Role of the ubiquitin-selective CDC-48$^{UFD-1/NPL-4}$ chaperone in DNA replication

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· Abbreviations ·

°C	degree Celsius
µg	microgramme
µl	microlitre
µm	micrometre
9-1-1 complex	Rad9-Rad1-Hus1 complex (HPR-9-MRT-2-HUS-1 in *C. elegans*)
aa	amino acids
AAA	ATPases associated with diverse cellular activities
ATM / ATM-1	ataxia telangiectasia, mutated, protein 1
ATP	adenosine triphosphate
ATPase	ATP hydrolase
ATR / ATL-1	ATM and Rad3-related
ATRIP	ATR interacting protein
aU	arbitrary units
BRCA1 / BRC-1	breast cancer 1
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CDC-45	cell division cycle protein 45
CDC-48 / Cdc48	cell division cycle protein 48
CDC-6	cell division cycle protein 6
CDK	cyclin dependent kinase
CDT-1 / Cdt1	chromatin licensing and DNA replication factor 1
CDT-2	chromatin licensing and DNA replication factor 2
CGC	*Caenorhabditis* Genetics Center
CHK-1	checkpoint kinase 1
cm	centimetre
CSN-5	COP9 signalosome subunit 5
C-terminal	carboxy-terminal
CUL-1	cullin protein 1
CUL-2	cullin protein 2
CUL-4	cullin protein 4
DAPI	4',6-diamidino-2-phenylindole
DCN-1	defective in cullin neddylation protein 1
DDB-1	damage specific DNA binding protein 1
DDK	Dbf4-dependent Cdc7 kinase
DIV-1	DNA polymerase α-subunit
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
DUB	de-ubiquitylating enzyme, ubiquitin hydrolase
E. coli	*Escherichia coli*
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E4	ubiquitin-chain elongation factor
ER	endoplasmic reticulum
ERAD	ER associated degradation
et al.	*et alii* / *et aliae*, and others
FACT / DUF	facilitates chromatin transcription / DNA unwinding factor
G phase	gap phase
GFP	green fluorescent protein
GINS	go-ichi-ni-san complex, Japanese for 5-1-2-3

GMN-1	geminin homolog 1
h	hour(s)
H2B	histone 2 B
HECT	homologous to E6-AP carboxyl terminus domain
HeLa	Henrietta Lacks
HIM-6	high incidence of males protein 6, ortholog of human Bloom syndrome helicase
His-tag	translational histidine fusion, 5-6 residues
HPR-9	homolog of *Schizosaccharomyces pombe* Rad9
HU	hydroxyurea
HUS-1	HU sensitive protein 1
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranosid
K11	lysine 11 of ubiquitin
K48	lysine 48 of ubiquitin
K63	lysine 63 of ubiquitin
kb	kilobases
L3MBTL1	lethal (3) malignant brain tumor-like protein 1
LB	lysogeny browth
M phase	mitosis, mitotic phase
MAD	mitochondria associated degradation
Matα2	mating-type protein α2
mCherry	monomeric Cherry
MCM complex	minichromosome maintenance complex (MCM-2-7)
MCM-2	minichromosome maintenance protein 2
MCM-3	minichromosome maintenance protein 3
min	minutes
ml	millilitre
mM	millimolar
MRT-2	mortal germline protein 2, ortholog of *Schizosaccharomyces pombe* Rad1
ms	milliseconds
MUS-101	mutagen sensitive 101
NC	nitrocellulose
NED-8	NEDD8, ubiquitin-like protein
NF-κB	nuclear factor κB
ng	nanogramme
NGM	nematode growth medium
nm	nanometre
NPL-4 / Npl4	nuclear protein localization 4
N-terminal	amino-terminal
OD_{600}	optical density at 600 nm
ORC	origin recognition complex (ORC-1-6)
ORC-1	origin recognition complex protein 1
ORC-2	origin recognition complex protein 2
P1 delay	time between division of AB and P1 cell
p47	protein 47
p97	protein 97
PBS	phosphate buffered saline
PCNA / PCN-1	proliferating cell nuclear antigen
PCR	polymerase chain-reaction
PIP	PCNA interacting motif
pre-RC	pre-replicative complex
PRI-1	DNA α-primase subunit 1
PSF-1	partner of sld five 1

PSF-2	partner of sld five 2
PSF-3	partner of sld five 3
PUB	PNGase/ubiquitin associated
PUL	PLAP/UFD3/Lub1
P-value	probability-value
RAD-51	radiation sensitive protein 51
RBX-1	RING-box protein 1
rcf	relative centrifugal forces
RING	really interesting new gene domain
RNA	ribonucleic acid
RNAi	RNA interference
RPA	replication protein A
Rpb1	RNA polymerase subunit 1
rpm	rounds per minute
s	seconds
S phase	DNA synthesis phase
s.e.m.	standard error of the mean
SCF	Skp2, cullin, and F-box protein containing complex
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
seq(RNAi)	sequential RNAi
SHP	Shp1-like domain
siRNA	small interfering RNA
SKP2 / SKPT-1	S phase kinase associated protein 2
SLD-5 / Sld5	synthetic lethal with dpb11-1 protein 5
ssDNA	single stranded DNA
SUMO	small ubiquitin-like modifier
TAE	Tris base, acetic acid, EDTA buffer
TER94	transitional ER ATPase
TRA-1	XX animals transformed into males protein 1
T-test	students T-test
UBC-12	ubiquitin conjugating enzyme 12, NED-8 ligase
U-box	specialized RING finger domain
UBX	ubiquitin regulatory x
Ubx5 / UBXN7	UBX domain containing protein 5 / 7
UBXD1 / UBXN-6	UBX domain containing protein 1 / 6
UBXN-1	UBX domain containing protein 1
UBXN-2	UBX domain containing protein 2
UBXN-3	UBX domain containing protein 3
UFD-1 / Ufd1	ubiquitin fusion degradation protein 1
UFD-2	ubiquitin fusion degradation protein 2
UPS	ubiquitin proteasome system
UV	ultraviolet light
VAT	VCP-like ATPase
VBM	VCP binding motif
VCP	valosin containing protein
VIM	VCP interacting motif
WRN-1	ortholog of human WRN, Werner syndrome helicase
z.B.	zum Beispiel

· Summary ·

Faithful transmission of genomic information requires tight spatiotemporal regulation of DNA replication factors. Posttranslational modifications, such as ubiquitylation, constitute a fast and effective mechanism to control such complex protein function. The AAA-ATPase CDC-48 plays an essential role in selective protein degradation triggered by ubiquitylation. While initial studies reported a crucial function of CDC-48 in the regulation of mitotic events, an essential role of the CDC-48$^{UFD-1/NPL-4}$ complex in DNA replication has been revealed in *Caenorhabditis elegans* (*C. elegans*) recently. Since the mechanistic details of CDC-48 activity remained to be elucidated, the identification of key substrates playing a vital role during DNA duplication is of major interest.

This work describes a regulatory function of CDC-48 in the coordination of licensing and elongation events of DNA replication in *C. elegans*. In the licensing step of DNA replication, CDT-1 is loaded onto chromatin to subsequently promote the recruitment of relevant replication factors, including CDC-45 and the GINS complex. Throughout the elongation step CDC-45 and the GINS complex move with the replication fork, however, it is largely unknown how their chromatin association is controlled. CDC-48$^{UFD-1/NPL-4}$ deficient embryos stabilize the licensing factor CDT-1 exclusively on mitotic chromatin. Furthermore, worm embryos lacking *cdc-48*, *ufd-1*, or *npl-4*, show persistent chromatin association of CDC-45 and the GINS complex. Notably, the protein levels of CDC-45 and the GINS subunits SLD-5 and PSF-3 are not affected by *ufd-1* and *npl-4* (RNAi), suggesting a non-proteolytic regulation. Down-regulation of CDT-1 suppresses the chromatin association of the GINS complex in embryos disrupted for a functional CDC-48$^{UFD-1/NPL-4}$ complex. Hence, CDC-48 is supposed to orchestrate both, CDT-1 degradation and chromatin dissociation of the CDC-45/GINS complex.

In conclusion, this work describes a novel role of the ubiquitin-selective chaperone CDC-48$^{UFD-1/NPL-4}$ in the context of chromatin associated processes. Elucidating the key substrates of CDC-48 during DNA replication illustrates a critical function in safeguarding genomic stability by an unexpected principle of target protein regulation.

· Zusammenfassung ·

Die verlässliche Weitergabe der genetischen Information bedarf strikter räumlicher und zeitlicher Regulierung von Replikationsfaktoren. Derart komplexe Proteinfunktionen sind meistens durch postranslationale Modifikationen, wie z.B. Ubiquitylierung, kontrolliert. Die AAA-ATPase CDC-48 ist essentiell am Ubiquitin-vermittelten Proteinabbau beteiligt. Während CDC-48 zunächst eine zentrale Funktion in der Regulierung der Mitose zugesprochen wurde, konnte kürzlich eine entscheidende Rolle des CDC-48$^{UFD-1/NPL-4}$-Komplexes bei der DNA-Replikation in *Caenorhabditis elegans* (*C. elegans*) gezeigt werden. Die mechanistischen Details der CDC-48-Aktivität während der DNA-Replikation sind bisher unbekannt. Daher ist es von besonderem Interesse, die ausschlaggebenden Substrate zu entschlüsseln.

Diese Arbeit beschreibt die regulatorische Funktion von CDC-48 in der Koordinierung von Lizensierungs- und Elongationsprozessen bei der DNA-Replikation in *C. elegans*. Im Lizensierungsschritt der DNA-Replikation bindet CDT-1 an das Chromatin und leitet die Rekrutierung wichtiger Replikationsfaktoren, darunter CDC-45 und der GINS-Komplex, ein. CDC-45 und GINS bewegen sich während des Elongationsschrittes zusammen mit der Replikationsgabel. Es ist jedoch weitgehend unbekannt, wie deren Chromatinassoziierung kontrolliert wird. In *cdc-48, ufd-1* oder *npl-4* defizienten Embryonen ist der Lizensierungsfaktor CDT-1 spezifisch auf mitotischem Chromatin stabilisiert. Weiterhin zeigen Wurmembryonen andauernde Chromatinassoziierung von CDC-45 und des GINS-Komplexes, wenn *cdc-48*, *ufd-1* oder *npl-4* durch RNAi ausgeschaltet wurden. Bemerkenswerterweise sind die Proteinlevel von CDC-45 und der GINS-Untereinheiten SLD-5 und PSF-3 nicht durch *ufd-1* oder *npl-4* (RNAi) beeinträchtigt. Dies legt eine Regulierung nahe, die ohne Proteolyse von CDC-45 und GINS abläuft. Verminderte CDT-1 Level unterdrücken die Chromatinassoziierung des GINS-Komplexes in Embryonen, in denen CDC-48$^{UFD-1/NPL-4}$ nicht funktionsfähig ist. Folglich koordiniert CDC-48 vermutlich beide Prozesse, sowohl den Abbau von CDT-1, als auch die Chromatindissoziierung von CDC-45 und des GINS-Komplexes.

Diese Arbeit beschreibt somit eine neue Funktion des Ubiquitin-selektiven Chaperons CDC-48$^{UFD-1/NPL-4}$ in der Regulierung chromatinassoziierter Prozesse. Die Identifizierung der relevanten Faktoren während der DNA-Replikation belegt eine wesentliche Role in der Beibehaltung der genetischen Information durch ein unerwartetes Prinzip der Substrat-Regulierung.

· Chapter 1 · Introduction ·

1.1. The cell division cycle.

Cell division is an essential process in all living organisms. In order to reproduce, all organisms undergo a highly organized repeated sequence of cellular events which are summarized as the cell division cycle (cell cycle). During the DNA synthesis phase (S phase) the genomic information of a cell is accurately duplicated before chromosomes start to condense into compact units. Condensed sister chromatids will be faithfully transmitted to the daughter cells in mitosis (M phase) (Figure 1.1). Both, DNA replication and mitosis require extensive regulation to ensure genomic integrity. Thus, perturbance of the cell cycle severely affects cell division and may cause cell death or occurrence of disease due to chromosomal instability [1].

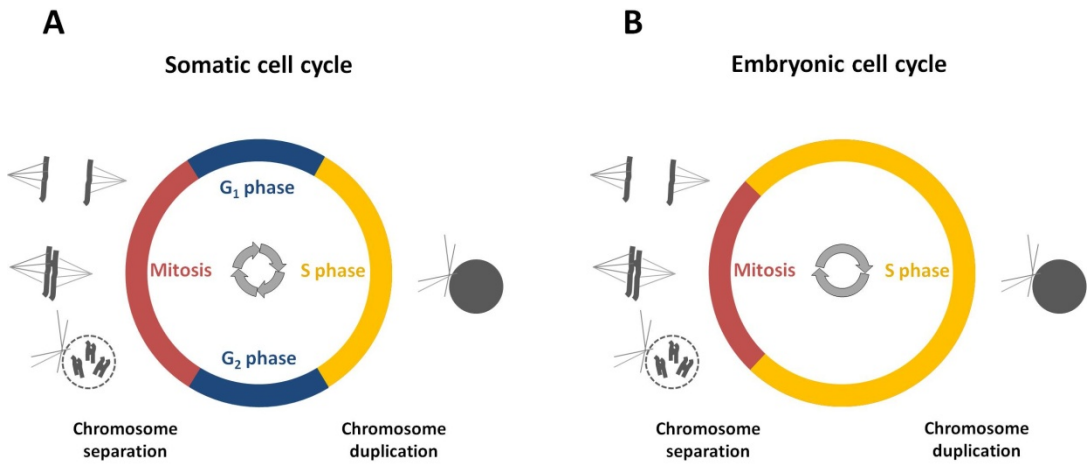


Figure 1.1. Schematic representation of the cell division cycle.
(A, B) The cell cycle consists of repeated alternation of chromosome duplication in S phase and chromosome separation in mitosis. In somatic tissues S phase and mitosis are separated by G phases (A) that are absent in early embryonic divisions (B). The morphology of microtubule organization, and S phase nuclei or condensed chromosomes during mitosis is indicated in the periphery of the cycle. Modified from [2].

In somatic cells, S phase and mitosis are separated by gap phases (G_1 and G_2 phases respectively) during which cells increase in size and execute their physiological tasks (Figure 1.1.A). This paradigm of cell cycle progression including repeated cycles of G_1, S, G_2 and M phase is modified in certain cell types [3]. Embryonic cell divisions represent one example for a cell type specific accommodation of the cell cycle, lacking apparent gap phases (Figure 1.1.B) [4]. Thereby, rapid alternation of S phase and mitosis ensures fast and efficient increase in cell number. The key factors that have been identified to drive cell cycle progression are highly

conserved in all eukaryotic organisms. Consequently, the basic regulatory mechanisms apply to all eukaryotic cell cycles, while species and cell type specific differences adapt to distinct requirements. The following paragraphs illustrate principles of eukaryotic cell cycle regulation and for simplification name the *Caenorhabditis elegans* (*C. elegans*) proteins whenever applicable [2].

1.1.1. Duplication of genomic information.

The duplication of genomic information is a prerequisite for the formation of two identical daughter cells from one single cell. This highly organized process involves spatiotemporal coordination of more than 20 essential proteins known today [5, 6]. An essential principle of regulation is achieved by subdividing the replication process in three timely separated events, the licensing, activation, and elongation steps (Figure 1.2.) [5].

In the licensing step the origins of replication are bound by the origin recognition complex (ORC). ORC serves as a loading platform for the recruitment of CDC-6 and CDT-1 together with the minichromosome maintenance (MCM) complex (Figure 1.2.). Conjointly, ORC, CDC-6, CDT-1, and the MCM complex constitute the pre-replication complex (pre-RC), which qualifies replication origins for subsequent activation (origin firing). At this step, however, the MCM complex, which is the major DNA helicase that opens the DNA duplex during the replication process, is not active [7, 8]. Cell cycle dependent kinases initiate the activation step by triggering the recruitment of replication factors, including CDC-45 and the Go-Ichi-Ni-San (GINS) complex (Figure 1.2.) [8-12]. The tetrameric GINS complex comprises the subunits SLD-5, PSF-1, PSF-2 and PSF-3 [13, 14].

Initially identified to be required for the activation of origin firing, CDC-45 and the GINS complex have been proven as essential components of the active replisome [15-20]. Whereas the exact function of CDC-45 and GINS during DNA synthesis remains elusive, functional and proteomic approaches reveal that both proteins are required for the stable assembly of the active replisome by facilitating binding of further replisome components to the chromatin [19, 21-24]. Strikingly, binding partners of CDC-45 and the GINS complex are essential elongation factors, as well as several factors that are involved in replication fork integrity and progression [19, 21, 24]. Therefore, CDC-45 and the GINS complex are recognized as central components that function as a scaffold to assemble and maintain the active replisome [15, 18-20].

Once a functional replisome has been assembled on the chromatin, the MCM helicase opens the DNA duplex and the replication fork progresses bi-directionally while the DNA polymerases catalyze DNA synthesis (Figure 1.2.) [25].

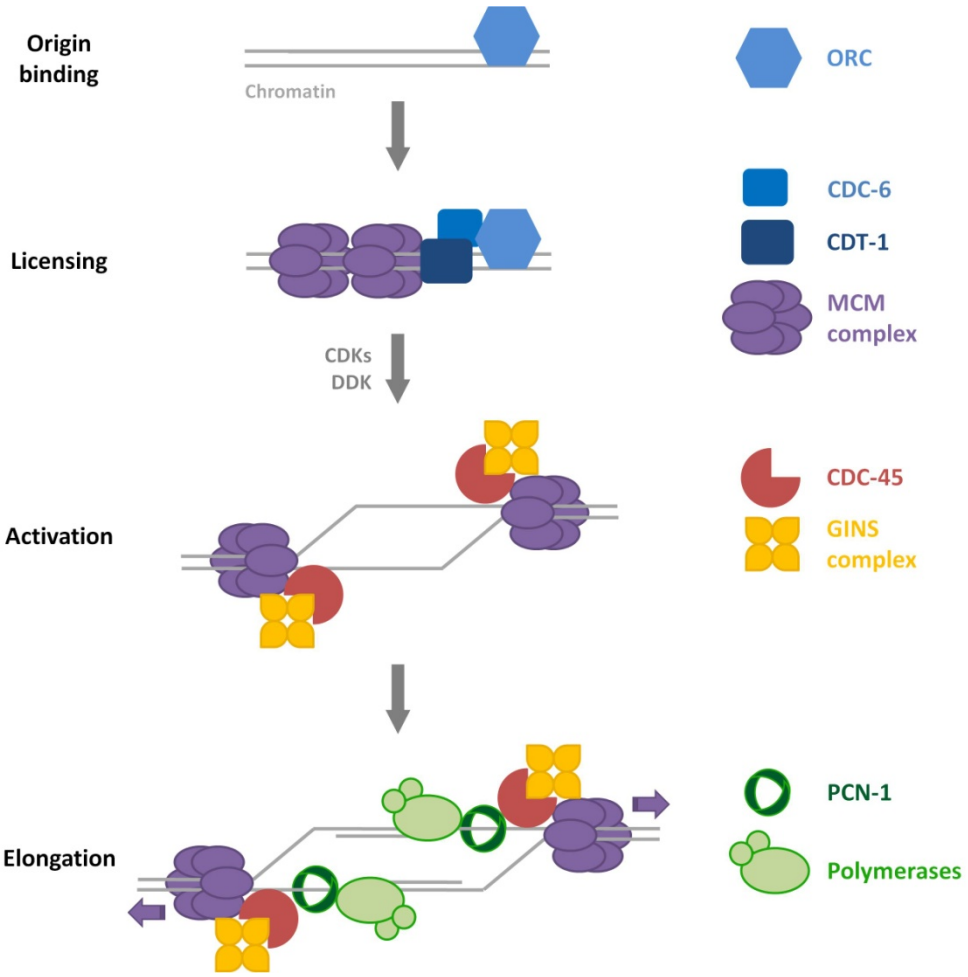


Figure 1.2. Schematic representation of distinct steps during DNA replication.
After the binding of ORC to the origins of replication, DNA replication occurs in three timely separated steps: licensing, activation, and elongation. In the licensing step origins of replication are primed by the assembly of the pre-RC. The transition from the licensing to the activation step is triggered by the cell cycle dependent kinases CDKs and DDK. Binding of CDC-45 and the GINS complex promotes the formation of a functional replisome and initiates the elongation phase. Replication forks move bi-directionally during the elongation phase (indicated by arrows) while polymerases catalyze DNA synthesis. Essential proteins that function in respective phases of DNA replication are shown on the right. See text for further details. Modified from [26].

1.1.2. Maintenance of genomic information requires spatiotemporal regulation of replication factors.

Accurate duplication of DNA is a central and challenging task in dividing cells that requires the hierarchical order of timely separated events. Defects in chromosomal DNA replication can severely impair cellular physiology and lead to cell death. Strikingly, failure in the assembly, fidelity or integrity of the replication machinery threatens genomic stability and is therefore recognized as a cause of a variety of diseases and cancer development [27-38]. Attachment of signalling molecules constitutes a fast and effective mechanism to control such

complex protein function. Hence, the following paragraph highlights key functions of DNA replication that are triggered by posttranslational modification.

1.1.2.1. Posttranslational modifications of licensing factors ensure genomic stability.

Cell cycle progression greatly depends on the activity of cell cycle dependent kinases [39]. In particular, phosphorylation of target proteins is essential for the activation of DNA replication. An important example is the phosphorylation of the MCM helicase [40]. Cyclin dependent kinases (CDKs) and the Dbf4-dependent Cdc7 kinase (DDK) trigger the activation of replication by phosphorylation of the MCM complex [10, 11]. This initiates the recruitment of CDC-45 and the GINS complex to the chromatin, thereby promoting the activity of the MCM helicase and facilitating DNA synthesis [8, 9, 41].

Over-replication by re-initiation of replication (re-replication) can severely affect genomic stability [37]. In order to ensure that replication origins are used only once per cell cycle and thus cannot be reused to re-initiate replication, the licensing and elongation processes are temporally separated [42]. Once the MCM helicase complex has been assembled on the chromatin with the help of the licensing factors ORC, CDC-6, and CDT-1, these assisting factors are not further required for the DNA synthesis process [43]. Consequently, the essential licensing factors ORC-1, CDC-6 and CDT-1 are tightly controlled in protein abundance, localization, and activity to prohibit the re-initiation of origin licensing.

In yeast a general inhibition of licensing factors is coordinated through phosphorylation of licensing factors by CDKs and DDK [44]. CDKs and DDK mediated phosphorylation events inhibit replication licensing by diverse mechanisms. Phosphorylation of ORC inhibits pre-RC assembly [45] while phosphorylation of CDC-6 and CDT-1 [46-49] or MCM components [50, 51] trigger protein degradation or nuclear export, respectively.

In higher eukaryotes, additional mechanisms have evolved to restrict replication licensing. ORC-1, an essential component of the ORC, is targeted for protein degradation through ubiquitylation in *Drosophila melanogaster* and human cell culture [52-54]. In contrast, other ORC subunits remain stable throughout the cell cycle [52]. The best studied mechanism to avoid re-replication in all eukaryotes, however, is the control of the activity and proteolysis of CDT-1. As research in diverse model organisms points towards differences in CDT-1 regulation between species and cell types, the following paragraph emphasizes the principles of CDT-1 surveillance.

1.1.2.2. Mechanisms of CDT-1 regulation.

The regulation of CDT-1 activity and protein abundance during cell cycle progression is supposed to be the major mechanism to restrict replication licensing to occur only once per cell cycle. Two basic principles ensure that CDT-1 protein is only active during the licensing period of DNA replication [55].

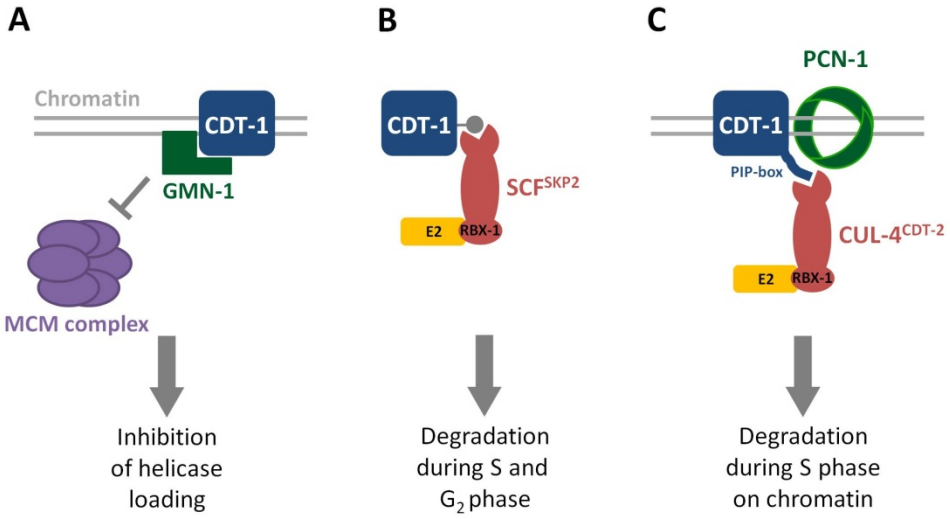


Figure 1.3. Distinct mechanisms of CDT-1 regulation.
(A) GMN-1 binds to CDT-1 and thereby inhibits the loading of the MCM helicase complex during S phase.
(B) Phosphorylated CDT-1 is recognized by the SCF^{SKP2} ligase, which facilitates CDT-1 ubiquitylation by recruiting the E2 enzyme. Ubiquitylation results in CDT-1 degradation during S and G2 phases.
(C) Chromatin bound CDT-1 binds PCN-1 (C. elegans ortholog of PCNA) and the $CUL\text{-}4^{CDT\text{-}2}$ ligase via the PIP-box. The $CUL\text{-}4^{CDT\text{-}2}$ ligase promotes CDT-1 ubiquitylation and degradation in S phase. See text for further details. Modified from [56].

One strategy to restrict CDT-1 activity is through binding by geminin (GMN-1 in *C. elegans*) that directly interacts with CDT-1 and inhibits its licensing activity by prohibiting interaction with the MCM complex and helicase loading onto chromatin (Figure 1.3.A.) [57, 58]. To allow MCM complex loading by CDT-1 during the licensing process the inhibitory function of geminin is abrogated by temporally protein degradation through ubiquitylation [59]. While CDT-1 regulation by geminin is crucial in higher eukaryotes no functional orthologs have been identified in yeast.

The second mechanism that regulates CDT-1 activity acts through targeted protein turnover depending on protein ubiquitylation [56]. The conjugation of ubiquitin to target proteins is catalyzed by substrate specific ubiquitin-ligases (E3's) (see paragraph 1.2. for details). Two distinct E3 ubiquitin-ligases have been linked to CDT-1 degradation that apparently target slightly different subsets of CDT-1 in the cell. In mammalian cell culture

experiments the SCF^{SKP2} ligase is required for efficient CDT-1 degradation throughout S and G_2 phases of the cell cycle depending on CDT-1 phosphorylation (Figure 1.3.B) [33, 60-63]. Interestingly, the SCF^{SKP2} mediated CDT-1 degradation represents an evolutionary young mechanism that could so far only be shown in mammalian cells [64]. Indeed, the human SCFSKP2 ligase appears to work redundantly with the CUL-4^{CDT-2} ubiquitin ligase that plays a crucial role in CDT-1 turnover in all eukaryotes [63].

