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Caractérisation de la fonction de OBI1, une E3 ubiquitine ligase, dans la réplication de l'ADN.

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*"…The set of possible people allowed by our **DNA** so massively exceeds the set of actual people. Certainly, those unborn ghosts include poets greater than Keats and scientists greater than Newton…In the teeth of these stupefying odds, it is you and I, in our ordinariness, that are here..."*

Richard Dawkins

“Part of the journey, is the end”

Tony Stark

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List of Abbreviations

AD	Alzheimer's Disease
APC/C	Anaphase Promoting Complex or Cyclosome
APOE4	Apolipoprotein Epsilon 4 allele
ARS	Autonomously Replicating Sequence
BARD1	BRCA1 Associated RING Domain 1
BLM	Bloom syndrome protein
BRCA1	Breast Cancer 1
BS	Bloom Syndrome
CDC	Cell Division Cycle
CDH1	CDC20 Homolog 1
CDK	Cyclin Dependent Kinase
CDT1	CDC10 Dependent Transcript 1
CKI	CDK Inhibitor
CMG	CDC45, MCM2-7, GINS
CRL	Cullin RING Ligases
CSF	Cytostatic Factor
DDK	Dbf4 Dependent Kinase
DNA	Deoxyribonucleic Acid
DUB	DeUbiquitylating enzyme
EMI1	Early Mitotic Inhibitor
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
GCN5	General Control of amino acid synthesis protein 5
GINS	Go Ichi Ni San
G1	Gap 1
G2	Gap 2
G4	G quadruplex

HAT	Histone Acetyl Transferase
HBO1	Histone acetyltransferase Binding ORC1
HECT	Homologous to the E6AR Carboxyl Terminus
HMT	Histone Methyl Transferase
HP1	Heterochromatin Protein 1
HSE	High Speed Extract
H1	Histone 1
IAP	Inhibitors of Apoptosis Proteins
IKK	IKB Kinase
KAT2A	Lysine Acetyl Transferase 2A
LOAD	Late Onset Alzheimer's Disease
LRWD1	Leucine rich Repeat and WD repeat-containing protein 1
LSE	Low Speed Extract
LUBAC	Linear Ubiquitin Assembly Complex
M	Mitosis
MBT	Mid Blastula stage
MCM	Mini Chromosome Maintenance
NEMO	NF-Kappa-B Essential Modulator
NF-KB	Nuclear Factor Kappa B
MGS	Meier- Gorlin Syndrome
NMJ	Neuro- Muscular Junction
MMR	Miss Match Repair
NPE	Nucleoplasmic Extract
N/C	Nucleus/ Cytoplasm
OGRE	G-rich Repeated Elements
ORC	Origin Recognition Complex
ORCA	ORC-Associated protein
ORF	Open Reading Frame
PCNA	Proliferating Cell Nuclear Antigen
PIP-box	PCNA Interaction Motif
Pre-IC	Pre-Initiation Complex

Pre-RC	Pre-Replication Complex
PR-set7	PR/SET domain containing protein 7
PTM	Post-Translational Modification
RAD	Radiation- Repair
RBR	RING-Between-RING ligase
RFC	Replication Factor C
RING	Really Interesting New Gene
RNA	Ribonucleic Acid
RNF219	Ring Finger Protein 219
RPA	Replication Protein A
RTS	Rothmund-Thomson syndrome
S	Synthesis
SCF	SKP1 Cullin F-box
SCF dia2	SKP1 Cullin F-box- Dig Into Agar 2
SMAD	Mothers Against Decapentaplegic
ssDNA	Single Stranded DNA
TOPBP1	DNA topoisomerase 2 binding protein 1
TRIM	Tripartite Motif containing protein
TSS	Transcription Starting Site
UBD	Ubiquitin Binding Domain
WS	Werner syndrome
XE	Xenopus Egg Extract
0K	Zero lysine

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Introduction

Chapter 1 Introduction

Part 1 DNA replication

1.1 The cell cycle

The cell is the smallest unit of life with the striking capacity of reproducing. For this to happen, the cell has to copy its genetic material and divide it, along with its cytoplasm and organelles to produce two identical daughter cells. During this so-called “Cell Cycle”, cells undertake several biochemical reactions. These reactions are divided into four stages and are controlled by several regulatory mechanisms, to allow accurate cell division.

The stages of the cell cycle

The cell cycle is divided into two major parts, interphase and mitosis (See Figure 1). Cells double in size during interphase stages before dividing in mitosis. The duration of the cell cycle is variable between different cell types. Some cells like embryonic or cancer cells proliferate rapidly, while other cell types in the human body can live without necessarily dividing. Some cells stay in a quiescent state (called G0), and occasionally, depending on the right stimuli (inflammation, growth factors, nutrients, hormones, etc.) these cells can exit G0 and enter the cell cycle in the first phase, called the Gap-1 phase or G1. During this phase, cells grow and prepare to undertake the duplication of their genome in the following step named S phase (or Synthesis phase). The completion of DNA replication is then followed by the last stage of interphase, the Gap-2 or G2 phase when the cell synthesizes proteins in preparation for mitosis (M-phase), which corresponds to the separation of replicated chromosomes and ends with the cell division.

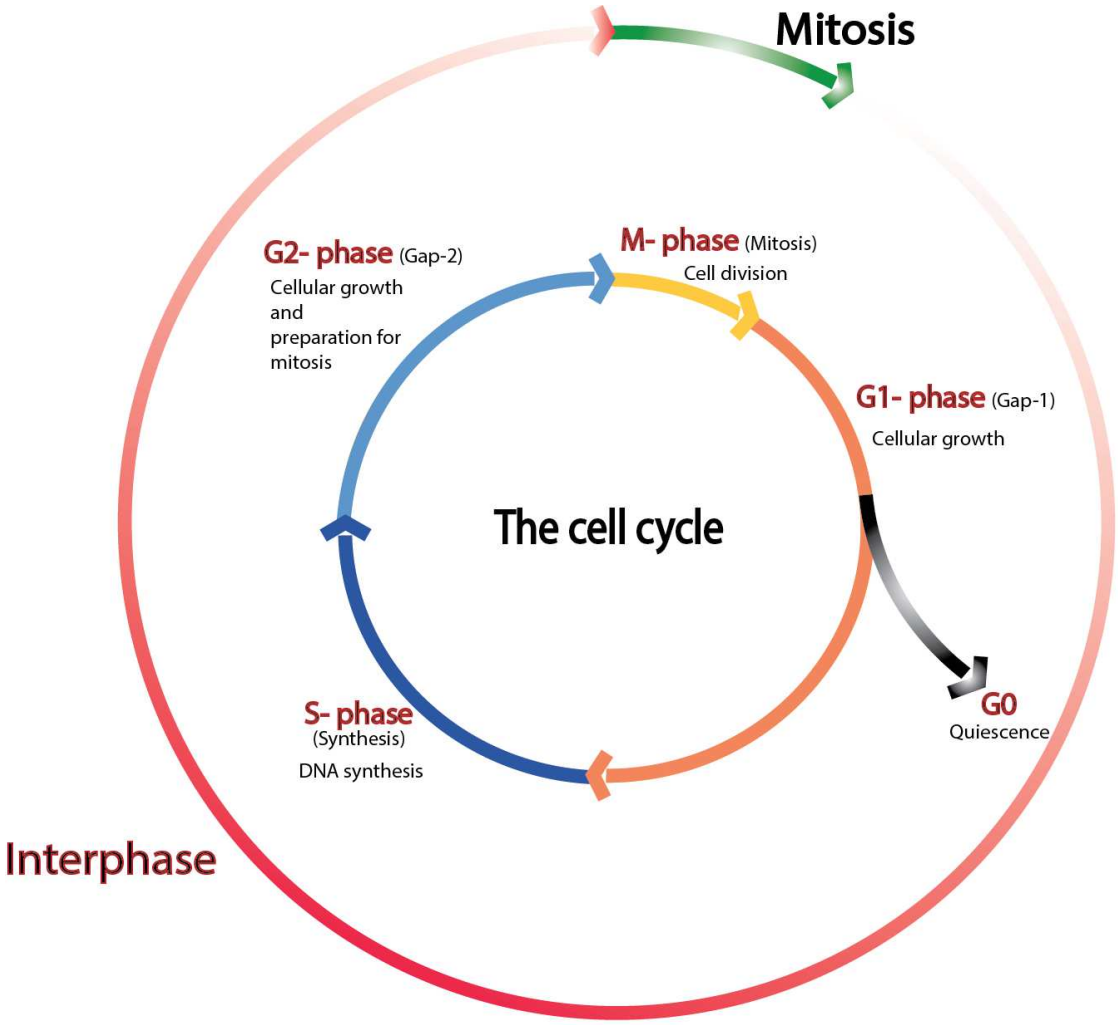


Figure 1. The cell cycle

Recapitulation of the different stages of the cell cycle and the main events that characterize each stage.

1.2 DNA replication

The genetic material copying process, known as DNA replication, takes place during only one phase of the cell cycle, the S-phase. DNA replication is one of the most fundamental processes a cell undergoes, as its life depends on its ability to accurately replicate the entirety of its DNA, and transmit error free genetic information to the next generation. During this process, the DNA double helix is unwound, and each strand serves as a template for replicating its counterpart. As a result, the new DNA molecule will be composed of the mother

strand as well as the newly synthesized strand. A process known as semi-conservative DNA replication (See Figure 2).

Briefly, DNA replication is completed in 3 stages, first the ‘’initiation’’ step launches this process by the binding of protein complexes onto DNA initiation sites and their activation, which will allow the unwinding of the DNA double helix and the initiation DNA synthesis at these sites. Next, the ‘’elongation’’ step allows the entire DNA within a cell to be replicated, before the disassembly of the replication forks during the final step of DNA replication known as ‘’termination’’(Pray 2008).

Interestingly, in eukaryotes, while the process of DNA synthesis takes place in the S phase of the cell cycle, the initiation of this process, named origin licensing, is restricted to G1. The uncoupling of these two processes prevents replication of already replicated DNA.

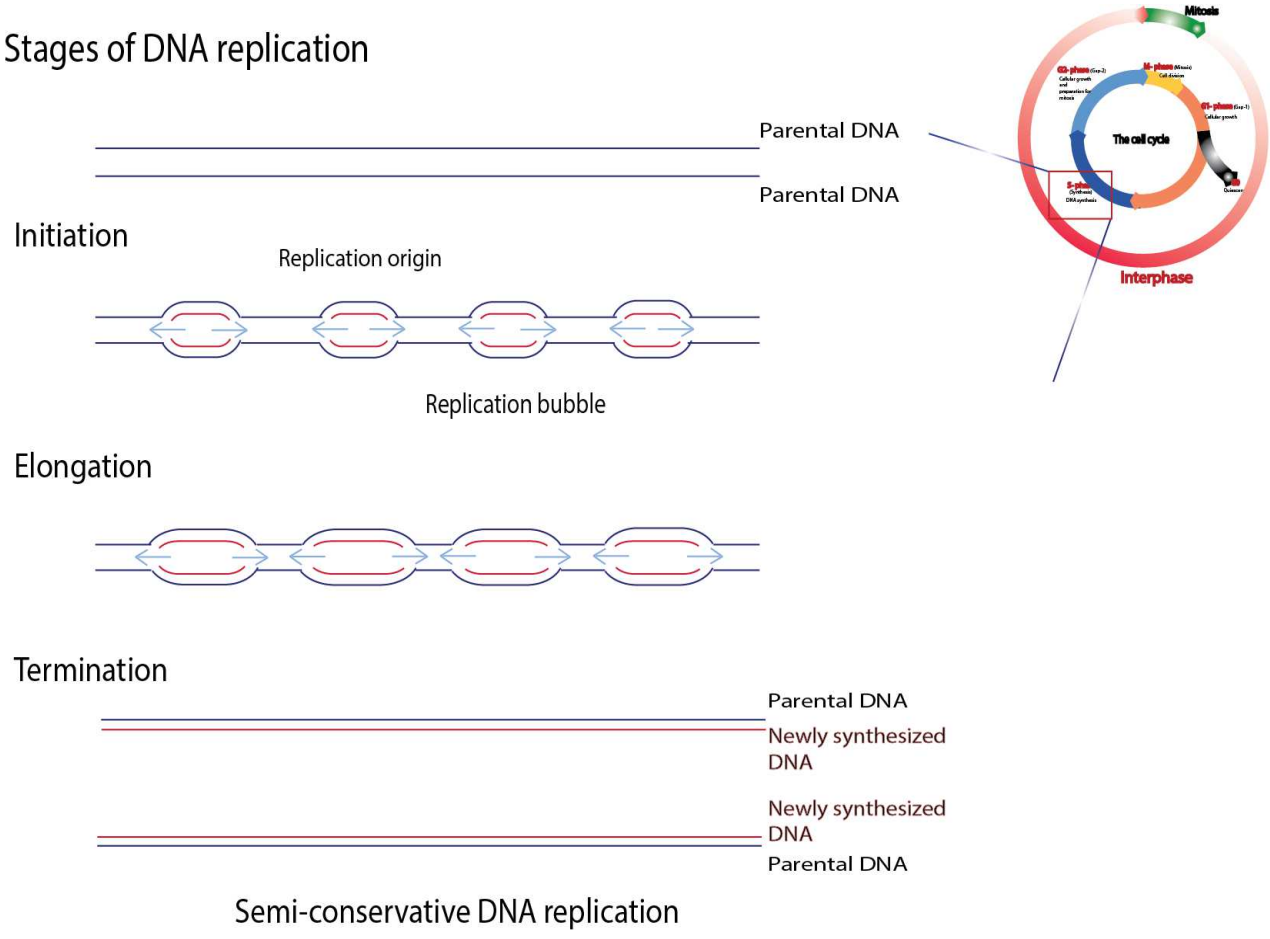


Figure 2. The stages of DNA replication.

Schematic recapitulation of the different steps of DNA replication, from initiation till the completion of the synthesis of the new DNA stands.

Initiation of DNA replication

In 1963, Jacob, Brenner and Cuzin proposed the "Replicon model" to explain DNA replication initiation in prokaryotes. This model suggests the existence of (at least) two entities: the replicator and the initiator. The replicator is any cis-acting DNA sequence sufficient to direct DNA replication initiation, while the initiator is a replication factor binding to the replicator (Jacob, Brenner et al. 1963). This model was later shown to apply to both prokaryotes and eukaryotes. The replicator is known as the "Origin of replication" in both models, while the initiator varies from DnaA in prokaryotes to the Origin Recognition Complex (ORC) in eukaryotes (Bell and Stillman 1992).

Interestingly, in Bacteria, DNA replication was shown to start at a single origin in the unique circular chromosome. While in eukaryotes, as the genome grew larger there was a requirement for multiple origins of replication to achieve the full duplication of the genome.

Replication initiation: a two-step mechanism

To ensure the replication of their large genomes before each cell division, eukaryotic cells start their replication at multiple origins, with mammalian cells utilizing tens of thousands of origins that are spread throughout the genome (Huberman and Riggs 1968). However, this increase in the number of origins also raised numerous regulatory issues that were not apparent in bacterial systems with a single origin per chromosome. Consequently, eukaryotic cells have also developed regulatory mechanisms to ensure the fidelity of this process and its coordination with others DNA related processes such as transcription.

In order for eukaryotic cells to control the activation of thousands of potential initiation sites, none of which should fire more than once, origin activation was divided into two distinct steps separated in time. During the G1 phase of each cell cycle, as cells emerge from mitosis, the ORC complex, a heteromeric six-subunit complex with ATPase activity (ORC1-6), binds the origins of replication (Bell and Stillman 1992, Bell 1995). This is followed by the licensing factors CDC6 (Diffley and Cocker 1992) and CDT1 (Maiorano, Moreau et al. 2000) (Cell Division Cycle 6 and Cdc10-Dependent Transcript 1, respectively), which allow the

loading onto the chromatin of the MCM2-7 (Mini Chromosome Maintenance 2-7) helicase in its inactive state (Evrin, Clarke et al. 2009). This reaction results in the formation of the Pre-Replicative Complex (or Pre-RC), which marks the end of the first step of replication initiation known as origin ''licensing''. The second step of origin activation or ''firing'' takes place later, at the G1/S transition and throughout S phase and is driven by the kinases DBF4-dependent kinase (DDK) and Cyclin dependent Kinases (CDKs)(Depamphilis, de Renty et al. 2012). During this step, the Pre-RCs are converted into Pre Initiation Complexes (or Pre-IC) by the phosphorylation dependent recruitment of additional factors to the origins. DDK and CDK phosphorylate several replication factors, such as CDC45 (Cell Division Cycle 45) (Pacek and Walter 2004), MCM10 (Wohlschlegel, Dhar et al. 2002), GINS (Go-ichi-ni-san) (Takayama, Kamimura et al. 2003), Treslin and TOPBP1 (DNA topoisomerase 2-binding protein 1) (Kumagai, Shevchenko et al. 2010) and promote their binding onto origins. Moreover, the chromatin bound but inactive MCM2-7 is also phosphorylated and interacts with components CDC45 and GINS to form the CMG (CDC45, MCM2-7, GINS) complex (Ilves, Petojevic et al. 2010). Following the assembly of the CMG active helicase, DNA is unwound, allowing the recruitment of additional elongation factors such as RFC (Replication Factor C), PCNA (Proliferating Cell Nuclear Antigen), and RPA (Replication Protein A)… In order to create a complete replisome at each replication site. The mentioned proteins, alongside DNA polymerases then initiate DNA replication at the concerned origin.(Masai, Matsumoto et al. 2010, Fragkos, Ganier et al. 2015) (Replication initiation steps recapitulated in Figure 3, below).

These steps recapitulate the main steps and factors involved in DNA replication in eukaryotes. Interestingly, along with the conserved replication factors between eukaryotes, many new factors involved in DNA replication emerged in mammals. An example is ORC-associated protein or ORCA (also known as Leucine-rich Repeat and WD repeat-containing protein 1, LRWD1). ORCA was shown to collaborate with the ORC complex during mammalian replication initiation. This protein seems to facilitate/ stabilize ORC binding to the chromatin with a more specific role in heterochromatic regions, such as centromeres and telomeres (Shen, Sathyan et al. 2010). Other factors, such as Geminin (Wohlschlegel, Dwyer et al. 2000),

are also found to have an important role in mammalian DNA replication (will be further discussed in parag 2.6 “Regulation of DNA replication”)

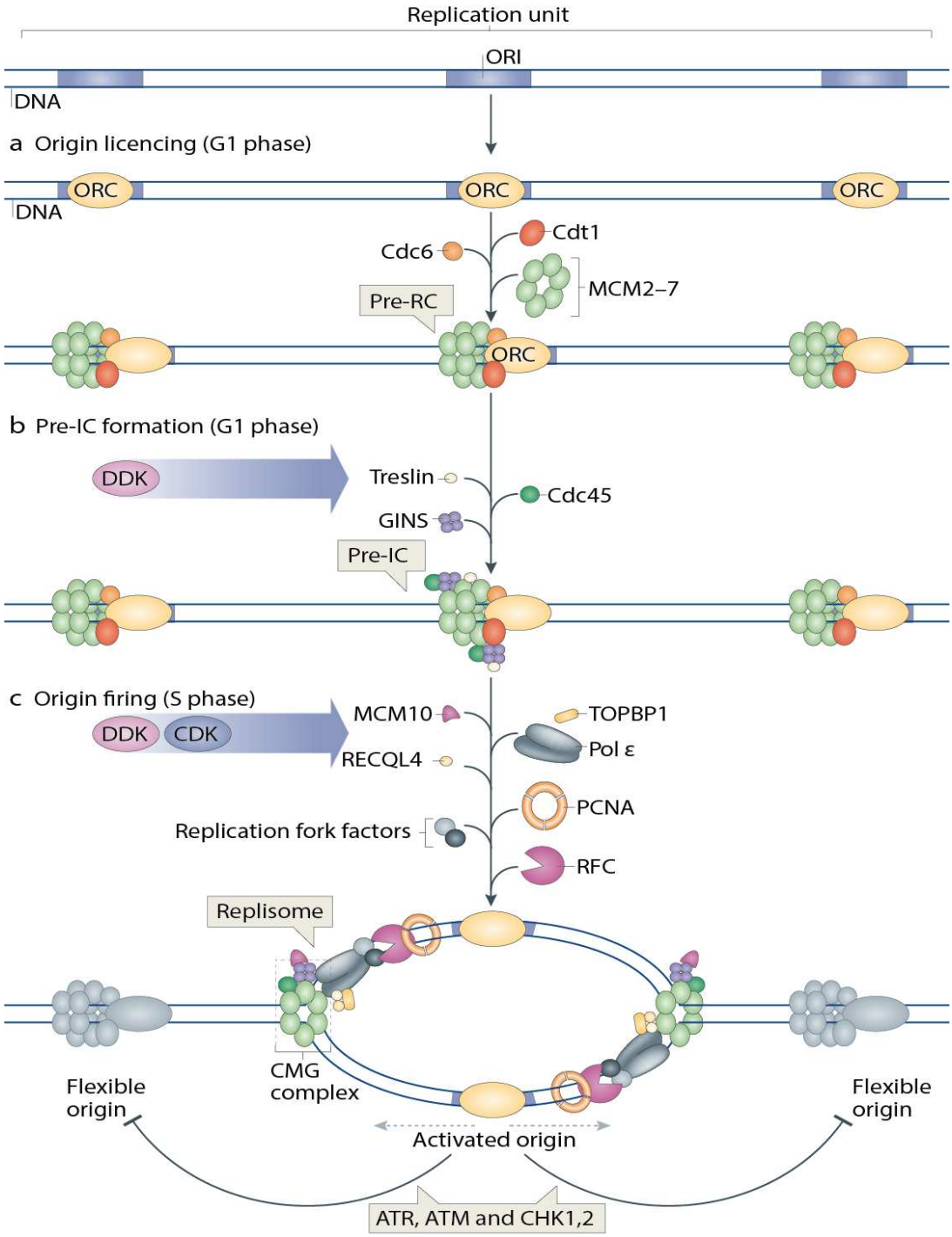


Figure 3: Initiation of DNA replication in mammals (Fragkos, Ganier et al. 2015)

a *Origin licensing taking place during the G1 phase of the cell cycle, marked by the formation of the Pre-RC* ***b, c,*** *Pre-RC activation into Pre-IC during the G1/ S transition, with the elevated levels of DDK and CDKs.* ***c*** *Origin firing in S phase with complete replisome assembly.*

1.3 DNA Replication origins

As previously mentioned, in Bacteria, DNA replication was shown to start at a single origin in a chromosome while in eukaryotes replication initiation starts at multiple sites per chromosome. Interestingly, bacterial origins additionally have a defined consensus sequence for their replication origins, while in eukaryotes, this sequence specificity seemed to be reduced with evolution. Accordingly, budding yeast replication starts at multiple origins that share an AT rich consensus sequence, called Autonomously Replicating Sequences or ARS. (Brewer and Fangman 1987). However, in higher eukaryotes, such as human cells, replication origins did not reveal the strong sequence specificity found in budding yeast and Bacteria.

Indeed, metazoan replication origin sequences exhibit a high heterogeneity and do not share a clear consensus. Until now, the exact sequence(s) or chromatin features that mark these origins of replication remain unclear.

a. Origin determinants

Although the absence of sequence specificity in mammalian replication origins suggests that replication initiation could be stochastic. Studies done on replication origins in the genome using cellular models show that their distribution is not random, but depends on an affinity for particular DNA sequences. However, these sequences could not account for all the origins in the genome. Consequently, other determinants, genetic and epigenetic, could play as well a role in origin distribution and activation, making replication initiation an interplay between cis-acting elements and trans-acting factors that ensure the initiation of DNA replication at specific sites in the genome (Prioleau and MacAlpine 2016).

Cis-elements

Although no sequence specificity was shown, a sequence preference was observed around eukaryotic replication origins. Indeed, many studies showed the presence of origins around G-rich regions. These sequences were called OGRE, for Origin G rich Repeated

Elements (Cayrou, Coulombe et al. 2012) and were potentially thought to form secondary structures known as G quadruplexes or G4s (Cayrou, Coulombe et al. 2012, Cayrou, Ballester et al. 2015). G4s are conserved structures formed of four stranded nucleic acid structure -with bonds between guanines- and were shown to associate with highly efficient origins in metazoans. Moreover, it was shown that this element was predominantly found at a relatively precise distance, in a nucleosome free region, upstream of the initiation sites of DNA replication. G4s were subsequently observed at replication origins (Besnard, Babled et al. 2012, Valton, Hassan-Zadeh et al. 2014, Foulk, Urban et al. 2015). Analyses at single loci showed that such G4s can create a replication origin when placed in an ectopic region and that their deletion strongly inhibits the corresponding replication origin activity (Valton, Hassan-Zadeh et al. 2014, Prorok, Artufel et al. 2019). Other features that exhibit strand asymmetry, such as GC rich regions, were also shown to play a role in origin distribution.

Trans-factors

The chromatin plays an important role in origin distribution (Smith and Aladjem 2014). Indeed, many studies have correlated replication origins and open chromatin domains. Accordingly, open chromatin marks were detected at replication origins and were shown to correlate with replication origin locations. The major mechanism of chromatin influence is through nucleosome positioning. By compacting chromatin, nucleosomes prevent DNA related processes and they must be disassembled or displaced in order to allow the replication machinery to access DNA.

Histone AcetylTransferases (HATs) have been implicated in nucleosome disassembly and catalyze distinct histone modifications that can modulate nucleosomes and therefore influence initiation protein access and origin positioning. An example of HATs implicated in origin positioning is HBO1 (Histone acetyltransferase Binding ORC1). HBO1 was found to bind near replication origins and to associate with licensing factors ORC1 and CDT1, which was shown to be required for MCM assembly (Iizuka and Stillman 1999, Iizuka, Matsui et al. 2006, Miotto and Struhl 2010) this HAT catalyzes H4K5 and H4K12 modifications promoting chromatin de-condensation. In addition, HBO1 overexpression was shown to induce CDT1-

dependent re-replication (Miotto and Struhl 2008) suggesting a role of HBO1 in pre-RC formation and origin licensing.

As mentioned, histone marks could indicate origin location, an example of such marks are H3K4me3, H3K9ac and H3K27ac which were shown to associate with origins and more specifically early replicating origins(Smith, Kim et al. 2016). Indeed chromatin marks are not only correlated with origin distribution, but they also play an important role in replication timing. Early replicating origins tend to associate with open chromatin marks. In contrast, late replicating regions tend to associate with heterochromatin marks such as histone hypo-acetylation, and histone methylation marks such as H3K9 and H3K27 (Cayrou, Ballester et al. 2015) (Smith, Kim et al. 2016). Accordingly, HBO1, which promotes chromatin de-condensation, associates with early replicating origins, while Histone methyltransferase PR-Set7 (PR/SET domain containing protein 7) on the other hand, was shown to associate with heterochromatin along with ORCA and HP1 (Heterochromatin Protein 1) (Giri, Aggarwal et al. 2015) (Chakraborty, Shen et al. 2011)

Interestingly, open chromatin regions are also rich in TSS and in somatic cells replication origins are often associated with TSS (Sequeira-Mendes, Diaz-Uriarte et al. 2009). However, this is not the case in highly transcribed regions, therefore preventing a transcription- replication conflict (Martin, Ryan et al. 2011). In agreement with the role of the chromatin and transcription state in origin distribution and replication timing, studies on Xenopus embryos show that in early development, where the chromatin is relatively de-condensed, and transcription is inhibited, origin preference is not detected and no significant replication timing is observed (Mechali and Kearsey 1984, Hyrien, Maric et al. 1995). However, inducing transcription or tethering of transcription factors resulted in a more localized initiation (Hyrien, Maric et al. 1995).

b. Origin efficiency

Although the events leading to the initiation of replication (Origin licensing) occur at all potential origins, not all potential origins initiate replication in all cells at each cell cycle, nor do they all initiate at the same time (DePamphilis 1993, Smith, Kim et al. 2016). Indeed, in addition to origin distribution, origin activation or firing also shows an important

heterogeneity, with a large fraction of replication origins exhibiting consistent initiation in particular cell types and not in others (Cayrou, Coulombe et al. 2011, Cayrou, Ballester et al. 2015). In most somatic metazoan cells, only 10–20% of all potential origins actually initiate replication each cell cycle, suggesting that most origins exhibit flexible initiation patterns. While some origins remain "dormant" and do not initiate during normal replication, they replicate passively from adjacent replication forks. This difference in origin usage allows their classification roughly into three categories: Constitutive origins, flexible origins and dormant origins. With constitutive origins consistently activated in all cells of a cell population and forming only a small subset of total origins in a cell. Meanwhile, flexible origins constitute the most abundant origins in a cell and their usage varies from one cell to another. Finally, dormant origins are defined as replication origins that are licensed but almost never fired under normal replication conditions, these dormant origins play a role in genome stability, as they might serve as back up origins during replication stress, fork stalling or following DNA damage (Ge, Jackson et al. 2007).

Although current knowledge about replication origins allows their classification, the exact mechanism that accounts for their flexibility and determines which origins are to be activated from all the potential licensed origins remain silenced remains unclear.

Interestingly, HBO1 described above for its role in promoting licensing and pre-RC formation was recently also shown to have a potential role in origin activation. Recent reports show that the HBO1-BRPF3 complex regulates origin activation through H3K14 acetylation, by allowing CDC45 loading onto chromatin (Feng, Vlassis et al. 2016) therefore implicating HBO1 in both origin licensing and firing. Moreover, recent studies suggest that the role of open chromatin is more likely to affect origin activation than origin distribution (Giri and Prasanth 2015, Feng, Vlassis et al. 2016). Accordingly, GCN5 (General Control of amino acid synthesis protein 5, also known as KAT2A or lysine AcetylTransferase 2A) a HAT previously described for its role in promoting transcription by providing an open chromatin around gene promoters, was recently found to be involved in DNA replication and more specifically in origin firing. Indeed a study showed that the ORC5 subunit of the ORC complex, promotes local chromatin de-compaction around the origin, through GCN5 recruitment, providing a favorable environment for efficient origin activation. Probably by facilitating the recruitment of limiting factors involved in origin firing (Giri, Chakraborty et al. 2016).

In conclusion, open chromatin seems to facilitate DNA replication and correlates with replication origins. However whether this directly affects licensing by favoring pre-RC loading or firing by enhancing recruitment of firing components is still debatable. Interestingly, chromatin remodelers such as HBO1 and GCN5 were shown to influence origin distribution/activation through binding to the ORC complex. This suggests an additional role of this complex in origin licensing or firing through chromatin remodeling.

1.4 The ORC complex

The ORC complex is a complex of six subunits (ORC1-6) that binds DNA in an ATP dependent manner. ORC was initially identified in budding yeast as an origin binding complex (Bell and Stillman 1992). It is highly conserved in eukaryotes and homologues of its six subunits were identified in all eukaryotes, from yeast to Humans (Bell 2002).

a. Role of ORC in DNA replication

ORC plays a major role in DNA replication initiation, as it launches the first step of this process and recruits the licensing factors CDC6 and CDT1, which allow pre-RC formation. ORC is conserved throughout evolution allowing it to maintain its role. Despite conservation, some elements of its structure evolved differently along with its regulation and behavior on chromatin.

Indeed, in budding and fission yeast, ORC remains in a complex and bound to the chromatin throughout the entire cell cycle; however the complex is regulated by cell cycle dependent phosphorylation, from G1/ S transition until M phase in order to prevent re-replication (Makise, Takehara et al. 2009, Chen and Bell 2011). Similarly to yeast, in Xenopus, ORC was found in a stable complex as well. Studies using interphase Xenopus egg extracts show that the ORC complex remains on the chromatin in S phase, and is later released in Mitosis (Romanowski, Madine et al. 1996, Rowles, Tada et al. 1999). In mammalian cells, the level of ORC bound to chromatin was shown to remain stable with the exception of ORC1 that

is selectively degraded in S phase (Natale, Li et al. 2000, Li and DePamphilis 2002, Tatsumi, Ohta et al. 2003).

Another feature of the ORC complex that evolved differently is its binding specificity for DNA. While in S. cerevisiae ORC was shown to recognize a specific DNA sequence, this specificity has been reduced during evolution, and to date, no specific sequence recognition has been clearly demonstrated for ORC in mammalian cells. In Xenopus early development ORC also shows reduced affinity to chromatin (Mechali and Kearsey 1984), and origins of replication were shown to be equally spaced, suggesting that ORC binding does not show any sequence preference. However, contrary somatic cells, Xenopus sperm chromatin lacks histone H1, suggesting that the random binding of ORC could be facilitated by the chromatin's reduced condensation state. A similar observation was recently made in H1-depleted mouse ES cells, where the level of initiation of replication is largely increased (Almeida, Fernandez-Justel et al. 2018).

This heterogeneity in ORC behavior in different models presented important variations in studying origin selectivity in eukaryotes. Indeed, certain findings show that two subunits of the ORC complex, ORC1 and ORC2, where dispensable for DNA replication (Shibata, Kiran et al. 2016). While other studies have showed that ORC's strength in binding the DNA had an important role in origin licensing (Gardner, Gillespie et al. 2017).

None the less, the ORC complex's role in DNA replication, although variable, remains essential, this variability leaves the door open for further characterization of this complex, its role in licensing and more recently in firing and its regulation.

b. ORC's role in other cellular processes

The ORC complex is best known for its role in DNA replication. However, ORC also plays additional roles independently of DNA replication, such as heterochromatin formation.

In mammalian cells, the ORC complex binds an additional protein termed ORCA that facilitates pre-RC assembly and stabilizes ORC on the chromatin (Shen, Sathyan et al. 2010). ORCA additionally interacts with Histone MethylTransferases (HMTs) and repressive chromatin marks such as H3K9me3, H3K27me3, and H4K20me3 suggesting that ORCA could

likely act as a complex scaffold at heterochromatic sites. (Bartke, Vermeulen et al. 2010, Giri, Aggarwal et al. 2015, Wang, Khan et al. 2017).

Subunit 2 of the ORC complex was also shown to have a role in heterochromatin, mainly in the maintenance of centromeric methylation, where it was also shown to be SUMOylated (Prasanth, Prasanth et al. 2004, Huang, Cheng et al. 2016, Wang, Liu et al. 2017) (Craig, Earle et al. 2003). In yeast and drosophila, The ORC complex was also related to chromosomal organization, where mutations in ORC led to mitotic defects (Suter, Tong et al. 2004). In accordance, studies in Xenopus Laevis described a role of the ORC complex in sister chromatid cohesion through the involvement of the pre-RC in cohesin recruitment to the chromatin(Bermudez, Farina et al. 2012). However, although already described in other systems, there is no strong relationship observed between chromatid cohesion and the ORC complex in mammalian cells.

In addition to ORC's participation in nuclear processes, ORC has also a number of functions in the cytoplasm. One of which is the regulation of centrosome duplication. Indeed, ORC subunits, ORC1 in particular, were shown to co-localize at the centrosomes and to be directly involved in centrosomal division (Hemerly, Prasanth et al. 2009, Hossain and Stillman 2012). Furthermore, ORC2 depletion also leads to a defect in cell division and centrosome division (Prasanth, Prasanth et al. 2004). On another note, ORC6 was also shown to be present in the cytoplasm and in the cell membrane (Chesnokov, Chesnokova et al. 2003). Accordingly, in drosophila and mammals ORC6 was shown to have a role in cytokinesis (Prasanth, Prasanth et al. 2002).

Amongst all the non-replicating functions of the ORC complex, one of the most unexpected is its role in neurogenesis. ORC3 is expressed in the adult central nervous system and it localizes in the neuro-muscular junctions (NMJ) in drosophila (Rohrbough, Pinto et al. 1999). In mammals, the ORC2–5 subunits are highly expressed in the adult brain (Huang, Zang et al. 2005). In addition, neural cells grown in culture demonstrate a high ORC3 expression, ORC3 was also detected the cytoplasm of Purkinje cell during prenatal development (Cappuccio, Colapicchioni et al. 2010). In mammals, knockout of either ORC3 or ORC5 leads to severe impairment of dendrite growth and branching in cultured hippocampal neurons (Huang, Zang et al. 2005). It is therefore apparent that in higher eukaryotes, subunits of the

ORC complex are also involved in neurogenesis, regulation of dendritogenesis and synapse formation in the post-mitotic neurons (Stiff, Alagoz et al. 2013).

The role of the ORC complex in DNA replication, and other nuclear and cytoplasmic processes allows this complex to have a major role in the cell. Consequently, a defect in ORC could act on different levels causing important damage and leading to diseases.

1.5 DNA Replication and Disease

a. Cancer

As described above, DNA replication is a tightly regulated process which is crucial in order to preserve genetic stability. Although the DNA replication error rate is minimal, taking in account the amount of DNA replicated during an organism's lifetime and that not all mutations are harmful (polymorphism, evolution…), the replication machinery is not always error free and some mutations are harmful. An error could occur during DNA replication due to the incorporation of a wrong nucleotide, if this error remains after Miss Match Repair (or MMR), it becomes a permanent mutation after the next cell cycle. Indeed, the erroneous DNA sequence can no longer be recognized as a mistake and will later serve as a template for future replication events leading to genetic instability and mutation accumulation, a hallmark of cancer. The most common cause of cancerous mutations are the ones that lead to proto-oncogenes or defective tumor suppressors. These mutations lead to aberrant cellular proliferation eventually leading to neoplastic growth. In these cases, replication factors such as PCNA, MCMs and Geminin, were shown to provide good biomarkers for cancer detection and prognosis (Tachibana, Gonzalez et al. 2005).

Interestingly, a defective replication machinery is not the only way replication proteins are involved in cancer. Indeed, it has been recently proposed that oncogenes themselves might induce firing of origins inside genes, generating replication-transcription conflicts and replication stress, leading to genomic instability and cancer development (Macheret and Halazonetis 2015).

b. Other diseases

Mutations in genes coding for DNA replication factors were shown to cause developmental disorders in diseases other than cancer. Examples include mutations in RecQ helicases and in major components of the replication initiation machinery.

RecQ helicases

RecQ helicases diseases are autosomal recessive mutations in genes that encode three of the five RecQ helicases. Mutations in RecQ helicases are responsible for Bloom syndrome (BS, mutation in BLM helicase) (Bloom 1954, German 1997). Werner syndrome (WS, mutation in WRN helicase, and Rothmund-Thomson syndrome (RTS, mutation in RecQL4) RecQ diseases are associated with reduced growth, premature aging and cancer predisposition (Monnat 2010, Croteau, Popuri et al. 2014)

Knowing that RecQ helicases play a major role in DNA replication and repair, defect in these fundamental processes could be responsible for the symptoms observed. However, interestingly, recent studies raised the hypothesis that G4 structures are physiologic substrates for WRN helicase, WRN could use these structures to modulate gene expression in human cells, which could cause also be responsible for the disease's symptoms (Tang et al., 2016). G4 structures are also the substrates for BLM helicase (Nguyen, Tang et al. 2014).

Initiation factors

Meier-Gorlin syndrome (MGS) (Gorlin, Cervenka et al. 1975, de Munnik, Hoefsloot et al. 2015) is a rare inherited condition caused by genetic mutations in any of eight different genes, all involved in DNA replication. Orc1, Orc4, Orc6, Cdt1, Cdc6, Cdc45L, Mcm5 and Geminin *(Meier- Gorlin Syndrome 1 Online Mendelian Inheritance in Man OMIM*). These mutations lead to eight different types of MGS, MGS type 1 for Orc1 mutation, type 2 for Orc4, type 3 for Orc6, type 4 for Cdt1, type 5 for Cdc6, type 6 for Geminin, type 7 for Cdc45

and type 8 for Mcm5. MGS is inherited in an autosomal recessive pattern, except for type 6 (Geminin mutation) which is inherited in an autosomal dominant pattern (de Munnik, Hoefsloot et al. 2015).

MGS is considered a form of primordial dwarfism, characterized by growth retardation, microcephaly, aplasia or hyplasia of the patellae (absent or very small kneecaps), and skeletal abnormalities, with feeding and breathing problems in early infancy, and respiratory problems later in life (Fryns 1998, Shalev and Hall 2003). Interestingly, since some of the ORC subunits were shown to localize outside the nucleus and therefore to be implicated in cellular processes distinct from DNA replication, such as synapse formation, these processes could also be responsible for the disease's phenotype.

Accordingly, ORC's abnormal cellular distribution was also linked to Alzheimer's disease (AD) through regulation of neuronal plasticity and cognitive functions (Arendt and Bruckner 2007).

In conclusion, DNA replication is a fundamental process that is highly complex. It involves many steps and several factors involved in each of these steps. As described here, a defect in the replication machinery could be result in many diseases. The importance and complexity of this system requires a high level of regulation. Accordingly, this process is regulated in several and sometimes overlapping mechanisms. These regulations include many post translational modifications such as phosphorylation, SUMOylation and ubiquitylation.

Part 2 Ubiquitylation

2.1 Ubiquitin

Ubiquitin is a highly conserved protein, formed of a compact β-grasp fold and a flexible six residue C-terminal tail. Ubiquitin is so highly conserved that it is almost invariant from yeast to humans. This evolutionary conservation of the structure of this protein shows the importance of its surface for its recognition by ubiquitin binding domains (or UBDs).

Ubiquitin is often recognized through its hydrophobic surface that consists of four different patches (Dikic, Wakatsuki et al. 2009). These patches allow ubiquitin to be bound by the proteasome, UBDs, (Sloper-Mould, Jemc et al. 2001, Dikic, Wakatsuki et al. 2009) deubiquitylating enzymes (or DUBs) (Hu, Li et al. 2002) and HECT (Homologous to the E6AR carboxyl Terminus) E3 ubiquitin ligases (see paragraph 2.4, "E3 ubiquitin ligases") (Kamadurai, Souphron et al. 2009). Importantly, the divergence between the four patches of ubiquitin and its closest homologue Nedd8 enables the DUBs to distinguish between these two modifiers (Ye, Akutsu et al. 2011). These patches alongside additional surfaces might also fulfill functions that are not yet identified. However, the most important features of ubiquitin are its N terminus and its seven lysines (K6, 11, 27, 29, 33, 48 and 63), which are the attachment sites for chain assembly. These lysine residues cover all surfaces of ubiquitin and point into distinct directions allowing different ubiquitin chain configurations.

2.2 Ubiquitin conjugation (ubiquitylation)

Ubiquitylation is catalyzed by the sequential action of three enzymes: The ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligase enzymes (E3s) (see Figure 4, below). The human genome encodes two E1s, fewer than 60 E2s and more than 600 different E3s, in which the E3s determine the specificity for the different substrates (Deshaies and Joazeiro 2009, Michelle, Vourc'h et al. 2009).

Briefly, the ubiquitylation reaction goes as follows. First, the E1 enzyme activates the ubiquitin and transfers it to the E2 conjugating enzyme. With the help of the E3, the E2- E3

complex catalyzes the formation of an isopeptide bond between the C terminal glycine residue of ubiquitin and usually a substrate's lysine, leading to the attachment of a single ubiquitin on the substrate, a process known as monoubiquitylation. It is also possible that multiple lysine residues on a single substrate become modified with one ubiquitin each, leading to what is called multi-monoubiquitylation (see Figure 5).

Further modification of these substrate-attached ubiquitin or "ubiquitin chain elongation" leads to formation of polymeric chains a process known as polyubiquitylation.

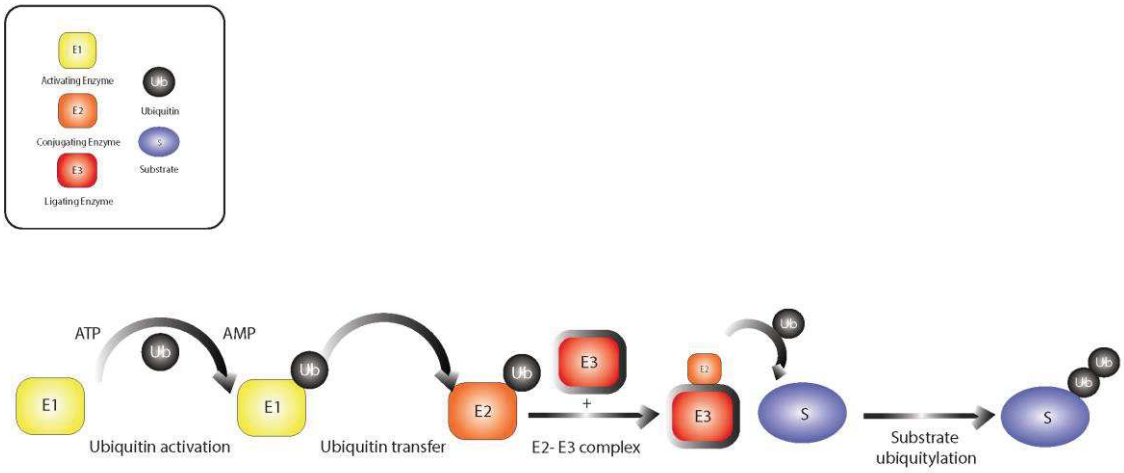


Figure 4. The ubiquitylation reaction

Figure recapitulating the three steps of the ubiquitylation reaction. From ubiquitin activation to conjugation and ligation, using the three respective enzymes E1, E2, and E3s.

2.3 Types of ubiquitylation

a. Monoubiquitylation

Monoubiquitylation is the attachment of a single ubiquitin on a substrate. Monoubiquitylation can occur at a defined residue, such as the conserved lysine, which is the

case of Lys164 in PCNA, (Nikolaishvili-Feinberg, Jenkins et al. 2008) or it might be confined to a domain, as is the case of the transcription factor p53 (Carter, Bischof et al. 2007).

The enzymes catalyzing monoubiquitylation have to recognize substrate lysine residues, while sparing those of ubiquitin from modification. This specificity can be determined by the E2 or the E3. In order for the E3 to determine monoubiquitylation it has to block the ability of E2s to catalyze chain formation. An example of E3 determining monoubiquitylation is RAD18. RAD18 binds the E2 RAD6, which can synthesize mixed ubiquitin chains or Lys48-linked chains, when interacting with other E3s (Hibbert, Huang et al. 2011). However, when interacting with RAD18 it promotes monoubiquitylation of PCNA (Hoege, Pfander et al. 2002). Since RAD6 depends on a noncovalent ubiquitin-binding site for chain formation (Similarly to other E2s) RAD18 occupies this site, thereby blocking chain formation without interfering with monoubiquitylation (Hibbert, Huang et al. 2011).

In other cases, the E2 determines monoubiquitylation, yet the molecular basis for this specificity are poorly understood.

b. Polyubiquitylation

Polyubiquitylation is the formation of polymeric chains on a substrate. These chains can be short and contain only two ubiquitin molecules or can incorporate more than ten molecules. Interestingly, ubiquitin chains are very diverse, they can be linear (Met 1) or homogenous, if the same lysine residue is modified during elongation (See figure 5). Polyubiquitin chains could also be heterogeneous or have mixed topology if different linkages alternate at succeeding ubiquitins in the chain, as seen per example in NF-κB signaling (Nuclear Factor Kappa B) or protein trafficking (Boname, Thomas et al. 2010, Goto, Yamanaka et al. 2010, Gerlach, Cordier et al. 2011). Branched ubiquitin chains can also be generated if a single ubiquitin is modified at different lysines. However these branched chains are still of unknown functions.

Compared to monoubiquitylation, the enzymes that catalyze polyubiquitylation chains face a different specificity issue as they need to modify a specific lysine residue on ubiquitin. Per example, RING (Really Interesting New Gene) E3 ligases and their E2s initiate

chain formation on a substrate lysine, which can occur at random positions or in preferred sequences, a process referred to as chain initiation motifs, then, the initiating E2s cooperate with a specific chain-elongating E2, which allows for assembly polyubiquitin chains.

