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CRL4^{DCAF12} Ubiquitin Ligase: A Novel Factor in DNA Replication Control

Ubikvitin ligáza CRL4^{DCAF12}: Nový faktor kontroly replikace DNA

Diploma Thesis

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V Praze, 12. 12. 2024

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Poděkování

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Abstrakt

Tato diplomová práce se zaměřuje na úlohu ubikvitinové ligázy CRL4^{DCAF12} v regulaci replikace DNA a její potenciální roli v nádorové supresi. V rámci předběžných dat jsme proteomickou analýzou identifikovali chaperonový protein MCMBP jako pravděpodobný substrát CRL4^{DCAF12}. Chaperonový protein MCMBP je klíčovým faktorem pro sestavení a jadernou lokalizaci komplexu MCM helikázy. V průběhu S fáze zajišťuje MCM komplex separaci dvouvláknové DNA, a je tak nezbytný pro funkci DNA polymerázy a průběh replikace. Interakci mezi MCMBP a DCAF12 jsme potvrdili afinitní purifikací. Dále jsme pomocí CRISPR metody vytvořili DCAF12-deficientní buněčnou linii a ověřili funkční vliv DCAF12 na proteinovou hladinu a stabilitu MCMBP. Kvantitativní mikroskopie ukázala, že DCAF12 reguluje nukleární pool MCMBP proteinu. Experimenty zaměřené na synchronizaci buněčného cyklu odhalily, že DCAF12 působí jako regulátor MCMBP proteinu během S a G2 fází, kdy je MCMBP translokován do jádra v komplexu MCM3-7.

Další část výzkumu byla zaměřena na analýzu myšího modelu deficientního v *Dcaf12*. Tento myší model nevykazoval výrazné fenotypové rozdíly, avšak detailní analýza odhalila signifikantní rozdíly v populacích hematopoetických buněk. Analýzou proteinových lyzátů izolovaných z různých tkání jsme potvrdili vliv exprese DCAF12 na proteinovou hladinu MCMBP. Dysregulace funkce MCM helikázy může vést k vyšší mutagenezi a potenciálně k rozvoji nádorového onemocnění. Pro potvrzení tumorsupresorové funkce DCAF12 jsme zkřížili *Dcaf12* a *Trp53*-deficientní myší kmeny. *Trp53*-deficientní kmen vede k rozvoji lymfomů ve stáří přibližně 25 týdnů. Haploinsuficience v *Trp53* vede k rozvoji solidních nádorů přibližně v 60 týdnech. V obou případech se nádory rozvíjely signifikantně rychleji na pozadí *Dcaf12*-deficientního kmene.

Souhrnně jsme v této práci identifikovali DCAF12 jako potenciální nový tumorsupresor, který reguluje kvalitu replikace DNA.

Klíčová slova: ubikvitin ligáza, DCAF12, MCM, MCMBP, replikace

Abstract

This thesis focuses on the role of the ubiquitin ligase CRL4^{DCAF12} in the regulation of DNA replication and its potential role in tumor suppression. Based on preliminary data, we have identified the chaperone protein MCMBP as a likely substrate of CRL4^{DCAF12} through proteomic analysis. The chaperone protein MCMBP is a key factor in the assembly and nuclear localization of the MCM helicase complex. During the S phase, the MCM complex ensures the separation of double-stranded DNA, which is essential for the function of DNA polymerase and the process of replication. We confirmed the interaction between MCMBP and DCAF12 through affinity purification. Furthermore, we created a DCAF12-deficient cell line using the CRISPR method and evaluated the functional impact of DCAF12 on the protein levels and stability of MCMBP. Quantitative microscopy revealed that DCAF12 regulates the nuclear pool of the MCMBP protein. Experiments aimed at synchronizing the cell cycle revealed that DCAF12 acts as a regulator of the MCMBP protein during the S and G2 phases, when MCMBP is translocated to the nucleus in a complex with MCM3-7.

The next part of the research focused on analyzing a mouse model deficient in *Dcaf12*. This mouse model did not exhibit significant phenotypic differences; however, detailed analysis revealed significant differences in hematopoietic cell populations. Analysis of protein lysates isolated from various tissues confirmed the influence of DCAF12 expression on the protein levels of MCMBP. Importantly, dysregulation of MCM helicase function may lead to increased mutagenesis and potentially to the cancerogenesis. To confirm the tumor suppressor function of DCAF12, we crossed *Dcaf12* and *Trp53*-deficient mouse strains. The *Trp53*-deficient strain leads to the development of lymphomas at approximately 25 weeks of age. Haploinsufficiency in *Trp53* leads to the development of solid tumors around 60 weeks. In both cases, tumors developed significantly faster in the background of the *Dcaf12*-deficient strain.

Overall, this work provides new insights into the regulation of DNA replication and potentially identifies DCAF12 as a novel tumor suppressor.

Key words: ubiquitin ligase, DCAF12, MCM, MCMBP, replication

Abbreviations

ACK	Ammonium-chloride-potassium
AMPK	AMP-activated protein kinase
AP	Affinity purification
APC/C	Anaphase promoting complex/ cyclosome
ATCC	American Type Culture Collection
bp	Base pair
BPA	Betta-propeller A
BPB	Betta-propeller B
BPC	Betta-propeller A
BSA	Bovine serum albumin
CRISPR	Clustered regularly interspaced short palindromatic repeats
CRLs	Cullin RING Ubiquitin ligases
CUL	Cullin
DAPI	4',6-diamino-2-phenylindole
DCAF12	DDB1-CUL4- associated factor 12
DCAF12L1	DDB1- and CUL4-associated factor 12-like protein 1
DCAF12L2	DDB1- and CUL4-associated factor 12-like protein 2
DDB1	DNA damage-binding protein 1
DKO	Double knock-out
DMEM	Dulbecco's Modified Eagle's medium
DUBs	Deubiquitinating enzymes
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FBP1	Fructose-1,6-bisphosphatase 1
GART	Glycineamide ribonucleotide transformylase
HECT	Homologous to E6-AP carboxyl terminus
HRP	Horseradish peroxidase
IP	Immunoprecipitation
KO	Knock-out

LLR1	Leucine-rich repeat protein 1
MAGE	Melanoma antigen genes
MCM	Minichromosome maintenance protein
MCMBP	Minichromosome maintenance binding protein
MEF	Mouse embryonic fibroblasts
MOV10	Moloney leukemia virus 10
NAE	NEDD8-activating enzyme
NEDD8	Neural precursor cell expressed developmentally downregulated protein 8
NLS	Nuclear localization signal
NTD	Neural tube defects
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RBR	RING-in- between-RING
Rbx	RING-box protein
RING	Really interesting new gene
SDS	Sodium dodecyl sulphate
SF tag	Twin-Strep-FLAG tag
sgRNA(s)	single guide RNA(s)
siRNA(s)	small interfering RNA(s)
TOPBP1	Topoisomerase II- β binding protein 1
TRAF	TNF receptor-associated factor
TRP53	Tumor protein p53
Ub	ubiquitin
UPS	Ubiquitin proteasome system
VCP	Valosin-containing protein
WT	Wild type

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1. Introduction

Dynamic protein turnover, a key aspect of proteostasis, is crucial for maintaining the balance between life and death in cells. Proteins can be degraded through two primary pathways. The first, non-specific lysosomal degradation, targets protein aggregates, organelles, and intracellular pathogens, such as bacteria. The second pathway, the ubiquitin-proteasome system (UPS), is a selective mechanism that primarily relies on post-translational modification by a small protein called ubiquitin. Ubiquitinated substrates are then translocated to the 26S proteasome for degradation. The UPS is frequently dysregulated in various pathological states, including metabolic disorders, neurodegenerative diseases, and tumorigenesis^{1,2}.

The selectivity of the UPS is orchestrated by ubiquitin ligases, with cullin-RING ubiquitin ligases being the most prominent. This thesis focuses on the function of CRL4 and the role of its substrate receptor, DCAF12, in DNA replication. Our hypothesis posits that DCAF12 regulates DNA replication by influencing the MCM complexes through MCMBP, which acts as a chaperone for MCM transport into the nucleus. Precise and timely regulation of MCM loading onto DNA is critical for maintaining genome stability. Dysregulation of DCAF12 could alter the composition of the MCM complex, potentially increasing the mutational burden and promoting tumorigenesis. Therefore, we propose that *DCAF12* may act as a novel tumor suppressor.

2. Theoretical Introduction

2.1. Ubiquitin-proteasome System

The UPS is a pivotal mechanism responsible for selective and specific protein degradation. It is primarily targeting damaged, misfolded, or unneeded proteins³. Ratio between protein synthesis and destruction, e.g. protein half-life, is a very important and highly regulated process. It is involved in every aspect of cell homeostasis, including processes controlling the cell cycle, cell growth, differentiation, cell survival and others⁴⁻⁶. In contrast to UPS, which is mostly targeting single proteins, lysosome, the other center of protein catabolism, is targeting complex substrates, such as protein aggregates or organelles⁷. Lysosomes are acidic organelles containing over 60 hydrolyses, including proteases called cathepsins, which are responsible for the catalytic destruction of the peptidic bond in proteins⁸. When compared to the UPS, where proteins must bear a specific type of signals to be destructed, the lysosomal degradation is mostly nonspecific⁹.

The specificity of UPS system is dependent on targeted and selective modification of its substrates by a chain of small protein called ubiquitin - polyubiquitination. The polyubiquitination is a process catalyzed by a cascade system of three enzymes: E1-activating enzyme, E2-conjugating enzyme, and an E3 ubiquitin ligase, which mediates the ATP-dependent transport of the ubiquitin chain onto the substrate. In specific situation, single attachment of ubiquitin to substrate, monoubiquitination, can also lead to degradation¹⁰. Ubiquitin modified substrate is actively transported to a large protein complex called 26S proteasome which contains proteases that are involved in dismantling of the substrate into small peptides or amino acids (Fig. 1)¹¹.

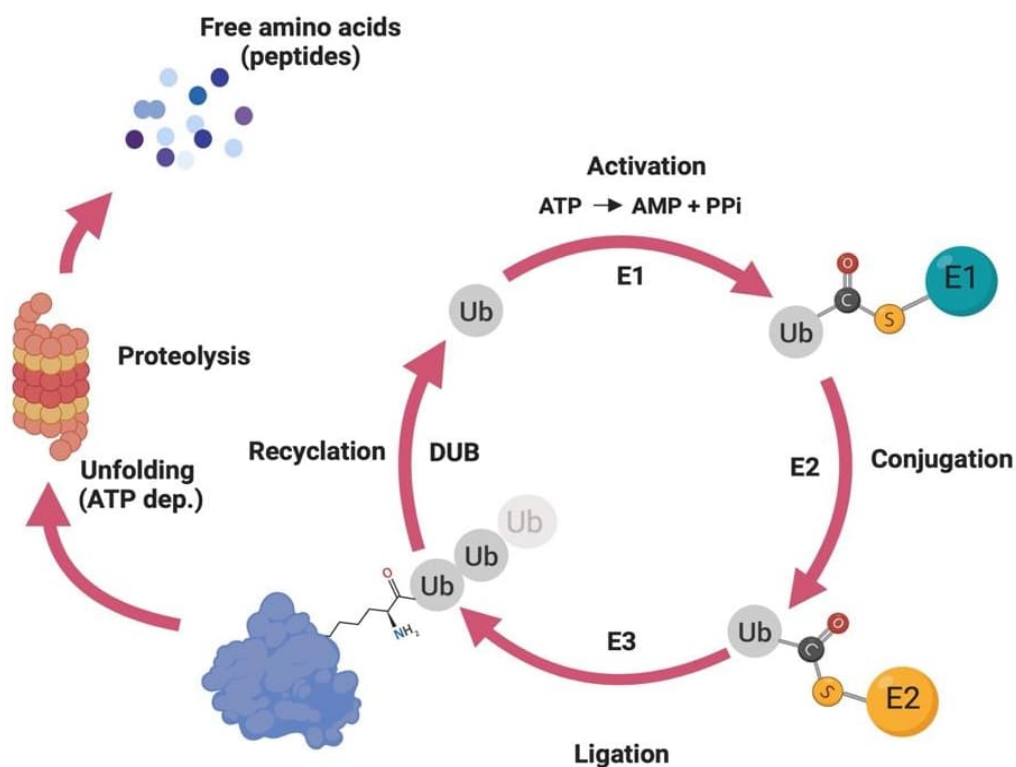


Figure 1: Scheme of UPS

Proteins are ubiquitinated in three main steps. First, the E1 enzyme activates the ubiquitin by creating a thioester bond and transporting it to the E2-conjugating enzyme. The substrate is then through transesterification moved to the E3 ligase, which creates an isopeptide bond between the ubiquitin and the substrate. Modified protein is degraded in the proteasome. Adapted from Baloghova et al⁴.

2.1.1. Proteasome

Proteasome, a 2,5-MDa multicatalytic protease complex, was first isolated from rabbit reticulocytes lysates¹². The catalytic function of the proteasome is evolutionarily conserved from Archaea to Eukaryotes, but the substrate targeting mechanism, subunit composition and complexity of proteasome itself differ in Prokaryota¹³.

The eukaryotic proteasome structure (Fig. 2) is divided into the regulatory and catalytic part. The catalytic part has a barrel-like shape made of 28 subunits that form four heptamer stacked rings with a central proteolytic cavity¹¹. The outer rings of the barrel are composed of α -subunits and two inner rings are made of β -subunits. Of the 7 β -subunits, only three of them bear the proteolytic activity- β 1, β 2, and β 5. β 1 has a caspase-like activity with a typical preference for cleaving after acidic amino acids, mostly aspartate¹⁴. β 2 and β 5 proteases are serine-type and cleave substrates in vicinity of basic or hydrophobic amino acids, respectively¹⁵.

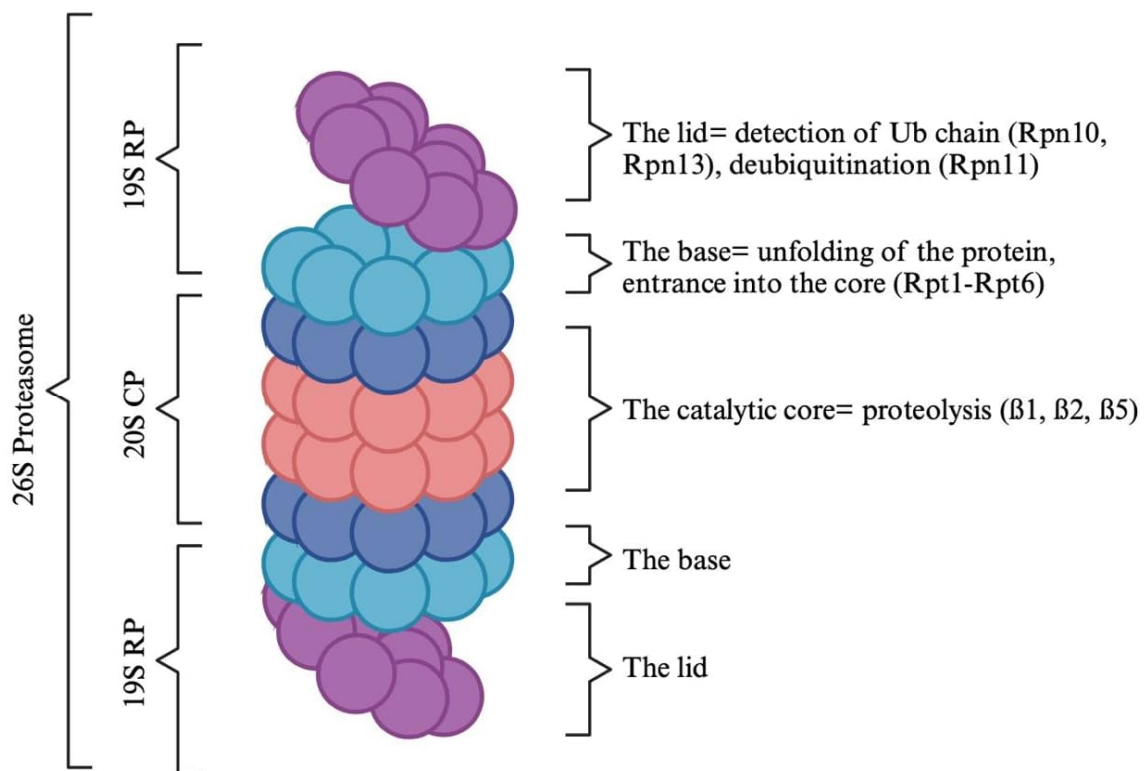


Figure 2: The Proteasome

The 26S proteasome constitutes from two subunits- the 19S regulatory particle and the 20S catalytic core. The lid is responsible for recognition of polyubiquitinated proteins and recycling of the ubiquitin. In the base part of 19S RP are proteins unfolded in ATP-dependent manner and pushed into the core for the proteasomal degradation. Adapted and edited from¹⁶.

The regulatory particle is responsible for binding, deubiquitinating and unfolding substrates before they are actively moved into the catalytic cavity. Polyubiquitinated substrates are brought to proteasome by ubiquitin-binding shuttle proteins such as RAD23a or BAG1^{17,18}. Proteasome proteins - RPN10 and RPN13 serve as substrate receptors mainly recognizing polyubiquitin chain¹⁶. Subsequently the ubiquitin chain is removed. The removal and recycling of the ubiquitin (Ub) chain is mediated by RPN11, an enzyme from the deubiquitinase family⁶. Additional deubiquitinases- USP14 and UCH37 support the cleavage of polyUb chain from the substrate¹⁹. Deubiquitinated substrate is then moved to the base, which is localized between the lid and the catalytic core²⁰. Six subunits of the base which all belong to family of AAA+ATPases, form ATP-dependent motor with central α -channel. Substrate binding to proteasome leads to opening of this channel which is otherwise in a closed position preventing the unspecific degradation²¹. In the following steps substrate is unfolded and actively moved into the catalytic part. Both steps are ATP-dependent²².

Proteasomes also have tissue-specific roles which differ from basic protein catabolism. In the immune cells, the immunoproteasome is responsible for cleaving proteins into small peptides that are presented through the MHC I. type (major histocompatibility complex I). The difference from a standard proteasome is in the catalytic composition. In immunoproteasome, the β 1, β 2, and β 5 are substituted for interferon-gamma inducible subunits β 1i, β 2i, and β 5i²³. Other conditions which lead to increased activity of immunoproteasome are various stress conditions such as oxidative stress or cytokine storm²⁴. In spleen, the immunoproteasome makes up the majority and standard proteasome activity is mostly downregulated²⁵. Another subtype of proteasome is found in the thymus. Cortical thymic cells express a thymoproteasome, with a structure very similar to the immunoproteasome. Subunits β 1i, β 2i or β 1, β 2 are the same but there is a special β 5t subunit. β 5t presence in cells is responsible for the processing of MHC I. presented peptides and for the positive selection of CD8⁺ T cells^{26,27}.

Importantly, cancer cells require high proteasomal activity²⁸. Currently, this become a target of biological therapy as several proteasome inhibitors, including Bortezomib, Carfilzomib, or Ixazomib, were developed²⁹. Typical example is the Bortezomib treatment of multiple myeloma and mantle-cell lymphoma, which results in a slower progression of cancer stage and prolonging of the survival period. The fact that patients can rather quickly develop a resistance to the drugs or negative side effects, such as neuropathy or thrombocytopenia arises a matter for a better strategy of treatment in the future³⁰⁻³².

2.1.2. Ubiquitination

Ubiquitination was discovered in the 1970^{33,34}. It represents the covalent attachment between the C-terminal glycine of 76-amino acids long ubiquitin and the side chain of the substrate's lysines. This process is orchestrated by a cascade of enzymes- E1, E2, and E3, the activating enzyme, the conjugating enzyme, and a ligase, respectively. E1 activating enzyme is responsible for the activation of ubiquitin by an ATP-dependent adenylation, which results in the formation of a thioester bond between the ubiquitin's C-terminus, and the catalytic cysteine located in the E1 enzyme. In second step, the activated ubiquitin is transferred onto the E2 conjugating enzyme, specifically E2 catalytic cysteine via trans-thioesterification³⁵. Finally, the E3 ligase, mediates the conjugation of activated ubiquitin onto substrate lysines through an isopeptide bond³⁶. Ubiquitin itself can be conjugated with other ubiquitin via several of lysines located in its primary structure, ultimately creating a polyubiquitin chain.

Ubiquitination is reversible modification. DUBs can reverse the process of ubiquitination or polyubiquitination, as they cleave out the ubiquitin from the substrate or polyubiquitin chain³⁷. One of DUBs, RPN11, plays a special role as it is in the regulatory subunit of proteasome and is responsible for cleavage of polyubiquitin chains from proteasome substrates¹⁹. This is necessary for ubiquitin recycling and substrate unfolding.

The primary structure of ubiquitin contains seven lysines—K6, K11, K27, K29, K33, K48, and K63 (Fig. 3)—which enable a variety of chain formation combinations and influence the functional outcomes of these modifications^{38,39}. Ubiquitin chains can form both homotypic and heterotypic structures, leading to the creation of branched chains⁴⁰. In addition to ubiquitination, ubiquitin itself can undergo modifications such as acetylation or phosphorylation, which can also impact the functionality of ubiquitin chains^{41,42}.

Among these modifications, homotypic polyubiquitination at K48 is the most common and typically marks substrates for proteasomal degradation. Ubiquitination at K63, on the other hand, does not associate with proteasomal degradation and usually serves as a signal for diverse cellular processes, including DNA damage repair and activation of the immune response. Other ubiquitin linkages play roles in specific cellular functions: K29 is associated with lysosomal degradation, K11 with cell cycle regulation, K6 with DNA repair^{43,44}. In addition to isopeptide modification of substrate lysines, first methionine of certain substrates can be linked to ubiquitin using peptidic bond. This modification plays a similar role like K63 and is involved in many biochemical pathways including NF-kappaB signaling⁴⁵.

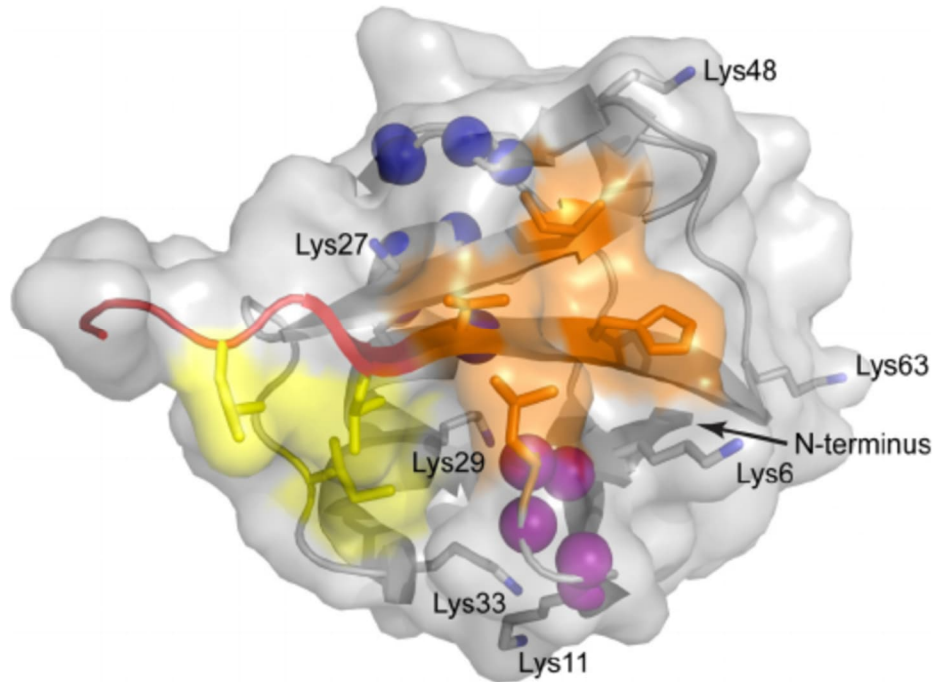


Figure 3: Ubiquitin Structure

The ubiquitin is a protein consisted of 76 amino acids. In its structure are seven lysines distinguishable. These lysines are crucial for ubiquitination and each of them represents different cell signaling role. The most common ubiquitination is the homotypic polyubiquitination of K48 which signals for protein degradation. Taken from¹⁹⁷.

2.1.3. E3 Ubiquitin Ligases

The specificity of the UPS is driven by E3 ubiquitin ligases. In comparison to E1 or E2 enzymes which are encoded by two and ~thirty genes, respectively, there is currently more than ~600 genes in human genome encoding E3 ubiquitin ligases or their functional subunits^{46,47}. There are three main groups of E3 ubiquitin ligases (RING, HECT and RBR) which differ in the mechanism of transferring the ubiquitin chain from the E2 enzyme onto the substrate. The largest group of E3 ligases is called the RING ligases (Really interesting new gene) (Fig. 4a). RING domain is responsible for the recruitment of E2 enzyme and the direct transfer of activated ubiquitin to the substrate⁴⁸. RING domain is coordinating two Zn^{2+} ions in similar fashion as in zinc fingers^{49,50}. These ions are necessary for interaction between the E3 and E2 enzymes. Upon binding to RING E3 ligase E2 enzyme adopt close conformation allowing activated ubiquitin to be conjugated to substrate⁵¹. RING-type ubiquitin ligases can act as monomers, homodimers, heterodimers, or multi-subunit complexes. Other members

of this group, the U-box ligases, have a structurally similar domain to the RING, only without the coordinating Zn^{2+} ions⁵².

The other two groups of E3 ubiquitin ligases belong to HECT (homologous to E6AP C-terminus), the RBR (RING-between-RING) and RCR (RING-Cys-relay) ligases. Their mechanism of action is different as they create an intermediate of ubiquitin and a catalytic cysteine in their own structure before transferring ubiquitin on the substrate (Fig. 4b)³⁵.

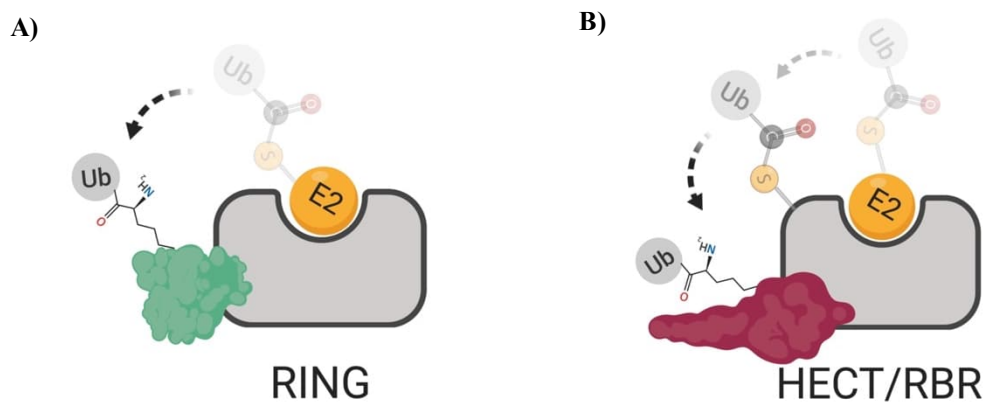


Figure 4: Ubiquitin Ligases

E3 ligases differ in the mechanism of ubiquitin transport onto the substrate. A) RING type ligases move the ubiquitin to the substrate directly, on the other hand, HECT and RBR ligases shown in B) create an intermediate before the final transfer to the substrate.

2.1.4. Cullin-dependent Ring Ligases

CRLs (cullin-RING ligases) are multi-subunit ubiquitin ligases. They are composed from several functional components. CULLINs, long protein containing several repeats, serve as a platform. Their C-termini are associated with catalytic part composed of RING-type ubiquitin ligase RBX1 (RING-Box protein 1) or RBX2 (RING-Box protein 2), whose if active, bind E2 enzymes⁵³. The N-termini of CULLINs provide basis for substrate recognition. CULLIN protein family consists of eight members- CUL1, 2, 3, 4A, 4B, 5, 7 and 9 (Fig. 5). Each of CULLIN has a different binding partner at its N-terminus. Besides CUL3 they use adaptor proteins such as SKP1 (CUL1) or DDB1 (CUL4A/B), which connect complex to different substrate receptors. CUL3 directly bind to substrates via BTB-motif containing protein⁵⁴. CUL1 and CUL7 bind F-box motif containing substrate receptors via, above mentioned, adaptor protein SKP1^{55,56}. CUL4A/B-dependent CRLs use DCAF substrate receptors which are linked to CUL4A/B via DDB1 (DNA damage-binding protein 1) adaptor.

Finally, ELONGIN B/C partners with CUL2 and CUL5 serving as a platform for SOCS-containing substrate receptors⁵⁷. The diverse composition of CRLs enables them to target a wide range of substrates, even though there are only eight cullins⁵³.

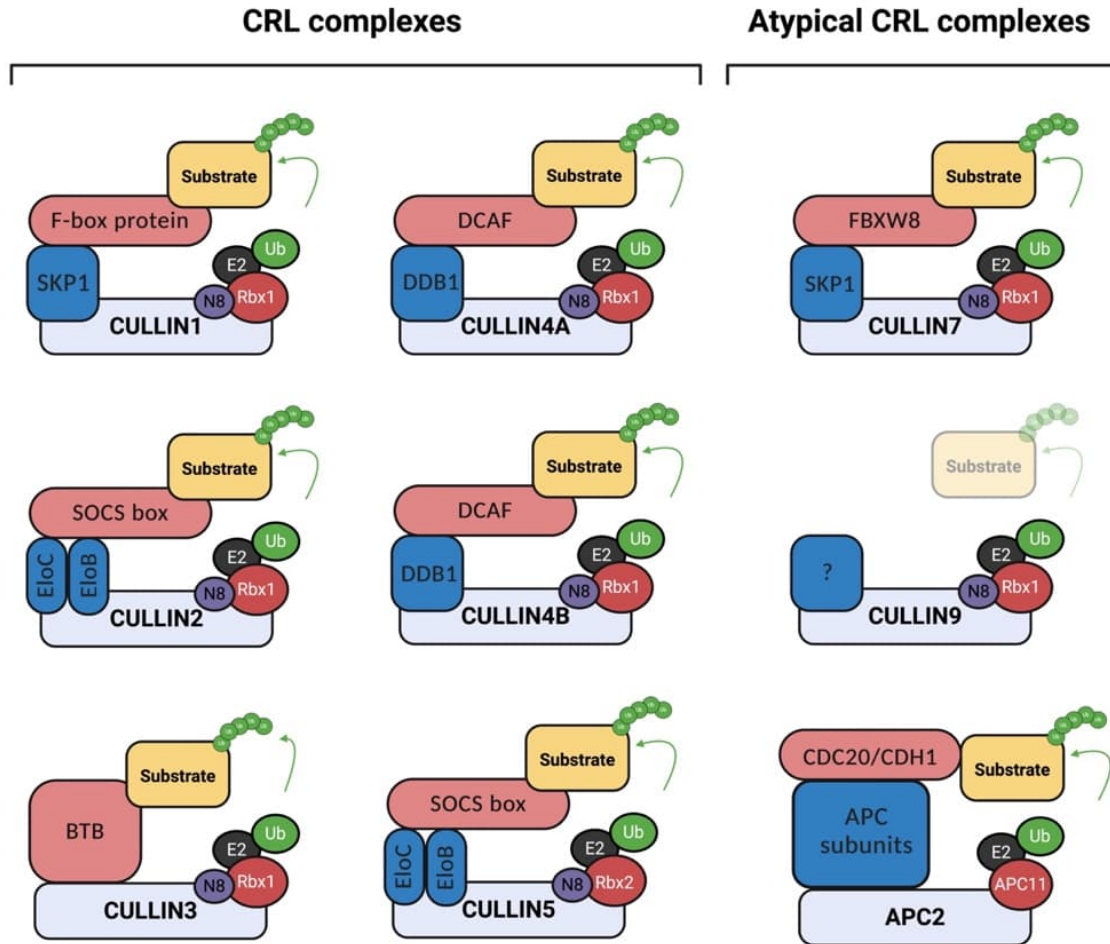


Figure 5: Multisubunit CRL Complexes

Multisubunit E3 ligases dependent on scaffold proteins Cullins, are represented in humans by five standard subunits- Cullin1, 2, 3, 4A, 4B and Cullin 5. Atypical CRL complexes are formed with Cullin 7, Cullin 9 and APC2, a mitotic anaphase checkpoint protein. Adapted from¹⁹⁸.

For activity of CULLIN-dependent ligases the modification of CULs C-terminus with ubiquitin-like protein NEDD8 (Neural Precursor cell expressed developmentally down-regulated protein 8), is necessary⁵⁸. The neddylation, induces a slight bend in the CULLIN, allowing the E2-Ub to be brought to the proximity of the substrate. It provides the CRL complex with a necessary conformational adjustment, without which the ligase complex is autoinhibited. Similarly to ubiquitin, NEDD8 must be activated by NEDD8-activating enzyme (NAE)⁵⁹. This activity can experimentally be exploited by using NAE inhibitor MLN4924 (Pevonedistat)⁶⁰. Upon treatment of cells with MLN4924 all CULs dependent

ubiquitination and degradation is blocked. Neddylation of CULs can be reversed by the COP9 signalosome complex only when a substrate is not bound. The presence of the substrate changes the steric conformation of CULs and blocks the access of the COP9 signalosome to the CRL, prohibiting its inactivation^{61,62}. When substrate is not bound, COP9 cleaves the NEDD8, inactivates the CRL and allows the remodeling protein CAND1/2 to dismantle the adaptor-substrate complex⁶³. The dynamic process of cullin neddylation, along with CAND1/2-facilitated exchange of substrate receptors, preserves the wide range of CRL repertoire and provide necessary feedback to avoid non-specific or untimely degradation⁶⁴.

Beyond the various combinations of CRL components, the mechanism for identifying numerous substrates lies in the degron—a sequence or structural motif within the substrate that mediates the recognition by the substrate receptor^{65,66}. The degrons varied across the CRLs from simple linear motifs such as C-terminal degrons to much more complex situations when CRL recognizes its substrate in context of larger proteins assembly or through post-translational modifications such as phosphodegrons^{64,67}.

2.1.5. CRL4

CUL4, which is involved in biochemical processes described in experimental part of this thesis, is encoded by two genes: CUL4A and CUL4B. These are orthologous genes with approximately 60% sequence similarity, but they differ in cellular localization. CUL4B contains an N-terminal nuclear localization signal (NLS), making it predominantly nuclear, whereas CUL4A is found in both the nucleus and the cytoplasm.

As noted above, CRL4 complexes rely on the adaptor protein DDB1, which consists of three β -propeller domains—BPA, BPB, and BPC—each containing seven β -sheets⁶⁸. The BPB domain binds CUL4A/B, while substrate binding is facilitated by the BPA and BPC domains⁶⁹. DDB1 also has a flexible range of 150°, which enhances its ability to recognize substrates.

The substrate receptors in CRL4 are called DCAFs (DDB1 and CUL4-associated factors) and contain a WD40 domain that binds to DDB1 through an α -helical H-box⁷⁰. Two CUL4 ubiquitin ligase complexes, CRL4^{TRPC4AP} and CRL4^{DCAF12}, are known to target C-terminal degrons, which require specific amino acids—arginine at position -3 and glutamic acid at position -2, respectively⁶⁵. The C-terminal structure of proteins is often more rigid, which may facilitate these protein interactions.

CRL4 complexes coordinate and regulate multiple cellular processes, such as the cell cycle regulation and the DNA damage response. Due to the extensive number of CRL substrate

receptors, it has not yet been possible to fully elucidate their roles in cell biology. To better understand the wide-ranging functions of CRL4 components, numerous knockout studies in mice have been conducted. Germ-line deletions of *Ddb1* result in embryonic lethality, while the absence of *Ddb1* in other cell types, such as those in the brain, leads to neonatal death due to apoptosis of proliferating cells and subsequent neurodegeneration⁷¹. Other non-fatal consequences of *Ddb1* inactivation include impaired hematopoiesis and disrupted oocyte meiosis⁷². Deletion of *Cul4a* also results in embryonic lethality and abnormalities in placental development⁷³. In conditional mouse models where *Cul4a* and *Cul4b* are deleted in the epiblast, embryonic lethality does not occur, but the models exhibit male infertility⁷⁴. These studies underscore the critical role of *Cul4b* in extraembryonic development⁷⁵. *Cul4a* and *Cul4b* play crucial yet distinct roles in spermatogenesis, making them both indispensable for male reproduction⁷⁶. Apart from roles in reproduction mechanisms, mutations in *CUL4B* had been associated to X-linked mental retardation in humans, exhibiting many other symptoms, for example a macrocephaly, hypogonadism, gynecomastia, aggressivity or central obesity⁷⁷. The proper function of CRL4 complex is a key factor in avoiding many detrimental and fatal symptoms, including cancer, which is also caused by anomalous expression of CRL4 components⁷⁸.

2.1.6. DCAFs

Currently there are ~29 CRL4-dependent substrate receptors. They are mostly from DCAF family characterized by presence of H-box responsible for DDB1 binding and WD40 repeats involved in substrates binding. There are some exceptions like CEREBLON (CRBN) protein which does not bear WD40 repeats but contains H-box.

Following is short introduction into the function of few well describer CRL4 substrate receptors:

CRL4^{CDT2} is a ubiquitin ligase involved in the S phase of cell cycle with a major role in the DNA damage response mechanism. It is targeting many proteins such as CDT1, SET8, CDC6, P21 for ubiquitination and subsequent proteasomal degradation^{79,80}. These proteins function as replication regulators, essential for the timely and precise licensing of replication origins. Additionally, CDT2 (DCAF2) facilitates the activation of translesion synthesis through the mono-ubiquitination of PCNA (proliferating cell nuclear antigen) and contributes to antiviral defense by targeting HBV DNA polymerase for degradation⁸¹. CDT2 recognizes its substrates only when they are in a complex with a DNA-bound PCNA protein. Both, CDT2 and its substrates, contain a PCNA interacting motive, called PIP box, and a downstream

sequence forming a C-terminal PIP degron, obligatory factor for their interaction⁸². The trimeric protein PCNA acts normally as a clamp protein during DNA replication. Dysregulation or overexpression of CDT2 leads to genomic instability and many pathological states, since it is responsible additionally for degradation of CDT1, a protein essential for correct origin licensing⁸³. Overexpression of CDT2 is found in HPV induced cancers and can serve as a prognostic marker in hepatocellular cancer and melanoma, pointing to a worse outcome, while exerting replication stress that is put on the cells, promoting tumorigenesis⁸⁴. Moreover, the overexpression of CDT2 has been implicated in Alzheimer's disease. Hyperactivity of CDK kinases, resulting from CRL4^{CDT2}-dependent degradation p21- CDK inhibitor, leads to the hyperphosphorylation of TAU and accumulation of amyloid- β , contributing to the pathogenesis of the disease⁸⁵.

Importantly, CRL4^{DDB2} and CRL4^{CSA} ligases are essential for nucleotide excision repair (NER) DNA damage response pathway, especially for DNA damage induced by ultraviolet light. NER exists in two types, an all-time active global-genome (GG-NER) and transcription-coupled (TC-NER) pathway⁸⁶. GG-NER operates continuously, while TC-NER specifically surveys the genome during transcription, stalling RNA polymerase II to facilitate repair mechanisms. The mechanism of repair in both types of NER lays in the recruitment of transcription factor IIIH (TFIIH), excision of the damage, filling in the nucleotides by DNA polymerase δ and finally, the nick ligation. Together, these processes are crucial for protecting cells against skin cancer caused by sun exposure⁸⁷. In GG-NER, DDB2 mediates the interaction with XPC (Xeroderma Pigmentosum group C), a sensor of DNA lesions, bringing it to the proximity of DNA⁸⁸. Additionally, active CRL4^{DDB2} monoubiquitinates histones near the UV damage, leading to chromatin decondensation⁸⁹. After recognizing the lesion, DDB2 itself is ubiquitinated and released from the DNA, allowing XPC to effectively manage the repair of the damage⁹⁰. TC-NER is initiated when RNA polymerase II encounters a DNA lesion, causing it to stall during transcription. This stalled polymerase then binds to Cockayne syndrome protein B (CSB), which subsequently recruits CRL4^{CSA} and TFIIH to initiate the DNA repair process. CRL4^{CSA} is responsible for the ubiquitination of RNA polymerase II, facilitating the recruitment of TFIIH⁹¹. If the repair process fails, CRL4^{CSA} can degrade the stalled RNA polymerase II.

Mutations of *DDB2* are the cause of autosomal recessive disorder Xeroderma pigmentosum group E, causing the skin hypersensitivity to sunlight and significantly increases the susceptibility to developing, apart from other cancer types, melanoma up to 2000 times⁹². Similarly, mutations in *CSA* lead to Cockayne syndrome, characterized by a range of symptoms

such as developmental delay, microcephaly, retinal atrophy, and deafness. Patients with Cockayne syndrome typically have a life expectancy of approximately 12 years⁹³.

Furthermore, in 1950s a new drug called Thalidomide was introduced to the market initially believed to be an effective treatment of nausea, pregnancy morning sickness and sleeping disorders. However, it had the unexpected consequence of being teratogenic, resulting in thousands of babies being born with deformed limbs or, in some cases, no limbs at all^{94,95}. After this disaster, the use of thalidomide was banned. CEREBLON acts as a CRL4-dependent substrate receptor. Interestingly, when thalidomide is present in cells, it binds CRBN and mediates its interaction with wide varieties on neosubstrates⁹⁶. Notable examples of these neosubstrates include the transcription factors Casein kinase 1 α , IKAROS, AIOLOS or SALL4⁹⁷⁻⁹⁹. Interestingly, SALL4 degradation is responsible for limb development arrest. IKAROS and AIOLOS as well as CK1 α degradation leads to immunomodulatory or growth-inhibitory effects. Recently the drug has been given a second chance because of its effect on certain cancer types, especially multiple myeloma. Extensive research focus on CRBN functions has led to derivatives with a greater affinity for CRBN, namely Lenalidomide, Pomalidomide and Iberdomide, all used in multiple myeloma cases¹⁰⁰. Notably, cells deficient in CRBN show resistance to Lenalidomide in multiple myeloma cell lines, suggesting that CRBN plays a crucial role in growth inhibition through protein ubiquitination and degradation¹⁰¹. Mutations in CRBN have been linked to seizures, establishing an epilepsy in adult mice, however these mutations, have not been identified in human epilepsy patients¹⁰².

2.1.7. DCAF12

DCAF12, the major subject of this thesis belongs also to the DCAF family. DCAF12 is a CRL4 substrate receptor originally identified in *Drosophila melanogaster*, where is responsible for activating the apoptosis, thereby controlling tissue growth, completion of metamorphosis and continued life with intact tissue homeostasis¹⁰³. Tissue growth is also regulated by the Hippo pathway, in which DCAF12 plays a vital role by degrading YKI/YAP/TAZ, which leads to a reduction in organ size and inhibits tumor progression¹⁰⁴. In addition, DCAF12 regulates synaptic plasticity, T-cell homeostasis, spermatogenesis and autophagy¹⁰⁵. *DCAF12* has two mammalian orthologs- *DCAF12L1* (DCAF12-like protein 1) and *DCAF12L2* (DCAF12-like protein 2), which are not functionally overlapping. The structure of DCAF12 (Fig. 6) consists of two N-terminal nuclear localization signals (NLS), an H-box, and WD40 repeats. The presence of two NLS suggests that DCAF12

predominantly localizes to the nucleus, a conclusion supported by observations of cytoplasmic localization in cells with DCAF12 mutated NLS. DCAF12 also associates with some mitochondrial and secretory proteins in the cytoplasm, pointing to an ability of DCAF12 to degrade mislocalized proteins¹⁰⁶. DCAF12, along with other substrate receptors (Fig. 7), recognizes its targets through a degron, a short sequence/motive at the substrate's terminus.

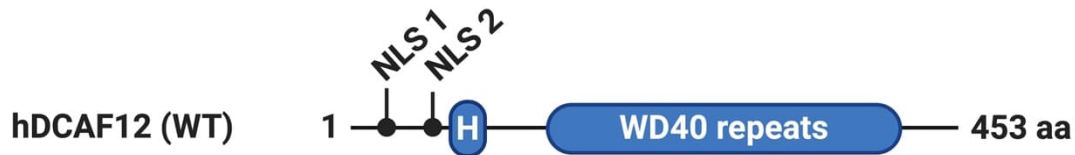


Figure 6: hDCAF12 Gene Structure

Schematic representation of DCAF12 gene. Nuclear localization signals are located on the N- terminus followed by the H-box motive, detrimental for DDB1 binding. WD40 repeats enable the substrate binding.

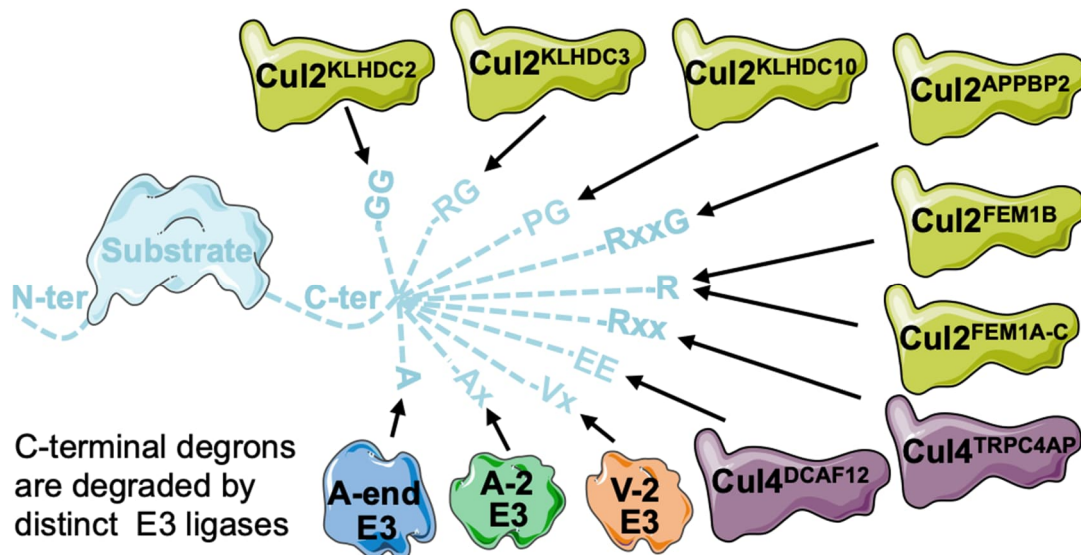


Figure 7: E3 Ligases Recognize C-terminal Degron

E3 ligases dependent on a Cullin 2 and Cullin 4 were detected to be able to recognize, apart from N-terminal degrons, also C-terminal degrons as the scheme suggests. Taken from⁶⁶.

The glutamic acid at the -2 position of the C-degron is specifically recognized by the WD40 repeats of DCAF12, facilitating this interaction. While the C-terminal position of the degron can vary, indicating flexibility regarding the specific residue, human DCAF12 demonstrates a strong preference for a C-terminal double glutamic acid (-EE) motif⁶⁶.

