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The molecular mechanisms underlying erroneous segregation on Warsaw Breakage Syndrome

Faculdade de Medicina e Ciências Biomédicas

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Master's in Oncobiology – Molecular Mechanisms of Cancer
Work under the supervision of:
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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

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(Xavier Santana Jorge)

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Abstract

Given the vast number of mechanisms where cohesin is involved, problems that affect the structure of cohesin or its cofactors can lead to a variety of pathologies, namely cancer and some rare syndromes. The term cohesinopathies is used to describe rare syndromes that share mutations in the cohesin complex or at its regulators. Warsaw Breakage Syndrome (WABS) belongs to the group of cohesinopathies due to overlapping clinical features and to be caused by a bi-allelic mutation in the protein DDX11, known to regulate cohesin. In addition, DDX11 functions as a DNA helicase during replication. The etiology of this disease is still unknown, but two no-mutual exclusive hypotheses have been proposed: the first is that it can be caused by problems in DNA replication and reparation, and the second is that it can be caused by problems during mitosis. In this project, we aim to try to better understand the mechanisms that underline WABS etiology using WABS patient cell lines and RPE-1 CRISPR DDX11 KO cell lines in combination with imaging techniques. For that, we capitalized in ongoing research in the lab to evaluate three possible outcomes of how mis-regulate mitosis contributes to WABS etiology. We evaluated the localization of the DDX11 during mitosis, but unspecific binding of the antibody made it impossible for us to draw conclusions about the DDX11 importance during this cell cycle stage. Then we studied the localization of the Aurora B kinase, an important protein involved in the metaphase checkpoint. Despite previous publications suggesting that DDX11 impacts Aurora B, in our system the impact on Aurora B was more subtle. Finally, we studied the localization of Topoisomerase II a, a protein responsible for resolve the DNA catenation. This isomerase has a later localization across the chromosomes upon DDX11 perturbations. Topoisomerase typical more centromeric localization changed to an almost even distribution along all the chromosomes in WABS models. From this work execution, we identified at least one very promising candidate to explore, yet more experiments are necessary in the future to strengthen results and final conclusions about the mechanisms underlining this disease.

Keywords: Warsaw Breakage Syndrome (WABS), DDX11, Cohesinopathies, Cohesin, Aurora B, Topoisomerase II α (TOPO2A).

Resumo

O ciclo celular é uma sequência de processos, pelos quais uma célula é capaz de crescer e se dividir em duas copias idênticas. Este processo pode ser dividido em duas grandes fases, a interfase, que é composta pela fase G1, S e G2 e a mitose, esta composta por seis estádios, a prófase, prometáfase, metáfase, anáfase, telófase e citocinese. De um modo simplista, pode-se dizer que a interfase é onde as células passa a maior parte da sua vida, crescem e duplicam o seu genoma para depois entrarem em mitose onde efetivamente a células divide o seu genoma duplicado nas duas células filhas de um modo equitativo. A passagem e cada fase do ciclo celular é altamente regulado para garantir que nenhum erro ocorra em nenhum dos processos, e que culmine com a formação de células com números anormais de cromossomas, isto é aneuploidia. A presença de um conteúdo genético anormal (quem em número ou organização) levam a que estas células tenham funções alteradas que na maioria são alterações nocivas para o organismo. Por isso, não é surpreendente que aneuploidia esteja tão frequentemente associadas a patologias como o cancro e doenças do desenvolvimento. A aneuploidia pode surgir por alterações ao nível ou durante a replicação do ADN ou por exemplo devido a agente exógenos mutagénicos. Durante a mitose, problemas ao nível na maquinaria do fuso mitótico, cinetócoros, centrossomas ou em estruturas cromossómicas podem levar a células aneuploides. Assim de modo impedir que estes erros ocorram há diversos mecanismos para proteger a célula, os chamados “checkpoints”, os quais estão distribuídos ao longo do ciclo. Estes têm o grande objetivo de garantir a integridade genómica das células, só deixando a célula progredir pelo ciclo se cumprir todos os requerimentos associados. Desde a replicação do ADN até a sua separação nas células filhas formadas, é necessário assegurar que cromatídeos irmãos se mantenham juntos. A coesina é um complexo proteico composto por 3 subunidades, a SMC1, a SMC3 e a Rad21, que forma um anel proteico que tem a capacidade de aprisionar ambas as cadeias de ADN irmãs, desta forma prevenindo que haja uma separação prematura dos dois cromatídeos irmãos. Nos últimos anos foi descoberto que a coesina é capaz de desempenhar funções em outras fases do ciclo celular, nomeadamente na regulação da expressão génica, na organização e integridade do genoma. Diversas patologias, como cancro ou síndromes raras têm associadas alterações nas subunidades da coesina ou nos seus reguladores. Aliás as coesinopatias é conjunto de síndromes raras que todas têm em comum o facto de todas serem originadas por problemas na coesina, ou nos seus cofatores e reguladores. Neste projeto procurou-se

aprofundar o conhecimento dos mecanismos biológicos que levam ao desenvolvimento de uma coesinopatia chamada de Síndrome de rutura de Varsóvia (do inglês *Warsaw Breakage Syndrome*, tipicamente denominada por WABS). WABS é uma síndrome rara identificada pela primeira vez em 2010, sendo que até hoje apenas 23 casos foram reportados. Esta é causada por uma mutação bialélicas no gene que codifica a proteína DDX11, que é uma helicase de ADN e um cofator do *loading* coesina, embora se desconheça os mecanismos exatos. A etiologia desta doença ainda é desconhecida, mas duas hipóteses não mutuamente exclusivas são propostas: a primeira pressupõe problemas durante a replicação ou nos mecanismos de reparação do ADN são responsáveis pelo desenvolvimento da WABS. A segunda, sugere que problemas ao nível da segregação cromossómica duranta a mitose causa o desenvolvimento desta. Neste trabalho recorreu-se a linhas humanas para investigação a segunda hipótese, em particular células de fibroblastos obtida de dois indivíduos diagnosticados com WABS (e respetiva linha célula de controlo obtida de um individuo saudável), assim como a células RPE-1 modificadas geneticamente utilizando a técnica CRISPR no qual o gene DDX11 foi *knockout*. Assim, utilizando estas ferramentas testamos: (i) localização do DDX11 nas células WABS durante a mitose. Porém, devido a problemas de inespecificidade do anticorpo foi nos impossível retirar quaisquer conclusões sobre a localização e inferir sob a importância do DDX11 durante a mitose. (ii) Perceber a localização e distribuição, em cromossomas mitóticos, da cináse Aurora B, uma proteína necessária à correção e aferição da ligação entre os cromatídeos irmão e os microtúbulos do fuso mitótico prevenindo erros na segregação cromossómica. Num estudo prévio observou-se em células de galinha (DT40) que o *knockout (KO)* de DDX11 causa uma deslocalização desta proteína ao nível centromérico. Neste trabalho realizado, ao contrário do estudo prévio, observou-se um aumento da distribuição de Aurora B ao nível da região peri- e centromérica. Por fim, (iii) estudou-se a distribuição da enzima Topoisomerase II, uma responsável por resolver os emaranhamentos do ADN, que ocorrem de forma natural durante o processo de replicação do ADN. Sabe-se que a não expressão de DDX11 leva a baixos níveis da proteína coesina o que em certos contextos pode levar a uma separação precoce dos cromatídeos irmãos. Em células de indivíduos com WABS os nossos estudos laboratoriais mostraram que não era o caso, pelo que se colocou a hipótese que problemas ao nível da catenação do ADN (por exemplo pela Topoisomerase II) poderiam prevenir a precoce separação dos cromatídeos irmãos.

Pela realização deste projeto verificou-se que em células de pacientes WABS e células DDX11 KO a distribuição da Topoisomerase II se distribuída de forma homogénea por todo o cromossoma ao invés de um enriquecimento na zona centromérica. A partir dos resultados obtidos neste projeto, identificou-se um candidato promissor para a etiologia desta doença, no entanto muitas mais experiências são necessárias realizar no futuro para fortalecer estes resultados e nos permitir retirar conclusões finais sobre os mecanismos subjacentes a esta doença.

Palavras-chave: Síndrome de rutura de Varsóvia (do inglês Warsaw Breakage Syndrome, WABS), DDX11, Coesinopatias, Coesina, Aurora B, Topoisomerase II.

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List of Abbreviations

APC/C - Anaphase Promoting Complex

AurB - Aurora B

CAID - Chronic Atrial and Intestinal Dysrhythmia

Cdk1 - Cyclin-Dependent Kinase 1

CdLS - Cornelia de Lange Syndrome

CHLR - human Chl1-related

CPC - Chromosomal Passenger Complex

CUL3 - E3 ubiquitin ligase cullin 3

DNA - ácido desoxirribonucleico

EV - Empty vector

FA - Fanconi anemia

FANCJ - Fanconi anemia group J protein

Fe-S - Iron-sulfur cluster

G1 - Gap phase 1

G2 - Gap phase 2

G4 - G-quadruplex

HD1 and HD2 - Homologous domain 1 and 2

HP1 - Heterochromatin protein 1

HR - Homologous recombination

MAPs - Microtubule-associated proteins

MT - Microtubules

PCM - pericentriolar matrix of proteins

RBS - Roberts Syndromes

RTEL1 - Regulator of telomere elongation helicase 1

S - Synthesis phase

SAC - spindle assembly checkpoint

SAFs - Spindle assembly factors

SF2 - DNA helicase super-family 2

Sgo1 - Shugoshin

SMC - Structural maintenance of chromosomes

TGF-β - Transforming growth factor β

TOP2A - Topoisomerase II a

Triplex - triple-strands

WABS - Warsaw Breakage Syndrome

WAPL - Wings-apart like protein

WT - Wild type

XPD - *Xeroderma pugmentosum* group D proteins

1. Introduction

1.1 Cell cycle

The cell cycle is characterized as an orderly sequence of events, by which the cells can grow and duplicates its contents. This process culminates in the division of themselves into two identical cells, the daughter cells. For that, it is necessary to accurately replicate the DNA of each chromosome and all their organelles and macromolecules, this way ensuring that the newly born cells do not lose any content and information.

The cell cycle is composed of four phases that cycle along the life of the cells, each phase only starts when the last one is completed correctly: Gap 1 phase (G1), DNA Synthesis phase (S); Gap 2 phase (G2); and mitosis (M) (Figure 1), there is also the possibility that during G1 the cell enters in Gap 0 phase (G0).

1.2 Cell cycle phases

The cell cycle can be grouped into two major groups, the interphase, and the mitosis. The interphase is the longest phase in which cells pass most of their lives and is in they which cells grow and duplicate their DNA, their organelles, and macromolecules, and the mitosis is a shorter phase that only takes hours and is where the cells divide their genetic material and organelles into the two daughter cells. The interphase is composed of three major phases the G1 phase, the S phase, and the G2 phase.

The G1 phase or gap phase 1, is the gap between the finish of the last cycle and the start of the S phase, in this phase while the Cdk-cyclin D is active the cell could decide to continue the process or to leave the cycle and enter a quiescent state, also called the G0 which is a resting phase, normally the cell enters in this state when the environmental conditions are not favorable for them to continue to divide or for certain types of cells that reach their fully differentiated state. During this phase is where cells grow and prepare all their mechanisms to start to duplicate the DNA.

The S phase or synthesis phase, this phase starts with the activations of the Cdk-cyclin E, and during this phase, the cell starts duplicating its genome with the objective of creating two exact copies of all its chromosomes.

The G2 phase or gap phase 2, is the second gap in the cycle, this time is between the finish of the DNA duplication and the start of the mitotic phase, during this gap the Cdk-cyclin A is active and during this phase the cell starts to condensate the chromatin and prepare/make all the needed proteins to start in the mitosis.

Then starts the last phase, the M phase or mitotic phase, this phase starts when the Cdk-cyclin B is active and is during this phase that the cells separate their genome and in the end divide into two new cells (Matthews et al., 2022; Yang, 2018), this phase will be described in detail in the next section.

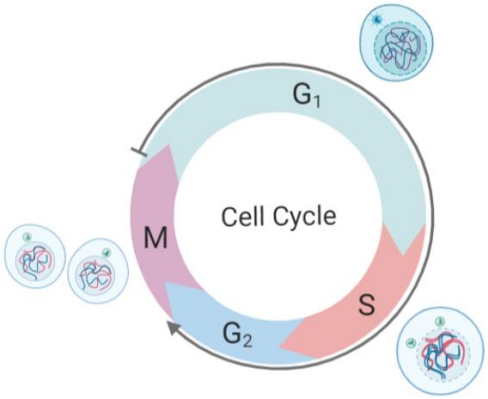

Figure 1: The cell cycle phases. Schematic representation of the four division of the cell cycle. The G1 phase where the cell increase in size and prepare all organelles and genome to start to be duplicated; the S phase where all the genome of the cell is duplicated in two exact copies; the G2 phase where cell prepare all molecular component to entry in mitosis; and Mitosis or M phase where cells separate all the genetic material in two identical daughter cells (Made by Beatriz Rodrigues, a previous student of the lab).

To control cell cycle progress through the different phases the cells have a system composed of a family of serine/threonine protein kinases called cyclin-dependent kinases (Cdks) and their regulators, the cyclins.

The Cdks have similar levels along all the cycles and while the different cyclins suffer from a cycle of synthesis and degradation. Exist around 9 different Cdks, but only four are directly responsible for the cell cycle control (Cdk1, Cdk2, Cdk4, and Cdk6), the other ones are involved in processes like gene transcription and differentiation process. The Cdks impair the control of this system by phosphorylating several proteins in the cell, but Cdks only have their enzymatic activity when they are in complex whit one of the cyclins and the different combinations of this complex (Cdks-Cyclins) is the responsible

for allowing the progression through the different phases (Alberts et al., 2008; Petsko et al., 2007).

It is possible to group the cyclins in four groups, the first are the cyclins D, which are responsible for the progression of the cells through G1, and for the transition between G0 to G1; the second are the cyclins E, which are responsible for controlling the S phase; the third are the cyclins A, which are responsible for controlling the progression through G2; and the fourth are the cyclins B, which are responsible to controls the entry in mitosis (M) (Figure 2) (Martínez-Alonso & Malumbres, 2020).

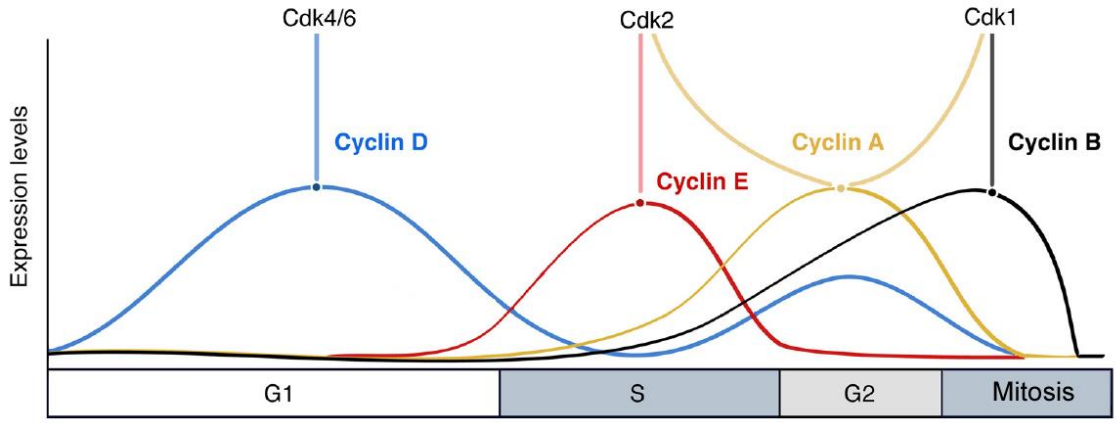

Figure 2: Cyclin expression levels. Schematic representation of the different levels of cyclin throughout the cell cycle and the respective Cdks with whom they interact (Figure adapted from Martínez-Alonso & Malumbres, 2020).

Besides the control of the Cdks-cyclins, cells keep many mechanisms like regulators and checkpoints to ensure the correct conclusion of each phase and prevent the prevalence of genetic mutations. These checkpoints are distributed along the different phases of the cycle, and each of them can monitor different types of problems, e.g during G1 and G2 phases the checkpoints monitor for DNA damage, during S phase monitor the loss of DNA replication fork integrity, and during mitosis, they monitor the incorrect spindle assembly.

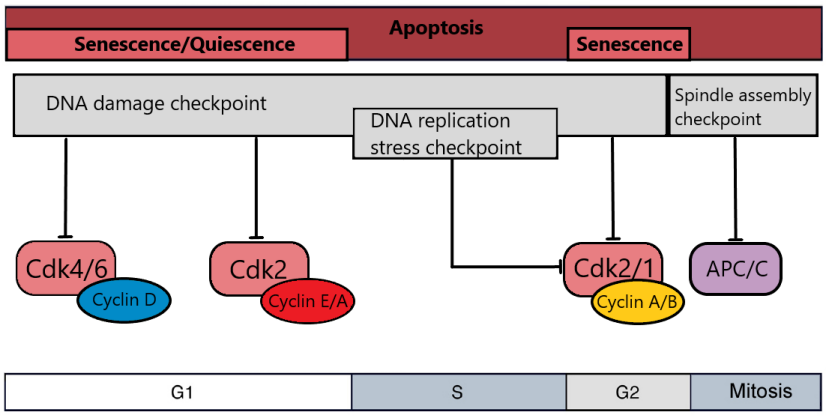

Figure 3: Cell cycle checkpoint and fates. Cell cycle checkpoints and how they can block the cell cycle, and respectively impact the cell fate (see top panel). The DNA damage checkpoint can block the cell cycle in interphase in three different moments, first during G1 can block the cycle by inhibiting the Cdk4/6-cyclin D, second in the transition to S phase by inhibiting the Cdk2-cyclin E/A this way blocking replication initiation, and third in G2 can bock the mitotic entry by inhibiting the Cdk1/2-cyclinA/B, in all the cases can leading cell to a quiescent or senescent state. The DNA replication stress checkpoint can block the progression to G2 and entry in mitosis in a similar way to the DNA damage checkpoint in the G2 phase by inhibiting the Cdk1/2-cyclin A/B and leading the cell to an apoptotic fate. The spindle assembly checkpoint can block the mitotic exit by inhibiting the activation of the APC/C complex and leading the cell to an apoptotic fate (Figure adapted from Matthews et al., 2022).

These checkpoints are capable of arresting or slowing down the progression of the cycle by inhibiting the activity of Cdks and the APC (anaphase-promoting complex). For example, the DNA damage checkpoint that, when find a double-strand DNA break, blocks the progression of the cell cycle through the ATM (ataxia telangiectasia mutated) that will inhibit the Cdk2; the replication stress checkpoint that when find single-strand DNA breaks, blocks the progression by the ATR (ataxia telangiectasia and Rad3-related protein) that will inhibit the Cdk1 and 2. Another example is the spindle assembly checkpoint that when find an incorrect attachment of a chromosome to the mitotic spindle, blocks the progression of mitosis by the inhibiting the APC complex.

After checkpoint arrest the progression of cell cycle, and cell fate depend on the severity of the damage. For instance, whether the cell is capable of repairing a problem defines if a cell can reenter cell cycle, but if the cell cannot repair it, then the cell would permanently exit the cycle and become senescent or enter in apoptosis - if the damages are to danger the cell (Figure 3) (Matthews et al., 2022).

1.3 Phases of mitosis

Mitosis is the last phase before cell division, this phase is composed of six stages. During this process, the cell suffers several changes in its composition and overall structure to ensure the correct division of the duplicated chromatin into the two daughter cells. This process needs to be highly regulated to ensure genome integrity, but also the equal division of the others many components known to participate in it (Rehman et al., 2022).

Overall mitosis can be divided into six stages: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis (Figure 4). In this section we described these stages (Rehman et al., 2022; Yang, 2018). In the following section, section 1.3.1, it is described the major components participating in the process of mitosis.

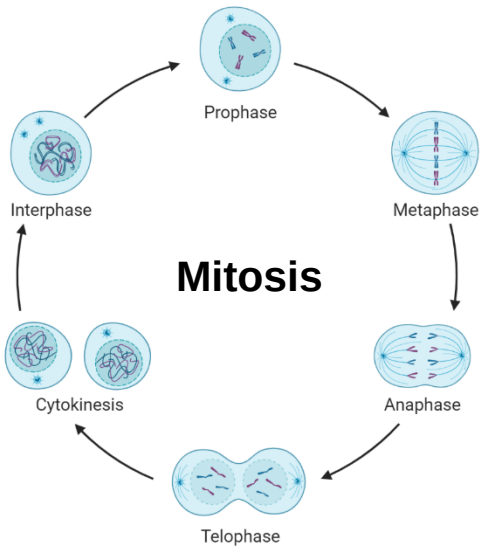

Figure 4: Mitotic stages. Representation of all stages of mitosis, initiating in prophase where the chromatin is condensed into chromosomes, the nuclear envelop is break down, and the duplicated centrosomes move to the opposite poles of the cell. Followed by metaphase where the chromosomes align at the metaphase plate with help of the mitotic spindle, and anaphase is when the sister chromatids are separated and each one goes to each mitotic spindle pole. Then in telophase chromosomes start to de-condense and a new nuclear envelop start to be form in each pole of the cell, to finish is the cytokinesis where each daughter cell is physically split creating that way two new completed separated cell, that then enter in interphase. Figure made using BioRender®.

During prophase, the chromatin starts to condense into the chromosome shape with the two sister chromatids are kept together at their centromeric region and the centrosomes begin to move into the opposite poles of the cell starting to reorganize the microtubules to create the mitotic spindle.

Prometaphase, in most of the cases is marked by the nuclear envelope disassembling, which allows the microtubules to bind to the kinetochores, proteins localized in the centromeric region of the chromosomes that allow the microtubules to attach to the chromosomes.

During metaphase, the chromosomes reach their maximum condensation and all of them are fully attached to the mitotic spindle. Then, they start to align at the metaphase plate (plane equidistant to both centrosomes) where chromosomes are in presence of equal pulling and pushing forces.

Anaphase is marked by the cleavage of the protein complex cohesin which has kept the sister chromatids together. Each sister chromatid begins to move to the opposite poles because of the pulling forces caused by the mitotic spindle.

During telophase, the mitotic spindle depolymerizes, and both sets of chromosomes already separate for each pole start to dissociate and one nuclear envelope begins to form around each one.

In the end, cytokinesis happens, which consists of the cleavage of the cytoplasm and cellular membrane forming two independent (and equal) cells (Rehman et al., 2022; Yang, 2018).

1.3.1 Overview of the components of mitosis spindle apparatus

1.3.1.1 Chromosomes

Chromosomes are thread-like structures present in the nucleus of the cells while mitotic chromosomes are made of condensed chromatin. Their basic organization is the double DNA strand wrapped around the histones - small proteins that organize themselves in octamers where the DNA can wrap around. Histones have N- and C- terminal tails that can be covalently modified to change the interactions between them, which will change the structure of the chromatin. This basic conformation is called nucleosome (one nucleosome is one octamer of histones with two spirals of DNA wrapped around it), and the nucleosome can be further compacted until it reaches the known form of the chromosomes (Figure 5)(Biterge & Schneider, 2014; Ohta et al., 2011). Yet remains

unknown how exactly the (mitotic) chromosome structure is nucleosomes pack together. Current field supports the loop extrusion hypothesis. In the most recent models, it is proposed that the DNA is extruded by the SMC proteins (e.g cohesin) creating cis DNA loops (more described at the section 1.5.5.2) (Davidson & Peters, 2021). Also, other proteins (e.g Topoisomerase II) can associate with the DNA and change their composition to increase the fidelity of the segregated chromosomes (Aragon et al., 2013; Baxter & Aragón, 2012). Yet, all the sequencing-based methods for characterizing 3D genome structure – like Hi-C- have been unveiling a much more complex and hierarchical organization within the chromosomes, and of these with the nucleosomes and linked proteins than previously anticipated (McCord et al., 2020).

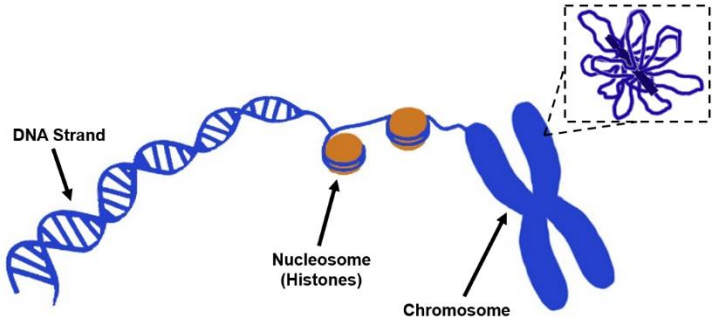

Figure 5: DNA composition. Schematic representation of the DNA compaction into the mitotic chromosome shape, from their double-strand format to the model of loop extrusion supported.

At this thesis we will focus on the structure of the chromosomes during mitosis. Upon replication, the chromosomes are composed of two sister chromatids, that are one exact copy of each other joint together at the centromeric region (see below the definition). Upon cell division, each chromatid goes to one of the daughter cells. In each chromatid, we can distinguish two main structures, the centromeres, and the telomeres.

1.3.1.2 Centromeres

The centromere is the region where the sister chromatids are connected (Figure 6 a)), this region shares a widely difference in their organization and structure across species. In human cells, a common definition of this region is based on epigenetics. This region is defined by an enriched in a specific variant of histone H3 called CENPA. The main function of the centromere in to guide the correct segregation of the sister chromatids by

serving as an anchoring place for the kinetochores, a complex of proteins that assembles on the centromeric region of the chromosomes that are involved in the process of attachment of the microtubules to the chromosomes (Ohta et al., 2011; Schalch & Steiner, 2017).

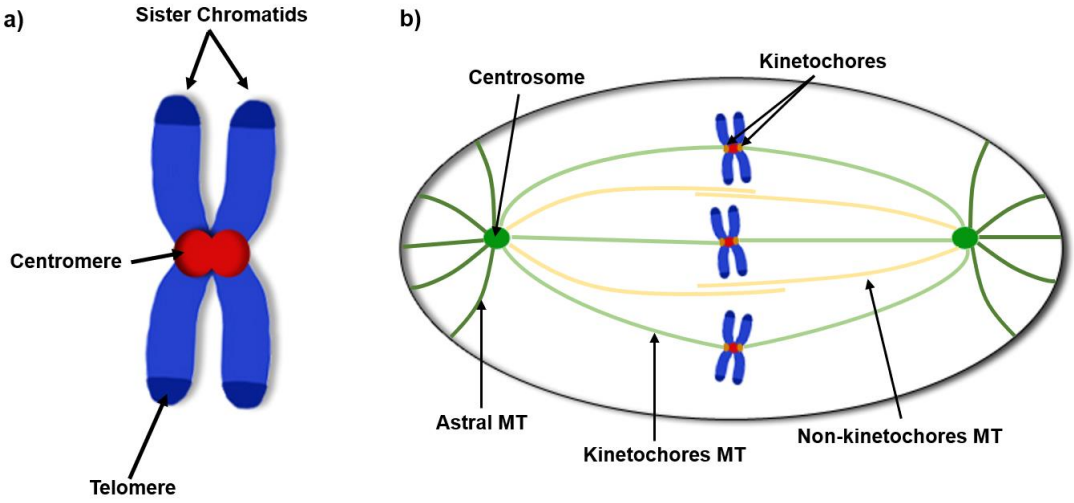

Figure 6: Components of mitosis. a) Schematic representation of a chromosome and its structures; b) Schematic representation of the mitotic spindle. MT means microtubules. Representation is not at scale.

1.3.1.3 Telomeres

The telomeres are the end region of each chromatid (Figure 6 a)), they are composed of repetitive regions rich in guanines that end in one single strand that folds back onto a duplex telomeric DNA. The length of this region is different according to different species, types of cells, and the age of the organism. Each time a cell replicates its DNA one little part of this region is lost leading to a shrinking of this region throughout the life of the organism. Their function is to protect the genome from degradation and prevent the cell mechanisms to recognize the extremity of the chromosome as a double-strand break (Aubert & Lansdorp, 2008; Blackbum, 1991).

1.3.1.4 Centrosomes

The centrosomes are a structure composed of two centrioles (cylindrical structures composed of microtubes) surrounded by a mass of pericentriolar matrix of proteins (PCM). Each centriole is in most cases is formed by nine microtubule triplets, organized in a symmetric way, creating a cylindrical shape. Both centrioles display structural and functional differences due to their time of creation, the older centriole is called mother centriole and present their full length and two sets of nine appendages at is distal end, while the younger centriole called daughter centriole only present around 80% of their length and do not display appendages, both centrioles are connected from their proximal ends and despite both being capable of nucleate microtubules in their vicinity only the mother centriole is capable of anchoret them (Figure 7)(Bornens, 2012).

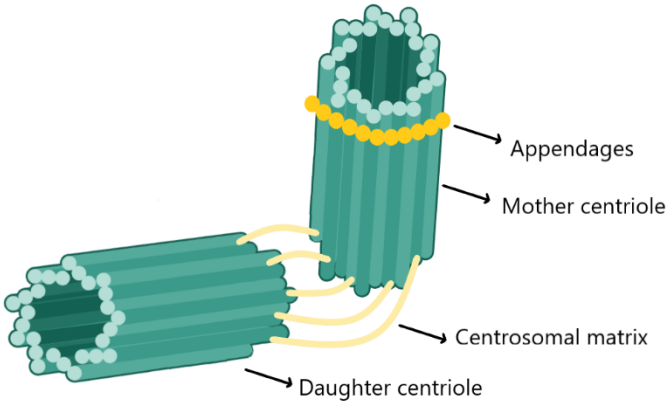

Figure 7: Centrosome structure. Representation of the main components of the centrosome.

The centrosomes are major microtubule organization center and are the responsible for the assembling of the mitotic spindle. At the centrosomes there is a plethora of centrosomal interactors - still being identified - yet, of interest there is a macro complex called γ-tubulin ring complex (γ-TuRC) that functions as a microtubule nucleator capping the minus end of the microtubules, this way stabilizing and anchoring the microtubules to the centrosomes.

During most of the cell's life, it only has one centrosome but when the cell transit from G1 to S phase, start to happen the duplication of the centrioles. During the first stages of mitosis the two-centrosome star to go in the opposite direction to form the bipolar spindle (Figure 6 b)) (Arquint et al., 2014; Azimzadeh & Marshall, 2010; Guilloux & Gibeaux, 2020). It is now appreciated that the regulation of the centrosome number

and duplication is highly connect with the regulation and progression of the cell cycle. This has opened interesting link between pathologic conditions and abnormal centrosome function and regulation beyond centrosome canonical role as a microtubule organizing center (more described at the section 1.4.2).

1.3.1.5 Kinetochores

The kinetochores are a platform of protein complexes composed of many proteins, that are assembled at the chromosomes on the centromeric region. This structure has the function of linking the chromosomes to the microtubules. The establishment of appropriate kinetochore microtubules attachments supports faithful chromosome segregation.

The kinetochores can be divided into two main regions, the inner kinetochore and the outer kinetochore, each one having its specific function, the inner kinetochores are assembled on the centromeric region of the chromatid and work as a scaffold to the assembly of the outer kinetochore, while the outer kinetochore is responsible for the interaction with the microtubule (Figure 8).

