. Furthermore, we identified Repo-Man as a marker of malignancy in tripe egative breast cancer, which controls cell movement and levels of important oncogenic markers Aurora A and C-Myc, and propose Repo-Man/PP1 complex as a therapeutic target for the treatment of triple negative breast cancer through the newly identified RepoSLIM.

1. INTRODUCTION

1.1 Cell Cycle

The term cell cycle summarizes the life of a cell and the events that take place during that time. It is defined as a cycle because a cell goes through the exact same process over and over again resulting in dividing and proliferating until the cell dies or exit it to become a differentiated or quiescent cell. The cell cycle is separated into several phases. These are G1, S, G2 phases, which are also collectively called the interphase, and M phase (Figure 1.1). These phases represent the growth and division of the cell. At interphase the cell grows, produces energy, handles transportations and other duties as well as transcription of proteins. The first phase within interphase is the G1 (Gap1), where the cell activity is at its highest as the majority of the cell growth happens at this stage. The second phase is the S phase, which stands for synthesis, where the DNA replication occurs. At the third phase, G2 (Gap 2), with the duplicated DNA the cell continues to grow and synthesise proteins to prepare for the M phase (Cooper, 2000).

M phase stands for mitosis and this is the stage where the cell divides. In mitosis all the genetic information is equally distributed between the two daughter cells. After mitosis, the two new daughter cells start their new cycle. M phase itself has several stages: prophase, where the replicated chromatin starts to condense and the nuclear lamina is dis-assembled; pro-metaphase, where the chromosomes are condensed and ready to form the metaphase plate, the mitotic spindle is formed; metaphase, where the chromosomes are aligned on the metaphase plate and fibres of the mitotic spindles are attached to each single chromatid at the kinetochore; anaphase, where the sister chromatids separate and move towards opposite spindle poles; telophase, where sister chromatids reach the poles and, nuclear lamina starts to reform and the cleavage furrow ingresses in the middle of the cell;

cytokinesis, where the division of the cytoplasm occurs and the daughter cells separate from each other completely (O'Connor, 2008).

Sometimes cells enter a phase called G0. This is the phase where the cell does not grow or proliferate and is called the resting phase or quiescence. This is usually due to lack of nutrients, oxygen or other environmental factors; this status is usually reversible and, when the necessary conditions are provided the cell can re-enter the cycle again. When the cell accumulates too much DNA damage or somehow loses its function it can enters a similar stage called senescence, where the cell does not proliferate and is committed to die (Sharpless and Sherr, 2015).

The cell cycle is a highly regulated and controlled process with monitoring stages to ensure that some events do not occur until the previous ones are successfully completed: these are called check points. There are many important events that need to be controlled within the cell cycle including DNA replication and mitosis. After division, the very first checkpoint is when the cell goes from G1 to S phase. This is when the cell decides if it is ready to commit to the DNA replication. In the presence of appropriate growth factors and nutrients the cell decides that it needs to divide in order to allow further growth. Another important checkpoint is at the G2, when the cell needs to make sure that the whole genome is replicated without any errors. This also prevents the cell from going through mitosis with a semi-replicated DNA. At this stage, any DNA damage is also detected and the cell repairs these errors before mitosis. If, in any way the damage is not repairable, the cell may go through apoptosis, a programmed cell death, in order not to carry the same error to the subsequent cell cycle (Figure 1.1).

After a successful DNA replication the cell prepares for mitosis at G2. During mitosis there is a very important checkpoint where the cell needs to make sure that each sister chromatid is successfully attached to the spindle fibres coming from opposite poles to ensure and equal distribution of the genetic information between the two daughter cells. If this attachment is not successful, it may result in lagging chromosomes and different chromosome numbers in the two daughter cells (explained in the SAC section). When all of these checkpoints are satisfied, the cell successfully divides and continues to grow (Cooper, 2000).

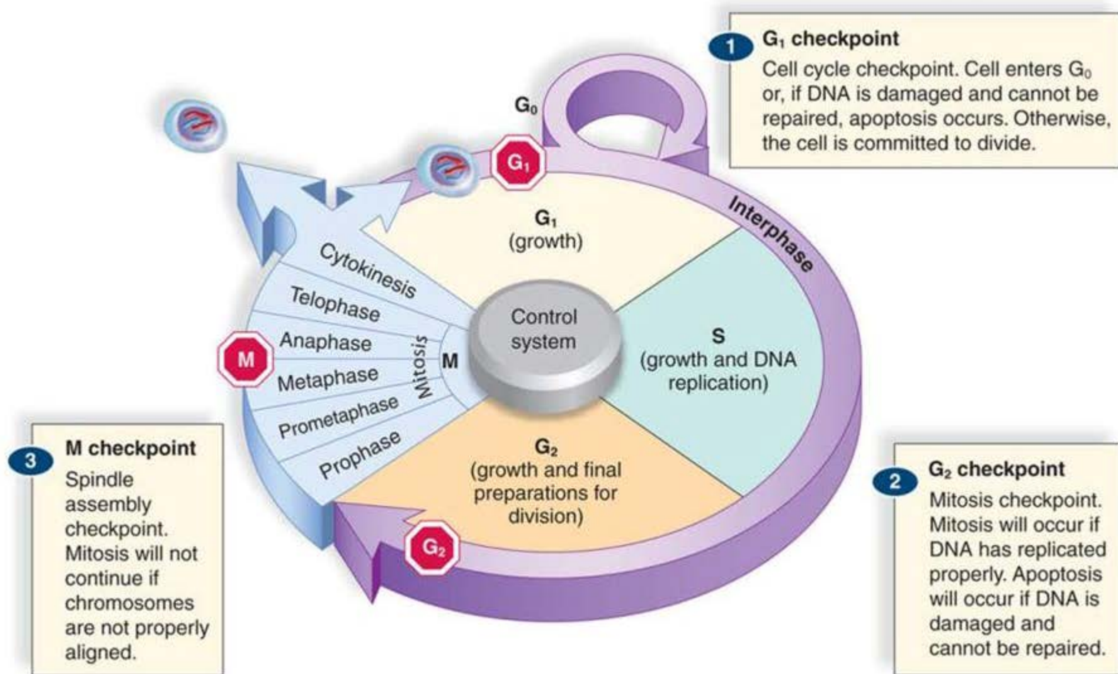


Figure 1.1: Cell cycle and checkpoints explained. Diagram showing the cell cycle stages G1, S, G2 and M as well as stages of mitosis and checkpoints. G0, quiescence/senescence stage is also shown, where the cell does not divide and lives on minimum metabolism. There are three major checkpoints during cell cycle (shown in red), at G1, G2-S and M. These are designed to make sure each stage is completed without any errors. (Image taken from McGraw-Hill, Chapter 8 Cell Division and Reproduction.)

1.1.1 Role of kinases and phosphatases

Kinases and phosphatases are catalytic proteins that transfer phosphate groups to and from their substrates. A kinase transfers a phosphate group from ATP or GTP to its substrate, whereas a phosphatase opposes this activity by catalysing the removal of a phosphate group from its substrates. Phosphorylation and de-phosphorylation are without a doubt the most predominant post-translational modifications that regulate structures and functions of a wide range of proteins by activating or inactivating their substrates (Ubersax and Ferrel, 2007). Phosphorylation acts as a molecular switch, affecting important cellular processes from cell division to cell metabolism and cell death. When a protein is phosphorylated, this may result in conformational changes allowing or blocking its interactions with other proteins (Holt et al., 2009). Phosphorylation can also affect protein-protein interactions by blocking or releasing the interactive or active surfaces of target proteins (Serber and Ferrel, 2007). The functions of both kinases and phosphatases are mediated by their substrate proteins and understanding how they recognise their targets is important to have a better understanding their role in the cell cycle.

Kinases either recognise their target proteins phosphorylation sequence with their active site or use a docking motif that may be on the kinase or on the target substrate (Turk, 2008). These interactions allow kinases to bind to their substrates with excellent specificity. Similarly, phosphatases also recognise their substrates via an interaction or docking motif on their substrates. These motifs are usually located at a distance from the active site of the kinase or phosphatase, since the active site is where the phosphorylation or de-phosphorylation is achieved and it should not be blocked unless the kinase or the phosphatase activity is being inhibited willingly (Roy and Cyert, 2009). Some phosphatases

like protein phosphatase 1 (PP1) bind to proteins that target them to their actual substrates. These are called regulatory or targeting subunits and they can either increase the specificity of PP1 or direct it to de-phosphorylate a different substrate (Bollen et al., 2010).

When a cell proceeds from one phase to another, it needs to make sure that this transition is irreversible. This is achieved by activating specific kinases to phosphorylate specific targets for that stage of the cell cycle (Novak et al., 2010). What makes these transitions irreversible, is the degradation of certain proteins that can reverse these reactions at this stage, so that the necessary phosphorylation and de-phosphorylation processes remain. Cyclins are the best example for this type of degradation. Along with cyclin dependent kinases (CDKs) are the cell cycle regulators that are activated at different stages of the cell cycle. CDKs are activated only when they are bound to cyclins, when they phosphorylate their target proteins. At each stage a different cyclin is activated so a different pair of cyclin/CDK is responsible for the progression of each stage. CDKs 1, 2, 4 and 6 along with cyclins A, B, D and E are involved in this process (Bonelli et al., 2014). Cyclin/CDK activity starts a phosphorylation cascade that leads to the activation of necessary transcription factors and therefore transcription of targeted genes. For example, the CDK4-CyclinD complex phosphorylates the retinoblastoma (Rb) protein along with CDK2-Cyclin E in G1, which then activates the transcription factors necessary for the S phase (Dyson, 1998; Hatakeyama and Weinberg, 1995). Similarly, CDK1-Cyclin B phosphorylates a wide range of proteins at the beginning of mitosis to make sure the process is completed without any problems (Errico et al., 2010); these multiple phosphorylations are then removed by different phosphatases at the end of mitosis so that the cell can go back to its essential functions (Nilsson and Hoffmann, 2000; Domingo-Sananes et al., 2011; Swaffer et al., 2016).

Other kinase cascades activate transcription factors and lead to cell differentiation, proliferation or apoptosis. Mitogen-activated protein- kinase (MAPK), phosphoinositide 3-kinase (PI3K), Akt, mammalian target of rapamycin (mTOR) as well as protein kinase A (PKA) and protein kinase C (PKC) pathways are involved in the regulation of fundamental cellular processes. These pathways crosstalk between each other and they importance for cell survival is highlighted by the fact their mis-regulation is part of important oncogenic pathways; overexpression of some of these kinases can lead to an overgrown population of cells that could potentially turn into cancer cells, whereas the opposing phosphatases are considered as tumour suppressors because they can de-phosphorylate these oncogenic targets (Bononi et al., 2011).

The opposing activity between kinases and phosphatases ensures that each stage of the cell cycle is highly regulated and controlled so that the each cell cycle is properly completed and a new one can re-started without any problems: this is particularly important when the cells exit mitosis and enter G1.

1.1.2 From mitosis to G1

Mitosis is a complex, well-structured and highly organised process that allows a cell to divide and form two genetically identical daughter cells. It includes a number of vital steps like chromosome condensation, nuclear envelope disassembly, chromosome segregation, reformation of the nuclear envelope and chromosome de-condensation. All these events are controlled by activation and inactivation of certain proteins, which is achieved by phosphorylation and de-phosphorylation processes throughout mitosis.

Resetting the cell cycle and resuming all the functions in the cell after mitosis depends on the reversal of the events mentioned above and re-establishing protein-protein interactions as well as epigenetic changes. These should happen in the correct order and in a regulated manner. The preparation for the start of the next cycle starts towards the end of mitosis, and is called the mitotic exit. Mitotic exit begins with the inactivation of mitotic kinases and activation of relevant phosphatases for the reversal of the phosphorylations. These de-phosphorylations occur in many components of the cell including the nuclear lamina for the re-formation of the nuclear envelope, histone proteins for the re-packaging of the DNA and several transcription factors to trigger cell growth (de Castro et al. 2015).

It is well-known that entry into mitosis depends on the activation of CDK1-Cyclin B complex, which initiates phosphorylation of a broad range of proteins. The activation of this complex is achieved by the phosphatase Cdc25 through de-phosphorylation of its inhibitory residues, Thr14 and Thr15 (Draetta et al., 1988; Gould et al., 1989; Krek et al., 1991).Phosphorylation by CDK1-Cyclin B has an inhibitory effect on its substrates, making sure that these substrates do not interfere with the progression of mitosis.

The first sign of mitotic exit is the inactivation of CDK1-Cyclin B by degradation of Cyclin B. This is achieved by the proteasome after being marked by the ubiquitin ligase anaphase promoting complex (APC), and results in chromosome segregation as well as CDK1-Cyclin B inactivation (Glotzer et al., 1991). When CDK1-Cyclin B is inactivated, the de-phosphorylation of its substrates begins. Although the inactivation of CDK1-Cyclin B is crucial for mitotic exit, the activity of phosphatases is equally important to prepare the cell for a functional G1 nucleus (Sullivan and Morgan, 2007). The best example of this is the identification of PP2A as an essential mitotic exit phosphatase. Activity of PP2A-B55δ was reported to be essential for mitotic entry and exit in Xenopus egg extracts (Mochida and Hunt, 2012; Mochida et al., 2009) and furthermore, this activity was found to be regulated by the Greatwall kinase through the activation of PP2A-B55δ inhibitors (Mochida et al., 2010; Gharbi-Ayachi et al., 2010; Lorca and Castro, 2012).

1.2 Ser/Thr phosphatases

The Ser/Thr phosphatase family is one of the two major families of phosphatases along with protein tyrosine phosphatases (PTPs). Both families are named according to the type of amino acid they de-phosphorylate. It has been well established that Ser/Thr phosphorylation and de-phosphorylation regulates more important processes during cell division than PTPs (Barr et al., 2011). They influence many pathways involving in cell proliferation, apoptosis, embryonic development, cell motility and cell differentiation (Janssens and Goris, 2001; Barford, 1996). The effect of Ser/Thr phosphatases on multiple important processes was proven by their inhibition using okadaic acid, an algal toxic specific for Ser/Thr phosphatases, which triggered many changes in mitosis like chromatin condensation and re-arrangement of organelles (Lucocq et al., 1991; Yamashita et al., 1990). This also indicates that inhibiting Ser/Thr phosphatase activity is as important as activating protein kinases at mitotic entry, and mitotic exit requires the reversal of these activities as mentioned before.

The two most abundant Ser/Thr phosphatases in most of the cell types, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), both form holoenzyme complexes with their associated regulatory or scaffolding subunits. These holoenzymes are then targeted to the substrate for de–phosphorylation process to occur. This regulatory system is designed to increase target specificity and to prevent random binding of PP1 or PP2A to any other protein at any given time. In this section, molecular structures of both PP1 and PP2A will be explained in detail.

1.2.1 Structure and function of PP1

PP1 is a 38.5 kDa protein that is coded by three different genes giving rise to four different isoforms: PP1α, PP1β/δ, PP1γ1/γ2. There is 85-93% similarities between these isoforms and the majority of differences are at the N and C terminal ends. All PP1 isoforms are ubiquitous except for PP1γ2, which is restricted to the testis. The interaction and activity of PP1 is strictly regulated by its interaction with more than 200 targeting proteins (Bollen et al., 2010). These targeting subunits localise PP1 to certain regions of the cell to de-phosphorylate its targets. For example, when PP1 binds to Repo-Man it is recruited to chromatin at anaphase onset to de-phosphorylate histone H3 (Vagnarelli et al., 2006; Trinkle-Mulachy et al., 2006); when PP1 binds to PNUTS it stimulates chromosome de-condensation when the cells re-enter interphase (Landsverk et al., 2005) or when it binds to AKAP149 it helps the formation of nuclear lamina (Steen et al., 2000). The plethora of subunits also include some inhibitors like the inhibitor 2 (I-2) and DARPP-32, which bind to and block the active site of PP1.

The active site, also called the catalytic site, is at the intersection of the substrate binding hydrophobic, acidic and C-terminal grooves (Figure 1.2, A and B) and it contains two metal ions (Mn^{+2}) (Dancheck et al., 2011; Kelker et al., 2009). The interaction with the targeting subunits occurs through a motif called the RVxF (x stands for any amino acid except for Phe, Ile, Met, Tyr, Asp or Pro), which is common to almost all targeting subunits and inhibitors of PP1 (Wakula et al., 2003; Meiselbach et al., 2006; Bollen et al., 2010).

The interaction through the RVxF motif is necessary but it does not affect the enzymatic activity of PP1, since it is away from the active site. There are also other docking motifs like SILK and MyPhoNE that contribute to the regulation of PP1 substrate specificity (Hendrickx et al., 2009).

As a phosphatase that interacts with a large number of targeting subunits and substrates, the remaining question about PP1 is the molecular mechanism of holoenzyme and substrate recognition. There are only a few well-studied examples of PP1 holoenzymes up to date. These are: PP1/spinophilin in neurons (Ragusa et al., 2010); PP1/MYPT1 in muscle (Terrak et al., 2004; Yamashiro et al., 2008); PP1/inhibitor-2 (Hurley et al., 2007; Marsh et al., 2010); PP1/neurabin (Ragusa et al., 2010). In addition, toxin bound and inhibited PP1 crystal structures have also been obtained. These include PP1 bound to okadaic acid (Maynes et al., 2001), caluculin A (Kita et al., 2002) and nodularin R (Kelker et al., 2009). These crystal structure studies have also revealed that the majority of PP1 binding subunits are highly disordered when they are not bound to PP1. They do not have a well-defined three dimensional structure and these are defined as intrinsically disordered or unstructured proteins (IDPs/IUPs) (Hurley at el., 2007). Being intrinsically disordered gives them some sort of flexibility that enables them to form unique interactions with PP1 (Ragusa et al., 2010; Terrak et al., 2004) and they can adopt different protein surfaces binding motifs.

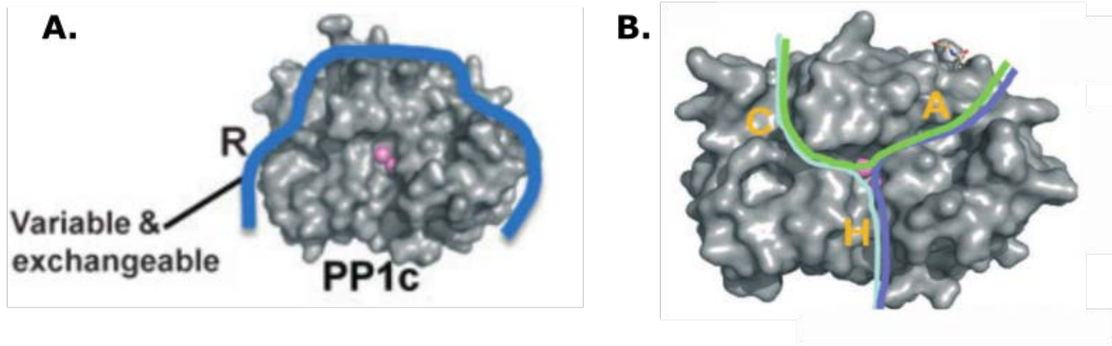


Figure 1.2: Structure of PP1. A. Crystallized PP1 catalytic subunit (PP1c), showing the active/catalytic site with two Mn^{+2} ions (in pink) and the binding of a variable and exchangeable regulatory or targeting subunit (in blue). **B.** Crystallized PP1, showing the active site at the intersection of three grooves. C, H and A represent C-terminal, hydrophobic and acidic grooves respectively. (Both images taken from Peti et al., 2013).

PP1 substrate-specificity depends highly on the targeting subunit as the targeting subunit may block or extend certain grooves on PP1, which might then allow PP1 to interact with a limited number of substrates via the remaining grooves. An example for this type of interaction is Spinophilin, which blocks the C-terminal groove of PP1, restricting its interaction to the substrates that can interact with the acidic and hydrophobic grooves (Ragusa et al., 2010). Similarly, MYPT1 extends the acidic groove, creating more space to bind for the substrate, which then makes the acidic groove more preferable than the others (Terrak et al., 2004).

Another important question is: How is the selectivity achieved between PP1 and its interacting proteins? The selectivity is achieved through the regulation of PP1 interacting proteins. PP1 activity depends highly on the interacting proteins, as it is never found on its own in the cell. The regulation might happen by the phosphorylation of the interacting protein, which changes the binding affinity, or in some cases, the activation and expression of the binding protein is cell type specific (Moorhead et al., 2007; Virshup and Shenolikar, 2009). Even with phosphorylation, it is still hard for the cell to control the pool of PP1 interacting proteins. It was shown that to solve this problem, the concentration of PP1 binding proteins in the cell is regulated by proteolysis: this is true for GADD34 and R5/PTG (Brush et al., 2003; Vernia et al., 2009). Overall, the concentration and affinities of PP1 interacting proteins determine which PP1 holoenzymes are being formed in the cell.

1.2.2 Structure and function of PP2A

PP2A is a heterodimer formed by a 36 kDa catalytic subunit (C subunit), a 65 kDa structural subunit (A subunit) and a variable regulatory subunit (B subunit), which can vary in size (55 to 130 kDa) (Figure 1.3, A and B). The A and C subunits form the catalytic complex which then binds to different variable B subunits (B55/B56 and PR72/PR130). PP2A holoenzymes are vastly diverse due to several isoforms of each subunit and different combinations of these isoforms (Bononi et al., 2011). The regulatory B subunits play a crucial role in PP2A activity and they achieve this by regulating substrate selectivity. Some of the B subunits target PP2A to multiple places like the microtubules, nucleus or mitochondria, whereas some B subunits have a more restricted number of targets inside the nucleus (Shi, 2009; Janssens et al., 2005; Forester et al., 2007). This different localisation patterns are caused by different signals carried by different B isoforms.

For example, in the beta isoform of B56 (B56β) only a nuclear export signal is present whereas in alpha, and epsilon isoforms (B56α and B56ε) both nuclear export and nuclear localisation signals are present. As a consequence, B56 proteins that have nuclear export signals are shuttled to the cytoplasm after mitosis. The different localisation of B56 subunits therefore allow PP2A to target a wide range of proteins distributed not only in the nucleus but also in the cytoplasm (Flegg et al., 2010; Jin et al., 2009; Riedel et al., 2006). As clearly understood from the given examples, most PP2A regulatory subunits do not have a targeting motifs but their presence help PP2A compartmentalization within the cell.

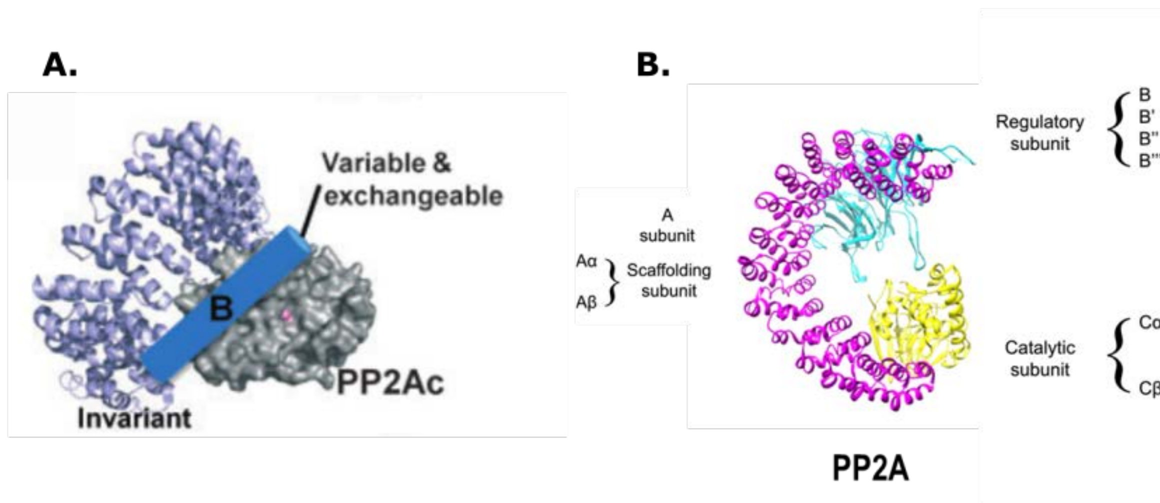


Figure 1.3: Structure of PP2A. A. Crystal structure of PP2A, showing the catalytic C subunit with two metal ions (in pink) A subunit and variable B subunit (in blue) (c represents catalytic subunit) (Image taken from Peti et al., 2012). **B.** Secondary structure of PP2A, showing different isoforms of each subunit. (Image taken from Sangodkar et al., 2015).

PP2A influences and controls many important cellular processes in the cell. These include glycolysis and lipid metabolism (Tung et al., 1985) as well as cell proliferation, DNA replication, cytoskeleton dynamics signal transduction, cell mobility and transcription and translation (Alberts et al., 1993; Glenn and Eckhart, 1993; Ronne et al., 1991; Schonthal, 2001). Moreover, PP2A is involved in intrinsic apoptosis pathway by de-phosphorylating Bcl-2 and creating an anti-proliferative signal, therefore it is considered as a tumour suppressor and targeted for the treatment of several cancers (Ruvolo et al., 2002).

As mentioned before, regulation of PP2A mostly depends on the diversity of its subunits. But, apart from subunit diversity, PP2A is also regulated by phosphorylation and methylation. The TPDYFL conserved amino acid sequence on PP2A can be phosphorylated and methylated at tyrosine (Y) and leucine (L) residues. This effectively decreases PP2A activity and prevents its interaction with regulatory subunits (Longin et al., 2007). PP2A is also phosphorylated by a kinase capable of performing auto-phosphorylation, which alters PP2A efficacy (Guo, H. and Damuni, Z., 1993). Methylation of PP2A is achieved by PP2A-methyltransferase (PPMT). Addition of a methyl group to L309, enhances the binding affinity of the catalytic complex (A and C subunits) for certain regulatory subunits (B-alpha), hence provides specificity (Leulliot et al., 2004). From all of the information above, we can conclude that both PP1 and PP2A are important phosphatases that regulate essential pathways in the cell. Although their regulation or holoenzyme structures are not the same, they work in similar ways and they both have a wide range of substrates. From here onwards, I will focus specifically on phosphatases at mitotic exit and explain mitotic exit proteins as well as the proteins of relevance for this thesis.

1.3 Important players of mitotic exit

1.3.1 PP1 and PP2A at mitotic exit

Role of phosphatases in mitotic entry and exit was reported in two papers in the early 90s by using the PP1 and PP2A inhibitor Okadaic Acid (OA) on Xenopus egg extracts. In both papers PP2A is suggested to de-phosphorylate Cdc25 and maintain it at a low activity state, which negatively regulates the cell cycle (Felix M. A. et al., 1990; Clarke P.R., 1993). Later on, the importance of PP2A in mitotic exit was further proven by studies performed in budding yeast. Wang and Ng reported that PP2A negatively regulates mitotic exit by regulating the de-phosphorylation of Tem1, which acts at the very top of the MEN pathway (Wang and Ng, 2005), while Queralt and colleagues found a down-regulatory pathway of PP2A by separase that initiates mitotic exit by the activation of mitotic exit network (MEN) (Queralt et al., 2006).

The complex structure of PP2A clearly links this phosphatase to the regulation of different specific actions by its multiple subunits and isoforms. Unsurprisingly, PP2A-B56δ was then reported to be the only member of PP2A-B56 to directly bind to Cdc25C in human cells in vivo and negatively regulate it by de-phosphorylating Cdc25C at Thr130 and maintaining the binding of 14-3-3 to Cdc25C at interphase (Margolis et al., 2006). Also at M phase, the PP2A-B56δ was reported to interact with Cdc25C, which leads to the inactivation of the Cdk1-Cyclin B complex and makes PP2A-B56δ and important regulator of mitotic exit (Forester et al., 2007). Forester and colleagues proved this further by generating a stable knockdown of B56δ in mammalian cells and observed that Cdc25C is hyper-phosphorylated and Cyclin B degradation is delayed, resulting in the delays of mitotic exit. The importance of PP2A-B56δ was also proven by its depletion from Xenopus egg extracts.

It was observed that PP2A-B56δ depleted cells at interphase go into mitosis faster and remain in mitosis indefinitely, whereas PP2A-B55δ depletion in mitotic extracts did not have an effect on mitotic exit (Mochida et al., 2009). These observations suggest that B56δ and B55δ are crucial subunits of PP2A for entry into and exit from mitosis but other phosphatases must also be involved in the process and, considering the complex structure of PP2A, these subunits cannot be the only regulators. Soon after the discovery of the roles of PP2A-B55δ and B56δ another subunit of PP2A was reported to be a key regulator of mitotic exit. Using RNAi knockdown and live cell imaging, Schmitz and colleagues screened a genome-wide protein phosphatase library to identify mitotic exit proteins and they found PP2A-B55α along with importin-β1 as a key factor in mitotic spindle breakdown and nuclear envelope reassembly (Schmitz et al., 2010). The dynamics and mathematical model of PP2A control over the cell cycle and cell cycle transitions revealed that PP2A controls the separation of S phase and mitosis by counteracting Cdk1 activation and activity of mitotic kinases (Krasinska et al., 2011).

It is important that early phosphatase activity and early entry into mitotic exit is prevented: this is achieved by the accumulation of phosphorylations. Most of these phosphorylations are carried out by Cdk1 to assure correct entry into mitosis but there are other kinases as well, which make sure mitotic exit proteins are inactive during this stage (Vigneron et al., 2009; Castilho et al., 2009). Greatwall kinase is one of these kinases known to act on PP2A and inhibit its activity upon mitotic entry. It was found to phosphorylate two other proteins arpp19 and ensa, which associate with and inhibit PP2A at its B55 type regulatory subunits (Mochida et al., 2010; Gharbi-Ayachi et al., 2010). A human homologue of the Greatwall kinase was identified as a microtubulue-associated-serine/threonine kinase-

like (MASTL) but how this protein is related to other phosphatases in human cells is not completely established (Voets and Wolthuis, 2010; Burgess et al., 2010). In addition, PP1 activity is also supressed upon mitotic entry both by direct phosphorylation or binding of the inhibitor-1. Direct phosphorylation of PP1 is achieved in mammalian cells by Cdk1 and the binding of inhibitor-1 depends on its phosphorylation by PKA (Kwon et al., 1997; Dohadwala et al., 1994). Inhibition of PP1 is important to protect mitotic phosphosites from early de-phosphorylation before mitotic exit.

As mentioned above, PP1 and PP2A are largely inactive at the beginning of mitosis but they should be activated at mitotic exit onset to regulate de-phosphorylations and allow the cell to finalise mitosis. An important investigation on how these phosphatases are activated at mitotic exit was led by Grallert and colleagues in early 2015 and an important regulatory cascade was discovered. They report an interaction between PP1 and PP2A-B55/B56, which leads to a relay of de-phosphorylations, activating both phosphatases (Figure 1.4). This process is reported to be essential for chromosome segregation and therefore for cell division (Grallert et al., 2015). This study was carried on in fission yeast but they report a similar interaction in human cells as well. The discovery of this cascade raised new questions about the B55 and B56 subunits of PP2A. Structurally, these subunits have hydrophobic cores and their PP1 binding sites are folded in these cores (Shi, 2009). Therefore, it seems impossible for these proteins to interact with PP1 without disrupting the well-folded domains in the core. One possible explanation, still to be demonstrated, is that the proteins possibly change confirmation and expose their PP1 binding sites upon binding and activation. There are proteins known to undergo conformational changes (Whisstock and Bottomley, 2006) but it is not clear if this is the case in the interaction of PP1 and PP2A.

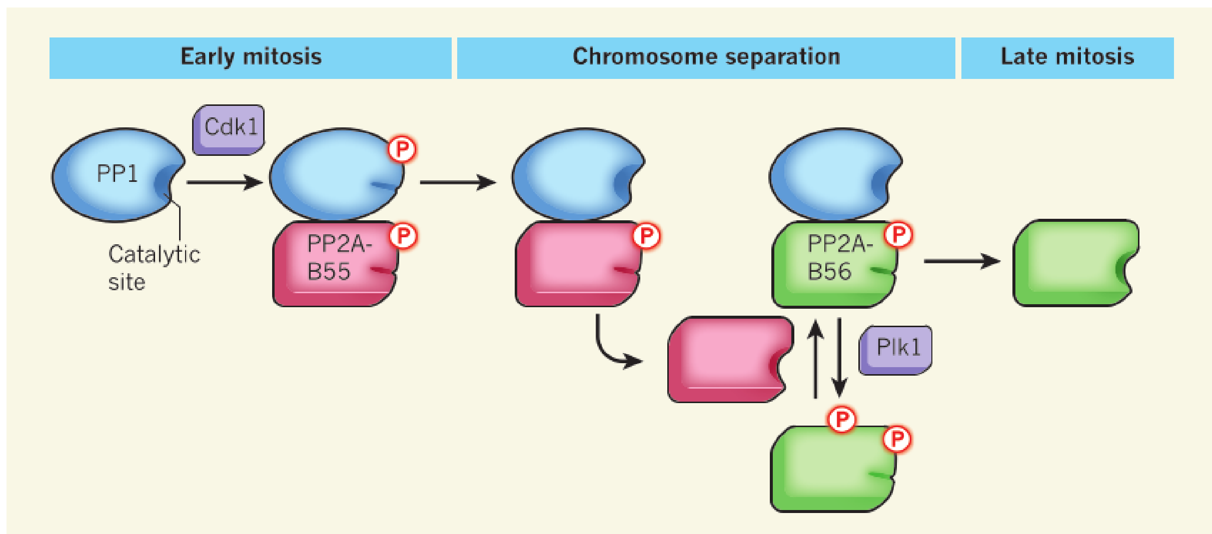


Figure 1.4: The PP1-PP2A phosphatase relay at mitosis. At early mitosis PP1 is phosphorylated by CDK1. Phosphorylated PP2A-B55 is associated with phosphorylated PP1 and when CDK1 is inactivated, PP1 activates itself by removing its phosphate group. Active PP1 then de-phosphorylates bound PP2A-B55 to activate it. Active PP2A-B55 removes one of the phosphate groups on PP2A-B56 and allows it to bind to PP1. This is may be delayed because of Plk1 activity which counteracts PP2A-B55 but when Plk1 is inactivated in late mitosis PP2-B56 is successfully de-phosphorylated and binds to PP1. PP2A-B56 is then de-phosphorylated once again by PP1 and released. (As explained in Grallert et al., 2015, image taken from Bollen, 2015)

1.3.2 Spindle assembly checkpoint (SAC)

In eukaryotic cells, the spindle assembly checkpoint (SAC) acts as a control mechanism by monitoring chromosome bi-orientation and alignment on the mitotic spindle at metaphase. The purpose of this is to detect any unattached or improperly attached chromosomes on the metaphase plate. At this stage the cell continues to remain in mitosis until the problem is fixed and all the chromosomes are aligned correctly. SAC prevents the cell from losing or gaining any of the sister chromatids, therefore inheriting wrong number of chromosomes in the daughter cells. This is achieved by a sensory apparatus that monitors the chromosome attachment to the mitotic spindle and an effector complex that targets the mitotic machinery. Like all other cell cycle mechanisms, SAC is also regulated by reversible phosphorylations (Foley and Kapoor, 2013).

The effector complex of SAC is called the mitotic checkpoint complex (MCC), which targets the anaphase-promoting complex (APC) and is activated by the unattached kinetochores. The APC is crucial for mitotic exit as it is responsible for ubiquitination of Cyclin B and Securin, promoting their destruction. Cyclin B, as mentioned before, keeps its targets phosphorylated with CDK1 and a successful mitotic exit requires its inactivation as well as de-phosphorylation of all its targets. Securin on the other hand, is a protein that blocks the Separase enzyme, which targets cohesin molecules (a complex that holds sister chromatids together) and promotes sister chromatid separation. By targeting and inhibiting APC, MCC stabilizes Cyclin B and Securin, which effectively prevents mitotic exit (Figure 1.5) (Primorac and Musacchio, 2013).

Chromosome attachment to microtubules is mediated by the kinetochores. These are multi-subunit structures, where the mitotic spindle is attached during cell division to pull the sister chromatids apart. They have an inner and outer layer. The inner layer interacts with the centromeres of the chromosomes, whereas the outer layer is involved in mitotic spindle binding and SAC activation (Fukagawa and Earnshaw, 2014; McKinley and Cheeseman, 2014). Specifically, the KMN network, formed of kinetochore null protein 1 (KNL1), mis-segregation 12 (MIS12) complex and the nuclear division cycle 80 (NDC80) complex, is responsible for kinetochore attachment to the spindle and also for MCC activation in case of an unattached chromatid (Cheeseman et al., 2006). One of the key points of MCC activation is the recruitment of all MCC proteins to kinetochores at the early stages of kinetochore-microtubule attachment (Howell et al., 2004). This close proximity allows KNM to signal about the attachment status and generates a dynamic control system for the SAC.

The sensory apparatus of the SAC is Mps1, which is recruited to the kinetochores by the help of Aurora B (Vigneron et al. 2004; Santaguida et al. 2011), the Ser/Thr kinase within chromosome passenger complex (CPC, explained in detail in the next section). Mps1 phosphorylates KMN and creates docking sites for SAC proteins that are important in MCC assembly (Krenn et al., 2014). The main responsibility of Aurora B is sensing the unattached or abnormally attached kinetochores to the mitotic spindle (Lampson and Cheeseman, 2011). The ability of Aurora B to do this does not depend on its kinase activity but depends more on its localisation and close proximity to the kinetochores. Although the contribution of Aurora B is controversial, in terms of direct or indirect activation of SAC, it certainly has a huge influence on the system. Furthermore, Aurora B counteracts PP1, which is considered as the SAC silencing phosphatase (Lampson and Cheeseman, 2011).

The SAC is overall a feedback mechanism generated by the unattached kinetochores, creating a negative signal towards the progression of mitosis. And because it is a feedback mechanism, it has the potential to be repeated several times in a loop, until the negative feedback is lost from the attached kinetochore. This may extend the prometaphase or metaphase stage of mitosis and delay cell division, but ensures proper distribution of chromosomes.

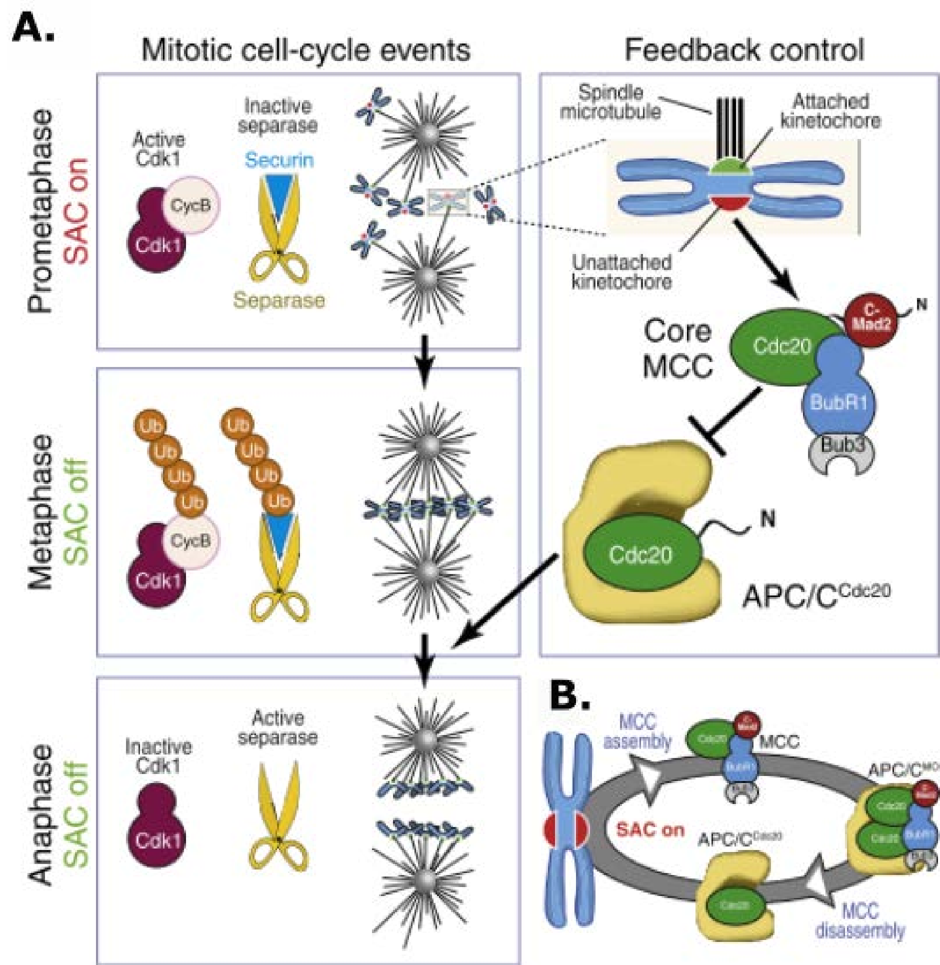


Figure 1.5: The components and mechanism of SAC. A. SAC components and activation explained. At prometaphase when the kinetochores start to attach to the spindle, any kinetochore that it not attached (in red) activates the SAC and MCC is assembled. APC is inhibited until the unattached kinetochore is safely attached. Then the SAC is turned off and degradation of Cyclin B and Securin is achieved, which then promotes sister chromatid separation. **B.** The cycle of MCC assembly and inactivation. The presence of unattached kinetochores trigger MCC assembly and after APC inactivation, MCC disassembly is triggered. However, this cycle is repeated as many times as necessary until the kinetochore is safely attached to the spindle. (taken from Musacchio, 2015).