The C-terminal double glutamic acid (-EE) motif is found in proteins that promote immune responses, such as GART, as well as in melanoma antigen genes (MAGE). MAGE genes are normally expressed only in male germ cells; however, their abnormal activation is associated with tumorigenesis¹⁰⁷. The role of MAGE in tumorigenesis is linked to its ability

to degrade 5' adenosine monophosphate-activated protein kinase (AMPK) and fructose-1,6-bisphosphatase 1 (FBP1)¹⁰⁸. Under starvation conditions, DCAF12 targets MAGE-A3/6 for degradation, resulting in unchanged levels of AMPK, which promotes macro-autophagy to obtain energy from catabolic processes, and FBP1, which inhibits glycolysis and the Warburg effect¹⁰⁹. Overexpression of MAGE-A3/6 inhibits autophagy and promotes glycolysis, contributing to the Warburg effect¹¹⁰. Elevated levels of MAGE proteins are correlated with cancer progression and poor prognosis^{111,112}.

A study conducted by our group found that DCAF12 also recognizes a glutamic acid-leucine (-EL) motif present in MOV10 (Moloney leukemia virus 10) RNA helicase¹⁰⁶. MOV10 plays an important, though not yet fully understood, role in the host defense system through RNA interference, inhibition of transposon activity, and viral reverse transcription^{113,114}. Furthermore, MOV10 is stabilized in *Dcaf12*-deficient cells, and high levels of MOV10 correlate with a lower percentage of mature sperm, increased levels of γ -H2AX, and higher apoptotic activity in splenocytes, leading to disruption of T cell populations.

2.2. Eukaryotic Replication

The cell cycle is essential for organism development, ensuring that cells divide accurately and at the appropriate time to support tissue formation and overall growth. During the cell cycle, a cell progresses through four key phases: the synthesis phase (S), where DNA is duplicated; the second gap phase (G2), where the fidelity of DNA replication is checked; the mitotic phase (M), where the mother cell divides into two daughter cells; and the first gap phase (G1), where the cell monitors mitotic accuracy and prepares for the next round of DNA replication. Together, G1, S, and G2 make up interphase, the preparatory stage for mitosis.

Additionally, cells can enter a quiescent phase (G0), a resting stage where they may re-enter the cell cycle, differentiate, or become senescent¹¹⁵. Each phase is tightly regulated by oscillating levels of cyclins and their catalytic subunits, known as cyclin-dependent kinases (CDKs). Cyclins, recognized for their cyclical activation patterns, are regulated by the ubiquitin-proteasome system. The timely degradation of cyclins, along with the inactivation of CDKs, is essential for passing cell cycle checkpoints and ensuring proper cell division.

Maintaining genomic stability is a critical cellular function, ensuring that DNA replication occurs only once per cell cycle. This process is tightly regulated by multiple pathways to prevent genetic aberrations, which can result from unscheduled replication initiation, replication errors, and other disruptions. Robust DNA repair mechanisms are

necessary to correct these errors, ultimately supporting cellular growth and homeostasis. Without these safeguards, accumulated mutations and genetic abnormalities significantly increase the risk of carcinogenesis.

During each cell division, DNA replication produces a newly synthesized DNA strand, using the parental strands as templates. The initiation of DNA replication depends on the formation of DNA helicase, an ATP-dependent hexameric enzyme that unwinds the double-stranded DNA. In late M phase (telophase) through G1 phase, MCM (minichromosome maintenance) proteins are loaded onto the DNA in a process called origin (Ori) licensing. This loading is restricted to ensure that replication occurs only once per cell cycle. The hexameric ATPase complex, composed of MCM2-7 subunits, forms an inactive helicase that binds to replication origins but cannot yet unwind dsDNA. In the S phase, the MCM helicase converts into two active, head-to-head CMG helicases, enabling the initiation of replication¹¹⁶. The separated single-stranded DNA (ssDNA) strands are coated with replication protein A (RPA), which serves as a chaperone to prevent strand reannealing¹¹⁷. This arrangement forms a Y-shaped replication fork, where the replication machinery initiates DNA synthesis. The unwinding of DNA generates topological stress both ahead of the replication fork and on the newly synthesized segments¹¹⁸. To relieve the supercoiled pressure, the replication fork can either rotate or rely on topoisomerases I and II, which act as molecular "scissors" to untangle the double-stranded DNA (dsDNA).

To initiate the synthesis of a new DNA strand, DNA polymerases, which operate in the 5' to 3' direction, require a free 3' hydroxyl group to begin adding nucleotides. This need is addressed by a primer- a short RNA sequence synthesized by the DNA polymerase α /primase complex, which provides the starting point for DNA polymerases ϵ and δ to continue strand synthesis¹¹⁹. On the leading strand, DNA synthesis proceeds continuously in the 5' to 3' direction, driven by DNA polymerase ϵ , which has proofreading activity in the 3' to 5' direction, allowing it to correct errors on the leading strand¹²⁰. DNA polymerase ϵ has high processivity due to its interactions with various other factors. One key factor is the trimeric protein PCNA, recruited to the chromatin by replication factor C (RFC). It acts as a sliding clamp, holding the replication machinery close to the DNA and enhancing the stability of polymerase and other replication factors¹²¹.

The lagging strand template is oriented in the opposite direction and must be synthesized discontinuously. This process relies on a series of primers that provide the necessary 3' hydroxyl group for DNA polymerase δ . During synthesis, short RNA-DNA segments called Okazaki fragments are formed along the lagging strand. Once synthesis is complete, FEN1 (Flap Endonuclease 1) cleaves the RNA primers, and DNA ligase I joins the fragments in a process called Okazaki fragment maturation^{122,123}. PCNA stabilizes the replication machinery on the lagging strand to facilitate the formation of the new double-stranded DNA (Fig. 8).

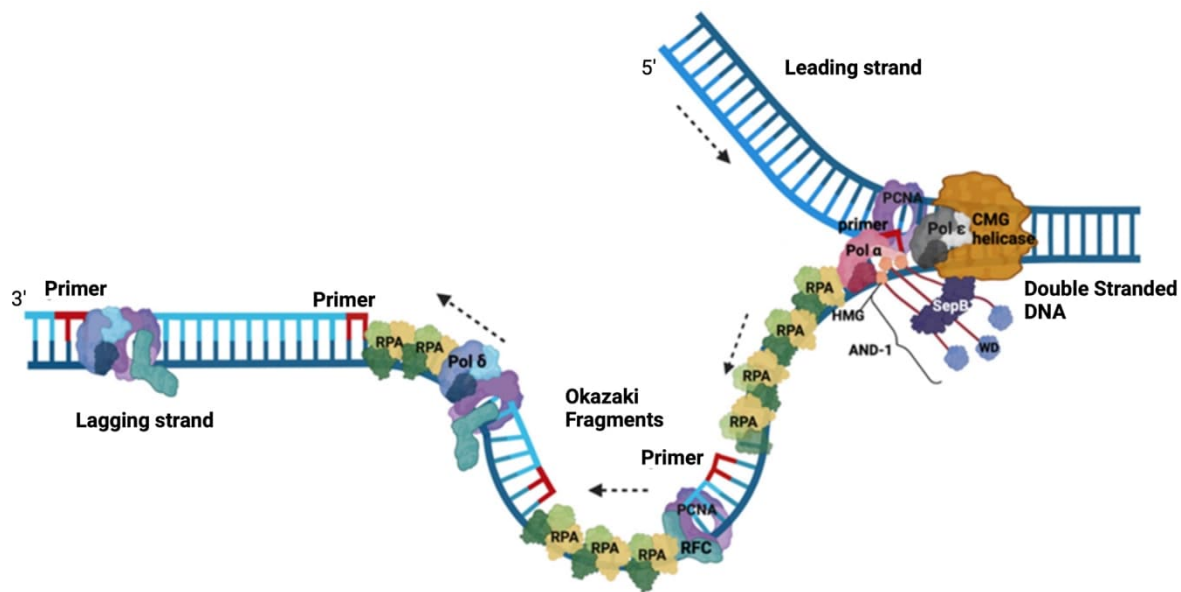


Figure 8: Eukaryotic Replication Fork

Schematic depiction of replication fork components on the leading and lagging strand. Synthesis of the leading strand is continuously done by DNA polymerase ϵ . Synthesis of the lagging strand is dependent on primers, which provide a free 3' hydroxyl group utilized by the DNA polymerase δ , which discontinuously creates a short DNA segments, called the Okazaki fragments throughout the strand. Adapted from¹³⁰.

Termination of the replication is assured by collision of two replication forks in a head-to-head formation. During this process the DNA synthesis is completed, and the replication machinery is disassembled to prevent re-replication¹²⁴. The disassembly of the replisome is executed by the K48-linked ubiquitination of MCM7, leading to CMG (CDC45, MCM2-7, GINS) unloading and its recycling¹²⁵.

The initiation of replication is dependent on a formation of the DNA helicase. In late M to G1 phase, there is a loading of MCMs (Minichromosome maintenance proteins) to the DNA, which is restricted only for this period, to assure that replication happens only once-per cell cycle¹²⁶. It is also called the licensing. Hexameric ATPase complex composed of MCM2-7

creates a premature helicase that is loaded on the replication origins but is not yet able to dismantle the dsDNA. In the S phase, the MCM helicase is transformed into two head-to-head active CMG helicases, which can initiate the replication firing¹²⁷.

2.2.1. MCM

The replication bubble contains numerous proteins that stabilize the replication machinery on DNA, enabling smooth and uninterrupted synthesis. Among these is the MCM protein family, whose members share a conserved domain structure. The AAA+ ATPase domain in MCM proteins facilitates ATP hydrolysis, driving DNA unwinding. The N-terminal domain, which includes a helical subdomain, promotes oligomerization of the MCM2-7 complex and binds to single-stranded DNA, allowing stable loading at replication origins. Additionally, the N-terminal domain contains a zinc finger motif essential for forming the double-headed helicase structure, while other N-terminal extensions mediate interactions with DDK (Dbf4-dependent kinase), crucial for regulating initiation¹²⁸. The C-terminal domain, organized into a winged helix structure, interacts with CDC6 and CDT1 to assemble the pre-replication complex¹²⁹. Together, these domains form a functional DNA helicase complex responsible for separating double-stranded DNA.

The loading of helicases begins with the formation of the pre-replication complex, which includes the ORC1-6 (Origin recognition complex 1-6), CDC6 (Cell division cycle 6), and CDT1 (CDC10-dependent transcript 1). Initially, ORC and CDC6 recognize and bind to replication origins, creating a foundation for replisome assembly. CDT1 then binds to the core helicase proteins, the MCMs, and recruits them to complete the pre-replicative complex¹³⁰.

The MCM complex forms a double-hexamer ring, made of two MCM2-7 complexes, which relies on ATP hydrolysis for proper loading onto the DNA. Without ATP hydrolysis, only a single MCM hexamer can be loaded, blocking pre-initiation complex formation¹³¹. The specific arrangement of MCM subunits, with an open interface between MCM2 and MCM5, allows double-stranded DNA to enter the helical channel¹³². CDT1 stabilizes this open conformation by binding to MCM2, MCM4, and MCM6¹³³. The MCM2-7 helicase then closes when CDT1 is removed in an ATP-dependent manner¹³⁴.

Once the MCM helicase is closed, additional replication proteins bind, and the helicase is activated via phosphorylation by CDK and DDK kinases (Fig. 9)¹³⁵. DDK acts first by recruiting CDC45 (Cell division cycle 45), while CDK activity in the S phase prevents re-licensing of replication origins by phosphorylating ORC, CDC6, and CDT1. CDK also phosphorylates TRESLIN, enabling its interaction with TOPBP1 and forming a scaffold that

attracts firing factors such as MTBP (Mdm2-binding protein), RECQL4 (RecQ like helicase), and DONSON¹³⁶. The final step in activating the MCM helicase involves transforming it into the CMG (Cdc45-MCM2-7-GINS) complex, a highly active helicase, through the recruitment of GINS (Go-ichi-ni-san proteins) and CDC45 to the outer structure of MCM. GINS binding to MCM4 enhances CMG's affinity for chromatin, ensuring robust initiation of DNA replication¹³⁷.

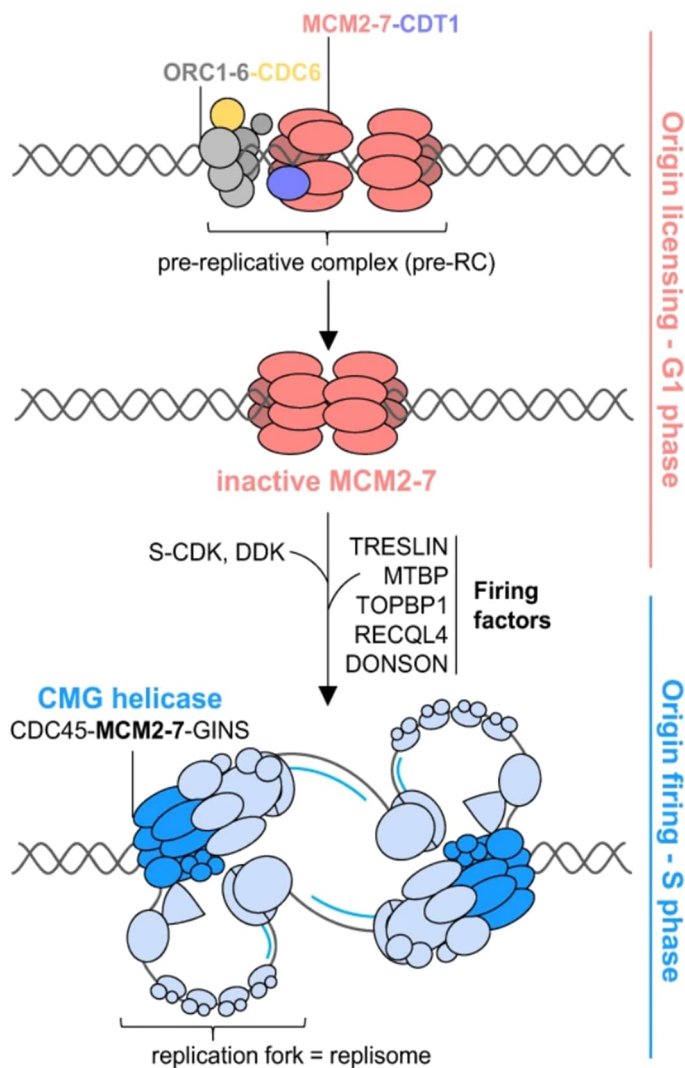


Figure 9: MCM Helicase Activation

Origin licensing during G1 phase is orchestrated by several licensing factors (ORC1–6, CDC6, and CDT1), which attach the inactive MCM2–7 complex to the DNA. In the S phase, these inactive MCM complexes are transformed into active replicative CMG helicases through the combined actions of S phase-specific CDK, DDK, and firing factors. Taken from¹⁵³.

In addition to firing factors such as TRESLIN, TOPBP1, RECQL4, MTBP, and DONSON, DNA polymerase ϵ and MCM10 are also recruited, which are essential for the recruitment of PCNA and other polymerases. Once these proteins are bound, the DNA helicase becomes fully active, unwinding the double-stranded DNA and allowing one strand to be sterically excluded from the helicase channel. The final step of initiation involves the binding of DNA polymerases α and δ to the DNA, forming a complete replisome (Fig. 10)¹³⁸.

The absence of MCM10 in the replisome results in the disassociation of the polymerase α /primase complex, inhibiting primer synthesis¹³⁹.

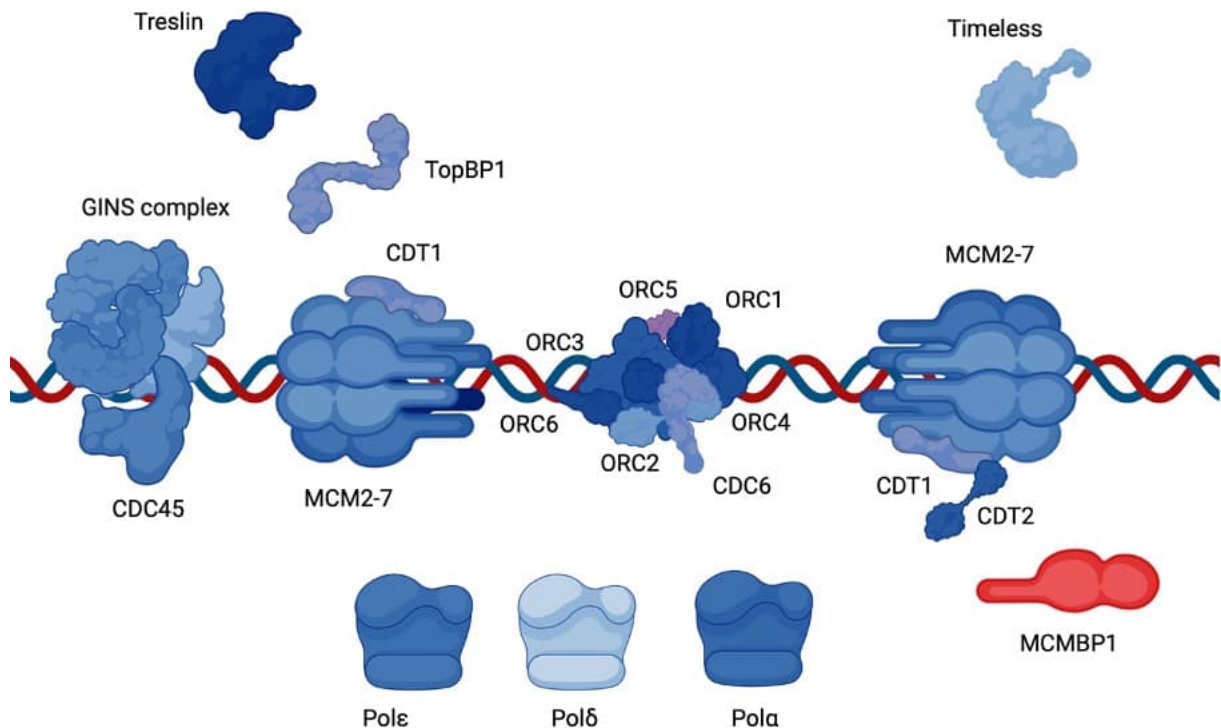


Figure 10: Replisome

The machinery required to form the replisome begins with the binding of the ORC protein, which recognizes the origins of replication on the DNA and recruits Cdc6 and Cdt1. This complex then associates with the MCM proteins, facilitating their loading onto the chromatin, where they are subsequently transformed into the active CMG helicase. The binding of DNA polymerases is dependent on scaffold proteins associated with the helicases, which enhance the affinity of these factors for the DNA.

The disassembly of the CMG helicase occurs when two replication forks collide or at the ends of chromosomes, leading to the release of CMG. This process is orchestrated by E3 ubiquitin ligases, specifically SCF^{Dia2} (SKP1-Cullin-F-BOX E3 ligase) in yeast and CRL2^{LLR1} (DDB1-CUL2- Leucine-rich repeat protein 1 E3 ligase) in metazoans, which are active during the S phase¹⁴⁰. TRAIP, an E3 ubiquitin ligase, also plays a role in this disassembly, acting as a “backup” mechanism during mitosis¹⁴¹. Beyond its mitotic function, TRAIP is involved in the DNA damage response during interstrand cross-linking, providing time for repair mechanisms to act. LLR1 and TRAIP are responsible for the polyubiquitination of the MCM7 subunit in organisms ranging from worms and frogs to humans¹⁴². Studies on human cells have shown that LLR1 is crucial for the unloading of CMG; its depletion blocks the transition

to M phase by activating the ATR checkpoint pathway¹⁴³. Additionally, LLR1 and TRAIP recruit VCP (Valosin-containing protein), also known as p97 or CDC48 in *Saccharomyces cerevisiae*, an ATPase that unfolds the MCM7 subunit and facilitates the disassembly of CMG from the DNA (Fig. 11)¹⁴⁴. Notably, CUL2^{LLR1} interacts only with terminated CMGs, as the recognition site on CMG is obscured during replication by the excluded ssDNA from the replication fork.

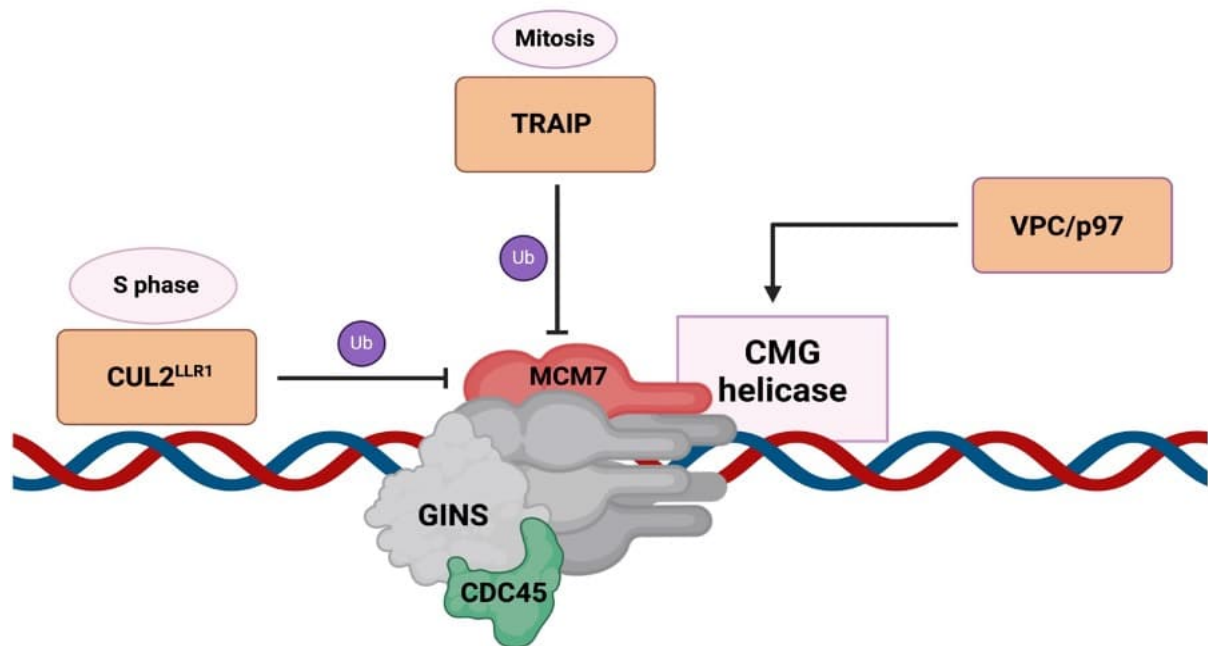


Figure 11: CMG Termination

Termination of DNA replication is orchestrated by CUL2^{LLR1}-dependent ubiquitination of MCM7 subunit of CMG helicase during the S phase. MCM7 can also be ubiquitinated by the activity of TRAIP E3 ligase during mitosis, where it acts as a back-up mechanism. After polyubiquitination of MCM7, the CMG is unloaded by Cdc48/p97 segregase. Adapted from¹⁹⁹.

In *C. elegans* and *X. laevis*, CMG disassembly during M phase can occur without the termination of replication, allowing the unreplicated genome to undergo division. The interval between TRAIP activation by CDKs and the polyubiquitination of MCM7 is quite short, but the unreplicated regions of DNA can be rapidly broken, potentially leading to genome instability¹⁴⁵.

To address these problematic unreplicated DNA regions, a process known as MiDAS (mitotic DNA repair synthesis) has evolved; however, the mechanisms controlling the switch to MiDAS in mammals remain unknown. TRAIP plays a critical role in recognizing PCNA-associated DNA damage sites and interacts with DNA damage response (DDR) factors. Studies have shown that the depletion of *Traip* through siRNA increases the formation of DNA bridges and micronuclei, highlighting its importance in maintaining replication stability¹⁴⁶.

Additionally, mutations in *TRAIP* in humans are linked to defects in the DNA damage response, resulting in conditions such as dwarfism¹⁴⁷.

2.2.2. MCM Biogenesis

During each cell cycle approximately 30,000 to 50,000 replication origins with MCM proteins are loaded, yet only about 15% of these are activated into CMGs (the active DNA helicases). This phenomenon is known as the “MCM paradox,” as it remains unclear why cells invest energy into loading complexes that remain unused¹⁴⁸.

A significant aspect of the MCM pools lifecycle is the existence of two forms: parental and nascent MCMs. Parental MCMs are present in the nucleus and have already undergone a full cell cycle in the mother cell and they are primarily responsible for forming CMG helicases. In contrast, nascent MCMs are newly synthesized in the mother cells and passed to daughter cells without prior loading onto the chromatin. The daughter cells inherit MCMs in a 1:2 ratio, with nascent MCMs predominating¹⁴⁹. MCM proteins are produced during G1 and S phases to ensure a sufficient pool is available for all replication origins. During the S phase, MCMs are distributed throughout the DNA, but only a small fraction is converted into CMGs and the rest remains inactive. Active CMGs traverse the DNA, facilitating the removal of inactive MCMs. Both MCM complexes and CMG helicases function in a sliding manner during replication and can be moved through the chromatin by cohesin loops or polymerases¹⁵⁰.

Throughout the cell cycle, the overall levels of MCMs remain relatively constant, though the percentage bound to chromatin varies. The absence of inactive MCMs on chromatin signals the transition to the G2 phase. Removed MCMs are not degraded; instead, they are retained in the nucleus for reuse in the next cycle. Once the nuclear envelope disassembles, all MCMs relocate to the cytoplasm and must be subsequently translocated back to the nucleus by specific chaperones, as they lack a nuclear localization signal, except for MCM2. Additionally, MCM2 serves as a histone-binding protein, facilitating critical interactions between the helicase and chromatin¹⁵¹. A surplus of MCMs is essential for efficient replication, not only for licensing purposes but also because a higher abundance of loaded MCMs provides flexibility in selecting which origins will fire. Research has demonstrated a clear correlation between MCM levels and the assembly and progression of the replisome¹⁵².

The functional role of parental MCMs has been established, as they are converted into CMG helicases. In contrast, the nascent MCMs, which are excessively loaded onto chromatin, create a speed barrier that regulates replication speed¹⁴⁹. This suggests that nascent MCMs enhance replication fidelity and prevent DNA damage. A study by Sedlackova et al. found that

depleting nascent MCMs during the S phase leads to unregulated replication fork speed, ultimately resulting in genome instability¹⁵³. The presence of DNA damage can cause fork stalling, activating the ATR-CHK1 (Ataxia telangiectasia and Rad3 related-Checkpoint kinase 1) pathway, which triggers the DNA damage response (DDR)¹⁵⁴. This response inhibits cell cycle progression and provides time for DNA repair. In severe cases of persistent fork stalling, dormant origins can be activated to rescue the replication process¹⁵⁵.

Any inconsistencies in the DDR mechanism can lead to increased level of the mutagenic burden and could commonly results in cancer¹⁵⁶. Overexpression of MCMs is linked to various cancer types, including prostate, breast, cervical carcinoma, glioma, renal cell carcinoma, and lung squamous cell carcinoma¹⁵⁷.

Moreover, MCMs have been identified as potential biomarkers or prognostic indicators, as their overexpression correlates with the proliferative state of cells¹⁵⁸. Beyond their function in replication, MCMs are also recognized as interactors with cohesin, suggesting a role in chromatin organization¹⁵⁹. Discovering new insights into the regulation of MCM equilibrium could pave the way for more targeted treatments in cancer therapy.

The chaperone protein called MCMBP, has been proposed by Sedlackova et al. to promote the transportation and assembly of nascent MCM 3-7, since MCM2 contains its own NLS¹⁵³. Another study done by Saito et al. came up with a different results, implying that MCMBP interacts only with MCM3/5 subcomplex and the transfers is dependent on the MCM3 interaction¹⁶⁰. Taken together, MCMBP is required for the MCM complex to be translocated into nucleus and keeping their concentration balanced, but unfortunately, it is not yet known what exact mechanism stands behind it. MCMBP depletion, but also overexpression, in HEK293 cells leads to abnormal chromatid segregation through cohesin defectivity, overall, to nuclear malformations and aneuploidy¹⁶¹. In cancer patients, both low and high expression of MCMBP bear an increased probability of future relapses. The discovery of new insights to the regulation of MCM equilibrium and MCMBP could offer more specific treatment in the cancer therapy.

3. Hypothesis and Aims of Thesis

In the preliminary phase of our project, we identified an interaction between DCAF12 and MCMBP, a chaperone protein essential for the biogenesis and nuclear translocation of the MCM helicase complex. Our working hypothesis proposes that MCMBP is a target of the CRL4^{DCAF12} ubiquitin ligase as it enters the nucleus in complex with the immature MCM helicase (MCM3-7). We hypothesize that inactivation of DCAF12 would reduce the levels of mature, chromatin-associated MCM helicase due to decreased exchangeability of the final subunit, MCM2, with MCMBP, resulting from its increased stability. Since the quantity of dormant, chromatin-bound MCM complexes is known to correlate with the speed and fidelity of DNA polymerase, we propose that DCAF12 functions as a tumor suppressor. To test this hypothesis, we crossed *Dcaf12*-deficient mice with a cancer-prone mouse model to observe the effects on tumor development.

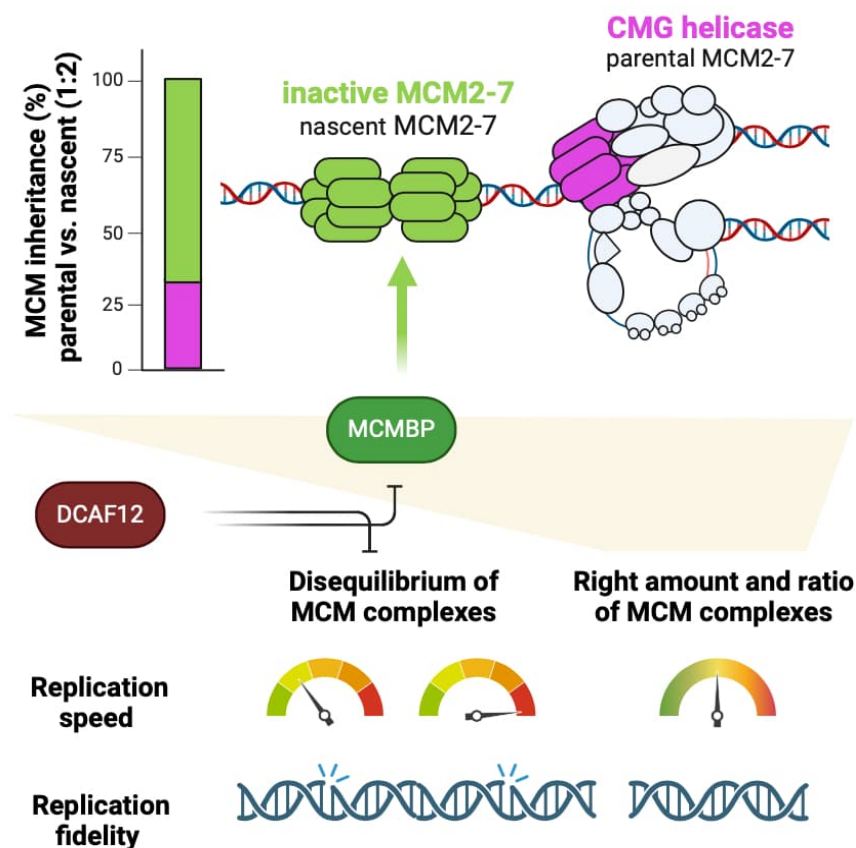


Figure 12: Working Hypothesis

We propose that DCAF12 targets MCMBP in complex with MCM3-7 and subsequently influences the nuclear pool of MCM complexes, resulting in DNA replication alteration. Modified from¹⁵³.

Aims of this thesis:

- 1. Generation of *DCAF12*-deficient HEK293 and U-2 OS Clones**
- 2. Validation of the Interaction Between DCAF12 and MCMBP**
- 3. Establishment of an Experimental Mouse Cohort Deficient in *Dcaf12* and *Trp53* Genes**
- 4. Phenotypic Characterization and Survival Analysis of DKO Mice**

4. Methods and Materials

4.1. Mouse Model

C57Bl/6NCrl strain mice were selectively bred within the controlled environment of the Institute of Molecular Genetics at the Academy of Sciences of the Czech Republic in Vestec (BIOCEV) and Krč. All animal experiments underwent approval from the Animal Care and Use Committee of the Institute of Animal Care and Use of the CAS, following both Czech Act No. 246/1992 and European Directives 2010/63/EU. Animals were checked daily with significant attention given to their health and well-being, to prevent any suffering during the period of our study. To qualify for working with mice, I completed an intensive course accredited by the Ministry of Agriculture and obtained a certificate (n. CZ04866) of professional competence for designing experiments and experimental projects under Section 15d (3) of Act No 246/1992 Coll., on the Protection of Animals against Cruelty, as amended.

4.1.1. Genotyping

To confirm the results of our breeding plans, DNA was isolated from the tip of a mouse tail, measuring no more than 3 mm. The tissue was incubated in 500 µl of DNA isolation buffer containing 0.2 mg/ml Proteinase K. Samples were placed on a heated shaker at 55 °C for 1 hour. After incubation, samples were neutralized by adding 500 µl of a phenol/chloroform/isoamyl alcohol solution (in a 25:24:1 ratio) and gently mixed. The samples were centrifuged at 18 000 rpm for 5 minutes, and the upper aqueous phase containing the DNA was transferred to a new tube. DNA precipitation was carried out by adding 500 µl of 98 % ethanol to the upper phase, followed by centrifugation. The resulting pellet was washed with cold 70 % ethanol, centrifuged and then dried at 37 °C for 15 minutes. The dried DNA was resuspended in 500 µl of TE buffer and incubated at 65 °C for 15 minutes.

DNA isolation buffer
100 mM Tris HCl (pH 8,5)
200 mM NaCl
5 mM EDTA
0,2 % SDS
TE buffer
10 mM Tris-HCl (pH 8)
1 mM EDTA

To confirm the genotype of the mice, PCR reactions were prepared and run in a thermocycler (T100, Bio-Rad). The PCR products were separated on a 1 % agarose gel containing ethidium bromide. Electrophoresis was carried out at 110 V for 25 minutes. The band sizes were determined by the 1 kb GeneRuler DNA ladder (Thermo Fisher). The WT product for *mDcaf12* is 1032 bp, while the knockout (KO) product is 612 bp. For *mTrp53*, the WT product is 616 bp, and the KO product is 400 bp.

PCR reaction (14µl)		
2x DreamTaq Green PCR Master Mix (Thermo Scientific)		
Diluted primers with a final concentration of 0,5µM		
0,2 µl DNA sample		
RNase-free water (Thermo Scientific)		
Primer sequence for <i>mDcaf12</i>		
Forward	ACTGCCAGGCGTATTGTACC	
Reverse	GCACAGGCAGGTGGATCTAT	
Primer sequence for <i>mTrp53</i>		
Forward 1	ATCCCGAGTATCTGGAAGACAG	
Forward 2	AATGCCTGCTCTTTACTGAAGG	
Reverse	AAACAGGCTAACCTAACCTACCAC	
Primer sequence for <i>SRY</i>		
Forward 1	CAGCCCTACAGCCACATGAT	
Reverse	GAGTACAGGTGTGCAGCTCTA	
PCR programme settings		
Initial denaturation	95 °C	3 min
Denaturation	95 °C	20 s
Primer annealing	60 °C	20 s
Elongation	72 °C	70 s
Final extension	72 °C	5 min
Number of cycles		33

4.1.2. Hematocrit Analysis

In collaboration with the Czech Centre for Phenogenomics (CCP) in Biocev, selected mice underwent hematocrit analysis using 20 µl of peripheral blood sample. The analysis included whole cell counts, and the resulting data were analyzed in GraphPad Prism.

4.1.3. Mouse Dissection

To analyze tissue-specific protein expression, various organs were isolated from mice. Small tissue fragments (3 mm x 3 mm) were dissected, weighed, and lysed for protein extraction. Bone marrow was extracted separately from the femur and tibia by flushing with PBS, followed by centrifugation and washing with PBS. The lysis buffer contained Benzonase and an EDTA-free Protease Inhibitor Cocktail tablet. The volume of lysis buffer was adjusted according to the sample type: 50 µl for splenocytes, 100 µl for bone marrow, and 200 µl for other tissues. Samples were incubated in the lysis buffer on ice for 40 minutes, with sonication performed twice during the incubation time.

After the incubation, SDS was added to achieve a final concentration of 1 %, followed by boiling at 95 °C for 5 minutes. The samples were then cleared by centrifugation at maximum speed for 15 minutes. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and measured with the Multiskan FC™ Microplate photometer (Thermo Fisher Scientific). Based on the concentrations, the loading volumes for each sample were adjusted to contain 20 µg of protein. The samples were mixed with Bolt™ LDS Sample Buffer (Thermo Fisher Scientific) and 10 % β-mercaptoethanol (Sigma Aldrich). The prepared samples were then boiled again at 95°C for 5 minutes and loaded onto gels for Western blotting.

For splenocyte isolation, the ACK buffer was used to lyse red blood cells. After dissecting the spleen, the tissues were placed in cold PBS and kept on ice. To ensure proper dissociation, the spleen was gradually minced using small scissors, starting with small cuts and progressively increasing the area. The dissociated tissue suspension was filtered through a cell strainer and centrifuged at $400 \times g$ for 3 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of ACK lysis buffer. The samples were incubated for 3 minutes at room temperature to ensure effective lysis of red blood cells. To end the lysis process, 3 ml of PBS was added to each sample, followed by centrifugation under the same conditions. The resulting pellet contained purified white blood cells.

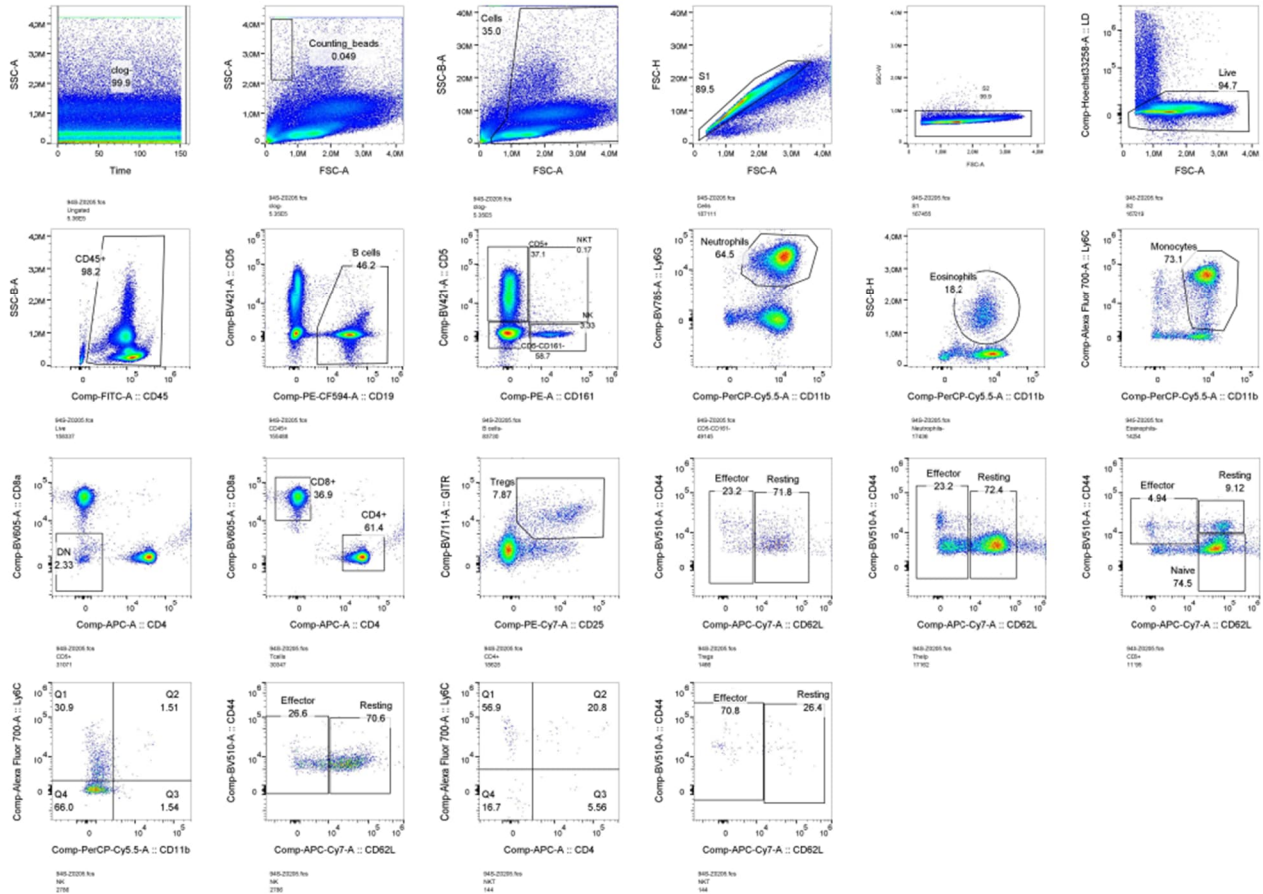
ACK buffer
150 mM NH ₄ Cl
10 mM KHCO ₃
0,1 mM Disodium EDTA
pH 7,2-7,4

4.1.4. Hematology, Immunophenotyping

In collaboration with the CCP in BIOCEV, we have analyzed several cohorts of mice to evaluate their blood and immune cell profiles. Approximately 20 μ l of blood from each sample was processed and analyzed by flow cytometry, employing the following gating strategy using listed antibodies (Tab. 1).

Antibody	Vendor	Cat.#.		Antibody	Vendor	Cat.#.
Hoechst 33258	BD	562739		CD11b-PerCPCy5.5	Biolegend	101228
CD5-BV421	BD	103043		CDC161-PE	SONY	1143540
CD44-BV510	Biologend	100744		CD19-PE-CF594	BD	562329
CD8a-BV605	Biologend	563390		CD25-PC7	BD	552880
GITR-BV711	BD	563390		CD4-APC	Biologend	100411
Ly6G-BV785	Biolegend	127645		Ly6C-AF700	BD	561237
CD45-FITC	Biolegend	103108		CD62L-APC-Cy7	BD	560514

Table 1: List of Used Fluorescent Antibodies



4.2. Cell Culture

Cell lines were cultured in a 37 °C incubator with a 5 % CO₂ atmosphere. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS) and a 5 % antibiotic solution containing penicillin (BB Pharma), gentamycin (Sandoz), and streptomycin (Sigma Aldrich). Cells were passaged using a solution of 0.25 % trypsin and 0.01 % EDTA.

The cell line used in this study were human osteosarcoma cell line U-2 OS, human kidney cancer cell line HEK293T (ATCC #CRL-1573), colon cancer cell line HCT116 (ATCC CCL-247), and their derivatives. All cell lines were routinely tested for mycoplasma contamination using the Mycoplasma Detection Kit (InvivoGen, rep-mys-50), with consistently negative results.

To synchronize the cell cycle, U-2 OS cells were treated with 2 mM hydroxyurea (Sigma, H8627-1G) for 16 hours. After the treatment, cells were washed twice with warm PBS, followed by the addition of fresh medium. Cells were collected at specified time points, and samples were prepared for Western blot analysis as described above.

For the cycloheximide chase assay, U-2 OS cells were treated with 355µM cycloheximide (Sigma, C7698). Cells were collected at specific timepoints, and protein samples were prepared following the previously described protocol.

4.2.1. Cell Lines Preparation

Generation of Knockouts Using CRISPR-Cas9

A U-2 OS cell line with a knockout of the *Dcaf12* gene was generated using the CRISPR/Cas9 genome editing system. Single-guide RNAs (sgRNA) were designed to target introns flanking exon 5 of the *Dcaf12* gene. A mixture of lentiCRISPR plasmids (pXPR001) encoding these sgRNAs and Cas9 was transfected into the cells. After brief selection with puromycin (1 µg/ml), single-cell clones were isolated, and the exon deletions were confirmed by PCR.

Sequence of sgRNAs	
sgRNA target 1	AAGCCCTCTTTGGCGTCTGTGGG
sgRNA target 2	AAGCAAGAGGAATACACGTCAGG
Sequence of CRISPR verifying primers	
DCAF12 forward	GAGGATGATGCACAAACAGC
DCAF12 reverse	CCAGAATACAGCACCTGCAA

Generation of Cell Lines with Ectopic Protein Expression

The HEK293T cell line was transiently transfected with pcDNA3-based vectors expressing Strep II-FLAG-tagged substrate receptors, HA-tagged MOV10, MCMBP, or their respective C-terminal deletion mutants. Stable cell lines expressing Strep II-FLAG-tagged DCAF12 under the control of a doxycycline (DOX)-inducible promoter were generated using the Sleeping Beauty transposon system. Briefly, cells were transfected with a pSBtet vector containing *Dcaf12* cDNA or its mutants lacking the NLS, along with the transposase-expressing vector pSB100X, using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Positive clones were selected with puromycin 48 hours after transfection. HCT116 cells ectopically expressing Strep II-FLAG-tagged DCAF12, and its truncated variants DCAF12Δ(aa 1-11), and DCAF12Δ(aa 1-38), were prepared and validated in a previous study¹⁰⁶.

The following chemical reagents were used in this study: For proteasome inhibition, U-2 OS cells were treated with MG132 (Merck, 474790-1MG) at a final concentration of 5 μM for 6 hours. Where indicated, cycloheximide (Sigma; C7698; 100 μg/ml; 355 μM), MLN4924 (Medchemexpress; HY-70062; 1 μM) or hydroxyurea (Sigma, H8627-1G, 2 mM, 16 h) were used.

4.2.2. Mouse Embryonic Fibroblasts

Females in the breeding cages intended for the preparation of MEFs were closely monitored for the presence vaginal plugs. Upon pregnancy confirmation, females were euthanized at embryonic day 13.5 (E13.5). Embryos were dissected from the placenta and organs rich in blood (heart and liver), as well as head were removed. Remaining embryo tissue was washed with PBS, homogenized, and trypsinized with a 0,25 % trypsin and 0,01% EDTA solution for 15 minutes in 37 °C. After trypsinization, larger tissues fragments were removed by washing. Isolated MEFs were cultured in DMEM with the addition of 2 mM L-glutamine and 2 mM non-essential amino acids. The MEFs genotype was analyzed by PCR.

4.2.3. Protein Analysis, Western Blotting

Cells collected by scraping were centrifuged at 300 × g for 5 minutes at 4 °C and then washed with cold PBS. Cell pellet was lysed in lysis buffer on ice for 30 minutes. For whole-cell lysate preparation, Benzonase was added into the lysis buffer. Subsequently, SDS was added to the samples to achieve final concentration 1 % and the samples were boiled at 95 °C

for 5 min. Samples were then precleared by centrifugation at maximum speed for 5 minutes, and protein lysates were transferred to a new tube.

