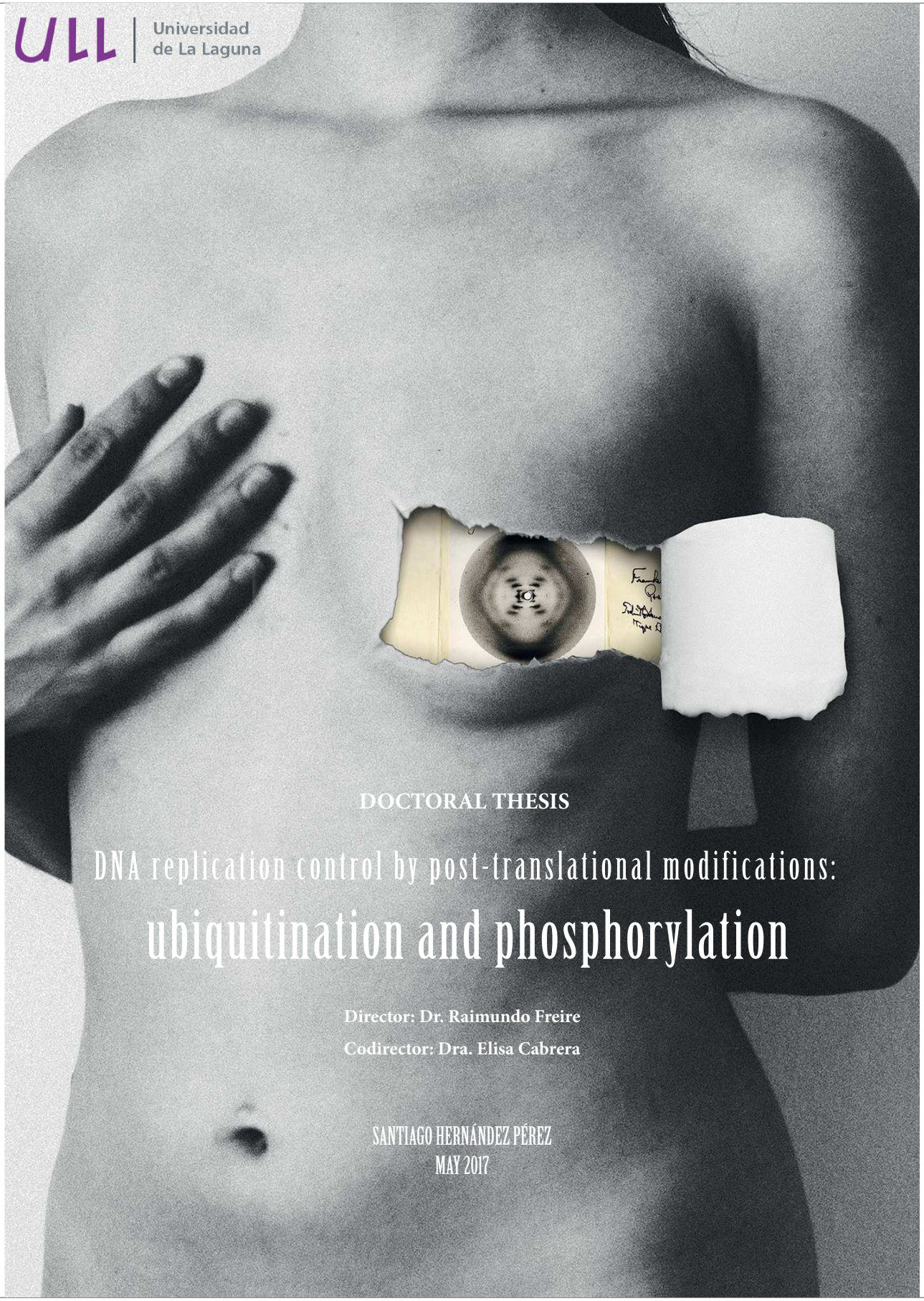

DOCTORAL THESIS

DNA replication control by post-translational modifications: ubiquitination and phosphorylation

Director: Dr. Raimundo Freire

Codirector: Dra. Elisa Cabrera

SANTIAGO HERNÁNDEZ PÉREZ

MAY 2017

"Every science begins as philosophy and ends as art."

William James Durant

Este trabajo ha sido financiado a través de los proyectos SAF2013-49149-R y BFU2014-51672-REDC del Ministerio de Economía y Competitividad, el proyecto AP2015/008 de la Fundación Cajacanarias y el proyecto BA15/00092 del Instituto de Salud Carlos III.

Santiago Hernández Pérez ha sido contratado como investigador a través del proyecyo SAF2013-49149-R del Ministerio de Economía y Competitividad.

Dr. Raimundo Freire Betancor y la **Dra. Elisa Cabrera Afonso, director** y **co-directora** de la tesis doctoral presentada por el licenciado Santiago Hernández Pérez

Certifica que:

La memoria presentada por el Licenciado en Biología **Santiago Hernández Pérez** titulada **"DNA replication control by post-traslational modifications: ubiquitination and phosphorylation"** ha sido realizada bajo su dirección en la Unidad de Investigación del Hospital Universitario de Canarias, y considerando que reúne las condiciones de calidad y rigor científico, se autoriza para que pueda ser presentada y defendida ante la comisión nombrada al efecto para optar al grado de doctor con **mención internacional** por la Universidad de La Laguna.

La Laguna, 22 MARZO 2017

Fdo: Dr. Raimundo Freire Betancor

Dra. Elisa Cabrera Afonso

Dr. Raimundo Freire Betancor y la **Dra. Elisa Cabrera Afonso, director** y **co-directora** de la tesis doctoral presentada por el licenciado **Santiago Hernández Pérez**

Certifica que:

El Licenciado en Biología **Santiago Hernández Pérez** ha realizado la parte fundamental de los artículos incluidos en la memoria titulada **"DNA replication control by post-traslational modifications: ubiquitination and phosphorylation"** con lo que se autoriza para que pueda ser presentada y defendida como tesis doctoral por **compendio de publicicaciones** por la Universidad de La Laguna.

La Laguna, 22 MARZO 2017

Fdo: Dr. Raimundo Freire Betancor

Dra. Elisa Cabrera Afonso

ACKNOWLEDGMENTS

"Errors are notoriously hard to kill. But an error that ascribes to a man what was actually the work of a woman has more lives than a cat."

Hertha Ayrton

Una vez llegado a este punto te das cuenta que esta tesis no sólo recoge el trabajo propio de años, sino que para que haya salido adelante son muchas las personas que han tenido que estar involucradas. Además, es inevitable no ver en ella una evolución profesional y personal donde quedará reflejada tanto lo que he sido capaz de aprender en mi trabajo como las experiencias vividas en esta etapa. Por ello, es vital agradecer tanto a mis jefes, compañeros de trabajo, amigos y familia ya que son con los que he compartido los buenos momentos, son los que en los malos me han ayudado, son los que han hecho posible esto.

…You can't always get what you want... En mi caso no me puedo quejar. A pesar de la época que le ha tocado vivir a mi generación, ha habido una persona que ha apostado por mí. Gracias Raimundo, gracias sinceramente por el esfuerzo que has hecho en buscar siempre financiación y tener un sueldo digno, es algo que todo doctorando merece. Gracias por haberme formado profesionalmente y sobre todo gracias por hacerme ver que valgo para la ciencia, espero haber estado a la altura de lo que me han dado. También agradecer a los grupos de investigación de Veronique Smits y David Gillespie con los que me reunía semanalmente. Sois una referencia profesional, gracias por vuestros conocimientos, por estar siempre a disposición, por escuchar opiniones, porque a pesar de vuestro curriculum haceis sentir a la otra persona como un igual.

*Quisiera agradecer a todos los que hayan participado en los artículos incluidos en esta tesis. Gracias a las colaboraciones con otros laboratorios hemos podido sacar adelante un trabajo con mucha mayor calidad.

*I would like to thank all people who have participated in any of the acticles of this thesis. Thanks to the collaborations we have done with other laboratories, we have been able to carry out a work of a higher standard.

The Rolling Stones -"You can´t always get what you want"

Me gustaría agradecer al profesor que hizo que me picara la curiosidad por la investigación. Mariano Hernández, eres un ejemplo como investigador y docente.

Django Reinhardt - "Minor Swing"

Humildad, compañerismo y serenidad son valores que por sí solos son difíciles de encontrar en el mundo de la investigación, sin embargo mi codirectora Elisa Cabrera nació con ellos. Humildad: siempre escuchó mi opinión, dándome la confianza de que mi manera de ver las cosas puede ser muchas veces igual de válida o incluso mejor. Esto es algo que necesita todo doctorando; Compañerismo: siempre me echaste un cable con cualquier experimento, siempre estuviste ahí. Gracias por animarme cuando más desmotivado estaba, gracias por tirar del carro; Serenidad: a pesar de que muchos días deseabas que bajara la escalera rodando, nunca diste la patada. Gracias por calmarme en los momentos más tensos y hacer que respirara.

Porque si a los doctorando se les pidieran cartas de recomendaciones, la tuya Elisa sería un libro. Que me envidien todos, porque puedo decir que he tenido la mejor codirectora. MIL GRACIAS.

Lenny Kravitz -"Looking Back On Love"

Es difícil no pasarlo bien en tu trabajo si tus compañeros son el "pulguita time". Hugo, Nacho y Elisa (otra vez). Tener compañeros como ustedes debería ser un requisito indispensable para poder hacer un doctorado. He pasado muchísimos buenos momentos, como los kiwis pelados del Hugo y su imitación del paracaidista, los besitos a Nacho o la tocaya de Elisa. En serio, sois unos personajes!! Muchas gracias por ser amigos y no compañeros.

The wiseguys - "Ooh La La"

El trabajo de Cdt1 fue inicialmente desarrollado por Hugo como parte de su tesis, sin embargo decidió dejar el laboratorio para empezar una nueva tesis fuera de España donde las condiciones no eran tan precarias. Gracias a su esfuerzo pudimos seguir desarrollando el proyecto hasta publicarlo. Espero que te sientas identificado tú también con esta tesis, muchísimas gracias.

Jimi Hendrix - "Born Under a Bad Sign"

Une partie de cet travail à était développé dans le département "Dynamics of Genetic Information" dans l'institut Curie à Paris. Je voudrais remercier le Dr. Michelle Débâtisse et à l'ensemble de son département pour l'accueil et pour l'enseignement du DNA Combing. Je voudrais remercier aussi d'une manière spéciale aux Anges de Charlie: Sandra Carignon, Anne Letessier, Dana Azar y Marie-France. J'ai eu des bons moments avec vous, vous êtes uniques. D'une autre façon, je voudrais remercier particulièrement à Stephane Koundrioukoff, qui a guidé mon séjour, qui a eu la patience pour moi d'apprendre, pas à pas la fastidieuse technique du DNA combine. Merci pour tout à tous. Je n'ai pas assez des mots pour exprimer ma gratitude en travaillant avec vous et pour l'apprentissage.

Plastic Bertrand - "Ca plane pour moi"

Aunque no salga en ningún artículo, gran parte de esta tesis es suya. Mi madre, bueno y la de mi hermana… y la madre de todos, porque quien la conoce sabe que es alguien que lo da todo por los demás. Eres la persona que más valoro, por tu dedicación a nosotros, a tu trabajo, porque eres el ejemplo de persona luchadora, de que tú sola te bastas. Gracias por sacarnos adelante, gracias por dejarnos ser lo que queríamos ser, por no exigirnos nada, solo ser buenas personas. Gracias porque a pesar de estar independizado, mi nevera sea tu cocina. Asi que como me decías tú… TE QUIERO… como miles, de millones de montañas. Gracias a ti Bea porque tu mirada siempre ha reflejado lo orgulloso que estas de tu hermano, a Adán por ser como un hermano mayor durante muchos años. A Pablo porque sé que me querías como a un hijo, y sé que te hubiera gustado poder estar el día que me doctorase. A mi padre, porque soy quien soy ya que su caracter corre por mis venas y es la mejor manera de recordarle para siempre.

Carlos Varela - "Una palabra"

Esta tesis me trae grandes recuerdos con Cati, con la que pasé gran parte de esta etapa. Quiero darte las gracias por todos ellos, por lo bien que lo pasamos, por ayudarme tanto en los días más tristes, por acompañarme al laboratorio los fines de semana para no estar solo, por todos esos desayunitos y viajes que hicimos. Gracias por dejarme ser un apoyo también en tu tesis y en tu vida personal.

The XX - "Infinity"

Grandes personas, grandes nombres, como es el caso de Henna Ana Rajan Harjani Sánchez-Gijón. Apareciste en un momento muy delicado y fuiste de gran apoyo. Gracias por fortalecer mi autoestima, por alegrarte tanto por mí. Haces que la gente crea que pueda ser un superhéroe.

Raphael Gualazzi - "Reality and Fantasy"

Al final de esta tesis me he encontrado con una persona increíble. Marta Gerster, eres un ejemplo de fortaleza y bondad. Gracias por enseñarme tanto sin decir nada, gracias por ayudarme en la escritura y montaje de la tesis, gracias por valorarme tanto.

Ludovico Einaudi -"Una Mattina"

Quería reflejar de alguna manera la importancia y el olvido de la mujer en la ciencia, el granito de arena que hemos aportado en esta tesis para un mejor entendimiento de la regulación en el control de la replicación del ADN y su relación con el cancer de mama entre otros. Y esto ha sido posible gracias a la pedazo de portada que ha hecho la fotógrafa Lucia Petters. Muchísimas gracias.

Nicola Cruz - "Cumbia del olvido"

Hay gente que nunca te pregunta que tal la tesis, otros ni siquiera entienden muy bien que haces e incluso algunos piensan que sigues estudiando biología. Tener amigos así es lo que te permite desconectar de tu trabajo. Estoy orgulloso de contar con todos ellos, pero me gustaría agradecer especialmente a Manón y Leo, por las aventuras vividas en mis primeros años de tesis. A Tomi, Denis, Cristo y el resto de amigos de la Orotava por todo los buenos momentos vividos, las fiestas, los fresquitos… Laura gracias por presentarme a todos ellos. Y Tomi, espero que después de 5 años leas la tesis y te quede claro que no estudio ni el comportamiento del erizo de mar ni sus heces. A Yeray por estar ahí SIEMPRE desde que éramos "más niños". A niñati-Nacho por que una cerveza, un tequila o un mezcal a veces es la mejor manera de desconectar. A Marcos, Manu, Carvallo, Aarón, Jonás y Marc por ser personas que aunque no he tenido la posibilidad de verles tanto durante la tesis, los valores que he aprendido de ellos me han servido para crecer como persona estos años.

Justice vs Simian - " We Are Your Friends"

Los Espiritus - "La Crecida"

Pendulum - "Tarantula"

Y por último gracias a ti Tesis, por darme el mayor reto de superación hasta ahora, contigo he aprendido infinidad de cosas, pero como dice Amy … We only said goodbye with words…

Amy Winehouses - "Back to Black"

A MI PADRE
A MI MADRE

Led Zeppelin - "Stairway To Heaven"

"Como te sigas portando mal, llamo a tu padre, te coge como a un conejo y sanseacabó"

Ana Maria Pérez Gutierrez, *"Mi Madre"*.

TABLE OF CONTENTS

ABBREVIATIONS

λPPase	Lambda phosphatase
53BP1	p53 Binding Protein 1
9-1-1	Rad9/Hus1/Rad1 complex
APC	Anaphase Promoting Complex
Asyn	Asynchronous
ATF6	Activating Transcription Factor 6
ATRIP	ATR Interacting Protein
ATG8	Autophagy Related Protein 8
ATM	Ataxia Telangiectasia-mutated
ATR	ATM and Rad3-related
BIP	Immunoglobulin Binding protein
BRCA1	Beast Cancer Type 1 Susceptibility Protein
BrdU	Bromo Deoxyuridine
cAMP	Cyclic AMP (Adenosine Monophosphatase)
Cdc	Cell Division Cycle protein
Cdc6	Cell Division Cycle 6
Cdh1	Cadherin 1
CDK	Cyclin Dependent Kinase
Cdt1	Cdc10-Dependent Transcript 1
CFSs	Common Fragile Sites
CHX	Cycloheximide
CHOP	C/EBP Homologous Protein
CI	Catalytic Inactive
CIN	Chromosomal Instability
CldU	Chloro Doxyuridine
CMG	Cdc45/MCM 2-7/GINS Proteins
CPT	Camptothecin
CUE	Coupling of Ubiquitin conjugation to ER degradation
Cul4	Cullin 4 protein
Cys	Cysteine
DAB	3-3' Diaminobenzidine
DDB1	DNA Damage Binding protein 1
DDK	Dbf4 Dependent Kinase
DMEM	Dulbecco´s Modified Eagle´s Medium
DNA pol ε	DNA Polymerase Epsilon

DSB	Double Strand Breaks
DUB	Deubiquitinating enzymes
DUIM	Double Sided Ubiquitin Interacting Motif
DTT	Dithiothreitol
E1	Activating enzyme E1
E2	Conjugating enzyme E2
E3	Ubiquitin ligase E3
EGF	Epidermal Growth Factor
eIF2A	Eukaryotic Initiation Factor 2 Alpha
Emi1	Early Mitotic Inhibitor 1
ER	Endoplasmic Reticulum
ERFSs	Early Replicating Fragile Sites
EV	Empty Vector
FBXO31	F-Box protein 31
FBS	Fetal Bovine Serum
GADPH	Glyceraldehyde 3 Phosphate Dehydrogenase
GADD34	Growth Arrest and DNA Damage Inducible 34
GAT	Golgi localized, gamma ear containing, ADP ribosylation Factor (ARF) binding protein
GFP	Green Fluorescent Protein
GINS	Go-Ichi-Ni-San protein complex
GLUE	GRAM Like Ubiquitin Binding in Eap 45
HBO1	Histone Acetyltransferase Binding to ORC
HDAC11	Histone De-Acetylase 11
HU	Hydroxyurea
HR	Homologous Recombination
HRP	Horseradish Peroxidase
IdU	Iodo Deoxyuridine
IHC	Immunohistochemistry
IP	Immunoprecipitation
IRE1	Inositol Requiring Enzyme 1
ISG15	Interferon Stimulated Gene 15
ISRIB	Integrated Stress Response inhibitor
JAMM	JAB1/MPN/Mov34 proteases
Josephin	Machado-Joseph domain-containing proteins
Luc	Luciferase
Lys	Lysine
MCM	Mini-Chromosome Maintenance

MCS	Molecular Combing System
MDM2	Murine Doble Minute 2 protein
MIU	Motif Interacting with Ubiquitin
NEDD8	Neural Precursor Cell Expressed Developmentally Downregulated protein 8
NEM	N-Ethylmaleimide
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Signals
ns	Statistically nonsignificant
NZF	Npl4 Zinc Finger
ORC	Origin Recognition Complex
OTU	Ovarian Tumor Domain
P	P-value
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PERK	Protein Kinase RNA-like Endoplasmatic Reticulum Kinase
PERKi	PERK inhibitor
P-Chk1	Phospho Chk1
PFU	PLAA Family Ubiquitin binding
PI	Propidium iodide
PIP	PCNA-interaction motif
PK	Proteinase K
PKA	Protein Kinase A
Plk1	Polo Like Kinase 1
PMSF	Phenylmethylsulfonyl fluoride
PP1C	Protein Phosphatase 1C
pre-IC	Pre-Initiation Complex
pre-RC	Pre-Replicative Complex
PTEN	Phosphatase and Tensin Homolog protein
QFU	Queensland Breast Cancer Follow-Up
RECQL4	RecQ Like Helicase 4
RFC	Replication Factor C
RPA	Replication Protein A
RRM1	Ribonucleoside Diphosphate Reductase subunit 1
RRM2	Ribonucleoside Diphosphate Reductase subunit 2
S1P	Site 1 protease
S2P	Site 2 protease

SCF	Skp1/Cullins/F-box proteins
Ser	Serine
SIM	SUMO-Interacting Motifs
siRNA	short interference RNA
SKP2	S-phase Kinase-associated Protein 2
ssDNA	Single-stranded DNA
SUMO	Small Ub like modifier
TBS	Tris Buffered Saline
Thap	Thapsigargin
Thr	Threonine
TMA	Tissue Microarray
TOPBP1	DNA Topoisomerase 2 Binding Protein 1
TRAF	Tumour Necrosis Receptor Associated Factor
Ub	Ubiquitin
UBA	Ubiquitin Associated Domain
Ubc	Ubiquitin conjugating enzyme
UBD	Ubiquitin Binding Domain
Ubl	Ubiquitin like proteins
UBL	Ubiquitin Like Domain
UBM	Ubiquitin Binding Motif
UBZ	Ubiquitin Binding Zinc Finger
UCH	Ubiquitin C-terminal Hydrolases
UEV	Ubiquitin Conjugating Enzyme E2 variant
UIM	Ubiquitin Interacting Motif
UPR	Unfolded Protein Response
USP	Ubiquitin Specific Proteases
UV	Ultraviolet light
WHD	Winged Helix Domains
XBP1	X-Box Binding Protein
ZnF	Zinc Finger Based

INTRODUCTION

"Medical science has made such tremendous progress that there is hardly a healthy human left."

Aldous Huxley

1. Cell cycle control

The cell division occurs by the coordination of two events: the replication of the DNA (S phase) and the segregation of the DNA (M phase) in two daughter cells. Between those two phases there are two "gaps" phases (G1 and G2) that connects them. Central to these processes are the Cyclin-Dependent Kinases (CDKs) and the Cyclin proteins. The CDKs are a family of serine/threonine protein kinases that become activated at specific points of the cell cycle and, in a complex with specifics Cyclin proteins regulate the cell progression through the different stages of the cell cycle.

The G1 phase represents the first "gap" of the cell cycle, where the cell is prepared for the DNA replication in the next stage, the S phase. To promote the cell division and transition to the S phase, the CDK4 and CDK6 bind to three D types of Cyclins, (Cyclins D1, D2 and D3) during G1. Then, Cyclin E is assembled with the CDK2 to trigger the S phase start. Later, the formation of the complex Cyclin A-CDK2 is required to the complete replication of the DNA. When the DNA is fully replicated, the cell prepares for the DNA segregation during the G2 phase, the second "gap". For this, Cyclin A exchanges its partner and binds to CDK1 allowing the progression until the M phase. During mitosis Cyclin B-CDK1 controls events that allow the physical segregation of sister chromatids to daughter cells (Vermeulen et al., 2003) **(Fig. 1)**.

Alterations in the genetic control of cell division could results in an unrestrained cell proliferation leading in pathologies as cancer.

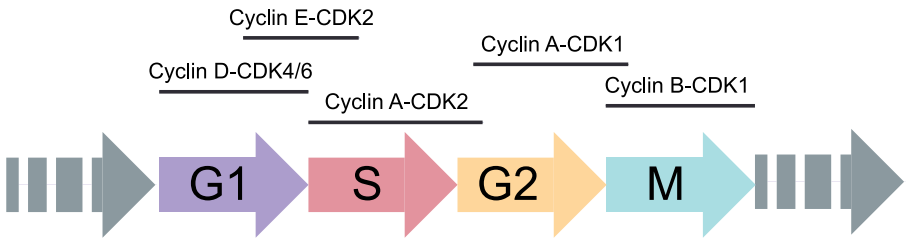

Figure 1. Cyclins-CDKs during the cell cycle phases. The balance between different Cyclins-CDKs must oscillate as shown in order to allow cell cycle progression. Cell cycle progression is triggered by high levels of Cyclins D complexes with CDK4 and CDK6. At the end of G1 phase Cyclin E-CDK2 allows early S phase entry. S phase is controlled by the Cyclin A-CDK2 complex and during G2 phase the Cyclin A binds to CDK1 until early mitosis. The Cyclin B-CDK1 regulates mitotic progression.

2. DNA replication control

DNA replication is the process by which a double stranded DNA molecule is copied to produce two identical DNA molecules. Replication is a critical process because, whenever a cell divides, the two new daughter cells must contain the same genetic information as the parent cell. Errors in the DNA replication process result in genomic alterations and those include mistakes introduced by the DNA polymerase, under or over-replication events. The replication origins are the regions where the replication starts and to avoid under/over-replication, its activity must be carefully regulated. The under-replication is prevented by the presence of many origins in the DNA molecule, and the over-replication is avoided by

restricting origin activation once per cell cycle (DePamphilis et al., 2006). Therefore, the DNA replication control is a key process in maintaining genomic integrity.

In contrast to prokaryotic organisms, eukaryotes contain multiple DNA replication origins grouped in so called replicons, and with the exception of yeast, the origins seem to be more defined by a local DNA structure and chromatin environment than a specific sequence (Costa et al., 2013; Méchali, 2010). In eukaryotes the replication begins with the process called "origing licencing", in which a group of proteins binds in an ordered manner to a sequence of DNA to allow it starting DNA replication. Importantly, among all the licensed origins, only a subset of origins is activated and fired in each S phase of the cell cycle. This choice between the origins that are fired and those that are not differs between the type of cells, but even varies within the same cell population. This is an indicative of the flexibility feature of eukaryotes, allowing a possible adaptation to environmental signals and different types of cell stress (Cayrou et al., 2011; Fragkos et al., 2015).

Origin licensing starts in late mitosis and early G1 phase when the initiator proteins ORC (Origin Recognition Complex) recognize and bind origins. This coupling allows the recruitment of two proteins, Cdc6 (Cell Division Cycle 6) and Cdt1 (Cdc10-Dependent Transcript 1) enhancing the loading of the MCM 2-7 (Mini-Chromosome Maintenance complex) complex in an inactive head-to-head double hexamer at the origins. This protein structure is called the pre-Replicative Complex (pre-RC) and when it is fully assembled the DNA replication origins are licensed and they are capable to replicate **(Fig. 2.1)**. Activation of the replication origins requires the dissociation of the double MCM hexamer into two active MCM hexamers, those form the replisomes that can unwind DNA and start the replication (Fragkos et al., 2015). This step is triggered by the transient binding of DDK (Dbf4 Dependent Kinase) and CDKs to the chromatin, which phosphorylates the MCM 2-7 complex (Heller et al., 2011; Jares and Blow, 2000; Tanaka et al., 2007). The phosphorylation of the MCM 2-7 complex allows the subsequent recruitment and formation of the CMG complex constituted by Cdc45 (Cell division cycle protein 45), the MCM complex and the DNA replication complex GINS, and also participates in the formation of the pre-Initiation complex (pre-IC). The pre-IC is defined as the protein complex preceding the activation of the DNA helicase (Fragkos et al., 2015). In adittion of the CMG complex, other proteins are components of the pre-IC such as, TOPBP1 which interacts with the C-terminal region of topoisomerase II beta (Szambowska et al., 2014), Treslin that interacts with TOPBP1, RECQL4 that belongs to the RecQ helicase (Kitao et al., 1998), MCM 10 homologous to the other MCMs members and DNA polymerase ε. They are phosphorylated also by CDKs and DDK in order to promote their loading onto the chromatin and enhance the formation and activation of the CMG complex (Boos et al., 2011; Gros et al., 2014; Im et al., 2009; Izumi et al., 2001; Kumagai et al., 2010; Kumagai et al., 2011; Thu and Bielinsky, 2013) (**Fig. 2.2)**. The helicase activation induce the recruitment of other proteins such as PCNA, RPA, RFC and the DNA polymerase δ allowing the beginning of the DNA synthesis (Fragkos et al., 2015)**(Fig. 2.3)**. The licensing of the DNA replication origins must occur once per cell cycle to prevent over-replication of the genome. To avoid over-replication, cells have developed several mechanisms to regulate origin licensing, such as to prevent the re-licensing of the origins that have already been fired. One of these mechanisms is the targeting of Cdt1 for degradation, and the interaction between the residual Cdt1 with the Cdt1 inhibitor Geminin during the S phase.

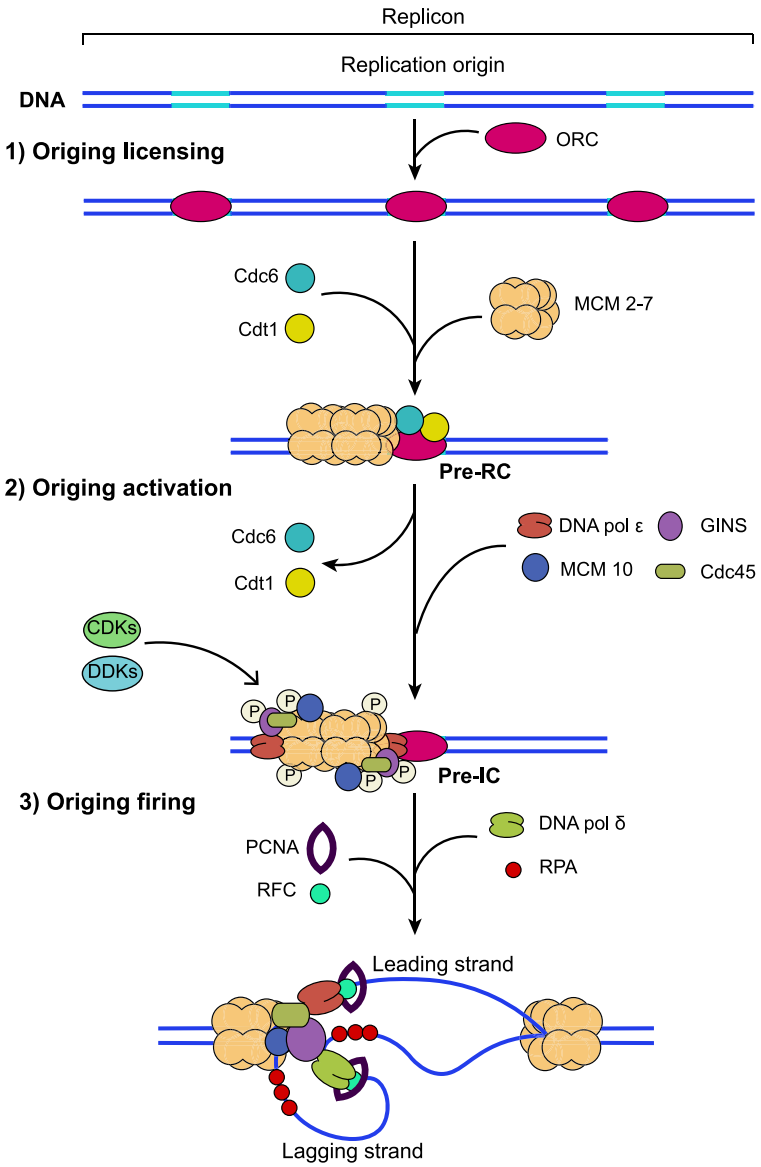

Figure 2. Formation and activation of the pre-RC. The figure shows a replicon unit containing three replication origins (top). **1.** The origin licensing begins with the loading of the origin recognition complex (ORC) into the origins, followed by the recruitment of the Cdc6 and Cdt1. The last step of the origin licensing is the loading of the two MCM 2-7 helicase complex into the origins, forming the pre-RC. **2.** The origin activation need the formation of the pre-IC. Different proteins, such as, GINS, DNA polymerase ε, MCM 10, and Cdc45 are phosphorylated by DDKs and CDKs and recruited to the DNA replication origins. At the same time, Cdc6 and Cdt1 leave the complex. The kinases activate by phosphorylation different residues within of the MCM 2-7 complex that allow the DNA unwinding. **3.** Before to the origin firing, the MCM 2-7 complex divides into two hexamers, one for each emanating replicative fork. The activation of the MCM 2-7 complex induces the recruitment of PCNA, RPA, RFC and DNA polymerase δ. At this point the pre-IC is converted in two active replication forks that move in opposite directions, where the DNA polymerase ε carry out the leading strand, while the DNA polymerase δ carry out the lagging strand.

3. Post-translational modifications

Post-translational modifications often refer to the covalently linkage of a small molecule to the protein in order to switch its function, distribution, folding or stability control. Some of the most studied modification are: phosphorylation, methylation, acetylation, ubiquitination, sumoylation and neddylation. This thesis focussed on the study of replication proteins that are modified by ubiquitin molecules (ubiquitination) and phosphate groups (phosphorylation).

3.1. Ubiquitination

The ubiquitination is a reversible process where a protein is labelled by an isopeptide link between the C-terminal glycine of the ubiquitin molecule (Ub) and the ε-amino group of a substrate lysine residue (Hershko and Ciechanover, 1998; Hochstrasser, 1995). The Ub is a highly conserved protein that exists in all eukaryotic cells. Yeast ubiquitin only differs in three of the 76 residues when it is compared to ubiquitins in higher eukaryotes (Jentsch and Pyrowolakis, 2000). Initially, this modification was identified to regulate the half-life of proteins through proteasome, a protein complex that degrade unneeded or damage proteins by proteolysis. In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for their discovery of the ubiquitin system (Giles, 2004; Komander, 2009). One interesting characteristic of ubiquitin is its high stability, related with its secondary structure. Ub adopts a β-grasp fold that consists of a central five stranded β-sheet wrapping one α-helix and short 3/10 helix (Vijay-Kumar et al., 1987). In spite of it small size, Ub structure is stable. The Ub stability is due to the hydrophobic core constituted by three hydrophobic residues of the α-helix and 11 of the 13 hydrophobic residues of the β-sheet and besides by a lot of hydrogen-bonding interactions (Vijay-Kumar et al., 1987) **(Fig. 3)**. Also, ubiquitin molecule itself contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) where additional ubiquitin chains can be elongated with different length and linkages (Komander, 2009) **(Fig. 3).**

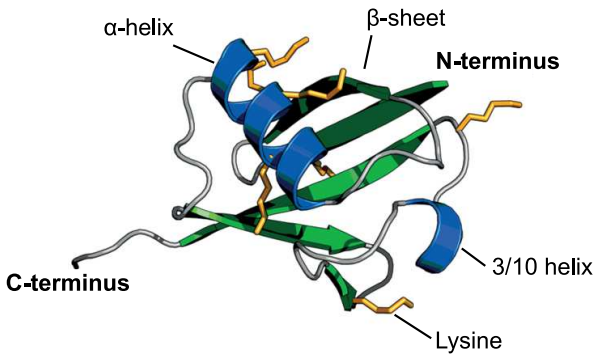

Figure 3. Secondary ubiquitin structure. The crystal structure of human has been refined at 1.8 A resolution. The β-grasp fold is shown: the α-helix and 3/10 helix colored in blue, the five β-sheet colored in green and the seven lysine residues colored in yellow. (Taken from Vijay-Kumar et al., 1987).

3.1.1. Ubiquitination pathway and the ubiquitin machinery

Ubiquitination is catalyzed by a sequential action of three enzimes (Ikeda and Dikic, 2008; Miranda and Sorkin, 2007; Pickart and Eddins, 2004). The enzymes are called E1, E2 and E3. The activating enzyme E1 catalyzes the initial step in the ubiquitination reaction by activation of the ubiquitin molecule with ATP **(Fig. 4.1)**. The conjugating enzyme E2 is involved in an intermediate step in which the ubiquitin molecule is transferred from the E1 to the E2 via a thioester linkage between the ubiquitin C-terminus and a catalytic cysteine residue in the E2 **(Fig. 4.1)**. The ubiquitin ligase E3 is involved in the conjugation of ubiquitin to the target protein. Depending on the E3, sometimes the ubiquitin ligase recognizes the target protein and then, the E2 transfers the ubiquitin to the substrate **(Fig. 4.2A and 4.3)** or, the E3 itself has the dual role of recognizing the substrate and binding the ubiquitin to it **(Fig. 4.2B and 4.3)**. The human genome encodes two E1s, less than sixty E2s and more than 600 different E3 given that confer the specificity with the different substrates (Deshaies and Joazeiro, 2009; Markson et al., 2009; Michelle et al., 2009).

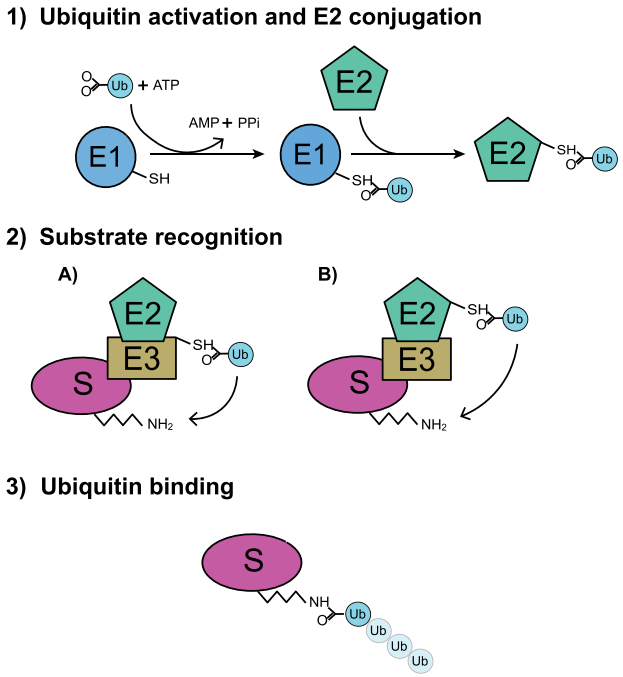

Figure 4. Ubiquitination pathway. 1. The pathway begins with the E1 enzyme activating the ubiquitin molecule "Ub" (small blue circle) with ATP. This ubiquitin activated molecule is then transferred to the E2 enzyme. **2.** The E3 and E2 enzymes interact and the E3 recognizes a specific substrate (S, pink circle) and transfer the ubiquitin to it in two ways. **A.** The E2 transfer the ubiquitin activated molecule to the E3 and then, the E3 transfer the ubiquitin activated molecule to the substrate. **B.** The E2 conjugates the ubiquitin activated molecule directly to the substrate. **3.** The substrate could be targeted with more ubiquitin activated molecules (shown with faint blue circles "ub").

The proteins can be modified in four different types of ubiquitinations. 1) Monoubiquitination: the protein is labelled just by a single Ub molecule. 2) Multiple monoubiquitination: multiple single Ub molecules are attached in a several lysine (Lys) residues of the substrate. 3) Diubiquitination: two Ub molecules are conjugated into the substrate. 4) Polyubiquitination: Ub chains of different lengths and

linkages could be conjugated into the substrate. The polyubiquitin chain can be elongated in a homochain manner, formed by the conjugation of ubiquitins in the same lysine. Also chains can be assembled through different lysine and they are called mixed-linkage chains.

In addition to the ubiquitin, several ubiquitin like proteins (Ubl) have been identified, such as small Ub like Modifier (SUMO), Interferon Stimulated Gene 15 (ISG15), Autophagy-Related protein 8 (ATG8) and Neural Precursor Cell Expressed Developmentally Downregulated 8 (NEDD8). The Ubls show similar structure to Ubs and they are also conjugated to proteins, however they require other, but similar specific conjugation machineries in order to be linked to proteins. Interestingly, heterologous Ub chains can be formed through a covalent link between Ubl and Ub (Haglund and Dikic, 2012; Ikeda and Dikic, 2008; Miranda and Sorkin, 2007).

While the monoubiquitination, multiple monoubiquitination and diubiquitination always leads to functional effects in the modified proteins, such as activation, inactivation or change of the subcellular location, the effect of polyubiquitination in proteins depends on the lysines by whose the Ub chain has been elongated, giving a wide range of molecular signals. One of the most studied Ub chains is the elongation using the conjugation via Lys48 that sends the proteins to be degraded by proteasome. Also Lys29 and Lys11-linked polyubiquitin chains have been involved in proteasome-dependent protein degradation (Jin et al., 2008; Johnson et al., 1995; Xu et al., 2009). On the other hand, the chains that involve conjugation via Lys63 are not associated with degradation, but with protein function changes, for example Lys63 conjugation is associated with coordination of DNA repair, endocytic trafficking, inflammation and translation (Miranda and Sorkin, 2007). The function of the others lysine chains remains relatively unclear.