1.3.3 The chromosome passenger complex (CPC)

The chromosome passenger complex (CPC) is a four subunit complex responsible for a successful mitotic progression. The CPC localisation changes during mitosis, where it regulates different events like SAC and cytokinesis. The four subunits of CPC are the catalytic subunit Aurora B (a Ser/Thr kinase), and other regulatory components Survivin, inner centromeric protein (INCENP) and Borealin (Ruchaud et al., 2007).

The assembly of CPC starts on the INCENP subunit, which is the assembly point. The N-teminus of INCENP along with Survivin and Borealin form a three helix bundle, which links the baculovirus IAP repeat (BIR) domain of Survivin and the C-terminus of Borealin (Jeyaprakash et al., 2007). Aurora B on the other hand, binds to the IN-BOX on the INCENP C-terminus and gets activated (Adams et al., 2000) (Figure 1.6).

INCENP is considered as the platform on which all other CPC components are assembled and it is regulated by both Aurora B and CDK1 (Cooke et al., 1987). When bound to INCENP, Aurora B phosphorylates it, which also leads to the full activation of Aurora B (Honda et al., 2003). Furthermore, phosphorylation of INCENP by CDK1 is required for polo-like kinase 1 (PLK1) localisation to the centromere, where the activation of PLK1 is achieved by Aurora B at Thr210 (Goto et al., 2006; Carmena et al., 2012).

Once assembled, CPC phosphorylates its targets through Aurora B and its dynamic localisation during mitosis enables CPC to achieve these phosphorylations at the appropriate time and space for mitotic progression (van der Horst and Lens, 2014). At the beginning of mitosis, CPC is localised at the centromeres, which is necessary for a successful kinetochore-microtubule attachment. This is achieved by activating the SAC through Mps1 (as mentioned in the SAC section). At metaphase-anaphase transition, CPC localizes to the equator of the cell (Earnshaw and Cooke, 1991), where it controls cytokinesis and the timing of nuclear envelope reformation after mitosis (Kitagawa and Lee, 2015; Ruchaud et al., 2007).

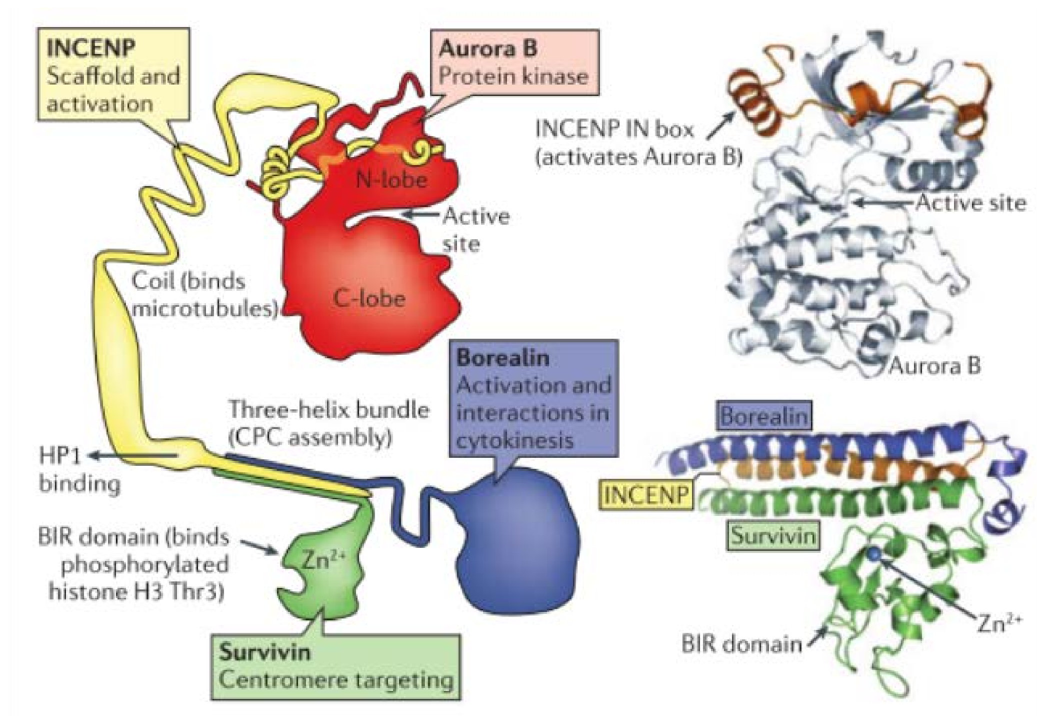


Figure 1.6: CPC components and assembly mechanism. Components of CPC and their functions are explained. INCENP (yellow), Survivin (green) and Borealin (blue) forming the three helix bundle and Aurora B (red) binding to the IN box of INCENP. The secondary structures are also shown. (Image taken from Carmena et al., 2012)

The activity of Aurora B is highly regulated as it has so many targets and phosphorylation of these targets must be done in a timely manner for a mitotic progression. An important aspect of Aurora B regulation is the counteracting phosphatases PP1 and PP2A-B56. At early mitosis, PP1 is recruited to the outer kinetochores by its related targeting subunits SDS22 (Posch et al., 2010) and CENP-E (Kim et al., 2010), while its localization to the bulk chromatin by Repo-Man is suppressed (Trinkle-Mulachy et al., 2006; Vagnarelli et al., 2006). PP2AB56 on the other hand, counteracts Aurora B phosphorylation and stabilizes kinetochore-microtubule attachments (Foley et al., 2011).

When the cells enter mitosis, Aurora B phosphorylates histone H3 at Ser10 and Ser28 (Hsu et al., 2000; Murnion et al., 2001; Goto et al., 2003). This causes the release of heterochromatin protein 1 (HP1) from the adjacent trimethylated Lys9 of H3 (H3K9me3) (Fischle et al., 2005; Hirota et al., 2005). CPC localizes at the centromeres from this point onwards and stays there until anaphase. This localization at the centromere requires the mitosis specific phosphorylation of two histone tails: H3T3 by Haspin kinase and H2AT120 by Bub1 kinase (Yamagishi et al., 2010). H3T3 phosphorylation localizes CPC, therefore Aurora B, to the centromeres via the BIR domain of Survivin, which recognises H3T3ph specifically (Kelly et al., 2010; Wang et al., 2010; Dai and Higgins, 2005). In addition, recognition of the H2AT120 by Shugoshin and its interaction with CPC also promotes CPC recruitment to centromeres. CPC localization at centromeres creates a positive feedback loop that increases H3T3 phosphorylation (Figure 1.7). This positive feedback loop includes phosphorylation of Haspin by CDK1-Cyclin B at early mitosis, when this phosphorylation is recognised by Plk1 to further phosphorylate Haspin (Zhou et al., 2014: Ghenoiu et al., 2013).

These series of phosphorylations trigger the H3T3 phosphorylation and after being recognised by CPC, Haspin is even further phosphorylated by Aurora B and this enhances the H3T3 phosphorylation (Wang et al., 2011; Zhou et al., 2014). In the meantime the Repo-Man/PP1 complex, that is responsible for removing H3T3 phosphorylation, is kept inactive until anaphase onset when CPC is released from the mitotic chromatin. The inactivation of the complex is achieved by CDK1-Cyclin B phosphorylation (at T412 and T419) and is kept away from the substrate by Aurora B phosphorylation (at S893); these phosphorylations are removed by PP1 and PP2A respectively (Vagnarelli et al., 2011; Qian et al., 2013).

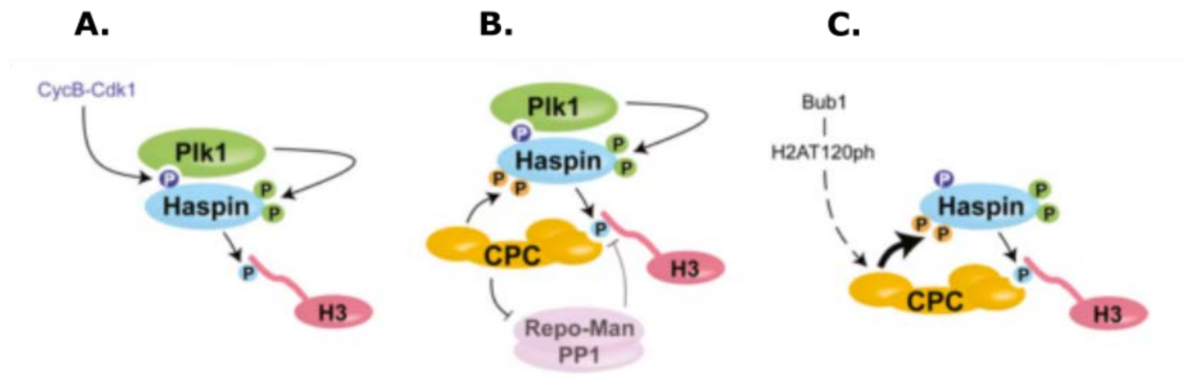


Figure 1.7: CPC-Haspin-H3T3ph feedback loop. A. Early mitosis signal when Haspin is phosphorylated by CDK1-Cyclin B as well as Plk1 and H3T3 phosphorylation is achieved. **B.** Signal amplification when CPC is recruited to the H3T3ph and further phosphorylates Haspin to enhance H3T3ph even further. Repo-Man/PP1 complex is inhibited at this stage. **C.** CPC accumulation at the centromere is increased with the contribution of H2AT120 phosphorylation by Bub1. (image taken from Zhou et al., 2014)

1.4 Histone modifications

Genomic DNA in eukaryotic cells is packed around histones to form nucleosomes and histones undergo further modifications for proper packaging of DNA into euchromatic and heterochromatic regions. One of the challenges for the cell when preparing for mitosis is to resolve all these levels of organisation on the histones for timely condensation of chromosomes then to re-organise the interphase chromatin. The cell also needs to have a memory of the previous modifications so that the exact same ones could be maintained (Egli et al., 2008).

Histone modifications include phosphorylation, methylation, acetylation and ubiquitylation (Kouzarides, 2007). These modifications change the biophysical properties of the nucleosomes, exposing parts of DNA that need to be transcribed and recruiting necessary proteins or hiding the parts of DNA that are not transcribed, therefore preventing any other protein to reach that particular part of the genome. Most of these modifications occur at the N or C terminal tail of histones, which allows other proteins to access and recognise them (Wang and Higgins, 2013). For each type of modifications, there are specific group of proteins that create the mark and another group of proteins that recognise that mark. In other words, histones are marked at specific places for specific proteins to recognise and follow orders. Some modifications are increased as the cells go into mitosis and similarly some are decreased but for a successful mitotic exit, several modifications are rapidly removed at appropriate times. Table 1.1. summarise some important histone modifications, the proteins responsible for these and their significance.

Table 1.1: Important histone marks and their significance

Modification	Enzyme	Comments
H2AT120ph	Bub1	Shugoshin recruitment, CPC localisation onto chromatin
H3T3ph	Haspin	CPC(Survivin) recruitment onto chromatin
H3S10ph	Aurora B	Displacement of HP1 from chromatin
H3S28	Aurora B	Displacement of PRC1
H3K4me2/3	Set1, MLL	Bookmarking active genes CENP-A loading
H3K9me2/3	Suv39H1/2, G9a	HP1 binding
H3K27me3	Ezh2	Bookmarking polycomb genes, PRC1 and PRC2 binding

Phosphorylation of histones usually results in either recruitment of a specific protein to the area or release of a protein from the same area. For example, H3T3 phosphorylation by Haspin triggers recruitment of CPC to centromeres (Dai et al., 2005; Wang et al., 2011), whereas H3S10 phosphorylation by Aurora B triggers the release of HP1 from the adjacent H3K9me3 and this is referred to as a phosphor-methyl switch (Fischle et al., 2005; Hirota et al., 2005; Wang and Higgins, 2013). Similarly, recruitment of multiple proteins as a result of

histone phosphorylation may result in a series of phosphorylations between the proteins, which then activates or inactivates several signalling cascades.

Methylation of lysine (mono, di and tri) and arginine residues on the histones are mostly repressive markers that form heterochromatin, while acetylation on the other hand, marks the regions that are actively transcribed. Acetylation therefore is a lot more dynamic than methylation (Kouzarides, 2007). Ubiquitinylation of histones does not trigger degradation as it does with other proteins; instead, it may trigger gene repression and heterochromatin maintenance (Wang et al., 2004; Racine et al., 2012).

At mitotic exit, the re-establishment of histone acetylation and removal of phosphorylation are very important in order to resume gene transcription. PP1 plays an important role, counteracting most of the mitotic kinases including CDK1-Cyclin B, Aurora B, Haspin and Plk1. PP1, along with its targeting subunit Repo-Man, is recruited to anaphase chromatin at high levels to remove H3T3, H3S10 and H3S28, as the CPC is removed from chromatin (Vagnarelli et al, 2011, Qian 2011, Qian 2015, De Castro et al, 2016).

It has been well documented that histone modifications trigger replacement and recruitment of proteins to the chromatin and these facilitate chromosome condensation and segregation. This information is also transmitted to the daughter cells for the exact same histone modifications to take place.

1.5 Function and regulation of Repo-Man

1.5.1 Repo-Man as a PP1 targeting subunit

Repo-Man is a PP1 targeting subunit which is coded by the CDCA2 (Cell division cycle associated 2) gene on chromosome 8 (8p21.2). It is named so, because it is responsible for recruiting PP1 onto mitotic chromatin at anaphase onset (*Re*cruits *P*P1 *o*nto *M*itotic chromatin at *an*aphase) (Trinkle-Mulcahy 2006). Repo-Man coordinates chromosome organisation and dynamics during mitosis and nuclear envelope reassembly during mitotic exit (Vagnarelli et al., 2011). The C terminal domain localizes Repo-Man to bulk chromatin in early anaphase to target PP1 for de-phosphorylation of histone H3 and the N terminal domain localizes Repo-Man to the chromosome periphery in late anaphase where it helps reassembly of the nuclear envelope by recruiting nuclear envelope proteins like Importinβ and Nup153 in a PP1 independent manner (Vagnarelli et al., 2011).

At the onset of mitosis, Repo-Man is found to be dispersed in the cytoplasm, whereas at anaphase onset it is observed to be highly associated with chromatin, where it targets PP1 through its RVTF motif (Figure 1.8). It was reported that when the RVTF motif is mutated, Repo-Man PP1 binding is completely abolished but this mutation only affects Repo-Man binding to PP1 and not to chromatin. Furthermore, Repo-Man was proven to be vital for cell viability as its absence induces apoptosis and this was shown by RNA interference (Trinkle-Mulachy et al., 2006).

Repo-Man/PP1 complex de-phosphorylates histone H3 at Thr3 (Vagnarelli et al, 2011, Qian et al 2011) Ser10 (Vagnarelli et al, 2011) and Ser28 (de Castro et al, 2016) which are substrates for Haspin and Aurora B kinases respectively (Dai and Higgins, 2005; Dai et al., 2006; Dai et al., 2005). Association of Repo-Man with the chromatin is very dynamic in early mitosis and the system favours Repo-Man to stay OFF the chromatin, whereas towards mitotic exit the system favours Repo-Man to be loaded ON the chromatin (Figure 1.9). This was shown by FRAP analysis using interphase and metaphase chromatin in HeLa cells that transiently express GFP:Repo-Man. The recovery of the signal in metaphase was reported to be a lot more dynamic than in interphase (Vagnarelli and Earnshaw, 2012).

Repo-Man contributes to a successful mitotic exit not only by de-phosphorylating histone H3, but also by regulating chromosome architecture along with condensin during mitosis. When chromosome condensation is promoted by an unknown RCA factor (Regulator of chromosome architecture), condensin complex (1 and 2) associates with chromosomes and its absence causes abnormalities in chromosome condensation. These abnormalities can be prevented by either blocking PP1 recruitment onto chromatin by Repo-Man as PP1 recruitment inactivates RCA and blocks condensin accumulation, or by inducing high CDK1 activity (Vagnarelli et al., 2006). Blocking PP1 recruitment by Repo-Man is achieved by mutating the RVTF motif (RAXA mutant) (Trinkle-Mulachy et al., 2006).

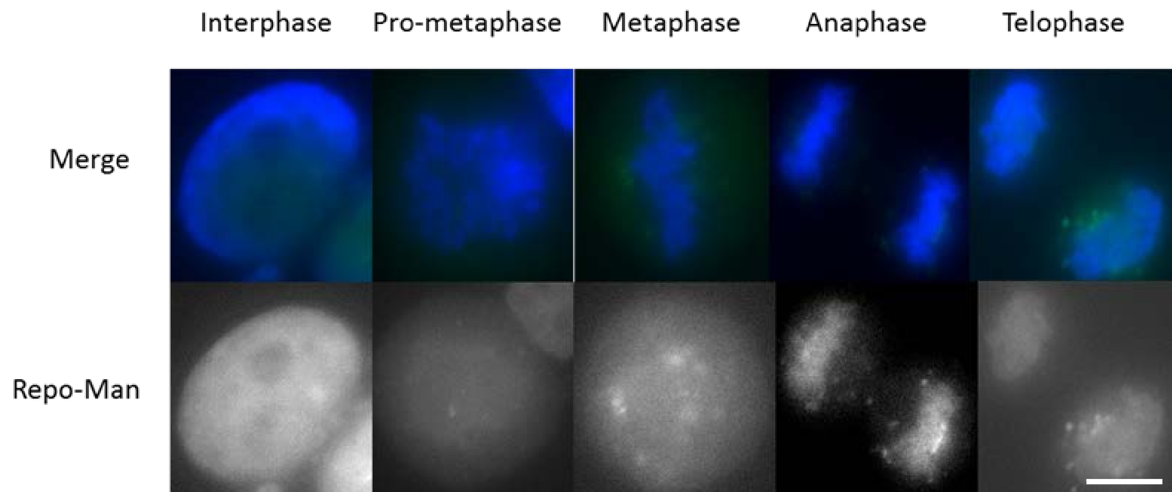
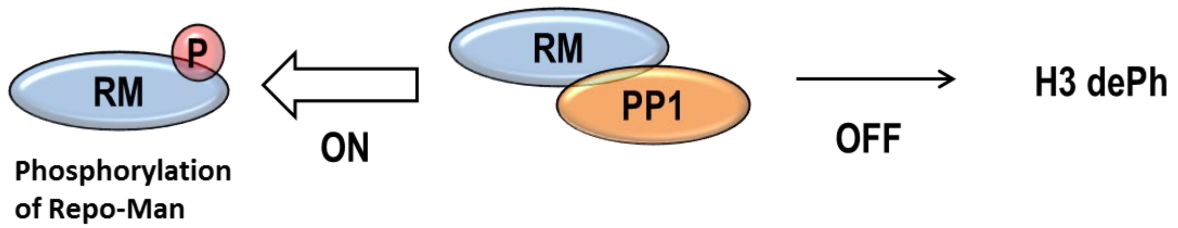


Figure 1.8: Repo-Man localisation throughout the cell cycle. Repo-Man (green) is nuclear at interphase and disperses as the cells enter mitosis. Repo-man is kept away from the mitotic chromatin until anaphase, when where it is stably associated with mitotic chromatin. DNA (blue) Repo-Man (green-upper panels; grayscale- bottom panel) Scale bar 10µm.



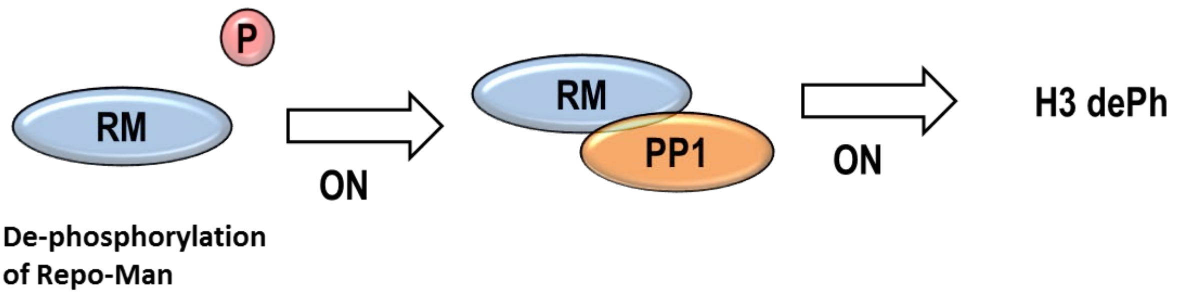


Figure 1.9: A diagram showing the regulation of Repo-Man/PP1 complex throughout mitosis. At early mitosis Repo-Man phosphorylation occurs at a high level, which favours towards keeping Repo-Man OFF the chromosomes, whereas at late mitosis, Repo-Man is released from phosphorylations, which favours the Repo-Man/PP1 complex to load onto chromosomes and de-phosphorylate histone H3.

1.5.2 Regulation of Repo-Man throughout mitosis

Repo-Man activity is controlled by CDK1-Cyclin B phosphorylation on T412 and T419 (Vagnarelli et al., 2011). Phosphorylated Repo-Man has low affinity for chromosomes and this prevents early recruitment of Repo-Man to mitotic chromatin. In fact, it was shown that when CDK1-Cyclin B phosphorylation sites are mutated to phospho-deficient Alanine, Repo-Man localizes on mitotic chromatin early at prometaphase/metaphase (Vagnarelli et al., 2011). CDK1-Cyclin B phosphorylation is also important for regulating the binding of PP1 to Repo-Man. It is, in a way, a mechanism to make sure the Repo-Man/PP1 holoenzyme is not fully activated before anaphase onset. The activation of the complex at the right time is important to maintain sufficient H3 phosphorylation on chromosomes. Full activation of the complex is achieved when Repo-Man is de-phosphorylated by PP1 itself. This makes Repo-Man both a substrate and a targeting subunit for PP1 (Vagnarelli, 2014).

Another phosphorylation of Repo-Man is achieved by Aurora B kinase at Ser893, which was reported to be de-phosphorylated by PP2A and play and important role in Repo-Man recruitment on chromosomes (Qian et al., 2013). When phosphorylated at this site, Repo-Man localisation on mitotic chromatin is blocked. Similar to the CDK1 sites, when this Aurora B site is mutated to the phospho-mimetic Aspartic Acid, Repo-Man did not localise on chromatin even when it is meant to at mitotic exit. These regulation sites on Repo-Man indicate that the control of the complex is achieved through phosphorylation of Repo-Man rather than PP1 and there might be more sites that regulate Repo-Man PP1 binding and localisation.

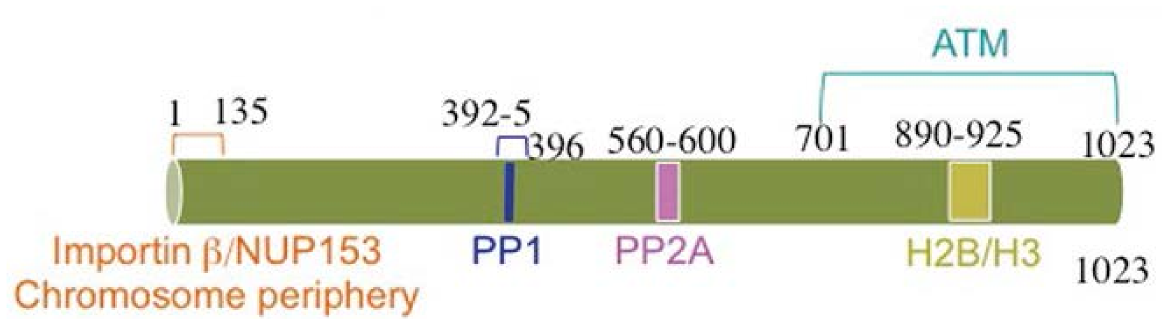


Figure 1.10: A diagram showing Repo-Man binding domains. N terminal domain of Repo-Man regulates its interaction with nuclear membrane proteins, whereas the C terminal domain regulates its binding to chromatin as well as its interaction with ATM kinase. Furthermore, two more important domains are placed towards the C terminal site where the binding with PP1 and PP2A occurs.

In addition, a recent study showed that the S400 residue on Repo-Man might contribute to its localisation and might be phosphorylated as well. Indeed, phospho-mimetic mutations on this site combined with the CDK1 sites (T412 and T419) keep Repo-Man OFF the mitotic chromatin and similarly the phospho-deficient mutations on the same sites cause pre-mature localisation of Repo-Man onto the chromatin (Qian et al., 2015). Although this suggests that S400 phosphorylation may contribute towards Repo-Man localisation on chromatin, it is still not clear which kinase is responsible from its phosphorylation.

The sites that are de-phosphorylated by Repo-Man/PP1 complex have different functions. Among these sites Thr3 represents the docking site for the Chromosome Passenger Complex (CPC) thus de-phosphorylation of this site is important for the inactivation of the spindle assembly checkpoint (Yamagishi et al., 2010; Wang et al., 2010; Kelly et al., 2010; Jeyaprakash et al., 2007). The Ser10 mark on the other hand, is involved in the regulation of heterochromatin protein 1 (HP1) binding. HP1 recognizes the methylated H3K9 and directs heterochromatin formation (Fischle, 2005; Hirota et al., 2005). When Ser10 is phosphorylated by Aurora B, HP1 is dissociated from heterochromatin and re-associated after de-phosphorylation of Ser10 by Repo-Man/PP1 complex. This is an important function of Repo-Man in organizing heterochromatin environment in post-mitotic cells (Vagnarelli et al, 2011; De Castro et al, 2016).

Overall, Repo-Man is considered to be one of the important players in mitotic exit, as its activity is necessary for a successful mitotic exit as well as cell viability. It has also been reported to play an important role in nuclear envelope reformation (explained in detail in the Repo-Man and nuclear envelope section) and to be overexpressed in several cancers (explained in detail in the Repo-Man and cancer section).

1.6 Nuclear envelope dynamics during mitosis

1.6.1 Nuclear envelope proteins

The nuclear envelope (NE) is formed of the inner nuclear membrane (INM), the outer nuclear membrane (ONM) and the perinuclear space in between. INM is a complex structure, which is composed of lamins (type A and B), nuclear lamin-associated membrane proteins (LAP1/LAP2), lamin B receptor (LBR) and emerin. INM serves as a dock for proteins like BAF and HP1 (heterochromatin protein 1) and helps these proteins to provide a link between the lamina and the chromatin, whereas ONM extends to the endoplasmic reticulum (ER) towards the cytoplasm (Figure 1.10). The open spaces in the NE are occupied by the nuclear pore complexes (NPCs), which provide the rapid transport of proteins out of the nucleus, into the cytoplasm, and are formed of nucleoporins (Nups) (de Castro et al., 2016).

Mammalian cells require a series of complicated events during mitosis. Since they undergo an open mitosis, disassembly and reassembly of the nuclear envelope are critical to let the chromosomes interact with the mitotic spindle and both require major reorganization and instrumentation of related proteins. Nuclear envelope breakdown (NEBD) is achieved by phosphorylation of the NPCs and lamins and by mechanical disruption, achieved by the mitotic spindle (Burke and Ellenberg, 2002). At the end of this process, the mitotic chromatin is free to attach the mitotic spindle. However, it is also critical that after mitosis, the phosphorylations are reversed for the re-formation of the NE.

Several kinases are involved in NEBD. One of them is cyclin dependent kinase 1 (CDK1), which targets the type-A lamins in early prophase (Peter et al. 1990) and another one is protein kinase C, which targets type-B lamins in late prophase (Heald and McKeon 1990). The phosphorylation dynamics on lamins are important for the dissociation of chromatin from the NE. Nucleoporins of the NPC are also phosphorylated by several kinases to complete the full breakdown and dissociation of NE (Laurell et al. 2011).

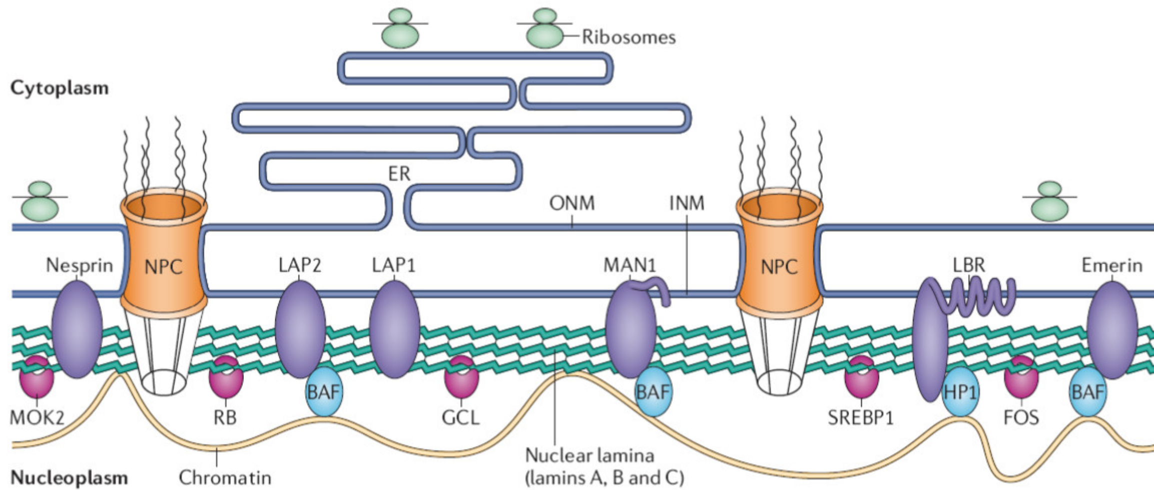


Figure 1.11: Structure and layers of the nuclear envelope. The structural units of the nuclear envelope (NE) are shown. The inner (nucleus) and outer (cytoplasm) nuclear membrane (INM and ONM) are separated by the nuclear pore complexes (NPC) and pore proteins as well as INM proteins and lamins. Lamins form the nuclear lamina underlying the INM, and these are anchored by transmembrane proteins to the peripheral chromatin. Nesprin, lamina-associated proteins 1 and 2 (LAP1 and LAP2), emerin, the lamin B receptor (LBR) and MAN1 are nuclear envelope proteins that are bound to the lamina. Transcription factors are also present, including sterol response element binding protein (SREBP1), the retinoblastoma transcriptional regulator (RB), germ cell-less (GCL), FOS and MOK2. Barrier to autointegration factor (BAF) also binds to the nuclear lamina. Heterochromatin protein 1 (HP1) binds both chromatin and the LBR. (Image taken from Coutinho et al., 2009)

1.6.2 Repo-Man and nuclear envelope

PP1 and PP2A are known to be involved in NE reassembly at M/G1 transition. Especially PP1 was reported to be involved in the de-phosphorylation of Lamin B (Thompson et al., 1997) and with the help of its targeting subunit A-kinase anchoring protein (AKAP149). PP1 is recruited at the nuclear envelope for a successful reassembly (Steen et al., 2000). Hence, PP1 targeting to the NE is a pre-requisite for NE reassembly. The phosphatase responsible for the de-phosphorylation of NUPs on the other hand, remains unknown. However, there is evidence that NUP153 and NUP50 interacts with PP1 and its targeting subunit Repo-Man (Moorhead et al., 2007; Vagnarelli et al 2011; de Castro et al, 2016).

Repo-Man was found to interact directly with Importin β and this binding was abolished upon phosphorylation of Repo-Man by CDK1 at the N terminal domain. This interaction was reported to be important for the targeting of Importin β to the chromosome periphery and it does not depend on the catalytic activity of Repo-Man/PP1 complex (Vagnarelli and Earnshaw, 2012; Vagnarelli et al., 2011). In addition, Repo-Man was also found to interact with NUP153 and NUP50 at mitotic exit, and this interaction is important to maintain a pool of Repo-man a repressive chromatin environment at the nuclear periphery (de Castro et al, 2016).

Since Importin β levels are critical for NE reassembly (Walther et al., 2003; Harel et al., 2003), targeting it to the NE is a crucial step and this makes Repo-Man an important player of NE reassembly after mitosis. Moreover, a pool of Repo-Man was observed to localize at the chromosome periphery after anaphase, and at the rim of the NE at interphase. Furthermore, Repo-Man depletion compromises the morphology of the NE

possibly due to the inability of targeting Importin β to the chromosome periphery during NE reassembly, as shown by Lamin A/C staining (Vagnarelli et al., 2011).

Although there is sufficient data to conclude that Repo-Man is important for NE reassembly, it is not the only regulator and Repo-Man recruitment only is not sufficient to trigger the complete reassembly pathway (Vagnarelli et al, 2011). There are other key proteins to consider like ELYS-MEL28, which mediates incorporation of some NUPs into the NE (Guttinger et al., 2009), and the lamins. However, it is tempting to speculate that the localisation of a phosphatase to the NE could potentially be important to de-phosphorylate other relevant NE targets.

1.6.3 Regulation of Lamin A/C throughout the cell cycle

Lamin A/C, coded by the *LMNA* gene, is a member of intermediate filament proteins (IFs), which share a structurally conserved rod domain, and variable head and tail regions (Eriksson et al., 2009). Expression of Lamin A/C is highly regulated and it depends on differentiation. Further to its role as a nuclear shaper, Lamin A/C also has other defined binding partners that are related to transcription and signalling, including DNA and histone proteins (Simon and Wilson, 2013). Its ability to bind to DNA directly or indirectly, may indicate that it is one of the important regulators of the chromatin environment (Dechat et al., 2008).

Regulation of Lamin A/C is achieved via post-translational modifications throughout the cell cycle. The most substantial of these is phosphorylation but other modifications like sumoylation, acetylation and farnesylation were also reported (Snider and Omary, 2014; Simon and Wilson, 2013). There are 70 unique phosphorylation sites on Lamin A/C and it is well-known that phosphorylation of lamins occurs continuously throughout the cell cycle, during both interphase and mitosis. So far, functions of these phosphorylations have been explained in terms of nuclear targeting or assembly/disassembly of lamins (Luscher et al., 1991; Ottaviano and Gerace, 1985; Rzepecki et al., 2002).

As mentioned before, CDK1 directly phosphorylates Lamin A/C during mitosis (Peter et al., 1990). The mitotic sites S22 (N terminus) and S392 (C terminus) have been shown to promote disassembly of nuclear lamina when phosphorylated (Heald and McKeen, 1990). Among these sites, S22 has been shown to be more important as it promotes head-to-tail de-polymerization, whereas the N terminal S392 is not sufficient on its own (Luscher et al., 1991).

CDK5 (cyclin dependent kinase 5) has also been reported to phosphorylate Lamin A/C at the mitotic sites to induce neuronal apoptosis (Chang et al., 2011), and AKT has been shown to phosphorylate Lamin A/C at S404 to regulate *LNMA* gene expression (Bertacchini et al., 2013). Considering the number of phospho-sites on Lamin A/C, it is likely that there are more kinases to be identified which are involved in its regulation.

A recent study showed that Lamin A/C is phosphorylated not only in mitosis but also in interphase. Interphase phosphorylation is concentrated on the head, the beginning of the tail and the end of the tail (Kochin et al., 2014). It was also reported in this study that some of the interphase phospho-sites are also the sites known as the mitotic sites (S22 and S392). Western blot and immunofluorescence analyses on these sites revealed that they are indeed phosphorylated at interphase, but as expected, at a much lower rate than during mitosis. Furthermore, mutation analyses showed that several serine residues on Lamin A/C are important for its association with the nuclear lamina as well as its mobility and localization. Phosphorylated LaminA/C was found to be more mobile than the un-phosphorylated protein (Kochin et al., 2014).

After mitosis, reversal of all the events depends on de-phosphorylation of all targets, so that re-association of the NE can take place. It is known that Protein phosphatase 1 (PP1) is recruited at the periphery of the chromosomes at telophase to direct de-phophorylation of Lamin B by its targeting subunit AKAP149 (Thompson et al. 1997; Steen et al. 2000). Another targeting subunit Repo-Man de-phosphorylates importin β and targets it to NE (Vagnarelli et al. 2011; Qian et al, 2015). Protein phosphatase 2A (PP2A) is also involved by de-phosphorylating Barrier to autointegration factor (BAF) and allowing it to re-associate with chromatin (Asencio et al. 2012). Although Lamin A/C was observed to localize at some

chromosome regions at early anaphase, where it extends at a later stage, (Haraguchi et al. 2008; Dechat et al. 2004), there is not information on its de-phosphorylation.

It has been recently reported that the re-assembly of Lamin A/C after mitosis depends on its SUMO-interacting motif (SIM). In this study, Moriuchi et al. identified one of the SIMs on Lamin A/C as an important facilitator for its interaction with SUMO isoforms. They also report that this motif is essential for Lamin A/C recruitment on the chromosomes but most importantly, it is necessary for de-phosphorylation of the S22 at the end of mitosis (Moriuchi et al., 2016). Although it is still not quite clear which phosphatase or phosphatases are involved, this study proves that sumoylation of Lamin A/C may serve as a specific signal and promote its interaction with phosphatases at the end of mitosis.

1.7 Repo-Man and cancer

The first correlation between Repo-Man and cancer progression was proposed when expression profiling of more than a hundred neuroblastoma tumours was published (Krasnoselsky et al., 2005). This study showed Repo-Man among the top scored genes that are overexpressed in stage 4 neuroblastoma tumours. Furthermore, at protein level, Repo-Man overexpression was found to correspond with N-Myc expression in neuroblastoma cells and the survival curve of neuroblastoma patients with high and low levels of Repo-Man showed that high levels of Repo-Man corresponds with poor prognosis (Vagnarelli, 2014). Although the mechanism behind this effect is not known, this is the indication that Repo-Man may be used as a diagnostic marker for neuroblastoma.

Another gene expression profiling on melanoma cell lines revealed signature genes that are associated with melanoma progression. These genes include the cell cycle progression genes that are upregulated and genes related to DNA replication and repair. Repo-Man was included in this list of signature genes but no further studies were done to understand the relevance in detail (Ryu et al., 2007).

Repo-Man was identified as a gene that correlates with the metastatic behaviour of synovial sarcoma (SS) as well. The importance of the t(X;18) translocation in SS is well established however the genetic basis is not clear. With the study that was performed on metastatic and non-metastatic SS tumours for a gene expression profiling comparison, Repo-Man, together with KIF14, was identified as a top ranked gene that drives metastasis of SS, which is more common in adults than children (Legarde et al., 2013).

