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STUDY OF UBAP2L ROLE IN CELL HOMEOSTASIS

ETUDE DU ROLE DE UBAP2L DANS L'HOMEOSTASIE CELLULAIRE

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A ma mère,

Du plus profond de mon cœur, merci ...

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RESUME DE THESE EN FRANCAIS

INTRODUCTION

La mitose est un processus cellulaire conservé au cours de l'évolution qui assure la répartition égale de l'information génétique d'une cellule mère à deux cellules filles. D'importants changements morphologiques ont lieu au cours de la mitose, et ces changements sont largement médiés par de multiples modifications post-traductionnelles (PTMs) des facteurs mitotiques. En effet, l'ubiquitylation et la phosphorylation sont des événements clés dans le processus de mitose et sont médiées par les actions étroitement régulées des Ubiquitine (Ub) ligases, des enzymes de déubiquitylation (DUBs), des kinases et des phosphatases. L'une des kinases les mieux caractérisées est la Polo-Like Kinase 1 (PLK1), qui joue un rôle essentiel tout au long de la mitose, en régulant l'assemblage précoce du fuseau mitotique ainsi que la ségrégation des chromosomes et la cytokinèse, préservant ainsi la stabilité du génome et la survie des cellules (Schmucker and Sumara, 2014). L'expression de PLK1 est régulée par le cycle cellulaire, étant faible pendant l'interphase, augmentant en phase G2, atteignant un pic pendant la mitose et chutant à nouveau en sortie de mitose via sa dégradation protéolytique médiée par l'ubiquitine ligase E3 du complexe promoteur de l'anaphase/cyclosome (APC/C) (Lindon and Pines, 2004; Bruinsma et al., 2012). PLK1 se compose d'un domaine kinase (KD) abritant son activité catalytique et de deux domaines polo-box (PBD) contrôlant la spécificité du substrat de PLK1 et son auto-inhibition (Zitouni et al., 2014). Il est intéressant de noter que PLK1 se localise dynamiquement à différentes structures mitotiques dépendamment des phosphorylations et ubiquitylations (Schmucker and Sumara, 2014). D'autre part, le complexe $CRL3^{KLHL22}$ monoubiquityle PLK1, déclenchant sa dissociation de ses phosphorécepteurs du kinétochore (KT) avant l'anaphase (Beck et al., 2013). Hormis ces études, la régulation spatio-temporelle ubiquitine-dépendante de PLK1 pendant la mitose reste mal définie. Dans le but de mieux caractériser les voies d'ubiquitylation contrôlant la division cellulaire, notre laboratoire a réalisé un criblage visuel à haut contenu de petits acides ribonucléiques interférents (siRNA) pour les protéines de liaison à l'ubiquitine (UBP) connues et prédites et a évalué l'effet de leur déplétion sur la progression mitotique en examinant la forme du noyau des cellules (Krupina et al., 2016). En effet, les atypies nucléaires sont très souvent le résultat de défauts de ségrégation chromosomique. Parmi les meilleurs résultats de ce criblage, la déplétion de la protéine de liaison à l'ubiquitine 2-Like (UBAP2L), également appelée NICE-4, provoque de graves irrégularités nucléaires telles que la multinucléation et des noyaux polylobés, très similaires à

celles observées lors de la régulation négative du contrôle positif Cullin3 (CUL3), ce qui nous a incité à étudier le rôle potentiel d'UBAP2L pendant la mitose. UBAP2L est composé d'un domaine d'ubiquitine (UBA) et d'un domaine de liaison à l'ARN (RGG) dans sa partie N-terminale (NT) et d'un domaine de fonction inconnue (DUF) dans sa partie C-terminale (CT) (Guerber et al., 2022). Des données récentes suggèrent que UBAP2L pourrait réguler la progression mitotique via la méthylation de son domaine RGG (Maeda et al., 2016). Cependant, le mécanisme sous-jacent et les cibles mitotiques potentielles en aval d'UBAP2L n'ont pas encore été identifiés.

Au cours de mon doctorat, j'ai tenté d'élucider les fonctions d'UBAP2L dans l'homéostasie cellulaire et plus précisément, j'ai étudié le rôle d'UBAP2L pendant la division cellulaire.

RÉSULTATS

UBAP2L régule la ségrégation correcte des chromosomes pendant la mitose

Afin de corroborer les données publiées précédemment et nos résultats de criblage, des expériences de vidéo-microscopie en direct ont été menées pour visualiser la progression mitotique des cellules HeLa de type sauvage (WT) et de type Knock-Out (KO) d'UBAP2L. De manière frappante, la déplétion d'UBAP2L entraîne un retard de l'entrée en mitose, un allongement de la durée de la prophase à l'anaphase, des désalignements chromosomiques sévères en métaphase et des ponts d'ADN de l'anaphase à la télophase. Ces défauts mitotiques conduisent à la formation de multiples micronoyaux (MN) après la sortie de la mitose ou à la mort cellulaire après un arrêt mitotique prolongé. La présence de MN et la forme anormale des noyaux ont été confirmées dans des lignées cellulaires dérivées du cancer colorectal et de l'ostéosarcome, suggérant que UBAP2L est cruciale pour la progression mitotique normale.

UBAP2L régule spécifiquement les niveaux protéiques et l'activité de PLK1 par l'intermédiaire de son domaine C-terminal

Les phénotypes stringents observés lors de la déplétion d'UBAP2L nous ont incité à analyser si UBAP2L pourrait réguler des facteurs mitotiques clés afin d'assurer la fidélité de la ségrégation des chromosomes. À cette fin, j'ai analysé l'effet de la déplétion d'UBAP2L sur les facteurs mitotiques Aurora A (AURA), Aurora B (AURB), PLK1 et Cyclin B1 ainsi que d'autres membres de la famille PLK. De manière surprenante, bien que la régulation à la baisse ou la déplétion de UBAP2L n'affectent pas les niveaux protéiques et la localisation de AURA, AURB, Cyclin B1 et d'autres membres de la famille PLK, elles augmentent considérablement les niveaux protéiques et l'activité de PLK1. L'absence d'UBAP2L induit également l'accumulation nucléaire de PLK1 sous la forme de « points » correspondant aux

centromères/KTs, suggérant que UBAP2L régule spécifiquement PLK1. De plus, j'ai démontré que les cellules KO pour UBAP2L présentent des niveaux accrus de protéine PLK1 pendant les phases G1 et S mais pas en G2 par rapport aux cellules WT. En outre, après l'inhibition de la traduction, PLK1 reste stable jusqu'à 8 heures après le traitement au cycloheximide (CHX) dans les cellules dépourvues d'UBAP2L et l'inhibition du protéasome n'augmente pas davantage les niveaux de PLK1 observés dans les cellules UBAP2L KO, suggérant que UBAP2L favorise la dégradation de PLK1. De manière importante, j'ai prouvé que la régulation de PLK1 par UBAP2L est spécifiquement médiée via son domaine CT et n'est pas liée à sa fonction précédemment rapportée dans l'assemblage des granules de stress (SGs) (Huang et al., 2020). Étant donné qu'une division cellulaire erronée entraîne souvent la mort cellulaire, j'ai caractérisé la capacité de prolifération et la viabilité à long terme des cellules UBAP2L KO et j'ai constaté de nombreuses déficiences par rapport à la lignée cellulaire témoin, un phénotype qui peut être attribué au domaine CT de UBAP2L.

UBAP2L se localise aux kinétochores pendant la mitose et favorise l'élimination de PLK1 de ces structures

Pour mieux comprendre comment UBAP2L régule la localisation de PLK1 aux KTs, j'ai étudié la localisation d'UBAP2L pendant la mitose et j'ai montré qu'UBAP2L est recruté aux KTs de la prométaphase à la métaphase et qu'il est éliminé de l'anaphase à la télophase. Il est important de noter qu'alors que les niveaux globaux de protéine UBAP2L diminuent lors de l'abrogation de PLK1 ou de son inhibition catalytique avec l'inhibiteur BI2536, sa localisation aux KTs augmente dans les mêmes conditions, suggérant une boucle de rétroaction positive dans laquelle PLK1 favoriserait le recrutement de UBAP2L aux KTs pour assurer sa propre régulation. Enfin, j'ai pu montrer que l'accumulation de PLK1 aux KTs observée lors de la déplétion d'UBAP2L est due à sa non-élimination pendant la mitose plutôt qu'à un recrutement accru en G1 où il a été établi que PLK1 joue un rôle clé dans le dépôt *de novo* de CENP-A (McKinley and Cheeseman, 2014).

UBAP2L pourrait être impliqué dans la voie PLK1-CUL3^{KLHL22} pour assurer la stabilité du génome

Pour évaluer plus en détail l'implication de UBAP2L dans le retrait de PLK1 des KTs, j'ai réalisé des expériences d'immunoprécipitation (IP) endogène pour analyser tout effet sur la voie de signalisation PLK1-CUL3^{KLHL22}. J'ai démontré que UBAP2L interagit avec PLK1, CUL3 et KLHL22 mais pas avec AURB, confirmant à nouveau sa spécificité envers PLK1. De manière importante, la déplétion d'UBAP2L perturbe l'interaction entre PLK1 et CUL3, pouvant

expliquer, au moins partiellement, les défauts d'éviction de PLK1 des KT observés dans les cellules déplétées pour UBAP2L. De plus, la polyubiquitylation de PLK1 est nettement diminuée dans les cellules UBAP2L KO par rapport aux cellules WT, suggérant que UBAP2L pourrait réguler la signalisation protéolytique et non protéolytique de PLK1 pendant la sortie mitotique. Il est important de noter que le rétablissement de l'activité enzymatique de PLK1 à des niveaux basaux à l'aide de faibles doses de BI2536 dans les cellules déplétées de UBAP2L permet de corriger toutes les erreurs de ségrégation observées dans ces cellules, à savoir les mauvais alignements chromosomiques, les ponts d'ADN et la formation de MN. Les résultats décrivant le rôle direct de UBAP2L dans la régulation de PLK1 durant la mitose sont inclus dans un manuscrit soumis pour publication (Guerber et al., soumis pour publication à *Journal of Cell Biology*).

UBAP2L inhibe les dommages à l'ADN causés par des facteurs endogènes

Comme mentionné ci-dessus, la déplétion d'UBAP2L entraîne de graves erreurs de ségrégation, une altération de la prolifération et de la survie cellulaires à long terme et la formation de MN. Les MN sont généralement considérés comme des caractéristiques typiques de l'instabilité génomique. En effet, les cellules UBAP2L KO ont montré une augmentation frappante de γH2AX, un marqueur commun des cassures double-brin de l'ADN (DSB) par rapport aux cellules témoins. En outre, la mise sous silence de PLK1 ou son inhibition par le BI2536 ne permettent pas de corriger l'augmentation des dommages observés dans les cellules UBAP2L KO, indiquant une fonction de UBAP2L indépendante de PLK1 dans la signalisation des dommages à l'ADN.

UBAP2L est un régulateur négatif de l'autophagie

Enfin, j'ai découvert que la déplétion de UBAP2L induit de forts défauts d'autophagie, évalués par l'accumulation de granules autophagiques. Plus précisément, je fournis des preuves de défaillance de fusion autophagosome-lysosome et d'une légère déficience de l'initiation de l'autophagie dans les cellules UBAP2L KO par rapport au contrôle, des phénotypes qui devront être étudiés plus en détail à l'avenir.

CONCLUSIONS

Nos données suggèrent un modèle dans lequel UBAP2L exerce des rôles clés dans divers processus cellulaires distincts. Nous montrons que UBAP2L régule spécifiquement la localisation mitotique de PLK1 en permettant son retrait des KTs pendant la métaphase, servant probablement de co-adaptateur ou chaperon pour la reconnaissance de PLK1 dépendante de

CUL3 au niveau des KTs. Cependant, d'autres travaux sont nécessaires pour comprendre le mécanisme moléculaire précis qui conduit au désassemblage de PLK1 des KTs. De plus, nous fournissons des preuves que le domaine C-terminal de UBAP2L est crucial pour cette fonction sur PLK1 et pour la survie cellulaire alors que le domaine UBA-RGG ne semble pas jouer un rôle majeur dans ces processus. Ceci est en accord avec les données précédemment publiées suggérant que la partie NT d'UBAP2L n'est pas suffisante pour restaurer une progression mitotique normale (Maeda et al., 2016). En outre, nous démontrons que UBAP2L régule la stabilité de la protéine PLK1 à la sortie de la mitose. Il serait fascinant d'élucider si et comment UBAP2L coopère avec les machineries de dégradation pour assurer la dégradation correcte de PLK1 et la sortie mitotique. Par ailleurs, nous présentons des preuves que UBAP2L se localise aux KTs d'une manière dépendante de PLK1, étant progressivement recruté de la prométaphase à la métaphase. Des efforts supplémentaires seront nécessaires à l'avenir pour disséquer comment exactement PLK1 favorise ce recrutement. De manière intéressante, il a été rapporté que UBAP2L est phosphorylée pendant la mitose mais la kinase impliquée n'a pas encore été identifiée. De plus, nous avons montré que la déplétion d'UBAP2L provoque des altérations chromosomiques sévères telles que des désalignements, des ponts ADN et des MN, tous ces phénotypes étant dus à l'activité enzymatique aberrante de PLK1 dans ces cellules. Enfin, nous ouvrons un large champ de recherche sur UBAP2L en identifiant plusieurs phénotypes frappants résultant de la déplétion d'UBAP2L, telles qu'une instabilité génomique élevée et des perturbations de l'autophagie. D'autres recherches seront nécessaires afin de clarifier l'implication précise d'UBAP2L dans ces processus.

THESIS SUMMARY IN ENGLISH

INTRODUCTION

Mitosis is an evolutionary conserved cellular process ensuring equal partitioning of genetic information from one mother cell to two genetically identical daughter cells. Extensive morphological changes need to take place during mitosis and this is largely mediated by the cooperative actions of multiple post-translational modifications (PTMs) on mitotic factors. Indeed, ubiquitylation and phosphorylation are key events in the process of mitosis and they are mediated by the tightly-regulated actions of Ubiquitin (Ub) ligases, deubiquitylating enzymes (DUBs), kinases and phosphatases. One of the most well-characterized kinases is the Polo-Like Kinase 1 (PLK1), which executes essential roles throughout mitosis, regulating early mitotic spindle assembly as well as chromosome segregation and subsequent cytokinesis, thereby safeguarding genome stability and cell survival (Schmucker and Sumara, 2014). PLK1 expression is cell cycle regulated, being low during interphase, increasing in G2 phase, peaking during mitosis and dropping again during mitotic exit via proteolytic degradation mediated by the anaphase promoting complex/cyclosome (APC/C) E3 ubiquitin ligase (Lindon and Pines, 2004; Bruinsma et al., 2012). PLK1 consists of a kinase domain (KD) harboring the kinase catalytic activity and two polo-box domains (PBDs) controlling PLK1 substrate specificity and self-inhibition (Zitouni et al., 2014). Interestingly, PLK1 dynamically localizes to different mitotic structures in phosphorylation- and ubiquitylation-dependent manners (Schmucker and Sumara, 2014). Of note, the Cullin3 (CUL3)-based complex $CRL3^{KLHL22}$ has been proposed to monoubiquitylate PLK1 and triggers its dissociation from its kinetochore (KT) phosphoreceptors prior to anaphase (Beck et al., 2013). Despite these research efforts, spatio-temporal, ubiquitin-dependent regulation of PLK1 during mitosis remains ill-defined. In order to better characterize ubiquitylation pathways controlling cell division, our lab performed a high-content visual small interfering ribonucleic acid (siRNA) screen for known and predicted ubiquitin-binding proteins (UBPs) and assessed the effect of their depletion on mitotic progression by looking at cell nuclear shape (Krupina et al., 2016). In fact, nuclear atypia is very often the result of chromosome segregation defects. Among the top hits of this screen, we found the Ubiquitin-Binding Protein 2-Like (UBAP2L), also called NICE-4, to cause severe nuclear shape irregularities such as multinucleation and polylobed nuclei very similar to those observed upon downregulation of the positive control CUL3, which prompted us to investigate the potential role of UBAP2L during mitosis. UBAP2L is composed of an ubiquitin (UBA) and a RNA-binding (RGG) domain in its N-terminal part (NT) and a domain of unknown function

(DUF) in its C-terminal part (CT) (Guerber et al., 2022). Interestingly, recent data suggested that UBAP2L may regulate mitotic progression via the methylation of its RGG domain (Maeda et al., 2016). However, the underlying mechanism and potential UBAP2L downstream mitotic targets have not yet been identified.

During my PhD, I attempted to elucidate UBAP2L functions in cellular homeostasis and more precisely, I investigated the role of UBAP2L during cell division.

RESULTS

UBAP2L regulates proper chromosome segregation during mitosis

To corroborate previously published data and our screen results, live-video microscopy experiments were conducted to visualize mitotic progression in UBAP2L wild-type (WT) and Knock-Out (KO) HeLa cells. Strikingly, UBAP2L depletion led to mitotic entry delay, extended prophase to anaphase length, severe chromosome misalignments during metaphase and DNA bridges during anaphase and telophase. These mitotic defects led to the formation of multiple micronuclei (MN) after mitotic exit or cell death after prolonged mitotic arrest. The presence of MN and abnormal nuclear shape were further confirmed in colorectal cancer and osteosarcoma-derived cell lines, strongly suggesting that UBAP2L is important for normal mitotic progression.

UBAP2L specifically regulates PLK1 levels and activity through its C-terminal domain

The strong phenotypes observed upon UBAP2L depletion prompted us to analyze whether UBAP2L might regulate key mitotic factors as a means to ensure fidelity of chromosome segregation. To this end, I analyzed the effect of UBAP2L depletion on the key mitotic factors Aurora A (AURA), Aurora B (AURB), PLK1 and Cyclin B1 as well as other members of the PLK family. Surprisingly, although UBAP2L downregulation or depletion did not affect the protein levels and localization of AURA, AURB, Cyclin B1 and other PLK family members, it dramatically increased PLK1 protein levels and activity. UBAP2L depletion also induced the nuclear accumulation of PLK1 in a dotty pattern corresponding to centromeres/KTs, suggesting that UBAP2L may specifically regulate PLK1. Further on, I demonstrated that UBAP2L KO cells display increased PLK1 protein levels during G1 and S phases but not during G2 compared to WT cells. Interestingly, following translation inhibition, PLK1 remained stable up to 8h after cycloheximide (CHX) treatment in UBAP2L-depleted cells and proteasomal inhibition did not further increase PLK1 levels observed in UBAP2L KO cells relative to WT cells, suggesting that UBAP2L may promote PLK1 degradation. Importantly, I proved that UBAP2L-dependent PLK1 regulation is mediated by its CT domain and not related to its previously reported

function in stress granules (SGs) assembly (Huang et al., 2020). Because erroneous cell division often leads to cell death, I characterized the long-term proliferation capacity and viability of UBAP2L KO cells and found that these cells display proliferation and cell survival deficiencies compared to the isogenic-control cell line, a phenotype that can be attributed to the CT domain of UBAP2L.

UBAP2L localizes to kinetochores during mitosis and promotes PLK1 removal from these structures

To gain insights into how UBAP2L regulates PLK1 KT localization, I investigated UBAP2L localization during mitosis and showed that UBAP2L is recruited to KTs from prometaphase to metaphase and removed from anaphase to telophase. Interestingly, while UBAP2L global protein levels were decreased upon PLK1 silencing or inhibition with the small molecule inhibitor BI2536, its KT localization was increased under the same conditions, suggesting that there might exist a positive feedback loop in which PLK1 promotes UBAP2L recruitment to KT to ensure its own finetuning. Finally, I could show that PLK1 KT accumulation observed upon UBAP2L depletion is due to its non-removal during mitosis rather than an increased recruitment in G1 where it has been established to play key roles in CENP-A *de novo* deposition (McKinley and Cheeseman, 2014).

UBAP2L may be involved in the PLK1-CUL3^{KLHL22} pathway to ensure genome stability

To further assess the involvement of UBAP2L into PLK1 KT removal, I performed endogenous immunoprecipitation (IP) experiments to analyze any effects on the PLK1-CUL3^{KLHL22} pathway. I first demonstrated that UBAP2L interacts with PLK1, CUL3 and KLHL22 but not AURB, again confirming specificity towards PLK1. Importantly, UBAP2L depletion disrupted the interaction between PLK1 and CUL3, which could at least partially explain PLK1 KT removal defects observed in UBAP2L-depleted cells. Moreover, PLK1 polyubiquitylation was markedly decreased in KO cells relative to WT cells, suggesting that UBAP2L may regulate PLK1 proteolytic and non-proteolytic signaling during mitotic exit. Importantly, restoring PLK1 enzymatic activity to basal levels using low doses of BI2536 in UBAP2L-downregulated cells rescued all segregation errors observed in UBAP2L-depleted cells, namely chromosome misalignments, DNA bridges and MN formation, providing evidence that the chromosomal abnormalities characterizing UBAP2L-downregulated cells are the direct consequence of PLK1 aberrant enzymatic activity. The results describing the direct role of UBAP2L in the regulation

of PLK1 during mitosis have been included in a submitted manuscript (Guerber et al., submitted to *Journal of Cell Biology*).

UBAP2L inhibits DNA damage caused by endogenous factors

As mentioned above, UBAP2L depletion leads to severe segregation errors, impaired long-term cellular proliferation and survival and MN formation. MN are generally considered as typical features of genomic instability. Indeed, UBAP2L KO cells displayed a striking increase of γH2AX, a common marker of DNA double-strand breaks (DSBs) relative to control cells. Interestingly, PLK1 silencing or inhibition by BI2536 did not rescue the increased damage observed in UBAP2L KO cells, pointing to a PLK1-independent function of UBAP2L in DNA damage signaling.

UBAP2L is a negative regulator of autophagy

Moreover, I found that UBAP2L depletion induces strong autophagy defects as assessed by the accumulation of autophagic granules. More precisely, I provide evidence for autophagosome-lysosome fusion defects and mild impairment of autophagy initiation in UBAP2L KO cells relative to control, phenotypes that will need to be further studied in the future.

CONCLUSIONS

Our data suggest a model in which UBAP2L exerts key roles in various distinct cellular processes. We show that UBAP2L specifically regulates PLK1 localization during mitosis by allowing its removal from KTs during metaphase possibly serving as a co-adaptor or chaperone for CUL3-dependent PLK1 recognition at KTs. However, further work is needed to understand the precise molecular mechanism driving PLK1 disassembly from KTs. Moreover, we provide evidence that the C-terminal domain of UBAP2L is crucial to mediate its function on PLK1 and for cell survival whereas the UBA-RGG domain does not seem to play a major role in these processes. This is in line with previously published data which suggested that the N-terminal part of UBAP2L is not sufficient to restore normal mitotic progression (Maeda et al., 2016). Furthermore, we demonstrate that UBAP2L regulates PLK1 protein stability at mitotic exit. It would be fascinating to elucidate if and how UBAP2L cooperates with degradation machineries to ensure proper PLK1 degradation and mitotic exit. In addition, we present evidence that UBAP2L localizes to KTs in a PLK1-dependent manner, being gradually recruited from prometaphase to metaphase. More efforts will be needed in the future to dissect how exactly PLK1 promotes UBAP2L recruitment to kinetochores. Interestingly, UBAP2L was reported to be phosphorylated during mitosis but the involved kinase has not yet been identified. Furthermore, we showed that UBAP2L depletion causes severe chromosomal alterations such

as misalignments, DNA bridges and MN, all phenotypes owing to PLK1 aberrant enzymatic activity in these cells. Finally, we open a broad research area on UBAP2L by identifying several striking phenotypes arising upon UBAP2L depletion such as high genomic instability and autophagy perturbations. Further investigations will be required in order to clarify the precise involvement of UBAP2L in these processes.

LIST OF ABBREVIATIONS

µm: micrometer

µM: micromolar

53BP1: p53-Binding Protein 1

A.U.: Arbitrary Unit

aa: aminoacid

AKT: serine/threonine protein kinase

ANOVA: Analysis Of Variance

APC/C: Anaphase-Promoting Complex/Cyclosome

APC11: Anaphase-Promoting Complex subunit 11

ASB7: Ankyrin repeat and SOCS Box protein 7

ATM: Ataxia Telangiectasia Mutated

ATP: Adenosine Triphosphate

ATR: Ataxia Telangiectasia and Rad3-related protein

AUR: Aurora

BafA1: Bafilomycin A1

BI2536 : PLK1 inhibitor

BORA: Protein Aurora Borealis

BSA: Bovine Serum Albumine

BTB: Bric-a-brac-Tramtrack-Broad complex

BUB1/3: Budding Uninhibited by Benzimidazoles 1/3

BUBR1: BUB1-Related Protein 1

CAP: Chromosome-Associated Protein

Cas9: CRISPR associated protein 9

CCAN: Constitutive Centromere-Associated Network

CCNB1: Cyclin B1

CDC: Cell division cycle protein

CDE: Cell cycle-dependent element

CDH1: CDC20 homolog 1

CDK: Cyclin-Dependent Kinase

cDNA: complementary DNA

CENP: Centromere Proteins

CEP55: Centrosomal Protein 55 kDa

CFA: Colony Formation Assay

CHK: Checkpoint kinase

CHR: Cell cycle genes homology region

CHX: Cyclohexamide

C-Mad2: Closed-Mad2

CP110: Centriolar coiled-coil protein of 110 kDa

CPC: Chromosome Passenger Complex

C-RAF: Cellular-Rapidly Accelerated Fibrosarcoma

CREST: Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CRL: Cullin-RING Ligase

CRM1: Chromosomal Region Maintenance 1

CT: C-Terminal

CtBP: C-terminal-binding protein

CtIP: CtBP-interacting protein

CUL: Cullin

DAPI: 4',6-Diamidino-2-phenylindole dihydrochloride

DCAF: DDB1- and CUL4-Associated Factor

DDA3: proline/serine-rich coiled-coil protein 1 (PSRC1)

DDB1: DNA Damage-Binding Protein 1

DDR: DNA Damage Response

DLD-1: Colorectal adenocarcinoma cell line

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DSB: Double Strand Break

DTB: Double Thymidine Block

DTBR: DTB and Release

DUB: Deubiquitinating enzyme

DUF: Domain of Unknown Function

Dvl2: Dishevelled 2

E1: Ub-activating enzyme

E2: Ub-conjugating enzyme

E3: Ub-ligase

ECL: Enhanced Chemiluminescence

EDTA: Ethylenediaminetetraacetic Acid

Eg5: Kinesin-5 or KIF-11

eGFP: enhanced GFP

EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EMI1: Early Mitotic Inhibitor 1

EMT: Epithelial-Mesenchymal Transition

ERK: Extracellular signal-Regulated Kinase

FACS: Fluorescence-Activated Cell Sorting

FBXW8: F-box/WD repeat-containing protein 8

FCS: Foetal Calf Serum

FGFR1: Fibroblast growth factor receptor 1 protein

FK2: Anti-Ubiquitinylated proteins Antibody, clone FK2

FL: Full Length

FOP: FGFR1 Oncogene Partner

FOR20: FOP-related protein of 20 KDa

FOXM1: Forkhead box protein M1

Fw: Forward

FZR1: Fizzy And Cell Division Cycle 20 Related 1

G1: Gap phase 1

G2: Gap phase 2

G3BPs: Ras GTPase-activating protein-binding proteins

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDP: Guanosine Diphosphate

GFP: Green Fluorescent Protein

gRNA: guide RNA

GTP: Guanosine Triphosphate

h: hour

H2A: Histone 2A

H2AX: Histone H2A Variant X

H3: Histone 3

HBO1: Histone acetyltransferase Binding to ORC1

HCl: Hydrogen Chloride

HECT: Homologous to E6-AP Carboxyl Terminus

HEF1: Human Enhancer of Filamentation 1

HeLa K: HeLa Kyoto

HeLa: *Henrietta Lacks*, cervical cancer cell line

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His: Histidine

HR: Homologous recombination

HRP: Horseradish Peroxidase

HU: Hydroxyurea

IF: Immunofluorescence

IgG: Immunoglobulin G

INCENP: Inner Centromere Protein

IP: Immunoprecipitation

JUNB: Transcription factor jun-B

K: Lysine

KD: Kinase Domain

kDa: Kilodalton

K-fibers: kinetochore fibers

KI: Knock-In

KLHL: Kelch-Like proteins

Klp2: Kinesin-like protein 2-A

KMN: KNL1, Mis12, Ndc80 complex

KNL1: Kinetochore-Null Protein 1 or Kinetochore-scaffold protein 1

KO: Knock-Out

KT: Kinetochore

l.e.: long exposure

LB: Laemmli Buffer

LC3: Microtubule-associated protein 1A/1B-light chain 3

LMB: Leptomycin B

M: Mitosis

Mad1/2: Mitotic Arrest Deficient 1/2

MCAK: Mitotic Centromere-Associated Kinesin

MCC: Mitotic Checkpoint Complex

MCM: Minichromosome Maintenance

MES: 2-(N-morpholino)ethanesulfonic acid

MG132: Proteasome inhibitor, carbobenzoxy-l-leucyl-l-leucyl-l-leucinal

Min: minutes

Mis: MIS kinetochore complex component

MKLP2: Mitotic Kinesin-Like Protein 2

MN: Micronuclei

MPS1: Monopolar Spindle 1

MR: Monastrol Release

MRE11: Meiotic Recombination 11

MRFAP1: Mof4 Family Associated Protein 1

mRFP: monomeric RFP

MRN: MRE11, RAD50, NBS1

MST2: Mammalian STE20-like protein kinase 2

MT: Microtubule

MTOC: Microtubule Organizing Center

mTOR: mammalian Target of Rapamycin

mTORC: mTOR complex

MW: Molecular Weight

MYT1: Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase

NaCl: Sodium Chloride

NaF: Sodium Fluoride

Na-Pyr: Sodium-Pyruvate

NBS1: Nijmegen Breakage Syndrome protein 1

NCS: Neocarzinostatin

NDC80: Kinetochore protein NDC80

NEDD: Neural precursor cell Expressed Developmentally Downregulated protein

NEK2: NIMA-related kinase 2

Nek2A: NIMA-related kinase 2A

NES: Nuclear Export Signal

NIPA: Non-Imprinted in Prader-Willi/Angelman syndrome region protein

NLS: Nuclear Localization Signal

nM: nanomolar

NP-40: Nonyl Phenoxypolyethoxylethanol

ns: non-significant

NT: N-Terminal

NUMB: Protein numb homolog

O-Mad2: Open-Mad2

ORC2: Origin Recognition Complex subunit 2

P: P value

PAM: Protospacer Adjacent Motif

PBD: Polo-Box Domain

PBIP1: Polo-Box Interacting Protein 1

PBS: Phosphate-Buffered Saline

PBS-T: PBS-0,01% Triton X-100

PCM: Pericentriolar Material

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

pH: potential of Hydrogen

PI3K: Phosphoinositide 3-kinase

PIC: Protease Inhibitor Cocktail

PLK: Polo-Like Kinase

Plx1: PLK1 *Xenopus* ortholog

PMSF: Phenylmethylsulfonyl Fluoride

PP1: Protein Phosphatase 1

PP2A: Protein Phosphatase 2A

PRC1: Protein Regulator of Cytokinesis 1

PRMT1: Protein Arginine N-Methyltransferase 1

PTM: Post-Translational Modification

pULK1: Ser757 phosphorylated ULK1

PVDF: Polyvinylidene Difluoride

RADs: DNA repair proteins

RAP1: Ras-related Protein 1

RAPTOR: Regulatory-Associated Protein of mTOR

RBBP7: Retinoblastoma-Binding Protein 7

RBR: RING-in-Between-RING

RBX: RING-box protein

RFP: Red Fluorescent Protein

RGG: Arginine–Glycine–Glycine

RICTOR: Rapamycin-insensitive companion of mammalian target of rapamycin

RIF1: Rap1-Interacting Factor 1

RING: Really Interesting New Gene

RIPA: Radioimmunoprecipitation Assay buffer

RNA: Ribonucleic Acid

RNAP: RNA Polymerase

RO3306: CDK1 inhibitor

ROI: Region Of Interest

RPMI: Roswell Park Memorial Institute

RT: Room Temperature

Rv: Reverse

s.e.: short exposure

S: DNA synthesis phase

SAC: Spindle Assembly Checkpoint

SCC: Sister Chromatid Cohesion proteins

SCF: Skp, Cullin, F-box containing complex

SD: Standard Deviation

SDS: Sodium Dodecyl Sulfate

Ser: Serine

SG: Stress Granule

sgRNA: single guide RNA

SHH: Sonic Hedgehog

siNT : Non-Targeting siRNA

SiR-DNA: Silicon Rhodamine DNA

siRNA: small interfering RNA

SKP: S-phase Kinase-associated Protein

SMC: Structural Maintenance of Chromosomes

SMU1: Suppressor of Mec-8 and Unc-52 protein

SOCS: Suppressor Of Cytokine Signaling

SQSTM1 (P62): Sequestosome 1

STLC: S-trityl-L-cysteine

SUMO: Small Ubiquitin-related Modifier

Taxol: Paclitaxel

TBS: Tris-Buffered Saline

TBS-T: TBS-0,05% Tween

Thr: Threonine

Topo II: Topoisomerase II

Torin1: specific ATP-competitive mTOR inhibitor

TPX2: Targeting protein for Xenopus Klp2

t-test: Student's t test

U2OS : Human U-2 Osteaosarcoma cell line

Ub: Ubiquitin

UBA: Ubiquitin Associated domain

UBAP2: Ubiquitin-Binding Protein 2

UBAP2L: UBAP2-Like

UBASH3B: Ubiquitin-Associated and SH3 domain-containing protein B

UBD: Ubiquitin-Binding Domain

UBP: Ubiquitin-Binding Protein

ULK1: Unc-51–like kinase 1

UPS: Ubiquitin Proteasome System

USP16: Ubiquitin Specific Peptidase 16

VHL: von Hippel-Lindau disease tumor suppressor

WB: Western Blotting

WEE1: Wee1-Like Protein Kinase

WNT: Contraction of "*Wg, wingless*" and "*Int, integration site*"

WT: Wild Type

Xatr: ATR *Xenopus* ortholog

Xchk1: CHK1 *Xenopus* ortholog

ZYG11: Protein ZYG11

β-TRCP1: β-Transducin Repeat Containing Protein 1

γH2AX: Ser139 phosphorylation of the histone variant H2AX

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MATERIAL AND METHODS

INTRODUCTION

I. THE CELL CYCLE AND MITOSIS

A. General principles

The human body is made of trillions of cells cooperating together in order to form a fully functional organism. Cell maintenance is driven by their accurate division for most cell types. The ultimate goal of cell division is to give rise to two genetically identical daughter cells through the faithful segregation of the mother cell's genetic information encoded by DNA. In eukaryotes, the cell cycle is divided into four stages. First, cells grow during the gap phase 1 (G1) and prepare for DNA replication occurring in S phase. After DNA synthesis, the gap phase 2 (G2) enables the cell to prepare for the ultimate step of the cell cycle, cell division also called mitosis. G1, S and G2 phases form together the so-called interphase which generally occupies 90% of the cell cycle duration and its length is variable depending on the cell type. Interphase is crucial for cell division as it ensures the synthesis of DNA, proteins and most importantly organelles. The last step of the cell cycle is mitosis which generally requires around one hour in human cells. Mitosis is conventionally subdivided into five stages: prophase, prometaphase, metaphase, anaphase and telophase. The mitotic phase is completed with cytokinesis (**Figure 1**) (McIntosh, 2016; Urry et al., 2020).

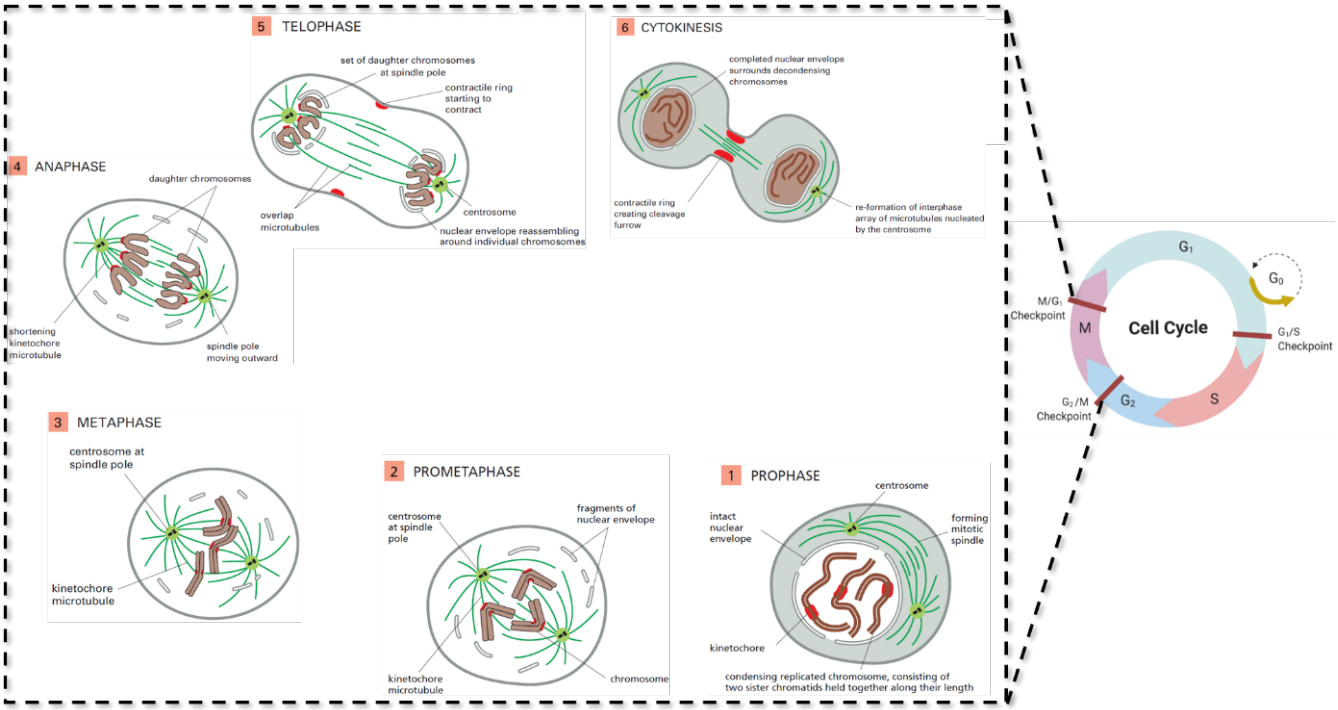

Figure 1: The cell cycle and mitosis

In eukaryotes, the cell cycle is divided into four stages, namely the gap phases 1 and 2 (G1 and G2), the DNA synthesis phase (S) and cell division also called mitosis (M). Mitosis is subdivided into five stages. First, the nuclear envelope breaks down at the same time as chromosomes start condensing during prophase. The centrosomes duplicate creating a bipolar spindle and enabling Microtubule-Kinetochore (MT-KT) attachments to form during prometaphase. Chromosomes align at the equatorial zone during metaphase when the proper attachment and alignment is controlled by the Spindle Assembly Checkpoint (SAC) signaling before anaphase onset. During anaphase, sister chromatids of the same chromosome are pulled apart towards opposite spindle poles. Finally, chromosomes decondense during telophase while the nuclear envelope reassembles around DNA. Mitosis is completed thanks to an actinomyosin contractile ring creating a cleavage furrow in order to separate the cytoplasm of the two genetically identical daughter cells (*adapted from Alberts, 2015, created with Biorender.com*).

B. G2 preparation

The cell division is achieved through the collaboration of a myriad of proteins with the cytoskeleton, in particular with microtubules (MTs). MTs are filamentous-like structure made of α- and β-tubulin heterodimers. These subunits confer polarity to the formed tubule, the α-tubulin marking the minus (-) and the β-tubulin the plus (+) end of the MTs. The latter are very dynamic tubular structures, constantly subjected to polymerization and depolymerization cycles from both ends in a GTP-dependent manner (**Figure 2**) (Nogales and Wang, 2006). MTs are essential for the spindle formation and function. The organelle responsible for the precise organization of MTs, especially during mitosis, is the centrosome, functioning as the Microtubule Organizing Center (MTOC). Centrosomes consist of a pair of centrioles surrounded by Pericentriolar Material (PCM) (Lawo et al., 2012). Nine MTs triplets assemble together forming a barrel-like structure called centriole while PCM consists of specific proteins driving the recruitment of γ-tubulin in order to meet the needs for the formation of new MT fibers during a process called MT nucleation (Moritz et al., 1995). After cell division, daughter cells contain only one centriole which needs to be duplicated and elongated during G1/S transition. The full centrosome containing a centriole pair is then duplicated during G2, forming a bipolar spindle. Each centrosome will form one pole towards which chromosomes will be segregated during mitosis.

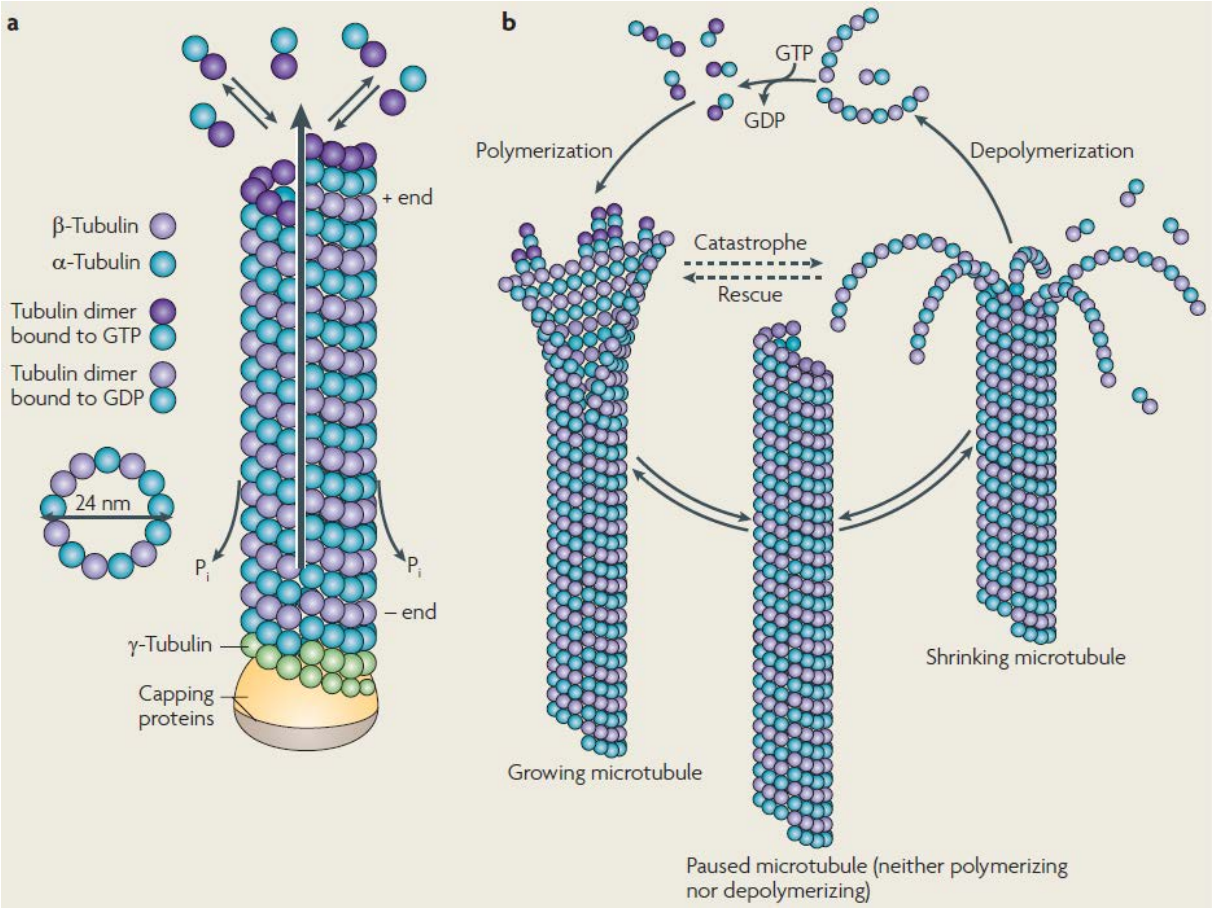

Figure 2: Microtubules dynamics

a. Schematic view of γ-tubulin mediated MT nucleation from centrosomes. **b.** MTs are dynamic filament-like structure undergoing constant polymerization and depolymerization cycles in a GTP-dependent manner. Elongation and shrinkage steps are reversible and MTs can transiently pause between the two illustrated states (*From Conde and Cáceres, 2009*).

C. Mitosis

1. Prophase

1.1 Chromatin condensation

Prophase is the first stage of mitosis during which chromatin starts condensing to form the mitotic chromosomes. Although the mechanism of chromatin condensation has not yet been fully understood, the cooperative actions of Condensins I and II that are triggered by Cyclin-Dependent Kinase 1 (CDK1) activity are essential for DNA compaction (Abe et al., 2011). Condensin II binds to DNA in the nucleus and Condensin I interacts with DNA after the nuclear envelope breakdown, with both proteins being responsible for DNA loops formation and a helical-like arrangement called the loop exclusion model (**Figure 3A**) (Gibcus et al., 2018).

Interestingly, sister chromatids are often joined all along their arms by Cohesin (**Figure 3B**). The resolution of sister chromatids progressively occurs during prophase triggered by PLK1 and AURB cleavage-independent removal of Cohesin from chromosome arms with the exception of the very particular centromeric region holding both chromatids together until onset of the anaphase (Waizenegger et al., 2000; Losada et al., 2002; Sumara et al., 2002).

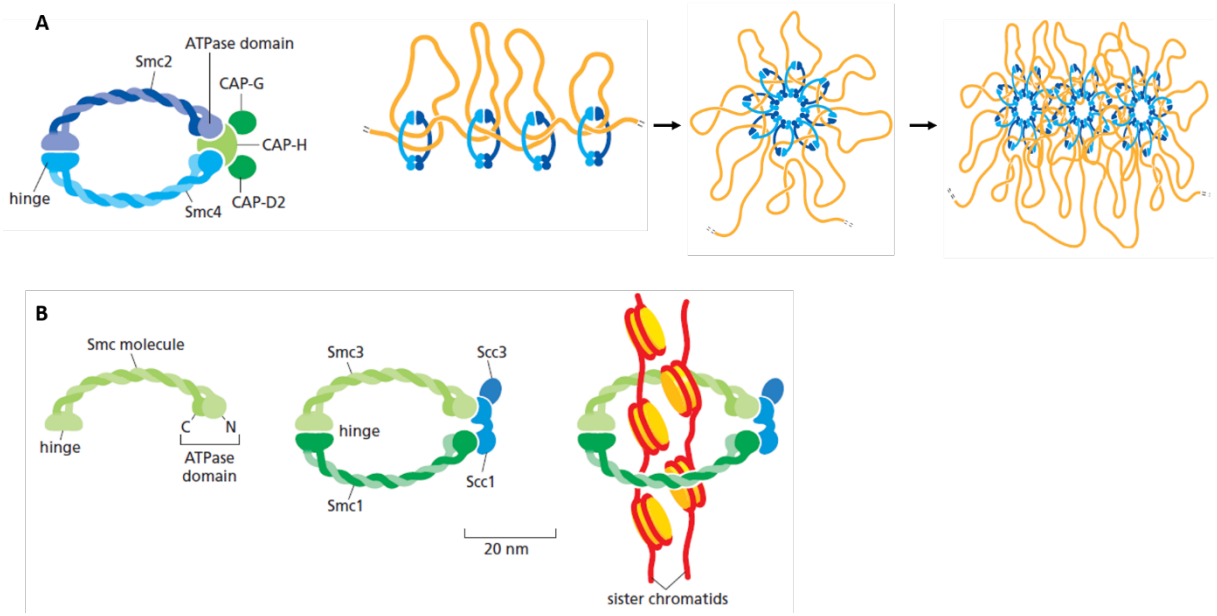

Figure 3: Condensin and Cohesin structures

A. Condensin complexes consist of five subunits: a coiled-coil heterodimer of Structural Maintenance of Chromosomes 2 and 4 (SMC2 and SMC4) containing an ATPase domain and three additional proteins (Chromosome-Associated Proteins CAP-G, H and D2) forming a ring that might encircle DNA to promote its compaction. Different CAP proteins assemble to form Condensin I and II. **B.** Cohesin is a tetrameric complex composed of two coiled-coil proteins, SMC1 and SMC3, Sister Chromatid Cohesion proteins 1 and 3 (SCC1 and SCC3) which give the ring structure to the complex and the ability to wrap around sister chromatids (*adapted from Alberts, 2015*).