3.1.2. De-ubiquitination and the ubiquitin hydrolases

As it occurs in the Kinase/Phosphatase regulatory pathways, the Ubiquitin hydrolases or Deubiquitinating enzymes (DUBs) antagonize the Ub conjugation carried out by the E3 Ubiquitin ligases. The human genome encodes nearly 100 DUBs grouped into five families: Otubain Domain Containing Proteases (OTU), Ubiquitin Specific Proteases (USP), Ubiquitin C-terminal Hydrolases (UCH), Machado-Joseph domain-containing proteins (Josephin) and JAB1/MPN/Mov34 proteases (JAMM). The first four families are cysteine proteases whereas last one are metalloproteases (Reyes-Turcu et al., 2009).

As well as ubiquitination, de-ubiquitination is highly regulated. This regulation is in part due to the fact that DUBs are modular and contain distinct domains that allow their activity to be controlled in different ways preventing adventitious cleavage of inappropriate substrates (Liz and Sousa, 2005). In addition to their catalytic domain, ubiquitin hydrolases contain insertions and N or C-terminal extensions such as Ubiquitin Binding Domains (UBDs) and Ubiquitin Like Domains (UBLs), protein-protein interaction domains or substrate adapters, to allow regulate its cellular location, catalytic activity, substrate recognition and protein-protein interaction (Reyes-Turcu and Wilkinson, 2009; Reyes-Turcu et al., 2009).

Nowadays, how the DUBs recognize and discriminate between the different types of ubiquitination is an active field of research. Most of the studies of DUB specificity have focused predominantly on de-ubiquitination of monoubiquitin and Lys48 and Lys63 polyubiquitin chains (Reyes-Turcu and Wilkinson, 2009). Although some DUBs seems to have low polyubiquitin isoform specificity (Row et al., 2006), some have been shown to be more specific for one kind of chain isoform over another (Komander and Barford, 2008). For instance, the tumor suppressor CYLD acts exclusively on K63-linked chains (Komander et al., 2008), yeast OTU1 prefers long K48-linked chains (Messick et al., 2008) and USP5 cleaves both linkages

(Reyes-Turcu et al., 2008). In addition, the DUB specificity can be regulated by additional domains, including UBDs and UBLs. There are at least 16 types of UBDs including UBA (Ubiquitin Associated Domain), UIM (Ubiquitin Interacting Motif), MIU (Motif Interacting with Ubiquitin), DUIM (Double Sided Ubiquitin Interacting Motif), CUE (Coupling of Ubiquitin conjugation to ER degradation), GAT (Golgi localized, gamma ear containing, ADP ribosylation Factor binding protein), NZF (Npl4 Zinc Finger), the Zinc Finger based UBDs, such as, A20 ZnF and UBP ZnF, UBZ (Ubiquitin Binding Zinc Finger), Ubc (Ubiquitin conjugating enzyme), UEV (Ubiquitin Conjugating Enzyme E2 variant), UBM (Ubiquitin Binding Motif), GLUE (GRAM Like Ubiquitin Binding in Eap 45), Jab1/MPN, and PFU (PLAA Family Ubiquitin binding) (Reyes-Turcu and Wilkinson, 2009). These domains are generally small (less than 100 amino acids) being the UIM and MIU domains the simplest, and formed by a single 20 residues helix (Komander, 2009). The UBLs are still poorly characterized, however, multiple studies have described specifically motifs for SUMO, termed SUMO Interacting Motifs (SIM) (Grabbe and Dikic, 2009). Four mechanisms were suggested for the ubiquitin recognition by DUBs **(Fig. 5)**. First, DUBs can interact with both the target protein and the proximal end of polyubiquitin **(Chain amputation Fig. 5A)**. Second, when the DUBs interact with the distal ubiquitin **(Distal trimming Fig. 5B)**. Third, the DUBs could bind simultaneously to two ubiquitins by interacting with surfaces on both ubiquitins that surround the isopeptide bond **(Endo trimming, Fig. 5C)**. And fourth, the DUBs recognize the polyubiquitin chain through the use of multiple UBDs **(Long chain binding Fig. 5A)** (Reyes-Turcu and Wilkinson, 2009).

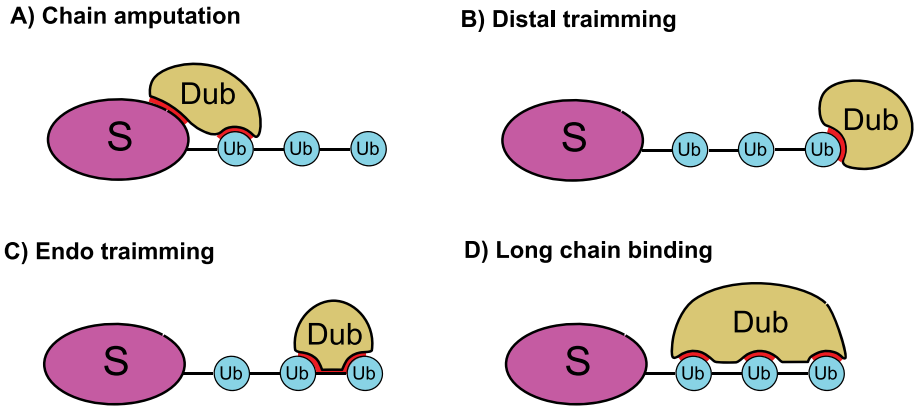

Figure 5. DUB recognition. Different possibilities of DUB recognition for ubiquitinated substrate: **A.** Chain amputation. **B.** Distal trimming. **C.** Endo trimming. **D.** Long chain binding. (Adapted from Reyes-Turcu and Wilkinson, 2009).

DUB activation can be also regulated by transcription. For instance, DUB-1 is induced by interleukins 3 and 5 and the granulocyte macrophage colony stimulating factor GM-CSF (Jaster et al., 1999; Zhu et al., 1996). Also, DUB-2 expression is stimulated by interleukin 2 (Zhu et al., 1997). Moreover, both DUB-1 and DUB-2 are rapidly degraded when the cytokine response is inactive, probably by ubiquitin-proteasome pathway (Baek et al., 2004; Lee et al., 2008). Also, the binding to the substrate can lead to conformational changes in the DUB structure that enhances the protease activity, as it happens in some proteins of the USP family. For example, USP7 aligns the catalytic triad (see below at section 3.1.2.1) in a productive conformation upon substrate binding (Hu et al., 2002). Also USP14 displace the active site loops that otherwise block the binding with the C-terminus of the ubiquitin of the substrate

(Hu et al., 2005). USP18 also moves the finger domain (see below 3.1.2.1) outwards to accommodate the globular body of ubiquitin (Avvakumov et al., 2006). Moreover, some DUBs are regulated by binding with a scaffold protein(s) that enhances its activity, an example are the USP14, UCH13 and POH1 that are inactive until they complex with the proteasome (Chernova et al., 2003; Crosas et al., 2006; Hanna et al., 2006; Yao et al., 2006). In this way, USP14 and UCH13 may limit their activity as both bound and unbound forms co-exist in the cell (Crosas et al., 2006; Yao et al., 2008). In some DUBs, the interaction with a scaffolding protein(s) allow a proper location with their substrate (Ventii and Wilkinson, 2008). Also, DUBs are modulated by transduction pathways. For example, studies have been shown that Ataxia Telangiectasia Mutated (ATM) and ATM Rad3-related (ATR) kinases phosphorylate USP15, USP19 and USP28 upon DNA damage (Matsuoka et al., 2007; Mu et al., 2007). However, although the majority of DUBs are phosphorylated, not much is known of the consequences of this modification (Reyes-Turcu et al., 2009). Proteasome degradation by polyubiquitination is another modification that have been shown in a small number of DUBs. USP4 is a curious case, which de-ubiquitinate the oncoprotein E3 ligase Rho52. USP4 is inhibited when it binds to Rho52 in the presence of the E3 ligase substrates. However, at the lack of substrate, Rho52 is auto-ubiquitinated and USP4 can reverse the ubiquitination. Nevertheless, Rho52 also can ubiquitinate USP4 and send it to be degraded (Wada and Kamitani, 2006). Finally, it was shown that the sumoylation of USP25 impairs its binding to polyubiquitin and therefore its activity (Meulmeester et al., 2008).

The balance between ubiquitination and de-ubiquitination is an important process for cell, including roles in cell cycle regulation, proteasome and lysosome dependent protein degradation, DNA repair, gene expression and kinase activation. Interestingly, recent studies shown that some pathogenic microorganisms have acquired genes encoding DUBs. That suggests that disruption of ubiquitination/de-ubiquitination balance in the cell may confer a selective advantage for these bacteria (Fischer, 2003; Le Negrate et al., 2008; Misaghi et al., 2006; Rytkönen and Holden, 2007) and possibly to viruses (Arguello and Hiscott, 2007; Lindner, 2007). As expected for such important process in the cell, mutations in several DUBS have been linked to pathological conditions including cancer or neurological disorders among others (Fischer, 2003; Jiang and Beaudet, 2004; Shanmugham and Ovaa, 2008).

3.1.2.1. Ubiquitin Specific Proteases family (USPs)

In this thesis we studied three USP family members of the DUBs: USP37, DUB3 and USP7/HAUSP. The USP family comprises 56 members in human cells and is the largest single group of DUBs. The USPs contain a conserved catalytic core comprising between 295 and 850 residues, however 27 of 56 USPs contain a catalytic core of ~350 amino acids. Six conserved regions (named box 1 until box 6) have been identified in all USP domains **(Fig. 6A).** Box 1 contains the catalytic cysteine residue, box 5 contains the catalytic histidine, and box 6 contains the catalytic aspartate acid/asparagine residue. 45 of 56 human USP domains contain in the box 3 and box 4 a conserved Cys-X-X-Cys motif, which is most commonly associated with a zinc-binding site (Ye et al., 2009). Moreover, most USPs contain a catalytic domain with a terminal extension and insertions between the individual boxes of a wide range size giving them unique structural features **(Fig. 6B)**. These terminations and insertions explain the large size differences in USP catalytic domains (Ye et al., 2009).

Based in the USP7 catalytic domain studies, the USPs domain resemble an open hand with three sub-domains: Thumb, Palm and Fingers **(Fig. 6A)**. The Thumb consists of eight α-helices with the N-terminal cysteine box adopting an extended conformation (box 1 and box 2). The Palm contains eight central β-strands buttressed by two α-helices and several surface loops (box 5 and box 6). An anti-parallel β-sheet, formed by six of the eight β-strands from the Palm, intimately packs against the globular Thumb

and gives rise to an inter-domain deep cleft. The cysteine box and the histidine box are positioned on the opposing sides of this cleft. The Fingers are four β-strands in the center of the USP domain and two at the tip of the domain (box 3 and box 4). The catalytic triad resides between the Thumb (cysteine) and Palm sub-domains (histidine/aspartic acid) **(Fig. 6A)**. All de USPs show this particular folding, and only the CYLD de-ubiquitinase enzyme, the most divergent USP at the sequence level, lacks the Finger sub-domain (Avvakumov et al., 2006; Hu et al., 2002; Hu et al., 2005; Komander et al., 2008; Renatus et al., 2006; Ye et al., 2009).

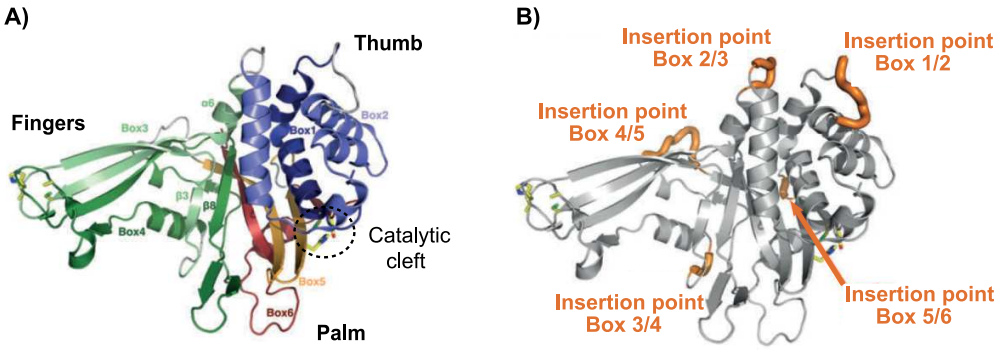

Figure 6. Structure of the USP family based USP7. A. The crystal structure of USP7 catalytic domain contoured at 1.5 at 2.3A° resolution. The sub-domains are colored in red and orange (Palm), green (Fingers) and blue (Thumb). The catalytic cleft is enclosed in a black circle. **B.** The cartoon representation of USP7 catalytic domain showing the boxes domains and insertion points (taken from Ye et al., 2009).

The different aforementioned insertions can impact in the structure of the USP core domain with different effects on USP function. USP7, another DUB studied in this thesis is an example of catalytic activation by UBLs insertion. USP7 has five consecutive C-terminal UBL domains (HUBLs), of which the last two (HUBL-45) are sufficient to activate USP7. These HUBL-45 domains promote conformational changes allowing the organization of the catalytic center and the ubiquitin binding. Moreover, the activation of USP7 can be enhanced allosterically by the metabolic enzyme GMP-synthetase. It bind to the first three HUBLs (HUBL-123), in turn, stabilize the HUBL-45 and hyperactivate USP7 (Faesen et al., 2011; Faesen et al., 2012). Some of the insertions might modulate the distal or proximal ubiquitin binding site of the USP domain itself, but others, located further from the catalytic core, probably would not affect the ubiquitin binding site. Insertions between boxes 3 and 4, and boxes 4 and 5 occur at highest frecuency. An insertion between boxes 3 and 4 could interfere with the ubiquitin binding at the distal ubiquitin binding site, and could affect catalytic efficiency (Ye et al., 2009). For example, eight USPs contain a UBL motif between boxes 3 and 4 (Zhu et al., 2007). The insertions between boxes 4 and 5 might extend the ubiquitin binding platform (Ye et al., 2009). For instance, USP5 and USP13 present UBA domains that provide additional distal binding sites (Reyes-Turcu et al., 2008), or USP37, an DUB studied in this thesis, contains three UIMs inserted at this position (Ye et al., 2009). Insertions between boxes 1 and 2 are near to the proximal binding site and therefore might shape it allowing different chain specificity. The insertions between box 2 and 3, and box 5 and 6 are located on a surface remote from the catalytic domain (Ye et al., 2009).

3.1.2.1.1. USP7/HAUSP

The herpesvirus-associated ubiquitin specific peptidase USP7 was first identified as a DUB for p53, a tumour suppressor mutated or inactivated in over 50% of human cancers (Li et al., 2002), but later studies have also shown that USP7 can control the auto-ubiquitination, and thus stability of MDM2, the E3 ligase that promote p53 degradation (Hu et al., 2006). USP7 has been reported to stabilize others E3 ligases, such as UHRF1 through an interaction with the complex USP7-Dnmt1 (Felle et al., 2011), moreover, this interaction also promotes the stabilization of Dnmt1 and its DNA methylation activity (Du et al., 2010; Qin et al., 2011). USP7 controls the stability of the E3 ligases Chfr, a regulator of many cell cycle proteins (Oh et al., 2007) and Rad18 involved in stability and reduction of the DNA damage tolerance (Zlatanou A et al., 2016). Importantly, USP7 promote the Chk1 and Claspin stability, regulating the DNA damage checkpoint response (Alonso-de Vega et al., 2014; Faustrup et al., 2009; Zhang et al., 2014) and the transcriptional-coupled nucleotide excision repair by stabilization of ERCC6 (Schwertman et al., 2012). Furthermore, USP7 modulates the stress-tolerance pathways by promoting the DNA pol η stability (Qian et al., 2015). USP7 also play a role in the regulation of several transcription factors, for instance, stabilizing Foxp3 (van Loosdregt et al., 2013), decreasing FOXO4 monoubiquitination (van der Horst et al., 2006) or de-ubiquitinating NF-kB leading to increase DNA binding (Colleran et al., 2013). Moreover USP7 regulates the polycomb mediated gene silencing by regulation of the PRC1 ubiquitination status (Lecona et al., 2015; Maertens et al., 2010) and the stabilization of RINGB1 (de Bie et al., 2010). Recent work from the Fernandez-Capetillo group points USP7 as a new critical regulator of DNA replication (Lecona et al., 2016). The inhibition of USP7 decreases DNA replication by reducing both DNA replication fork progression and firing of new DNA replication origins. In this work it is shown a general enrichment of proteins modified by SUMO and diminished in Ub at active replication forks. In contrast, this balance is reversed (high Ub, low SUMO) in already replicated regions near to the replication forks (Lopez-Contreras et al., 2013). Interestingly, USP7 is able to de-ubiquitinate SUMO-ubiquitin chains at the chromatin (Lecona et al., 2016). These data suggest that USP7 controls DNA replication by maintaining an enrichment of SUMOylation with lower ubiquitination at nascent DNA.

Beside the typical USP structure and the HUBL domains aforementioned, USP7 also contains a N-terminal TRAF (tumour-necrosis-receptor-associated-factor) domain that is important for the target proteins recruitment (Hu et al., 2005; Sheng et al., 2006).

3.1.2.1.2. USP37

USP37 was first identified as key enzyme that stabilizes Cyclin A counteracting the ubiquitination by the Anaphase Promoting Complex APC^{Cdh1}. Moreover, the phosphorylation of USP37 by CDK2 stimulate its full activity. Curiously, APC^{Cdh1} also target USP37 for proteasomal degradation (Huang et al., 2011). USP37 protein levels oscillate during cell cycle, USP37 is degraded in G2/M boundary in a $SCF^{\beta TrCP}$ and Plk1-dependent manner and remains low in G1 phase of the cell cycle (Burrows et al., 2012). The RE1 silencing transcription factor (REST) represses USP37 gene expression as knockdown of REST increases the USP37 transcription, that also de-ubiquitinates and stabilizes p27 to block cell proliferation (Das et al., 2013). Also USP37 regulates PLZF/RARA fusion protein stability, it is involved in acute promyelocytic leukemia (Yang and Shih, 2013). The c-Myc protein, overexpressed in many cancers, is also de-ubiquitinated and stabilized by USP37. Moreover, high levels of both USP37 and c-Myc expression are correlated in human lung cancer (Pan et al., 2015). USP37 is able to regulate genomic stability by controlling DNA double strand break repair by homologous recombination by counteracting RAP80 (an ubiquitin binding protein, that specifically recognizes and binds Lys 63 linked ubiquitin) (Typas et al.,

2015) or proper mitotic progression, centrosome integrity, and chromosome alignment by controlling WAPL, a negative regulator of chromatin cohesion (Yeh et al., 2015). USP37 was also reported to stabilize the highly conserved protein 14-3-3γ, whose overexpression causes changes of the morphologic characteristics of cell transformation and promotes cell migration and invasion (Kim et al., 2015).

3.1.2.1.3. DUB3/USP17L2

Little is known about DUB3 yet. It was firstly described as an important regulator of the cell cycle progression through stabilization of the CDK activating phosphatase Cdc25. DUB3 knockdown cells show increased Cdc25A ubiquitination and degradation, leading to a reduction of CDK/Cyclin activity and arrest at the G1/S and G2/M phase transitions of the cell cycle (Pereg et al., 2010). DUB3 was also reported as an important regulator of genomic stability through post-translational control of H2AX (Delgado-Díaz et al., 2014). In DNA damage response the phosphorylation and post monoubiquitination of H2AX is a crucial step for the recruitment of DNA repair proteins at the lesion place. The ectopic expression of DUB3, decreases H2AX monoubiquitination, and abrogates focus formation of 53BP1 and BRCA1 upon genotoxic stress (Delgado-Díaz et al., 2014). Possibly related with its role controlling cell cycle and genomic instability, a work showed little or no DUB3 expression in normal ovarian tissue but DUB3 was overexpressed in ovarian carcinomas (Zhou et al., 2015). Furthermore, in cancer cells, high DUB3 levels correlated with high Cdc25 levels. Therefore it was suggested DUB3 as a possible biomarker of ovarian cancer prognosis (Zhou et al., 2015).

3.2. Phosphorylation

Phosphorylation is the most extensively studied post-translational modification. In spite of Phoebus Levene in 1906 indentified the phosphate in the amino acid composition of the egg yolk protein vitellin (Levene and Alsberg, 1906), it was not until 1932 when Lavene and Fritz isolated phosphoserine from vitellin (Lipmann and Levene, 1932). However, it took another 20 years before Eugene Kennedy described the first "enzymatic phosphorylation of proteins" (Burnett and Kennedy, 1954). In 1955 Edmond Fischer and Edwin Krebs described a phosphorylation/dephosphorylation mechanism (Kresge et al., 2011), and both researchers were awarded with the nobel prize in Physiology or Medicine in 1992 for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism.

Nowadays, we know that the phosphorylation is a reversible process present in both prokaryotic and eukaryotic organisms (Barford et al., 1998; Chang and Stewart, 1998; Cozzone, 1988). The enzymatic process is catalyzed by kinases that add a phosphate group via an ester bond, whereas phosphatases carry out the reverse enzymatic action. The phosphorylation of a target protein often affects its activity due to conformational changes. In eukaryotes, the phosphate can be linked to serine, threonine and tyrosine residues of the substrate protein (Cieśla et al., 2011). Similar to ubiquitination, phosphorylation affects every basic processes in the living organisms, including metabolism and growth, cell division and differentiation, cell cycle, motility, organelle trafficking, membrane transport, muscle contraction, immunity, and memory and learning (Manning et al., 2002).

The modification by phosphorylation has an important role into the cascade of many cellular signaling pathways such as the intracellular signal transduction. For example, the cyclic AMP was one of the first phosphorylation pathways, among many discovered. The cyclic AMP is synthesized from ATP by the enzyme adenylyl cyclase. These cAMP molecules activate the enzyme protein kinase A (PKA),

which it is responsible of the multiple phosphorylations in downstream substrates. Each step in the cascade further amplifies the initial signal, and the phosphorylation reactions mediate both short- and long-term responses in the cell (Cooper and Hausman, 2000).

4. DNA replication licensing proteins

Most of the work of this thesis focuses in the identification and characterization of DUBs that regulate the stability of two proteins involved in the control of DNA replication licensing: Cdt1 and Geminin. Both Cdt1 and Geminin are controlled by ubiquitin-dependent proteasomal degradation and although several E3 ubiquitin ligases were identified to ubiquitinate them, no DUBs were reported before our work to control both DNA replication proteins.

4.1. Cdt1

As mentioned, the DNA replication factor Cdt1 is key regulator for the formation of the pre-RC of DNA replication in mammalian cells. The orthologue protein was first identified in *Schizosaccharomyces pombe* by Hofmann and Beach in 1994 by searching for novel target genes of the Cdc10 transcription factor (Hofmann and Beach, 1994). Cdt1 structure is divided in three domains **(Fig. 7)**, a N-terminal domain (residues 1–166), two Winged Helix Domains (WHD) at the middle of the protein (residues 167–351) and a C-terminal domain (residues 352–546) (Caillat and Perrakis, 2012).

4.1.1. Cdt1 N-terminal region

The N-terminal domain of Cdt1 mediates the interaction with different proteins involved in multiple mechanisms of regulation. Four E3 ligases recognize the N-terminal domain during S and G2 phases to promote its proteasome-dependent degradation (Truong and Wu, 2011). First, the SCF-Skp2 E3 recognize the Skp2 SPARPALR site (residues 31–38) (Nishitani et al., 2006). The phosphorylation of the nearby Thr29 promotes the recognition of SCF-Skp2 to Cdt1. This phosphorylation is performed by Cyclin A-CDK that itself is binding to the Cyclin binding sequence Cy-motif RRL (residues 68–70) of the N-terminus of Cdt1 (Liu et al., 2004; Sugimoto et al., 2004). Second, APC^{Cdh1} recognizes three destruction boxes of Cdt1(Sugimoto et al., 2008), third, the E3 ligase Cul4–DDB1–Cdt2 target Cdt1 in a PCNA dependent manner. PCNA binds to Cdt1 through a consensus PCNA-interaction motif (PIP) located in the first 28 amino acids of Cdt1 and allows Cdt2 recognition of the degron motif adjacent to the PIP box (Arias and Walter, 2006; Roukos et al., 2011). And fourth the E3 ligase FBXO31 that target Cdt1 through the N-terminus of Cdt1 regulating Cdt1 at G2 phase of the cell cycle (Johansson et al., 2014).

Whereas phosphorylation promotes its degradation, the N-terminus can also be acetylated by the histone acetylase HBO1 preventing the ubiquitination (Glozak and Seto, 2009). Interestingly, the Geminin-Cdt1 binding promotes the recruitment of the histone de-acetylase 11 (HDAC11) that de-acetylates Cdt1 and stimulate its degradation (Wong et al., 2010). Cdc7 also binds to the N-terminal domain of Cdt1, which is important to regulate the Cdt1 chromatin binding (Ballabeni et al., 2009). Finally, Cdt1 has two NLS (Nuclear Localization Signals) at the N terminus that are required for its nuclear import (Arentson et al., 2002).

4.1.2. Cdt1 Winged Helix Domains (WHD) and C-terminal region

The WHDs are also in other pre-RC proteins such ORC1, ORC2, Cdc6 and MCM 6 (Khayrutdinov et al., 2009) and they are mediating both DNA recognition and protein-protein interactions (Gajiwala and Burley, 2000). The middle domain of WHD contains the binding site for Geminin and can also bind to the DNA (Caillat and Perrakis, 2012). The C-terminal does not bind to the DNA, but mediates the interaction with the C-terminal WHD domain of MCM 6 (Wei et al., 2010; Yanagi et al., 2002), important for the MCM 2-7 recruitment at the DNA replication origins for the origin licensing (Maiorano et al., 2000; Nishitani et al., 2000).

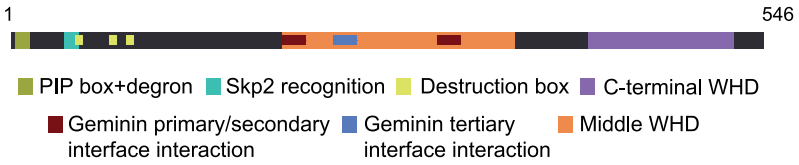

Figure 7. Cdt1 molecule. Cartoon depicting domains and motifs of Cdt1. (Adapted from Caillat and Perrakis, 2012).

4.2. Geminin

Geminin was originally described in *Xenopus* egg extracts as an inhibitor of the DNA replication initiation that is destabilized in mitosis (McGarry and Kirschner, 1998). Geminin is known for the ability to directly interact with Cdt1 inhibiting its function from S phase until early mitosis (Wohlschlegel et al., 2000). Studies suggest that this inhibitory licensing complex is formed on the chromatin (Xouri et al., 2007). Both Geminin and Cdt1 regulate histone acetylation, involved in the control of the MCMs loading at the origins of replication and replication licensing. Geminin inhibits licensing by histone acetylation suppression via Cdt1 in two different ways. On one hand, in G1 phase Cdt1 promotes the loading of MCM complex by recruiting histone acetylase HBO1, which acetylates histone H4 and thereby induce chromatin de-condensation (Miotto and Struhl, 2008; Wong et al., 2010). Geminin was reported to repress the acetylation by HBO1-Cdt1 complex (Miotto and Struhl, 2010). On the other hand, the interaction of Geminin with Cdt1 promote histones de-acetylation by HDAC11 in S phase (Wong et al., 2010).

The Geminin molecule **(Fig. 8)** contains an N-terminal region (residues 1–95), a central coiled-coil domain (residues 96–160) and a C-terminal domain of uncharacterized function (residues 160–209).

4.2.1. Geminin N-terminal region

The N-terminus of Geminin contains a destruction box (residues 23-31) that is recognized by the E3 ligase APC^{Cdh1} and attaches ubiquitin to nearby lysine residues (King et al., 1996; McGarry and Kirschner, 1998). Geminin localizes in the nucleus (Kroll et al., 1998; McGarry and Kirschner, 1998) and contains a NLS. Whereas non mammalian Geminin shows a NLS located in the N-terminal domain (Benjamin et al., 2004; Boos et al., 2006), the mammalian Geminin NLS is located in the coiled-coil domain in an arginine-arginine-lysine sequence (RRK, residues 106–108). Importantly arginine 106 and 107 also interact with Cdt1, therefore Cdt1 could interfere with the nuclear localization of Geminin (Boos et al., 2006; Caillat and Perrakis, 2012; Sakaue-Sawano et al., 2008).

4.2.2. Geminin coiled-coil domain

Although the coiled-coil domains are simple structures that include two or more α-helical peptides, they are functionally highly versatile, and very important for protein oligomerization. The specificity and features of the domain depends on the nature of the amino acids at each position. Normally they are formed of a repetition of seven amino acids (heptads), which are denoted "abcdefg". The amino acids at position "a" and "d" are mainly hydrophobic and these amino acids of both α-helixes form the interface core. The amino acids at position "e" and "g" are often charged residues and play a role in the specificity binding (Caillat and Perrakis, 2012).

The Geminin α-helices forms a head-to-head homodimer in solution with seven heptads repetition (residues 96–144) that differs to the canonical coiled-coil domains (Saxena et al., 2004). Although, the amino acids at the position "a" and "d" seems to be not ideal for the stabilization suggesting that Geminin homodimer is unstable, three amino acids at the position "d" and "e" are exposed to the solvent and mediate a hydrophobic interaction with Cdt1, suggesting that Geminin homodimer is probably stable when interacts with Cdt1. The unstability of the Geminin homodimer allows Geminin to heterodimerize with other partners providing additional mechanism for Geminin regulation (Caillat and Perrakis, 2012). For example, the ERNI protein, involved in establishing neural plate identity, can interact with itself and with the coiled-coil of Geminin (Papanayotou et al., 2008). Similarly, the coiled-coil of Idas, a phylogenetically conserved Geminin related protein can also interact with itself but preferentially with the coiled-coil of Geminin reducing the affinity of Cdt1 to Geminin (Pefani et al., 2011).

Figure 8. Geminin molecule. Representation of Geminin domains and motifs. (Adapted from Caillat and Perrakis, 2012).

4.3. The Cdt1-Geminin interaction

The interaction reported by Lee and collaborators (Lee et al., 2004) in mice showed a heterotrimer between one Cdt1 and two Geminin molecules (Cdt1:2xGeminin). This interaction occurs through the middle domain of Cdt1 (amino acids 167–353) and the Geminin dimer (amino acids 86–160) by primary and secondary interfaces. The primary interface, the most important, is formed by the two small α-helices located in the N-terminus of the middle WHD of Cdt1 (residues 167–182) and the N-terminal regions of both monomers of the Geminin coiled-coil (residues 106–121). The secondary interface is formed by a loop located towards the C-terminus of the Cdt1 domain (residues 310–336) and a loop in the N-terminal of one monomers of the Geminin coiled-coil (residues 86–101). However, the C-terminal coiled-coil of Geminin, known to be essential to inhibit the Cdt1 function, was not observed to be in contact with Cdt1 in that structure (Benjamin et al., 2004; McGarry and Kirschner, 1998; Thépaut et al., 2004) and the explanation of how Geminin inhibits Cdt1 activity remained unclear. However, afterwards V. de Marco (De Marco et al., 2009) showed that the heterotrimer forms an heterohexamer that consist in a head-to-tail interacting heterodimers by a tertiary interface **(Fig. 9)**, indicating that the Cdt1-Geminin complex can exist in at least two distinct quaternary forms. In this structure, the C-terminal coiled-coil of Geminin directly interacts with Cdt1, being essential for the heterohexamer formation. It is possible that heterotrimer and heterohexamer both co-exist in the cells, and the equilibrium between the two forms

change during the cell cycle. Changes in the heterotrimer-heterohexamer might be a molecular switch between "licensing-permissive form" (heterotrimer) and "licensing-inhibitory form" (heterohexamer) states of the Cdt1-Geminin complex (De Marco et al., 2009). Possibly related with this, the Cdt1-Geminin interaction positively regulates the stabilization of Cdt1 basal levels during S phase and its acumulation during mitosis (Ballabeni et al., 2004).

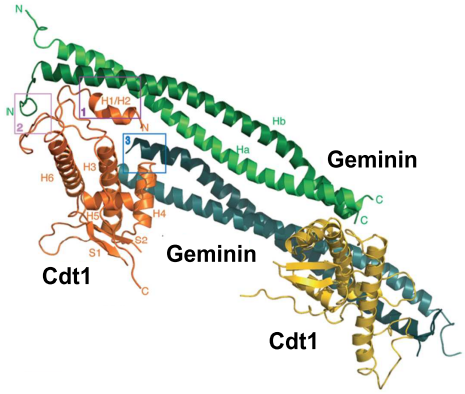

Figure 9. Structure of the human heterohexamer 2x[Cdt1:2xGeminin] complex. Geminin is coloured in green and blue, Cdt1 is coloured in orange and yellow. Two molecules of Geminin interact with one molecule of Cdt1 (heterotrimer) through the primary and secondary interfaces. The interface regions are boxed in magenta and pink. Two heterotrimers interact forming a heterohexamer through the tertiary interfaces. The interface region is boxed in blue. (Taken from De Marco et al., 2009)

4.4. Cdt1 and Geminin regulation prevents re-replication

The eukaryotic cells have the ability to duplicate vast amounts of genetic information quickly and accurately in each cell division. That is due to the large number of origins distributing around the genome and the capacity of the cell regulating their activation. Cells have developed multiple overlapping mechanisms in order to prevent the re-licensing of the replicated origins, because the continuous re-activation of the origins leads accumulation of re-replication events resulting in genomic instability (Arias and Walter, 2007) **(Fig. 10)**.

Cdt1 regulation plays a key role in the re-replication prevention. The regulation of Cdt1 is carried out at two levels: through stability and the regulation of the activity of Cdt1 **(Fig. 11)**. Cdt1 levels change during the different stages of the cell cycle. Cdt1 shows the highest levels in late mitosis and G1 phases when its activity is high because it is required for the formation of the pre-RC. However, when the cells entry to S phase, Cdt1 is targeted for proteasomal degradation to inhibit the activation of excess origins. The Cul4-DDB1 (PCNA dependent) and the SCF-Skp2 (PCNA independent) are the main E3 ligases responsible of this proteasomal dependent degradation, although another E3 ligase, the FBXO31 has been identified. FBXO31 interacts with Cdt1 and regulates its abundance by ubiquitination, leading to subsequent degradation (Johansson et al., 2014). The Cul4-DDB1 complex is active in S phase, during ongoing replication, while SCF-Skp2 operates both in S and G2 phases (Nishitani et al., 2006). The FBXO31 is limited to G2 phase of the cell cycle and its independent for the other degradation pathways (Johansson et al., 2014). In agreement with the role of Cul4-DDB1 in Cdt1 degradation, studies in *Caenorhabditis elegans* reported that the inactivation of Cul4 induces accumulation of Cdt1 and also re-replication events (Zhong et al., 2003). At the end of the S phase Cdt1 starts to be stabilized in order to be ready for the next licensing cycle. Importantly, a residual active form of Cdt1 persists during S phase

and to avoid new licensing of replicated origins, the cell developed a second mechanism involving the protein Geminin that represses the Cdt1 activity by physically interacting with it (Wohlschlegel et al., 2000) **(Fig. 11)**.

Addition of recombinant Geminin protein to *Xenopus* eggs extracts blocks the loading of MCM 2-7 onto chromatin and licensing of the origins (Tada et al., 2001). Moreover, Geminin has a positive role in the pre-RC formation, as during late G2 phase and mitosis, Geminin protects Cdt1 from proteasome-mediated degradation by inhibiting its ubiquitination, allowing the accumulation of Cdt1 in an inactive form for the next pre-RC assembly in late M/G1 phase (Ballabeni et al., 2004) **(Fig. 11)**.

As occurs with Cdt1, Geminin levels oscillate during the cell cycle **(Fig. 11)**. At the metaphase/anaphase transition, Geminin is ubiquitinated and targeted for degradation by the E3 ligase APC^{Cdh1}, whose activation depends on CDK1 (Li and Blow, 2004; McGarry and Kirschner, 1998). However, in the previous steps of mitosis, the degradation of Geminin is protected by two mechanisms: the APC^{Cdh1} inhibitor Emi1 blocks APC^{Cdh1} activity and Aurora A phosphorylates Geminin to prevent its APC^{Cdh1} mediated proteolysis **(Fig. 11)**. Geminin stabilization at those stages of cell cycle ensures that Cdt1, although at higher levels, does not re-activate the DNA replication origins (Reimann et al., 2001; Tsunematsu et al., 2015). Several studies have shown that the depletion of Geminin leads re-replication in many, but not all cell types (Li and Blow, 2005; Melixetian et al., 2004; Mihaylov et al., 2002; Quinn et al., 2001; Zhu et al., 2004).

Therefore, the Cdt1-Geminin interaction, together with the activation of the E3 ligase APC^{Cdh1} by CDK1 and the degradation of Cdt1 is one of the main molecular mechanisms by which origin licensing is regulated during S, G2 and mitosis (Ballabeni et al., 2004; Tada et al., 2001) **(Fig. 11).**

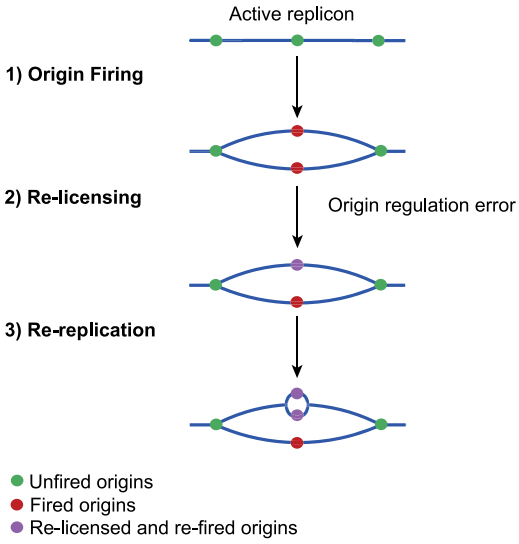

Figure 10. Re-replication. Example of how re-replication events can occur in a replicon. **1.** One origin is fired (red) and the DNA synthesis occurs bidirectionally. **2.** Origin regulation fails and the origin already fired is re-licensensed and re-fired (purple). **3.** The nascent DNA is at least once more replicated generating duplications of the genome.