Unlike monoubiquitylation, which could be determined by the E3, studies done on polyubiquitylation show that the linkage specificity is likely to be determined by the E2 (Ye and Rape 2009), since these E3 ligases can synthetize different chain types depending on the E2 they interact with. An example of this change in chain catalysis is BRCA1-BARD1 (Breast Cancer 1- BRCA1 associated RING domain 1). BRCA1, an E3 ubiquitin ligase involved in DNA damage response and considered as a tumor suppressor, assembles K63 chains when associated with the E2 heterodimer enzyme Ube2N-Uev1A, however, when bound to Ube2K E2 (Ubiquitin- conjugating Enzyme E2 K) it assembles K48 linkages (Christensen, Brzovic et al. 2007). Importantly, RING E3s that interact with a single E2 only, generally display the specificity of this E2. An example of which is the APC/C complex (or the Anaphase Promoting Complex/Cyclosome described later in parag 2.6), which produces k11 linked chains, using the k11 specific E2 Ube2S (Ubiquitin- conjugating Enzyme E2 S) (Williamson, Wickliffe et al. 2009).

All the ubiquitin linkages described above have been detected in cells (Peng, Schwartz et al. 2003, Xu, Duong et al. 2009), many of them have been well established, such as monoubiquitylation, K48 and k63 chains, however some chains such as K6 and k27 remain poorly understood (Komander 2009).

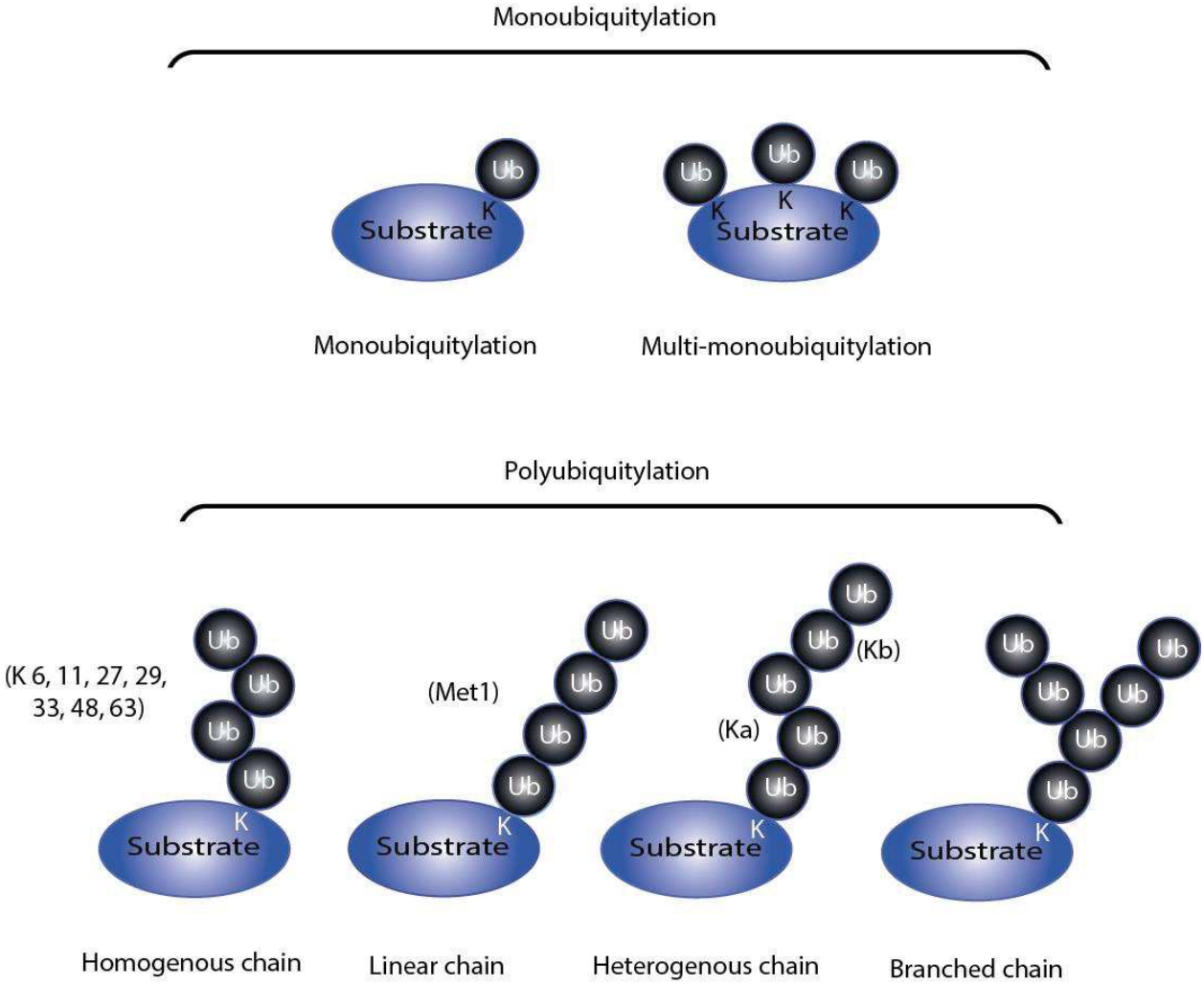


Figure 5: Ubiquitin linkages.

Figure recapitulating the different ubiquitin linkages catalyzed on a substrate's lysine(s) (K) and their different conformation.

2.4 E3 ubiquitin ligases

E3 ubiquitin ligases represent the enzymes required for the final step of the ubiquitylation reaction, where they are responsible for transferring the ubiquitin molecule to the substrate. E3 ubiquitin ligases are grouped into two major classes: the RING E3 ligases and the HECT E3 ligases (See Figure 5). Depending on its class, the E3 ubiquitin ligase can either bind both the E2 enzyme and the target protein (characteristic of RING E3s) after which the E2 transfers the ubiquitin to the substrate, or, the E3 ligase itself can have a dual role in substrate recognition and ubiquitin conjugation (characteristic of HECT E3s).

RING E3 ligases constitute the largest family of ubiquitin ligases, they are characterized either by a RING domain which is a type of Zinc binding domain with eight cysteines and histidines that coordinate two zinc ions (Borden and Freemont 1996, Deshaies and Joazeiro 2009) or by a U-box catalytic domain which is similar to the RING domain and with similar function, however it has a hydrophobic core instead of the Zinc ions. RING E3s catalytic domain can be found in a single protein, as is the case of RNF 219 (Described in project 1) or in a separate component of the E3 complex, such as CRLs (Cullin-RING ligases) and the APC/C complexes (Zimmerman, Schulman et al. 2010, Chang and Barford 2014). The RING domain allows RING E3s to promote the transfer of ubiquitin directly from the E2 to the substrate.

Conversely to RING E3s, which catalyze a direct reaction of the substrate lysine on the E2~ Ub, HECT E3 ligases catalyze two reactions: in the first one, the ubiquitin is transferred from the E2's cysteine to a cysteine in the HECT domain of the E3 and this is followed by a second reaction that allows HECT~Ub to bind a substrate lysine (See figure6) (Rotin and Kumar 2009).

A third smaller class of ubiquitin ligases, distinct from the two major classes mentioned above, was also defined (Morett and Bork 1999). This class represents a unique family of RING and HECT hybrids and are called RBR (or RING-between-RING E3 ligases). RBR ligases are characterized by two RING fingers and a central-in between RINGs or IBR- Zinc domain and include the Parkin E3 ubiquitin ligase that was shown to be involved in Parkinson's disease (Marin, Lucas et al. 2004).

The human genome encodes approximately 600 RING E3s (Li, Bengtson et al. 2008), 30 HECT E3s (Rotin and Kumar 2009)and 10 RBR type E3s (Li, Bengtson et al. 2008).

The function of E3 ligases is not restricted to their catalytic activity since E3 ligases also provide substrate specificity. The involvement of ubiquitylation in diverse cellular pathways provides thousands of ubiquitylated substrates where it is the E3's role to distinguish them. E3 ligases can recognize their substrate proteins via a short consensus sequence termed degrons (Ravid and Hochstrasser 2008). In most of the proteins, Degron peptides adopt an extended conformation to maximize their interactions with the ligase surface residues, therefore, post translational modification of these degrons also play an

important role in regulating the E3/ Substrate interaction (Hao, Zheng et al. 2005, Hao, Oehlmann et al. 2007). Interestingly, many E3 ligases, such as TRIMs (Tripartite Motif containing proteins) have evolved to form homo/ hetero-dimers improving the substrate specificity by recognizing multiple degrons in a single substrate (Zhuang, Calabrese et al. 2009, Li, Wu et al. 2014). Multiple degron binding could also influence the efficiency of ubiquitin binding by modulating the conformation-topology of the E3 bound substrate (Tang, Orlicky et al. 2007). It is important to mention that E3's specificity is not only narrowed to degrons as E3s can recognize other features of a protein that could be perceived as substrate determinants, such as the N terminus of a substrate (Choi, Jeong et al. 2010, Matta-Camacho, Kozlov et al. 2010).

There is still a lot to be learned about E3 ligases specificity. Since these proteins dictate the specificity of the whole ubiquitin system, they could be targeted by pharmacological agents in the aim of therapeutic approaches. Therefore, a therapy consisting on deregulating an E3 ligase in order to alter their substrate concentration such as IAPs (inhibitors of apoptosis proteins), SCF ligase (SKP1 Cullin F-box ligase), and the APC, would be expected to have less side effects than targeting other less specific factors of the ubiquitin pathway (Aghajan, Jonai et al. 2010, Orlicky, Tang et al. 2010, Wu, Grigoryan et al. 2012, Chan, Morrow et al. 2013).

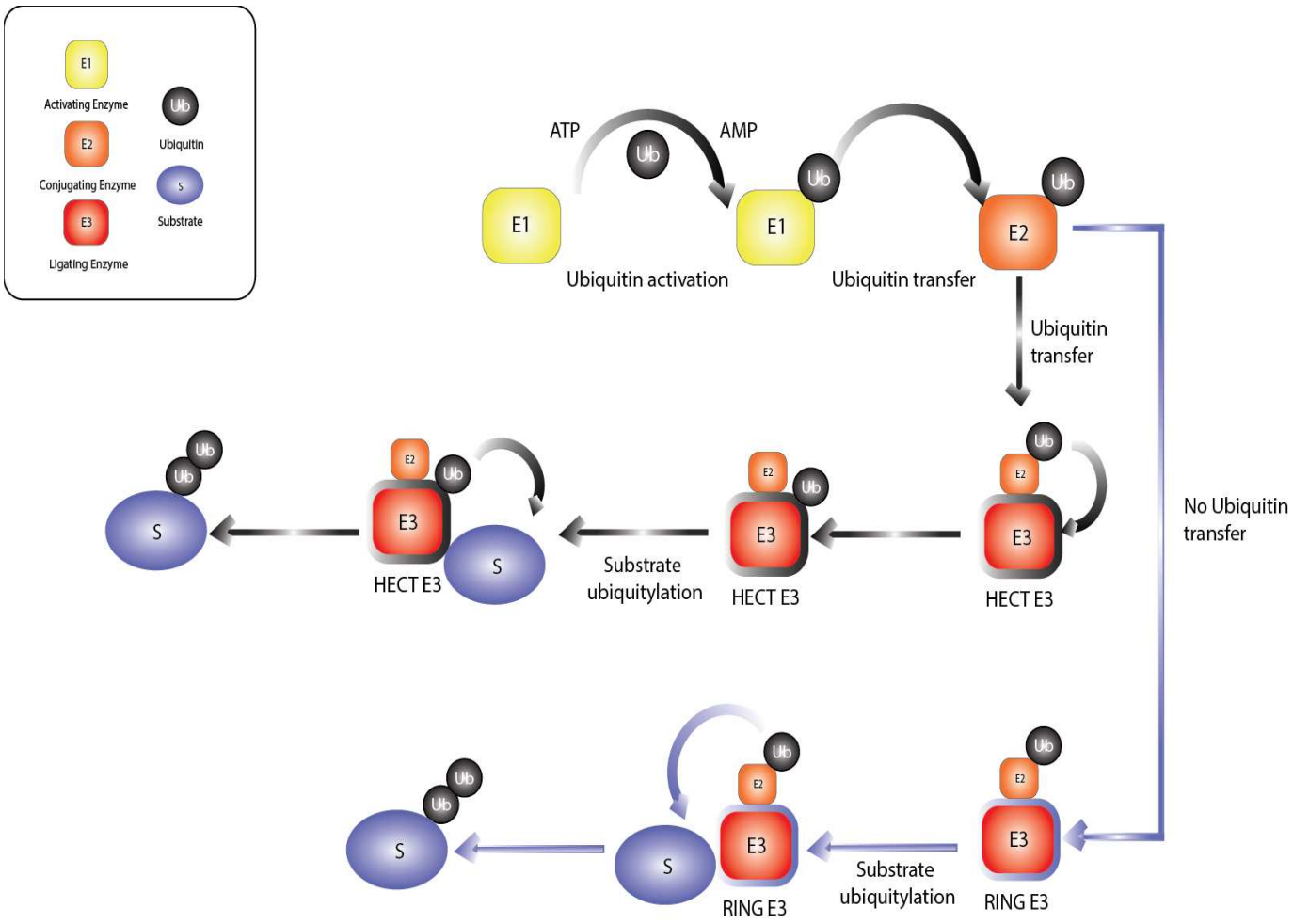


Figure 6: The ubiquitylation reaction: RING E3s Vs HECT E3s

Figure recapitulating the three steps of the ubiquitylation reaction. From ubiquitin activation to conjugation and ligation, using the three respective enzymes E1, E2, and E3s. The figure highlights the two major types of E3 ubiquitin ligases and their operating mechanism, with HECT E3s first transferring ubiquitin from the E2 and later ubiquitylating their substrate. While RING E3s catalyze a direct reaction on the substrate, using the E2 bound ubiquitin.

2.5 Cellular functions of ubiquitylation

The ubiquitylated substrate could be destined for different outcomes, according to which ubiquitin lysine is being targeted, therefore, which type of chains is being catalyzed. Ubiquitylation is best known as a signal for proteosomal degradation, however, ubiquitylation is also able to regulate proteins non-proteolytically. Indeed, ubiquitylation is able to regulate signaling, it could be used to recruit proteins, to attract trafficking factors that can consequently affect a protein's localization. Ubiquitylation could also control a substrate

activity. These non-proteolytic functions are often induced by monoubiquitylation, Met1 or K63 linked chains. (See figure 7)

a. Regulation of protein stability (Proteasomal degradation)

One of the main roles of ubiquitylation is protein degradation, it is well established that ubiquitylation can target proteins to the 26S proteasome (Finley 2009). The role of ubiquitylation in proteasomal targeting was first assigned to K48 chains (Chau, Tobias et al. 1989). Many E3 ligases, including SCF (More details in parag.2.6) trigger substrate turn over by catalyzing K48 linked chains (Li, Tu et al. 2007). As a result, K48 linkages are the most abundant ubiquitin chains and their levels increase rapidly when the proteasome is inhibited (Peng, Schwartz et al. 2003, Kaiser, Riley et al. 2011, Kim, Bennett et al. 2011). Interestingly, consistent with that fact, K48 is the only essential lysine of ubiquitin in yeast (Xu, Duong et al. 2009). Later on, other linkages were shown to be also recognized by the proteasome, and these linkages also accumulate upon proteasome inhibition (Xu, Duong et al. 2009, Matsumoto, Wickliffe et al. 2010), such as K11 chains, suggesting that they also contribute to proteasomal degradation (Xu, Duong et al. 2009, Matsumoto, Wickliffe et al. 2010). An example of an E3 catalyzing K11 linkages in human cells is the APC/C complex (More details in parag. 2.6) inhibition of K11 chain formation stabilizes APC/C substrates and leads to cell cycle arrest (Jin, Williamson et al. 2008, Matsumoto, Wickliffe et al. 2010). APC/C, often interacts with the proteasome to efficiently couple ubiquitylation and degradation (Verma, Chen et al. 2000). Less frequently K29 and K63 chains were also shown to contribute to substrate degradation (Johnson, Ma et al. 1995, Kirkpatrick, Hathaway et al. 2006, Saeki, Kudo et al. 2009).

b. Regulation of Protein-protein interactions

The attachment of a single ubiquitin molecule on the substrate, also known as monoubiquitylation is often sufficient to recruit binding partners. An example of which is the elongation factor PCNA, in response to DNA damage, PCNA is shown to be monoubiquitylated (Hoege, Pfander et al. 2002), a modification that recruits Y family of DNA polymerases

(Bienko, Green et al. 2005, Freudenthal, Gakhar et al. 2010). These polymerases recognize PCNA through a PCNA- interaction motif (PIP-Box) and recognize ubiquitin through Ubiquitin binding domains, leading to a high affinity interaction that replaces replicative polymerases binding to PCNA. In this manner, monoubiquitylation of PCNA contribute to a ubiquitin-dependent polymerase switch, crucial in the regulation of DNA synthesis (Bienko, Green et al. 2010).

Interestingly, monoubiquitylation can also impair interactions, indeed, monoubiquitylation of SMAD4 (Mothers Against Decapentaplegic Homolog 4), a protein involved in signal transduction, blocks its association with its cofactor SMAD2 (Mothers Against Decapentaplegic Homolog 2). Later, deubiquitylation of SMAD4 relieves this inhibition and triggers transcriptional activation by re-allowing cofactor binding (Dupont, Mamidi et al. 2009).

c. Regulation of protein activity

Ubiquitylation can affect a protein's activity by different means. The easiest straight forward way, its inhibitor or activator is sent for degradation. However, in a non-proteolytic manner, some modification such K11, K63 or Met1 linked chains were shown to cause conformational changes in substrate proteins (Tokunaga, Sakata et al. 2009, Xu, Skaug et al. 2009, Dynek, Goncharov et al. 2010), leading to a change in their activity as a consequence. An interesting example is LUBAC (Linear Ubiquitin Assembly Complex). LUBAC catalyzes Met1-linked chains on NEMO (NF-Kappa-B Essential Modulator) a subunit of the IKK complex (IkB Kinase) involved in cellular response to inflammation. Interestingly this modification is recognized by NEMO itself, which causes conformational changes that might lead to allosteric activation of IKK (Rahighi, Ikeda et al. 2009).

d. Regulation of protein localization

The role of ubiquitylation in regulating protein localization is also an additional example of the diverse non-proteolytic functions of ubiquitylation. Indeed, ubiquitylation can

directly affect protein localization, an example is the transcription factor p53. P53 is monoubiquitylated on several lysine residues and this modification drives its nuclear export (Carter, Bischof et al. 2007) likely by inducing changes in the accessibility of p53's nuclear export sequence. Following its multi-monoubiquitylation, p53 is exported out of the nucleus (Li, Brooks et al. 2003). P53 was also shown to be polyubiquitylated, however, p53's polyubiquitylation regulates its degradation and as opposed to it monoubiquitylation, this polyubiquitylation is irreversible (Yuan, Luo et al. 2010).

During the past few years, the ubiquitin field has been highly explored, allowing a dissection of the molecular mechanisms involved in this reaction. It was shown that different chain linkages adapt different conformations, that specific ubiquitin modifications are assembled by specific enzymes and are recognized by specific ubiquitin binding proteins and destined for specific outcomes. Later this modification can be erased by specific enzymes that act as erasers. However, because of the complexity of this system, a little is known about the biological functions of many ubiquitin chains such as K6, 27, 29 and 33 chains, without mentioning the more complex structures such as mixed and branched chains with still unknown outcomes.

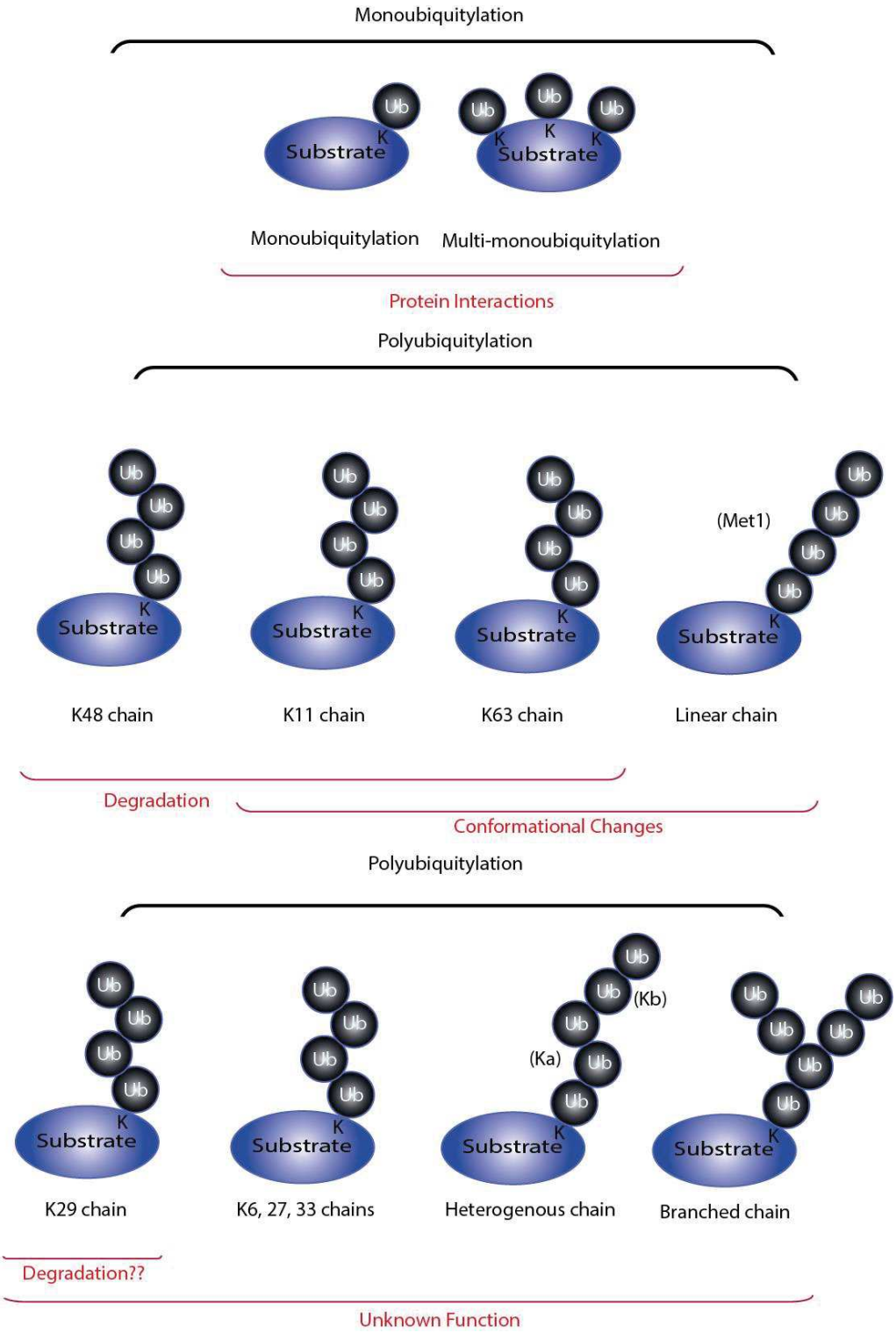


Figure 7: Cellular functions of the different ubiquitin chains

Figure recapitulating the main cellular function (s) of each type of ubiquitin linkages, described until now, with some chains remaining with unknown functions.

2.6 Regulation of DNA replication by the ubiquitin pathway

As previously discussed, DNA replication initiates at multiple sites along the DNA, which is likely to create problems for the cell if not properly controlled. Eukaryotic cells have developed many overlapping regulatory mechanisms. Mentioned previously were few examples of regulation such as the timely step-wise activation of replication origins, the presence of dormant origins. Moreover, the tight regulation of initiation proteins includes the activation of the pre-RC under high cyclin levels, while its formation requires low cyclin levels making it impossible to form new pre-RCs in S-phase a thus avoiding re-replication. In addition, another important feature is the low abundance of some of the firing proteins, crucial to avoid genetic instability and replication stress. However, the major regulation of DNA replication is through post translational modification (PTMs) of the components of the DNA replication machinery. Indeed, all three steps of normal DNA replication were shown to be regulated by PTMs, especially ubiquitylation, as two classes of E3 ubiquitin ligases, the SCF and the APC/C complexes play a central role in replication and cell cycle regulation (See Figure 8).

a. G1 regulation

Initiation

Following initiation of DNA replication it becomes essential that no more replication origins are able to be licensed. Accordingly, cells have developed multiple overlapping mechanisms to degrade or inhibit the activity of licensing factors upon S phase entry.

The Anaphase Promoting Complex or Cyclosome (APC/C) plays an important role in replication protein regulation. APC/C is a multi-subunit ubiquitin ligase that polyubiquitylates proteins targeting them for proteasomal degradation (Schreiber, Stengel et al. 2011). APC/C mainly controls the G1 phases of the cell cycle and utilizes two substrate recognition adaptor proteins, Cdh1 (CDC20 Homolog 1) in G1 and Cdc20 (Cell Division Cycle 20) in G2/M (Jaspersen, Charles et al. 1999, Shirayama, Toth et al. 1999).

APC/C-Cdh1 mainly regulates the activity of CDC6 and CDK, however it was also shown to play a role in ORC1, CDT1 and Geminin degradation.

APC/C-Cdh1 polyubiquitylates and targets licensing factor CDC6 for proteasomal degradation in G1. However, upon S phase entry, APC/C-Cdh1 becomes inactive and no longer induces CDC6 degradation (Petersen, Wagener et al. 2000, Mailand and Diffley 2005), CDC6 then becomes phosphorylated and is therefore protected from ubiquitin dependent degradation, consequently CDC6 level increase in S phase compared to G1 when licensing occurs. However, further regulatory mechanisms target CDC6 for cytoplasmic re-localization, where it cannot participate in origin licensing and allow re-licensing. Human CDC6 activity is inhibited by nuclear export stimulated by CDK (Saha, Chen et al. 1998, Kim and Kipreos 2008).

Moreover, the licensing factor CDT1, which is mainly regulated by the SCF ligase, was interestingly also shown to be degraded in mammalian cells, by the APC/C-Cdh1 ubiquitin ligase in early G1 (Sugimoto, Kitabayashi et al. 2008). Similarly to CDT1, during Drosophila development, ORC1 also mainly described as a substrate of the SCF ligase was shown to be ubiquitylated by the APC/C complex (Narbonne-Reveau, Senger et al. 2008).

APC/C-Cdh1 also plays a key role in regulating CDK activity. Indeed, since pre-RC formation cannot take place under high CDK activity, CDK activator and CDK inhibitors (or CKI) levels are tightly controlled. First APC/C-Cdh1 ubiquitylates CDK activator Cdc25 targeting it for degradation (Donzelli, Squatrito et al. 2002). Second, APC/C-Cdh1 mediates the accumulation of CKIs such as P21 and P27, by promoting the degradation of cofactors and subunits of the SCF ubiquitin ligase which is responsible for the ubiquitylation and degradation of these CKIs (Bashir, Dorrello et al. 2004), thus leading to both inhibition of CDKs and S phase entry.

Therefore, in order to allow entry is S phase APC/C-Cdh1 activity has to be inhibited. This inhibition happens in a number of ways, Cdh1 was found to be phosphorylated in order to block its activity or in order to be targeted for polyubiquitylation. These two events are independent and mediated by two different kinases (Fukushima, Ogura et al. 2013, Lau, Inuzuka et al. 2013). APC/C-Cdh1 is also inhibited as a consequence of the accumulation of its inhibitor EMI1 (Eldridge, Loktev et al. 2006) and finally APC/C-Cdh1 was also found to auto-ubiquitylate and therefore auto-inhibit itself (Rape and Kirschner 2004). As a result, the

transition from G1 to S phase can happen, ending with it the dominance of APC/C ubiquitin ligase and marking the beginning of the rule of the CRL ubiquitin ligases.

b. G1/ S transition

Cullin-RING ligases (or CRLs) are multisubunit ubiquitin ligases built around a scaffold cullin subunit, which interacts at its C-terminus with RING domain ubiquitin ligase subunits and at its N-terminus with substrate adaptor and substrate receptor. Higher eukaryotes express seven different cullins (Cul1, 2, 3, 4A, 4B, 5 and 7) with each of them interacting with a specific set of substrate adaptor and receptor proteins (Zimmerman, Schulman et al. 2010). Once entry in S phase a wide number of SCF ligases (CRL1) are used to ubiquitylate and degrade essential factors required for G1/ S transition but are now dispensable, such as cyclin D and cyclin E (Koepp, Schaefer et al. 2001, Lin, Barbash et al. 2006) and essential licensing factors such as ORC1 and CDT1.

Accordingly, in mammalian cells during G1/S transition, ORC1, the subunit responsible for origin recognition is ubiquitylated by the SCF-Skp2 complex and targeted for degradation, the levels of this subunit oscillate throughout the cell cycle while the other ORC subunits levels remain stable. This process avoids ORC1 recognizing DNA replication origins on newly synthetized DNA therefore preventing re-licensing (Mendez, Zou-Yang et al. 2002). In addition, ORC1 was shown to be regulated by phosphorylated in yeast, Xenopus and mammalian cells (Li, Vassilev et al. 2004).

CRL4, a cullin 4 based E3 Ligase, plays an important role in origin activation regulation. Mammalian cells degrade CDT1 in a PCNA depended manner thought ubiquitylation by CRL4-Cdt2 (Arias and Walter 2006), this interaction requires CDT1 phosphorylation by S phase CDKs (Sugimoto, Tatsumi et al. 2004). Interestingly, CDT1 is also regulated through an additional mechanism involving SCF-Skp2. Indeed, CDT1 is highly regulated in mammalian cells with overlapping pathways, as an inhibition of one pathway is not sufficient to inhibit CDT1 degradation, while in S. pombe CDT1 is only ubiquitylated by the CRL4-Cdt2 complex.

Finally, CDK inhibitor p21 is also ubiquitylated and degraded by both CRL4-Cdt2 and SCF-skp2 allowing full activity of S phase CDKs and leading to CDK driven phosphorylation and nuclear export of CDC6 (Kim and Kipreos 2008).

c. S phase

Elongation

During the progression of replication forks many of the replisome factors are affected by ubiquitylation, especially during DNA damage, such as PCNA ubiquitylation in eukaryotic cells. PCNA is monoubiquitylated by RAD18- RAD6 ubiquitin ligase in response to replication fork block (Mailand, Gibbs-Seymour et al. 2013).

However, besides DNA damage, some factors are also subjected to ubiquitylation in normal DNA replication. In Xenopus Laevis and fission yeast PCNA can be monoubiquitylated on lysine 164 (K164) during normal DNA replication. This ubiquitylation was shown to be important for efficient DNA replication by increasing the amount of chromatin bound PCNA and influencing pol δ recruitment (Leach and Michael 2005, Daigaku, Etheridge et al. 2017). Additionally, MCM10 was also shown to be ubiquitylated in unperturbed S phase, during both initiation and elongation, this ubiquitylation is thought to promote its interaction with PCNA (Das-Bradoo, Ricke et al. 2006).

Finally, lagging strand DNA polymerase Pol δ was also shown to be monoubiquitylated on two of its subunit in unperturbed replication in U2OS cells (Liu and Warbrick 2006).

Termination

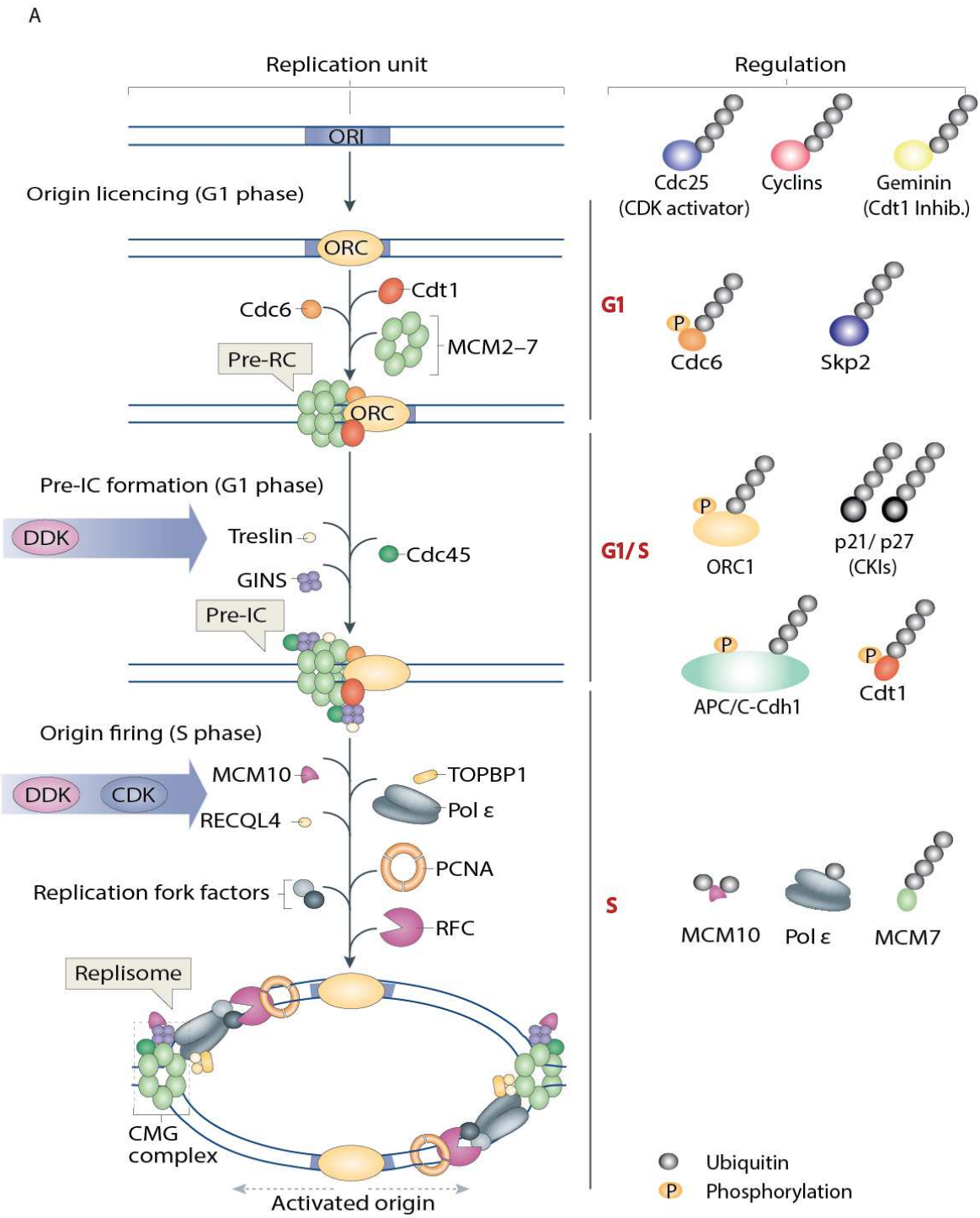
Ubiquitylation was shown to have a major role in the final step of DNA replication, more importantly in replisome disassembly. Indeed, MCM7 polyubiquitylation was observed in Xenopus Laevis, S. Cerevisae, and in higher eukaryotes, leading to the disassembly of the CMG replicative helicase. MCM7 ubiquitylation was shown to be catalyzed by SCF-Dia2 E3 ubiquitin ligase in S Cerevisae (Maric, Maculins et al. 2014). In higher eukaryotes its

ubiquitylation involved a different E3 ligase, termed CRL2-Lrr1, although it also leads to CMG complex dissolution (Dewar, Low et al. 2017).

d. G2- M phase regulation

Origin licensing is inhibited during G2 and M phase by several mechanisms. With Geminin accumulation throughout S phase, this protein was shown to reach a peak in late G2, allowing it to bind to CDT1 and inhibit its activity by interfering with its interaction with the MCM complex. Consequently, Geminin blocks origin licensing during G2 and mitosis (Yanagi, Mizuno et al. 2002, Cook, Chasse et al. 2004). In addition, CDT1 was also found to be phosphorylated during G2/M, which interferes with its function, later, in early G1 of the following cell cycle CDT1 is dephosphorylated (Chandrasekaran, Tan et al. 2011, Coulombe, Gregoire et al. 2013).

Finally, the APC/C-Cdh1 complex targets Geminin, the CDT1 inhibitor, for proteolysis during late mitosis (McGarry and Kirschner 1998), which allows CDT1 to recruit the MCM2-7 complex to the origins during the next cell cycle.


A
Replication unit
Regulation
ORI
Origin licencing (G1 phase)
ORC
Cdt1
Cdc6
MCM2–7
Pre-RC
ORC
Pre-IC formation (G1 phase)
DDK
Treslin
Cdc45
GINS
Pre-IC
Origin firing (S phase)
DDK
CDK
MCM10
TOPBP1
RECQL4
Pol ε
Replication fork factors
PCNA
RFC
Replisome
CMG complex
Activated origin
Cdc25 (CDK activator)
Cyclins
Geminin (Cdt1 Inhib.)
G1
P
Cdc6
Skp2
G1/S
P
ORC1
p21/ p27 (CKIs)
P
APC/C-Cdh1
P
Cdt1
S
MCM10
Pol ε
MCM7
Ubiquitin
P
Phosphorylation

B

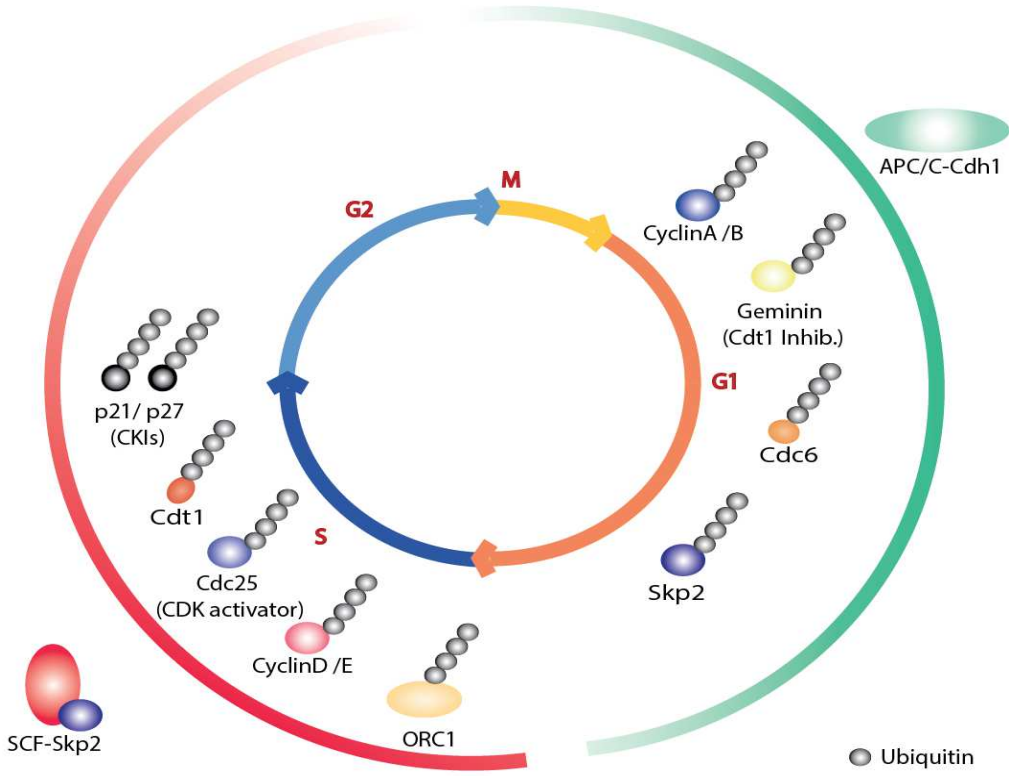


Figure 8: Regulation of DNA replication initiation

a, *(Adapted from Fragkos et al 2015) summarizes the main Post-Translational Modifications (PTMs) of the factors involved in the initiation of DNA replication during each phase of DNA replication* ***b,*** *Recapitulates the substrates of the two major regulators of replication during G1 and S phase of the cell cycle.*

Results

Project 1

Chapter 2 Results

Project 1: “The ORC ubiquitin ligase OBI1 promotes DNA replication origin firing”

1.1 Article Introduction

In eukaryotic cells, including human cells, the initiation of DNA replication starts at thousands of pre-defined sites in the genome, named "origins of replication". These origins are spread throughout the genome, without any apparent consensus sequence. Interestingly, studies done on replication origins show that origin distribution is not completely random, suggesting that origins are marked at certain sites on the DNA. Indeed, studies show a sequence preference that correlate with initiation sites. Origins were found in proximity to G4s, CpG islands and OGRE elements. However, this sequence preference alone could not account for the distribution of all the origins in the genome. Origin distribution was further correlated with open chromatin marks, and TSSs, suggesting a role of transcription and the chromatin state in origin distribution.

Origin distribution does not represent the only variant in replication origin biology. While all origins in the genome are licensed in G1, only a subset will be activated in S phase, the mechanism behind this selection also remains unclear. Interestingly, the origins marked for activation are not all activated at the same time.

Many studies aimed to explain origin heterogeneity in eukaryotes, however the mechanisms behind their distribution, selection for activation and temporal regulation remain unclear. In order to understand more how replication origins are regulated, our group performed an unbiased proteomic approach to study the interactome of the main factors forming the pre-RC, in order to identify new factors that could be involved in origin regulation. Using this approach our laboratory identified a novel protein (RNF 219), that binds the origin recognition complex (ORC complex), and that was named OBI1 (for ORC-ubiquitin-ligase-1).

RNF219

RNF 219 or RING Finger Protein 219 is a gene found in the open reading frame of Chromosome 13 in Humans (C13orf7). This gene codes for a protein of 726 amino acids and approximately 81 kDa, with a conserved RING domain in its N-terminus, a Coiled Coil domain and a C terminal end with no specific conserved domains. RNF219 is shown to be conserved in higher eukaryotes (Using BLAST, blast.ncbi.nlm.nih.gov), with its RING domain showing the highest conservation. However, Ecdysozoans D. Melanogaster and C. elegans seem to have lost this protein during evolution.

RNF219 was shown to be ubiquitously expressed in the brain and the testis in normal tissue. During development, RNF219 was shown to express mostly in the intestines and the heart (ncbi.nlm.gov/gene). Most importantly RNF219 was shown to be deregulated in several cancers, such as colorectal cancer, lymphoma and renal carcinoma (expression examined using the ONCOMINE server, oncomine.org)

RNF219 is poorly characterized, previous genome wide studies suggest that this gene may be implicated in late onset Alzheimer's disease (LOAD). RNF219 was shown to interact with APOE4 or apolipoprotein Epsilon 4 allele and its genetic variant was found to affect amyloid deposition in the human brain (Mosca, Sperduti et al. 2018).

On a cellular level, RNF219 was shown to interact with proteins mainly localized in the nucleoplasm (using the Reactome open server, reactome.org). However, BioID experiments showed also the presence of RNF219 in the cytoplasm and nuclear bodies (Youn, Dunham et al. 2018).

Genome wide studies may have identified variants of this gene in different diseases, and proteomic studies show a possible protein localization in the nucleus and the cytoplasm, however no functional data was revealed on this protein, and its role in the cell remains unknown.

1.2 Article Objectives

In this project (Article: “The ORC ubiquitin ligase OBI1 promotes origin firing”), the aim was to identify and characterize a novel interactor of the human pre-RC complex that could play a role in DNA replication and possibly provide further information on the biology of replication origins. Protein purification led to the identification of OBI1 an E3 ubiquitin ligase found to bind to the ORC complex. In vivo experiments were already initiated in the lab using human cells in order to characterize OBI1’s role in DNA replication.

During this project, the main objective was to decipher the exact role of OBI1 in DNA replication and how this protein was involved in this process.

OBI1, being an E3 ubiquitin ligase, it was important to study the role of OBI1’s ligase activity and the specificity of its catalytic domain in its role in DNA replication. In addition, it was important to focus on OBI1’s substrates and their implication in OBI1’s role. Finally, we aimed to study the ubiquitin chain type catalyzed by OBI1 on its substrates in the aim of understanding how this modification is leading to the observed phenotypes.

1.3 The Article “The ORC ubiquitin ligase OBI1 promotes DNA replication origin firing”

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The ORC ubiquitin ligase OBI1 promotes DNA replication origin firing

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DNA replication initiation is a two-step process. During the G1-phase of the cell cycle, the ORC complex, CDC6, CDT1, and MCM2–7 assemble at replication origins, forming pre-replicative complexes (pre-RCs). In S-phase, kinase activities allow fork establishment through (CDC45/MCM2–7/GINS) CMG-complex formation. However, only a subset of all potential origins becomes activated, through a poorly understood selection mechanism. Here we analyse the pre-RC proteomic interactome in human cells and find C13ORF7/RNF219 (hereafter called OBI1, for ORC-ubiquitin-ligase-1) associated with the ORC complex. OBI1 silencing result in defective origin firing, as shown by reduced CMG formation, without affecting pre-RC establishment. OBI1 catalyses the multi-mono-ubiquitylation of a subset of chromatin-bound ORC3 and ORC5 during S-phase. Importantly, expression of non-ubiquitylable ORC3/5 mutants impairs origin firing, demonstrating their relevance as OBI1 substrates for origin firing. Our results identify a ubiquitin signalling pathway involved in origin activation and provide a candidate protein for selecting the origins to be fired.

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DNA replication is initiated at defined genomic sites called origins of replication (see refs. [1,2] for review). Potential DNA replication origin sites (hereafter called origins) are established during the G1 phase of the cell cycle through a highly regulated process known as replication licensing[3,4] (for review). In eukaryotes, origins are recognised by the ORC complex, a multi-subunit (ORC1–6 and LRWD1/ORCA in vertebrates) AAA + ATPase[5]. After ORC binding, CDC6 and CDT1 assemble at origins and permit the licensing reaction that culminates with the loading onto chromatin of the replicative helicase MCM2/7 in its inactive state, defining the pre-replicative complexes (pre-RCs). Although ORC binding is sequence-specific in *S. cerevisiae*, it is unclear how ORC recognises origins in metazoans.

As cells enter S phase, the MCM2/7 helicase is activated at pre-RCs through CDK (Cyclin-dependent kinase) and DDK (DBF4-dependent kinase) activities[6]. This leads to the association of CDC45 and the GINS complex to chromatin-bound MCM2/7 and, consequently, to the formation of the active CMG complex[7]. CMG catalyses the unwinding of the DNA, allowing origin firing and the establishment of active replication forks. In mammals, origins are relatively inefficient and only a fraction of pre-RCs (5–40% of all potential origins) are activated, in a flexible manner, during each cell cycle[8–10]. Moreover, the repertoire of origin usage can differ from cell to cell[11]. The mechanisms involved in selective activation of origins are poorly understood.

To gain further insights into origin establishment and activation, we characterised the human pre-RC interactome using a proteomic approach. In addition to already known interacting partners, we identified previously undescribed pre-RC associated proteins. Among these, we focused on C13ORF7/RNF219, an uncharacterised potential E3 ubiquitin ligase that we called OBI1 (ORC ubiquitin-ligase-1). The properties of OBI1 suggest that it could be a replication origin selector essential for DNA replication origin activation during S phase.