1.2 Preparation for microtubule-kinetochore attachment

Centromeres are constitutive heterochromatin regions which consist of megabase-long arrays of repetitive sequences called α-satellites. The peculiar structural organization of centromeres resides in its unique nucleosome composition containing the histone H3 variant Centromere protein-A (CENP-A) (Müller and Almouzni, 2017). Centromeres can be very different from one species to another, ranging from point centromeres in budding yeast (very short) to meta-polycentromeres in *P. sativum* (3-5 centromeres) or holocentromeres in *C. elegans* (whole

chromosome). Humans or *D. melanogaster* for instance have monocentromeres spanning a larger region than point centromeres and for this reason are called regional centromeres (**Figure 4**) (Steiner and Henikoff, 2014; Wong et al., 2020). This region is the assembly platform for kinetochore (KT) proteins.

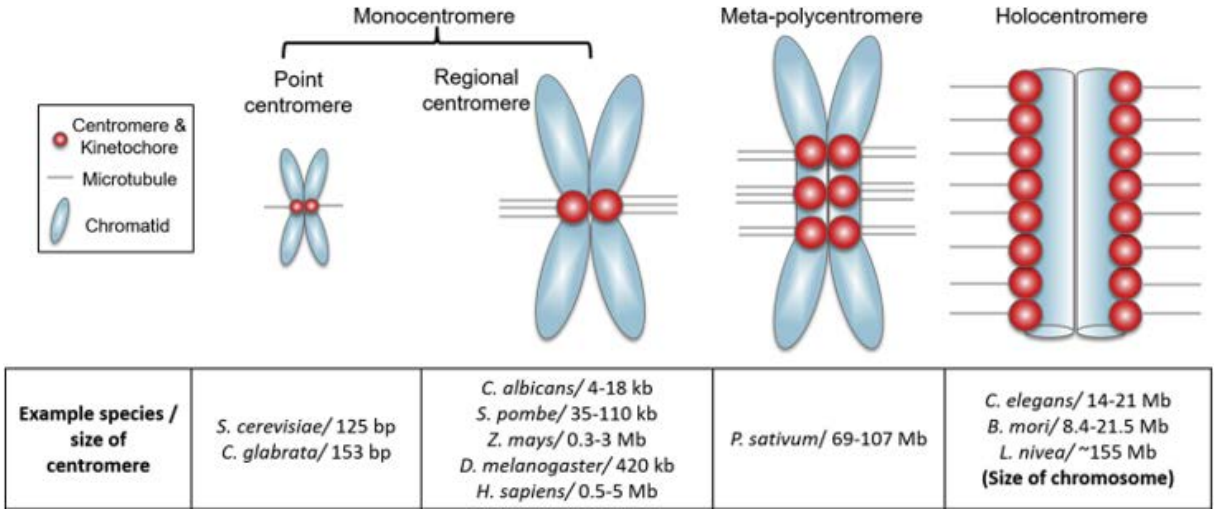

Figure 4: Types of centromeres

Centromeres structure is variable through species ranging from monocentromeres with variable size to holocentromeres spread along the whole chromosome arms (*adapted from Wong et al., 2020*).

In fact, CENP-A nucleosomes are specifically recognized by the KT proteins CENP-C and CENP-N "priming" centromeric regions for subsequent KT assembly (Carroll et al., 2010). CENP-C and CENP-N are part of a key structural complex called the Constitutive Centromere-Associated Network (CCAN) made of 16 subunits in vertebrate KTs and constitutively localized to the centromere throughout the cell cycle (**Figure 5**) (Cheeseman and Desai, 2008). The CCAN can be subdivided into five groups: CENP-C, the CENP-L-N complex, the CENP-H-I-K-M complex, the CENP-O-P-Q-U-R complex and the CENP-T-W-S-X complex (McKinley and Cheeseman, 2016). The highly organized structure of the CCAN allows the recruitment of additional proteins during prophase, often referred to as the outer KT, building a robust platform for MT-KT attachment. The outer KT consist of three main complexes (KNL1, Mis12 and NDC80) often referred to as the KMN network (Cheeseman et al., 2006). The KNL1 complex serves as a scaffold for protein binding. The Mis12 complex is a tetrameric complex (Mis12, PMF1, Nsl1, Dsn1) which makes the link with the CCAN through CENP-C and CENP-T binding. NDC80 is the complex responsible for MTs binding (Wei et al., 2007).

Importantly, NDC80 is excluded from the nucleus during interphase and is recruited to the inner kinetochore exclusively during mitosis through the phosphorylation of several kinetochore substrates by CDK1 (Gascoigne and Cheeseman, 2013). The outer kinetochore serves itself as a recruitment platform for additional regulatory factors. Regulators of stable MT-KT attachment, SAC components and motor proteins such as dynein or kinesins are recruited to this structure. The correct MT-KT attachment is pivotal for proper chromosome alignment during metaphase and faithful chromosome segregation during anaphase.

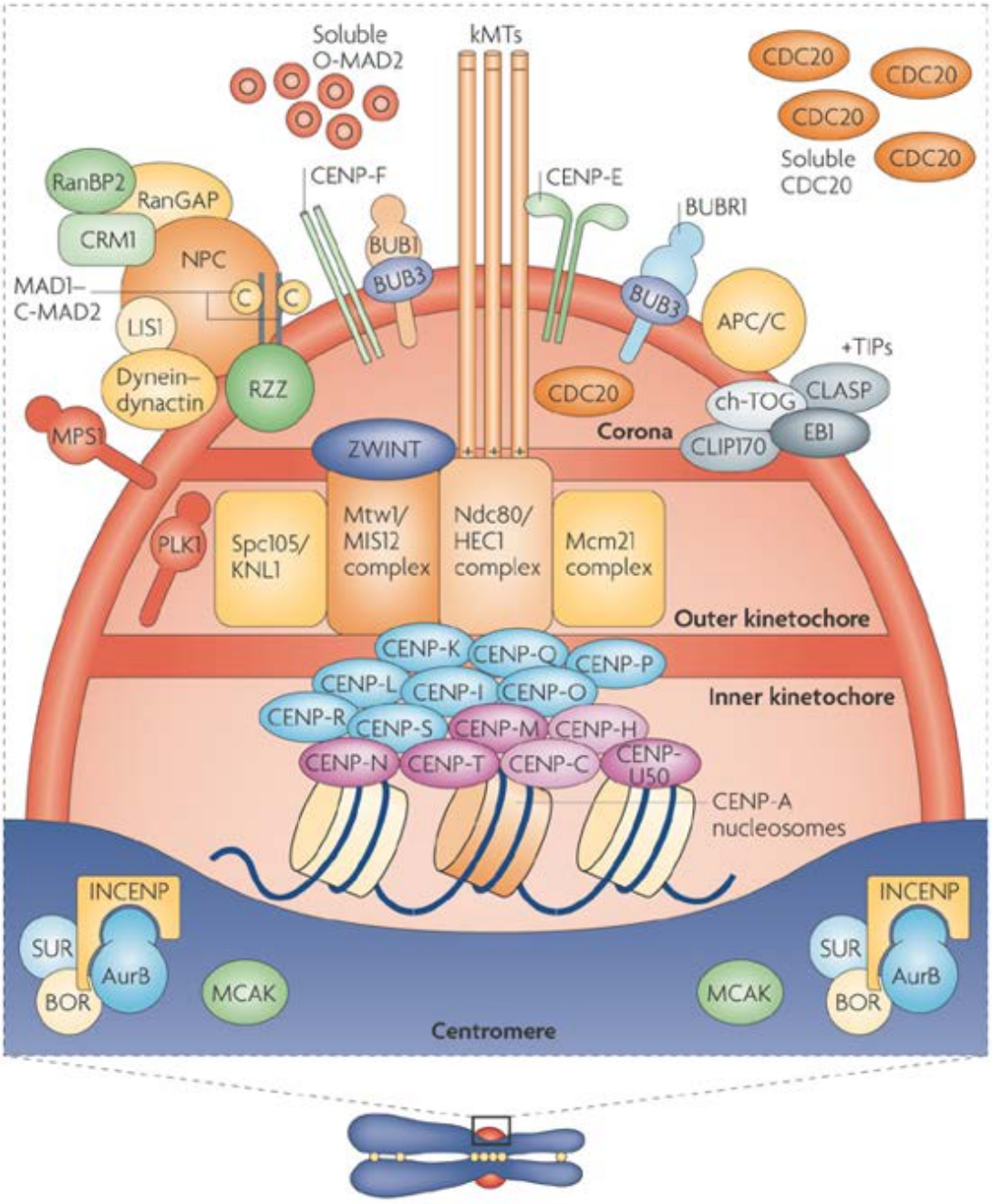

Figure 5: The human kinetochore

In humans, the kinetochore is a huge assembly platform gathering more than one hundred proteins. It can be divided into four different parts. First, the centromere is a typical region mainly characterized by the presence of CENP-A nucleosome. The latter is associated with the inner kinetochore made of the CCAN, a big network of about 16 subunits. CCAN recruits outer kinetochore proteins such as the KMN network which in turns triggers the recruitment of tens of regulatory factors such as motor proteins (CENP-E, dynein), transporters, kinases or SAC components forming the corona (*from O'Connor, C. (2008)).*

1.3 Centrosome positioning

In parallel to chromosome condensation, the two centrosomes move away from each other and migrate to opposite poles of the cell, defining the polarity, motility and shape of the cell (Desai and Mitchison, 1997; Keating and Borisy, 1999). However, despite centrosome positioning being crucial for faithful chromosome segregation, the underlying molecular mechanisms remain an area of debate in the field (Tang and Marshall, 2012). It is yet commonly accepted that the pulling forces generated by the lengthening MTs arising from both centrosomes would partially drive centrosomes to opposite directions (Zhu et al., 2010). This marks the beginning of the mitotic spindle formation.

2. Prometaphase

During prometaphase, the size of the helical turns increases until 150 DNA loops per turn (12Mb/turn) enabling chromatin to adopt the very compacted and peculiar X shape of mitotic chromosomes (Gibcus et al., 2018). The nuclear envelope fragments, thus enabling the invasion of the nuclear area by the MTs growing from the centrosomes. Distinct populations of MTs are part of the mitotic spindle. Among them, the non-KT MTs are not attached to KT whereas Kinetochore fibers (K-fibers) are stably bound to the KT. Both populations grow from the centrosome and have stable orientation, the (-) ends facing the poles on the contrary to the (+) ends directed towards the future equatorial zone or the cell cortex. The non-KT MTs are as important as K-fibers as they promote stability of the spindle (Booth et al., 2011; Deutsch and Lewis, 2015). When MTs emanating from opposite spindle poles meet, they form antiparallel bundles stabilized by several regulatory proteins such as Protein Regulator of Cytokinesis 1 (PRC1) forming the “mesh network” (Nixon et al., 2015). At the end of prometaphase, KTs are assembled at centromeres and some MTs attach to KTs.

3. *Metaphase*

Thanks to the coordinated action of all previously mentioned protein networks, chromosomes are being aligned at the metaphase plate, an equatorial zone which is generally equidistant from the two spindle's poles. The inter-polar MTs, by growing and sliding on each other, determine the spindle length (**Figure 6A**) (Deutsch and Lewis, 2015). The KT of each sister chromatid is attached to MTs emanating from the opposite poles forming amphitelic attachment (Urry et al., 2020). Three incorrect types of attachment can also occur (monotelic, syntelic, merotelic), triggering the prolongation of metaphase until all erroneous attachments are corrected. Monotelic attachment represents the situation when only one KT of the two sister chromatid is correctly attached to MT, syntelic attachment when the two KT of the same chromosome are attached to MTs emanating from the same spindle pole and merotelic attachment when one of the two KT of one chromosome is attached to both poles (**Figure 6B**). Of note, it is very frequent that KTs first bind the side of MTs (side-on attachment) before being pulled towards the (+) end of MTs, enabling a correct and stable end-on attachment (**Figure 6C**) (Itoh et al., 2018). The proper MT-KT attachment is sensed by the SAC also named mitotic checkpoint and is discussed in the next section (Musacchio and Salmon, 2007). It is very important that all attachment errors are corrected prior to anaphase onset as merotelic attachments are the most frequent cause of aneuploidy in mammalian cells (Cimini et al., 2001), subsequently increasing the chromosomal instability (CIN) (Thompson and Compton, 2008).

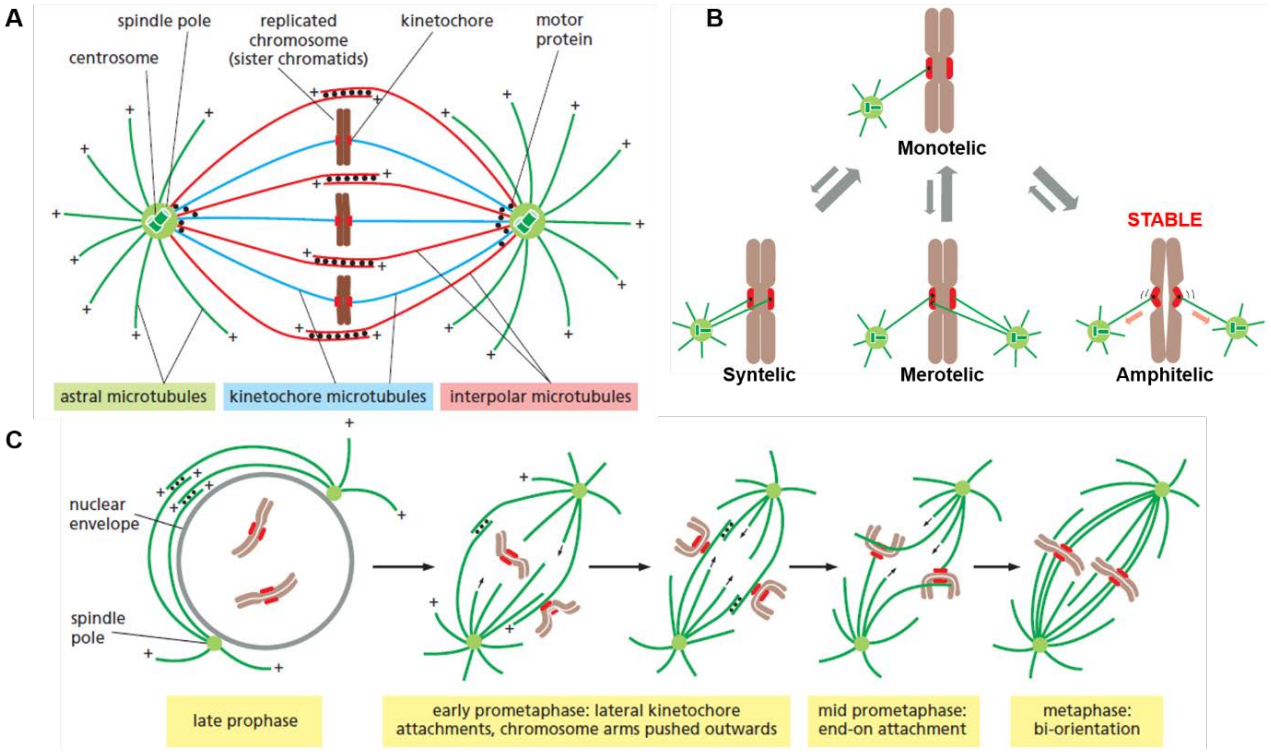

Figure 6: The mitotic spindle and MT-KT attachments

A. Schematic representation of the mitotic spindle highlighting the main types of MTs. **B.** Types of MT-KT attachments. The stable form is the amphitelic attachment while syntelic, merotelic and monotelic attachments potentiate SAC activity. **C.** Illustration of chromosome attachment to MTs in animal cells. Chromosomes are first laterally attached before being dragged to promote end-on attachment (*adapted from Alberts, 2015*).

4. Anaphase

Anaphase is characterized by the separation of the two sister chromatids of each chromosome due to the cleavage of the remaining Cohesin at centromeres, which is achieved by Separase in a precisely time-regulated manner (Silva et al., 2018). The entangled DNA generated during prophase by extensive looping needs to be decatenated to avoid the formation of chromosome bridges during anaphase. Chromatin relaxing is achieved through the action of Topoisomerase II (Topo II) whose absence has been linked with increased genomic instability assessed by the number of bridges during anaphase (Clarke et al., 1993). Each chromatid becomes an independent chromosome part and is pulled towards one pole of the cell as KT-bound MTs shorten. On the contrary, non-KT MTs grow thus enabling the cell to elongate.

5. Telophase and cytokinesis

During telophase, the two pools of daughter chromosomes progressively decondense and the nuclear envelope reassembles around them. The remaining spindle MTs depolymerize and nucleoli reform. Finally, the separation of the cytoplasm giving rise to two independent daughter cells is achieved through the ingression of the cleavage furrow during cytokinesis (Urry et al., 2020).

II. REGULATION OF CELL DIVISION

Cell survival depends on its ability to faithfully segregate its genetic material. Such an important process needs to be tightly regulated in time and space thanks to multiple control processes. Indeed, perturbation in proliferation signaling has been identified as one of the six primary hallmarks of cancer (Hanahan and Weinberg, 2000). Progression through the cell cycle and especially mitosis is regulated by numerous Post-Translational Modifications (PTMs) such as phosphorylation or ubiquitylation events. In this section, I will give an overview of the major mitotic regulators and linked signaling pathways.

A. The Spindle Assembly Checkpoint

The SAC also called the mitotic checkpoint is the major sensor of chromosome aberrations occurring during cell division such as misalignments and incorrect MT-KT attachments. When such abnormalities occur, the active SAC blocks premature anaphase onset until chromosomes are properly attached to MTs, thus preventing loss of cohesion between sister chromatids and subsequent segregation errors.

More precisely, unproperly attached kinetochores trigger the transformation of Open-Mitotic Arrest Deficient 2-like Protein 1 (O-MAD2) into Closed-MAD2 (C-MAD2) by the MAD1/C-MAD2 complex. The increased affinity of the C-MAD2 form for Cell Division Cycle Protein 20 (CDC20) enables the assembly of the Mitotic Checkpoint Complex (MCC) composed of C-MAD2, Budding Uninhibited by Benzimidazoles 3 (BUB3), BUB-related protein 1 (BUBR1) and CDC20 (Musacchio, 2015). The trapping of CDC20 in the MCC hinders its association with its main target, the Anaphase- Promoting Complex/Cyclosome (APC/C) E3 ubiquitin ligase, thus preventing the ubiquitylation and subsequent degradation of the Separase inhibitor Securin and the CDK1 co-factor Cyclin B1 which remain bound to their respective partners (Sudakin et al., 2001). Under these conditions, mitosis remains arrested until all MT-KT attachments are properly formed. At the same time, the major mitotic kinase AURB allows for the correction of erroneous attachments (merotelic, syntelic) (Cimini et al., 2006) by targeting the Monopolar Spindle 1 (MPS1) kinase to the kinetochore and potentiating its enzymatic activity leading to the phosphorylation of KNL1 subsequently triggering the recruitment of additional SAC components such as BUB1, BUB3 and BUBR1 (**Figure 7A**) (Ditchfield et al., 2003; Saurin et al., 2011). The cascade of phosphorylation events amplifies the SAC response.

Once all KTs are stably attached to MTs, the SAC sensor machinery needs to be silenced. The inactivation of the SAC occurs at multiple levels simultaneously. On one hand, SAC components are removed from KTs in a dynein-dependent manner (Howell et al., 2001) and the AURB-opposing Protein Phosphatase 1 (PP1) dephosphorylates its substrates, blocking further amplification of the SAC signal (Rosenberg et al., 2011). On the other hand, the conversion of MAD2 to its closed form is inhibited by the $p31^{Comet}$ protein by steric hindrance (Yang et al., 2007). $P31^{Comet}$ would also be involved in a parallel pathway by promoting APC/C dependent CDC20 ubiquitylation and degradation, thus contributing to MCC disassembly and APC/C activation ultimately enabling Cyclin B1 and Securin degradation and anaphase onset (**Figure 7B**) (Westhorpe et al., 2011; Musacchio, 2015).

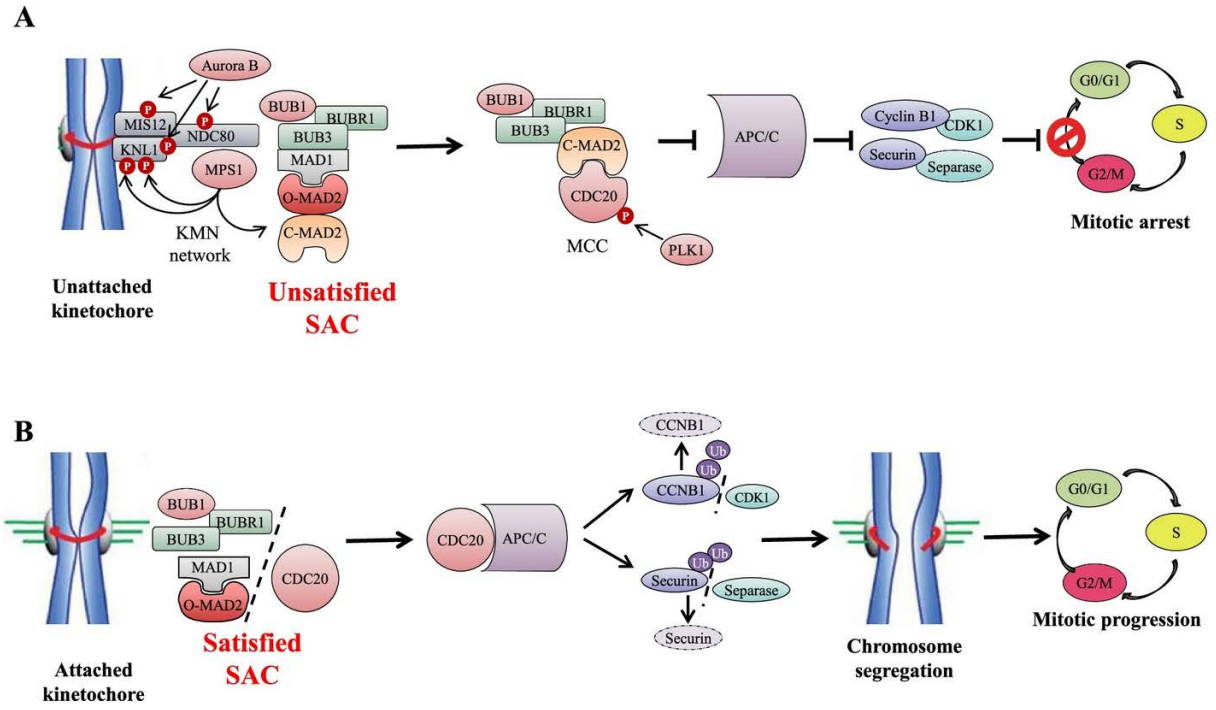

Figure 7: The Spindle Assembly Checkpoint

A. In the presence of unattached kinetochores, a phosphorylation cascade targets MCC assembly and conversion of O-MAD2 into C-MAD2, enhancing its affinity for CDC20 thus inhibiting APC/C activity. **B.** When all kinetochores are properly attached, the SAC is satisfied and as a consequence removed from kinetochores. MAD2 adopts its opened form again, enabling CDC20 association with APC/C and subsequent degradation of Cyclin B1 (CCNB1) and Securin to promote anaphase onset (*from Bruno et al., 2022*).

B. Regulation of mitosis by ubiquitylation

Traditionally, ubiquitylation is associated with proteasomal degradation of target proteins but it can also be involved in many different cellular processes known as non-proteolytic pathways (Liao et al., 2022). Ubiquitin (Ub) is a small protein which can be covalently attached to lysine (K) residues of specified substrate proteins thanks to the cooperation of three types of enzymes. First, a Ub-activating enzyme (E1) activates the C-terminal carboxyl group of Ub thanks to ATP hydrolysis. Ub is in turn transferred to a Ub-conjugating enzyme (E2). Finally, a Ub-ligase (E3) is responsible for identifying the substrate, recognizing the E2 enzyme and catalyzing the transfer of Ub to the targeted protein (**Figure 8A**). This dynamic process can be reversed through the action of Deubiquitylating enzymes (DUBs). Ub itself can be modified by additional Ub molecules forming various types of isopeptide-linked Ub chains but also by Small Ubiquitin-related Modifier (SUMO)-, Neural precursor cell Expressed Developmentally Downregulated protein 8 (NEDD8)-, acetylated- or phosphorylated-modified chains,

conferring ubiquitylated substrates the potential to regulate multiple biological functions (**Figure 8B**) (Komander and Rape, 2012; Kwon and Ciechanover, 2017; Mulder et al., 2020). Whereas few E1 and E2 enzymes have been ascribed specific functions in human cells, E3 ligases have been extensively studied the past years. E3 ligases can be classified into three main families: the Homologous to E6 Carboxy Terminus (HECT) domain, the Really Interesting New Gene H2 (RING-H2) ligases and the RING-in-Between-RING (RBR) ligases (Kee and Huibregtse, 2007; Deshaies and Joazeiro, 2009).

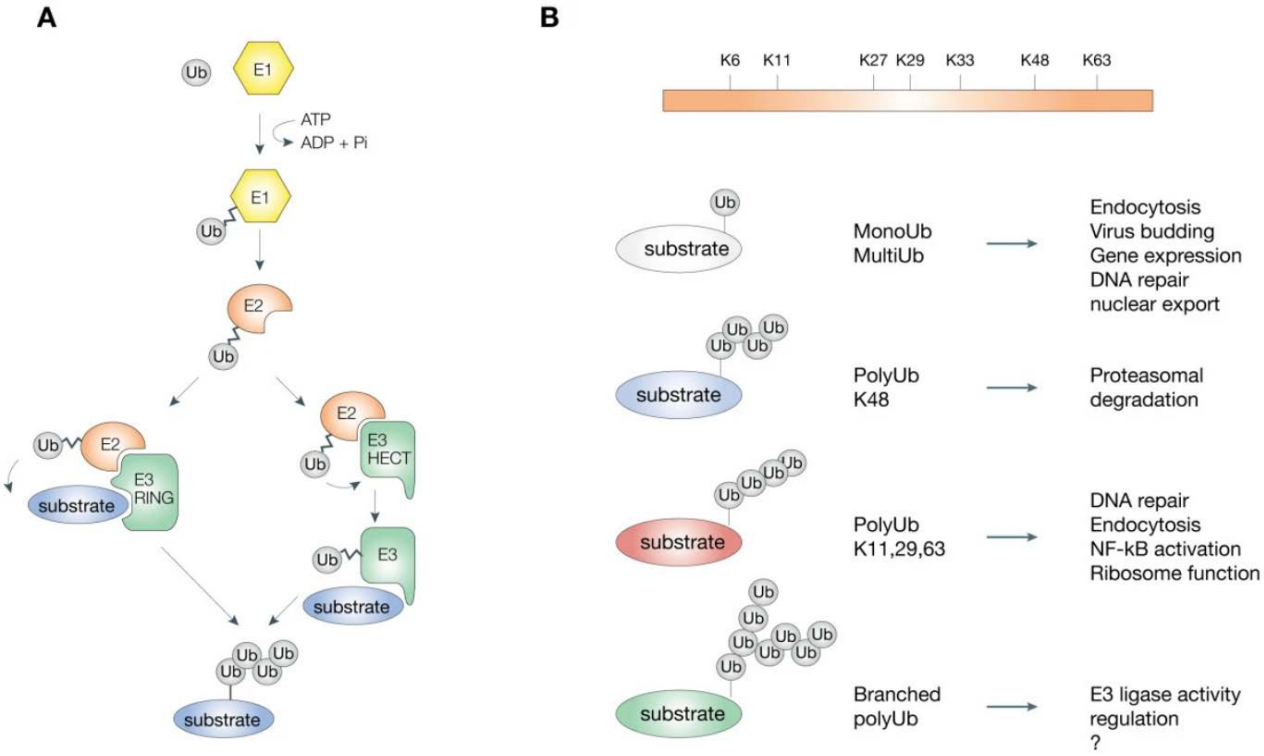

Figure 8: The ubiquitin code

A. Representative scheme of the signaling cascade mediated by E1, E2 and E3 enzymes to promote specific substrate ubiquitylation. **B.** Main Ub chains that can be covalently attached to a substrate and their corresponding physiological consequences (*from Woelk et al., 2007*).

1. Cullin-RING ubiquitin ligases

The largest human E3 ligase family, harboring a RING domain, contains more than 600 members (Morreale and Walden, 2016). Among them, eight classes of Cullin-RING ubiquitin ligases (CRLs) have been described in mammals, all working as part of a complex to catalyze ubiquitylation of specific substrates. Each complex comprises a Cullin (CUL) isoform (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 or CUL9) being the scaffold protein, an adaptor protein binding to the N-terminal part of CUL, a RING-containing E2 enzyme binding to the

C-terminal part of CUL and finally a receptor which gives the substrate specificity to the complex and binds the target which needs to be ubiquitylated (**Figure 9**) (Bulatov and Ciulli, 2015). The large variety of possible CRL complexes composition enables CRLs' involvement in a myriad of cellular processes ranging from cell division to metabolism, DNA replication and repair, chromatin remodeling and cell differentiation (Jang et al., 2018).

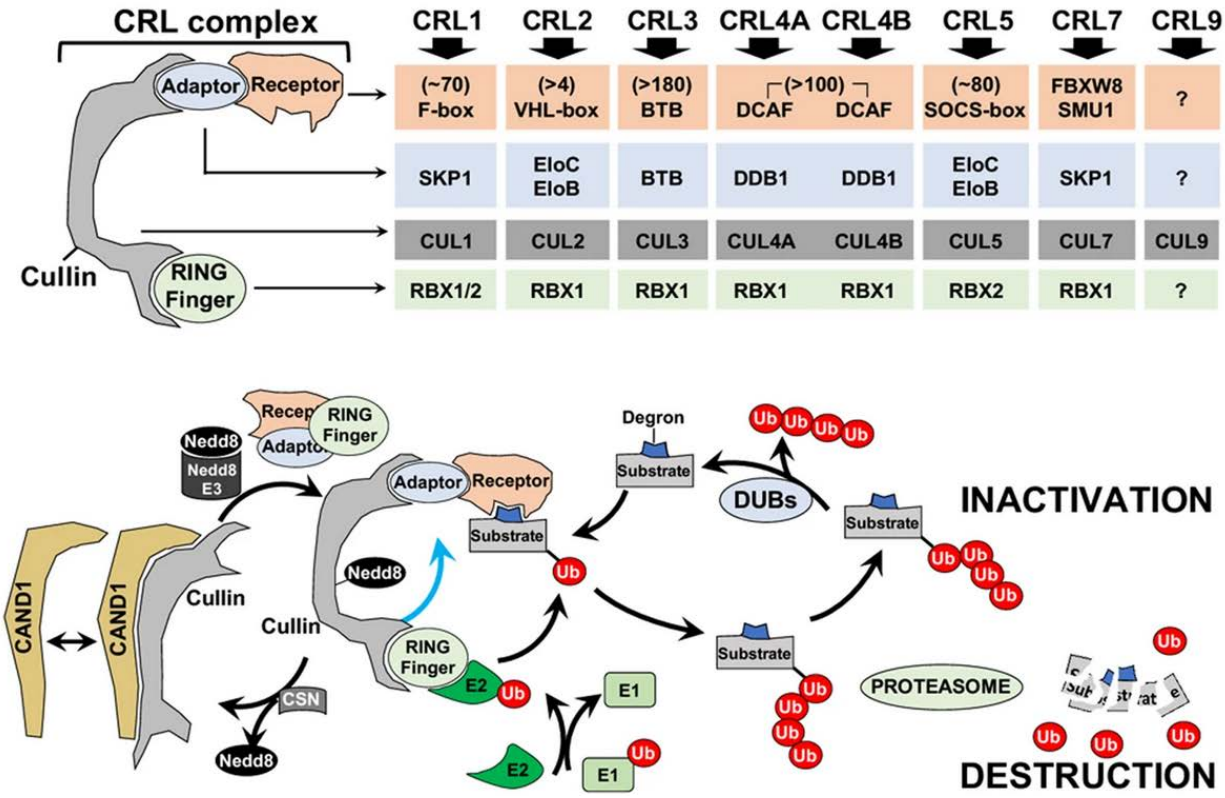

Figure 9: Schematic structure and mode of action of Cullin-based complexes

Upper part: The basic CRL complex structure is depicted and corresponding adaptors, receptors and RING finger proteins are indicated for each complex specifically. As highlighted by the question marks, CRL9 complexes are poorly characterized and no adaptor, receptor or RING finger protein have been identified to date. **Lower part**: Cullins are activated by neddylation inducing a conformational change enabling interactions with their partners. Ub transfer to the substrate is catalyzed, often (but not systematically) leading to the substrate inactivation or degradation (*from Jang et al., 2020*).

1.1 Proteasomal degradation

One of the most important features of mitotic regulation is the fluctuation of protein abundance and availability. The APC/C and CRL complexes are responsible for the proteasomal degradation of many key mitotic factors. The ultimate step of proteasomal degradation is

achieved in an ATP-dependent manner by the 26S proteasome, a large multiprotein complex consisting of three main parts: (1) the core 20S particle harboring the proteolytic active sites and organized as a barrel-like structure, (2) the base including ubiquitin-binding proteins and ATPases, and (3) the lid acting mainly as a scaffold (**Figure 10**). Interestingly, (2) and (3) do not always coexist as in fact, some proteasomes only have a lid. Proteins targeted to proteolytic degradation are covalently attached to polyubiquitin chains, conferring a relative selectivity to the 26S proteasome. These Ub chains are removed prior to the substrate destruction, the protein is unfolded in order to penetrate the barrel and get degraded (Bard et al., 2018).

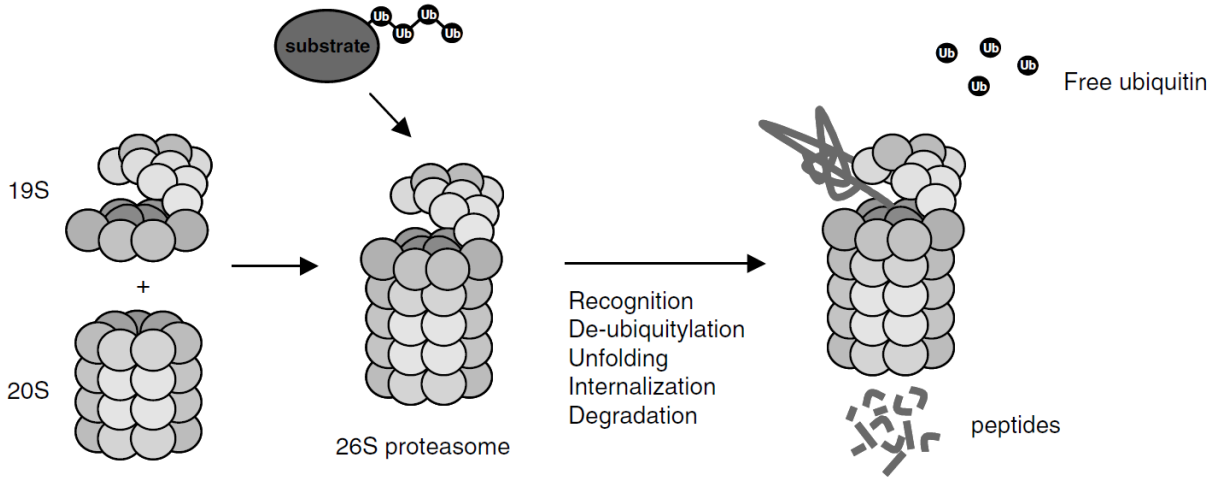

Figure 10: The 26S proteasome

The proteasome is made of 20S subunit carrying the catalytic activity and flanked by one or two 19S subunits helping for protein recognition, unfolding, deubiquitylation and degradation (*from Marteijn et al., 2006*).

Cullin1 (CUL1)-based complexes

Cullin1-based complexes also called S-phase Kinase-associated Protein (SKP), Cullin, F-box containing complex (SCF) are certainly the most well studied and characterized E3 ubiquitin ligases. They are structurally made of the core CUL1 protein, a recognition module made of the linker protein SKP1 and a substrate-specific adaptor protein containing an F-box motif and the RING-finger protein RING-box protein 1 (RBX1) (Gilberto and Peter, 2017). As mentioned in the SAC section, reactivation of APC/C is essential for progression through and completion of mitosis. Among multiple layers of regulation, the CRL1 with its adaptor β-Transducin Repeat Containing Protein 1 (β-TRCP1) contributes to this reactivation by proteasomal degradation of the APC/C inhibitor Early Mitotic Inhibitor 1 (EMI1) during early mitosis (Guardavaccaro et al., 2003). In addition, $CRL1^{\beta\text{-}TRCP1}$ has been shown to ubiquitylate WEE1-Like Protein Kinase

(WEE1) in a PLK1 phosphorylation priming-dependent manner, promoting its degradation and normal mitosis onset *in vivo* (Watanabe et al., 2004). Moreover, additional SCF complexes such as $CRL1^{FBXL2,7}$ and $CRL1^{FBXW7}$ have been implicated in AURB and Transcription factor Jun-B (JUNB) degradation therefore promoting proper cytokinesis and preventing premature sister chromatid separation, respectively (Chen et al., 2013; Pérez-Benavente and Farràs, 2013). Targeted degradation of substrates can often indirectly regulate mitosis as it has been shown for $CRL1^{FBXO31}$-mediated degradation of the transcription factor Forkhead box protein M1 (FOXM1) during G2/M transition, resulting in the transcription regulation of key mitotic factors (Jeffery et al., 2017). Furthermore, the centrosomal protein Centriolar coiled-coil protein of 110 kDa (CP110) destruction by $CRL1^{CyclinF}$ in a timely-regulated manner limits the aberrant centrosome duplication, thereby ensuring genome integrity (D'Angiolella et al., 2010). Another well characterized proteolytic-dependent regulation of mitotic entry by SCF complex is Cyclin B1 degradation during interphase by the $CRL1^{NIPA}$ complex which is inactivated prior to mitosis by Non-Imprinted in Prader-Willi/Angelman syndrome region protein (NIPA) CDK1-mediated phosphorylation (**Figure 12**) (Bassermann et al., 2005).

Cullin2 (CUL2)-based complexes

Similarly to CRL1-based complexes, CRL2 complexes consist of the scaffold protein Cullin2, the RBX1 protein and the substrate recognition Suppressor of Cytokine Signaling (SOCS)-box proteins linked to Cullin by ElonginC (Sumara et al., 2008). Interestingly, CRL2 complexes seem to redundantly function with the APC/C ligase. $CRL2^{ZYG11}$ is a good example of this dual degradation of important mitotic factors. In fact, both $CRL2^{ZYG11}$ and APC/C are responsible for CyclinB1 degradation. Although the CRL complex depletion does not seem to affect the progression of mitosis, when APC/C is absent or inactive (as it is the case during SAC activation), or if CyclinB1 is overexpressed, $CRL2^{ZYG11}$ is essential for progression through mitosis, often favoring a process called "mitotic slippage", overcoming the mitotic arrest caused by the SAC activation (Balachandran et al., 2016). Another CRL2 complex including the von Hippel-Lindau disease tumor suppressor (VHL) acting as an adaptor triggers the Topoisomerase IIα (TopoIIα) degradation (**Figure 12**) (Yun et al., 2009). TopoIIα being essential for mitotic chromosome compaction and segregation (Uemura et al., 1987; Ishida et al., 1994; Escargueil et al., 2000), it is reasonable to hypothesize that $CRL2^{VHL}$-dependent TopoIIα degradation has an effect on mitotic progression.

Cullin3 (CUL3)-based complexes

CRL3 complexes also associate with RBX1 but on the contrary to previously cited ligases, the recognition module and substrate specificity functions are executed by a single protein belonging to the Bric-a-brac-Tramtrack-Broad complex (BTB) domain-containing family (Sumara et al., 2008). The proteolytic function of CRL3 complexes during mitosis is limited to the promotion of the p60/katanin degradation therefore allowing normal mitotic progression (**Figure 12**) (Cummings et al., 2009). However, Cullin3 is crucial for mitotic progression, especially via its non-proteolytic functions as discussed in the next chapter (**Figure 11**).

Cullin4 (CUL4)-based complexes

CRL4 complexes have a very similar structure as CRL1 complexes. They are composed of a DNA Damage-Binding Protein 1 (DDB1)- and one of the CUL4-Associated Factor (DCAF) proteins (Lydeard et al., 2013) making the link to Cullin4 and providing the substrate specificity to the complex, respectively (Gilberto and Peter, 2017). Although CRL4 are mainly reported to regulate DNA replication (Jackson and Xiong, 2009), $CRL4^{RBBP7}$ mediates the ubiquitylation of BUB3 during mitosis, one of the major SAC components, triggering its silencing and subsequent anaphase onset (**Figure 12**) (Jang et al., 2020).

Cullin5 (CUL5)-based complexes

CRL5 complexes are structurally composed of the core CUL5 protein, the linker proteins ElonginB/C, a substrate-specific adaptor protein containing a SOCS box motif and the RING-finger protein RBX2 (Bano et al., 2022). Despite the lack of sufficient knowledge about Cullin5-based complexes, recent evidence suggests that the Cullin 5–interacting suppressor of cytokine signaling box protein Ankyrin repeat and SOCS Box protein 7 (ASB7) promotes the spindle dynamics regulator DDA3 ubiquitylation and subsequent proteasomal degradation, thus sustaining genome integrity (**Figure 12**) (Uematsu et al., 2016).

Cullin7 (CUL7)-based complexes

Like almost all other CRLs, CRL7 are built around the Cullin7 scaffold protein linked to FBXW8, which provides the substrate specificity, by the linker SKP1 and works together with the E2 recruiter RBX1 (Sarikas et al., 2008). $CRL7^{FBXW8}$ is responsible for proteasomal degradation of the histone marks balancer Mof4 Family Associated Protein 1 (MRFAP1). Surprisingly, even-though no direct implication in cell division has been published for this

protein, MRFAP1 overexpression causes mitotic aberrations leading to cell death, highlighting the importance of balancing its protein levels through CRL7 ^{FBXW8} (**Figure 12**) (Li et al., 2017).

CRLs	Substrates	Receptors	Substrate roles	Reference
CRL1	EMI1	β-TRCP1	Inhibits APC/C. Its destruction allows progression through mitosis.	Guardavaccaro et al., 2003
CRL1	WEE1	β-TRCP1	Inhibits CDK1 in mitosis. Its degradation allows progression through mitosis.	Watanabe et al., 2004
CRL1	AURB	FBXL2,7	Regulates Aurora kinases abundance required for normal mitosis.	Chen et al., 2013
CRL1	JUNB	FBXW7	Destruction inhibits premature sister chromatid separation.	Pérez-Benavente and Farràs, 2013
CRL1	FOXM1	FBXO31	Transcription factor active in G2 required for transcription of genes crucial for progression through G2/M.	Jeffery et al., 2017
CRL1	CP110	Cyclin F	Required for normal centrosome duplication.	D'Angiolella et al., 2010
CRL1	Cyclin B1	NIPA	Cyclin B1 regulates CDK1 activity and function in G2/M phases.	Bassermann et al., 2005
CRL2	Cyclin B1	ZYG11	Cyclin B regulates CDK1 activity and function in G2/M phases.	Balachandran et al., 2016
CRL2	TopoIIα	VHL	Alters DNA topology. Drives progression through mitosis.	Yun et al., 2009

CRL3	P60/katanin	KLHDC5	Ubiquitylation controls microtubule levels necessary for normal mitosis.	Cummings et al., 2009
CRL4	BUB3	RBBP7	Ubiquitylation allows metaphase to anaphase transition.	Jang et al., 2020
CRL5	DDA3	ASB7	Degradation controls microtubule polymerization. Required for normal mitotic progression.	Uematsu et al., 2016
CRL7	MRFAP1	FBXW8	Promotes anaphase to telophase transition.	Li et al., 2017

Table 1: Summary of known CRLs' proteolytic roles in mitotic regulation (*adapted from Jang et al., 2020*)

1.2 Non-proteolytic pathways

Although Ub signaling is very frequently associated to proteolytic degradation of targeted substrates, increasing evidence highlights the key roles of E3 Ub-ligases in non-proteolytic signaling pathways (**Figures 11 and 12**) (Liao et al., 2022). The most striking example of non-degradative regulation by Ub ligases during mitosis is certainly the one of Cullin3-RING ligases. In fact, CRL3 are mainly regulating key mitotic factors localization and activation during cell division, ensuring faithful chromosome segregation and maintaining genome integrity. First, $CRL3^{KLHL18}$ has been reported to monoubiquitylate AURA specifically at centrosomes during mitotic entry promoting its activation and subsequent initiation of mitosis as discussed in the "Aurora family" section (Moghe et al., 2012). Second, the CUL3/KLHL9/KLHL13 E3 ligase controls AURB dynamic localization on the mitotic chromosomes and proper midzone and midbody organization during anaphase and telophase, respectively, thereby ensuring completion of mitosis (Sumara et al., 2007). Similarly, $CRL3^{KLHL21}$ complex regulates AURB localization but in contrast to the CUL3/KLHL9/KLHL13 complex, KLHL21 first localizes to the midzone MTs during anaphase, recruiting AURB and CUL3 to this region and mediating AURB ubiquitylation. As a result, combined mechanisms might be responsible for the ubiquitylation of different pools of AURB, promoting both its removal from chromosomes as well as increasing its retention at the midzone (Maerki et al., 2009). Lastly, CUL3 together with its adaptor KLHL22 has been

demonstrated to monoubiquitylate PLK1, triggering its dissociation from its kinetochore phosphoreceptors, satisfying the SAC checkpoint and ultimately promoting faithful partition of the genetic material (**Figure 11**) (Beck et al., 2013).

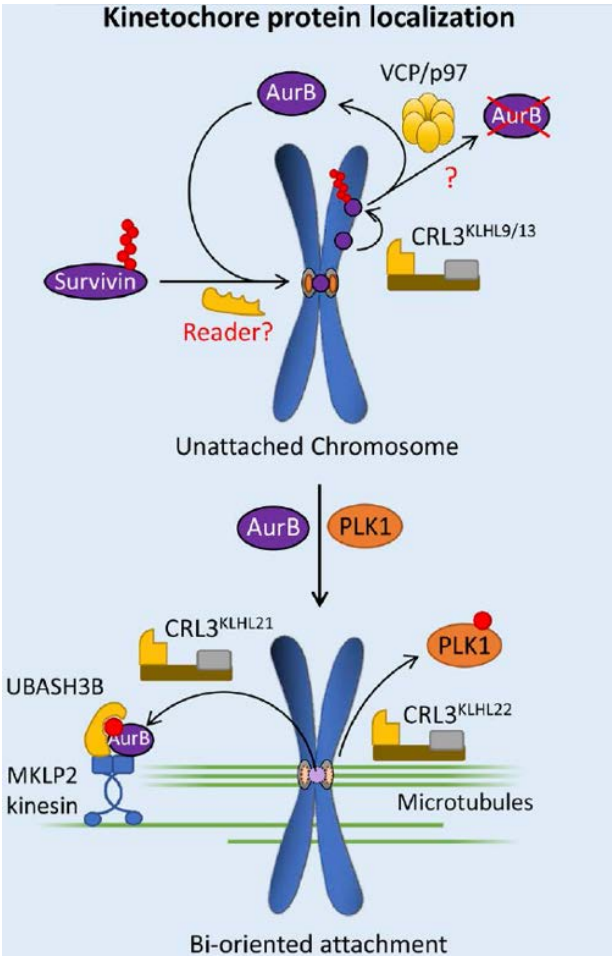

Figure 11: Non-proteolytic CUL3 functions during mitosis

CRL3 complexes are involved in kinetochore protein localization such as AURB and PLK1 in a non-proteolytic-dependent manner through monoubiquitylation of the substrates (*adapted from Gilberto and Peter, 2017*).