The most striking study that links Repo-Man with cancer progression was on breast cancer cells. Normal, malignant and metastatic breast cancer cells were compared for Repo-Man expression and the results showed that malignant and metastatic breast cancer tumours have high levels of Repo-Man. Furthermore, depletion of Repo-Man stopped growth of malignant MCF10A-CA1h breast cancer cell line in soft agar (Peng et al., 2010), and arrested squamous cell carcinoma cells at G1, leading to apoptosis (Uchida et al., 2013). These were the first studies suggesting that Repo-Man could potentially become a therapeutic target for breast cancer and other cancers, as its absence was strikingly effective towards negatively regulating cell proliferation in cancer cells.

Repo-Man has also been suggested to be involved in DNA damage response and was shown to interact with ATM kinase in a PP1 dependent manner. In Xenopus oocytes and egg extracts, Repo-Man was shown to interact with ATM but this interaction does not seem to be conserved in other systems as the same results were not confirmed in DT40 and HeLa cells (Peng et al., 2010). In Xenopus system, Repo-Man was found to target PP1γ at the DNA damage site and negatively regulate the DNA damage response. The release of Repo-Man from the site and dissociation from active ATM leads to an increased DNA damage response. Repo-Man role in DNA repair mechanism can also contribute to its properties as a cancer driver gene, as it can provide a platform for mutations and chromosome rearrangements and therefore trigger tumour formation.

In addition, Repo-Man also controls chromosome segregation and counteracts Aurora B kinase, which may lead to aneuploidy and lagging chromosomes. As mentioned before, Repo-Man is one of the important players of mitotic exit and any defect in its function, is likely to make the cells more susceptible to accumulate mutations due to hyper-phosphorylation of Histone H3. Moreover, since Repo-Man is involved in de-phosphorylating histone H3, its loss of function could cause problems in chromatin organization after mitosis and defects in heterochromatin environment.

2. MATERIALS AND METHODS

2.1 Tissue Culture

HeLa cells were cultured in DMEM high glucose, GlutaMAX™ supplement (Gibco) with addition of 10% foetal bovine serum (Labtech) and 5% penicillin/streptomycin (Gibco), at 37^0C in 5% CO_2 humid incubator. The cells were passaged (1:10) every time they reached 80-90% confluency by trypsinization (TryplE, Gibco).

Chicken B cell lymphoma DT40 cells were cultured in RPMI 1640 Medium, L-Glutamine (Gibco) with addition of 10% foetal bovine serum (Labtech), 5% penicillin/streptomycin (Gibco) and 1% chicken serum, at 39^0C in 5% CO_2 humid incubator. The cells were passaged (1:25) once every two days when they reached 90% confluency.

HCC1143 triple negative breast cancer and CA1h malignant breast cancer cell lines were cultured by Lorena Ligammari. Wound healing assays with these cell lines were also performed by her.

2.2 Site Directed Mutagenesis

All mutations were performed by using GENEART® Site-Directed Mutagenesis System and AccuPrime™ Pfx DNA Polymerase (Invitrogen). The DNA concentration to be used was optimised to 100ng/µl and a PCR mixture was prepared for each reaction (Table 2.1) by using specifically designed oligonucleotides (Eurofins Genomics, Germany) (Table 2.5) and the reaction was performed by following suggested PCR parameters (Table 2.2).

Table 2.1: Components of the PCR mix

Component	Volume	Final Concentration
10X AccuPrime™ Pfx Reaction mix	5 µL	1X
10X Enhancer	5 µL	1X
Primer mix (10 µM each)	1.5 µL	0.3 µM each
Plasmid DNA (100 ng/µL)	1 µL	100ng
DNA Methylase (4 U/µL)	1 µL	4 units
25X SAM	2 µL	1X
AccuPrime™ Pfx (2.5 U/µL)	0.4 µL	1 unit
PCR water	to 50 µL	

Following the PCR, a recombination reaction (20µl total volume) was performed (Table 2.3) for 10 min at room temperature (stopped by adding 1 µL 0.5 M EDTA) and 2µl of this reaction was used for the transformation reaction.

Table 2.2: Parameters for PCR

Temperature	Duration	Number of Cycles
37^{0}C	20 min	1
94^{0}C	2 min	
94^{0}C	20 sec	18
57^{0}C	30 sec	
68^{0}C	30 seconds/kb of plasmid	
68^{0}C	5 min	1
4^{0}C	forever	1

Table 2.3: Components of the recombination reaction mix

Component	Volume	Final Concentration
5X Reaction Buffer	4 μL	1X
PCR water	10 μL	-
PCR Sample	4 μL	-
10X Enzymer Mix	2 μL	1X

One Shot® MAX Efficiency® DH5α™-T1R competent cells (provided with the kit) were used for all transformation reactions. After adding 2μl of the recombination reaction on competent cells, the cells were incubated on ice for 12 min, at 42^{0}C for 30 sec and again on ice for 2 min. Samples were then removed from ice and 250 μL of pre-warmed SOC medium was added. After one hour of incubation at 37^{0}C (shaking), the samples were plated on LB-Agar plates containing the correct antibiotic without performing any dilutions and left overnight at 37^{0}C to grow.

The next day, colonies were picked and grown in LB media overnight with the appropriate antibiotic. DNA was extracted from these cultures and sent for sequencing. The colony that had the mutation was then grown further and the mutated plasmid DNA was extracted by following a midi prep kit without any optimisations (Qiagen midi prep kit, USA).

Table 2.4: List of the mutations performed.

Name	Mutation	Plasmid
3A	S400A:T412A:T419A	GFP:RMwt (pEGFPc1)
3D	S400D:T412D:T419D	GFP:RMwt (pEGFPc1)
FA	F404A	GFP:RMwt (pEGFPc1)
Res2 (oligo res)	T177T:L179L:T180T:R181R	GFP:RMwt (pEGFPc1)
3ARes	T177T:L179L:T180T:R181R	GFP:3A (pEGFPc1)
3DRes	T177T:L179L:T180T:R181R	GFP:3D (pEGFPc1)
4ARes	T177T:L179L:T180T:R181R	GFP:3ARes (pEGFPc1)
4DRes	T177T:L179L:T180T:R181R	GFP:3DRes (pEGFPc1)
FARes	T177T:L179L:T180T:R181R	GFP:FA (pEGFPc1)
IRCE	K762R	GFP:RMwt (pEGFPc1)
IKCA	E764A	GFP:RMwt (pEGFPc1)
GST-CtermIRCE	K762R	GST:CtermRM (pGEX-4T)
GST-CtermIKCA	E764A	GST:CtermRM (pGEX-4T)
RVDF	T394D	GFP:Laci:RMwt (pEGFPc1)
2ARes	S400A:S407A	GFP:Res2 (pEGFPc1)
IRCERes	T177T:L179L:T180T:R181R	GFP:IRCE (pEGFPc1)
IKCARes	T177T:L179L:T180T:R181R	GFP:IKCA (pEGFPc1)
1ARes	S407A	GFP:Res2 (pEGFPc1)
PP1gR20Q	R20Q	RFP:PP1gwt (pEGFPc1)

Table 2.5: List of oligonucleotide sequences used for mutation analyses.

Mutation	Oligonucleotide sequence 5' - 3'
S400A	FW- ttggagaggacttagccccggaagtgtttga
	Rev- tcaaacacttccggggctaagtcctctccaa
S400D	FW- ttggagaggacttagacccggaagtgtttga
	Rev- tcaaacacttccgggtctaagtcctctccaa
F404A	FW- taagcccggaagtggctgatgaatctttgcc
	Rev- ggcaaagattcatcagccacttccgggctta
T419D	FW- tgcgtaaaggaggagatcctgtttgtaaaaa
	Rev- tttttacaaacaggatctcctcctttacgca
Oligo res (Res2)	FW- aaagagtccgagatgactgacttaacgaggaaggaaggtctcagcg
	Rev- cgctgagaccttccttcctcgttaagtgagtcatctcggactcttt
S407A	FW- aagtgtttgatgaagcattgccagcaaat
	Rev- atttgctggcaatgcttcatcaaacactt
PP1gR20Q	FW- cggctgctggaggtgcaaggatcaaaaccaggt
	Rev- acctggttttgatcctggcacctccagcagccg
T394D	FW- aagaggaagagagttgcatttggagaggactta
	Rev- taagtcctctccaaatgcaactctcttcctctt
T419A	FW- ttgcgtaaaggaggagcacctgtttgtaaaaaa
	Rev-ttttttacaaacaggtgctcctcctttacgcaa
T412D	FW- tctttgccagcaaatgatccattgcgtaaagga
	Rev-tcctttacgcaatggatcatttgctggcaaaga
T412A	FW- tctttgccagcaaatgatc cattgcgtaaagga
	Rev- tcctttacgcaatggatcatttgctggcaaaga
IRCE	FW- ccagatttaaacataaggtgtgaaagaaaggat
	Rev- atcctttctttcacaccttatgtttaaatctgg
S407D	FW- aagtgtttgatgaagacttgccagcaaat
	Rev- atttgctggcaagtcttcatcaaacactt

2.3 Transfections

2.3.1 Transfections using jetPRIME® reagent

All transient transfections with HeLa cells were performed by using the jetPRIME® reagent (Polyplus). Cells were seeded on 6-well tissue culture plates (10^5 cells/well) on the first day and left to grow for 24 hrs in 2ml media. On the second day, the transfection mixture was prepared for each reaction (2µg DNA diluted in 200µl of jetPRIME® buffer) correct amount of siRNA (110pmoles) was also added to this mixture if necessary (Table 2.6) and 4µl of jetPRIME® reagent was added to each mixture. After 10 min of incubation at room temperature, the transfection mixture was added directly to the media and the cells were left to grow for 48 hrs.

Table 2.6: List of siRNA sequences used in transfection experiments.

Name	Sequence 5'-3'
Repo-ManUL	UGACAGACUUGACC
Repo-Man 5	Hs_CDCA2_5 (Qiagen)
Control	CGUACGCGGAAUACUUCGAdTdT

2.3.2 Electroporation

All transient transfections with chicken B cell lymphoma DT40 cell line were performed by using Neon™ transfection system. Correct volume of cells (containing $5x10^6$ cells/transfection) was harvested and washed with PBS. The cell pellet was resuspended in 100µl of Buffer R (provided with the kit) and 10µg of DNA was added to the mixture. The cells were picked up with the Neon™ pipette by using Neon™ tips and exposed to electric current (1600V, 10ms, 3 pulses) in the Neon™ cuvette filled with 3ml of Buffer E (provided with the kit). Cells were then left to grow in 10ml of media without antibiotics in T25 tissue culture flasks for 24 hrs.

2.3.3 Generating stable cell lines

HeLa stable cell lines were generated using the jetPRIME® reagent. The cells on 6-well tissue culture plates were transfected with a plasmid DNA coding fluorescent tagged protein of interest as described before and left to grow for 24 hrs. After 24 hrs, the cells were trypsinized and resuspended in 2ml of media and transferred to 100mm tissue culture dishes containing 10ml of media (5 dishes for each well, 400µl of cells in each dish). Correct amount of G418 selection (Gibco, 1:25, 2mg/ml final concentration) was then added to each dish and the cells were left to grow for a minimum of 10 days. At the end of the selection process, the colonies were collected and further grown separately. Each colony was observed under the fluorescent microscope and colonies that expressed the fluorescent tag were selected.

DT40 stable cell lines were generated using Neon™ transfection system. The cells were transfected with the desired plasmid that contains a fluorescent tagged protein as described previously and left to incubate for 24 hrs. At the 24 hr time point, cells were put in G418 selection (Gibco, 1:25, 2mg/ml final concentration) in a total volume of 50ml and seeded onto 96-well plates (100µl/well) and left to grow for a minimum of 10 days. At the end of the selection process, the colonies were collected and further grown. Each colony was observed under the fluorescent microscope and positive colonies were selected.

2.4 Immunofluorescence

After transfecting the cells on tissue culture plates as described previously and applying drug treatments if necessary (Table 2.8), they were fixed with 4% PFA for 5 min. HeLa cells were transfected in tissue culture plates containing cover slips and fixed directly on the plate, whereas DT40 cells were transferred to a poly-l-lysine microscope slides to provide surface attachment 24hrs after the transfection. After fixation, cells were washed with PBS and blocked in 1% BSA for 30 min at 37^0C (100µl/slide).

Antibody dilutions were prepared in 1% BSA (Table 2.7) and the primary antibody was applied for 30 min at 37^0C after blocking (80µl/slide). The cells were then washed with PBS (3 times for 5 min) and incubated with the secondary antibody for 30 min at 37^0C (80µl/slide). After another PBS wash, HeLa cover slips were mounted on slides and DT40 slides were covered with cover slips with the addition of Vectashield. All the slides were kept at 4^0C to be observed and imaged with the fluorescent microscope.

Table 2.7: List of antibodies used for immunostaining experiments.

Antibody	Species	Dilution	Company	Catalog number
H3T3ph	Rabbit	1:1000	Millipore	04-746
ACA	Human	1:1000	-	-
c-myc	Rabbit	1:800	Abcam	ab32072
Aurora A	Rabbit	1:200	Abcam	ab12875
Lamin A/Cph	Rabbit	1:500	Cell Signaling Technologies	13448
Lamin A/C total	Rabbit	1:250	Abcam	ab108595
γTubulin	Mouse	1:1000	Cell Signaling Technologies	5886
αTubulin	Mouse	1:1000	Cell Signaling Technologies	3873
Texas Red	Anti-rabbit Anti-mouse	1:200	Jackson Immunoresearch	711-585-152 115-076-062
Alexa Fluor 488/FITC	Anti-rabbit Anti-mouse	1:200	Jackson Immunoresearch	715-095-150 715-545-150
Cy5	Anti-human	1:200	Jackson Immunoresearch	709-175-149

Table 2.8: List of drugs used for immunostaining experiments.

Name	Function	Final Concentration
Nocadazole	Blocks polymerization of microtubules	20µM
ZM447439	Aurora B inhibitor	20µM

2.5 Cloning

2.5.1 Primer design and PCR

All cloning primers were designed specifically for each experiment and ordered from Eurofins Genomics (Table 2.11). PCR was prepared by using the standard Taq DNA polymerase and buffer (NEB, UK), dNTP mix (NEB,UK), appropriate plasmid DNA and primers designed previously to a final volume of 50µl (Table 2.9). The reaction was carried out following the correct parameters (Table 2.10). When the reaction was terminated, 5µl of all PCR samples were run on an agarose gel (1%).

Table 2.9: PCR ingredients and concentrations.

Name	Stock Concentration	Amount Used (µl)	Final Concentration
Standard taq buffer	10X	5	1X
dNTP	10mM	1	0.2mM
Forward primer	100µM	1	2µM
Reverse Primer	100µM	1	2µM
Standard taq polymerase	5000 units/ml	0.25	25 units/ml
Nuclease free water	-	Up to 50µl	-

Table 2.10: PCR parameters for cloning experiments.

Temperature	Duration	Number of Cycles
95^0C	30 sec	1
95^0C	30 sec	30
Tm of the primer pair (see Table 2.11)	1 min	
68^0C	30 seconds/kb of plasmid	
68^0C	5 min	1
4^0C	forever	1

Table 2.11: List of oligonucleotides used for cloning experiments.

Name	Sequence	Enzyme Sequence Added (if applicable)	Tm (^0C)
GSTRMFW	tttgagtccggagaggacttaagcccggaa	BamHI	57
GSTRMRev	Attctcgagtcttctgttagaagtccttgttattct	XhoI	55

2.5.2 Cloning into pGEM®-T Easy vector system

The amount of PCR reaction to be ligated into pGEM®-T Easy vector system (Promega, USA) was calculated by referring to the 5µl PCR product run on the agarose gel. The weight and intensity of the band were used to calculate the concentration of the PCR product and the ligation reaction was prepared as recommended and left overnight at 4^0C (table x). All reagents, except the PCR product, were provided with the kit.

After the overnight incubation, all 15µl of the ligation reaction was used to transform E.Coli DH5α competent cells (see later for protocol). The ligation reaction was added directly on top of 100µl of competent cells and mixed gently. The cells were then incubated on ice for 30 min, heat shocked at 42^0C for 30 sec, put back on ice for 5 min and diluted in 250µl of SOC medium (see later for recipe). After one hour incubation at 37^0C (shaking, 225rpm) all of the transformation reaction was plated on LB agar plates (see later for recipe) with Ampicillin (50µg/µl, Fisher, UK), X-gal (1.25%, Fisher, UK) and IPTG (1M, Fisher, UK) and left overnight in the 37^0C incubator to grow. The next day white colonies were collected to be grown overnight in LB and plasmid DNA was extracted from grown cultures (MoBio mini prep kit, USA). The extracted plasmid DNA was then used for a digestion reaction to check if the insert was successfully ligated into pGEM®-T Easy vector system. A reaction was prepared by using the appropriate enzymes (1µl each, NEB, UK), reaction buffer (6µl, NEB, UK), BSA (3µl, NEB, UK), plasmid DNA (1µg) and nuclease free water (up to 50µl, Thermo Scientific, UK) and incubated at 37^0C for 3 hours. After the incubations, samples were run on agarose gel (1%) and positive clones were grown further to get high quality DNA (Qiagen midi prep kit, USA).

Table 2.12: Reagents for pGEM®-T Easy vector system ligation reaction.

Reagent	Amount required for ligation reaction (μl)	Amount required for control reaction (μl)
2X Rapid Ligation Buffer	7.5	7.5
pGEM®-T Easy Vector (50ng)	1	1
PCR product	Calculated for 1:3 ratio	-
T4 DNA Ligase	1	1
Nuclease free water	Up to 15μl	Up to 15μl

2.5.3 Cloning from pGEM®-T Easy vector system into another plasmid

To clone a fragment from pGEM®-T Easy vector system into another plasmid, the pGEM®-T Easy vector and the recipient plasmid were digested separately with appropriate enzymes. A reaction was prepared by using the enzymes (2µl each, NEB, UK), reaction buffer (6µl, NEB, UK), BSA (3µl, NEB, UK), plasmid DNA (10.5µg insert, 5µg recipient vector) and nuclease free water (up to 50µl, Thermo Scientific, UK) and incubated at 37^0C for 3 hours. Digested plasmids were then run on agarose gel (1%, 5µl of each sample) to confirm the size of the products. All the remaining digested product of the donor plasmid was then run separately and the correct sized band was excised. The excised band was then used to extract the DNA (Qiagen gel extraction kit, USA) and 5µl of it was run on gel along with 5µl of the recipient vector. The gel was used to calculate the concentrations of the insert and the vector and a ligation reaction was prepared accordingly. Vector DNA (50ng), insert DNA (calculated for 1:3 ratio), T4 ligase buffer (2µl, Thermo Scientific, UK), T4 ligase (0.2µl, Thermo Scientific, UK), nuclease free water (up to 20µl, Thermo Scientific, UK) was used to prepare the ligation reaction and a control reaction without the insert was also prepared (Table 2.12). Reactions were then left overnight at 4^0C.

The next day, transformation reaction was prepared as described previously and colonies were collected after 24 hrs. The colonies were grown in 5ml of LB media overnight and the DNA was extracted (MoBio mini prep kit, USA) and digested to check for the correct insert as described previously. The positive colony or colonies were sent to sequencing (Source Biosciences, Cambridge, UK) and grown further to obtain high quality plasmid DNA with high concentration (Qiagen midi prep kit, USA)

2.6 Western Blotting

2.6.1 Solutions and reagents

- 10X Running buffer:
  - 0.25M Tris (Sigma, Germany)
  - 1.92M Glycine (Sigma, Germany)
  - dH_2O to 1L
- 1X Running buffer:
  - 100ml 10X running buffer
  - 5ml 20% SDS (Fisher Scientific, UK)
  - dH2O to 1L
- 10X Transfer buffer:
  - 0.25M Tris
  - 1.92M Glycine
- 1X Transfer buffer:
  - 100ml 10X transfer buffer
  - 5ml 20% SDS
  - 200ml methanol (Fisher Scientific, UK)
  - H_2O up to 1L
- 30% Acrylamide (Severn Biotech, UK)
- TEMED (Fisher Scientific, UK)
- 10% APS:
  - 1g APS (Sigma, Germany)
  - dH2O to 10ml

- PBS-Tween
    - 1X PBS (tablets, Fisher Scientific, UK)
    - 0.1% Tween 20 (Fisher Scientific, UK)
    - dH2O to 1L
- SDS gel buffer for running gel:
    - 1.5M Tris-HCl (pH 8.8)
    - 0.4% SDS
    - dH2O to 500ml
- Stacking gel buffer
    - 0.5M Tris-HCl (pH 6.5)
    - 0.4% SDS
    - H2O to 100ml
- 10% Protein gel
    - 2.5ml SDS gel buffer for running gel
    - 3.3ml 30% Acrylamide stock
    - 4.2ml H_2O
    - 100µl 10% APS
    - 10µl TEMED

2.6.2 SDS-PAGE gel electrophoresis, transfer onto membrane and visualization

All western blot protocols were performed with 10% protein gels (except for sumoylation assay) and assembled in BioRad Mini-PROTEAN Electrophoresis System. After loading, gels were run for 2 hrs in 1X running buffer (constant 130V) then transferred onto the nitrocellulose membrane (GE, USA) (constant 200mA) for 2hrs in 1X transfer buffer. The membrane was blocked overnight with 5% milk in PBS-Tween at 4^0C (shaking) and the appropriate primary antibody (diluted in 3% milk in PBA-Tween) was applied for an hour at room temperature the next day (see table 2.7 for antibody dilutions). Fluorescently labelled secondary antibodies (LI-COR, US) were applied (1:5000) in 1% milk in PBS-Tween for an hour at room temperature. The membrane was visualised by using the LI-COR Odyssey imaging system.

2.7 In-vitro sumoylation assay

2.7.1 Solutions and reagents

- 1M Tris-HCl pH 8
    - 121.1g Tris base (Sigma, Germany)
    - Adjust pH with concentrated HCl
    - H_2O up to 1L
- 5M NaCl
    - 292g NaCl (Sigma, Germany)
    - H_2O up to 1L
- 10% NP-40
    - 1g of 100% NP-40 (Roche, Germany)
    - H_2O up to 10ml
- 1M PMSF
    - 17.42g PMSF (Fisher Scientific, UK)
    - DMSO up to 100ml
- CLAP
    - Chymostatin at 1mM (Sigma, Germany)
    - Leupeptin at 2 mM (Sigma, Germany)
    - Antipain at 80 µM (Sigma, Germany)
    - Pepstatin A at 1.5 mM (Sigma, Germany)

- Lysis buffer
    - 5ml 1M Tris-HCl pH 8
    - 8ml 5M NaCl
    - 10ml 10% NP-40
    - 5ml 10% NaDeox
    - 200µl 1M PMSF
    - 100µl CLAP
    - H_2O up to 100ml
- Lysozyme
    - 0.2g Lysozyme (Sigma, Germany)
    - H_2O up to 10ml
- Coomassie blue stain
    - 0.1% Coomassie Brilliant Blue R-250,
    - 50% methanol
    - 10% glacial acetic acid
- De-stain
    - 40% methanol
    - 10% glacial acetic acid

2.7.2 Protein expression and purification from bacteria

GST tagged plasmids were transformed into E.Coli Rosetta strain and single colonies were picked to grow in 10ml of LB overnight with the appropriate antibiotic at 37^0C. The next day a 1:100 dilution of the culture was grown in a total 300ml until an OD of 0.7 - 0.9 and the cells were induced with IPTG (1:1000 from 1M stock, Fisher Scientific) at 18^0C overnight (shaking). The next day cell pellets were collected (4000g for 20min) and each pellet was resuspended in the lysis buffer and 1ml of lysozyme. After 30min incubation on ice, the samples were sonicated at full power at least 3 times (30sec ON, 30sec OFF). Sonicated material was transferred into 2ml Eppendorf tubes and centrifuged for 10min at 14000rpm. The supernatant was collected into a new tube. Glutathine beads (Sigma, Germany; 420µl for each sample) were centrifuged (500g for 5min) and washed with 1ml of lysis buffer. The beads were resuspended with the cell lysate and incubated at 4^0C for an hour (rotating). The tubes were then centrifuged (500g for 5min) and washed with the lysis buffer for 3 times. Beads were then resuspended in 100µl elution buffer (10mM Tris-HCL pH7.4, 150mM NaCl, reduced glutathione, dH_2O) and incubated at 4^0C for 30min (rotating). The samples were then centrifuged (500g for 5min) and a second elution was performed. After the second elution, 30µl of the samples were mixed with 1X sample buffer and run on SDS-PAGE gel, stained with the Coomassie blue dye and de-stained.

2.7.3 In-vitro sumoylation reaction

In-vitro sumoylation reaction was performed by using the corresponding kit from Enzo Life Sciences, USA. For each sample three positive reactions were prepared for each SUMO protein (SUMO1, 2 and 3) as well as a negative reaction without ATP. RanGAP1 was used as a control as it gets sumoylated by all SUMO isoforms. A negative reaction for RanGAP1 without ATP was also prepared. The reactions were incubated at 37^0C for 1hr and 20µl of 3X sample buffer was added to all reactions after. All samples were incubated at 95^0C for 5min and loaded onto the 12% SDS-PAGE gel for separation. The proteins were then transferred onto a PVDF membrane (as described before) and blocked with 1% BSA in PBS-Tween for an hour at room temperature. The membrane was then washed for 10min (3 times) in PBS-Tween and anti-SUMO1 anti-SUMO 2/3 and antibodies (provided with the kit) were added (1:1000 in 1%BSA in PBS-Tween) for overnight incubation at 4^0C. After the antibody incubation, the membrane was washed in PBS-Tween for 10min (3 times). Secondary anti-rabbit IgG (HRP linked) antibody (Abcam, UK) was applied in 1% BSA in PBS-Tween (1:2000) for 1h at room temperature and the membrane was washed with PBS-Tween for 10min (6 times). ECL detection reagent (Fisher Scientific, UK) was prepared and applied. The 5min exposure signal was detected on X-ray film (GE, USA).

2.8 Proximity ligation assay

Duolink® (Sigma, Germany) was used for the proximity ligation assay. Cells were grown, fixed and blocked on cover slips and primary antibodies were applied as described in immunofluorescence section. Cover slips were then washed with PBS for 5min (2 times). PLA probes (provided with the kit) were diluted as described (1:5) and 80µl were applied on each cover slip. Samples were incubated for 1h at 37^0C and washed with 1X wash buffer A (provided with the kit) for 5min (2 times). The ligation stock (1:5) and ligase (1:40) mix were diluted as instructed and applied on the cover slips. After 1h incubation at 37^0C, 1X wash buffer A was applied for 2min (2 times). Amplification stock (1:5) and the Polymerase (1:80) were diluted as instructed and applied on cover slips. After a 100min incubation at 37^0C, samples were washed in 1X wash buffer B for 10min (2 times) and 0.01X wash buffer B for 1min. Mounting medium (Vectashield) with DAPI was applied and samples were prepared for imaging.

2.9 Fluorescence microscopy and analysis

All images presented in this report were taken using the Nikon Eclipse Ti-E inverted microscope system. For every image, Z stacks (0.300 µm steps, approx. 20 stacks for each image) captured the signal and the stacks were projected (maximum intensity projection) to get the whole signal for analyses and presentation. For single cell analyses, 100X objective was used, whereas for the analysis of a group of cells, 40X objective was used. The fluorescence intensity analyses were conducted with the Nikon software which helped us measure ROI sum intensity in a chosen area or in the whole image for the desired channel. For single locus analysis circular ROI was defined and only the intensity on the locus was detected. For whole image analysis all nuclei in the image was automatically detected by the software and the intensity was measured. All analyses were done subtracting the background, where there was not any cells or fluorescence signal related to the cells. Live cell imaging was also performed with the same system where we maintained the cells at 37^0C in Leibovitz's L-15 Medium (Gibco).

The statistical analyses were done using the Mann-Whitney U test as Gaussian distribution was not expected. For PLA analysis, Chi-Square test was used. P values were represented as follows: ns ($P > 0.05$), * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), **** ($P \leq 0.0001$).

2.10 Preperation of competent E.Coli cells

2.10.1 Solutions and media

- TFB1 (pH 5.8):
    - 30Mm KAc (Fisher Scientific, UK)
    - 100mM RbCl (Fisher Scientific, UK)
    - 10mM $CaCl_2$ (Fisher Scientific, UK)
    - 50mM $MnCl_2$ (Fisher Scientific, UK)
    - 15% Glycerol (Fisher Scientific, UK)
- TFB2 (pH 6.5):
    - 10mM MOPS
    - 75mM $CaCl_2$
    - 10mM RbCl
- LB media:
    - 10g Bacto-tryptone (Fisher Scientific, UK)
    - 5g Bacto-yeast extract (Fisher Scietific, UK)
    - 500mg NaCl (Fisher Scientific, UK)
    - Up to 1L dH_2O
- LB-Agar (1L):
    - 10g Bacto-tryptone (Fisher Scientific, UK)
    - 5g Bacto-yeast extract (Fisher Scientific, UK)
    - 10g NaCl (Fisher Scientific,UK)
    - 15g Agar per 500ml (Fisher Scientific, UK)
- 1M $MgSO_4$ (Fisher Scientific, UK)

2.10.2 Preparation

E.Coli-DH5α strain cells were plated on an antibiotic free LB-Agar plate and incubated overnight at 37^{0}C. A single colony from the plate was inoculated in 3ml of LB and left overnight at 37^{0}C (shaking). The culture was diluted 1:100 the next day and 5ml of $MgSO_4$ was added (total volume 250ml). It was left grown to an OD of 0.4-0.6 at 37^{0}C (shaking). The culture was then centrifuged at 3000rpm for 5min at 4^{0}C and the supernatant was removed. The pellet was resuspended in 100ml of TFB1 and incubated on ice for 5min. After centrifuging for 5min at 3000rpm, 4^{0}C, the supernatant was removed and the pellet was resuspended in 5ml of TFB2. The samples were incubated on ice for 30min and aliquoted into 1.5ml Eppendorf tubes (200μl). The tubes were then fresh frozen in liquid nitrogen and stored at -80^{0}C.

2.11 Other solutions

- SOC media:
    - 2g Bacto-tryptone (Fisher Scientific, UK)
    - 0.5g Bacto-yeast extract (Fisher Scientific, UK)
    - 0.05g NaCl (Fisher Scientific, UK)
    - 2.5M KCl (Fisher Scientific, UK)
    - 10mM $MgCl_2$ (Fisher Scientific, UK)
    - 10mM $MgSO_4$ (Fisher Scientific, UK)
    - 20mM Glucose (Fisher Scientific, UK)
    - H_2O up to 100ml

3. STRUCTURE AND REGULATION OF REPO-MAN/PP1 COMPLEX

3.1 Introduction

Phosphorylated Repo-Man has low affinity for chromosomes and this prevents early binding of Repo-Man on chromosomes. In fact, it was shown that when CDK1-Cyclin B phosphorylation sites are mutated, Repo-Man localizes on mitotic chromatin early at prometaphase/metaphase (Vagnarelli et al., 2011). Aurora B phosphorylation is also important for regulating the binding of PP1 to Repo-Man (Qian et al., 2013). It is, in a way, a mechanism to make sure the Repo-Man/PP1 holoenzyme is not fully activated before anaphase onset. The activation of the complex at the right time is important to maintain sufficient H3 phosphorylation on chromosomes. Full activation of the complex is achieved when Repo-Man is de-phosphorylated by PP1 itself. This makes Repo-Man both a substrate and a targeting subunit for PP1 (Vagnarelli, 2014). All the information obtained so far about the regulation of Repo-Man activity indicates that the control of the complex is achieved through phosphorylation and de-phosphorylation of Repo-Man rather than PP1.

The RVTF flanking region was proven to be important with previous studies but the actual confirmation of the complex and how the two proteins bind to each other through this RVTF flanking site and how this site affects localisation and function of the complex is still unknown. Here we hypothesize that the RVTF flanking region is regulated via multiple kinases and phosphorylation of this region disrupts Repo-Man and PP1 binding, therefore changes the timing of Repo-Man localisation and Histone H3 phosphorylation. In this chapter, with the help of the crystal structure our collaborators (Peti and Page laboratories, Brown University, U.S) kindly provided, we identify new unique sites on Repo-Man (T394, S400 and S407) other than the RVTF motif by NMR crystallography and show that these sites are important for the complex formation and localisation by mutation analyses. Phospho-

deficient and phospho-mimetic mutations were performed on newly identified sites along with previously identified CDK1-Cyclin B sites and the effect of the mutant Repo-Man was investigated in terms of localisation, H3T3 phosphorylation and affinity for PP1.

We also introduce Repo-Man as a Plk1 substrate and propose a model for the regulation of the complex throughout cell cycle, and show a mechanism to explain how the cells maintain H3T3 phosphorylation at the centromeres. In addition, we show the specificity of Repo-Man and PP1γ by a single point mutation (R20), which abolishes the binding. Our findings explain Repo-Man/PP1 complex structure and regulation in detail and introduce a new targetable area for the complex other than the RVTF motif, which is not shared with any other PP1 binding subunits.

3.2 Results

3.2.1 Identification of novel sites on Repo-Man

Repo-Man, like the majority of PP1 binding subunits, has an RVxF (RVTF in case of Repo-Man) motif to facilitate its binding with PP1 and this motif is conserved in different species. RVTF motif allows Repo-Man to recruit PP1 onto mitotic chromatin and mutating this domain abolishes PP1 binding completely, which also results in the abolished recruitment of PP1 onto mitotic chromatin (Trinkle-Mulachy et al., 2006). Furthermore, this mutation does not disrupt chromatin binding of Repo-Man at mitosis, which shows the possibility of other regulatory sites for Repo-Man localisation. Recently, the region flanking the RVTF motif has been shown to play an important role in the localisation and function of the complex. Moreover, comparison of SILAC (Stable isotope labelling of amino acids in culture) based pull-down experiments of mitotic versus roscovitin-treated mitotic cells, have shown that this complex is more stable in anaphase than in early mitosis. Initial studies have shown that phosphorylation at two conserved sites (T412 and T419) by CDK1-Cyclin B was important for the localisation of Repo-Man on chromosomes (Vagnarelli et al., 2011).

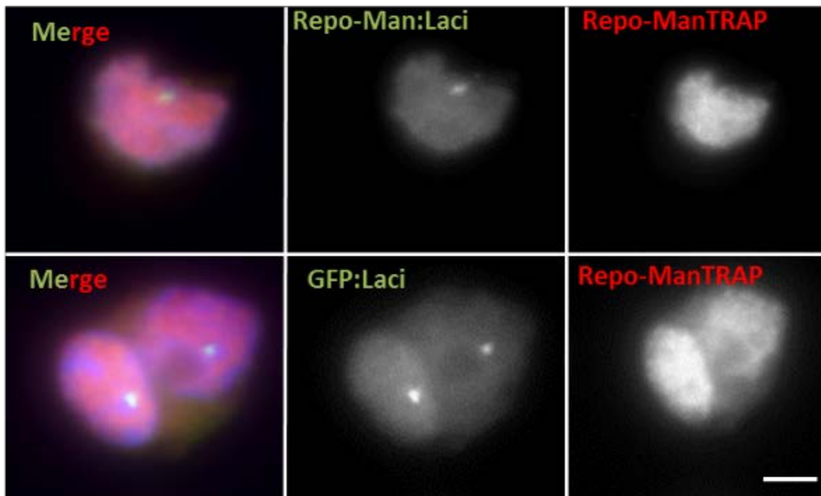
Subsequent studies, published during the course of my PhD, have also shown the importance of the CDK1 phospho-sites T412 and T419 in complex regulation. In addition, S400 was also proposed to be important for Repo-Man localisation onto mitotic chromatin. By mutation analyses on all three sites, it was shown that not only T412 and T419, but also S400 also contribute to the regulation of the complex (Qian et al., 2015). This study also proposed S400 as a putative CDK1 phospho-site, however, the molecular basis for the regulation of the complex structure and assembly were not known.

In order to get more insight into this aspect, we wanted to obtain a crystal structure of the minimal interaction domain between Repo-Man and PP1. To achieve this goal we established a collaboration with Professor Page and Professor Peti (Brown University, USA) who have previously solved several structure of PP1 complexes (Choy et al., 2014; Kelker et al., 2009; Ragusa et al., 2010).

Using NMR spectroscopy and isothermal titration calorimetry (ITC) approaches, our collaborators identified the minimal binding region of Repo-Man to PP1γ and showed that this contains the previously identified sites shown to be important for the complex function (T412, T419 and S400) (Vagnarelli et al, 2011; Qian et al., 2015). The crystal structure revealed that Repo-Man stretches along a large area on PP1 surface and these sites represent contact points between Repo-Man and PP1.

The structure obtained has also revealed other additional interesting features: for example other contact points S407 and F404 (Figure 3.2, A), which are also conserved (Figure 3.2, B). To have a better understanding on the function of these contact points, mutation analyses were undertaken. Traditionally, the experiments to assess the function of a mutant are conducted in the absence of the endogenous proteins since not all the mutations will be dominant-negative and also because we do not know if this complex acts as a monomer or it can multimerise.

A.



B.

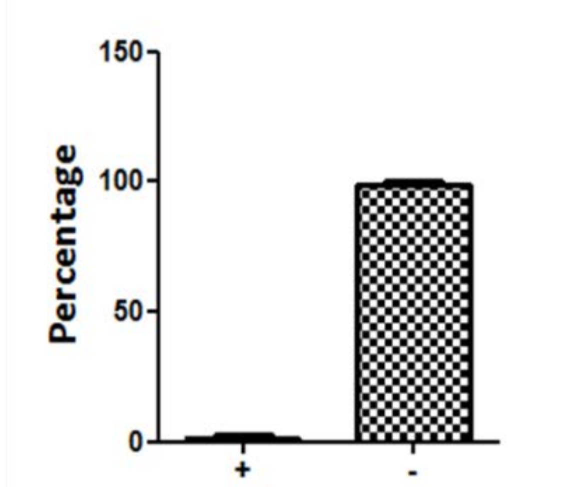


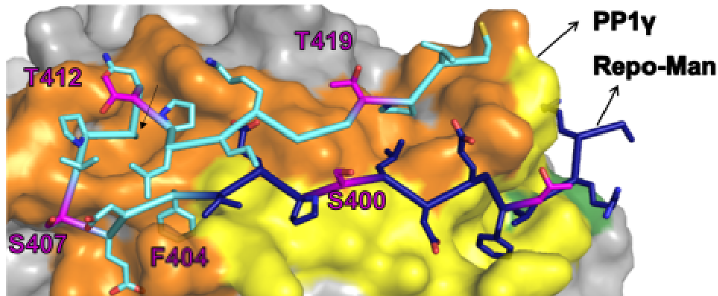
Figure 3.1: Repo-Man does not dimerize. A. DT40 cells with LacO array inserted in their genome (Vagnarelli, 2006) were co-transfected with GFP:Laci:Repo-Man and Repo-ManTRAP constructs and stained with anti-SBP antibody (in red). A single Repo-Man:Laci signal in green was observed but a red signal could not be detected. GFP:Laci only, was used as a control. Scale bar 5µm. **B.** Quantification of the experiment in A. Graph shows the percentage of the cells that have both signals together (+) or just have one signal (-) in Repo-Man:Laci and Repo-ManTRAP co-transfected cells. Images are representative of a single experiment. Total cell count is 100. Fluorescent microscope is used.

Although my experiments suggest that the complex likely acts as a monomer (Figure 3.1), I decided to test the function of these sites in absence of the endogenous protein. In order to perform these experiments, I have first generated an RNAi-resistant version of the GFP-tagged full length Repo-Man construct (GFP:RM^{res2}) (Figure 3.2, C) by site directed mutagenesis. The mutations were designed to change the codon but not the amino acid sequence so that the constructs could be expressed in an RNAi background.

To prove that the oligo-resistant construct worked, HeLa cells were transfected with the GFP:RM^{res2} and GFP:RM^{wt} together with control and Repo-Man siRNA oligos (Figure 3.3, A). GFP:RM^{res2} transfection was detectable in both control and treatment, whereas GFP:RM^{wt} was only present in control siRNA treated cells. Moreover the localisation of the GFP:RM^{res2} construct was undistinguishable from the GFP:RM^{wt} .