Results

The human pre-RC interactome. To gain further insights into replication origin biology, we characterised the human pre-RC proteomic interactome. In HeLa S3 cell lines that stably express several pre-RC components (ORC1, ORC2, ORCA/LRWD1, CDC6 and CDT1) C-terminally tagged with Flag/HA. The expression levels of these baits and of the endogenous proteins were comparable (Supplementary Fig. 1a). After purification of baits and the associated proteins from Dignam nuclear extracts using a sequential immunoprecipitation/peptide elution approach (TAP-TAG), see Supplementary Fig. 1b, c and Methods), we analysed a fraction of the final eluates by gel electrophoresis/silver staining (Fig. 1a), and by mass spectrometry for protein identification (Fig. 1a, b and Supplementary Tables 1 and 2). Mass spectrometry identified previously known pre-RC interactors. Players and regulators of origin licensing and firing (ORC/ORCA complexes, CDKs, geminin, SCF^{SKP2} and ubiquitin) as well as factors involved in chromatin transactions (G9a/b, shelterin complex, Ku proteins and histones) were amongst these known interactors (more details in Supplementary Discussion). Western blot analysis confirmed the presence of selected interactors in the purified complexes (Fig. 1c). Among the identified pre-RC interactors that were previously undescribed, we found factors associated with the nuclear matrix (RIF1, NUMA and AKAP8), RNA metabolism (DDX5, DDX39B, FXR1, HNRPM, RBM10 and PRMT5), transcription (NFIL3 and TRIM29), and the ubiquitin pathway (C13ORF7/RNF219 and PSME3).

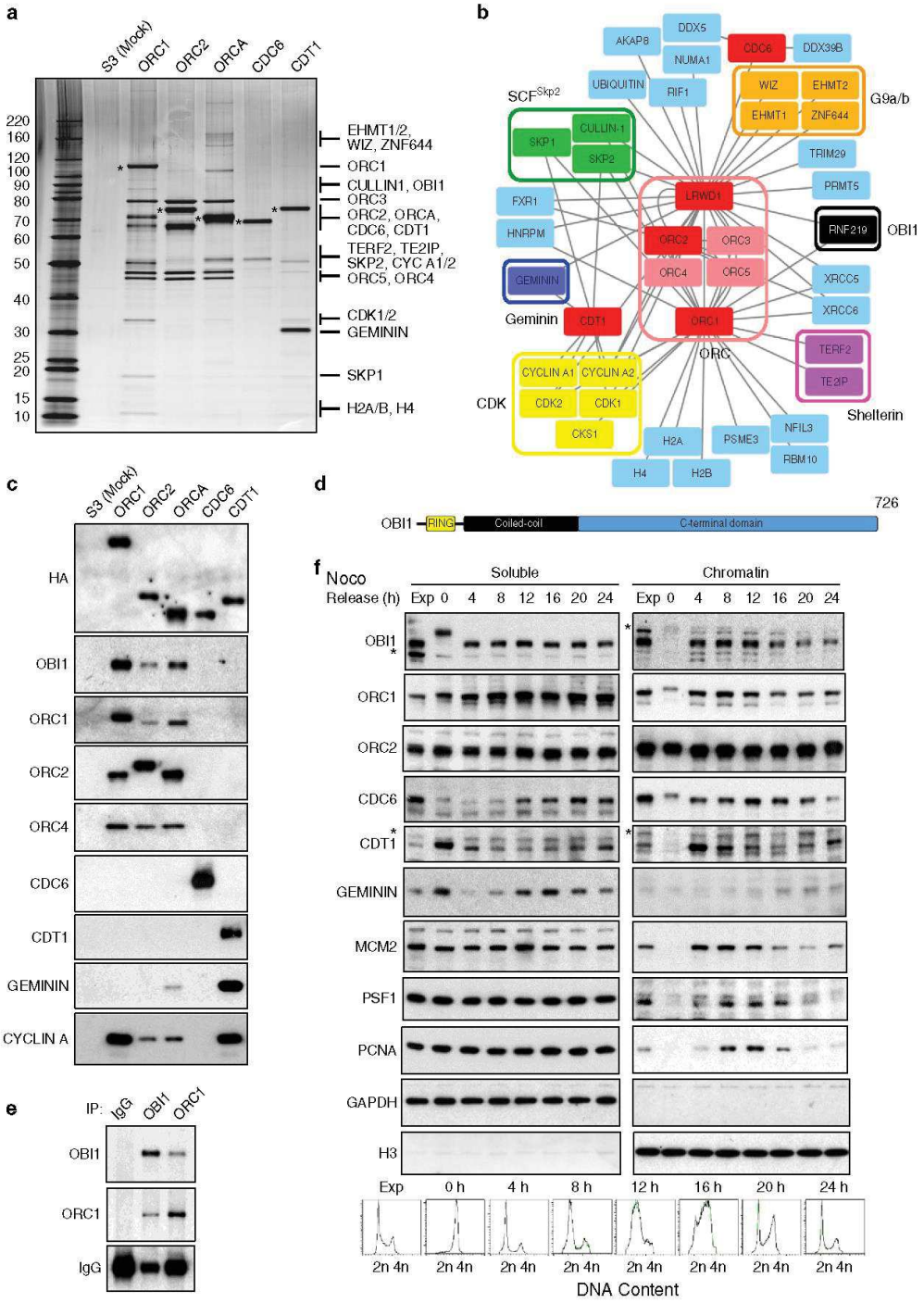
OBI1, an ORC complex binding protein. This study focuses on C13ORF7/RNF219, a putative E3 ubiquitin ligase with unknown function(s). Based on the biochemical function of C13ORF7/RNF219 reported herein, we propose to name this enzyme OBI1, for ORC-ubiquitin-ligase-1. OBI1 has an N-terminal RING domain followed by a coiled-coil domain and a large C-terminal extension without any recognisable domain (Fig. 1d). We found homologues of this putative ubiquitin ligase in all metazoans, with the same overall architecture (Supplementary Fig. 2a). Phylogenetic analysis suggested that ecdysozoans, which include arthropods and nematodes, lost this enzyme during evolution. OBI1's RING domain showed the strongest conservation (Supplementary Fig. 2a, b).

Western blot analysis confirmed the presence of endogenous OBI1 in purified ORC complexes (ORC1, ORC2 and ORCA baits, Fig. 1c), but not with the CDC6 or CDT1 baits. Both endogenous and ectopic OBI1 were associated with the ORC complex in proliferating cells (Fig. 1e, Supplementary Figs. 3a–c and 10d). OBI1 association with various ORC complex subunits suggested that it interacts with the whole ORC complex. To characterise OBI1 domain(s) required for ORC binding, we generated OBI1 deletion mutants and assessed their association with ORC1. OBI1 C-terminal region and coiled-coil domain were both required for ORC interaction (Supplementary Fig. 3a), but not the RING domain. Moreover, a catalytically inactive OBI1 mutant (Cys38Ser, see Supplementary Fig. 2b, further details later) interacted with the ORC complex (Supplementary Fig. 3d), showing that OBI1 ubiquitin ligase activity is not required for ORC association.

We then monitored the expression and chromatin association of endogenous OBI1 in U2OS cells synchronised in mitosis and then released in the cell cycle. Western blot analysis revealed that OBI1 expression was fairly constant throughout the cell cycle, while its chromatin association was cell cycle-regulated (Fig. 1f). OBI1 was absent from mitotic chromosomes, but was associated with chromatin from G1, and partially released from chromatin from mid S-phase. Moreover, in mitotic cell extracts, OBI1 band showed a molecular weight shift (Fig. 1f, left panel), suggesting a possible cell cycle regulation through phosphorylation, as documented for several replication origin factors[4].

OBI1 is involved in cell growth and transformation. Proper DNA replication is needed for cell growth. Indeed, siRNA-mediated silencing of ORC1 and CDC7, which are essential factors for origin licensing and firing respectively, impaired cell proliferation (Fig. 2a, siORC1- and siCDC7-transfected cells), compared with cells transfected with a non-targeting siRNA (siMock, see Supplementary Table 3 for sequences). Similarly, *OBI1* knockdown using siRNA pools targeting the coding sequence (siOBI1) or the 3′UTR (siUTR) significantly reduced cell proliferation (Fig. 2a). As OBI1 is a positive cell growth regulator, we evaluated its expression in human cancer samples using the ONCOMINE server[12]. OBI1 was overexpressed in different tumours, particularly colorectal adenocarcinoma (Supplementary Fig. 4a). We then investigated OBI1 potential oncogenic properties using classical transformation assays in non-transformed mouse NIH 3T3 cells[13]. OBI1 overexpression abrogated contact inhibition and allowed anchorage-independent cell growth (Supplementary Fig. 4b–d), two hallmarks of cell transformation. In these conditions, control NIH 3T3 cells did not form foci at confluence and colonies in soft-agar.

OBI1 is essential for origin firing but not for origin licensing. We then assessed whether OBI1 was involved in DNA replication. Cell cycle profile analysis by flow cytometry of siRNA-transfected U2OS cells pulsed with the thymidine analogue BrdU showed that *OBI1* knockdown (siOBI1 and siUTR) resulted in a



noticeable accumulation of cells in the S- and G2/M-phases, compared with control (siMock, Fig. 2b). BrdU incorporation per cell, reflecting the overall DNA synthesis, was reduced by ~50% in *OBI1* knockdown cells (BrdU fluorescence intensity quantified by flow cytometry) (Fig. 2b, right panel). *OBI1* knockdown led to a similar DNA synthesis defect also in HCT 116 and T98G cells (Supplementary Fig. 5). ORC1 and CDC7 depletion, which decreases the number of licensed and fired origins, respectively, led to a similar reduction of BrdU fluorescence intensity level (Fig. 2b). Silencing of treslin or its associated protein MTBP, which are essential components of origin firing, also caused a similar DNA synthesis defect[14,15].

Fig. 1 The pre-RC interactome analysis identifies OBI1 (C13ORF7/RNF219) as an ORC-associated protein. **a** The indicated baits were TAP-TAG purified from Dignam nuclear extracts of stably expressing HeLa S3 cell lines. Then, 20% of the final eluates were resolved on gradient acrylamide gels and visualised by silver staining. Asterisks mark the Flag-HA-tagged baits. The molecular weight markers are indicated in kDa. **b** Schematic representation of the human pre-RC interactome. Baits used in this study are marked in red. Known pre-RC interactors are highlighted in different colours, while novel binders are in light blue. OBI1 (C13ORF7/RNF219) is highlighted in black. Interactors are grouped based on their known interactions. **c** The TAP-TAG final eluates were analysed by western blotting using antibodies against the indicated proteins. **d** Schematic representation of human OBI1 with the RING, coiled-coil and C-terminal domains highlighted. **e** Immunoprecipitation/western blotting analysis confirmed the association of endogenous OBI1 and ORC1 in HeLa S3 cell Dignam extracts. **f** Cell cycle regulation of OBI1 expression and chromatin association. Exponentially growing U2OS cells (Exp) were synchronised in mitosis by incubation with nocodazole and then released in normal growth medium for the indicated times. Soluble and chromatin fractions were analysed by western blotting with antibodies against the indicated proteins. Synchronisation was evaluated by flow cytometry (lower panels). Asterisks mark non-specific bands

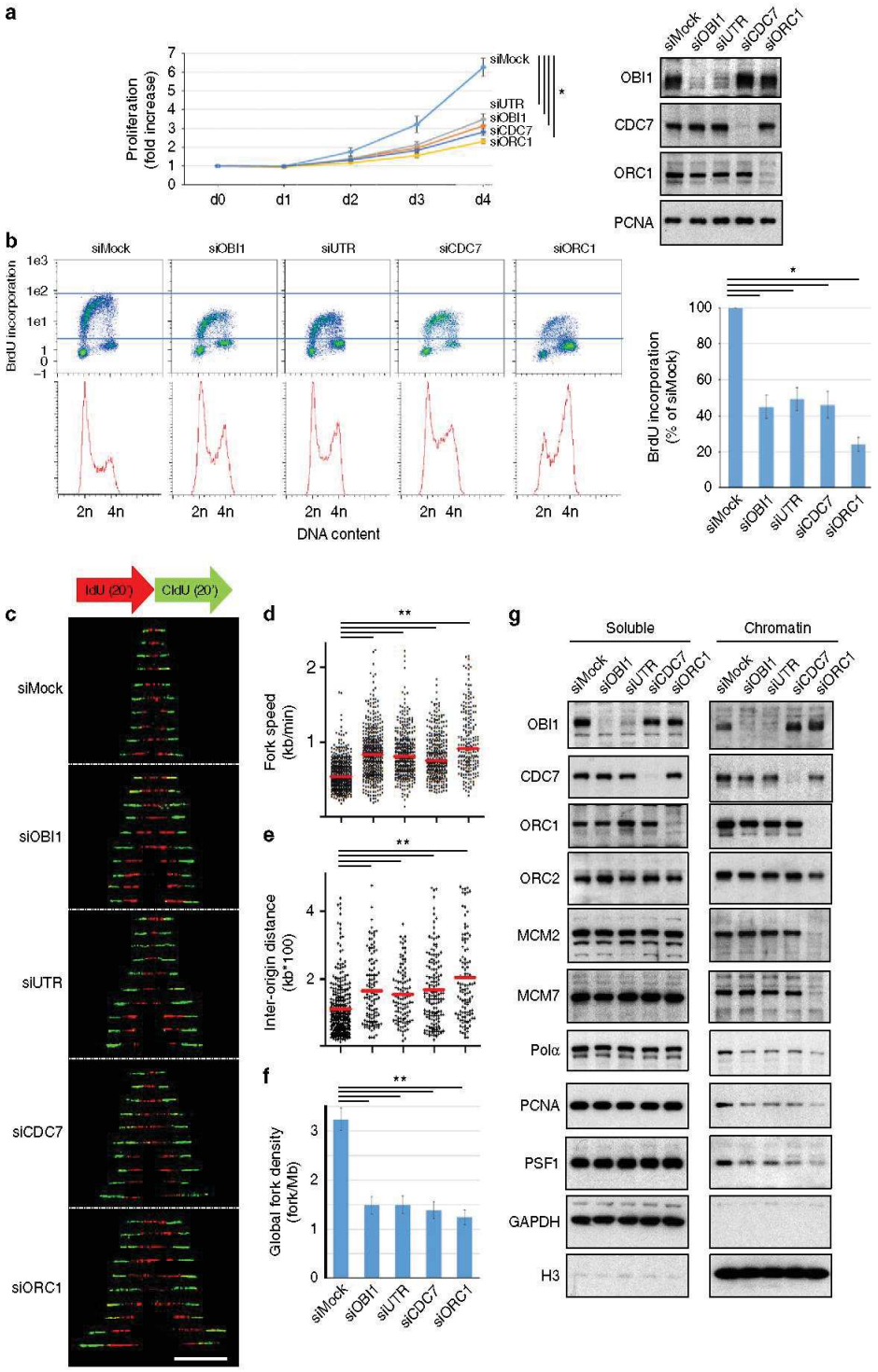
To further characterise the DNA synthesis defects induced by *OBI1* silencing, we studied DNA replication dynamics using DNA combing and DNA stretching assays (Fig. 2c–f and Supplementary Fig. 6a, b, respectively; see Methods). It is well established that reducing DNA replication initiation events results in higher replication fork speed, as a compensatory mechanism[15–19]. In agreement, fork speed was increased upon ORC1 or CDC7 depletion (Fig. 2c, d), as previously observed[17], and also upon *OBI1* silencing (Fig. 2c, d). Quantification of the origin firing rate by measuring the Inter-Origin Distances (IOD) and Global Fork Densities (GFD) in silenced cells showed that the reduction in DNA synthesis initiation events, as expected in *ORC1* and *CDC7* knockdown cells, led to larger IOD and lower GFD values (Fig. 2e, f), as previously reported[16,17]. *OBI1* silencing also resulted in higher IOD and lower GFD values compared with control cells (Fig. 2e, f). Similarly, DNA fibre stretching analysis showed replication fork acceleration, consistent with the activation defect associated with *OBI1* silencing (Supplementary Fig. 6a, b).

Then, we analysed the expression and chromatin association of key replication factors to determine which step of replication origin activity (origin licensing or firing) is regulated by OBI1. Upon ORC1 depletion, recruitment of the MCM2/7 complex to chromatin was impaired (Fig. 2g, Supplementary Figs. 6c and 11), confirming defective origin licensing. Consequently, chromatin binding of factors involved in the subsequent firing step, such as DNA polymerase-α, RPA32, PSF1/GINS1, the processivity factor PCNA and its loader RFC2, also was reduced (Fig. 2g, Supplementary Figs. 6c and 11). On the other hand, *OBI1* knockdown did not affect ORC1, ORC2, ORC4, CDC6 or MCM2/7 recruitment to chromatin (Fig. 2g, Supplementary Figs. 6c, d and 11), demonstrating that OBI1 is not required for licensing. However, chromatin recruitment of replisome components, such as DNA polymerase α, RPA32, PSF1/GINS1, PCNA and RFC2, was impaired by ~50% (Fig. 2g, Supplementary Figs. 6d and 11). We observed a similar phenotype upon silencing of the firing factor CDC7 (Fig. 2g and Supplementary Fig. 11), consistent with impaired CMG complex formation. The observed phenotype was not a consequence of inappropriate activation of the DNA damage checkpoint, as indicated by the absence of CHK1 phosphorylation or CHK1 induction upon OBI1 knockdown (Supplementary Fig. 6c). Altogether, these findings indicate that OBI1 is critical for origin firing, but not for origin licensing.

Chromatin-bound ORC3 and ORC5 are multi-mono-ubiquitylated in a cell cycle regulated manner. As OBI1 is an ORC interactor and a predicted ubiquitin ligase, it could positively regulate origin firing by direct ubiquitylation of the ORC complex. To test this hypothesis, we first assessed the ubiquitylation status of the ORC complex in vivo (see Methods). In these experiments, we co-expressed each tagged ORC subunit with HA-ubiquitin, and analysed their ubiquitylation status by western blotting. We detected ORC1 ubiquitylation, as previously described[20,21], while ORC2, ORC4 and ORC6 ubiquitylation was found to be very low (Fig. 3a and Supplementary Fig. 7 for controls). Strikingly, we found that ORC3 and ORC5 subunits (ORC3/5) were strongly ubiquitylated in vivo (Fig. 3a). In agreement, published large-scale proteomic studies to identify ubiquitylated proteins found many ubiquitylation sites on endogenous ORC3/5 in human cells[22,23] (see Supplementary Fig. 10a).

We further studied this previously uncharacterised post-translational modification of the ORC complex. We found that ORC3/5 purified from cellular fractions under denaturing conditions were ubiquitylated mainly on chromatin (Fig. 3b). Then, we studied the linkage type of ubiquitin assembled on ORC subunits using different approaches. Indeed, besides being covalently attached to substrates (known as mono-ubiquitylation), ubiquitin can be linked to any of its seven internal lysine residues (poly-ubiquitylation), yielding different outcomes depending on the nature of the ubiquitin linkage type[24,25]. For example, lysine 48-linked poly-ubiquitin chains target for proteasomal degradation. First, we co-expressed ORC3/5 with single-lysine ubiquitin mutants and analysed their ubiquitylation status. These ubiquitin mutants are proficient for a single linkage type poly-ubiquitylation, and can also be attached to substrates, yielding mono-ubiquitylation. All seven single-lysine ubiquitin mutants ubiquitylated ORC3/5, like wild type (WT) ubiquitin (Fig. 3c and Supplementary Fig. 8a). Moreover, lysine-less ubiquitin (0 K), which cannot sustain poly-ubiquitylation, but is still proficient for mono-ubiquitylation, was efficiently linked to ORC3/5 (Fig. 3c and Supplementary Fig. 8a). These results suggested that ORC3/5 are modified by mono-ubiquitylation. This conclusion was also supported by the results obtained with the UbiCRest assay, based on treatment of ubiquitylated substrates with linkage-specific deubiquitinating enzymes (DUBs)[26]. The universal DUB $USP2_{CD}$, which can cleave any kind of ubiquitin attachment, efficiently eliminated high molecular weight species of immunoprecipitated ORC3/5, releasing free ubiquitin in the supernatant (Fig. 3d and Supplementary Fig. 8b), illustrating again that these proteins are ubiquitylated in vivo. Conversely, ubiquitin linkage-specific DUBs did not affect ORC3/5 ubiquitylation (Fig. 3d and Supplementary Fig. 8b). Nevertheless, linkage-specific DUBs displayed activity in extracts supplemented with HA-ubiquitin that showed various types of ubiquitylation (Supplementary Fig. 8c), indicating that linkage-specific DUBs were active. The apparent molecular weight of ubiquitylated ORC3/5 species suggested that many ubiquitin molecules (2 to ~20) are conjugated to these substrates. This modification is referred to as multi-mono-ubiquitylation (or multi-ubiquitylation)[24], and is believed to have signalling roles, for instance in regulating protein/protein interactions[25].

Finally, to study the regulation of ORC3/5 ubiquitylation during the cell cycle, we generated stable U2OS cell lines that express tagged ORC3 or ORC5, and assessed ORC3/5 ubiquitylation and cell cycle profile at different times points after their



synchronisation in mitosis with nocodazole and release in fresh medium. ORC3/5 ubiquitylation, as observed by the appearance of high molecular weight species, was low in mitotic and early G1-phase cells, whereas it was induced in late G1-/early S-phase, peaked in S-phase, and then decreased toward the end of the cell cycle (Fig. 3e). Also, these experiments showed that only a subset of ORC3/5 became ubiquitylated (estimated to be 5–10%) during S-phase. Altogether, these results showed that chromatin-bound ORC3/5 become multi-mono-ubiquitylated concomitantly with origin firing.

Fig. 2 OBI1 is required for replication origin firing. **a** Involvement of OBI1 in cell proliferation. U2OS cells were transfected with siRNA pools targeting OBI1 3′ UTR (siUTR) or coding sequence (siOBI1), ORC1 (siORC1), CDC7 (siCDC7) or a non-targeting siRNA (siMock) (sequences in Supplementary Table 3). Cell proliferation (fold-increase relative to day 0) was evaluated by counting cells every day after transfection. The mean results of three independent experiments are shown. Expression of endogenous OBI1, ORC1, CDC7 and PCNA was monitored by western blotting at day 3 (right). **b** U2OS cells were transfected with siRNAs as in **a**. Three days post-transfection, cells were incubated with BrdU for 15 min. BrdU incorporation and DNA content were analysed by flow cytometry (left panels). Lines delimiting BrdU-positive siMock-treated cells are shown. BrdU incorporation fluorescence signal was quantified from three independent experiments (right panel). **c** U2OS cells were transfected with siRNAs as in **a**. Three days post-transfection, cells were incubated with IdU (20 min) followed by CldU (20 min) and processed for DNA combing analysis (see Methods). Representative images of bidirectional forks labelling are shown. **d** Analysis of replication fork speed (in kb/min) in the cells described in **c**, based on the measurement of CldU tracks preceded by the IdU signal (two independent experiments). Red bars indicate median values. **e** Inter-origin distances (in kb) in the cells described in **c** were quantified from two independent experiments. Red bars indicate median values. **f** The mean global fork density (in fork/Mb) in the cells described in **c** was quantified by measuring the number of labelled forks per megabase of combed DNA, normalised to the percentage of S-phase cells (two independent experiments). **g** U2OS cells were transfected with siRNAs as in **a**. Three days later, chromatin and soluble fractions were isolated and analysed by western blotting with antibodies against the indicated proteins. $^{*}p < 0.01$; $^{**}p < 0.001$

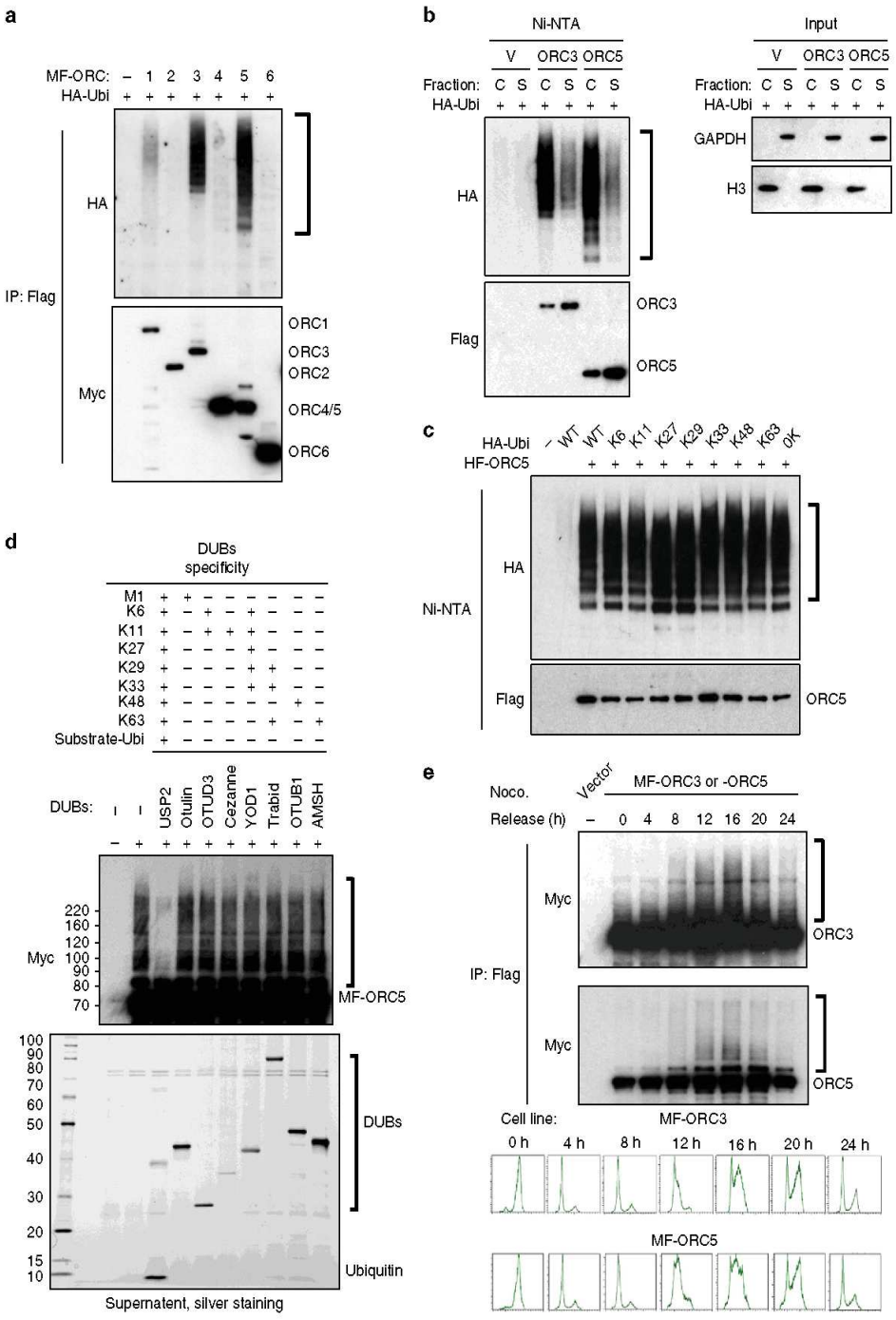
OBI1 catalyses ORC3 and ORC5 multi-mono-ubiquitylation. We next assessed the possible involvement of OBI1 in ORC3/5 ubiquitylation using gain and loss of function experiments and in vitro assays. First, OBI1 overexpression stimulated mainly ORC3/5 ubiquitylation, leaving the other tested subunits largely unaffected (Fig. 4a). Although we cannot exclude that OBI1 might modify the other ORC subunits, we decided to focus on OBI1-induced ORC3/5 ubiquitylation because it was very robust. We observed this enhanced ubiquitylation when ORC3/5 were purified in native or in denaturing conditions (Fig. 4b–c and Supplementary Fig. 9a, b), showing that the modification is specific to these ORC subunits. OBI1-induced ORC3/5 ubiquitylation required its intrinsic ubiquitin ligase activity, because the OBI1 Cys38Ser (CS) and RING-deleted (ΔRING) mutants were inactive (Fig. 4b–c and Supplementary Figs. 2b and 9a–c). OBI1 mutants lacking the coiled-coil or the C-terminal extension (unable to bind to the ORC complex, see Supplementary Fig. 3a) also were inactive (Supplementary Fig. 9c). OBI1 ubiquitin ligase activity toward the ORC complex is a conserved feature because the *Xenopus laevis* OBI1 homologue also could catalyse human ORC5 ubiquitylation (Supplementary Fig. 9d). Importantly, *OBI1* siRNA-mediated knockdown in human U2OS cells drastically reduced ORC3/5 ubiquitylation (Fig. 4d–e). These findings identified OBI1 as a major ORC3/5 ubiquitin ligase in vivo. Nevertheless, we do not formally exclude the possibility that other E3 ligase(s) could be involved in ORC3/5 ubiquitylation, depending on the physiological and cellular context. Finally, we performed in vitro ubiquitylation assays to test whether OBI1 could directly modify ORC3/5. WT OBI1 but not the CS mutant purified from cell extracts ubiquitylated in vitro translated ORC3/5 (Fig. 4f–g), yielding a signal very similar to the one observed in cells. In these in vitro conditions, we found that OBI1 auto-ubiquitylates, as previously described for other E3 ligases[27,28]. In addition, in vitro assays using WT or 0 K ubiquitin gave very similar ORC3/5 ubiquitylation patterns (Fig. 4h), showing that OBI1 directly catalysed ORC3/5 multi-mono-ubiquitylation. Altogether, these results indicate that OBI1 is a major cellular ORC3/5 ubiquitin ligase capable of catalysing their multi-mono-ubiquitylation.

ORC3 and ORC5 ubiquitylation is important for origin firing. If ORC3/5 were functionally relevant OBI1 substrates for origin activation, then expression of non-ubiquitylable ORC mutants should mimic the effect of OBI1 depletion. Mutation of the lysine residues identified in large-scale ubiquitylome studies[22,23] (nine and seven lysine residues in ORC3 and ORC5, respectively, Supplementary Fig. 10a) into arginine to hinder ubiquitin attachment did not affect ORC3/5 ubiquitylation in vivo (ORC3–9R and ORC5–7R versus WT proteins; Supplementary Fig. 10b), suggesting a strong flexibility in the choice of the ubiquitin attachment sites by OBI1. Indeed, OBI1 overexpression stimulated the ubiquitylation of ORC3–9R and ORC5–7R (Supplementary Fig. 10c), showing that OBI1 could target other lysine residues. To obtain definitive non-ubiquitylable mutants, dominant negative experiments were then performed using lysine-less (0 K) versions of ORC3 and ORC5 (see Supplementary Fig. 10a) that were no longer ubiquitylated in vivo (Fig. 5a).

Functional characterisation of the lysine-less ORC3/5 variants showed that ORC3/5 0 K could bind to chromatin (Supplementary Fig. 10d), and associate with the ORC complex (Fig. 5b) and OBI1 (Supplementary Fig. 10e), like the WT counterparts. The unrelated protein EGFP did not displayed these properties. Moreover, the ORC3/5 0 K mutants did not perturb pre-RC formation (see Fig. 5d), as indicated by the similar amount of ORC1, ORC2 and the MCM2/7 complex on chromatin of control (Vector), WT ORC3/5, and ORC3/5 0K-expressing cells. As a functional control, expression of the CDT1 inhibitor geminin impaired MCM2/7 chromatin recruitment (Fig. 5d), as previously observed[29]. These results suggested that the conservative lysine-to-arginine substitutions introduced in ORC3/5 0 K do not affect the essential function of the ORC complex in origin licensing. However, ORC3/5 0 K expression resulted in defective DNA synthesis (BrdU fluorescence intensity) associated with accumulation of S-phase cells (Fig. 5c), as observed with *OBI1*-silenced cells (see Fig. 2b). In addition, cell fractionation revealed defective chromatin recruitment of proteins involved in DNA replication activation, such as DNA polymerase α, PSF1 and PCNA (Fig. 5d). Moreover, DNA stretching experiments revealed that inhibition of ORC3/5 ubiquitylation resulted in faster replication forks (Fig. 5e), as observed when origin firing is impaired (see Fig. 2d and Supplementary Fig. 6b). These results indicated that OBI1-catalysed ORC3/5 multi-mono-ubiquitylation per se is important for efficient origin activation.

Discussion

The ubiquitin pathway is generally recognised as an important regulator of DNA replication. Indeed, during the licensing reaction, the pre-RC factors ORC1[18,19], CDC6[30], CDT1[31,32] and the licensing inhibitor geminin[29] are controlled by K48-linked poly-ubiquitylation and proteasomal degradation. Chromatin unloading of the replicative helicase at the end of replication involves K48-linked MCM7 poly-ubiquitylation[33,34]. Replicative DNA methylation maintenance relies on the action of the ubiquitin ligase UHRF1 to catalyse the mono-ubiquitylation of histone H3 lysine 23[35]. Here, we show that DNA replication origin activation is positively regulated by a novel ubiquitin signalling pathway that involves ORC3 and ORC5 multi-mono-ubiquitylation catalysed



by OBI1 (C13ORF7/RNF219), a previously poorly characterised E3 ligase.

We found that OBI1 can interact with several ORC subunits (ORC1, ORC2, ORC3, ORC5 and ORCA/LRWD1, see Fig. 1c, e, Supplementary Fig. 3 and Supplementary Fig 10e), suggesting that it interacts with the whole complex. The fact that OBI1 ubiquitylates ORC3 and ORC5 in vitro also indicates that OBI1 can directly interact with these ORC subunits.

Replication origin selection is required for choosing the licensed origins to be activated amongst all potential origins and their timing of activation during S-phase. Our results suggest that both selection processes could be regulated by OBI1, as proposed in the following model (Fig. 6a). When cells exit mitosis and enter the G1-phase, ORC complexes catalyse the licensing of all potential origins, independently of OBI1 action, leading to the chromatin recruitment of the replicative helicase MCM2/7 in an

Fig. 3 Chromatin-bound ORC3 and ORC5 are multi-mono-ubiquitylated in S-phase. **a** U2OS cells were co-transfected with the indicated Myc-Flag (MF)-tagged ORC subunits and HA-tagged ubiquitin. Two days post-transfection, cells were lysed in NEM-containing lysis buffer and tagged proteins were purified by anti-Flag immunoprecipitation. Expression and ubiquitin conjugation of the immunoprecipitates were monitored by western blotting, as indicated. **b** U2OS cells were co-transfected with the indicated His_6-Flag (HF)-tagged ORC subunits and HA-tagged ubiquitin. Chromatin (C) and soluble (S) fractions were isolated and ORC subunits were purified by nickel beads (Ni-NTA) under denaturing conditions. Purified proteins and input extracts were analysed by western blotting with antibodies against the indicated proteins. **c** U2OS cells were co-transfected with His_6-Flag-ORC5 (HF-ORC5) and the indicated HA-tagged ubiquitin single-lysine (K) mutants. In the 0 K mutant, all lysine residues were replaced by arginine residues. ORC5 ubiquitylation was detected by purification in denaturing conditions on nickel beads (Ni-NTA). Expression and ubiquitin conjugation of the isolated proteins were monitored by western blotting. **d** UbiCRest analysis of ubiquitylated ORC5. U2OS cells were transfected with Myc-Flag (MF)-tagged ORC5, without ectopic ubiquitin. Two days post-transfection, MF-ORC5 was immunoprecipitated (IP: Flag) and incubated with the indicated deubiquitylating enzymes (DUBs). Supernatants were recovered and analysed by silver staining. DUBs and released endogenous ubiquitin are indicated. Ubiquitylation was revealed by the presence of high molecular weight forms detected by western blotting against tagged ORC subunits. The ubiquitin linkage specificity of the used DUBs is indicated. **e** U2OS cells stably expressing Myc-Flag (MF)-tagged ORC3 or ORC5 were synchronised in mitosis by incubation with nocodazole and then released in normal growth medium for the indicated times. Tagged proteins were immunoprecipitated (IP: Flag) and ubiquitylation was assessed by anti-Myc antibody (upper panels). Synchronisation was evaluated by flow cytometry (lower panels)

inactive state. This is in agreement with the cell cycle-regulated OBI1 binding to chromatin and with the finding that OBI1 knockdown does not affect pre-RC formation. At S-phase onset, OBI1 could play the role of a replication origin selector involved in the selection of the origins to be fired by modifying a pool of replication origins through ORC multi-mono-ubiquitylation that would favour their preferential activation (Fig. 6b).

As it is well known that mono-ubiquitylation regulates protein/protein interactions[25], this modification might allow the recruitment of limiting firing factor(s) with ubiquitin-binding capacity to the pre-RCs to be fired. Mechanistically, multi-mono-ubiquitylation of the ORC complex may also alter the local chromatin environment around origins, making them more accessible to limiting firing factors. Consistent with this idea, it was recently reported that ORC5 interacts with the histone acetyltransferase GCN5/KAT2A, making origins more accessible for activation[36]. ORC5 multi-mono-ubiquitylation could promote this interaction, opening the local origin chromatin environment and stimulating origin activation. Although we cannot exclude that other factors involved in replication origin activation might also be ubiquitylated by OBI1, ubiquitylation of ORC3/5 is essential because non-ubiquitylable ORC3/5 mimic OBI depletion effects on DNA replication. Importantly, our findings suggest that, in addition to its essential and well-established role in origin licensing during G1-phase, the ORC complex also has a second crucial role in origin activation in S-phase, as the substrate of OBI1 activity.

Methods

Reagents and antibodies. The following reagents were purchased from Sigma-Aldrich: BrdU (B5002), IdU (I7125), N-ethylmaleimide (E3876), hydroxyurea (H8627), polybrene (H9268), puromycin (P9620), propidium iodide (P4864), Hoechst dye (H6024), anti-Flag coupled agarose beads (A2220), Flag peptide (F3290), anti-Flag (M8823), -Myc (M4439), -CDC6 (C0224), -cyclin A (C4710), -ORC1 (PLA0221), -MCM7 (M7931) and -PCNA (P8825) antibodies. CldU (105478) was from MP Biomedicals; magnetic affinity beads (Dynabeads M-450 goat anti-mouse) from Invitrogen; HA peptide from Roche; anti-HA coupled agarose beads (sc-7392 AC), anti-HA (sc-805), -ORC2 (sc-13238), -MCM2 (sc-10771), -ORC4 (sc-20634), -geminin (sc-74456; sc-74496), -OBI1/RNF219 (sc-84039), -RPA70 (sc-48425) and -CHK1 (sc-8408) antibodies were purchased from Santa-Cruz. Anti-RFC2 (ab88502), -histone H3 (ab62642), -RPA32 (ab76420), -polymerase alpha (ab176734), -CDC7 (ab10535), -His_6-tag (ab18184), -GAPDH (ab9484) and -PSF1 (ab181112) antibodies were from Abcam. Anti-BrdU and anti-IdU mouse IgG1 were from Becton Dickinson; anti-γ-H2AX (9718) and -phospho-CHK1 (2341) antibodies were from Cell Signaling; HRP-coupled anti-mouse, -rabbit and -goat antibodies from Amersham; TrueBlot HRP-coupled antibodies from Rockland; anti-CldU rat and anti-ssDNA mouse IgG2a antibodies from AbCys SA and Chemicon, respectively; anti-mouse FITC-coupled, anti-mouse IgG1 Alexa 546-coupled, anti-rat Alexa 488-coupled, anti-mouse IgG2a Alexa 647-coupled antibodies from Molecular Probes; anti-IL2Rα mouse monoclonal antibody from Millipore. Anti-CDT1 and anti-RNF219 rabbit polyclonal antibodies were generous gifts by M. Fujita and B. Sobhian, respectively. In-house anti-OBI1 rabbit polyclonal antibodies were generated against recombinant OBI1 N- and C-terminus. siRNAs against human OBI1 and ORC1 (SmartPool) and negative control (siGENOME Non-Targeting siRNA #2, D-001210–02) were purchased from Dharmacon. Boston Biochem provided the UbiCRest kit as well as E1, UbcH5, wild type and lysine-less ubiquitins and ubiquitin-aldehyde proteins. TNT reticulocyte in vitro translation kit was from Promega.

Cell culture, infection and transfection. T98G, HeLa S3, U2OS, NIH 3T3 and EcoPlatinum cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS), glutamine and antibiotics. HCT116 cells were grown in McCoy's 5 A medium supplemented with 10% FBS, glutamine and antibiotics. Plasmids (2–6 μg/6 cm dishes) and siRNAs (5–10 nM) were transfected using Lipofectamine (Invitrogen) or JetPEI (polyplus) and Interferin (Polyplus) reagents, respectively. To generate HeLa S3 cell lines that stably express tagged baits, cells were first transfected with a vector that expresses mouse cationic amino acid transporter-1 (mCAT1) to render them susceptible to ecotropic retroviruses. Two days after transfection, HeLa S3 cells were incubated with retroviruses produced in EcoPlatinum cells in the presence of 4 μg/mL Polybrene for 24 h. Transduced cells were selected using magnetic beads (Invitrogen) coupled to anti-IL2Rα antibodies and enriched populations were expanded. For cell transformation experiments, NIH 3T3 cells were co-transfected with OBI1-expressing plasmids (or empty vector as control) and the puromycin resistance vector (pBabe-puro). Two days after transfection, cells were selected in 2.5 μg/mL puromycin. To generate stable U2OS cell lines expressing Myc-Flag-ORC3 or –ORC5, cells were co-transfected with tagged-ORC expression plasmid along with a puromycin resistant vector in a ratio 10:1. Two days after transfection, cells were passaged in puromycin containing media. 2–3 days later, resistant transfected cells were seeded at low confluence and individual colonies were isolated, expanded and monitored for ectopic protein expression. Similarly, in dominant-negative experiments, U2OS cells were co-transfected with His_6-tagged ORC3/5 WT or 0 K (or the empty vector) and pBabe-puro. One day after transfection, cells were passaged in puromycin containing medium for two days. Selected cells were further cultured for 18–24 h without puromycin and processed for assays.

Plasmid constructs. The coding sequences of human ORC1, ORC2, LRWD1, CDC6 and CDT1 were cloned in the retroviral vector pOZ-FH-C[37]. For transient expression experiments, the described coding sequences were cloned in derivatives of the mammalian expression vector pCS3 to express N-terminally Myc_6-, Myc_5-Flag-, His_6- or Flag-tagged proteins, as indicated. OBI1 mutants were generated using PCR mutagenesis. EGFP and CDT1 expression vectors were previously described[38]. HA-tagged ubiquitin expression vectors were previously described[39]. The pBabe-puro retroviral vectors have been described[40]. Ubiquitin single-lysine mutants or lysine-less (0 K) expression vectors were obtained from Addgene. ORC3 and ORC5 lysine mutants were produced through gene synthesis (MWG). Details about DNA constructs available on request.

Cell proliferation and transformation assays. Proliferation was measured by counting viable cells after trypan blue staining each day for 4 days. The fold-increase in cell proliferation compared to day zero (siRNAs) is expressed as the mean of three independent experiments. The oncogenic properties of NIH 3T3 cells stably expressing OBI1 or empty vector were characterised by foci formation and soft agar growth assays, as previously described[38]. Briefly, for formation assays, stable populations were plated in 60 mm plates at equivalent cells density. Confluent cells were cultured for 2–3 weeks and the culture medium was changed every 2–3 days. Foci were stained with crystal violet dye (0.5% crystal violet, 4% formaldehyde, 30% ethanol and 0.17% NaCl). For quantification, the number of stained foci was counted from two independent experiments. For soft-agar growth, cells (10^5) were mixed with medium supplemented with 0.4% agarose and placed

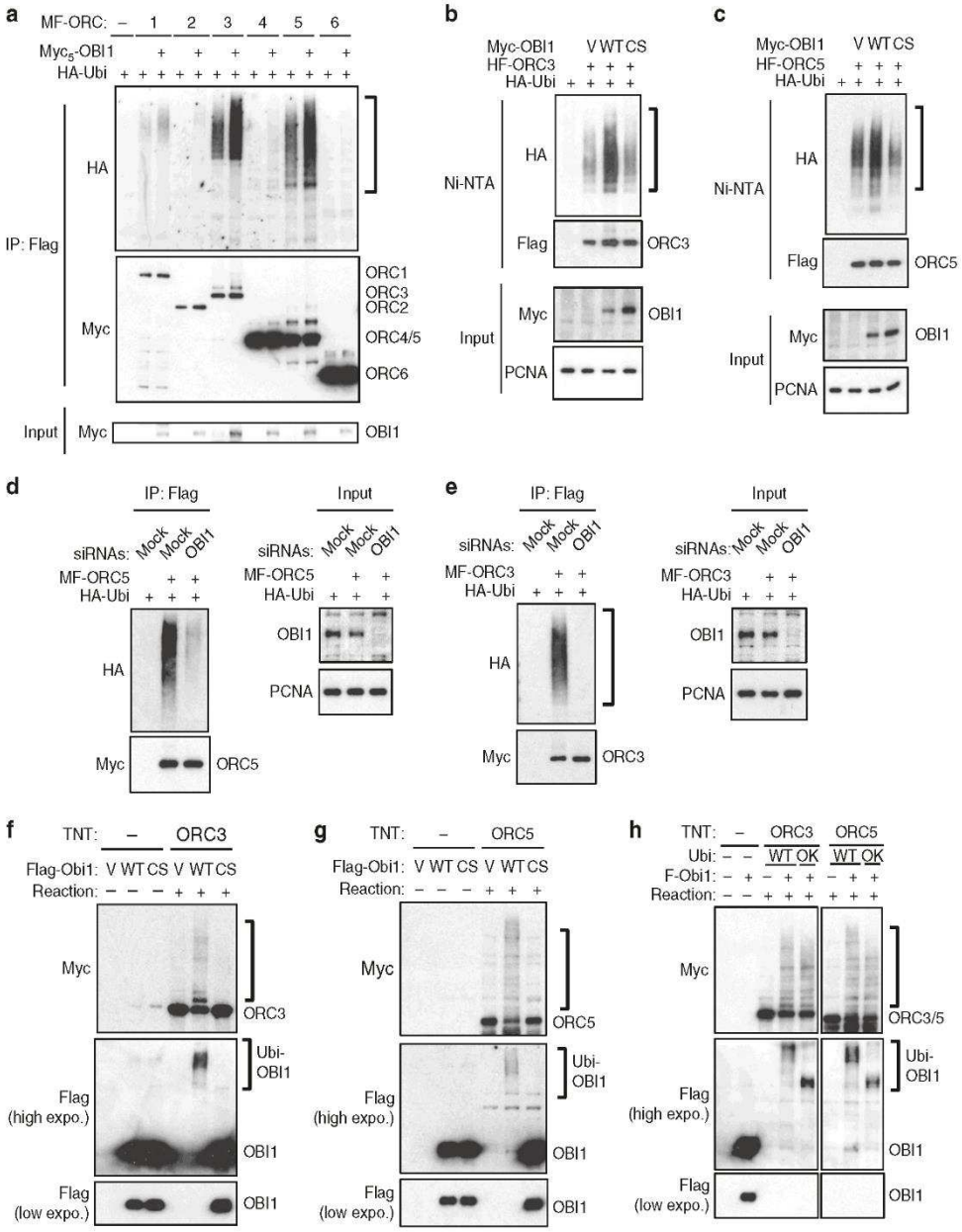
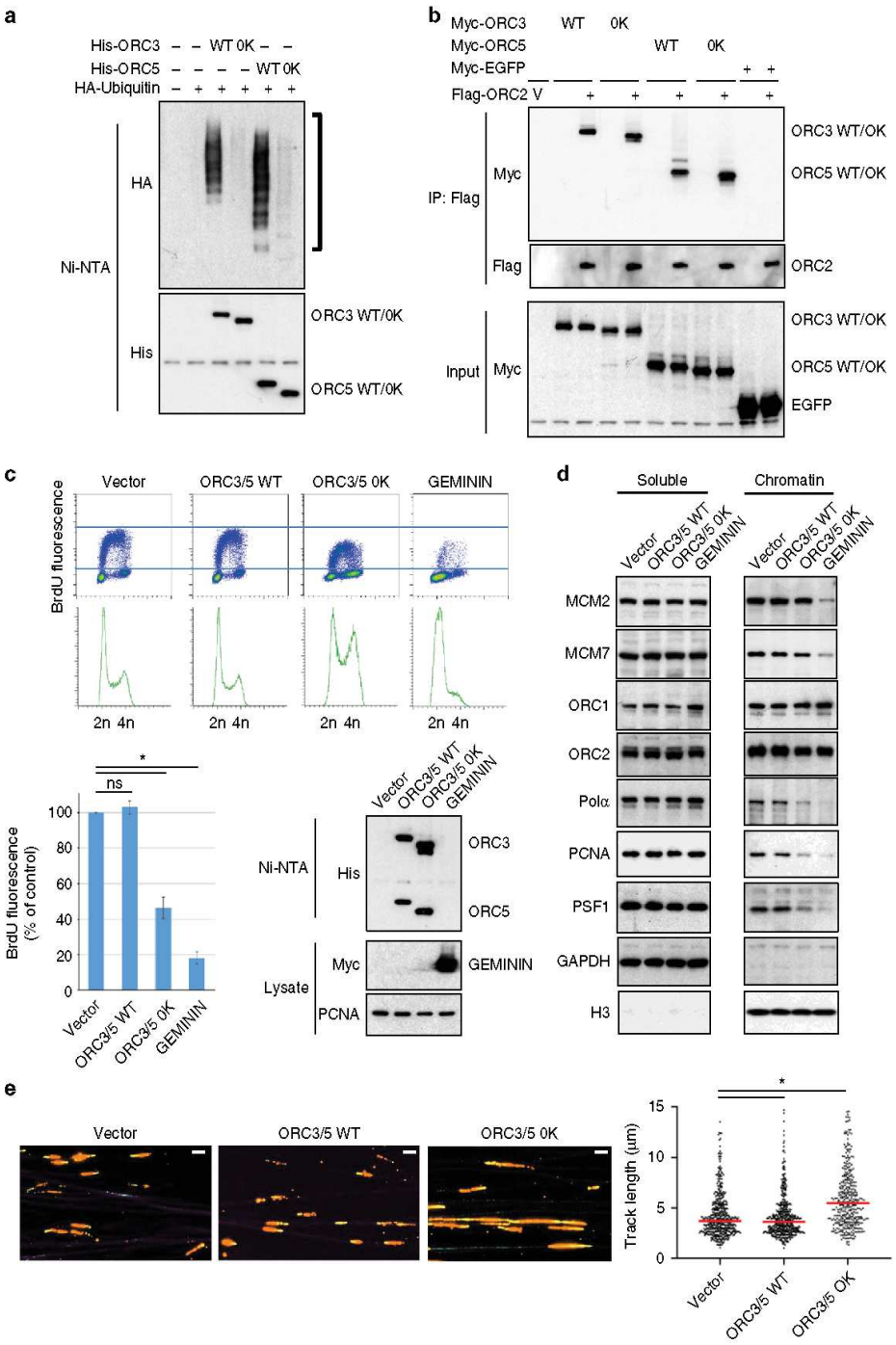


Fig. 4 OBI1 catalyses ORC3 and ORC5 multi-mono-ubiquitylation. **a** U2OS cells were co-transfected with the indicated Myc-Flag (MF)-tagged ORC subunits and HA-tagged ubiquitin. When indicated (+), Myc-tagged OBI1 was also co-expressed. Two days post-transfection, cells were lysed in NEM-containing lysis buffer and tagged proteins were purified by anti-Flag immunoprecipitation. Expression and ubiquitin conjugation of the immunoprecipitates were monitored by western blotting, as indicated. Myc-OBI1 expression was also analysed in input extracts. **b**, **c** U2OS cells were co-transfected with His_6-Flag-ORC3 (HF-ORC3) or His_6-Flag-ORC5 (HF-ORC5) and either wild type (WT) or inactive (CS) Myc-OBI1 along with HA-ubiquitin as indicated. ORC3/5 ubiquitylation was detected by purification in denaturing conditions on nickel beads (Ni-NTA). Expression and ubiquitin conjugation of the isolated proteins were monitored by western blotting. Myc-OBI1 expression was also analysed in input extracts. **d**, **e** U2OS cells were transfected with Mock or *OBI1*-specific siRNAs. The next day, cells were co-transfected with Myc-Flag (MF)-tagged ORC3 or ORC5 and HA-tagged ubiquitin, as indicated. Ubiquitylation was detected 2 days later as in **a**. Expression of endogenous OBI1 was monitored in input extracts by western blotting. **f**, **g** OBI1 in vitro assay. U2OS cells were transfected with wild type Flag-OBI1 (WT) or inactive mutant (CS) for two days. Immunoprecipitated OBI1 (IP: Flag) was incubated with in vitro translated Myc-ORC3 or -ORC5 along with E1, E2 (UbcH5a), ubiquitin and ATP for 30 min. at 37 °C. Tagged proteins were detected by western blotting as indicated. **h** OBI1 in vitro assay performed as in **f**, **g**, but with wild type (WT) or lysine-less (0 K) ubiquitin. Tagged proteins were detected by western blotting as indicated



on top of the 1% agarose layer with growth medium. One millilitre medium was added to the solidified layer and changed every 2–3 days. After 3–4 weeks, colonies were photographed in randomly selected fields using a phase-contrast microscope.