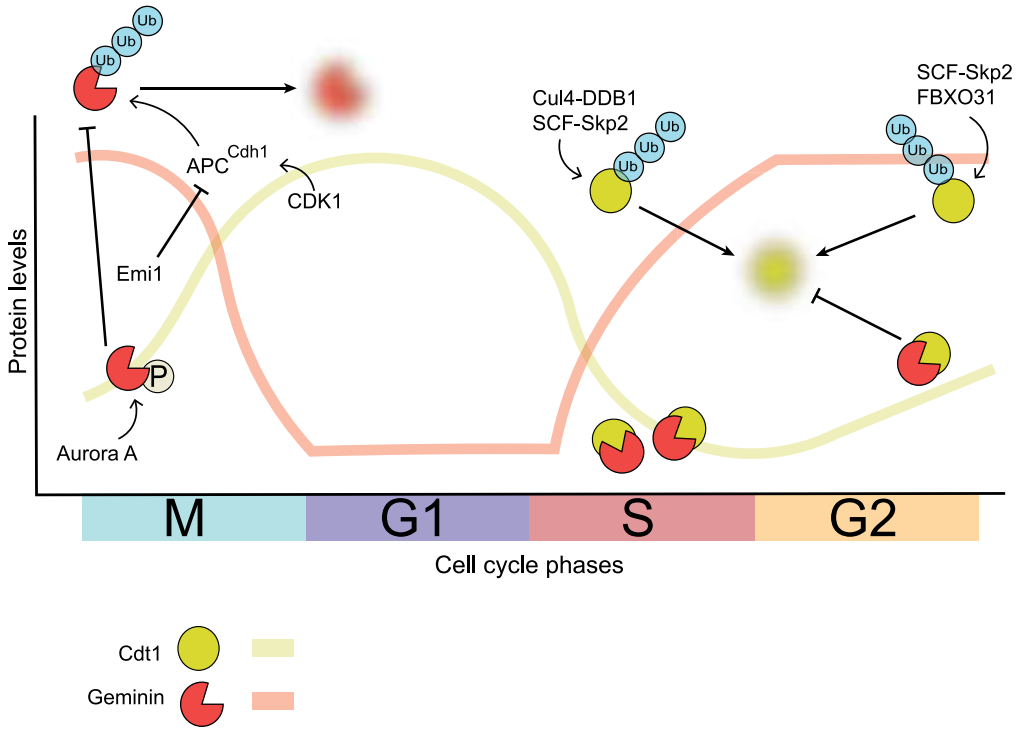

Figure 11. Cdt1 and Geminin regulation throughout the cell cycle. The Cdt1 (yellow line) and Geminin (red line) protein levels are shown during the cell cycle. Both unfocused Cdt1 and Geminin show degraded proteins by the proteasome. The Cdt1 protein levels are high during mitosis and are the highest in G1 phase and during S phase when Cdt1 is targeted by Cul4-DDB1 and SCF-Skp2 for ubiquitination and degradation. Whereas, the abundances levels of Cdt1 during G2 phase are controlled by SCF-Skp2 and FBXO31. Basal Cdt1 protein levels during S and G2 phases are inhibited by physical interaction with Geminin. Geminin protein levels are highest in G2 and early mitosis because Aurora A phosphorylates Geminin and protects it to be degraded by APC^{Cdh1} E3 ligase, that is also inhibited by Emi1 in early mitosis. In late G2 phase and mitosis, the interaction of Geminin with Cdt1 allows the accumulation of Cdt1 because protects the Cdt1 degradation by ubiquitination. At the end of mitosis, CDK1 activated APC^{Cdh1} that ubiquitinates Geminin. Geminin levels remain low during G1 phase until the entry of S phase when it increases.

4.5. Pre-RC and cancer

The DNA replication must be strictly regulated to ensure the complete transfer of the genome and its viability from the parent cells to the daughter cells. Alterations in the control of the DNA replication can result in detrimental gains or losses of genomic DNA that commonly occurs in cancer and developmental diseases. The pre-RC proteins play an important role in preventing genomic instability, and therefore their deregulations are linked to a wide variety of cancer and developmental diseases (Blow and Gillespie, 2008; Lau et al., 2007).

Eric Lau and colleagues reviewed the alterations in pre-RC proteins in cancers (Lau et al., 2007). For example, both Cdt1 and Cdc6 are up-regulated in cervical, lung and brain cancer. MCM 2 was observed to be up-regulated in oligodendriomas, breast, esophageal, renal and lung cancers, while MCM 5 was overexpressed in cervical and esophageal cancers (Lau et al., 2007). Importantly, regulators of pre-RC such as Geminin were up-regulated in breast, colorectal cancers, oligodendriomas and astrocytic brain tumours (Lau et al., 2007). Increasing tumour grade and poor prognosis are also correlated with overexpression of pre-RC proteins. For instance, genes that encode MCMs are up regulated at the mRNA

level in breast cancer, medulloblastoma and mantle cell lymphoma (Gonzalez et al., 2005). Moreover, the overexpression of pre-RC proteins affects the spatial distribution of proliferating cells, since the proliferation restriction in normal tissues is lost in tissues with abnormal pre-RC proteins expression (Lau et al., 2007). Studies in transgenic mice strains that expresses Cdc6 in stratified epithelia shown that the overexpression of Cdc6 enhances the loading of MCM complexes onto the chromatin. Also the overexpression of Cdc6 enhanced the formation of carcinogen-induced papillomas (Búa et al., 2015).

5. Replicative stress

Replicative stress is understood as the slowing or stalling of the replication fork progression leading an improper DNA synthesis. There are several exogenous and endogenous sources/factors that can trigger replicative stress by compromising DNA synthesis such as limiting nucleotides, ribonucleotide misincorporation, misreplication of telomeres or repetitive DNA elements, the presence of DNA lesions, secondary DNA structures or the formation of DNA–RNA hybrids, misactivation of dormant replication origins, collisions between the replication and transcription forks, replication on chromatin highly compacted and hypo-acetylated, early-replicating fragile sites (ERFSs) or common fragile sites (CFSs) and oncogene induced stress (Mazouzi et al., 2014; Zeman and Cimprich, 2014).

DNA replication stress normally results in the formation of single-stranded DNA (ssDNA) leading the recruitment of the single strand binding protein complex, RPA. This generates a signal for the activation of the replication stress response (Byun et al., 2005). Then, ATR plays a crucial role as a master regulator of this response (Maréchal and Zou, 2013; Zou and Elledge, 2003). In this pathway, RPA has two functions: first, RPA helps to recruits ATRIP (ATR Interacting Protein) to the ssDNA and second, RPA also recruits the cell cycle checkpoint protein Rad17 which loads the Rad9/Hus1/Rad1 (9-1-1) complex onto ssDNA. The 9-1-1 complex is essential to recruit and place TOPBP1 in close proximity to ATR to activate it (Mazouzi et al., 2014). Then, among other proteins, ATR phosphorylates and activates Chk1 in a manner dependent on the checkpoint adaptor protein, Claspin. ATR also phosphorylates Rad17 that now interacts with Claspin and regulate its phosphorylation after Hydroxyurea (HU) treatment (Wang et al., 2006). ATR also promotes the phosphorylation of substrates that act in the Nucleotide Excision Repair (NER), a DNA replication stress that generate ssDNA (Liu et al., 2000; Lopez-Contreras and Fernandez-Capetillo, 2010).

The activation of the ATR-Chk1 pathway leads to a several effector pathways in the cell. First, ATR-Chk1 triggers a block in cell cycle progression, promotes stalled fork stabilization, suppresses recombination, and inhibits late origin firing. And second, ATR regulates the repair by homologous recombination, which is essential to restarts collapsed forks (Mazouzi et al., 2014; Zeman and Cimprich, 2014). Consequences of impaired ATR signalling leads to chromosome instability, developmental defects, and accelerated aging (Brown and Baltimore, 2000; Murga et al., 2009). Importantly, if the replication stress persists, the stalled forks can collapse and then degenerate in DNA double strand breaks (DSB) compromising even more the genome integrity of the cell (Lambert and Carr, 2005; Tercero et al., 2003). Upon persistent DNA damage, ATR and Chk1 also phosphorylate p53, which contribute to the cell cycle arrest and/or activation of apoptosis (Taylor and Stark, 2001). Studies suggest that under such conditions of persistent DNA damage, the ATR-Chk1 pathway might be critical for induction of senescence, which could be the case for the damage induced by oncogenes (Di Micco et al., 2006; Mallette et al., 2007; Bartkova J et al., 2006). In fact, prolonged ATR activation, even in the absence of DNA damage, is enough to promote senescence in a p53-dependent manner (Toledo et al., 2008). Other studies have shown that loss of Chk1 function can lead to similar defects. For example, Chk1 depletion

results in accumulation of DNA damage in replicating cells (Syljuåsen et al., 2005). Also, similar to ATR (Brown and Baltimore, 2000; de Klein et al., 2000), Chk1 depletion is early embryonic lethal in mice (Liu et al., 2000; Takai et al., 2000). Importantly, ATR hypomorphism results in Seckel Syndrome in humans, a disease which is associated with dwarfism, craniofacial abnormalities and an increase in DNA replication stress (Murga et al., 2009; O'Driscoll et al., 2003).

6. Regulation of origin usage

Since cells are continuously exposed to stresses that can threaten the total replication of the genome, cells license in late mitosis and G1 phase a higher number of the origins than finally will be activated and fired. It is important that the origin licensing is restricted at this point, because during S phase in response to replication stress, the new DNA synthesized could potentially be re-licensed. Related with this process, the DNA replication origins can be classified in three different types: the constitutive origins that are activated all the time in the same cell type, the flexible origins whose activation differs in unknown manner (the most common type) and the dormant origins that are activated after stress when the replication has been compromised in order to ensure the total replication of the genome (Fragkos et al., 2015).

Dormant origins are licensed in each cell cycle due to the excess of the MCM 2-7 complex loaded on the chromatin, but once an adjacent replicon is activated they are not fired. The inhibition of dormant origins within an active replicon in normal conditions seem to mediated mainly by a temporal issue, because the dormant origins have a short time to fire before they are passively replicated by a replication fork arising from an adjacent fired origin (Ge et al., 2007). Moreover, the effect in unperturbed replication of S-phase checkpoint machinery (ATR-Claspin-Chk1) within the active replicon is not clear, but evidence suggests that these proteins may also play a critical role in the regulation of fork progression, stability and density during a physiological S phase (Petermann et al., 2008; Scorah and McGowan, 2009) **(Fig. 12A)**. For example, inhibition of Chk1 or depletion of Claspin strongly decrease the replication fork progression (Petermann et al., 2008; Petermann et al., 2006). Interestingly, Chk1 or Claspin inhibition also results in greatly increased replication origin firing that under some circumstances at least can compensate for reduced fork progression rate to maintain normal levels of bulk DNA synthesis (Maya-Mendoza et al., 2007; Scorah and McGowan, 2009). Studies of ATR-Claspin-Chk1 in unperturbed replication conditions support a model in which Claspin plays a role regulating fork stability independent of its function mediating Chk1 phosphorylation (Scorah and McGowan, 2009).

Under stress condition **(Fig. 12B)** fork progression is slowed, replication time increases and dormant origins have more probability to be fired in the active replicon cluster. Also there is an increase of ATR activity that is recruited at the stalled forks and phosphorylation of Chk1 with the help of Claspin to stabilize the forks (Ge et al., 2007; Smith et al., 2010) **(Fig. 12B.1)**.

Interestingly, at the same time, ATR inhibits the late origin firing strongly. As ATR is physically bound at the stalled fork, Chk1 is released to diffuse the inhibitory signal throughout the nucleus. Chk1 phosphorylates and inhibits DDK and Cdc25 blocking the formation of the pre-IC in late origins (Ge et al., 2007) **(Fig. 12B.2)**.

In parallel, the mitotic entry is delayed to help to resolve the DNA damage (Fragkos et al., 2015) **(Fig. 12B.3)**. The exact mechanism how ATR-Claspin-Chk1 regulates replication fork stability and the origins dynamic remains largely unknown.

As mentioned, there are different sources of replicative stress with post-ATR-Claspin-Chk1 pathway activation and can be linked to pathological conditions such as cancer. But also the same sources can trigger other effects in the cell. For example, oncogene activation can lead to intracellular oxidative stress and extracellular acidification. This metabolic alteration together with an abnormal vascularisation that lead to hypoxia and nutrient deprivation results in a suboptimal microenvironment that can potentially limit tumour cell proliferation (Hanahan and Weinberg, 2011).

All of these adverse conditions disturb protein folding in the endoplasmic reticulum (ER) leading activation of the Unfolded Protein Response (UPR). In this thesis we demonstrate that the UPR is involved in DNA synthesis control upon DNA stress throughout of the phosphorylation and the activation of Chk1 and Claspin triggered by protein kinase RNA-like endoplasmatic reticulum kinase (PERK) in an ATR- independent manner.

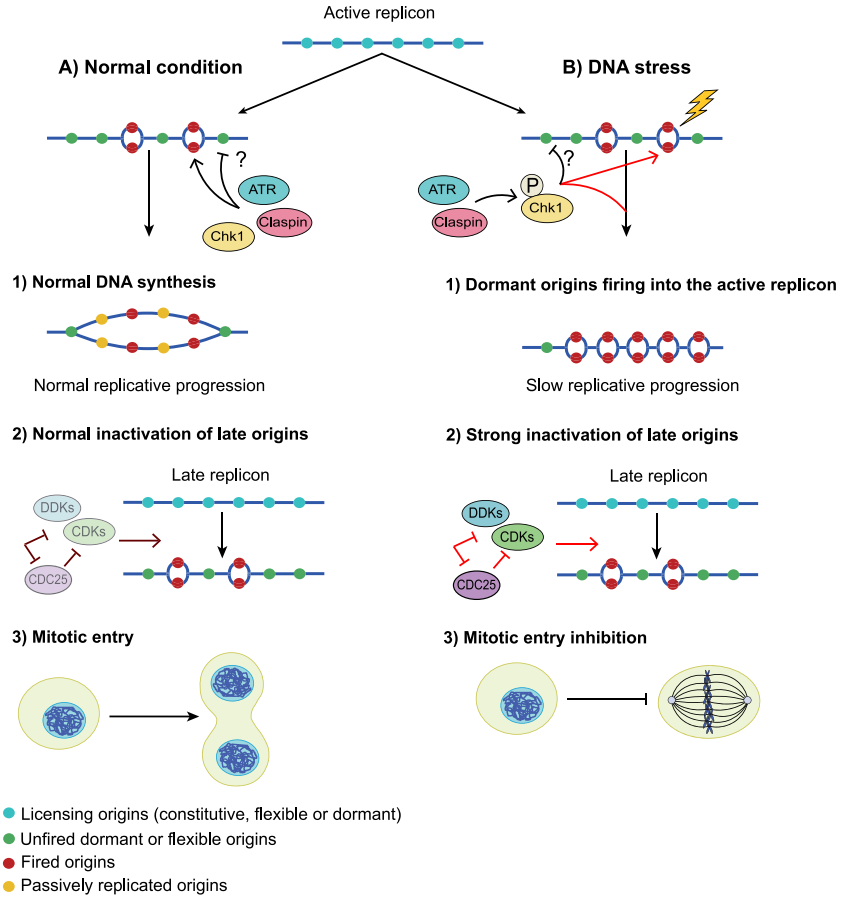

Figure 12. DNA synthesis control by ATR-Claspin-Chk1. A. Under normal conditions, the dormant origins are passively replicated by the activation of nearby origins that are required to duplicate the replicon. **1.** Basal levels of ATR-Claspin-Chk1 act in greater extend in fork progression and stability and perhaps in the inhibition of the dormant origin firing **2.** ATR-Chk1 inhibit weakly the late origin firing. **3.** When all the genome is duplicated, the cell is divided correctly. **B.** Upon DNA stress, ATR phosphorylates and activates Chk1 helped by Claspin. ATR-Claspin-Chk1 stabilize the stalling forks. **1.** To abolish the delay in the DNA synthesis, dormant origins are activated. **2.** Claspin and phosphorylated Chk1 avoid DNA synthesis in late origins (under DNA stress conditions, Chk1 blocks DDK directly and CDK by the inhibition of Cdc25). **3.** Cells do not enter mitosis under these conditions.

7. Unfolded Protein Response (UPR)

The UPR is an adaptive, homeostatic mechanism that acts to transiently inhibit protein synthesis, thus alleviating the burden of unfolded proteins entering the ER, and simultaneously to enhance the inherent capacity of the protein folding machinery (Wang and Kaufman, 2014). These responses are controlled through the principal UPR effectors that span the ER membrane: Inositol-Requiring Enzyme 1α (IRE1α), Protein RNA-like ER Kinase (PERK) and Activating Transcription Factor 6 (ATF6), through a combination of global protein synthesis inhibition and selective transcription/translation of positively acting UPR components (Wang and Kaufman, 2014) **(Fig. 13)**. The initial consequences of UPR activation allow cells to adapt to ER stress and thus are pro-survival factors, however chronic UPR activation can also promote autophagy (Kouroku et al., 2007) and cell death via apoptosis (Wang and Kaufman, 2014).

In this thesis we have identified a new role of UPR in the inhibition of the DNA replication through its effector PERK. PERK is a transmembrane serine/threonine protein kinase and contains a N-terminal luminal domain and a cytoplasmic protein kinase domain (Bobrovnikova-Marjon et al., 2010; Harding et al., 1999; Shi et al., 1998). The ER stress induces the oligomerization of N-terminal luminal domain of PERK promoting itself the trans-phosphorylation of the C-terminal cytoplasmic kinase domain at multiple residues including Thr980 on the kinase activation loop. Activated PERK phosphorylates Ser51 of the α-subunit of translation Initiation Factor 2 (eIF2α) (Cui et al., 2011) that leads to the attenuation of translation initiation of most transcripts, but increases the translation of selected mRNAs such as the ATF4 transcription factor, which encodes a transcription factor that induces the expression of genes involved in antioxidant responses, amino acid metabolism, autophagy and apoptosis (Bobrovnikova-Marjon et al., 2010; Harding et al., 2000; Harding et al., 2003; Lange et al., 2008; Vattem and Wek, 2004). Moreover ATF4 controls the expression of the pro-apoptotic components GADD34 and CHOP. GADD34 participates in a feedback loop to dephosphorylate eIF2α by interacting with protein phosphatase 1C (PP1C), which restores protein synthesis (Novoa et al., 2001). The phosphorylation of eIF2α also inhibits the initiation of the protein synthesis and decreases the load in the ER of unfolded protein (Cui et al., 2011) **(Fig 13B)**.

Evidence suggests that the UPR is frequently activated in tumour cells and that it can promote cancer cell proliferation and survival by alleviating ER stress (Wang and Kaufman, 2014). Firstly, over-expression of functional UPR components such as BIP, ATF6, and CHOP has been documented in a wide range of human primary tumour types (Wang and Kaufman, 2014). Secondly, activation of oncogenes such HRas and c-Myc (Denoyelle et al., 2006; Hart et al., 2012) or loss of tumour suppressors such as BRCA1 and PTEN (Fang et al., 2010; Yeung et al., 2008), increases protein synthesis leading to UPR activation to cope with the increased demand for protein folding during oncogenic transformation. Finally, UPR activation can promote cancer development in mouse models (Hart et al., 2012), whilst pharmacological inhibitors of UPR effectors such as PERK have anti-tumour activity in xenograft models (Wang and Kaufman, 2014). As a result the UPR is a promising new anti-cancer target, either as a monotherapy or in combination with existing therapeutic modalities (Wang and Kaufman, 2014).

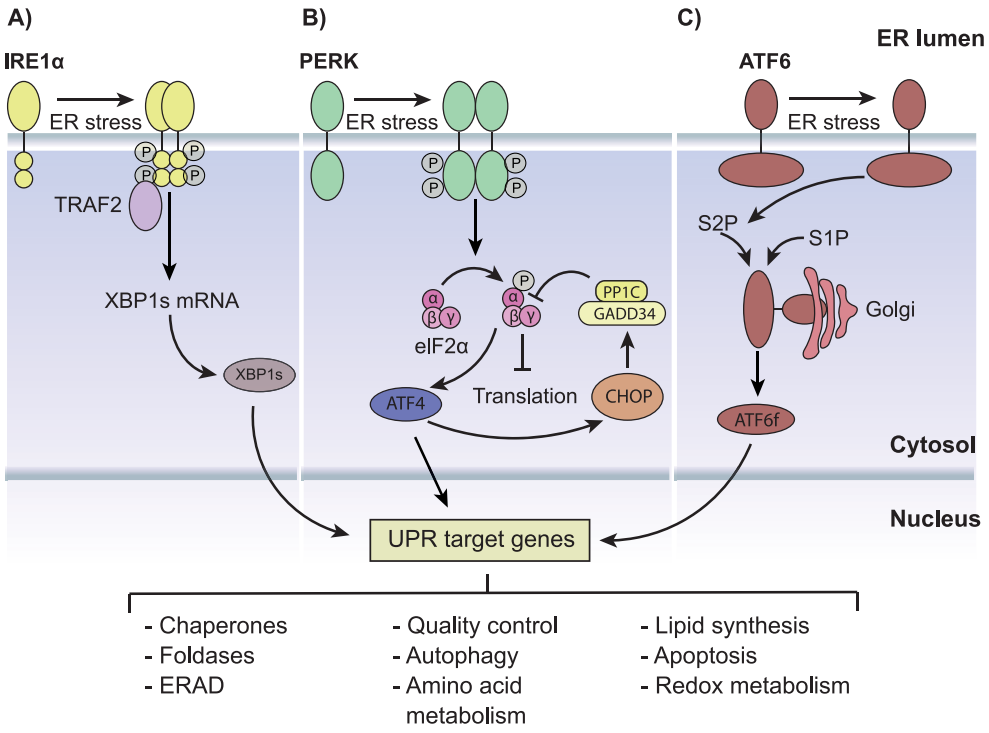

Figure 13. Unfolded protein response (UPR). ER stress induces the adaptative response UPR. Three major stress effectors control the UPR: IRE1α, PERK and ATF6. These transmembrane proteins transduce the signal from the cytosol to the nucleus to restore the protein folding through different pathways. **A.** The RNAse activity of IRE1α processes the mRNA that encode the transcription factor XBP1, leading the expression of an active X-Box Binding Protein 1 (XBP1) that up-regulate a subset of UPR target genes related to protein folding. **B.** PERK activation phosphorylate the eIF2α leading in a general attenuation of the protein synthesis. Moreover, the phosphorylation of eIF2α allows the specific traslation of the ATF4 mRNA, which encondes a transcription factors that induces the expression of genes involved in antioxidant responses, amino acid metabolism, autophagy and apoptosis. In addition, ATF4 controls the expression of the pro-apoptotic components GADD34 and CHOP. A protein phosphatase PP1C binds to GADD34 to dephosphorylate eIF2α. **C.** After ER stress, ATF6 translocate to the Golgi apparatus to be processed by a site 1 protease (S1P) and site 2 protease (S2P) releasing its cytosolic domain (ATF6f). ATF6f controls the upregulation of select UPR target genes. (Adapted from Hetz et al., 2013).

MATERIALS AND METHODS

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie

1. Cell lines, medium and culture conditions

The following human cell lines were used:

Name	Origin
HEK 293T	Derived from embryonic kidney, with epithelial morphology
U2OS	Osteosarcoma
MCF7	Breast adenocarcinoma
MCF10A	Non-tumorigenic epithelial breast
HCT116	Colon cancer
A549	Adenocarcinoma alveolar basal epithelial
BJ	Fibroblast
RPE	Retinal pigment epithelial

All cell lines except MCF10A were grown in Dulbecco´s Medium.

Modified Eagle´s Medium (DMEM, Lonza/Gibco), supplemented with 10% Fetal Bovine Serum (FBS, Gibco-Thermo Fisher Scientific), 1.5 mM L-glutamine (Sigma-Adrich), 150 U/ml penicillin and 150 µg/ml streptomycin (Sigma-Adrich). MCF10A were grown in DMEM/F12 (Gibco-Thermo Fisher Scientific) supplemented with 5% horse serum (Sigma-Aldrich), 20 ng/ml of Epidermal Growth Factor (EGF) (Enzo), 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin (Enzo), 10 µg/ml insulin (Sigma-Adrich), 1.5 mM L-glutamine (Sigma-Adrich), 150 U/ml penicillin and 150 µg/ml streptomycin (Sigma-Adrich).Cells were grown at 37ºC in a 5% of CO_2 atmosphere in 6 and 10 cm cell culture dishes (VWR). The plasmids used are shown in the appendix 1.

2. Cell handling

2.1. Plasmid transfection

Transfection of plasmids was carried out with the calcium phosphate method (the volumes and amount of DNA described below are for a 10 cm dish). 10 µg of DNA plasmid were mixed with 450 µl water and 50 µl of 2.5 M $CaCl_2$. Then 500 µl of HBS 2X buffer were slowly added to the mix, after which this was added to cells cultured at 40-60% of confluence containing 8 ml of medium. After 16 hours of incubation, the cells were washed with PBS and incubated in fresh medium for 24 hours, after which the cells were harvested.

HBS 2X buffer pH 7.02
280 mM NaCl
1.5 mM Na_2HPO_4
12 mM Glucose
10 mM KCl
50 mM Hepes

2.2. siRNA oligo transfection

siRNA transfection was carried out following the next steps (volumes for a 6 cm dish). In a tube is mixed 5 µl of Lipofectamine RNAiMAX (Invitrogen) with 250 µl of Opti-MEM (Gibco-Thermo Fisher Scientific). In another tube, 5 µl of oligonucleotide from a 20 µM stock were mixed to 250 µl of Opti-MEM. Then the content of the two tubes was mixed and incubated for 20 minutes at room temperature. During this incubation, the medium of the cells (20-60% confluency) was refreshed and the mix was added drop by drop into the plate and incubated at 37ºC. Cells were collected 24-72 hours later. The siRNA oligos (Thermo Fisher Scientific, Microsynth) used are shown in the appendix 2.

2.3. Cells treatment

Ultraviolet (UV) light causes thymine base pairs next to each other to bind together into pyrimidine dimers and 6-4 photoproducts. For UV treatment cells were incubated with 40 J/m^2 (Philips UV lamp, UVA) and were collected 1 hour later.

Thapsigargin is an inhibitor of the sarcoplasmic or endoplasmic reticulum Ca^{2+} ATPase. Blocks the transient increase in intracellular Ca2+ induced by angiostatin and endostatin. Cells were treated with 0.25 µM or 2 µM Thapsigargin (Cayman Chemical Company) for 1 hour.

Dithiothreitol (DTT) is a reducing agent. Causes a redox imbalance in the endoplasmic reticulum and produced an improperly formed disulphide bonds and unfolding proteins. Cells were treated with 2 mM DTT (Sigma-Aldrich) for 1 hour.

Camptothecin (CPT) is a cytotoxic quinoline alkaloid which binds irreversibly to the DNA-topoisomerase I complex, inhibiting the reassociation of DNA after cleavage by topoisomerase I and traps the enzyme in a covalent linkage with DNA. Cells were treated with 2 µM CPT (Sigma-Aldrich) for 1 hour.

Cychloheximide (CHX) is a eukaryote protein synthesis inhibitor. CHX exerts its effect by interfering with the translocation step in protein synthesis, thus blocking translational elongation. Cells were treated with 50 µg/ml of CHX (Sigma-Aldrich) at different times.

3. Protein detection

3.1. Whole cell extracts

Whole cell extracts were made using a Urea/SDS buffer and removing the lysate with a cell scraper (Greiner bio-one). After that, samples were sonicated for 15 seconds at 80% frequency (UP1000H, Hielsher Ultrasonic) and stored at -20ºC until they used for analysis. Before loading on SDS-Page gel, samples were quantified using the Bicinchoninic Acid method (BCA) assay kit (Sigma-Aldrich) and equal amounts of total protein were analysed after adding loading buffer at each sample and heating at 95ºC during 5 minutes.

UREA-SDS buffer	Loading buffer (4x)
6 M UREA	5% β-Mercaptoethanol
1% SDS	20% Glicerol
125 mM NaCl	0.1% Bromophenol Blue
25 mM TRIS pH 8	25% Stacking buffer

3.2. Western blot

3.2.1. Electrophoresis

Protein analysis was performed in SDS polyacrylamide gels containing different percentages of acrylamide (between 6% and 12%) depending on the size of analysed proteins. Those gels contain a stacking and separating layers (see composition below). Between 20 and 40 µg of total protein were loaded per sample. Electrophoresis was carried out using a mini-PROTEAN 3 system (BioRad), at 120-160 V in electrophoresis buffer, until the front left the gel.

Stacking gel	Separating gel
125 mM TRIS pH 6.8	0.75 M TRIS pH 8.8
0.1% SDS	0.2% SDS
5% acrylamide:bisacrylamide (29:1)	6-12% acrylamide:bisacrylamide (29:1)

Electrophoresis buffer
25 mM TRIS
192 mM Glycine
0.1% SDS

3.2.2. Gel transference and blocking

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Protran BA 85, GE Healthcare), using the mini-PROTEANS trans-blot module (BioRad), in transfer buffer at 265-295 mA for 1 hour. Blocking of the membranes was performed in a 5% skimmed milk (Sveltesse, Nestlé) in TBS supplemented with 0.1% Tween 20 (Sigma-Aldrich) (TBS-T) for 1 hour while gently shaking at room temperature.

Transfer buffer	TBS pH 7.4
25 mM TRIS	25 mM TRIS
192 mM Glycine	10 mM KCL
20% Ethanol	270 mM NaCl

3.2.3. Immunodetection and antibodies

Immunodetection of transferred proteins was performed by incubating the membrane with antibodies or (purified) immune serum (Appendix 3) diluted in 5% skimmed milk in TBS-T overnight at 4ºC under gentle shaking. Then, membranes were washed in TBS-T five times for 5 minutes each and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson InmunoResearch) diluted 1:10,000 in TBS-T for 1 hour at room temperature. After this incubation, five washes of 5 minutes in TBS-T were performed and finally the blots were incubated in a 1:1 mix of LumiSensor Chemiluminescent HRP solutions (GensScript) for 5 minutes at room temperature. Chemiluminescence detection was carried out using X-ray films (Kodak) or the ImageQuant LAS 4000 mini equipment (GE Healthcare).

3.3. Immunoprecipitation

For immunoprecipitations cells were tripsinized and washed with cold PBS 1X by centrifugation at 1,500 rpm for 5 minutes. Then the cells were lysed in 1 ml EB150 (from a 10 cm dish) and incubated for 20 minutes on ice.

EB150 buffer
50 mM Hepes pH 7.5
150 mM NaCl
2 mM $MgCl_2$
5 mM EGTA
1 mM DTT
0.5% NP-40
10% Glycerol
1 mM Na_3VO_4
5 mM NaF
Protease inhibitor cocktail (Calbiochem)

Lysates were cleared by centrifugation at 18,000 rpm at 4ºC for 20 minutes. 100 µl of supernatant were taken for input, to which sample buffer was added. The rest of the lysate was incubated with 25 µl of anti-Flag M2 Affinity Agarose Gel (SigmaAldrich) for 2 hours with agitation at 4ºC. The resin was collected by centrifugation at 5,000 rpm for 1 minute, and washed 4 times with EB150 buffer. Proteins bound to the resin were eluted using 50 µl of sample buffer and heated at 96ºC for 5 minutes.

3.4. His-ubiquitin pull down

Before lysis, cells were incubated with proteasome inhibitor MG123 (Calbiochem) for 16 hours (5 µM). Then, cells were tripsinized, harvested by centrifugation at 1,500 rpm for 5 minutes and washed in PBS. To prepare the input, from 1 ml of cells (from a 10 cm dish) resuspended in PBS, 100 µl were centrifuged and lysed with 80 µl of Urea/SDS buffer and store at -20 ºC. The pellet corresponding to the remaining 900 µl was lysed with 1 ml of buffer A and samples were sonicated for 30 seconds at 50% of frequency 3 times (UP1000H, Hielsher Ultrasonic). Afterwards, we added 50 µl of Nickel NTA agarose (Qiagen) previously washed 3 times with buffer A. The samples were in agitation for 2 hours at room temperature and then washed several times: first with 1 ml of buffer A 3 times, second with 1 ml of a buffer composed by buffer A and buffer B in a 1:3 ratio twice, and third, with 1 ml of buffer B twice. The samples were then eluted from the resin with 50 µl of buffer C, heated at 95ºC for 5 minutes and centrifuged at maximum speed. The supernatant was then stored at -20ºC.

Buffer A	Buffer B	Buffer C
6 M Guanidinium-HCL	25 mM TRIS pH 6.8	0.2 M Imidazol
0.1 Na_2HPO_4	20 mM Imidazole	50% Sample buffer 4X
10 mM Imidazol		
10 mM TRIS-HCl pH 8		

3.5. Chromatin fractionation

From 10 cm dish, cells were tripsinized and harvested in 1 ml of PBS. From those cells, we obtained three different types of extracts: whole cell extracts (WC), chromatin fraction (P) and soluble fraction (S). To prepare "WC", the pellet corresponding to 100 µl of the cell suspension in PBS was lysed in 80 µl of Urea/SDS buffer and stored at -20ºC. To isolate "P" and "S", the rest of the cells was resuspended in 150 µl of buffer D. 0.1% of Triton X-100 (final concentration) was added, and the cells were incubated for 5 minutes on ice. Cells were then centrifuged at 3,800 rpm at 4ºC for 4 minutes, and the supernatant was collected ("S" fraction). The nuclei (pellet) was washed once with buffer D and centrifuged again. 125 µl of buffer E were then added and left on ice for 10 minutes. After that, extracts were centrifuged at 4,300 rpm at 4ºC for 4 minutes and the supernatant was mixed with the "S" fraction, obtaining the total "S" fraction. The insoluble chromatin (pellet) was washed once with buffer E and centrifuged at 10,000 rpm at 4ºC for 2 minutes. The final chromatin pellet ("P") was obtained by resuspending with 100 µl of Urea/SDS buffer, heated at 95ºC for 5 minutes and sonicated for 15 seconds.

Buffer D	Buffer E
10 mM Hepes pH 7.5	3 mM EDTA
10 mM KCl	0.2 mM EGTA
1.5 mM $MgCl_2$	1 mM DTT
0.35 M Sucrose	100 mM NaF
10% Glycerol	1 mM Na_3VO_4
1 mM DTT	Protease inhibitor cocktail (Calbiochem)
10 mM NaF	
1 mM Na_3VO_4	
Protease inhibitor cocktail (Calbiochem)	

4. Protein purification and enzymatic assays

4.1. Protein purification

To purify ubiquitinated GFP-Geminin, Flag-DUB3 and Flag-USP7, 293T cells were first transfected with the adequate expression vectors (Appendix 1). Then, cells were washed in PBS and lysed in EB150 lysis buffer as described before for 20 minutes on ice. In the case of cells overexpressing DUBs, no protease inhibitors were added. In case of GFP-Geminin, the buffer was supplemented with protease inhibitors and 2 mM of DUB inhibitor NEM (Sigma Aldrich) and extracts were sonicated for 15 seconds (UP1000H, Hielsher Ultrasonic). After centrifugation at 13,000 rpm for 20 minutes, Flag-DUBs and GFP-Geminin extracts were incubated with anti-Flag M2 agarose (Sigma Aldrich) or anti-GFP in protein A beads (GE Healtcare) respectively for 2 hours at 4ºC, followed by 4 washes with EB150 lysis buffer and 1 wash in elution buffer (50 mM TRIS pH 7.5). DUBs proteins were eluted in elution buffer supplemented with 330 μg/ml 3x Flag peptide (DYKDDDDK, Generon Ltd) three times for 10 minutes at room temperature. The supernatant was collected and samples to check for expression and purification were taken before and after purification by western blot or left at -20 ºC supplemented with 50% glyclerol.

4.2. *In vitro* de-ubiquitination assay

To investigate de-ubiquitination *in vitro*, ubiquitinated Geminin and different DUBs were purified as described above. Then Geminin-protein A beads and DUB3 or USP7 were mixed in de-ubiquitin assay buffer and incubated for 2 hours at 37ºC. Sample buffer was added to stop the reaction. Geminin ubiquitination was subsequently analysed by western blotting using an FK2 antibody.

Deubiquitin assay buffer
50 mM TRIS pH 7.5
4 mM DTT

5. Flow cytometry

5.1. Propidium Iodide (PI) staining

For PI staining cells were harvested after trypsinization and washed once with PBS. The cells were centrifugated at 1,500 rpm, 5 minutes and fixed with cold ethanol 70% added drop by drop using a vortex at low speed. Cells were then stored at 4ºC for 2-16 hours. Then, the cells were centrifuged, washed with PBS and incubated with 500 µl of staining solution for 20 minutes at 37ºC in dark.

Staining solution
20 µg/µl Propidium Iodide (Sigma Aldrich)
40 µg/µl RNAse (Sigma Aldrich)

Cells where then analyzed with a MACSQuant VYB flow cytometer (Miltenyi Biotec).

5.2. BrdU staining

BrdU was added into the cell plated in a 1:1,000 concentration (from a 10 mM stock) and incubated for 15 minutes at 37ºC in a 5% of CO_2 atmosphere. The BrdU was washed off twice with PBS. Then cells were harvested and fixed as performed for the PI staining. The cells were then washed once with PBS and centrifuged gently (1,500 rpm, 5 minutes) and 500 µl of 2 M HCl in 0.5% of Triton-X 100 were added to permeabilized and denatured the DNA for 30 minutes at 37ºC. To neutralize the HCl, 10 ml of 1M TRIS pH 7.5 were added, centrifuged and washed once with PBS. After removing the supernatant, the cells were resuspended in 100 µl of a solution containing 1:100 anti-BrdU mouse primary antibody (GenScript) with 1% BSA in 0.5% PBS-T (Tween 20%) and incubated 16 hours at 4ºC. Afterwards, the cells were washed once with 1% BSA in 0.5% PBS-T. After gentle centrifugation, the cells were resuspended in 100 µl of a solution containing 1:400 Alexa 647 secondary antibody (Life Technologies) and 1% BSA in 0.5 % PBS-T and incubated for 45 minutes at 37ºC in dark. Cells were then washed once with 1% BSA in 0.5 % PBS-T and centrifuged. Then the cells were stained with PI following the final step of the described protocol. Cells where analyzed with a MACSQuant VYB flow cytometer (Miltenyi Biotec).

6. DNA fiber analysis

For this technique, cells were grown until 80% confluence. The cells were pulsed with 50 µM CldU diluted in DMEM medium for 20 minutes. The chloro-deoxyuridine CldU (Sigma-Aldrich C6891) was washed 3 times with warm PBS and immediately the cells were pulsed with 250 µM iodo-deoxyuridine IdU (Sigma-Aldrich I7125) diluted in DMEM medium. The cells were trypsinazed and harvested, washed them with PBS. The cells were then resuspended in cold PBS at aprox. 0.25 x 10^6 cells/ml **(Fig. 14.1).** The cell suspension was placed on a regular microscope slide pre-cleaned with 70% EtOH. Drop the cell suspension was dropped near one end of the slide and the cells were lysed by adding 10 µl of fresh pre-

warmed (30ºC) spreading buffer and the lysing was incubated for 6 min at RT in humidity chamber.

Spreading buffer
0.5% SDS
200 mM TRIS 7.4
50 mM EDTA

The slides were tilted at 10º-15º angle to allow the DNA suspension to run slowly down the slide trying to make it run straight **(Fig. 14.2)**. Then, the slides were air dried at least 20 min and then fixed with cold (-20ºC) 3:1 methanol:acetic acid at room temperature for 2 minutes. The slides were again air dried and incubated in 2.5 M HCl for 30 minutes at room temperature to denature the DNA. After this, the slides were well rinsed with PBS 3 times in order to completely remove the HCl and protect the antibodies used in the next steps. Slides were then blocked with 1% BSA with 0.1% Triton X-100 in PBS (Blocking Solution) for 1 hour at room temperature in a humidity chamber before incubating with 75 µl (per slide) of the indicated primary antibodies diluted in blocking solution (1:100) for 1 hour at room temperature in a humidity chamber.

	Antibody	Origin	Source
CldU	anti-BrdU (sc-56258)	Rat	Santa Cruz Biotechnology
IdU	anti-BrdU (B44)	Mouse	BD Biosciences
ssDNA	IgG2a (MAB3034)	Mouse	Merck-Millipore

Then slides were rinsed 3 times with PBS before incubating (1 hour at room temperature) with the indicated secondary antibodies diluted in blocking solution (1:300) in a opaque humidity chamber **(Fig. 14.3)**.