CDT-1 turnover mediated by CUL-4^{CDT-2} is coordinated with active DNA replication, and therefore operates exclusively during the S phase of the cell cycle in order to prevent re-licensing (Figure 1.3.C) [65-67]. PCNA (PCN-1 in *C. elegans*) is an essential factor during active DNA replication and plays a central role in CDT-1 degradation because it interacts with both the CUL-4^{CDT-2} ubiquitin-ligase and a peptide signature named PIP-box in the CDT-1 protein itself [68-71]. By bringing the substrate CDT-1 and the ubiquitin-ligase CUL-4^{CDT-2} into close proximity, PCNA coordinates CDT-1 degradation in time and space and thereby represents an intriguing mechanism that prevents re-licensing during active DNA synthesis and on chromatin (Figure 1.3.C.).

It is worth to note that analogous mechanisms of CDT-1 degradation are also operating when genomic stability is challenged by DNA damaging agents [48, 72, 73], indicating that CDT-1 regulation is vital for both, unperturbed cell cycles, and DNA damage repair.

1.1.3. External and internal assaults interfere with the fidelity of DNA replication.

The fidelity of DNA replication and therefore genomic stability is constantly challenged by external or internal factors resulting in replication stress [74]. External effectors that manipulate the integrity of the DNA molecule or associated proteins are for example ultraviolet-(UV)-light, ionizing radiation, or alkylating agents [75]. Moreover, internal sources have been identified to cause replication fork stalling [76]. These physiological obstacles often arise at dedicated regions of the genome for example transposable elements, repetitive DNA stretches, or highly transcribed genes. These regions are intrinsically difficult to replicate and therefore susceptible to replication errors [77-81]. Reduction in the availability of replication factors as well as limitation of the deoxyribonucleotide triphosphate (dNTP) pool can also limit fork progression and result in paused replication forks [82, 83]. Stalling of replication forks is strongly associated with unusual DNA rearrangements which are linked to DNA breaks and genomic instability and therefore described as fragile sites [84]. Strikingly, fragile sites are known as a source of numerous genomic disorders [85].

In order to counteract the genotoxic threat that emanates from replication stresses, eukaryotes have evolved genome maintenance mechanisms that can sense paused replication and initiate appropriate cellular responses. The respective molecular mechanisms are commonly described as the replication checkpoint [74].

1.1.3.1. The replication checkpoint controls fidelity and progression of the replication fork.

Occurrence of replication stress generally results in the impairment of replication fork progression. When replication forks are forced to stall at a barrier, this results in the uncoupling of the replisomes at the leading and lagging strands or the uncoupling of the DNA helicase from the replisome (Figure 1.4.A) [86, 87]. Consequently, while DNA synthesis by the DNA polymerases is blocked, the unwinding of the DNA duplex continues and thereby generates excessive single stranded DNA (ssDNA) (Figure 1.4.A).

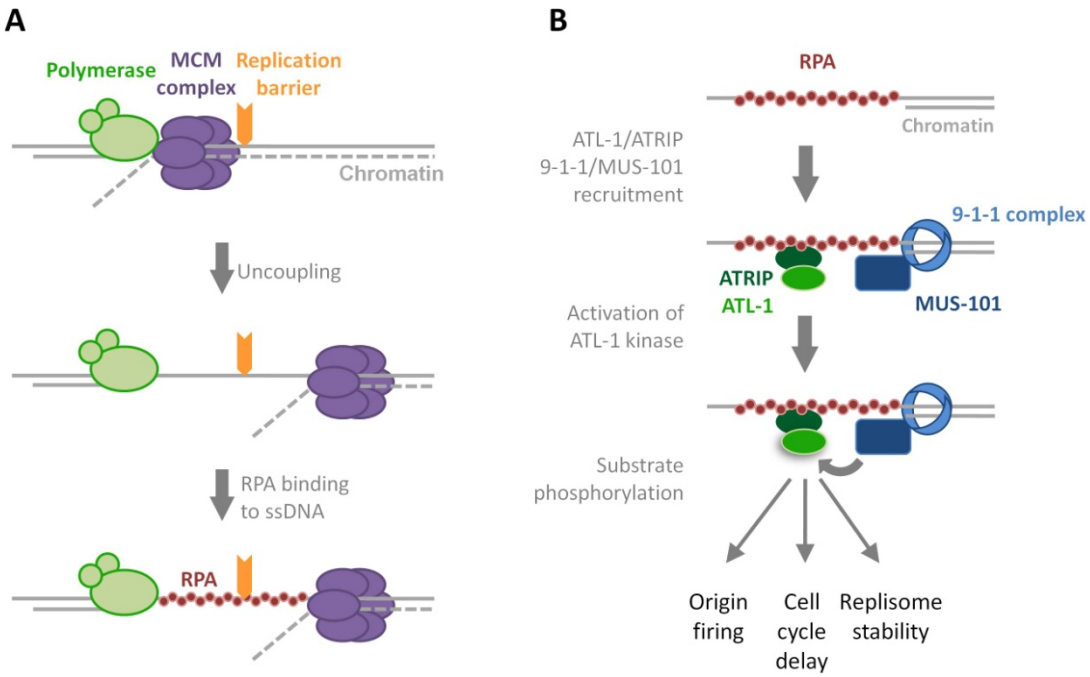


Figure 1.4. Occurrence of replication stress and initiation of ATL-1 mediated checkpoint response.
(A) Replication barriers result in uncoupling of the MCM helicase complex from the replisome, including the DNA polymerases. ssDNA generated by DNA unwinding is bound by RPA. Scheme represents replication stress situation on one DNA strand (continuous line) while the situation on the other strand (discontinuous line) is not shown. Modified from [88].
(B) ssDNA that is coated with RPA recruits ATL-1/ATRIP and the 9-1-1 complex together with MUS-101. Close proximity to MUS-101 activates the kinase activity of ATL-1 which promotes distinct cellular responses through phosphorylation of target proteins. Modified from [89].

Interestingly, the generation of ssDNA and not replication barriers themselves represents the common structural basis of how cells sense stalled replication forks [90, 91]. ssDNA is bound by the trimeric single-strand binding replication protein A (RPA, RPA-1, RPA-2, and RPA-3 in *C. elegans*) [92]. The decoration of excess ssDNA with RPA initiates a signalling pathway which is coordinated around the checkpoint kinase ATR (ATL-1 in *C. elegans*) and the 9-1-1 complex (HPR-9, HUS-1, and MRT-2 in *C. elegans*) (Figure 1.4.B.) [93]. Upon ATL-1 activation replication forks are stabilized [94-96] and progression through the cell cycle is delayed [97-99] while repair mechanisms are initiated to promote subsequent re-start and completion of replication [100] (Figure 1.4.B.). If the ATL-1 signalling pathway is compromised the cellular response to replication stress is impaired, and aberrant DNA structures accumulate, resulting in genomic instability [79, 86, 101].

1.1.3.3. Posttranslational modifications of PCNA determine distinct pathways to solve replication stress.

Since diverse response mechanisms are initiated upon ATL-1 phosphorylation [102], it is of great interest how cells eventually decide between distinct processes in order to accomplish a replication stress situation. As a central component of the replisome PCN-1 is required for the processivity of DNA polymerases (Figure 1.2.) [103, 104]. Besides its basic function in DNA replication, PCN-1 acts as an integrator of distinct signalling events and thereby determines the response to replication stress.

When a replication fork stalls, PCN-1 is ubiquitylated [105]. Interestingly, two distinct modifications with ubiquitin have been observed that promote two different modes of replication fork progression (Figure 1.5.). Modification of PCNA with one single ubiquitin (mono-ubiquitylation) results in an exchange of polymerases towards translesion DNA polymerases that are capable of reading through replication barriers (Figure 1.5) [106-108]. DNA synthesis is re-initiated, however, the probability to incorporate false nucleotides into the DNA renders the translesion synthesis error prone. In contrast, the modification of PCNA with a chain of ubiquitin moieties (poly-ubiquitylation) promotes an error free pathway that involves a template switch mechanism enabling DNA synthesis based on the unaffected sister strand (Figure 1.5.) [105, 109-111]. Conjugation of SUMO to PCN-1 occurs at unperturbed replication forks and inhibits recombination events to allow normal progression of the replisome (Figure 1.5.) [112, 113].

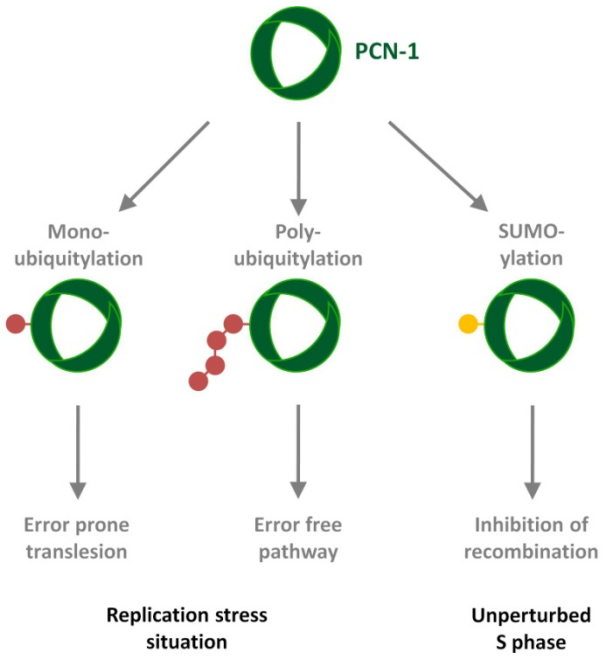


Figure 1.5. Ubiquitylation and SUMOylation of PCN-1 determine essential processes at the replication fork. In response to replication stress PCN-1 is ubiquitylated. Mono-ubiquitylation promotes translesion DNA synthesis. Poly-ubiquitylation initiates an error free repair pathway. In unperturbed S phases SUMOylation of PCN-1 inhibits recombination events. Modified from [114].

In conclusion, crucial events at the replication fork that safeguard genomic stability involve ubiquitylation of target proteins. Therefore, the following paragraphs focus on protein modification with ubiquitin and its physiological relevance in more detail.

1.2. Protein ubiquitylation.

Posttranslational modifications such as phosphorylation, methylation, or acetylation are observed for the majority of the cellular proteome [115]. Remarkably, attachment of the signalling peptide ubiquitin to target proteins is emerging as one of the most important mechanisms that regulate cellular physiology [116, 117].

Ubiquitin is highly conserved among all eukaryotes. An enzymatic cascade of ubiquitin-activating E1, ubiquitin-conjugating E2, and ubiquitin-ligating E3 catalyzes the conjugation of ubiquitin to lysine residues of a target protein (Figure 1.6.) [118]. Ubiquitylation can occur in distinct modes and modifications, thereby initiating diverse cellular processes. A single attachment of one ubiquitin moiety to a substrate protein (mono-ubiquitylation) is commonly thought to regulate protein interaction and to affect protein localization [115]. In contrast, poly-ubiquitylation is achieved by successive attachment of ubiquitin molecules to one of

seven internal lysine residues of the previous ubiquitin, thereby forming chains of different topology. Depending on the linkage between the ubiquitin molecules, the chain on a target protein can be recognized by destined adaptor molecules and thereby promote distinct events [119]. Chains that are constructed via lysine 48 or lysine 11 (K48, K11) linkages between ubiquitin moieties target proteins for their degradation by the 26S proteasome [120-122]. In contrast, lysine 63 (K63) linked ubiquitin does not promote degradation [123] but initiation of the assembly of protein complexes which are for example involved in signalling events such as the DNA damage response [124, 125]. Other linkage types have been described, for example linear ubiquitin functioning in NF-κB signalling [126, 127]. Ubiquitin specific hydrolases (or de-ubiquitylating enzymes, DUBs) counteract the formation of ubiquitin chains and consequently influence the ubiquitylation status of substrate proteins (Figure 1.6.) [128].

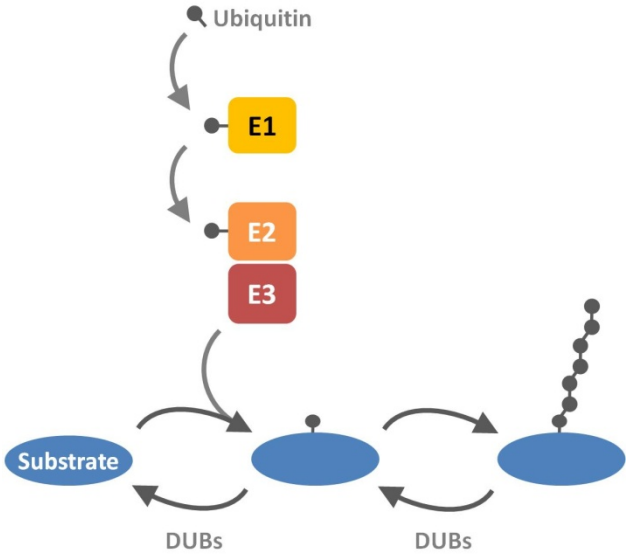


Figure 1.6. Protein ubiquitylation by an enzymatic cascade of E1, E2, and E3 enzymes.
Substrate proteins are attached to ubiquitin by an enzymatic cascade of E1, E2, and E3 enzymes. Ubiquitylation can occur by a single ubiquitin molecule or through the formation of poly-ubiquitin chains. DUBs counteract the ubiquitylation reaction. See text for further details.

1.2.1. The Ubiquitin Proteasome System – UPS.

Protein degradation is vital for the regulation of protein homeostasis, and moreover, triggers a variety of processes in eukaryotic cells. Autophagosomes or lysosomes contribute to general protein degradation, however, these mechanisms are rather unselective regarding the targeted substrates that are mainly macromolecules or organelles [129]. The ubiquitin

proteasome system (UPS) represents the major pathway that determines selective proteolysis. A poly-ubiquitin chain is assembled on target proteins, or substrates, labelling these proteins for their subsequent degradation by the 26S proteasome (Figure 1.7.) [120, 121]. The 26S proteasome represents a multi-catalytic protease chamber that recognizes, unfolds, and degrades ubiquitylated proteins into peptide fragments in both cytosol and nucleus [130, 131]. Strikingly, as the proteasome demonstrates the terminal degradation step of thousands of proteins, malfunction is linked to a variety of different diseases [132, 133].

1.2.1.1. E2 and E3 enzymes determine substrate specificity.

The E1, E2, and E3 enzymes that catalyze the ubiquitylation reaction represent protein classes of increasing complexity and specificity. The E1 enzyme (two E1 enzymes are known in humans, one in *C. elegans*) is required and sufficient for the activation reaction of ubiquitin that precedes the subsequent conjugation to a substrate protein [134-136]. In turn, activated ubiquitin can be conjugated to several E2's (38 identified in humans, about 20 in *C. elegans*) [127, 136]. E2 enzymes cooperate with different E3 ligases (up to 1000 identified in humans, about 170 in *C. elegans*) adding a high layer of complexity to the system [137, 138]. Whereas the E2 conjugating enzyme represents the catalytic core of the ubiquitylation reaction that determines processivity and linkage type specificity [54, 139, 140], the E3 ligase mainly defines the target protein for ubiquitylation [141]. Interestingly, so called E4 enzymes have been described that are required for the assembly of poly-ubiquitin chains on certain substrates [142].

E3 ligases are grouped into four categories: HECT-domain, U-Box-domain, monomeric RING finger, and multi-subunit RING finger ligases. Despite the differences in the modular architecture, all E3 ligases connect the E2 conjugating-enzyme with the substrate and therefore facilitate specific protein ubiquitylation (Figure 1.6.) [141].

Downstream of the ubiquitylation reaction earmarked proteins are recognized by dedicated adaptor proteins that eventually promote their delivery to the 26S proteasome where protein degradation occurs. The ubiquitin-selective chaperone CDC-48 plays a crucial role in the mobilization of target proteins and their transfer to the proteasome [143].

1.2.2. CDC-48 – a ubiquitin selective chaperone.

CDC-48 is a highly conserved and abundant protein in all eukaryotes. Orthologs of *C. elegans* CDC-48 are called VAT in archaea, Cdc48 in yeast, TER94 in insects, p97 in vertebrates, or VCP in mammals. Initially described as a crucial factor in ubiquitin mediated

protein degradation [144], CDC-48 and it orthologs are emerging as fundamental regulators of various cellular processes that are governed by ubiquitylation [145, 146]. CDC-48 recognizes ubiquitylated proteins and subsequently regulates their activity, localization, or protein stability.

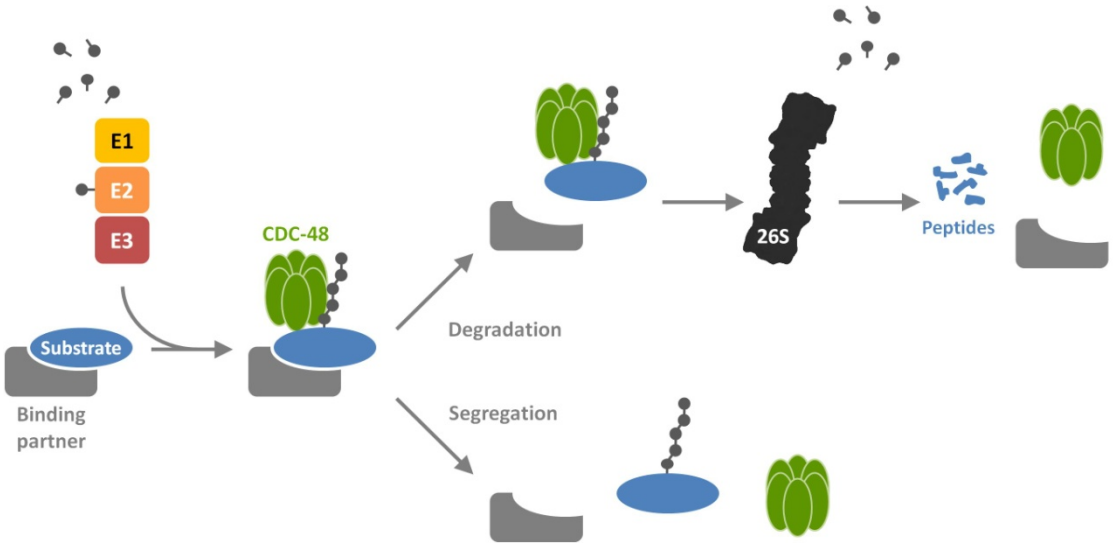


Figure 1.7. Functional role of the ubiquitin-selective chaperone CDC-48 in the UPS and in complex segregation.
CDC-48 binds ubiquitylated substrates with the help of dedicated cofactors (not shown). Depending on its ATPase activity, CDC-48 segregates target proteins from tight binding partners. Target proteins are then either transferred to the 26S proteasome for degradation (upper row) or show altered localization or activity (lower row) after the segregation process. CDC-48 acts on protein complexes or immobile structures such as membranes or chromatin (illustrated as binding partner).

CDC-48 belongs to the ATPases associated with diverse cellular activities (AAA) protein family that shares common composition of distinct functional domains [147]. As typical for AAA proteins, CDC-48 assembles in homo-hexamers, forming a barrel-like structure around a central pore [148, 149]. Two ATPase domains, namely D1 and D2, are oriented towards the inside of the barrel, whereas the structurally defined N-terminal domain (N-domain) is positioned at the outer surface (Figures 1.7., 1.8.) [148, 149]. The N-domain shows a high degree of flexibility that is intimately linked to the ATPase cycle of the D2 domain [54, 150]. Upon hydrolysis of ATP, conformational changes occur in the CDC-48 structure [54, 151]. Although the exact mechanism still remains to be solved, these conformational rearrangements are supposed to account for the chaperone activity of CDC-48 [152]. Recognizing ubiquitylated substrates with the help of dedicated cofactors [153, 154], CDC-48 is thought to provide

unfolding activity required to mobilize client proteins out of protein complexes and eventually promote the transfer to the proteasome for degradation (Figure 1.7.) [155]. The unfolding activity of CDC-48 is required for functional proteolysis, as proteasomes can stall in the absence of the CDC-48 chaperone, emphasizing a general function of CDC-48 in proteasomal processivity [156, 157].

1.2.2.1. CDC-48 – a general chaperone with distinct cellular functions.

While a general role in ubiquitin-mediated protein degradation is well documented, a challenging task remains the identification of cellular pathways and crucial substrates that are regulated by CDC-48. Due to its central role in protein degradation by the proteasome, CDC-48 is implicated in the maintenance of cellular protein homeostasis and avoidance of aggregates [158-160]. Besides the rather broad function in protein turnover, a variety of cellular pathways such as endoplasmic reticulum associated degradation (ERAD), mitochondria associated degradation (MAD), membrane fusion, autophagy, endosomal trafficking, neurogenesis, transcription, DNA damage response, and cell cycle regulation have been described to require CDC-48 as an essential component [145, 146, 161, 162]. In order to decide about a particular process to occur additional determinants need to be involved. Accordingly, CDC-48 associates with alternative cofactors that are supposed to form distinct functional sub-complexes defining the specificity for distinct cellular pathways (Figure 1.8.) [145, 163, 164].

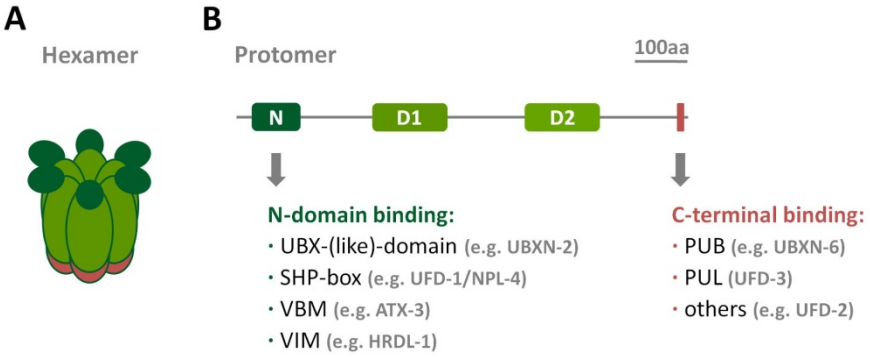


Figure 1.8. Alternative cofactors of CDC-48 interact with distinct domains.
(A) CDC-48 forms homo-hexamers.
(B) The domain structure of the CDC-48 protomer bares two ATPase domains (D1 and D2). Cofactors either interact with the N-terminal or the C-terminal domain of CDC-48 depending on distinct interaction motifs (UBX: ubiquitin regulatory x; SHP: Shp1; VIM: VCP interacting motif; VBM: VCP binding motif; PUB: PNGase/ubiquitin associated; PUL: PLAP/UFD3/Lub1). Scale bar represents 100 aa. Information taken from [165]

Depending on their activities, CDC-48 cofactors are subdivided into substrate recruiting and substrate processing factors [143]. The substrate recruiting factors p47 and UFD-1/NPL-4 are believed to represent distinct core complexes of CDC-48 that determine its involvement in certain cellular pathways [153, 166]. Substrate processing factors actively influence the fate of target proteins. UFD-2 is a cofactor of CDC-48 possessing ubiquitin-chain elongation (E4) activity, thus facilitating substrate degradation [142]. In contrast, different de-ubiquitylating enzymes (DUB's) associate with CDC-48 that can alter the fate of a substrate by editing the length or linkage type of the ubiquitin chain [167, 168]. Cofactors mainly interact with the N-domain or C-terminal domain of CDC-48 depending on distinct interaction motifs (Figure 1.8.).

1.2.2.3. CDC-48 functions in the regulation of cell division.

Initially, CDC-48 has been identified in a screen for yeast mutants that are defective in cell cycle progression [169]. Although the understanding of CDC-48 function is steadily increasing, certain facets of CDC-48 in cell cycle regulation remain elusive. Remarkably, while initial studies reported a crucial function of CDC-48 in the regulation of mitotic events [170-175], a role of the CDC-48$^{UFD-1/NPL-4}$ complex in DNA replication has been identified in *C. elegans* recently [162, 176].

In *C. elegans* two homologues of CDC-48, namely CDC-48.1 and CDC-48.2, exist that show differences in their expression pattern during nematode development (in the following text both transcripts will be referred to as CDC-48) [177]. However, both transcripts are abundantly expressed in *C. elegans* embryos. Depletion of CDC-48 using RNA interference (RNAi) causes severe defects during cell division [176]. Surprisingly, the observed defects do not reflect the mitotic function of CDC-48 reported from other model systems, but instead revealed pronounced defects in DNA synthesis which is also observed for the RNAi-mediated depletion of the cofactors UFD-1 and NPL-4 (both homologs, NPL-4.1, and NPL-4.2 will be referred to as NPL-4 in the following text). A series of functional assays convincingly showed that DNA replication does not occur efficiently in CDC-48$^{UFD-1/NPL-4}$ depleted embryos. In line with compromised DNA replication, activation of the replication checkpoint kinases ATL-1 and CHK-1 as well as focal accumulation of the DNA repair protein RAD-51 indicate severe problems in DNA synthesis and the occurrence of replication stress [178-180]. Moreover, worms depleted for the CDC-48$^{UFD-1/NPL-4}$ complex are hypersensitive towards treatment with hydroxyurea (HU), a replication-blocking agent [181].

1.3. Aim of the thesis.

CDC-48 is an essential protein in ubiquitin-mediated cellular processes. Following the recognition of ubiquitylated proteins, CDC-48 mobilizes substrates out of higher order complexes and thereby facilitates their degradation by the proteasome [182]. A variety of cellular pathways depend on the ubiquitin-selective chaperone activity of CDC-48 [146]. Therefore, the identification of target proteins regulated by CDC-48 is of major interest.

The observation that CDC-48 together with the cofactors UFD-1 and NPL-4 is required for effective DNA synthesis, established a so far unknown function in cell cycle regulation [176]. As mechanistic details of CDC-48 activity remained to be elucidated, the aim of this work is the identification of key substrates of the CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ complex that play a vital role during DNA duplication. A combination of microscopic, genetic, and biochemical analysis was used in order to gain insight into the mechanism of CDC-48 during eukaryotic DNA replication. Thereby, this work provides further understanding of the regulation of crucial cell cycle events involving ubiquitylation, in the context of chromatin associated proteins. Moreover, elucidating the mechanistic function of the CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ complex during DNA replication will emphasize a critical role in the maintenance of genomic stability.

· Chapter 2 · Results ·

2.1. CDC-48$^{UFD-1/NPL-4}$ specifically regulates early steps in DNA replication.

Elucidating the time point of CDC-48 activity during DNA replication was the first aim of this work. To this end, a systematic analysis of the subcellular localization and dynamics of several conserved replication factors was carried out in dividing *C. elegans* embryos. Essential replication factors were fluorescently labeled, in order to recapitulate the licensing, activation and elongation phases of DNA replication. A functional analysis of the replication factor fusions to green fluorescent protein (GFP) and their biological relevance has recently been validated [183]. The dynamic distribution of replication factors was followed in embryos that were depleted for a functional CDC-48$^{UFD-1/NPL-4}$ complex by time-lapse microscopy (Figure 2.1.). As a control an *empty* RNAi vector without a gene-specific targeting sequence was used. Depletion of *ufd-1* by RNAi results in the disruption of a functional CDC-48$^{UFD-1/NPL-4}$ complex. To allow precise discrimination of distinct cell cycle phases chromatin was visualized by co-expression of mCherry-tagged histone 2B (mCherry::H2B) during these experiments.

Phase of replication	Licensing					Activation		Elongation		
GFP fusion protein	**ORC-1**	**ORC-2**	**CDC-6**	**MCM-2**	**MCM-3**	**CDC-45**	**SLD-5**	**RPA-1**	**PCN-1**	**DIV-1**
***empty* (RNAi)**	Mitosis	Mitosis	Mitosis	Mitosis & S phase	Mitosis & S phase	S phase	S phase	S phase	S phase	S phase
***ufd-1* (RNAi)**	Mitosis	Mitosis	Mitosis	Mitosis & S phase	Mitosis & S phase	Mitosis & S phase	Mitosis & S phase	S phase	S phase	S phase

Figure 2.1. Systematic analysis of replication factors fused to GFP.
Subcellular distribution and dynamics of indicated replication factors fused to GFP were monitored in *empty* (control) and *ufd-1* (RNAi) treated embryos. Subcellular localization was either classified as mitotic chromatin association (Mitosis) or nuclear localization during S phase. Involvement in distinct phases of respective replication factors is indicated. Green letters reveal wild-type localization, red letters indicate altered localization upon *ufd-1* (RNAi).