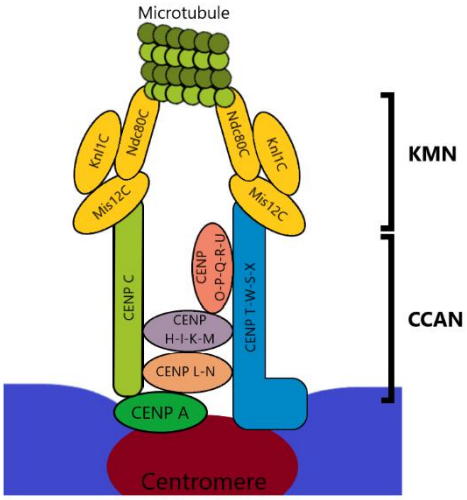

Figure 8: Kinetochore structure. Representation of the two main region of the kinetochores, CCAM, KMN, and their localization at the centromere, more specifically at the CENPA sites. The CCAM is assembled at the CENPA and then KMN is recruited to the CCAM and mediates the binding to the microtubules (Figure adapted from Dong & Li, 2022).

At the inner kinetochores can be found 16 proteins that can be divided into five groups (CENP-L-N, CENP-H-I-K-M, CENP-O-P-Q-R-U, CENP-T-W-S-X, and CENP-C), that together are known as constitutive centromere-associated network (CCAN) in vertebrates, it is known the CCAN is constitutively found at the centromeric region throughout all the stages of the cell cycle, but the mechanisms underlying the dynamic behavior of the CCAN is still barely known (Figure 8).

And at the outer kinetochores can be found 10 proteins that can be divided into three complexes (Ndc80C, Mis12C, and Knl1C), together these complexes are known as the KMN network, this complex is assembled on top of the inner kinetochore and by the Ndc80C complex, it is capable of bind the microtubules. The KMN network unlike the CCAN only is assembled at the onset of the mitosis (Figure 8) (Dong & Li, 2022).

The kinetochores are involved in the checkpoint that ensures the correct chromosome segregation - the SAC checkpoint and the error-correction mechanism mediated by the Aurora B kinase (Saurin, 2018)(see section 1.7.3.1 for further details).

1.3.1.6 Microtubules

The microtubules are composed of dimers of α- and β- tubulins. The microtubules are a dynamic structure that is continuously polymerizing and depolymerizing at a rapid cycle which is an important characteristic because allows the microtubules to remodel very fast. They are polar structures with two different ends, the minus end where the polymerization of the microtubules is slower, and the plus end where the polymerization is faster (Figure 9) (Prosser & Pelletier, 2017).

The dynamic of the microtubules is based on the velocity of growth and shrinkage and the frequencies of the transition between the growth and shrinkage phases, this way we can define two phenomena, the catastrophe when they start to depolymerize and shrink, and the rescue when they start to polymerize and growth.

During interphase, the microtubules work like a cytoskeleton to the cell, giving a structural conformation to the cell and also serving as a track for vesicles and organelles to be transported on by motor proteins, then in the onset to mitosis, this structure is disassembled and will be used to originate the mitotic spindle (more described in the next section) (Guilloux & Gibeaux, 2020).

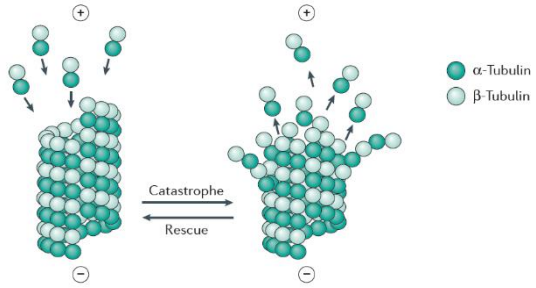

Figure 9: Microtubules structure and dynamics. The microtubules are composed of dimer of α- and β- tubulins and have two different poles a positive pole and a negative pole. They pass through a dynamic switching between growing (Rescue) and shrinking phases (Catastrophe) (Figure adapted from Prosser & Pelletier, 2017).

1.3.1.7 Mitotic spindle

The mitotic spindle is a dynamic structure composed of microtubules and associated proteins. It assembles during mitosis and is essential for the orientation of the chromosomes and their segregation, this way orchestrating the equal division of the chromosomes into the daughter cells. The spindle is composed of two different poles, one on each side of the cell where the microtubule-organizing center or centrosomes is present. On the onset of mitosis, after the microtubule cytoskeleton structure is depolymerized, the microtubules rearrange into a new conformation, that emerges from both centrosomes, and change their dynamics to facilitate the capture of the chromosomes.

In this new structure is possible to identify three different groups of microtubules, each one with distinct functions, the k-fibers or kinetochores microtubules, the astral microtubules and the non-kinetochore microtubules (Figure 6 b)).

(i) K-fibers which attach the spindle poles to the chromosomes via the kinetochores, mediating the chromosomes movements and alignment at the metaphase plate;

(ii) Astral microtubules localized at the spindle poles and work to attach the spindle poles to the cell cortex, helping the positioning of the spindle;

(iii) Non-Kinetochore microtubules which attach to other microtubules coming from the opposite pole in an antiparallel manner forming an overlapping between them, this way providing stability to the spindle, helping to create tension between the sister chromatids, and having roles in the spindle elongation.

(Guilloux & Gibeaux, 2020; Lacroix & Dumont, 2022; Prosser & Pelletier, 2017).

The assembly of the mitotic spindle is modulated by a theory called "search-and-capture" (Kifschner & Mitchison, 1986), this theory says that the kinetochores microtubules probe the cytoplasm until they are captured and stabilized by one kinetochore (when the chromosome is only captured by one pole they are called mono-oriented and then when the chromosome is captured by both poles star to be called bi-oriented). But this model only relies on the dynamic instability of the microtubules, and this will make this process take a long search time, which is not seen, then was identified that the chromatin has a biochemical environment that helps the polymerization of the microtubules. In this environment exist several factors, but some of them have a more prominent function like the RAS-related nuclear protein (RAN), and chromosome passenger complex (CPC).

RAS has two forms, the RAN-GTP and the RAN-GDP (depending on what it is bounded to a GTP or to a GDP), and is regulated by two factors, in the cytoplasm is by the RAN GTPase-activating protein 1 (RANGAP1) and on the chromatin by the guanine nucleotide exchanger factor RCC1 (regulator of chromosome condensation 1). This dual regulation creates a gradient of RAN-GTP around the chromosomes, this RAN-GTP binds to the importin-β which is one inhibitor of the SAFs (more described in the next section), like TPX2 (a recruiter of Aurora A kinase) and NLS (a stabilizer of the kinetochore microtubules) that which will promote the polymerization of the microtubules in the proximities of the chromosomes.

CPC (described in detail at section 1.7.3.1) via Aurora B will inactivate microtubule destabilizing proteins such as the MCAK and the STMN1, which will promote the polymerization of the microtubules at the centromeric region (Guilloux & Gibeaux, 2020; Prosser & Pelletier, 2017).

1.3.1.8 Mitotic spindle associated proteins

The proteins associated with the mitotic spindle can be divided into spindle assembly factors (SAFs) and microtubule-associated proteins (MAPs), the assembly of microtubules is a dynamic process, and depended on the local concentration of tubulin dimers, SAF proteins can change this special concentration of tubulin to regulate the spindle assembly, and the MAP that can be a regulator of the polymerization and depolymerization of the microtubules or can be motor proteins that can move along the microtubules.

The MAPs can be divided into several categories: (i) stabilizers that increase the polymerization; (ii) destabilizers that decrease the polymerization; (iii) capping that binds to the ends of the microtubules and stops both the polymerization and depolymerization, (iv) bundlers or cross-linkers that bind two microtubules laterally; (v) cytoskeletal integrators that bind the microtubules to the other cytoskeletal elements, and (vi) motor proteins. These last one can associates to the microtubules and allow other microtubules, proteins or organelles to use the microtubules as a road map inside the cell. Under this last category we also can highlight kinesins that only goes in the microtubules plus direction and dyneins that only go in the minus direction (Goodson & Jonasson, 2018; Prosser & Pelletier, 2017).

1.3.2 Cell cycle Checkpoints

The checkpoints are specific moments in the cell cycle where the cell as the name suggest checks the integrity of the cell before proceeding to the next phase, which will not happen until satisfying all the requirements of the specific checkpoint. If the cell cannot repair the problems a specific mechanism marks the cell to apoptosis, as a way to maintain genome integrity.

During the cell cycle, four main checkpoints exist, three of them in the interphase and one in mitosis (Figure 10). In the interphase, there are two DNA damage checkpoints, one on G1/S which arrests the cell in G1, just before the entry into S phase in case the environmental conditions are unfavorable to the division (like the presence of DNA damage or lack of growth factors), and other on G2 which stops the cell to enter in mitosis

if exist any error in the DNA or are any incomplete replication, then exist one DNA replication stress checkpoint in the S phase, which delays the progression through the S phase while the cell tries to repair any errors that occur during the replication process. Then in mitosis are a spindle assembly checkpoint, that arrests the cell between metaphase and anaphase until all chromosomes are correctly attached to the mitotic spindle (Rehman et al., 2022; Yang, 2018).

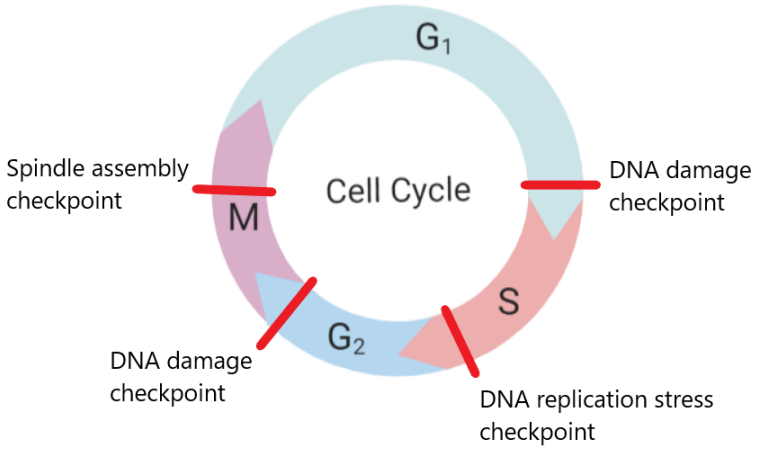

Figure 10: Cell cycle checkpoint in the cycle. Schematic representation of the localization of the cell cycle checkpoint through the four phases (G1, S, G2, and M).

DNA damage checkpoint: this checkpoint is triggered by the occurrence of DNA double-strand breaks. Upon these breaks via the protein kinase ataxia telangiectasia mutated (ATM) activates a signaling pathway trigger by and as a result cell cycle progression slows down or arrest. When occurring a break, the DNA damage sensor complex MRN is activated leading to a phosphorylation/activation of the ATM, that creates a successive activation of protein ending with the activation of the p21 (ATM → CHK2 → p53 → p21, as represented on the Figure 11). Then, the p21 inhibits the Cdk-cyclin complex (Cdk2-cyclin E) stopping the cell cycle progression to the S phase.

In the G2 phase, the activation process is the same but instead of the CHK2 activating the p53, the CHK2 will inhibit the CDC25, one of the activators of the Cdk1-cyclin B (the Cdk-cyclin complex responsible for the initiation of the mitosis).

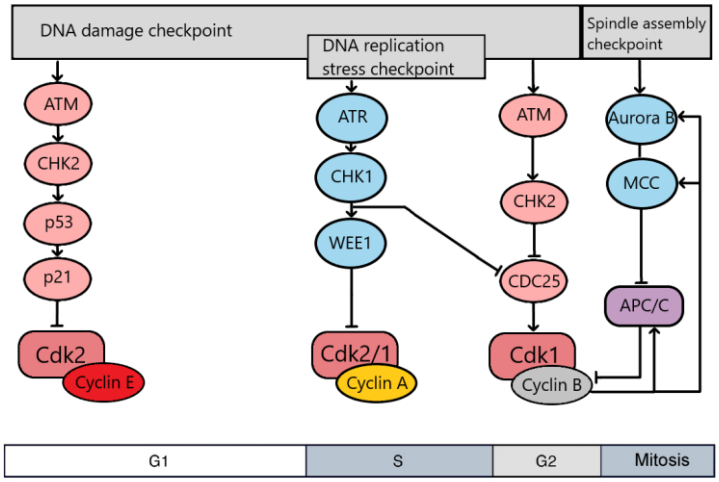

Figure 11: Cell cycle checkpoints pathways. Representation of the pathways by each cell cycle checkpoint act to block the progression of the cycle. In the DNA damage checkpoint, the damage activates ATM which will lead to the activation of p21 that inhibits the Cdk2-cyclin E during G1, and in G2 the ATM will lead to the inhibition of the CDC25 what will prevent the activation of the Cdk1-cyclin B. In the DNA replication stress checkpoint, the replication stress will activate ATR that will inhibit the Cdk2/1-cyclin A and at mitosis the spindle assembly checkpoint by the Aurora B will inhibit the activation of the APC/C complex preventing the progression of mitosis (Figure adapted from Matthews et al., 2022).

DNA replication stress checkpoint: this checkpoint is triggered when the replication fork encounters an obstruction that slows or stalls its progress (creating replication stress). It can be caused by obstruction from endogenous or exogenous sources, like the deregulation of components of the DNA replication machinery, unusual DNA structures, DNA damage and interference of the transcription machinery. This checkpoint has as its main objective preventing the creation of DNA damage because when the fork progression is slowed or stalled this will leads to the exposure of single-strand DNA, that are more prone to be damaged.

This accumulation of single-strand DNA will activate the protein kinase ATR, that are responsible for activating the CHK1, which is involved in the activation of WEE1 an inhibitor of the Cdks-cyclins complexes. But CHK1 is also capable of inhibiting the CDC25, like the CHK2 in the DNA damage checkpoint in the G2 phase, that way both CHK1 and CHK2 are responsible for inhibiting the CDC25, preventing the progression to mitosis (Figure 11).

Spindle assembly checkpoint (SAC): this checkpoint has the function of delaying mitosis progression until the proper kinetochores-microtubules attachment, it is constitutively triggered while existing unattached or incorrectly attached kinetochores, working like a surveillance mechanism that prevents incorrect chromosome segregation. This checkpoint is composed of a multiprotein complex that is present in all kinetochores and has as one of its main components the protein kinase Aurora B (which will be more extensively described in section 1.7.3.1),

After the microtubule attaches to the kinetochore, it will create a pulling force in the direction of the pole, when both kinetochores are connected to the correct opposite spindle poles will make the opposing forces compete creating a tension force through the kinetochores and centromere. That tension can act as a signal capable of destabilizing erroneous kinetochore-microtubules attachments and benefitting the correct attachments.

The cells only can pass and continue through mitosis when they can create a robust tension, if not they will stay locked in mitosis, and the longer time they have arrested the higher risk of suffering from chromosome mis-segregation. This happens because tension is capable of altering the kinetochores and centromere structure, which can modulate the Aurora B and SAC activity (the main regulator of the metaphase to anaphase onset).

Aurora B is one of the main parts of this process, being the major component of the error correction mechanism, a mechanism that destabilizes the erroneously attached microtubules giving them a second chance to reattach to the correct kinetochore. This happens because Aurora B is capable of phosphorylating components of the kinetochores, this way impossibility the stable attachment of the microtubules. The process by which the tension is capable of avoiding this Aurora B activity is still a debate, some models have been proposed but one of the most popular suggests that the structural changes created by the tension are capable of physically displacing the kinetochore components that are phosphorylated by the Aurora B from the range of action of the Aurora B, allowing the stable connection (Bunning & Gupta Jr., 2023; Matthews et al., 2022; Yang, 2018).

1.4 Aneuploidy

The cell cycle is a complex process that needs to be highly regulated. So many proteins and checkpoints (as described in the previous section) exist to control this whole process and prevent disinherited DNA in the formed cells. If problems occur in these control points, then erroneous segregation at the daughter cells with loss or gain of parts or entire chromosomes may arise if, daughter cells survive. This erroneous segregation can be caused by several problems, either mitotic or pre-mitotic problems. Pre-mitotic problems can come from abnormalities in the DNA structure originated during DNA replication and repair. Mitotic problems can come from failures during mitosis like incorrect attachment of the mitotic spindle to the chromosomes, defects in the SAC checkpoint and cohesion defects. Depending on the origin of the problem the cell can suffer from different outcomes, in pre-mitotic cases, the cells can suffer from the gain or loss of parts of the chromosomes while in mitotic cases the cell can suffer from the gain or loss of entire chromosomes (Garribba & Santaguida, 2022; Vasudevan et al., 2021).

These daughter cells with abnormal karyotypes - defined as the number and appearance of the chromosomes present in one specific species (Tosh et al., 2022) - are called aneuploid cells. The origin of aneuploidy cells can be multi factorial and there is still some debate about its source. Related to aneuploidy is the concept of, chromosomal instability (CIN) which is the frequency of mis-segregate chromosomes, that means of having aneuploidy (Ben-David & Amon, 2020; Vasudevan et al., 2021).

Aneuploidy can be characterized by a wide range of genomic abnormalities, like polyploidy (presence of copies of all chromosomes), whole chromosome aneuploidy (presence of copies of only one chromosome), partial aneuploidy (presence of copies of a part of one chromosome) (Martin et al., 2015).

1.4.1 Aneuploidy and human diseases

Aneuploidy is associated with abnormalities in cell function (Orr et al., 2015) because genomic abnormalities may result in the gene expression changes and/or alterations that are propagated to the daughter cells which affect many cellular mechanisms like cellular proliferation, protein stability, and genomic stability and cellular signaling. Although these changes are deleterious to the fitness of the cells, in most cases

harmful, some types of cells present in a “natural way” aneuploidy (examples of human tissues that present this are the liver and the brain). Still unclear the why these specific types of cells beneficiate (if at all) of these changes, but some speculations say that they help the cell to adapt to nutritional and noxious stresses (Garribba & Santaguida, 2022).

Yet in most cases aneuploidy is involved in the development of certain pathologies like developmental diseases and cancer

1.4.1.1 Cancer

In the majority of the cancer is possible to identify some type of aneuploidy and studies have demonstrated that these karyotype changes confer resistance to chemotherapy because this increasing karyotype heterogeneity can be used to find the correct karyotypic landscape to help the cell survive adverse environments (Garribba & Santaguida, 2022). Cancer is originated from genetic alterations, that can vary from single mutations to the loss or gain of complete chromosomes. These genetic alterations change the behavior of the cells making them grow and proliferate uncontrollably, creating a mass of cells called tumors, then when this mass gains the capability to grow and spread to other surrounding organs, it starts to be called cancer.

The CIN promotes tumorigenesis (the process of development of a tumor) because they contribute to the genetic heterogeneity of the cell this way helping the tumor cell to gain or lose some functions, and creates abnormalities in the cellular transcriptome and proteome, these changes lead to alterations in the levels of factors that could impair several mechanisms like DNA replication and repair this way creating more CIN which leads to more aneuploidy and tumorigenesis. CIN promotes aneuploidy, so in some way, aneuploidy is related to tumorigenesis, and in fact, aneuploidy is a hallmark of tumorigeneses. In fact, approximately 90% of tumors display some degree of aneuploidy. The degree of aneuploidy seen in the cancer is different depending on the type of cancer, in some cases, it is possible to see some specific patterns of aneuploidies more frequently while in other types of cancer they have no specific patterns of aneuploidy and have low levels of aneuploidy. The range of frequency of aneuploidy in cancer can change from 25% (e.g thyroid carcinomas) to nearly 100% (e.g glioblastomas) (Vasudevan et al., 2021).

The appearance of aneuploidy in cancers arises frequently in the first stages of tumorigenesis, but in some cancer, specific aneuploidies are only seen in late stages, possibly because only in that stages the cells have acquired some mutations that allow them to survive to this aneuploidy.

The aneuploidies lead to changes in gene expression. As an example, the gain or loss of part or whole chromosomes leads to an increase or decrease in the expression of the genes present in that part or whole chromosome (Vasudevan et al., 2021).

But how the aneuploidy itself contributes to the development of the tumor is still an area of investigation. During the development of the tumor while having an increase of the aneuploids also occurs an increase of the mutations so it is hard to understand if the problems and changes seen are caused by the mutations that are appearing or due to the mutations. Also, unlike the oncogenes and tumor suppressor genes (genes involved in the development of the tumor: oncogenes when mutated will lead to the development of a tumor, and tumor suppressor genes when intact they prevent the development of the tumor) that lead to direct changes in all cell types, aneuploidy genomic alterations can have different behavior depending on the situations and the cell type. Aneuploidy can be beneficial - helping the progression of cancer - or deleterious. Recent studies demonstrated that aneuploidy could act as an oncogene or a tumor suppressor gene. The changes in the karyotype present in the aneuploidy cells make them have delays in the cell cycle, metabolic alterations, and genomic instability. Due these changes in some cases this cell ends up being overcome by the normal cell. This way there is a behavior like a tumor suppressor gene because these cells present a decreased fitness and tumorigenicity. On another side in some cases, these changes in the karyotype of the cell end up providing a selective advantage to the cell making them more resistant to certain environments and in some cases resistant to certain drugs (Ben-David & Amon, 2020; Vasudevan et al., 2021; Garribba & Santaguida, 2022)

1.4.1.2 Developmental and Rare Disease

Aneuploidy cells beside are involved in the susceptibility to cancer also have implications for the development of other diseases, like developmental and rare diseases.

The definition of rare diseases has some variations between the different regions, but by the legislation of the European Union, a disease is considered rare when it affects fewer than 1 in 2000 people. The majority of them are originated by genetic problems but exist also some infectious diseases and cancers that enters in this classification. The bigger problem with this type of diseases is the difficulties in the diagnostic, that is often long and in some cases wrong, this problem came first because of the few information available for each disease and because of problems in the consistency in defining the disease, so far the most common estimation of how many rare diseases exist is that vary between 5000 and 8000, but some recent studies start to points to more than 10000 (Haendel et al., 2020).

Approximately one-third of all spontaneous abortions are caused by aneuploidies, and between 10 to 30 % of all fertilized eggs are aneuploid prior to implantation (Hassold & Hunt, 2001). Aging infertility in women is also related to aneuploidy because with the age the levels of aneuploidy increase this way compromising the successful survival of the fetus. In women's under the age of 25, the percentage of trisomic pregnancies is 2% while in women's over the age of 40 this percentage increase to 35% (Oromendia & Amon, 2014; Tosh et al., 2022).

Aneuploidy in most cases is not compatible with human life, only a few aneuploidies are supported. Aneuploidy in eggs is a leading cause of miscarriages and infertility as referred before, this aneuploidy comes from chromosome segregation errors that in the case of the oocytes can occur during the two meiotic divisions. Exist a high occurrence of aneuploidy in the human oocytes because in these cells it is seen an inherent instability of the spindle allowing erroneous kinetochores-microtubules attachments, and the SAC at the human oocytes lacks stringency allowing the progression through mitosis even with incorrect attachments. The human oocyte spindle does not display centrosomes or any type of microtubule organizer, so their spindle assembly is more slow and only dependent of the chromosomes, this way reflecting in a more frequent appearance of errors. Also, the oocytes in it form of the primordial follicle are arrested for long periods of time, when a female born already has all of their oocytes, so at the moment of pregnancy the oocytes already have several years, and during all that time it can be exposed to several exogenous sources of damages that deteriorate the integrity of the egg, also in the mouse, oocytes have been seen with the age exists a gradual dissociation of the cohesin from the chromosomes, leading to premature separation of the chromosomes,

which could be a driver of the aneuploid phenomenon seen at the oocytes (Thomas et al., 2021).

How these aneuploidies can lead to specific phenotypes is still not understood. Although aneuploidy in most cases leads to spontaneous abortions, some studies in mice have shown that if only a part of the embryonic cells is aneuploidy, the embryo has the chance of self-correct towards euploidy and continuing the gestation, but so far this mechanism exists in human embryos and if exist what is its frequency of occurring (Bolton et al., 2016).

In humans, it is possible to divide the aneuploidies into two major groups, depending on which type of chromosome has been gained or lost, it can be in the 22 autosomal chromosomes – called autosomal aneuploidy or in the X or Y sex chromosomes – sex linked chromosome aneuploidy (Tosh et al., 2022). Also, aneuploidy type can be distinguished by where the defect has occurred, if occur in meiosis or in mitosis. When fertilization of an aneuploid gamete, is generated by defective chromosome segregation in meiosis creates a constitutional/germinal aneuploidy. When defective chromosome segregation takes place in mitosis creates a somatic aneuploidy (Orr et al., 2015).

Autosomal aneuploidies:

In humans, the most common autosomal aneuploidy is the trisomy of chromosome 16. This trisomy is incompatible with life and is responsible for 6% of all spontaneous aborts between 8 and 15 weeks of gestation. Only in some cases of mosaicism, the body is capable of tolerate this aneuploidy leading to the born of children with developmental issues that depend on the level of mosaicism (Tosh et al., 2022). Like with the case of the trisomy of chromosome 16, the majority of trisomies are incompatible with life in humans, only the 13, 18, and 21 trisomies are tolerated.

Trisomy 21 also known as Down syndrome (DS) is the most known trisomy, having an incidence that vary between 1 in 1 000 to 1 in 1 100 live births worldwide. The patients have several clinical features, some of them invariable (that are present in all patients, only differing in severity) like learning and memory defects, characteristic facial dysmorphology, high-risk of early onset Alzheimer type dementia, and some of them

variable (not all patients have) like congenital heart defects, increased incidence of blood cancer, and elevated risk of autoimmune disease.

Trisomy 18 also known as Edwards syndrome is the second most known, having an incidence of 1 in 6 000 live births, but its overall incidence counting the fetal loss and pregnancy termination after prenatal diagnosis is 1 in 2500. These patients have as clinical features several prenatal growth deficiencies, characteristic facial features, and a distinctive hand posture with overlapping fingers, and normally present heart malformations.

Trisomy 13 also known as Patau syndrome is the third most known and has an incidence of 1 in 10 000 living births. These patients have as clinical features slow prenatal development, holoprosencephaly, heart defects, cleft palate, and these children have a mortality rate between 90 and 95% one year after birth.

Sex chromosomes aneuploidies:

Unlike autosomal aneuploidies, sex chromosome aneuploidies are more tolerated by humans. The five most known are the Klinefelter syndrome, only seen in men and is characterized by the presence of two X chromosomes (XXY) and having an incidence of 1 in 750 live births; the Turner syndrome, only seen in women and is characterized by the presence of only one X chromosome (X) and having an incidence of 1 in 2500 live births; the trisomy X, only seen in women and is characterized by the presence of tree copies of the X chromosome (XXX) and having an incidence of 1 in 1000 live births; the XYY and XXYY syndromes, only seen in men and being characterized by the presence of a second Y chromosome and a second pair of X and Y chromosomes respectively, they have an incidence of 1 in 1000 and 1 in 20 000 live births respectively.

These syndromes present mild phenotypes, most likely because of the random inactivation of the X chromosomes, because of that no matter how many X chromosomes the cell has, only one stays fully activated, and the other ones only have approximately 15% active. On another side, the absence of X chromosomes is incompatible with life, most likely because the X chromosomes are responsible for approximately 5% of the genome (Tosh et al., 2022).

1.4.2 Causes of chromosome instability and aneuploidy

Genomic instability is associated with genetic changes that can be from single nucleotide loss/modification to elimination or gain of entire chromosomes. CIN is one type of genomic instability and is responsible for an increased rate of changes both in chromosome number and chromosome structure, these changes then result in karyotypic alterations like whole-chromosome aneuploidy or partial aneuploidy. CIN can come from different points of the cell cycle, and it is possible to distinguish between mitotic and pre-mitotic defects. The mitotic defects are normally characterized by whole-chromosome aneuploidies, that result from erroneous chromosome segregation caused by the incorrect attachment of chromosomes to the mitotic spindle. In another hand, pre-mitotic defects are normally characterized by partial aneuploidies, that result from defective repairs or replication of DNA, these defects increased the generation of DNA damage that can affect the integrity of the chromosome structure. The DNA damage can be caused by exogenous sources like radiation and chemical compounds, and also by endogenous sources like telomere dysfunctions, defective DNA repair, and defective DNA replication (which can be identified by the enrichment of chromosomal breakpoints and loss of heterozygosity). Recently it has been also appreciated partial aneuploidies can arise from anaphase bridges breakages and a consequence of chromothripsis of whole-chromosome aneuploidies (Burrell et al., 2013; Siri et al., 2021).

The CIN can be distinguished into two types, the numerical CIN and the structural CIN, the numerical CIN is characterized as the loss or gain of entire chromosomes and leads to aneuploidy, this gain or loss can be caused by several problems since chromosomes segregation errors, defective mitotic spindle, cohesion defects, erroneous microtubule-kinetochore attachment, chromosome condensation defects, and defective cytokinesis. While structural CIN is characterized as the structural changes in the chromosomes, which can be chromosomic rearrangements like chromothripsis, amplifications, deletions, and translocations of part of chromosomes, and are caused by DNA replication stress, DNA repair problems, defective telomeres or a mitotic defects like lagging chromosomes (Kwon et al., 2020).The mitotic defects, as was said before, result in defects of chromosome segregation such as lagging chromosomes, DNA bridges, micronucleus, multipolar spindle, among others (Figure 12).

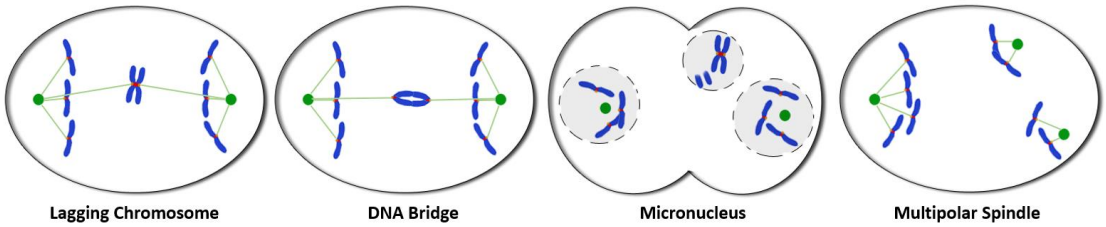

Figure 12: Chromosome segregation errors. Representation of different chromosome segregation errors.

Lagging chromosomes: as the name suggests chromosomes lag in the metaphase plate during anaphase, they appear mainly because of problems during mitosis like the abnormal microtubule-kinetochore attachment (merotelic attachment), centrosome amplification problems (multipolar spindle), aberrant spindle assembly checkpoint, and defects in sister chromatid cohesion. After the formation of these chromosomes, if they could not rejoin their mass of chromosomes, they end up being lost, or in some cases, they form a micronucleus.

DNA bridges: make a connection of DNA – bridge – between the two masses of chromosomes present in the cells during the last phases of mitosis, they are created when part of the chromosome is simultaneously pulled from both mitotic spindle poles during chromosome segregation. There are two types of DNA bridges.

- The Bulky DNA bridges are composed of double-stranded DNA and have the presence of nucleosomes. They can be originated from the erroneous binding of two DNA ends that have lost their end-caping (telomeres), dicentric chromosomes by the erroneous binding of two non-homologous chromosomes, from chromosomes with Holliday junction structure (an X-shape chromosome where the strands of two chromosomes are intertwined) that can happen when replications fork stall and then the homologous recombination repair joint by mistake two different chromosomes, and from chromosomes with cohesion defects, in all these cases because the two chromatids are in some way untangles when the cell start to pole both chromatids to the opposite poles will create a bridge between them.
- The ultra-fine bridges on the other side are mainly composed only of single strands of DNA (in recent studies also have been detected some ultrafine bridges composed of double strands, originated by homologous recombination events

(Chan & West, 2018)) without any nucleosomes, they can be originated mainly from the accumulation of under-replicated DNA that occurs when the DNA replication is interrupted by the stall of the replication forks in both ends of the replication bubble, also can be originated from the centromeric and telomeric regions, because they tend to be replicated late and have a high chance of creating secondary DNA structures that end up stalling the replication fork, in both this cases because the transition from S to G2 phase only requires the absence of replication and not the correct end of replications, the cells can progress throw the cycle and enter in mitosis with accumulated regions of single-strands DNA, and lastly, the unresolved DNA catenation also can be responsible for originating some ultra-fine bridges.