Lysis buffer
150mM NaCl 50mM Tris (pH 7,5)
1mM EDTA
0,4% Triton X-100 2mM CaCl ₂
2mM MgCl ₂
Phosphatases and proteases inhibitors (MedChem Express)
Benzonase (0,125U/μl; Santa Cruz)

To separate soluble and insoluble fractions, samples were first incubated in the lysis buffer without Benzonase for 10 min on ice and subsequently centrifuged at maximum speed for 10 minutes at 4 °C. This process allowed cytoplasmic proteins to remain in the supernatant which was collected and stored on ice in strip tubes. Proteins associated with DNA were retained in the pellet, which was subjected to the second lysis step with the addition of Benzonase for 10 minutes on ice. All samples were then mixed with SDS in 50 mM Tris-HCl to a final concentration of 1 % and boiled for 5 minutes at 95 °C. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and a Multiskan FC™ Microplate photometer (Thermo Fisher Scientific). Based on the protein concentrations, the loading volume for each sample was adjusted to achieve a protein content of 20 μg per sample. The samples were then mixed with Bolt™ LDS Sample Buffer (Thermo Fisher Scientific) and 10% β-mercaptoethanol (Sigma Aldrich) and boiled again at 95 °C for 5 minutes. Proteins were separated using a NuPAGE™ 4-12 % gradient Bis-Tris gel (Invitrogen) in a NuPAGE MES SDS Running buffer. The protein transfer to a polyvinylidene fluoride (PVDF) membrane (Amersham) was performed with a Bio-Rad Criterion™ Blotter at 65 V for 1 hour in a cold transfer buffer. Prior to transfer, the PVDF membrane was activated by immersion in methanol for 3-5 minutes.

Transfer buffer	TBS-T
25 mM Tris	20 mM Tris
192 mM Glycin	135 mM NaCl
20 % methanol	0,05 % Tween-20
dH ₂ O	dH ₂ O
	pH 7,6

Following protein transfer, Ponceau S staining was performed to assess protein loading. The membrane was then blocked for 1 hour at room temperature in 5% milk (Santa Cruz) in TBS-T (Tris-buffered saline with Tween). After blocking, the membrane was washed with TBS-T and incubated overnight at 4 °C with primary antibodies diluted to 1:1000-5000 in 3 % BSA (Applichem, Darmstadt, Germany) in TBS-T. The following day, the membrane was washed three times in TBS-T for 10 minutes and incubated for 1 hour at room temperature with secondary antibodies conjugated with horseradish peroxidase (HRP). Following this, the membrane was washed again three times in TBS-T for 10 minutes each. Signal detection was performed using either SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher) or WesternBright ECL (Advansta). After developing the signal, the membrane was stripped of antibodies using a stripping buffer, then re-blocked in 5 % milk, washed in TBS-T, and incubated overnight with a new set of primary antibodies.

Stripping buffer
25mM glycine - HCl 1%
SDS
pH 2

4.2.4. Affinity Purification, Immunoprecipitation (IP)

The cells were collected and lysed on ice for 10 minutes in lysis buffer. For Strep-tagged constructs, the lysates were cleared by centrifugation and subsequently incubated with Strep-Tactin beads (IBA Lifesciences; 2-1206-025) for 2 hours at 4 °C. After incubation, the beads were washed three times with lysis buffer containing Benzonase (Santa Cruz). Purified proteins were eluted with Buffer E (IBA Lifesciences) by incubating the beads for 5 minutes at room temperature. The inputs (0.5%) and eluates were analyzed by SDS-PAGE followed by western blotting.

Following the same protocol, HA-tagged proteins were immunoprecipitated using anti-HA magnetic beads (Pierce™; Thermo Fisher Scientific). Immunoprecipitated proteins were eluted by adding 1× Bolt LDS Sample Buffer (Thermo Fisher Scientific) supplemented with β-mercaptoethanol, followed by incubation at 95 °C for 5 minutes.

4.2.5. Immunofluorescence (IF) Staining

A total of 10⁴ U-2 OS cells were seeded in a 96-well plate (Celvis; P96-1.5H-N). Following treatment, the cells were washed with 1× PBS, fixed with 3.2% paraformaldehyde,

permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and blocked in 3% BSA in PBS for 1 hour. The cells were then incubated with the indicated primary antibodies for 2 hours, followed by washing and subsequent incubation with the corresponding anti-mouse or anti-rabbit secondary antibodies for 1 hour. Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI). The cells were mounted with AD-Mount F (ADVI, CZ). Images were acquired using the Olympus ScanR-2 high-content microscope.

4.3. Antibodies Used for Protein Staining

Primary antibodies used for western blotting/IF were diluted at a ratio of 1:1,000-1:5,000 ratio in 3% BSA in TBS-T containing 0,05% azide. Secondary antibody conjugates used for western blotting were HRP-conjugated horse anti-mouse IgG antibody (Vector Laboratories, PI-2000) and HRP goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000), both diluted at a ratio of 1:10,000. Secondary antibodies for immunofluorescence were used at a ratio of 1:1,000- 1:5,000 ratio, listed below. For DNA visualization, DAPI was used in a final concentration 5 µg/ml.

	Antigen	Vendor	Cat. #.	Use
Primary antibodies	β-actin	Santa Cruz	sc-69879	WB
	CCNA	serum		WB
	CCNB1	serum		WB
	CCNB1	SCBT	sc-245	WB
	DDB1	Zymed	34-2300	WB
	GART	SCBT	sc-166379	WB
	HA	Cell Signalling	3724	WB
	MCM2	ABClonal	A1056	WB
	MCM3	ABClonal	A1060	WB
	MCM4	Proteintech	13043-1-AP	WB
	MCM5	Proteintech	11703-1-AP	WB
	MCM6	Proteintech	13347-2-AP	WB
	MCMBP	Atlas Abs	HPA038481	WB/IF
	p53 (DO-1)	SCBT	sc-126	WB
	PARP1	Proteintech	66520-1-Ig	WB
	PCNA	SCBT	sc-56	WB
	RPS6	SCBT	sc-74459	WB
	SKP1	Cell Signaling	12248	WB
	STREP II Tag	Novus	NBP2-43735	WB/IF
	SMAC	BD	612246	WB
	α-Tubulin	Proteintech	66031-1-Ig	WB

Table 2: List of Used Primary Antibodies

Secondary antibodies	Antibody	Vendor	Cat. #.	Use
	DAPI	Sigma-Aldrich	D9542-5mg	IF
	Phalloidin-iFluor 488 conjugate	Abcam	ab176753	IF
	anti-Mouse anti-IgG (DyLight 488)	Thermo FS	35503	IF
	Donkey anti-Mouse anti-IgG (Alexa Fluor® 555)	Abcam	ab150110	IF
	Goat anti-Mouse anti-IgG (Alexa Fluor® 647)	Abcam	ab150115	IF
	Donkey anti-Rabbit IgG (Alexa Fluor® 488)	Abcam	ab150061	IF
	Donkey anti-Rabbit IgG (Alexa Fluor® 555)	Abcam	ab150070	IF
	Donkey anti-Rabbit IgG (Alexa Fluor® 647)	Abcam	ab150067	IF
	donkey anti-Human (Alexa Fluor® 647)	Jackson IR	709-605-149	IF
	anti-mouse IgG, HRP-linked	Cell signaling	7076	WB
	anti-rabbit IgG, HRP-linked	Cell signaling	7074	WB

Table 3: List of Used Secondary Antibodies

4.4. Used software, Microscopes and Statistical Analysis

Affinity purification experiments were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Proteomics Facility in BIOCEV, following their standardized workflow. Protein densitometry was conducted using Image Lab software (Bio-Rad), and figures were created in CorelDRAW. All graphs were generated using Prism version 10 (GraphPad Software). Schemes, unless otherwise specified, were created using Biorender.com.

For statistical analysis, a student's t-test was employed when comparing two groups. For comparisons involving three or more groups, an ordinary one-way ANOVA was utilized.

Volcano plots were generated using multiple unpaired t-tests, adhering to the standard protocols implemented in GraphPad.

The mouse cohorts were managed in Pyrat (Python-based Relational Animal Tracking, Scionics), a breeding control system. All experiments involving mice were conducted in compliance with ethical guidelines, following the 3R principle (Replacement, Reduction, Refinement) with a commitment to minimize animal usage and reduce harm, while ensuring reproducible and statistically significant results.

5. Results

5.1. DCAF12-dependent Control of MCMBP Stability

5.1.1. Preliminary Data

5.1.1.1. Identification of Novel DCAF12-interacting Partners

Previous research has shown that CRL4^{DCAF12} ubiquitin ligase recognizes and binds to its protein substrates through short C-terminal glutamate residue-containing motifs. To explore a potential new substrates, we have re-analyzed mass spectrometry (MS) data from affinity purified DCAF12 complexes. We used affinity purification of StrepII-Flag-tagged DCAF12, which was followed by immunoprecipitation of HA-tagged CUL4A. Non-related ubiquitin ligases CRL4^{DCAF4} or SCF^{FBXL6} were used as a control (Fig. 12A and 12B).

To specifically identify protein substrates that interact with CRL4^{DCAF12}, we plotted the identified proteins as the ratio between their intensities in CRL4^{DCAF12} purifications and cognate controls (Fig. 13). First, we analyzed all identified proteins and successfully recovered previously described substrates, such as MOV10 and GART, as well as canonical components of CRL ligases, including DDB1 and CULs (Fig. 13A). Since DCAF12 is a nuclear protein, we refined this dataset by excluding proteins that were neither detected in the nucleus nor contained a nuclear localization signal (NLS) (Fig. 13B). Finally, we narrowed our selection to proteins containing a C-terminal motif with a glutamyl residue in the -2 position.

AP(STREP): CUL4A-DCAF4/CUL4A-DCAF12 AP(STREP): CUL1-FBXL6/CUL4A-DCAF12

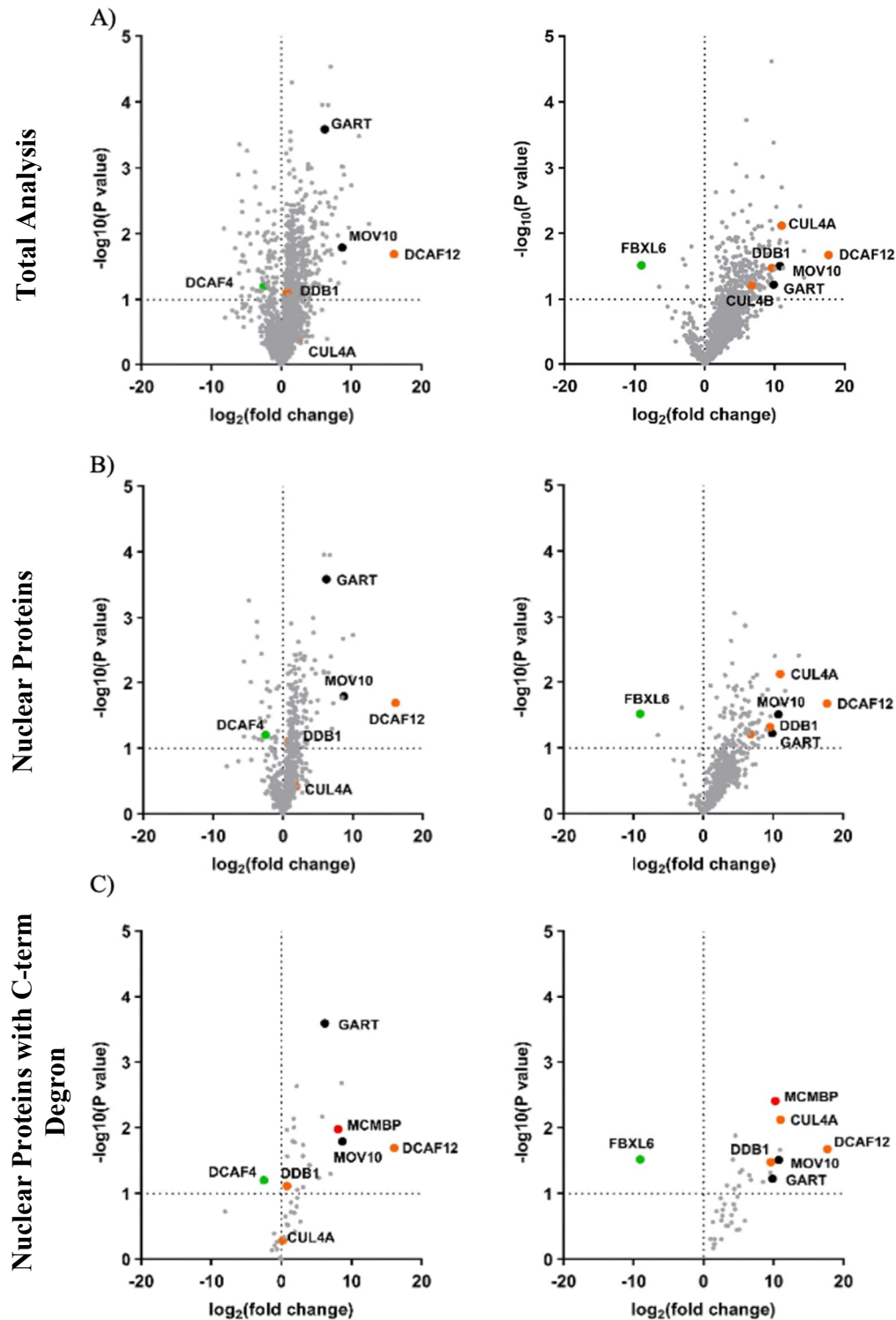


Figure 13: Affinity Purification of CUL4A^{DCAF4/12} and CUL1^{FBXL6}

To confirm the interaction between DCAF12 and MCMBP, an AP through StrepII-Flag-tagged DCAF12 isolation was conducted (Fig. 15). Total analysis is shown in 15A). To see only proteins containing the NLS we have subtracted cytosolic proteins in 15B). In 15C) we have subtracted only nuclear proteins containing a C-terminal degon.

This analysis revealed that the MCM helicase chaperone MCMBP is likely a substrate of CRL4^{DCAF12}, as it was highly enriched in DCAF12 purifications, is a nuclear protein, and possesses a C-terminal degron (Fig. 13C).

To determine if MCMBP interacts with the whole CRL4A^{DCAF12} complex (Fig. 12B), we further analyzed co-purified proteins from HA-immunopurified CRLs (Fig. 14). Consistently, this analysis confirmed MCMBP as a prominent CRL4^{DCAF12} interacting partner.

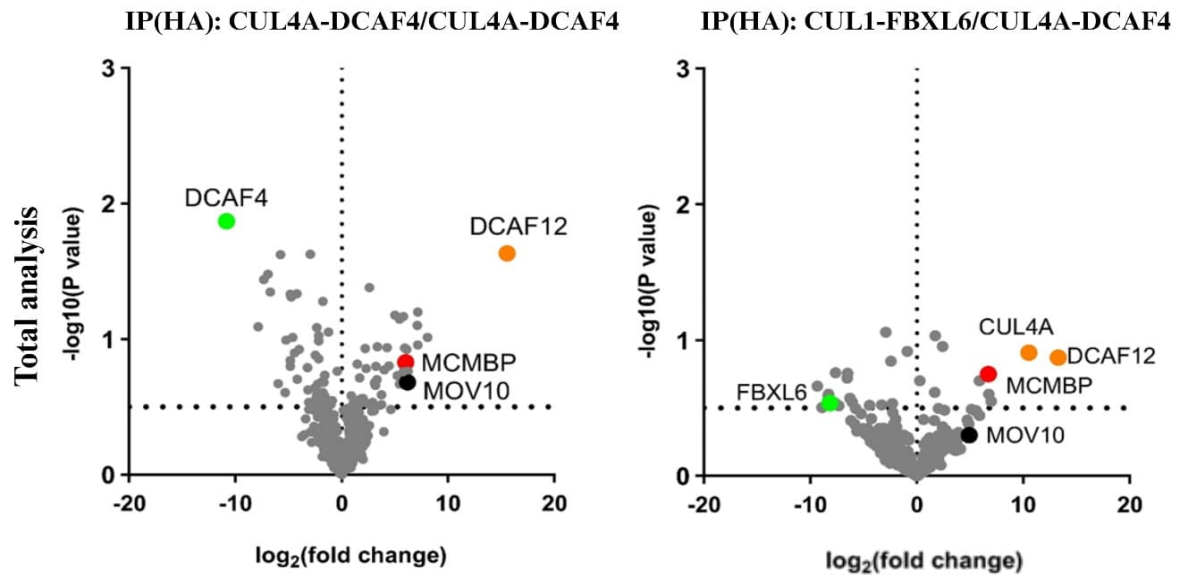


Figure 14: CRL4^{DCAF12}-associated Proteins Identified by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)

The composition of purified complexes from both steps was analyzed by LC-MS/MS. Non-related ubiquitin ligases CRL4^{DCAF4} or SCF^{FBXL6} were used as a control. Proteins that were significantly enriched with CRL4^{DCAF12} compared to controls are in the upper right quadrants.

5.1.1.2. MCMBP Protein Level is Controlled by CRL-dependent Mechanism

The activity of the CRL ubiquitin ligases is controlled by neddylation, post-translational modification of C-terminal parts of cullins. The neddylation inhibitor, MLN4924, effectively blocks CRL complex activity, leading to substrate stabilization. To investigate the role of CRL activity in MCMBP stability, we treated U-2 OS cells with MLN4924. The impact of MLN4924 treatment is shown in Figure 15, where quantitative image-based cytometry revealed a strong stabilization of MCMBP levels. This finding supports that MCMBP levels are controlled by CRL-mediated degradation.

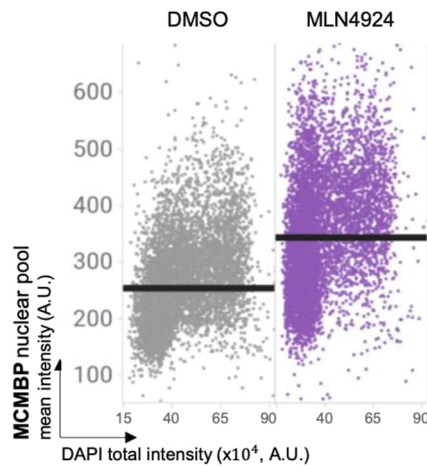


Figure 15: MCMBP Protein Level is Controlled by CRL-dependent Mechanism

Quantitative image-based cytometry (QIBC) plots of total nuclear MCMBP levels. U-2 OS cells were treated with DMSO or MLN4924 (5 μ M, 6 h). Lines denote medians; $n \approx 6,800$ cells per condition. Experiment performed and analyzed by Hana Polasek-Sedlackova.

5.1.1.3. MCMBP Protein Level is Controlled by CRL4-dependent Mechanism

To identify the specific CRL complex responsible for MCMBP protein levels control, we transfected U-2 OS cells with siRNAs targeting *CUL1-5* genes. Quantitative image-based cytometry, as used in Figure 15, was employed to assess MCMBP levels. The results indicated that depletion of CUL1, CUL2, CUL3, and CUL5 had minimal impact, with MCMBP levels remaining the same or even decreasing. However, a significant upregulation of MCMBP protein level was observed upon downregulation of *CUL4A/B* genes, suggesting that the CUL4-based CRL complex plays a key role in MCMBP degradation (Fig. 16).

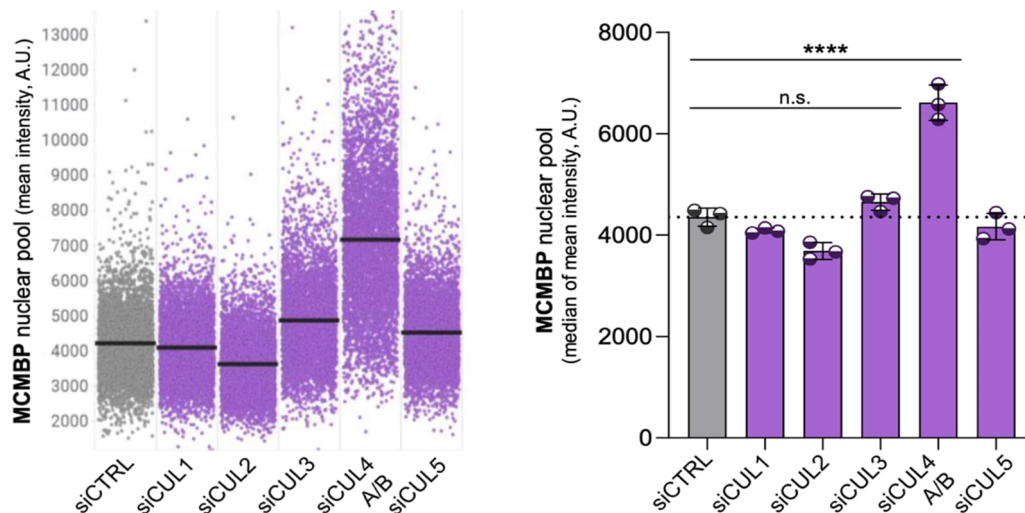


Figure 16: MCMBP Protein Level is Controlled by CRL4-dependent Mechanism

QIBC analysis plots of MCM4-Halo cells immunostained for MCMBP after treatment with siRNAs against cullins. Lines denote medians; $n \approx 5,000$ cells per condition. Cells were treated with siRNAs for 72 hours. Significance value of ****= $p < 0,0001$, n.s.= non-significant. Experiment performed and analyzed by Hana Polasek-Sedlackova.

Altogether, preliminary results show MCMBP chaperone as a potential substrate of E3 ubiquitin ligase CRL4^{DCAF12}.

5.1.2. DCAF12 Specifically Interacts with MCMBP Protein

Our preliminary results clearly demonstrated the association between MCMBP and DCAF12 within the fully assembled CRL4 complex. This interaction was additionally validated using small purification of set of CRL1/4 substrate adapters. The HEK293T cells were transfected with either an empty vector or the indicated Twin-Strep-FLAG-tagged substrate receptor subunits of the Cullin-RING ubiquitin ligase. Six hours prior to cell collection, cells were treated with MLN4924, harvested and lysed. Whole-cell lysates were subjected to affinity purification using Strep-TactinXT resin and purified complexes analyzed using PAGE and immunodetection. Results showed that MCMBP interacted only with DCAF12 substrate adapter (Fig. 17). As a controls we used SKP1 (CRL1) or DDB1 (CRL4) adapter proteins and PCNA as a negative control.

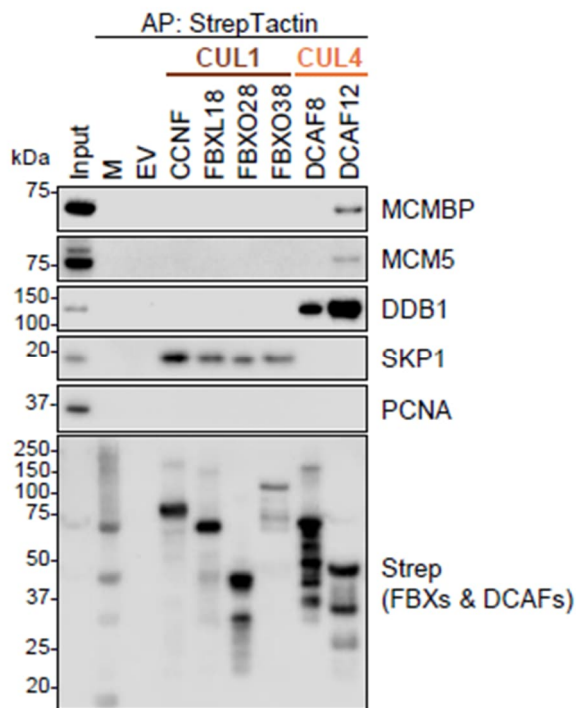


Figure 17: DCAF12 Specifically Interacts with MCMBP Protein

HEK293T cells were transfected with an empty vector or the indicated StrepII-FLAG-tagged CRL constructs and treated with MLN4924 for 6 h before harvesting. Eluates were immunoblotted with indicated antibodies. DDB1 and SKP1 were used as positive controls, PCNA stands as a negative control. SMAC is a positive control of DCAF12 since the discovery of their interactions have been published already by T.Lidak¹⁰⁶.

5.1.3. MCMBP C-terminal Motif is Conserved in Vertebrates

To confirm if interaction between MCMBP and DCAF12 is facilitated by the C-terminal degron motif we first analyzed conservation of this motif between animal species. Recent research has shown that, in addition to the typical twin-glutamic acid (-EE*) degron motif, CUL4^{DCAF12} can also bind to protein substrates with alternative motifs such as -EI*, -EM*, -ES*, and -EL*^{106,162}. Bioinformatics analysis identified a potential -EL* degron motif in the MCMBP protein, which is conserved across most vertebrates. Interestingly, fish and

lampreys have leucine in -1 position changed to glutamine or proline. Early chordates as lancelets or sponges do not have this motif (Fig. 18).

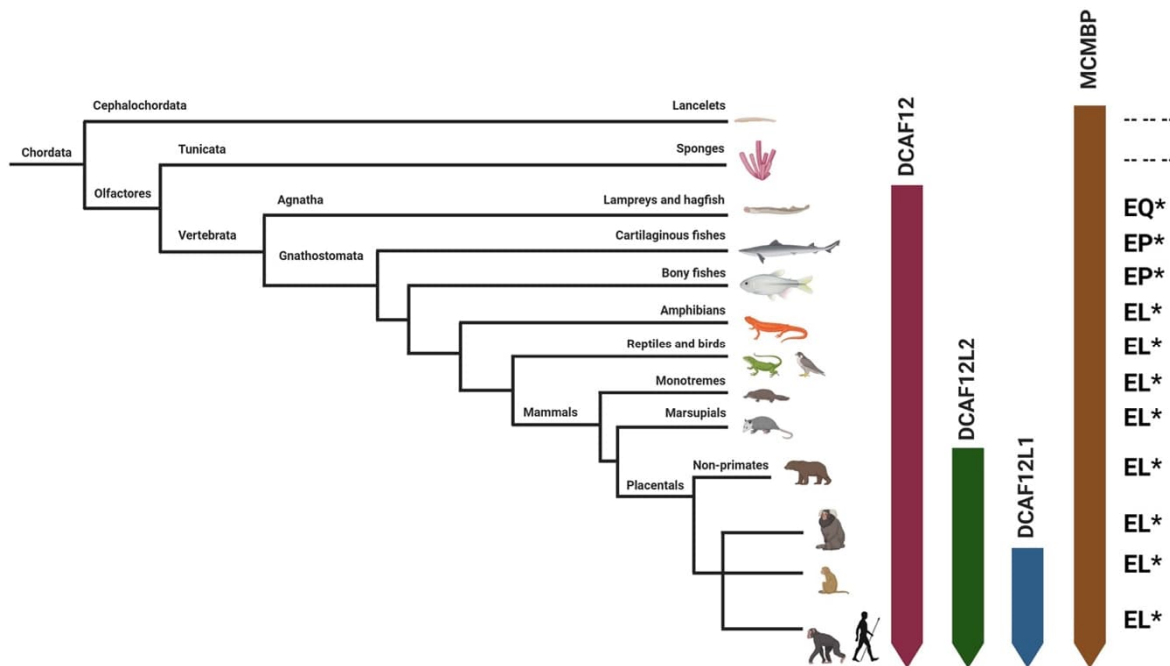


Figure 18: MCMBP C-terminal Motif is Conserved in Vertebrates

A simplified evolutionary diagram shows the development of DCAF12, its paralogs, and MCMBP in chordates. DCAF12 is found in both vertebrates and insects, while DCAF12L2 is present across all placental mammals, and DCAF12L1 is exclusive to euarchontoglires. MCMBP exists in all chordates, species containing a C-terminal degon of MCMBP are indicated.

5.1.4. DCAF12 Recognizes MCMBP via C-terminal Motif

To confirm the role of the C-terminal motif as a necessary element for the interaction between CRL4^{DCAF12} and its substrates, we conducted an immunoprecipitation using HEK293 cells co-expressing HA-tagged MOV10 and MCMBP. Both of these proteins were expressed in either wild type form or truncated C-terminal forms lacking the motifs. Before harvesting, cells were treated with MLN4924 for 6 hours. When the C-terminal -EL* degon was removed from MOV10, the mutation disrupted the interaction with DCAF12, consistent with the effect observed in truncated MCMBP (Fig. 19).

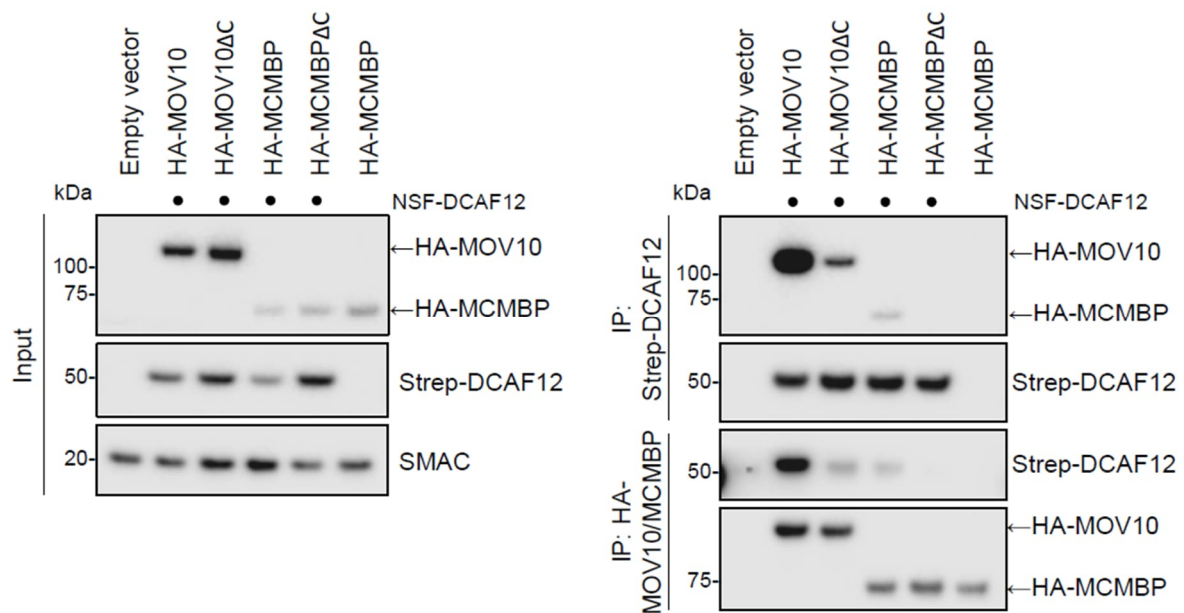


Figure 19: DCAF12 Recognizes MCMBP via C-terminal Motif

HEK293T cells were co-transfected with StrepII-FLAG-tagged DCAF12 and either an empty vector or constructs expressing HA-tagged MOV10, MOV10ΔC, MCMBP, or MCMBPΔC. MLN4924 was added for the final 6 hours before collecting. Whole-cell lysates were subjected to affinity purification or anti-HA immunoprecipitation and immunoblotted as indicated. The left panel shows 1% input samples, the upper right panel shows Strep-Tactin AP, and the lower right panel shows anti-HA IP.

5.1.5. DCAF12 Orthologs Do Not Interact with MCMBP

In addition to DCAF12, the mammalian proteome contains two DCAF12 orthologs: DCAF12L1 and DCAF12L2, which share around 60% amino acid sequence identity. However, immunoprecipitation experiments showed that only the canonical DCAF12 interacts with MCMBP, suggesting that DCAF12 orthologues do not have identical functions with DCAF12 (Fig. 20). These findings led us to conclude that CRL4^{DCAF12} specifically recognizes MCMBP through a C-terminal degron.

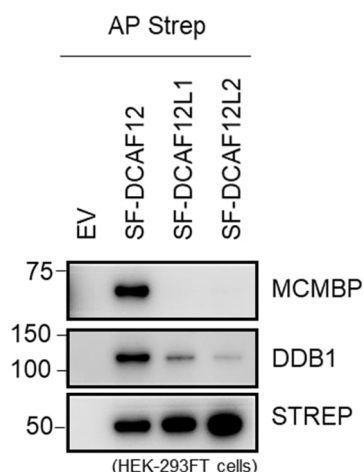


Figure 20: DCAF12 Orthologs Do Not Interact with MCMBP

HEK293T cells were co-transfected with StrepII-FLAG-tagged DCAF12, its orthologs and an empty vector. WCL were subjected to affinity purification and immunoblotted as indicated.

5.1.6. Exogenously Expressed DCAF12 Mediates Degradation of MCMBP

DCAF12 contains two nuclear localization signals, hence its functionality should occur in the nucleus. To prove this, we have used HCT116 cells expressing either wild type form of DCAF12 or its variant with truncated N-terminus containing NLSs¹⁰⁶. Truncated version of DCAF12 should be located mostly in cytoplasm. In contrast to nuclear substrates GART, an exclusive cytoplasmic protein containing a C-terminal degron reacted only to expression of DCAF12 without functional NLS. Interestingly, MCMBP and MCM3 (both nuclear proteins) protein levels decreased upon expression of both full length and truncated forms of DCAF12 (Fig. 21).

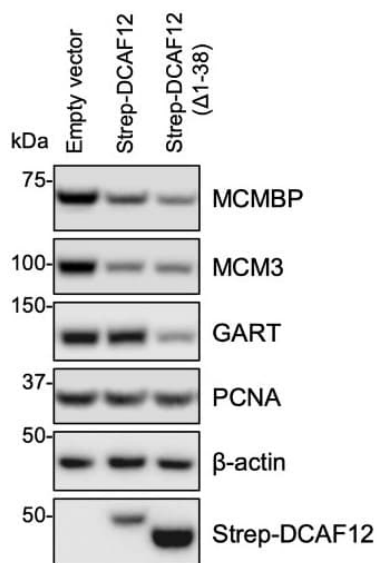


Figure 21: DCAF12 Mediates Degradation of MCMBP

HCT116 cells expressing N-terminally StrepII-FLAG-tagged DCAF12, WT or variant lacking both NLS motifs were prepared for WCL and immunoblotted as indicated. PCNA and β-actin were used as loading controls.

5.1.7. Endogenous DCAF12 Mediates Degradation of MCMBP

To further confirm that DCAF12 control MCMBP we prepared DCAF12-deficient U-2 OS cells using CRISPR-methodology. We employed powerful analysis using high content microscopy. This allowed us to determine the differences of MCMBP levels in both cytoplasmic and nuclear compartments. Treatment of wild type cells with MLN4924 led to significant upregulation of MCMBP protein levels only in nuclear compartment. Comparison of WT, KO, and KO cells with inducible DCAF12, showed no significant changes in MCMBP levels within the cytoplasmic pool (Fig. 22B). In contrast, the nuclear pool of MCMBP was upregulated either upon MLN4924 treatment or in KO cells.

Rescue of DCAF12 expression resulted in significant decrease of nuclear pool MCMBP, especially in KO cells. The concomitant expression of DCAF12 and inhibition of CRLs activity did not affect MCMBP protein levels (Fig. 22C,D). As expected, most of induced DCAF12 protein localized to nucleus.

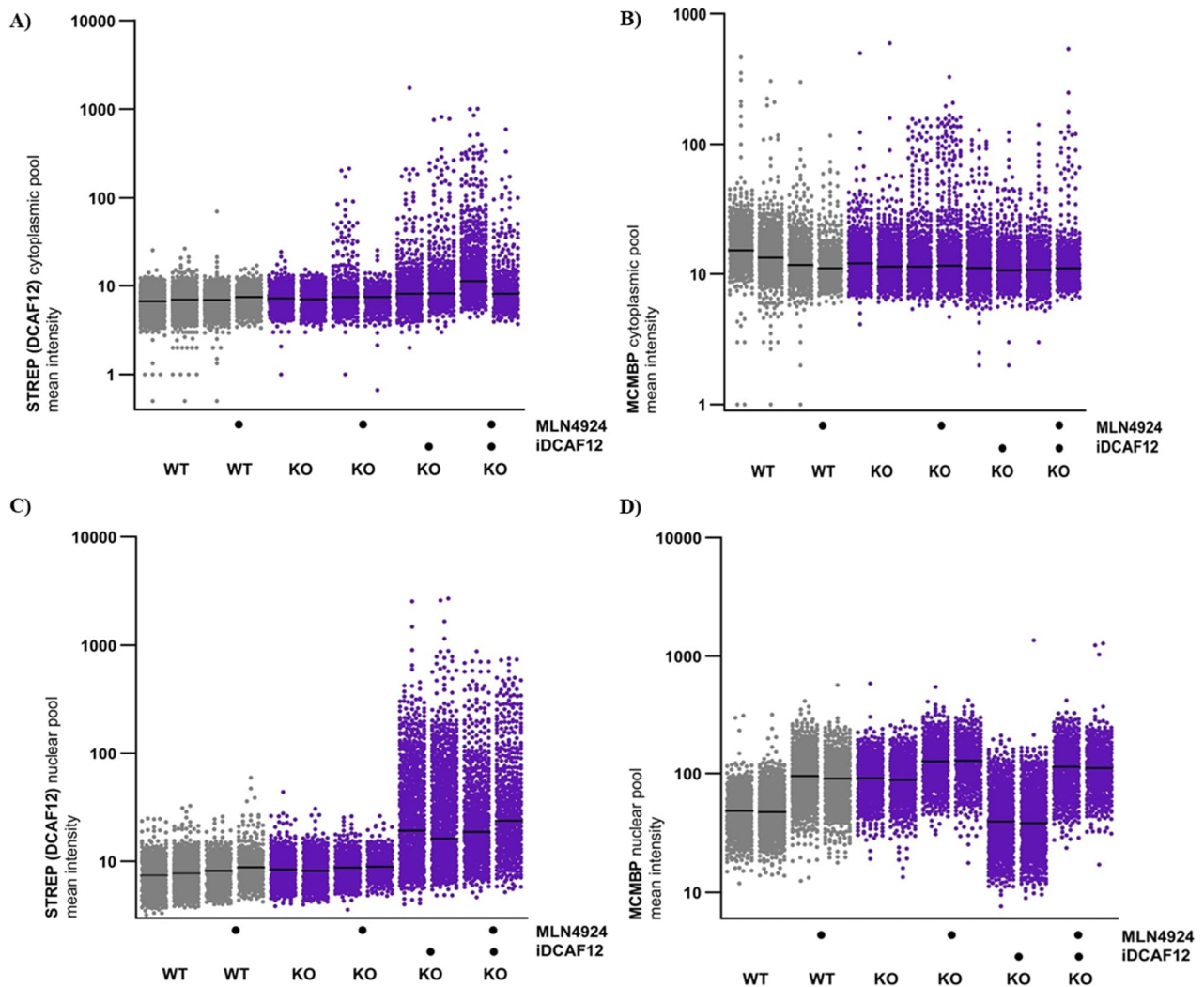


Figure 22: DCAF12 Influence on MCMBP Level in Cytoplasm and Nucleus

QIBC of U2-OS WT, DCAF12^{KO} and where indicated iDCAF12 cells upon inhibition of neddylation with MLN4924 for 6 hours. Lines denote medians; $n \approx 10,000$ cells per condition.

This method allowed us to revisit results from Figure 20. To see how the presence of NLS in DCAF12 influences the levels of MCMBP, we used truncated forms of DCAF12, either with mutated one Δ (aa 1-11), or both Δ (aa 1-38) NLS. We observed a significant increase in the cytoplasmic levels of DCAF12 mutants compared to the wild type (Fig. 23A). However, despite lacking the nuclear localization signal, these mutants were still found in the nuclear

fraction (Fig. 23C). Again, we did not observe any changes in cytoplasmic MCMBP levels, concluding that MCMBP is primary targeted by CRL4^{DCAF12} in the nucleus (Fig. 23B, D).

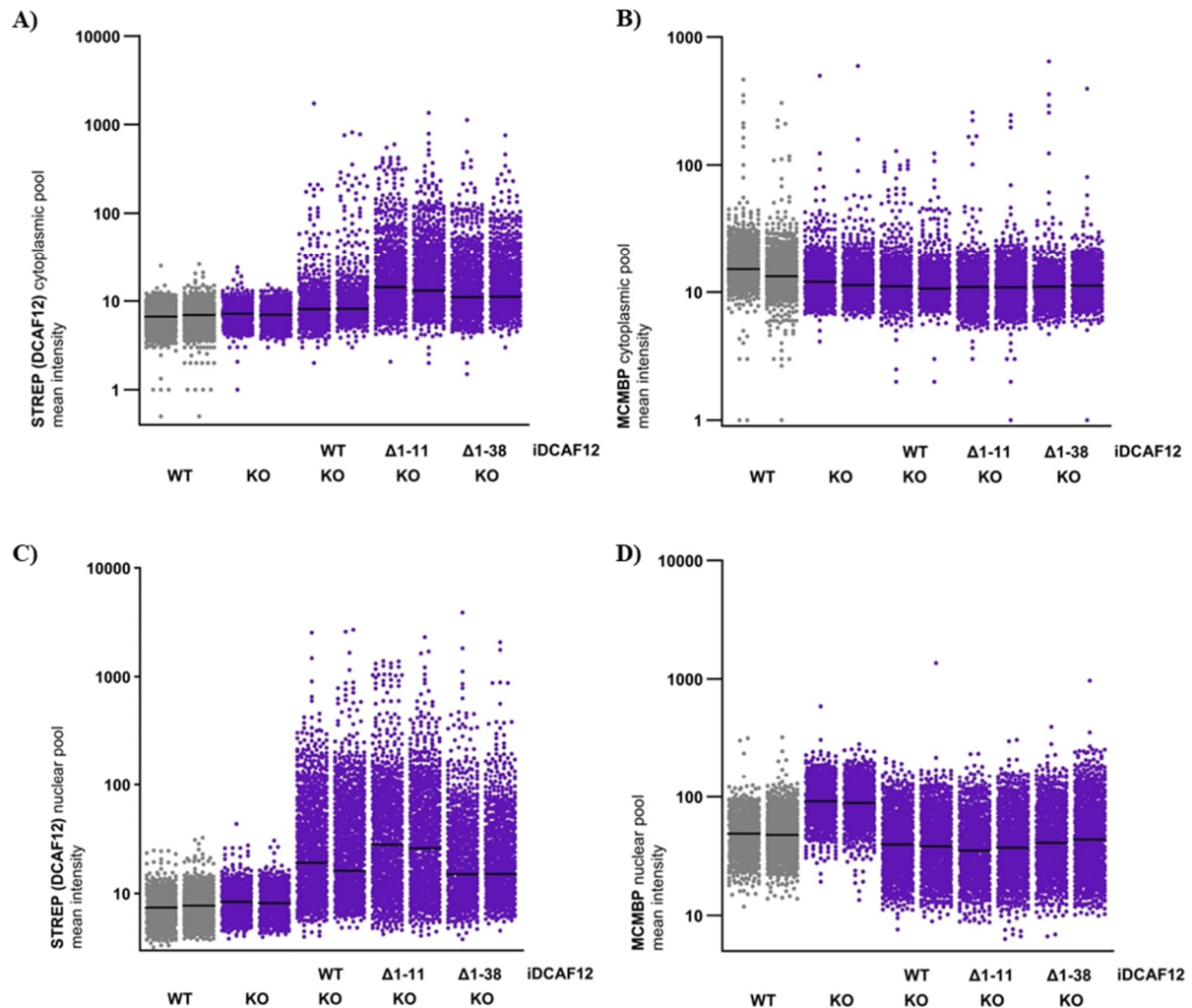


Figure 23: DCAF12 Regulates the Nuclear Pool of MCMBP

QIBC plots of U2-OS WT and truncated forms of DCAF12 expressing doxycycline (DOX, 48 h) inducible Strep-DCAF12 (where indicated) with one NLS (ΔAA1-11), or both (ΔAA1-38) NLS immunostained for MCMBP. Lines denote medians; $n \approx 10,000$ cells per condition.

5.1.8. Lack of DCAF12 Expression Increases MCMBP Protein Stability

To assess the stability of MCMBP, we employed a cycloheximide (CHX) chase assay. CHX, a ribosome inhibitor, allowed us to observe only previously synthesized proteins, already present in the cells¹⁶³. Again, we employed WT, KO and iDCAF12 U-2 OS cells. Cells were treated with CHX and collected at indicated time points. Confirming our previous observation, DCAF12^{KO} cells had increased MCMBP protein levels as well as its stability. Notably, this effect was reversible upon re-expression of exogenous DCAF12 (Fig. 24, 25).

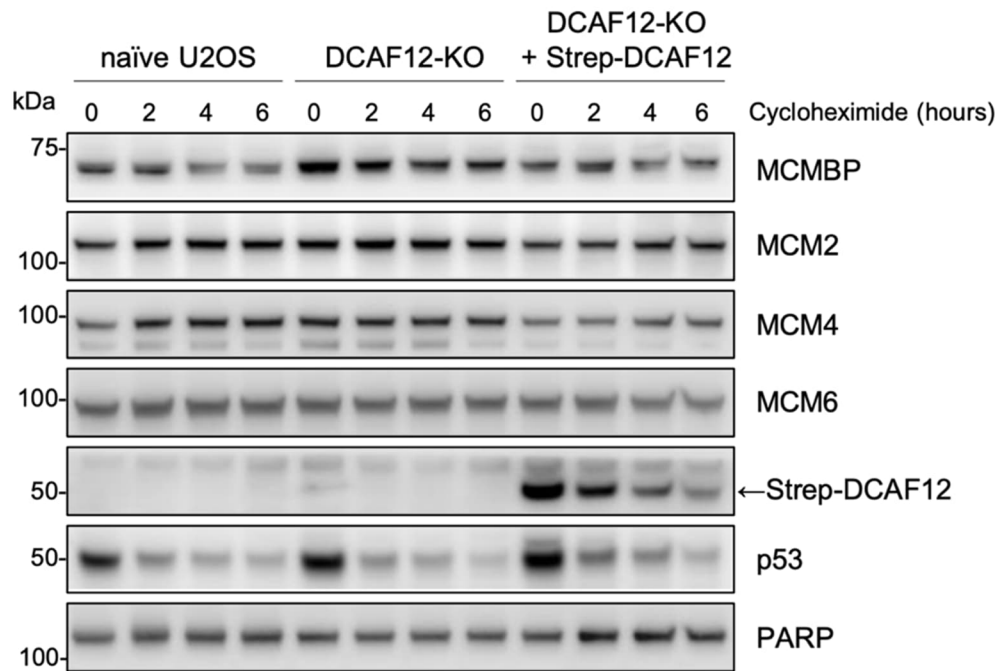


Figure 24: Lack of DCAF12 Expression Increases MCMBP Protein Stability

StrepII-FLAG-tagged DCAF12 was inducibly expressed in DCAF12^{KO} U-2 OS cells using the Sleeping Beauty transposon System Cells were treated with cycloheximide for the indicated time. Whole-cell lysates were immunoblotted as indicated. Knockdown of DCAF12 led to increased stability of its substrate MCMBP, an effect that was rescued by the inducible expression of an DCAF12 construct.

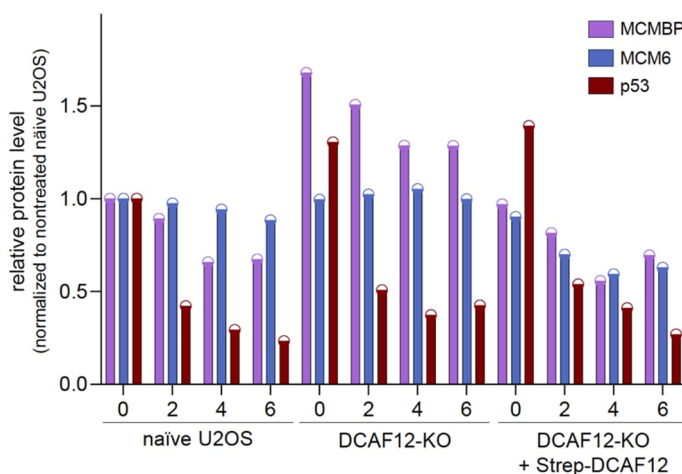


Figure 25: Densitometry of MCMBP, MCM6 and P53

Results from densitometry of MCMBP, MCM6, P53 levels from Fig. 24.