	Antibody	Origin	Source
CldU	Alexa Fluor 555 (A21434)	Goat	Life Technology
IdU	Alexa Fluor 488 (A21121)	Goat	Life Technology
ssDNA	Alexa Fluor 647 (A21241)	Goat	Life Technology

The slides were then rinsed 3 times with PBS and air dried before mounting them with Prolong (Invitrogen P36930). Slides were air dried overnight in dark.

The slides were examined with a Leica DM6000 B microscope. The conversion factor used was 1 µm= 2.59 kb. In each assay, at least 200 tracks were measured to estimate fork rate and around 300 tracks were analyzed to estimate the frequency of origin firing **(Fig. 14.4)**.

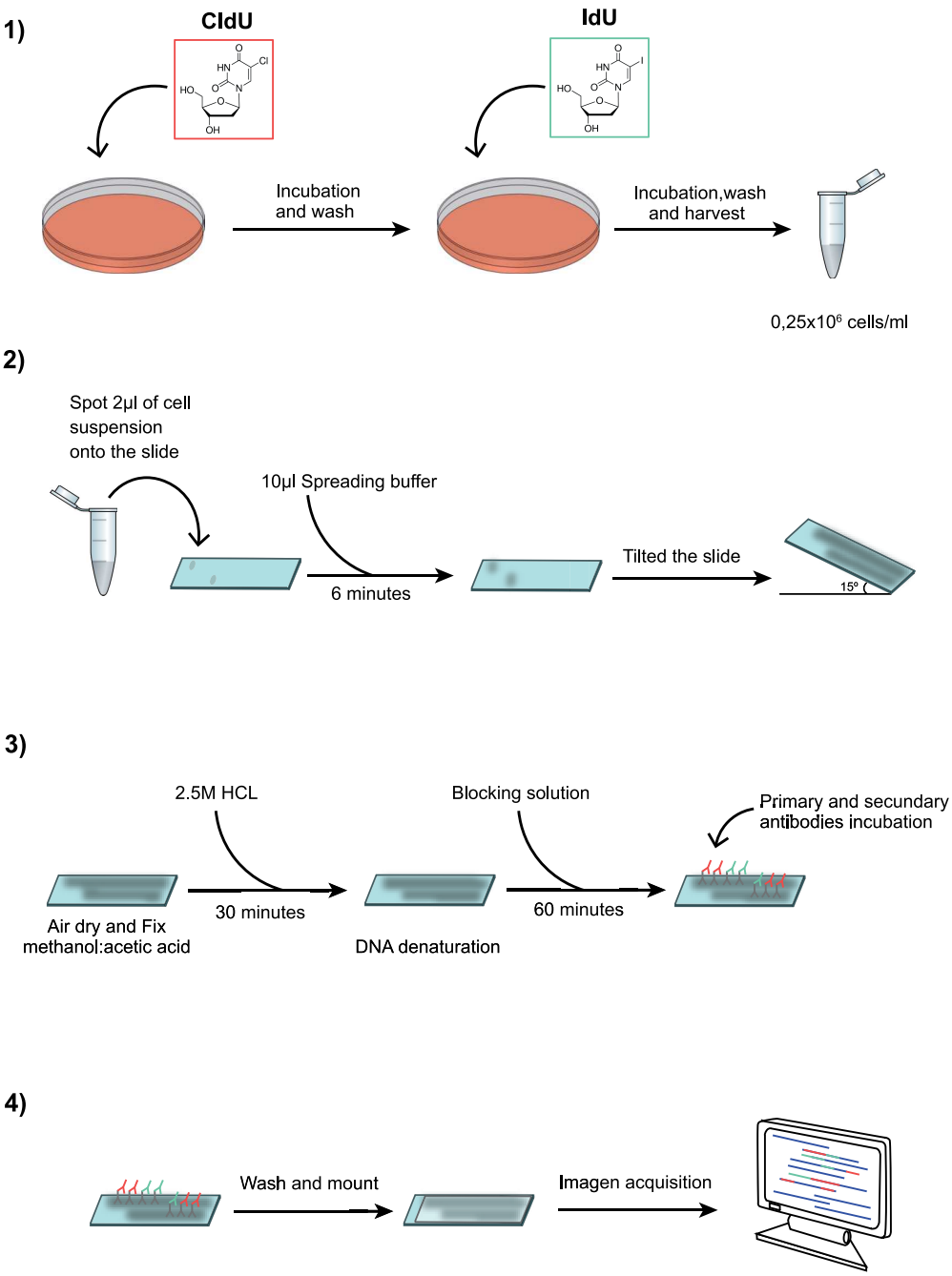

Figure 14. DNA Fiber protocol. 1. The cells were labelled with CldU first and second with IdU, then the cells were harvested. **2.** and **3.** The cells were stretched, then, the antibodies were incubated. **4.** The images were acquired and analyzed using a microscope and PC.

7. DNA combing analysis

7.1. DNA labelling and blocks preparation

Cells were grown until a maximal 80% confluence. Then, 20 µM IdU diluted in warm DMEM medium were added to the cells for 30 minutes. The cells were rapidly washed with warm DMEM medium and immediately 100 µM CldU were added incubated again for 30 minutes. In order to inhibit the replication after the second labelling, 1 mM thymidine (Sigma Aldrich T9250) was added for 5 minutes before harvesting them by trypsinization and centrifugation **(Fig. 15)**.

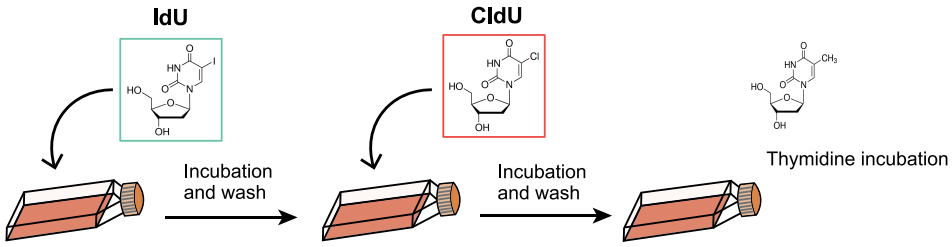

Figure 15. DNA combing protocol, DNA replication labelling. The cells were labelled with IdU first, and second with CldU.

Once the thymidine has blocked the DNA replication, the cells were harvested with trypsin and washed then with PBS. Afterwards, the trypsin was neutralized with DMEM medium. Then cells were washed with PBS and centrifuge at 1,500 rpm for 5 minutes. Cells were then resuspended in PBS at 7 x 10^6 cells/ml after counting them. Afterwards, 300 µl of cell suspension were taken and warmed 10 seconds at 45ºC, then cells were mixed with 300 µl of 2% agarose low melting (2-Hydroxyethylagarose, A4018- Sigma-Aldrich) and loaded 80 µl of the mix in a well of a Plug Mold (Bio Rad – 1703706), to load 5 wells. The Plug Mold was left at 4°C for 15 minutes in order to solidify. Firstly, the 2% agarose low melting in PBS was prepared and kept the liquid in a water bath at 45°C before being used **(Fig. 16)**.

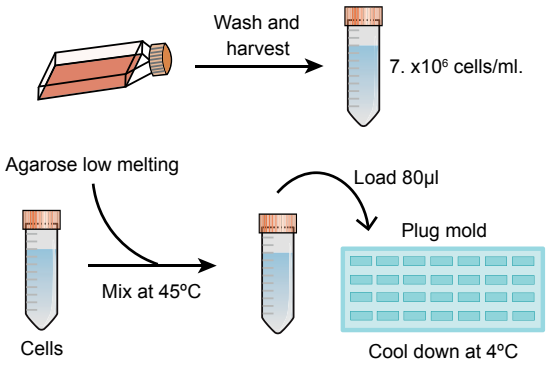

Figure 16. DNA combing protocol. Preparation of agarose blocks. Agarose blocks were prepared using a Plug Mold.

The 5 blocks were incubated in 50 ml falcon tubes with 10 ml of proteinase K (PK) solution at 42ºC for 48 hours.

PK solution
1% SDS
0.25 M EDTA
1 mg/ml PK (Roche)

After 2 days, the blocks were incubated in a wheel for 20 minutes with 10 ml of TE (TRIS 10 mM pH 8, EDTA 1mM) with 1 mM of Phenylmethylsulfonyl fluoride PMSF (Sigma Aldrich) to inhibit the PK. The blocks were incubated twice in a rotating wheel for 20 minutes with 10 ml of TE. At this point the blocks can be store at 4ºC with TE **(Fig. 17)**.

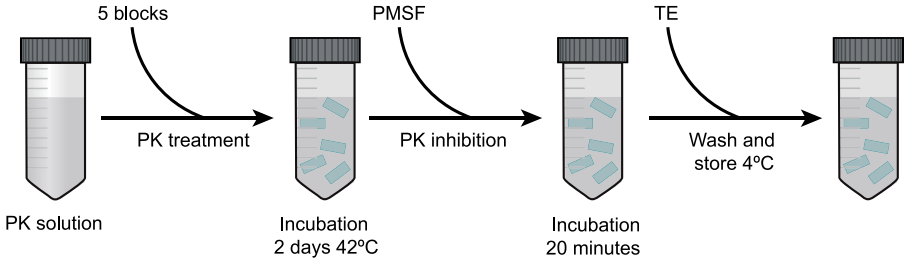

Figure 17. DNA combing protocol. Proteinase K treatment.

One block was moved to a 2 ml tube together with 280 µL of TE and 40 µl of 10X β- Agarase buffer (New England Biolabs). Then the tube was heated at 68ºC for 15 minutes and cooled down to 42ºC. Afterwards, 2.5 µl of β- Agarase (New England Biolabs M0392) were added and incubated 1 additional hour at 42ºC. Two additional incubations with 2.5 µl of β- Agarase were done for 24 hours at 42ºC. At this point the DNA is dissolved and unprotected and thus, carefully, 1.6 ml of 0.25 M MES pH 5.5 were added and the tube was incubated at 65ºC for 30 minutes to force the untangling of the DNA. Then, the tube was cooled down at room temperature and stored at 4ºC. The DNA was then kept at 4ºC in MES solution at least 3-5 weeks in order to allow a well DNA disentanglement **(Fig. 18)**.

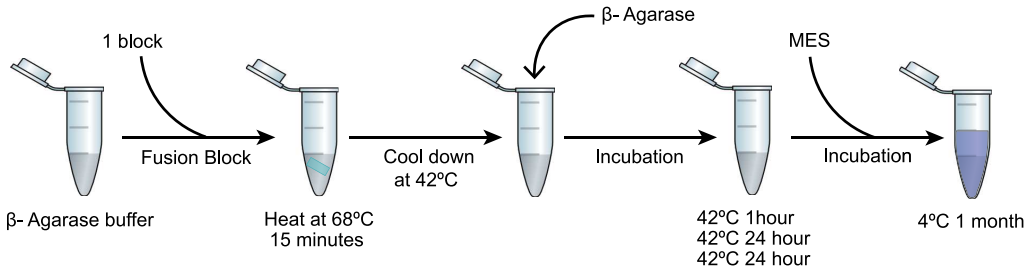

Figure 18. DNA combing protocol. Fusion of agarose block.

7.2. Coverslips preparation. Silanization

The stretching/combing of the DNA must be done in previously silanized coverslips. The quality of the stretching and post-immunodetection depend greatly on the quality of the coverslips. For silanization, coverslips are pre-treated and during all those steps the coverslips were on a teflon rack. First, the coverslips were washed and the rack was immersed in a beaker, and the beaker was placed in an ultrasonic bath with water. The coverslips were sonicated for 20 min in isopropanol first, then in 50% metanol /50% water and finally in chloroform and were dried. Next, the coverslips were placed at high power for 1 minute in the Plasma Cleaner device (PDC-32G- Harrick Plasma) that cleans dust particles on coverslipts. Finally, in order to remove all the water of the coverslips before silanization, coverslips were dried in an oven at 110ºC for 1 hour. When the coverslips were totally dried, they are silanized in liquid phase, for that, the coverslips were introduced in a beaker containing 150 ml of n-Heptane (Sigma-Aldrich - H9629) together with 0.1% of Trimethoxy (7-octen-1-yl) silane (Sigma-Aldrich - 452815). In order to protect for the air and water the coverslips were left in a desiccator overnight. After silanization, coverslips were sonicated for 5 minutes first with n-Heptane, then with water and last with chloroform. At the end, the coverslips were air dried and kept in aluminium paper.

7.3. DNA fiber stretching

The DNA stretching was performed using the FiberComb Molecular Combing System (MCS) (Genomic Vision). The DNA solution was warmed at room temperature and placed in the reservoir container of the MCS. Two silanized coverslip were clipped to the MCS and rapidly dipped into the reservoir containing the DNA solution. The coverslips were incubated briefly with the DNA solution to allow that DNA attaches to the glass surface and then the coverslips were automatically raised up at constant speed (300 μm/s), thereby stretching the DNA in a controlled and consistent manner. Stretching is achieved by the air-liquid meniscus, which results in the extension and parallel alignment of DNA molecules. Each DNA molecule is combed with a constant stretching factor (2 kb/μm), enabling precise and direct measurement along the length of the DNA molecules. After this, the coverslips were sticked on a glass slide with SuperGlue-3 (Loctite) and before storing them at -20ºC, the slides were heated at 60ºC for 2 hours in a humidity oven **(Fig. 19)**.

7.4. Immunodetection

The slides were incubated at room temperature before the immunodetection started. In the staining process, first the DNA was denatured with NaOH 1N for 5-10 minutes (depending on of the silanized coverslips quality) at room temperature. Then, the slides were well washed with cold (4ºC) PBS in coplins jars twice: first for 10 seconds to remove the excess of NaOH, and then for 5 minutes. Then, to fix the DNA, the slides were incubated with 70% ethanol (-20ºC) for 5 minutes at 4ºC followed by an incubation with 85% ethanol for 5 minutes at room temperature. To dry the surface, the slides were incubated with 100% ethanol for 5 minutes at room temperature. Next, the samples were blocked to improve the quality of the antibody detection. To prepare the blocking solution, 0.375 g of blocking reagent (DIG DNA labelling and detection Kit, Boehringer Mannheim) were dissolved in 25 ml PBS containing 0.05% Tween 20. The mixture was heated at 68ºC for 2 hours and then centrifuged 2 times at 4,000 rpm. The supernatant was filtered, aliquoted and stored at -20ºC. The slides were incubated with 30 μl of blocking solution for 30 minutes at 37ºC. Then, the slides were incubated with each specific antibodies, that were diluted in blocking solution. First, the primary antibodies recognizing IdU and CldU were incubated for 30 minutes at 37ºC (for IdU was used mouse α-BrdU FITC (Becton-

Dickinson-347583) diluted 1:5 and for CldU was used rat α-BrdU Abd (Serotec-OBT0030) diluted 1:25). Since both antibodies recognize BrdU, they crossreact both IdU and CldU. However, the bound α-BrdU FITC recognizes IdU with higher affinity than CldU. The α-BrdU Abd recognizes CldU better than IdU. To increase the specificity of each antibody against the selective analogue, the slides were washed for 6 minutes at room temperature with a solution 0.5 M NaCl, 20 mM Tris pH 7.8 and 0.5% Tween 20. This solution allows remove the weak bounds between the analogues and the antibodies. Prior incubation with the secondary antibodies, the slides were washed with PBS for 1 minute and then were incubated with goat α-mouse Alexa 488 (Invitrogen-A11029) and goat α-rat Cy3 (Invitrogen-A21434) diluted at 1:50 and incubated for 30 minutes at 37ºC. Then, the slides were washed 3 times with PBS for 5 minutes at room temperature. To improve the detection of the ssDNA, a mouse primary antibody was used, anti-ssDNA (Milipore-MAB3034) at 1:50 for 1 hour at room temperature. Then it was used a secondary antibody at 1:100 (goat anti-mouse Cy5.5 (Abcam-ab6947)) for 30 minutes at 37ºC, and another secondary antibody at 1:100 dilution (donkey anti-goat Cy5.5.5 (Abcam-ab6951)) for 30 minutes at 37ºC to increase the Cy5.5 signal. After each incubation step, the slides were washed 3 times with PBS for 5 minutes at room temperature and were mounted using VectaShield (without DAPI). The slides were examined with a similar method aformentioned in th DNA fiber analysis section.

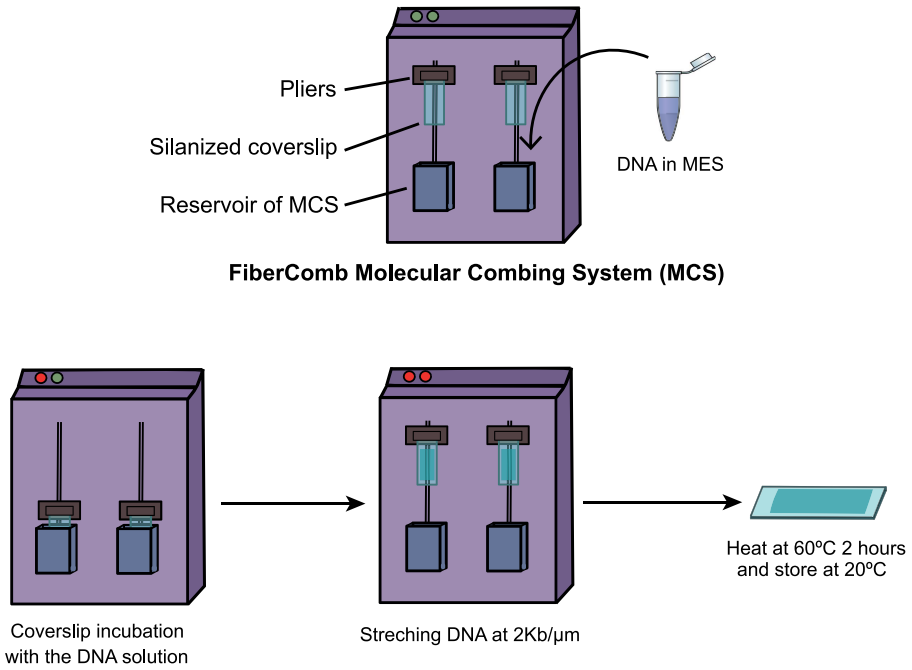

Figure 19. DNA Combing protocol. DNA fiber streching.

8. Quantitative real-time RT-PCR

8.1. RNA isolation

The cells from 10 cm dish were harvested and resuspended in 250 µl Buffer F and were homogenized by sonication on ice for 15 seconds (UP1000H, Hielsher Ultrasonic). The homogenized was mixed with 25 µl of 2 M sodium acetate pH 4, 250 µl of bidistilled phenol saturated with DEPC-treated water, and 100 µl of chloroform-isoamyl alcohol mix 49:1. The samples were then cooled 10 minutes at -20ºC. The aqueous phase was collected and mixed with the same volume of isopropanol and kept during 1 hour at -20ºC to precipitate the RNA. The solution was centrifuged at 9,000 rpm 20 minutes at 4ºC and the pellet was collected. The pellet (RNA) was dissolved in 150 µl of Buffer F and then 150 µl of isopropanol were added. The samples were incubated 1 hour at -20ºC, centrifuged at 9,000 rpm for 20 minutes at 4ºC and the RNA pellet was collected and the supernatant was discarded. 200 µl of 75% ethanol in DEPC-treated water were added. The RNA pellet was stored 1 hour at -20ºC and then was detached carefully and centrifuged at 1,300 rpm for10 minutes at 4ºC. The ethanol was totally removed and the pellet was air dry. The RNA pellet was then dissolved in DEPC-treated water and stored at -80ºC.

Buffer D
4 M Guanidinium thiocyanate
25 mM Sodium citrate
0.5% Sarkosyl pH 7
0.1 M β-Mercaptoethanol

The purity and concentration of RNA were determined by Nano-drop 2000 (Thermo-Fisher). The quantification of relative abundance of Cdt1 mRNA was carried out using quantitative PCR and the SYBR green detection method.

8.2. Retro-transcription

Total RNA was reverse transcribed using a cDNA synthesis Kit ImProm-II™ Reverse Transcription System (Promega) and was carried out with 1 µg of RNA. The cycles of the RT were the next ones:

25ºC	5 minutes
42ºC	1 hour
70ºC	15 minutes

8.3. cDNA amplification

The cDNA was PCR amplified using gene-specific primers. The Cdt1 oligonucleotides used were: 5´TAATCTGACCTCCTGGTGCC 3´ (forward primer), and 5´GTAGGCGTTTTGAGGAGTGC 3´ (reverse primer). The resulting increase in fluorescence during the PCR reaction was detected in the iQ5 system (Bio-Rad, Hercules, CA) using the IQ SYBR Green Supermix (Bio-Rad). Four serial dilutions of cDNA, were amplified in triplicate for each amplicon in a volume of 20 µl. The relative changes in

gene expression from qRT-PCR experiments were analyzed using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), GADPH was used as a reference gen.

The cycles of the qRT-PCR were:

Denaturation	2 minutes	95ºC	1 cycle
Amplification	15 seconds 20 seconds 20 seconds	95ºC 60ºC 72ºC	40 cycles
Melting	Δ0.5ºC/s	60ºC-90ºC	

9. Histological staining

Samples were obtained from the Queensland Breast Cancer Bank collected from consenting patients. The study was approved by the local ethics committees. Tissue microarrays were constructed from duplicate cores of formalin-fixed, paraffin-embedded breast tumor samples.

9.1. Tissue processing

The samples were introduced briefly in 3% of paraformaldehyde for 6 hours to fix both the cell and the original structures. Then, the samples were dehydrated following the next steps: the samples were incubated in crescent ethanol percentages (70°, 96°and 100°) allowing the total dehydration of the tissue. Next, tissues were cleared with xylene and finally embedded in paraffin that provide consistency to be cut with the microtome. These are the different steps followed:

Ethanol 70°	2 hours
Ethanol 96°	4 hours
Ethanol 100°	6 hours
Xylol	2 hours
Paraffin	5 hours

Blocks of paraffin embedded tissue were prepared and stored at room temperature.

Tissue microarrays (TMAs) were generated from selected cases in order to have multiple samples in the same block. Paraffin sections, 3 µm-thick, were cut using a microtome, floated on a warm water bath and mounted on slides. The slides were air dried and baked at 65°C for 3 hours. Tissue slides were dewaxed in xylene and rehydrated with ethanol baths as follows: the samples were washed in xylene twice for 10 minutes, and then with a decrescent percentage of ethanol (100°, 96°and 76°), finally they were washed with water.

9.2. Immunohistochemistry

Four TMA sections were processed in an antigen de-cloaker (Biocare Medical) for antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 20 minutes, and then the immunohistochemistry (IHC) analysis was performed.

The TMAs were permeabilized with 0.1% Triton X-100-PBS, for 15 minutes in shaking. The excess Triton X-100 was washed with PBS three times for 5 minutes each one and then the TMAs were blocked with 3% BSA in PBS (blocking solution) for 30 minutes. Next, sections were incubated with the USP7 or Geminin antibodies (ab119364, ab175799 from Abcam), diluted 1:400 in blocking solution, at 4°C for 18 hours in humidity chamber. The primary antibody was washed twice with PBS 5 minutes. The endogenous peroxidases were inhibited with 0.3% H_2O_2 in ethanol for 15 minutes and then, they were washed with PBS again. The secondary species-specific antibody, (HRP)-conjugated, was incubated for 45 minutes and washed three times with PBS. The developing was performed with Polymer Detection kit (Biocare Medical), which produces an insoluble brown precipitate where the HRP is located. Finally, the excess of HRP was washed with PBS and counterstained with hematoxylin, as explained in the section 9.3.

To perform the study of the TMAs in the optic microscope, the samples were dehydrated again in a crescent ethanol percentage (70°, 96°and 100°) for 10 seconds each one, then they were incubated 10 seconds in a carboxyl bath and two baths of xylene. The TMAs were mounted sticking a cover with the Eukit.

9.3. Hematoxylin dye

It is a easy staining which allows differentiate the histological structures. The hematoxylin dyes acids regions like the nucleus.

The staining was performed with the rehydrated samples. The TMAs were incubated with a bath of hematoxylin for 30 seconds and then washed with water. After the staining, the samples were dehydrated and mounted with the same method explained before (section 9.2).

9.4. Data acquisition and interpretation

The samples were scanned at 40X magnification on an Aperio AT Turbo slide scanner (Leica Biosystems). Digital images were scored by an experienced molecular pathologist (Jodi Saunus), taking into account both percentage of cells stained and the intensity of immunostaining. The percentages of tumour cells stained (%TC; 0-100%) and intensities (0-3+) were multiplied to derive an IHC score (0-300). Each core in the TMA was scored and each case was assigned the maximum score from its duplicate cores.

AIMS

"There are in fact two things, science and opinion; the former begets knowledge, the latter ignorance."

Hippocrates

DNA replication control is essential for the maintenance of genomic integrity and for avoiding pathogenic conditions. Dysregulations in this control can lead to cancer. Therefore, to characterize new players involved in DNA replication is important for diagnosis, prognosis and even treatment of cancer. For example the inhibition of enzymes involved in DNA replication control could stop DNA replication in cancer cells and/or also sensitize cancer cells to different treatments that interfere with DNA replication. In this thesis, we will focus our work in trying to identify new enzymes that regulate DNA replication proteins controlled by post-translational modifications.

First, Cdt1 and Geminin, crucial proteins for initiation of replication control, are regulated by ubiquitination and degradation during the cell cycle. Although several ubiquitin ligases have been identified to regulate both proteins, there are yet not know ubiquitin hydrolases that regulate them. Second, preliminary results indicate that Claspin and Chk1, who are known controllers of DNA replication, become modified after treatment with drugs that activate the Unfolded Protein Response (UPR).

Therefore the aims of this thesis are:

1- To isolate and characterize the specific ubiquitin hydrolase/s for Cdt1 and Geminin and study their possible relationship with cancer.

2- To characterize how Claspin and Chk1 become modified after activation of the UPR and the functional consecuences of this modification for the cell.

RESULTS

"An expert is a man who has made all the mistakes which can be made, in a narrow field"

Niels Bohr

MOLECULAR ONCOLOGY 10 (2016) 1196–1206

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USP37 deubiquitinates Cdt1 and contributes to regulate DNA replication

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ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
23 May 2016
Accepted 26 May 2016
Available online 3 June 2016

Keywords:
Initiation of DNA replication
DNA damage response
Ubiquitin hydrolase
DNA damage
Protein degradation
Proteasome

ABSTRACT

DNA replication control is a key process in maintaining genomic integrity. Monitoring DNA replication initiation is particularly important as it needs to be coordinated with other cellular events and should occur only once per cell cycle. Crucial players in the initiation of DNA replication are the ORC protein complex, marking the origin of replication, and the Cdt1 and Cdc6 proteins, that license these origins to replicate by recruiting the MCM2-7 helicase. To accurately achieve its functions, Cdt1 is tightly regulated. Cdt1 levels are high from metaphase and during G1 and low in S/G2 phases of the cell cycle. This control is achieved, among other processes, by ubiquitination and proteasomal degradation. In an overexpression screen for Cdt1 deubiquitinating enzymes, we isolated USP37, to date the first ubiquitin hydrolase controlling Cdt1. USP37 overexpression stabilizes Cdt1, most likely a phosphorylated form of the protein. In contrast, USP37 knock down destabilizes Cdt1, predominantly during G1 and G1/S phases of the cell cycle. USP37 interacts with Cdt1 and is able to de-ubiquitinate Cdt1 *in vivo* and, USP37 is able to regulate the loading of MCM complexes onto the chromatin. In addition, downregulation of USP37 reduces DNA replication fork speed. Taken together, here we show that the deubiquitinase USP37 plays an important role in the regulation of DNA replication. Whether this is achieved via Cdt1, a central protein in this process, which we have shown to be stabilized by USP37, or via additional factors, remains to be tested.

Abbreviation: DUB, deubiquitinating enzyme.

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http://dx.doi.org/10.1016/j.molonc.2016.05.008
1574-7891/

1. Introduction

Replicating the genome is an essential process for living organisms. DNA replication needs to be tightly regulated and monitored in order to preserve cellular genomic stability. First, because the genome needs to be replicated only once per cell cycle to avoid differences in genome content between the mother and daughter cells and second, because while DNA synthesis occurs, the genome is particularly vulnerable to damage and errors. In eukaryotic cells, complex mechanisms control and monitor DNA replication. A critical regulated step to avoid DNA replication associated instability occurs at the initiation of DNA replication. In eukaryotes, the control of DNA initiation requires the coordination of several proteins/protein complexes (reviewed in Costa et al., 2013; Fragkos et al., 2015). The origin recognition complex (ORC), a heterohexamer with DNA-dependent ATPase activity, directly recognizes and binds origins of replication. Subsequently, Cdc6 and Cdt1 are recruited to the origins and are able to load the minichromosome maintenance protein (MCM) complex MCM2–7, a heterohexamer that has ATPase-dependent DNA helicase activity, onto the replication origin. The binding of the helicase to the DNA starts the licensing of replication origins and forms the so-called prereplicative complex (pre-RC). In this loading process, ATP hydrolysis by Cdc6 helps the stable association of MCM2-7 with the DNA, after which the interaction with two additional factors enhance its helicase activity: the heterotetrameric GINS complex (formed by Psf1, Psf2, Psf3 and Sld5 proteins) and CDC45, forming the CMG complex. This complex is subsequently activated by S phase CDKs and CDC7–DBF4. Then, the replication protein A complex (RPA) binds to and stabilizes the single-stranded DNA, interacts with the DNA polymerase-α-DNA primase complex (Pol α complex) and acts as a "fidelity-clap" for the polymerase (Bochkareva et al., 1998; Fanning et al., 2006; Maga et al., 2001). Polymerization by the Pol α complex marks the start of DNA replication in the cell that continues with the recruitment of other DNA polymerases and the help of associated factors.

Cdt1 is a major regulatory factor during the initiation of DNA replication. Cdt1 is inhibited by Geminin, but regulation of Cdt1 protein levels also critically depends on ubiquitin-mediated degradation by the proteasome during cell cycle progression (Saxena and Dutta, 2005). Cdt1 levels are high during mitosis and G1 phase of cell cycle and low during S and G2 phases (Nishitani et al., 2001; Rialland et al., 2002; Wohlschlegel et al., 2000). DNA origin licensing starts during metaphase (Dimitrova et al., 1999), which coincides with drop of Geminin levels that continue to be low during G1 and rise again during S/G2 phases, thereby avoiding origin licensing after G1 (Clijsters et al., 2013; McGarry and Kirschner, 1998). Moreover, Cdt1 was shown to be degraded in the presence of DNA damage as Cdt1 levels drastically drop after a genotoxic insult (Higa et al., 2003; Hu et al., 2004). Together these regulatory mechanisms help to restrict the replication licensing to only once per cell cycle and/or avoid DNA replication in the presence of damage.

Several E3 ligase complexes were described to target Cdt1 for proteasomal degradation in different conditions. Cdt1 degradation during the cell cycle was shown to depend on the SCF–Skp2, the CRL4-Cdt2 E3 ubiquitin ligase complexes and on SCF-FBXO31 (Chandrasekaran et al., 2011; Higa et al., 2006; Jin et al., 2006; Johansson et al., 2014; Nishitani et al., 2006; Sansam et al., 2006). Moreover, CRL4-Cdt2 dependent degradation of Cdt1 depends on DNA-bound PCNA, which occurs during S phase and after DNA damage (Arias and Walter, 2006; Senga et al., 2006).

Although relatively much is known about the ubiquitination of Cdt1, the reverse process or Cdt1 deubiquitination is less studied. In this article we find USP37 as a ubiquitin hydrolase for Cdt1. USP37 was first identified as key enzyme that stabilizes Cyclin A counteracting the ubiquitination by the anaphase-promoting complex APC/CCdh1 (Huang et al., 2011). USP37 itself is a substrate of the APC/CCdh1 and its regulated during the cell cycle as USP37 levels increase at the G1/S boundary, and remain high during S and G2 phases (Huang et al., 2011). USP37 is degraded at the G2/M boundary in a SCF-β-TRCP and Plk1-dependent manner and remains low in G1 phase of cell cycle (Burrows et al., 2012). Since its first description in 2011, USP37 function has been linked to a number of different proteins. These include important pro-division regulators (Cyclin A, c-Myc or 14-3-3γ) but also USP37 is able to regulate genomic stability by controlling DNA double strand break repair by homologous recombination or proper mitotic progression by controlling WAPL, a negative regulator of chromatin cohesion (Huang et al., 2011; Kim et al., 2015; Pan et al., 2014; Typas et al., 2015; Yeh et al., 2015).

In this article we describe a new role of USP37 controlling Cdt1 and DNA replication. USP37 overexpression increases Cdt1 protein levels and knock down of this deubiquitinating enzyme (DUB) leads to lower Cdt1 levels. Importantly, USP37 depletion impacts on the loading of the MCM2-7 replication helicase and on the replication fork speed demonstrating its critical role in regulating DNA replication.

2. Materials and methods

2.1. Cell lines and plasmids

U2OS and 293T cells were grown using standard procedures.

The Addgene expression plasmid #22602 for Flag-HA-USP37 was obtained from JW Harper (Harvard Medical School, Boston, USA) (Sowa et al., 2009). A catalytic inactive version of USP37 was obtained by changing the Cysteine 350 to Serine into the Flag-HA-USP37 expressing plasmid, using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). The same kit was used to generate an siRNA#1 resistant version of the Flag-HA-USP37 by introducing the following 4 silent mutations (shown in lower case) in the cDNA: CAGCTgtcTCAcAA-CATT. Cdt1 cDNA was cloned into the pEXPR-IBA103 (Novagen) vector to obtain a Strep-Cdt1 expressing plasmid. An expression plasmid for His-Ubiquitin was a gift from D. Bohmann (Rochester, New York, USA) (Salghetti et al., 1999).

2.2. Cell synchronizations

Cells in G1 or mitosis were synchronized with a single thymidine block (2.5 mM thymidine for 24 h) and release. Also to

enrich cells in G1 we incubated with 10 μM Lovastatin (Cayman) for 20 h. After 8 h, 0.1 μg/ml nocodazole (Sigma–Aldrich) was added for 16 h. Mitotic cells were then isolated by shake off and lysed (mitotic cells) or nocodazole was washed off and cells were replated in fresh medium for 5 h (G1 cells). For synchronization at G1/S, a single thymidine block was used. To obtain S and G2 cells, thymidine was subsequently washed off and cells were collected after 4 and 12 h, respectively.

2.3. Antibodies and reagents

Antibodies obtained from commercial sources were: anti-β-actin (clone AC-15, Sigma–Aldrich), anti-USP37 (ab190184), anti-Cdt1 (ab70829) and anti-Kap1 (ab190178) antibodies from Abcam, anti-γ-H2AX (Clone JBW301) and anti-ubiquitinylated proteins (clone FK2, #04-263) from Merck-Millipore, anti-Chk1 (G-4), anti-Cdt1 (H-300), anti-RRM2 (N-8) and anti-GAPDH (FL-335) from Santa Cruz Biotechnology, anti-pSer317-Chk1 from R&D, anti-pSer10 histone H3 and anti-Flag from Genscript and anti-RRM1 and mouse anti-Ubiquitin from Cell Signaling. The anti-Cdt1 antibody was raised against amino acids 1–230 of human Cdt1. Anti-MCM7 and anti-Rad9 were previously described (Méndez and Stillman, 2000; Toueille et al., 2004).

Thymidine and nocodazole were purchased from Sigma Aldrich and MG132 (used at 5 μM for 16 h or 6 h at 20 μM) from Calbiochem. Lambda phosphatase was purchased from New England Biolabs.

2.4. Cell transfections

Transfections of plasmids were performed using standard calcium phosphate method (Pérez-Castro and Freire, 2012). For downregulation, siRNA oligos were transfected using Lipofectamine RNAiMAX (Thermo Fisher) according to the manufacturers instructions. The following siRNA oligos were used (Thermo Fisher and GenePharma):

Luciferase	UCGAAGUAUUCCGCGUACGdTdT
USP37#1	CAGCUAAGUCAUAACAUUAdTdT
USP37#2	CCAAGGAUAUUUCAGCUAAdTdT
USP37#3	GAAUAAAGUCAGCCUAGUAdTdT
Cdt1	AACGUGGAUGAAGUACCCGACdTdT

2.5. Immunoprecipitations and ubiquitin assays

Immunoprecipitations were carried out with anti-Flag M2-agarose (Sigma–Aldrich) or with Cdt1/control antibody crosslinked to protein A sepharose CL-4b (GE Healthcare) as previously described (Pérez-Castro and Freire, 2012). His-Ubiquitin pull downs were carried out using Nickel-NTA agarose (Qiagen) as described before (Mamely et al., 2006).

2.6. Chromatin fractionation

Biochemical fractionation of cells was performed as previously described (Méndez and Stillman, 2000; Smits et al., 2006).

2.7. Flow cytometry

Cells were collected by trypsinization and fixed in 70% ethanol at 4 °C for minimal 2 h. After fixation, cells were washed with PBS and the DNA was stained with propidium iodide. For BrdU staining, cells were incubated with BrdU 10 μM for 30 min. After fixation, cells were washed with 0.5% PBS-T (0.5% Tween-20 in PBS) and then incubated in denaturing solution (0.5% Triton X-100, 2 M HCl) for 30 min at 37 °C. Then cells were neutralized with 1M Tris–HCl pH 7.5. After washing with PBS, cells were incubated with anti-BrdU antibody (GenScript) in BSA-T-PBS (1% BSA, 0.5% Tween 20 in PBS) for 16 h at 4 °C. After washing with BSA-T-PBS, cells were incubated with Alexa 647 secondary antibody (Life Technologies) followed by staining with 25 μg/ml propidium iodide. The samples were analyzed using a MACSQuant Analyzer flow cytometer using MACSQuantify software (Miltenyi Biotec).

2.8. DNA fiber analysis

Exponentially growing cells were pulse-labeled with 50 uM CldU (20 min) followed by 250 uM IdU (20 min). Labeled cells were collected and DNA fibers were spread in buffer containing 0.5% SDS, 200 mM Tris pH 7.4 and 50 mM EDTA. For immunodetection of labeled tracks, fibers were incubated with primary antibodies (for CldU, rat anti-BrdU; for IdU, mouse anti-BrdU) and developed with the corresponding secondary antibodies conjugated to Alexa dyes. Mouse anti-ssDNA antibody was used to assess fiber integrity. Slides were examined with a Leica DM6000 B microscope, as described previously (Mourón et al., 2013). The conversion factor used was 1 μm = 2.59 kb (Jackson and Pombo, 1998). In each assay, at least 200 tracks were measured to estimate fork rate and around 300 tracks were analyzed to estimate the frequency of origin firing (first label origins – green–red–green – are shown as percentage of all red – CldU – labeled tracks) (Petermann et al., 2010).

2.9. Quantitative real-time RT-PCR

Total RNA was isolated from cells by the Chomczynski method (Chomczynski and Sacchi, 1987). The purity and concentration of RNA was determined by Nano-drop 2000 (Thermo-Fisher). The quantification of relative abundance of Cdt1 mRNA was carried out using quantitative PCR and the SYBR green detection method. Total RNA was reverse transcribed using a cDNA synthesis kit (Promega, Madison, WI), following the manufacturers instructions, and the Cdt1 cDNA was PCR amplified using gene-specific primers. The oligonucleotides used were: 5′ TAATCTGACCTCCTGGTGCC 3′ (forward primer), and 5′ GTAGGCGTTTTGAGGAGTGC 3′ (reverse primer). The resulting increase in fluorescence during the PCR reaction was detected in the iQ5 system (Bio-Rad,

Hercules, CA) and gene expression was normalized with the reference gen GADP. The data were analyzed using 2 (-Delta Delta C(T)) Method (Livak and Schmittgen, 2001).