In early *C. elegans* embryonic cell cycles S and M phases rapidly alternate without apparent gap phases (Figure 1.1.B) [3]. Progression through these cell cycles can be monitored by time-lapse microscopy. Figure 2.1. shows a summary of the replication reporter fusions that were initially analyzed. Strikingly, specific defects were observed in the regulation of GFP::CDC-45 and GFP::SLD-5, two factors that are crucial during the activation process (Figure 1.2., 2.2.).

However, the dynamics of replication factors that are essential in the licensing and elongation steps of DNA replication were found to be unaffected by *ufd-1* (RNAi) (Figure 2.1., 2.3.).

2.2. Chromatin dissociation of CDC-45 and GINS subunits requires CDC-48$^{UFD-1/NPL-4}$.

In wild-type embryos, CDC-45 and the GINS subunit SLD-5 similarly accumulate in the nucleus during S phase but never co-localize with H2B on mitotic chromosomes (Figure 2.2.). In contrast, co-depletion of CDC-48.1 and CDC-48.2 by RNAi (hereafter referred to as CDC-48 depletion or *cdc-48* (RNAi)) or depletion of its cofactors UFD-1/NPL-4 specifically affects the localization of GFP::CDC-45 and GFP::SLD-5. Time-lapse microscopy revealed that CDC-45 and SLD-5 remain associated with chromatin throughout cell cycle progression in *cdc-48*, *ufd-1*, and *npl-4* (RNAi) embryos (Figure 2.2.A, 2.2.B). However, down-regulation of *ufd-1* does not change the cellular distribution of the pre-RC proteins ORC-2 and CDC-6 or the DNA helicase subunit MCM-2 (Figure 2.1., 2.3.). As CDC-48 is also involved in diverse biological processes, likely cooperating with other cofactors than UFD-1/NPL-4, its complete down-regulation blocks embryonic cell division (Figure 2.11.C) [160, 176, 184]. I order to specifically analyze the replication related phenotype, *ufd-1* and/or *npl-4* were depleted in further experiments.

Consistent with its role in DNA replication, disruption of the CDC-48$^{UFD-1/NPL-4}$ complex results in the activation of the DNA replication checkpoint kinases ATL-1 and CHK-1, eventually leading to a pronounced delay in cell cycle progression [176, 178, 185]. Accordingly, the altered localization of CDC-45/GINS might be a consequence of an activated replication checkpoint triggered by replication stress. However, checkpoint activation caused by depletion of the DNA polymerase α-subunit DIV-1 did not generate a comparable effect (Figure 2.2.B) [179]. Down-regulation of *atl-1* suppresses the cell division delay of *ufd-1* (RNAi) embryos [176], but it did not reverse the GFP::SLD-5 mislocalization (Figure 2.2.B). Together, these data suggest that false regulation of the CDC-45/GINS complex is caused by CDC-48$^{UFD-1/NPL-4}$ depletion and is not a secondary effect of checkpoint activation.

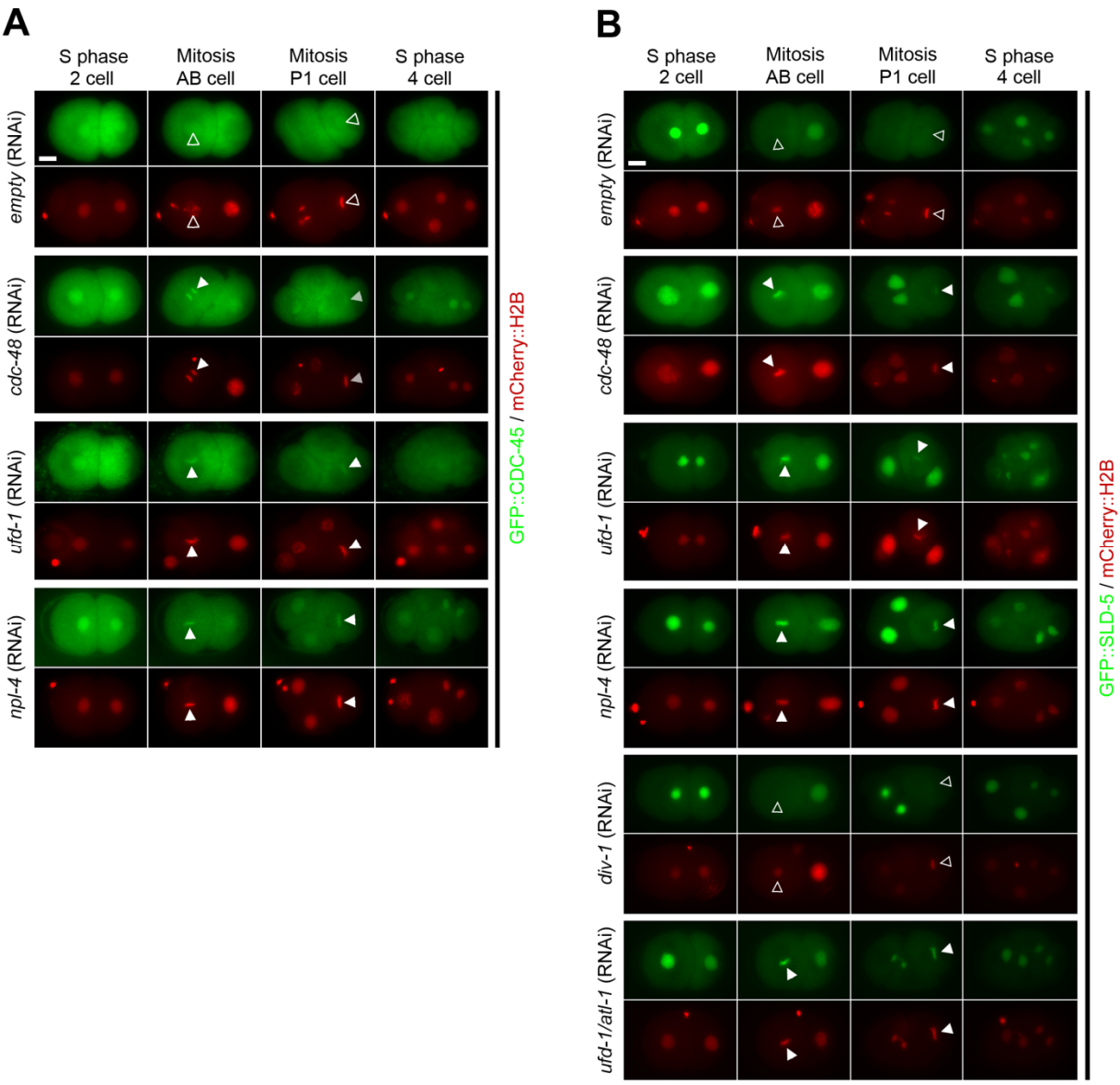


Figure 2.2. CDC-45 and subunits of the GINS complex persist on mitotic chromatin in embryos with a disrupted CDC-48$^{UFD-1/NPL-4}$ complex.

(A, B) Selected pictures of time-lapse recordings of embryos expressing GFP::CDC-45 or GFP::SLD-5 (green) and mCherry::H2B (red) depleted for *empty* control, *cdc-48* and/or *ufd-1*, *npl-4*, *div-1*, or co-depleted for *ufd-1/atl-1* by RNAi. Each image series represents cell cycle phases (Mitosis or S phase) at distinct time points of embryonic development (2 to 4 cell stage) of one single *C. elegans* embryo. Empty arrows indicate wild-type like mitotic localization, filled arrows indicate persistent association of the respective proteins with mitotic chromatin. Anterior is to the left. Scale bars represent 5 µm.

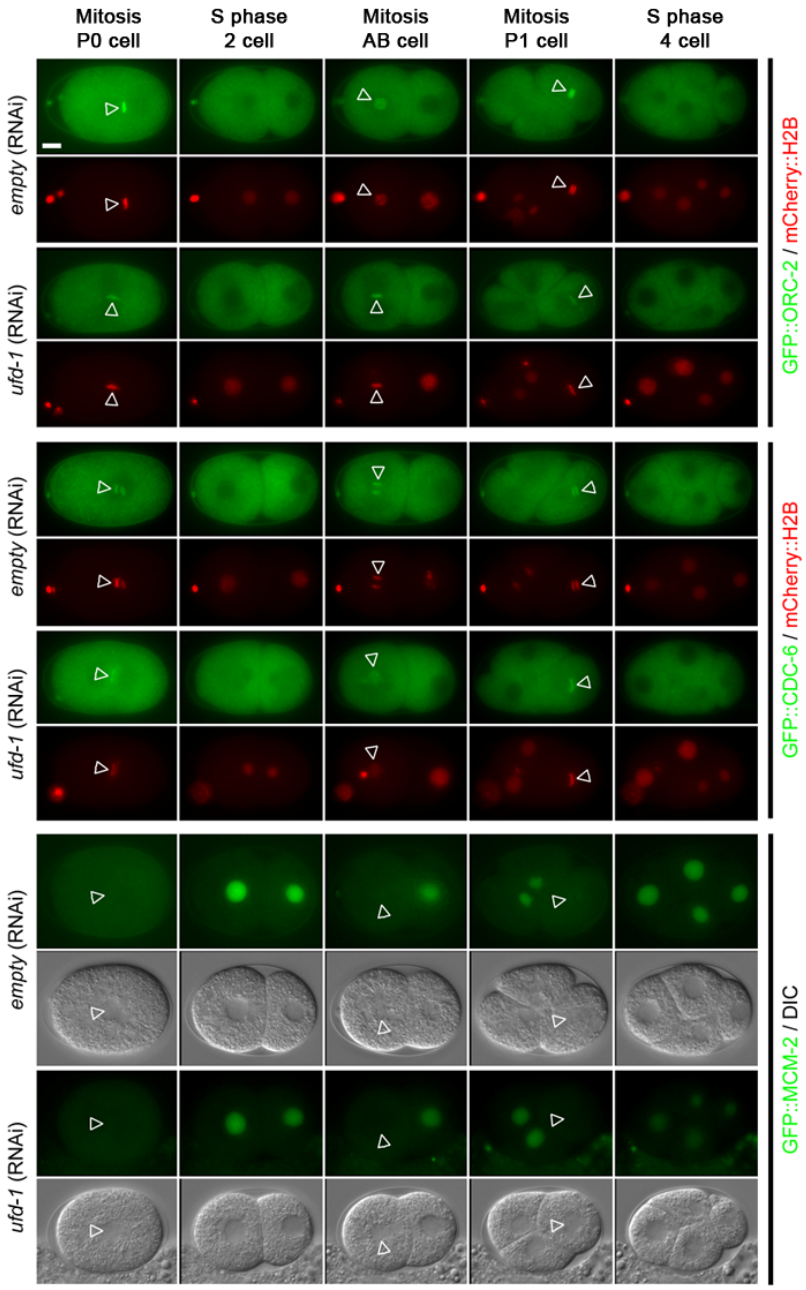


Figure 2.3. ORC-2, CDC-6, or MCM-2 localization is not affected in embryos depleted for the CDC-48$^{UFD-1/NPL-4}$ complex.

Selected pictures of time-lapse recordings of embryos expressing GFP::ORC-2, GFP::CDC-6, or GFP::MCM-2 (green) and mCherry::H2B (red) that are depleted for *empty* control or *ufd-1*. Representative pictures of indicated cell cycle phases (Mitosis or S phase) at distinct time points of embryonic development (1 to 4 cell stage) of one single embryo are shown. Empty arrows indicate wild-type localization. Anterior is to the left. The scale bar represents 5 µm.

2.3. Cell cycle progression defects of embryos lacking UFD-1 or NPL-4 depend on CDC-45/GINS.

Embryonic divisions in *C. elegans* occur in a remarkably stereotypic manner which led to the identification of invariant cell lineages arising from the one cell zygote [186]. The defined division pattern and timing has established embryonic development in *C. elegans* as an excellent model to study cell cycle regulation [3, 187]. Perturbance of the scheduled division pattern usually leads to embryonic lethality [3].

After fertilization the first division of the Po zygote is carried out asymmetrically, generating an anterior AB cell, and a smaller posterior P1 cell (Figure 2.4.). These cells have different developmental fates and division timing [188]. In the subsequent division cycle the AB cell divides approximately 2 min before P1, resulting in a four cell embryo [179]. Activation of the replication checkpoint by down-regulation of *cdc-48, ufd-1,* and *npl-4* further increases the cell division delay of P1 in comparison to AB, leading to a prolonged three-cell stage (Figure 2.4.) [176]. Considering the persistent chromatin association of CDC-45 and SLD-5 in embryos lacking CDC-48$^{UFD\text{-}1/NPL\text{-}4}$, this raised the question whether CDC-45 and GINS have a direct impact on checkpoint activation.

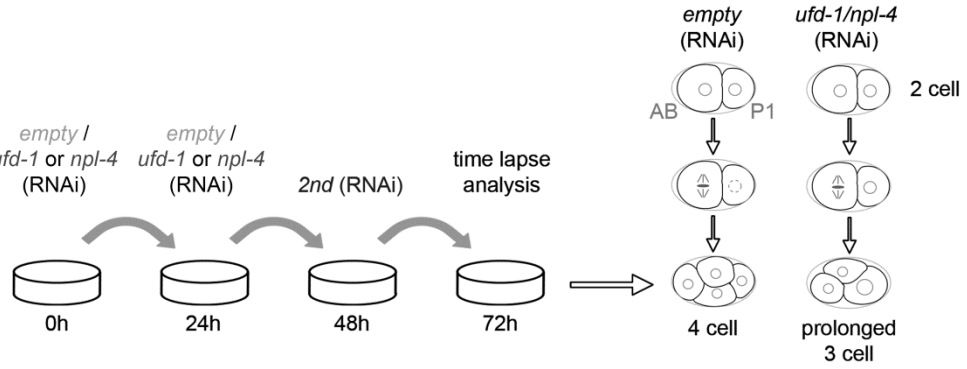


Figure 2.4. RNAi feeding procedure for sequential RNAi-mediated gene depletion.
Schematic illustration of the RNAi feeding procedure to achieve a sequential depletion of *empty* (light grey), *ufd-1* or *npl-4* (dark grey) for the first 48 h and *cdc-45, sld-5, psf-3* or *cdc-45+sld-5* (*2nd* (RNAi)) for the last 24 h. Subsequent time-lapse analysis was performed to visualize the previously described delay of *ufd-1* and *npl-4* RNAi embryos in cell cycle progression of the P1 cell [176].

To test this hypothesis, a RNAi protocol has been established to deplete UFD-1 or NPL-4 in the first place, followed by the depletion of CDC-45 or different GINS subunits in a second step (Figure 2.4.). Subsequent measurement of the cell cycle delay of the P1 cell (P1 delay)

allowed drawing conclusions about the importance of CDC-45 or GINS components for checkpoint activation.

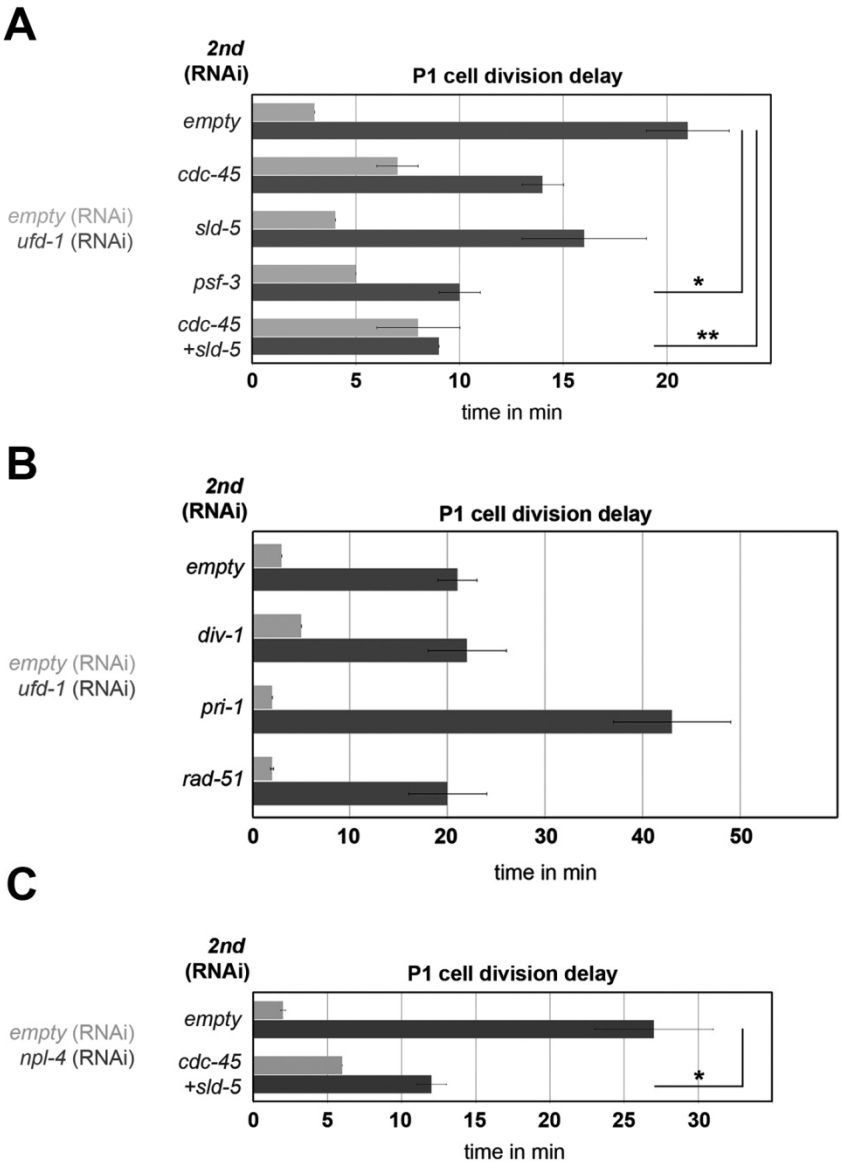


Figure 2.5. Suppression of the cell cycle progression delay of embryos lacking UFD-1 or NPL-4 by *cdc-45, sld-5* or *psf-3* (RNAi).

(A, C) Quantification of the time between division of AB and P1 cell (P1 division delay) of embryos depleted first for *empty* (light grey) and *ufd-1* or *npl-4* (dark grey) and sequentially for *empty, cdc-45, sld-5*, *psf-3* or co-depleted for *cdc-45+sld-5* (*2nd* (RNAi)).

(B) Quantification of P1 division delay of embryos depleted first for *empty* (light grey) and *ufd-1* (dark grey) and sequentially for *empty, div-1*, *pri-1*, or *rad-51* (2^{nd} (RNAi)).

Data are mean values. Error bars show standard error of the mean (s.e.m.). Statistical significance between cell division timings are indicated by asterisks in A and C. The single asterisk indicates $P \leq 0.05$ and the double asterisk indicates $P \leq 0.001$.

Indeed, depletion of the GINS component *psf-3* or co-depletion of both *cdc-45* and *sld-5* together significantly suppressed the P1 cell division delay of *ufd-1* and *npl-4* (RNAi) embryos (Figure 2.5.A, 2.5.C). Given that single RNAi depletion of CDC-45/GINS obviously affects DNA replication and delays S phase, it is reasonable that the suppression does not restore the delay in cell division and embryonic development completely to the wild-type level. At the same time, RNAi-mediated down-regulation of the DNA polymerase subunits DIV-1 and PRI-1, or the DNA repair protein RAD-51 [189] had no effect or even increased the defect in cell cycle progression of embryos lacking UFD-1 (Figure 2.5.B) [178].

2.4. Depletion of CDC-45 and SLD-5 does not suppress defects in cell cycle progression in general.

CDC-45 and the GINS complex are essential for the activation of the MCM helicase [8-10]. This raises the possibility that suppression of checkpoint activation by depletion of CDC-45 and GINS components in *ufd-1* and *npl-4* (RNAi) embryos could be due to reduced generation of ssDNA. In turn, reduced amounts of ssDNA might cause a milder activation of the replication checkpoint. A temperature sensitive mutant of *div-1* was used to test whether depletion of CDC-45/SLD-5 is able to generally reduce S phase progression defects that are not linked to CDC-48. The *div-1(or148)* allele shows activation of the replication checkpoint when cultivated at the restrictive temperature due to a mutation in the DNA polymerase α-primase subunit [179]. Strikingly, *cdc-45*/*sld-5* co-depletion significantly enhanced rather than reduced the P1 delay phenotype of *div-1(or148)* (Figure 2.6.). In conclusion, persistent chromatin association of CDC-45/GINS in embryos lacking a functional CDC-48$^{UFD-1/NPL-4}$ complex seems to contribute directly to the activation of the DNA replication checkpoint.

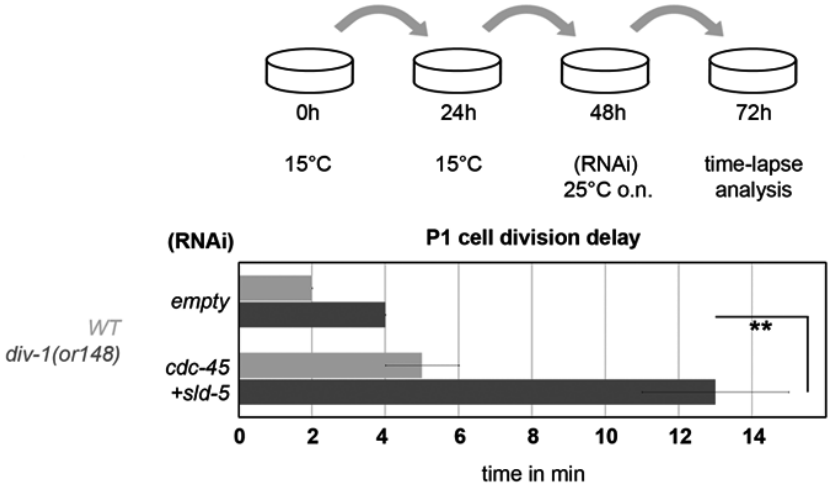


Figure 2.6. *cdc-45* and *sld-5* (RNAi) do not suppress cell division delay in *div-1(or148)* temperature-sensitive mutants.
Quantification of the cell division delay between AB and P1 cell of wild-type or the temperature sensitive *div-1* allele *or148* depleted for *cdc-45+sld-5*. WT and *div-1(or148)* were shifted from 15°C to the restrictive temperature 25°C over night (o. n.) before time-lapse analysis was performed. Values are shown in hours for schematic illustration of the experiment or in minutes for quantification of cell division timing. Data are mean values. Error bars show standard error of the mean (s.e.m.). Statistical significance between cell-division timings are indicated by asterisks. The double asterisk indicates $P \leq 0.001$.

2.5. Depletion of a functional CDC-48$^{UFD-1/NPL-4}$ complex does not alter CDC-45 or GINS protein levels.

Recent data on the regulation of the RNA polymerase II subunit Rpb1 as well as protein turnover in response to DNA damage indicate a general function of CDC-48 in the degradation of chromatin bound proteins [190-192]. Because CDC-48 has also been linked to non-proteolytic removal of chromatin associated proteins [172, 193], a CDC-48$^{UFD-1/NPL-4}$ dependent regulation of the stability of CDC-45 and GINS subunits was analyzed. The protein levels of CDC-45, SLD-5, and PSF-3 were detected by western blotting of embryonic lysates after removal of somatic tissues. Notably, the protein levels of CDC-45, SLD-5, and PSF-3 were not significantly changed in *ufd-1,* or *npl-4* (RNAi) embryos (Figure 2.7.).

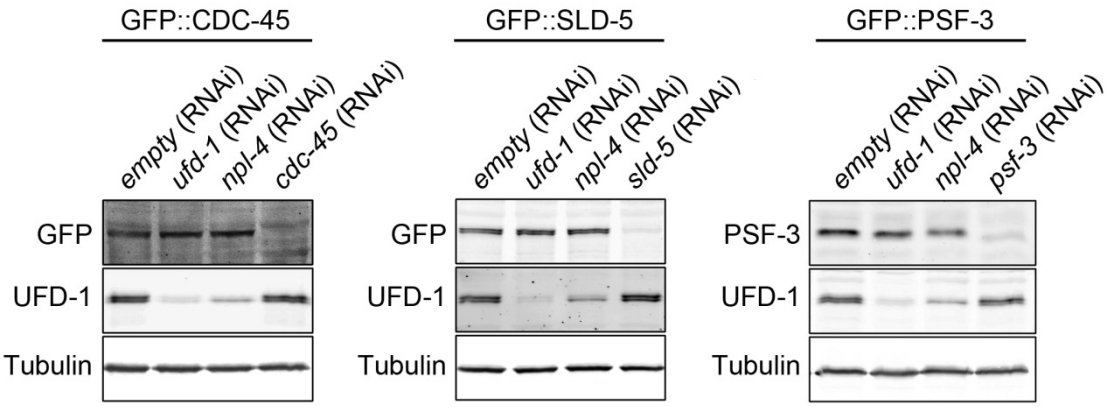


Figure 2.7. CDC-45, SLD-5, and PSF-3 protein levels are unaffected by depletion of *ufd-1* or *npl-4*.
Western blot analysis of GFP fusions of CDC-45 or SLD-5, and PSF-3 protein in *C. elegans* embryonic extracts depleted for *empty, ufd-1, npl-4* and *cdc-45, sld-5, psf-3* respectively. Detection of UFD-1, GFP, and PSF-3 proteins validates effective RNAi-mediated depletion and specificity of respective protein bands. Tubulin was used as loading control.

2.6. Depletion of *cdt-1* suppresses cell cycle delay of *ufd-1* (RNAi) embryos.

Once per cell cycle CDT-1 participates in the formation of the pre-RC at origins of replication. To avoid re-initiation of replication origins, CDT-1 is targeted for degradation during the elongation phase of replication [56, 66]. As CDT-1 turnover is regulated via ubiquitylation, the CDC-48$^{UFD-1/NPL-4}$ complex might be involved. Interestingly, similar to *cdc-45*, *sld-5*, and *psf-3* depletion, down-regulation of the licensing factor CDT-1 partially reduced the P1 cell division delay of *ufd-1* (RNAi) embryos (Figure 2.8.). This genetic interaction raised the possibility, that the regulation of CDT-1 might involve the function of the CDC-48$^{UFD-1/NPL-4}$ complex.

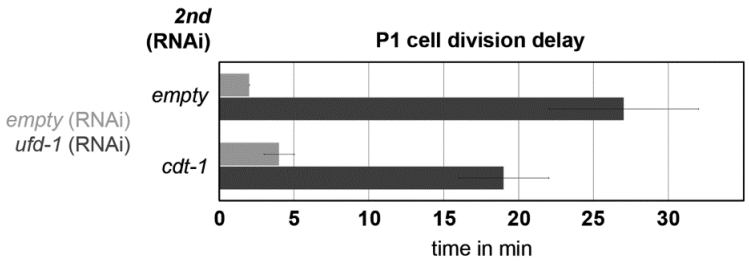


Figure 2.8. *cdt-1* (RNAi) suppresses the cell division delay of *ufd-1* depleted embryos.
Quantification of the time between division of AB and P1 cell (P1 division delay) of embryos depleted first for *empty* (light grey) and *ufd-1* (dark grey) and sequentially for *empty* or *cdt-1* (*2nd* (RNAi)). Data are mean values. Error bars show standard error of the mean (s.e.m.).