5.1.9. DCAF12 Regulates MCMBP During S/G2 Phase

To elucidate if DCAF12-dependent degradation of MCMBP in the cell cycle-dependent manner, we have synchronized WT and DCAF12^{KO} cells with hydroxyurea (HU) (Fig. 26, 27). HU inhibits the DNA synthesis by blocking the activity of ribonucleotide reductase, an enzyme responsible for creating deoxyribonucleotides¹⁶⁴. Upon release from hydroxyurea cells enter the S-phase. Levels of MCMBP were comparable in this stage in both cells. Interestingly, we saw destabilization of MCMBP protein levels in wild type cells in late S and G2 phases, indicating specific degradation of MCMBP in these cell cycle phases. In contrast, DCAF12 knockout cells did not show such decrease proving that DCAF12 most probably targeted MCMBP protein in these stages. These results were also seen on densitometry analysis in Figure 27.

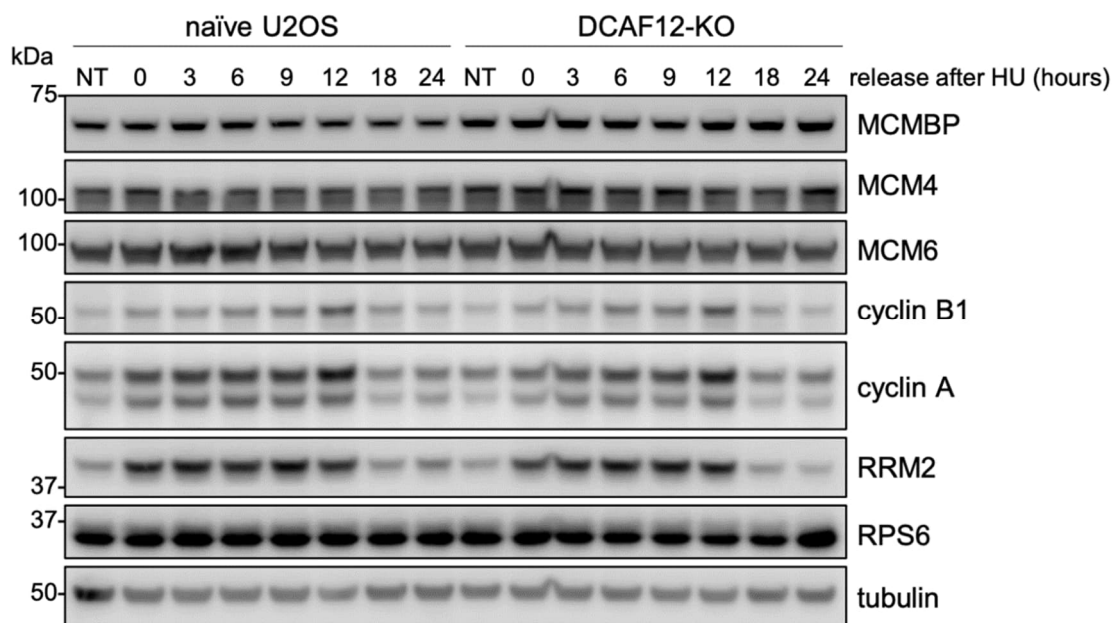


Figure 26: DCAF12 Regulates MCMBP During S/G2 Phase

U-2 OS DCAF12 WT and KO cells were synchronized by hydroxyurea treatment (2 mM; 16h) and then released in fresh medium for the indicated hours. Cells were collected at indicated times and processed for western blot. Whole-cell lysates were immunoblotted as indicated.

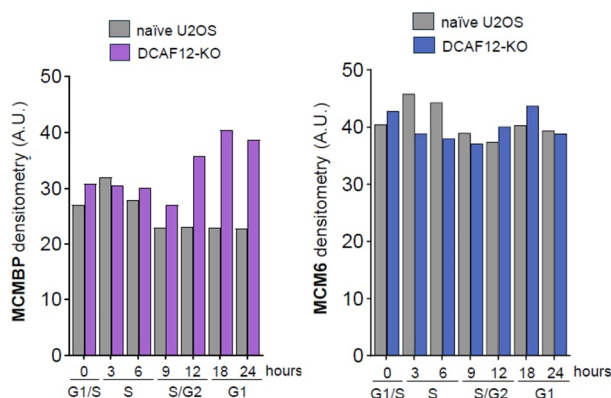


Figure 27: Densitometry Analysis of MCMBP and MCM6 from Figure 26

Results from densitometry of MCMBP and MCM6 levels from Fig. 26.

5.2. Impact of *Dcaf12* Deficiency on Mouse Model

5.2.1. Preliminary Data

Our laboratory previously generated a mouse model deficient in *mDCAF12* to investigate its physiological and functional significance¹⁰⁶. The *Dcaf12* gene was targeted using CRISPR-Cas9 genome editing technology at exon 4, resulting in a deletion of 420 nucleotides. Comprehensive physiological evaluations of the *Dcaf12* knockout mice revealed no overt phenotypic differences compared to wild-type controls (Fig. 28). Additionally, deletion of *Dcaf12* did not disrupt the Mendelian ratio of offspring, despite the observation that *Dcaf12* depletion slightly impaired the sperm count but also spermatogenesis, together associated with increased expression of γ -H2AX, indicative of DNA damage¹⁰⁶.

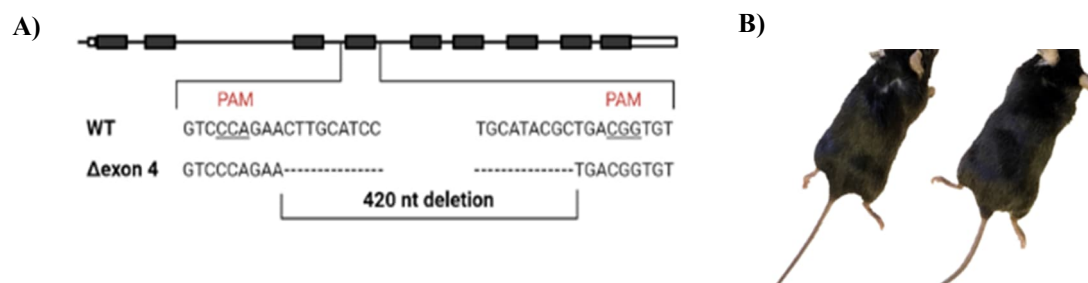


Figure 28: Mouse Model Deficient in *mDcaf12*

A) The deficiency in DCAF12 has been created by targeting of the exon 4 by CRISPR/Cas9 method.
B) Mice *DCAF12*^{KO} show no phenotypical change in comparison with WT mice. Taken from¹⁰⁶.

The aims:

- Validating the function of CRL4^{DCAF12} as the ubiquitin ligase responsible for MCMBP proteasomal degradation
- Assessing the impact of DCAF12 loss on the composition of the hematopoietic system.
- Evaluating the role of *Dcaf12* expression in cancer-prone mice to determine its potential as a tumor suppressor

5.2.2. DCAF12 Controls MCMBP Level in Mice

To investigate the proposed role of DCAF12 in MCMBP degradation *in vivo*, we have selected a cohort of 22-weeks old WT and DCAF12^{KO} *C57Bl/6NCrl* mice and isolated proteins from several organs. We observed that MCMBP was expressed in all selected tissues and its levels were significantly higher in *Dcaf12*-deficient strain, especially in high proliferating tissues such as bone marrow and splenocytes (Fig. 29; densitometry in Fig. 31).

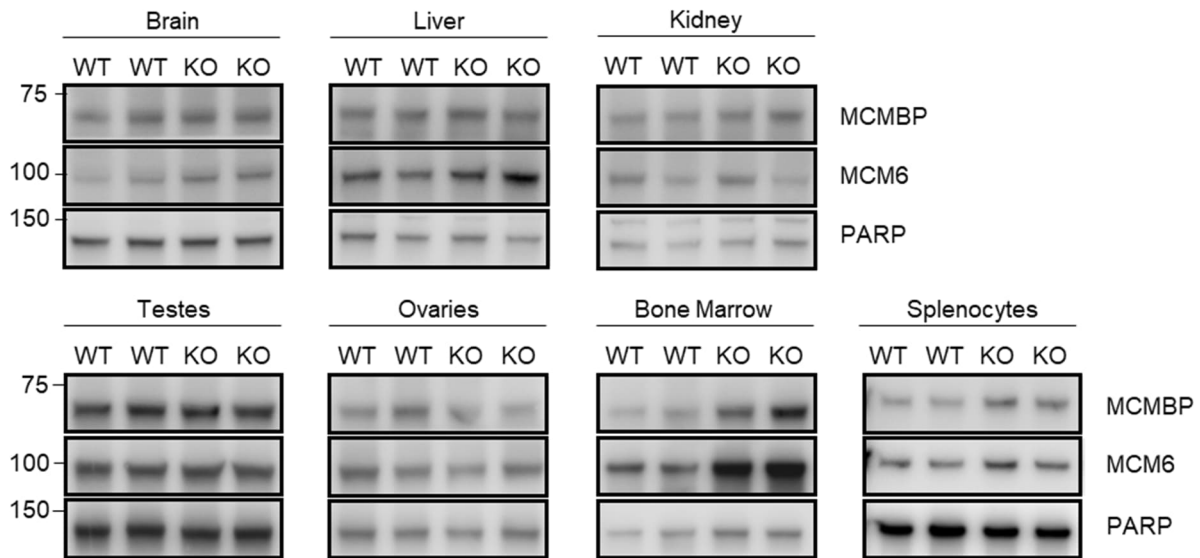


Figure 29: DCAF12 Controls MCMBP Level in Mice

WCL were prepared from multiple organs of 22-weeks old WT and DCAF12 KO male mice. MCMBP expression is observed in all tissues, but overall, most significant stabilization of MCMBP is seen in *Dcaf12* KO bone marrow and splenocytes, both high-replicative tissues. PARP was used as a loading control.

Next, we tested the MCMBP protein levels in fetal mouse tissues (20-days old). Embryos isolated from pregnant females were dissected and their organs lysed in presence of Benzonase. We used PCR analysis to distinguish sex and genotype. WCLs from both males and females (lungs, liver and brain- all high-proliferative organs) were analyzed by PAGE and immunoblotted (Fig. 30). Again, we used PARP and MCM6 staining as loading controls. We added TUBA staining to avoid differential loading. We observed, similarly as in the adult tissues, that MCMBP was stabilized in *Dcaf12*^{KO} strain independently of sex (Fig. 30; densitometry in Fig. 31).

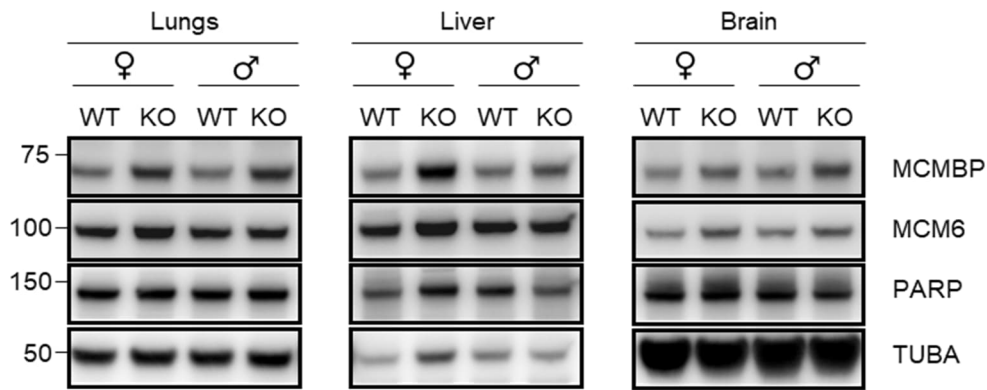


Figure 30: DCAF12 Regulates MCMBP Level in Fetal Mouse Tissues

Mouse organs were prepared for WCL and immunoblotted as indicated. PARP and TUBA were used as loading controls.

The densitometry analysis of WB results showed the normalized levels of MCMBP (Fig. 31). Significant difference in MCMBP level was seen in all fetal tissues and in adult bone splenocytes (although in bone marrow was the difference between WT and KO tissues clear), again pointing to the importance of DCAF12-dependent control of MCMBP protein levels in highly proliferative tissues. Altogether, analysis of adult and fetal tissues proved that DCAF12 controls the MCMBP protein level *in vivo*.

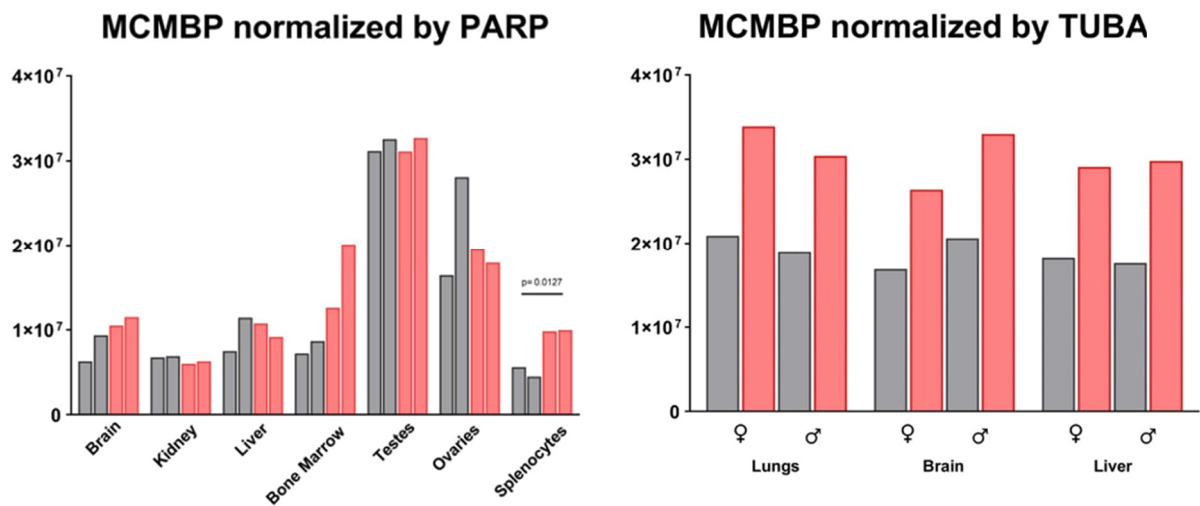


Figure 31: Densitometry Analysis of MCMBP Levels in Mouse Model

Densitometry analysis of MCMBP levels (Fig. 29,30) in mouse tissues normalized by PARP/TUBA. *Dcaf12*^{WT} in grey and *Dcaf12*^{KO} in red.

5.2.3. DCAF12 Deficiency Modifies Immune Cell Distribution

Following the observation that, in adult animals, *Dcaf12*-deficiency has the greatest impact on MCMBP protein levels in the bone marrow and spleen, we sought to investigate potential phenotypic changes in the hematopoietic system of *Dcaf12*-deficient mice. To begin, we quantified specific immune cell populations in peripheral blood to evaluate immune system abnormalities associated with *Dcaf12*-deficiency (Fig. 32). This analysis involved immunophenotyping peripheral blood samples collected from approximately 14-week-old male mice, with a focus on basic lymphocyte populations.

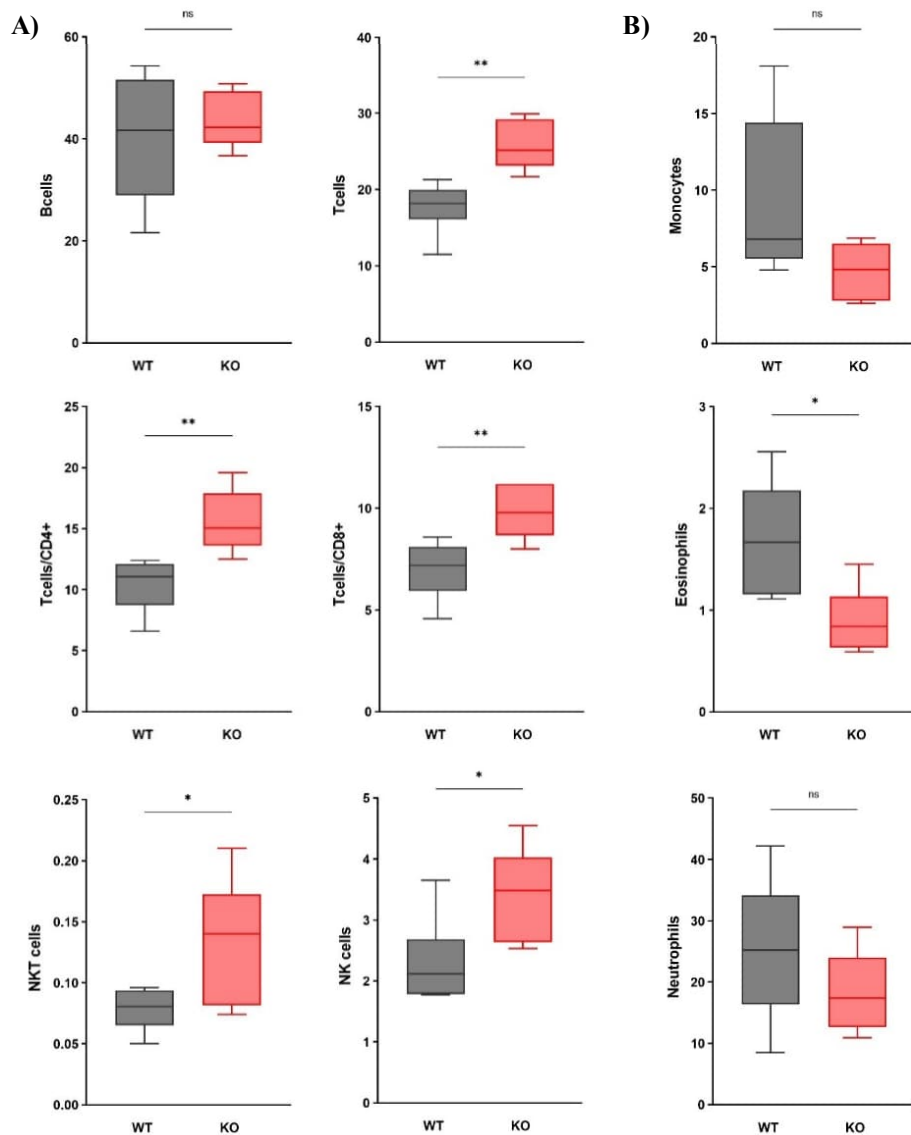


Figure 32: DCAF12 Deficiency Modifies Immune Cell Distribution

Immunophenotyping analysis was performed from blood samples of ~14-week-old male *Dcaf12*^{WT} (grey) and *Dcaf12*^{KO} (red) mice. Cell analysis was conducted by CCP at Biocev using flow cytometry, and statistical significance was evaluated with an unpaired two-tailed t-test. Ns= non-significant, *= <0,1, **<0,01 significance value.

While the B-cell population remained unaffected, we observed significant hyperproliferation of T-cells in *Dcaf12*-deficient mice, with all T-cell subsets showing a comparable level of hyperproliferation. Additionally, hyperproliferation was detected in natural killer (NK) cells. In contrast, a marked reduction in myeloid cell populations was observed in the peripheral blood of *Dcaf12*-deficient mice.

Collectively, these findings demonstrate that *Dcaf12* deficiency profoundly impacts immune cell development, leading to distinct alterations in both lymphoid and myeloid compartments.

5.2.4. The Effect of DCAF12 Expression in a Cancer-prone Mouse Model

Previous studies have shown that altered MCM levels are associated with increased mutagenesis frequency and tumorigenesis^{157,165–167}. Based on this, we sought to investigate the potential tumor suppressor role of DCAF12 *in vivo*. To achieve this, we utilized our *Dcaf12*-deficient strain and intercrossed it with tumor-prone *Trp53*-deficient mice (Jackson Laboratory, strain 002101), as illustrated in the breeding scheme (Fig. 33). The breeding strategy was designed to generate mice with a double-knockout (DKO) genotype for *Dcaf12* and *Trp53*. Initially, mice heterozygous for *Dcaf12* and *Trp53* were intercrossed, and genotypes were confirmed via PCR analysis (Fig. 34). This initial cross produced offspring with various genotypes. From these, animals with a *Dcaf12*^{-/-} *Trp53*^{+/-} genotype were selected for following breeding to obtain double-knockout mice in the next generation. This DKO model enabled us to investigate the impact of DCAF12 loss on tumor development.

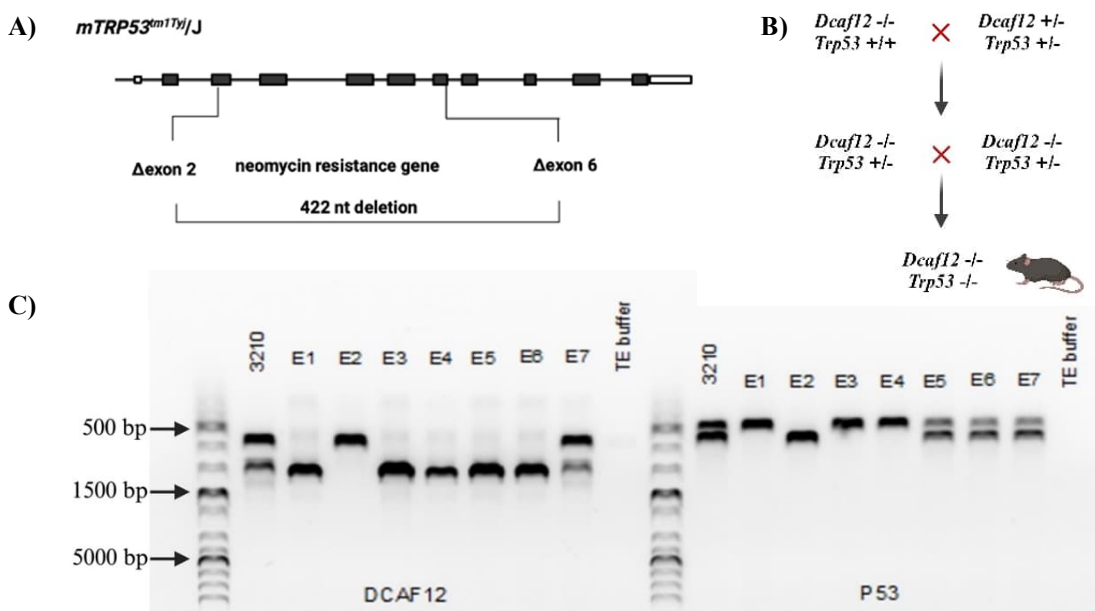


Figure 33: *mTrp53* Deletion and Strategy of DKO Breedings

- A)** *Trp53* deficient mice were prepared by replacing of exon 2-6 with a neomycin resistance cassette.
- B)** The DKO genotype was created by multiple breedings, starting with crossing of *Dcaf12* heterozygotes and *Trp53* heterozygotes. Subsequently obtained *Dcaf12*^{-/-} mice were intercrossed with *Trp53* heterozygotes.
- C)** Genotyping example. The genotype in both DKO mice was confirmed by a standard PCR method. The band sizes were compared to the 1 kb GeneRuler (Thermo Fisher). The wild-type product of *Dcaf12* is 1032 bp, while the knockout product measures 612 bp. For *Trp53*, the WT product is 616 bp, and the KO product is 400 bp.

5.2.5. The Sex Ratio is Impaired in DKO Mice

Trp53 deficiency has been linked to alterations in sex-biased birth ratios. In alignment with previous findings, we observed a significant reduction of approximately 50% in female births, resulting in a male-to-female ratio of 3:1¹⁶⁸. During embryogenesis, female embryos frequently exhibit neural tube closure defects, such as exencephaly or anencephaly, which contribute to high mortality rates¹⁶⁹. Notably, in the combined *Dcaf12*- and *Trp53*-deficient model, we observed a significant decrease of approximately 80% female births, leading to a striking male-to-female ratio of 9:1 (Fig. 34).

	All	Males		Females		Chi-square	p-value
Genotype	Observed	Expected	Observed	Expected	Observed		
<i>Dcaf12</i> ^{-/-} <i>Trp53</i> ^{+/+}	95	48	47	47	48	0	1
<i>Dcaf</i> ^{+/+} <i>Trp53</i> ^{-/-}	29	15	23	14	6	0.9675	0.3253
<i>Dcaf12</i> ^{-/-} <i>Trp53</i> ^{-/-}	27	14	25	13	2	3.6872	0.0548

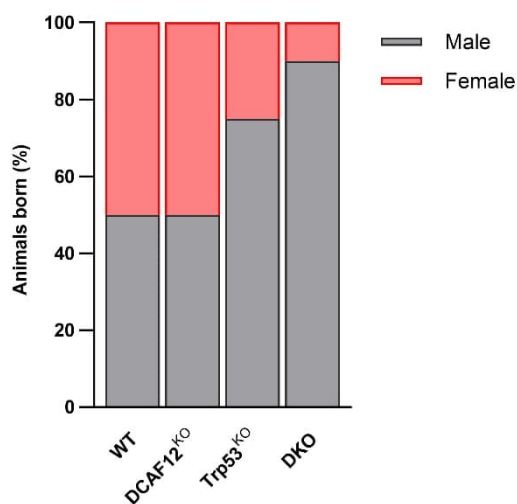


Figure 34: The Sex Ratio is Influenced by Double Deficiency in *Dcaf12* and *Trp53*

The sex ratio was statistically measured by the Chi-square test. The male-female ratio was impaired in *Trp53*^{-/-} by ~25 %, consistently with already known effect of *Trp53* loss. A significant decline in the females born was found in DKO mice.

5.2.6. Survival Rates Decline with DCAF12 Deficiency

To observe the role of *Dcaf12* in the survival tendencies we have created a cohort of DKO mice, deficient in both *Dcaf12* and *Trp53*. The *Trp53*-deficient strain typically led to the development of aggressive T-cell lymphomas at approximately 20 weeks of age. Haploinsufficiency in *Trp53* led to the development of solid tumors around 60 weeks¹⁷⁰. In both cases, tumors developed significantly faster on the background of the *Dcaf12*-deficient strain (Fig.35).

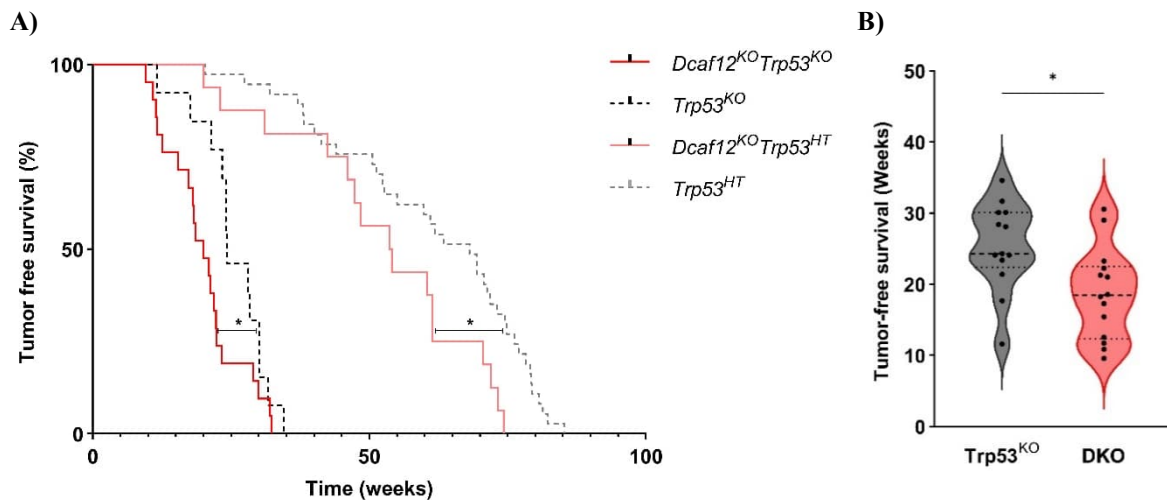


Figure 35: *Dcaf12*-deficiency Leads to Shorter Lifespan

A) Cohorts of approximately 20 mice from *Dcaf12*- and *Trp53*-deficient strains were monitored, and their survival data were analyzed and visualized using Kaplan-Meier survival curves. Statistical significance was assessed using the Mantel-Cox log-rank test and the Gehan-Breslow-Wilcoxon test to evaluate differences in survival distributions between the groups. Significance value for $\ast = <0,1$. **B)** Separate analysis of *Trp53* KO and DKO groups was done by unpaired two-tailed t-test. Significance value for $\ast = <0,1$.

5.2.7. DCAF12 Deficiency Causes Abnormal Blood Cell Profiles

The role of DCAF12 in immune system regulation has been shown in various studies. To further investigate how blood cell profiles might differ in a tumor-prone mouse model, we conducted a comprehensive hematological screening (Fig. 36). Our analysis revealed significant changes, particularly in white blood cell and lymphocyte counts. Mice deficient in *Dcaf12* displayed substantial hyperproliferation in white blood cells, lymphocytes, and monocytes. In *Trp53*-deficient mice, alterations were observed across nearly all blood cell types, except for monocytes.

When examining the combined *Dcaf12/Trp53* double knockout model, we observed mild but noticeable changes in white blood cell, lymphocyte, and monocyte counts. These findings suggest that *Dcaf12* and *Trp53* deficiencies uniquely influence blood cell composition and may interact to shape immune cell populations in tumor-prone environments.

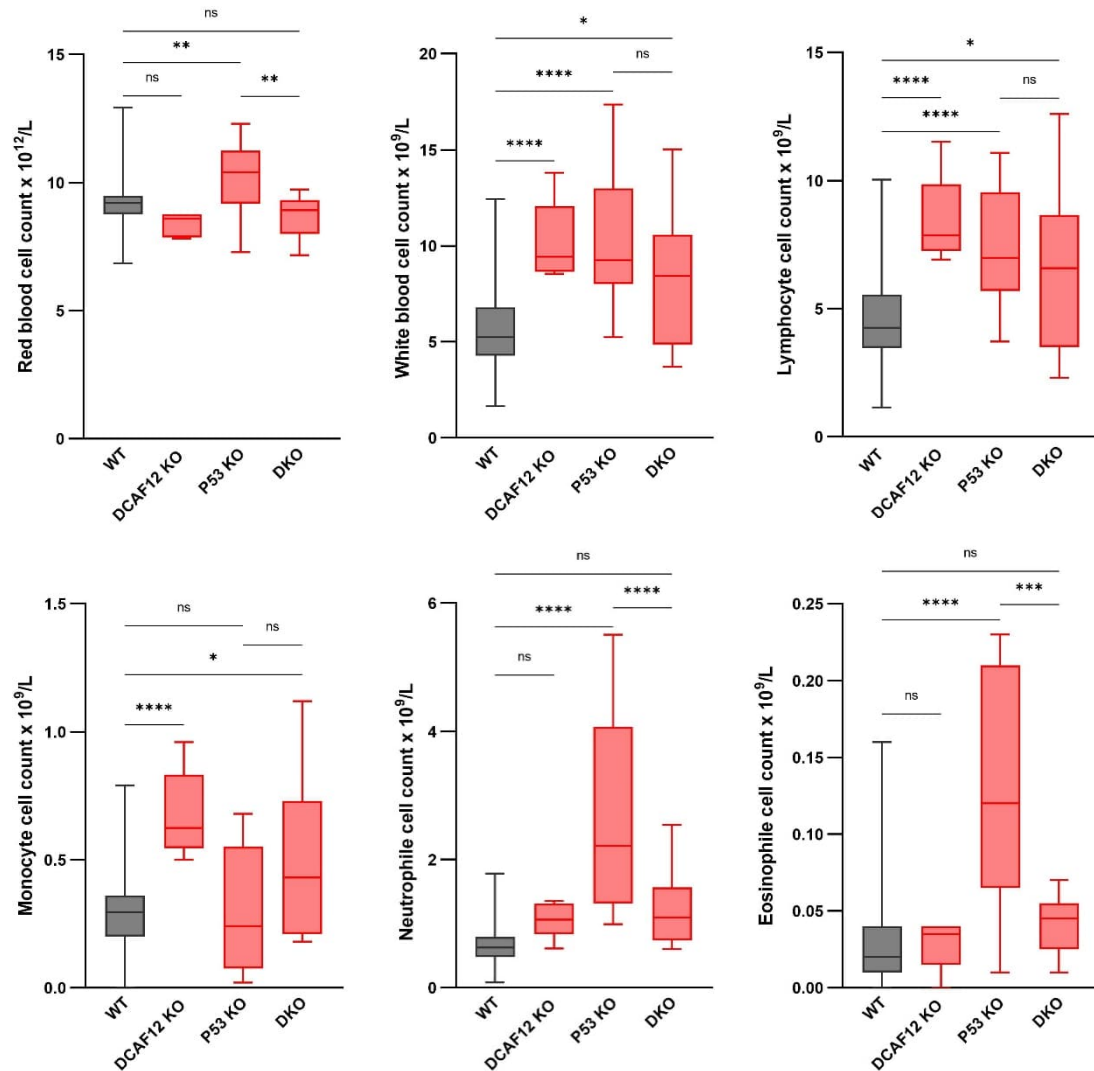


Figure 36: DCAF12 Deficiency Causes Abnormal Hematology Profiles

Blood samples from ~19-25 week-old mice were analysed by CCP at Biocev using flow cytometry, and statistical significance was evaluated with an unpaired two-tailed t-test. Ns= non-significant, *= <0,1, **<0,01, ***<0,001, ****<0,0001 significance value. Data for WT mice were used from publicly available Phenotyping module at <https://ccp-tools.img.cas.cz/apps/phenolyze/>.

6. Discussion

This project explores a novel factor in the pathway of controlling DNA replication: an E3 ubiquitin ligase, CRL4^{DCAF12}. Previous research has highlighted the critical roles of various Cullin-RING E3 ubiquitin ligases in regulating genome duplication. For instance, CRL4^{CDT2} degrades CDT1 following MCM loading onto chromatin, thereby preventing re-replication and ensuring genome stability^{80,171}. Similarly, CUL2^{LRR1} has been shown to facilitate replisome disassembly by ubiquitinating MCM7 subunit during replication termination¹⁴³. KEAP1-mediated ubiquitination of the MCM3 subunit has also been observed in the cytoplasm, though it does not lead to its proteasomal degradation¹⁷². Our work contributes to this field by elucidating the role of CRL4^{DCAF12} ubiquitin ligase in controlling the stability of MCM helicase chaperone protein MCMBP.

A recent study identified numerous novel C-terminal degrons within the human genome, recognized by various CRL2 and CRL4 complexes⁶⁶. Among these, CRL4^{DCAF12} specifically targets acidic C-terminal degrons containing a double glutamate motif. Building on this, our laboratory has extensively investigated the ubiquitin ligase function of CRL4^{DCAF12}, uncovering that it recognizes a broader range of C-terminal degrons than previously thought¹⁰⁶. Specifically, we demonstrated that a glutamate residue at the -2 position is essential for recognition, while the terminal position exhibits greater flexibility. Notably, most substrates contain either a double glutamate motif or a glutamate followed by leucine, highlighting the diversity of degron recognition by DCAF12¹⁰⁶.

Largest obstruction identified during our research stems from the observation that most of the DCAF12 interaction partners we identified did not act as substrates for DCAF12, despite possessing C-terminal degrons and demonstrating *in vitro* interaction with DCAF12. Specifically, we observed DCAF12-dependent degradation of MOV10 and GART proteins, both of which are recognized through distinct C-terminal degrons. Interestingly, both of these proteins were strongly degraded upon expression of DCAF12 lacking nuclear localization signal¹⁰⁶. Interestingly, the behavior of MOV10 differed from that of GART under physiological conditions. While GART predominantly localizes to the cytosol, MOV10 is found in both nuclear and cytosolic compartments. This compartmental difference suggests that DCAF12 functions as a ubiquitin ligase primarily within the nucleus in our cellular system. Supporting this hypothesis, MOV10 was observed to act as a DCAF12 substrate under specific physiological conditions, such as in the testes, whereas GART did not. These findings highlight

the compartment-specific nature of DCAF12's ubiquitin ligase activity and underscore the complexity of substrate recognition in this context.

Contrary to the expected nuclear activity of DCAF12, we detected its interaction with many cytosolic proteins. Initially, we considered the possibility that these interactions were artifacts arising from post-lysis conditions, as the use of whole-cell lysates in our experiments results in the mixing of nuclear and cytosolic compartments¹⁷³. Additionally, the simplicity of the degron sequence recognized by DCAF12 raised the likelihood of non-specific binding. A potential contributor to these observations is the presence of C-terminal KDEL motifs in a subset of cytosolic proteins¹⁷⁴. This motif is well-characterized for its role in regulating protein trafficking by retrieving mislocalized secretory proteins back to the ER, suggesting that sequence similarity may underlie the observed cytosolic interactions of DCAF12.

This assumption resulted in re-analysis of our mass spectrometry data to identify only nuclear DCAF12-interacting proteins. These results form part of our preliminary findings, where we explore the DCAF12 interactome through LC-MS protein mass spectrometry analysis. To interpret the identified proteins, we curated a comprehensive list of their compartmental localizations. Given that DCAF12 is a nuclear protein, we subtracted from our analysis those proteins lacking nuclear localization signals (NLS), those reported to be predominantly cytosolic, or those which do not contain C-terminal degron motifs. We also took into account that nuclear localization of proteins is not directed only by NLS presence but also through passive diffusion for proteins of appropriate molecular size. After excluding non-nuclear candidates, our analysis revealed a more refined subset of potential substrates. Among those, we choose to validate MCMBP protein, previously characterized as a chaperone for MCM helicase involved in the replication machinery (Fig. 13)^{149,160}.

Next step was to isolate the whole CRL complex and confirm the interaction. The reason we chose to isolate the whole CRL complex was to identify specific substrates. We detected MCMBP peptides not only in the affinity purification of DCAF12 but also in subsequent immunopurification of CUL4A using affinity-purified DCAF12 complexes, demonstrating that MCMBP associates with the CRL4^{DCAF12} complex (Fig. 14). This comprehensive approach allowed us to capture the full range of interactions and ensure the identification of relevant substrates within the complex. Additional experiments revealed that MCMBP protein levels were stabilized following treatment of U-2 OS cells with the neddylation inhibitor MLN4924 (Fig. 15). MLN4924 is a selective inhibitor of the NEDD8-activating enzyme (NAE), which is responsible for the neddylation process. Neddylation involves the attachment of the small ubiquitin-like protein NEDD8 to cullin proteins, a key

step for the activation of cullin-RING ligase (CRL) complexes. By inhibiting NAE, MLN4924 prevents CUL neddylation, leading to the inactivation of CRL complexes¹⁷⁵. This disruption of CRL activity can result in the stabilization of certain proteins, like MCMBP, because CRLs are typically responsible for the ubiquitination and subsequent proteasomal degradation of these substrates. Therefore, treatment with MLN4924 prevents the degradation of MCMBP, causing its accumulation. To confirm the specific role of CRL4 in MCMBP degradation, we assessed the effects of siRNA-mediated depletion of CUL1–5. Notably, MCMBP stabilization occurred only upon CUL4A/B knockdown, reinforcing the role of CRL4-dependent degradation (Fig. 16). Similar approach of ubiquitin ligases knockdown was used in the research of CRL4^{AMBRA1} role in the Cyclin D degradation¹⁷⁶.

The chaperone protein MCMBP has been proposed by Sedlackova et al. to facilitate the transport and assembly of MCM3-7 subcomplexes, as MCM2 has its own nuclear localization signal¹⁵³. After delivery of MCMBP-MCM3-7 complex to the nucleus, MCM2 integrates into the MCM3-7 complex while MCMBP, not part of pre-replicative chromatin-loaded complexes, is removed. Sedlackova et al. proposed that MCMBP interacts with MCM3/5, others have shown the affinity of MCMBP to be variable, as it interacts with various MCM subunits with different affinity^{160,177}. MCMBP is crucial for MCM nuclear translocation and balance, but the precise mechanism behind the final step, where MCMBP is exchanged for MCM2 to form the mature nascent MCM2-7 complex, remains unclear. Following our hypothesis, that CRL4^{DCAF12} could be the factor behind the correct MCM2-7 biogenesis, we aimed to confirm this interaction and investigate its spatiotemporal context.

To validate the interaction between DCAF12 and MCMBP, we prepared an affinity purification panel of various CUL1/4 substrates receptor (Fig. 17). As MCM complexes are often appearing in interaction studies we first decided to validate its interaction with DCAF12 in small scale purification. This experiment resembles similar approach shown in our previous publications^{178,179}. Among CUL1/4 substrates receptors tested, only DCAF12 was able to interact with both MCMBP and MCM5. Importantly we did not detect any PCNA in DCAF12 complex. PCNA is a part of replication fork- acting as a clamp protein. This result provided us with assumption that DCAF12 recognizes MCMBP and MCMs in single complex but is not part of chromatin associated complex.

As previously described, DCAF12 recognizes its substrates through C-terminal degrons. To investigate the evolution of the C-terminal degron in MCMBP across eukaryotes, we created a phylogenetic analysis highlighting the presence of acidic degrons in MCMBP (Fig. 18). Our findings indicate that the presence of DCAF12 in eukaryotes (since vertebrates)

correlates with the occurrence of the C-terminal degron in MCMBP. Interestingly, C-terminus of MCMBP originally consisted in lampreys of glutamate followed by glutamine, at penultimate and ultimate position, respectively. In fish, the ultimate glutamine was exchanged for proline and in amphibians to mammals for leucine. We will follow this analysis in our future research trying to uncover if amino acids in last position correlate with changes in DCAF12 primary structure – especially involved in binding substrates. The function of the MCMBP chaperone in assembling MCM complexes appears to be conserved from fission yeast to eukaryotes, raising intriguing questions about the absence of MCMBP in budding yeast¹⁸⁰.

As expected, additional experiments confirmed that the C-terminal degron is crucial for the interaction with DCAF12. HA-tagged variants of MOV10 and MCMBP- both the wild-type and truncated forms lacking the C-terminus were used in affinity purification assay. We observed that the interaction between DCAF12 and MOV10 was disrupted in the truncated form, as was the interaction with the MCMBP Δ C mutant (Fig. 19). In this experiment, both truncated forms were more expressed than their wild type alternatives. That is not surprising because they should not be targeted for proteasomal degradation via DCAF12. Interestingly, also DCAF12, which was overexpressed, was on protein level higher in samples with truncated forms of its substrates. This could be the result of autoubiquitination and self-degradation following overexpression of its substrates. Autoubiquitination is common between ubiquitin ligases. It was reported for example in the case CRL3 or SMURF1, a HECT ubiquitin ligase. Autoinhibition can prevent autoubiquitination, thereby avoiding proteasomal degradation. Autoinhibition of ubiquitin ligases has been proposed to maintain the ideal ratio of E3 ligases in the cells^{181–184}.

We also investigated the interaction between MCMBP and two orthologs of DCAF12: DCAF12L1 and DCAF12L2 (Fig. 20). As expected, no binding between MCMBP and the DCAF12 orthologs was observed. This aligns with previous findings showing that neither DCAF12L1 nor DCAF12L2 can bind to the C-terminal acidic end¹⁰⁶. Furthermore, we did not detect significant binding of the adaptor protein DDB1 to DCAF12L1 or DCAF12L2. Consequently, it remains unclear whether these orthologs can form a fully functional CRL4 complex. Interestingly, we observed significantly higher expression levels of DCAF12L1 and DCAF12L2 compared to DCAF12. This difference in expression levels might result from stronger autoubiquitination of DCAF12 compared to its orthologs.

As demonstrated, DCAF12, a nuclear protein, specifically interacts with MCMBP via the C-terminal degron. Following this observation, we proved that this interaction promotes the assembly of nascent MCM2-7 subcomplexes in the nucleus, as detailedly shown in our

preprint paper¹⁸⁵. In contrast, the MCM2 subunit's cellular level and nuclear import appear to be independent of MCMBP. Although the rationale behind the separation of the MCM complex during nuclear transport remains unclear, a recent study suggested that certain protein cargos may enhance the mechanoselectivity of the nuclear pore complex, thereby increasing nuclear import rates¹⁸⁶.

To further analyze how MCMBP protein levels are influenced by the subcellular localization of DCAF12, we utilized HCT116 cells expressing either WT or a truncated form of NSF-DCAF12 that lacks the nuclear localization signal Δ (aa 1-38). Western blot analysis indicated DCAF12-dependent degradation of MCMBP and MCM3. Unexpectedly, MCMBP and MCM3 levels were also low in cells expressing DCAF12 Δ (aa 1-38) variant lacking the NLS. This finding may be explained by several hypotheses: (1) DCAF12 might have some NLS-independent nuclear transport mechanism; (2) the truncated DCAF12 may diffuse passively through the nuclear pore complex; or (3) MCMBP could be targeted co-translationally when DCAF12 is over expressed. To verify the functionality and localization of mutant DCAF12 Δ (aa 1-38), we used as a control a known cytoplasmic substrate of DCAF12, the GART protein. Importantly, and in accordance with our previous results, expression of DCAF12 lacking NLS destabilized GART protein (Fig. 21). This result supported hypothesis that, even if NLS is deleted, DCAF12 enter nucleus independently and thus MCMBP is degraded in nuclear compartment.

To confirm compartment-specific degradation of MCMBP, we utilized high content quantitative microscopy assays (Figs. 22, 23). Our initial objective was to assess the CRL4^{DCAF12}-dependent degradation of MCMBP in the cytosolic and nuclear compartment. The effect of MLN4924, which inhibits all CUL-dependent pathways is shown in Fig. 22. Upon overexpression of DCAF12, most of the DCAF12 protein level was found in the nucleus, and only a small fraction in the cytosol, due to the non-physiological overexpression induced by doxycyclin. Expression of DCAF12, both endogenous and exogenous, did not affect the cytosolic MCMBP level. However, the nuclear pool of MCMBP was slightly increased in KO cells, possibly due to the MLN4924-driven synchronization to S-phase. It was previously shown that MCMBP could be target of E2F factors, since MCMBP gene contains a putative E2F1 transcription binding site, and thus its levels could be sensitive to S-phase synchronization¹⁸⁷. Importantly, in inducible DCAF12 KO cells, re-expression of DCAF12 led to a destabilization of MCMBP levels. The inhibition of CUL-dependent activity by MLN4924 blocked the effect of DCAF12 re-expression and further proved that CRL4^{DCAF12} is bona fide ubiquitin ligase of MCMBP in nuclear compartment.

Interestingly, as previously shown, MCMBP levels were destabilized even when we expressed DCAF12 lacking both nuclear localization signals (NLS) (Fig. 21). To investigate further, we examined whether the deletion of either NLS (Δ aa 1–11) or both NLS (Δ aa 1–38) in the N-terminal region of DCAF12 was critical for its localization and subsequent DCAF12-dependent MCMBP degradation. In Fig. 23A, we compared the expression of wild-type and cytoplasmic variants of DCAF12. The expression of neither variant affected MCMBP cytoplasmic protein levels. Furthermore, we observed that even when both NLS were deleted, DCAF12 was still predominantly localized to the nucleus. This supports our hypothesis that truncated DCAF12 translocates to the nucleus either via passive diffusion or through mediation by another factor. However, this result requires cautious interpretation, as we used a ScanR microscope, which is not confocal. Consequently, the nuclear localization of DCAF12 lacking NLS may be misidentified due to technical limitations. Another limitation arises from our experimental design. We were unable to detect DCAF12 using any commercial antibody or successfully implement endogenous tagging. As a result, our experiments rely on visualizing tagged forms of DCAF12, which may introduce artifacts.