Micronucleus: are little nucleus that can be formed from chromosome fragments or whole chromosomes that fail to be absorbed by the chromosome mass during the end of mitosis, these fragments or whole chromosomes can come from DNA bridges that after cytokinesis end up being cut off the nucleus, or from lagging chromosomes like referred before. In the end, this extra nucleus could end up being reincorporated into the main nucleus by for example chromothripsis or expelled from the cell (Siri et al., 2021).

Multipolar spindle: are aberrant mitotic spindle that have supernumerary centrosomes, this way losing the bi-orientation necessary to the correct chromosome segregation. These spindles may result from two different events: centrosome overduplications or loss of spindle pole integrity.

In the centrosome overduplication we see the appearance of multiple copies of the centrosome. In normal conditions the centrosome replication only occurs once every cell cycle and by duplicating an existent centriole (mother centriole). When problems like de novo centriole assembly occurs in the cell create a new centriole without a template centriole. This defect can also be caused by cytokinesis failure where the duplicated centrioles that are supposed to be separated to the new cells end up going both to the same cell. Another process is by mitotic slippage, when the cell ends up leaving mitosis without being separated in two new cell (occurs due to the reduction of the level of cyclin B), this way creating a cell with a double of its DNA (tetraploid cell). Trough cell fusion, similar to cytokinesis failure in a way, two cell come together thus creating a cell with twice the

genome and internal organelle leading also to the formation of multiples centrosomes (Brownlee & Rogers, 2013; Maiato & Logarinho, 2014).

In the case of loss of spindle pole integrity - instead of having the appearance of a whole new centrosome - what happen is the appearance of multiples centrioles and premature centriole disengagement. This creates multiples “centrosomes” composed of only one centriole. This can happen because in normal conditions the centriole was supposed to be joined since the S phase until end of mitosis where they need to be separated to allow them to be duplicated. Duplicated centrosomes are kept together by the cohesin, so their separation is dependent of the separase. If any problem interferes with e.g the inhibition of separase to prevent an early separation will lead to an premature separation creating multipolar spindles (Maiato & Logarinho, 2014).

1.4.3 Mitotic defects that cause chromosome instability and aneuploidy

It has long been appreciated that distinct problems at mitosis - as briefly referred above - can contribute to aneuploidy and chromosome instability. In here we enumerated known main causes of erroneous segregation of chromosomes mainly based on two revisions on the topic Orr et al., 2015 and Prosser & Pelletier, 2017.

Defects in the spindle assembly checkpoint (SAC) stops the progression of the mitosis until all the chromosomes are correctly attached to bought centrosomes. While kinetochores are unattached the SAC is active, which goes produce an inhibitor complex that prevents the binding of Cdc20 to the Anaphase Promoting Complex (APC/C) which is responsible for ubiquitinate the Securin this way freeing the Separase, an important enzyme involved in the anaphase onset. As such, it is necessary a correct attachment to deactivate SAC allowing the progression of mitosis from metaphase to anaphase (Varetti et al., 2011). Importantly the cell does not have an ability to arrest in mitosis permanently. Therefore, cells after some time arrested are capable of bypassing the checkpoint. The mechanism underlining this bypass are not completely understood (Sinha et al., 2019) by e.g SAC positive or negative feedback by be altered due impaired or hyper-activated signaling. SAC arrest can also be overcome by degradation of cyclin B1 leading to the liberation of functional Separase. This escape of the checkpoint is called mitotic slippage and when this occurs the sister chromatids will not be correctly separated from the

daughter cells leading to chromosome mis-segregation (Brito & Rieder, 2006; Orr et al., 2015).

Defects in Kinetochore-microtubule attachment. For correct segregation to take place, its necessary that both kinetochores in each chromosome are attached in a bioriented way where each kinetochore is bound to microtubules that come from opposite spindle poles. Kinetochore-microtubule attachments are a stochastic process and error-prone, where alternative attachments can culminate in erroneous segregation of the chromosomes during anaphase. Incorrect attachments at the kinetochore level are sensed by a mechanism mediated by Aurora B which will undo the incorrect attachments kinase - this mechanism will be further detailed in a later section of this introduction (Carmena et al., 2012). This way, the kinetochores can identify if chromosomes are attached correctly (amphitelic attachment, when sister kinetochores are anchored to microtubules from opposite poles) or incorrectly (monotelic attachment, when only one kinetochore is anchored; syntelic attachment, when both kinetochores are anchored to microtubules coming from the same pole; merotelic attachment, when more than two microtubules are anchored to the sister kinetochores). If this mechanism fails, it will lead to incorrect segregation and chromosome breakages. Also, prevention and/or correction of improper kinetochore-microtubule attachments are dependent of proteins associated to the spindle such as MCAK (Orr et al., 2015; Prosser & Pelletier, 2017).

Supernumerary centrosomes, centrosomes are responsible for organizing the microtubules into the bipolar spindle, if during the centrosome duplication any defect occurs that causes over-duplication, it will create a multipolar spindle during mitosis. When this occurs the chromosomes will be submitted to merotelic kinetochore-microtubules attachments (incorrect attachment) and then during the separation, they will experience pulling forces from multiple directions which will lead to chromosome breakage.

Tetraploidy, the tetraploid cells are cells with duplicated chromosome content. These cells can be caused by several events like defective cytokinesis, mitotic slippage, a non-programmed fusion of diploid cells, and endoreplication (replication of the nuclear genome in the absence of mitotic cell division). In any condition, if one cell like that enters mitosis this will always lead to problems in chromosome segregation.

Telomere dysfunction, this structure is at the extremities of all chromosomes markings the end of the chromosome and protecting their integrity. But in its absence, the chromosome ends become exposed which will activate the non-homologous end joining the DNA repair pathway, then this pathway will fuse two extremities of chromosomes that do not have telomeres creating a dicentric chromosome (aberrant chromosomes that have two centromeres). Because of its aberrant form if this chromosome enters mitosis, when separated, it will lead to chromosomal breakage (Orr et al., 2015).

Cohesion defects. During the cell cycle, both sister chromatids are kept together until they are properly aligned within the spindle to then be segregated. The separation of the sister chromatids and spindle forces are countered by the protein complex called cohesin. This complex is present during all the cell cycle, and during replication in the S phase, it is responsible for physically entrapping bought DNA strands, the template, and the newly replicated, by forming a ring around the sister chromatid's chains. But despite the cohesin being the main contributor of forces that counteracts the mitotic spindle, it is argued not the only contributor. Is argued that the DNA catenation (the entanglement created between the fibers of DNA during the replication) also contributes to cohesion, but how much it helps is still in debate. Nonetheless is known that by itself it is not capable of resisting the spindle forces. Yet besides that, before anaphase, the catenation needs to be resolved otherwise it will lead to problems in the chromosome segregation like chromosome bridges or DNA breaks, so to help in this point exist the enzyme Topoisomerase II which has the function of decatenate the DNA (this mechanism will be further detailed in another section of this introduction) (Reviewed in Díaz-Martínez et al., 2008; Mirkovic & Oliveira, 2017)

The point between metaphase and anaphase is marked as the point of no return, because after the cohesin cleavage it is impossible to rejoin the sister chromatids, so this point needs to be highly regulated. The main regulator of this point is the SAC, which only lets the process continues when all the chromosomes are bioriented on the metaphase plate, as previously discussed the role of SAC on Separase (Reviewed in Mirkovic & Oliveira, 2017; Brito & Rieder, 2006; Orr et al., 2015). So, during the passage from metaphase to anaphase the cohesin needs to be cleaved correctly to allow the separation of the sister chromatids and the DNA catenation needs to be resolved completely, if this cleavage and decatenation does not occur or occurs too soon this will lead to chromosome mis-segregation (Orr et al., 2015; Prosser & Pelletier, 2017)).

1.5 Cohesin

1.5.1 Cohesin structure and composition

Cohesin is a protein complex composed of four subunits, the Smc1, Smc3, Rad21 (or kleisin in yeast), and the STAG1/2 (or Scc3 in yeast), they can be divided into two domains the hinge and the head, that are connected by two flexible rods in the shape of a coil (see Figure 13). Due the presence of Smc1 and 3, it part of the family of structural maintenance of chromosomes (SMC) complexes where condensin I and II also belong, two complexes involved on the proper structure of the chromosomes. Both Smc1 and Smc3 subunits are joined together at the hinge domains forming a V shape structure, then the Rad21 subunit bond to the heads domains of both Smc1 and Smc3 creates a ring structure. In the heads domains are present two incomplete ATP binding domains that in the presence of an ATP molecule are capable of engaging and activating their ATPase activity, then with the binding of a DNA molecule between the Smc3 and the Rad21 will lead to the hydrolysis of the ATP that will create a conformational change that makes the molecule bend over leaving the hinge close to the heads domains.

Besides the four subunits, many other factors interact with this complex, namely the loading factors NIPBL (or Scc2 in yeast) and MAU2; the regulating factors ESCO1, ESCO2 and HDAC8; the inducing cohesin modificatory factor Pds5 and Sororin (CDC5A); the removal factor WAPL; and protectors factors PLKs, SGO1, and SOG2. See Figure 14. (Chandrasekaran et al., 2022; Matityahu & Onn, 2022).

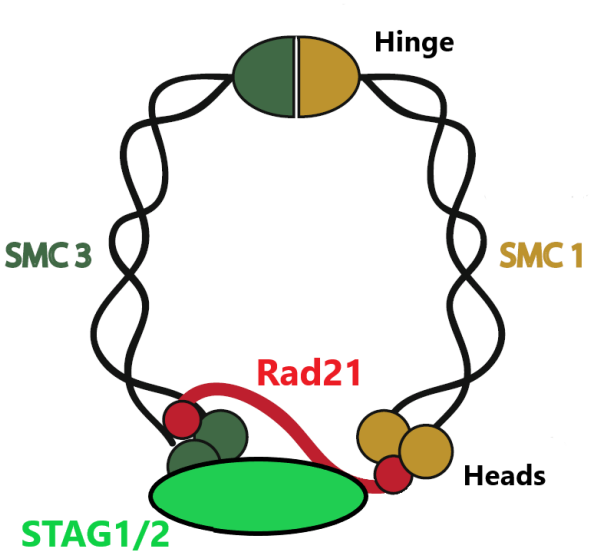

Figure 13: Cohesin. The cohesin complex structure with its four subunits, the Smc1, Smc2, Rad21, and Stage1/2, and the representation of the hinge and heads domains (Figure adapted from Mirkovic & Oliveira, 2017).

1.5.2 Cohesin distribution

In mammalians the cohesin is present in the DNA since the beginning of the cell cycle, at the end of mitosis the cohesin is loaded into the DNA mediated by the NIPBL, then during the DNA replication at the S phase, the cohesin in coordination with the replication fork is capable of capture both the old and the newly synthesized DNA strands. But until that point the cohesin has a volatile confirmation being possible to be unloaded from the chromatin by the WAPL, which is capable of open the ring of cohesin by disrupting the interface between Smc3/Rad21. These dynamic properties can lead to unwanted disruptions of the ring, and it may cause problems with chromosome cohesion, so to prevent this after capturing the new strand, the ESCOs will acetylate two lysine sites at the Smc3 subunit, that will recruit another protein called Sororin, which will compete for the binding site of the Pds5, inhibiting the interaction of WAPL with the SMC3/Rad21 interface preventing the cohesin to be unloaded by the WAPL. This establishment pass ensures the stability to make the cohesin resist until mitosis (Matityahu & Onn, 2022; Mirkovic & Oliveira, 2017).

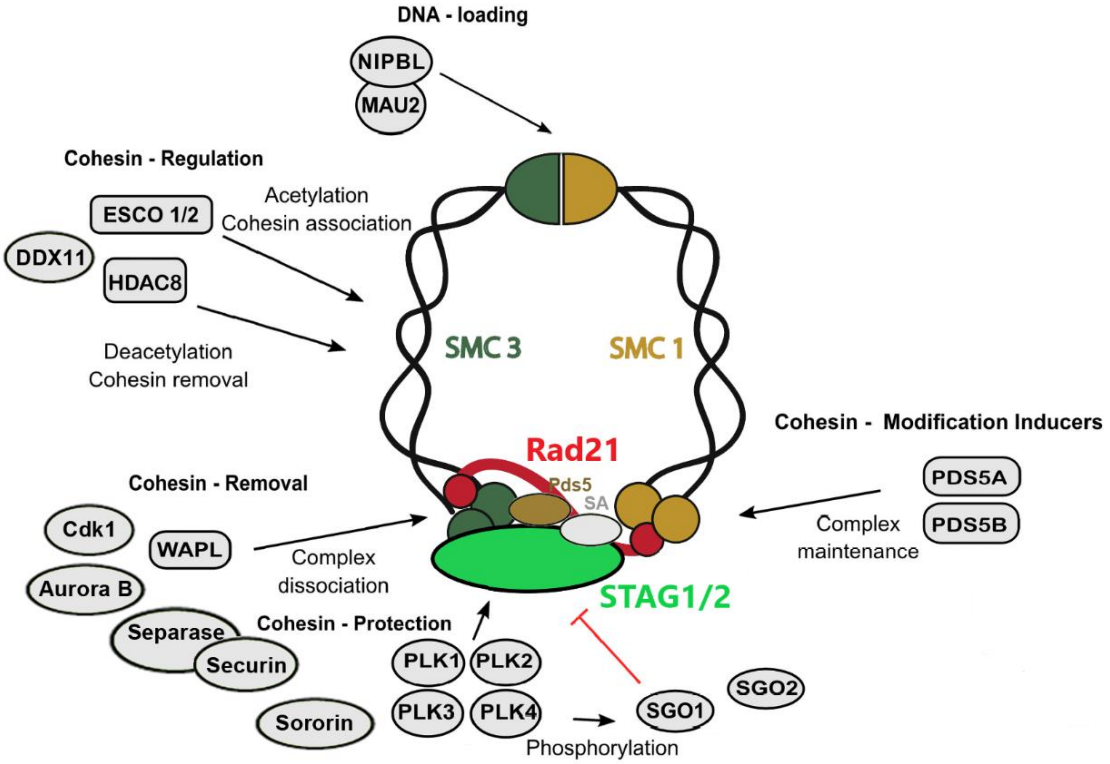

Figure 14: Cohesin-associated proteins. Schematic representation of all the diverse cofactors that interact with the cohesin complex, divided in groups according to their functions, of loading, regulation, removal, protection, and modifiers (Figure adapted from (Chandrasekaran et al., 2022; Mirkovic & Oliveira, 2017).

The cohesin is seen in a bigger amount at the pericentromeric region of the mitotic chromosomes, where it is responsible for ensuring cohesion between both chromatids and also responsible for giving to that region the correct organization and strength, allowing the supporting need for the chromosomes to be capable of holding the mitotic spindle forces without being tear apart. Cohesin is enriched pericentromeric region that is responsible for leading into an X shape the mitotic chromosome. Besides the pericentromeric region, cohesin also is seen in multiples loci along all the chromosome arms but in must smaller quantities, between 3 to 20 cohesin per site according to review (Matityahu & Onn, 2022).

1.5.3 Cohesin functions

Cohesin has originally linked with functions in mitosis - capacity of tethering the sister chromatids, preventing early separation of the sister chromatids before the correct alignment during metaphase. Recently, cohesin has been associated with new functions outside of mitosis, in areas namely genome organization, genome integrity, and gene transcription regulation (see Figure 15).

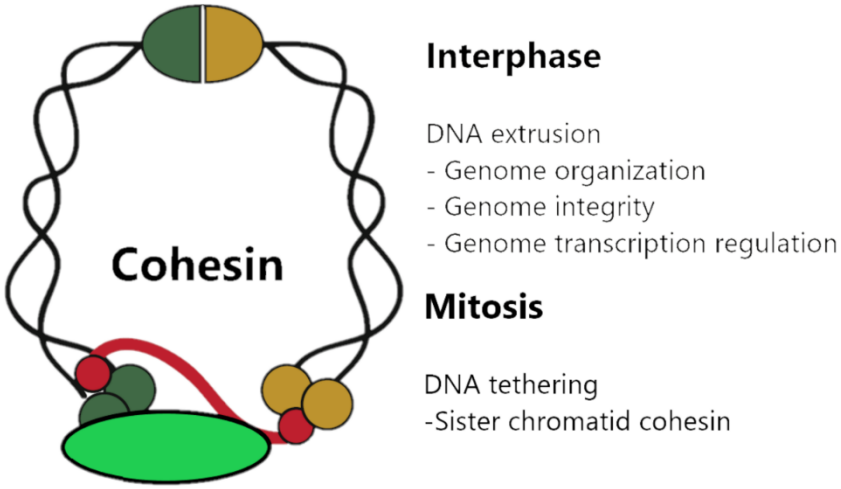

Figure 15: Cohesin functions summary. Summary of the cohesin function in interphase and mitosis. In interphase it has functions related through the DNA extrusion which are involved in the genome organization, genome integrity, and genome transcription regulation. In mitosis it has functions related through the DNA tethering which are mainly involved in the siter chromatid cohesin.

1.5.4 Cohesin functions during interphase

Right at the end of mitosis, at the beginning of G1 when the cohesin starts to be loaded onto the chromatin, cohesin is important to regulate transcription and the genome architecture. Then during the S phase, it already entrapped the DNA in the newly replicated fibers. Some of the evidence that supports interphase cohesin functions are the requirement of a cohesin complex during DNA damage repair (Ström et al., 2004), and the capacity of the cohesin complex to create chromosome loops (Figure 16) (DNA extrusion) or topology-associated domains (TADs) that will affect the interaction between the enhancer and the promotor. These mechanisms will namely inhibit a specific gene transcription or an inactivator thus allowing the gene transcription (Piché, van Vliet, et al., 2019). Examples of that type of interaction are the co-localization of cohesion biding sites with CCCTC binding factor (CTCF) sites in mammals (Parelho et al., 2008; Wendt et al., 2008). CTCF is an insulator protein that participates in blocking enhancer promotor interaction (Barbero, 2013) that forms a complex formation with Mediator (transcriptional coactivator) to activate gene transcription. This complex brings enhancer and promoter regions together by forming a loop connecting two DNA segments (Chandrasekaran et al., 2022; Kagey et al., 2010; Matityahu & Onn, 2022).

CTCF biding sites

The first impression about DNA loops is that they are dynamic structures and can have multiple positions, but when observed with more detail is possible to identify some patterns and is possible to notice that are associated with the TADs. Near the TADs are found the CTCF binding sites, which are required for the Cohesin recruitment, which is one of the main responsible for the loop creation.

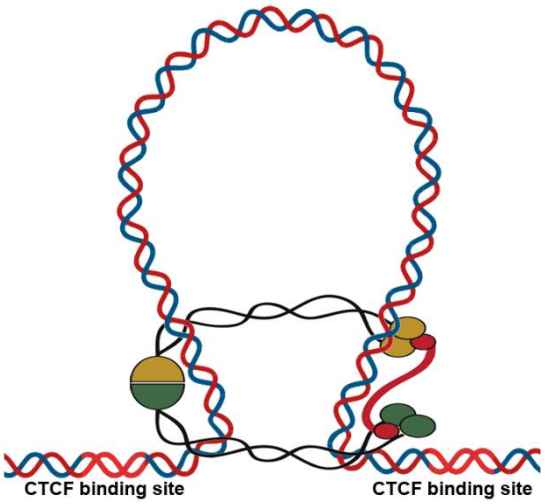

Figure 16: DNA loop and CTCF binding sites. Schematic representation of the position of the cohesin complex in an DNA loop and a possible localization of the CTCF binding sites.

The CTCF are unstructured structures, with N- and C- ends that are associated with eleven zinc fingers, which confers direction to this structure, normally the N-terminal is pointed in a convergent way to the TAD, suggesting that the CTCF works as a boundary to the DNA loop. The CTCFs have been shown to be required for several functions, since controlling enhancer-promotor interactions, cell differentiation, nuclear reprogramming, recombination of antigen receptor genes, and timing of DNA replication.

But how this works mechanically is still doubted, some suggestions of how CTCF prevents the loop extrusion are that binding to the cohesin (cohesin can bind to the N-terminal of the CTCF), that CTCF work as a barrier that blocks the progression, or that CTCF prevents the release of cohesin from DNA by interfering with the binding of some factor to the cohesin, like inhibiting the WAPL or preventing the NIPBL binding.

The CTCF molecule has been shown to after binding to their site can block the cohesin diffusion, just in its monomer conformation, which goes according to the accumulation of cohesin seen at the TADs boundaries. This blocking capacity has been shown to be more efficient at the N-terminal and to be regulated by the tension of the DNA that CTCF and cohesin are bounded, this way altering the tension will modulate the permeability of the CTCF and influence the length of the loop.

In recent studies (Davidson et al., 2022), they defend that CTCF not only works as a barrier to the DNA loop extrusion but also can work as a switcher in the direction of extrusion, this way allowing to the loop be extruded from the opposite direction or invent to shrink (Figure 17). This suggests that CTCF can have functions of a failsafe mechanism because that way it allows repeated interactions between the genomic region until it finds a productive loop. They defend that CTCF can control cohesin and that way influencing the genome architecture via multiples modes (Davidson et al., 2022).

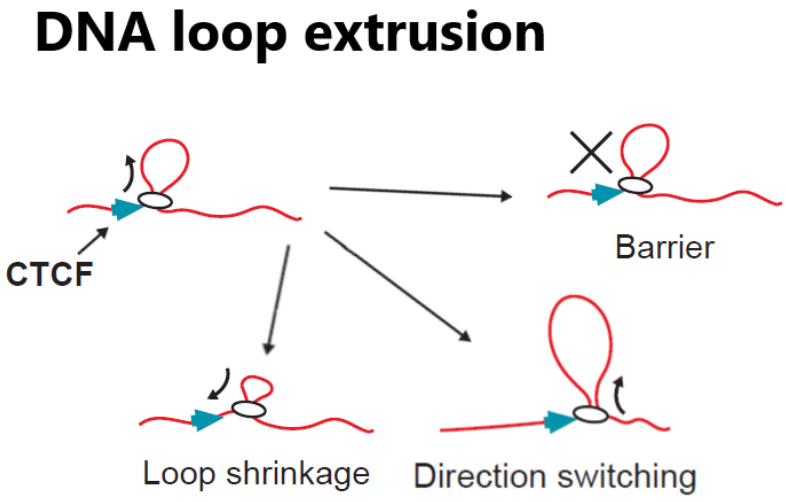

Figure 17: CTCF interactions with the DNA loop. The different interactions that CTCF can made and what it the outcomes. CTCF can act as a barrier, stopping the DNA extrusion, can act as a switch and change the direction of extrusion, and can act as a loop shrinker forcing the loop to shrink (Figure adapted from Davidson et al., 2022).

1.5.5 Cohesin functions during mitosis

At the start of mitosis, the cohesin present in the chromosome arm is removed to help in the resolution of the sister chromatid. It is believed that this removal helps in the resolution because the presence of cohesin was shown to interfere with the topoisomerase II activity (Farcas et al., 2011; Sen et al., 2016), possibly because, in the presence of the cohesin, the sister chromatids are too close not letting the topoisomerase II successfully decatenate the DNA. The removal of the cohesin is done by the WAPL, which is dependent on removal of Sororin. The removal of Sororin is done mainly by two kinases, the Aurora B and the Cyclin-Dependent Kinase 1 (Cdk1), by phosphorylating the Sororin and other key proteins involved in the cohesin cycle like the SA.

The remaining cohesin (at the chromosome pericentromeric region) during the metaphase needs protection to ensure that centromeric cohesin is not removed, and the consequent loss of cohesion leads to aneuploidy. This protection is conferred by a protein called Shugoshin (Sgo1), that in complex with the PP2A phosphatase will antagonize the phosphorylation made by the Aurora B and the Cdk1, favoring the Sororin interaction with Pds5, preventing the WAPL action (Mirkovic & Oliveira, 2017).

Once chromosomes are properly aligned (as discussed above), cohesin will be completely removed. This process is very fast and conducted by a cysteine protease called Separase, which cleaves the Rad21 subunit. The cleavage of the Rad21 will separate the SMC1 and SMC3 subunits opening the ring. To prevent any early separation the Separase is regulated by two main mechanisms. The Securin binds to the Separase at its active site, preventing the interaction between Separase and cohesin. Also, Securin is inhibited by the Cdk1-Cyclin B complex. This complex phosphorylates and binds to the Separase inhibiting its functions. As such, the progression through the cell cycle - and cleavage of cohesin – happens when APC/C ubiquitin ligase ubiquitinate Securin and the Cyclin B, releasing Separase.

APC/C is also highly regulated to prevent an early release of the Separase, this regulation is made by the SAC. While the microtubules are not attached correctly to the kinetochore, the SAC checkpoint is active leading to the kinetochores catalyzing one inhibitor that will attach to the Cdc20 (required molecule to the activation of the APC/C), creating a complex called MCC that inhibits the APC/C. When the kinetochores are all correctly attached the SAC is inactivated Cdc20 is released, and consequently the APC/C activated leading to Separase release (Mirkovic & Oliveira, Maria Clara Sancadas Pereira 2017).

In 2018 studies in *Drosophila* using systems to acutely decrease the level of cohesin in metaphase arrest chromosomes have shown that only when the loss of cohesin is above 80% we start to see disjunction problems, but sister chromatid cohesion is maintained in the majority of the cells. Yet, this reduction of cohesin levels in fly embryos that have sister chromatids tethered have defects at attachment and alignment and erroneous chromosome segregation (Carvalhal et al., 2018). A parallel study in human cells, also reached the same conclusion partial loss of chromatid separation also leads to predisposition to chromosome mis-segregation (Sapkota et al., 2018). These studies both

two unrecognized sources of chromosome instability related with cohesion loss before chromosome disjunction.

1.5.5.1 Cohesin DNA tethering

In the case of DNA tethering three main models are suggested (Figure 18), where the difference between them is the number of cohesin complexes involved.

The first model is the Ring model (Haering et al., 2008), in this model is suggested that the entrapment of the sister chromatids is done by only one cohesin complex (monomer form), but this model has problems explaining some dynamics in the cohesin activity like the loading and unloading of cohesin during different stages of the cell cycle and how to maintain the sister chromatid cohesin during the DNA replication.

The second model is the Handcuff model (Guacci, 2007) and suggests that the entrapment of the sister chromatids is done by two cohesin complexes (dimer form) where each cohesin only entraps one DNA strand and both cohesin is bonded to each other by the Rad21 subunit, but for a long time was a lack of evidence to support this model.

The third model is the Bracelet model and suggests that the entrapment of the sister chromatids is done by multiple complexes forming an oligomer in the shape of a bracelet where the Smc1 subunit of one cohesin is bonded to the Smc3 subunit of another cohesin by the Rad21.

Although the Bracelet model is one of the most accepted, they are not mutually exclusive, because of the dynamic behavior of the cohesin complex it is possible that all these different states where only conditional dependent and depending on the occasion all of them can exist (Chandrasekaran et al., 2022; Matityahu & Onn, 2022).

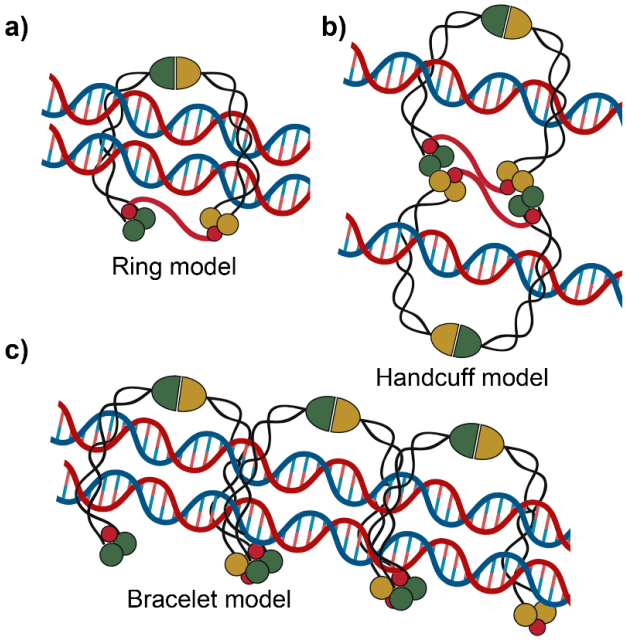

Figure 18: Cohesin DNA tethering models. Different models of how cohesin entrap the DNA. a) the one ring model, that propose that a single ring of cohesin entrap both DNA strands; b) the handcuff model, that propose that two cohesin rings work as a dimer (being connected by the Rad21 subunits) and each cohesin ring only entrap one DNA strand; c) the bracelet model, that propose thar multiple cohesin rings working as a oligomer (being connected by the Smc1 subunit of one cohesin that is bonded to the Smc3 subunit of another cohesin by the Rad21), creates and bracelet shape structure around the two DNA strands.

1.5.5.2 Cohesin DNA extrusion

One big doubt involving all these functions of cohesin (DNA extrusion and DNA tethering) is whether it plays its role as a monomer, dimer, or even oligomer.

In the case of DNA extrusion, some studies have shown that cohesin can extrude the DNA bi-directionally, but these observations are not compatible with the translocation rate of a cohesin monomer, only being possible of having that rate in the presence of a cohesin dimer. Many models of how the cohesin extrudes the DNA has arisen, some suggesting an active extrusion and other an non-active extrusion (Matityahu & Onn, 2022).

The non-active extrusion models suggest that the extrusion only relies on biophysics principles, in this model the extrusion is mediated by cohesin motor activity. They suggest that cohesin binds to two DNA regions. By Brownian motion they come together stabilizing them self, then the cohesin unbinds that region and binds to another and repeats this process in a cycle. By this way it is creating a loop that will increase due to the

fluctuations of the chromatin. Yet, this model does not answer how many cohesins are involved in this process because it could be applied to a monomer situation - where one ring captures the two DNA strands - or to a dimer situation - where each cohesin only capture one DNA strand (Matityahu & Onn, 2022).

The active extrusion models on the other side suggest multiple different ways of cohesin to extrude the DNA. The most known are the "Walking" model, "pumping" model and "scrunching" model (see Figure 19). All these models suffer from limitations, because of a yet lack of (total) experimental support and rigorous tests (Nichols & Corces, 2018; Marko et al., 2019; Ryu et al., 2020).

The "Walking" or Winchworm" model proposes that the cohesin hinge binds to the DNA and then the heads domains walk along DNA. This movement can be compared to the movement of motor proteins on a microtubule (Nichols & Corces, 2018).

The "pumping" model proposes that cohesin heads domains clamp the DNA. Then, by the stretching of the coiled coil push another DNA region from the hinge to the heads, and the repetition of this pumping motion leads to the increase of the loop (Marko et al., 2019).

The "scrunching" model, suggests that a cohesin hinge binds to DNA, bent over, and transfers the DNA to the head domains. After the hinge reaches DNA, the coiled coil distends again and binds to other DNA regions. This repetition in a cycle creates movements by which increases the loop dimension (Ryu et al., 2020).