The new identified sites were then mutated to Alanine (A) and Aspartic Acid (D) on the oligo-resistant mutant (Res2) along with previously identified amino acids to mimic the phospho-deficient and phosphorylated states of the protein (Figure 3.2, D).

A.



B.

Repo_Human	386	NMRKRKRVTFGEDLSPEVFDESLPANTPLRKGGTPVCI	422
Repo_Mouse	382	NPRKRKRVTFGEDLSPEVFDESLPANTPLCKGGTPVRI	412
Repo_Chicken	330	KRAKKKKVTFGDDLSPEIFDKTLPANTPLRKGSTPVCI	360
Repo_Rat	373	NLRKRKRVTFGEDLSPEVFDESLPANTPLCKGGTPVHI	403

C.

	175 … 186
Repo-Man wt	gagatgacagacttgaccagaaaggaaggtctcagc
	E M T D L T R K E G L S
Oligo res (Res2)	GAGATGACTGACTTAACGAGGAAGGAAGGTCTCAGC
	E M T D L T R K E G L S

D.

	S400 F404 S407 T412 T419
Res2	taagcccggaagtgtttgatgaatctttgccagcaaatactccattgcgtaaaggaggaacacct
	L S P E V F D E S L P A N T P L R K G G T P
3DRes	TAGACCCGGAAGTGTTTGATGAATCTTTGCCAGCAAATGATCCATTGCGTAAAGGAGGAGATCCT
	L D P E V F D E S L P A N D P L R K G G D P
3ARes	TAGCCCCGGAAGTGTTTGATGAATCTTTGCCAGCAAATGCTCCATTGCGTAAAGGAGGAGCACCT
	L A P E V F D E S L P A N A P L R K G G A P
FARes	TAAGCCCGGAAGTGGCTGATGAATCTTTGCCAGCAAATACTCCATTGCGTAAAGGAGGAACACCT
	L S P E V A D E S L P A N T P L R K G G T P
4ARes	TAGCCCCGGAAGTGTTTGATGAAGCATTGCCAGCAAATGCTCCATTGCGTAAAGGAGGAGCACCT
	L A P E V F D E A L P A N A P L R K G G A P
4DRes	TAGACCCGGAAGTGTTTGATGAAGACTTGCCAGCAAATGATCCATTGCGTAAAGGAGGAGATCCT
	L D P E V F D E D L P A N D P L R K G G D P
2ARes	TAGCCCCGGAAGTGTTTGATGAAGCATTGCCAGCAAATACTCCATTGCGTAAAGGAGGAACACCT
	L A P E V F D E A L P A N T P L R K G G T P

Figure 3.2: First crystal structure of Repo-Man/PP1 complex and identification of a novel interaction site. A. Crystal structure of a fraction of Repo-Man bound to PP1γ, showing the newly identified residues S400, F404, S407 as well as previously identified ones T412 and T419 (Provided by Peti and Page laboratories at Brown University, USA). **B.** Alignment showing the conservation of identified sites (S400 and S407 in orange; T412 and T419 in blue) as well as the RVTF (green) motif among species. **C.** Introduced oligo-resistant mutations (T177, L179, T180 and R181) on GFP:RM^{wt} changed the codons but not the amino acids, which prevented the siRNA binding but conserved wild type phenotype. **D.** Mutations of identified sites performed on the Res2, including A and D mutations on four (S400, S407, T412, T419) and three (S400, T412, T419) of the sites; D mutation on two (S400, S407) of the sites; and A mutation on F404.

3.2.2 Mutations on identified sites affect Repo-Man localisation on chromosomes

In order to assess the function of the new residues identified in the stability of the complex, HeLa cells were transfected with all the Alanine mutants, in a Repo-Man RNAi background. Since these mutants are phospho-deficient, the Repo-Man/PP1 complex was expected to be hyper-active in early mitosis (Vagnarelli et al., 2011; Qian et al., 2015). H3T3ph staining was used to measure the activity of the complex in comparison with the wild type protein. Images of both Repo-Man transfected and un-transfected cells were taken to show the differences of activity, and the H3T3ph staining was normalised against the un-transfected cells on the same slide (Figure 3.4, A).

As expected, depletion of Repo-Man causes a hyper-phosphorylation of H3T3 with a distribution that covers all the chromosomes instead of being restricted to the centromeric region. All the mutants were able to rescue reduce the H3T3 hyper-phosphorylation levels caused by depletion of Repo-Man and the remaining H3T3 phosphorylation on Repo-Man transfected cells was not significantly different than that of wild type Repo-Man (Figure 3.4, C), showing that the complex was indeed active.

Also, transfected cells showed a distinct H3T3ph pattern and to address if the residual phosphorylation was indeed centromeric, GFP:Repo-Man^{FA} transfected cells were stained with the anti-centromeric antibody (ACA). The staining clearly showed that the remaining H3T3 phosphorylation overlaps with the centromeres (Figure 3.5, A).

Although I did not observe dramatic differences between the mutants in terms of activity, I observed significant differences in localisations with some mutants being localised on chromosomes in early mitosis and others not. To quantify these differences, Repo-Man signal intensity was measured on the chromosomes and the cytoplasm at 3 different circular points each, with an additional spot in the background. The background was subtracted and the average signal was calculated for chromosomes and cytoplasm. The ratio of the signal intensity on the chromosomes to the cytoplasm was then calculated for each individual cell. H3T3 phosphorylation was also calculated in a similar way: The overall signal was measured with a single circular ROI, covering all chromosomes, with an additional spot in the background that covers the same area. The background was subtracted and the signal from transfected cells were normalised against the average signal from the un-transfected cells.

These experiments have shown that the 3Ares and 4Ares mutants prematurely localised on the chromosomes at pro-metaphase/metaphase, whereas FAres mimicked the localisation of the wild type protein (Figure 3.4, B).

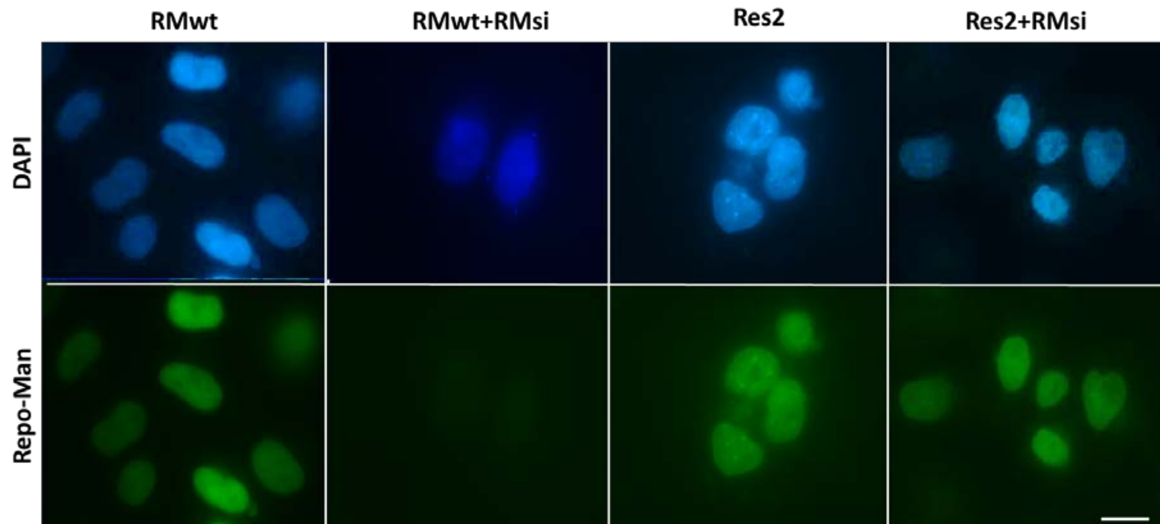


Figure 3.3: Oligo resistant Repo-Man is still expressed in the presence of Repo-Man siRNA. Wild type (RMwt) and oligo resistant mutant (Res2) expression in HeLa cells with control and Repo-Man siRNA (48 hrs), showing the abolished expression of the wild type with Repo-Man siRNA, whereas the Res2 expression is still present. Scale bar 10µm. Images are representative of a single experiment.

A. Repo-Man un-transfected — Merge, Repo-Man, H3T3ph; Repo-Man transfected — Merge, Repo-Man, H3T3ph (wt; S400A T412A T419A; F404A; S400A S407A T412A T419A)

B. Repo-Man Enrichment on Chromosomes (RM wt, 3A, 4A, FA; ***, ***, ns)

C. H3T3 phosphorylation (RMwt, 3A, 4A, FA)

Figure 3.4: Phospho-deficient mutations on newly identified sites cause pre-mature localisation of Repo-Man on chromosomes. A. HeLa cells transfected with the A mutants and stained for H3T3ph in RNAi background, showing the pre-mature localisation of 3ARes and 4ARes mutants on chromosomes and wild type-like localisation of the FARes mutant. H3T3 phosphorylation in transfected cells is visibly less than un-transfected cells. Sacle bar 10µm. **B.** Graph showing mutant and wild type Repo-Man enrichment on chromosomes. 3ARes and 4ARes are significantly higher than the wild type and FARes. **C.** Graph showing the remaining H3T3 phosphorylation, normalised against the un-transfected cells. The phosphorylation does not significantly differ from that of wild type with any of the mutants. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used. Mann-Whitney test is used for significance.

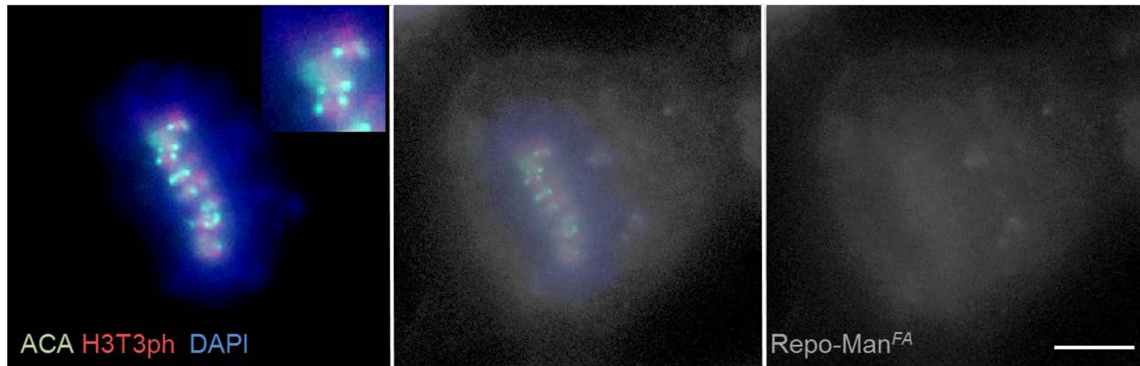


Figure 3.5: Remaining H3T3 phosphorylation overlap with the centromeres. HeLa cells transfected by wild type Repo-Man were stained for H3T3ph (Red) and ACA (Green). Remaining H3T3 phosphorylation on the chromosomes, overlap with the centromeric staining, whereas the chromosome arms (Blue) are completely free of the phosphorylation. (Scale bar 5µm). Images are representative of a single experiment. Fluorescent microscope is used.

3.2.3 PP1 binding is important for Repo-Man localisation on chromosomes

Repo-Man localisation on chromosomes does not happen until anaphase onset and it was shown that this localisation is a dynamic event, depending on the activity of Repo-Man activating and inactivating proteins. So far, Aurora B activity and PP2A-B56 binding has been reported to be important for the regulation of Repo-Man localization on chromosomes. By phosphorylating Repo-Man at S893, Aurora B prevents premature localization until the phosphorylation is removed by PP2A-B56 and if Aurora B is inhibited, premature localization of Repo-Man on chromosomes is observed (Qian et al., 2011). Also a phospho-mimetic mutation on S893 abolishes Repo-man localisation on chromosomes and causes H3T3 phosphorylation to remain (Qian et al., 2011; Qian et al., 2015).

However, the data I have obtained using several mutants I have generated indicate that the region flanking the RVTF motif on Repo-Man is important for the activation of the complex and the localisation of Repo-Man can be controlled on this region. Since we have shown that these amino acids are contact points within the complex, it can be hypothesised that PP1 pays a significant role in the regulation of chromosome targeting of Repo-Man. It is also known that PP1 binding is a major factor for Repo-Man localisation on chromosomes and a mutated RVTF motif (RAXA) abolishes the binding between Repo-Man and PP1, but does not affect Repo-Man binding to mitotic chromatin (Trinkle-Mulcahy et al., 2006).

To test the contribution of these mechanisms, HeLa cells were transfected with wild type and 3A (S400, T412 and T419) mutants along with their non-PP1 binding versions (RM_RAXA and 3A_RAXA), in the presence or absence of Aurora B inhibitor (ZM447439) and their chromosomal localisation was analysed.

In the untreated cells, the 3A mutant localised on the chromosomes at early mitosis as expected, whereas the 3A_RAXA mutant rescued the pre-mature localisation, showing a wild type-like phenotype (Figure 3.6, A). In the ZM treated cells on the other hand, the pre-mature localisation was observed with all mutants as well as the wild type protein. Repo-Man signal intensity on the chromosomes and in the cytoplasm were measured and similar to the previous analyses, the ratio of signal intensity on the chromosomes over the cytoplasm was calculated. Analysis of these localisations shows that there is a significant difference between the RM_RAXA and 3A_RAXA mutants in the untreated cells, as well as the wild type and the RM_RAXA mutant in the treated cells (Figure 3.6, B and C). These datasets clearly indicate that both PP1 and Aurora B contribute to the localisation of Repo-Man to chromosomes.

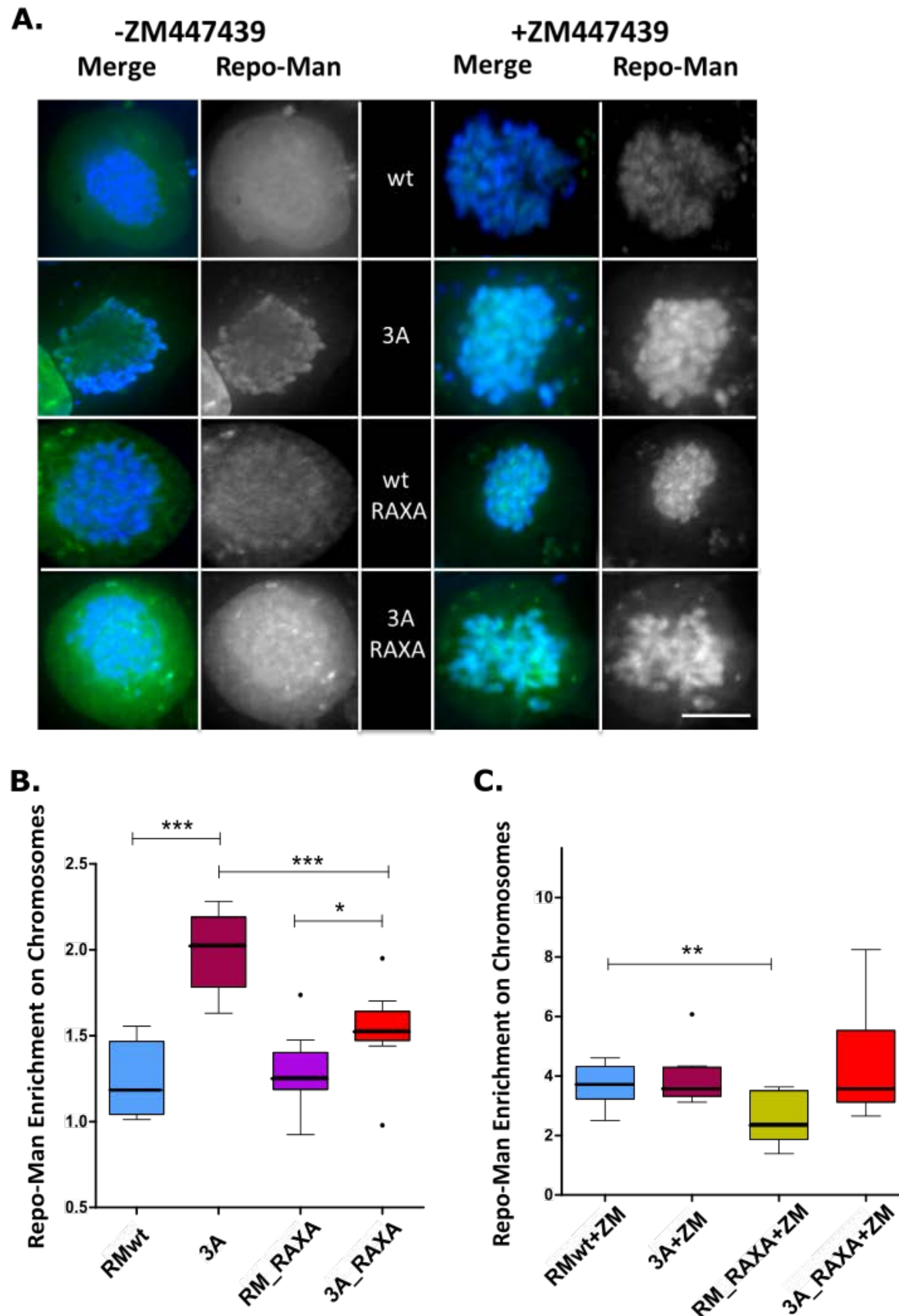


Figure 3.6: PP1 binding is important for Repo-Man localisation on chromosomes. A. HeLa cells transfected with wild type Repo-Man and the 3A mutant along with RM_RAXA and 3A_RAXA in the presence or absence of the Aurora B inhibitor ZM447439. Cells treated with 20µM of inhibitor for 3 hrs. Scale bar 10µm. **B.** Graph showing Repo-Man enrichment on chromosomes on untreated cells. 3A is significantly more on the chromosomes than the wild type, whereas 3A_RAXA is significantly less on the chromosomes when compared to 3A. **C.** Graph showing Repo-Man enrichment on ZM447439 treated cells. There is no significant difference between the mutants and the wild type protein except for RM_RAXA, which is significantly less accumulated on chromosomes. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used.

3.2.4 Phosphorylation of the newly identifies Repo-Man SLIM motif regulates PP1 binding.

After establishing that PP1 is important for Repo-Man localisation on chromosomes, we wanted to directly prove that phosphorylation of these residues were indeed important for PP1 binding, independently of RVTF motif. For this purpose we have mutated the newly identified sites to Aspartic Acid (3D) and cloned the Laci sequence in the construct to generate the GFP:Laci:RM^{3D}. Then we have used a tethering system with chicken DT40 cell lines carrying a LacO array inserted in their genome (Vagnarelli et al., 2006) (Figure 3.7, A) and co-transfected these cells with wild type and mutant Repo-Man along with RFP tagged PP1γ. The transfected cells showed a single spot of wild type or mutant Repo-Man on the chromosomes, where the LacO/Laci interaction occurs, and a red spot was observed on the same spot, if PP1γ was recruited as well. As expected, wild type Repo-Man recruited PP1γ on the same spot, whereas the 3D mutant abolished the binding and did not have any PP1γ signal (Figure 3.7, B). The non-PP1 binding RAXA mutant was used as a control, which also did not recruit PP1. This shows that the complex can be inactivated through the RVTF flanking region.

To see if the phospho-mimetic mutants differ from the wild type protein in terms of localisation and H3T3 phosphorylation, HeLa cells were transfected with 3DRes and 4DRes mutants in RNAi background (Figure 3.8, A). The localisation of the phospho-mimetic mutants showed a dispersed phenotype, which , as expected, did not differ from the wild type, and the remaining H3T3 phosphorylation was not significantly different from that of wild type in prometaphase cells (Figure 3.8, B and C).

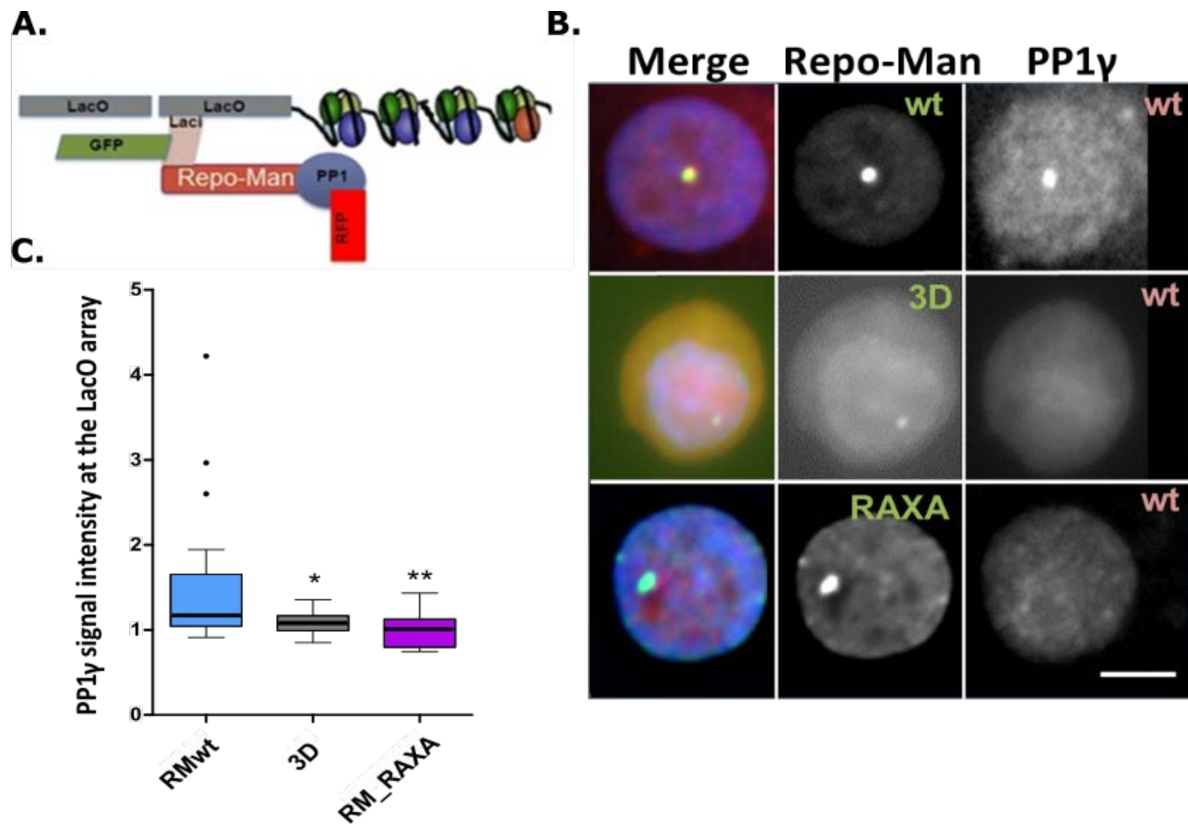


Figure 3.7: Phospho-mimetic mutations on Repo-Man, inactivate the complex by abolishing PP1 binding. A. The tethering system used to assess the formation of the complex in vivo. DT40 cells containing a LacO array inserted at a single locus were co-transfected with Laci:GFP:Repo-Man^{wt} or mutant forms and RFP tagged PP1γ. Using the LacO/Laci interaction, a single Repo-Man-enriched spot was obtained and recruitment of PP1γ was measured. Scale bar 5µm. **B.** PP1γ recruitment on wild type Repo-Man and the non PP1 binding RAXA mutant were used as controls. PP1γ accumulation at the LacO array is significantly reduced with the phospho-mimetic 3D mutant. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 50 for each experiment. Fluorescent microscope is used.

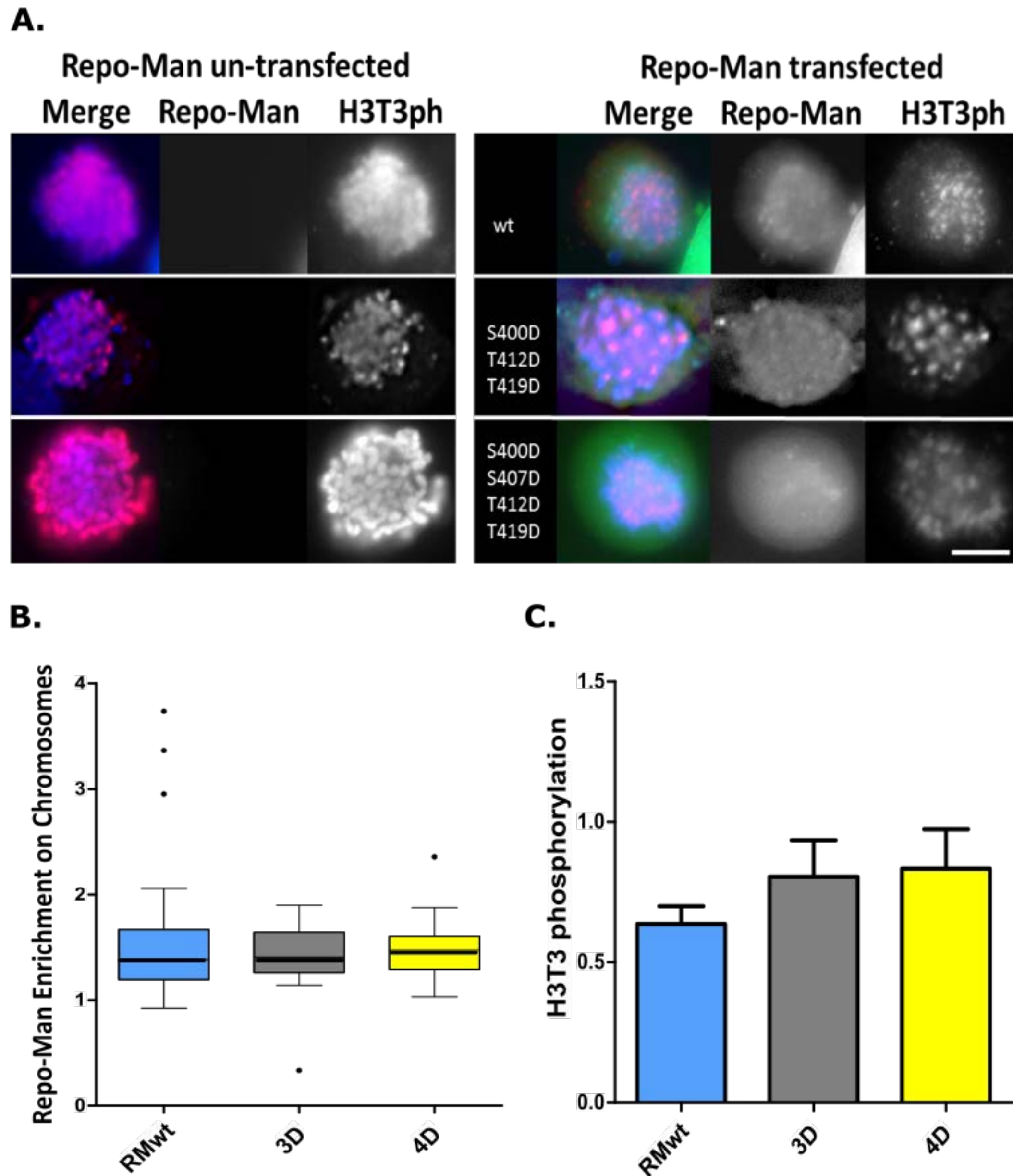


Figure 3.8: Phospho-mimetic mutants do not affect localisation or H3T3 phosphorylation. A. Hela cells transfected with the 3D and 4D mutants as well as the wild type in RNAi background (48 hrs), and stained for H3T3ph. Localisation of the mutants are not different than the wild type. Un-transfected cells were used as a control. Scale bar 10µm. **B.** Graph showing Repo-Man enrichment on chromosomes. There is no significance between the wild type and the mutants. **C.** Quantification of the remaining H3T3 phosphorylation, normalised against the un-transfected cells. There is no significant difference between wild type and mutant proteins. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used.

3.2.5 Phosphorylation of the RVTF motif abolishes the binding between Repo-Man and PP1

We have so far shown that the RVTF motif is responsible for Repo-Man/PP1 binding and the localisation of Repo-Man onto mitotic chromatin along with Aurora B. We have also reported the regulation of a unique region flanking the RVTF motif by phosphorylation in order to keep the complex inactive and off the chromosomes. All these analyses clearly show the importance of both the RVTF motif and the flanking region in complex formation and localisation. In addition, proteomic screenings have also identified previously that T394 of the RVTF motif is also getting phosphorylated (Dephoure et al., 2008; Nousiainen et al., 2006), but the kinase responsible for this phosphorylation remained elusive.

By using in-vitro phosphorylation assay, coupled with NMR spectroscopy and mass spectrometry, our collaborators (Peti and Page laboratories, Brown University, USA) identified Aurora B as the kinase responsible for the phosphorylation of T394 in the RVTF motif. To see if this phosphorylation affects PP1 binding, I have generated a phosphor-mimetic mutant of this site (RVDF) and performed a recruitment experiment. Using the Laci/LacO tethering system, I have recruited the wild type PP1 on wild type Repo-Man but not with the RVDF mutant (Figure 3.9, A). The mutation on this Aurora B site, completely abolished the recruitment of PP1 like the RAXA mutant, which was used as a control.

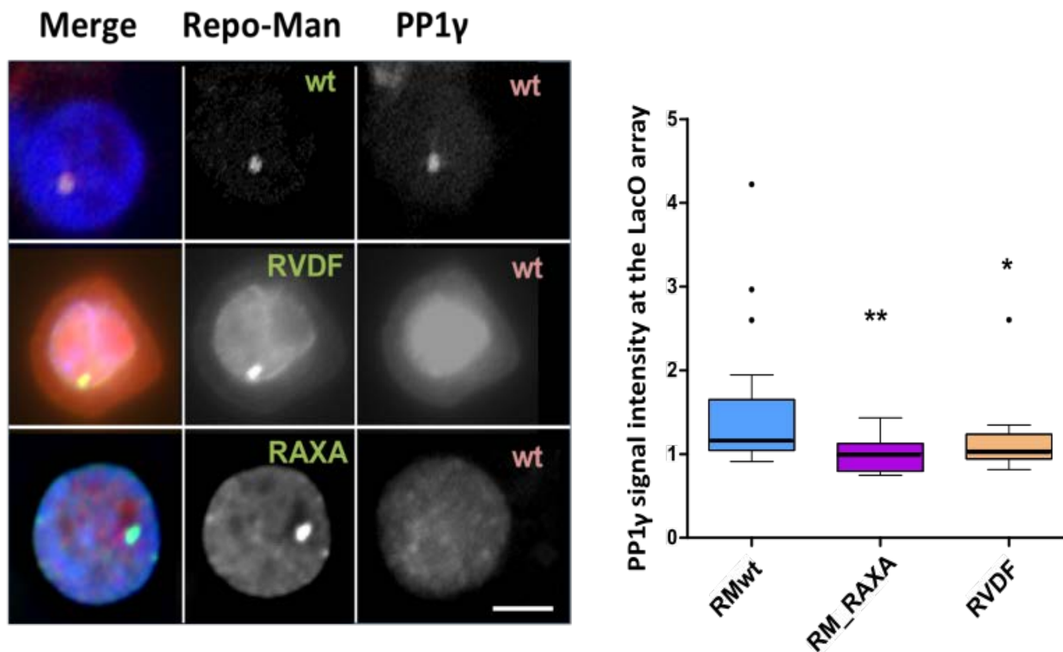


Figure 3.9: Phosphorylation of T394 by Aurora B abolishes the binding between Repo-Man and PP1. A. Chicken DT40 cells were transfected with wild type Repo-Man and RVDF mutant as well as the RAXA mutant, using the Laci/LacO tethering system. Scale bar 5µm. **B.** Analysis of the experiment in A. PP1 signal intensity at the LacO array was measured. Data was analysed using Mann-Whitney test. A significant decrease is observed with the RVDF mutant. Statistics show the comparison with the wild type protein. Images are representative of three repeats. Total cell count is 50 for each experiment. Fluorescent microscope is used. Mann-Whitney test is used for significance.

3.2.6 Is Plk1 involved in the local regulation of Repo-Man/PP1 complex in early mitosis?

I have so far shown that phosphorylation of the newly identified SLIM on Repo-Man is important in regulating the complex at mitosis, therefore it also important to identify how this regulation occurs and which kinases are involved in the process. As mentioned earlier, it is already known that two of the sites in this region (T412 and T419) are phosphorylated by CDK1-CyclinB at early mitosis (demonstrated by in vitro phosphorylation experiments) (Vagnarelli et al, 2011). No experiments have been conducted on the other sites. We have therefore run a database search on this region to identified kinases that might be involved in regulating Repo-Man. Our database research (GPS-Polo) suggested that Plk1 has potential priming (T412 and T419) and phosphorylation (S400 and S407) sites on this region (Figure 3.10, B). Moreover, in a phospho-proteomic screening for PLK1-dependent phosphorylations in mitosis by Santamaria et al. (2010) (Figure 3.10, A), these sites were also identified.

To observe the effect of Plk1 on Repo-Man localisation, the predicted phosphorylation sites of Plk1 (S400 and S407) were mutated to Alanine on the oligo resistant construct (2Ares) and HeLa cells were transfected with either this mutant and or wild type Repo-Man in an RNAi background. The cells were also stained for H3T3ph. The results of these experiments show that the 2Ares mutant does not affect the localisation. Transfected cells showed a dispersed phenotype like the wild type protein (Figure 3.10, C) and do not accumulate on the chromosomes. Nevertheless, these cells showed a difference in terms of the H3T3 phosphorylation. Cells transfected with the 2Ares mutant did not show an accumulation of the remaining H3T3 phosphorylation at the centromeres. Instead, they showed a dispersed although reduced H3T3 phosphorylation (Figure 3.10, D, E).

To analyse this aspect in more detail, HeLa cells stably expressing GFP:CENP-A were transfected with the 2Ares mutant and stained for H3T3ph in an RNAi background. The remaining H3T3 phosphorylation at the CENP-A and distal regions were measured and compared to the wild type. The results showed that the remaining phosphorylation with wild type Repo-Man transfected cells were mostly on the centromeres, whereas with the 2Ares mutant transfected cells it was dispersed and not accumulated at the centromeres (Figure 3.11, A, B). Total H3T3 phosphorylation was again observed to be less with the 2ARes mutant (Figure 3.11, C).

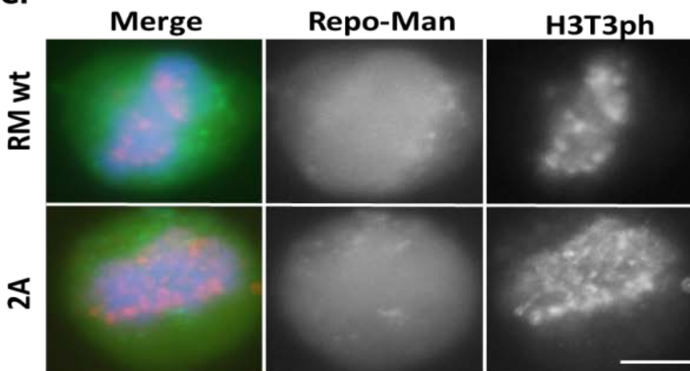
A.

Protein	IPI:IPI00794105
Name	Repo-Man
Position	400
Sequence Window	TFGEDLSPEVFDE
Positions	401, 400
Gene Names	CDCA2
Protein Descriptions	Isoform 1 of Cell division cycle-associates protein 2; 113kDa protein

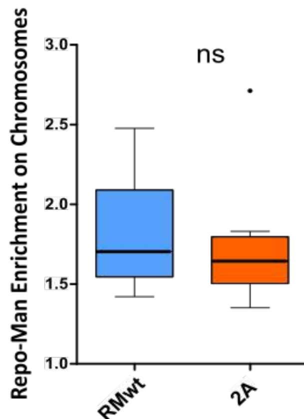
B.

Predicted Plk1 sites (GPS Polo)	
Position	Function
S400	Phosphorylation
S407	Phosphorylation
T412	Phospho-binding
T419	Phospho-binding

C.



D.



E.

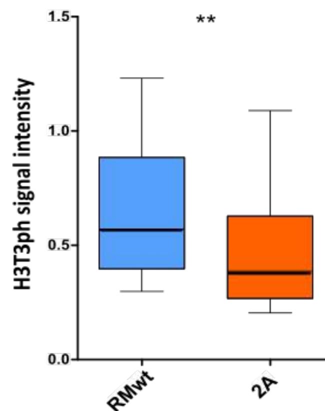


Figure 3.10: Phospho-deficient mutations on Plk1 sites do not affect localisation but makes a difference with H3T3 phosphorylation. A. Predicted Plk1 phosphorylation sites as shown in Santamaria et al. (2010). **B.** Predicted Plk1 binding and phosphorylation sites on GPS Polo database. T412 and T419 are shown as phospho-binding sites, whereas S400 and S407 are shown as phosphorylation sites. **C.** HeLa cells transfected with wild type (RMwt) and mutant (2A) Repo-Man in RNAi background (48 hrs). The 2A mutant shows the same localisation as the wild type protein. Scale bar 10µm. **D.** Quantification of Repo-man localisation on chromosomes. No significant difference between wild type and mutant. **E.** Graph showing the total H3T3ph. The phosphorylation is significantly lower with the 2A mutant. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used.

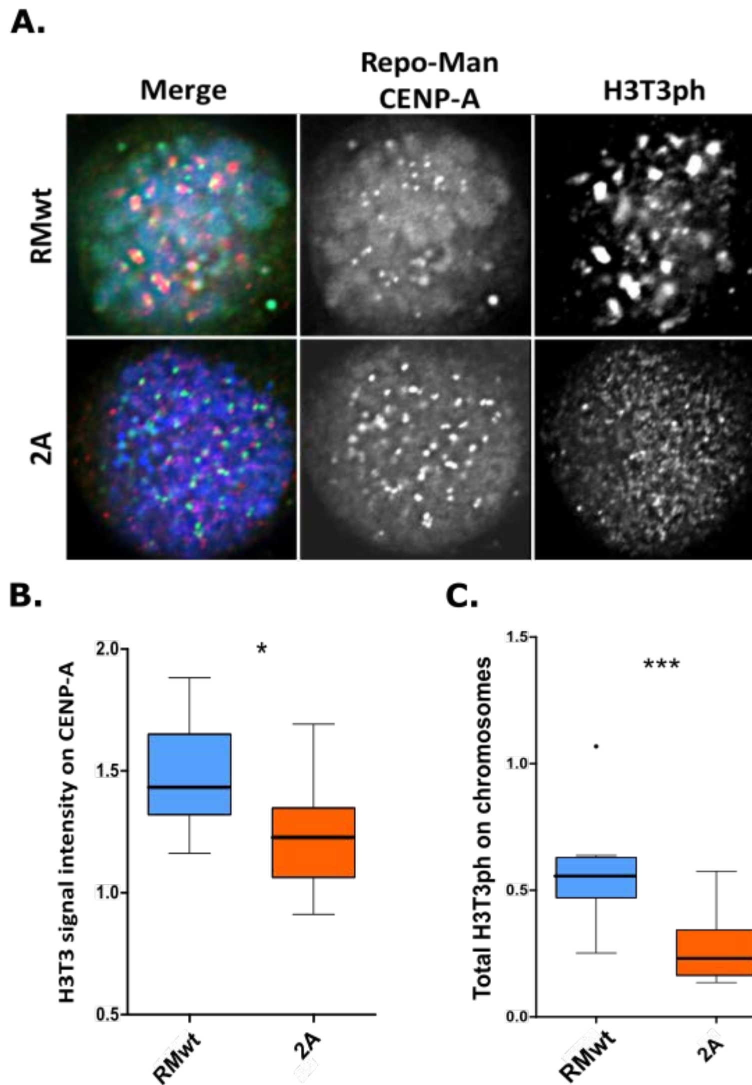


Figure 3.11: Plk1 phsophorylates Repo-Man and maintains H3T3 phosphorylation at the centromeres. A. HeLa cells stably expressing GFP:CENP-A, were transfected with oligo resistant wild type (Res2) and mutant (2ARes) Repo-Man in RNAi background (48 hrs), incubated with 75mM KCl before fixation and stained for H3T3ph. **B.** Graph showing the H3T3ph enrichment at the centromeres for wild type and mutant Repo-Man (n=15). Remaining H3T3ph is significantly higher at the centromeres with the wild type protein. **C.** Total H3T3ph measured all over the chromosomes with wild type and mutant Repo-Man, showing a significantly less phosphorylation with the 2ARes mutant. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used. Mann-Whitney test is used for significance.