Altogether, these experiments highlight the role of DCAF12 in nuclear MCMBP turnover and emphasize the importance of its NLS for efficient MCMBP degradation. To elucidate the role of DCAF12 in regulating the MCMBP stability, we employed a cycloheximide chase assay (Fig. 24, densitometry in Fig. 25), a standard method for assessing protein stability by inhibiting protein synthesis^{106,178}. In DCAF12 knockout cells, MCMBP degradation was markedly slower and significantly reduced compared to wild-type cells, suggesting that DCAF12 is critical for MCMBP degradation. The re-expression of DCAF12 in KO cells restored MCMBP stability and protein levels to patterns like those observed in naïve U-2 OS cells. This confirmed that MCMBP degradation is DCAF12-dependent. Interestingly, despite the absence of DCAF12, MCMBP protein levels decreased upon cycloheximide treatment in KO cells. This observation suggested the involvement of an alternative ubiquitin ligase system in MCMBP regulation. While no specific ubiquitin ligase has been definitively identified for this role, public database searches revealed potential candidates such as PARK2 and RNF40, which were co-isolated with the MCMBP protein in high-throughput experiments^{188,189}. These findings warrant additional validation. To ensure the correct loading, PARP-1, a protein known to remain stable in the absence of caspase activation, was included as a control. PARP-1 levels were consistently stable, and no caspase-specific degradation product was observed. Additionally, P53, a central regulator of the DNA damage response and

apoptosis, exhibited DCAF12-independent degradation, which is provided by DCAF12-independent E3 ubiquitin ligase- MDM2^{190,191}.

Given MCMBP's essential role in MCM helicase loading onto chromatin during S-phase, we examined its degradation dynamics throughout the cell cycle (Fig. 26, densitometry in Fig. 27). Using hydroxyurea (HU) to arrest cells at the G1/S phase transition, we observed a rise in MCMBP levels upon release from synchronization in WT cells. This is consistent with prior findings that the MCMBP promoter contains E2F binding sites, suggesting transcriptional upregulation during S-phase entry¹⁸⁷. Newly translated MCMBP forms complexes with MCM3-7 in the cytosol and translocates to the nucleus. Our data indicated that DCAF12 interacted with the MCMBP-MCM3-7 complex upon its entry into the nucleoplasm, facilitating its timely degradation towards the end of S-phase and the start of G2-phase. In contrast, DCAF12 KO cells maintained or even upregulated MCMBP protein levels during this period, underscoring the role of DCAF12 in regulating MCMBP turnover.

This prolonged stability of MCMBP in DCAF12-deficient cells likely disrupts the balance of dormant and active MCM complexes in the nucleoplasm, preventing proper chromatin loading. Although these experiments were beyond the scope of this thesis, complementary studies in our laboratory have demonstrated that the absence of DCAF12 led to an imbalance in nascent-parental MCM complexes, resulting in accelerated replication speed and increased mutagenesis¹⁸⁵.

The proposed tumor-suppressor role of DCAF12 was additionally confirmed on a *Dcaf12*-deficient mouse strain developed by our laboratory¹⁰⁶. We observed that MCMBP levels were stabilized in high-proliferation tissues such as bone marrow, and splenocytes of *Dcaf12*-deficient adult mice (Fig. 29). Similarly, fetal tissues—including the liver, lungs, and brain—exhibited MCMBP stabilization across both sexes (Fig. 30). This stabilization highlights DCAF12's essential role in regulating MCMBP degradation with a particular influence in proliferative environments. These observations are in accordance with fact that DCAF12 regulates MCMBP stability in the S/G2 phases of the cell cycle. The destabilization of MCMBP in tissues such as bone marrow, fetal liver, and splenocytes likely reflects their high proportion of actively cycling cells^{192,193}. Interestingly, no significant stabilization of MCMBP was detected in the intestines of *Dcaf12*-deficient mice, despite the presence of proliferative stem cells in intestinal crypts (result not shown). This discrepancy may result from the heterogeneous nature of the sampled tissue or methodological limitations in isolating specific cell types. Additionally, the observed disparity between MCMBP stabilization in the fetal and adult brain likely reflects differences in neurogenesis rates, as the fetal brain

is characterized by robust cell proliferation, whereas the adult brain exhibits limited regenerative capacity.

Building on previous studies linking DCAF12 to T-cell activation¹⁰⁶, our immunophenotyping screen revealed significant alterations in immune cell populations in *Dcaf12*-deficient mice, with the pronounced changes in CD8⁺ T-cells (Fig. 32). These cells, essential for targeting infected and cancerous cells, exhibited hyperproliferation in *Dcaf12*-deficient mice, suggesting potential compensatory immune responses to tumorigenic activity.

The tumor-suppressor role of DCAF12 was supported by our analysis of the *Dcaf12/Trp53* DKO mouse model. Interestingly, the DKO model also revealed a striking male-to-female sex ratio imbalance, with only two females born among 29 DKO animals (Fig. 34). This finding was consistent with previously reported neural tube defects (NTDs) in *Trp53*-deficient homozygous females, which contribute to high embryonic mortality. Additionally, NTDs are more frequently found in females of mice and human embryos, too¹⁶⁸. The exacerbated sex ratio imbalance in our DKO cohort suggested that *Dcaf12* deficiency may amplify developmental vulnerabilities linked to *Trp53* loss. While the precise mechanisms underlying this phenomenon remain unclear, they may involve disruptions in female-specific gene regulation or heightened sensitivity of embryonic neuroderm to combined deficiencies.

Trp53-deficient mice typically develop aggressive T-cell lymphomas within 25 to 30 weeks, while DKO mice exhibited significantly shorter survival times, highlighting an accelerated tumor onset (Fig. 35). This outcome aligns with increased genomic instability and mutation rates resulting from the combined loss of *Dcaf12* and *Trp53*, underscoring DCAF12's critical role in maintaining genomic integrity. Similar results were obtained in *Pten*-depleted mouse model. The combined model showed accelerated tumor progression in prostate cancer, emphasizing their cooperative role in driving aggressive disease¹⁹⁴.

In contrast to *Dcaf12*-deficient, *Trp53*-deficient mice showed broader alterations across nearly all blood cell types, reflecting P53's critical roles in apoptosis, genomic stability, and hematopoiesis. The combined *Dcaf12/Trp53* DKO model demonstrated milder changes in white blood cell, lymphocyte, and monocyte counts compared to single knockouts, suggesting a potential compensatory interplay between DCAF12 and P53 in hematopoiesis (Fig. 36). This interplay underscored the complex interactions between these genes in maintaining immune cell homeostasis, with implications for understanding immune pathologies and tumor progression. Importantly, immunophenotyping was done on 14 weeks old males and hematology screen was performed on blood from ~25 weeks old males

suggesting that there could be compensatory mechanism on monocytes and neutrophil levels. In both situations DCAF12 deficient mice showed increased lymphocyte population.

Our findings confirmed that DCAF12 is a crucial regulator of MCMBP stability, both *in vitro* and *in vivo*. The stabilization of MCMBP in the absence of DCAF12 and its broader effects on replication dynamics highlighted DCAF12 as a potential therapeutic target in cancer. Dysregulated MCMBP levels may disrupt the MCM complex equilibrium, impairing replication origin licensing and accelerating replication stress. While normal cells can temporarily arrest the cell cycle to address replication stress, cancer cells, often operating under reduced replication origins, are more vulnerable to replication-induced DNA damage. Targeting this vulnerability by modulating DCAF12 activity or the MCMBP degradation pathway could enhance the efficacy of existing cancer therapies. DCAF12's role in immune cell regulation indicates potential connections to immune-related pathologies. The observed *Dcaf12*-deficiency induced hyperproliferation of white blood cells may predispose to immune dysregulation, necessitating further investigation into the gene's broader implications in immune homeostasis and inflammation-driven tumorigenesis^{195,196}.

7. Conclusion

The precise maintenance of cellular MCM equilibrium is crucial for error-free genome duplication. Our study provides new insights into the MCM biogenesis pathway, emphasizing the role of MCMBP, a dedicated chaperone, in assembling nascent MCM3-7 subcomplexes in the cytoplasm and facilitating their nuclear translocation. We identify CRL4^{DCAF12} as a key regulator of this process, mediating the proteasomal degradation of MCMBP via its C-terminal degron motif to ensure the proper integration of MCM2 into the MCM2-7 complex within the nucleus. This tightly regulated interaction represents a critical step for origin licensing during replication and introduces an additional quality control layer for genome stability. Furthermore, we propose that targeting the MCM biogenesis pathway could have therapeutic potential, particularly in cancer cells, which are more susceptible to replication stress and DNA damage due to disrupted MCM homeostasis.

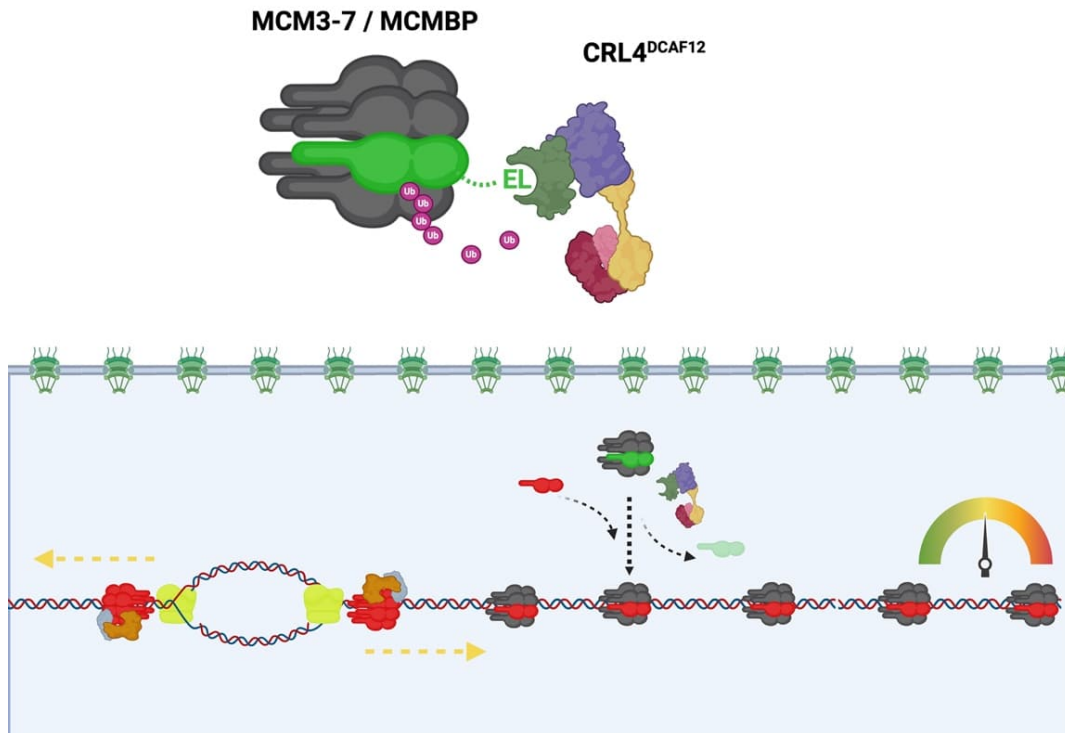
To conclude our findings:

- MCMBP chaperone interacts exclusively with DCAF12.
- Overexpression of DCAF12 resulted in decreased MCMBP protein levels.
- Inactivation or inhibition of DCAF12 stabilized MCMBP in both *in vivo* and *in vitro* models.
- The tumor-suppressor role of DCAF12 was described in a *Dcaf12*-deficient mouse model in the context of *Trp53* haploinsufficiency.
- CRL4^{DCAF12} is bona fide ubiquitin ligase of MCMBP.

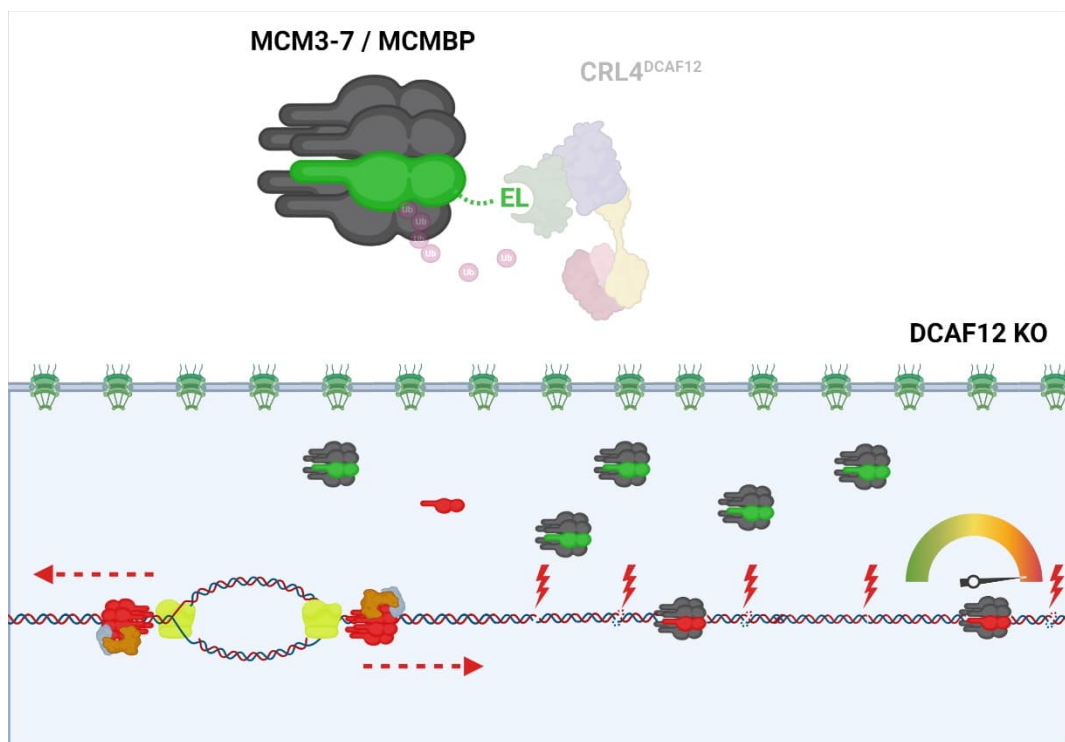
In conclusion, CRL4^{DCAF12} serves as an E3 ubiquitin ligase for the chaperone protein MCMBP, thereby influencing DNA replication through its regulation of MCM complex levels.

8. Model of DCAF12-dependent Control of DNA Replication

- DCAF12 regulates MCMBP levels during S/G2 phase and controls DNA replication and its fidelity.



- DCAF12 absence leads to DNA replication alteration and genomic instability.



9. Literature

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10. Addendum

a) CRL4^{DCAF12} regulation of MCMBP ensures optimal licensing of DNA replication

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b) FBXO38 is dispensable for PD-1 regulation

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a)

1 **CRL4^{DCAF12} regulation of MCMBP ensures optimal licensing of DNA replication**

2

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15

16 **ABSTRACT:** The minichromosome maintenance (MCM2-7) protein complexes are central drivers of
17 genome duplication. Distinct protein pools, parental and nascent MCMs, and their precise
18 equilibrium are essential to sustain error-free DNA replication¹. However, the mechanism responsible
19 for generating these pools and maintaining their equilibrium remains largely unexplored. Here, we
20 identified CRL4^{DCAF12} as a new factor controlling the assembly of nascent MCM complexes. During
21 MCM biogenesis, MCMBP facilitates the assembly and transport of newly synthesized MCM3-7
22 subcomplexes into the nucleus^{2,3}. Once in the nucleus, the MCM2 subunit must be incorporated into
23 the MCM3-7 subcomplex, while MCMBP needs to be removed. CRL4^{DCAF12} facilitates the degradation
24 of MCMBP and thereby regulates the assembly of MCM2-7 complexes. The absence of CRL4^{DCAF12}
25 adversely affects the level of chromatin-bound nascent MCMs, resulting in accelerated replication
26 forks and genome instability. Collectively, our findings uncovered the molecular mechanism
27 underlying nascent MCM production essential to counteract genome instability and tumor
28 formation.

29

30 **MAIN:**

31 Accurate and complete genome duplication is a vital process that relies on error-free regulation of
32 DNA replication. In eukaryotic cells, DNA replication is initiated in the G1 phase by origin licensing,
33 during which inactive MCM2-7 protein complexes are loaded on replication origins⁴. Subsequently,
34 during the S phase, a small fraction of inactive MCM complexes is converted to active replicative
35 CMG (CDC45-MCM2-7-GINS) helicases, around which the entire replisome responsible for genome
36 duplication is assembled. Although the majority of MCM complexes remain inactive throughout the
37 DNA replication program, their precise regulation and maintenance during the cell cycle are critical

for cellular fitness and survival. Our recent work provided mechanistic insights into this MCM paradox². We found that two distinct MCM protein variants sustain optimal origin licensing and error-free genome duplication in daughter cells: 1) parental MCMs, which represent a protein pool already involved in the DNA replication process in mother cells; and 2) nascent MCMs, which were newly formed in mother cells, but not used in the replication process. Notably, these two MCM protein forms differ not only in age but also in their engagement during the replication program. While parental MCM complexes are preferentially converted to active replicative CMG helicases, the nascent MCMs remain largely inactive but function as natural replisome pausing sites¹. Such pausing sites help to regulate the physiological replication fork progression and thereby maintain error-free genome duplication. Therefore, even a minor imbalance in MCM levels can cause replication-induced DNA damage, which, if left unrepaired, can lead to genome instability and severe diseases, including cancer⁵⁻⁹. Although numerous regulatory pathways that oversee MCM chromatin binding have been identified in eukaryotic cells^{10,11}, the apical mechanism responsible for the generation and maintenance of MCM equilibrium across generations of dividing cells remains largely unexplored. In this work, using a set of screens against cullin (CUL)-RING E3 ubiquitin ligases (CRLs) and their receptors, we identified the DCAF12 substrate receptor as a novel regulator of the assembly of nascent MCM complexes. During the MCM biogenesis pathway, the MCMBP, a specific chaperone, promotes the assembly and nuclear transport of newly formed MCM3-7 complexes^{2,3}. Subsequently, MCMBP needs to be removed as it is not part of pre-replicative complexes loaded on chromatin. We showed that this step is regulated by CRL4^{DCAF12}, which facilitates the formation of a complete MCM2-7 ring. Inactivation of the MCM biogenesis pathway leads to suboptimal origin licensing, which results in fast replication fork progression and genome instability. Collectively, these findings reveal a cellular mechanism important to sustaining optimal MCM equilibrium and, thus, error-free genome duplication.

RESULTS:

siRNA-based screening identified CRL4^{DCAF12} as a novel regulator of MCM equilibrium

The ubiquitin-proteasome system (UPS) is a central mechanism through which cells achieve selective protein degradation, necessary to sustain the homeostasis of essential proteins within a cell¹². Therefore, we aimed to investigate the potential role of UPS in the generation and maintenance of MCM equilibrium. To this end, we treated U2OS cells with MLN4924, an inhibitor of all CRLs, and monitored the level of MCMBP, a specific chaperone for the nascent MCM complexes during their biogenesis^{2,3}. Quantitative image-based cytometry (QIBC) revealed a higher protein level of MCMBP

72 upon MLN4924 inhibitor treatment (Extended Data Fig. 1a). Next, we opted for a siRNA-based
 73 screening approach to directly target individual cullins (CUL1, CUL2, CUL3, CUL4A/B, and CUL5).
 74 CRL4^{AMBRA1} has previously been identified as a regulator of D-type cyclins^{13,14}; thus, the cyclin D1
 75 marker was utilized as a positive control for siRNA cullin screening (Extended Data Fig. 1b, c).
 76 Notably, the depletion of CUL4A/B resulted in a massive stabilization of MCMBP, indicating a
 77 putative regulatory role of CRL4A/B on MCMBP (Fig. 1a, b).

78 The C-terminus of CUL4A/B interacts with RBX1 to recruit the E2 enzyme, while the N-terminus binds
 79 specific substrate receptors via DNA-damage-binding protein 1 (DDB1). Previous research identified
 80 DDB1- and CUL4A/B-associated factors (DCAFs), which mediate substrate protein recognition and its
 81 recruitment^{15,16}. To determine the substrate receptors of CUL4A/B complexes that are involved in the
 82 regulation of MCMBP, we conducted a siRNA screen targeting DCAFs substrate receptors. In this
 83 screen, CDT2 (DCAF2) and AMBRA1 (DCAF3) receptors served as positive controls. Aligned with the
 84 previous observations^{13,14,17,18}, depletion of CDT2 and AMBRA1 resulted in higher stabilization of
 85 CDT1 and cyclin D1 proteins, respectively (Extended Data Fig. 1d-g). Notably, the depletion of
 86 DCAF12 and, to a lesser extent, CDT2 and AMBRA1 led to elevated MCMBP levels (Fig. 1c-d). To
 87 clarify whether this effect was directly caused by increased MCMBP stabilization due to impaired
 88 protein degradation or indirectly due to cell cycle disturbances or elevated transcriptional activity,
 89 we analyzed the cell cycle phases and relative mRNA level of MCMBP. Our findings indicate that
 90 CDT2 depletion led to a substantial accumulation of cells in the G2 phase (Fig. 1e). Such dramatic
 91 change in the cell cycle likely contributes to the elevated levels of MCMBP following CDT2 depletion,
 92 as MCMBP levels increase during the cell cycle (Fig. 1f) aligned with MCM biogenesis occurring during
 93 the S phase and reaching its maximum in the G2 phase^{2,19}. In contrast, no cell cycle changes were
 94 observed in AMBRA1 and DCAF12-depleted cells (Fig. 1e). Nevertheless, increased cyclin D1 levels
 95 upon AMBRA1 depletion could activate the E2F-dependent transcription program. Indeed, MCMBP
 96 gene contains a putative E2F1 transcription binding site²⁰, indicating that the increased MCMBP
 97 mRNA levels following AMBRA1 depletion are likely attributable to elevated protein synthesis rather
 98 than stabilization (Fig. 1g). Importantly, DCAF12 depletion did not show any impact on cell cycle or
 99 transcription activity (Fig. 1e, g), suggesting that CRL4^{DCAF12} acts as a specific regulator of MCMBP,
 100 through which it can potentially modulate the cellular MCM equilibrium.

101

102 CRL4^{DCAF12} specifically interacts with MCMBP through the C-terminal degron motif

103 Prior research demonstrated that DCAF12 recognizes and interacts with its protein substrates
 104 through their short peptide motifs, termed degrons, located at their C-terminus²¹. To investigate the

potential interaction between MCMBP and DCAF12, we first re-analyzed mass spectrometry (MS) data from our affinity purification of StreptII-Flag-tagged DCAF12²², which was followed by immunoprecipitation of HA-tagged CUL4A (Fig. 1h). To enrich for protein substrates specifically interacting with CRL4A^{DCAF12}, the resulting interactomes were plotted as the ratio of either of two control CRLs (CRL4A^{DCAF4} or CRL1^{FBXL6}) to CRL4A^{DCAF12} (Fig. 1i, j). This analysis pinpointed MCMBP as one of the top interacting partners of the fully assembled CRL4A^{DCAF12} complex (Fig. 1i, j). To further understand whether the association of MCMBP with CRL4A^{DCAF12} is mediated directly through DCAF12, we analyzed co-purified proteins using MS immediately after affinity purification with StreptII-Flag-tagged DCAF12. Similarly to the tandem approach, this analysis revealed MCMBP among prominent DCAF12 interacting partners localized in the nucleus (Extended Data Fig. 2a-f). Our extensive MS analysis demonstrated the association of MCMBP with DCAF12 within a fully assembled CRL4 complex. The interaction between MCMBP and DCAF12 was recapitulated by western-blotting-based immunoprecipitation, confirming the binding between DCAF12 and MCMBP is unique among different CRL1 and CRL4 adapters (Fig. 1k).

Next, we sought to explore whether the interaction between MCMBP and DCAF12 is directly mediated through the C-terminal degron motif. Recent studies identified that beyond the canonical twin-glutamic acid -EE* degron motif, CRL4^{DCAF12} can recognize protein substrates ending with alternative motifs, such as -EI*, -EM*, -ES*, and -EL* [refs.^{22,23}]. Indeed, the bioinformatic analysis revealed a putative -EL* degron motif in MCMBP protein conserved across higher eukaryotes (Extended Data Fig. 2g, h), the deletion of which abrogated the interaction between MCMBP and DCAF12, similarly as deletion of C-terminal -EL* degron in MOV10, a previously identified DCAF12 substrate (Fig. 1l)²². Besides DCAF12, the mammalian proteome contains two paralogs, DCAF12L1 and DCAF12L2, which share approximately sixty percent amino-acid sequence identity with canonical DCAF12 protein²⁴. Nevertheless, the immunoprecipitation of DCAF12 and its paralogs revealed the interaction only between canonical DCAF12 and MCMBP, suggesting DCAF12 paralogs are not functionally redundant with DCAF12 (Extended Data Fig. 2i). Based on these data, we conclude that CRL4^{DCAF12} selectively recognizes MCMBP as a C-terminal degron substrate.

132

133 MCMBP protein levels are tightly regulated by CRL4^{DCAF12}-mediated proteasomal degradation

Multiple siRNA screens (Figure 1) showed that DCAF12 depletion increased MCMBP protein levels. Similar results from pharmacological inhibition or CUL4A/B knockdown suggest that this effect is due to impaired CRL4-dependent protein degradation. This observation was reproduced with two independent siRNAs targeting different regions of the DCAF12 gene (Extended Data Fig. 3a-f) and

138 further corroborated in non-cancerous RPE1 cells (Extended Data Fig. 3g-j) as well as in various
 139 mouse tissues (Extended Data Fig. 3k-l). Moreover, the ablation of the DCAF12 gene by the CRISPR-
 140 Cas9 knockout approach led to the stabilization of endogenous MCMBP protein levels. This effect
 141 was reversed by the doxycycline-inducible re-expression of StrepII-Flag-tagged DCAF12 protein in
 142 DCAF12-knockout cells, as evidenced by QIBC (Fig. 2a-e). Additionally, a cycloheximide chase
 143 experiment demonstrated that MCMBP protein stability was greater in DCAF12-knockout cells, which
 144 was again reversible with exogenous DCAF12 re-expression (Fig. 2f, g). Notably, overexpression of
 145 wild-type DCAF12, greatly exceeding its endogenous levels, reduced MCMBP protein below its basal
 146 levels, indicating precise cellular regulation of DCAF12 to achieve optimal MCMBP levels. Consistent
 147 with this, QIBC analysis showed that the increased levels of MCMBP following DCAF12 depletion
 148 were predominantly localized in the nucleus and not in the cytoplasm (Fig. 2a-d, Extended Data Fig.
 149 3a-d, g-j), aligning with our previous findings that DCAF12 is predominantly a nuclear protein²².
 150 Moreover, cell-cycle-specific analysis revealed stabilization of MCMBP protein levels in late S/G2
 151 phases upon DCAF12 depletion, indicating specific degradation of MCMBP in these cell cycle phases
 152 (Fig. 2h-j). Finally, the QIBC analysis demonstrated that DCAF12-depleted cells in the presence of
 153 proteasome inhibitor MG132 showed a similar stabilization effect on nuclear MCMBP protein levels
 154 as in DCAF12-depleted cells without MG132 treatment (Fig. 2k, l). Altogether, we conclude that
 155 cellular levels of MCMBP protein are spatiotemporally regulated by CRL4^{DCAF12}-mediated proteasomal
 156 degradation.

157

158 **Epistatic relationship between CRL4^{DCAF12} and MCMBP in the MCM biogenesis pathway**

159 Recent studies have reported that MCMBP acts as the specific chaperone of nascent MCM helicases,
 160 and its cellular levels and activity are needed for optimal origin licensing^{2,3}. Indeed, reduced levels of
 161 MCMBP were shown to impair MCM loading on chromatin^{2,3}. Therefore, it is reasonable to anticipate
 162 that elevated MCMBP levels resulting from DCAF12 depletion would lead to increased origin
 163 licensing. However, the depletion of DCAF12 unexpectedly led to reduced chromatin binding of all
 164 MCM subunits (Extended Data Fig. 4a-l). This phenotype was further recapitulated with two
 165 independent siRNAs against the DCAF12 gene (Extended Data Fig. 4m-o) and was not caused by the
 166 cell cycle changes (Extended Data Fig. 5a, b). To confirm the potential role of DCAF12 in the stability
 167 and loading of nascent MCMs on chromatin, a dual HaloTag labeling protocol (Extended Data Fig. 5c)
 168 was employed to distinguish the chromatin binding of parental and nascent MCM complexes. While
 169 the loading of parental MCMs on chromatin was not altered by depletion of DCAF12 or MCMBP, the
 170 chromatin licensing of nascent MCMs was impaired in both DCAF12- and MCMBP-depleted cells (Fig.

3a-d). Notably, the simultaneous depletion of DCAF12 alongside MCMBP did not exacerbate the inability to load the optimal levels of nascent MCM complexes (Fig. 3e-g). These findings suggest that CUL4-DCAF12 is not responsible for the degradation of the freely available MCMBP protein. Instead, they imply a complex regulatory mechanism wherein DCAF12 modulates MCMBP chaperone activity during the biogenesis of nascent MCM complexes occurring from the S phase until the G2 phase^{2,19}. This is also supported by the S/G2-phases-specific regulation of MCMBP levels by DCAF12 (Fig. 2h-j). Collectively, these observations reinforce the notion that MCMBP is a specific chaperone for nascent MCM complexes and identify CRL4^{DCAF12} as a novel regulator of the MCM biogenesis pathway.

179

180 **DCAF12 depletion impairs origin licensing but not localization or total nuclear levels of MCMs**

Before delving into the role of CRL4^{DCAF12} in MCM2-7 production, we shortly summarize the key findings that lead us to propose a model for the MCM biogenesis pathway (Fig. 4a). The presented model is built on the previous experiments revealing that shortly after MCMBP depletion or mutating its nuclear localization signal (NLS), nascent MCM3-7 subunits are rapidly mislocalized in the cytoplasm, where they undergo proteasomal degradation². Compared to MCM3-7 subunits, MCM2 remains orphaned in the nucleus, and its levels do not change. These observations align with previous studies showing a strong association of MCMBP with MCM3-7 subunits, as opposed to a weak interaction with MCM2^{25,26}. Based on this, we postulate a model in which MCMBP facilitates the assembly of the nascent MCM3-7 subcomplex and contributes to its transport to the nucleus while the MCM2 subunit autonomously enters the nucleus. Once all subunits are in the nucleus, the entire MCM2-7 ring is assembled and can be loaded onto chromatin by licensing factors during the G1 phase (Fig. 4a).

To identify the specific step of the MCM biogenesis pathway regulated by CRL4^{DCAF12}, we examined the assembly of nascent MCM subcomplexes in the cytoplasm and their transport to the nucleus. Using a dual HaloTag labeling protocol (Extended Data Fig. 5c) and QIBC, we tracked the levels of parental and nascent MCM complexes across both cellular compartments. Consistent with our previous analysis of parental MCM loading on chromatin (Fig. 3a, b), we observed that neither nuclear nor cytoplasmic levels of parental MCMs were affected by DCAF12 or MCMBP depletion (Extended Data Fig. 5d-g). Notably, QIBC analysis revealed that MCMBP depletion resulted in reduced nuclear levels of nascent MCMs due to their mislocalization in the cytoplasm (Fig. 4b-f), where they ultimately undergo proteasomal degradation². In contrast, DCAF12 downregulation did not impact the overall levels of nascent MCMs in either the nucleus or the cytoplasm (Fig. 4b-f). In fact, no change in nuclear and cytoplasmic levels upon DCAF12 depletion was observed for all MCM subunits

204 (Extended Data Fig. 5h, i). These findings indicate that CRL4^{DCAF12} does not regulate the assembly of
205 the MCM3-7 subcomplex or its transport to the nucleus (steps i and ii in Fig. 4a) but is likely involved
206 in downstream steps of this pathway. The experiments in the following paragraphs indicate that this
207 is indeed the case.

208 To further reinforce our conclusions and eliminate the possibility that the observed phenotype of
209 MCM loading following DCAF12 depletion is an indirect consequence of prolonged exposure to siRNA
210 treatment, we seek to develop a cell system that enables the examination of the effect of DCAF12 on
211 MCM levels and their loading on chromatin within shorter time periods. Despite several unsuccessful
212 attempts to produce the functional auxin-inducible degron of DCAF12, we leveraged MCMBP-KO
213 cells generated in our earlier study². In this genetic background, we prepared two stable cell lines re-
214 expressing HA-tagged MCMBP wild-type and a mutant lacking the -EL* C-terminal degron motif
215 (designated as ΔC), essential for interaction with DCAF12, under the control of a doxycycline-
216 inducible promoter. Intriguingly, 24-hour doxycycline treatment of these cells showed that both wild-
217 type MCMBP and MCMBP-ΔC mutant equally restored nuclear and cytoplasmic levels of MCM4-Halo
218 (Fig. 4g, h). Nevertheless, only wild-type MCMBP, not the MCMBP-ΔC, rescued the chromatin loading
219 of MCM4-Halo (Fig. 4i, j). These observations with inducible systems align with our siRNA findings,
220 suggesting that CRL4^{DCAF12} is not involved in the assembly of the MCM3-7 subcomplex or its nuclear
221 transport, but it may regulate the MCMBP binding during the assembly of the nascent MCM2-7
222 complex (step iii in Fig. 4a).

223

224 CRL4^{DCAF12} regulates the removal of MCMBP during the assembly of nascent MCM2-7 complexes

225 As shown previously, MCMBP functions as a dedicated chaperone for the nascent MCM helicases,
226 but it is not part of pre-replicative complexes loaded on chromatin^{2,3}. This underscores the necessity
227 to tightly regulate the interaction of MCMBP with MCMs. If the removal of MCMBP is either impaired
228 or delayed, it may hinder the assembly of the complete MCM2-7 ring. Consequently, this would lead
229 to a reduced MCM2-7 loading on chromatin, while the localization and total nuclear levels of MCM2-
230 7 would remain unaffected, as indicated by the experiments in Figures 3 and 4. Based on this, we
231 hypothesized that CRL4^{DCAF12} may regulate the interaction of MCMBP with MCMs during the
232 assembly of a complete MCM2-7 ring (step iii in Fig. 4a). To test this, we monitored the interaction
233 between MCMBP and MCM subcomplexes in control and DCAF12-depleted cells using
234 immunoprecipitation. We considered two potential scenarios: (1) MCMBP and MCM2 are mutually
235 exclusive, implying that MCMBP needs to be removed in order to incorporate MCM2 into the MCM3-
236 7 subcomplex (2) MCMBP and MCM2 are not mutually exclusive, meaning that they occupy different

237 structural surfaces in the MCM3-7 subcomplex. However, to achieve the complete formation of the
 238 MCM2-7 complex that can be loaded on chromatin, MCMBP needs to be removed from the MCM2-7
 239 structure (Fig. 5a). If the first scenario holds true, then DCAF12-depleted cells should manifest
 240 enhanced interaction between MCMBP and the MCM3-7 subcomplex and diminished interaction
 241 between MCM2 and MCM3-7. If the second scenario is valid, we should observe an increased
 242 interaction between MCMBP and MCM3-7 and an unchanged interaction between MCM2 and
 243 MCM3-7. The immunoprecipitation of MCM4-Halo did indeed show an increased association of
 244 MCMBP with MCMs upon DCAF12 depletion (Fig. 5b, c). Nevertheless, the interaction with MCM2
 245 remained unchanged even when using a higher salt concentration in the immunoprecipitation
 246 reaction (Fig. 5b-e). To further confirm that DCAF12 recognizes MCMBP when it is part of the MCM2-
 247 7 complex, we analyzed the DCAF12 interactome under normal conditions and after MG132
 248 treatment (Fig. 5f; Extended Data Fig. 6a). Our analysis revealed that DCAF12 interacts with the
 249 MCMBP-MCM2-7 complex, with MCM2 interaction becoming more prominent upon MG132
 250 treatment. These findings indicate that MCMBP and MCM2 lack mutually exclusive binding surfaces
 251 within the MCM structure and suggest that CRL4^{DCAF12} may facilitate the removal of MCMBP from the
 252 MCM2-7 complex.

253 Throughout our study, we demonstrated that stabilization of MCMBP upon DCAF12 depletion is
 254 confined to the cell nucleus and not the cytoplasm. This suggests that CRL4^{DCAF12} regulates MCMBP in
 255 the context of the MCM2-7 complex within the nucleus, which is consistent with previous findings
 256 identifying DCAF12 as a nuclear protein²². To further support our observations, we utilized our
 257 previously published cell systems HCT116 expressing DCAF12 variants, including wild-type and
 258 mutants lacking one or two NLS motifs (Fig. 5g; Extended Data Fig. 6b)²². Although the mutants
 259 lacking NLS were still detected in the nuclear fraction, presumably due to the involvement of other
 260 proteins in DCAF12 nuclear transport, we observed an approximately threefold increase in
 261 cytoplasmic signal of DCAF12 mutants in comparison to wild type (Fig. 5h; Extended Data Fig. 6c). To
 262 control functionality of this system, we used GART protein that has previously been shown to be
 263 exclusively located in the cytoplasm and its C-terminal degron motif is recognized by DCAF12 when it
 264 is forced into the cytoplasm (Extended Data Fig. 6b)²². In this experimental system, we sought to
 265 investigate whether the forced mislocalization of DCAF12 in the cytoplasm would also lead to the
 266 degradation of MCMBP in this cellular compartment. QIBC analysis revealed that DCAF12 does not
 267 degrade MCMBP in the cytoplasm but targets it for degradation exclusively in the nucleus (Fig. 5i;
 268 Extended Data Fig. 6d). This suggests that during the assembly and transport of MCM3-7
 269 subcomplexes, the acidic tail of MCMBP may be shielded from DCAF12. Analogous to the behavior of
 270 the acidic tail of CCT5, a component of the chaperonin complex under the regulation of DCAF12, the

271 acid tail of MCMBP may be concealed within the MCM3-7 structure²⁷. Once in the nucleus,
272 conformational changes during the final MCM2-7 complex assembly might release the acidic tail,
273 allowing it to be recognized by DCAF12. Such regulatory mechanism, facilitated by CRL4^{DCAF12}, further
274 refines the chaperone function of MCMBP while concurrently introducing an additional quality
275 control layer into the process of assembling nascent MCM complexes.

276

277 **DCAF12 is an important regulator of optimal MCM equilibrium**

278 Using various cellular systems and approaches to manipulate DCAF12 protein in this study, we noted
279 that tight cellular regulation of DCAF12 is essential to achieve optimal MCMBP levels and, thus,
280 proper MCM equilibrium. We showed that the downregulation of DCAF12 leads to impaired removal
281 of MCMBP during the nascent MCM2-7 complex assembly (Fig. 5a-i), which phenotypically appears
282 as increased MCMBP levels and reduced origin licensing (Extended Data Fig. 3a, b; Extended Data Fig.
283 4a-c). Nevertheless, the up-regulation of DCAF12 levels also poses a threat to optimal MCMBP levels.
284 Indeed, overexpression of DCAF12, approximately tenfold above its endogenous levels, led to
285 substantial degradation of MCMBP below its basal levels (Fig. 2a, b, e). This, in turn, caused the
286 massive degradation of MCM complexes in an MCMBP-dependent manner (Extended Data Fig. 6e-h),
287 which, as shown previously, results in impaired origin licensing². Interestingly, previous proteomic
288 datasets showed that CUL4A/B are highly abundant proteins in the cellular environment, reaching a
289 similar copy number as MCM proteins²⁸. However, the cellular level of DCAF12 protein is about a
290 hundred times lower than the amount of CUL4A/B. Our findings demonstrated that too little or too
291 high cellular levels of DCAF12 are detrimental to origin licensing, explaining the necessity to tightly
292 regulate DCAF12 protein level, reminiscent of the well-known Goldilocks principle, to ensure its fine
293 specificity under physiological conditions. Based on this, we conclude that DCAF12 is a critical
294 regulator of the MCM biogenesis pathway in mammalian cell systems.

295

296 **Misregulation of the MCM biogenesis pathway leads to genome instability**

297 Prior research demonstrated that even minor imbalances in MCM equilibrium can lead to genome
298 instability and severe diseases, including cancer^{2,5-9}. Although nascent MCMs remain largely in the
299 inactive chromatin-bound state during the DNA replication program, they serve as natural replisome
300 pausing sites, helping to coordinate replisome movement through chromatin^{2,29,30}. These pausing
301 sites are part of the multilayer replication fork speed control, which recently emerged as a pivotal
302 genome surveillance mechanism protecting cells against replication stress and genome instability

303 integrity³¹⁻³⁴. Notably, reducing natural replisome pausing sites by direct depletion of nascent MCMs
 304 or their chaperone MCMBP led to accelerated replication fork speed and generation of DNA damage
 305 without a major impact on CMG formation and the origin firing program². In light of these findings,
 306 we sought to explore whether impaired origin licensing caused by the downregulation of DCAF12
 307 leads to similar hallmarks of replication stress. Using the DNA fiber method, we observed that the
 308 depletion of DCAF12 resulted in increased replication fork speed without alteration of chromatin-
 309 bound CDC45 or PCNA (Fig. 6a; Extended Data Fig. 7a-e). Similarly to MCMBP depletion, unrestrained
 310 fast fork progression due to DCAF12 depletion led to an elevated incidence of fork asymmetry,
 311 pointing toward a higher frequency of replication fork pausing or stalling events (Fig. 6b).
 312 Importantly, neither the replication fork speed nor the fork asymmetry phenotypes were
 313 exacerbated when DCAF12 and MCMBP were co-depleted, supporting the notion that DCAF12 and
 314 MCMBP operate within the same signaling pathway (Fig. 6a, b).

315 As shown previously, loss of replication fork speed control can lead to replication-born DNA
 316 damage^{2,31,32}. Our QIBC analysis revealed an elevated level of γH2AX during the S phase, alongside an
 317 increased number of spontaneous RAD51 foci, well-established markers of DNA damage (Fig. 6c, d;
 318 Extended Data Fig. 7f-j). Notably, the generated DNA damage can be mitigated by re-expression of
 319 MCMBP wild type, but not MCMBP-ΔC mutant, which lacks the DCAF12 binding site and therefore
 320 cannot restore origin licensing in MCMBP-KO cells (Fig. 4i, j; 6e). Previous studies have demonstrated
 321 that increased replication stress can lead to genome instability^{2,31-34}. This phenomenon is also
 322 observed in DCAF12-depleted cells, as indicated by abnormalities in cell division and elevated levels
 323 of micronuclei formation (Fig. 6f, Extended Data Fig. 7k). Ultimately, the accumulation of genome
 324 instability over multiple generations of dividing cells may account for the reduced colony formation
 325 observed in DCAF12 and MCMBP-depleted cells (Fig. 6g). Collectively, our findings suggest that
 326 precise regulation of MCMBP chaperone function through CRL4^{DCAF12} is important to sustain optimal
 327 levels of inactive nascent MCMs on chromatin, essential to preserve physiological replication fork
 328 speed and genome stability.

329 To investigate the potential tumor-suppressor role of DCAF12, we compared survival rates of *Trp53*-
 330 deficient mice and double knockout mice lacking both *Trp53* and *Dcaf12* (Fig. 6h). Typically, *Trp53*
 331 knockout mice survive for only 25 to 30 weeks, with most succumbing to aggressive T-cell
 332 lymphomas, which are characteristic of this cancer-prone genotype (Fig. 6h). In contrast, mice
 333 deficient in *Dcaf12* demonstrated a significantly shorter survival duration, suggesting an accelerated
 334 onset of tumor development (Fig. 6h). This earlier manifestation of tumors in *Dcaf12* knockout mice
 335 is consistent with an increased mutational burden and impaired genomic stability due to the

336 concurrent loss of both *Trp53* and *Dcaf12* genes. These results provide compelling evidence that
337 DCAF12 acts as a tumor suppressor, further underscoring its critical role in cancer progression.

338

339 **DISCUSSION:**

340 The precise maintenance of cellular MCM equilibrium is crucial to ensure error-free genome
341 duplication. Our current work provides new insights into the molecular mechanism of the MCM
342 biogenesis pathway, essential for optimal loading of nascent MCM on chromatin, thereby mitigating
343 replication stress and genome instability (Fig. 6i). Building on our current and previous findings², we
344 propose a model in which MCMBP, a dedicated chaperone for nascent MCM complexes, fosters the
345 assembly of nascent MCM3-7 subcomplexes in the cytoplasm and facilitates their translocation to
346 the cell nucleus. In contrast to the MCM3-7 subcomplex, the cellular level and nuclear transport of
347 the MCM2 subunit are independent of MCMBP. Although the reason for the separation of the MCM
348 complex during nuclear transport is not yet clear, a recent study suggested that certain protein
349 cargos can enhance the mechano-selectivity of the nuclear pore complex and facilitate nuclear
350 import rates³⁵. Further elucidation will be needed to determine if MCMBP may potentially possess
351 analogous attributes.

352 After the MCMBP delivers MCM3-7 subcomplexes into the nucleus, the MCM2 subunit needs to be
353 integrated into the MCM complex structure, while the MCMBP must be removed as it is not part of
354 pre-replicative complexes loaded on chromatin^{2,3}. This highlights the need to tightly regulate the
355 interaction between the MCMBP and MCMs. In this work, we have uncovered that CRL4^{DCAF12} is a
356 bona fide regulator of this interaction (Fig. 6i). Our findings suggest that CRL4^{DCAF12} recognizes and
357 interacts with the C-terminal degron motif of MCMBP, resulting in the proteasomal degradation of
358 MCMBP. Specifically, we have demonstrated that CRL4^{DCAF12} facilitates the removal of MCMBP from
359 the MCM2-7 complex in the nucleus, representing an important step in the assembly of a complete
360 MCM2-7 ring that can be subsequently loaded onto chromatin during origin licensing. Notable is our
361 observation that the activity of DCAF12 does not impede the assembly and transportation of MCM3-
362 7 subcomplexes, even when DCAF12 is artificially delocalized to the cytoplasm (Fig. 5g-i; Extended
363 Data Fig. 6b-d). This suggests that during these steps, the acidic tail of MCMBP may be shielded from
364 DCAF12, similar to the protective mechanism described previously for the acidic tail of CCT5, a
365 component of the chaperonin complex regulated by DCAF12²⁷. Upon delivery of the MCMBP-MCM3-
366 7 complex to the nucleus, it is feasible that engagement of the MCM2 subunit with the complex can
367 induce a conformational alteration in the MCMBP binding. This, in turn, may facilitate the release of
368 the acidic tail, enabling its recognition by DCAF12. However, further structural studies are necessary

369 to gain detailed insights into the assembly of the MCM2-7 complex, particularly in terms of the
 370 molecular interactions between MCMBP and this complex. Current experimental studies diverge in
 371 this regard, with one study reporting a direct interaction between MCMBP and MCM3 and another
 372 reporting a direct interaction between MCMBP and MCM7^{3,26}. In addition to structural insights,
 373 exploring the evolutionary perspective on MCMBP function could be another exciting area for future
 374 research. MCMBP chaperone function during assembly of MCM complexes appears to be conserved
 375 from fission yeast to higher eukaryotes³⁶, prompting the need to investigate why MCMBP is absent in
 376 budding yeast. Notably, certain species, such as *S. pombe* or *A. thaliana*, possess MCMBP and CUL4
 377 but lack DCAF12 (Extended Data Fig. 2h). Our experiments measuring the stability of MCMBP in
 378 DCAF12 knockout cells indicate a certain degree of adaptability in this process (Fig. 2f, g), suggesting
 379 that, in the absence of DCAF12, alternative biochemical pathways can govern this process. Exploring
 380 how the interaction between MCMBP and MCM complexes is regulated in different species
 381 represents an intriguing avenue for future research.