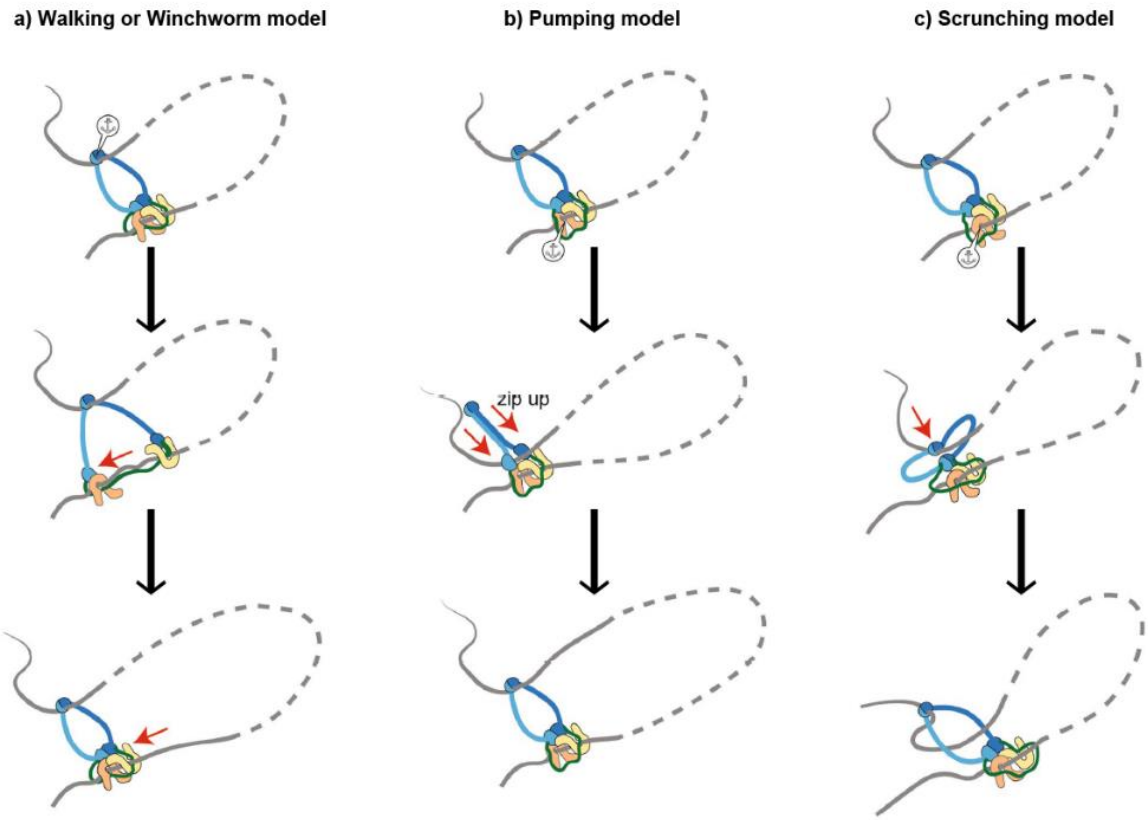

Figure 19: Cohesin DNA extrusion models. a) Walking or Winchworm model, where the hinge domain anchors the DNA and both heads in an ATP dependent manner pass through a cycle of binding and unbinding the DNA to pull the DNA, looking like if the heads are "walking" along the DNA. b) Pumping model, where the heads domains anchor the DNA and then by repeated conformational changes of the coiled-coils structures of the Smc1 and Smc3, from the ring shape to the rod shape, the Closing of the ring pump the DNA. c) Scrunching model, where the heads domains anchor the DNA and then the hinge bind to another DNA, then the coiled coil bend pushing the DNA closer to the heads domains and release the DNA, then repeat this movement in cycle (Figure adapted from Ryu et al., 2020).

Besides these models, a new model has surged in 2021, the "swing and clamp" model. This model says that the DNA extrusion is mediated by a cohesin-NIPBL complex and that the extrusion is mediated by swing movements of the cohesin molecule. These movements are characterized of three main conformations. The ring conformations where both coiled coils (the Smc1 and Smc3 subunits) are totally separated and distended. The rod conformations where both the coiled coils are distended and aligned together. Finally, the bent conformations where both coiled coils are bent allowing the hinge to be close to the heads domains (see Figure 20).

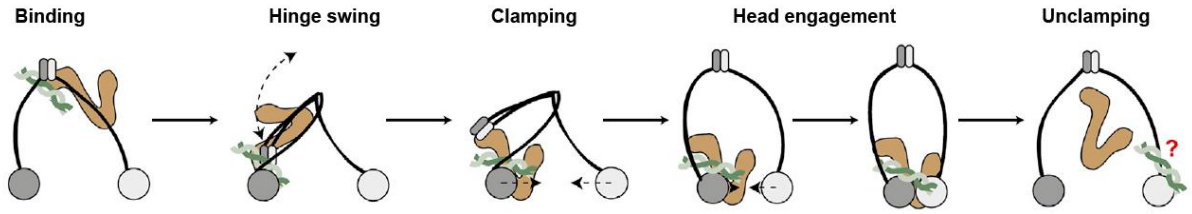

Figure 20: Cohesin DNA extrusion "Swing and Clamp" model. Representation of the Swing and Clamp model of extruding DNA. In this model the hinge domains bind to the DNA with the help of the NIPBL, then the coiled coil structure bends pushing the DNA close to the Smc3 head, where the DNA will be clamped with the help of the NIPBL. This leads to the release of the hinge domains, then both heads domains come close activating the ATP hydrolysis resulting into the release of the DNA and the NIPBL. After, it is ready again to bind another DNA part together with the hinge, and this movements repeats in cycle creating a swing movement that pulls the DNA. As for the unclamping, the final step, remains unclear what happens to the DNA. It has been suggested that DNA could remain bonded to the Smc1 head (Figure adapted from B. W. Bauer et al., 2021).

The "Swing and Clamp" model suggests that the cohesin hinge helped by the NIPBL bind to the DNA. Then, the cohesin coiled coil bent dislocating the hinge to the Smc3 head, where the DNA is transfer to the Smc3 head and clamped with the NIPBL. This makes the hinge free and that the coiled coil come back to the original form. In the end, the heads come together activating the ATP hydrolysis what will result in the disengagement of the DNA from the heads freeing the NIPBL. Next, NIPBL together with the hinge will bind to a new DNA region, repeating these movements which create a swing movement. This movement will push the DNA into an increasing loop. It is important to refer that after the heads release the DNA remains what happens to the DNA. A speculative reasoning is that DNA is transferred to the Smc1 head, but this was not proved yet. While this model is quite elucidating how the cohesin could extrude the DNA, it remains not fully understood (B. W. Bauer et al., 2021).

All these models show different ways of how the cohesin could extrude the DNA but in the end, one can say that it is still impossible to decipher if the cohesin works alone or not because all these models could be applied both to the monomer state or to the dimer state (Matityahu & Onn, 2022).

1.5.6 Cohesin gates

Studies have shown that cohesin has two entry gates to inside the ring, one at the interface between the Smc3 and the Rad21, and the other in the hinge domains (between the Smc1 and Smc3). As is seen in the Smc3/Rad21 gate, the Smc1/Smc3 gate also needs interaction with the STAG and with the NIPBL to allow the passage of the DNA to the inside of the ring (Figure 21). But from the result of the James E Collier 2022 study, have shown that the hinged gate is essential to the loading of the cohesin, while the Smc1/Smc3 gate is more involved it the unloading of the cohesin most specifically in the WAPL-dependent way. In that study, they propose that the passage of the DNA through the hinge gate is most likely dependent on the entrapment of the DNA by the clamp created between the Rad21 and the heads domains in the presence of the NIPBL, then after the DNA is clamped, the DNA will be passed through the hinge in a process dependent of the STAG factor, but the role of the STAG at this stage is still unclear (Beckouët et al., 2016). On the other side in the absence of the NIPBL is presumable that occur the entrapment of the DNA by the Smc3/Rad21 gate, this could occur because in these circumstances the Rad21 dissociates from the Smc3 making the ring open. This could work as an entry or an exit to the DNA, but so far it is not known if this entrapment of the DNA through the Smc3/Rad21 gate happens *in vivo* or is an *in vitro* artifact. From what is known, the co-entrapment of the unreplicated DNA and the newly replicated DNA do not require the NIPBL (Gao et al., 2019) but from their results (Collier & Nasmyth, 2022), they show that the NIPBL is necessary to the DNA pass through the hinge, so these results suggest that during replication no DNA pass through the hinge, what opens the question of how the newly replicated DNA enter the cohesin ring? or through the Smc3/Rad21 gate or through another mechanism where it can enter without opening the ring (Collier & Nasmyth, 2022). Yet, many other cohesin associated proteins, like DDX11, are missing from this equation, which may unveil new ways on cohesin co-entrap DNA during the different phases of mitosis.

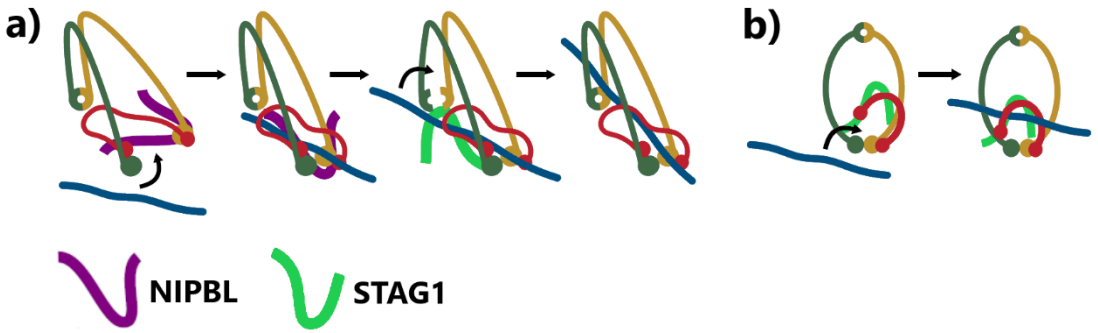

Figure 21: Cohesin gates models. Model of how DNA enter in the cohesin ring. a) The DNA is clamped by the heads domains hit the help of the NIBPL, then the hinge opens and with the help of STAG1 the DNA pass through the hinge being that way entrapped inside the ring; b) The DNA pass through the Smc3-Rad21, the Smc3-Rad21 gate opens by the interaction with an ATP, then the DNA pass through the opens space with the help of the STAG1 (Figure adapted from Collier & Nasmyth, 2022).

1.6 Cohesin and human diseases

As previously mentioned, cohesin canonical function is established sister chromatid cohesion. Nowadays, cohesin has functions in the regulation of several cellular mechanisms like gene expression, DNA repair, fork replication stability, and recruitment of proteins involved in the activation of cell cycle checkpoints (di Nardo et al., 2022).

As such, one can say that cohesin is essential to prevent genome instability, one of the hallmarks of cancer and other pathologies, so its deregulation is a driver event to cancer development because if cohesin fails it will affect the chromosome segregation originating an imbalance that will affect all the gene expression, both by increasing or decreasing the number of certain genes and also by affecting the genome organization, like DNA loops and promotor enhancer interaction, leading to alteration in the expression of the genes. Given the vast number of mechanisms where cohesin is involved, it is not surprised that problems that affect the structure of cohesin or its cofactors can lead to a variety of pathologies, namely cancer, and other rare syndromes.

In terms of rare syndromes, it is possible to highlight a class of rare diseases that are characterized by all being caused by mutations in the cohesin complex or at its regulators. This group of rare syndromes is called cohesinopathies (this topic will be discussed in more detail in the following section) (di Nardo et al., 2022; J. Liu & Krantz, 2008; Singh & Gerton, 2015).

In terms of cancer, it is suggested that cohesin mutations can lead to tumorigenesis. To support this assumption is STAG2, a subunit that is one of the top 12 genes found mutated in four or more types of cancer (Romero-Pérez et al., 2019). Other mutated variants of cohesin are presented in several types of cancer. STAG1, STAG2, NIPBL, and Pds5 the most frequent, varying between 3.4% to 5%, but also is possible to see mutations in other components like the Smc1 and the Rad21 (di Nardo et al., 2022). The incidence of these (or others) mutated cohesin variants changes according to the type of cancer. Some specific subunits are more frequently mutated in some types of cancers, these changes probably came because of the different roles of cohesin and most likely depending on the tissue, the level of activity of cohesin and the factor with which it interacts changes.

So, depending on the locality where the mutation is, it can affect the interaction with different factors having different outcomes, for example, if cohesin complex with STAG1 or to STAG2 will lead to the complex having different biological functions. Although the mutation can be seen in the different parts of the complex, it starts to be evident that some regions are more frequent to suffer mutations, like the hinge and heads domains.

Although not all cancer present cohesin defects, cancer that is seen more commonly with cohesin defects are colorectal carcinoma, breast cancer, lung cancer, urothelial bladder carcinoma, Ewing's sarcoma, glioblastoma, melanoma, and myeloid neoplasms (di Nardo et al., 2022).

1.6.1 Cohesinopathies

Among the cohesinopathies known to date, two of the most described are Cornelia de Lange Syndrome (CdLS) and the Roberts Syndromes (RBS). But others have been identified in the last years -despite the low number of cases diagnosed to today's date - namely Chronic Atrial and Intestinal Dysrhythmia (CAID) and the Warsaw Breakage Syndrome (WABS) in which I will focus on my project (Piché, van Vliet, et al., 2019). Cohesinopathies share mutations in the complex and associated or regulatory proteins of the cohesin complex. In addition, many patients also share railroad mitotic chromosome phenotype, a chromosome structure typical of a chromosome with lower levels of cohesion, where the chromosomes have the appearance of a train railroad.

Cornelia de Lange Syndrome (CdLS) is the most known cohesinopathies with a prevalence of 1 in every 10 000 live births (Krantz et al., 2004; Opitz, 1985). CdLS is an autosomal dominant disorder caused by mutations in genes encoding for cohesin regulators: 50-70% on NIPBL (4% HDAC8, 3-5% on SMC1A, 3% on SMC3 and 1% on RAD21 gene. CdLS patients are characterized by mental retardation, craniofacial abnormalities (e.g microcephaly, facial dysmorphism), upper limb abnormalities, and growth delay, cardiovascular and gastrointestinal disorders are also frequent in patients. At the cellular level, most of the CdLS patients do not exhibit mitotic defects or railroad mitotic. Given the absence of problems at sister chromatids cohesion in the majority of patients, it is believed that CdLS are caused by problems in the regulatory functions of the cohesin (Banerji et al., 2017; Barbero, 2013; Piché, van Vliet, et al., 2019). Yet, recent studies (Carvalhal et al., 2018; Sapkota et al., 2018) have shown that before loss of cohesion sister chromatids, decay of cohesin can already cause significant problems in terms of genome instability.

Robert's Syndrome (RBS) is a rare autosomal recessive disorder caused by mutations in the gene encoding for cohesin regulation ESCO2. RBS patients have an overlap of clinical features with CdLS including mental retardation, craniofacial abnormalities (like microcephaly), limb abnormalities, and growth delay. Yet RBS patients (but not CdLS) also have cardiovascular disorders but not gastrointestinal. At the cellular level, RBS patients exhibit railroad mitotic chromosomes due to the lack of cohesin at the centromeric region, which leads to premature centromere separation and consequently mitotic defects (lagging chromosomes, aneuploidy, and micronucleus) (Banerji et al., 2017; Barbero, 2013; Piché, van Vliet, et al., 2019).

Chronic Atrial and Intestinal Dysrhythmia (CAID) syndrome is another very rare disease, caused by a homozygous recessive mutation in SGO1 (K23E), one modulator of the cohesin complex. CAID patients have as clinical features failure of pacemaking tissues (tissues that create rhythmic impulses) in the heart and gut, this creates problems like the sinoastrial node and Interstitial network of Cajal. Even though there is some clinical overlap with other cohesinopathies like heart and gut defects, none of CAID patients exhibit intellectual or growth defects, and unlike other cohesinopathies, CAID syndrome does not manifest phenotypes at birth, only occurring after 6 years of age. At a cellular level, CAID patient cells display railroad chromosomes like other cohesinopathies. These cells also exhibit enhanced activation of Transforming growth

factor β (TGF-β) signaling, DNA methylation, chromatin compaction, and disruption of potassium currents. First studies suggest a lack of major mitotic defects unlike other cohesinopathies, but further studies are needed to understand if CAID phenotype comes from SGO1 non-cohesin-related functions (Piché, Gosset, et al., 2019; Piché, van Vliet, et al., 2019).

Warsaw breakage syndrome is a less known cohesinopathies. It is a very rare autosomal recessive disease caused by a bi-allelic mutation in the gene encoding for a DNA helicase DDX11. WABS patients have as clinical features growth retardation, craniofacial abnormalities (facial dysmorphia, microcephaly), deafness, abnormal skin pigmentation, extremity impairments (clinodactyly), and in some cases heart defects (as summarized in Table 1). At a cellular level, WABS patients cells display sister chromatin cohesion defects and railroad mitotic chromosomes like the RBS (Banerji et al., 2017; Piché, van Vliet, et al., 2019; van Schie et al., 2020).

More recently, a possible new cohesinopathie also was identified. There were found two patients with a biallelic mutation in the gene BUB1. Both patients have as common clinical features to cohesinopathies microcephaly, intellectual disability, and developmental delay. At the cellular level, both patients show problems at the mitotic level, namely problems in the regulation of cohesin, chromosome segregation errors, prolonged mitosis duration, railroad mitotic chromosomes, and *in vitro* premature sister chromatid separation. These are also common features to WABS and RBS (Carvalhal et al., 2022).

1.7 Warsaw Breakage Syndrome (WABS)

So far only 23 cases have been diagnosed with WABS (van Schie et al., 2020). As previously mentioned, WABS patients share clinical features with CdLS and RBS, but they also have some overlap of clinical features with Fanconi anemia (FA), a recessive genetic disorder where patients display growth retardation, microcephaly, skeletal malformations, progressive bone marrow failure, and cancer predisposition. Yet, it has not been described WABS patients with cancer predisposition (van Schie et al., 2020). Due to the diversity of symptoms, the diagnosis of FA is based on analysis of increased chromosome breakage on a culture of patients lymphocytes. In this analysis, cell are treated with DNA crosslinking agents (diepoxy butane and methyl methane sulfonate), as

FA patients cells exhibit a highly sensitive response to these agents. This test has been used as a gold standard to diagnose FA. Curiously, WABS patients cells also display this highly sensitive response to these DNA crosslinking agents, which may give rise to wrong diagnoses, especially due to the overlap of clinical features with FA. Also, the heterozygosity condition of the DDX11 can give rise to a lower penetrance phenotype, which could create very mild clinical features that can be confounded with the FA features. Overall, these arguments support that the incidence of WABS is currently underestimated (Banerji et al., 2017; Piché, van Vliet, et al., 2019; Pisani, 2019).

Table 1: Clinical features in WABS. Description of the clinical features and other information about WABS. The color codes represent: red means that is present in more than 50% of the cases, rosy means that is present in less than 50% pf the cases, and blue means that is not present. The select features are typical clinical features presented in several cohesinopathies or related syndromes (Table adapted from Carvalhal et al., 2022).

Syndrome	Warsaw Breakage Syndrome
OMIM	613398
Reference	Carvalhal et al., 2022
Number of reported cases	23
Gege	DDX11
Growth retardation	
Microcephaly	
Intellectual disability	
Clinodactyly	
Brain morphology abnormalities	
Heart abnormalities	
Urogenital abnormalities	
Eye abnormalities	
Lung abnormalities	
Retrognathia	
Cleft lip and palate	
Abnormal skin pigmentation	
Abnormal ear morphology	
Deafness	
Gastrointestinal abnormalities	
Phocomelia	
Cancer	

1.7.1 DDX11, WABS pathogenic gene

So far, all known cases diagnosed with WABS carry mutations in the gene DDX11.

DDX11 stands for DEAD/H-Box Helicase 11 or ChlR1 and it is an ATP-dependent DNA helicase. The Human DDX11 was discovered by Frank and Werner in 1996, in that study they isolated a complementary DNA (cDNA) that shows a high similarity with the *Saccharomyces cerevisiae* CHL1 gene (Frank & Werner, 1996). In that same year, two others human cDNAs were found to share high similarities with the CHL1 gene. After genomic studies, the human Chl1-related (CHLR) cDNA have been shown to be encoded by two similar genes that were named CHLR1 and CHLR2 (DDX11 and DDX12), and they are located in chromosome 12 in the region 12p11 and 12p13 (Amann et al., 1996). The Chrl1/DDX11 was later produced and characterized *in vitro* for ATP-dependent DNA helicase activity. Nonetheless, the Chrl2/DDX12 is today considered a pseudogene with predicted activity (Santos et al., 2021).

1.7.1.1 DDX11 Structure

DDX11 is an ATP-dependent DNA helicase with 5´to 3´directionality that belongs to the DNA helicase super-family 2 (SF2) and has an iron-sulfur cluster (Fe-S). These characteristics are similar to other three human DNA helicases: the *Xeroderma pugmentosum* group D proteins (XPD), the Fanconi anemia group J protein (FANCJ), and the Regulator of telomere elongation helicase 1 (RTEL1) (Pisani et al., 2018). All have the main role the genome maintenance more precisely in the DNA replication and repair (Reviewed in Brosh, 2013).

By crystallographic and sequencing studies (Fan et al., 2008; H. Liu et al., 2008) is know that DDX11 have around 970 amino acids and in its composition presents two RecA like domains (HD1 and HD2) characteristic from SF2 proteins and two accessory domains, the Fe-S cluster and the Arch domains (see Figure 22). The two RecA like domains are known to be involved in the ligand binding and ATP hydrolysis, which contain eight conserved helicase motifs: the HD1 contain the motifs Q, I, Ia, II and III and the HD2 contains the IV, V and VI motifs. Each of this helicase motifs have their main role and together they accomplish their main functions (as summarized in Table 2) (Bansal et al., 2018).

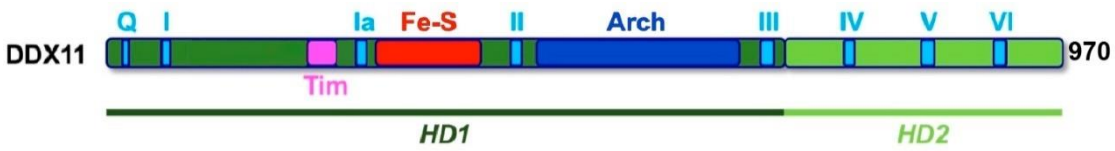

Figure 22: DDX11 structure. Schematic representation of the DDX11 composition, based on the paper by Pisani et al., 2018 (Figure adapted from Pisani et al., 2018).

Table 2: Helicase Motifs. Different helicase motifs and their respective known functions (Bansal et al., 2018).

Helicase Motif	Function
Q	Involved in DNA binding
I	Important for ATP hydrolysis and mediates ATP binding
II	Interact with magnesium ion
III	Sensor for ATP hydrolysis
IV	Interact with the phosphate blackbone of the DNA
V	Interact with DNA
VI	Interact with DNA

The iron sulfur cluster (Fe-S) belongs to the HD1 domain, between the helicase motif Ia and II. Its exact role is still an object of study, but it has been proposed to be involved in the structural and functional roles. It is hypothesized that proteins from SF2 proteins that exhibit the Fe-S cluster may use the redox properties of thus domains to scan the genome for DNA damage by sensing DNA charge transport that is mediated by base pair stacking (Genereux et al., 2010; Wu & Brosh, 2012), or to find unusual DNA structures like covalent base adducts like triplex and G-quadruplex (Suhasini et al., 2009; Wu & Brosh, 2012).

The Arch domain is also located in the HD1 domains, between the helicase motif II and III, by studies in the DNA helicase XPD, that also pretense to the SF2 Fe-S proteins,

it was proposed that the Arch domain interact with the MAT1 protein (one component of the CDK-activating kinase (CAK), that are one component of the TFIIH that is involved in the transcription initiation and DNA repair), and after this interaction Arch domains is capable of engage with DNA and separate the DNA strands. They suggest that Arch domains are involved in the strands separation and this way mediating the helicase activity (Peissert et al., 2020). Beside these domains commons between the member of the SF2 Fe-S family, in the DDX11 is also find on Timeless binding site (Tim), that is one a component of the replication fork-protection complex and this association is essential for the recruitment of DDX11 to the replication fork (Calì et al., 2015; Cortone et al., 2018).

1.7.1.2 DDX11 localization

In 2006, the Androphy group used indirect immunofluorescence experiments in RPE-1 cells studied the DDX11 localization for the first time. DDX11 has a dynamic localization during the cell cycle - In interphase, DDX11 appears in a sparse localization in the nucleus, then in the first stages of mitosis it is in the condensed chromatin, at metaphase it is associated with the centrosomes and spindle poles. At the end of mitosis, DDX11 is at the midbody (Parish et al., 2006).

In 2015, Sun and colleagues using immunofluorescence experiments with specific DDX11 antibodies confirmed that DDX11 is a nucleolar protein, and reported that DDX11 colocalizes with nucleolin, a nucleolar protein (Sun et al., 2015).

Nevertheless, in the last years, two groups using the western blot technique, found that a great part of the DDX11 is present in the cytoplasmic fraction of the human cell extracts, and not in the nuclear fraction as in the past believed (Bottega et al., 2021; Simon et al., 2020).

1.7.1.3 DDX11 functions

DDX11 was first characterized to be an ATP-dependent DNA helicase, that is capable of translocating on single-stranded DNA with a 5´to 3´direction and needs as the requirement to be loaded one single-stranded 5’ tail with at least 15 nucleotides (Amann et al., 1997; Farina et al., 2008).

DNA helicases have their core function helping to counteract replication stress, by their ability to untangle alternative DNA structures. These structures can arise in regions with repetitive sequences like the centromeres, telomeres, ribosomal RNA gene clusters, and fragile sites.

DDX11 helicase activity shows substrate preferences for structures like three-strands D-loops. This suggests a role in homologous recombination (HR) reactions (Shah et al., 2013) or telomere metabolism, due to the structural similarity between D-loops and T-loops present in the chromosome ends (Santos et al., 2021). DDX11, unlike the other DNA helicases from the same family (FANCJ and RECQ1), can bypass and unwind DNA substrates containing cPu (8.5' cyclosporine deoxynucleotide adduct) adducts on the translocating strand. These adducts cause modifications on the DNA helix twist and base pairing stacking that affect DNA replication and transcription (Khan et al., 2014; Shah et al., 2013) DDX11 repair of DNA damages by HR occurs by association with the 9-1-1 checkpoint clamp and its loader, Rad17 (Abe et al., 2018). DDX11 is involved in the replication fork protection, releasing the stress created by the alternative DNA structures that block the replication process. To do that, DDX11 interacts with Timeless, a component of the replication fork-protection complex (Calì et al., 2015). This association is essential for the recruitment of DDX11 to the replication fork and believe to be important to sister chromatid cohesion, and the stable binding of the cohesin complex onto chromatin in the S phase (Cortone et al., 2018).

Given that DDX11 associates with the heterochromatin protein 1 (HP1, protein involved in the assembly of higher-order chromatin structure and epigenetic inheritance) at pericentric and telomeric sites of chromosomes, DDX11 was proposed to be also involved in the organization of the chromosomal territories, influencing gene expression (Inoue et al., 2011).

DDX11 also is known to unwind G-quadruplex (G4) structures (van Schie et al., 2020). G4 structures is one of the DNA secondary structures together with triple-strands (triplex). The G4 structures can have different compositions like tetramolecular G4 which is composed of four different parallel DNA strands, bimolecular G4 which is composed of two different DNA strands, and unimolecular G4 that are formed by a unique strand folding on itself. These structures can be formed in the same molecular (intramolecularly) or between two different molecules (intermolecularly) (Reviewed in Santos et al., 2021).

DDX11 has a higher catalytic efficiency to unwind G4 than the other DNA helicases present in humans. Yet, other members of the SF2 Fe-S DNA helicases family are also capable of unwind G4 structures. Many studies have highlighted that these structures like the G4 might impact many aspects of the genome metabolism, including gene transcription regulation, replication origin definition and activation, DNA replication, and telomere maintenance (Varshney et al., 2020). Recently it has been described that the Timeless protein has a G-quadruplex binding domain that works together with the DDX11 helicase to facilitate replication of G4 DNA structures, which seems to support sister chromatid cohesion (Lerner et al., 2020; Santos et al., 2021; van Schie et al., 2020).

DDX11 also is proposed to work in sister chromatid cohesion, which is supported by several evidence (Reviewed in Santos et al., 2021). The presence of railroads chromosomes in WABS patients suggests that DDX11 is involved in the loading of the cohesin to the chromosomes; similarly, DDX11 siRNA in HeLa cells shown abnormal sister chromatid cohesion (Parish et al., 2006). Another evidence is that DDX11 binds to Fen1 (Flap endonuclease, responsible for Okazaki fragment maturation) and induces its catalytic activity. This opened the hypothesis that DDX11 can impair cohesin establishment by executing a function at the replication fork in the lagging strand (Parish et al., 2006). DDX11 yeast ortholog helps to anchor the cohesin ring to the replication fork and facilitate the SMC3 acetylation Cohesion establishment depends on de novo acetylation during DNA replication and contributes to the maintenance of sister chromatid cohesion (Beckouët et al., 2010; Minamino et al., 2023; Terret et al., 2009). This will stabilize the cohesin ring to do not be unloaded by the WAPL (as referred in the section 1.5.3). This acetylation during in S-phase is on charge of the enzyme ESCO2. Of interest, RPE-1 cells knockout for DDX11 predominantly rely on ESCO2, not ESCO1, for residual sister chromatid cohesion, growth and survival (Faramarz et al., 2020). Also, DDX11 ability to resolve secondary DNA structures is necessary to the replication fork progression, because this way facilitates the progression of the replication fork, and the sister chromatid cohesion, because facilitates the loading of both strands into the cohesin ring, which was reported to require single-strand DNA (Murayama et al., 2018). It was shown that both DDX11 and ESCO2 knockdown are lethal to the cells and in the loss of only one the other is capable of lead to a level of cohesin that can still be tolerated. This shows that both act in different pathways, but both promote cohesin loading and establishment in synchrony with DNA replication fork passage (Faramarz et al., 2020).

1.7.1.4 DDX11 in cancer

In the context of cancer, problems with DNA helicases, like DDX11, can have different outcomes depending on the genetic context and the tissue type. In some cases, the inactivation of the DNA helicases leads to an increment in the predisposition to cancer, acting as a tumor suppressor gene. In other cancers, we can see an increment in the level of DNA helicases expressed, indicating an oncogenic activity (Dhar et al., 2020).

In 2021, Pisani laboratory published a thorough review on the link between DDX11 and cancer and its potential functions and implications in cancer progression. Below is a summary of the relevant findings described in this review (Mahtab et al., 2021).

Genome sequencing data has highlighted DDX11 levels are altered in many cancer types. In many cases, DDX11 is over-expressed in the tumor tissues relative to the healthy ones. This is observed in cancers such as hepatocellular carcinomas, osteosarcoma, melanoma, and lung adenocarcinoma. Over-expression seems to be associated with poor survival rates, which led to speculation that DDX11 has an oncogenic behavior.

In hepatocellular carcinoma (HCC), DDX11 appears to have roles in the onset and progression of the disease. While upregulation of DDX11 appears to have a direct correlation with lower survival rates when DDX11 is downregulated it is seen a suppression of the proliferation. Studies, described in better detail at (Mahtab et al., 2021), suggest that DDX11 acts as an oncogene in HCC by interacting with the PI3K/AKT/mTOR and E2F1/EZH2/p21 pathways.

In Osteosarcoma, it was identified a DDX11 antisense RNA 1, DDX11-AS1. Long noncoding RNA (LncRNA) has an important role in the onset of the development of osteosarcomas. Despite that, very few LncRNA have been characterized even if 25% of the human genome have LncRNA. It is speculated that LncRNA may have roles in the regulation of multiples pathways involved in functions like cell cycle progression, nuclear transport, gene transcription, and chromatin remodeling along others, and because of that if occurred deregulation of this LncRNA, they could be responsible for the development of tumors (Sanchez Calle et al., 2018). The DDX11-AS1 in this type of cancer is a transcript that is harbored at the same site of the DDX11 at chromosome 12 but is transcribed in the opposite direction without overlapping with the DDX11. In osteosarcomas DDX11-AS1was overexpressed of the. It was suggested that DDX11-AS1 may be suppressor of the Epithelial-Mesenchymal Transition (EMT) due a link with miR-

873-5p known to regulate EMT in many cancers. Also, DDX11-AS1 has been found to be able to interact with the insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2), a protein that is known to have oncogenic roles in many cancers that are capable of stabilize the DDX11 mRNA this way increasing their levels.