TAP-TAG purification. Dignam nuclear extracts from 60 15-cm dishes of cells for each HeLa S3 cell line were obtained, as previously described[37,41]. Briefly, cells were collected and washed twice in ice-cold PBS. Cell pellets were resuspended in 10 cell pellet volume of ice-cold Hypotonic Buffer (HB: 10 mM Tris-HCl pH 7.3, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM PMSF) and spun at 190 g at 4 °C for 5 min. Cell pellets were resuspended in two cell pellet volume of HB and incubated on ice for 10–15 min. for the cells to swell. Cells suspensions were transferred to an ice-cold Dounce, lysed with 10–15 strokes with the tight pestle and spun at 3000×*g* at 4 °C for 20 min to pellet nuclei. Nuclei were resuspended in half of the nuclei pellet volume (NPV) of ice-cold Low Salt Buffer (20 mM Tris-HCl pH 7.3, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM KCl, 1 mM DTT, 0.3 mM PMSF). While gently vortexing, half of NPV of ice-cold High Salt Buffer (20 mM Tris-HCl pH 7.3, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.2 M KCl, 1 mM DTT and 0.3 mM PMSF) was added dropwise. The extraction continued on a rotary wheel at 4 °C for 30 min. The suspensions were spun at 15,700 g at 4 °C in microcentrifuge tubes for 30 min. The

Fig. 5 ORC3 and ORC5 multi-mono-ubiquitylation is essential for origin firing. **a** U2OS cells were co-transfected with His_6-tagged wild type (WT) ORC3 and ORC5 or lysine-less versions (0 K, see Supplementary Fig. 10a) and HA-ubiquitin, as indicated. Two days post-transfection, cells were lysed in denaturing conditions for His_6-tagged protein purification. Expression and ubiquitin conjugation of the isolated proteins were monitored by western blotting. **b** U2OS cells were co-transfected with Myc-tagged (WT or 0 K) ORC3 and ORC5 or EGFP and Flag-ORC2. Two days after transfection, cell lysates were Flag-immunoprecipitated to purify the ORC subunit, and the association of co-expressed proteins was analysed by western blotting. Expression of Myc-tagged proteins in input extracts was also monitored. **c** U2OS cells were transfected with ORC3 and ORC5 (WT or 0 K), Myc-geminin or empty vector and with a puromycin-resistance plasmid. The next day, transfected cells were selected in puromycin medium for 2 days, and grown for another day without selection. Cells were then pulsed with BrdU for 15 min and analysed by flow cytometry (upper panels). BrdU incorporation fluorescence signal was quantified from three independent experiments (lower left panel). Expression of ectopic proteins and endogenous PCNA was assessed by western blotting (lower right panels). ns, non-significant; $^*p < 0.01$. **d** U2OS cells were treated as in **c**. Chromatin and soluble fractions were isolated and analysed by western blotting with antibodies against the indicated proteins. **e** DNA fibre stretching analysis. U2OS cells were treated as in **c**. Cells were incubated with IdU (15 min; red) followed by CldU (15 min; green) and processed for DNA spreading. Representative images are shown. Scale bars, 5 µm. The length of CldU tracks after the IdU signal was measured. More than 200 measurements are shown from two independent experiments. Red bars indicate median values. ns, non-significant; $^*p < 0.01$

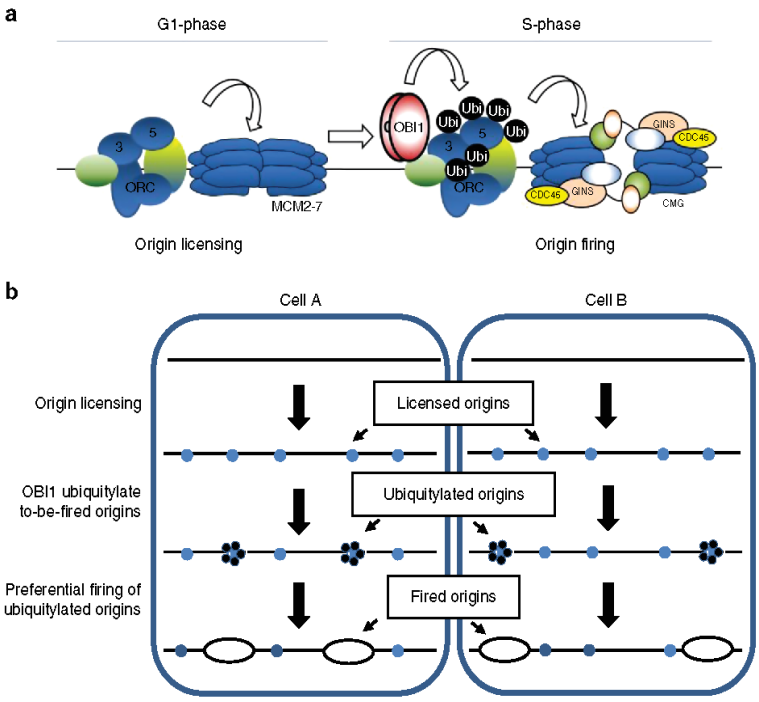


Fig. 6 Proposed role of OBI1 in DNA replication origin activation. **a** In the G1-phase of the cell cycle, potential origins are licensed independently of OBI1 action, leading to the chromatin recruitment of the replicative helicase MCM2/7 in an inactive state. In S-phase, OBI1 catalyses the multi-mono-ubiquitylation of origin-bound ORC complexes, a modification that promotes origin firing. **b** OBI1 might select the origins to be activated in S-phase among all the potential origins licensed in G1 by marking them through ORC3 and ORC5 multi-mono-ubiquitylation. OBI1-selected pool of origins might differ from cell to cell, explaining the relatively low firing efficiency of mammalian origins. This modification of the ORC complex could act by recruiting putative ubiquitin-binding activation factors, or alternatively by opening the local chromatin environment, making it more accessible to limiting firing factors

supernatants (i.e. Dignam extracts) were frozen in liquid nitrogen at stored at −80 °C. TAP-TAG purification was performed as previously described[42,37]. Briefly, 8–10 mL of Dignam nuclear extracts were incubated with 80–100 µL of anti-Flag agarose beads at 4 °C for 4 h on a rotary wheel. After extensive washes in HA-IP buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 10% glycerol, 0.1% Tween20, 0.05% NP-40, 1 mM DTT, 0.2 mM PMSF, 1 mM ATP), beads were eluted twice by competition using 200 ng/mL Flag peptide in 300 µL HA-IP buffer for 1 h at 4 °C. Pooled eluates were then incubated with 10 µL anti-HA beads at 4 °C for 2 h. After extensive washes in HA-IP buffer, complexes were eluted twice with 30 µL of the same buffer containing 400 ng/mL HA peptide for 1 h, as before. Purified complexes were then analysed by silver staining, western blotting or mass spectrometry.

Mass spectrometry analysis. Mass spectrometry (MS) analysis was performed at the Functional Proteomic Platform (IGF, Montpellier, France). TAP-TAG purified complexes were separated on NuPAGE gradient gels (Invitrogen) and stained using colloidal Coomassie (Biorad). Bands were excised, digested with trypsin (Promega) and processed for MS analysis. Samples (1 µl) were analysed online using a nanoflow HPLC system (Ultimate 3000, Dionex) coupled to a mass spectrometer with a nanoelectrospray source (LTQ-Orbitrap VELOS, Thermo Fisher Scientific). Peptides were separated on a capillary column (C18 reverse phase, Pepmap®, Dionex) with a gradient of 0–40% Buffer B in Buffer A for 150 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in 100% acetonitrile) at a flow rate of 300 nl/min. Spectral data were analysed using the Proteome Discoverer 1.2 software (Thermo Fisher Scientific) and Mascot (Matrix Science) version 2.3. The SwissProt CPS HUMAN databases, including the Cardation (M) modification, was used. The false discovery rate (FDR) was set at 1%. In Fig. 1b, interactions were visualised using Cytoscape 3.3.0 (cytoscape.org).

Protein biochemistry. To monitor protein expression, cells were lysed in Triton-lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 40 mM β-glycero-phosphate, 50 mM NaF, 1% Triton X-100 and protease inhibitors), as

described previously[38]. In brief, cells were washed in ice-cold PBS and lysed in Triton buffer for 30 min. on ice. The crude lysates were spun at 15,700 × *g* for 10 min. at 4 °C to obtain the final lysates. Protein concentration was evaluated using the BCA kit (Pierce). Soluble and chromatin fractions were obtained using the CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM Sucrose, 1 mM $MgCl_2$, 0.1–0.3% Triton X-100, 1 mM ATP, 0.5 mM DTT and protease inhibitors), as described previously[38]. In brief, cells were washed in ice-cold PBS and spun at 190 × *g* for 5 min at 4 °C. Cell pellets were resuspended in 100–500 µL CSK buffer for 10 min on ice. Crude lysates were spun in microcentrifuge tubes at 1000 × *g* at 4 °C for 3 min. The supernatants were collected and corresponded to the soluble fractions. The chromatin pellets were washed with the same volume of CSK buffer and incubated again 10 min. on ice. After another centrifugation, the supernatants were discarded, yielding the chromatin pellets. Both fractions were denatured in Laemmli sample buffer. For Flag-ORC/OBI1 co-immunoprecipitation experiments, cells were lysed in TK300 lysis buffer[43] (20 mM Tris-HCl pH 7.9, 300 mM KCl, 5 mM $MgCl_2$, 10% glycerol; 0.5% NP-40 plus protease inhibitors cocktail) on ice for 30 min. Equivalent amounts of protein samples were incubated at 4 °C with anti-Flag coupled agarose beads (10 µL) for 4 h. After extensive washing with TK300 lysis buffer, bound proteins were eluted in boiling Laemmli buffer. Samples were denatured in Laemmli sample buffer, ran on gradient gel (NuPAGE, Invitrogen) and transferred to nitrocellulose membrane. For western blot analysis, membranes were blocked with 5% milk in TBS/T buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween20). Primary antibodies were diluted in 2% BSA in TBS/T buffer and incubated overnight with the membranes. Western blots were revealed using ECL Plus (Pierce) chemiluminescence reagent. The shown western blots are representative of two to three independent experiments. Silver staining was done using the SilverQuest silver staining kit (Invitrogen), following the recommended procedures.

Uncropped blots of main figures can be found in Supplementary Figs. 11, 12, 13 and 14.

Ubiquitylation-related assays. For native purification of ubiquitylated proteins, cells were lysed in Triton X-lysis buffer containing 10mM *N*-ethylmaleimide (NEM), as described previously[44]. Equal amounts of protein samples were incubated with anti-Flag agarose beads (10 µL) at 4 °C for 4 h. After extensive washes in lysis buffer, samples were analysed by western blotting. For purification of ubiquitylated proteins in denaturing conditions, cells were lysed in 500 µL of denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris base, 6 M guanidine hydrochloride, 10 mM imidazole, pH 8.0) for 30 min. on ice. Lysates viscosity was reduced by trituration pipetting. Clarified lysates were incubated with 10 µL Ni-NTA beads (Qiagen) at 4 °C for 2–3 h, and washed in denaturing washing buffer (100 mM NaH_2PO_4, 150 mM NaCl, 8 M urea, 22.5 mM imidazole, pH 8.0) 5 times. Samples were eluted in Laemmli buffer and analysed by western blotting. For purification of ORC3 and ORC5 from chromatin and soluble fractions, cells were processed using the CSK buffer as described above. Equivalent amounts of chromatin and solubles fractions (~100 µL) were denatured in denaturing lysis buffer (500 µL) and ORC subunits were purified as stated above. The UbiCRest assay was performed following the recommended conditions (Boston Biochem). Briefly, Myc-Flag-ORC3 and ORC5 transiently expressed in U2OS cells were purified in native conditions using 10 µL of Flag-beads, as described above. After extensive washes in lysis buffer followed by PBS, beads were incubated with a panel of deubiquitylases (DUBs) at 37 °C for 60 min. in supplied reaction buffer. Samples were vortexed and centrifuged to collect the supernatants. Beads and supernatants were analysed by western blotting and silver staining, respectively. To control for DUBs activity, Xenopus egg extract[45] were supplemented with recombinant HA-ubiquitin (200 ng/mL) and ATP (10 mM) for 15 min. at 30 °C. 10 µg of extracts were incubated with individual DUBs in 20 µL reaction buffer for 60 min. at 37 °C. Samples were denatured in Laemmli buffer and analysed by anti-HA western blot. For in vitro OBI1 ubiquitylation assay (based on)[46] Flag-OBI1 was immunoprecipitated from transiently transfected cells using 10 µL Flag-beads in TK300 lysis buffer supplemented with 2 mM DTT. After extensive washing in TK300 buffer, beads were washed twice with ubiquitylation reaction buffer (40 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT). Myc-tagged ORC3 and ORC5 cloned in pCS3 were in vitro translated using SP6 polymerase and cold aminoacids provided in the TNT kit as specified by the manufacturer. Purified OBI1 on beads was incubated with ORC substrates (1–3 µL), E1 (100 ng), UbcH5a (1 µM), wild type or lysine-less ubiquitin (1 mg/mL), ubiquitin-aldehyde (1 µM) and ATP (10 mM) in ubiquitylation reaction buffer (20 µL final) for 30 min. at 30 °C with agitation. Reactions were stopped with Laemmli buffer and analysed by western blotting.

Flow cytometry. For cell cycle analysis, ethanol-fixed cells were incubated with RNase A and propidium iodide, and analysed by flow cytometry using a MACSQuant cytometer (Miltenyi) as described[38]. Alternatively, cells were pulsed with 100 nM BrdU for 15 min prior to fixing. After HCl denaturation, BrdU incorporation was detected using a mouse anti-BrdU antibody followed by an anti-mouse FITC-coupled antibody. BrdU incorporation was quantified by measuring the fluorescence intensity of BrdU-positive cells. The value of control cells (siMock) was normalised to 100%. The fluorescence value of BrdU-positive cells from experimental conditions was expressed as the percentage of fluorescence relative to that of control cells. The summary graph is the mean of three independent experiments.

DNA fibre analysis. *Plugs preparation*: Asynchronous U2OS cells transfected with siRNAs for three days were labelled with two modified nucleosides: IdU and CldU. Cells were sequentially labelled with 30 µM IdU and 300 µM CldU for 20 min each, without intermediate washing. After labelling, cells were immediately placed on ice to stop DNA replication. Cells were then centrifuged (300 × *g* at 4 °C for 5 min) and washed three times with 1 × phosphate-buffered saline (PBS); 3×10^6 cells were resuspended in 100 µl of 1 × PBS with 1% low-melting agarose to embed cells in agarose plugs. Plugs were incubated in 2 mL of proteinase K buffer (10 mM Tris-Cl, pH 7.0, 100 mM EDTA, 1% N-lauryl-sarcosyl and 2 mg/mL proteinase K) at 45 °C for 2 days (fresh solution was added on the second day).

DNA molecular combing: Digested proteins and other degradation products were completely removed by washing the plugs several times in TE_{50} buffer (50 mM EDTA, 10 mM Tris-Cl, pH 7.0). Protein-free DNA plugs were then stored in TE_{50} buffer at 4 °C, or used immediately for combing. Agarose plugs were stained with the YOYO-1 fluorescent dye (Molecular Probes) in TE_{50} buffer for 1 h, washed few times with TE_{50} buffer, resuspended in 100 µl of TE_{50} buffer and melted at 65 °C for 15 min. The solution was maintained at 42 °C for 15 min, and then 10 units of β-agarase (Sigma Aldrich) was added overnight. After digestion, 4 ml of 50 mM MES (2-(*N*-morpholino) ethanesulfonic acid, pH 5.7) was added very gently to the DNA solution and then DNA fibres were combed and regularly stretched (2 kb/µm) on silanised coverslips, as described previously[47]. Combed DNA was fixed at 65 °C for at least 2 h, denatured in 1 N NaOH for 20 min, and washed several times in 1 × PBS. After denaturation, silanised coverslips with DNA fibres were blocked with 1% BSA and 0.1% Triton X100 in PBS. Immuno-detection was done with antibodies diluted in 1 × PBS, 0.1% Triton X100, 1% BSA and incubated at 37 °C in a humid chamber for 60 min. Each incubation step with antibodies was followed by extensive washes with 1 × PBS. Immuno-detection was done with an anti-ssDNA antibody (1/100 dilution, Merck Milipore), the mouse (1/20 dilution, clone B44 from Becton Dickinson) and rat anti-BrdU antibodies (1/20 dilution, clone BU1/75 Thermo Fisher Scientific) that recognise the IdU and CldU tracks, respectively. The mouse anti-BrdU antibody, clone B44, is derived from hybridisation of mouse Sp2/0-Ag14 myeloma cells with spleen cells from BALB/c mice immunised with iodouridine-conjugated ovalbumin. It reacts with iodouridine and BrdU[48,49]. The rat anti-BrdU antibody, clone BU1/75 (ICR1), cross-reacts with CldU, but not with thymidine or iododeoxyuridine[49]. The secondary antibodies were: goat anti-rat antibody coupled to Alexa 488 (1/50 dilution, Molecular Probes), goat anti-mouse IgG1 coupled to Alexa 546 (1/50 dilution, Molecular Probes), and goat anti-mouse IgG2a coupled to Alexa 647 (1/100 dilution, Molecular Probes). Coverslips were mounted with 20 µl of Prolong Gold Antifade (Molecular Probes), dried at room temperature for 12 h, and processed for image acquisition using a fully motorised Leica DM6000 microscope equipped with a CoolSNAP HQ2 1 CCD camera and controlled by MetaMorph (Roper Scientific). Images were acquired with a ×40 objective, where 1 pixel corresponds to 335 bp. As at the ×40 magnification, one microscope field of view corresponds to ~450 kb, observation of longer DNA fibres required the capture of adjacent fields. Inter-origin distances and replication fork speed were measured manually using the MetaMorph software. Statistical analyses of inter-origin distances and replication fork speed were performed using Prism 5.0 (GraphPad).

DNA spreading was performed as previously described[50–52]. Briefly, cells were pulsed with 20 µM iodo-deoxyuridine (IdU) for 15 min. followed by 200 µM chloro-deoxyuridine (CldU) for 15 min, trypsinised and counted. Two thousand cells were lysed in SDS spreading buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.5% SDS) and deposited on glass slides (StarFrost). The slides were tilted slightly and the drops were allowed to run down the slides slowly, then air dried, fixed in 3:1 methanol:acetic acid for 10 min, and allowed to dry. Glass slides were processed for immunostaining as for DNA combing analysis. Coverslips were mounted with Prolong Gold anti-fading agent. Images were acquired by immunofluorescence microscopy (Leica DM6000 or Zeiss ApoTome, RIO imaging facility) and a Coolsnap HQ CCD camera 25 (Photometrics), and analysed with the Metamorph software (Molecular Devices). Fork speed was measured in more than 200 forks using the ImageJ software.

Statistical analysis. No statistical methods were used to predetermine sample size. The experiments were not randomised, and investigators were not blinded to allocation during experiments and outcome assessment. Replication fork speed was estimated for individual forks with IdU track flanked by a CldU track. Only uninterrupted forks, as confirmed by DNA counterstaining, were analysed. Inter-origin distances were measured as the distance (kb) between the centres of two adjacent progressing forks located on the same DNA fibre. For the analysis of fork density, images were acquired in an unbiased manner (randomly), without first searching for the presence of IdU-CldU tracts. GraphPad Prism (GraphPad Software) and Excel (Microsoft) were used to generate graphs and perform statistical analyses. DNA replication parameters generally do not display a Gaussian distribution[53]. Therefore, distributions were compared using the non-parametric Mann–Whitney two-tailed test that does not assume a Gaussian distribution. For statistical significance, differences between two experimental groups were examined using Student's *t*-test. Statistical significance was set at $^*P \le 0.01$, $^{**}P \le 0.001$.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this study are available within the article and its supplementary information files. All data are available from the corresponding authors upon reasonable request.

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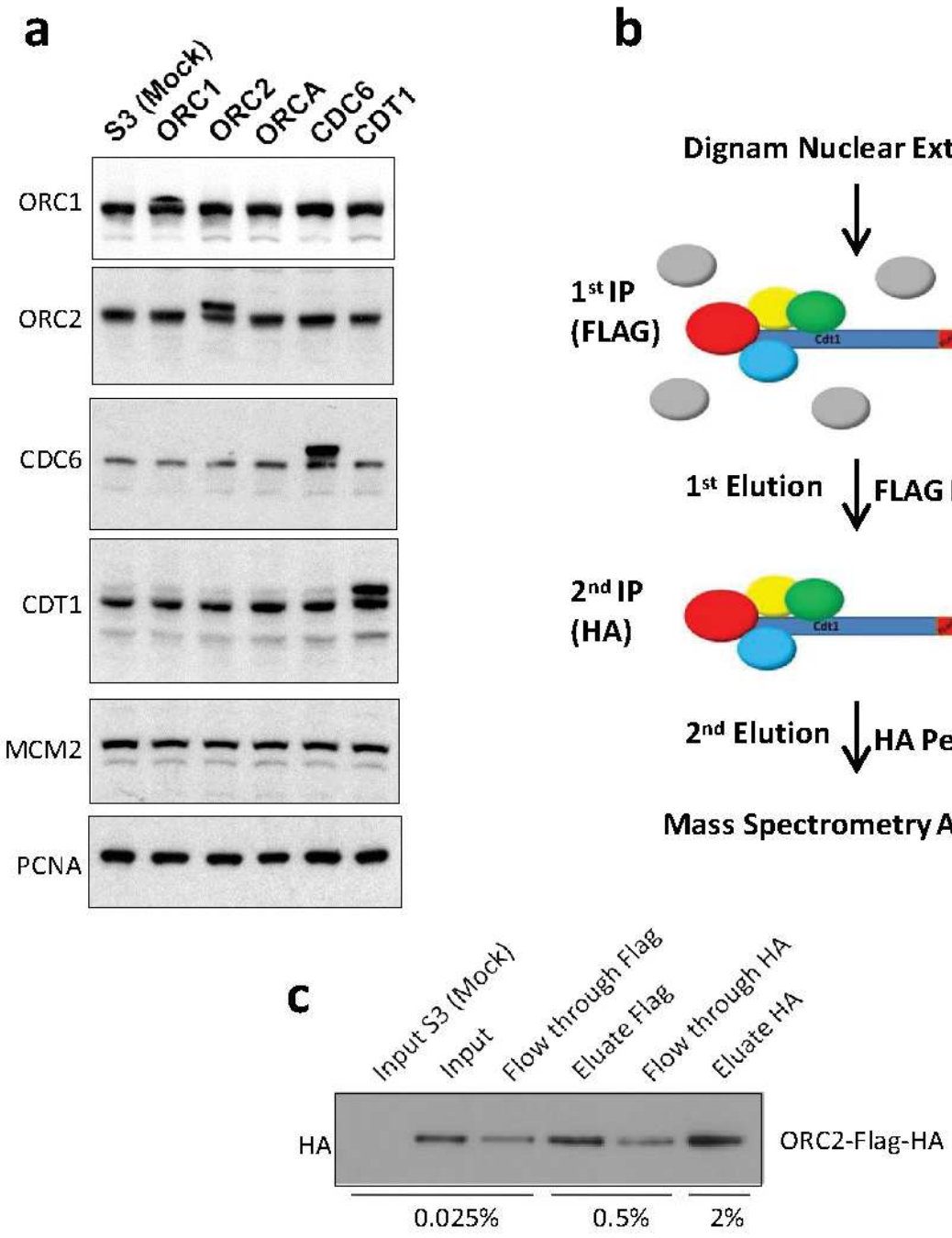
References

1. Pragkos, M., Ganier, O., Coulombe, P. & Mechali, M. DNA replication origin activation in space and time. *Nat. Rev. Mol. Cell Biol.* **16**, 360–374 (2015).
2. Marks, A. B., Smith, O. K. & Aladjem, M. I. Replication origins: determinants or consequences of nuclear organization? *Curr. Opin. Genet. Dev.* **37**, 67–75 (2016).
3. Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N. & Oda, M. Eukaryotic chromosome DNA replication: where, when, and how? *Annu. Rev. Biochem.* **79**, 89–130 (2010).
4. Riera, A. et al. From structure to mechanism-understanding initiation of DNA replication. *Genes Dev.* **31**, 1073–1088 (2017).
5. Bleichert, F., Botchan, M. R. & Berger, J. M. Crystal structure of the eukaryotic origin recognition complex. *Nature* **519**, 321–326 (2015).
6. Heller, R. C. et al. Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. *Cell* **146**, 80–91 (2011).
7. Ilves, I., Petojevic, T., Pesavento, J. J. & Botchan, M. R. Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. *Mol. Cell* **37**, 247–258 (2010).
8. Dijkwel, P. A. & Hamlin, J. L. The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. *Mol. Cell Biol.* **15**, 3023–3031 (1995).
9. Cayrou, C. et al. Genome-scale analysis of metazoan replication origins reveals their organization in specific but flexible sites defined by conserved features. *Genome Res.* **21**, 1438–1449 (2011).
10. DePamphilis, M. L. Origins of DNA replication that function in eukaryotic cells. *Curr. Opin. Cell Biol.* **5**, 434–441 (1993).
11. Mechali, M. Eukaryotic DNA replication origins: many choices for appropriate answers. *Nat. Rev. Mol. Cell Biol.* **11**, 728–738 (2010).
12. Rhodes, D. R. et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* **6**, 1–6 (2004).
13. Todaro, G. J. & Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17**, 299–313 (1963).
14. Sansam, C. G., Goins, D., Siefert, J. C., Clowdus, E. A. & Sansam, C. L. Cyclin-dependent kinase regulates the length of S phase through TICRR/TRESLIN phosphorylation. *Genes Dev.* **29**, 555–566 (2015).
15. Kumagai, A. & Dunphy, W. G. MTBP, the partner of Treslin, contains a novel DNA-binding domain that is essential for proper initiation of DNA replication. *Mol. Biol. Cell* **28**, 2998–3012 (2017).
16. Zhong, Y. et al. The level of origin firing inversely affects the rate of replication fork progression. *J. Cell Biol.* **201**, 373–383 (2013).
17. Montagnoli, A. et al. A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. *Nat. Chem. Biol.* **4**, 357–365 (2008).
18. Cottineau, J. et al. Inherited GINS1 deficiency underlies growth retardation along with neutropenia and NK cell deficiency. *J. Clin. Invest.* **127**, 1991–2006 (2017).
19. Boos, D., Yekezare, M. & Diffley, J. F. Identification of a heteromeric complex that promotes DNA replication origin firing in human cells. *Science* **340**, 981–984 (2013).
20. Li, C. J. & DePamphilis, M. L. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. *Mol. Cell Biol.* **22**, 105–116 (2002).
21. Mendez, J. et al. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Mol. Cell* **9**, 481–491 (2002).
22. Wagner, S. A. et al. A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteom.* **10**, M111–013284 (2011).
23. Kim, W. et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* **44**, 325–340 (2011).
24. Sadowski, M., Suryadinata, R., Tan, A. R., Roesley, S. N. & Sarcevic, B. Protein monoubiquitination and polyubiquitination generate structural diversity to control distinct biological processes. *IUBMB Life* **64**, 136–142 (2012).
25. Jadhav, T. & Wooten, M. W. Defining an embedded code for protein ubiquitination. *J. Proteomics Bioinform.* **2**, 316 (2009).
26. Hospenthal, M. K., Mevissen, T. E. T. & Komander, D. Deubiquitinase-based analysis of ubiquitin chain architecture using Ubiquitin Chain Restriction (UbiCRest). *Nat. Protoc.* **10**, 349–361 (2015).
27. Wirbelauer, C. et al. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. *EMBO J.* **19**, 5362–5375 (2000).
28. Ranaweera, R. S. & Yang, X. Auto-ubiquitination of Mdm2 enhances its substrate ubiquitin ligase activity. *J. Biol. Chem.* **288**, 18939–18946 (2013).
29. McGarry, T. J. & Kirschner, M. W. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**, 1043–1053 (1998).
30. Petersen, B. O. et al. Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. *Genes Dev.* **14**, 2330–2343 (2000).
31. Sugimoto, N. et al. Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. *J. Biol. Chem.* **279**, 19691–19697 (2004).
32. Arias, E. E. & Walter, J. C. PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. *Nat. Cell Biol.* **8**, 84–90 (2006).
33. Maric, M., Maculins, T., De Piccoli, G. & Labib, K. Cdc48 and a ubiquitin ligase drive disassembly of the CMG helicase at the end of DNA replication. *Science* **346**, 1253596 (2014).
34. Moreno, S. P., Bailey, R., Campion, N., Herron, S. & Gambus, A. Polyubiquitylation drives replisome disassembly at the termination of DNA replication. *Science* **346**, 477–481 (2014).
35. Nishiyama, A. et al. Uhrf1-dependent H3K23 ubiquitylation couples maintenance DNA methylation and replication. *Nature* **502**, 249–253 (2013).
36. Giri, S., Chakraborty, A., Sathyan, K. M., Prasanth, K. V. & Prasanth, S. G. Orc5 induces large-scale chromatin decondensation in a GCN5-dependent manner. *J. Cell Sci.* **129**, 417–429 (2016).
37. Nakatani, Y. & Ogryzko, V. Immunoaffinity purification of mammalian protein complexes. *Methods Enzymol.* **370**, 430–444 (2003).
38. Coulombe, P., Gregoire, D., Tsanov, N. & Mechali, M. A spontaneous Cdt1 mutation in 129 mouse strains reveals a regulatory domain restraining replication licensing. *Nat. Commun.* **4**, 2065 (2013).
39. Sobhian, B. et al. RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science* **316**, 1198–1202 (2007).
40. Morgenstern, J. P. & Land, H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**, 3587–3596 (1990).
41. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
42. Traver, S. et al. MCM9 is required for mammalian DNA mismatch repair. *Mol. Cell* **59**, 831–839 (2015).
43. Ghosh, S., Vassilev, A. P., Zhang, J., Zhao, Y. & DePamphilis, M. L. Assembly of the human origin recognition complex occurs through independent nuclear localization of its components. *J. Biol. Chem.* **286**, 23831–23841 (2011).
44. Coulombe, P., Rodier, G., Pelletier, S., Pellerin, J. & Meloche, S. Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. *Mol. Cell Biol.* **23**, 4542–4558 (2003).
45. Nishiyama, A., Frappier, L. & Mechali, M. MCM-BP regulates unloading of the MCM2-7 helicase in late S phase. *Genes Dev.* **25**, 165–175 (2011).
46. Rodier, G., Coulombe, P., Tanguay, P. L., Boutonnet, C. & Meloche, S. Phosphorylation of Skp2 regulated by CDK2 and Cdc14B protects it from degradation by APC(Cdh1) in G1 phase. *EMBO J.* **27**, 679–691 (2008).
47. Michalet, X. et al. Dynamic molecular combing: stretching the whole human genome for high-resolution studies. *Science* **277**, 1518–1523 (1997).
48. Gratzner, H. G. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**, 474–475 (1982).
49. Elovaara, H. et al. Primary amine oxidase of Escherichia coli Is a metabolic enzyme that can use a human leukocyte molecule as a substrate. *PLoS ONE* **10**, e0142367 (2015).
50. Lutzmann, M. et al. MCM8- and MCM9-deficient mice reveal gametogenesis defects and genome instability due to impaired homologous recombination. *Mol. Cell* **47**, 523–534 (2012).
51. Jackson, D. A. & Pombo, A. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J. Cell Biol.* **140**, 1285–1295 (1998).
52. Coquel, F. et al. SAMHD1 acts at stalled replication forks to prevent interferon induction. *Nature* **557**, 57–61 (2018).
53. Bianco, J. N. et al. Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. *Methods* **57**, 149–157 (2012).

ORC ubiquitin ligase OBI1 promotes DNA replication origin firing

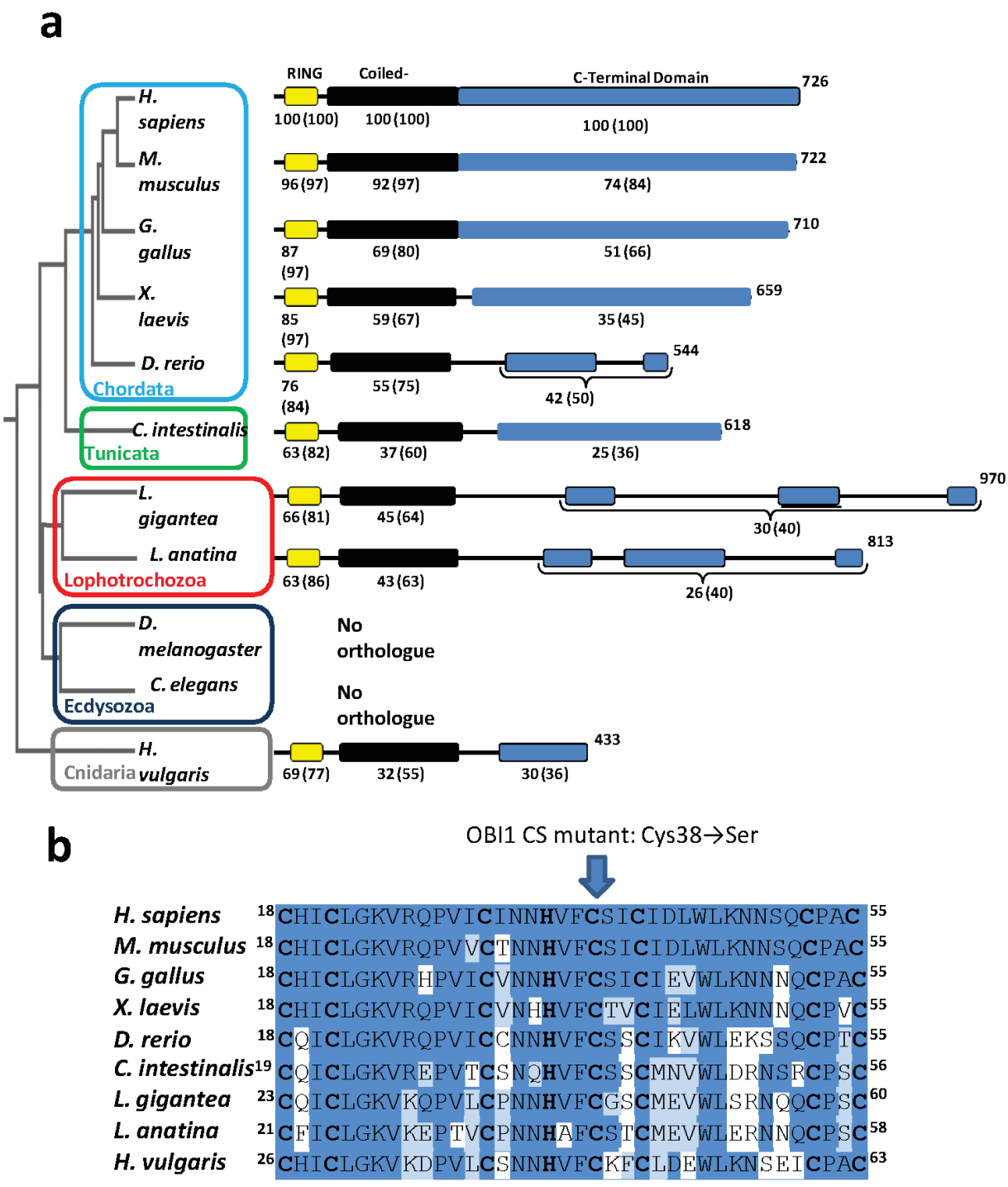
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Supplementary Information



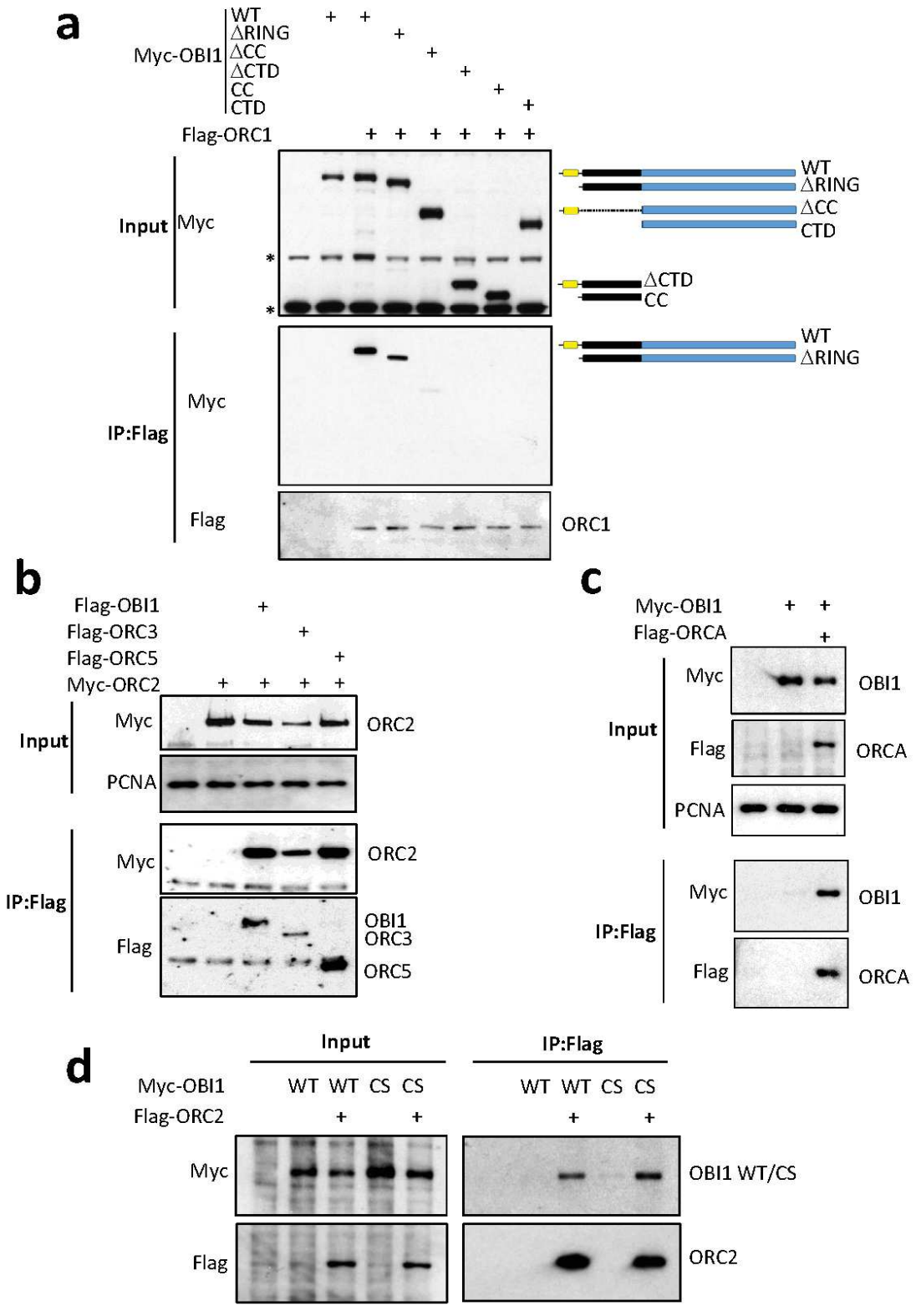
Supplementary Figure 1: Characterization of the TAP-TAG procedure

a) Expression level of pre-RC baits. Dignam extracts from parental HeLa S3 cells (Mock) and from cells transduced to express the indicated pre-RC factors were analysed by western blotting. **b)** Schematic representation of the TAP-TAG purification procedure. **c)** TAP-TAG purification efficiency. A Dignam extract from ORC2-Flag-HA expressing HeLa S3 cells was processed for TAP-TAG purification. Aliquots (in percentage) of fractions collected during the procedure were analysed by western blotting.



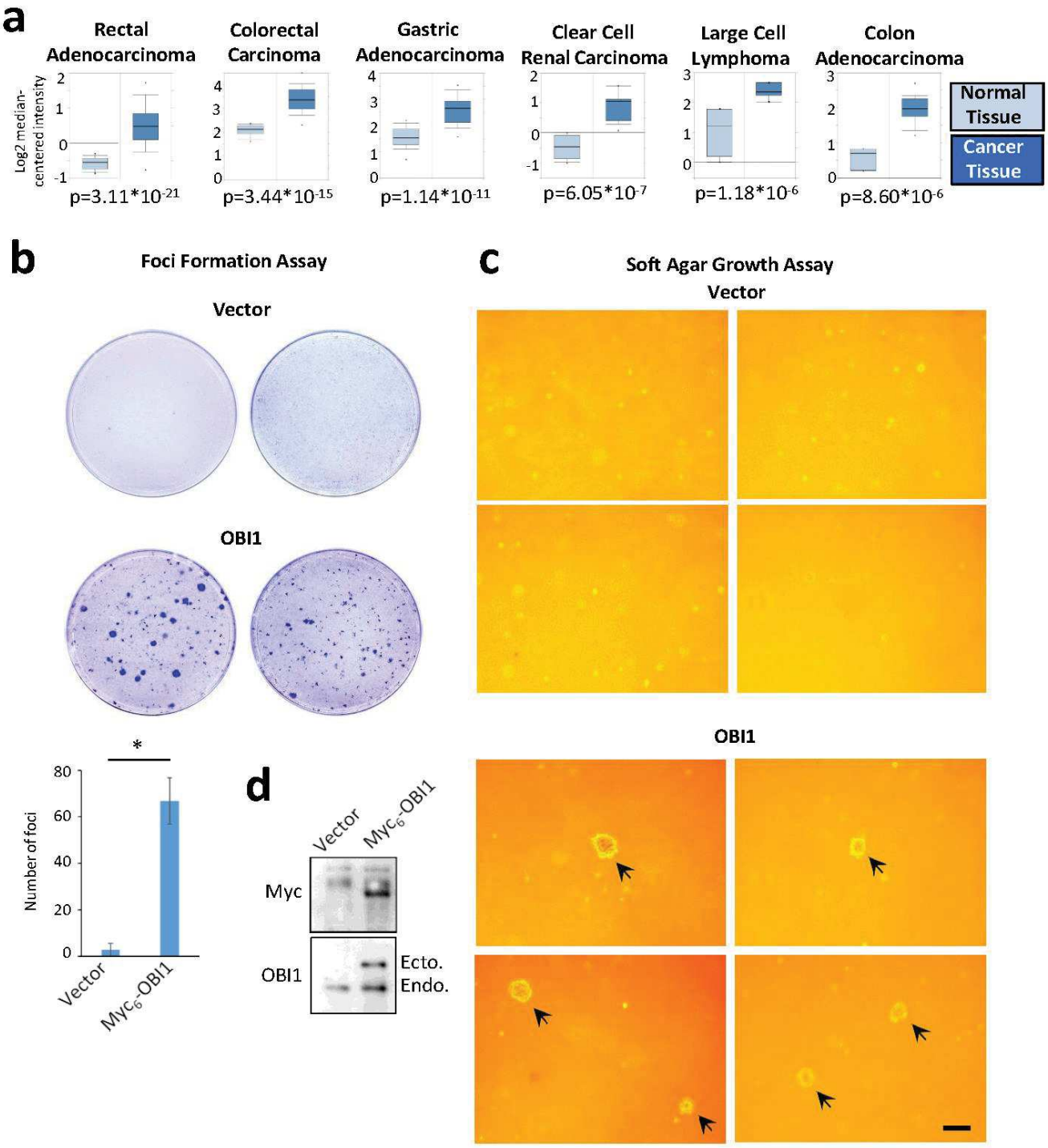
Supplementary Figure 2: OBI1 conservation

a, Phylogenetic tree showing the evolutionary relationship between species harbouring OBI1 homologues (left panel). Schematic representation of OBI1 homologues showing the conserved domains. The percentage of identity and similarity (in brackets) of OBI1 homologues compared with the human isoform is indicated. Phylogenetic tree and sequence alignments were generated using the SuperFamily (supfam.cs.bris.ac.uk) and BLAST (blast.ncbi.nlm.nih.gov) servers, respectively. **b,** Alignment of the RING domain of OBI1 homologues. Residues conserved relative to the human isoform are highlighted. Residues involved in coordinating the two zinc ions are in bold. The OBI1 RING domain point mutant Cys38Ser is highlighted.