382 Previous research has underscored the crucial roles of various cullin E3 ubiquitin ligases in regulating
 383 genome duplication. For instance, CRL4^{CDT2} plays a key role in degrading CDT1 after MCM loading on
 384 chromatin is completed, preventing re-replication and genome instability^{11,17,18}. Furthermore,
 385 CUL2^{LRR1} has been identified as a driver of replisome disassembly during replication termination^{37,38}.
 386 Our work contributes to this exciting area by understanding the role of CRL4^{DCAF12} in regulating the
 387 MCMBP chaperone function during the assembly of nascent MCM2-7 complexes. Additionally, this
 388 tight regulation of MCMBP interaction introduces an additional quality control layer in the
 389 maintenance of cellular MCM equilibrium important to maintain genome stability. We envisage that
 390 future studies exploring the regulatory pathways governing replication protein homeostasis may
 391 yield valuable insights into error-free genome duplication and bring new cancer treatment strategies.
 392 Given the frequent overproduction of MCMs in various cancer types³⁹, along with supporting
 393 evidence for DCAF12 tumor suppressor activity^{40,41}, the strategic targeting of MCM equilibrium holds
 394 promise for cancer therapy. We hypothesize that pharmacological inhibition of the MCM biogenesis
 395 pathway may impair origin licensing, leading to increased replication stress attributable to
 396 accelerated fork progression. While normal cells can activate the licensing checkpoint and
 397 temporarily arrest in the G1 phase, cancer cells replicate their DNA with reduced replication origins
 398 and accumulate DNA damage¹. Consequently, the amplified burden of DNA damage in cancer cells
 399 may render them more susceptible to currently available treatments.

400

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420

421 **AUTHOR CONTRIBUTIONS:**

422 H.P.-S. and L.C. conceived the project and wrote the manuscript. H.P.-S. lab contribution: A.K.Y.
423 generated cell lines, performed the majority of QIBC, immunoprecipitation, analyzed data, and
424 contributed to experimental design and concept development. S.N. performed colony formation
425 assay and contributed to cell line generation. J.K. contributed to cell line generation. H.P.-S. devised
426 the MCM biogenesis concept, designed and performed QIBC, DNA fibers, and genome instability
427 experiments, analyzed data, prepared figures, wrote an original draft, and reviewed and edited the
428 manuscript. L.C. lab contribution: L.C.: Conceptualization; Investigation; Methodology; Writing—
429 original draft; Writing—review and editing; Supervision; Funding acquisition. A.A.: Investigation;
430 Methodology. K.O.: Investigation; Methodology; Visualisation. K.K.: Investigation; Methodology;
431 Visualisation. N.D.: Investigation; Methodology. All authors read and commented on the manuscript.

432

433 **COMPETING INTERESTS:**

434 The authors declare no competing interests.

435

436 **FIGURE LEGENDS:**

437 **Fig.1: CRL4^{DCAF12} regulates MCMBP cellular level through direct interaction with its C-terminal**
438 **degron motif. a,** QIBC plots of MCM4-Halo cells immunostained for MCMBP after treatment with
439 siRNAs against cullins (as indicated). Lines denote medians; n ≈ 5,000 cells per condition. **b,**
440 Quantification of QIBC plots in **a**; each bar indicates the median of mean intensity (data are mean ±
441 s.d.; n = 3 technical replicates). **c,** QIBC plots of MCM4-halo cells immunostained for MCMBP after
442 treatment with siRNAs against the DCAF family of adaptors (as indicated). Lines denote medians; n ≈
443 5,000 cells per condition. **d,** Quantification of QIBC plots in **c**; each bar indicates the median of mean
444 intensity (data are mean ± s.d.; n = 3 technical replicates). **e,** Percentage distribution of individual cell
445 cycle phases of MCM4-Halo cells after treatment with control siRNA or siRNAs against *CDT1*,
446 *AMBRA1*, or *DCAF12*. Individual phases were defined according to the DAPI profile based on sub-
447 stratification shown in [Extended Data Fig. 1h](#). **f,** QIBC plots showing the percentage distribution of
448 MCMBP in indicated cell cycle phases. Lines denote medians; n ≈ 6,800 cells divided into G1, S, and
449 G2 phases. **g,** qPCR-based analysis of MCMBP mRNA levels after indicated siRNA treatments;
450 normalized with respect to siCTRL treatment as 1 (n = 3 technical replicates). **h,** Schematic
451 representation of the tandem affinity purification strategy. **i-j,** CRL4^{DCAF12}-associated proteins
452 identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The composition of
453 purified complexes from both steps was analyzed by LC-MS/MS. Non-related ubiquitin ligases (**i**)
454 CRL4^{DCAF4} or (**j**) SCF^{FBXL6} were used as a control. Proteins that were significantly enriched with
455 CRL4^{DCAF12} compared to controls are in the upper right quadrants. **k,** Affinity purification (AP) of a
456 panel of cullin-RING ubiquitin ligases (CRLs). HEK293T cells were transfected with an empty vector
457 (EV) or the indicated StrepII-FLAG-tagged CRL constructs and treated with MLN4924 for 6 hours
458 before harvesting. Forty-eight hours after transfection, cells were harvested and lysed, and whole-
459 cell lysates (WCL) were subjected to AP with Strep-TactinXT resin. Eluates were immunoblotted with
460 indicated antibodies. **l,** Affinity purification of N-terminal STREP-FLAG-tagged DCAF12 and
461 immunopurification of HA-tagged MCMBP, MOV10, and C-terminal degron-lacking mutants.
462 HEK293T cells were co-transfected with StrepII-FLAG-tagged DCAF12 and either an empty vector (EV)
463 or constructs expressing HA-tagged MOV10, MOV10ΔC, MCMBP, or MCMBPΔC. MLN4924 was added
464 for the final 6 hours before harvest. Following lysis, whole-cell lysates (WCL) were subjected to
465 affinity purification (AP) with Strep-TactinXT resin or anti-HA immunoprecipitation (IP) and
466 immunoblotted as indicated. The left panel shows 1% input samples, the upper right panel shows
467 Strep-Tactin AP, and the lower right panel shows anti-HA IP. P values were calculated by ordinary
468 one-way ANOVA with Dunnett's test (**b, d**); ****P<0.0001, **P<0.01, *P<0.1, n.s. (not significant)
469 indicates P>0.1. (A.U. Arbitrary Units).

470

471 **Fig. 2: MCMBP protein levels are spatiotemporally regulated by CRL4^{DCAF12}-mediated proteasomal**
472 **degradation. a,** QIBC plots of naïve U2OS cells and DCAF12 knock-out U2OS cells (DCAF12-KO#1,
473 DCAF12-KO#2) expressing doxycycline (DOX, 48 hours) inducible Strep-DCAF12 (where indicated)
474 immunostained for MCMBP. Lines denote medians; n ≈ 9,200 cells per condition. **b,** Quantification of

QIBC plots in **a**; each bar indicates the median of mean intensity (data are mean $\pm$ s.d.; $n = 2$ technical replicates). **c**, Mean fluorescent intensity (MFI) of cytoplasmic MCMBP under similar conditions as in **a**. Central lines of box plots denote medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate 5 and 95 percentile values; $n = 200$ cells per condition. **d**, Quantification of the MFI of cytoplasmic MCMBP in **c**; each bar indicates the median of mean intensity normalized with respect to naïve U2OS as 100 percent (data are mean $\pm$ s.d.; $n = 2$ technical replicates). **e**, RT-PCR-based analysis of MCMBP mRNA levels in naïve U2OS cells and DCAF12 knock-out U2OS cells (DCAF12-KO#1, DCAF12-KO#2) expressing DOX inducible Strep-DCAF12 (where indicated); normalized with respect to naïve U2OS cells. $n = 3$ technical replicates. **f**, StrepII-FLAG-tagged DCAF12 (SF-DCAF12) was inducible expressed in DCAF12 WT and KO U2OS cells using the Sleeping Beauty Transposon System. Where indicated, the expression of DCAF12 was induced with doxycycline for 24 h. Cells were treated with cycloheximide (CHX) for the indicated time. Whole-cell lysates were immunoblotted as indicated. **g**, Densitometry of selected protein staining in **f**; **h**, U2OS DCAF12 WT or KO cells were synchronized in G1/S by a hydroxyurea treatment (2 mM; 16 hours) and then released in fresh medium for the indicated hours. Cells were collected at the indicated time and processed for western blot. Whole-cell lysates were immunoblotted as indicated. **i**, Densitometry of MCMBP staining in **h**; **j**, Densitometry of MCM6 protein staining in **h**. **k**, QIBC plots of MCM4-Halo U2OS cells treated with MG123 inhibitor and control siRNA (siCTRL) or siRNA against DCAF12 (siDCAF12). Lines denote medians; $n \approx 5,000$ cells per condition. **l**, Quantification of QIBC plots in **k**; each bar indicates the median of mean intensity normalized with respect to siCTRL as 100 percent (data are mean $\pm$ s.d.; $n = 3$ technical replicates). P values were calculated by ordinary one-way ANOVA with Tukey's test (**b**, **d**) or Dunnett's test (**l**); *** $P < 0.001$, ** $P < 0.01$, * $P < 0.1$, n.s. (not significant) indicates $P > 0.1$. (A.U. Arbitrary Units).

498

Fig. 3: CRL4^{DCAF12} is a novel regulator of the MCM biogenesis pathway. **a**, **c**, QIBC plots of pre-extracted MCM4-Halo U2OS cells stained for parental (**a**) and nascent (**c**) MCM4 after indicated siRNA treatments. DAPI counterstains nuclear DNA; $n \approx 4,000$ cells per condition. **b**, **d**, MFI of parental and nascent MCM4 in G1/S cells based on QIBC in **a** and **c**, respectively. Lines denote medians; $n \approx 2,000$ cells per condition. **e**, **f**, Quantification of the MFI of parental and nascent MCM4 (in G1/S) in **b** and **d**, respectively. Each bar indicates the median of mean intensity normalized with respect to siCTRL as 100 percent (data are mean $\pm$ s.d.; $n = 3$ biological replicates). **g**, Quantification of chromatin-bound levels of parental and nascent MCMs in G1/S phase MCM4-Halo cells after indicated siRNA treatments (data are mean $\pm$ s.d.; $n = 3$ biological replicates). P values were calculated by ordinary one-way ANOVA with Šidák's test (**e**, **f**), **** $P < 0.0001$, n.s. (not significant) indicates $P > 0.1$. (A.U. Arbitrary Units).

510

Fig. 4: The assembly of the nascent MCM3-7 subcomplex or its transport to the nucleus is not affected by DCAF12 depletion. **a**, Proposed model of MCM biogenesis pathway (see text for details). **b**, QIBC plots of MCM4-Halo U2OS cells stained for nascent MCM4 after indicated siRNA treatments. Lines denote medians; $n \approx 3,400$ cells per condition. **c**, Quantification of QIBC plots in **b**; each bar indicates the median of mean intensity (data are mean $\pm$ s.d.; $n = 3$ biological replicates). **d**, MFI of cytoplasmic nascent MCM4 after indicated siRNA treatments. Central lines in box plots denote

517 medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate 5 and 95 percentile
518 values; n = 500 cells per condition. **e**, Quantification of MFI of cytoplasmic nascent MCM4; each bar
519 indicates the median of mean intensity (data are mean $\pm$ s.d.; n = 2 biological replicates). **f**,
520 Representative images of immunostained MCM4 after indicated siRNA treatments (without pre-
521 extraction); the pseudo-color gradient indicates the MFI. Scale bar, 20 μ m. **g**, QIBC of MCMBP knock-
522 out (MCM4-Halo) U2OS cells stably expressing wild-type MCMBP or C-terminal deleted MCMBP
523 (MCMBP- Δ C) stained for MCM4. Lines denote medians; n $\approx$ 5,000 cells per condition. **h**, MFI of
524 cytoplasmic MCM4 under similar conditions as in **g**; Central lines in box plots are medians, the boxes
525 indicate the 25th and 75th centiles, the whiskers indicate 5 and 95 percentile values; n $\approx$ 200 cells per
526 condition. **i**, QIBC plots of pre-extracted MCMBP-KO, MCMBP-wt (MCMBP-KO), MCMBP- Δ C
527 (MCMBP-KO) stained for MCM4. DAPI counterstained nuclear DNA; n $\approx$ 3,000 cells per condition. **j**,
528 Quantification of MFI of MCM4 for G1/S cells from QIBC in **i**; each bar indicates the median of mean
529 intensity normalized with respect to MCMBP-KO as 100 percent; data are mean $\pm$ s.d.; n = 2 technical
530 replicates. P values were calculated by ordinary one-way ANOVA with Tukey's test (**c**, **j**) or Šidák's
531 test (**e**), ***P<0.001, **P<0.01, *P<0.1, n.s. (not significant) indicates P>0.1. (A.U. Arbitrary Units).

532

533 **Fig. 5: CRL4^{DCAF12} regulates the interaction between MCMBP and nascent MCM complexes.** **a**, A
534 schematic describing the experimental approach for understanding the role of CUL4-DCAF12 in
535 MCM2-7 assembly (see text for details). **b**, Halo-immunoprecipitation (Halo IP) of whole cell lysates
536 followed by western blotting, cells were treated with siRNA against DCAF12 (siDCAF12) as indicated.
537 This IP was done at low salt concentration (150 mM NaCl) **c**, Quantification of Halo-IP products based
538 on western blots in **b**; IP products upon control siRNA treatments have been considered as 100%
539 (data are mean $\pm$ s.d.; n = 2 biological replicates). **d**, Halo IP of whole cell lysates followed by western
540 blotting, cells were treated with siRNA against DCAF12 (siDCAF12) as indicated. This IP was done at a
541 high salt concentration (500 mM NaCl). **e**, Quantification of Halo-IP products based on western blots
542 in **d**; IP products upon control siRNA treatments have been considered as 100% (data mean $\pm$ s.d.; n =
543 2 biological replicates). **e**, Heatmap, derived from mass spectrometry analysis of HEK293T cells co-
544 transfected with StreptII-FLAG-tagged DCAF12, shows log2 intensities of indicated proteins. Cells were
545 treated with either DMSO (CTRL) or 10 μ M MG132 for 6 hours prior to protein purification. **g**,
546 Schematic representation of human DCAF12 and its N-terminally truncated mutants. Two predicted
547 nuclear localization signals (NLS) and H-box are highlighted. **h**, **i**, QIBC plots of the cytoplasm of U2OS
548 cells stained for StreptII-tagged DCAF12 (**h**) or MCMBP (**i**) protein after indicated DCAF12 constructs
549 were inducibly expressed. Lines denote medians; n $\approx$ 1,000 cells per condition.

550

551 **Fig. 6: Shortage of nascent MCM complexes on chromatin leads to fast fork progression and**
552 **genome instability.** **a**, Left, labeling protocol for DNA fiber experiments and a panel of representative
553 images of ongoing replication forks per conditions as indicated. Right, replication fork speed in U2OS
554 cells treated with indicated siRNAs. Lines represent medians; n = 500 fibers per condition. **b**, Left,
555 representative images of bidirectional replication forks; right, quantification of sister fork ratio in
556 U2OS cells following indicated siRNA treatments. In box plots, central lines denote medians, the
557 boxes indicate the 25th and 75th centiles, and the whiskers indicate Tukey values; n = 50 fibers per
558 condition. **c**, Quantification of the level of γ H2AX in the nuclei of S phase cells after indicated siRNA

559 treatments. Each bar indicates the median of mean intensity normalized with respect to siCTRL as
560 100 percent (data are mean $\pm$ s.d.; n = 3 biological replicates). See QIBC in [Extended Data Fig. 7f. d](#),
561 Quantification of number of RAD51 foci per cell nucleus after indicated siRNA treatments. Each bar
562 indicates the median of the mean foci count (data are mean $\pm$ s.d.; n = 3 biological replicates). See
563 QIBC in [Extended Data Fig. 7j. e](#), Left, QIBC plots of MCMBP-KO, MCMBP-wt (in MCMBP-KO),
564 MCMBP- Δ C (in MCMBP-KO) stained for γ H2AX. DAPI counterstains nuclear DNA; n $\approx$ 5,000 cells per
565 condition. Right, MFI of γ H2AX in S phase cells based on QIBC on the left. Lines denote median, n $\approx$
566 5,000 cells per condition. **f**, Representative images (left), quantification (right) of micronucleation in
567 U2OS cells after indicated siRNA treatments (n $\approx$ 500 cells per condition) (data are mean $\pm$ s.d.; n = 3
568 biological replicates). Scale bar, 20 μ m. **g**, Clonogenic survival of U2OS cells after 10 days of siRNA
569 treatments, as indicated. Left, representative images; right, quantification. Each bar indicates the
570 average of observed colonies normalized with respect to control siRNA treatment as 100 percent; n =
571 4 biological replicates. **h**, Tumor-free survival in *Trp53* homozygous knockout mice group (TP53-KO)
572 and double knockout (TP53+DCAF12-KO) mice lacking both *Trp53* and *Dcaf12* genes. P value was
573 calculated by a two-tailed unpaired t-test (P = 0.0283). **i**, A model depicting the critical role of the
574 MCM biogenesis pathway to support optimal levels of origin licensing. The assembly of nascent MCM
575 complexes is regulated by the MCM biogenesis pathway (i). Precise control of the MCM biogenesis
576 pathway ensures optimal levels of inactive nascent MCMs on chromatin (ii), which facilitates normal
577 replication fork speed and stable genomes. Conversely, inhibition of this pathway results in a
578 shortage of inactive nascent MCMs on chromatin, leading to accelerated fork progression and
579 genome instability (see text for details). P values were calculated by one-way ANOVA with Tukey's
580 test (**a**, **b**, **c**) or Šidák's test (**d**, **f**, **g**), ***P<0.0001, **P<0.01, *P<0.1, n.s. (not significant) indicates
581 P>0.1. (A.U. Arbitrary Units).

582

583 **METHODS:**

584 **Cell culture**

585 The human immortalized retinal epithelial cell line hTERT-RPE1 (ATCC, CRL-4000), osteosarcoma
586 cancer cell line U2OS (ATCC, HTB-96), and its derivatives were cultured under standard sterile
587 conditions at 37°C and 5% CO₂ level in Dulbecco's modified Eagle's medium (DMEM) containing high
588 glucose and GlutaMAX (Thermo Fischer Scientific, 31966047) supplemented with 10% Fetal Bovine
589 Serum (FBS; Thermo Fischer Scientific, A5256801) and 0.5% penicillin-streptomycin (Thermo Fischer
590 Scientific, 15140122). The human kidney cancer cell line HEK293T (ATCC, CRL-1573), colon cancer cell
591 line HCT116 (ATCC, CCL-247), and its derivatives were maintained in Dulbecco's Modified Eagle's
592 Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and penicillin (BB Pharma),
593 streptomycin (Merck), and gentamycin (Sandoz), and cultured in a humidified incubator at 37 °C with
594 5% CO₂. All cell lines and their derivatives were routinely tested for mycoplasma using Mycoplasma
595 Detection Kit (InvivoGen, rep-mys-50) and always found negative.

596

597 **Generation of endogenously tagged cell lines and knockouts using CRISPR-Cas9**

598 A derivative of the U2OS cell line expressing C-terminally endogenously Halo-tagged MCM4 and GFP-
599 tagged CDC45 was generated using CRISPR-Cas9 and validated in the previous study². A U2OS cell
600 line containing knockout of the MCMBP gene (U2OS MCMBP-KO cells) and C-terminal endogenous
601 Halo-tagged MCM4 was prepared and validated in the previous study². A U2OS cell line with a
602 knockout of the DCAF12 gene was generated using the CRISPR-Cas9 genome editing system. The
603 sgRNAs were designed to target introns flanking exon 5 of the DCAF12 gene. A mixture of lentiCRISPR
604 plasmids (pXPR001) encoding these sgRNAs and Cas9 was transfected into the cells. After a 48-hour
605 selection with puromycin (1 µg/ml), single-cell clones were selected, and the exon deletions were
606 confirmed by PCR.

607

608 **Generation of cell lines with ectopic protein expression**

609 The HEK293T cell line was transiently transfected with pCDNA3-based vectors expressing Strep II-
610 FLAG-tagged substrate receptors, HA-tagged MOV10, MCMBP, or their C-terminal deletion mutants.
611 The stable cell lines expressing Strep II-FLAG-tagged DCAF12 under the control of a doxycycline
612 (DOX)-inducible promoter were generated using the Sleeping Beauty system as described
613 previously²². Briefly, cells were transfected with a pSBtet vector containing DCAF12 cDNA or its
614 mutants lacking the NLS, along with the transposase-expressing pSB100X, using Lipofectamine 2000
615 (Invitrogen) according to the manufacturer's protocol. Positive clones were selected with puromycin
616 48 hours after transfection. Derivatives of HCT116 ectopically expressing StrepTactin-tagged DCAF12,
617 DCAF12(Δ1-11), and DCAF12(Δ1-38) were prepared and validated in the previous study²².

618 To generate stable cell lines expressing doxycycline-inducible variants of MCMBP, U2OS MCMBP-KO
619 cells containing endogenously Halo-tagged MCM4 were transfected with either HA-tagged MCMBP-
620 wt or MCMBP-ΔC constructs using Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific,
621 15338100). After 24 hours of post-transfection, the cells were selected with DMEM containing
622 puromycin (0.1 mg per ml, InvivoGen, ant-pr-1) for 3 days and then serially diluted onto 100 mm
623 dishes. The cells were grown under puromycin selection for 12 days. The isolated colonies were
624 individually expanded and treated with doxycycline (1 µg per ml, Merck, D3072) for 24 hours to
625 induce expression of MCMBP-wt or MCMBP-ΔC prior to QIBC-based analysis. Afterward, the cells
626 were pulsed with fluorescent HaloTag ligand as described in the section *HaloTag labeling protocol*
627 and the nuclear pool of labeled MCM4-Halo was analyzed by QIBC.

628

629 **Mouse lines**

630 DCAF12 knockout mice (Δ exon4) were generated in a C57BL/6N background using the CRISPR/Cas9
631 genome-editing system as described^{22,42}. Shortly, the Cas9 protein and gene-specific sgRNAs
632 (Integrated DNA Technologies) were used for zygote electroporation. The genome editing was
633 confirmed in the founder mouse using PCR. The mutant allele was subsequently backcrossed for two
634 generations to the C57BL/6N strain. To further investigate our hypothesis regarding the tumor-
635 suppressor role of DCAF12, we created a new DCAF12-deficient mouse model on the background of
636 tumor-prone TP53-deficient mice (Trp53^{tm1Tvj}, Jackson Laboratory, strain 002101). Trp53^{tm1Tvj} mice⁴³.
637 were obtained from the Jackson Laboratories (Bar Harbor, USA) and were backcrossed and
638 maintained on a C57BL/6N background.

639

640 **Chemical reagents, siRNAs, and antibodies**

641 Chemical reagents were used in this study as follows. Cycloheximide (Merck, C7698, 100 μ g/ml, 355
642 μ M), MLN4924 (Medchemexpress, HY-70062, 1 μ M), hydroxyurea (Merck, H8627-1G, 2mM, 16
643 hours), and doxycycline hyclate (Merck, D9891, 0.1 μ g/ml, 48 hours) were used as indicated in
644 figures and respective figure legends. For experiments with MG132, U2OS cells were treated with
645 MG132 (Merck, 474790-1MG) at a final concentration of 5 μ M for 6 hours. For cell cycle
646 synchronization, U2OS cells were incubated with 2 mM hydroxyurea (Sigma, H8627-1G) for 16 hours.

647 All siRNA transfections were performed using the Lipofectamine RNAiMAX Transfection Reagent
648 (Thermo Fisher Scientific, 13778075) according to the manufacturer's recommendation. Cells were
649 treated with siRNAs against CUL1, CUL2, CUL3, CUL4A, CUL4B, and CUL5 at a final concentration of
650 20 nM for 72 hours (Dharmacon/Horizon Discovery, L-004086-00-0005, L-007277-00-0005, L-010224-
651 00-0005, L-012610-00-0005, L-017965-00-0005, L-019553-00-0005). For remaining experiments,
652 siRNAs were procured from Ambion Silencer Select: DCAF1 (s18762), CDT2 (s226738), AMBRA1
653 (s31112), DCAF4 (s25085), DCAF6 (s31604), DCAF8 (s27089), DCAF10 (s35726), DCAF12#1 (s24628),
654 DCAF12#2 (s24627), DCAF14 (s30012), DCAF16 (s29650), and MCMBP (s36586). All transfections
655 were performed at a final concentration of 10 nM siRNA for 72 hours. Ambion negative control #1 (a
656 non-targeting siRNA) was used as the control siRNA.

657 Primary antibodies used for immunofluorescence (IF) were as follows: CDT1 (rabbit, Abcam,
658 ab202067, 1:2,000), Cyclin D1 (rabbit, Proteintech, 26939-1-AP, 1:1,000), GFP (rabbit, Proteintech,
659 PABG1, 1:5,000), MCMBP (rabbit, Novus Biologicals, NBP1-90746, 1:1,000), MCM2 (rabbit,
660 Proteintech, 10513-1-AP, 1:1,000), MCM3 (mouse, Santa Cruz, sc-390480, 1:1,000), MCM4 (rabbit,

661 Proteintech, 13043-1-AP, 1:1,000), MCM5 (rabbit, Proteintech, 11703-1-AP, 1:1,000), MCM6 (mouse,
662 Novus Biologicals, H00004175-M04, 1:1,000), MCM7 (mouse, Santa Cruz, sc-9966, 1:1,000), PCNA
663 (human, Immuno Concepts, 2037, 1:1,000), RAD51 (rabbit, BioAcademia, 70-012, 1:1,000), Strep II
664 Tag (mouse, Novus Biologicals, NBP2-43735, 1:1,000), γ H2AX (Ser139) (rabbit, Abcam, ab81299,
665 1:1,000).

666 Primary antibodies used for western blotting were as follows: α -tubulin (mouse, Proteintech, 66031-
667 1-Ig, 1:1,000), β -actin (mouse, Santa Cruz, sc-69879, 1:1,000), CCNA (home-made serum), CCNB1
668 (home-made serum and Santa Cruz Biotechnology, sc-245, 1:1,000), DDB1 (rabbit, Zymed, 34-2300,
669 1:1,000), GART (mouse, Santa Cruz, sc-166379, 1:1,000), γ H2AX (rabbit, Proteintech, 83307-2-RR,
670 1:1,000; mouse, Milipore, 05-636, 1:1,000), HA (rabbit, Cell Signaling, 3724, 1:1,000), MCMBP (rabbit,
671 Proteintech, 19573-1-AP, 1:1,000; rabbit, Atlas Antibodies, HPA038481, 1:1,000), MCM2 (rabbit,
672 ABClonal, A1056, 1:1,000), MCM3 (rabbit, ABClonal, A1060, 1:1,000), MCM4 (rabbit, Proteintech,
673 13043-1-AP, 1:1,000), MCM5 (rabbit, Proteintech, 11703-1-AP, 1:1,000), MCM6 (rabbit, Proteintech,
674 13347-2-AP, 1:1,000), MCM7 (mouse, Santa Cruz, sc-9966, rabbit, Proteintech, 11225-1-AP, 1:1,000),
675 p53 (mouse, Santa Cruz, sc-126, 1:1,000), PARP1 (mouse, Proteintech, 66520-1-Ig, 1:1,000), PCNA
676 (mouse, Santa Cruz, sc-56, 1:1,000), pH3 (Ser10) (rabbit, Cell Signaling, 53348, 1:1,000), RRM2
677 (mouse, Santa Cruz, sc-398294, 1:1,000), RPS3A (rabbit, ABClonal, A5885, 1:1,000), RPS6 (mouse,
678 Santa Cruz, sc-74459, 1:1,000), SKP1 (rabbit, Cell Signalling, 12248, 1:1,000).

679 Secondary antibody conjugates for IF were goat anti-rabbit Alexa Fluor 488 (A11034), goat anti-rabbit
680 and goat anti-mouse Alexa Fluor 568 (A11036, A11031), goat anti-rabbit and goat anti-mouse Alexa
681 Fluor 647 (A21245, A21236) (all from Thermo Fischer Scientific, 1:2,000); donkey anti-human Alexa
682 Fluor 647 (Jackson Immuno Research, 709-605-149, 1:2,000); goat anti-mouse (DyLight 488, Thermo
683 Fisher, 35503, 1:1,000); and donkey anti-rabbit IgG (Alexa Fluor® 555, Abcam, ab150070, 1:1,000).
684 Secondary antibody conjugates used for western blotting were HRP-linked horse anti-mouse IgG
685 (Vector Laboratories, PI-2000, 1:10,000), goat anti-rabbit IgG (Vector Laboratories, PI-1000,
686 1:10,000), horse anti-mouse IgG (Cell Signaling, 7076, 1:5,000), goat anti-rabbit IgG (Cell Signaling,
687 7074, 1:5,000) antibodies.

688

689 **HaloTag labeling protocol**

690 For single-fluorescence HaloTag labeling, the U2OS or MCMBP-KO cells with Halo-tagged MCM4 and
691 its derivatives were pulsed with JFX554-HaloTag ligand at a final labeling concentration of 50 nM for
692 30 minutes prior to fixation. For all dual HaloTag labeling, MCM4-Halo U2OS cells were incubated

693 with JF549 HaloTag ligand (50 nM) for 30 minutes, followed by three washes with phosphate-
694 buffered saline (PBS) buffer (Thermo Fisher Scientific, 14190250). The cells were then incubated with
695 complete DMEM medium for 1 hour, washed three times with PBS, and incubated in fresh DMEM
696 with JF646 HaloTag ligand (200 nM) for 24 hours. After HaloTag labeling, the cells were processed for
697 immunofluorescence staining.

698

699 **Immunofluorescence (IF) staining**

700 The cells were grown on round glass coverslips (12 mm diameter, #1.5, VWR, MENZCB00120RAC20).
701 For IF staining of chromatin-bound proteins, ice-cold cytoskeleton (CSK) buffer (10 mM Hepes pH 7.5;
702 300 mM sucrose; 100 mM NaCl; 3 mM MgCl₂ and 0.5% Triton X-100) was used to pre-extract the cells
703 for 10 minutes at room temperature. After CSK buffer incubation, coverslips were washed three
704 times with PBS buffer and fixed using 4% buffered formaldehyde (VWR, 9713.1000) for 20 minutes at
705 room temperature. For staining the total cellular pool of proteins, 4% buffered formaldehyde was
706 added to coverslips to fix the cells for 20 minutes at room temperature without pre-extraction. After
707 fixation, the cells were washed three times with PBS and permeabilized using ice-cold PBS containing
708 0.2% TritonX-100 for 5 minutes at room temperature. Primary and secondary antibodies were diluted
709 in fresh DMEM supplemented with 10% FBS. The coverslips were first incubated with the primary
710 antibody cocktail for 90 minutes at room temperature, washed two times with PBS, and then
711 incubated with the secondary antibody cocktail supplemented with 0.5 µg per ml 4',6-Diamidino-2-
712 Phenylindole, Dihydrochloride (DAPI; Thermo Fisher Scientific, D1306) for 45 minutes at room
713 temperature. After primary and secondary antibody staining, the coverslips were washed three times
714 with PBS and twice with distilled water, air dried, and mounted on grease-free slides using Mowiol-
715 based mounting medium (12% Mowiol 4-88 (Merck, 81381-250G), 30% glycerol, 0.12 M Tris-HCl pH
716 8.5).

717 For IF staining in [Fig. 5h, i](#) and [Extended Data Fig. 6c, d](#) samples were prepared as follows. A total of
718 10⁴ U2OS cells were plated in a 96-well plate (Corning; P96-1.5H-N). Following treatment, the cells
719 were washed with 1× PBS, fixed with 3.2% paraformaldehyde, permeabilized with 0.5% Triton X-100
720 in PBS for 10 minutes, and blocked in 3% BSA in PBS for 1 hour. The cells were then incubated with
721 the indicated primary antibodies for 2 hours, followed by the corresponding anti-mouse or anti-
722 rabbit secondary antibodies for 1 hour. Nuclei were visualized using DAPI (Merck, D9542-5mg,
723 1:1,000). The cells were mounted with AD-Mount F (ADVI, CZ).

724

725 Quantitative image-based cytometry

726 The images for experiments (Fig. 1a-d; 2a-d, k, l; 4g-j; Extended Data Fig. 1b-g; 2g-j; 6g,h) were
727 acquired using an inverted screening microscope ScanR (IX83, Evident), equipped with a UPLXAPO
728 dry objective (20X, 0.8 NA), fast excitation and emission filter wheel for DAPI, FITC, Cy3, and Cy5
729 wavelengths, Lumencor Spectra X led fluorescence light source, digital monochromatic sCMOS ORCA-
730 Flash 4.0 LT Plus camera, and ScanR acquisition software (Evident, v.3.4.1). The remaining
731 experiments were acquired using an inverted screening microscope ScanR (IX81, Evident), equipped
732 with UPLSAPO dry objective (20X, 0.75 NA), fast excitation and emission filter wheel for DAPI, FITC,
733 Cy3, and Cy5 wavelengths, MT20 illumination system, digital monochrome Hamamatsu ORCA-R2 CCD
734 camera, and ScanR acquisition software (Evident, v.2.7.1). During image acquisition, the laser
735 intensity was set at 100% and exposure times were adjusted individually for each fluorophore. Image
736 analysis was performed in ScanR analysis software (Evident, v.3.4.1). Automated dynamic
737 background correction was applied to all images for each fluorescent channel separately, maintaining
738 a threshold at a minimum of 5-fold pixel intensity above background levels for each channel. To
739 identify individual nuclei as main objects, an intensity-threshold-based mask was generated based on
740 the DAPI signal, which was then applied to analyze pixel intensities in different channels for each
741 nucleus. The multiparameter analysis was exported as a table and further analyzed in Spotfire
742 software (TIBCO, v.11.8.0). Within a single experiment, a similar number of cells were analyzed and
743 compared for each condition. The mean fluorescence intensity of cytoplasmic MCMBP or MCMs was
744 quantified using ImageJ software (Fiji, version 1.54i).

745

746 Immunoprecipitation (IP)

747 The cells were collected and lysed in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.4% Triton X-
748 100, 2 mM CaCl₂, 2 mM MgCl₂, 1 mM EDTA, 5 mM NaF, supplemented with phosphatase and
749 protease inhibitors) for 10 minutes on ice. For Strep-tagged constructs, the lysates were cleared by
750 centrifugation and incubated with Strep-Tactin beads (IBA Lifesciences; 2-1206-025) for 2 hours at
751 4°C. After incubation, the beads were washed three times with lysis buffer containing Benzonase
752 (Santa Cruz). Purified proteins were then eluted with Buffer E (IBA Lifesciences) for 5 minutes at
753 room temperature. The inputs (0.5%) and eluates were analyzed by SDS-PAGE and western blotting.
754 HA-tagged proteins were immunoprecipitated using anti-HA magnetic beads (Pierce™; Thermo Fisher
755 Scientific). Immunoprecipitated proteins were eluted by adding 1× Bolt LDS Sample Buffer (Thermo
756 Fisher Scientific) mixed with β-mercaptoethanol, followed by incubation at 95°C for 5 minutes. To
757 investigate the protein interactome of DCAF12, HEK293T cells were co-transfected with StrepII-FLAG-

758 tagged DCAF12 and cultured for 48 hours. After 48 hours, cells were treated with either DMSO or 10
759 μ M MG132 for 6 hours prior to harvesting. Collected cells were lysed as described above. Protein
760 complexes associated with DCAF12 were purified using Strep-TactinXT Superflow resin and eluted
761 with desthiobiotin. The eluates were analyzed by LC-MS/MS at the Proteomics facility of the
762 Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University (BIOCEV)
763 in Vestec, Czech Republic.

764 For MCM4-Halo IP, the cells were lysed in low salt lysis buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl,
765 0.5 mM EDTA, 0.5% NP-40; in Fig. 5b) or in high salt lysis buffer (10 mM Tris-Cl pH 7.5, 500 mM NaCl,
766 0.5 mM EDTA, 0.5% NP-40, in Fig. 5d) supplemented with protease and phosphatase inhibitors
767 (ROCHE, 4693116001 and 4906845001) and 250 U per ml of benzonase (Merck, E1014-25KU) on ice
768 for 45 minutes. Whole-cell lysates were then incubated with 20 μ l of Halo-Trap agarose beads
769 (Proteintech, ota-20) for 2 hours at 4°C. After incubation, the beads for low salt IP were washed twice
770 with low salt lysis buffer and additionally twice with high salt lysis buffer. For MCM4-Halo IP under
771 high salt conditions, the beads were washed four times with a high salt lysis buffer. The bound
772 proteins were eluted by incubating the beads with 60 μ l of Laemmli loading buffer containing
773 NuPAGE LDS Sample Buffer (Thermo Fisher Scientific, NP0007) and NuPAGE Sample Reducing Agent
774 (Thermo Fisher Scientific, NP0009) for 20 minutes at 95°C. The inputs and elutes were processed by
775 SDS-PAGE and western blotting.

776

777 **SDS-PAGE and western blotting**

778 Protein samples were separated by SDS-PAGE using NuPAGE 4–12% Bis-Tris gels and NuPAGE MES
779 SDS Running Buffer (Thermo Fisher Scientific). Typically, 15–30 μ g of total protein was loaded per
780 well. After separation, proteins were transferred to an Amersham Hybond P 0.45 μ m PVDF
781 membrane (GE Healthcare). Membranes were blocked with 5% milk (Santa Cruz Biotechnology) in
782 PBS supplemented with 0.1% Tween 20 (PBS-T) for 20 minutes and incubated with the indicated
783 primary antibodies diluted in 3% bovine serum albumin (BSA; AppliChem) in PBS-T overnight at 4°C.

784 Protein samples in Fig. 5b, d were separated on mPAGE™ 4-12% Bis-Tris Precast Gels (Merck,
785 MP41G10 or MP41G12) through standard SDS-PAGE protocol. The iBlot (Thermo Fisher Scientific,
786 IB21001) system was used to transfer the separated proteins onto the nitrocellulose membrane
787 (Thermo Fisher Scientific, IB23002X3). The membrane was blocked with PBS buffer containing 5%
788 milk (Merck, 70166-500G) and 0.1% Tween-20 (Merck, P1379-500ML) for 1 hour at room
789 temperature. The blocked membrane was incubated with primary antibody diluted in the blocking

790 buffer overnight at 4°C, followed by secondary antibody staining (diluted in blocking buffer) for 2
791 hours at room temperature. HRP-signal was then detected by ECL Select Western Blotting Detection
792 Reagent (Merck, GERPN2235). Following the detection of the target protein, Restore PLUS Western
793 Blot Stripping Buffer (Thermo Fischer Scientific, 46430) was used to strip the membrane and stain for
794 other proteins.

795 For tissue-specific protein expression analysis, organs were isolated from wild-type mice and
796 DCAF12-deficient strains²². Tissue samples (3 mm x 3 mm) were weighed for consistency and
797 prepared for whole-cell lysis. Bone marrow was extracted from the femur and tibia by flushing with
798 PBS, followed by cell centrifugation and washing. A lysis buffer containing Benzonase and EDTA-free
799 Protease Inhibitor Cocktail was added (100 µl for bone marrow and 200 µl for other tissues). Samples
800 were incubated on ice for 40 minutes with several rounds of sonication. SDS was then added to a
801 final concentration of 1%, and the samples were boiled at 95°C for 5 minutes before centrifugation.
802 For each sample, 15-20 µg of total protein was analyzed using western blot.

803

804 **DNA fiber assay**

805 DNA fiber experiments were performed as previously described³¹ using the following antibodies for
806 CldU (anti-BrdU, rat, Abcam, ab6326, 1:200), IdU (anti-BrdU, mouse, Becton Dickinson, 347580,
807 1:200), goat anti-rat AlexaFluor 594 IgG (Thermo Fisher Scientific, A21209, 1:200), and goat anti-
808 mouse AlexaFluor 488 IgG (Thermo Fisher Scientific, A11029, 1:200). The stained slides were
809 mounted using Mowiol-based mounting medium (12% Mowiol 4-88 (Merck, 81381-250G), 30%
810 glycerol, 0.12 M Tris-HCl pH 8.5). DNA fiber images were acquired using an inverted screening
811 microscope ScanR (IX81, Evident) equipped with UPLSAPO dry objective (20X, 0.75 NA), appropriate
812 excitation and emission filters, and ScanR acquisition software (Evident, v.2.7.1). The lengths of CldU
813 (red) and IdU (green) labeled tracks were measured using the ImageJ software (version 1.54i). For
814 the calculation of replication fork speed (kb per min), the measured length (in µm) was multiplied by
815 2.59 (kb/µm, conversion factor)⁴⁴ and divided by the duration of labeling by CldU and IdU.

816

817 **Reverse transcriptase quantitative PCR (RT-qPCR)**

818 Total RNA was isolated using a NucleoSpin RNA isolation kit (Macherey-Nagel, 740955.50). The cDNA
819 was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific,
820 4387406) according to the manufacturer's recommendations. The RT-qPCR was performed using the

821 Brilliant II SYBR Green qPCR Master Mix (Agilent, 600828) and primers (MCMBP primer pair #1:
822 forward, ATGCCCTTCTGGGGATAGT; reverse, CTATCCGTGGGAACCACT; MCMBP primer pair #2:
823 forward, ACAGCCCCAGCTAATCCAC; reverse, AGGGTCGTTCTCCGCATT; DCAF12 primer pair #1:
824 forward, TGGTATCCATGCCATCGAGC; reverse, AGAACCATCACGTGAGCCAG; DCAF12 primer pair #2:
825 forward, GATCCACGGCAGCCATCATA; reverse, CTGCTAGTCTGGGCTTGGAC.

826

827 **Colony formation assay**

828 U2OS cells were transfected with control siRNA or siRNA against DCAF12, MCMBP, or both. After 24
829 hours of siRNA transfection, the cells were seeded onto 6-well plates (400 cells per well). After ten
830 days, cells were fixed with 4% buffered formaldehyde and stained with 0.1% crystal violet diluted in
831 20% ethanol for 30 minutes at room temperature. The plates were washed twice with distilled water
832 and air-dried overnight before colony counting.

833

834 **Statistical analysis**

835 Statistical analysis was performed using GraphPad Prism 10.1.2. Experiments were not randomized,
836 and no blinding was used during data analysis. Sample size, statistical tests, and number of replicates
837 for all experiments are specified in the figure legends.

838

839 **Reporting summary**

840 Further information on research design is available in the Nature Research Reporting Summary linked
841 to this paper.

842

843 **Data availability statement**

844 All the source data, including numerical and statistical source data, will be deposited appropriately at
845 the European Bioinformatics Institute (EBI) BioStudies database (<https://www.ebi.ac.uk/biostudies/>)
846 and will be immediately accessible as an open resource pending acceptance of this manuscript. The
847 data presented in Fig. 1i-j and Extended Data Fig. 3a-f were extracted from ref.[22]. Any additional
848 data or information in support of this study will be available from corresponding authors upon
849 reasonable request.

850

851 **Ethics statement**

852 All animal experiments were approved by the Resort Professional Commission for Approval of
853 Projects of Experiments on Animals of the Czech Academy of Sciences, Czech Republic (protocols
854 115/2016 and AVCR 1667/2022 SOV II) and were in accordance with the Czech Act No. 246/1992
855 Coll. and European directive 2010/63/EU. The mice developed for this study are part of the
856 International Mouse Phenotyping Consortium (IMPC) project (MGI:1916220; Dcaf12em1(IMPC)Ccpz
857 allele) and are available upon request. Animals were maintained in a controlled, specific pathogen-
858 free environment at the Animal Facilities of the Institute of Molecular Genetics of the Czech Academy
859 of Sciences, Czech Republic. Mice were provided food and water ad libitum and kept in the animal
860 facility with a 12-h light-dark cycle.