In lung adenocarcinoma (ADC) was identified an increase of the levels of DDX11 mRNA and like in other cancers the overexpression of the DDX11 is correlated with a decrease in the survival rate in the ADC, and the levels of DDX11 also show a correlation with the tumor size and stage.

In melanoma also was found the same increase in the levels of DDX11 mRNA, and also the correlation with the tumor size and stage. When DDX11 is silenced in melanoma models, using siRNA, it has observed sister chromatid cohesion anomalies, chromosome breakages, telomere shortening, cell proliferation inhibition, and high levels of apoptosis, which suggests that DDX11 could be a good therapeutic target to cure melanomas.

The behavior of DDX11 in these cancers indicates an oncogenic activity but remains speculative if DDX11 could act as a tumor suppressor gene in other conditions and tissues, due to the fact that loss of DDX11 lead to sister chromatid cohesion defects, chromosomal breakage, and genome instability, which are important propelling factor to tumorigenesis.

While this review (Mahtab et al., 2021) points into the importance of DDX11 in cancer, interestingly none of the WABS patients identified so far, have been diagnosed with any type of cancer (van Schie et al., 2020). This could be due to the fact that from the studies performed in cancer, DDX11 is overexpression when compared with control condition, while in the WABS patients, the described pathologic mutations typically lead to destabilization or a decrease in the levels of DDX11 (Pisani, 2019; van Schie et al., 2020).

1.7.2 Etiology of WABS

The etiology of WABS and in general cohesinopathies is supported by two non-exclusive hypotheses. The first hypothesis is that cohesinopathies are caused by problems during replication and DNA repair. In the case of WABS, it is a disease that can be caused by dysregulation in the genes expression due the fact that cohesin complex has important roles in the organization of the chromatin loops that are believed to play a role in

regulating gene transcription. Also, the interaction of the DDX11 with the HP1 factor suggests the involvement of DDX11 in the chromosome architecture maintenance (Reviewed in Santos et al., 2021).

Alternatively, in light with Carvalhal and Sapkota paper (Carvalhal et al., 2018; Sapkota et al., 2018), a second hypothesis has been proposed. This hypothesis supports that cohesinopathies can also be caused by problems during mitosis. While loss-of-cohesin is incompatible with life, these studies showed that cohesion-loss before disjunction of the sister chromatids causes segregation errors – which is overall the case in WABS - crucial to maintenance of genome stability. On the following section we will detail some ongoing research in the lab that support this hypothesis. In this case, pathologic DDX11 may cause loading of cohesion problems, as previously suggest (Rudra & Skibbens, 2013), which will lead to cell enter in mitosis with a compromised cohesion leading to mitotic segregation errors and defective attachment/ chromosome structure. A less obvious link with mitosis would be the fact that DDX11 has been described with a centrosomal localization in metaphase, where can hold unforeseen functions in mitosis that also contribute to WABS etiology.

While etiology of WABS is still an open question, we should consider the limited disease models currently available. Many have attempted to create mouse models for the WABS have shown to be lethal at post-embryonic stages (Santos et al., 2021). Yet currently there are zebrafish DDX11 KO model embryos have many phenotypes that resemble the ones seen in some WABS patients, namely microcephaly (Sun et al., 2015). At the cellular level, WABS immortalized patient cells and DDX11 CRISPR depletion have been used to understand the role of DDX11 (van Schie et al., 2020). Ongoing research at Pisani lab are generating iPSC from patient cells (personal communication).

1.7.3 Ongoing research on WABS etiology in the lab

Analysis of segregation errors in WABS patient cells, made in our laboratory, has shown that immortalized fibroblast from patients have an increased number of segregation errors, namely lagging chromosomes and DNA bridges (see Figure 23, unpublished results). While DNA bridges may be associated with replication defects, lagging chromosomes are more often associated with problems in kinetochore-microtubules attachments (Orr et al., 2015). Due to the presence of this type of errors, one

of the mitotic players that stands out is the Aurora B, that are involved in the correction of aberrant kinetochores-microtubules (see below for a description of Aurora B function). This hypothesis is supported by previous studies on chicken DDX11 KO cells where Aurora B was shown to be defective (Abe et al., 2016).

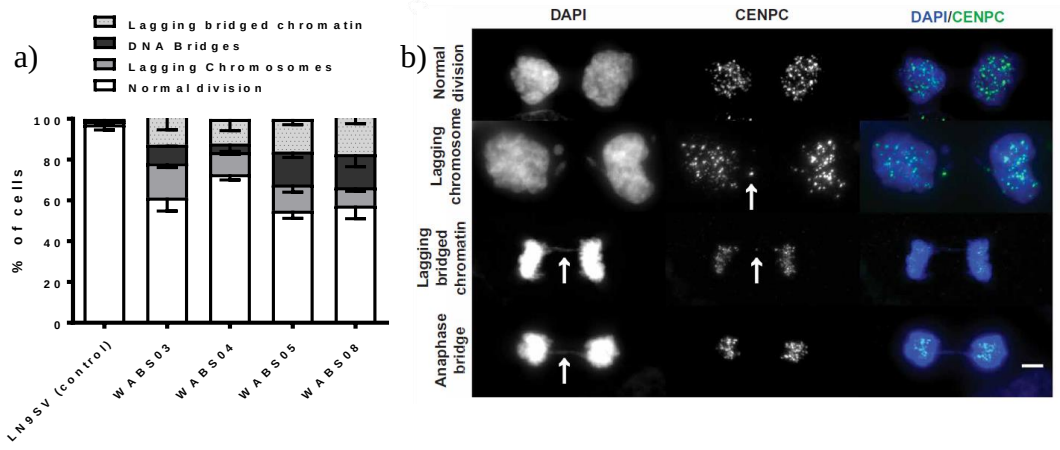

Figure 23: WABS patients' cells display chromosome segregation errors. Chromosome segregation was evaluated by fixed imaging of unsynchronized cells stained by tubulin, CENPC and DNA. More than 99 mitosis cells were analyzed, derived from at least three independent experiments. a) Percentage of segregation error observed in the control cells and WABS patient cells available in our lab; b) Images depict examples of each category scored: lagging chromosomes, lagging bridged chromatin (DNA bridge with centromere staining), and anaphase bridges (defined when no centromere staining was visible within the DNA bridge), category differences are highlighted by white arrows; Images used are from (Carvalhal et al., 2022). Shared with consent. WABS cells used in this experiment have been previously described in van Schie et al., 2020). Carvalhal, unpublished results.

In certain cohesinopathies, including WABS, mitotic chromosomes display railroads morphology, which has have suggested to be caused by loss of cohesin (Kawasumi et al., 2021; Rudra & Skibbens, 2013). To assess the *in vivo* impact of loss of cohesin can be infer by a live imaging assay where it is measuring the strength of cohesion by causing cohesion fatigue (a term used to describe when cohesion forces surrender to mitotic spindle forces). This is done by the use of two inhibitors of APC to arrest cells specifically in mitosis (without perturbing interphase). Once cells are arrested the spindle pulling forces will be counteracted by the strengthen of cohesion. As such, low level of cohesin will give rise to shorter time to enter in cohesin fatigue. Using this approach, research in the lab, we evaluated timing of WABS patient cells (same as described in Figure 23) to enter in cohesion fatigue state as a proxy to evaluate the impact of cohesion loss in these cells, see Figure 24.

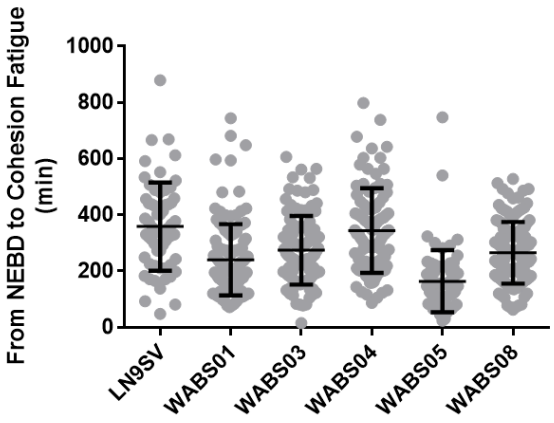

Figure 24: Cohesion fatigue is different across WABS patient cells. Cells were treated with 20 μM proTAME and 100 μM apcin to specially arrest cells at metaphase without perturb interphase. The time from NEB to the moment at which single chromatids start escaping the metaphase plate (cohesion fatigue) was assessed by live-cell imaging. The time is used as a proxy of the *in vivo* cohesion strength. Despite an overall reduction of timing, WABS04 does not show a significant difference when compared with control conditions (LN9SV cells). In fact, only WABS05 cells shows reduce time, which is similar to the timing measure in DDX11 KO cells (Carvalhal et al., 2022). Each dot represents one cell. Results from at least three independent experiments. Statistical analysis was performed using one-way ANOVA. Carvalhal, unpublished results.

From this assay, it can be appreciated that WABS patient cells do not have a severe reduction in the time to enter in cohesion fatigue. As such, one can ask, how cells have lower cohesin levels and holdup a stronger cohesion? A hypothesis explore in (Carvalhal et al., 2022) where a similar phenotype was observed, was that defective resolution of the DNA catenation, ensured by TOP2A, mimics a strong cohesion even with low levels of cohesion. As such we decide to explore if these was the case in WABS cell and explore the localization of TOP2A in the WABS system models available in the lab.

On the following section we described in more detail Aurora B kinase and TOP2A as we decided to investigate the localization of these on my thesis work.

1.7.3.1 Aurora B

Aurora B is a serine/threonine kinase that belongs to a highly conserved family of three kinases: Aurora A, B, and C. All of them have a function in mitosis or meiosis. Aurora A is involved in the mitotic spindle, and Aurora B and C are involved in the centromere and anaphase spindle. While Aurora B works in mitosis and Aurora C works in meiosis (Carmena et al., 2012). Aurora B is part of a larger protein complex called Chromosomal Passenger Complex (CPC). This complex is composed of four subunits: the Aurora B (kinase subunit), the INCENP (scaffold subunit), and the Survivin and Borealin (regulator subunits). Together these subunits create two modules, the localization modules and activity modules. The first is composed of the Survivin and Borealin associated with the N-terminal CEN domain of INCENP and have the function of controlling the localization of the CPC. The second is composed of the Aurora B associated with the C-terminal In-box domain of INCENP and have the functional part of this complex (Hindriksen et al., 2017; Yi et al., 2019).

Aurora B by itself does not have a kinase activity, it needs first to be activated. The processes of activation are dependent on phosphorylation events that occur in trans (between two different molecules). First the INCENP binds to Aurora B activating the Kinase activity, after that Aurora B is now capable of phosphorylating the TSS (Thr-Ser-Ser) motif present in the C-terminal of the INCENP and the Threonine 232 present in T-loop of its kinase domain, leading to Aurora B fully activated (Carmena et al., 2012).

But this full activation of Aurora B also has another intervenient, involved in its activation but also in its localization. For example, increasing the local density of the CPC stimulates Aurora B activity, so in regions like the inner centromere and the spindle midzone full activity of Aurora B is stimulated. Aurora B upon posttranslational modifications, like the phosphorylation of the Serine 311 by the Chk1, leads into the activation of the Plk1 that is a regulator of the microtubule-binding at the kinetochores; the ubiquitination by the E3 ubiquitin ligase cullin 3 (CUL3) that promotes relocalization of the CPC in anaphase; and the sumoylation of the kinase domains, necessary to the mitotic progression (Carmena et al., 2012). This phosphorylation only occurs when Aurora B is close to the kinetochores. As such, phosphorylation of Aurora B needs to be proper regulated to ensure a correct cell division as well as correction of erroneous attachments between kinetochore and microtubules. When the kinetochores are not

correctly attached to microtubules, Aurora B phosphorylates several substrates at the kinetochores, which destabilizes erroneous attachments. So is necessary some type of regulation that when kinetochores are correctly attached to the microtubules will inhibit the activity of the Aurora B (Carmena et al., 2012). Aurora B is recruited to the inner centromere region at the first stages of mitosis, where it will phosphorylate several proteins at the kinetochores. When the correct attachment of kinetochore-microtubules occurs, the pulling forces created by the spindle will put away the kinetochores from the Aurora B pool, easing the kinase gradient generated on Aurora B to act on the kinetochores. However, Aurora B not only accumulates at the inner centromere region but also at the region of the kinetochores, as such, it is currently assumed that Aurora B is capable of phosphorylate kinetochores substrates in an independent manner of the inner centromeres pool (Broad et al., 2020; Hadders et al., 2020).

Aurora B recruitment is mediated by two different pathways, the Haspin, which phosphorylates the histone H3 at the Thr3 (pH3-T3) this way creating a binding site for the Survivin (a component of the CPC); and the Bub1, which phosphorylates the histone H2A at the Thr120 (pH2A-T120) this way recruiting the Shugoshin 1 (Sgo1) that is a binding site to the Borealin (another component of the CPC) (Figure 25). These two phosphorylations lead to the recruitment of the Aurora B to the centromeric region. But these modifications are not overlapped, each one is spatially distinct, the pH3-T3 are localized in the inner centromere and the pH2A-T120 are localized in the kinetochores proximal outer centromere, and both are capable of recruiting the Aurora B individually, this way creating two distinct pools of Aurora B. Recent studies found a third pool that is independent of the Haspin and the Bub1, located in the inner kinetochore close to the centromeric protein CenpC. This Aurora B pool can phosphorylate kinetochores substrates (Broad et al., 2020; Hadders et al., 2020).

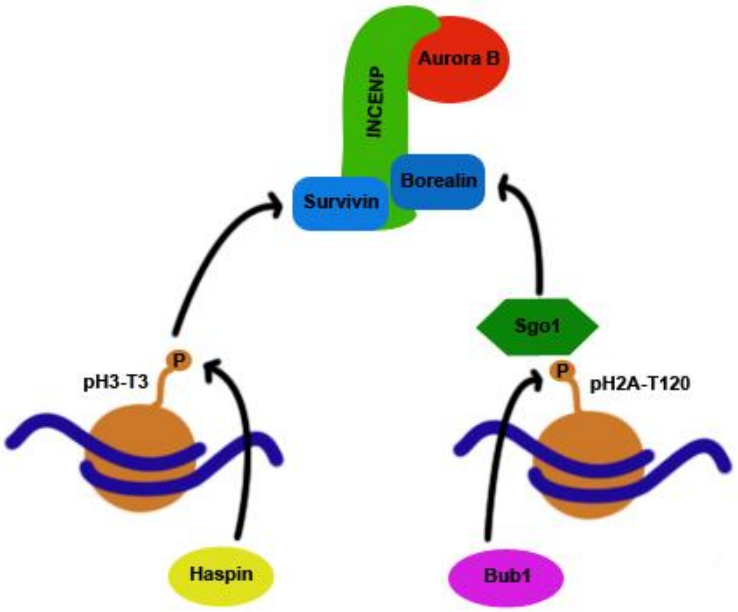

Figure 25: CPC complex and the cofactors that recruit it. Representation of the CPC complex (Survivin, Borealin, INCENP and Aurora B) and its regulators responsible for recruit the complex to their respective pools.

Lens laboratory showed that when Aurora B stops to localize at this centromeric region increases the frequency of anaphases with lagging chromosomes, but surprisingly did not compromise the mitotic checkpoint or the phosphorylation of the Aurora B kinetochores substrates. This suggests that the correction of erroneous kinetochore microtubule attachments is more sensitive to reductions in the centromeric Aurora B levels than activation of the mitotic checkpoint and the phosphorylation of the kinetochore substrates. Probably because both individual pools of Aurora B are capable of support faithful chromosome segregation in normal cells, and the third pool, by phosphorylating the kinetochores contributes to mitotic checkpoint signaling and also to the regulation of the kinetochore-microtubule attachments (Hadders et al., 2020).

The recruitment of Aurora B to the kinetochores by the Bub 1 modification in the pH2A-T120, is also crucial for stabilization at the centromeric region. The pH2A-T120 will lead to the recruitment of the Sgo1, that is an antagonize of the Aurora B and the Cdk1 phosphorylation of the Sororin, which is responsible for inhibiting the cohesin unloading by the WAPL (Nishiyama et al., 2013).

1.7.3.2 Topoisomerase II

Topoisomerase II (TOP2A) is an enzyme that belongs to the family of topoisomerases, whose function is to modulate the supercoiled state of DNA. In eukaryotes, TOP2A is the only enzyme capable of untangle topologically intertwined DNA molecules or creating new entanglements. In other words, the TOP2A is capable of decatenate or catenate the DNA. TOP2A is composed of two equal subunits (homodimer) and can change the entanglement of the DNA by allowing one DNA double strand to pass through another. To do that, TOP2A binds to one of the double strands, cut them to allow the other doubles strand to pass through and then rejoin the cleaved strands (Figure 26) (Piskadlo & Oliveira, 2017).

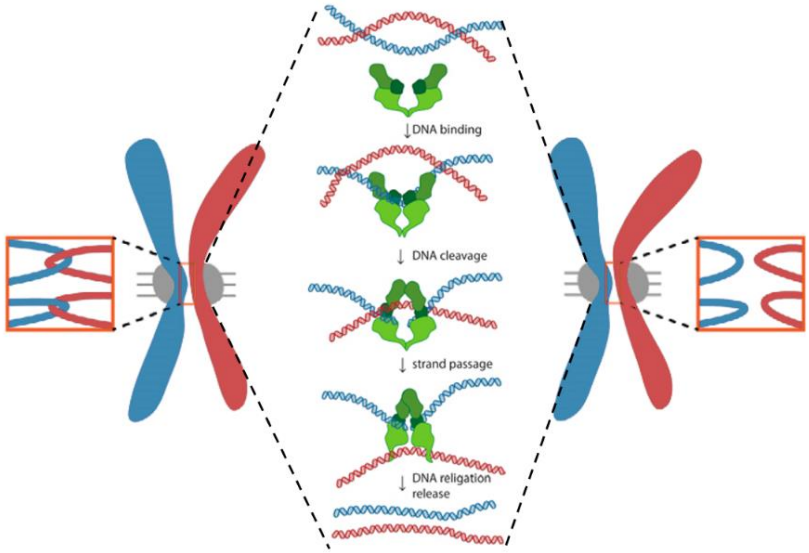

Figure 26: Mechanism of DNA decatenation by topoisomerase II. TOP2A is capable of decatenate DNA by bindings to one of the DNA strands, cleave that strand and pass the cleaved strand through the other DNA strand, then rejoin the cleaved strand and releases the DNA molecules (Figure adapted from Piskadlo & Oliveira, 2017 and Zhang et al., 2020).

During DNA replication, the DNA is inevitably entangled creating catenation. As consequence, if these catenations are not resolved until the first stages of mitosis will lead to errors in chromosome segregation that can cause DNA bridges. So, sister chromatid resolution is the process that will lead to the chromosome X shape structure, composed of two sister chromatids, it is critical to faithful segregation, chromosome individualization, and chromosome compaction. But to achieve all these things is necessary to have an equilibrium between the DNA catenation: it is necessary decatenate the DNA to allow the resolution of the chromosomes, but it is also necessary some catenation to allow the DNA compaction (Reviewed in Piskadlo & Oliveira, 2017). In these processes proteins like the condensin are necessary. Condensin is a protein complex

that has roles in the formation of the chromosome structure and in ensuring the integrity of condensation remains until the right moment (Hudson et al., 2009). But in this work, we will not delve too deeply into this topic, as it is not part of the scope of this work.

The sister chromatid resolution is essential for mitosis to ensure the correct individualization of the chromosomes, studies show that all the catenation is, in normal conditions, totally resolved until the first stages of mitosis. The absence of TOP2A will lead to problems in anaphase that will result in DNA bridges and consequently DNA breaks (Charbin et al., 2014). This is a gradual process where first the chromosome arms will be decatenated progressively leaving to the end the centromeric region that will be decatenated only close to the onset of anaphase.

Maintenance of the catenation is important to ensure the correct chromosome condensation, the absence of TOP2A activity leads to problems in the sister chromatid disjunction but one overactivity of the TOP2A can lead to significant changes in the chromosome shape. By these studies it was found that TOP2A also can create catenation. This finding together with classic studies that show that TOP2A is one of the most abundant proteins present in the chromosomes, corroborate that TOP2A is involved in DNA compaction together with the condensin maintaining condensation (Piskadlo & Oliveira, 2017). Yet the role of TOP2A catalytic activity or non-enzymatic plays into chromosome compaction remains unclear. So far what has been more believed is that the catalytic activity of TOP2A introduces self-entanglement to promote compaction (D. L. V. Bauer et al., 2012).

Due to the TOP2A having both the function of decatenate and catenate is necessary some type of regulation to ensure the decatenation between the sister chromatids (trans) and the catenation in each chromatid (cis). This regulation is made by post-translational modifications, like phosphorylation which will increase the catalytic activity of TOP2A (Chikamori et al., 2003), and sumoylation which will decrease the decatenation activity (Agostinho et al., 2008). But this modification only affects the activity of TOP2A and not the way how the TOP2A identifies what is trans and cis. Some proteins are proposed to be involved in this point namely Structural maintenance of chromosomes (SMC) complexes: the SMC5/6, the cohesin, and the condensing. Yet, how these proteins interact with TOP2A are still unknown (Reviewed in Piskadlo & Oliveira, 2017).

In the case of cohesin, it is hypothesized that the presence of cohesin “inhibits” the activity of TOP2A, as cohesin holds the sister chromatids together, so even if TOP2A tries to untangle the DNA, it ends up re-entangling itself by the reverse activity of its own. So one way of cohesin can impair the sister chromatid resolution is by only allowing the resolved when it is removed, and two possible ways that it can impair the sister chromatid resolution is by working as a physical barrier, preventing the TOP2A from interacting with the chromatin or it can hold the sister chromatids close “inhibiting” the activity of TOP2A (Farcas et al., 2011b; Sen et al., 2016).

2. Objectives

Throughout this thesis my overall goal is to give a step further in the understanding etiology of WABS. To achieve it, I focused on tree main questions:

(i) Does DDX11 have mitotic roles? The DDX11 is known to have function in the DNA repair and transcription, and in the loading of cohesin, yet no mitotic specific roles have been described. It has been shown previously that DDX11 during interphase have a nuclear localization and in mitosis it has a centrosomal localization (Parish et al., 2006). If DDX11 holds some mitotic specific roles has never been study, and this role could contribute to the observed mitotic errors in WABS patient cells. As such, we aimed to studying the localization of the DDX11 at the patient cell to understand this mitotic role.

(ii) Is Aurora B defective in WABS patient cells? Aurora B is one of the components of the mitotic spindle assembly checkpoint, and problems on its localization leads to mitotic segregation errors (Broad et al., 2020; Carmena et al., 2012; Hadders et al., 2020). It has also previously shown that Aurora B localization is affected in DT40 cells upon DDX11 knockout (Abe et al., 2016), but this analysis was never performed in cells expressing any mutant version of DDX11, including DDX11 pathological mutation. As such, under the work of this thesis is was study the localization of the Aurora B at cellular WABS system models with the of understand if Aurora B localization is affected which could contribute to the mitotic segregations errors observed.

(iii) Is TOP2A a possible explanation of robust cohesion fatigue observed in WABS cells? TOP2A have function of resolving DNA catenation, but in some condition also is capable of create more catenation, that could have impact at the cohesion strength and in the chromosome segregation fidelity. The WABS patient cells are associated with decreased levels of cohesin, and DDX11 have been shown to have impact in the levels of cohesin subunits; e.g Smc3 as described in (Kawasumi et al., 2021; Rudra & Skibbens, 2013). But overall, WABS immortalized patients' cells still

present a robust cohesion strength. One possible hypothesis for explaining this phenotype could be a defective TOP2A localization that could lead to DNA catenation, this way preventing the disjunction of the sister chromatids even with lower levels of cohesin. So, by studying the TOP2A localization we could have a lead if TOP2A localization is affected at the WABS patient cell.

To achieve answer these questions I used two cell system models: WABS patient cells that have been immortalized using SV40 and a CRISPR DDX11 model generated in RPE-1 p53 KO cells.

3. Methods

3.1 Cell Lines and Cell Cultures

A total of seven cell lines were used in this project, all kindly gifted by our collaborator Job de Lange (Vanderbilt university medical center), see Table 3. These cells have been described before. In summary in this project were used two different sets of cells: the skin fibroblast set LN9SV SV40 immortalized cell line obtained from a healthy donor (healthy control) (Digweed et al., 1995), WABS03 and WABS04 patient cell lines. The last two were generated from fibroblast of two different WABS patients. WABS03 cells are from a male presenting respiratory problems, several developmental delays, microcephaly, sensorineural deafness, hyperactivity, congenital hypothyroidism, low set ears and retrognathia as clinical features and present a biallelic mutations on the DDX11 gene, in one copy have one duplication of thymine in the position 1403 and in the second copy have a substitution of a guanine for a adenine in the position 419. The second cell line WABS04 is from a female presenting hyperactivity, growth and mental retardation, deafness, microcephaly, skin pigmentation, facial dysmorphy, bulbous nose, clinodactyly, insulin-dependent diabetes mellitus and respiratory problems as clinical features and biallelic mutations on the DDX11 gene. One copy has one substitution of a guanine for an adenine in the position 1930 and in the second copy have a substitution of a guanine for a adenine in the position 2114. All cells have been described in (van Schie et al., 2020).

The second set of cells is four clonal knockouts in RPE-1 cells, constructed using CRISPR-Cas9 technology. RPE-1-hTERT_TetOn-Cas9_TP53KO (control cell, with a TP53 KO), the RPE-1-hTERT_TetOn-Cas9_TP53KO_DDX11KO (control cell with a DDX11 KO transfected with an empty vector (EV) used on the followed two cell lines), the RPE-1-hTERT_TetOn-Cas9_TP53KO_DDX11KO transfected with a wild type version of DDX11 (DDX11 KO + DDX11 WT), and RPE-1-hTERT_TetOn-Cas9_TP53KO_DDX11KO transfected with a mutant version of DDX11 (DDX11 KO + DDX11 K50R). DDX11 K50R has null helicase activity (Farina et al., 2008b; Wu et al., 2012). Expression of EV, DDX11 WT and DDX11 K50R is kept under blasticidin pressure. Cells have been previously described in (Benedict et al., 2020).

All the cell lines were cultured in Dulbecco´s Modified Eagle's Medium (DMEM) high glucose (BioWest) supplemented with 10% Fetal Bovine Serum (FBS) Premium (BioWest), 1% Sodium Pyruvate (BioWest), 1% Glutamax (BioWest) and 1% Penicillin/Streptomycin (BioWest). Media of RPE-1 carrying DDX11 KO and variable transfections were supplemented with 2 µg/mL of Blasticidin. Cells grown in a humidified incubator at 37ºC with a 5% CO_2 atmosphere.

Table 3: Cell lines. Different cell lines used in this thesis, with a brief description and a type of cell.

Cell line	Cell type
LN9SV (control) SV40 immortalized cell line obtained from a healthy donor	Fibroblast
WABS03 Patient cell line with biallelic mutations on the DDX11 gene (V1. 1403dupT; V2. 419G>A)	
WABS04 Patient cell line with biallelic mutations on the DDX11 gene (V1. 1930G>A; V2. 2114G>A)	
TP53 KO CRISPR-Cas9 TP53 knockout	RPE-1
DDX11 KO + EV CRISPR-Cas9 p53 and DDX11 knockout	
DDX11 KO + DDX11 K50R CRISPR-Cas9 p53 and DDX11 knockout, transfected with a DDX11 mutant variant (K50R) with helicase dead activity	
DDX11 KO + DDX11 WT CRISPR-Cas9 p53 and DDX11 knockout, transfected with a Wildtype DDX11 variant	

3.2 Chromosome spreads

3.2.1 Manual Preparation

Culture cells with a confluence of around 80-90% were treated with 10 µM of Colchicine for 30 minutes in optimal growth conditions to arrest the cells in metaphase. After incubation, medium was carefully removed, and warm PBS added to harvest the cell by a process called mitotic shake-off loosely attached rounded-up cells will be detached. Mitotic shake-off was achieved by applying slight mechanical force with the flow of the up and down of pipetting. Collected suspension media with mitotic cells was centrifuged at 1200 rpm for 8 minutes in a 15 mL Falcon. The majority of supernatant was removed (around 50 µL left), then the pellet was gently resuspended in 4 mL 75 mM KCl and left to incubate for 30 minutes at room temperature (± 25ºC) to enlarge the cell which will help separate the metaphase chromosome in the next steps. Upon second centrifugation the cells are resuspended in a fixing/permeabilizing solution, composed of 4% PFA and 1% Triton x-100 in PBS, for 10 minutes. Then was centrifuged a third time: cells were resuspended in low quantities of the fixing solution (approximately 150 µL) to increase the concentration of cells per volume. A 50 µL of solution was dropped into a glass coverslip, treated with poly-L-lysin to increase the adherence, from approximately 60 cm high. After air dry the coverslip, it was washed two times in TBS-wash and one time in PBS-wash for 5 minutes each. The coverslip was incubated for 30 minutes in blocking solution (TBS, 0.1% Triton X-100, 2% bovine serum albumin (BSA, NzyTech), and 0.1% sodium azide, also called Abdil). The coverslip was then mount in Vectashield Mounting Medium with DAPI in glass slides and sealed.

3.2.2 Mechanized Preparation (Cytospin)

Culture cells with a confluence of around 80-90% were treated with 10 µM of Colchicine for 30 minutes in optimal growth conditions to arrest the cells in metaphase. After incubation, medium was carefully removed, and warm PBS added to harvest the cell by mitotic shake-off. Collected suspension media was centrifuged at 800 rpm for 4 minutes and are resuspended in 75 mM KCl in the proportion of 1:1 (usually 50 µL of media and 50 µL of KCl) and left to incubate for 7 minutes at room temperature. After that was added in the proportion of 1:1 2% BSA. A total of 100 µL of the solution was loaded into a cytospin funnel. This mixture was centrifuge in a cytospin at 800 rpm for 4

minutes into a glass coverslip treated with poly-L-lysine (Gibco). Coverslips were let to air dry for 1 minute. After a fixing/permeabilizing solution (4%PFA, 1% Triton x-100 in PBS) was added to the coverslip and incubate for 10 minutes at room temperature. This was followed by wash the coverslip two times in TBS-wash and one time in PBS-wash for 5 minutes each. Coverslip was incubated in blocking solution (Abdil) for 30 minutes and immunofluorescence processed as described in the next section. During optimization, coverslips were mount in Vectashield Mounting Medium with DAPI in glass slides and sealed.

This protocol has been described in Carvalhal et al., 2022.

3.3 Immunofluorescence

3.3.1 DDX11 localization

For study DDX11 localization, cells were seeded on glass coverslips and fix at a confluence of 80% - 90%. Cells were fixed and permeabilized in 4% PFA, 1% Triton x-100 and blocked in Abdil (TBS, 0.1% Triton X-100, 2% bovine serum albumin (BSA), and 0.1% sodium azide) for 30 min at room temperature. Coverslips were incubated overnight at 4ºC in a mixed solution with all the primary antibodies in Abdil (DDX11 (1:100; H00001663-B01P, Abnova) alone or together with Anti-Pericentrin (1:1000; Abcam), in a dark humidified chamber, then the coverslips were washed three times in PBS-wash for 5 minutes each and then incubated for 1 hour at room temperature in a mix solution with all the secondary antibodies Alexa 488 and Alexa 549 (1:500) and one DNA intercalator-dye (33342 Hoechst (Thermo)), in a dark humidified chamber. Coverslips were washed three times with PBS-wash for 5 mins each and mounted with Vectashield Mounting Medium in glass slides and sealed.