2.7. CDC-48$^{UFD-1/NPL-4}$ is required for CDT-1 turnover.

Whether the CDC-48$^{UFD-1/NPL-4}$ complex might regulate CDT-1 degradation was tested by western blotting of embryonic extracts depleted for *cdc-48*, *ufd-1*, *npl-4*, or *cdt-1* as control. Strikingly, quantification of the immunoblot relative to tubulin levels identified stabilization of CDT-1 upon down-regulation of CDC-48, UFD-1, and NPL-4 (Figure 2.9.A, 2.9.B).

Given the high abundance of CDC-48 protein in eukaryotic cells (up to 1% of all cellular proteins [194]), RNAi treatment is not sufficient to completely down-regulate CDC-48 in wild-type. Hence, the *cdc-48.1(tm544)* deletion mutant lacking one of two genes encoding CDC-48 was used to achieve complete down-regulation by RNAi treatment. Efficient depletion of CDC-48 also results in the stabilization of CDT-1 protein levels (Figure 2.9.B).

Contrary to stabilization in embryos, CDT-1 did not accumulate significantly in whole worm lysates of a similar experiment, suggesting that CDT-1 degradation by the CDC-48$^{UFD-1/NPL-4}$ complex is especially important in actively dividing tissues [176].

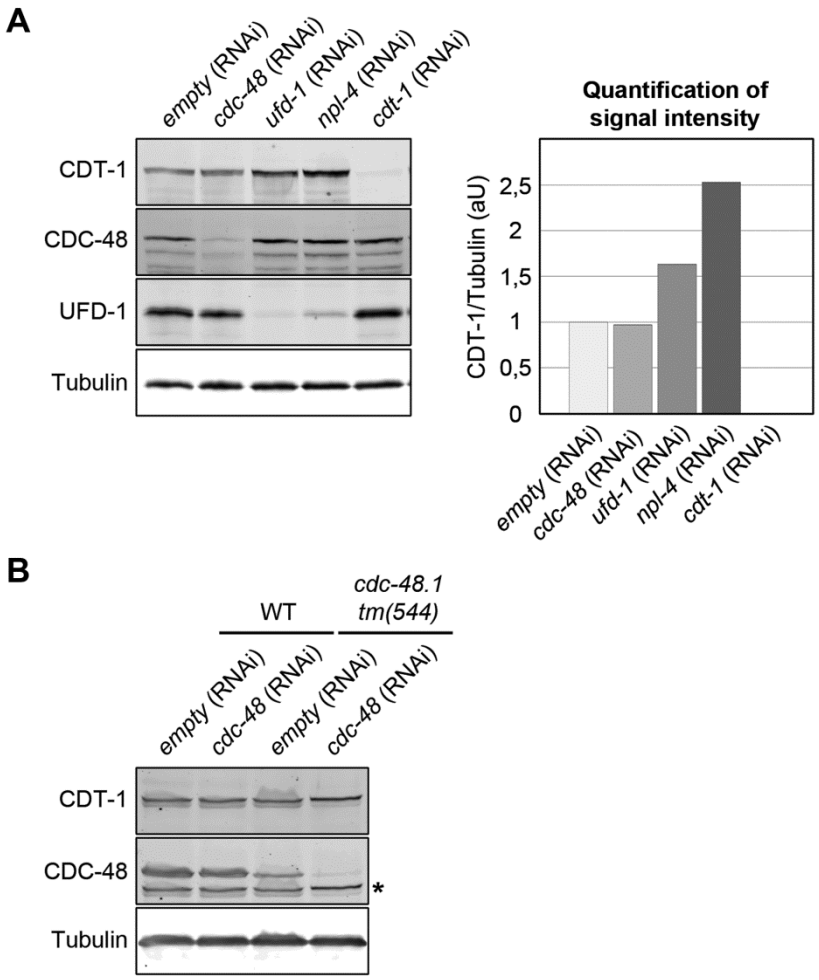


Figure 2.9. CDC-48$^{UFD-1/NPL-4}$ depleted embryos show elevated levels of CDT-1 protein.
(A, B) Western blot analysis of CDT-1 protein levels in embryonic extracts that were depleted for the indicated gene products by RNAi. In (B) embryos were depleted for *empty* or *cdc-48* (RNAi) in wild-type or *cdc-48.1(tm544)* mutant background. Quantification of the signal intensity was calculated relative to the tubulin levels and normalized to the protein levels of the *empty* (RNAi) control.

2.8. CDT-1 protein levels are additively stabilized by *ufd-1* and *rbx-1* (RNAi).

In *C. elegans*, the targeted degradation of CDT-1 is achieved by its ubiquitylation through the CUL-4^{CDT-2} cullin based RING finger ligase [66, 195]. RBX-1 is an essential component of cullin based RING finger ligases and has thus been identified to be required for CDT-1 degradation in somatic and germline cells in *C. elegans* [196, 197]. As the data presented above implicate a role for the CDC-48$^{UFD-1/NPL-4}$ chaperone complex in CDT-1 turnover, this raises the question whether CDT-1 degradation involving CDC-48 is initiated by ubiquitylation through the CUL-4$^{CDT-2/RBX-1}$ ligase.

To answer this question CDT-1 levels were monitored in embryonic lysates treated with *ufd-1* and *rbx-1* (RNAi) separately and in combination. Surprisingly, a combination of *ufd-1* (RNAi) together with *rbx-1* (RNAi) enhanced the stabilization of CDT-1 compared to separated RNAi treatment (50% (RNAi)), indicating that they might affect parallel degradation pathways (Figure 2.10.).

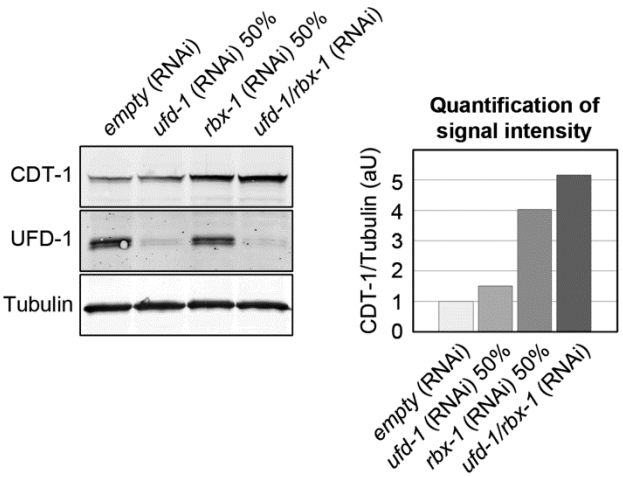


Figure 2.10. *ufd-1* and *rbx-1* (RNAi) additively stabilize CDT-1 protein levels.
Western blot analysis of CDT-1 protein levels in embryonic extracts that are depleted for *empty, ufd-1, rbx-1,* or *ufd-1* and *rbx-1* simultaneously by RNAi. *ufd-1* and *rbx-1* (RNAi) bacteria were equally mixed either with *empty* control (50% (RNAi)) bacteria or together.

2.9. CDC-48$^{UFD-1/NPL-4}$ specifically restricts CDT-1 level on mitotic chromatin.

CDT-1 degradation during S phase involves the CUL-4$^{CDT-2/RBX-1}$ ligase as well as the PCN-1 sliding clamp in *C. elegans* (Figure 1.3.C) [68, 195, 196]. To test, whether CDC-48$^{UFD-1/NPL-4}$ mediated CDT-1 turnover represents a so far undefined pathway, CDT-1 protein was analyzed by immunostainings of embryos depleted for *cdc-48, ufd-1*, *npl-4*, *rbx-1*, or *pcn-1,* respectively. Consistent with the reported function of RBX-1 and PCN-1 in CDT-1 degradation during S phase, CDT-1 staining was observed in S phase nuclei when embryos were treated with *rbx-1* or *pcn-1* (RNAi), while CDT-1 staining was absent in control embryos (Figure 2.11.A) [68, 196]. This is in contrast to *cdc-48*, *ufd-1*, and *npl-4* RNAi embryos which do not accumulate CDT-1 protein in S phase nuclei. Surprisingly, depletion of the CDC-48$^{UFD-1/NPL-4}$ complex specifically resulted in a distinct time-pattern of CDT-1 stabilization (Figure 2.11.A, 2.11.B). Here, a strong accumulation of CDT-1 became obvious on mitotic chromatin, after *cdc-48*, *ufd-1*, and *npl-4* depletion, which was not observed for *rbx-1* or *pcn-1* (RNAi).

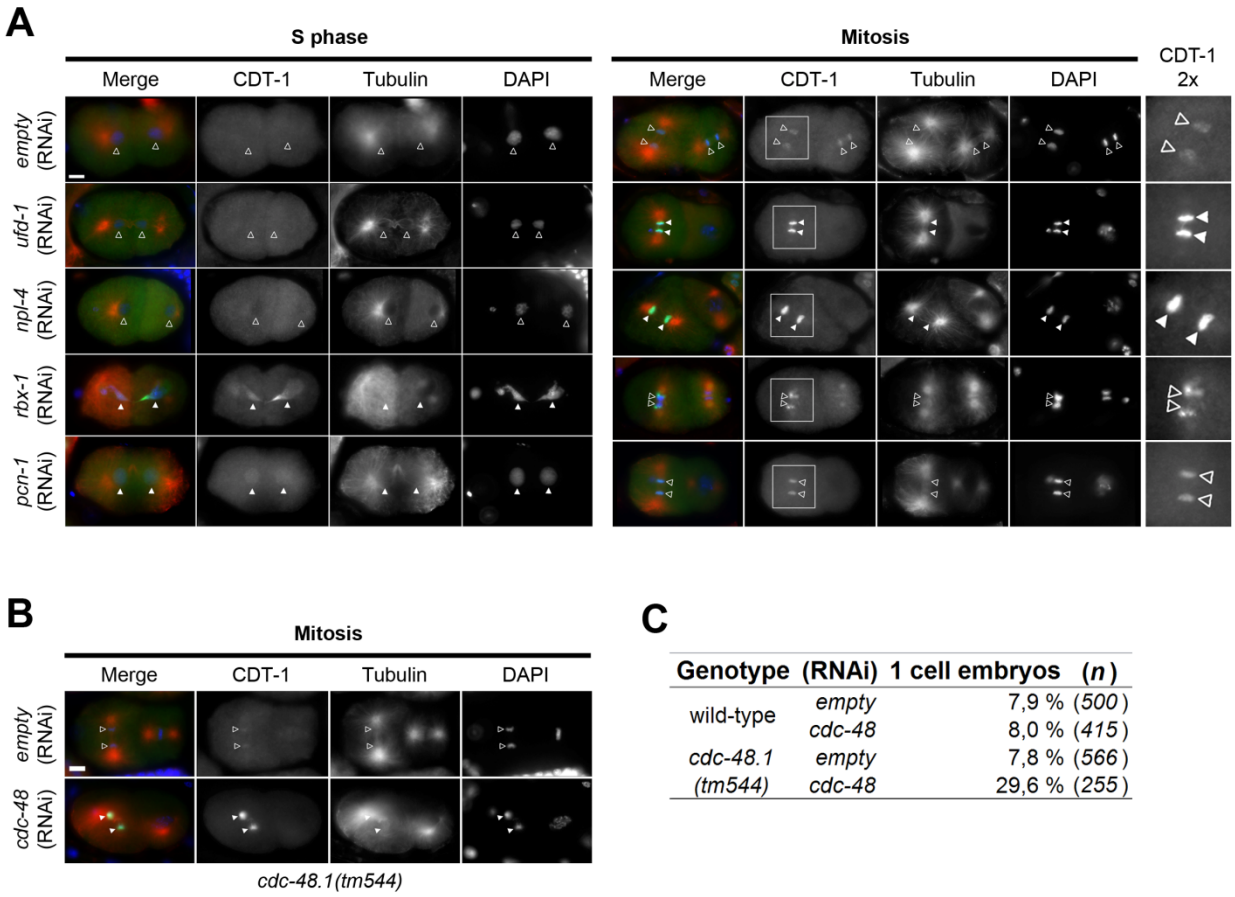


Figure 2.11. CDT-1 accumulates on mitotic chromatin in *cdc-48, ufd-1* and *npl-4* (RNAi) embryos.
(A, B) Immunostainings of early *C. elegans* embryos treated with *empty, cdc-48, ufd-1, npl-4, rbx-1,* or *pcn-1* (RNAi). CDT-1 (green), tubulin (red) and DAPI (blue) staining is shown as merge images and in separate channels. Distinct cell cycle phases are indicated as mitosis or S phase. Empty arrowheads indicate wild-type CDT-1 levels, whereas filled arrowheads indicate enhanced signal intensity on mitotic chromatin. The region marked by squares was magnified twice for better visualization of the CDT-1 staining in A (CDT-1 2x). In B *cdc-48* (RNAi) was performed on *cdc-48.1(tm544)* mutant background. Scale bar represents 5 µm.
(C) Quantification of one cell stage embryos. Embryos were classified as one cell or beyond one cell stage judged by CDT-1, tubulin, or DAPI staining. Respective genotypes and RNAi conditions as well as the number of quantified embryos (*n*) are indicated.

As cell division during *C. elegans* embryonic development occurs unsynchronized, embryonic lysates represent cells engaged in both S phase and mitosis. However, S phase constitutes the mayor part of early embryonic cell cycles compared to mitosis (S phase approximately 12 min, mitosis 2 min) [178]. Considering that CDT-1 accumulates exclusively on mitotic chromatin after depletion of CDC-48$^{\text{UFD-1/NPL-4}}$, the stabilization of CDT-1 in asynchronous embryonic lysates appears weak but obvious, given the low proportion of embryos undergoing mitosis, particularly when the progression through S phase is delayed due to activation of the replication checkpoint. Moreover, quantification of one-celled embryos after complete down-regulation of CDC-48 revealed that embryos appear to arrest at

early stages of development, providing an explanation for the rather weak stabilization of CDT-1 protein due to limitation in cellular material (Figure 2.11.C).

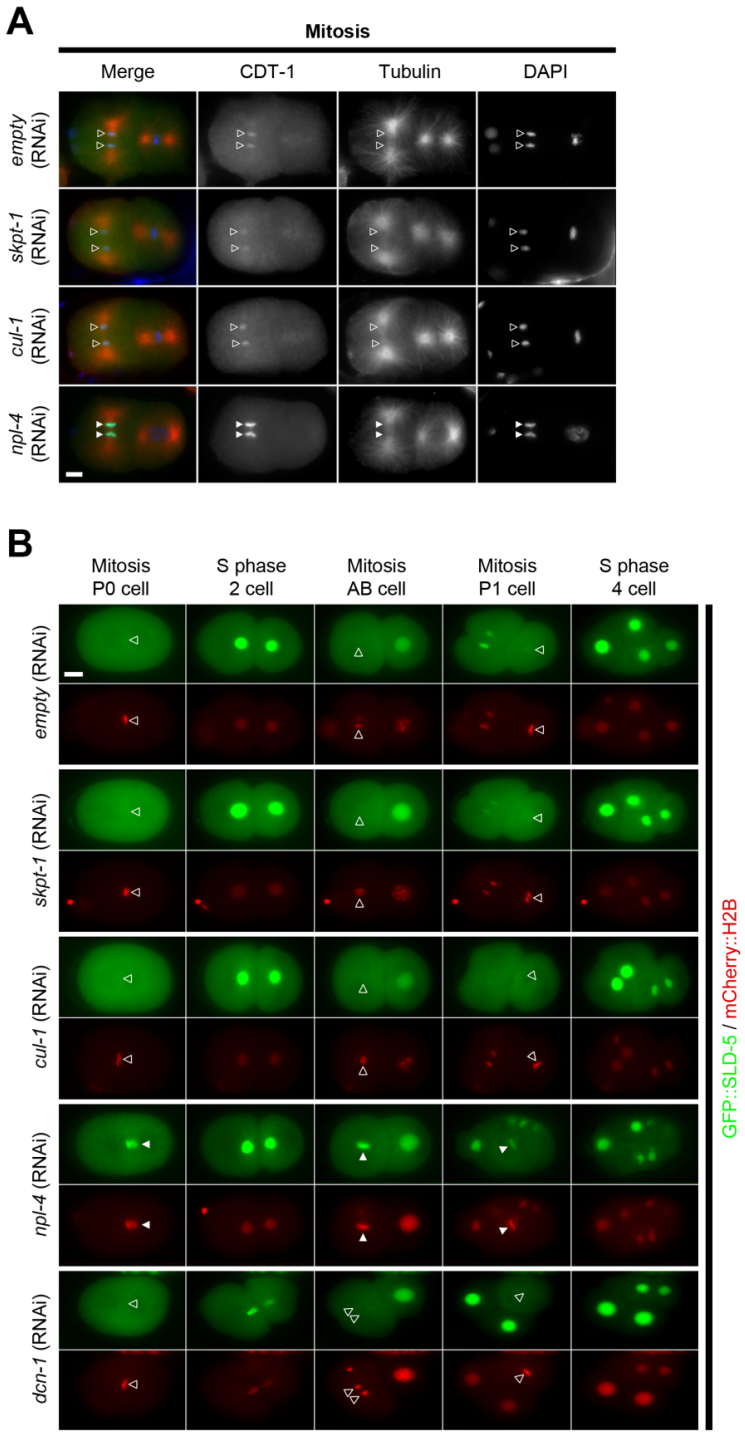
2.10. CDT-1 degradation by the CUL-1^{SKPT-1} E3 ligase is not crucial in *C. elegans.*

The cullin E3 ligase SCF^{SKP2} has been identified to contribute in CDT-1 turnover during the S and G_2 phases of the cell cycle in mammalian cell culture (Figure 1.3.B) [33, 56, 63]. The closest *C. elegans* ortholog of human SCF^{SKP2} is the CUL-1^{SKPT-1} ligase. To reveal a putative function of CUL-1^{SKPT-1} in CDC-48 mediated CDT-1 degradation, *cul-1* and *skpt-1* depleted embryos were analysed by immunostainings and time-lapse microscopy. Neither CDT-1 immunostainings nor GFP::SLD-5 localization were found to be affected by *cul-1* or *skpt-1* (RNAi), indicating that the CUL-1^{SKPT-1} ligase is not crucial for CDT-1 regulation in *C. elegans* (Figure 2.12.A, 2.12.B).

Indeed, *skpt-1* deletion mutants have been shown to be viable without any obvious defects in cell cycle regulation [195]. In line with this, the phosphorylation recognition sequences required for SCF^{SKP2} targeted degradation of CDT-1 in human cell culture appear not to be conserved in the *C. elegans* CDT-1 protein, underscoring that CUL-1^{SKPT-1} is not essential for CDT-1 degradation in the worm. [62, 63, 198-200]

The activity of cullin ligases is regulated by conjugation and de-conjugation of the ubiquitin-like molecule NEDD8 (NED-8 in *C. elegans*) [201, 202]. However, down-regulation of DCN-1 which is required for cullin neddylation [203] does not result in persistent chromatin association of SLD-5, further supporting the observation that CDC-48$^{UFD-1/NPL-4}$ mediated CDT-1 degradation is different from the described turnover involving the cullin based ligases CUL-4^{CDT-2} or CUL-1^{SKPT-1} (Figure 2.12.B).

Figure 2.12. Depletion of *skpt-1* or *cul-1* does not alter CDT-1 and SLD-5 localization.
(A) Immunostainings of early *C. elegans* embryos treated with *empty, skpt-1, cul-1,* and *npl-4* (RNAi). CDT-1 (green), tubulin (red) and DAPI (blue) staining is shown as merge images and in separate channels. Empty arrowheads indicate wild-type CDT-1 levels, whereas filled arrowheads indicate enhanced signal intensity.
(B) Selected pictures of time-lapse recordings of *C. elegans* embryos expressing GFP::SLD-5 (green) and mCherry::H2B (red) that are depleted for *empty* control, *skpt-1, cul-1, npl-4,* and *dcn-1*. Each image series shows representative cell-cycle phases (Mitosis or S phase) at distinct times of embryonic development (1 to 4 cell stage) of one single *C. elegans* embryo. Empty arrowheads point to wild-type like SLD-5 localization, filled arrowheads indicate persistent chromatin association in mitosis. Scale bars represent 5 µm.



2.11. Impaired CDT-1 turnover in S phase does not result in persistent chromatin association of SLD-5.

Whereas down-regulation of *pcn-1* or *rbx-1* by RNAi stabilized CDT-1 protein in S phase nuclei as expected (Figure 2.10., 2.11.), the observation that CDC-48$^{UFD-1/NPL-4}$ restricts the amount of CDT-1 on mitotic chromatin was surprising (Figure 2.9., 2.10., 2.11.). Considering

that depletion of CDC-48$^{UFD-1/NPL-4}$ not only results in CDT-1 degradation defects but in addition shows failure in chromatin dissociation of CDC-45 and the GINS complex, raises the question, whether general impairment of CDT-1 turnover results in persisting chromatin association of CDC-45/GINS. In contrast to CDC-48$^{UFD-1/NPL-4}$ depletion, however, S phase stabilization of CDT-1 by *pcn-1* or *rbx-1* (RNAi) leaves chromatin association of SLD-5 unaffected (Figure 2.13., 2.10., 2.11.). This observation suggests that stabilization of CDT-1 does not alter CDC-45/GINS regulation in general. Consequently, CDC-48$^{UFD-1/NPL-4}$ defines CDT-1 degradation in mitosis, which correlates with chromatin dissociation of the CDC-45/GINS complex.

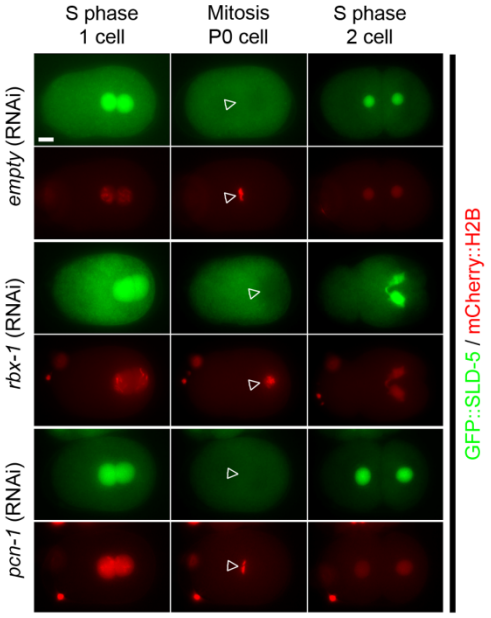


Figure 2.13. S phase stabilization of CDT-1 by *pcn-1* or *rbx-1* (RNAi) does not result in persistent chromatin association of SLD-5.
Selected pictures of time-lapse recordings of *C. elegans* embryos expressing GFP::SLD-5 (green) and mCherry::H2B (red) that are depleted for *empty* control, *rbx-1,* and *pcn-1*. Each image series shows representative cell cycle phases of the first mitotic division of one single embryo. Empty arrowheads point to wild-type like SLD-5 localization. Scale bar represents 5 µm.

2.12. Persistent chromatin binding of SLD-5 in *ufd*-1 (RNAi) embryos depends on CDT-1.

Binding of the CDT-1 licensing factor to origins of replication precedes the recruitment of CDC-45 and the GINS complex to chromatin. In fact, CDT-1 has been shown to promote the chromatin recruitment of CDC-45 [204]. This raises the hypothesis that CDT-1 stabilization by CDC-48$^{UFD-1/NPL-4}$ depletion might directly affect the chromatin association of CDC-45/GINS. To address this assumption, the sequential RNAi feeding protocol introduced in Figure 2.4. was used. First *ufd-1* was depleted to induce the stabilization of CDT-1 and the persistent chromatin association of CDC-45 and the GINS complex. Subsequently *cdt-1* (RNAi) was applied to artificially reduce CDT-1 protein in the embryos. The impact of *cdt-1* (RNAi) on *ufd-1* depleted embryos was tested by following the chromatin association of GFP::SLD-5 in dividing

embryos. Then, the fluorescence intensity of chromatin associated SLD-5 was quantified relative to the mCherry::H2B signal in the area of interest.

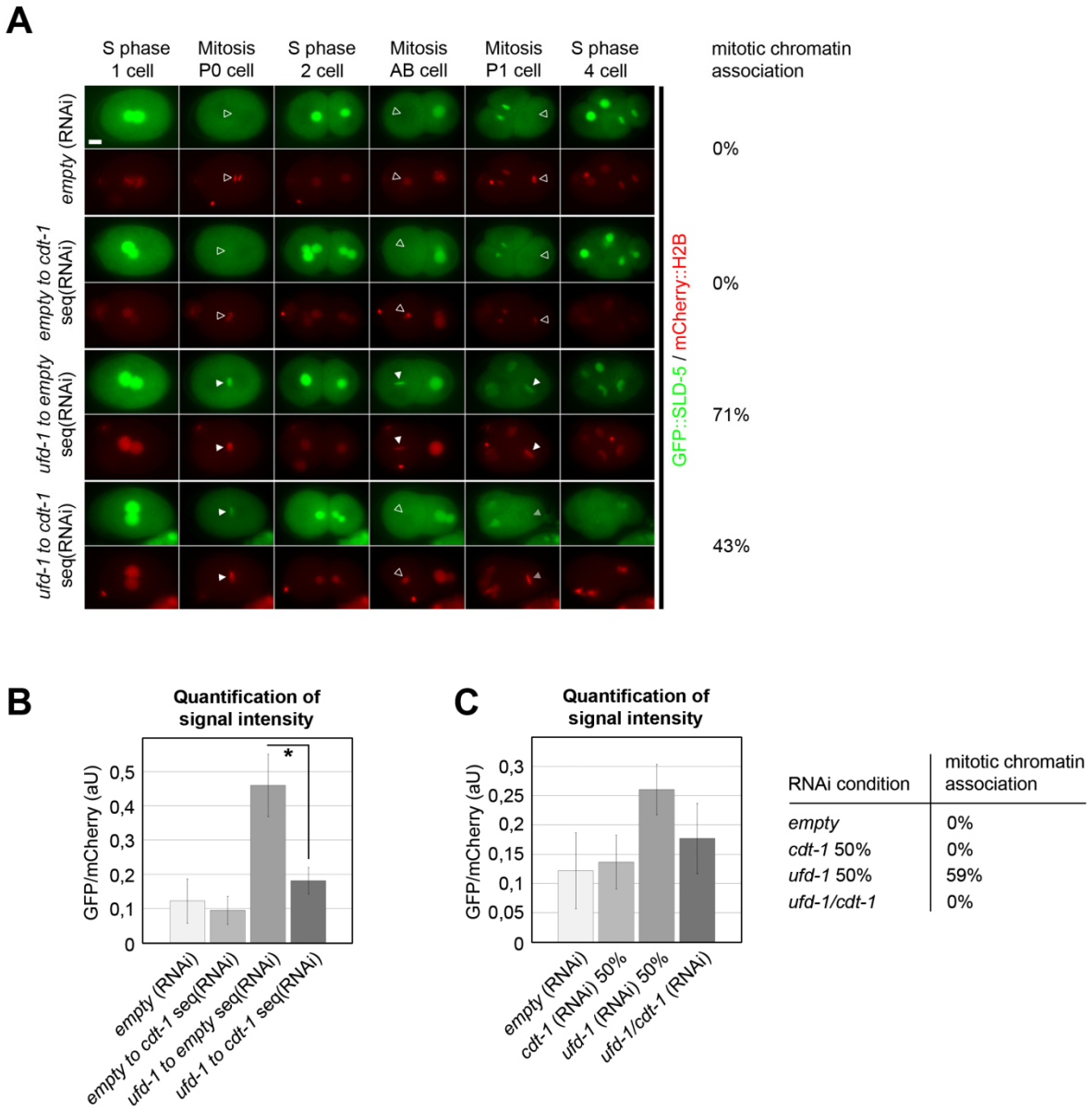


Figure 2.14. Depletion of CDT-1 suppresses persistent SLD-5 chromatin association in *ufd-1* (RNAi) embryos.
(A) Selected pictures of time-lapse recordings of embryos expressing GFP::SLD-5 (green) and mCherry::H2B (red) that are depleted first for *empty* or *ufd-1* followed by *empty* or *cdt-1* (seq(RNAi)). Representative pictures of indicated cell cycle phases (Mitosis or S phase) at distinct time points of embryonic development (1 to 4 cell stage) of one single *C. elegans* embryo are shown. Empty arrows indicate wild-type like mitotic localization, filled arrows indicate persistent association with mitotic chromatin, shaded arrowheads indicate partial mislocalization. Percentage values represent the number of mitotic divisions where SLD-5 chromatin association was monitored under indicated experimental conditions.
(B) Quantification of the GFP signal intensity on mitotic chromatin in embryos treated with *empty, cdt-1, ufd-1* or *ufd-1/cdt-1* seq(RNAi) shown in (A). GFP::SLD-5 signal intensity is shown relative to the intensity for mCherry::H2B in the same area.