Surprisingly, a Cullin7-based complex is also involved in non-proteolytic regulation of mitosis and more specifically of sister chromatids cohesion. Indeed, $CRL7^{SMU1}$ catalyzes the monoubiquitylation of the histone H2B at the lysine residue K120 corresponding to the Structural Maintenance of Chromosomes protein 1A (SMC1a) locus, a central component of the Cohesin complex, thereby ensuring the maintenance of sister chromatid cohesion during mitosis (**Figure 12**) (Shah and Maddika, 2018). Further effort is needed in order to identify potential additional targets of these Cullin-based E3 ligases, as the described substrates might not be the only targets of CRLs during mitosis.

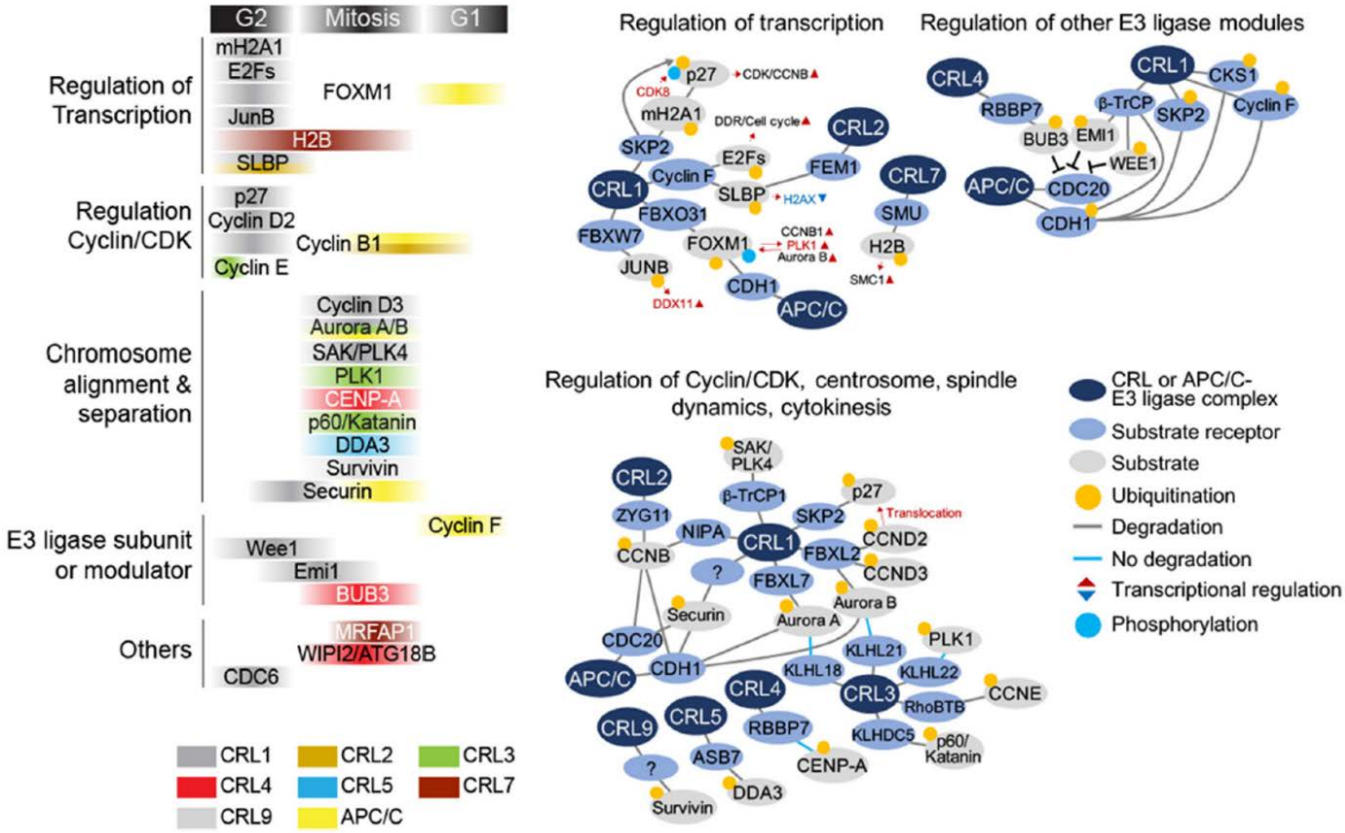

Figure 12: Regulation of the G2/M transition by CRLs

Left panel: Diagram depicting CRLs targets during G2/M. **Right panel**: Schematic representation of CRL functions through G2/M. The types and fates of the substrates are indicated as well as the different Cullins and CRLs' targets have been classified into three main sub-groups: regulation of transcription, regulation of other E3 ligase modules and regulation of mitotic dynamics (*from Jang et al., 2020*).

2. *The Anaphase Promoting Complex/Cyclosome*

The APC/C is a 1,5 MDa E3 Ub-ligase consisting of around a dozen subunits forming a complex machinery acting in concert to promote the ubiquitylation of key substrates during cell division and beyond. During mitosis, the APC/C is essential to promote sister chromatid separation prior to anaphase and mitotic exit (Peters, 2006). The catalytic module of APC/C is made of Anaphase-Promoting Complex subunit 11 (APC11) and the RING-domain and the Cullin-like APC2 subunits which catalyze Ub transfer but give rather poor substrate specificity (Gmachl et al., 2000). This function is rather conferred by the WD-40 domain-containing coactivators CDC20 homolog 1 (CDH1) in late mitosis and in interphase and CDC20 in mitosis (reviewed

in Yamano, 2019). Although the best characterized role of APC/C in mitosis is its function in promoting metaphase to anaphase transition, the complex is already required at earlier mitotic steps (**Figure 13**). Indeed, Cyclin A degradation at early mitosis is mediated by APC/C^{CDC20} (Geley et al., 2001) and, likewise, the APC/C-dependent destruction of the NIMA-related kinase 2A (Nek2A) is essential for proper centrosome separation (Hames, 2001).

Importantly, APC/C^{CDC20} mediates Securin polyubiquitylation after all chromosomes are properly aligned and attached to MTs, which triggers its degradation, liberating the protease Separase which in turns cleaves Cohesin maintaining sister chromatids together at the centromeric regions (Uhlmann et al., 1999, 2000). This event marks the onset of anaphase. Interestingly, APC/C activation by its coactivators CDC20 and CDH1 strongly depends on phosphorylation of APC/C subunits by the kinases PLK1 and CDK1 (Golan et al., 2002). Whereas CDC20 is targeted to APC/C when the latter is hyperphosphorylated, CDH1 phosphorylation prevents its association with APC/C (Jaspersen et al., 1999; Kramer et al., 2000; Zhang et al., 2016). Therefore, the phosphorylation events need to be tightly regulated in time in order to promote the degradation of the correct proteins in a very precise manner. For example, Cyclin B1 degradation occurring during anaphase is initiated by APC/C^{CDC20} and completed by APC/C^{CDH1} (Raff et al., 2002). Upon lowering of CDK1 activity, CDH1 is dephosphorylated and associates with APC/C, mediating CDC20 degradation (Robbins and Cross, 2010). APC/C^{CDH1} is essential for mitotic exit as it promotes ubiquitylation and subsequent degradation of the key mitotic kinases PLK1, AURA and AURB in a sequential-manner (Lindon and Pines, 2004; Lindon et al., 2016). Finally, APC/C^{CDH1} may contribute to the proteolysis of additional important cell cycle-regulated proteins to promote mitotic exit as it is the case for the MT-associated protein Targeting protein for Xenopus Kinesin-like protein 2 Klp2 (TPX2) or the cleavage furrow protein Anillin (Stewart and Fang, 2005; Zhao and Fang, 2005).

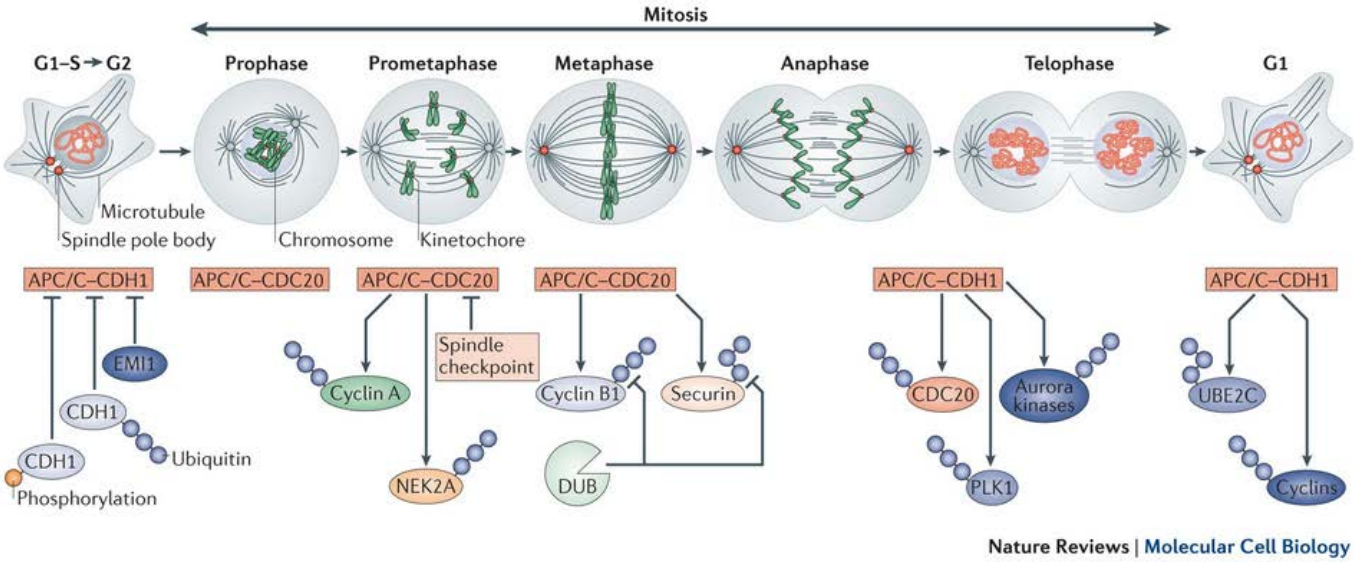

Figure 13: Sequential degradation of major APC/C substrates

The timely-regulated switch from CDH1 to CDC20 association with APC/C triggers the degradation of key substrates in order to promote faithful mitotic progression. Key APC/C regulators as well as major targets are indicated on the scheme (*from Sivakumar and Gorbsky, 2015*).

3. Remarks

Although our knowledge about E3 ligases is constantly growing, additional studies are needed to dissect the molecular mechanisms by which APC/C and Cullin-based complexes regulate mitosis in proteolytic and non-proteolytic manners. It is crucial to note that all the above-mentioned ubiquitylation pathways are counteracted or supported by specific DUBs and additional Ubiquitin-binding proteins (UBPs) which are not reviewed here but play key roles in ubiquitin signaling during mitosis. More information about DUBs as critical regulators of mitosis can be found in review articles such as Park et al., 2019.

C. Regulation of mitosis by phosphorylation

Mitosis is synonymous with dramatic morphological changes that need to be achieved very rapidly. Phosphorylation, representing a fast and reversible PTM, is a key modification in this process. In fact, more than 1000 phosphorylated proteins have been identified during mitosis (Dephoure et al., 2008; Kettenbach et al., 2011). Here, I aim at summarizing the main mitotic kinases responsible for these phosphorylation events and their roles during cell division, with a particular focus at PLK1. As discussed in the previous section, like E3 Ub ligases, phosphorylation events are not only dependent on kinases but also, and as importantly, on

phosphatases that oppose their roles, finetuning the rearrangements necessary for the faithful completion of mitosis (Bollen et al., 2009; De Wulf et al., 2009). The essential roles and regulation of phosphatases during mitosis have been summarized by Moura and Conde (2019).

1. The CDK family

The CDK family consists of around 20 members in humans. These serine/threonine (Ser/Thr) kinases are characterized by their dependency on a regulatory subunit, a cyclin, to promote their catalytic activity by inducing a conformational change. Although CDKs protein levels are not regulated in a cell-cycle dependent manner, cyclins are, thus adjusting CDKs kinase activity by cyclins availability during the different cell cycle phases (Malumbres and Barbacid, 2005). CDKs are crucial cell cycle regulators as they control cell cycle checkpoints (Barnum and O'Connell, 2014) but CDK1 has been shown to be sufficient to drive the mammalian cell cycle (Santamaría et al., 2007) whereas the second main kinase CDK2 is dispensable for mitosis (Ortega et al., 2003).

CDK1 is a highly conserved protein which was first discovered in yeast and named Cell division control protein 2 (cdc2) in a screen showing that its mutation led to severe cell cycle defects (Russell and Nurse, 1987). As a primary regulatory mechanism, CDK1 associates with both Cyclins A and B which triggers its activation, underlying its rise and fall during mitotic entry and exit (Crncec and Hochegger, 2019). The initiation of mitosis resides in the removal of critical phosphates from two CDK1 residues, acting as an inhibitory signal (**Figure 14**). This is achieved by the inhibition of the kinases responsible for the deposition of these phosphates, WEE1/Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (MYT1), and in parallel the activation of the counteracting phosphatase CDC25, catalyzing the dephosphorylation of the two residues (Dunphy and Kumagai, 1991; Kumagai and Dunphy, 1991). WEE1/MYT1 and CDC25 inactivation and activation respectively are regulated in multiple ways involving other phosphorylation and dephosphorylation events, protein-protein interactions, proteolysis and proline isomerization as elegantly reviewed by Perry and Kornbluth (2007). Once active, CDK1 is responsible for the major morphological rearrangements observed during early mitosis until chromosomes align properly. As mitosis progresses, Cyclins A and B are sequentially degraded, shutting down CDK1 activity and promoting anaphase onset and subsequent mitotic exit (Primorac and Musacchio, 2013).

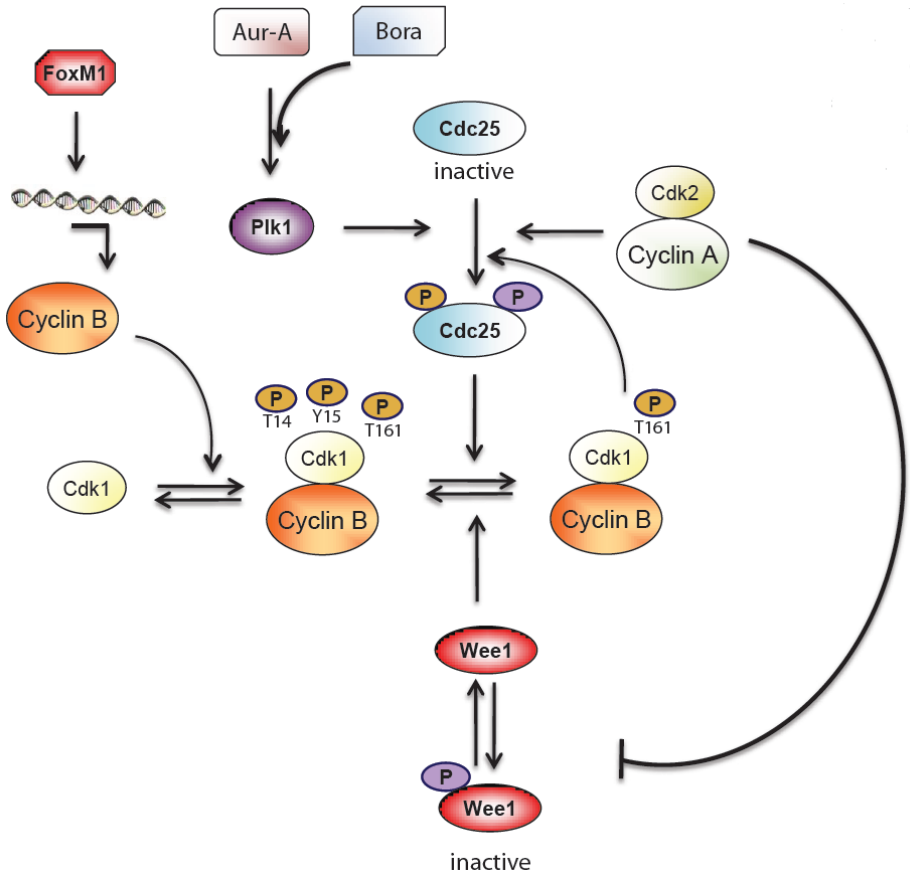

Figure 14: Schematic view of mitotic entry regulation

Mitotic entry is ensured by finetuning CDK1 phosphorylation status. This is achieved by the opposing functions of CDC25 and WEE1. Internal and external feedback loops regulate this activation to safeguard mitotic entry at the appropriate time, adding an extra layer of complexity. For clarity and simplicity, only few mitotic kinases and phosphatases contributing to mitosis onset have been depicted on this scheme (*from Vigneron et al., 2016*).

2. *The Aurora family*

The Aurora family also belongs to Ser/Thr kinase superfamily and contains three members in mammals: Aurora A (AURA), Aurora B (AURB) and Aurora C (AURC). Although AURC expression is limited to germ cells and is important for meiosis (Kimmins et al., 2007), AURA and AURB are ubiquitously expressed and play crucial roles during mitosis (Fu et al., 2007). AURA and AURB have distinct roles during cell division, partly due to their different localizations.

First, AURA contributes to the previously discussed CDK1 activation, therefore promoting G2/M transition. Indeed, depletion of AURA causes G2/M arrest in HeLa cells (Du and Hannon, 2004). Mechanistically, AURA is responsible for CDC25 phosphorylation at the

centrosomes, triggering Cyclin B1 recruitment to the nucleus and as a consequence contributing to CDK1 activation (Cazales et al., 2005). Moreover, AURA is involved in centrosome maturation by directly phosphorylating or recruiting essential centrosomal components (Berdnik and Knoblich, 2002; Terada et al., 2003) as well as promoting the nucleation and polymerization of centrosomal MTs (Giet et al., 2002; Barros et al., 2005; Kinoshita et al., 2005). At a later stage, AURA regulates centrosome separation (Marumoto et al., 2003), and importantly promotes MTs nucleation at the midzone during anaphase (Courthéoux et al., 2019), thereby exerting crucial roles in mitotic spindle assembly (**Figure 15**) (Fu et al., 2007; Magnaghi-Jaulin et al., 2019).

AURB is a major mitotic kinase as it displays crucial functions ranging from chromosome condensation to SAC and MT-KT attachment regulation and cytokinesis (Vagnarelli and Earnshaw, 2004). It exerts its role as part of the Chromosome Passenger Complex (CPC), harboring the catalytic activity of the complex. In addition to AURB, the CPC consist of the inner centromere protein (INCENP), Survivin and Borealin (known as Dasra B) (Jeyaprakash et al., 2007; Carmena et al., 2012). During early mitosis, the CPC needs to be targeted to the inner centromere which is mediated through several histones phosphorylations: H2A (Thr120) and H3 (Thr3) by BUB1 and Haspin kinases respectively thereby creating docking sites for Borealin and Survivin (Kawashima et al., 2010). Moreover, CENP-A phosphorylation by AURA has been shown to be required for proper CPC centromeric localization (Kunitoku et al., 2003). This recruitment is further increased by the positive feedback loop generated by Haspin AURB-mediated phosphorylation (Wang et al., 2011). AURB full activation, triggered by autophosphorylation at Thr232 residue, promotes Condensin I but not Condensin II association with mitotic chromosomes during prophase (Lipp et al., 2007), thereby ensuring proper chromosome condensation. Furthermore, AURB is actively involved in the regulation of MT-KT attachment status, on one hand, by maintaining the active SAC until correct attachments are achieved and on the other hand by promoting the selective disassembly of syntelic and merotelic MT-KT attachments (Hauf et al., 2003; Cimini et al., 2004; Lampson et al., 2004). Additional phosphorylation of Mitotic Centromere-Associated Kinesin (MCAK or KIF2C) by AURB ensures that correct attachments are stabilized whereas incorrect ones are repaired through MTs depolymerization by MCAK (Andrews et al., 2004; Lan et al., 2004). An extra-layer of regulation mediated by AURB, is SAC regulation as discussed in the chapter IIA. Once all chromosomes are properly aligned, AURB is relocalized to MTs through the coordinated action of the Ubiquitin-Associated and SH3 domain-containing protein B

(UBASH3B) ubiquitin receptor and the Mitotic Kinesin-Like Protein 2 (MKLP2 or kinesin-6) motor protein (Krupina et al., 2016). Finally, during telophase AURB is localized to the midbody where it precisely regulates the actinomyosin contractile ring assembly, abscission and cytokinesis, thereby inhibiting chromosome breakage (**Figure 15**) (Norden et al., 2006; Basant et al., 2015).

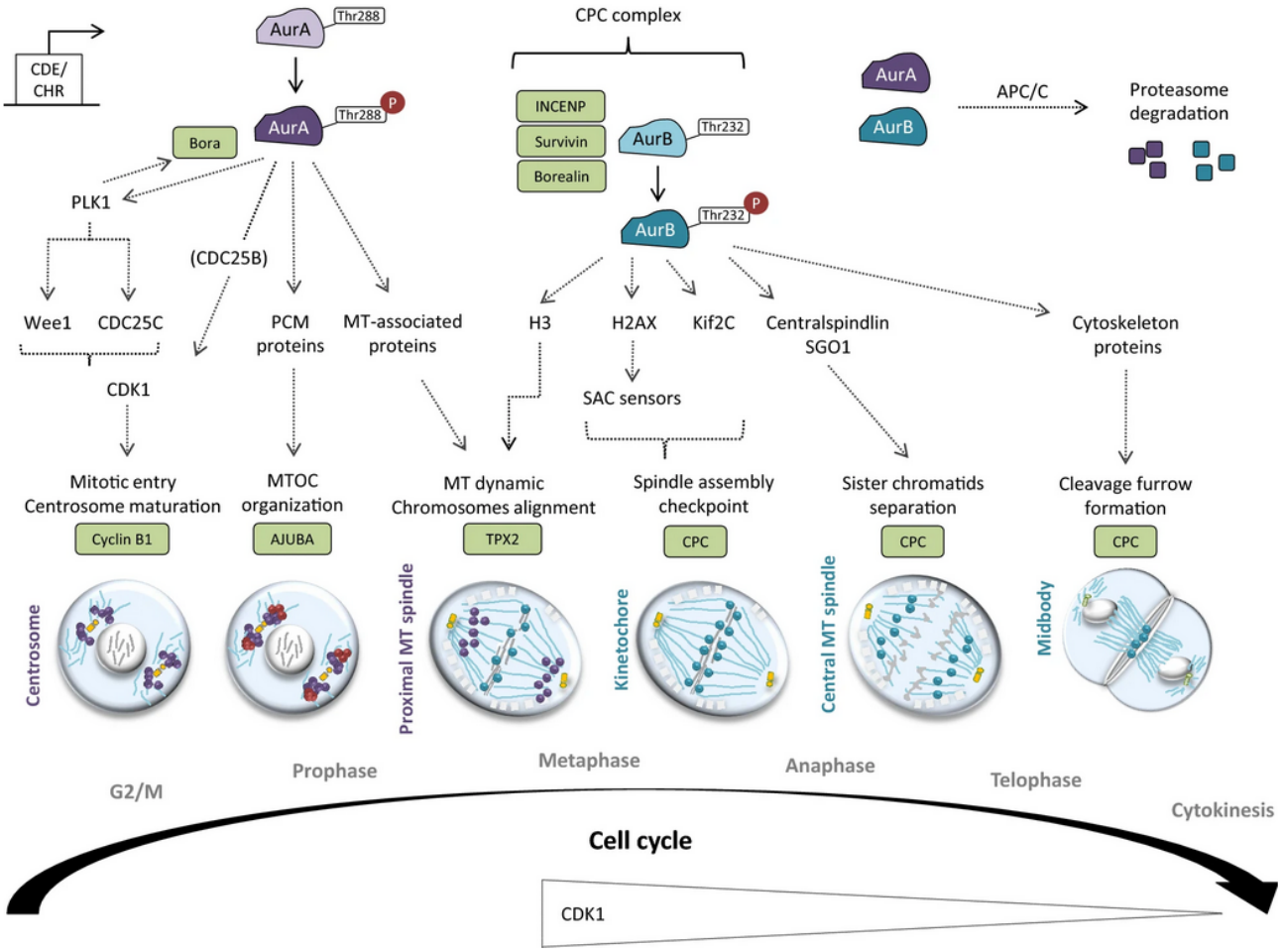

Figure 15: Various roles of Aurora kinases during mitosis

The Cell cycle-Dependent Element (CDE)/Cell cycle genes Homology Region (CHR) elements control the cell cycle-dependent expression of AURA and AURB. As detailed in the chapters IIC2 and IIC3, AURA is responsible for PLK1 activation, centrosome maturation and MTOC organization during early mitosis while AURB, as part of the CPC complex, regulates multiple steps of cell division such as chromosome alignment, SAC response, anaphase onset and cytokinesis (*from Willems et al., 2018*).

3. *The PLK family*

Similarly to the two previous families, PLKs are also Ser/Thr protein kinases playing crucial roles in cell cycle regulation (Zitouni et al., 2014). In vertebrates, the PLK family consists of five, structurally similar members numbered from 1 to 5, with an N-terminal catalytic domain and one or several C-terminal Polo-Box Domains (PBD) which mediate their dynamic localization to distinct substructures, substrate specificity and self-priming (**Figure 16**) (Park et al., 2010). Whereas PLK1 has been extensively studied over the past years mainly due to its importance for mitotic progression but also for its interphasic roles, the roles of the other PLK family members remain less explored. PLK2 has been described as an important factor for centriole duplication (Warnke et al., 2004) and mitotic spindle orientation in mammary gland (Villegas et al., 2014) as well as spindle damage recovery (Burns et al., 2003). Similarly, PLK4 is a key regulator of centriole duplication (Habedanck et al., 2005). Despite the poor characterization of PLK3, it seems that it could also be important for mitosis through CDC25C phosphorylation and subsequent translocation to the nucleus during G2/M transition to trigger Cyclin B1 recruitment and CDK1 activation (Bahassi et al., 2004). PLK5 is the only member with no catalytic activity and has not been linked to mitosis yet but to the regulation of neuron differentiation (**Figure 16**) (de Cárcer et al., 2011).

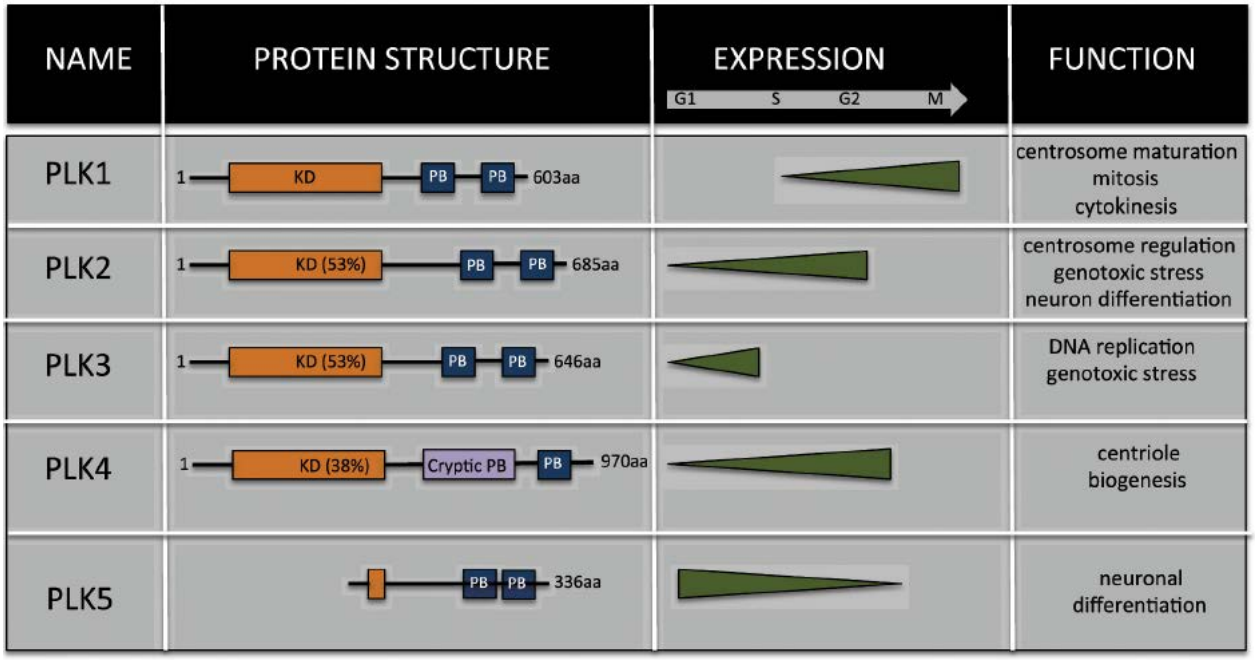

Figure 16: The human PLK family

The protein structure, expression through the cell cycle and the functions of PLK family members are summarized. Orange boxes represent the Kinase Domain (KD), blue boxes represent the Polo-Box Domains (PBDs). The indicated percentage inside the KD represents the conservation of the KD sequence with PLK1's KD. aa stands for aminoacids (*from Raab et al., 2021*).

PLK1 is the most conserved PLK family member through species. It dynamically localizes to different mitotic structures as cell division progresses thanks to its two PBDs and phosphorylation of specific substrates (Hanisch et al., 2006; Schmucker and Sumara, 2014). During G2/M transition, PLK1 is strongly enriched at centrosomes. Its recruitment is thought to be mediated through CDK1-mediated phosphorylation of the centrosomal component Cenexin1 (Soung et al., 2006). PLK1 presence at the centrosome triggers Pericentrin phosphorylation and subsequent PCM components recruitment such as γ-tubulin and AURA among others (Lee and Rhee, 2011). Interestingly, PLK1 binding to Protein Aurora Borealis (BORA) changes its conformation allowing the phosphorylation of the Thr210 residue of PLK1 by AURA and thus enabling its full activation (Seki et al., 2008). Not surprisingly, Parrilla and colleagues reported that BORA is primarily phosphorylated by CDK1 in G2, supporting AURA-mediated PLK1 phosphorylation (Parrilla et al., 2016). Once fully activated, PLK1 indirectly boosts CDK1 activity through CDC25 phosphorylation and subsequent nuclear translocation (**Figure 17**) (Toyoshima-Morimoto et al., 2002). PLK1 promotes centrosome maturation and separation by phosphorylating the Mammalian STE20-like protein kinase 2 (MST2)-NIMA-related kinase 2 (NEK2A) kinase module and the kinesin-5 Eg5 (Mardin et al., 2011; Smith et al., 2011). During prometaphase, PLK1 progressively accumulates at kinetochores thanks to its interaction with Polo-Box Interacting Protein 1 (PBIP1) (Kang et al., 2006) where it regulates proper MT-KT attachment possibly through its interaction with kinetochore receptors including BUB1, BUBR1, INCENP and protein phosphatase 2A (PP2A) counteracting AURB (Sumara et al., 2004; Goto et al., 2006; Qi et al., 2006; Elowe et al., 2007; Lénárt et al., 2007; Foley et al., 2011). Recently, PLK1 recruitment mechanism to kinetochores has been further studied and identified BUB1 and CENP-U as the major receptors of PLK1 to the outer and inner kinetochore, respectively, in a process driven by CDK1- and PLK1-dependent phosphorylation events (Singh et al., 2021). Yet another mechanism where PLK1 is removed from kinetochores during metaphase by the CUL3/KLHL22 complex exists and contributes to SAC satisfaction and mitotic exit as described in the chapter IIB1.2 (Beck et al.,

2013). PLK1 subsequently migrates to the spindle midzone during anaphase through interaction and phosphorylation of PRC1 (Hu et al., 2012). Finally, during telophase and cytokinesis, PLK1 accumulates at the midbody, regulating crucial abscission factors such as Centrosomal Protein 55 kDa (CEP55) in complement to AURB late mitotic functions (Bastos and Barr, 2010).

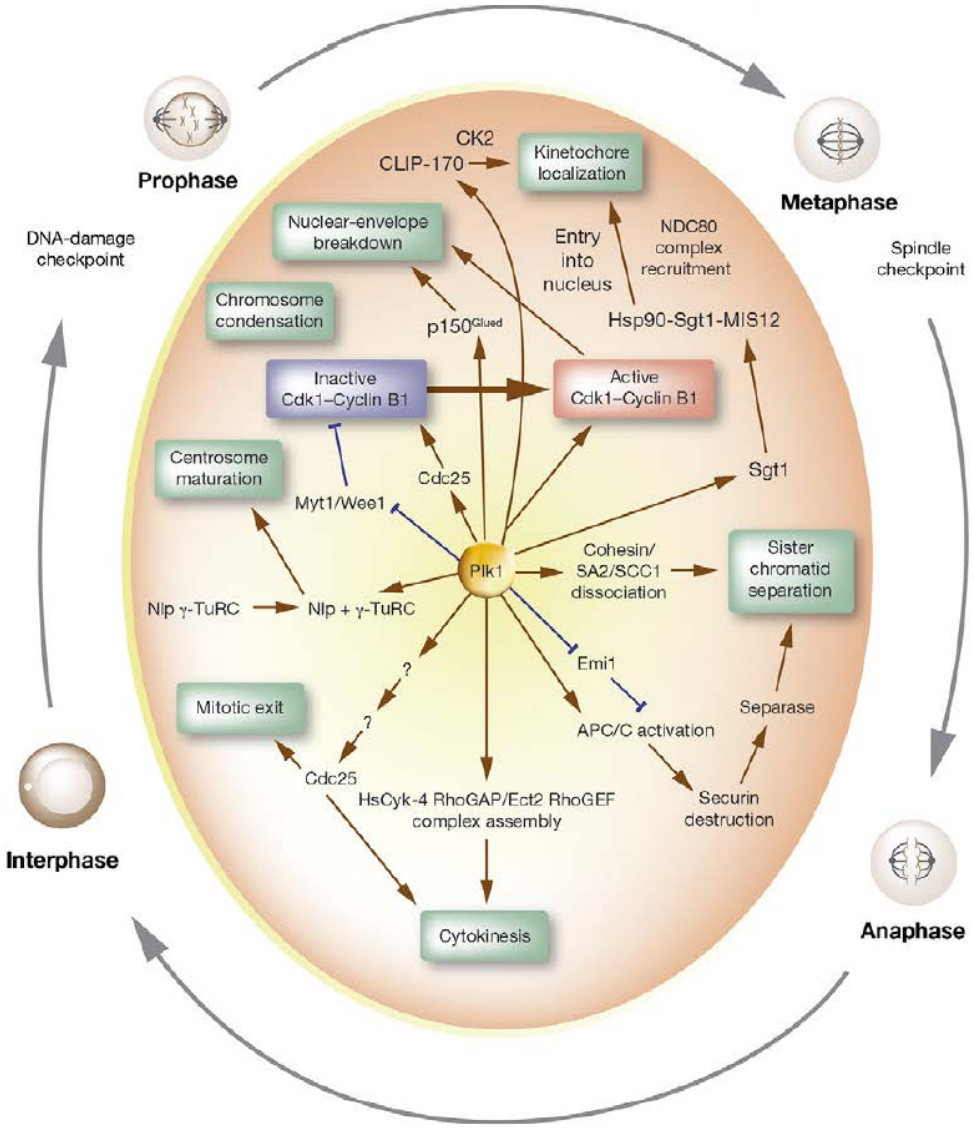

Figure 17: Functional roles of PLK1 during cell division

PLK1 is a master regulator of mitosis as depicted by these non-exhaustive roles. Among others, it regulates mitotic entry, MT-KT attachment, sister chromatids separation and subsequent anaphase onset and cytokinesis (*from Liu, 2015*).

4. Remarks

Here, I aimed at describing the major molecular mechanisms that fine-tune mitosis, especially in the context of PTMs. It is important to keep in mind that most of these pathways are interconnected and regulate each other in space and time in order to promote faithful partition of the genome.

III. NON-MITOTIC FUNCTIONS OF PLK1

Except its well-established role in cell division, PLK1 emerges as a critical regulator of a plethora of cellular processes not linked to mitosis.

A. PLK1 regulates cilium disassembly

Primary cilia are small protrusions of the plasma membrane which are important extracellular fluid propulsion, linked to WNT and Sonic hedgehog (SHH) signaling pathways and autophagy among others (Pampliega et al., 2013; Anvarian et al., 2019). Cilium assembly and disassembly are cell cycle-regulated and are crucial for cell survival (Jeffries et al., 2019; Doornbos and Roepman, 2021). Interestingly, PLK1 has been shown to contribute to primary cilia disassembly by interacting with Dishevelled 2 (Dvl2) which in turn stabilizes and activates the Human Enhancer of Filamentation 1 (HEF1)/AURA complex triggering cilia disassembly (Lee et al., 2012).

B. PLK1 and autophagy

Autophagy is a conserved cellular process involving self-degradation of cellular components in order to maintain cell homeostasis under nutrient stress conditions (Cooper, 2018). The very basic concept triggering autophagy activation is stress sensing mainly by two complexes: mammalian Target of Rapamycin (mTOR) complexes 1 and 2 (mTORC1 and mTORC2). While mTORC1 is sensitive to nutrients level variations, mTORC2 is rather responsive to Phosphoinositide 3-kinase (PI3K) and growth factor signaling pathways (Jhanwar-Uniyal et al., 2019). mTORC1 complex, composed of the Ser/Thr kinase mTOR and its regulatory partner Regulatory-Associated Protein of mTOR (RAPTOR), inhibits autophagy under normal conditions. Interestingly, in HeLa cells PLK1 directly interacts with mTOR as part of the mTORC1 complex, and its inhibition promotes mTORC1 localization to lysosomes, thereby restraining autophagy (Ruf et al., 2017). Consistently, the same phenotype was observed in various cancer cell lines as summarized by Chiappa and colleagues (2022).

C. PLK1 and DNA replication

Studying a key primary cilium formation factor, Fibroblast growth factor receptor 1 protein (FGFR1) Oncogene Partner (FOP)-related protein of 20 KDa FOR20 protein, Shen and co-workers identified PLK1 as a crucial DNA replication regulator, exerting its function at the S-phase centrosome. PLK1 recruitment to centrosomes was mediated in a FOR20-dependent manner, ensuring normal S-phase progression (Shen et al., 2013). This is consistent with the fact that PLK1-depleted cells exhibit longer S-phase although the most evident phenotype triggered by PLK1 depletion is a mitotic arrest (Lei and Erikson, 2008). Additional evidence of PLK1-mediated DNA replication regulation comes from the demonstrated interactions between PLK1 and key replication fork factors such as Minichromosome Maintenance (MCM) complex proteins and the DNA replication machinery component Origin Recognition Complex subunit 2 (ORC2) (Tsvetkov and Stern, 2005; Stuermer et al., 2007; Mandal and Strebhardt, 2013). Furthermore, PLK1 phosphorylates Histone acetyltransferase Binding to ORC1 (HBO1) thereby regulating the loading of pre-replicative complexes (pre-RCs) onto the origins (Wu and Liu, 2008). A recent study underlined the importance of PLK1 in controlling DNA replication origin firing in collaboration with the Ras-related Protein 1 (RAP1)-Interacting Factor 1 (RIF1) in *Xenopus* (Ciardo et al., 2021). Finally, PLK1 is proposed to regulate DNA replication under various stress conditions (Song et al., 2011, Song et al., 2012) and, more specifically, PLK1 is recruited to the broken replication forks in an Ataxia Telangiectasia Mutated (ATM)-dependent manner to inhibit the Double Strand Break (DSB) ubiquitylation response, catalyzing DNA end resection by C-terminal-binding protein (CtBP)-Interacting Protein (CtIP) to ensure accurate DNA repair specifically by Homologous Recombination (HR) (Nakamura et al., 2021).

D. PLK1 and the DNA damage checkpoints

As previously discussed, the final aim of the cell cycle is the faithful segregation of the genetic material into two daughter cells. Cellular integrity is achieved thanks to the existing surveillance mechanisms throughout the cell cycle. Inevitably, DNA damage caused by either endogenous or exogenous factors may occur during cell cycle progression. In the presence of DNA damage, the cell is faced with three possible outcomes: **(1)** DNA repair before erroneous segregation also called checkpoint recovery, **(2)** checkpoint adaptation in which cell decides to ignore and overpass the damage and progress through the cell cycle and **(3)** apoptosis in cases when damage is irreparable, triggering programmed cell death (Bartek and Lukas, 2007). Three main DNA damage checkpoints have been described in humans occurring in G1, S and G2 phases,

respectively, ensuring genome integrity prior to DNA replication (G1 checkpoint), after DNA replication (S checkpoint) and before cell division (G2/M checkpoint) (Barnum and O'Connell, 2014). The ultimate goal of the checkpoints is to prevent DNA replication and segregation until the damage is fixed by inducing cell cycle arrest. This is mainly achieved through the regulation of key cell cycle drivers such as the well-known tumor suppressor p53 and CDC25 proteins. Briefly, the presence of DNA damage is sensed by the Meiotic Recombination 11 (MRE11), the DNA repair protein RAD50 and the Nijmegen Breakage Syndrome protein 1 (NBS1) (MRN) sensor complex which recruits the ATM and/or Ataxia Telangiectasia and Rad3-related protein (ATR) kinases, driving the recruitment of key DNA repair factors and subsequently defining the appropriate DNA repair pathways to use (Ciccia and Elledge, 2010). This is the DNA Damage Response (DDR). To summarize, ATM and ATR phosphorylate and activate the Checkpoints kinases CHK2 and CHK1 respectively which in turn regulate p53 and CDC25 in order to block the progression of the cell cycle mediated by their Cyclin/CDKs targets (Bartek and Lukas, 2003; Giono and Manfredi, 2006).

(1) After DNA damage has been fixed, cells have to restart cycling. Interestingly, PLK1 has been demonstrated to have crucial function in cell cycle restart after DNA damage-mediated G2 arrest in mammalian cells. Indeed, PLK1 inactivates the CDK1 inhibitor WEE1 and promotes its degradation, thereby triggering CDK1 activation and mitotic entry (van Vugt et al., 2004). Additional studies from the same authors further show that PLK1 phosphorylates the checkpoint adaptor protein p53-Binding Protein 1 (53BP1) as well as CHK2 to inactivate the checkpoint signaling (**Figure 18**) (van Vugt et al., 2010). Importantly, ATR-dependent CHK1 activation requires the essential mediator Claspin. The latter needs to get degraded in order to promote checkpoint silencing and cell cycle restart and this is mediated through PLK1-mediated phosphorylation of Claspin leading to its ubiquitylation by $SCF^{\beta TrCP}$ and subsequent proteolysis (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006).

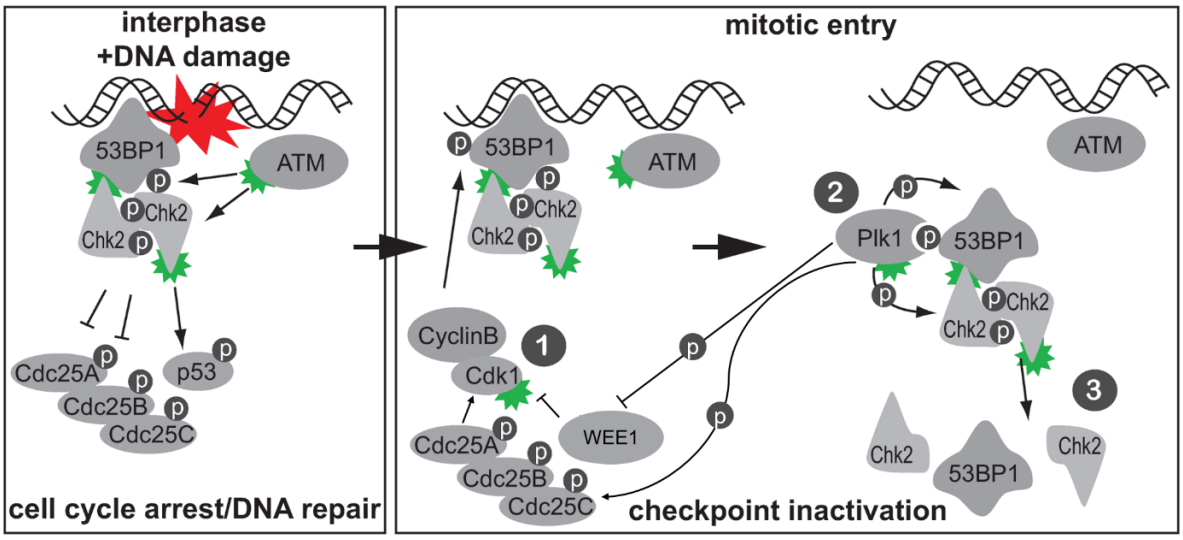

Figure 18: Checkpoint recovery regulation by PLK1

Left panel: When DNA damage occurs, the repair machinery is recruited to the break and activates p53 while inhibiting CDC25 to promote cell cycle arrest. **Right panel**: Once the break is repaired, CyclinB/CDK1 complex ❶ is activated and phosphorylates components of the repair machinery including 53BP1, creating a docking site for PLK1 PBD and leading to its recruitment ❷. PLK1 subsequently phosphorylates WEE1 and CDC25 promoting CDK1 full activation while phosphorylating 53BP1 and CHK2 ultimately leading to the inactivation of the checkpoint ❸ and mitotic entry. The green stars indicate enzymatically active kinases (*modified from van Vugt et al., 2010*).

(2) Intriguingly, PLK1 displays multifaceted functions as it has also been proposed to regulate checkpoint adaptation, a mechanism allowing cell cycle to progress regardless of the presence of unrepaired DNA breaks. Preliminary work from Yoo and colleagues highlighted the role of Plx1, the *Xenopus* PLK1 ortholog, in bypassing the S-phase checkpoint response by directly interacting with and phosphorylating Claspin, thus triggering its removal from chromatin, CHK1 inactivation and cell cycle progression (**Figure 19**) (Yoo et al., 2004). This result has further been reinforced in yeast where Cdc5 has been shown to display similar functions (Donnianni et al., 2010) and in human cells where the subunit of the DNA clamp complex 9-1-1 RAD9 was phosphorylated by both CDK1 and PLK1 to limit its efficacy in recognizing DNA damage under low doses of hydroxyurea (HU) mimicking replication stress conditions. S-phase DNA damage checkpoint was therefore overridden to drive proliferation under stress conditions (Wakida et al., 2017). G2/M checkpoint adaption in higher organisms is thought to be detrimental as it leads to mitotic entry in the presence of DNA damage often associated with

carcinogenesis. However, an interesting study showed that human cells can indeed enter mitosis with γH2AX foci, a DSB marker, following ionizing radiation and G2 arrest, suggesting that human cells can also exit G2/M checkpoint with unrepaired DNA and this is mediated by CHK1 and PLK1 (Syljuåsen et al., 2006).