3. Results and discussion

3.1. *USP37 regulates Cdt1 protein levels*

Cdt1 levels are regulated by ubiquitin-dependent proteasomal degradation during the cell cycle and after DNA damage. Since so far no enzymes were described to regulate Cdt1 deubiquitination, we carried out an overexpression screen with a library of human ubiquitin and ubiquitin-like hydrolases. We first raised an anti-Cdt1 antibody and the specificity of this antibody was checked using extracts of Cdt1 protein-depleted cells and cells overexpressing a Flag-tagged version of Cdt1 (Supplemental Figure 1A and B). The antibody recognized a specific signal both in 293T and U2OS cells. The screen was then carried out with a collection of 78 different hydrolases, mainly ubiquitin hydrolases but also Sumo hydrolases in both control conditions and after DNA damage (30 min after UV light) and Cdt1 protein levels were studied by Western blot. We expected the overexpressed hydrolase enzyme targeting Cdt1 to decrease ubiquitination and therefore to increase Cdt1 protein levels. The result of the full screen is shown in Supplemental Figures 2 and 3. Surprisingly, overexpression of only one DUB in our library, USP37, was able to increase levels of Cdt1 (Supplemental Figure 3 and Figure 1A). The effect of Cdt1 upregulation after USP37 overexpression was observed under conditions of DNA damage. In unperturbed cells, Cdt1 expression levels similar to the control were observed for all deubiquitinases tested. No significant changes in Cdt1 mRNA levels were observed after USP37 overexpression, indicating that the increase of Cdt1 was due to changes at protein level only (Figure 1B). A more detailed study shows that levels of Cdt1 were upregulated after USP37 overexpression also basal conditions and at different times after DNA damage (Figure 1C). Since cell cycle progression could be affected by USP37 overexpression and Cdt1 levels change during the cell cycle, the experiment was repeated in cells synchronized in S phase. Figure 1D shows an USP37-dependent stabilization of Cdt1 in cells with similar DNA profiles, excluding the possibility of an indirect cell cycle effect. In this experiment also the levels of the RRM1 and RRM2 subunits of the ribonucleotide reductase and phosphorylated H2AX (γH2AX) were analyzed, as changes in these proteins might be indicative of anomalous S phase progression. However, no significant differences were found after USP37 overexpression (Figure 1D). Moreover, USP37 overexpression did not affect cell cycle progression judged by BrdU/PI flow cytometry analysis (Figure 1E). If the overexpression of USP37 increases Cdt1 protein levels by affecting its ubiquitination- and proteasomal-dependent degradation, lack of USP37 should have the opposite consequence. Depletion of USP37 by siRNA indeed resulted in decreased Cdt1 protein levels in basal conditions, as shown in Figure 1F. Nevertheless, the Cdt1 degradation kinetics after DNA damage is similar in Luc and USP37 siRNA treated cells, indicating that only the overexpression of the USP37 can stabilize Cdt1 after DNA damage. Also, overexpression of an siRNA resistant version of Flag-USP37 rescued the drop in Cdt1 levels caused by USP37 depletion (Supplemental Figure 4A).

Western blot analysis using the Cdt1 antibody showed two Cdt1 bands in most of the experiments, of which predominantly the upper band becomes stabilized after USP37 overexpression. We therefore pursued to characterize the nature of the upper band. Our antibody was raised against the N-terminus of Cdt1. To explore if the upper band was a Cdt1 splice variant, two other commercial antibodies were used that recognize the Cdt1 C-terminus. As shown in Figure 2A, the three antibodies detected both Cdt1 bands, suggesting that the upper band is a modified version of Cdt1 rather than a splice form. Since USP37 catalytic activity removes ubiquitin and Cdt1 is target for degradation by the proteasome, we reasoned that the upper band might be an unstable form of Cdt1. Indeed, addition of the proteasome inhibitor MG132 increases the level of the band that moves with lower mobility (Figure 2B) and interestingly, USP37 knock down decreased the upper band under such conditions indicating that even in the presence of MG132, the lack of USP37 destabilizes the Cdt1 upper band. The experiment was repeated with 3 different USP37 siRNA oligos with a similar outcome, indicating that the observed decrease in Cdt1 stability was due to USP37 depletion and not the result of an off target effect (Figure 2C). Next, to further investigate the nature of the unstable upper band, the possibility of an (mono-)ubiquitin modification was tested. For that, endogenous Cdt1 was immunoprecipitated from USP37 overexpressing cells. Even though both forms of Cdt1 were efficiently immunoprecipitated (Supplemental Figure 4B and Figure 2D), no signal was observed after probing the immunoprecipitate with anti-ubiquitin or conjugated ubiquitin antibodies (Figure 2D). This result argues against the Cdt1 slow mobility being the result of ubiquitination. Then the extracts were treated with lambda phosphatase, to examine possible phosphorylation of Cdt1. Rad9, 45 kDa in unphosphorylated form, but up to 60 kDa when phosphorylated (St Onge et al., 2001, 1999; Volkmer and Karnitz, 1999), was used as a positive control for lambda phosphatase activity (Figure 2E). Treating the extracts with phosphatase resulted in a reduction of the Cdt1 upper band that is induced by USP37 overexpression (Figure 2E). As an additional approach, Cdt1 was immunoprecipitated from USP37 overexpressing cells and the purified immunoprecipitates were treated with lambda phosphatase. Again an increase of the low mobility band was observed after USP37 overexpression. This band disappeared completely after phosphatase treatment (Figure 2F). These experiments strongly suggest that the Cdt1 mobility shift is due to phosphorylation. Instability of a low mobility, phosphorylated form of Cdt1 was also described by others and an additional study showed that this Cdt1 phosphorylation is dependent on CDKs. This phosphorylated form of Cdt1 interacts with the SCF-Skp2 complex that targets Cdt1 for ubiquitination (Li et al., 2003; Liu et al., 2004). Therefore, is likely that USP37 counteracts this ubiquitination and degradation by binding with higher affinity.

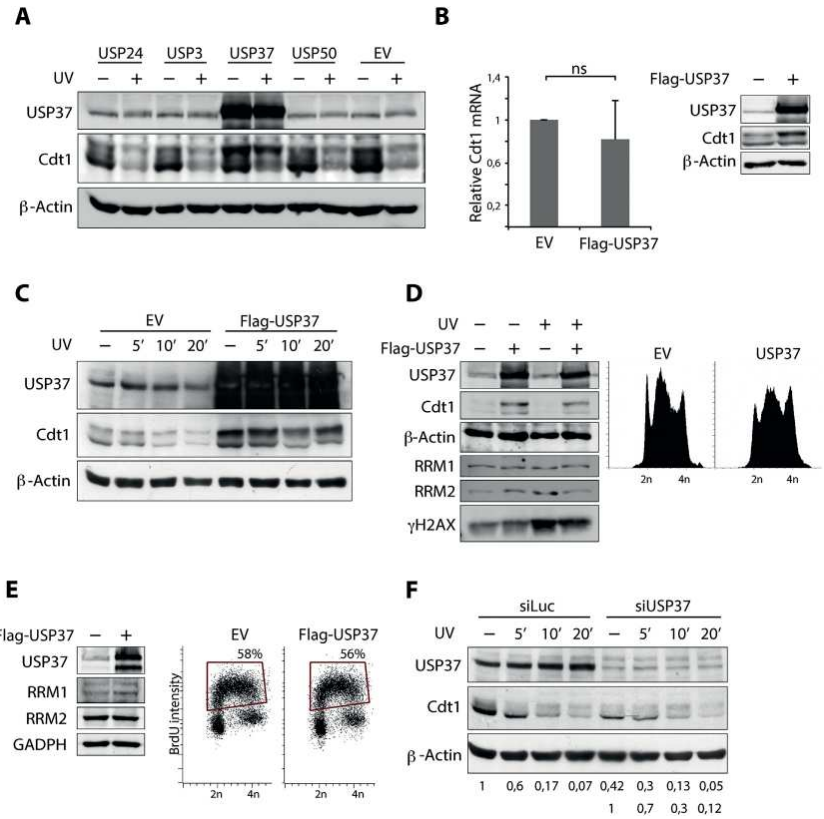

Figure 1 – USP37 controls Cdt1 levels. (A) 293T cells were transfected with an empty vector (EV) or expression plasmids for the indicated DUBs and when indicated treated with UV light (40 J/m^2) and collected 30 min later. Cell lysates were analyzed by Western blot with the indicated antibodies. (B) 293T cells transfected with EV or Flag-USP37 were lysed for analysis of mRNA levels by real-time PCR or by Western blot with the indicated antibodies. The graph shows the average Cdt1 mRNA levels of 3 independent experiments. (C) 293T cells were transfected with EV control or Flag-USP37. Then, cells were left untreated or treated with 40 J/m^2 UV light before collecting them at the indicated times (min). Extracts were analyzed by Western blot. (D) 293T cells transfected with Flag-USP37 were incubated with thymidine for 24 h and released in fresh medium for 4 h. Then cells were left untreated or treated with UV light (40 J/m^2) and collected 30 min post-treatment before Western blot (left) or by flow cytometry (propidium iodide) analysis. (E) 293T cells were transfected with Flag-USP37 or empty vectors and were labeled with BrdU (30 min) for FACS analysis. The percentage of BrdU positive cells is indicated. (F) U2OS cells were transfected with the indicated siRNA oligos and left untreated or UV irradiated (40 J/m^2) before collection at the indicated times and analysis by Western blot. Quantification of Cdt1 levels compared to the loading control β-actin is shown at the bottom. In this quantification, Cdt1 levels in all samples were compared to those in siLuc cells without damage (set as 1, upper row). Also, samples of USP37 depleted cells were compared to the undamaged control (set to 1, bottom row).

3.2. *USP37 de-ubiquitinates Cdt1*

Next, to examine if USP37 was directly affecting Cdt1 levels, the interaction between the two proteins was analyzed by immunoprecipitation. Endogenous Cdt1 co-immunoprecipitated with overexpressed Flag-USP37 (Figure 3A). Although overexpression of USP37 stabilizes Cdt1 after DNA damage, Figure 3B shows that the interaction between the two proteins did not significantly change under these conditions, suggesting that Cdt1 stability after UV light is not due to changes in affinity towards USP37. Interestingly, Cdt1 upper band is enriched in the immunoprecipitations, indicating USP37 regulation preferentially over that isoform.

The interaction data suggest that USP37 directly affects Cdt1 ubiquitination. We therefore tested if the ubiquitination status of Cdt1 is affected by USP37 overexpression. As a control a catalytic inactive version of USP37 (Cys350Ser) was used. After expression of His-Ubiquitin in 293T cells and performing a pull down for His containing proteins, ubiquitinated forms of Cdt1 were detected (Figure 3C, second lane). Ubiquitinated forms of Cdt1 notably decreased upon co-expression of wild type USP37. In contrast, Cdt1 ubiquitination increased

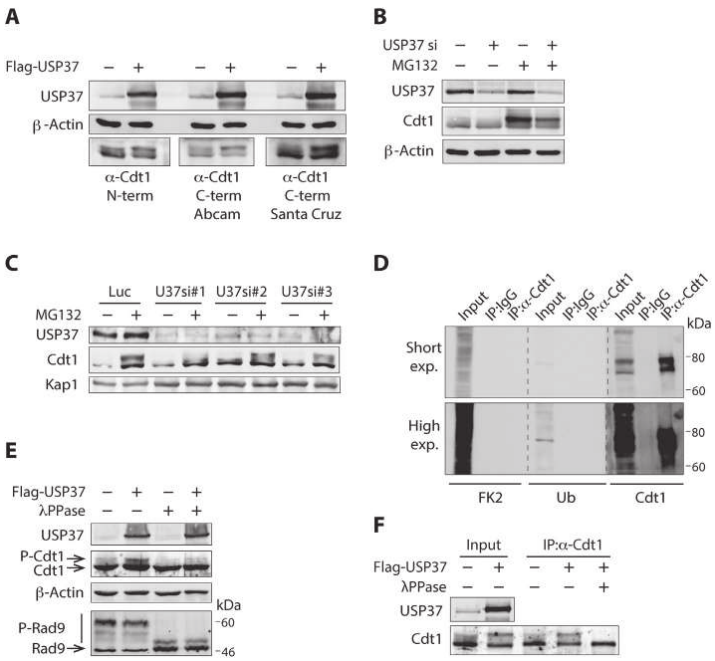

Figure 2 – USP37 stabilizes a phosphorylated form of Cdt1. (A) 293T cells transfected with empty or Flag-USP37 vector were lysed and loaded three times in the same gel for parallel Western blot analysis with three different Cdt1 antibodies. The anti-Cdt1 immunoblot intensities were different and comparable exposures in each case are shown. (B) 293T cells were transfected with control or USP37 siRNA oligos and left untreated or treated with MG132 for 6 h when indicated. Cell lysates were analyzed with the indicated antibodies. (C) The same as (B), but using U2OS cells. (D) Endogenous Cdt1 was immunoprecipitated with the anti-Cdt1 antibody from lysates of 293T cells overexpressing USP37. Input and immunoprecipitates were analyzed by Western blot with the indicated antibodies. Two different exposures of the same blot are shown. (E) 293T cells transfected with empty or Flag-USP37 overexpression vectors were lysed. Extracts were treated or not with lambda phosphatase for 60 min prior analysis by Western blotting with the indicated antibodies. (F) 293T cells overexpressing Flag-HA-USP37 were lysed and anti-Cdt1 immunoprecipitations were carried out. The indicated immunoprecipitates were incubated with lambda phosphatase for 60 min before analysis by Western blot with the indicated antibodies.

in the presence of Cys350Ser USP37, suggesting that overexpression of the catalytic inactive protein antagonizes the endogenous wild type USP37. Altogether these data strongly indicate that Cdt1 is a target substrate for USP37.

3.3. *USP37 regulates Cdt1 during G1 and G1/S*

To understand the biological significance of the Cdt1 regulation by USP37, first the levels of both proteins during the cell cycle were examined. It is documented that during the cell cycle, both proteins are controlled by ubiquitin-mediated proteasomal degradation, but in a distinct way. While Cdt1 levels are starting to raise during mitosis, continue high in G1 and decrease in S and G2 phases, USP37 levels are low in G1 and increase in S and G2 phases (Huang et al., 2011; Nishitani et al., 2000). Figure 4A confirms that USP37 levels were mainly high at the G1/S transition and in S and G2, in agreement to its described role to promote S phase entry (Huang et al., 2011). In contrast, Cdt1 protein levels were high in G1 and mitosis and low in S/G2 phases. Cdt1 damage-specific degradation occurred during all stages of cell cycle except mitosis, similar to recently reported (Morino et al., 2015). Interestingly, after UV irradiation in S and G2 phases USP37 levels showed a slight decrease and also a mobility shift, suggesting a DNA damage-induced posttranslational modification specifically in these phases. Importantly, USP37 protein was low but detectable during the G1 phase and likewise, Cdt1 levels were low but detectable during S/G2 (Figure 4A). We therefore questioned during what phase of cell cycle USP37 regulates Cdt1. Cells were synchronized in different stages of the cell cycle and at the same time USP37 was depleted by siRNA. Interestingly, knock down of USP37 reduced the levels of Cdt1 in G1 and G1/S but not during S phase, indicating that although USP37 levels are low in G1, this DUB still functions to stabilize Cdt1 at this moment of the cell cycle (Figure 4B). USP37 depletion clearly affected the levels of the lower Cdt1 band, whereas the upper band is not detected here. This adds proof to our hypothesis that the upper phosphorylated band of Cdt1 is an unstable form of Cdt1.

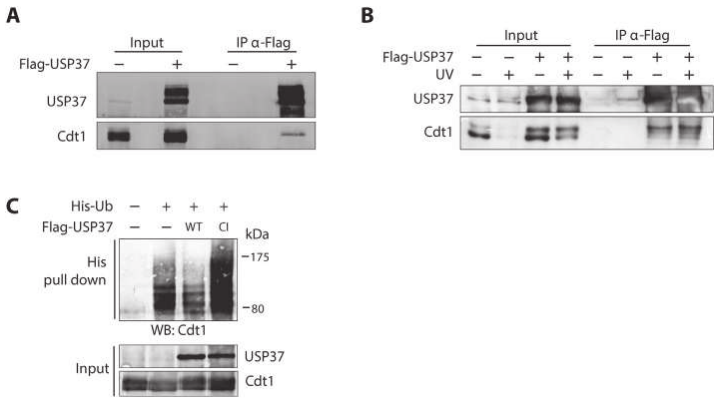

Figure 3 – USP37 interacts with and deubiquitinates Cdt1. (A) 293T cells transfected with an empty vector or Flag-USP37 were lysed and immunoprecipitations were carried out with anti-Flag beads. Immunoprecipitates were analyzed by Western blot with anti-Cdt1 and anti-USP37 antibodies. (B) As in (A), but when indicated, cells were UV irradiated (40 J/m^2, 1 h). (C) 293T cells were transfected with a plasmid expressing Strep-Cdt1 together with control or His-Ubiquitin expression plasmids, and wild type or catalytic inactive Flag-USP37. 20 h after transfection, cells were incubated with MG132 for 16 h before lysis under denaturing conditions. Western blot analysis of input and His-pull downs were carried out with the indicated antibodies.

3.4. *USP37 regulates DNA replication at different levels*

Since the role of USP37 was linked to the transition from G1 to S phase, but not much was known about its role during DNA replication, replication fork speed was measured accurately by DNA fiber analysis. As shown in Figure 5A, replication fork progression rate was significantly delayed in USP37-depleted cells as compared to control cells and consequently

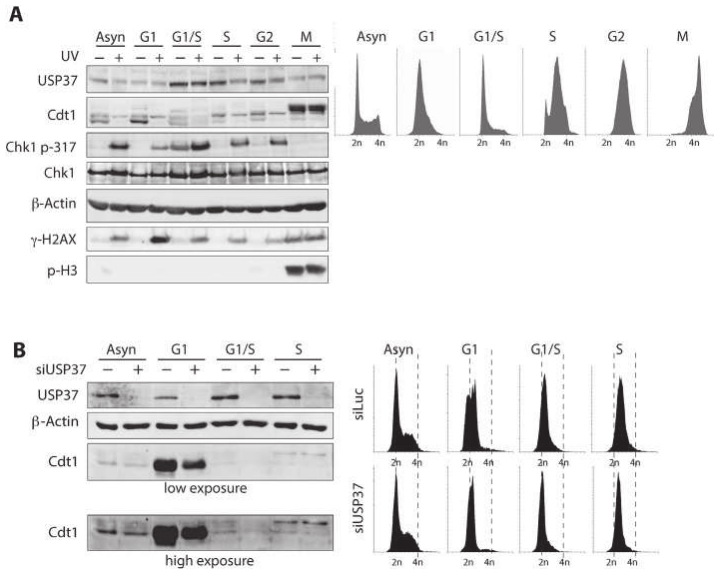

Figure 4 – USP37 controls Cdt1 levels during G1 and G1/S. (A) U2OS were left asynchronous or synchronized using different protocols as described in Materials and Methods. When indicated, 30 min before collecting, cells were treated with 40 J/m^2 UV light. Then samples were collected for propidium iodide analysis by flow cytometry (right) or Western blot with the indicated antibodies (left). (B) U2OS were transfected with control or USP37 siRNA oligos and synchronized at the same time as described. A fraction of cells was lysed for Western blot analysis with the indicated antibodies (left) and another fraction of cells was collected for flow cytometry analysis after propidium iodide staining (right).

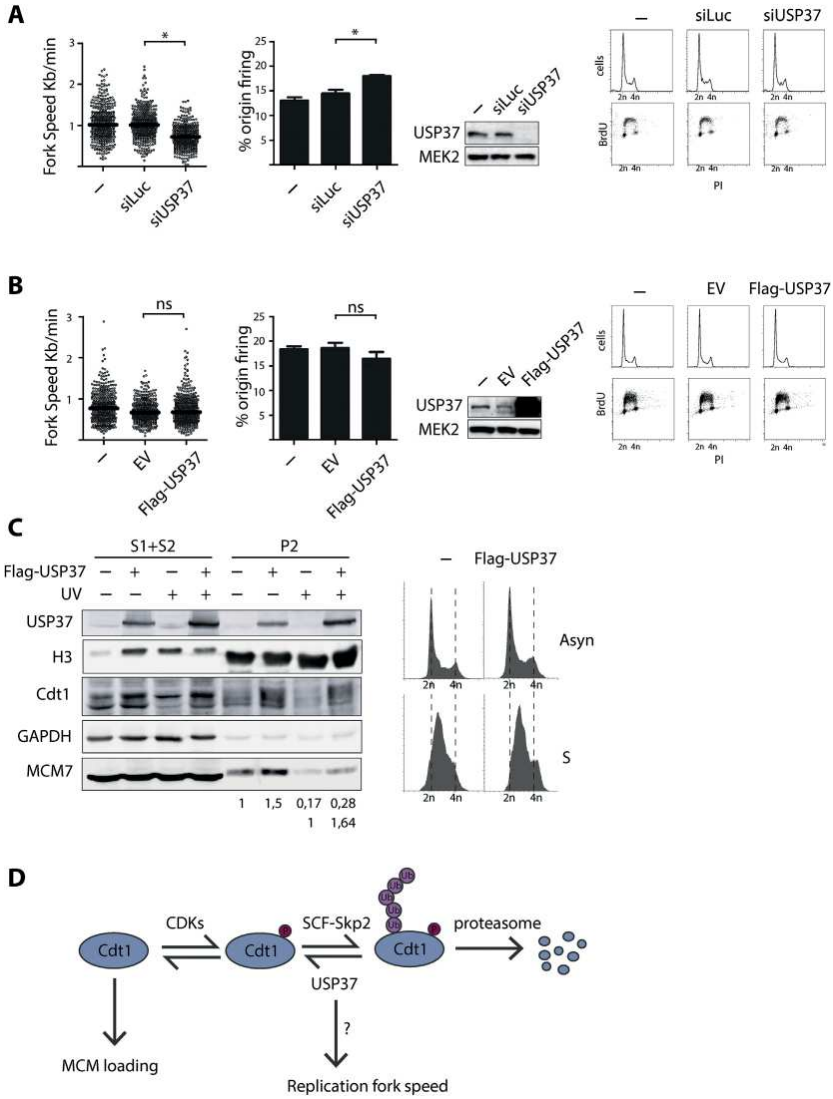

Figure 5 – USP37 controls replication fork speed and impacts on MCM loading. U2OS cells were left untreated or transfected with control or USP37 siRNA oligos (A) or 293T were left untreated or transfected with an empty or Flag-USP37 expressing plasmid (B). 48 h after transfections cells were labeled with nucleotide analogues as described in materials and methods. Cells were then collected for DNA fiber analysis, for Western blotting with the indicated antibodies and for flow cytometry analysis (BrdU/PI). Depicted is the replication fork speed and the percentage of origin firing from two different experiments. Horizontal lines represent the median of relative fork speed distribution. Western blots and flow cytometry graphs are shown from a representative experiment. (C) 293T cells transfected with an empty vector or Flag-USP37 expressing plasmid were synchronized by thymidine block and collected 4 h after thymidine release for flow cytometry analysis or for biochemical fractionation and Western blot analysis with the indicated antibodies. S1 + S2 represent soluble proteins, P2 the chromatin fraction. Quantification of the MCM7 levels in the chromatin fraction compared to the levels of histone H3 is represented. In the first row the MCM7 levels are compared to EV without damage, the second row compares −/+ Flag-USP37 expression in UV-treated cells. (D) Model summarizing previously known data and the new data from this article. USP37 deubiquitinates a phosphorylated, unstable form of Cdt1. USP37 also delays replication fork speed by a mechanism likely independent of Cdt1.

an increase of the percentage of replication firing was observed under these conditions. These data strongly suggest that USP37 plays an active role in replication fork progression. It is tempting to speculate that USP37 could stabilize one of the important proteins at the replication fork, and since there was no reduction of the percentage of replication origins firing, this effect is unlikely to be due to controlling Cdt1 directly. As shown in Figure 1D and E, this effect is also not the result of a reduced availability of nucleotides by controlling the levels of the subunits RRM1 or RRM2 of the ribonucleotide reductase. As USP37 was described to control Cyclin A, known regulator of DNA replication, this Cyclin is a candidate target for the decrease on replication fork speed after USP37 depletion (Huang et al., 2011).

However, USP37 overexpression did not lead to a significant change in the replicative parameters compared to control conditions (Figure 5B). Nevertheless, we continued to study the role of USP37 in modification of Cdt1 particularly, and the effect of USP37 overexpression on the loading of the MCM2-7 complex, the major function of Cdt1 in initiation of DNA replication, was examined. To avoid any undesired cell cycle effects, cells synchronized in early S phase were used. Notably, USP37 overexpression increased MCM7 loading onto the insoluble nuclear fraction (P2; chromatin) of the chromatin fractionation (Figure 5C). This effect was also observed in UV-irradiated S phase cells (Figure 5C), strongly suggesting that USP37 can regulate the initiation of DNA replication. Similar experiments were performed after USP37 depletion, resulting in no changes on MCM7 loading, suggesting that the there is an excess of Cdt1 to achieve the loading. Another possibility to explain the uploading of MCM7 after USP37 overexpression is a direct change in the ubiquitination status of MCM7 by USP37. However, *in vivo* ubiquitination experiments did not show changes in MCM7 ubiquitination status after USP37 overexpression (Supplemental Figure 4C). Interestingly, similar to others, an increase on the ubiquitination status of the USP37 catalytic inactive version comparing to the wild type UPS37 was observed, suggesting that USP37 could auto-deubiquitinate (Tanno et al., 2014).

The fact that overexpression of USP37 did not change replication parameters (Figure 5B) or cell cycle profiles (Figures 1E and 5B) in spite of increasing MCM7 loading suggest that the other known mechanisms controlling Cdt1/initiation of replication are able to maintain replication levels normal after even when Cdt1 protein is upregulated. Future work will investigate this into detail. Similarly, the increase of MCM7 loading after UV treatment might not have an effect on replication parameters, as recently it was shown that a non-degradable version of Cdt1 does not induce additional DNA synthesis after DNA damage (Tsanov et al., 2014).

Altogether, we show that USP37 is a DUB that modifies Cdt1, a central protein in DNA replication initiation, by stabilizing mainly a low mobility form that is likely a phosphorylated form of Cdt1 (Figure 5D). Our data also demonstrate that USP37 controls DNA replication fork speed (Figure 5D) and it is expected that this effect on DNA fork speed is by controlling different target proteins than Cdt1, as Cdt1 itself is not predicted to have a major role in replication fork speed regulation. This adds USP37 to USP7 and USP29, recently demonstrated by us and others, as other important DUBs in DNA replication (Jagannathan et al., 2014; Martín et al., 2015).

Further studies are needed to establish the details of regulation of USP37 itself, although our data indicate that this protein is actively controlling Cdt1 function during G1 and S phases. It is possible that the USP37 mobility change after UV light, shown in Figure 3, reflects changes in its regulation. The fact that USP37 is involved in DNA replication, and particularly in the control of the protein levels of Cdt1, a potential oncogenic protein, makes it a putative therapeutic target in cancer treatment. USP37 was shown to also stabilize other known oncogenes like c-Myc or Cyclin A, strengthening the relevance of USP37 inhibition in cancer therapy (Huang et al., 2011; Pan et al., 2014).

3.5. *Conclusions*

We identified a new regulator of DNA replication: USP37. USP37 stabilizes the licensing factor Cdt1 and also plays a further role in the progression of DNA replication.

Acknowledgments

The authors are grateful to V. Smits for careful reading of the manuscript. This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (SAF2013-49149-R, BFU2014-51672-REDC), Instituto de Salud Carlos III (BA15/00092) and Fundación CajaCanarias (AP2015/008) to RF.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.05.008.

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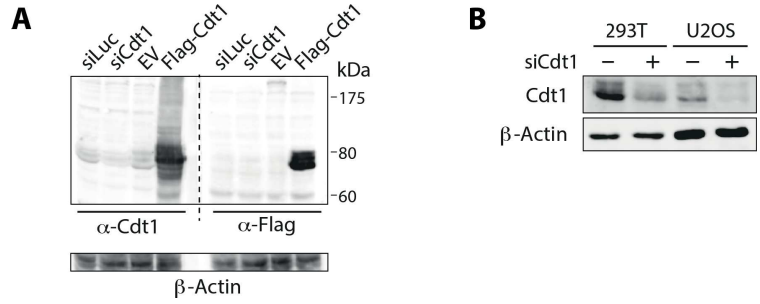
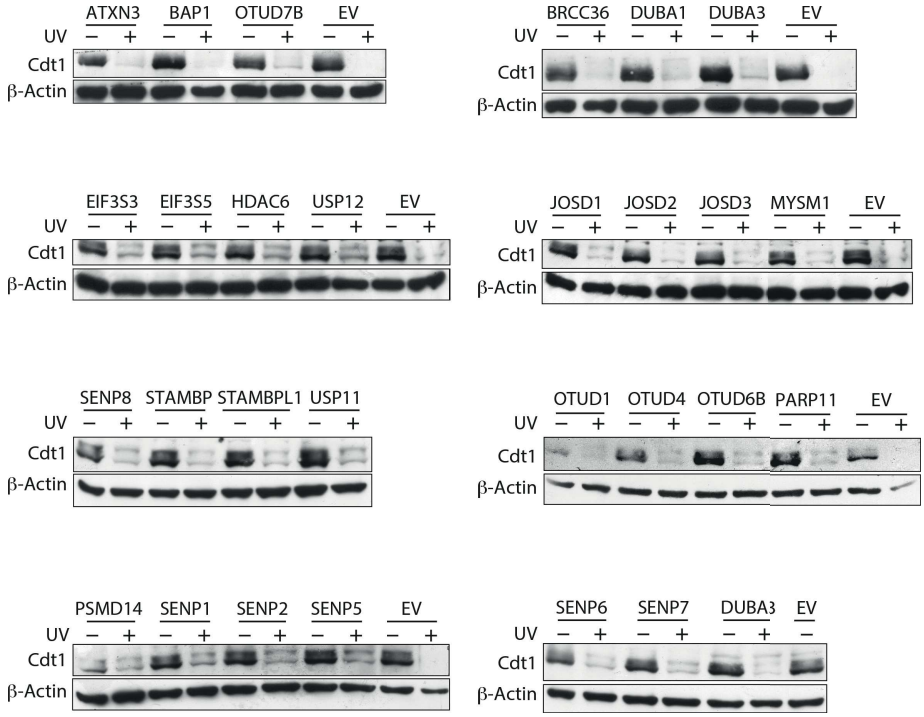
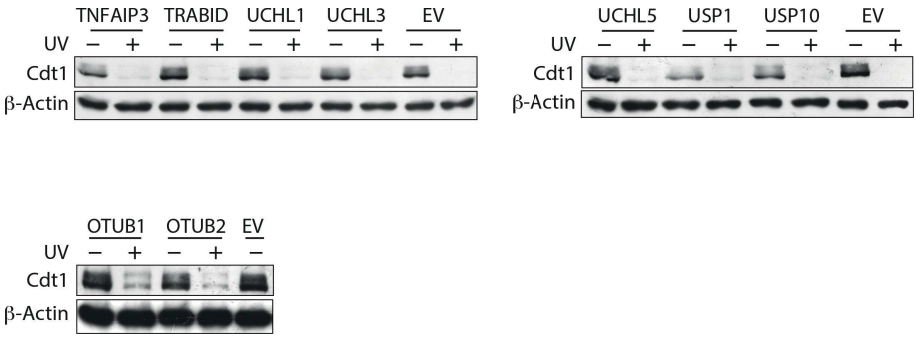
SUPPLEMENTARY FIGURES

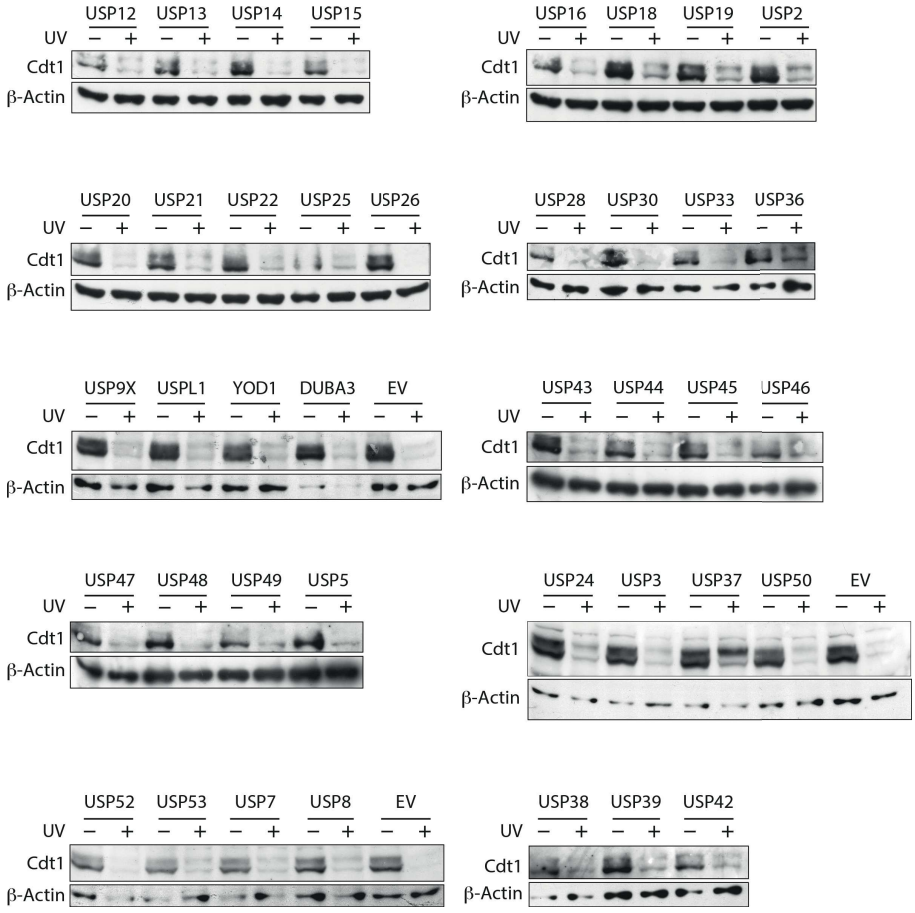


Supplemental Figure 1 Homemade anti-Cdt1 antibody specifically recognizes human Cdt1. **(A).** 293T cells were transfected with control or Cdt1 siRNA oligos or with empty vector (EV) or a vector expressing Flag-Cdt1. Duplicate samples were ran on a gel and Western blot analysis was carried out using the anti-Cdt1 homemade antibody and anti-Flag as indicated. Dotted line indicates where the membrane was cut to incubate with the different antibodies. **(B)**. 293T and U2OS cells were transfected with control or Cdt1 siRNAs and extracts were made for Western blot analysis with the indicated antibodies.

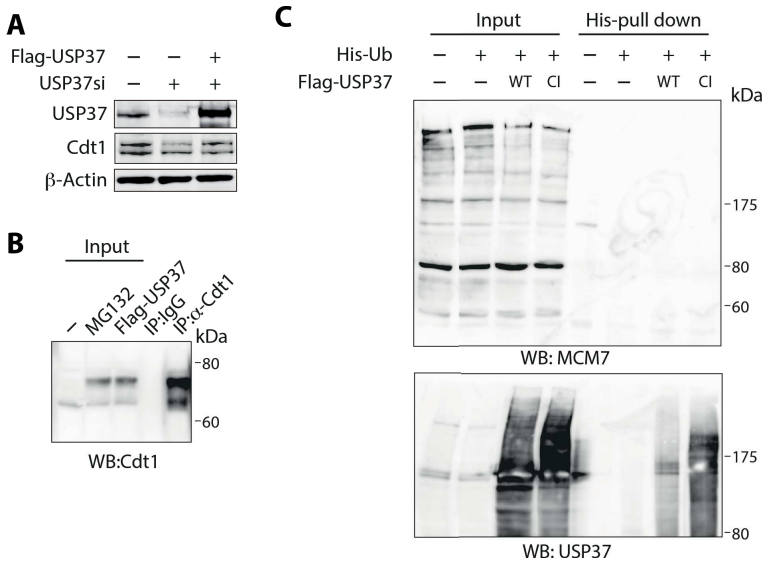




Supplemental Figure 2. No change in Cdt1 levels after overexpression of many ubiquitin or ubiquitin-like hydrolases. 293T cells were transfected with expression plasmids for the indicated hydrolases (or empty vector) and were left untreated or UV irradiated (40 J/m^2). Cells were lysed 30 min after treatment and analyzed by Western blot with anti-Cdt1 antibodies and β-actin as loading control. The expression plasmids for (tagged) ubiquitin or ubiquitin-like hydrolases were kindly provided by several collaborating laboratories.



Supplemental Figure 3. Increased Cdt1 levels after USP37 overexpression. 293T cells were transfected, lysed and analyzed as in Supplemental Figure 2.



Supplemental Figure 4. Flag-USP37 overexpression rescues Cdt1 levels in USP37 depleted cells and does not affect MCM7 ubiquitination. **(A)** 293T cells were transfected 2 times with siRNA against Luc or USP37#1 and with siRNA resistant Flag-USP37 and were treated with thymidine 24 h, released for 6 h and incubated with Lovastatin 10 μM for extra 20 h before being analyzed by Western blot with the indicated antibodies **(B)** 293T cells were transfected with Flag-USP37, treated with MG132 for 6 h or left untreated. Immunoprecipitations with control or anti-Cdt1 antibodies were carried out using extracts of the Flag-USP37 expressing cells. All samples were analyzed by Western blot with the anti-Cdt1 antibody. **(C)** 293T cells were transfected when indicated with control, His-Ubiquitin, wild type or catalytic inactive Flag-USP37 plasmids. 20 h after transfection, cells were incubated with MG132 for 16 h before lysis under denaturing conditions. Western blotting analysis of input and His pull downs were performed with the indicated antibodies.

Oncogene (2017), 1–8

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SHORT COMMUNICATION

DUB3 and USP7 de-ubiquitinating enzymes control replication inhibitor Geminin: molecular characterization and associations with breast cancer

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Correct control of DNA replication is crucial to maintain genomic stability in dividing cells. Inappropriate re-licensing of replicated origins is associated with chromosomal instability (CIN), a hallmark of cancer progression that at the same time provides potential opportunities for therapeutic intervention. Geminin is a critical inhibitor of the DNA replication licensing factor Cdt1. To properly achieve its functions, Geminin levels are tightly regulated through the cell cycle by ubiquitin-dependent proteasomal degradation, but the de-ubiquitinating enzymes (DUBs) involved had not been identified. Here we report that DUB3 and USP7 control human Geminin. Overexpression of either DUB3 or USP7 increases Geminin levels through reduced ubiquitination. Conversely, depletion of DUB3 or USP7 reduces Geminin levels, and DUB3 knockdown increases re-replication events, analogous to the effect of Geminin depletion. In exploring potential clinical implications, we found that USP7 and Geminin are strongly correlated in a cohort of invasive breast cancers ($P < 1.01E-08$). As expected, Geminin expression is highly prognostic. Interestingly, we found a non-monotonic relationship between USP7 and breast cancer-specific survival, with both very low or high levels of USP7 associated with poor outcome, independent of estrogen receptor status. Altogether, our data identify DUB3 and USP7 as factors that regulate DNA replication by controlling Geminin protein stability, and suggest that USP7 may be involved in Geminin dysregulation during breast cancer progression.