(C) Quantification of the GFP signal intensity on mitotic chromatin in embryos simultaneously depleted for *ufd-1/cdt-1*. GFP::SLD-5 signal intensity is shown relative to the intensity for mCherry::H2B in the same area. Percentage values represent the number of mitotic divisions where SLD-5 chromatin association was monitored under indicated experimental conditions. Anterior is to the left. Data are mean values. Error bars show standard error of the mean (s.e.m.). Statistical significance relative signal intensities are indicated by asterisks. The single asterisk indicates P ≤ 0.05. Scale bar represents 5 µm.

Depletion of *cdt-1* alone did not affect the localization of SLD-5, whereas depletion of *ufd-1* resulted in persistent chromatin association (Figure 2.14.A, 2.14.B). In contrast, sequential co-depletion of *ufd-1* and *cdt-1* significantly reduced the amount of SLD-5::GFP that remained bound to chromatin after S phase (Figure 2.14.A, 2.14.B), indicating that CDT-1 is necessary for the persistent chromatin association of SLD-5. In conclusion, the degradation of CDT-1 and the chromatin release of SLD-5 are coordinated via CDC-48$^{UFD-1/NPL-4}$.

Noteworthy, an analogues observation was made using a RNAi protocol that facilitates the simultaneous depletion of *ufd-1* and *cdt-1* by a mixture of respective RNAi constructs. In this experimental setup co-depletion of *ufd-1* und *cdt-1* again reduced the amount of GFP::SLD-5 that was associated with mitotic chromatin after *ufd-1* (RNAi) (Figure 2.14.C). In conclusion, CDT-1 turnover by the CDC-48$^{UFD-1/NPL-4}$ complex is directly linked to chromatin dissociation of CDC-45 and the GINS complex.

2.13. Down-regulation of ORC-2 and CDC-6 does not reduce the accumulation of SLD-5 on mitotic chromatin.

Functional pre-RCs consists of ORC, CDC-6, and CDT-1 that together load the MCM helicase onto chromatin [205]. ORC-2, a subunit of ORC, and CDC-6 are essential for the licensing process. In order to reveal, whether depletion of licensing factors apart from CDT-1 can also reduce the chromatin association of SLD-5, sequential RNAi was performed for *ufd-1* and *orc-2* or *cdc-6* respectively. Down-regulation of the licensing factors ORC-2 and CDC-6 did not result in reduced amounts of GFP::SLD-5 on mitotic chromatin (Figure 2.14., 2.15.B). Moreover, neither *orc-2* nor *cdc-6* (RNAi) suppressed the P1 division delay of *ufd-1* depleted embryos as it is seen for *cdt-1* (RNAi) (Figure 2.8., 2.15.A). This underlines the conclusion that persisting chromatin association of CDC-45/GINS is directly linked to CDT-1 degradation mediated by the CDC-48$^{UFD-1/NPL-4}$ complex during mitosis.

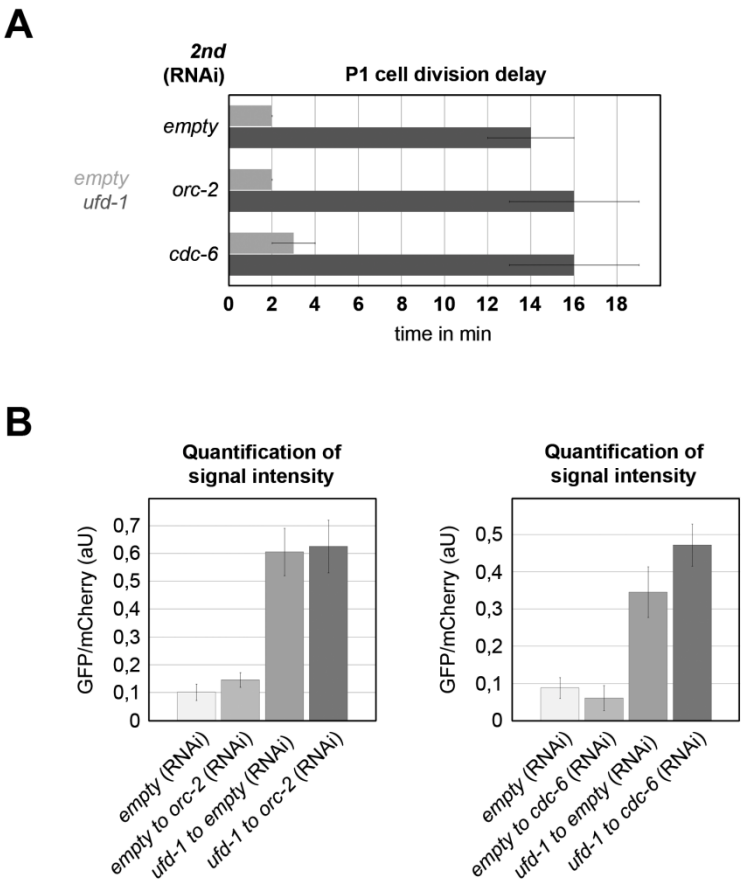


Figure 2.15. Neither *orc-2* nor *cdc-6* (RNAi) suppresses the cell division delay or SLD-5 chromatin association of *ufd-1* depleted embryos.

(A) Quantification of the time between division of AB and P1 cell (P1 division delay) of embryos depleted first for *empty* (light grey) or ufd-1 (dark grey) and sequentially for *empty, orc-2,* or *cdc-6*. Data are mean values. Error bars show standard error of the mean (s.e.m.).

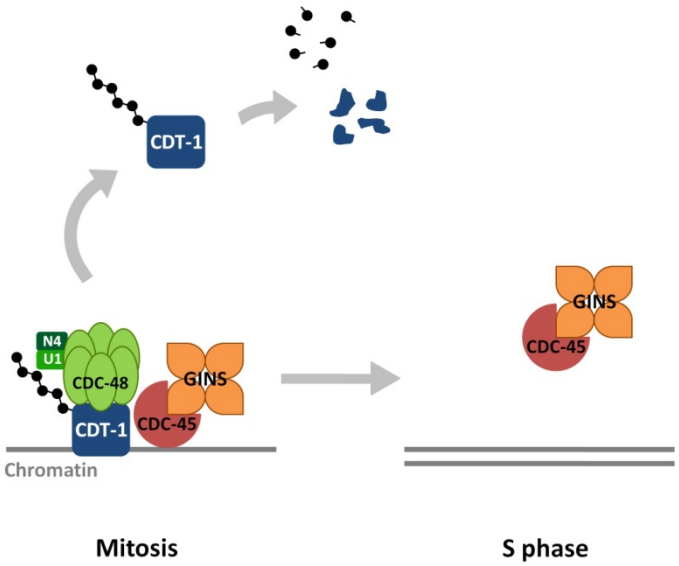
(B) Quantification of the GFP signal intensity on mitotic chromatin in embryos first treated with *empty* or *ufd-1* (RNAi) and sequentially for *empty, orc-2,* or *cdc-6* (RNAi). GFP::SLD-5 signal intensity is shown relative to the intensity for mCherry::H2B in the same area.

· Chapter 3 · Discussion ·

Reliable propagation of the genomic information depends on tight regulation of protein function throughout the DNA duplication process [206]. Posttranslational modification of target proteins is a powerful mechanism triggering rapid and effective control of protein activity [207]. For instance, attachment of the signalling peptide ubiquitin to substrate proteins is inevitably linked to the maintenance of cellular physiology [116, 117]. An essential factor mediating protein degradation of ubiquitylated substrates is the CDC-48 chaperone [182]. Remarkably, CDC-48 activity is required in a variety of cellular processes governed by ubiquitin [145, 146]. Initially identified as a crucial factor for cell cycle progression in yeast, the precise role of CDC-48 during the cell cycle remained unclear [169]. Temperature sensitive *cdc48* mutants in budding yeast arrest at the transition from G_2 to M phase, which is indicative of failures occurring during the S phase of the cell cycle [169, 208]. Interaction with several proteins involved in DNA metabolism has been reported in diverse organisms, supporting the recently described requirement of CDC-48 for effective DNA replication [162, 176]. In order to unravel the mechanistic details of CDC-48 activity, the key substrates playing a crucial role during DNA duplication need to be identified.

This work discovered a regulatory function of CDC-48 in the coordination of licensing and elongation events during eukaryotic DNA replication in *C. elegans* (Figure 3.1.). CDC-48 together with the cofactors UFD-1 and NPL-4 promotes the degradation of the licensing factor CDT-1 during mitosis. Moreover, the CDC-$48^{UFD\text{-}1/NPL\text{-}4}$ chaperone is required for the dissociation of CDC-45 and the GINS complex, which appears to be connected to mitotic CDT-1 turnover. In conclusion this work reveals a regulatory mechanism of the ubiquitin-selective CDC-$48^{UFD\text{-}1/NPL\text{-}4}$ chaperone in dynamic chromatin association of essential DNA replication factors, underscoring a central role in the maintenance of genomic integrity (Figure 3.1.).

Figure 3.1. A model for the regulation of replication factor dynamics by the CDC-$48^{UFD\text{-}1/NPL\text{-}4}$ complex.
CDC-48/p97 together with the cofactors UFD-1/NPL-4 (U1/N4) coordinates the turnover of ubiquitylated CDT-1 during the licensing phase with chromatin dissociation of CDC-45/GINS. Failure in CDT-1 turnover keeps CDC-45/GINS tightly associated with chromatin, becoming visible with condensing chromosomes at the end of S phase. CDC-45/GINS malregulation may interfere with dynamic progression of the replication fork in S phase. See text for further details. Modified from [209].



3.1. Proteolytic regulation of the CDT-1 protein by CDC-48$^{UFD-1/NPL-4}$.

3.1.1. CDC-48$^{UFD-1/NPL-4}$ restricts CDT-1 protein specifically on mitotic chromatin.

The licensing factor CDT-1 is required for the initial assembly of the pre-RC at origins of replication [205, 210]. It is worth to note that CDT-1 protein activity needs to be tightly controlled in order to ensure faithful replication and chromosomal stability [32, 33, 37]. This study identified a role of the ubiquitin-selective chaperone CDC-48 together with its cofactors UFD-1 and NPL-4 in the degradation of CDT-1 in *C. elegans* embryos (Figure 2.9., 2.10. 2.11., 2.12.). Accordingly, disruption of the CDC-48$^{UFD-1/NPL-4}$ complex by RNAi results in stabilization of CDT-1 protein levels in embryonic lysates (Figure 2.9., 2.10.). Targeted degradation of CDT-1 during S phase is important to prevent re-replication and chromosomal instability in eukaryotic cells [60, 63, 65-71, 195, 211]. S phase degradation of CDT-1 mainly depends on two crucial factors, the CUL-4$^{CDT-2/RBX-1}$ ubiquitin ligase, and the PCN-1 sliding clamp (Figure 1.3.). This study confirms that depletion of *rbx-1* or *pcn-1* by RNAi stabilizes CDT-1 in S phase nuclei of *C. elegans* embryos (Figure 2.10., 2.11.) [68, 69, 71, 196]. Strikingly, *cdc-48*, *ufd-1*, or *npl-4* depletion results in a different temporal pattern of CDT-1 stabilization (Figure 2.10., 2.11., 2.12.). In this case CDT-1 protein is specifically stabilized on mitotic chromatin (Figure 2.9., 2.11., 2.12.).

In order to verify, whether mitotic CDT-1 turnover by CDC-48$^{UFD-1/NPL-4}$ represents a hitherto not described regulatory mechanism, a careful comparison to the known degradation pathways during S phase was done. Coincidently, none of the factors that were analyzed recapitulated the phenotype of CDC-48$^{UFD-1/NPL-4}$ complex disruption (Figure 2.2., 2.9., 2.10., 2.11., 2.12., 2.13.). Depletion of *rbx-1* and *pcn-1* results in CDT-1 stabilization in S phase nuclei,

instead leaving mitotic CDT-1 levels unaffected (RNAi) (Figure 2.10., 2.11.). In human cells the SCF^{SKP2} ubiquitin ligase is involved in CDT-1 turnover during S phase [63]. However, this work reveals that the CUL-1^{SKPT-1} ligase, orthologous to mammalian SCF^{SKP2}, does not markedly contribute to CDT-1 degradation in *C. elegans* embryos (Figure 2.12.) [56, 195]. In addition to RBX-1, PCN-1, and, CUL-1^{SKT-1}, factors required for UV induced CDT-1 degradation were tested for putative contribution to CDT-1 turnover in unperturbed cell cycles [212]. Neither *cdt-2*, *ddb-1*, *ubc-12*, *csn-5*, nor *dcn-1* (RNAi) (as an example *dcn-1* (RNAi) is shown in Figure 2.12.) reflected the phenotype of *cdc-48, ufd-1,* or *npl-4* depletion (Figure 2.11., 2.12., 2.13.). In conclusion, mitotic turnover via CDC-$48^{UFD-1/NPL-4}$ represents an unanticipated regulatory mechanism of CDT-1 activity.

Supporting these findings, a study in human cell culture reported Cdt1 accumulation in mitosis, when its ubiquitylation is inhibited in the presence of geminin [213]. The data of Ballabeni and colleagues implies the existence of a CDT-1/Cdt1 degradation pathway besides its known turnover during S phase. Nevertheless, it remains to be determined how mitotic CDT-1 ubiquitylation is catalyzed [213] and whether subsequent degradation is also connected to CDC-48 activity.

3.1.2. Requirement for mitotic CDT-1 regulation by CDC-$48^{UFD-1/NPL-4}$.

CDT-1 is an essential component of the pre-RC required for loading of the MCM helicase complex during the licensing step of DNA replication [210, 214]. In *C. elegans* embryonic cell cycles the licensing of origins takes place during mitosis [66, 215]. Therefore, the observation that CDC-$48^{UFD-1/NPL-4}$ is required to limit the amount of CDT-1 protein in mitosis (Figure 2.9., 2.10., 2.11.) is unexpected [66, 215].

Recently, a detailed *in vivo* analysis of Cdt1 protein during the licensing process revealed that association of Cdt1 with chromatin is far more dynamic than previously anticipated [216]. In this study, Cdt1 fused to GFP was followed by live-imaging techniques in mammalian tissue cell culture. Analysis of Cdt1 during the licensing process revealed that about 30% of chromatin associated CDT-1 protein is highly dynamic and shuttling between chromatin and nucleoplasm. Interestingly, a likewise dynamic behavior has been observed for the ORC during the licensing process [217]. It appears feasible that elevated CDT-1 protein levels on mitotic chromatin upon *cdc-48, ufd-1,* or *npl-4* (RNAi) (Figure 2.9., 2.11.) reflect defects in dynamic chromatin association. The CDC-$48^{UFD-1/NPL-4}$ chaperone might promote rapid release and re-binding of CDT-1 throughout the licensing process, thereby ensuring efficient origin licensing [162, 176].

The precise physiological function of dynamic chromatin association of the licensing factors ORC and CDT-1 remains elusive. One possible scenario is the repetitive loading of the MCM complex to origins of replication [218]. Worth mentioning, repeated recruitment of the MCM helicase is strongly dependent on ORC and CDT-1 activity [219-222]. It is likely that limited amounts of MCM complexes are not sufficient to complete DNA replication when MCM loading is impaired [218, 219]. Consistently, efficient loading of the MCM helicase is required to overcome situations of replication stress [222-224]. Therefore, repetitive CDT-1 binding cycles depending on CDC-48 activity might be required to promote sufficient MCM loading to the chromatin (Figure 2.9., 2.11.). This would be in line with the initial observation that the CDC-48$^{UFD-1/NPL-4}$ complex is required for efficient DNA synthesis [162, 176].

Another hypothetical situation requiring dynamic CDT-1 association with the chromatin during the licensing process is the requirement for flexible and rapid response to internal or external causes of replication stress, ensuring efficient initiation of replication [225]. Current reports indeed show that an adaptable control of origin firing is detrimental for genome stability, particularly under situations of replication stress [223, 224, 226]. This might be especially important in early embryonic cell cycles that are supposed to rapidly synthesize DNA and utilize a high density of replication origins [227, 228].

3.1.3. De-regulation of CDT-1: Induction of re-replication versus impairment of faithful DNA duplication.

Tight regulation of replication licensing is critical to ensure that the duplication of DNA is limited to occur only once per each cell cycle. Re-initiation of origin licensing can result in over-replication of DNA segments which threatens genome integrity [37]. The depletion of *cdc-48, ufd-1,* or *npl-4* was shown to result in inefficient DNA synthesis, rather than increased DNA content caused by over-replication [162, 176]. At a first glance, this appears inconsistent, considering the elevated levels of the CDT-1 licensing factor accompanied with CDC-48$^{UFD-1/NPL-4}$ complex disruption, revealed by this thesis (Figure 2.9., 2.10.).

Research in the past 15 years mainly addressed the regulation of the licensing factor CDT-1 as over-expression conditions in *Xenopus* egg extracts and in certain mammalian cancer cell lines are sufficient to promote re-initiation of replication in a single cell cycle [70, 211, 229-231]. However, functional licensing, and hence re-licensing, requires the availability of all three components ORC, CDC-6, and CDT-1 in order to promote MCM helicase loading [232]. Accumulating evidence suggests that cultured mammalian cells that are not derived from transformed cancer tissue indeed require the simultaneous de-regulation of at least two of the

licensing factors ORC, CDC-6 or CDT-1 to induce detectable re-replication [233-235]. This also applies for *C. elegans* embryonic divisions. Here, CDT-1 together with CDC-6 needs to be de-regulated to induce re-replication [236]. The initial finding that the CUL-4 ubiquitin ligase is critical to restrict DNA synthesis by targeting CDT-1 protein for degradation, is thus explained by the observation that CUL-4 regulates other essential cell cycle proteins involving CDC-6 (Figure3.2.) [66, 236, 237].

In spite of initiation of re-replication, a recent study emphasizes the occurrence of chromosomal aberrations and chromosomal damage in different non cancer cell lines exclusively stabilizing CDT-1 protein levels [33]. Strikingly, no re-replication was detectable in these experiments when measuring the DNA contents. However, CDT-1 stabilization resulted in the activation of the DNA replication and damage checkpoints in line with chromosomal aberrations and segregation defects [33]. Chromosomal instability by fragmentation of DNA resulting in the activation of the ATL-1 replication checkpoint is also observed in *Xenopus* [32]. However, occurrence of fragmented chromosomes likely results from re-replication events in this system.

Detailed analysis of DNA integrity will be required to decipher, whether the specific accumulation of CDT-1 on mitotic chromatin after *cdc-48*, *ufd-1* and *npl-4* (RNAi) (Figure 2.9, 2.10., 2.11.) leads to DNA fragmentation and loss of chromosomal integrity. *C. elegans* embryos without a functional CDC-48$^{UFD-1/NPL-4}$ complex stabilize CDT-1 protein causing ATL-1 checkpoint activation and replication defects probably due to chromosomal alterations (Figure 2.8., 2.14., 2.15.). Chromosomal bridges visible in the anaphase of mitosis and focal accumulation of the DNA repair protein RAD-51 might be indicative of likewise chromosomal aberrations in *C. elegans* embryos lacking CDC-48$^{UFD-1/NPL-4}$ activity [176].

3.2. Mechanisms of CDC-45 and GINS regulation.

3.2.1. CDC-45/GINS chromatin association is regulated by CDC-48$^{UFD-1/NPL-4}$.

This study reveals that CDC-48 not only restricts chromatin bound CDT-1 levels but in addition promotes the chromatin dissociation of CDC-45 and the GINS complex (Figure 3.1., 3.2.). Consistently, worm embryos lacking *cdc-48*, *ufd-1*, and *npl-4*, show persistent chromatin association of CDC-45 and the GINS subunit SLD-5, becoming visible on condensing chromosomes after S phase is completed (Figure 2.2., PSF-3 is not shown). Interestingly, CDC-45, SLD-5, and PSF-3 protein levels were found to be unaffected by *ufd-1* (RNAi), suggesting a non-proteolytic regulation by CDC-48$^{UFD-1/NPL-4}$ (Figure 2.7.). It is worth to note, that the dynamics of other essential replication factors were found to be unaffected by *ufd-1* (RNAi),

emphasizing a specific regulation of CDT-1 and CDC-45/GINS by the CDC-48$^{UFD-1/NPL-4}$ chaperone (Figure 2.1., 2.2., 2.3.).

In contrast to the control of CDT-1, the regulation of CDC-45 and the GINS complex is much less explored. Chromatin recruitment of CDC-45/GINS is triggered by phosphorylation events involving CDK and DDK kinases at the transition from replication licensing to elongation [8, 10, 238, 239]. Once recruited, both CDC-45 and the GINS complex promote the assembly of a functional replisome and are required for replication fork progression [8, 19].

In addition to its regulation through phosphorylation in regular cell cycles, CDC-45 is also known to be controlled upon replication stress in mammalian cell culture [240-242]. Activation of the replication checkpoint results in reduced association of CDC-45 with chromatin. However, the soluble fraction of CDC-45 appears to remain unaffected [240]. Instead CDC-45 degradation is supposed to be induced by terminal differentiation into post-mitotic cells [243]. Here, treatment with proteasomal inhibitors during the differentiation process delays the degradation of CDC-45 protein. Although, this initial observation might suggest a targeted CDC-45 degradation by the proteasome, an indirect effect of proteasomal inhibition on CDC-45 stability, for example through transcriptional regulation [244], is also plausible. Whether CDC-45 regulation is linked to proteasomal degradation and whether this has an impact on cell cycle progression demands further investigation. The data presented here suggest, that the ubiquitin-selective chaperone CDC-48 is not essential for CDC-45 protein degradation during *C. elegans* embryonic divisions (Figure 2.2., 2.7.).

The regulation of the GINS complex is even less investigated than it is the case for CDC-45. So far, it has only been shown in a proteomic study that the PSF-2 subunit is phosphorylated by the replication and damage checkpoint kinases ATL-1 and ATM-1 [102]. A recent report proposes, that the GINS complex dissociates from the replisome at stalled replication forks in the *Xenopus* egg extract system [245]. In turn reassembly of an active replisome requires the reincorporation of the GINS complex [245]. Disruption of a functional CDC-48$^{UFD-1/NPL-4}$ complex in *C. elegans* embryos results in the occurrence of replication stress and pronounced ATL-1 checkpoint activation (Figure 2.5) [176, 179]. However, both CDC-45 and GINS remain associated with the chromatin throughout cell cycle progression, even after ATL-1 down-regulation (Figure 2.2.). This could suggest distinct regulatory mechanisms for CDC-45 and GINS upon replication fork stalling in mammals, *Xenopus,* and *C. elegans* (Figure 2.2) [240, 246, 247]. Alternatively, CDC-45 and GINS removal from stalled replisomes might be actively catalyzed by CDC-48$^{UFD-1/NPL-4}$.

Considering the current literature and the data presented here, the control of CDC-45 and the GINS complex by CDC-48$^{UFD-1/NPL-4}$ likely does not involve targeted proteolysis of these replication factors (Figure 2.7.). This is in agreement with a number of studies implicating a function of CDC-48 that is triggered by ubiquitylation but does not involve subsequent degradation of the substrate protein [172, 173, 193, 248]. This raises the possibility that CDC-45 and the GINS complex might be regulated by a non-proteolytic segregation mechanism (Figure 1.8., 2.2., 2.7.). In the course of this work Paul Pirson could show that CDC-45 interacts with the CDC-48 cofactor UFD-1 in a yeast-two-hybrid assay [209]. This might hint at a direct role of CDC-48$^{UFD-1/NPL-4}$ in dissociating CDC-45 and GINS from the chromatin in mitosis.

In order to elucidate the contribution of CDC-45 and GINS to the replication defects of CDC-48$^{UFD-1/NPL-4}$ depleted embryos in more detail, it will be of great interest to analyze the role of reported binding partners. In this regard it appears eligible that the FACT chromatin remodelling complex (also called DUF) has been identified as an interaction partner of both CDC-48 and CDC-45/GINS [19, 249]. Intriguingly, ubiquitylation of FACT is important for its involvement in DNA replication [250]. Moreover, CDC-48 was found as an interactor of the chromatin helicases HIM-6 and WRN-1 [251, 252]. Chromatin remodelling by DNA helicases is required to overcome physiologically or exogenously induced replication barriers [253]. Therefore, one can speculate about a function of CDC-48 in cooperation with CDC-45/GINS in the handling of replication barriers by regulating chromatin remodelling activity.

3.2.2. Chromatin dissociation of CDC-45 and GINS is linked to mitotic CDT-1 degradation.

CDC-48 activity is required for both restriction of chromatin bound CDT-1 levels in mitosis and chromatin dissociation of CDC-45 and the GINS complex (Figure 2.2., 2.9., 2.11.). Binding of the CDT-1 licensing factor to origins of replication precedes the recruitment of CDC-45 and the GINS complex to the chromatin. In fact CDT-1 has been shown to facilitate the recruitment of CDC-45 to the chromatin [204], raising the possibility that persistent chromatin association of CDC-45/GINS might be a consequence of mitotic CDT-1 stabilization upon *cdc-48, ufd-1,* or *npl-4* depletion.

Down-regulation of *cdt-1* suppresses the chromatin association of the GINS complex and the cell division delay phenotype in embryos that were disrupted for a functional CDC-48$^{UFD-1/NPL-4}$ complex (Figure 2.8., 2.14.). Conversely, RNAi-mediated depletion of the licensing factors ORC-2 and CDC-6 neither suppress the division delay induced by *ufd-1* (RNAi), nor reduced the amount of SLD-5::GFP on mitotic chromatin (Figure 2.15.). Reduction of chromatin associated GINS in *ufd-1* (RNAi) embryos is specific to *cdt-1* depletion, implying that decreased

GINS is not secondarily caused by impaired recruitment during the licensing process. Thus, these findings suggest that CDC-48 orchestrates both, CDT-1 degradation and chromatin dissociation of the CDC-45/GINS complex during eukaryotic DNA replication (Figure 2.9., 2.11., 2.14.). Accordingly, chromatin dissociation of CDC-45 and GINS requires CDT-1 degradation during mitosis (Figure 2.14., 2.15., 3.1., 3.2.).

As a consequence of CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ disruption, stabilization of CDT-1 in mitosis might result in tight CDC-45/GINS chromatin association. Stable association of CDC-45/GINS with chromatin may sterically interfere with dynamic progression of the replication fork in S phase, preventing efficient DNA synthesis [254]. In line with this, pre-RCs that have not been initiated were found to cause stalling of the replication fork in budding yeast [255]. Alternatively, ectopic CDC-45/GINS on mitotic chromatin might disturb physiological processes at the replication fork, thereby generating replication stress [19, 24]. This hypothesis is supported by the genetic suppression of the cell cycle delay in *ufd-1* or *npl-4* depleted embryos by *cdc-45*/*sld-5* or *psf-3* (RNAi), implicating a critical role of CDC-45/GINS in the activation of the replication checkpoint (Figure 2.5.). Thus, malfunction of CDC-45 and GINS contributes to replication defects in embryos lacking a functional CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ complex.