3.3.2 Aurora B & TOP2A localization

For processing chromosome spreads for immunofluorescence upon 30 min incubation with AbDil, coverslips were incubated overnight at 4ºC with the first primary antibody: anti-Aurora B or anti-TOP2A. In the following day, coverslips were incubated

for 1 hour at room temperature with a suitable secondary Ab. Then incubated again with the second primary Ab, in this case anti-CENPC for one hour at room temperature. Upon that period the corresponding second secondary antibody and the DNA intercalator-dye was incubated for 1 hour at room temperature. Between each incubation, excess of antibody was removed by washing three times with PBS-wash for 5 mins each. Coverslips were mounted with Vectashield Mounting Medium in glass slides and sealed.

The antibodies used were anti-TOP2A (1:1000; M042-3, MBL), anti-Aurora B (1:200; AIM-1, BD Biosciences), anti-CENPC (1:500; MBL International), and secondaries Alexa 488 and Alexa 549 (1:500), DNA intercalator-dye (33342 Hoechst (Thermo)) (1:1000).

3.4 Imaging

Images were acquired on a Delta-Vision Elite microscope (IMSOL) with a 100X oil-immersion 1.35 Na objective lens (Olympus), a cMOS camera (Photometrics), Standard filter sets, and SoftWoRx software (version 5.5.0; Applied Precision). All the images were three-dimensional acquisitions (Z-stack) with between 20 to 30 slices (defined by the DNA channel) with a distance between them of 0.2 μm, in all acquisitions used the same settings of exposure time and laser intensity. And all cells selected are selected based on the DNA staining.

3.5 Imaging analysis / Quantifications

All acquired images were processed the images acquired in FIJI (Image J). All present images are z-stack projections without subtraction background.

3.5.1 Distribution of proteins along chromosomes

Analysis of protein distributions were performed in z-stack projections made based on DNA channel. I have adapted an in-house-developed macro to measure mean intensity of the signal at specific regions defined in a semi-manually mode (Region of interest, ROI). Four regions of interest were used: two centromeres region defined by the two CENPC signals, the inner centromeric region defined by the region between the two

CENPC signals, and the arm region defined by the DAPI staining. The mean intensity of the signal in each region was measured using a circle of 6 pixel diameter placed in each respective region of the same chromosome, and the signal was measured in all the different channel of fluorescence (CENPC, DAPI, and TOP2A or Aurora B). Per image 3 to 5 chromosomes were measures, the selection of the chromosome was random only requiring them to be perfectly isolated. All the collected the data was later analyzed in FIJI and GraphPad software. Below is the macro used.

Macro to create ratios between two ROIs:

```
roiManager("Show All");
run("Clear Results");
setSlice(3);
for (i=0;i<10;i++){
  makeOval(256,256,10,10);
  setSlice(3);
  waitForUser("Select chromosome");
  setSlice(3);
  roiManager("Add");
//measures
  setSlice(3);
  resetThreshold();
  run("Measure");
  setSlice(2);
  resetThreshold();
  run("Measure");
  setSlice(1);
  resetThreshold();
  run("Measure");
  }
roiManager("Reset");
setSlice(3);
String.copyResults();
run("Open Next");
```

Macro to create multiple ROIs at the same image:

```
roiManager("Show All");
  var radius = 3;
```

```
        getCursorLoc(x, y, z, flags);
        makeOval(x-radius, y-radius, radius*2, radius*2);
    setSlice(3);
    roiManager("Add");
    resetThreshold();
    run("Measure");
    setSlice(2);
    roiManager("Add");
    resetThreshold();
    run("Measure");
    setSlice(1);
    roiManager("Add");
    resetThreshold();
    run("Measure");
String.copyResults();
```

Macro to measure the distance between centromeres:

```
//AutoReset
Property.set("CompositeProjection", "Sum");
Stack.setDisplayMode("composite");
target=round(nSlices*0.17);
Stack.setPosition(1,target,1);
resetMinAndMax();
Stack.setPosition(2,target,1);
resetMinAndMax();
Stack.setPosition(3,target,1);
resetMinAndMax();
//Select Chanel 2, and make a mask
run("Duplicate...", "duplicate channels=2");
setAutoThreshold("Default dark");
run("Threshold...");
//save the lines and make a multiplot
setTool("line");
roiManager("Show All");
//add all the lines (t to add)
```

```
waitForUser("Draw the Line");
rename("Imagem");
selectWindow("Imagem");
roiManager("Multi Plot");
Plot.showValues();
selectWindow("Results");
String.copyResults();
```

3.6 Statistics

Plots shown have been made in Prism 7 (GraphPad). To test significance, one-way analysis of variance (ANOVA) was followed by Dunnet's multiple comparison, to conduct an analysis of all possible pairwise means.

4. Results and Discussion

Part I – Selection of WABS patient cell to use

The work performed under my dissertation underlies in the use of WABS patient cells. In our lab we have access to a panel of SV40 immortalized skin fibroblast cell lines derived from five WABS patients (described in van Schie et al., 2020), yet we have decided to focus our study in two cell lines to perform my studies. For that, we evaluate the available data on structure and function to select these cell lines.

4.1 Comprehensive analysis of DDX11 mutations from the WABS patient cell panel

4.1.1 DDX11 Structure

To better understand the impact of DDX11 mutations in WABS etiology it was performed a comprehensive analysis of DDX11 secondary structure taking into account the described mutations in the cell panel (see Table 4). For that I collect information about DDX11 sequence from two databases, InterPro and UniProt using DDX11 ID: Q96FC9, and combined with the information about the domains available in the literature (Figure 27)(Pisani et al., 2018; Santos et al., 2021), as previously mentioned in section 1.7.1.1 at Introduction.

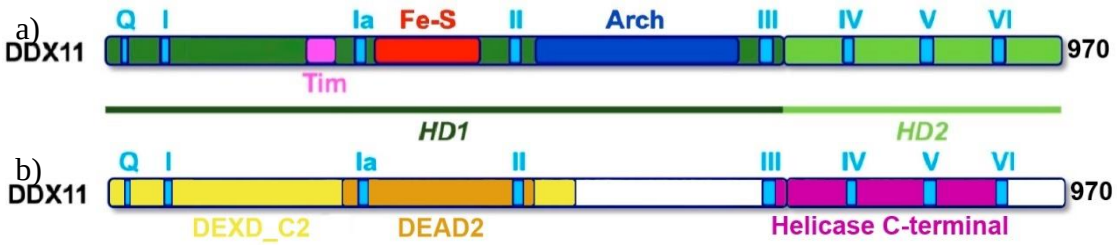

Figure 27: DDX11 composition. a) Schematic representation of the DDX11 composition, based on the Pisani et al., 2018 and Santos et al., 2021 (Figure adapted from Pisani et al., 2018); b) Schematic representation of the DDX11 composition, based on the InterPro DDX11 (ID: Q96FC9). In dark green are presented the homologous domain 1 (HD1), in light green the homologous domain 2 (HD2), in light blue are represented the Helicase motifs (Q, I, Ia, … VI), in red the iron-sulfur (Fe-S) cluster, in dark blue the arch domains (Arch), in pink the Timeless binding site (Tim), in yellow the DEXD C2 domains (DEXD-C2), in orange the DEAD2 domains (DEAD2), and in purple the Helicase c terminal domains (Helicase C-terminal).

From the information collected from InterPro I have identified three “new” domains, not referred in the literature (Pisani et al., 2018; Santos et al., 2021). Within the HD1 domain there is a DEXD_c2 domain (InterPro IPR006554) located between 11 and 437 amino acids and are known to have role in the DNA helicase activity and in hydrolase activity. DEAD2 domain (InterPro IPR010614) is located inside the DEXD_C2 domains between 231 and 415 amino acids and is known to have role in DNA binding, DNA helicase activity and ATP binding. Within the HD2, between 692 and 859 amino acids there is the Helicase C-terminal domain (InterPro IPR006555) known to have a role in nucleic acid binding, helicase activity, ATP binding and hydrolase activity.

Table 4: Mutations present in WABS patient cells. Five WABS patient cell lines available in our lab and their respective mutations presents in the DDX11 sequence and protein. Codename are attributed by (van Schie et al., 2020), where these cell lines have been firstly described. V1 and V2 are variants presented in each patient DNA coding sequence (c.). Impact of these mutations are described for their coding DNA (c.) and protein (p.) sequences.

Codename	Mutation		
	-	Coding DNA sequence (c.)	Protein sequence (p.)
WABS01	V1 – c.**2271+2T>C** / p.C754Pfs*9 V2 – c.**2689-2691del** / p. K897del	Substitution of a thymine for a cytosine in the second position of the intron started in 2271 Deletion of 3 nucleotides, from position 2689 to 2691	Substitution of a Cysteine 754 for a Proline, which make a frameshift with 9 amino acids of lengths Deletion of the Lysine 897
WABS03	V1 – c.**1403dupT** / p.S469V fs*31 V2 – c.**419G>A** / p. R140Q	Duplication of a thymine in position 1403 Substitution of a guanine for an adenine in position 419	Substitution of a Serine 469 for a Valine, which make a frameshift with 31 amino acids of length. Substitution of an Arginine 140 for a Glutamine
WABS04	V1 – c.**1930G>A** / p.V644M V2 – c.**2114G>A** / p.C705Y	Substitution of a guanine for an adenine in position 1930 Substitution of a guanine for an adenine in position 2114	Substitution of a Valine 644 for a Methionine Substitution of a Cysteine 705 for a Tyrosine
WABS05	V1 – c.**2571C>A** / p.S857R V2 – c.**1672C>T** / p.R558*	Substitution of a cytosine for an adenine in position 2571 Substitution of a cytosine for a thymine in position 1672	Substitution of a Serine 857 for an Arginine Substitution in the Arginine 558 that is nonsense
WABS08	V1 – c.**1946-1948del** / p. G650del V2 – c.**1763-1G>C** / p.G588Afs*6	Deletion of 3 nucleotides, from position 1946 to 1948 Substitution of a guanine for a cytosine in position 1763	Deletion of the Glycine 650 Substitution of a Glycine 588 for an Alanine, which make a frameshift with 6 amino acids length.

All available cell lines in the lab are compound heterozygous variants (both alleles of a gene harbor mutations, which are different). Next, I crossed the position of the mutations presented in these five cell lives with the location of all domains, both the literature and the database available (see Figure 28). From that, I identifiedWABS01 (with the mutations 2271+2T>C and 2689-2691del) presents both mutations in HD2 domains, more specifically one in the helicase domain IV in the Helicase-C terminal domain and a second close to the end of the protein. The WABS03 (with the mutations 1403dupT and 419G>A) present both mutations in the HD1 domains, one at the DEXD C2 domains and other at the Arch domains. The WABS04 (with the mutations 1930G>A and 2114G>A) present both mutation at the HD1, one at the Arch domains and other in the Helicase-C terminal. The WABS05 (with the mutations 2571C>A and 1672 C>T) present one mutation in each HD, at the HD1 is in the Arch domains and at the HD2 is in the helicase motif VI at the end on the Helicase-C terminal. And then the WABS08 (with the mutations 1946-1948del and 1763-1G>C) present both mutations at the HD1 and both present at the Arch domains. From this analysis, we observe most of the mutations are present at the HD1 domains and more specifically at the Arch domain.

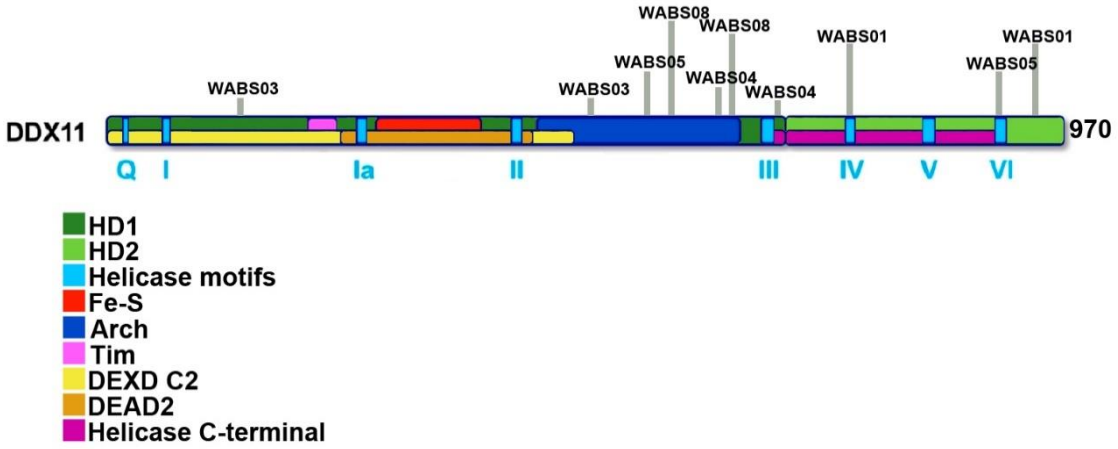

Figure 28: Localization of the mutations present in the WABS patient cells. Schematic representation of all DDX11 domains and its interceptions with WABS mutations presented in five WABS patients cell lines available in the lab. Mutations carried by these patients are described in Table 4.

From that information, we decided to focus our study in WABS03 and the WABS04 cell lines. This was due the fact that both present one mutation in the same domain - the Arch domain - and one mutation in opposite domains. WABS03 have one mutation in the DEXD c2 domains and the WABS04 have one mutation in the Helicase C-terminal. Moreover, WABS03 presents a slightly reduced protein stability, but present

DDX11 mRNA level similar to the control cells and study proposed these DDX11 variants do not affect their helicase activity at the replication fork speed (van Schie et al., 2020). WABS04 presents a substantially increased cytoplasmic retention, and reduced DDX11 mRNA level. Contrary to WABS03, it has an hypomorphic helicase activity at the replication fork (van Schie et al., 2020). In addition, this decision was also based on the on-going studies in the lab as summarized in the Table 5. Both cell lines present mitotic segregation errors at late anaphase and telophase, yet the timing of mitotic progression is only significantly affected in WABS04 despite an almost similarly mean delay in minutes, WABS03 most frequent mitotic error is lagging chromosomes while WABS04 is lagging bridged chromatin (see Table 5 and Figure 23, unpublished results). In addition, cohesion fatigue assay showed a significant decreased timing in WABS03, but not in WABS04 (see Figure 24).

Table 5: Resume of characteristic of WABS03 and WABS04. Resume of the available information about the WABS patient cells used in this study. The first three rows comparison were obtained using LN9SV as a control, and the other four rows came from published data in van Schie et al., 2020, where these cells were described for the first time. N.s means no significant.

	WABS03	**WABS04**
Mutation	V1 – c.**1403dupT** / p.S469V fs*31 V2 – c.**419G>A** / p. R140Q	V1 – c.**1930G>A** / p.V644M V2 – c.**2114G>A** / p.C705Y
Mitotic timing	+ 3 min (n.s)	+ 6 min ($p \leq 0.001$)
% Chromosome segregation defects	+ 39 %	+ 28 %
Cohesion fatigue timing	- 84 min ($p \leq 0.002$)	- 9 min (n.s)
DDX11	Reduce stability	Increased cytoplasmic retention
DDX11 mRNA level	Similar to WT	Decreased
DDX11 protein levels by western blot analysis	Not detected (↓↓↓)	Decreased (↓↓)
Helicase *in vitro* activity	Similar to WT	Hypomorphic

Part II – Unveiling new aspects of WABS etiology

After we have decided the two cell lines from the panel of SV40 immortalized skin fibroblast cell lines derived from five WABS patients (described in van Schie et al., 2020), we next focused our attention to unravel new aspects on WABS etiology linked with mitosis.

4.1.2 DDX11 Localization

DDX11 have a dynamic localization during the cell cycle. DDX11 is at the nucleus and chromatin during interphase as review in, and at first stages of mitosis DDX11 goes to the centrosomes and in the end of mitosis going to the midbody (Parish et al., 2006).

Have in mind this published information about the localization of the DDX11 across the cell cycle, namely in mitosis (Parish et al., 2006), we aimed to test whether the mutations present in the mutated DDX11 proteins at WABS03 and WABS04 altered their cellular location, namely at the centrosome. Centrosomes together with the mitotic spindle ensure the equal distribution of chromosomes between the daughter cells. As such, we to understand how pathological DDX11 variants affects the centrosome and the observed defective chromosome segregation. As previously published, we decide to evaluate DDX11 localization by immunofluorescence analysis at metaphase. For that, we used a specific antibody to label DDX11 (H00001663-B01P, Abnova) and DNA intercalator-dye to mark the DNA, which help to identify the cell cycle stage.

The LN9SV (SV40 immortalized cell line obtained from a healthy donor (Digweed et al., 1995)) was used as a control to evaluate DDX11 dynamic localization at different phases of the cell cycle (see Figure 29). From this analysis we observed specific signals coming from what resemble a centrosome, yet we did not observe a mid-body localization contrary to what was been published by Parish et al., 2006.

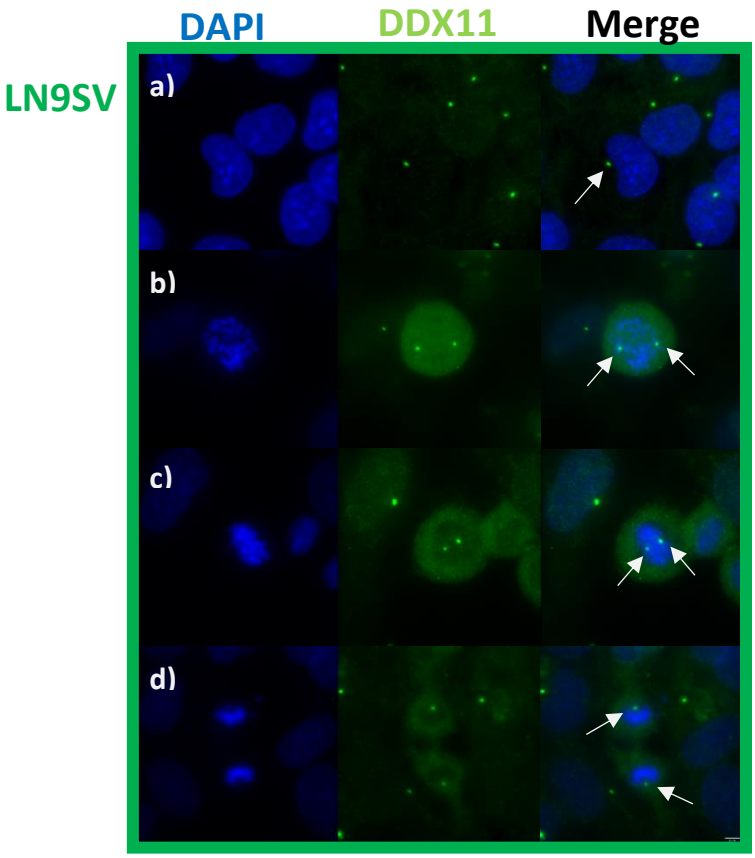

Figure 29: DDX11 localization in control cells. Representative imagens of the DDX11 staining in LN9SV (control cells) in different stages of the cell cycle, a) interphase; b) prophase; c) metaphase; and d) anaphase. Green shows obtained signal for DDX11 antibody (H00001663-B01P, Abnova) and blue depicts DNA staining. Scale bar, 5µm. White arrows depict DDX11 localization on what resemble the centrosome localization.

To confirm if DDX11 signal comes from the centrosomes, we repeat the staining in the LN9SV control cells, but this time we added a specific antibody that recognizes pericentrin, a centrosome component. Figure 30 shows a representative image of the obtained results, where we observed the co-colocalization of both signals. This result indicates that the DDX11 has a centrosomal localization.

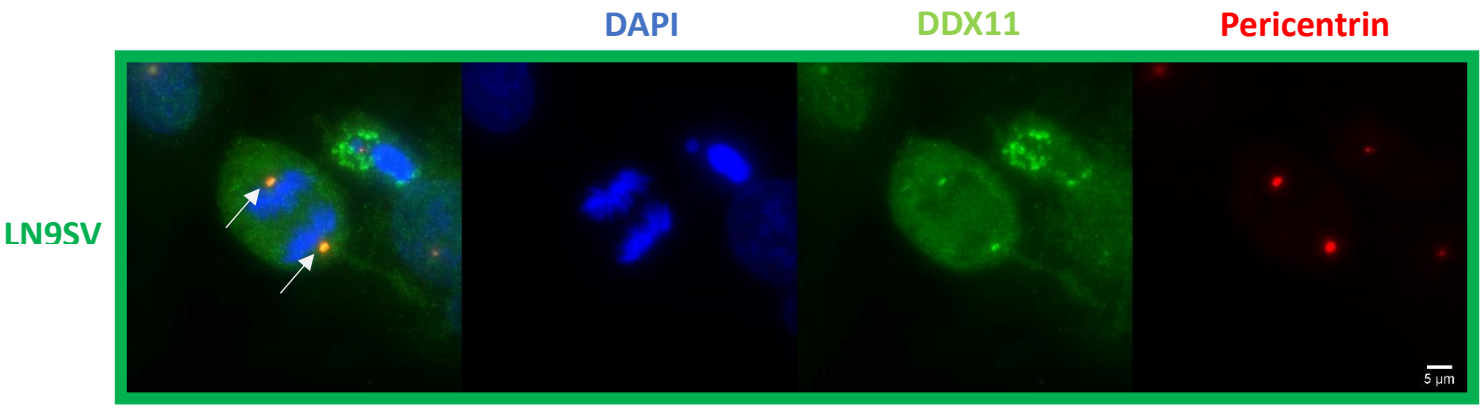

Figure 30: DDX11 has a centrosome localization. Representative image of the DDX11 and pericentrin co-staining (green and red respectively) in LN9SV anaphase cell. Scale bar, 5 µm. Arrow indicates centrosomal localization.

Next, we evaluate DDX11 localization analysis in our patient cells, WABS03, and WABS04. Similar to LN9SV, we also identify intensity signals at centrosome (Figure 31). Interesting both patient cell lines have a apparent similar intensity as the observed in control cell line, LN9SV. This suggesting that the level and distribution of the DDX11 are not changed at the patient cell comparing to the control LN9SV, which was somehow unexpected since the protein is unstable (van Schie et al., 2020).

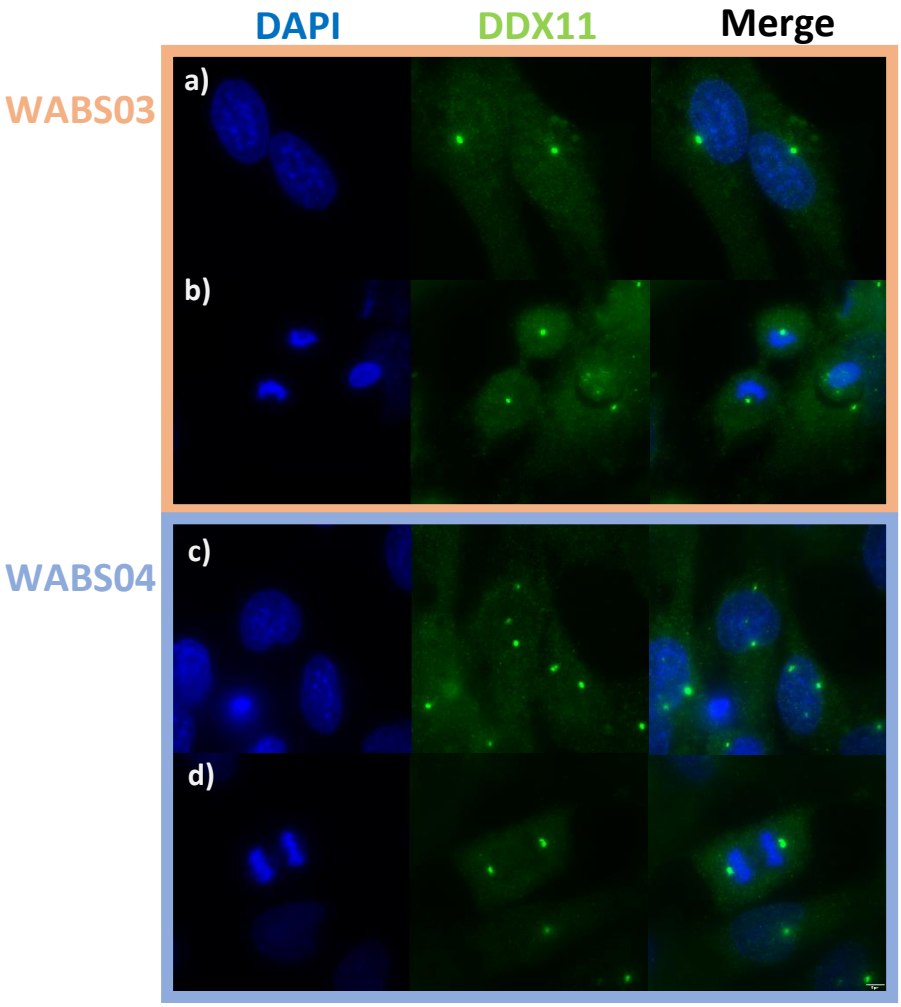

Figure 31: DDX11 localization in patient cells. Representative imagens of the DDX11 staining in WABS03, and WABS04 (WABS patient cells) in different stages of the cell cycle, a) and c) are interphase; and b) and d) are metaphase. Green shows obtained signal for DDX11 antibody (H00001663-B01P, Abnova) and blue depicts DNA staining. Scale bar, 5µm.

Concomitantly with this analysis, together with Cristina Patrício from the lab we performed one extra assay to validate the specificity of DDX11 antibody. For that, we made use of a CRISPR-Cas9 system model available in the lab for DDX11: hTERT RPE-1 (retinal epithelial, RPE-1) knockout TP53 and DDX11. We performed the same staining in RPE-1 cell line p53 KO with and without DXX11 KO, cell lines already validated in the lab and previously describe in van Schie et al., 2020 (Figure 32). From these assays, we observed in both conditions a DDX11 labeling (using H00001663-B01P, Abnova, antibody) is visible at the centrosome. This staining is similar to the previously observed in the LN9SV and both WABS patient cells. This assay shows us that centrosomal staining are not specific to the DDX11, because even in cell with absence of DDX11

(RPE-1 DDX11-KO cells) we still see the same signal. As such, from these results we were unable to conclude the centrosome localization of the DDX11 in the patient cell lines. Also, further analysis is necessary to confirm if DDX11 has a centrosome localization in certain cell types, as previously described in Parish et al., 2006, or due the absent controls for the staining the attribute centrosome localization is an artifact (see Figure 33 for a summary of published results and results described in this dissertation).

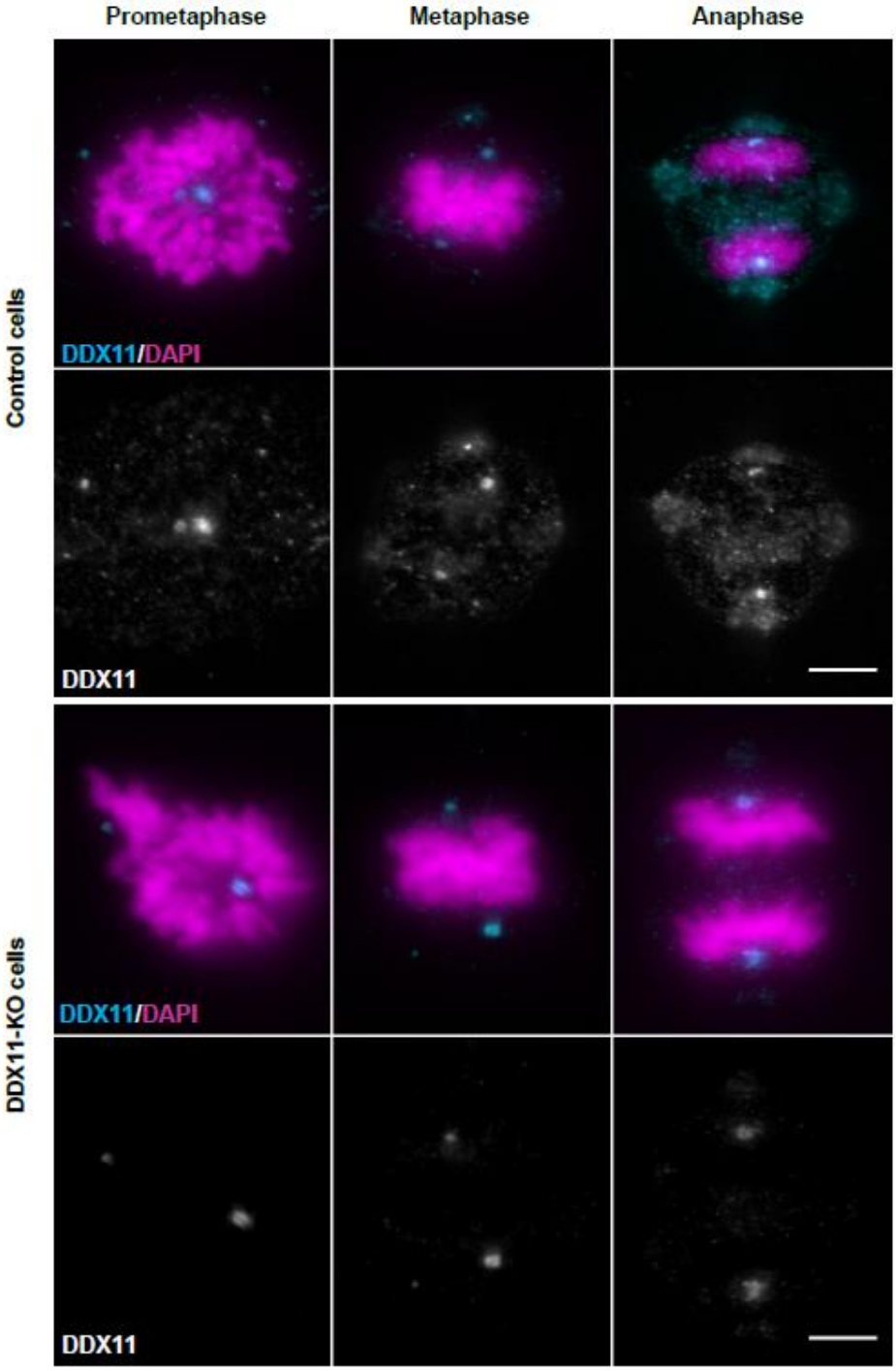

Figure 32:DDX11 staining in DDX11-KO cells. Representative images of the DDX11 in RPE-1 p53 KO (Control cells) and RPE-1 p53 and DDX11 KO (DDX11-KO cells), at different stages

of mitosis. Scale bar, 5 µm. (Image acquired by Cristina Patrício, Carvalhal lab, unpublished data).