Oncogene advance online publication, 13 March 2017; doi:10.1038/onc.2017.21

INTRODUCTION

Initiation of DNA replication is highly conserved and tightly regulated in eukaryotes. As accurate genome replication should only occur once per cell division cycle to preserve genome integrity, cells have evolved mechanisms to prevent re-replication events.[1] For instance, replication origin sites need to be 'licensed' only in late mitosis and early G1 phase when the origin recognition complex docks and recruits Cdc6 and Cdt1, followed by the mini-chromosome maintenance MCM2-7 helicase complex.[2,3] This pre-replication complex licenses the chromatin for replication.[2,3] Dysregulation of DNA replication licensing is associated with replication stress, which is one of the emerging causes of chromosomal instability (CIN) in sporadic cancers, including breast cancer.[4] Many oncogenes induce replication stress by directly having an impact on the assembly of pre-replication complexes, origin licensing and origin firing, resulting in replication-related errors and genomic lesions.[5–7]

Control of Cdt1 expression and activity is critical to prevent re-replication and genome instability.[8] Proteolytic control through E3 ubiquitin ligase-mediated proteasomal degradation ensures that Cdt1 levels remain high during mitosis and G1, but decrease sharply during S/G2 phases of cell cycle.[9,10] Any residual Cdt1 present during S/G2 is inhibited by direct interaction with Geminin.[1] Geminin is also tightly controlled throughout the cell cycle by ubiquitin-dependent proteasomal degradation. Steady-state levels are high in S/G2 to suppress Cdt1 activity,[11,12] and the protein is degraded in late mitosis/G1 by the E3 ubiquitin ligase APC/C to facilitate origin licensing and another cell division cycle.[11] Alterations in the Cdt1–Geminin balance can cause replication-associated genome instability, with several groups demonstrating that Geminin deficiency can produce re-replication events, resulting in genomes that are replicated more than once per cell cycle.[13,14] APC/C is known to tag Geminin for degradation,[11] but the de-ubiquitinating enzyme(s) (DUBs) or ubiquitin hydrolase(s) that counteract this step to facilitate precise control of Geminin remain unknown.

In this study, we identified the ubiquitin hydrolases DUB3/USP17L2 and USP7/HAUSP as novel regulators of Geminin stability. DUB3 was recently described as an important regulator of genomic stability through post-translational control of histone H2AX and Cdc25 phosphatase.[15,16] USP7 controls multiple substrates important for accurate cell cycle control, including p53, MDM2 and PTEN.[17–20] Additional work linked USP7 with regulation of replication-associated substrates, such as Claspin, Chk1 and chromatin binding by the MCM2-7 complex.[21–24] Most importantly, USP7 was recently found to be a SUMO de-

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Received 29 July 2016; revised 15 December 2016; accepted 2 January 2017

ubiquitinase associated with replisomes and required for DNA replication fork progression.[20,25]

High expression of Geminin has been linked to genome instability, DNA replication alterations and aneuploidy.[26] Geminin levels are deregulated in human tumors and its expression is prognostic in several cancers, including breast cancer.[27–29] USP7 regulates many proteins linked to genome maintenance, and aberrant expression has been linked to progression of prostate, liver and colon cancers,[30] making USP7 a conceptually attractive antitumor target.[31]

Here we identified DUB3 and USP7 as regulators of Geminin stability. Overexpression of either DUB stabilizes Geminin by increasing its de-ubiquitination. DUB3 knockdown increases the number of re-replicating cells, to a similar extent as Geminin depletion. Notably, we found that USP7 expression levels are linked to Geminin levels in a collection of invasive breast cancers, and the USP7 level has prognostic value for this cancer type. Altogether, these data identify DUB3 and USP7 as regulators of DNA replication by controlling Geminin levels and point to a novel role of USP7 in breast cancer progression.

RESULTS AND DISCUSSION

DUB3 and USP7 control Geminin stability

Levels of the replication inhibitor Geminin are regulated by ubiquitin-dependent proteosomal degradation during the cell cycle, yet the ubiquitin hydrolases responsible for re-establishing Geminin levels have not been described. We reasoned that overexpression of a hydrolase that deubiquitinates Geminin would increase its stability and steady-state levels in the cell. Taking an approach analogous to previous studies, we performed an ectopic DUB expression screen, followed by measuring of Geminin levels by western blot analysis.[15,32] Of the 78 DUBs tested, overexpression of DUB3 and USP7 most distinctly increased Geminin protein levels (Figure 1a). As Geminin levels oscillate during the cell cycle, the changes observed could be due to an indirect effect on the cell cycle. However, cell cycle profiles of DUB3 and USP7-overexpressing cells presented only small changes as compared to the control cells, transfected with empty vector, suggesting a direct effect on Geminin levels (Figure 1a).

Next, changes in Geminin protein stability in DUB3- and USP7-overexpressing cells were studied by treating cells with cycloheximide to block protein synthesis, and assaying Geminin protein decay rates in time. As shown in Figure 1b, overexpression of wild-type (WT) DUB3 increased Geminin stability compared to control cells. Cells overexpressing a DUB3 version mutated in the catalytic site (CI) and unable to de-ubiquitinate were not able to stabilize Geminin, indicating that the increased levels of Geminin are caused by increased protein stability and DUB3 enzymatic activity. Similarly, USP7 WT overexpression also augmented Geminin stability compared to control cells or to cells expressing USP7 with a mutation in the catalytic site (CI) of the protein (Figure 1c). A slower gel mobility of the CI versions of both DUBs as compared to WT was observed. We speculate that in the case of DUB3 this might be related to the three point mutations in the CI version. The higher mobility of USP7 CI has previously been reported to be due to a modification of the protein by ubiquitin. [23]

If ectopic expression of DUB3 and USP7 increases Geminin protein, DUB depletion should have the opposite effect. Indeed, transfection of U2OS cells with DUB3 or USP7 short interfering RNA oligos significantly decreased Geminin levels (Figures 1d and e). As DUB3 antibodies were unable to detect endogenous DUB3, we confirmed the efficacy of our DUB3 short interfering RNA oligo in cells with Flag-DUB3 expression (Figure 1d, right panel).

DUB3 and USP7 de-ubiquitinate Geminin

To investigate whether the effects of DUB3 and USP7 on Geminin are direct, protein–protein interactions were examined by co-immunoprecipitation experiments. WT as well as CI mutant versions of DUB3 and USP7 physically interacted with both ectopically expressed green fluorescent protein (GFP)-Geminin (left panels, Figures 2a and b) and the endogenous protein (right panels, Figures 2a and b). Next, to test whether USP7 and DUB3 affect the ubiquitination status of Geminin, we performed a His-Ubiquitin pull-down assay using cells expressing WT or CI DUBs and GFP-Geminin. The extent of Geminin ubiquitination was significantly lower in cells co-expressing DUB3 WT, but not CI, indicating that DUB3 is able to de-ubiquitinate Geminin *in vivo* (Figure 2c). Similarly, expression of USP7 WT strongly decreased Geminin ubiquitination *in vivo* compared to control cells. However, USP7 CI produced a similar phenotype as USP7 WT (Figure 2d). We speculate that this behavior is associated to a non-enzymatic function of USP7 in cells overexpressing GFP-Geminin and His-Ubiquitin. For example, both WT and CI USP7 could bind to exogenous Geminin, and thereby avoid its ubiquitination. In fact, USP7 is able to regulate cellular processes independently of its catalytic activity.[24]

Finally, in order to assess whether both DUBs directly de-ubiquitinating Geminin, we performed an *in vitro* (de)ubiquitination assay with purified ubiquitin hydrolases and ubiquitinated Geminin. Interestingly, DUB3 is able to remove ubiquitin from Geminin *in vitro* as judged by the western blot analysis for conjugated ubiquitin (FK2). However, we were unable to show Geminin de-ubiquitination *in vitro* by USP7, showing that either the purified protein is not fully active in this experimental setup and/or that USP7 regulates Geminin ubiquitination and stability via a different mechanism. Altogether, these data indicate that Geminin is a target substrate of DUB3 and that USP7 contributes to the control of Geminin stability.

DUB3 depletion increases re-replication events

The functional relevance of DUB3- and USP7-related de-ubiquitination of Geminin was subsequently examined. Geminin is critical for maintaining DNA replication fidelity, and previous studies have shown that its insufficiency increases polyploidy (cells with >4N DNA content), resulting at least partly from re-replication events.[13,14] Consistent with these reports, we found that short interfering RNA-mediated knockdown of Geminin in U2OS cells increased the >4N subpopulation that are actively replicating (5-bromodeoxyuridine-positive; Figure 3a). Notably, DUB3 depletion resulted in a comparable increase in the percentage of re-replicating cells (>4N DNA content, 5-bromodeoxyuridine-positive). This strongly indicates that DUB3 controls re-replication, most likely by regulating Geminin (Figure 3a). DUB3 downregulation also induced a S/G2 phase arrest, possibly related to its role in controlling the cell cycle activator Cdc25A.[16] Depleting USP7 resulted in strong inhibition of S phase and a G1 arrest, consistent with recent reports,[20,25] and therefore, the assay was not appropriate for measuring effects of USP7 on re-replication (Figure 3a).

To investigate whether control of Geminin protein levels by DUB3 and USP7 is a general phenomenon, we studied the effects observed in the breast-derived non-tumorigenic cell line MCF10A and the breast tumor cell line MCF7. Interestingly, depletion of DUB3 or USP7 also decreased Geminin levels in both these cell lines. USP7 downregulation also decreased the number of replicating (5-bromodeoxyuridine-positive) cells in these breast-derived cell lines (Figures 3b and c), similar to the observation in U2OS. In addition, like in U2OS, knockdown of USP7 did not affect re-replication in MCF10A or MCF7 cells (Figures 3b and c), likely due to the lack of replicating cells. The effect of DUB3 depletion on re-replication is different though. Whereas knocking down

DUB3 increased the amount of re-replicating cells in the transformed MCF7 cells (Figure 3c), DUB3 depletion did not affect re-replication in the non-tumorigenic MCF10A (Figure 3b). This might be due to the fact that after depleting DUB3 in MCF10A, less replicating (5-bromodeoxyuridine-positive cells) are present, which is incompatible with measuring re-replication. Alternatively,

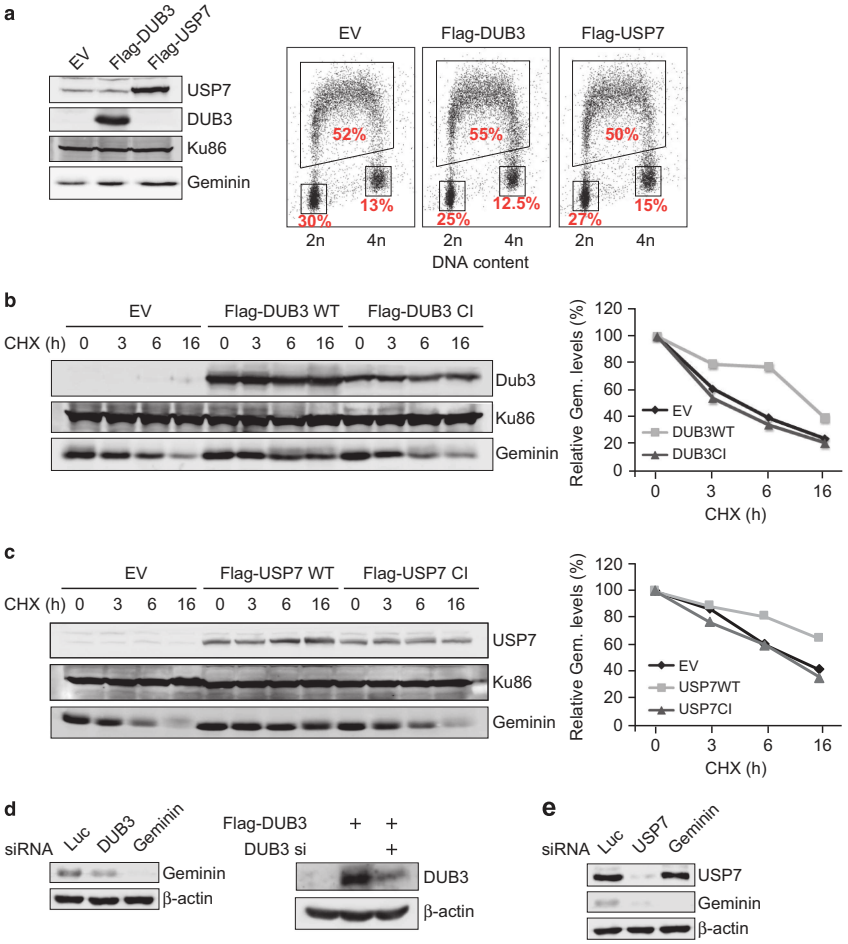

Figure 1. DUB3 and USP7 control Geminin levels. (**a**) 293T cells were transfected (calcium phosphate method)[47] with empty vector (EV) or ectopic expression plasmids encoding Flag-DUB3 (JF Burrows-Queen's University, Belfast, Northern Ireland) or Flag-USP7 (Professor Everett-MRC University of Glasgow Centre for Virus Research). Thirty-six hours after transfection, cells were incubated with 5-bromodeoxyuridine (BrdU) for 15 min and then either collected for western blot (left) or fixed and analyzed by flow cytometry for propidium iodide and BrdU (mouse anti-BrdU from Genscript, Piscataway, NJ, USA)[48] using a MACSQuant Analyzer flow cytometer and MACSQuantify software (Miltenyi Biotec, GmbH, Bergish Gladbach, Germany). The percentage of cells in the different cell cycle stages is shown. Immunoblot analysis was performed with antibodies against USP7 (Abcam, Cambridge, UK, ab190183), DUB3,[15] Geminin (Abcam ab175799) and β-actin (Genscript). (**b**, **c**) 293T cells were transfected with the indicated vectors (WT, wild type; CI, catalytically inactive, that contains the mutations Cys223Ser or Cys89Ser/His334Gln/Asp350Asn[15] in Flag-USP7 and Flag-DUB3, respectively, which prevents the ubiquitination hydrolyze activity of the DUBs) and incubated with cycloheximide (50 μg/ml; Sigma-Aldrich, Madrid, Spain) for the indicated time points. Immunoblot analysis with the indicated antibodies (anti-Ku86, Santa Cruz Biotechnology, Heidelberg, Germany, C-20). Right panels in **b**, **c** show quantification of Geminin western blots (left panels) using the ImageQuant TL Software (GE Healthcare, Madrid, Spain). Geminin levels were compared to loading control Ku86 and the amount of Geminin at $t = 0$ h was put to 100%. (**d**) U2OS cells were transfected with short interfering RNA (siRNA) oligos using Lipofectamine RNAiMAX (Life Technologies, Madrid, Spain; left panel) or the Flag-DUB3 expression plasmid together with the shown siRNAs for 72 h using Metafectene Pro (Biontex, München, Germany; right panel). Cell lysates were analyzed by western blotting. siRNA oligo (Thermo-Fisher, Madrid, Spain) sequences: Luciferase CGUACGCGGAAUACUUCGAdTdT, DUB3 CCUCCGUGAUGUUGCUUGAdTdT, Geminin UGCCAACUCUGGAAUCAAAdTdT. (**e**) U2OS were transfected with the indicated siRNAs and analyzed as in **d**. siRNA oligo sequence for USP7: GGCAACCUUUCAGUUCACUdTdT.

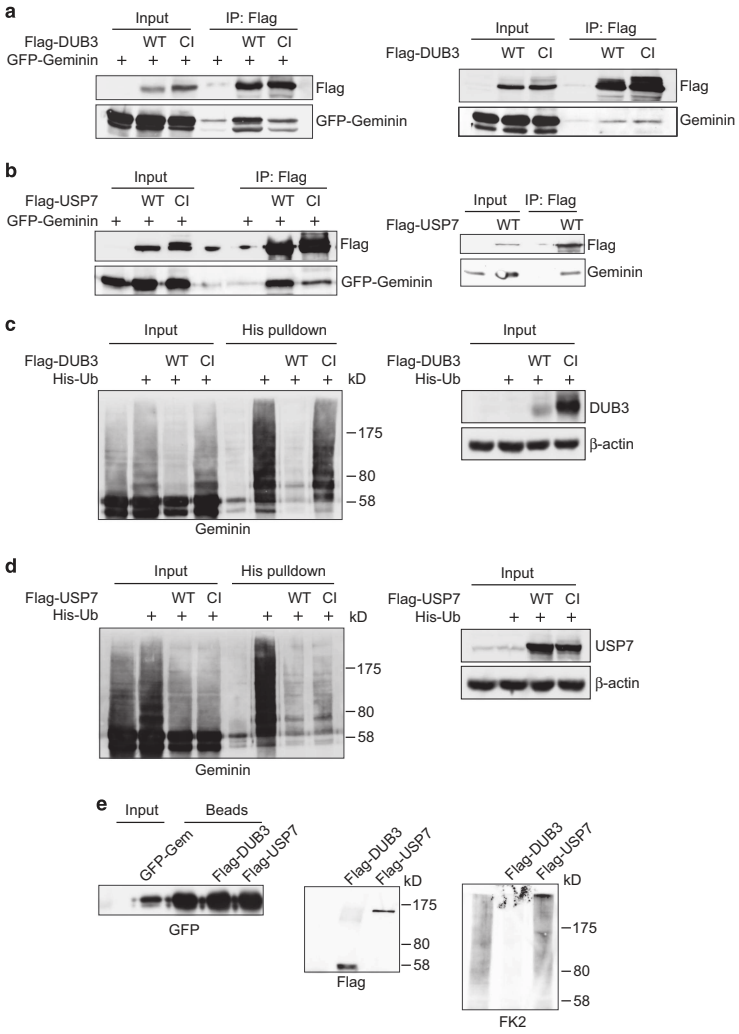

Figure 2. DUB3 and USP7 interact with Geminin and stimulate Geminin de-ubiquitination. (**a, b**) 293T cells transfected when indicated with a GFP-Geminin expressing plasmid (obtained by cloning human Geminin into peGFP-C1) and/or with plasmids encoding Flag-DUB3 or Flag-USP7. Thirty-six hours after transfection, cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris pH 7.5, 0.5% NP40, 150 mM NaCl) supplemented with protease inhibitors (complete-Roche, Madrid, Spain), and IP were performed using anti-Flag M2-agarose (Sigma-Aldrich). In the IPs of endogenous Geminin with USP7 WT, the lysis buffer II contained 20 mM Tris pH 7.5, 135 mM NaCl, 1.5 mM MgCl, 1 mM EGTA, 10% Glycerol and 1% NP40 supplemented with protease inhibitors. Western blot analysis was performed with the indicated antibodies (mouse anti-Flag from Genscript). (**c**, **d**) 293T cells were transfected with expression plasmids for the indicated proteins and GFP-Geminin (PMT107 expressing His-Ubiquitin was a gift from D Bohmann, Rochester, New York, NY, USA). Twenty-four hours post transfection, cells were incubated with 5 μM MG132 (Sigma-Aldrich) for 16 h before lysis under denaturing conditions, and His-Ubiquitin pull downs were carried out using Nickel-NTA agarose (Qiagen, Madrid, Spain) as described.[49] Analysis by western blot analysis with the indicated antibodies. (**e**) An *in vitro* de-ubiquitination assay[50] was performed incubating beads with immunoprecipitated ubiquitinated GFP-Geminin with purified DUBs. Flag-USP7 and Flag-DUB3 were purified from 293T after incubation with lysis buffer II, then with anti-Flag M2-agarose and elution with 3x-Flag-peptide (Generon Ltd, Maidenhead, UK, 200 μg/ml). Ubiquitinated GFP-Geminin was purified from 239T cells transfected with GFP-Geminin and His-Ubiquitin. Twenty-four hours post transfection, cells were incubated with 5 μM MG132 overnight before lysis in native conditions (lysis buffer II) and incubation with rabbit anti-GFP antibody[51] and protein A-sepharose beads (GE Healthcare). Beads were incubated as before,[50] before being analyzed with the indicated antibodies (anti-GFP mouse from Genscript and the multi-ubiquitin chain monoclonal antibody from Cayman Chemical, Ann Arbor, MI, USA, clone FK2).

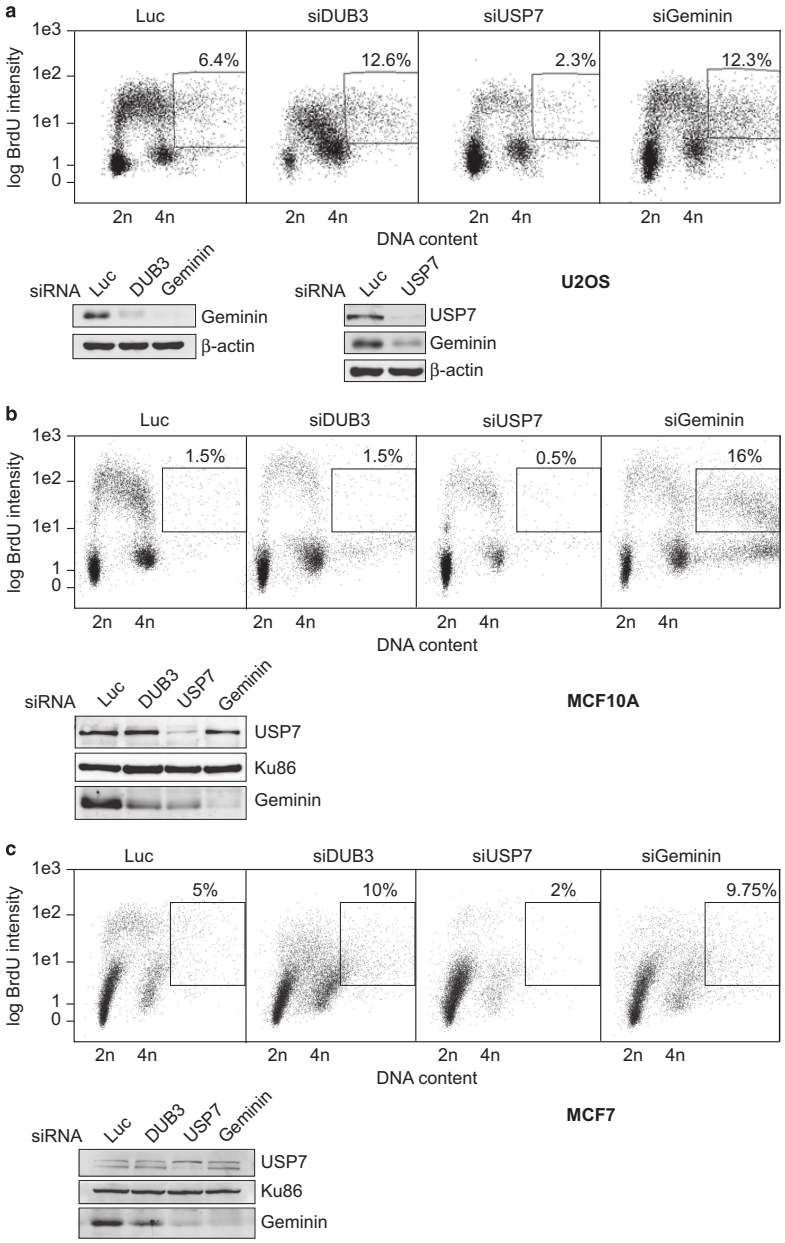

Figure 3. DUB3 depletion induces re-replication events. U2OS (**a**) MCF10A (**b**) or MCF7 (**c**) cells were transfected with the indicated siRNA oligos for 48 h, incubated for 15 min with BrdU then fixed and analyzed by flow cytometry for propidium iodide and BrdU. The percentage of cells with re-replicated DNA is indicated in the top right panels of PI-BrdU dot blots. Analysis of Geminin, USP7, β-actin or Ku86 levels in each cell line were monitored by western blot analysis (lower panels).

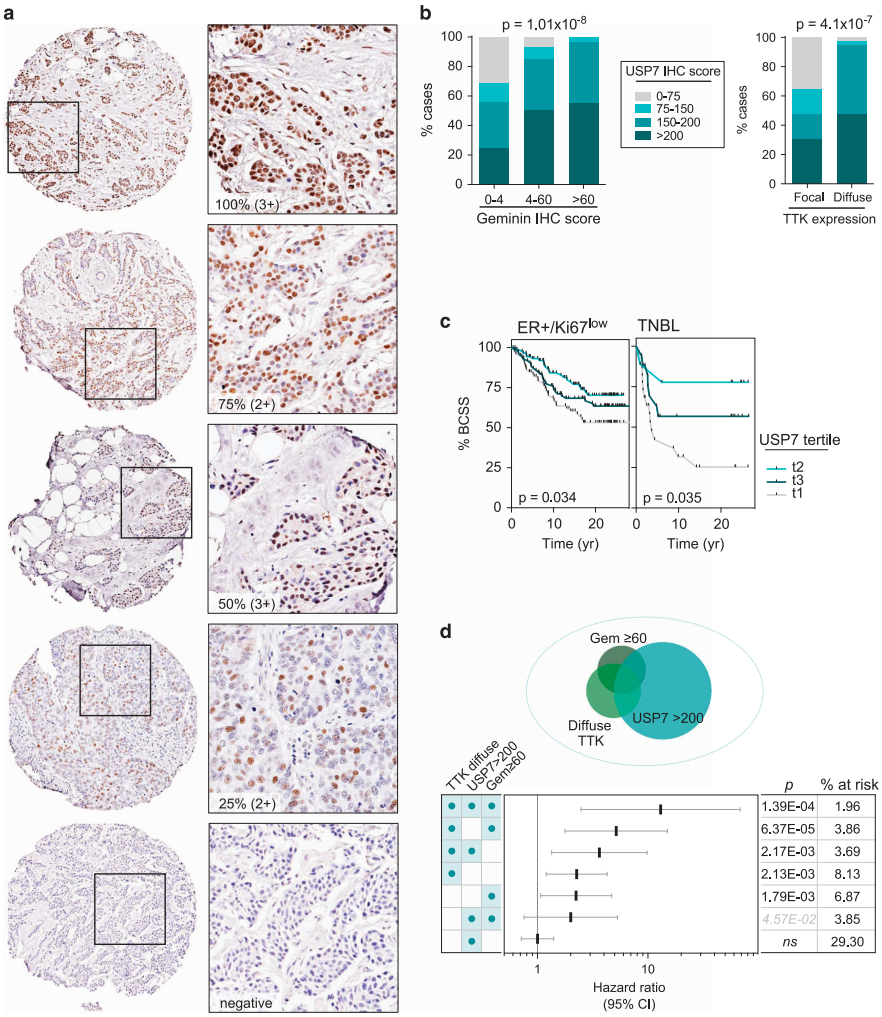

Figure 4. Expression of Geminin and USP7 is strongly correlated in invasive breast cancer, and predicts poor clinical outcome in cells with chromosomal instability. (**a**) Representative breast tumor tissue cores from the Queensland breast cancer follow-up (QFU) cohort, demonstrating the range of USP7 staining observed in immunohistochemical (IHC) analysis. Four micrometer tissue microarray (TMA) sections were processed in a de-cloaker for antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 20 min, and then IHC was performed using USP7 antibody (Abcam, ab119364; 1:400) and the Mach-1 Universal HRP-Polymer Detection kit (Biocare Medical, Concorde, CA, USA). Hematoxylin-counterstained, mounted sections were then scanned at ×40 magnification on an Aperio AT Turbo slide scanner (Leica Biosystems) for scoring of digital images by an experienced molecular pathologist (JMS). The percentages of tumor cells stained (0–100%) and intensities (0–3+) were recorded for each core, and multiplied to derive an IHC score (0–300). Each case was assigned the maximum score from its duplicate cores. For Geminin IHC examples and methods refer to Supplementary Figure S1. (**b**) Proportion bar charts showing co-expression of USP7 with Geminin (left) and TTK (right), according to USP7 and Geminin IHC scores or the expression pattern of TTK (focal staining indicates the normal expression of TTK in mitotic cells, diffuse staining refers to more widespread, mitosis-independent expression of TTK in tumor cells[35]). *P*-values are from X^2/Fisher's exact tests (GraphPad Prism v7, La Jolla, CA, USA). (**c**) Kaplan–Meier analysis of USP7 expression in ER+/Ki67low and triple-negative, basal-like breast cancers (cohorts stratified by IHC score tertiles; BCSS, breast cancer-specific survival). Log-rank *P*-values are shown. (**e**) Venn diagram showing the proportions of breast tumors co-expressing high levels of USP7, Geminin and diffuse TTK within the QFU cohort, the hazard ratios (with 95% confidence intervals) of each biomarker combination, Gehan–Breslow–Wilcoxon *P*-values indicating the significance of differences in survival between each subgroup and its reciprocal, and the percentages of assessable cases for each subgroup ($n = 362$ cases with follow-up and informative IHC for all three markers).

untransformed cells, like MCF10A, might have other mechanisms (intact) to avoid DNA re-replication that the tumor cells used here might have lost. As a consequence, re-replication in MCF10A might only occur upon very low cellular levels of Geminin, whereas a small reduction in Geminin levels in U2OS and MCF7 is sufficient to trigger re-replication.

Expression of Geminin is strongly correlated with USP7 and markers of CIN in breast cancer

Although we could not study the effects of USP7 on re-replication *in vitro*, we hypothesized that if USP7 is an important regulator of Geminin in cancer tissues, we may see a relationship between expression of the two proteins in a human clinical sample cohort. To investigate this, we performed immunohistochemical analysis of the Queensland breast cancer follow-up resource, which comprises tissue microarrays of 449 invasive breast carcinomas with clinical data (median follow-up 14.1 years, range 0.03–41.75).[33–35] During our validation of primary antibodies for this study, we were satisfied with the specificity of antibodies against Geminin and USP7. However, we were unable to source a satisfactory DUB3 antibody for immunohistochemistry. We observed predominantly nuclear staining of Geminin and USP7 in breast tumor cells, and quantified expression according to both the intensity of the staining and the percentage of cells stained. These parameters were subsequently multiplied to generate an immunohistochemical score. Each sample was assigned the maximum score from its duplicate tissue cores (Figure 4a and Supplementary Figure S1A).

Consistent with previous reports,[27–29] Geminin expression was strongly and directly correlated with features of proliferative tumors: grade, mitotic score, nuclear pleomorphism, Ki67 expression, triple-negative phenotype and pushing margins ($P < 1.0E-8$; Supplementary Table S1). High Geminin expression was also strongly predictive of poor clinical outcome ($P = 1.53E-04$; hazard ratio 3.24 (1.39–7.52)). Consistent with a separate study,[36] USP7 expression was associated with high histological grade in the Queensland breast cancer follow-up (Supplementary Table S2). Interestingly, high USP7 expression was less frequent in invasive lobular compared to ductal carcinomas (~30 versus 67% of cases, respectively). Of all the parameters analyzed, the most significant association was between Geminin and USP7 ($P = 1.53E-8$), indicating that they are frequently co-expressed in breast cancer (Figure 4b, left panel; Supplementary Table S1 and Supplementary Table S2). We next performed Kaplan–Meier analysis to investigate the prognostic significance of USP7, separating the cohort into clinically relevant subgroups based on estrogen receptor (ER), progesterone receptor, HER2 and Ki67 status. There was a significant relationship between USP7 expression and breast cancer-specific survival in both low-proliferative (luminal-A, ER +/Ki67low) and high-proliferative (triple-negative, basal-like) disease subtypes. However, the association was non-monotonic, with both low and high levels of USP7 associated with less favorable prognosis than moderate expression (Figure 4c). This suggests that too much, or too little USP7 is associated with therapy resistance and metastatic progression in breast cancer.

It is noteworthy that the relationship between CIN and clinical outcome is also non-monotonic in multiple cancers independent of treatment, suggesting that there may be a level of CIN that surpasses a threshold required for treatment resistance, and instead is catastrophic to the cell.[37] Therefore, to explore the relationships between Geminin, USP7 and CIN in breast cancer, we made use of existing Queensland breast cancer follow-up data on TTK,[35] a dual specificity kinase and component of the spindle assembly checkpoint, with critical roles in centrosome duplication and chromosome alignment. Diffuse, mitosis-independent expression of TTK is a marker of CIN and poor clinical outcome that occurs in ~8% of breast tumors.[35,37,38] Despite this relatively low frequency, ~94% of TTK-diffuse tumors exhibited high USP7 expression (Figure 4b right panel/Supplementary Table S2; $P = 4.1E-07/1.12E-07$), and was associated with high Geminin expression (Supplementary Table S1; $P < 1.0E-15$). Hypothesizing that tumors with all three features would be associated with poor clinical outcomes, we examined the impact of co-expression on breast cancer-specific survival using Kaplan–Meier analysis. This showed that Geminin, its stabilizing DUB USP7 and the CIN marker TTK had additive prognostic power—co-expression was more strongly associated with poor outcome than the individual proteins, and the triple combination was the most prognostic, despite small case numbers in the combination cohorts (Figure 4d).

Multiple studies have demonstrated post-translational regulation of checkpoint and caretaker proteins by USP7 (for example, RAD18, DNA polymerase eta, UHRF1, p53).[39–43] Evidence is accumulating to suggest USP7 could be a master regulator that preserves genome stability by maintaining a low-ubiquitin environment around replication forks, and that when this occurs inappropriately in the face of genotoxic insult, it can promote DNA damage tolerance, perpetuate CIN and aggressive clinical behavior. For example, with elegant mechanistic experiments, Wang *et al*.[36] recently showed that USP7 confers resistance to ionizing radiation and the radiomimetic neocarzinostatin in breast cancer cell lines. This was primarily achieved by stabilizing the histone demethylase PHF8, which is recruited to double stranded DNA breaks where it promotes repair by both homologous recombination and non-homologous end joining. USP7 was one of only a handful of genes predicted to be synthetic lethal with DNA damage-response genes,[44] and has been a strong focus in the ongoing discussion on targeting DUBs as a therapeutic strategy for breast and other cancers.[31,45,46]

In summary, in this study we identified DUB3 and USP7 as novel regulators of Geminin stability. Given that dysregulation of Geminin is linked to DNA re-replication, polyploidy, CIN and cancer progression, we also analyzed relationships between expression USP7, Geminin, CIN and survival in human breast carcinomas, and found strong new correlations. Altogether, the data point to a role for USP7-mediated regulation of Geminin that could be relevant in understanding breast cancer progression and/or developing novel therapeutic strategies.

ABBREVIATION

DUB, de-ubiquitinating enzyme.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to V Smits for critical reading of the manuscript, J Moreira, E Lam, M van Vugt for sending cell lines and A Bravo for logistic help. This work was supported by grants from Instituto de Salud Carlos III (BA15/00092), the Spanish Ministry of Economy and Competitiveness (SAF2013-49149-R, BFU2014-51672-REDC), Fundación CajaCanarias (AP2015/008) to RF and the Australian National Health and Medical Research (NHMRC program grant to SRL and KKK (APP1017028)).

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

SUPPLEMENTARY FIGURES

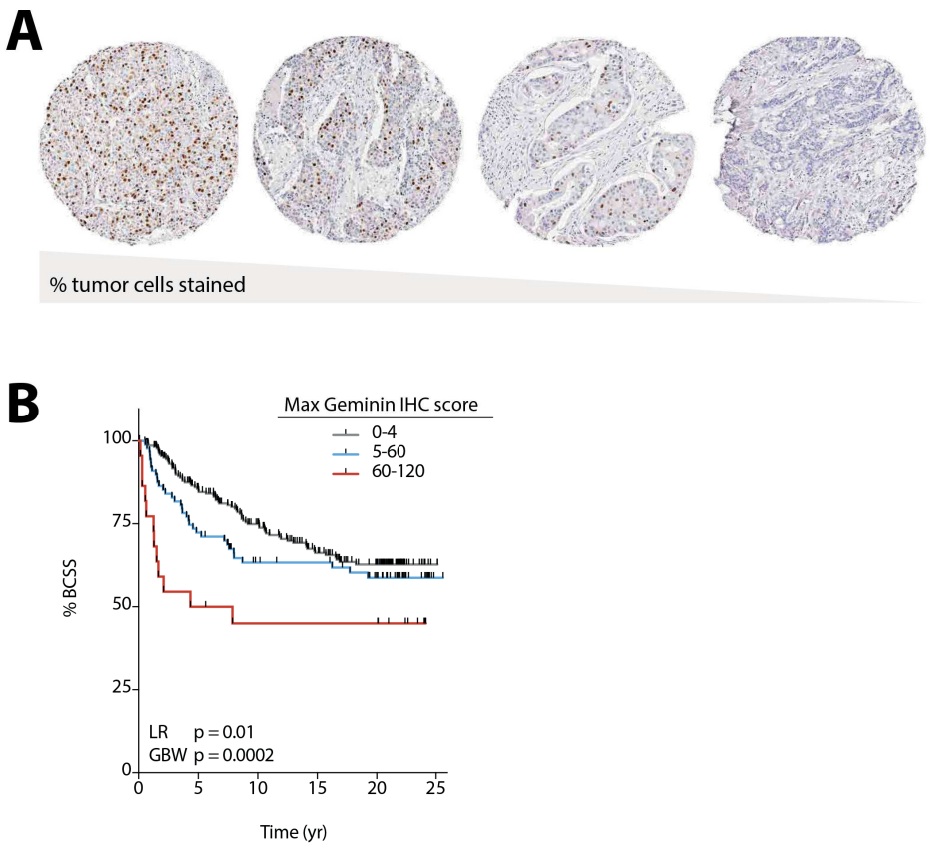

Figure S1. Expression and prognostic significance of Geminin in the Queensland Follow-UP (QFU) cohort. (A) Representative breast cancer tumour tissue cores, demonstrating the range of Geminin staining observed by immunohistochemistry (IHC) analysis. Four µm tissue microarray (TMA) sections were processed in a de-cloaker for antigen retrieval in EDTA buffer (pH 8.8) for 15 minutes, and then IHC was performed using a Geminin antibody (Abcam a 175799; 1:2000) and the Mach-1 Universal IIRP-Polymer Detecction kit (Biocarc Medical). Hematoxylin-counterstained, mounted sections were then scanned at 40X magnification on an Aperio AT Turbo slide scanner (Leica Biosystems) for scoring of digital images by an experienced molecular pathologist (JMS). The percentages of tumor cells stained (%TC; 0-100%) and intensities (0-3+) were recorded for each core, and multiplied to derive an IHC score (0-300). Each case was assigned the maximum score from its duplicate cores. Geminin IHC scores were mostly driven by %TC. (B) Kaplan Meier analysis of the relationship between Geminin expression and breast cancer-specific survival (BCSS). Geminin was strongly prognostic, consistent with previous reports. I.R, log-rank test; GBW, Gehan-Breslow-Wilcoxon test.

Table S1: Associations between Geminin expression and histopathological parameters in breast cancer.