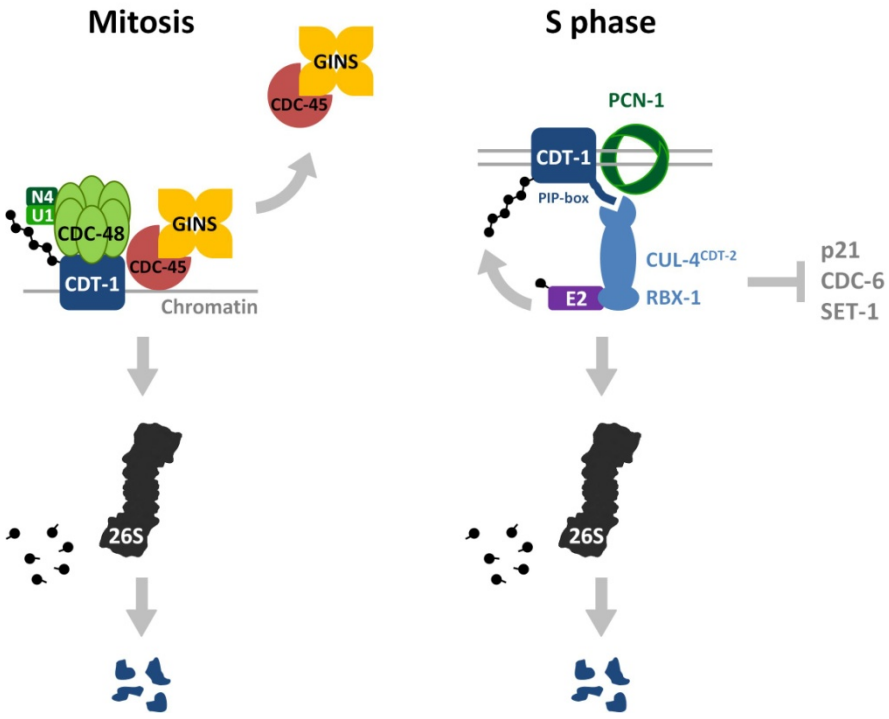


Figure 3.2. Distinct CDT-1 degradation pathways ensure chromosomal stability.
The licensing factor CDT-1 requires tight regulation throughout the cell cycle in order to maintain genomic stability. During mitosis the CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ chaperone mediates CDT-1 degradation by the 26S proteasome. Mitotic CDT-1 turnover is closely linked to chromatin dissociation of CDC-45 and the GINS complex. Conversely, CDT-1 degradation during the S phase requires two essential components the CUL-4$^{CDT\text{-}2/RBX\text{-}1}$ ligase and the

PCN-1 sliding clamp. The PIP-box domain in the CDT-1 protein mediates close proximity to PCN-1 and recruitment of the CUL-4 ligase, thereby facilitating CDT-1 turnover during the S phase. The CUL-$4^{CDT\text{-}2}$ ligase also controls p21, CDC-6, and SET-1 activities, thereby prohibiting re-replication and ensuring genome integrity. The S phase control of CDT-1 degradation in mammalian cells by the SCF^{SKP2} ligase is not shown. Modified from [56, 256].

The data presented in this thesis reveal a CDT-1 degradation pathway that acts specifically during mitosis and is required for chromatin dissociation of CDC-45 and GINS (Figure 2.2., 2.9., 2.11., 2.14.). Strikingly, CDT-1 regulation by CDC-$48^{UFD\text{-}1/NPL\text{-}4}$ is different from its turnover during S phase involving the CUL-$4^{CDT\text{-}2/RBX\text{-}1}$ ligase and PCN-1 (Figure 2.10., 2.11.). The fact that the GINS subunit SLD-5 does not persist on chromatin in the absence of *rbx-1* and *pcn-1* supports the idea that stabilization of CDT-1 in S phase does not enhance CDC-45/GINS recruitment (Figure 2.13.), emphasizing that this phenotype is specific for *cdc-48*, *ufd-1* and *npl-4* (RNAi) (Figure 2.2., 2.13.).

These findings demonstrate that the spatial and temporal regulation between dynamic protein complexes at the replication fork is governed by CDC-48. Elevated CDT-1 protein levels during mitosis results in replication defects including the persistent association of CDC-45/GINS with the chromatin, which represents a regulation of replication factor dynamics not described so far. Future experiments will address the surveillance of CDT-1, CDC-45, and GINS by the ubiquitin-selective chaperone CDC-48 in more detail.

3.3. Evolutionarily conserved regulation of replication factors by CDC-48.

3.3.1. p97 regulates CDT-1 and GINS chromatin association in *Xenopus laevis.*

Identification of a crucial function of CDC-48 in the dynamic regulation of replication factors in *C. elegans* raises the question whether the underlying mechanisms are conserved in other species. The *Xenopus laevis* egg extract system is a powerful tool to study cell cycle related processes at the biochemical level [257, 258]. Therefore, collaboration with the laboratory of Olaf Stemmann at the University of Bayreuth was initiated. The experiments were carried out by Michael Orth.

The aim of the collaboration was to investigate whether also the CDC-48 ortholog in vertebrates, p97, together with its cofactors Ufd1/Npl4 is required to coordinate dynamics of replication factors. Remarkably, co-immunoprecipitation (co-IP) experiments revealed that Ufd1 interacts with Cdt1 in egg extracts [259]. Sequential pulldowns of Ufd1 and His-tagged ubiquitin indeed suggest the interaction between Ufd1 and ubiquitylated Cdt1 species, implicating the existence of a conserved regulation of Cdt1 by $p97^{Ufd1/Npl4}$ in vertebrates.

Using affinity purified antibodies [171], Ufd1 or Npl4 proteins were specifically immunodepleted from egg extracts and the progression of chromatin through the cell cycle was monitored. Chromatin re-isolated during S phase or mitosis was examined for the presence of Cdt1 and the GINS subunit Sld5, both by immunofluorescence experiments and western blot analysis. Strikingly, persistent binding of Cdt1 and Sld5 on mitotic chromatin isolated from Ufd1/Npl4 depleted extracts was observed [259]. However, in the *Xenopus* system Cdt1 also accumulated on S phase chromatin upon Ufd1 or Npl4 depletion.

In conclusion, these data and the data presented here suggest an evolutionarily conserved function of CDC-48/p97 in the degradation of Cdt1 linked to dissociation of GINS from chromatin, which seems to be a prerequisite for accurate eukaryotic DNA replication (2.2., 2.9., 2.11., 2.14.). Demonstrating the interaction between Ufd1 and ubiquitylated Cdt1 further underscores Cdt1 as a direct target of CDC-48/p97 and provides mechanistic insight into the process of Cdt1 regulation. Since CDC-48/p97/VCP has not been implicated in the regulation of DNA replication in mammalian cell culture yet, it remains to be shown whether an analogous mechanism also operates in somatic tissues. Down-regulation of mammalian CDC-48 ortholog, VCP, by RNAi results in significant accumulation of cells in the S phase or at the G_2 to M phase transition in HeLa cells [260]. Thus, a conserved and critical role of CDC-48/p97/VCP in the regulation of S phase processes might also exist in somatic cells.

3.3.2. CDC-48 dependent turnover of CDT-1 in unperturbed cell cycles and upon DNA damage induction.

In addition to its degradation during the S phase, CDT-1 is also rapidly degraded upon induction of DNA damage [261]. Targeted proteolysis of CDT-1 in the presence of DNA damage in human cells has been linked to the CUL-4^{CDT-2} and SCF^{SKP2} ubiquitin ligases that also operate in unperturbed cell cycles [262, 263]. Interestingly, a recent study identified the requirement of the human CDC-48 ortholog VCP for Cdt1 degradation upon UV irradiation [212]. A genome wide siRNA (small interfering RNA) screen was performed in order to unravel crucial factors for damage induced CDT-1 turnover. Besides the validation of several components around the CUL-4^{CDT-2} ligase and the neddylation pathway, a so far not described function of VCP and Ufd1 could be shown. From a series of *in vitro* experiments using the *Xenopus* egg extract system, the authors conclude that the ATPase activity of $p97/VCP^{UFD-1}$ is required to mobilize ubiquitylated Cdt1 from the chromatin facilitating its subsequent degradation by the proteasome after UV treatment.

The basic observation of CDC-48$^{UFD-1/NPL-4}$ mediated CDT-1 regulation being also conserved in human cell culture support the data presented in this work (Figure 2.8, 2.9., 2.10.). However, apparent differences emerge concerning CDC-48/p97/VCP mediated CDT-1 turnover in unperturbed cell cycles in comparison to the DNA damage response. Noteworthy, UV induced Cdt1 degradation inevitably requires PCN-1 and subsequent CUL-4^{CDT-2} recruitment to DNA damage sites [212, 264], whereas the experiments in *C. elegans* embryos strongly suggest that the CDC-48 dependent CDT-1 turnover is different from the PCN-1 and CUL-4$^{CDT-2/RBX-1}$ pathway in unperturbed cell cycles (Figure 2.10., 2.11., 2.13.). This discrepancy becomes even more evident by the fact that the PCN-1 sliding clamp is loaded onto chromatin after mitosis at the beginning of the DNA synthesis phase (Figure 2.1.) [265]. This clearly distinguishes UV-induced Cdt1 degradation from CDT-1 turnover in regular cell cycles in *C. elegans* embryos, as PCN-1 is supposed not be present on the chromatin in mitosis when CDT-1 stabilization occurs upon CDC-48$^{UFD-1/NPL-4}$ disruption (Figure 3.2.).

Intimate interdependencies exist between the DNA replication machinery and the DNA repair pathways [206, 266]. This might account for the differences observed by Raman and colleagues and this work. Malfunction of DNA replication result in the formation of DNA damage and subsequent initiation of repair pathways. In turn the repair of damaged DNA involves the DNA replication machinery [267]. In line with this, the replication checkpoint controls origin firing and the velocity of replication forks also in regular cell cycles [90, 268-270]. It seems possible that the regulatory mechanisms operating in unperturbed cell cycles become more accentuated upon treatment with DNA damaging agents, thus enforcing phenotypic outcomes. Elucidation of the precise CDT-1 degradation pathways in unperturbed versus damage induced cells requires future research.

3.3.3. Implication of CDC-48 as a ubiquitin-selective chaperone at the chromatin.

Based on its function as a ubiquitin-selective chaperone, CDC-48 is thought to provide segregase activity that separates ubiquitylated proteins from tightly bound partners (Figure 1.7.) [163]. The best studied segregase-like function is described for the ERAD pathway, where CDC-48$^{UFD-1/NPL-4}$ mediates the re-translocation of damaged proteins from the ER lumen to the cytosol for subsequent proteasomal degradation [271, 272]. Considering recent findings, it is intriguing to speculate that CDC-48 activity is generally required to extracts client proteins from chromatin associated complexes [172, 174, 190, 191, 193, 212, 273, 274].

The first study implicating a crucial function of CDC-48/p97 at the chromatin, was the requirement for the dissociation of the Aurora-B (AIR-2 in *C. elegans*) kinase after mitosis in *Xenopus* egg extracts [172]. The removal of Aurora-B from the chromatin allows subsequent chromatin de-condensation and was shown to occur without its protein degradation. In line with this, Cdc48 regulates the mating type identity of budding yeast cells by removal of the transcriptional repressor Matα2 from the chromatin [193]. Thereby, expression of mating type specific genes is initiated in a non-proteolytic manner. One example for a chromatin associated process of CDC-48 involving protein degradation was recently discovered in yeast. Here, CDC-48 is required for the turnover of the RNA Polymerase II subunit Rpb1 upon UV irradiation [191]. Interestingly, CDC-48 appears to play a general role in the response to DNA damage [275]. In human cell culture, for example, CDC-48 together with its cofactors UFD-1 and NPL-4 is recruited to DNA damage sites and facilitates the chromatin assembly of DNA repair factors, by promoting the degradation of ubiquitylated L3MBTL1 [190, 273]. Moreover, CDC-48 was identified as a binding partner of BRC-1, HIM-6, and WRN-1 (*C. elegans* orthologs of human BRCA1, BLM, and WRN), three proteins important for the repair of damaged DNA [251, 252, 276].

These studies support an essential role of CDC-48, either by extracting substrates from protein/DNA complexes or by promoting the degradation of chromatin associated proteins that are otherwise not directly accessible for the proteasome. This is in line with the data presented in this work. CDC-48 probably facilitates the extraction of ubiquitylated CDT-1 from mitotic chromatin, resulting in degradation of CDT-1 and subsequent dissociation of CDC-45/GINS (Figure 3.1., 3.2.). Chromatin segregation of CDC-45/GINS likely is connected to CDT-1 degradation by CDC-48$^{UFD\text{-}1/NPL\text{-}4}$, nevertheless, a CDT-1 independent mechanism seems also feasible.

3.3.4. Putative involvement of CDC-48 accessory factors in the regulation of DNA replication.

CDC-48 and its orthologs in diverse species partake in a variety of cellular processes [146]. Alternative cofactors have been identified to bind to distinct domains of CDC-48, thereby providing specificity for particular processes to occur [164, 165, 277]. Depending on their activities, CDC-48 cofactors have been subdivided into substrate recognition and substrate processing factors (Figure 3.3.) [143].

The substrate recruiting factors p47 and UFD-1/NPL-4 are believed to represent distinct core complexes of CDC-48 that determine its involvement in certain cellular pathways [153,

166]. UBX domain proteins appear to further elevate the level of substrate specificity, either by promoting binding of the substrate, determination of CDC-48 localization, or by recruitment of specific ubiquitin ligases [164, 278, 279]. Ubx5 in yeast (or UBXN7 in humans) has been shown to provide substrate specificity by bringing together CDC-48 and substrate specific ubiquitin ligases [191, 278, 279]. UBXD1 (UBXN-6 in *C. elegans*), in turn, determines the role of CDC-48 in the endosomal pathway [280]. In *C. elegans* the three UBX domain proteins UBXN-1, UBXN-2, and UBXN-3 have been shown to direct CDC-48's involvement in sex determination of germline cells, together with the CUL-2 ligase, by promoting the degradation of the transcription factor TRA-1 [281, 282].

Substrate processing factors actively influence the fate of target proteins. UFD-2 is a cofactor of CDC-48 possessing ubiquitin-chain elongation (E4) activity, thus facilitating substrate degradation [142]. Worth to mention, Ufd2 was found among the factors involved in UV-induced Cdt1 degradation in human cell culture [212]. Different de-ubiquitylating enzymes (DUB's) associate with CDC-48 that can alter the fate of a substrate by editing the length or linkage type of the ubiquitin chain [167, 168]. Thus, DUB's may also be involved in the regulation of DNA replication by CDC-48.

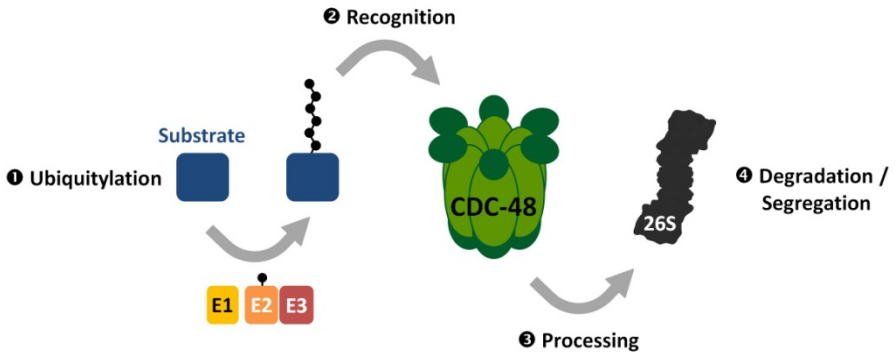


Figure 3.3. Four steps in CDC-48-mediated protein degradation: Ubiquitylation, recognition, processing, and degradation.
CDC-48-mediated protein degradation involves four steps. First, proteins are ubiquitylated by substrate specific E3 ligases. Subsequently ubiquitylated substrates are recognized by CDC-48 with the help of dedicated cofactors. Cofactors can also harbour substrate processing activity, for instance elongation or shortening of the ubiquitin-chain on the substrate. Moreover, substrate processing cofactors can promote contact to the 26S proteasome. Proteasomal proteolysis of the substrate presents the final step in protein destruction. Strikingly, substrates may also be segregated from binding partners without their final destruction by the proteasome. Information taken from [182].

Thus far, the known cofactors of CDC-48 have not been linked to the regulation of DNA replication. Having identified crucial substrates of CDC-48 in DNA replication will allow the identification of accessory factors that determine the substrate specificity and substrate

processivity in cell cycle control (Figure 2.2., 2.9., 2.11.). This will provide understanding of the molecular mechanism and the selectivity of the ubiquitin-selective chaperone CDC-48 in more detail. In addition, further biochemical analysis of replication intermediates and chromatin structure will elucidate the precise requirement of CDC-48$^{UFD\text{-}1/NPL\text{-}4}$ dependent regulation of CDT-1 and CDC-45/GINS and thereby highlight its role in ensuring faithful duplication of genomic information.

· Chapter 4 · Material & Methods ·

4.1. Resources and software.

4.1.1. Reagents and instruments.

The microbiological, molecular, and biochemical experiments of this study were based on routine techniques [283] or on the manuals provided by the manufacturer's.

Consumable plasticware, reagents, chemicals and instruments were purchased from the following suppliers, unless otherwise indicated: Amersham Biosciences, Applied Biosystems, Biomol, Bio-Rad, Carl Roth, Clontech, Eppendorf, Fermentas, GE Healthcare, Greiner Bio-One, Invitrogen, Metabion, Millipore, New England Biolabs, Peqlab, Qiagen, Roche, Sarstedt, Serva, Sigma Aldrich, VWR.

De-ionized sterile water, sterile solutions, and sterile flasks were used whenever applicable.

4.1.2. Software, databases, and resources.

This work was prepared using the Microsoft XP operating system installed on a DELL Latitude E6400 notebook. Standard software was used for data analysis or visualization, and text preparation: Microsoft Office (Microsoft Corp.), EndNote X2 (Thomson Reuters), Adobe Creative Suite CS4 (Adobe Systems Inc), ImageJ (National Institutes of Health), AxioVision (Carl Zeiss), Odyssey Application Software (Free Software Foundation, Inc), VectorNTI (Invitrogen), GENtle (Free Software Foundation, Inc), PerlPrimer (Owen Marshall).

The homepage of the National Center for Biotechnology Information (NCBI) was used as a database for scientific literature, DNA and protein sequences, and DNA and protein sequence analysis and alignment (http://www.ncbi.nlm.nih.gov/). Information about *C. elegans* specific genes and proteins, as well as general information on nematode handling and techniques was obtained from the Wormbase (http://www.wormbase.org/) and the *Caenorhabditis elegans* WWW server (http://elegans.som.vcu.edu/). The Phenobank website was used as a resource for gene function in embryonic and germline cell cycles (http://www.worm.mpi-cbg.de/phenobank/cgi-bin/MenuPage.py). Protein domain, sequence, and alignment analysis was done using the Expasy (http://expasy.org/) or ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) web pages. Identification of orthologs in different species was done using the Princeton Protein Orthology Database (http://ppod.princeton.edu/).

Sequencing of DNA fragments was routinely done by GATC Biotech.

4.2. Microbiology.

4.2.1. Media and Solutions.

The following table summarizes the media and solutions that were used for microbiology.

Table 4.1. Solutions for microbiology.

Solution	Ingredients
LB (lysogeny browth)	1 % (w/v) bacto-trypton 0,5 % (w/v) yeast extract 1 % (w/v) NaCl adjusted to pH 7,5 sterilized by autoclaving
LB agar	LB supplemented with 1,5 % (w/v) agar sterilized by autoclaving
TBF1	30 mM KAc 10 mM CaCl2 50 mM MnCl2 100 mM RbCl 15 % (v/v) glycerol adjusted at pH 8,5 filter sterilized
TBF2	10 mM MOPS at pH 6,5 75 mM CaCl2 10 mM RbCl 15 % (v/v) glycerol adjusted at pH 8,5 filter sterilized

4.2.2. Bacteria strains.

Escherichia coli (*E.coli*) bacteria strains that were used in this study and their respective genotypes are listed below.

Table 4.2. List of *E. coli* strains and genotypes.

Strain	Genotype	Source
DH5α	F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 dam^+ *deoR*	Invitrogen
OP50	uracil auxotroph, *E. coli* B strain	Caenorhabditis Genetics Center (CGC)
HT115(DE3)	F^-, *mcrA, mcrB,* IN(rrnD-rrnE)1, lambda -, *rnc14::*Tn*10* (DE3 lysogen: *lacUV5* promoter-T7 polymerase) (IPTG-inducible T7 polymerase) (RNAse III minus)	Caenorhabditis Genetics Center (CGC)

4.2.3. Cultivation of bacteria.

Liquid bacterial cultures were grown in LB medium shaking at 180 rpm at 37°C. Agar plate cultures were incubated at 37°C. Short term storage of bacterial cultures was done at 4°C. For long term storage a liquid culture was grown overnight and mixed 1:1 with 50 % glycerol, allowing preservation at -80°C. Selection of transformed bacteria was achieved by cultivation

in LB media containing respective antibiotics. Ampicillin was used at a final concentration of 100 µg/µl, Tetracycline was used at 12,5 µg/µl.

The density of liquid cultures was determined by the absorbance of light at 600 nm wavelength (optical density, OD_{600}) using the Ultrospec 10 (Amersham Biosciences).

4.2.4. Production of chemo-competent bacteria.

In order to obtain chemo-competent bacteria, a starting culture of respective bacteria was grown overnight using respective antibiotics. The following day, 250 ml LB medium was inoculated with the overnight culture. Bacteria were grown to an OD_{600} of 0,4 to 0,6. Bacteria cells were harvested by centrifugation (15 min at 4.500 rcf). The following steps were performed with ice-cold solutions. The bacterial pellet was resuspended in 100 ml TBF1 buffer. Bacteria were again centrifuged (15 min at 4.500 rcf, 4°C) and resuspended in 10 ml TBF2 buffer. Incubation on ice was done for 15-60 min, followed by freezing of aliquots in liquid nitrogen. Competent bacteria were stored at -80°C.

4.2.5. Transformation of plasmid DNA into competent bacteria.

Chemo-competent bacteria were allowed to thaw on ice. Approximately 1 µl plasmid DNA or 5 µl of a ligation reaction was mixed with the bacteria cells and incubated on ice for 15-20 min, followed by an incubation at 42°C for 80 s. 800 µl LB medium without antibiotics was added and bacteria were allowed to recover, shaking at 37°C for 45 to 60 min. Afterwards, bacteria were pelleted by centrifugation (2.000 rpm for 2 min). The pelleted bacteria were resuspended in approximately 50 µl LB and streaked out on LB agar plates containing respective antibiotics for the selection of transformants.

4.3. Molecular biology.

4.3.1. Buffers and solutions.

The following table summarizes the media and solutions that were used for molecular biology.

Table 4.3. Solutions for molecular biology.

Solution	Ingredients
TAE	40 mM TRIS 1,14 % (v/v) acetic acid 1 mM EDTA pH adjusted to 8,5
2x Laemmli buffer	125 mM TRIS, pH 6,8 4 % (w/v) SDS 20 % (v/v) glycerol 0,03 % (w/v) bromophenol blue 0,02 % (v/v) β-mercapthoethanol
SDS-PAGE running buffer	25 mM TRIS 1,88 % (w/v) glycine 0,1 % (w/v) SDS
Semi-dry blotting buffer	48 mM TRIS 0,293 % (w/v) glycine 0,00375 % (w/v) SDS add 20 % (v/v) methanol before use
Colloidal Coomassie	10% (w/v) ammonium sulfate 12% (v/v) phosphoric acid 0,12% (w/v) brilliant blue G-250 add 20% (v/v) methanol before use

4.3.2. Isolation of plasmid DNA from bacteria.

Isolation of plasmid DNA from transformed bacteria was done using the Plasmid Mini Kit (Qiagen). A 5 ml liquid culture of respective bacteria was grown over night at 37°C. Bacteria cells were pelleted by centrifugation (11.000 rcf for 1 min). Cell lysis and DNA preparation was performed according to the instructions by the manufacturer. Plasmid DNA was eluted in water. DNA concentration and purity were determined by absorbance of light at 260 nm wavelength using the NanoDrop 8000 (Thermo Scientific). The identity of the purified DNA was validated by PCR amplification, or DNA restriction followed by agarose gel electrophoresis, or sequencing.

4.3.3. Agarose gel electrophoresis.

The size of DNA fragments was determined by separation through agarose gel electrophoresis. Routinely, agarose gels were prepared using 0,8 to 1,0 % agarose (w/v) solved in TAE buffer containing 1 µg/ml ethidium bromide. Agarose gels were assembled in gel electrophoresis chambers (Peqlab). DNA was loaded using according amounts of 6x loading buffer (Fermentas) and separated electrophoretically (Peqlab). DNA fragments were detected using an UV trans-illuminator (Wealtec). A standard marker (Fermentas 1 kb marker) allowed estimation of the size of respective DNA fragments.

4.3.4. SDS-PAGE.

Proteins were separated under denaturating conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the discontinuous system by Laemmli [284]. SDS-PAGE gels were prepared with a stacking phase and a separation phase. The stacking gel was prepared with 3 % acrylamide, the separation gels contained 9 to 12 % acrylamide. Mini-gel pouring devices and electrophoresis chambers were purchased from Amersham Biosciences. Protein samples were prepared with according amounts of 2x Laemmli buffer. Before loading to the gel, samples were incubated for 5 min at 95°C, centrifuged for 5 min at 14.500 rpm. After electrophoresis gels were either used for western blotting or protein bands were detected by staining with coomassie-brilliant-blue. The coomassie staining was done overnight according to the colloidal coomassie staining procedure [285]. The size of respective protein bands was estimated according to the standard marker (PageRuler plus prestained, Fermentas).

4.3.5. Western blotting.

For western-blotting, proteins were separated by SDS-PAGE and transferred to nitrocellulose (NC) membranes (Protran, 0,2 µm pore size, Whatman) using a semi-dry blotting device (Trans-Blot SD, Bio-Rad). The SDS gel, the filter membranes and the NC membrane were briefly incubated in Semi-dry blotting buffer. Filter membranes, SDS gel, and transfer membrane were assembled in the blotting machine according to the manufacturer's instructions. Protein transfer onto the NC membrane was done at constant 20 to 25 volts for 60 to 90 min. After the blotting, protein transfer was validated by Ponceau S staining (Bio-Rad) of the NC membrane. NC membranes were blocked in 3 % (w/v) milk powder solution and incubated with the primary antibodies over night at 4°C in RotiBlock solution (Carl Roth) (anti-CDT-1 (Rabbit) 1:300 [66], anti-PSF-3 (Rabbit) 1:1.000 (from Lionel Pintard), anti-GFP (Mouse) 1:5.000, Clonetech, anti-Tubulin (Mouse) 1:5.000, Sigma, clone DM1A, anti-UFD-1 (Rabbit) 1:50.000, anti-CDC-48 (Rabbit) 1:50.000). Incubation with fluorescently labelled secondary antibodies (anti-Mouse/Rabbit 1:10.000, Li-cor) was done at room temperature, before signals were detected and documented using an Odyssey scanner (Li-cor).

Quantification of signal intensities was done using the Odyssey V3.0 software. Background signal in the surrounding area was subtracted from values in the area of interest.

4.3.6. PCR.

The polymerase chain-reaction was used for the specific amplification of DNA. Analytical DNA amplification was routinely carried out in a reaction volume of 25 µl. The PCR reaction contained approximately 10 to 100 ng template DNA, as well as dNTP mix, forward and reverse primers, reaction buffer and the DNA polymerase according to the requirements in the manufacturers' instructions. PCR reactions were performed using a DNA Engine or S1000Thermo Cycler (Bio-Rad). The specific requirements for timing and temperature of distinct steps in the PCR reaction vary with the DNA polymerase and the size and sequence of the template DNA. Therefore, the following PCR reaction protocol serves as an example that needs to be adjusted to the specific needs of the polymerase, the primers, and the DNA template.