3.2.7 Repo-Man specifically binds to PP1γ and not PP1α

It was already shown that Repo-Man successfully recruits PP1γ with high affinity and it also binds to PP1β with a lower affinity but not to PP1α (Booth et al., 2014; Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2011). However, the question about isoform specificity of the targeting subunits is still very much an open question. Based on the amino acid sequence of the different PP1 isoforms (PP1α and PP1γ), it would be expected that the 15 amino acids C-terminal domain is involved in conferring specificity since is the most diverse region among the different isoforms (Figure 3.12). Having generated the crystal structure of PP1 together with the interacting domain of Repo-Man we can start looking into this question: How does Repo-Man differentiate between PP1γ and PP1α?

The N terminus on the other hand, has 4 amino acid differences. Our collaborators (Peti and Page Laboratories, Brown University) have deleted the 15 amino acids at the C terminus and showed that this region does not have an effect on the binding affinity. However, looking in details at the contact point between the two subunits, only the Arg20 in PP1γ is in close proximity to Repo-Man. This Arg is a unique difference among a series of conserved residues. Interestingly, it was previously shown that a mutation of this site had an effect on PP1γ localisation throughout the cell cycle (Lesage et al., 2005), however no molecular clues were available to understand its specific function.

We have therefore mutated the Arg20 residue on the N terminus PP1γ to Gly. This way, we have transformed PP1γ into the exact sequence of PP1α. Our results confirm that this mutant changes localisation throughout the cell cycle. HeLa cells were transfected with both wild type and mutant PP1γ and the cellular distribution of the construct was analysed during the cell cycle.

In early mitosis the mutant showed a dispersed localisation (Figure 3.12, A) and in anaphase, it was not recruited to the chromosomes. Since several studies have shown that Repo-Man is the targeting subunit that is responsible for the recruitment of PP1 to the chromosomes in anaphase (hence the name) we hypothesised that this mutation could indeed disrupt the binding between the targeting and cathalytic subunit.

In order to prove this, I have used the Laci/LacO tethering system. Chicken DT40 cells were transfected with wild type and RAXA mutant Repo-Man along with wild type and mutant PP1γ. Wild type Repo-Man successfully recruited wild type PP1γ, whereas mutant PP1γ was not recruited at all or recruited less than the wild type. RAXA mutant was used as a control, since we have already established that it does not bind to PP1 (Figure 3.13, C).

Since these residues involved in the isoform selection are highly conserved in another PP1 targeting subunit (Ki-67), we tested if the behaviour was the same also for Ki-67. Chicken DT40 cells and the Laci/LacO system were used for this purpose. The cells were transfected with GFP:Laci, as a control, and GFP:Laci:Ki67 constructs as well as wild type and mutant RFP tagged PP1γ. A single GFP or Ki67 locus was generated and PP1γ recruitment on both was calculated. Results show that the R20 on PP1γ is important also for Ki67 binding and changes the behaviour completely. Wild type Ki67 successfully recruited wild type PP1γ, whereas mutant PP1γ was not recruited at all Figure 3.14,A). These experiments altogether represent the first demonstration of the molecular basis for the isoform specificity between a targeting subunit and PP1.

Figure 3.12: Alignment of PP1γ and PP1α protein sequences. Alignment of two PP1 isoforms on T-Coffee online alignment tool. PP1α is represented with PP1a, PP1γ is represented with PP1g and both sequences are the human isoforms. Differences on the C - terminal area are more noticeable than the differences on the N-terminal area, where we expected to see a difference in Repo-Man binding.

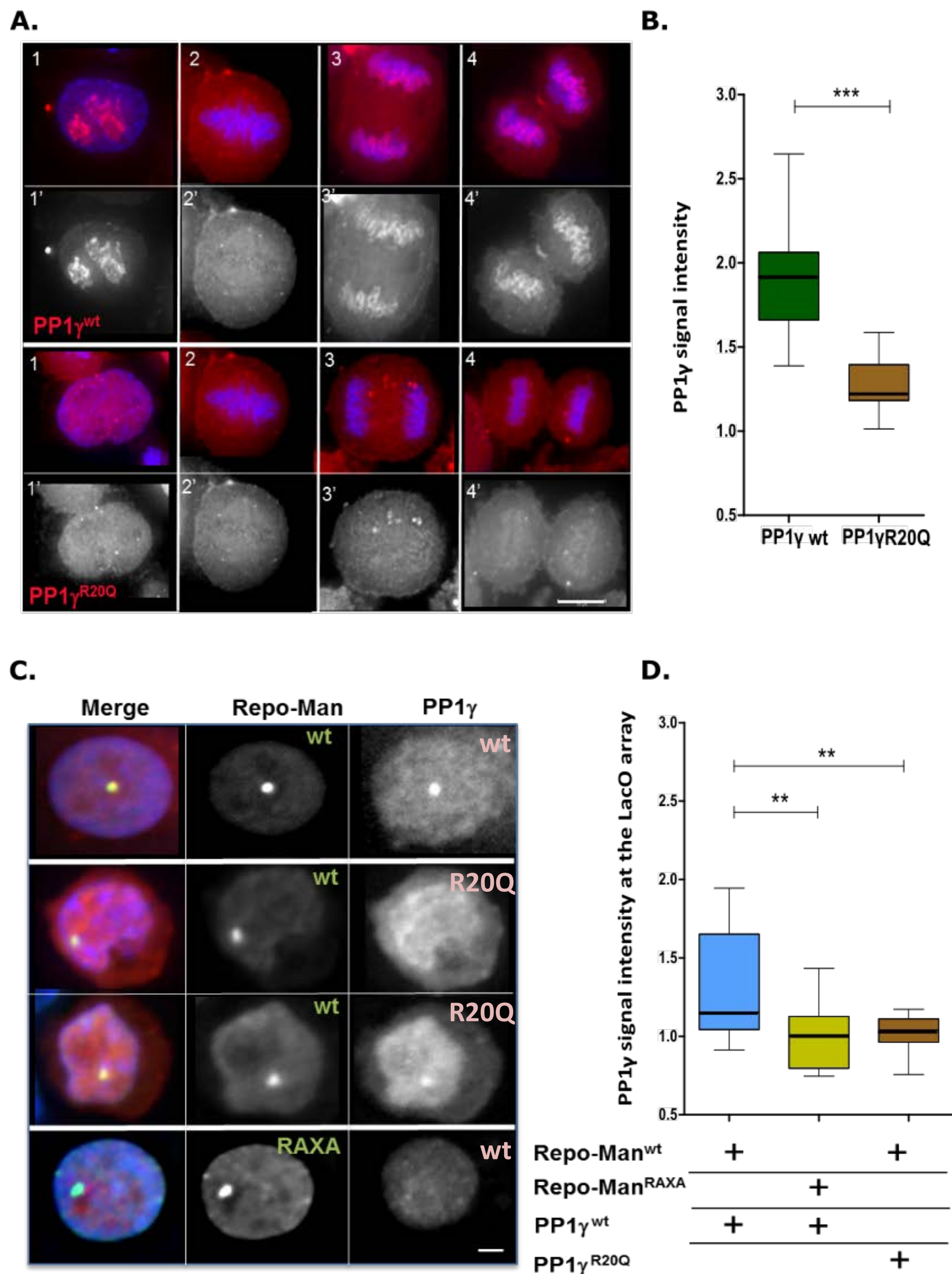


Figure 3.13: R20Q mutation on PP1γ abolishes the localisation and prevents Repo-Man from recruiting PP1. A. HeLa cells transfected with wild type (upper plane) and mutant PP1γ (lower plane) at different stages of the cell cycle. Mutant PP1 changes the localisation completely (scale bar 10µm). **B.** Quantification of PP1γ on chromosomes. R20 mutant is significantly less recruited. **C.** Chicken DT40 cells transfected with wild type Repo-Man along with wild type and mutant PP1γ. Repo-Man cannot recruit mutant PP1γ. RAXA mutant was used as a control (scale bar 5µm). **D.** Quantification of PP1γ recruitment on single Repo-Man focus, showing the significant decrease with PP1γ mutant. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 30 (for section A) and 50 (for section C) for each experiment. Fluorescent microscope is used.

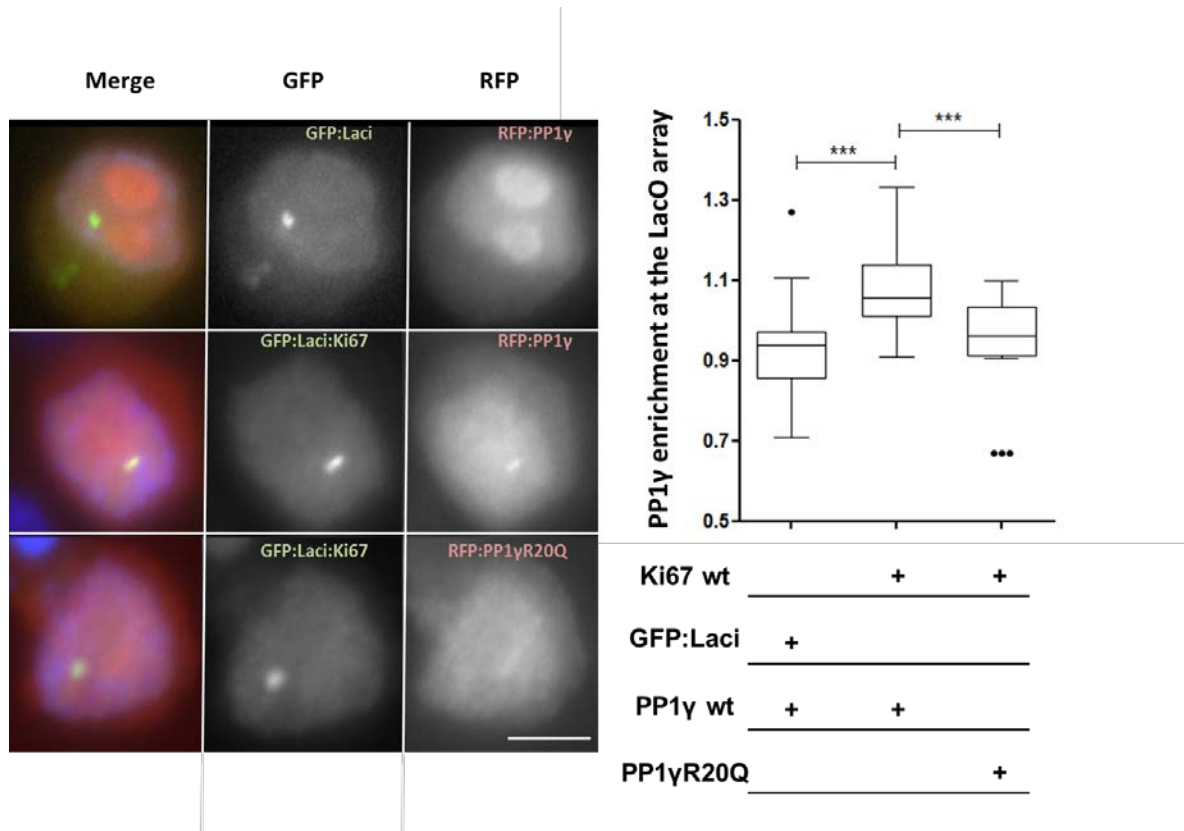


Figure 3.14: Ki67 does not recruit mutant PP1γ. A. Fluorescent images of DT40 cells transfected with GFP:Laci only and GFP:Laci:Ki67 as well as RFP tagged wild type and mutant PP1γ. Mutant PP1γ is not recruited by wild type Ki67. Recruitment on GFP alone was used as a control. Scale bar 5µm. **B.** Quantification of the experiment in A. Mutant PP1γ is significantly less recruited by Ki67. Mann-Whitney test was used for statistical analysis. Images are representative of three repeats. Total cell count is 50 for each experiment. Fluorescent microscope is used.

3.3 Discussion

Repo-Man is a multifunctional PP1 targeting subunit that controls chromosome organisation and dynamics as well as NE dynamics during mitosis. In this chapter, we focused on identifying the molecular basis regulating the complex formation in order to better understand how the complex is regulated throughout mitosis. It was already known that, like most PP1 binding subunits, Repo-Man binds to PP1 through an RVxF motif (RVTF). This motif is highly conserved and shared by many targeting subunits and therefore it does not represent a useful Protein-Protein Interaction (PPI) surface that will be amenable to target for clinical purposes. However, previous studies had suggested that other regions outside the RVxF could have been important for the complex assembly and stability.

The first studies on the RVTF flanking region showed that T412 and T419 residues are phosphorylated by CDK1 at the beginning of mitosis and that this phosphorylation is important in localising Repo-Man onto chromosomes at mitotic exit (Vagnarelli et al., 2011). Here we have identified S400, F404 and S407 as possible regulatory regions with the help of our collaborators. With NMR crystallography, the minimal binding region of Repo-Man to PP1 was identified and these residues were in critical positions to stabilise the binding. Mutation analyses have shown that phospho-deficient mutations to Alanine (3A and 4A) on this region localise pre-maturely on chromosomes: this suggests that phosphorylations on these sites keep Repo-Man from localising onto mitotic chromatin earlier than it should. The phospho-mimetic mutations to Aspartic Acid (3D and 4D) showed a wild type-like phenotype but also a decreased affinity for PP1 even in interphase. Taken together these data strongly

indicate that phospho-switches of the SLIM motif are essential for the complex formation and stability.

Repo-Man localisation onto chromosomes at mitosis has been previously investigated by the Bollen lab. In these studies, Repo-Man phosphorylation at S893 by Aurora B was reported to be important for its localisation (Qian et al., 2011). Our results show that the contribution of Aurora B phosphorylation at S893 is significant but PP1 binding is also necessary for Repo-Man localisation onto chromosomes. In normal cells, the RAXA mutant rescued the pre-mature localisation of the 3A mutant. Similarly, in cells where Aurora B is inhibited (by ZM447439 treatment), even though wild type Repo-Man was localised on chromosomes as well as the 3A, the RAXA mutant showed a significant decrease in its chromosome localisation. The fact that RAXA mutant decreases but cannot rescue the localisation completely, tells us that Aurora B phosphorylation is significantly important but PP1 binding is priority.

However, we have now also discovered that AuroraB phosphorylates a residue within the RVxF and that this phosphorylation diminished PP1 binding. In light of this, Aurora B inhibition is affecting both targeting systems: the C-terminus histone binding domain and PP1 binding.

Our results clearly showed that the region flanking the RVTF motif is important in regulation of the complex. We already know CDK1 is responsible for phosphorylation of T412 and T419 and in addition, T394 (T in RVTF motif) is phosphorylated by Aurora B (Kumar et al., 2016). This was demonstrated via in-vitro phosphorylation coupled with NMR spectroscopy and mass spectrometry. Our in silico analyses revealed that the remaining two residues (S400 and S407) could be phosphorylated by Plk1.

The Phospho-deficient mutants on Plk1 sites have also an interesting phenotype in early mitosis: they abolish the centromeric accumulation of the remaining H3T3 phosphorylation; this suggests that Plk1 phosphorylation on these sites keep Repo-Man/PP1 complex inactive at the centromeres but not at the chromosome arms.

We can then imagine that different kinases contribute to the full inactivation of the complex in mitosis (CDK1, AuroraB, Plk1). A total inactivation would results in a hyperphosporylation of H3T3 with a distribution all over the chromosomes (as seen in the case of Repo-Man RNAi). However, in normal mitosis, the H3T3ph is retained at centromere indicating that somehow Repo-Man cannot de-phosphorylate these centromeric residues. We already know that the centromeric region is where Aurora B is localised and active PLK1 is localised. Therefore, we hypothesise that there is a gradient of active kinases at the centromere that make sure that all the sites that contribute to the stability of the complex are phosphorylated: this will produce a local full inactivation of the complex (Figure 3.15, C). Still, the overall H3T3 phosphorylation was lower with the Plk1 mutant (2A), proving that the complex is more active than the wild type.

We have also showed the mechanism of how Repo-Man recognises PP1γ selectively. With the help from our collaborators, we have identified that the selection occurs through the N terminal region of PP1. The R20 residue was shown to have an effect on PP1 localisation previously (Lesage et al., 2005), but the crystal structure that we have provided also shows the R20 residue in close proximity of Repo-Man when bound to PP1γ (Kumar et al., 2016). By mutating this residue to Gly to mimic PP1α, we abolished the binding of Repo-Man to PP1 and showed that Repo-Man's affinity to PP1γ occurs through the R20 residue.

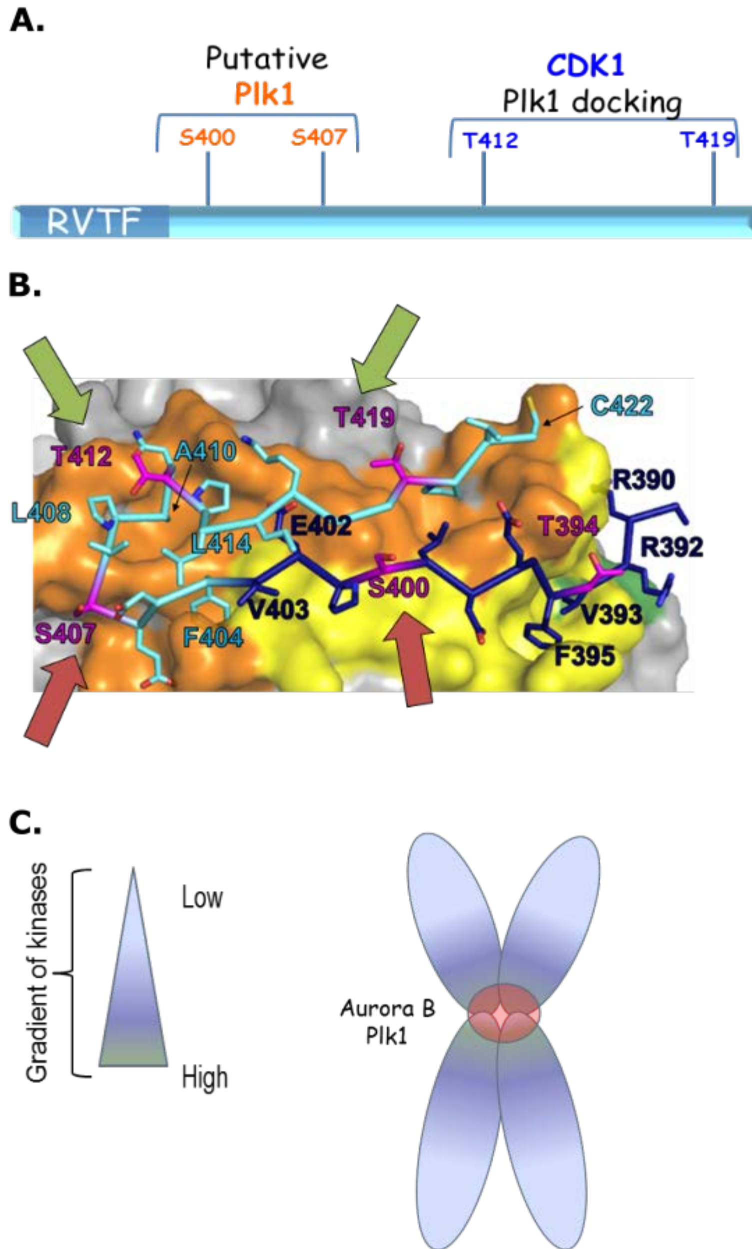


Figure 3.15: Working model. A. Showing the putative Plk1 phospho and docking sites on a linear representation. The sites look far away from each other in this representation. **B.** Crystal structure of Repo-Man bound to PP1, showing the real representation of the Plk1 sites. The docking and phospho sites are exactly opposite each other. **C.** Our hypothesis on the regulation of H3T3 phosphorylation. The kinase gradient on the chromosomes is lower at the arms and higher at the centromere, keeping the complex inactive at the centromere and keeping the H3T3 phosphorylated.

4. REPO-MAN REGULATES LAMIN A/C DE-PHOSPHORYLATION

4.1 Introduction

Repo-Man has been shown to interact with nuclear envelope (NE) proteins like Nup153 and Importinβ and help nuclear envelope reassembly (Vagnarelli et al., 2011). It was also shown that Repo-Man knockdown causes abnormal NE. Here we show that it also interacts with Lamin A/C and regulates its de-phosphorylation for a successful NE reassembly and it may depend on the sumoylation of Repo-Man.

The mitotic sites S22 (N terminus) and S392 (C terminus) have been shown to promote disassembly of nuclear lamina when phosphorylated (Heald and McKeen, 1990). It is well-known that phosphorylation of lamins occur continuously throughout the cell cycle, during both interphase and mitosis. So far, functions of these phosphorylations were explained in terms of nuclear targeting or assembly/disassembly of lamins (Luscher et al., 1991; Ottaviano and Gerace, 1985; Rzepecki et al., 2002) and knowing that the absence of Repo-Man causes abnormal lamina and Lamin A/C localisation, gave us a hint that Lamin A/C and Repo-Man may be interacting.

Furthermore, it has been reported recently that the re-assembly of Lamin A/C after mitosis depends on its SUMO-interacting motif (SIM). This motif is essential for Lamin A/C recruitment on the chromosomes but most importantly, it is necessary for de-phosphorylation of the S22 at the end of mitosis (Moriuchi et al., 2016). Here we hypothesize that Repo-Man is involved in de-phosphorylation of Lamin A/C. In this chapter we show that Repo-Man and Lamin A/C interact and this interaction may be through sumoylation, although we could not detect Repo-Man sumoylation in vitro. In addition, we prove that absence of Repo-Man affects Lamin A/C phosphorylation levels at interphase.

4.2 Results

4.2.1 Repo-Man knockdown causes abnormal nuclear lamina that lack stiffness.

Repo-Man effect on the nuclear envelope reassembly and nuclear lamina and its interaction with some nuclear lamina proteins has already been established. Repo-Man directly binds to Importinβ and recruits it to the chromatin at anaphase to initiate nuclear lamina re-assembly. Furthermore, depletion of Repo-Man causes dramatic changes in the nuclear lamina; this could be a consequence of insufficient loading of Importinβ around the chromatin at anaphase. It was also reported that HP1 localisation (Vagnarelli et al., 2011) and, more recently heterochromatin at the nuclear periphery are affected by Repo-Man depletion as well (DeCastro et al, 2016 in press).

To investigate the effect of Repo-Man on the nuclear lamina in more details, we have performed Repo-Man knock-down experiments in HeLa cells and stained for Lamin A/C. As previously reported, upon Repo-Man RNAi, nuclei have an irregular shape compared to the Control RNAi nuclei (Figure 4.1, A_ No KCl). This phenotype can be quantified by using the "circularity" descriptor within the analyses software package of AS-Nikon (Figure 4.1, B). This phenotype could be caused by different problems both on the Lamina per se or in the underlying chromatin. However, because both these structure have been shown to contribute to provide structure and rigidity to the nuclear lamina, experiments were carried out to test if Repo-Man depleted cells present lack of rigidity in their NE. After RNAi, the cells were incubated in hypotonic solution (75mM KCl) or PBS and stained for total Lamin A/C (Figure 4.1, A) and then the nuclei were analysed for circularity.

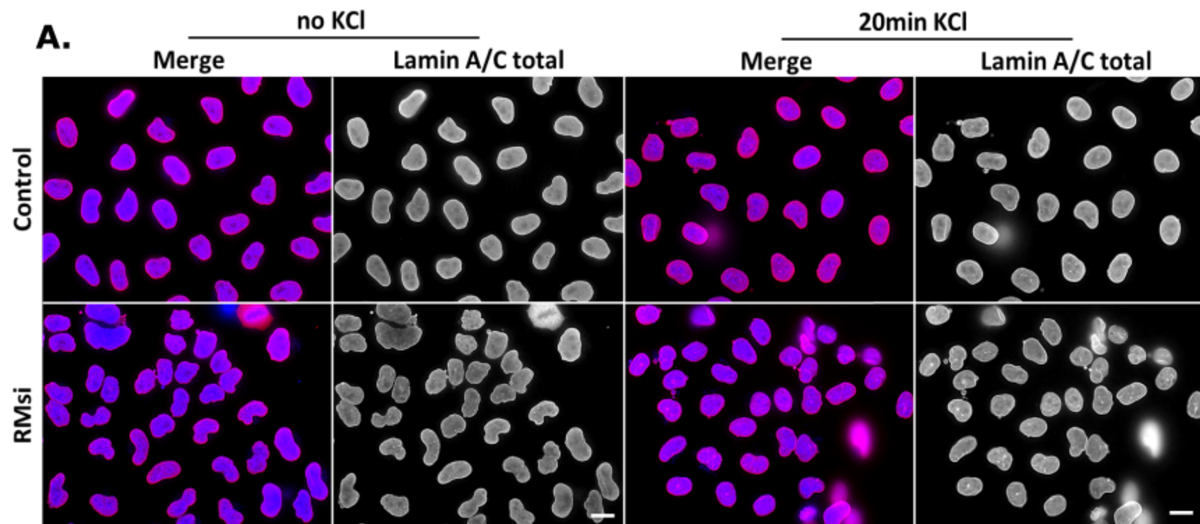
As expected, Control nuclei become more circular after KCl treatment but, more importantly, the hypotonic treatment rescued this abnormal nuclear morphology of Repo-Man RNAi treated cells to the point that in these conditions, they are not distinguishable from the control cells (Figure 4.1, A and B). In addition, total Lamin A/C levels are also shown to emphasise that Repo-Man knockdown does not change total Lamin A/C levels in the cell.

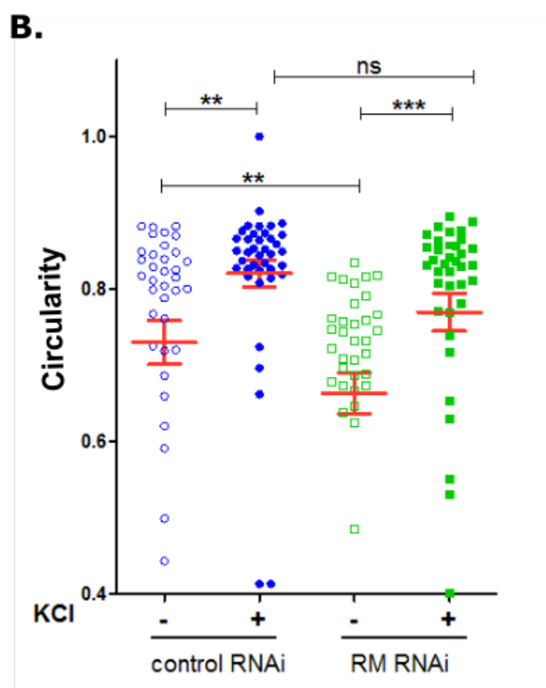
Our results not only prove the consequences of Repo-Man knock-down once again, but also show that this defect in Lamin A/C causes the formation of a NE that lacks stiffness. Therefore the multiple invaginations observed in the Lamina in the absence of Repo-Man are the consequence of lack of rigidity.

As mentioned before, Lamin A/C is highly phosphorylated at mitosis and this phosphorylation triggers its dissociation from the nuclear lamina, but a low level of phosphorylation at interphase is also observed (Heald and McKeen, 1990; Kochin et al., 2014). Similarly, reversal of this phosphorylation towards the end of mitosis triggers re-incorporation of Lamin A/C to the nuclear lamina but there is no information on the phosphatase responsible for this de-phosphorylation. These data indicate that the Lamina structure is affected by phosphorylation.

Since Repo-Man is a phosphatase that is loaded on the chromatin during anaphase and its absence causes defects in Lamin A/C re-assembly, potentially it could represent a plausible candidate for Lamin A/C de-phosphorylation.

Based on this, I set up to answer the question: “Does Repo-Man affect de-phosphorylation of Lamin A/C?”





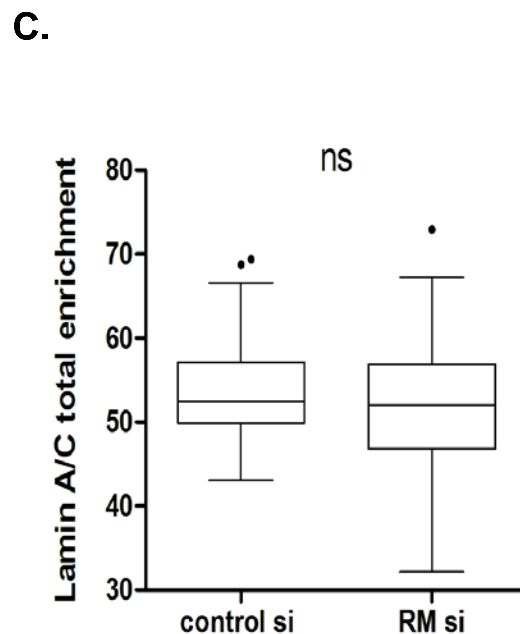


Figure 4.1: Repo-Man knock-down affects cell circularity through Lamin A/C. A. HeLa cells transfected with Repo-Man (RMsi) and control siRNA (48h) were incubated with or without KCl (75mM) for 20min and stained for total Lamin A/C (in red) and DAPI (in blue). Cells with "no KCl" were incubated in PBS for equal amount of time. The images clearly show the abnormal lamina morphology in the Repo-Man siRNA transfected cells and the rescued phenotype after KCl treatment. Scale bar 10μm. **B.** Quantification of the experiment in A, calculated on Image J, showing the significant difference before and after the hypotonic treatment as well as between control and Repo-Man siRNA. KCl treatment abolished the significant difference as shown on the graph (n=35). Mann-Whitney test is used for statistics. Images are representative of a single experiment. **C.** Total Lamin A/C enrichment is shown to prove that Repo-Man knockdown does not affect total Lamin A/C levels.

4.2.2 Repo-Man knockdown affects Lamin A/C phosphorylation.

Using a commercial antibody against Lamin AS22Ph, I have analysed the dynamic of Lamin A phosphorylation during mitosis (Figure 4.2). As reported before, phospho-Lamin A/C is at low levels at interphase but increases dramatically during mitosis until cytokinesis. I could reproduce the exact same localisation pattern. An interesting observation is that de-phosphorylation of LaminA starts occurring upon binding of the Lamina to the chromatin (late anaphase). In interphase, Lamin A/C phosphorylation was still present but at low levels, whereas in mitosis it was at high levels until cytokinesis, when the nuclear lamina is reformed.

If Repo-Man is involved in de-phosphorylation of Lamin A, then it would be expected to observe a higher level of Lamin A/C S22ph also in interphase. Repo-Man RNAi treated cells were therefore analysed for Lamin A/C phosphorylation in interphase and were compared to the untreated cells at the same stage (Figure 4.3, A). Lamin A/C signal intensity was measured in a defined ROI covering the whole nucleus and these analyses indicated that in the absence of Repo-Man, there is increased Lamin A/C phosphorylation at interphase (Figure 4.3, C).

These results indicate that Repo-Man directly or indirectly affects de-phosphorylation of Lamin A/C and the higher level of phosphorylated Lamin A/C could be responsible for the improper assembly of the NE observed after Repo-Man knock-down. Whether this is a result of a direct interaction between the proteins or an indirect consequence of Repo-Man activity is still unknown at this stage.

Furthermore, the normal level of Lamin A/C S22ph in interphase was restored when HeLa cells were transfected with oligo resistant wild type Repo-Man (Res2) in RNAi background; these cells showed less Lamin A/C phosphorylation in interphase than the un-transfected cells, proving that presence of Repo-Man can rescue the normal levels of Lamin A/C phosphorylation (Figure 4.3, B and D). This clearly shows that the effect is indeed Repo-Man dependent.

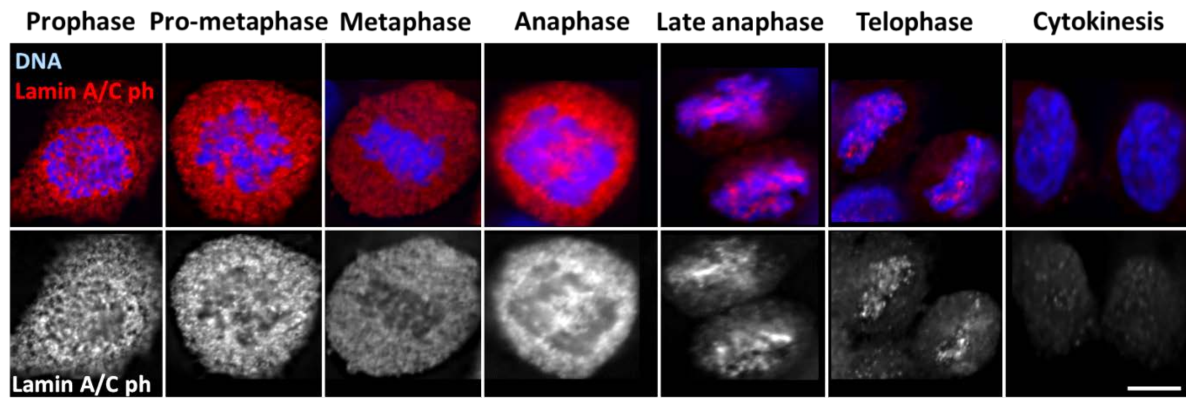


Figure 4.2: Phospho-Lamin A/C localisation throughout cell cycle. Lamin A/C phosphorylation (red) is high during mitosis (prophase-anaphase), it decreases after anaphase and goes back to the normal levels at cytokinesis, when the nuclear lamina is reformed (Scale bar 10µm). Images are representative of a single experiment. Fluorescent microscope is used.

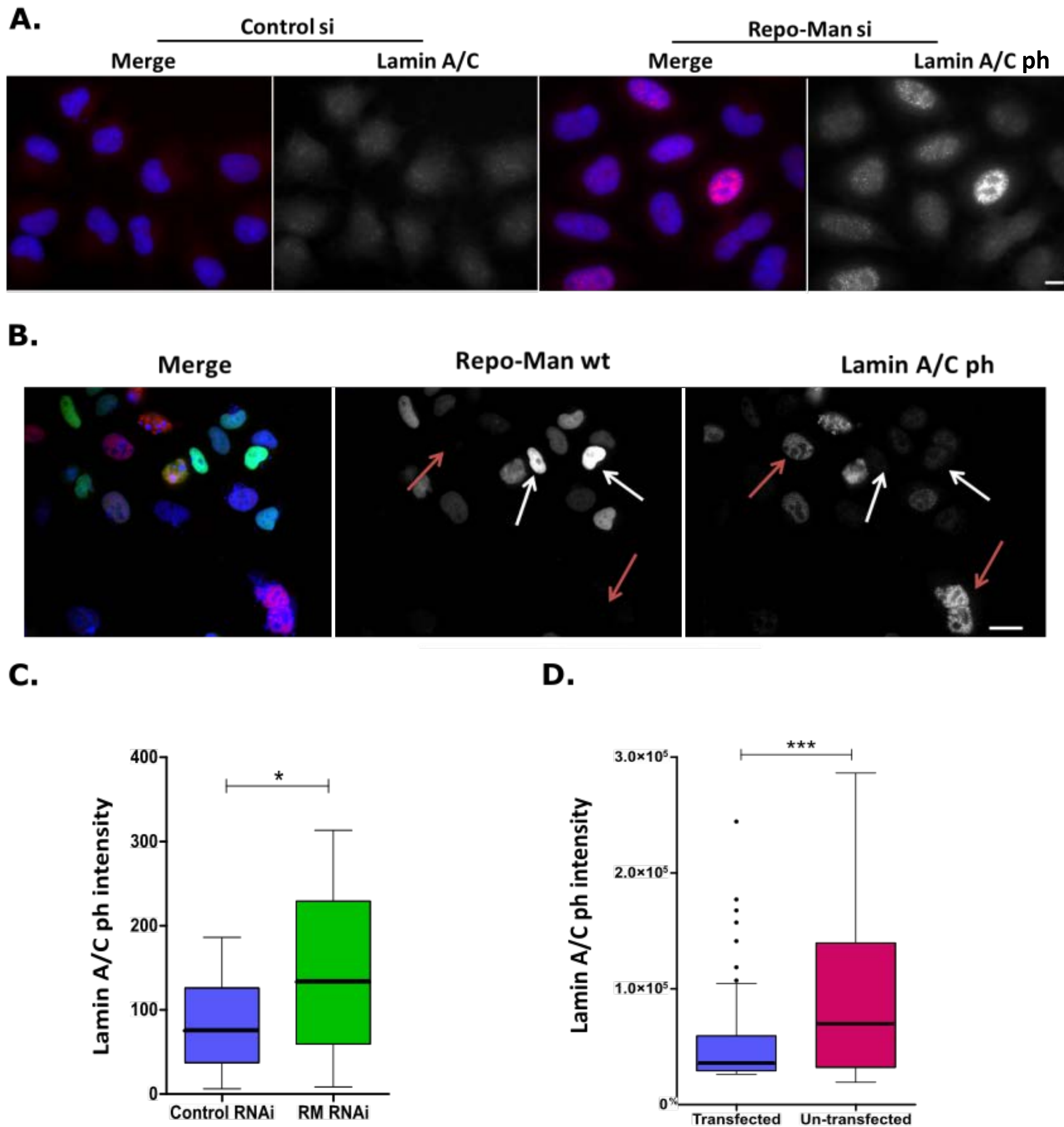


Figure 4.3: Repo-Man knock-down causes increased phospho-Lamin A/C levels at interphase. A. HeLa cells transfected with control and Repo-Man siRNA and stained for Lamin A/C (phSer22). Increase levels of phospho-Lamin A/C were observed in the absence of Repo-Man at interphase (Scale bar 10µm). **B.** HeLa cells transfected with oligo-resistant wild type Repo-Man (Res2) in RNAi background (48h) and stained for phospho-Lamin A/C; phospho-Lamin A/C levels in interphase were rescued in Repo-Man transfected cells (Scale bar 10µm). **C.** Quantification of the experiment in A. Significant increase in phopsho-Lamin A/C levels with Repo-man RNAi. Mann-Whitney test is used for significance. **D.** Quantification of the experiment in B. Cells that lack Repo-Man (red arrows) have higher amount of phospho-Lamin A/C than the cells that are transfected with Repo-Man (white arrows). Mann-Whitney test is used for significance. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used.

4.2.3 Sumoylation of Repo-Man is essential for Lamin A/C de-phosphorylation.

As mentioned before, SIM3 of Lamin A/C is important to facilitate the de-phosphorylation of the Ser22 residue and re-incorporation of Lamin A/C to the nuclear envelope after mitosis. Live cell imaging and immunofluorescence experiments showed that SIM3 of Lamin A/C is required for Lamin A/C accumulation on chromosomes. In addition, mutation of SIM3 abolished de-phosphorylation of S22 residue of Lamin A/C (Moriuchi et al., 2016). Repo-Man is a chromatin associated phosphatase, active during mitotic exit whose depletion causes a persistance of hyperphosphorylated LaminA. Moriuchi et al.,suggested that plusible candidates for the S22 de-phosphorylation could be BAF, emerin, and Repo-Man.

Repo-Man was reported as a SUMO 2/3 substrate in two different studies (Schou et al., 2014; Scimmel et al., 2014). We therefore analysed if Repo-Man could be sumoylated. Indeed K762 residue on Repo-Man is a plausible candidate for the SUMO2/3 modification, as shown in the GPS-SUMO database (An online database that predicts sumoylation sites) (Figure 4.4, A).