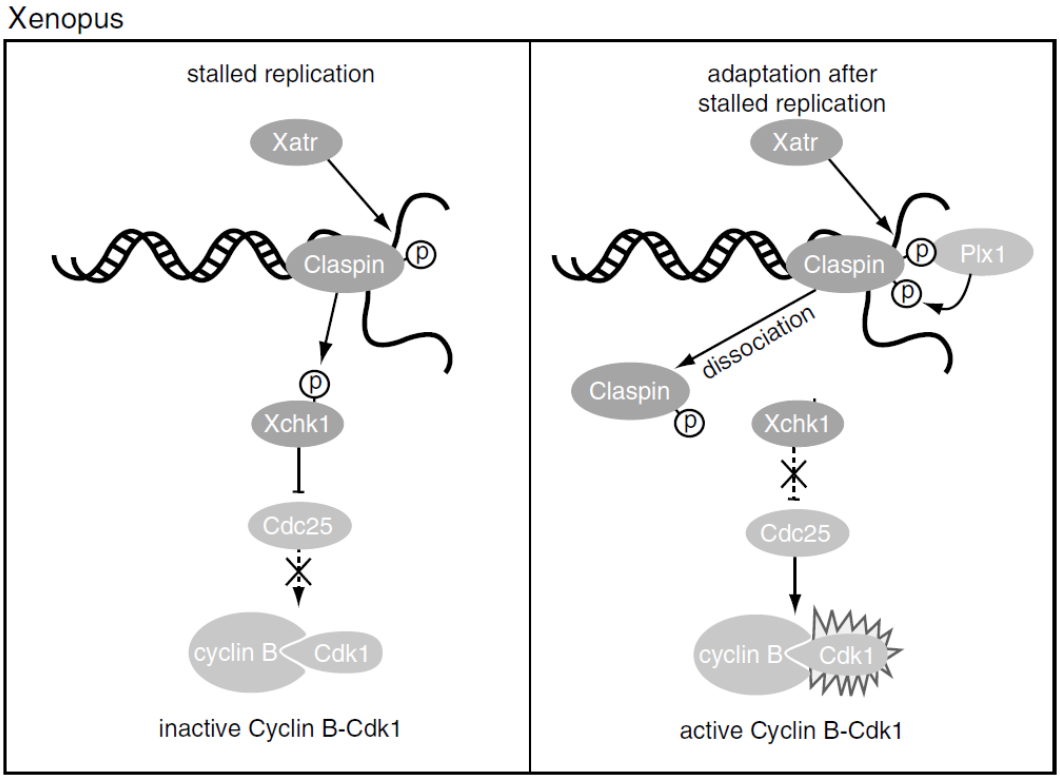

Figure 19: Checkpoint adaptation regulation by Plx1 in *Xenopus*

In *Xenopus*, when the replication fork stalls, Xatr mediates the phosphorylation of Xchk1 in a Claspin-dependent manner to arrest the cell cycle. During checkpoint adaptation, Plx1 phosphorylates Claspin leading to its dissociation from chromatin and silencing of the checkpoint. Xchk1 is inactive, Cdc25 phosphatase mediates Cdk1 activation and cell cycle restart (*adapted from van Vugt and Medema, 2005*).

(3) Third, PLK1 displays anti-apoptotic activity in both p53-dependent and -independent manners. On one hand PLK1 directly binds the DNA-binding domain of p53, thus blocking its well-established pro-apoptotic and transactivation properties (Ando et al., 2004). On the other hand, PLK1 interacts with and phosphorylates the pro-apoptotic factor p73 therefore inhibiting p53-independent apoptosis (Koida et al., 2008; Soond et al., 2008). This is consistent with previously published data showing that PLK1 inhibition induces apoptosis in several human cancer cell lines (Liu and Erikson, 2003; Fan, 2005).

Overall, PLK1 stands as a master regulator of the cell cycle (Bahassi, 2011) and as such PLK1 is very often hijacked by cancer cells as discussed in the following chapter IIIE.

E. PLK1 and cancer

Not surprisingly, PLK1 is found overexpressed in various human cancers where it displays oncogenic properties (Holtrich et al., 1994; Yamada et al., 2004; Strebhardt and Ullrich, 2006). As mentioned above, PLK1 negatively regulates p53 expression and activity while the latter represses PLK1 expression. This mutual inhibition is misregulated in cancer cells, promoting their proliferation and survival in spite of the accumulation of aberrations (reviewed in Louwen and Yuan, 2013 and Chiappa et al., 2022). Strikingly, PLK1 directly interacts with 75% of the twelve signaling pathways components regulating the three milestones of cell biology: cell survival, cell fate and genomic maintenance (Cholewa et al., 2013; Vogelstein et al., 2013). In addition to the previously discussed roles in cell cycle regulation, PLK1 can promote Epithelial-Mesenchymal Transition (EMT) and metastasis in prostate cancer cells and gastric carcinoma through the activation of the Cellular-Rapidly Accelerated Fibrosarcoma (C-RAF)/ Extracellular signal-Regulated Kinase (ERK) and AKT signaling pathways, respectively (Cai et al., 2016; Wu et al., 2016; Fu and Wen, 2017). Due to its ability to evade growth suppressors, activate EMT and metastasis, resist cell death while sustaining proliferative signaling, on top of its crucial roles in cell division and genome stability, PLK1 is implicated in most of the pathways that have been described as hallmarks of cancer (**Figure 20**) (Hanahan and Weinberg, 2011). Plethora of potent PLK1-targeted small molecules and siRNAs have been developed and are currently under different phases of clinical trials (Kumar et al., 2016). However, because the majority of these drugs target PLK1 PBD or kinase activity, off-target effects on other PLK family members have been observed as well as strong side effects such as hematological toxicities, nausea, fatigue, and more importantly limited efficacy was observed on advanced or relapsed tumors. This highlights the importance of developing novel PLK1 inhibition strategies that will exploit the PLK1 interactome and rely on synergistic targeting and synthetic lethality approaches (Chiappa et al., 2022).

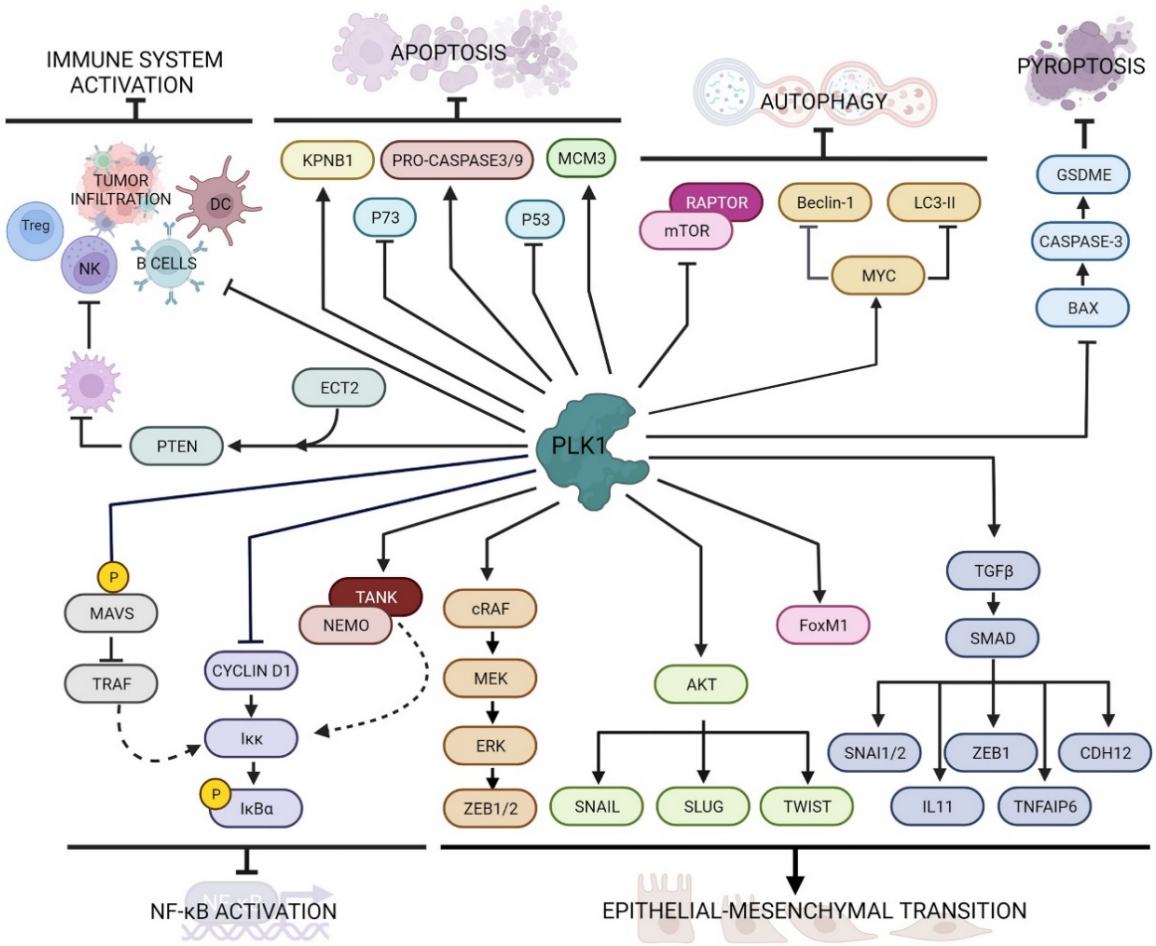

Figure 20: Non-exhaustive view of PLK1 non-mitotic roles

A small fraction of PLK1 substrates is depicted, outside of the well-established roles in mitosis and DNA damage checkpoints. As shown in the upper part, PLK1 regulates the cell degradation machineries as well as the immune system activation. PLK1 favors cancer progression partially through the finetuning of key proliferative EMT signaling pathways (*from Chiappa et al., 2022)*

IV. REVIEW: UBIQUITIN-BINDING PROTEIN 2-LIKE

A. Relevance of the work

In line with my project and because to date no review summarizing the various roles of UBAP2L existed, I decided to describe Ubiquitin-Binding Protein 2-Like (UBAP2L)'s implication in various cellular processes and compile it into this mini-review which was published in June 2022 in Frontiers in Cell and Developmental Biology in the section Cell Growth and Division as part of the research topic *Editors' Showcase 2021: Insights in Cell Growth and Division*.

B. Published manuscript: Ubiquitin-Binding Protein 2-Like (UBAP2L): is it so NICE after all?

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Ubiquitin Binding Protein 2-Like (UBAP2L): is it so NICE After All?

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Ubiquitin Binding Protein 2-like (UBAP2L, also known as NICE-4) is a ubiquitin- and RNA-binding protein, highly conserved in metazoans. Despite its abundance, its functions have only recently started to be characterized. Several studies have demonstrated the crucial involvement of UBAP2L in various cellular processes such as cell cycle regulation, stem cell activity and stress-response signaling. In addition, UBAP2L has recently emerged as a master regulator of growth and proliferation in several human cancers, where it is suggested to display oncogenic properties. Given that this versatile protein is involved in the regulation of multiple and distinct cellular pathways, actively contributing to the maintenance of cell homeostasis and survival, UBAP2L might represent a good candidate for future therapeutic studies. In this review, we discuss the current knowledge and latest advances on elucidating UBAP2L cellular functions, with an aim to highlight the importance of targeting UBAP2L for future therapies.

Keywords: UBAP2L, mitosis, cancer, ubiquitin, stress signaling

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INTRODUCTION

Ubiquitin Associated Protein 2-Like (UBAP2L) or NICE-4 is a highly conserved protein in vertebrates (Chang et al., 2018). Encoded by the KIAA0144 gene located on the chromosomal region 1q21, NICE-4 was originally identified by Marenholz and colleagues in an effort to discover new Human Epidermal Differentiation Complex (EDC)-encoded genes (Marenholz et al., 2001). Five different isoforms produced by alternative splicing have been reported for UBAP2L, that are broadly expressed in nearly all tissues. Despite its abundant expression, UBAP2L has only recently attracted attention of broad scientific community which led to the discovery of its highly versatile roles. Interestingly, UBAP2L orthologs have been identified in metazoans such as Prion-like (Q/N-rich)-domain-bearing protein (PQN-59) in *Caenorhabditis elegans* and lingerer in *Drosophila melanogaster* (Uhlén et al., 2015).

UBAP2L is a 1,087 amino-acid (aa)-long protein, structurally composed of a N-terminal Ubiquitin-Associated Domain (UBA; aa 49-89), an Arginine-Glycine-Glycine (RGG; aa 131-190) domain and three predicted RNA-Binding regions (aa 239-257, aa 282-290 and aa 850-864) (Castello et al., 2016) (**Figure 1**). SILAC analysis demonstrated that UBAP2L cofractionates with ubiquitin in aggregates following proteasomal inhibition, emphasizing the functionality of its UBA domain (Wilde et al., 2011). Moreover, ribosome profiling studies demonstrated that UBAP2L promotes translation of target mRNAs suggesting that it can act as a ribosome-binding protein essential for protein synthesis (Luo et al., 2020). In addition, UBAP2L harbors a Domain of Unknown Function (DUF; aa 495-526). Prediction tools have unraveled several disordered regions prone to undergo Liquid-Liquid Phase Separation (LLPS) as well as several Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES), suggesting that UBAP2L is shuttling between the cytoplasm and the

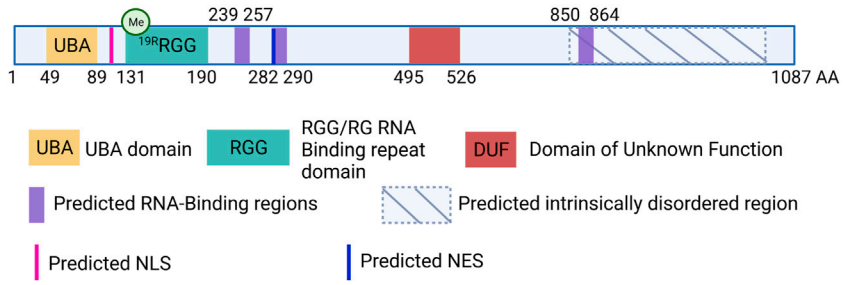

FIGURE 1 | Schematic view of the human UBAP2L protein and its domain organization. UBAP2L (1087 AA) is composed of a Ubiquitin-associated domain (UBA, yellow), an Arginine-Glycine-Glycine (RGG, green) domain and a Domain of Unknow Function (DUF, red). Additional RNA-binding regions have been predicted and are painted in purple. Moreover, UBAP2L is predicted to contain several Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES) (pink and dark blue respectively). Several UBAP2L regions have been proposed to be intrinsically disordered (IDR), and prone to liquid-liquid phase-separation. The most conserved ones are shown with hatched lines. It is important to note that for simplicity we chose to depict only some of the predicted NLS, NES and IDR regions of UBAP2L in the scheme, and that this does not exclude the existence of other similar motifs or regions. Similarly, documented methylation modification on 19 different arginines (19R) present within the RGG domain has been indicated schematically.

nucleus. Such atypical domain organization classifies UBAP2L in both Ubiquitin-binding and RNA-binding proteins superfamilies, highlighting its potential involvement in a plethora of cellular processes.

Although UBAP2L was initially described as an interactor of the Human Zona Pellucida Sperm-binding protein 3 (ZP3) (Naz and Dhandapani, 2010), during the last decade additional studies have demonstrated its direct involvement in cell growth, mitotic progression, stem cell activity, apoptosis and stress response signaling (Bordeleau et al., 2014; Li and Huang, 2014; Chai et al., 2016; Maeda et al., 2016; Youn et al., 2018; Huang et al., 2020). Moreover, UBAP2L is overexpressed in different types of cancer, displaying oncogenic potential and often correlating with poor prognosis (Li and Huang, 2014; Zhao et al., 2015; Bai et al., 2016; Chai et al., 2016; Aucagne et al., 2017; He et al., 2018; Yoshida et al., 2020; Guan et al., 2021). Of note, UBAP2L KO mice die before birth or within minutes after surgical delivery from acute respiratory failure, demonstrating that UBAP2L holds housekeeping functions, essential for living organisms (Aucagne et al., 2017). This review discusses the current knowledge and the latest advances on elucidating NICE-4 cellular functions, with an aim to highlight the importance of targeting NICE4 for future therapies.

UBAP2L AND CELLULAR HOMEOSTASIS

UBAP2L and Stem Cell Activity

As mentioned above, UBAP2L KO mice die prematurely, pointing to a potential role for UBAP2L during development. Interestingly, in *C. elegans*, PQN-59 has been shown to modulate gene expression thus playing a key role in cell fate specification during development (Carlston et al., 2021). In an embryo, undifferentiated cells, called stem cells, give rise to one or several types of differentiated cells which later form mature tissues and organs. UBAP2L was proposed to be modified by O-Linked N-Acetylglucosamine (O-Glc-NAc) in mouse MC3T3E1 differentiating osteoblasts (Nagel et al., 2013). Interestingly, UBAP2L is found enriched in osteoblasts and as such it is used as an osteoblast marker (Guan et al., 2021). More globally, UBAP2L expression is increased in other types of undifferentiated cells such as mouse and human hematopoietic and leukemic stem cells. In the above study, Bordeleau and colleagues propose a model in which UBAP2L forms a complex with the Polycomb group (PcG) proteins BMI1 and Ring Finger Protein 2 (RNF2), thereby regulating long-term repopulating hematopoietic stem cells (LT-HSCs) independently of Ink4a/Arf locus repression, a popular target of BMI1. The authors suggest that at least two Polycomb-repressive complexes can assemble in order to regulate HSC function, which are distinguishable by the presence or the absence of UBAP2L (Bordeleau et al., 2014). Further investigations are needed in order to elucidate UBAP2L's precise role as part of the Polycomb complex since the exact mechanism has not been fully understood yet. A partial answer has been provided by Lin et al. who used rat bone marrow mesenchymal stem cells (BMSCs) overexpressing UBAP2L to transplant it to rats suffering from semi-sectioned spinal cord injury (SPI) and to monitor the recovery of the injured tissue (Lin et al., 2018). UBAP2L overexpressing cells exhibited stronger neuronal differentiation potential, which led to faster spinal cord function recovery. Mechanistically, UBAP2L overexpression results in increased expression of the cell cycle related protein cyclin D1 and of p38 MAPK, and more importantly to decreased expression of Caspase 3, a key apoptotic factor responsible for the majority of post-SCI neuronal death (Yu and Fehlings, 2011). Overall, the authors propose that UBAP2L overexpression in BMSCs promotes neuronal proliferation and survival, limits contingent damage like post-SCI inflammation and eventually leads to SCI repair (Lin et al., 2018). Given that the UBAP2L locus has been associated with other neuronal disorders such as bipolar or anorexia nervosa disorders (eQTLGen Consortium et al., 2019; Iranzo-Tatay et al., 2022), it would be of great interest to further investigate its potential role in the development of

other neurological and aging-related neurodegenerative diseases.

UBAP2L and Cell Division

In eukaryotes, mitosis is a crucial process which needs to be tightly regulated in time and space to allow for faithful division of a mother cell into two identical daughter cells (McIntosh, 2016). UBAP2L has been proposed to regulate cell division. Its depletion impairs chromosome alignment during metaphase and potentiates Spindle Assembly Checkpoint (SAC) response. Chromosome misalignment phenotypes upon UBAP2L depletion occur due to the disruption of stable k-fibers, suggesting defects in proper microtubule-kinetochore (MT-KT) attachment, which in turn hinders proper chromosome segregation and mitosis completion (Maeda et al., 2016). Maeda and colleagues further showed that UBAP2L RGG/RG domain is responsible for the multi- and micronucleation phenotypes observed in UBAP2L downregulated HeLa cells and more importantly that this function is mediated by the methylation of the arginines within the RGG/RG domain by the methyl-transferase PRMT1. Although the construct lacking this post-translational modification is properly localized at the spindle, it cannot rescue chromosome misalignment during metaphase observed in UBAP2L depleted cells suggesting that UBAP2L RGG/RG domain methylation is essential for proper MT-KT attachments, accurate chromosome distribution and proper mitotic progression. Consistently, UBAP2L depletion leads to an enrichment of G2/Mitotic (G2/M) population in HeLa cells (Maeda et al., 2016), in ZR-75-30 and in T-47D breast cancer cells (He et al., 2018) and in DU145 prostate cancer cells (Li and Huang, 2014) pointing to an important role of UBAP2L as a cell cycle regulator.

UBAP2L and Stress Signaling

An interesting feature of UBAP2L protein is its ability to aggregate and to regulate protein synthesis as indicated above (Wilde et al., 2011; Luo et al., 2020). mRNA turnover and protection under stress conditions have been associated with the formation of Stress Granules (SG) (Parker and Sheth, 2007). In an attempt to identify new components and/or regulators of cytosolic RNA granules, Youn and colleagues performed proximity-based proteomics and identified UBAP2L as a critical factor for efficient SG assembly following stress induced by the arsenite treatment. Importantly, the DUF domain of UBAP2L containing an phenylalanine-glycine phenylalanine-glycine (FG-FG) motif is critical for G3BP1 (Ras GTPase-activating protein-binding protein 1) recognition and binding in flies (Baumgartner et al., 2013) and is responsible for G3BP1 assembly in HeLa cells. In contrast, UBA and RGG domains of UBAP2L seem to be dispensable for SG formation (Youn et al., 2018). Subsequent studies by another group demonstrated the crucial role of the RGG domain of UBAP2L for SG competence under stress-null and stress conditions (Huang et al., 2020). More precisely, under stress conditions, UBAP2L methylation by PRMT1 is decreased, enabling UBAP2L's interaction with SG components and subsequently promoting SG assembly. The authors show that UBAP2L's DUF domain is still very important for G3BP1/2 NTF2-like domain binding and localization. In fact, depletion of the DUF domain promotes UBAP2L shuttling from the cytoplasm to the nucleus, impeding its interaction with G3BP1/2 and consecutively abolishes SG formation (Huang et al., 2020). Further work from Gotta group, propose that UBAP2L forms SG cores to which G3BP1 is subsequently recruited to allow for SG maturation, suggesting that UBAP2L acts upstream of G3BP1 in SG nucleation (Cirillo et al., 2020). Intriguingly, this phenomenon seems to be specific to human cells as a recent study from the same group established that PQN-59 and GTBP-1 (the human UBAP2L and G3BP1/2 orthologs respectively) are not essential for SG assembly in C. elegans (Abbatemarco et al., 2021). Interestingly, additional types of subcellular complexes can be assembled under stress conditions. Among them, the nuclear "twins" of SG are called paraspeckles (PS). These ribonucleoproteins (RNP) granules assemble around the long noncoding RNA (lncRNA) NEAT1 (Fox et al., 2018). Upon stress induction, SGs regulate PSs assembly via the sequestration of important negative regulators of PS formation such as UBAP2L (An et al., 2019). For the moment, we still lack sufficient knowledge to explain the molecular mechanism behind this regulation and it would be important to understand if and how UBAP2L acts as a global regulator of stress-induced complex assemblies, in addition to its well-established role in SGs.

UBAP2L AND CANCER

Recent work has demonstrated that UBAP2L is overexpressed in a variety of cancers and as such it has gained significant attention of researchers over the past years. Although its aberrant expression is a common feature of very different types of tumors, the way UBAP2L acts to promote carcinogenesis appears to be highly variable (**Figure 2**), highlighting UBAP2L's versatile functions not only in healthy tissues but also under pathological conditions. As mentioned above, UBAP2L is broadly expressed in almost all tissues. Likewise, this abundance is also found and exacerbated in distinct tumor types such as prostate, breast, uterine, cervical, non-small cell lung and gastric cancers, glioma, colorectal and hepatocellular carcinoma (HCC) and lung adenocarcinoma (Li and Huang, 2014; Zhao et al., 2015; Bai et al., 2016; Chai et al., 2016; Aucagne et al., 2017; Wang et al., 2017; Ye et al., 2017; He et al., 2018; Li et al., 2018; Lin et al., 2018; Pan et al., 2020; Yoshida et al., 2020; Guan et al., 2021; Yang et al., 2021; Li et al., 2022). In nearly all cited cancer studies, UBAP2L is suggested to act as an oncogene promoting cancer cell proliferation and growth *in vitro* and *in vivo*, thus providing an explanation to the existing negative correlation between UBAP2L expression and patients' prognosis.

UBAP2L Promotes Cell Proliferation and Growth

In prostate, breast cancers and HCC, UBAP2L depletion leads to an accumulation of G2/M cell population (Li and Huang, 2014; He et al., 2018; Li et al., 2018), whereas it was shown to increase

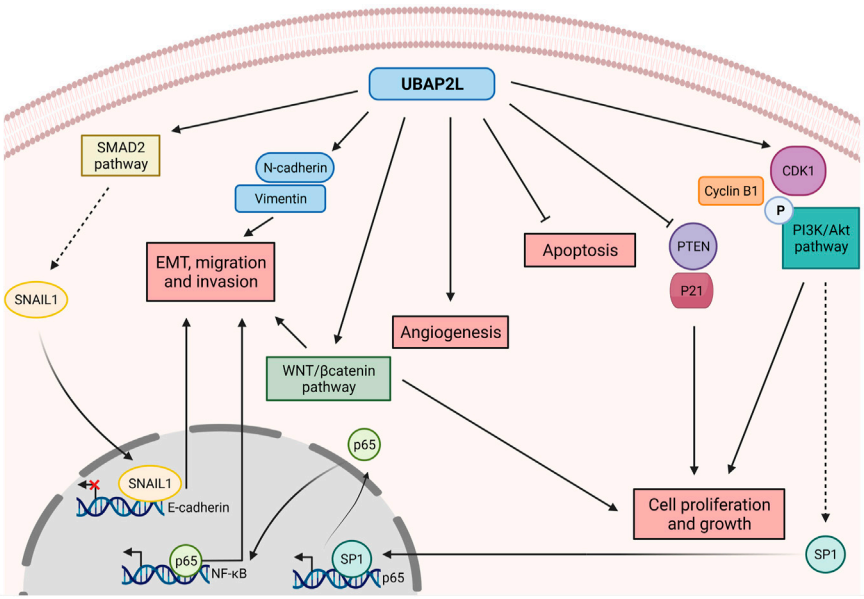

FIGURE 2 | Versatile roles of UBAP2L in promoting cancer disease. UBAP2L upregulates key cell cycle regulators such as CyclinB1, CDK1 and the PI3K/Akt pathway, while it inhibits the expression of tumor suppressors such as PTEN and P21, thereby promoting cell proliferation and growth. PI3K/Akt activation enhances SP1 levels which in turn activates P65 expression, thereby activating NF-κB pathway and favoring epithelial-mesenchymal transition (EMT), migration and invasion. The metastatic potential of UBAP2L-overexpressing cells is also sustained by the activation of the SMAD2 pathway, triggering the transcriptional repressor SNAIL1 to the E-cadherin promoter, shutting down its expression. Cancer cells overexpressing UBAP2L are characterized by hyperactivation of the WNT/βcatenin pathway and by upregulation of mesenchymal factors such as N-cadherin and Vimentin, resulting in increased invasion and proliferation. Finally, UBAP2L favors tumor vascularization while inhibiting cancer cells apoptosis. Overall, UBAP2L promotes cancer progression by regulating various axes of tumorigenesis known as the hallmarks of cancer.

the G0/G1 cells rate in Glioma and colorectal carcinoma, suggesting that UBAP2L may act during several cell cycle stages (Zhao et al., 2015; Chai et al., 2016). Additionally, UBAP2L is responsible for the multifaceted regulation of tumors' cellular and molecular properties in order to promote cellular survival as well as migration. Compelling evidence suggests that oncogenic pathways rely on the establishment of a suitable micro-environment that provides nutrients and supports tumor development and survival as elegantly summarized in 2011 (Hanahan and Weinberg, 2011). Intriguingly, UBAP2L seems to be involved in the regulation of several hallmarks of cancer.

Firstly, as mentioned above, UBAP2L sustains cell proliferation potentially via the regulation of cell cycle signaling pathways. For instance, it has been observed that knockdown of UBAP2L increases p21 and decreases CDK1 and CyclinB1 expression in breast cancer cells (He et al., 2018). This observation was further confirmed in HCC in a study showing a gene enrichment analysis after UBAP2L depletion. As previously demonstrated, the authors found PTEN and p21 among the most upregulated genes, while CDK1, CyclinB1, p-PI3K and p-AKT were among the most downregulated genes following UBAP2L silencing (Li et al., 2018). The signaling pathways downstream of PTEN, TP53 and PI3K/Akt are commonly dysregulated and hijacked in cancerous cells in order to promote their growth as extensively reviewed in the past years (Hollander et al., 2011; Khemlina et al., 2017; Levine, 2020). Of particular interest, the PI3K/Akt pathway is implicated in a broad range of cellular processes including cell proliferation but also apoptosis, angiogenesis, replicative immortality, invasion and metastasis, pointing out to UBAP2L oncogene as a golden target for future anti-cancer therapies (Lien et al., 2017). The molecular mechanism of how UBAP2L might regulate the PI3K/Akt pathway can be partially explained by a study suggesting that UBAP2L activates the PI3K/Akt pathway by promoting a phosphorylation cascade which in turn triggers SP1 binding to P65 promoter, inducing its expression. UBAP2L enables P65 translocation into the nucleus and possibly activates NF-KB (Li et al., 2022), a pathway strongly associated to cancer progression (Zinatizadeh et al., 2021). However, further efforts are required in order to dissect how UBAP2L precisely regulates signaling pathways to enable cancer progression.

UBAP2L Promotes Epithelial-Mesenchymal Transition, Migration, Invasion and Metastasis

An additional common feature of cancer cells is the ability to undergo epithelial-mesenchymal transition (EMT) as a means to promote effective invasion and metastasis (Hanahan and Weinberg, 2011). Interestingly, wound-healing assays of HCC cells lacking UBAP2L, revealed defects in migration and invasion. Consistently, cells lacking UBAP2L harbor increased epithelial (E-cadherin, CK-18) and decreased mesenchymal markers (N-cadherin, vimentin) (Ye et al., 2017), highlighting

UBAP2L's crucial role in regulating the metastatic potential of cancer cells. In addition to HCC, the promotion of EMT by UBAP2L has also been reported in prostate, lung and gastric cancers (Li and Huang, 2014; Aucagne et al., 2017; Lin et al., 2021). Complementary studies verified these conclusions *in vivo* where inhibition of UBAP2L led to defective cancer invasion in xenografts (Guan et al., 2021). In addition, mice injected with Ubap2l−/− A549 cells show less nodules in their lungs, lighter lungs and increased survival 3 weeks after injection in contrast to mice injected with Ubap2l+/+ A549 cells (Aucagne et al., 2017), while the opposite result is observed in gastric cancer when UBAP2L is overexpressed (Li et al., 2022). Finally, it was recently suggested that UBAP2L positively regulates the expression of the transcriptional repressor SNAIL1 *via* the SMAD2 signaling pathway which subsequently binds to and inhibits the promoter of E-cadherin, hindering the expression of this epithelial marker in favor of mesenchymal ones, ultimately leading to EMT, invasion and metastasis (Ye et al., 2017).

As previously discussed, cancer cells must use many diverse strategies to escape the cellular surveillance mechanisms in order to survive and migrate. To this end, most of the signaling pathways exploited by normal cells have to be hijacked, to favor cancer progression. For example, components of the Wnt/β-catenin signaling which is a highly conserved pathway regulating fundamental developmental processes, has been frequently observed to be mutated in cancer (Nusse and Clevers, 2017). Not surprisingly, UBAP2L has been proposed to activate the Wnt/β-catenin signaling cascade in gastric cancer cells, leading to the expression of downstream pathway targets, known to be implicated in tumorigenesis and metastasis (Yook et al., 2006; Liu et al., 2010; Damsky et al., 2011; Lin et al., 2021). However, the precise molecular mechanisms driving UBAP2L's oncogenic potential are not yet defined. UBAP2L has been reported as a BMI1 interactor as cited before (Bordeleau et al., 2014). Although BMI1 is essential for the activity of hematopoietic stem cells, it has also been suggested as a Wnt signaling activator by regulating the Wnt antagonist IDAX (Yu et al., 2018). Therefore, one hypothesis that could be further explored, might be that Wnt/β-catenin hyperactivity in UBAP2L-overexpressing tumors could be attributed to UBAP2L/BMI1 interaction.

UBAP2L Prevents Apoptosis of Cancer Cells and Promotes Tumor Vascularization

Cancer cells must acquire resistance to cellular death to ensure their survival and expansion (Hanahan and Weinberg, 2011). In this context, UBAP2L is suggested to act as an anti-apoptotic factor possibly by regulating, through yet unknown mechanisms, the expression of crucial apoptotic factors such as Bad/Bax and the cleavage of PARP and caspase 3 (Li and Huang, 2014; Chai et al., 2016). Bypassing all checkpoints employed by the cellular machinery is a challenge for cancer cells. Nevertheless, tumor microenvironment is crucial for proper cancer dissemination across tissues. For instance, cancerous cells require a certain amount of nutrients and oxygen to function properly and these components are efficiently brought to the cells only if the tumor is properly vascularized. Interestingly, samples from HCC patients revealed a positive correlation between UBAP2L and VEGF expression, a crucial protein for angiogenesis. Consistently, micro vessel density was also found to be increased in UBAP2L overexpressing tumors (Wang et al., 2017) and a complementary study from another laboratory reported that UBAP2L downregulation decreases the average vascular length and number of vascular branches (Li et al., 2018), once more pointing to a potential role for UBAP2L in favoring angiogenesis.

UBAP2L AND RNAS

Incremental studies were conducted on microRNAs (miRNAs), small nucleotides duplexes which post-transcriptionally regulate gene expression of their targets, being involved in general biological processes such as cell proliferation, apoptosis or brain development among others (Ambros, 2004). Intriguingly, UBAP2L was demonstrated to be targeted by different miRNA. First, in non-small cell lung cancer (NSCLC), miR-19a-3p directly inhibits UBAP2L, resulting in similar phenotypes as those observed upon UBAP2L downregulation, mainly inhibition of cell proliferation, migration and invasion (Pan et al., 2020). Similarly, UBAP2L was silenced by miR-148b-3p in gastric cancer cells leading to the same phenotypes as in NSCLC (Lin et al., 2021). Interestingly, the UBAP2L ortholog PQN-59 stabilizes several miRNAs involved in various cellular functions and interacts with RNA metabolism, transcription and translation cellular components similarly to UBAP2L, highlighting the importance of this protein in RNA regulation (Carlston et al., 2021). Supporting this hypothesis, UBAP2L localizes to stress granules and P-bodies under certain conditions, two structures highly linked to RNA turnover, miRNA or gene expression regulation (Leung et al., 2006).

Concluding Remarks

Conclusively, although UBAP2L has been identified more than 20 years ago, its extremely versatile roles in various signaling pathways have been elucidated only recently. It would therefore be fascinating that future studies address the underlying precise molecular mechanisms that govern and direct UBAP2L's functions towards such distinct signaling nodes to ensure cellular homeostasis. Our review aimed at highlighting the growing evidence on the oncogenic potential of UBAP2L that may identify UBAP2L as a promising target and stimulate research on UBAP2L-based future cancer therapies.

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Conceptualization, Writing, Review and Editing: LG, EP, and IS.

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RESULTS

I. SUBMITTED MANUSCRIPT: UBAP2L-DEPENDENT COUPLING OF PLK1 LOCALIZATION AND STABILITY DURING MITOSIS

The work related to the first part of the results section is entitled "UBAP2L-dependent coupling of PLK1 localization and stability during mitosis" and it has been submitted to *Journal of Cell Biology* (Guerber et al., 2022). The last version of the manuscript is included and corresponds to the initial submitted draft before rounds of revision. Main figures and legends have been properly positioned to facilitate readers' comprehension. Supplementary figures and legends mentioned are located following the main text. "Material and methodology" part of the manuscript has been omitted in this chapter and has been included in the general "Materials and methods" section of the thesis.

A. Aims of the study

Background: Mitosis is a fundamental process during which the genetic information encoded by DNA of one mother cell is partitioned into two identical daughter cells, ensuring its multiplication. Thus, cell division is subjected to a very precise spatio-temporal regulation. PLK1 is a crucial factor actively contributing to mitotic control but how exactly its timely localization and function are regulated needs further elucidation.

Previous work: This project is based on preliminary visual siRNA screen from the laboratory for known and predicted UBPs and DUBs with a role in mitosis. Among the top candidates, UBAP2L depletion led to the accumulation of polylobed nuclei and multinucleated cells, suggesting a potential function in cell division. Therefore, the main aim of my PhD is to understand if and how UBAP2L regulates mitotic progression.

Aim 1: To confirm the mitotic defects observed upon UBAP2L depletion

Aim 2: To identify downstream partners of UBAP2L

Aim 3: To examine UBAP2L localization

Aim 4: To study the mechanism by which UBAP2L regulates PLK1 localization and stability

Aim 5: To investigate the physiological consequences of PLK1 aberrant activity in UBAP2L-depleted cells

B. Manuscript title and authors affiliations

Title: **UBAP2L-dependent coupling of PLK1 localization and stability during mitosis**

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C. Author's contribution to the manuscript "UBAP2L-dependent coupling of PLK1 localization and stability during mitosis"

While I write my thesis, our manuscript has been submitted to *Journal of Cell Biology*. I will list here author's contribution for the initial submission. This might change after rounds of revision. In the *Journal of Cell Biology* manuscript, each author contributed in the following manner:

Evanthia Pangou initiated and designed this project and performed the pilot experiments showing PLK1 specific increased protein levels and activity in UBAP2L depleted cells. She designed and helped generating the different plasmids used in this study. She performed the endogenous IP shown in Figure 9B, the cycloheximide experiment of Figure S3B and helped performing and imaging experiments shown in Figures 6 and 7. Overall, the majority of the experiments shown in this manuscript were done in collaboration with Evanthia. Finally, she wrote the manuscript and contributed to the figures' creation.

Aurore Vuidel did the majority of the cloning necessary for this project, she generated the different UBAP2L plasmids and confirmed the basic phenotypes of UBAP2L depletion on PLK1.

Yongrong Liao generated the UBAP2L Knock-Out (KO) clones used in this study and performed the IP under denaturing conditions shown in Figure 9C.

Charlotte Kleiss contributed to the Green Fluorescent Protein (GFP)-PLK1 plasmid generation and helped with experiments.

Erwan Grandgirard generated the Fiji pipelines used to quantify the colony formation assays (area and number of colonies). He optimized the setups used for live-microscopy imaging and helped with microscopy in general.

Izabela Sumara coordinated the project, wrote the manuscript and prepared the figures.

I designed the experiments and confirmed all preliminary results upon UBAP2L siRNA and in UBAP2L KO cells, namely PLK1 increased protein levels and activity, PLK1 stability, PLK1 interaction with UBAP2L and mitotic defects. I determined the cell cycle phases during which UBAP2L regulates PLK1 and confirmed that UBAP2L specifically regulates PLK1 and not other mitotic factors nor PLK family members. I performed the rescue experiments and showed that UBAP2L-mediated PLK1 regulation is owed to UBAP2L C-terminal part in a Ras GTPase-activating protein-binding protein (G3BPs)-independent manner, which also regulated long-term proliferation and cell survival. I performed live-video experiments to show the severe defects in UBAP2L KO cells and rescue experiments demonstrating that these abnormalities are the direct consequence of PLK1 aberrant activity in UBAP2L-depleted cells. I produced the data showing that PLK1 is not removed from kinetochores upon UBAP2L downregulation, including the monastrol release experiment in fixed cells and the live-video experiment presented in Figure 8 as well as experiments excluding PLK1 kinetochore recruitment problems during G1 in UBAP2L KO cells. I performed the experiments of Figures 6 and 7 with the help of Evanthia. Finally, I did all initial data processing and quantifications shown in our manuscript, I wrote the manuscript and prepared the figures.

D. Key words and highlights of the manuscript

Key words: Polo-like kinase 1 (PLK1), mitosis, UBAP2L, kinetochore, chromosome segregation

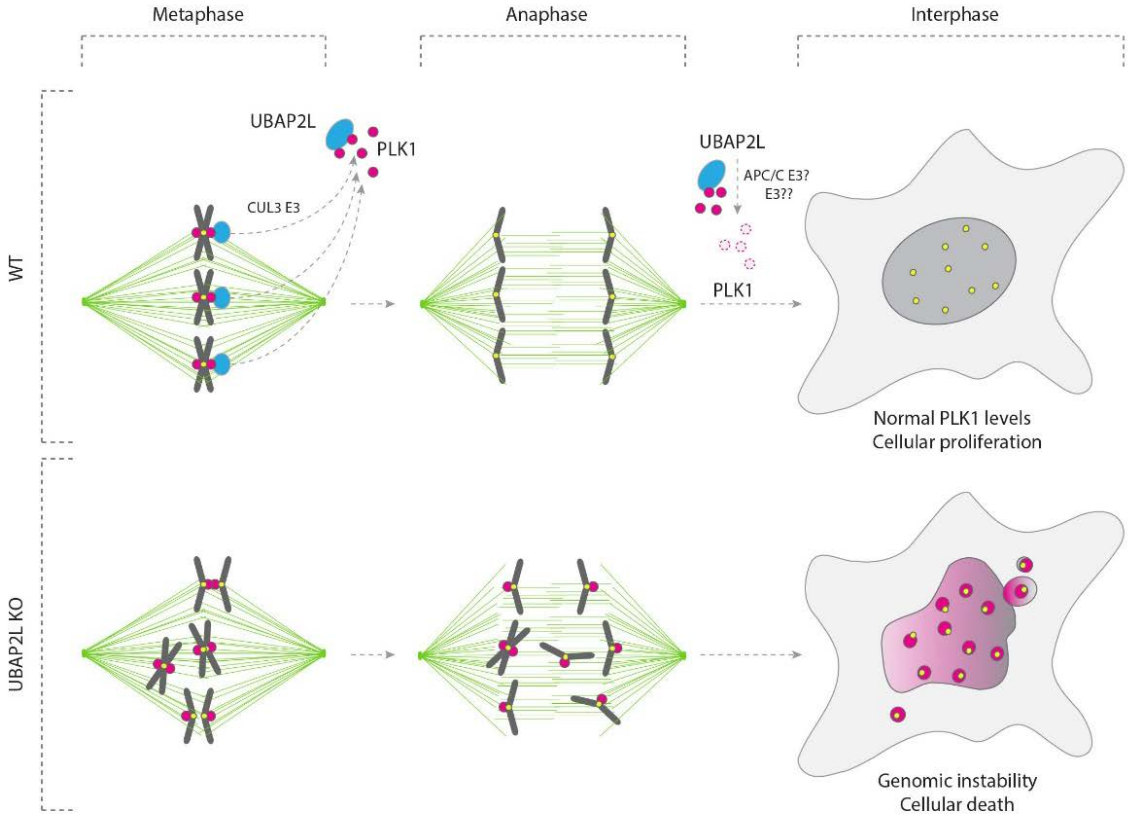
Highlights:

- UBAP2L regulates PLK1 kinetochore localization and protein stability
- UBAP2L depletion leads to aberrant PLK1 kinase activity in interphase
- UBAP2L localizes at the kinetochore in a PLK1-dependent manner
- Genomic instability of UBAP2L depleted cells is rescued upon PLK1 inhibition

E. Abstract

Polo-like kinase 1 (PLK1) is a key regulator of eukaryotic cell division and an attractive target for cancer therapies. PLK1 levels and activity fluctuate during cell cycle, increasing in G2 phase, peaking during mitosis and decreasing during mitotic exit and G1. Dynamic mitotic regulation of PLK1 is crucial for its roles in spindle assembly, chromosome segregation and cytokinesis. PLK1 localizes to kinetochores during prometaphase and is removed from these structures during metaphase to allow for anaphase onset and proper segregation of chromosomes. However, the molecular mechanisms linking localized activity of PLK1 to its protein stability remain elusive. Here, we identify the Ubiquitin-Binding Protein 2-Like (UBAP2L) protein that regulates dynamic removal of PLK1 from kinetochores prior to anaphase and its protein stability upon mitotic exit. We demonstrate that UBAP2L localizes to kinetochores in a PLK1-dependent manner during mitosis and regulates timely dissociation of PLK1 from these structures and proper progression through mitosis. UBAP2L depletion inhibits PLK1 dissociation from kinetochores prior to anaphase leading to alignment and segregation defects. We show that C-terminal domain of UBAP2L mediates its function on PLK1 and that UBAP2L specifically regulates PLK1 localization and not of other mitotic factors such as Aurora B, Aurora A and Cyclin B1, or other PLK family members. Interestingly, we demonstrate that inhibited kinetochore removal of PLK1 increases its stability after mitosis completion, resulting in aberrant PLK1 kinase activity in interphasic cells, ultimately causing genomic instability and cellular death. Our data thus suggest that UBAP2L can regulate PLK1 localization and stability during mitosis ensuring proper chromosome segregation and normal PLK1 signaling in human cells.

F. Graphical abstract



G. Introduction

Protein kinases represent key regulatory elements of the mitotic cycle, transferring phosphorylation signals to critical effectors (Nigg, 2001). Polo-like kinase 1 (PLK1) represents one of the key mitotic enzymes ensuring both mitotic entry as well as fidelity of genome segregation, mitotic exit and cytokinesis (Petronczki et al., 2008; Schmucker and Sumara, 2014; Combes et al., 2017) and remains an attractive target for anticancer therapies (Strebhardt, 2010; Chiappa et al., 2022). PLK1 is a serine/threonine kinase with an enzymatic domain at its N-terminal and a Polo-Box domain (PBD) at its C-terminal part, the latter representing a unique feature of the PLK kinase family and conferring specificity to phosphorylation substrates (Barr et al., 2004; Strebhardt, 2010; Zitouni et al., 2014). Its expression is cell cycle dependent, with PLK1 levels peaking at G2/M transition and dropping during mitotic exit and in early G1 (Golsteyn et al., 1995; Bruinsma et al., 2012) owing to the proteasomal degradation of PLK1 mediated through proteolytic ubiquitylation by the anaphase promoting complex/cyclosome (APC/C) E3 ubiquitin ligase (Lindon and Pines, 2004).

During mitosis PLK1 undergoes several post-translational modifications which fine-tune its dynamic localization, stability and activation/inactivation at several structures including the centrosomes, the kinetochores, the central spindle and the midbody (Schmucker and Sumara, 2014). PLK1 is enriched at kinetochores from prometaphase till metaphase stages through the interaction of its PBD with phosphorylated kinetochore receptors including budding uninhibited by benzimidazole 1 homolog (BUB1), BUBR1, and inner centromere protein (INCENP) (Goto et al., 2006; Qi et al., 2006; Elowe et al., 2007). At kinetochores, PLK1 regulates stability of kinetochore-microtubule (KT-MT) attachments and correct chromosome alignment (Elowe et al., 2007). Consequently, downregulation of PLK1 levels or inhibition of its kinase activity leads to spindle assembly checkpoint (SAC) potentiation and mitotic death (Sumara et al., 2004; Lénárt et al., 2007). Interestingly, most of the PLK1 protein is removed from kinetochores during metaphase upon establishment of stable KT-MT attachments to allow for SAC silencing and anaphase onset (Elowe et al., 2007; Liu et al., 2012; Maia et al., 2012). Our previous studies have shown that PLK1 is a substrate for non-proteolytic CUL3-mediated ubiquitylation (Beck et al., 2013; Metzger et al., 2013) prior to anaphase. CUL3 in complex with the substrate specific adaptor protein KLHL22 mono-ubiquitylates PLK1 within its PBD domain and interferes with phospho-receptors' binding, leading to the timely removal of PLK1 from kinetochores and faithful genome segregation. This modification is counteracted by the opposing function of the deubiquitylase (DUB) USP16 (Zhuo et al., 2015) that promotes proper chromosome alignment in early mitosis. Thus, both dynamic localization and protein stability of PLK1 are tightly regulated by phosphorylation- and ubiquitylation-based signals to ensure proper mitotic progression and genome stability. However, the exact molecular mechanisms linking the regulation of localized activity of PLK1 to its protein stability remain elusive.