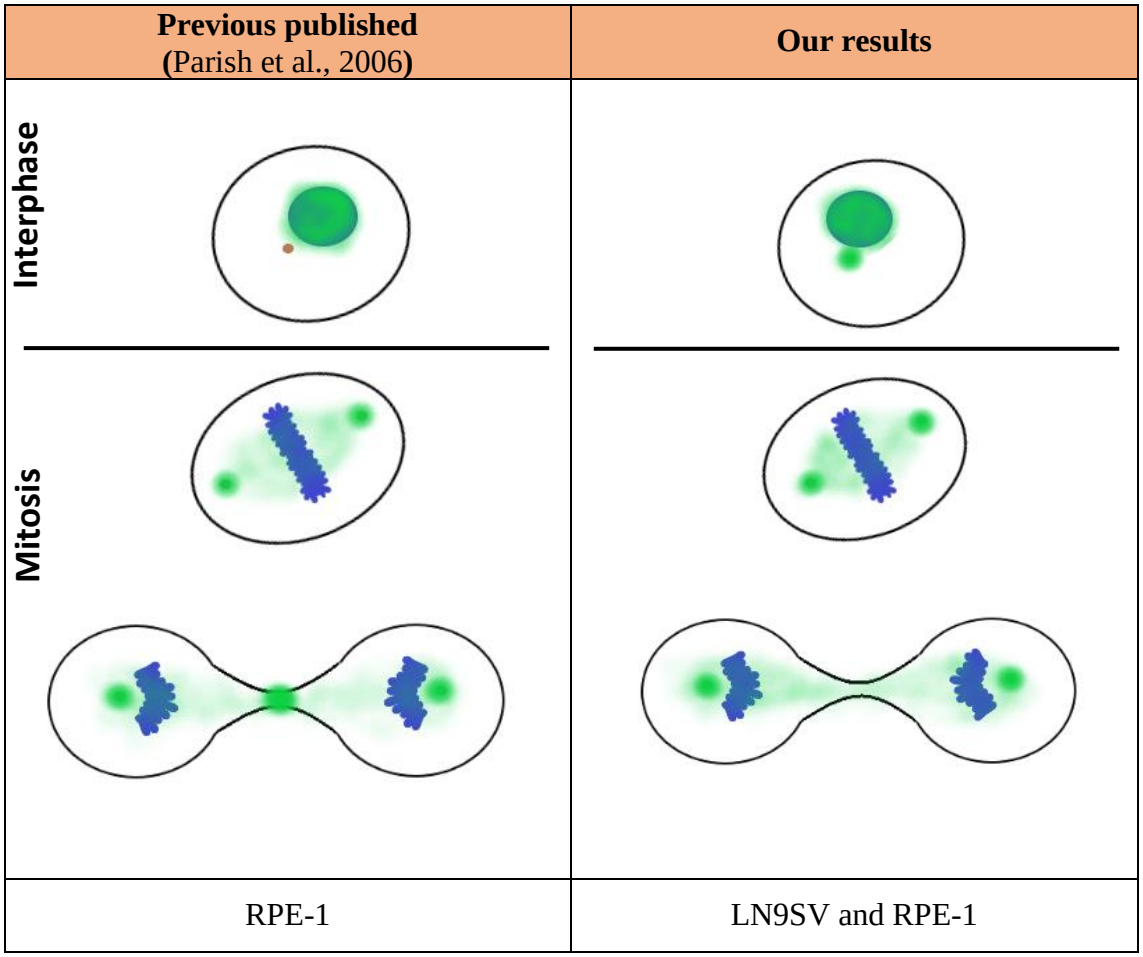

Figure 33: Summary of DDX11 localization. Published information in RPE-1 cell have shown a nuclear localization of the DDX11 during interphase and a centromeric and midbody localization during mitosis. Our results in LN9SV (our control cells) and in RPE-1 cells have shown a nuclear and centromeric localization during all the cell cycle. In the representations the blue depicts DNA, red centrosome and green DDX11 protein.

4.2 Promising candidates on the etiology of WABS

4.2.1 Optimization of chromosome spreads

Given our findings on the DDX11 centrosomal localization (section 4.1.2), we stop to seek the impact of the centrosome of the mechanisms behind WABS etiology. Instead, we focused our attention in two promising candidates, Aurora B and Topoisomerase II alpha. This was based on previous assays performed in the lab (Figure 23 and Figure 24, unpublished results) and in previous published literature (Abe et al., 2016; Carvalhal et al., 2022). Both candidates have a mitotic chromosomic localization, as such was necessary to firstly optimize a technique to assess protein localization in mitotic chromosome spreads.

There are two methods to prepare mitotic chromosome spreads, the manually preparation (taking advantage of gravity to spread the chromosomes in the cover slip) and the mechanized preparation (using a dedicated centrifuge, the cytospin, to spread the chromosomes). To decided which method to use, we performed the preparation of mitotic chromosomes using both methods (described in detail on the section 3.2, Methods) in LN9SV cells. We observed the mechanized preparation causes a better individualization of the chromosomes while with the manual preparation creates mass of condensed chromosomes. To access the localization of our candidates we need individualized chromosomes to better understand their localization and distributions, as such we decided to use the mechanized process to prepare mitotic chromosome spreads on the upcoming analysis. Ither advantage is that the mechanized method is more environmentally friendly (as it uses less reagents) and a short preparation time.

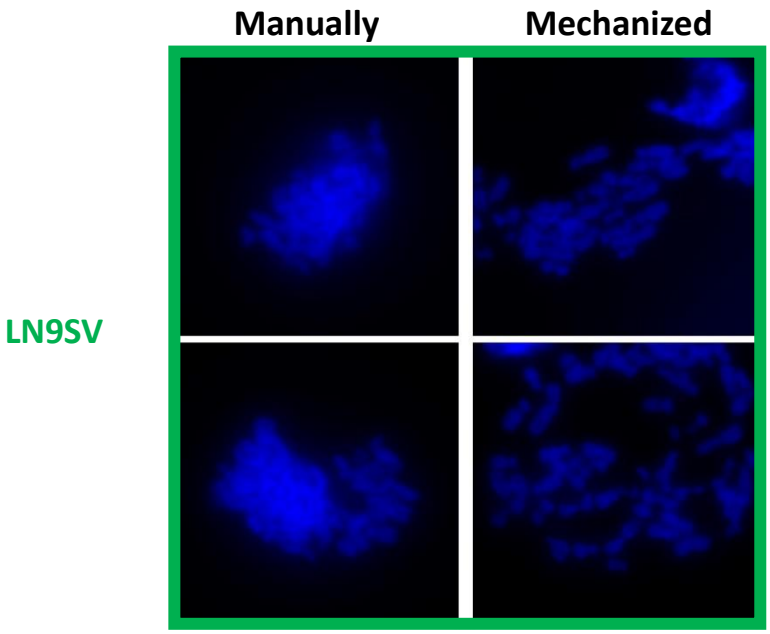

Figure 34: Chromosome spreads following manually or a mechanized preparation. Two representative imagens of each technique used to prepare mitotic chromosome spreads. the left images are manually prepared chromosomes spreads, and the right images are mechanized prepared chromosome spreads using a cytospin. In both conditions was used LN9SV cells which were stained for DNA using DAPI., Scale not shown.

4.2.2 Aurora B

Aurora B kinase is a main regulator of the kinetochore – microtubule attachment and proper tension (Carmena et al., 2012; Carvalhal et al., 2022). Interestingly, Aurora B was observed to be affected in DT40 cells with depletion of both copies of DDX11 (Abe et al., 2016). When Aurora B function is affected, it can lead to lagging chromosomes (Carvalhal et al., 2022). In the absence of DDX11, Aurora B distribution changed from a more centromeric specific localization to a more diffuse along all chromosome arms (Abe et al., 2016), making this kinase a very promising candidate to explore in WABS patient cells.

Chromosome fidelity has been previously evaluated in WABS patient cells (see Figure 23 and Table 5 with summarized data for WABS cell lines used in this work). From this analysis (Figure 23) we observed that WABS03 and WABS4 have a high percentage of cells with lagging chromosomes alone (LN9SV = 1.6% ± 1.3; WABS03= 16.9% ± 2.2; WABS04= 10.8% ± 1.2 of lagging chromosomes) or combined with a DNA bridge (LN9SV= 0%, WABS03 =12.7% ± 5.3 and WABS04 = 12.1% ± 5.8 of chromatin bridged with lagging chromosomes). As such, to address if the Aurora B localization is changed at mitotic chromosomes of WABS patients cell lines, we performed mechanized

chromosome spreads of synchronized cells with 10 μM of colchicine during 30 min, as previously described. Next these chromosome spreads were processed for immunofluorescence to reveal Aurora B and CENPC localization. CENPC was used a marker for the centromere localization. See Figure 35 a) for two representative images of each condition studied. We observed that cells at this stage have the Aurora B signal in chromosome spreads mainly accumulate at the centromeric region (between the two dots of CENPC signal, which marks both centromeres). These observations are in alignment with previous studies done to access Aurora B localization in mitotic chromosomes on LN9SV cell lines (Carvalhal et al., 2022). As far, patient cell staining for Aurora B, we could also observe a staining of Aurora B at both cell lines. To better evaluate whether Aurora B localization was changed, we performed quantitative analysis using homemade macro in FIJI. In summary, Aurora B was measured at the arm and inner centromeric region using fixed ROI sizes, see Figure 35 b). These results show a slight increase of the Aurora B signal at the inner centromeric region more evident to WABS03 (see Figure 35 c)). Nonetheless Aurora B localization found in patient cells is distinct on the studies previous done in chicken cell with depletion of DDX11 (Abe et al., 2016), where the Aurora B signal were diffuse along the whole chromosomes arm in the absence of DDX11.

When considering these results in Aurora B, we have to consider that the layout of this experiment does not allow to quantitatively measure the quantity of Aurora B, because our images of fixed chromosomes, do not have an internal control to normalize the intensity of the signal (as CENPC was changed, data not shown), as such a possible alternative would be to live imaging.

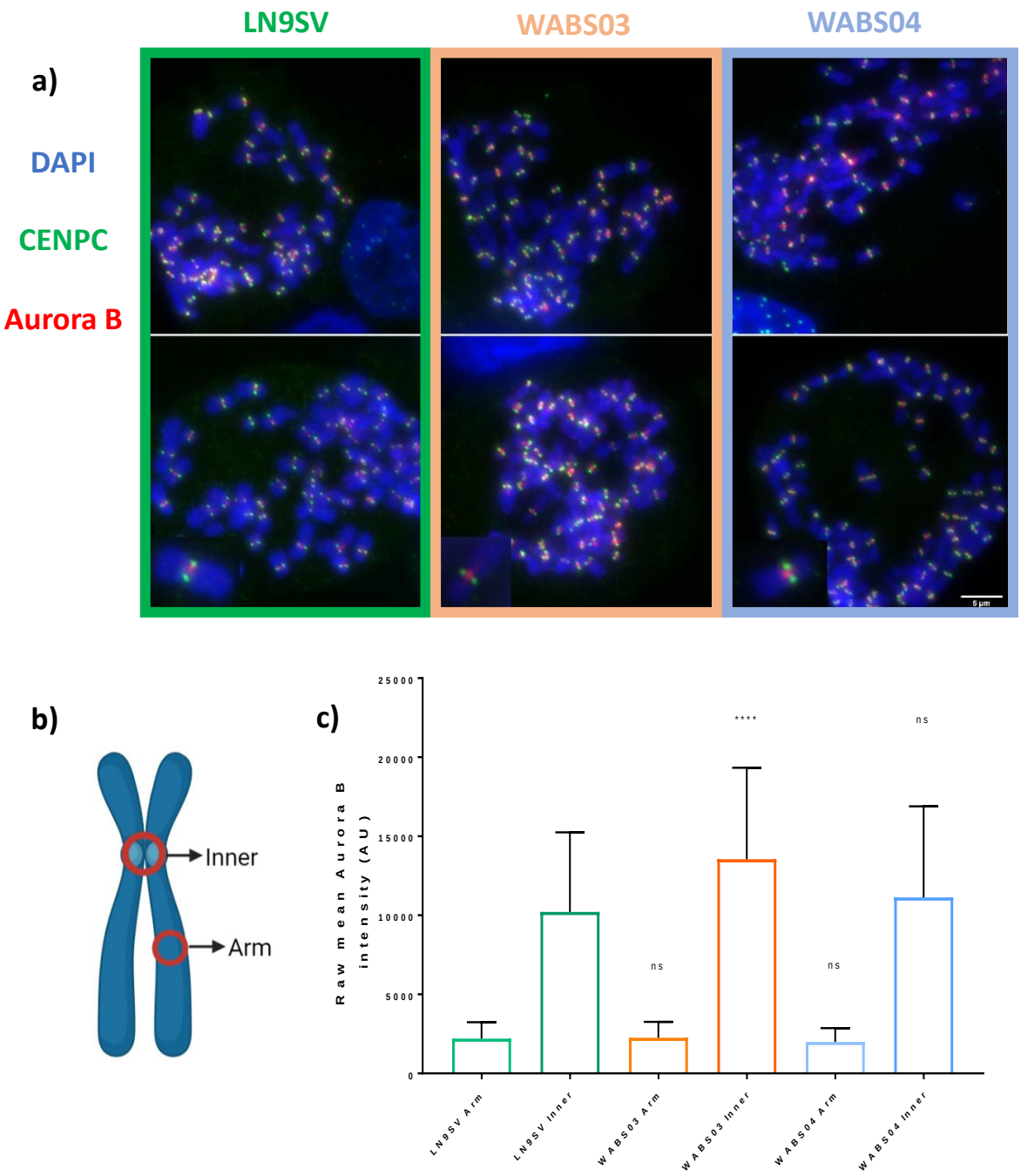

Figure 35: Aurora B signal distribution. a) Representative imagens of the Aurora B staining in LN9SV (control cells), WABS03, and WABS04. Blue shows DNA, green shows CENPC, and red shows Aurora B staining in chromosome spreads. Scale bar, 5µm; b) Schematic representation of the ROI measured in the chromosomes; c) Quantification of the raw mean Aurora B intensity (AU) for each cell line in two ROIs, the inner centromeric region and the arm region, this result came from 3 independent experiments (n=3). A total of 5 individual chromosomes coming for 30 mitotic chromosome was analyzed.

The previous studies to access the localization of the Aurora B on cell with DDX11 depletions have been made in cell with complete knockout of the DDX11 (Abe et al., 2016), while our last results have been done in cell whit mutated variants of the DDX11, so to better understand the localization of Aurora B it was decided to also study Aurora B localization in the DDX11 deletion CRISPR-Cas9 system, using 4 different lines (Figure 36). Control condition hTERT RPE-1 TP53 knockout (because DDX11 knockout was lethal in RPE-1 cells without the p53 knockout). RPE-1 p53 and DDX11 KO stable transfected with Empty Vector (EV), mutated version of a helicase dead form of DDX11 (K50R) or a wild type form of DDX11 protein (WT). This system model has been previously validated and used (Benedict et al., 2020; Carvalhal et al., 2022; van Schie et al., 2020). The same procedure as described above was repeated but this time in all the four RPE-1 CRISP cell lines (see Figures 36).

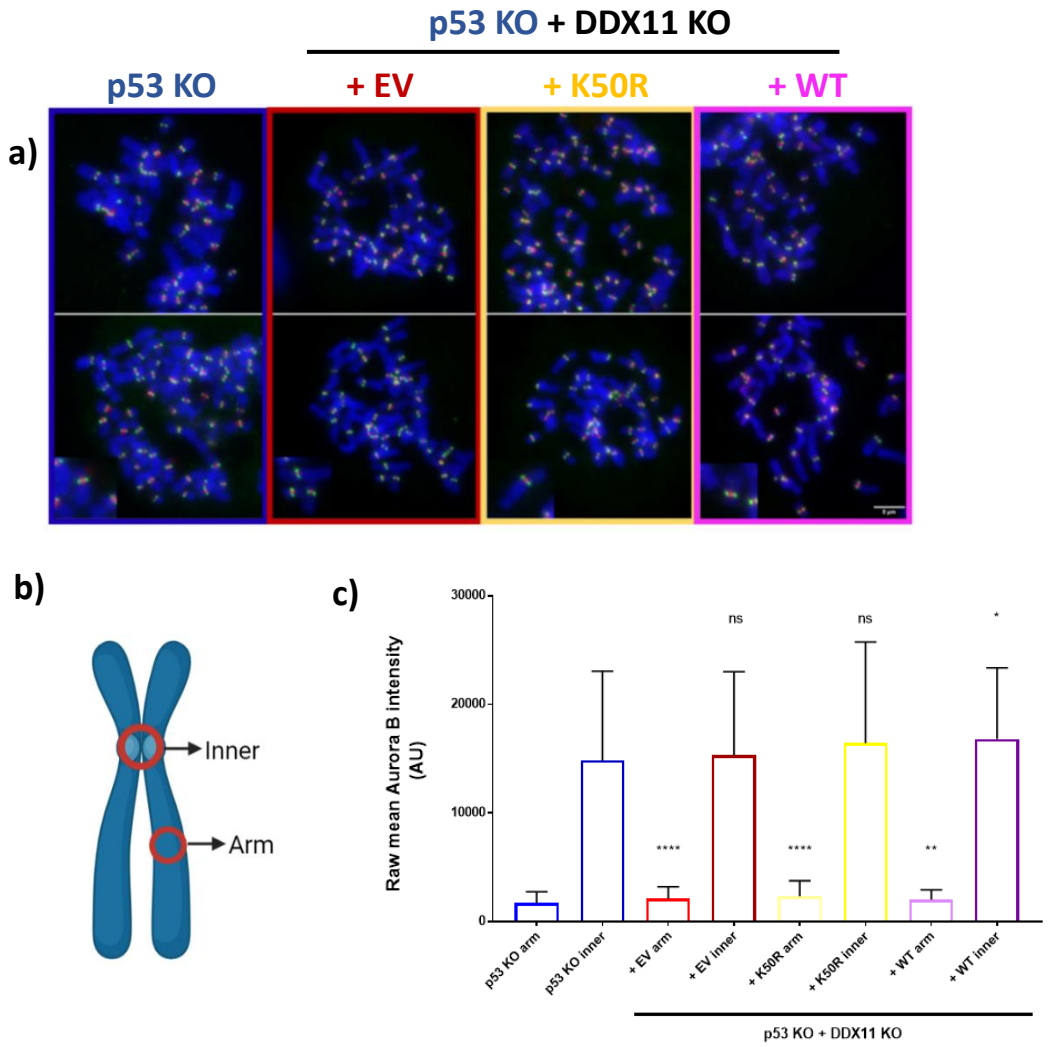

Figure 36: Aurora B signal distribution in RPE-1 CRISPR cells. a) Representative imagens (2 for each condition) of chromosome spreads at the indicated conditions: RPE-1 TP53 KO (p53 KO) (control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53

and DDX11 KO transfected with a mutant variant of the DDX11 (K50R) with null helicase activity, and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT). Blue shows DNA, green shows CENPC, and red shows Aurora B, all merged at the same condition. Scale bar, 5µm; b) Schematic representation of the ROI measured in the chromosomes; c) Quantification of the raw mean Aurora B intensity (AU) for each cell line in two ROIs, the inner-centromeric region and the arm region, this result came from 3 independent experiments (n=3).

Observing the results, we can appreciate that the RPE-1 p53 KO DDX11 KO (+EV) condition did not have a similar localization to the one described in DT40 DDX11 KO cells where Aurora B in the absent of DDX11 had a very weak inner-centromeric localization (Abe et al., 2016)). In our case, all DDX11 conditions evaluated (depletion or in the presence of a DDX11 dead helicase form) Aurora B retained a strong inner-centromeric localization. Despite this difference, several isolated chromosomes had a "less focused" Aurora B staining at this region. This is highly relevant as for proper Aurora B function functionally relevant pool of Aurora B resides near or at kinetochores or rather at the centromeres (Hindriksen et al., 2017). To better dissect this localization in a more unbiased, we used our homemade macro in FIJI to measure the intensity at the chromosome arms, inner-centromeric region, but also at the centromere region as a proxy for the kinetochore localization (see scheme at Figure 37). This assay was repeat in four independent experiments for all the conditions.

In order to compare Aurora B intensities across the distinct parts of the chromosome, their intensities were normalized by CENPC intensity (see Annex Figure 1 and 2 for all the raw data) assuming that CENPC should be an independent variable on our study. From this normalization (Aurora B intensity/ CENPC intensity) we could observed an overall significance increase of Aurora B at the inner centromeric (all DDX11 KO cell lines p-value < 0.001 comparing to RPE-1 p53 KO). Yet remains to be understand if is an increased on the overall poll of Aurora B in the cell. As for the chromosome arms, it was marginally observed a changed in Aurora B intensity when the DDX11 dead helicase was expressed (p-value no significant for +EV and +WT DDX11; p-value <0.01 for DDX11 KO cell line expressing DDX11 K50R mutant). For the centromere/kinetochore region (defined by the CENPC region), we observed an Aurora B overall significant increase in DDX11 KO condition (+EV) which could only be rescued by the expression of DDX11 WT (DDX11 KO cell line +EV and DDX11 KO + DDX11 K50R mutant both p-value <0.01; DDX11 KO + DDX11 WT not significant

when comparing to centromeric Aurora B intensity/ CENPC intensity of control condition). The performed assays suggested Aurora B is possibly spread outwards the centromere-kinetochore region, yet other markers are necessary to stablish a firm conclusion.

While we assume CENPC should not change, we observed a CENPC variability across experiments, which could be due technical variability. As such, to overcome CENPC variability, we performed analysis on Aurora B distribution using ratios (Aurora B intensity inner centromeric region / arm and inner centromeric region / centromere region, which will be control for intrinsic variability across experiments), see Figure 38. By this analysis we can see that DDX11 KO depletion, and their rescue have a changed distribution of Aurora B from control conditions. Moreover, we observed that the presence of WT rescued this centromere-kinetochore miss-distribution of Aurora B. Contrary to the previous method of quantification, the ratio of Aurora A can be used to address defective localization but not levels. As such, it can only be said that Aurora B is slightly (yet significantly affect) upon DDX11 depletion or in a system that only express DDX11 dead helicase form.

More experiments are needed to better understand whether and how Aurora B is altered upon DDX11 absent and/or in the presence of mutant forms of DDX11. Importantly, on these experiments inner centromere and kinetochore markers should be use as in the performed studies the characterization of the inner centromeric and centromeric-kinetochore region was based in a single staining, CENPC. In fact, the inner centromeric came from the prevision that it localizes between both centromeres, but this could lead to some miss-readouts (e.g histones could be used as a specific marker to the inner centromeric region to increase precision).

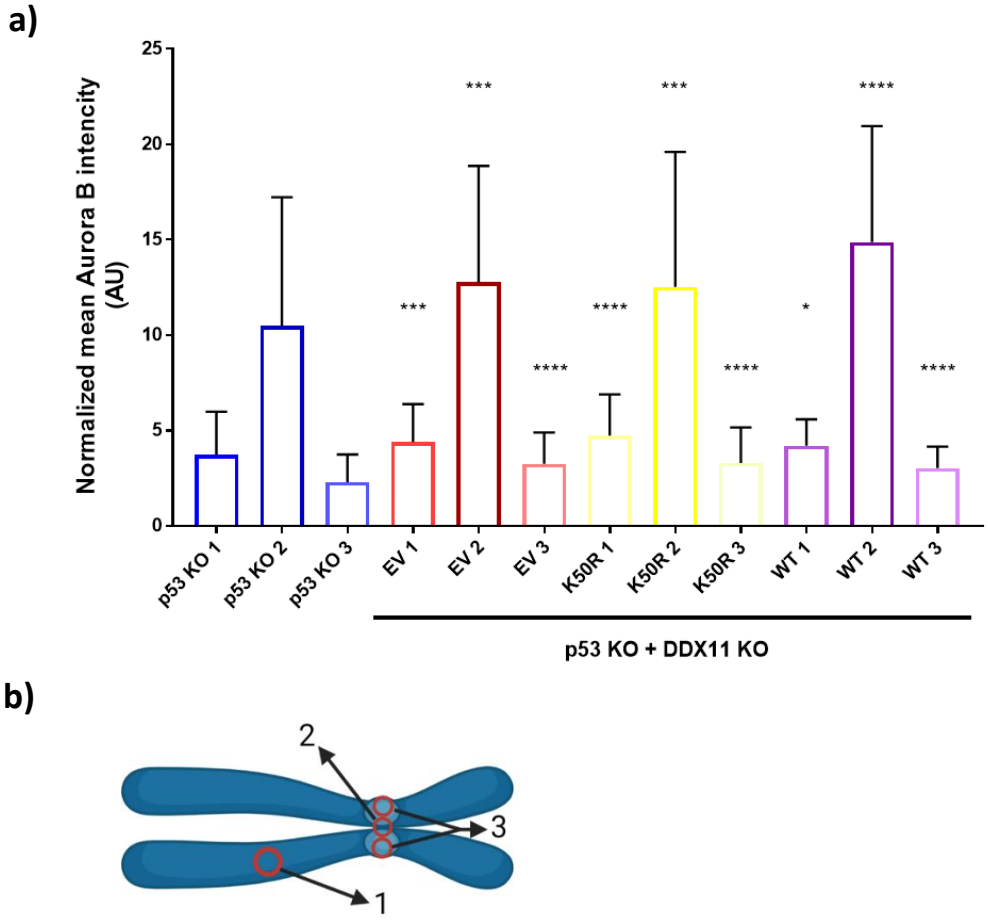

Figure 37: Normalized Aurora B signal distribution in RPE-1 CRISPR-Cas9 cells. a) Mean Aurora B intensity (AU) normalized by the CENPC mean intensity obtained at centromeric ROIs average value for all indicated conditions: RPE-1 TP53 KO (p53 KO, control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53 and DDX11 KO transfected with a mutant variant of the DDX11 K50R with dead helicase activity (K50R, Mutant), and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT). In four ROIs, the arm region, the inner centromeric region, and the centromeric region (mean of the signal from the two centromere), this result came from 3 independent experiments (n=3); b) Schematic representation of the ROI measured in the chromosomes, 1 represent arm, 2 represent inner centromeric region, and 3 represent both centromeres.

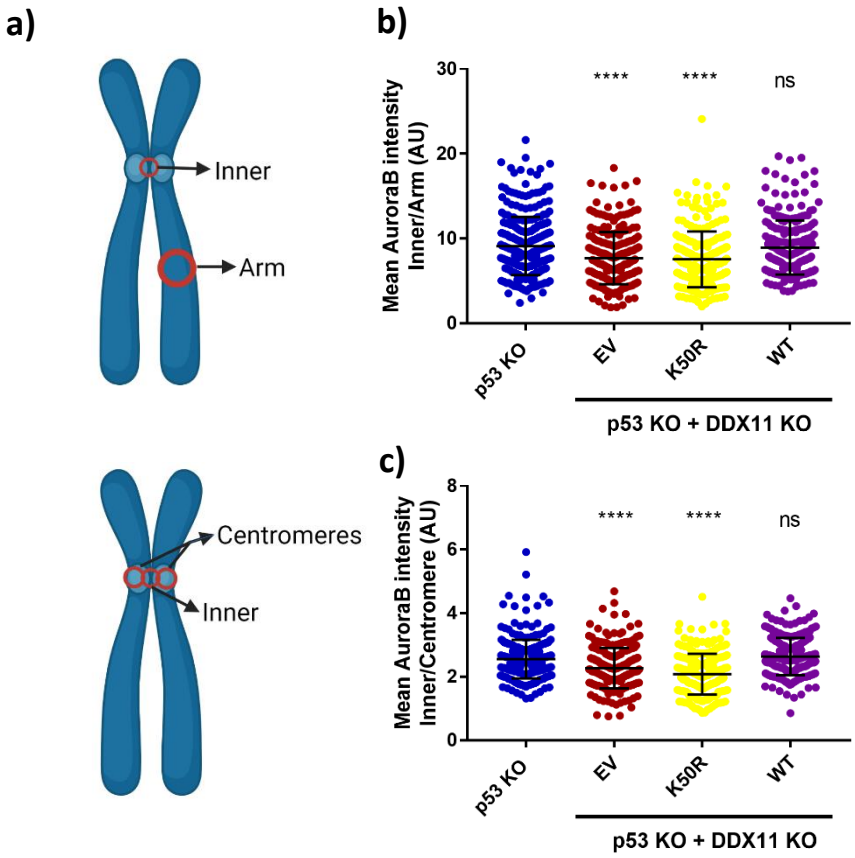

Figure 38: Ratios of Aurora B signal in RPE-1 CRISPR cells. a) Schematic representation of the ROI measured in the chromosomes; b) Quantifications of the ratio between the mean of Aurora B signal in the inner-centromeric region and the arm region, in RPE-1 p53 KO (p53 KO) (control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53 and DDX11 KO transfected with a mutant variant of the DDX11 (K50R) with dead helicase activity (Mutant), and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT); c) quantifications of the ratios between the mean of Aurora B signal in the inner-centromeric region and the centromeric region, in the same cell lines. Results are from 3 independent experiments (n=3).

4.2.3 Topoisomerase II alpha

Strong cohesion could be mimicked by abnormal levels of catenation (see section 1.7.3 introduction) so one can hypothesize that despite lower levels of cohesin typical in cohesinopathies (never addressed in mitosis), strong cohesion observed upon cohesion fatigue live imaging assay is due to abnormal TOP2A. As such, we hypotheses WABS cell we will have some abnormalities on the TOP2A localization due to the fact that WABS cell presented strong cohesion even having supposedly low level of cohesin. A similar behavior to what has been seen in studies in cells with BUB1 mutations, where the cell also has lower level of cohesin but still present a strong cohesion, and in these cell lines TOP2A show a clear delocalization from a centromeric localization towards the chromosome arms, creating almost an evenly distribution of TOP2A on mitotic chromosomes (Carvalhal et al., 2022).

TOP2A have as main function the resolution of the DNA catenation, that is naturally created by the DNA replication, when the TOP2A functions is affected, it will lead to errors in chromosome segregation (Piskadlo & Oliveira, 2017). In the abscess of TOP2A cell are not capable of reach mitosis and in depleted conditions the cells suffer from problems in mitosis like DNA bridges that consequently result in DNA breaks (Charbin et al., 2014). As such, in my thesis I aimed to understand if TOP2A localization was changed using two WABS system model: WABS patient immortalized fibroblasts and DDX11 KO CRISPR-Cas9 system in RPE-1 cells. To do that we resort to immunofluorescence experiments performed upon mitotic chromosome spreads. We used an antibody that specifically recognize TOP2A, and an antibody specific to CENPC (centromere marker).

As far as WABS cell panel from patient fibroblast is concern, in LN9SV, our control conditions, the TOP2A signal is defined as a bigger signal intensity coming from the centromeric region and a slightly fainter signal along the arms of the chromosome, according to what is expect for this protein for mitotic chromosomes and to what is was described for this cell line in Carvalhal et al., 2022 (see Figure 39).