Step of PCR reaction	Temperature (°C)	Time in s	
Initial denaturation	95	120	
Denaturation	95	30	30 cycles
Primer annealing	55	30	
Extension	72	60 (per kb)	
Final extension	72	300	
Cooling	10	forever	

4.4. *Caenorhabditis elegans* techniques.

Nematodes were handled according to the standard protocols for *C. elegans* [286-288]. Briefly, maintenance of *C. elegans* strains was achieved by feeding with the *E. coli* strain OP50, growing on nematode growth medium (NGM). Worms were cultivated at 15°C or 20°C unless otherwise stated. The N2 Bristol strain was used as the wild-type reference.

Buffers and solutions used for C. elegans techniques are listed as follows.

Table 4.4. Solutions for *C. elegans* techniques.

Solution	Ingredients
M9	20 mM KH_2PO_4 40 mM Na_2HPO_4 80 mM NaCl 1 mM $MgSO_4$
NGM agar	0,25 % (w/v) bacto-peptone 0,3 % (w/v) NaCl 1,7 % (w/v) serva agar 1 mM $CaCl_2$ 1 mM $MgSO_4$ 5 µg/ml cholesterol 25 mM KPO_4 buffer nystatin 25 units/ml
1 M KPO_4 buffer	10,83 % (w/v) KH_2PO_4 3,53 % (w/v) K_2HPO_4 (3xH_2O) adjusted at pH 6
Bleaching solution	250 mM KOH 2,5 % (v/v) NaClO
PBS	137 mM NaCL 2,7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4 adjusted at pH 7,4

4.4.1. *C. elegans* strains.

Nematode strains carrying mutations or transgenes used in this study are listed as follows:

div-1(or148)III [179];

cdc-48.1(tm544)II [177];

gtIs60 [Ppie-1 LAP-orc-1::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs61 [Ppie-1 LAP-orc-2::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs62 [Ppie-1 LAP-cdc-6::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs63 [Ppie-1 LAP-mcm-2::cDNA, unc-119(+)], unc-119(ed3)III;

gtIs64 [Ppie-1 LAP-mcm-3::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs65 [Ppie-1 LAP-cdc-45::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs67 [Ppie-1 LAP-sld-5::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs66 [Ppie-1 LAP-div-1::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

gtIs [Ppie-1 LAP-rpa-1::cDNA, unc-119(+)], odIs57[Ppie-1 mCherry::histoneH2B unc-119(+)], unc-119(ed3)III;

isIs17 [Ppie-1::GFP::pcn-1], unc-119(ed3)III (received from Pierre Gönczy)

leaIs [Ppie-1::GFP::psf-3], unc-119(ed3)III (received from Lionel Pintard)

The generation of *orc-1, orc-2, cdc-6, mcm-2, mcm-3, cdc-45, sld-5, div-1,* and *rpa-1* transgenes fused to *gfp* and controlled by the *pie-1* promoter was done by Remi Sonneville (Dundee, Scotland). Detailed description of the generation and validation of the fusion constructs was recently published [215]. The GFP::RPA-1 fusion is not yet published, contact Remi Sonneville before using this strain for any experiments. The strain carrying the GFP::PSF-3 was obtained from Lionel Pintard (Paris, France) and is not published so far.

4.4.2. RNAi-mediated depletion.

RNAi-mediated depletion was achieved using the feeding method [289-291]. The *E. coli* strain HT115(DE3) was transformed with the Isopropyl-β-D-thiogalactopyranosid (IPTG) inducible vector pPD129.36. IPTG induction results in the production of double-stranded (ds) RNA against the respective target gene. In RNAi control experiments, bacteria only contained the *empty* vector pPD129.36. Bacteria were grown in liquid culture until reaching an OD_{600} of about 1 before they were seeded onto IPTG containing NGM plates. Induction of dsRNA expression was done either over night at room temperature or, if required for proper bacterial growth, for several days. IPTG was added to NGM plates at a final concentration of 1 mM for single gene depletion or 2 mM for sequential or simultaneous gene depletion. NGM IPTG plates were supplemented with 100 µg/µl Ampicillin.

RNAi bacteria were fed to L3/L4 larvae at 15°C for 72 h. To induce stronger expression of the fluorescent reporter constructs, worms were shifted to 20°C or 25°C over night the day before time-lapse analysis. Alternatively, RNAi was fed from the L3/L4 larval stage and worms were kept at 20°C until reaching adulthood. For sequential RNAi (seq(RNAi)) depletion worms were fed with the dsRNA-containing bacteria against the first target gene for 48 h and then switched to bacteria containing dsRNA against the second target by picking. For the simultaneous depletion of two genes the respective bacteria were mixed 1:1 in cell density. Worms were transferred to fresh RNAi plates each day by picking.

For the preparation of embryonic lysates worms were kept at 20°C during the entire experimental procedure (see following paragraphs for details).

4.4.3. Synchronization of worm cultures.

Gravid adult worms were washed from plates using cold M9 buffer. Somatic tissue was then removed by incubation in bleaching solution. Extruded embryos were harvested by centrifugation (1 min at 2.000 rpm) and washed three times in water. Embryos were then transferred to a fresh reaction tube in M9 buffer and incubated shaking over night at room temperature. The following day, a synchronized culture of starved L1 larvae was obtained und used for subsequent experiments.

4.4.4. Preparation of embryonic lysates.

Synchronized L1 larvae were cultured on plates with *empty* control bacteria from the L1 to the L3 larval stage (about 1500-2000 L1 larvae per 10 cm petri dish, seeded with 850-900 µl bacteria culture). Then L3 larvae were washed from the plates using cold M9 buffer. Worms

were washed twice in M9 buffer or water to remove bacteria. An aliquot of the resulting worm suspension was used to determine the density of worms per volume. About 350 to 400 L3 larvae were seeded onto respective RNAi bacteria (10 cm diameter petri dishes) and kept at 20°C until reaching adulthood. The embryos of gravid adults were harvested as described above. Embryos were washed twice in water before they were resuspended in 2x Laemmli Buffer and frozen in liquid nitrogen. Before SDS-PAGE analysis embryos were sonicated twice for 15 s (Microtip MS 1,5, Bandelin), incubated at 95°C for 5 min and centrifugated at 14.000 g for 5 min. Embryos and lysates were stored at -20°C.

4.5. Microscopy.

General handling and observation of Nematodes was achieved using Leica M80 stereomicroscopes (Leica Microsystems).

4.5.1. Time-lapse analysis of early embryogenesis.

In order to determine the timing of the first embryonic divisions gravid adults were dissected and extruded embryos were analyzed by time-lapse microscopy. Embryos were mounted on 3 % agar pads essentially as described before [176]. An Axio-Imager.M1 microscope equipped with an AxioCam MRm camera and a HXP 120 mercury short arc light source (Carl Zeiss) was used for image acquisition. Time-lapse recordings in 90 s intervals were acquired using 2x2 mono binning, in order to minimize light exposure and reduce photobleaching and -toxicity. To allow direct comparison of signal intensities, images were recorded under identical illumination conditions (GFP and mCherry channels were acquired using an exposure time of 400 ms). Analysis of time-lapse recordings was done in the AxioVision 4.7 software (Carl Zeiss). Timing of cell division was estimated by morphological criteria of distinct cell cycle events, essentially as described previously [176, 292]. Processing of selected pictures was done in Adobe Photoshop CS4.

4.5.2. Immunostainings of embryos.

Immunostaining of early embryos was done according to the “freeze-crack” protocol [293]. Briefly, gravid worms were dissected onto poly-lysine coated slides (Thermo Scientific) and frozen in liquid nitrogen. Directly after taking the slides out of the liquid nitrogen, cover slips were removed with the help of a razor blade. Slides were then immediately transferred to -20°C methanol for 20 min followed by 5-20 min incubation in -20°C acetone. Acetone was allowed to evaporate at room temperature, followed by rehydration in PBS three times for 5

min. After a 30-60 min blocking step in 5 % (w/v) BSA (in PBS), embryos were incubated with the primary antibody over night at 4°C (anti-CDT-1 (Rabbit) 1:300 [66], anti-αTubulin (Mouse) 1:200 Sigma, clone DM1A). Slides were washed three times in PBS. Incubation with the secondary antibodies (anti-Mouse/Rabbit (Donkey/Goat) Alexa488 or Alexa 594 conjugated 1:400, Invitrogen) was done at room temperature for one h followed by three washing steps in PBS. Embryos were then mounted in Dapi Fluoromount G medium (SouthernBiotech). Slides were sealed with nail polish and stored at 4°C. Images of immunostainings were acquired with AxioImager.M1 microscope and AxioCam MRm camera using full resolution of the camera. The CDT-1 antibody appears to be sensitive to treatment with detergent, therefore, Tween-20 was included in the washing buffers at a concentration of 0,01-0,05 % (v/v) only in two washing steps after incubation with the secondary antibodies.

4.5.3. Fluorescence signal quantification of immunostainings.

In order to quantify the amount of GFP::SLD-5 that was associated with mitotic chromatin respective pictures were selected from the time lapse analysis. A composite of these images was created using Adobe Photoshop CS4. Composites were then imported into ImageJ (National Institutes of Health). The region of mitotic chromatin was selected based on the mCherry::H2B signal and marked with an oval selection tool. The signal intensities for mCherry::H2B and GFP::SLD-5 were measured in the same region of interest. Cytosolic background signal in the surrounding area was subtracted from values in the area of interest. Finally, the ratio of mCherry::H2B and GFP::SLD-5 signal intensities was calculated and compared for distinct RNAi conditions.

4.6. Statistical Analysis.

Statistical analysis was performed in Microsoft Excel. Statistical significance was calculated with two-tailed paired student's T-test. P-values of $P \leq 0.05$ are indicated with a single asterisk and double asterisks indicate $P \leq 0.001$. Comparison of cell division timings was done for experiments that were done on one single day, whereas Figure 2.5.A/B show summarized values for the control conditions of all experiments for better visualization.

· References ·

1. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. **432**(7015): p. 316-23.
2. Kipreos, E.T., *C. elegans cell cycles: invariance and stem cell divisions.* Nat Rev Mol Cell Biol, 2005. **6**(10): p. 766-76.
3. Budirahardja, Y. and P. Gonczy, *Coupling the cell cycle to development.* Development, 2009. **136**(17): p. 2861-72.
4. Ballabeni, A., et al., *Cell cycle adaptations of embryonic stem cells.* Proc Natl Acad Sci U S A. **108**(48): p. 19252-7.
5. Bell, S.P. and A. Dutta, *DNA replication in eukaryotic cells.* Annu Rev Biochem, 2002. **71**: p. 333-74.
6. Masai, H., et al., *Eukaryotic chromosome DNA replication: where, when, and how?* Annu Rev Biochem, 2010. **79**: p. 89-130.
7. Takahashi, T.S., D.B. Wigley, and J.C. Walter, *Pumps, paradoxes and ploughshares: mechanism of the MCM2-7 DNA helicase.* Trends Biochem Sci, 2005. **30**(8): p. 437-44.
8. Ilves, I., et al., *Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins.* Mol Cell, 2010. **37**(2): p. 247-58.
9. Mimura, S., et al., *Central role for cdc45 in establishing an initiation complex of DNA replication in Xenopus egg extracts.* Genes Cells, 2000. **5**(6): p. 439-52.
10. Masuda, T., S. Mimura, and H. Takisawa, *CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in Xenopus egg extracts: possible activation of MCM helicase by association with Cdc45.* Genes Cells, 2003. **8**(2): p. 145-61.
11. Sheu, Y.J. and B. Stillman, *Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression.* Mol Cell, 2006. **24**(1): p. 101-13.
12. Yabuuchi, H., et al., *Ordered assembly of Sld3, GINS and Cdc45 is distinctly regulated by DDK and CDK for activation of replication origins.* EMBO J, 2006. **25**(19): p. 4663-74.
13. Choi, J.M., et al., *Crystal structure of the human GINS complex.* Genes Dev, 2007. **21**(11): p. 1316-21.
14. Chang, Y.P., et al., *Crystal structure of the GINS complex and functional insights into its role in DNA replication.* Proc Natl Acad Sci U S A, 2007. **104**(31): p. 12685-90.
15. Moyer, S.E., P.W. Lewis, and M.R. Botchan, *Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase.* Proc Natl Acad Sci U S A, 2006. **103**(27): p. 10236-41.
16. Kubota, Y., et al., *A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication.* Genes Dev, 2003. **17**(9): p. 1141-52.
17. Takayama, Y., et al., *GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast.* Genes Dev, 2003. **17**(9): p. 1153-65.
18. Aparicio, T., et al., *The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication.* Nucleic Acids Res, 2009. **37**(7): p. 2087-95.
19. Gambus, A., et al., *GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks.* Nat Cell Biol, 2006. **8**(4): p. 358-66.
20. Pacek, M., et al., *Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication.* Mol Cell, 2006. **21**(4): p. 581-7.
21. Gambus, A., et al., *A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome.* EMBO J, 2009. **28**(19): p. 2992-3004.
22. Aparicio, O.M., D.M. Weinstein, and S.P. Bell, *Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase.* Cell, 1997. **91**(1): p. 59-69.
23. Aparicio, O.M., A.M. Stout, and S.P. Bell, *Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication.* Proc Natl Acad Sci U S A, 1999. **96**(16): p. 9130-5.
24. Calzada, A., et al., *Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork.* Genes Dev, 2005. **19**(16): p. 1905-19.

25. Kriegstein, H.J. and D.S. Hogness, *Mechanism of DNA replication in Drosophila chromosomes: structure of replication forks and evidence for bidirectionality.* Proc Natl Acad Sci U S A, 1974. **71**(1): p. 135-9.
26. Labib, K. and A. Gambus, *A key role for the GINS complex at DNA replication forks.* Trends Cell Biol, 2007. **17**(6): p. 271-8.
27. Di Micco, R., et al., *Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication.* Nature, 2006. **444**(7119): p. 638-42.
28. Gorgoulis, V.G., et al., *Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions.* Nature, 2005. **434**(7035): p. 907-13.
29. Bicknell, L.S., et al., *Mutations in the pre-replication complex cause Meier-Gorlin syndrome.* Nat Genet. **43**(4): p. 356-9.
30. Bicknell, L.S., et al., *Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome.* Nat Genet. **43**(4): p. 350-5.
31. Sangrithi, M.N., et al., *Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome.* Cell, 2005. **121**(6): p. 887-98.
32. Davidson, I.F., A. Li, and J.J. Blow, *Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision.* Mol Cell, 2006. **24**(3): p. 433-43.
33. Tatsumi, Y., et al., *Deregulation of Cdt1 induces chromosomal damage without rereplication and leads to chromosomal instability.* J Cell Sci, 2006. **119**(Pt 15): p. 3128-40.
34. Pruitt, S.C., K.J. Bailey, and A. Freeland, *Reduced Mcm2 expression results in severe stem/progenitor cell deficiency and cancer.* Stem Cells, 2007. **25**(12): p. 3121-32.
35. Goldsby, R.E., et al., *Defective DNA polymerase-delta proofreading causes cancer susceptibility in mice.* Nat Med, 2001. **7**(6): p. 638-9.
36. Venkatesan, R.N., et al., *Mutation at the polymerase active site of mouse DNA polymerase delta increases genomic instability and accelerates tumorigenesis.* Mol Cell Biol, 2007. **27**(21): p. 7669-82.
37. Blow, J.J. and P.J. Gillespie, *Replication licensing and cancer--a fatal entanglement?* Nat Rev Cancer, 2008. **8**(10): p. 799-806.
38. Bartkova, J., et al., *Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints.* Nature, 2006. **444**(7119): p. 633-7.
39. Bloom, J. and F.R. Cross, *Multiple levels of cyclin specificity in cell-cycle control.* Nat Rev Mol Cell Biol, 2007. **8**(2): p. 149-60.
40. Lei, M. and B.K. Tye, *Initiating DNA synthesis: from recruiting to activating the MCM complex.* J Cell Sci, 2001. **114**(Pt 8): p. 1447-54.
41. Mimura, S. and H. Takisawa, *Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk.* EMBO J, 1998. **17**(19): p. 5699-707.
42. Blow, J.J. and A. Dutta, *Preventing re-replication of chromosomal DNA.* Nat Rev Mol Cell Biol, 2005. **6**(6): p. 476-86.
43. Lydeard, J.R., et al., *Break-induced replication requires all essential DNA replication factors except those specific for pre-RC assembly.* Genes Dev. **24**(11): p. 1133-44.
44. Diffley, J.F., *Quality control in the initiation of eukaryotic DNA replication.* Philos Trans R Soc Lond B Biol Sci. **366**(1584): p. 3545-53.
45. Chen, S. and S.P. Bell, *CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6.* Genes Dev. **25**(4): p. 363-72.
46. Drury, L.S., G. Perkins, and J.F. Diffley, *The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast.* EMBO J, 1997. **16**(19): p. 5966-76.
47. Drury, L.S., G. Perkins, and J.F. Diffley, *The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle.* Curr Biol, 2000. **10**(5): p. 231-40.
48. Ralph, E., E. Boye, and S.E. Kearsey, *DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1.* EMBO Rep, 2006. **7**(11): p. 1134-9.
49. Tanaka, S. and J.F. Diffley, *Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase.* Nat Cell Biol, 2002. **4**(3): p. 198-207.

50. Liku, M.E., et al., *CDK phosphorylation of a novel NLS-NES module distributed between two subunits of the Mcm2-7 complex prevents chromosomal rereplication.* Mol Biol Cell, 2005. **16**(10): p. 5026-39.
51. Nguyen, V.Q., et al., *Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7.* Curr Biol, 2000. **10**(4): p. 195-205.
52. Mendez, J., et al., *Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication.* Mol Cell, 2002. **9**(3): p. 481-91.
53. Araki, M., et al., *Degradation of origin recognition complex large subunit by the anaphase-promoting complex in Drosophila.* EMBO J, 2003. **22**(22): p. 6115-26.
54. Rouiller, I., et al., *Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle.* Nat Struct Biol, 2002. **9**(12): p. 950-7.
55. Chang, A.S., et al., *Visible three-dimensional metallic photonic crystal with non-localized propagating modes beyond waveguide cutoff.* Opt Express, 2007. **15**(13): p. 8428-37.
56. Kim, Y. and E.T. Kipreos, *Cdt1 degradation to prevent DNA re-replication: conserved and non-conserved pathways.* Cell Div, 2007. **2**: p. 18.
57. Tada, S., et al., *Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin.* Nat Cell Biol, 2001. **3**(2): p. 107-13.
58. Wohlschlegel, J.A., et al., *Inhibition of eukaryotic DNA replication by geminin binding to Cdt1.* Science, 2000. **290**(5500): p. 2309-12.
59. McGarry, T.J. and M.W. Kirschner, *Geminin, an inhibitor of DNA replication, is degraded during mitosis.* Cell, 1998. **93**(6): p. 1043-53.
60. Sugimoto, N., et al., *Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding.* J Biol Chem, 2004. **279**(19): p. 19691-7.
61. Li, X., et al., *The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation.* J Biol Chem, 2003. **278**(33): p. 30854-8.
62. Liu, E., et al., *Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation.* J Biol Chem, 2004. **279**(17): p. 17283-8.
63. Nishitani, H., et al., *Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis.* EMBO J, 2006. **25**(5): p. 1126-36.
64. Fujita, M., *Cdt1 revisited: complex and tight regulation during the cell cycle and consequences of deregulation in mammalian cells.* Cell Div, 2006. **1**: p. 22.
65. Jin, J., et al., *A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1.* Mol Cell, 2006. **23**(5): p. 709-21.
66. Zhong, W., et al., *CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing.* Nature, 2003. **423**(6942): p. 885-9.
67. Sansam, C.L., et al., *DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint.* Genes Dev, 2006. **20**(22): p. 3117-29.
68. Havens, C.G. and J.C. Walter, *Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2.* Mol Cell, 2009. **35**(1): p. 93-104.
69. Arias, E.E. and J.C. Walter, *PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication.* Nat Cell Biol, 2006. **8**(1): p. 84-90.
70. Arias, E.E. and J.C. Walter, *Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts.* Genes Dev, 2005. **19**(1): p. 114-26.
71. Senga, T., et al., *PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination.* J Biol Chem, 2006. **281**(10): p. 6246-52.
72. Ishii, T., et al., *Proliferating cell nuclear antigen-dependent rapid recruitment of Cdt1 and CRL4Cdt2 at DNA-damaged sites after UV irradiation in HeLa cells.* J Biol Chem. **285**(53): p. 41993-2000.
73. Higa, L.A., et al., *L2DTL/CDT2 interacts with the CUL4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage.* Cell Cycle, 2006. **5**(15): p. 1675-80.
74. Branzei, D. and M. Foiani, *Maintaining genome stability at the replication fork.* Nat Rev Mol Cell Biol. **11**(3): p. 208-19.

75. Sancar, A., et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. **73**: p. 39-85.
76. Franchitto, A. and P. Pichierri, *Understanding the molecular basis of common fragile sites instability: role of the proteins involved in the recovery of stalled replication forks.* Cell Cycle. **10**(23): p. 4039-46.
77. Argueso, J.L., et al., *Double-strand breaks associated with repetitive DNA can reshape the genome.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11845-50.
78. Deshpande, A.M. and C.S. Newlon, *DNA replication fork pause sites dependent on transcription.* Science, 1996. **272**(5264): p. 1030-3.
79. Cha, R.S. and N. Kleckner, *ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones.* Science, 2002. **297**(5581): p. 602-6.
80. Takeuchi, Y., T. Horiuchi, and T. Kobayashi, *Transcription-dependent recombination and the role of fork collision in yeast rDNA.* Genes Dev, 2003. **17**(12): p. 1497-506.
81. Roeder, G.S. and G.R. Fink, *DNA rearrangements associated with a transposable element in yeast.* Cell, 1980. **21**(1): p. 239-49.
82. Lemoine, F.J., et al., *Chromosomal translocations in yeast induced by low levels of DNA polymerase a model for chromosome fragile sites.* Cell, 2005. **120**(5): p. 587-98.
83. Poli, J., et al., *dNTP pools determine fork progression and origin usage under replication stress.* EMBO J. **31**(4): p. 883-94.
84. Admire, A., et al., *Cycles of chromosome instability are associated with a fragile site and are increased by defects in DNA replication and checkpoint controls in yeast.* Genes Dev, 2006. **20**(2): p. 159-73.
85. Orr, H.T. and H.Y. Zoghbi, *Trinucleotide repeat disorders.* Annu Rev Neurosci, 2007. **30**: p. 575-621.
86. Sogo, J.M., M. Lopes, and M. Foiani, *Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects.* Science, 2002. **297**(5581): p. 599-602.
87. Byun, T.S., et al., *Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint.* Genes Dev, 2005. **19**(9): p. 1040-52.
88. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.
89. Nam, E.A. and D. Cortez, *ATR signalling: more than meeting at the fork.* Biochem J, 2011. **436**(3): p. 527-36.
90. Tercero, J.A., M.P. Longhese, and J.F. Diffley, *A central role for DNA replication forks in checkpoint activation and response.* Mol Cell, 2003. **11**(5): p. 1323-36.
91. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
92. Bochkarev, A., et al., *Structure of the single-stranded-DNA-binding domain of replication protein A bound to DNA.* Nature, 1997. **385**(6612): p. 176-81.
93. Recolin, B., S. Van Der Laan, and D. Maiorano, *Role of replication protein A as sensor in activation of the S-phase checkpoint in Xenopus egg extracts.* Nucleic Acids Res.
94. Lopes, M., et al., *The DNA replication checkpoint response stabilizes stalled replication forks.* Nature, 2001. **412**(6846): p. 557-61.
95. Tercero, J.A. and J.F. Diffley, *Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint.* Nature, 2001. **412**(6846): p. 553-7.
96. De Piccoli, G., et al., *Replisome stability at defective DNA replication forks is independent of s phase checkpoint kinases.* Mol Cell. **45**(5): p. 696-704.
97. Jin, J., et al., *SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase.* Genes Dev, 2003. **17**(24): p. 3062-74.
98. Xiao, Z., et al., *Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents.* J Biol Chem, 2003. **278**(24): p. 21767-73.
99. Zegerman, P. and J.F. Diffley, *Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation.* Nature. **467**(7314): p. 474-8.
100. Kawabata, T., et al., *Stalled fork rescue via dormant replication origins in unchallenged S phase promotes proper chromosome segregation and tumor suppression.* Mol Cell. **41**(5): p. 543-53.

101. Casper, A.M., et al., *ATR regulates fragile site stability.* Cell, 2002. **111**(6): p. 779-89.
102. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.
103. Li, X., et al., *Lagging strand DNA synthesis at the eukaryotic replication fork involves binding and stimulation of FEN-1 by proliferating cell nuclear antigen.* J Biol Chem, 1995. **270**(38): p. 22109-12.
104. Burgers, P.M., *Mammalian cyclin/PCNA (DNA polymerase delta auxiliary protein) stimulates processive DNA synthesis by yeast DNA polymerase III.* Nucleic Acids Res, 1988. **16**(14A): p. 6297-307.
105. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
106. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
107. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
108. Barbour, L. and W. Xiao, *Regulation of alternative replication bypass pathways at stalled replication forks and its effects on genome stability: a yeast model.* Mutat Res, 2003. **532**(1-2): p. 137-55.
109. Torres-Ramos, C.A., S. Prakash, and L. Prakash, *Requirement of RAD5 and MMS2 for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae.* Mol Cell Biol, 2002. **22**(7): p. 2419-26.
110. Branzei, D., F. Vanoli, and M. Foiani, *SUMOylation regulates Rad18-mediated template switch.* Nature, 2008. **456**(7224): p. 915-20.
111. Minca, E.C. and D. Kowalski, *Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks.* Mol Cell. **38**(5): p. 649-61.
112. Krejci, L., et al., *DNA helicase Srs2 disrupts the Rad51 presynaptic filament.* Nature, 2003. **423**(6937): p. 305-9.
113. Veaute, X., et al., *The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments.* Nature, 2003. **423**(6937): p. 309-12.
114. Bergink, S. and S. Jentsch, *Principles of ubiquitin and SUMO modifications in DNA repair.* Nature, 2009. **458**(7237): p. 461-7.
115. Deribe, Y.L., T. Pawson, and I. Dikic, *Post-translational modifications in signal integration.* Nat Struct Mol Biol. **17**(6): p. 666-72.
116. Kerscher, O., R. Felberbaum, and M. Hochstrasser, *Modification of proteins by ubiquitin and ubiquitin-like proteins.* Annu Rev Cell Dev Biol, 2006. **22**: p. 159-80.
117. Mukhopadhyay, D. and H. Riezman, *Proteasome-independent functions of ubiquitin in endocytosis and signaling.* Science, 2007. **315**(5809): p. 201-5.
118. Scheffner, M., U. Nuber, and J.M. Huibregtse, *Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade.* Nature, 1995. **373**(6509): p. 81-3.
119. Behrends, C. and J.W. Harper, *Constructing and decoding unconventional ubiquitin chains.* Nat Struct Mol Biol. **18**(5): p. 520-8.
120. Chau, V., et al., *A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein.* Science, 1989. **243**(4898): p. 1576-83.
121. Thrower, J.S., et al., *Recognition of the polyubiquitin proteolytic signal.* EMBO J, 2000. **19**(1): p. 94-102.
122. Jin, L., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex.* Cell, 2008. **133**(4): p. 653-65.
123. Jacobson, A.D., et al., *The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome.* J Biol Chem, 2009. **284**(51): p. 35485-94.
124. Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain.* Cell, 2000. **103**(2): p. 351-61.
125. VanDemark, A.P., et al., *Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer.* Cell, 2001. **105**(6): p. 711-20.