Ubiquitin-Binding Protein 2-Like (UBAP2L, also known as NICE-4) is a highly conserved ubiquitin- and RNA-binding protein with versatile roles in multiple signaling cascades and cellular functions (Guerber et al., 2022). While UBAP2L has been mostly studied in the context of stress response signaling (Cirillo et al., 2020; Huang et al., 2020), recent evidence suggests that it can be involved in regulating mitotic progression (Maeda et al., 2016). UBAP2L is methylated within its RGG domain located at the N-terminal part and this modification was shown to promote the stability of KT-MT attachments, ensuring accurate chromosome distribution (Maeda et al., 2016). However, it remains unknown whether additional mechanisms to the reported methylation can actively drive the role of UBAP2L in cell division and what is the identity of direct downstream targets of UBAP2L during mitosis. In this study we provide

evidence that UBAP2L can regulate both the dynamic localization of PLK1 and its protein stability in an RGG-domain independent manner. We demonstrate that UBAP2L localizes to kinetochores in a PLK1-dependent manner during mitosis and regulates timely dissociation of PLK1 from these structures and proper mitotic progression. Cells depleted for UBAP2L are characterized by mitotic delay, aberrant chromosome segregation, micronuclei and nuclear atypia. UBAP2L depletion impairs the removal of PLK1 from kinetochores prior to anaphase, increases its stability after mitosis completion and results in elevated PLK1 kinase activity in interphasic cells. Importantly, several defective mitotic phenotypes in UBAP2L depleted cells can be fully restored upon PLK1 inhibition, suggesting that the genomic instability observed upon UBAP2L depletion can be directly coupled to aberrant PLK1 mitotic signaling.

H. Results

1. UBAP2L regulates proper chromosome segregation during mitosis.

To identify novel ubiquitin-related factors with a potential role in mitosis, we previously performed a high-content visual siRNA screen in HeLa cells for known and predicted human ubiquitin-binding domain (UBD) proteins (Krupina et al., 2016) and we assessed phenotypes of irregular nuclear shape which is often the result of chromosome segregation defects (Jevtić et al., 2014). UBAP2L was among the top hits of the screen (Krupina et al., 2016), as its depletion led to increased number of cells displaying polylobed nuclei and multinucleation, phenotypes highly comparable to those observed upon down-regulation of the positive control CUL3 (**Fig. S1A**) (Sumara et al., 2007; Maerki et al., 2009). Interestingly, UBAP2L has been proposed to be involved in mitotic progression via its methylation by the arginine methyltransferase PRMT1 which is required for the formation of KT-MT attachments and chromosome alignment (Maeda et al., 2016) but the direct downstream targets of UBAP2L important for mitotic progression are currently unknown.

In order to corroborate our screening results and to further dissect the precise role of UBAP2L during mitosis, we deleted UBAP2L in HeLa cells using CRISPR/Cas9-mediated gene editing (**Fig. S1B and S1C**) and performed time-lapse live video microscopy (**Fig. 1A and Videos S1-4**). UBAP2L Knock-Out (KO) cell line displayed significant delay in mitotic entry and in timing from prophase to anaphase relative to isogenic wild-type (WT) control cell line (**Fig. 1A-C**). Moreover, UBAP2L KO cells were characterized by chromosome alignment defects and DNA bridges during anaphase and telophase, after which cells either exited mitosis as polyploid cells

in the presence of accumulated micronuclei or died after prolonged mitotic arrest (**Fig. 1A, and 1D-G**).

The presence of micronuclei and nuclear atypia in UBAP2L depleted cells was further confirmed in additional cell lines derived from colorectal cancer (DLD-1) (**Fig. S1D-E**) and osteosarcoma (U2OS) (**Fig. S1F-G**), respectively. Importantly, the mitotic defects observed in UBAP2L KO cells did not seem to be the consequence of pre-existing genomic instability, since UBAP2L KO cells that entered mitosis with both normal (**Fig. 1A, second row and Video S2**) and abnormal (**Fig. 1A, third row and Video S3**) nuclear shape, displayed equally severe segregation errors. Our results suggest that UBAP2L regulates proper and timely chromosome segregation during mitosis.

Figure 1.

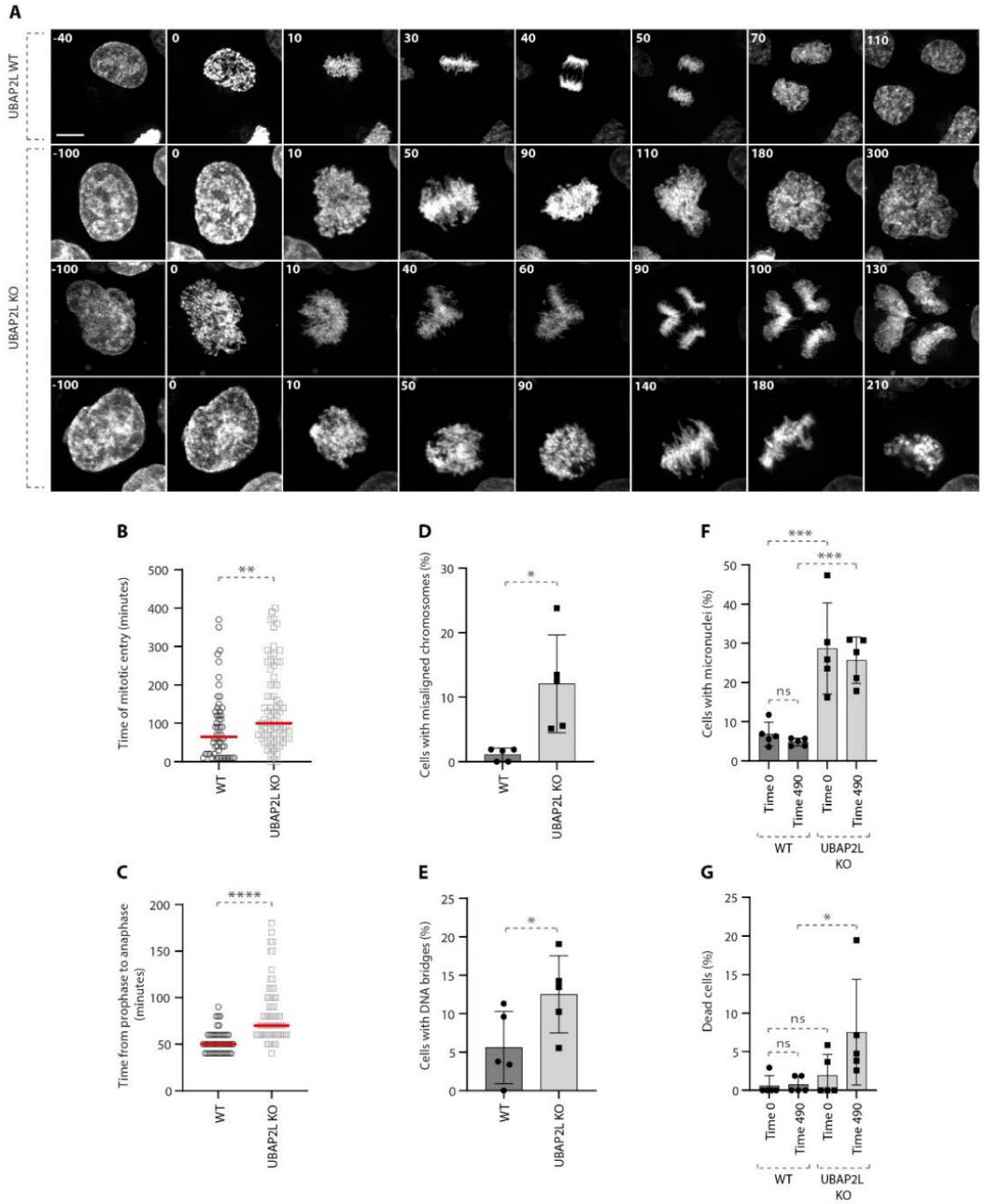


Fig. 1. UBAP2L regulates proper chromosome segregation during mitosis.

(A) Spinning disk time-lapse microscopy of WT and UBAP2L KO HeLa cells synchronized with double thymidine block and release (DTBR) in mitosis. The selected frames of the movies are depicted and the corresponding time is indicated in minutes. SiR-DNA was used for DNA staining. Scale bar, 8µm.

(B and C) The time of mitotic entry **(B)** and from prophase to anaphase **(C)** was quantified. At least 50 cells per condition were analyzed for each experiment. Red bar represents the mean. **(D-G)** The percentages of cells with misaligned chromosomes **(D)**, DNA bridges **(E)**,

micronuclei **(G)** and dead cells **(H)** were quantified. At least 50 cells per condition were analyzed. Graphs represent the mean of five replicates ± standard deviation (SD) (two sample two-tailed t-test or one-way ANOVA with Dunnett's correction *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001, ns=non-significant).

2. UBAP2L regulates PLK1 levels and activity.

Considering the fact that UBAP2L has been proposed to interact with CUL3 complexes (Bennett et al., 2010), we next aimed to understand if components of the CUL3 mitotic signaling (Jerabkova and Sumara, 2019) are linked to UBAP2L and its function during mitotic progression. While UBAP2L depletion did not affect the expression and localization of Aurora A (AURA) and Aurora B (AURB) in mitotically arrested cells, it increased the levels of PLK1 (**Fig. S2A**). UBAP2L depletion did also not affect the localization of other mitotic factors such as Cyclin B1 (**Fig. S2A**), suggesting that deletion of UBAP2L affects specifically PLK1 and not as an indirect effect of perturbed cell cycle progression. Western Blot analysis of cells synchronized in G1/S phase, revealed that although UBAP2L downregulation by specific siRNA (Cirillo et al., 2020) had no effect on the protein levels of Cyclin B1, AURA and AURB, it resulted in increased levels of PLK1 relative to control-depleted cells (**Fig. 2A**), confirming dysregulation of PLK1 signaling in the absence of UBAP2L. Consistently, immunofluorescence analysis showed that UBAP2L downregulation led to an increased number of cells with enriched nuclear localization of PLK1 (**Fig. 2B-D**). These results were confirmed in UBAP2L KO cells which displayed increased PLK1 protein levels and nuclear localization during interphase (**Fig. 2E-H**), without affecting Cyclin B1, AURA and AURB expression (**Fig. 2E and Fig. S2B-E**). Subcellular fractionation assays further confirmed nuclear accumulation of PLK1 during interphase in UBAP2L KO cells relative to WT cells (**Fig. 2I**). The effect of UBAP2L on PLK1, prompted us to test whether UBAP2L might also regulate additional PLK family members but no detectable changes were observed upon UBAP2L downregulation in the total protein levels of PLK2, PLK3 and PLK4 (**Fig. 2J**). Interestingly, PLK1 activatory phosphorylation on Thr210 as well as the PLK1 phospho-substrate BubR1 (Elowe et al., 2007) were increased in the absence of UBAP2L (**Fig. 2J**) in interphasic cells upon UBAP2L depletion.

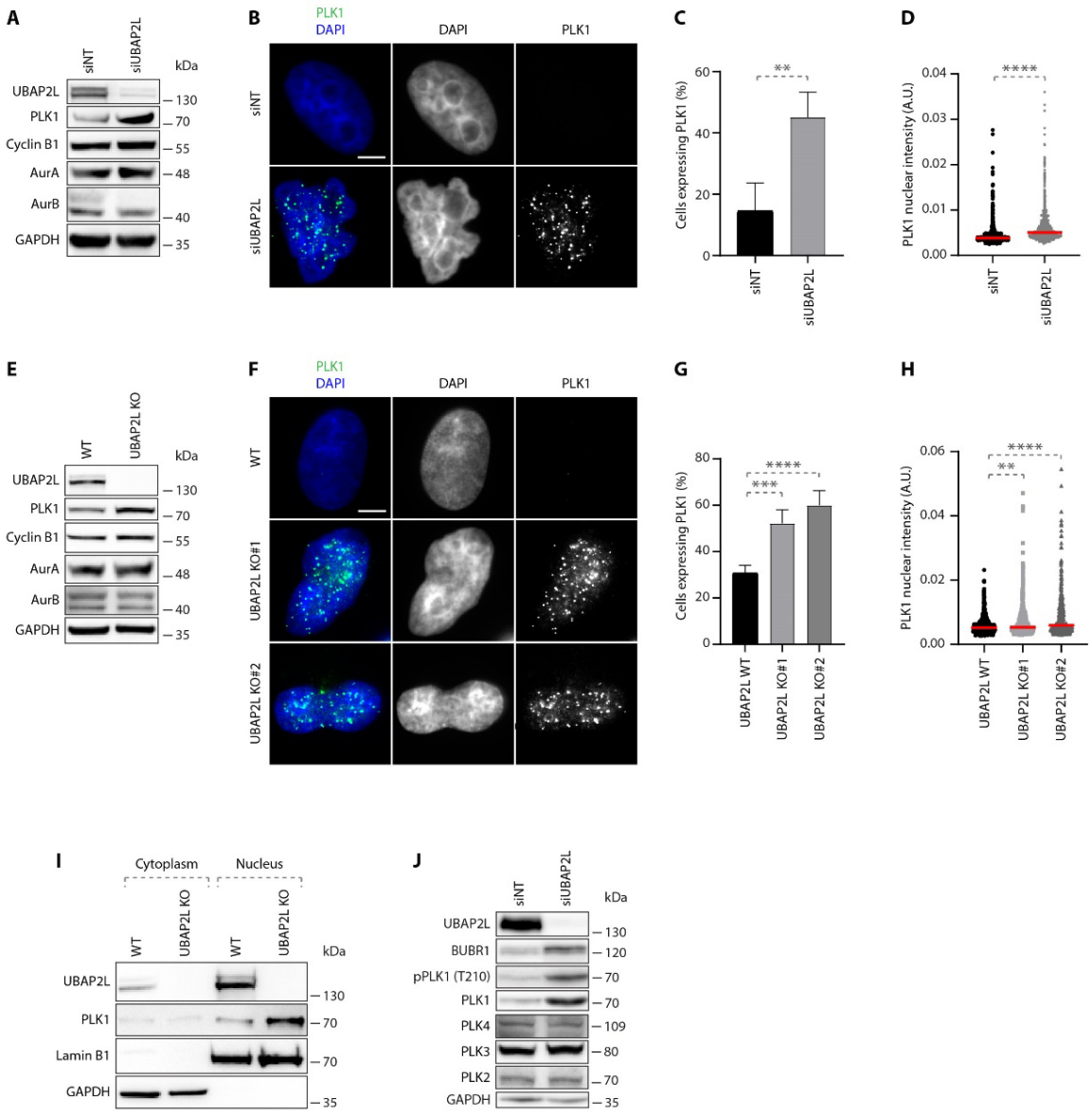


Fig. 2. UBAP2L regulates PLK1 levels and activity.

(A) Western blot (WB) analysis of G1/S synchronized HeLa cells lysates using DTB treated with non-targeting (siNT) or UBAP2L siRNA. Proteins molecular weight (MW) is indicated in kilo Daltons (kDa). WB is representative of three independent replicates.

(B-D) Immunofluorescence (IF) representative pictures of G1/S synchronized HeLa cells treated with the indicated siRNAs and quantification of the percentage of cells expressing PLK1 **(C)** or PLK1 nuclear intensity **(D)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Graphs depicted in **(C)** represent the mean of three replicates ± SD (two sample two-tailed t-test). Each dot of graphs **(D)** represents PLK1 nuclear intensity in a

single nucleus. The measurements of three biological replicates are combined, red bars represent the mean (Mann-Whitney test). **P<0,01, ***P<0,001, ****P<0,0001).

(E) WB analysis of G1/S synchronized WT or UBAP2L KO HeLa cells lysates using DTB. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(F-H) IF representative pictures of G1/S synchronized WT or UBAP2L KO HeLa cells and quantification of the percentage of cells expressing PLK1 **(G)** or PLK1 nuclear intensity **(H)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Graphs depicted in **(G)** represent the mean of four replicates ± SD (one-way ANOVA with Dunnett's correction). Each dot of graphs **(H)** represents PLK1 nuclear intensity in a single nucleus. The measurements of four biological replicates are combined, red bars represent the mean (Kruskal-Wallis test with Dunn's correction). **P<0,01, ***P<0,001, ****P<0,0001).

(I) WT or UBAP2L KO G1/S synchronized HeLa cells were lysed and fractionated into cytoplasmic and nuclear fractions and analyzed by WB. Proteins MW is indicated in kDa.

(J) WB analysis of unsynchronized HeLa cells lysates treated with the indicated siRNAs. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

Overall, our results suggest that UBAP2L regulates PLK1 protein levels and activity without affecting other major mitotic factors.

The increased PLK1 levels observed in UBAP2L KO cells could be either due to enhanced protein translation or reduced protein degradation. To distinguish between the two possibilities, we analyzed PLK1 protein levels in a time course of WT and UBAP2L KO cells treated either with the translation inhibitor cycloheximide (CHX), or with the proteasomal inhibitor MG132. In contrast to AURB, PLK1 protein levels remained stable up to 8h of CHX treatment in the absence of UBAP2L, while AURB and PLK1 were gradually degraded in WT cells, both during interphase (**Fig. S3A**) and in cells arrested in mitosis using the microtubule stabilizing agent paclitaxel (**Fig. S3B**). MG132 treatment increased the levels of total ubiquitin as expected but no additive effect was observed in PLK1 levels in UBAP2L depleted cells relative to WT cells (**Fig. S3C**). Taken together, our results suggest that UBAP2L may promote degradation of PLK1 and its function on PLK1 might be uncoupled from the regulation of cell cycle progression.

3. The C-terminal domain of UBAP2L mediates its function on PLK1.

Next, we aimed to understand if effects of UBAP2L on PLK1 levels and localization are specific to downregulation of UBAP2L and which functional domain of UBAP2L mediates its function on PLK1. Rescue experiments in UBAP2L KO cells ectopically expressing flag-tagged UBAP2L full length (FL) and/or UBAP2L protein fragments (**Fig. 3A and 3B**), revealed that nuclear accumulation of PLK1 in interphase could be efficiently restored by re-expression of UBAP2L FL or the UBAP2L C-terminal fragment but not the N-terminal fragment of UBAP2L (**Fig. 3C and 3D**). These findings argue that the function of UBAP2L on PLK1 is mediated through its C-terminal part and it might be disconnected from the reported role of the RGG domain on mitosis (Maeda et al., 2016), which may regulate other, yet to be identified mitotic factors.

The Domain of Unknown Function (DUF) located within the C-terminal part of UBAP2L is responsible for its interaction with core components of stress granules (SGs) such as the Ras GTPase-activating protein-binding protein (G3BPs), thus enabling their correct assembly upon stress signaling (Huang et al., 2020). In order to exclude the possibility that UBAP2L-mediated regulation of PLK1 is linked to stress signaling, we performed similar rescue experiments in the presence and absence of G3BP1 and G3BP2 (**Fig. 4A and 4D**). Importantly, G3BPs depletion by specific siRNAs (Cirillo et al., 2020) (**Fig. 4D**) did not abolish the rescue potential of UBAP2L FL and C-terminal part on PLK1 nuclear accumulation (**Fig. 4A-C**), suggesting that UBAP2L-mediated regulation of PLK1 can be uncoupled from the previously established function of the UBAP2L C-terminal domain in G3BP1/G3BP2-dependent SGs assembly.

Since absence of UBAP2L led to segregation errors frequently followed by cellular death (**Fig. 1A fourth row, G and Video S4**), we tested whether UBAP2L might also regulate cell proliferation. In accordance with studies showing that cells harboring accumulated errors during cell division often display reduced survival (Cheng and Crasta, 2017), UBAP2L KO cells displayed significantly reduced long-term proliferation capacity and viability (**Fig. 3E-G**). Re-expression of UBAP2L FL or the UBAP2L C-terminal fragment but not the UBAP2L N-terminal protein part fully rescued cell survival and partially rescued cell proliferation (**Fig. 3E-G**). These results further strengthen our hypothesis that UBAP2L emerges as an important factor for fine-tuning PLK1 levels and localization and ultimately cellular proliferation and survival.

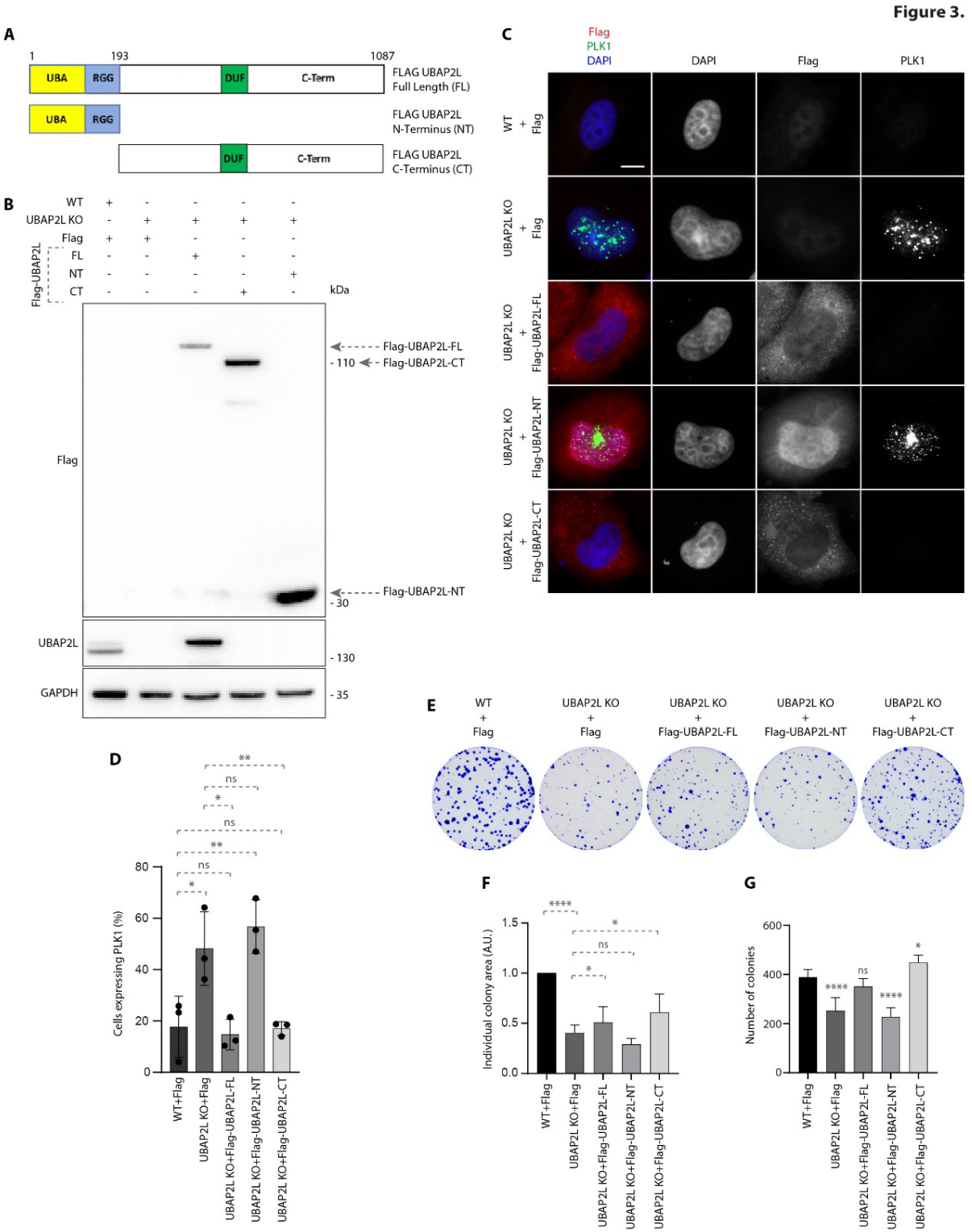


Fig. 3. The C-terminal domain of UBAP2L mediates its function on PLK1.

(A) Schematic representation of UBAP2L protein fragments. Indicated numbers stand for aminoacids (aa).

(B) WB analysis of G1/S synchronized WT or UBAP2L KO HeLa cells lysates transiently transfected with the indicated flag-tagged UBAP2L protein fragments. Proteins MW is

indicated in kDa. Arrows point to the migration of each fragment. WB is representative of three independent replicates.

(C-D) IF analysis of G1/S synchronized WT or UBAP2L KO HeLa cells transiently transfected with the indicated flag-tagged UBAP2L protein fragments and quantification of the percentage of cells expressing PLK1 **(D)**. Scale bar, 5µm. At least 100 cells per condition were quantified for each experiment. Graphs represent the mean of three replicates ± SD (one-way ANOVA with Sidak's correction *P<0,05, **P<0,01, ns=non-significant).

(E-G) Colony formation assay of WT or UBAP2L KO HeLa cells transiently transfected with the indicated flag-tagged UBAP2L protein fragments and quantification of the individual colony area **(F)** and of the number of colonies **(G)** after 7 days of culture. Graphs represent the mean of three replicates ± SD (one-way ANOVA with Sidak's correction *P<0,05, **P<0,01, ****P<0,0001, ns=non-significant).

4. UBAP2L does not regulate PLK1 levels and localization in G2 cell cycle stage.

Since we observed increased protein levels of PLK1 in interphasic cells (**Fig. 2**) likely due inhibition of protein degradation (**Fig. S3A and S3B**) in the absence of UBAP2L, we next aimed to understand during which cell cycle stage UBAP2L controls PLK1 stability. Indeed, PLK1 protein levels strongly fluctuate during cell cycle progression, increasing in G2 phase, peaking during mitosis and decreasing again during mitotic exit and in early G1 (Golsteyn et al., 1995; Bruinsma et al., 2012). For this purpose, we analyzed PLK1 levels and localization by synchronizing cells in different cell cycle stages using several treatments: double thymidine block for G1/S transition, hydroxyurea for the S phase and CDK1 inhibitor RO3306 for G2 (**Fig. 5A**), as previously described (Agote-Arán et al., 2021). Western blotting with antibodies to several cell cycle markers confirmed efficient synchronization of cells where Cyclin E was accumulated during G1/S transition and decreased along the S phase, Cyclin A levels gradually increased peaking in the S phase, and Cyclin B1 gradually increased reaching the highest concentration in G2 (**Fig. 5B**). Interestingly, the number of cells expressing PLK1, as well as PLK1 nuclear accumulation, were increased in UBAP2L KO cells during G1 and S phases, but no changes were detected during G2 stage relative to WT cells (**Fig. 5A, 5C and 5D**). These results suggest that UBAP2L rather seems to regulate PLK1 levels during or after mitotic exit and not prior to mitotic entry.

Figure 4.

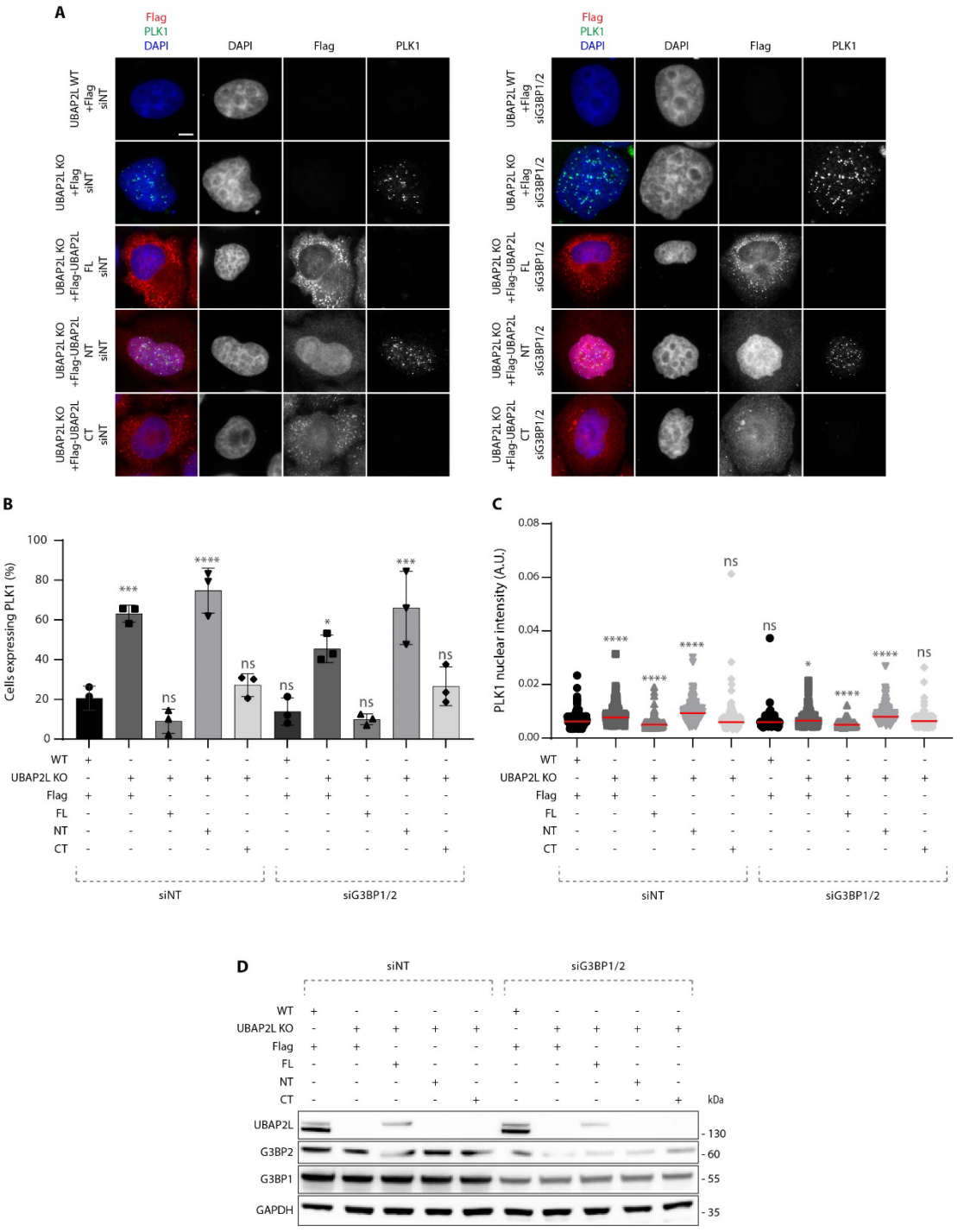


Fig. 4. UBAP2L-mediated PLK1 regulation is G3BP1/2 independent.

(A-C) Representative IF images of WT or UBAP2L KO HeLa cells transiently transfected with the indicated flag-tagged UBAP2L constructs and control or G3BP1/2 siRNAs. Scale bar, 5µm. Quantification of the percentage of cells expressing PLK **(B)** and of PLK1 nuclear intensity **(C)** At least 150 cells were quantified per condition for each replicate. Graphs depicted in **(B)** represent the mean of three replicates ± SD (one-way ANOVA with Sidak's correction). Each

dot of graphs **(C)** represents PLK1 nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, red bars represent the mean (Kruskal-Wallis test with Dunn's correction) *P<0,05, ***P<0,001, ****P<0,0001, ns=non-significant.
(D) WB analysis of the experiment described in **(A)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

The finding that PLK1 accumulates in the nucleus in a dotty pattern in G1/S when UBAP2L is depleted, triggered us to investigate in more detail how UBAP2L regulates the spatiotemporal dynamics of PLK1. Is PLK1 nuclear enrichment enhanced specifically during G1 or is it a consequence of its aberrant expression and localization during mitosis? To test the possibility that UBAP2L might regulate the recruitment of PLK1 at the kinetochores during G1 which is known to occur in order to promote faithful CENP-A deposition at the centromeres in a Mis18 complex-dependent manner (McKinley and Cheeseman, 2014), we depleted Mis18α and CENP-A in G1 synchronized WT and UBAP2L KO cells (**Fig. S4A**) and quantified the percentage of cells displaying PLK1 kinetochore enrichment. Interestingly, neither Mis18α nor CENP-A depletion could rescue the PLK1 kinetochore accumulation observed in UBAP2L KO cells (**Fig. S4A-E**), thereby suggesting that UBAP2L depletion does not seem to trigger the premature kinetochore recruitment of PLK1 during G1 but might rather regulate its removal prior to anaphase and mitotic exit.

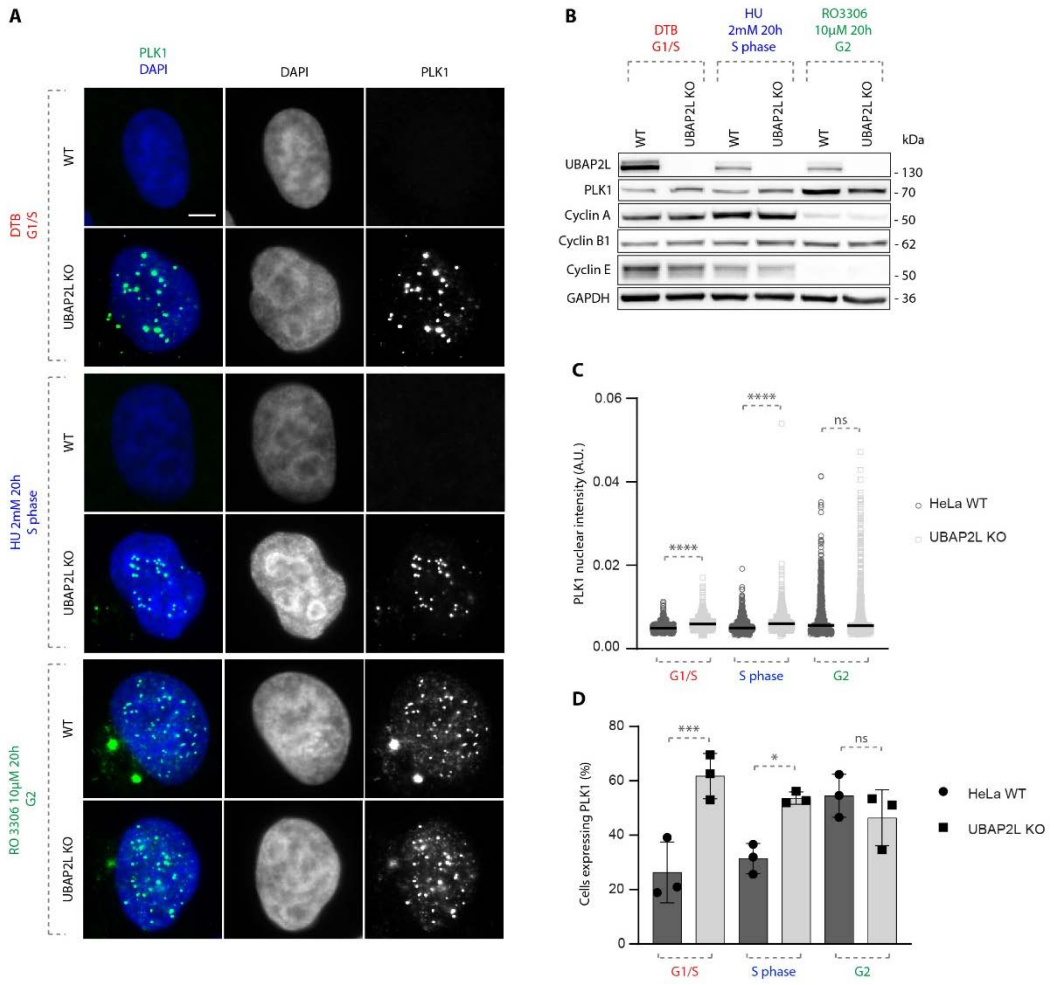


Fig. 5. UBAP2L does not regulate PLK1 levels and localization in G2 cell cycle stage.

(A) Representative IF pictures of WT or UBAP2L KO HeLa cells synchronized in G1/S using double thymidine block, in S using hydroxyurea or in G2 using CDK1 inhibitor RO 3306. Scale bar, 5µm.

(B) WB analysis of the experiment depicted in **(A)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(C-D) Quantification of PLK1 nuclear intensity **(C)** and of the percentage of cells expressing PLK1 **(D)**. At least 200 cells per condition were quantified for each replicate. Each dot of graphs **(C)** represents PLK1 nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean. Graphs depicted in **(D)** represent the

mean of three replicates ± SD (two-sample two-tailed t-test or Mann-Whitney test *P<0,05, ***P<0,001, ****P<0,0001, ns=non-significant).

5. UBAP2L localizes to kinetochores during mitosis.

First, we tested if UBAP2L may directly regulate kinetochore dynamics of PLK1 by localizing to these structures during mitosis. Immunofluorescence microscopy analysis of endogenous UBAP2L revealed that this protein despite being mostly cytoplasmic, can also weakly localize to kinetochores in cells arrested in prometaphase using the Eg5 inhibitor STLC (**Fig. 6A**). Intriguingly, decrease of PLK1 activity using the specific kinase inhibitor BI2536 (Lénárt et al., 2007), led to increased recruitment of UBAP2L to kinetochores (**Fig. 6A and 6B**), despite the total levels of UBAP2L being reduced upon BI2536 treatment (**Fig. 6C**). The kinetochore enrichment of UBAP2L in prometaphase arrested cells was more pronounced upon PLK1 down-regulation, with endogenous UBAP2L accumulating in cytoplasmic and/or chromosomal aggregates which often but not always co-localized with individual pairs of sister kinetochores (**Fig. 6D and 6E**), while the total levels of UBAP2L were reduced in PLK1-downregulated cells (**Fig. 6F**). These results suggest that the kinetochore localization pattern of UBAPL2 might be dynamic and dependent on presence and localized activity of PLK1 and possibly on microtubule attachment status.

To further test the hypothesis that the association of UBAP2L to kinetochores is PLK1- and attachment-dependent, we synchronized cells in several mitotic stages using the Eg5 inhibitor Monastrol block and release protocol as described previously (Pangou et al., 2021) and analyzed the localization of endogenous UBAP2L. We observed increased recruitment of UBAP2L to kinetochores during metaphase relative to prometaphase stages (**Fig. 7A and 7B**), which correlates with reported decrease in localized PLK1 activity upon attachment stabilization and represents the mitotic stage when PLK1 undergoes removal from kinetochores. Our findings on the kinetochore-associated fraction of endogenous UBAP2L were also confirmed by analyzing the mitotic localization of ectopically expressed flag-tagged UBAP2L FL and UBAP2L protein fragments. Interestingly, both UBAP2L FL and the UBAP2L C-terminal fragment mimicked the phenotype observed for the endogenous UBAP2L upon PLK1 depletion, forming aggregates on chromosomes, a fraction of which accumulated on individual kinetochores, while the N-terminal fragment of UBAP2L was not detected at the kinetochores and rather displayed a diffused localization pattern in the cytoplasm (**Fig. 7C**).

Overall, these results are in line with the fact that UBAP2L mediates its function on PLK1 via its C-terminal domain (**Fig. 3**) specifically at kinetochores.

Figure 6.

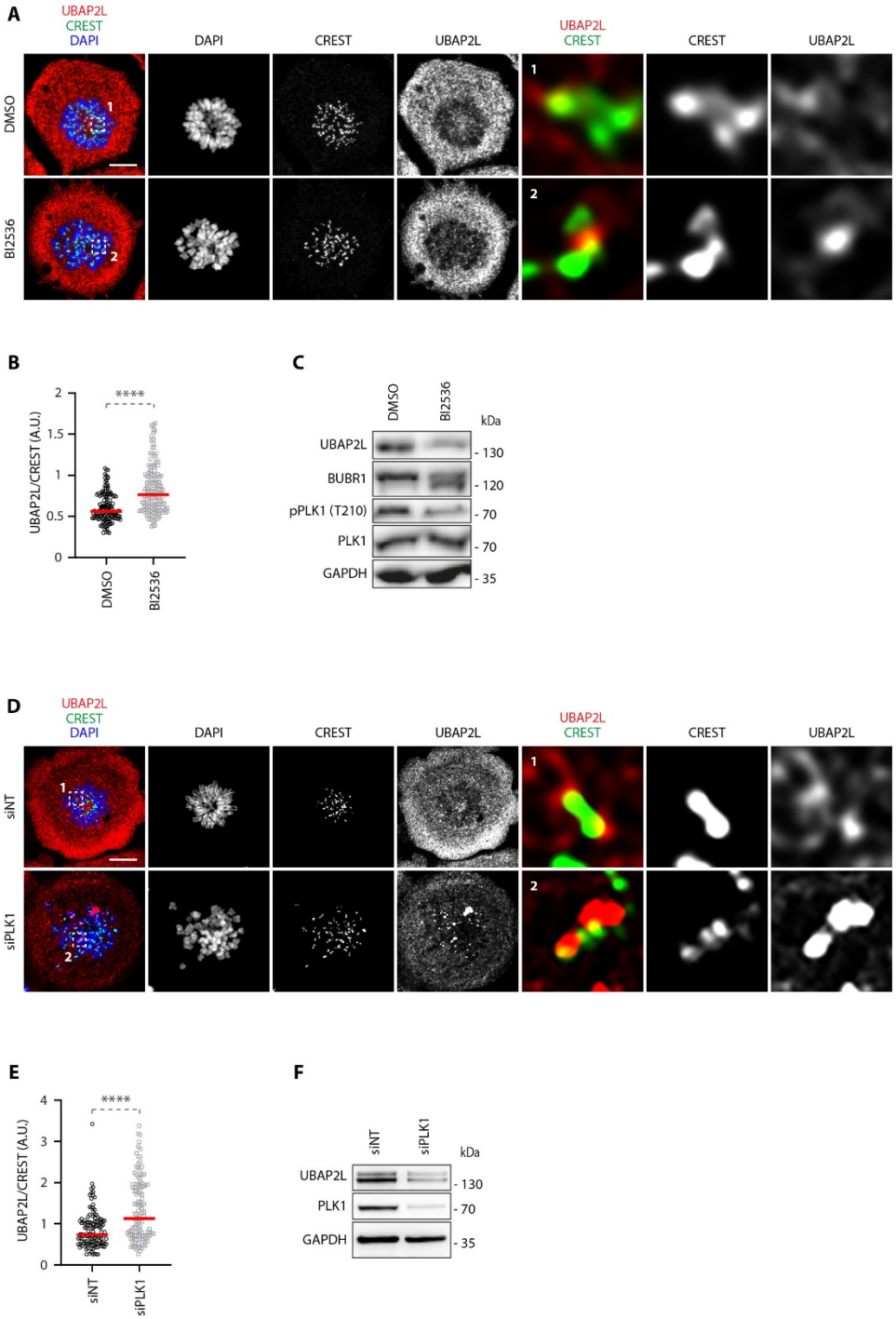


Fig. 6. UBAP2L localizes to kinetochores during mitosis in a PLK1-dependent manner.

(A-B) Representative IF pictures of HeLa cells synchronized in mitosis using STLC and treated with DMSO or 50nM BI2536 **(A)** and quantification of the relative UBAP2L intensity at

kinetochores (arbitrary units A.U.) **(B)**. ROIs are shown in the corresponding numbered panels. Scale bar, 5µm. At least 50 cells were quantified per condition for each experiment. Each dot represents UBAP2L/CREST intensity ratio at a single pair of kinetochores. The measurements of three biological replicates are combined, red bars represent the mean (Mann-Whitney test ****P<0,0001).

(C) WB analysis of the experiment depicted in **(A)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) Representative IF images of HeLa cells synchronized in mitosis using STLC and transfected with siNT or siPLK1 **(D)** and quantification of the relative UBAP2L intensity at kinetochores (arbitrary units A.U.) **(E)**. Regions of interest (ROIs) are shown in the corresponding numbered panels. Scale bar, 5µm. At least 50 cells were quantified per condition for each experiment. Each dot represents UBAP2L/CREST intensity ratio at a single pair of kinetochores. The measurements of three biological replicates are combined, red bars represent the mean (Mann-Whitney test ****P<0,0001).

(F) WB analysis of the experiment depicted in **(D)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

6. UBAP2L removes PLK1 from kinetochores.

Having demonstrated that UBAP2L does not interfere with the kinetochore recruitment of PLK1 during G1 and that UBAP2L localizes to kinetochores preferentially during metaphase, we then wondered whether UBAP2L is involved in dissociating PLK1 from kinetochores prior to anaphase onset. To this end, we assessed the effect of UBAP2L depletion on PLK1 localization in mitotically synchronized cells treated with Monastrol and collected at different time points after the release. Immunofluorescence analysis revealed that as early as in prometaphase, PLK1 displayed increased levels as well as cytoplasmic aggregates upon UBAP2L depletion relative to control cells (**Fig. 8A**). Moreover, during telophase and cytokinesis stages (1h and 30 min post release), UBAP2L depletion not only led to enrichment of PLK1 signals at the midbody, but also PLK1 was aberrantly retained at the kinetochores relative to control cells (**Fig. 8A-C**). Finally, when UBAP2L depleted cells exited mitosis and entered into the subsequent interphase (3h, 4 h and 30min post release), PLK1 was still highly enriched at the kinetochores compared to control cells in which PLK1 was no longer detected

at these structures (**Fig. 8A and 8C**). These results suggest that UBAPL2 is required for the efficient removal of PLK1 from the kinetochores during mitosis. Interestingly and consistent with previous results on PLK1 stability in G1 cells, UBAP2L downregulation led to reduced PLK1 degradation after release from Monastrol (**Fig. 8D**). To further corroborate these findings, we generated a HeLa PLK1-eGFP knock in (KI) cell line, which displayed no aberrant phenotypes in terms of PLK1 expression, localization and mitotic progression relative to isogenic PLK1-WT control cell line (**Fig. S5A-E**). Live video imaging in the PLK-eGFP KI cells synchronized with double thymidine block and release, further confirmed the enhanced expression of PLK1 from prophase to cytokinesis at the kinetochores, spindle poles, midzone and midbody, as well as its aberrant accumulation on kinetochores from anaphase to cytokinesis in the absence of UBAP2L (**Fig. 8E and Videos S5, S6**). Altogether, our results indicate that UBAP2L emerges as an important factor for the efficient and timely removal of PLK1 from the kinetochores during metaphase to anaphase transition and for the regulation of PLK1 protein stability.