Geminin IHC score		*n*				% cases			*p* value
		total	low (0-4)	mod (5-59)	high (≥60)	low (0-4)	mod (5-59)	high (≥60)	
Age	>40 yr	316	209	84	23	66,1	26,6	7,3	*ns*
	≤40 yr	39	22	16	1	56,4	41,0	2,6	
	n	*355*							
Lymph node status	Negative	108	70	30	8	64,8	27,8	7,4	*ns*
	Positive	101	61	34	6	60,4	33,7	5,9	
	n	209							
Tumour size	<2 cm	152	111	34	7	73,0	22,4	4,6	**3,64E-03**
	2-5 cm	142	85	46	11	59,9	32,4	7,7	
	>5 cm	29	13	10	6	44,8	34,5	20,7	
	n	*323*							
Histological type	IC NST	219	127	68	24	58,0	31,1	11,0	**1,20E-03**
	lobular/variants	48	39	8	1	81,3	16,7	2,1	
	mixed ducto-lob	30	23	7	0	76,7	23,3	0,0	
	mixed	34	18	14	2	52,9	41,2	5,9	
	special types	42	35	7	0	83,3	16,7	0,0	
	n	*373*							
Grade	1	47	42	5	0	89,4	10,6	0,0	**1,40E-14**
	2	181	140	39	2	77,3	21,5	1,1	
	3	145	60	60	25	41,4	41,4	17,2	
	n	*373*							
Mitotic score	1	208	170	36	2	81,7	17,3	1,0	**1,00E-15**
	2	51	34	17	0	66,7	33,3	0,0	
	3	113	38	50	25	33,6	44,2	22,1	
	n	*372*							
Nuclear pleomorphism	1-2	183	145	35	3	79,2	19,1	1,6	**9,95E-09**
	3	190	97	69	24	51,1	36,3	12,6	
	n	*373*							
Tubule formation	1-2	77	60	16	1	77,9	20,8	1,3	**1,09E-02**
	3	296	182	88	26	61,5	29,7	8,8	
	n	*373*							
Lymphovascular invasion	Absent	282	190	69	23	67,4	24,5	8,2	**2,62E-02**
	Present	91	52	35	4	57,1	38,5	4,4	
	n	*373*							
Lymphocytic infiltrate	Absent	130	104	24	2	80,0	18,5	1,5	**5,75E-07**
	Mild	152	104	35	13	68,4	23,0	8,6	
	Moderate-severe	80	34	35	11	42,5	43,8	13,8	
	n	*362*							
Central scarring/fibrosis	Absent	335	228	87	20	68,1	26,0	6,0	**2,31E-04**
	Present	38	14	17	7	36,8	44,7	18,4	
	n	373							
Tumour border	infiltrative	323	226	83	14	70,0	25,7	4,3	**3,52E-10**
	pushing	49	15	21	13	30,6	42,9	26,5	
	n	372							

Ki67 expression (10% threshold)	Low	229	176	43	10	76,9	18,8	4,4	**2,26E-12**
	High	125	47	61	17	37,6	48,8	13,6	
	n	*354*							
HER2 status (SISH)	Negative	337	224	89	24	66,5	26,4	7,1	*ns*
	Positive	39	20	16	3	51,3	41,0	7,7	
	n	*376*							
ER status	Positive	89	36	36	17	40,4	40,4	19,1	**2,56E-09**
	Negative	284	205	69	10	72,2	24,3	3,5	
	n	*373*							
TN status	Non-TNBC	306	217	77	12	70,9	25,2	3,9	**1,64E-09**
	TNBC	68	25	28	15	36,8	41,2	22,1	
	n	*374*							
Prognostic subgroups	HER2+	39	20	16	3	51,3	41,0	7,7	**4,00E-15**
	HR+/HER2-neg (Ki67-low)	232	179	50	3	77,2	21,6	1,3	
	HR+/HER2-neg (Ki67-high)	21	4	11	6	19,0	52,4	28,6	
	TN (basal-like)	51	15	23	13	29,4	45,1	25,5	
	TN (non-basal)	13	7	4	2	53,8	30,8	15,4	
	n	*356*							
USP7 expression	IHC score <150	125	109	15	1	87,2	12,0	0,8	**1,14E-09**
	IHC score >150	251	136	89	26	54,2	35,5	10,4	
	n	*376*							
TTK expression	Negative-moderate (0-2)	340	238	89	13	70,0	26,2	3,8	**1,00E-15**
	Diffuse, mitosis-independent (3)	35	6	15	14	17,1	42,9	40,0	
	n	*375*							
p53 expression	Neg-moderate (IHC score<240)	241	159	72	10	66,0	29,9	4,1	**1,05E-08**

Table S2: Associations between USP7 expression and histopathological parameters in breast cancer.

USP7 IHC score		*n*			% cases		*p* value[#]
		total	low (0-6)	high (6-12)	low (0-6)	high (6-12)	
Age	>40 yr	376	168	208	44,7	55,3	**4,99E-02**
	≤40 yr	43	12	31	27,9	72,1	
	n	*419*					
Lymph node status	Negative	135	61	74	45,2	54,8	*ns*
	Positive	115	43	72	37,4	62,6	
	n	250					
Tumour size	<2 cm	177	76	101	42,9	57,1	*ns*
	2-5 cm	167	74	93	44,3	55,7	
	>5 cm	34	14	20	41,2	58,8	
	n	*378*					
Histological type	IC NST	254	84	170	33,1	66,9	**1,04E-06**
	lobular/variants	64	45	19	70,3	29,7	
	mixed ducto-lob	39	22	17	56,4	43,6	
	mixed	37	14	23	37,8	62,2	
	special types	47	22	25	46,8	53,2	
	n	*441*					
Grade	1	52	32	20	61,5	38,5	**6,29E-04**
	2	219	102	117	46,6	53,4	
	3	160	53	107	33,1	66,9	
	n	*431*					
Mitotic score	1	257	128	129	49,8	50,2	**6,69E-04**
	2	58	22	36	37,9	62,1	
	3	125	37	88	29,6	70,4	
	n	*440*					
Nuclear pleomorphism	1-2	233	120	113	51,5	48,5	**4,89E-05**
	3	208	67	141	32,2	67,8	
	n	*441*					
Tubule formation	1	13	5	8	38,5	61,5	*ns*
	2	82	34	48	41,5	58,5	
	3	346	148	198	42,8	57,2	
	n	*441*					
Lymphovascular invasion	Absent	337	154	183	45,7	54,3	**6,10E-03**
	Present	102	31	71	30,4	69,6	
	n	*439*					
Lymphocytic infiltrate	Absent	165	75	90	45,5	54,5	*ns*
	Mild	186	75	111	40,3	59,7	
	Moderate-severe	88	36	52	40,9	59,1	
	n	*439*					
Central scarring/fibrosis	Absent	394	168	226	42,6	57,4	*ns*
	Present	47	19	28	40,4	59,6	
	n	441					
Tumour border	infiltrative	384	169	215	44,0	56,0	*ns*
	pushing	56	17	39	30,4	69,6	

	n	*440*					
Ki67 expression (10% threshold)	Low	253	110	143	43,5	56,5	**1,13E-03**
	High	142	38	104	26,8	73,2	
	n	*395*					
HER2 status (SISH)	Negative	396	171	225	43,2	56,8	*ns*
	Positive	43	13	30	30,2	69,8	
	n	*439*					
ER status	Positive	332	137	195	41,3	58,7	*ns*
	Negative	100	42	58	42,0	58,0	
	n	*432*					
TN status	Non-TNBC	355	144	211	40,6	59,4	*ns*
	TNBC	75	32	43	42,7	57,3	
	n	*430*					
Prognostic subgroups	HER2+	45	15	30	33,3	66,7	*ns*
	HR+/HER2-neg (Ki67-low)	262	105	157	40,1	59,9	
	HR+/HER2-neg (Ki67-high)	25	6	19	24,0	76,0	
	TN (basal-like)	56	22	34	39,3	60,7	
	TN (non-basal)	14	5	9	35,7	64,3	
	n	*402*					
Geminin expression	IHC score 0-4	245	109	136	44,5	55,5	**1,10E-09**
	IHC score 4-60	104	15	89	14,4	85,6	
	IHC score >60	27	1	26	3,7	96,3	
	n	*376*					
TTK expression	Negative-moderate (0-2)	385	201	184	52,2	47,8	**1,12E-07**
	Diffuse, mitosis-independent (3)	36	3	33	8,3	91,7	
	n	*421*					
p53 expression	Neg-moderate (IHC score<240)	273	131	142	48,0	52,0	**4,79E-03**
	Overexpressed (IHC score >240)	55	15	40	27,3	72,7	
	n	*328*					

Oncogene (2016), 1–9

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ORIGINAL ARTICLE

PERK inhibits DNA replication during the Unfolded Protein Response via Claspin and Chk1

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Stresses such as hypoxia, nutrient deprivation and acidification disturb protein folding in the endoplasmic reticulum (ER) and activate the Unfolded Protein Response (UPR) to trigger adaptive responses through the effectors, PERK, IRE1 and ATF6. Most of these responses relate to ER homoeostasis; however, here we show that the PERK branch of the UPR also controls DNA replication. Treatment of cells with the non-genotoxic UPR agonist thapsigargin led to a rapid inhibition of DNA synthesis that was attributable to a combination of DNA replication fork slowing and reduced replication origin firing. DNA synthesis inhibition was dependent on the UPR effector PERK and was associated with phosphorylation of the checkpoint adaptor protein Claspin and activation of the Chk1 effector kinase, both of which occurred in the absence of detectable DNA damage. Remarkably, thapsigargin did not inhibit bulk DNA synthesis or activate Chk1 in cells depleted of Claspin, or when Chk1 was depleted or subject to chemical inhibition. In each case thapsigargin-resistant DNA synthesis was due to an increase in replication origin firing that compensated for reduced fork progression. Taken together, our results unveil a new aspect of PERK function and previously unknown roles for Claspin and Chk1 as negative regulators of DNA replication in the absence of genotoxic stress. Because tumour cells proliferate in suboptimal environments, and frequently show evidence of UPR activation, this pathway could modulate the response to DNA replication-targeted chemotherapies.

Oncogene advance online publication, 4 July 2016; doi:10.1038/onc.2016.239

INTRODUCTION

Owing to the abnormal morphology, vascularization and metabolism typical of tumours, cancer cells must necessarily proliferate and survive under conditions of environmental stress, including hypoxia, nutrient deprivation and acidification.[1] Each of these, and other intracellular stresses, can disturb the folding of proteins in the endoplasmic reticulum (ER), resulting in an excess of unfolded proteins and ultimately ER stress or dysfunction.[2] ER stress triggers the activation of a complex homoeostatic mechanism, the Unfolded Protein Response (UPR), which acts to remediate the accumulation and deleterious effects of unfolded proteins in the ER.[3] The UPR is mediated through three principal effectors, the protein kinase PERK, the protein kinase and RNA processing enzyme IRE1, and the transcription factor ATF6.[3] Collectively these mechanistically diverse effectors act to inhibit global translation, thus reducing the burden of unfolded proteins entering the ER, but also to enhance the transcription and translation of specific genes that increase the protein folding capacity of the ER.[3]

Under conditions of ER stress the UPR is thought capable of exerting both pro-survival and pro-death effects depending on the duration and intensity of the stimulus. Its initial homoeostatic role is thought to favour cell survival under conditions of moderate or transient ER stress, whereas acute or chronic activation can promote cell death by apoptosis or other mechanisms.[3] As a result, numerous studies have sought to determine whether activation of the UPR favours or suppresses tumour progression. The results of these studies are complex and suggest that UPR activation can both promote and suppress carcinogenesis according to context.[4,5] Despite this complexity the UPR is considered to be a valid anti-cancer target, and drugs inhibiting PERK in particular have been developed for this purpose.[6] The exact circumstances under which PERK inhibition might have beneficial therapeutic effects however remain unclear.

Radiation and genotoxic chemotherapies are important cancer treatments and are likely to remain so for the foreseeable future, despite limited efficacy and severe side effects. As with ER stress, exogenous DNA-damaging agents elicit a complex cellular response termed the DNA damage response that seeks to limit toxicity and promote DNA repair and cell survival.[7] Prominent among the components of the DNA damage response is the ATR–Claspin–Chk1 pathway, which is activated by both DNA damage and DNA replication arrest.[7] The Chk1 protein kinase is the ultimate effector in this pathway and is capable of both imposing cell cycle arrest in G2 and modulating DNA replication *per se*.[8] Chk1 has also attracted interest as an anti-cancer drug target and a number of inhibitors are currently under preclinical investigation or early-stage clinical trials.[9] As with PERK however, rational strategies for the application of Chk1 inhibitor drugs remain under development.[9]

Many genotoxic chemotherapies act by inhibiting DNA replication through polymerase inhibition or chain termination, such as 5-fluorouracil and gemcitabine, or by inducing replication fork-blocking DNA damage lesions, such as camptothecin (CPT). When

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Received 15 January 2016; revised 6 May 2016; accepted 3 June 2016

replication forks are impeded by such agents, Chk1 is activated by its upstream kinase, ATR, in conjunction with the checkpoint adaptor protein, Claspin.[7] Under such conditions of acute replication inhibition Chk1 acts to stabilize stalled replication forks and to suppress late replication origin firing, functions that are important for cell survival.[8] Chk1 also plays an important role during unperturbed replication, since inhibition of Chk1 or depletion of Claspin has been shown to markedly slow replication fork progression.[10,11] Interestingly, however, Chk1 or Claspin inhibition also results in greatly increased replication origin firing that under some circumstances at least can compensate for reduced fork progression rate to maintain normal levels of bulk DNA synthesis.[12,13]

One of the canonical functions of the UPR is to inhibit global translation when unfolded proteins accumulate in the ER.[14,15] This response is mediated primarily via PERK, which phosphorylates and inhibits eukaryotic initiation factor 2 alpha (eIF2A), which is required for translational initiation.[15,16] Although a small number of mRNAs are preferentially translated under conditions of ER stress, these encode specialized products generally involved in the restoration of ER homoeostasis.[3] During DNA replication however abundant new histone protein translation is necessary to allow the incorporation of newly synthesized DNA into chromatin. It is known that UPR activation can trigger cell cycle arrest in the G1 phase via PERK,[17] leading us to postulate that other cell cycle events might also be subject to negative regulation by this pathway. Here, we show that under conditions of UPR activation DNA replication is rapidly suppressed at the level of both fork progression and origin firing by a mechanism that is initiated by PERK and acts in part through Claspin and Chk1. A remarkable feature of this novel mechanism is that Claspin and Chk1 function in the absence of genotoxic stress or DNA damage as measured by conventional markers.

PERK suppresses DNA replication during UPR activation

To determine whether UPR activation affects DNA replication we pre-treated U2OS cells with the non-genotoxic UPR agonist thapsigargin or solvent control for 1 h, pulsed with bromodeoxyuridine (BrdU) for 15 min, and then quantified BrdU incorporation by flow cytometry. As shown in Figures 1a and b,

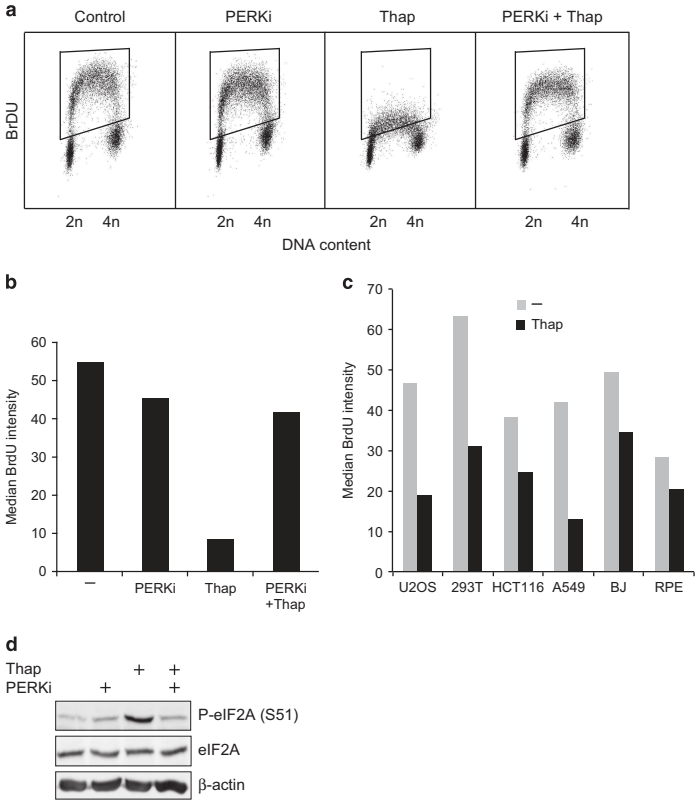

Figure 1. Thapsigargin induces PERK-dependent suppression of DNA synthesis. (**a**) Replicate cultures of U2OS cells in the exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 min after which all cultures were treated with 2 μM thapsigargin for 1 h. BrdU was added for the last 15 min of the thapsigargin treatment after which cells were fixed and processed for flow cytometry. (**b**) Quantification of mean BrdU fluorescence as shown in (**a**). The experiment has been repeated at least three times with similar results. (**c**) Replicate cultures of the indicated cell lines in the exponential growth phase were treated with 2 μM thapsigargin for 1 h. BrdU was added for the last 15 min of the thapsigargin treatment after which cells were fixed and processed for flow cytometry. (**d**) U2OS cell cultures were treated as in (**a**), lysed and analysed by western blotting using the indicated antibodies.

this analysis revealed that thapsigargin treatment induced a large decrease in BrdU incorporation into the S-phase population. This was a highly specific effect as 1 h of thapsigargin treatment did not alter the overall cell cycle distribution of the cultures, nor was there any detectable increase in cell death. To determine if this was a general phenomenon we exposed a panel of tumour and normal cell lines to thapsigargin for 1 h and measured the effect on BrdU incorporation by flow cytometry (Figure 1c). This analysis revealed that thapsigargin suppressed DNA synthesis in all cell lines tested, although the scale of the reduction varied from greater than 50% in U2OS and A549 cells to approximately 25% in HCT116, BJ fibroblasts and RPE cells (Figure 1c).

The UPR effector kinase PERK is rapidly activated in response to thapsigargin treatment and has previously been implicated in cell cycle arrest in the G1 phase.[17] To assess the possible involvement of PERK in suppressing DNA replication we pre-treated cells with GSK2606414, a selective PERK inhibitor, for 30 min prior to thapsigargin exposure and BrdU pulse-labelling. Remarkably, pre-treatment with PERK inhibitor completely blocked the suppression of DNA synthesis by thapsigargin, although the inhibitor had no effect on BrdU incorporation alone (Figures 1a and b).

To ensure that both thapsigargin and the PERK inhibitor were active under our experimental conditions we examined the phosphorylation of eIF2A at serine 51 (S51), a well-characterized PERK target site.[16] As expected, 1 h of thapsigargin treatment resulted in a substantial increase in eIF2A S51 phosphorylation that was completely blocked by pre-treatment with PERK inhibitor (Figure 1d).

To determine if these effects were specific to thapsigargin we next investigated the effects of dithiothreitol (DTT) and tunicamycin, two well-characterized UPR and PERK agonists with modes of action distinct both from one another and from thapsigargin. As shown in Figures 2a and b, a 1 h treatment with 2 mM DTT strongly suppressed DNA synthesis in U2OS cells and, as with thapsigargin, this decrease was blocked by pre-treatment with PERK inhibitor. Western blot analysis of eIF2A S51 phosphorylation confirmed that DTT activated PERK and that activation under these conditions was blocked by pre-treatment with PERK inhibitor (Figure 2c). We also observed that DNA synthesis was inhibited when cells were treated with tunicamycin; however, this response took much longer to develop (7 h) and resulted in a much smaller increase in eIF2A S51phosphoryation, indicative of weak PERK activation (Supplementary Figure S1A). DNA synthesis inhibition in response to tunicamycin treatment could nevertheless also be partially reversed by pre-treatment with PERK inhibitor (Supplementary Figure S1A). We conclude that UPR activation using two well-characterized and powerful agonists, thapsigargin and DTT, leads to a rapid suppression in the rate of DNA replication that is mediated, at least in large part, via the effector kinase PERK. In subsequent mechanistic experiments we decided to focus on thapsigargin, since it has a well-defined and highly specific mechanism of UPR activation and no known genotoxic effects.

PERK inhibits both replication fork progression and replication origin firing

At the molecular level DNA synthesis could be inhibited by slowing the progression of active replication forks, reducing the number of active replication forks by suppressing replication origin firing, or a combination of both. To distinguish between these possibilities we pre-labelled cells with a pulse of iodo-deoxyuridine for 30 min and then administered a second pulse of chloro-deoxyuridine for 30 min in replicate cultures of U2OS cells treated or not with thapsigargin. Cells were then lysed and the length and spacing of incorporation tracts visualized as previously described.[18]

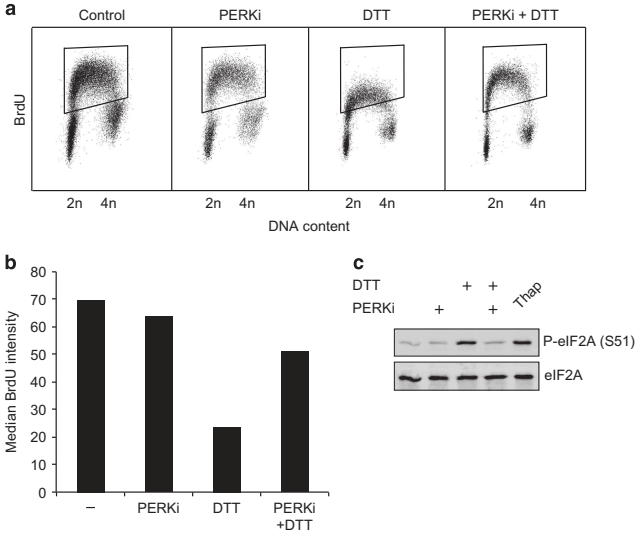

Figure 2. DTT induces PERK-dependent suppression of DNA synthesis. (**a**) Replicate cultures of U2OS cells in the exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 min after which all cultures were treated with 2 mM DTT for 1 h. BrdU was added for the last 15 min of the DTT treatment after which cells were fixed and processed for flow cytometry. (**b**) Quantification of mean BrdU fluorescence as shown in (**a**). The experiment has been repeated at least three times with similar results. (**c**) U2OS cell cultures were treated as in (**a**), lysed and analysed by western blotting using the indicated antibodies.

This analysis (Figures 3a and b) revealed that thapsigargin treatment markedly decreased both the rate of replication fork progression (from 0.92 to 0.28 kb/min) and also the density of active forks (from 3.75 to 2.4 forks/Mb), indicative of a reduction in replication origin firing. Strikingly, pre-treatment with PERK inhibitor prevented the decrease in fork density with an almost complete rescue of fork rate (Figures 3a and b). Taken together, these data demonstrate that thapsigargin suppresses DNA replication by slowing the progression of active forks and by suppressing the formation of new forks, and that both of these responses are mediated largely or completely via PERK.

PERK-dependent Claspin phosphorylation and Chk1 activation during UPR activation

It is known that Chk1 can suppress replication origin firing under conditions of DNA damage or replication arrest.[8] Accordingly, next we investigated whether thapsigargin might activate Chk1 under these conditions. Cells treated with the conventional genotoxic agents ultraviolet (UV) light and CPT were used for comparison.

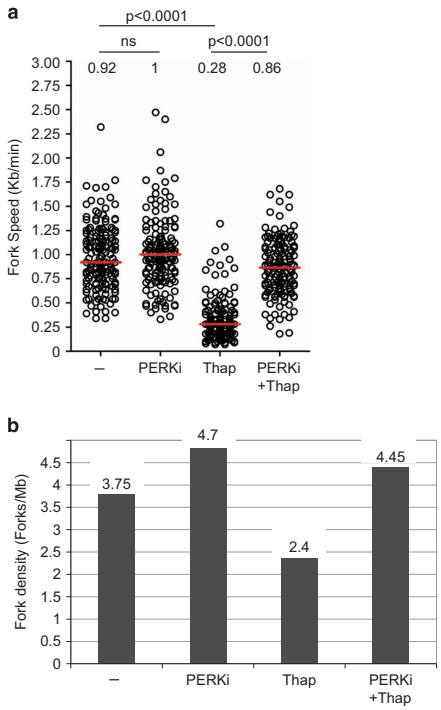

Figure 3. Suppression of DNA synthesis by thapsigargin results from replication fork slowing and suppression of replication origin firing. Replicate cultures of U2OS cells in the exponential growth phase were pre-treated with 0.5 μM PERK inhibitor for 30 min then 0.25 μM thapsigargin or solvent control for 1 h as indicated. Cells were then sequentially pulse labelled for 30 min with iodo-deoxyuridine (IdU) and then chloro-deoxyuridine (CldU). Cells were then harvested, lysed and DNA fibres analysed to determine replication fork speed (**a**) and fork density (**b**) as described in Materials and methods. *P* = *P*-value, NS = statistically nonsignificant.

As shown in Figure 4a, this analysis revealed that treatment with thapsigargin resulted in activation of Chk1 as judged by an increase in phosphorylation of the positive regulatory sites pS317 and pS345. This increase was less than that induced by UV or CPT; however, it was completely blocked by pre-treatment with GSK2606414 (Figure 4a), indicating that it was dependent on activation of PERK. Interestingly, thapsigargin did not induce any detectable phosphorylation of H2AX (γ-H2AX) or of replication protein 32 (RPA32), well-established markers of DNA damage and PI3 kinase-like kinase (PIKK) activation, both of which were strongly induced by UV and CPT (Figure 4a). Neither did thapsigargin treatment result in the formation of γ-H2AX foci detectable by immunofluorescence microscopy, whereas both UV and CPT did (Supplementary Figure S2). Taken together, these results indicate that thapsigargin treatment leads to Chk1 activation in the absence of detectable DNA damage.

The adaptor protein Claspin is required for Chk1 activation under conditions of genotoxic stress[19] and we noted that Claspin underwent a pronounced electrophoretic mobility shift in response to thapsigargin treatment that was blocked by pre-treatment with PERK inhibitor (Figure 4a). This shift was due to phosphorylation as it could be reversed when thapsigargin-treated extracts were incubated with lambda phosphatase (Figure 4b). Interestingly, a qualitatively similar mobility shift was also induced by UV, which in addition to inducing DNA damage is a potent UPR and PERK agonist,[20] whereas CPT, which does not activate PERK as judged by eIF2A phosphorylation (Figure 4a), did not. A similar Claspin gel mobility shift was observed in cells treated with DTT (Figure 4c), and as with thapsigargin, this also was blocked by pre-treatment with PERK inhibitor (Figure 4c). Thus, thapsigargin and other mechanistically distinct UPR agonists induce PERK-dependent phosphorylation of Claspin that is detectable as a gel mobility shift.

As an independent approach we next used siRNA depletion to assess the requirement for PERK in Chk1 activation and Claspin phosphorylation. Cells were treated with PERK-specific siRNA (siPERK) or luciferase control (siLuc) for 48 h, after which they were treated with thapsigargin or CPT for comparison. PERK depletion in this experiment was very efficient as indicated by an almost complete absence of detectable PERK protein expression and a large reduction in the induction of eIF2A S51 phosphorylation (Figure 4d). Importantly, thapsigargin treatment failed to induce activation of Chk1 or Claspin phosphorylation in PERK-depleted cells, corroborating our results with the PERK inhibitor.

Claspin is required for PERK-mediated Chk1 activation and DNA synthesis suppression

The correlation between the PERK-dependent phosphorylation gel shift of Claspin, activation of Chk1 and suppression of DNA synthesis raised the question of whether these phenomena were causally linked. To test this we first used siRNA to deplete Claspin and determined whether this had any effect on Chk1 activation and DNA synthesis inhibition by thapsigargin. As shown in Figure 5a, activation of Chk1 in response to thapsigargin was eliminated in cells depleted of Claspin, while DNA synthesis became almost completely resistant to suppression under these conditions (Figure 5b). Strikingly, depletion of Chk1 also rendered DNA synthesis significantly resistant to thapsigargin, although the phosphorylation shift of Claspin persisted (Figures 5a and b).

As an independent approach to siRNA depletion, we also investigated the effect of two selective small-molecule inhibitors of Chk1 catalytic activity, UCN01 and AZD7762. As shown in Figures 5c and d, pre-treatment with both Chk1 inhibitors for 30 min rendered DNA synthesis significantly resistant to thapsigargin treatment. Taken together, these data demonstrate that both Claspin and Chk1 are necessary for maximal suppression of DNA synthesis downstream of PERK activation by thapsigargin,

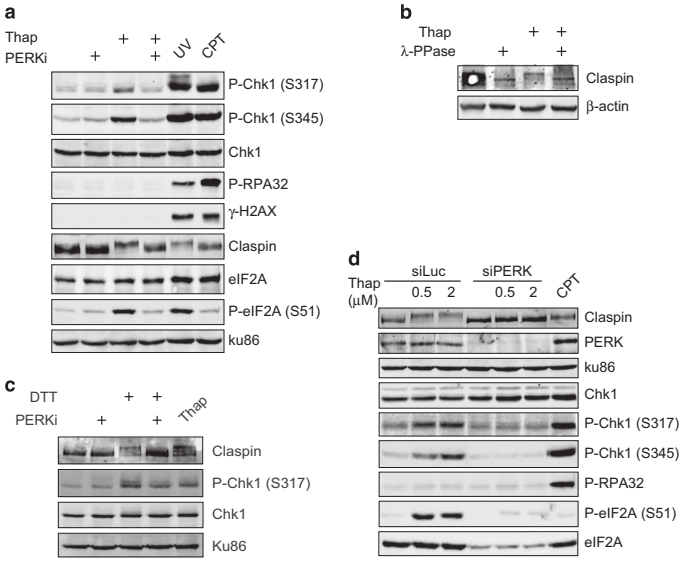

Figure 4. Thapsigargin induces PERK-dependent activation of Chk1 and phosphorylation of Claspin. (**a**) Replicate cultures of U2OS cells in exponential growth were exposed to the indicated treatments (2 μM camptothecin (CPT), 40 J/m^2 UV) for 1 h (or harvested 1 h after exposure to UV), lysed and analysed by western blotting using the indicated antibodies. Where appropriate cells were pre-treated for 30 min with 0.5 μM PERK inhibitor. (**b**) Replicate cultures of U2OS cells were treated with 2 μM thapsigargin or solvent control for 1 h as indicated, lysed and the extracts treated with lambda (λ) phosphatase for 30 min prior to analysis by western blotting. (**c**) Replicate cultures of U2OS cells in the exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 min after which all cultures were treated with 2 mM DTT for 1 h, lysed and analysed by western blotting using the indicated antibodies. (**d**) Replicate cultures of U2OS cells in exponential growth were treated with siRNA against PERK or luciferase as control. After 48 h cells were exposed to the indicated concentrations of thapsigargin or camptothecin (CPT, 2 μM) for 1 h, lysed and analysed by western blotting using the indicated antibodies.

while the protective effect of UCN01 and AZD7762 argues that Chk1 catalytic activity is likely also required.

Claspin and Chk1 are required for PERK-mediated replication origin suppression

The finding that depletion of Claspin or inhibition of Chk1 rendered bulk DNA synthesis partially or completely resistant to inhibition by thapsigargin raised the question of whether this was mediated at the level of replication fork progression, origin firing or a combination of both. To this end we examined fork progression rates and origin density in cells depleted for Claspin of Chk1 with or without thapsigargin treatment.

This analysis revealed that Claspin depletion alone resulted in a severe decrease in fork progression rate (Figure 6a), even though Claspin-depleted cells showed no diminution of BrdU uptake as judged by flow cytometry analysis (Figure 5b). We believe this is because, as previously reported,[11] fork density was significantly increased after Claspin depletion (Figure 6b), thus compensating for slower fork progression and maintaining bulk DNA synthesis at normal levels. Similar but less extreme effects on fork progression and density were observed after Chk1 depletion (Figures 6a and b). We next examined how Claspin or Chk1 depletion affected replication dynamics in response to thapsigargin treatment. Strikingly, although depletion of Claspin or Chk1 did not increase replication fork progression rate in thapsigargin-treated cells (Figure 6a), replication fork density was greatly increased and rendered resistant to suppression by thapsigargin. In contrast, both fork progression and fork density were suppressed in control cells exposed to thapsigargin (Figure 6b). Thus, we conclude from these data that Claspin and Chk1 are required specifically for PERK-dependent suppression of replication origin firing.

eIF2A inhibition is required for PERK-mediated suppression of DNA replication and Claspin phosphorylation

Many of the biological effects of PERK result from eIF2A S51 phosphorylation.[3] This modification inhibits eIF2A function by dissociating the active, dimeric form into inactive monomers, thus inhibiting the initiation of protein translation.[21] Recently a compound termed 'Inhibitor of the Integrated Stress Response' (ISRIB) was identified that prevents dissociation of eIF2A dimers after S51 phosphorylation.[21] ISRIB thus counters translational inhibition and other downstream effects of PERK activation that normally arise as a consequence of eIF2A inhibition via S51 phosphorylation.

To determine if the effects of PERK on DNA replication are a consequence of eIF2A inhibition, we treated U2OS cells with thapsigargin for 1 h with or without pre-treatment with ISRIB and measure the rate of DNA synthesis by flow cytometry. Remarkably, this analysis revealed that pre-treatment with ISRIB completely blocked both DNA synthesis inhibition and the PERK-dependent Claspin mobility shift in response to thapsigargin treatment (Figures 7a and b). ISRIB alone had little or no effect on DNA synthesis, and importantly, PERK remained active in cells treated with thapsigargin and ISRIB as judged by increased levels of eIF2A S51 phosphorylation (Figure 7b). Thus, we conclude that PERK-mediated DNA synthesis inhibition and Claspin phosphorylation depend on inhibition of eIF2A activity.

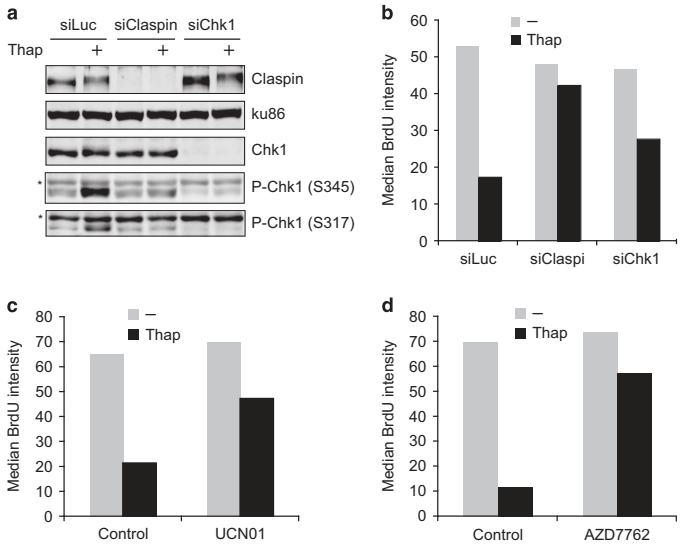

Figure 5. Claspin and Chk1 are required for PERK-mediated DNA synthesis inhibition. (**a**) Replicate cultures of U2OS cells were treated with siRNAs against Claspin, Chk1 or luciferase control for 24 h, treated with 2 μM thapsigargin for 1 h as indicated, then lysed and analysed by western blotting using the indicated antibodies. Nonspecific bands are indicated by asterisks. (**b**) Replicate cultures of U2OS cells were treated with siRNAs against Claspin, Chk1, or luciferase control for 24 h, treated with 2 μM thapsigargin for 1 h as indicated, pulse labelled with BrdU for 15 min, then harvested and analysed by flow cytometry as described in Materials and methods. (**c**) Replicate cultures of U2OS cells in exponential growth were treated with 2 μM thapsigargin for 1 h with or without pre-treatment with 1 μM UC01. Cultures were pulse labelled with BrdU during the final 15 min of treatment, then harvested and analysed by flow cytometry. (**d**) Replicate cultures of U2OS cells in exponential growth were treated with 2 μM thapsigargin for 1 h with or without pre-treatment with 0.1 μM AZD7762. Cultures were pulse labelled with BrdU during the final 15 min of treatment, then harvested and analysed by flow cytometry.

DISCUSSION

The UPR is increasingly recognized as a major determinant of cell survival under diverse conditions of external or endogenous stress and is considered to play a role in numerous pathological conditions, including cancer.[3,5,22] Many of the key functions of the UPR relate to the restoration or maintenance of ER homoeostasis; however, the UPR, and in particular its effector kinase PERK, can also affect cell cycle progression. Prolonged activation of PERK results in cell cycle arrest in the G1 phase owing to depletion of the highly labile cyclin D1 protein as a result of global protein synthesis inhibition.[17] Cyclin D1 is required for retinoblastoma protein phosphorylation, a key regulatory step in the G1–S transition, and in its absence cells become incapable of initiating DNA replication. More recently it has also been reported that PERK can also trigger arrest in the G2 phase of the cell cycle via Chk1 as part of the 'Integrated Stress Response' (ISR).[23]

Here we document an additional cell cycle effect of the UPR: inhibition of DNA replication. Unlike arrest in G1, which takes many hours to develop,[17] inhibition of DNA synthesis in response to UPR activation with thapsigargin or DTT is rapid and occurs within 1 h. We observed that thapsigargin inhibited DNA synthesis in multiple cancer cell lines and in normal BJ fibroblasts and RPE cells, indicating that it is a general phenomenon. The scale of DNA synthesis inhibition induced by thapsigargin varied between cell lines; however, we saw no obvious distinction between normal and transformed cells. DNA fibre combing experiments revealed that thapsigargin-induced DNA synthesis inhibition results from a combination of replication fork slowing and suppression of replication origin firing. As far as we are aware this is the first time a functional connection between the UPR and DNA replication has been described. Importantly, DNA synthesis became resistant to thapsigargin when PERK was inhibited using either a selective small-molecule inhibitor or siRNA-mediated depletion, indicating that this UPR effector kinase is the principal mediator of replication fork slowing and origin suppression under conditions of ER stress.

In considering potential downstream targets through which PERK could suppress DNA replication, we chose to examine the ATR–Claspin–Chk1 pathway for several reasons. Firstly, it is known that Chk1 and Claspin can regulate replication origin firing and fork stability,[10,11,13] and secondly, Claspin is both required for Chk1 activation[19] and may also play a direct role in DNA replication fork progression *per se*.[11]

We observed that Chk1 was activated in response to thapsigargin as judged by phosphorylation of two positive regulatory sites, S317 and S345, that are normally modified by the upstream regulatory kinase ATR under conditions of genotoxic stress.[7] The scale of Chk1 activation by thapsigargin was less than that induced by conventional genotoxic agents such as UV or CPT, but crucially, thapsigargin-induced Chk1 S317/S345 phosphorylation occurred in the absence of DNA damage as judged by conventional surrogate markers such as γ-H2AX and phospho-RPA32.[7] These findings argue that Chk1 S317/S345 phosphorylation in response to thapsigargin is unlikely to be catalysed by ATR or the related PIKK ATM. Consistent with this interpretation, small-molecule inhibition of ATM, depletion of ATR or both together had no effect on Chk1 activation in response to thapsigargin treatment (Supplementary Figure S3). Crucially, however, Chk1 activation

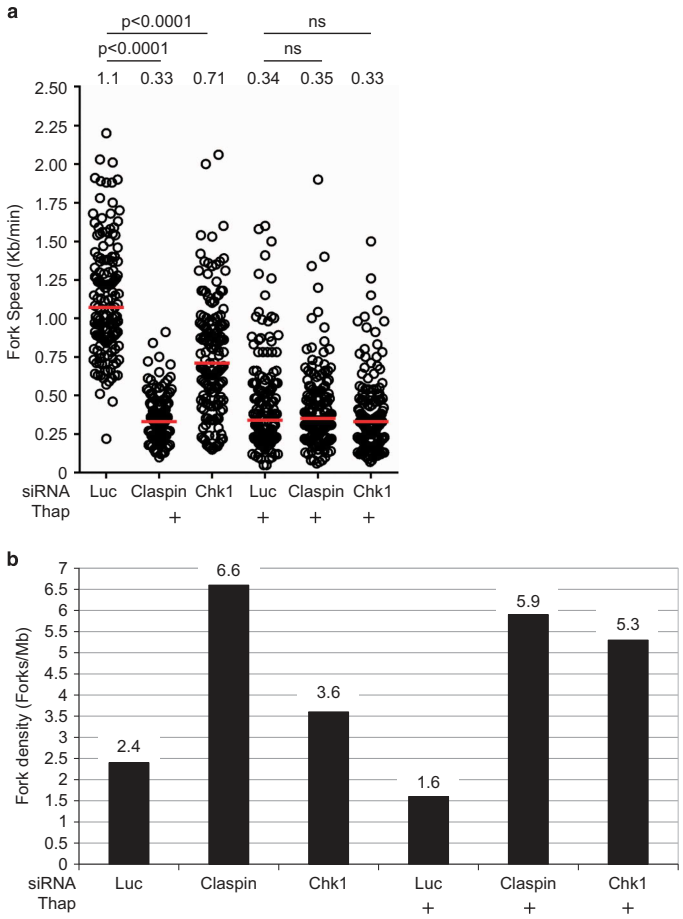

Figure 6. Depletion of Claspin or Chk1 increases thapsigargin-resistant replication origin firing. Replicate cultures of U2OS cells were treated with siRNAs against Claspin, Chk1 or luciferase control for 48 h, then treated with 0.25 μM thapsigargin for 1 h as indicated. During the final hour cells were sequentially pulse labelled for 30 min with iodo-deoxyuridine (IdU) and then chloro-deoxyuridine (CldU). Cells were then harvested, lysed and the DNA fibres analysed to determine replication fork speed (**a**) and density (**b**) as described in Materials and methods. *P* = *P*-value, NS = statistically nonsignificant.

was effectively blocked both by chemical inhibition or by depletion of PERK (Figure 4 and Supplementary Figure S3).