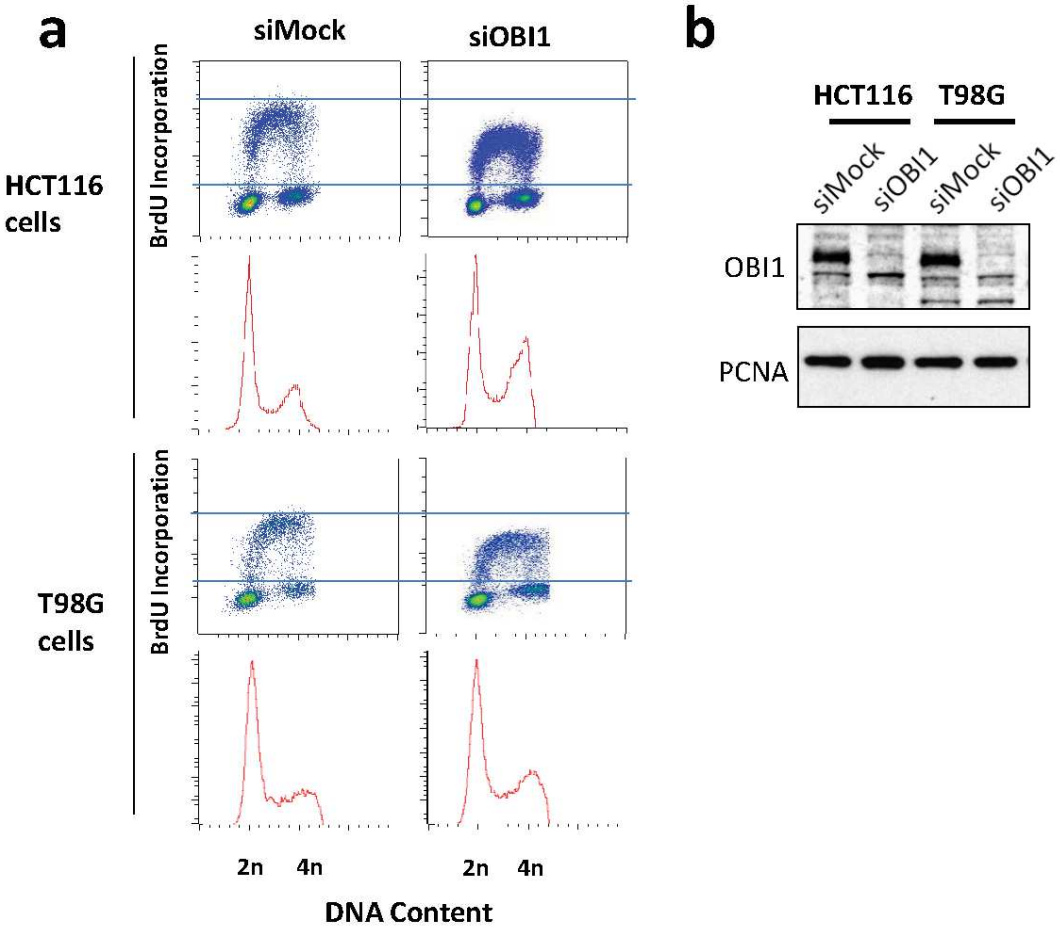
Supplementary Figure 3: Characterization of OBI1-ORC interaction

a-d, Association of OBI1 with the ORC complex. U2OS cells were transfected with tagged versions of ORC subunits and OBI1, as indicated. Input lysates and Flag-immunoprecipitates were analysed by western blotting.

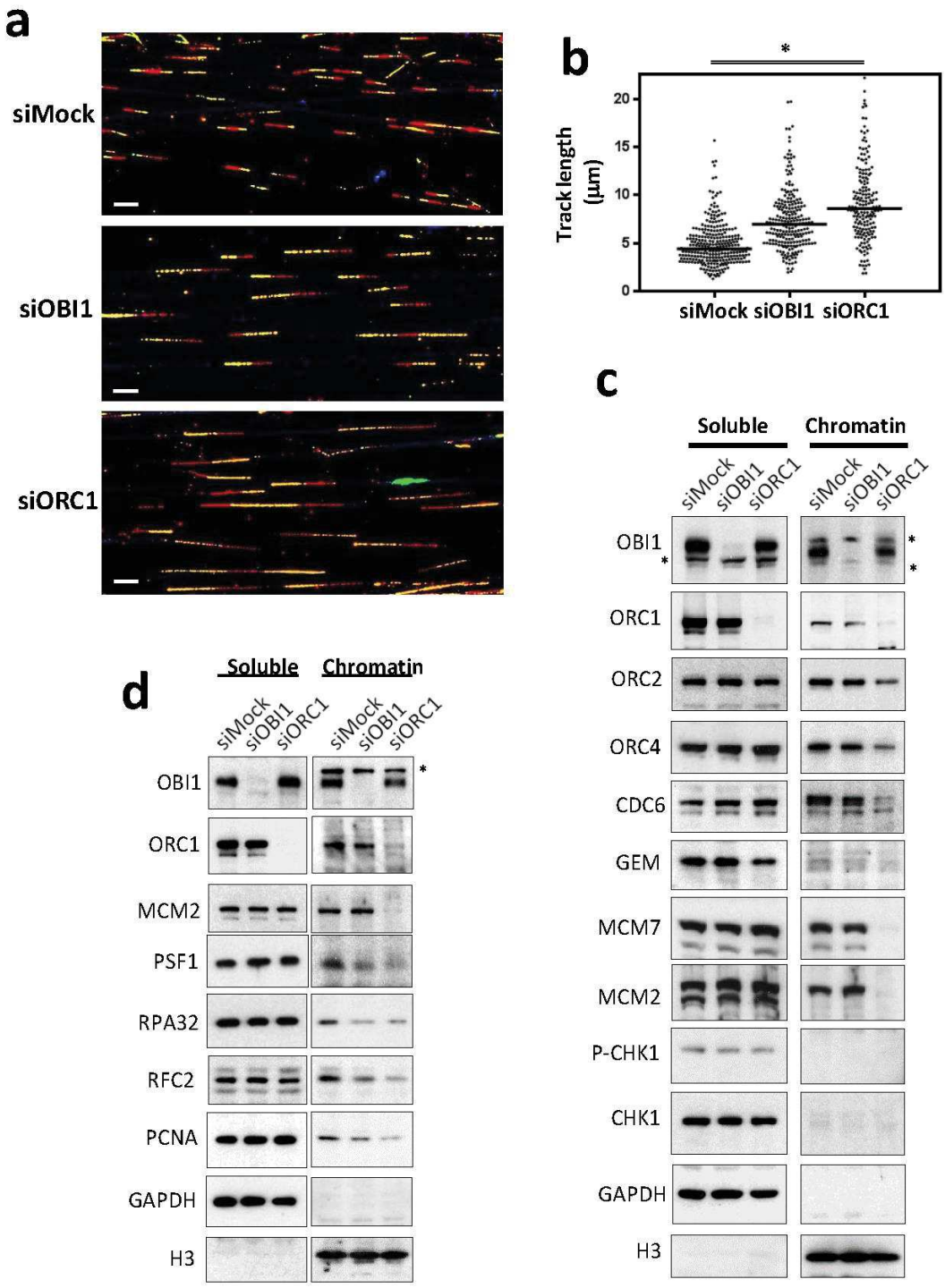


Supplementary Figure 4: OBI1 involvement in oncogenic transformation

a, OBI1 expression in human primary tumour samples and matched normal tissues was examined using the ONCOMINE server (oncomine.org). Cases in which expression was significantly different between tumour and normal tissue are shown. P-values are indicated. **b-d,** NIH 3T3 cells were stably transfected with empty vector or OBI1 expression plasmid and a puromycin-resistance plasmid. The oncogenic properties of cell populations were evaluated by foci formation assay (**b**, quantified in lower left panel) and soft agar growth assay (**c**). Arrows show OBI1-expressing colonies growing in soft agar. Scale bar, 300 mm. Endogenous (Endo) and ectopic (Ecto) OBI1 expression was evaluated in puromycin-selected cell cultures by western blotting (**d**).

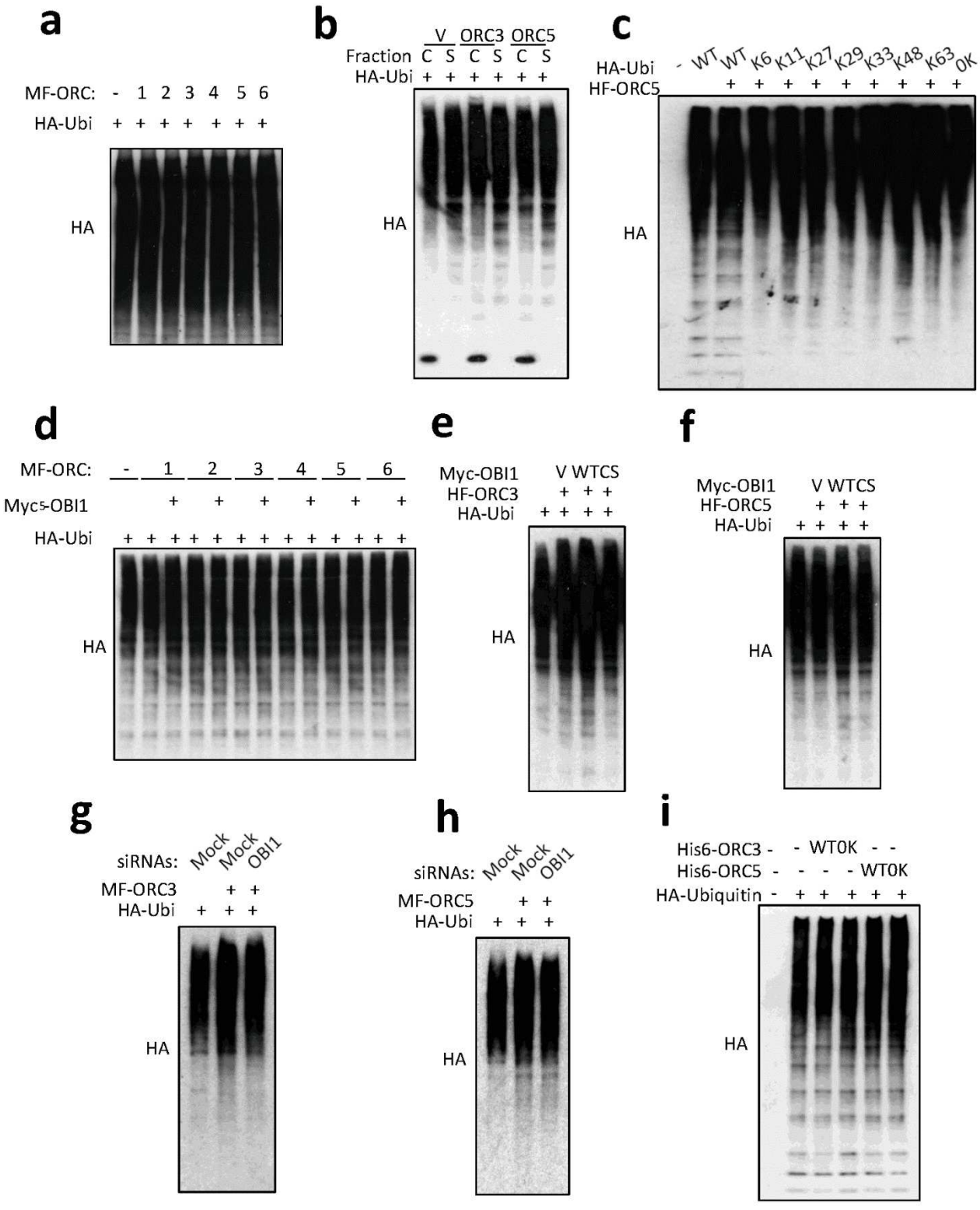


Supplementary Figure 5: OBI1 depletion in HCT116 and T98G cells results in DNA synthesis defects
a, Human colon cancer HCT116 and non-transformed hTERT-immortalized RPE1 cells were transfected with siRNAs targeting OBI1 (siOBI1) or mock (siMock), as indicated. Three days after transfection, cells were pulsed with BrdU for 15min and processed for flow cytometry analysis. Lines delimiting BrdU-positive siMock-treated cells are shown. **b,** Expression of endogenous OBI1 and PCNA was analysed in aliquots of cells described in **a** by western blotting.

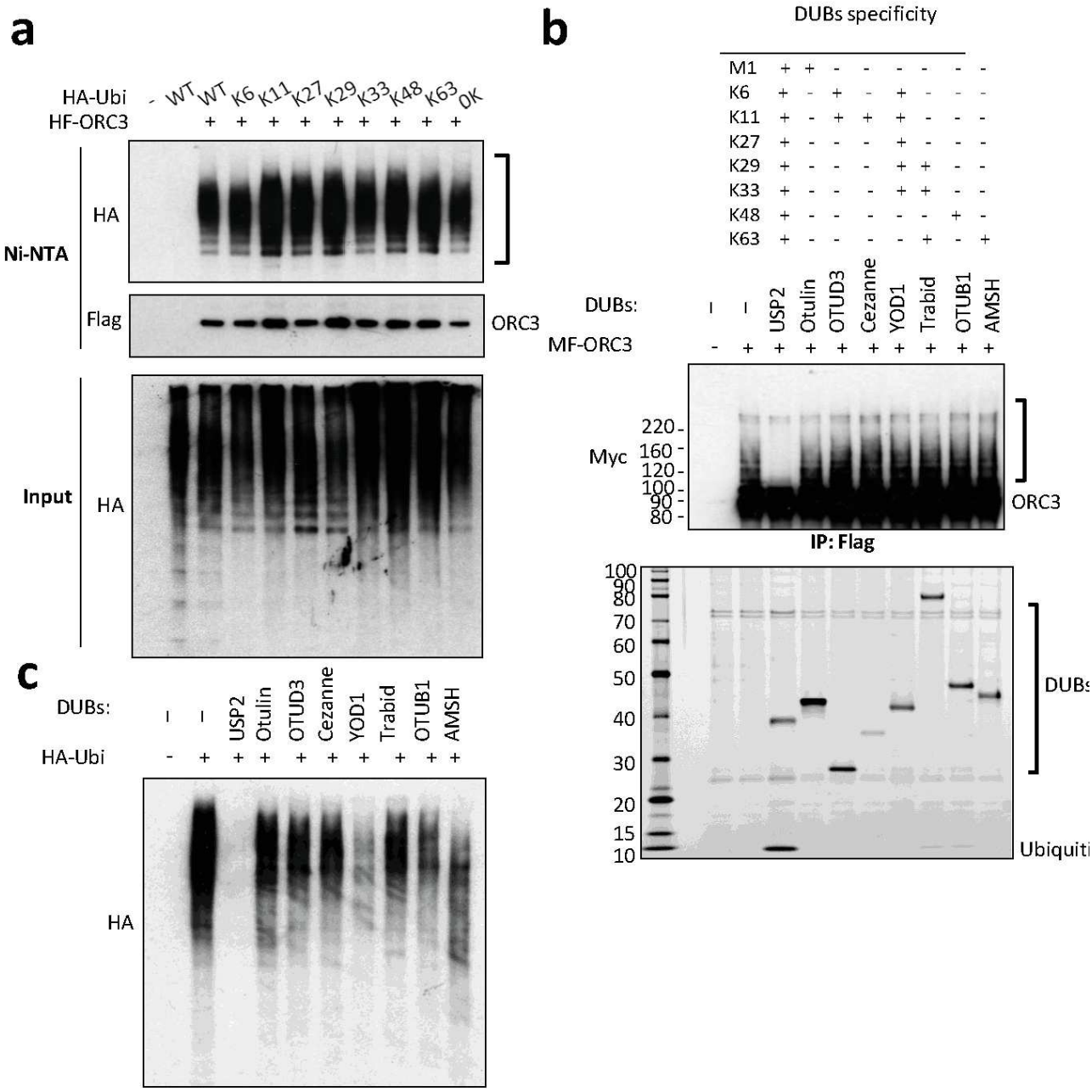


Supplementary Figure 6: OBI1 involvement in origin activation

a, DNA fibre stretching analysis. U2OS cells were transfected with Mock, *OBI1*- or *ORC1*-specific siRNAs. Three days later, cells were incubated with IdU (15min; red) followed by CldU (15min; green) and processed for DNA spreading. Representative images are shown. Scale bars, 5 mm. **b,** Fork speed analysis in Mock, *OBI1* and *ORC1* siRNA-transfected cells. The length of CldU tracks after the IdU signal was measured. More than 200 measurements are shown from two independent experiments. Bar indicates the median length. **c-d,** OBI1 is involved in origin firing, but not in origin licensing. U2OS cells were transfected with Mock, *OBI1*- or *ORC1*-specific siRNAs. Three days later, chromatin and soluble fractions were isolated and analysed by western blotting with antibodies against the indicated proteins. Asterisks mark non-specific bands.

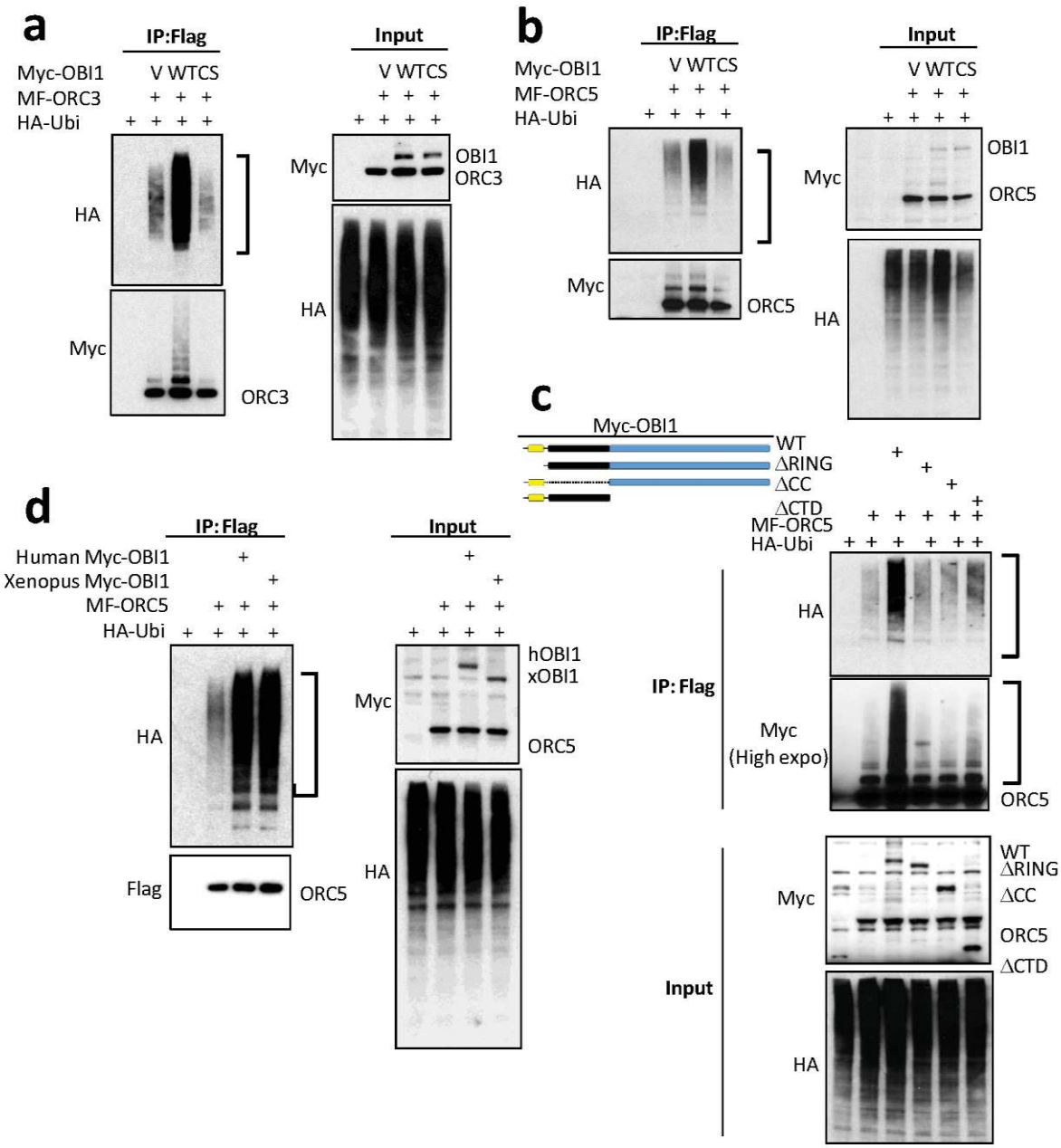


Supplementary Figure 7: Characterization of ORC ubiquitylation; complementary to Figure 3-5
a-c, Expression of HA-tagged ubiquitin in the experiments described in Figure 3a-c was evaluated by western blotting of input extracts. **d-h,** Expression of HA-tagged ubiquitin in the experiments described in Figure 4a-e was evaluated by western blotting of input extracts. **i,** Expression of HA-tagged ubiquitin in the experiment described in Figure 5a was evaluated by western blotting of input extracts.



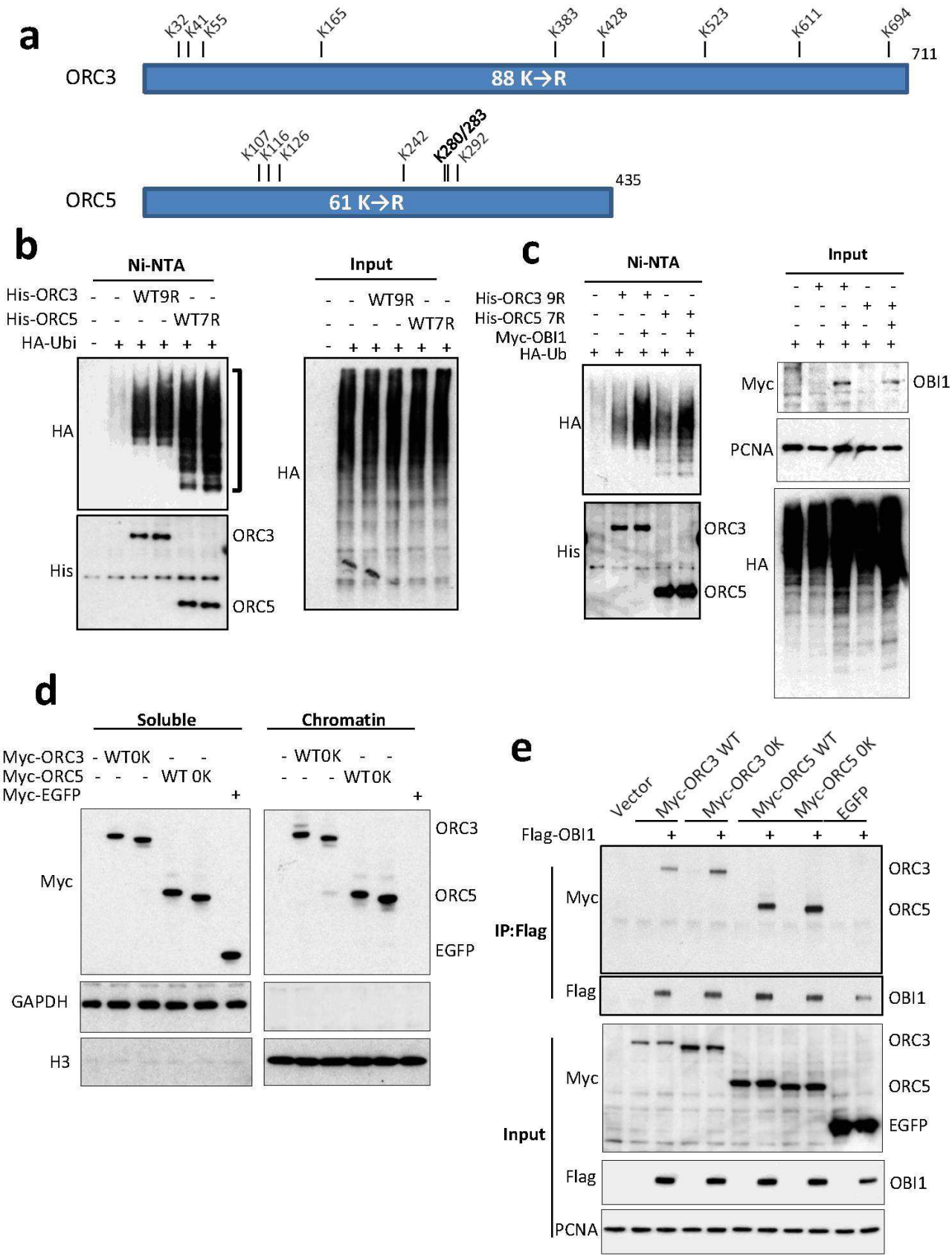
Supplementary Figure 8: Multi-mono-ubiquitylation of ORC3

a, U2OS cells were co-transfected with HF-ORC3 and the indicated HA-tagged ubiquitin single-lysine (K) mutants. In 0K ubiquitin, all lysine residues are replaced by arginine residues. Cells were lysed in denaturing conditions for His_6-tagged protein purification. Expression and ubiquitin conjugation of isolated proteins were monitored by western blotting, as indicated. **b,** UbiCRest analysis of ubiquitylated ORC3. U2OS cells were transfected with Myc-Flag (MF)-tagged ORC3 (A), without ectopic ubiquitin. Two days post-transfection, cells were lysed in NEM-containing buffer and Flag-tagged proteins were immunoprecipitated. After extensive washes, proteins bound to beads were incubated with the indicated deubiquitylating enzymes (DUBs), as specified by the manufacturer. At the end of the incubation, supernatants were recovered and analysed by silver staining. DUBs and released endogenous ubiquitin are indicated. Ubiquitylation was revealed by the presence of high molecular weight forms detected by western blotting against tagged-ORC3. **c,** UbiCRest assay on cellular ubiquitylated proteins. DUBs were incubated with extract expressing HA-ubiquitin as indicated. Ubiquitylation was revealed by anti-HA western blotting. Note the partial digestion of ubiquitylated proteins by several DUBs showing their activity.



Supplementary Figure 9: Characterization of OBI1 ubiquitin ligase activity

a-b, U2OS cells were co-transfected with Myc-Flag (MF)-tagged ORC3 or ORC5, HA-ubiquitin and wild type (WT) OBI1 or the C38S (CS) mutant, as indicated. Two days post-transfection, cells were lysed in NEM-containing lysis buffer and tagged proteins were purified by immunoprecipitation. Expression and ubiquitin conjugation of the immunoprecipitates were monitored by western blotting, as indicated. Expression of tagged ORC and OBI1 was monitored in input extracts by western blotting. **c,** U2OS cells were co-transfected with Myc-Flag (MF)-ORC5 and the indicated OBI1 variants along with HA-ubiquitin. Two days post-transfection, cells were lysed in NEM-containing lysis buffer and ORC5 was purified by immunoprecipitation. Ubiquitin conjugation of immunoprecipitates and expression of ectopic proteins were monitored by western blotting, as indicated. **d,** U2OS cells were co-transfected with Myc-Flag (MF)-tagged ORC5 and human or *X. laevis* Myc-tagged OBI1 and HA-tagged ubiquitin. Two days post-transfection, cells were lysed in NEM-containing lysis buffer and ORC5 was purified by immunoprecipitation. Ubiquitin conjugation of immunoprecipitates and expression of ectopic proteins were monitored by western blotting, as indicated.



Supplementary Figure 10: Generation and characterization of non-ubiquitylable ORC3 and ORC5 variants

Supplementary Figure 10: Generation and characterization of non-ubiquitylable ORC3 and ORC5 variants

a, The lysine residues modified by ubiquitylation on ORC3 (n=9) and ORC5 (n=7) identified by large-scale mass spectrometry-based approaches (see main text for references) are highlighted. Also, the number of lysine to arginine (K→R) substitutions introduced in the 0K variant of ORC3 and ORC5 is indicated. **b,** U2OS cells were co-transfected with His_6-tagged wild type (WT) ORC3 and ORC5 or mutants for identified ubiquitylation sites (ORC3-9R and ORC5-7R) and with HA-ubiquitin, as indicated. Two days post-transfection, cells were lysed in denaturing conditions for His_6-tagged protein purification. Expression and ubiquitin conjugation of isolated proteins were monitored by western blotting, as indicated. **c,** U2OS cells were co-transfected with His_6-tagged-ORC3-9R and -ORC5-7R with Myc-OBI1 and HA-ubiquitin as indicated. Two days post-transfection, cells were lysed in denaturing conditions for His_6-tagged protein purification. Expression and ubiquitin conjugation of isolated proteins were monitored by western blotting, as indicated. **d,** U2OS cells were transfected with the indicated Myc-tagged ORC3 and ORC5 variants (WT or 0K) or EGFP as indicated. Two days after transfection, chromatin and soluble fractions were isolated and analysed by western blotting with the indicated antibodies. **e,** U2OS cells were co-transfected with the indicated Myc-tagged ORC3 and ORC5 variants (WT or 0K) or EGFP and with Flag-OBI1, as indicated. Two days after transfection, cell lysates were Flag-immunoprecipitated to purify the ORC subunit, and the association of co-expressed proteins was analysed by western blotting, as indicated. Expression of Myc-tagged proteins in input extracts was also monitored.

Supplementary Table 1: The human pre-RC interactome

ORC1		ORC2		LRWD1/ORCA		CDC6		CDT1	
Protein	**ΣPSM**	**Protein**	**ΣPSM**	**Protein**	**ΣPSM**	**Protein**	**ΣPSM**	**Protein**	**ΣPSM**
ORC1	312	ORC2	178	LRWD1/ORCA	358	CDC6	103	GEMININ	117
LRWD1/ORCA	86	ORC3	154	ORC1	108	DDX39B	9	CDT1	68
ORC2	85	LRWD1/ORCA	134	ORC3	101	LRWD1/ORCA	6	CDK1	28
ORC5	78	ORC4	81	ORC4	86	DDX5	6	CYCLIN A2	27
ORC4	73	ORC5	75	ORC5	76			CDK2	21
ORC3	72	FXR1	60	WIZ	66			SKP2	13
CYCLIN A2	64	ORC1	14	ORC2	65			SKP1	6
CULLIN-1	48	CYCLIN A2	6	ZNF644	55			CYCLIN A1	5
CDK2	46	XRCC6	5	EHMT1	43				
CDK1	37			HNRPM	30				
SKP2	30			RIF1	29				
XRCC5	19			EHMT2	29				
H4	16			FXR1	21				
XRCC6	14			CYCLIN A2	14				
TERF2	9			CDK2	13				
HNRPM	9			CDK1	10				
PSME3	9			CULLIN-1	9				
H2B	9			NUMA1	8				
OBI1/C13ORF7 /RNF219	8			GEMININ	8				
				SKP2	8				
TE2IP	8			XRCC6	6				
RBM10	6			PRMT5	6				
H2A	6			TRIM29	6				
CKS1	5			XRCC5	6				
NFIL3	5			OBI1/C13ORF7 /RNF219	5				
SKP1	5			UBIQUITIN	5				
				AKAP8	5				

List of the proteins identified by mass spectrometry as associated with the indicated baits. The total number of identified peptide sequences (peptide spectrum matches, ΣPSM) for each protein, including those redundantly identified, is indicated. Only proteins with ΣPSM ≥5 are shown.

Supplementary Table 2: Description of the human pre-RC interactome

Baits	Preys		
	Gene name	Description(s)	Function(s)
ORC1	ORC1	Origin recognition complex subunit 1	Pre-RC formation (origin licensing), AAA+ ATPase
	LRWD1 /ORCA	Leucine-rich WD40-containing protein 1 /ORC associated protein 1	ORC binding protein; Binds to heterochromatin epigenetic marks; heterochromatin formation
	ORC2	Origin recognition complex subunit 2	Pre-RC formation (origin licensing)
	ORC5	Origin recognition complex subunit 5	Pre-RC formation (origin licensing)
	ORC4	Origin recognition complex subunit 4	Pre-RC formation (origin licensing)
	ORC3	Origin recognition complex subunit 3	Pre-RC formation (origin licensing)
	CYCLIN A2	Cyclin A2	Binds and activates CDK; cell cycle regulation
	CULLIN-1	Cullin 1	SCF ubiquitin ligase complex; cell cycle regulation
	CDK2	Cyclin dependent kinase 2	Proline-directed kinase, cell cycle regulation
	CDK1	Cyclin dependent kinase 1	Proline-directed kinase, cell cycle regulation
	SKP2	S-phase kinase associated protein 2	SCF^{SKP2} ubiquitin ligase complex; cell cycle regulation
	XRCC5	X-ray repair cross complementing 5/Ku80	Associates with Ku70; DNA repair, Transcription
	H4	Histone H4	Nucleosome constituant
	XRCC6	X-ray repair cross complementing 6/Ku70	Associates with Ku80; DNA repair, Transcription
	TERF2	Telomeric repeat binding factor 2	Shelterin complex; telomere homeostasis
	HNRPM	Heterogeneous nuclear ribonucleoprotein M	RNA binding protein; mRNA metabolism
	PSME3	Proteasome activator subunit 3	Constituant of the proteasome 11S regulator
	H2B	Histone H2B	Nucleosome constituant
	OBI1/C13ORF7 /RNF219	ORC ubiquitin ligase 1/Chromosome 13 open reading frame 7/Ring finger protein 219	Previously uncharacterized; ORC complex ubiquitin ligase, promotes origin firing (present study)
	TE2IP	TERF2-interacting protein 2	Shelterin complex; telomere homeostasis
	RBM10	RNA binding motif protein 10	RNA binding protein; mRNA metabolism
	H2A	Histone H2A	Nucleosome constituant
	CKS1	Cyclin-dependant kinase regulatory subunit 1	Binds to CDK; cell cycle regulation
	NFIL3	Nuclear factor, interleukin 3 regulated	Transcription factor
	SKP1	S-phase kinase associated protein 1	SCF ubiquitin ligase complex; cell cycle regulation
ORC2	ORC2	Origin recognition complex subunit 2	Pre-RC formation (origin licensing)
	ORC3	Origin recognition complex subunit 3	Pre-RC formation (origin licensing)
	LRWD1 /ORCA	Leucine-rich WD40-containing protein 1 /ORC associated protein 1	ORC binding protein; Binds to heterochromatin epigenetic marks; heterochromatin formation

Supplementary Table 2: Description of the human pre-RC interactome

Baits	Preys		
	Gene name	Description(s)	Function(s)
ORC2	ORC4	Origin recognition complex subunit 4	Pre-RC formation (origin licensing)
	ORC5	Origin recognition complex subunit 5	Pre-RC formation (origin licensing)
	FXR1	FMR autosomal homolog 1	RNA binding protein, mRNA metabolism
	ORC1	Origin recognition complex subunit 1	Pre-RC formation (origin licensing), AAA+ ATPase
	CYCLIN A2	Cyclin A2	Binds and activates CDK; cell cycle regulation
	XRCC6	X-ray repair cross complementing 6/Ku70	Associates with Ku80; DNA repair, Transcription
LRWD1 /ORCA	LRWD1 /ORCA	Leucine-rich WD40-containing protein 1 /ORC associated protein 1	ORC binding protein; Binds to heterochromatin epigenetic marks; heterochromatin formation
	ORC1	Origin recognition complex subunit 1	Pre-RC formation (origin licensing), AAA+ ATPase
	ORC3	Origin recognition complex subunit 3	Pre-RC formation (origin licensing)
	ORC4	Origin recognition complex subunit 4	Pre-RC formation (origin licensing)
	ORC5	Origin recognition complex subunit 5	Pre-RC formation (origin licensing)
	WIZ	Widely Interspcaced Zinc Finger Motifs	Multiple Zinc finger protein, associates with EHMT1/2
	ORC2	Origin recognition complex subunit 2	Pre-RC formation (origin licensing)
	ZNF644	Zinc Finger Protein 644	Transcription factor, associates with EHMT1/2
	EHMT1	Euchromatic histone lysine methyltransferase 1	H3K9 methyltransferase, heterochromatin
	HNRPM	Heterogeneous nuclear ribonucleoprotein M	RNA binding protein; mRNA metabolism
	RIF1	Replication Timing Regulatory Factor 1	Nuclear matrix component, replication timing regulator
	EHMT2	Euchromatic histone lysine methyltransferase 2	H3K9 methyltransferase, heterochromatin
	FXR1	FMR autosomal homolog 1	RNA binding protein, mRNA metabolism
	CYCLIN A2	Cyclin A2	Binds and activates CDK; cell cycle regulation
	CDK2	Cyclin dependant kinase 2	Proline-directed kinase, cell cycle regulation
	CDK1	Cyclin dependant kinase 1	Proline-directed kinase, cell cycle regulation
	CULLIN-1	Cullin 1	SCF ubiquitin ligase complex; cell cycle regulation
	NUMA1	Nuclear Mitotic Apparatus Protein 1	Nuclear matrix component
	GEMININ	Geminin	CDT1 inhibitor, APC substrate
	SKP2	S-phase kinase associated protein 2	SCF^{SKP2} ubiquitin ligase complex; cell cycle regulation
	XRCC6	X-ray repair cross complementing 6/Ku70	Associates with Ku80; DNA repair, Transcription
	PRMT5	Protein Arginine Methyltransferase 5	Transcription regulation, ribonucleoprotein assembly
	TRIM29	Tripartite Motif Containing 29	Zinc Finger protein, transcription regulation
	XRCC5	X-ray repair cross complementing 5/Ku80	Associates with Ku70; DNA repair, Transcription

Supplementary Table 2: Description of the human pre-RC interactome

Baits	Preys		
	Gene name	Description(s)	Function(s)
LRWD1 /ORCA	OBI1/C13ORF7 /RNF219	ORC-ubiquitin-ligase-1/Chromosome 13 Open Reading Frame 7/RING Finger Protein 219	Previously uncharacterized; ORC complex ubiquitin ligase, promotes origin firing (present study)
	UBIQUITIN	Ubiquitin	Protein degradation, signaling
	AKAP8	A-Kinase Anchoring Protein 8	Bind PKA, associates with nuclear matrix
CDC6	CDC6	Cell Division Cycle 6	Pre-RC formation (origin licensing), AAA+ ATPase
	DDX39B	DExD-box Helicase 39B	ATP-dependant RNA helicase, mRNA splicing and export
	LRWD1 /ORCA	Leucine-rich WD40-containing protein 1 /ORC associated protein 1	ORC binding protein; Binds to heterochromatin epigenetic marks; heterochromatin formation
	DDX5	DExD-box Helicase 39B	ATP-dependant RNA helicase
CDT1	GEMININ	Geminin	CDT1 inhibitor, APC substrate
	CDT1	CDC10 Dependent Transcript 1	Pre-RC formation (origin licensing)
	CDK1	Cyclin dependant kinase 1	Proline-directed kinase, cell cycle regulation
	CYCLIN A2	Cyclin A2	Binds and activates CDK; cell cycle regulation
	CDK2	Cyclin dependant kinase 2	Proline-directed kinase, cell cycle regulation
	SKP2	S-phase kinase associated protein 2	SCF^{SKP2} ubiquitin ligase complex; cell cycle regulation
	SKP1	S-phase kinase associated protein 2	SCF ubiquitin ligase complex; cell cycle regulation
	CYCLIN A1	Cyclin A1	Binds and activates CDK; cell cycle regulation

Supplementary Table 3: Sequence of siRNAs used in this study

siRNAs	Sequences
siMock	UAAGGCUAUGAAGAGAUAC
siOBI1	GUAGAAGUAAUGUUAGAUG
	CCAAAGGUUCUCUAACUAA
	GGAUUUGGAUGGGUUAUCA
	GAACGAAGUGAUAAGUAUA
siUTR	GCACUUGGGUUUAGAGGAA
	GUCAGGAUGGUGGAUUAAC
siCDC7	CAGGAAAGGUGUUCACAAA
	CUACACAAAUGCACAAAUU
	GUACGGGAAUAUAUGCUUA
	GCAUUCAUCAGUUUGGUAU
siORC1	UAAGAAACGUGCUCGAGUA
	GAGAUCACCUCACCUUCUA
	GCAGAGAGCCCUUCUUGGA
	CCUCAGAUCCGUAGUCGAA

Supplementary Discussion: Information on the known interactors found associated with pre-RC components (related to Fig. 1a,b and Supplementary Table 1 and 2)

The ORC complex. All ORC subunits (with the exception of ORC6, see below) were associated with the baits ORC1, ORC2 and ORCA (Figure 1A-C and Table 1). Tagged ORCA strongly associated with the ORC subunits, while the endogenous protein was found in ORC1 and ORC2 complexes, confirming previous reports that ORCA is a *bona fide* additional ORC subunit [1-3]. Figures 1a-c and Supplementary Table 1 and 2 show ORC2 presence in ORC1 and ORCA baits, and ORC1 presence in ORC2 and ORCA baits specifically. The amount of ORC1 was lower in purified ORC2 and ORCA complexes. We think that the main reason is the known fate of ORC1 and ORC2 during the cell cycle in mammals [4]. In contrast to yeast in which all six ORC subunits are stably bound to chromatin throughout the cell cycle [5-9], the affinity of mammalian ORC1 for chromatin is selectively reduced during S-phase. Thus, ORC1 binds to chromatin during G1, and is then largely released from chromatin during S- [10] and M-phase [11], whereas ORC2 remains constantly bound to chromatin during S-phase. Therefore, in exponentially growing cells (our experimental condition in Fig. 1c), ORC2 will be in excess relative to ORC1.
ORC6 was not associated with ORC1, ORC2 or ORCA using our purification conditions. This finding could be explained by the fact that the ORC1-5/ORC6 interaction is salt-sensitive [12] and that our nuclear extraction was performed in high-salt buffer (see Methods section). This could also explain why we observed few interactions with the CDC6 bait.

Geminin. Geminin is an important negative regulator of origin licensing through direct binding to and inhibition of CDT1 [13]. Accordingly, endogenous geminin strongly and specifically interacted with the CDT1 bait (Fig. 1c and Supplementary Table 1 and 2). Low amount of geminin was also associated with the ORCA bait (Fig. 1c and Supplementary Table 1 and 2), as previously reported [14].

Cyclin-dependent kinases (CDKs). CDKs are important regulators of origin licensing and activation (for review [17]). CDKs (CDK1 and CDK2) as well as regulatory (cyclin A1 and A2) and accessory (CKS1) subunits were associated with the ORC complex and CDT1 baits (Fig. 1a-c and Supplementary Table 1 and 2). Both ORC1 and CDT1, which directly interact with cyclin A through their cyclin binding (Cy) motif [15,16], associated with significant amount of cyclin A. ORC2 and ORCA complexes included less ORC1 and also contained significantly lower amounts of cyclin A (Fig. 1c).

SCF^{Skp2} ubiquitin ligase. ORC1 and CDT1 are negatively regulated via degradation [17] by the cullin-based SCF^{Skp2} ubiquitin ligase [18-20]. In agreement, components of this E3 ligase were associated with the ORC1, ORCA and CDT1 baits (Fig. 1a,b and Supplementary Table 1 and 2).

Shelterin complex. The shelterin complex plays an important role in ensuring telomere integrity and genome stability [21]. TERF2 and TE2IP bind to telomere repetitive DNA and recruit the shelterin complex to telomeres. TERF2 also recruits the ORC complex to telomeres, thus facilitating telomere replication and stability. As previously reported [22,23], ORC1 specifically associated with the shelterin complex subunits TERF2 and TE2IP (Fig. 1a,b and Supplementary Table 1 and 2).

G9a/b H3K9 methyltransferase complexes. The ORC complex associates with heterochromatin marks, including H3K9me3 and H3K27me3 [2,3]. ORCA plays a role in recruiting the ORC complex to heterochromatin [24]. It was shown recently that ORCA interacts with the H3K9 methyltransferase (HMT) G9a/b [24], and is important for heterochromatin formation and histone methyltransferase stability. We also observed that the ORCA bait strongly interacted with several subunits of this HMT complex (G9a, G9b, WIZ and ZNF644) (Fig. 1a,b and Supplementary Table 1 and 2). WIZ and ZNF644 facilitate G9a/b interaction with chromatin [25].

Ku proteins. The heterodimeric Ku proteins Ku70 and Ku86 are DNA binding proteins involved in many chromatin transactions, notably in DNA repair pathways [26]. It is reported that Ku70/86 could play a role in DNA replication, by interacting with the ORC complex and facilitating origin establishment [27,28]. Here, we observed a specific association of Ku70 and Ku86 with the ORC1 and ORCA baits (Fig. 1a,b and Supplementary Table 1 and 2).

Histones. The association between histones and ORC1 observed in our pre-RC interactome study (Fig. 1a,b and Supplementary Table 1 and 2) could be related to the finding that ORC1, through its BAH domain, directly interacts with histone K4 dimethylated on lysine 20 (H4K20me2) and the nucleosome [29,30].

SUPPLEMENTARY REFERENCES

1 Shen, Z. *et al.* A WD-repeat protein stabilizes ORC binding to chromatin. *Mol Cell* **40**, 99-111, doi:10.1016/j.molcel.2010.09.021 (2010).

2 Bartke, T. *et al.* Nucleosome-interacting proteins regulated by DNA and histone methylation. *Cell* **143**, 470-484, doi:10.1016/j.cell.2010.10.012 (2010).

3 Vermeulen, M. *et al.* Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. *Cell* **142**, 967-980, doi:10.1016/j.cell.2010.08.020 (2010).

4 Li, C. J. & DePamphilis, M. L. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. *Mol Cell Biol* **22**, 105-116 (2002).

5 Diffley, J. F., Cocker, J. H., Dowell, S. J. & Rowley, A. Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* **78**, 303-316 (1994).

6 Fujita, M. *et al.* Cell cycle dependent topological changes of chromosomal replication origins in Saccharomyces cerevisiae. *Genes Cells* **3**, 737-749 (1998).

7 Kong, D. & DePamphilis, M. L. Site-specific DNA binding of the Schizosaccharomyces pombe origin recognition complex is determined by the Orc4 subunit. *Mol Cell Biol* **21**, 8095-8103, doi:10.1128/MCB.21.23.8095-8103.2001 (2001).