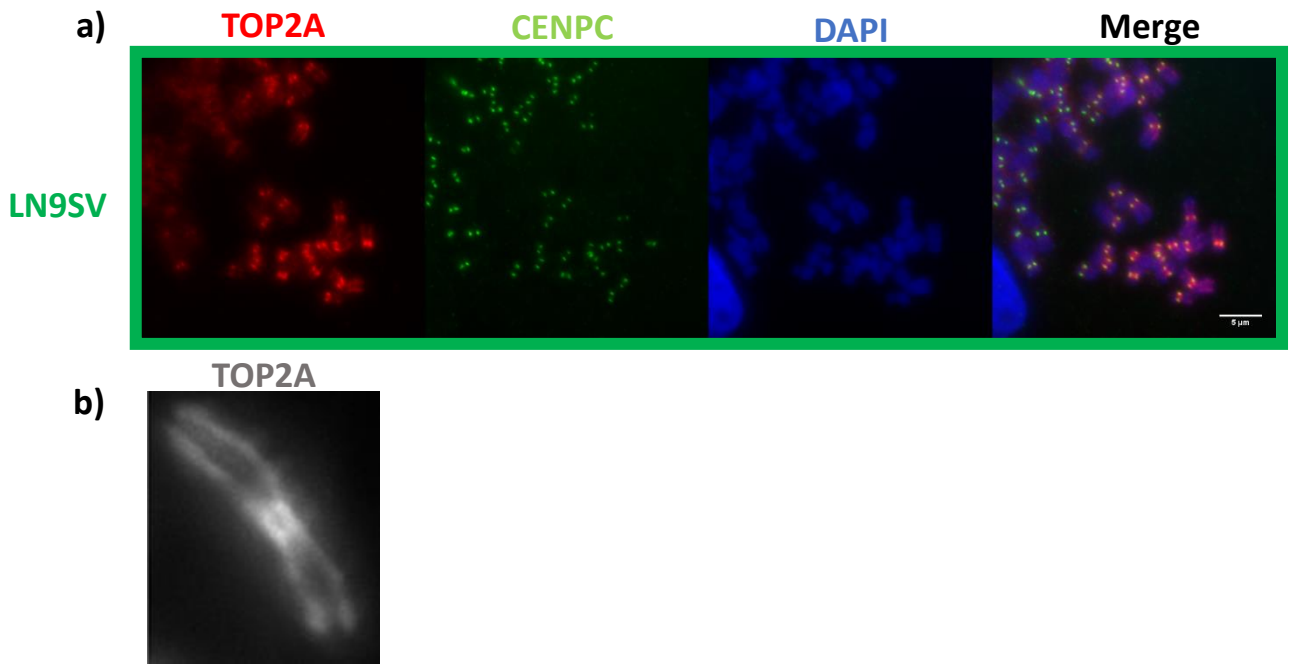

Figure 39: TOP2A staining in the control cells. a) Representative image of the TOP2A staining in LN9SV (control cell) for the indicated antibodies and dyes. Last panel on the right shows the merge of them. Scale bar, 5 µm; b) Representation of the TOP2A staining in control conditions in gray scale image.

Next, to assess impact of DDX11 pathological mutations in TOP2A, we performed this assay in WABS patient cell lines and control condition in parallel (see Figure 40 from representative images solely showing the localization of TOP2A at the chromosomes). We could observe that both patient cells displayed a distinct distribution of the signal. In WABS03 cell line we can see some chromosomes with a "*wild-type* like distribution" yet, we can also observed several chromosomes have a TOP2A signal more homogeneous along chromosome arms and centromeric region. In case of WABS04, TOP2A signal had a more even distribution across the chromosomes than WABS03. Yet, chromosome spreads depicting TOP2A localization in WABS patients on average show a distinct localization from control cell line LN9SV.

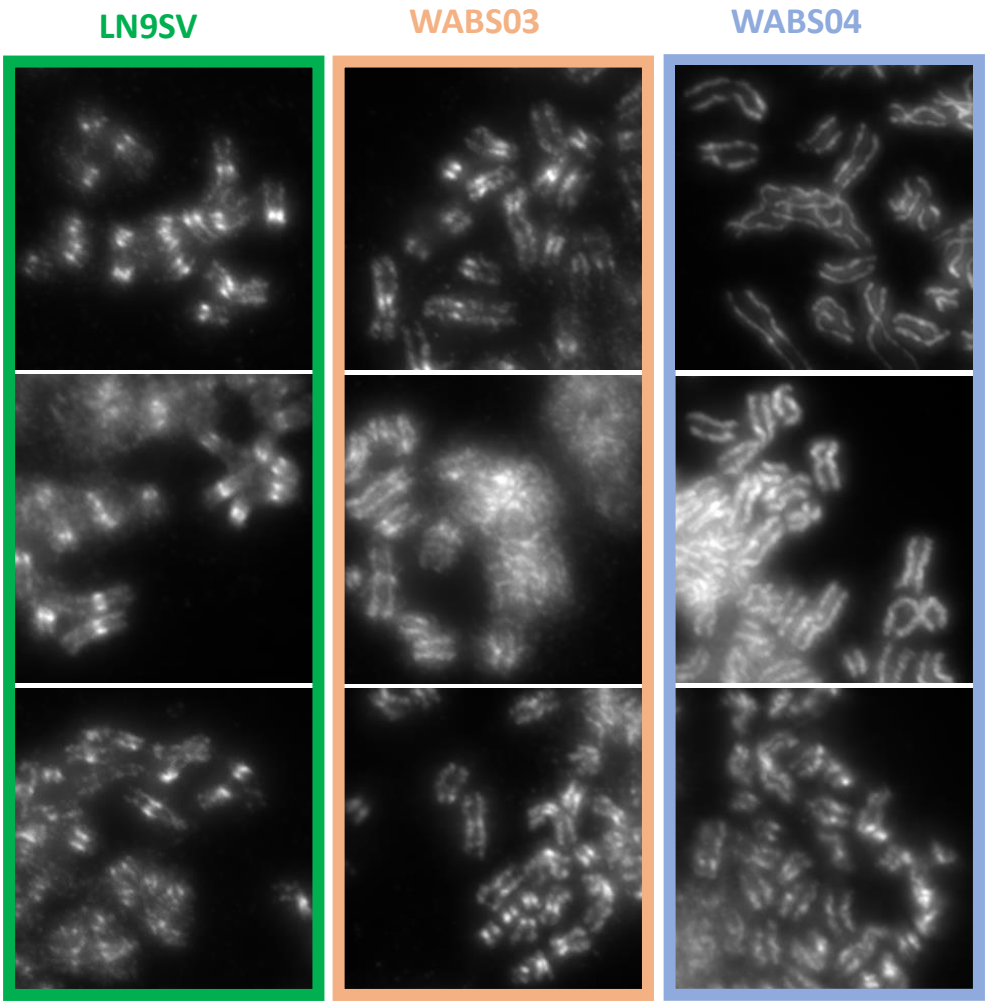

Figure 40: TOP2A signal distribution in LN9SV (control cells), WABS03, and WABS04. Chromosome spreads proceeded as shown in Figure 34. In here, only TOP2A is shown using grayscale for 3 distinct spreads as representative images TOP2A staining in grayscale. Images presented at the same scale.

To evaluate this localization in a more quantitative way, we performed imaging analysis, using a homemade macro for the ImageJ software (see method section 3.4 and 3.5). In summary, in each chromosome spread acquired 5 chromosomes (completed isolated) were measured for their TOP2A signal intensity in the centromeric region (using CENPC staining as a reference point) and the arm (using DNA as a marker). Selected chromosomes were chosen based solely on their DNA staining to make the result less biased.

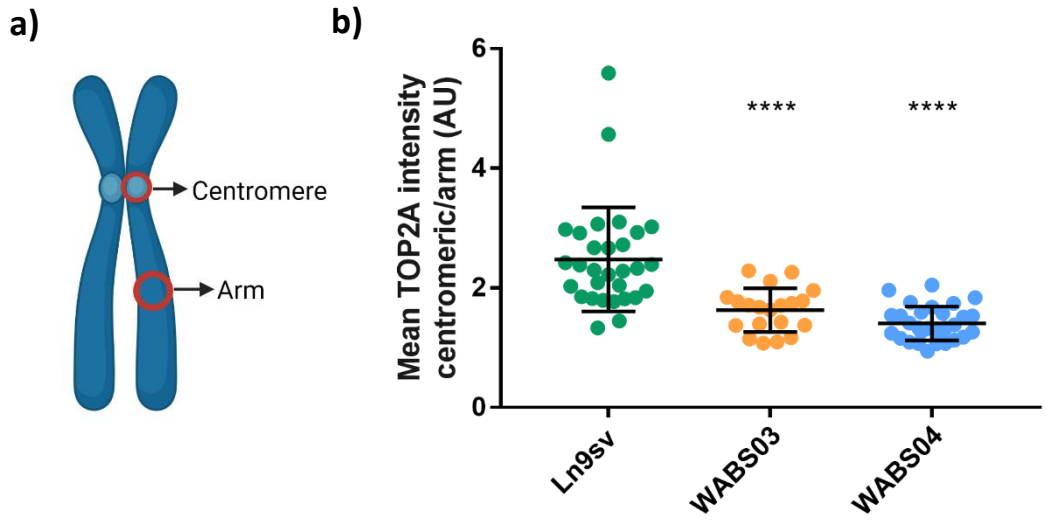

Figure 41: TOP2A ratio between centromeric intensity and arm intensity. a) Schematic representation of the ROI measured in the chromosomes; b) LN9SV (control cells), WABS03, and WABS04 were evaluated for the TOP2A in chromosome spreads stained for TOP2A, CENPC and DNA. Each dot represents a chromosome measure that come from LN9SV= 30, WABS03=20 and WABS04=30 mitotic chromosome spreads. These results came from one independent experiments (n=1).

To evaluate TOP2A distribution, for each chromosome, it was calculated its TOP2A ratio between centromeric region and arm (see Figure 41). This preliminary result (N=1) indicates that TOP2A distribution is highly affected in WABS patient cells, having a more homogeneous distribution of TOP2A along all the chromosomes, in concordance with our hypothesis (Carvalhal et al., 2022).

In order to strengthen these findings, TOP2A localization was equally analyzed in DDX11 KO CRISPR-Cas9 system cell lines (see Figure 42). In addition, we will take advantage of the cell line DDX11 KO rescued with a dead helicase form of DDX11 (DDX11 K50R) to evaluate if suggestive TOP2A defective localization is dependent on DDX11 helicase activity.

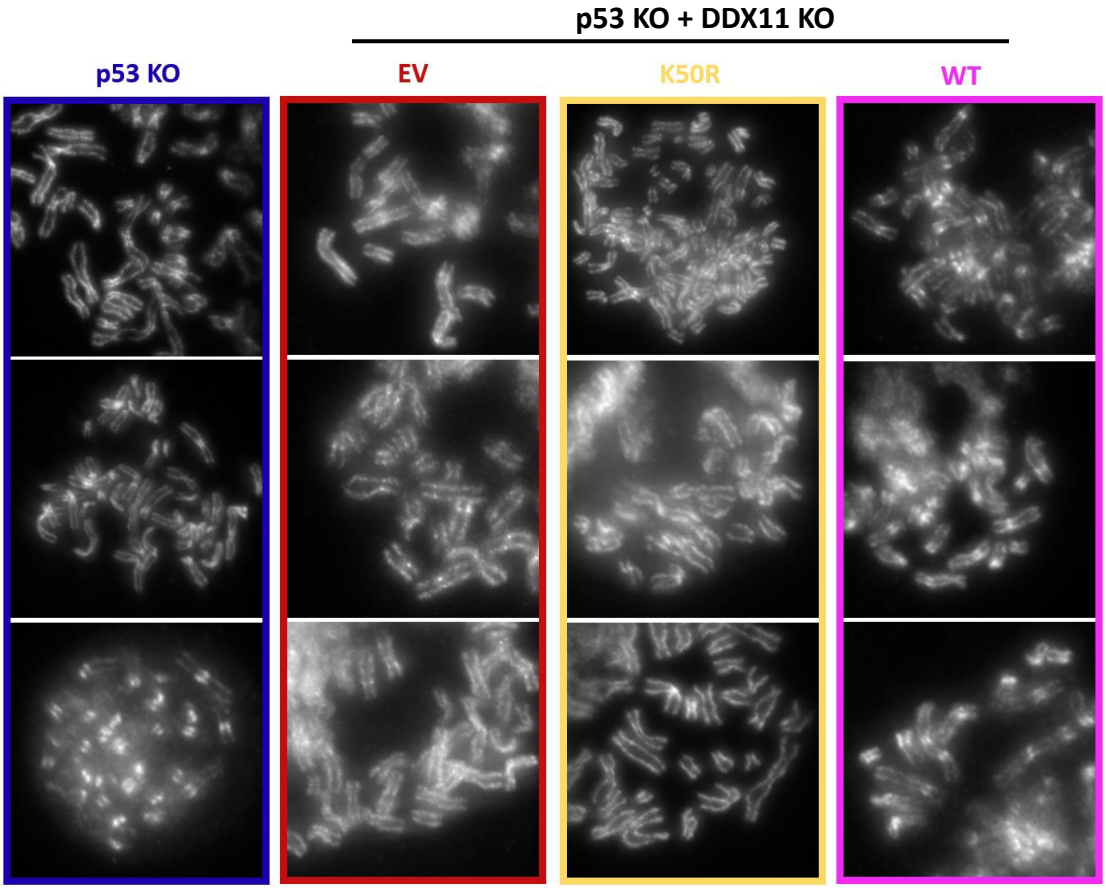

Figure 42: TOP2A signal distribution in RPE-1 CRISPR DDX11 model system and its controls. Representative imagens of the TOP2A staining in RPE-1 p53 KO (p53 KO) (control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53 and DDX11 KO transfected with a mutant variant of the DDX11 (K50R) with null helicase activity (Mutant), and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT) in grayscale. These results came from one independent experiments (n=1).

From these results we can see that in the absence of DDX11 and in the presence of a DDX11 with dead helicase activity, we observed a similar phenotype. In both cases, TOP2A is evenly distributed across chromosomes. As expected upon DDX11 WT re-expression in the KO cell the TOP2A misdistribution is rescued. Unfortunately, due a technical problem with the immunofluorescence I could not repeat this experiment again, yet from these preliminary analysis in the presence of the DDX11 dead helicase the phenotype of TOP2A localization is more severe than in the absence of the DDX11. This could indicate that a cell behaves different when DDX11 is present or absent, despite defective helicase activity in both causes. This could be due DDX11 has some type of

redundance and in it abscess other mechanism can counteract their fault (e.g with ESCO2 described in Faramarz et al., 2020), but when it is present and have defective activity this mechanism could not act, but is necessary to make more repeats to validate this finding and this is not an variability of the phenotype (due the lower number of mitotic spreads evaluated).

Despite both systems point that TOP2A is defective in WABS cells, remains unknow whether TOP2A defective localization is cause by defective replication defects (caused by DDX11) or instead due to decreased levels of cohesin, more experiments are needed to better understand these mechanisms in the future.

4.2.4 Centromeric distance

As referred in the section 1.7.3, the WABS cell present mitotic defects and chromosome structure normally seen in cell with low level of cohesin, but when accessed that by a cohesin fatigue assay, it was seen that the WABS patients cell have similar times to enter cohesin fatigue, what opened the hypothesis that this cohesion can come from catenation created from the abnormal activity of the TOP2A and not from cohesin, and in fact the results of the TOP2A showed abnormal distribution of the TOP2A in the patient cell.

To better understand this hypothesis, we access the centromeric distance in mitotic chromosome spreads upon a 30 min treatment with colchicine (which disrupt microtubules). If a cell presents lower levels of cohesin (and TOP2A does not play a role in the overall mitotic chromosome structure) is expected that the mitotic chromosome will present a bigger distance between the centromeres. Yet, if TOP2A plays a role, due to its miss localization towards the arms, new DNA catenations will take place at the arms causing a closer proximity between the centromeres. To access that, I recurred to the previous obtained imagens from Aurora B signaling and performed measurement of the distance between the centromeres using homemade macro in FIJI, and to do that used the CENPC signal which mark both centromeres (Figure 43). The use of Aurora B signal ensures that both CENPC signals are correspondent of the same chromosome.

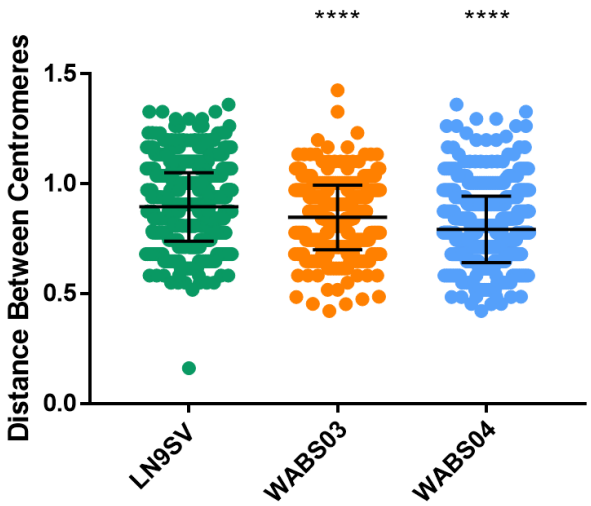

Figure 43: Intra centromeric distance in WABS patients cells. Measurement of the distance between the centromeres (signal of CENPC as a marker and Aurora B as a guide for correct assessment of independent chromosomes). These results came from three independent experiments (n=3) where at least x mitotic chromosome was measure in each condition.

If we would take into account solely the loss of cohesion, then, opposing to what expected the centromeric distance in the WABS cell showed a are shorter intra-centromeric distance than the control conditions. As such, this result favors that abnormal distribution of TOP2A could been creating more catenation do not allowing the chromatids to separate, promoting the formations of railroad chromosomes, and possibly creating mitotic defects such as DNA bridges.

In the future it is need more studies to understand how this abnormal TOP2A distribution arise if due defective cohesin or DDX11.

5. Conclusion & Futures Perspectives

From the execution of this thesis work my overall goal was to give one step further in understanding the mechanisms that can cause WABS etiology. For that I aimed to answer, or provide first evidence – to three specific questions: (i) Does DDX11 hold a centrosomal mitotic role? (ii) Is Aurora B defective in WABS patient cells explaining observed lagging chromosomes? (iii) Is TOP2A a possible explanation of robust cohesion fatigue observed in WABS cells? (Figure 44)

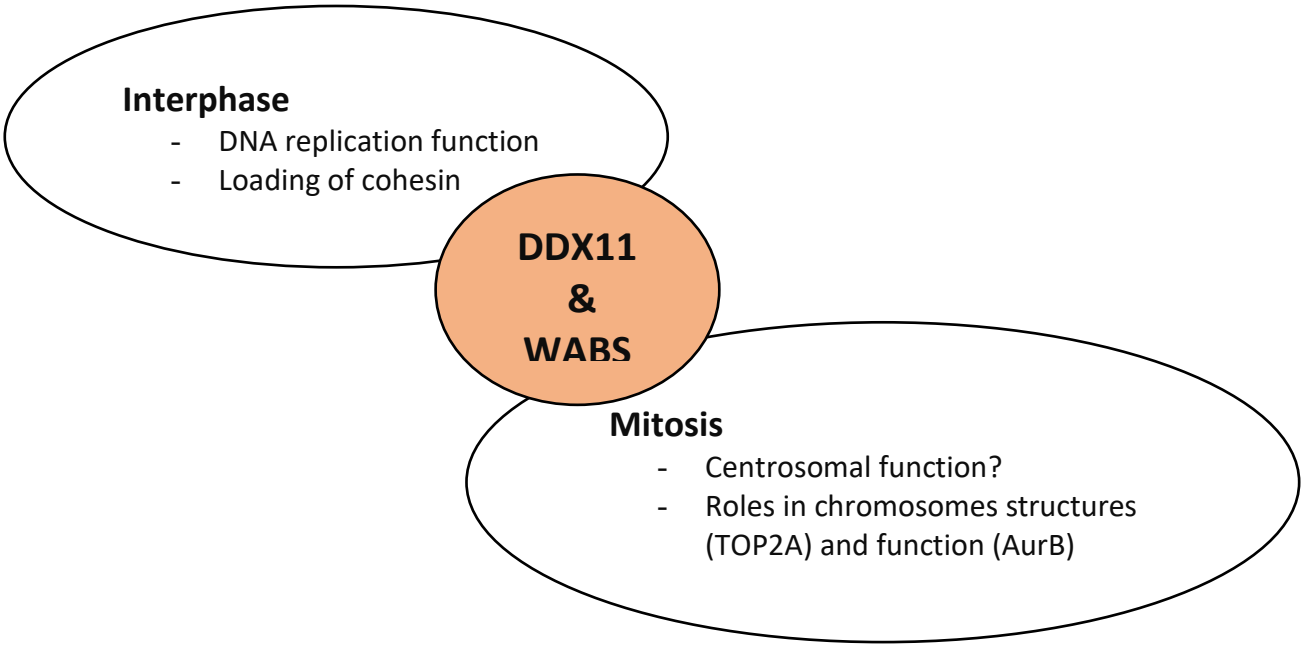

Figure 44: Summary of WABS possible etiologies study on this work. New possible candidates to the etiology of WABS were discussed in this thesis, both DDX11 roles are not possible to be concludes due to the unspecificity of the antibody available, Aurora B do not show the same behavior of other cell lines submitted to the same conditions but more studies are needed, and TOP2A show a promising result by it localization changes but need more experiment to improve the statistic power.

From our studies in DDX11 localization during mitosis, we can conclude that t commercially available anti-DDX11 antibody used in this study non-specifically detect the centrosomes, even if the DDX11 protein is absent. It is important to note that we have used different antibody from the one used in the previous study where DDX11 was described to be at centrosomes during metaphase (Parish et al., 2006). As such it remains unclear whether DDX11 localization correctly published as validations where not performed on Parish 2006 study. In the future, would be important to repeat DDX11 localization assays with an alternative antibody and/or with a DDX11 fused with a GFP

as a tag protein. The last approach will allow to study DDX11 localization in live cells excluding artifacts from the antibodies or fixatives used. Due to the non-specificity of the antibody, we could not identify any change at the DDX11 localization, so no further conclusion about if DDX11 could have roles in the centrosome could be taken, and if it that could account for the observed mitotic errors in the WABS patient cells. Centrosomal unspecific localization is frequent in several immunofluorescences with distinct proteins (e.g Suzuki et al., 1981) as such, when controls are not present the description of this protein's localization should be addressed with caution.

Our Aurora B results on WABS etiology suggest a middle participation when compared to great changes in the Aurora B distribution described in (Abe et al., 2016). It is important to note that previous results were obtained in different cell type, DT40 cells, which may explain the distinct pattern observed upon DDX11 KO conditions. Despite that, it is possible that Aurora B distribution is more spread outwards the centromere/kinetochore region. In the future would be important to better characterize this Aurora B phenotype for example quantifying the impact of known Aurora B substrates (e.g HEC-1). Yet, this would only point to a possible importance of Aurora B in the observed chromosome segregation defects. To directly prove the importance of Aurora B, miss localization at centromeric/kinetochore region to WABS chromosome segregation it would be necessary rescue of its localization, which at the moment it difficult to envision a suitable strategy to use. Nonetheless, it is known that Aurora B has several pools at the chromosome and (Broad et al., 2020; Hadders et al., 2020) as such would be important to understand if upstream regulators of this pathway (e.g Haspin and BUB1) are affected in WABS cells.

As for TOP2A, this isomerase shows a very distinct phenotype in WABS patient cells when compared with control cells. In this case, we see a reduced TOP2A centromeric/arm ration due an even distribution of the TOP2A along all the chromosomes while in wild-type conditions is normally seen a bigger pool of TOP2A at the centromeric region, and reduced levels at the arms. However, because of the problems we faced with the TOP2A staining (data not shown), our quantification statistical power is very low, and much more cells need to be analyzed. The same limitation is observed in TOP2A analysis done in DDX11 CRISPR-Cas9 cell lines. As such, it is necessary to increase the number of independent experiments performed. Also, an important aspect to address in the future is to understand how the changes in the cohesin affect the TOP2A localization, or instead

they are caused by DDX11 functions during replication. One possible experiment is use a Rad21-AID system to perturb artificially the level of cohesin upon replication and see how this impairment affects TOP2A localization. TOP2A mis-localization was found to be also changed in a rare syndrome with overlap features with cohesinopathies (Carvalhal et al., 2022). It would be interesting to analyzed whether TOP2A perturbations are often associated with the different types of cohesinopathies. A tempting assumption is to assume that railroads chromosome is caused by a configuration exacerbated by defects in chromosome architecture like the abnormal TOP2A localization instead of a reduced centromeric cohesion (Chang et al., 2003; Farr et al., 2014; Nielsen et al., 2020; Piskadlo & Oliveira, 2017). Also, this TOP2A mis-localization seen at the WABS patient cell could be related to the results of the centromeric distance, that show a shorter distance at the patient cells, that are cells associated to low levels of cohesin. The TOP2A abnormal localization could be leading to the creation of abnormal catenation that can be the responsible for the strong cohesion, the mitotic segregation errors (lagging chromosomes and DNA bridges), and railroad mitotic chromosomes seen at the WABS patient cell. Interestingly there is a study describing a TOP2A SUMOylation-dependent mechanism of Aurora B recruitment to mitotic centromeres (Johansson et al., 2021; Pandey et al., 2020). It would be relevant to explore the common regulators in both processes, e.g Haspin (Johansson et al., 2021; Pandey et al., 2020). Also, while DDX11 is known to be involved in effective cohesion in the chromosomes (van Schie et al., 2020). We still do not understand exact mechanism Aurora B is an important regulator of cohesin establishment and maintenance because of its requirement to the centromeric region by the Bub1 modification that will recruit the Sgo1 (Nishiyama et al., 2013) which will protect cohesion. (Johansson et al., 2021; Pandey et al., 2020) This demonstrates the complex network involved in a healthy sister chromatid cohesion and correct sister chromatid segregation. This may also explain why loss of cohesin is not compatible with life, yet cohesinopathies are known to have low levels of cohesion despite being compatible with life in the majority of the cases. This may be due the complex network feedback that exist in the cohesin pathway. Many questions are still open where many studies need to be done until it all could be confirmed and unveil.

Beside all this also one last doubt remains, in the WABS and in other cohesinopathies like Roberts syndrome, one of the recurrent phenotypes is the microcephaly, but a clear driver line between the origin of the cohesinopathies and the

development of microcephaly is still not fully defined. The main origin of microcephaly development comes from mutation that encode centrosomal proteins, proteins of the mitotic spindle and chromosome structure. These defects on mitotic system end up affecting with several other cellular functions leading to spindle problems (misorientations, and assembly delays) which all typically result in chromosomes mis-segregation and possible cell death. Yet why is the brain so susceptible to chromosome mis-segregation remains unclear. Recently a distinct model proposed that microcephaly is caused because, longer mitosis, which trigger the mitotic surveillance pathway induce cell death. Increase cell death also reduces proliferation ratios, which could result in a lower grow of the brain. Another large group of mutant genes causing microcephaly is on the DNA repair and replication functions. For the case of the WABS, the most promising link between the current proposals and microcephaly is for the DNA repair and replication, and the chromatin organization and maintenance mechanisms, due to the fact that DDX11 is directly involved at the DNA repair and replication, and in the chromatin organization by is function in loading the cohesin. Further studies are necessaries to understand is this is a mutual exclusive role of not (Phan & Holland, 2021). The field currently support the importance of DDX11 in replication to underlie brain defective size in WABS (van Schie et al., 2020), yet my study supports that mitosis play a role in the etiology. Yet remains unclear if due specific changes during mitosis and/or due a consequence of defective replication.

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Appendices

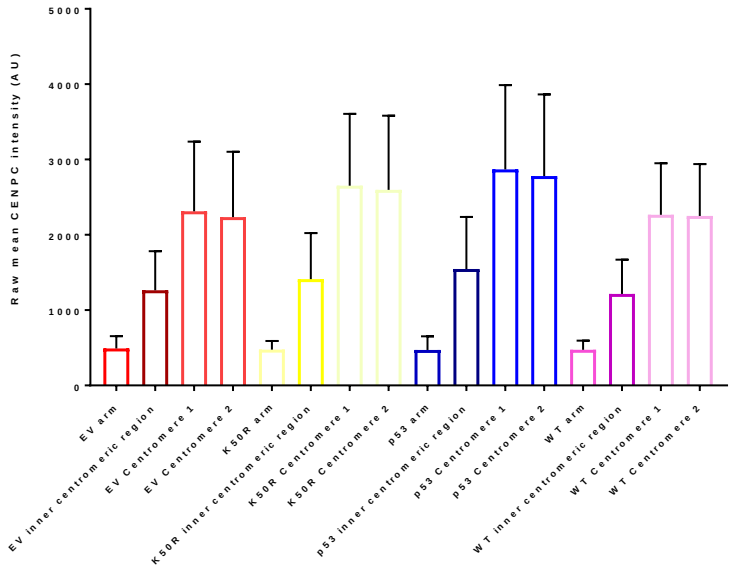

Figure 1: Quantification of the raw mean CENPC intensity (AU) in RPE-1 p53 KO (p53 KO) (control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53 and DDX11 KO transfected with a mutant variant of the DDX11 (K50R) with null helicase activity (Mutant), and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT). For each cell line were measured four ROIs, the inner centromeric region, the arm region, and both centromeres, this result came from 3 independent experiments (n=3).

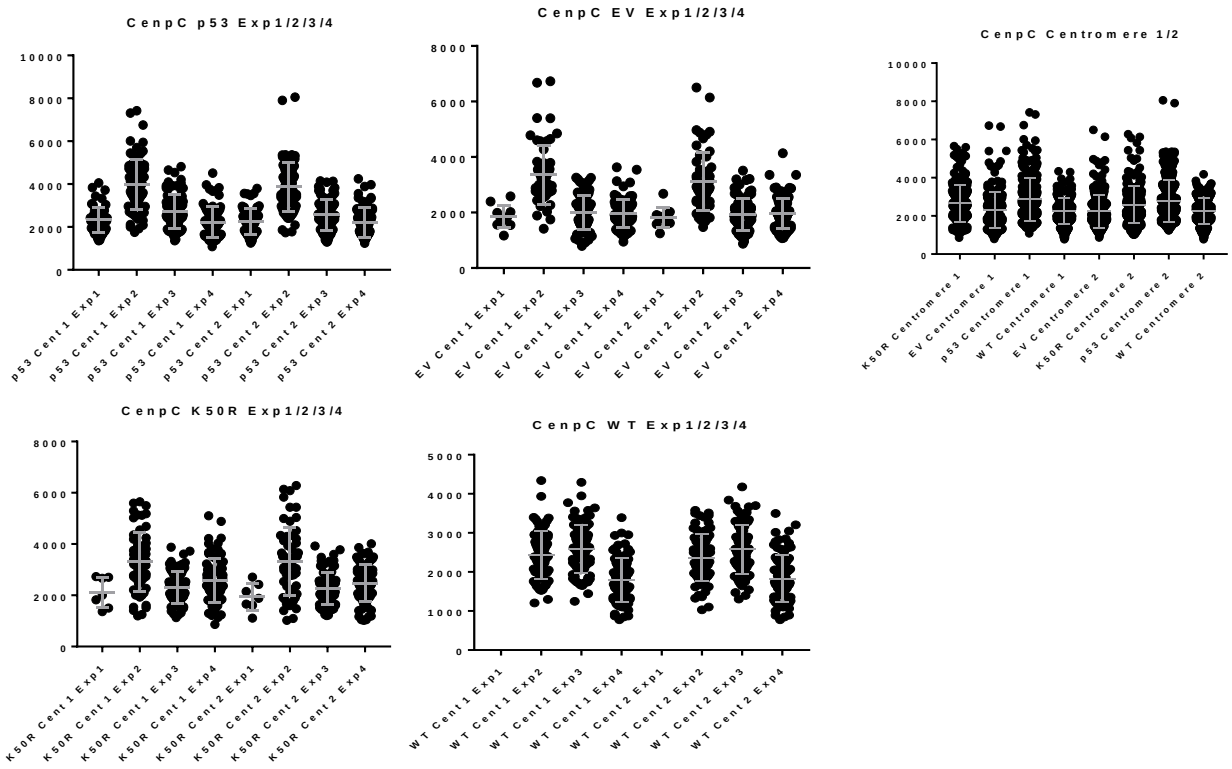

Figure 2: Quantification of the raw mean CENPC intensity (AU) in RPE-1 p53 KO (p53 KO) (control), RPE-1 p53 and DDX11 KO transfected with an empty vector (EV), RPE-1 p53 and DDX11 KO transfected with a mutant variant of the DDX11 (K50R) with null helicase activity (Mutant), and RPE-1 p53 and DDX11 KO transfected with a wild type variant of the DDX11 (WT). a) Raw mean CENPC intensity of each centromere in each separated experiments; b) Raw mean CENPC intensity of each centromere merged.