126. Rahighi, S., et al., *Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation.* Cell, 2009. **136**(6): p. 1098-109.
127. Ye, Y. and M. Rape, *Building ubiquitin chains: E2 enzymes at work.* Nat Rev Mol Cell Biol, 2009. **10**(11): p. 755-64.
128. Singhal, S., M.C. Taylor, and R.T. Baker, *Deubiquitylating enzymes and disease.* BMC Biochem, 2008. **9 Suppl 1**: p. S3.
129. Mizushima, N., *Autophagy: process and function.* Genes Dev, 2007. **21**(22): p. 2861-73.
130. Bedford, L., et al., *Assembly, structure, and function of the 26S proteasome.* Trends Cell Biol, 2010. **20**(7): p. 391-401.
131. Wojcik, C. and G.N. DeMartino, *Intracellular localization of proteasomes.* Int J Biochem Cell Biol, 2003. **35**(5): p. 579-89.
132. Ciechanover, A., *Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting.* Cell Death Differ, 2005. **12**(9): p. 1178-90.
133. Goldberg, A.L., *Functions of the proteasome: from protein degradation and immune surveillance to cancer therapy.* Biochem Soc Trans, 2007. **35**(Pt 1): p. 12-7.
134. Ciechanover, A., D. Finley, and A. Varshavsky, *Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85.* Cell, 1984. **37**(1): p. 57-66.
135. Schulman, B.A. and J.W. Harper, *Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways.* Nat Rev Mol Cell Biol, 2009. **10**(5): p. 319-31.
136. Jones, D., et al., *Functional and phylogenetic analysis of the ubiquitylation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins.* Genome Biol, 2002. **3**(1): p. RESEARCH0002.
137. Deshaies, R.J. and C.A. Joazeiro, *RING domain E3 ubiquitin ligases.* Annu Rev Biochem, 2009. **78**: p. 399-434.
138. Kipreos, E.T., *Ubiquitin-mediated pathways in C. elegans.* WormBook, 2005: p. 1-24.
139. Eddins, M.J., et al., *Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation.* Nat Struct Mol Biol, 2006. **13**(10): p. 915-20.
140. Rodrigo-Brenni, M.C., S.A. Foster, and D.O. Morgan, *Catalysis of lysine 48-specific ubiquitin chain assembly by residues in E2 and ubiquitin.* Mol Cell, 2010. **39**(4): p. 548-59.
141. Passmore, L.A. and D. Barford, *Getting into position: the catalytic mechanisms of protein ubiquitylation.* Biochem J, 2004. **379**(Pt 3): p. 513-25.
142. Koegl, M., et al., *A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly.* Cell, 1999. **96**(5): p. 635-44.
143. Richly, H., et al., *A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting.* Cell, 2005. **120**(1): p. 73-84.
144. Ghislain, M., et al., *Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in Saccharomyces cerevisiae.* EMBO J, 1996. **15**(18): p. 4884-99.
145. Meyer, H., M. Bug, and S. Bremer, *Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system.* Nat Cell Biol, 2012. **14**(2): p. 117-23.
146. Yamanaka, K., Y. Sasagawa, and T. Ogura, *Recent advances in p97/VCP/Cdc48 cellular functions.* Biochim Biophys Acta, 2011. **1823**(1): p. 130-7.
147. Ammelburg, M., T. Frickey, and A.N. Lupas, *Classification of AAA+ proteins.* J Struct Biol, 2006. **156**(1): p. 2-11.
148. Huyton, T., et al., *The crystal structure of murine p97/VCP at 3.6A.* J Struct Biol, 2003. **144**(3): p. 337-48.
149. DeLaBarre, B. and A.T. Brunger, *Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains.* Nat Struct Biol, 2003. **10**(10): p. 856-63.
150. Niwa, H., et al., *The role of the N-domain in the ATPase activity of the mammalian AAA ATPase p97/VCP.* J Biol Chem.
151. Beuron, F., et al., *Conformational changes in the AAA ATPase p97-p47 adaptor complex.* EMBO J, 2006. **25**(9): p. 1967-76.
152. Pye, V.E., et al., *Going through the motions: the ATPase cycle of p97.* J Struct Biol, 2006. **156**(1): p. 12-28.

153. Meyer, H.H., Y. Wang, and G. Warren, *Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4.* EMBO J, 2002. **21**(21): p. 5645-52.
154. Kloppsteck, P., et al., *Regulation of p97 in the ubiquitin-proteasome system by the UBX protein-family.* Biochim Biophys Acta. **1823**(1): p. 125-9.
155. Beskow, A., et al., *A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation.* J Mol Biol, 2009. **394**(4): p. 732-46.
156. Isakov, E. and A. Stanhill, *Stalled proteasomes are directly relieved by P97 recruitment.* J Biol Chem. **286**(35): p. 30274-83.
157. Dai, R.M. and C.C. Li, *Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation.* Nat Cell Biol, 2001. **3**(8): p. 740-4.
158. Kitami, M.I., et al., *Dominant-negative effect of mutant valosin-containing protein in aggresome formation.* FEBS Lett, 2006. **580**(2): p. 474-8.
159. Fernandez-Saiz, V. and A. Buchberger, *Imbalances in p97 co-factor interactions in human proteinopathy.* EMBO Rep. **11**(6): p. 479-85.
160. Yamanaka, K., et al., *Analysis of the two p97/VCP/Cdc48p proteins of Caenorhabditis elegans and their suppression of polyglutamine-induced protein aggregation.* J Struct Biol, 2004. **146**(1-2): p. 242-50.
161. Stolz, A., et al., *Cdc48: a power machine in protein degradation.* Trends Biochem Sci. **36**(10): p. 515-23.
162. Deichsel, A., J. Mouysset, and T. Hoppe, *The ubiquitin-selective chaperone CDC-48/p97, a new player in DNA replication.* Cell Cycle, 2009. **8**(2): p. 185-90.
163. Ye, Y., *Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase.* J Struct Biol, 2006. **156**(1): p. 29-40.
164. Schuberth, C. and A. Buchberger, *UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97.* Cell Mol Life Sci, 2008. **65**(15): p. 2360-71.
165. Madsen, L., et al., *New ATPase regulators--p97 goes to the PUB.* Int J Biochem Cell Biol, 2009. **41**(12): p. 2380-8.
166. Kondo, H., et al., *p47 is a cofactor for p97-mediated membrane fusion.* Nature, 1997. **388**(6637): p. 75-8.
167. Kuhlbrodt, K., et al., *The Machado-Joseph disease deubiquitylase ATX-3 couples longevity and proteostasis.* Nat Cell Biol, 2011. **13**(3): p. 273-81.
168. Sowa, M.E., et al., *Defining the Human Deubiquitinating Enzyme Interaction Landscape.* Cell, 2009. **138**(2): p. 389-403.
169. Moir, D., et al., *Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies.* Genetics, 1982. **100**(4): p. 547-63.
170. Cao, K., et al., *The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis.* Cell, 2003. **115**(3): p. 355-67.
171. Heubes, S. and O. Stemmann, *The AAA-ATPase p97-Ufd1-Npl4 is required for ERAD but not for spindle disassembly in Xenopus egg extracts.* J Cell Sci, 2007. **120**(Pt 8): p. 1325-9.
172. Ramadan, K., et al., *Cdc48/p97 promotes reformation of the nucleus by extracting the kinase Aurora B from chromatin.* Nature, 2007. **450**(7173): p. 1258-62.
173. Dobrynin, G., et al., *Cdc48/p97-Ufd1-Npl4 antagonizes Aurora B during chromosome segregation in HeLa cells.* J Cell Sci. **124**(Pt 9): p. 1571-80.
174. Cheng, Y.L. and R.H. Chen, *The AAA-ATPase Cdc48 and cofactor Shp1 promote chromosome bi-orientation by balancing Aurora B activity.* J Cell Sci. **123**(Pt 12): p. 2025-34.
175. Ikai, N. and M. Yanagida, *Cdc48 is required for the stability of Cut1/separase in mitotic anaphase.* J Struct Biol, 2006. **156**(1): p. 50-61.
176. Mouysset, J., et al., *Cell cycle progression requires the CDC-48UFD-1/NPL-4 complex for efficient DNA replication.* Proc Natl Acad Sci U S A, 2008. **105**(35): p. 12879-84.
177. Yamauchi, S., K. Yamanaka, and T. Ogura, *Comparative analysis of expression of two p97 homologues in Caenorhabditis elegans.* Biochem Biophys Res Commun, 2006. **345**(2): p. 746-53.
178. Brauchle, M., K. Baumer, and P. Gonczy, *Differential activation of the DNA replication checkpoint contributes to asynchrony of cell division in C. elegans embryos.* Curr Biol, 2003. **13**(10): p. 819-27.

179. Encalada, S.E., et al., *DNA replication defects delay cell division and disrupt cell polarity in early Caenorhabditis elegans embryos.* Dev Biol, 2000. **228**(2): p. 225-38.
180. Rinaldo, C., et al., *Roles for Caenorhabditis elegans rad-51 in meiosis and in resistance to ionizing radiation during development.* Genetics, 2002. **160**(2): p. 471-9.
181. Rosenkranz, H.S. and J.A. Levy, *Hydroxyurea: A Specific Inhibitor of Deoxyribonucleic Acid Synthesis.* Biochim Biophys Acta, 1965. **95**: p. 181-3.
182. Stolz, A., et al., *Cdc48: a power machine in protein degradation.* Trends Biochem Sci, 2011. **36**(10): p. 515-23.
183. Sonneville, R., et al., *The dynamics of replication licensing in live Caenorhabditis elegans embryos.* J Cell Biol. **196**(2): p. 233-46.
184. Sasagawa, Y., et al., *Caenorhabditis elegans p97/CDC-48 is crucial for progression of meiosis I.* Biochem Biophys Res Commun, 2007. **358**(3): p. 920-4.
185. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
186. Deppe, U., et al., *Cell lineages of the embryo of the nematode Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 1978. **75**(1): p. 376-80.
187. Schierenberg, E., J. Miwa, and G. von Ehrenstein, *Cell lineages and developmental defects of temperature-sensitive embryonic arrest mutants in Caenorhabditis elegans.* Dev Biol, 1980. **76**(1): p. 141-59.
188. Pellettieri, J. and G. Seydoux, *Anterior-posterior polarity in C. elegans and Drosophila--PARallels and differences.* Science, 2002. **298**(5600): p. 1946-50.
189. Wicky, C., et al., *Multiple genetic pathways involving the Caenorhabditis elegans Bloom's syndrome genes him-6, rad-51, and top-3 are needed to maintain genome stability in the germ line.* Mol Cell Biol, 2004. **24**(11): p. 5016-27.
190. Acs, K., et al., *The AAA-ATPase VCP/p97 promotes 53BP1 recruitment by removing L3MBTL1 from DNA double-strand breaks.* Nat Struct Mol Biol, 2011. **18**(12): p. 1345-50.
191. Verma, R., et al., *Cdc48/p97 mediates UV-dependent turnover of RNA Pol II.* Mol Cell, 2011. **41**(1): p. 82-92.
192. Meerang, M., et al., *The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks.* Nat Cell Biol. **13**(11): p. 1376-82.
193. Wilcox, A.J. and J.D. Laney, *A ubiquitin-selective AAA-ATPase mediates transcriptional switching by remodelling a repressor-promoter DNA complex.* Nat Cell Biol, 2009. **11**(12): p. 1481-6.
194. Peters, J.M., M.J. Walsh, and W.W. Franke, *An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative vesicle fusion proteins Sec18p and NSF.* EMBO J, 1990. **9**(6): p. 1757-67.
195. Kim, Y. and E.T. Kipreos, *The Caenorhabditis elegans replication licensing factor CDT-1 is targeted for degradation by the CUL-4/DDB-1 complex.* Mol Cell Biol, 2007. **27**(4): p. 1394-406.
196. Jia, L., et al., *RBX1 (RING box protein 1) E3 ubiquitin ligase is required for genomic integrity by modulating DNA replication licensing proteins.* J Biol Chem, 2010. **286**(5): p. 3379-86.
197. Zheng, N., et al., *Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex.* Nature, 2002. **416**(6882): p. 703-9.
198. Takeda, D.Y., J.D. Parvin, and A. Dutta, *Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase.* J Biol Chem, 2005. **280**(24): p. 23416-23.
199. Adams, P.D., et al., *Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors.* Mol Cell Biol, 1996. **16**(12): p. 6623-33.
200. Schulman, B.A., D.L. Lindstrom, and E. Harlow, *Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10453-8.
201. Ohh, M., et al., *An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells.* EMBO Rep, 2002. **3**(2): p. 177-82.

202. Pintard, L., et al., *Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in C. elegans.* Curr Biol, 2003. **13**(11): p. 911-21.
203. Kurz, T., et al., *The conserved protein DCN-1/Dcn1p is required for cullin neddylation in C. elegans and S. cerevisiae.* Nature, 2005. **435**(7046): p. 1257-61.
204. Ballabeni, A., et al., *Human CDT1 associates with CDC7 and recruits CDC45 to chromatin during S phase.* J Biol Chem, 2009. **284**(5): p. 3028-36.
205. Tsakraklides, V. and S.P. Bell, *Dynamics of pre-replicative complex assembly.* J Biol Chem, 2010. **285**(13): p. 9437-43.
206. Branzei, D. and M. Foiani, *Maintaining genome stability at the replication fork.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 208-19.
207. Deribe, Y.L., T. Pawson, and I. Dikic, *Post-translational modifications in signal integration.* Nat Struct Mol Biol, 2010. **17**(6): p. 666-72.
208. Rhind, N. and P. Russell, *Mitotic DNA damage and replication checkpoints in yeast.* Curr Opin Cell Biol, 1998. **10**(6): p. 749-58.
209. Franz, A., et al., *CDC-48/p97 coordinates CDT-1 degradation with GINS chromatin dissociation to ensure faithful DNA replication.* Mol Cell, 2011. **44**(1): p. 85-96.
210. Nishitani, H., et al., *The Cdt1 protein is required to license DNA for replication in fission yeast.* Nature, 2000. **404**(6778): p. 625-8.
211. Li, A. and J.J. Blow, *Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus.* EMBO J, 2005. **24**(2): p. 395-404.
212. Raman, M., et al., *A genome-wide screen identifies p97 as an essential regulator of DNA damage-dependent CDT1 destruction.* Mol Cell, 2011. **44**(1): p. 72-84.
213. Ballabeni, A., et al., *Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis.* EMBO J, 2004. **23**(15): p. 3122-32.
214. Takara, T.J. and S.P. Bell, *Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 helicases.* EMBO J. **30**(24): p. 4885-96.
215. Sonneville, R., et al., *The dynamics of replication licensing in live Caenorhabditis elegans embryos.* J Cell Biol, 2012. **196**(2): p. 233-46.
216. Xouri, G., et al., *Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin.* EMBO J, 2007. **26**(5): p. 1303-14.
217. McNairn, A.J., et al., *Chinese hamster ORC subunits dynamically associate with chromatin throughout the cell-cycle.* Exp Cell Res, 2005. **308**(2): p. 345-56.
218. Edwards, M.C., et al., *MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts.* J Biol Chem, 2002. **277**(36): p. 33049-57.
219. Bowers, J.L., et al., *ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication.* Mol Cell, 2004. **16**(6): p. 967-78.
220. Takara, T.J. and S.P. Bell, *Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 helicases.* EMBO J, 2011. **30**(24): p. 4885-96.
221. Waga, S. and A. Zembutsu, *Dynamics of DNA binding of replication initiation proteins during de novo formation of pre-replicative complexes in Xenopus egg extracts.* J Biol Chem, 2006. **281**(16): p. 10926-34.
222. Chen, S., M.A. de Vries, and S.P. Bell, *Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading.* Genes Dev, 2007. **21**(22): p. 2897-907.
223. Ge, X.Q., D.A. Jackson, and J.J. Blow, *Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress.* Genes Dev, 2007. **21**(24): p. 3331-41.
224. Woodward, A.M., et al., *Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress.* J Cell Biol, 2006. **173**(5): p. 673-83.
225. Xouri, G., et al., *Cdt1 interactions in the licensing process: a model for dynamic spatiotemporal control of licensing.* Cell Cycle, 2007. **6**(13): p. 1549-52.
226. Gilbert, D.M., *Replication origin plasticity, Taylor-made: inhibition vs recruitment of origins under conditions of replication stress.* Chromosoma, 2007. **116**(4): p. 341-7.
227. Maric, C., B. Levacher, and O. Hyrien, *Developmental regulation of replication fork pausing in Xenopus laevis ribosomal RNA genes.* J Mol Biol, 1999. **291**(4): p. 775-88.

228. Marheineke, K. and O. Hyrien, *Control of replication origin density and firing time in Xenopus egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint.* J Biol Chem, 2004. **279**(27): p. 28071-81.
229. Vaziri, C., et al., *A p53-dependent checkpoint pathway prevents rereplication.* Mol Cell, 2003. **11**(4): p. 997-1008.
230. Saxena, S. and A. Dutta, *Geminin-Cdt1 balance is critical for genetic stability.* Mutat Res, 2005. **569**(1-2): p. 111-21.
231. Nishitani, H., Z. Lygerou, and T. Nishimoto, *Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region.* J Biol Chem, 2004. **279**(29): p. 30807-16.
232. Tsakraklides, V. and S.P. Bell, *Dynamics of pre-replicative complex assembly.* J Biol Chem. **285**(13): p. 9437-43.
233. Sugimoto, N., et al., *Redundant and differential regulation of multiple licensing factors ensures prevention of re-replication in normal human cells.* J Cell Sci, 2009. **122**(Pt 8): p. 1184-91.
234. Yanow, S.K., Z. Lygerou, and P. Nurse, *Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication.* EMBO J, 2001. **20**(17): p. 4648-56.
235. Nguyen, V.Q., C. Co, and J.J. Li, *Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms.* Nature, 2001. **411**(6841): p. 1068-73.
236. Kim, J., H. Feng, and E.T. Kipreos, *C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway.* Curr Biol, 2007. **17**(11): p. 966-72.
237. Kim, Y., N.G. Starostina, and E.T. Kipreos, *The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing.* Genes Dev, 2008. **22**(18): p. 2507-19.
238. Zou, L. and B. Stillman, *Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase.* Mol Cell Biol, 2000. **20**(9): p. 3086-96.
239. Tanaka, S., et al., *CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast.* Nature, 2007. **445**(7125): p. 328-32.
240. Liu, P., et al., *The Chk1-mediated S-phase checkpoint targets initiation factor Cdc45 via a Cdc25A/Cdk2-independent mechanism.* J Biol Chem, 2006. **281**(41): p. 30631-44.
241. Liu, H., et al., *Phosphorylation of MLL by ATR is required for execution of mammalian S-phase checkpoint.* Nature. **467**(7313): p. 343-6.
242. Pryde, F., et al., *H3 k36 methylation helps determine the timing of cdc45 association with replication origins.* PLoS One, 2009. **4**(6): p. e5882.
243. Pollok, S. and F. Grosse, *Cdc45 degradation during differentiation and apoptosis.* Biochem Biophys Res Commun, 2007. **362**(4): p. 910-5.
244. DeGregori, J., T. Kowalik, and J.R. Nevins, *Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes.* Mol Cell Biol, 1995. **15**(8): p. 4215-24.
245. Hashimoto, Y., F. Puddu, and V. Costanzo, *RAD51- and MRE11-dependent reassembly of uncoupled CMG helicase complex at collapsed replication forks.* Nat Struct Mol Biol. **19**(1): p. 17-24.
246. Liu, H., et al., *Phosphorylation of MLL by ATR is required for execution of mammalian S-phase checkpoint.* Nature, 2010. **467**(7313): p. 343-6.
247. Hashimoto, Y., F. Puddu, and V. Costanzo, *RAD51- and MRE11-dependent reassembly of uncoupled CMG helicase complex at collapsed replication forks.* Nat Struct Mol Biol, 2011. **19**(1): p. 17-24.
248. Ramanathan, H.N. and Y. Ye, *The p97 ATPase associates with EEA1 to regulate the size of early endosomes.* Cell Res. **22**(2): p. 346-59.
249. Yamada, T., et al., *p97 ATPase, an ATPase involved in membrane fusion, interacts with DNA unwinding factor (DUF) that functions in DNA replication.* FEBS Lett, 2000. **466**(2-3): p. 287-91.

250. Han, J., et al., *Ubiquitylation of FACT by the cullin-E3 ligase Rtt101 connects FACT to DNA replication.* Genes Dev. **24**(14): p. 1485-90.
251. Partridge, J.J., et al., *DNA damage modulates nucleolar interaction of the Werner protein with the AAA ATPase p97/VCP.* Mol Biol Cell, 2003. **14**(10): p. 4221-9.
252. Caruso, M.E., et al., *GTPase-mediated regulation of the unfolded protein response in Caenorhabditis elegans is dependent on the AAA+ ATPase CDC-48.* Mol Cell Biol, 2008. **28**(13): p. 4261-74.
253. Labib, K. and B. Hodgson, *Replication fork barriers: pausing for a break or stalling for time?* EMBO Rep, 2007. **8**(4): p. 346-53.
254. McGlynn, P. and C.P. Guy, *Replication forks blocked by protein-DNA complexes have limited stability in vitro.* J Mol Biol, 2008. **381**(2): p. 249-55.
255. Anand, R.P., et al., *Overcoming natural replication barriers: differential helicase requirements.* Nucleic Acids Res, 2012. **40**(3): p. 1091-105.
256. Abbas, T. and A. Dutta, *CRL4Cdt2: master coordinator of cell cycle progression and genome stability.* Cell Cycle. **10**(2): p. 241-9.
257. Tutter, A.V. and J.C. Walter, *Chromosomal DNA replication in a soluble cell-free system derived from Xenopus eggs.* Methods Mol Biol, 2006. **322**: p. 121-37.
258. Maresca, T.J. and R. Heald, *Methods for studying spindle assembly and chromosome condensation in Xenopus egg extracts.* Methods Mol Biol, 2006. **322**: p. 459-74.
259. Franz, A., et al., *CDC-48/p97 coordinates CDT-1 degradation with GINS chromatin dissociation to ensure faithful DNA replication.* Mol Cell. **44**(1): p. 85-96.
260. Wojcik, C., M. Yano, and G.N. DeMartino, *RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis.* J Cell Sci, 2004. **117**(Pt 2): p. 281-92.
261. Roukos, V., et al., *Dynamic recruitment of licensing factor Cdt1 to sites of DNA damage.* J Cell Sci. **124**(Pt 3): p. 422-34.
262. Kondo, T., et al., *Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex.* J Biol Chem, 2004. **279**(26): p. 27315-9.
263. Hu, J., et al., *Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage.* Nat Cell Biol, 2004. **6**(10): p. 1003-9.
264. Roukos, V., et al., *Dynamic recruitment of licensing factor Cdt1 to sites of DNA damage.* J Cell Sci, 2011. **124**(Pt 3): p. 422-34.
265. Kisielewska, J., P. Lu, and M. Whitaker, *GFP-PCNA as an S-phase marker in embryos during the first and subsequent cell cycles.* Biol Cell, 2005. **97**(3): p. 221-9.
266. Branzei, D. and M. Foiani, *The checkpoint response to replication stress.* DNA Repair (Amst), 2009. **8**(9): p. 1038-46.
267. Hashiguchi, K., Y. Matsumoto, and A. Yasui, *Recruitment of DNA repair synthesis machinery to sites of DNA damage/repair in living human cells.* Nucleic Acids Res, 2007. **35**(9): p. 2913-23.
268. Petermann, E., M. Woodcock, and T. Helleday, *Chk1 promotes replication fork progression by controlling replication initiation.* Proc Natl Acad Sci U S A. **107**(37): p. 16090-5.
269. De Piccoli, G., et al., *Replisome stability at defective DNA replication forks is independent of s phase checkpoint kinases.* Mol Cell, 2012. **45**(5): p. 696-704.
270. Santocanale, C. and J.F. Diffley, *A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication.* Nature, 1998. **395**(6702): p. 615-8.
271. Ye, Y., H.H. Meyer, and T.A. Rapoport, *The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol.* Nature, 2001. **414**(6864): p. 652-6.
272. Jarosch, E., et al., *Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48.* Nat Cell Biol, 2002. **4**(2): p. 134-9.
273. Meerang, M., et al., *The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks.* Nat Cell Biol, 2011. **13**(11): p. 1376-82.
274. Dobrynin, G., et al., *Cdc48/p97-Ufd1-Npl4 antagonizes Aurora B during chromosome segregation in HeLa cells.* J Cell Sci, 2011. **124**(Pt 9): p. 1571-80.
275. Ramadan, K., *p97/VCP- and Lys48-linked polyubiquitination form a new signaling pathway in DNA damage response.* Cell Cycle, 2012. **11**(6).

276. Zhang, H., et al., *VCP, a weak ATPase involved in multiple cellular events, interacts physically with BRCA1 in the nucleus of living cells.* DNA Cell Biol, 2000. **19**(5): p. 253-63.
277. Kloppsteck, P., et al., *Regulation of p97 in the ubiquitin-proteasome system by the UBX protein-family.* Biochim Biophys Acta, 2011. **1823**(1): p. 125-9.
278. Alexandru, G., et al., *UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1alpha turnover.* Cell, 2008. **134**(5): p. 804-16.
279. Besten, W.D., et al., *NEDD8 links cullin-RING ubiquitin ligase function to the p97 pathway.* Nat Struct Mol Biol, 2012.
280. Ritz, D., et al., *Endolysosomal sorting of ubiquitylated caveolin-1 is regulated by VCP and UBXD1 and impaired by VCP disease mutations.* Nat Cell Biol, 2011. **13**(9): p. 1116-23.
281. Sasagawa, Y., et al., *Caenorhabditis elegans p97 controls germline-specific sex determination by controlling the TRA-1 level in a CUL-2-dependent manner.* J Cell Sci, 2009. **122**(Pt 20): p. 3663-72.
282. Sasagawa, Y., et al., *Caenorhabditis elegans UBX cofactors for CDC-48/p97 control spermatogenesis.* Genes Cells. **15**(12): p. 1201-15.
283. Sambrook, J. and D.W. Russell, *Molecular cloning : a laboratory manual*. 3rd ed. 2001, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
284. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
285. Candiano, G., et al., *Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis.* Electrophoresis, 2004. **25**(9): p. 1327-33.
286. Wood, W.B., *The Nematode Caenorhabditis elegans*. Cold Spring Harbor monograph series 17. 1988, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory. xiii, 667 p.
287. Riddle, D.L., *C. elegans II*. Cold Spring Harbor monograph series,. 1997, Plainview, N.Y.: Cold Spring Harbor Laboratory Press. xvii, 1222 p.
288. Hope, I.A., *C. elegans : a practical approach*. The practical approach series. 1999, Oxford ; New York: Oxford University Press. xxi, 281 p.
289. Kamath, R.S., et al., *Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans.* Genome Biol, 2001. **2**(1): p. RESEARCH0002.
290. Kamath, R.S., et al., *Systematic functional analysis of the Caenorhabditis elegans genome using RNAi.* Nature, 2003. **421**(6920): p. 231-7.
291. Timmons, L. and A. Fire, *Specific interference by ingested dsRNA.* Nature, 1998. **395**(6705): p. 854.
292. Gonczy, P., et al., *Dissection of cell division processes in the one cell stage Caenorhabditis elegans embryo by mutational analysis.* J Cell Biol, 1999. **144**(5): p. 927-46.
293. Kemphues, K.J., et al., *Two loci required for cytoplasmic organization in early embryos of Caenorhabditis elegans.* Dev Biol, 1986. **113**(2): p. 449-60.

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