8 Liang, C. & Stillman, B. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes & development* **11**, 3375-3386 (1997).

9 Lygerou, Z. & Nurse, P. The fission yeast origin recognition complex is constitutively associated with chromatin and is differentially modified through the cell cycle. *Journal of cell science* **112 (Pt 21)**, 3703-3712 (1999).

10 Kreitz, S., Ritzi, M., Baack, M. & Knippers, R. The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. *The Journal of biological chemistry* **276**, 6337-6342, doi:10.1074/jbc.M009473200 (2001).

11 Natale, D. A., Li, C. J., Sun, W. H. & DePamphilis, M. L. Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals. *The EMBO journal* **19**, 2728-2738, doi:10.1093/emboj/19.11.2728 (2000).

12 Ghosh, S., Vassilev, A. P., Zhang, J., Zhao, Y. & DePamphilis, M. L. Assembly of the human origin recognition complex occurs through independent nuclear localization of its components. *The Journal of biological chemistry* **286**, 23831-23841, doi:10.1074/jbc.M110.215988 (2011).

13 McGarry, T. J. & Kirschner, M. W. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**, 1043-1053 (1998).

14 Shen, Z. *et al.* Dynamic association of ORCA with prereplicative complex components regulates DNA replication initiation. *Mol Cell Biol* **32**, 3107-3120, doi:10.1128/MCB.00362-12 (2012).

15 Hossain, M. & Stillman, B. Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. *Genes & development* **26**, 1797-1810, doi:10.1101/gad.197178.112 (2012).

(2012).

15 Hossain, M. & Stillman, B. Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. *Genes & development* **26**, 1797-1810, doi:10.1101/gad.197178.112 (2012).
16 Sugimoto, N. *et al.* Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. *The Journal of biological chemistry* **279**, 19691-19697, doi:10.1074/jbc.M313175200 (2004).
17 Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N. & Oda, M. Eukaryotic chromosome DNA replication: where, when, and how? *Annu Rev Biochem* **79**, 89-130, doi:10.1146/annurev.biochem.052308.103205 (2010).
18 Mendez, J. *et al.* Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Mol Cell* **9**, 481-491 (2002).
19 Li, X., Zhao, Q., Liao, R., Sun, P. & Wu, X. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *The Journal of biological chemistry* **278**, 30854-30858, doi:10.1074/jbc.C300251200 (2003).
20 Nishitani, H. *et al.* Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *The EMBO journal* **25**, 1126-1136, doi:10.1038/sj.emboj.7601002 (2006).
21 Hockemeyer, D. & Collins, K. Control of telomerase action at human telomeres. *Nat Struct Mol Biol* **22**, 848-852, doi:10.1038/nsmb.3083 (2015).
22 Deng, Z., Norseen, J., Wiedmer, A., Riethman, H. & Lieberman, P. M. TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. *Mol Cell* **35**, 403-413, doi:10.1016/j.molcel.2009.06.025 (2009).
23 Tatsumi, Y. *et al.* Involvement of human ORC and TRF2 in pre-replication complex assembly at telomeres. *Genes Cells* **13**, 1045-1059, doi:10.1111/j.1365-2443.2008.01224.x (2008).
24 Giri, S. & Prasanth, S. G. Association of ORCA/LRWD1 with repressive histone methyl transferases mediates heterochromatin organization. *Nucleus* **6**, 435-441, doi:10.1080/19491034.2015.1102814 (2015).
25 Bian, C., Chen, Q. & Yu, X. The zinc finger proteins ZNF644 and WIZ regulate the G9a/GLP complex for gene repression. *eLife* **4**, doi:10.7554/eLife.05606 (2015).
26 Fell, V. L. & Schild-Poulter, C. The Ku heterodimer: function in DNA repair and beyond. *Mutat Res Rev Mutat Res* **763**, 15-29, doi:10.1016/j.mrrev.2014.06.002 (2015).
27 Sibani, S., Price, G. B. & Zannis-Hadjopoulos, M. Ku80 binds to human replication origins prior to the assembly of the ORC complex. *Biochemistry* **44**, 7885-7896, doi:10.1021/bi047327n (2005).
28 Sibani, S., Price, G. B. & Zannis-Hadjopoulos, M. Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80+/- cells. *Journal of cell science* **118**, 3247-3261, doi:10.1242/jcs.02427 (2005).
29 Kuo, A. J. *et al.* The BAH domain of ORC1 links H4K20me2 to DNA replication licensing and Meier-Gorlin syndrome. *Nature* **484**, 115-119 (2012).
30 Zhang, W., Sankaran, S., Gozani, O. & Song, J. A Meier-Gorlin syndrome mutation impairs the ORC1-nucleosome association. *ACS Chem Biol* **10**, 1176-1180, doi:10.1021/cb5009684 (2015).

Project 2

Project 2: "Studying the function (s) of OBI1, in DNA replication, using Xenopus Egg Extracts."

2.1 Project introduction

To preserve genome stability, origins of replication must be activated only once per cell cycle. Origin activation is tightly coordinated, first origins are licensed in G1, starting by the recruitment of the ORC complex, followed by licensing factors CDC6 and CDT1 and ends with the recruitment of MCM2-7 which marks the formation of the pre-RC. Later in S phase, phosphorylation dependent loading of CDC45 and GINS converts the pre-RC into a pre-IC. Finally, the CMG active helicase (CDC45, MCM2-7, GINS) promotes DNA unwinding at the origins of replication allowing the initiation of DNA synthesis. Interestingly, only a limited amount of chromatin loaded MCM2-7 will be activated in S phase. How these origins are chosen from all licensed origins in G1 is still unclear. Our previous work done in human cells, described a role of the E3 ubiquitin ligase OBI1 in origin activation or selection, through the monoubiquitylation of a subset of chromatin bound ORC3 and ORC5.

In order to decipher the mechanisms behind OBI1's function, we aimed to use an in-vitro system derived from Xenopus eggs as this system is perfectly adapted to dissect the molecular mechanisms involved in the function of replication proteins. It allows studies on endogenous proteins, in a synchronized system, without the involvement of other major DNA related processes. These advantages allowed this system to play a major role, both in identifying and characterizing DNA replication factors, such as CDT1 and in studying ubiquitylation and ubiquitin ligases (Arias and Walter 2005, Moreno, Bailey et al. 2014, Larsen, Gao et al. 2019).

It is important to mention that similarly to mammalian cells, ORC3/5 ubiquitylation has not been described in Xenopus extracts and the extent of the implication of ubiquitylation in general in the initiation of DNA replication in this system is still unclear. Consequently, the study on the function of OBI1 in Xenopus laevis will allow us to provide more information on OBI's role in origin activation/ selection and on the implication of ubiquitylation in replication initiation in this system.

2.2 System description and uses

Xenopus egg extracts (XE) are cytoplasmic fractions that are isolated from intact Xenopus eggs using centrifugation. This cytoplasm is concentrated in proteins, organelles and other cellular structures that allow it to carry out many processes associated with DNA replication and the cell cycle in vitro (Murray 1991, Hannak and Heald 2006).

Xenopus eggs are arrested in metaphase of meiosis II, fertilization promotes completion of meiosis, and progression into interphase of the mitotic cell cycle. In 7 hours after fertilization xenopus embryos undergo approximately 11 synchronous rounds of cell division in the absence of significant transcriptional activity. This is allowed by the accumulation of a stockpile of maternal proteins and RNA previously synthesized during oogenesis. It is only after the Mid-Blastula transition or MBT that transcription occurs (Newport and Kirschner 1982, Newport and Kirschner 1982). Consequently, Xenopus eggs provide a concentrated pool of replication proteins, which allows studies on DNA replication in the absence of other cellular processes.

a. Xenopus extracts preparation

The preparation of extracts from eggs of Xenopus Laevis, is important since different types of extract can be obtained, depending on the mechanism to analyze. This process briefly consists on inducing ovulation in female frogs by injecting them with Chorulon (Chorionic Gonadotrophin), eggs are then collected dejellied, packed, crushed, and fractioned using centrifugation. Typically, the cytoplasmic layer in the middle is collected (with or without activation) and supplemented with protease inhibitors and energy mixture (Gillespie, Gambus et al. 2012, Good and Heald 2018) (See Figure 1).

CSF extracts

Egg extract, more specifically low speed extracts (LSE), can be prepared by low speed centrifugation of metaphase arrested eggs, which will give rise to a mitotic extract (CSF-arrested extract) that can support spindle assembly, chromosome condensation and segregation.

LSE extracts

Xenopus eggs can be driven into interphase by addition of Ca2+ (resulting in interphase extracts, LSE). The addition of exogenous Ca2+ mimics the calcium wave generated during fertilization, and ensures the exit from meiosis and entry in the first mitotic interphase. LSE can further be driven into mitosis by addition of cyclin B.

HSE extracts

An additional ultracentrifuge step to clarify the crude extracts described above will result in high-speed extracts or HSE. HSEs were the first DNA replication system developed from Xenopus eggs, able to support the complete replication of single-stranded DNA (ssDNA) plasmid substrates. Although they cannot replicate duplex DNA, they can assemble chromatin on such templates and form Pre-RCs on replication origins.

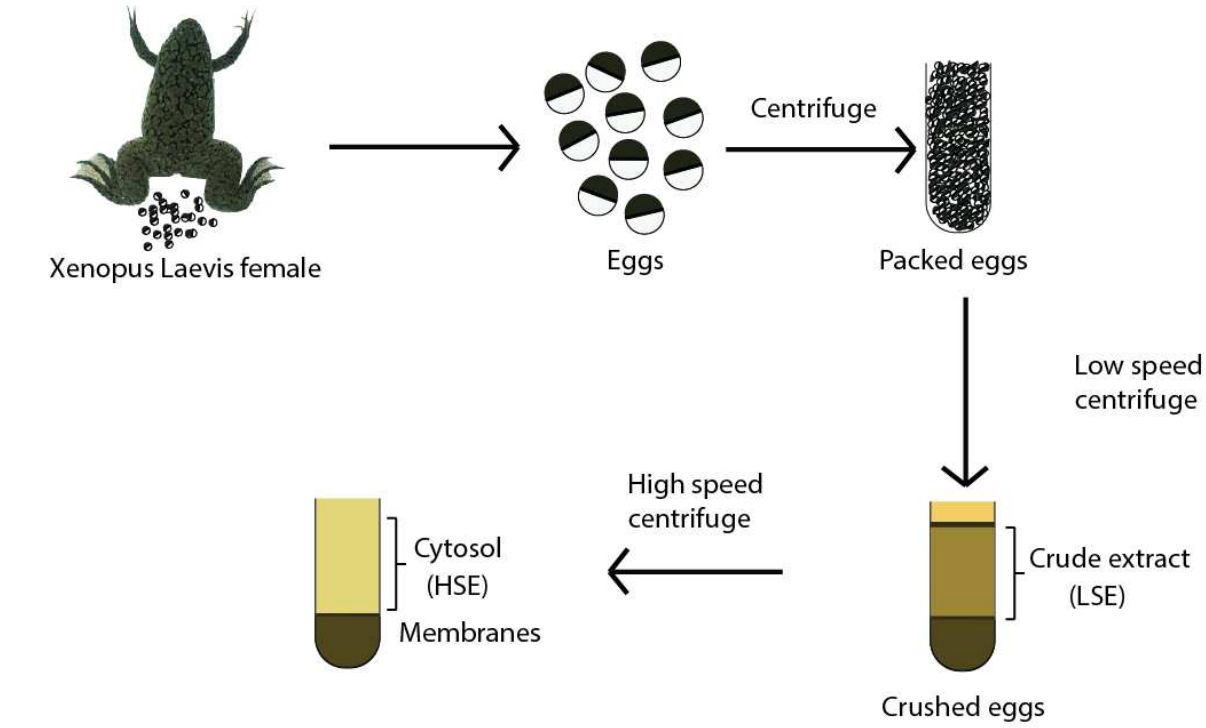
NPE extracts

More recently, additional systems prepared from Xenopus eggs have also been described, expanding the scope of biological processes that can be studied in vitro, such as nucleoplasmic extract or NPE, which contains CDK and DDK (Dbf4-Dependent Kinase) activities. NPEs allow the replication of templates previously licensed in HSE. The use of this system allows the uncoupling between DNA replication initiation and elongation (Walter and Newport 2000).

Once these extracts are prepared, DNA or demembranated sperm nuclei extracted from male frogs is added to the extract, where they can be assembled into a nucleus and then

efficiently replicated. These extracts recapitulate key nuclear functions of the eukaryotic cell cycle, and are therefore uniquely suited to study mechanisms and dynamics of cell cycle processes in vitro.

Interestingly, extract preparation is not only limited to the egg stage, since an embryo extract system also enables the characterization of specific biological processes at different stages of early embryogenesis (Good 2016).



B

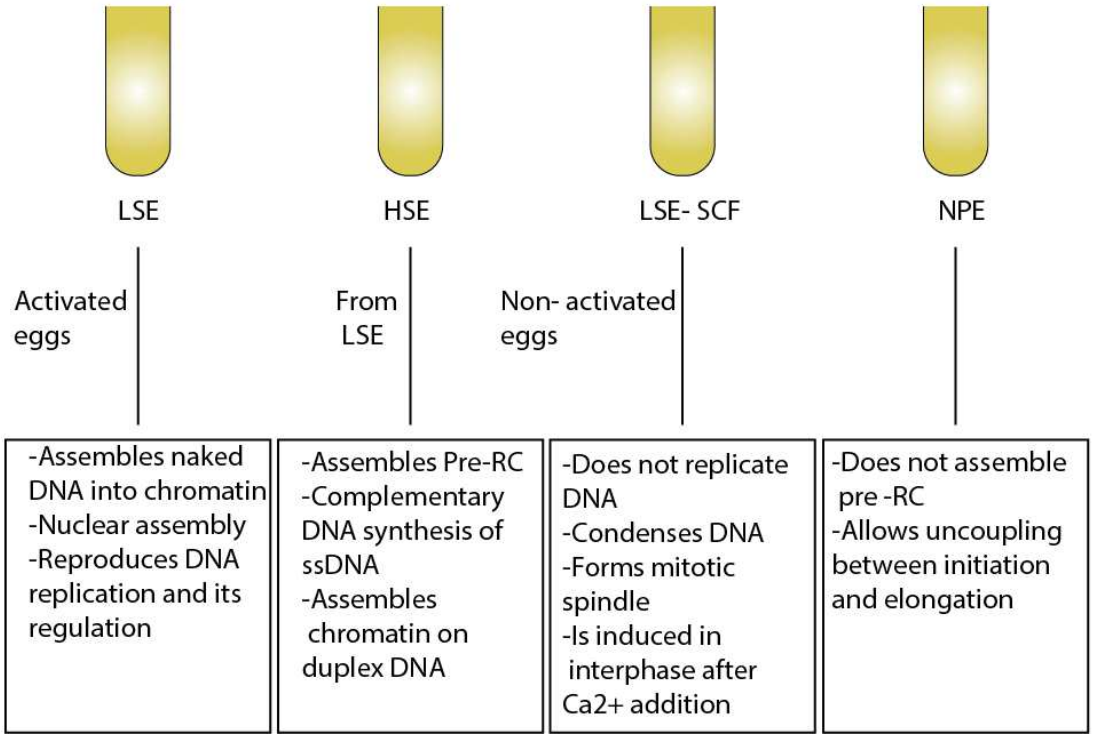


Figure 1: Xenopus egg extracts: preparation and uses.

a *resumes the steps of extract preparation form egg collection to HSE* ***b*** *represents different types of extracts, their origin and the nuclear function each can sustain*

b. System advantages

There are many advantages to using Xenopus egg extracts when compared to studies in cell culture. Studies done on essential proteins in living cells are difficult as knocking down an essential gene often results in cell cycle arrest or cell death, which could as well be due to indirect transcription effects. This cell-free egg extract represents an ideal system to overcome these issues and to facilitate the biochemical study of replication-associated functions of essential proteins in vertebrate organisms (Blow and Laskey 1986, Almouzni and Mechali 1988, Gillespie, Gambus et al. 2012).

This system is suitable to dissect the different steps of DNA replication for several reasons:

- Absence of transcription
- Availability of standardized treatments and inhibitors. Natural synchronization of the cell cycle without the use of drugs.
- This system recapitulates a complete round of cell-cycle regulated semi-conservative DNA replication in vitro under most of the controls present in a vertebrate cell (Harland and Laskey 1980).
- The high degree of genetic conservation of the most essential cellular and molecular mechanism between Xenopus and mammals, facilitates the detailed biochemical study of essential proteins and complexes present in egg extract. These proteins can be easily isolated and characterized.
- The absence of a cell membrane offers unique manipulation opportunities, proteins and nucleic acids can be directly added into the extract without any special delivery technique, it is possible to monitor the kinetic of the incorporation of modified nucleotides using fluorescent or radiolabelled nucleotides.
- Possibility to perform immediate addition of exogenous components at any time to the extract and to deplete proteins directly from the extract using antibodies to study their impact on nuclear processes. Most importantly, depletion experiments can be rescued by adding back recombinant version of the protein, in order to exclude the presence of off-target effects. Addition of an excess of recombinant protein can also be used as an overexpression tool.

- Finally, one female frog can be the source of thousands of unfertilized eggs where most of the proteins required for cell cycle progression are already formed and stocked, providing a large volume of extract which allows for many reactions to be carried out in parallel.

c. Ubiquitylation in Xenopus egg extracts

As mentioned previously, protein degradation plays a key role in regulation of DNA replication. This has also been characterized in Xenopus extracts. The Xenopus system played a major role in studying the ubiquitin system. Indeed, the APC/C complex was discovered using Xenopus egg extracts (King, Peters et al. 1995) it was discovered at the same time in clam oocyte extracts and named the Cylcosome (Sudakin, Ganoth et al. 1995) which led to its current nomenclature as the APC/C complex.

Proteasomal degradation

As in mammalian cells, Geminin, an important negative regulator of DNA replication (McGarry and Kirschner 1998) was also shown to be ubiquitylated by the APC/C complex in xenopus, resulting in the degradation of a fraction of the protein. Additionally, the licensing factor CDT1 was shown to be ubiquitylated and targeted for degradation in Xenopus extract in order to prevent additional rounds of DNA replication (Arias and Walter 2005). Finally, Cyclin B was also shown to be degraded in a ubiquitin dependent manner in this system (Glotzer, Murray et al. 1991).

Non-proteolytic ubiquitylation

In xenopus Laevis egg extracts a proportion of PCNA was shown to be constitutively ubiquitylated on K164 during unperturbed DNA replication. This ubiquitylation was shown to be important for efficient DNA replication (Leach and Michael 2005).

As in mammalian cells, recent studies in Xenopus laevis egg extract have also shown that polyubiquitylation plays a key role in disassembly of the replisome machinery at the termination of DNA replication forks. Studies done in xenopus laevis on replisome machinery disassembly at the termination of DNA replication, showed the MCM7 subunit of the active helicase to be polyubiquitylated with K48 linked chains upon replication fork termination, by the CRL ubiquitin ligase complex (Moreno, science 2014). MCM ubiquitylation is then followed by dissolution of the CMG replicative helicase, which is dependent on the activity of the protein segregase, Cdc48/p97/VCP. The fate of ubiquitylated Mcm7 upon its recognition and remodeling by the segregase has not yet been established. The ubiquitylated protein could be directed for proteasomal degradation or deubiquitylated by one of the DUBs interacting with the segregase.

Ubiquitylation and initiation of DNA replication

Interestingly, early work done on the implication of ubiquitylation in the initiation step of DNA replication was inconclusive.

The different conclusions were based mainly on studying the role of CDK inhibitor P27/XIC1 in replication initiation. For instance, in one study, blocking ubiquitylation mediated proteasomal degradation resulted in DNA replication inhibition. This was shown to be related to cell cycle progression through p27's ubiquitin regulation and degradation (Yew and Kirschner 1997).

On the other hand, other studies showed that p27 appears to be significantly expressed only at later stages in development (Shou and Dunphy 1996). Moreover, when a truncated version of p27 was injected into early Xenopus embryos, it did not inhibit DNA

replication, but caused longer cell cycles, suggesting that it may normally begin functioning at the MBT (Hartley, Sible et al. 1997). These studies concluded with the fact that inhibition of p27 proteolysis does not inhibit DNA replication in early Xenopus cell cycles (Chuang and Yew 2001, You, Harvey et al. 2002) and that Ub-dependent proteolysis is not required for the initiation of DNA replication in the Xenopus early embryonic cell cycle (Mahaffey, Gorbea et al. 2003).

Interestingly, more recent studies made on ubiquitylation in Xenopus Laevis egg extract (although not targeting directly the initiation step), showed that the use of proteasome inhibitor or methylated ubiquitin (which inhibits ubiquitin chain assembly) affected re-replication without showing any defect in replication initiation (Arias and Walter 2005). Moreover, the use of lysine-less (0K) ubiquitin, which inhibits polyubiquitylation, in a study made on replisome disassembly in XE also did not show any defect in replication initiation (Moreno, Bailey et al. 2014).

In conclusion, further insight on the role of ubiquitylation in the initiation of DNA replication is needed.

2.3 Project objectives

In this work, our first aim was to address the role of ubiquitylation in the initiation of DNA replication in Xenopus eggs. Using E1 inhibitors in order to abolish de novo ubiquitylation, we wished to get more information on the implication of this process in origin activation. Additionally, we aim to study the conservation of OBI1, its catalytic activity and its role in origin selection in this system.

In the pursuit of our objectives, in addition to ubiquitylation inhibition, chromatin isolation will allow us to sequentially monitor OBI1 recruitment on replicating chromatin and depletion experiments should allow us to assess the loss of OBI1 on DNA replication efficiency. Later, rescue experiments will provide a specificity to the observed phenotype. Immuno-precipitation experiments will allow us to study the conservation of ORC

ubiquitylation and confirm the interaction between OBI1 and the ORC complex in Xenopus laevis.

2.4 Materials and methods

Xenopus Laevis egg extract preparation

Xenopus laevis frogs were purchased from the "Centre de Ressources Biologiques Xenopes", at the University of Rennes, France. LSE were prepared as previously described (Lutzmann and Mechali 2008). Briefly, eggs were collected in 1x HSB buffer (15 mM Tris pH 7.5, 110 mM NaCl, 2 mM KCl, 1 mM MgSO4, 0.5 mM Na2HPO4, 2 mM NaHCO3). Eggs were then dejellied in 0.2x HSB buffer, pH 7.9, containing 2% cysteine, for 5- 6min before they are washed five times in 0.2x HSB and twice in 1x MMR buffer (5 mM HEPES pH 7.8, 100 mM NaCl, 2 mM KCl, 0.1 mM EDTA, 1 mM MgCl2, 2 mM CaCl2). Eggs are then activated in 0.2x MMR supplemented with 0.3 µg ml –1 calcium ionophore and packed in order to remove the excess of buffer. Packed eggs were then crushed in 1x XB buffer (100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 50 mM Sucrose) supplemented with protease inhibitors cocktail (Leupeptine, Pepstatine, Aprotinine, 5 ug/mL each), by centrifugation (Sorvall HB6 swinging rotor) for 20 min at 10 000 rpm. After addition of cytochalasin B (100 µg ml –1), low speed supernatants were finally clarified by centrifugation (SW 55Ti rotor) for 20 min at 20 000 rpm. Extracts were aliquoted, and stored at –80 °C after snap freezing.

DNA replication kinetics

Xenopus extracts were supplemented with cycloheximide (250 ug/mL) and energy mix (1mM ATP, 2 mM MgCl2, 10 mM creatine kinase, 10 mM creatine phosphate). DNA replication efficiency was assessed by adding demembranated X. laevis sperm nuclei (1 300 nuclei/uL of extract or 10 000 nuclei/uL of extract for high sperm nuclei experiments) to OBI1 depleted, E1 inhibitor (1 mM MLN 7243) treated or mock treated egg extracts supplemented with [α–32P]-dCTP. Mock treated samples were incubated with preimmune serum (for depletion experiments) and with XB buffer or DMSO (For E1 inhibitor experiments). DNA synthesis was monitored by TCA precipitation. Briefly, at the indicated times points samples were

neutralized in 80 mM Tris pH8, 8 mM EDTA pH8 and 1% SDS supplemented with 400 ug/mL Proteinase K and incubated at 37°C overnight. Incorporated acid-insoluble material was spotted onto GF/C Whatman glass microfiber filters and then precipitated with 5% TCA solution containing 2% tetra- sodium pyrophosphate. After 5% TCA and 96% ethanol washes, filters were dried and the incorporated TCA precipitated radioactivity was counted in scintillation liquid using liquid scintillation analyzer (PerkinElmer, Tri-Carb 2910 TR). Replication kinetic values are displayed in percentage of replicated DNA compared to total amount of added DNA.

Chromatin purification

Upon thawing, xenopus extracts were supplemented with cycloheximide (250 ug/mL) and energy mix (1mM ATP, 2 mM MgCl2, 10 mM creatine kinase, 10 mM creatine phosphate) before sperm chromatin incubation and purification as previously described (Maiorano, Moreau et al. 2000). Briefly, at the indicated time points after sperm addition (3000 nuclei/uL of extract or 10000 nuclei/uL of extract for high sperm nuclei experiments), samples were diluted four folds in XB (100mM KCl, 50 mM HEPES, 2.5 mM MgCl2) supplemented with 0.25% NP-40, incubated on ice for 5min and centrifuged through a sucrose cushion (100 mM KCl, 50 mM HEPES, 2.5 mM MgCl2, 0.25% NP-40 and 0.88 M Sucrose) in a swinging bucket rotor micro centrifuge at 10 000 rpm for 5 min at 4°C. The supernatant is then removed and the chromatin pellet is washed twice with XB buffer, then centrifuged in fixed angle micro-centrifuge at 10 000 rpm for 5 min at 4°C Chromatin pellets were finally re-suspended in 2× LB (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-β-mercaptoethanol and 0.8% bromophenol blue), denatured at 95°C for 5 min and immediately loaded in SDS page gel (Invitrogen, NuPAGE 4-12% 1.0 mm Bis-Tris Gel) for western blot analysis or stored at −20°C. When required, extracts were pre-incubated with E1 inhibitors (1 mM MLN-7243), aphidicolin (25ug/mL), p27 (40 ug/mL), Geminin (60 nM), HA-ubiquitin, DMSO (1%) or XB buffer for mock conditions.

Recombinant proteins.

N- Termini and C- termini GST tagged fragments of ORC3, ORC5 (for antibody production) and HA tagged full length ubiquitin (for E1 inhibitor experiments) wild type proteins were expressed in E. coli BL21 cells after induction with 0.8mM IPTG at room temperature over-night. Purification of recombinant proteins was done in native conditions as described

previously (Lutzmann and Mechali 2008). Briefly, the cells were re-suspended and washed with 1x PBS, after washes the cells are lysed in TB buffer (1xPBS, 10 mM Tris- HCL pH 7.5, 1 mM EDTA and 1% Triton X- 100), supplemented with protease inhibitors cocktail (Leupeptine, Pepstatine, Aprotinine, 5 ug/mL each) and PMSF (Phenylmethylsulfonyl fluoride). The mixture was then kept at -80°C for 2 to 4 hours then thawed before the addition of 1% Sarkozyl. The mix is then subjected to 3 or 4 freeze/thaw cycles. After the last thaw, the samples are sonicated to finish lysis and cleared by centrifugation at 4 000 rpm for 10 min at 4°C. Cleared samples are incubated with GSH- beads for two to four hours on a rotary wheel at 4°C. Later, the beads are pelleted by centrifugation (4000 rpm for 10 min at 4°C) and washed three times with 1x PBS 1% Triton X-100. Finally, the proteins were eluted by GST competition. Protein quantification was done using SDS page gel (Invitrogen, NuPAGE 4-12% 1.0 mm Bis-Tris Gel) and Coomassie staining (50% methanol, 10% Acetic acid, 0.1% Coomassie R.250 powder).

Antibodies.

In house: Rabbit polyclonal anti- xenopus ORC3 antibodies were raised against GST-ORC3-Cter and GST-ORC3-Nter recombinant proteins, ORC5 rabbit polyclonal antibodies were also raised against GST-ORC5-Cter and GST-ORC5-Nter recombinant proteins, expressed and purified from bacteria (As described above).

Acquired: Anti-H3 (Abcam, ab1791, dilution 1/2000), Anti- PCNA (Sigma, P8825, dilution 1/2500), Anti- HA(Santa- Cruz, Sc- 805), Streptavidin (Generous gift from Constantinou's lab at the institute of Human Genetics), Anti- RPA 32 (Francon, Lemaitre et al. 2004) (dilution 1/500), Anti-Geminin, Anti- CDT1, Anti- ORC2 (Lutzmann and Mechali 2008) (dilution 1/1000), Anti-MCM3 (Maiorano, Cuvier et al. 2005) (dilution 1/2000), Anti- CDC45 (Walter and Newport 2000) (dilution 1/1000). In house rabbit polyclonal antibodies: Anti- OBI1 (1:1000), Anti- ORC3 (1:1000) and Anti- ORC5 (1:1000).

A fraction of OBI1 crude serum was also purified by affinity chromatography using the same antigen used to immunize the rabbits coupled to CNBr- activated sepharose beads (Pharmacia- 52-1153-00-AI).

Xenopus embryos

Xenopus Laevis embryos were prepared by in vitro fertilization using standard procedures (Sive, Grainger et al. 2007). Briefly, freshly collected testis from a male frog is rubbed over eggs freshly collected form a female frog. The eggs are then incubated for 1 min for activation with calcium ionophore. After 0.1x MBS (8.8 mM NaCl,0.1 mM KCl, 0.1 mM MgSO4, 0.5 mM HEPES and 0.25 mM NaHCO3, pH 7.8 with NaOH) addition the eggs are incubated for 35 min at room temperature and then dejellied in 0.2x HSB buffer, pH 7.9, containing 2% cysteine solution. The cysteine solution is then discarded and the eggs are washed extensively with 1x MBS and left at 23°C. The staged embryos are then collected according to Nieuwkoop and Faber normal tables, around twenty embryos were collected at each stage and stored an -80°C after snap freezing. For western blot analysis, the embryos were thawed, re-suspended in 1x XB buffer (100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 50 mM Sucrose) supplemented with protease inhibitors cocktail (Leupeptine, Pepstatine, Aprotinine) and cytochalasin B and crushed by centrifugation at 13000 rpm for 10 min at 4°C in a fixed rotor micro-centrifuge. The cytoplasmic fraction is then recovered and denatured in 2x LB (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-β-mercaptoethanol and 0.8% bromophenol blue) at 95°C for 5 min and loaded in SDS page gel for western blot analysis.

Immunodepletion experiments

OBI1 was depleted from the extracts after three rounds of depletion (20 min/ round) with crude OBI1 serum coupled to rProtein A beads (rProtein A sepharose fast flow, GE healthcare). Briefly, egg extracts were thawed, supplemented with cycloheximide and incubated with beads that were are extensively pre-washed in 1x XB buffer (100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 50 mM Sucrose) and excess buffer was removed thoroughly to avoid the dilution of the extract during the depletion procedure. At each round the extract-beads mix is kept on ice and homogenized every 2 minutes for 20 min, the extract is then passed through a “glass bead column” in order to remove the old set of beads before the following round. The procedure is repeated three times, after the last round of depletion, the beads are removed and the extract is processed for replication kinetic assay and western blot analyses.

Immunoprecipitation experiments

Extracts were thawed, diluted four folds in 1x XB buffer (100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 50 mM Sucrose) and cleared by centrifugation at 8000 rpm for 2 min at 4°C. The diluted extracts are then incubated with the respective antibody coupled to protein A beads (Protein A agarose, sigma) for three to four hours at 4°C. The immunoprecipitates are collected by 8000 rpm centrifuge for 1 min and washed twice with 1x XB buffer, once with 1x XB supplemented with 0.1% triton X-100 and finally once with 1x XB before denaturation in 2x LB at 95°C for 5 min.

For ubiquitylation experiments all buffers used were supplemented with 10 mM N-ethylmaleimide (NEM)

Sucrose gradient

Sucrose density gradient centrifugation was performed as described (Lutzmann and Mechali 2008). Briefly, egg extracts were diluted two folds in XB and centrifuged at 10000 rpm for 5 min at 4°C. 200uL of cleared egg extract was laid on a 5 mL 7-19% gradient prepared in XB buffer supplemented with protease inhibitors (Leupeptine, Pepstatine, Aprotinine, 5 ug/mL each). The gradient was then centrifuged at 26000 rpm for 22 h at 4°C in a SW 55Ti rotor. Finally, 200 uL fractions were collected and stored at -80°C or processed for western blot analyses.

E1 inhibitor experiments

Egg extracts were thawed and incubated with E1 inhibitors (Aobious USA, MLN 7243, NCS 624206, PYR 41 or PYZD 4409, 1 mM each), 1% DMSO or 1x XB for mock treated extracts. After 30 min incubation, HA-tagged WT ubiquitin (purified from E. coli) was added in excess to the extracts to monitor de novo ubiquitylation. After E1 inhibitor or mock treatment the extracts were processed for chromatin purification, replication kinetic assay and total extract western blot analyses.

Immunoprecipitation of chromatin fraction

Upon thawing, xenopus extracts were supplemented with cycloheximide (250 ug/mL) and energy mix (1mM ATP, 2 mM MgCl2, 10 mM creatine kinase, 10 mM creatine phosphate) before sperm chromatin incubation and purification as previously described (Maiorano,

Cuvier et al. 2005, Lutzmann and Mechali 2008). Briefly, at the indicated time points after sperm addition (3000 nuclei/uL of extract), samples were diluted four folds in XB (100mM KCl, 50 mM HEPES, 2.5 mM MgCl2, and 0.25% NP-40) incubated on ice for 5min and centrifuged through a sucrose cushion (100 mM KCl, 50 mM HEPES, 2.5 mM MgCl2, 0.25% NP-40 and 0.88 M Sucrose), in a swinging bucket rotor micro centrifuge at 10 000 rpm for 5 min at 4°C. The supernatant is then removed and the chromatin pellet is washed twice with XB buffer, then centrifuged in fixed angle micro-centrifuge at 10 000 rpm for 5 min at 4°C. Chromatin pellets were finally solubilized in Foz- lysis buffer (50 mM Tris pH7.4, 100 mM NaCl, 5 mM EDTA, 40 mM b-glycerolphosphate, 50 mM NaF and 1% Triton X-100) for 10 min on ice, before incubation with the respective antibody coupled to protein A beads (Protein A agarose, sigma) for three to four hours at 4°C. The immunoprecipitates are collected by 8000 rpm centrifuge for 1 min and washed with Foz- buffer before addition of 2x LB and denaturation at 95°C for 5 min.

For ubiquitylation experiments all buffers used were supplemented with 10 mM N-ethylmaleimide (NEM)

Western blot analysis

Total egg extract or purified chromatin were denatured as mentioned above, ran on a SDS page gel (Invitrogen, NuPAGE 4-12% 1.0 mm Bis-Tris Gel) and transferred to nitrocellulose membrane (0.45 um nitrocellulose membranes, GE healthcare). Membranes were then blocked with 5% milk in TBS/T buffer (20 mM Tris-HCL pH 7.6, 137 mM NaCl and 0.1% Tween 20) for one hour, washed with TBST/T and incubated with primary antibodies diluted in 2% BSA- TBS/T solution overnight. Membranes were later washed with TBS/T and incubated with secondary antibodies diluted in 5% milk TBS/T solution for one hour before final washes with TBS/T and revelation using ECL chemiluminescence solution (ECL Plus, Pierce).

2.5 Results

Ubiquitylation in early development

Before studying the role of OBI1, first we had to understand the involvement of ubiquitylation in DNA replication; therefore, we started by addressing the role of ubiquitylation, in general, in the initiation of DNA replication. Many studies aimed to establish the role of ubiquitylation in DNA replication in Xenopus egg extracts. However, most of these studies mainly focused on the role of ubiquitylation in the elongation and termination steps of DNA replication, while the implication of this process in the initiation step remains elusive. While these studies did not directly target replication initiation, they showed that the use of proteasome inhibitor, methylated ubiquitin or lysine-less ubiquitin in order to inhibit ubiquitin chain assembly affected re-replication (Arias and Walter 2005) and replisome disassembly (Moreno, Bailey et al. 2014) without showing any major defects in replication initiation. Interestingly, the use of proteasome inhibitor or mutated ubiquitin do not exclude the possibility of monoubiquitylation or multimonoubiquitylation reactions. Therefore we aimed to completely abolish de novo ubiquityaltion, by inhibiting the first step in the ubiquitylation reaction (using E1 inhibitors), in order assess the implication of this process in origin activation.

In order to study de novo ubiquitylation, we used an exogenous tagged ubiquitin (HA-tagged wild type ubiquitin). HA-ubiquitin was produced and purified from BL-21 strain of E. coli (see materials and methods). The capacity of the extract to incorporate the tagged ubiquitin was then tested by adding HA-ubiquitin to total extract and the ubiquitylation of total endogenous proteins was checked by western blotting. This allowed us to detect total ubiquitylation in LSE extract in the absence of DNA (Fig2-a). Next, we aimed to see if de novo protein ubiquitylation could be detected on replicating chromatin. For this, LSE was supplemented with HA-ubiquitin and de-membranated sperm nuclei. Chromatin fractions were then collected and purified at different time points after sperm chromatin addition, to monitor ubiquitylation of chromatin bound proteins. LSE incubated with HA-ubiquitin without sperm DNA addition was used to monitor background signal. This allowed us to detect specific ubiquitylation of chromatin associated proteins starting from 10 minutes after sperm addition

(Fig2-b), suggesting that protein ubiquitylation starts very early upon fertilization concomitantly with chromatin remodeling and licensing.

In order to further address whether ubiquitylation had an impact on DNA replication we used E1 inhibitors (Fig2-c) targeting the first step of the ubiquitylation reaction. In this aim, LSE were incubated with 1 mM of the respective E1 inhibitor for 30 min (see materials and methods), then the extract was supplemented with HA-ubiquitin to monitor de novo ubiquitylation. Fig 2-d shows that the E1 inhibitor MLN 7243 (hereafter called MLN) was able to efficiently inhibit the incorporation of HA-ubiquitin in the extract. Similar experiments with longer time monitoring allowed us to conclude that the inhibition efficiency of MLN remains until at least 150 min after HA-Ubiquitin addition (Fig2-e). Using this setup, we analyzed the impact of ubiquitylation inhibition on DNA replication efficiency. Xenopus extracts were incubated for 30 min with MLN or HA-ubiquitin, DMSO and XB buffer as controls, then the extract replication capacity was monitored by [α– 32P]-dCTP incorporation upon sperm addition. Interestingly, inhibition of ubiquitylation resulted in a delay in replication initiation (Fig2-f). This result suggested a potential effect of ubiquitylation on replication initiation.

In order to establish the reason behind the effect of ubiquitylation inhibition on origin activation, we analyzed the loading of licensing and firing factors onto replicating chromatin by western blot (Fig2-g). Our experiment shows that ORC1 and ORC5 were normally loaded on chromatin in presence of the inhibitor. On the other hand, MCM3's release from the chromatin, characteristic of completion of DNA replication, was delayed compared to the control conditions (Fig2-g). This observation first confirmed a previous study reporting that the MCM complex needs to be ubiquitylated in order to be removed from chromatin (Moreno, Bailey et al. 2014) while also confirming the suitability of our experimental condition. In contrast, we observed an important delay of CDC45 recruitment when compared to both control conditions (Fig2- g). These findings suggested that the delay observed in replication initiation after incubation with MLN could be resulting from a defective origin activation, translated by a defect in the recruitment of factors involved in origin firing.

We further investigated the effect of MLN on origin firing by pre-incubating the extract with a DNA polymerase inhibitor, Aphidicolin, and asked whether the chromatin loading of factors involved in origin activation was affected by the E1 inhibitor MLN. Addition of aphidicolin alone resulted in higher chromatin recruitment of RPA (data not shown) as a result

of exposure of single stranded DNA resulting from the replication fork block induced by this inhibitor. Interestingly, when the aphidicolin-treated extract is incubated with E1 inhibitor MLN, the chromatin loading of RPA was significantly lower (Fig2-h). This observation suggests that the inhibition of ubiquitylation resulted in less single stranded DNA exposed and recognized by RPA. This result is in agreement with less replication forks activated with the E1 inhibited treatment.

Altogether, our observations suggest that ubiquitylation inhibition resulted in a decrease in origin activation events, without however affecting pre-RC assembly.

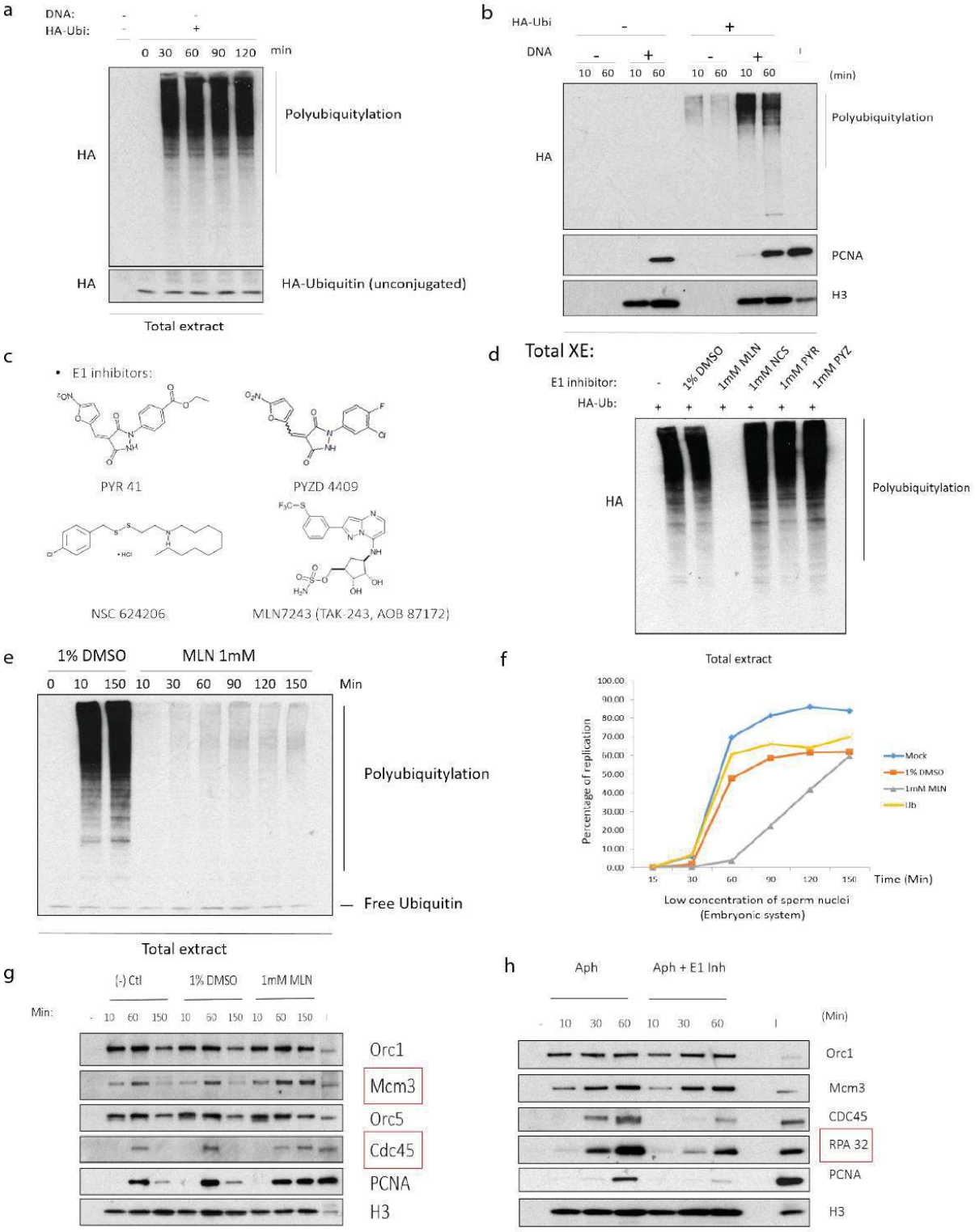


Figure 2. Ubiquitylation is important for timely origin activation in Xenopus Laevis egg extracts.

a *Western blot analysis of Xenopus interphase egg extract (LSE) time course, supplemented with HA-tagged Wild type ubiquitin.* ***b*** *Time course of ubiquitylated proteins binding to the chromatin. Sperm nuclei (3000 nuc/uL of XE) were added to LSE and detergent- resistant chromatin fractions were isolated at the indicated time points during DNA replication and analyzed by western blotting. A sample of total extract (I, input) (0.5uL) or insoluble material obtained by centrifugation of LSE (- DNA)*

were used as control. ***c*** *The structure and nomenclature of the E1 inhibitors tested in (**d**).* ***d*** *LSE were incubated with 1 mM of the indicated E1 inhibitor for 30 min, western blot analysis was done on total extracts (0.5uL) after addition of HA-tagged ubiquitin, LSE incubated with 1% DMSO or 1x XB buffer were used as control.* ***e*** *Western blot analysis of total egg extract after a 30 min incubation with 1 mM of E1 inhibitor (MLN7243) or 1% DMSO as control. LSE were then supplemented with HA-Tagged ubiquitin and 0.5uL of extract were collected and the indicated time points.* ***f*** *Replication efficiency of the reaction described in (**e**) after addition of 1300 sperm nuclei/uL. Extracts incubated with 1x XB buffer or HA- ubiquitin were added as control.* ***g*** *Time course of licensing and firing proteins binding the chromatin after sperm nuclei (3000 nuc/uL) addition to LSE. Detergent resistant fractions were isolated at the indicated time point during DNA replication and analyzed by western blotting. Prior to sperm addition, LSE were pre-incubated for 30 min with 1 mM E1 inhibitor or 1% DMSO and 1x XB buffer (Ctl-) as controls. Total extract (I, input, 0.5uL) was also added as control.* ***h*** *Time course of licensing and firing proteins binding to the chromatin after sperm addition (3000 nucl/uL) to LSE pre-incubated with Aphidicolin or Aphidicolin and E1 inhibitor. Detergent resistant fractions were isolated at the indicated time points during DNA replication and analyzed by western blot. Prior to sperm nuclei addition LSE were pre-incubated with Aphidicolin (25ug/mL) or Aphidicolin (25ug/uL) and E1 inhibitor (1mM) for 30 min. Total extract (I, input) was added as control.*

OBI1- ORC interaction is conserved in Xenopus Laevis

OBI1's sequence and domains are conserved in higher eukaryotes, with its RING domain showing the most conservation throughout evolution (Fig3-a). In order to study OBI1's activity, our first experiments were done using human cells. U2OS cells were co-transfected with Myc-FlagORC5 and HA-ubiquitin, with or without the over expression of either the human Myc-OBI1 or the Xenopus Myc-OBI1. ORC5 Flag pull down showed an increased ORC5 ubiquitiylation with human-OBI1 over-expression (Fig3-b) as previously described in project 1. Interestingly, ORC5 ubiquitylation was increased to the same extent after the over-expression of xenopus-OBI1, when compared to our control. This experiment suggested that OBI1's ligase activity is conserved in Xenopus.

To investigate this further, we derived three in-house rabbit serums raised against Xenopus-OBI1 that will allow to study OBI1 in xenopus extracts and analyze the dynamics of its recruitment to the chromatin. First, we performed immunoprecipitation experiments on total extract (LSE). All three anti-OBI1 crude sera, but not the pre-immune serum, specifically recognized a protein with a molecular weight of ~80 kDa in xenopus egg extracts (Fig3-c). Which allowed us to conclude that OBI1 is present in the extract.