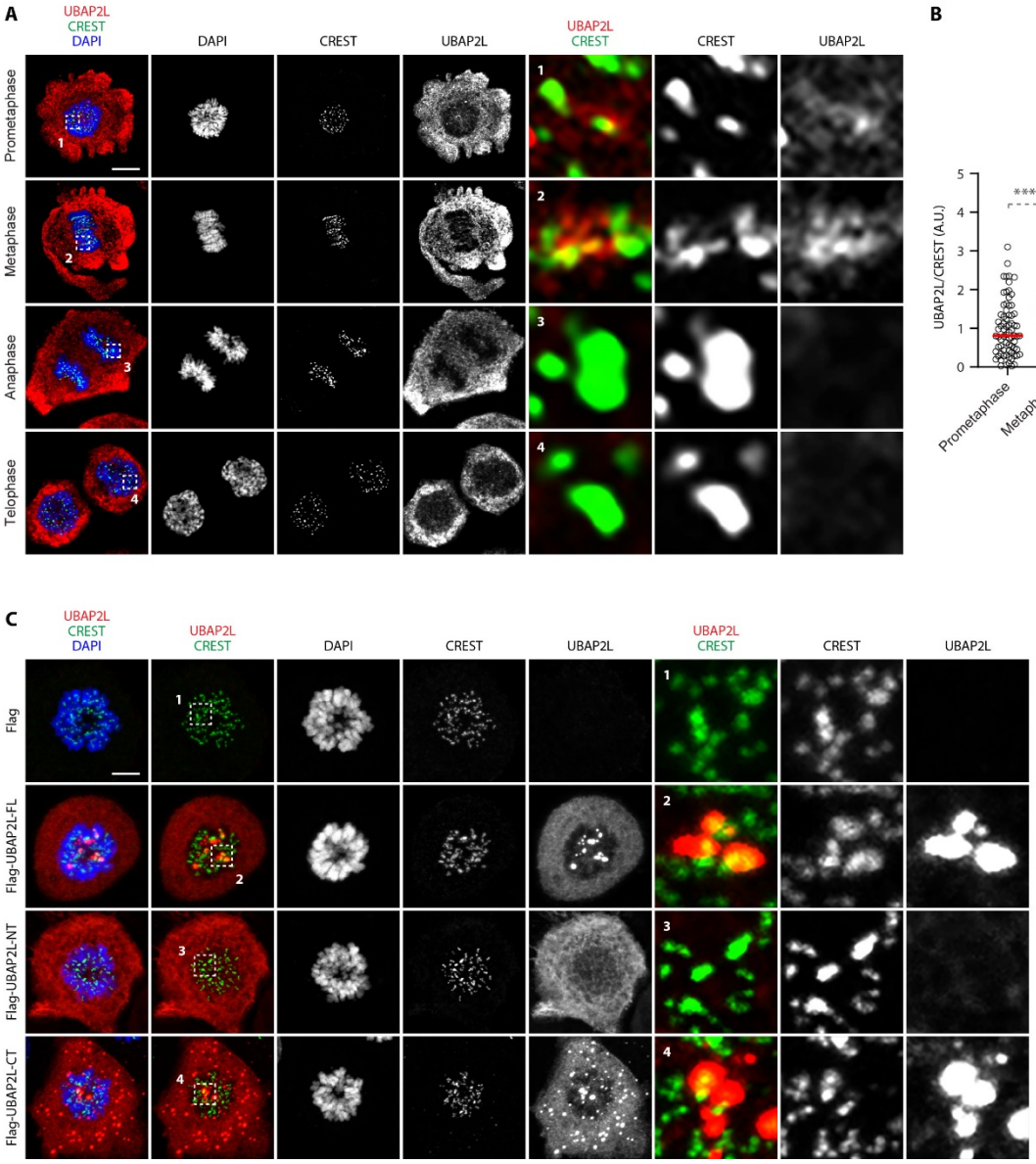


Fig. 7. UBAP2L localizes to kinetochores before anaphase onset.

(A-B) Representative IF images of HeLa cells synchronized in mitosis using Monastrol and released for 0h, 45min or 1h30 to visualize all mitotic stages **(A)** and quantification of the relative UBAP2L recruitment to kinetochores (arbitrary units A.U.) **(B)**. ROIs are shown in the corresponding numbered panels. Scale bar, 5µm. At least 50 cells were quantified per cell cycle stage for each experiment. Each dot represents UBAP2L/CREST overlapping area at a single pair of kinetochores. The measurements of three biological replicates are combined, red bars represent the mean (Kruskal-Wallis test with Dunn's correction ****P<0,0001, ns=non-significant).

(C) Representative IF pictures of HeLa cells synchronized in mitosis using STLC and transfected with the indicated UBAP2L flag-tagged constructs. ROIs are shown in the corresponding numbered panels. Scale bar, 5µm.

7. UBAP2L may regulate interaction of PLK1 with CUL3 to ensure faithful chromosome segregation.

Timely kinetochore removal of PLK1 during metaphase and chromosome segregation is regulated by CUL3-mediated mono-ubiquitylation of PLK1 (Beck et al., 2013). Although this modification does not affect the protein stability of PLK1 (Beck et al., 2013), we reasoned that possible involvement of UBAP2L in CUL3 pathway could explain, at least partially, the observed localization defects of PLK1 in UBAP2L-depleted cells. Indeed, a proteomics study has suggested that UBAP2L interacts with CUL3 complexes in human cells (Bennett et al., 2010).

Co-immunoprecipitation (co-IP) assays in mitotically synchronized cells showed that endogenous UBAP2L could efficiently interact with CUL3 and its substrate specific adaptor KLHL22 as well as with PLK1, relative to IgG control, but not with AURB which is another known mitotic ubiquitylation substrate of CUL3 (Sumara and Peter, 2007; Sumara et al., 2007; Maerki et al., 2009; Krupina et al., 2016) (**Fig. 9A**). To test the hypothesis that CUL3-mediated regulation of PLK1 during mitosis could be, at least to some extent, dependent on UBAP2L, endogenous co-IP of PLK1 was performed in the presence or absence of UBAP2L. UBAP2L depletion reduced the PLK1 interaction with CUL3 relative to control cells expressing UBAP2L (**Fig. 9B**), indicating that UBAP2L may be an essential component of this pathway.

Since UBAP2L, but not CUL3 (Beck et al., 2013), can also regulate stability of PLK1, we next aimed at understanding if polyubiquitylation status of PLK1 can be regulated by UBAP2L. To this end, we performed co-IP of GFP-PLK1 in the presence of proteasomal inhibitor MG132 under denaturing conditions in UBAP2L KO and in WT cells. Interestingly, we observed a significantly less pronounced polyubiquitin modification on immunoprecipitated GFP-PLK1 in cells depleted for UBAP2L (**Fig. 9C**). These results suggest that UBAP2L may control both timely non-proteolytic removal of PLK1 from kinetochores with help of CUL3 E3-ligase as well as ubiquitin-mediated proteolysis of PLK1 during mitotic exit. The identity and precise mechanism of the possible additional E3-ligase involved in UBAP2L regulation of PLK1 stability remains to be determined in future.

Figure 8.

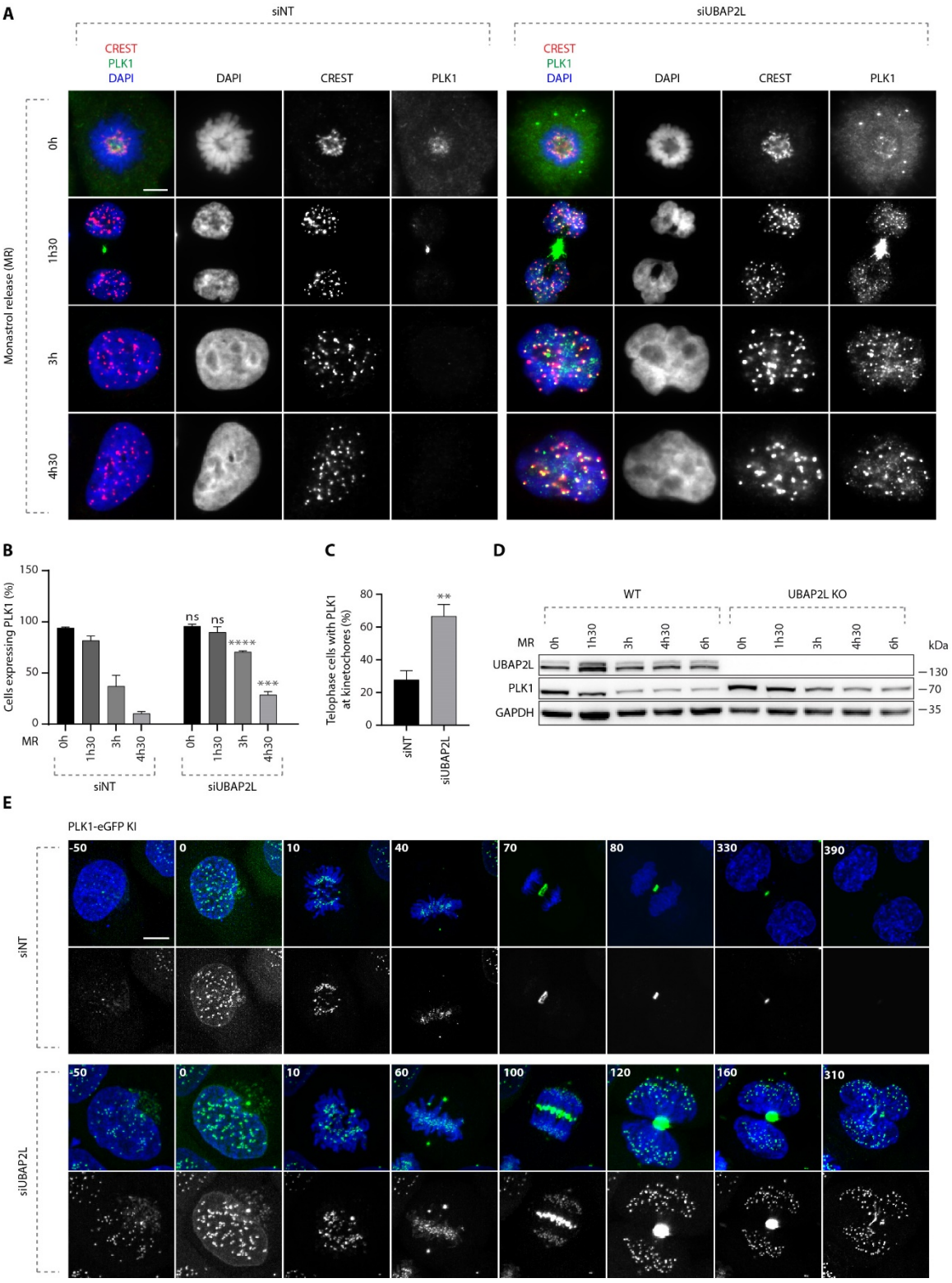


Fig. 8. UBAP2L removes PLK1 from kinetochores.

(A-C) Representative IF images of control (siNT) or UBAP2L-downregulated cells synchronized in prometaphase using monastrol and released at the indicated time points. Scale bar, 5µm. Quantification of the percentage of cells expressing PLK1 **(B)** and of telophase cells

with PLK1 at kinetochores **(C)**. At least 250 cells per condition were quantified for each replicate. Graphs represent the mean of three replicates ± SD (two sample two-tailed t-test or one-way ANOVA with Dunnett's correction **P<0,01, ***P<0,001, ****P<0,0001, ns=non-significant).

(D) WB analysis of WT or UBAP2L KO HeLa cells lysates after monastrol release at the indicated time points. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(E) Spinning disk time-lapse microscopy of PLK1-eGFP Knock-In (KI) HeLa cells synchronized with DTBR in mitosis. The selected frames of the movies are depicted and the corresponding time is indicated in minutes. SiR-DNA was used for DNA staining. Scale bar, 8µm.

To prove that the chromosome segregation and other mitotic errors observed in cells lacking UBAP2L could be directly linked to increased levels and activation of PLK1 we performed rescue experiments using the chemical inhibitor BI2536 of PLK1 kinase (Lénárt et al., 2007). To this end, we inhibited PLK1 activity after release from Monastrol treatment at different time points and we compared the rate of segregation errors in UBAP2L-downregulated cells relative to control cells. BI2536 efficiently restored PLK1 activity to basal levels in UBAP2L depleted cells as verified by its auto-phosphorylation on Thr210 (**Fig. 9D**). Interestingly, BI2536 treatment fully rescued all types of erroneous mitotic phenotypes observed in UBAP2L depleted cells, including chromosome misalignment in metaphase, DNA bridges in anaphase and telophase and micronuclei formation after cytokinesis completion (**Fig. 9E-H**). Our results suggest that aberrant PLK1 activity resulting from increased stability of this kinase is the leading cause for mitotic defects observed in UBAP2L-depleted cells.

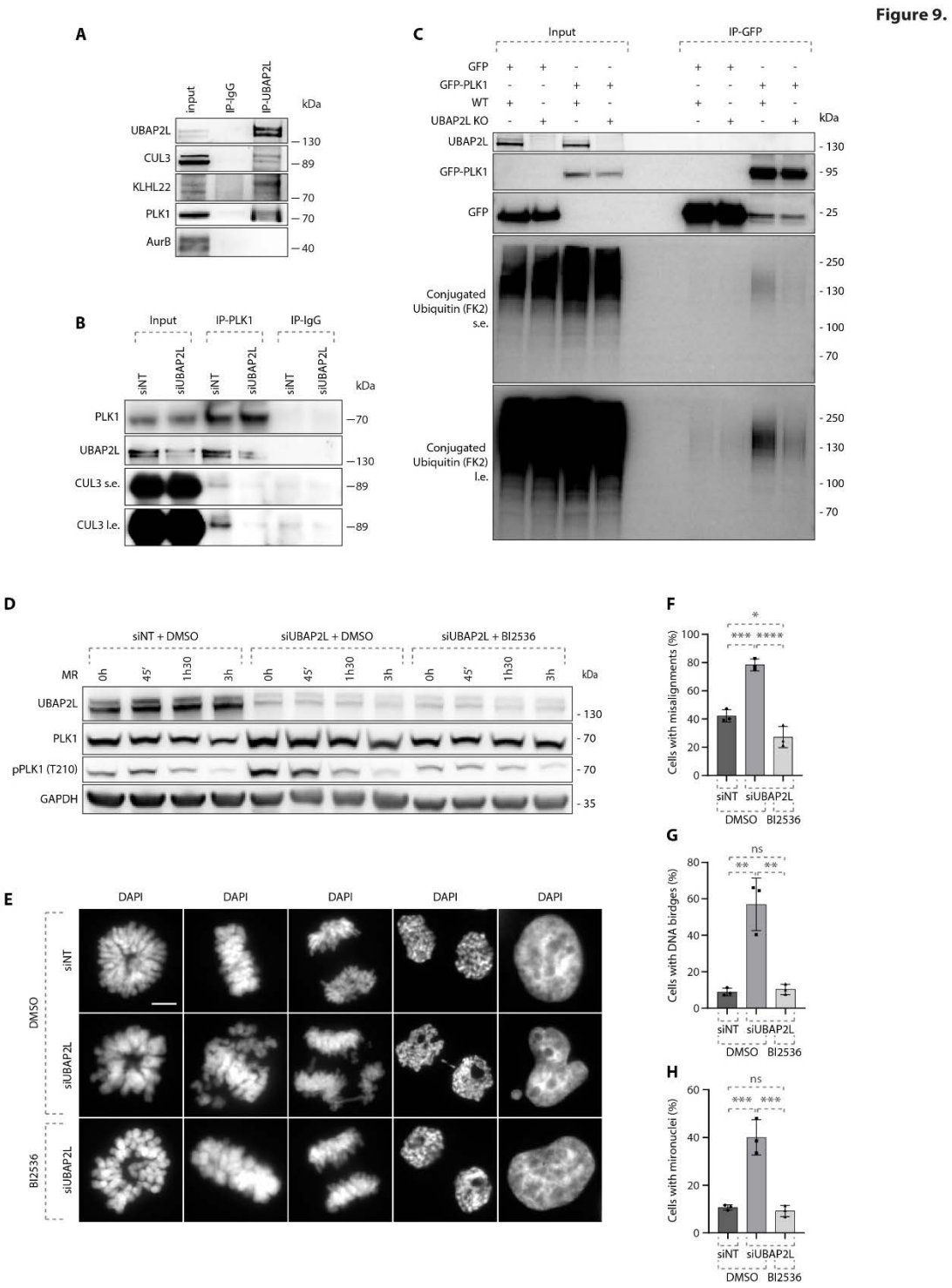


Fig. 9. UBAP2L may regulate interaction of PLK1 with CUL3 to ensure faithful chromosome segregation.

(A) WB analysis of endogenous immunoprecipitation (IP) of IgG or UBAP2L from HeLa cells synchronized in mitosis using STLC. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(B) WB analysis of endogenous IP of IgG or PLK1 from HeLa control or UBAP2L-downregulated cells synchronized in mitosis using STLC. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(C) WB analysis of IP under denaturing conditions of WT or UBAP2L KO HeLa cells transiently transfected with plasmids encoding for GFP-PLK1 and His-Ubiquitin. The short exposure (s.e.) and long exposure (l.e.) of the membrane blotted against the FK2 antibody that specifically recognizes conjugated but not free ubiquitin are shown. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D) WB analysis of control (siNT) or siUBAP2L treated HeLa cells were synchronized with monastrol, treated with DMSO or with 10nM of the PLK1 inhibitor BI2536 for 45min and subsequently washed out from monastrol. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(E-H) DAPI staining of the experiment described in **(D)** showing different mitotic stages **(E)**. Scale bar, 5µm. Quantification of the percentage of cells with misalignments **(F)**, DNA bridges **(G)** and micronuclei **(H)**. At least 100 cells from each mitotic stage were quantified for all conditions. Graphs represent the mean of three replicates ± SD (one-way ANOVA with Sidak's correction *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001, ns=non-significant).

I. Discussion

In summary, our study provides novel insights into how PLK1 by UBAP2L is spatiotemporally regulated during mitotic progression. We propose that UBAP2L associates with kinetochore structures during metaphase in order to efficiently promote both the kinetochore removal and the degradation of PLK1 prior to anaphase as a means to ensure faithful chromosome segregation. We demonstrate that UBAP2L depleted cells are characterized by significant mitotic delay, severe segregation errors and micronuclei formation, phenotypes that can be directly linked to aberrant PLK1 kinase activity. We provide evidence that in the absence of UBAP2L-mediated signaling, PLK1 is abnormally retained at the kinetochore and fails to get degraded during mitotic exit, resulting in excessive PLK1 expression and kinase activity in the subsequent interphase, which may ultimately cause genomic instability and cell death.

1. *How does UBAP2L regulate mitosis?*

Mitosis is a fundamental process in eukaryotes, where steps such as chromosome congression and chromosome alignment need to be precisely fine-tuned to ensure high fidelity of cell division (McIntosh, 2016). Phosphorylation and ubiquitylation pathways are tightly interconnected during mitosis, however how exactly these signaling cues are integrated and orchestrated in a space–time-dependent manner remains not fully understood. Here, we identify the ubiquitin-binding protein UBAP2L as a novel regulator of PLK1 controlling both its localization and protein stability. We show that UBAP2L regulates PLK1 in a cell-cycle specific manner, with UBAP2L depletion leading to enhanced protein levels and kinetochore enrichment of PLK1 in mitosis and in the subsequent G1/S, while we did not observe any effect during G2 (**Fig. 5**). UBAP2L interacts with PLK1 in mitotically synchronized cells (**Fig. 9A),** thereby licensing the kinetochore removal of PLK1 prior to anaphase (**Fig. 8**), while having no effect on the kinetochore recruitment of PLK1 during G1/S (**Fig. S4**). Importantly, we show that the regulatory effect of UBAP2L towards PLK1 is specific and can be uncoupled from cell cycle progression, since UBAP2L does not interact (**Fig. 9A**) and does not modulate the protein levels and/or localization of other mitotic factors including AurA, AurB, and Cyclin B1 (**Fig. 2 and S2**), nor other PLK family members (**Fig. 2J**).

This specific UBAP2L-PLK1 signaling could potentially be explained by the fact that a fraction of UBAP2L dynamically localizes at the kinetochore during prometaphase and metaphase (**Fig. 7**), indicating that UBAP2L exerts its mitosis-related functions specifically at these mitotic structures and stages. PLK1 is known to be enriched at kinetochores from prometaphase till metaphase (Elowe et al., 2007), while at these early mitotic stages AURB mostly localizes at the inner centromere (Yamagishi et al., 2010) and AURA, Cyclin B1, PLK2, PLK3 and PLK4 are mostly enriched at the mitotic spindle and the centrosomes (Pines, 1997; Sugimoto et al., 2002; Warnke et al., 2004; Jiang et al., 2006; Fournier et al., 2016). We could therefore speculate that the mitotic role of UBAP2L can be attributed to its kinetochore associated fraction which provides access to kinetochore substrates such as PLK1. Therefore, it would be worth investigating whether additional kinetochore proteins might be under UBAP2L regulation to ensure mitotic fidelity.

Of interest, UBAP2L has also been proposed to be phosphorylated during mitosis (Dephoure et al., 2008; Maeda et al., 2016), but the kinase involved or the underlying mechanisms are currently unknown. Our results demonstrate that the kinetochore associated fraction of UBAP2L is dependent on PLK1 activity/expression (**Fig. 6**). Given that the C-terminal domain

of UBAP2L is predicted to harbor several PLK1 consensus motifs (Santamaria et al., 2011), it would be interesting to address the possibility of UBAP2L being a direct phosphorylation target of PLK1 or an indirect substrate via CDK1 priming phosphorylation (Parrilla et al., 2016). Such a regulatory feedback loop has already been described for PLK1/USP16 (Zhuo et al., 2015) and would advance our understanding on how PLK1 can dynamically drive its own localized activity to ensure fidelity of cell division.

2. Role of UBAP2L C-terminal domain in the regulation of PLK1

Our data demonstrate that the uncontrolled kinetochore PLK1 retainment and the elevated PLK1 protein stability during mitotic exit observed in UBAP2L depleted cells are mediated specifically and exclusively through UBAP2L. The phenotypes described for segregation errors, polyploidy, specific effect on PLK1 and not on other mitotic factors are corroborated by specific siRNAs against UBAP2L (Cirillo et al., 2020) and by CRISPR-mediated genetic depletion of UBAP2L, excluding the possibility of an off-target or a compensatory effect. Our rescue experiments provide evidence that both PLK1 aberrant kinetochore accumulation (**Fig. 3C**) and cell survival (**Fig. 3E**) can be entirely rescued by overexpression of the C-terminal domain of UBAP2L, but are not dependent on its N-terminal domain that was until now considered to mediate the mitotic role of UBAP2L (Maeda et al., 2016). Moreover, we show that the accumulated micronuclei observed in UBAP2L depleted cells during mitotic exit is directly linked to aberrant PLK1 expression/activity in these cells (**Fig. 9E**). However and in line with our results, the study by Maeda and colleagues reported that an extra sequence after the UBA-RGG domain is essential for proper mitotic progression, while overexpression of the UBA-RGG domain alone cannot restore the multinuclear phenotype observed in UBAP2L-depleted cells (Maeda et al., 2016). Altogether, these results argue for the existence of at least two distinct pathways responsible for mediating the role of UBAP2L during mitosis. One dependent on PRMT1 methylation with yet unknown UBAP2L downstream mitotic targets (Maeda et al., 2016) and one dependent on UBAP2L kinetochore localization and on PLK1 activity as proposed in this study.

UBAP2L and in particular its C-terminus domain have been mostly studied in the context of SGs signaling (Youn et al., 2018; Cirillo et al., 2020; Huang et al., 2020). Our results show that depletion of core SGs components had no effect on the ability of UBAP2L FL and/or UBAP2L C-terminal fragment to fully restore the aberrant kinetochore accumulation of PLK1 (**Fig. 4B-C**), thus suggesting that the C-terminus domain of UBAP2L has an unexpected new role during

mitosis that seems unrelated to its established role of G3BP1/G3BP2-dependent SGs signaling. Furthermore, the PRMT1-dependent UBAP2L methylation that is linked to accurate chromosome segregation, was recently reported to impair SG assembly (Huang et al., 2020), again indicating that the role of UBAP2L in mitosis and its effect on PLK1 does not interfere with its role is SGs signaling. Interestingly, SGs cannot be formed during mitosis and membraneless organelles (apart from centrosomes) are dissolved at G2/M transition in a kinase-dependent manner (Rai et al., 2018). It would be intriguing to speculate that UBAP2L may be subjected to phosphorylation during mitotic entry as a means to promote the dissolution of SGs, thereby shifting the interactions and functions of UBAP2L towards components of the mitotic machinery.

3. UBAP2L regulates both PLK1 localization and stability

How exactly does UBAP2L regulate PLK1 to ensure fidelity of cell division? Our data demonstrate that in cells lacking UBAP2L, not only PLK1 is abruptly retained at the kinetochore throughout mitosis (**Fig. 8**), but is also protected from degradation (**Fig. S3**), resulting in persistent PLK1 protein stability and activity in the interphasic cells. More specifically, we show that in the absence of UBAP2L, PLK1 is resistant to CHX treatment both in interphasic (**Fig. S3A**) and mitotic cells (**Fig. S3B**) and that the number of cells expressing PLK1 during interphase is significantly increased compared to control WT cells where PLK1 is only detected at basal levels (**Fig. 2**). Furthermore, we show that UBAP2L depletion does not interfere with the kinetochore recruitment of PLK1 in early G1/S (**Fig. S4**), but it impairs PLK1 kinetochore removal during mitosis (**Fig. 8**). Finally, we observe that loss of UBAP2L weakens the mitotic interaction between PLK1 and CUL3 (**Fig. 9B**) and results in markedly decreased polyubiquitin modification of PLK1 under denaturing conditions (**Fig. 9C**).

Cullin-RING ubiquitin ligases (CRLs) are the largest family of E3 ubiquitin ligases that regulate both proteolytic and non-proteolytic ubiquitin signals in a large variety of cellular processes (Jerabkova and Sumara, 2019; Jang et al., 2020). Accumulating evidence suggests that CUL3 emerges as a critical regulator of cell division by regulating critical mitotic kinases such as PLK1, AURA and AURB (Sumara et al., 2007; Maerki et al., 2009; Moghe et al., 2012; Beck et al., 2013; Courtheoux et al., 2016; Krupina et al., 2016a). However, we still lack sufficient knowledge regarding the molecular identity and function of additional factors that act in concert with CUL3 to precisely define the cellular fate of mitotic substrates and subsequently cell cycle progression. It was recently proposed that both CRL substrate recruitment as well as CRL

complex assembly are dependent on the coordinated actions of specific co-adaptors and inhibitors to ensure their function in time and space (Akopian et al., 2022). Here, we demonstrate that UBAP2L specifically regulates the protein levels and localization of PLK1 but of no other mitotic targets of CUL3 including AURA and AURB (**Fig. 2 and S2**). Moreover, UBAP2L directly interacts with PLK1, CUL3 and KLHL22, but not with AURB during mitosis (**Fig. 9A**). Given the loss of interaction between CUL3 and PLK1 observed upon UBAP2L depletion (**Fig. 9B**), our data indicate that UBAP2L might be important for the recognition of PLK1 by the KLHL22/CUL3 complex. This could, at least to some extent, explain the phenotype of PLK1 being unable to get efficiently removed from kinetochores in the absence of UBAP2L and could suggest that UBAP2L might act as a co-adaptor for CUL3 to ensure its access to PLK1 at the kinetochore prior to anaphase. Further studies are needed to explore whether UBAP2L might decipher the versatility of the CUL3-based ubiquitin code during cell division.

Intriguingly, the additional regulation of PLK1 by UBAP2L at the level of protein stability, suggests that UBAP2L might also regulate PLK1 independently of the CUL3-based pathway via yet uncharacterized mechanisms. PLK1 is ubiquitylated by the APC/C E3 ubiquitin ligase in anaphase via its interaction with FZR1/CDH1, which provides the signal for the proteasomal-dependent degradation of PLK1 during mitotic exit (Lindon and Pines, 2004). One possibility would be that in the absence of UBAP2L the affinity of PLK1 towards CDH1 is reduced or shifted towards CDC20, therefore leading to increased PLK1 protein stability during mitotic exit. Still, we cannot exclude that the UBAP2L-driven proteolytic signals on PLK1 might involve other E3 ligases independent of the APC/C established mechanism, or that CUL3 might associate with unknown adaptors/inhibitors (Akopian et al., 2022) which in turn activate proteolytic ubiquitylation on PLK1. To our knowledge, such a dual regulation for PLK1 in terms of both stability and localization has only been described in one more study which addressed the role of NUMB in mitosis, a protein mostly known for its function in progenitor cell fate determination (Gulino et al., 2010). The authors show that NUMB depletion resulted in reduced PLK1 protein stability and in aberrant centrosomal localization of PLK1 at both metaphase and anaphase, leading to disorganized γ-tubulin recruitment to centrosomes (Schmit et al., 2012). Our work is the first to report a unique role for UBAP2L in converging both proteolytic and non-proteolytic ubiquitin signals on PLK1 in order to ensure fidelity of mitotic progression. How exactly those two UBAP2L-dependent signaling cascades communicate with

each other to precisely regulate PLK1 in time and space remains to be addressed in future studies.

4. Possible consequences of aberrant PLK1 signaling

Mitotic perturbations are causally linked to aneuploidy and genomic instability (S. Pedersen et al., 2016). Phosphorylation and ubiquitylation pathways are tightly interconnected in mitosis and it is important to understand these links in the context of carcinogenesis. PLK1 is misregulated in human cancers and small molecule inhibitors targeting PLK1 are currently being explored for cancer treatment (Chiappa et al., 2022). However, preclinical success with currently available PLK1 inhibitors has not translated well into clinical success, highlighting the need for a complete understanding of upstream PLK1 regulatory mechanisms. In our view, combined therapies targeting other relevant pathways together with PLK1 may be vital to combat issues observed with monotherapy, especially resistance. In addition, research should also be directed towards understanding the mechanisms regulating localized activity of PLK1 and designing additional next generations of specific, potent PLK1 inhibitors to target cancer (Gutteridge et al., 2016). Of interest, the signaling pathways mediating the recruitment and the removal of PLK1 at and from kinetochore structures are characterized by several layers of regulation and complexity, raising the possibility that distinct pools of PLK1 may exist at kinetochores (Lera et al., 2016).

We provide evidence that UBAP2L depletion inhibits the kinetochore removal of PLK1 and increases its stability after mitosis completion, resulting in aberrant PLK1 kinase activity in interphasic cells, which may ultimately cause genomic instability and cellular death. What could be the potential consequences for cells entering the subsequent cell cycle in the presence of high PLK1 activity? PLK1 has a largely unexplored and unconventional functional territory beyond mitosis especially in processes such as DNA replication, transcription and damage checkpoint recovery (Kumar et al., 2017). Our study suggests that the accumulated micronuclei observed in UBAP2L depleted cells during mitotic exit is directly linked to aberrant PLK1 expression/activity in these cells (**Fig. 9E and 9H**). Micronuclei display highly heterogeneous features regarding the recruitment or retainment of replication, transcription and DNA damage response factors, ultimately being associated with chromosomal instability (Krupina et al., 2021). Interestingly, PLK1 has been shown to regulate RNAPIII-dependent transcription, switching from activation to repression based on its activatory status (Fairley et al., 2012), while a recent study implicated UBAP2L in the ubiquitylation and degradation of RNAPII through

the recruitment of a Cullin-based ubiquitin complex (Herlihy et al., 2022). We could therefore speculate that cells with defective UBAP2L-PLK1 signaling would be more prone to unbalanced transcription which would further hijack their genome fidelity, a concept worth to be investigated in the future.

Finally, growing evidence suggests that UBAP2L is overexpressed in a variety of cancers where it displays oncogenic properties by interfering with signaling pathways that promote cancer cell proliferation, tumor vascularization, migration, invasion and metastasis (Guerber et al., 2022). While the oncogenic potential of UBAP2L renders it an attractive candidate for therapy, the results presented in our study linking its depletion to aberrant PLK1 activation and perturbed cell division, rather indicate that targeting UBAP2L might be a strategy that should be applied with caution. The pathway described in our study could maybe direct research efforts towards the synergistic inhibition of UBAP2L and PLK1 in specific cancer types.

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K. Acknowledgments and funding

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L. Declaration of interests

The authors declare no competing interests.

M. Supplementary figures and legends

Figure S1.

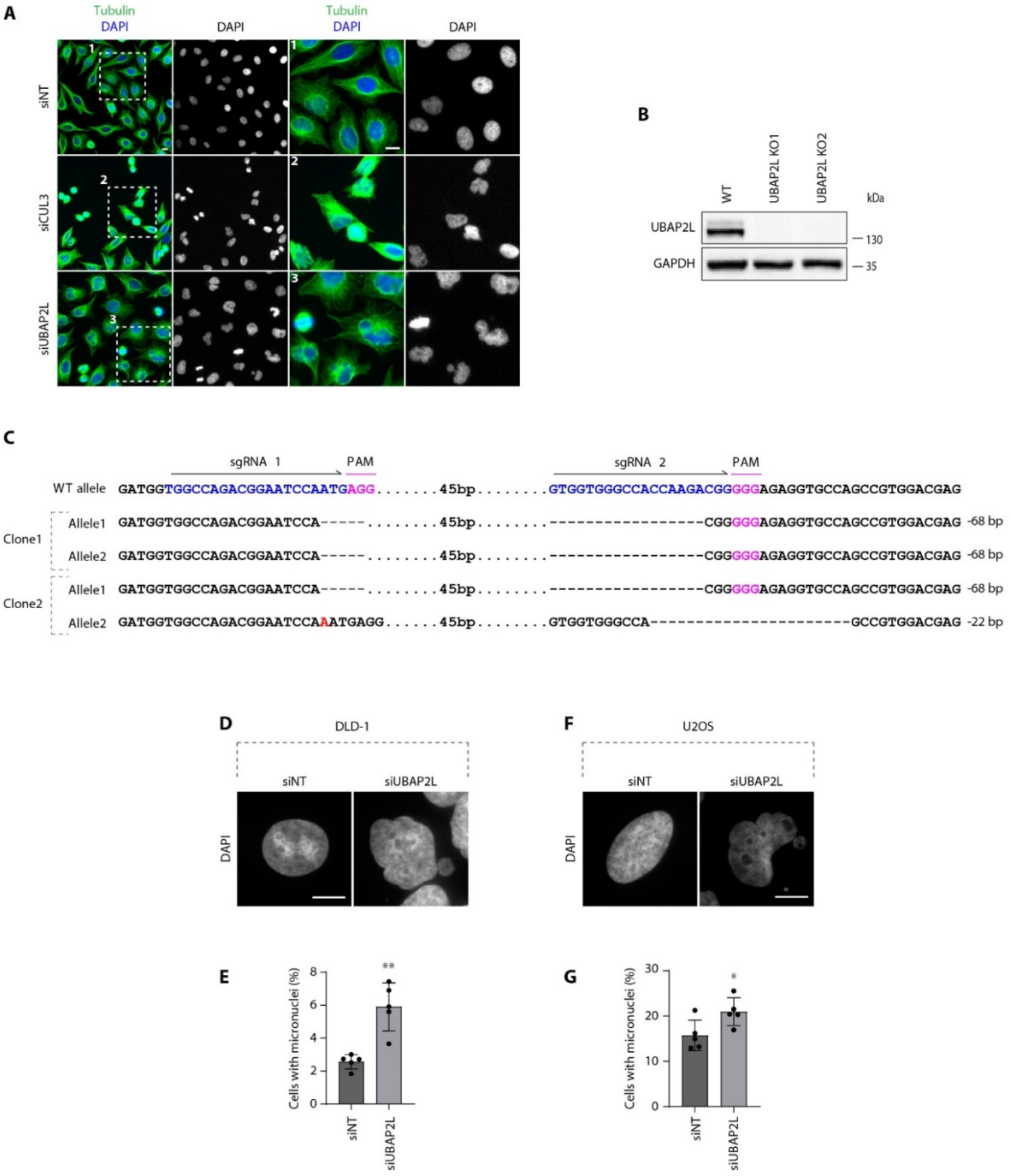

Fig. S1. UBAP2L regulates proper chromosome segregation during mitosis.

(A) Representative microscopy images from high-content visual validation siRNA screen in HeLa cells for known and predicted human UBD proteins (Krupina et al., 2016). ROIs are shown in the corresponding numbered panels. Scale bars, 10µm.

(B-C) Validation of CRISPR-Cas9 mediated UBAP2L KO HeLa cell clones by WB analysis **(B)** and Sanger-sequencing **(C)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) DLD-1 cells were transfected with the indicated siRNAs and the presence of micronuclei was assessed by IF microscopy **(D)** and quantified in **(E)**. Scale bar, 10µm.

(F-G) U2OS cells were transfected with the indicated siRNAs and the presence of micronuclei was assessed by IF microscopy **(F)** and quantified in **(G)**. Scale bar, 10µm. Graphs represent the mean of three replicates ± standard deviation (SD) (two sample two-tailed t-test *P<0,05, **P<0,01).

Figure S2.

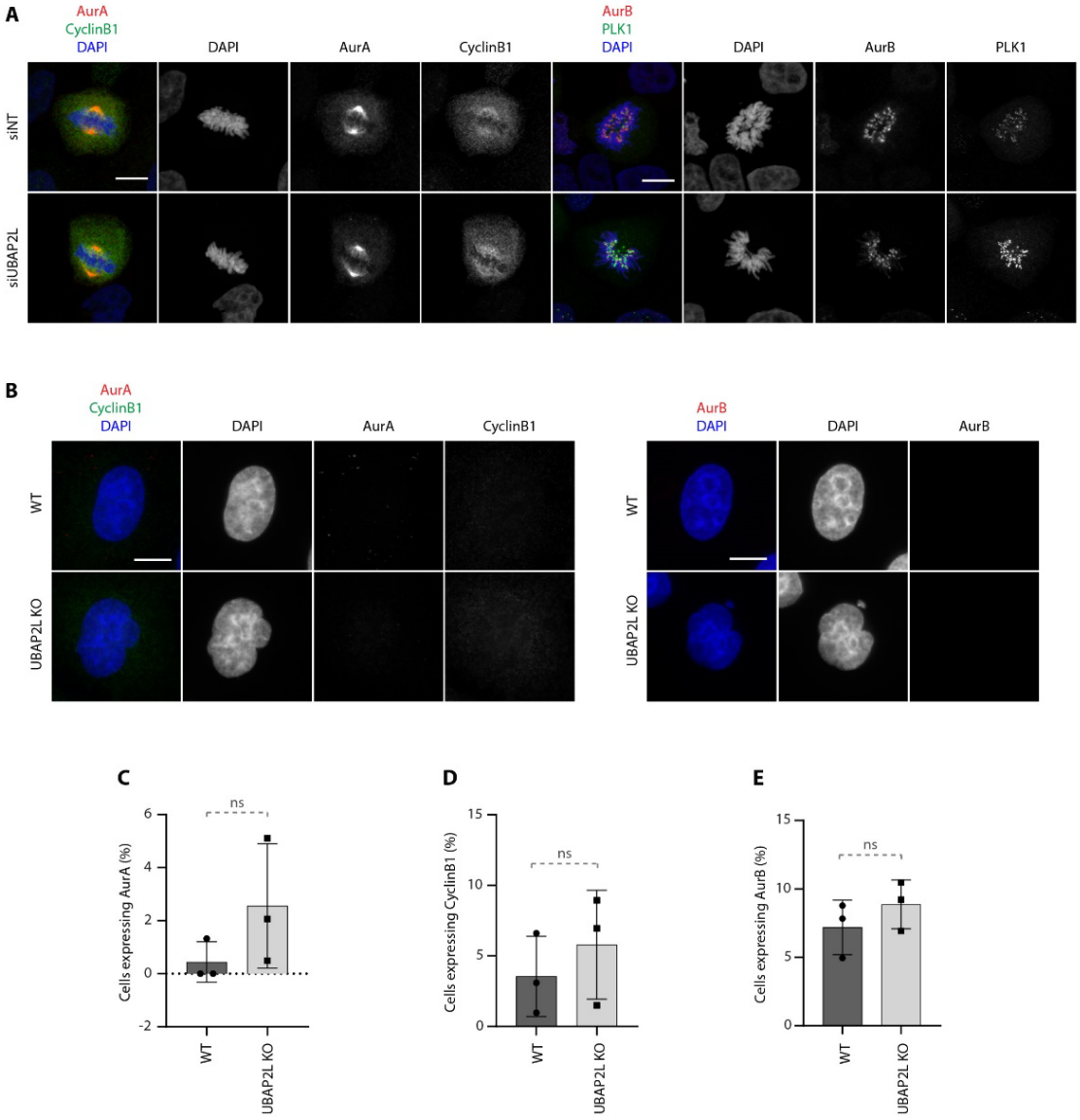

Fig. S2. UBAP2L regulates PLK1 levels and activity.

(A) Representative IF images of control or UBAP2L-downregulated HeLa cells synchronized in mitosis using DTBR. Scale bar, 10µm.

(B-E) Representative IF images of WT or UBAP2L KO HeLa cells synchronized in G1/S using DTB. Scale bar, 10µm. The percentage of cells expressing AURA, CyclinB1 or AURB was quantified in **(C)**, **(D)** and **(E)** respectively. Graphs represent the mean of three replicates ± standard deviation (SD) (two sample two-tailed t-test ns=non-significant).

Figure S3.

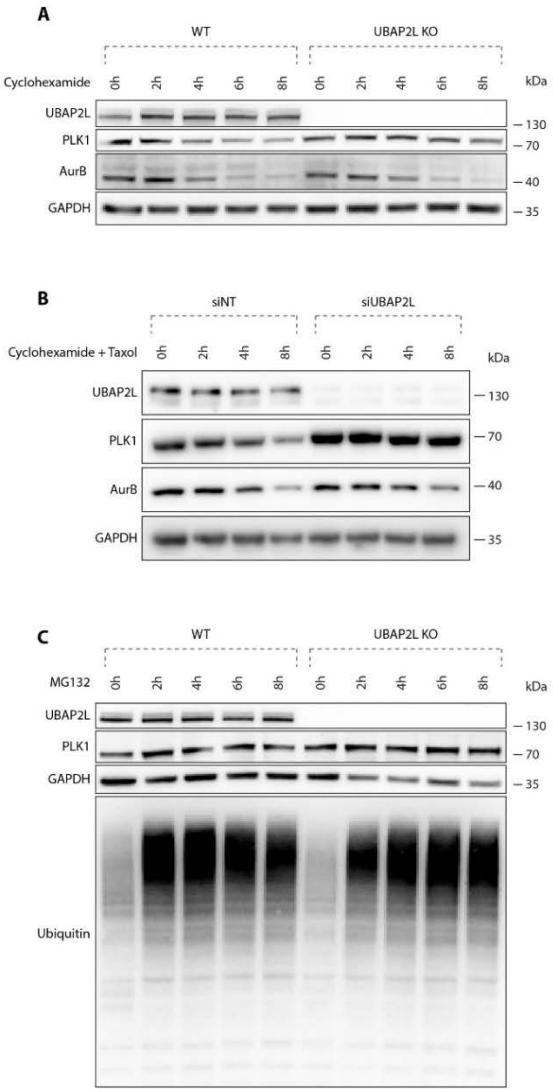

Fig. S3. UBAP2L regulates PLK1 levels and activity.

(A) WB analysis of WT or UBAP2L KO HeLa lysates of interphasic cells treated with 100µg/mL cycloheximide (CHX) for the indicated times. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(B) WB analysis of control or UBAP2L-silenced HeLa lysates of mitotic cells treated with 100µg/mL CHX for the indicated times. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(C) WB analysis of WT or UBAP2L KO HeLa lysates of interphasic cells treated with 25µM of the proteasomal inhibitor MG132 for the indicated times. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

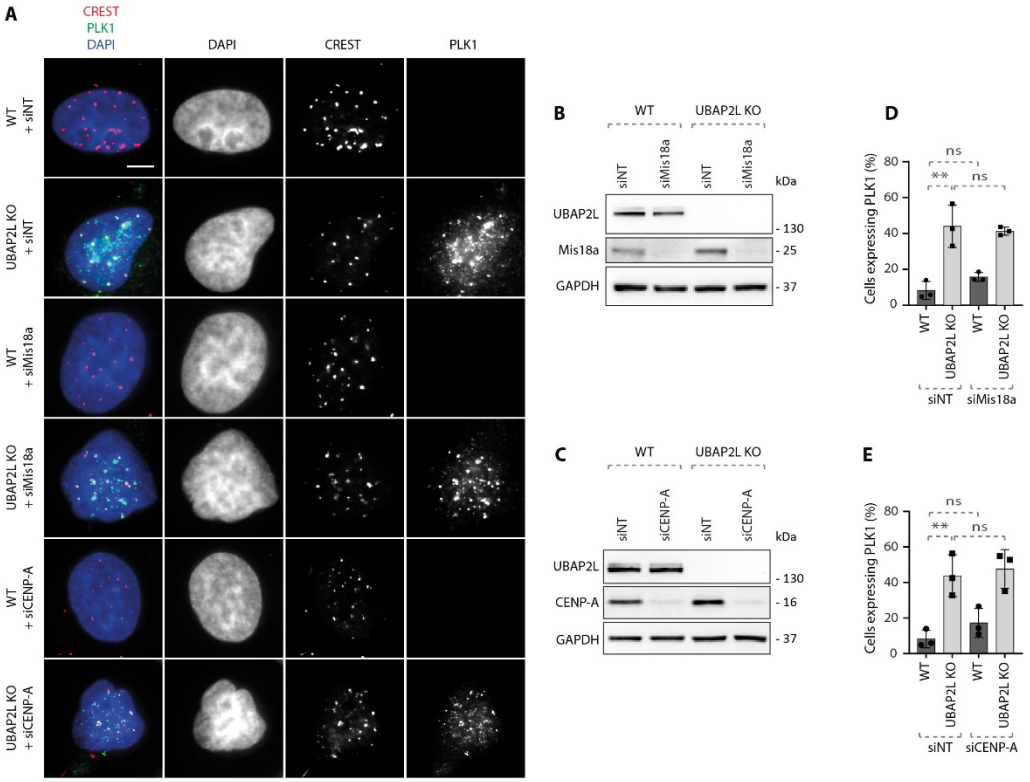

Fig. S4. UBAP2L does not regulate PLK1 kinetochore recruitment.

(A) Representative IF images of WT or UBAP2L KO G1/S synchronized HeLa cells treated with the indicated siRNAs. Scale bar, 5µm.

(B-C) WB analysis of the experiment described in **(A)**. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) Quantification of the percentage of cells expressing PLK1 of the experiment described in **(A)**. At least 250 cells per condition were quantified for each experiment. Graphs represent the mean of three replicates ± SD (one-way ANOVA with Sidak's correction **P<0,01, ns=non-significant).

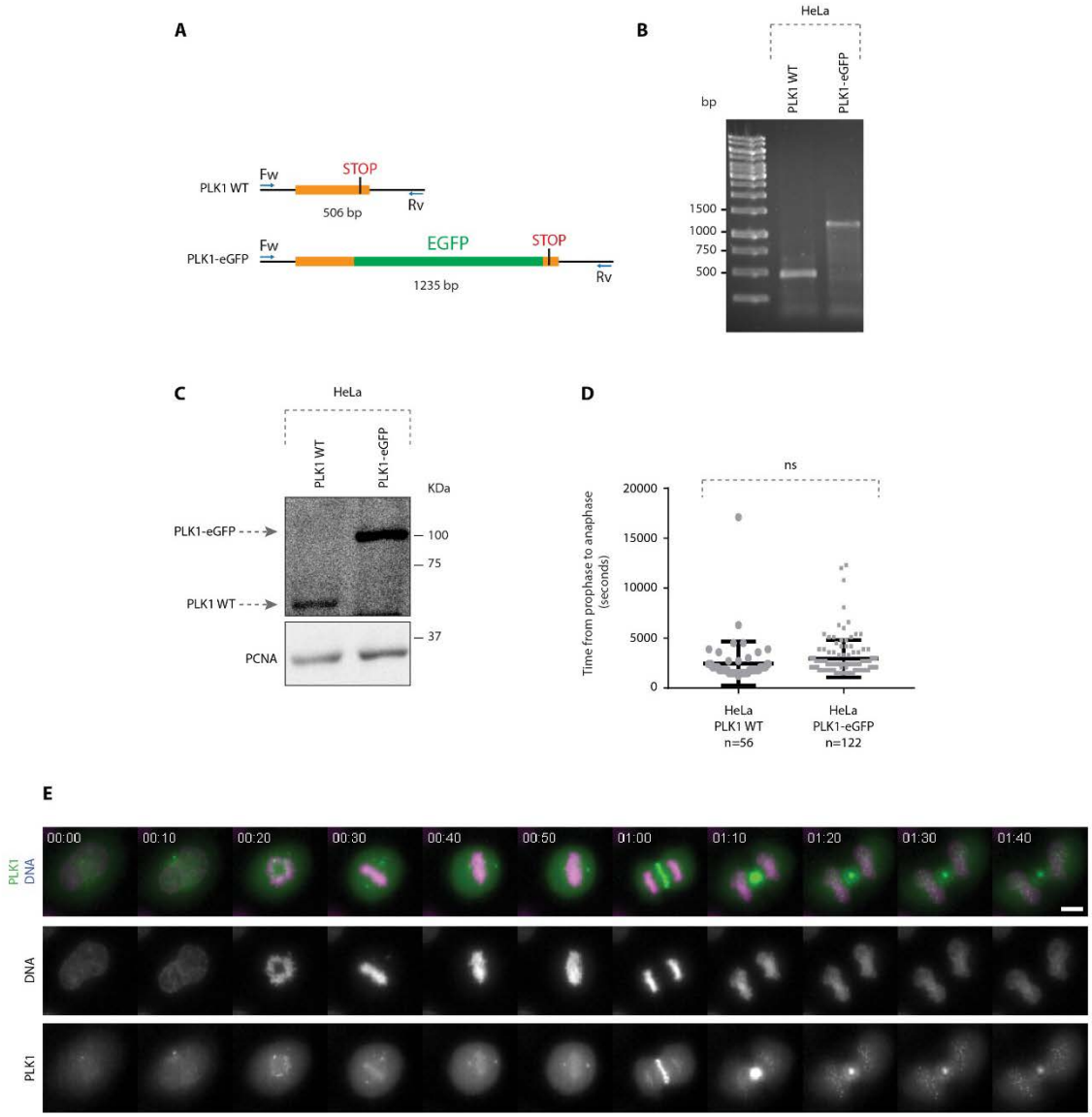

Fig. S5. Generation of the PLK1-eGFP cell line.