The K762 residue was then mutated to Arginine (R) (Figure 4.4, B) and made oligo-resistant (IRCE Res). HeLa cells were transfected with GFP:IRCE Res and wild type oligo-resistant Repo-Man GFP:Res2 in RNAi background, to observe the localisation during mitosis. The IRCE mutant did not show any difference in localisation compared to the wild type during mitosis (Figure 4.4, C); this shows that this mutant does not affect Repo-Man accumulation on chromosomes and both constructs expressed at a similar level, as assessed by wetsern blotting of HeLa transfected extracts (Figure 4.4, D).

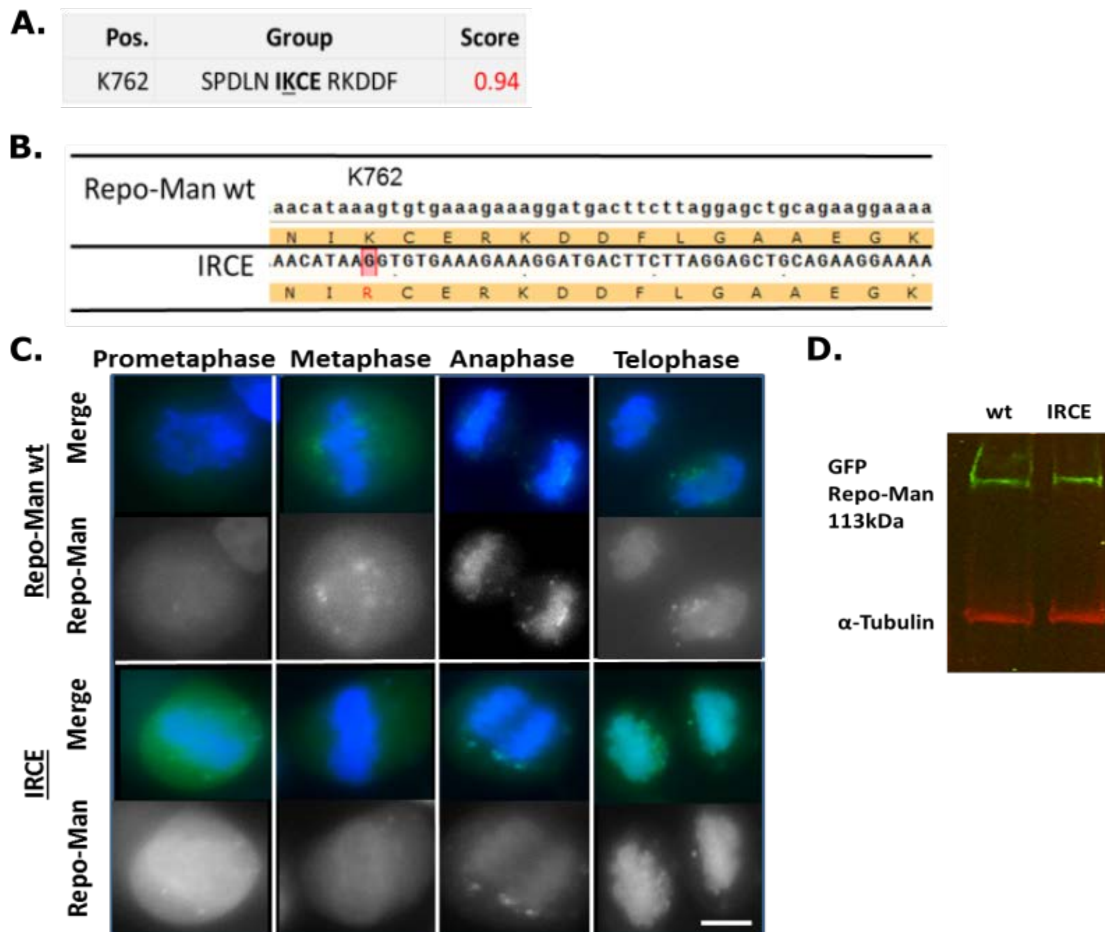


Figure 4.4: Repo-Man sumo mutant IRCE shows wild type-like localisation. A. Predicted sumoylation site on Repo-Man. **B.** Lys to Arg (K to R) mutation on Repo-Man K762. **C.** Oligo resistant Repo-Man wild type and IRCE mutant transfected HeLa cells in the presence of Repo-Man siRNA (48h) are shown at different stgaes of mitosis. IRCE mutant behaves exactly as the wild type protein in terms of localisation (Scale bar 10µm). **D.** Western blot showing the full length wild type and mutant Repo-Man, extracted from Repo-Man siRNA treated and Repo-Man wild type and IRCE (oligo resistant) transfected HeLa cells. Detected with anti-GFP antibody. α-Tubulin was used as loading control. Images are representative of a single experiment. Fluorescent microscope is used.

The sumo mutant was then used for a rescue experiment to test its effect on Lamin A/C de-phosphorylation. HeLa cells were transfected with the wild type and mutant oligo-resistant constructs in an RNAi background and the cells were analysed in interphase. The un-transfected cells in both, showed a high level of phospho-Lamin A/C as observed before and Repo-Man wild type transfected cells could rescue the phenotype (Figure 4.5, A Repo-Man wt 1-3, 1'-3'). However, the sumo mutant transfected cells still had a certain amount of phospho-Lamin A/C present at interphase (Figure 4.5, A IRCE 1-3, 1'-3'), showing that the sumo mutant cannot rescue the phospho-Lamin A/C levels and therefore sumoylation of Repo-Man may have an effect on Lamin A/C de-phosphorylation.

The IRCE mutant represents the non-sumoylated version of the protein and it cannot rescue the Lamin A/C phosphorylation back to the normal levels, suggesting that the wild type protein gets sumoylated and this has an effect on amin A/C S22ph levels.

We wanted to show that indeed Repo-Man can be sumoylated in vitro using an in-vitro sumoylation assay. GST tagged C terminal region (amino acids 391-1023) of wild type Repo-Man and sumo mutant (IRCE) were expressed in E.Coli Rosetta strain and the proteins were extracted by using Gluthatione agarose beads (Figure 4.6, A). The purified proteins were then used for the assay, to test if Repo-Man gets sumoylated and which SUMO protein is involved. For each sumo protein, reactions with and without ATP were prepared and the RanGAP1 protein, which is known to be modified by all three SUMO proteins, was used as a positive control. The results show a positive band for the control protein for each SUMO protein at 64kDa. However, neither wild type nor mutant Repo-Man had a positive band with any of the SUMO proteins (Figure 4.6, B). Overall, the sumoylation of Repo-Man was not detectable in-vitro, but I have strong evidence in favour of Repo-Man sumoylation.

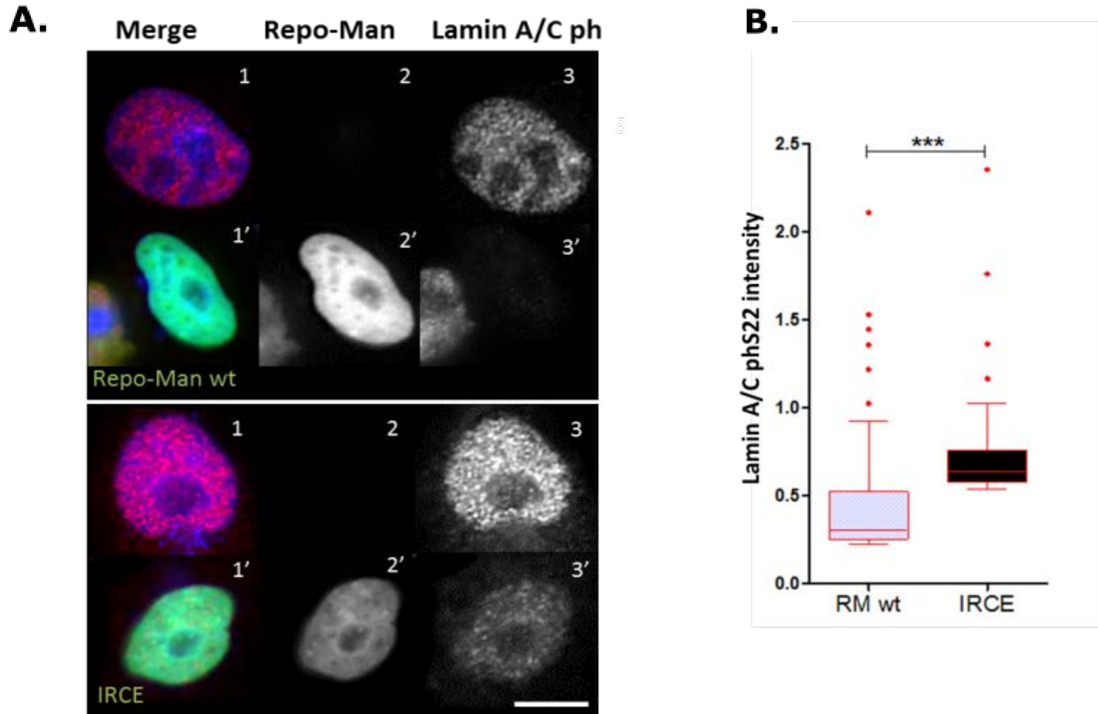


Figure 4.5: IRCE mutant cannot rescue phospho-LaminA/C levels at interphase. A. HeLa cells transfected with wild type and mutant oligo resistant Repo-Man and treated with Repo-man siRNA (48h). Un-transfected cells (1,2,3 both panels) showing high levels of Lamin A/C phosphorylation, whereas wild type Repo-Man expression decreases these levels (1', 2', 3' upper panel). The IRCE mutant however, cannot reverse the effect and rescue Lamin A/C phosphorylation levels (1', 2', 3' lower panel) (Scale bar 10µm). **B.** There is significantly higher amount of phospho-Lamin A/C in IRCE transfected cells than Repo-Man wild type transfected cells (n=30). Total signal from transfected cells was normalised against the signal from the un-transfected cells. Images are representative of three repeats. Total cell count is 30 for each experiment. Fluorescent microscope is used. Mann-Whitney test is used for significance.

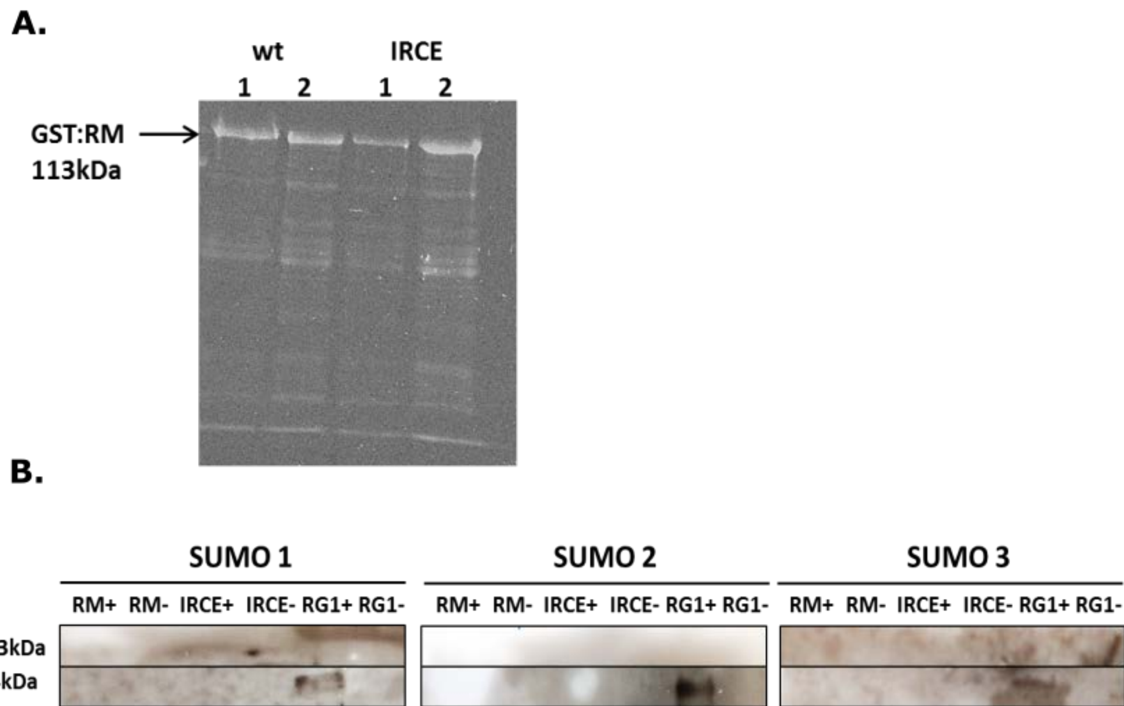


Figure 4.6: Repo-Man sumoylation cannot be detected in-vitro. A. GST tagged wild type and mutant Repo-Man extracted from E.Coli Rosetta strain by using Glutathione beads. Samples were run in duplicates for both proteins (1 and 2). **B.** Western blots showing the results for the in-vitro sumoylation assay. Sumoylation of wild type or mutant Repo-Man (RM and IRCE) could not be detected but sumoylation of RanGAP1 (RG1) was observed with all SUMO proteins. (+) indicates the positive reaction in the presence of ATP, whereas (-) indicates the negative reaction in the absence of ATP. RG1 sumoylation was observed at 64kDa.

4.2.4 Sumoylation of Repo-Man is important for the interaction with Lamin A/C

Although sumoylation of Repo-Man could not be detected in-vitro, there was clear evidence of the effect of sumoylation Lamin A/C phosphorylation in the knock-down experiments. Further optimization was needed for the in-vitro sumoylation assay as the the control reactions worked for all SUMO proteins but amount of protein could have been insufficient for the other reactions to work.

Due to the time constraints we could investigate this aspect further so we followed a different approach. Another system to investigate protein-protein interactions in vivo is the proximity ligation assay (PLA), which determines if two proteins are closer than 16nm. The assay makes use of primary antibodies of two different species that detects target proteins and secondary antibodies that have short sequences of oligonucleotides. The oligonucleotides ligate in close proximity and the signal is amplified with fluorescently labelled oligonucleotides.

A PLA assay was conducted using a validated antibody against GFP (mouse) and the Lamin A/C S22ph antibody (rabbit) on HeLa cells. HeLa cells were transfected with GFP:Repo-Man^{wt} and GFP:Repo-Man^{IRCE} in a Repo-Man RNAi background. Since the difference in phospho-Lamin A/C levels was detectable at interphase, we focused on the end of mitosis where we expected to see the interaction between Repo-Man and phospho-Lamin A/C.

Our results show that the interaction occurs in telophase and increases in interphase (Figure 4.7, A). The localisation of the interaction is not just confined to the nuclear lamina, but is spread all over the nucleus. Interestingly, the sumo mutant (IRCE) showed much less interactions with phospho-Lamin A/C then the wild type Repo-Man in interphase (Figure 4.7, A). However in telophase, the interaction between the mutant and phospho-Lamin A/C was not significantly different than that of wild type protein (Figure 4.7, B).

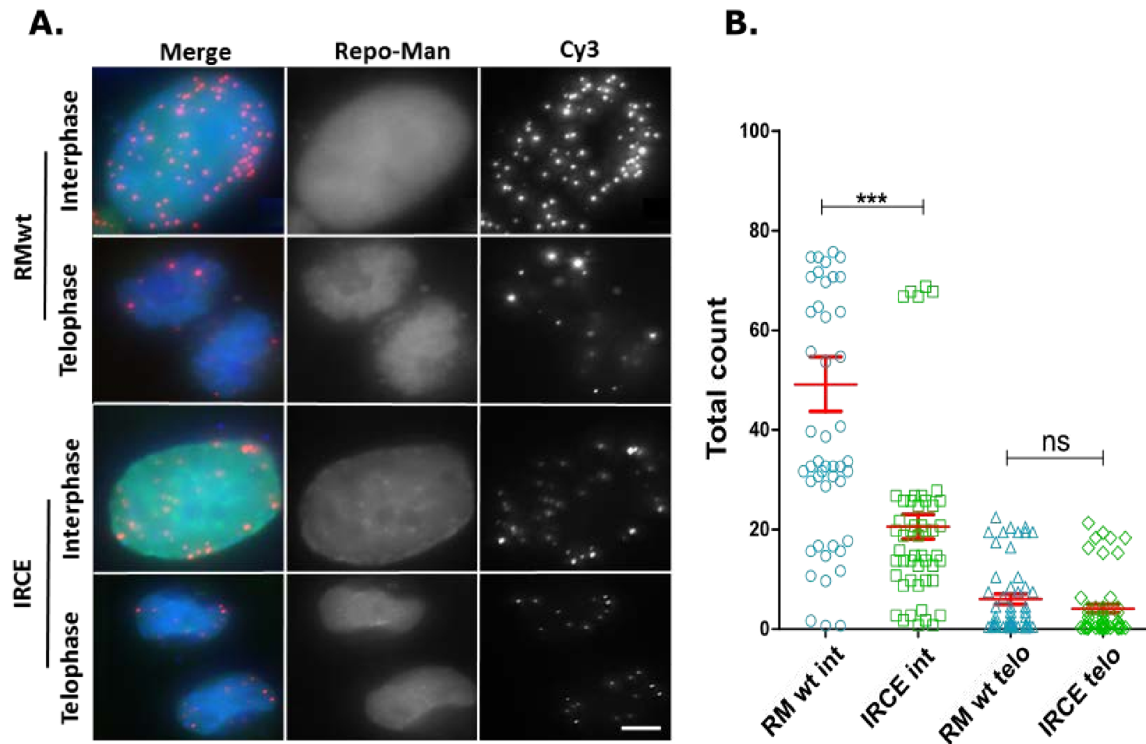


Figure 4.7: Repo-Man interacts with phospho-Lamin A/C at interphase and the sumo mutant IRCE decreases this interaction. A. HeLa cells transfected with oligo resistant wild type and mutant Repo-Man and with Repo-Man siRNA were blocked at with Nocadazole for 3hrs and released for 2hrs before fixation to obtain high number of telophase cells. Each Cy3 spot represents an interaction between the two proteins. The sumo mutant decreases the interaction both at interphase and telophase (Scale bar 10µm). **B.** Analysis of the experiment. Significant decrease with the sumo mutant was observed at interphase and not in telophase (n=50) (Chi-square test was used for significance) Images are representative of three repeats. Fluorescent microscope is used.

4.3 Discussion

Dis-assembly and re-assembly of the NE at mitosis depends,among other important factors, on phosphorylation and de-phosphorylation of NE commponents including Nups and lamins. Repo-Man has already been shown to interact with some of these components like Importinβ (Vagnarelli et al., 2011; Qian et al., 2015). In addition, Repo-Man knockdown has an effect on the nuclear shape. However, the only proven NE-associated substrate for Repo-Man/PP1 complex so far reported is ImportinB.

Here, guided by some recent discoveries on Lamin A/C de-phoshorylation, we have investigated the molecular mechanism that could be at the base of the occurrence of a mis-shaped lamina following Repo-man depletion.

Upon Repo-Man knock-down, the nuclei lost circularity as assessed by nuclear Lamin A/C staining. Furthermore, we rescued the abnormal lamina by using 75mM KCl and creating an hypotonic environment: this shows that the abnormal nuclear lamina can be reversed by an external force and that the phenotype observed is possibly caused by a weak NE that folds on itself thus producing the observed invaginations.

Considering the phosphatase properties of Repo-Man/PP1, it would be plausible to infer that Repo-Man effect on Lamin A/C occurs through phosphorylation. In particular, phosphorylation of Lamin A/C had been implicated in changes of its properties and linked to an increased mobility and a softer nucleus (Harada et al., 2014). To investigate if this was the case, we have used an antibody against Lamin A/C S22ph, as this is the most important mitotic phosphorylation on Lamin A/C and it has been related to chnages in Lamina mobility and assembly (Luscher et al., 1991; Harada et al., 2014).

Indeed, our studies showed that absence of Repo-Man affects de-phosphorylation of Lamin A/C at S22, and causes high levels of phosphorylated Lamin A/C to remain after mitosis. This phenotype is rescued and phospho-Lamin A/C levels decrease when Repo-Man is externally re-introduced. These data strongly support and unveil an important and new role for Repo-Man in controlling Lamin A/C de-phopshorylation.

Another important aspect of this interaction is the mechanism. It was already shown that SIM3 of Lamin A/C promotes an interaction with a sumoylated protein that causes Lamin A/C de-phosphorylation at S22 (Moriuchi et al., 2016). This brought into question if Repo-Man could be acting directly on the Lamina. In order to do so, Repo-Man must be sumoylated. Previous report have identified Repo-man as sumoylated protein and have also identified a single sumoylation site within its the C-terminus domain. Although the the SUMO mutant IRCE that we generated does not change the property of Repo-Man in terms of its localisation in the cells, it provides evidendence that sumoylation of Repo-Man may be the mechanism behind this interaction. The IRCE mutant was generated by performing the common Lys to Arg (K to R) muatation, according to the data we gathered from previous studies on sumoylation of different proteins. This mutant, unlike the wild type, failed to rescue the Lamin A/C phosphorylation levels in interphase, suggesting that this mutation affects the interaction of Repo-Man with either Lamin A/C directly or with another phosphatase that then has Lamin A as a substrate. The PLA experiments seem to support the former hypothesis of a direct interaction between the two proteins, as the interaction decreases with the SUMO mutant both at telophase and interphase. Although we could not detect the in-vitro sumoylation of Repo-Man, we have several evidence that support this hypothesis including the PLA experiment and the rescue experiments with SUMO mutants.

Our working hypothesis is therefore the following: Repo-Man via its Sumoylated C-teminus interacts directly with the SIM of LaminA and mediates the de-phosphorylation of the S22 during mitotic exit. Since Repo-Man persists in interphase as well and it is enriched at the nulcear periphery (De Castro et al, in press) and LaminA is phsophorylated also in interphase (Kochin et al., 2014) this could be a general mechanism that controls nuclear elasticity during several processes (Figure 4.8).

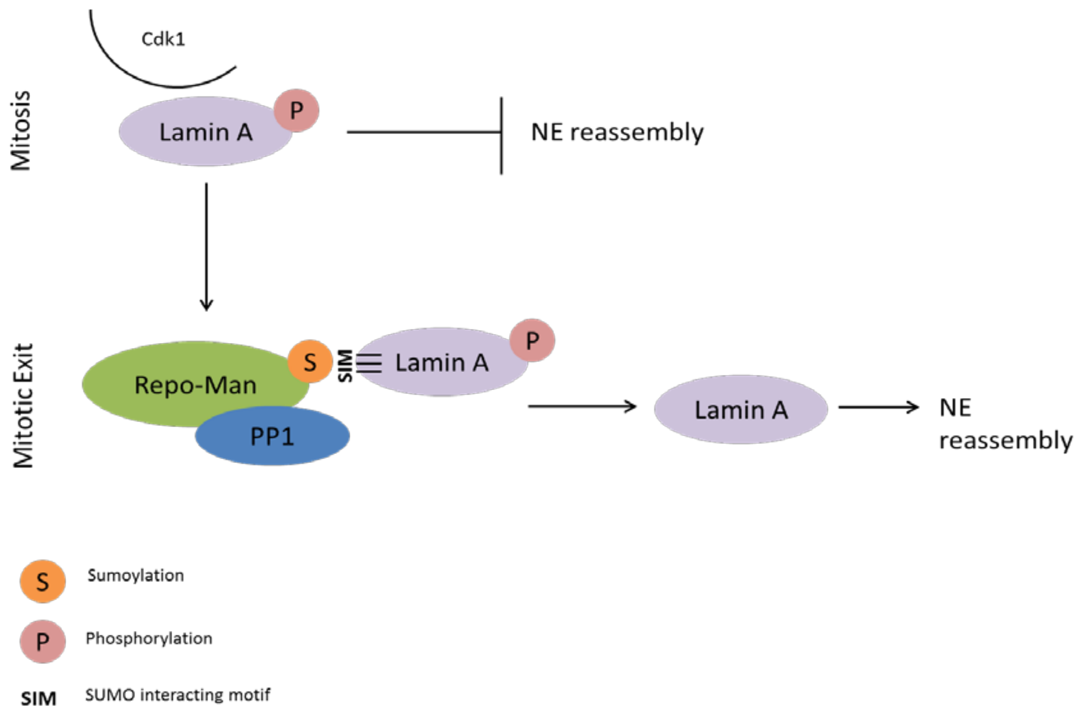


Figure 4.8: Working model. Lamin A/C phosphorylation by CDK1 at the beginning of mitosis promotes NE dis-assembly and at mitotic exit Repo-Man reverses this by de-phosphorylating Lamin A/C for its re-integration to the nuclear lamina. Our hypothesis is that Repo-Man accomplishes this by directly binding to the SIM of Lamin A/C after going through the sumoylation process itself. Sumoylation of Repo-Man promotes Lamin A/C binding and de-phosphorylation is achieved.

5. REPO-MAN/PP1 COMPLEX AS A THERAPEUTIC TARGET FOR TRIPLE NEGATIVE BREAST CANCER

5.1 Introduction

Repo-Man is up-regulated in several cancer types. Using an isogenic breast cancer model system (MCF10A and its derivatives), malignant (MCF10A-TK1) and metastatic (MCF10A-CA1h) breast cancer cells were compared to the normal cell line (MCF10A) for Repo-Man expression and it was shown that malignant and metastatic breast cancer tumours have high levels of Repo-Man (Vagnarelli, 2014). Furthermore, depletion of Repo-Man stopped growth of malignant MCF10A-CA1h breast cancer cell line in soft agar (Peng et al., 2010), and arrested squamous cell carcinoma cells at G1, leading to apoptosis (Uchida et al., 2013).

Repo-Man levels appear to be related to cancer progression in several ways. First, Repo-Man is known to be involved in DNA damage response (Peng et al., 2010), which can provide a platform for mutations and chromosome rearrangements. Second, Repo-Man controls chromosome segregation and counteracts Aurora B kinase, which may lead to aneuploidy and lagging chromosomes. Moreover, since Repo-Man is involved in de-phosphorylating histone H3, its loss of function could cause problems in chromatin organization after mitosis and defects in heterochromatin environment and gene expression (DeCastro et al, 2016).

In this chapter we focused on the effect of Repo-Man in breast cancer and, using the invasive MCF10A-CA1h and the triple negative HCC1143 cell lines as models, we tried to investigate the molecular mechanisms that link Repo-Man overexpression and cancer progression.

Since we found that Repo-Man is highly overexpressed in triple negative breast cancers (TNBC), we focused on this type as it represents the most aggressive type of breast cancer with no current therapeutic targets. TBNC lacks oestrogen receptor (ER), progesterone receptor and Her2/neu. The absence of these markers makes TNBC not targetable or treatable and currently is the most aggressive type of breast cancer.

Here we show that the high levels of Repo-Man occurs in different breast cancer cell lines when compared to the normal breast tissue; in addition, we monitor the wound healing capability of these cell lines using live cell imaging in the presence and absence of Repo-Man. We also report that Repo-Man expression levels affect the levels of important cancer markers like C-Myc and Aurora A. Our results show that Repo-Man as an important cancer marker especially for triple negative breast cancer, which regulates malignancy through C-Myc and Aurora A; most importantly, Repo-Man depletion halts the mobility of these breast cancer cells.

5.2 Results

5.2.1 Repo-Man is overexpressed in triple negative breast cancer

The idea of Repo-Man being a cancer driver gene is a fairly new idea and there is not much research done on Repo-Man and cancer. The few studies that were published are mentioned above and these all suggest that overexpression of Repo-Man correlates with the aggressive behaviour of the tumour in certain types of cancers. Among these, the most striking study was from Peng et al., which reported that absence of Repo-Man causes breast cancer cells (MCF10A-CA1h and Tk1 cell lines) to stop growing on soft agar due to the cells being more susceptible to DNA damage. Taking this study as a starting point, we wanted to further investigate the effect of Repo-Man in different breast cancers and see if Repo-Man can be a therapeutic target for breast cancer.

We started our research by conducting a database search on Repo-Man expression levels in different grades of breast cancer. The data we obtained from the Oncomine database showed that as the grade gets higher, Repo-Man expression levels increase proportionally (Figure 5.1, A). Furthermore, there is a dramatic increase in Repo-Man expression in basal-like breast cancers, which includes the triple negative breast cancer, when compared to the normal breast tissue (Figure 5.1, B). Another important information we have come across during our database search was the survival curve of the lymph node positive patients. In patients that had low Repo-Man expression, the survival rate was significantly higher than the patients that had high Repo-Man expression (Figure 5.1, C). This clearly shows us the potential of Repo-Man as a diagnostic and prognostic marker.

Using UCSC cancer browser, we analysed Repo-Man expression (high seq dataset) in different types of breast cancers as well as the normal breast tissue. These include luminal A (ER+, PR+/-, HER2-) and luminal B (ER+, PR+/-, HER2+) types; basal, which include triple negative; and others which are ER and PR negative and HER2 positive. The data we obtained clearly shows that Repo-Man expression is higher in triple negative type of breast cancers (Figure 5.2, A).

Since these datasets are only available for mRNA levels, we wanted to analyse also the protein levels in different breast cancer cell lines. A comprehensive analysis of a wide range of breast cancer cell lines in terms of Repo-Man expression has already been done previously in our lab. The cell lines that were used and their properties are listed in table 5.1. Among these, here I only show the HCC1143 triple negative cell line and the MCF10A derivative CA1h cell line, because I have used these two cell lines for further experiments. We were able to detect high Repo-Man levels in all breast cancer cell lines including the triple negative cell lines HCC1143 and BT20 and the malignant CA1h. MCF10A and HMEC184 (human mammary epithelial cells) have normal levels of Repo-Man and were used as controls, and tubulin staining was used as loading control (Figure 5.2, B). This western blot analysis showed high Repo-Man protein levels in these cancer cell lines.

Table 5.1: List of cell lines used for western blot experiments to detect Repo-Man levels.

Name	Characteristics	Receptors
MCF10A	Normal human epithelial breast cells	-
CA1h	Derived from MCF10A	HER2 positive
HMEC184	Normal mammary cells	-
BT474	Invasive ductal carcinoma	ER, PR, HER2 positive
HCC1143	Non-invasive ductal carcinoma	Triple negative
BT20	Invasive ductal carcinoma	Triple negative
MCF7	Invasive ductal carcinoma	ER, PR positive

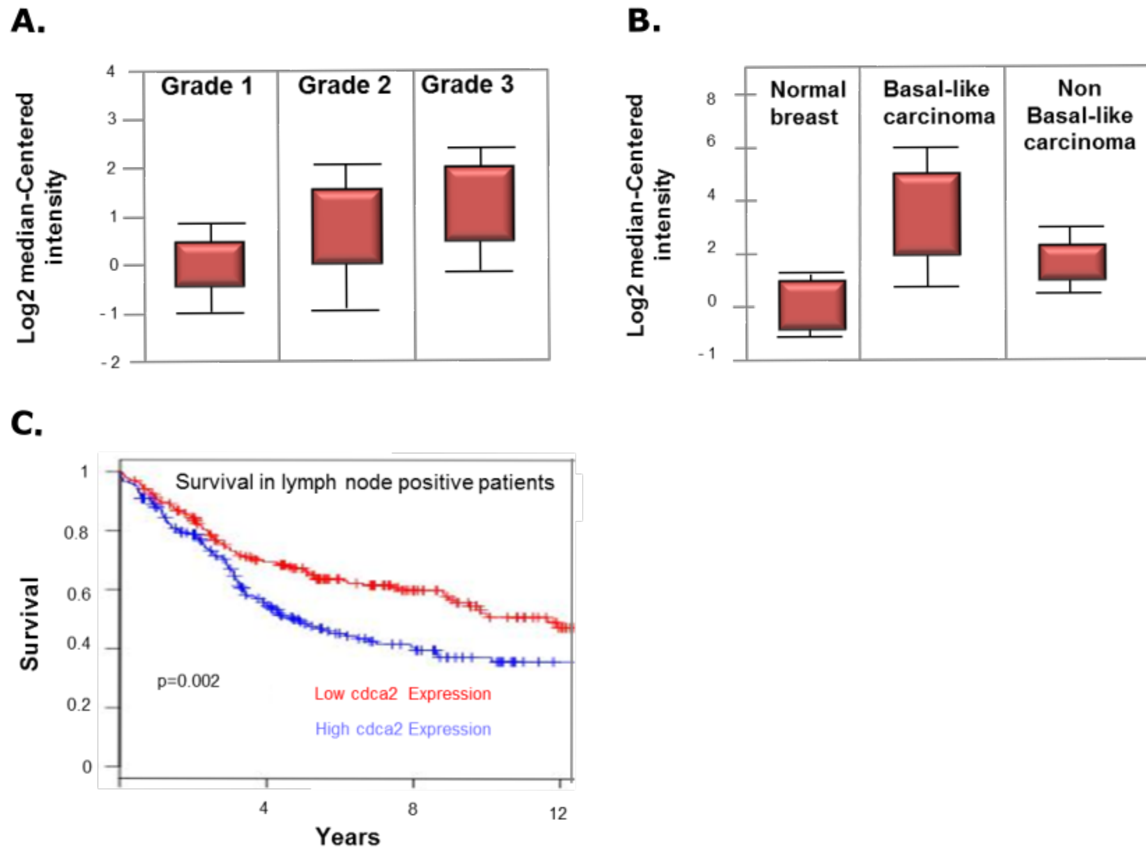


Figure 5.1: Repo-Man is overexpressed in high grade aggressive breast cancer cell lines and decreases survival rates of breast cancer patients. A. Comparison of different grades of breast cancer in terms of Repo-Man expression (data obtained from Oncomine). Grade 1 represents initial cancerous tissue, grade 2 represents malignancy and grade 3 represents metastatic behaviour. Repo-Man expression is highest in grade 3 breast cancers. **B.** Repo-Man gene expression is high in basal-like (triple negative) breast cancers, when compared to normal breast tissue and non-basal like breast cancers (data obtained from Oncomine). **C.** Survival rates of lymph node positive breast cancer patients with high or low Repo-Man expression. High Repo-Man levels cause low survival rates.

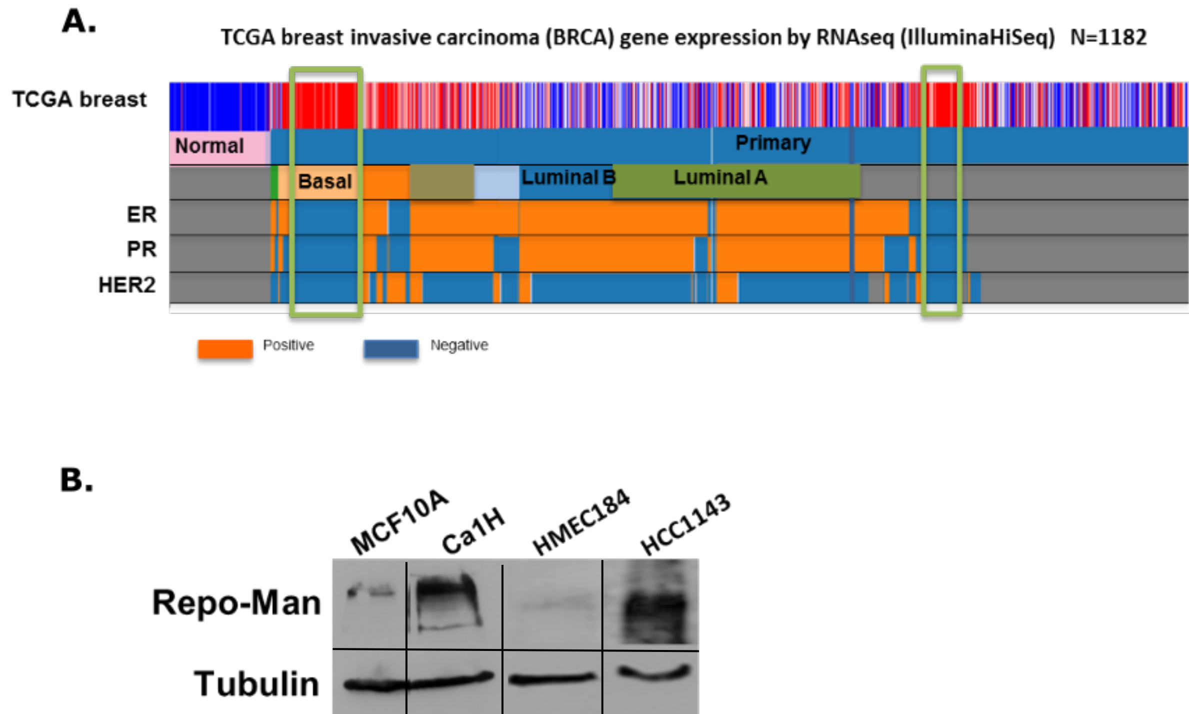


Figure 5.2: Repo-Man is overexpressed in triple negative breast cancer. A. Repo-Man expression levels in different types of breast cancer (UCSC cancer browser). Repo-Man expression is high in triple negative breast cancer (green boxes). **B.** Western blot showing Repo-Man levels in different breast cancer cell lines. MCF10A and HMEC184 normal epithelial cells were used as controls. HCC1143 triple negative cell line and CA1h malignant cell line (derived from MCF10A) have high levels of Repo-Man.

5.2.2 Repo-Man knock-down blocks wound healing ability in CA1h and HCC1143.

As mentioned before Repo-Man depletion has previously been reported to block the growth of malignant breast cancer cells on soft agar (Peng et al., 2010). Although the mechanism behind this was not clear, it was reported to be related with the interaction between Repo-Man and ATM kinase. Since Repo-Man negatively regulates the DNA damage response, its depletion causes a more efficient activation of the pathway and stops cell growth on soft agar (Peng et al, 2010).

We wanted to analyse how absence of Repo-Man would affect the growth and mobility of the two breast cancer cell lines we selected as a model (CA1h and HCC1143). For this purpose we depleted Repo-Man by RNA interference (control RNAi was used in parallel) and performed a wound healing assay. The wound was created after 24h of RNAi interference and the cells were observed for the next 24h by live cell imaging. Control cells were able to close the wound very efficiently within 20h however, the cells treated with Repo-Man siRNA could not close the wound in both cell lines within the same time frame (Figure 5.3, A and C).

We have then asked the question “What is making the cells delay the wound closure in the absence of Repo-Man?”, and considered two possible answers: The first possibility is that cells stop dividing (effect on proliferation) and the second one that cells divide normally but move slower (effect on motility) in the absence of Repo-Man.

To address the two possibilities, I have conducted two different analyses: the mitotic index and the velocity of the cell movement. I have first calculated the mitotic index of control and Repo-Man siRNA treated cells in both cell lines. For the mitotic index, cells were fixed at 20h after generating the wound and stained with H3T3ph (a marker for mitotic cells); H3T3ph positive cells were counted as well as the total cell number within 50µm radius around the wound at 3 different positions along the wound and the percentage of mitotic cells was calculated. The results show that there is no difference in the number of mitotic cells between control and siRNA treated cells as the mitotic index was the same in both conditions for both of the cell lines (Figure 5.3, E and F). This argues against the possibility of an inhibition of mitosis.

I then calculated the velocity of cell movement. The calculations were conducted between time points 0 and 20h. The distance between the two edges of the wound was calculated every 30min between 0 and 20h and the velocity was calculated by dividing the difference in the distance by the time. Results showed a significant decrease in the velocity in Repo-Man depleted cells when compared to control cells (Figure 5.3 B and D). Moreover, it was observed that the triple negative cells were a lot slower than CA1h cells even without siRNA treatment.

All these results suggest that the absence of Repo-Man slows down the ability of migration both on the triple negative and CA1h breast cancer cells; taken our results together with the previous findings of a reduction of growth in soft agar, we can propose that Repo-Man can potentially be used as a therapeutic target for the treatment of breast cancer, including the triple negative.

Now that we know the mechanism of action and relate high levels of Repo-Man with malignancy, we can look at other cancer markers and their relation with Repo-Man levels in the cell to see if we can control the levels of these markers through Repo-Man.