To study the ORC3 and ORC5 subunits of the ORC complex in Xenopus laevis, we produced and characterized polyclonal antibodies against GST-ORC3 and GST-ORC5 (see

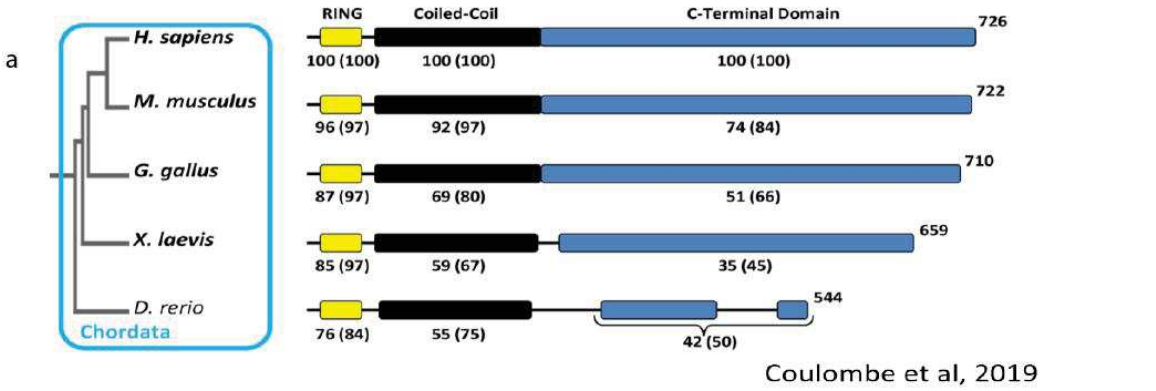
materials and methods). The crude serum raised against ORC3, but not the pre-immune serum, specifically recognized a protein with a molecular weight of 80 kDa in xenopus egg extracts. Similarly, the crude serum raised against ORC5, but not the pre-immune serum, specifically recognized a protein with a molecular weight of 50 kDa (Data not shown).

To confirm OBI1- ORC interaction, xenopus interphase egg extract protein complexes were fractionated by sucrose gradient density centrifugation and the fractionation was checked using Ponceau staining (Data not shown) and western blotting (Fig3-d). Subunit 1 of the ORC complex, was detected in fractions 13 to 17 (Fig3-d), while OBI1 was mainly present in lower density fractions 5 to 7. Interestingly, some OBI1 was also detected in fraction 13 to 15 suggesting a possible complex formation with the ORC complex. However, this needs to be confirmed by looking for interaction after co-immunoprecipitating ORC1 and OBI1 in the respective fractions.

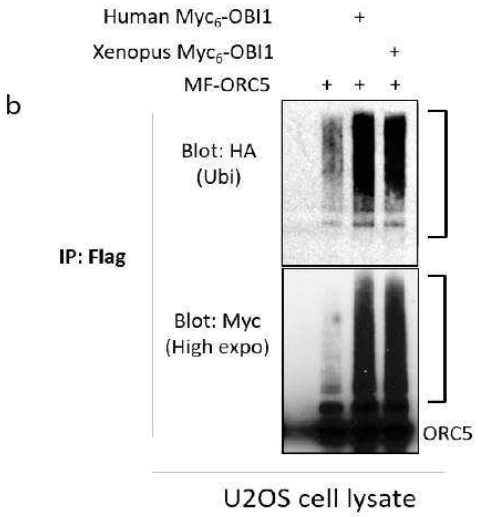
In order to investigate this further, subunits 1 and 2 (Fig3-e) were checked for an interaction with OBI1 in total LSE extract. In this aim, we first immunoprecipitated OBI1, ORC1 and ORC2 separately and by western blot we checked for OBI1 co-precipitation (Fig 3-e, Left panel), ORC1 co-precipitation and ORC2 co-precipitation (Fig 3-e, Right panel). ORC1 and ORC2 pull-down resulted in co-precipitation of OBI1 (Fig3-e, left panel, high expo), conversely, OBI1 pull-down did not specifically result in ORC1 and ORC2 co-precipitation (Fig3-e, right panel). Additional experiments were done using ORC3 and ORC5 immunoprecipitation yielded similar results (Data not shown). Taken together, these experiments allowed us to detect interaction between OBI1 and components of the ORC complex in xenopus laevis egg extracts.

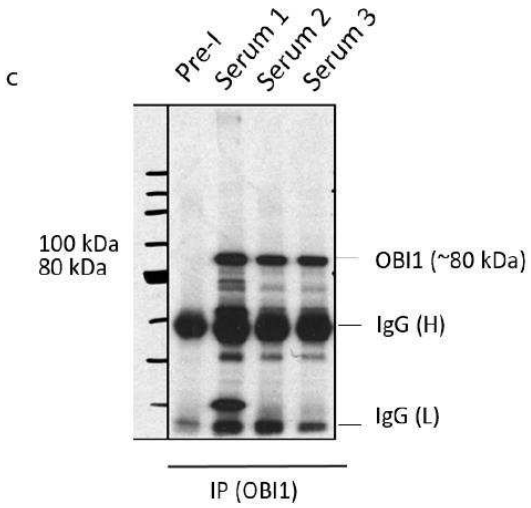
Finally, we wanted to investigate the ubiquitylation status of ORC3 and ORC5 in xenopus laevis. In this aim, LSE was supplemented with sperm nuclei DNA, with or without addition of biotin-ubiquitin. The chromatin was then isolated at different time points and the proteins bound to the chromatin were solubilized in lysis buffer. The lysate was then subjected to immunoprecipitation using anti-ORC3 antibody and then ubiquitylation was monitored using streptavidin anti-biotin antibody. Using this protocol, we were able to efficiently immunoprecipitate ORC3 recruited to replicating chromatin (Fig3-f, Right panel). Interestingly, blotting the same samples against ubiquitin allowed us to detect specific ubiquitylation (Fig3-f, Left panel) in our samples when compared to our two control

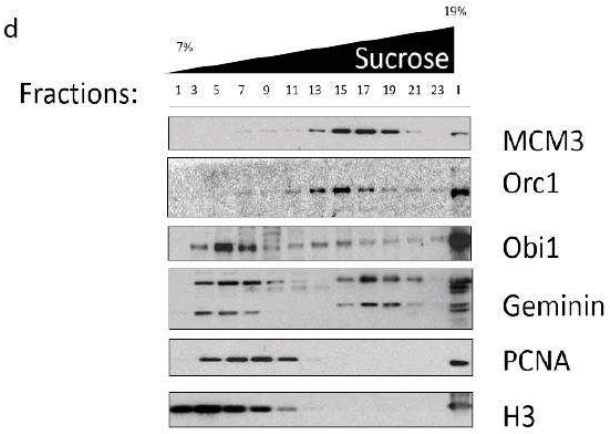
conditions (using pre-I serum for mock immunoprecipitation and ORC3 immunoprecipitation without addition of biotin ubiquitin for background), suggesting that ORC3 ubiquitylation might be conserved in Xenopus laevis.

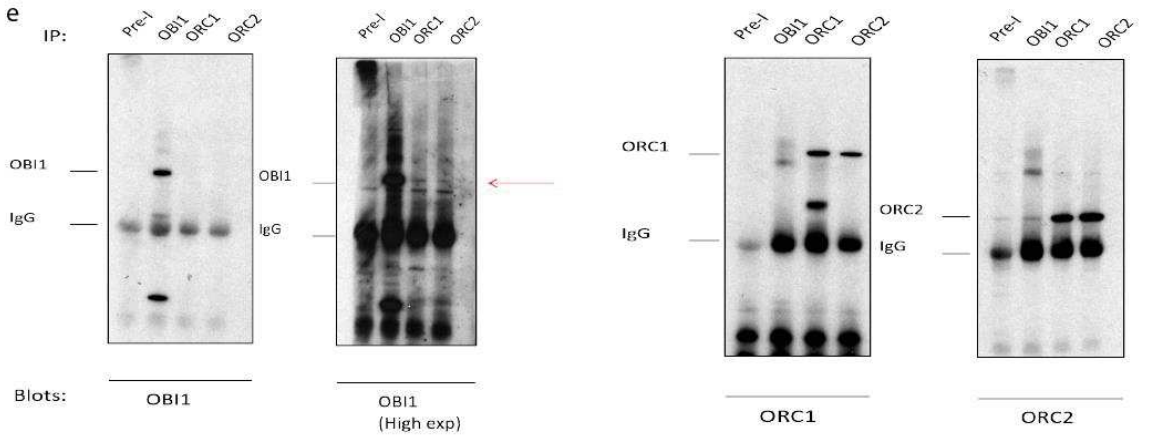


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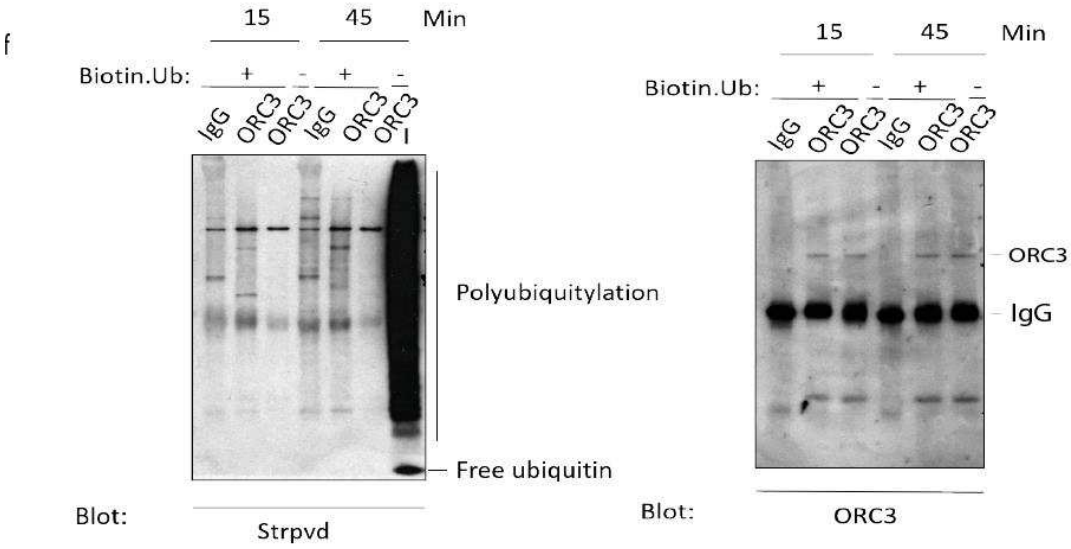


Figure 3. OBI1- ORC interaction is conserved in Xenopus laevis

a *Phylogenic tree showing the evolutionary conservation of OBI1 (Coulombe et al, 2019).* ***b*** *U2OS cells were co-transfected with Myc-tagged OBI1 (Human or xenopus), Myc-Flag ORC5 and HA-tagged ubiquitin. Cell lysates were later Flag- immunoprecipitated to purify ORC5. The immunoprecipitates were analyzed by western blotting using anti-Myc and anti-HA antibodies. U2OS cells transfected with an empty vector or Myc-Flag-ORC5 alone were used as controls.* ***c*** *LSE were immunoprecipitated using three different crude sera raised against OBI1. The immunoprecipitates were analyzed by western blotting using anti-OBI1 (serum 1) antibody. Pre-immune serum (Pre-I) was used as control.* ***d*** *Western blot analysis of LSE following fractionation on 7%- 19% sucrose gradient, without sperm addition. The total fractionated proteins were analyzed by western blotting.* ***e*** *LSE were immunoprecipitated using anti-OBI1, anti-ORC1 and anti-ORC2 crude sera. The immunoprecipitates were analyzed by western blotting using anti-OBI1 (left panel) or anti-ORC1 and anti-ORC2 (right panel) antibodies. Mock depletion using pre-immune serum (Pre-I) was used as a control.* ***f*** *Western blot analysis of ORC3 ubiquitylation on chromatin. Sperm nuclei (3000 nucl/uL of XE) were added to LSE, with (ORC3+) or without (ORC3-) the addition of wild type biotin-ubiquitin. Detergent- resistant chromatin fractions were isolated at the indicated time points during DNA replication. Chromatin fraction were then immunoprecipitated using anti-ORC3 crude serum. The immunoprecipitates were analyzed by western blotting using streptavidin antibody (left panel) or anti-ORC3 serum (right panel). Pre-immune serum (Pre-I) was used as control. A sample of total extract (I, input) (0.5uL) was also added as control.*

OBI1's role in DNA replication and in vivo characterization

We finally asked whether OBI1 had an active role in DNA replication as it is the case in human cells. First OBI1's recruitment to the chromatin was assessed upon addition of sperm nuclei to LSE. Chromatin purification assay showed replication initiation starting at 15 min through the recruitment of the ORC complex (Fig4-a). Interestingly, OBI1 shows a specific recruitment to replicating chromatin starting 30 min, when compared to the control condition

(-DNA), with a probability (because of the background signal) of a small amount already being loaded at 15 min.

In order to more deeply decipher the role of OBI1 in DNA replication, we also performed loss of function experiments: using the immune anti-OBI1 serum we aimed to deplete OBI1 from LSE, then assess the influence of this depletion on DNA replication efficiency after sperm nuclei addition. Our anti-OBI1 serum efficiently depleted OBI1 from total egg extract (Fig4-b). However, replication kinetic assay (Fig4-c) did not show any severe defect in DNA replication after OBI1 removal, when compared to mock depleted extract, which led us to suggest that OBI1 might not be crucial for DNA replication in early development, and an alternative approach to target this question was needed.

These observations and the potential involvement of OBI1 in origin selection also led us to investigate OBI1's behavior in late developmental stages (post-MBT). Initiation of DNA replication occurs at an accelerated rate in early Xenopus embryos and the corresponding egg extracts (Mechali and Kearsey 1984). However, when the embryo reaches the mid-blastula stage, after 12 divisions, a somatic cell cycle is introduced, origin selection becomes more pronounced and inter-origin distance is increased (Hyrien, Maric et al. 1995). We thus asked whether the results observed would be different in conditions mimicking late embryos, than in early embryo conditions. These conditions can be mimicked in vitro by adding a high concentration of sperm nuclei in Xenopus LSE extracts, allowing the establishment of a high Nuclear/ cytoplasm ratio mimicking late developmental stages. In this aim LSE were supplemented with a high concentration of sperm nuclei, chromatin purification shows OBI1 recruitment to the chromatin at 30 min (Fig4-d).

In parallel we also investigated OBI1's expression in xenopus embryos, which allowed us to detect OBI1 at a constant level at all pre-MBT embryonic stages, with an increased amount after MBT (stage 9), (Fig4-e, f). However, this observation may be due to new OBI1 synthesis after MBT, when transcription resumes in the embryo.

Finally, in parallel to our work on OBI1 in post-MBT, we wanted to know if the phenotype observed in our early experiments on E1 inhibitor were also affected by the nuclear/ cytoplasm ratio (N:C ratio). Therefore, after pre-incubation of extract with MLN or DMSO for control, we decided to monitor DNA replication after addition of a high

concentration of sperm nuclei, repeating our experiments at a high nuclear/ cytoplasm ratio (N:C ratio). Interestingly, closer to somatic-like conditions ubiquitylation inhibition resulted in a more severe defect and the extract's capacity to replicate DNA was nearly abolished (Fig4-g).

We concluded that ubiquitylation was more crucial for DNA replication in conditions of late development, than in early development, when replication proteins are in excess.

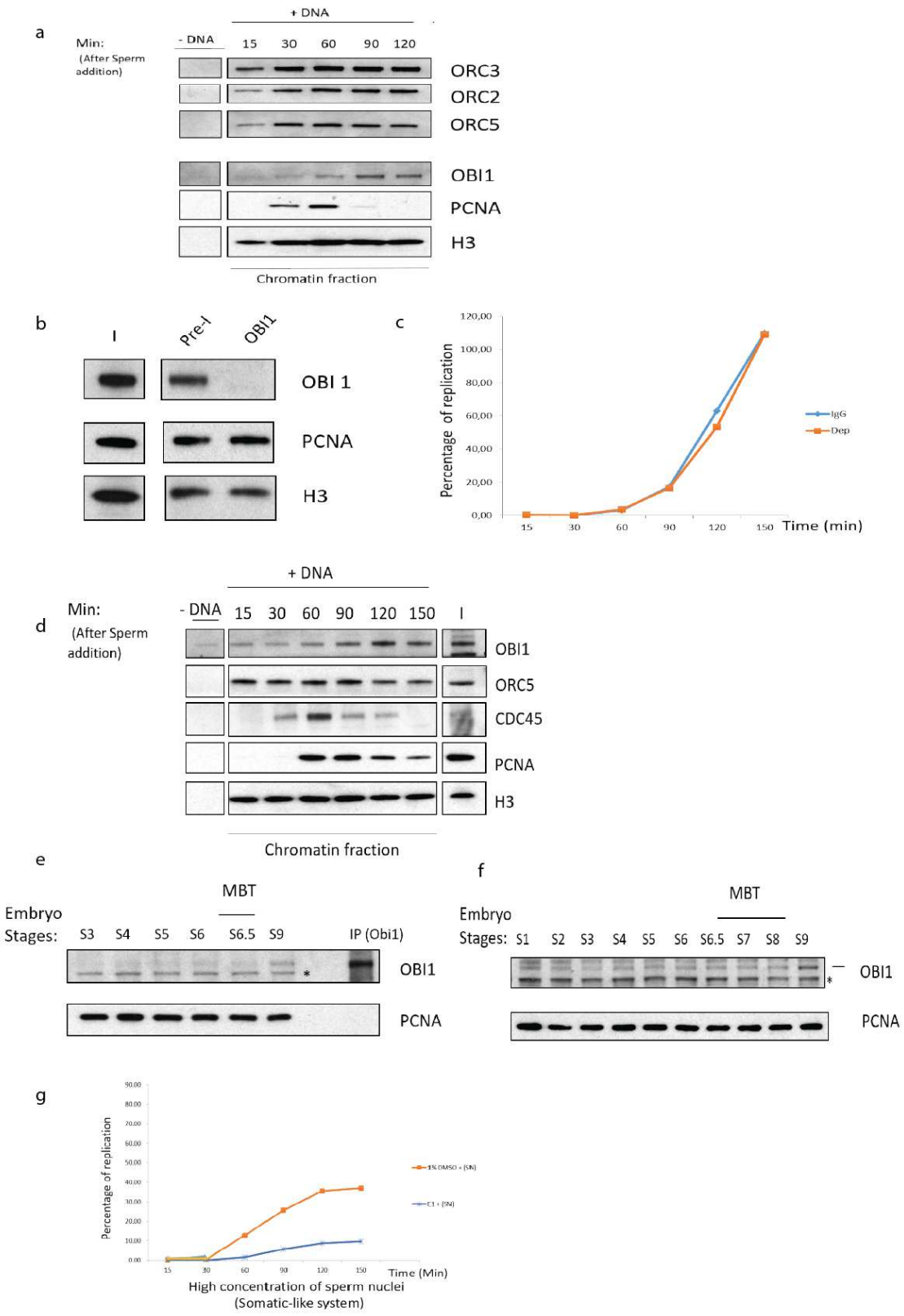


Figure 4. OBI1 interacts with the ORC complex in Xenopus egg extracts

a *Sperm nuclei (3000 nucl/uL) were added to LSE and the chromatin fractions were isolated at the indicated time points during DNA replication. OBI1 and chromatin bound proteins were analyzed by western blotting using the indicated antibodies. Total extract (I, input) or insoluble material obtained after centrifugation of LSE (-DNA) were used as control.* ***b*** *LSE were depleted using anti-OBI1 crude*

serum or pre-immune serum (Pre-I) for mock depletion. 0.5uL of total extract was analyzed by western blot with the indicated antibodies to confirm depletion. ***c*** *Replication efficiency of the reaction described in* ***b.*** *Sperm nuclei* ***d*** *(10000 nucl/uL) were added to LSE and the chromatin fractions were isolated at the indicated time points during DNA replication. OBI1 and chromatin bound proteins were analyzed by western blotting using the indicated antibodies. Total extract (I, input) or insoluble material obtained after centrifugation of LSE (-DNA) were used as control.* ***e*** *Western blot analysis of OBI1 expression in total embryo protein extracts at the indicated stages of development, before and after mid-blastula transition (MBT). Immunoprecipitates of OBI1 (IP OBI1) was added as control. Asterisk marks the unspecific bands.* ***f*** *Western blot with higher exposure and additional development stages of the experiment described in* ***e.*** ***g*** *LSE were supplemented with (10000 nucl/uL) and replication efficiency was assessed after a 30 min pre-incubation with 1 mM of E1 inhibitor (MLN7243) or 1% DMSO as control.*

2.6 Discussion

While several previous studies aiming to understand the role of ubiquitylation in xenopus laevis mainly showed that this modification was not necessarily needed for the initiation of DNA replication, these studies focused on the inhibition of ubiquitylation using methylated ubiquitin, 0K ubiquitin or proteasome inhibitors. However, these methods did not exclude the possibility that monoubiquitylation or multimonoubiquitylation was taking place. We aimed to abolish ubiquitylation using another strategy based on the inhibition of the first step that takes place during the ubiquitylation reaction (using E1 inhibitor). This strategy allowed a total inhibition of ubiquitylation and a better understanding of its implication in DNA replication. Ubiquitylation inhibition resulted in a delayed replication, which was narrowed down to a defect in origin firing, as seen by the delayed CDC45 recruitment to the chromatin. However, this defect in origin firing in low N/C ratio conditions seemed to be later compensated as replication was eventually completed at the end of the kinetic, similarly to our control. This observation could be either explained by a non-detectable re-start of ubiquitylation, however this is unlikely since our data show MCM stabilization on chromatin, or by the fact that the replication proteins present in the extract are in excess which allows the extract to eventually compensate the lack of origin activation. This hypothesis is also supported by the fact that the same experiment done with a high N/C ratio showed a more sever phenotype in response to the lack of initiation events. In these conditions, with more than three folds the amount of sperm nuclei, replication factors become more limiting and the extract is less able to compensate for the defective initiation. Interestingly, this delay in

origin activation could also be attributed to a delay in chromatin remodeling or nuclear assembly. DNA combing experiments after E1 inhibition could highlight more information on the defective initiation.

In addition, our data showed an interaction between OBI1 and the ORC complex, suggesting a conservation of OBI1's interactome and maybe its catalytic activity. Concerning the conservation of ORC ubiquitylation, we studied ORC3/5 ubiquitylation in total extract (data not shown) and on replicating chromatin (Fig3-f) assuming that this reaction takes place during DNA replication as in human cells. However, our results are still preliminary and the possibility of contaminating proteins is still not excluded since ORC5 precipitation was done in native conditions.

Finally, we were able to experimentally confirm OBI1's conservation in Xenopus egg extracts and in xenopus embryos at different developmental stages. OBI1 was also found to be recruited to replicating chromatin in embryonic and somatic-like conditions, suggesting a role of OBI1 in DNA replication. Interestingly, while experiments done on human cells using Xenopus OBI1 showed a conservation in activity, OBI1 depletion in xenopus extracts did not yield the expected effect on DNA replication. This observation could be explained by the extract's ability to compensate the lack of ubiquitylation events. While the answer is still not clear, it is important to keep in mind that OBI1's activity in xenopus does not necessarily take place in early development. Indeed, typically replication origins in xenopus embryos are regularly spaced which contributes to the accelerated rate of S phase, it is only close to MBT that initiation of DNA replication becomes restricted to specific sites, which resembles more the somatic cell system. Therefore if OBI1 is implicated in origin selection, its role could be more important in later stages of development. A potential way to target this hypothesis could be by immunodepleting OBI1 and supplementing the extract with a high concentration of sperm nuclei. The high concentration of DNA and the limiting replication factors along with the start of a possible origin selection should aggravate the phenotype of OBI1 depletion (similarly to what was observed in the E1 inhibitor experiments) and therefore allow us to detect the defect easier than in early development conditions.

Overall, our data suggest that ubiquitylation is important for origin firing in xenopus, however this phenomenon seems to be more crucial in somatic-like replication rather than embryonic conditions. In addition, we were able to confirm OBI1's presence in the extract, its

interaction with the ORC complex and recruitment to replicating chromatin, while its functional role needs to be further investigated.

Discussion

Chapter 3 Discussion

The ubiquitin pathway plays a major role in the regulation of several cellular processes, including DNA replication. Our study provided key information on OBI1, a previously undescribed ubiquitin ligase, and its role in origin firing.

As previously mentioned, DNA replication is regulated through the ubiquitylation of several factors involved in the licensing and the firing reactions. In the first part of this manuscript, I presented the study initiated in the laboratory that led to the discovery of OBI1 that was found to positively regulate DNA replication origin activation. In this project we also showed that OBI1 binds the ORC complex and ubiquitylates two of its subunits and finally established a direct link between ORC's ubiquitylation and origin firing. In the second part of this thesis, we showed that some aspects of this regulation were conserved in the in vitro system based on Xenopus laevis egg extracts. Our findings also highlighted many aspects that remain to be investigated.

OBI1's role in origin selection

In human cells (project 1), OBI1 was shown to be crucial for efficient origin firing, since OBI1's knockdown resulted in approximately 50% less DNA synthesis and a similar decrease was shown in total fork density. This observation suggests that some replication origins managed, none the less, to fire allowing for replication to take place

One interpretation of these observations could be that the excess of potential replication origins set in GI phase of the cell cycle, allowed the cell to partly compensate for the inhibition of OBI1. This could be helped as well by the increase in the replication fork speed that we observed.

Another interpretation could be that OBI1's role in origin activation is attributed to the selection of a subset of replication origins. This selection operates at two levels, during G1 and during S phase. Origins can be selected during early G1, at the so-called timing decision point (Gilbert 2010, Wilson, Elefanty et al. 2016) where the timing of replication is set. Or in late G1, at the so-called Origin Decision Point where the selection of origins to be fired in the next S phase is decided. OBI1 could be involved in this origin selection, by selecting a subset

of origins to fire amongst all licensed origins, through ORC ubiquitylation. Consistently with the idea, previous studies on E3 ligases, such as the CRL4 ubiquitin ligase complex component, RepID, suggested a role of this subunit in origin selection. Interestingly, cells knocked down for RepID were able to compensate the phenotype by relying on an alternative E3 ligase, the SCF-Skp2 complex. However, the origins activated by repID were distinct from those activated by SCF-Skp2, showing different selectivity of two ubiquitin ligases towards replication origins, despite the overlapping mechanisms (Jang, Zhang et al. 2018).

OBI1 could also be involved in the replication temporal program by selecting at which time during S phase these origins are supposed to fire. The role of OBI1 in replication timing could also explain the subset of origins regulated by OBI1 and it can also be supported by the finding in our early mass spectrometry data (Project 1, fig1-a, b), that OBI1 interacts with ORCA, a subunit of the ORC complex. ORCA was shown to stabilize methylation on heterochromatic regions and its depletion was found to disturb the replication timing of late replicating origins (Giri, Aggarwal et al. 2015). The interaction between OBI1 and ORCA could suggest the presence of OBI1 in late replicating domains where this ligase could have a possible role in the activation of late replication origins.

Interestingly, the potential role of OBI1 in origin selection or replication timing is also supported by our results obtained in Xenopus laevis. Indeed, as previously mentioned, in the early stages of embryonic development, DNA replication is extremely fast, with replication origins regularly spaced at short intervals, suggesting that origin selection is bypassed. Also in accordance with our hypothesis, these early stages of development lack an obvious temporal regulation. Therefore, origin activation doesn't become restricted to specific sites or to a regulated timing until post-MBT stages, where in our study, OBI1 was shown to be over-expressed (Project 2, fig4-e, f) and in the condition mimicking post-MBT (high N/C ratio) where ubiquitylation inhibition was shown to have a more pronounced effect on replication.

OBI1's mechanism of action

OBI1 was found to catalyze ORC3 and ORC5 multi-monoubiquitylation. The E2 conjugating enzyme acting with OBI1 on this modification was later found to be UbcH5a (Project1, fig4-f, g, h). It is known that RING E3 ligases, unlike HECT ligases, do not transfer the ubiquitin directly to the substrates, thus allowing the E2 to have more influence on chain

formation. However, in most monoubiquitylation cases, the specificity of this modification is determined by the E3 ligase. In our case we strongly believe that OBI1 is responsible for the ubiquitylation specificity since UbcH5a was shown to help catalyze polyubiquitylation when interacting with other E3s (Windheim, Peggie et al. 2008).

In addition to chain specificity, it was also described that for many E3 ligases, working as a homodimer provides more specificity and efficiency towards their substrates. Accordingly, many RING ligases were shown to dimerize, either through the RING domain itself which is the case of RAD18 dimerization, or through another structure found in the protein (Metzger, Pruneda et al. 2014). Interestingly, OBI1's structure includes a coiled coil domain, which is known to enforce oligomerization. This information led us to further investigate the possibility of OBI1 dimerization. Our preliminary investigation suggests that wild type OBI1 is able to dimerize (and oligomerize) in vitro, while an OBI1 mutant with a coiled coil deletion was not able to dimerize (Data not shown) suggesting that this protein could potentially act as a dimer in vivo.

OBI1's regulation

On replicating chromatin, OBI1's loading follows ORC1's kinetic of recruitment, as they are both recruited in early S and found to be released from the chromatin at the end of S phase (Project 1, Fig1-f). OBI1's pattern on chromatin suggests a possible regulation in a cell cycle dependent manner. Indeed, OBI1 could be released from the chromatin at the end of S phase through ubiquitylation, as is the case of ORC1. Interestingly, our in vitro experiments aiming to characterize ORC3/5 ubiquitylation also show OBI1 auto-ubiquitylation (Project 1, Fig4-f, g, h), this ubiquitylation is specific to OBI1's catalytic domain since OBI1 inactive mutant (CS mutant) was unable to auto-ubiquitylate itself. This auto-ubiquitylation signal could be responsible for OBI1's release from the chromatin, or possibly for its degradation.

Interestingly, chromatin study of OBI1 in mitosis show a shift in the migration of the protein when compared to interphase chromatin. This observed shift could be due to a possible phosphorylation event (Project 1, Fig1-f). This signal could be either triggered by OBI1's ubiquitylation or it could act as an independent overlapping regulation mechanism. Indeed, phosphorylation was shown to act as an additional regulatory mechanism, by

regulating a protein's activity through changes in their cellular localization, such as the case of the licensing factor CDC6.

ORC ubiquitylation

OBI1 was found to regulate DNA replication by selecting and ubiquitylating a pool of chromatin bound ORC complex. However, how exactly this modification would favor the preferential activation of the ubiquitylated ORC related origins is still unclear.

One explanation could be the by the stabilization of the ORC complex and the tightening of its interaction with the chromatin. Indeed it was previously shown that a tight interaction between the ORC complex and the DNA is important for origin firing efficiency (Gardner, Gillespie et al. 2017). Another explanation, could be through "ORC activation". Work done on the ORC complex in drosophila shows different states of the ORC complex activity, an auto-inhibited state and an active state, the factors behind this activation remain unknown (Bleichert, Botchan et al. 2015). Interestingly, since a modification of the ORC complex is needed for efficient licensing, another modification event, involving OBI1, could be needed efficient firing.

In addition, multi-monoubiquitylation could mechanistically, or by modifying protein-protein interactions, alter the chromatin environment around the origins and allow the recruitment of factors involved in origin firing. This modification catalyzed by OBI1 could attract limiting factors involved in origins firing such as firing factors (CDC45, CDKs…) or chromatin regulators. Consistently, chromatin modulators and the chromatin environment were shown to be involved in replication initiation. The chromatin in some cases was shown to be responsible for origin selection, such as in yeast, enforcing origin specificity by preventing unspecific MCM binding (Kurat, Yeeles et al. 2017) or through facilitating pol alpha recruitment. The chromatin state is also important by ensuring proper replication timing in heterochromatic regions (Brustel, Kirstein et al. 2017) and for efficient origin activation by providing an open chromatin environment, which is the case of HBO1 (Feng, Vlassis et al. 2016) and GCN5 (Giri, Chakraborty et al. 2016).

These observations led to ask if ORC5 multi-monoubiquitylation could play a role in the interaction between the ORC complex and certain chromatin modifiers such as HBO1, PCAF and GCN5. By ubiquitylating a pool chromatin bound ORCs, OBI1 could allow the

recruitment of HATs, which would then provide an open local chromatin environment around the targeted origins and consequently stimulate origin activation. In order to test this hypothesis, we started a set of experiments that could shed some light on this matter. Interestingly, our preliminary experiments showed a specific interaction between ORC5 and GCN5 in comparison to other tested HATs (Supplementary fig1-a- b) and suggest a role of ORC5 ubiquitylation, mediated by OBI1, in increasing GCN5 -ORC5 interaction (Supplementary fig1-c-d). However, OBI1 role was not restricted to its catalytic activity suggesting that this protein could possibly act though another mechanism or simply as a platform for ORC5- GCN5 interaction. This observation could also be related to OBI1's dimerization proprieties, where the inactive mutant over expression could still provide a functional dimer responsible for the phenotype observed in this condition.

Curiously, GCN5 was found to interact with the ubiquitylated form of ORC5 but also with the unmodified ORC5. This suggests that GCN5 is able to recognize unmodified ORC5 by an ORC5 binding domain as well a potential ubiquitin binding domain which eventually allows it to increase the interaction affinity. This characteristic is observed in many ubiquitin dependent interactors such as the previously mentioned polymerase affinity to monoubiquitylated PCNA. OBI1 and unmodified ORC5 interaction could also suggest a possible interaction with a ubiquitylated ORC3 in complex with an unmodified ORC5, however at this point, our knowledge of the homogeneity of the ubiquitylated ORC subunits within the ORC complex is still narrow and needs further investigation.

Finally, it is important to highlight that the GCN5- ORC5 work is also in accordance with the results obtained in project 2. Indeed, in Xenopus laevis early development the chromatin is mainly in a relaxed state due to the lack of histone H1, replaced by histone B4, and down regulation of DNA methyltransferases. In these conditions, it is not surprising that there might be no need for GCN5 recruitment in order to open the chromatin environment and allow access of limiting factors. Therefore, if this "OBI1 pathway" is conserved in Xenopus laevis, the impact of its inhibition, would not cause major consequences during early development, as is the case in somatic cells with a more compact chromatin.

OBI1 and disease

As mentioned in the introduction, previous genome wide studies suggest that OBI1 might be implicated in late onset Alzheimer's disease. In addition, the ORC complex was also linked to Alzheimer's disease and to Meier- Gorlin syndrome.

Interestingly, in some patients with MGS, none of the mutations thought to be responsible for the disease where detected. This could suggest a defect in ORC at the protein level rather than the gene level, due to a possible defect in ORC ubiquitylation caused by an OBI1 mutation.

Conclusion and perspectives

In conclusion, this study provided new information on the implication of ubiquitylation in general and OBI1 in particular in DNA replication initiation. The characterization of OBI1 in human cells showed its implication in origin firing with a possible role in origin selection through chromatin remodeling. Further experiments done in Xenopus laevis supported OBI1's findings and hypotheses in human cells.

This study also highlighted a previously undescribed post translational modification of the ORC complex. More interestingly, it also added an important role for ORC in origin firing, while this complex's implication in DNA replication was mainly restricted to origin licensing.

Our findings also provide further implication of ubiquitylation in DNA replication in Xenopus laevis. In addition, they provide information on origin selection, replication timing and the chromatin environment in this system, and it reinforce the observation that all these processes might be less influential in early development.

Finally, there is still a lot to be learned concerning OBI1's role in human cells, therefore a dedicated study on the "OBI1 pathway" focused on GCN5 is essential in order to decipher how OBI1 is regulating replication origins. This future study could include interaction experiments done using non ubiquitylable ORC3/5 mutants and wild type GCN5 in order to provide strong proof of the implication of ubiquitylation in this process. Experiments aiming to study local chromatin environment, around replication origins, after OBI1 knockdown

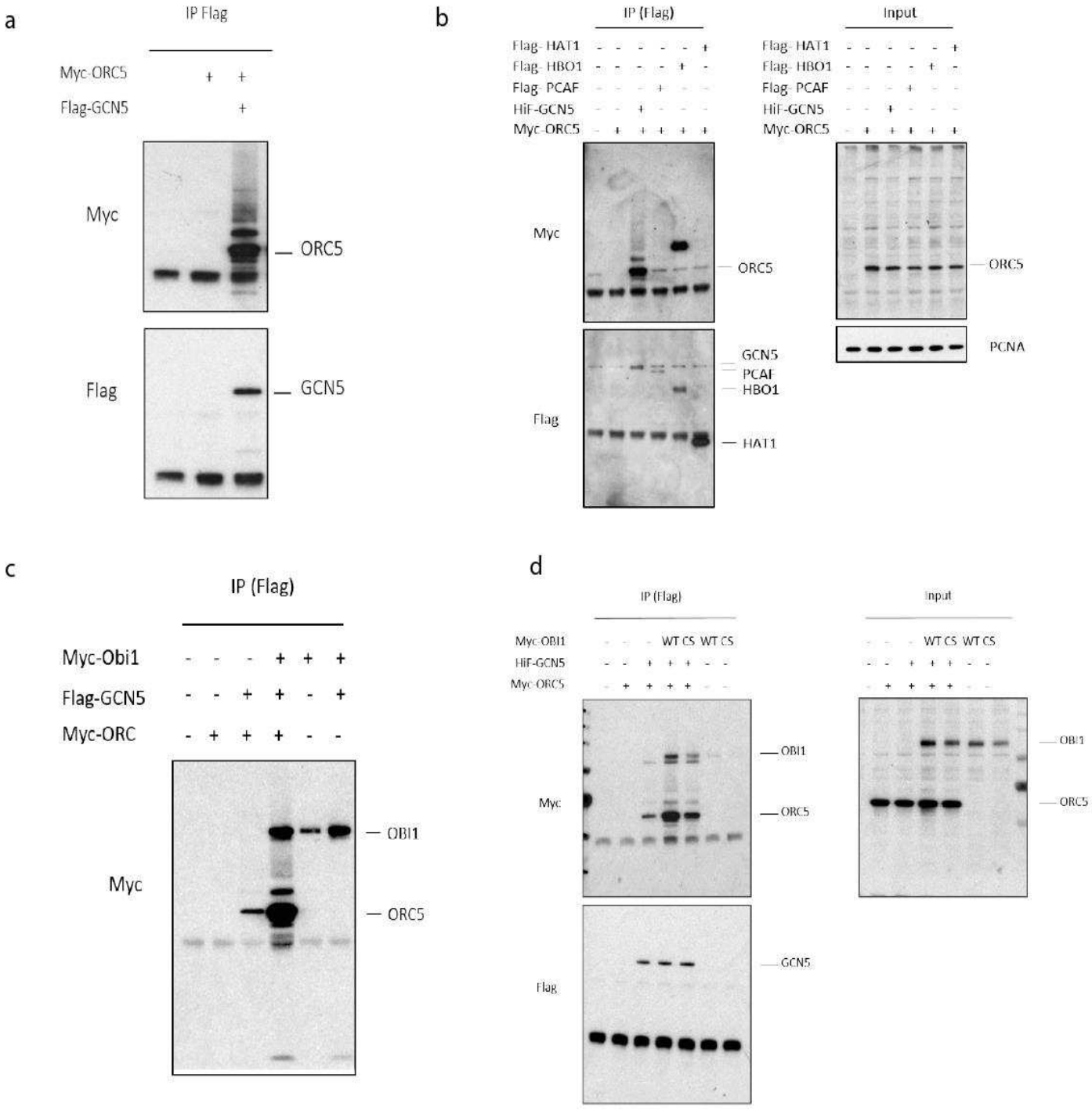
could provide a direct link between OBI1 and GCN5 induced modifications (By checking acetylation around the initiation sites that were already shown to be targeted by GCN5).

OBI1's potential role in origin selection could be studied by nascent stand purification analysis in cells that lack OBI1 compared to control cells. Furthermore, OBI1's suggested a role in replication timing could also be investigated by profiling the replication origin timing throughout the S phase after OBI1 knockdown.

In Xenopus Laevis, further analyses may also reveal how OBI1 is implicated in later stages of development. This could be achieved by depleting OBI1 in Xenopus extracts in post-MBT experimental conditions.

Finally, a proteomic approach on OBI1 in order to identify other binding partners of this protein could also be interesting.

Supplementary materials



Supplementary figure 1.ORC5-GCN5 interaction is specific and OBI1 dependent

a *U2OS cells were co-transfected with Flag-GCN5 and Myc-ORC5. 48 hours post-transfection cells were lysed and tagged GCN5 was immunoprecipitated using Flag-beads. Immunoprecipitated GCN5 and co-immunoprecipitated ORC5 were analyzed by western botting.* ***b*** *U2OS cells were co-transfected with*

the indicated Flag-tagged HATs and Myc-ORC5. 48 hours post-transfection cells were lysed and the tagged HATs were immunoprecipitated using Flag-beads. Immunoprecipitated HATs and co-immunoprecipitated ORC5 were analyzed by western botting. Expression of ORC5 in input extract was monitored by western blot. ***c*** *U2OS cells were co-transfected with Flag-GCN5, Myc-ORC5 and Myc-OBI1. 48 hours post-transfection cells were lysed and tagged GCN5 was immunoprecipitated using Flag-beads. Co-immunoprecitated ORC5 was analyzed by western botting.* ***d*** *U2OS cells were co-transfected with Flag-GCN5, Myc-ORC5, Myc-Wild type OBI1 and Myc-mutant OBI1 (CS). 48 hours post-transfection cells were lysed and tagged GCN5 was immunoprecipitated using Flag-beads. Precipitated GCN5 and co-immunoprecitated ORC5 and OBI1 were analyzed by western botting. Expression of ORC5 and OBI1 in input extract was monitored by western blot*

References

References

Aghajan, M., N. Jonai, K. Flick, F. Fu, M. Luo, X. Cai, I. Ouni, N. Pierce, X. Tang, B. Lomenick, R. Damoiseaux, R. Hao, P. M. Del Moral, R. Verma, Y. Li, C. Li, K. N. Houk, M. E. Jung, N. Zheng, L. Huang, R. J. Deshaies, P. Kaiser and J. Huang (2010). "Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase." Nat Biotechnol **28**(7): 738-742.

Almeida, R., J. M. Fernandez-Justel, C. Santa-Maria, J. C. Cadoret, L. Cano-Aroca, R. Lombrana, G. Herranz, A. Agresti and M. Gomez (2018). "Chromatin conformation regulates the coordination between DNA replication and transcription." Nat Commun **9**(1): 1590.

Almouzni, G. and M. Mechali (1988). "Xenopus egg extracts: a model system for chromatin replication." Biochim Biophys Acta **951**(2-3): 443-450.

Arendt, T. and M. K. Bruckner (2007). "Linking cell-cycle dysfunction in Alzheimer's disease to a failure of synaptic plasticity." Biochim Biophys Acta **1772**(4): 413-421.

Arias, E. E. and J. C. Walter (2005). "Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts." Genes Dev **19**(1): 114-126.

Arias, E. E. and J. C. Walter (2006). "PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication." Nat Cell Biol **8**(1): 84-90.

Bartke, T., M. Vermeulen, B. Xhemalce, S. C. Robson, M. Mann and T. Kouzarides (2010). "Nucleosome-interacting proteins regulated by DNA and histone methylation." Cell **143**(3): 470-484.

Bashir, T., N. V. Dorrello, V. Amador, D. Guardavaccaro and M. Pagano (2004). "Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase." Nature **428**(6979): 190-193.

Bell, S. P. (1995). "Eukaryotic replicators and associated protein complexes." Curr Opin Genet Dev **5**(2): 162-167.

Bell, S. P. (2002). "The origin recognition complex: from simple origins to complex functions." Genes Dev **16**(6): 659-672.

Bell, S. P. and B. Stillman (1992). "ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex." Nature **357**(6374): 128-134.

Bermudez, V. P., A. Farina, T. L. Higashi, F. Du, I. Tappin, T. S. Takahashi and J. Hurwitz (2012). "In vitro loading of human cohesin on DNA by the human Scc2-Scc4 loader complex." Proc Natl Acad Sci U S A **109**(24): 9366-9371.

Besnard, E., A. Babled, L. Lapasset, O. Milhavet, H. Parrinello, C. Dantec, J. M. Marin and J. M. Lemaitre (2012). "Unraveling cell type-specific and reprogrammable human replication origin signatures associated with G-quadruplex consensus motifs." Nat Struct Mol Biol **19**(8): 837-844.

Bienko, M., C. M. Green, N. Crosetto, F. Rudolf, G. Zapart, B. Coull, P. Kannouche, G. Wider, M. Peter, A. R. Lehmann, K. Hofmann and I. Dikic (2005). "Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis." Science **310**(5755): 1821-1824.

Bienko, M., C. M. Green, S. Sabbioneda, N. Crosetto, I. Matic, R. G. Hibbert, T. Begovic, A. Niimi, M. Mann, A. R. Lehmann and I. Dikic (2010). "Regulation of translesion synthesis DNA polymerase eta by monoubiquitination." Mol Cell **37**(3): 396-407.

Bleichert, F., M. R. Botchan and J. M. Berger (2015). "Crystal structure of the eukaryotic origin recognition complex." Nature **519**(7543): 321-326.

Bloom, D. (1954). "Congenital telangiectatic erythema resembling lupus erythematosus in dwarfs; probably a syndrome entity." AMA Am J Dis Child **88**(6): 754-758.
Blow, J. J. and R. A. Laskey (1986). "Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs." Cell **47**(4): 577-587.
Boname, J. M., M. Thomas, H. R. Stagg, P. Xu, J. Peng and P. J. Lehner (2010). "Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains." Traffic **11**(2): 210-220.
Borden, K. L. and P. S. Freemont (1996). "The RING finger domain: a recent example of a sequence-structure family." Curr Opin Struct Biol **6**(3): 395-401.
Brewer, B. J. and W. L. Fangman (1987). "The localization of replication origins on ARS plasmids in S. cerevisiae." Cell **51**(3): 463-471.
Brustel, J., N. Kirstein, F. Izard, C. Grimaud, P. Prorok, C. Cayrou, G. Schotta, A. F. Abdelsamie, J. Dejardin, M. Mechali, G. Baldacci, C. Sardet, J. C. Cadoret, A. Schepers and E. Julien (2017). "Histone H4K20 tri-methylation at late-firing origins ensures timely heterochromatin replication." EMBO J **36**(18): 2726-2741.
Cappuccio, I., C. Colapicchioni, V. Santangelo, P. Sale, F. Blandini, M. Bonelli, C. Niccolini, C. Busceti, D. Bucci, F. Nicoletti and D. Melchiorri (2010). "The origin recognition complex subunit, ORC3, is developmentally regulated and supports the expression of biochemical markers of neuronal maturation in cultured cerebellar granule cells." Brain Res **1358**: 1-10.
Carter, S., O. Bischof, A. Dejean and K. H. Vousden (2007). "C-terminal modifications regulate MDM2 dissociation and nuclear export of p53." Nat Cell Biol **9**(4): 428-435.
Cayrou, C., B. Ballester, I. Peiffer, R. Fenouil, P. Coulombe, J. C. Andrau, J. van Helden and M. Mechali (2015). "The chromatin environment shapes DNA replication origin organization and defines origin classes." Genome Res **25**(12): 1873-1885.
Cayrou, C., P. Coulombe, A. Puy, S. Rialle, N. Kaplan, E. Segal and M. Mechali (2012). "New insights into replication origin characteristics in metazoans." Cell Cycle **11**(4).
Cayrou, C., P. Coulombe, A. Puy, S. Rialle, N. Kaplan, E. Segal and M. Mechali (2012). "New insights into replication origin characteristics in metazoans." Cell Cycle **11**(4): 658-667.
Cayrou, C., P. Coulombe, A. Vigneron, S. Stanojcic, O. Ganier, I. Peiffer, E. Rivals, A. Puy, S. Laurent-Chabalier, R. Desprat and M. Mechali (2011). "Genome-scale analysis of metazoan replication origins reveals their organization in specific but flexible sites defined by conserved features." Genome Res **21**(9): 1438-1449.
Chakraborty, A., Z. Shen and S. G. Prasanth (2011). ""ORCanization" on heterochromatin: linking DNA replication initiation to chromatin organization." Epigenetics **6**(6): 665-670.
Chan, C. H., J. K. Morrow, C. F. Li, Y. Gao, G. Jin, A. Moten, L. J. Stagg, J. E. Ladbury, Z. Cai, D. Xu, C. J. Logothetis, M. C. Hung, S. Zhang and H. K. Lin (2013). "Pharmacological inactivation of Skp2 SCF ubiquitin ligase restricts cancer stem cell traits and cancer progression." Cell **154**(3): 556-568.
Chandrasekaran, S., T. X. Tan, J. R. Hall and J. G. Cook (2011). "Stress-stimulated mitogen-activated protein kinases control the stability and activity of the Cdt1 DNA replication licensing factor." Mol Cell Biol **31**(22): 4405-4416.
Chang, L. and D. Barford (2014). "Insights into the anaphase-promoting complex: a molecular machine that regulates mitosis." Curr Opin Struct Biol **29**: 1-9.
Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda and A. Varshavsky (1989). "A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein." Science **243**(4898): 1576-1583.