To gain further insight into the pathway leading from PERK to Chk1, we investigated the upstream adaptor protein, Claspin. Strikingly, thapsigargin induced a pronounced electrophoretic mobility shift of the Claspin protein that was due to phosphorylation. As with DNA synthesis inhibition and Chk1 activation, thapsigargin-induced Claspin phosphorylation was blocked by inhibition of PERK. Furthermore, Chk1 S317/S345 phosphorylation in response to thapsigargin required Claspin, strongly arguing that PERK-dependent Chk1 activation is mechanistically linked to Claspin phosphorylation.

Depletion or inhibition of either Claspin or Chk1 rendered bulk DNA replication significantly resistant to inhibition by thapsigargin; however, this was not due to reversal of replication fork slowing, but instead to an increase in replication origin firing that was now resistant to inhibition by thapsigargin. Based on these observations we propose the model shown in Figure 8. In response to ER stress induced by the non-genotoxic UPR agonist thapsigargin, PERK is activated and acts to suppress DNA synthesis in S-phase cells by both slowing the rate of replication fork progression and suppressing replication origin firing. Downstream of PERK we propose that Claspin and Chk1 together mediate PERK-dependent replication origin suppression, whereas because replication fork slowing is unaffected by Chk1 or Claspin depletion (Figure 8) it seems probable a distinct mechanism is engaged.

A number of questions arise from these findings. Firstly, how does PERK activate Chk1 in conjunction with Claspin in the absence of genotoxic stress? Direct phosphorylation of Claspin and/or Chk1 by PERK itself seems unlikely, since PERK resides in the lumen of the ER while Claspin and Chk1 are both considered to be nuclear proteins. Indeed, our finding that

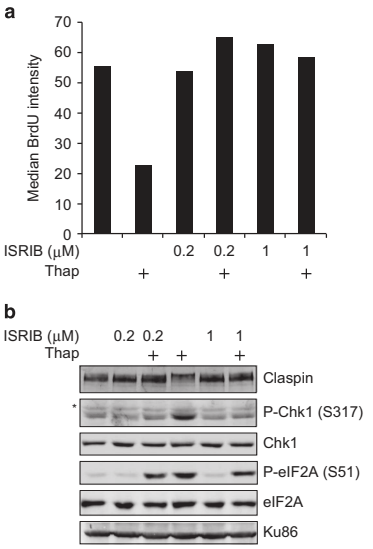

Figure 7. eIF2A inhibition is required for PERK-mediated suppression of DNA replication and Claspin phosphorylation. (**a**) Replicate cultures of U2OS cells in the exponential growth phase were pre-treated with 0.2 or 1 μM ISRIB inhibitor or solvent control for 30 min after which all cultures were treated with 2 μM thapsigargin for 1 h. BrdU was added for the last 15 min of the thapsigargin treatment after which cells were fixed and processed for flow cytometry. (**b**) U2OS cell cultures were treated as in (**a**), lysed and analysed by western blotting using the indicated antibodies.

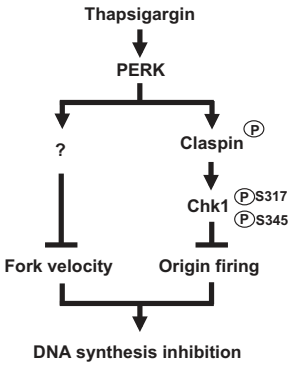

Figure 8. Hypothetical model depicting how PERK regulates DNA replication. Please refer to the text for additional details and explanation.

the ISRIB inhibitor blocks Claspin phosphorylation in thapsigargin-treated cells while PERK remains active argues that the kinase responsible for this modification is likely activated downstream of PERK-mediated phosphorylation and inhibition of eIF2A. The sites of PERK-dependent phosphorylation of Claspin are currently unknown; however, Chk1 S317/S345 are proceeded by glutamine residues (SQ), a motif preferred by PIKKs such as ATR and ATM, suggesting that a kinase with similar substrate specificity may catalyse phosphorylation of Chk1 in response to thapsigargin treatment. Further work will be required to identify the kinase(s) responsible for PERK-dependent Claspin and Chk1 phosphorylation and to elucidate the detailed molecular mechanisms involved.

Secondly, how does PERK slow replication fork progression? Replication fork slowing via DNA polymerase inhibition classically results in exposure of single-stranded DNA due to polymerase–helicase uncoupling leading to activation of ATR and Chk1 via a well-defined checkpoint mechanism. In this situation replication fork slowing or stalling is invariably accompanied by ectopic origin firing as cells presumably attempt to maintain DNA synthesis at normal levels.[10,11] By contrast, PERK-mediated replication fork slowing occurs without evidence of single-stranded DNA generation or ATR activation as judged by conventional markers such as formation of γ-H2AX and phosphorylation of RPA32, or crucially, of ectopic origin firing. These observations argue strongly that PERK controls a mechanism that can slow replisome progression without the pathological consequences of polymerase–helicase uncoupling. Interestingly, a recent study reported that DNA replication can be suppressed in the absence of conventional checkpoint activation when histone biosynthesis is inhibited.[24] In this study replication fork slowing was also observed to occur without compensatory origin firing.[24] However, we did not observe any change in histone levels in response to thapsigargin treatment in our experiments (Supplementary Figure S4), indicating that histone depletion cannot explain the phenomenon we document here. Taken together, these and other observations suggest the existence of an intrinsic checkpoint-like mechanism that can slow replication fork progression when protein synthesis rates are depressed or when protein folding is compromised. The precise nature and molecular targets of this mechanism remain to be elucidated.

Finally, it is thought that UPR signalling is frequently active in tumours owing to a variety of adverse extracellular and intracellular stress conditions.[4] It seems likely therefore that the phenomenon we document here, namely suppression of DNA replication via PERK, may occur naturally in at least a proportion of tumours. Studies have shown that UPR activation can diminish the toxicity of replication-directed chemotherapeutics, such as topoisomerase inhibitors[25] and gemcitabine.[26] It seems possible therefore that UPR inhibition using for example PERK inhibitors might provide a strategy for enhancing the efficacy of such treatments.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

U2OS cells were grown using standard procedures as described previously.[27] Antibodies obtained from commercial sources were as follows: eIF2A (FL-315), Ku86 (C-20), Chk1 (G-4) and PERK (H-300) from Santa Cruz Biotechnology (Dallas, TX, USA); γ-H2AX and β-actin from Genscript (Jiangning, China); p-Ser51-eIF2A and pSer345-Chk1 from Cell Signaling Technology (Boston, MA, USA); pSer317-Chk1 from R&D Systems (Mineapolis, MN, USA); and pSer4,8-RPA32 from Bethyl Laboratories (Montgomery, TX, USA). Antiserum against Claspin was generated in-house as described.[28] Lambda (λ) phosphatase was purchased from New England Biolabs (Ipswich, MA, USA), thapsigargin from Cayman Chemical Company (Ann Arbor, MI, USA), PERK Inhibitor I (GSK2606414) from Calbiochem (San Diego, CA, USA) and UCN01, AZD7762 and ISRIB from Sigma-Aldrich (St Louis, MO, USA).

Transfection

The following siRNA oligonucleotides were used: Luciferase 5′-UCGAAGUA UUCCGCGUACG-3′ (Thermo Fisher Scientific, Waltham, MA, USA), Claspin 5′-GCACAUACAUGAUAAAGAA-3′ (Dharmacon, Lafayette, CO, USA), Chk1 5′-GCGUGCCGUAGACUGUCCA-3′ (Thermo Fisher Scientific) and PERK 5′-GCAUGCAGUCUCAGACCCA-3′ (Thermo Fisher Scientific). Cells were transfected with siRNAs using lipofectamine RNAiMax (InVitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Flow cytometry

For cell cycle analysis, cells were collected by trypsinization and fixed in 70% ethanol at 4 °C for a minimum of 2 h. After fixation, cells were washed with phosphate-buffered saline (PBS), and the DNA was stained with 25 μg/ml propidium iodide. For BrdU staining, cells were incubated with BrdU 10μM for 15 min. After fixation, cells were washed with 0.5% PBS-T (0.5% Tween-20 in PBS) and then incubated in denaturing solution (0.5% Triton X-100, 2 M HCl) for 30 min at 37 °C. Then cells were neutralizated with 1 M Tris-HCl pH 7.5. After washing with PBS, cells were incubated with anti-BrdU antibody (GenScript) in BSA-T-PBS (1% BSA, 0.5% Tween-20 in PBS) for 16 h at 4 °C. After washing with BSA-T-PBS, cells were incubated with Alexa 647 secondary antibody (Life Technologies, Carlsbad, CA, USA) followed by staining with 25 μg/ml propidium iodide. The samples were analysed by flow cytometry using a MacsQuant Analyzer (Miltenyi Biotec, San Diego, CA, USA).

DNA combing and imagine acquisition

Molecular combing and immunodetection was performed as previously described.[29,30] Cells were pulse labelled 30 min with iodo-deoxyuridine, and then chloro-deoxyuridine. Next, incorporation of halogenated nucleotides was blocked by thymidine addition, cells were harvested and DNA fibres were purified by digestion of proteins in agarose plugs and subsequently stretched at a rate of 2 kb/μm on silanized coverslips prepared as previously described.[31] Immunodetection of neo-synthesized DNA and DNA fibres was performed as previously described.[18] Measurements of fork speed and fork density were performed with MetaMorph software (Roper Scientific, Martinsried, Germany). We systematically used DNA counterstaining to ensure that replication signals belong to the same fibre, that two fibres are not overlapping and that signals were contiguous.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (SAF2013-49149-R, BFU2014-51672-REDC to RF) and Fundacion CajaCanarias (AP2015/008 to RF), the IMBRAIN Project (FP7-REGPOT-2012-CT2012-31637-IMBRAIN to DAG) funded by EU FP7 and Gobierno de Canarias, and World Wide Cancer Research (WWCR; Project grant no. 12-0149 to DAG).

AUTHOR CONTRIBUTIONS

EC, SH, SK, MD, DK and MS performed experiments and data analysis. EC, SH, RF and DAG designed experiments and prepared the manuscript.

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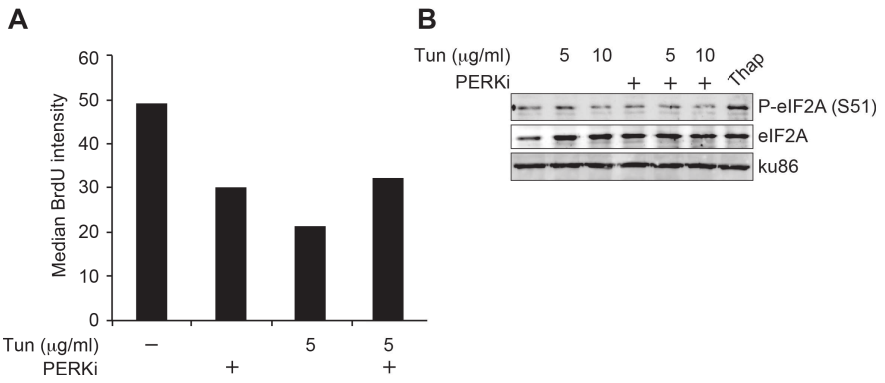
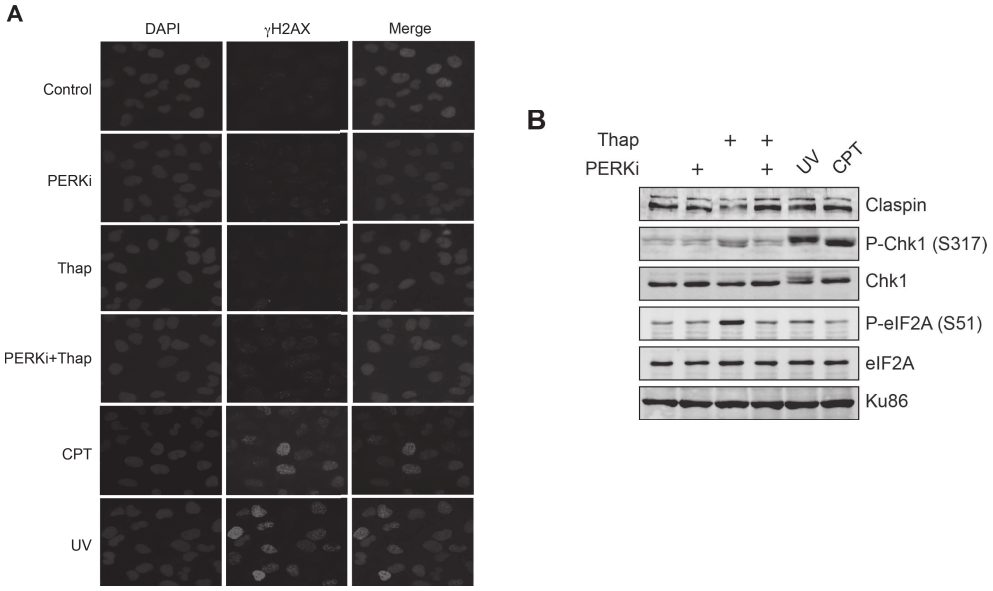
Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

SUPPLEMENTARY FIGURES

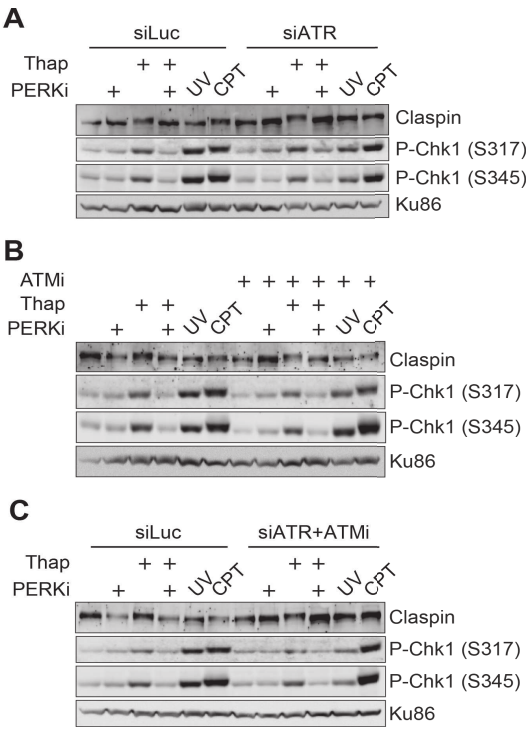


Supplementary Figure 1. Tunicamycin inhibits DNA synthesis. A) Replicate cultures of U2OS cells in exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 minutes after which all cultures were treated with 5 μg/ml tunicamycin for 7 hours. BrdU was added for the last 15 minutes of the tunicamycin treatment after which cells were fixed and processed for flow cytometry. **B)** U2OS cells cultures were treated as in (A), lysed and analysed by western blotting, using the indicated antibodies.

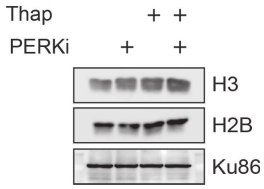
Suppl. Figure 2



Supplementary Figure 2. Thapsigargin does not induced γ-H2AX DNA damage foci.. A) Replicate cultures of U2OS cells in exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 minutes. Cultures were then treated with camptothecin (CPT; 2 μM), UV light (40J/m^2), or thapsigargin (2 μM) for 1 hour prior to harvest. Cells were fixed, processed for immunofluorescence using antibodies specific for γ-H2AX as described previously[1], counterstained with DAPI, and visualised using a Zeiss Axiovert fluorescence microscope. B) U2OS cells cultures were treated as in (A), lysed and analysed by western blotting, using the indicated antibodies.



Supplementary Figure 3. ATM or ATR activity is not required for Chk1 activation in response to thapsigargin treatment. Replicate cultures of U2OS celss were treated with siRNA against ATR or luciferase control (LUC) for 48 hours. Cultures were treated with campthothecin (CPT; 2 μM), UV light (40J/m^2), or thapsigargin (2 μM) for 1 hour prior to harvest. Where indicated cells were pre treated with the ATM inhibitor KU-55933 (10 μM) for 2 hours or PERK inhibitor GSK2606414 (0.5 μM) for 30 minutes. Cells were then harvested and analysed by western blotting using the indicated antibodies.



Supplementary Figure 4. Replicative culture of cells in exponential growth phase were pre-treated with 0.5 μM PERK inhibitor or solvent control for 30 minutes after which all cultures were treated with 2 μM thapsigargin for 1 hour. Cells were then harvested and analysed by western blotting using the indicated antibodies.

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DISCUSSION

"The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom."

Isaac Asimov

1. The ubiquitin hydrolase USP37 stabilizes a phosphorylated form of Cdt1

USP37 was firstly described to control the stabilization of Cyclin A by counteracting the ubiquitination by APC^{Cdh1} (Huang et al., 2011). Later, USP37 was shown to regulate other pro-division regulators such as c-Myc or 14-3-3γ (Kim et al., 2015; Pan et al., 2014). Moreover, USP37 regulates genomic stability by controlling WAPL, a negative regulator of chromatin cohesion (Typas et al., 2015; Yeh et al., 2015).

In this thesis we identified a Cdt1 slow electrophoretic mobility form that is stabilized by USP37. This Cdt1 form is also detected by treating cells with the proteasome inhibitor MG132 suggesting that is an unstable isoform of Cdt1. Moreover, we also observe that USP37 interacts with the slow migrating form with higher affinity than the non-modified faster migrating form of Cdt1. Phosphorylation of Cdt1 by CDKs was described before (Liu et al., 2004) and it was shown that this phosphorylation enhanced the interaction with the SCF-Skp2 ubiquitin ligase complex and targets Cdt1 for ubiquitination and degradation (Li et al., 2003). To explore if USP37 was mainly stabilizing this phosphorylated form of Cdt1, we performed treatment with lambda phosphatase. Indeed, the treatment decreased the slow migrating band stabilized after USP37 overexpression. Altogether our data indicate that the Cdt1 mobility shift is due to a phosphorylation that triggers Cdt1 polyubiquitination and proteasomal dependent degradation and USP37 is able to reverse this last step.

2. USP37 regulates Cdt1 during G1 and G1/S phases

To understand the biological significance of the Cdt1 regulation by USP37, the levels of both proteins were examined during the cell cycle as levels of the two proteins are controlled by proteasome degradation during the cell cycle (Huang et al., 2011; Nishitani et al., 2000). Indeed, we observed that both USP37 and Cdt1 shown different levels during the cell cycle and while USP37 levels were high at the G1/S transition and in S and G2, Cdt1 protein levels were high in G1 and mitosis and low in S/G2 phases. However, although low in different stages of cell cycle, the levels of Cdt1 and USP37 were detectable throught the cell cycle. To study in which stage of cell cycle USP37 stabilizes Cdt1, we downregulated USP37 in synchronized cells and study Cdt1 levels. Interestingly downregulation of USP37 reduced the Cdt1 levels in G1 and G1/S but not during S phase. This indicates that although USP37 levels are low in G1, the DUB is important to regulate Cdt1 in that phase, but not in others.

3. USP37 controls the DNA replication by different mechanisms

In this work, we were interested to investigate the role of USP37 in DNA replication, as Cdt1 is a main player on DNA replication licensing. On one hand, we could detect a delay in replication fork progression measured by DNA fiber analysis in USP37 depleted cell and this effect was likely to be independent of a reduced availability of nucleotides, as the USP37 siRNA did not decrease the levels of the subunits RRM1 or RRM2 of the ribonucleotide reductase. Also, the overexpression of USP37 did not lead a significant change in fork speed parameters. On another hand, overexpression of USP37 in cells synchronized in S phase leads a increment of MCM 7 loading into the chromatin independent of changes in the ubiquitination of the MCM 7 complex. The fact that overexpression of USP37 did not change replication parameters or cell cycle profiles in spite of increasing MCM 7 loading suggest that

other known mechanisms controlling initiation of replication are able to maintain replication initiation levels normal after even if Cdt1 protein is upregulated. If the only effect on replication of USP37 is the control of Cdt1, the downregulation of USP37 would decrease the number of initiations. On the contrary, USP37 siRNA increases initiation of replication and decreases replication fork speed. This suggest that USP37 regulates one/several unidentified replication proteins involved in DNA replication fork progression and this will be an interesting subject to follow up. Taken the data together, we conclude that USP37 seems regulate the DNA replication at least in two levels. First, by controlling the loading of MCM 2-7 into the chromatin by regulating the stability of Cdt1 and second, by controlling the replication speed by regulating other target protein/s involved in fork progression different than Cdt1.

4. The ubiquitin hydrolases DUB3 and USP7 stabilize Geminin

In this thesis we have shown that depletion of both DUBs decreases Geminin levels/stability and overexpression of both DUBs increases Geminin stability. Moreover, both DUB3 and USP7 interact with Geminin and both wild type DUB3 and USP7 decrease the ubiquitination status of Geminin *in vivo*. However, overexpression of a catalytic version of USP7 was also able to decrease ubiquitinated Geminin levels, whereas overexpression of the inactive version of DUB3 was unable to do so. This would indicate that the mechanism by which DUB3 stabilizes Geminin is mainly by the enzymatic activity of the protein and USP7 might stabilize Geminin by an additional mechanism. Moreover, only wild type DUB3 (and not USP7) was able to de-ubiquitinate Geminin *in vitro*. We speculate with a non-enzymatic function of USP7, where the physical interaction of both wild type or the catalytic inactive versions of USP7 avoid the ubiquitination of Geminin.

5. DUB3 but not USP7 increases re-replicative events

Geminin depletion increase the re-replicative events in some human cancer cell lines. In this research we measured re-replication by BrdU incorporation and flow cytometry analysis. In U2OS cells both Geminin and DUB3 depletion increase re-replicative events, however USP7 depletion produced a strong inhibition of the S phase and arrest at the G1 phase of the cell cycle, consistent with recent reports (Lecona et al., 2016; Smits and Freire, 2016). We obtained similar results using the breast tumour derived cell line MCF7. However, using the non-transformed breast cell line MCF10A the re-replicative events appears only in Geminin depleted cells. Increased re-replication was not observed in DUB3 or USP7 depleted cells. It is possible that normal cell lines, such as MCF10A, have extra mechanisms to avoid re-replication of the DNA and only re-replicative events can occur when very low levels of Geminin are present in the cell, like it occurred in siRNA Geminin cells but did not occur in DUB3 or USP7 siRNA treated cells. Also, we noticed that DUB3 siRNA increased the percentage of MCF10A G1 cells, so it is also possible that DUB3 depletion affect cell cycle in a similar way as USP7 depletion in the other tested cell lines. It is interesting to notice that other articles do not report changes in re-replication as measured with PI/BrdU in Geminin depleted cells by in several human cell lines including Hela (Kulartz and Knippers, 2004; Machida and Dutta, 2007; Nishitani et al., 2004) and 293T (Teer and Dutta, 2008), or in NHF1-hTert fibroblasts (NFH1) cells (Dorn et al., 2009). However, depletion of Geminin in NFH1 cells increased re-replication events when re-replication was measured by DNA fiber analysis (Dorn et al., 2009). Therefore, it is also possible that downregulation of DUB3 in MCF10A cells produces an increase of re-replicative that can not be detected by flow cytometry analysis.

6. USP7 as a marker of chromosomal instability in cancer

Since control of replication is key for genomic stability and several DUBs control Geminin/Cdt1, it was possible that expression of the DUBs could be related with cancer progression. We described that USP37 controls Cdt1 and DUB3 and USP7 control Geminin and the three DUBs are candidate markers for cancer prognosis. Although we tried, we could not find antibodies that work to detect DUB3 and USP37 by immunochemistry and therefore we focussed to study the levels of USP7 and Geminin in a human clinical sample cohort. Interestingly, expression of USP7 is strongly correlated with the expression of Geminin in breast cancer. Consistent with previous reports (Gonzalez et al., 2004; Sundara Rajan et al., 2014; Yagi et al., 2016), Geminin expression strongly and directly correlated with features of proliferative tumours: grade, mitotic score, nuclear pleomorphism, Ki67 expression, triple-negative phenotype and pushing margins. In addition, low and high levels of USP7 is associated with less favorable prognosis than moderate expression suggesting that abnormal expression of USP7 is associated with therapy resistance and metastatic progression in breast cancer.

Moreover our data reveals the complex role of USP7 in replication as although USP7 controls the proliferation, we have shown that USP7 as well controls the inhibition of the replication. Therefore, low levels of USP7 could inhibit the replication progression at the same time that activate the replication initiation, leading a critical scenario for the cell. We found that low and high levels of USP7 were associated with a bad prognosis, and this fact fit with the idea that both low and high levels misregulate DNA replication in different ways.

7. UPR controls the DNA replication

UPR maintains ER homeostasis, but one of its effectors, the kinase PERK, additionally plays a role in the cell cycle progression. Prolonged activation of PERK leads cell cycle arrest in G1 phase and synthesis inhibition due to a depletion of Cyclin D1, a critical activator of G1-S progression (Brewer and Diehl, 2000). Moreover, PERK is required to arrest in the G2 phase via Chk1 as part of the 'Integrated Stress Response' (ISR) (Malzer et al., 2010).

In this thesis we document the inhibition of DNA replication as an additional cellular effect of UPR activation by thapsigargin. We observed that thapsigargin inhibited DNA synthesis in multiple cancer cell lines and in normal BJ fibroblasts and RPE cells, indicating that it is a general phenomenon. The inhibition of DNA replication by thapsigargin occurs at two levels, by slowing replication fork speed and decreasing the replication origin firing. Moreover, the suppression of replication origin firing by UPR occurs via its effector kinase PERK, and interestingly Claspin and Chk1 acts downstream. However, the slow in replication fork speed depends on PERK and not on Claspin or Chk1. Moreover, in response to thapsigargin Claspin become phosphorylated in a PERK dependent manner. However, PERK localizes at the ER and Claspin in the nucleus and therefore, we speculate with an intermediate kinase that is able to phosphorylate Claspin and possibly mediate in the inhibition of initiation of DNA replication. Our work leaves open the question on how PERK slows replication fork progression. The classical slow-down of replication fork progression via DNA polymerase also activates the checkpoint mechanism ATR-Claspin-Chk1 due to an increment of single stranded DNA produced by the helicase uncoupling to the delayed replication fork (Petermann et al., 2006; Petermann et al., 2008). However, after thapsigargin treatment, no single-strand generation was observed judged by γ-H2AX or P-RPA signal, indicating that PERK controls a mechanism that can slow replisome progression without polymerase-helicase

uncoupling mechanism. Interestingly, recent studies shown that the fork slowing progression and post compensation with origin firing can occur in the absence of DNA checkpoint activation when histone biosynthesis is inhibited (Mejlvang et al., 2014). However, we did not observe any change in histone levels in response to thapsigargin treatment in our experiments. Therefore, a different, yet unidentified mechanism is activated by thapsigargin to control replication speed progression.

We have shown the phosphorylation of Claspin after thapsigargin treatment, however we have unable to identified a possible kinase for Claspin. We tested if p38, a MAPK implicated in different type of stress, PKR and GSK3β, also involved in phosphorylation of the eIF2α, were responsible of the Claspin shift after thapsigargin treatment, but withouth success.

In order to identify possible kinases for Claspin, we will perform a screening using kinases inhibitors and, we will measure the EdU incorporation in the presence of thapsigargin. PERKi will be the positive control. In addition, we will test by mass spectometry Claspin interactors in the presence or not of thapsigargin inn order to know the interactors and phosphorylation sites of Claspin.

8. UPR inhibition as a possible tumour therapy

It is thought that UPR signalling is frequently active in tumours owing to a variety of adverse extracellular and intracellular stress conditions (Ballabeni et al., 2004). It seems likely therefore that the phenomenon we document here, namely suppression of DNA replication via PERK, may occur naturally in at least a proportion of tumours. Studies have shown that UPR activation can diminish the toxicity of replication directed chemotherapeutics, such as topoisomerase inhibitors (Mann et al., 2012) and gemcitabine (Palam et al., 2015). It seems possible therefore that UPR inhibition using for example PERK inhibitors might provide a strategy for enhancing the efficacy of such treatments. In a future, we would like to analyze DNA replication in tumoral cells with high rate of UPR activation. Thus, we will study the cell lines where the inhibition of PERK increases the DNA replication rate and test the sensitivity to different cytotoxic agents that produce DNA replication stress such as gemcitabine.

CONCLUSIONS

"You do not really understand something unless you can explain it to your grandmother."

Albert Einstein

1. The ubiquitin hydrolase USP37 interacts and stabilizes directly a phosphorylated form of Cdt1.

2. Wild type USP37 but not a catalytic inactive version de-ubiquitinates Cdt1 *in vivo*.

3. USP37 controls replication fork speed.

4. Ectopic overexpression of USP37 increases the MCM 7 loading into the chromatin.

5. The ubiquitin hydrolases DUB3 and USP7 interact and stabilize directly to Geminin.

6. Wild type DUB3 but not a catalytic inactive version de-ubiquitinates Geminin *in vivo*.

7. Wild type USP7 but not a catalytic inactive version to lesser extent deubiquitinate Geminin *in vivo*.

8. Wild type DUB3 deubiquitinates Geminin *in vitro*.

9. The depletion of DUB3 increase re-replication events in U2OS and MCF-7 cells.

10. Geminin and USP7 expression strongly correlates in breast cancer.

11. Geminin and USP7 expression strongly correlates with markers of chromosomal instability in breast cancer.

12. PERK controls the DNA replication after activation the Unfolded Protein Response (UPR) with thapsigargin by reduction of fork speed and origin firing.

13. PERK inhibits DNA replication after UPR activation involves the phosphorylation of the checkpoint adaptor protein Claspin and activation of the Chk1 effector kinase.

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"Most people say that it is the intellect which makes a great scientist. They are wrong: it is character."

Albert Einstein

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APPENDIX

1.Plasmids

The ubiquitin hydrolase screening has been performed using the following plasmid:

Enzyme	Original vector
ATXN3	pcDNA3.1/Hygro+ (Flag/Myc)
BAP1	pDEST-LTR-N-FLAG-HA-IRES -puro
CEZANNE	pHM6-HA
COPS5	pDEST-LTR-N-FLAG-HA-IRES -puro
CXORF53	pOZ-FH-C (Flag/HA)
CYLD	pDEST-LTR-N-FLAG-HA-IRES -puro
DUB3	pDEST-LTR-N-FLAG-HA-IRES -puro
DUBA1	pDEST-LTR-N-FLAG-HA-IRES -puro
DUBA3	pDEST-LTR-N-FLAG-HA-IRES -puro
EIF3S3	pDEST-LTR-N-FLAG-HA-IRES -puro
EIF3S5	pDEST-LTR-N-FLAG-HA-IRES -puro
HDAC6	pCDNA3.1 (Flag/HA)
JOSD1	pDEST-LTR-N-FLAG-HA-IRES -puro
JOSD2	pDEST-LTR-N-FLAG-HA-IRES -puro
JOSD3	pDEST-LTR-N-FLAG-HA-IRES -puro
OTUB1	pDEST-LTR-N-FLAG-HA-IRES -puro
OTUB2	pDEST-LTR-N-FLAG-HA-IRES -puro
OTUD1	pDEST-LTR-N-FLAG-HA-IRES -puro
OTUD3	pCMV-Tag2B (Flag)
OTUD4	pDEST-LTR-N-FLAG-HA-IRES -puro
OTUD6A	pCMV-Tag2B (Flag)
OTUD6B	pDEST-LTR-N-FLAG-HA-IRES -puro
PARP11	pDEST-LTR-N-FLAG-HA-IRES -puro
PRPF8	pCMV-Tag2B (Flag)
PSMD14	pDEST-LTR-N-FLAG-HA-IRES -puro
SENP1	pDEST-LTR-N-FLAG-HA-IRES -puro
SENP2	pEGFP-C1
SENP5	p3xFLAG-CMV-10
SENP6	pEGFP-C1

Enzyme	Original vector
SENP7	peGFP-C1
SENP8	pDEST-LTR-N-FLAG-HA-IRES -puro
STAMBP	pME-FLAG
STAMBPL1	pME-FLAG
TNFAIP3	pDEST-LTR-N-FLAG-HA-IRES -puro
TRABID	pHM6-HA
UCHL1	pOZ-N (Flag/HA)
UCHL3	pOZ-N (Flag/HA)
UCHL5	pCDNA3 (Flag/HA)
USP1	pCMV-Tag2B (Flag)
USP10	pCMV-Tag2B (Flag)
USP11	pCMV-Tag2B (Flag)
USP12	pDEST-LTR-N-FLAG-HA-IRES -puro
USP13	pDEST-LTR-N-FLAG-HA-IRES -puro
USP14	p pCDNA3.1 (Flag/HA)
USP15	pDEST-LTR-N-FLAG-HA-IRES -puro
USP16	pOZ-FH-N (Flag/HA)
USP18	pFLAG-CMV5-USP18
USP19	His-3xFLAGUSP19-WT-pcDNA3
USP2	pDEST-LTR-N-FLAG-HA-IRES -puro
USP20	pDEST-LTR-N-FLAG-HA-IRES -puro
USP21	pDEST-LTR-N-FLAG-HA-IRES -puro
USP22	pcDNA3.1 /Flag/HA)
USP24	pCMV-Tag2B (Flag)
USP25	pCMV-Tag2B (Flag)
USP26	pDEST-LTR-N-FLAG-HA-IRES -puro
USP28	pCMV-Tag2B (Flag)
USP29	pDEST-LTR-N-FLAG-HA-IRES -puro
USP3	pOZ-FH-N /Flag/HA)
USP30	p3xFLAG-CM-14- hUSP30-Flag
USP31	pDEST-LTR-N-FLAG-HA-IRES -puro
USP33	pDEST-LTR-N-FLAG-HA-IRES -puro
USP34	pCMV-Tag2B (Flag/HA)
USP35	pCMV-Tag2B (Flag/HA)
USP36	pDEST-LTR-N-FLAG-HA-IRES -puro
USP37	pDEST-LTR-N-FLAG-HA-IRES -puro

Enzyme	Original vector
USP38	pDEST-LTR-N-FLAG-HA-IRES -puro
USP39	pDEST-LTR-N-FLAG-HA-IRES -puro
USP4	pCMV-Tag2B (Flag/HA)
USP42	pDEST-LTR-N-FLAG-HA-IRES -puro
USP43	pDEST-LTR-N-FLAG-HA-IRES -puro
USP44	pDEST-LTR-N-FLAG-HA-IRES -puro
USP45	pDEST-LTR-N-FLAG-HA-IRES -puro
USP46	pDEST-LTR-N-FLAG-HA-IRES -puro
USP47	pcDNA 3.0 (Flag)
USP48	pDEST-LTR-N-FLAG-HA-IRES -puro
USP49	pDEST-LTR-N-FLAG-HA-IRES -puro
USP5	pDEST-LTR-N-FLAG-HA-IRES -puro
USP50	pDEST-LTR-N-FLAG-HA-IRES -puro
USP52	pDEST-LTR-N-FLAG-HA-IRES -puro
USP53	pDEST-LTR-N-FLAG-HA-IRES -puro
USP54	pCMV-Tag2B (Flag/HA)
USP6	pcDNA3 (Flag/HA)
USP7	pCMV-Tag2B (Flag/HA)
USP8	pDEST-LTR-N-FLAG-HA-IRES -puro
USP9X	pDEST-LTR-N-FLAG-HA-IRES -puro
USPL1	pcDNA3.1 (Flag/HA)

2. siRNA oligos

siRNA oligo	Sequence 5'- 3`
Luciferase	UCGAAGUAUUCCGCGUACGdTdT
USP37#1	CAGCUAAGUCAUAACAUUAdTdT
USP37#2	CCAAGGAUAUUUCAGCUAAdTdT
USP37#3	GAAUAAAGUCAGCCUAGUAdTdT
Cdt1	AACGUGGAUGAAGUACCCGACdTdT
USP7	GGCAACCUUUCAGUUCACUdTdT
Dub3	CCUCCGUGAUGUUGCUUGAdTdT
Geminin	UGCCAACUCUGGAAUCAAAdTdT
PERK	GCAUGCAGUCUCAGACCCAdTdT
Claspin	GCACAUACAUGAUAAAGAAdTdT
Chk1	GCGUGCCGUAGACUGUCCAdTdT

3. Primary antibodies

Antibody	Origin	Source
β-actin (AC-15)	Mouse	Sigma-Aldrich
USP37 (ab190184)	Rabbit	Abcam
Cdt1 (ab70829)	Rabbit	Abcam
Cdt1 (H-300)	Rabbit	Santa Cruz Biotechnology
Cdt1 serum	Rabbit	Homemade
Kap1 (ab190178)	Rabbit	Abcam
ɣ-H2AX (JBW301)	Mouse	Merck-Millipore
Ubiquitinated protein FK2 (#04-263)	Mouse	Merck-Millipore
Chk1 (G4)	Mouse	Santa Cruz Biotechnology
RRM2 (N-8)	Goat	Santa Cruz Biotechnology
GADPH (FL-335)	Rabbit	Santa Cruz Biotechnology
pSer317-Chk1 (AF2054)	Rabbit	R&D Systems
pSer10 histone H3	Rabbit	GenScript
Flag	Mouse	GenScript
RRM1 (#3388)	Rabbit	Cell Signalling Technology
Ubiquitin (P4D1)	Mouse	Cell Signalling Technology
Rad 9	Rabbit	Homemade
eIF2A (FL-315)	Rabbit	Santa Cruz Biotechnology
PERK (H-300)	Rabbit	Santa Cruz Biotechnology
ɣ-H2AX	Rabbit	GenScript
β-actin	Mouse	GenScript
pSer51-eIF2A	Rabbit	Cell Signalling Technology
pSer345-Chk1	Rabbit	Cell Signalling Technology
pSer317-Chk1	Rabbit	R&D Systems
pSer4,8-RPA32	Rabbit	Bethyl Laboratories
Claspin serum	Rabbit	Homemade
DUB3	Rabbit	Homemade
USP7 (ab190183)	Rabbit	Abcam
Geminin (ab175799)	Rabbit	Abcam
Ku86 (C-20)	Goat	Santa Cruz Biotechnology
USP7 IHC (ab119364)	Mouse	Abcam

"Hakuna Matata"

Timón y Pumba.