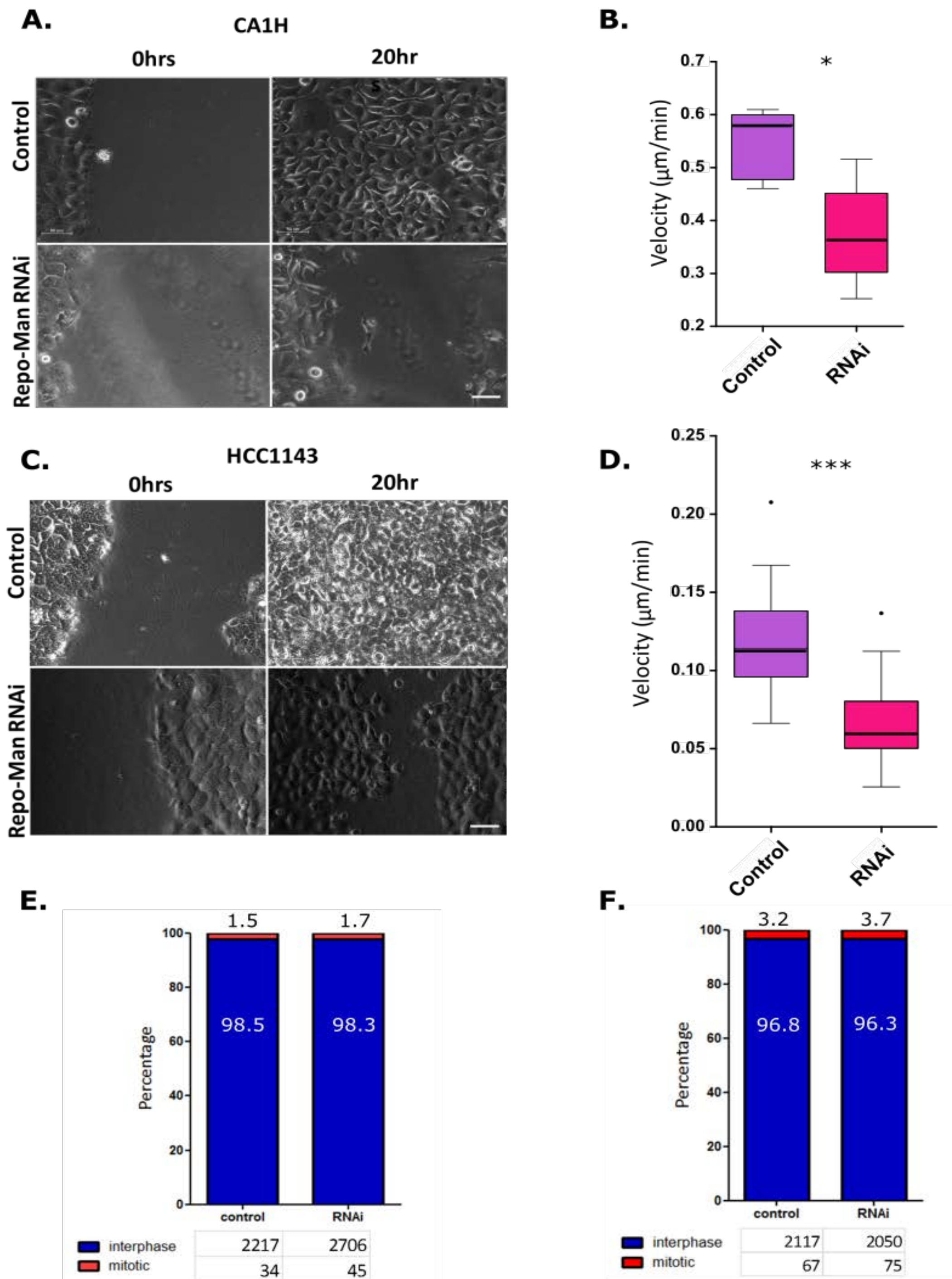


Figure 5.3: Repo-Man knockdown decreases CA1h and HCC1143 cells motility. A and C. Wound healing assay of CA1h and HCC1143 cells that are treated with control and Repo-Man siRNA. This experiment was done by Lorena Ligammari. Repo-Man depleted cells cannot close the wound, whereas control cells efficiently do so. Scale bar 50µm. **B and D.** Velocities of CA1h and HCC1143 cell lines with control and Repo-Man siRNA treatments. There is a significant decrease in velocity for both cell lines when Repo-Man is depleted. **E and F.** Mitotic index of the wound healing assay for CA1h and HCC1143 cell lines respectively. The mitotic index is the same in control and Repo-Man siRNA treated cells in both cell lines. Images are representative of five repeats. Fluorescent microscope is used. Mann-Whitney test is used for significance.

5.2.3 Repo-Man knock-down decreases Aurora A levels in CA1h and HCC1143 cell lines

Since we have already established Repo-Man overexpression in triple negative breast cancer and results from our lab have shown that Repo-Man dosage contributes to the regulation of expression of Polycomb repressed genes (De Castro et al, 2016), we wanted to see how Repo-Man affects gene expression in breast cancer cells. A microarray analysis was performed in our lab and showed that, among the genes controlled by Repo-Man dosage, the expression of an inhibitor of Aurora A (AURKAIP1) was inversely proportional with Repo-Man expression. AURKAIP1 was previously reported to interact with Aurora A, leading to its ubiquitin-dependent degradation (Kiat et al., 2002; Lim and Gopalan, 2007) Therefore, Repo-Man expression may indirectly affect Aurora A levels.

Aurora A has previously been reported to be involved in oncogenic pathways in several ways. By activating NK-κB transcription factor, Aurora A counteracts apoptosis and promotes cell survival (Sun C, et al., 2007). Similarly, by inhibiting the tumour suppressor gene p53, Aurora A blocks the intrinsic apoptotic pathway (Katayama, et al., 2004). Most importantly, Aurora A amplification is very common in breast cancers and by phosphorylating the breast cancer gene BRCA1, Aurora A contributes to the silencing of the gene, causing the cells to proliferate (Ouchi M, et al., 2004).

We therefore decided to analyse the expression levels of Repo-Man, Aurora A and AURKAIP1 using the UCSC cancer browser database and found that AURKAIP1 expression is inversely proportional to Repo-Man levels, although the difference is not striking. However, we can argue that small differences in expression of a regulator could generate a much bigger downstream effect. However, what was really impressive was the correlation between Repo-Man expression and Aurora A (Figure 5.4, A). I have therefore investigated this relationship between Repo-Man and Aurora A with further immunostaining experiments. Repo-Man depleted and control CA1h and HCC1143 cells were stained with an Aurora A antibody and the signal intensity of Aurora A was calculated for both conditions.

Aurora A is localised near the centrosome in late G1/early G2. With the progression of the cell cycle, Aurora A associates with the mitotic poles and the spindle. This association continues until through telophase until G1, when Aurora A gets degraded. Degradation of Aurora A is achieved in multiple ways. First way is through Cdh1 subunit of the APC (Crane et al., 2004); second is de-phosphorylation at Ser51 by PP2A (Horn et al., 2007); and third is by its interactor AURKAIP1 (Fumoto et al., 2008).

Aurora A signal intensities at the two poles were measured in metaphase cells with a defined ROI and compared with the control cells. Unfortunately we were not able to detect Aurora A levels in triple negative breast cancer cells but with CA1h cells we observed a clear decrease in Aurora A levels when Repo-Man was depleted (Figure 5.4, B). With this experiment, we have shown that Repo-Man affects Aurora A also at protein level possibly via regulating its inhibitor AURKAIP1 expression; more interestingly these experiments show that we can control Aurora A levels via Repo-Man.

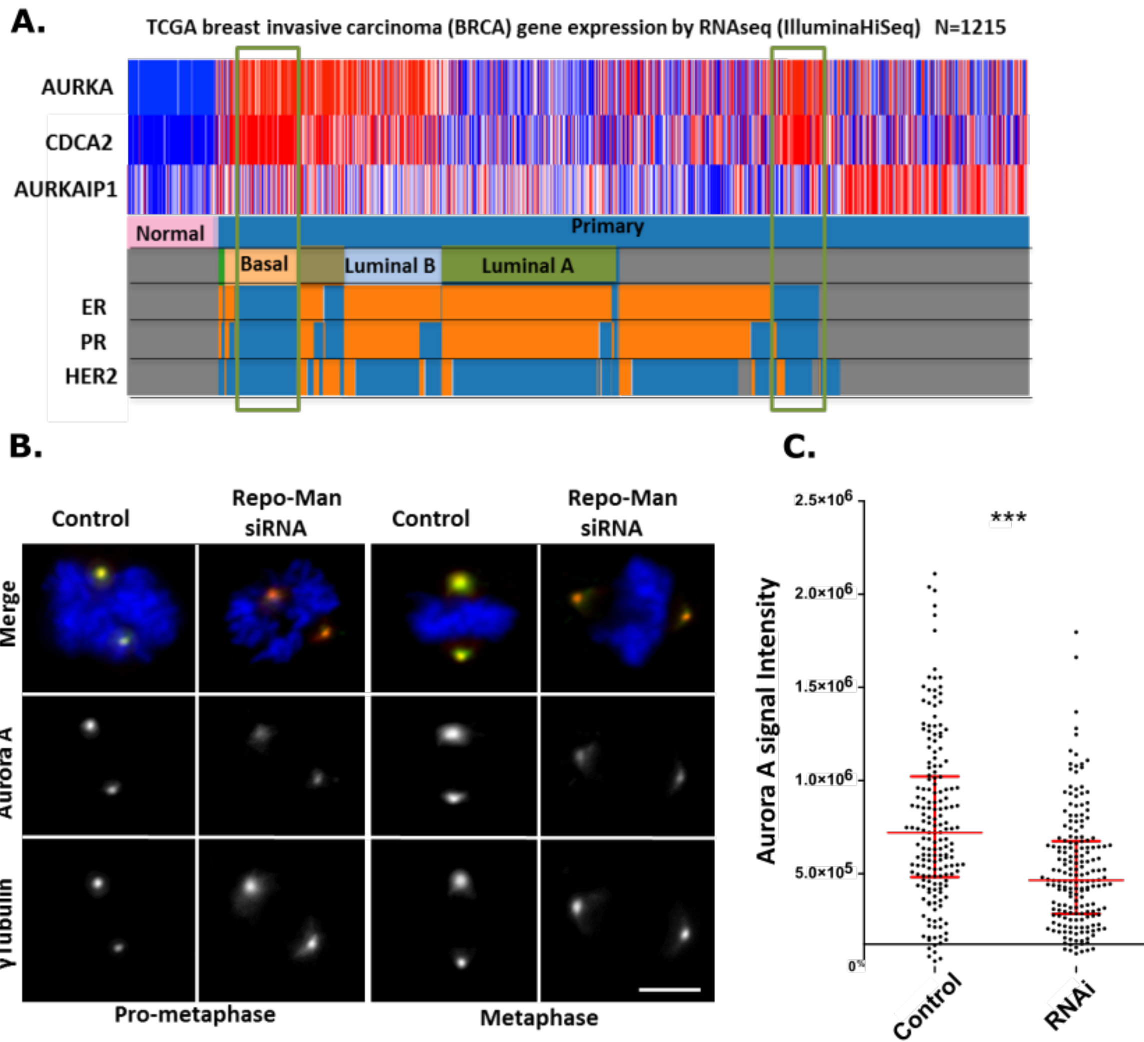


Figure 5.4: Repo-Man affects Aurora A levels through its inhibitor AURKAIP1. A. Expression levels of Repo-Man, Aurora A and AURKAIP1 in different breast cancers (UCSC cancer browser database). Orange areas are positive and blue areas are negative for the corresponding marker. Repo-Man overexpression in triple negative breast cancer corresponds with the low expression of AURKAIP1 and high expression of Aurora A. **B.** Control and Repo-Man siRNA treated CA1h cells were stained with Aurora A antibody. Both pro-metaphase and metaphase cells show low Aurora A levels. Scale bar 10μm. **C.** Quantification of the immunostaining experiment in B. Repo-Man siRNA treated cells have significantly lower levels of Aurora A. Mann-Whitney test is used. 30 cells counted in two repeats.

5.2.4 Repo-Man controls C-Myc levels in CA1h and HCC1143 cells

Being one of the most important transcription factors, C-Myc has already been established as an oncogene that controls growth regulation and cellular metabolism. Overexpression of C-Myc causes dramatic changes in gene expression levels, resulting in increased cell proliferation (Miller et al., 2012). Another member of the Myc family of proteins is the N-Myc, overexpression of which is observed mostly in neuroblastoma.

An important study showed that Aurora A serves as a stabilizer for N-Myc in neuroblastoma, preventing its degradation by ubiquitination. Otto et al., describe a pathway for N-Myc degradation (explained in Figure 5.5, A), where N-Myc is phosphorylated by GSK3 at G1/S by CDK1/Cyclin B as well at G2/M. The second phosphorylation causes N-Myc to be degraded but this process is prevented by Aurora A binding (Otto et al., 2009).

Since we have observed that we can control Aurora A levels in breast cancer cells through Repo-Man, and since N-Myc and C-Myc belong to the same family of proteins and they are structurally very similar, we wanted to find out if Repo-Man depletion also affects C-Myc levels. For this purpose, control and Repo-Man depleted CA1h and HCC1143 cells were stained for total C-Myc and the signal were measured in both cell lines. Our results show that when Repo-Man is depleted, C-Myc levels decrease significantly in both cell lines. This indicates that Repo-Man controls two of the most important oncogenes and especially for triple negative breast cancer this property of Repo-Man could represents a potential therapeutic target

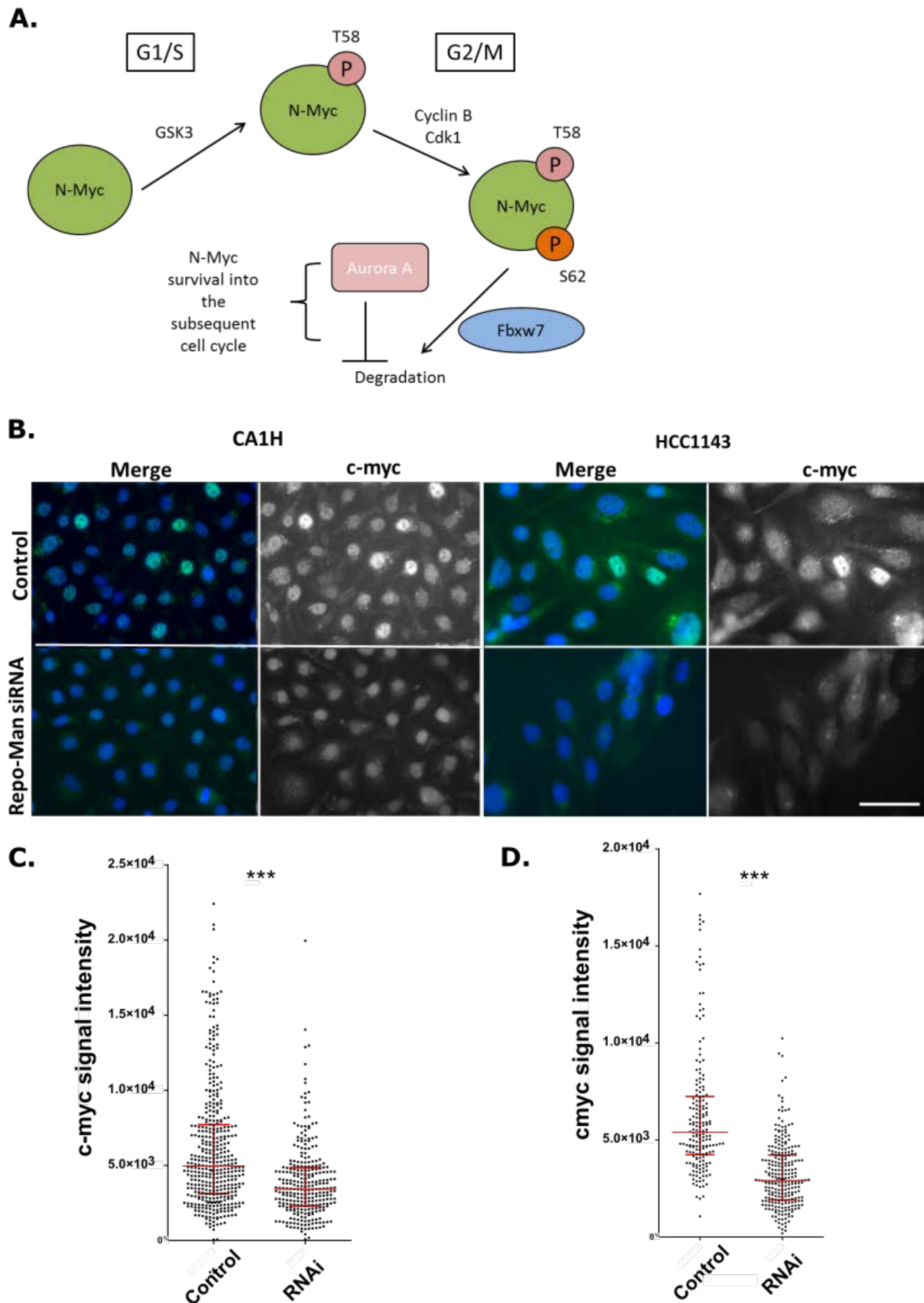


Figure 5.5: Repo-Man controls C-Myc levels in malignant and triple negative breast cancer cells. A. Mechanism of action that explains how Aurora A prevents degradation of N-Myc in neuroblastoma (as described in Otto et al., 2009). B. Control and Repo-Man siRNA transfected CA1h and HCC1143 cells stained with C-Myc antibody, showing a decreased level of C-Myc in Repo-Man depleted cells for both cell lines. Scale bar 20µm. **C and D.** Quantification of the C-Myc staining of CA1h and HCC1143 respectively. C-Myc levels are significantly less in Repo-Man depleted cells. 30 cells counted in 10 different images.

5.3 Discussion

Although Repo-Man has not been defined as a cancer driver gene, it has been reported to be overexpressed in many cancers including synovial sarcoma, squamous cell carcinoma, neuroblastoma, lung cancer and breast cancer (Vagnarelli, 2014). In this chapter we focused on the role of Repo-Man/PP1 complex in breast cancer, specifically on the triple negative breast cancer, because we have shown increased levels of Repo-Man in this type of breast cancer. Another important reason to focus on breast cancer is the fact that Repo-Man depletion has been previously reported to stop the growth of malignant MCF10A-CA1h breast cancer cells on soft agar (Peng et al., 2010). Our aim was to understand the mechanism behind the effect of Repo-Man depletion and test Repo-Man as a therapeutic target for triple negative breast cancer. For this purpose, we have chosen MCF-10A-CA1h and HCC1143 cell lines as models, which represent malignant and triple negative breast cancer cells respectively.

Our starting point was to deplete Repo-Man and observe the behaviour of our model cell lines in a wound healing assay. Control and Repo-Man depleted cells were observed for 24h with live cell imaging and the behaviour of both cell lines were analysed. The results were very striking in terms of cell movement. For both cell lines control cells did not have any difficulties closing the wounds but Repo-Man depleted cells could not close the wound. Here we show the results in the first 20 h because control cells efficiently close the wound within this time point and we took this time point as the reference as to how the cells should have behaved normally. In addition, both cell lines were moving visibly slow around the wound, not wanting to stretch or move across the wound; creating a heartbeat-like movement. Indeed, velocity analysis showed that Repo-Man depleted cells move

significantly slower than the control cells in both cell lines. Our first conclusion to explain Repo-Man mechanism of action is that it affects cell movement but we have also looked at the mitotic index to eliminate the possibility of Repo-Man affecting the ability of cells to divide.

Our studies on Repo-Man and breast cancer revealed another important role for Repo-Man: It affects the levels of two of the most important the important oncogenes, Aurora A and C-Myc. These two genes have already been shown as tumour inducing when overexpressed, as both of them affect important pathways that lead to apoptosis, cell proliferation and differentiation (Miller et al., 2012; Katayana H, et al., 2004). We have revealed this effect of Repo-Man by building on a previously performed microarray in our laboratory, which showed a decreased expression of an Aurora A inhibitor AURKAIP1 when Repo-Man was overexpressed. We have also analysed the expression profile on the UCSC database and furthermore, we have observed that Aurora A levels are directly proportional to Repo-Man levels. Indeed, our immunostaining experiments showed that Repo-Man depletion in breast cancer cells decreases Aurora A levels as well. Although we could not detect Aurora A levels in triple negative breast cancer, this was a particularly important result to explain how important Repo-Man is, in terms of driving malignancy in breast cancer. Moreover, it was shown that Aurora A prevents degradation of N-Myc in neuroblastoma (Otto et al., 2009) and the structural similarities between N-Myc and C-Myc made us wonder if the same prevention happens for C-Myc as well. With another immunostaining experiment we revealed that Repo-Man controls C-Myc levels and depending on the study that connects two proteins and our own results so far, we can hypothesize that this effect might happen through Aurora A. We have also conducted the

same immunostaining experiment with C-Myc in the presence and absence of an Aurora A inhibitor, however these experiments was inconclusive due to different results each time we repeated the experiment. This might have been due to the inhibitor that was used but the length of my PhD was not enough for me to test different inhibitors.

Nevertheless, our overall results on Repo-Man and breast cancer show that Repo-Man has the potential to be used as a therapeutic target especially for triple negative breast cancer, as this type of breast cancer does not currently have reliable target molecules. Here we report a very significant and important role of Repo-Man in breast cancer: It controls the levels of important oncogenes, Aurora A and C-Myc, through an inhibitor of Aurora A and slows down the growth of cells in culture in its absence. These properties of Repo-Man certainly point out that it might be a cancer driving gene and, although we do not have further proof, Repo-Man may also control malignancy as well as metastasis.

6. DISCUSSION

6.1 Repo-Man binds to PP1 via a novel interaction motif: RepoSLIM

With this project, we aimed to reveal how Repo-Man and PP1 bind to each other and identify regions within the molecules that can potentially be used to target and block the complex for the treatment of cancers where Repo-Man is overexpressed. In this thesis, we also propose a novel regulatory mechanism for Repo-Man at mitosis. Furthermore, we explain the selective binding of Repo-Man to PP1γ and propose Repo-Man as a malignancy marker for triple negative breast cancer.

Repo-Man, a multifunctional PP1 targeting subunit, is an important mitotic exit protein that regulates histone H3 de-phosphorylation at anaphase onset and is necessary for cell viability (Trinkle-Mulachy et al., 2006). The C terminal domain localizes Repo-Man to bulk chromatin in early anaphase to target PP1 for de-phosphorylation of histone H3 and the N terminal domain localizes Repo-Man to the chromosome periphery in late anaphase where it helps reassembly of the nuclear envelope by recruiting Importinβ and Nup153 in a PP1 independent manner (Vagnarelli et al., 2011).

Repo-Man counteracts Haspin and Aurora B kinase by de-phosphorylating their targets on H3, which are Thr3 (Haspin), Ser10 and Ser28 (Aurora B) in a PP1 dependent manner (Qian et al., 2011; Dai and Higgins, 2005; Dai et al., 2006; Dai et al., 2005). Its absence was shown to leave H3 hyperphosphorylated, displace the CPC and, in turn, to cause lagging chromosomes (Qian et al., 2013; Qian et al., 2011; Vagnarelli et al., 2011; Qian et al., 2015).

Repo-Man was known to bind PP1 via the canonical RVTF motif, which is common to the majority of PP1 binding subunits (more than 200 identified) (Bollen et al., 2010). Therefore, the RVTF motif does not represent a useful interaction domain to target for therapeutical purposes. Mutation on the RVTF motif (the RAXA mutant), abolishes PP1 binding completely and delays (but does not abolish) Repo-Man localisation on the mitotic chromatin (Trinkle-Mulachy et al., 2006), suggesting that there might be other important regulatory mechanisms. Indeed, the CDK1-Cyclin B phosphorylation sites T412 and T419 as well as S400 were shown to affect Repo-Man localisation on the chromatin, causing pre-mature localisation at their phospho-deficient state (Vagnarelli et al., 2011, Qian et al., 2015).

To reveal the molecular basis for Repo-Man/PP1 complex, we collaborated with the Peti and Page laboratories (Brown University, USA) and identified the minimal binding region of Repo-Man/PP1 complex by NMR and crystallography (explained in detail in Kumar et al., 2016). In addition to the previously identified points S400, T412 and T419, we have identified F404 and S407 as other potential regulatory sites on Repo-Man, according to their conformation when bound to PP1.

These sites on Repo-Man are on the region flanking the RVTF motif that forms a hairpin-like structure on PP1 surface. They (apart from the F404) are not hidden on PP1 surface, instead, they are exposed and they extend away from the PP1 surface. We have called this unique region the RepoSLIM (Small Linear Interacting Motif).

The RepoSLIM is unique and not shared with any other PP1 binding subunits except for Ki67, which has been shown to be is the ancestor of Repo-Man (Booth et al., 2014). Indeed, the crystal structure of Ki67 bound to PP1 revealed that Ki67 and RepoSLIM matches perfectly in terms of the hairpin-like structure, with only a few small differences (explained in Kumar et al., 2016). We should emphasise here that the amino acid sequence of RepoSLIM is not shared with Ki67 but the similarity is caused by how the binding regions of both proteins align on PP1 surface. The differences on the other hand, are mostly angular, caused by the differences in the amino acid sequences.

Overall, the RepoSLIM represents a unique interaction surface that could be a useful target for chemical disruption of protein-protein interaction (PPI). After the discovery of RepoSLIM, we have asked the question: Do the new sites on RepoSLIM have an effect on Repo-Man/PP1 complex formation and function, and also on Repo-Man localisation on chromatin.

6.2 Repo-Man is regulated by multiple kinases via the novel RepoSLIM

Repo-Man has a characteristic localisation throughout the cell cycle. It changes from nuclear (interphase) to dispersed (Prometaphase/metaphase) and it localises on the mitotic chromatin at anaphase; this very dynamic localisation pattern is controlled by phosphorylations (Vagnarelli and Earnshaw, 2012). When Repo-Man is phosphorylated at mitotic entry, it loses its affinity for PP1 as well as to chromatin. As mitosis progresses, the phosphorylations are removed (by PP1 and PP2A) and Repo-Man localises on the chromosomes to de-phosphorylate histone H3 (Vagnarelli et al., 2011; Qian et al, 2011; Qian et al., 2015).

It has been previously reported that CDK1-Cyclin B is responsible for phosphorylating Repo-Man at T412 and T419 and these phosphorylations affect Repo-Man affinity for chromosomes (Vaganrelli et al., 2011). The S400 residue was shown to have the exact same effect together with the CDK1 sites previously reported. Although S400 has been considered another CDK1 substrate (Qian et al., 2015), however there is no evidence provided to support CDK1 involvement for this phosphorylation (unlike the other 2 sites that were shown to be in vitro substrates of CDK1 (Vagnarelli et al, 2011). These data are nevertheless a strong indication of how Repo-Man regulations occur on the RepoSLIM. Furthermore, my mutation analyses on the RepoSLIM sites showed that the new sites are extremely important both for PP1 binding and Repo-Man localisation on chromosomes. This agrees with a recent analysis published by the Bollen laboratory (Qian et al, 2015). The S400, T412 and T419 and S400, S407, T412 and T419 combinations were mutated to Alanine and Aspartic Acid (3A and 3D; 4A and 4D respectively). In addition, investigations of published databases suggested that S400 and S407 are predicted Plk1 phospho-sites, therefore these sites were mutated to

Alanine only (2A) on their own. These two amino acid mutations are commonly used to mimic the phospho-deficient (Alanine) and phospho-mimetic (Aspartic Acid) states, because of their chemical confirmations.

The 3A and 4A mutants had a strong premature localisation on chromosomes supporting the idea that these sites are clearly phosphorylated in vivo. The FA mutant on the other hand, behaved exactly like the wild type allele and did not fail to localise on the chromosomes in anaphase suggesting that the F404 does not play a major role in stabilising the interaction domain with PP1. The differences in localisation between 3A/4A and 2A mutants show that phosphorylation of S400 and S407, the predicted Plk1 phosho-sites, on their own is not enough to block its localisation onto chromosomes and the CDK1 phosphorylation is the pre-requisite for the full Repo-Man inactivation. However, although the 2A mutant did behave like the wild type in terms of localisation, the total H3T3ph was much less than that of wild type suggesting that was still hyper-active.

Interestingly, the localisation of the remaining H3T3ph with the 2A was entirely different than the wild type and no published mutant of Repo-Man has so far been reported to change this pattern. The normal localisation of H3T3ph is at the centromeres and the 2A mutant abolishes this localisation pattern. This result was further investigated in HeLa cells that stably express CENP-A, where I quantified the H3T3ph signal intensity at the CENP-A region and showed that the 2A mutant prevents the centromeric accumulation of H3T3ph. These data suggest that S400 and S407 phosphorylation contribute to maintain the H3T3ph at the centromeres. Moreover, the 2A mutant also showed that, when these sites cannot be phosphorylated, Repo-Man activity is higher but lacks localisation. We can therefore interpret that, whichever kinase is responsible for this phosphorylation, its activity is

somehow limited at the centromere during mitosis, hence we can observe a gradient of Repo-Man activity. Although we have not formally proven that the responsible kinase is Plk1, there is important evidence in favour of this hypothesis. First, active Plk1 is localised at centromeres during mitosis (Beck et al., 2013); second, Plk1 phosphorylates Haspin kinase and contributes towards its activation and promotes H3T3 phosphorylation (Zhou et al., 2014). Therefore, as a kinase that regulates H3T3 phosphorylation, it would possibly be reasonable to postulate that it also blocks the removal of the exact same phosphorylation via inactivation of the responsible phosphatase: these mechanisms are very common in mitosis; third, Plk1 often acts by docking on previous phosphorylations on proteins, which are called priming sites, with its PBD (polo box domain) and CDK1 sites, especially at mitosis, serve as priming sites for Plk1 (Kishi et al., 2009). On Repo-Man, the CDK1 sites are the potential priming sites for Plk1 (as predicted on GPS-Polo), and S400 and S407 are identified as potential phospho-sites. Normally these priming and phospho-sites would be too far from each other but the hairpin-like binding of Repo-Man on PP1 surface brings these sites exactly opposite to each other, making it possible for Plk1 to function.

In addition to CDK1 and putative Plk1 sites, our collaborators also identified T394 in the RVTF motif to be phosphorylated by Aurora B using in-vitro phosphorylation coupled to NMR spectroscopy and mass spectrometry (explained in Kumar et al., 2016). This finding is also very exiting since it shows that Repo-Man is potentially regulated by three mitotic kinases (CDK1, Plk1 and Aurora B) (Figure 6.1) on the RepoSLIM and that these phosphorylations contribute to prevent early Repo-Man localisation on chromosomes but also that are important for topological information.

In light of our data, we hypothesise that, since Aurora B and active Plk1 are centromeric kinases at mitosis, it is more likely that their activity forms a gradient on the chromosomes, which keeps Repo-Man really OFF at the centromeres. However, at the chromosome arms, the kinase activity is not present and Repo-Man can be active (still in a very dynamic status until anaphase onset). This gradient of kinase activity might be contributing to the generation of the localisation pattern of the remaining H3T3ph at the centromeres in mitosis.

An alternative explanation for the mitotic H3T3ph pattern could be the CPC localisation per se. The CPC recognises and binds to H3T3ph via its subunit survivin (Kelly et al., 2010; Wang et al., 2010; Dai and Higgins, 2005). CPC binding to H3T3ph may physically block Repo-Man from removing this phosphorylation. And since Plk1 and CPC recruitment at the centromeres are dependent on each other (Zhou et al., 2014), these two mechanisms might be linked. Further experiments are needed to identify the mechanism behind the H3T3ph localisation.

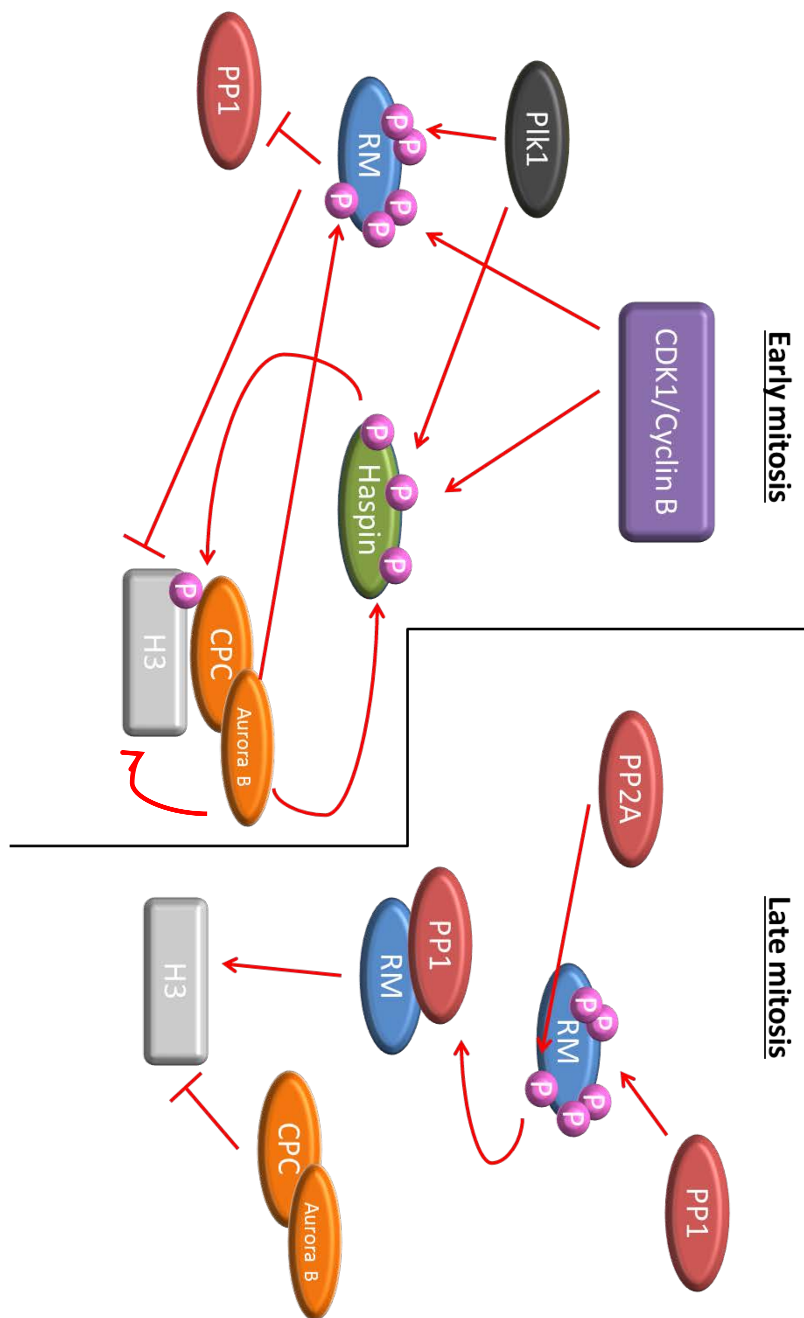


Figure 6.1: Overall schematic review of Repo-Man regulation along with Haspin and Aurora B kinases. In early mitosis phosphorylation of CDK1/Cyclin B targets have different effects on Repo-Man and Haspin. Recognition of the CDK1 phosphorylation on both proteins by Plk1 causes further phosphorylation of both proteins. The phosphorylation has an activating effect on Haspin, and an inactivating effect on Repo-Man. Furthermore, phosphorylation of Repo-Man by Aurora B, keeps Repo-Man away from chromatin and CDK1 phosphorylation along with Plk1, prevents the binding of Repo-Man to PP1. After being activated by CDK1 and Plk1, Haspin phosphorylates H3 at T3, which recruits CPC and a further phosphorylation by Aurora B enhances the T3 phosphorylation by Haspin. Recruitment of CPC also allows Aurora B to further phosphorylate T3 at S10 and S28. In late mitosis on the other hand, de-phosphorylation of Repo-Man by both PP1 and PP2A activates the Repo-Man/PP1 complex, which then de-phosphorylates H3 at T3 along with S10 and S28. This causes the release of CPC from chromatin and allows chromosome segregation.

6.3 Repo-Man selectively binds to PP1γ

Repo-Man localisation on the chromosomes is tightly regulated and it occurs in a timely manner exactly at anaphase onset. It is a dynamic event, depending on the activity of Repo-Man activating and inactivating proteins. We have already shown that the phospho-sites on the RepoSLIM are important in facilitating Repo-Man localisation on chromosomes. In addition, Aurora B activity and PP2A-B56 binding has been reported to be important for the regulation of Repo-Man localization on chromosomes. By phosphorylating Repo-Man at S893, Aurora B prevents premature localization until the phosphorylation is removed by PP2A-B56 and if Aurora B is inhibited, Repo-Man localizes on chromosomes at an early stage (Qian et al., 2011). Also a phospho-mimetic mutation on S893 abolishes Repo-Man localisation on chromosomes and causes H3T3 phosphorylation to remain (Qian et al., 2011; Qian et al., 2015).

I have tested the contribution of these two mechanisms by using an inhibitor of Aurora B (ZM447439) on 3A and wild type Repo-Man transfected HeLa cells. The non PP1 binding RAXA version of 3A and wild type Repo-Man were also used. The results confirmed that PP1 binding is important for Repo-Man localisation on chromosomes, as the RAXA version of both constructs (3A and wild type Repo-Man) abolished the pre-mature localisation in the absence of Aurora B inhibitor. However, in the presence of the Aurora B inhibitor, both 3A and wild type Repo-Man, as well as their RAXA versions, heavily localised on the chromosomes, suggesting that both mechanisms contribute to Repo-Man localisation on chromosomes.

The phospho-mimetic mutants of Repo-Man confirmed further that PP1 contribution is important for Repo-Man localisation on chromosomes. The 3D and 4D mutants behave like the wild type protein and do not show a pre-mature localisation on chromosomes. These mutants also cannot bind to PP1 in interphase, as shown with the Laci/LacO tethering experiments. Along with the Aurora B site T394, which also cannot bind to PP1, these mutants show that phospho-sites on RepoSLIM regulate PP1 binding.

Despite the fact that PP1 isoforms have very little differences in their amino acid sequence, Repo-Man selectively binds to PP1γ and not any other PP1 isoform (Trinkle-Mulcahy 2006). Most of these differences are at the C terminal region end and very few of them are at the N terminal region. Our collaborators have deleted the 15 amino acids that are different on PP1α and found that it does not affect PP1 binding (explained in Kunal et al., 2016). However, they noticed that the R20 residue on PP1γ was in close proximity to Repo-Man when the two proteins are bound to each other. This residue was also previously reported to effect PP1γ localisation throughout the cell cycle. I have therefore mutated this site in PP1γ and shown that this affects its localisation. Moreover, using the Laci/LacO tethering system, I observed that Repo-Man binding to PP1γ was abolished completely in the R20Q mutant (to mimic the residue present in PP1 alpha). The same is true also for Ki67 showing that both these PP1 interacting proteins use the same binding mechanism to direct their specificity. The same mutation on PP1γ abolished the binding of Ki67 as well. These findings confirm that Repo-Man selectively binds to PP1γ and a single amino acid change abolishes the binding and represent the first molecular demonstration of isoform specificity for PP1 targeting subunits.

6.4 Lamin A/C is a substrate of Repo-Man at interphase

Although the S22 phosphorylation on Lamin A/C is mostly known as a mitotic marker that favours nuclear lamina disassembly (Heald and McKeen, 1990), recent studies have shown that it also gets phosphorylated in interphase (Kochin et al., 2014). Furthermore, de-phopshorylation of S22 of Lamin A/C has been shown to be dependent on its SIM3 (Moriuchi et al., 2016).

Repo-Man has been shown to interact with nuclear envelope (NE) proteins like Nup153 and Importinβ and help nuclear envelope reassembly (Vagnarelli et al., 2011). It was also shown that Repo-Man knockdown causes abnormal NE.