Chen, S. and S. P. Bell (2011). "CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6." Genes Dev 25(4): 363-372.
Chesnokov, I. N., O. N. Chesnokova and M. Botchan (2003). "A cytokinetic function of Drosophila ORC6 protein resides in a domain distinct from its replication activity." Proc Natl Acad Sci U S A 100(16): 9150-9155.
Choi, W. S., B. C. Jeong, Y. J. Joo, M. R. Lee, J. Kim, M. J. Eck and H. K. Song (2010). "Structural basis for the recognition of N-end rule substrates by the UBR box of ubiquitin ligases." Nat Struct Mol Biol 17(10): 1175-1181.
Christensen, D. E., P. S. Brzovic and R. E. Klevit (2007). "E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages." Nat Struct Mol Biol 14(10): 941-948.
Chuang, L. C. and P. R. Yew (2001). "Regulation of nuclear transport and degradation of the Xenopus cyclin-dependent kinase inhibitor, p27Xic1." J Biol Chem 276(2): 1610-1617.
Cook, J. G., D. A. Chasse and J. R. Nevins (2004). "The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells." J Biol Chem 279(10): 9625-9633.
Coulombe, P., D. Gregoire, N. Tsanov and M. Mechali (2013). "A spontaneous Cdt1 mutation in 129 mouse strains reveals a regulatory domain restraining replication licensing." Nat Commun 4: 2065.
Craig, J. M., E. Earle, P. Canham, L. H. Wong, M. Anderson and K. H. Choo (2003). "Analysis of mammalian proteins involved in chromatin modification reveals new metaphase centromeric proteins and distinct chromosomal distribution patterns." Hum Mol Genet 12(23): 3109-3121.
Croteau, D. L., V. Popuri, P. L. Opresko and V. A. Bohr (2014). "Human RecQ helicases in DNA repair, recombination, and replication." Annu Rev Biochem 83: 519-552.
Daigaku, Y., T. J. Etheridge, Y. Nakazawa, M. Nakayama, A. T. Watson, I. Miyabe, T. Ogi, M. A. Osborne and A. M. Carr (2017). "PCNA ubiquitylation ensures timely completion of unperturbed DNA replication in fission yeast." PLoS Genet 13(5): e1006789.
Das-Bradoo, S., R. M. Ricke and A. K. Bielinsky (2006). "Interaction between PCNA and diubiquitinated Mcm10 is essential for cell growth in budding yeast." Mol Cell Biol 26(13): 4806-4817.
de Munnik, S. A., E. H. Hoefsloot, J. Roukema, J. Schoots, N. V. Knoers, H. G. Brunner, A. P. Jackson and E. M. Bongers (2015). "Meier-Gorlin syndrome." Orphanet J Rare Dis 10: 114.
DePamphilis, M. L. (1993). "Origins of DNA replication in metazoan chromosomes." J Biol Chem 268(1): 1-4.
Depamphilis, M. L., C. M. de Renty, Z. Ullah and C. Y. Lee (2012). ""The Octet": Eight Protein Kinases that Control Mammalian DNA Replication." Front Physiol 3: 368.
Deshaies, R. J. and C. A. Joazeiro (2009). "RING domain E3 ubiquitin ligases." Annu Rev Biochem 78: 399-434.
Dewar, J. M., E. Low, M. Mann, M. Raschle and J. C. Walter (2017). "CRL2(Lrr1) promotes unloading of the vertebrate replisome from chromatin during replication termination." Genes Dev 31(3): 275-290.
Diffley, J. F. and J. H. Cocker (1992). "Protein-DNA interactions at a yeast replication origin." Nature 357(6374): 169-172.
Dikic, I., S. Wakatsuki and K. J. Walters (2009). "Ubiquitin-binding domains - from structures to functions." Nat Rev Mol Cell Biol 10(10): 659-671.
Donzelli, M., M. Squatrito, D. Ganoth, A. Hershko, M. Pagano and G. F. Draetta (2002). "Dual mode of degradation of Cdc25 A phosphatase." EMBO J 21(18): 4875-4884.

Dupont, S., A. Mamidi, M. Cordenonsi, M. Montagner, L. Zacchigna, M. Adorno, G. Martello, M. J. Stinchfield, S. Soligo, L. Morsut, M. Inui, S. Moro, N. Modena, F. Argenton, S. J. Newfeld and S. Piccolo (2009). "FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination." Cell **136**(1): 123-135.
Dynek, J. N., T. Goncharov, E. C. Dueber, A. V. Fedorova, A. Izrael-Tomasevic, L. Phu, E. Helgason, W. J. Fairbrother, K. Deshayes, D. S. Kirkpatrick and D. Vucic (2010). "c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling." EMBO J **29**(24): 4198-4209.
Eldridge, A. G., A. V. Loktev, D. V. Hansen, E. W. Verschuren, J. D. Reimann and P. K. Jackson (2006). "The evi5 oncogene regulates cyclin accumulation by stabilizing the anaphase-promoting complex inhibitor emi1." Cell **124**(2): 367-380.
Evrin, C., P. Clarke, J. Zech, R. Lurz, J. Sun, S. Uhle, H. Li, B. Stillman and C. Speck (2009). "A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication." Proc Natl Acad Sci U S A **106**(48): 20240-20245.
Feng, Y., A. Vlassis, C. Roques, M. E. Lalonde, C. Gonzalez-Aguilera, J. P. Lambert, S. B. Lee, X. Zhao, C. Alabert, J. V. Johansen, E. Paquet, X. J. Yang, A. C. Gingras, J. Cote and A. Groth (2016). "BRPF3-HBO1 regulates replication origin activation and histone H3K14 acetylation." EMBO J **35**(2): 176-192.
Finley, D. (2009). "Recognition and processing of ubiquitin-protein conjugates by the proteasome." Annu Rev Biochem **78**: 477-513.
Foulk, M. S., J. M. Urban, C. Casella and S. A. Gerbi (2015). "Characterizing and controlling intrinsic biases of lambda exonuclease in nascent strand sequencing reveals phasing between nucleosomes and G-quadruplex motifs around a subset of human replication origins." Genome Res **25**(5): 725-735.
Fragkos, M., O. Ganier, P. Coulombe and M. Mechali (2015). "DNA replication origin activation in space and time." Nat Rev Mol Cell Biol **16**(6): 360-374.
Francon, P., J. M. Lemaitre, C. Dreyer, D. Maiorano, O. Cuvier and M. Mechali (2004). "A hypophosphorylated form of RPA34 is a specific component of pre-replication centers." J Cell Sci **117**(Pt 21): 4909-4920.
Freudenthal, B. D., L. Gakhar, S. Ramaswamy and M. T. Washington (2010). "Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange." Nat Struct Mol Biol **17**(4): 479-484.
Fryns, J. P. (1998). "Meier-Gorlin syndrome: the adult phenotype." Clin Dysmorphol **7**(3): 231-232.
Fukushima, H., K. Ogura, L. Wan, Y. Lu, V. Li, D. Gao, P. Liu, A. W. Lau, T. Wu, M. W. Kirschner, H. Inuzuka and W. Wei (2013). "SCF-mediated Cdh1 degradation defines a negative feedback system that coordinates cell-cycle progression." Cell Rep **4**(4): 803-816.
Gardner, N. J., P. J. Gillespie, J. T. Carrington, E. J. Shanks, S. P. McElroy, E. J. Haagensen, J. A. Frearson, A. Woodland and J. J. Blow (2017). "The High-Affinity Interaction between ORC and DNA that Is Required for Replication Licensing Is Inhibited by 2-Arylquinolin-4-Amines." Cell Chem Biol **24**(8): 981-992 e984.
Ge, X. Q., D. A. Jackson and J. J. Blow (2007). "Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress." Genes Dev **21**(24): 3331-3341.
Gerlach, B., S. M. Cordier, A. C. Schmukle, C. H. Emmerich, E. Rieser, T. L. Haas, A. I. Webb, J. A. Rickard, H. Anderton, W. W. Wong, U. Nachbur, L. Gangoda, U. Warnken, A. W. Purcell, J. Silke and H. Walczak (2011). "Linear ubiquitination prevents inflammation and regulates immune signalling." Nature **471**(7340): 591-596.

German, J. (1997). "Bloom's syndrome. XX. The first 100 cancers." Cancer Genet Cytogenet **93**(1): 100-106.
Gilbert, D. M. (2010). "Cell fate transitions and the replication timing decision point." J Cell Biol **191**(5): 899-903.
Gillespie, P. J., A. Gambus and J. J. Blow (2012). "Preparation and use of Xenopus egg extracts to study DNA replication and chromatin associated proteins." Methods **57**(2): 203-213.
Giri, S., V. Aggarwal, J. Pontis, Z. Shen, A. Chakraborty, A. Khan, C. Mizzen, K. V. Prasanth, S. Ait-Si-Ali, T. Ha and S. G. Prasanth (2015). "The preRC protein ORCA organizes heterochromatin by assembling histone H3 lysine 9 methyltransferases on chromatin." Elife **4**.
Giri, S., A. Chakraborty, K. M. Sathyan, K. V. Prasanth and S. G. Prasanth (2016). "Orc5 induces large-scale chromatin decondensation in a GCN5-dependent manner." J Cell Sci **129**(2): 417-429.
Giri, S. and S. G. Prasanth (2015). "Association of ORCA/LRWD1 with repressive histone methyl transferases mediates heterochromatin organization." Nucleus **6**(6): 435-441.
Glotzer, M., A. W. Murray and M. W. Kirschner (1991). "Cyclin is degraded by the ubiquitin pathway." Nature **349**(6305): 132-138.
Good, M. C. (2016). "Encapsulation of Xenopus Egg and Embryo Extract Spindle Assembly Reactions in Synthetic Cell-Like Compartments with Tunable Size." Methods Mol Biol **1413**: 87-108.
Good, M. C. and R. Heald (2018). "Preparation of Cellular Extracts from Xenopus Eggs and Embryos." Cold Spring Harb Protoc **2018**(6).
Gorlin, R. J., J. Cervenka, K. Moller, M. Horrobin and C. J. Witkop, Jr. (1975). "Malformation syndromes. A selected miscellany." Birth Defects Orig Artic Ser **11**(2): 39-50.
Goto, E., Y. Yamanaka, A. Ishikawa, M. Aoki-Kawasumi, M. Mito-Yoshida, M. Ohmura-Hoshino, Y. Matsuki, M. Kajikawa, H. Hirano and S. Ishido (2010). "Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex class I internalization." J Biol Chem **285**(46): 35311-35319.
Hannak, E. and R. Heald (2006). "Investigating mitotic spindle assembly and function in vitro using Xenopus laevis egg extracts." Nat Protoc **1**(5): 2305-2314.
Hao, B., S. Oehlmann, M. E. Sowa, J. W. Harper and N. P. Pavletich (2007). "Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases." Mol Cell **26**(1): 131-143.
Hao, B., N. Zheng, B. A. Schulman, G. Wu, J. J. Miller, M. Pagano and N. P. Pavletich (2005). "Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase." Mol Cell **20**(1): 9-19.
Harland, R. M. and R. A. Laskey (1980). "Regulated replication of DNA microinjected into eggs of Xenopus laevis." Cell **21**: 761-771.
Hartley, R. S., J. C. Sible, A. L. Lewellyn and J. L. Maller (1997). "A role for cyclin E/Cdk2 in the timing of the midblastula transition in Xenopus embryos." Dev Biol **188**(2): 312-321.
Hemerly, A. S., S. G. Prasanth, K. Siddiqui and B. Stillman (2009). "Orc1 controls centriole and centrosome copy number in human cells." Science **323**(5915): 789-793.
Hibbert, R. G., A. Huang, R. Boelens and T. K. Sixma (2011). "E3 ligase Rad18 promotes monoubiquitination rather than ubiquitin chain formation by E2 enzyme Rad6." Proc Natl Acad Sci U S A **108**(14): 5590-5595.
Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis and S. Jentsch (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-141.

Hossain, M. and B. Stillman (2012). "Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication." Genes Dev **26**(16): 1797-1810.
Hu, M., P. Li, M. Li, W. Li, T. Yao, J. W. Wu, W. Gu, R. E. Cohen and Y. Shi (2002). "Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde." Cell **111**(7): 1041-1054.
Huang, C., J. Cheng, T. Bawa-Khalfe, X. Yao, Y. E. Chin and E. T. H. Yeh (2016). "SUMOylated ORC2 Recruits a Histone Demethylase to Regulate Centromeric Histone Modification and Genomic Stability." Cell Rep **15**(1): 147-157.
Huang, Z., K. Zang and L. F. Reichardt (2005). "The origin recognition core complex regulates dendrite and spine development in postmitotic neurons." J Cell Biol **170**(4): 527-535.
Huberman, J. A. and A. D. Riggs (1968). "On the mechanism of DNA replication in mammalian chromosomes." J Mol Biol **32**(327): 327-341.
Hyrien, O., C. Maric and M. Mechali (1995). "Transition in specification of embryonic metazoan DNA replication origins." Science **270**(5238): 994-997.
Hyrien, O., C. Maric and M. Mechali (1995). "Transition in specification of embryonic metazoan DNA replication origins." Science **270**(5238): 994-997.
Iizuka, M., T. Matsui, H. Takisawa and M. M. Smith (2006). "Regulation of replication licensing by acetyltransferase Hbo1." Mol Cell Biol **26**(3): 1098-1108.
Iizuka, M. and B. Stillman (1999). "Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein." Journal of Biological Chemistry **274**(33): 23027-23034.
Ilves, I., T. Petojevic, J. J. Pesavento and M. R. Botchan (2010). "Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins." Mol Cell **37**(2): 247-258.
Jacob, F., J. Brenner and F. Cuzin (1963). "On the regulation of DNA replication in bacteria." Cold Spring Harbor Symp. Quant. Biol. **28**: 329-348.
Jang, S. M., Y. Zhang, K. Utani, H. Fu, C. E. Redon, A. B. Marks, O. K. Smith, C. J. Redmond, A. M. Baris, D. A. Tulchinsky and M. I. Aladjem (2018). "The replication initiation determinant protein (RepID) modulates replication by recruiting CUL4 to chromatin." Nat Commun **9**(1): 2782.
Jaspersen, S. L., J. F. Charles and D. O. Morgan (1999). "Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14." Curr Biol **9**(5): 227-236.
Jin, L., A. Williamson, S. Banerjee, I. Philipp and M. Rape (2008). "Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex." Cell **133**(4): 653-665.
Johnson, E. S., P. C. Ma, I. M. Ota and A. Varshavsky (1995). "A proteolytic pathway that recognizes ubiquitin as a degradation signal." J Biol Chem **270**(29): 17442-17456.
Kaiser, S. E., B. E. Riley, T. A. Shaler, R. S. Trevino, C. H. Becker, H. Schulman and R. R. Kopito (2011). "Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools." Nat Methods **8**(8): 691-696.
Kamadurai, H. B., J. Souphron, D. C. Scott, D. M. Duda, D. J. Miller, D. Stringer, R. C. Piper and B. A. Schulman (2009). "Insights into ubiquitin transfer cascades from a structure of a UbcH5B approximately ubiquitin-HECT(NEDD4L) complex." Mol Cell **36**(6): 1095-1102.
Kim, J. and E. T. Kipreos (2008). "Control of the Cdc6 replication licensing factor in metazoa: the role of nuclear export and the CUL4 ubiquitin ligase." Cell Cycle **7**(2): 146-150.
Kim, W., E. J. Bennett, E. L. Huttlin, A. Guo, J. Li, A. Possemato, M. E. Sowa, R. Rad, J. Rush, M. J. Comb, J. W. Harper and S. P. Gygi (2011). "Systematic and quantitative assessment of the ubiquitin-modified proteome." Mol Cell **44**(2): 325-340.

King, R. W., J. M. Peters, S. Tugendreich, M. Rolfe, P. Hieter and M. W. Kirschner (1995). "A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B." Cell 81(2): 279-288.
Kirkpatrick, D. S., N. A. Hathaway, J. Hanna, S. Elsasser, J. Rush, D. Finley, R. W. King and S. P. Gygi (2006). "Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology." Nat Cell Biol 8(7): 700-710.
Koepp, D. M., L. K. Schaefer, X. Ye, K. Keyomarsi, C. Chu, J. W. Harper and S. J. Elledge (2001). "Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase." Science 294(5540): 173-177.
Komander, D. (2009). "The emerging complexity of protein ubiquitination." Biochem Soc Trans 37(Pt 5): 937-953.
Kumagai, A., A. Shevchenko, A. Shevchenko and W. G. Dunphy (2010). "Treslin collaborates with TopBP1 in triggering the initiation of DNA replication." Cell 140(3): 349-359.
Kurat, C. F., J. T. P. Yeeles, H. Patel, A. Early and J. F. X. Diffley (2017). "Chromatin Controls DNA Replication Origin Selection, Lagging-Strand Synthesis, and Replication Fork Rates." Mol Cell 65(1): 117-130.
Larsen, N. B., A. O. Gao, J. L. Sparks, I. Gallina, R. A. Wu, M. Mann, M. Raschle, J. C. Walter and J. P. Duxin (2019). "Replication-Coupled DNA-Protein Crosslink Repair by SPRTN and the Proteasome in Xenopus Egg Extracts." Mol Cell 73(3): 574-588 e577.
Lau, A. W., H. Inuzuka, H. Fukushima, L. Wan, P. Liu, D. Gao, Y. Sun and W. Wei (2013). "Regulation of APC(Cdh1) E3 ligase activity by the Fbw7/cyclin E signaling axis contributes to the tumor suppressor function of Fbw7." Cell Res 23(7): 947-961.
Leach, C. A. and W. M. Michael (2005). "Ubiquitin/SUMO modification of PCNA promotes replication fork progression in Xenopus laevis egg extracts." J Cell Biol 171(6): 947-954.
Li, C. J. and M. L. DePamphilis (2002). "Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle." Mol Cell Biol 22(1): 105-116.
Li, C. J., A. Vassilev and M. L. DePamphilis (2004). "Role for Cdk1 (Cdc2)/cyclin A in preventing the mammalian origin recognition complex's largest subunit (Orc1) from binding to chromatin during mitosis." Mol Cell Biol 24(13): 5875-5886.
Li, M., C. L. Brooks, F. Wu-Baer, D. Chen, R. Baer and W. Gu (2003). "Mono- versus polyubiquitination: differential control of p53 fate by Mdm2." Science 302(5652): 1972-1975.
Li, W., M. H. Bengtson, A. Ulbrich, A. Matsuda, V. A. Reddy, A. Orth, S. K. Chanda, S. Batalov and C. A. Joazeiro (2008). "Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling." PLoS One 3(1): e1487.
Li, W., D. Tu, A. T. Brunger and Y. Ye (2007). "A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate." Nature 446(7133): 333-337.
Li, Y., H. Wu, W. Wu, W. Zhuo, W. Liu, Y. Zhang, M. Cheng, Y. G. Chen, N. Gao, H. Yu, L. Wang, W. Li and M. Yang (2014). "Structural insights into the TRIM family of ubiquitin E3 ligases." Cell Res 24(6): 762-765.
Lin, D. I., O. Barbash, K. G. Kumar, J. D. Weber, J. W. Harper, A. J. Klein-Szanto, A. Rustgi, S. Y. Fuchs and J. A. Diehl (2006). "Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex." Mol Cell 24(3): 355-366.
Liu, G. and E. Warbrick (2006). "The p66 and p12 subunits of DNA polymerase delta are modified by ubiquitin and ubiquitin-like proteins." Biochem Biophys Res Commun 349(1): 360-366.

Lutzmann, M. and M. Mechali (2008). "MCM9 binds Cdt1 and is required for the assembly of prereplication complexes." Mol Cell **31**(2): 190-200.
Macheret, M. and T. D. Halazonetis (2015). "DNA replication stress as a hallmark of cancer." Annu Rev Pathol **10**: 425-448.
Mahaffey, D. T., C. Gorbea and M. Rechsteiner (2003). "Evidence that DNA replication is not regulated by ubiquitin-dependent proteolysis in Xenopus egg extract." Exp Cell Res **288**(2): 225-234.
Mailand, N. and J. F. Diffley (2005). "CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis." Cell **122**(6): 915-926.
Mailand, N., I. Gibbs-Seymour and S. Bekker-Jensen (2013). "Regulation of PCNA-protein interactions for genome stability." Nat Rev Mol Cell Biol **14**(5): 269-282.
Maiorano, D., O. Cuvier, E. Danis and M. Mechali (2005). "MCM8 is an MCM2-7-related protein that functions as a DNA helicase during replication elongation and not initiation." Cell **120**(3): 315-328.
Maiorano, D., J. Moreau and M. Mechali (2000). "XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis." Nature **404**(6778): 622-625.
Makise, M., M. Takehara, A. Kuniyasu, N. Matsui, H. Nakayama and T. Mizushima (2009). "Linkage between phosphorylation of the origin recognition complex and its ATP binding activity in Saccharomyces cerevisiae." J Biol Chem **284**(6): 3396-3407.
Maric, M., T. Maculins, G. De Piccoli and K. Labib (2014). "Cdc48 and a ubiquitin ligase drive disassembly of the CMG helicase at the end of DNA replication." Science **346**(6208): 1253596.
Marin, I., J. I. Lucas, A. C. Gradilla and A. Ferrus (2004). "Parkin and relatives: the RBR family of ubiquitin ligases." Physiol Genomics **17**(3): 253-263.
Martin, M. M., M. Ryan, R. Kim, A. L. Zakas, H. Fu, C. M. Lin, W. C. Reinhold, S. R. Davis, S. Bilke, H. Liu, J. H. Doroshow, M. A. Reimers, M. S. Valenzuela, Y. Pommier, P. S. Meltzer and M. I. Aladjem (2011). "Genome-wide depletion of replication initiation events in highly transcribed regions." Genome Res **21**(11): 1822-1832.
Masai, H., S. Matsumoto, Z. You, N. Yoshizawa-Sugata and M. Oda (2010). "Eukaryotic chromosome DNA replication: where, when, and how?" Annu Rev Biochem **79**: 89-130.
Matsumoto, M. L., K. E. Wickliffe, K. C. Dong, C. Yu, I. Bosanac, D. Bustos, L. Phu, D. S. Kirkpatrick, S. G. Hymowitz, M. Rape, R. F. Kelley and V. M. Dixit (2010). "K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody." Mol Cell **39**(3): 477-484.
Matta-Camacho, E., G. Kozlov, F. F. Li and K. Gehring (2010). "Structural basis of substrate recognition and specificity in the N-end rule pathway." Nat Struct Mol Biol **17**(10): 1182-1187.
McGarry, T. J. and M. W. Kirschner (1998). "Geminin, an inhibitor of DNA replication, is degraded during mitosis." Cell **93**(6): 1043-1053.
Mechali, M. and S. Kearsey (1984). "Lack of specific sequence requirement for DNA replication in Xenopus eggs compared with high sequence specificity in yeast." Cell **38**(1): 55-64.
Mendez, J., X. H. Zou-Yang, S. Y. Kim, M. Hidaka, W. P. Tansey and B. Stillman (2002). "Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication." Mol Cell **9**(3): 481-491.
Metzger, M. B., J. N. Pruneda, R. E. Klevit and A. M. Weissman (2014). "RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination." Biochim Biophys Acta **1843**(1): 47-60.

Michelle, C., P. Vourc'h, L. Mignon and C. R. Andres (2009). "What was the set of ubiquitin and ubiquitin-like conjugating enzymes in the eukaryote common ancestor?" J Mol Evol 68(6): 616-628.
Miotto, B. and K. Struhl (2008). "HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1." Genes Dev 22(19): 2633-2638.
Miotto, B. and K. Struhl (2010). "HBO1 histone acetylase activity is essential for DNA replication licensing and inhibited by Geminin." Mol Cell 37(1): 57-66.
Monnat, R. J., Jr. (2010). "Human RECQ helicases: roles in DNA metabolism, mutagenesis and cancer biology." Semin Cancer Biol 20(5): 329-339.
Moreno, S. P., R. Bailey, N. Campion, S. Herron and A. Gambus (2014). "Polyubiquitylation drives replisome disassembly at the termination of DNA replication." Science 346(6208): 477-481.
Morett, E. and P. Bork (1999). "A novel transactivation domain in parkin." Trends Biochem Sci 24(6): 229-231.
Mosca, A., S. Sperduti, V. Pop, D. Ciavardelli, A. Granzotto, M. Punzi, L. Stuppia, V. Gatta, F. Assogna, N. Banaj, F. Piras, F. Piras, C. Caltagirone, G. Spalletta and S. L. Sensi (2018). "Influence of APOE and RNF219 on Behavioral and Cognitive Features of Female Patients Affected by Mild Cognitive Impairment or Alzheimer's Disease." Front Aging Neurosci 10: 92.
Murray, A. W. (1991). "Cell cycle extracts." Methods in Cell Biology 36: 581-605.
Narbonne-Reveau, K., S. Senger, M. Pal, A. Herr, H. E. Richardson, M. Asano, P. Deak and M. A. Lilly (2008). "APC/CFzr/Cdh1 promotes cell cycle progression during the Drosophila endocycle." Development 135(8): 1451-1461.
Natale, D. A., C. J. Li, W. H. Sun and M. L. DePamphilis (2000). "Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals." EMBO J 19(11): 2728-2738.
Newport, J. and M. Kirschner (1982). "A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage." Cell 30(3): 675-686.
Newport, J. and M. Kirschner (1982). "A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription." Cell 30(3): 687-696.
Nguyen, G. H., W. Tang, A. I. Robles, R. P. Beyer, L. T. Gray, J. A. Welsh, A. J. Schetter, K. Kumamoto, X. W. Wang, I. D. Hickson, N. Maizels, R. J. Monnat, Jr. and C. C. Harris (2014). "Regulation of gene expression by the BLM helicase correlates with the presence of G-quadruplex DNA motifs." Proc Natl Acad Sci U S A 111(27): 9905-9910.
Nikolaishvili-Feinberg, N., G. S. Jenkins, K. R. Nevis, D. P. Staus, C. O. Scarlett, K. Unsal-Kacmaz, W. K. Kaufmann and M. Cordeiro-Stone (2008). "Ubiquitylation of proliferating cell nuclear antigen and recruitment of human DNA polymerase eta." Biochemistry 47(13): 4141-4150.
Orlicky, S., X. Tang, V. Neduva, N. Elowe, E. D. Brown, F. Sicheri and M. Tyers (2010). "An allosteric inhibitor of substrate recognition by the SCF(Cdc4) ubiquitin ligase." Nat Biotechnol 28(7): 733-737.
Pacek, M. and J. C. Walter (2004). "A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication." EMBO J 23(18): 3667-3676.
Peng, J., D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley and S. P. Gygi (2003). "A proteomics approach to understanding protein ubiquitination." Nat Biotechnol 21(8): 921-926.

Petersen, B. O., C. Wagener, F. Marinoni, E. R. Kramer, M. Melixetian, E. Lazzerini Denchi, C. Gieffers, C. Matteucci, J. M. Peters and K. Helin (2000). "Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1." Genes Dev **14**(18): 2330-2343.
Prasanth, S. G., K. V. Prasanth, K. Siddiqui, D. L. Spector and B. Stillman (2004). "Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance." EMBO J **23**(13): 2651-2663.
Prasanth, S. G., K. V. Prasanth and B. Stillman (2002). "Orc6 involved in DNA replication, chromosome segregation, and cytokinesis." Science **297**(5583): 1026-1031.
Pray, L. (2008). "Major molecular events of DNA replication." Nature Education **1(1):99**.
Prioleau, M. N. and D. M. MacAlpine (2016). "DNA replication origins-where do we begin?" Genes Dev **30**(15): 1683-1697.
Prorok, P., M. Artufel, A. Aze, P. Coulombe, I. Peiffer, L. Lacroix, A. Guedin, J. L. Mergny, J. Damaschke, A. Schepers, B. Ballester and M. Mechali (2019). "Involvement of G-quadruplex regions in mammalian replication origin activity." Nat Commun **10**(1): 3274.
Rahighi, S., F. Ikeda, M. Kawasaki, M. Akutsu, N. Suzuki, R. Kato, T. Kensche, T. Uejima, S. Bloor, D. Komander, F. Randow, S. Wakatsuki and I. Dikic (2009). "Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation." Cell **136**(6): 1098-1109.
Rape, M. and M. W. Kirschner (2004). "Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry." Nature **432**(7017): 588-595.
Ravid, T. and M. Hochstrasser (2008). "Diversity of degradation signals in the ubiquitin-proteasome system." Nat Rev Mol Cell Biol **9**(9): 679-690.
Rohrbough, J., S. Pinto, R. M. Mihalek, T. Tully and K. Broadie (1999). "latheo, a Drosophila gene involved in learning, regulates functional synaptic plasticity." Neuron **23**(1): 55-70.
Romanowski, P., M. A. Madine, A. Rowles, J. J. Blow and R. A. Laskey (1996). "The Xenopus origin recognition complex is essential for DNA replication and MCM binding to chromatin." Curr Biol **6**(11): 1416-1425.
Rotin, D. and S. Kumar (2009). "Physiological functions of the HECT family of ubiquitin ligases." Nat Rev Mol Cell Biol **10**(6): 398-409.
Rowles, A., S. Tada and J. J. Blow (1999). "Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins." J Cell Sci **112 (Pt 12)**: 2011-2018.
Saeki, Y., T. Kudo, T. Sone, Y. Kikuchi, H. Yokosawa, A. Toh-e and K. Tanaka (2009). "Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome." EMBO J **28**(4): 359-371.
Saha, P., J. Chen, K. C. Thome, S. J. Lawlis, Z. H. Hou, M. Hendricks, J. D. Parvin and A. Dutta (1998). "Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase." Mol Cell Biol **18**(5): 2758-2767.
Schreiber, A., F. Stengel, Z. Zhang, R. I. Enchev, E. H. Kong, E. P. Morris, C. V. Robinson, P. C. da Fonseca and D. Barford (2011). "Structural basis for the subunit assembly of the anaphase-promoting complex." Nature **470**(7333): 227-232.
Sequeira-Mendes, J., R. Diaz-Uriarte, A. Apedaile, D. Huntley, N. Brockdorff and M. Gomez (2009). "Transcription initiation activity sets replication origin efficiency in mammalian cells." PLoS Genet **5**(4): e1000446.
Shalev, S. A. and J. G. Hall (2003). "Another adult with Meier-Gorlin syndrome--insights into the natural history." Clin Dysmorphol **12**(3): 167-169.

Shen, Z., K. M. Sathyan, Y. Geng, R. Zheng, A. Chakraborty, B. Freeman, F. Wang, K. V. Prasanth and S. G. Prasanth (2010). "A WD-repeat protein stabilizes ORC binding to chromatin." Mol Cell 40(1): 99-111.
Shibata, E., M. Kiran, Y. Shibata, S. Singh, S. Kiran and A. Dutta (2016). "Two subunits of human ORC are dispensable for DNA replication and proliferation." Elife 5.
Shirayama, M., A. Toth, M. Galova and K. Nasmyth (1999). "APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5." Nature 402(6758): 203-207.
Shou, W. and W. G. Dunphy (1996). "Cell cycle control by Xenopus p28Kix1, a developmentally regulated inhibitor of cyclin-dependent kinases." Mol Biol Cell 7(3): 457-469.
Sive, H. L., R. M. Grainger and R. M. Harland (2007). "Dejellying Xenopus laevis Embryos." CSH Protoc 2007: pdb prot4731.
Sloper-Mould, K. E., J. C. Jemc, C. M. Pickart and L. Hicke (2001). "Distinct functional surface regions on ubiquitin." J Biol Chem 276(32): 30483-30489.
Smith, O. K. and M. I. Aladjem (2014). "Chromatin structure and replication origins: determinants of chromosome replication and nuclear organization." J Mol Biol 426(20): 3330-3341.
Smith, O. K., R. Kim, H. Fu, M. M. Martin, C. M. Lin, K. Utani, Y. Zhang, A. B. Marks, M. Lalande, S. Chamberlain, M. W. Libbrecht, E. E. Bouhassira, M. C. Ryan, W. S. Noble and M. I. Aladjem (2016). "Distinct epigenetic features of differentiation-regulated replication origins." Epigenetics Chromatin 9: 18.
Stiff, T., M. Alagoz, D. Alcantara, E. Outwin, H. G. Brunner, E. M. Bongers, M. O'Driscoll and P. A. Jeggo (2013). "Deficiency in origin licensing proteins impairs cilia formation: implications for the aetiology of Meier-Gorlin syndrome." PLoS Genet 9(3): e1003360.
Sudakin, V., D. Ganoth, A. Dahan, H. Heller, J. Hershko, F. C. Luca, J. V. Ruderman and A. Hershko (1995). "The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis." Mol Biol Cell 6(2): 185-197.
Sugimoto, N., I. Kitabayashi, S. Osano, Y. Tatsumi, T. Yugawa, M. Narisawa-Saito, A. Matsukage, T. Kiyono and M. Fujita (2008). "Identification of novel human Cdt1-binding proteins by a proteomics approach: proteolytic regulation by APC/CCdh1." Mol Biol Cell 19(3): 1007-1021.
Sugimoto, N., Y. Tatsumi, T. Tsurumi, A. Matsukage, T. Kiyono, H. Nishitani and M. Fujita (2004). "Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding." J Biol Chem 279(19): 19691-19697.
Suter, B., A. Tong, M. Chang, L. Yu, G. W. Brown, C. Boone and J. Rine (2004). "The origin recognition complex links replication, sister chromatid cohesion and transcriptional silencing in Saccharomyces cerevisiae." Genetics 167(2): 579-591.
Tachibana, K. E., M. A. Gonzalez and N. Coleman (2005). "Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology." J Pathol 205(2): 123-129.
Takayama, Y., Y. Kamimura, M. Okawa, S. Muramatsu, A. Sugino and H. Araki (2003). "GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast." Genes Dev 17(9): 1153-1165.
Tang, X., S. Orlicky, Z. Lin, A. Willems, D. Neculai, D. Ceccarelli, F. Mercurio, B. H. Shilton, F. Sicheri and M. Tyers (2007). "Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination." Cell 129(6): 1165-1176.
Tatsumi, Y., S. Ohta, H. Kimura, T. Tsurimoto and C. Obuse (2003). "The ORC1 cycle in human cells: I. cell cycle-regulated oscillation of human ORC1." J Biol Chem 278(42): 41528-41534.

Tokunaga, F., S. Sakata, Y. Saeki, Y. Satomi, T. Kirisako, K. Kamei, T. Nakagawa, M. Kato, S. Murata, S. Yamaoka, M. Yamamoto, S. Akira, T. Takao, K. Tanaka and K. Iwai (2009). "Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation." Nat Cell Biol 11(2): 123-132.
Valton, A. L., V. Hassan-Zadeh, I. Lema, N. Boggetto, P. Alberti, C. Saintome, J. F. Riou and M. N. Prioleau (2014). "G4 motifs affect origin positioning and efficiency in two vertebrate replicators." EMBO J.
Valton, A. L., V. Hassan-Zadeh, I. Lema, N. Boggetto, P. Alberti, C. Saintome, J. F. Riou and M. N. Prioleau (2014). "G4 motifs affect origin positioning and efficiency in two vertebrate replicators." EMBO J 33(7): 732-746.
Verma, R., S. Chen, R. Feldman, D. Schieltz, J. Yates, J. Dohmen and R. J. Deshaies (2000). "Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes." Mol Biol Cell 11(10): 3425-3439.
Walter, J. and J. Newport (2000). "Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha." Mol Cell 5(4): 617-627.
Wang, R., F. Liu, Y. Zhao, D. Wu, L. Chen, E. T. H. Yeh and C. Huang (2017). "Reversible regulation of ORC2 SUMOylation by PIAS4 and SENP2." Oncotarget 8(41): 70142-70155.
Wang, Y., A. Khan, A. B. Marks, O. K. Smith, S. Giri, Y. C. Lin, R. Creager, D. M. MacAlpine, K. V. Prasanth, M. I. Aladjem and S. G. Prasanth (2017). "Temporal association of ORCA/LRWD1 to late-firing origins during G1 dictates heterochromatin replication and organization." Nucleic Acids Res 45(5): 2490-2502.
Williamson, A., K. E. Wickliffe, B. G. Mellone, L. Song, G. H. Karpen and M. Rape (2009). "Identification of a physiological E2 module for the human anaphase-promoting complex." Proc Natl Acad Sci U S A 106(43): 18213-18218.
Wilson, K. A., A. G. Elefanty, E. G. Stanley and D. M. Gilbert (2016). "Spatio-temporal re-organization of replication foci accompanies replication domain consolidation during human pluripotent stem cell lineage specification." Cell Cycle 15(18): 2464-2475.
Windheim, M., M. Peggie and P. Cohen (2008). "Two different classes of E2 ubiquitin-conjugating enzymes are required for the mono-ubiquitination of proteins and elongation by polyubiquitin chains with a specific topology." Biochem J 409(3): 723-729.
Wohlschlegel, J. A., S. K. Dhar, T. A. Prokhorova, A. Dutta and J. C. Walter (2002). "Xenopus Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45." Mol Cell 9(2): 233-240.
Wohlschlegel, J. A., B. T. Dwyer, S. K. Dhar, C. Cvetic, J. C. Walter and A. Dutta (2000). "Inhibition of eukaryotic DNA replication by geminin binding to Cdt1." Science 290(5500): 2309-2312.
Wu, L., A. V. Grigoryan, Y. Li, B. Hao, M. Pagano and T. J. Cardozo (2012). "Specific small molecule inhibitors of Skp2-mediated p27 degradation." Chem Biol 19(12): 1515-1524.
Xu, M., B. Skaug, W. Zeng and Z. J. Chen (2009). "A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta." Mol Cell 36(2): 302-314.
Xu, P., D. M. Duong, N. T. Seyfried, D. Cheng, Y. Xie, J. Robert, J. Rush, M. Hochstrasser, D. Finley and J. Peng (2009). "Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation." Cell 137(1): 133-145.

Yanagi, K., T. Mizuno, Z. You and F. Hanaoka (2002). "Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity." J Biol Chem **277**(43): 40871-40880.
Ye, Y., M. Akutsu, F. Reyes-Turcu, R. I. Enchev, K. D. Wilkinson and D. Komander (2011). "Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21." EMBO Rep **12**(4): 350-357.
Ye, Y. and M. Rape (2009). "Building ubiquitin chains: E2 enzymes at work." Nat Rev Mol Cell Biol **10**(11): 755-764.
Yew, P. R. and M. W. Kirschner (1997). "Proteolysis and DNA replication: the CDC34 requirement in the Xenopus egg cell cycle." Science **277**(5332): 1672-1676.
You, Z., K. Harvey, L. Kong and J. Newport (2002). "Xic1 degradation in Xenopus egg extracts is coupled to initiation of DNA replication." Genes Dev **16**(10): 1182-1194.
Youn, J. Y., W. H. Dunham, S. J. Hong, J. D. R. Knight, M. Bashkurov, G. I. Chen, H. Bagci, B. Rathod, G. MacLeod, S. W. M. Eng, S. Angers, Q. Morris, M. Fabian, J. F. Cote and A. C. Gingras (2018). "High-Density Proximity Mapping Reveals the Subcellular Organization of mRNA-Associated Granules and Bodies." Mol Cell **69**(3): 517-532 e511.
Yuan, J., K. Luo, L. Zhang, J. C. Cheville and Z. Lou (2010). "USP10 regulates p53 localization and stability by deubiquitinating p53." Cell **140**(3): 384-396.
Zhuang, M., M. F. Calabrese, J. Liu, M. B. Waddell, A. Nourse, M. Hammel, D. J. Miller, H. Walden, D. M. Duda, S. N. Seyedin, T. Hoggard, J. W. Harper, K. P. White and B. A. Schulman (2009). "Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases." Mol Cell **36**(1): 39-50.
Zimmerman, E. S., B. A. Schulman and N. Zheng (2010). "Structural assembly of cullin-RING ubiquitin ligase complexes." Curr Opin Struct Biol **20**(6): 714-721.

Abstract: Cell division is one of the most complex processes a cell undergoes. For this to happen properly, the genetic material stored in a cell must be faithfully copied or *replicated*. During this process, DNA replication is initiated at pre-defined sites in the genome, called "origins of replication". The activation of these origins is highly regulated, as a dysfunction in origin activity is linked to several human pathologies. Several proteins have been found at replication origins, but none of them explain how replication to be activated origins are recognized and selected. Our research group aims to understand how DNA replication origins are regulated in metazoan cells, to this aim, a proteomic approach was performed to define the interactome at human replication origins. Our goal was to identify new factors that could be involved in replication origin regulation. Using this methodology, a novel E3 ubiquitin ligase, named OBI1 (for ORC-ubiquitin-ligase-1), was identified prior to my arrival in the laboratory. OBI1 binds the origin recognition complex (ORC complex) and my project aimed at further characterizing the role of this new protein in DNA replication. Our experimental strategy used two different model systems: an in-vivo model based on human cells in culture, and an in-vitro DNA replication system derived from Xenopus eggs. Our analyses in human cells first revealed that OBI1 was a crucial gene involved in cellular proliferation, this observation was later attributed to OBI1's role in DNA replication and more specifically, to replication origin activation. Indeed, OBI1 knockdown resulted in a deficient origin firing and a decrease in the chromatin recruitment of factors involved in origin firing. A further functional analysis showed that OBI1 multiubiquitylates two subunits of the ORC complex, ORC3 and ORC5. This ubiquitylation was directly linked to OBI1's role in origin firing, after the over-expression of non-ubiquitylable ORC3/5 mutants yielded similar results to OBI1's knock down. Altogether, our results demonstrated that OBI1 encoded for a protein essential for origin activation, and allowed us to propose its main role: by multiubiquitylating a subset of the ORC complex, OBI1 could select the replication origins to be activated amongst all the potential replication origins set in G1 phase of the cell cycle. After this set of experiments, now published, we wanted to address the mechanistic impact of the multiubiquitylation of ORC on origin activation. Our preliminary experiments suggest a role of the histone acetyl-transferase (HAT) GCN5 in the "OBI1 pathway". In the second part of my project, we used the *in vitro* DNA replication system, based on Xenopus laevis egg extracts, to study the role of OBI1 and ubiquitylation in origin activation. Our in-vitro analyses confirmed the conservation of OBI1 and its recruitment to the chromatin during DNA replication. Moreover, using E1 inhibitors, we found that active ubiquitylation is important for efficient origin firing. Interestingly, our loss of function experiments suggested that OBI1's impact on origin activation could defer in early development when compared to somatic-like conditions. Taken together, the discovery of this new replication initiation factor provided key information on the role of ubiquitylation in and OBI1 in on origin activation and selection, in embryonic and somatic systems.

Résumé: La division cellulaire est l'un des processus cellulaires les plus complexes. Pour que cette division se déroule correctement, la cellule doit répliquer de manière fiable l'intégralité de son génome. Durant ce processus, la réplication de l'ADN est initiée a des sites prédéfinis du génome, appelés « origines de réplication ». Vu qu'un dysfonctionnement de l'activité des origines est lié à plusieurs pathologies humaines, leur activation doit être hautement régulée. Plusieurs protéines ont été trouvées aux origines de la réplication, mais aucune n'explique comment les origines sont reconnues et sélectionnées pour l'activation. Notre groupe de recherche vise à comprendre comment les origines de réplication sont régulées dans les cellules métazoaires. Dans ce but, une approche protéomique a été réalisée pour définir l'interactome des origines de réplication humaine, dans l'objectif d'identifier de nouveaux facteurs qui pourraient être impliqués dans la régulation des origines. À l'aide de cette approche, une nouvelle ubiquitine ligase, nommée OBI1 (ORC-ubiquitine-ligase-1), a été identifiée avant mon arrivée au laboratoire. OBI1 se lie au complexe de reconnaissance des origines (complexe ORC) et mon projet vise à mieux caractériser le rôle de cette nouvelle protéine dans la réplication de l'ADN. Notre stratégie expérimentale est basée sur deux modèles différents: un modèle in vivo de cellules humaines en culture et un système de réplication d'ADN in vitro dérivé d'œufs de Xénope. Nos analyses sur des cellules humaines ont d'abord révélé qu'OBI1 était crucial pour la prolifération cellulaire. Cette observation a été ensuite attribuée à son rôle dans la réplication de l'ADN et plus précisément dans l'activation des origines de réplication. En effet, la déplétion d'OBI1 a montré une diminution de recrutement à la chromatine de facteurs impliqués dans l'activation des origines. De plus, une analyse fonctionnelle a montré qu'OBI1 multiubiquitine ORC3/5, deux sous-unités du complexe ORC. Cette ubiquitination a été ensuite liée au rôle d'OBI1 dans le l'activation des origines de réplication, après que la surexpression de mutants ORC3 / 5 non-ubiquitinables ait donné des résultats similaires à ceux observés lors de la déplétion d'OBI1. Dans l'ensemble, nos résultats ont démontré qu'OBI1 est une protéine essentielle à l'activation des origines et nous ont permis de mettre en place une hypothèse suggérant qu'en ubiquitinant ORC3/5, OBI1 pourrait jouer un rôle dans la sélection des origines destinées à l'activation, parmi les origines définies antérieurement. Après cette étude, maintenant publiée, nous avons voulu aborder le rôle de la multiubiquitination des ORC dans l'activation des origines. Nos expériences préliminaires suggèrent un rôle de l'histone acétyl-transférase (HAT) GCN5. Dans la deuxième partie de mon projet, nous avons utilisé le système in vitro, basé sur des extraits d'œufs de xénope, pour étudier le rôle de l'OBI1 et de l'ubiquitination dans l'activation des origines de réplication. Nos analyses ont confirmé la conservation d'OBI1 et son recrutement a la chromatine lors de la réplication. De plus, en utilisant des inhibiteurs de E1, nous avons constaté que l'ubiquitination est importante pour l'activation des origines. De façon intéressante, la déplétion de OBI1 dans ce système a suggéré un rôle diffèrent d'OBI1 dans l'activation des origines dans le système embryonnaire comparé aux conditions plus somatiques. Finalement, la découverte de ce nouveau facteur d'initiation a fourni des informations essentielles sur le rôle de l'ubiquitination et d'OBI1 sur l'activation et la sélection des origines de réplication.