(A) Schematic representation of the screening strategy used to identify PLK1-eGFP positive clones. Forward (Fw) and Reverse (Rv) primers used are annotated.

(B-C) Agarose gel electrophoresis **(B)** and WB analysis **(C)** of PLK1 WT and PLK1-eGFP cells lysates.

(D) Scatterplot representing the time from prophase to anaphase (seconds) in HeLa PLK1 WT and PLK1-eGFP cell lines.

(E) Representative time frames of a 12 hours movie of HeLa PLK1-eGFP. Scale bar, 10µm. Error bars indicate Standard Error of the Mean. The number of analyzed cells is indicated in

the graph. Statistical significance was determined using Mann-Whitney test (ns=non-significant). Time is indicated as hh:mm.

Supplementary Videos S1-S4. UBAP2L regulates proper chromosome segregation during mitosis. Related to Figure 1.

(S1-S4) Spinning disk time lapse microscopy of WT **(S1)** or UBAP2L KO HeLa cells **(S2-S4)** synchronized in mitosis with DTBR and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z-stacks (25µm range, 2µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S1.

Time lapse of WT HeLa cells, related to Figure 1. Spinning disk time lapse microscopy of WT HeLa cells synchronized in mitosis with DTBR and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (25µm range, 2µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S2.

Time lapse of UBAP2L KO HeLa cells, related to Figure 1. Spinning disk time lapse microscopy of UBAP2L KO HeLa cells synchronized in mitosis with DTBR and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (25µm range, 2µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S3.

Time lapse of UBAP2L KO HeLa cells, related to Figure 1. Spinning disk time lapse microscopy of UBAP2L KO HeLa cells synchronized in mitosis with DTBR and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (25µm range, 2µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S4.

Time lapse of UBAP2L KO HeLa cells, related to Figure 1. Spinning disk time lapse microscopy of UBAP2L KO HeLa cells synchronized in mitosis with DTBR and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (25µm range, 2µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Supplementary Videos S5-S6. UBAP2L regulates proper chromosome segregation during mitosis. UBAP2L removes PLK1 from kinetochores. Related to Figure 6.

(S4-S6) Spinning disk time lapse microscopy of PLK1-eGFP KI HeLa cells synchronized in mitosis with DTBR and transfected with control **(S5)** or UBAP2L siRNA **(S6)** and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z-stacks (12µm range, 0,5µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S5.

Time lapse of PLK1-eGFP KI HeLa cells, related to Figure 6. Spinning disk time lapse microscopy of PLK1-eGFP KI HeLa cells synchronized in mitosis with DTBR, transfected with control siRNA (siNT) and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (12µm range, 0,5µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

Video S6.

Time lapse of PLK1-eGFP KI HeLa cells, related to Figure 6. Spinning disk time lapse microscopy of PLK1-eGFP KI HeLa cells synchronized in mitosis with DTBR, transfected with UBAP2L siRNA and analyzed by spinning disk live-video microscopy for 8 hours. SiR-DNA was used to stain DNA. Z stacks (12µm range, 0,5µm step) were acquired every 10 minutes and maximum intensity projection images are shown at speed 7 frames per second.

II. UNPUBLISHED RESULTS: PHYSIOLOGICAL CONSEQUENCES OF UBAP2L DEPLETION

The results presented in the second part of the thesis document were not included in the previously presented manuscript. Nevertheless, these results may create a basis for potential future projects and can be of interest for the scientific community.

NOTE: Some of the following experiments do not allow to perform statistical tests due to their preliminary character. More generally, all data described in this section should be considered as preliminary.

A. UBAP2L might regulate the nucleo-cytoplasmic transport of proteins

Recently, UBAP2L emerged as a candidate in a genome-wide CRISPRi screen in mammalian cells for new factors with potential role in protein transport (Bassaganyas et al., 2019). In fact, I noticed that UBAP2L can shuttle between the cytoplasm and the nucleus (**Fig. 10A**) which is not surprising considering that several Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES) have been predicted within UBAP2L sequence (Guerber et al., 2022). In particular, there seems to be a NES located in the C-terminal part of UBAP2L since abolishment of export using the Chromosomal Region Maintenance 1 (CRM1) inhibitor Leptomycin B (LMB) leads to accumulation of the C-term but not of the N-term UBAP2L protein fragment in the nucleus (**Fig. 10A**). More precisely, the NES may be located within the UBAP2L DUF domain as confirmed by IF (**Fig. 10B**). If and how this NES is important for mediating protein export will need further investigation.

Next, given my hypothesis that UBAP2L might regulate the interaction between PLK1 and CUL3 via a yet unknown mechanism, I decided to check if the lack of interaction could be the indirect effect of deficient protein transport. In fact, subcellular fractionation experiments revealed that CUL3 nuclear protein levels are reduced in UBAP2L KO cells (**Fig. 10C**), a result which was confirmed by the ectopic expression of flag-tagged CUL3 in UBAP2L WT and KO cells that showed mild decrease of nuclear CUL3 (**Fig. 10D**), suggesting that UBAP2L might play a role in CUL3 nuclear translocation. However, these experiments will need to be repeated in the future using LMB treatment and different UBAP2L NES mutants in rescue experiments to firmly establish a role for UBAP2L in CUL3 nucleo-cytoplasmic transport.

Figure 10

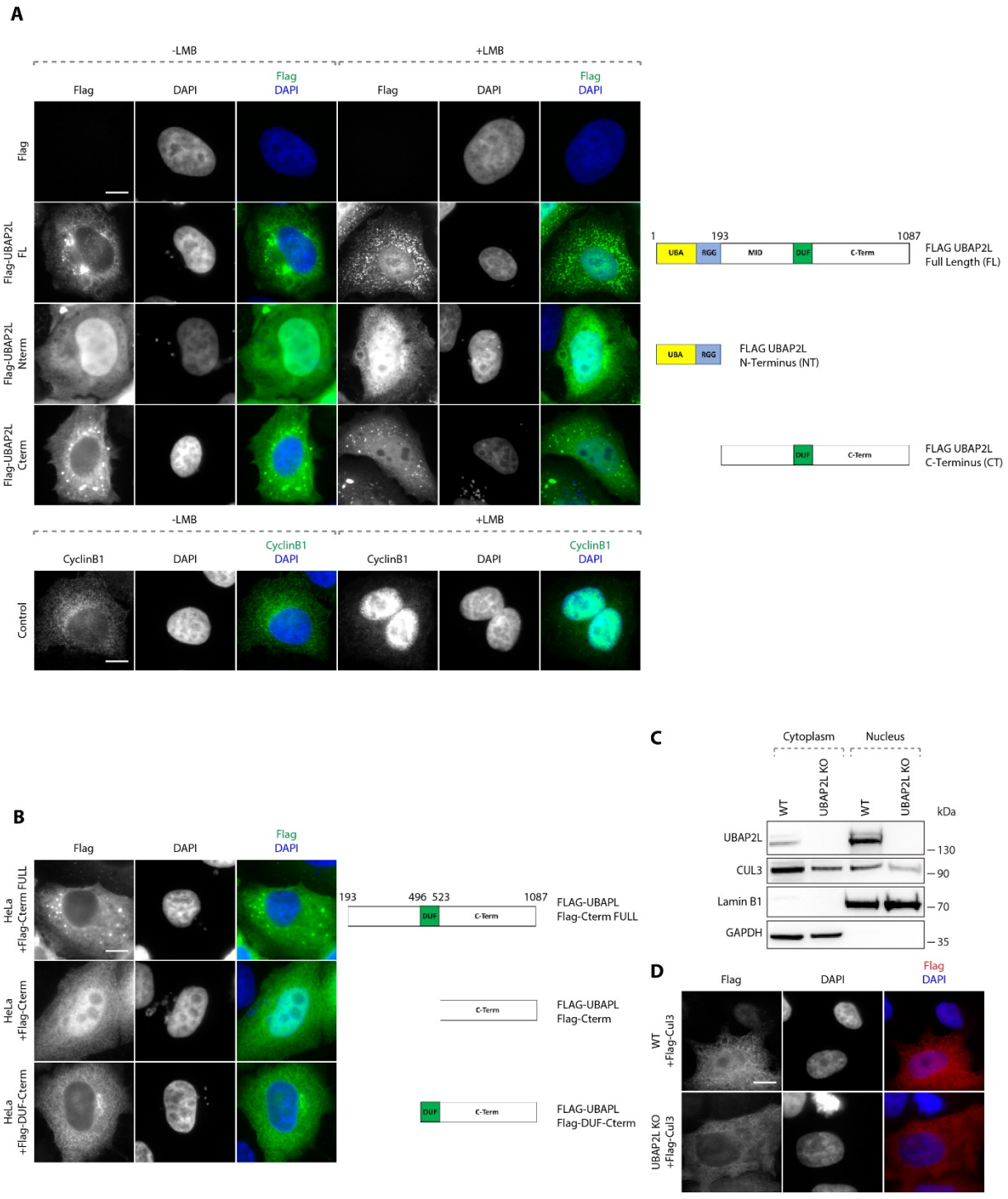

Fig. 10. UBAP2L might regulate CUL3 subcellular localization

(A) Representative IF pictures of HeLa cells transfected with the indicated UBAP2L flag-tagged constructs and treated with vehicle or 10ng/mL LMB for 4h. The schematic structures of the used constructs are represented facing the corresponding images. Scale bars, 8µm. Cyclin B1 staining has been used here as a control to verify the efficacy of LMB treatment.

(B) Representative IF pictures of HeLa cells transected with the indicated flag-tagged UBAP2L fragments. The schematic structures of the used constructs are represented facing the corresponding images. Scale bars, 8μm.

(C) WB analysis of WT or UBAP2L KO cell lysates fractionated into cytoplasmic and nuclear fractions. Proteins MW is indicated in kDa. WB is representative of two independent replicates.

(D) Representative IF picture of WT or UBAP2L KO cells transfected with flag-tagged CUL3. Scale bar, 8μm.

B. UBAP2L depletion enhances basal DNA damage levels

As described in our manuscript, UBAP2L-depleted cells which manage to complete cell division, exit mitosis with severe genomic aberrations resulting in MN formation in the next interphase. MN have long been associated with genomic instability as the result of segregation defects and they have recently been shown to display excessive chromosomal rearrangements termed chromothripsis, ultimately having a central role in tumorigenesis as reviewed by Krupina and colleagues (2021). Newly formed MN generally undergo deficient DNA replication leading to DSBs (Crasta et al., 2012). This led me to investigate if UBAP2L-depleted cells also accumulate extensive DNA damage. To this end, I examined γH2AX levels, a marker of DNA DSBs in UBAP2L KO cells (Kuo and Yang, 2008). Interestingly, apart from the increased number of cells with MN (**Fig. 11D-E**) and nuclear atypia as assessed by the nuclear form factor (**Fig. 11F**), two different UBAP2L KO clones displayed strong nuclear accumulation of γH2AX as visualized both by IF and WB relative to WT cells (**Fig. 11A-C**). I thus asked if this phenotype is solely occurring in G1 after numerous segregation errors can be observed but I found that γH2AX levels are higher in UBAP2L KO cells relative to WT during the whole cell cycle with the most vigorous differences occurring during G1 and G2 exactly after the key cell cycle steps of cell division (M) and DNA replication (S) (**Fig. 11G**), possibly due to numerous defects during these processes. In order to exclude the possibility that the observed γH2AX accumulation is an unspecific effect of UBAP2L KO generation in HeLa cells, I repeated the experiment in UBAP2L-downregulated U2OS and DLD-1 cells. Consistently, similar phenotypes were observed in UBAP2L-downregulated HeLa and UBAP2L KO U2OS cells (**data not shown**). Importantly, accumulation of cells with MN as shown in our manuscript (**Fig. S1D-G**), the abnormal nuclear shape (**Fig. 12D and D'**) and

γH2AX accumulation as quantified by measuring γH2AX nuclear intensity (**Fig. 12A-C and A'-C'**) were observed in all UBAP2L-depleted cell lines compared to control cells.

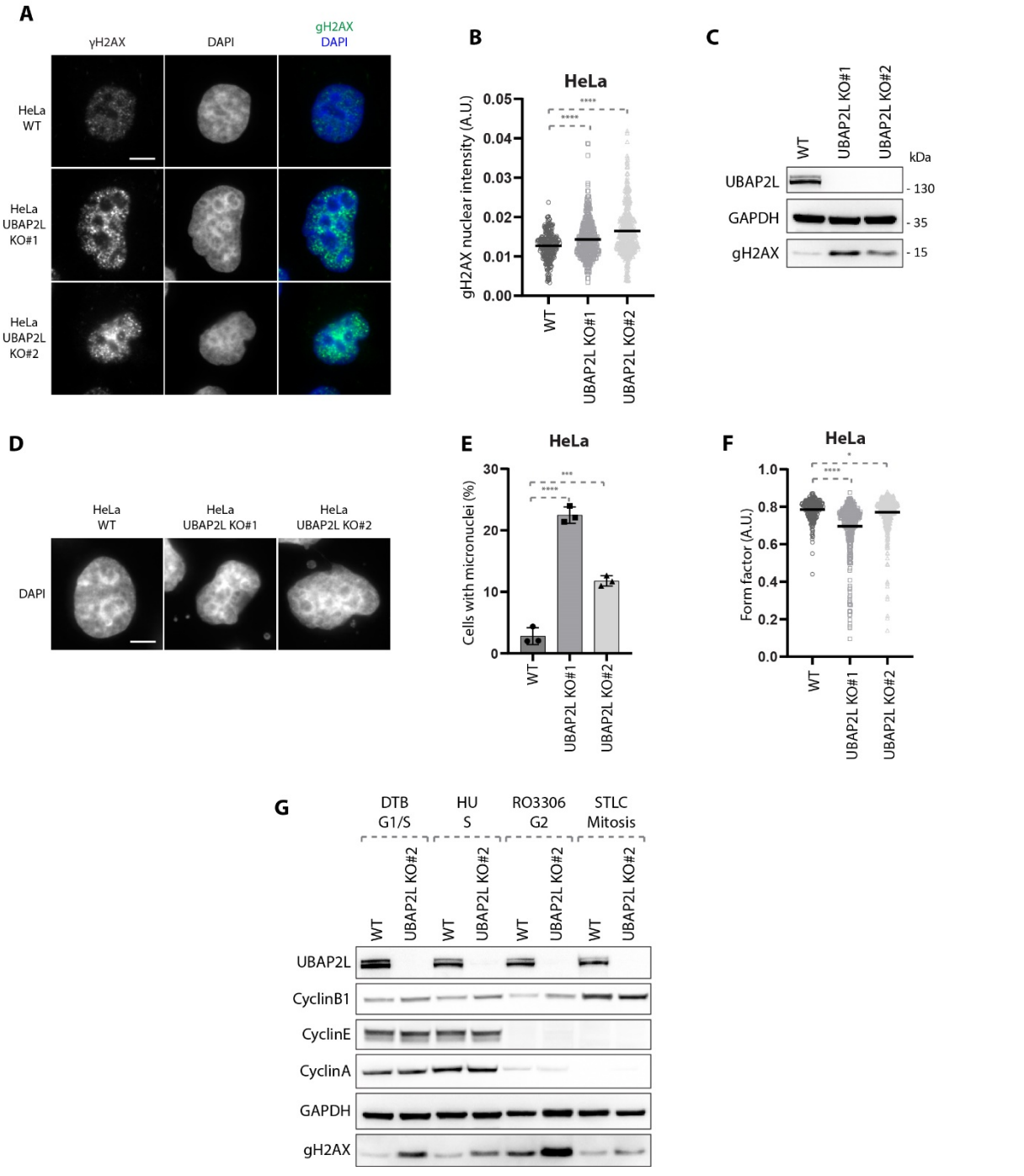

Fig. 11. Genomic stability profile of UBAP2L KO cells

(A-B) Representative IF pictures of unsynchronized WT or UBAP2L KO HeLa cells **(A)** and quantification of γH2AX nuclear intensity **(B)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Each dot of graphs **(B)** represents γH2AX nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) ****P<0,0001.

(C) WB analysis of unsynchronized WT or UBAP2L KO cell lysates. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) Representative IF images of unsynchronized WT or UBAP2L KO HeLa cells **(D)** and quantification of the percentage of cells with MN **(E)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Graphs represent the mean of three replicates ± SD (one-way ANOVA with Dunnett's correction ***P<0,001, ****P<0,0001).

(F) Quantification of the form factor of WT or UBAP2L KO nuclei. Each dot represents the form factor of a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) *P<0,05, ****P<0,0001.

(**G**) WB analysis of WT or UBAP2L KO cells synchronized in G1 (DTB), S (HU), G2 (RO3306) or mitosis (STLC). Proteins MW is indicated in kDa. WB is representative of three independent replicates.

The increased levels of γH2AX in UBAP2L-depleted cells could be the result of DNA repair defects or of increased endogenous damage. To distinguish between the two possibilities, I treated cells with the chemical neocarzinostatin (NCS) in order to induce DNA DSBs and analyzed γH2AX levels. I assume that in the absence of repair defects, induced DNA breaks should be repaired as efficiently in WT as in UBAP2L KO cells. NCS efficiently induced DSBs in both control and UBAP2L-depleted U2OS and in DLD-1 cells but I could no longer detect a difference in γH2AX levels between the two cell lines (**Fig. 12A-C and A'-C'**), suggesting that UBAP2L depletion likely promotes an internal source of damage rather than impairing the DNA repair machinery. This prompted me to investigate if, in a similar manner to what I observed for chromosome segregation defects (**Fig. 9E-H**), PLK1 depletion or inhibition could reverse γH2AX accumulation in UBAP2L KO cells. However, unlike mitotic abnormalities, PLK1 silencing (**Fig. 13A-C**) or inhibition (**Fig. 13D-F**) not only did not rescue γH2AX accumulation in UBAP2L KO cells but also showed a tendency to worsen the phenotype. Altogether, my data suggest that UBAP2L depletion causes DNA DSBs accumulation in a PLK1-independent manner, which might arise from an internal source of damage.

Figure 12

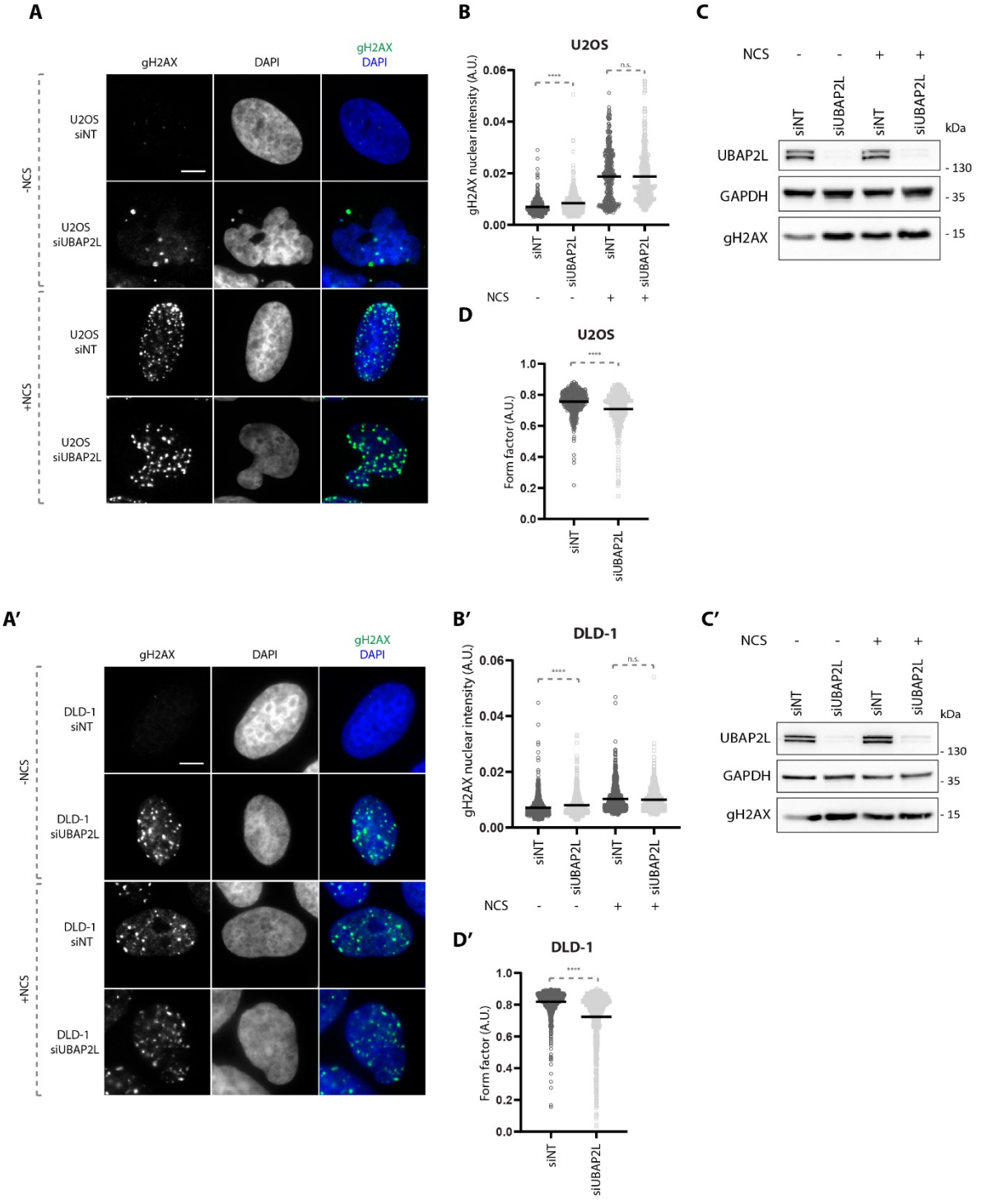

Fig. 12. Genomic stability profile of UBAP2L-downregulated U2OS and DLD-1 cells

(A-B) Representative IF pictures of unsynchronized U2OS cells transfected with the indicated siRNAs and treated with MES buffer or NCS **(A)** and quantification of γH2AX nuclear intensity **(B)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Each dot of graphs **(B)** represents γH2AX nuclear intensity in a single nucleus. The measurements

of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) ****P<0,0001, ns=non-significant.

(C) WB analysis of unsynchronized U2OS cells transfected with the indicated siRNAs. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D) Quantification of the form factor of control or siUBAP2L U2OS nuclei. Each dot represents the form factor of a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Mann-Whitney test) *P<0,05, ****P<0,0001.

(A'-B') Representative IF pictures of unsynchronized DLD-1 cells transfected with the indicated siRNAs and treated with MES buffer or NCS **(A')** and quantification of γH2AX nuclear intensity **(B')**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Each dot of graphs **(B')** represents γH2AX nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) ****P<0,0001, ns=non-significant.

(C') WB analysis of unsynchronized DLD-1 cells transfected with the indicated siRNAs. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D') Quantification of the form factor of control or siUBAP2L DLD-1 nuclei. Each dot represents the form factor of a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Mann-Whitney test) *P<0,05, ****P<0,0001.

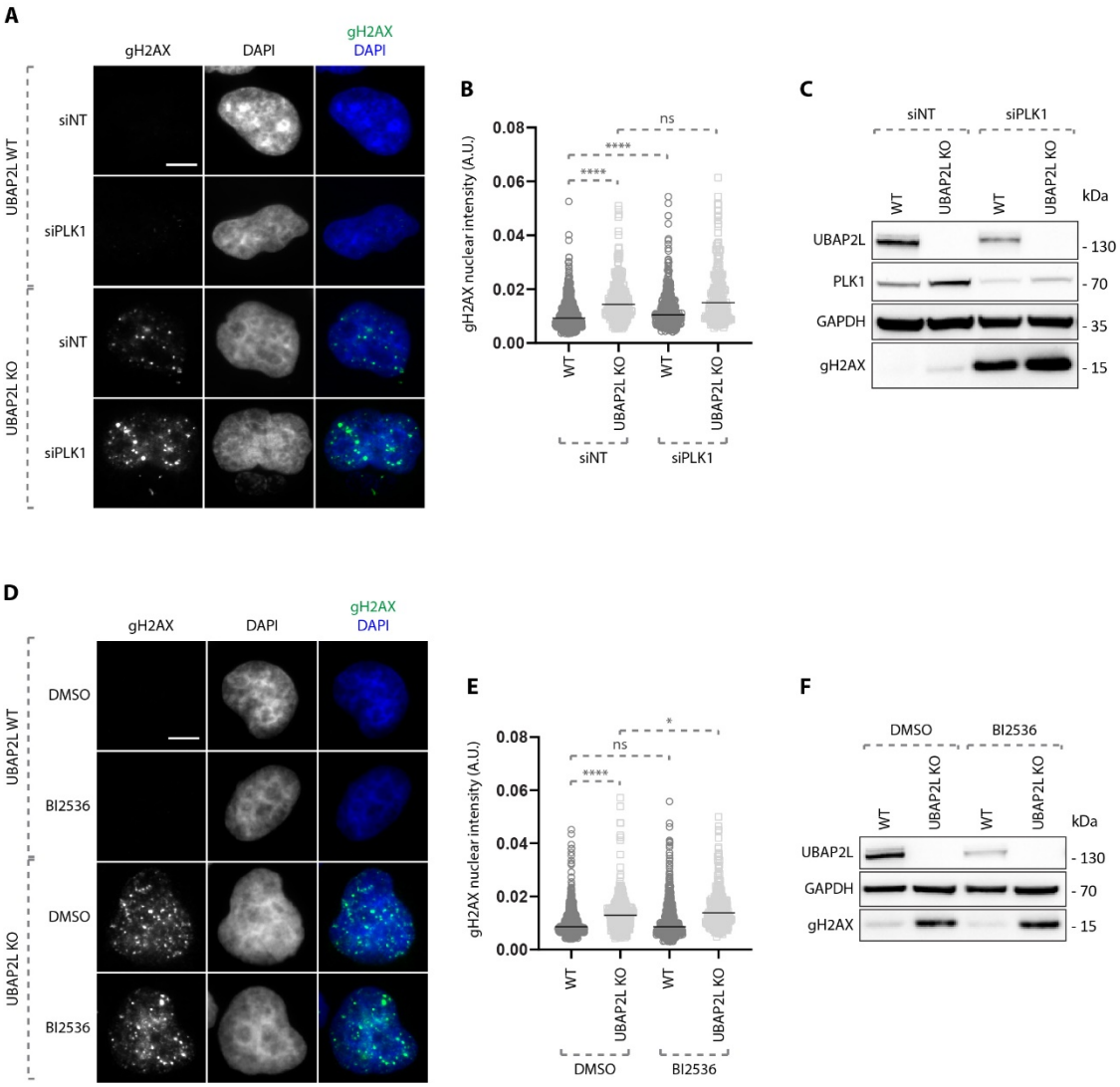

Fig. 13. UBAP2L regulates γH2AX in a PLK1-independent manner

(A-B) Representative IF pictures of unsynchronized WT or UBAP2L KO HeLa cells transfected with the indicated siRNAs **(A)** and quantification of γH2AX nuclear intensity **(B)**. Scale bar, 5µm. At least 250 cells were quantified per condition for each replicate. Each dot of graphs **(B)** represents γH2AX nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) ****P<0,0001, ns=non-significant.

(C) WB analysis of unsynchronized WT or UBAP2L KO cells transfected with the indicated siRNAs. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) Representative IF pictures of unsynchronized WT or UBAP2L KO HeLa cells treated with DMSO or 50nM BI2536 **(D)** and quantification of γH2AX nuclear intensity **(E)**. Scale bar,

5µm. At least 250 cells were quantified per condition for each replicate. Each dot of graphs **(E)** represents γH2AX nuclear intensity in a single nucleus. The measurements of three biological replicates are combined, black bars represent the mean (Kruskal-Wallis test with Dunn's correction) *P<0,05, ****P<0,0001, ns=non-significant.

(F) WB analysis of unsynchronized WT or UBAP2L KO cells treated with DMSO or 50nM BI2536. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

C. UBAP2L inhibits autophagy

In UBAP2L-depleted cells, MN and DNA damage accumulate while PLK1 displays kinetochore localization enrichment, increased enzymatic activity and increased protein stability. In cancer cells, MN have been shown to be cleared out by autophagy, which acts as a tumor-suppressor mechanism to eliminate pre-cancerous cells displaying high genomic instability and cell cycle checkpoints perturbations, thus resulting in extensive cell death (Rello-Varona et al., 2012; Nassour et al., 2019). On the other hand, PLK1 is believed to activate the autophagic-lysosomal pathway by interacting with and phosphorylating the mTORC1 component RAPTOR, thereby inhibiting mTORC1 lysosomal association and subsequently abrogating mTORC1-dependent inhibition of autophagy (Ruf et al., 2017). For these reasons, I aimed at studying the autophagy signaling in UBAP2L-depleted cells.

To this end, I first performed IF experiment, staining for the autophagy receptor Sequestosome 1 (SQSTM1, more broadly known as P62), which links cargos to the autophagic machinery targeting their specific degradation (Gubas and Dikic, 2022). However, P62 is also involved in multiple cellular processes such as inflammation and oxidative defense system among others (Sánchez-Martín et al., 2019). For this reason, I performed a co-staining with Microtubule-associated protein 1A/1B-light chain 3 (LC3), a crucial component of autophagic structures such as autophagosomes and autolysosomes (Tanida et al., 2008). Interestingly, both UBAP2L KO clones displayed an enrichment for P62/LC3 containing granules, suggesting that autophagy flux might be perturbed in these cells (**Fig. 14A**). This prompted me to investigate which step of autophagy is disrupted upon UBAP2L depletion. Recent work from Wang and colleagues proposed that UBAP2L regulates mTORC1 activity specifically through binding to mTOR and RAPTOR but not Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), a subunit of mTORC2 (Wang et al., 2021). Given that mTORC1 is known to be maintained inactive through the inhibitory phosphorylation of Unc-51–like kinase

1 (ULK1) at Serine 757 residue by mTOR (Kim et al., 2011), I decided to verify if UBAP2L regulates mTORC1 activity by inhibiting this phosphorylation event which, in turn could lead to the aberrant induction of autophagy. Importantly, UBAP2L depletion resulted in a mild decrease of ULK1 phosphorylation (S757) but not of ULK1 total protein levels (**Fig. 14C**), which could only partially explain the strong autophagy defects observed in UBAP2L KO cells and suggesting an additional layer of autophagy regulation in these cells. To gain further insights, I used Bafilomycin A1 (BafA1) and Torin1 to inhibit and induce autophagy respectively in WT and UBAP2L KO cells. BafA1 blocks the terminal step of autophagy by inhibiting autophagosome-lysosome fusion and autolysosome acidification (Huss and Wieczorek, 2009) whereas Torin1 is a specific ATP-competitive mTOR inhibitor thereby indirectly inducing autophagy (Liu et al., 2011). Interestingly, BafA1 led to the accumulation of autophagy vesicles as expected in WT as well as in both UBAP2L KO clones. However, it did not further increase aberrant levels of P62 observed upon UBAP2L depletion (**Fig. 14B**). Similarly, Torin1 efficiently targeted autophagy induction but did not lead to reduction in LC3 protein levels compared to WT levels (**Fig. 14B**). Increased LC3 protein levels observed by IF and WB under both normal conditions and upon induction with BafA1 (**Fig. 14A-B**) suggest increased autophagy flux in UBAP2L KO cells relative to WT cells as described by Yoshii and Mizushima (2017). However, the fact that Torin1 does not completely rescue the accumulation of autophagic granules in UBAP2L KO cells prompted me to investigate if later autophagy steps could be affected in these cells. Intriguingly, UBAP2L-depleted cells displayed similar phenotypes as those observed upon BafA1 treatment, pointing to potential autophagosome-lysosome fusion defects. Hence, I transfected WT and UBAP2L KO cells with a mRFP-GFP-LC3 tandem fluorescent probe which is a sensitive indicator of acidity levels. More specifically, GFP fluorescence is quenched when the pH acidifies, namely after autophagosome-lysosome fusion (Kimura et al., 2007). Strikingly, while WT cells undergo normal fusion under physiological conditions and potent autophagy activation by Torin1 but not upon BafA1, as expected, both UBAP2L KO clones displayed equally enriched RFP/GFP colocalization upon all three treatments, suggesting that these cells indeed exhibit autophagosome-lysosome fusion defects (**Fig. 14D-E**).

Overall, my data suggest that UBAP2L depletion does not regulate a single step of autophagy but rather multiple events such as initiation and autophagosome-lysosome fusion, highlighting the crucial involvement of UBAP2L in distinct and multiple cellular processes and the need to further investigate this oncogene in the future.

Figure 14

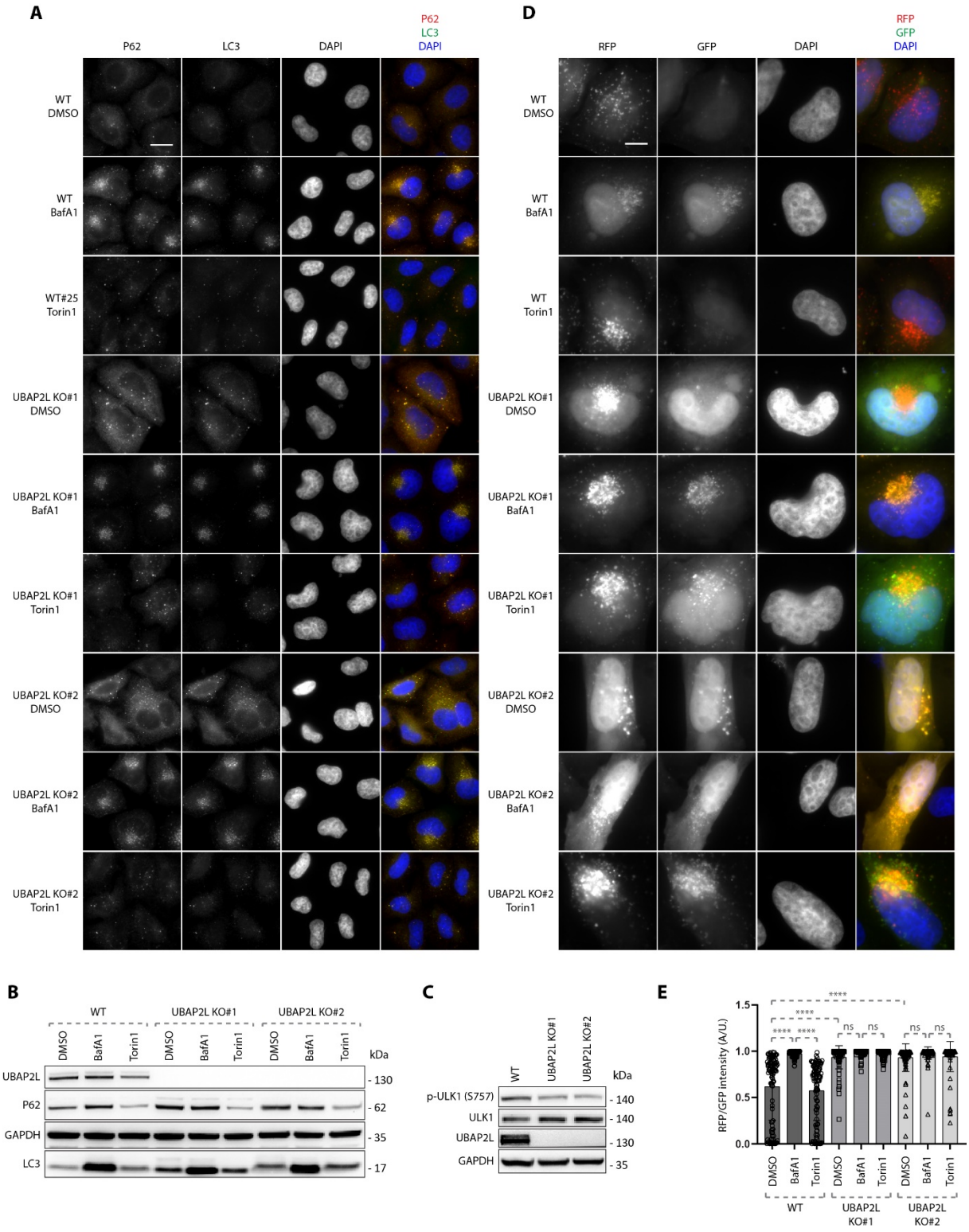

Fig. 14. UBAP2L-depleted cells display strong autophagy defects

(A) Representative IF images of WT or UBAP2L KO cells treated with DMSO, 50nM BafA1 or 250nM Torin1 for 4h. Scale bar, 8µm.

(B) WB analysis of WT or UBAP2L KO cells treated with DMSO, 50nM BafA1 or 250nM Torin1 for 4h. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(C) WB analysis of WT or UBAP2L KO cell lysates. Proteins MW is indicated in kDa. WB is representative of three independent replicates.

(D-E) Representative IF pictures of unsynchronized WT or UBAP2L KO cells treated with DMSO, 50nM BafA1 or 250nM Torin1 **(D)** and quantification of RFP/GFP colocalization **(E)**. Scale bar, 5µm. At least 50 cells were quantified per condition for each replicate. Each dot of graphs **(E)** represents RFP/GFP intensity (A.U.) in a single cell. The graphs represent the mean ± SD. The measurements of three biological replicates are combined (Kruskal-Wallis test with Dunn's correction) ****P<0,0001, ns=non-significant.

DISCUSSION

During my PhD I attempted to dissect the functions of UBAP2L protein in multiple cellular processes. First, our manuscript provides direct evidence that UBAP2L regulates mitotic progression by controlling PLK1 localization and stability in a cell cycle-dependent manner. We show that UBAP2L is recruited to KT from prometaphase to metaphase in a PLK1-dependent manner and finetunes the proper removal of PLK1 from these structures at a precise time possibly by regulating PLK1/CUL3 interaction and subsequent PLK1 ubiquitylation. Importantly, the timely dissociation of PLK1 by UBAP2L ensures faithful chromosome segregation. In fact, cells depleted from UBAP2L display severe mitotic delay and segregation errors driving the formation of MN in the following interphase. Of note, all chromosomal abnormalities that characterize UBAP2L-depleted cells could be directly linked to aberrant PLK1 enzymatic activity induced upon loss of UBAP2L. We further demonstrate that the genomic instability that characterizes UBAP2L KO or downregulated cells causes long-term proliferation and cell survival defects. PLK1 localization and cell survival are directly controlled by UBAP2L C-terminal domain, as overexpression of this domain could fully rescue both PLK1 KT accumulation and cell death and partially rescue proliferation defects observed in UBAP2L KO cells.

A. How is UBAP2L recruited to kinetochores?

Intriguingly, UBAP2L specifically regulates PLK1 but not other mitotic kinases nor other PLK family members in terms of protein levels or localization (**Fig. 2 and S2**). How exactly this specificity is conferred remains a mystery but several hypotheses have emerged from our data. On one hand UBAP2L has been suggested to be phosphorylated during mitosis (Dephoure et al., 2008; Maeda et al., 2016). However, the kinase responsible for this phosphorylation has not yet been identified. Our data suggest that UBAP2L recruitment to KT is PLK1 dependent (**Fig. 6A-B and D-E**). Given that UBAP2L harbors several predicted PLK1 and CDK1 consensus motifs in its CT part, we could speculate that UBAP2L is phosphorylated by one or both kinases to ensure its proper mitotic localization and functions. PLK1 recruitment to KT has been shown to be mainly mediated through CDK1-dependent priming phosphorylation on BUB1 and CENP-U, which is a pre-requisite signaling for PLK1 to positively regulate its localization and activity (Singh et al., 2021). It is therefore crucial to gain further insights into this putative regulatory loop in the future by performing *in vitro* kinase assays as well as by investigating UBAP2L interactions with other kinetochore proteins and mitotic kinases.

We showed that endogenous UBAP2L specifically interacts with PLK1 but not with AURB (**Fig. 9A**), however if and how UBAP2L regulates additional KT-associated proteins is still unclear. It would be fascinating to explore if UBAP2L could have similar functions to testis expressed protein 14 (Tex14), a PLK1-regulated protein ensuring proper MT-KT attachment and SAC components KT recruitment. Interestingly, Tex14 is localized to KT by PLK1 in a CDK1-dependent manner to drive recruitment of the outer KT machinery during early mitosis and its impaired localization drives chromosome aberrations highly reminiscent to those observed upon UBAP2L depletion (Mondal et al., 2012). Similarly, the DUB USP16 is recruited to kinetochore through both CDK1 priming and PLK1 phosphorylation, triggering PLK1 deubiquitylation and leading to its enhanced recruitment and maintenance at KT (Zhuo et al., 2015). If and how PLK1 could promote the recruitment of UBAP2L to KTs in a similar manner, in order to finetune its own localization and activity remains to be determined. UBAP2L is a very abundant protein whose major pool is cytoplasmic. Our observation that a small fraction of UBAP2L is able to localize to KT and that its depletion can induce strong phenotypes during mitosis, makes it appealing to investigate a more general role of UBAP2L in the recruitment of additional KT components as means to ensure fidelity of cell division.

B. What is the role of UBAP2L in PLK1/CUL3 pathway?

Our data suggest an exciting dual role of UBAP2L in regulating both PLK1 localization and stability. In fact, upon translation inhibition, PLK1 is much more stable in UBAP2L-depleted cells relative to controls (**Fig. S3A-B**). This suggests that UBAP2L may control both proteolytic and non-proteolytic signals on PLK1. More specifically, we show that PLK1 interaction with CUL3 is decreased upon UBAP2L depletion (**Fig. 9B**), favoring a role for UBAP2L in CUL3-dependent recognition of PLK1 to promote its KT removal which could partially explain the observed phenotypes. However, further effort is needed to dissect the precise molecular mechanisms involved in the regulation of this pathway. Does UBAP2L mediate PLK1/CUL3 interaction directly? Does UBAP2L enable PLK1 recognition by CUL3 or does it drive the assembly of CUL3 complexes during mitosis? Could UBAP2L be a general co-adaptor of CUL3 complexes? These are important questions which will need to be addressed in the future. PLK1 has been shown to be recruited to and maintained at KTs by the DUB USP16, that counteracts the actions of CUL3. In fact, USP16-dependent deubiquitylation of PLK1 increases its interaction with KT receptors such as BUBR1, retaining PLK1 at these structures until proper MT-KT is achieved (Zhuo et al., 2015). We could thus speculate that UBAP2L depletion may promote the recognition of PLK1 by USP16, subsequently triggering its recruitment to

KTs. Furthermore, proteomics studies suggest that UBAP2L interacts with Cullin3-based E3 ligase but not with other CRLs (Bennett et al., 2010). It would therefore be interesting to investigate if UBAP2L interacts with other CUL3 complexes thus regulating their mitotic functions. Our data suggest that other known CUL3 mitotic substrates such as AURA and AURB are not regulated by UBAP2L, arguing for a specificity towards PLK1/CUL3/KLHL22 complex (**Fig. 2 and S2**), however we cannot exclude that UBAP2L might be implicated in the recognition of yet unidentified CUL3 targets to ensure proper mitotic progression. Another possibility would be that UBAP2L could regulate CRL3 macromolecular complexes assembly by specifying which KLHL adaptor has to bind to Cullin3 at specific time and space. In fact, very preliminary results indicate that UBAP2L might interact with KLHL21 (**data not shown**), the CRL3 co-adaptor responsible for AURB localization during mitosis. However, this experiment has to be repeated in order to confirm this result which drew our attention as UBAP2L does not interact with AURB. Could UBAP2L localization influence which substrate specifier to attach to CUL3? This exciting hypothesis will need further efforts to be affirmed.

Additionally, it is well established that a large variety of ubiquitin chains can be attached to proteins and that each type of Ub chain is involved in various cellular processes (Liao et al., 2022). If and how UBAP2L can recognize and bind different kinds of Ub chains remains unknown and would be an important direction to follow in order to uncover the role of UBAP2L in decoding diverse Ub signals. Ubiquitin chains adopt different structures ranging from quite flexible (K63 chains) to more compact (K48 chains) conformations (Winget and Mayor, 2010), regulating their specific recognition and binding by key DUBs and UBPs and determining the activity of these interacting partners (Ye et al., 2012). It would thereby be important to study which chain types can be specifically recognized by UBAP2L and if both its UBA domain as well as its highly disorganized sequences can contribute to the flexible recognition of various Ub chain conformations. Additionally, as mentioned in the introduction, Ub can itself be modified by Ub-like molecules (SUMO, NEDD8) and other PTMs (Komander and Rape, 2012; Kwon and Ciechanover, 2017; Mulder et al., 2020). An interesting perspective would be to study UBAP2L recognition and binding affinity to these modified Ub. Recently, significant advances in the Ubiquitin field resulted in the invention of an inducible and linkage-selective ubiquitylation tool called Ubiquiton which allows the *de novo* addition of polyubiquitin chains to proteins of interest *in vitro* and in cells (Wegmann et al., 2022). It would therefore be fascinating to apply this new method to PLK1 and study if UBAP2L can bind ubiquitinated PLK1 and if yes if it has some preferences for specific Ub chains types.

C. How does UBAP2L C-terminal domain exert its functions?

Intriguingly, the C-terminal fragment but not the UBA-RGG fragment of UBAP2L specifically regulates PLK1 kinetochore localization (**Fig. 3A-D**). This result was unexpected because while UBA and RGG domains have been well characterized for their ability to bind Ubiquitin and RNAs respectively, much less is known about the CT domain. It harbors a DUF domain which has been proved to play a role in stress signaling by regulating UBAP2L interaction with the core components of SGs such as G3BPs (Huang et al., 2020). However, in our hands, these interactions do not seem to be important for mediating its function in PLK1 regulation (**Fig. 4**). Except this available study, no evident function for UBAP2L CT part has been described. A large portion of the CT fragment is predicted to be intrinsically disordered and no structure is available to date. Interestingly, it is excluded from the nucleus and several NES were predicted among which one is located within the DUF domain as confirmed by IF in my hands (**Fig. 10A-B**). On the contrary, the UBA-RGG fragment is enriched in the nucleus, fitting with the presence of a predicted NLS (Guerber et al., 2022). This suggests that UBAP2L is a very dynamic protein, shuttling between the nucleus and the cytoplasm. It is very intriguing that the fragment responsible for PLK1 KT localization regulation is the CT as it is the one excluded from the nucleus. This raises the question whether UBAP2L CT could mediate the transport and, more specifically the export of key proteins responsible for PLK1 KT removal prior to mitotic entry. In fact, preliminary results indicate that CUL3 nuclear localization seems to be decreased upon UBAP2L depletion (**Fig. 10C-D**) but further quantitative analyses are needed in order to confirm this observation. Moreover, CUL3 total protein levels seem to be decreased in UBAP2L-depleted cells (**Fig. 10C**) which likewise requires further investigation. In fact, given that UBAP2L is both an RNA- and Ubiquitin-binding protein, extensive analysis is required to elucidate if UBAP2L could regulate CUL3 on a mRNA and/or protein level. Further optimization of IF protocols allowed us to detect UBAP2L CT fragment aggregating both in the cytoplasm and at KTs (**Fig. 7C**), suggesting that, at least during mitosis, this fragment can localize to nuclear structures. At which precise moment it is translocating to the cytoplasm during interphase and how will need further clarifications in the future.