Here we have shown that Repo-Man depletion causes increased Lamin A/C phosphorylation at S22 in interphase and that we can rescue this phenotype by transfecting the cells with the oligo resistant wild type Repo-Man in RNAi background. This clearly shows that Repo-Man somehow affects Lamin A/Cph levels in interphase but the mechanism is not yet clear.

Since we know that de-phsophorylation of S22 is SIM3 mediated, we wanted to know if Repo-Man effect on Lamin A/C de-phosphorylation is through Repo-Man sumoylation. The proposed SUMO-site on Repo-Man (K762) (Schou et al., 2014, GPS-SUMO) was mutated to R (IRCE) and the behaviour of the mutant was observed in terms of localisation and Lamin A/C de-phosphorylation in RNAi background. The IRCE did not behave any different than the wild type in terms of localisation, suggesting that this region at the C terminal tail of Repo-Man is not responsible for localisation and the mutant can be expressed in full length. However, the IRCE could not rescue the S22ph levels at interphase, showing that sumoylation may indeed be the mechanism of how the two proteins interact. Although we could not detect

sumoylation of Repo-Man in-vitro, there is solid evidence that Repo-Man sumoylation mediates de-phosphorylation of Lamin A/C. the fact that IRCE mutant could not rescue the phenotype is a strong argument in favour of this mechanism. In addition, the interaction of two proteins, which was detected by PLA, is much less with the IRCE mutant at interphase. Since S22 phosphorylation also happens in interphase, this interaction could potentially meant that Repo-Man de-phosphorylates the pool of Lamin A/C that gets phosphorylated at interphase, or this interaction could be the result of the timely integration of the nuclear lamina proteins to the NE. Therefore, Lamin A/C integration might be later than other proteins like Importin β, hence the de-phosphorylation continues at interphase.

Although we are still not clear about the exact mechanism, we have shown that Lamin A/C is a potential substrate of Repo-Man/PP1 complex. This fits perfectly well within the role of the complex at mitotic exit, as it is an established regulator of both chromatin environment and NE reassembly.

6.5 Repo-Man could represent a therapeutic target for the treatment of triple negative breast cancer

Repo-Man has been shown to be overexpressed in many cancers including neuroblastoma (Krasnoselsky et al., 2005), melanoma (Ryu et al., 2007), synovial sarcoma (Wozniak et al., 2013), squamous cell carcinoma (Uchida et al., 2013) and breast cancer (Peng et al., 2010). Furthermore, its depletion was shown to stop the growth of malignant breast cancer cells on soft agar, and induce apoptosis of squamous cell carcinoma cells. These studies also revealed that high level of Repo-Man is correlated with poor prognosis and metastatic tumours.

Here we used the malignant CA1h and the triple negative HCC1143 cell lines as models to show Repo-Man overexpression and the effect of its depletion. Using a wound-healing assay, we have shown that, in the absence of Repo-Man, the two cell lines had impaired movement. Which indicates that Repo-Man depletion could potentially inhibit or delay metastasis.

Moreover, Repo-Man depletion caused a decreased protein level of Aurora A and C-Myc and this could represent and interesting signalling cascade. Aurora A was shown to act as a stabilizer for N-Myc, preventing its degradation (Otto et al., 2009) and since C and N-Myc have structural similarities, this role could be expected for c-myc as well although it has never been demonstrated. Here we have observed the same effect in our model cell lines.

According to a microarray experiment conducted in the lab upon Repo-Man depletion on the Ca1H cell line, Repo-Man knock down causes up-regulation of AURKAP1 gene expression. AURKAP1 has been proposed to regulate Aurora A degradation (Fumoto et al., 2008).

These findings are quite important in terms of treatment for especially the triple negative breast cancer, as it does not express any of the markers that are currently used to treat breast cancer (ER, PR, Her2). We hypothesise that targeting the RepoSLIM and abolishing the Repo-Man PP1 binding through a peptide specifically designed to compete with Repo-Man for PP1 binding, could decrease the malignant and metastatic properties of triple negative breast cancers (Figure 6.2). When used in combination with other drugs, this could serve as an effective treatment.

Up to date, no chemical or peptide has been reported to block the binding of Repo-Man/PP1 complex except for one (Chatterjee et al., 2012) but we could not see the reported results with this peptide as it was designed to block the RVTF motif and was not specific to Repo-Man. Clearly a more specific approach must be undertaken to identify small molecules that can target the RepoSLIM. A possible approach could be the split-intein circular ligation of peptides and proteins (SICLOPPS), which releases a linear product and a circularized peptide and can be manipulated to express a target protein inside the cell (Gohard et al., 2014; Tavassoli and Benkovic, 2007). This would eliminate the cellular intake problems but a more specific targeting is needed.

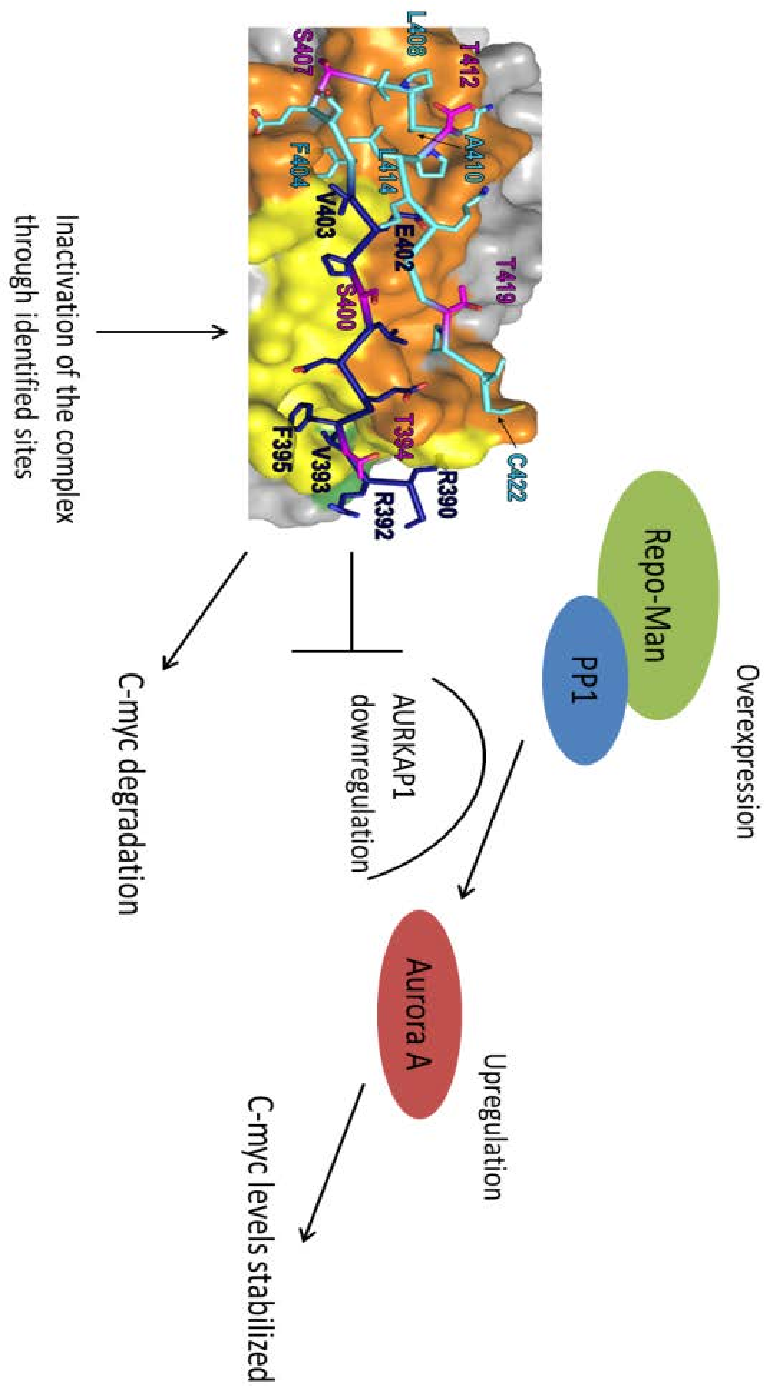


Figure 6.2: The overall working model. Targeting Repo-Man through the RepoSLIM in Repo-Man overexpressed triple negative breast cancer cells would potentially decrease Aurora A levels through the Aurora A inhibitor AURKAP1, causing C-Myc degradation.

7. References

Adams, R.R. et al., 2000. INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavege furrow. *Current Biology*, 10(17), pp.1075–1078.

Alberts, A.S. et al., 1993. Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proceedings of the National Academy of Sciences of the United States of America*, 90(2), pp.388–92. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=45667&tool=pmcentrez&rendertype=abstract.

Asencio, C. et al., 2012. Coordination of kinase and phosphatase activities by Lem4 enables nuclear envelope reassembly during mitosis. *Cell*, 150, pp.122–135.

Barr, F.A., Elliott, P.R. & Gruneberg, U., 2011. Protein phosphatases and the regulation of mitosis. *Journal of Cell Science*, 124(Pt 14), pp.2323–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21709074.

Beck, J. et al., 2013. Ubiquitylation-dependent localization of PLK1 in mitosis. Nature Cell Biology, 15(4), pp.430–439. Available at: http://www.nature.com/doifinder/10.1038/ncb2695%5Cnpapers3://publication/doi/10.1038/ncb2695.

Bollen, M., Peti, W., Ragusa, M. and Beullens, M. (2010). The extended PP1 toolkit: designed to create specificity. Trends in Biochemical Sciences, 35(8), pp.450-458.

Bonelli, P. et al., 2014. CDK/CCN and CDKI alterations for cancer prognosis and therapeutic predictivity. *BioMed Research International*, 2014(January 2015).

Bononi, A. et al., 2011. Protein kinases and phosphatases in the control of cell fate. *Enzyme research*, 2011(1), p.329098. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84857895434&partnerID=40&md5=d31e6373260ebc53970955ab2371d754%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3166778&tool=pmcentrez&rendertype=abstract%5Cnhttp://dx.medra.org/10.4061/2011/329098.

Bononi, A. et al., 2011. Protein kinases and phosphatases in the control of cell fate. *Enzyme Res*, 2011, p.329098. Available at: http://downloads.hindawi.com/journals/er/2011/329098.pdf.

Booth, D.G. et al., 2014. Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *eLife*, 2014, pp.1–22.

Brush, M.H., Weiser, D.C. & Shenolikar, S., 2003. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Molecular and cellular biology*, 23(4), pp.1292–303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12556489%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC141149.

Burgess, A. et al., 2010. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proceedings of the National Academy of Sciences of the United States of America*, 107(28), pp.12564–12569.

Burke, B. & Ellenberg, J., 2002. Remodelling the walls of the nucleus. *Nature reviews. Molecular cell biology*, 3(July), pp.487–497.

Carmena, M. et al., 2012. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nature reviews. Molecular cell biology*, 13(12), pp.789–803. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3729939&tool=pmcentrez&rendertype=abstract.

Carmena, M. & Wheelock, M., 2012. The Chromosomal Passenger Complex (CPC): From Easy Rider to the Godfather of Mitosis. *Nature Review. Molecular Cell Biology*, 13(12), pp.789–803. Available at: http://www.nature.com/nrm/journal/v13/n12/abs/nrm3474.html.

de Castro, I., Gokhan, E. and Vagnarelli, P. (2016). Resetting a functional G1 nucleus after mitosis. Chromosoma, 125(4), pp.607-619.

Chalovich, J.M. & Eisenberg, E., 2005. NIH Public Access. *Biophysical Chemistry*, 257(5), pp.2432–2437.

Chang, K.-H. et al., 2011. Nuclear envelope dispersion triggered by deregulated Cdk5 precedes neuronal death. *Molecular biology of the cell*, 22, pp.1452–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3084668&tool=pmcentrez&rendertype=abstract.

Chatterjee, J. et al., 2012. Development of a peptide that selectively activates protein phosphatase-1 in living cells. *Angewandte Chemie - International Edition*, 51(40), pp.10054–10059.

Cheeseman, I.M. et al., 2006. The Conserved KMN Network Constitutes the Core Microtubule-Binding Site of the Kinetochore. *Cell*, 127(5), pp.983–997.

Cheng, H.C. et al., 2011. Regulation and function of protein kinases and phosphatases. *Enzyme Res*, 2011, p.794089. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22195276.

Choy, M.S. et al., 2014. Understanding the antagonism of retinoblastoma protein dephosphorylation by PNUTS provides insights into the PP1 regulatory code. *Proceedings of the National Academy of Sciences of the United States of America*, (19), pp.1–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24591642.

Clarke, P.R. et al., 1993. Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in Xenopus egg extracts. *Molecular biology of the cell*, 4(April), pp.397–411.

Cooke, R.M. et al., 1987. The solution structure of human epidermal growth factor. *Nature*, 327(6120), pp.339–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3495735.

Coutinho, H.D.M. et al., 2009. Molecular ageing in progeroid syndromes: Hutchinson-Gilford progeria syndrome as a model. *Immunity & ageing : I & A*, 6, p.4.

Crane, R., Kloepfer, A. & Ruderman, J. V, 2004. Requirements for the destruction of human Aurora-A. *Journal of cell science*, 117(Pt 25), pp.5975–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15536123.

Dai, J. et al., 2005. The kinase haspin is required for mitotic histone H3 Thr 3 phosphorylation and normal metaphase chromosome alignment. *Genes and Development*, 19, pp.472–488.

Dai, J. & Higgins, J.M.G., 2005. Haspin: A mitotic histone kinase required for metaphase chromosome alignment. *Cell Cycle*, 4(5), pp.665–668.

Dancheck, B. et al., 2011. Molecular investigations of the structure and function of the protein phosphatase 1-spinophilin-inhibitor 2 heterotrimeric complex. *Biochemistry*, 50(7), pp.1238–1246.

Dechat, T. et al., 2004. LAP2alpha and BAF transiently localize to telomeres and specific regions on chromatin during nuclear assembly. *Journal of cell science*, 117, pp.6117–6128.

Dechat, T. et al., 2008. Nuclear lamins: Major factors in the structural organization and function of the nucleus and chromatin. *Genes and Development*, 22, pp.832–853.

Dohadwala, M. et al., 1994. Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. *Proceedings of the National Academy of Sciences of the United States of America*, 91(14), pp.6408–6412.

Dudek, S.M. et al., 2010. The M Phase Kinase Greatwall (Gwl) Promotes Inactivation of PP2A/B55d , a Phosphatase Directed Against CDK Phosphosites. *Molecular biology of the cell*, 21(22), pp.4042–4056.

Egli, D., Birkhoff, G. & Eggan, K., 2008. Mediators of reprogramming: transcription factors and transitions through mitosis. *Nature reviews. Molecular cell biology*, 9(7), pp.505–516.

Eriksson, J.E. et al., 2009. Introducing intermediate filaments: From discovery to disease. *Journal of Clinical Investigation*, 119, pp.1763–1771.

Félix, M. a, Cohen, P. & Karsenti, E., 1990. Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the Xenopus early embryonic cell cycle: evidence from the effects of okadaic acid. *The EMBO journal*, 9(3), pp.675–683.

Fischle, W. et al., 2005. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature*, 438(7071), pp.1116–1122. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16222246.

Foley, E. a., Maldonado, M. & Kapoor, T.M., 2011. Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nature Cell Biology*, 13(10), pp.1265–1271. Available at: http://dx.doi.org/10.1038/ncb2327.

Forester, C.M. et al., 2007a. Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proceedings of the National Academy of Sciences of the United States of America*, 104(50), pp.19867–19872.

Forester, C.M. et al., 2007b. Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proceedings of the National Academy of Sciences of the United States of America*, 104(50), pp.19867–19872.

Fukagawa, T. & Earnshaw, W.C., 2014. The centromere: Chromatin foundation for the kinetochore machinery. *Developmental Cell*, 30(5), pp.496–508.

Fumoto, K. et al., 2008. AIP Regulates Stability of Aurora-A at Early Mitotic Phase Coordinately with GSK-3beta. *Oncogene*, 27(32), pp.4478–4487.

Gharbi-Ayachi, Jean-Claude Labbé, Andrew Burgess, Suzanne Vigneron, Jean-Marc Strub, Estelle Brioudes, Alain Van-Dorsselaer, Anna Castro, T.L. & Initiation, 2010. The Substrate of Greatwall Kinase, Arpp19, Controls Mitosis by Inhibiting Protein Phosphatase 2A. Science, 330(December), pp.1673–1677.

Ghenoiu, C., Wheelock, M. and Funabiki, H. (2013). Autoinhibition and Polo-Dependent Multisite Phosphorylation Restrict Activity of the Histone H3 Kinase Haspin to Mitosis. Molecular Cell, 52(5), pp.734-745.

Glenn, G.M. & Eckhart, W., 1995. Amino-terminal regions of polyomavirus middle T antigen are required for interactions with protein phosphatase 2A. *Journal of Virology*, 69(6), pp.3729–3736.

Gohard, F., St-Cyr, D., Tyers, M. and Earnshaw, W. (2014). Targeting the INCENP IN-box-Aurora B interaction to inhibit CPC activity in vivo. Open Biology, 4(11), pp.140163-

140163.

Goto, H. et al., 2003. Aurora-B regulates the cleavage furrow-specific vimentin phosphorylation in the cytokinetic process. *Journal of Biological Chemistry*, 278(10), pp.8526–8530.

Grallert, A. et al., 2014. A PP1/PP2A phosphatase relay controls mitotic progression. *Nature*, 517(7532), pp.94–98. Available at: http://dx.doi.org/10.1038/nature14019.

Guo, H. & Damuni, Z., 1993. Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. *Proc Natl Acad Sci U S A*, 90(6), p.2500–4.

Güttinger, S., Laurell, E. & Kutay, U., 2009. Orchestrating nuclear envelope disassembly and reassembly during mitosis. *Nature reviews. Molecular cell biology*, 10(3), pp.178–191.

Harada, T. et al., 2014. Nuclear lamin stiffness is a barrier to 3D migration, but softness can limit survival. *Journal of Cell Biology*, 204(5), pp.669–682.

Haraguchi, T. et al., 2008. Live cell imaging and electron microscopy reveal dynamic processes of BAF-directed nuclear envelope assembly. *Journal of Cell Science*, 121, pp.2540–2554.

Harel, A. et al., 2003. Importin beta negatively regulates nuclear membrane fusion and nuclear pore complex assembly. *Molecular biology of the cell*, 14(11), pp.4387–96. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=266759&tool=pmcentrez&rendertype=abstract.

Heald, R. & McKeon, F., 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell*, 61, pp.579–589.

Hendrickx, A., Beullens, M., Ceulemans, H., Den Abt, T., Van Eynde, A., Nicolaescu, E., Lesage, B. and Bollen, M. (2009). Docking Motif-Guided Mapping of the Interactome of Protein Phosphatase-1. Chemistry & Biology, 16(4), pp.365-371.

Hirota, T. et al., 2005. Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature*, 438(7071), pp.1176–1180.

Honda, R., Körner, R. & Nigg, E., 2003. Exploring the Functional Interactions between Aurora B, INCENP, and Survivin in Mitosis. *Molecular biology of the cell*, 14(August), pp.3325–3341.

Horn V, Thélu J, Garcia A, Albigès-Rizo C, Block MR, V.J., 2007. Functional Interaction of Aurora-A and PP2A during Mitosis. *Molecular biology of the cell*, 18(8), pp.2991–3001.

Hurley, T., Yang, J., Zhang, L., Goodwin, K., Zou, Q., Cortese, M., Dunker, A. and DePaoli-Roach, A. (2007). Structural Basis for Regulation of Protein Phosphatase 1 by Inhibitor-2. Journal of Biological Chemistry, 282(39), pp.28874-28883.

Van Der Horst, A. & Lens, S.M.A., 2014. Cell division: Control of the chromosomal passenger complex in time and space. *Chromosoma*, 123(1–2), pp.25–42.

Howell, B.J. et al., 2004. Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr Biol*, 14(11), pp.953–964. Available at: http://www.hubmed.org/display.cgi?uids=15182668.

Hsu, J.-Y. et al., 2000. Mitotic Phosphorylation of Histone H3 Is Governed by Ipl1/aurora Kinase and Glc7/PP1 Phosphatase in Budding Yeast and Nematodes. *Cell*, 102(3), pp.279–291. Available at: http://www.sciencedirect.com/science/article/pii/S0092867400000349.

Janssens, V. & Goris, J., 2001. Phosphatases Implicated in Cell Growth and Signalling. *Society*, 439, pp.417–439. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1221586/.

Janssens, V., Goris, J. & Van Hoof, C., 2005. PP2A: The expected tumor suppressor. *Current Opinion in Genetics and Development*, 15(1), pp.34–41.

Jeyaprakash, A.A. et al., 2007. Structure of a Survivin-Borealin-INCENP Core Complex Reveals How Chromosomal Passengers Travel Together. *Cell*, 131(2), pp.271–285.

Jin, Z. et al., 2009. The 48-kDa alternative translation isoform of PP2A:B56?? is required for Wnt signaling during midbrain-hindbrain boundary formation. *Journal of Biological Chemistry*, 284(11), pp.7190–7200.

Katayama, H. et al., 2004. Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. *Nat Genet*, 36(1), pp.55–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14702041.

Kelker, M.S., Page, R. & Peti, W., 2009. Crystal Structures of Protein Phosphatase-1 Bound to Nodularin-R and Tautomycin: A Novel Scaffold for Structure-based Drug Design of Serine/Threonine Phosphatase Inhibitors. *Journal of Molecular Biology*, 385(1), pp.11–21. Available at: http://dx.doi.org/10.1016/j.jmb.2008.10.053.

Kelly, A.E. et al., 2010. Survivin Reads Phosphorylated Histone H3 Threonine 3 to Activate the Mitotic Kinase Aurora B. *Science*, 330(6001), pp.235–239. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21959306%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3259195%5Cnhttp://www.sciencemag.org/cgi/doi/10.1126/science.1189505.

Kiat, L.S., Hui, K.M. & Gopalan, G., 2002. Aurora-A kinase interacting protein (AIP), a novel negative regulator of human Aurora-A kinase. *Journal of Biological Chemistry*, 277(47), pp.45558–45565.

Kim, Y., Holland, A., Lan, W. and Cleveland, D. (2010). Aurora Kinases and Protein Phosphatase 1 Mediate Chromosome Congression through Regulation of CENP-E. Cell, 142(3), pp.444-455.

Kishi, K. et al., 2009. Functional dynamics of Polo-like kinase 1 at the centrosome. *Molecular and cellular biology*, 29(11), pp.3134–3150.

Kita, A. et al., 2002. Crystal structure of the complex between calyculin A and the catalytic subunit protein phosphatase 1. [Erratum to document cited in CA137:121557]. *Structure (Cambridge, MA, United States)*, 10(8), p.71149.

Kitagawa, M. & Lee, S.H., 2015. The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis. *Frontiers in cell and developmental biology*, 3(March), p.14. Available at: http://journal.frontiersin.org/article/10.3389/fcell.2015.00014/abstract.

Kochin, V. et al., 2014. Interphase phosphorylation of lamin A. *Journal of cell science*, 127, pp.2683–96. Available at: http://jcs.biologists.org/content/127/12/2683.long.

Kouzarides, T., 2007. Chromatin Modifications and Their Function. *Cell*, 128(4), pp.693–705.

Krasinska, L. et al., 2011. Protein phosphatase 2A controls the order and dynamics of cell-cycle transitions. *Molecular Cell*, 44, pp.437–450.

Krasnoselsky, A.L. et al., 2005. Altered expression of cell cycle genes distinguishes aggressive neuroblastoma. *Oncogene*, 24(9), pp.1533–1541. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=15592497.

Krasnoselsky, A.L. et al., 2005. Altered expression of cell cycle genes distinguishes aggressive neuroblastoma. *Oncogene*, 24, pp.1533–1541.

Krenn, V. et al., 2014. KI motifs of human Knl1 enhance assembly of comprehensive spindle checkpoint complexes around MELT repeats. *Current Biology*, 24(1), pp.29–39. Available at: http://dx.doi.org/10.1016/j.cub.2013.11.046.

Kwon, Y.G. et al., 1997. Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 94(6), pp.2168–2173.

Landsverk, H., Kirkhus, M., Bollen, M., Küntziger, T. and Collas, P. (2005). PNUTS enhancesin vitrochromosome decondensation in a PP1-dependent manner. Biochemical Journal, 390(3), pp.709-717.

Lagarde, P. et al., 2013. Chromosome instability accounts for reverse metastatic outcomes of pediatric and adult synovial sarcomas. *Journal of Clinical Oncology*, 31(5), pp.608–615.

Lampson, M.A. & Cheeseman, I.M., 2011. Sensing centromere tension: Aurora B and the regulation of kinetochore function. *Trends in Cell Biology*, 21(3), pp.133–140.

Laurell, E. et al., 2011. Phosphorylation of Nup98 by multiple kinases is crucial for NPC disassembly during mitotic entry. *Cell*, 144, pp.539–550.

Lesage, B. et al., 2005. Determinants of the nucleolar targeting of protein phosphatase-1. *FEBS Letters*, 579, pp.5626–5630.

Leulliot, N. et al., 2004. Structure of Protein Phosphatase Methyltransferase 1 (PPM1), a Leucine Carboxyl Methyltransferase Involved in the Regulation of Protein Phosphatase 2A Activity. *Journal of Biological Chemistry*, 279(9), pp.8351–8358.

Lim, S.K. & Gopalan, G., 2007. Antizyme1 mediates AURKAIP1-dependent degradation of Aurora-A. *Oncogene*, 26(46), pp.6593–6603.

Longin, S. et al., 2007. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic subunit. *Journal of Biological Chemistry*, 282(37), pp.26971–26980.

Lucocq, J., Warren, G. & Pryde, J., 1991. Okadaic acid induces Golgi apparatus fragmentation and arrest of intracellular transport. *Journal of cell science*, 100 (Pt 4, pp.753–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1667660.

Lüscher, B. et al., 1991. A role for the p34cdc2 kinase and phosphatases in the regulation of phosphorylation and disassembly of lamin B2 during the cell cycle. *The EMBO journal*, 10(4), pp.865–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452727&tool=pmcentrez&rendertype=abstract.

Mackay, A.M. et al., 1998. A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis. *Journal of Cell Biology*, 140(5), pp.991–1002.

Margolis, S.S. et al., 2006. Role for the PP2A/B56?? Phosphatase in Regulating 14-3-3 Release from Cdc25 to Control Mitosis. *Cell*, 127(4), pp.759–773.

Maynes, J.T. et al., 2001. Crystal Structure of the Tumor-promoter Okadaic Acid Bound to Protein Phosphatase-1. *Journal of Biological Chemistry*, 276(47), pp.44078–44082.

McKinley, K.L. & Cheeseman, I.M., 2014. Polo-like kinase 1 licenses CENP-a deposition at centromeres. *Cell*, 158(2), pp.397–411.

Meiselbach, H., Sticht, H. & Enz, R., 2006. Structural analysis of the protein phosphatase 1 docking motif: Molecular description of binding specificities identifies interacting proteins. *Chemistry and Biology*, 13(1), pp.49–59.

Michael Glotzer, Andrew W. MURRAY, M.W.K., 1991. Cyclin is degraded by the ubiquitin pathway. *Nature*, 354, pp.56–58.

Miller, D.M. et al., 2012. c-Myc and cancer metabolism. *Clinical Cancer Research*, 18(20), pp.5546–5553.

Mochida, S. et al., 2009. Regulated activity of PP2A-B55 delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts. *The EMBO journal*, 28(18), pp.2777–2785. Available at: http://dx.doi.org/10.1038/emboj.2009.238.

Moorhead, G., Trinkle-Mulcahy, L. & Ulke-Lemee, A., 2007. Emerging roles of nuclear protein phosphatases. *Nature Reviews Molecular Cell Biology*, 8(3), pp.234–244. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17318227%5Cnhttp://www.nature.com/nrm/journal/v8/n3/abs/nrm2126.html.

Moriuchi, T. et al., 2016. Lamin A reassembly at the end of mitosis is regulated by its SUMO-interacting motif. *Experimental Cell Research*, 342(1), pp.83–94. Available at: http://dx.doi.org/10.1016/j.yexcr.2016.02.016.

Murnion, M.E. et al., 2001. Chromatin-associated Protein Phosphatase I Regulates Aurora-B and Histone H3 Phosphorylation. *Journal of Biological Chemistry*, 276(28), pp.26656–26665.

Musacchio, A., 2015. The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *Current Biology*, 25(20), pp.R1002–R1018. Available at: http://dx.doi.org/10.1016/j.cub.2015.08.051.

Novak, B. et al., 2010. Regulated protein kinases and phosphatases in cell cycle decisions. *Current Opinion in Cell Biology*, 22(6), pp.801–808. Available at: http://dx.doi.org/10.1016/j.ceb.2010.07.001.

Ottaviano, Y. & Gerace, L., 1985. Phosphorylation of the nuclear lamins during interphase and mitosis. *Journal of Biological Chemistry*, 260, pp.624–632.

Otto, T. et al., 2009. Stabilization of N-Myc Is a Critical Function of Aurora A in Human Neuroblastoma. *Cancer Cell*, 15(1), pp.67–78.

Ouchi, M. et al., 2004. BRCA1 Phosphorylation by Aurora-A in the Regulation of G2 to M Transition. *Journal of Biological Chemistry*, 279(19), pp.19643–19648.

Peng, A. et al., 2010. Repo-Man Controls a Protein Phosphatase 1-Dependent Threshold for DNA Damage Checkpoint Activation. *Current Biology*, 20(5), pp.387–396. Available at: http://dx.doi.org/10.1016/j.cub.2010.01.020.

Peter, M. et al., 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell*, 61, pp.591–602.

Peti, W., Nairn, A.C. & Page, R., 2013. Structural basis for protein phosphatase 1 regulation and specificity. *FEBS Journal*, 280(2), pp.596–611.

Posch, M. et al., 2010. Sds22 regulates aurora B activity and microtubule-kinetochore interactions at mitosis. *Journal of Cell Biology*, 191(1), pp.61–74.

Primorac, I. & Musacchio, A., 2013. Panta rhei: The APC/C at steady state. *Journal of Cell Biology*, 201(2), pp.177–189.

Qian, J. et al., 2013. Aurora B defines its own chromosomal targeting by opposing the recruitment of the phosphatase scaffold Repo-Man. *Current Biology*, 23(12), pp.1136–1143. Available at: http://dx.doi.org/10.1016/j.cub.2013.05.017.

Qian, J. et al., 2015. of a Chromosome-Associated Phosphatase Switch. *Nature Communications*, pp.1–13. Available at: http://dx.doi.org/10.1038/ncomms10215.

Qian, J. et al., 2011. PP1/repo-man dephosphorylates mitotic histone H3 at T3 and regulates chromosomal aurora B targeting. *Current Biology*, 21(9), pp.766–773. Available at: http://dx.doi.org/10.1016/j.cub.2011.03.047.

Queralt, E. et al., 2006. Downregulation of PP2ACdc55 Phosphatase by Separase Initiates Mitotic Exit in Budding Yeast. *Cell*, 125, pp.719–732.

Racine, A. et al., 2012. Histone H2B ubiquitylation promotes activity of the intact set1 histone methyltransferase complex in fission yeast. *Journal of Biological Chemistry*, 287(23), pp.19040–19047.

Ragusa, M.J. et al., 2010. Spinophilin directs protein phosphatase 1 specificity by blocking substrate binding sites. *Nature structural & molecular biology*, 17(4), pp.459–64. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2924587&tool=pmcentrez&rendertype=abstract.

Riedel, C.G. et al., 2006. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature*, 441(7089), pp.53–61.

Ronne, H. et al., 1991. Protein phosphatase 2A in Saccharomyces cerevisiae: effects on cell growth and bud morphogenesis. *Mol Cell Biol*, 11(10), pp.4876–4884. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1656215.

Ruchaud, S., Carmena, M. & Earnshaw, W.C., 2007. Chromosomal passengers: conducting cell division. *Nat Rev Mol Cell Biol*, 8(10), pp.798–812. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17848966.

Ruvolo, P.P. et al., 2002. A functional role for the B56 ??-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *Journal of Biological Chemistry*, 277(25), pp.22847–22852.

Ryu, B. et al., 2007. Comprehensive expression profiling of tumor cell lines identifies molecular signatures of melanoma progression. *PLoS ONE*, 2(7).

Sangodkar, J. et al., 2016. All roads lead to PP2A: Exploiting the therapeutic potential of this phosphatase. *FEBS Journal*, 283(6), pp.1004–1024.

Santaguida, S. et al., 2011. Evidence that Aurora B is implicated in spindle checkpoint signalling independently of error correction. *EMBO J*, 30(8), pp.1508–1519. Available at: http://www.hubmed.org/display.cgi?uids=21407176.

Schimmel, J. et al., 2014. Uncovering SUMOylation dynamics during cell-cycle progression reveals foxM1 as a key mitotic SUMO target protein. *Molecular Cell*, 53(6), pp.1053–1066.

Schmitz, M.H. a et al., 2010. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. *Nature cell biology*, 12(9), pp.886–893. Available at: http://dx.doi.org/10.1038/ncb2092.

Schou, J. et al., 2014. Comprehensive identification of SUMO2/3 targets and their dynamics during mitosis. *PLoS ONE*, 9(6).

Schönthal, A.H., 2001. Role of serine/threonine protein phosphatase 2A in cancer. *Cancer Letters*, 170(1), pp.1–13.

Shi, Y., 2009. Serine/Threonine Phosphatases: Mechanism through Structure. *Cell*, 139, pp.468–484.

Simon, D.N. & Wilson, K.L., 2013. Partners and post-translational modifications of nuclear lamins. , 122(0), pp.13–31.

Snider, N.T. & Omary, M.B., 2014. Post-translational modifications of intermediate filament proteins: mechanisms and functions. *Nature reviews. Molecular cell biology*, 15, pp.163–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4079540&tool=pmcentrez&rendertype=abstract.

Steen, R.L. et al., 2000. Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly. *Journal of Cell Biology*, 150(6), pp.1251–1261.

Sullivan, M. & Morgan, D.O., 2007. Finishing mitosis, one step at a time. *Nature reviews. Molecular cell biology*, 8(november), pp.894–903.

Sun, C. et al., 2007. Aurora kinase inhibition downregulates NF-kappaB and sensitises tumour cells to chemotherapeutic agents. *Biochemical and biophysical research communications*, 352(1), pp.220–225.

Takagi, M. & Imamoto, N., 2014. Cancer Biology and the Nuclear Envelope. , 773, pp.571–591. Available at: http://link.springer.com/10.1007/978-1-4899-8032-8.

Tavassoli, A. & Benkovic, S.J., 2007. Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in E. coli. *Nature protocols*, 2(5), pp.1126–1133.

Terrak, M. et al., 2004. Structural basis of protein phosphatase 1 regulation. *Nature*, 429(6993), pp.780–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15164081.

Thompson, L.J., Bollen, M. & Fields, A.P., 1997. Identification of protein phosphatase 1 as a mitotic lamin phosphatase. *Journal of Biological Chemistry*, 272, pp.29693–29697.

Trinkle-Mulcahy, L. and Lamond, A. (2006). Mitotic phosphatases: no longer silent partners. Current Opinion in Cell Biology, 18(6), pp.623-631.

Uchida, F. et al., 2013. Overexpression of CDCA2 in human squamous cell carcinoma: correlation with prevention of G1 phase arrest and apoptosis. *PLoS One*, 8(2), p.e56381.

Vagnarelli, P. et al., 2006. Condensin and Repo-Man-PP1 co-operate in the regulation of chromosome architecture during mitosis. *Nature cell biology*, 8(10), pp.1133–1142.

Vagnarelli, P. et al., 2011. Repo-Man Coordinates Chromosomal Reorganization with Nuclear Envelope Reassembly during Mitotic Exit. *Developmental Cell*, 21(2), pp.328–342. Available at: http://dx.doi.org/10.1016/j.devcel.2011.06.020.

Vagnarelli, P. & Earnshaw, W.C., 2012. Repo-Man-PP1: A link between chromatin remodelling and nuclear envelope reassembly. *Nucleus*, 3(2), pp.25–29.

Vagnarelli, P. (2014). Repo-Man at the Intersection of Chromatin Remodelling, DNA Repair, Nuclear Envelope Organization, and Cancer Progression. In: E. Schirmer and J. de las Heras, ed., Cancer Biology and the Nuclear Envelope, 1st ed. New York: Springer Science+Business Media.

Vernia, S. et al., 2009. Increased endoplasmic reticulum stress and decreased proteasomal function in Lafora disease models lacking the phosphatase laforin. *PLoS ONE*, 4(6).

Vigneron, S. et al., 2009. Greatwall maintains mitosis through regulation of PP2A. *The EMBO journal*, 28(18), pp.2786–93.

Vigneron, S. et al., 2004. Kinetochore localization of spindle checkpoint proteins: who controls whom? *Mol Biol Cell*, 15(10), pp.4584–4596.

Virshup, D.M. & Shenolikar, S., 2009. From Promiscuity to Precision: Protein Phosphatases Get a Makeover. *Molecular Cell*, 33(5), pp.537–545.

Voets, E. & Wolthuis, R.M.F., 2010. MASTL is the human orthologue of Greatwall kinase that facilitates mitotic entry, anaphase and cytokinesis. *Cell Cycle*, 9(17), pp.3591–3601.

Wakula, P. et al., 2003. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. *Journal of Biological Chemistry*, 278(21), pp.18817–18823.

Walther, T.C. et al., 2003. The conserved Nup107-160 complex is critical for nuclear pore complex assembly. *Cell*, 113(2), pp.195–206.

Wang, F., Ulyanova, N.P., et al., 2011. A positive feedback loop involving haspin and aurora B promotes CPC accumulation at centromeres in mitosis. *Current Biology*, 21(12), pp.1061–1069. Available at: http://dx.doi.org/10.1016/j.cub.2011.05.016.

Wang, F., Dai, J., et al., 2011. Histone H3 Thr-3 phosphorylation by Haspin positions Aurora B at centromeres in mitosis. , 330(6001), pp.231–235.

Wang, F. et al., 2010. Histone H3 Thr-3 phosphorylation by Haspin positions Aurora B at centromeres in mitosis. *Science (New York, N.Y.)*, 330(6001), pp.231–235.

Wang, F. & Higgins, J.M.G., 2013. Histone modications and mitosis: Countermarks, landmarks, and bookmarks. *Trends in Cell Biology*, 23(4), pp.175–184. Available at: http://dx.doi.org/10.1016/j.tcb.2012.11.005.

Wang, H. et al., 2004. Role of histone H2A ubiquitination in Polycomb silencing. *Nature*, 431(7010), pp.873–8. Available at: http://dx.doi.org/10.1038/nature02926%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/15386022.

Wang, Y. & Ng, T.-Y., 2006. Phosphatase 2A Negatively Regulates Mitotic Exit in Saccharomyces cerevisiae. *Molecular biology of the cell*, 17(January), pp.80–89.

Whisstock, J.C. & Bottomley, S.P., 2006. Molecular gymnastics: serpin structure, folding and misfolding. *Current Opinion in Structural Biology*, 16, pp.761–768.

Wurzenberger, C. & Gerlich, D.W., 2011. Phosphatases: providing safe passage through mitotic exit. *Nature reviews. Molecular cell biology*, 12(8), pp.469–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21750572.

Yamagishi, Y. et al., 2010. Two histone marks establish the inner centromere and chromosome bi-orientation. *Science*, 330(6001), pp.239–243.

Yamashiro, S., Yamakita, Y., Totsukawa, G., Goto, H., Kaibuchi, K., Ito, M., Hartshorne, D. and Matsumura, F. (2008). Myosin Phosphatase-Targeting Subunit 1 Regulates Mitosis by Antagonizing Polo-like Kinase 1. Developmental Cell, 14(5), pp.787-797.

Yamashita, K. et al., 1990. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *The EMBO journal*, 9(13), pp.4331–8.

Zhou, L. et al., 2014. Polo-like kinase-1 triggers Histone phosphorylation by Haspin in mitosis. *EMBO Reports*, 15(3), pp.273–281.