861

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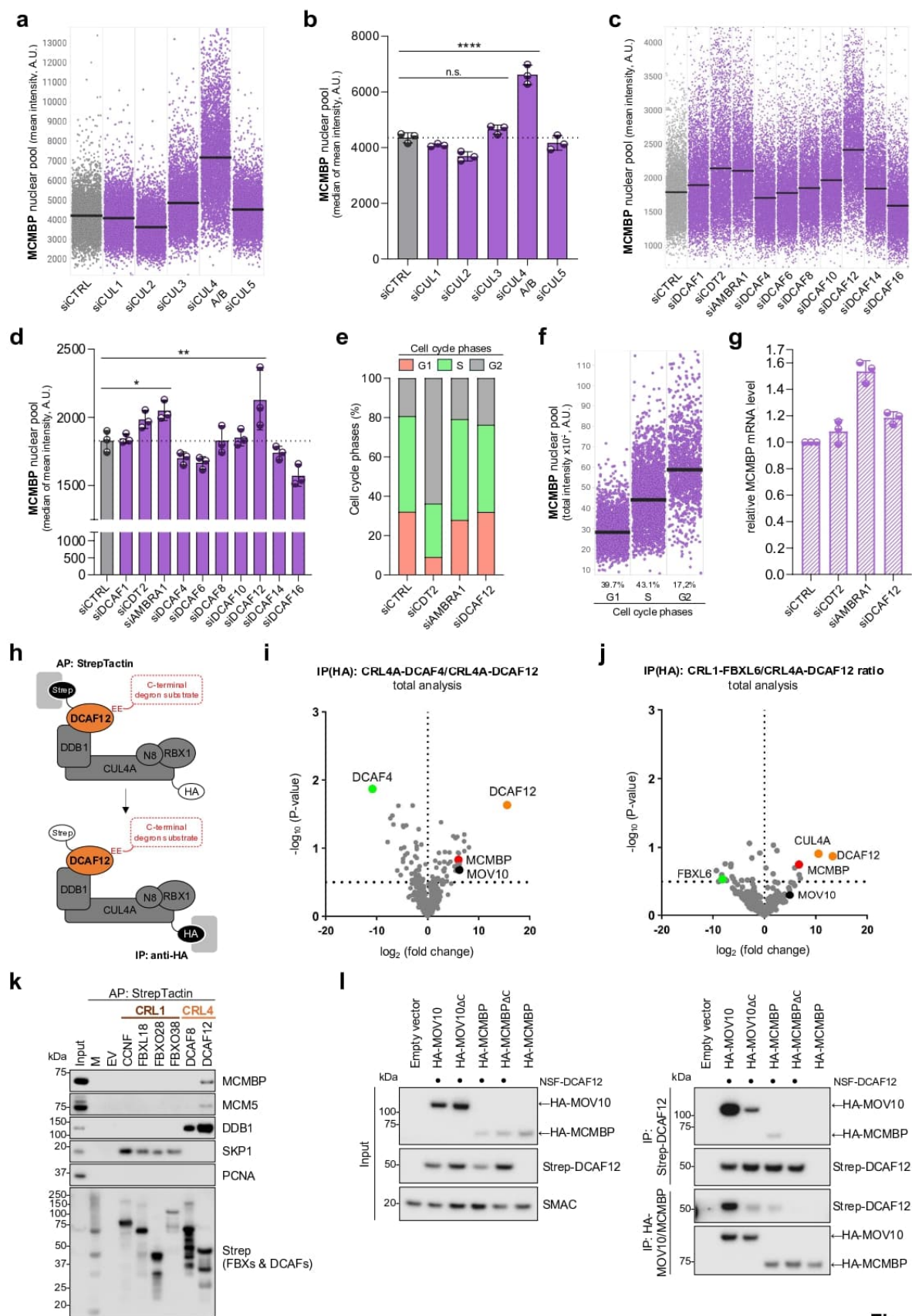


Fig. 1

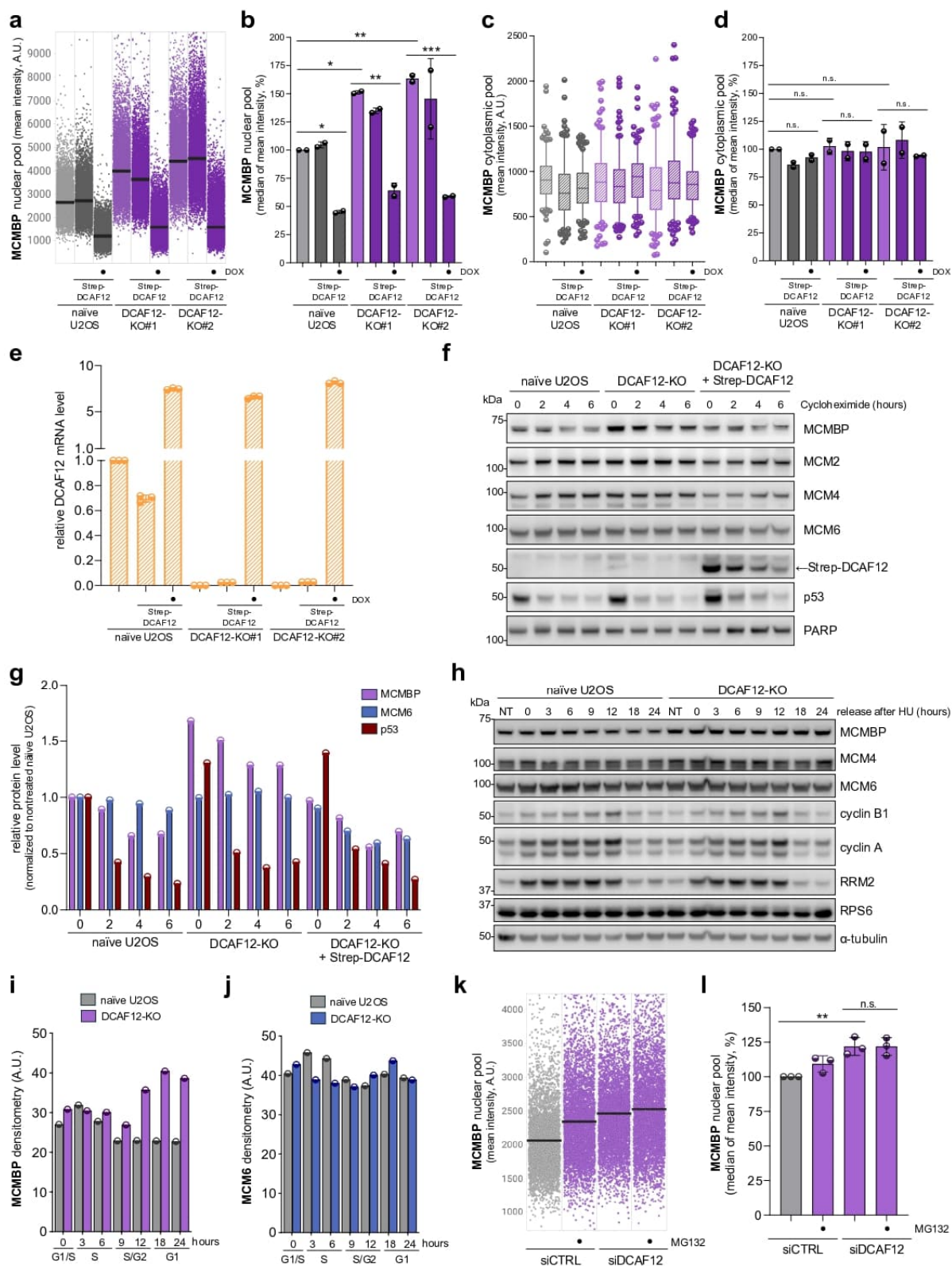


Fig. 2

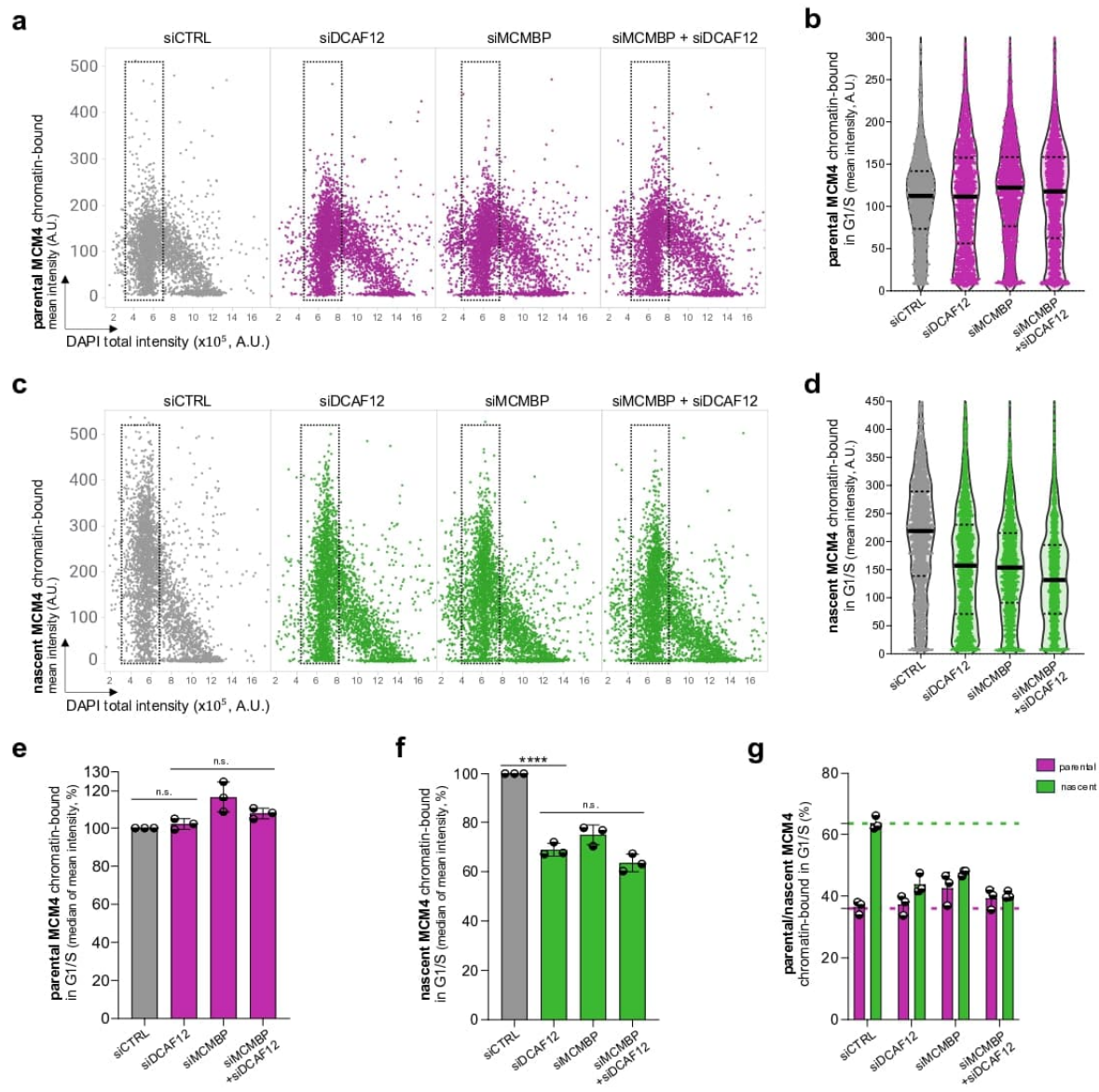


Fig. 3

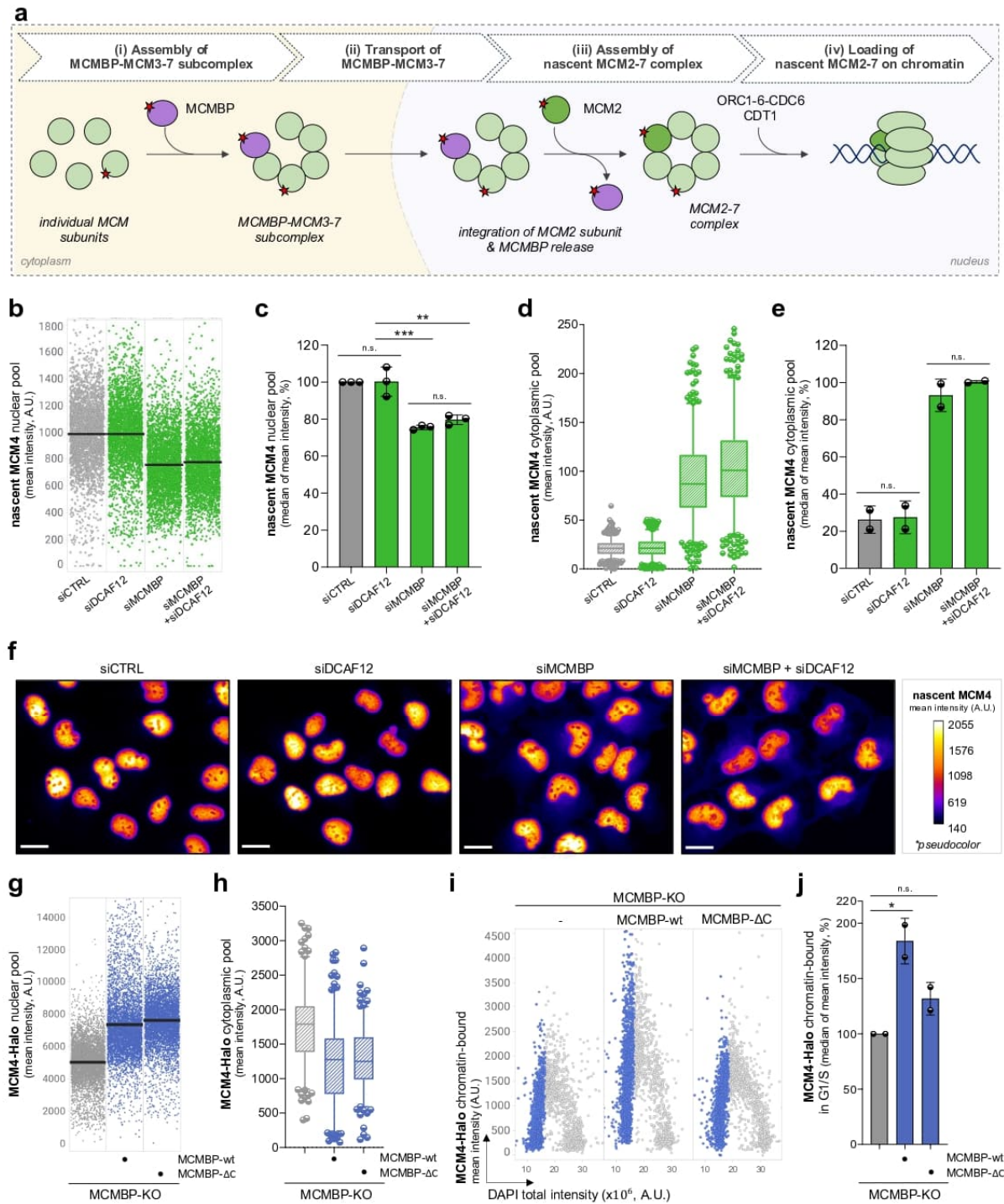


Fig. 4

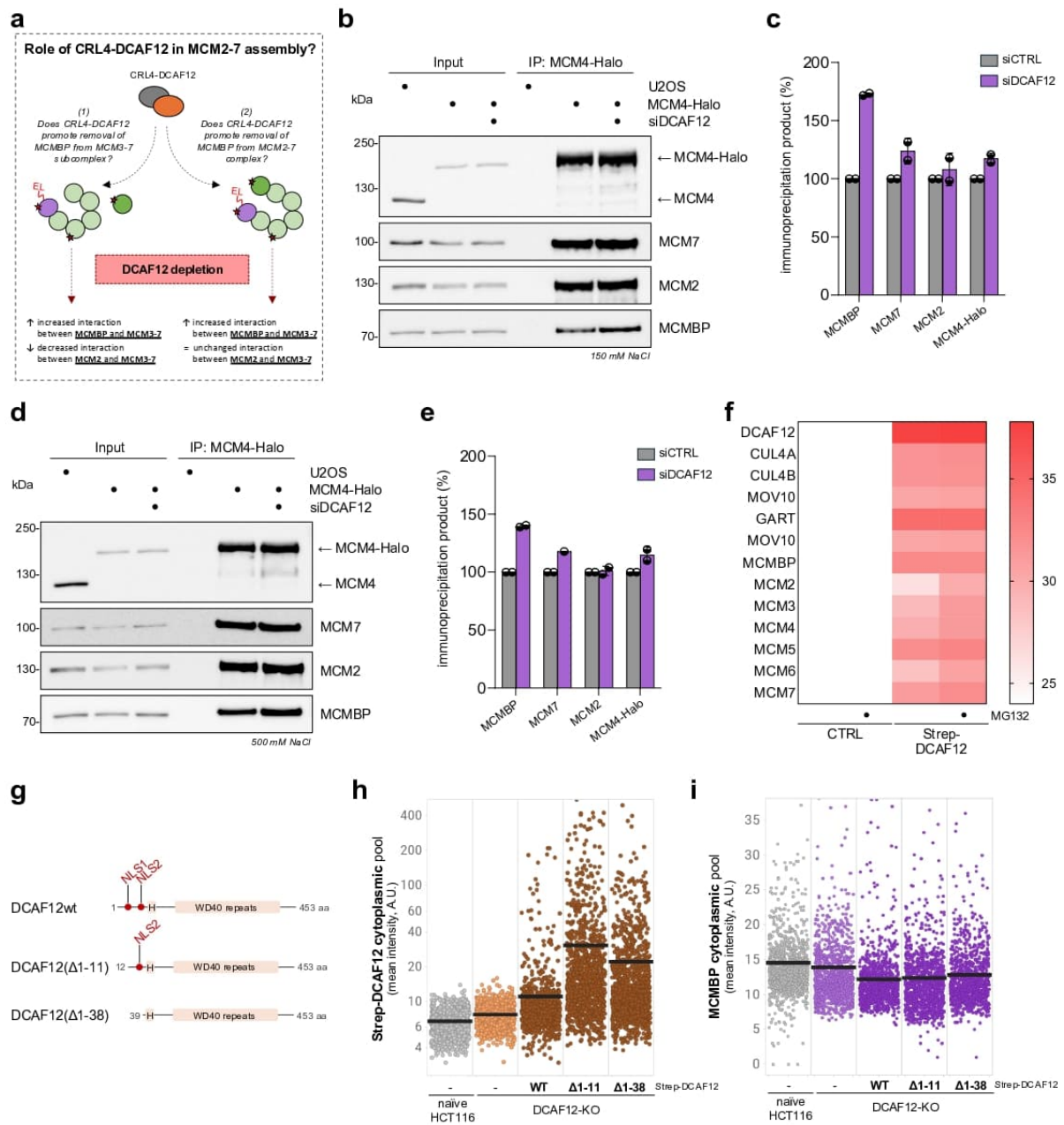


Fig. 5

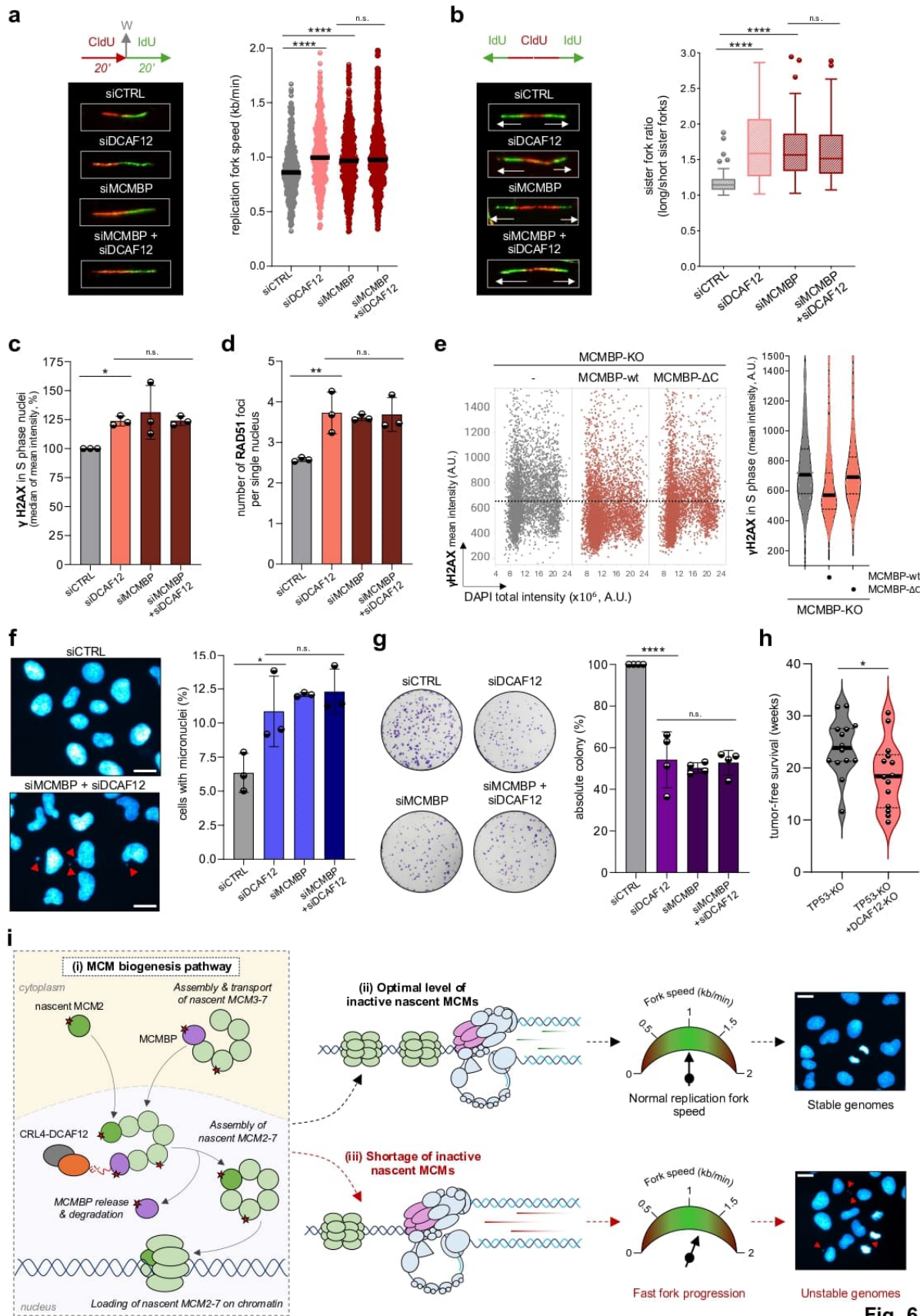


Fig. 6

b)

Report

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FBXO38 is dispensable for PD-1 regulation

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Abstract

SKP1-CUL1-F-box protein (SCF) ubiquitin ligases are versatile protein complexes that mediate the ubiquitination of protein substrates. The direct substrate recognition relies on a large family of F-box-domain-containing subunits. One of these substrate receptors is FBXO38, which is encoded by a gene found mutated in families with early-onset distal motor neuropathy. SCF^{FBXO38} ubiquitin ligase controls the stability of ZXDB, a nuclear factor associated with the centromeric chromatin protein CENP-B. Loss of FBXO38 in mice results in growth retardation and defects in spermatogenesis characterized by deregulation of the Sertoli cell transcription program and compromised centromere integrity. Moreover, it was reported that SCF^{FBXO38} mediates the degradation of PD-1, a key immune-checkpoint inhibitor in T cells. Here, we have re-addressed the link between SCF^{FBXO38} and PD-1 proteolysis. Our data do not support the notion that SCF^{FBXO38} directly or indirectly controls the abundance and stability of PD-1 in T cells.

Keywords PD-1; FBXO38; Cullin; Immune Checkpoint; Protein Degradation

Subject Categories Cancer; Immunology; Post-translational Modifications & Proteolysis

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Introduction

Cullin-RING ubiquitin ligases (CRLs) constitute a major group of enzymes responsible for targeting various proteins for ubiquitin- and proteasome-dependent degradation (Skaar et al, 2013). These multi-subunit E3 ubiquitin ligases have separate modules for substrate recognition and catalysis. Many of their substrate-binding receptors are dysregulated in various human diseases, including cancer and neurodegenerative syndromes (Skaar et al, 2014).

F-box-containing protein 38 (FBXO38), also known as MoKA, is a substrate receptor for one of many CRLs (CUL1-RING ligases), also known as SCFs (for SKP1, CUL1, F-box protein ubiquitin ligases) (Jin et al, 2004; Smaldone et al, 2004). Several FBXO38 gene

mutations were found in patients with early-onset distal hereditary motor neuropathy (Sumner et al, 2013). Initially, FBXO38 was discovered as a modulator of the transcription factor Krüppel-like factor 7 (KLF7) activity (Smaldone et al, 2004), but its role as a CRL1 substrate receptor was poorly characterized. Then, the programmed cell death 1 (PD-1, also known as PDCD1, CD279) protein involved in immune-checkpoint control was identified as its substrate (Meng et al, 2018). Subsequently, SCF^{FBXO38} was shown to play a role in centromeric chromatin control by targeting two zinc finger proteins, ZXDA and ZXDB, for degradation both in vivo and in vitro, impacting the stabilization of CENP-B protein at centromeres (Dibus et al, 2022a; Dibus et al, 2022b).

As these discoveries pointed to a potentially interesting crosstalk between different cellular processes occurring in different sub-cellular domains, we decided to investigate the role of FBXO38 in the control of PD-1 and connect it to its nuclear functions. Importantly, beside the original observation (Meng et al, 2018), there was no conclusive information in literature to support the role of FBXO38 in PD-1 biology. A comprehensive CRISPR/Cas9-based genomic-wide screen exploring positive and negative regulators of PD-1 protein expression identified several known ubiquitin ligases potentially linked to PD-1 ubiquitination and degradation, including SCF substrate receptors FBXO28 and FBXO47, as well as the Cullin-independent membrane-localized ubiquitin ligase RNF43 (Okada et al, 2017). Notably, FBXO38 did not emerge as a hit in this screen, a result consistent with its absence in other screens focused on PD-1 protein or the T-cell exhaustion pathway (Belk et al, 2022; Dong et al, 2019). Moreover, the FBXO38-dependent degron composition identified in our previous study was confirmed through large-scale ubiquitin ligase degron identification (Dibus et al, 2022a; Zhang et al, 2023). Yet, the primary sequence in the intracellular domain of PD-1 did not contain any similar motifs. Additionally, individuals harboring FBXO38 mutations exhibit neurodegenerative syndromes without concomitant immune deficiencies or alterations in the immune system (Akcimen et al, 2019; Sumner et al, 2013).

While the non-identification of FBXO38 in above-mentioned screens does not definitively rule out its role as a PD-1 regulator, it aligns with the findings of our targeted approach. Our attempts to reproduce key experimental outcomes that substantiate SCF^{FBXO38} function as a ubiquitin ligase for the PD-1 receptor were unsuccessful. Instead, our results indicate that SCF^{FBXO38} is not involved in PD-1 regulation.

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Results and discussion

FBXO38 and the PD-1 receptor localize to distinct subcellular compartments

To validate the findings by previous study (Meng et al, 2018), we performed a partial replication involving PD-1 overexpression. However, upon PD-1 overexpression in HEK293FT cells (lacking endogenous expression of PD-1 protein), we observed a significant accumulation of PD-1 in the cytoplasm (Fig. 1A; white arrowheads).

To minimize such overexpression artifacts, we utilized a Sleeping Beauty transposon-based system to generate HEK293FT-PD-1. The addition of doxycycline induced human PD-1 expression at levels comparable to or lower than those in T cells. In this optimized system, approximately 10^6 copies of *hPDCD1* mRNA are expressed per 1 μ g of total RNA (Appendix Table S1). In contrast, highly expressed genes like *ACTB* or *GAPDH*, are consistently expressed at levels between 10^7 and 10^8 copies of mRNA per 1 μ g (Glare et al, 2002).

Moreover, instead of using tagged PD-1, we optimized the use of a commercially available antibody to detect PD-1 protein in western blots and paraformaldehyde-fixed cells (Fig. 1B,C). This allowed us to determine whether FBXO38 and PD-1 are located in the same cellular compartments. As with endogenous PD-1, doxycycline induction led to PD-1 localization predominantly at cellular membranes, including the plasma membrane (Fig. 1C) (Pentcheva-Hoang et al, 2007). Our previous studies demonstrated that FBXO38 is localized in the nucleus both in cultured cells and in vivo (Dibus et al, 2022a; Dibus et al, 2022b). Exogenous FBXO38 localizes to the nucleus when not overexpressed above the physiological level, which is a consequence of its strong nuclear localization signal (Fig. 1D). To test whether PD-1 expression affects FBXO38 localization or whether PD-1 could be stabilized in the nucleus, we detected both FBXO38 and PD-1 by immunofluorescence upon inhibition of the proteasome (with MG-132) or inhibition of neddylation (using MLN4924, which prevents SCF-mediated degradation) (Duda et al, 2008). In contrast to plasma membrane-associated PD-1, endogenous FBXO38 was strictly nuclear. Incubation with MG-132 or MLN4924 did not lead to colocalization of FBXO38 and PD-1 in any subcellular compartment (Fig. 1E).

FBXO38 neither associates with the PD-1 receptor nor influences its protein levels

Next, we tested the interaction between FBXO38 and PD-1. We pretreated the cells with MLN4924 to avoid the degradation of PD-1, which could be potentially caused by FBXO38 overexpression. Consistent with our previous observations (Dibus et al, 2022a; Dibus et al, 2022b), FBXO38 interacted with its substrate ZXDB, and deletion of either its C-terminus or its F-box motif disrupted this interaction (Fig. 2A). However, we did not detect any interaction between PD-1 and FBXO38. To verify that inducible PD-1 correctly folds and associates with its established interactors (Okazaki et al, 2001), we immunoprecipitated it from HEK293FT-PD-1 cells. Immunoprecipitated PD-1 interacted with its canonical partner SHP2, but not with FBXO38 or SKP1 (Fig. 2B).

The discrepancy between our findings and those of Meng et al, in the analysis of the FBXO38:PD-1 interaction may stem from variations in experimental protocols. Overexpression of large nuclear proteins can lead to mislocalization, as observed in previous studies (Bolognesi and Lehner, 2018). Additionally, SCF substrate receptors may associate with potential substrates post-lysis (Reitsma et al, 2017), potentially yielding false positive results when proteins, which cannot interact in vivo due to being localized into different cellular compartments, meet after cell lysis. Even mild concentrations of non-ionic detergents, such as NP-40 or Triton X-100, can cause that nuclear proteins not tightly associated with chromatin intrude into cytosolic and membrane fractions. To circumvent these artifacts, reliable separation methods, such as mechanical cell disruption in hypotonic conditions or digitonin-based lysis, are essential. The use of turbonuclease in all our experiments effectively prevented nonspecific interactions potentially caused by nucleic acid contamination. Furthermore, given the absence of the FBXO38:PD-1 interaction, we found it unnecessary to replicate the in vitro ubiquitination reactions performed in the previous study.

Subsequently, we evaluated the impact of protein degradation inhibition on PD-1 protein levels. We observed that they remained unchanged following the inhibition of SCF or the proteasome with MLN4924 and MG-132, respectively (Fig. 2C). In contrast, the protein level of p27, an established substrate of SCF^{FBXL1}, increased (Carrano et al, 1999). The previous study showed that overexpression of FBXO38 in HEK293FT cells leads to increased PD-1 degradation (Meng et al, 2018). This contrasts with our previous observation that FBXO38 overexpression does not enhance the degradation of its substrates (Dibus et al, 2022a). Accordingly, we observed only a modest decrease in ZXDB protein levels and no changes in PD-1 levels upon FBXO38 overexpression. However, inhibition of the SCF-dependent degradation led to an increase in FBXO38, ZXDB, and p27 levels, with no effects on PD-1 protein levels (Fig. 2D).

We also monitored the levels of ZXDB and PD-1 in control and FBXO38-depleted cells with or without cycloheximide treatment to inhibit protein synthesis. We observed a substantial increase in the protein levels and stability of ZXDB, but not PD-1 in FBXO38 knocked-down cells (Fig. 2E). Expression of an FBXO38 construct restored ZXDB degradation, but exhibited no effect on PD-1 protein levels.

Quantifying PD-1 protein through western immunoblotting presents a challenge due to its significant posttranslational modifications. Studies indicate the importance of PD-1 fucosylation (N-type glycosylation) for proper trafficking and plasma membrane localization (Okada et al, 2017). Additionally, recent findings describe substantial O-glycosylation in the stalk region of PD-1 (Tit-Oon et al, 2023). As shown in Fig. 1B, these glycosylation types are not prevalent in HEK293FT cells. However, for experiments investigating PD-1 in T cells, potential post-lysis deglycosylation using PNGase is necessary. PNGase selectively removes specific N-type modifications, not O-type, possibly resulting in multiple PD-1 variants with distinct molecular weights. To ensure methodological precision, we adhered to established field standards, employing flow cytometry analysis with specific antibodies, as demonstrated in the following results. This approach alleviated potential selectivity issues associated with the incomplete deglycosylation of specific PD-1 protein fractions.

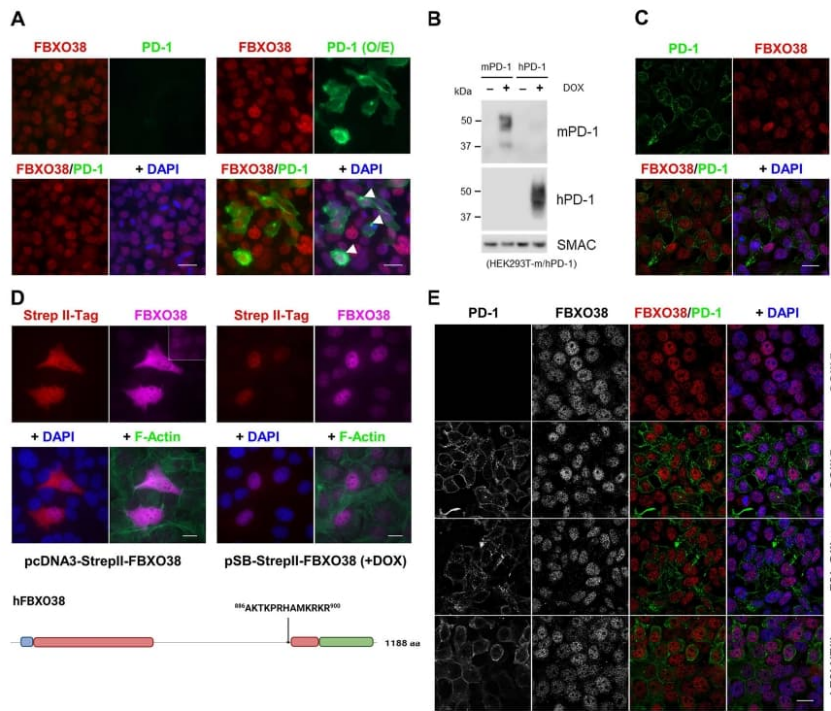


Figure 1. FBXO38 and the PD-1 receptor localize to distinct subcellular compartments.

(A) HEK293FT cells were transfected with a pcDNA3.1 vector expressing human PD-1. The cells were fixed and permeabilized 48 h after transfection and subsequently incubated with FBXO38 and PD-1 antibodies. DNA was stained with DAPI. White arrowheads mark PD-1 cytosolic accumulation. Scale bar, 20 μ m. (B) HEK293FT-mPD-1 and HEK293FT-hPD-1 cells were incubated with doxycycline, lysed, and immunoblotted as indicated. SMAC staining was used as a loading control. (C) HEK293FT cells with the inducible expression of human PD-1 (HEK293FT-hPD-1) were incubated with doxycycline for 48 h and subsequently fixed, permeabilized, and incubated with anti-FBXO38 and PD-1 antibodies. DNA was stained with DAPI. Scale bar, 20 μ m. (D) HEK293FT cells were transfected with pcDNA3.1 or pSB vectors expressing human FBXO38. The cells were fixed and permeabilized 48 h after transfection and subsequently incubated with FBXO38 and Strep II-tag antibodies. Prior to fixing, cells transfected with pSB vector were treated for 16 h with doxycycline. DNA was stained with DAPI. Polymerized actin was visualized using phalloidin. The intensity of overexpressed FBXO38 (pcDNA3), both Strep II tag and FBXO38 staining, was reduced to facilitate a comparison of localization. In the upper right corner, the staining from FBXO38 shows an image subset with comparable intensity. Scale bar, 20 μ m. In the lower panel, the schematic representation of the structure of FBXO38 is depicted. Following the F-box motif (blue), the structured leucine repeat-rich region (red) is divided into two parts by the unstructured serine- and threonine-rich regions. The C-terminal part, which is involved in substrate binding, is colored green. The position of the nuclear localization signal (NLS) is shown above. (E) HEK293FT-hPD-1 cells were incubated with doxycycline and then treated with MG-132 or MLN4924. Cells were fixed 6 h after treatments, permeabilized, and incubated with FBXO38 and PD-1 antibodies. DNA was stained with DAPI. Scale bar, 20 μ m. Source data are available online for this figure.

FBXO38 does not alter PD-1 receptor membrane levels or stability in T cells

Next, we assessed the proposed role of SCF^{FBXO38} in regulating PD-1 levels in T-cell leukemia cell lines and primary mouse T cells. First, we induced the expression of endogenous PD-1 in Jurkat and HPB-ALL cell lines using ionomycin and phorbol myristyl acetate (PMA) in the presence or absence of proteasome and neddylation inhibitors. Flow cytometry analysis showed that upon activation, the majority of the cells exhibited strong surface PD-1 expression (Fig. EV1A,B). Proteasome inhibition by MG-132 had no significant effect in Jurkat cells, but increased the percentage of

PD-1-positive cells and the level of PD-1 surface expression in the HPB-ALL cell line (Figs. 3A–D and EV1A,B). The inhibition of neddylation did not induce an increase in the PD-1 surface expression and the percentage of PD-1 positive cells in both cell lines (Figs. 3A–D and EV1A,B), which contradicts the hypothesis that PD-1 is degraded via SCF ubiquitin ligases and other CRLs, whose activity is neddylation-dependent. On the contrary, it resulted in decreased expression, suggesting a potential interplay between CRL-dependent degradation and other CRL-independent mechanisms controlling PD-1 protein levels. On the other hand, we do not dispute the regulation of PD-1 protein via proteasome-based degradation since we also found that the inhibition of the

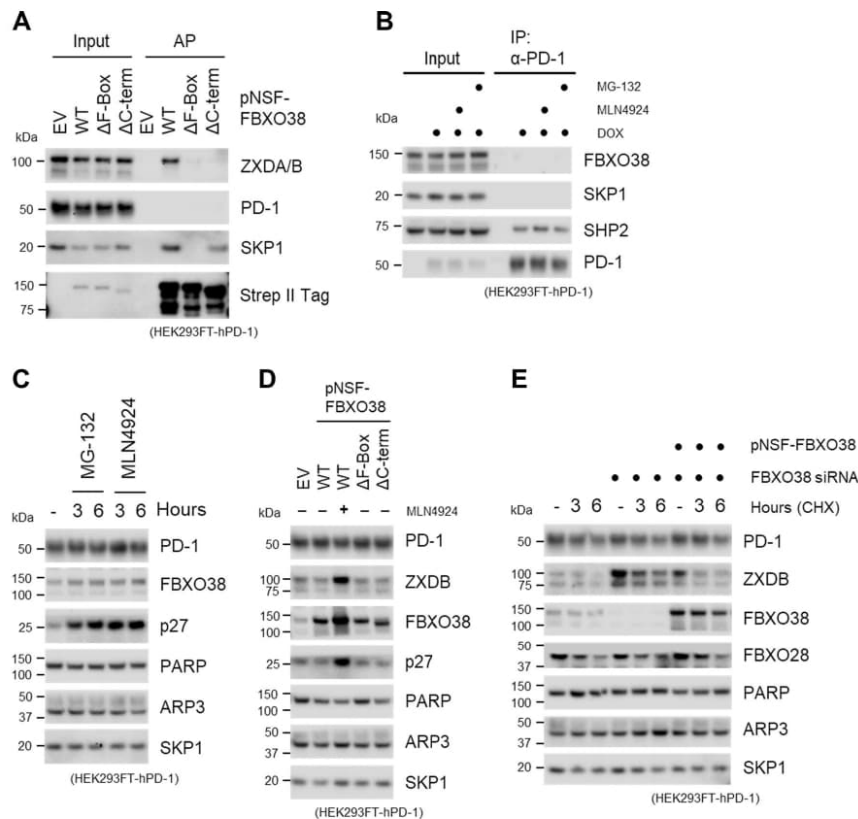


Figure 2. FBXO38 neither associates with the PD-1 receptor nor regulates its protein levels.

(A) HEK293FT-hPD-1 cells were transfected with Strep II-FLAG-tagged WT FBXO38 (pNSF-FBXO38 WT), FBXO38 lacking the F-box motif (Δ F-Box) or C-terminus (Δ C-term). The cells were treated with MLN4924 inhibitor 6 h prior to collecting, lysed, and subjected to affinity purification (AP) using Strep-Tactin resin and immunoblotted as indicated. SKP1 or ZXDB staining was used as the positive control. Inputs represent 1% of the whole-cell lysates subjected to AP. (B) HEK293FT-hPD-1 cells were treated with doxycycline, MG-132, and MLN4924 where stated. Whole-cell lysates were subjected to immunoprecipitation (IP) and immunoblotted as indicated. SHP2 staining was used as a positive control. Inputs represent 1% of the whole-cell lysate subjected to IP. (C) HEK293FT-hPD-1 cells were treated with MLN4924 and MG-132 for 3 or 6 h, lysed, and immunoblotted as indicated. p27 staining was used as a positive control, and PARP, ARP3, and SKP1 as the loading controls. (D) HEK293FT-hPD-1 cells were transfected with Strep II-FLAG-tagged WT FBXO38 (pNSF-FBXO38 WT), FBXO38 lacking the F-box motif (Δ F-Box) or C-terminus (Δ C-term). Where stated, the cells were treated with MLN4924 inhibitor 6 h prior to collection. Cells were lysed and immunoblotted as indicated. ZXDB and p27 stainings were used as positive controls, and PARP, ARP3 and SKP1 as loading controls. (E) HEK293FT-hPD-1 cells were transfected with non-targeting siRNA or a mixture of three different siRNAs targeting FBXO38 along with an empty pcDNA3.1 vector or SF-FBXO38 WT vector. Cells were then treated with cycloheximide (CHX) for the indicated time. Whole-cell lysates were immunoblotted as indicated. ZXDB and FBXO28 stainings were used as positive controls, and PARP, ARP3, and SKP1 as loading controls. Source data are available online for this figure.

proteasome leads to an increased level of membrane-bound PD-1 protein in the HPB-ALL T-cell line.

To assess the impact of FBXO38 deficiency on PD-1 expression in primary T cells, we employed our established *Fbxo38* knockout (KO) mouse model. While the *Fbxo38* mRNA is expressed in this mouse strain, a CRISPR-mediated deletion targeting the F-box motif led to the generation of the strain incapable of producing functional SCF^{FBXO38} ubiquitin ligase (Dibus et al, 2022b). First, we assessed the immune system of the *Fbxo38*^{KO/KO} mouse by

immunophenotyping of splenocytes. Our analysis encompassed a comprehensive assessment of T cells, B cells, NKT cells, monocytes, eosinophils, and neutrophils, yet, no significant differences were noted within these cell populations between the wild-type (WT) and *Fbxo38*^{KO/KO} mice (Fig. EV1C). Intriguingly, NK-cell percentages consistently exhibited a modest decrease in *Fbxo38*^{KO/KO} splenocytes. While this observation may hold interest within the context of FBXO38 role in the immune system, it is unrelated to PD-1 regulation in T cells.

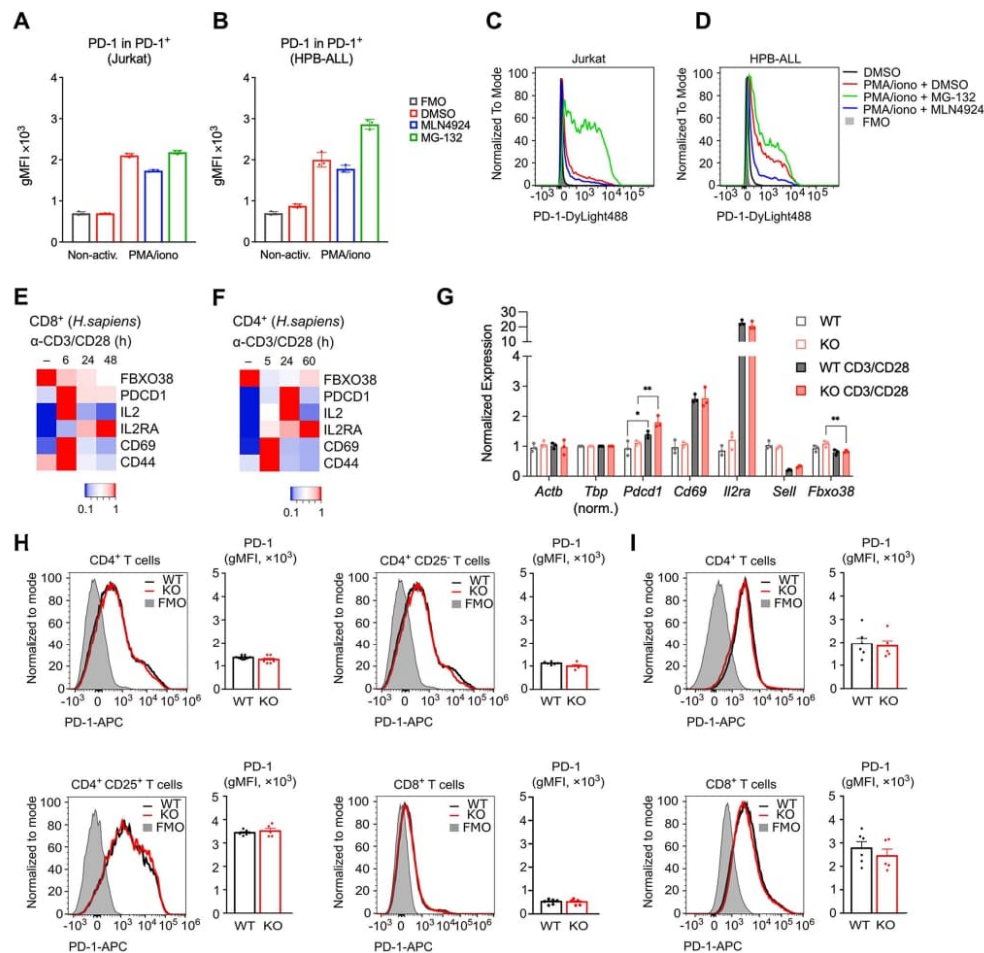


Figure 3. FBXO38 does not control the levels and stability of PD-1 in T cells.

(A–D) Jurkat (A) or HPB-ALL (B) T-cell leukemia cell lines were incubated for 72 h with phorbol myristyl acetate (PMA) and ionomycin (iono) followed by 6 h treatments with MG-132 or MLN4924 ($n = 3$; biological replicates). Surface PD-1 expression was analyzed by flow cytometry. (A, B) Bars represent the geometric mean of PD-1 fluorescence intensities in the PD-1⁺ population (gMFI + SD). (C, D) Histograms represent the analysis of representative samples. (E, F) Publicly available data of gene expression changes upon activation of CD8⁺ T cells (E; GSE122149; mean from triplicate) (Data ref: Ogando et al, 2019a; Ogando et al, 2019b) and CD4⁺ T cells (F; GSE116697; mean from duplicate) (Data ref: Yukawa et al, 2020a; Yukawa et al, 2020b) using anti-CD3 and anti-CD28 antibodies. Data are visualized as a heatmap showing gene expression normalized to the highest obtained count (or average count from replicates). Publicly available data were normalized using a GREIN application (<http://www.ilincs.org/apps/grein/>) (Mahi et al, 2019). (G) Relative expression of markers in B-cell-depleted splenocytes. mRNAs isolated from naive or activated T cells from WT and Fbxo38 KO animals ($n = 3$; biological replicates) were reverse transcribed and subjected to RT-qPCR analysis. Expression levels were normalized to Tbp gene expression. Error bars indicate SD, and each data point represents an individual animal. (H, I) Cell-surface expressions of PD-1 in T cells isolated from the spleens of Fbxo38^{WT/WT} (WT) and Fbxo38^{KO/KO} (KO) mice. Histograms of representative mice and the quantification of the geometric mean fluorescence intensities (gMFI) for all mice (mean + SEM) are shown. (H) Steady-state surface levels of PD-1 in naive CD4⁺, CD4⁺ CD25⁺, and CD8⁺ T cells. $n = 6$ Fbxo38^{WT/WT} and 5 Fbxo38^{KO/KO} mice in two independent experiments. (I) Surface levels of PD-1 in CD4⁺ (upper) and CD8⁺ T (lower) cells stimulated with anti-CD3/CD28 beads (1:1 bead-to-cell ratio) in the presence of IL-2 for 96 h. $n = 6$ Fbxo38^{WT/WT} and 5 Fbxo38^{KO/KO} mice in two independent experiments. Source data are available online for this figure.