Interestingly, recent genome-wide CRISPRi screen found UBAP2L as a new factor mediating protein transport and more specifically transport through the ER-Golgi membranes (Bassaganyas et al., 2019). Besides their role in the secretory pathways, both organelles have crucial roles in protein translation and folding, as PTMs factories and trafficking hubs to target proteins to their final destination (Vitale et al., 1993; Banfield, 2011). In parallel, UBAP2L has

been proposed to regulate the translation of target mRNAs where it is suggested to act as a ribosome-binding protein (Luo et al., 2020). Hence, it would be fascinating to study if and how exactly UBAP2L regulates the temporal translation of specific mRNAs such as CUL3 mRNA. Some preliminary data from our lab indeed suggest that PLK1 mRNA is upregulated upon UBAP2L depletion (**data not shown**), a result that requires further confirmation by performing for example Fluorescence *in situ* hybridization (FISH) with PLK1 and CUL3 mRNA specific probes, which would allow us to monitor the expression and localization pattern of PLK1 and CUL3 mRNA during mitosis in the absence of UBAP2L.

D. How does UBAP2L regulate PLK1 degradation?

Additionally, we show that UBAP2L depletion triggers a decrease of PLK1 polyubiquitylation implying that PLK1 degradation might be perturbed in the absence of UBAP2L (**Fig. 9C**). PLK1 is gradually degraded from anaphase to mitotic exit through APC/C^{CDH1}-mediated ubiquitylation and subsequent targeting to the proteasome (Lindon and Pines, 2004). As previously introduced, the switch from CDH1 to CDC20 and to CDH1 again is essential for the sequential degradation of APC/C substrates and faithful mitotic progression and exit (Sivakumar and Gorbsky, 2015). Interestingly, CDH1 has been proposed to be phosphorylated by PLK1, thereby preventing its association with APC/C, favoring its ubiquitylation by $SCF^{\beta\text{-}TRCP}$ and subsequent degradation (Fukushima et al., 2013). Based on these findings, it is reasonable to hypothesize that accumulation of PLK1 protein and activity observed in UBAP2L-depleted cells could therefore inhibit its own degradation by hyperphosphorylating its substrate CDH1. In line with this assumption, APC/C^{CDH1} assembly blockade could partially explain the mitotic delay characterizing UBAP2L KO cells. Alternatively, APC/C^{CDH1} association could be affected by CDK1 enzymatic activity. Given that we did not observe any differences in Cyclin B1 levels nor localization in UBAP2L-depleted cells relative to control (**Fig. 2A, 2E and S2**), we do not expect any defects in lowering CDK1 activity during anaphase. However, we never formally assessed CDK1 activity in UBAP2L KO cells which is a crucial experiment to perform in order to explore UBAP2L-dependent PLK1 stability regulation. We can also not exclude the possibility that other E3 ligases play important roles in PLK1 degradation process or that CUL3, in spite of its major role in non-proteolytic events, might exert proteolytic activity by associating with yet unidentified adaptors or inhibitors as it has recently been shown for KLHL12- and Lunapark (LNP)-dependent regulation of CUL3 (Akopian et al., 2022). Importantly, UBAP2L has been proposed to interact with the WW domain-containing E3 ligases NEDD4-1 and Atrophin-1-interacting Protein 4 (AIP4) through

its PY motifs for which no mitotic functions have been reported to our knowledge (Ingham et al., 2005). Emerging evidence suggests important crosstalk between Cullin-based E3 ligases and APC/C machinery to orchestrate mitotic progression (Vodermaier, 2004; Watson et al., 2019). It is thus tempting to speculate that UBAP2L could be an important linker of these signaling pathways. As UBAP2L contains a UBA domain, it would be worth assessing if PLK1 stability is regulated by the UBA-RGG domain of UBAP2L, highlighting its dual and independent roles in PLK1 regulation. For instance, similar experiment as in Fig. 9C could be performed in a rescue context using UBAP2L different constructs to check PLK1 poly-ubiquitylation status. Similarly, CDH1 phosphorylation levels could be analyzed under the same conditions to confirm a potential hyperphosphorylation hindering its association with APC/C to achieve PLK1 degradation.

E. Does UBAP2L play a role during DNA replication and DNA damage signaling?

In our manuscript, we described a novel function for UBAP2L in safeguarding genomic stability. Indeed, cells depleted for UBAP2L display strong chromosomal abnormalities such as multipolar spindles as suggested by the formation of multiple axes during metaphase, lagging chromosomes and DNA bridges (**Fig. 1 and 9E-H**) which are considered as hallmarks of CIN. Although the most common cause of lagging chromosomes and DNA bridges are merotelic MT-KT attachments (Gregan et al., 2011), mild replication stress has been also shown to cause similar phenotypes as well as multiple mitotic spindle poles (Naim and Rosselli, 2009; Wilhelm et al., 2014), suggesting that UBAP2L depletion could generate replication stress. In line with this hypothesis, I demonstrated that UBAP2L KO or downregulated cells strongly accumulate DNA DSBs compared to WT cells as assessed by monitoring γH2AX signals by IF and WB (**Fig. 11A-C and G**), a phenotype that was no longer observed upon chemical DSB induction using the drug NCS (**Fig. 12**). This implies that UBAP2L KO cells suffer from an intrinsic, metabolic source of damage rather than DNA repair defects. Replication stress is one of the major endogenous sources of damage and is the result of several cellular stresses occurring during replication such as abnormal replication forks formation, exposition of single-stranded DNA or chromatin inaccessibility (Zeman and Cimprich, 2014). It is important that these problems are rapidly repaired because persistence of stalled forks leads to their breakage. ATM and ATR kinases are crucial to relieve cells from replication stress (Marechal and Zou, 2013). Intriguingly, both UBAP2L and its homolog UBAP2 have been identified as ATM/ATR phosphorylation targets following DNA damage induction by ionizing radiation (IR) and UV (Matsuoka et al., 2007). Moreover, UBAP2 was demonstrated to be recruited at broken

replication forks to promote their error-free DSBs repair as its absence led to aberrant γH2AX accumulation and impaired HR repair (Nakamura et al., 2021). Albeit UBAP2L and UBAP2 only sharing 42% of identity, these proteins seem to exert redundant roles and compensate for each other. In fact, UBAP2L depletion leads to a marked increase of UBAP2 protein levels and both proteins redundantly regulate RNAPII ubiquitylation and degradation, possibly by recruiting $CRL5^{Elongin}$ to sites of UV-induced damage, thus triggering its degradation as means to alleviate fork stalling or arrest (Herlihy et al., 2022).

Overall, UBAP2L seems to play key roles in DNA damage signaling and it is crucial to determine in the future how it could promote faithful DNA replication to hinder aberrant checkpoint adaptation and carcinogenesis. For instance, fork degradation and restart assays could be performed in order to investigate potential involvement of UBAP2L at the replication forks. More precisely, isolation of Proteins On Nascent DNA (iPOND) experiments as well as DNA fiber assay which allow to discriminate between fork degradation after stalling and fork restart defects would be of great interest (Sirbu et al., 2013; Quinet et al., 2017). Furthermore, given the preliminary character of our unpublished data, IF microscopy analysis to evaluate the recruitment of the key replication factors in UBAP2L WT and KO cells upon induction of stress using high doses of HU would be essential. Importantly, in our experiments, we used γH2AX to assess DSBs levels. However, phosphorylation of H2AX can be driven by many various kinases except for ATR (Sharma et al., 2012). Therefore, more appropriate markers should be used to monitor replication such as the measurement of ssDNA content via ATR-dependent phosphorylation of RPA (Ser33) and Chk1 (Ser345) or RPA foci formation (Nam and Cortez, 2011). Substantial work is needed to study more deeply if UBAP2L could also have similar or overlapping functions with UBAP2 in DNA repair. Similar repair factors recruitment experiments could be performed upon DNA damage induction using IR or Cas9 coupled to specific gRNAs to target precise sites. An alternative method using GFP reporter constructs could be used to assess the efficiency of different repair pathways in UBAP2L KO cells (Gunn et al., 2011; Gunn and Stark, 2012).

My unpublished data point to a role for UBAP2L in DNA damage signaling independently of PLK1 (**Fig. 13**). Previous studies showed that PLK1 may restrict NHEJ repair pathway at broken forks to promote error-free repair by HR (Nakamura et al., 2021). Thus, the increased PLK1 protein levels observed during S phase in UBAP2L KO cells relative to control (**Fig. 5**) would be expected to be beneficial for DSBs repair. This is consistent with my data demonstrating that PLK1 inhibition or downregulation not only do not rescue γH2AX levels

but also tend to worsen the phenotype (**Fig. 13**). In addition, PLK1 phosphorylates numerous DNA repair factors thereby promoting or inhibiting their recruitment to DNA lesions to ensure fidelity of DNA repair (van Vugt et al., 2010; Yata et al., 2012; Chabalier-Taste et al., 2016). Aberrant protein levels and activity of PLK1 that characterize UBAP2L KO cells are not sufficient to inhibit the accumulation of DSBs, highlighting an essential role for UBAP2L in DNA damage signaling, independently of PLK1.

F. How does UBAP2L regulate autophagy?

My unpublished results clearly demonstrate that autophagy is perturbed upon UBAP2L depletion. More precisely, I showed that UBAP2L KO cells display an accumulation of P62/LC3 containing vesicles (**Fig. 14A**) and increased protein levels of P62 and LC3 under physiological conditions relative to controls (**Fig. 14B**). Moreover, indirect induction of autophagy by Torin1 did not completely restore LC3 high protein levels in UBAP2L KO cells relative to control (**Fig. 14B**), which could be the result of several potential problems. On one hand, I noticed a mild autophagy initiation defect as assessed by the phosphorylation of ULK at the S757 residue (**Fig. 14C**) and on the other hand I proved the existence of severe autophagosome-lysosome fusion deficiencies in UBAP2L-depleted cells relative to WT cells (**Fig. 14D-E**). Further investigation is needed to elucidate UBAP2L functions in autophagy and particularly to understand the exact autophagy steps that are affected upon UBAP2L depletion. To this end, the autophagosome-lysosome fusion defect hypothesis could be reinforced by performing Fluorescence-activated Cell Sorting (FACS) experiments with WT and UBAP2L KO cells transfected with LC3-GFP construct upon DMSO, BafA1 and Torin1 treatment. The GFP signal intensity is expected to decrease when autophagy is induced and granules are properly degraded whereas it is high when autophagic vesicles accumulate because of fusion problems. In addition, alternative methods have been optimized to monitor autophagy such as the measurement of degradation of long-lived proteins labeled with radioisotopes, the use of the fluorescent protein Keima which has bi-modal excitation spectra depending on the pH or the use of GFP-LC3-RFP-LC3ΔG to assess proper cleavage by the autophagy factor ATG4 and subsequent degradation of GFP-LC3 (Yoshii and Mizushima, 2017).

Additionally, it is important to uncover which domain of UBAP2L could mediate its functions in autophagy. Interestingly, UBAP2L sequence harbors a predicted LC3-interacting region (LIR) within the DUF domain, a motif which is responsible for targeting autophagy receptors to phagophore's membrane-anchored LC3 (Birgisdottir et al., 2013). Deletion or point

mutations of the predicted LIR motif within UBAP2L CT will be essential to perform rescue experiments in UBAP2L KO cells. Intriguingly, the LIR motif is located within the same UBAP2L domain as the one mediating its function on PLK1. As previously introduced, PLK1 has been proposed to interact with the mTORC1 complex and phosphorylate RAPTOR *in vitro*, thereby preventing mTORC1 association with lysosomes where it is active, thus indirectly promoting autophagy (Ruf et al., 2017). Importantly, some preliminary data revealed that in some cells, PLK1 colocalizes with P62 in cytoplasmic aggregates highly similar to autophagosomes upon UBAP2L depletion both in mitosis and in interphase (**data not shown**). Moreover, UBAP2L has emerged as a novel mTOR and RAPTOR interactor and specific regulator of mTORC1. In fact, UBAP2L might positively regulate mTORC1 activity, thus inhibiting autophagy (Wang et al., 2021). However, these findings need to be corroborated and robust experiments will be crucial in order to confirm UBAP2L potential involvement in mTORC1 regulation. Based on available data and published findings, it is tempting to speculate that PLK1 and UBAP2L could exert opposing functions in mTORC1 regulation thereby finetuning autophagy induction.

Our manuscript described UBAP2L-dependent regulation of PLK1 localization and stability thereby regulating its mitotic functions. During mitosis, autophagy is believed to be inhibited as a protective mechanism to prevent the degradation of chromosomes and organelles (Mathiassen et al., 2017). In line with this assumption, several studies reported decreased autophagy flux during mitosis and reappearance of autophagosomes at mitotic exit/G1 (Eskelinen et al., 2002; Furuya et al., 2010). Nevertheless, contradictory studies showed efficient accumulation of LC3/P62 puncta in mitotic cells (Liu et al., 2009; Loukil et al., 2014; Li et al., 2016). Despite the controversial status of autophagy signaling during mitosis, the most commonly accepted concept supports autophagy repression during cell division. Interestingly, mitotic autophagy inhibition seems to be dependent on CDK1-mediated autophagy factor vacuolar protein sorting 34 (VSP34) phosphorylation, disabling its interaction with Beclin1 and ultimately autophagy induction (Furuya et al., 2010). In addition, NEDD4-1 acts in concert with USP13 to promote VSP34 stabilization by removing the K48-linked poly-ubiquitin chains at its K419 residue thereby blocking its proteasomal degradation and enabling autophagy initiation (Xie et al., 2020). As mentioned above, UBAP2L has been shown to interact with NEDD4 (Ingham et al., 2005). Could UBAP2L inhibit NEDD4-1/USP13-mediated deubiquitylation of VSP34 to prevent aberrant autophagic activity during mitosis? It would be fascinating to

investigate whether UBAP2L is recruited to autophagy structures in a CDK1-priming and PLK1-dependent manner prior to mitosis in order to inhibit autophagy.

MATERIAL AND METHODS

I. GENERATION OF STABLE CELL LINES AND CELL CULTURE

HeLa WT and UBAP2L KO cell lines were generated using CRISPR/Cas9-mediated gene editing as described in (**Fig. S1C**). Two gRNAs targeting UBAP2L were cloned into pX330-P2A-EGFP/RFP (**Tables S1 and S2**) (Zhang et al., 2017) through ligation using T4 ligase (New England Biolabs). HeLa cells were transfected and GFP and RFP double positive cells were collected by FACS (BD FACS Aria II), cultured for 2 days and seeded with FACS into 96-well plates. Obtained UBAP2L KO single-cell clones were validated by Western blot and sequencing of PCR-amplified targeted fragment by Sanger sequencing (GATC).

For the generation of PLK1-eGFP KI cell line, HeLa Kyoto cells were transfected with Cas9-eGFP, a sgRNA targeting PLK1 (**Table S2**) and a repair templates (Genewiz). The repair template was designed as a fusion of 5xGly-eGFP flanked by two 500 bp arms, homologous to the genomic region around the Cas9 cutting site. 5 days after transfection eGFP positive cells were sorted and expanded for one week before a second sorting of single cells in a 96 well plates. After 2-3 weeks cells were screened by PCR.

All cell lines were cultured in 5% CO_2 humidified incubator at 37°C. Most of the cell lines were trypsinized and seeded into a new plate three times per week. Culture conditions for each cell lines are listed below:

- **HeLa Kyoto and derived stable cell lines** (UBAP2L KOs, UBAP2L WT#25, PLK1-eGFP) were kept in culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4,5g/L Glucose, 10% Foetal Calf Serum (FCS), 1% Penicillin and 1% Streptomycin.
- **Human U-2 Osteosarcoma (U2OS) cells** were cultured in DMEM supplemented with 1g/L Glucose, 10% FCS and 40µg/mL Gentamycin
- **U2OS derived stable cell lines** (U2OS GFP-Nup96 KI WT, U2OS GFP-Nup96 KI UBAP2L KOs) were cultured in DMEM supplemented with 1g/L Glucose, 10% FCS, AANE, 1mM Sodium-pyruvate (Na-Pyr) and 40µg/mL Gentamycin
- **DLD-1** cells were kept in culture in Roswell Park Memorial Institute (RPMI) medium without HEPES supplemented with 10% FCS and 40µg/mL Gentamycin

II. CLONING

Human UBAP2L isoform1 of 1087aa was isolated from HeLa cDNA and amplified by PCR. UBAP2L NT and CT fragments were generated using the primers listed in **Table S1**. PCR products were cloned into pcDNA3.1 vector.

III. CELL SYNCHRONIZATION

Cell cycle experiments were carried out as previously described (Agote-Arán et al., 2021; Pangou et al., 2021).

A. Double Thymidine Block and Release

Cells were synchronized by addition of 2mM Thymidine (Sigma-Aldrich) for 16 hours, released 8 hours and re-blocked using 2mM Thymidine for 16hours. For G1/S synchronization, cells were not released. For other cell cycle stages, cells were washed out with warm medium to allow synchronous progression through the cell cycle and collected at the desired timepoint. For example, to have enrichment of mitotic population, cells were collected 8 to 9 hours after release.

B. Paclitaxel

Cells were treated with 1µM Taxol (Sigma-Aldrich) for 16 hours which inhibits microtubule depolymerization and subsequently blocks cells at G2/M transition.

C. Monastrol

Cells were treated with 1mM Monastrol (Euromedex) for 16 hours which specifically inhibits the Kinesin-5 family member Eg5, subsequently blocking cells in prometaphase. For monastrol release experiments, cells were collected by centrifugation at 1500 rpm (room temperature RT) and washed out with warm media three times before being seeded in a new culture plate and collected at the desired timepoint.

D. S-Trityl-L-Cysteine (STLC)

Cells were treated with 5µM STLC (Enzolifesciences) for 16 hours which acts similarly as Monastrol and blocks cells in prometaphase.

E. Hydroxyurea

Cells were treated with 2mM HU (Sigma-Aldrich) for 20 hours which reduces the intracellular deoxynucleotide triphosphate pools inhibiting DNA synthesis and subsequently blocking the cells in S phase.

F. RO-3306

Cells were treated with 10µM RO-3306 (VWR INTERNATIONAL) for 16h which inhibits CDK1 and subsequently prevents mitotic entry, blocking cells in G2 phase.

IV. CELL TREATMENTS

A. BI2536

Cells were incubated with 100nM BI2536 (Euromedex) for 1h in the incubator in order to inhibit PLK1 enzymatic activity and immediately collected for further experiments. For rescue experiments presented in Fig. 9, cells were treated with 10nM BI2536 for 45min.

B. Torin1

Cells were treated with 250nM Torin1 (BIO-TECHNE) or the appropriate vehicle (DMSO) for 4h in the incubator to induce autophagy.

C. Bafilomycin A1

Cells were treated with 50nM Bafilomycin A1 (Sigma-Aldrich) or the appropriate vehicle (DMSO) for 4h in the incubator to inhibit autophagy.

D. MG132

Cells were treated with 25µM MG132 (Tocris bioscience) for 4h in the incubator to inhibit proteasomal degradation.

E. Cyclohexamide

Cells were treated with 100µg/mL CHX (Sigma-Aldrich) for 4h in the incubator in order to inhibit translation.

F. Neocarzinostatin

Cells were treated with 100ng/mL NCS (Sigma-Aldrich) for 15min in the incubator, washed out with warm medium and collected 2 to 4h later to perform appropriate experiments.

G. Leptomycin B

Cells were treated with 10ng/mL LMB (Merck) or Methanol/H2O (3:7) vehicle for 4h in the incubator and collected to perform appropriate experiments.

V. PLASMID AND SIRNA TRANSFECTION

A. siRNA

Lipofectamine RNAiMAX (Invitrogen) was used to transfect siRNAs according to the manufacturer's instructions. Final concentration of siRNA used varies from 20 to 40nM. All used oligonucleotides are listed in **Table S2**.

B. Plasmids

Jetpei (Polyplus transfection) or X-tremeGENE9 (Roche) DNA transfection reagents were used to perform plasmid transfections according to the supplier's instructions. All used oligonucleotides are listed in **Table S3**.

VI. SAMPLES PREPARATION FOR IMMUNOFLUORESCENCE MICROSCOPY

A. Standard IF protocol on coverslips

After the appropriate treatments, coverslips were washed with 1X Phosphate-Buffered Saline (PBS), fixed for 10min using 4% Paraformaldehyde (PFA, Electron Microscopy Sciences), washed three times with PBS and permeabilized with 0,5% NP-40 (Sigma-Aldrich) in 1X PBS for 5min under agitation. Cells were washed three times with 1X PBS under agitation and blocked for 1h at RT with 3% Bovine Serum Albumin (BSA, Millipore) diluted in PBS. Appropriate primary antibodies diluted in blocking buffer were added to the coverslips for 1h at RT. After the incubation, the coverslips were washed 3 times with 1X PBS-0,01%Triton X-100 (Sigma-Aldrich) (PBS-T) for 5min each at RT under agitation. Secondary antibodies diluted in blocking buffer were then added to the coverslips for 45min in the dark at RT followed by 3 washes with PBS-T as performed for the previous step. Finally, the coverslips were mounted on glass slides using Mowiol containing DAPI (Calbiochem) and stored until imaging using Zeiss epifluorescence microscope with oil x63 objective.

B. IF protocol for lysosomal staining

The standard IF protocol was performed except that the permeabilizing reagent used was 0,1% Triton X-100, the blocking buffer includes 3% BSA in PBS supplemented with 0,02% saponin (Sigma-Aldrich) and the washes were done with the blocking solution.

C. IF protocol for γH2AX staining

The standard IF protocol was performed except that the permeabilizing reagent used was 0,1% Triton X-100 and the incubation time with primary and secondary antibodies was reduced to 45min and 30min, respectively.

D. IF on slides

For IF on mitotic synchronized cells, after the appropriate treatments, cells were detached by slightly tapping the culture plate and a small number of cells was taken and spread on a glass slide using a cytocentrifuge (Epredia, Thermo Scientific Shandon Cytospin 4) at 1000rpm RT for 5min. Cells were immediately fixed using 4% PFA for 10min at RT and the normal IF protocol was performed.

E. Live-imaging microscopy

Cells were grown on 35/10mm four compartments glass bottom dishes (Greiner Bio-One). After appropriate treatments and synchronization, SiR-DNA and Verapamil were added to the medium 1h before filming. The acquisition was done by the Yokogawa W1 rotating disk combined with a Leica 63x/1.0 water lens. To assess mitotic progression, HeLa WT and UBAP2L KO cells were filmed during 8h using the following parameters: 25µm range, 2µm step, 1 picture every 10min. For PLK1-eGFP cell line experiment, cells were synchronized using DTBR protocol and were filmed 10h after release using a 63x water immersion objective for a total time frame of 8h. Images were acquired every 10 min in stacks of 12µm range (0,5µm steps). Image analysis was performed using ImageJ software. For both experiments, maximum intensity projection pictures were selected and movies were created with a 7 frames/sec speed.

All used antibodies are listed in **Table S4**.

VII. WESTERN BLOTTING

For protein extraction, cells were harvested by scraping, centrifuged at 1500rpm for 5min at 4°C and washed three times using cold 1X PBS. Pelleted cells were lysed in RIPA buffer (50

mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM Sodium pyrophosphate, 1 mM NaVO4 (Na3O4V) and 1 mM NaF) supplemented with protease inhibitor cocktail (PIC) during 30min on ice with periodic vortexing before being centrifuged at 14 000rpm for 30min at 4°C. Protein concentration was determined using Bradford assay by Bio-Rad Protein Assay kit (Bio-Rad). Samples were boiled at 96°C for 10min, loaded into pre-casted 4-12% Bis-Tris gradient gels (Thermo Scientific) and run at 100V for 1h30. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) using wet transfer modules (BIO-RAD Mini-PROTEAN® Tetra System) for 1h30 at 100V. Membranes were blocked in 5% non-fat milk or 3% BSA diluted in 1X TBS - 0,05% Tween (TBS-T) for at least 1h RT shaking. Membranes were then incubated with the appropriate primary antibody diluted in blocking solution overnight at 4°C shaking. The next day, membranes were washed 3 times 10min with TBS-T and incubated with secondary antibodies for 1h RT shaking before being washed as mentioned before. Finally, membranes were developed using ECL Western blotting substrate (Thermo Scientific) or Luminata Forte Western HRP substrate (Millipore).

All used antibodies are listed in **Table S4**.

VIII. SUBCELLULAR FRACTIONATION

After indicated treatments, cells were washed with 1X PBS and harvested by scraping in cold 1X PBS. Cells were pelleted by centrifugation at 1500rpm for 5min at 4°C and washed three times using cold 1X PBS. The cytosolic fraction was removed by incubation in hypotonic buffer 1 (10mM HEPES pH7, 50mM NaCl, 0,3M Sucrose, 0,5% Triton X-100) supplemented with PIC for 10min on ice and centrifuged at 1500rpm for 5min at 4°C. The supernatant was collected and referred to as the cytosolic fraction. The pellet was washed by resuspension in buffer 2 (10mM HEPES pH7, 50mM NaCl, 0,3M Sucrose) supplemented with PIC and centrifuged at 1500rpm for 5min at 4°C. The soluble nuclear fraction was removed by incubation with the nuclear buffer 3 (10mM HEPES pH7, 200mM NaCl, 1mM EDTA, 0,5% NP-40) supplemented with PIC for 10min on ice and centrifuged at 14000rpm for 2min at 4°C. The supernatant was collected and referred to as the soluble nuclear fraction. The pellet was washed by resuspension in buffer 2 (10mM HEPES pH7, 50mM NaCl, 0,3M Sucrose) supplemented with PIC and centrifuged at 14000rpm for 2min at 4°C. The pellets were resuspended in lysis buffer 4 (10mM HEPES pH7, 500mM NaCl, 1mM EDTA, 1% NP-40) supplemented with PIC and Benzonase (Merck), incubated for 15min on ice and sonicated at low amplitude (10amp, 1sec ON, 1sec OFF, 3 times), incubated again 15min one ice after

sonication and centrifuged for 1min at 14000rpm 4°C. The supernatant was collected and referred to as the chromatin-bound fraction. Total protein was quantified at least twice through Bradford assay and a total of 10µg from each fraction was used for subsequent Western Blot.

IX. IMMUNOPRECIPITATIONS

A. Endogenous immunoprecipitation

For endogenous IP, cells were scraped and washed 3 times in 1X PBS before being lysed in lysis buffer (25 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Triton X-100, 5mM MgCl2, 2mM EDTA, 2mM PMSF and 10mM NaF) supplemented with PIC for 30min on ice. Samples were centrifuged at 14 000rpm for 30min at 4°C and protein concentration was quantified through Bradford assay. Lysates were equilibrated to volume and concentration. IgG and target specific antibodies (anti-UBAP2L or anti-PLK1) as well as protein G sepharose 4 Fast Flow beads (GE Healthcare Life Sciences) were used. Samples were incubated with the IgG and specific antibodies overnight at 4°C under rotation. Beads were blocked with 3% BSA diluted in 1X lysis buffer and incubated for 4h at 4°C with rotation. Next, the IgG/specific antibodies-samples and blocked beads were incubated together to a final volume of 1 ml for 4h at 4°C under rotation. The beads were washed with lysis buffer 4 to 6 times for 10 min each at 4°C under rotation. Notably, beads were pelleted by centrifugation at 1500rpm for 5 min at 4°C. The washed beads were directly eluted in 2X LB with β-Mercaptoethanol (Biorad) and boiled for 10 min at 96°C and samples were resolved by WB as described above.

B. Denaturing IP

For denaturing IP experiments, HeLa cells were transfected with His/Biotin Ubiquitin and pEGFP-PLK1 or with His/Biotin Ubiquitin and pEGFP-N1 for 30h. Cells were treated with 50µM MG132 and lysed on ice with denaturing buffer (8M Urea, 300 mM NaCl, 50 mM Na2HPO4, 50 mM Tris-HCl, 1 mM PMSF, pH 8) supplemented with PIC for at least 1 hour and supernatants were cleared by centrifugation at 14 000rpm for 15 minutes at 4°C. The mixed GFP-Trap A agarose beads (Chromotek) and proteins were incubated overnight at 4°C under rotation and washed with the same denaturing buffer using the same method as for GFP-IP. Finally, GFP beads were eluted in 2X LB with β-Mercaptoethanol and boiled at 96°C for 10 minutes to isolate proteins for WB.

X. COLONY FORMATION ASSAY

Colony Formation Assay (CFA) was carried out as previously described (Pangou et al., 2021). Briefly, 500 cells of each cell line were seeded in triplicates in 6-well plates. Following appropriate treatment, cells were incubated at 37°C 5% CO_2 for 7 days until colonies form. Cells were washed with 1X PBS, fixed with 4% PFA and stained with 0,1% Crystal Violet (Thermo Fischer Scientific) for 30min. The number of colonies was first manually counted and then automatically quantified with Fiji software. A Fiji pipeline was created to quantify the number of colonies per well, the individual and total colony area. Three biological replicates were performed.

XI. AUTOMATIC MEASUREMENTS

A. Measurement of nuclear intensity

A pipeline allowing to measure nuclear intensity of the protein of interest was previously generated (Agote-Aran et al., 2020) using CellProfiler software. Briefly, the pipeline automatically recognizes cell nuclei based on the DAPI-fluorescent channel. Several parameters are then systematically measured such as the area and the form factor (roundness of the nucleus) which is helpful to quantify abnormal nuclear shape in an unbiased way. Moreover, the intensity of the fluorescence in the delimited nuclear area is also measured in an unbiased and automatic way. Depending on the experiment, 100 to 500 cells were quantified per condition.

B. Measurement of UBAP2L kinetochore intensity

To assess UBAP2L kinetochore localization upon siNT/siPLK1 or DMSO/BI2536 treatments, UBAP2L signal intensity overlapping CREST signal was measured at single pairs of sister kinetochore. All pictures were taken randomly and constant exposure time was kept for all pictures. The ROI (one pair of kinetochores) was selected and intensity of the two channels in this precise area was quantified.

To assess UBAP2L kinetochore localization in different mitotic stages, colocalization of UBAP2L signal with CREST was measured in an intensity-independent manner, measuring the overlapping area of the two channels. To simplify, any pixel different than black was considered as a positive signal independently of its intensity in order to overcome CREST intensity variance throughout mitosis.

C. Automatic measurement of colocalization (JacoP ImageJ plugin)

For RFP/GFP colocalization measurement, the JacoP ImageJ plugin was used. Briefly, a signal recognition threshold was defined and remained constant for all conditions and Mander's coefficient 1 (RFP overlapping GFP) was measured for each cell.

XII. STATISTICAL ANALYSIS

At least three independent biological replicates were performed for each experiment. Graphs were made using GraphPad Prism and Adobe illustrator softwares. Schemes were created using BioRender.com. Normal distribution was assessed using Shapiro-Wilk test for each experiment. Normal data was analyzed using two sample two-tailed T-test or One-way ANOVA with Dunnett's or Sidak's correction, in case of multiple group analysis. For non-normally distributed data, Mann-Whitney's or Kruskal-Wallis test with Dunn's correction tests were performed. On graphs, error bars represent Standard Deviation (SD) and in all experiments, significance stars were assigned as following: * P<0,05, ** P<0,01, *** P<0,001, **** P<0,0001, ns=non-significant.

Primers for cloning and sequencing	
Cloning hUBAP2L fragments in pcDNA3.1	
hUBAP2L-FL-Flag-Fwd	5'-TTTGAATTCTTATGACATCGGTGGGCACTAACC-3'
hUBAP2L-FL-Flag-Rv	5'-TTTCTCGAGTCAGTTGGCCCCCCAGC-3'
hUBAP2L-NT-Flag-Fwd	5'-TTTGAATTCTTATGACATCGGTGGGCACTAACC-3'
hUBAP2L-NT-Flag-Rv	5'-TTTCTCGAGTTAAGCAGAAAACCTTCCTCCTCG-3'
hUBAP2L-CT-Flag-Fwd	5'-TTTGAATTCTTATGCAAGGAATGGGAACCTTTAACCCAGC-3'
hUBAP2L-CT-Flag-Rv	5'-TTTCTCGAGTCAGTTGGCCCCCCAGC-3'
Cloning hUBAP2L KO sgRNAs in pX330-P2A-EGFP/RFP	
hUBAP2L KO exon5 sgRNA-1-Fwd	5'-CACCGTGGCCAGACGGAATCCAATG-3'

hUBAP2L KO exon5 sgRNA-1-Rv	5’-AAACCATTGGATTCCGTCTGGCCAC-3’
hUBAP2L KO exon5 sgRNA-2-Fwd	5’-CACCGGTGGTGGGCCACCAAGACGG-3’
hUBAP2L KO exon5 sgRNA-2-Rv	5’-AAACCCGTCTTGGTGGCCCACCACC-3’
Sequencing of UBAP2L KO clones - cloning of genomic DNA in pUC57	
hUBAP2L KO exon5-DNA sequencing-Fwd	5’-CGAATGCATCTAGATATCGGATCCCTGCTGAGTG GAGAATGGTTA-3’
hUBAP2L KO exon5-DNA sequencing-Rv	5’-GCCTCTGCAGTCGACGGGCCCGGGAGACTGGTGG CAGTTGGTAG-3’

Table S1: List of primers used for cloning and sequencing

Oligonucleotide	Sequence	Manufacturer
UBAP2L	5’CAACACAGCAGCACGUUAU-3’	Eurogentec
PLK1	5’-GGGGUUGCUGUGUAAGUUA-3’	Eurogentec
CUL3	5′-CAACACUUGGCAAGGAGAC- 3′	Eurogentec
Mis18α	5’-CAGAAGCUAUCCAAACGUGUU-3’	Eurogentec
CENP-A	5'-CCGCCUGGCAAGAGAAAUAUU-3’	Eurogentec
G3BP1	5’-ACAUUUAGAGGAGCCUGUUGCUGAA-3’	Eurogentec
G3BP2	5’-GAAUAAAGCUCCGGAAUAU-3’	Eurogentec
siGENOME Non-targeting individual siRNA-2 (Ctrl siRNA)	5’-UAAGGCUAUGAAGAGAUAC-3’	Dharmacon
sgRNA: PLK1	5’-TCGGCCAGCAACCGTCTCA-3’	This study

gRNA1: UBAP2L	5'-TGGCCAGACGGAATCCAATG-3'	This study
gRNA2: UBAP2L	5'-GTGGTGGGCCACCAAGACGG-3'	This study
Primers used for Cloning and sequencing are included in Table S1		

Table S2: List of used siRNAs and gRNAs

Recombinant DNA	Source	Cat. Number
pEGFP-N1	Clontech	Cat# 6085-1
pEGFP-N1-PLK1 WT	From Metzger et al., 2013	N/A
pcDNA3.1-Flag-N	This study	N/A
pcDNA3.1-Flag-N-UBAP2L FL	This study	N/A
pcDNA3.1-Flag-N-UBAP2L-NT	This study	N/A
pcDNA3.1-Flag-N-UBAP2L-CT	This study	N/A
pAAV-MCS-His/Biotin-Ubiquitin-WT	From Magliarelli et al., 2016	N/A

Table S3: List of used plasmids

Antibody	Host specie	IF Dilution	WB Dilution	Supplier	Reference
PLK1	Mouse	1:200	/	Santa Cruz Biotechnology	Cat# sc-17783, RRID:AB_628157

PLK1	Mouse	/	1:1000	Abcam	Cat# ab17057, RRID:AB_443613
PLK1	Rabbit	/	1:1000	Cell Signaling Technology	Cat# 4513, RRID:AB_2167409
AurB	Mouse	1:500	1:1000	BD Biosciences	Cat# 611083, RRID:AB_398396
PLK2	Rabbit	/	1:1000	GeneTex	Cat# GTX112022, RRID:AB_10623592
PLK3	Rabbit	/	1:1000	Novus Biologicals	Cat# NBP2-32530, RRID: N/A
PLK4	Rabbit	/	1:1000	Novus Biologicals	Cat# NB100-894, RRID:AB_2284148
AurA	Rabbit	1:500	1:1000	Cell Signaling Technology	Cat# 4718, RRID:AB_2061482
BubR1	Mouse	/	1:1000	BD Biosciences	Cat# 612502, RRID:AB_399803
Cyclin B1	Mouse	1:500	1:1000	Santa Cruz Biotechnology	Cat# sc-245, RRID:AB_627338
Cyclin A	Rabbit	/	1:1000	Santa Cruz Biotechnology	Cat# sc-751, RRID:AB_631329
Cyclin E	Mouse	/	1:1000	Santa Cruz Biotechnology	Cat# sc-247, RRID:AB_627357
GAPDH	Rabbit	/	1:10000	Sigma-Aldrich	Cat# G9545, RRID:AB_796208

G3BP1	Rabbit	/	1:3000	BD Biosciences	Cat# 611126, RRID:AB_398437
G3BP2	Rabbit	/	1:1000	Thermo Fischer Scientific	Cat# A302-040A-M, RRID:AB_2780294
KLHL22	Rabbit	/	1:1000	Proteintech	Cat# 16214-1-AP, RRID:AB_2131201
LaminB1	Rabbit	/	1:1000	Abcam	Cat# ab16048, RRID:AB_443298
CREST	Human	1:500	/	Antibodies Incorporated	Cat# 15-234-0001, RRID:AB_2687472
Flag	Rabbit	1:1500	1:1500	Sigma-Aldrich	Cat# F7425, RRID:AB_439687
Flag M2	Mouse	1:1500	1:1500	Sigma-Aldrich	Cat# F1804, RRID:AB_262044
GFP	Rabbit	/	1:2000	Abcam	Cat# ab290, RRID:AB_303395
β-Actin	Mouse	/	1:5000	Sigma-Aldrich	Cat# A2228, RRID:AB_476697
α-tubulin	Mouse	1:2000	/	Sigma-Aldrich	Cat# T9026, RRID:AB_477593
Ubiquitin (P4D1)	Mouse	/	1:1000	Cell Signaling Technology	Cat# 3936, RRID:AB_331292
Anti-ubiquitylated proteins clone FK2	Mouse	/	1:1000	Millipore	Cat# ST1200, RRID:AB_10681625

Anti-gamma H2A.X (phospho S139) antibody [3F2]	Mouse	1:1000	1:1000	Abcam	Cat# ab22551, RRID:AB_447150
Phospho-Histone H2A.X (Ser139) (20E3)	Rabbit	1:500	/	Cell Signaling Technology	Cat# 9718, RRID:AB_2118009
C21orf45 Polyclonal antibody (Mis18α)	Rabbit	/	1:500	Proteintech	Cat# 25832-1-AP, RRID:AB_2880259
CENP-A	Rabbit	1:250	1:500	Thermo Fisher Scientific	Cat# PA5-17194, RRID:AB_10987425
Phospho-PLK1 (Thr210)	Rabbit	/	1:1000	Cell Signaling Technology	Cat# 5472, RRID:AB_10698594
CUL3	Rabbit	/	1:1000	From (Sumara et al., 2007)	
P62	Guinea pig	1:500	1:2000	Progen	Cat# GP62-C, RRID:AB_2687531
P62	Rabbit	1:500	1:1000	GeneTex	Cat# GTX100685, RRID:AB_2038029
ULK1	Rabbit	/	1:1000	Santa Cruz Biotechnology	Cat# sc-33182, RRID:AB_2214706

Phospho-ULK1 (Ser757)	Rabbit	/	1:1000	Cell Signaling Technology	Cat# 6888, RRID:AB_10829226
LC3B	Rabbit	1:500	1:1000	Novus Biologicals	NB100-2220SS, RRID:AB_791015
LC3B	Mouse	1:500	1:1000	Nanotools	Cat# 0260-100/LC3-2G6, RRID: N/A
UBAP2L	Rabbit	1:500	1:1000	Homemade (IGBMC)	
Goat anti-mouse Alexa Fluor 488	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-11029, RRID:AB_2534088
Goat anti-rabbit Alexa Fluor 488	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-11034, RRID:AB_2576217
Goat anti-human Alexa Fluor 555	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-21433, RRID:AB_2535854
Goat anti-mouse Alexa Fluor 568	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-11031, RRID:AB_144696
Goat anti-rabbit Alexa Fluor 568	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-11036, RRID:AB_10563566
Goat anti-human Alexa Fluor 568	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-21090, RRID:AB_2535746

Goat anti-guinea pig Alexa Fluor 568	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-11075, RRID:AB_141954
Goat anti-rabbit Alexa Fluor 647	N/A	1:500	/	Thermo Fischer Scientific	Cat# A-21245, RRID:AB_2535813
Goat anti-rabbit IgG-HRP conjugate	N/A	/	1:5000	Biorad	Cat# 170-6515, RRID:AB_11125142
Goat anti-mouse IgG-HRP conjugate	N/A	/	1:5000	Biorad	Cat# 170-6516, RRID:AB_11125547

Table S4: List of used antibodies

LIST OF PUBLICATIONS AND COMMUNICATIONS

Publications:

- Pangou E.#, Bielska O.#, **Guerber L.**, Schmucker S., Agote-Arán A., Ye T., Liao Y., Puig-Gamez M., Liu Y., Compe E., Zhang, Z., Grandgirard E., Aebersold R., Ricci R and Sumara I. A PKD-MFF signaling axis couples mitochondrial fission to mitotic progression, Cell Reports, 2021, 35(7): 109129. #equal contribution

- **Guerber L.**, Pangou E. and Sumara I., Ubiquitin Binding Protein 2-Like (UBAP2L): is it so NICE After All?, Front. Cell Dev. Biol. 10:931115. doi: 10.3389/fcell.2022.931115

- Liao Y., Andronov L., Liu X., Lin J., **Guerber L.**, Lu L., Agote-Arán A., Kleiss C., Qu M., Schmucker S., Pangou E., Cirillo L., Zhang Z., Riveline D., Gotta M., P. Klaholz B. and Sumara I., Mechanism driving scaffold assembly of nuclear pore complexes at the intact nuclear envelope, submitted to the EMBO Journal.

- **Guerber L.#**, Pangou E.#, Vuidel A., Liao Y., Kleiss C., Grandgirard E. and Sumara I., UBAP2L regulates PLK1 localization and stability and ensures proper mitotic progression, submitted to Journal of Cell Biology, #equal contribution

- Pangou E.#, Awal S.#, Kleiss C., **Guerber L.**, Da Costa P., Villa P., Bonnet D., Sumara I., Targeting SAC-regulator UBASH3B for future cancer therapies, manuscript in preparation for Cancer research, #equal contribution

Oral communications and posters:

- Unexpected role of NICE4 in the regulation of PLK1 during interphase, IGBMC, April 2021 (IGBMC department seminar)

- Unexpected role of NICE4 in the regulation of PLK1 during interphase, IGBMC, Octobre 2021 (IGBMC department seminar)

- UBAP2L is a novel regulator of PLK1 dynamics at the kinetochore. **Guerber L.#**, Pangou E.#, Liao Y., Kleiss C. and Sumara I. Dynamic kinetochore, EMBO Workshop, June 2022, Oslo, Norway (Poster)

- Coupling of PLK1 localization and stability during mitosis. **Guerber L.#**, Pangou E.#, Vuidel A., Liao Y., Kleiss C., Grandgirard E. and Sumara I. IMCBio Master day 2022, IGBMC, September 2022 (Poster)

- UBAP2L is a novel regulator of PLK1 dynamics at the kinetochore. **Guerber L.#**, Pangou E.#, Vuidel A., Liao Y., Kleiss C., Grandgirard E. and Sumara I. Ubiquitin and ubiquitin-like proteins in health and disease, EMBO Workshop, September 2022, Cavtat, Croatia (Poster)

Attended conferences (without communication):

- Ubiquitin, Autophagy & Disease (Virtual) CSH meeting, Cold Spring Harbor, April 2021, NY, USA.
- IGBMC-FMI Graduate Student Symposium, IGBMC, April 2021, Illkirch, France
- Salk Cell Cycle Symposium meeting (Virtual) by the Salk Institute, June 2021, California, USA

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Agote-Arán, A., Lin, J., and Sumara, I. (2021). Fragile X–Related Protein 1 Regulates Nucleoporin Localization in a Cell Cycle–Dependent Manner. *Front. Cell Dev. Biol.* 9, 755847. doi: 10.3389/fcell.2021.755847.

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ETUDE DU ROLE DE UBAP2L DANS L'HOMEOSTASIE CELLULAIRE

Résumé

La polo-like kinase 1 (PLK1) est un régulateur de la division cellulaire eucaryote. Au cours de la mitose, la régulation dynamique de PLK1 est cruciale pour ses rôles dans l'assemblage du fuseau, la ségrégation des chromosomes et la cytokinèse. Elle est médiée par des voies non protéolytiques et protéolytiques de l'ubiquitine. Cependant, les mécanismes moléculaires régissant ces différents signaux sur PLK1 restent mal définis. Ici, nous identifions la protéine de liaison à l'ubiquitine 2-Like (UBAP2L) régulant spécifiquement PLK1 et non d'autres facteurs mitotiques ou membres de la famille PLK par son domaine C-terminal. Nous démontrons que UBAP2L est recruté aux kinétochores (KTs) en métaphase grâce à PLK1, favorisant son retrait des KTs et sa dégradation correcte en fin de mitose, probablement en assurant l'interaction de PLK1 avec la ligase E3 CULLIN3 (CUL3), dont nous avons démontré qu'elle régule la localisation de PLK1 aux KTs et la ségrégation fidèle des chromosomes d'une manière non protéolytique. La déplétion d'UBAP2L cause une activité aberrante de PLK1, provoquant des erreurs de ségrégation, l'instabilité génomique et la mort cellulaire. Finalement, nous apportons des résultats préliminaires montrant l'implication de UBAP2L dans le signalement des dommages à l'ADN et dans l'autophagie.

Mots clés : PLK1, UBAP2L, mitose, ubiquitine, kinétochores, instabilité génomique

Résumé en anglais

Polo-Like Kinase 1 (PLK1) is a key regulator of eukaryotic cell division. During mitosis, dynamic regulation of PLK1 is crucial for its roles in spindle assembly, chromosome segregation and cytokinesis and is mediated by ubiquitin-dependent pathways where both non-proteolytic and proteolytic ubiquitylation has been implicated. However, the molecular mechanisms governing these different ubiquitin signals on PLK1 remain ill-defined. Here, we identify the Ubiquitin-Binding Protein 2-Like (UBAP2L) that specifically regulates PLK1 and not other mitotic factor nor PLK family members through its C-terminal domain. We demonstrate that UBAP2L is recruited to kinetochores (KTs) during metaphase in a PLK1-dependent manner, promoting PLK1's removal from KTs and proper degradation after mitosis completion possibly by ensuring interaction of PLK1 with CULLIN3 (CUL3) Ring E3-Ligase, which we previously demonstrated to regulate timely localization of PLK1 to KTs and faithful chromosome segregation in a non-proteolytic manner. UBAP2L depletion leads to an aberrant PLK1 activity, causing severe segregation errors, genomic instability and cell death. Finally, we provide preliminary evidence that UBAP2L is involved in DNA damage signaling and autophagy.

Key words: PLK1, UBAP2L, mitosis, ubiquitin, kinetochores, genomic instability