Next, we analyzed T cells from WT and *Fbxo38*^{KO/KO} mice at the steady state and upon activation. First, we analyzed publicly available data and found that *FBXO38* is consistently down-regulated at the transcriptional level during the early time points following T-cell activation via CD3 and CD28 receptor engagement (Fig. 3E,F) (Data ref: Ogando et al, 2019a; Ogando et al, 2019b; Data ref: Yukawa et al, 2020a; Yukawa et al, 2020b). In contrast, *Pdcd1* (PD-1) mRNA levels mimicked other known markers of T cell activation *Cd44*, *Cd69*, *Il2ra*, and *Il2* (Fig. 3E,F). In both CD4⁺ and CD8⁺ T cells, *Pdcd1* level peaked early and declined during the second and third days of activation. Therefore, day four was selected as the most promising time point to monitor the putative effect of *FBXO38* expression on membrane PD-1 levels. Moreover, this time point was also used in the previous study (Meng et al, 2018).

In the next step, we compared the response of WT and *Fbxo38*^{KO/KO} T cells to the anti-CD3/CD28 activation in the presence of IL-2. The RT-qPCR analysis revealed that the pattern of *Pdcd1*, *Cd69*, *Sell*, and *Il2ra* expression at 96 h post-activation was consistent with T-cell activation and comparable in both strains. The level of *Fbxo38* mRNA did not change significantly upon activation (Fig. 3G). We observed that a small fraction of activated T cells was still progressing through the cell cycle at 96 h post-activation with no difference between the WT and *Fbxo38*^{KO/KO} T cells (Fig. EV1D). These experiments revealed that there are no apparent differences between the response of WT and *Fbxo38*^{KO/KO} T cells to the activation, at least on the transcriptional level.

Finally, we analyzed PD-1 and *FBXO38* levels in the WT and *Fbxo38*^{KO/KO} mice. At the steady state, PD-1 was expressed almost exclusively in regulatory CD4⁺ CD25⁺ T cells at similar levels in both strains (Figs. 3H and EV2A). Upon activation, the *FBXO38* protein was upregulated (Fig. EV2B), suggesting that it is regulated post-translationally. The canonical SCF^{*FBXO38*} substrate ZXDB was upregulated in the activated *Fbxo38*^{KO/KO} T cells, which validated our experimental approach (Fig. EV2B). We hypothesize that ZXDB is ubiquitinated by another ubiquitin ligase in naive cells, which becomes inactivated upon T-cell receptor engagement. This results in increased degradation of *FBXO38* through self-ubiquitination (de Bie and Ciechanover, 2011). Upon activation and entry into the cell cycle, *FBXO38* likely becomes the primary ubiquitin ligase for ZXDB.

Ex vivo activation with anti-CD3/CD28 beads induced the upregulation of PD-1 surface levels in CD4⁺ and CD8⁺ T cells (Fig. 3I). However, *Fbxo38*^{KO/KO} T cells did not exhibit higher PD-1 surface levels than their WT counterparts, as it would have been expected if PD-1 was a substrate for SCF^{*FBXO38*}. If anything, the PD-1 surface levels were rather slightly lower in *Fbxo38*^{KO/KO} than in WT T cells (Fig. 3I).

To assess the potentially differential impact of *FBXO38* deficiency on extracellular and intracellular PD-1, we employed flow cytometry to analyze cell-surface and total PD-1 levels in T cells at the steady state and upon activation. Analysis of differences between surface and whole-cell PD-1 staining revealed that there is a minor fraction of PD-1 in the intracellular compartment. However, we did not observe any increase in total PD-1 levels in *Fbxo38*^{KO/KO} T cells compared to WT (Fig. EV2C–E).

FBXO38 does not alter PD-1 receptor membrane levels upon viral infection

Next, we analyzed how *FBXO38* expression affects PD-1 levels in T cells responding to the lymphocytic choriomeningitis virus (LCMV) infection in vivo. First, we analyzed publicly available data from the time course of LCMV infection and found that *Pdcd1* mRNA begins to decline between the third and seventh days after infection (Fig. 4A) (Data ref: Zhai et al, 2021; Zhai et al, 2021). This decline is followed by a similar decrease in mRNA for *Irfng*, indicating the successful clearance of the virus and termination of the immune response. Furthermore, *Fbxo38* mRNA is slightly inhibited during the first three days, coinciding with the activation of *Il2ra* (Fig. 4A). For this reason, we decided to monitor the PD-1 levels on T cells on day eight post-infection. A relatively large fraction of CD4⁺ and CD8⁺ T cells upregulated PD-1 during the LCMV infection (Figs. 4B and EV3A–C). In agreement with the steady-state and ex vivo activation data, PD-1 surface levels on PD-1⁺ CD4⁺ or CD8⁺ T cells were equal or slightly lower in *Fbxo38*^{KO/KO} than in WT mice (Fig. 4C). Using D^b-NP396 tetramer, we gated on LCMV-specific CD8⁺ T cells. We observed a comparable frequency of D^b-NP396 in WT and *Fbxo38*^{KO/KO} mice (Fig. EV3C). These D^b-NP396 tetramer⁺ CD8⁺ T cells were almost all PD-1 positive. Similar to the previous results, we observed no differences in the surface PD-1 expression in CD8⁺NP396 tetramer⁺ T cells from WT and *Fbxo38*^{KO/KO} mice (Figs. 4C and EV3A,B).

In summary, our investigation revealed no upregulation of surface PD-1 levels on T cells from *Fbxo38* KO mice, both in steady-state conditions and following ex vivo activation or viral infection. Analyses of CD8⁺ T cells, conventional CD4⁺ T cells, and regulatory CD25⁺ CD4⁺ T cells did not support the notion that *FBXO38* acts as a negative regulator of PD-1. Notably, Meng et al, employed conditional *Fbxo38* KO mice driven by *Cd4*-CRE, potentially contributing to the discrepancy between our results. However, we find this unlikely. First, detailed immunophenotyping of the *Fbxo38* KO mice showed that these mice do not exhibit a severe phenotype in the leukocyte populations. Accordingly, the frequency of virus-specific CD8⁺ T cells was comparable in LCMV-infected WT and *Fbxo38* KO mice, indicating that the whole-body *FBXO38* deficiency does not interfere with normal anti-viral immune response. Second, there is a hypothetical possibility of compensatory mechanisms in the T-cell compartment in the whole-body KO mice. However, it is unclear why these putative mechanisms would not apply as well in the *Cd4*-CRE-driven conditional KO mice, which deletes *Fbxo38* early in the T-cell development in the thymus.

A low amount of intracellular PD-1 within the T cells suggest that it undergoes either rapid degradation or recycling back to the plasma membrane. Crucially, our experiments demonstrated that the intracellular fraction of PD-1 remained unaffected by *FBXO38* expression. While we cannot entirely rule out the possibility that a small subset of intracellular PD-1 protein is regulated by *FBXO38* (e.g., in the nuclear compartment), our data strongly indicate that such regulation does not exert a significant impact on the overall abundance of PD-1 or its localization at the plasma membrane.

Meng et al, concluded that *FBXO38*-deficient T cells exhibit impaired response to IL-2 immunotherapy, which is based on the

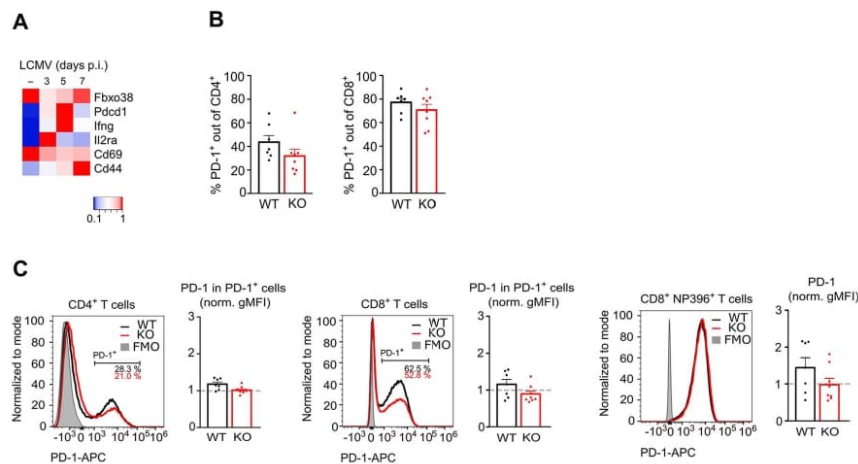


Figure 4. FBXO38 does not regulate the levels of PD-1 upon viral infection.

(A) Publicly available data of gene expression changes in CD8⁺ T cells (GSE176310) in mice infected with LCM virus. Each time point represents the mean value of two different CD8⁺ T cell isolations (GEO accession number GSE176310) (Data ref: Zhai et al, 2021; Zhai et al, 2021). Data are visualized as a heatmap showing gene expression normalized to the highest obtained mean from replicates. Publicly available data were normalized using the GREIN application (<http://www.ilincs.org/apps/grein/>) (Mahi et al, 2019). (B) Percentage of PD-1⁺ cells out of CD4⁺ and CD8⁺ T cells from *Fbxo38*^{WT/WT} and *Fbxo38*^{KO/KO} mice infected with LCMV. The bars represent mean + SEM. *n* = 7 *Fbxo38*^{WT/WT}, 8 *Fbxo38*^{KO/KO} mice in two independent experiments. (C) Cell-surface expressions of PD-1 in T cells isolated from the spleens of LCMV-infected *Fbxo38*^{WT/WT} and *Fbxo38*^{KO/KO} mice. The results of two independent experiments were normalized to the PD-1 gMFI of *Fbxo38*^{WT/WT} T cells (set as 1). Histograms represent results from littermate mice. Bars show normalized cell-surface PD-1 levels in CD4⁺ PD-1⁺, CD8⁺ PD-1⁺, and CD8⁺ D⁺-NP396 tetramer⁺ T cells (all PD-1⁺ gate). *n* = 7 *Fbxo38*^{WT/WT}, 8 *Fbxo38*^{KO/KO}, and 8 *Fbxo38*^{WT/KO} mice in two independent experiments. Individual experiments are shown in Fig. EV3. Source data are available online for this figure.

evidence that IL-2 inhibited tumor growth in 2 out of 10 WT mice, but not in *Fbxo38* cKO mice. However, the primary distinction between WT and *Fbxo38* cKO mice in their experiment appears to be the faster tumor growth in *Fbxo38* cKO mice. We do not challenge the general observations by Meng et al, that FBXO38-deficient T cells elicit impaired anti-tumor protection, which was shown in multiple experiments throughout their paper. However, the underlying mechanism is still to be elucidated as our data suggest that it does not involve the regulation of PD-1 levels by FBXO38. Notably, recent reports showed that FBXO38 positively controls the cGAS–STING pathway, crucial in regulating anti-tumor responses (Wu et al, 2024).

Despite the central role of the immune-checkpoint inhibitory receptor PD-1 in cancer immunotherapy, the regulation of the PD-1 levels and posttranslational modifications is incompletely understood. Meng et al, proposed an important mechanism of PD-1 regulation via SCF^{FBXO38}-mediated degradation. Our results challenge their observations on two different levels. First, we could not detect the interaction and colocalization of FBXO38 and PD-1 even when we pharmacologically inhibited the proteasome or neddylation, which is necessary for the SCF activity. Second, we could not observe any effect of FBXO38 on the levels and stability of PD-1 in vitro and in vivo.

Collectively, our data indicate that FBXO38 does not regulate the ubiquitination and overall levels of PD-1 receptor in T cells.

Methods

Methods and protocols

Cell culture procedures

Human cell line HEK293FT (ATCC #CRL-1573) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and penicillin, streptomycin, and gentamicin, and cultured in a humidified incubator at 37 °C with 5% CO₂. Where indicated, cycloheximide (100 µg/ml), MLN4924 (1 µM; 6 h), MG-132 (10 µM; 6 h), or doxycycline hyclate (0.1 µg/ml; 48 h) were used. Cells were transfected with pSBtet containing PD-1 and the transposase-containing pSB100X using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Positive clones were selected by puromycin 48 h after transfection. Transient transfections were carried out using polyethylenimine (PEI MW 25000, Polysciences). Jurkat E6 (ATCC #TIB-152) and HPB-ALL (DSMZ #ACC-483) were cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics. All cell lines were routinely cultured in the presence of potent anti-mycoplasma antibiotics (Plasmocure; Invivogen) and subsequently tested under laboratory conditions for the presence of mycoplasma using qPCR, along with the assessment of membranous DAPI positivity.

Reagents and tools table

Reagent/resource	Reference or source	Identifier or catalog number
2-Mercaptoethanol	Sigma	M6250
4',6-diamidin-2-fenylindol (DAPI)	Sigma	D9542
Benzonase	Santa Cruz	Sc-391121
Cycloheximide	Sigma	C7698
Desthiobiotin	IBA Lifesciences	2-1000-025
Dithiothreitol	Sigma	1019777001
Doxycycline hyclate	Sigma	D9891
Formaldehyde	Thermo Fisher	28908
Gentamycin	Sandoz	
mIL-2 Recombinant Protein	Gibco	PMC0024
Ionomycin calcium salt	Sigma	10634
Lipofectamine 2000	Thermo Fisher	11668019
LIVE/DEAD fixable near-IR-dye	Thermo Fisher	L34976
Methanol	Penta	67-56-1
MG132	Medchemexpress	HY-13259
MLN4924	Medchemexpress	HY-70062
Paraformaldehyde	Electron Microscopy Sciences	15710
Penicillin	BB Pharma	
Phorbol myristyl acetate (PMA)	Sigma	P1585
Plasmocure	Invivogen	ant-pc
Polyethyleneimine (MW 25 K)	Polysciences	23966
Ponceau S	VWR	K793
Propidium iodide	Sigma	537059
Protease Inhibitors Mini Tablets	Pierce	A32955
Proteinase K	Sigma	P2308
Puromycin	Sigma	P8833
RNAiMax	Thermo Fisher	13778150
Sodium dodecyl sulfate	Sigma	71736
Sodium fluoride	Sigma	S7920
Sodium orthovanadate	Sigma	450243
Streptomycin	Sigma	S9137
Triton X-100	Sigma	T8787
Experimental models		
<i>E. coli</i>	New England Biolabs	C29871
HEK293FT	ATCC	CRL-1573
HPB-ALL	DSMZ	ACC-483
Jurkat E6	ATCC	TIB-152
C57BL/6N	Czech Centre for Phenogenomics	
LCMV (Armstrong)	European Virus Archive Global	
Recombinant DNA		
Expression vectors	Backbone	N-term. tag
FBXO38	pcDNA3.1 (pNSF)	1xFLAG-2xStreptII
hPD-1	pcDNA3.1	
hPD-1	pSB	
mPD-1	pSB	

Reagent/resource	Reference or source	Identifier or catalog number	
Sleeping beauty system	Vendor	Cat. number	
FBXO38-containing plasmids	Dibus et al, 2022a	N/A	
pSBtet-Pur	Addgene	60507	
pSB100X	Addgene	34879	
Antibodies			
Antigen	Vendor	Catalog number	RRID
ARP3	Santa Cruz	ab49671	AB_2257830
SH-PTP2	Santa Cruz	sc-280	AB_632401
FBXO28	Bethyl	A302-377A	AB_1907260
FBXO38	Atlas Antibodies	HPA041444	AB_2677484
FLAG	Cell Signaling	14793	AB_2572291
p27	Santa Cruz	sc-528	AB_632129
PARP-1	Santa Cruz	sc-8007	AB_628105
SKP1	Cell Signaling	12248	AB_2754993
Strep II Tag	Novus Biologicals	NBP2-43735	AB_2916323
ZXDA/B	Atlas Antibodies	HPA043789	AB_2678673
mPD-1	BioLegend	109111	AB_10613470
hPD-1	ExBio	11-176	AB_2687629
Cyclin A	Pagano Lab		
B220	BioLegend	103204	AB_312989
CD19	BioLegend	152402	AB_2629714
CD8a-BV421	BioLegend	100753	AB_2562558
CD4-BV650	BioLegend	100546	AB_2562098
TCRβ-FITC	BioLegend	109206	AB_313429
CD25-PE	BioLegend	102008	AB_312857
CD25-PE-Cy7	BioLegend	102016	AB_312865
PD-1-APC	BioLegend	135209	AB_2251944
B220-BV510	BioLegend	103248	AB_2650679
Streptavidin, R-Phycoerythrin	Thermo Fisher	S866	
Viability (SytoxBlue)	Thermo Fisher	S34857	
Fc Block	BD Biosciences	553142	AB_394657
CD4-BUV496	BD Biosciences	741050	AB_2870665
CD5-BV421	BD Biosciences	562739	AB_2737758
CD44-BV510	Biolegend	103043	AB_2561391
CD8a-BV605	Biolegend	100744	AB_2562609
GITR-BV711	BD Biosciences	563390	AB_2738176
Ly6G-BV785	Biolegend	127645	AB_2566317
CD45-FITC	Biolegend	103108	AB_312973
CD11b-PerCPCy5.5	Biolegend	101228	AB_893232
CD161-PE	SONY	1143540	
CD19-PE-CF594	BD Biosciences	562291	AB_11154223
CD25 -PC7	BD Biosciences	552880	AB_394509
PD1-APC	Biolegend	109111	AB_10613470
Ly6C-AF700	BD Biosciences	561237	AB_10612017
CD62L-APC-Cy7	BD Biosciences	560514	AB_10611861

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Reagent/resource	Reference or source	Identifier or catalog number	
Anti-rabbit IgG (Alexa Fluor® 555)	Abcam	ab150070	AB_2783636
Anti-mouse anti-IgG (DyLight 488)	Thermo Fisher	35503	AB_1965946
Anti-mouse IgG, HRP-linked	Cell signaling	7076	AB_330924
Anti-rabbit IgG, HRP-linked	Cell signaling	7074	AB_2099233
Beads/resin	Vendor	Catalog number	
Dynabeads® Mouse T-Activator CD3/CD28	Thermo Fisher	11453D	
Dynabeads Untouched Mouse CD8 Cells Kit	Thermo Fisher	11417D	
Protein G (magnetic)	Dynabeads	10004D	
Strep-Tactin® Superflow resin	IBA	2-1206-025	
Oligonucleotides and other sequence-based reagents			
Cloning primers	Sequence	RE site	
hPD-1 (FWD)	AAAGGCCTCTGAGGCCACCATGCAGATCCCACAGGCGCCCTGG	Sfil	
hPD-1 (RVS)	CTTGGCCTGACAGGCCTCAGAGGGGCCAAGAGCAGTGTCC	Sfil	
mPD-1 (FWD)	AAAGGCCTCTGAGGCCACCATGTGGTCCGGCAGGTACCCTGG	Sfil	
mPD-1 (RVS)	CTTGGCTGACAGGCCTCAAGAGGCCAAGAACAATGTCC	Sfil	
CRISPR PCR verification primers	Sequence	Position	
mFBXO38 (FWD)	GGCTGTTCTCCTCTTTGTG	Exon 3	
mFBXO38 (REV)	TCCTTCCTCTGCTAGACTCT	Exon 3	
RT-qPCR primers	Sequence		
hPD-1 (FWD)	CAGATCCCACAGGCGCCCTGG		
hPD-1 (REV)	GAGGGGCCAAGAGCAGTGTCC		
hRPL23A (FWD)	TACGATGCTTTGGATGTTGC		
hRPL23A (REV)	GGCAGCTGGACTCAGTTTAGA		
mActb (FWD)	CTAAGGCCAACCGTGA AAAAG		
mActb (REV)	ACCAGAGGCATACAGGGACA		
mTbp (FWD)	GGCGGTTTGGCTAGGTTT		
mTbp (REV)	GGGTTATCTTCACACCATGA		
mCd69 (FWD)	ACTGGAACATTGGATTGGGCT		
mCd69 (REV)	CCCGTCAAGTTGAACGAGC		
mPdcd1 (FWD)	TGCAGTTGAGCTGGCAAT		
mPdcd1 (REV)	GGCTGGGTAGAAGGTGAGG		
mIl2ra (FWD)	AGAACACCACCGATTTC TG		
mIl2ra (REV)	GGCAGGAAGTCTCACTCTCG		
mSell (FWD)	GGTCATCTCCAGAGCCAATC		
mSell (REV)	TCCATGGTACCAACTCAGG		
mFbxo38 (ALL ISO- FWD)	GGGGCATATTTACGCGAGTA		
mFbxo38 (ALL ISO- REV)	GGCTCTCCATTGACATCACA		
mFbxo38 (ALL ISO- FWD)	CCAGGGAAACGGCAGTAAAGT		
mFbxo38 (ALL ISO- REV)	GTGGGAGTTGTTGCACCTCT		
Small interfering RNAs	Sequence	Catalog number	
FBXO38#1	GGGUGUAUUUCAGCGAGUAUU		
FBXO38#2	GGACUCGAUUGGUUGAUUUUU		
FBXO38#3	GAGCGAAGCUGUUUGAGUAUU		
CTRL_#1	AUGAACGUGAAUUGCUCAAUU	D-001210-04	
CTRL_#2	AUGUAUUGGCCUGUAUUAGUU	D-001210-03	

Reagent/resource	Reference or source	Identifier or catalog number
Chemicals, enzymes and other reagents	Vendor	Catalog number
4X Bolt™ LDS Sample Buffer	Invitrogen	B0007
Albumin (BSA) Fraction V	PanReac AppliChem	9048-46-8
Amersham™ Hybond® P Western blotting membranes, PVDF	Merck	GE10600023
Biotinylated MHC I monomer H-2Db-NP396 (FQPNQGQFI)	NIH Tetramer Core Facility	
Blotto, non-fat dry milk	Santa Cruz Biotechnology	sc-2324
Buffer E	IBA	2-1000-025
DpnI	Thermo Fisher	FD1703
DMEM high glucose	Sigma- Aldrich	D6429
Fetal bovine serum (FBS)	Thermo Fisher	10270106
LightCycler 480 SYBR Green I master mix	Roche	04887352001
Maxima™ H Minus cDNA Synthesis Master Mix	Thermo Fisher	M1662
NuPAGE™ 4-12% Bis-Tris gel	Invitrogen	NP0323BOX
PfuX7 DNA polymerase	Addgene	182364
RPMI 1640 medium	Gibco™	11875093
Sall	Thermo Fisher	FD0644
SfiI	Thermo Fisher	FD1824
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher	34095
WesternBright™ ECL HRP Substrate Kits	Advansta	490005-016
ProLong Gold Antifade Mountant	Invitrogen	P36930
Pierce™ BCA Protein Assay Kits	Thermo Fisher	23225
RNeasy Plus Kit	Qiagen	74134
Software	Vendor/link	
FlowJo 10.6.2	BD Biosciences	
GraphPad Prism 5.0	GraphPad Software	
GREIN application	http://www.ilincs.org/apps/grein/	
Heatmapper	www.heatmapper.ca	
ImageJ	https://imagej.net	
Image Lab	Bio-Rad	
Zen 2.3	Zeiss	
LAS	Leica	
Other (hardware)	Vendor	
Axio Imager Zeiss 2	EC Plan-Nefluar objectives	
LSRII	BD Biosciences	
Cytek Aurora	Cytek	
FACSSymphony	BD Biosciences	
TCS SP8	Leica	

Plasmid construction

cDNAs of human and mouse *PDCD1* were amplified by PCR from human peripheral blood and mouse lymph node cDNAs, respectively. Prepared cDNAs were cloned into the pSBtet-Pur plasmid using SfiI restriction sites.

FBXO38-containing plasmids were created as described previously (Dibus et al, 2022a). Briefly, human FBXO38 cDNA (Origene #RC204380) was cloned into pcDNA3.1 containing N-terminal twinStrepII-FLAG-tag (NSF). For the F-box-lacking FBXO38 variant, PCR mutagenesis was carried out using PfuX7 DNA polymerase with subsequent DpnI digestion.

Gene silencing

Small interfering RNAs (Sigma Aldrich) were transfected into the subconfluent HEK293FT cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Non-targeting siRNAs were used as negative controls.

Immunoblotting

For whole-cell lysates, cells were washed with ice-cold PBS and lysed in a lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.4% Triton X-100, 2 mM CaCl_2 , 2 mM MgCl_2 , 1 mM EDTA) in the presence of phosphatase a protease inhibitor cocktail (Pierce) and Benzonase nuclease (0.125 U/ μl ; Santa Cruz) for 30 min on ice. Lysates were mixed with an equal volume of 2% SDS in 50 mM Tris-HCl, pH 8, heated for 5 min at 95 °C, and cleared by centrifugation.

Protein concentration was determined by the BCA method (Thermo Fisher Scientific). Samples were prepared by mixing with Bolt™ LDS Sample Buffer (Thermo Fisher Scientific) supplemented with 10% β -mercaptoethanol (Sigma Aldrich), heated for 5 min at 95 °C, and then separated using NuPAGE™ 4–12% gradient Bis-Tris gel (Invitrogen). Afterward, the samples were transferred to PVDF membrane (Amersham), blocked with 5% non-fat milk (Chem-Cruz), and incubated with indicated antibodies diluted in 3% BSA (PanReac AppliChem) in tris-buffered saline with Tween® 20 (TBST) overnight at 4 °C. The HRP-conjugated secondary antibodies (Cell Signaling) were diluted in 5% milk in TBS-T. The membranes were developed using WesternBright ECL (Advanta) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Protein affinity purification and immunoprecipitation

The cells were collected and lysed in the lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.4% Triton X-100, 2 mM CaCl_2 , 2 mM MgCl_2 , 1 mM EDTA, supplemented with phosphatase and protease inhibitors) for 10 min on ice. For Strep-tagged FBXO38, the lysates cleared by centrifugation were incubated with Strep-Tactin® Sepharose resin (IBA Lifesciences). Purified proteins were then eluted by desthiobiotin using Buffer E (IBA Lifesciences) and subsequently prepared for immunoblotting as described above. For immunoprecipitation of PD-1 protein from 1×10^7 HEK293FT-PD-1 cells, lysates were incubated with the PD-1 antibody (1 μg ; Exbio #11–176) and mixed with Dynabeads® Protein G. Parental HEK293FT cells were used as a negative control. Elution of immunoprecipitated proteins was carried out with 1× Bolt™ LDS Sample Buffer (Thermo Fisher Scientific), and samples were prepared for immunoblotting as described above.

Immunocytochemistry

Cells were washed with PBS, fixed with 3% PFA in PBS for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked for 1 h (3% BSA, 0.1% Triton X-100 in PBS). Incubation with indicated primary antibodies was carried out for 2 h at RT, followed by incubation with Alexa Fluor-conjugated secondary antibodies (Abcam) for 30 min at RT. DAPI was used to stain DNA. Slides were mounted with ProLong Gold Antifade Mountant (Invitrogen). Images were acquired either using Axio Imager Zeiss 2 (EC Plan-Nefluar objectives) and analyzed with ZEN 2.3 or ImageJ software. Confocal images were acquired using Leica TCS

SP8 Confocal Microscope (HC PL APO CS2 40×/1.30 OIL objective) and analyzed with LAS software.

Mouse model

Fbxo38^{KO/KO} mice were generated in a C57BL/6N background using the CRISPR/Cas9 genome-editing system as described (Dibus et al, 2022b). Shortly, the Cas9 protein and gene-specific sgRNAs (Integrated DNA Technologies) were used for zygote electroporation. The genome editing was confirmed in the founder mouse using PCR.

Immunophenotyping

Single-cell suspensions were prepared from spleens using a 100 μm cell strainer. RBCs were lysed with ACK buffer. In total, 2×10^6 cells were stained with 50 μl FACS buffer (0.5 mM EDTA, 1 M HEPES and 2% FBS in HBSS without Mg^{2+} and Ca^{2+}) supplemented with 20% BD Horizon Brilliant Stain Buffer Plus, Fcblock (1:200) and fluorochrome conjugated antibodies. After 30 min, cells were washed with FACS buffer. SYTOX Blue (1:40,000) was added and 300,000 live leukocytes were acquired on Cytex Aurora spectral flow cytometer.

In vitro activation assay

T cells were isolated from mouse splenocytes using erythrocyte lysis (ACK buffer), followed by B220⁺ or CD19⁺ B-cells depletion with specific antibodies and anti-rat magnetic beads (Invitrogen 11417D). In all, 2×10^6 of the remaining cells were stimulated with 2×10^6 anti-CD3/CD28 beads (Gibco, 11453D) in 5 ml of IMDM medium supplemented with 10% FBS, antibiotics, and 10 ng/ml IL-2 (Gibco) for 96 h at 37 °C and 5% CO_2 .

LCMV infection

Mice were infected intraperitoneally with LCMV (Armstrong) dose of 2×10^5 PFU. The spleens were harvested on day 8 post-infection. Splenocytes were depleted of erythrocytes by incubation in ACK buffer. The samples were analyzed using Cytex Aurora (Cytex) or FACSSymphony (BD Biosciences) flow cytometers.

Flow cytometry analysis

For the analysis of mouse T cells, the following antibodies were used: anti-CD8a-BV421 (clone 53–6.7, BioLegend 100753, diluted 200×), anti-CD4-BV650 (clone RM4-5, BioLegend 100546, diluted 200×), anti-TCR β -FITC (clone H57-597, BioLegend 109206, diluted 400×), anti-CD25-PE-Cy7 (clone PC61, BioLegend 102016, diluted 200×) or anti-CD25-PE (clone PC61, BioLegend 102008, diluted 200×), anti-PD-1-APC (clone 29F.1a12, BioLegend 135209, diluted 200×), anti-B220-BV510 (clone RA3-6B2, BioLegend 103248, diluted 200×). LIVE/DEAD fixable near-IR dye (Thermo Fisher Scientific, L34976) was used for the viability staining.

LCMV-specific CD8⁺ T cells were identified by specific H-2d^b-NP396 tetramer staining (diluted 200×). The tetramers were produced by incubating biotinylated H-2D^b-NP396-PE (FQPQNGQFI) monomers (from NIH Tetramer Core Facility) with streptavidin-PE (Thermo Fisher Scientific, S866) at a molar ratio of 3:1. Streptavidin-PE was added in two doses with 1 h incubation on ice after each step. Staining was performed in 2% FBS, 2 mM EDTA, 0.1% sodium azide in PBS on ice for 30 min.

Fluorescence minus one (FMO) stained samples without anti-PD-1 antibody were used to gate PD-1⁺ cells.

For the analysis of human T cell-leukemia cell lines, anti-PD-1 (clone EH12-2H7; Exbio 11-176, diluted 500×) in combination with anti-mouse IgG secondary (Thermo Fisher Scientific 35503; DyLight 488) was used. Propidium iodide (1 µg/ml in PBS; Sigma) was used for the viability staining.

To perform intracellular staining, activated and naive cells were first fixed with 3% paraformaldehyde (PFA) and then stained with T-cell markers (anti-CD4/8). Subsequently, cells were either directly stained with anti-PD-1 antibody or permeabilized with 0.5% Triton X-100 (TX-100). After permeabilization, cells were further stained with anti-PD-1 antibody or 4',6-diamidino-2-phenylindole (DAPI) for subsequent cell cycle analysis.

cDNA preparation and RT-qPCR analysis

Synthesis of cDNA was performed using B-cell-depleted splenocytes that were either naive or activated as described in detail above. Total RNA (1 µg) isolated from these cells using the RNeasy Plus kit (Qiagen, 74134) was subjected to reverse transcription using Maxima[™] H Minus cDNA Synthesis Master Mix (Thermo Fisher, M1662).

For RT-qPCR analysis, the LightCycler 480 SYBR Green I master mix (Roche; 04887352001) was used and gene expression was analyzed using the LC480 Real-Time PCR system. Biological triplicate analyses were performed for each condition and relative expression levels of target genes were determined using the $2^{-\Delta(Ct^{Act} - Ct^{Naive})}$ method with means of normalized Ct values. TATA-binding protein (Tbp) cDNA levels were used for normalization. Detailed information regarding qPCR primers is given in the Reagents and Tools table.

Ethics statement

All animal experiments were approved by the Resort Professional Commission for Approval of Projects of Experiments on Animals of the Czech Academy of Sciences, Czech Republic (protocols 115/2016 and AVCR 1667/2022 SOV II) and were in accordance with the Czech Act No. 246/1992 Coll. and European directive 2010/63/EU.

Animals were maintained in a controlled, specific pathogen-free environment at the Animal Facilities of the Institute of Molecular Genetics of the Czech Academy of Sciences, Czech Republic. Mice were provided food and water ad libitum and kept in the animal facility with a 12-h light-dark cycle.

Quantification and statistical analysis

Graphs were generated using Prism version 10 (GraphPad Software). The quantity/densitometry of protein in western blots was analyzed using Image Lab software (Bio-Rad). Flow cytometry data were analyzed using FlowJo version 10.6.2 (BD Biosciences). A representative experiment out of three is shown for all western blots and immunofluorescence pictures. The geometric mean was used for the bar charts, and the error bars show the standard deviation or standard error mean. If not stated otherwise, all reported experiments were replicated at least twice in the laboratory. In mouse experiments, the sample size was estimated as a minimal number of mice which can provide conclusive results based on our previous experience. Experiments were not randomized because they were structured based on specific genotypes. Blinding was not

implemented, as the experiments involved objectively measured parameters using devices (such as FACS) that were independent of the researchers' subjective judgment. Student's t-test was employed to compare the means of the two groups in the RT-qPCR analysis, following confirmation that the assumptions of normality and equal variances were met.

Publicly available data from the GEO database were normalized using the GREIN application (<http://www.ilincs.org/apps/grein/>) (Mahi et al, 2019). A heatmap was created with Heatmapper (www.heatmapper.ca) (Babicki et al, 2016).

Data availability

All original uncut blots images, Ponceau S stainings, western blots densitometry, normalized Ct and expression values, and immunofluorescence images files are available as a Source data associated with Figures and Expanded View Figures. All flow cytometry files and gating strategies were deposited in BioStudies under accession number S-BSST1326 and are accessible via the following link <https://www.ebi.ac.uk/biostudies/studies/S-BSST1326>. All other raw data are available upon request.

The source data of this paper are collected in the following database record: [biostudies:S-SCDT-10_1038-S44319-024-00220-8](https://www.ebi.ac.uk/biostudies/studies/S-SCDT-10_1038-S44319-024-00220-8).

Expanded view data, supplementary information, appendices are available for this paper at <https://doi.org/10.1038/s44319-024-00220-8>.

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Author contributions

Nikol Dibus: Conceptualization; Investigation; Methodology; Writing—original draft; Writing—review and editing. **Eva Salyova:** Investigation. **Karolina Kolarova:** Investigation. **Alikhan Abdirov:** Investigation. **Michele Pagano:** Conceptualization; Supervision; Writing—review and editing. **Ondrej Stepanek:** Conceptualization; Supervision; Funding acquisition; Methodology; Writing—review and editing. **Lukas Cermak:** Conceptualization; Supervision; Funding acquisition; Investigation; Methodology; Writing—original draft; Writing—review and editing.

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Disclosure and competing interests statement

MP is a scientific cofounder of SEED Therapeutics; receives research funding from and is a shareholder in Kymera Therapeutics; and is a consultant for, a member of the scientific advisory board of, and has financial interests in CullGen, SEED Therapeutics, Triana Biomedicines, and Umbra Therapeutics; however, no research funds were received from these entities, and the findings presented in this manuscript were not discussed with any person in these companies. The remaining authors declare no competing interests.

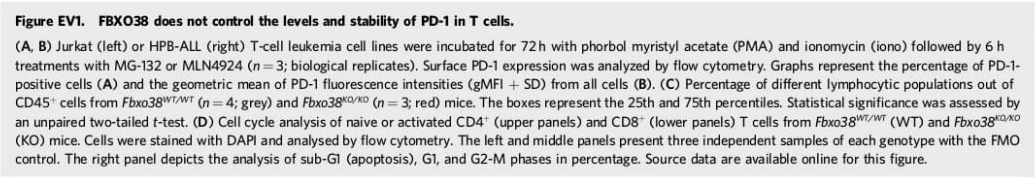
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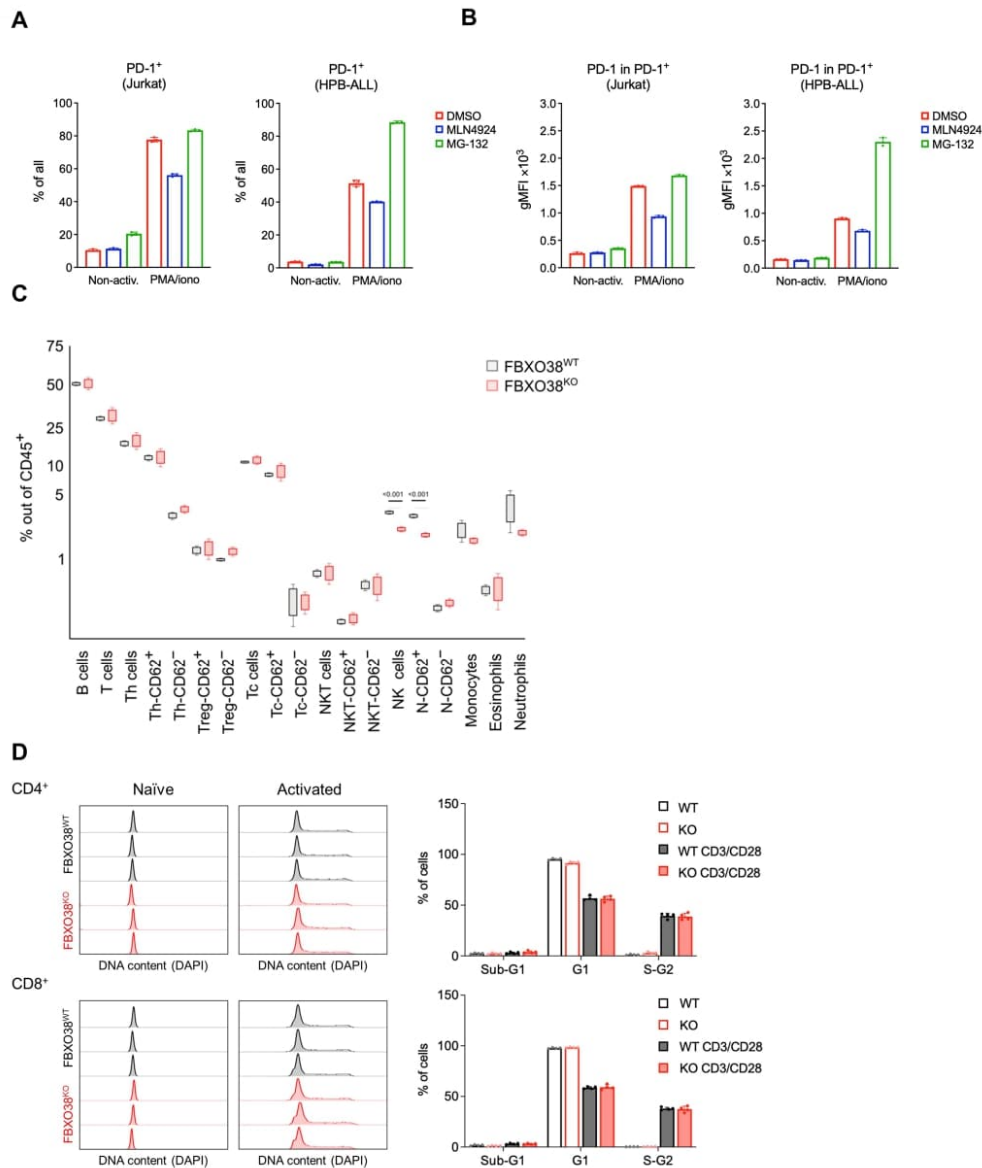
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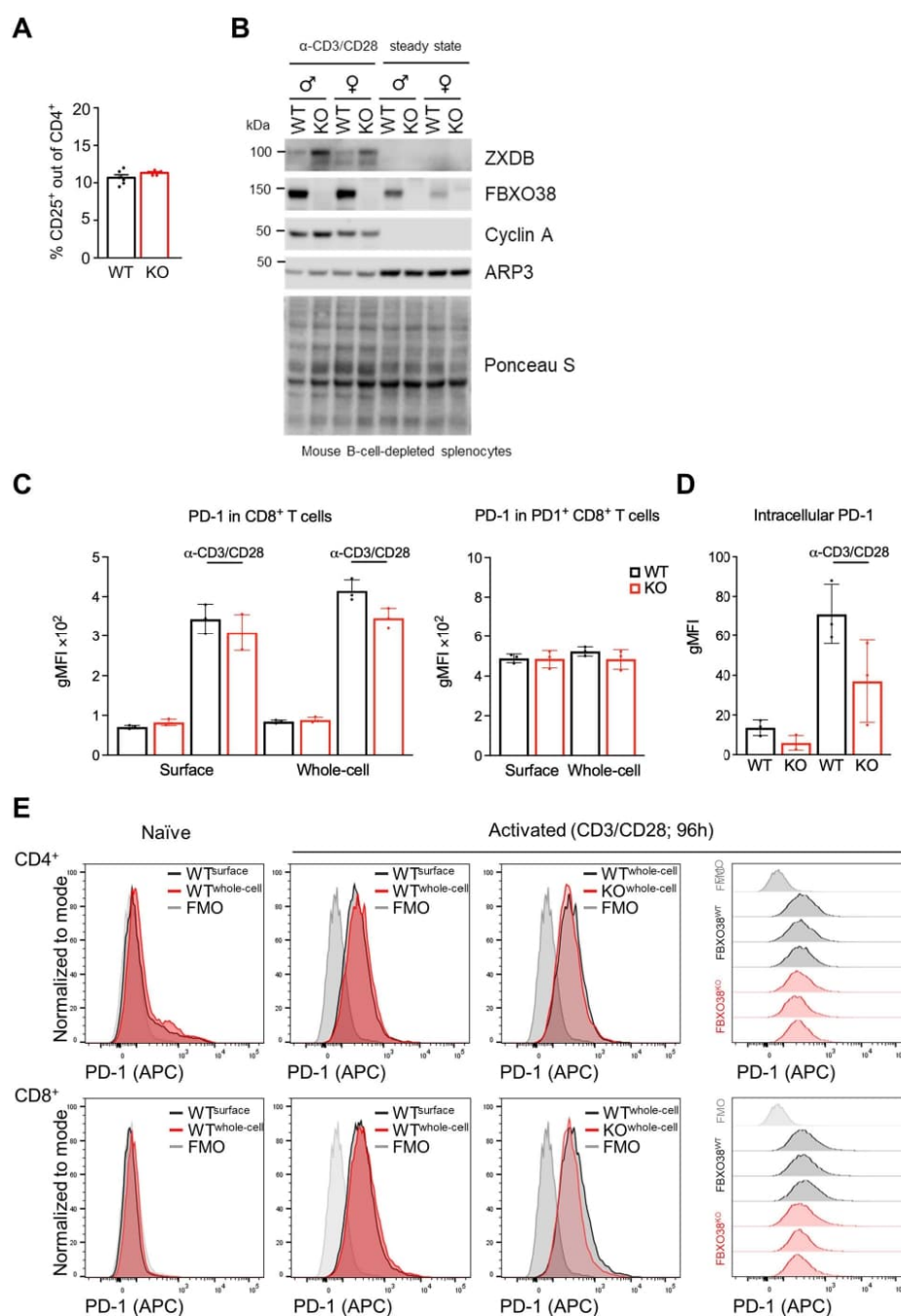
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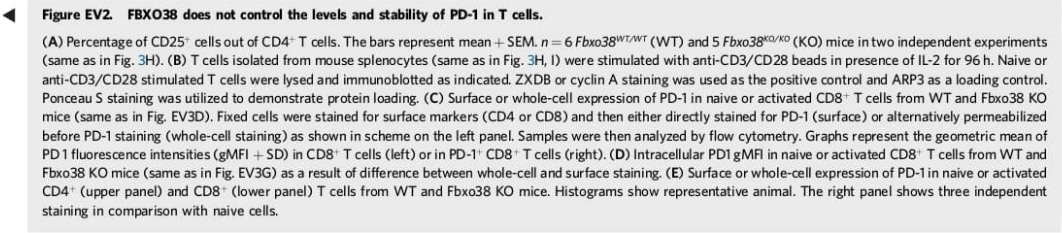
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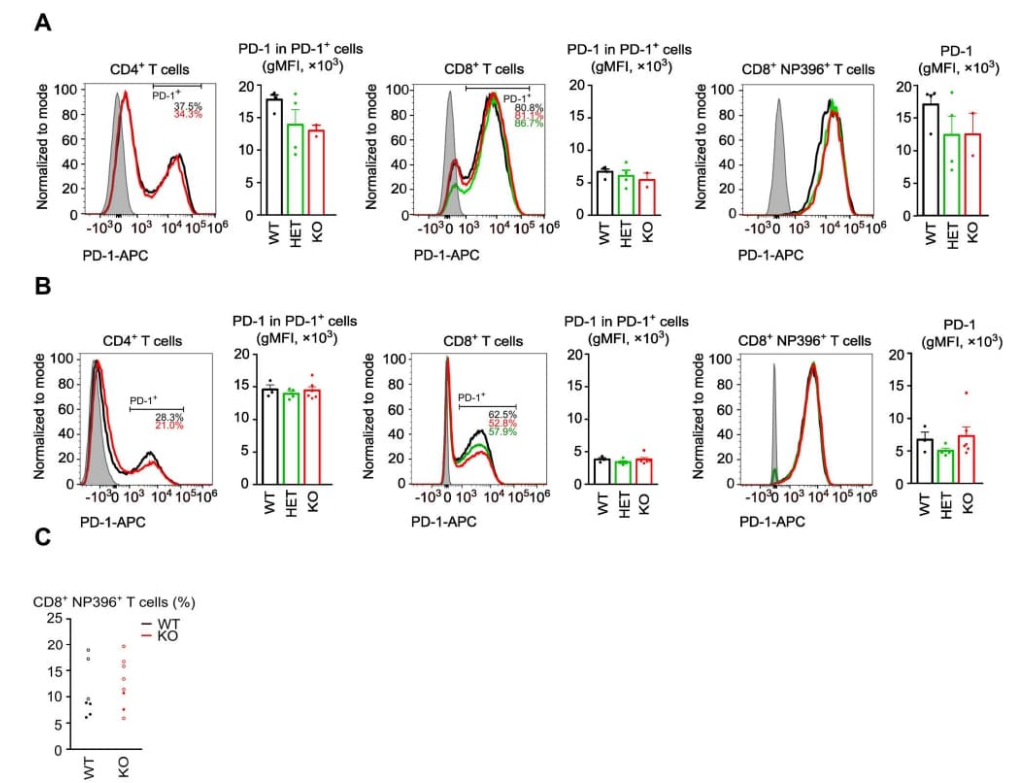


Figure EV3. Fbxo38 does not control the levels or stability of PD-1 upon viral infection.

(A, B) Cell-surface expressions of PD-1 in T cells isolated from the spleens of LCMV-infected *Fbxo38*^{WT/WT} and *Fbxo38*^{KO/KO} mice. The results of two independent experiments (A, B) are presented as normalized data in Fig. 4C. Histograms represent the analysis of representative samples. Bars show cell-surface PD-1 levels in CD4⁺ PD-1⁺, CD8⁺ PD-1⁺ and CD8⁺ D^b-NP396 tetramer⁺ T cells (all PD-1⁺ gate). *n* = 7 *Fbxo38*^{WT/WT}, 8 *Fbxo38*^{KO/KO}, and 8 *Fbxo38*^{WT/KO} mice. (C) WT and KO mice were infected with LCMV. The percentage of Db-NP396 tetramer⁺ LCMV-specific T cells out of splenic CD8⁺ T cells was quantified 8 days post-infection by flow cytometry (same experiments as shown in Fig. 4C).

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